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TNF Mediates the Sustained Activation of Nrf2 in Human Monocytes

Stuart A. Rushworth, Suharsh Shah, and David J. MacEwan

Modulation of monocyte function is a critical factor in the resolution of inflammatory responses. This role is mediated mainly by the production of TNF-α. Investigations of the actions of TNF have mostly focused on acute activation of other cell types such as fibroblasts and endothelial cells. Less is known about the effects of TNF on monocytes themselves, and little is known about the regulation of cell responses to TNF beyond the activation of NF-κB. In this study, we investigated the regulation of NF-E2–related factor 2 (Nrf2) cytoprotective responses to TNF in human monocytes. We found that in monocytes TNF induces sustained Nrf2 activation and Nrf2 cytoprotective gene induction in a TNFR1-dependent manner. Under TNF activation, monocytes increased their expression of Nrf2-dependent genes, including NAD(P)H:quinone oxidoreductase 1 and glutamyl cysteine ligase modulatory, but not heme oxygenase-1. We also showed that autocrine TNF secretion was responsible for this sustained Nrf2 response and that Nrf2 activation by TNF was mediated by the generation of reactive oxygen species. Moreover, we showed that Nrf2-mediated gene induction can modulate TNF-induced NF-κB activation. These results show for the first time, to our knowledge, that TNF modulates prolonged Nrf2-induced gene expression, which in turn regulates TNF-induced inflammatory responses. The Journal of Immunology, 2011, 187: 702–707.

Monocytes are the main producers of TNF in innate immune responses and in chronic inflammatory diseases such as rheumatoid arthritis. Investigations of the actions of TNF have mostly focused on acute activation of other cell types such as endothelial cells (1). Less is known about the effects of TNF on monocytes themselves, and little is known about the regulation of cell responses to TNF beyond the initial acute and transient activation of signaling pathways and transcription factors such as NF-κB. Studies have emphasized the importance of mechanisms and autocrine loops that sustain and regulate the activity of signaling pathways and transcription factors after the initial and typically transient response to an extracellular ligand (2).

TNF is a pleiotropic cytokine that regulates a broad range of biological activities, including cell differentiation, proliferation, and death, as well as inflammation, innate and adaptive immune responses, and tissue development (3). TNF binds to two distinct receptors on the cell surface, TNFR1 and TNFR2 (4). TNFR1 is constitutively expressed by most cell types, and TNFR2 expression is mainly in immune cells. Most of the biological effects of TNF are attributed to TNFR1, but there is also evidence that both receptors transduce signals cooperatively (5, 6). Ligation of both receptors results in the activation of downstream kinase pathways, NF-κB and the AP-1 transcriptional activator (6). Cellular responses to TNF vary depending on the cell type and microenvironment and are controlled by the balance of activation of various signaling pathways (6, 7).

Recently, the transcription factor NF-E2–related factor 2 (Nrf2) has emerged as a key regulator of inflammation (8, 9). The main role of Nrf2 is to protect cells from oxidative damage by inducing the expression of various cytoprotective and detoxification genes (10). The genes regulated by Nrf2 include the cytoprotective enzyme heme oxygenase-1 (HO-1), as well as the phase II enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and GSTα1 (GSTA1), which detoxify endogenous and exogenous chemicals through reduction and conjugation reactions (10). It also includes the regulation of glutamyl cysteine ligase modulatory (GCLM) and glutamyl cysteine ligase catalytic (GCLC) units, the two subunits of the rate-limiting enzyme in glutathione biosynthesis. Several of these Nrf2-regulated genes have been shown to have anti-inflammatory actions (8, 9, 11–15), which suggests that Nrf2 mediates its control on inflammation by inducing the expression of any number of ARE-dependent genes.

