A Nitric Oxide Storage and Transport System That Protects Activated Macrophages from Endogenous Nitric Oxide Cytotoxicity

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 53, pp. 27042–27061, December 30, 2016

A Nitric Oxide Storage and Transport System That Protects Activated Macrophages from Endogenous Nitric Oxide Cytotoxicity*

Received for publication, October 27, 2016, and in revised form, November 16, 2016 Published, JBC Papers in Press, November 19, 2016, DOI 10.1074/jbc.M116.763714

Hiu Chuen Lok[‡], Sumit Sahni[‡], Patric J. Jansson[‡1], Zaklina Kovacevic[‡2], Clare L. Hawkins[§3], and Des R. Richardson[‡1,4]

From the [‡]Molecular Pharmacology and Pathology Program, Department of Pathology and Bosch Institute, University of Sydney, Sydney, New South Wales 2006 and the [§]Heart Research Institute, Sydney, New South Wales 2042, Australia

Edited by F. Peter Guengerich

Nitric oxide (NO) is integral to macrophage cytotoxicity against tumors due to its ability to induce iron release from cancer cells. However, the mechanism for how activated macrophages protect themselves from endogenous NO remains unknown. We previously demonstrated by using tumor cells that glutathione *S*-transferase P1 (GSTP1) sequesters NO as dinitrosyl-dithiol iron complexes (DNICs) and inhibits NO-mediated iron release from cells via the transporter multidrug resistance protein 1 (MRP1/ABCC1). These prior studies also showed that MRP1 and GSTP1 protect tumor cells against NO cytotoxicity, which parallels their roles in defending cancer cells from cytotoxic drugs. Considering this, and because GSTP1 and MRP1 are up-regulated during macrophage activation, this investigation examined whether this NO storage/transport system protects macrophages against endogenous NO cytotoxicity in two well characterized macrophage cell types (J774 and RAW 264.7). MRP1 expression markedly increased upon macrophage activation, and the role of MRP1 in NO-induced ^{59}Fe release was demonstrated by *Mrp1* siRNA and the MRP1 inhibitor, MK571, which inhibited NO-mediated iron efflux. Furthermore, *Mrp1* silencing increased DNIC accumulation in macrophages, indicating a role for MRP1 in transporting DNICs out of cells. In addition, macrophage ^{59}Fe release was enhanced by silencing *Gstp1*, suggesting GSTP1 was responsible for DNIC binding/storage. Viability studies demonstrated that GSTP1 and MRP1 protect activated macrophages from NO cytotoxicity. This was confirmed by silencing nuclear factor-erythroid 2-related factor 2 (*Nrf2*), which decreased MRP1 and GSTP1 expression, concomitant with reduced ^{59}Fe release and macrophage survival. Together, these results demonstrate a mechanism by which macrophages protect themselves against NO cytotoxicity.

Nitric oxide (NO) is a short-lived diatomic effector and messenger molecule (1, 2) that is responsible for a wide range of biological processes, such as macrophage cytotoxicity (3, 4), carcinogenesis (5), blood pressure regulation (6), and neurotransmission (7). Physiologically, NO is produced by the NO synthase (NOS; EC 1.14.13.39) family of enzymes (8), which consist of inducible NOS (iNOS),[5] endothelial NOS, and neuronal NOS (9). This group of enzymes is responsible for the catalysis of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent conversion of L-arginine and O_2 to L-citrulline and NO (10).

Although endothelial NOS and neuronal NOS are expressed constitutively in cells, iNOS expression and activity can be stimulated by lipopolysaccharide (LPS) or cytokines such as interferon-γ (IFN-γ), triggering NO generation in macrophages, and subsequent apoptosis of target pathogens (11–13) and tumor cells (14–16). The metabolic damage inflicted by macrophages on neoplastic cells has been well demonstrated in the seminal studies of Hibbs *et al.* (15). These investigators showed that co-cultivation of tumor cells with activated macrophages led to an inhibition of mitochondrial respiration and suppression of DNA synthesis. Furthermore, these effects occurred concomitantly with a significant loss of iron from tumor target cells (64% of cellular iron over 24 h) (15). The pivotal role of NO in macrophage cytotoxicity has been highlighted by studies demonstrating that the anti-tumor activity of macrophages is replicated by NO gas (3).

The high affinity of NO for intracellular iron(II) results in the interaction of NO with iron-sulfur clusters in proteins, leading to their degradation and the formation of dinitrosyl-dithiol iron complexes (DNICs (3)). This process is reflected by a substantial loss of iron from tumor target cells (15). Furthermore, formation of DNICs with the formula $Fe(RS)_2(NO)_2$ has been reported in activated macrophages (4) and tumor cells co-cultured with activated macrophages (17). These complexes are readily detected by electron paramagnetic resonance (EPR) spectroscopy with the unique signal of $g = 2.04$ (18, 19). Importantly, DNICs are a highly bioavailable source of iron and NO

* This work was supported in part by National Health and Medical Research Council of Australia Project Grant 1062026 (to D. R. R. and C. L. H.). The authors declare that they have no conflicts of interest with the contents of this article.
[1] Supported by the National Breast Cancer Foundation and Avner Pancreatic Cancer Foundation.
[2] Supported by National Health and Medical Research Peter Doherty Early Career Fellowship 1037323 and Cancer Institute New South Wales Early Career Fellowship 12-ECF2-17.
[3] Supported by Australian Research Council Future Fellowship FT120100682.
[4] Supported by the National Health and Medical Research Council of Australia Senior Principal Research Fellowship. To whom correspondence should be addressed. Tel.: 61-2-9036-6548; Fax: 61-2-9351-3429; E-mail: d.richardson@med.usyd.edu.au.

[5] The abbreviations used are: iNOS, inducible nitric-oxide synthase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DNDGIC, dinitrosyl diglutathionyl iron complex; DNIC, dinitrosyl-dithiol iron complex; L-NAME, L-N^G-nitroarginine methyl ester hydrochloride; MEF, murine embryonic fibroblast; NOS, nitric oxide synthase; Tf, transferrin.

and constitute a major proportion of the NO adducts within cells (20, 21), demonstrating their crucial biological relevance.

Studies from our laboratory showed that NO-induced iron efflux, in the form of DNICs, is mediated by the glutathione (GSH) transporter, multidrug resistance protein 1 (MRP1/ABCC1) (22, 23). Originally associated with multidrug resistance in cancer as part of the cell detoxification system (24, 25), MRP1 is also well known to interact synergistically with members of the glutathione *S*-transferase (GST) protein family to confer resistance to cancer therapeutics (26–33). This is important because GSTs catalyze nucleophilic attack of reduced GSH on non-polar compounds, leading to glutathione conjugates that are effluxed out of cells via MRP1 (34, 35). It has been conclusively demonstrated that GST family members bind dinitrosyl-diglutathionyl iron complexes (DNDGIC) with high affinity (K_d 10^{-7} to 10^{-10} M) (36–39). Of additional significance, an X-ray crystal structure of the DNDGIC with glutathione *S*-transferase P1 (GSTP1) has been reported (37).

Considering the cooperation between MRP1 and GSTs in mediating drug resistance (26–30, 33), we showed that GSTP1 can prevent NO-induced iron release by binding DNICs and preventing their release via MRP1 in tumor cells (23). This effect was demonstrated using cells incubated with NO-generating agents or transfected with iNOS (23). Further studies using cellular fractionation, fast protein liquid chromatography (FPLC), and EPR spectroscopy demonstrated that DNIC binding by GSTP1 prevented DNIC efflux from tumor cells by MRP1 (23).

