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Nrf2 expression and activity in human T lymphocytes: stimulation by T cell receptor activation and priming by inorganic arsenic and *tert*-butylhydroquinone

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Abbreviations: Nrf2: Nuclear Factor-erythroid 2-related-2, NQO1: NAD(P)H quinone oxidoreductase 1, HO-1: heme oxygenase 1, γ-GCSm: γ-glutamylcysteine synthase, Keap1: Kelch-like ECH-associated protein 1, ARE: antioxidant responsive elements, Th: T helper, IFN-γ: interferon-γ, TNF-α: tumor necrosis factor-a, TCR: T cell receptor, Ab: antibody, aCD3/aCD28: monoclonal antibodies against CD3 and CD28 proteins, PMA: phorbol myristate acetate, As(III): arsenite, NAC: N-acetylcysteine, CHX: cycloheximide, IL: interleukin, SDS-PAGE: sodium dodecyl sulfate polymerase gene electrophoresis, TBS: tris-buffered saline, H_2-DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate, ROS: reactive oxygen species, GSH: glutathione, tBHQ: *tert*-butylhydroquinone, RT-qPCR: real-time quantitative polymerase chain reaction.

Abstract

The transcription factor Nuclear Factor-erythroid 2-related-2 (Nrf2) controls cellular redox homeostasis and displays immunomodulatory properties. Nrf2 alters cytokine expression in murine T cells, but its effects in human T lymphocytes are unknown. This study investigated the expression and activity of Nrf2 in human activated CD4+ T helper lymphocytes (Th cells) that mediate the adaptive immune response. Th cells were isolated from peripheral blood mononuclear cells and activated with antibodies against CD3 and CD28, mimicking physiologic Th cell stimulation by dendritic cells. Nrf2 is hardly detectable in non-stimulated Th cells. Activation of Th cells rapidly and strongly increases the levels of Nrf2 protein by increasing NRF2 gene transcription. Th cell activation also enhances mRNA and protein levels of Nrf2 target genes coding antioxidant enzymes. Blocking Nrf2 expression using chemical inhibitors or siRNAs prevents these gene inductions. Pre-treatment with inorganic arsenic, a Nrf2 inducer that does not alter NRF2 gene expression, increases protein level and transcriptional activity of Nrf2 induced by Th cell stimulation. Inorganic arsenic enhances nuclear translocation of Nrf2, its interaction with the coactivator protein p300 and its DNA binding activity. Inhibition of Nrf2 expression abrogates the effects of inorganic arsenic on mRNA levels of antioxidant genes, but does not alter the expression of IL-2, TNF-α, interferon-γ, and IL-17 in Th cells activated in the absence or presence of the metalloid. In conclusion, this study demonstrates for the first time that stimulation of human Th cells increases the transcription of NRF2 gene and the activity of Nrf2 protein. However, modulation of Nrf2 levels does not modify the secretion of inflammatory cytokines from these T lymphocytes.

Keywords: Nrf2, T lymphocytes, arsenic, antioxidant genes, cytokines.

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Nuclear factor-erythroid 2-related-2 (Nrf2) is a leucine zipper transcription factor that plays a central role in cellular responses to oxidative insults induced by chemicals, radiations and electrophilic stress [1]. Nrf2 controls basic and inducible expression of several antioxidant genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), and modifier subunit for glutamate-cysteine ligase (GCLM) [1]. The NRF2 gene is expressed in most cell types and independently of inducers [1, 2]. Under basal conditions, Nrf2 is mainly sequestrated in the cytoplasm by the cytoskeleton-associated protein Kelch-like ECH-associated protein 1 (Keap1). Keap1 forms an E3 ubiquitin ligase complex with Cullin 3 and Ring-box 1, and triggers polyubiquitination of Nrf2 by promoting its binding to Cullin 3 [3]. Ubiquitinated Nrf2 is then degraded by the 26S proteasome with a half-life of approximately 20 min. Consequently, levels of Nrf2 protein are generally very low in basal state cells. Pro-oxidant chemicals classically increase intracellular Nrf2 levels through oxidation of thiol groups in pivotal cysteine residues of Keap1. Such oxidations probably cause conformational changes of the Keap1-E3 ubiquitin ligase complex that block Nrf2 ubiquitination and favor its accumulation. Nrf2 then rapidly translocates into the nucleus, dimerizes with the small Maf protein and binds to its antioxidant responsive elements (ARE) in gene promoters [2]. In nuclei, the coactivator CBP/p300 can cooperatively interact with specific domains of Nrf2 [3]. These interactions increase Nrf2 DNA binding and synergistically activate transcription of Nrf2 target genes [4]. A noncanonical mechanism of Nrf2 activation has recently emerged with the discovery that the Sesquestosome 1 (p62/SQSTM1) protein directly interacts with Keap1 at the Nrf2 binding sites [5]. When overexpressed, p62 can compete with the interaction between Nrf2 and Keap1, resulting in accumulation and activation of Nrf2.

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Besides the control of intracellular redox status, Nrf2 also exerts a protective role towards several inflammatory disorders induced in murine models [6]. Using Nrf2 knockout mice, different groups have demonstrated that the loss of Nrf2 expression not only triggers oxidative damage, but also promotes the development of inflammation-related diseases, such as sepsis [7], atherosclerosis [8], chronic obstructive pulmonary diseases [9, 10], asthma [11], chemical-induced skin allergy [12], and autoimmunity [13]. Disruption of Nrf2 markedly increases the mortality of mice in response to endotoxin- and cecal ligation-induced septic shock [7]. Conversely, enhancement of Nrf2 expression by deleting Keap1 protein significantly reduces mortality, organ injury, and bacteremia [14]. In these murine models, the loss of Nrf2 increases the levels of inflammatory cytokines, chemokines, and adhesion molecules, suggesting that Nrf2 can limit the development of inflammatory diseases by regulating the immune response [7, 8, 13]. Analysis of immune cell function in $Nrf2^{-/-}$ mice has revealed that inhibition of Nrf2 expression alters the physiology of CD4+ T helper (Th) lymphocytes, which play a major role in the adaptive immunity [15]. Ex-vivo, activated Th cells from $Nrf2^{-/-}$ mice exhibit a stronger ability to produce interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α) [16]. This suggests that Nrf2 may thus repress the expression of major pro-inflammatory cytokines in murine T cells.

The role of Nrf2 on redox homeostasis and immune gene expression in human activated T lymphocytes is widely unknown, in contrast to murine models. The present study was thus designed to determine firstly whether Nrf2 is expressed in human Th lymphocytes, and secondly whether Nrf2 can up-regulate antioxidant genes and repress genes encoding cytokines in these cells. Th cells are specifically activated by antigen-presenting cells, such as dendritic cells. Their optimal activation requires a double signal that involves engagement of the CD3-T cell receptor

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(TCR) and stimulation of the CD28 receptor [17]. The present results demonstrate that, *in vitro*, stimulation of human Th lymphocytes either with monoclonal antibodies (Abs) directed against CD3 and CD28 proteins (aCD3/aCD28) that mimic the physiologic activation of Th cells [18], or with phorbol myristate acetate (PMA) in the absence or presence of ionomycin, strongly increases Nrf2 protein levels, likely through activation of NRF2 gene transcription. Th cell activation also markedly enhances, at both mRNA and protein levels, the Nrf2-dependent expression of several antioxidant genes, including that of NQO1, HMOX1, and GCLM genes. Interestingly, cell pre-treatment with inorganic arsenic (arsenite, As(III)), a reference post-transcriptional Nrf2 inducer that also represses cytokine expression in activated human T lymphocytes [19, 20], primes costimulated Th cells for expression of these antioxidant genes. As(III) increases Nrf2 expression in activated Th cells and potently enhanced transcriptional activity of a Nrf2-driven luciferase reporter vector in activated leukemic T Jurkat cells. Genetic invalidation of Nrf2 markedly reduces the expression of both Nrf2 and antioxidant genes in human Th cells activated in the absence or presence of As(III). However, this loss of Nrf2 neither prevents the induction of genes encoding cytokines nor their repression by As(III) in activated Th cells.

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Materials and Methods

Chemical reagents and antibodies (Abs)

As(III), *tert*-butylhydroquinone (tBHQ), phorbol myristate acetate (PMA), ionomycin, cycloheximide (CHX), MG132, actinomycin D, and N-acetylcysteine (NAC) were from Sigma-Aldrich. BAY 11-7082 was purchased from Calbiochem. Primary Abs directed against Nrf2 (H-300, sc-13032), p300 (C-20, sc-585), Hsc70, NQO1, γ-GCS, and p38-kinase were from Santa

Cruz whereas HO-1 antibody was from Stressgene Biotechnologies. Ab against phospho-IκBα was purchased from Cell Signaling Technology.

Cell culture

To isolate human Th cells, peripheral blood mononuclear cells were first isolated from blood buffy coats from healthy donors through Ficoll gradient centrifugation. After separation of monocytes in a 1 h adhesion step, Th cells were purified from non-adherent cells by negative selection using Dynabeads T cell kits specific for human CD4+ cells (Invitrogen). The purity of the Th cell suspension was determined by flow cytometry (FC500, Beckman), using a monoclonal fluorescein isothiocyanate-conjugated Ab directed against human CD4 (Miltenyi) and the corresponding control isotype (IgG1) Ab. Analysis reveals that more than 93% of the selected cells were CD4-positive cells (Fig. S1). Cells were then cultured in RPMI 1640 GlutaMAX medium (Gibco) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were activated with Dynabeads coated with aCD3/aCD28 (Invitrogen) (one bead per cell 1:1, or otherwise indicated). The leukemic T-cell line Jurkat was cultured in RPMI 1640 GlutaMAX medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C.

