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Brief Conclusive Report

A novel p38-MAPK signaling axis modulates neutrophil biology in head and neck cancer

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RECEIVED APRIL 11, 2011; REVISED DECEMBER 26, 2011; ACCEPTED DECEMBER 29, 2011. DOI: 10.1189/jlb.0411193

ABSTRACT

Neutrophils are emerging as important mediators in cancer progression. Recent studies associated neutrophils with poor clinical outcome of HNC patients and showed that HNC induces recruitment, survival, and release of proinflammatory factors by neutrophils in vitro. The molecular mechanisms through which HNC and other cancers modulate neutrophil biology are currently unknown. To explore these mechanisms, we used an in vitro system that models the interaction between human HNC cells and neutrophils or neutrophilic-differentiated HL-60 cells, respectively. We show that HNC-derived factors activate p38-MAPK in neutrophils, which partly promotes neutrophil survival, but not neutrophil recruitment and motility. Most importantly, HNC-induced p38-MAPK activation strongly stimulates the release of CCL4, CXCL8, and MMP9 by neutrophils. We identify CREB and interestingly, p27 phosphorylated at T198 as downstream members of the HNC-induced p38-MAPK signaling cascade. Using siRNA technology, we demonstrate that p27 and CREB mediate the release of CCL4 and CXCL8 and that CREB, additionally, mediates the release of MMP9. These data unravel novel molecular mechanisms involved in regulation of neutrophil proinflammatory functions. Our studies on human HNC tissues indicate that tumor-infiltrating neutrophils might be a major source of CCL4 and particularly, MMP9 in cancer patients. Thus, our findings provide novel, mechanistic insights relevant for the pathophysiology of HNC and possibly, other types of cancer as well. *J. Leukoc. Biol.* 91: 591–598; 2012.

Introduction

Solid tumors frequently display an inflammatory microenvironment, characterized by large numbers of tumor-associated immune cells [1, 2]. Within this microenvironment, tumor cells can "educate" the immune cells of the host to down-regulate their immune functions and to aquire tumor-promoting activities. Accumulating evidence indicates that neutrophils and other myeloid cells play an important role in cancer progression. In melanoma, elevated neutrophil counts appear in the peripheral blood of patients, and high pretreatment neutrophil counts are an independent prognostic factor for poor overall survival of patients treated with IL-2 immunotherapy [3, 4]. In hepatocellular carcinoma, tumor-associated neutrophils were positively correlated with tumor progression and angiogenesis [5]. Additionally, it has been suggested that neutrophils are immunosuppressive, as they inhibit T cells functions and influence the pathophysiology and the course of disease in several carcinomas and cutaneous T cell lymphoma [6, 7]. Recent studies from our group indicated a potential role of neutrophils in HNC pathophysiology [8, 9]. We found that a strong neutrophilic infiltration of the tumor tissue was associated with poor survival in HNC patients with advanced disease [8]. In vitro, we demonstrated that HNC alters the biology of neutrophils by promoting their motility, survival, and release of proinflammatory factors, such as CCL4 and MMP9. Furthermore, we demonstrated that the factors released by neutrophils upon HNC stimulation have a feedback effect on the tumor cells by enhancing their migratory properties [9]. The exact molecular mechanisms responsible for modulation of neutrophil functions in cancer are, however, poorly understood thus far.

MAPKs are central components of signal transduction pathways that regulate multiple cell functions, such as proliferation and differentiation, cytokine production, and apoptosis [10]. In mammalian cells, three distinct subgroups of MAPK families have been identified: ERK1/2, JNK, and p38-MAPK. The canonical p38-MAPK signaling pathway involves phosphorylation of p38-MAPK at threonine and tyrosine residues by small G proteins and Src-like tyrosine kinases. Downstream, p38-MAPK can activate several kinases, such as MAPKAPK2 or MNK1; transcription factors, such as activation transcription factor 1/2, p53, or STAT1; and cytosolic proteins, such as caspase

Abbreviations: 7-AAD=7-aminoactinomycin D, APC=allophycocyanin, HNC=head and neck cancer, MMP=matrix metalloproteinase, RSK=ribosomal S6 kinase, S133=serine 133, SB 202190=4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, siRNA=small inhibitory RNA, SN=supernatants, T198, 180, 157=threonine 198, 180, 157, UM-SCC=University of Michigan squamous cell carcinoma, UT-SCC=University of Turku squamous cell carcinoma, Y182=tyrosine 182

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3/6 [10]. In neutrophils, p38-MAPK activation has been often linked to oxidative (respiratory) burst, chemotaxis, and rolling on endothelial cells [11–13].

A p38-MAPK-dependent modulation of neutrophil biology and functions by tumor-released factors has not been shown previously. Here, we demonstrate that supernatants (SN) derived from HNC cells induce a rapid and sustained phosphorylation of p38-MAPK in neutrophils. We show that HNC-induced p38-MAPK activation results in prolonged survival of neutrophils and enhanced release of CCL4, CXCL8, and MMP9, factors that are highly expressed in HNC tissue-infiltrating neutrophils. We demonstrate that HNC induces phosphorylation of CREB and of p27 via p38-MAPK and provides the first evidence that p38-MAPK is an upstream activator of p27 through phosphorylation at T198. Furthermore, we characterize the roles of p27 and CREB in HNC-induced neutrophil activation. Our study is also the first to demonstrate that p27, apart from its role in neutrophil differentiation, is involved in regulation of neutrophil proinflammatory functions.

