Published in final edited form as:

J Neuroimmunol. 2008 August 30; 200(1-2): 71–76. doi:10.1016/j.jneuroim.2008.06.014.

Restraint Stress Induces Lymphocyte Reduction through p53 and PI3K/NF-κB Pathways

Yi Zhang^{a,*}, Robert Foster^{a,*}, Xiuli Sun^{b,*}, Qiaoqiao Yin^a, Yi Li^a, Gregory Hanley^c, Charles Stuart^a, Yili Gan^a, Chuanfu Li^d, Zhiyong Zhang^{e,**}, and Deling Yin^{a,**}

^aDepartment of Internal Medicine, Medicine, College of Medicine, East Tennessee State University, Johnson City, TN 37614, United States

^bDepartment of Obstetrics and Gynecology, Peking University People's Hospital, Beijing, China

^cDepartment of Laboratory Animal Resources, College of Medicine, East Tennessee State University, Johnson City, TN 37614, United States

^dDepartment of Surgery, College of Medicine, East Tennessee State University, Johnson City, TN 37614, United States

^eShanghai Public Health Clinical Center, Fudan University, Shanghai, China

Abstract

Restraint stress, either physical or psychological, can modulate immune function. However, the mechanisms associated with stress-induced lymphocyte reduction remains to be elucidated. We have previously shown that chronic stress induces Fas-mediated lymphocyte reduction. Here, we investigated the mechanisms by which restraint stress modulates lymphocyte reduction. Our data have shown that inhibition of p53 by the p53 inhibitor PFT-α attenuates stress-induced reduction in lymphocyte numbers. These results were verified using p53 knockout mice, suggesting a pivotal role of p53 in this process. In addition our data have indicated that PI3K/ nuclear factor kappa B (NF-κB) signaling pathway plays an important role in the stress-induced lymphocyte reduction. Our study thus demonstrates that restraint stress promotes lymphocyte reduction through p53 and PI3K/NF-κB pathways.

Keywords

Restraint stress; Mice; Lymphocyte reduction; p53; PI3K; NF-κB

1. Introduction

The effects of stress, which may occur on a daily basis, on the immune system vary with the severity of the stress. It is generally believed that moderate stress, such as routine exercise,

^{**}Corresponding author: Deling Yin, M.D., Ph.D., Department of Internal Medicine, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, Phone: 423 439 8826 (o); Fax: 423 439 6387, E-mail: yin@etsu.edu. Corresponding author: Zhiyong Zhang, MD., Shanghai Public Health Clinical Center, Fudan University, Shanghai, China, Phone: 86-21-37990333, E-mail: Zhangzy@shaphc.org.

^{*}These authors contributed equally to this project

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

enhances immune response, while chronic stress, such as long-term emotional stress, can decrease immune function and increase susceptibility to diseases (Dhabhar and McEwen, 1997; Shi et al., 2003; Reiche et al., 2004; Quan et al., 2001). This effect is at least in part due to the reduction of lymphocytes (Zorrilla et al., 2001; Yin et al., 2000; Yin et al., 2006b). Dhabhar and McEwen have shown that acute restraint stress could significantly enhance delayed-type hypersensitivity reactions (Dhabhar and McEwen, 1999), while chronic stress could decrease immune function and enhance susceptibility to diseases (Yin et al., 2000; Dhabhar and McEwen, 1997). We and others have reported that stressors modulate immune regulation through stress hormones such as endogenous opioids, rather than exclusively through glucocorticoids (Yin et al., 2000; Shi et al., 2003; Moynihan et al., 2000; Minton, 1994). Moreover, we found that physical restraint stress modulates the immune system through the cell death receptor Fas-mediated apoptotic mechanism (Yin et al., 2000; Shi et al., 2003; Yin et al., 2006b). However, the mechanisms associated with stress-induced lymphocyte reduction remains to be elucidated.