Our laboratory also demonstrated the DNIC-binding/storage function of GSTP1 and the ability of MRP1 to efflux DNIC, which leads to tumor cell resistance against NO challenge (23). Hence, the storage function mediated by GSTP1 of DNICs and the ability of MRP1 to efflux DNICs are both critical in preventing NO cytotoxicity and constitute an integrated NO storage and transport system (23). It is notable in this regard that GSH conjugates are transported by MRP1 (40) and that DNICs, composed of GSH, are also a type of GSH conjugate (37). Collectively, the role of GSTP1 and MRP1 within tumor cells in protecting them from NO by storing and transporting DNICs, respectively, may be similar to their function in preventing drug cytotoxicity.

In view of the cooperative relationship of MRP1 and GSTP1 in protecting human tumor cells against NO, it is notable that MRP1 and GSTs are both positively regulated by nuclear factor-erythroid 2-related factor 2 (NRF2) (41, 42), a redox-sensitive leucine zipper transcription factor that is activated by NO (43). Importantly, NRF2 maintains redox homeostasis through the transcriptional regulation of genes related to anti-oxidant protection, detoxification, and inflammation (44–46). The role of NRF2 in anti-oxidant defense has been demonstrated in activated macrophages, where it controls the expression of oxidative stress-inducible proteins, including heme oxygenase-1 (44), sequesterome 1 (SQSTM1/A170/p62) (47), peroxiredoxin (44), etc. The critical role of NRF2 in anti-oxidant defense is demonstrated by the fact that macrophages deficient in NRF2 are rendered more vulnerable to toxic electrophiles (44).

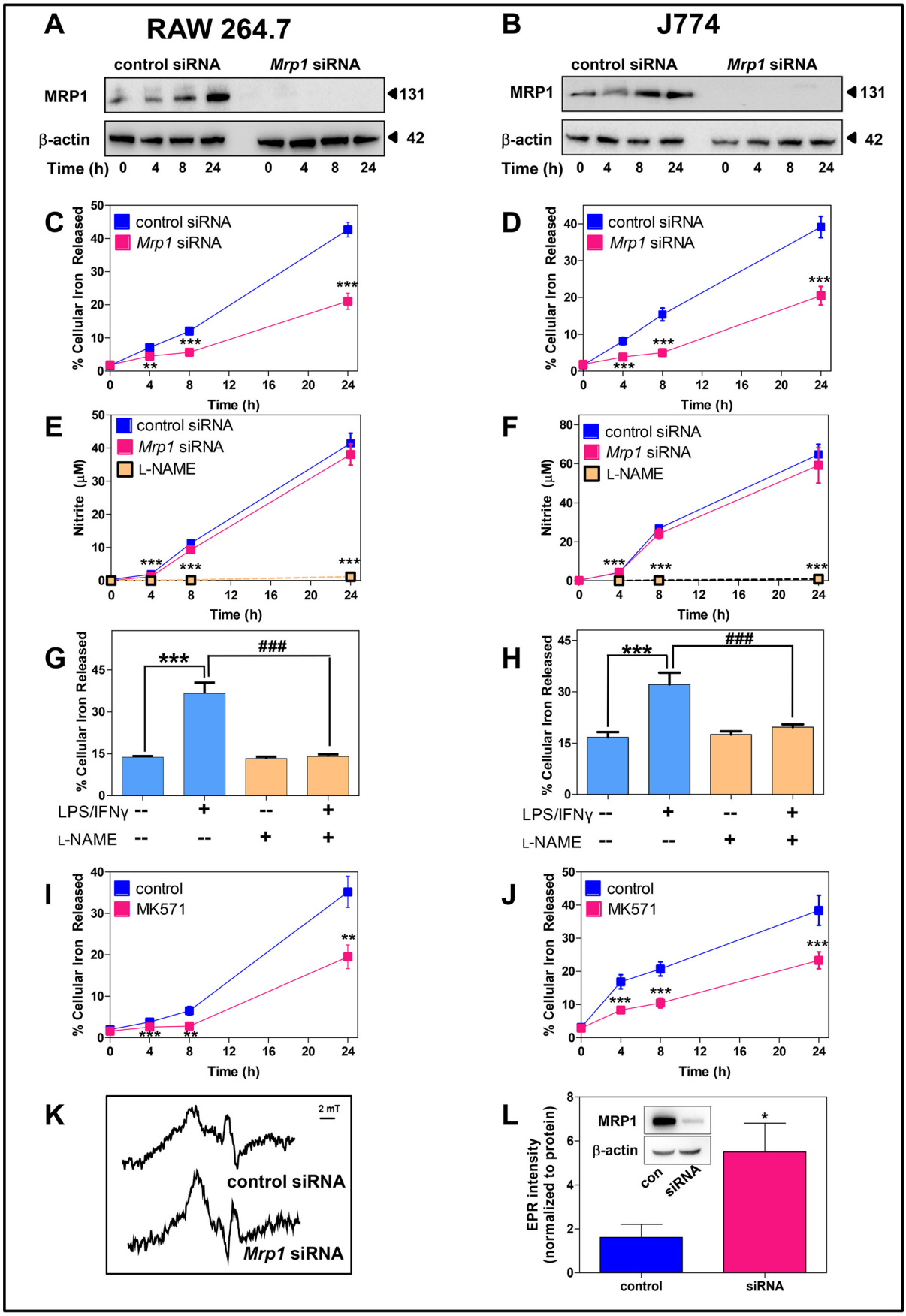
Considering the role of NRF2 as the "master regulator" of anti-oxidant defense and its positive regulation by NO (43), it was important to examine whether the GSTP1 and MRP1 storage and transport system for DNICs is part of the NRF2-controlled cellular defense mechanism against elevated endogenous NO in activated macrophages. For the first time, we demonstrate the existence of the GSTP1/MRP1 integrated storage and transport system in two well characterized murine macrophage models (*i.e.* J774 and RAW 264.7 cells) and its crucial role in preventing self-inflicted NO-mediated cytotoxicity.

Results

NO-induced ^{59}Fe Release from Activated RAW 264.7 and J744 Macrophages Is Markedly Reduced by Mrp1 Silencing—To evaluate the role of MRP1 in ^{59}Fe release from activated macrophages, we used the RAW 264.7 and J774 cell types, which become activated and generate NO via iNOS after incubation with LPS (100 ng/ml) and IFNγ (50 units/ml) (Fig. 1, *A–L*) (48–52). Initially, to assess the role of MRP1 in NO-mediated ^{59}Fe release from macrophages, RAW 264.7 or J774 cells were transiently transfected with *Mrp1* siRNA or, alternatively, control siRNA with no sequence homology to MRP1. These cells were then incubated for 24 h at 37 °C with ^{59}Fe-transferrin (^{59}Fe-Tf; 0.75 μM) to physiologically label intracellular iron pools (22, 23, 53, 54). After this, ^{59}Fe-labeled cells were then subsequently stimulated by incubation for up to 24 h at 37 °C with LPS (100 ng/ml) and IFNγ (50 units/ml), and ^{59}Fe release was assessed during this incubation at 0, 4, 8, and 24 h at 37 °C. The generation of nitrite (a product of NO oxidation) as a function of time was simultaneously measured as an indication of iNOS activation (49).