MATERIALS AND METHODS

Cell lines and HNC SN

FaDu and HL-60 were from American Type Culture Collection (Manassas, VA, USA). UT-SCC 24A/50 and UM-SCC 17B/22B have been described elsewhere [14]. All cells were cultured and maintained as described previously [9].

To obtain HNC SN, we incubated 2×10^6 HNC cells/mL for 24 h at 37°C in culture medium and removed cellular debris by centrifugation. To induce neutrophilic differentiation of HL-60, we stimulated 5×10^6 cells/mL with 1.25% DMSO for 5 days. To knock down p27 and CREB, HL-60 cells were transfected with 33 nM validated p27 siRNA, 200 nM validated CREB siRNA, or AllStars Negative Control siRNA (Qiagen, Hilden, Germany) using the NEON transfection system (Invitrogen, Karlsruhe, Germany).

Isolation and culture of neutrophils

Neutrophils were isolated and cultured as described previously [9].

Study subjects

Neutrophils were isolated from the peripheral blood of healthy volunteers. Tissue samples were collected from 14 patients with head and neck SCC. All experiments were approved by the local ethics committee, and informed, written consent was obtained from each individual.

Antibodies and inhibitors

Mouse anti-CD66b antibodies were from Immunotech (Marseille, Cedex, France). Rabbit antiphospho-p38-MAPK, p38-MAPK, p27, and CREB antibodies were from Cell Signaling Technology (Danvers, MA, USA). Rabbit antiphospho-p27 (T198) and CREB (S133) and goat anti-CCL4 antibodies were from R&D Systems (Wiesbaden-Nordenstadt, Germany). Goat anti-MMP9 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-anti-CD71 and APC-anti-CD11b were from BD Biosciences (Heidelberg, Germany). All secondary antibodies (FITC donkey anti-goat IgG, DyLight 488 donkey anti-rabbit IgG, Cy5 goat anti-mouse IgG, and alkaline phosphatase goat anti-rabbit IgG) were from Jackson ImmunoResearch (West Grove, PA, USA). SB 202190 and the protease inhibitor cocktail sets I and III were from Merck (Darmstadt, Germany). PhosStop was from Roche (Mannheim, Germany).

Detection of phosphorylated proteins

Neutrophils (10^6 cells/sample) were incubated in Lysis Buffer 6 (R&D Systems) containing 10% PhosStop and 4% each of protease inhibitor cocktails I and III. The phospho-kinase array was performed according to the manufacturer's protocol (R&D Systems).

For Western blot, cells were lysed as above and incubated with SDS-sample buffer (final concentrations, 50 mM Tris, pH 6.8, 4% glycerin, 0.8% SDS, 1.6% β-ME, and 0.04% bromophenol blue). Samples were boiled and analyzed by SDS-PAGE, followed by transfer to PVDF membranes (Roche). Incubation with primary and secondary antibodies was performed for 2 h and 1 h, respectively, at room temperature. Chemiluminescent detection was performed with a ChemiDoc-It imaging system (UVP, LLC, Upland, CA, USA).

Immunofluorescence and flow cytometric assays

Neutrophils and HNC frozen sections were stained and analyzed as described elsewhere [9].

HL-60 cells were incubated with FITC-anti-CD71 or APC-anti-CD11b antibodies for 30 min at 4°C and were analyzed immediately by flow cytometry with a FACSCanto II flow cytometer (Becton Dickinson, Heidelberg, Germany).

Chemotaxis assay

Neutrophil chemotaxis was assessed in a transwell system, as described previously [9].

Quantification of CCL4 and CXCL8

Levels of CCL4 and CXCL8 released by neutrophils and HL-60 cells were analyzed by ELISA, according to the manufacturer's protocol (R&D Systems). A Synergy 2 microplate reader (BioTek, Bad Friedrichshall, Germany) was used to determine sample absorbance at 450 nM.

Gelatin zymography

The release of MMPs by neutrophils or HL-60 was analyzed as described previously [9].

Apoptosis assays

Neutrophils were stained with PE-conjugated Annexin-V and 7-AAD (BD Biosciences) and analyzed with a BD FACSCanto II flow cytometer.

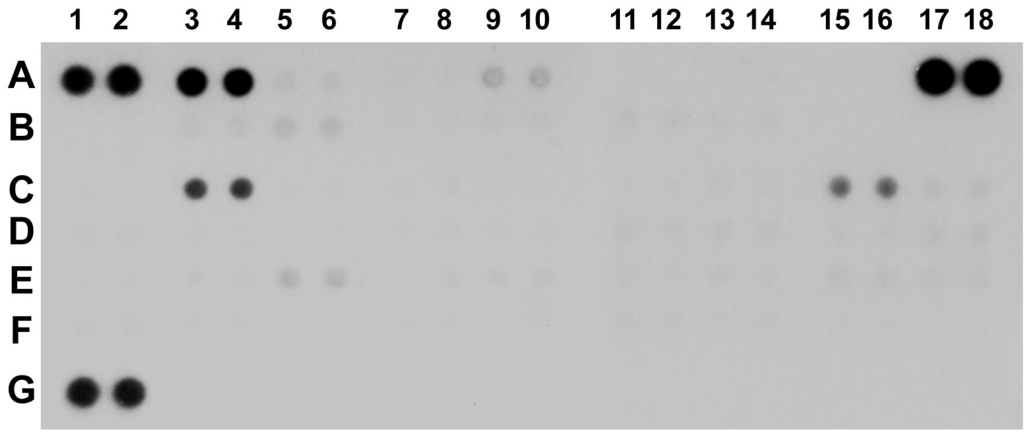
Statistical analysis

Data are presented as means and SDs. Statistical analysis was performed with two-tailed paired Student's t test. The level of significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