Western blot analysis

For whole cell lysates, cells were harvested and lysed for 20 min on ice in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM orthovanadate, and a cocktail of protein inhibitors (Roche). Cells were then centrifugated at 13000 × g for 15 min at 4°C. The resulting supernatants were collected and frozen at −80°C or used immediately. For nuclear cell extracts, cells were lysed and nuclear proteins were prepared

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using the Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. 10 to 30 µg of whole cell, cytoplasmic, or nuclear cell extracts were heated for 5 min at 100°C, loaded in a 4% stacking gel and then separated by a 10% sodium dodecyl sulfate polymerase gel electrophoresis (SDS-PAGE). Gels were electroblotted overnight onto nitrocellulose membranes (Bio-Rad). After blocking the membrane with a Tris-buffered saline (TBS) solution supplemented with 0.1% tween-20 and 5% bovine serum albumin, membranes were hybridized with primary Abs overnight at 4°C and incubated with appropriate horseradish peroxidase-conjugated secondary Abs. For Nrf2 detection, membranes were first blocked with 5% skim milk in TBS and then hybridized overnight at 4°C with the H-300 Nrf2 Ab (1/1000) in TBS. Immunolabeled proteins were visualized by chemiluminescence. When indicated, a densitometry analysis was carried out to normalize the expression of each protein studied by that of p38-kinase. Independent experiments were repeated at least three times.

Immunoprecipitation

After treatment, Jurkat cells were collected, washed in cold PBS, and lysed for 30 min on ice in a buffer containing TBS pH 7.6, Triton X100 1%, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1% protease inhibitor cocktail. Lysates were next centrifuged at 13000 × g for 5 min at 4°C and pre-cleared for 1 h at 4°C with 50 µl protein G–Sepharose beads (GE Healthcare) to reduce nonspecific protein binding. Then, 1 mg of each sample was incubated overnight at 4°C in the presence of 2 µg anti-p300 Ab. The resulting immunocomplexes were next bound to Dynabeads® Protein G (50 µl) for 4 h at 4°C. At the end of incubation, the beads were washed five times and heated at 100°C for 5 min. The samples were analyzed by a 10% SDS-PAGE and immunoblotted with Nrf2 and p300 Abs. Finally, a

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densitometry analysis was carried out for each experiment to normalize the expression of Nrf2 to that of p300. Independent experiments were repeated four times.

Total RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR).

Total RNAs were extracted by the TRIzol method (Invitrogen) and then analyzed by RT-qPCR using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem), as previously described [19]. Specific gene primer sets were from Qiagen. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA to a 18S RNA endogenous reference. Independent experiments were repeated at least three times.

Transfection of siRNA

Two sets of siRNAs from Dharmacon (Thermofischer) were used: control siRNAs (ON-TARGETplus Non-targeting Pool) and NRF2 siRNAs (ON-TARGETplus SMART pool, L-003755-00-0005). Transfection of siRNAs into Th cells was performed using the P3 Primary Cell 4D-Nucleofector™ X Kit (Lonza) with the Amaxa nucleofection technology. Briefly, ten million Th cells were incubated with 30 pmol of control or NRF2 SiRNAs and then electroporated, using the E0.115 program, on the 4D-Nucleofector™ System. After 24 h, the cells were washed and used for experiments. Independent experiments were repeated four times.

Measurement of Nrf2 DNA binding

Nrf2 DNA binding was analyzed using a specific ELISA-based TransAM™ kit from Active Motif. In brief, nuclear cell extracts (5 µg) were incubated for 1 h in a 96-well plate to which oligonucleotides, containing a Nrf2 consensus binding site, had been immobilized. After washing, the plate was incubated for 1 h with the primary Ab (TransAM Nrf2 kit, Active Motif,

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1:1000), which specifically detected an epitope accessible only when Nrf2 is activated and bound to its cognate oligonucleotide. The plate was then washed and incubated with horseradish peroxidase-conjugated secondary Ab (1:1000) for 1 h at room temperature. Colorimetric readout was quantified by spectrophotometry at 450 nm. Independent experiments were repeated four times.

Luciferase assays

Jurkat cells (5 million) were electroporated with plasmids at 300V with one pulse of 10 ms using the BTM 830 electroporation generator (BTX Instrument Division, Holliston, MA) [21]. Cells were cotransfected with 10 µg of the NF-κB p65-driven luciferase reporter vector from Panomics or an Nrf2-driven luciferase reporter vector (a gift from Dr Ahn, Korea Institute of Radiological and Medical Sciences, Seoul, Korea), and 40 ng of a pRL CMV-Renilla reporter vector, used as an internal control. The Nrf2-driven luciferase reporter vector contains four repeats of the ARE consensus binding sequences (GCTGAGTCA) ligated in a pGL3 plasmid [22]. At 24 h after transfection, cells were pre-treated for 2 h with 20 µM As(III) or 50 µM tBHQ and then stimulated for 6 h with PMA or aCD3/aCD28. After treatment, cell extracts were assayed for luciferase activity with the Dual-Luciferase® Reporter assay system from Promega, according to the manufacturer's instructions. Independent experiments were repeated at least four times.

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Measurement of intracellular production of reactive oxygen species (ROS) by flow cytometry

Cells were stimulated with aCD3/aCD28 or PMA (20 ng/ml) for 2 h then incubated with the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCF-DA, 10 µM), an oxidation-sensitive probe, for 30 min at 37°C. After treatment, cells were washed with PBS and analyzed

using a FC500 flow cytometer (Beckman Coulter). Fluorescence emission from oxidized H2-DCF-DA was detected at 525 nm. Each measurement was conducted on 10000 events and analyzed using CXP analysis software. Two independent experiments were performed.

Measurement of intracellular glutathione (GSH)

Levels of intracellular GSH were quantified using the ThiolTracker™ Violet dye (Molecular probed, Invitrogen), which reacts actively with reduced thiols, and mainly with GSH, in intact cells. After treatments, cells were incubated with ThiolTracker™ Violet dye (10 µM) for 30 min at 37°C and then analyzed on a FACSAria cytometer (BD Biosciences) at 405 nm excitation. Each measurement was conducted on 10000 events and analyzed with CXP analysis software. Two independent experiments were performed.

Quantification of cytokine levels

Levels of IL-2, TNF-α, IFN-γ, and IL-17 secreted in culture medium were quantified by ELISA using specific Duoset ELISA development system kits (R&D Systems). Independent experiments were repeated four times.

Statistical analysis.

Data are expressed as means ± SD. Significant differences were evaluated using Student's t-test or the multi-range Dunnett's test when multiple comparisons were studied. Criterion of significance of the difference between means was $p < 0.05$.

Results

Activation of human Th cells potently increases Nrf2 expression

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First, to detect Nrf2 in Th cells, cells were treated for 6 h with 10 μM MG132, a proteasome inhibitor that blocks Nrf2 degradation. Using the H300 Nrf2 Ab from Santa Cruz, and whole cell lysates from MG132-treated Th cells, Nrf2 was detected as a single band at approx. 110 kDa on a 10% SDS-PAGE (Fig. 1A). This result is in accordance with the recent demonstration that the biological relevant species of Nrf2 migrate between average molecular weights of 95 kDa and 110 kDa [23]. Nrf2 was barely detectable in non-stimulated Th cells, but activation with aCD3/aCD28 (1:1) for 6 h induces its expression (Fig. 1A). In addition, kinetic analysis reveals that Nrf2 expression was increased by 2 h and is still measurable in cells stimulated for 24 h (Fig. 1B). Activation also regulates Nrf2 expression in a dose-dependent manner. Maximal induction was observed in cells stimulated with the ratio 1:1, aCD3/aCD28 at the lowest ratio used in this study (1:30) also slightly increased Nrf2 protein levels (Fig. 1C). In addition, Nrf2 expression was induced in Th cells activated with PMA and ionocymin, or PMA alone (Fig. 1D). This costimulation bypasses aCD3/aCD28-dependent engagement of the TCR, but activates the same downstream signaling pathways [24]. PMA also increased Nrf2 levels in the leukemic Jurkat T-cell line. In these activated cells, Nrf2 was weakly detectable in cytosol but strongly expressed in nuclear extracts (Fig. 1E).

Up-regulation of Nrf2 expression in Th cells results from an increase of NRF2 mRNA levels

Nrf2 expression is commonly increased by post-transcriptional events that inhibit its degradation [1]. Particularly, ROS and pro-oxidant compounds can promote the dissociation of Nrf2 from the ubiquitin ligase complex through oxidation of thiol groups in Keap1, and can secondarily induce its translocation to the nucleus. Figure 2A indicates that NAC, a potent thiol reductive compound, did not inhibit the induction of Nrf2 expression in Th cells activated by aCD3/aCD28 or PMA (Fig. 2A). In addition, neither aCD3/aCD28 nor PMA increased ROS

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levels in Th cells stimulated for 2 h (Fig. S2). By contrast, both aCD3/aCD28 and PMA rapidly and significantly increased mRNA levels of NRF2 gene in Th cells (Fig. 2B). Notably, NRF2 mRNA levels were increased in Th cells activated for only 2 h. Moreover, the inhibition of NRF2 gene transcription by actinomycin D prevents expression of Nrf2 protein in activated Th cells. Figure 2C shows that addition of this inhibitor into culture medium, 2 h after the beginning of Th cell activation by aCD3/aCD28 or PMA, blocked the increase of Nrf2 protein levels detected at 6 h. Expression of the human NRF2 gene is positively regulated by NF-κB [25], a major transcription factor that controls several responses of activated Th cells and the production of many cytokines, such as IL-2 and TNF-α [26]. To test the involvement of NF-κB pathway in the induction of NRF2 gene expression, the IκBα inhibitor BAY 11-7082 was used [27]. At 5 μM, this compound completely blocked the increase of IκBα phosphorylation (Fig. 2D) and the induction of IL2 and TNF-α mRNA levels, in activated Th cells (Fig. 2E). BAY 11-7082 also prevented the increase of both NRF2 mRNA and Nrf2 protein levels induced by aCD3/aCD28 (Fig. 2F and 2G).

