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## The relationship of host immune cells, cytokine and nitric oxide production to tumor cells in ovarian carcinoma

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### ABSTRACT

**Aims:** This brief review focuses on the current understanding of the complex relationship of tumor-associated mononuclear cells (TAMs) with neoplastic cells, summarizing their immunological efficiency, cytokine profile and production of nitric oxide (NO) in the tumor microenvironment, with current insights on how this might affect tumor growth.

**Data source:** Data was obtained through Medline from articles indexed during the last 10 years. The main key words used in the research were: cancer, ovarian cancer, cytokine, nitric oxide (NO), mononuclear cell, lymphocyte, macrophage.

**Selection of studies and data collection:** 30 studies were reviewed, which contained data regarding the production of cytokines and NO by TAMs or malignant cells, and tried to establish a correlation between these mediators and tumor growth, especially in ovarian carcinoma.

**Data summary:** TAMs consist mainly of macrophages and T lymphocytes which present lower proliferative indices and cytotoxicity compared to autologous blood monocytes, although they are able to release various cytokines. The profile of cytokine expression could help to explain both the immunological impairment observed in patients with advanced carcinoma diseases and the potential of TAMs to exert antitumor activity, which makes these cells an attractive target for therapeutic intervention. NO is also produced in the tumor microenvironment. Several reports in animals suggest a tumoricidal role for NO, but in human tumors its role has not been well-established and may change during tumor progression.

**Key words:** Ovarian carcinoma. Tumor-associated mononuclear cells. Cytokines. Nitric oxide.

### REVIEW

#### *Tumor-associated mononuclear cells (TAMs)*

Solid tumors consist of malignant cells and stroma. Malignant cells elicit stroma formation and this is essential for neoplasia growth.<sup>1</sup> Many tumors of epithelial origin contain, in the tumor stroma, a significant number of infiltrating host leukocytes, especially macrophages and lymphocytes.<sup>2</sup> Also, these cells obtained from neoplastic effusions are extremely useful in evaluating the interactions between immune and cancer cells in the tumor microenvironment.<sup>3</sup> Phenotypic studies (by flow cytometry) and functional studies of the so-called tumor-infiltrating leukocytes (recovered from tumor) or tumor-associated leukocytes (recovered from malignant effusions) have detected no differences between these cells,<sup>3,4</sup> which will be referred to here as tumor-associated mononuclear cells (TAMs).

In terms of characterization of the leukocyte infiltrate, ovarian cancer is one of the most extensively studied human tumors.<sup>5</sup> Immunohistochemistry analysis of tissue sections has shown that the cells present in large numbers are mainly macrophages and T cells.<sup>2,6</sup> B cells,

natural killer (NK) cells and mast cells are present in low numbers. Neutrophils are largely confined to blood vessels and eosinophils are seen occasionally. In general, the infiltrating cell density is higher in the stroma than in the tumor compartment.<sup>2</sup> In peritoneal or pleural effusions secondary to cancers at different sites, including ovarian carcinoma, cells consist mainly of T lymphocytes, but NK cells, B cells and macrophages are also present.<sup>3</sup> It has also been reported that neutrophils are frequently found in the ascites of ovarian carcinoma.<sup>5</sup>

### ***Immunological efficiency of TAMs***

It is not known whether TAMs are a cell population that participates in the immune response against the tumor, or whether these cells might even enhance the development of the tumor.<sup>7</sup> In this context, it is known that the recruited leukocytes can both recognize and kill malignant cells and establish an immune memory against them or produce factors that help tumor growth and vascularization through paracrine loops.<sup>8</sup> The concept of a macrophage balance was introduced by Mantovani et al. to encapsulate the notion that macrophages may aid or inhibit tumor growth according to their state of activation.