HNC-induced phosphorylation pattern in neutrophils

To determine which signaling pathways might be involved in HNC-induced activation of neutrophils, we used a phospho-kinase array kit, which simultaneously detects the relative phosphorylation levels of 46 proteins. To this end, we stimulated neutrophils with FaDu SN for 30 min, 1 h, and 2 h. The samples were pooled and analyzed as above. The results showed that the strongest phosphorylation levels were displayed by p38-MAPK at T180/Y182 (**Fig. 1**). Strong phosphorylation levels were also displayed by CREB at S133 and by p27 at T198 but interestingly, not by p27 at T157 (Fig. 1).



	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	positive control	**p38 α** T180/Y182	**ERK 1/2** T202/Y204, T185/Y187	**JNK pan** T183/Y185, T221/Y223	**GSK-3 α/β** S21/S9		**p53** S392		positive control
B		**MEK 1/2** S218/S222, S222/S226	**MSK 1/2** S376/S360	**AMPK α 1** T174	**Akt** S473	**Akt** T308	**p53** S46		
C	**TOR** S2448	**CREB** S133	**HSP27** S78/S82	**AMPK α 2** T172	**β-Catenin**	**p70 S6 Kinase** T389	**p53** S15	**p27** T198	**Paxillin** Y118
D	**Src** Y419	**Lyn** Y397	**Lck** Y394	**STAT 2** Y689	**STAT 5a** Y699	**p70 S6 Kinase** T421/S424	**RSK 1/2/3** S380	**p27** T157	**PLC λ-1** Y783
E	**Fyn** Y420	**Yes** Y426	**Fgr** Y412	**STAT 3** Y705	**STAT 5b** Y699	**p70 S6 Kinase** T229	**RSK 1/2** S221	**c-Jun** S63	**Pyk 2** Y402
F	**Hck** Y411	**Chk-2** T68	**FAK** Y397	**STAT 6** Y641	**STAT 5a/b** Y699	**STAT 1** Y701	**STAT 4** Y693	**eNOS** S1177	PBS negative control
G	positive control		PBS negative control						

Figure 1. HNC strongly phosphorylates p38-MAPK (T180/Y182), CREB (S133), and p27 (T198) in neutrophils. Neutrophils were stimulated with FaDu SN for 30 min, 1 h, and 2 h and pooled, and the relative phosphorylation levels of the proteins were assesed with a phospho-kinase array kit, followed by chemiluminescence detection. The results of a 2-min exposure are shown in the upper panel, and the coordinates for each capture spot are indicated accordingly. The legend of the array membrane is displayed in the lower panel and shows the name of the target protein as well as the phosphorylated amino acid residue. MSK, Mitogen- and stress-activated protein kinase; TOR, target of rapamycin; HSP27, heat shock protein 27.

HNC-induced p38-MAPK (T180/Y182), p27 (T198), and CREB (S133) activation in neutrophils

To confirm that HNC induces phosphorylation of p38-MAPK in neutrophils and elucidate the kinetics of p38-MAPK activation, we stimulated neutrophils with FaDu SN for 30 min, 2 h, and 4 h. Western blot analysis demonstrated that p38-MAPK (T180/Y182) was strongly phosphorylated already at 30 min after stimulation and that it remained constantly activated until 4 h poststimulation (**Fig. 2A**). In addition, fluorescence microscopy analysis of neutrophils stimulated with FaDu SN for 30 min confirmed that p38-MAPK (T180/Y182) is phosphorylated and localizes to the nucleus upon stimulation (Fig. 2B).

Next, we determined the kinetics of HNC-induced p27 (T198) and CREB (S133) phosphorylation in neutrophils. Furthermore, we tested whether p27 and CREB are downstream targets of p38-MAPK. To this end, neutrophils were stimulated with FaDu SN for 30 min, 2 h, and 4 h in the presence or absence of the p38-MAPK-specific inhibitor SB 202190, which was used at a concentration of 10 μM. Western blot analysis using p27 (T198) phospho-specific antibodies revealed that p27 was strongly activated already at 30 min after stimulation and decreased afterward (Fig. 2C). Inhibition of p38-MAPK by SB 202190 reduced the levels of p27 phosphorylation at each time-point (Fig. 2C). HNC-induced CREB phosphorylation showed similar kinetics to p27 phosphorylation and was, likewise, dependent on p38-MAPK (Fig. 2C). These data indicate that HNC-triggered signaling cascade in neutrophils involves p27 (T198) and CREB (S133) as downstream targets of p38-MAPK. Although CREB is a known downstream target of p38-MAPK [15], previous studies showed that p27 (T198) is activated via PI3K/PKB and RSK [16, 17]. To the best of our knowledge, we are the first to identify p38-MAPK as an upstream activator of p27 (T198).

