Analysis of circulating CD14+/CD16+ monocyte-derived macrophages (MDMs) in the peripheral blood of patients with oral squamous cell carcinoma

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Objectives. Monocytes/macrophages are regarded as the first line of defense in tumors. Therefore, analyzing monocyte subtypes in oral squamous cell carcinoma (OSCC) may be of value in disease monitoring and to explore immunotherapeutic strategies for cancer patients.

Study Design. Circulating peripheral blood CD14+/CD16+ monocyte-derived macrophages (MDMs) were evaluated in OSCC patients with oral squamous cell carcinoma (n = 44) compared with controls (n = 85). Moreover, epitope detection in monocytes (EDIM) technology was used to detect biomarkers Apo10 and transketolase-like-1 in CD14+/CD16+ MDMs.

Results. Compared with controls, no significant ($P = .3646$) difference (control group 9.8%, OSCC group 8.8%) in CD14+/CD16+ MDM were noted in OSCC. However, EDIM-Apo10 and EDIM-TKTL1 scores detected in the CD14+/CD16+ MDMs were increased in OSCC compared with controls ($P < .0001$).

Conclusions. Analyzing CD14+/CD16+ MDMs represents a stable cell population for detecting biomarkers in cancer disease monitoring. (Oral Surg Oral Med Oral Pathol Oral Radiol 2016;121:301-306)

Improving the understanding of the host immune system and, in general, the immunologic characteristics of oral squamous cell carcinoma (OSCC) is necessary to explore and test immunotherapeutic strategies for patients with cancer.[1-5] The immune system plays an important role in the elimination of cancer cells.[6] Innate and adaptive immune cells participate in the surveillance and the elimination of tumor cells. Monocytes/macrophages are regarded as the first line of defense in tumors because they colonize rapidly and secrete cytokines/chemokines, which attract and activate other antigen-presenting cells (APCs), such as dendritic cells (DCs), as well as natural killer (NK) cells. In turn, activated DCs and NK cells initiate the immune response against the transformed cells.[7]

The presence of different populations of monocytes in the blood is well established.[8] They may participate in anti-inflammatory or proinflammatory processes, depending on their state of activation and differentiation.[9] The "classic" monocytes are strongly positive for the CD14 cell surface molecule (CD14++/CD16− monocytes) and represent 90% to 95% of total monocytes in a healthy person.[10,11] In 1988, a subpopulation of monocytes co-expressing CD16 and low numbers of CD14 antigens (CD14+/CD16+ monocytes) was discovered.[12] This CD14+/CD16+ subpopulation varies in healthy individuals from 3% to 13%[13] and embodies a unique type of monocytes[14] that resemble certain types of mature macrophages.[15] Indeed, this subset of CD14+/CD16+ monocyte-derived macrophages (MDMs), which was detected in inflammatory diseases (e.g., patients with chronic periodontitis[16]), showed a higher phagocytic potential[17,18] and was described as superior APCs,[8] which makes them attractive for monitoring disease progression[18] and for establishing potential immunotherapies.[19] Furthermore, it has been demonstrated that elevated blood levels of the CD14+/CD16+ MDM subset are associated with cancer progression.[13,20]

Therefore, analyzing monocyte subtypes may help to identify patients likely to benefit from cancer immunotherapy strategies. This is the first study analyzing the

Statement of Clinical Relevance

Understanding immunologic characteristics by analyzing monocyte subtypes in oral squamous cell carcinoma is necessary for disease monitoring and to explore immunotherapeutic strategies for cancer patients. Peripheral blood CD14+/CD16+ monocyte-derived macrophages represent a stable cell population for detecting biomarkers in disease monitoring.

OF and JFC are employees and shareholders of Zyagnum AG, Pfungstadt, Germany, and declare a potential conflict of interest due to the possible utilization of Apo10 and TKTL1 for diagnostic and/or therapeutic purposes.

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CD14+/CD16+ MDM subset in patients with oral squamous cell carcinoma (OSCC) compared with controls. Moreover, using the epitope detection in monocytes (EDIM) technology, two biomarkers—Apo10 and transketolase-like-1 (TKTL1)—were analyzed in CD14+/CD16+ MDMs.[21,22]

MATERIALS AND METHODS

Patients, blood samples, and flow cytometric analysis of monocyte-derived macrophages

Patients (cases collected from August 2014 to August 2015) with histopathologically confirmed primary and/or recurrent OSCC (n = 44 cases before surgery; Table I) and controls (blood donors, healthy individuals, n = 85) were prospectively enrolled in this study. Patients with nonresectable disease and patients who had received preoperative antineoplastic therapies (radiation/chemoradiation) were excluded from the study. Written informed consent to participate was obtained prospectively from all patients (Ethics Committee Tuebingen, Germany, approval number: 562-2013 BO2). The diagnosis of SCC was confirmed by the department of Pathology, University Hospital Tuebingen.