Nrf2 protein controls the induction of antioxidant gene expression in activated Th cells

Nrf2 transcriptional activity was further investigated in activated Jurkat cells transfected with an Nrf2-luciferase reporter vector. Jurkat cell responsiveness to PMA and aCD3/aCD28 stimulations was controlled for by quantifying the transcriptional activity of NF-κB. As previously described [28, 29], PMA induced the activity of a NF-κB p65-luciferase reporter vector in Jurkat cells (Fig. 3A). By contrast, costimulation with aCD3/aCD28 only slightly increased vector activity, which suggests that the TCR cannot be fully engaged by these Abs in these Jurkat cells. PMA also induced transcriptional activity of the Nrf2-luciferase reporter

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vector. Although responsiveness of Jurkat cells to aCD3/aCD28 is low, this costimulation also significantly increased Nrf2-dependent luciferase activity (Fig. 3A), but this activity was lower than that induced by PMA. To better characterize Nrf2 activity, the effects of Th cell stimulation on the expression of various antioxidant genes classically up-regulated by this transcription factor were studied. Figure 3B demonstrates that aCD3/aCD28 or PMA significantly increased mRNA levels of GCLM and NQO1 genes in Th cells activated for 6 h or 24 h (Fig. 3B). If NRF2 gene activation subsequently controls the induction of GCLM and NQO1 genes, inhibition of NRF2 mRNA translation should prevent expression of the two genes. In fact, the translation inhibitor CHX blocks the aCD3/aCD28-dependent expression of Nrf2 (Fig. S3), and this significantly reduced GCLM and NQO1 mRNA levels in aCD3/aCD28-costimulated Th cells (Fig. 3C). BAY 11-7082 also totally blocks expression of these genes (Fig. 3C). In addition, stimulation of Th cells with aCD3/aCD28 or PMA for 2 h, 6 h, and 24 h induces increased mRNA levels of the genes HMOX1 and TRX1, which encode heme oxygenase-1 and thioredoxin-1, respectively (Fig. S2). Similarly to the transcription of NQO1 and GCLM genes, transcription of HMOX1 and TRX1 genes is mainly controlled by Nrf2. To confirm that Nrf2 actually controls up-regulation of these genes in activated human Th cells, the impact of genetic invalidation of Nrf2 by SiRNA technology was assessed. CTR and NRF2 SiRNAs were electroporated into Th cells using a 4D-Nucleofector™ System from Lonza. After 24 h of transfection, the cells were washed and costimulated for 6 h. Figure 3D demonstrates that NRF2 SiRNAs decreased both NRF2 mRNA and Nrf2 protein levels in costimulated Th cells. Nrf2 extinction also significantly reduced the up-regulation of mRNA levels of NQO1, GCLM, HMOX1, and TRX1 genes (Fig. 3D). Th cell activation is therefore likely to increase antioxidant gene expression by inducing the transcription of NRF2.

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As(III) increases Nrf2-dependent expression of antioxidant genes in activated Th cells

A classical Nrf2 inducer that acts at a post-transcriptional level may be able to prime Th cells to express NRF2 and antioxidant genes in response to aCD3/aCD28 or PMA stimulation. Th cells were exposed to 2 μM As(III) for 2 h before stimulation; at this concentration, the metalloid does not alter the viability of activated Th cells [19] and, as expected, did not modulate NRF2 mRNA levels (Fig. 4A). However, As(III) significantly increased Nrf2 protein expression in non-stimulated (Fig. 4B) and aCD3/aCD28-stimulated Th cells (Fig. 4C). The metalloid also potently up-regulated mRNA levels of NQO1, GCLM, and HMOX1 genes induced by aCD3/aCD28 or PMA stimulation (Fig. 5A). In addition, As(III) significantly increased NQO1, γ-glutamylcysteine synthase regulatory subunit (γ-GCSm, coded by GCLM gene), and heme oxygenase-1 (HO-1) expression in Th cells costimulated with aCD3/aCD28 for 24 h (Fig. 5B). As γ-GCS is a rate-limiting enzyme that controls the synthesis of glutathione (GSH) [30], intracellular amounts of this peptide were quantified by flow cytometry, using the fluorescent ThiolTracker violet dye. Figure S5 shows that, as previously reported [31], costimulation of Th cells with aCD3/aCD28 increases intracellular GSH levels. However, cell pre-treatment with As(III) did not further enhance the amounts of GSH in activated Th cells. This result can be explained by the fact that As(III) forms stable complexes with GSH, and thereby diminishes the level of reduced GSH being able to react with the ThiolTracker dye.

Given that inhibition of Nrf2 expression blocks the induction of antioxidant genes in activated Th cells, NRF2 gene repression might also prevent overexpression of these genes in cells pre-treated with As(III). Figure 6A clearly shows that BAY 11-7082 completely inhibits the increase of NQO1 and GCLM mRNA levels induced by the metalloid in aCD3/aCD28-stimulated cells. In addition, genetic invalidation of Nrf2 also significantly reduced mRNA levels

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of these two genes in metalloid-exposed cells (Fig. 6B). Altogether, these results support a major role for Nrf2 in As(III)-induced overexpression of antioxidant genes in activated Th cells.

As(III) increases transcriptional activity of Nrf2 in Jurkat cells

To specify the molecular mechanisms by which As(III) can strengthen Nrf2-dependent expression of antioxidant genes, Nrf2 activity was analyzed in Jurkat cells activated with PMA, in the absence or presence of As(III). As shown in Figure 7A, pre-treatment of Jurkat cells with 20 µM As(III) for 2 h significantly increased the levels of Nrf2 in nuclear extracts of PMA-stimulated Jurkat cells. In nuclei, the coactivator p300 can associate with Nrf2 and promote its transcriptional activity. The results of coimmunoprecipitation experiments performed with p300 Ab show that As(III) significantly increased physical interaction of p300 with Nrf2 in PMA-activated Jurkat cells (Fig. 7B). In addition, the ELISA-based Nrf2 TransAM kit (Active Motif) showed that the metalloid significantly enhanced Nrf2 DNA binding in activated cells (Fig. 7C). Finally, As(III) augmented the Nrf2-dependent luciferase activity induced by PMA and aCD3/aCD28 in Jurkat cells (Fig. 7D); the metalloid also slightly increased luciferase activity in non-stimulated cells.

The potent Nrf2 inducer tBHQ mimics the effects of As(III) towards antioxidant genes

Like As(III), tBHQ potently activates Nrf2 expression, at a post-transcriptional level, in several cell types [32]. Additional experiments were performed with this compound to confirm the priming of activated Th cells for antioxidant gene expression. As observed with As(III), tBHQ did not alter NRF2 mRNA levels (Fig. S6A) but it increased total Nrf2 levels in aCD3/aCD28-stimulated Th cells (Fig. S6B). Similarly, pre-treatment with tBHQ significantly up-regulated NQO1 and GCLM mRNA levels in Th cells stimulated with aCD3/aCD28 or PMA

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(Fig. S6C). It also enhanced Nrf2-dependent luciferase activity in Jurkat cells stimulated with PMA or aCD3/aCD28 (Fig. S6D).

Extinction of Nrf2 does not modify the expression of genes coding cytokines in Th cells

Besides its role on redox homeostasis, Nrf2 was shown to exert immunomodulatory effects in several murine models. The regulation by Nrf2 of the expression of immune-related genes in human Th cells activated with aCD3/aCD28 was examined. Costimulation increased mRNA levels of IL2, INFG, TNF, and IL17 genes in Th cells stimulated for 6 h (Fig. S7). However, genetic invalidation of Nrf2 expression with NRF2 SiRNAs did not alter the levels of mRNA of these four genes (Fig. 8A) or the levels of IL-2, TNF-α, IFN-γ, and IL-17 secreted in culture medium (Fig. 8B). We previously reported that cell pre-treatment with As(III) reduces the aCD3/aCD28-induced expression of IL-2, IFN-γ, and IL-17 [19, 20]. As(III)-dependent activation of Nrf2 could therefore mediate these inhibitory effects. The results show that the loss of Nrf2, in transfected Th with NRF2 SiRNA cells, does not inhibit the effects of the metalloid on IL2, TNF, IFNG, and IL17 genes in Th cells (Fig. 8A and 8B).

Discussion

The present study demonstrates that activation of human Th lymphocytes increases expression and activity of Nrf2, likely through the induction of NRF2 gene transcription. In addition, pre-treatment with electrophilic compounds that stabilize the Nrf2 protein primes Th cells for the expression of antioxidant genes. However, induction of NRF2 does not alter the expression of genes coding inflammatory cytokines in Th lymphocytes.

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The results demonstrate that Nrf2 protein expression is low and hardly detectable in non-stimulated Th cells. This data was expected since, under basal conditions, Nrf2 is rapidly degraded by the proteasome in most cells. However, activation of human Th cells with aCD3/aCD28 or PMA ± ionomycin strongly increases the level of Nrf2 protein. Classic regulation of Nrf2 expression occurs at a post-transcriptional level through oxidation of critical cysteine residues in Keap1 [1]. This mechanism is unlikely to occur in activated Th lymphocytes because NAC, a potent thiol reductive compound known to prevent oxidative stress-dependent activation of Nrf2 [33], does not block Nrf2 expression in activated Th cells; moreover, neither aCD3/aCD28 nor PMA increases the levels of ROS which markedly contribute to Keap1 oxidation [2]. By contrast, activation of Th cells probably induces the transcription of NRF2 gene. Indeed, the results demonstrate that activation of Th cells rapidly and significantly increases NRF2 mRNA levels with a kinetic very similar to that observed for Nrf2 protein expression. In addition, inhibition of gene transcription by actinomycin D, after the beginning of Th cell activation, totally blocks induction of Nrf2 protein expression.