Regulation of neutrophil functions by p38-MAPK

In a next set of experiments, we tested which neutrophil functions are modulated by HNC via the p38-MAPK signaling cascade. To this end, we stimulated neutrophils with FaDu SN in the presence or absence of the p38-MAPK-specific inhibitor SB 202190 (10 μM) and determined neutrophil chemotaxis, survival, and release of proinflammatory factors (CCL4, CXCL8, and MMP9). Initial studies were performed to exclude a potential toxic effect of SB 202190 and of its solvent (DMSO) on the basal responses of neutrophils (data not shown). Our results demonstrated that inhibition of p38-MAPK did not affect HNC-induced neutrophil che-

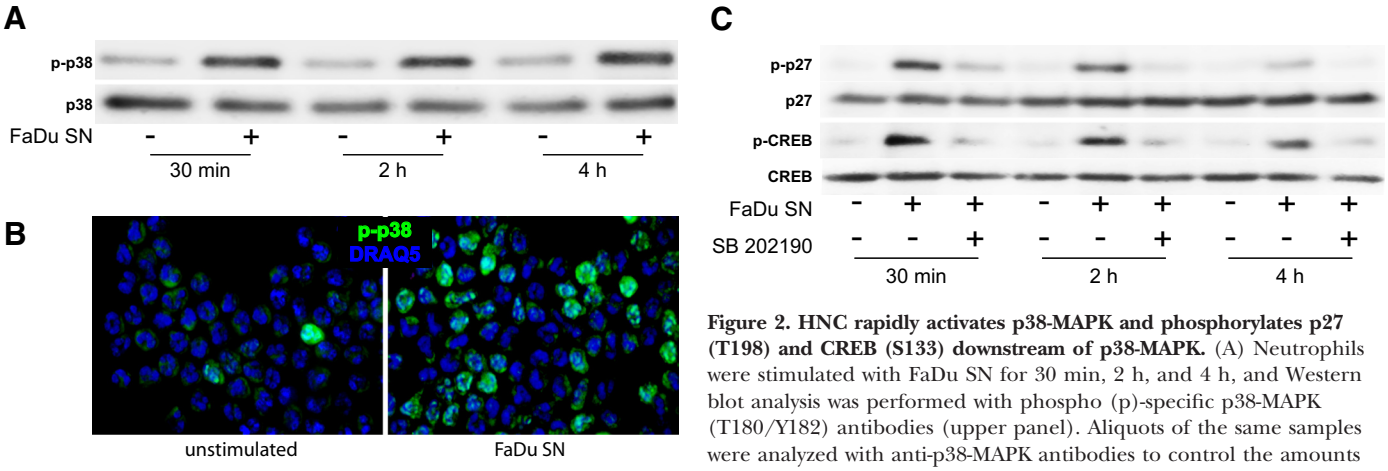


Figure 2. HNC rapidly activates p38-MAPK and phosphorylates p27 (T198) and CREB (S133) downstream of p38-MAPK. (A) Neutrophils were stimulated with FaDu SN for 30 min, 2 h, and 4 h, and Western blot analysis was performed with phospho (p)-specific p38-MAPK (T180/Y182) antibodies (upper panel). Aliquots of the same samples were analyzed with anti-p38-MAPK antibodies to control the amounts of proteins in these samples (lower panel). The results are representative of three independent experiments. (B) Neutrophils were stimulated with FaDu SN for 30 min. After fixation and permeabilization, neutrophils were stained with antiphospho-p38-MAPK (T180/Y182) antibodies and then with DyLight 488-conjugated secondary antibodies (green). Nuclei were visualized by DRAQ5 staining (blue pseudocolor). Turquoise indicates a colocalization of phospho-p38 with the nuclei of the neutrophils. The results are representative of two independent experiments. (C) Neutrophils were stimulated with FaDu SN in the presence or absence of the p38-MAPK-specific inhibitor SB 202190 (10 μM) for 30 min, 2 h, and 4 h. Cells were lysed, and the samples were analyzed by Western blot using phospho-specific antibodies against p27 (T198) and CREB (S133). As control, samples were analyzed with anti-p27 and anti-CREB antibodies, respectively. The results are representative of three independent experiments.

motaxis (**Fig. 3A**) nor chemokinesis (data not shown). However, p38-MAPK inhibition reduced HNC-mediated neutrophil survival ($P \leq 0.01$) and importantly, the release of proinflammatory factors, such as CCL4 ($P \leq 0.01$), CXCL8 ($P \leq 0.05$), and MMP9 (Fig. 3B–D).

To verify the involvement of p38-MAPK as a regulator of neutrophil functions in HNC, we stimulated neutrophils with SN from four additional HNC cell lines in the presence or absence of 10 μM SB 202190. Similar to FaDu SN (Fig. 3B and C), addition of SB 202190 to these new SN resulted in strong inhibition of CCL4 release by neutrophils and in roughly 35–50% inhibition of survival (**Fig. 4A** and **B**). Furthermore, Western blot analysis confirmed the phosphorylation of p38-MAPK in neutrophils upon stimulation with all HNC SN (Fig. 4C).