Blood samples (2.7 mL) were collected in vials treated with ethylenediaminetetraacetic acid before surgery. The blood samples were collected, anonymized, and processed on the following day, blinded to the clinical data. After counting the cells via a peripheral blood count, flow cytometric analysis of whole blood samples was performed. Antibodies conjugated with CD14 (PerCP) and CD16 allophycocyanin (APC) were purchased from BD Biosciences (Heidelberg, Germany). An aliquot of the cell suspension was incubated without fluorophore-conjugated antibodies as a negative control (fluorescence minus one) to reveal background staining by the conjugated primary antibody. Samples were analyzed by fluorescence-activated cell sorting, using a fluorescence-activated cell sorting device (CantoII, BD Biosciences).[23] In brief, 50,000 to 100,000 total cells were collected, and the monocyte population was gated on the basis of cell size; stop criteria were determined by reaching 1000 $CD14^+/CD16^+$ MDMs. Monocytes were characterized for total amount and proportion of CD14+/CD16+ MDMs. Gating for the identification of circulating MDMs is shown in Figure 1A. The absolute cell amounts of the subpopulations are determined by calculating the relative amounts related to their absolute amount in the peripheral blood count.

Flow cytometric analysis of EDIM blood tests and determining EDIM scores

EDIM-TKTL1 and EDIM-Apo10 blood tests were performed to determine the presence of Apo10 and TKTL1 in CD14+/CD16+ monocytes in 44 patients (n = 44) with primary and/or recurrent OSCC and in 85 healthy blood donors (n = 85, blood donation service, Darmstadt, Germany), as described previously by flow cytometric analysis.[23] FITC- and PE-conjugated Apo10/TKTL1 antibodies were provided by Zyagnum AG (Pfungstadt, Germany).

The result of the EDIM test is given as a relative score, indicating the relative amount of CD14/CD16 double positive monocytes harboring Apo10 (or TKTL1) compared with the total amount of CD14/CD16 double positive monocytes multiplied with 10.[21] For example, the Apo10 score of 140 means that 14% of CD14/CD16 positive monocytes harbored Apo10 intracellularly above their own control (fluorescence minus one) as the cutoff criteria.

Statistical analysis

Statistical analysis was performed with MedCalc Software, Version 15.6.1 (Mariakerke, Belgium). The

Table I. Clinicopathologic characteristics of 44 patients with oral squamous cell carcinoma (OSCC)

Characteristics	*Number of patients N = 44*
Age (years)	
Mean 65 (range 48-84)	
Gender	
Male	28
Female	16
Histologic grading	
G1	5
G2	35
G3	4
G4	0
Depth of invasion	
pT1	15
pT2	18
pT3	1
pT4	10
Cervical lymph node metastasis	
pN0	28
pN1	12
pN2	4
pN3	0
Union for International Cancer Control (UICC) stage	
UICC I	15
UICC II	10
UICC III	4
UICC IV	15
Distant metastasis	
Yes	0
No	44
Site distribution of OSCC	
Tongue	14
Floor of the mouth	12
Palate	8
Buccal mucosa	2
Alveolar ridge	8

