Published in final edited form as:

Cytokine. 2009 June ; 46(3): 359–369. doi:10.1016/j.cyto.2009.03.010.

Tumor necrosis factor (TNF)- α -induced IL-8 expression in gastric epithelial cells: Role of reactive oxygen species and AP endonuclease-1/redox factor (Ref)-1

Ann M. O'Hara^{a,1}, Asima Bhattacharyya^a, Jie Bai^a, Randy C. Mifflin^b, Peter B. Ernst^a, Sankar Mitra^c, and Sheila E. Crowe^{a,*}

^a Department of Medicine, University of Virginia, Charlottesville, VA 22908, USA

^b Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA

^c Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, USA

Abstract

TNF- α contributes to oxidative stress via induction of reactive oxygen species (ROS) and pro-inflammatory cytokines. The molecular basis of this is not well understood but it is partly mediated through the inducible expression of IL-8. As redox factor-1 (Ref-1), is an important mediator of redox-regulated gene expression we investigated whether ROS and Ref-1 modulate TNF- α -induced IL-8 expression in human gastric epithelial cells. We found that TNF- α treatment of AGS cells enhanced nuclear expression of Ref-1 and potently induced IL-8 expression. Overexpression of Ref-1 enhanced IL-8 gene transcription at baseline and after TNF- α treatment whereas Ref-1 suppression and antioxidant treatment inhibited TNF- α -stimulated IL-8 expression. TNF- α -mediated enhancement of other pro-inflammatory chemokines like MIP-3 α and Gro- α was also regulated by Ref-1. Although TNF- α increased DNA binding activity of Ref-1-regulated transcription factors, AP-1 and NF- κ B, to the IL-8 promoter, promoter activity was mainly mediated by NF- κ B binding. Silencing of Ref-1 in AGS cells inhibited basal and TNF- α -induced AP-1 and NF- κ B DNA binding activity, but not their nuclear accumulation. Collectively, we provide the first mechanistic evidence of Ref-1 involvement in TNF- α -mediated, redox-sensitive induction of IL-8 and other chemokines in human gastric mucosa. This has implications for understanding the pathogenesis of gastrointestinal inflammatory disorders.

Keywords

ROS; Ref-1; TNF- α ; IL-8; AGS cell

1. Introduction

ROS such as superoxide radical (O₂⁻) are ubiquitous, highly diffusible molecules that are increased during inflammatory processes with activated phagocytes, neutrophils, and macrophages recruited to sites of inflammation serving as potent sources of ROS. An excess of ROS *in vivo* can adversely alter intracellular reduction/oxidation (redox) homeostasis and

ROS have been implicated as a major cause of cellular and tissue damage associated with chronic inflammation [1,2]. In the human gastric mucosa, elevated levels of ROS have been associated with *Helicobacter pylori*-infection [3] and it is thought that this leads to oxidative DNA damage in the gastric mucosa thereby contributing to mucosal injury and promoting carcinogenesis [4,5]. *H. pylori* infection is also associated with increased gastric mucosal cytokine expression including interleukin (IL)-8 [6,7] and tumor necrosis factor (TNF)- α [8–11].

TNF- α is an endogenous mediator of pro-inflammatory cytokine stimulation and other cellular responses, including lymphocyte activation and migration, and cell proliferation, differentiation and apoptosis [12–14]. Moreover, TNF- α can induce ROS [15,14] and stimulate the induction of various genes involved in inflammation [16–18] including interleukin-8 (IL-8). IL-8 (CXCL8), shows potent chemotactic activity for neutrophils [19] and is an important mediator of *H. pylori*-associated neutrophil infiltration and gastric inflammation [6]. IL-8 gene expression is regulated primarily at the transcriptional level in a stimulus- and cell type-specific manner [20] while the promoter contains binding sites for several inducible transcription factors including activator protein (AP)-1, nuclear factor (NF)- κ B and NF-IL-6 [21,22]. Studies show that ROS modulates IL-8 secretion in gastric epithelial cells, suggesting that IL-8 gene expression in the gastric mucosa is redox-sensitive [23].

Oxidative changes modulate activity of several transcription factors. A key mediator of redox-sensitive transcription factor activation and gene expression is redox factor-1 (Ref-1) [24], also referred to as apurinic/apyrimidinic endonuclease-1. Ref-1 is a ubiquitous multi-functional protein initially characterized as a DNA repair enzyme [25] that is also involved in the redox regulation of transcription factor DNA binding activity via the reduction of a conserved cysteine residue in the DNA binding domain of several transcription factors. These include AP-1, NF- κ B, p53, Myb, hypoxia-inducible factor-1- α and others [26,24]. Studies have shown that ROS mediate and enhance Ref-1 expression and activity in fibroblasts, HeLa, B cells and other cell types [27–30]. We previously demonstrated a redox-sensitive increased expression of Ref-1 in ROS-stimulated and *H. pylori*-infected human gastric epithelial cells and reported that Ref-1 activates AP-1 and NF- κ B in these cells [31,32].

The present study aims to investigate the mechanism of IL-8 induction by TNF- α and examine whether Ref-1 plays a role in the modulation of TNF- α -induced IL-8 in human gastric epithelial cells. We identified that TNF- α -mediated IL-8 expression involves ROS and that the major regulators of IL-8 transcription, NF- κ B and AP-1 are themselves regulated by induction of Ref-1 in TNF- α -treated AGS cells. In addition, we found that other immunomodulatory cytokines including a CC chemokine, MIP-3 α , and a CXC chemokine, Gro- α , are also upregulated by Ref-1 in TNF- α -treated AGS cells. Thus, this study establishes a novel role of Ref-1 in regulation of TNF- α -induced inflammatory reactions in gastric epithelial cells.

2. Methods

2.1. Cell culture and treatments

AGS cells (human gastric epithelial cells) were grown as previously described [31]. Cell viability was determined by trypan blue exclusion. AGS cells were seeded into 75-cm² culture flasks or 4-cm² 12-well plates 24 h prior to treatment. All treatments were performed in serum-free Ham's F12 to minimize serum-induced IL-8 release. Recombinant human TNF- α (Peprotech Inc., Rocky Hill, NJ) was used at 20 ng/ml. To examine the effect of the antioxidant *N*-acetyl-L-cysteine (NAC) on TNF- α -induced IL-8 expression, AGS cells were pre-incubated with varying concentrations of NAC (Sigma Chemical Company, St. Louis, MO) for 30 min prior to TNF- α treatment.

2.2. Primers and PCRs

Total RNA was extracted from AGS cells using the RNeasy kit (Qiagen, Valencia, CA) and was reverse transcribed to get cDNA using the SuperScript First Strand synthesis system (Invitrogen, Carlsbad, CA). Unless mentioned otherwise, primers were from Integrated DNA Technologies, Inc. (Coralville, IA). Real-time dual-labeled probe RT-PCR for human Ref-1, IL-8, or the epithelial cell-derived neutrophil-activating protein-78 chemokine (ENA-78 or CXCL5) was performed in a SmartCycler (Cepheid, Sunnyvale, CA). A pre-optimized FAM-labeled primer and probe set (Applied Biosystems, Foster City, CA) was used to amplify ENA-78. The Ref-1 and IL-8 primer and TET-labeled probe sets were designed in our laboratory. The IL-8 primer sequences have previously been described [33] and sequences for the Ref-1 primers were 5'-TGGATTGTGGATGGGCTTCGAGCC-3' (forward), 5'-AAGGAGCTGACCAGTATTGATGA-3' (reverse) and 5'-/5TET/TAAAGGAAGAAGCCCCAGATATACTGT/3BHQ-1/-3' (probe). Real-time RT-PCR reaction mixtures were prepared and PCR amplified as described [32]. Following RT-PCR amplification, Ref-1 or IL-8 mRNA expression levels were determined semi-quantitatively by comparing the critical threshold (C_T) values to a standard curve and normalizing these C_T values to 18S rRNA C_T values generated from the same cDNA samples using the pre-optimized 18S rRNA primer and probe set (Applied Biosystems). Expression levels of ENA-78 were determined using hypoxanthine phosphoribosyl-transferase (HPRT) as the endogenous control reference gene and normalized levels of ENA-78 expression were calculated according to the formula $2^{(R_t - E_t)}$, where R_t is the threshold cycle of HPRT and E_t is the threshold cycle of the experimental gene ($\Delta\Delta C_T$ method). Sequences for the HPRT primers were 5'-TGCCGAGGATTTGGAAAAGTG-3' (forward) and 5'-CACAGAGGGCCACAATGTGATG-3' (reverse).

