Wspolczesna Onkol 2012; 16 (4): 279–284

DOI: 10.5114/wo.2012.30054

Review

Pleural macrophages are involved in local defense mechanisms against environmental pollution, bacteria and cancer. Their main function encompasses phagocytosis of degenerated mesothelial cells. In human pleural effusions macrophages represent more than half of all cells. A model of polarized macrophage activation (M1 and M2) was proposed, which defines a functionally different macrophage populations generated in response to various factors present in the inflamed environment. Tumor associated macrophages are a major component of the inflammatory infiltrate of most cancers. They can promote the proliferation and spread of cancer cells in the early stages of carcinogenesis and during metastasis. Macrophages isolated from malignant pleural effusions as well as tumor associated macrophages exhibit weak cytotoxic activity against tumor cells, increase their proliferative activity and may protect tumor cells from apoptosis. Defining biology of macrophages present in specific environment of the pleural effusion could allow the introduction of innovative diagnostic and therapeutic strategies.

Key words: pleural macrophages, malignant pleural effusion, M1/M2, tumor associated macrophages, TAM.

Macrophages in malignant pleural effusions – alternatively activated tumor associated macrophages

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Pleural macrophages

Macrophages are phagocytic cells of myeloid origin that possess the ability of antigen presentation. They can be classified as resident (attached to a particular tissue), or migrating (free) cells. Resident macrophages adapt to the local environment in order to carry out specific functions, e.g. control inflammation or restore damaged tissue [1]. Despite heterogeneity resulting from the settlement of various organs, these cells also show diversity within a single organ. Pulmonary macrophages, according to location, can be divided into alveolar, airway, interstitial, and those present in the vessels of pulmonary circulation. Other mediastinal macrophage populations include pleural macrophages residing in the pleural space and macrophages resident in the local lymph nodes that collect lymph from the lungs. In humans, macrophages constitute over a half of all cells in the pleural fluid (Figure). Pleural macrophages differentiate from peripheral blood monocytes migrating through the lining of the pleural mesothelium. Their increased influx occurs during the parenchymal inflammatory response [2]. Normally, they proliferate locally to maintain a constant number in the pleura. Presumably, similarly to the peritoneum, a constant pool of macrophages resides on the edge of the pleura. These macrophages are believed to accumulate in the pleural fluid during inflammation associated with cancer. Rat pleural macrophages are less frequent in numbers than the alveolar macrophages, have limited capacity of Fcγ receptor-dependent phagocytosis and weaker non-specific esterase activity. They express fewer surface antigens, which conforms them to peritoneal macrophages [3]. Like monocytes, pleural macrophages show an intermediate level of expression of CD16 and increased expression of CD14 antigens. The level of expression of CD11b, CD33 and CD64 is similar to that observed for $CD14^{++}$ monocytes, while expression of CD32, CD54 and HLA-DR is higher [4]. Due to the high density of HLA-DR and ICAM-1 molecules, pleural macrophages exhibit enhanced ability to present antigens. They participate in the local defense against environmental pollution, bacteria and neoplastic cells. Inside the pleural space, they are the active helper cells of the immune response. Through cytokine secretion, they participate in generating an inflammatory and fibrotic response. In comparison to monocytes they live longer, show more efficient phagocytosis and perform more diverse functions, and their biological activity depends mainly on the microenvironment in which they are located [5]. Macrophages contribute to maintaining tissue homeostasis, and their main function is phagocytosis of degenerated mesothelial cells. They also phagocytose biotic material penetrating into the pleural space, and granulocytes which previously absorbed particles [2]. In response to deposition of foreign particles in the lungs, the number of pleural macrophages increases, which is related to increased concentrations of chemotactic factors and mitogens potentiating the penetration of alveolar macrophages into the pleural space [6]. After lipopolysaccharide (LPS) stimulation, pleural macrophages secrete

greater amounts of tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10) than monocytes, although the expression of mRNA encoding the IL-10 in non-stimulated cells remains constantly high. By secreting IL-1β, TNF-α, IL-6, and GRO-β, macrophages support pleural neutrophils influx [7]. Inflammatory cytokines produced by macrophages that reside in the pleura, e.g. TNF-α and IL-1β, are necessary for the secretion of chemokines of CXC (e.g. GRO-β, IL-8) and CC (e.g. MCP1) families by mesothelial cells [8].

