ORIGINAL ARTICLE

# Pleural Effusion of Patients with Malignant Mesothelioma Induces Macrophage-Mediated T Cell Suppression

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**ABSTRACT**

**Introduction:** Clinical studies have demonstrated beneficial effects of immunotherapy in malignant pleural mesothelioma. The pleural cavity seems an attractive compartment to administer these types of therapies; however, local immunosuppressive mechanisms could hamper their efficacy. Macrophages are abundantly present within the mesothelioma microenvironment. This study investigates the influence of the macrophage phenotype, macrophages' capacity to inhibit local immune responses, and the decisive role of pleural effusion (PE) in this regard.

**Methods:** We cultured macrophages in the presence of PEs and investigated their phenotype. Macrophages and T cells were cocultured in the presence of PEs and tumor cell line supernatants. The levels of 11 cytokines and the prostanoid prostaglandin $E_2$ were measured in PEs and supernatants. The presence and phenotype of macrophages and T cell subsets was measured in the PE of patients with mesothelioma.

**Results:** PE induced a tumor-promoting M2 phenotype in macrophages, which was confirmed by the suppressive activity of macrophages on T cell proliferation during coculture. Prostanoid prostaglandin E2 was identified as a potential inducer of the suppressive capacity of macrophages in PE. Macrophages isolated from PEs displayed an M2 phenotype and were negatively correlated with T cells in vivo.

**Conclusions:** The current study demonstrates that macrophages in PE can play a pivotal role in directly hampering the antitumor T cell immune response. This emphasizes the potential of macrophages as a therapeutic target in mesothelioma and indicates that the presence and phenotype of macrophages in PE should be taken into consideration in the application of (intrapleural) immunotherapies.



*Keywords:* Mesothelioma; Pleural cavity; Tumor-associated macrophages; Immunosuppression; Microenvironment

## Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive cancer with currently limited treatment options. The immune system is considered to play a major role in the pathogenesis and prognosis, and potentially, in the treatment of this devastating disease.[1–6]

Despite encouraging results of immunotherapeutic approaches, responses are hampered by local and systemic immunosuppressive mechanisms.[7,8] Therefore, attention is being focused on the cellular and molecular mechanisms, which play a role in the immunosuppressive tumor environment. The pleural cavity is a convenient compartment to administer different treatment modalities in close approximation to the tumor and potentially minimize systemic toxicities. Different intrapleural treatment options are now under investigation.[9] Pleural effusion (PE) accompanies mesothelioma in

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approximately 70% of the cases, predominantly in the epithelioid subtype.[10] PE consists of tumor cells and numerous types of immune cells and stromal cells.[11,12]

Immune cells invade both the tumor and PE of patients with MPM.[13–15] These infiltrating immune cells can exert either beneficial or detrimental effects, depending on their phenotype.[16] Tumor-associated macrophages (TAMs) are a major component of the immune cell infiltration of the tumor microenvironment in mesothelioma patients.[17] Under the influence of various stimuli within the tumor microenvironment, TAMs can develop into a tumor-inhibitory (M1) or tumor-promoting (M2) phenotype.[18,19] Others and we have found that the presence and M2 phenotype of TAMs in MPM tumor biopsy specimens is related to a worse survival.[20,21]

Given the close proximity between PE and the pleural tumor, the pleural space is a pivotal part of the tumor environment in MPM and characterization of the local immunosuppressive mechanisms is essential to improve (local) immunotherapeutic approaches. The aim of the present study was to investigate the immunosuppressive properties of PE and its effect on the phenotype and function of macrophages.

## Materials and Methods

### Collection and Processing of PEs

Thoracocentesis was performed using fine-needle aspiration of the pleural cavity; the PE was collected in sterile containers without anticoagulant. Pleural cells were pelleted from PE, and Ficoll density gradient centrifugation was applied to separate the red blood cells from the leukocytes as previously described.[22] Six PE supernatants (SNs) were selected for the in vitro experiments because accompanying long-term MPM cell lines were established from the cellular fractions of these PEs.[13] To obtain MPM cell lines, the original cellular fractions of the PEs were cultured in culture medium (Roswell Park Memorial Institute medium) supplemented with 10% normal human serum. Cell line SNs were collected around passage number 70 for all cell lines at 80% confluency. SNs were collected during passaging and centrifuged at 1200 *g* for 10 minutes before use.

### Isolation of Healthy Monocytes and T Cells

Peripheral blood mononuclear cells were isolated from a buffy coat of a healthy donor (Sanquin, Amsterdam, The Netherlands) using Ficoll density gradient centrifugation.[22] Monocytes and T cells were isolated with magnetic activated cell sorting separation using a Monocyte Isolation Kit followed by a Pan T Cell Isolation Kit (all from Miltenyi Biotec, San Diego, CA). Purity of the isolated fractions was confirmed using flow cytometry (>97% pure [data not shown]).

### Macrophage Cultures

For all conditions, normal monocytes from a healthy donor were differentiated to macrophages during a 6-day culture in the presence of 10% normal healthy AB serum and macrophage colony-stimulating factor (20 ng/mL [R&D Systems, Minneapolis, MN]) in Roswell Park Memorial Institute-1640 medium containing GlutaMAX (GIBCO/ThermoFisher, Waltham, MA). Subsequent polarization to the M1 or M2 phenotype occurred in the presence of lipopolysaccharide (100 ng/mL [Sigma-Aldrich, St. Louis, MO]) and interferon-$\gamma$ (IFN-$\gamma$) (20 ng/mL [R&D Systems]) for M1 or interleukin-10 (IL-10) (40 ng/mL [R&D Systems]) for M2 for 2 days. For the PE conditions, the differentiated macrophages were subsequently cultured for 2 days in the presence of 10% PE SN.

### Gene Expression Analysis

Gene expression analysis of selected genes was performed on the macrophages after 8 days of culture as described earlier.[21] Specificity of the amplification product was confirmed by examination of dissociation curves. Expression levels were normalized to the internal control $\beta$-actin. The primer sequences are depicted in Supplementary Table 1.

### T Cell Coculture and Proliferation Assay

Monocytes were seeded at $5 \times 10^4$ cells in wells of a 96-well plate and differentiated to macrophages for 6 days, as described earlier. T cells isolated from the same healthy donor were labeled with carboxyfluorescein succinimidyl ester (CFSE) molecular probes (ThermoFisher) as previously described.[22] The CFSE-labeled T cells were cocultured in a 1:1 ratio with the differentiated macrophages and stimulated using anti-CD3/anti-CD28 beads (Invitrogen Dynabeads [ThermoFisher]) for 4 days. T cells and macrophages were cocultured for 4 days in 10% normal healthy AB serum, 10% PE SNs ($n = 6$), or 30% MPM cell line SNs ($n = 6$). Cell division was quantified on the basis of serial halving of CFSE intensity; algorithms provided by FlowJo software (Tree Star, Inc., Ashland, OR) were used. Proliferation percentages were calculated as the percentage of T cells recruited into cell division, as previously described.[23]

### Cytokine Measurements

The levels of 12 cytokines were measured by a magnetic bead–based multiplex assay in the six PE SNs and accompanying MPM cell line SNs used for the in vitro experiments (11-plex and single-plex [transforming