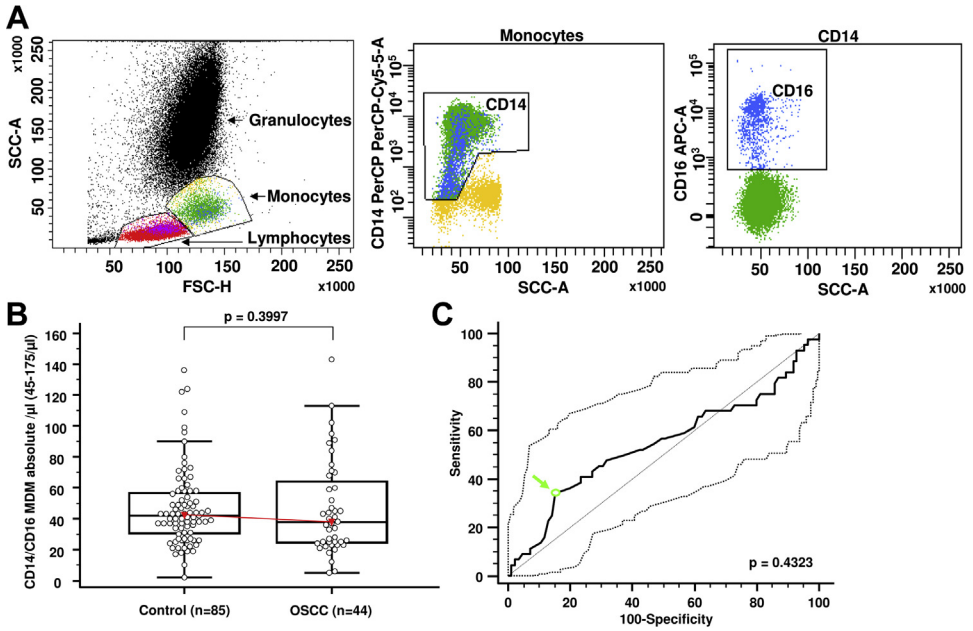

Fig. 1. Gating strategy for the identification of CD14+/CD16+ monocyte-derived macrophages (MDMs), box plot of MDM numbers, and receiver operating characteristics (ROC) analysis showing MDM cutoff value with highest diagnostic accuracy in patients with oral squamous cell carcinoma compared with controls. **A**, Gating strategy (*dot plots, upper panel*) demonstrates analysis of CD14+/CD16+ MDMs in a representative OSCC tumor sample derived from leukocytes (lymphocytes, monocytes, and granulocytes). Cy5-A, Cyanine5 area; FSC, forward scatter; SSC, side scatter; APC-A, allophycocyanin area. **B**, Compared with controls, no significant difference is assessed for total MDM numbers. The median value (±95% confidence interval, CI) for the total number of MDMs in the control group is calculated at 42 (±38-45) cells/µK (arithmetical mean: 46 cells/µL) and at 35 (±26-45) cells/µL (arithmetical mean: 46 cells/µL) for the OSCC group ($P = .3997$). The red line indicates the course of the median numbers (controls vs patients with OSCC). Black circles show single numbers. The red triangle indicates the median value. General accepted normal value of the total MDM number is given in brackets (vertical axis). **C**, The true positive rates (sensitivity) of ROC analysis are plotted in function of the false positive rate (100-specificity) for measurement of the MDM cutoff point (*green circle and arrow*) to distinguish controls and patients with OSCC. Black dotted lines show 95% CI. No significant MDM cutoff point (associated criterion: ≤25 cells/µL, sensitivity 34.1% and specificity 84.7%, area under the curve (AUC) = 0.545; $P = .4323$) is detected.

D'Agostino-Pearson test was performed to test normal distribution of the data. Median numbers of MDM and EDIM scores were determined with 95% confidence interval (CI). The Mann-Whitney U test was used for unpaired (control group vs OSCC groups) nonparametric quantitative data of MDM and EDIM scores.

To analyze differences in MDMs between controls (blood donors) and patients with OSCC, receiver operating characteristics (ROC) analysis was performed.[24] ROC analysis was plotted to allow sensitive and specific discrimination between patients with cancer and healthy individuals and to determine the best cutoff range for healthy group compared with the cancer group. Area under the curve (AUC) analysis was performed for quality measurement. The cutoff points were determined as the values corresponding to the highest diagnostic average of sensitivity and specificity (highest diagnostic accuracy, highest Youden index). The calculated cutoff value of MDM was used for the association of clinicopathologic characteristics by using Fisher's exact test in patients with OSCC.

In our previous work,[21] when screening for EDIM-Apo10 and EDIM-TKTL1, the cutoff point for the control group compared with OSCC group was determined by ROC analysis. Cutoff points[21] showing high sensitivity and specificity were determined at the score of greater than 109 for EDIM-Apo10 and at the score of greater than 117 for EDIM-TKTL1.

All P values presented were two sided, and $P < .05$ was considered statistically significant.

