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J Immunol published online 6 December 2013
http://www.jimmunol.org/content/early/2013/12/06/jimmunol.1301397

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/12/06/jimmunol.1301397.DC1

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The p53 Transcription Factor Modulates Microglia Behavior through MicroRNA-Dependent Regulation of c-Maf

Wei Su,* Stephanie Hopkins,* Nicole K. Nesser,*† Bryce Sopher,* Aurelio Silvestroni,* Simon Ammanuel,*2 Suman Jayadev,*† Thomas Möller,*3 Jonathan Weinstein,*† and Gwenn A. Garden*†

Neuroinflammation occurs in acute and chronic CNS injury, including stroke, traumatic brain injury, and neurodegenerative diseases. Microglia are specialized resident myeloid cells that mediate CNS innate immune responses. Disease-relevant stimuli, such as reactive oxygen species (ROS), can influence microglia activation. Previously, we observed that p53, a ROS-responsive transcription factor, modulates microglia behaviors in vitro and in vivo, promoting proinflammatory functions and suppressing downregulation of the inflammatory response and tissue repair. In this article we describe a novel mechanism by which p53 modulates the functional differentiation of microglia both in vitro and in vivo. Adult microglia from p53-deficient mice have increased expression of the anti-inflammatory transcription factor c-Maf. To determine how p53 negatively regulates c-Maf, we examined the impact of p53 on known c-Maf regulators. MiR-155 is a microRNA that targets c-Maf. We observed that cytokine-induced expression of miR-155 was suppressed in p53-deficient microglia. Furthermore, Twist2, a transcriptional activator of c-Maf, is increased in p53-deficient microglia. We identified recognition sites in the 3' untranslated region of Twist2 mRNA that are predicted to interact with two p53-dependent microRNAs: miR-34a and miR-145. In this article, we demonstrate that miR-34a and -145 are regulated by p53 and negatively regulate Twist2 and c-Maf expression in microglia and the RAW macrophage cell line. Taken together, these findings support the hypothesis that p53 activation induced by local ROS or accumulated DNA damage influences microglia functions and that one specific molecular target of p53 in microglia is c-Maf. The Journal of Immunology, 2014, 192: 000–000.
as a novel candidate modulator of microglia behavior and demonstrated ongoing p53 transcriptional activity in microglia cultured under basal conditions (11–13). Genetic knockout (KO) or pharmacological inhibition of p53 results in microglia that are less neurotoxic in response to proinflammatory stimuli (11, 14). We also observed that p53-deficient microglia express genes associated with the anti-inflammatory/tissue repair phenotype of alternatively activated macrophages (13). In addition, p53 is activated in a subpopulation of microglia in the inflamed human brain (12), but p53 activation is excluded from the population of microglia labeled by CD163 (13), a marker of macrophages that have adopted anti-inflammatory or tissue repair phenotypes (15).

MicroRNAs (miRNAs) are small noncoding RNAs (~22 nucleotides long) involved in many physiological and pathological processes (16–18). A single miRNA can regulate hundreds of genes, and ∼90% of human genes are potentially regulated by miRNAs (19, 20). MiRNAs downregulate the expression of mRNA targets by binding to complementary sequences in the 3′ untranslated region (UTR). Several reports suggest that miRNAs play an important role in regulating the responses of innate immune cells (21), including microglia (18). For example, recent reports suggest that miR-124 is a key regulator of microglia quiescence in the CNS, thereby helping to prevent CNS inflammation (18, 22). Because a single miRNA can modulate expression of many genes during inflammation, miRNAs may serve as epigenetic regulators of microglia behavior (23).

As a transcriptional activator, p53 exerts its function through promoting expression of target genes to initiate cellular responses. Recent work has revealed that miRNAs are important components in the p53 transcriptional network (24). Maturation of miRNAs is promoted by p53-mediated transcription (25), and p53 also induces expression of specific miRNAs. For example, several groups reported that p53 directly regulates the expression of the miR-34 family (26–30). The miR-34 family consists of miR-34a, miR-34b, and miR-34c, which are encoded by two different genes. In family (26–30). The miR-34 family consists of miR-34a, miR-34b, and miR-34c, which are encoded by two different genes. In addition, p53 activates the expression of several additional miRNAs, including miR-145 (24, 25). Overexpression of miR-145 reduces c-Myc expression, whereas suppression of endogenous miR-145 enhances c-Myc expression (24), which partially accounts for p53-mediated inhibition of tumor cell growth both in vitro and in vivo (31, 32).

