Can inflammatory cells affect activities of tongue cancer cells and vice versa?

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Abstract

Background: 5-year survival rate of oral tongue squamous cell carcinoma (OTSCC) has been low (less than 60%) despite developing treatment modalities. A previous research revealed that different populations of inflammatory cells infiltration in OTSCC were associated with different clinical outcomes. On the other hand, extracellular vesicles (EVs) secreted by OTSCC cells suggested crosstalk between OTSCC cells and tumor infiltrating inflammatory cells.

Study aims: This study aims to investigate the interaction between OTSCC cells and inflammatory cells and answer 3 questions: (1) Can human peripheral blood mononuclear cells (MNCs) affect activities of OTSCC cells such as proliferation, migration and invasion? (2) Can EVs of OTSCC cells affect polarization of macrophages? (3) Can EVs of OTSCC cells affect cytotoxic activity of CD8+ T cells and NK cells?

Materials and methods: Two OTSCC cell lines (HSC-3 and SCC-25) were used. OTSCC cells and human peripheral blood MNCs were co-cultured using a 3D organotypic myoma model. Proliferation and invasion into myoma tissue of OTSCC cells were detected by Immunohistochemical staining of pan-cytokeratin and Ki67. Invasion area and depth of OTSCC cells were measured using ImageJ software. Migration of OTSCC cells in the presence of MNCs was monitored using a scratch wound healing assay with IncuCyte™ system. OTSCC EVs were isolated with ultracentrifugation and characterized with NTA and Immuno-EM. Human primary monocytes, CD8+ T cells and NK cells were isolated using MACS, and their purity was checked using FACS. Expression of macrophage phenotypic markers was checked with qPCR. Cytotoxic activity was evaluated using an IncyCyte™ cell killing assay.

Results: Activated human peripheral blood MNCs significantly reduced proliferation of both OTSCC cell lines, and invasion area of only HSC-3. None of the inflammatory cells in the experiment had any effect on invasion depth and migration of OTSCC cells. On the other hand, OTSCC cell-derived EVs didn’t influence macrophage
polarization, but had heterogeneous modulating effects on cytotoxic activity of CD8⁺ T cells and NK cells.

Conclusion: We detected effects of OTSCC cells and inflammatory cells on each other by secreted molecule mediators or EVs, but the results were not uniform and varied in different OTSCC cell lines or inflammatory cell populations and sources. The outcome of the study emphasizes the importance of a personalized design of cancer treatment, which takes other components in tumor microenvironment such as inflammatory cells and EVs into consideration.

Key words: oral tongue squamous cell carcinoma, cancer immunity, extracellular vesicles

Additional information: Based on the data in this project one manuscript was written and now it is accepted for publication in Oncotarget ('Crosstalk between tongue carcinoma cells, extracellular vesicles, and immune cells in in vitro and in vivo models')
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>cancer associated fibroblast</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>DSS</td>
<td>disease-specific survival</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECS</td>
<td>extracapsular spread</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EV</td>
<td>extracellular vesicle</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell separation</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic-activated cell sorting</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>natural killer group 2D</td>
</tr>
<tr>
<td>NTA</td>
<td>nanoparticle tracking analysis</td>
</tr>
<tr>
<td>OSCC</td>
<td>oral squamous cell carcinoma</td>
</tr>
<tr>
<td>OTSCC</td>
<td>oral tongue squamous cell carcinoma</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>q-PCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution(s) per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SLN</td>
<td>sentinel lymph node</td>
</tr>
<tr>
<td>TAM</td>
<td>tumor associated macrophage</td>
</tr>
<tr>
<td>Tfh</td>
<td>follicular T helper</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TME</td>
<td>tumor microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WPOI</td>
<td>worst pattern of invasion</td>
</tr>
</tbody>
</table>
1. Literature review

1.1. Oral tongue squamous cell carcinoma

Over 90% of oral cancers are squamous cell carcinomas (Beenken & Urist, 2003). Possible sites of oral squamous cell carcinoma (OSCC) include gums, palate, floor of mouth, inner aspects of lips, as well as oral and basal tongue. Among those the most common site is the oral tongue (the anterior two thirds of the tongue, defined from basal tongue by a V-shaped groove named sulcus terminalis), accounting for over 40% of all OSCC cases (Kirita & Omura, 2015).

The tongue is an essential part in the oral cavity which functions in speech, eating, tasting and deglutition. It has a central core of a bunch of skeleton muscle bundles and is covered with a layer of epithelium. Tongue squamous cell carcinoma usually develop within the stratum spinosum layer of the epithelium. Like other cancers, normal keratinocytes of the tongue epithelium undergo a series of DNA mutations and acquire the ability to proliferate in an uncontrolled fashion. Once these keratinocytes invade through the basement membrane into the connective, muscle and adipose tissue, and even metastasize through the blood and lymph vessels, the malignancy process begins (Scully & Bagan, 2009; Kato & Shimizu, 2012; Weinberg, 2013).

1.1.1. Epidemiology and etiology of OSCC and OTSCC

Oral cancer is globally among the eight most common cancers, with an increasing incidence. Each year over 300,000 new cases are diagnosed worldwide (Torre et al., 2015). Specifically, the number of Finnish patients diagnosed for oral tongue squamous cell carcinoma (OTSCC) annually has approximately doubled from 1980 to 2010 (Ng et al., 2016). Smoking and alcohol use are identified globally as the most representative risk factors for OSCC. Other etiological factors include smokeless tobacco, human papilloma virus (HPV), betel chewing, and poor dental health condition. Smoking is the top causative agent of oral cancer and it has been firmly
epidemiologically established (Murti et al., 1995; Kumar et al., 2016). The overall oral cancer risk of smokers is about 1.4 to 1.7 times higher than non-smokers (Hashibe et al., 2007). Additionally, when smoking and alcohol drinking are acting synergistically, the probability of developing an oral cancer is significantly raised (Radoi & Luce, 2013).

Historically, males have been more susceptible to OSCC than females. The male-to-female ratio was once as high as 6:1, yet gradually it has been now decreased to 2:1. The most probable explanation for the increased incidence among females is the growing number of female smokers and alcohol consumers (The Oral Cancer Foundation, 2017). In fact, in recent decades there has been an obvious trend that the average annual incidence increase of OSCC is higher in young female population (under 45) in many European countries and the States (Ng et al., 2016). Nowadays HPV, especially subtype 16, is becoming the primary carcinogenic factor of oropharyngeal cancers in non-smoking younger group (The Oral Cancer Foundation, 2017). HPV is sexually transmitted between partners, also causing more than 90% of cervical cancers. The prevalence of HPV as an oral cancer carcinogenic factor reveals the change in sexual practice of young people. However, in case of OTSCC, HPV has not shown to be an etiological factor; the reason for the increased incidence of OTSCC among non-drinkers and non-smokers is not known (Salem, 2010). Conclusively, people's choices of life style play important roles in the development of oral cancer.

1.1.2. TNM staging, invasion and metastasis of OTSCC

1.1.2.1 TNM staging system of OTSCC

Clinically, OTSCC can be classified by TNM staging system according to 3 factors, which are the size of the primary tumor (T), location of the metastasis in regional lymph nodes (N) and presence of the distant metastasis (M) (Table 1).
<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>T1</td>
<td>Tumor 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor more than 2 cm but not more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T4a</td>
<td>Moderately advanced local disease; tumor invasion into the mandibular bone marrow, invasion into the submandibular space and invasion into the extrinsic muscles of the tongue</td>
</tr>
<tr>
<td>T4b</td>
<td>Very advanced local disease; tumor invasion into the masticator space, invasion into the pterygoid plate, invasion into the skull base, and invasion circumferentially surrounding the internal carotid artery</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph Nodes</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis as specified in N2a, 2b, 2c below</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2c</td>
<td>Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in a lymph node more than 6 cm in greatest dimension</td>
</tr>
</tbody>
</table>

| Distant Metastasis | |
|-------------------| |


Table 1. TNM staging system of OTSCC tumors. (Modified from Kirita & Omura, 2015; Compton et al., 2012)

<table>
<thead>
<tr>
<th>Stage Grouping</th>
<th>TNM Staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis, N0, M0</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1, N0, M0</td>
</tr>
<tr>
<td>Stage II</td>
<td>T2, N0, M0</td>
</tr>
<tr>
<td>Stage III</td>
<td>T3, N0, M0</td>
</tr>
<tr>
<td></td>
<td>T1-3, N1, M0</td>
</tr>
<tr>
<td>Stage IVA</td>
<td>T4a, N0-1, M0</td>
</tr>
<tr>
<td></td>
<td>T1-4a, N2, M0</td>
</tr>
<tr>
<td>Stage IVB</td>
<td>Any T, N3, M0</td>
</tr>
<tr>
<td></td>
<td>T4a, any N, M0</td>
</tr>
<tr>
<td>Stage IVC</td>
<td>Any T, any N, M1</td>
</tr>
</tbody>
</table>

Since TNM staging system was established in 1950s, it has been universally accepted and used in many kinds of solid tumors to give a clear staging evaluation of clinical cases. Nevertheless, its guidance on prognosis determination might not be informative enough. Other factors are being taken into account in order to facilitate the prognostic predication.

In terms of OTSCC, these factors include tumor proximity to midline, tongue base involvement, and other pathological parameters like invasion depth and so on (Sahin et al., 2016).