In a study of 25 patients with gynecological tumors, of whom 15 had ovarian carcinoma, the autologous peripheral blood mononuclear cells (PBMCs) differed from TAM subset populations in having a significantly higher potential to proliferate in response to mitogen stimulus and also in presenting higher cytotoxicity.<sup>7</sup> Agreeing with this data, TAMs obtained from peritoneal or pleural effusions secondary to cancer at different sites had a lower proliferative response to mitogens than autologous PBMCs, which in turn was lower than that of control PBMCs, indicating a functional immunological impairment in patients with advanced carcinoma disease.<sup>3</sup>

Another indicator of the immune state is the pattern of cytokine release by TAMs. Most of the available data was obtained from advanced ovarian carcinoma. In a recent

study, it was found that the cultured supernatants of mitogen-stimulated TAMs produced less IL-1 $\alpha$  and  $\beta$ , TNF- $\alpha$ , IL-4 and IL-10, compared to those of autologous and control PBMCs, as assessed by the ELISA test.<sup>3</sup> In a similar study, IL-4 was the major cytokine expressed by TAMs, while IFN- $\gamma$  production was predominant in PBMCs.<sup>7</sup> The analysis of cytokine expression by reverse transcriptase polymerase chain reaction (RT-PCR) showed that TAMs had reduced expression of genes for IL-2, IFN- $\gamma$  and IL-4, and inversely, had increased IL-10 gene expression relative to normal PBMCs.<sup>4</sup> Taken together, these results suggest that the incomplete activation of TAMs in vivo may be due to the accumulation of Th2 cells instead of Th1 cells, and it is plausible that the increased IL-10 contributes to downwards regulation of the Th1 cytokines.

But the role of TAMs studied to date varies considerably, even in ovarian carcinoma. In a study of advanced ovarian carcinoma, TAMs produced appreciable amounts of IL-6 and spontaneously released significantly higher amounts of IL-8, compared to PBMCs.<sup>5</sup> In patients with ovarian or breast cancer, cytotoxic T cell cultures, isolated from tumors and then further stimulated with autologous tumor cells, lysed these cells and also secreted cytokines such as TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF.<sup>10</sup> In a prospective study of 17 ovarian carcinomas, it was observed that patients in relapse had a significant reduction in TAMs, which were unable to respond to the tumor as evidenced by the correlation between tumor growth and a decreased number of infiltrating cells.<sup>6</sup>

These differences in cellular composition and the variable prognostic significance of leukocytes that infiltrate many human tumors suggest that different types of interactions are possible between tumor and host cells, possibly resulting in heterogeneous responses.<sup>8</sup> Since TAMs are located at the tumor-host interface and have the potential to exert anti-tumor activity, these cells may constitute an attractive target for therapeutic intervention.<sup>5</sup>

### ***The role of cytokine expression in the tumor***

Although the role of infiltrating cells in malignant tumors is controversial, a likely stimulus for their presence is the local production of chemokines, so that the leukocyte content of a tumor may depend on the expressed cytokines.<sup>2</sup> In this context, a variety of human and murine tumor cells produce monocyte chemoattractant factors and there is a correlation between the amount of activity in cultured supernatants and the number of TAMs, when these cells produce tumors in vivo.<sup>11</sup> Several lines of evidence suggest that monocyte chemoattractant protein-1 (MCP-1) is an important determinant of macrophage infiltration into tumors (review<sup>9</sup>). The presence of messenger RNA for MCP-1 in ovarian carcinoma was first demonstrated by an in situ hybridization technique,<sup>11</sup> and this study also demonstrated the expression of chemokines such as macrophage inflammatory protein-1 (MIP-1 $\alpha$ ), MIP-1 $\beta$  and RANTES activity (regulation upon activation: normal T cell expression and stimulation) by these tumors.<sup>2</sup> Furthermore, a direct topographical association was observed between the number of chemokine-expressing cells and the leukocyte infiltrate at the epithelial-stroma interface.<sup>11</sup>

In general, the major components of TAMs have been described as cells resembling Th0 cells, i.e. producing both Th1- and Th2-type cytokines, or with a gradual shift from Th1 to Th2 cells occurring during progressive tumor growth.<sup>3</sup> The secretion of Th1-like cytokines, as opposed to Th2, could potentially further enhance the endogenous immune response to ovarian cancer.<sup>10</sup> Analysis of cytokine expression using RT-PCR techniques on total RNA isolated from ovarian carcinoma showed that the majority expressed TGF- $\beta$  and IL-10, with absence of expression of IFN- $\gamma$ . Half of these tumors expressed GM-CSF and IL-8,<sup>6</sup> which has also been described in vitro.<sup>8</sup> In accordance with this, higher concentrations of IL-10 have been demonstrated in neoplastic effusions secondary to cancers at different sites, including ovarian carcinoma and, surprisingly, higher IFN- $\gamma$  compared to autologous serum.<sup>3</sup> It

is known that IL-10 strongly inhibits the production of TNF- $\alpha$  and  $\beta$ , GM-CSF and IFN- $\gamma$  by peripheral blood monocytes.<sup>6</sup>

