Bioorganic & Medicinal Chemistry 17 (2009) 4378–4382

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Synthetic resveratrol aliphatic acid inhibits TLR2-mediated apoptosis and an involvement of Akt/GSK3β pathway

Lin Chen [a,b,†], Yi Zhang [a,†], Xiuli Sun [c,†], Hui Li [a], Gene LeSage [a], Avani Javer [d], Xiumei Zhang [b], Xinbing Wei [b], Yulin Jiang [d,*], Deling Yin [a,*]

[a] Department of Internal Medicine, College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA
[b] Department of Pharmacology, Shandong University School of Medicine, Jinan 250012, China
[c] Department of Obstetrics and Gynecology, Peking University People's Hospital, Beijing, China
[d] Department of Chemistry, East Tennessee State University, Johnson City, TN 37614, USA

ARTICLE INFO

Article history:
Received 6 March 2009
Revised 5 May 2009
Accepted 9 May 2009
Available online 18 May 2009

Keywords:
TLR2
Resveratrol
Apoptosis
Akt
GSK3β

ABSTRACT

As resveratrol derivatives, resveratrol aliphatic acids were synthesized in our laboratory. Previously, we reported the improved pharmaceutical properties of the compounds compared to resveratrol, including better solubility in water and much tighter binding with human serum albumin. Here, we investigate the role of resveratrol aliphatic acids in Toll-like receptor 2 (TLR2)-mediated apoptosis. We showed that resveratrol aliphatic acid (R6A) significantly inhibits the expression of TLR2. In addition, overexpression of TLR2 in HEK293 cells caused a significant decrease in apoptosis after R6A treatment. Moreover, inhibition of TLR2 by R6A decreases serum deprivation-reduced the levels of phosphorylated Akt and phosphorylated glycogen synthase kinase 3β (GSK3β). Our study thus demonstrates that the resveratrol aliphatic acid inhibits cell apoptosis through TLR2 by the involvement of Akt/GSK3β pathway.

1. Introduction

Resveratrol is a phytoalexin polyphenolic compound, 3,4′,5-trihydroxy-*trans*-stibene (Fig. 1), which exists in many plants, including grapes, berries and peanuts. The compound attracts great attention due to its wide range of biological properties including antitumoric, antileukemic, and antibacterial activities. Also, it has strong antioxidative and anti-inflammatory activities associated with chemopreventive properties.[1,2] For example, resveratrol inhibits the growth of breast cancer cells.[2–4] It has also been found that the compound has potential inhibitory effects on cyclooxygenase-2.[5] Resveratrol is currently in clinical phase II trials as an anticancer drug for treatment of human colon cancer.[6] However, the mechanisms by which resveratrol affects on cell apoptosis remain to be elucidated.

Although resveratrol is a potential therapeutic agent for cancer and cardiovascular disease treatment, its solubility in water is low.[7] Thus, the derivatives of resveratrol, resveratrol aliphatic acids have been synthesized in our laboratories (Fig. 2).[7] The resveratrol aliphatic acids have been found to have greater solubility in water. In addition, the binding affinity of the compound resveratrol hexanoic acid (R6A) to human serum albumin, a drug carrier, has been increased as high as 40-fold. To explore the biological effects of the resveratrol derivative, resveratrol carboxylic acids R2A and R6A were investigated in the inhibition of Toll-like receptor 2 (TLR2).

TLRs are recognitions of pathogens in the innate immune system aimed as defending the survival of the host.[8–11] Thirteen TLRs have been identified and 10 of them are widely expressed in the human cells.[8,12–14] Mammalian TLRs play a critical role in induction of innate immune and inflammatory responses.[8,15] TLRs recognize cell wall products from various pathogens and transduce an activation signal into the cell.[8,10,16,17] For example, TLR2 and TLR4, which are expressed on the cell surface, are involved in the recognition of structures unique to bacteria or fungi. TLR2 was identified as a key immune receptor in the TLR family with a large repertoire of ligands. Many classes of microorganisms, as well as the bacterial cell wall components peptidoglycan and lipoteichoic, have been found to activate TLR2.[10,18] Overexpression of TLR2

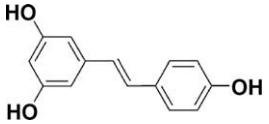

Figure 1. Structural formula of resveratrol (Res).

* Corresponding authors.
E-mail addresses: jiangy@etsu.edu (Y. Jiang), yin@etsu.edu (D. Yin).
† These authors contributed equally to this project.

