

Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages

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Summary

Age-associated immune dysfunction, characterized by increased systemic levels of cytokines, manifests as an increased susceptibility to infections. Thus, understanding these negative regulators of the immune response has paved the way to delineating signaling pathways that impact immune senescence. In the present study, we found that miR-146a, which negatively regulated the expression of IL-1 β and IL-6, was highly expressed in aged mice. However, there was a lack of response to the stimulation of lipopolysaccharide (LPS) and proinflammatory cytokines in macrophages of aged mice. As a result, the negative feedback regulation loop with miR-146a involving down-regulation of inflammation factors was interrupted in aged mice. Aberrant NF- κ B binding to the miR-146a promoter was demonstrated to be associated with the abnormal expression of miR-146a in aged mice. The DNA methyltransferase inhibitor (5-aza-2-deoxycytidine) and the histone deacetylase inhibitor [trichostatin A (TSA)] both significantly up-regulated miR-146a transcriptional activation by altering the DNA-binding activity of NF- κ B in macrophages isolated from aged mice, which suggests that DNA methylation and histone acetylation are involved in the suppression of age-dependent miR-146a expression. Additionally, high levels of histone deacetylase (HDACs) expressions contributed to the inhibition of miR-146a expression in LPS-stimulated macrophages from aged mice *in vitro*. While the suppression of HDACs activities by TSA could improve LPS-induced inflammatory responses owing to up-regulation of miR-146a expression in macrophages from aged mice. These data indicate that the dysregulated expression of miR-146a results in the age-associated dysfunction of macrophages, and miR-146a may be a good target for the treatment of age-related inflammatory diseases.

Key words: miR-146a; immunosenescence; macrophage; inflammation; IL-6; IL-1 β .

Introduction

Aging is associated with the dysregulation of immune responsiveness, which is termed 'immunosenescence'. The elderly are more susceptible to peripheral infections with increased incidence of disability and mortality

rates for individuals 65 years of age and older (Plackett *et al.*, 2004; Plowden *et al.*, 2004). Aging is related to a proinflammatory environment characterized by constitutively elevated levels of several NF- κ B-driven proinflammatory cytokines, such as IL-6, IL-1 β , and TNF α , in the absence of overt disease conditions, the so-called inflamm-aging (Franceschi *et al.*, 2000). A higher level of proinflammatory cytokines is associated with a number of age-related degenerative diseases, such as atherosclerosis, arthritis, cancer, diabetes, osteoporosis, obesity, and metabolic syndrome. However, it remains controversial whether inflammatory mediators have a primary causal or consequential relationship or simply aggravate the pathologies of age-related disease. Thus, understanding the molecular mechanisms that regulate the expression of proinflammatory cytokines in association with aging is important for understanding the development of age-related diseases.

MicroRNAs (miRNAs) are an abundant class of highly evolutionarily conserved small (18–25 nt long) noncoding RNAs that are involved in posttranscriptional gene silencing by binding to the 3'-untranslated region of target mRNA (Ambros, 2004). MiRNAs play a key role in the regulation of diverse biological processes, such as embryonic development, infection, immune response, inflammation, and tumorigenesis (Bartel, 2004; Sempere *et al.*, 2004). MiRNAs in the innate immune response were initiated by a report identifying miR-146a as a negative feedback regulator in Toll-like receptor (TLR) signaling that targets IL-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) (Taganov *et al.*, 2006). MiRNAs have also been implicated in the control of aged and cellular senescence (Boehm & Slack, 2005; Mudhasani *et al.*, 2008; Poliseno *et al.*, 2008; Bhaumik *et al.*, 2009). Bhaumik *et al.* (2009) demonstrated that the levels of miR-146a and miR-146b increased in senescent human fibroblasts in an IL-1 α -dependent manner and inhibited excessive secretion of the inflammatory cytokines IL-6 and IL-8, thereby limiting senescence-associated inflammation. However, to date there have been few reports on the involvement of miRNAs in aging processes in mammals.

In the present study, we went to determine whether age affects the expression profile of miRNAs in mouse peritoneal macrophages stimulated with or without lipopolysaccharide (LPS), one of the most powerful bacterial virulence factors in terms of proinflammatory properties (Blais *et al.*, 2005). We found that seven miRNAs (miR-101b, miR-223, miR-142-3P, miR-M1-8, miR-146a, miR-26b, and miR-191) were up-regulated in the LPS-stimulated macrophages of young mice but not in aged group. The regulatory mechanisms of miR-146a in the LPS-stimulated responses of macrophages from aged mice were further investigated. This study demonstrated that dysregulated expression of miR-146a is involved in the age-associated dysfunction of macrophages, and miR-146a might be a good target for the treatment of age-related inflammatory diseases.

Results

MiR-146a is highly expressed in the macrophages of aged mice and does not respond to LPS stimulation

To identify miRNAs potentially involved in age-associated immune dysfunction, total RNA was isolated from the peritoneal macrophages of young and aged mice after stimulation by intraperitoneal injection of LPS.

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Using the miRCURY LNA Array platform, we profiled the expression of miRNAs in untreated and LPS-treated macrophage of two age groups. Significantly, there was a pronounced difference in miRNA profiling changes between the young and aged groups following LPS stimulation (Fig. S1A). There were 16 miRNAs up-regulated more than 1.5-fold and 17 miRNAs down-regulated less than the threshold level (0.67-fold) set in LPS-treated macrophage as compared to that untreated macrophage in young mice; however, there were only 16 miRNAs (seven up-regulated and nine down-regulated) differentially in aged mice stimulated with or without LPS, much less than those of young mice (Table S1). Interestingly, the miRNAs profile of aged groups was precisely overlapped with young group. It was supposed that the complement set of two lists, as changed miRNAs in young groups but not in aged groups, contributed to dysfunction in immune response. Six changed miRNAs were validated by real-time quantitative RT-PCR (q-PCR). As shown in Fig. S1B (Supporting information), the results confirmed by q-PCR were in agreement with those by the microarray hybridization assay.

As miR-146a has been identified as a negative feedback regulator of TLR signaling, and its expression is increased in senescent human fibroblasts (Bhaumik *et al.*, 2009), we therefore explored the function and regulation mechanism of miR-146a expression in aged mice stimulated with LPS. As shown in Fig. 1A, the mature form of miR-146a expression in macrophages from aged mice without LPS stimulation was approximately a sixfold increase as compared to young counterparts ($P < 0.01$). With LPS stimulation, miR-146a expression in the

peritoneal macrophages from young mice was up-regulated 12-fold ($P < 0.01$). However, there was no change in the miR-146a expression in macrophages from LPS-stimulated aged mice ($P > 0.05$). To estimate whether mature miR-146a expression is regulated at the miRNA processing level, miR-146a precursor (pre-miR-146a) expression was detected. As shown in Fig. 1B, the expression pattern of pre-miR-146a in young and aged animals exactly matched with that of mature miR-146a, suggesting that the abnormal expression of miR-146a in the macrophages of aged mice is largely because of aberrant regulation of miR-146a at its transcriptional level. Similar characteristics of the mature miR-146a expression in the splenocyte and serum from young and aged mice stimulated with or without LPS were also observed (Fig. 1C,D), indicating that aberrant miR-146a expression may be a systemic feature in aged mice.

miR-146a regulates the expression of IRAK1, IL-1 β , and IL-6 in aged mice

As miR-146a expression was abnormal in response to LPS stimulation in aged animals, we further determined whether miR-146a could modulate the production of the inflammatory cytokines IL-6 and IL-1 β in the peritoneal macrophage from aged mice. Target prediction algorithms and reports in the literature (Perry *et al.*, 2008; Hou *et al.*, 2009) demonstrate that IRAK1 and TRAF6, two important adaptor molecules, are the major target genes of miR-146a in human lung alveolar epithelial cells. We showed that miR-146a regulated the pro-inflammatory cytokines IL-1 β and IL-6 negatively by regulating IRAK1 expression in macrophages from aged mice (Fig. 2) with the same function as in young mice (Fig. S2), suggesting that abnormal expression of miR-146a in the macrophages of aged mice is associated with age-related defects in cytokine production.

miR-146a expression cannot be induced by high level of proinflammatory cytokine in aged mice

Given that miR-146a could regulate proinflammatory cytokine expression regardless of age, we were interested in whether the aberrant expression of miR-146a was because of the stimulation of a proinflammatory cytokine environment in aged mice. We tested the effect of IL-1 β , TNF α , and IL-6 on the expression of miR-146a in peritoneal macrophages isolated from young and aged mice by q-PCR. As shown in Fig. 3, miR-146a expression increased significantly in young mice in a cytokine dose-dependent manner, whereas much less miR-146a was induced with the same concentrations of cytokines in the macrophages of aged mice. These data suggest that because of the loss of response to inflammatory cytokines, miR-146a function in a negative feedback loop controlling the high-level expression of inflammatory cytokines fails to be exerted in aged mice.

