Immunobiology 222 (2017) 1–10

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Hyaluronan carried by tumor-derived microvesicles induces IL-10 production in classical ($CD14^{++}CD16^{-}$) monocytes *via* PI3K/Akt/mTOR-dependent signalling pathway

Marzena Lenart[a], Magdalena Rutkowska-Zapala[a], Monika Baj-Krzyworzeka[a], Rafał Szatanek[a], Kazimierz Węglarczyk[a], Timothy Smallie[b], Löms Ziegler-Heitbrock[c,d], Marek Zembala[a], Maciej Siedlar[a,*]

[a] *Department of Clinical Immunology, Polish-American Institute of Paediatrics, Jagiellonian University Medical College, Wielicka 265, 30-663 Krakow, Poland*
[b] *Centre for Translational Inflammation Research, School of Immunity and Infection, University of Birmingham, Edgbaston B15 2TT, United Kingdom*
[c] *Asklepios-Fachklinik and Helmholtz Zentrum München, German Research Center for Environmental Health, Robert-Koch-Allee 1, D-82131, Gauting, Germany*
[d] *Department of Infection, Immunity and Inflammation, University of Leicester, University Road, Leicester LE1 9HN, United Kingdom*

ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form 11 June 2015
Accepted 23 June 2015
Available online 17 July 2015

Keywords:
Hyaluronan
IL-10
Monocytes
Monocyte subsets
PI3K/Akt/mTOR pathway
Tumor-derived microvesicles,

ABSTRACT

Tumor-derived microvesicles (TMV) can mimic effects of tumor cells leading to an increased anti-inflammatory cytokine production, such as interleukin 10 (IL-10), by tumor-infiltrating monocytes and macrophages. Yet, the mechanism of IL-10 induction by TMV in monocytes remains unclear.

The co-incubation of TMV derived from the human pancreas carcinoma cell line (HPC-4) with human monocytes resulted in a nearly 30-fold increase in IL-10 protein production. This effect operates at the level of transcription since monocytes transduced with an adenovirus containing IL-10-promoter luciferase reporter gene showed a 5-fold induction of luciferase activity after treatment with TMV. Since tumor cells can express hyaluronan (HA), which participates in tumor invasion and metastases, we have tested its effect on IL-10 expression. We showed that HA at the concentration of 100 μg/ml induces IL-10 protein expression and the IL-10 promoter activation in monocytes. Moreover, hyaluronidase treatment of TMV reduced IL-10 protein production by 50% and promoter activity by 40%. Inhibitors of the PI3K/Akt/mTOR pathway reduced both, TMV-induced IL-10 promoter activity and protein production, and the same was observed in monocytes when stimulated by HPC-4 cells or HA. Inhibition of PI3K activity down-regulated phosphorylation of the Akt and (to a lesser extent) mTOR proteins in monocytes following TMV or HA stimulation. When comparing monocyte subsets, TMV induced IL-10 protein and mRNA synthesis only in classical $CD14^{++}CD16^{-}$ but not in CD16-positive monocytes. Our data show that TMV induce IL-10 synthesis in human classical monocytes *via* HA, which, in turn, activates the PI3K/Akt/mTOR pathway.

1. Introduction

Interleukin 10 (IL-10) is synthesized *in vivo* by a broad variety of immune cells (Chomarat et al., 1993; Siewe et al., 2006; Sabat et al., 2010) and inhibits the release of Th1 cytokines, antigen presentation, expression of co-stimulatory molecules, phagocytosis, but enhances B cell survival and antibody production (Sabat et al., 2010; Itoh and Hirohata, 1995). IL-10 production is elevated in various types of cancer, where is produced by both tumor cells and/or tumor-infiltrating monocytes/macrophages (TIM), and is being associated with tumor-mediated immunosuppression. IL-10 production by monocytes/macrophages following

Abbreviations: DCs, dendritic cells; HA, hyaluronan; IRF, interferon regulatory factor; MOI, multiplicity of infection; PKB, protein kinase B; RNI, reactive nitrogen intermediates; ROI, reactive oxygen species; mTOR, mammalian target of rapamycin; TIM, tumor-infiltrating monocytes/macrophages; TMV, tumor microvesicles.