We previously reported that microglia from p53-deficient mice had altered gene expression and inflammatory behaviors suggesting that p53 transcriptional activity is a critical activator of proinflammatory responses in microglia (13). In this article, we identify c-Maf, a transcription factor known to promote functional differentiation and anti-inflammatory responses in both lymphocytes and myeloid cells (33, 34), as a downstream p53 target in microglia. We determined that p53 negatively regulates c-Maf in microglia by enabling the induction of miR-155, a miRNA that suppresses c-Maf. In addition, Twist2, a transcriptional activator of c-Maf, is suppressed by p53-regulated miR-34a and miR-145. Taken together, we propose that p53 activation may influence microglia responses to injury by suppressing c-Maf expression and the subsequent protective inflammatory response.

Materials and Methods

Animals

Mice deficient in p53 (p53<sup>−/−</sup>) (35) and miR-155 (miR-155 KO) (36), and strain-matched p53-expressing mice (p53<sup>+/+</sup>) as well as miR-155-expressing mice (C57BL6 from The Jackson Laboratory), were maintained in a specific pathogen-free facility under the guidance of an Institutional Animal Care and Use Committee–approved protocol.

Middle cerebral artery occlusion

Middle cerebral artery occlusion (MCAO) was performed on anesthetized 12- to 16-wk-old male C57/BL6 mice obtained from The Jackson Laboratory, using the transient intraluminal filament method (37) with laser Doppler cerebral blood flow monitoring. The filament was inserted to obtain a ≥70% occlusion for 15 min, followed by filament removal and documentation of reperfusion; a paradigm developed to result in ischemic preconditioning (38). Mice with cerebral blood flow profiles outside a predetermined expected range were excluded. Mice were monitored by neurobehavioral assessments using a published scoring system (39) for 72 h following creation of the lesion. Animals with a detectable behavioral deficit were excluded from further study. Using this paradigm, we could find no detectable region of infarction in the cortex by 2,3,5-triphenyltetrazolium chloride staining.

Ex vivo flow cytometry

Adult mice (12–16 wk old, n = 4 mice per genotype) were intracardially perfused with cold saline solution. Cortical tissue was separated from whole brain and dissociated using the MACS Neural Dissection Kit (Miltenyi Biotec) and a modified version of the Miltenyi gentleMACS protocol. Preparation of single-cell suspensions from mouse neural tissue was done according to the manufacturer’s instructions. Depletion of myelin and positive selection of myeloid cells were performed in sequential myelin and CD11b magnetic selection steps (Miltenyi Biotec). The remaining cells were centrifuged at 300 × g for 10 min at 4°C; then the pellet was resuspended in FACS medium (FM) [10% FCS (Atlanta Biologicals), 10 mM HEPES pH 7.3 (Life Technologies), in HBSS buffer without calcium or magnesium (Life Technologies)] and incubated with FC block (BD Pharmingen). Cells were next stained with fluorochrome-conjugated Abs according to the manufacturer’s protocol: FITC-CD45 (BD Pharmingen, Transduction Labs), APC-F4/80 (Affymetrix eBio-science), or isotype control and DAPI (Sigma-Aldrich). Cells with Abs were incubated on ice for 45 min, washed three times with FM, and resuspended in FM. All cells were sorted on a BD ARIA II cell sorter directly into TRIZol LS reagent (Invitrogen). Microglia were defined as the population of cells that were F4/80<sup>+</sup> and CD45<sup>+</sup> by gating on a previously published approach (40).

Cell culture

The RAW cell line was grown in high-glucose DMEM supplemented with 10% FBS (Life Technologies), 25 U/ml penicillin, and 25 mg/ml streptomycin and incubated in a 37°C cell incubator supplemented with 5% CO<sub>2</sub>. RAW cells were plated onto a 24-well dish at a density of 5 × 10<sup>4</sup> cells per well with 200 μl media. Cells were treated with IFN-γ (10 U/ml) for 24 h before harvest. For nutlin treatment, cells were treated with DMSO, 10 μM, 40 μM, or 80 μM nutlin, for 6 h before harvest. Cells were lysed for total RNA 24 h post transfection and for total protein 72 h post transfection. Mixed glia cultures were generated, using previously published methods, from cortical tissue dissected on postnatal day 3 or 4 (13). Cells were cultured in high-glucose DMEM (HyClone, Thermo Scientific) supplemented with 10% heat-inactivated horse serum (Life Technologies), 10% nutrient mixture F-12 (Gibco), and 20% L929-conditioned medium. Microglia were isolated from the cultures 7–10 d post dissection by collecting floating cells. Primary microglia were plated on poly-γ-lysine–coated plates at a density of 5 × 10<sup>4</sup> per 35-mm plate or 1 × 10<sup>5</sup> per 60-mm plate in DMEM media. Cells were treated with IFN-γ (10 U/ml) for 24 h, nutlin-3 (40 μM) for 6 h, or vehicle control.

