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Soluble factor from tumor cells induces heme oxygenase-1 by a nitric oxide-independent mechanism in murine peritoneal macrophages

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Abbreviations: HO, heme oxygenase; iNOS, inducible NO synthase; NO, nitric oxide; RNS, reactive nitrogen species; TBS, Tris-buffered saline; TG, thioglycollate; TMAF, tumor-derived macrophage-activating factor

Abstract

Tumor target-derived soluble secretary factor has been known to influence macrophage activation to induce nitric oxide (NO) production. Since heme oxigenase-1 (HO-1) is induced by a variety of conditions associated with oxidative stress, we questioned whether soluble factor from tumor cells induces HO-1 through NO-dependent mechanism in macrophages. We designated this factor as a tumor-derived macrophage-activating factor (TMAF), because of its ability to activate macrophages to induce iNOS. Although TMAF alone showed modest activity, TMAF in combination with IFN-γ significantly induced iNOS expression and NO synthesis. Simultaneously, TMAF induced HO-1 and this induction was slightly augmented by IFN-γ. Surprisingly, however, induction of HO-1 by TMAF was not inhibited by the treatment with the highly selective iNOS inhibitor, 1400 W, indicating that TMAF induces the HO-1 enzyme by a NO-independent mechanism. While rIFN-γ alone induced iNOS, it had no effect on HO-1 induction by itself. Collectively, the current study reveals that soluble factor from tumor target cells induces HO-1 enzyme in macrophages. However, overall biological significance of this phenomenon remains to be determined.

Keywords: heme oxygenase; interferon type II; macrophages; neoplasms; nitric oxide; nitric-oxide synthase

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme of heme degradation yielding iron, carbon monoxide (CO) and biliverdin, subsequently reduced to the antioxidant bilirubin by biliverdin reductase (Abraham, *et al.,* 1988). Three isozymes have been characterized: HO-2 and HO-3 are constitutively expressed (Abraham, *et al.,* 1988, McCoubrey, *et al.,* 1997), whereas HO-1 is inducible by different stimuli (Terry, et al., 1998). The induction of HO-1 expression may be protective against oxidative stress. It was shown that overexpression of HO-1 moderated the severity of cell damage produced by heme and hemoglobin in endothelial cells (Abraham, *et al.,* 1995). In addition, HO-1 may exert anti-inflammatory effects likely due to CO production (Otterbein, *et al.,* 2000). However, detrimental biological activity of HO-1 also has been suggested in human gliomas (Nishie, *et al.,* 1999; Deininger, *et al.,* 2000).

Accumulated evidence indicates that free radical species and nitric oxide (NO) or its derivatives are found in tumor microenvironment (Maeda and Akaike, 1998). Since tumor-associated macrophages make up a significant proportion of the total cell population of many neoplasias (Manthey, *et al.,* 1994), direct interaction between resident macrophages and tumor target cells may contribute to the generation of these potentially harmful free radicals. Accordingly, tumor-derived soluble products have been known to induce inducible NO synthase (iNOS) gene expression and

NO secretion by murine macrophages (Jiang, *et al.*, 1992; Isobe and Nakashima, 1993). However, because higher levels of NO can also mediate tissue injury, in part, through the interaction with superoxide anion to form peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS) (MacMicking, *et al.*, 1997), mammalian cells may have inducible defense mechanisms to protect host against these toxic radicals. In good agreement with this hypothesis, recent report demonstrated that NO induces HO-1 expression in various cell types, including murine macrophages (Alcaraz, *et al.*, 2000, Bouton and Demple, 2000; Alcaraz, *et al.*, 2001). Interestingly, previous report demonstrated that HO-1 expressing macrophages/microglia cells accumulate during oligodendroglioma progression in areas of focal necrosis (Deininger, *et al.*, 2000).

In this study, we questioned whether tumor-derived soluble secretary factor could induce HO-1 expression in murine peritoneal macrophages. We used the supernatants from the superficial murine bladder tumor-2 (MBT-2) cell line, as our unpublished observation (Choi, *et al.*, 2003 in press) demonstrated that these supernatants alone could stimulate macrophages to induce iNOS expression and NO synthesis. In this paper, we named this factor as a tumor-derived macrophage-activating factor (TMAF), because of its ability to activate macrophages to induce iNOS expression. We also addressed whether the expression of HO-1 is dependent on the secretion of NO in murine macrophages, by using the highly selective iNOS inhibitor, 1400 W [*N*-(3-(aminomethyl)benzyl)-acetamidine].