BAY 11-7082, a potent inhibitor of IκBα, also prevents Nrf2 expression. This result suggests that induction of NRF2 gene transcription can be mediated by the NF-κB transcription factor in activated Th cells. Two arguments support this idea: first, it has recently been demonstrated that the NF-κB subunits p50 and p65 control the inducible levels of NRF2 mRNAs in human acute myeloid leukemia cells through activation of a κB site in the promoter of NRF2 gene [25]. Second, NF-κB activity is well-known to be rapidly and potently induced in T lymphocytes stimulated with aCD3/aCD28 or PMA [26, 29]. Indeed, TCR engagement by costimulation triggers several signaling pathways that notably activate the protein kinase C theta. Through activation of the IκB-kinase complex, this kinase increases the proteasome-dependent

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degradation of phosphorylated-IκBα and allows the activation of NF-κB [26]. Stimulation of T lymphocytes with PMA mimics the effect of costimulation and also potently activates protein kinase C theta [26]. Although Nrf2 expression is not commonly induced at the transcriptional level, this mechanism of regulation was observed in other human immune cells. Activation of human macrophages by lipopolysaccharide, a bacterial endotoxin, up-regulates Nrf2 levels by a NF-κB-dependent signaling pathway that increases the amounts of NRF2 mRNAs [25]. Collectively, these results suggest that the immune response may result in the induction of Nrf2 expression in key immune cells, including, at least, T lymphocytes and macrophages.

This study demonstrates that the induction of Nrf2 levels in Th cells is associated with the expression of several genes encoding antioxidant proteins. Costimulation of Th cells with aCD3/aCD28 notably increases the expression of NQO1, HMOX1 and GCLM genes at both mRNA and protein levels. Moreover, Th cell activation markedly stimulates GSH synthesis which is limited, in part, by transcription of the GCLM gene. Nrf2 is likely to control expression of these antioxidant genes in activated Th cells since i) aCD3/aCD28 and PMA increase Nrf2 transcriptional activity and ii) pharmacological (by CHX and BAY 11-7082) and genetic (by NRF2 SiRNAs) inhibition of Nrf2 protein expression prevents the increase of NQO1 and GCLM mRNA levels. Consequently, the activation of Th lymphocytes may strengthen redox homeostasis by promoting Nrf2 expression and the subsequent induction of antioxidant genes. These regulations may thus constitute a general mechanism to prevent deleterious effects of electrophilic and pro-oxidant compounds in activated Th lymphocytes.

In this context, we previously reported that 2 μM As(III) induces the apoptosis/necrosis of human monocytes differentiating into macrophages or dendritic cells [34, 35], whereas it is non-cytotoxic, and unable to stimulate ROS production, in activated human Th cells [19]. The

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present results show that pre-treatment with As(III) or tBHQ prime activated Th cells for expression of antioxidant genes by strengthening Nrf2 activation. Indeed, As(III) and tBHQ increase Nrf2 protein levels and potentiate Nrf2 transcriptional activity in PMA- and aCD3/aCD28-stimulated Jurkat cells. Moreover, inhibition of NRF2 expression blocks metalloid effects towards antioxidant genes. As(III) and tBHQ do not enhance the transcription of NRF2, but probably reduce the proteasome-dependent degradation of Nrf2 in Th cells. These two electrophilic compounds are thought to trigger Nrf2 stabilization through oxidations of critical thiol groups in Keap1, which diminish the affinity of Keap1 for Nrf2 [32, 36]. Recent studies demonstrate that the metalloid can also increase Nrf2 protein levels, either by impairing the Keap1-Cul3 E3 ubiquitin ligase complex activity or by inhibiting autophagic flux in a p62-dependent manner [37, 38] .

Experiments on Jurkat cells further specify the mechanism by which As(III) can enhance Nrf2 transcriptional activity. The metalloid increases nuclear concentrations of Nrf2, its interaction with the coactivator p300 and the DNA binding activity of Nrf2 in activated Jurkat cells. The coactivator p300 promotes transcription by acetylating core histones to decondensate chromatin and facilitate recruitment of the RNA polymerase machinery [4]. Also, p300 directly acetylates Nrf2 on lysine residues and thereby augments the binding of Nrf2 to specific AREs [39]. Altogether, these results show that optimal expression and activity of Nrf2 occur in human T cells when strong transcription of the NRF2 gene and stabilization of Nrf2 protein are simultaneously operant.

Studies on Nrf2 knockout mice have suggested that Nrf2 can modulate different functions of murine T lymphocytes, specifically their ability to express pro-inflammatory cytokines [16]. Activated Th cells from Nrf2 knockout mice secrete higher amounts of IFN-γ and TNF-α than

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their control counterparts from wild-type mice. The results of this study do not support a role for Nrf2 in the regulation of cytokine synthesis in human Th cells, since genetic invalidation of Nrf2 expression does not up-regulate mRNA levels of IL2, IFNG, TNF, and IL17 genes in Th cells activated in the absence or presence of As(III). The different technologies used to repress Nrf2 expression may explain the divergent results obtained with murine and human T cells. Indeed, the stable disruption of Nrf2 in Th cells from KO mice deeply affects cell homeostasis and may indirectly impair the expression of inflammatory genes in response to costimulation. Particularly, this loss of Nrf2 may stimulate the production of intracellular ROS at a level sufficient to increase both the aCD3/aCD28-dependent activity of NF-κB and the subsequent transcription of immune genes controlled by this transcription factor [6, 15]. Conversely, SiRNA-mediated repression of Nrf2 is transient and is unlikely to alter the global physiology of human Th cells. Thus, the silencing RNA technology allows the study of direct, rather than indirect, consequences of protein extinction. In this context, the present results suggest that Nrf2, expressed in activated human T lymphocytes, cannot directly interfere with the transcription of these immune genes. The fact that Nrf2 extinction does not prevent the repressive effects of As(III) on immune genes suggests that the modulation of Nrf2 levels may not constitute a promising therapeutic approach to limit or prevent the excessive T lymphocyte-derived production of inflammatory cytokines observed in chronic inflammatory diseases [40, 41].

In conclusion, this study demonstrates for the first time that costimulation of human Th lymphocytes up-regulates the expression and activity of Nrf2 at the transcriptional level. Induction of Nrf2 strengthens the redox status of Th cells, but does not impair their ability to express inflammatory cytokines.

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Legends

Figure 1. Activation of human T lymphocytes increases Nrf2 protein levels. Th cells were either untreated (NS) or stimulated for 6 h (A, C, D), or indicated times (B), with 10 μM MG132 (A), aCD3/aCD28 (A-D), or 20 ng/ml PMA ± 0.5 μM ionomycin (iono) (D). In (E) Jurkat cells were stimulated with 20 ng/ml PMA for up to 4 h. Nrf2 protein expression in whole cell lysates (Th cells), cytosol and nuclear extracts (Jurkat cells) was analyzed by Western blot. In (B, C) densitometric analysis of Nrf2 expression is presented in the right panel and the results are expressed as means ± SD of 5 (B) and 4 (C) independent experiments (*$p<0.05$, **$p<0.01$ versus "NS"). Equal gel loading was verified by re-probing nitrocellulose membrane with p38 kinase (A, B, C, D), Hsc70 or p300 (E) Abs. In (A, D, E), immunoblots are representative of at least three independent experiments.

Figure 2. Up-regulation of Nrf2 protein results from an increase of NRF2 mRNA levels. Th cells were either untreated (NS) or pre-treated with 10 mM NAC or 5 μM BAY 11-7082 (Bay) for 2 h then left unstimulated or stimulated with aCD3/aCD28 or PMA for 1 h (D), 6 h (A, E, F, G) or the indicated times (B, C). In (C), 2 h after the start of cell stimulation, 3 μg/ml actinomycin D (Act) was added to the culture medium. Levels of Nrf2 (A, C, G) and phospho (p)-IκBα (D) in whole cell lysates were then analyzed by Western blot. Equal gel loading was verified by re-probing nitrocellulose membrane with p38 kinase Ab. In (A) immunoblots are representative of

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three independent experiments. In (C, D, G), densitometric analysis is presented in the right panel and the results are expressed as means ± SD of four independent experiments. In (B, E, F), data are expressed relatively to mRNA levels found in NS Th cells, arbitrarily set at the value of 1. Results are expressed as means ± SD of at least four independent experiments. *p<0.05, *p<0.01 versus "NS"; #p<0.05, ##p<0.01 vs aCD3/aCD28 or PMA.

Figure 3. Stimulation of T lymphocytes increases Nrf2 activity. In (A), Jurkat cells were transiently cotransfected for 24 h with a p65- or a Nrf2-driven luciferase reporter vector (10 µg) and a CMV-Renilla reporter vector (40 ng). Cells were left untreated (NS) or stimulated with 20 ng/ml PMA or aCD3/aCD28 for 6 h. Luciferase activity was determined as indicated in "Materials and Methods". In (B, C), Th cells were left untreated (NS) or pre-treated with 5 µg/ml CHX or 10 µM BAY 11-7082 (Bay) for 30 min or 2 h, respectively. In (D), Th cells were transiently transfected with CTR SiRNAs (Si CTR) of NRF2 SiRNAs (Si NRF2) for 24 h. Cells were then left untreated or stimulated with aCD3/aCD28 (B, C, D) or 20 ng/ml PMA (B) for 6 h (C, D) or the indicated times (B). In (B, C, D), mRNA levels were determined by RT-qPCR assays. Data are expressed relatively to mRNA levels found in NS Th cells (B, C) or costimulated Th cells transfected with Si CTR (D), arbitrarily set at the value of 1. In (D), levels of Nrf2 and p38-kinase in transfected cells were analyzed by Western blot. (left bottom panels). The immunoblot is representative of three independent experiments. In (A, B, C, D), results are expressed as means ± SD of at least four independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus "NS" (A, B, C), #p<0.05, ##p<0.01, ###p<0.001 versus "aCD3/aCD28" (C) or "Si CTR" (D).

