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Research article

Impact of macrophages on tumor growth characteristics in a murine ocular tumor model

Marta M. Stei [a, *], Karin U. Loeffler [a], Christian Kurts [b], Tobias Hoeller [c], Christiane Pfarrer [d], Frank G. Holz [a], Martina C. Herwig-Carl [a]

[a] Department of Ophthalmology, University of Bonn, Bonn, Germany
[b] Institute of Experimental Immunology, University of Bonn, Bonn, Germany
[c] Institute for Medical Biometry, Informatics and Epidemiology, University of Bonn, Bonn, Germany
[d] Department of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany

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ABSTRACT

Tumor associated macrophages (TAM), mean vascular density (MVD), PAS positive extravascular matrix patterns, and advanced patients' age are associated with a poor prognosis in uveal melanoma. These correlations may be influenced by M2 macrophages and their cytokine expression pattern. Thus, the effect of TAM and their characteristic cytokines on histologic tumor growth characteristics were studied under the influence of age.

Ninety five $CX_3CR1^{+/GFP}$ mice (young 8–12weeks, old 10–12months) received an intravitreal injection of 1×10^5 HCmel12 melanoma cells. Subgroups were either systemically macrophage-depleted by Clodronate liposomes ($n = 23$) or received melanoma cells, which were pre-incubated with the supernatant of M1- or M2-polarized macrophages ($n = 26$). Eyes were processed histologically/immunohistochemically ($n = 75$), or for flow cytometry ($n = 20$) to analyze tumor size, mean vascular density (MVD), extravascular matrix patterns, extracellular matrix (ECM) and the presence/polarization of TAM.

Prognostically significant extravascular matrix patterns (parallels with cross-linkings, loops, networks) were found more frequently in tumors of untreated old compared to tumors of untreated young mice ($p = 0.024$); as well as in tumors of untreated mice compared to tumors of macrophage-depleted mice ($p = 0.014$). Independent from age, M2-conditioned tumors showed more TAM ($p = 0.001$), increased collagen IV levels ($p = 0.024$) and a higher MVD ($p = 0.02$) than M1-conditioned tumors. Flow cytometry revealed a larger proportion of M2-macrophages in old than in young mice.

The results indicate that TAM and their cytokines appear to be responsible for a more aggressive tumor phenotype. Tumor favoring and pro-angiogenic effects can be directly attributed to a M2-dominated tumor microenvironment rather than to age-dependent factors alone. However, an aged immunoprofile with an increased number of M2-macrophages may provide a tumor-favoring basis. Further, old mice represent a more suitable tumor model instead of young mice since their histologic tumor pattern better resembles human tumors.

1. Introduction

Several histologic tumor characteristics such as angiogenesis, vascular-like structures as well as inflammatory cells, can be found in various tumors, such as uveal melanoma (UM), cutaneous melanoma, breast cancer and others (Allavena et al., 2008; Mantovani et al., 2007). Inducing new blood vessels that deliver nutrients and oxygen (angiogenesis) is crucial for tumor progression and metastasis and thus, tumor mean vascular density (MVD) is associated with patients' prognosis in various tumors. However, tumor

Abbreviations: AEC, 3-amino-9-ethylcarbazole; ECM, Extracellular matrix patterns; FCS, Fetal Calf Serum; GFP, Green fluorescent protein; H&E, Hematoxylin & Eosin; IL, Interleukin; LPS, Lipopolysaccharide; MVD, Mean vascular density; NK, Natural killer cells; PAS, Periodic acid-Schiff reaction; PFA, Paraformaldehyde; TAM, Tumor associated macrophages; UM, Uveal melanoma; VM, Vasculogenic mimicry.

* Corresponding author. Department of Ophthalmology, Ernst-Abbe-Str. 2, 53127, Bonn, Germany.
E-mail address: marta.stei@ukb.uni-bonn.de (M.M. Stei).

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vasculature is very heterogeneous and apart from "true" endothelial vessels it also may comprise vascular-like structures. Such have been suggested to be fluid conducting but non-endothelial channels which are rich in Periodic acid-Schiff reaction (PAS) positive extracellular matrix (ECM), laminin, collagen IV, and are termed vasculogenic mimicry (VM) (Hendrix et al., 2003). These vasculogenic-like networks may form patterns (extravascular matrix patterns) and are also associated with outcome in many tumors (Cao et al., 2013). In UM, outcome further correlates with patients' age, tumor size, and extraocular extension (Bronkhorst et al., 2011; Folberg et al., 1997; Foss et al., 1996; Kaliki et al., 2015; Shields et al., 2013, 2015). However, such histopathologic factors are no longer crucial for prognosis since chromosomal analysis and genetic profiling were found to be more reliable indicators for patients' outcome (Field and Harbour, 2014; Onken et al., 2012; Prescher et al., 1990; Sisley et al., 1990). On the other hand, those histologic parameters reflect tumor growth characteristics which can be studied in a defined and controlled microenvironment in animal models.