RESULTS

Comparison of circulating CD14+/CD16+ monocyte-derived macrophages in controls and in patients with OSCC

Compared with controls, no significant difference was assessed for total MDM numbers (Figure 1B). The median value (±95% CI) for the relative number of MDMs in the control group was calculated at 9.8%

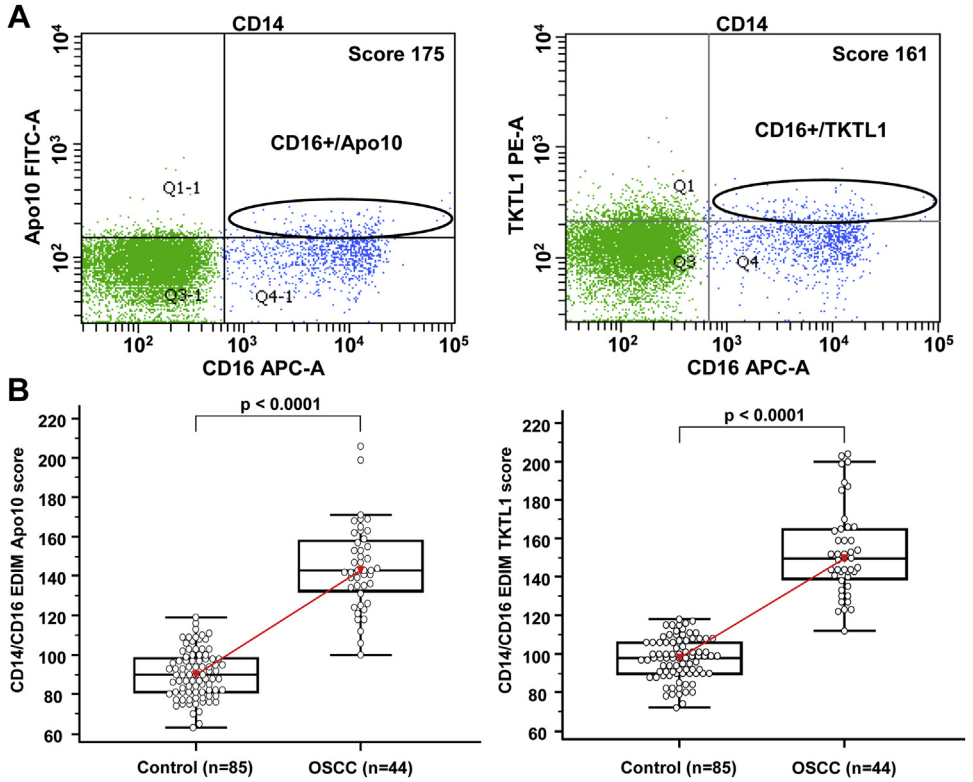

Fig. 2. Epitope detection in monocytes (EDIM) dot plots of Apo10 and TKTL1 staining, and box plots of EDIM values. **A**, Dot plots (upper panel) show increased Apo10 and TKTL1 values in a patient with OSCC. Score values indicate the relative number of positive monocyte-derived macrophages (MDMs). FITC-A, Fluorescein isothiocyanate area; APC-A, allophycocyanin area. Compared with controls, a significant difference is assessed for EDIM-Apo10 and EDIM-TKTL1. **B**, The median value (±95% confidence interval, CI) for EDIM-Apo10 score in the control group is calculated at 90 (±87-94) and at 143 (±139-150) for the OSCC group ($P < .0001$). The median value (±95% CI) for EDIM-TKTL1 score in the control group is calculated at 98 (±95-100) and at 149 (±143-154) for the OSCC group ($P < .0001$). The red line indicates the course of the median numbers (controls vs patients with OSCC). The black circles show single values. The red triangles indicate median values.

(±8.7-10.6) (arithmetical mean: 10.9%) and at 8.8% (±7.4-11.6) (arithmetical mean: 10.6%) for the OSCC group ($P = .3646$). ROC analysis was performed for a closer discrimination of MDMs in patients with OSCC compared with controls.

Association of circulating CD14+/CD16+ monocyte-derived macrophages in patients with OSCC with different clinicopathologic characteristics

ROC analysis (Figure 1C) was performed to associate "low" or "high" absolute numbers (associated criterion 25 cells/μL) of MDMs with clinicopathologic characteristics. No significant associations were found between low or high numbers of MDMs and advanced tumor size (pT3/4), positive cervical lymph node metastasis (pN+), grading (G3/4), advanced tumor stages (UICC III/IV), gender, or mean age (≥65 years).