Apoptotic cell death is an active process mediated by various signaling pathways. P53 is a potent inhibitor of cell growth that can induce cell growth arrest and apoptosis (Tamura et al., 1995). Expression of wild-type p53 up-regulates the expression of Fas and renders cells sensitive to Fas mediated apoptosis in various types of tumor cells (Owen-Schaub et al., 1995). The role of p53 in the regulation of Fas expression was also observed in chemotherapy-induced apoptosis (Muller et al., 1997). These findings suggest that a Fas-mediated pathway is linked to a p53-dependent apoptotic pathway.

The phosphatidylinositol 3-kinases (PI3K) are a conserved family of signal transduction enzymes which are involved in regulating cellular proliferation and inhibit cell apoptosis (Franke et al., 1997). PI3K is an enzyme complex composed of a regulatory subunit (p85) and a catalytic subunit (p110) (Cantley, 2002). There may exist a reciprocal regulation between the PI3K pathway and p53 protein. In the immune system, impaired PI3K signaling leads to immunodeficiency, whereas unrestrained PI3K signaling contributes to autoimmunity and leukemia (Okkenhaug and Vanhaesebroeck, 2003). The PI3K inhibitors LY294002 and wortmannin have been used extensively as research tools. Intriguingly, recent studies reported that inhibition of the PI3K/Akt signaling pathway increases the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cells (Osaki et al., 2004). It has been reported that inhibition of PI3K with LY294002 or wortmannin completely eliminated the protection against septic mortality which resulted in increased serum levels of IL-2, IL-6, and TNF- α in septic mice. They also reported that inhibition of PI3K in septic mice resulted in increased splenocyte apoptosis (Williams et al., 2004). Recent studies have shown that pretreatment of neutrophils with the PI3K inhibitor LY294002 totally reversed the delay of apoptosis promoted by heme (Arruda et al., 2004). NF- κ B, the ubiquitously expressed and highly regulated dimeric transcription factor, plays a fundamental role in modulating the expression of genes responsible for innate and adaptive immunity, cell proliferation and apoptosis (Li and Verma, 2002). Among five NF- κ B isoforms in mammalian cells, the p50/p65 heterodimer is predominant in many cell types (Ghosh and Karin, 2002).

In this study, we investigated the involvement of the pro-apoptotic p53 and anti-apoptotic PI3K/NF- κ B pathways. Our data showed that restraint stress induced lymphocyte reduction is mediated through p53-mediated pathway. Furthermore, restraint stress modulates the number of lymphocytes through PI3K/NF- κ B-mediated signaling.

2. Materials and Methods

2.1. Mice

Balb/c mice were obtained from Harlan (Indianapolis, IN), p53 knockout mice on a C57BL/6 background and wild type C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

2.2. Physical restraint stress and administration of reagents

Six- to seven-week-old male mice were subjected to an established chronic physical restraint protocol used in our laboratory as well as others (Yin et al., 2000; Yin et al., 2006b; Sheridan et al., 1998; Zhang et al., 2008). Briefly, mice were placed in a 50-ml conical centrifuge tube with multiple punctures to allow ventilation. Mice were held horizontally in the tubes for 12 h followed by a 12-h rest. During the rest period food and water were provided ad libitum. Control littermates were kept in their original cage and food and water were provided only during the 12 h rest. Animal received a single intraperitoneal (ip) injection of one of the following: p53 inhibitor pifithrin- α (PFT- α) (60 μ g/mouse; Calbiochem, San Diego, CA), PI3K inhibitor LY294002 (1 mg/25 g body weight; Sigma, St. Louis, MO), or pyrrolidine dithiocarbamate (PDTTC), an antioxidant which has been shown to inhibit NF- κ B activation (Muller et al., 2000; Li et al., 2004b) (120 mg/kg/day; Sigma, St. Louis, MO). Two days after physical restraint, mice were sacrificed by CO₂ asphyxiation, and the spleens were harvested.

