BMB
Reports

BMB Rep. 2014; 47(5): 286-291
www.bmbreports.org

Enhancement of phagocytosis and cytotoxicity in macrophages by tumor-derived IL-18 stimulation

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Inoculation of mice with the murine NFSA cell line caused the formation of large tumors with necrotic tumor cores. FACS analysis revealed accumulations of CD11b$^+$ cells in the tumors. Microarray analysis indicated that the NFSA cells expressed a high level of the pro-inflammatory factor *interleukin-18* (*il-18*), which is known to play a critical role in macrophages. However, little is known about the physiological function of IL-18-stimulated macrophages. Here, we provide direct evidence that IL-18 enhances the phagocytosis of RAW264 cells and peritoneal macrophages, accompanied by the increased expression of *tumor necrosis factor* (*tnf-α*), *interleukin-6* (*il-6*) and *inducible nitric oxide synthase* (*Nos2*). IL-18-stimulated RAW264 cells showed an enhanced cytotoxicity to endothelial F-2 cells via direct cell-to-cell interaction and the secretion of soluble mediators. Taken together, our results demonstrate that tumor-derived IL-18 plays an important role in the phagocytosis of macrophages and that IL-18-stimulated macrophages may damage tumor endothelial cells. [BMB Reports 2014; 47(5): 286-291]

INTRODUCTION

Tumor promotion relies on the formation of new blood vessels and lymphatic vessels to provide an adequate supply of oxygen and nutrients (1, 2). This process is regulated by the remodeling of pre-existed vasculatures and the recruitment of cells originating from bone marrow (3). Macrophages constitute a large portion of the infiltrated heterogeneous cells at tumor sites. Over the past decade, macrophages have been increasingly recognized as critical regulators that break the balance between pro- and anti-angiogenesis through the secretion of multiple cytokines, matrix metalloproteinases (MMPs) (4), reactive oxygen species (ROS) and nitric oxide (NO) (5).

Among the cytokines, interleukin-18 (IL-18) was initially characterized as a potent inducer of interferon-gamma (IFN-γ). It is produced by T cells (6). IL-18 is first synthesized as a bio-inactive precursor, which undergoes proteolytic cleavage by the intracellular protease caspase-1 (Casp-1) to generate a mature, biologically active cytokine (7, 8). As an immunostimulatory cytokine, IL-18 plays a pivotal role in connecting inflammatory immune responses with tumor progression. IL-18 in the tumor environment significantly potentiates anti-tumor immunity mediated by innate and adaptive immune mechanisms in murine prostate carcinomas (9). In murine melanoma, IL-18 exerts potential anti-tumor activity via the inhibition of angiogenesis (10). In addition, the injection of IL-18 into tumors inhibits tumor growth, which is associated with an increase in intratumoral macrophages cells (9). IL-18 increases the expression of the ICAM-1 in monocytes (11), and ICAM-1 and the VCAM-1 in endothelial cells (12), thus accelerating tissue infiltration by immune cells. IL-18, alone or in combination with IL-12, stimulates macrophages to produce IL-6, TNF-α (13, 14), Cxcl10 (15) and Nos2 (16), which are typically recognized as markers of classically activated macrophages and which perform important anti-tumor functions. Increasingly, the evidence suggests that IL-18 plays a critical role in the behavior of macrophages. However, few studies have focused on the function of IL-18-stimulated macrophages, especially phagocytosis and interactions with the endothelium.

Recently, we found that NFSA-generated fibrosarcomas showed poor blood vessel formation and necrosis in their cores, accompanied by the recruitment of CD11b$^+$ cells. Microarray analysis revealed a high level expression of *il-18* in NFSA cells. In this report, we show that IL-18 enhances phagocytosis in both RAW264 cells and CD19-negative peritoneal macrophages and enhances their cytotoxicity to endothelial cells.