These findings indicate that p38-MAPK is an important regulator of neutrophil biology and functions upon stimulation by HNC. However, the extent of p38-MAPK involvement in individual neutrophil functions seems to vary. For instance, using lower doses of SB 202190, we observed that CCL4 release was still strongly reduced, whereas survival of neutrophils was less so (data not shown). Although difficult to prove experimentally, these observations could indicate that SB 202190 has slight off-target effects. On the other hand, pyridinyl imidazole compounds (such as SB 202190) are widely used in similar studies to block p38-MAPK and

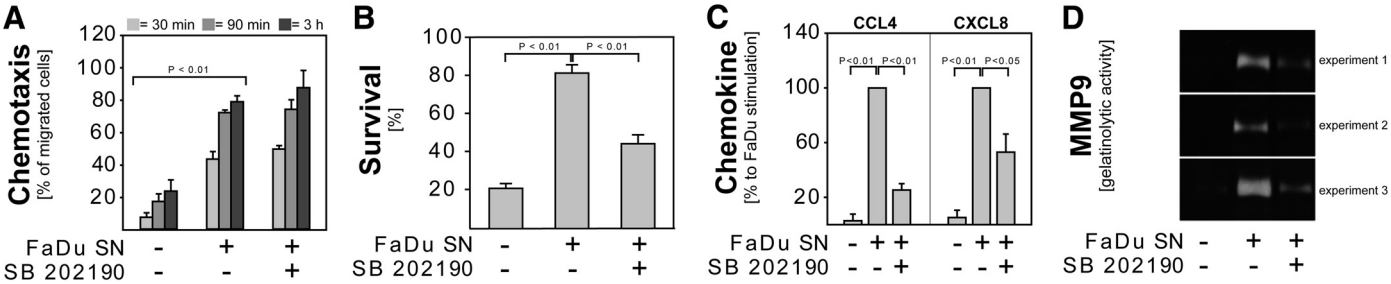


Figure 3. HNC promotes neutrophil survival and release of proinflammatory factors via p38-MAPK. (A) Neutrophils were incubated or not with 10 μM SB 202190 and allowed to migrate toward FaDu SN in a transwell culture system. Migrated cells were counted after 30 min, 90 min, and 3 h, respectively. Data are means ± SD of three independent experiments. (B) Neutrophils were stimulated with FaDu SN in the presence or absence of 10 μM SB 202190 for 24 h at 37°C. Cells were stained with PE-conjugated annexin-V and 7-AAD and analyzed by flow cytometry. Data are means ± SD of four independent experiments. (C) Neutrophils were stimulated as in B. Neutrophil SN were collected, and the levels of CCL4 and CXCL8 were determined by ELISA. Chemokine levels produced by neutrophils stimulated with FaDu SN without SB 202190 were set as 100%. Data are means ± SD of three independent experiments. (D) Neutrophils were stimulated with FaDu SN in the presence or absence of 10 μM SB 202190 for 1 h at 37°C. Neutrophil SN were collected, and MMP9 release was determined by gelatin zymography. The results of three independent experiments are shown.