2.3. Western blot analysis

Cytoplasmic proteins were prepared from splenic tissues and immunoblots were performed as described previously (Yin et al., 1999; Yin et al., 2006a; Hua et al., 2007). Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ, USA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1x TBS (10 mM Tris, pH 8.0, and 140 mM NaCl) for 1 hour. The membrane was then incubated with the blocking solution containing the first antibody overnight at 4°C. The expression of p53 was detected with antibody specific to p53 (Santa Cruz Biotechnology, CA). After washing three times with TBS for 5 min, the blot was then incubated with a second antibody. The blot was again washed three times with TBS before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL).

2.4. Electrophoretic mobility shift assay (EMSA)

NF- κ B binding activity was determined as described previously (Zhang et al., 2007; Li et al., 2004a) in 15 μ l of binding reaction mixture containing 1x binding buffer, 15 μ g of nuclear proteins, and 35 fmol of double-stranded NF- κ B consensus oligonucleotide end-labeled with [γ -³²P]ATP (Amersham) using T4 polynucleotide kinase (Promega). After incubation at room temperature for 20 min, the binding reaction mixture was analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels, and the gels will be dried by Gel-Drier, scanned, and quantified by a phosphor-imaging system (Bio-Rad).

2.5. Statistical analysis

The results were presented as mean $\pm$ S.D. The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to determine where differences among groups existed. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of p53 attenuates stress-induced reduction of splenocyte numbers

To examine the role of pro-apoptotic p53 signaling in physical restraint, we subjected Balb/c mice to a 12 hours physical restraint daily for 2 days, and administrated pifithrin- α (PFT- α) as a p53 inhibitor (Liu et al., 2004) prior to physical restraint. Although treatment of mice with PFT- α did not alter the number of splenocytes in unstressed mice (Fig. 1A), administration of PFT- α partially blocked stress-induced lymphocyte reduction in the spleen (Fig. 1B), suggesting that p53 plays a role in the physical restraint stress. We next examined the level of p53 expression in splenocytes with or without physical restraint. As shown in Figure 1C, the expression of p53 in splenocytes was significantly increased at 12 hours after physical restraint. To further verify the role of p53 in restraint stress, p53 knockout mice and wild type mice (control) were subjected to a 12 hours physical restraint daily for 2 days. Dysfunction of p53 in knockout mice partially inhibits stress-induced reduction in lymphocyte numbers in the spleen (Fig. 1D). P53 knockout mice have similar splenocyte numbers to the wild type mice in the absence of restraint stress (Fig. 1E). These data indicate that restraint stress-induced lymphocyte reduction is mediated through an apoptotic p53 signaling.

3.2. Inhibition of PI3K exerts an additive effect on stress-induced splenocyte reduction

PI3K is generally considered to promote cell survival and inhibit cell apoptosis (Franke et al., 1997). We recently reported that inhibition of PI3K with LY294002 or wortmannin administration to mice significantly increased splenocyte apoptosis and cardiac myocyte apoptosis (Williams et al., 2004; Hua et al., 2007). Our published data support that PI3K plays an important role in anti-apoptotic signaling (Yin et al., 2006a). However, the role of PI3K in restraint stress-induced reduction in lymphocyte numbers is not known. To answer this question, we injected mice with the PI3K inhibitor LY294002. We found that LY294002 did not alter splenocyte numbers in unstressed mice (Fig. 2A). Interestingly, administration of LY294002 before physical restraint caused a greater reduction in splenocyte numbers than stress alone (Fig. 2B). Similar results were obtained when we employed wortmannin as a PI3K inhibitor to instead of LY294002 (data not shown). These results demonstrate that the PI3K mediated signaling pathway plays an important role in stress-induced lymphocyte reduction.