RESULTS

Effects of tumor-derived factors on macrophages

After inoculation, both the MS-K and NFSA cells formed large tumors (Fig. 1A). Histological analysis revealed necrosis in the

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http://dx.doi.org/10.5483/BMBRep.2014.47.5.152

Received 2 July 2013, Revised 20 July 2013, Accepted 5 September 2013

Keywords: IL-18, macrophages, phagocytosis, *Nos*2, angiogenesis

ISSN: 1976-670X (electronic edition)

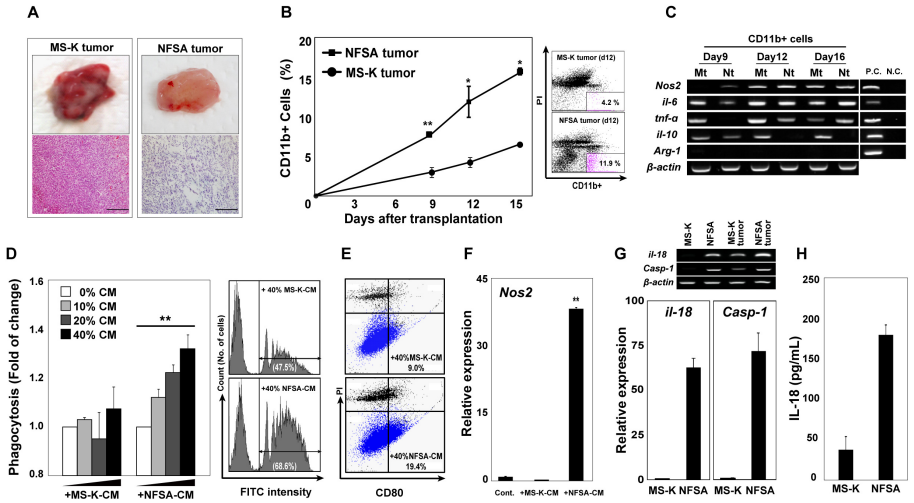

Fig. 1. Effects of tumor-derived factors on macrophages. (A) MS-K tumor and NFSA tumor on day 16. The tumor sections were stained with hematoxylin and eosin (lower). (B) The accumulation of CD11b$^+$ cells in each tumor. The data are the mean percentages of CD11b$^+$ cells in the tumors (n=3). Scatter diagrams from the FACS analysis are shown. The number represents the percentage of CD11b$^+$ cells in the tumors on day 12. (C) The expression of genes in tumor-derived CD11b$^+$ cells. Mt: MS-K tumor-derived CD11b$^+$ cells. Nt: NFSA tumor-derived CD11b$^+$ cells. P.C.: positive control. N.C.: negative control. (D) The effect of CM on the phagocytic activity of RAW264 cells. RAW264 cells were stimulated with increasing concentrations of CM. The uptake of microspheres was determined by FACS (right). (E) The CD80-positive RAW264 cells were analyzed by FACS. The number in each panel represents the percentage of CD80-positive RAW264 cells. (F) The relative expression of *Nos2* in RAW264 cells (qPCR). (G) RT-PCR (upper) and qPCR (lower) analysis of il-18 and *Casp-1*. The relative expression of each gene in the MS-K cells was set to 1 in the qPCR analysis. (H) Quantification of the IL-18 in the CM. All bars show the mean ± S.E. Asterisks denote significant differences, *P < 0.05, **P < 0.005.

Table 1. Comparison of gene expression in NFSA and MS-K

	Gene	Accession No.	Signal		Log2 ratio (NFSA/MS-K)
			NFSA	MS-K	
Chemokine ligand	Chemokine (C-C motif) ligand 11 (Ccl11)	NM_011330	9.13E+03	6.15E+00	10.54
Cytokine	Chemokine (C-C motif) ligand 8 (Ccl8)	NM_021443	1.49E+04	2.28E+02	6.03
	Chemokine (C-C motif) ligand 7 (Ccl7)	NM_013654	1.86E+05	3.00E+03	5.95
	Chemokine (C-X-C motif) ligand 3 (Cxcl3)	NM_203320	3.72E+02	7.85E+00	5.57
	Chemokine (C-C motif) ligand 2 (Ccl2)	NM_011333	4.05E+05	1.22E+04	5.05
	Interleukin 18 (il-18)	NM_008360	1.96E+03	5.90E+01	5.06
Enzyme	Vascular endothelial growth factor-A(Vegfa)	NM_00102525	3.28E+03	7.29E+03	−1.15
	Caspase 1 (Casp1)	NM_009807	2.77E+04	3.34E+01	9.7