1.1.2.2. Invasion pattern and depth of OTSCC

As for the clinical types of OTSCC, there are superficial, exophytic and endophytic type which describe different growth patterns of the primary tumor (Fig.1).
Fig. 1 Clinical types of OTSCC. (a) Superficial type. (b) Exophytic type. (c) Endophytic type. (Taken from Kirita & Omura, 2015)

A previous study analyzed 2,224 tongue cancer patients with T1-2 tumors and saw significant differences in prognostic parameters like local recurrence rate, lymph node metastasis, distant metastasis, and 5-year survival rate among the three types, which showed the potential use of clinical types as a prognostic indicator. OTSCC of
endophytic type have higher rate of local recurrence, lymph node and distant metastasis, and lower 5-year survival rate than the other two types (Japan Society for Oral Tumors, 2005).

In their invasive front, OTSCC cells can expand with a pushing border, a finger-like pattern or some small cell islands (Ahmed Haji Omar, 2015). Worst pattern of invasion (WPOI), namely whether cohesive pattern (pushing border, finger-like pattern, big cell islands) or invasive pattern (small cell islands, tumor satellites), is strongly predictive for prognosis. OTSCC with an invasive pattern WPOI is found to be more likely to cause local recurrence and cancer death (Almangush et al., 2015; Almangush et al., 2014).

**Fig.2** (a) Ultrasonographic image of normal tongue. (b) Measurement of tumor depth. (Modified from Kirita & Omura, 2015)

Besides, invasion depth is another factor associated with OTSCC prognosis. Studies showed significantly more frequent local recurrence and cervical lymph node
metastasis in early OTSCC tumors with deeper invasion (≥4 mm) (Sahin et al., 2016; Almangush et al., 2015).

Under ultrasonography scanning, the lateral border of the oral tongue (the most common site of OTSCC) shows three heterogeneous layers from outmost to inside: surface layer, mucosal layer, submucosal and muscular layer (Fig.2a). Tongue cancer invasion depth should be measured from the assumed normal mucosal surface to the deepest anatomical location, rather than the tumor thickness, since swelling and invagination may exist (Fig.2b).

1.1.2.3 Biology of metastasis

Over 90% of cancer deaths are caused by distant metastasis other than the primary tumors. Metastasis means cancer spreading from the primary tumor to another organ. It has several steps: cancer (carcinoma) cells invade through the basement membrane reaching nearby stroma from the epithelium, intravasate into lymphatic or blood vessels, travel to regional lymph nodes or distant anatomical sites, extravasate and eventually colonize there, forming a macroscopic metastasis (Spano et al., 2012).

To get motile and invasive, carcinoma cells need to undergo an epithelial-mesenchymal transition (EMT), by which their morphology, motility, invasive ability and gene expression profile change. Expression of characteristic epithelial cell proteins, such as E-cadherin, is suppressed, while production of matrix metalloproteinases (MMPs) and proteins only made by mesenchymal cells like fibronectin are upregulated (Weinberg, 2013; Irani et al., 2014). Thus cell-cell adhesion is lost and the extracellular matrix (ECM) is degraded, which facilitates the carcinoma invasion.

Once normal epithelial cells are detached from the ECM, anoikis, a special form of apoptosis is triggered. This process prevents re-adhesion to ectopic locations and
uncontrolled proliferation of suspending cells. However, invasive carcinoma cells survive from anoikis program and benefit from this disparity (Paoli et al., 2013).

Even before carcinoma cells break through the basement membrane, they start to secret angiogenic factors to stimulate angiogenesis in the stromal part (Weinberg, 2013). With the overexpressed vascular endothelial growth factors (VEGFs), cancer cells attract excess capillaries and get access to the vessels. After the intravasation, the disseminated cancer cells travel inside the blood and lymph circulation. During the travelling, these cells have to escape from immune surveillance in order to finally find a new target tissue to colonize in.

1.1.2.4. Tongue cancer metastasis

Tongue cancer metastasizes relatively early. Over 30% patients are with at least one enlarged lymph nodes when they first come to have a clinical check (Kirita & Omura, 2015). Compared with blood vessels, OTSCC is more metastasized via lymphatic vessels to regional lymph nodes (Sano & Myers, 2007). Cervical lymph nodes are located at seven levels divided by the anatomic regions (Table 2, Fig.3).

<table>
<thead>
<tr>
<th>Level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level IA</td>
<td>Submental lymph nodes</td>
</tr>
<tr>
<td>Level IB</td>
<td>Submandibular lymph nodes</td>
</tr>
<tr>
<td>Level IIA</td>
<td>Superior deep cervical lymph nodes (anterior)</td>
</tr>
<tr>
<td>Level IIB</td>
<td>Superior deep cervical lymph nodes (posterior)</td>
</tr>
<tr>
<td>Level III</td>
<td>Middle deep cervical lymph nodes</td>
</tr>
<tr>
<td>Level IV</td>
<td>Inferior deep cervical lymph nodes</td>
</tr>
<tr>
<td>Level VA</td>
<td>Spinal accessory lymph nodes</td>
</tr>
<tr>
<td>Level VB</td>
<td>Supraclavicular lymph nodes</td>
</tr>
<tr>
<td>Level VI</td>
<td>Pre-laryngeal, pre-tracheal, and para-tracheal lymph nodes</td>
</tr>
<tr>
<td>Level VII</td>
<td>Superior mediastinal lymph nodes</td>
</tr>
</tbody>
</table>

Table 2. Classification of cervical lymph nodes. (Modified from Kirita & Omura, 2015; Sano & Myers, 2007)
The lymphatic vessels in the tongue compose a dense network. In different areas of the tongue, lymphatic fluid flows in various drainage patterns (Werner et al., 2003). There are basically three main lymphatic drainage pathways which lead to sentinel lymph nodes (SLNs) at different levels in the neck region. The anterior part of the tongue has SLNs principally at level I and II. SLNs of the lateral one-third of the dorsum of the tongue are mainly at levels I, II, or III. The central pathway drains to levels I and III from the central part of the tongue (Sano & Myers, 2007; Han et al., 2005). Lymph nodes at level I and II receive most of the lymph from the oral tongue. And most OTSCC primary lymph node metastases occur at levels I, II, and III (Sano & Myers, 2007).

![Levels of cervical lymph nodes. (Taken from Carolyn et al., 2012)](image)

The presence, level, and number of nodal metastases are powerful prognostic factors of OTSCC. According to Spiro et al. (1986) and Grandi et al. (1985), 2-year survival rate of primary OTSCC patient decreases from over 85% to about 50% when cervical modal metastasis is found, and 5-year survival rate from 65% to 29%. Furthermore,
an extracapsular spread (ECS) means worse prognosis. ECS happens when the cancer cells penetrate the metastasized lymph node and are disseminated to the neighboring connective tissue. ECS represents higher probability of regional recurrence and distant metastasis, which cause most treatment failures of OTSCC. In a retrospective study, OTSCC patients with ECS had lower 5-year overall and disease-specific survival (DSS) rates than those with nodal metastasis but without ECS (Myers et al., 2001).

Distant metastases of OSCC are rather uncommon compared with other cancers (Irani, 2016). The incidence of distant metastasis is associated with the stage and location of the primary tumor and the presence of nodal metastasis (Ferlito et al., 2001). The gingiva is among the most highly metastatic parts in oral cavity along with the tongue and the floor of the mouth (Irani, 2016; Osaki et al., 2000). In a retrospective study on 332 OTSCC patients treated at Washington University School of Medicine, 9.6% of the patients developed distant metastasis, while 21% had second primary cancers (Sessions et al., 2002).

The most common organ of distant metastasis from head and neck region is the lungs, followed by the bone, heart and liver (Irani, 2016). Other less frequently seen sites include the brain and the skin (Probert et al., 1974). The mechanism of this tissue specificity of metastasis remains unclear. Several theories have been proposed to debate on how the metastatic cancer cells are directed to their metastasis sites. In 1889, Paget first proposed the concepts of ‘seed’ and ‘soil’, standing for metastatic cancer cells and metastasis site respectively. According to ‘seed and soil’ theory, the location and histopathological property of the primary tumor is the determining factors of metastasis pattern. Another theory suggests that the C-C chemokine receptors (CCRs) of cancer cells help them to anchor and invade into the defined niches where the corresponding chemokines are expressed (Ben-Baruch, 2009). There are also new theories that emphasize the effects of both primary and metastasis sites, and describe metastasis as a multidirectional process (Comen et al., 2011).
1.1.3. Treatment modalities of OTSCC

There are various treatment modalities of OTSCC: surgery (local resection, elective neck dissection, etc.), radiotherapy, chemotherapy and other composite therapies. The treatment plan for an OTSCC patient depends very much on his or her disease situation: the location, size and extension of the tumor, and the presence of lymph node metastasis.

It’s very important for local resection surgery to diagnose the invasive range of the malignant tumor accurately enough to leave a clear and wide resection margin. Previous studies show patients with clear margins had significantly higher 5-year DSS rate and were less likely to develop local recurrence (Sessions et al., 2002; Al-rajhi et al., 2000).

Radiotherapy is as curative as surgical resection regarding early stage cancer. Meanwhile, post-operative radiotherapy is recommended for late stage patients with close or positive resection margins, more than one nodal metastases and advanced invasion (Kirita & Omura, 2015). For those patients whose bodies cannot take an operation, radiotherapy or composite chemoradiotherapy is the prime treatment.