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Figure 4. As(III) increases Nrf2 protein levels in costimulated Th cells. Th cells were untreated (NS) or pre-treated with 2 μM As(III) for 2 h (A, B, C) and 8 h (B), then left unstimulated or stimulated with aCD3/aCD28 for 6 h. In (A), data are expressed relatively to mRNA levels found in NS Th cells, arbitrarily set at the value of 1. In (B, C), Nrf2 expression was analyzed by Western blot. Equal gel loading was verified by re-probing nitrocellulose membrane with p38 kinase Ab. In (B, C), densitometric analysis of Nrf2 expression is presented in the right panel. Results are expressed as means ± SD of four independent experiments. *p<0.05, **p<0.01 versus "NS", #p<0.05 versus "aCD3/aCD28".

Figure 5. As(III) increases the expression of stress-related genes in costimulated Th cells. Th cells were either untreated (NS) or pre-treated with As(III) for 2 h. Cells were then left unstimulated or stimulated with aCD3/aCD28 or 20 ng/ml PMA for 6 h (A) or 24 h (B). In (A), data are expressed relatively to mRNA levels found in NS Th cells, arbitrarily set at the value of 1. In (B), protein expression was analyzed by Western blot. Equal gel loading was verified by re-probing nitrocellulose membrane with p38 kinase Ab. Densitometric analysis of protein expression is presented in the panel below the immunoblot. Results are expressed as means ± SD of four independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus "NS", #p<0.05, ##p<0.01, ###p<0.001 versus "aCD3/aCD28" or "PMA".

Figure 6. Inhibition of Nrf2 expression prevents the effects of As(III) on NQO1 and GCLM gene in activated Th cells. In (A), cells were either untreated or pre-treated for 2 h with 5 μM BAY 11-7082 and/or 2 μM As(III). In (B), cells were transfected for 24 h with CTR SiRNAs (Si CTR) or NRF2 SiRNAs (Si NRF2) and then, untreated, or pre-treated with 2 μM As(III) for 2 h. Next, Th cells were, non stimulated, or stimulated for 6 h with aCD3/aCD28. Data are expressed

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relatively to mRNA levels found in NS Th cells (A) or costimulated Th cells transfected with Si CTR (not shown), arbitrarily set at the value of 1. Results are expressed as means ± SD of four independent experiments. #p<0.05, ###p<0.001 versus "As(III) + aCD3/aCD28" (A) or "Si CTR" (B).

Figure 7. As(III) enhances Nrf2 activity induced by Jurkat cell stimulation. Cells were either untreated (NS) or pre-treated for 2 h with 20 μM As(III) and then left unstimulated or stimulated with 20 ng/ml PMA or aCD3/aCD28 (D) for 2 h (A, B, C) or 6 h (D). After preparation of cytosol and nuclear extracts (A), or immunoprecipitation of p300 as described in Materials in Methods (B), Nrf2 protein present in cell extracts or bound to p300 was analyzed by Western blot, and quantified by densitometric analysis (A, right panel; B, bottom panel) Equal gel loading was verified by re-probing nitrocellulose membrane with Hsc70 and/or p300 Abs. In (C), DNA binding activity of Nrf2 protein, present in nuclear extracts of Jurkat cells, was determined using the ELISA-based Nrf2 TransAM kit from Active Motif. In (D), before pre-treatment, cells were transiently cotransfected for 24 h with a Nrf2-driven luciferase reporter vector (10 μg) and a CMV-Renilla reporter vector (40 ng). After stimulation, luciferase activity was determined as indicated in "Materials and Methods". In (A, B, C, D), the results are expressed as means ± SD of four independent experiments. ***p<0.001 versus NS; #p<0.05, ##p<0.01, ###p<0.001 versus "PMA" (A, B, C, D) or "aCD3/aCD28 (D).

Figure 8. SiRNA-mediated invalidation of Nrf2 protein does not prevent expression of immune genes in Th cells activated in the absence or presence of As(III). Th cells were first transiently transfected for 24 h with CTR SiRNA (Si CTR) or NRF2 SiRNA (Si NRF2). Then, cells were either untreated or pre-treated with 2 μM As(III) for 2 h and left unstimulated or stimulated with

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aCD3/aCD28 for 6 h (A) or 24 h (B). In (A), data are expressed relatively to mRNA levels found in stimulated Th cells transfected with Si CTR, arbitrarily set at the value of 1. In (B), the levels of cytokines secreted in culture medium were quantified by Elisa. Results are expressed as means ± SD of four independent experiments. #p<0.05, ##p<0.01, ###p<0.001 versus activated Th cells non-exposed to As(III).

Highlights

Expression and activity of Nrf2 in human primary T lymphocytes are unknown
Activation of human T helper lymphocytes induces expression of NRF2 gene
Nrf2 invalidation blocks induction of antioxidant genes in activated T lymphocytes
Inorganic arsenic primes activated T lymphocytes for Nrf2 activity
Nrf2 does not modulate cytokine expression in human activated T lymphocytes

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Graphical Abstract (for review)