Aberrant NF- κ B binding to the miR-146a promoter contributes to the abnormal expression of miR-146a in macrophages from aged mice

To clarify the molecular mechanisms involved in the regulation of miR-146a expression in aged mice, we analyzed the levels of transcription factor binding to miR-146a promoter. Two NF- κ B-binding sites, site I and II, are crucial for LPS-induced miR-146a expression (Taganov *et al.*, 2006; Cameron *et al.*, 2008; Lukiw *et al.*, 2008) (Fig. 4A). Using a chromatin immunoprecipitation (ChIP) assay, we observed that the binding of NF- κ B p65 to both sites I and II of the miR-146a promoter was significantly increased in macrophages of LPS-stimulated young

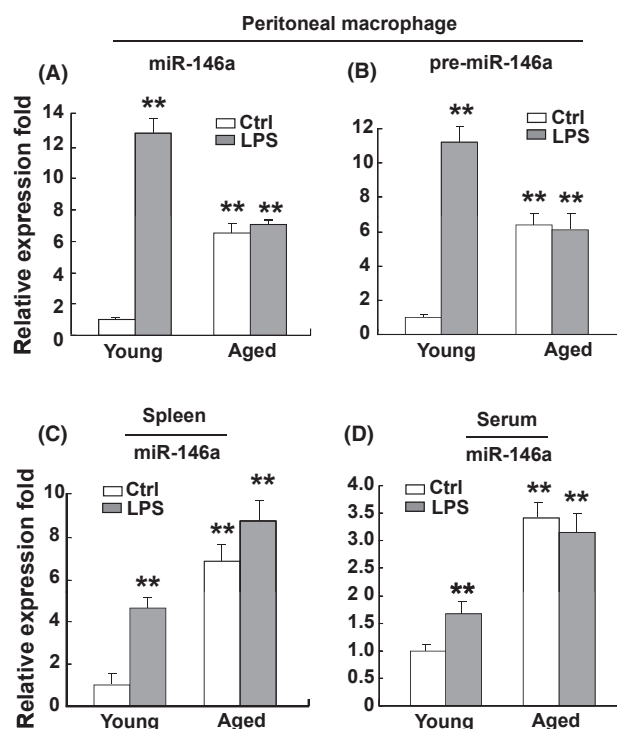


Fig. 1 Effect of LPS on miR-146a expression in macrophages from young and aged mice. The expression of mature miR-146a (A) and pre-miR-146a (B) in peritoneal macrophages, and mature miR-146a in splenocytes (C) and in serum (D) from young and aged mice ($n = 3$) stimulated with or without LPS was measured by q-PCR analysis. Total RNA was purified from the respective cell pellets and analyzed by q-PCR for the expression of miR-146a. Mature miR-146a expression was normalized to the expression of U6, and pre-miR-146a expression was normalized to the expression of β -actin. Data are the mean \pm SD of one representative experiment. Similar results were obtained in three independent experiments. ** $P < 0.01$ compared with young control cells (ctrl).

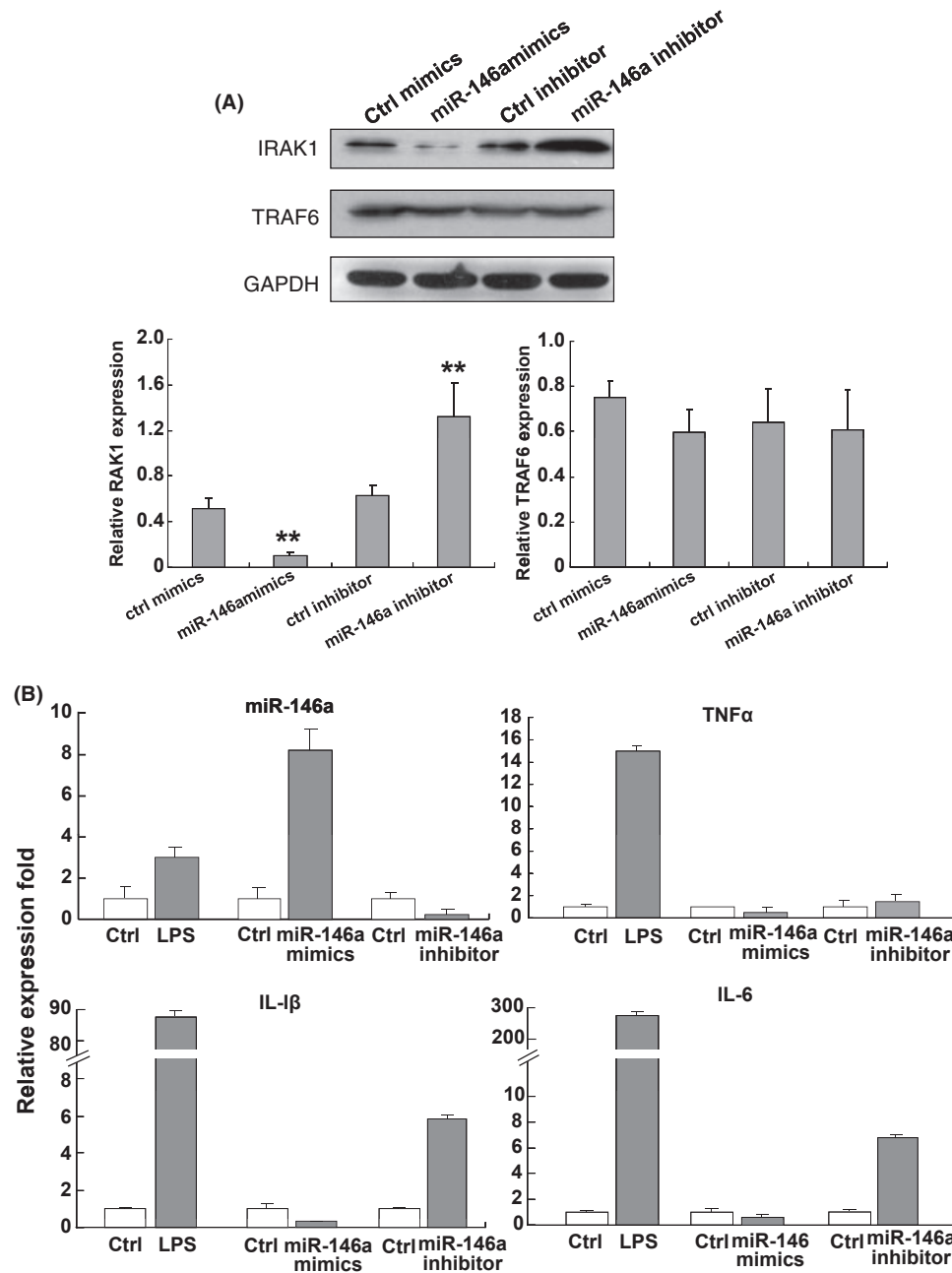


Fig. 2 MiR-146a targeted mouse IRAK1 and negatively regulated the expression of inflammatory cytokines IL-1 β and IL-6 in mouse peritoneal macrophages from aged mice. Macrophages were transfected with control (ctrl) mimics or miR-146a mimics, ctrl inhibitor or miR-146a inhibitor as indicated in material and methods at a final concentration of 10 nM. After 48 h, cells were collected for Western blot (A) and real-time PCR (B). Western blot analysis of total protein lysates was probed for IRAK1, TRAF6, and GAPDH in macrophages from young mice. GAPDH protein levels served as a loading control. The ratio of IRAK1 and TRAF6 to GAPDH band intensity for each lysate was normalized to the control ratio. IL-1 β and IL-6 were measured by q-PCR and normalized to the expression of β -actin in each sample of aged mice. Data are the mean \pm SD of one representative experiment. Similar results were obtained in three independent experiments. ** $P < 0.01$ compared with ctrl mimics or inhibitor.

mice compared with the control; however, there was a significant decrease in LPS-stimulated aged mice compared with the control ($P < 0.05$) (Fig. 4B). Interestingly, the macrophages of aged mice without stimulation with LPS showed stronger site I-binding abilities of NF- κ B than those of young mouse cells ($P < 0.01$) (Fig. 4B). Taken together, these data demonstrate that aberrant NF- κ B-binding activity to the miR-146a promoter contributes to the abnormal expression of miR-146a in the macrophages of aged mice.