Materials and Methods

Mice

The original stock of C57BL/6 mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and the mice were maintained in the Department of Microbiology and Immunology, Wonkwang University School of Medicine. To obtain peritoneal macrophages, mice were used at 8 to 12 wk of age.

Reagents

Murine IFN-γ (1×10^5 u/mg) was purchased from Genzyme (Munich, Germany). Rabbit anti-mouse iNOS monoclonal antibody (mAb), anti-actin mAb, and peroxidase-labeled anti-rabbit IgG (H+L) were purchased from Affinity Bioreagents, Inc. (Golden, CO). Goat anti-HO-1 polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). 1400 W [*N*-(3-(aminomethyl)benzyl) acetamidine] was a generous gift from Dr. Hyun-Ock Pae (Medicinal Resources Research Center, Wonkwang University). Dithiothreitol, PMSF, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sodium nitrite, and sulfanilamide were purchased from Sigma (St. Louis, MO). N^G-monomethyl-L-arginine (N^GMMA) was purchased from Calbiochem-Boehring Corp. (La Jolla, CA). All reagents and media for tissue culture experiments were tested for their LPS content with use of a clolorimetric Limulus amoebocyte lysate assay (detection limit 10 pg/ml; Whittaker Bioproducts, Walkersville, MD). Six-well tissue culture plates and 100-mm diameter petri dishes were purchased from Nunc (Naperville, IL). RPMI 1640 containing L-arginine (200 mg/l), HBSS, FBS and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). Amincon concentrators, centriprep, and centricon were purchased from Millipore (Bedford, MA).

Cell cultures

MBT-2 cells and human leukemic HL-60 cells were maintained in complete medium of RPMI 1640 with 10% FBS supplemented with penicillin G (100 u/ml), streptomycin (100 u/ml), and L-glutamine (2 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Thioglycollate (TG)-elicited macrophages were harvested 3 days after i.p. injection of 2.5 ml TG to 8- to 12-wk-old mice and isolated, as reported previously (Jun, *et al.*, 1994). Peritoneal lavage was performed by using 8 ml of HBSS, which contained 10 u/ml heparin. Then, cells were distributed in RPMI 1640, which was supplemented with 10% (v/v) FBS, in 6-well tissue culture plates (5×10^6 cells/well), incubated for 3 h at 37°C in an atmosphere of 5% CO_2, washed three times with HBSS to remove nonadherent cells, and equilibrated with RPMI 1640 that contained 10% FBS before treatment, as indicated in the text.

Measurement of nitrite concentration

Accumulation of nitrite in the medium was measured by a colorimetric assay method based on the Griess reaction (Jun, *et al.*, 1994). Briefly, samples were reacted with 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid at room temperature for 10 min, and the nitrite concentration was determined by absorbance at 540 nm in comparison with sodium nitrite as a standard.

MBT-2 and HL-60 cell supernatant preparation and concentration

MBT-2 and HL-60 cells were cultured at an initial density of 1×10^6/ml in RPMI. After 48 h, the medium was centrifuged (2,500 rpm, 20 min, 4°C) and the

supernatant was sterilized by filtration (0.2 μm) and stored at 4°C. Samples were concentrated up to 10-fold by using centriprep or centricon membrane (YM-3). Final samples were filtered (0.2 μm) and stored at -70°C.

Western blot analysis

Protein samples (50-100 μg) were mixed with an equal volume of 2x SDS sample buffer, boiled for 5 min, and then separated through 8-15% SDS-PAGE gels. After electrophoresis, proteins were transferred to nylon membranes. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with primary antibodies (diluted at 1:500-1:1000) in tris-buffered saline (TBS) overnight at 4°C. Primary antibody was then removed by washing the membranes four times in TBS, and labeled by incubating with 0.1 μg/ml peroxidase-labeled secondary antibodies (against rabbit and goat) for 1 h. Following three washes in TBS, bands were visualized by ECL and exposed to X-ray film.