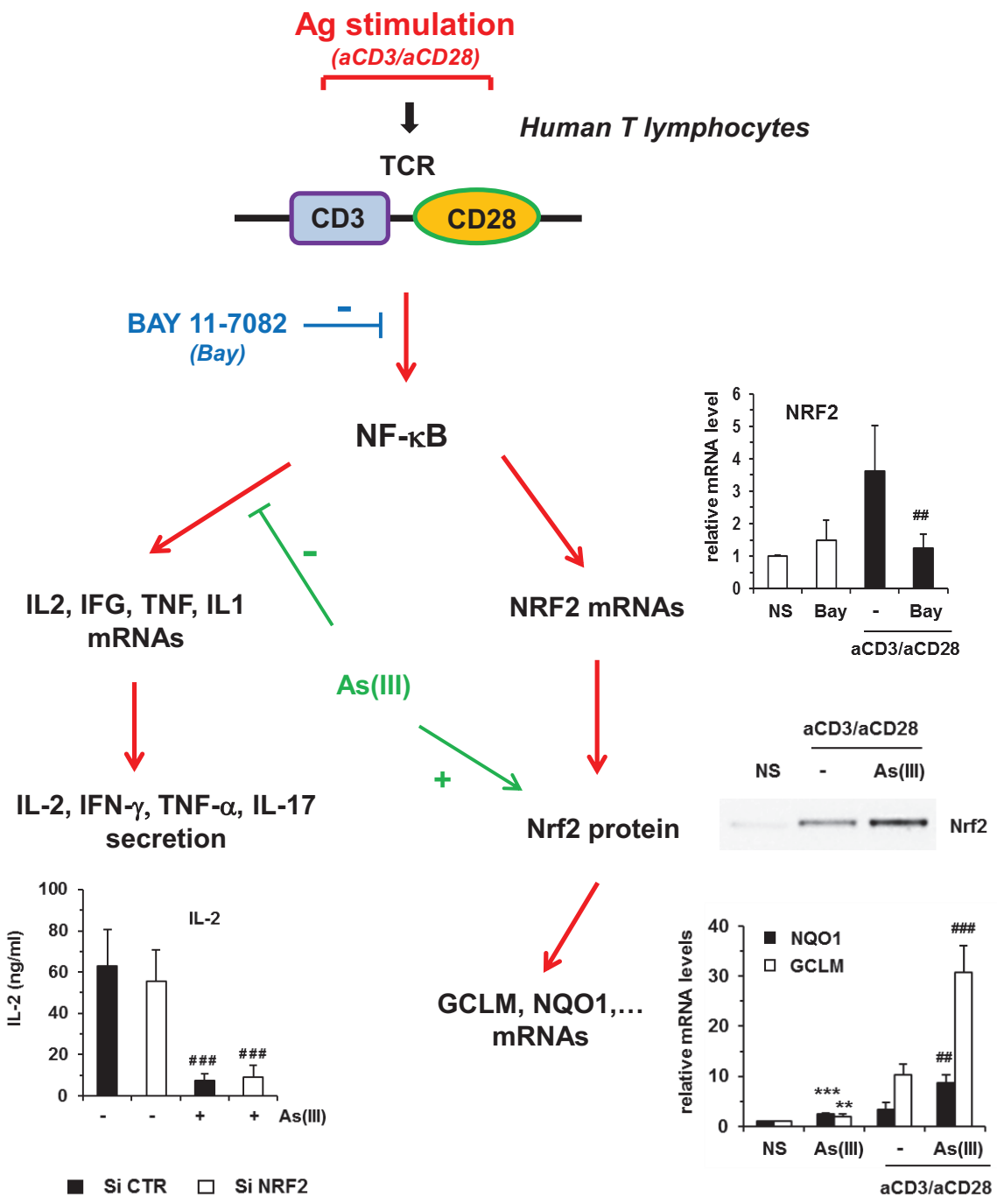

Figure 1

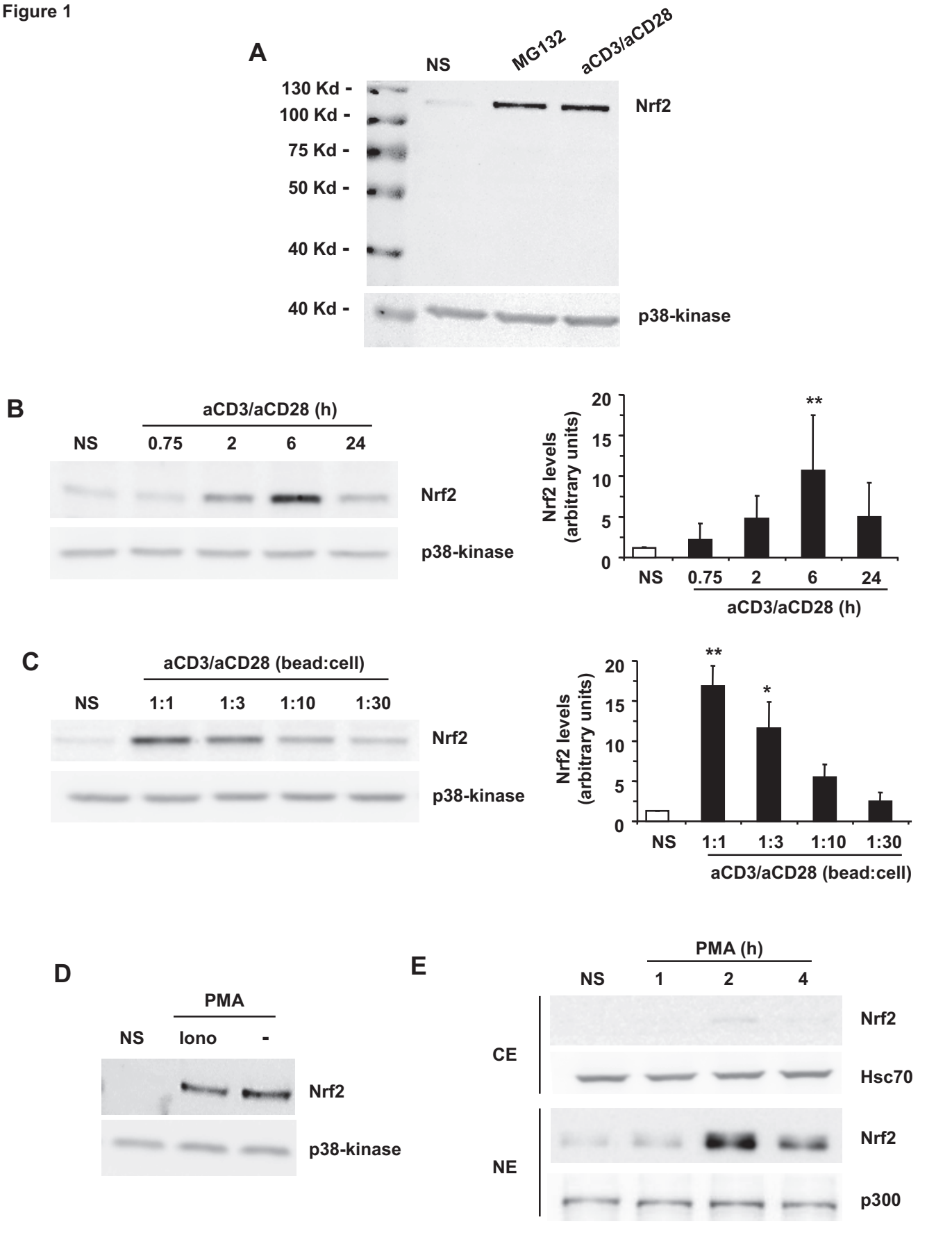

Figure 1

Figure 2

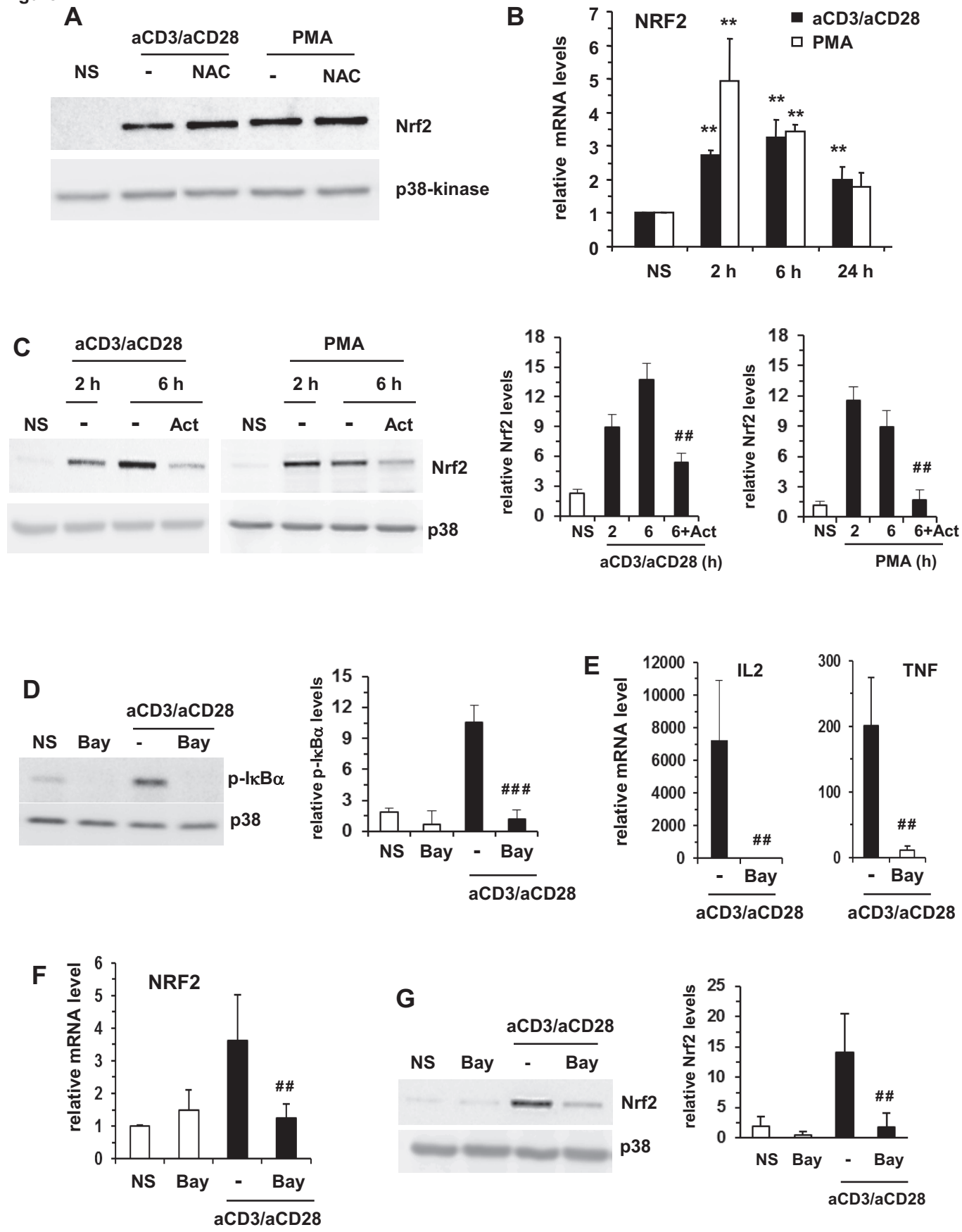

Figure 2

Figure 3