DNA methylation and histone acetylation are involved in the regulation of miR-146a expression by altering the DNA-binding activity of NF- κ B p65 in aged mice *in vitro*

To clarify how aging affects miR-146a expression in response to LPS, the peritoneal macrophages were stimulated with various concentrations of LPS for a time course. As shown in Fig. 5A, the expression of miR-146a was almost not changed in the unstimulated macrophages from young

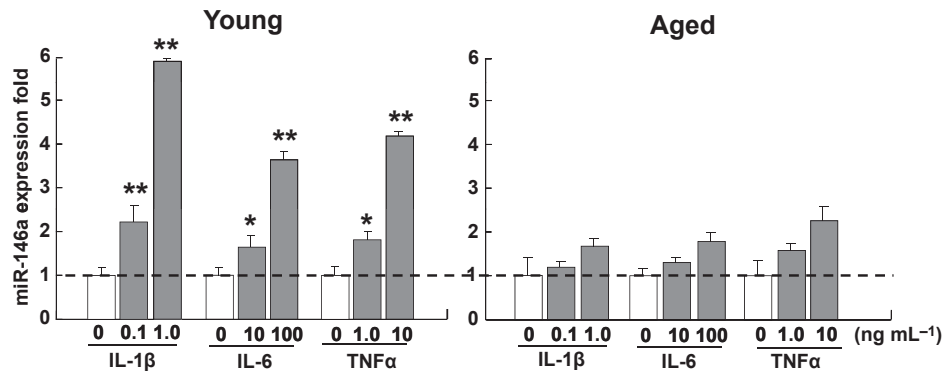


Fig. 3 Concentration dependency of IL-1 β , TNF α , and IL-6-induced miR-146a expression in young and aged mice. Macrophages were exposed to either buffer or different concentrations of IL-1 β , TNF α , and IL-6 for 24 h before the measurement of miR-146a expression. Data are the mean \pm SD of one representative experiment. Similar results were obtained in three independent experiments. * P < 0.05 and ** P < 0.01 compared with control group.

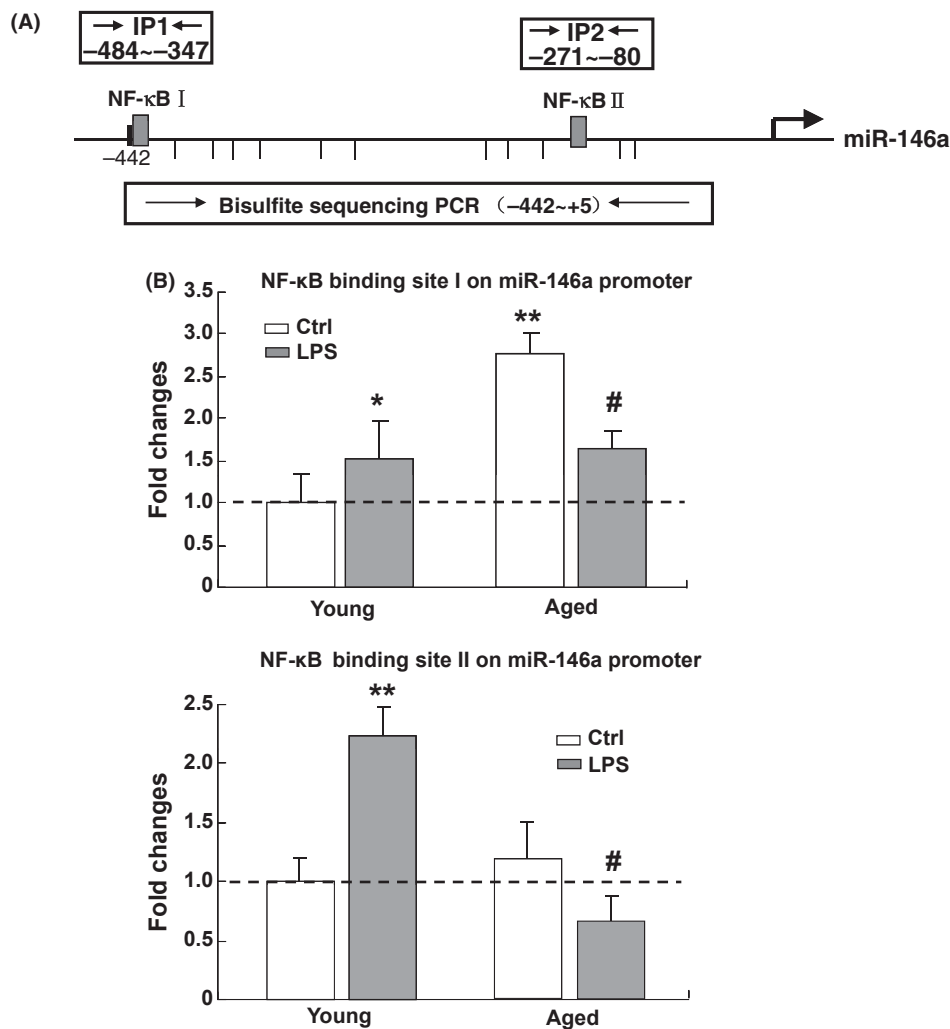


Fig. 4 (A) Schematic diagrams of the miR-146a genomic loci on mouse chromosome 5. Putative binding sites of the NF- κ B transcription factor (gray) are shown as boxes. PCR fragments amplified in bisulfite sequencing PCR and the two chromatin immunoprecipitation (ChIP) assays are also shown (blank boxes). Short vertical lines indicate the CpG sites. (B) ChIP analysis of NF- κ B p65-binding activity at the miR-146a promoter with or without LPS stimulation of macrophages. Levels of NF- κ B at the miR-146a promoter were determined by q-PCR. Data are the mean \pm SD of one representative experiment. N = 3 mice per group. Similar results were obtained in three independent experiments. * P < 0.05 and ** P < 0.01 compared with control young cells; # P < 0.05 compared with control aged cells.