In the retrospective study done on 332 OTSCC patients treated during 1957 to 1996, researchers found no significant increase of 5-year DSS within a clinical stage by any certain treatment modalities. The 5-year DSS over the whole study was 57.3% (Sessions et al., 2002). Another study in Finland investigated the outcome of treatment modalities of OTSCC from 1995 to 1999 and from 2005 to 2009, and found the 5-year recurrence-free survival rate improved from 47% to 65%, which may be attributed to the use of combination treatment of chemoradiotherapy and surgery (Mroueh et al., 2017).

Despite of remarkable improvement of treatment modalities nowadays, the survival rate of OTSCC is still unsatisfyingly less than 60%, probably because of the late
(stage III and IV) diagnosis of the disease (Rivera, 2014; Bell et al., 2007; Mehrotra & Gupta, 2011).

1.1.4. Shortage of prognostic biomarkers of OTSCC

Unlike in several other malignancies, there are so far no specific OSCC biomarkers in clinical use (Rivera, 2014). Nonetheless, recent researches brought us hope with some promising predictive and prognostic biomarkers. For instance, circulating miRNAs in blood, serum and plasma show potential to predict OSCC recurrence and metastasis (Troiano et al., 2016). Additionally, as a study reported, a loss-of-heterozygosity status of chromosomes 3p and 9p could be predictive for oral premalignant lesions (Zhang et al., 2012). On the other hand, biomarkers found in saliva are of great interest due to its direct contact with OSCC lesions (Radhika et al., 2016). Pro-inflammatory cytokines in saliva such as interleukin-1α (IL-1α), IL-6, IL-8, tumor necrosis factor-α (TNF-α) and VEGF-α were suggested as proangiogenic biomarkers for OTSCC screening (Korostoff et al., 2011). After all, the prognostic value of these potential biomarkers on the progression of OTSCC still need further investigation (Zheng et al., 2010).

1.2. Link between inflammation and OTSCC

1.2.1. Tumor microenvironment

By the end of last century, more and more researchers realized that cancer progression is not only controlled by intrinsic genetic changes of cancer cells, but also by a dynamic dialogue between cancer cells and its neighbors (Weinberg, 2013). Tumor microenvironment (TME), composed of all the non-neoplastic cells present in the solid tumor and the proteins produced by them, in many ways contributes to cancer growth, invasion and metastasis (Casey et al., 2015). These non-neoplastic cells include immune cells, fibroblasts and many other cells (Pottier et al., 2015). As an essential part of TME, immune cells play a dual role as anti-tumorigenesis
guardians and pro-tumorigenesis accomplices, depending on which immune mechanism is triggered.

1.2.2. Cancer-related inflammation

1.2.2.1. ‘Good’ and ‘bad’ inflammation in cancer

Since 1863 when Rudolph Virchow first suggested the potential relationship between cancer and inflammation with the presence of leukocytes in neoplasm, there has been a good amount of evidence to validate his hypothesis.

Cancer has many similar properties to wound healing. Malignant cells are like invading parasites that rely on the host (stroma) to provide them with nutrients, oxygen and room to proliferate. The supply is done through abundantly vascularized connective tissue stroma, which is induced by VEGF, the growth factor both cancer cells and macrophages secrete. The active cellular inflammation components in wound healing, namely neutrophils, macrophages and lymphocytes, are also common in TME (Dvorak, 2015). Together with mediators they release, the recruited inflammatory cells composite an immunological environment, or so-called ‘smoldering’ inflammation that favors cancer cells’ proliferation, metastasis, escaping from immune response, and angiogenesis (Mantovani et al., 2008). In that sense, cancer resembles a persistent infection in wound that continuously fails to heal (Dvorak, 2015).

Different inflammatory cells in TME have different effects on tumorigenesis. Generally they can be divided into two groups: ‘good’ inflammatory cells that kill cancer cells and evoke anti-tumorigenic immune responses, and ‘bad’ ones that harbor both immunosuppression and pro-tumorigenic activities (Shiao et al., 2011).

\textit{CD4^+ T lymphocytes}
Adaptive immune response is the major part of cancer recognition and rejection, involving antigen presenting cells (APCs), T and B lymphocytes, cytokines and major histocompatibility complex (MHC) system. MHC system is the cell surface proteins that help binding peptide antigen of pathogens and present them on cell surface in order to be recognized by T cells (Janeway et al., 2001). MHC are classified to class I (with β2 subunits) and II (without β2 subunits). Most cancer cells are positive for MHC I, which is only targeted by CD8+ T cells to induce direct cancer cell killing (Pardoll & Topalian, 1998). On the other hand, CD4+ T cells specifically recognize MHC class II molecules. That’s why CD8+ T cells have been considered the major component in adaptive immunity against cancer for quite a long time. However, from 1994 and on, researchers have demonstrated more and more tumor antigens recognition by CD4+ T cells, suggesting induction of CD4+ T cells by tumor is also an indispensable arm of antitumor adaptive immunity (Topalian et al., 1994).

CD4+ T cells are central mediators in immune system. They regulate, aid and balance other immune cells’ activities, like antibody production by B cells, cytotoxic activity of CD8+ T cells and functions of macrophages. They also suppress immune response to regulate autoimmunity and control the response shutdown (Zhu et al., 2009). As for cancer immunity, tumor infiltrating CD4+ T cells subsets have complicated effects on the immune response to cancer cells.

Upon recognition of MHC class II molecules presented by APCs, CD4+ T cells are activated and differentiated into subsets controlled by different cytokines induction. These subsets include T helper 1 (Th1), Th2, T regulatory (Treg), Th17, Th22 and follicular T helper (Tfh) (Protti et al., 2014). The correlation between cancer and Th1, Th2 and Treg is the most discovered and characterized.

Induced by IL-12 and IFN-γ, naïve CD4+ T cells differentiate to Th1 cells, and characteristically secrete TNF-α and IFN-γ. Th2 differentiation is induced by IL-4 and thymic stromal lymphopoietin. IL-4, IL-5 and IL-13 are cytokines production of
Th2 cells. Treg cells differentiate by TGF-β, and release TGF-β and IL-10 (Zhu et al., 2009).

Higher concentration of tumor infiltrating Th1 cells and its production of cytokines are strongly related with better prognosis of cancers. In contrast, Th2 and its products including immunosuppressive cytokine IL-10, are associated with tumor progression, with a few exceptions (Fridman et al., 2012). Treg cells infiltration into a tumor suppresses antitumor immune response and also corresponds to poor prognosis in many cancers (Takeuchi & Nishikawa, 2016).

**CD8$^+$ T cells and NK cells**

Naïve CD8$^+$ T cells are activated to cytotoxic T lymphocytes (CTLs) by tumor-associated antigen co-presented with MHC class I molecules by APCs. CTLs target cancer cells by interaction between T cell receptor and antigenic peptide on the surface of cancer cell, and release cytotoxic granules that contain granzymes in order to break down proteins inside cancer cells and induce apoptosis. CTLs also target cancer cells by Fas/Fas ligand (Fasl) interactions, which activate the pathway of caspase cascade, resulting in apoptosis of cancer cells as well (Ruddon, 2007).

NK cells are stimulated by cytokines like IFN-γ, secreted by both T cells and activated NK cells. Cytotoxicity of NK cells doesn't rely on MHC antigen expression (Trinchieri, 1989). NK cells receptors sense activating or inhibitory signals by interaction with cancer cells ligands, thus targeting cancer cells that have reduced ligands to trigger inhibitory signals and increased ligands for activation signals (Pahl & Cerwenka, 2017). Like CD8$^+$ T cells, NK cells kill cancer cells by releasing cytotoxic granules and Fas/Fasl interactions (Smyth et al., 2005).

**Macrophages: M1 or M2?**

Macrophages are the most abundant immune cells infiltrating in TME. Tumor associated macrophages (TAMs) have shown promoting effects on cancer
progression in most of the cases (Qian & Pollard, 2010). The high density of macrophages in tumor is correlating with poor prognosis of a number of cancers (Bingle et al., 2002). Macrophages can be recruited and regulated by tumor cells via secretion of cytokines such as VEGF, platelet-derived growth factor (PDGF), C-C chemokine ligands (CCL)-2, TGF-β and TNF-α (Mantovani & Sica, 2010). Local TAMs support tumor-associated angiogenesis, promote invasion and migration of cancer cells, and suppress immune response against tumor.

Although considerable amount of clinical and experimental evidences show that TAMs are in favor of malignancy, there are exceptions. For example, in a rat model, liver macrophages killed circulating cancer cells and inhibited metastasis (Heuff et al., 1993). Accumulating research results suggest that macrophages play multifaceted roles in the progress of malignancy. As in wound healing, TAMs are dynamically activated and differentiate into M1 or M2 phenotypes according to the signals in TME.