Expression levels of the chemokines macrophage inflammatory protein (MIP)-3 α (CCL20) and growth-related oncogene (GRO)- α (CXCL1) were analyzed by real-time RT-PCR using the MJ Research Opticon system with SYBR Green I (Molecular Probes, Eugene, OR) as a fluorescent reporter. The primer sequences used were: MIP-3 α (forward, 5'-CTGGCCAATGAAGGCTGTGA-3'; reverse, 5'-ACCTCC AACCCCAGCAAGGT-3') and GRO- α (forward, 5'-AAACCGAAGTCATAGCCACACT-3'; reverse, 5'-CAGGGCCTCCTTCAGGAACA-3'). Calculations were performed using HPRT as the reference gene and the $\Delta\Delta C_T$ method described above.

GAPDH and β -actin mRNA expression were assessed by usual PCR amplification in a DNA Engine ThermoCycler (MJ Research Inc., Watertown, MA) and by running the PCR products on 2% agarose gel. The β -actin primers were from Applied Biosystems. GAPDH primers were 5'-GGC-GTCTTCACCACCATGGAG-3' (forward) and 5'-AAGTTGTCATGGATTGACCTTGG-3' (reverse). PCR cycling conditions for GAPDH consisted of 95 °C for 5 min, followed by 34 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, whereas those for β -actin consisted of 95 °C for 5 min, followed by 39 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min.

2.3. IL-8 protein determination by ELISA

IL-8 protein levels in TNF- α -treated cell-culture supernatants were quantified using an ELISA DuoSet kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The assay has a sensitivity of approximately 10–50 pg/ml.

2.4. Silencing of Ref-1 by RNA interference

The siRNA sequences used to silence Ref-1 in AGS cells {5'-GUCUGGUACGACUGGAGUACC-3' (sense) and 5'-UACUCCAGUCGUACCAGACCC-3' (antisense) (Dharmacon Research, Lafayette, CO)}

were described earlier by Fan et al. [34]. The scrambled siRNA sequences were 5'-ACGGUGUAG-GUCGACUGUAdTdT-3' (sense) and 5'-UACAGUCGACCUACACCGUdTdT-3' (antisense) (Dharmacon). siControl RNATM (Dharmacon), a non-targeting siRNA, was used instead of scrambled siRNA in some experiments. siRNAs were introduced in AGS cells and after 24 h, the transfection reagent was removed and cells were incubated in fresh medium for additional 24–48 h before analyzing Ref-1 expression levels by real time RTPCR and western blotting as previously described [32].

2.5. Electrophoretic mobility shift assay (EMSA)

AGS cells seeded in 75-cm² tissue culture flasks in 10 ml of growth media were incubated for 24 h. After replacing media with 8 ml of fresh media cells were transfected with 50 nM Ref-1 siRNA using 16 µl of Lipofectamine 2000 per flask with non-transfected cells used as controls. After 24 h the media was removed and replaced with 10 ml of serum-free Ham's F12 and cells were incubated for a further 24 h prior to treatment with 20 ng/ml TNF- α for varying times. Subsequently, cells were scraped, washed with ice cold PBS, and the cells from three wells were pooled and nuclear extracts were prepared as described [35]. After extraction, nuclear proteins were normalized using the BCA protein assay reagent kit (Pierce, Rockford, IL). EMSA was done using Gel Shift assay system (Promega, Madison, WI) using [γ -³²P] ATP (Amersham) end-labeled consensus AP-1 or NF- κ B oligonucleotides (Promega). Each binding reaction mixture contained 8.5 µg of nuclear extract in gel shift binding buffer (Promega). Reactions were incubated at room temperature for 10 min and then 0.06 pmol of γ -³²P-labeled oligonucleotide was added. AP-1 DNA binding reactions were incubated for additional 20 min at room temperature: NF- κ B DNA binding reactions were incubated for 20 min at room temperature followed by overnight incubation at 4 °C. Subsequently, the samples were fractionated through 4% non-denaturing polyacrylamide gels. In competition assays, 3.5 pmol of cold AP-1 or NF- κ B consensus (specific competitors) or cold oligos were added simultaneously with the labeled probes. Following electrophoresis, gels were dried and exposed for autoradiography at -70 °C.

2.6. Effect of Ref-1 suppression on TNF- α -induced nuclear accumulation of NF- κ B and AP-1

Western blot was performed to determine whether TNF- α induced an accumulation of Ref-1 in the nucleus and to examine expression of NF- κ B subunits, p50 and p65 and AP-1 subunits, c-Jun and c-Fos in the nucleus of AGS cells. Ref-1 protein levels were also analyzed by western blot of whole cell lysates. AGS cells were seeded and mock-transfected or transfected with siRNA or siControl RNATM and 48 h later were treated with 20 ng/ml TNF- α for 1 h (for NF- κ B) or 2 h (for API-1). Subsequently, whole cell lysates were prepared or nuclear proteins were extracted as described previously [31] and the protein concentration of the resulting lysates or nuclear extracts was measured using the BCA protein assay reagent kit. For transcription factor assays, protein samples (8 µg) were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Immunodetection analysis was performed by incubating the membrane with rabbit anti-p50, -p65, -c-Fos, or -c-Jun antibody (Cell Signaling Technology) at a 1:1000 dilution. HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) was used as a secondary antibody. To examine total or nuclear Ref-1 expression, 5 µg of the relevant protein sample was fractionated by 10% SDS-PAGE, immunoblotted and the membrane was then incubated with Ref-1 MAb (Novus Biologicals) at 1:3000 dilution, followed by HRP-conjugated goat anti-mouse IgG (Cell Signaling Technology) as a second antibody. Immunoreactions were visualized by enhanced chemiluminescence (Amersham) and autoradiographed.

2.7. Determination of the effect of Ref-1 silencing on TNF- α -induced IL-8

The effect of TNF- α treatment on IL-8 mRNA expression was determined in mock-transfected AGS cells and cells transfected with 50 nM Ref-1 siRNA. We previously demonstrated that IL-8 expression was no different in AGS cells that were transfected with scrambled siRNA versus mock-transfected cells [32]. Increased IL-8 mRNA expression in gastric epithelial cell lines can occur within 1 h of TNF- α treatment [36]. Preliminary time course experiments showed that treatment of AGS cells (48 h post-transfection) with 20 ng/ml TNF- α for 3 h resulted in increased IL-8 protein production as reported for other gastric epithelial cell lines [37]. For subsequent studies IL-8 protein was determined by ELISA 3 h following TNF- α treatment. To normalize for cell number between control- and siRNA-transfected cells, the total protein concentration of the lysates was determined by Bradford assay and results were expressed as pg of IL-8/mg total protein concentration.

2.8. Luciferase assay

Human IL-8 promoter luciferase constructs were prepared as described earlier [38] and four hIL-8 promoter constructs were used. The -1498/+44 hIL-8/Luc and -162/+44 hIL-8/Luc plasmids contained binding sites for AP-1, NF-IL-6 and NF- κ B. In the -99/+44 hIL-8/Luc plasmid the AP-1 binding site was deleted and -54/+44 hIL-8/Luc plasmid lacked all three transcription factor binding sites. Site-directed mutations of the IL-8 AP-1 and NF- κ B binding sites in the -162/+44 hIL-8 promoter were introduced by the PCR overlap extension mutagenesis technique as described [39]. To induce the over-expression of Ref-1, pFLAG-REF-1 cDNA3.1 was used [31].

