Short Communication

TLR2-Mediated Expansion of MDSCs Is Dependent on the Source of Tumor Exosomes

Xiaoyu Xiang,* Yuelong Liu,[†] Xiaoyin Zhuang,*
Shuangqin Zhang,[†] Sue Michalek,[‡]
Douglas D. Taylor,* William Grizzle,[§]
and Huang-Ge Zhang*[¶]

From the Department of Microbiology & Immunology,* James Brown Cancer Center, University of Louisville, Louisville, Kentucky; the Division of Clinical Immunology and Rheumatology,[†] and the Departments of Medicine, Microbiology,[‡] and Pathology,[§] University of Alabama at Birmingham, Birmingham, Alabama; and the Robley Rex Medical Center,[¶] Louisville, Kentucky

Exosomes released from tumor cells having been shown to induce interleukin-6 release from myeloid-derived suppressor cells in a Toll-like receptor 2/Stat3-dependent manner. In this study, we show that exosomes released from tumor cells re-isolated from syngeneic mice are capable of inducing interleukin-6 in a Toll-like receptor 2-independent manner, whereas the data generated from exosomes of tumor cells having undergone numerous *in vitro* passages induce interleukin-6 in a Toll-like receptor 2-dependent manner. This discrepancy may be due to the source of tumor cells used to generate the exosomes for this study. These results suggest that exosomes released from tumor cells that are not within a tumor microenvironment may not realistically represent the role of tumor exosomes *in vivo*. This is an important consideration since frequently passing tumor cells *in vivo* is an accepted practice for studying tumor exosome-mediated inflammatory responses. (Am J Pathol 2010, 177:1606–1610; DOI: 10.2353/ajpath.2010.100245)

Communication between cells through exosomes has recently become a phenomenon of increasing interest. In particular, tumor cells communicate with immune cells, leading to immune suppression.^{1–6} One of the challenging issues is that the data generated from tumor cell-derived exosomes released from *in vitro* cultured tumor cells may not represent the real effects caused by exosomes released from tumor cells being grown under immune pressure (a true physiological microenviron-

ment). However, due to technical obstacles, it is difficult to isolate exosomes from solid tumor tissues.

The tumor microenvironment is enriched with the inflammatory factors that promote angiogenesis, turnover of the extracellular matrix, and tumor cell motility.^{7,8} Understanding the molecular mechanisms of how surrounding inflammatory factors promote tumor progression represents one of the major challenges in cancer research.

It is well-known that Toll-like receptor (TLR) stimulation leads to the induction of pro-inflammatory factors. The adaptor protein myeloid differentiation antigen 88 (MyD88) is a central adaptor required by all TLRs except TLR3. MyD88 has been found to play a critical tumorigenic role in a mouse model by upregulating the expression of tumor promoting genes, growth factors and various cytokines and chemokines.^{9,10} However, it remains to be determined whether these tumor promoting effects are mediated by specific TLRs.

Myeloid-derived suppressor cells (MDSCs) contribute to the negative regulation of immune responses during tumor progression. In addition to their suppressive effects on T cell immune responses,¹¹ MDSCs have also been reported to regulate innate immune responses by modulating natural killer cell tumor cytotoxicity¹² and macrophage cytokine profiles.^{13–15} There are a number of host factors that contribute to the expansion of MDSCs. Exosomes released from tumor cells have been shown to induce the production of MDSCs.^{5,6} Further, our data¹⁶ and others¹ support the view that tumor exosomes play a role in the expansion of MDSCs in a MyD88 dependent manner. However, our data¹⁶ does not support the idea that TLR2 plays a role in the induction of MDSCs. We used tumor exosomes released from re-isolated tumor cells from

Supported by grants from the National Institutes of Health (NIH) (R01CA116092, R01CA107181, R01AT004294 and R01CA137037); Louisville Veterans Administration Medical Center (VAMC) Merit Review Grants (H.-G.Z.); and a grant from the Susan G. Komen Breast Cancer Foundation.

Accepted for publication June 4, 2010.

None of the authors disclosed any relevant financial relationships.

Address reprint requests to Dr. Huang-Ge Zhang, Department of Microbiology & Immunology, James Brown Cancer Center, University of Louisville, Louisville, KY 40202. E-mail: H0zhan17@louisville.edu.

syngeneic mice¹⁶ to test whether the tumor microenvironment plays a role in causing this discrepancy.