In this study, we report that TNF induces prolonged Nrf2 transcriptional activation in human monocytes. We demonstrate that TNF induced autocrine TNF upregulation, which was responsible for prolonged activation of Nrf2. Previously, we have shown that LPS can induce the expression of HO-1 and NQO1 in human monocytes by an Nrf2-dependent mechanism (8). In this study, we show that TNF can mediate the upregulation of NQO1, GCLM, and GSTA1, but not HO-1. We show that silencing TNF-induced Nrf2 responses leads to a greater induction of proinflammatory cytokines mediated by NF-κB. We also show that TNF induces autocrine TNF expression that further prolongs Nrf2 activation and cytoprotective gene induction. These data suggest that a functional autocrine loop is evident in human monocytes in response to TNF, thus providing new insight into the regulation of inflammatory processes regulated by human monocytes.

Materials and Methods

Materials

All Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Control Nrf2, Bach1 p50, and p65 small interfering RNA (siRNA) were

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Abbreviations used in this article: GCLC, glutamyl cysteine ligase catalytic; GCLM, glutamyl cysteine ligase modulatory; H2DCFDA, dichlorodihydrofluorescein diacetate; HO-1, heme oxygenase-1; NAC, N-acetyl cysteine; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2–related factor 2; ROS, reactive oxygen species; siRNA, small interfering RNA.

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purchased from Applied Biosystems (Foster City, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless indicated.

**Cell culture**

Primary human monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM l-glutamine (Invitrogen). Cells were maintained in a humidified atmosphere at 37°C and 5% CO2. For monocyte isolation, heparinized blood was collected from healthy volunteers and human PBMCs isolated by Histopaque (Sigma-Aldrich) density gradient centrifugation. PBMCs (4 × 10⁶/ml) were incubated in complete medium for 2 h at 37°C to allow adherence of monocytes (16). Cell type was confirmed by microscopy and flow cytometry.

**RNA extraction and real-time PCR**

Total RNA was extracted from 5 × 10⁶ cells using the nucleic acid PrePStation from Applied Biosystems, according to the manufacturer’s instructions. Reverse transcription was performed using the RNA PCR core kit (Applied Biosystems). Real-time PCR primers were purchased from Invitrogen. Sequences of primers were as follows: GAPDH, forward 5'-ACCAGCTC-CAAGATCATCAGC-3' and reverse 5'-TCTAAGCAGTGGTGTCGC-3'; HO-1, forward 5'-ATGCGCTCTTGAACATC-3' and reverse 5'-TTGGGTCTCAATTTCCTCT-3'; Nrf2, forward 5'-AAACAGTTG-GATCTGGCAAC-3' and reverse 5'-AGCATCTGATTTGGGAATGTG-3'; GCLM, forward 5'-GGCGAGGACTTGTATGATGG-3' and reverse 5'-CTTGAGAACCTCGCTGACCAAA-3'; NQO1, forward 5'-GCCGAGGATCTTGGCAGAA-3' and reverse 5'-TTCCCTCCCCCTTTCTTG-3'; ferritin, forward 5'-CTCGTGAAGCTA-GCCGCTCTA-3' and reverse 5'-TGGTCGGGCTTACCATCAG-3'; IL-1β, forward 5'-AGCTGATGCGCTTAAACAGA-3' and reverse 5'-GGAGATCTGAGCTGATGC-3'; and Nrf2, forward 5'-GCCGAGGACTTGAGTTGGA-3' and reverse 5'-CGGTTGCGCTTACCATCAG-3'.

Quantitative real-time PCR was performed using SYBR Green technology (TaqMan) on cDNA generated from the reverse transcription of purified RNA. After preamplification (95°C for 2 min), the PCRs were amplified (Roche) on cDNA generated from the reverse transcription of purified RNA. After preamplification (95°C for 2 min), the PCRs were amplified for 45 cycles (95°C for 15 s, 60°C for 10 s, and 72°C for 10 s) on a Light-Cycler 480 (Roche). Each mRNA expression was normalized against GAPDH mRNA expression using the standard curve method.