For the luciferase assays, 24 h prior to transfection, AGS cells (1×10^5) were seeded in triplicate in 12-well plates. Subsequently, cells were transiently transfected with equimolar amounts of one of the four hIL-8/Luc promoter constructs and pRL-TK *Renilla* luciferase construct (Promega) using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). For each transfection, 3 μ l of Fugene 6 and 1 μ g of DNA was used. When needed, 0.25 μ g of pFLAG-Ref-1 cDNA3.1 was also transfected to overexpress Ref-1. The transfection reagent was removed 24 h post-transfection and cells were incubated for a further 48 h in 2 ml of Ham's F12 with 0.2% heat-inactivated FCS. After 3 h TNF- α in serum-free media cells were lysed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luminescence signals were quantified using a MircoBeta TriLux luminescence counter (Wallac, Turku, Finland). Firefly luciferase activity was normalized to the *Renilla* luciferase activity and then normalized to protein determined by Bradford assay as previously reported [32]. Overexpression of Ref-1 in cells transfected with pFLAG-Ref-1 cDNA3.1 was confirmed by comparing with control vector-transfected cells and analyzed by western blot as described above.

2.9. Statistical analysis

Depending on sample type, two-tailed Student's *t* test, Mann-Whitney Rank Sum test or the Kruskal-Wallis One-Way Analysis of Variance were used for data analysis. The Signed Wilcoxon's Rank Sum test was used to analyze the mRNA expression levels of MIP-3 α , ENA-78 and GRO- α . Data are expressed as the mean $\pm$ SEM. *P* values <0.05 were considered significant.

3. Results

3.1. NAC inhibits TNF- α -induced IL-8 expression

In the absence of serum, IL-8 protein was not detectable in untreated AGS cell supernatants, but within 3 h of TNF- α treatment a significant level of IL-8 protein (772 ± 135.2 pg/ml) was

detected. To determine whether ROS play a role in mediating TNF- α -induced IL-8 protein expression, AGS cells were pre-treated with varying concentrations of NAC, a commonly used agent with antioxidant properties [40], for 30 min prior to treatment with TNF- α . As shown in Fig. 1A, pre-treatment with NAC attenuated TNF- α -induced IL-8 secretion in a dose-dependent manner. At the lowest concentration of NAC tested (10 mM), TNF- α -stimulated IL-8 was suppressed by 38% and a significant inhibition was observed when cells were pre-treated with 20 mM (62% inhibition) or 40 mM (78% inhibition) NAC. Low but detectable levels of IL-8 mRNA were measured in untreated AGS cells and treatment with TNF- α for 1 h resulted in a significant increase in IL-8 mRNA expression that was dose-dependently suppressed by NAC (Fig. 1B). Compared with TNF- α alone, pre-treatment with 10, 20, or 40 mM NAC significantly inhibited TNF- α -induced IL-8 mRNA expression in AGS cells by approximately 50%, 70%, or 88%, respectively.

3.2. Silencing of Ref-1 inhibits TNF- α -induced IL-8 secretion in gastric epithelial cells

The inhibition of TNF- α -induced IL-8 by NAC implied that this response in AGS cells was mediated by ROS. As Ref-1 is an important mediator of redox-regulated gene expression in other cell types, we investigated whether Ref-1 modulates TNF- α -induced IL-8 in human gastric epithelial cells by silencing Ref-1 expression using RNA interference. Silencing Ref-1 gene using siRNA suppressed mRNA expression by >90% and protein expression by 75% compared to mock-transfected or scrambled siRNA-transfected cells as we have reported earlier [32].

The effects of Ref-1 gene silencing on inducible IL-8 expression in TNF- α -treated AGS cells 48 h after siRNA transfection are depicted in Fig. 2A and B. The increased IL-8 mRNA expression that was detected in mock-transfected AGS cells exposed to TNF- α for 1 h was significantly inhibited (>76%) in TNF- α -treated cells with silenced Ref-1 expression (Fig. 2A). Moreover, in Ref-1 siRNA-transfected cells, TNF- α -stimulated IL-8 protein secretion was suppressed by >83% compared to TNF- α -treated cells that were transfected with Lipofectamine 2000 alone (Fig. 2B). The data implicate Ref-1 as a key regulatory factor in the induction of IL-8 by TNF- α in human gastric epithelial cells.

3.3. Ref-1 suppression inhibits DNA binding activity of transcription factors

The human IL-8 promoter contains binding sites for AP-1 and NF- κ B and both of these elements have been implicated in TNF- α -induced IL-8 gene transcription in gastric epithelial cells [23]. Given that Ref-1 regulates AP-1 and NF- κ B DNA binding activity [26], we used gel shift assays to examine the effect of Ref-1 gene silencing on the DNA binding activity of these transcription factors in AGS cells. As shown in Fig. 3A, high basal AP-1 DNA binding activity was observed in untreated non-transfected AGS cells and a small additional increase in AP-1 activation was detected in nontransfected cells that were treated with TNF- α for 1 h. In cells with silenced Ref-1 expression, both basal and TNF- α -induced AP-1 DNA binding activity were markedly inhibited compared to cells that expressed normal levels of Ref-1. Competition with an unlabeled AP-1 probe or unrelated oligomer demonstrated that the AP-1 complexes were specific.

As shown in Fig. 3B, control non-transfected AGS cell extracts contained little nuclear NF- κ B binding activity, but treatment with TNF- α for 2 h resulted in a marked augmentation of NF- κ B DNA binding. The complex induced by TNF- α was abolished by a specific oligomer, but not by an unrelated one, demonstrating the specificity of the NF- κ B complex. The small amount of activated NF- κ B that was detected in control non-transfected cells was almost completely abolished in untreated cells transfected with Ref-1 siRNA. Moreover, TNF- α -induced NF- κ B DNA binding activity was substantially inhibited in cells with silenced Ref-1 expression compared to TNF- α -treated non-transfected cells. These data demonstrate that both

basal and TNF- α -induced transcription factor DNA binding activity in gastric epithelial cells is suppressed in cells with silenced Ref-1 expression, indicating a role of Ref-1 in the regulation of AP-1 and NF- κ B activation in these cells.

3.4. TNF- α -induced nuclear translocation of transcription factors is not inhibited by Ref-1 gene silencing

We next examined whether the accumulation of transcription factors in the nucleus is also regulated by Ref-1. Western blot was used to examine the effect of Ref-1 silencing on the levels of c-Jun, c-Fos, p50 and p65 in nuclear extracts prepared from untreated or TNF- α -treated AGS cells. In non-transfected cells or siControl RNATM-transfected cells, increased levels of p65, c-Jun, and c-Fos were detected in the nucleus following treatment with TNF- α (Fig. 3C). A lesser increase of p50 was seen in the TNF- α -treated cells. In addition, elevated levels of nuclear Ref-1 were observed in TNF- α -treated cells. Decreased Ref-1 expression was confirmed in both nuclear extracts and whole cell lysates of cells transfected with Ref-1 siRNA. In the cells with silenced Ref-1 expression, the levels of p50, p65, c-Jun and c-Fos detected in the nucleus following TNF- α treatment were similar to those detected in non-transfected or siControl RNATM-transfected cells (Fig. 3C). Our results demonstrate that TNF- α induces the nuclear translocation of Ref-1 along with transcription factors. Collectively, the findings indicate that Ref-1 modulates transcription factor DNA binding activity in the nucleus while the translocation of these transcription factors to the nucleus is not dependent on Ref-1.