Two models of macrophage activation: M1 vs. M2

A polarized model of macrophages activation differentiates functionally macrophages formed in response to various inflammatory environmental factors. According to the proposed mechanism, macrophages secrete either IL-12 necessary for the synthesis of interferon γ (IFN-γ) and induction of the Th1 response in lymphocytes, or IL-10 enhancing production of IL-4/IL-13 and inducing the Th2 response. Macrophage phenotype M1 is characterized by IL-12^{high} IL-23^{high} IL-10^{low}. Its development is dependent on IFN-γ, IL-12 and IL-18 [9, 10]. Macrophages type M1 have increased expression of HLA-DR, CD80, CD86, TLR2, TLR4 and TLR6 molecules on their surface [11]. They secrete significant amounts of pro-inflammatory cytokines (i.e. IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, IL-18) and produce increased amounts of cytotoxic reactive oxygen and nitrogen species (ROS and RNS) [12].

At the same time they demonstrate a reduced ability to absorb apoptotic cells [13]. Inside the M1 population, two subtypes, M1a and M1b, can be distinguished. Unlike the typical M1, M1b macrophages exhibit reduced phagocytic properties and their main marker is the receptor MARCO [14].

Macrophages type M2 are activated by IL-4, IL-10, IL-13, immune complexes, glucocorticoids (GC) or secosteroid hormones (i.e. vitamin D_3). They exhibit a common phenotype, IL-12^{low} IL-23^{low} IL-10^{high}, but differ in the ability to produce pro-inflammatory cytokines. They have immunoregulatory functions and participate in the Th2 response [15]. Macrophages are activated in the direction of M2 during parasitic infections, development of cancer, atherosclerosis, and central nervous system repair. They participate in wound healing, removing dead tissue, fibroblast activation, as well as in growth and tissue remodeling during angiogenesis [10]. Macrophages M2 predominantly express nonopsonic receptors, mainly scavenger receptors (e.g., SR-A, SR-B) and membrane C-type lectin receptors [16]. These cells exhibit increased expression of CD62L, an adhesion molecule involved in the process of colonization of secondary lymphoid organs [17]. They induce signals that inhibit expression of proinflammatory chemokines and secrete post-inflammatory chemokines associated with an alternative type of immune response (e.g. MCP-4, HCC-1, TARC) [15]. Three variants of M2 polarization can be distinguished: M2a (alternative activation) induced by IL-4 or IL-13; M2b (activation of type II; innate activation) resulting from activation of Fcγ receptors in the presence of Toll-like receptor (TLR) stimulants; and M2c (deactivation), which includes a variety of stimuli-induced inactivation of macrophages, e.g. IL-10, transforming growth factor β (TGF-β) or GC. M2a macrophages do not express inducible nitric oxide synthase (iNOS) but are characterized by high expression of arginase 1 (ARG1), and thereby stimulate cell growth, collagen formation and tissue repair. Macrophages type M2b secrete chemokine I-309, stimulate the CCR8 receptor present on eosinophils, Th2 and T-regulatory lymphocytes (T-reg), and thus enhance Th2 polarization and immunoregulation [10, 12, 18]. However, expression of sphingosine kinase 1 (SPHK1) assimilates them to type M1 [16]. In contrast to M2a, M2b macrophages, despite large amounts of IL-10, also produce TNF-α, IL-1β and IL-6 [19]. M2c macrophages are distinguished by reduced expression of proinflammatory cytokines and increased cleaning of tissue and cell residues. On their surface, they exhibit increased expression of CD14 antigen, which indicates a return from the activated status to equilibrium [20]. Macrophages from malignant and non-malignant pleural effusions differ from each other. The phenotype of macrophages isolated from malignant effusions moved in the direction of M2 activation profile [21].

Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are a major component of the inflammatory infiltrate in most cancers. In breast cancer, their proportion reaches up to 80% of all leukocytes [22]. The number of macrophages present in infiltrates of esophageal, breast, pancreatic and ovarian cancers as well as melanoma has been correlated with the level of CCL2 [23–25]. A relationship between infiltration of macrophages, expression of colony stimulating factor 1 (CSF-1), and late phase breast cancer or the formation of lung metastases, has also been reported [26]. *In vitro*, TAMs exhibit a reduced ability to lyse tumor cells, impaired function of presenting tumor-associated antigens to T cells, and expression of cytokines inducing antitumor activity of T lymphocytes and natural killer (NK) cells. Tumor microenvironment rich in IL-4, IL-6, IL-10, TGFb1 and CSF-1 polarizes them toward the M2 phenotype. Interleukin-10 induces features of TAMs related to phenotype M2c, such as phagocytosis of cell residues, participation in angiogenesis and tissue repair and remodeling [27]. Their phenotype depends on the type and stage of cancer development. In areas of chronic inflammation associated with less mature cancer they exhibit a phenotype similar to M1, while in areas of mature and more diverse cancer they exhibit a phenotype similar to M2. They may also exhibit a mixed phenotype of both M1 and M2 [28]. TAMs exhibit defective activation of NFκB transcription factor associated with its constitutive translocation of the inhibitor to the nucleus [29]. Their numbers in the specific tissue also depend on tumor hypoxia (low tissue oxygen concentration). In-situ environment demonstrating insufficient oxygen concentration reduces the mobility of TAMs and causes their immobilization. Tumor hypoxia also enhances the secretion of HIF-1-dependent proteins associated with the angiogenic, invasive and immunosuppressive phenotype [e.g. vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietin 1, cyclooxygenase 2 (COX-2) [30]. TAM accumulation in hypoxic/necrotic areas correlates with increased risk of metastasis and poor prognosis in breast and endometrial cancers [31, 32]. Depending on the type of secret-

ed regulators, TAMs may act anti- or pro-malignantly. Their activation may result from direct contact with a tumor cell, or from mediators secreted into the environment by cells involved in immunological processes, mainly T helper cells. Macrophages activated by IL-2, interferon γ (IFN-γ) and IL-12 can kill cancer cells, yet they can effectively limit the antitumor cytotoxic T lymphocyte (CTL) response by secreting IL-10 [33]. TAMs are able to eliminate cancer cells by macrophage-mediated tumor cytotoxicity (MTC) or antibody-dependent cellular cytotoxicity (ADCC) [34, 35]. They may also inhibit cancer development by secreting TNF-α or nitric oxide (NO), which induce apoptosis in many tumor types *in vitro* [36]. The presence of macrophages in the leucocyte infiltrate may be associated with a successful outcome for stomach or colon cancers and melanoma [37–39]. However, TAMs more often lead to cancer growth and progression and immunosuppression. They can promote proliferation and spread of cancer cells in early stages of carcinogenesis and during formation of metastases [40]. TAMs play a central role in the relationship between inflammation and initiation of various types of neoplasms. Macrophages present in the areas of inflammation can cause extensive damage to the surrounding epithelial tissue, mutations and damage to cellular DNA, and defective activity of p53. Persistent changes resulting from their activities may lead to the transformation of premalignant epithelial cells and initiate tumor formation. The presence of TAMs may be a poor prognostic factor in the course of cancer of the esophagus, lung, breast, kidney, bladder, endometrium, prostate, uveal melanoma as well as follicular lymphomas [41–48]. TAMs secrete factors stimulating tumor cell proliferation and survival (e.g. TGF-β1, VEGF, PDGFB) [49]. TAMs producing estrogen stimulate breast cancer cell proliferation and enhance its invasiveness [50]. They are involved in tumor invasion to the surrounding healthy tissue and in tumor stroma formation, through releasing mediators affecting the dissolution of the connective tissue, mainly extracellular matrix degrading enzymes (matrix metalloproteinases and serine proteases) and activators of these enzymes [51, 52]. TAMs are involved in the release of metastatic cells from primary tumors as well as in stabilizing the position of metastases in distant places. A so-called "wolf in sheep's clothing" model was proposed in which to produce diversity, preservation of chromosomal aberrations, epigenetic regulation, and metastasis, tumor cells fuse with macrophages. Such hybrids gain the ability of tropism, like macrophages [53]. TAMs are important modulators of angiogenesis enabling the formation of new blood vessels and converting them into coherent functional networks. They release many angiogenic cytokines (e.g. TNF-α, IL-1α, IL-1β, IL-6), chemokines (e.g. CXCL1, CXCL2, CXCL8), growth factors (e.g. TGF-α, VEGF, PDGF, angiopoietins), and enzymes that modulate angiogenesis (e.g. COX-2, iNOS, MMP2, MMP7, MMP9) [54]. The number of TAMs correlates with increased angiogenesis in gliomas, cancer of the esophagus, breast, bladder, prostate, and uveal melanoma [34, 41, 44, 46, 47, 55]. Secreting immunosuppressive factors, such as prostaglandins (PGs), IL-10, and TGF-β, they can indirectly promote tumor growth. These mediators impair the cytotoxic activity of TAMs, and limit their ability to present tumor-associated antigens of cytotoxic T lymphocytes, as well as the capacity for phagocytosis [56, 57].