Here, we demonstrate that exosomes released from tumor cells passaged *in vitro* 40 or more times function in a TLR-dependent manner; whereas, exosomes released from tumor cells passed *in vivo* in immune competent mice expand MDSCs numbers in a MyD88-dependent manner.

Materials and Methods

Mice

C57BL/6j (B6) and BALB/c mice were purchased from The Jackson Laboratory. TLR2 and MyD88 knockout mice on a B6 background were provided by Dr. Shizuo Akira (University of Osaka, Japan). All animal experiments were performed under protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Cell Culture

The TS/A cell line, 4T-1 cell line, murine mammary adenocarcinomas of spontaneous BALB/c origin, B16 melanoma tumor cells, and EL4 lymphoma cell lines of C57BL/6 origin were maintained *in vitro* at 37°C in a humidified 5% CO₂ atmosphere in air in complete medium (Dulbecco's modified Eagle's medium with 5% fetal bovine serum) as described previously.^{5,6,17} The fetal bovine serum used in cell cultures was exosome depleted by differential centrifugation using a method described previously.⁶ TS/A, 4T-1, B16, or EL4 tumor cells from 80% confluent cultures were injected subcutaneously into mice.

Mice received 1.2×10^5 tumor cells in 50 μ l of PBS per site at three sites in the flank regions. Tumor tissue was removed from mice at day 15 after injection. Tumor tissue was minced into small pieces (~ 1 mm³) in prewarmed Dulbecco's modified Eagle's medium (Gibco Invitrogen, Carlsbad, CA). The small pieces of tumor tissue were incubated for 2 hours at 37°C in the presence of 1 mg/ml of type I collagenase (Sigma, St. Louis, MO). After dissociation of the tumor cells, the primary tumor cells were then washed extensively in Dulbecco's modified Eagle's medium. The cells were cultured to 80% confluency before frozen in liquid nitrogen and stored at -80°C until used for exosome production according to a previously described method.⁶

Co-Culture of B16 Tumor Cells with CD3 Activated Spleen T Cells

Spleen T cells were isolated from 7-week-old C57BL/6j mice and purified using a previously described method.¹⁸ Purified T cells were cultured at 2×10^6 /ml in RPMI 1640 medium containing 10% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, penicillin (100 U/ml), streptomycin (100 mg/ml), and 50 μ mol/L 2-mercaptoethanol in the presence of soluble anti-CD3 (2

μ g/ml clone; 145 2C11, BD Pharmingen, San Diego, CA) for 3 days. Then, B16 tumor cells were co-cultured with 3-day cultured spleen T cells (B16:spleen T cells = 10:1) as described above for an additional 1 day. The T cells were removed from the culture media. Adhered tumor cells were washed and trypsinized. Trypsinized tumor cells were then replated for exosome production using a previously described method.¹⁷

Lipid Extraction and Heat Inactivation of Exosomes

Exosomal lipids were extracted as described¹⁹ and were analyzed by thin layer chromatography. Lipid extracts (normalized based 100 μ g of protein/sample) were resolved on silica gel plates (Fisher Scientific), and lipid analysis was performed using a solvent system of chloroform/methanol/water (65:25:4, v/v/v). The relative amount of lipids extracted from 10 μ g of exosomes or 10 μ g of exosomes heated at 100°C for 15 minutes was added to bone marrow cell cultures in the presence of 20 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF). On day 7 of culture, the amount of interleukin (IL)-6 was determined using a standard enzyme-linked immunosorbent assay (ELISA). In addition, the biological effects of the exosomes were determined by two different measurements: (1) expansion of MDSCs; and (2) quantification of phosphorylated Stat3. Details of each method used in this study are previously described.⁶

Results

Recent reports by Chalmin et al¹ suggest that tumor exosomes trigger MDSC expansion via activation of Stat3. Tumor exosomal Hsp72 triggered Stat3 activation in MDSCs in a TLR2/MyD88-dependent manner through autocrine production of IL-6.¹ However, we continue to have concerns regarding the conclusion that MDSC expansion is TLR2-dependent. It is our opinion that exosomes released from *in vitro* cultured tumor cells lacking a corresponding tumor/immune microenvironment could function differently than exosomes from tumor cells having been grown under immune pressure; therefore, we did the following experiments for comparison.