3.5. Ref-1 modulates the expression of other TNF- α -induced chemokines

In addition to IL-8, TNF- α is known to stimulate the secretion of a variety of other immunomodulatory chemokines including Gro- α , MIP-3 α and ENA-78 [41,42]. To determine whether Ref-1 played a role in the expression of other gastric epithelial chemokines RT-PCR was performed on cells treated with Ref-1 siRNA or siControl RNATM then treated with TNF- α for 1 h or left untreated. Chemokine expression in siControl RNATM-transfected cells was not significantly different than in non-transfected cells (data not shown) and thus only data from Ref-1 siRNA and siControl RNATM-transfected cells are included in Table 1. Increased levels of IL-8, MIP-3 α and Gro- α mRNA were detected in AGS cells within 1 h of TNF- α treatment. However, at this time-point ENA-78 mRNA levels were not significantly increased compared to untreated cells and the effect of silencing of Ref-1 could not be evaluated for this chemokine with very low level mRNA expression (data not shown). In TNF- α -stimulated cells with silenced Ref-1 expression, the levels of IL-8, MIP-3 α , or Gro- α mRNA expression were significantly inhibited by 62%, 49%, or 33%, respectively ($P < 0.05$) compared to scrambled siRNA-transfected cells (Table 1). A functional role of Ref-1 in the modulation of chemokine expression in human gastric epithelial cells is suggested by these findings.

3.6. TNF- α -induced IL-8 promoter activation in AGS cells

In order to define the regions of the IL-8 promoter involved in regulating inducible gene expression in gastric epithelial cells, AGS cells were transiently transfected with luciferase-linked plasmids containing serial deletions of the 5' flanking region of the IL-8 gene (Fig. 4). In untreated cells transfected with the -1498/+44 hIL-8/Luc construct, high levels of basal IL-8 luciferase activity were detected (Table 2). Deletion from -1498 to -99 bp reduced basal IL-8 luciferase activity by 15.8-fold, suggesting that the AP-1 binding site is important in basal IL-8 promoter activation and the deletion to -54 bp reduced basal promoter activity by 69.8-fold compared to baseline activity detected in cells transfected with the -1498/+44 hIL-8/Luc construct (Table 2).

The effect of these promoter deletions on TNF- α -induced IL-8 promoter activity is shown in Fig. 5A. Treatment with TNF- α induced the luciferase activity of the -1498/+44 and -99/+44 hIL-8/Luc constructs by 4.75-fold and 6.0-fold, respectively, compared to untreated cells.

Thus, the deletion to -99 bp did not affect the inducibility of the promoter, although the overall basal activity of the promoter was reduced (Table 2). However, further deletion to -54 bp completely abolished TNF- α -induced IL-8 luciferase activity (Fig. 5A), indicating that the region from -99 to -54 bp, a region that contains important regulatory elements including the NF- κ B binding site, is absolutely required for IL-8 gene activation TNF- α . The data also implicate a more important role for NF- κ B than AP-1 in TNF- α -induced IL-8 promoter activation in AGS cells.

We next determined the relative role of the Ref-1-regulated transcription factors, AP-1 or NF- κ B, on IL-8 transcription by examining the effect of site-directed mutations of the AP-1 or NF- κ B sites on inducible promoter activity in AGS cells with basal or over-expressed levels of Ref-1. The constructs bearing site-directed mutations of the AP-1 and NF- κ B sites were prepared from the -162/+44 hIL-8/Luc construct. Although the deletion from -1498 to -162 bp reduced the basal activity of the promoter by 177-fold compared to the baseline activity of the -1498/+44 hIL-8/Luc construct, the deletion did not affect the ability to induce the -162 bp promoter. In cells with basal levels of Ref-1, treatment with TNF- α significantly elevated the luciferase activity of the -162/+44 hIL-8/Luc plasmid by 515% compared to control cells (Fig. 5B). Although mutation of the AP-1 binding site did not significantly affect TNF- α -stimulated promoter activity, mutation of the NF- κ B binding site completely abrogated TNF- α -induced luciferase activity (Fig. 5B). In cells with normal levels of Ref-1, these data suggest that the NF- κ B binding site, but not the AP-1 site, is critically involved in IL-8 transcriptional activation following TNF- α stimulation.

In Ref-1 over-expressing cells, treatment with TNF- α increased by >200-fold the luciferase activity of the parent -162/+44 hIL-8/Luc plasmid, compared to untreated cells with basal levels of Ref-1 (Fig. 5C). In TNF- α -treated cells that over-expressed Ref-1, mutation of the AP-1 binding site significantly decreased luciferase activity by 13.7-fold compared to the parent construct. Although mutation of the NF- κ B binding site further halved this activity in TNF- α -stimulated Ref-1 over-expressing cells, the difference in IL-8 luciferase activity between the two mutated constructs was not significant (Fig. 5C). These results demonstrate that in Ref-1 over-expressing cells, the relative involvement of AP-1 in inducible IL-8 promoter activation is more important than in cells that express constitutive levels of Ref-1 and indicate that Ref-1 modulates both NF- κ B and AP-1 regulatory activity in human gastric epithelial cells exposed to TNF- α .

3.7. Ref-1 modulates IL-8 promoter activity in AGS cells

Role of Ref-1 in TNF- α -induced IL-8 gene activation was examined by co-transfecting AGS cells with the -1498/+44 hIL-8/Luc plasmid and pFLAG-Ref-1 cDNA 3.1. Western blot confirmed the over-expression of Ref-1 in cells transfected with pFLAG-Ref-1 cDNA3.1 compared to non-transfected cells (Fig. 6A). Increased Ref-1 protein expression was detected in pFLAG-Ref-1 cDNA3.1-transfected AGS cells, compared to non-transfected cells, or those transfected with an inert control vector. The very small shift in mobility evident in pFLAG-Ref-1 cDNA3.1-transfected cells is probably due to the FLAG tag on the expression vector.

In untreated AGS cells transfected with the -1498/+44 hIL-8/Luc plasmid, Ref-1 over-expression significantly increased luciferase activity by almost 5-fold compared to cells with basal levels of Ref-1 (Fig. 6B). Similarly, at baseline, Ref-1 over-expression significantly enhanced IL-8 promoter activation in untreated cells transfected with any one of the plasmids containing serial deletions of the IL-8 gene (data not shown). This demonstrated that the over-expression of Ref-1 significantly augmented basal IL-8 promoter activity. Moreover, as shown in Fig. 6B, the IL-8 luciferase activity induced by TNF- α in cells with basal levels of Ref-1 was further enhanced following stimulation of Ref-1 over-expressing cells bearing the -1498/+44 hIL-8/Luc construct. In these cells, Ref-1 over-expression augmented TNF- α -stimulated

IL-8 luciferase activity from 4.1-fold to 10.1-fold. These results implicate Ref-1 in the regulation of IL-8 promoter activation in gastric epithelial cells at baseline and following treatment with TNF- α .

4. Discussion

TNF- α contributes to several pathophysiological states initiated by infectious or inflammatory agents by producing ROS and inducing pro-inflammatory chemoattractant cytokine cascades. Although a regulatory role of TNF- α in epithelial cell repair has been described [43], it is well established that TNF- α stimulates IL-8 and contributes to epithelial cell injury and apoptosis [17,14]. The cysteine-donating reducing agent NAC is widely used as an antioxidant and previous studies have shown that IL-8 secretion is inhibited by NAC or its lysine derivative, nacystelyn, in airway epithelial cells [40,44]. In this study, we report for the first time that NAC inhibits TNF- α -induced IL-8 mRNA expression in gastric epithelial cells. Although NAC has other actions, our results imply that TNF- α -induced IL-8 expression is mediated at least in part by ROS. Our study also shows that TNF- α induces an accumulation of Ref-1 in the nucleus of gastric epithelial cells. Moreover, this study demonstrates that the Ref-1-regulated transcription factor, NF- κ B, is required for TNF- α -induced IL-8 promoter activity and that inhibition of Ref-1 diminished AP-1 and NF- κ B DNA binding activity in gastric epithelial cells at baseline and following TNF- α treatment. In addition, silencing of Ref-1 inhibited the redox-sensitive induction of IL-8 by TNF- α , whereas Ref-1 over-expression significantly enhanced basal and TNF- α -induced IL-8 gene transcription. Collectively, these findings provide mechanistic information regarding TNF- α -induced IL-8 promoter activation in gastric epithelial cells and functionally implicate Ref-1 as an important determinant of gastric epithelial cell function following exposure to TNF- α (Fig. 7).