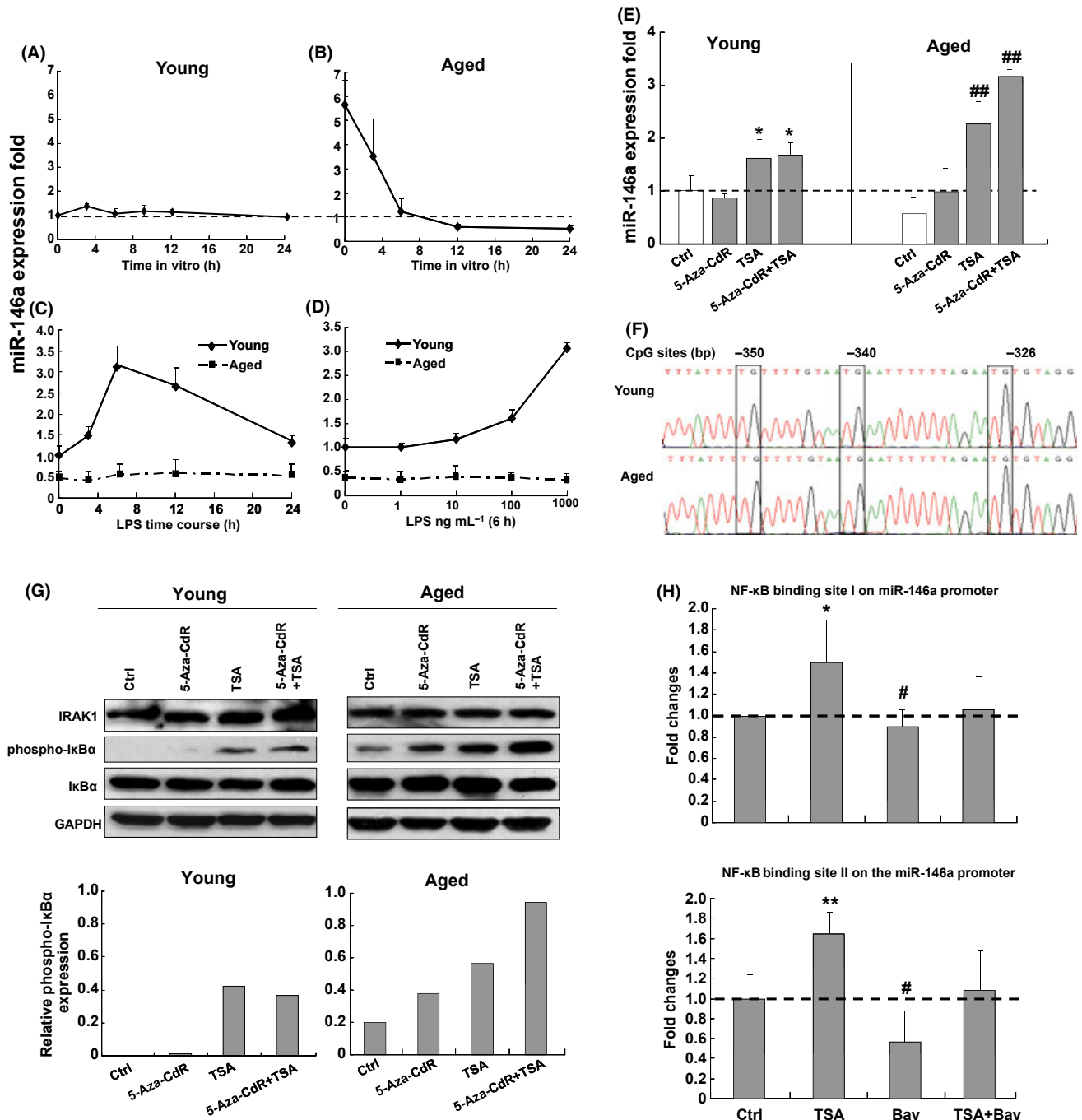


Fig. 5 Regulation of miR-146a expression by histone acetylation and DNA methylation in macrophages. (A–D) The expression of miR-146a in peritoneal macrophages from young and aged mice *in vitro*. Mouse macrophages from both age group animals were pooled and cultured in the absence (A and B) and presence of LPS (C and D). (E) Macrophages were treated with trichostatin A (TSA), 5-Aza-CdR or the combination of both for 72 h. MiR-146a levels were quantitated by q-PCR. * $P < 0.05$ compared with control young cells; ** $P < 0.01$ compared with control aged cells. (F) Representative genomic sequencing chromatograms of the miR-146a promoter from nt –350 to nt –326 for the two age groups. DNA was first treated with sodium bisulfite, and the amplified PCR products were then directly subjected to sequencing. The open boxes indicate CpG sites. (G) Phosphorylation of the NF- κ B inhibitor I κ B α and IRAK1 protein in macrophages from young and aged mice. Western blot of total protein lysates prepared from macrophages of the four groups was probed for phosphorylated I κ B α protein and for total I κ B α protein. The ratio of phosphorylated band intensity to total band intensity was normalized. (H) Chromatin immunoprecipitation analysis of NF- κ B p65-binding activity at the miR-146a promoter after treatment of the macrophages with TSA, BAY 11-7082 or the combination of both. Levels of NF- κ B at the miR-146a promoter were determined by q-PCR. Data are the mean \pm SD of one representative experiment. Similar results were obtained in three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control. # $P < 0.05$ compared with TSA-treated cells.

mice at all time points tested, but significantly higher in the unstimulated macrophages from aged mice at 0 h (when adherent macrophages were separated from peritoneal exudate cells just after nonadherent cells were

removed) (Fig. 5B), which was consistent with the result by miRNA array assay (Fig. 1). However, miR-146a expression in the cells from aged mice was sharply decreased, reaching a minimum concentration, only about

50% relative to that from young mice at 12 h and thereafter *in vitro* incubation (Fig. 5B). Additionally, a ChIP assay demonstrated that the binding of NF- κ B p65 to both sites I and II of the miR-146a promoter was significantly decreased in 12-h *in vitro* incubation of macrophages from aged mice compared with young cells ($P < 0.05$) (Fig. 53), which were quite different from those results obtained *in vivo* (Fig. 4B). The rapid decline of miR-146a expression in *in vitro* culture of the first 12 h in macrophages from aged mice may be due to that the medium used to culture macrophages could affect macrophage activation differently than autologous serum from aged mice (Gomez *et al.*, 2005). Accordingly, in the following experiments, the peritoneal macrophages had to be incubated at least for 12 h to adapt to the *in vitro* environment and then were treated with various reagents. With LPS stimulation, miR-146a expression in the macrophages from young mice transiently peaked from 4 to 6 h and then declined to control level at 24 h, while in the cells from aged mice, miR-146a expression was not altered at any time points with LPS stimulation ($P > 0.05$) (Fig. 5C). Similarly, miR-146a expression was up-regulated in a LPS concentration-dependent manner in the macrophages from young mice, but no changes were observed in the macrophages from aged counterparts ($P > 0.05$) (Fig. 5D). Taken together, these data provide evidences that aging attenuates miR-146a expression in macrophages in response to LPS stimulation *in vitro*, which were consistent with the characteristic of the macrophages from LPS-treated aged mice (Fig. 1).

To determine whether epigenetic modification is involved in the regulation of miR-146a expression in aged mice, the peritoneal macrophages were cultured respectively in the presence of DNMT inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR), histone deacetylase (HDAC) inhibitor trichostatin A (TSA), and the combination of both inhibitors. As shown in Fig. 5E, miR-146a expression in the macrophages from young mice was not affected by 5-Aza-CdR, but there was a 1.6-fold increase in expression either with TSA alone or with the combination of the two inhibitors, indicating that TSA, not 5-Aza-CdR, interferes with miR-146a expression in young mouse macrophages. Furthermore, miR-146a expression in aged mouse macrophages treated with 5-Aza-CdR or TSA increased 1.5- or 3-fold, respectively. The combination of both inhibitors resulted in a 4.2-fold increase in miR-146a expression. These data indicate that miR-146a expression is suppressed to a greater extent by epigenetic modifications in aged mouse macrophages than in young animals.

To determine whether DNA methylation is involved in the regulation of miR-146a expression, we assessed and compared DNA methylation in the miR-146a promoter region of both age groups by bisulfite sequencing PCR (BSP). As expected, all of the 11 CpG sites (Fig. 4A) were unmethylated in the miR-146a promoter of both age mice, suggesting that DNA methylation is not related to the transcriptional repression of miR-146a. Two representative DNA sequencing chromatograms containing three CpG sites are shown in Fig. 5F.