TS/A cells, 4T-1 mouse breast carcinoma tumor cells, B16 mouse melanoma cells, or EL4 lymphoma cells (1×10^5 of each) were subcutaneously injected into syngeneic mice. Tumor cells were re-isolated from tumor bearing mice 15 days after challenges. Exosomes were isolated from these tumor cells having been passed *in vitro* less than five passages and referred to as primary exosomes (P-exo). As controls, each of the cell lines cultured at least more than 40 passages before the cells were expanded on a large scale for exosome production using a method as described.⁶ The exosomes isolated from tumor cells passaged 40 or more times were referred to as cultured exosomes (C-exo).

Figure 1. TLR2-mediated tumor exosome blocking of the differentiation of GM-CSF-stimulated BM precursor cells is dependent on the source of tumor exosomes. Erythrocyte-depleted BM cells of WT B6 mice or B6 mice with MyD88 or TLR2 knockout were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 20 ng/ml recombinant mouse GM-CSF. B16 exosomes or bovine serum albumin (10 μ g/ml) was added at day 0 after the addition of the GM-CSF to the cell cultures. After 7 days in culture, the cells were analyzed by fluorescence-activated cell sorting for the expression of CD11b and Gr-1. One representative of five independent experiments is shown (A, left panel). All data are given as the average values $\pm$ SEM obtained for five samples in three independent experiments (A, right panel). B: Supernatants of 7-day cultures treated as described in A were collected, and the amount of IL-6 was measured using a standard ELISA. Data represent the mean $\pm$ SEM of five samples in three independent experiments, ** P < 0.01. To determine the effect of B16 exosome-mediated induction on phosphorylated Stat3, erythrocyte-depleted BM cells of WT B6 mice or B6 mice with a MyD88 or TLR2 knockout were treated with B16 exosomes or bovine serum albumin (10 μ g/ml) in the presence of 20 ng/ml recombinant mouse GM-CSF. After 48 hours in culture, phosphorylated Stat3 and total Stat3 were detected in cells by Western blot analysis (C, left panel). The signal intensity of each protein analyzed was quantified using an Odyssey infrared imaging system (LI-COR). The ratios of signal intensity of phosphorylated Stat3 (after normalized with total Stat3) in cells treated with either C-exo or P-exo against bovine serum albumin were calculated and plotted (C, right panel). The data are presented as the mean of three independent experiments.

Bone marrow (BM)-derived precursor cell cultures were prepared from wild-type (WT) mice or mice with a MyD88 or TLR2 knockout (KO). The precursor cells were differentiated in the presence of GM-CSF (20 ng/ml) plus either P-exo or C-exo isolated from B16 tumor cells as described previously.⁶ After seven days, cell cultures were stained with anti-CD11b and Gr-1 antibodies and analyzed using FACS. Cell culture supernatants were collected and assayed by ELISA for cytokine induction. P-exo-induced CD11bGr-1 (Figure 1A), IL-6 (Figure 1B), and phosphorylated Stat3 (Figure 1C) were fully dependent on MyD88 but not on TLR2 (Figure 1). In contrast, C-exo induced CD11bGr-1 (Figure 1A), IL-6 (Figure 1B)

and phosphorylated Stat3 (Figure 1C) were both TLR2 and MyD88 dependent.

To further determine which C-exo factors play a role in induction of IL-6, C-exo and P-Exo were heat-inactivated (100°C for 15 minutes) or were extracted with chloroform. Our data further indicate that C-exo derived factor(s) that cause the induction of IL-6 in a TLR2 dependent fashion is heat-resistant and soluble in chloroform (Figure 2A). The results were reproducible when exosomes purified from the cultured supernatants of TS/A cells, 4T-1 breast tumor cells, and EL4 lymphoma cells were used (Data not shown). Moreover, when exosomes isolated from B16 tumor cells