Real-time quantitative RT-PCR

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) or miRNeasy kit (QIAGEN) according to the manufacturers’ instructions. cDNA was generated using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences). cDNA from adult mRNA transcripts was amplified using the Ovation quantitative (q)PCR system (NuGEN) at the University of Washington Center on Human Development and Disability Microarray and Bioinformatics Core Laboratory; cDNA from adult mouse miRNA was generated using miRNA-specific primers. qPCR was performed using the StepOnePlus Real Time PCR Instrument (Applied Biosciences) with the TaqMan Gene Expression Assay, TaqMan MicroRNA
Assay, or Roche Universal Probe Library. TaqMan probe and primers used are as follows; cMaf: Mm02581355_s1; Twist2: Mm00492147_m1; miR-34a: 000426; miR-145: 002278; and miR-155: 002571. PCR products were normalized to TBP (Mm01277042_m1) or 18S (Mm03928990_q1) for TaqMan gene expression assays, and Sno202 (001232) and Sno234 (001234) for miRNA assays. Roche UPL primer probe sets are listed in Table 1.

**Western blot**

RAW cells were lysed with RIPA buffer (Thermo Scientific) containing protease inhibitor mixture (Sigma-Aldrich). A total of 10 µg total proteins were run on NuPage 4–12% Bis Tris gel (Invitrogen) and transferred according to the manufacturer’s protocol (Invitrogen). Membranes were probed for Twist2 (Ab66031, Rabbit polyclonal; Abcam), c-Maf (Ab76817, mouse monoclonal; Abcam), and β-actin (SC-7778, mouse monoclonal; Santa Cruz Biotechnology) as primary Abs (1:1000 dilution), and anti-Rabbit (SC2301; Santa Cruz Biotechnology) or anti-mouse (SC2302; Santa Cruz Biotechnology) as a secondary Ab (1:5000 dilution). Twist2, c-Maf protein levels were normalized to β-actin. All films were scanned and analyzed using ImageJ 1.47 software.

**Results**

**c-Maf expression is suppressed by p53 in microglia**

We previously reported that p53 supports the proinflammatory response in microglia (13). We observed that p53 KO (p53<sup>−/−</sup>) microglia in neuron/microglia cocultures are functionally distinct from wild-type (WT) primary microglia in response to HIV gp120-coated protein (11). We further found that p53<sup>−/−</sup> microglia demonstrated a blunted response to IFN-γ, failing to increase expression of genes associated with classical macrophage activation or secrete proinflammatory cytokines, and tend to have gene expression patterns and functions similar to those of alternatively activated macrophages (13).

The c-Maf transcription factor has been reported to promote expression of anti-inflammatory cytokines and suppress expression of proinflammatory cytokines in macrophages (33, 34, 42). We hypothesized that p53 promotes microglia proinflammatory behaviors by suppressing c-Maf-mediated gene transcription. We therefore measured c-Maf mRNA in microglia cultured from day 3–4 postnatal p53<sup>/−</sup> or p53<sup>−/−</sup> mice, and found that c-Maf was elevated by 2-fold in p53<sup>−/−</sup> cells compared with p53<sup>/−</sup> cells (data not shown), indicating a regulatory connection between p53 and c-Maf. However, cultured microglia are exposed to cytokines that promote cell division, and c-Maf expression may be regulated by these cytokines (43). To determine whether the regulation of c-Maf expression by p53 in cultured microglia was unique to the in vitro environment, we asked whether c-Maf expression was specifically regulated by p53 in microglia from the adult brain. We obtained microglia from 12- to 16-wk-old adult mice by ex vivo flow cytometry, based on previously published criteria (44–46), and measured c-Maf mRNA by qRT-PCR. c-Maf mRNA was elevated by 2.5-fold in p53<sup>−/−</sup> microglia (Fig. 1), compared with p53<sup>/−</sup> cells, suggesting that basal p53 activity in normal microglia is sufficient to suppress c-Maf expression in vivo.