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.05.029

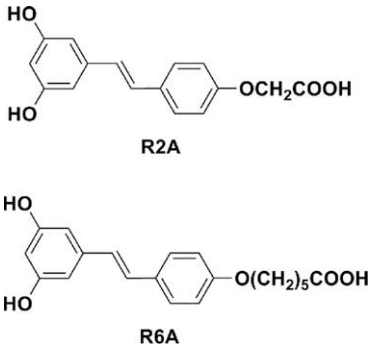

Figure 2. Structural formulae of resveratrol derivatives.

resulted in cells susceptible to serum deprivation-induced apoptosis.[19,20] However, the role of synthetic resveratrol aliphatic acids in TLR2-mediated apoptosis is currently unknown.

Recent studies have suggested that stimulation of TLRs can result in apoptosis by triggering pro-apoptotic signaling. TLR2 stimulation activates the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway.[21,22] Akt regulates cellular activation, inflammatory response, and apoptosis. Activated Akt phosphorylates several downstream targets of the PI3 K signaling pathway such as GSK3β. GSK3β is a constitutively active enzyme that is inactivated by Akt.[22,23] GSK3β plays a pivotal role in regulating many cellular functions, including cell survival and apoptosis.[22–24]

In the present study, the role of resveratrol aliphatic acids in TLR2-mediated cell apoptosis has been investigated. We showed here that resveratrol aliphatic acid R6A inhibits TLR2-mediated apoptosis involving Akt/GSK3β pathway.

2. Results and discussion

2.1. R6A inhibits the expression of TLR2

Although it is established that resveratrol inhibits cell growth,[2–4] the mechanisms by which synthetic resveratrol aliphatic acids may affect TLR2-mediated apoptosis is not yet known. As reported previously, the inhibitory effects of resveratrol in proliferation had a dose-dependent manner.[1,2] We selected 10 μM and 100 μM of compounds R2A and R6A as the appreciate concentrations for the following study. We treated HEK293 and HEK293 cells stably transfected with TLR2 (TLR2/HEK293) with R2A and R6A at different concentrations for 24 h and then subjected to serum deprivation for different time periods. As shown in Figure 3, R6A treatment dramatically decreased the expression of TLR2. However, the treatment with R2A could not alter the expression level of TLR2 (data not shown). We then focused on the study of the role of R6A in TLR2-mediated apoptosis.

2.2. R6A attenuates the number of apoptotic cells through TLR2

To examine whether R6A plays a role in TLR2-mediated apoptosis, TLR2/HEK293 and HEK293 cells were treated with or without 100 μM of R6A for 24 h and then the experimental cells subjected to serum deprivation for 0, 12, and 24 h, respectively. The apoptotic cells were determined by TUNEL assay. As shown in Figure 4, overexpression of TLR2 was susceptible to serum deprivation-induced cell apoptosis. After serum deprivation for 12 and 24 h, the percentages of the apoptotic cells in control HEK293 cells were 11% and 25%, respectively, while 20% and 49% cell apoptosis was observed in TLR2/HEK293 cells. Interestingly, the TLR2/HEK293 cells treated with R6A and exposed to serum deprivation have a significantly lower number of apoptotic cells than those exposed to serum deprivation alone (Fig. 4). These data clearly show that R6A inhibits TLR2-mediated cell apoptosis.