The mechanisms of IL-8 gene induction have been investigated in a variety of cell types and studies indicate a stimulus- and cell-type-specific activation [20] suggesting that the differential regulation of AP-1 or NF-IL-6, together with NF- κ B, is important for IL-8 gene transcription. Our observation that deletion of the IL-8 promoter to -99 or -54 bp reduced basal promoter activity in untreated AGS cells in MKN45 gastric epithelial cells [45] and was attributed to the involvement of AP-1 in basal IL-8 transcriptional activity [46]. In contrast, these previous studies did not demonstrate an effect on basal promoter activity with the -162 bp construct, but this difference may be due to the different epithelial cell lines used. In most cell types, NF- κ B cooperates preferentially with NF-IL-6 in TNF- α -induced IL-8 expression [47], but the mechanism by which TNF- α stimulates IL-8 in gastric epithelial cells is less well established. The current study demonstrates that TNF- α -induced IL-8 promoter activity in AGS cells leads to IL-8 mRNA and protein synthesis and that TNF- α -stimulated IL-8 production is mediated by ROS. The data show that the IL-8 promoter region containing the NF- κ B and NF-IL-6 binding sites (-99 to -54 bp) is required for TNF- α -induced IL-8 transcription. In agreement with others [45], the site-directed mutation data indicate that in gastric epithelial cells, only an intact NF- κ B site, not an AP-1 site, is essential for IL-8 promoter activation by TNF- α . Although we do not exclude an involvement of NF-IL-6 in this process in AGS cells, NF-IL-6 was not implicated in TNF- α induced IL-8 promoter activity in MKN45 gastric epithelial cells [45,37].

The current study demonstrates that silencing of Ref-1 expression significantly inhibited TNF- α -induced IL-8 mRNA and protein expression, as well as TNF- α -stimulated MIP-3 α and Gro- α mRNA expression. In contrast, the over-expression of Ref-1 significantly augmented basal IL-8 promoter activity and IL-8 promoter activity in cells treated with TNF- α . Reports suggest that the redox-sensitive induction of IL-8 by TNF- α may depend on ROS-mediated activation of NF- κ B and AP-1 in gastric epithelial cells [23]. While the mechanisms of oxidant mediated activation of NF- κ B have been debated in the literature, recent studies suggest that H₂O₂

activates NF- κ B mainly through the classical I κ K-dependent pathway [48]. Redox regulation of transcription factors occurs mostly in the nuclear compartment and is largely mediated by the reductive action of Ref-1 [24]. The DNA binding activity of both AP-1 and NF- κ B are redox-mediated by Ref-1 [26], although the precise amino acid residues of Ref-1 involved remain to be fully defined [49]. Here, we demonstrate that TNF- α increased NF- κ B, and to a lesser extent, AP-1, DNA binding activity in AGS cells. Furthermore, TNF- α induced an accumulation of p65, c-Jun and c-Fos, and to a lesser extent, p50, in the nucleus of AGS cells. Both AP-1 and NF- κ B are redox-responsive elements and the generation of ROS by TNF- α [15] may trigger their activation in gastric epithelial cells. Our results do not suggest a role for Ref-1 in the accumulation of AP-1 or NF- κ B in the nucleus of gastric epithelial cells, but rather indicate an important role of Ref-1 in the regulation of transcription factor DNA binding activity within the nucleus.

In AGS cells with silenced Ref-1 expression, we observed a suppression of both basal and TNF- α -induced AP-1 DNA binding activity. Moreover, silencing of Ref-1 abolished basal NF- κ B DNA binding activity and inhibited TNF- α -induced NF- κ B activation. These findings are supported by immunodepletion and antisense studies which have indicated that Ref-1 regulates inducible AP-1 DNA binding in rodent fibroblasts and HeLa cells [50] and that suppression of Ref-1 decreases both basal and mitogen- or oxidative stress-induced AP-1 DNA binding [51]. Furthermore, augmented Ref-1 expression increases AP-1 and NF- κ B DNA binding activity at baseline and in response to oxidative stimuli [52] and previously, we have demonstrated that Ref-1 over-expression enhanced AP-1-mediated transcription in AGS cells [31]. The observed incomplete inhibition of TNF- α -induced NF- κ B DNA binding activity may be due to the persistence of some Ref-1 protein in the cells, or may be due to the independent and synergistic roles that Ref-1 and thioredoxin have been reported to play in the promotion of NF- κ B DNA binding [24]. Considering that silencing of Ref-1 significantly inhibited TNF- α -induced IL-8, our data suggest that without Ref-1, nuclear AP-1 and NF- κ B are not reductively activated and thus, cannot bind to the promoter to drive transcription. On the other hand, the over-expression of Ref-1 modulates these transcription factors by promoting increased reductive activation, resulting in enhanced binding and increased IL-8 promoter activity. It may be that Ref-1 exerts a generalized effect on basal transcription, possibly through cryptic AP-1 sites associated with these control vectors. Nonetheless, the RNA interference assays verify the over-expression studies independently and together, our data show that Ref-1 over-expression has a greater, more specific effect, on IL-8 promoter activity.

The over-expression of Ref-1 in the site-directed mutation experiments presented here demonstrated that Ref-1 over-expression affected AP-1 and NF- κ B regulatory activity. In Ref-1 over-expressing cells, the relative involvement of AP-1 in TNF- α -inducible IL-8 promoter activity was significantly greater than in cells with basal levels of Ref-1. Reports suggest that Ref-1 regulates inducible, but not basal AP-1 DNA binding activity [50] and, c-Jun itself plays a central role in activation of the Ref-1 promoter while simultaneously being a target of redox regulation by Ref-1 [26]. Furthermore, Ref-1 itself is regulated by ROS [29] and we have reported a redox-sensitive increased expression of Ref-1 in ROS-stimulated or *H. pylori*-infected human gastric epithelial cells that was accompanied by *de novo* protein synthesis and a rapid cytoplasmic to nuclear translocation of Ref-1 [31]. Taken together with the current study, we believe that nuclear Ref-1 serves to activate responsive transcription factors, with the potential, as demonstrated here, to modulate redox-sensitive gene expression in human gastric epithelial cells (Fig. 7).

In summary, our data indicate that Ref-1 modulates basal and TNF- α -induced IL-8 promoter activation in gastric epithelial cells. Our findings implicate Ref-1 as an important functional regulator of the redox-sensitive induction of IL-8 and other inflammatory chemokines by TNF- α in the human gastric mucosa. Although this study was limited to TNF- α -treated AGS cells,

the ubiquitous expression of Ref-1 and the involvement of TNF- α and consequently, IL-8, in numerous inflammatory conditions, suggest that the regulatory effect of Ref-1 on IL-8 promoter activation may be relevant to the redox biology of other cell types.

Acknowledgments

We are grateful to Kieran A. Ryan for designing the Ref-1 and IL-8 primer and TET-labeled probe sets and to Elizabeth Wiznerowicz for her technical assistance. This work was supported by National Institutes of Health Grants RO1 DK61769 (to S.E. Crowe), RO1 ES 08457 (to S. Mitra), and RO1 DK51677 (to P.B.E.). Support from the Immunology and Cell Isolation Core of the University of Virginia Digestive Health Center (National Institutes of Health Grants DK67629) is gratefully acknowledged.