**MiR-155, a suppressor of c-Maf, is regulated by p53**

Because c-Maf is a potential downstream target of p53 in microglia, next we asked how p53 influences the expression of c-Maf. The transcriptional repertoire of p53 has been intensely studied, but direct binding of p53 to the c-Maf gene had not been identified (47). To assess whether p53 directly suppresses expression of c-Maf mRNA, we evaluated the c-Maf genomic sequence and observed several p53 consensus binding sites 2 Kb upstream of the translational start site. One recent study reported that p53 promoted transcriptional activation of c-Maf during the process of lens development (48). Because this report suggested that a direct interaction between p53 and the c-Maf gene led to regulation in the opposite direction of what was observed in microglia, we hypothesized that p53 represses c-Maf expression in microglia through an alternate mechanism. To determine how c-Maf expression levels are...
negatively regulated by p53, we assessed whether other known regulators of c-Maf expression are influenced by p53 in microglia.

Because p53 generally acts as a transcriptional activator, we hypothesized that suppression of c-Maf was mediated by a p53-induced miRNA. The proinflammatory miRNA miR-155 negatively regulates c-Maf expression in T cells (36). We therefore evaluated miR-155 expression in cultured primary microglia by qRT-PCR. Under resting conditions, very little miR-155 is expressed in microglia (Fig. 2A). Treatment with classical activation cytokine IFN-γ leads to a robust induction of miR-155 in p53+/+ cells. In contrast, induction of miR-155 in p53−/− cells was significantly repressed. Comparing IFN-γ–induced miR-155 expression between p53+/+ and p53−/− microglia cultures revealed that p53 significantly augments the induction of miR-155 (Fig. 2A).

We next measured c-Maf mRNA expression in microglia cultures from miR-155 KO mice and found that c-Maf mRNA was elevated by 4.5-fold over WT microglia (Fig. 2B). We also observed a 1.5-fold increase of c-Maf mRNA in microglia collected by ex vivo flow cytometry from miR-155 KO adult mice compared with WT mice (Fig. 2C), demonstrating that even under basal conditions c-Maf expression is suppressed by this pathway in vivo. Taken together, these data suggest that p53 negatively regulates c-Maf expression by transcriptional activation of miR-155.

miR-155 KO recapitulates a portion of the p53 KO phenotype in microglia

Previously, we reported that p53−/− microglia did not promote HIV-gp120 neurotoxicity (11) and have a muted transcriptional response to a classical activation signal (13). If miR-155 is the key mediator of the p53-dependent microglia proinflammatory response, we hypothesized that mediators of classical activation influenced by p53 would be similarly regulated by miR-155. To test this hypothesis, primary microglia cultured from WT and miR-155 KO mice were treated with 10 U/ml of IFN-γ for 24 h, and mRNAs associated with classical activation were evaluated.

We previously reported that three genes associated with a type I inflammatory response—IL-1α, IL-1β, and MARCO (49)—are not effectively induced by IFN-γ in p53−/− microglia (13). Similar results were observed in miR-155 KO microglia for IL-1α (Fig. 3A) and MARCO (Fig. 3C), but not for IL-1β (Fig. 3B). These results suggest that regulation of IL-1α and MARCO by p53 in microglia is mediated through miR-155; however, an alternative pathway might influence microglia IL-1β expression in response to IFN-γ.

Twist2, a positive regulator of c-Maf, is regulated by p53-inducible miRNAs

c-Maf expression is also transcriptionally regulated by Twist2, a basic helix-loop-helix transcription factor. Twist2 promotes myeloid differentiation and interacts with the 5′ regulatory region of the cMaf gene to increase c-Maf expression (50). We therefore investigated the relationship among p53, Twist2, and c-Maf in microglia. We observed that Twist2 was upregulated by 2.5-fold in cultured p53−/− microglia compared with p53+/+ cells on the microarray analysis previously reported (13). An even larger increase in Twist2 expression was confirmed by qPCR (Table I) on mRNA isolated from p53−/− and p53+/+ microglia (Fig. 4). This finding suggests an alternative mechanism by which c-Maf expression is regulated by p53 via p53-dependent suppression of Twist2 expression.

Because p53 is known as a transcriptional activator, we hypothesized that p53 also induces the expression of miRNAs that negatively regulate Twist2. The 3′-UTR of Twist2 was analyzed in silico for the identification of miRNA targets. Using two independent software platforms, we identified binding regions for two specific miRNAs, miR-34a and miR-145, which are known p53 transcriptional targets (26, 27, 31). We performed RT-PCR for these two miRNAs in both cultured microglia (Fig. 5A, 5B) and adult microglia extracted by ex vivo flow cytometry (Fig. 5C, 5D) and observed p53-dependent expression of these two miRNAs in both systems. Of interest, in vitro these two p53-dependent miRNAs are differentially regulated by p53, with miR-34a being turned on.