M1 phenotypic TAMs differentiation relies on cytokines like IFN-γ and TNF-α. The product of M1 TAMs include nitric oxide species, and pro-inflammatory mediators such as IL-12, IL-6 and TNF-α. They have tumouricidal properties and elicit tissue destruction (Mantovani & Sica, 2010). Nonetheless, the phenotype of TAMs that prevails in most cancer TME is M2. It is thought that cancer cells switch TAMs phenotypes from M1 to M2 through production of IL-10, colony stimulating factor (CSF)-1, CCL2, etc. (Mantovani & Sica, 2010; Roca et al., 2009). M2 phenotypic TAMs produce VEGF, MMPs, epidermal growth factor (EGF), which promote tumor growth, angiogenesis, invasion and migration (Qian & Pollard, 2010). Additionally, M2 TAMs suppress anti-tumor adaptive immune activities (cytotoxic T cell responses) and recruit Treg cells by CCL22 (Biswas & Mantovani, 2010), keeping an immune ecosystem that favors tumor progression.
1.2.2.2. Chemotaxis effect of cancer cells to attract inflammatory cells

Chemotaxis is the polarized movement of cells directed by the concentration gradient of an extracellular chemokine. In cancer, chemotaxis is involved in both cancer cells dissemination and TME shaping. This process is mediated by various chemokines (or growth factors) and chemokine receptors (or growth factor receptors) (Roussos et al., 2011).

Along with other stromal cells, cancer cells secret chemokines to recruit inflammatory cells which express the corresponding chemokine receptors. For instance, Treg cells expressing CCR4 are attracted by CCL22, a chemokine ligand secreted by ovarian cancer cells and TAMs (Curiel et al., 2004). Besides, in breast cancer, tumor-derived CCL2 and CCL5 attract T cells and other immune cells carrying CCR1 such as monocyte precursor cells, to accumulate in TME and suppress anti-tumor immune response (Soria & Ben-Baruch, 2008). On the other hand, colorectal cancer cells secret C-X-C chemokine ligand 16 (CXCL16) and attract cytotoxic effector cells such as CD8+ and CD4+ T cells with C-X-C chemokine receptor 6 (CXCR6), leading to a good prognosis (Koizumi et al., 2007).

In general, via chemotaxis of certain inflammatory cells, cancer cells establish a TME of particular design, which may have conflicting effects on immune evasion of cancer, depending on type of the infiltrating cells.

1.2.2.3. Inflammatory infiltrations and prognosis of OTSCC

Oral tongue tumors have more intense inflammatory response than tumors of the whole head and neck region (Lundqvist et al., 2012). Previous study showed that human oral cancer infiltrating lymphocytes injected into nude mice with transplanted human tongue squamous cell carcinoma cell lines Tca 8113 strongly inhibited the transplanted tumor growth (Liao et al., 1998). Further research found that dense lymphocytic infiltration correlated with better radiotherapy response and fewer recurrences in OTSCC (Lundqvist et al., 2012). These in vivo experimental
results and clinical facts suggest a positive effect of inflammatory infiltrations on prognosis of OTSCC.

Despite the overall positive impact, a study focusing on specific pro-tumorigenic components of inflammatory infiltrations in TME of OTSCC revealed that denser infiltrations of Treg cells (Foxp3⁺), M2 phenotypic TAMs (CD163⁺) and Treg-inducing immune cells (CD80⁺) were associated with higher locoregional recurrence. The recurrence hazard ratio of OTSCC with a high density of CD163⁺Foxp3⁺CD80⁺ infiltrations versus low density was 2.9 (Dayan et al., 2012).

The presence of TAMs plays a role in promotion of OSCC cells invasion and angiogenesis (Li et al., 2002). It is a predictor of poor prognosis of oral cavity cancers (Marcus et al., 2004). A significant correlation between TME rich in CD163⁺ TAMs and poor outcome of OSCC has been seen in an investigation including 108 OSCC patients (Fujii et al., 2012). More specifically, in vitro experiments with horizontal and 3D models showed M2 TAMs induced migration and invasion of OTSCC cells (HSC-3 cell line) and affected cytokine production of cancer cells in favor of tumor progression. But in the same experiments, M1 TAMs had a negative effect on OTSCC cells invasion (Pirilä et al., 2015).

The research results mentioned above suggest the necessity of looking at specific subsets of inflammatory infiltrations in OTSCC when investigating their impact on cancer progression.

1.2.3. Cancer cell extracellular vesicles

Extracellular vesicles (EVs) can be released to extracellular space by many cell types, with size from 40 to 200 nm. They originate from multivesicular endosomes that fuse with the plasma membrane (Raposo & Stoorvogel, 2013). EVs can contain proteins, lipids, RNA and DNA. They are found in diverse body fluids, including blood, urine, saliva, breast milk, and also conditioned cell culture media (van der Pol et al., 2012). Their function was earlier thought to be removal of excess cellular
constituents and recycling cell surface proteins in case to regulate outside-in signaling (Pan et al., 1985). What’s more, EVs deliver their contents to distant recipient cells by fusion with them, likely to mediate the recipient cells phenotypes, which indicates the role of EVs as mediators in cell-to-cell communication without direct interactions (De Toro et al., 2015).

Interestingly, blood of cancer patients contains approximately 2-fold more EVs than normal, and cancerous cells secret even more EVs, which is suspected to be due to a change of cellular physiology (Kalluri, 2016). Lots of *in vitro* and *in vivo* studies demonstrated the potential modulating effects of cancer-derived EVs on tumorogenesis, angiogenesis, fibroblasts activation, immune suppression, and even metastasis pre-niche formation (Beckler et al., 2013; Fernandes Ribeiro et al., 2013; Webber et al., 2015; Clayton et al., 2007; Costa-Silva et al., 2015).

1.2.3.1. Suppression of immune surveillance by cancer-derived EVs

Cancer-derived EVs can impair the maturation of APCs and inhibit anti-tumor immune responses. EVs of mammary cancel cells are able to induce myeloid precursor cells in the bone marrow to produce IL-6, which promotes blocking their differentiation into dendritic cells *in vitro* (Yu et al., 2007). EVs derived from human melanoma also impair differentiation of CD14+ monocytes into dendritic cells and even promote generation of a myeloid immunosuppressive cell subset (Valenti et al., 2006).

In mice model, cancer EVs decrease cytotoxicity and proliferation of both T cells and NK cells, benefiting tumor progression (Zhang & Grizzle, 2011). Many cancers have EVs that trigger the generation of extracellular adenosine, which suppresses T cell-mediated immune regulation (Clayton et al., 2011). *In vitro* experiment of EVs from diverse cancer cell lines showed that they downregulated expression of NKG2D, an activating receptor for cytotoxic inflammatory cells like NK cells and CD8+ T cells (Clayton et al., 2008). Cancer EVs carrying FasL can exert Fas-dependent apoptosis of Fas+ T cells (Andreola et al., 2002). Besides, cancer EVs promote Treg cells
expansion, and enhance their immune suppressing function and resistance against apoptosis, which are mediated by TGF-β1 and IL-10 (Clayton et al., 2007; Szajnik et al., 2010).

Macrophages polarization can also be affected by cancer EVs. After exposure to EVs derived from glioblastoma multiforme, monocytes were expressing significantly higher CD163, the M2 phenotypic marker (de Vrij et al., 2015).

1.2.3.2. Stimulation of immune response by cancer-derived EVs

As EVs contain proteins, nucleic acids and membrane-derived lipids from parental cells, they somewhat carry characteristic information of these cells. In fact, cancer EVs can bear cancer associated antigens with them, thus being potentially capable to be used as an anti-tumor vaccine (Kunigelis & Graner, 2015). For example, an in vitro model showed that EVs derived from many human cancer cell lines transferred cancer associated antigens to dendritic cells. In the same study, upon the taking of EVs antigens, mouse dendritic cells induced potent CD8+ T cell-dependent anti-tumor immune response (Wolfers et al., 2001). Heat shock protein 70-positive cancer EVs can also stimulate the migration and cytolytic activity of NK cells (Gastpar et al., 2005).

Although preclinical experiments done in animal models show upregulated anti-tumor immunogenicity by cancer EVs, the reported immune activities of cancer EVs indicate their overwhelming effect of immunosuppression in clinical trial and other experiments. The dichotomy of cancer EVs effects on cancer immunity remains to be investigated. More attention is supposed to be paid to the context where EVs are located and antigens are presented, which might explain the functional heterogeneity of cancer EVs (Kunigelis & Graner, 2015).
2. Aims of the study

This study aims to investigate the interaction between OTSCC cells and inflammatory cells and answer 3 questions:

1. How inflammatory cells affect OTSCC cells’ activities such as proliferation, migration and invasion?

2. How EVs of OTSCC cells affect macrophages polarization?

3. How EVs of OTSCC cells affect cytotoxic activity of CD8+ T cells and NK cells?
3. Materials and methods

3.1. Peripheral blood mononuclear cells isolation

Human buffy coat of three healthy donors was provided by Finnish Red Cross. We used Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA), a density gradient medium to isolate human peripheral blood mononuclear cells (MNCs).

To isolate cytotoxic CD8+ T cells, CD56+ NK cells and CD14+ monocytes from the MNCs, we used a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) with a negative selection. MNCs were resuspended in a buffer of PBS, BSA and EDTA to get a concentration of 10^7 cells in 40 µl. We added 10 µL of biotin-antibody cocktail and incubated the cells for 5 minutes at 4°C. After that, 30 µl of the buffer was added followed by 20 µl of the cell microbeads cocktail. The mixture was incubated for 10 minutes at 4°C and then it was loaded in the LS Column in the magnetic field. Unlabeled cells which passed through the column were collected.

A panel of antibodies (BD Biosciences, San Jose, CA, USA) were used to further check the purity of the isolated cells. The panel included anti-CD3-APC (T cells common antigen), anti-CD8-PerCP-Cy5.5 (CD8+ T cells), anti-CD14-FITC (monocytes) and anti-CD56-PE (NK cells). The different cell populations were stained with antibodies of the panel, and acquired by FACS-Verse, and then analyzed by BD FACSuite™ software.