Initially, to assess MRP1 expression under these conditions, immunoblotting was performed (Fig. 1, *A* and *B*). When RAW 264.7 and J774 macrophages were activated with LPS and IFNγ (hereafter referred to as the "activated" RAW 264.7 and J774 macrophages) and transfected with negative control siRNA, there was a time-dependent increase in MRP1 expression (Fig. 1, *A* and *B*). These observations regarding MRP1 were consistent with the studies of Silverstein *et al.* using RAW 264.7 macrophages (55). However, these authors did not assess the role of MRP1 in the integrated GSTP1-MRP1 NO storage and transport system. Transfection of RAW 264.7 and J774 macrophages with *Mrp1* siRNA almost totally inhibited MRP1 expression at all time points (Fig. 1, *A* and *B*).

Assessing ^{59}Fe efflux under these conditions, both cell types transfected with control siRNA exhibited a time-dependent increase in cellular ^{59}Fe release (Fig. 1, *C* and *D*), indicating that activation with LPS and IFNγ induced cellular ^{59}Fe mobilization. However, the stimulatory effect of LPS and IFNγ on ^{59}Fe efflux was markedly and significantly ($p < 0.001–0.01$) attenuated in cells transfected with *Mrp1* siRNA relative to the control siRNA at all time points (*i.e.* 4–24 h at 37 °C; Fig. 1, *C* and *D*). Thus, as demonstrated for a variety of tumor cell types after exogenous or endogenous NO generation (22, 23), these results for the first time indicate that ^{59}Fe release from activated macrophages occurs, at least in part, via MRP1. Significantly, this effect was also observed using another specific *Mrp1* siRNA (see under "Experimental Procedures"), which demonstrated comparable suppression of both MRP1 and ^{59}Fe efflux (data not shown).


A
RAW 264.7
control siRNA
Mrp1 siRNA
MRP1
β-actin
131
42
Time (h) 0 4 8 24 0 4 8 24
B
J774
control siRNA
Mrp1 siRNA
MRP1
β-actin
131
42
Time (h) 0 4 8 24 0 4 8 24
C
% Cellular Iron Released
control siRNA
Mrp1 siRNA
Time (h)
D
% Cellular Iron Released
control siRNA
Mrp1 siRNA
Time (h)
E
Nitrite (μM)
control siRNA
Mrp1 siRNA
L-NAME
Time (h)
F
Nitrite (μM)
control siRNA
Mrp1 siRNA
L-NAME
Time (h)
G
% Cellular Iron Released
LPS/IFNγ
L-NAME
H
% Cellular Iron Released
LPS/IFNγ
L-NAME
I
% Cellular Iron Released
control
MK571
Time (h)
J
% Cellular Iron Released
control
MK571
Time (h)
K
2 mT
control siRNA
Mrp1 siRNA
L
EPR intensity (normalized to protein)
MRP1
β-actin
con
siRNA
control
siRNA

To ascertain that the decrease observed in ^{59}Fe efflux from the *Mrp1* siRNA-treated cells (Fig. 1, *C* and *D*) was a direct result of the suppression of MRP1 expression, and not due to inadvertent and nonspecific inhibition of iNOS, the generation of nitrite in the overlying media of RAW 264.7 and J774 cells was evaluated (Fig. 1, *E* and *F*). Importantly, despite the pronounced decrease of ^{59}Fe efflux from cells incubated with *Mrp1* siRNA (Fig. 1, *C* and *D*), there was no significant ($p > 0.05$) difference in nitrite levels between the control siRNA and *Mrp1* siRNA-treated cells (Fig. 1, *E* and *F*). This observation suggested that iNOS activity was unaffected by *Mrp1* silencing, a conclusion also supported by immunoblotting studies that demonstrated that *Mrp1* silencing did not significantly ($p > 0.05$) affect iNOS expression (see below and Fig. 2, *A* and *B*).

To determine the effect of endogenously generated NO on nitrite generation, studies were conducted using activated RAW 264.7 and J774 macrophages co-incubated with the well characterized NOS inhibitor, L-N^G-nitroarginine methyl ester hydrochloride (L-NAME; 4 mM) (56, 57) and LPS/IFNγ for 4–24 h at 37 °C (Fig. 1, *E* and *F*). In these experiments, L-NAME markedly and significantly ($p < 0.001$) abrogated the ability of activated RAW 264.7 (Fig. 1*E*) and J774 (Fig. 1*F*) to generate nitrite. These results indicate the nitrite detected in the supernatants from activated macrophages was NOS-dependent and that *Mrp1* silencing did not affect iNOS activity.

To assess the role of NO in the ^{59}Fe release observed from activated RAW 264.7 and J774 macrophages (Fig. 1, *C* and *D*), these cells were co-incubated in the presence and absence of L-NAME (4 mM) and/or LPS/IFNγ for 24 h at 37 °C (Fig. 1, *G* and *H*). Incubation with L-NAME led to a pronounced and significant ($p < 0.001$) suppression in the ability of activated RAW 264.7 and J774 macrophages to efflux ^{59}Fe (Fig. 1, *G* and *H*). These results demonstrate that activation of iNOS by LPS and IFNγ plays a role in NO-mediated ^{59}Fe efflux from these activated macrophages. Notably, in the absence of stimulation with LPS and IFNγ, ^{59}Fe release from both RAW 264.7 and J774 cells after a 24-h incubation was approximately half that observed from their activated counterparts (Fig. 1, *G* and *H*). Furthermore, this ^{59}Fe release under non-stimulated conditions could not be inhibited with L-NAME (Fig. 1, *G* and *H*). Hence, ^{59}Fe release can occur from the control untreated RAW 264.7 and J774 cells by another mechanism that is NO-independent. This could be due to iron efflux via another carrier in these non-activated macrophages that is independent of NO and has been reported to play a role in iron efflux in many other cell types (58–60).

As an additional assessment of the function of MRP1 in NO-mediated ^{59}Fe efflux from activated RAW 264.7 and J774 macrophages, studies then examined the effect of the specific MRP1 inhibitor, MK571 (Fig. 1, *I* and *J*) (61). Similar to the results observed with *Mrp1* siRNA shown in Fig. 1, *C* and *D*, incubation with MK571 (20 μM) resulted in a marked and significant ($p < 0.001$–0.01) decrease in ^{59}Fe efflux from both activated RAW 264.7 and J774 cells (Fig. 1, *I* and *J*), suggesting a role of MRP1 in ^{59}Fe mobilization. Collectively, these results in Fig. 1, *A–J,* demonstrate that MRP1 plays a role in NO-mediated ^{59}Fe efflux from both RAW 264.7 and J774 macrophages.

Mrp1 Silencing Leads to DNIC Accumulation in Activated RAW 264.7 Cells—Having examined the role of MRP1 in NO-mediated ^{59}Fe release from activated RAW 264.7 and J774 macrophages (Fig. 1, *A–J*), it was important to determine the role of this transporter in intracellular DNIC distribution. Previous EPR studies from our laboratories demonstrated that inhibition of MRP1 transport activity by MK571 and other inhibitors of this protein led to DNIC accumulation in tumor cells incubated with exogenous NO donors (22, 23). Notably, EPR performed at 77 K provides a definitive "atomic fingerprint" of the presence of DNICs within cells (19).