As 5-Aza-CdR enhanced miR-146a expression in macrophages of aged mice but not in the young counterparts, we deduced that hypermethylation of certain genes in the NF- κ B pathway might contribute to decreased NF- κ B activity and thereby associate with the down-regulation of miR-146a transcription in aged mice. To determine the role of epigenetics in NF- κ B activation, I κ B α phosphorylation and NF- κ B DNA-binding activity were analyzed by Western blot in macrophages treated with 5-Aza-CdR, TSA or the combination of both inhibitors. As shown in Fig. 5G, the protein expressions of I κ B α and IRAK1, an upstream protein in the NF- κ B signaling pathway, were not affected by age. The effect of these treatments on the phosphorylation of I κ B α was almost the same as miR-146a expression in the two age groups. 5-Aza-CdR could not regulate phospho-I κ B α expression, but TSA alone or the combination of both inhibitors had similar activation effects in young mice. In aged mice, the phosphorylation of

I κ B α increased 2-, 3-, and 5-fold after the three types of treatments, respectively. Taken together, these data suggest that histone deacetylation influences translocation of the NF- κ B p65 subunit to the nucleus in both age groups of mice, whereas the inhibition in aged mice is much stronger than in the young group. Age-related increase in methylation is negatively associated with NF- κ B activity, and this effect is not a result of IRAK1 inhibition in the NF- κ B signaling pathway.

NF- κ B binding to the miR-146a promoter was further confirmed by a ChIP assay in macrophages treated with TSA. As shown in Fig. 5H, NF- κ B binding to both I and II sites in the miR-146a promoter region was increased 1.6-fold in TSA-treated young mouse macrophages and decreased significantly upon treatment with the NF- κ B inhibitor BAY 11-7082 ($P < 0.05$). However, there were no significant changes in the macrophages treated with the combination of TSA and BAY 11-7082. These data indicate that TSA-induced miR-146a expression is associated with enhanced binding capacity of NF- κ B to miR-146a promoter in mouse macrophages.

HDAC suppresses miR-146a expression more strongly in LPS-treated macrophages from aged mice than that of young mice *in vitro*

As TSA enhanced miR-146a expression in unstimulated macrophages, we hypothesized that HDAC expression might correlate with miR-146a expression in LPS-treated macrophages. As shown in Fig. 6A, mRNA expressions of all 11 HDAC members in macrophages from young mice showed a time-dependent change after treatment with LPS at 1000 ng mL⁻¹. These HDACs were transiently repressed within 3 h following stimulation with LPS and gradually increased thereafter. High levels of HDAC 1, 6, 9, and 11 reached higher levels, whereas expression of the remaining HDACs returned to baseline at 24 h of stimulation. In macrophages from aged mice, HDAC mRNA expressions exhibited a much greater fluctuation compared with young mice. The basal expression levels of HDACs in aged mouse macrophages were all higher than that in young mouse cells with the exception of HDAC 3 and HDAC 4. The decline of all HDAC mRNAs in aged mouse macrophages was very rapid, and their expressions reached the lowest levels at 3 or 6 h after LPS stimulation. However, at the lowest level, the expressions of HDAC 5, HDAC 6, HDAC 7, and HDAC 11 in the macrophages of aged mice were higher than those in young mouse cells at the same time point, even slightly higher than that of the basal level in young mouse cells. At 24-h post-LPS treatment, expressions of all HDAC mRNAs in macrophages from aged animals were significantly higher than those from young mice. Notably, HDAC 11 in aged mouse macrophages increased sevenfold compared with the young counterparts at 24-h post-LPS treatment.

To investigate whether mRNA expression of HDAC determines the acetylation status of histone in LPS-stimulated macrophages, we measured the amount of acetyl-histone H3 associated with two NF- κ B-binding sites in the promoters of miR-146a after LPS stimulation. Consistent with the opposite of up-regulated mRNA expressions of HDACs, histone H3 acetylation significantly decreased at 12 and 24 h post-LPS treatment compared with unstimulated young cells, and the extent of acetylation at 24 h was lower than that at 12 h ($P < 0.05$) (Fig. 6B). In addition, acetyl-histone H3 proteins on miR-146a promoter at the three time points (0, 12 and 24 h) in aged mice were all significantly lower than those in unstimulated young cells. Moreover, inhibition of HDAC activities by TSA, as expected, resulted in a significant increase in the amount of histone H3 acetylation on miR-146a promoter at 12 and 24 h post-LPS treatment in young mice, which was consistent with the previous report that inhibition