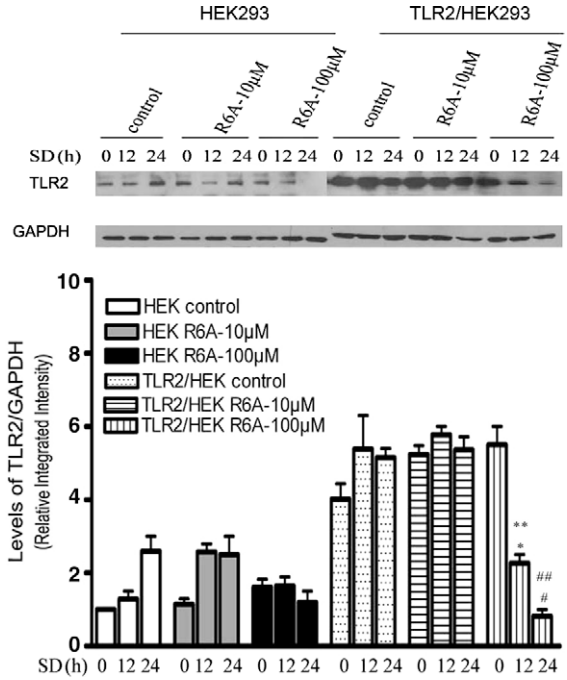

Figure 3. R6A decreases the expression of TLR2. HEK293 and TLR2/HEK293 cells were treated with R6A at 0, 10 μM, or 100 μM for 24 h and then subjected to serum deprivation (SD) for 0, 12, and 24 h, respectively. Cell lysates were probed for TLR2 expression by Western blot. Representative results of TLR2 immunoblotting are shown at the top of each pane. Results represent mean ± SEM of three independent experiments. $^{*}p < 0.01$ compared with TLR2/HEK293 cells for SD 12 h without R6A treatment group. $^{**}p < 0.01$ compared with TLR2/HEK293 cells for SD 12 h with 10 μM R6A treatment group. $^{\#}p < 0.01$ compared with TLR2/HEK293 cells for SD 24 h without R6A treatment group. $^{\#\#}p < 0.01$ compared with TLR2/HEK293 cells for SD 24 h with 10 μM R6A treatment group.

2.3. Inhibition of TLR2 by R6A inhibits serum deprivation-reduced the levels of phospho-Akt

Recent studies have revealed that phosphorylation of Akt activates the enzyme, which modulates cell growth and survival.[25,26] Akt is generally considered to promote cell survival and inhibit cell apoptosis.[26–29] We and others have demonstrated that inhibition of TLRs increases the level of phosphorylated Akt (phospho-Akt).[30,31] In addition, we and others have reported that TLRs negatively regulate Akt activity.[30,31] However, it seems that the function of TLR2, in some conditions, is not limited to negatively regulate the Akt pathway, since TLR2 also activates the Akt pathway. For example, stimulation of TLR2 induces the recruitment of active PI3K and Rac1 to the TLR2 cytosolic domain, resulting in activation of the Akt pathway. To determine whether Akt is involved in TLR2-mediated apoptosis after R6A treatment, we examined the levels of phospho-Akt at Ser^{473} in TLR2/HEK293 and HEK293 cells. We observed that serum deprivation significantly decreased the levels of phospho-Akt at Ser^{473} in the TLR2/HEK293 cells (Fig. 5). Interestingly, R6A dramatically blocked serum deprivation-reduced the levels of phospho-Akt in TLR2/HEK293 cells (Fig. 5). However, R6A did not significantly alter the level of phospho-Akt in HEK293 control cells (low level of endogenous TLR2 expression). This indicates that there are two possibilities. First, R6A also function through a pathway not involving alteration of the phospho-Akt. For example, TLR2-mediated MyD88 pathway is a key pathway in TLR2-mediated signaling pathways. While determining the exact pathway is beyond the scope of the current study it will be investigated in future. Second, our data indicated that the