Expression of genes in NFSA and MS-K was analyzed by SurePrint G3 (mouse). Table representspart of the data.
Signal means expression level of genes in NFSA or MS-K. Log2 ratio means the relative expression of genes in NFSA against in MS-K.

cores of the NFSA tumors. In contrast, a well-developed blood vessel network was observed in the MS-K tumors (Fig. 1A). The NFSA tumors recruited many more CD11b$^+$ cells than the MS-K tumors during tumor growth (Fig. 1B). In the CD11b$^+$ cells derived from NFSA tumors, the expression of *Nos2*, *il-6*, and *tnf-α* was increased with tumor growth (Fig. 1C). In contrast, in the CD11b$^+$ cells derived from MS-K tumors, the expression of *il-6* and *tnf-α* was down-regulated and the expression of *il-10* was up-regulated on day16. The expression of *arginase-I* (*Arg-1*), a typical M2 marker (17), was not detected in tumor-derived CD11b$^+$ cells. To determine the impact of tumor cells on macrophages, the macrophage cell line RAW264 was used. NFSA-CM enhanced phagocytosis in the RAW264 cells in a dose-dependent manner (Fig. 1D). Furthermore, NFSA-CM induced much higher expression of CD80, the well-established M1-macrophage marker (18), in RAW264 cells than did MS-K-CM (Fig. 1E). The expression of *Nos2* in the RAW264 cells was significantly up-regulated by NFSA-CM

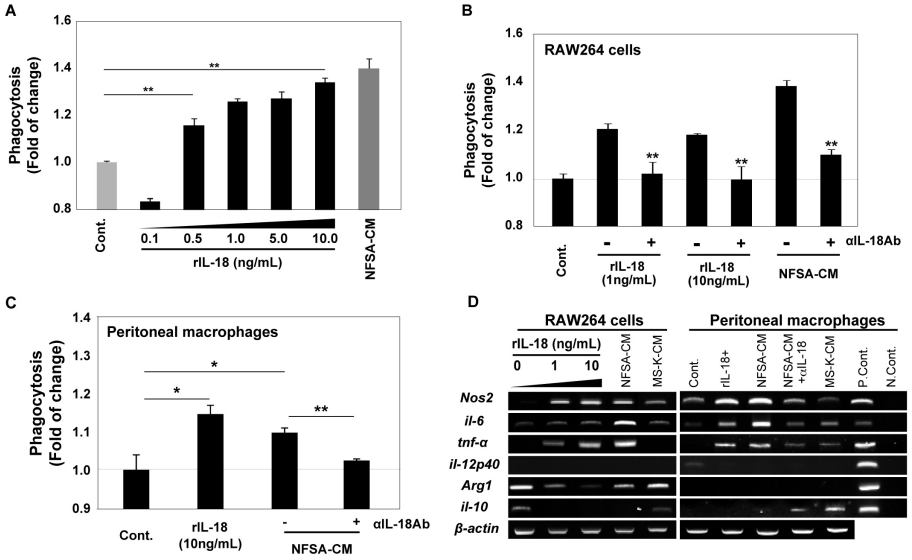

Fig. 2. Stimulation of phagocytosis in macrophages by rIL-18. (A) RAW264 cells were stimulated for 5 days with various concentrations of rIL-18 or NFSA-CM and phagocytosis was analyzed by FACS. (B) RAW264 cells were stimulated with rIL-18 or NFSA-CM, and phagocytosis was analyzed by FACS. In some assays, the neutralizing antibodies against IL-18 (αIL-18Ab) were added. (C) Peritoneal macrophages were stimulated with rIL-18 or NFSA-CM and phagocytosis was evaluated. In some assays, αIL-18Ab was added. (D) The expression of specific genes in stimulated RAW264 cells and the peritoneal macrophages was analyzed by RT-PCR. Asterisks denote significant differences, *P $<$ 0.05, **P $<$ 0.005.

but not by MS-K-CM (Fig. 1F). DNA microarray analysis revealed a higher level of expression of *il-18* and *Casp-1* in NFSA cells (Table 1). The differential expression of *il-18* and *Casp-1* was confirmed by qPCR and RT-PCR in MS-K and NFSA cells (Fig. 1G). ELISA revealed that the concentration of IL-18 in NFSA-CM was 179.4$\pm$7.8 pg/mL, and that in MS-K-CM was 36.9$\pm$16.6 pg/mL (Fig. 1H).