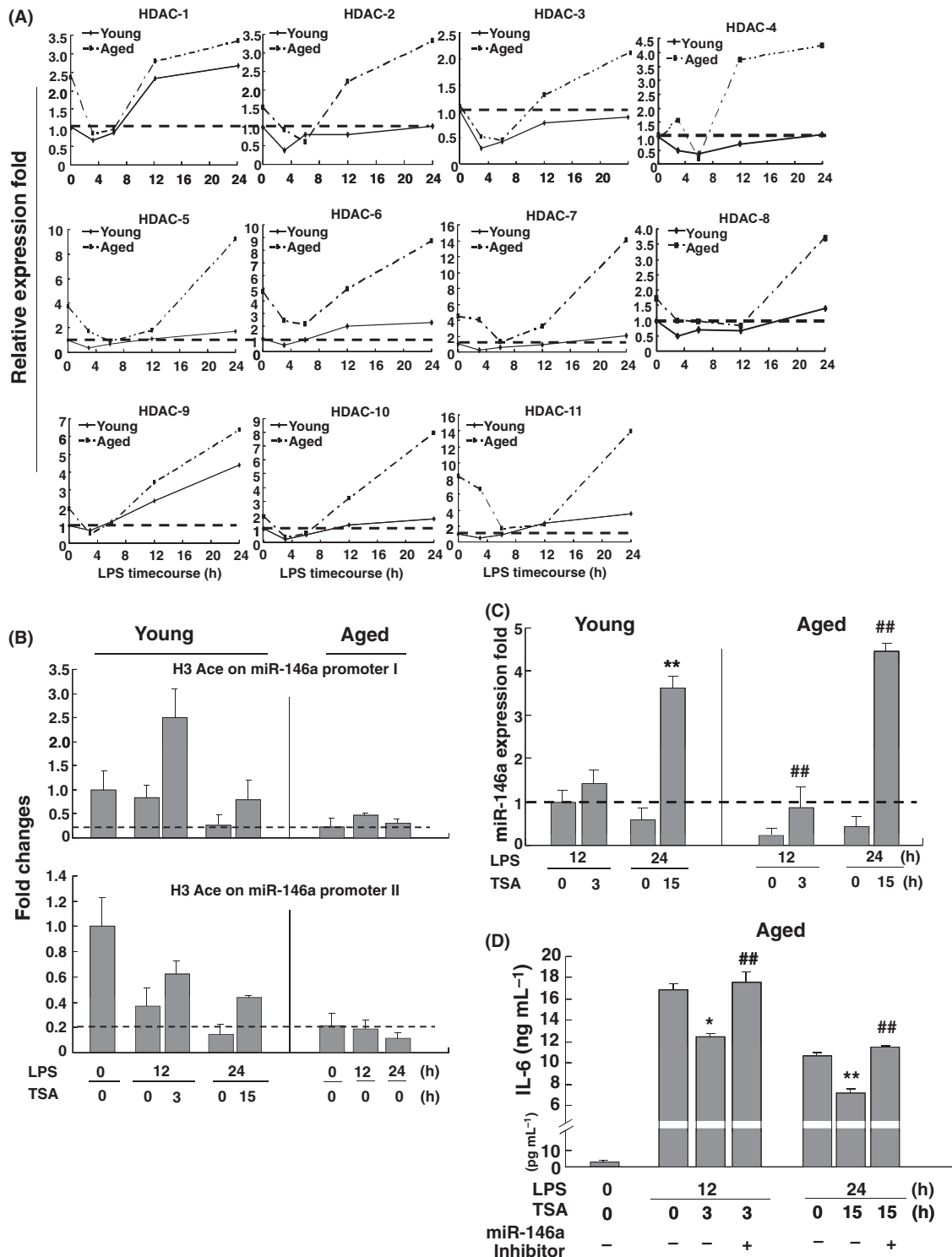


Fig. 6 The regulation of miR-146a expression by HDACs in LPS-treated macrophages from young and aged mice. (A) The HDACs mRNA expression during LPS stimulation. Macrophages were stimulated with LPS ($1.0 \mu\text{g mL}^{-1}$) for 0, 3, 6, 9, 12, or 24 h. HDAC-1-11 mRNA levels were quantitated by q-PCR. The levels of histone H3 acetylation at the miR-146a promoter were analyzed by chromatin immunoprecipitation assay (B). MiR-146a expression was measured by q-PCR (C), and IL-6 production was measured by ELISA (D) after treatment of the macrophages with LPS or trichostatin A (TSA) at 0, 12, and 24 h. After 24-h post-transfection with miR-146a inhibitor (D), macrophages were pretreated with LPS ($1.0 \mu\text{g mL}^{-1}$) for 9 h, and then treated with medium or TSA for 3 and 15 h. Data are the mean \pm SD of one representative experiment. Similar results were obtained in three independent experiments. $^{**}P < 0.01$ compared with LPS stimulation for 24 h but without TSA stimulation in young macrophages; $^{##}P < 0.01$ compared with LPS stimulation but without TSA stimulation in aged macrophages (C). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with LPS stimulation for 12 or 24 h but without TSA stimulation; $^{##}P < 0.01$ compared with TSA stimulation and LPS stimulation for 12 or 24 h in macrophages from aged mice (D).

of HDAC activity by TSA resulted in hyperacetylation of core histones in mammalian cells (Yoshida *et al.*, 1990).

To determine whether histone acetylation was responsible for a time-dependent alteration of miR-146a expression in LPS-stimulated macrophages, the effect of TSA on miR-146a expression was assessed with different time points following LPS stimulation. As shown in Fig. 6C, miR-146a expression in the macrophages from young mice increased a small amount at 12 h and then rapidly increased threefold at 24 h after TSA treatment ($P < 0.01$). These data suggest that histone modification is involved in the regulation of LPS-induced miR-146a expression in young macrophages in a time-dependent manner. However, TSA showed stronger reversal effects on miR-146a expression in macrophages from aged mice than those from young animals, increasing threefold at 12 h and then quickly reaching 21-fold at 24 h after stimulation ($P < 0.01$), suggesting that excessive suppression of miR-146a transcription by low levels of histone acetylation at the promoter resulted in miR-146a lack of response to LPS stimulation in macrophages of aged mice.

As miR-146a was associated with the cytokine production capacity in macrophages, we finally evaluated the effects of miR-146a on LPS-induced inflammatory responses in TSA-treated macrophages from aged mice. As shown in Fig. 6D, the production of IL-6 was elevated by LPS stimulation at either 12 or 24 h and was down-regulated by TSA, which showed stronger effect of inhibition at 24 than 12 h, in macrophages from aged animals. Knockdown of miR-146a expression by pretreatment with miR-146a inhibitor for 24 h followed by LPS and TSA treatment restored the levels of IL-6 in TSA/LPS-treated macrophages from aged mice, suggesting that TSA repression of IL-6 production in LPS-activated macrophages owes to up-regulation of miR-146a expression. Taken together, potent suppression of miR-146a transcription by histone deacetylation in aged mice was involved in the increased LPS-induced inflammatory response.

Discussion

The inflamm-aging process has been recognized as a part of the aged process in humans and experimental animals (Franceschi *et al.*, 2000). Serum levels of inflammatory cytokines, including IL-6 and TNF α , are constitutively elevated in the peripheral blood of older individuals. This increased production of inflammatory mediator is associated with persistent low-grade innate immune response that may augment tissue damage caused by infection in elderly individuals. Although this proinflammatory phenomenon in aged bodies is widely recognized, the mechanisms underlying this process remain largely unknown.

In the present study, we aimed to determine whether miRNAs are connected to the immune dysfunction associated with aging in macrophages. A miRNA array assay was used to examine which miRNA was associated with abnormal proinflammatory cytokine production in macrophages of aged mice. We showed that miR-146a, a feedback negative regulator of TLR-signaling pathways, could not be induced by LPS stimulation, but maintained at a higher expression level in macrophages of aged mice regardless of LPS stimulation compared with young cells. Abnormal binding of NF- κ B to the promoter of miR-146a was demonstrated to be associated with aberrant miR-146a expression in macrophages from aged mice *in vivo*. Furthermore, inhibition of HDAC activity by TSA contributed to significantly enhancing NF- κ B activity and hence up-regulating the NF- κ B-dependent miR-146a promoter activity in unstimulated macrophages from aged animals. Moreover, histone deacetylation was crucial for the inhibition of miR-146a expression in LPS-stimulated macrophages from aged mice, whereas inhibition of HDAC activity by TSA could improve LPS-induced inflammatory response

because of up-regulation of miR-146a expression in LPS-treated aged macrophages. These results indicated that miR-146a dysregulation failed to efficiently control the high level of proinflammatory cytokine expression in aged mice under normal or inflammatory conditions.

The innate immune system is composed of a network of cells including neutrophils, NK cells, macrophages, and dendritic cells that mediate the earliest interactions with pathogens. Age-associated defects in LPS-induced IL-6 and TNF α are observed in the activation of all of these cell types (Gomez *et al.*, 2007, 2009; Turnbull *et al.*, 2009). It is conceivable that age-associated *in vivo* defects in macrophage cytokine production can be reconciled with a proinflammatory environment that may result in enhanced basal levels of cytokines and chemokines. In our studies, the age-associated miR-146a expression in macrophages was also consistent with that in the serum of aged mice. Notably, there is a close parallel between miR-146a expression and inflammatory cytokine secretion in aged mice (both elevated inflammatory cytokine secretion and miR-146a expression), which seems that miR-146a is sensitive to proinflammatory environment and creates a negative feedback loop to limit cytokine expression. However, we demonstrated that the ability of macrophages to mount miR-146a expression response to proinflammatory cytokines was attenuated in aged mice (Fig. 3), which suggests that some crucial inhibitory mechanisms might be involved in the age-dependent alterations of miR-146a expression.

The current view suggests that miRNA expression is mainly controlled at its transcriptional level (O'Donnell *et al.*, 2005; Zhao *et al.*, 2005). However, like other RNAs, miRNA expression could potentially be controlled at its posttranscriptional level in *Caenorhabditis elegans* (Ambros *et al.*, 2003) and in mammals (Obernosterer *et al.*, 2006). In the present study, the expression trends of pre-miR-146a were similar to the mature miR-146a in young and aged mouse macrophages treated with or without LPS (Fig. 1), which suggests that there is no differential processing of precursor miRNAs into mature miRNAs with age. Recent reports demonstrated the NF- κ B-dependent induction of miR-146a expression by TLR signaling (Taganov *et al.*, 2006; Cameron *et al.*, 2008; Lukiw *et al.*, 2008). Although details about the mechanisms of immunosenescence have yet to be elucidated, many studies have been conducted in aged humans to establish a clear correlation between an increase in the constitutive activation of NF- κ B and chronic inflammation (Helenius *et al.*, 1996; Spencer *et al.*, 1997; Kim *et al.*, 2002; Sarkar & Fisher, 2006). Our studies demonstrated that an appreciable rise in miR-146a expression was correlated with an age-dependent augmentation of the binding of NF- κ B p65 to the miR-146a promoter in macrophages from aged mice (Fig. 4B). Moreover, aging decreased the binding ability of NF- κ B to miR-146a in LPS-treated macrophages (Fig. 4B), which is consistent with a lack of induction of miR-146a expression by LPS *in vivo* (Fig. 1). The result resembled the observations of Boehmer *et al.* (2005) who showed that NF- κ B activation was significantly decreased in macrophages from aged mice after exposure to LPS. The change in NF- κ B binding to the miR-146a promoter could explain to a large extent why miR-146a expression was abnormal in macrophages from aged animals with or without LPS stimulation.