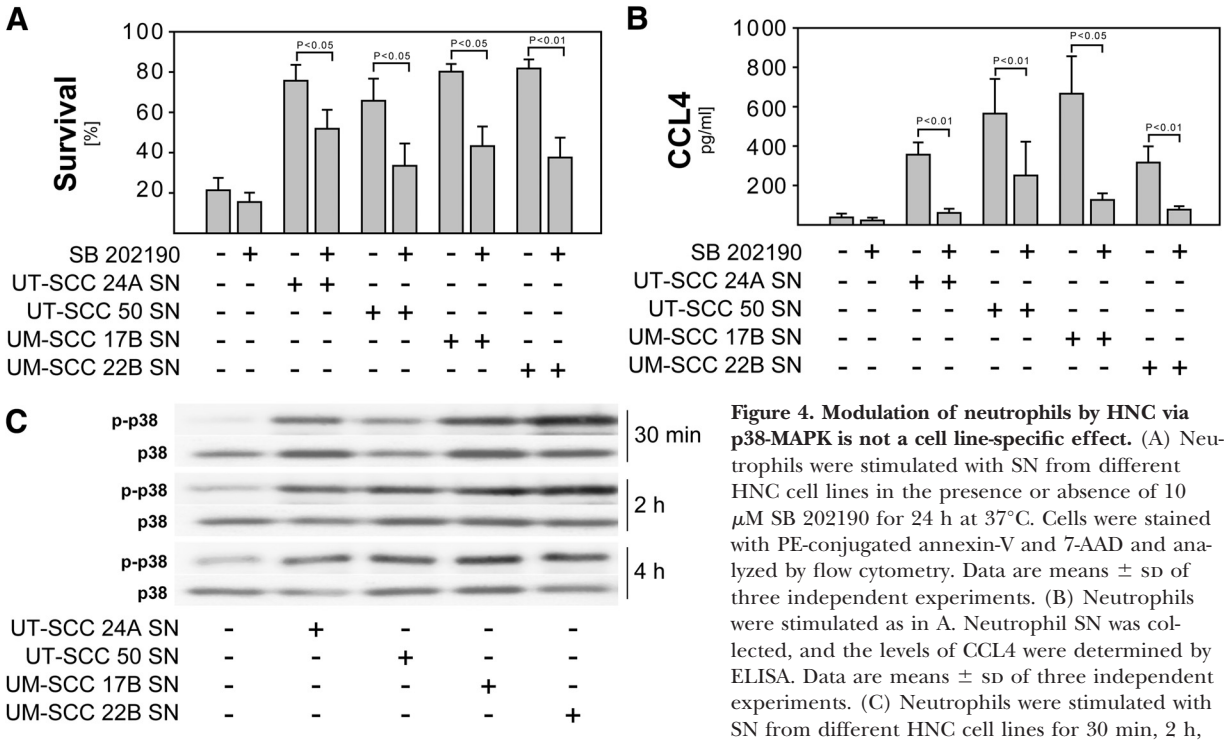


Figure 4. Modulation of neutrophils by HNC via p38-MAPK is not a cell line-specific effect. (A) Neutrophils were stimulated with SN from different HNC cell lines in the presence or absence of 10 μM SB 202190 for 24 h at 37°C. Cells were stained with PE-conjugated annexin-V and 7-AAD and analyzed by flow cytometry. Data are means ± SD of three independent experiments. (B) Neutrophils were stimulated as in A. Neutrophil SN was collected, and the levels of CCL4 were determined by ELISA. Data are means ± SD of three independent experiments. (C) Neutrophils were stimulated with SN from different HNC cell lines for 30 min, 2 h, and 4 h, and Western blot analysis was performed with phospho-specific p38-MAPK (T180/Y182) antibodies. As control, samples were analyzed with anti-p38-MAPK antibodies. The results are representative of three independent experiments.

were shown to be highly specific even at high doses [18, 19]. Therefore, future studies need to use (currently, technically problematic) genetic inhibition in primary neutrophils to elucidate the exact degree of p38-MAPK involvement in certain functions of these cells, such as survival.

Regulation of neutrophil functions by p27 and CREB

To assess the roles of p27 and CREB in HNC-mediated neutrophil activation, we established siRNA knock-down systems for these proteins using neutrophilic-differentiated HL-60 cells [20]. To test the response of neutrophilic-differentiated HL-60 to HNC stimulation, we incubated these cells with FaDu SN for 30 min, 2 h, and 4 h. Western blot analysis indicated that upon HNC stimulation, neutrophilic-differentiated HL-60 responded with p38 (T180/Y182), p27 (T198), and CREB (S133) phosphorylation in a manner comparable with primary neutrophils (**Fig. 5A**). Next, we transfected neutrophilic-differentiated HL-60 with p27, CREB, or AllStars Negative Control siRNAs and determined their functional response to HNC stimulation. Assessment of knock-down efficiency at protein level by flow cytometry indicated complete down-regulation of p27, already at 24 h post-transfection and ~60% down-regulation of CREB at 72 h post-transfection (data not shown). We found that HNC-induced release of CCL4 and CXCL8 was significantly inhibited by knock-down of p27 ($P \leq 0.05$ for CCL4; $P \leq 0.01$ for CXCL8) and CREB ($P \leq 0.01$ for CCL4; $P \leq 0.01$ for CXCL8; Fig. 5B and C). Silencing of CREB, but not of p27, also prevented the release of MMP9 by neutrophilic-differentiated HL-60 cells upon HNC stimulation (Fig. 5D and E). The effect of p27 and CREB on the survival of HL-60 cells could not be assessed, as differentiated HL-60, unlike primary neutrophils, exhibited very low levels of spontaneous apoptosis under standard culture conditions (data not shown).

Taken together, these data indicate that HNC modulates the release of proinflammatory chemokines by neutrophilic-differentiated HL-60 and most likely, by neutrophils via p27 and CREB activation. Our findings about the role of p27 in modulation of the above-mentioned neutrophil functions are of particular interest. In previous studies, p27 has been addressed solely in the context of the neutrophil differentiation process. Klausen and colleagues [21] showed that the total levels of p27 increased during neutrophil differentiation, with low levels until the metamyelocyte stage and high levels in band cells and mature neutrophils from peripheral blood. As p27 negatively regulates the CDKs, it has been proposed that the main role of p27 in neutrophils is to arrest these cells in the G1 phase of the cell cycle during their maturation process [21]. However, recent studies suggested that p27 may be involved in cell cycle-independent cellular functions, which interestingly, seem to be a consequence of p27 phosphorylation at T198. In an elegant study, Larrea and colleagues [22] demonstrated that phosphorylation of p27 at T198 promotes RhoA-p27 binding and results in increased cell motility of WM35 and WM239 cells. Other studies have linked p27 to cellular survival through