Thus, considering that activated macrophage-generated NO elicits ^{59}Fe release via MRP1 (Fig. 1, *A–J*), EPR spectroscopy was used to examine whether siRNA-mediated silencing of MRP1 expression in macrophages results in intracellular DNIC accumulation upon stimulation with LPS and IFNγ (Fig. 1, *K* and *L*). Briefly, in these studies, RAW 264.7 cells transiently transfected with siRNA specific for *Mrp1* or control siRNA were stimulated using LPS and IFNγ for 16 h at 37 °C, before being harvested and analyzed using EPR at 77 K. Both the *Mrp1* siRNA and control siRNA-treated RAW 264.7 cells exhibited the typical EPR signal at $g = 2.04$ that is unique for DNICs (Fig. 1*K*) (18, 19). However, importantly, the intensity of the signal in cells transfected with *Mrp1* siRNA was significantly ($p < 0.05$) greater when compared with the cells treated with control siRNA (Fig. 1, *K* and *L*). These results are consistent with suppression of MRP1 expression (Fig. 1*L, inset*) preventing DNIC transport out of cells, leading to a decrease in ^{59}Fe efflux (Fig. 1*C*), which then results in intracellular DNIC accumulation (Fig. 1*L*). Similar results were observed with activated J774 macrophages (data not shown). Hence, these EPR studies dem-

FIGURE 1. **MRP1 mediates NO-induced ^{59}Fe release from activated RAW 264.7 and J774 macrophages.** RAW 264.7 cells (*A*) and J774 cells (*B*) were transiently transfected with siRNA specific for *Mrp1* or control siRNA, and MRP1 levels were assessed by Western blotting analysis. RAW 264.7 cells (*C*) and J774 cells (*D*) were transiently transfected with *Mrp1* siRNA or control siRNA. The cells were then labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and re-incubated with media containing LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C, and cellular ^{59}Fe release was assessed. Results are mean ± S.D. (three experiments). RAW 264.7 cells (*E*) and J774 cells (*F*) as treated in *C* and *D* and nitrite were measured in the overlying media as a function of incubation time (4–24 h at 37 °C). As a negative control, cells treated with control siRNA were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C h in the presence or absence of the NOS inhibitor, L-NAME (4 mM). Concentrations of nitrite in the incubation media were then determined (see "Experimental Procedures"). RAW 264.7 cells (*G*) and J774 cells (*H*) were labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and re-incubated with media containing LPS (100 ng/ml) and IFNγ (50 units/ml) in the presence or absence of L-NAME (4 mM) for 24 h at 37 °C. The release of ^{59}Fe from cells was then determined as described under "Experimental Procedures." RAW 264.7 cells (*I*) and J774 cells (*J*) were labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and incubated with media in the presence or absence of the MRP1 inhibitor, MK571 (20 μM), for 30 min at 37 °C. Subsequently, the media were aspirated, and the cells were re-incubated with media containing LPS (100 ng/ml) and IFNγ (50 units/ml) in the presence or absence of MK571 (20 μM) for 0, 4, 8, and 24 h at 37 °C, and the release of cellular ^{59}Fe was assessed. Results are mean ± S.D. (three experiments). *K,* low temperature (77 K) EPR spectra of RAW 264.7 (10^{10} cells) transiently transfected with *Mrp1* siRNA or control siRNA that were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 16 h at 37 °C. *L,* quantification of EPR signals from *K*. EPR signal intensity is represented as normalized peak height in arbitrary units. The *inset* in *L* demonstrates MRP1 protein expression in cells transfected with *Mrp1* siRNA compared with control siRNA. Results are typical blots from three experiments, and the quantification represents mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control; ###, $p < 0.001$ relative to LPS/IFNγ.

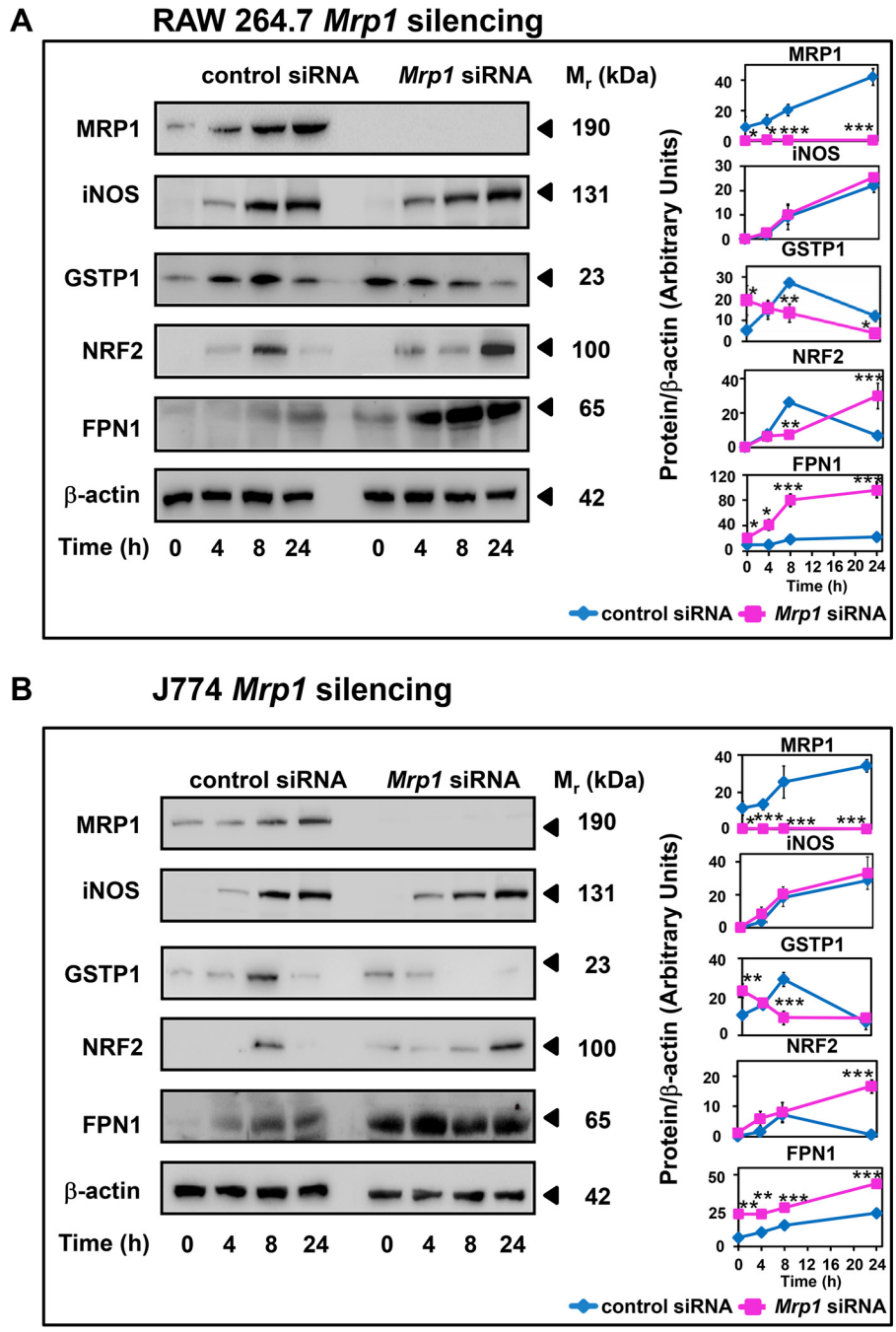


FIGURE 2. **Silencing *Mrp1* has no effect on iNOS expression but leads to alterations in GSTP1, FPN1, and NRF2 protein expression in activated macrophages.** RAW 264.7 cells and J774 cells were transiently transfected with siRNA specific for *Mrp1* or control siRNA. The cells were then stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C before the cells were harvested. Western blotting analyses were performed for RAW 264.7 cells (*A*) and J774 cells (*B*) to assess the expression of MRP1, iNOS, GSTP1, NRF2, and FPN1. Blots are typical of three experiments, and densitometry is mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

onstrate that MRP1 plays a role in the efflux of the DNIC, which consists of iron and NO. Taken together, these studies in Fig. 1, *A–L*, demonstrate that endogenous NO generated by activated macrophages leads to the formation of intracellular DNICs and that MRP1 plays an important role in their release out of these cells resulting in ^{59}Fe efflux.