Table I. Roche primer and probe sets used for qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer (5′–3′)</th>
<th>Reverse Primer (5′–3′)</th>
<th>Roche Probe No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGCTTGTCATCCACCGGAAA</td>
<td>TTGTATGTATGCGGTTCCTG</td>
<td>9</td>
</tr>
<tr>
<td>TBP</td>
<td>TGCCAGCCGAGCCCAAGA</td>
<td>CCAACACATGTTGTGTAGT</td>
<td>33</td>
</tr>
<tr>
<td>IL-1α</td>
<td>TTGGTTAAATGACCTGTCA</td>
<td>GGCGCTCCAGCAACAGTGG</td>
<td>52</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGTTGACGGACCCCAAAAG</td>
<td>AGTGGATGCCCTCTTATCAG</td>
<td>38</td>
</tr>
<tr>
<td>MARCO</td>
<td>ATGCTGGTTCATCCAGAGG</td>
<td>ATGTTCACCCAGATGTTC</td>
<td>10</td>
</tr>
<tr>
<td>c-Maf</td>
<td>CCTCGCTCTCCACCAGATT</td>
<td>CGACCGGACATTTAACAGAG</td>
<td>33</td>
</tr>
<tr>
<td>Twist2</td>
<td>CAGTGGCGCTCCCTCACCTA</td>
<td>GATGTGCAAGGGGTCCT</td>
<td>10</td>
</tr>
</tbody>
</table>

The qPCR primer sequences and corresponding probes were designed using the ProbeFinder Software available at http://www.roche-applied-science.com
by basal p53 activity and miR-145 being induced by higher level p53 activity following treatment with the MDM2 inhibitor nutlin. However, both miR-34a and miR-145 were significantly reduced in adult p53−/− microglia isolated by ex vivo sorting. Taken together, these findings support our hypothesis that p53 can suppress c-Maf expression through a pathway independent of miR-155: inducing miRNAs that suppress the c-Maf activator Twist2.

To further examine the hypothesis that p53 regulates Twist2 via induction of miR-145 and miR-34a, we employed the mouse leukemic monocyte macrophage cell line (RAW cells). To determine if activation of p53 lessened Twist2 protein in a myeloid cell line, we exposed RAW cells to increasing concentrations of nutlin. We observed greater p53 stabilization (Supplemental Fig. 1) and decreasing Twist2 protein detected by Western blot with increasing nutlin concentration (Fig. 6A, 6B). Next, miR-145 and miR-34a mimics (Sigma-Aldrich) were transfected into RAW cells, and Twist2 mRNA levels were determined by qRT-PCR. Twist2 mRNA levels were significantly reduced when either miR-145 or miR-34a was overexpressed in RAW cells. As shown in Fig. 6, 24 h post transfection, Twist2 mRNA was knocked down by 66% and 64%, when miR-145 and miR-34a were expressed, respectively (Fig. 6C). The levels of miR-145 and miR-34a before and after transfection were determined by the manufacturer’s recommended miRNA RT-PCR assay from the same extracted RNA samples (Supplemental Fig. 2). In addition, we checked the c-Maf mRNA level in RAW cells transfected with either miR-145 or miR-34a mimics, and observed > 60% reduction in c-Maf mRNA levels in both conditions (Fig. 6D). To ensure that the observed level of reduction in mRNA led to a physiologically relevant change in Twist2 or c-Maf protein, we performed Western blot for Twist2 or c-Maf protein, we performed Western blot for Twist2 mRNA on total RNA extracted from cultured microglia that were p53−/−, p53+/+, or p53+/+ following p53 induction with nutlin for 6 h (n = 3 separate cultures, statistics were determined from separate qPCR runs). *p < 0.01, **p < 0.0001 by one-way ANOVA. (A and B) qRT-PCR was performed to detect miR-34a and miR-145 on total RNA extracted from cultured microglia that were p53−/−, p53+/+, or p53+/+ following p53 induction with nutlin for 6 h (n = 3 separate cultures, statistics were determined from separate qPCR runs). *p < 0.01, **p < 0.0001 by one-way ANOVA. (C and D) qRT-PCR was performed for miR-34a and miR-145 on adult microglia extracted from p53−/− and p53+/+ mice. n = 3 from separate qPCR runs. *p < 0.02, **p < 0.002 by unpaired t test.