3.3. Inhibition of NF- κ B signaling exerts an additive effect on splenocyte reduction induced by stress

Nuclear factor-kappaB (NF- κ B) plays a central role in regulating the expression of genes responsible for innate and adaptive immunity, cell proliferation and apoptosis (Li and Verma, 2002; Burstein and Duckett, 2003). We have reported that NF- κ B plays an anti-apoptotic effect (Yin et al., 2006a). The effect of restraint stress on NF- κ B activation was determined by electrophoretic mobility shift assay (EMSA). As shown in Figure 3A, restraint stress significantly increased NF- κ B binding activity. We next examined the role of NF- κ B in restraint stress-induced lymphocyte reduction. Balb/c mice were subjected to a 12 hour physical restraint regimen daily for two days (Yin et al., 2000; Yin et al., 2006b). Administration of the NF- κ B inhibitor PDTC (Li et al., 2004b; Muller et al., 2000) before physical restraint caused a greater reduction in splenocyte numbers than stress alone (Fig. 3B). NF- κ B inhibitor PDTC did not change splenocyte numbers in unstressed mice (Fig. 3C). These results suggest that restraint stress-induced lymphocyte reduction is mediated through a NF- κ B signaling.

4. Discussion

Psychological and physical stress can alter the immune system in both humans and animals (Reiche et al., 2004; Frieri, 2003). Stress is a known risk factor for human diseases, such as

autoimmune diseases, infectious diseases and cancer (Shi et al., 2003; Dhabhar and McEwen, 1997; Hawkley and Cacioppo, 2004). Various experimental systems have been explored to investigate how stress changes immune responsiveness. However, little progress has been made in understanding restraint stress-induced lymphocyte reduction.

p53 has been established as an important apoptotic protein but the role of p53 in restraint stress-induced lymphocyte reduction is not known. We speculated that high levels of p53 may be responsible for the lymphocyte reduction induced by restraint stress. In this study, we demonstrated that inhibition of endogenously expressed p53 using a p53 inhibitor attenuated the stress-induced lymphocyte reduction (Fig. 1B). We confirmed this result using p53 knockout mice (Fig. 1D). Thus, these data indicate that restraint stress may initiate the apoptotic process in animals resulting in modulation of p53-mediated apoptotic signaling.

Anti-apoptotic genes inhibit cell apoptosis through activating specific signaling pathway(s), including the PI3K-mediated signaling. Activation of the PI3K-dependent signaling inhibits cardiac myocyte apoptosis (Li et al., 2004a; Matsui et al., 1999). Inhibition of PI3K-mediated pathway sensitizes cells to apoptotic stimuli (Osaki et al., 2004). It has been reported that LY294002, a PI3K inhibitor, caused inhibition of cell proliferation but not induction of cell apoptosis and that inhibition of the PI3K-mediated signaling dramatically enhanced the sensitivity of human gastric carcinoma cell apoptosis (Osaki et al., 2004). The role of PI3K-mediated signaling in stress-induced lymphocyte reduction remains to be elucidated. We speculated that inhibition of PI3K attenuates stress-induced lymphocyte reduction. To test this hypothesis we administered the PI3K inhibitor, LY294002, prior to restraint stress. We have observed that pharmacological inhibition of PI3K activity by LY294002 or wortmannin before physical restraint caused a greater reduction in lymphocyte numbers than stress did alone (Fig. 2). Therefore, restraint stress-induced lymphocyte reduction is mediated through a PI3K signaling.

NF- κ B, the ubiquitously expressed and highly regulated dimeric transcription factor, initiates transcription of genes associated with innate immune responses and inflammation (Hoshino et al., 2002). We observed that restraint stress significantly enhanced the level of NF- κ B binding activity, suggesting that restraint stress stimulates activation of the NF- κ B signaling pathway (Fig. 3A). NF- κ B is a transcription factor that controls the expression of a variety of target genes including those involved in cell proliferation, apoptosis, and inflammation (Li and Verma, 2002). We speculated that increased NF- κ B binding activity by stress as a means to reduce lymphocyte reduction in the stressed animals. We observed that inhibition of NF- κ B signaling exerts an additive effect on restraint stress-induced splenocyte reduction (Fig. 3B). Therefore, we conclude that restraint stress-induced lymphocyte reduction is mediated through a NF- κ B signaling.