The molecular mechanism that links aging and the change in NF- κ B activation is still poorly understood. Recent studies have shown that histone deacetylase inhibitors could prevent excessive inflammatory response by regulating the activation of the transcription factor NF- κ B in models of systemic immune activation *in vitro* and *in vivo* (Blanchard & Chipoy, 2005; Dokmanovic & Marks, 2005; Glozak *et al.*, 2005; Aung *et al.*, 2006; Calao *et al.*, 2008). To study epigenetic regulatory mechanism of miR-146a associated with NF- κ B activity during aging, we further confirmed the expression features of miR-146 in LPS-treated macrophage-

es from young and aged mice *in vitro*. MiR-146a expressions levels were relatively stable in macrophages from young mice during 24 h of unstimulated incubation (Fig. 5A). The small but not significant increase with time (3 h only) in culture is most likely due to the activation of NF- κ B during separation and the presence of FCS or other external signals in the culture. Surprisingly, miR-146a expression in macrophages isolated from aged mice rapidly declined within 12 h of incubation without stimulation, then stabilized at nearly half of the level of young mice (Fig. 5B). The decrease in miR-146a with time in cultured macrophages, perhaps, was because they were out of the stimulus in internal environment of old bodies (for example, radical oxidative species), which could induce constitutive activation of miR-146a, and therefore exhibited an original defect in the ability of miR-146a expression in aged macrophages. Many of the circulating levels of these agents and factors change with advanced age and can have profound effects on macrophage function. Hence, removing macrophages from the age-specific microenvironment by adherence to plastic or attachment to antibodies for magnetic bead selection could induce changes in the macrophage, rendering it unlike macrophages *in situ* (Gomez *et al.*, 2005).

The importance of histone deacetylation is evident from the ability of TSA to prevent the decline in miR-146a levels by increasing NF- κ B binding to miR-146a promoter in macrophages without LPS exposure from both age groups. Moreover, TSA showed a stronger effect on the induction of NF- κ B activity and miR-146a expression in macrophages from aged mice than those from young mice (Fig. 5G). It remains unclear whether up-regulated miR-146a by TSA is because of direct enhancing NF- κ B binding to miR-146a promoter associated with acetylated histone or increasing NF- κ B activity triggered by TSA and thus up-regulating NF- κ B/DNA binding. An interesting direction for further research would be to evaluate an association between histone acetylation and NF- κ B DNA-binding activity in unstimulated macrophages.

With LPS stimulation for 24 h, a significant negative correlation between expression profiles of HDACs and miR-146a was observed in young mice. Despite more intense of the fluctuations of HDACs mRNA levels in LPS-stimulated macrophages from aged mice than those from young mice, the lowest expression level of several HDACs was still higher than those of unstimulated young cells (Fig. 6A). The ChIP studies of the pattern of histone H3 acetylation across miR-146a promoter regions were further supported by our observation that the amount of histone acetylation at miR-146a promoter has a positive correlation with the levels of LPS-induced miR-146a expression in macrophages from young mice (Fig. 6B,C). Recent studies have demonstrated that NF- κ B activity was not altered by TSA in LPS-treated macrophage, including nuclear translocation of both p65 and p50 and activation of luciferase reporter expression driven by NF- κ B (Lu *et al.*, 2009). Therefore, we supposed that TSA enhanced miR-146a expression in LPS-treated macrophages by up-regulating the levels of histone acetylation and subsequently inducing the transcription of miR-146a. Accordingly, histone deacetylation in macrophages from aged mice might be involved in the transcriptional repression of miR-146a despite the induction of NF- κ B activity with LPS stimulation (data not shown). These observations suggest that histone modification is an important regulator of proinflammatory gene expression, which is consistent with reports by Sacconi *et al.* (2002).

Recent studies have shown that the activities of histone acetyltransferase (HAT) and HDAC change in response to LPS stimulation in macrophages (Barnes *et al.*, 2005). Thus, the response of a specific gene expression to LPS stimulation may be regulated by the balance of HAT and HDAC activities in the particular promoter region. Trichostatin A has been shown to enhance the LPS-induced expression of Cox-2, Cxcl2, and Lfit2 in bone marrow-derived macrophages

(Aung *et al.*, 2006). A new HDAC inhibitor KBH-A42 showed anti-inflammatory properties *in vitro* via suppression of the production of TNF α and nitric oxide (NO) by dose-dependent increase in DNA binding with AP-1 and decrease in p38 phosphorylation in macrophages (Choi *et al.*, 2008). In the present study, we found that knockdown of expression of miR-146a restored the levels of IL-6 in TSA/LPS-treated cells in macrophages from aged mice, suggesting that TSA diminishes the production of IL-6 in LPS-treated macrophages from aged mice by up-regulating miR-146a expression (Fig. 6D). This is the first report connecting age-associated changes in immune response with aberrant transcription regulation of miR-146a by histone modification.

In summary, we demonstrated for the first time that abnormal miR-146a expression contributes to the age-associated dysfunction of macrophages because of their attenuated functional adaptation to age-dependent changes in tissue environments. Using HDAC inhibitors, miR-146a may be a suitable therapeutic target for the maintenance of functional plasticity in macrophages and thus may enhance both innate and adaptive immunities in the elderly.

Materials and methods

Animal model and peritoneal macrophage isolation

Young (2-month-old) and aged (18- to 20-month-old) male BALB/c mice were obtained from the Institute of Zoological Sciences, Chinese Academy of Medical Sciences (Beijing). Animals were free of potential endemic viral pathogens that could influence their inflammatory response. They were housed and fed under similar conditions as at their respective sources in the Department of Laboratory Animal Research on a 12:12 h-light/dark cycle with *ad libitum* access to water and rodent chow. All animal procedures were approved by the Committee on the use and care of animals, Chinese Academy of Medical Sciences. For elicitation of peritoneal macrophages, all four groups of mice were injected intraperitoneally (i.p.) with 2 mL of 3% thioglycollate (Difco, Detroit, MI, USA). Two days later, mice of both ages were divided into two groups, the control group and the LPS-treated group. Mice were injected i.p. with 3 mg kg⁻¹ of LPS (*E. coli* 0111:B4 in sterile, pyrogen-free saline) (Sigma Chemical Co., St Louis, MO, USA). This dose has been shown to produce a mortality of approximately 50% in aged mice and no mortality in young animals. Control mice received saline alone. After 24 h, mice were killed by CO₂ inhalation followed by cervical dislocation. At the time of killing, cardiac blood was collected and serum was obtained, aliquoted, and stored at -80 °C until further use. Peritoneal exudate cells were enriched for macrophages using the method described by Kumagai *et al.*, 1979. Briefly, peritoneal cells were harvested by lavage and washed three times with complete culture medium. Approximately 1 × 10⁶ cells per well were then cultured in six-well culture plates. After 2 h of incubation to allow macrophages to adhere, each well was washed three times with warm Hank's balanced salt solution medium to remove nonadherent cells. The percentage of macrophages that adhered to the plates appeared equivalent by microscopic inspection regardless of whether the cells were derived from young or senescent mice (data not shown). Adherent macrophages were stimulated with various concentrations of activating stimuli as described later. Total RNA was later isolated from some cell samples for microarray analysis (*n* = 3) and q-PCR (*n* = 6–8). Other cells were used to determine total protein, cytokine proteins, and DNA methylation. After flush operation, mice spleens were removed and mechanically dissociated, subjected to red blood cell lysis, and then collected for q-PCR assay.

miRNA microarray assay

Total RNA was extracted from each cell sample using TRIzol (Invitrogen, Carlsbad, CA, USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After passing RNA measurement on the Nanodrop instrument, four group samples ($n = 3$, total 12 samples) were submitted to KangChen-Biotech (Shanghai, China) to be labeled using the miRCURY™ Hy3™/Hy5™ Power Labeling Kit and hybridized on the miRCURY™ LNA Array (v.11.0 – hsa, mmu & rno array; Exiqon, Vedbaek, Denmark). Scanning was performed with the Axon GenePix 4000B microarray scanner (Axon Instruments, Union City, CA, USA). GenePix Pro V6.0 software (Axon, Union City, CA, USA) was used to read the raw intensity of the image. Background subtraction and normalization were performed.