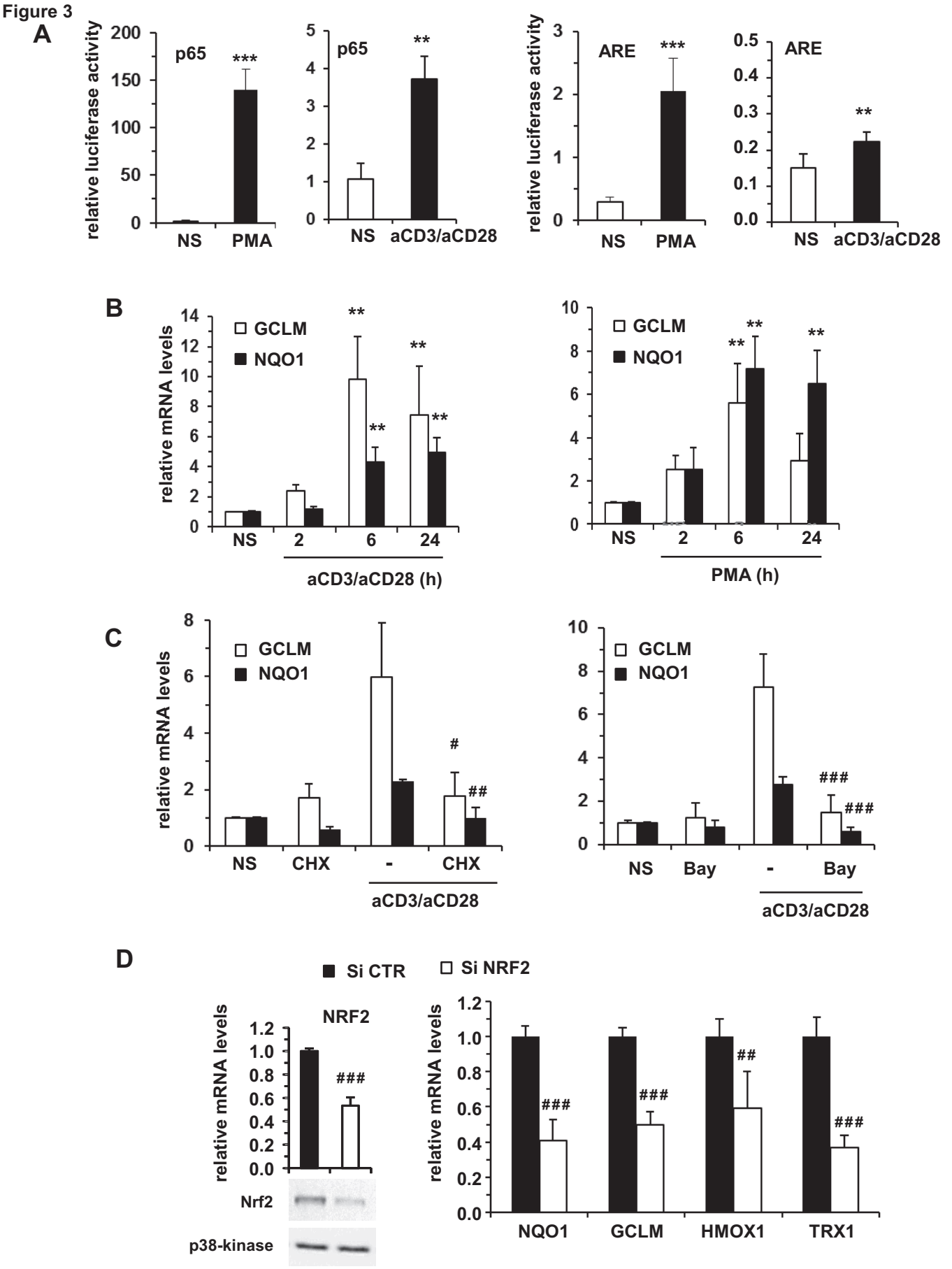

Figure 3

Figure 4

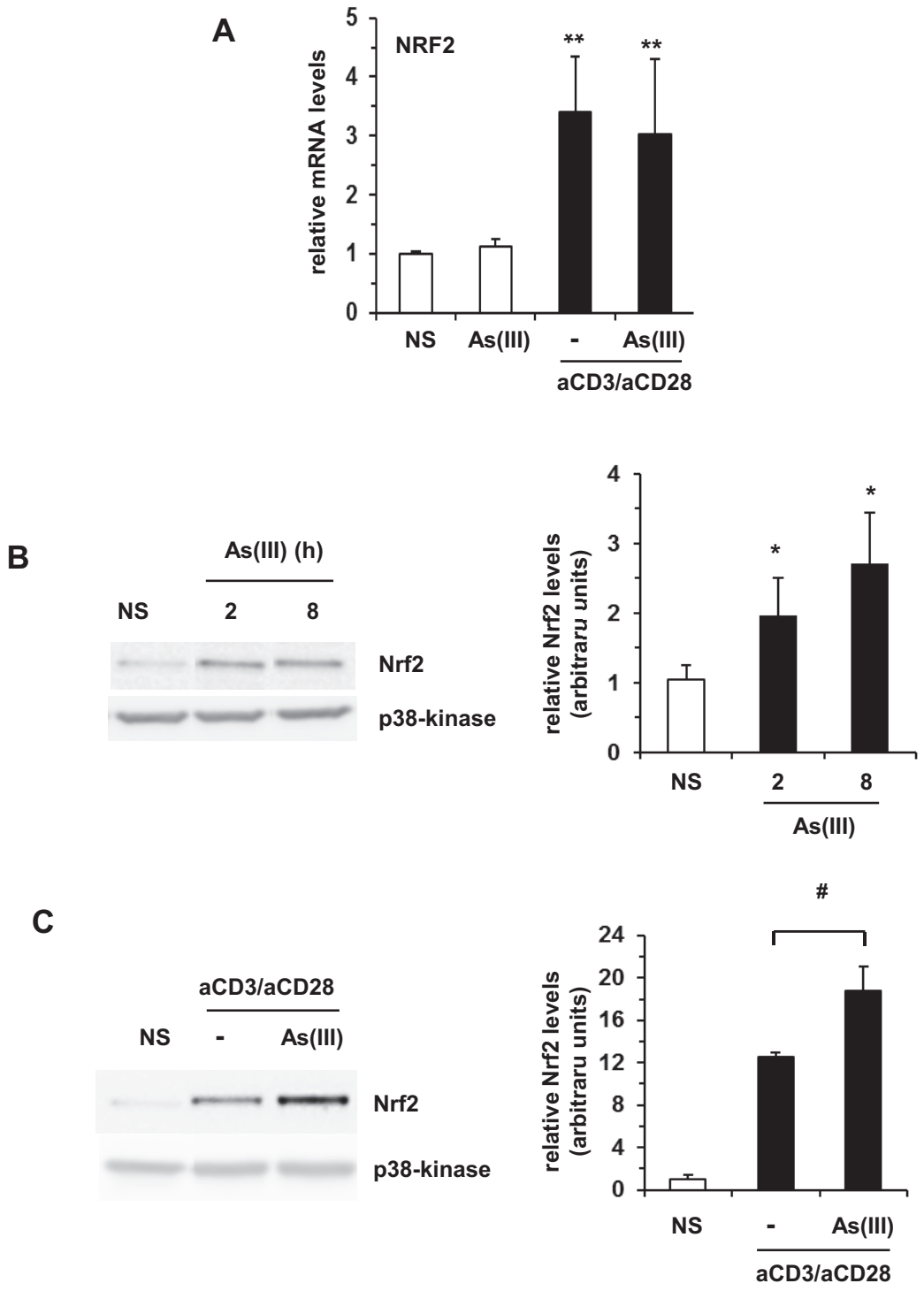

Figure 4

Figure 5

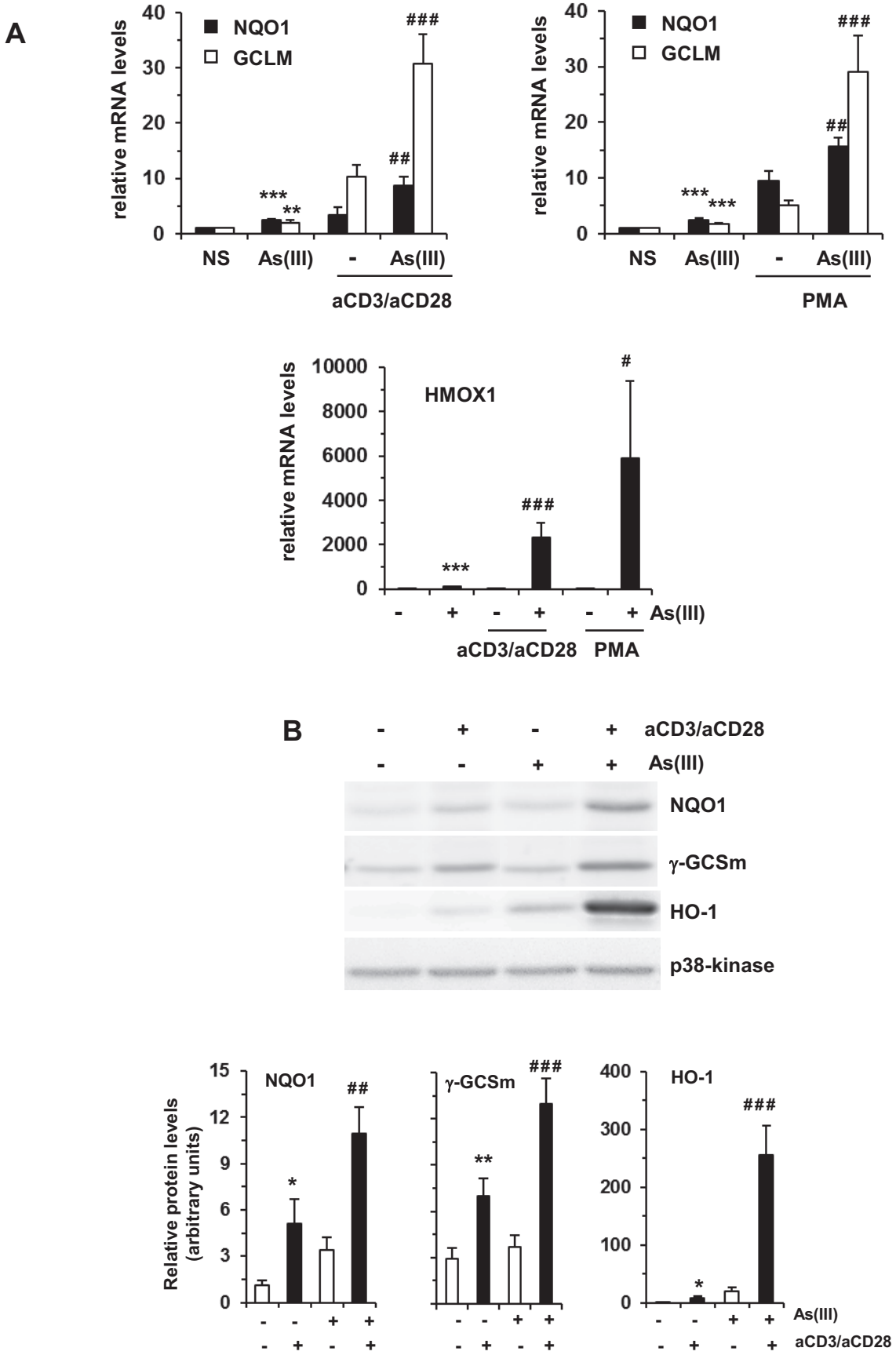

Figure 5

Figure 6