In summary, to our knowledge this study is the first to demonstrate that the p53 and PI3K/NF- κ B participate in restraint stress-mediated immune function. Our data suggest that inhibition of p53 pathway or modulation PI3K/NF- κ B pathway may be an effective approach for prevention and/or treatment of stress-induced lymphocyte reduction.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grant DA020120 and East Tennessee State University Research Development Committee (ETSU RDC) 0048 to D. Yin. This work was also supported in part by ETSU RDC grant 07-026M to G. Hanley.

References

- Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C, Graca-Souza AV. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. *J. Immunol* 2004;173:2023–2030. [PubMed: 15265937]
- Burstein E, Duckett CS. Dying for NF-kappaB? Control of cell death by transcriptional regulation of the apoptotic machinery. *Curr. Opin. Cell Biol* 2003;15:732–737. [PubMed: 14644198]
- Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002;296:1655–1657. [PubMed: 12040186]
- Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav. Immun* 1997;11:286–306. [PubMed: 9512816]
- Dhabhar FS, McEwen BS. Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc. Natl. Acad. Sci. U. S. A* 1999;96:1059–1064. [PubMed: 9927693]
- Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell* 1997;88:435–437. [PubMed: 9038334]
- Frieri M. Neuroimmunology and inflammation: implications for therapy of allergic and autoimmune diseases. *Ann. Allergy Asthma Immunol* 2003;90:34–40. [PubMed: 12839111]
- Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell* 2002;109:S81–S96. [PubMed: 11983155]
- Hawkey LC, Cacioppo JT. Stress and the aging immune system. *Brain Behav. Immun* 2004;18:114–119. [PubMed: 14986706]
- Hoshino K, Kaisho T, Iwabe T, Takeuchi O, Akira S. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol* 2002;14:1225–1231. [PubMed: 12356687]
- Hua F, Ha T, Ma J, Li Y, Kelley J, Gao X, Browder IW, Kao RL, Williams DL, Li C. Protection against myocardial ischemia/reperfusion injury in TLR4-deficient mice is mediated through a phosphoinositide 3-kinase-dependent mechanism. *J. Immunol* 2007;178:7317–7324. [PubMed: 17513782]
- Li C, Ha T, Kelley J, Gao X, Qiu Y, Kao RL, Browder W, Williams DL. Modulating Toll-like receptor mediated signaling by (1-->3)-beta-D-glucan rapidly induces cardioprotection. *Cardiovasc. Res* 2004a;61:538–547. [PubMed: 14962484]
- Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol* 2002;2:725–734. [PubMed: 12360211]
- Li Y, Ha T, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, Li C. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. *Am. J. Physiol Heart Circ. Physiol* 2004b;287:H1712–H1720. [PubMed: 15142841]
- Liu X, Chua CC, Gao J, Chen Z, Landy CL, Hamdy R, Chua BH. Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice. *Am. J. Physiol Heart Circ. Physiol* 2004;286:H933–H939. [PubMed: 14766674]
- Matsui T, Li L, del M, Fukui Y, Franke TF, Hajjar RJ, Rosenzweig A. Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* 1999;100:2373–2379. [PubMed: 10587343]
- Minton JE. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *J. Anim Sci* 1994;72:1891–1898. [PubMed: 7928769]
- Moynihan JA, Karp JD, Cohen N, Ader R. Immune deviation following stress odor exposure: role of endogenous opioids. *J. Neuroimmunol* 2000;102:145–153. [PubMed: 10636483]
- Muller DN, Dechend R, Mervaala EM, Park JK, Schmidt F, Fiebeler A, Theuer J, Breu V, Ganten D, Haller H, Luft FC. NF-kappaB inhibition ameliorates angiotensin II-induced inflammatory damage in rats. *Hypertension* 2000;35:193–201. [PubMed: 10642297]
- Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH, Galle PR. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/