The percentage of CD3+ CD8+ T cells, CD3- CD14+ monocytes, and CD3- CD56+ NK cells in the isolated population were 96%, 92% and 92% respectively (Fig.4).

3.2. Human OTSCC cell culture

Two OTSCC cell lines were used in this study. HSC-3 cells (Japan Health Sciences Foundation, Japan) are highly invasive cells while SCC-25 cells (American Type
Culture Collection, Manassas, VA, USA) are poorly invasive. The difference in the invasive properties of these cell lines was reported by Ramos et al. (1997).

Fig. 4 Inflammatory cells purity test with FACS. Isolated inflammatory cells were stained with anti-CD3-APC, anti-CD8-PerCP-Cy5.5, anti-CD14-FITC and anti-CD56-PE; and acquired
by FACS-Verse, and then analyzed by BD FACSuite™ software. The diagrams show the percentage of CD3⁺CD8⁺ T cells (A), CD3⁻CD14⁺ monocytes (B) and CD3⁻CD56⁺ NK cells (C). The 3 diagrams on the left show percentage of the positive cells after gating.

The medium used to culture the cell lines was Dulbecco's modified Eagle's medium (DMEM)-12 (Gibco, Paisley, UK) along with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml fungizone, and 50 μg/ml ascorbic acid. The cells were cultured in 75 cm² flasks at 37°C in 5% CO₂ incubator.

3.3. Co-culture of human OTSCC cells and human peripheral blood MNCs in an organotypic 3D myoma model

We developed a 3D in vitro organotypic model using human uterine leiomyoma discs to study the effects of human peripheral blood MNCs on proliferation and invasion of OTSCC cells, and the chemotaxis ability of cancer cells to attract MNCs. The myomas were taken from the by-product of routine surgical operations after getting the informed consent from the donors at the Oulu University Hospital, Department of Gynecology. The use of the myoma tissue was approved by the Ethics Committee of the Oulu University Hospital.

In a previous study, the myoma in vitro invasion model has been well established (Nurmenniemi et al., 2009). With some adjustment, we followed the described instructions to prepare the myomas and conduct the invasion experiments.

The uterine leiomyoma tissue was cut into macroscopically homogeneous discs which were 2 mm-thick and 8 mm in diameter. The discs were placed into the bottom of Transwell® inserts (Corning Inc., Corning, NY, USA). 5x10⁵ HSC-3 or SCC-25 cells were added on top of each myoma disc (Day 0). After overnight incubation (37°C, 5% CO₂) to let the cells attach to myoma, the discs were removed from the Transwell® inserts and transferred onto steel grids placed in 12-well plate (Day 1).
Each well was beforehand filled with 1.2 ml culture medium to reach the undersurface of myoma discs and the medium was replaced every three days.

On Day 4, 2x10^6 MNCs were added in the medium beneath the myoma discs either alone (Group 2, non-activated) or with different activators. To stimulate T cells, MNCs were added in medium with 2 µg/ml CD28 antibody (BD Bioscience) in wells coated with 5 µg/ml CD3 antibody (BD Bioscience, Group 3). To activate all MNCs, the medium beneath contained 5 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, Mo, USA) and 1 µM ionomycin (BD Bioscience, Group 4). For control without MNCs, we had medium alone (Group 1) and medium with 5 ng/ml PMA and 1 µM ionomycin (Group 5) beneath the myoma discs. The co-culture lasted until Day 10.

The myoma model set of the invasion experiment is demonstrated in Fig.5.

**Fig.5** Organotypic 3D myoma co-culture model of OTSCC cells and human peripheral blood MNCs. 5x10^5 HSC-3 or SCC-25 cells were added on top of each myoma disc. After 24-hour adhesion, the myoma discs were transferred onto metal grids placed in wells of 12-well plates. The wells were beforehand supplied with 1.2 ml medium. On the 4th day of the whole 10-day experiment, 2x10^6 MNCs were added beneath the discs with or without activators (CD3 and CD28 antibodies, or PMA and ionomycin).

3.4. Immunohistochemical (IHC) staining of myoma

After the co-culture, myoma discs were fixed in 10% formalin and embedded in paraffin blocks. The discs were then vertically cut into 6 µm-thick sections using a microtome. The slides were stained with a horseradish peroxidase–labeled dextran
polymer method using Leica BOND-MAX staining robot (Leica Microsystems, Wetzlar, Germany). The primary antibodies used here were monoclonal mouse anti-human pan-cytokeratin (0.7 µg/ml), monoclonal mouse anti-human Ki67 (0.8 µg/ml), monoclonal mouse anti-human CD45 (0.94 µg/ml), and monoclonal mouse anti-human CD8 (3 µg/ml; all from DAKO, Glostrup, Denmark).

3.5. Scratch wound OTSCC cell migration assay

Wells of a 96-well Imagelock plate (Essen Bioscience, Ann Arbor, MI, USA) were coated with 300 µg/ml Myogel, a cell culturing matrix formulated from human myoma tissue in order to substitute Matrigel® derived from mouse (Salo et al., 2015). After overnight incubation (37°C, 5% CO₂), excess myogel was removed and 3x10⁴ HSC-3 or SCC-25 cells were seeded to each well. The cells were left to adhere overnight.

Identical scratch wounds of 700-micron width were made in all wells using a WoundMaker™ (Essen Bioscience). Wells were washed with PBS and the medium was replaced by 100 µl of fresh medium with 8x10⁴ MNCs either alone or with activators (as in the invasion experiment). For control without MNCs, we replaced the medium with fresh medium only, and medium with 5 ng/ml PMA and 1 µM ionomycin.

The wound healing process in each well was monitored by an IncuCyte Live-Cell Imaging System (Essen Bioscience), and pictures were taken every an hour to measure the wound area over time.

3.6. EVs isolation and characterization

We seeded 1.3x10⁶ HSC-3 or SCC-25 cells per 75 cm² flask and left them to adhere overnight. We discarded the medium and washed the flasks with PBS, followed by adding serum-free DMEM-12 medium. After another 48-hour culturing, we
centrifuged the medium at 1000 rpm for 5 min, and collected the supernatant for EVs isolation.

We conducted a two-step ultracentrifugation (Beckman Coulter, Pasadena, CA, USA) to isolate EVs out of the medium. In the first step, the medium was centrifuged at 10 000 g for 1.5 hours at 4°C. Then the supernatant was collected and further centrifuged at 100 000 g for 1.5 hours at 4°C. After the second ultracentrifugation, we discarded the supernatant, re-suspended the EV-containing pellet with 200µl of PBS and kept it at -80°C for further use.

After EV isolation, the number and size of the particles were measured using nanoparticle tracking analysis (NTA). A Nanosight model LM14 (Nanosight) equipped with a blue laser (404 nm, 70 mW) and a SCMOS camera was used. We diluted the samples in DPBS and recorded three 90-second videos by camera level 14. The acquired data was analyzed by NTA software 3.0 with the optimized detection threshold and screen gain at 10.

To check the samples with immune-electron microscopy, we applied EV suspensions onto 200-mesh grids and fixed with 2% PFA. After blocking with 0.5% BSA in 0.1 M NaPO₄ buffer (pH 7.0) for 10 min, samples were stained with monoclonal mouse anti-human CD63 primary antibody (Pelicluster, Sanquin, Amsterdam, The Netherlands), and then anti-mouse secondary antibodies conjugated to colloidal gold particles (10 nm) in 0.1% BSA in 0.1 M NaPO₄ buffer (pH 7.0) for 30-60 min at room temperature. After extensive washing with 2% neutral uranyl acetate, the samples were embedded in methyl cellulose uranyl acetate mixture (1.8/0.4%). The samples were examine with a Tecnai 12 (FEI Company, Eindhoven, The Netherland) operated at 80 kV, equipped with Gatan Orius SC 1000B CCD-camera (Gatan Inc. USA) using 4008 x 2672 px image size and no binning.

According to the characterization outcome, most analyzed EVs were between 50-100 nm in diameter and CD63⁺ (Fig.6).
3.7. Monocytes differentiation and macrophage polarization

As mentioned, human peripheral blood primary monocytes (CD14+) were isolated and tested to be 92% purified. The monocytes were seeded in 24-well plates (2x10^5 cells per well) and the medium was advance RPMI-1640 (Gibco) supplied with 10% FBS, 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone. Monocytes were left to adhere for 1.5 hours and then unattached cells were washed away with PBS and fresh medium plus 100 ng/ml macrophage colony stimulating factor (M-CSF; R&D systems, Minneapolis, MN, USA) was added. The treatment lasted for 7 days to let the monocytes differentiate to macrophages, and images of the cells were taken daily with a Nikon DS-Fi2 camera (Nikon, Tokyo, Japan).

Fig.6 Characterization of the OTSCC cells EVs with NTA and electron microscope. Isolated EVs samples were analyzed by nanoparticle tracking analysis (NTA). To visualize their immune characteristic, the EVs were stained with CD63 and observed with electron microscope. Most analyzed EVs were between 50-100 nm in diameter for both HSC-3 (A) and SCC-25 (B), and CD63+ (pointed by arrow) for HSC-3 (C) and SCC-25 (D).