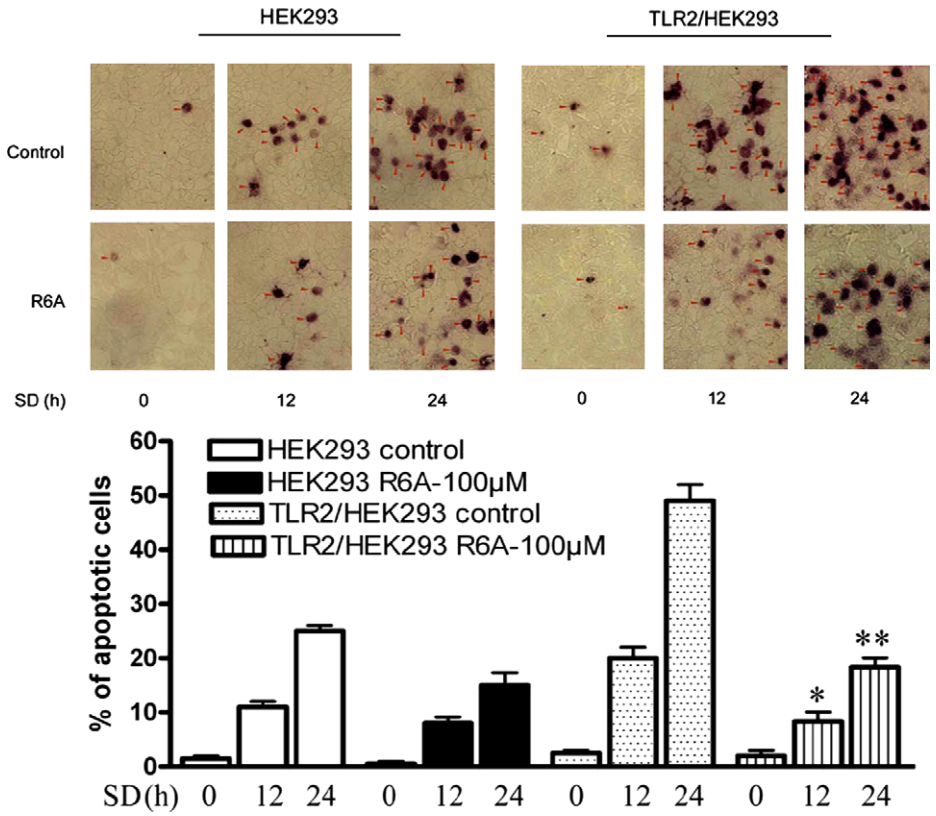

Figure 4. Role of R6A in TLR2-mediated apoptosis. We treated HEK293 and TLR2/HEK293 cells with or without 100 μM of R6A. After 24 h treatment, the cells were subjected to serum deprivation (SD) for 0, 12, and 24 h, respectively. Apoptotic cells (dark cells) were determined by TUNEL assay. Photographs of representative TUNEL-stained cells are shown at the top. Magnification 200×. The bar graph at the bottom shows the percentage of apoptotic cells. Results represent mean ± SEM of three independent experiments. $^{*}p < 0.01$ compared with TLR2/HEK293 cells for SD 12 h without R6A treatment group. $^{**}p < 0.01$ compared with TLR2/HEK293 cells for SD 24 h without R6A treatment group.

parental HEK293 cells possess a remarkable low level of endogenous TLR2 expression do not sensitize to TLR2-mediated Akt signaling. These data suggest that decrease of TLR2-mediated signaling will diminish Akt activity.

2.4. Effect of R6A on the levels of phospho-GSK3β in TLR2/HEK293 cells following serum deprivation

Glycogen synthase kinase-3β (GSK3β) is a constitutively active enzyme.[23,24] GSK3β is an important downstream target of the Akt signaling pathway.[23,24,30] Phosphorylation of GSK3β on the inactivating residue serine-9 by Akt results in GSK3β inactivation.[24,32] We determined the effect of R6A on phosphorylation of GSK-3β (phospho-GSK3β) following serum deprivation. As shown in Figure 6, serum deprivation dramatically decreased the levels of phospho-GSK3β in TLR2/HEK293 cells. Pretreatment of TLR2/HEK293 cells with R6A significantly attenuated serum deprivation-decreased the levels of phospho-GSK3β. However, neither serum deprivation nor R6A treatment could change the level of phospho-GSK3β in HEK293 control cells (Fig. 6). This suggests that R6A also function through a pathway not involving change of the phospho-GSK3β. This also suggests that HEK293 cells do not sensitize to TLR2-mediated GSK3β pathway. Since phospho-GSK3β is inhibitory for GSK3β activity, these results suggested that R6A decreases GSK3β activity through a TLR2-dependent manner.

Recent evidence demonstrated that TLRs and the Akt signaling pathways counter-regulate each other.[33,34] Our studies showed that inhibition of TLR2 by R6A increases the level of phosphorylation of both Akt and GSK3beta compared to that in the control. These data are consistent with our and other's previous findings that TLRs negatively regulates the Akt/GSK3β signaling pathways.[30,31,33,34]

3. Conclusion

In summary, our studies revealed that the compound R6A could effectively inhibit the expression of TLR2. Moreover, our results showed that R6A blocks TLR2-mediated apoptosis and an involvement of Akt/GSK3β pathway. Therefore, our data suggest that R6A is a novel and potent TLR2 inhibitor.