Silencing Mrp1 Expression Has No Effect on LPS and IFNγ-induced iNOS Activation—In Fig. 1, *C* and *D*, we demonstrated that silencing of MRP1 expression resulted in substantially reduced ^{59}Fe release from activated macrophages without affecting nitrite production (Fig. 1, *E* and *F*). Considering these results, we further examined the mechanism involved in endogenous NO-mediated ^{59}Fe efflux from activated macrophages. As iNOS is a crucial enzyme involved in NO generation, we examined the effect of *Mrp1* silencing on iNOS protein expression in RAW 264.7 (Fig. 2*A*) and J774 (Fig. 2*B*) macrophages activated over a period of 4–24 h at 37 °C.

As demonstrated in Fig. 1, *A* and *B*, in cells incubated with control siRNA, MRP1 expression increased as a function of activation time up to 24 h, while MRP1 expression was totally

ablated by *Mrp1* siRNA at all time points in RAW 264.7 and J774 cells (Fig. 2, *A* and *B*). In agreement with the increased nitrite levels in activated *Mrp1*-treated and control siRNA-treated RAW 264.7 and J774 cells (Fig. 1, *E* and *F*), there was also a comparable increase in iNOS expression as a function of time under both conditions (Fig. 2, *A* and *B*). Notably, throughout all studies, iNOS expression peaked at either 8 or 24 h during activation, and this varied between individual experiments. Nonetheless, in Fig. 2, *A* and *B*, iNOS increased as a function of time up to 24 h in the presence of control or *Mrp1* siRNA. This clearly demonstrated that effective suppression of MRP1 expression by siRNA in RAW 264.7 and J774 macrophages does not significantly ($p > 0.05$) affect iNOS expression, or NO generation, as also indicated by nitrite production (Fig. 1, *E* and *F*).

Silencing Mrp1 Expression Alters GSTP1 Expression in Activated Macrophages—Our laboratory has previously shown that MRP1 can form an integrated NO transport and storage system with GSTP1 to protect human neoplastic cells from NO-mediated cytotoxicity (23, 62, 63). However, the mechanisms of interaction between these two molecules have not been examined in cells endogenously generating large quantities of NO, such as macrophages. Thus, in this study, the effect of *Mrp1* silencing on GSTP1 expression was assessed in RAW 264.7 and J774 macrophages.

Interestingly, the effect of incubation of both cell types with LPS and IFNγ on GSTP1 expression was different after *Mrp1* silencing relative to control siRNA-treated cells (Fig. 2, *A* and *B*). In the control siRNA-treated cells, GSTP1 expression peaked at 8 h of incubation with LPS and IFNγ and then decreased at the 24-h time point in both cell types. In contrast to control siRNA-treated cells, GSTP1 expression decreased steadily over the 24-h incubation in *Mrp1* siRNA-treated cells.

The most notable difference in GSTP1 expression between the control siRNA and *Mrp1* siRNA-treated cells was that GSTP1 levels were significantly ($p < 0.01–0.05$) greater at the 0-h time point after incubation with *Mrp1* siRNA (Fig. 2, *A* and *B*). This latter observation at 0 h suggests that a reciprocal relationship may exist between the expression of MRP1 and GSTP1 that could be important in terms of the roles of these partner proteins in detoxification (23, 26–28), *i.e.* silencing of *Mrp1*, which transports DNICs out of cells (22, 23), could lead to a compensatory increase in GSTP1 that binds and stores intracellular DNICs (23, 37). Evidence for such a relationship was also demonstrated in murine embryonic fibroblasts (MEFs) from *Mrp1* knock-out mice, where there was a marked and significant ($p < 0.001$) increase in GSTP1 expression relative to wild-type MEFs (data not shown). Conversely, MEFs from *Gstp1/2* knock-out mice demonstrate a pronounced and significant ($p < 0.001$) up-regulation of MRP1 relative to wild-type MEFs (data not shown).

Mrp1 Silencing Leads to a Delay in NRF2 Expression in Activated Macrophages—Apart from the cooperative coordinate expression observed between MRP1 and GSTP1, both these proteins are positively controlled by NRF2 (41, 64), a crucial redox-sensitive transcription factor that can be activated by NO (65). These previous findings support the hypothesis that MRP1 and GSTs are part of the cellular response to protect macrophages from NO-inflicted oxidative stress. Thus, it was important to examine the expression of NRF2 in *Mrp1*-silenced J774 and RAW 264.7 macrophages after activation with LPS and IFNγ.

For activated RAW 264.7 and J774 cells treated with control siRNA, NRF2 expression peaked after an 8-h incubation and then decreased to near basal levels at 24 h (Fig. 2, *A* and *B*). This increase in NRF2 levels is consistent with previous studies examining its expression after macrophage activation (66). In contrast, the increase in NRF2 expression in *Mrp1* siRNA-treated cells was delayed and did not peak at 8 h; instead expression continued to increase up to 24 h (Fig. 2, *A* and *B*). In the absence of MRP1, both activated RAW 264.7 cells and J774 cells displayed significantly ($p < 0.001$) higher levels of NRF2 after a 24-h incubation relative to cells treated with control siRNA (Fig. 2, *A* and *B*). This alteration to NRF2 in response to *Mrp1* silencing may suggest a change in redox balance due to altered NO and iron metabolism resulting from a disturbance in the GSTP1/MRP1 storage and transport system. Surprisingly, despite the delay and marked increase in NRF2 expression after a 24-h incubation in *Mrp1* siRNA-treated macrophages (Fig. 2, *A* and *B*), GSTP1, which is positively regulated by NRF2 (42), was decreased at 24 h relative to the 0-h time point. This observation suggests an additional NRF2-independent mechanism of regulating GSTP1.