EDIM-Apo10 and EDIM-TKTL1 blood tests in patients with OSCC

EDIM blood tests (Figure 2A) were assessed in controls and patients with primary and/or recurrent OSCC. Preoperatively, 42 out of 44 patients (n = 42/44, 95%) with OSCC showed positive (>109) EDIM-Apo10 (Figure 2B) scores, and 43 out of 44 patients showed positive (>117) EDIM-TKTL1 scores (n = 43/44, 98%). No patient was negative for both values. The median values of EDIM-Apo10 and EDIM-TKTL1 scores were significantly elevated in the OSCC group compared with the control group ($P < .0001$; see Figure 2B).

DISCUSSION

This is the first study that has analyzed the CD14+/CD16+ MDM subset in the peripheral blood of patients with OSCC. As no significant difference of CD14+/CD16+ MDMs was detected in patients with OSCC compared with controls, analyzing this subtype

represents a stable cell population for detecting biomarkers (e.g., Apo10, TKTL1) in cancer disease monitoring.

Thus, the use of EDIM technology on MDMs, as illustrated in this study, could serve as a tool to analyze and/or to monitor cancers, as described in our previous work.[18,19] It is well known that MDMs show high phagocytic potential.[15,16] Therefore, this subpopulation seems to be highly suitable for detecting alternative tissue-specific biomarkers in OSCC and other tumor entities, such as prostate-specific antigen (PSA) in prostate cancer.[25]

In cancer, a chronic "smoldering" (subclinical)[26] inflammation has been described in the initiation and promotion of malignant disease. However, our results are in contradiction to Subimerb et al., who described elevated numbers of "inflammatory" CD14+/CD16+ MDMs in patients with cholangiocarcinoma and the MDMs decreasing after tumor resection. Moreover, elevation of MDMs was associated with rapid tumor progression and poor patient outcome.[13] Some authors hypothesized that the difference from our study could be the result of different chronic "smoldering" (subclinical)[26] inflammatory conditions measured in each tumor entity that may affect MDM amounts. In addition, strong inflammatory conditions of general diseases,[8] for example, those detected in rheumatoid arthritis and other systemic conditions, can clearly affect MDM amounts.

It is well established that monocytes can differentiate into "friendly" M1 macrophages, which initiate tumor rejection or "foe" M2 tumor-associated macrophages, which stimulate tumor growth, metastasis, and angiogenesis.[7,27,28] The functional significance of CD14+/CD16+ MDMs has been associated with both M1 and M2 cytokine profiles. On the one hand, CD14+/CD16+ MDMs have been documented to participate in anti-tumor responses (M1 macrophages), as judged by the enhance production of proinflammatory cytokines interleukin (IL)-12, tumor necrosis factor alpha (TNF-α), and reactive nitrogen intermediates, with demonstrating cytostatic and cytotoxic activity.[29] On the other hand, studies associated the CD14+/CD16+ MDM subtype with the M2 protumorigenic phenotype showing angiogenic properties—expression of angiopoietin 2, Tie 2,[30] vascular endothelial growth factor A, chemokine ligand 3,[13] and adhesion molecules CD11c, CD49d, CD54[13,31]—promoting tumor progression. Further studies with a larger patient cohort are necessary to describe the functional characterization of peripheral MDMs in OSCC (e.g., surface markers, vascular endothelial growth factor receptors, cytokine profiles, patient outcome).[8,11,32]

A potential weakness of our study was that we did not distinguish between the described "intermediate" CD14++/CD16+ and "nonclassic" CD14+/CD16++ monocytes (nomenclature proposal published in 2010[32]). The "classic" CD14++/CD16− monocytes account for about 90%[10,11] of the monocyte population, and the CD14+/CD16+ MDMs vary in healthy individuals from 3% to 13%[13] (∼10%[11,15]). Our results of 9.8% CD14+/CD16+ MDMs in the control group and 8.8% in the OSCC group are well in line with the published literature. For diagnostic purposes, it seems adequate to differentiate between CD14++/CD16− monocytes and solely CD14+/CD16+ MDMs.[18] However, the differentiation of "intermediate" CD14++/CD16+ and "nonclassic" CD14+/CD16++ MDMs[11,32] may be necessary to discuss functional properties[29,30] to try to enhance tumor-reactive immune response, influencing macrophage polarization by immunotherapeutic strategies, or vaccination therapies.

CONCLUSIONS

As no significant difference of CD14+/CD16+ MDMs has been detected in patients with OSCC compared with controls, analyzing this subtype represents a stable cell population for detecting biomarkers in cancer monitoring. EDIM technology using MDMs serves as a tool for analyzing OSCC.

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