**Western immunoblotting and flow cytometry**

NaDodSO₄-PAGE and Western analyses were performed, as described previously (7). Briefly, whole-cell lysates were extracted using radioimmunoprecipitation assay buffer method, and NaDodSO₄–PAGE separation was performed. Nuclear extracts were prepared, as previously described (17). Protein was transferred to nitrocellulose, and Western blot analysis of Nrf2 was performed with the indicated antisera, according to their manufacturer’s guidelines. Flow cytometry for measuring apoptosis was performed, data are representative of three independent experiments. Statistical analyses

**Statistical analyses**

Student t test was performed to assess statistical significance from controls. Results with p values < 0.05 were considered statistically significant (*). Results represent the mean ± SEM of three independent experiments. For Western blotting experiments, data are representative of three independent experiments.

**Results**

**TNF induces sustained Nrf2 activation in human monocytes**

Nrf2 is a key transcription factor in the regulation of antioxidant and cytoprotective genes, and through these genes we have previously shown that Nrf2 can regulate LPS-induced inflammatory processes in human monocytes (8). Our group’s focus is examining TNF-regulated signaling responses in human inflammatory cells with a view to understanding the basic mechanisms that control inflammatory responses in normal and disease states. To address this focus, we first examined the expression of Nrf2 in response to TNF in human monocytes. Fig. 1A shows that TNF can induce Nrf2 protein expression in human monocytes, and that this expression is stable up to and including 72 h. This Western blot for Nrf2 expression shows two bands at ~65 kDa and 110 kDa. These two bands equate to Nrf2 protein (~65 kDa) and ubiquitinated Nrf2 (~110 kDa). Both bands show an increase in response to TNF in human monocytes. We also examined monocyte viability by measuring apoptosis in response to TNF for 72 h. Supplemental Fig. 1A shows that no difference was apparent between the different treatment groups. Cytosolic and nuclear expression is an important marker for the activation of Nrf2. To determine whether TNF can induce Nrf2 nuclear expression, we examined the nuclear and cytosolic expression of Nrf2 in response to TNF. Fig. 1B shows that TNF induces nuclear accumulation of Nrf2 in human monocytes. Next, we examined Nrf2-regulated genes in response to TNF in human monocytes. We examined the mRNA expression of ferritin, GCLM, GCLC, HO-1, NQO1, and GSTA1 in response to TNF stimulation at 2, 24, and 72 h. TNF significantly increased NQO1, GSTA1, and GCLM mRNA expression in primary human monocytes.
monocytes (Fig. 1C). Unexpectedly, TNF stimulation did not induce HO-1 mRNA expression, but slightly reduced its expression. To confirm that Nrf2 was responsible for the induction of TNF-induced NQO1, GSTA1, and GCLM, we used Nrf2 siRNA to inhibit its expression. Fig. 2A shows that we can effectively knock down Nrf2 in human monocytes. Fig. 2B shows that Nrf2 siRNA blocked TNF-induced NQO1, GCLM, and GSTA1 expression in human monocytes. We next examined monocyte viability in Nrf2-silenced monocytes compared with control cells in response to TNF for 24 h. Supplemental Fig. 1B shows a slight decrease in monocyte viability in those cells treated with Nrf2 siRNA in combination with TNF when compared with control cells.

**ROS regulate TNF-induced Nrf2 activation**

Because it has been shown that Keap1 regulates the redox-sensitive activation of Nrf2 (19), and that ROS is known to be produced in response to TNF (20), the role of ROS was examined in regulating TNF-induced Nrf2 activation. We analyzed the production of ROS in monocytes with and without Nrf2 siRNA using dichlorodihydrofluorescein diacetate (H2DCFDA) in response to TNF. In response to TNF, H2DCFDA oxidation occurred in a time-dependent manner (Fig. 2C), suggesting that TNF induces ROS in monocytes. Furthermore, when we silence Nrf2, we get an increase in ROS production compared with control siRNA, when treated with TNF. The kinetics of ROS activation correlated with the nuclear accumulation of Nrf2 following TNF. The antioxidant N-acetyl cysteine (NAC) inhibited TNF-induced Nrf2 induction (Fig. 2D) at 4 and 24 h, suggesting that the production of ROS and the activation of Keap1 are required for the sustained TNF-induced Nrf2 activation in monocytes.