Ferroportin 1 Expression Is Markedly Up-regulated in Response to Mrp1 Silencing—Considering the delayed up-regulation of NRF2 after *Mrp1* silencing in activated macrophages (Fig. 2, *A* and *B*), studies then assessed the effect of *Mrp1* siRNA on another NRF2 downstream target, namely ferroportin 1 (FPN1) (67, 68). Examination of FPN1 expression was of particular interest, as it is up-regulated by NRF2 (67, 68) and NO (69) and is a well known iron exporter (70). In these studies, RAW 264.7 cells and J774 cells treated with control siRNA displayed a time-dependent increase in FPN1 expression when stimulated with LPS and IFNγ (Fig. 2, *A* and *B*). However, after *Mrp1* silencing, there was a pronounced and significant ($p < 0.001–0.05$) increase in FPN1 expression relative to the control siRNA-treated cells at all time points. In fact, after a 24-h incubation, FPN1 expression was 1.6–4.4-fold greater ($p < 0.001$) in *Mrp1*-silenced cells relative to those treated with control siRNA (Fig. 2, *A* and *B*). Importantly, it is notable that despite the pronounced increase in FPN1 expression after *Mrp1* silencing (Fig. 2, *A* and *B*), ^{59}Fe efflux was decreased in these activated macrophages (Fig. 1, *C* and *D*). These data again suggest that ^{59}Fe release is mediated by MRP1 and not FPN1 in these NO-generating cells.

In conclusion, the results in Fig. 2 indicate that *Mrp1* silencing in macrophages results in no change in iNOS expression but leads to increased NRF2 and FPN1 levels. Furthermore, despite the marked increase in FPN1 expression after *Mrp1* silencing, ^{59}Fe release was decreased, indicating the critical role of MRP1 in NO-mediated ^{59}Fe efflux.

Gstp1 Silencing Leads to Marked Up-regulation of MRP1 Protein Expression in Activated Macrophages—The data in Fig. 2 demonstrate that *Mrp1* silencing altered the levels and kinetics of GSTP1 expression in activated RAW 264.7 and J774 macrophages. Considering these results, studies then assessed the effect of silencing *Gstp1* in these cell types (Fig. 3, *A* and *B*). As

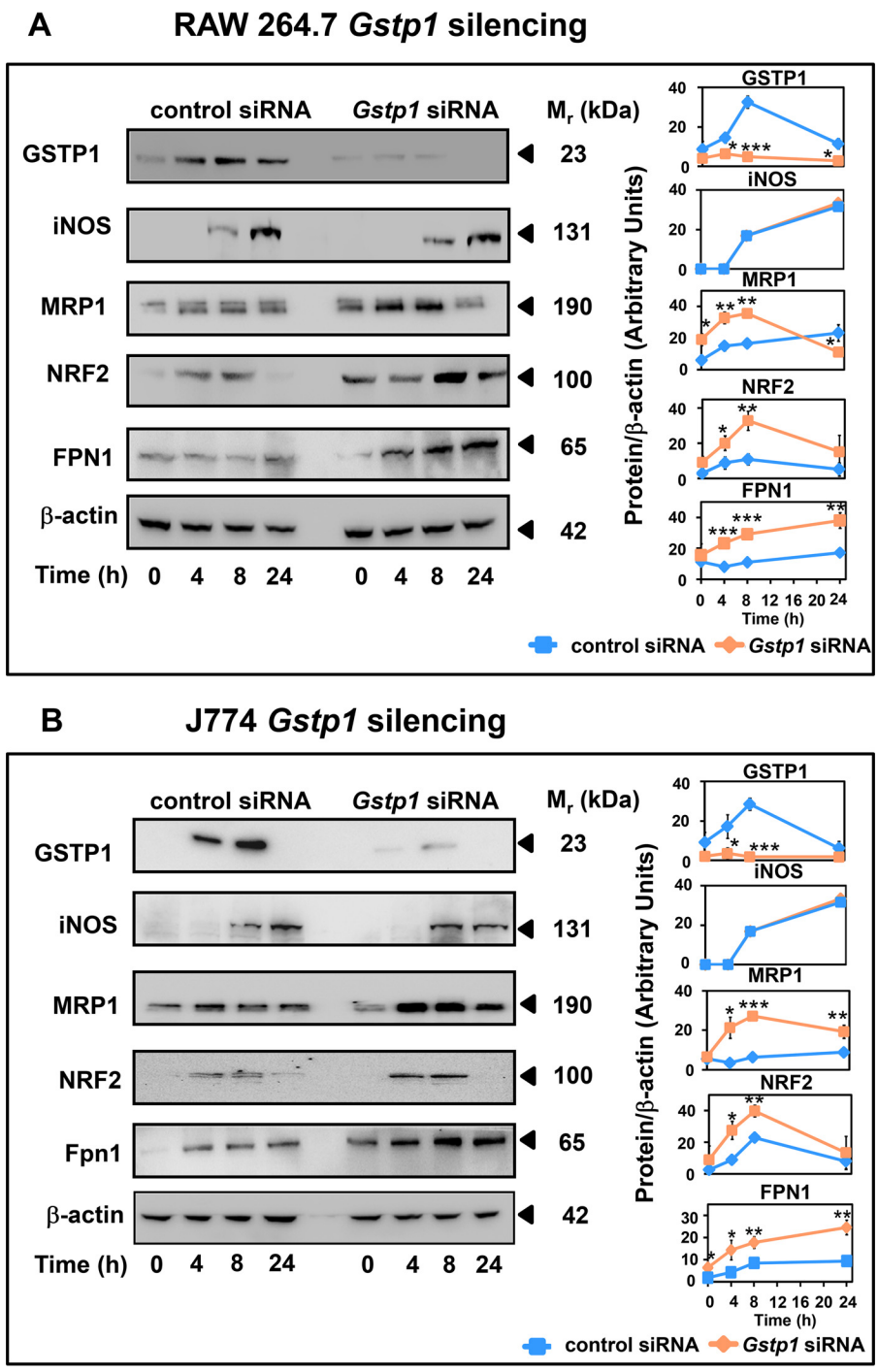


FIGURE 3. ***Gstp1* silencing in activated macrophages leads to increased MRP1, NRF2, and FPN1 expression.** RAW 264.7 cells and J774 cells were transiently transfected with siRNA specific for *Gstp1* or control siRNA. The cells were then stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C before the cells were harvested. Western blotting analyses were done for RAW 264.7 cells (*A*) and J774 cells (*B*) to assess the expression of GSTP1, iNOS, MRP1, NRF2, and FPN1. Blots are typical of three experiments, and densitometry is mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

shown in Fig. 2, *A* and *B*, after incubation with LPS and IFNγ, GSTP1 expression increased as a function of time up to an 8-h incubation and then decreased in the control siRNA-treated cells (Fig. 3, *A* and *B*). However, silencing *Gstp1* resulted in a marked and significant ($p < 0.001–0.05$) decrease in its expression after 4 and 8 h in both cell types, but this did not significantly ($p > 0.05$) affect iNOS expression relative to treatment with control siRNA (Fig. 3, *A* and *B*). In contrast, *Gstp1* silencing significantly ($p < 0.001–0.05$) increased MRP1 expression relative to control siRNA-treated cells up to an 8-h incubation and then decreased. These latter data further demonstrate the inverse relationship between GSTP1 and MRP1 expression shown in Fig. 2, *A* and *B*.