Cell culture, treatment, and transfection

To detect the time-dependent changes in miR-146a expression with LPS stimulation, cells stimulated with 0, 1, 10, 100, or 1000 ng mL⁻¹ LPS were cultured at 37 °C in 5% CO₂ for 0, 3, 6, 9, 12, and 24 h. These cells were then collected and immediately frozen and stored at -80 °C until further analysis of miR-146a expression by q-PCR. MiR-146a mimics (dsRNA oligonucleotides) and inhibitors (single-stranded chemically modified oligonucleotides) from GenePharma (Shanghai, China) were used for the overexpression and inhibition of miR-146a activity in mouse peritoneal macrophages, respectively. Negative control mimics or inhibitors (GenePharma Co.) were transfected to serve as matched controls. The macrophages described previously were transfected with RNAs at a final concentration of 10 nM using INTERFERin (Polyplus-Transfection SA, Illkirch, France) according to the manufacturer's instructions.

Measurement of miRNA and mRNA expression

Total RNA was extracted from cells and serum using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The Taqman miRNA assay system (Applied Biosystems, Foster City, CA, USA) was used to quantitatively detect the expression of miRNAs following the manufacturer's instructions. The SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) was used to analyze the expression of mRNA. The primer sequences for q-PCR are shown in Table S2. Quantification of miRNAs and mRNA by q-PCR was performed using an ABI 7300HT thermocycler at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in triplicate with mouse U6 or beta-actin as an internal control. Fluorescent signals were normalized to an internal reference (ΔR_n), and the threshold cycle was set within the exponential phase of PCR. The relative gene expression was calculated by comparing cycles for each target PCR. Cycle threshold values were converted to relative gene expression levels using the 2^{- $\Delta\Delta C_T$} method (Livak & Schmittgen, 2001).

Enzyme-linked immunosorbent assay (ELISA)

IL-6 protein concentration in the culture supernatants was measured by ELISA assay using an IL-6 ELISA kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Western blot analysis

Proteins extracted from cells were separated by electrophoresis on 12% SDS-polyacrylamide gels and 4% polyacrylamide gels and transferred

onto nitrocellulose. Proteins (60 µg) were detected by Western blotting using rabbit TRAF6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit IRAK1 antibody (Proteintech Group Inc, Chicago, IL, USA), and the I κ B α and p-I κ B α (Cell Signaling Technology, Inc., Beverly, MA, USA) antibodies. All primary antibodies were used at a dilution of 1:200 or 1:400 and were incubated overnight. After three washes with Tris-buffered saline with 0.05% Tween 20 (TBST), the membrane was incubated with biotinylated goat anti-rabbit IgG complex or goat anti-mouse IgG complex (Zhongshan Goldenbridge Biotechnology Co. Ltd., Beijing, China) for 2 h at room temperature. After thoroughly washing again with TBST buffer, specific proteins were visualized with enhanced chemiluminescence reagents (Hyperfilm ECL; Amersham Biosciences, Buckinghamshire, UK) followed by exposure to X-ray film for 1–3 min.

Treatment with 5-Aza-CdR and TSA in macrophages

After overnight incubation, macrophages were cultured for 48 h with media alone or 25.0 µM of the DNMT inhibitor 5-aza-2-deoxycytidine (5-Aza-CdR) (Sigma-Aldrich, St Louis, MO, USA) or 3 nM of the HDAC inhibitor TSA (Sigma-Aldrich). The transcription level of miR-146a and the protein levels of I κ B α , p-I κ B α , and IRAK1 were analyzed as described previously by q-PCR and Western blot, respectively. In some experiments, macrophages were pretreated with TSA 24 h before the addition of 1.0 µM NF- κ B inhibitor BAY 11-7082 (Santa Cruz Biotechnology). Next, the cells were incubated for 48 h and analyzed for NF- κ B binding to the miR-146a promoter by ChIP assay. In other experiments, macrophages of young and aged mice were exposed to TSA at 9 h post-LPS stimulation, and the levels of histone H3 acetylation were analyzed by ChIP assay at 12 and 24 h following stimulation with LPS.

Quantification of methylation level of miR-146a promoter

Genomic DNA was subjected to sodium bisulfite treatment (Grunau *et al.*, 2001). MiR-146a promoter regions essential for basal transcriptional activity occurred between nt -442 and nt +5 and included 11 CpG sites. The 447-bp region was amplified with PCR using primers designed with the help of METHPRIMER software (<http://www.urogene.org/methprimer/index1.html>) (Table S2). The BSP amplification for miR-146a promoter was performed using our novel program. In brief, the PCR (9 µL) contained 0.7 µL of modified genomic DNA and was overlaid with mineral oil to form a vapor barrier. The cycling conditions consisted of an initial denaturation at 96 °C for 5 min, at which point 0.5 µL reverse primer (10 µM) was added into the PCR mixture, followed by two cycles of 96 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min. When the temperature again rose to 96 °C, which was the beginning of the next phase of eight cycles (96 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min), 0.5 µL forward primer was added into the PCR mixture. Then 30 cycles of 96 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s were performed, followed by a final extension of 72 °C for 7 min. Then PCR products were sequenced using a DNA sequencer (ABI PRISM 3730, Foster City, CA, USA). Methylation level was measured according to our previously reported method (Jiang *et al.*, 2008, 2009).

ChIP assay and q-ChIP PCR

Chromatin immunoprecipitation assay was performed to determine the binding of NF- κ B p65 and Ace-H3 to the promoter of miR-146a using a commercially available ChIP Assay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, 1×10^7 peritoneal macrophages were cultured in 15-cm culture dishes and treated as

described earlier. Cells were fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min at 37 °C and collected by scraping and then sonicated to yield chromatin DNA fragments ranging in size from 200 to 1000 bp, followed by centrifugation for 10 min. Ten microliters of the cleared chromatin was reserved as input DNA sample. Immunoprecipitation analysis was carried out at 4 °C overnight using anti-NF- κ B p65 or anti-acetyl-histone H3 antibodies (Abcam, Cambridge, MA, USA), and anti-mouse IgG was used as a control for nonspecific binding. Immunoprecipitated DNA was analyzed by q-PCR using promoter-specific primer pairs for two NF- κ B-binding sites I and II in miR-146a promoter listed in Table S2 (Supporting information). The negative control primers, flanking a region of genomic DNA between GAPDH gene and the chromosome condensation-related structural maintenance of chromosomes (SMC)-associated protein (CNAIP1) gene, were used to represent specificity of ChIP reactions (Staub et al., 2007). The DNA purified from the sonicated nuclear lysate was analyzed by q-PCR using the same primer sets, which was used as an input control. Expression of a target DNA sequence was normalized to the input DNA and represented as fold enrichment compared with the nonLPS-treated control (set as onefold).

Statistical analysis

Data were expressed as the group's mean value \pm SD of one experiment representative of two to four identical experiments in which similar results were obtained. Data were considered significant at $P < 0.05$ as determined by *t*-tests or ANOVA with *post hoc* Newman–Keuls test.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials.

Fig. S1 (A) Microarray analysis of miRNA expression profile in primary peritoneal macrophages from young and aged mouse in absent or present LPS treatment. Each group includes three mice. Slide 1-1, 1-2, 1-3 represent no-treated young mice; slide 2-1, 2-2, 2-3 represent LPS-treated young mice; slide 3-1, 3-2, 3-3 represent no-treated old mice; slide 4-1, 4-2, 4-3 represent LPS-treated old mice. (B) Confirmation of miRNAs described in A by using two-step Taqman RT-PCR analysis and the expression of indicated miRNAs were normalized to that of U6 in each sample.

Fig. S2 MiR-146a targeted mouse IRAK1 and negatively regulated the expression of inflammatory cytokines IL-1 β and IL-6 in mouse peritoneal macrophages from young mice.

Fig. S3 ChIP analysis of NF- κ B p65 binding activity at the miR-146a promoter in 12 h *in-vitro* incubation of macrophages from young and aged mice.

Table S1 Differentially expressed miRNAs in LPS-treated young vs. young.

Table S2 List of primer sequences used for conventional PCR, q-PCR, BSP, ChIP-PCR assay.

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