Abbreviations

AP-1	activator protein-1
ENA-78	epithelial cell-derived neutrophil-activating protein-78
Gro- α	growth-related oncogene- α
IL	interleukin
MIP-3 α	macrophage inflammatory protein-3 α
NAC	<i>N</i> -acetyl-L-cysteine
NF- κ B	nuclear factor- κ B
Ref-1	redox factor-1
ROS	reactive oxygen species
TNF- α	tumor necrosis factor- α

References

1. Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 1996;313:17–29. [PubMed: 8546679]
2. Cooper D, Stokes KY, Tailor A, Granger DN. Oxidative stress promotes blood cell–endothelial cell interactions in the microcirculation. *Cardiovasc Toxicol* 2002;2(3):165–80. [PubMed: 12665663]
3. Ding SZ, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, et al. *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infect Immun* 2007;75(8):4030–9. [PubMed: 17562777]
4. Crowe SE. *Helicobacter* infection, chronic inflammation, and the development of malignancy. *Curr Opin Gastroenterol* 2005;21(1):32–8. [PubMed: 15687882]
5. Ernst PB, Peura DA, Crowe SE. The translation of *Helicobacter pylori* basic research to patient care. *Gastroenterology* 2006;130(1):188–206. [PubMed: 16401482]
6. Crowe SE, Alvarez L, Sherman PM, Jin Y, Dytoc M, Hunt RH, et al. Expression of interleukin-8 and CD54 by human gastric epithelium after *H. Pylori* infection in vitro. *Gastroenterology* 1995;108:65–74. [PubMed: 7806065]
7. Crabtree JE, Wyatt JI, Trejdosiewicz LK, Peichl P, Nichols PH, Ramsay N, et al. Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. *J Clin Pathol* 1994;47:61–6. [PubMed: 8132812]
8. Bamford KB, Fan XJ, Crowe SE, Leary JF, Gourley WK, Luthra GK, et al. Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. *Gastroenterology* 1998;114:482–92. [PubMed: 9496938]
9. Crabtree JE, Shallcross TM, Heatley RV, Wyatt JI. Mucosal tumour necrosis factor α and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. *Gut* 1991;32:1473–7. [PubMed: 1773951]

10. Houghton J, Macera-Bloch LS, Harrison L, Kim KH, Korah RM. Tumor necrosis factor alpha and interleukin 1beta up-regulate gastric mucosal Fas antigen expression in *Helicobacter pylori* infection. *Infect Immun* 2000;68:1189–95. [PubMed: 10678925]
11. Moss SF, Blaser MJ. Mechanisms of disease: inflammation and the origins of cancer. *Nat Clin Pract Oncol* 2005;2(2):90–7. [PubMed: 16264881]
12. Beutler BA. The role of tumor necrosis factor in health and disease. *J Rheumatol Suppl* 1999;57:16–21. [PubMed: 10328138]
13. Bradley JR. TNF-mediated inflammatory disease. *J Pathol* 2008;214(2):149–60. [PubMed: 18161752]
14. Shen HM, Pervaiz S. TNF receptor superfamily-induced cell death: redox-dependent execution. *FASEB J* 2006;20(10):1589–98. [PubMed: 16873882]
15. Vlahopoulos S, Boldogh I, Casola A, Brasier AR. Nuclear factor-kappaB-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood* 1999;94(6):1878–89. [PubMed: 10477716]
16. Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science* 2002;296(5573):1634–5. [PubMed: 12040173]
17. Mukaida N, Mahe Y, Matsushima K. Cooperative interaction of NF-κB and cis-regulatory enhancer binding protein-like factor binding elements in activating the IL-8 gene by pro-inflammatory cytokines. *J Biol Chem* 1990;265:21128–32. [PubMed: 2250017]
18. Russo MP, Bennett BL, Manning AM, Brenner DA, Jobin C. Differential requirement for NF-kappaB-inducing kinase in the induction of NF-kappaB by IL-1beta, TNF-alpha, and Fas. *Am J Physiol Cell Physiol* 2002;283(1):C347–57. [PubMed: 12055104]
19. Baggiolini M, Walz A, Kunkel SL. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J Clin Invest* 1989;84:1045–9. [PubMed: 2677047]
20. Mukaida N, Hishinuma A, Zachariae CO, Oppenheim JJ, Matsushima K. Regulation of human interleukin 8 gene expression and binding of several other members of the intercrine family to receptors for interleukin-8. *Adv Exp Med Biol* 1991:30531–8.
21. Chu SH, Kim H, Seo JY, Lim JW, Mukaida N, Kim KH. Role of NF-kappaB and AP-1 on *Helicobacter pylori*-induced IL-8 expression in AGS cells. *Dig Dis Sci* 2003;48(2):257–65. [PubMed: 12643600]
22. Aihara M, Tsuchimoto D, Takizawa H, Azuma A, Wakebe H, Ohmoto Y, et al. Mechanisms involved in *Helicobacter pylori*-induced interleukin-8 production by a gastric cancer cell line, MKN45. *Infect Immun* 1997;65:3218–24. [PubMed: 9234778]
23. Shimada T, Watanabe N, Hiraishi H, Terano A. Redox regulation of interleukin-8 expression in MKN28 cells. *Dig Dis Sci* 1999;44:266–73. [PubMed: 10063910]
24. Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. *Mutat Res* 2000;461(2):83–108. [PubMed: 11018583]
25. Izumi T, Hazra TK, Boldogh I, Tomkinson AE, Park MS, Ikeda S, et al. Requirement for human AP endonuclease 1 for repair of 3'-blocking damage at DNA single-strand breaks induced by reactive oxygen species. *Carcinogenesis* 2000;21(7):1329–34. [PubMed: 10874010]
26. Xanthoudakis S, Miao G, Wang F, Pan Y-CE, Curran T. Redox activation of Fos–Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* 1992;11:3323–35. [PubMed: 1380454]
27. Frossi B, Tell G, Spessotto P, Colombatti A, Vitale G, Pucillo C. H₂O₂ induces translocation of APE/Ref-1 to mitochondria in the Raji B cell line. *J Cell Physiol* 2002;193(2):180–6. [PubMed: 12384995]
28. Grosch S, Fritz G, Kaina B. Apurinic endonuclease (Ref-1) is induced in mammalian cells by oxidative stress and involved in clastogenic adaptation. *Cancer Res* 1998;58:4410–6. [PubMed: 9766671]
29. Ramana, Cv; Boldogh, I.; Izumi, T.; Mitra, S. Activation of apurinic/apyrimidinic endonuclease in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of free radicals. *Proc Natl Acad Sci* 1998;95:5061–5. [PubMed: 9560228]
30. Fung H, Kow YW, Van Houten B, Taatjes DJ, Hatahet Z, Janssen YMW, et al. Asbestos increases mammalian AP-endonuclease gene expression, protein levels, and enzyme activity in mesothelial cells. *Cancer Res* 1998;58:189–94. [PubMed: 9443389]