Results

Initially, we wished to determine whether murine peritoneal macrophages could be stimulated by the

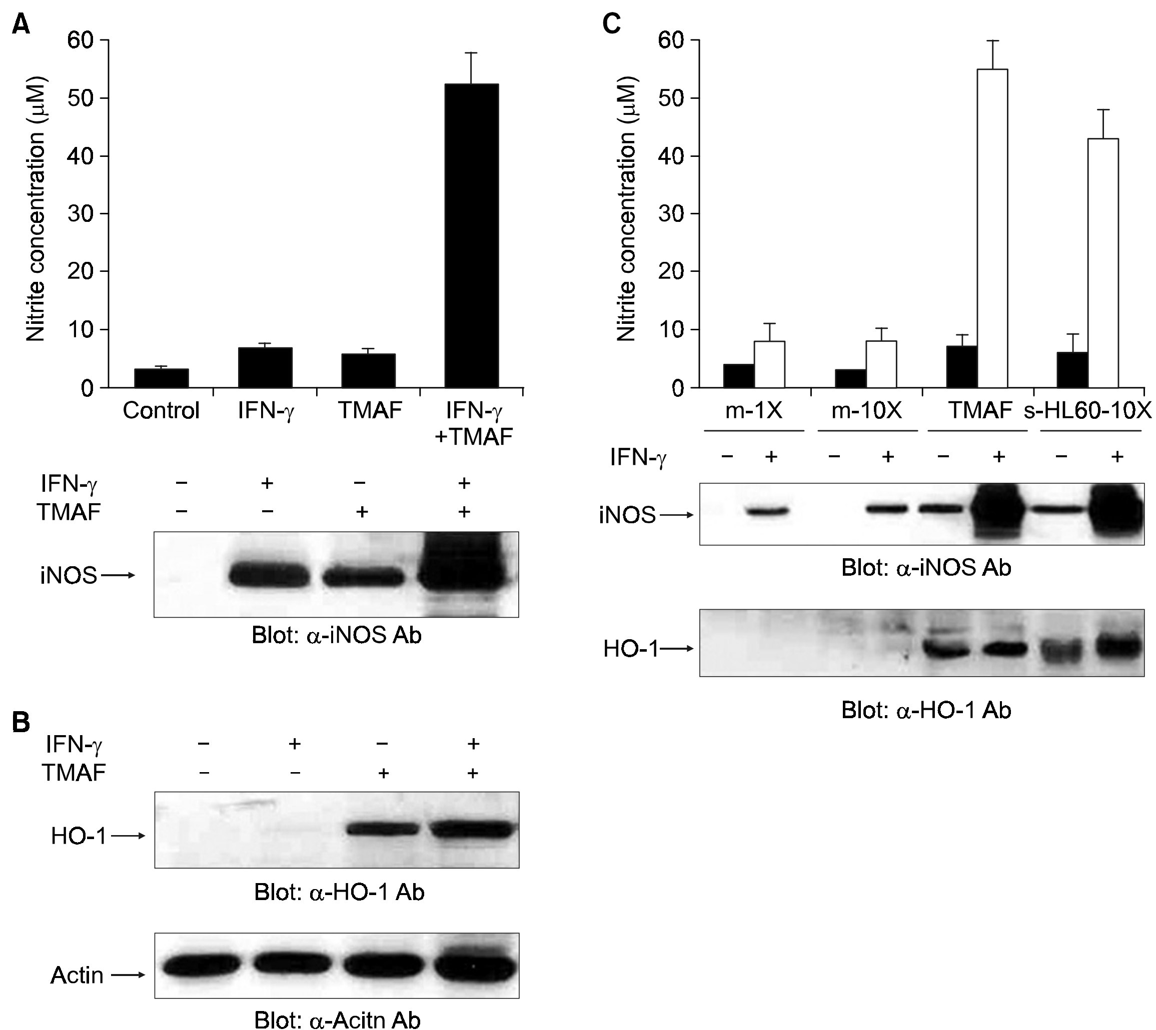

Figure 1. TMAF induces NO production and iNOS and HO-1 expression in murine macrophages. (A and B) TG-elicited macrophages (1×10^6) were cultured for 24 h with media alone (control) or media that contained with TMAF (40 μl/ml), rIFN-γ (5 u/ml), or TMAF plus rIFN-γ. After incubation, nitrite concentrations were determined by the method of Griess (*A, top*) and iNOS and HO-1 levels were detected by Western blot with the anti-iNOS (*A, bottom*) and anti-HO-1 antibodies (*B*). (C) TG-elicited macrophages (1×10^6) were cultured for 24 h with media alone (m-1X), 10-fold concentrated media (m-10X, 20 μl/ml), TMAF (40 μl/ml), or the supernatants of HL-60 cells (s-HL60-10X, 20 μl/ml) in the absence or presence of rIFN-γ (5 u/ml). Nitrite concentrations (*top*), and iNOS (*middle*) and HO-1 (*bottom*) expression were determined as described above (A and B). The experiment was repeated three times and results are expressed means ± SE for A (*top*) and C (*top*).

supernatants of cultured tumor cells either alone or in combination with rIFN-γ to induce the expression of iNOS enzyme. We utilized the supernatants of MBT-2 cells, designated as TMAF, because previously we found that these supernatants show relatively higher activity to induce NO synthesis than that of other tumor cell lines which we have screened (data not shown). Before use, the supernatants of MBT-2 cells (TMAF) were concentrated up to 10-fold as described in Materials and