Enhancement of phagocytosis in macrophages by IL-18

The effect of recombinant IL-18 on phagocytosis in RAW264 cells and CD19-negative peritoneal macrophages was analyzed. The rIL-18 enhanced the phagocytosis of RAW264 cells in a dose-dependent manner (Fig. 2A). When a neutralization antibody (αIL-18Ab) was used, it reduced phagocytosis in the rIL-18- or NFSA-CM-stimulated RAW264 cells (Fig. 2B). The phagocytosis of peritoneal macrophages was also up-regulated by rIL-18 or NFSA-CM, and αIL-18Ab reduced phagocytosis in the peritoneal macrophages (Fig. 2C). The expression of several pro-inflammatory- and anti-angiogenesis-related (that is, M-1 type) molecules, including *Nos2*, *il-6* and *tnf-α* (19, 20), and the anti-inflammatory factors *il-10* and *Arg-1* was analyzed (Fig. 2D). The expression of *Nos2*, *il-6* and *tnf-α* was enhanced, whereas the expression of *Arg-1* and *il-10* was suppressed in these cells (Fig. 2D). The addition of αIL-18Ab suppressed the induction of *Nos2* and *tnf-α* and induced *il-10* in peritoneal macrophages. In conclusion, these data suggest a direct effect of IL-18 on the enhancement of phagocytosis and the induction of pro-inflammatory factor expression in macrophages. Thus, IL-18 is one of the critical effectors in NFSA-CM.

Enhancement of cytotoxicity of IL-18-stimulated RAW264 cells

Direct co-culture of F-2-Orange cells with IL-18- or NFSA-CM-stimulated RAW264 cells led to severe death of the F-2-Orange cells (Fig. 3A, B). The survival ratio of F-2-Orange cells was also decreased significantly by the membrane-separated co-culture with RAW264 cells (Fig. 3C, D). Furthermore, the enhanced cytotoxicity of the stimulated RAW264 cells was abolished by the NOS2 inhibitor 1400w (21) (Fig. 3C, D). These results clearly demonstrate that rIL-18 and NFSA-CM stimulate RAW264 cells to damage the endothelium and that some soluble components, such as NO, secreted from the stimulated RAW264 cells damaged the F-2-Orange cells in our assay.

DISCUSSION

The phenotype of macrophages is dependent on the stage of solid tumor progression (17). In most established tumors, infiltrated macrophages are driven to acquire an M2-type, with a reduced expression of NOS2 and TNF-α (22), to provide an immunosuppressive microenvironment for tumor growth (23, 24). In NFSA tumors, an accumulation of $CD11b^+$ cells was observed. The expression of markers of M1-macrophages was increased in the $CD11b^+$ cells. In contrast, the expression of markers of M2-macrophages was suppressed. Furthermore, the macrophages, which were stimulated by soluble factors pro-