4. Materials and methods

4.1. Materials and instruments

Resveratrol was purchased from Sigma (St. Louis, MO, USA). Other chemicals for the synthesis and analysis were also from Sigma. The resveratrol derivatives R6A and R2A were synthesized and characterized as described previously by us.[7] The antibodies of total GSK3β, phospho-GSK3β, total Akt, and phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). The antibodies of TLR2, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

4.2. Cell culture

Human embryonic kidney HEK293 cells and HEK293 cells stably transfected with TLR2 (TLR2/HEK293) were kindly provided by Dr. Evelyn A. Kurt-Jones at the University of Massachusetts Medical School, Worcester, Massachusetts. These cell lines were grown in

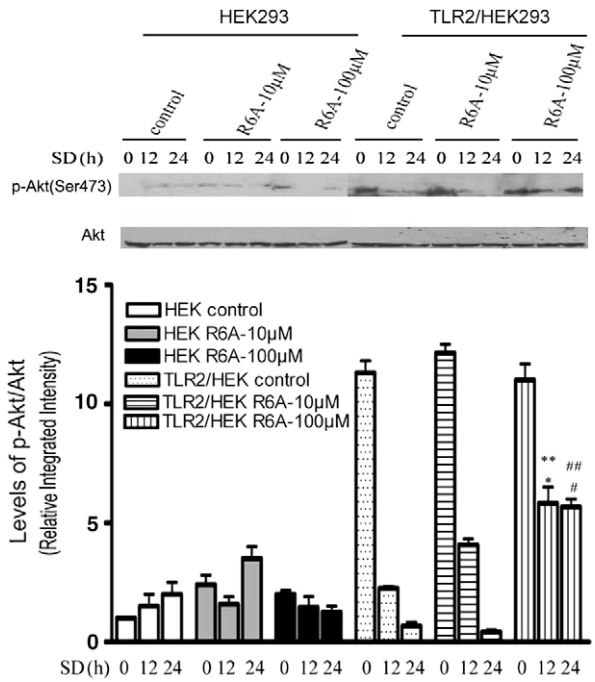

Figure 5. Effect of R6A on the levels of phospho-Akt in TLR2/HEK293 cells following serum deprivation. HEK293 and TLR2/HEK293 cells were treated with R6A at 0, 10 μM, or 100 μM. At 24 h after treatment, the cells were employed to serum deprivation (SD) for 0, 12, and 24 h, respectively. The levels of phospho-Akt and total Akt were determined by Western blot with specific antibodies. Representative results of phospho-Akt and total Akt immunoblotting are shown at the top of each pane. $^{*}p < 0.05$ compared with TLR2/HEK293 cells for SD 12 h without R6A treatment group. $^{**}p < 0.05$ compared with TLR2/HEK293 cells for 12 SD with 10 μM R6A treatment group. $^{\#}p < 0.01$ compared with TLR2/HEK293 cells for SD 24 h without R6A treatment group. $^{\#\#}p < 0.01$ compared with TLR2/HEK293 cells for SD 24 h with 10 μM R6A treatment group.