Figure 2. TLR2-dependent exosome factor is heat stable and chloroform soluble. Erythrocyte-depleted BM cells of WT B6 mice were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 20 ng/ml recombinant mouse GM-CSF. Lipids extracted from 10 μ g B16 exosomes, 100°C heat-inactivated B16 exosomes (10 μ g), or 10 μ g B16 exosomes without treatment were added at day 0 after the addition of GM-CSF to the cell cultures. The supernatants of 7-day cultures were collected, and the amount of IL-6 was measured using a standard ELISA (**A**). B16 exosomes purified from B16 tumor cells (passage number >40) pre-co-cultured with CD3-activated spleen T cells (CT-exo), C-exo, or bovine serum albumin (10 μ g/ml) were added at day 0 after the addition of GM-CSF to the cell cultures. Supernatants of 7-day cultures were collected, and the amount of IL-6 was measured using a standard ELISA (**B**). Data represent the mean $\pm$ SEM of five samples in three independent experiments, ** P < 0.01.

after being co-cultured with activated spleen T cells were used for induction of IL-6 released from bone marrow cells, TLR2 dependent induction of IL-6 was reversed (Figure 2B). In short, our current results are in agreement with our previously published results¹⁶ regarding the importance of MyD88 mediated activation of Stat3 and induction of IL-6 by both C-exo and P-exo tumor exosomes. However, tumor exosome-mediated activation of Stat3 and induction of IL-6 through a TLR2 dependent pathway does not occur when using tumor exosomes produced from tumor cells that are re-isolated from syngeneic mice. This conclusion also is supported by our recent finding that P-exo promote tumor metastasis in a MyD88-dependent manner.¹⁶

Discussion

The expansion of MDSCs in a tumor bearing host leading to immune suppression has been recognized as an important mechanism underlying tumor immune escape; however, the mechanisms underlying the expansion and activation of MDSCs in cancer remain unclear. Many of the mediators, including IL-6,^{1,5,6} PGE2,^{5,20} and IL-1 β ^{21–23} that are released in dysregulated chronic inflammation are found to promote the expansion of MDSCs. Our results and others¹ show that exosomes released from tumor cells promote the expansion of MDSCs, and the TLR pathway plays a role in the expansion of MDSCs. Both our data¹⁶ and data from another group¹ support the idea that MyD88, but not TRIP adaptor molecule, is required for tumor exosome-mediated expansion of MDSCs. However, unlike the results published by others,¹ we failed to demonstrate that TLR2 plays a role in tumor exosome-mediated expansion of MDSCs when P-exo was used in this study and a previous study.¹⁶ The discrepancy may be due to the source of tumor cells used to generate the exosomes for this study. One possible explanation is that *in vivo* immune pressure induces a factor(s) that plays a key role in tumor exosome-mediated activation of Stat3 and induction of IL-6 in a TLR2-independent manner whereas the driving force to induce these biological factors is lost by *in vitro* cultured tumor cells. This speculation was further supported by the fact that when exosomes isolated from B16 tumor cells, after

being co-cultured with activated T cells, were added to the bone marrow cell culture, TLR2 dependent induction of IL-6 was not observed any longer. A second possible explanation is that P-exo develops a mechanism(s) to activate the Stat3 pathway bypassing TLR2 crosslink. It is conceivable that once exosomes are taken up by recipient cells, MyD88 dependent activation of Stat3 signals may arise at the endosomal compartment instead of at the plasma membrane.^{24,25} A third possible explanation is the potential for some type of contamination of the *in vitro* cell culture, in particular, mycoplasma which leads to mycoplasma-derived lipid contaminated exosomes.^{26–28} The cell lines used in this study were checked every two passages for potential mycoplasma contamination using PCR technology and no mycoplasma were detected. However, we still cannot exclude the possibility of contamination with mycoplasma that may not be detected due to the limitation of sensitivity of the PCR or a species of mycoplasma not detectable by the PCR. Therefore, based on currently available technology, the possibility of contaminants cannot be excluded and there is no guaranteed procedure to eliminate potential mycoplasma contamination. To our knowledge, frequent re-isolation of tumor cell lines that have been injected into syngeneic mice is necessary for production of exosomes for the following reasons: 1) Exosomes released from *in vivo* passed tumor cells under immune pressure represents more closely the actual effects or functional roles of tumor exosomes, and 2) Potential contamination during cell culture, which can yield erroneous results in studies of tumor exosome-mediated inflammatory cytokine induction, can be avoided. Experiments and the results produced must mimic as closely as possible the actual *in vivo* activation to ensure interpretation of results can be trusted for application in tumor exosome-mediated chronic inflammatory disease.

Acknowledgments

We are indebted to Dr. Sam Hwang for providing the B16 and 4T-1 tumor cell lines. We thank Dr. Jerald Ainsworth for editorial assistance.