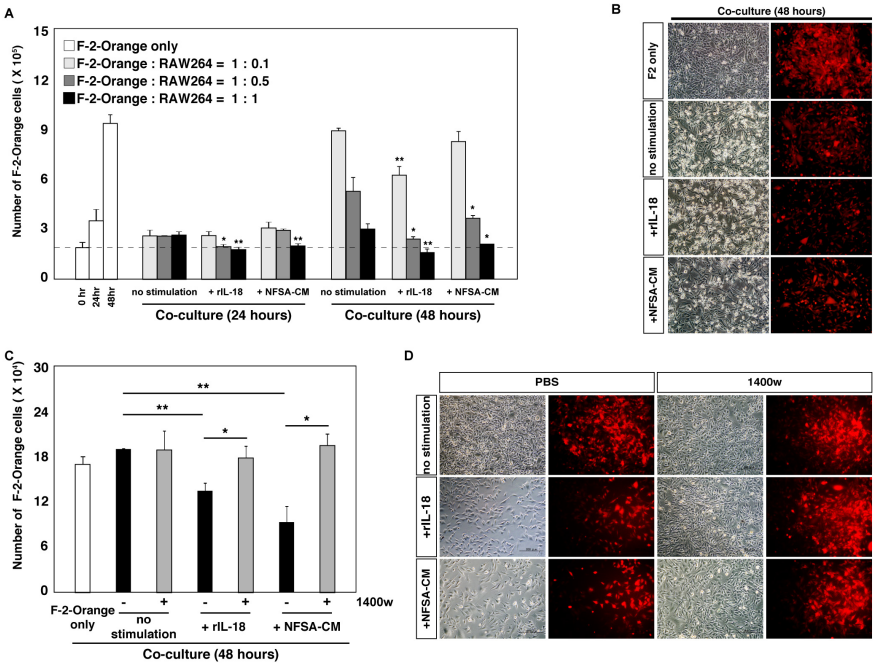

Fig. 3. Inhibition of F-2-Orange cells proliferation by stimulated RAW264 cells. (A) The histogram shows the number of surviving F-2-Orange cells after direct co-culture with RAW264 cells. (B) The photographs show the co-cultured cells at 48 hours. The left panels show the phase contrast images, and the right panels show the fluorescent images. (C) The histogram shows the number of surviving F-2-Orange cells after 48 hours by the membrane-separated co-culture with RAW264 cells. Effect of the NOS2 inhibitor (1400w) was also analyzed. Non-stimulated RAW264 cells were used as a control in all experiments. (D) The photographs show the co-cultured cells at 48 hours. The left panels show the phase contrast images and the right panels show the fluorescent images, with or without the 1400w. All bars show the mean ± S.E, n=3. Asterisks denote significant differences, *P < 0.05, **P < 0.005.

duced from NFSA cells but not from MS-K cells, showed enhanced phagocytosis and increased expression of CD80. These results suggest that the factors produced by NFSA cells have remarkable effects on the activation and polarization of the M1-like macrophages. DNA microarray analysis revealed a high level expression of *il-18* in NFSA cells. Thus, we investigated the effect of IL-18, which is a core effector in tumors, on macrophages.

As a central event of cellular protection, phagocytosis in phagocytic cells acts to eliminate foreign material and is sometimes accompanied by inflammation (25). Our results demonstrated that both NFSA-CM and IL-18 enhanced phagocytosis in both RAW264 cells and peritoneal macrophages. The stimulated RAW264 cells with 0.1 ng/mL of rIL-18 showed a reduction of phagocytosis in our study. It was possible that the induced phagocytosis in the RAW264 cells might depend on the amount of exogenous IL-18. The neutralizing antibody clearly blocked the phagocytosis induced by NFSA-CM and rIL-18. Consequently, we concluded that IL-18 was one of the critical factors in NFSA-CM, which enhanced the phagocytic activity of macrophages.

Five days of stimulation with rIL-18 or NFSA-CM increased the expression of *Nos2*, *il-6* and *tnf-α* in RAW264 cells and peritoneal macrophages. However, the expression of *Arg-1* and *il-10* decreased in RAW264 cells. The addition of aIL-18 Ab reduced the expression of some genes in peritoneal macrophages. These results indicate that IL-18 in NFSA-CM is responsible for polarization of M1-type macrophages.

Finally, we investigated the effect of IL-18 on the cytotoxicity of RAW264 cells to endothelial cells, because poor blood vessel formation was observed in NFSA tumors. Both NFSA-CM and IL-18-stimulated macrophages showed enhanced cytotoxicity to the F-2-Orange cells. The F-2-Orange cells were damaged more severely by direct co-culture compared to the membrane-separated co-culture. This result suggests that the direct cell-to-cell interaction between IL-18- stimulated macrophages and endothelial cells may play a critical role in damaging endothelial cells, in addition to soluble factors. Since the inhibition of NOS2 by the inhibitor clearly inhibited the damaging of the endothelial cells, the NO was certainly one of the factors.