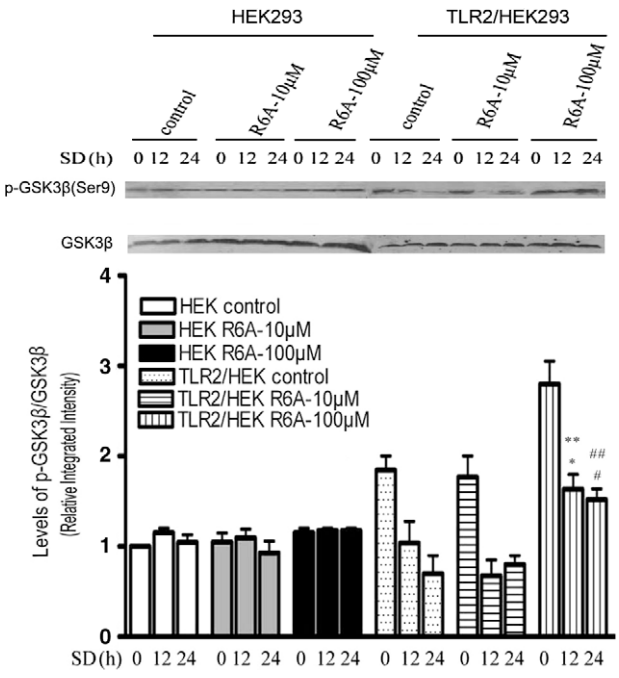

Figure 6. Inhibition of TLR2 by R6A inhibits serum deprivation-reduced the levels of phospho-GSK3β. We treated HEK293 and TLR2/HEK293 cells with R6A at 0, 10 μM, or 100 μM. After 24 h treatment, the cells were subjected to serum deprivation (SD) for 0, 12, and 24 h, respectively. The cells were harvested and the levels of phospho-GSK3β and total GSK3β were determined by Western blot with specific antibodies. Representative results of phospho-GSK3β and total GSK3β immunoblotting are shown at the top of each pane. Results represent mean ± SEM of three independent experiments. $^{*}p < 0.05$ compared with TLR2/HEK293 cells for SD 12 h without R6A treatment group. $^{**}p < 0.05$ compared with TLR2/HEK293 cells for 12 h with 10 μM R6A treatment group. $^{\#}p < 0.05$ compared with TLR2/HEK293 cells for SD 24 h in control group (without R6A treatment group). $^{\#\#}p < 0.05$ compared with TLR2/HEK293 cells for SD 24 h with 10 μM R6A treatment group.

RPMI-1640 medium (Invitrogen Corporation, Carlsland, CA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cultures were incubated at 37 °C and 5% CO_2 in a fully humidified incubator.

4.3. Detection of apoptosis by TUNEL assay

The experimental cells were treated with or without R6A for 24 h and then treated serum deprivation for 0, 12 h, and 24 h. The apoptotic cells were determined by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. Nucleosomal DNA fragmentation in cells was determined by the TUNEL assay using an in situ apoptosis detection kit (Roche Diagnostic, Indianapolis, IN) according to the manufacturer's instructions and our previous publication.[35] The number of apoptotic cells was counted in randomly selected fields to calculate the ratio of apoptotic cells and total cells.

4.4. Western blot

Western blotting was performed as described in our previous publications.[21,26] Briefly, equal numbers of cells were lysed in RIPA Lysis Buffer, which was composed of 1% Nonidet P-40, 50 mM HEPES, 150 mM NaCl, 500 μM orthovanadate (Fisher Scientific, Fairlawn, NJ USA), 50 mM $ZnCl_2$, 2 mM EDTA, 2 mM PMSF, 0.1% SDS, and 0.1% deoxycholate. The lysates were separated by 12% SDS–PAGE then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1 × TBS for 1 h. The membrane was then incubated with the blocking solution containing first antibody overnight at 4 °C. After washing three times with TBS-T for 5 min, the blot was incubated with a second antibody. The blot was again washed three times with TBS-T before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL).

4.5. Statistical analysis

All data were represented as means ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to determine where differences among groups existed. Differences were considered statistically significant for values of $p < 0.05$.

Acknowledgments

This work was supported in part by National Institutes of Health (NIH) Grant DA020120 to D. Yin. This work was also supported in part by East Tennessee State University Research Development Committee grant RD09010 and Department of Chemistry to Y. Jiang. We thank Honors College in the University for SFRA to A. Javer.

References and notes

1. Sexton, E.; Van Themsche, C.; LeBlanc, K.; Parent, S.; Lemoine, P.; Asselin, E. *Mol. Cancer* **2006**, *5*, 45.
2. Filomeni, G.; Graziani, I.; Rotilio, G.; Ciriolo, M. R. *Genes Nutr.* **2007**, *2*, 295.
3. Pozo-Guisado, E.; Alvarez-Barrientos, A.; Mulero-Navarro, S.; Santiago-Josefat, B.; Fernandez-Salguero, P. M. *Biochem. Pharmacol.* **2002**, *64*, 1375.