Similarly to *Mrp1* silencing, *Gstp1* silencing in both macrophage cell types led to a significant ($p < 0.01–0.05$) increase in NRF2 protein expression at 4 and 8 h, suggesting alterations in

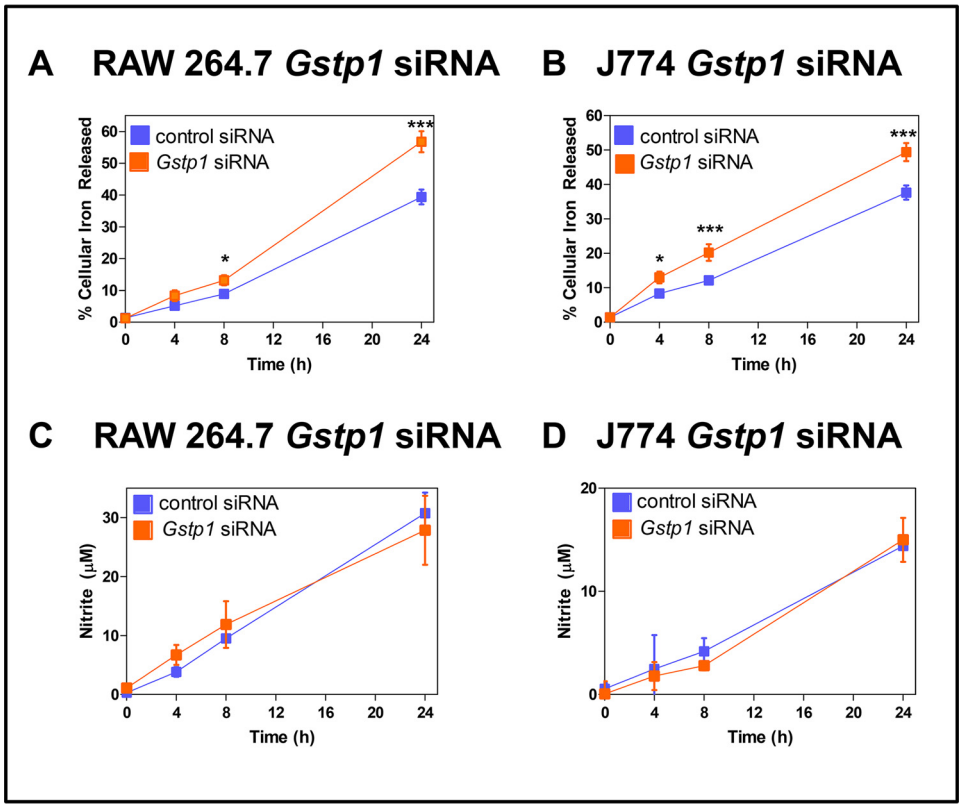


FIGURE 4. ***Gstp1* silencing in activated macrophages leads to increased ^{59}Fe release but does not affect nitrite production.** RAW 264.7 cells (*A*) and J774 cells (*B*) were transiently transfected with *Gstp1* siRNA or control siRNA. The cells were then labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then re-incubated with control media containing LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C, and ^{59}Fe release was assessed. RAW 264.7 cells (*C*) and J774 cells (*D*) were transfected with siRNA specific for *Gstp1* or control siRNA and were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h, and the presence of nitrite in the incubation media was determined. Results are mean ± S.D. (three experiments). *, $p < 0.05$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

GSTP1 expression led to changes in cellular redox state potentially due to the loss of DNIC storage capacity in GSTP1 (23, 37). Interestingly, similarly to *Mrp1* silencing (Fig. 2, *A* and *B*), *Gstp1* silencing significantly ($p < 0.001–0.05$) increased FPN1 expression relative to control siRNA-treated cells from 4 to 24 h (Fig. 3, *A*, and *B*). In summary, these data demonstrate the inverse relationship between GSTP1 and MRP1 expression. It is also relevant to note that as found for *Mrp1* silencing, both FPN1 and NRF2 expression were up-regulated by *Gstp1* silencing.

Gstp1 Silencing Results in Enhanced ^{59}Fe Efflux from Activated Macrophages—In this study, we showed that endogenous NO generated by iNOS in activated macrophages elicits ^{59}Fe release via MRP1 (Fig. 1, *C–J*) and that MRP1 expression is enhanced by *Gstp1* silencing (Fig. 3, *A* and *B*). Interestingly, we previously demonstrated in neoplastic cells that GSTP1 directly binds DNICs, which then prevents NO-induced iron release via MRP1 (23). In contrast, *Gstp1* silencing reduces DNIC binding and results in increased iron release via MRP1 (23). These results and others (23) demonstrated that GSTP1 bound DNICs to inhibit their efflux via MRP1 from human tumor cells. Considering this, and the possible effect of *Gstp1* silencing in macrophages on iron release experiments, we then examined the effect of decreasing GSTP1 expression on cellular ^{59}Fe efflux (Fig. 4, *A* and *B*).

Examining RAW 264.7 and J774 cells after *Gstp1* siRNA treatment, there was a significant ($p < 0.001–0.05$) increase in ^{59}Fe efflux after a 8 to 24- and 4 to 24-h incubation with LPS and IFNγ, respectively, relative to control siRNA (Fig. 4, *A* and *B*). These results were in agreement with the decrease in GSTP1 expression and increased MRP1 levels after *Gstp1* silencing (Fig. 3, *A* and *B*). Furthermore, *Gstp1* silencing resulted in no significant ($p > 0.05$) alteration in nitrite generation by RAW 264.7 and J774 cells relative to the control siRNA (Fig. 4, *C* and *D*). This observation was directly consistent with the fact that *Gstp1* silencing did not significantly ($p > 0.05$) affect iNOS expression (Fig. 3, *A* and *B*). Together with the results in Fig. 1, the studies in Figs. 3 and 4 strongly support the hypothesis that the integrated GSTP1 and MRP1 storage and transport system for DNICs in tumor cells (23) is also functional in activated macrophages.

Nrf2 Silencing Leads to Increased iNOS Expression but a Marked Decrease in MRP1, GSTP1, and FPN1 Expression in Activated Macrophages—As mentioned previously, *Mrp1*, *Gstp1*, and *Fpn1* are downstream targets of the transcription factor NRF2 (41, 42, 67). Considering this in conjunction with the fact that *Mrp1* silencing led to an alteration in the kinetics and levels of NRF2 expression in activated macrophages (Fig. 2, *A* and *B*), studies then assessed the effect of *Nrf2* silencing on iNOS, MRP1, GSTP1, and FPN1 expression (Fig. 5, *A* and *B*). Interestingly, *Nrf2* silencing resulted in a marked and significant ($p < 0.001–0.05$) increase in iNOS expression after a 4 to 24- and 4 to 8-h incubation with LPS and IFNγ in RAW 264.7 and J774 cells, respectively, relative to control siRNA (Fig. 5, *A*