**TNFR1 signaling is required for gene induction**

Next, we wanted to address the functional importance of TNF-induced Nrf2-mediated gene expression, as well as examining the different kinetics of Nrf2-mediated gene expression to TNF treatment. First, we wanted to determine whether the pattern of TNF-induced Nrf2-mediated gene expression was induced by TNF and not by contaminating microbial products. We prevented binding of TNF to its receptor using soluble TNF receptor (Humira). Fig. 3A and 3B shows that Nrf2 nuclear expression and Nrf2-mediated gene expression are attenuated with the cotreatment of TNF and anti-TNF (Humira).

It is important to establish the functional relevance of TNFR1 and TNFR2 in regulating TNF-mediated cytoprotective gene expression in human monocytes. Next, we used siRNA to knock down TNFR1 and TNFR2 (Fig. 3C). Targeting mRNA knockdown for TNFR1 and TNFR2 shows that inhibiting the expression of TNFR1 alone or in combination with TNFR2 blocks the TNF-induced Nrf2-mediated activation (Fig. 3D). Fig. 3E confirms that TNFR1 alone or in combination with TNFR2 blocks the TNF-induced Nrf2-mediated gene upregulation.

**Autocrine TNF responses mediate the prolonged activation of Nrf2**

To understand the kinetics of prolonged autocrine TNF responses, we first examined the mRNA expression of TNF to TNF treatment in human monocytes over a 48-h time period with a 1-h pretreatment or 2-h posttreatment with anti-TNF. Fig. 4A shows that TNF mRNA doesn’t return to basal levels even after 48 h. When we preincubated with anti-TNF for 1 h, prior to TNF treatment, unsurprisingly no TNF mRNA was induced. When we added anti-
TNF after 2 h of TNF treatment, this caused TNF mRNA levels to return to constitutive levels within 12 h. Next, we examined Nrf2 nuclear expression in response to TNF with a 1-h pretreatment or 2-h posttreatment with anti-TNF, and then RNA extracted and measured for TNF expression (A). B, Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2. C, Monocytes treated with TNF for 24 h were either untreated, or posttreated with anti-TNF, and then mRNA was prepared and analyzed for GCLM, NQO1, and GSTA1. D, Monocytes were either untreated or pretreated with BAY 11-7082 for 30 min before activation with TNF for 4 and 24 h. Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2, and mRNA was prepared and analyzed for NQO1 expression. Results are representative of similar findings from at least three separate experiments. Statistically significant difference of *p < 0.05 exists where indicated (*).

siRNA silencing of Nrf2 prolongs TNF-induced cytokine responses and induces proliferation

To understand the significance of TNF-induced Nrf2 activation in human monocytes, we used siRNA to target knockdown of Nrf2. Fig. 2A shows that we can effectively and specifically knock down mRNA levels of Nrf2 in human monocytes. Next, we examined the effect of Nrf2 siRNA on TNF-induced TNF and IL-1β mRNA expression. Fig. 5A shows that Nrf2 siRNA significantly prolonged the TNF-induced TNF and IL-1β expression in human monocytes. NF-κB is known to regulate the expression of several genes that are essential for initiating and promoting inflammation, including TNF and IL-1β. Because silencing Nrf2 in monocytes enhanced TNF-induced proinflammatory gene expression, their effects on NF-κB activation were then studied. Following TNF stimulation, NF-κB activation was examined after TNF stimulation in human monocyte cells using a p50 and p65 DNA-binding assay. Silencing Nrf2 resulted in significantly enhanced TNF-induced p50 and p65 DNA binding, suggesting an inhibitory role of Nrf2 on NF-κB activation (Fig. 5B).