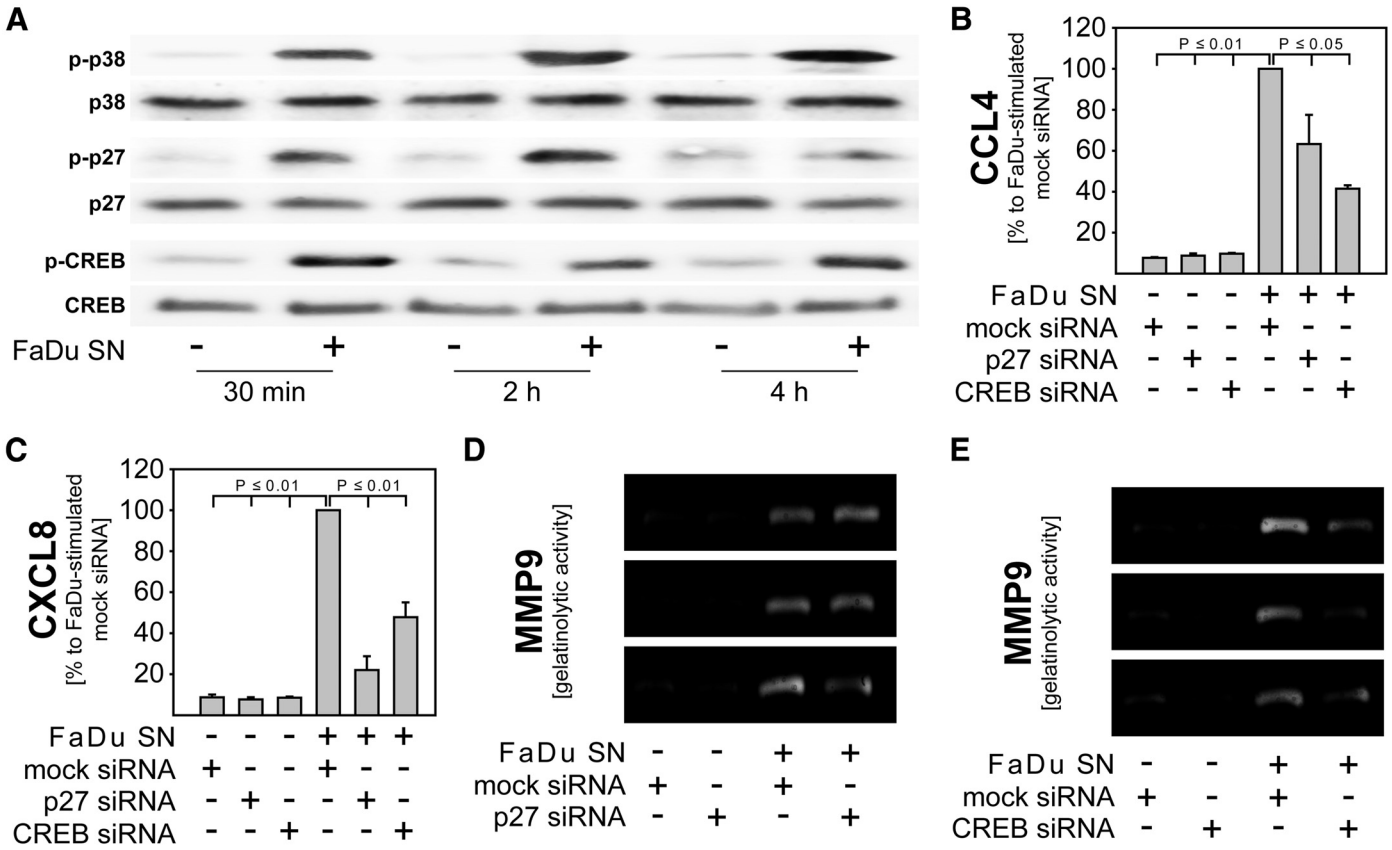


Figure 5. HNC induces the release of proinflammatory factors via p27 and CREB. (A) DMSO-differentiated HL-60 were stimulated with FaDu SN for 30 min, 2 h, and 4 h. Cells were lysed, and the samples were analyzed by Western blot using phospho-specific antibodies to assess the phosphorylation levels of p38-MAPK (T180/Y182), p27 (T198), and CREB (S133), respectively. As control, samples were analyzed with antibodies against the nonphosphorylated forms of these proteins. The results are representative of three independent experiments. (B) DMSO-differentiated HL-60 were transfected with validated p27 siRNA (33 nM) or CREB siRNA (200 nM). AllStars Negative Control (mock) siRNA was used at appropriate concentrations for each specific siRNA. Transfected cells were stimulated with FaDu SN for 24 h, and the levels of CCL4 released in the SN were determined by ELISA. Data are means ± SD of three independent experiments. (C) DMSO-differentiated HL-60 were transfected and stimulated as in B. The levels of CXCL8 released in the SN were determined by ELISA. Data are means ± SD of three independent experiments. (D) DMSO-differentiated HL-60 were transfected with validated p27 siRNA or AllStars Negative Control (mock) siRNA. Cells were stimulated with FaDu SN for 18 h at 37°C. SN were collected, and MMP9 release was determined by gelatin zymography. The results of three independent experiments are shown. (E) DMSO-differentiated HL-60 were transfected with validated CREB siRNA or AllStars Negative Control (mock) siRNA. Cells were stimulated with FaDu SN for 18 h at 37°C. SN were collected, and MMP9 release was determined by gelatin zymography. The results of three independent experiments are shown.

phosphorylation at T198 and stabilization of the protein [23]. Here, we identify a novel cellular role of p27 and show that this protein can also act as a regulator of proinflammatory chemokine release in neutrophils and perhaps, other cell types as well.