Pleural macrophages and tumor

Macrophages derived from pleural effusions are a convenient material to conduct the evaluation of existing relationships between immune cells and tumor cells. Pleural effusion constitutes a microenvironment rich in soluble factors devoid of connective barriers, which makes interactions between cells much easier. During the inflammatory response associated with infiltration of a primary bronchial cancer in pleura, macrophages from exudate and transudate fluids release IL-1β, TNF-α, and IL-8. Thereby, they affect T cell proliferation, enhance their accumulation and clonal expansion, and stimulate them to release local cytokines [58]. Additionally, a relationship between a decrease in expression of TCR ζ molecules on T cells, and an increase in the number of monocytes/macrophages in malignant effusions has been reported. In this case, cells with reduced expression of the ζ chain were the main subpopulation of T cells undergoing apoptosis [59]. The pivotal role of pleural macrophages is to phagocytose degenerated mesothelial cells. The phagocytosis of apoptotic cells changes classically activated macrophages from histotoxic inflammatory cells into alternatively activated anti-inflammatory cells that are similar to macrophages type M2c [10]. After the uptake of apoptotic cells, TAMs secrete mediators facilitating growth of cancer cells, and thereby their cytotoxicity is reduced. The impairment of natural cytotoxic activity and the ability to kill autologous tumor cells shown by macrophages isolated from malignant pleural effusions have been demonstrated [60]. Carcinogenesis is a multistep process in which cells undergo malignant transformation, autonomously promote their growth, produce their own circulation and resistance to apoptosis, escape from immune surveillance, and finally acquire the capacity for metastasis. IL-4, IL-10, PGE_2 and TGF-β secreted by TAMs affect the increased expression of anti-apoptotic proteins c-FLIP and BclxL [61, 62]. MMP7 digests the FasL molecules from the cell surface, thereby weakening the sensitivity of tumors to chemotherapeutic agents (i.e. doxorubicin) and cytotoxicity of CTLs and NK cells [63]. Soluble phosphatidylserine (sPS) exfoliated by TAM activity induces an