To study the effects of OTSCC cells derived EVs on macrophages polarization, the differentiated macrophages were then treated with EVs isolated from HSC-3 or SCC-
25 cells for 24 hours. For positive control, we used 10 ng/ml Lipopolysaccharides (LPS, Sigma-Aldrich) and 20 ng/ml IFN-γ (Prospec, Rehovot, Israel) to differentiate macrophages to M1 phenotype, and 20 ng/ml IL-4 and 20 ng/ml IL-13 (Prospec) to M2 phenotype. Primary macrophages without treatment were set as M0 control.

3.8. Quantitative real-time-PCR

The total RNA from macrophages was isolated using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). 200 ng of RNA was used for cDNA synthesis with an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For each PCR tube, 2 µl of cDNA sample along with 10 µl iQ SYBR green, 7 µl water and 1 µl of 250 nM primer solution were added. GAPDH and RPLP0 were used as housekeeping genes. Primer sequences are shown in Table 3:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL9</td>
<td>5'-ACCCAGATTCAGCAGATGTGAAGGA-3'</td>
<td>5'-GCCATCCTCCTTTGGGAATGATAGCG-3'</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5'-GCAAGCCAATTTTGCCACGTGTG-3'</td>
<td>5'-CAGCCTCTGTGTTGTCATCCTT-3'</td>
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<tr>
<td>CCL17</td>
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<td>5'-CCCTTGAAAGTACCTCAGCAGG-3'</td>
</tr>
<tr>
<td>CCL18</td>
<td>5'-TGCCGACATCCACAAAGTTCA-3'</td>
<td>5'-GGATGACACCTGGCTTG-3'</td>
</tr>
<tr>
<td>CCL22</td>
<td>5'-GTGGGCCTCTTACAGAATCTAG-3'</td>
<td>5'-GCCCAGACCGTAACCGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>RPLP0</td>
<td>5'-GGGGACCTGGAAGTCAACT-3'</td>
<td>5'-CCATCAGCACCACAGCCTTC-3'</td>
</tr>
</tbody>
</table>

Table 3. Sequences of the primers used in this study.

3.9. IncuCyte™ immune cell killing assay

The cytotoxic activity of CD8+ T cells and NK cells against OTSCC cells were measured using an IncuCyte™ immune cell killing assay.

First, we labeled HSC-3 and SCC-25 cells with fluorescent dye using a CellTraceTM Far Red Cell Proliferation Kit (Invitrogen, Carlsbad, CA, USA). The OSTCC cells were seeded in 96-well plates (5000 cells per well) and left to adhere overnight. CD8+ T
cells and NK cells were cultured in either medium alone (control) or medium supplied with EVs derived from HSC-3 or SCC-25 for 24 hours. The cytotoxicity activators were 100 ng/ml CD3 antibody and 10 ng/ml IL-2 (eBioscience, San Diego, CA, USA) for CD8+ T cells, and 10 ng/ml IL-2 for NK cells.

After the 24-hour culturing, 5x10^4 CD8+ T cells or NK cells were added to the OTSCC cells in the wells supplied with 2.5µM IncuCyte™ Caspase-3/7 Apoptosis Assay Reagent (Essen Bioscience). For controls, only corresponding cytotoxicity activators were added to cancer cells. Then the cytotoxic activities were monitored using IncuCyte Live-Cell Imaging System for 5 days and pictures were taken every two hours to measure the apoptosis of OTSCC cells induced by CD8+ T cells and NK cells.

3.10. Microscopic and histomorphometric analysis

Stained myoma slides were observed and photographed using Leica DM6000 B/M light microscope connected to a digital camera (DFC420; Leica Microsystems).

Invasion area and depth of OTSCC cells in the myomas were measured using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). At 10x magnification, the surface area of immunostained (pan-cytokeratin) invading cells into the myomas were measured. The maximal invasion depth (distance from the deepest invading cell to the lower surface of the non-invading cell layer) was also measured.

Percentage of Ki67+ OTSCC cells were calculated at 40x magnification from at least three randomly selected fields of the non-invading cell layer per slide. Numbers of CD45+ and CD8+ cells were counted at 10x or 20x magnification from at least three fields showing the most intensive infiltration per slide.
3.11. Statistical analysis

All experiments were done in duplicate or triplicate and repeated for 3 to 8 independently times. Values are provided as means ± standard deviation. One Way-Anova followed by Tukey's post hoc test was used to check statistical significance. P values less than 0.05 were regarded as statistically significant. Pearson correlation test was used to examine the correlations between the OTSCC cells' proliferation and the invasion area/depth.
4. Results

4.1. Human peripheral blood MNCs’ effects on proliferation, invasion area and depth of OTSCC cells in 3D organotypic myoma discs

After co-culturing OTSCC cells and human peripheral blood MNCs in the myoma invasion model, the myomas were cut into 6 µm-thick sections and immunohistochemically stained with pan-cytokeratin and Ki67. Staining of pan-cytokeratin displayed distribution of HSC-3 and SCC-25 cells in the myoma discs. As described in previous study, HSC-3 showed more aggressively invasion into the myoma tissue than SCC-25 (Fig.7A, B). The percentage of Ki67 positive (proliferative) cancer cells in the total non-invading cancer cells (cells on the surface layer, not inside myoma) for HSC-3 and SCC-25 cell lines was close to each other (Fig.7D, E). For controls, myoma discs without cancer cells were negative for both pan-cytokeratin and Ki67 (Fig.7C, F).

The proliferation of both OTSCC cell lines was decreased after adding activated MNCs (HSC-3, p=0.04; SCC-25, p=0.03). Interestingly, activated CD3+ T cells only lowered the proliferation percentage of SCC-25 (p=0.002, Fig.8A).

Activated MNCs reduced invasion area of HSC-3 cells (p=0.04), but showed no significant effects on invasion area of SCC-25, which had an overall much lower level of invasion area compared with HSC-3 (Fig.8B). Invasion depth of both OTSCC cell lines were not affected by adding inflammatory cells (Fig.8C).

For both OTSCC cell lines, proliferation showed positive correlation with the invasion area (HSC-3, r=0.5, p=0.002; SCC-25, r=0.5, p=0.02; Fig.8D), but no correlation with the invasion depth (HSC-3, r=0.2, p=0.1; SCC-25, r=0.1, p=0.6; Fig.8E).
**Fig. 7 Immunohistochemical staining with pan-cytokeratin and Ki67 of myoma sections.** HSC-3 cells showed more aggressively invasion into the myoma tissue than SCC-25 (A, B). The percentage of Ki67+ cells was similar for both cell lines (D, E). Myoma discs without cancer cells were negative for both pan-cytokeratin and Ki67 (C, F). Scale bar=100 µm.

4.2. Migration of peripheral blood MNCs towards OTSCC cells in myoma discs

The myoma slides were immunohistochemically stained with CD45 (leukocyte common antigen) and CD8 (cytotoxic T cells marker) antibodies. Since there were some inflammatory cells already present in myoma tissue, we set groups without co-cultured MNCs beneath as controls. The myomas used in this experiments were all derived from the same batch and macroscopically homogeneous.

From the stained slides we observed that when activated MNCs were added, CD45+ cells were more abundant in myoma (Fig.9A-D). However, this difference was not statistically significant (Fig.9I, J).
Fig. 8 Effects of activated MNCs on proliferation, invasion area and depth of OTSCC cells. The percentage of Ki67+ cells in the total non-invading cancer cells was counted from at least three randomly selected fields (A). Invasion area and invasion depth were measured using ImageJ software (B, C). Correlations between proliferation and invasion area and invasion depth were examined using Pearson correlation test (D, E), Statistical significance was checked using One Way Anova followed by Tukey’s post hoc test. *p ≤ 0.05, **p ≤ 0.01. All experiments were done in duplicate and repeated for 4 to 8 times independently.

CD8+ T cells density in myoma was also raised by adding activated MNCs, and they were mostly found at the bottom of the myoma and close to blood vessels (Fig. 9E-H). The increase was statistically significant only for HSC-3 cells (p=0.02) which were more invasive, but not for SCC-25 (p>0.05, Fig. 9K, L).

By adding inactivated MNCs, we didn’t see migration of the peripheral blood MNCs towards OTSCC cells in myoma (Fig. 9E-H).

4.3. Migration of OTSCC cells on top of Myogel in scratch wound healing assay

We co-cultured OTSCC cells and peripheral blood MNCs on top of Myogels in 96-well plate to study the effects of peripheral blood MNCs on migration of cancer cells. The wound closing process over time was recorded (Supplement 1).

The migration of HSS-3 or SCC-25 wasn’t affected by the presence of peripheral blood MNCs. MNCs activated by PMA and ionomycin impeded the wound closing process compared with control and other co-culture groups, nevertheless, the activators themselves (PMA and ionomycin) also inhibited the migration (Fig. 10).
**Fig. 9 Migration of peripheral blood MNCs towards OTSCC cells in myoma discs.** Myoma slides were stained with CD45 (A-D) and CD8 (E-H). The photos shown here are from experiment using HSC-3 cells. Numbers of CD45+ (I, J) and CD8+ cells (K, L) were counted at 10x or 20x magnification from at least three fields showing the most intensive infiltration per slide. Statistical significance was checked using One Way-Anova followed by Tukey’s post hoc test. *p ≤ 0.05. Scale bar=100µm.