The tumor microenvironment is characterized by a synergistic interplay between the tumor and the host. It comprises the tumor cells themselves, the stroma and ECM, different inflammatory cell types, as well as soluble factors such as cytokines or growth factors. Within the microenvironment, interactions between the tumor and the host may orchestrate tumor progression and metastasis (Catalano et al., 2013). Thus, a more detailed understanding of these interactions might help to interfere or even prevent tumor progression and metastasis. Tumor associated macrophages (TAM) are a major component of the inflammatory infiltrate in solid tumors and they are significantly involved in shaping the tumor microenvironment. High numbers of TAM were found to statistically correlate with poor prognosis in different tumor entities (Allavena et al., 2008). In UM, they are further associated with monosomy 3 and several histopathologic factors such as high mean vascular density (MVD) and extravascular matrix patterns/VM, and thus with an aggressive tumor phenotype (Folberg et al., 1997; Hendrix et al., 2003; Maat et al., 2008; Makitie et al., 2001; Toivonen et al., 2004). Generally, TAM functional polarization may range within a broad spectrum from pro-inflammatory tumoricidal M1 macrophages to anti-inflammatory tumor-favoring M2 macrophages (Jager et al., 2011). However, based on genetic analysis and different macrophage activators, a refined and extended spectrum model has been proposed (Xue et al., 2014). Cytokines produced by tumor cells and other cells in the tumor microenvironment determine whether TAM become M1 or M2 (Arnold et al., 2007). In response to the tumor microenvironment TAM tend to specialize predominantly into the M2-phenotype with pro-angiogenic and tumor-favoring properties (Mantovani et al., 2002). Also, a high M2/M1 ratio in UM was shown to be related to a worse prognosis, in particular to histologic negative prognostic parameters (Herwig et al., 2013). As the microenvironment and the capability of innate immunity changes with increasing age macrophages shift their secretory profile towards a M2-profile (Gomez et al., 2008). Macrophages from geriatric mice are particularly sensitive to signals that promote their M2 polarization which in turn may promote tumor development (Jackaman et al., 2013). Thus, the tumor microenvironment and advanced age have an influence on TAM polarization.

As macrophages play a pivotal role in the pathogenesis of several tumors and as they represent main orchestrators of the tumor microenvironment, we investigated their influence on growth characteristics in a new murine ocular tumor model. Herein, the tumor exhibits histologic features such as angiotropism, angiogenesis, extravascular matrix patterns/VM and inflammatory cell infiltration which can be influenced by systemic drug application or intraocular injections (Kilian et al., 2015). In this study, we focussed on the impact of cytokines produced by macrophages, on tumor progression in terms of proliferation rate *in vitro* and intraocular tumor size *in vivo*. Different forms of tumor vascularization as well as ECM were evaluated histologically. The role of macrophage cytokine expression was examined in macrophage-depleted mice in comparison to untreated mice. In addition, in order to address the impact of cytokines produced by M1- or M2-polarized macrophages, we investigated intraocular tumors grown from M1- or M2- conditioned melanoma cells. To study the influence of age on histologic tumor growth patterns, all experiments were conducted both in young and in old mice.

2. Materials and methods

2.1. Cell culture

Immortalized murine peritoneal macrophages from C57Bl/6 wildtype mice were obtained by courtesy from Prof. E. Latz, (Institute of Innate Immunity, University of Bonn, Bonn, Germany). They were examined after incubation in standard medium or after *in vitro* polarization. For polarization, they were first incubated in standard medium without fetal calf serum (FCS) for 2 h to avoid any impact of cytokines or chemokines within the FCS. Macrophage cultures were then treated with different cytokines and LPS to obtain polarized phenotypes according to a modified protocol (Mosser and Zhang, 2008). IFNγ (200 ng/ml) for M1-macrophages and Interleukin 4 (IL-4) (20 ng/ml) for M2-macrophages were added to the serum-free medium. After 10 h of incubation, LPS (100 ng/ml) was supplemented for each macrophage type for a total incubation time of 24 h. Functional polarization status of unfixed macrophages was verified by quantifying inflammatory cytokine production within the supernatant of unpolarized and of M1- and M2- polarized macrophages (inflammatory cytokines IL-6, IL-10, IL-12, IL-23, TNFα and IFNγ, Multi-Analyte ELISArray™ Kit, Mouse Mix-N-Match, Qiagen, Venlo, Netherlands). M1- specific expression of inflammatory cytokines comprises characteristic levels of IL-6^{high}, IL-10^{low}, IL-12^{high}, IL-23^{high}, TNFα^{high} and IFNγ^{high} (M2-specific expression levels vice versa) (Mantovani et al., 2004). Phenotypical macrophage polarization status of 4% paraformaldehyde (PFA)-fixed cells was examined immunocytologically (double staining kit, MultiVision Polymer Detection System, Thermo Scientific, Waltham, MA, USA) by using a F4/80- (for all macrophages) and CD163- (for M2-macrophages only; CD163, M-96, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody.

To investigate the impact of the inflammatory cytokines expressed by these macrophages on tumor growth properties, HCmel12 melanoma cells were cultured in different media or supernatants. HCmel12 melanoma cells were first treated with serum-free medium for 2 h and afterwards exposed to standard medium or to the supernatant of unpolarized or previously M1- or M2- polarized macrophages for 20 h, respectively. Cell proliferation rates of HCmel12 melanoma cells were then compared by a BrdU proliferation assay (BrdU cell proliferaton kit, EMD Millipore, Billerica, MA, USA).

2.2. Animals

Breeding, housing and investigations of $CX_3CR1^{+/GFP}$ mice were carried out and supervised according to accepted standards of the Policy on Humane Care and Use of Laboratory Animals of the National Institutes of Health, Bethesda, MD, USA.

In $CX_3CR1^{+/GFP}$ mice the transmembrane-receptor CX_3CR1 for CX_3C chemokine fractalkine is replaced by green fluorescent protein (GFP) reporter gene. Hence, these mice exhibit GFP expression