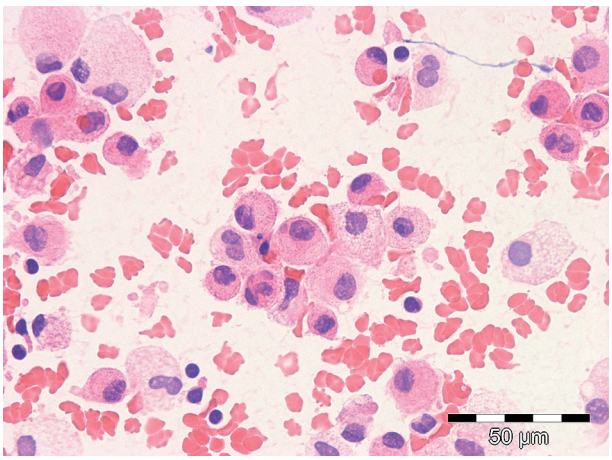

Fig. 1. Pleural effusion macrophages

inflammatory response of falsely activated TAMs. This process enhances tumor cell proliferation and escape of developing tumor cells from immune surveillance [56]. Conditioned media obtained from *in vitro* culture of macrophages isolated from malignant pleural effusions increase the proliferative activity of selected human cancer cell lines. Breast cancer (MDA-MB231), lung (A549), colon (HT29, HCT116, SW620) and hematopoietic (Jurkat, HL60) cell lines manifested an increased percentage of cells in S phase of the cell cycle when cultured in the conditioned media. Assessment of annexin V binding and TUNEL reaction demonstrated that pleural macrophages may protect cancer cells from apoptosis. The antiapoptotic properties of conditioned media correlated with their effect on the expression and activity of proteins regulating apoptosis (reduction in the expression of Fas receptor, expression and activity of caspase-3, and increased expression of Bcl2 and survivin) [64].

Tumor-associated macrophages in diagnosis and therapy

Macrophages are a significant component of the link between chronic inflammation and cancer development. Understanding their biology may largely benefit the knowledge of tissue reconditioning to a normal, pre-pathogenic state. Full determination of the mode of action in the tumor microenvironment requires precise information on specific areas of macrophage activity. Signals responsible for their polarization, identification of genes regulating their activation, determination of signals processed by them within the damaged tissue leading to their polarity, and development of systems for their manipulation *in vivo* are the most crucial elements [65]. Therapeutic strategies using TAMs involve inhibition of their recruitment and survival in locations of tumor development (e.g. trabectedin, brand name Yondelis), inhibition of their effects on angiogenesis and extracellular matrix remodeling (e.g. Linomid, zoledronic acid bisphosphate), and the reversal of immunosuppression and restoration of anticancer cytotoxicity [66–68]. Because of the correlation between the number of macrophages and the development of various types of cancer, TAM evaluation may be important for clinical or diagnostic purposes. Reducing the number of macrophages in glomerulonephritis decreased kidney damage, while in breast cancer it slowed pre-invasive change to full tumor, with simultaneous reduction of the formation of lung metastases [26]. Elimination of macrophages decreased the density of vascularity of advanced cancer by 50% [69]. Macrophage depletion may also be important due to their strong tendency to migrate to places of hypoxia, which may affect formation of relapses. Resulting from different therapy (e.g. radiotherapy, photodynamic therapy, antiangiogenic therapy), hypoxic areas associated with necrosis attract macrophages to these places [70–72]. In such situations, macrophages can switch their phenotype toward promalignant and instead of destroying cancer cells, promote revascularization and reoxygenation, and therefore re-contribute to tumor growth after therapy. Macrophages can be used in cellular immunotherapy, recovering autologous mononuclear cells from patients, activating them *in vitro* and switching back to the patient [73, 74]. Alternatively activated macrophages can be used to reduce injuries and promote repair processes (e.g. stabilization of atherosclerotic plaques) [10]. There has been an attempt to shift M2 polarization of TAMs into the M1 direction to restore inflammatory antitumor activity [75]. On the other hand, the natural ability of macrophages to migrate toward areas of hypoxia has been used for direct delivery of a cytotoxin during tumor development, to serve as hypoxia-induced vectors for genes encoding cytotoxic proteins [76]. Macrophages were transfected with the IFN-γ gene [77] and a hypoxia-induced gene encoding a pro-drug which activated cytochrome P450 [69].

The symbiotic relationship between cancerous disease and macrophages in the environment of a tumor, where cancer cells can affect the survival of macrophages by producing factors that cause tumor growth and angiogenesis, is highly unfavorable for therapeutic purposes. However, accurate characterization of this relationship may help to reverse this trend and enable the introduction of innovative diagnostic and therapeutic strategies.

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Submitted: 6.10.2011
Accepted: 7.02.2012