**4.4. Human primary macrophage polarization in the presence of OTSCC cells EVs**

Human primary monocytes were isolated and treated with M-CSF for 7 days for differentiation into macrophages (Fig.11A). Then the macrophages were treated with EVs isolated from HSC-3 or SCC-25 cells for 24 hours. For positive control, we used LPS and IFN-γ to polarize macrophages to M1 phenotype, and IL-4 and IL-13 to M2 phenotype. Primary macrophages without treatment were set as M0 control.
Fig.10 Peripheral blood MNCs showed no effects on migration of OTSCC cells. Scratch wound OTSCC cell migration assay on top of Myogel in 96-well plate was done. Pictures taken over time of wound closing process of HSC-3 cells alone (A), and HSC-3 cells with cocultured MNCs, PMA and ionomycin (B). Wound closing percentage over time of HSC-3 (C) and SCC-25 (D) with or without co-cultured MNCs.
Fig. 11 OTSCC cells EVs showed no effect on human primary macrophages polarization. Human peripheral blood primary monocytes were isolated and differentiated to macrophage for 7 days using M-CSF (A). The macrophages were cultured with medium alone (M0), medium with LPS and IFN-γ (M1), IL-4 and IL-13 (M2), EVs from HSC-3 or SCC-25 cells (B). Expression of M1 markers (CXCL9 and CXCL10) and M2 markers (CCL17, CCL18 and CCL22) was evaluated using q-PCR (C). Scale bar=200 μm.
Morphologically, M1 macrophages were spherical and M2 macrophages were more cone-shaped. Upon the treatment of HSC-3 and SCC-25 EVs, most of the macrophages looked like spindle-shaped M0. While some of them had M2 representative cone-shaped morphology (Fig.11B).

Quantitative real time-PCR of M1 and M2 macrophage markers was done to evaluate the polarization effects of EVs on macrophages. We included CXCL10 and CXCL9 as M1 markers, and CCL17, CCL18 and CCL22 as M2 markers. The outcome of q-PCR showed no significant difference of the expression of any phenotypic markers between M0 control and macrophages treated with EVs from any cell lines (Fig.11C).

4.5. Modulation of cytotoxic activity of CD8+ T cells and NK cells by OTSCC cell EVs

Human primary CD8+ T cells and NK cells were isolated from blood of three healthy donors and treated with EVs of HSC-3 or SCC-25 cells for 24 hours. Then the CD8+ T cells and NK cells were co-cultured with HSC-3 and SCC-25 cells to check their cytotoxic activity. Apoptosis of OTSCC cells induced by CD8+ T cells and NK cells was measured over time (Supplement 2).

Overall the cytotoxic activity of CD8+ T cells and NK cells were upregulated by treatment of OTSCC cells EVs (Fig.12). However, this upregulation was not seen in every circumstances. It varied from donor to donor, between different sources of EVs, different cytotoxic cells and different OTSCC cell lines they acted on.

In the case of donor 1, cytotoxic activity of CD8+ T cells against HSC-3 cells was upregulated by treatment of SCC-25 EVs, but the treatment had no effect on the killing activity against their source cancer cells, SCC-25 (Fig.12A, D). For donor 2, HSC-3 EVs were promoting the killing against only HSC-3 cells (Fig.12B, E). Meanwhile, for donor 3, EVs from both HSC-3 and SCC-25 cells were increasing the cytotoxic activity of CD8+ T cells against both OTSCC cell lines (Fig.12C, F).
Fig. 12 Modulation of OTSCC cells EVs on cytotoxic activity of CD8+ T cells and NK cells. Cytotoxic CD8+ T cells and CD56+ NK cells were and incubated with medium alone, or medium with EVs from HSC-3 and SCC-25 cells for 24 hours. Then the cytotoxic cells were co-cultured with HSC-3 or SCC-25 cells for 5 days and the number of apoptotic cancer cells were counted over time using IncuCyte Live-Cell Imaging System.

As for NK cells, after treatment with SCC-25 EVs, cytotoxic activity of NK cells to kill both HSC-3 and SCC-25 was enhanced for donor 2 and 3 but not donor 1 (Fig. 12H, I, K and L). HSC-3 EVs was only positively effective for NK cells killing ability against HSC-3 cells in donor 3 (Fig. 12I).
On the other hand, cytotoxic activity of CD8+ T cells against SCC-25 was even reduced by treatment of HSC-3 EVs for donor 1 (Fig.12D). SCC-25 EVs also reduced NK cells killing ability against both cell lines for donor 1 (Fig.12G, J).

<table>
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<th>Donor 1</th>
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</tbody>
</table>

Table 4. Modulatory effects of OTSCC cells EVs on cytotoxic activity of CD8+ T cells and NK cells against OTSCC cell lines. ‘SCC-25(+)/(−)’: SCC-25 EVs increased/ decreased cytotoxic activity; ‘HSC-3(+)/(−)’: HSC-3 EVs increased/decreased cytotoxic activity; ‘/’: EVs of neither cell lines had modulatory effect on cytotoxic activity.

The effects of EVs on the cytotoxic activity of CD8+ T cells and NK cells against OTSCC cell lines are briefly displayed in Table 4. To summarize up, EVs derived from more aggressive HSC-3 cells stimulated the increase of cytotoxic activity of inflammatory cells in 4 out of 12 circumstances (Fig.12B, C, F and I), while EVs from less aggressive SCC-25 cells showed this effect in 7 out of 12 (Fig. 12A, C, F, H, I, K and L).
5. Discussion

This study mainly investigated the crosstalk between OTSCC cells and inflammatory cells via extracellular cytokine/chemokine secretion, EVs production, or direct cell-cell contact. Firstly, with a 3D organotypic myoma model, we co-cultured OTSCC cells and MNCs and looked into the ability of the latter to affect the proliferation and invasion of the former, as well as the chemotaxis effect by the former attracting the latter into myoma tissue. In the second part of the study, we studied how EVs derived from OTSCC cells modulated the phenotypes and cytotoxic activity of selected inflammatory cells. The findings and significance of this study are discussed as following.

5.1. Effects of inflammatory cells on proliferation, invasion and migration of OTSCC cells

5.1.1. In vitro myoma model

We used an *in vitro* model made of human uterine leiomyoma which mimics the TME of OTSCC. Myoma is a more authentic alternative to traditionally used model composed of type I collagen and fibroblasts (Nurmenniemi et al., 2009; Nyström et al., 2005).

Compared with collagen model, myoma contains more various cell types and ECM components. Besides, in myoma, carcinoma cells behave more closely to how they do *in vivo*, and molecular activities that happen in TME such as MMP production and collagen degradation are enhanced (Nurmenniemi et al., 2009).

As discussed in literature review part, inflammatory cells present in TME and play a complicated and subtle role which is still of great debate. They can recognize certain antigen of cancer cells and induce cell death, or produce anti-tumor mediators (Mantovani et al., 2010; Ruddon, 2007). On the other hand, cancer cell recruit some
inflammatory cells to downregulate the immune surveillance and keep a TME favoring tumor progression (Qian & Pollard, 2010).

To study the mutual effects of cancer cells and inflammatory cells *in vitro*, it is important to co-culture them in an environment where they act as *in vivo* to a maximal extent, both physically and physiologically. As the fact that cancer cells of solid tumor are adherent while most immune cells are non-adherent, a multiphasic co-culture model is needed to provide a solid matrix that resembles TME as well as a medium for non-adherent immune cells. The myoma co-culture model used in this study meets the requirements.

5.1.2. Effects of activated MNCs on proliferation, invasion and migration of OTSCC cells

In the co-culture experiment, activated human peripheral MNCs significantly reduced proliferation of both HSC-3 and SCC-25 cells. This reduction was likely to be caused by certain extracellular signals such as anti-tumor mediators produced by MNCs and permeating into myoma tissue. Since CD8+ cells were only found at the bottom of the myoma discs while CD45+ cells distributed in all areas of the myoma, it seemed hard for CD8+ cells to kill cancer cells by releasing granules or Fas/Fasl interaction, thus other MNCs might turn to be predominant role to exert reduction effect on cancer cells proliferation in this case.

We saw the non-activated MNCs, which spontaneously produce little cytokines (Baran et al., 2001), had no significant effects on proliferation or any invasion parameters of OTSCC cells. The distinct performance of non-activated and activated MNCs indicates that the activation is the key and premise of MNCs function. Besides, activated T cells only partially showed inhibitory effects on proliferation of SCC-25 cells, which implies the considerable importance of other components of MNCs such as B cells, NK cells and monocytes in cancer immunity.
As for invasion and migration sections, the only significant effect witnessed in the experiment was the reduction of HSC-3 cells invasion area by activated MNCs. MNCs appeared to have little influence on invasion depth or migration of OTSCC cells. In fact, it was somehow obvious that the migration of OTSCC in the wound closing assay was impeded by PMA and ionomycin, the activators for MNCs, which could induce apoptosis of the migrating cancer cells (Han et al., 2013).

After all, as described earlier, cancer cells are adherent while immune cells are mostly non-adherent. This difference of adherence might cause insufficient interaction between two types of cells in our wound healing assays. The harmful MNCs activators (PMA and ionomycin) were also obstacle to acquire persuasive results.

In general, activated MNCs inhibited proliferation of both OTSCC cell lines and invasion area of only HSC-3 cells, but showed no influence on invasion depth or migration of OTSCC cells in this study. This may reveal the fact that these inflammatory cells are important in controlling tumor growth, yet they have little restriction effect of invasion and metastasis (Fukano et al., 1997).