Expression of CCL4 and MMP9 in tumor-infiltrating neutrophils

Proinflammatory mediators play critical roles within the tumor microenvironment [1]. Next, we determined the expression of CCL4 and MMP9 in tumor-infiltrating neutrophils, as high expression of these mediators is of potential relevance for the pathophysiology of HNC. For this purpose, frozen tissues obtained from 14 HNC patients were double-stained for CD66b (neutrophil marker) and CCL4 or MMP9, respectively. Fluorescent microscopy analysis showed a colocalization of CD66b-expressing cells with strong CCL4 and MMP9 signals in the tumor tissues of all patients analyzed (**Fig. 6A** and **B**), which indicates that tumor-infiltrating neutrophils express high levels of CCL4 and MMP9 in the HNC tissue. In particular, the expression of MMP9 in tumor-infiltrating neutrophils was higher than in any other cell type within the tumor tissue. These data suggest that neutrophils might partially contribute to the intratumoral levels of CCL4 and might even be a major source for MMP9 within the tumor microenvironment. Although the significance of CCL4 production for the tumor environment is not completely elucidated, several studies suggested that this chemokine may potentially play a pro-tumoral role. For instance, the expression of CCL4 has been found to be increased in some types of cancer, such as gastric and colon cancer [24, 25]. Additionally, Blum and coworkers [26]

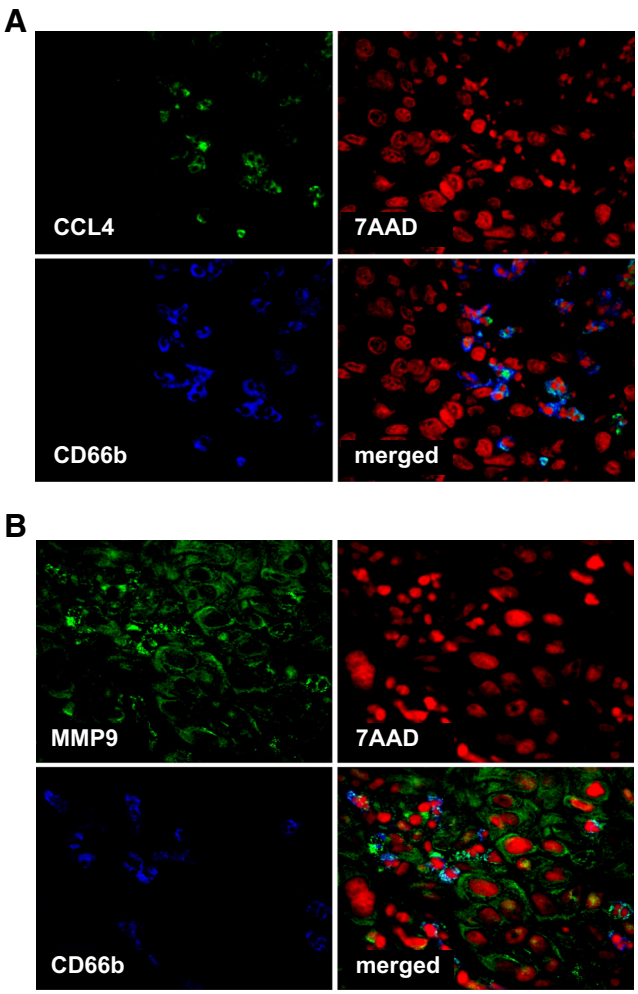


Figure 6. HNC tissue-infiltrating neutrophils express high levels of CCL4 and MMP9. Frozen sections of HNC tissues from 14 patients were double-stained against CCL4 (A) or MMP9 (B; green) and the neutrophil marker CD66b (blue pseudocolor). Nuclei were visualized by 7-AAD staining (red). Representative tissue sections are shown at 630-fold original magnification. Upper left, upper right, and lower left panels show single-color stainings, whereas the lower right panel (merged) shows the overlay of all three stainings. Turquoise in the merged figure indicates the colocalization of CD66b-positive cells with CCL4 or MMP9, respectively.

have shown recently that expression of CCL4 is linked to biochemical recurrence of prostate cancer following prostatectomy. In contrast to CCL4, the role of MMP9 in cancer has been investigated extensively, and previous studies found MMP9 to be one of the most important mediators of tumor progression [27, 28]. Thus, the molecular mechanisms described in our study may be of considerable relevance for the pathophysiology of this tumor entity.

In conclusion, our study elucidates novel signaling pathways through which HNC cells modulate the biology and functions of tumor-associated neutrophils. Understanding the exact molecular events used by tumors to skew the functions of immune cells may ultimately improve future therapeutic strategies.

AUTHORSHIP

C.A.D. performed most of the experiments and wrote the manuscript. M.K.F. performed part of the experiments. T.K.H. characterized and provided several of the HNC cell lines used in this study. S.L. provided clinical management. S.B. provided the conception and design and data interpretation and wrote the manuscript.

ACKNOWLEDGMENTS

This study was supported in part by the Internal Research Funding Essen program of the medical faculty of University Duisburg-Essen, by the Deutsche Forschungsgemeinschaft (Grant BR 2278/2-1), and by the Krebsgesellschaft Nordrhein-Westfalen. We are grateful to Dr. Alexander Schramm (University Children's Hospital Essen) for his help with the siRNA transfection studies. We thank Petra Altenhoff, Anne-Marie Heider, and Kirsten Bruderek (Department of Otorhinolaryngology, University of Duisburg-Essen) for excellent technical support.

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KEY WORDS:
p27 · CREB · inflammation · CCL4 · MMP9