The differences in the expression of cytokines described in the literature may be related to histological types of ovarian carcinoma analyzed in each study. For example, TNF- $\alpha$  and IL-2 are generally described as not being consistently detected in those tumors, in contrast to IL-10 and GM-CSF.<sup>6</sup> The analysis of 13 cases of ovarian carcinomas showed that in only four cases were cells expressing messenger RNA for TNF- $\alpha$  and IL-2 observed, a number considerably lower than that observed in inflammatory conditions such as salpingitis or in normal peripheral lymphoid tissue.<sup>12</sup> The gene for TNF was studied in biopsies of human epithelial ovarian cancer and a positive correlation was found between TNF expression and tumor grade, suggesting that TNF production may enhance tumor development.<sup>13</sup>

Ovarian carcinoma cells produce cytokines that attract monocytes and promote their survival; TAMs in turn produce cytokines which can stimulate cancer cell growth. So there is an ambivalent relationship between tumor cells and TAMs. In the absence of effective therapeutic intervention, evidence suggests that the balance is shifted in favor of the tumor.<sup>1</sup>

### ***A role for NO in tumor biology***

Nitric oxide (NO) is an essential physiological signaling molecule mediating various cell functions, including the cytotoxic/cytostatic effects of the immune system against debilitating factors like infection and tumor (review<sup>14</sup>). But, when produced for a long period and in high concentrations, an excess of NO could damage DNA leading to gene mutations and cancer.<sup>15</sup>

Several human cancers are associated with chronic viral, bacterial and parasitic infections, with NO formation being elevated in these infections.<sup>16</sup> Also, most of the cellular components of the tumor mass (tumor cells themselves and the immune cells infiltrate) have been shown to generate NO in vitro (review<sup>17</sup>).

The expression of NO synthase (NOS) in tumors provokes the question about a physiological role for tumor-associated NO production.

Several reports suggest a tumoricidal role for NO *in vivo*. A chronic inhibition of NO synthesis with N-monomethyl-L-arginine (L-NMA) resulted in increased tumor growth and delayed immune recognition in mice, implicating endogenous NO in the impaired ability of tumor cells to proliferate.<sup>18</sup> Moreover, the daily intraperitoneal administration of L-NMA prevented the tumoricidal activity in mice which were preinoculated with bacillus Calmette-Guérin (BCG) and subsequently transplanted with syngenic or xenogenic ovarian tumor cells. Since it has been demonstrated that NO mediates the BCG-induced host resistance to tumor grafts in mice, it is most likely that NO accounts for the tumoricidal activity.<sup>19</sup> Also in mice, the administration of cytokine-stimulated tumor cells caused a two-fold increase in subcutaneous tumor growth and experimental pulmonary metastases, in relation to control cells. N-monomethyl-L-arginine acetate reduced tumor size and the number of lung metastases to the control levels, suggesting that tumor cell NO production was responsible for this effect.<sup>20</sup>

In human tumors the role of NO has not been established. NO has been reported as being diminished or absent in premalignant lesions and tumors of the large intestine,<sup>14</sup> while, conversely, an increased level of NOS expression and/or activity was observed in human gynecological tumors, and this fact was inversely associated with the differentiation grade of the tumor.<sup>21</sup> In ovarian cancer, high levels of NOS activity were detected, while the enzyme activity was below detectable levels in gynecological tissue from non-cancer patients. In addition, the immunoreactive proteins in which NOS activity was detected were localized to the tumor cells.<sup>22</sup>

Since cytokines and hypoxia can synergistically induce NOS expression, the premalignant and malignant tumor tissue may establish sustained NO production in a variety

of tumor cells. However, the effect of NO production in tumor biology may change during tumor progression.<sup>21</sup> This hypothesis is supported by data investigating the role of NO in cancer metastasis. After *in vitro* incubation with cytokines or LPS, non-metastatic cells exhibited a high level of inducible NOS activity and NO production, whereas metastatic cells did not. The transfection of tumor cells produced fast-growing and highly metastatic tumors, whereas functional iNOS-transfected cells produced slow-growing and non-metastatic tumors in syngenic or nude mice. These data indicate that NO decreased survival of tumor cells in the circulation and inhibited tissue invasion.<sup>23</sup>

