Isolation of Human Myeloid Dendritic Cells from Tumor Tissue and Peripheral Blood

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Abstract. Background: Human myeloid dendritic cells (MDC) have been identified in human solid tumor tissue of head and neck squamous cell carcinoma (HNSCC), their cellular functions being strongly affected in this environment. The characterization of MDC differentiation and functions requires the establishment of highly efficient isolation procedures. Materials and Methods: Human MDC were isolated from peripheral blood and solid HNSCC using density gradient centrifugation or preparation of single cell suspensions, respectively. The cells were isolated by "magnetic bead separation" using magnetically-labelled antibodies and were analyzed by flow cytometry. Results: The isolation of human MDC from peripheral blood or solid HNSCC tissue resulted in highly enriched cell populations and revealed cell purities of about 90%. Conclusion: The scale of molecular investigations on human myeloid dendritic cells is restricted by the limited proportion of this cell type. Thus, highly efficient and gentle MDC isolation procedures are essential to characterize in vivo matured MDC.

Dendritic cells (DCs) are the most potent of the antigen-presenting cells and, therefore, essential for the initiation a primary immune response. DCs are CD34+ bone marrow-derived leukocytes and can be subdivided into subtypes, such as plasmacytoid and myeloid DCs (PDC, MDC), as well as the Langerhans cells of the skin (1-3). A complicated trafficking system leads them from the bone marrow, through the bloodstream to distinct peripheral tissues where they fulfill their antigen-capturing function. Finally they migrate to lymphoid organs in order to present processed antigens to lymphocytes and, thus, to stimulate adequate immune responses (2, 4-8). MDC show a monocytoid morphology and express CD11c and CD1c (human blood dendritic cell antigen-1, BDCA-1) and were determined to be negative for lineage markers (CD3, CD16, CD19, CD20, CD56) (2).

DCs, as well as other types of immune cells, have been shown to infiltrate human solid tumor tissues, such as head and neck squamous cell carcinoma (HNSCC) (9-11). HNSCC is one of the most frequent cancers in the world and, over the last 40 years, standard treatment has only marginally improved the 5-year survival rate of patients with this disease. It is supposed that tumor production of various immune suppressive mediators contributes to the massively impaired immune functions in patients with head and neck cancer (12, 13).

Since monocyte-derived DCs are known to show significant differences compared to in vivo matured MDCs (14, 15), it is essential to establish effective as well as gentle MDC isolation procedures to investigate the influence of HNSCC on distinct cellular functions of human MDCs.

In this work, human MDCs were directly isolated from peripheral blood and HNSCC tissue. Our data demonstrated the isolation yields and cell purities which finally define the scale of possible molecular investigations.

Materials and Methods

MDC isolation from peripheral blood. MDCs were isolated from human peripheral blood provided by the blood bank of the University Hospital, Lübeck, Germany. The blood donors were healthy 18-65 years old who were tested negative for allergies. Additional exclusion criteria were manifest infections during the previous 4 weeks, fever and medication of any kind. PBMCs (peripheral blood mononuclear cells) were obtained from buffy coats by Ficoll-Hypaque density gradient centrifugation, as described previously (16). The MDCs were isolated by magnetic bead separation using magnetically-labelled anti-BDCA-1 antibodies (Miltenyi, Bergisch Gladbach, Germany). The isolated cells were analyzed by flow cytometry using FSC and SSC properties and identified as a population of lineage-negative, CD11c-positive and HLA-DR-positive cells, as described previously (17).

To isolate more MDCs, leukaphereses can be used for automated cell isolation (autoMACS™ Separator, Miltenyi).