31. Ding SZ, O'Hara AM, Denning TL, Dirden-Kramer B, Mifflin RC, Reyes VE, et al. *Helicobacter pylori* and H₂O₂ increases AP endonuclease-1/redox factor-1 expression in human gastric epithelial cells. *Gastroenterology* 2004;127:845–58. [PubMed: 15362040]
32. O'Hara AM, Bhattacharya A, Bai J, Mifflin RC, Smith MF Jr, Ryan KA, et al. Interleukin-8 induction by *Helicobacter pylori* in human gastric epithelial cells is dependent on apurinic/aprimidinic endonuclease-1/redox factor-1. *J Immunol* 2006;177:7990–9. [PubMed: 17114472]
33. Ryan KA, Smith MF Jr, Sanders MK, Ernst PB. Reactive oxygen and nitrogen species differentially regulate Toll-like receptor 4-mediated activation of NF-kappa B and interleukin-8 expression. *Infect Immun* 2004;72(4):2123–30. [PubMed: 15039334]
34. Fan Z, Beresford PJ, Zhang D, Xu Z, Novina CD, Yoshida A, et al. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. *Nat Immunol* 2003;4:145–53. [PubMed: 12524539]
35. Smith MF Jr, Eidlen D, Arend WP, Gutierrez-Hartmann A. LPS-induced expression of the human IL-1 receptor antagonist gene is controlled by multiple interacting promoter elements. *J Immunol* 1994;153(8):3584–93. [PubMed: 7930581]
36. Brasier AR, Jamaluddin M, Casola A, Duan W, Shen Q, Garofalo RP. A promoter recruitment mechanism for tumor necrosis factor- α -induced interleukin-8 transcription in type II pulmonary epithelial cells. Dependence on nuclear abundance of Rel A, NF- κ B1, and c-Rel transcription factors. *J Biol Chem* 1998;273:3551–61. [PubMed: 9452482]
37. Yasumoto K, Okamoto S, Mukaida N, Murakami S, Mai M, Matsushima K. Tumor necrosis factor α and interferon γ synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kappaB-like binding sites of the interleukin 8 gene. *J Biol Chem* 1992;267:22506–11. [PubMed: 1331059]
38. Garofalo R, Sabry M, Jamaluddin M, Yu R, Casola A, Ogra P, et al. Transcriptional activation of the interleukin-8 gene by respiratory syncytial virus infection in alveolar epithelial cells: nuclear translocation of the RelA transcription factor as a mechanism producing airway mucosal inflammation. *J Virol* 1996;70:8773–81. [PubMed: 8971006]
39. Casola A, Garofalo RP, Jamaluddin M, Vlahopoulos S, Brasier AR. Requirement of a novel upstream response element in respiratory syncytial virus-induced IL-8 gene expression. *J Immunol* 2000;164(11):5944–51. [PubMed: 10820277]
40. Bartling TR, Drumm ML. Oxidative stress causes IL-8 promoter hyperacetylation in cystic fibrosis airway cell models. *Am J Respir Cell Mol Biol* 2009;40(1):58–65. [PubMed: 18635816]
41. Izadpanah A, Dwinell MB, Eckmann L, Varki NM, Kagnoff MF. Regulated MIP-3 α /CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. *Am J Physiol Gastrointest Liver Physiol* 2001;280(4):G710–9. [PubMed: 11254498]
42. Keates S, Keates AC, Mizoguchi E, Bhan A, Kelly CP. Enterocytes are the primary source of the chemokine ENA-78 in normal colon and ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol* 1997;273:G75–82.
43. Yan F, John SK, Wilson G, Jones DS, Washington MK, Polk DB. Kinase suppressor of Ras-1 protects intestinal epithelium from cytokine-mediated apoptosis during inflammation. *J Clin Invest* 2004;114(9):1272–80. [PubMed: 15520859]
44. Antonicelli F, Parmentier M, Drost EM, Hirani N, Rahman I, Donaldson K, et al. Nacystelyn inhibits oxidant-mediated interleukin-8 expression and NF-kappaB nuclear binding in alveolar epithelial cells. *Free Radic Biol Med* 2002;32(6):492–502. [PubMed: 11958950]
45. Yamaoka Y, Kudo T, Lu H, Casola A, Brasier AR, Graham DY. Role of interferon-stimulated responsive element-like element in interleukin-8 promoter in *Helicobacter pylori* infection. *Gastroenterology* 2004;126(4):1030–43. [PubMed: 15057743]
46. Casola A, Garofalo RP, Crawford SE, Estes MK, Mercurio F, Crowe SE, et al. Interleukin-8 gene regulation in intestinal epithelial cells infected with rotavirus: role of viral-induced IkappaB kinase activation. *Virology* 2002;298(1):8–19. [PubMed: 12093168]
47. Kunsch C, Lang RK, Rosen CA, Shannon MF. Synergistic transcriptional activation of the IL-8 gene by NF-kappa B p65 (RelA) and NF-IL-6. *J Immunol* 1994;153:153–64. [PubMed: 8207232]
48. Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol* 2006;72(11):1493–505. [PubMed: 16723122]

49. Ordway JM, Eberhart D, Curran T. Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding. *Mol Cell Biol* 2003;23(12):4257–66. [PubMed: 12773568]
50. Diamond DA, Parsian A, Hunt CR, Lofgren S, Spitz DR, Goswami PC, et al. Redox factor-1 (Ref-1) mediates the activation of AP-1 in HeLa and NIH 3T3 cells in response to heat shock. *J Biol Chem* 1999;274:16959–64. [PubMed: 10358044]
51. Ozolins TR, Hales BF. Post-translational regulation of AP-1 transcription factor DNA-binding activity in the rat conceptus. *Mol Pharmacol* 1999;56(3):537–44. [PubMed: 10462541]
52. Hall JL, Wang X, Van A, Zhao Y, Gibbons GH. Overexpression of Ref-1 inhibits hypoxia and tumor necrosis factor-induced endothelial cell apoptosis through nuclear factor-kappa β -independent and -dependent pathways. *Circ Res* 2001;88(12):1247–53. [PubMed: 11420300]

Fig. 1.

NAC inhibits TNF- α -induced IL-8 protein (A) and mRNA (B) production in AGS cells. AGS cells were pre-treated with 0 mM, 10 mM, 20 mM, or 40 mM NAC for 30 min prior to treatment with 20 ng/ml TNF- α or left untreated (controls). After 3 h the supernatants were assayed for IL-8 protein by ELISA (A). TNF- α stimulated the secretion of IL-8 protein by AGS cells, and pre-treatment with 20 mM and 40 mM NAC significantly suppressed TNF- α -induced IL-8 protein (* P < 0.05 versus cells that were treated with TNF- α only). (B) IL-8 mRNA expression was assayed by real-time RT-PCR 1 h post-treatment, and the graph depicts the levels of IL-8 mRNA (normalized to 18S rRNA) detected. IL-8 mRNA expression was increased by TNF- α treatment when compared to untreated cells, and pre-treatment with NAC at all

concentrations tested (10, 20, or 40 mM NAC) significantly decreased TNF- α -induced IL-8 mRNA (* $P < 0.05$ versus cells that were treated with TNF- α only). Results represent the mean $\pm$ SEM ($n = 3$).

Fig. 2.

(A) Effect of Ref-1 suppression on TNF- α -induced IL-8 expression in AGS cells. AGS cells transfected with 50 nM Ref-1 siRNA or mock-transfected cells were treated 48 h post-transfection with 20 ng/ml TNF- α or left untreated. Our previous studies demonstrated that IL-8 expression was no different in AGS cells that were transfected with scrambled siRNA versus mock-transfected cells [32]. IL-8 mRNA expression levels were assayed by real-time RT-PCR 1 h post-treatment. The graph depicts the percent IL-8 mRNA expression (normalized to 18S rRNA) of TNF- α -stimulated cells transfected with Ref-1 siRNA compared to TNF- α -stimulated cells transfected with Lipofectamine 2000 alone. TNF- α -induced IL-8 mRNA expression was significantly inhibited ($*P < 0.05$) in AGS cells transfected with Ref-1 siRNA compared to cells transfected with Lipofectamine 2000 alone. (B) Forty-eight hours post-transfection, cells were stimulated with TNF- α for 3 h and the supernatants were assayed for IL-8 by ELISA. Results are expressed as pg IL-8/mg total protein concentration. In cells expressing normal levels of Ref-1, TNF- α stimulated a significant increase in IL-8 protein. In cells transfected with Ref-1 siRNA, TNF- α -induced IL-8 protein was significantly inhibited ($*P < 0.05$ when compared to TNF- α -stimulated mock-transfected cells). All data represent the mean $\pm$ SEM of three separate experiments.