References

- Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, Boireau W, Rouleau A, Simon B, Lanneau D, De Thonel A, Multhoff G, Hamman A, Martin F, Chaffert B, Solary E, Zitvogel L, Garrido C, Ryffel B, Borg C, Apetoh L, Rebe C, Ghiringhelli F: Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J Clin Invest* 2010, 120:457–471
- Xie Y, Bai O, Yuan J, Chibbar R, Slattery K, Wei Y, Deng Y, Xiang J: Tumor apoptotic bodies inhibit CTL responses and antitumor immunity via membrane-bound transforming growth factor- β 1 inducing CD8+ T-cell anergy and CD4+ Tr1 cell responses. *Cancer Res* 2009, 69:7756–7766
- Xie Y, Bai O, Zhang H, Yuan J, Zong S, Chibbar R, Slattery K, Qureshi M, Wei Y, Deng Y, Xiang J: Membrane-bound HSP70-engineered myeloma cell-derived exosomes stimulate more efficient CD8(+) CTL- and NK-mediated antitumor immunity than exosomes released from heat-shocked tumor cells expressing cytoplasmic HSP70. *J Cell Mol Med* 2010, doi: 10.1111/j.1582-4934.2009.00851.x
- Thery C, Ostrowski M, Segura E: Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009, 9:581–593
- Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, Cheng Z, Shah SV, Wang GJ, Zhang L, Grizzle WE, Mobley J, Zhang HG: Induction of myeloid-derived suppressor cells by tumor exosomes. *Int J Cancer* 2009, 124:2621–2633
- Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, Li C, Cong Y, Kimberly R, Grizzle WE, Falkson C, Zhang HG: Tumor exosomes inhibit differentiation of bone marrow dendritic cells. *J Immunol* 2007, 178:6867–6875
- Kessenbrock K, Plaks V, Werb Z: Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141:52–67
- Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, Westmoreland SV, Chambon P, Scadden DT, Purton LE: A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. *Cell* 2007, 129:1097–1110
- Rakoff-Nahoum S, Medzhitov R: Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* 2007, 317:124–127
- Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M: Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007, 317:121–124
- Gabrilovich DI, Nagaraj S: Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009, 9:162–174
- Liu C, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR, Zhang HG: Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood* 2007, 109:4336–4342
- Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S: Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res* 70:68–77
- Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K: Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood* 2008, 111:5457–5466
- Umemura N, Saio M, Suwa T, Kitoh Y, Bai J, Nonaka K, Ouyang GF, Okada M, Balazs M, Adany R, Shibata T, Takami T: Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. *J Leukoc Biol* 2008, 83:1136–1144
- Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, Michalek S, Grizzle W, Zhang HG: Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. *Am J Pathol* 176:2490–2499
- Liu C, Yu S, Zinn K, Wang J, Zhang L, Jia Y, Kappes JC, Barnes S, Kimberly RP, Grizzle WE, Zhang HG: Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol* 2006, 176:1375–1385
- Grizzle WE, Xu X, Zhang S, Stockard CR, Liu C, Yu S, Wang J, Mountz JD, Zhang HG: Age-related increase of tumor susceptibility is associated with myeloid-derived suppressor cell mediated suppression of T cell cytotoxicity in recombinant inbred BXD12 mice. *Mech Ageing Dev* 2007, 128:672–680
- Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C, Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S: Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009, 284:34211–34222
- Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S: Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 2007, 67:4507–4513
- Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC: Overexpression of interleukin-1 β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 2008, 14:408–419
- Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S: Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res* 2007, 67:10019–10026
- Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S: Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol* 2006, 176:284–290
- Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R: TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon- β . *Nat Immunol* 2008, 9:361–368
- Xu F, Mukhopadhyay S, Sehgal PB: Live cell imaging of interleukin-6-induced targeting of "transcription factor" STAT3 to sequestering endosomes in the cytoplasm. *Am J Physiol Cell Physiol* 2007, 293:C1374–C1382
- Quah BJ, O'Neill HC: Mycoplasma contaminants present in exosome preparations induce polyclonal B cell responses. *J Leukoc Biol* 2007, 82:1070–1082
- Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS: Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood* 2007, 110:3234–3244
- Bhatnagar S, Schorey JS: Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. *J Biol Chem* 2007, 282:25779–25789