Discussion

Studies have emphasized the importance of mechanisms and autocrine loops that sustain and regulate the activity of signaling pathways and transcription factors after the initial and typically transient response to an extracellular ligand (2, 22). Research is limited in the study of TNF on monocytes, that is, not much is known about the regulation of monocyte responses to TNF beyond the initial acute and transient activation of signaling pathways (23, 24). The present study aimed to address the long-term exposure of monocytes to TNF. We showed that TNF induces the expression and activation of the transcription factor Nrf2, which leads to the upregulation of cytoprotective and detoxification genes, including NQO1, GCLM, and GSTA1. The regulation of such genes by Nrf2 has been shown to regulate innate immune responses in various experimental disease models (25, 26). This is the first time, to our knowledge, that TNF alone has been shown to regulate the expression and activation of Nrf2.

FIGURE 4. Autocrine TNF responses regulate sustained Nrf2 activation. TNF-treated monocytes were either untreated or pretreated, or posttreated with anti-TNF, and then RNA extracted and measured for TNF expression (A). B, Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2. C, Monocytes treated with TNF for 24 h were either untreated, or posttreated with anti-TNF, and then mRNA was prepared and analyzed for GCLM, NQO1, and GSTA1. D, Monocytes were either untreated or pretreated with BAY 11-7082 for 30 min before activation with TNF for 4 and 24 h. Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2, and mRNA was prepared and analyzed for NQO1 expression. Results are representative of similar findings from at least three separate experiments. Statistically significant difference of *p < 0.05 exists where indicated (*).

FIGURE 5. Silencing Nrf2 increases TNF-induced inflammatory responses in human monocytes. A, Monocytes were transfected with 30 nM of either control siRNA or Nrf2 siRNA for 24 h prior to treatment with TNF for various times. TNF or IL-1β mRNA expression levels were detected by real-time PCR. B, Monocytes were transfected with 30 nM of either control siRNA or Nrf2 siRNA for 24 h, and then stimulated with TNF for various times, and nuclear extracts were prepared. NF-κB DNA binding was assessed using the Panomics p50 and p65 transcription factor ELISA. Results are representative of similar findings from at least three separate experiments. Statistically significant difference of *p < 0.05 exists where indicated (*).
The control of Nrf2 transcription and its subsequent transcriptional responsibilities in controlling NQO1, GCLM, and GSTA1 is required for the dampening of TNF-induced inflammatory responses, without which inflammation could and probably would run unchecked. For example, NQO1 has been shown to be anti-inflammatory by modulating NF-κB (27). Moreover, other groups have shown that NQO1 is anti-inflammatory and can inhibit TNF-induced VCAM-1 expression in human endothelial cells (28). Interestingly, it was reported that this regulation of TNF-induced VCAM-1 expression was not through NF-κB inhibition. Thimmulappa et al. (9) reported that CDDO-Im (a synthetic triterpenoid) induces a number genes, including NQO1 in neutrophils, and was associated (coincided) with the inhibition of LPS-induced IL-6 and TNF induction. The results of the current study show that TNF-induced expression of NQO1 in combination with other Nrf2-regulated genes can inhibit TNF-induced TNF and IL-1β expression in human monocytes, and that this inhibition seems to be sustained and NF-κB dependent. Our results also suggest that Nrf2-regulated glutathione-induced signaling may also play an essential role in regulating immune response in human monocytes.

The present study demonstrates that ROS regulates the activation of Nrf2 in response to treatment with TNF; however, what is the significance of increased Nrf2-targeted antioxidant and detoxification genes in this system? One could argue that increasing antioxidant genes is to counteract increased ROS caused by TNF treatment, and thus protect monocytes from undergoing ROS-induced apoptosis. In this study, we showed a decrease in monocytes’ viability in response to silencing of Nrf2 in combination with TNF treatment. This is an important physiological finding and would certainly be beneficial to an inflammatory process in which monocytes are important mediators of such responses. However, if Nrf2 signaling is interrupted, then inflammation may well run unchecked and result in the progression to inflammation-associated diseases.