The in vivo response to CNS ischemia involves p53 activation, induction of p53-dependent miRNAs, and suppression of c-Maf

The data presented demonstrate that p53 influences c-Maf expression in several paradigms, including cultured microglia, RAW cells, and microglia extracted from normal adult mouse brain. The question remains whether these miRNAs modulate inflammatory gene expression in vivo during neuroinflammation. To address this, we induced neuroinflammation by transient MCAO, a model of CNS ischemia in which we have previously demonstrated that loss of p53 leads to increased microglia expressing CD206, a marker of the anti-inflammatory phenotype (13). We performed a Pscan promoter analysis (51) on microarray data obtained from forebrain microglia ex vivo extracted 3 d following 15-min MCAO [a less severe ischemic pulse that does not induce cortical infarction but does result in ischemic preconditioning (38)] or sham surgery. We observed that 15-min ischemia leads to a significant (p < 0.02) induction of p53 transcriptional targets in ex vivo sorted cortical microglia 3 d after the ischemic insult. These findings demonstrate that the proinflammatory response supported by p53 could be detected 3 d following this injury paradigm. To determine if this model of neuroinflammation involves activation of miR-155, miR-145, and miR-34a in microglia, we obtained forebrain microglia by ex vivo flow cytometry 3 d after 15-min MCAO. The presence of an inflammatory response to ischemia was confirmed by a 30% increase in microglia and a 10-fold increase in macrophages obtained from cortex ipsilateral to the MCAO, compared with the contralateral side. We observed an increase in the level of miR-155 (Fig. 7A). MiR-34a and MiR-145 were not induced. In addition, the expression of c-Maf mRNA was suppressed in microglia from the ischemic side of the brain at both time points (Fig. 7B). Twist2 mRNA was not detected. In summary, these data suggest that neuroinflammation in vivo is associated with induction of at least one p53-dependent miRNA that suppresses c-Maf expression in microglia (Fig. 8). The finding also suggests that miR-155 is the dominant mediator of c-Maf suppression during the response to CNS ischemia.

Discussion

Many studies suggest that inflammation influences tissue injury and degeneration in the CNS. It is now believed that neuroinflammation is an important contributor to neurodegeneration. Neurons are susceptible to oxidative damage from ROS induced by...
inflammation, and inflammatory mediators can be directly neurotoxic or can attract leukocytes with cytotoxic properties. However, microglia are also capable of promoting tissue repair in the CNS; thus the modulation of microglia behavior may have important influences on long-term functional outcome in CNS disease or injury states. Several studies have shown that microglia exhibit patterns of functional differentiation similar to those described for macrophages (52). Microglia express marker proteins associated with specific differentiation states in a number of CNS diseases and disease models (53, 54), demonstrating that microglia are capable of heterogeneous functional differentiation responses to disease-relevant stimuli.

Inflammation is a complex response that evolved to restore homeostasis post infection or injury, and microglia are key regulators of the inflammatory responses in CNS. However, little is known regarding the endogenous molecular signals that determine which components of the microglia functional repertoire will be expressed in response to a changing neural environment. Identifying the molecular signals that control whether microglia exhibit destructive or protective functions is likely to suggest novel therapeutic targets that may improve functional outcome in diseases associated with neuroinflammation, such as Alzheimer’s disease (55, 56), Parkinson’s disease (57), amyotrophic lateral sclerosis (58), multiple sclerosis (1), traumatic brain injury (59), and stroke (60). We previously identified p53 as a transcriptional regulator of microglia behavior (13) and now report that p53 acts through miRNA dependent regulation of c-Maf, a known transcriptional regulator of the inflammatory response, as diagramed in Fig. 8.

**FIGURE 6.** p53 activation suppresses Twist2 and c-Maf expression and transfection of miR-145 or miR-34a downregulates Twist2 and c-Maf in RAW cells. (A) p53 activation by nutlin causes a dose-dependent reduction of Twist2 protein in RAW cells. (B) Densitometry of Twist2-labeled bands relative to actin-labeled bands on Western blot of RAW cell lysates after treatment with DMSO or 10 μM, 40 μM, or 80 μM of nutlin. Data were plotted from three separate experiments. *p < 0.001, one-way ANOVA. (C) Transfection of miRNA mimics miR-145, and miR-34a significantly decreases Twist2 mRNA level in RAW cells at 24 h post transfection. n = 3 from separate transfections and qPCR runs. ***p < 0.001, one-way ANOVA. (D) c-Maf mRNA levels were determined by qRT-PCR in samples when cells were transfected with or without miRNA mimics. n = 3 from separate transfections and qPCR runs. **p < 0.01, one-way ANOVA. (E) Transfection of miR-145 and miR-34a mimics significantly decreased Twist2 and c-Maf protein in RAW cells, whereas IL-4 treatment increased both Twist2 and c-Maf protein. (F) Densitometry of Twist2 protein level in RAW cells transfected with miR-145 and miR-34a mimics, or treated with IL-4. Data were plotted from four separate experiments. *p < 0.05, **p < 0.001, one-way ANOVA. (G) c-Maf protein level in RAW cells when cells were transfected with miR-145 and miR-34a mimics, or treated with IL-4. Data were plotted from four separate experiments. *p < 0.05, one-way ANOVA.