- ligand system and involves activation of wild-type p53. *J. Clin. Invest* 1997;99:403–413. [PubMed: 9022073]
- Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol* 2003;3:317–330. [PubMed: 12669022]
- Osaki M, Kase S, Adachi K, Takeda A, Hashimoto K, Ito H. Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN-45. *J. Cancer Res. Clin. Oncol* 2004;130:8–14. [PubMed: 14605879]
- Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol. Cell Biol* 1995;15:3032–3040. [PubMed: 7539102]
- Quan N, Avitsur R, Stark JL, He L, Shah M, Caligiuri M, Padgett DA, Marucha PT, Sheridan JF. Social stress increases the susceptibility to endotoxic shock. *J. Neuroimmunol* 2001;115:36–45. [PubMed: 11282152]
- Reiche EM, Nunes SO, Morimoto HK. Stress, depression, the immune system, and cancer. *Lancet Oncol* 2004;5:617–625. [PubMed: 15465465]
- Sheridan JF, Dobbs C, Jung J, Chu X, Konstantinos A, Padgett D, Glaser R. Stress-induced neuroendocrine modulation of viral pathogenesis and immunity. *Ann. N. Y. Acad. Sci* 1998;840:803–808. [PubMed: 9629306]
- Shi Y, Devadas S, Greenelitch KM, Yin D, Allan MR, Zhou JN. Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain Behav. Immun* 2003;17:S18–S26. [PubMed: 12615182]
- Tamura T, Aoyama N, Saya H, Haga H, Futami S, Miyamoto M, Koh T, Ariyasu T, Tachi M, Kasuga M. Induction of Fas-mediated apoptosis in p53-transfected human colon carcinoma cells. *Oncogene* 1995;11:1939–1946. [PubMed: 7478511]
- Williams DL, Li C, Ha T, Ozment-Skelton T, Kalbfleisch JH, Preisner J, Brooks L, Breuel K, Schweitzer JB. Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis. *J. Immunol* 2004;172:449–456. [PubMed: 14688354]
- Yin D, Tuthill D, Mufson RA, Shi Y. Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J. Exp. Med* 2000;191:1423–1428. [PubMed: 10770807]
- Yin D, Woodruff M, Zhang Y, Whaley S, Miao J, Ferslew K, Zhao J, Stuart C. Morphine promotes Jurkat cell apoptosis through pro-apoptotic FADD/P53 and anti-apoptotic PI3K/Akt/NF-kappaB pathways. *J. Neuroimmunol* 2006a;174:101–107. [PubMed: 16529824]
- Yin D, Zhang L, Wang R, Radvanyi L, Haudenschild C, Fang Q, Kehry MR, Shi Y. Ligation of CD28 in vivo induces CD40 ligand expression and promotes B cell survival. *J. Immunol* 1999;163:4328–4334. [PubMed: 10510372]
- Yin D, Zhang Y, Stuart C, Miao J, Zhang Y, Li C, Zeng X, Hanley G, Moorman J, Yao Z, Woodruff M. Chronic restraint stress modulates expression of genes in murine spleen. *J. Neuroimmunol* 2006b; 177:11–17. [PubMed: 16814870]
- Zhang Y, Woodruff M, Zhang Y, Miao J, Hanley G, Stuart C, Zeng X, Prabhakar S, Moorman J, Zhao B, Yin D. Toll-like receptor 4 mediates chronic restraint stress-induced immune suppression. *J. Neuroimmunol* 2008;194:115–122. [PubMed: 18192029]
- Zhang Y, Zhou G, Wang H, Zhang X, Wei F, Cai Y, Yin D. Transcriptional Upregulation of Breast Cancer Resistance Protein by 17beta-Estradiol in ERalpha-Positive MCF-7 Breast Cancer Cells. *Oncology* 2007;71:446–455. [PubMed: 17878748]
- Zorrilla EP, Luborsky L, McKay JR, Rosenthal R, Houldin A, Tax A, McCorkle R, Seligman DA, Schmidt K. The relationship of depression and stressors to immunological assays: a meta-analytic review. *Brain Behav. Immun* 2001;15:199–226. [PubMed: 11566046]