It was previously reported that activated macrophages produce a variety of factors with the ability to lyse tumor cells, including TNF-α and NO (26-28). Heike et al. reported the induction of *Nos2* in murine peritoneal macrophages upon treatment with IL-18 and IL-12 was mediated by IFN-γ, but this phenomenon was not induced by IL-18 alone (16). Bogdan *et*

al. suggested the induction of Nos2 in macrophages requires the cooperation of other cells (29). In this study, we used a macrophage cell line to examine this idea, and determined that the expression of *Nos2* was induced only by stimulation with IL-18 for five days. Therefore, we believe that IL-18 alone could program macrophages, to express Nos2, because the expression of *il-12p40*, a subunit of IL12p70, was not detected in IL-18-stimulated RAW264 cells.

In conclusion, these results provide strong evidence that IL-18 secreted from tumor cells enhances the phagocytosis of macrophages, up-regulates some pro-inflammatory factors, and might inhibit angiogenesis *in vivo*.

MATERIALS AND METHODS

Mice
Eight- to 12-week-old C3H/HeN mice were used in compliance with the guidelines of Niigata University.

Cells
The murine monocyte/macrophage cell line RAW 264 (30) was used. The CD19-negative peritoneal macrophages were separated from peritoneal adherent cells using anti-CD19 microspheres (Miltenyi Biotec, CA, USA). The murine sarcoma cell lines NFSA (31) and MS-K (32) were also used. The F-2 cell line (33), was used for making the F-2-Orange cells.

Preparation of conditioned medium and DNA chip analysis
NFSA and MS-K cells were cultured for 3 days to prepare the conditioned medium (CM). The SurePrint G3 Mouse Gene Expression Microarray (Agilent, Tokyo, Japan) was used.

Analysis and sorting of $CD11b^+$ cells in tumors
MS-K or NFSA cells were inoculated into mice (1×10^6 cells/mouse/side). Tumors were excised at day 9, 12 and 16. A single cell suspension was treated with the anti-mouse CD11b antibody, then analyzed by FACS (BD, Tokyo, Japan).

Quantification of IL-18 by ELISA
IL-18 ELISA kit (MBL, Tokyo, Japan) was used.

Phagocytosis assay and cell characterization
RAW264 cells and CD19-negative macrophages were cultured with NFSA-CM, MS-K-CM or rIL-18 (MBL, Tokyo, Japan) for 5 days. In some experiments, the αIL-18Ab (MBL, Tokyo, Japan) was used. After the culture, fluoresbrite YG microspheres (Polysciences Inc., Tokyo, Japan) were added. The cells were harvested and analyzed by FACS.

Analysis of gene expression by RT-PCR and quantitative PCR (qPCR)
The preparation of cDNA was described previously (34). The expression of *Nos2*, *il-6*, *tnf-α*, *il-12p40*, *Arg-1*, *il-10* and *β-actin* was analyzed by RT-PCR. The qPCR was carried out in accordance with previous reports (34).

Direct co-culture of RAW264 cells with endothelial cells
F-2-Orange cells were seeded in dishes. The RAW264 cells were treated with NFSA-CM or rIL-18 for five days and then overlaid onto the monolayer of F-2-Orange cells. After the co-cilture, the number of surviving F-2-Orange cells was analyzed by FACS.

Membrane-separated co-cultures of RAW264 cells with endothelial cells
F-2-Orange cells were seeded in the lower chamber of a Transwell plate (Corning, NY, USA). The treated RAW264 cells were seeded in the upper chamber (F-2-Orange cells : RAW264 cells = 1 : 1). After the co-culture, the living F-2-Orange cells were counted. In some experiments, 1400 w (EMD chemicals, Inc, San Diego, CA), was used at 100 μM (21).

Statistical analysis
All of the data are expressed as the mean ± standard error of the mean (SEM). A Student's t-test was used. The data for multiple comparisons were analyzed using ANOVA and Dunnett's post hoc test.