**FIGURE 7.** In vivo CNS inflammation induces activation of miR-155 and suppression of c-Maf in microglia. (A) qPCR for miR-155, miR-145, and miR-34a expression in microglia extracted by ex vivo flow cytometry from mice that were 3 d after 15-min transient MCAO. miRNA levels were normalized to both Sno202 and Sno234 RNAs. *p < 0.001, two-way ANOVA. (B) qPCR for c-Maf mRNA relative to housekeeping gene expression in microglia extracted 3 d following 15 min. transient MCAO. *p < 0.001, unpaired t test.
The p53-mediated transcriptional activity is required for the induction of p53-dependent miRNAs (miR-145 and miR-34a). These miRNAs target a transcriptional regulator, Twist2, for suppression, thereby inhibiting the expression of the anti-inflammatory transcription factor c-Maf. In the absence of p53, microglia also fail to induce expression of miR-155, a miRNA that directly targets c-Maf. When p53 is activated in microglia by ROS, spontaneous DNA damage, or cellular stress associated with CNS disease and injury, the effectors of such activation work together to suppress the expression of c-Maf, thereby suppressing the anti-inflammation and tissue repair behaviors of microglia and making it more likely for microglia to adopt proinflammatory behaviors that can be injurious to surrounding neural cells.

A new role for p53 in promoting a proinflammatory response

P53 is a key modulator of stress responses becoming activated by physiological stressors, including but not limited to, DNA damage (induced by UV, ionizing radiation, or chemical agents such as hydrogen peroxide), oxidative stress, osmotic shock, ribonucleotide depletion, and deregulated oncogene expression (61, 62). Such activation is marked by two major events: a dramatic increase in the half-life of the p53 protein, and a conformational change in the structure that activates p53 transcriptional activity through phosphorylation of its N-terminal domain (63, 64). In microglia, however, it appears that p53 influences gene transcription even in the absence of cellular stress. We previously reported that cultured microglia have demonstrable p53 transcriptional activity under basal conditions (13), and in this article we show that microglia extracted from uninjured adult brain demonstrate differential expression of c-Maf and its regulatory miRNAs. We and others have reported that p53 also influences microglia responses to inflammatory stimuli, including the regulation of cytokine expression (13), inducible NO synthase expression, and TNF-α secretion (14). Inhibiting p53-mediated transcription in microglia prevented neurotoxicity, suggesting that targeting of p53-mediated pathways in microglia may have therapeutic benefit in CNS injury and disease exacerbated by an overexuberant inflammatory response.

**c-Maf transcription factor and its role in anti-inflammatory responses in CNS**

The Maf family of transcription factors consists of long and short members, as well as a viral oncogene, v-Maf (65). This family of Maf transcription factors has a variety of reported roles in regulating cellular differentiation (66). c-Maf is a long Maf family member that promotes the differentiation of Th2 lymphocytes (67) as well as expression of F4/80 (mouse homolog of EMR1: epidermal growth factor–like module-containing mucin-like hormone receptor–like 1) (68) and an anti-inflammatory cytokine pattern in macrophages (33, 34, 42). It was also demonstrated that c-Maf plays a role in regulating cell fate decision (69). Thus, c-Maf transcriptional activity is a critical molecular determinant of functional differentiation in two different classes of leukocytes. As a critical transcriptional activator of anti-inflammatory cytokine genes, c-Maf must be suppressed during proinflammation and activated during anti-inflammation. Oxidative stress has been shown to negatively regulate c-Maf expression, but the precise mechanism by which this occurs has not been determined (69). Our findings suggest that repression of c-Maf is one means by which p53 influences the functional differentiation of microglia.