Figure 1. Inhibition of p53 partially inhibits restraint stress-induced reduction of splenocyte numbers

A. Balb/c mice aged 7 weeks were administered with PFT-alpha (60 ug/ mouse) in the absence of stress. After 2 days PFT-alpha administration, mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated. Mean values were calculated from 5 mice per group. * $p > 0.5$ versus unstressed alone without PFT- α administration. B. Balb/c mice aged 7 weeks were subjected to a 12-hour physical restraint daily for 2 days. PFT-alpha (60 ug/ mouse) was administered at 1 hour before the initiation of each stress cycle. Mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated. Mean values were calculated from 6 mice per group. * $p < 0.01$ versus stressed control mice (without PFT- α administration). C.

Balb/c mice aged 7 weeks were subjected to a 12-hour restraint stress. The expression of p53 was determined by Western blot analysis as described under “Materials and Methods”. D. Wild type mice and p53 knockout mice aged 7 weeks were subjected to a 12-hour physical restraint daily for 2 days. Mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated (5 mice per group). * $p < 0.01$ versus stressed wild type control. E. P53 knockout mice and wild type mice aged 7 weeks were in the absence of stress and total splenocytes were enumerated (4 mice per group). * $p > 0.5$ versus unstressed wild type control mice.

Figure 2. Physical restraint-induced splenocyte reduction requires PI3K

A. Six to 7 week old Balb/c mice were intraperitoneally administered with LY294002 (single injection of 2 mg/ mouse). After 2 days LY294002 administration, mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated. Mean values were derived from 5 mice per group. * $p > 0.5$ versus unstressed alone without LY294002 administration. B. Six to 7 week old Balb/c mice were subjected to 12 h physical restraint daily for 2 days. LY294002 was intraperitoneally administered before physical restraint (single injection of 2 mg/ mouse at 1 hour before the initiation of each stress cycle). Mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated. Mean values were derived from 6 mice per group. * $p < 0.01$ versus stressed control mice (without LY294002 administration).

Figure 3. Inhibition of NF- κ B exerts an additive effect on stress-induced splenocyte reduction

A. Restraint stress enhances NF- κ B binding activity. Balb/c mice aged 6–7 week were subjected to a 12 h physical restraint daily. At one day after restraint stress, the spleens were harvested and nuclear proteins were isolated for NF- κ B binding activity by EMSA as described under “Materials and Methods”. There were five mice per group. Photographs of representative bands in the EMSA are shown at the right. * $p < 0.01$ compared with unstressed mice. B. Seven to 9 week old Balb/c mice were subjected to a 12 h physical restraint daily for 2 days. PDTC as an inhibitor of NF- κ B (120 mg/kg/day) was intraperitoneally administered 1 h before the initiation of each stress cycle. Mice were euthanized by CO₂ asphyxiation and total splenocytes were enumerated. Mean values were derived from three experiments (7 mice per group). * $p < 0.01$ versus stressed control mice (without PDTC administration). C. Seven to 9 week old Balb/c mice were intraperitoneally administered with PDTC as an inhibitor of NF- κ B (120 mg/kg/day). After 2 days PDTC administration, total splenocytes were enumerated. Mean values

were derived from three experiments (5 mice per group). * $p > 0.5$ versus unstressed alone without PDTC administration.