REFERENCES

1. Folkman, J. (1971) Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* pp 1182-1186.
2. Ding, Y. T., S. Kumar and D. C. Yu (2008) The Role of Endothelial Progenitor Cells in Tumour Vasculogenesis. *Pathobiology*. **75**, 265-273.
3. Murdoch, C., M. Muthana, S. B. Coffelt and C. E. Lewis (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nature Reviews Cancer*. **8**, 618-631.
4. Solinas, G., G. Germano, A. Mantovani and P. Allavena (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J. Leukocyte. Biol*. **86**, 1065-1073.
5. Sica, A., P. Larghi, A. Mancino, L. Rubino, C. Porta, M. G. Totaro, M. Rimoldi, S. K. Biswas, P. Allavena and A. Mantovani (2008) Macrophage polarization in tumour progression. *Semin. Cancer Biol*. **18**, 349-355.
6. Micallef, M. J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fuji, M. Ikeda, S. Fukuda and M. Kurimoto (1996) Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: Synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol*. **26**, 1647-1651.
7. Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micallef, M. Fujii, K. Torigoe, T. Tanimoto, S. Fukuda, M. Ikeda, H. Okamura and M. Kurimoto (1996) Cloning of the cDNA for human IFN-gamma-Inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. *J. Immunol*. **156**, 4274-4279.
8. Gu, Y., Kuida, K., Tsutsui, H. and H. K. Ku G (1997) Activation of interferon-gamma inducing factor mediated by inter-