In this study, we also showed that in human monocytes, HO-1 mRNA expression is reduced in response to TNF. These results corroborate work by Kirino et al. (29), who showed that TNF induces HO-1 downregulation in human monocytes by promoting the degradation of HO-1 mRNA. In our laboratory, we have shown that under normal conditions, human monocytes express significantly more HO-1 than other inflammatory cells (S.A. Rushworth and D.J. MacEwan, unpublished observations). This begs the question as to why these cells regulate this gene in such an aggressive way. We can certainly speculate that to get a significant inflammatory response to TNF, monocytes have to first control the expression of anti-inflammatory genes. However, if this is the case, then why does TNF not promote degradation of other protective genes? This concept that TNF can promote HO-1 mRNA degradation is very interesting and needs further investigation.

Another interesting finding of this study is that when block TNFR signaling or oxidant activity using NAC, this leads to inhibition of Nrf2 nuclear accumulation in monocytes. However, when we inhibit NF-κB using BAY 11-7082, this does not lead to inhibition of Nrf2 nuclear accumulation. This suggests that induction of ROS and subsequently Nrf2 nuclear accumulation in monocytes are independent of NF-κB activation. Moreover, blocking NF-κB activity alone or in combination with TNF has been shown to increase the levels of ROS, which can induce apoptosis (21, 30, 31). In this study, no apoptosis was observed in human monocytes by the treatment of NF-κB inhibition. Taken together, these results indicate that NF-κB does not play a role in regulating early Nrf2 nuclear accumulation in human monocytes. We have recently shown that LPS can induce Nrf2 expression and activation in human monocytes; moreover, we also show that HO-1 is induced in this system (8). This raises an important point for discussion, which is why does TNF have little effect on HO-1 expression, whereas LPS induces its induction within the same cells? The answer to that question may lie in the mechanism of HO-1 regulation. This is because the control of HO-1 gene is under the control of not just Nrf2, but many other transcription factors (30, 32–35). Among the transcription factors NF-κB, Nrf2, and AP-1, families are arguably the most important, and among the best studied; however, others, including heat shock factor, upstream stimulatory factors, Esz-2, hepatoocyte NF-4 with Sp1 or Sp3, and STAT proteins, have all been implicated in regulating HO-1 transcription (34).

In conclusion, the results of the current study show human monocytes depleted of Nrf2 have elevated levels of TNF-induced inflammatory cytokines, which may promote their recruitment of other leukocytes, thereby perpetuating inflammation and injury in response to infection. Furthermore, we show that TNF regulates the activation of the transcription factor Nrf2 and its associated gene targets. This occurs through a TNFR1-dependent mechanism with autocrine TNF production sustaining the Nrf2 activation up to 72 h posttreatment. Taken together, understanding of these mechanisms that protect against excessive inflammatory responses may pave the way to provide novel therapeutic strategies for the treatment of inflammation-associated disease processes.

Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In Fig. 4D, the incorrect immunoblot for the loading control was mistakenly used. The correct immunoblot has now been used in the updated Fig. 4. The results and the conclusions of the article remain unchanged. The corrected figure is shown below. The published figure legend is correct, but is shown below for reference. The online version of the article has been corrected and now differs from the print version as originally published.

**FIGURE 4.** Autocrine TNF responses regulate sustained Nrf2 activation. TNF-treated monocytes were either untreated or pretreated, or posttreated with anti-TNF, and then RNA extracted and measured for TNF expression (A). B. Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2. C, Monocytes treated with TNF for 24 h were either pretreated, or posttreated with anti-TNF, and then mRNA was prepared and analyzed for GCLM, NQO1, and GSTA1. D, Monocytes were either untreated or pretreated with BAY 11-7082 for 30 min before activation with TNF for 4 and 24 h. Nuclear extracts were prepared and separated by SDS-PAGE for Western blot analysis of Nrf2, and mRNA was prepared and analyzed for NQO1 expression. Results are representative of similar findings from at least three separate experiments. Statistically significant difference of *p* < 0.05 exists where indicated (*).

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