Preparation of single-cell suspension. The HNSCC specimens were washed several times and carefully minced into small pieces in sterile
serum-free RPMI medium (RPMI 1640 supplemented with 100 units/ml penicillin, 1 mM glutamine and 100 units/ml streptomycin). The tumor tissue was digested with collagenase type VIII (1.5 mg/ml; Sigma) and DNase type I (1.0 µg/ml) for 120 min at 37°C with gentle agitation. The resulting cell suspensions were washed in phosphate-buffered saline (PBS), resuspended in PBS containing trypsin/EDTA and filtered through a 40-µm nylon cell strainer (Falcon; Becton Dickinson Labware) into cold RPMI medium containing 10% fetal calf serum (FCS). Single cell suspensions were used for isolation of MDCs as described above.

Flow cytometry. Surface antigen staining was performed as described previously (4). The cells were stained with fluorescein-5-isothiocyanate (FITC-), phycoerythrin- (PE), peridinin-chlorophyll-protein- (PerCP) conjugated antibodies by incubation on ice for 15 min, followed by washing with PBS. Fluorescence-labelled monoclonal antibodies against CD11c, Lin-markers and HLA-DR were purchased from BD Biosciences (Becton Dickinson, Heidelberg, Germany). TO-PRO-3 iodide (2 nM; Molecular Probes, Leiden, The Netherlands) was used to determine dead cells. The samples were analyzed on a FACS Canto (Becton Dickinson). Data acquisition and analysis were performed using the FACS DIVA software.

Results

Isolation of MDCs from peripheral blood. MDCs were isolated from the peripheral blood of healthy donors by magnetic bead separation using magnetically-labelled anti-BDCA-1 antibodies after density gradient centrifugation (for a model see Figure 1). Apart from MDCs, CD1c (BDCA-1) is also expressed on a subpopulation of CD19+ small resting B lymphocytes. Therefore, CD19 MicroBeads were used for the depletion of B cells before enriching BDCA-1+ MDCs. Light microscopy was used to calculate cell numbers as well as to analyze the viability of the isolated cells by Trypan-blue staining of dead cells. Microscopic analysis illustrated the characteristic shape and morphology, as well as vitality, of the huge majority of cells (Figure 2). The isolated cells were analyzed by flow cytometry using FSC and SSC properties. The expression of characteristic surface antigens was investigated and MDCs were identified by their lack of lineage (lin) markers as well as their expression of HLA-DR and CD11c, as described before (Figure 3). Magnetic bead separation of MDCs resulted in average cell counts of about 3x10^8 cells per 500 ml of peripheral blood. Flow cytometric analysis revealed cell purities between 80 and 90%.

Isolation of MDCs from solid HNSCC. To analyze the frequency and function of tumor-infiltrating MDCs, single cell suspensions of solid HNSCC were prepared. Therefore, tissue specimens were split into small pieces, digested and filtered (for a model see Figure 1).

The frequencies of HNSCC-infiltrating MDCs were determined by flow cytometry of these single cell suspensions.
Our data revealed average MDC frequencies of about 0.2% of total cells (Figure 4), which was significantly lower compared to a frequency of about 0.5% in human peripheral blood. Single cell suspensions were used for magnetic bead separation as described for the isolation of MDC from peripheral blood.
Discussion

HNSCC is known to be infiltrated by various kinds of immune cells, but effective immune responses are greatly impaired by the HNSCC microenvironment (9-11). Thus, the DC function caused by abnormal differentiation of these cells represents an important immune escape mechanism. Previously, it was suggested that large numbers of immature MDCs participate in decreased Ag-specific T cell responses in cancer patients. It is supposed that an increased infiltration of HNSCC by DCs correlates with a worse prognosis (9,18). Correspondingly, the T-lymphocyte and monocyte functions of peripheral blood mononuclear cells from patients with HNSCC have been shown to be predictive factors for outcome (11).

Since patient survival in HNSCC has not changed significantly in many years, despite progress in surgical, radiotherapy and chemotherapy techniques, the development of immune-modulating therapies against HNSCC represents a rapidly progressing alternative cancer treatment (19). Therefore, the use of effective DC isolation techniques is essential for the investigation of the influence of the HNSCC microenvironment on DC function and differentiation, as well as for the modulation of DCs for novel immunotherapeutic strategies.

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