5.1.3. Correlation between OTSCC cell proliferation and invasion

For both HSC-3 and SCC-25 cells, proliferation was positively correlated with invasion area, but not correlated with invasion depth, suggesting thriving cancer cell reproduction doesn’t necessarily mean aggressive invasion and metastasis.

It will be easier to understand this conclusion in consideration of different clinical growth types (superficial, exophytic and endophytic) of OTSCC. It’s also in line with the finding by Hideo Fukano et al. that invasion mode and depth, other than tumor size, were two significant predictors of OTSCC regional metastasis (1997).
5.2. OTSCC cell chemotaxis attraction of inflammatory cells to infiltrate into myoma tissue

Cancer cells are a chemokine source which recruit various populations of immune cells to infiltrate via chemokines secretion, manipulating a particular immune TME (Koizumi et al., 2007). In our co-culture model, the chemotaxis of OTSCC cells to attract MNCs to infiltrate into myoma tissue in different conditions was studied.

In the IHC stained myoma section, more CD45* cells were found in myoma only when activated MNCs were added, at least from microscopic observation. However, this increase was not statistically significant for both OTSCC cell lines, perhaps due to the variation of the number of CD45* cells between samples. On the other hand, for HSC-3 experiment, significantly more CD8* T cells were infiltrating into myoma only when activated MNCs were added.

These findings suggest the OTSCC cells via chemokines recruit MNCs selectively. Considering the facts that dense lymphocytic infiltration correlated with better prognosis of OTSCC (Lundqvist et al., 2012), and CD8* T cells infiltration was also predictive for good prognosis for colorectal cancer (Naito et al., 1998), the activated MNCs added here may played an anti-tumor role of ‘good inflammation’ from this perspective.

Besides, chemotaxis ability varies between OTSCC cell lines. Although for more aggressive HSC-3 we witnessed significant difference while not in SCC-25, whether aggressiveness and chemotaxis tension are correlated is unknown.

The majority of CD8* T cells were located at the bottom of myomas. They appeared to be unable to migrate towards OTSCC cells, perhaps because of a relatively more powerful chemotaxis attraction from the MNCs in the bottom of this co-culture model (Roussos et al., 2011). Meanwhile, the CD8* T cells were mostly found close to blood vessels, suggesting they infiltrated into myoma through the vessels. This may provide another explanation of the accumulation of CD8* T cells at the bottom,
which is the loss of inflammatory vascular responses in myoma such as vascular permeability increase and oncotic pressure change.

There have been several methods and models to study different cell migrations in cancer, such as \textit{in vitro} 2D chemotaxis assays, Transwell assay, 3D culture and other \textit{in vivo} methods (Roussos et al., 2011). The results of this thesis also prove the practicability of myoma model especially to mimic the environment \textit{in vivo} to study immune cells recruitment. The advantages of myoma models include better simulation of TME as well as organotypic structure, and convenience of imaging.

5.3. Effects of OTSCC cell-derived EVs on different populations of inflammatory cells

It has been widely evidenced that cancer cells can secret extracellular vesicles to trigger cellular response and modulate activity and phenotype of TME cells (Kalluri, 2016). An \textit{in vitro} study showed that EVs of OTSCC cells (HSC-3) probably induced the transition from CaDEC fibroblastic cells to cancer associated fibroblast (CAF)-like cells (Dayan et al., 2012).

However, there is barely research looking into the function of OTSCC-derived EVs on particular populations of immune cells. Here, our \textit{in vitro} experiments in this thesis study firstly focused on how OTSCC-derived EVs influence macrophage polarization and cytotoxic activity of CD8$^+$ T cells and NK cells.

5.3.1. OTSCC cell-derived EVs and macrophage polarization

TAMs, as a significant component of TME cell community, are generally associated with cancer-promoting activities in most of the cases (Qian & Pollard, 2010). The induced polarization of TAMs from the anti-tumor phenotype M1 towards the pro-tumor phenotype M2 is suggested as a strategy of cancer cells to maintain an immune ecosystem that favors cancer cells (Mantovani et al., 2010).
An earlier *in vitro* research revealed the ability of breast cancer cell-secreted factors to educate human primary macrophages to differentiate towards M2 phenotype (Sousa et al., 2015). Another study got similar result with colorectal cancer cells and human monocytic cell line, THP-1 cells (Wu et al., 2014). Both experiment were conducted using cancer cell conditioned medium (supernatant).

In this thesis study, we aimed to verify whether OTSCC cell-derived EVs have the same educational ability. A pilot experiment indicated that HSC-3 EVs incubation polarized THP-1 cells towards M2 phenotype (data not shown). While EVs derived from both HSC-3 and SCC-25 cells did not polarize human primary macrophages towards any phenotypes.

Firstly, the failure of OTSCC cell-derived EVs to educate primary macrophage may be explained by the fact that the isolated EVs lack a variety of cytokines and growth factors present in the cancer cell conditioned medium, which results in the weakness of EVs in macrophage polarization (Mantovani et al., 2010).

Besides, we saw difference in the responses of THP-1 cells and primary macrophages to EVs treatment, which is in agreement with a previous finding that THP-1 cells were potently more responsive to toxic stimulation than primary monocytes (Heil et al., 2002). These suggest that THP-1 cells are not predictive enough for representing primary macrophages in terms of secretory response, and the results derived from THP-1 cells should always be followed by verification of the corresponding primary cells.

### 5.3.2. OTSCC cell-derived EVs and cytotoxic activity of CD8+ T cells and NK cells

As cancer cell EVs carry antigenic materials (proteins, nucleic acids and membrane-derived lipids from parental cells), they have been used as vaccination in research of *in vivo* murine cancer models and *in vitro* human immune cells, and showed potent immunogenic effects (Kunigelis & Graner, 2015; Wolfers et al., 2001; Andre et al., 2002). In this study, we treated two types of cytotoxic inflammatory cells,
namely CD8\(^+\) T cells and NK cells from three healthy donors, with EVs derived from two OTSCC cell lines (HSC-3 and SCC-25) of different aggressiveness, and then the treated cytotoxic cells were co-cultured with the two OTSCC cell lines to check whether their cytotoxic activity had been modulated by EVs.

We discovered that OTSCC cell-derived EVs to certain extent modulated cytotoxic activity of CD8\(^+\) T cells and NK cells, but the direction and responsiveness of modulation varied from case to case and depended on several factors. These factors included inflammatory cell sources (three donors), cytotoxic cell types (CD8\(^+\) T cells or NK cells), EVs sources (HSC-3 or SCC-25) and also targeted cell lines (HSC-3 or SCC-25) that were against. The diverse outcome in our experiment showed not only the individually distinguishing immune system phenotypes among different people, but also the controversial role (immune stimulator or suppressor) of cancer cell-derived EVs in cancer immunity.

In summary, as discussed in the review by Kunigelis & Graner (2015), the context where cancer cell EVs encounter immune cells, including both extracellular environment and specific immune cell type and condition, may be deciding factors of the immune response to cancer mediation by EVs.
6. Conclusion

This study investigated the crosstalk between OTSCC cells and inflammatory cells in an *in vitro* condition. We found that activated human peripheral blood MNCs significantly reduced proliferation of both OTSCC cell lines, and invasion area of only HSC-3. None of the inflammatory cells in the experiment had any effect on invasion depth and migration of OTSCC cells. On the other hand, OTSCC cell-derived EVs didn’t influence macrophage polarization, but had heterogeneous modulating effects on cytotoxic activity of CD8+ T cells and NK cells.

In conclusion, although we detected effects of OTSCC cells and inflammatory cells on each other by secreted molecule mediators or EVs, the results were not uniform and varied in different OTSCC cell lines or inflammatory cell populations and sources.

The outcome of the study emphasizes the importance of a personalized design of cancer treatment, which takes other components in TME such as immune cells and EVs into consideration. A pre-treatment *in vitro* test of patient’s cancer cells response to chemoradiotherapy or drug in the presence of patient’s serum may be useful.

The 3D organotypic model used in the study was a highlight as it offered a better simulation compared with previous 2D models or materials derived from murine, especially for studying cell activities like invasion and infiltration. Besides, it is suitable for co-culturing of different cell types in TME, thus providing a possible approach for the pre-treatment *in vitro* test of cancer patients.

Some improvement and further investigation that can be addressed in future are:

- The infiltration of immunosuppressive cells such as Treg upon the chemotaxis attraction from OTSCC cells.
• An OTSCC cell migration model that better mimics the authentic *in vivo* situation, and an inflammatory cell recruitment model that takes circulation into consideration

• The molecular mechanism of the crosstalk of OTSCC cells and inflammatory cells
Acknowledgement

This master thesis was conducted at Tuula Salo’s lab, Department of Oral and Maxillofacial Diseases, Clinicum, University of Helsinki. Based on the data in this project one manuscript was written and now it is accepted for publication in Oncotarget.

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Finally, I would like to thank my family, especially my mother, Yanling Zhang, for her endless love, support, believing in me, letting me choose my own path.

Thank you all!
Reference


stimulate migratory and cytolytic activity of natural killer cells. Cancer research, 65(12), 5238-5247.


Supplements

Supplement 1. HSC-3 cells migration in scratch wound healing assay

https://www.youtube.com/watch?v=LtcWEoGa5cc

Supplement 2. CD8+ T cells killing HSC-3 cells in cell killing assay

https://www.youtube.com/watch?v=NQE4gfl04mA