A hypothesis has been put forward suggesting that loss of NO from a biological cell could enable it to evade cell-cycle arrest and terminal differentiation, resulting in a premalignant cell or predisposed cell. A loss of NO from a malignant cell could result in uncontrolled cellular division. Since persistent vasodilatation is a specific feature in tumor vasculature and in the surrounding tissue, NO generated by the vascular endothelium in the proximity of or within the tumor, under the control of a local growth factor, could regulate the tumor blood flow via vasorelaxation.<sup>14,17</sup> In addition, the role of NO in angiogenesis is well documented.<sup>17</sup>

Cancer growth can be stimulated as well as inhibited by the immune system. The intratumor macrophage arginine metabolism is a molecular explanation for the dual ability of the immune system to inhibit or stimulate tumor growth. It has been proposed that arginine metabolism in the tumor bed yielding citrulline and NO favors tumor rejection, whereas production of ornithine and urea could promote tumor growth.<sup>24</sup>

### ***Interactions between cytokines and nitric oxide***

One of the first recognized natural mechanisms for regulating NO synthesis was IL-4. When macrophages were activated with IFN- $\gamma$  and a low dose of LPS, they produced significant amounts of NO and expressed high levels of NOS. This production and expression

were inhibited in a dose-dependent manner by preincubating the cells with IL-4. IL-10 and TGF- $\beta$  can also inhibit NO synthesis. In contrast, IFN- $\gamma$  and TNF- $\alpha$ , occupying their respective receptors, transmit a series of signals leading to the expression of NOS and the synthesis of NO.<sup>25</sup> In vitro, human MCP-1 was able to inhibit the production of NO by a macrophage cell line, suggesting that tumor-derived MCP-1 is likely to represent a mechanism for controlling NO-mediated macrophage cytotoxicity, and for the recruitment and concomitant partial functional deactivation of TAMs.<sup>26</sup>

Advanced neoplasia has long been associated with defective capacity to mount responses to inflammatory stimuli. Thus, a balance between chemotactic and inhibitory cytokines may regulate infiltrate in tissues, including neoplasms. In this context, it is known that in mice IL-8 and TNF- $\alpha$  cause defective neutrophil recruitment when administered in the systemic circulation,<sup>27-29</sup> and the production of NO is involved in this inhibitory activity.<sup>30</sup> This observation raises the possibility that cytokines, leaking from advanced tumors, play a role in systemic defects of inflammation and immunity associated with neoplasia.<sup>1</sup>

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## RESUMO

**Objetivos:** Analisar a complexa relação entre as células mononucleares associadas ao tumor (TAMs) e as células neoplásicas, sendo resumidos sua competência imunológica, perfil da produção de citocinas e de óxido nítrico (NO) no microambiente tumoral, com aspectos atuais de como a produção desses mediadores poderia afetar o crescimento tumoral. **Origem dos dados:** Os dados foram obtidos de artigos indexados através da rede Medline durante os últimos 10 anos. As palavras-chave utilizadas na pesquisa foram basicamente: câncer, carcinoma ovariano, citocina, óxido nítrico, células mononucleares, linfócito, macrófago. **Seleção dos estudos e coleta dos dados:** Foram revistos 30 trabalhos contendo dados relacionados à produção de citocinas e NO por TAMs e/ou células neoplásicas e que tentaram estabelecer uma correlação entre a produção desses mediadores e o crescimento tumoral, particularmente no carcinoma ovariano. **Resumo dos dados:** As TAMs consistem principalmente de macrófagos e linfócitos T que apresentam baixo índice proliferativo e baixa citotoxicidade comparada aos monócitos autólogos do sangue, embora sejam capazes de liberar várias citocinas. O perfil da expressão de citocinas poderia ajudar a explicar tanto a deficiência imunológica observada em pacientes com carcinoma em fase avançada como também o potencial das TAMs em exercer atividade antitumoral, o que torna essas células um alvo para intervenção terapêutica. Além das citocinas, o NO também é produzido no microambiente tumoral. Várias observações em animais sugerem um papel tumoricida para o NO, mas em tumores humanos seu papel não foi estabelecido podendo ser alterado durante a progressão do tumor.