**p53 promotes proinflammatory behavior in microglia through miR-155**

Initially, miR-155 was identified as a proinflammatory miRNA that contributes to macrophage activation by targeting anti-inflammatory genes (4, 22). Recent studies suggest that miR-155 promotes skewing toward proinflammatory behaviors by targeting anti-inflammatory genes like the IL-13R (IL13Ra1) for degradation (4). miR-155 also targets SMAD2, which is involved in the TGF-β anti-inflammatory signaling pathway (70, 71). Finally, miR-155 inhibits CEBPα, which was recently shown to be important for the expression of a number of genes associated with anti-inflammatory or “alternative” activation states, such as Arg1, IL-10, IL13Ra1, and CD206 (72). In microglia, miR-155 has been shown to downregulate suppressor of cytokine stimulus 1 expression (73), which also serves to promote proinflammatory behaviors. In this article, we show that in microglia, miR-155 is induced in a p53-dependent manner by proinflammatory cytokines. We also observed that in the absence of miR-155, c-Maf expression is increased, confirming that the previously reported role for miR-155 in lymphocytes is recapitulated in microglia. Thus, in the setting of oxidative stress (as occurs in chronic ischemia and neurodegenerative diseases), chronic p53 activation in microglia may suppress the anti-inflammatory response by promoting expression of miR-155. In this article, we demonstrate that neuroinflammation caused by brief CNS ischemia leads to activation of p53-mediated gene expression, induction of miR-155, and suppression of c-Maf mRNA in microglia. These findings support the hypothesis that p53 has a functional impact on an in vivo inflammatory response.

**How p53 may regulate miR-155**

MiRNAs have an established role in regulating inflammatory responses (23). For example, bic/miR155 plays an important role in the differentiation of B, T, and dendritic cells (36). In this article, we observed that induction of miR-155 expression by IFN-γ treatment was significantly higher in p53+/+ microglia than in p53−/− microglia. This finding suggests that induction of miR-155 is highly dependent on p53. The mechanism by which p53 regulates miR-155 expression has not been determined. It has been reported that p53 can directly regulate the transcription of certain miRNAs, but there is no clear consensus sequence for p53 binding to regulatory elements in proximity to the Bic/miR155 gene. An alternative mechanism by which p53 may modulate miR-155 expression is that p53 promotes posttranscriptional maturation of miRNAs (25). Drosha is a protein that mediates the processing of pri-miRNA transcripts into premiRNAs. For the maturation of a subset of miRNAs, Drosha requires RNA-associated proteins such as the microprocessor complex (74). Drosha and Pasha (the ICE matrix metalloproteinase) cleave the pri-miRNA transcript in the middle of the intron, generating a premiRNA. The Drosha/Pasha complex requires both RISC components and the double-stranded RNA-binding protein hTRBP (74).
as DEAD box RNA helicases p68 and p72 (also known as DDX17) to carry out its function. p53 promotes the Druska-mediated processing of certain miRNAs with growth-suppressive functions, such as miR-16-1, miR-143, and miR-145, in cells in response to DNA damage (25). Therefore, the regulation of miR155 by p53 in microglia could be through posttranscriptional modulation.

p53 modulates c-Maf in part through Twist2

The Twist family of basic helix-loop-helix transcription factors, including Twist1 and Twist2, are highly conserved, key regulators of mesodermal differentiation, embryogenesis (74), epithelial-mesenchymal transition (75), myoid lineage development, as well as inflammatory responses of mature myoid cells (50). Deficiency in Twist2 or Twist2 results in different phenotypes. Absence of Twist1 is embryonic lethal in mice, owing to a failure in neural tube closure, whereas Twist2 KO mice develop a severe inflammatory syndrome and die within 2 wk after birth (76, 77). In this article, we report a novel discovery that p53 regulates c-Maf expression in microglia through inducing miRNAs that target a c-Maf transcriptional activator, Twist2. Several groups have reported that p53 can directly promote the transcription of the miR-34 family members (26, 27, 30), and our data demonstrate a functionally significant regulation of miR-34a and miR-145 by p53 in the regulation of Twist2. Although Twist2 has not been specifically studied as a p53 target or in neuroinflammation, it was previously reported that Twist2 promotes the production of the anti-inflammatory cytokine IL-10 (50). Twist2 KO mice demonstrated enhanced production of proinflammatory cytokines in combination with the reduced production of IL-10 and develop uncontrolled and eventually lethal inflammation (76). Several recent studies showed that the transcription factor c-Maf promotes expression of IL-10 as well as IL-4 (42, 78). Accordingly, it was also found that c-Maf is decreased expression of p53 target genes. 

Acknowledgments

We thank Drs. Sean P. Murphy and Richard S. Morrison for helpful discussions during the performance of these experiments. Dr. Shahani Noor for training and assistance with ex vivo flow cytometry, Diana Chao for performing the MCAO procedure, Amanda Case for training and assistance with primary microglia culture, and Dr. David B. Wang for assistance with perfusions and editing the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