Methods, to minimize the volume for treatment. TG-elicited macrophages were cultured for 24 h with either TMAF (40 μl/ml) alone or TMAF in combination with rIFN-γ (5 u/ml), and then the iNOS levels and NO release were measured. As Figure 1A shows, while TMAF alone induced moderate amount of NO release and iNOS expression, TMAF in combination with rIFN-γ dramatically increased iNOS expression as well as NO synthesis. To address whether the induction of NO synthesis is correlated with HO-1 induction, we examined the expression of HO-1 after TMAF or TMAF plus rIFN-γ treatment. Western-blot analysis revealed that TMAF alone could induce HO-1 expression, and this induction was slightly increased when TMAF was combined with rIFN-γ (Figure 1B). Supernatants from HL-60 cells (s-HL60) also induced NO synthesis and iNOS and HO-1 expression, whereas the 10-fold concentrated media that were prepared as same as TMAF or s-HL60 had little effects (Figure 1C). Interestingly, while rIFN-γ-treated macrophages induced iNOS, they did not induce HO-1 expression (Figure 1B). These results let us to ask whether TMAF-mediated HO-1 induction is depended on NO production in murine macrophages. As shown in Figure 2A and B, however, HO-1 by TMAF was not affected in the presence of 1400 W (1 μg/ml), a highly selective iNOS inhibitor, indicating that NO production that was generated by either TMAF or rIFN-γ alone had no direct effect on HO-1 expression, and further suggest that TMAF by itself could induce HO-1 by a NO-independent mechanism. Treatment of macrophages with a general inhibitor for NOS, N^{G}MMA, also