4. Hudson, T. S.; Hartle, D. K.; Hursting, S. D.; Nunez, N. P.; Wang, T. T.; Young, H. A.; Arany, P.; Green, J. E. *Cancer Res.* **2007**, *67*, 8396.
5. Signorelli, P.; Ghidoni, R. *J. Nutr. Biochem.* **2005**, *16*, 449.
6. Schwedhelm, E.; Maas, R.; Troost, R.; Boger, R. H. *Clin. Pharmacokinet.* **2003**, *42*, 437.
7. Jiang, Y. L. *Bioorg. Med. Chem.* **2008**, *16*, 6406.
8. Aderem, A.; Ulevitch, R. J. *Nature* **2000**, *406*, 782.
9. Medzhitov, R.; Preston-Hurlburt, P.; Janeway, C. A., Jr. *Nature* **1997**, *388*, 394.
10. Doyle, S. L.; O'Neill, L. A. *Biochem. Pharmacol.* **2006**, *72*, 1102.
11. Gan, L.; Li, L. *Immunol. Res.* **2006**, *35*, 295.
12. Akira, S.; Hemmi, H. *Immunol. Lett.* **2003**, *85*, 85.
13. Kurt-Jones, E. A.; Popova, L.; Kwinn, L.; Haynes, L. M.; Jones, L. P.; Tripp, R. A.; Walsh, E. E.; Freeman, M. W.; Golenbock, D. T.; Anderson, L. J.; Finberg, R. W. *Nat. Immunol.* **2000**, *1*, 398.
14. Sutmuller, R. P.; den Brok, M. H.; Kramer, M.; Bennink, E. J.; Toonen, L. W.; Kullberg, B. J.; Joosten, L. A.; Akira, S.; Netea, M. G.; Adema, G. J. *J. Clin. Invest.* **2006**, *116*, 485.
15. Arnold, R.; Brenner, D.; Becker, M.; Frey, C. R.; Krammer, P. H. *Eur. J. Immunol.* **2006**, *36*, 1654.
16. Zhang, G.; Ghosh, S. *J. Clin. Invest.* **2001**, *107*, 13.
17. Krishnan, J.; Selvarajoo, K.; Tsuchiya, M.; Lee, G.; Choi, S. *Exp. Mol. Med.* **2007**, *39*, 421.
18. Hussain, T.; Nasreen, N.; Lai, Y.; Bellew, B. F.; Antony, V. B.; Mohammed, K. A. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2008**, *295*, L461.
19. Li, Y.; Sun, X.; Zhang, Y.; Huang, J.; Hanley, G.; Ferslew, K. E.; Peng, Y.; Yin, D. *Biochem. Biophys. Res. Commun.* **2009**, *378*, 857.
20. Fan, W.; Ha, T.; Li, Y.; Ozment-Skelton, T.; Williams, D. L.; Kelley, J.; Browder, I. W.; Li, C. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 840.
21. Zhang, Y.; Foster, R.; Sun, X.; Yin, Q.; Li, Y.; Hanley, G.; Stuart, C.; Gan, Y.; Li, C.; Zhang, Z.; Yin, D. *J. Neuroimmunol.* **2008**, *200*, 71.
22. Hu, X.; Paik, P. K.; Chen, J.; Yarilina, A.; Kockeritz, L.; Lu, T. T.; Woodgett, J. R.; Ivashkiv, L. B. *Immunity* **2006**, *24*, 563.
23. Jope, R. S.; Johnson, G. V. *Trends Biochem. Sci.* **2004**, *29*, 95.
24. Martin, M.; Rehani, K.; Jope, R. S.; Michalek, S. M. *Nat. Immunol.* **2005**, *6*, 777.
25. Cantley, L. C. *Science* **2002**, *296*, 1655.
26. Yin, D.; Woodruff, M.; Zhang, Y.; Whaley, S.; Miao, J.; Ferslew, K.; Zhao, J.; Stuart, C. *J. Neuroimmunol.* **2006**, *174*, 101.
27. Beaulieu, J. M.; Sotnikova, T. D.; Marion, S.; Lefkowitz, R. J.; Gainetdinov, R. R.; Caron, M. G. *Cell* **2005**, *122*, 261.
28. Franke, T. F.; Kaplan, D. R.; Cantley, L. C. *Cell* **1997**, *88*, 435.
29. Luo, H. R.; Hattori, H.; Hossain, M. A.; Hester, L.; Huang, Y.; Lee-Kwon, W.; Donowitz, M.; Nagata, E.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11712.
30. Zhang, Y.; Zhang, Y.; Miao, J.; Hanley, G.; Stuart, C.; Sun, X.; Chen, T.; Yin, D. *J. Neuroimmunol.* **2008**, *204*, 13.
31. Hua, F.; Ha, T.; Ma, J.; Li, Y.; Kelley, J.; Gao, X.; Browder, I. W.; Kao, R. L.; Williams, D. L.; Li, C. *J. Immunol.* **2007**, *178*, 7317.
32. Beurel, E.; Jope, R. S. *Prog. Neurobiol.* **2006**, *79*, 173.
33. Fukao, T.; Koyasu, S. *Trends Immunol.* **2003**, *24*, 358.
34. Fukao, T.; Tanabe, M.; Terauchi, Y.; Ota, T.; Matsuda, S.; Asano, T.; Kadowaki, T.; Takeuchi, T.; Koyasu, S. *Nat. Immunol.* **2002**, *3*, 875.
35. Yin, D.; Tuthill, D.; Mufson, R. A.; Shi, Y. *J. Exp. Med.* **2000**, *191*, 1423.