* Corresponding author. Fax: +48 12 658 17 56.
E-mail address: misiedla@cyf-kr.edu.pl (M. Siedlar).

http://dx.doi.org/10.1016/j.imbio.2015.06.019
0171-2985/

interaction with tumor cells and/or TMV results in alteration of their immunophenotype and biological activity (Mytar et al., 2003; Baj-Krzyworzeka et al., 2006, 2007). However, the signalling pathways responsible for IL-10 induction in monocytes/macrophages following their interactions with tumor cells are poorly characterised. IL-10 production by monocytes can be enhanced through the activation of phosphatidylinositol-3 kinase (PI3K) pathway (Guha and Mackman, 2002). PI3K converts phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-triphosphate, which recruits and activates downstream targets, including Akt, also termed protein kinase B (PKB). The other key cellular signalling pathway, depending on the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, affects broad aspects of cellular functions, such as metabolism, growth, and survival (Weichhart et al., 2008). Although, they were initially viewed as two separate pathways, it has been indicated that PI3K and mTOR are connected *via* Akt (Sekulic et al., 2000). PI3K/Akt/mTOR pathway has been reported to take part in the regulation of immune cells activity, including monocytes/macrophages (Rocher and Singla, 2013). In many types of cancer, PI3K pathway is activated by hyaluronan (HA) (Alaniz et al., 2006). Moreover, signal transducer and activator of transcription 3 (STAT3) and interferon regulatory factor 1 (IRF-1) have been characterised as important transcription factors inducing IL-10 promoter activation (Benkhart et al., 2000; Ziegler-Heitbrock et al., 2003).

Hyluronan (HA) is a glycosaminoglycan with a molecular weight ranging from 10^5 to 10^7 Da and is a major component of the extracellular matrix (Sironen et al., 2011). HA can be either attached directly to the cell surface by hyaluronan synthases or can bind to cell surface receptors, *i.e.*, CD44, activating intracellular signalling pathways associated with them (Sironen et al., 2011; Ahrens et al., 2001; Termeer et al., 2002). HA accumulates in the cellular division sites (Misra et al., 2011) and is one of the major extracellular matrix components in human malignancies (Itano et al., 2008), participating in tumor invasion and metastases (Sironen et al., 2011). TMV, originating from tumor cells, carry some tumor cell surface determinants, growth factors, nucleic acids, and tumor-associated antigens (Baj-Krzyworzeka et al., 2006; Dolo et al., 1995; Sidhu et al., 2004; Baran et al., 2010). It has been previously suggested that tumor-monocyte interactions may involve hyaluronan or other CD44 ligands carried by TMV (Mytar et al., 2001; Baj-Krzyworzeka et al., 2013). Therefore, TMV may affect monocytes/macrophages functions, altering their immunophenotype and biological activity. Moreover, TMV differently affect monocyte subsets (Baj-Krzyworzeka et al., 2007, 2010). Monocytes can be subdivided into three subsets: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) (Ziegler-Heitbrock et al., 2010), where the latter two may be collectively referred to as $CD16^{+}$ monocytes (Frankenberger et al., 2012). The $CD16^{+}$ monocytes are characterised by enhanced inflammatory cytokine secretion, including tumor necrosis factor α (TNF), and low secretion of IL-10; increased expression of human leukocyte antigens (HLA) class II and some adhesion molecules (Passlick et al., 1989; Steppich et al., 2000; Szaflarska et al., 2004). They are considered to be more mature than classical monocytes (Korkosz et al., 2012), and their numbers are elevated in inflammatory diseases (Ziegler-Heitbrock, 2006). In response to a contact with tumor cells, the $CD16^{+}$ monocytes produce more proinflammatory cytokines (TNF, IL-12) and show an increased cytotoxic/cytostatic activity towards tumor cells (Szaflarska et al., 2004). TMV-activated $CD16^{+}$ monocytes also show an increased release of TNF, IL-12p40 and reactive nitrogen intermediates (RNI), while $CD14^{++}CD16^{-}$ monocytes produce more reactive oxygen species (ROI) and IL-10 (Baj-Krzyworzeka et al., 2010).