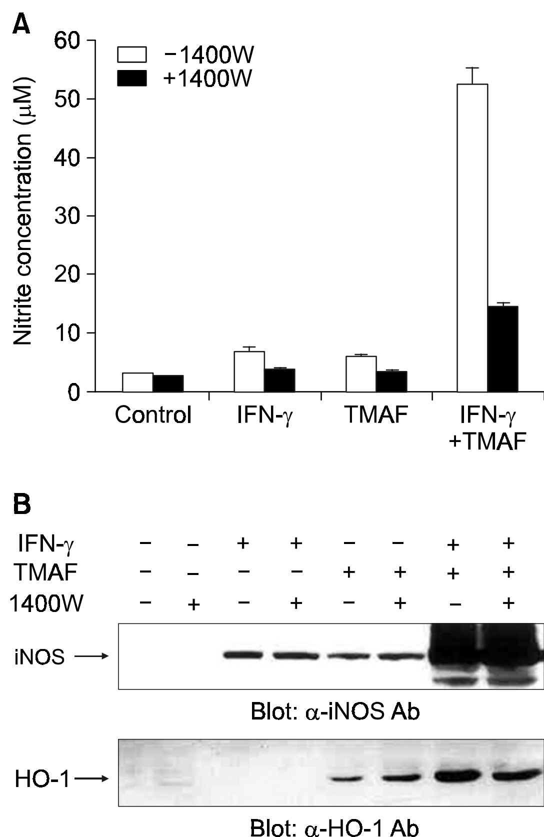

Figure 2. 1400 W, a selective iNOS inhibitor, does not affect TMAF-induced HO-1 expression in murine macrophages. TG-elicited macrophages (1×10^6) were cultured for 24 h with TMAF (40 μl/ml), rIFN-γ (5 u/ml), or TMAF plus rIFN-γ in the absence (-) or presence (+) of 1400 W (1 μg/ml). Nitrite concentrations (*A*), and iNOS (*B, top*) and HO-1 (*B, bottom*) expression were determined as described in Figure 1. The experiment was repeated three times and results are expressed means ± SE for A.

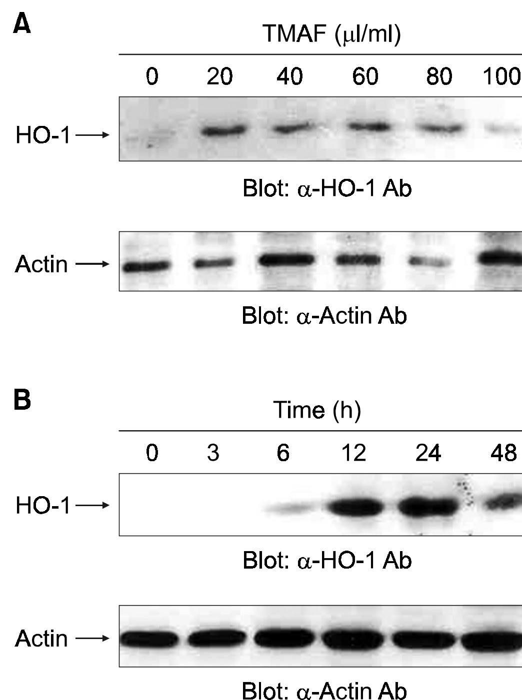

Figure 3. Dose- and time-dependent effects of TMAF on HO-1 induction in murine macrophages. (A) TG-elicited macrophages (1×10^6) were cultured for 24 h with various concentrations of TMAF (20-100 μl/ml). HO-1 expression was detected as described in Figure 1. (B) TG-elicited macrophages (1×10^6) were cultured for various times (0-48 h) with TMAF (40 μl/ml). At the indicated time points, the cells were harvested and HO-1 expression was detected as described above.

showed no effect on TMAF-induced HO-1 expression (data not shown). Dose response experiments revealed that the highest induction of HO-1 expression was seen at concentration of 60 μl/ml, and became less effective when the concentrations of TMAF reached over 60 μl/ml (Figure 3A). HO-1 was induced as early as 6 h, and reached a maximum after 24 h of treatment with TMAF (40 μl/ml) (Figure 3B).

Discussion

The present report illustrates the effect of tumor-derived soluble secretary factor on the induction of HO-1 protein in murine macrophages. HO-1 is ubiquitious and its mRNA and activity can be increased several-fold by heme, other metalloporphyrins, transition metals, and stimuli that induce cellular stress (Kutty, *et al.,* 1995; Maines, *et al.,* 1995; Matsuoka, *et al.,* 1999; Terry, *et al.,* 1999). Here, we report that a constitutively soluble activity, designated TMAF, from MBT-2 cells, induces HO-1 expression in murine macrophages. The induction of HO-1 was not reduced by the treatment with 1400 W, a highly specific iNOS inhibitor, indicating that TMAF induces the HO-1 enzyme by a NO-independent mechanism in murine macrophages.

Previous reports demonstrated that tumor cell lines, such as P815 and L1210, secrete tumor target-derived soluble factor and this factor could activate macrophages in the presence of IFN-γ or IL-2 (Jiang, *et al.,* 1992; Jiang, *et al.,* 1996). Since this soluble factor could be recognized by macrophages, they used the term 'tumor-derived recognition factor (TDRF)'. On the other hand, others also reported that the soluble products of P1HTR tumor cells could directly stimulate syngeneic or allogeneic peritoneal macrophages to produce NO (Isobe and Nakashima, 1993). Although the property of soluble factor that was generated from MBT-2 cells needs further characterization, direct activation of macrophages to induce iNOS expression is similar to that of P1HTR tumor cells.