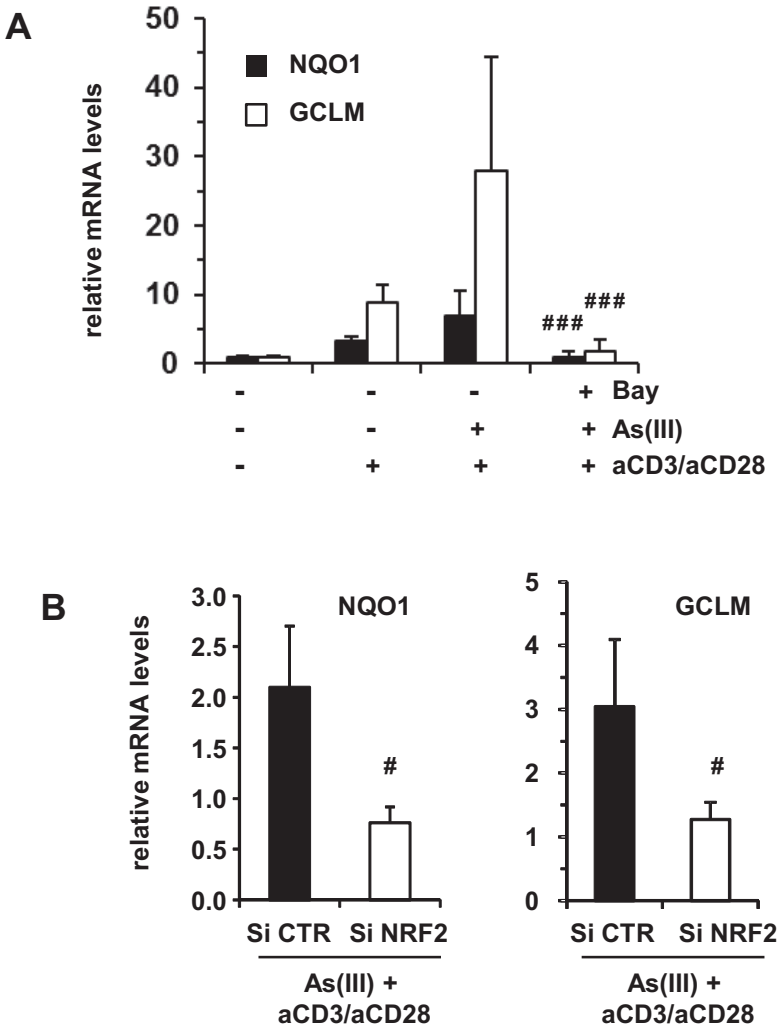

Figure 6

Figure 7

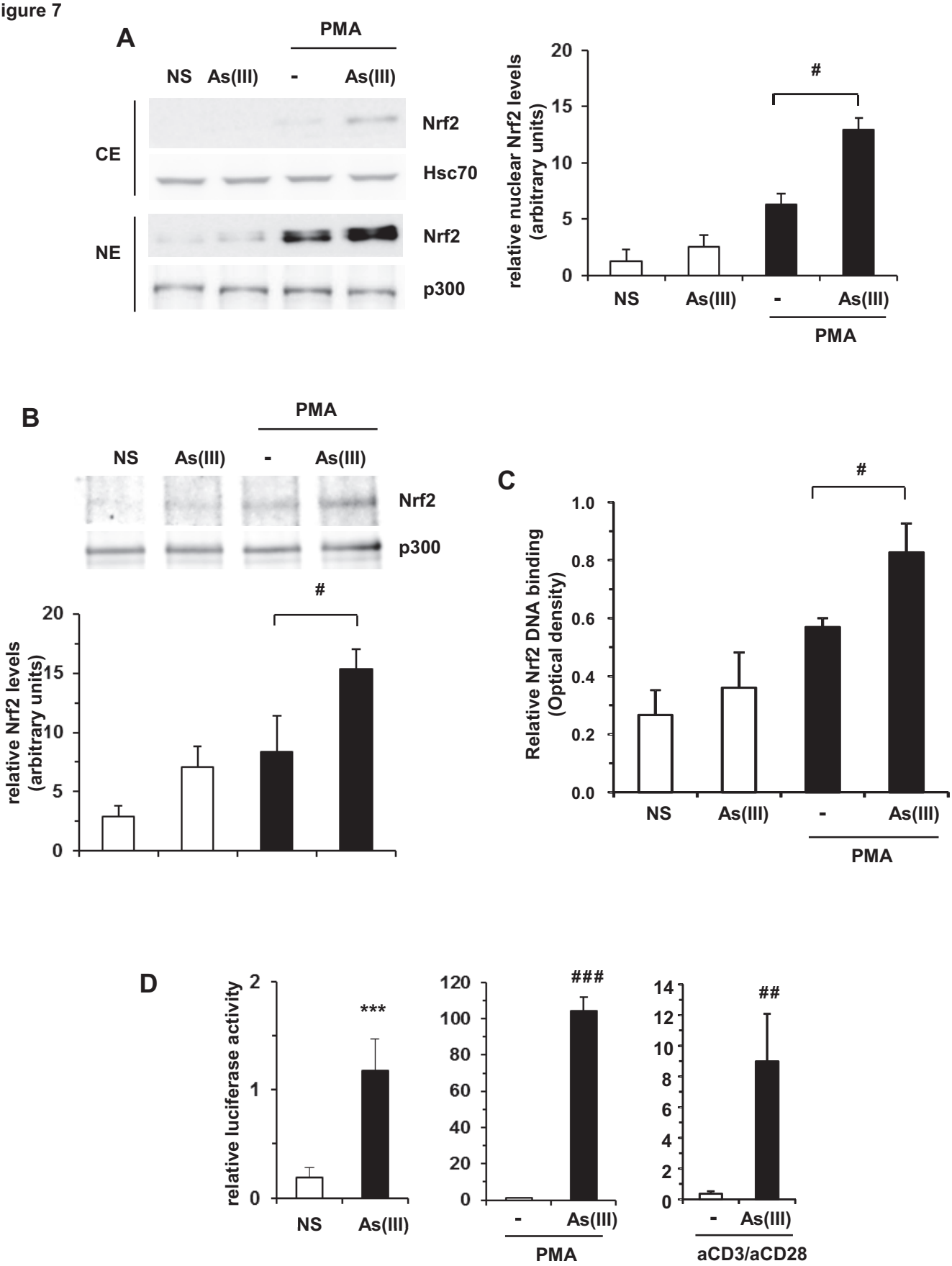

Figure 7

Figure 8

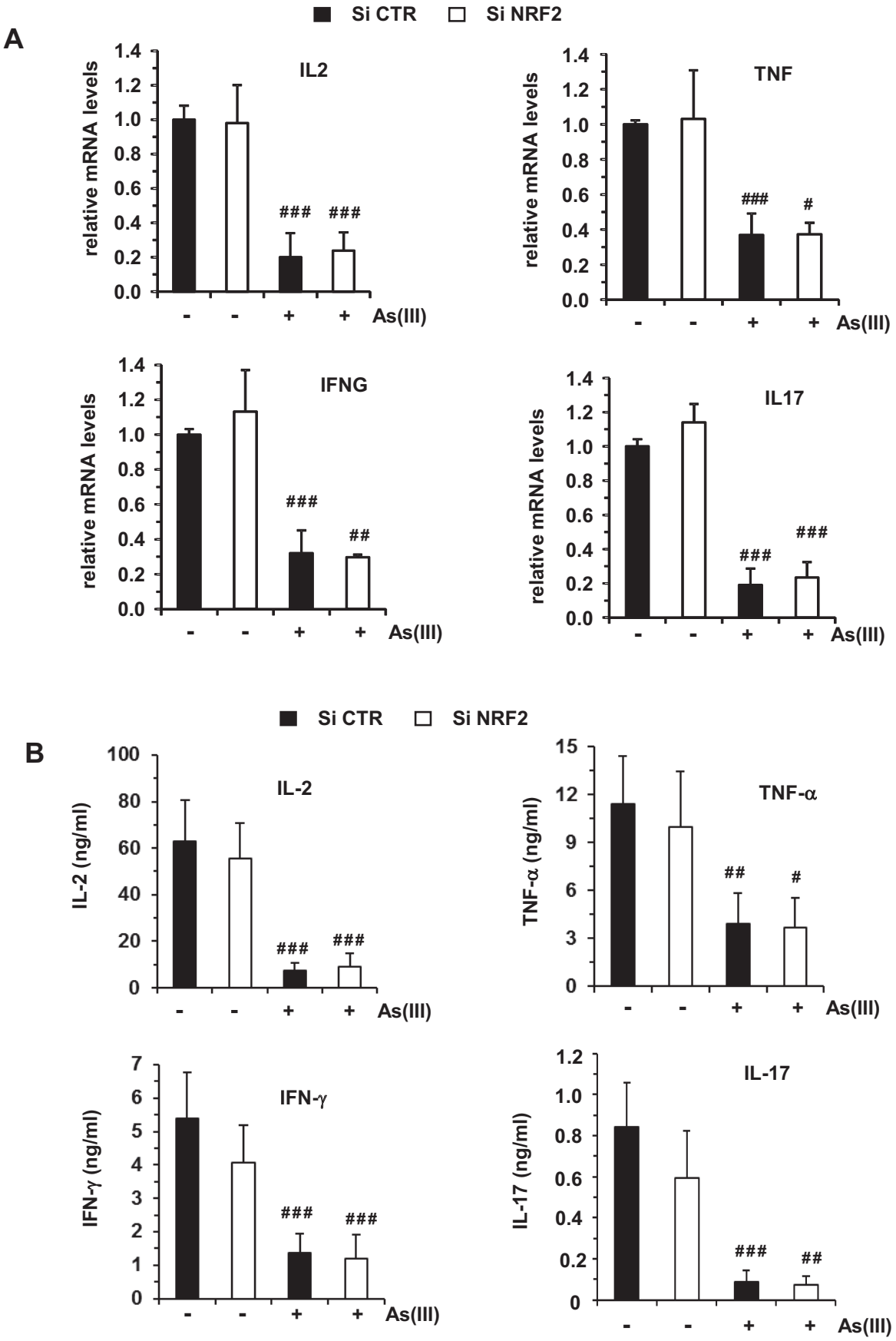

Figure 8