In this study, we have proposed a new possible mechanism responsible for the induction of IL-10 production in monocytes after stimulation with TMV. For the first time, we provide evidence that HA carried by TMV is able to induce IL-10 production in classical $CD14^{++}CD16^{-}$ (but not in $CD16^{+}$) monocytes *via* the PI3K/Akt/mTOR signalling pathway.

2. Materials and methods

2.1. Isolation of monocytes and their subsets

Monocytes were isolated by counter-flow centrifugal elutriation from peripheral blood mononuclear cells (PBMC) obtained from 10 healthy blood donors. Briefly, PBMC were isolated from EDTA-treated whole peripheral blood by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were then separated from PBMC with the JE-5.0 elutriation system, equipped with the Sanderson separation chamber (Beckman, Palo Alto, CA, USA), as described previously (Baran et al., 1996). Purity of isolation was above 95% as tested by staining with anti-CD14 mAb (BD Biosciences Pharmingen, San Diego, CA, USA) and flow cytometry analysis (FACSCanto flow cytometer, Becton Dickinson, San Jose, CA, USA).

The following monoclonal antibodies (mAbs) were used to stain $CD14^{++}CD16^{-}$ (further called: $CD14^{+}$) and $CD16^{+}$ monocytes: anti-CD14-APC (clone MφP9, BD Bioscience) and anti-CD16-PE-Cy7 (clone 3G8, BD Bioscience), in 1:25 dilution v/v. The stained monocytes were then incubated for 30 min at 4 °C after which they were sorted using the FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA) into foetal bovine serum (FBS)-coated polypropylene tubes (BD Biosciences), at 12.000 cells/s, in order to gain pure (usually 97–98%) $CD14^{+}$ and $CD16^{+}$ subsets. The $CD16^{+}$ monocytes were in the range 5–10%.

2.2. Cell culture

The HPC-4 cell line (Siedlar et al., 1995) was cultured by biweekly passages in RPMI 1640 supplemented with 5% FBS (Sigma, St. Louis, MO, USA). FBS used in all experiments was earlier microvesicles-depleted by appropriate centrifugation, as described previously (Baj-Krzyworzeka et al., 2006). Cell lines were regularly tested for *Mycoplasma* sp. contamination by polymerase chain reaction (PCR) ELISA test (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol.

Monocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-gLutamine (Invitrogen Life Technologies, Gaithersburg, MD, USA), and 25 μg/ml gentamycin (Invitrogen Life Technologies), 1–2 × nonessential amino acids (Invitrogen Life Technologies), and OPI supplement (contains oxalacetic acid, sodium pyruvate, and insulin; Sigma–Aldrich, Munich, Germany).

Monocytes were co-cultured with HPC-4 (4×10^4/well) cells (2,5:1 ratio) in supplemented medium overnight at 37 °C, 5% CO_2 in humidified atmosphere. Then, supernatants were collected and kept frozen in −20 °C.

2.3. TMV isolation

TMV were obtained from the HPC-4 cell line (TMV_{HPC}) as previously described (Baj-Krzyworzeka et al., 2007). Briefly, HPC-4 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) with 5% FBS and gentamycin (25 μg/ml). Supernatants from well grown cell cultures were collected and centrifuged at 300 × *g* for 20 min to remove cell debris. Then, supernatants were again centrifuged at 50,000 × *g* for 1 h at 4 °C. Pellets were washed several times in PBS and resuspended in serum-free RPMI 1640 medium. Protein concentration of TMV_{HPC} suspension was estimated by the Bradford method (BioRad, Hercules, CA, USA). TMV_{HPC} were tested for