It is particularly interesting to note that TMAF induces HO-1 protein directly in murine macrophages. Previous reports have demonstrated that HO-1 overexpression is cytoprotective by attenuating NO-mediated proinflammatory reaction cascades and HO-1 overexpression has consequently been suggested to constitute a novel therapeutic approach to disrupt inflammatory tissue deterioration in a wide range of diseases (Willis, *et al.,* 1996). Detailed analyses, however, are needed to reveal cell-type specific differences of HO-1 bioactivity. For example, in endothelial cells, HO-1 leads to a major resistance of the endothelium to oxidative stress (Motterlini, *et al.,* 1996). In macrophages, HO-1 is a physiological inhibitor of NO formation by decreasing heme availability for iNOS synthesis (Turcanu, *et al.,* 1998). In human gliomas, however, detrimental HO-1 biological activity has been described (Nishie, *et al.,* 1999). There is convincing evidence that accumulation of HO-1 expressing macrophages in the immediate vicinity of necroses in high grade gliomas is a rather detrimental mechanism that contributes to neoplastic outgrowth and tissue damage. Our current observations may appear to be most consistent with the latter finding. Moreover, because HO-mediated heme degradation is the primary mechanism for cellular CO production, reactive oxygen intermediates including CO may induce angiogenesis, and thus promote neoplastic outgrowth (Kuroki, *et al.,* 1996).

In our observations, rIFN-γ had no effect on HO-1 induction by itself, while it showed similar degree of iNOS induction and NO production as compared with TMAF. Furthermore, little effect of both 1400 W and N^GMMA on TMAF-mediated HO-1 induction indicates that the signaling pathway mediated by TMAF is distinctive from that of rIFN-γ. In an effort to understand the role of TMAF in producing NO, the supernatant obtained from MBT-2 culture medium was concentrated, fractionated by gel filtration, and then given to macrophages. The highest macrophage activating activity was obtained by the fraction with -66 kDa molecular weight (Choi, *et al.,* 2003, in press). Because above aspect of TMAF is similar to maleylated-BSA, which has been known to activate macrophages to induce NO via scavenger receptor (SR) (Matsuno, *et al.,* 1997, Alford, *et al.,* 1998), we also addressed the effect of oxidized-low density lipoprotein (Ox-LDL), an inhibitor for SR-mediated signals on TMAF-induced NO synthesis. While maleylated-BSA-induced NO release was greatly blocked by Ox-LDL, TMAF-induced NO release was not blocked by Ox-LDL (Choi, *et al.,* 2003, in press), indicating the existence of novel SR and IFN-γ-independent pathway to activate macrophages.

Although the present data provide evidence that HO-1 is expressed directly in murine macrophages by the treatment with TMAF, we can not convince whether the factor that induces HO-1 is same as TMAF, which has been named because of its property to induce iNOS expression and NO production. In addition, because TMAF is basically the mixtures of various components that were generated from MBT-2 cells, we are currently in the process of purification and characterization of TMAF activity to determine whether it consists of one or more novel components or previously characterized agents.

Proinflammatory cytokines such as IFN-γ, IL-1β, and other cytokines are modulators of the inflammatory reactions and many of them facilitate induction of the iNOS, thus could mediate excessive production

of NO (Nathan, 1992). Increased HO-1 expression by rIFN-γ may be of pathophysiological significance as a mechanism limiting the cytotoxicity of NO and reactive oxygen species generated during oxidative stress and inflammatory or immune responses. However, the role for high HO-1 expression in neoangiogenesis or detrimental effects could be considered.

In summary, our data provided convincing evidence for the *de novo* induction of HO-1 expression by TMAF through NO-independent mechanism in murine peritoneal macrophages. However, further studies are of need to resolve whether this phenomenon contributes to overall neoplastic out growth and cytoprotection or prolonged cytotoxicity.

Acknowledgement

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