Fig. 3. siRNA-mediated silencing of Ref-1 inhibits TNF- α -induced AP-1 and NF- κ B DNA binding activity but does not block nuclear accumulation of these transcription factors. AGS cells transfected with 50 nM Ref-1 siRNA (+) or non-transfected (-) cells were treated with 20 ng/ml TNF- α 48 h post-transfection. Untreated cells served as controls. Nuclear extracts were used to bind to radiolabeled AP-1 or NF- κ B probes. (A) A representative EMSA autoradiogram showing AP-1 activation in non-transfected or Ref-1 siRNA-transfected AGS cells treated for 1 h with TNF- α . The arrow indicates the activated AP-1 complex. (B) A representative EMSA autoradiogram showing NF- κ B activation in non-transfected or Ref-1 siRNA-transfected AGS cells treated with TNF- α for 2 h. The arrow indicates the activated NF- κ B complex. The specificity of the shifted AP-1 and NF- κ B complexes were analyzed using a 50-fold excess of unlabeled specific competitor or unlabeled non-specific competitor as indicated. (C) Non-transfected control cells (lanes 1 and 4), and cells transfected with 50 nM control siRNA (lanes 2 and 5) or Ref-1 siRNA (lanes 3 and 6) were treated with 20 ng/ml TNF- α or left untreated 48 h post-transfection for 1 or 2 h. After harvesting, nuclear extracts were analyzed by SDS-

PAGE immunoblotting for NF- κ B p50, p65, c-Jun, c-Fos. Ref-1 levels were assessed in nuclear and total cellular extracts. The data shown are representative of four separate experiments.

Fig. 4.

Schematic representation of the luciferase-linked human IL-8 promoter constructs used in this study. The plasmids contain sequentially deleted 5' flanking regions of the human IL-8 gene. Locations of transcription factor binding sites are indicated. The AP-1 binding site is located between -126 and -120 nucleotides, the NF-IL-6 binding site is between -94 and -81 nucleotides, and the NF-κB binding site is between -80 and -70 nucleotides. A consensus TATA box is located between -20 and -13 nucleotides.

Fig. 5.

Effect of 5' deletions and site-directed mutations in the human IL-8 promoter sequence on TNF- α -inducible IL-8 luciferase activity in cells with normal and over-expressed levels of Ref-1. AGS cells transfected with 5' deletions of the hIL-8 Luc promoter were treated with 20 ng/ml TNF- α for 3 h. Untreated AGS cells served as controls. IL-8 luciferase activity normalized to protein, was expressed as fold induction for each construct relative to untreated cells (A). In cells bearing the -1498/+44 or the -99/+44 hIL-8/Luc constructs, TNF- α significantly stimulated IL-8 luciferase activity (* P < 0.05 when compared to untreated cells). In contrast, TNF- α did not induce IL-8 luciferase activity in cells transfected with the -54/+44 hIL-8/Luc construct. All data represent the mean relative luciferase activity $\pm$ SEM of triplicate

samples from three separate experiments. AGS cells were transfected in triplicate with one of the site-mutated plasmids of the -162/+44 hIL-8/Luc promoter construct alone (B) or co-transfected with pFLAG-Ref-1 cDNA3.1 (C). Panel B depicts the percentage increase in normalized IL-8 luciferase activity of each plasmid in TNF- α treated compared to untreated cells. In cells with basal levels of Ref-1 bearing the -162/+44 hIL-8/Luc construct, TNF- α significantly stimulated IL-8 transcription ($*P < 0.05$). Following TNF- α stimulation, mutation of the NF- κ B binding site, but not the AP-1 binding site, significantly decreased TNF- α -induced promoter activity ($\#P < 0.05$ compared to TNF- α -treated cells transfected with -162/+44 hIL-8/Luc or -162/+44 hIL-8/Luc Δ AP-1). (C) The figure depicts the percentage increase in IL-8 luciferase activity of each plasmid in Ref-1 over-expressing cells compared to untreated cells with basal levels of Ref-1. In Ref-1 over-expressing cells, TNF- α significantly increased the IL-8 luciferase activity of the parent -162/+44 hIL-8/Luc plasmid, compared to untreated cells with constitutive levels of Ref-1 ($*P < 0.05$). Mutation of either the AP-1 or NF- κ B binding sites significantly reduced IL-8 luciferase activity in stimulated cells that over-expressed Ref-1 ($\#P < 0.05$ compared to TNF- α treated cells co-transfected with -162/+44 hIL-8/Luc and pFLAG-Ref-1 cDNA3.1), but the difference in the IL-8 luciferase activity between the two mutated constructs was not significant. All data represent the mean $\pm$ SEM of three separate experiments.

Fig. 6. Confirmation of Ref-1 over-expression and the effects of over-expression on TNF- α -inducible IL-8 promoter activity in AGS cells transfected with the -1498/+44 hIL-8/Luc construct. (A) The 37 kDa bands demonstrate increased Ref-1 expression in cells transfected with 0.25 μ g pFLAG-Ref-1 cDNA3.1 compared to non-transfected or mock-transfected cells by western blot. (B) The figure depicts the relative increase in normalized IL-8 luciferase activity of treated cells compared to untreated cells with basal levels of Ref-1. The over-expression of Ref-1 significantly (* $P < 0.05$) increased IL-8 luciferase activity in untreated control cells, and the IL-8 luciferase activity induced by TNF- α in cells with basal levels of Ref-1 was further enhanced in Ref-1 over-expressing cells following TNF- α stimulation (* $P < 0.05$). Data are

expressed as mean relative luciferase activity of the $-1498/+44$ hIL-8/Luc construct compared to untreated cells with constitutive levels of Ref-1, and results represent the mean $\pm$ SEM of three separate experiments.

Fig. 7.

Summary diagram illustrating a model for the mechanism by which Ref-1 regulates TNF- α -induced IL-8 in human gastric epithelial cells. TNF- α acts via its receptor, TNFR-1, on mitochondria to initiate caspase-mediated apoptosis that generates ROS, and/or on I κ B kinase to activate NF- κ B. ROS themselves can also activate NF- κ B, and once activated, free NF- κ B translocates into the nucleus. Similarly, either c-Jun/c-Jun homodimers or c-Jun/c-Fos heterodimers are activated by TNF- α or ROS through pathways involving mitogen-activated protein kinases leading to the activation of AP-1. Both ROS and TNF- α increase the expression of Ref-1 in gastric epithelial cells leading to nuclear translocation of Ref-1. The intranuclear presence of Ref-1 reductively modulates AP-1 and NF- κ B activation, and in the reduced form, these transcription factors activate transcription by binding to DNA binding sites upstream of several redox-sensitive pro-inflammatory cytokine genes including IL-8.

Table 1

Ref-1 siRNA suppresses TNF- α -induced chemokine mRNA expression in AGS cells.

Chemokine	Control siRNA TM	Ref-1 siRNA	% Suppression	<i>P</i> value
IL-8	206.55 $\pm$ 84.64	79.01 $\pm$ 32.74	62	<0.05
MIP-3 α	568.95 $\pm$ 227.17	288.92 $\pm$ 161.99	49	<0.05
Gro- α	28.23 $\pm$ 8.77	19.05 $\pm$ 7.59	33	<0.05

Human recombinant TNF- α treatment on Ref-1 siRNA transfected AGS cells suppressed different chemokine mRNA expression. Each TNF- α -treated sample was compared to its similarly transfected, uninfected control. Data are expressed as the mean fold increase over matched control $\pm$ SEM ($n = 6$).

Table 2

Effects of 5' deletions in the human IL-8 promoter sequence on basal and TNF- α -induced luciferase activity.

Treatment	-1498/+44 hIL-8/Luc	-99/+44 hIL-8/Luc	-54/+44 hIL-8/Luc
Untreated control	130820.2 $\pm$ 29420	7679.5 $\pm$ 1957.2	1745.2 $\pm$ 395.8
TNF- α	520462.3 $\pm$ 161438	50281.5 $\pm$ 17831.9	1977.5 $\pm$ 552.8

AGS cells were transfected in triplicate with 5' deletions of the hIL-8 Luc promoter and were treated with 20 ng/ml TNF- α for 3 h. Untreated AGS cells served as controls. Data are expressed as IL-8 luciferase activity normalized to total protein concentration (mean $\pm$ SEM, $n = 3$).