leukin-1beta converting enzyme. *Science.* **275(5297)**, 206-209.
9. Tse, B. W. C., P. J. Russell, M. Lochner, I. Forster and C. A. Power (2011) IL-18 Inhibits Growth of Murine Orthotopic Prostate Carcinomas via Both Adaptive and Innate Immune Mechanisms. *Plos One.* **6**, e24241-24212.
10. Zheng, J. N., D. S. Pei, L. J. Mao, X. Y. Liu, F. H. Sun, B. F. Zhang, Y. Q. Liu, J. J. Liu, W. Li and D. Han (2010) Oncolytic adenovirus expressing interleukin-18 induces significant antitumor effects against melanoma in mice through inhibition of angiogenesis. *Cancer Gene Ther.* **17**, 28-36.
11. Stuyt, R. J., M. G. Netea, T. B. Geijtenbeek, B. J. Kullberg, C. A. Dinarello and J. W. van der Meer (2003) Selective regulation of intercellular adhesion molecule-1 expression by interleukin-18 and interleukin-12 on human monocytes. *Immunology.* **110**, 329-334.
12. Morel, J. C., C. C. Park, J. M. Woods and A. E. Koch (2001) A novel role for interleukin-18 in adhesion molecule induction through NF kappa B and phosphatidylinositol (PI) 3-kinase-dependent signal transduction pathways. *J. Biol. Chem.* **276**, 37069-37075.
13. Netea, M. G., B. J. Kullberg, I. Verschueren and J. W. M. Van der Meer (2000) Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1 beta. *Eur. J. Immunol.* **30**, 3057-3060.
14. Hikosaka, S., I. Hara, H. Miyake, S. Hara and S. Kamidono (2004) Antitumor effect of simultaneous transfer of interleukin-12 and interleukin-18 genes and its mechanism in a mouse bladder cancer model. *Int. J. Urol.* **11**, 647-652.
15. Coma, G., R. Pena, J. Blanco, A. Rosell, F. E. Borras, J. A. Este, B. Clotet, L. Ruiz, R. M. E. Parkhouse and M. Bofill (2006) Treatment of monocytes with interleukin (IL)-12 plus IL-18 stimulates survival, differentiation and the production of CXC chemokine ligands (CXCL)8, CXCL9 and CXCL10. *Clin. Exp. Immunol.* **145**, 535-544.
16. Schindler, H., M. B. Lutz, M. Rollinghoff and C. Bogdan (2001) The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J. Immunol.* **166**, 3075-3082.
17. Biswas, S. K., A. Sica and C. E. Lewis (2008) Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J. Immunol.* **180**, 2011-2017.
18. Peng, J., J. Y. Tsang, D. Li, N. Niu, D. H. Ho, K. F. Lau, V. C. Lui, J. R. Lamb, Y. Chen and P. K. Tam (2013) Inhibition of TGF-beta signaling in combination with TLR7 ligation re-programs a tumoricidal phenotype in tumor-associated macrophages. *Cancer Lett.* **331**, 239-249.
19. Takeda, Y., S. Costa, E. Delamarre, C. Roncal, R. L. de Oliveira, M. L. Squadrito, V. Finisguerra, S. Deschoemaeker, F. Bruyere, M. Wenes, A. Hamm, J. Serneels, J. Magat, T. Bhattacharyya, A. Anisimov, B. F. Jordan, K. Alitalo, P. Maxwell, B. Gallez, Z. W. Zhuang, Y. Saito, M. Simons, M. De Palma and M. Mazzone (2011) Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. *Nature.* **479**, 122-U153.
20. Spence, S., A. Fitzsimons, C. R. Boyd, J. Kessler, D. Fitzgerald, J. Elliott, J. N. Gabhann, S. Smith, A. Sica, E. Hams, S. P. Saunders, C. A. Jefferies, P. G. Fallon, D. F. McAuley, A. Kissenpfennig and J. A. Johnston (2013) Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization. *Immunity.* **38**, 66-78.
21. Kim, S. F., D. A. Huri and S. H. Snyder (2005) Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science.* **310**, 1966-1970.
22. Saccani, A., T. Schioppa, C. Porta, S. K. Biswas, M. Nebuloni, L. Vago, B. Bottazzi, M. P. Colombo, A. Mantovani and A. Sica (2006) p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res.* **66**, 11432-11440.
23. Hao, N. B., M. H. Lu, Y. H. Fan, Y. L. Cao, Z. R. Zhang and S. M. Yang (2012) Macrophages in tumor microenvironments and the progression of tumors. *Clin. Dev. Immunol.* **2012**, 948098-948108.
24. Mantovani, A., S. Sozzani, M. Locati, P. Allavena and A. Sica (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23**, 549-555.
25. Suganuma, H., J. W. Fahey, K. E. Bryan, Z. R. Healy and P. Talalay (2011) Stimulation of phagocytosis by sulforaphane. *Biochem. Bioph. Res. Co.* **405**, 146-151.
26. Keller, R., R. Keist, A. Wechsler, T. P. Leist and P. H. Vandermeide (1990) Mechanisms of Macrophage-Mediated Tumor-Cell Killing - a Comparative-Analysis of the Roles of Reactive Nitrogen Intermediates and Tumor-Necrosis-Factor. *Int J Cancer.* **46**, 682-686.
27. Yamanishi, T., T. Hatakeyama, K. Yamaguchi and T. Oda (2009) CEL-I, an N-Acetylgalactosamine (GalNAc)-Specific C-Type Lectin, Induces Nitric Oxide Production in RAW264.7 Mouse Macrophage Cell Line. *J. Biochem.* **146**, 209-217.
28. Engin, A. B. (2011) Dual Function of Nitric Oxide in Carcinogenesis, Reappraisal. *Curr. Drug. Metab.* **12**, 891-899.
29. Bogdan, C. and U. Schleicher (2006) Production of interferon-gamma by myeloid cells - fact or fancy? *Trends Immunol.* **27**, 282-290.
30. Raschke, W. C., S. Baird, P. Ralph and I. Nakoinz (1978) Functional Macrophage Cell Lines Transformed by Abelson Leukemia-Virus. *Cell.* **15**, 261-267.
31. Ando, K., N. Hunter and L. J. Peters (1979) Immunologically Nonspecific Enhancement of Artificial Lung Metastases in Tumor-Bearing Mice. *Cancer Immunol. Immun.* **6**, 151-156.
32. Shirata, K., T. Suzuki, N. Yanaihara, K. Sugimoto and K. J. Mori (1991) Establishment of a Sarcoma Cell-Line, Ms-K, Expressing Ki-Ras Protooncogene Product from Mouse Bone-Marrow Stromal Cells. *Biomed. Pharmacother.* **45**, 1-8.
33. Toda, K. I., K. Tsujioka, Y. Maruguchi, K. Ishii, Y. Miyachi, K. Kuribayashi and S. Imamura (1990) Establishment and Characterization of a Tumorigenic Murine Vascular Endothelial-Cell Line (F2). *Cancer Res.* **50**, 5526-5530.
34. Zhong, X. Y., A. Yoshioka, Y. Mashio, T. Ikeda, H. Jiang, M. Touma, Q. Wu, C. Wang and K. Sugimoto (2011) Effect of vegf gene knockdown on growth of the murine sarcoma cell line MS-K. *Genes Cells.* **16**, 625-638.