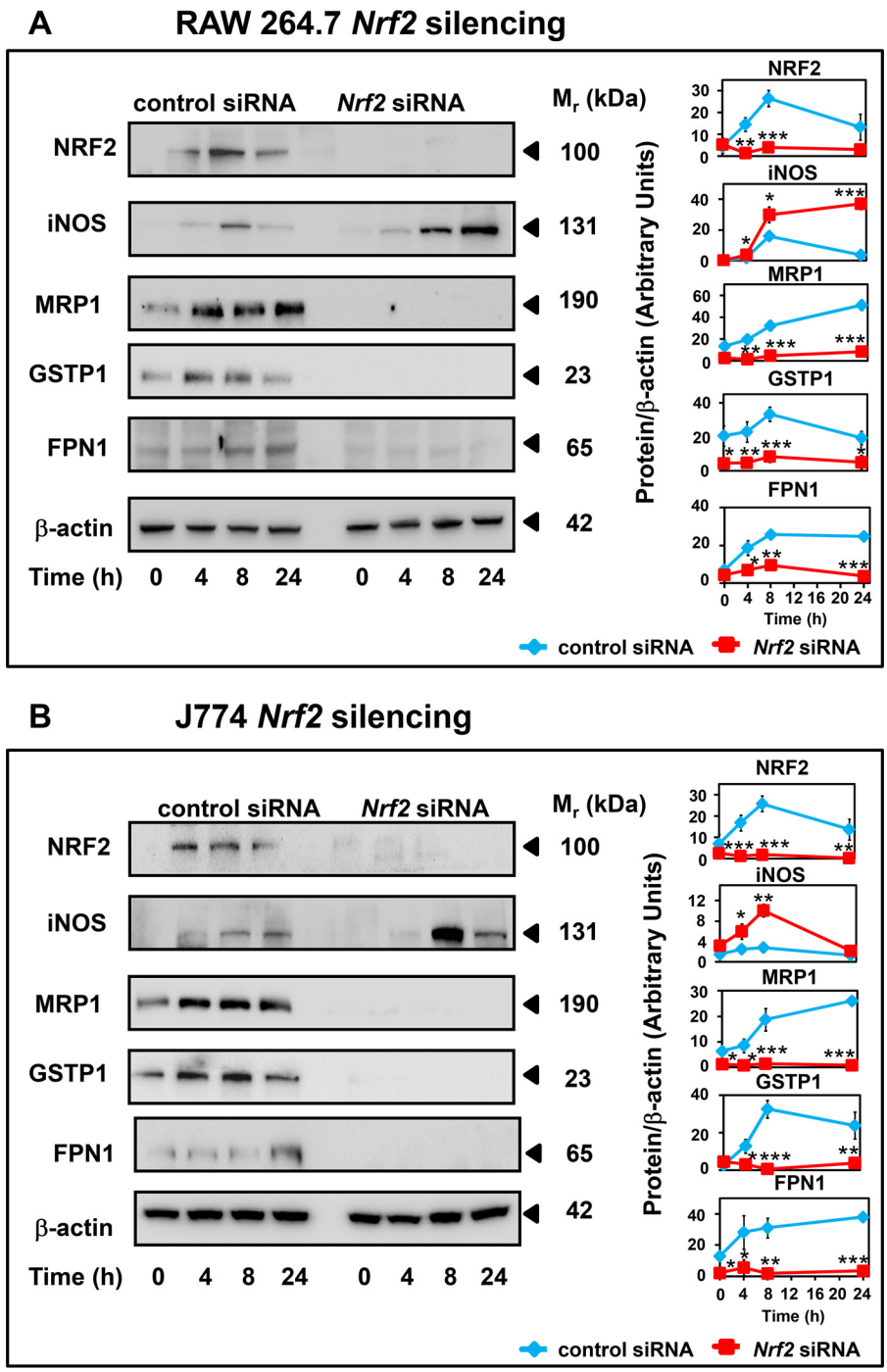


FIGURE 5. ***Nrf2* silencing in activated macrophages results in down-regulation of MRP1, GSTP1, and FPN1.** RAW 264.7 cells and J774 cells were transiently transfected with siRNA specific for *Nrf2* or control siRNA. The cells were then stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C before the cells were harvested. Western blotting analyses were done for RAW 264.7 (*A*) and J774 (*B*) cells for the assessment of NRF2, iNOS, MRP1, GSTP1, and FPN1 expression. Blots are typical of three experiments, and densitometry is mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

and *B*). A similar result has also been reported in alveolar macrophages from *Nrf2* knock-out mice (46, 71). In activated RAW 264.7 and J774 cells, silencing of *Nrf2* led to a marked and significant ($p < 0.001–0.05$) decrease in MRP1, GSTP1, and FPN1 expression relative to the control siRNA at incubations from 4 to 24 h (Fig. 5, *A* and *B*). These responses probably occurred as the genes encoding these proteins are downstream targets of NRF2 (41, 42, 67, 68).

Furthermore, considering that *Mrp1* or *Gstp1* silencing led to alterations in the kinetics and levels of NRF2 expression in macrophages (Figs. 2, *A* and *B*, and 3, *A* and *B*), these observations suggest interrelationships via the redox effects of NO. These data support the hypothesis that GSTP1 and MRP1 are part of the novel integrated NO storage and transport mechanism in macrophages that are expressed as part of the NRF2 response to redox imbalance.

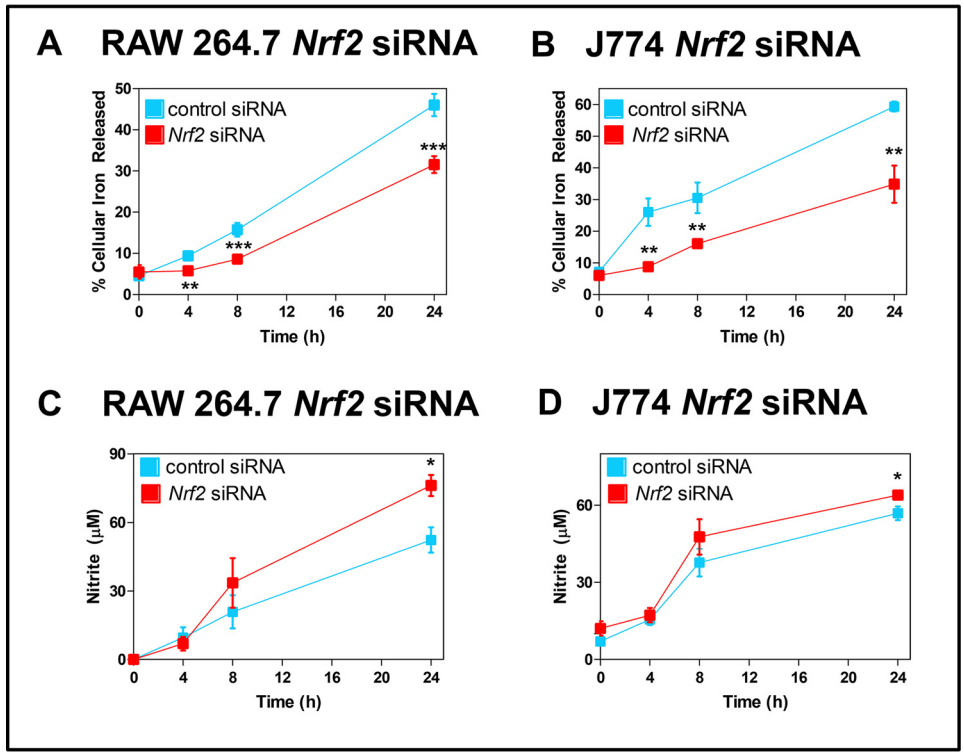


FIGURE 6. **Silencing of *Nrf2* in activated macrophages leads to decreased cellular ^{59}Fe release and increased nitrite production.** RAW 264.7 cells (*A*) and J774 cells (*B*) were transiently transfected with siRNA specific for *Nrf2* or control siRNA. The cells were then labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and re-incubated with control media containing LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C, and ^{59}Fe release into the overlying medium was assessed. Results are mean ± S.D. (three experiments). RAW 264.7 cells (*C*) and J774 cells (*D*) transfected with *Nrf2* siRNA or control siRNA were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C, and the presence of nitrite in the incubation media was determined. Results are mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

Nrf2 Silencing Results in a Decrease in ^{59}Fe Efflux and an Increase in Nitrite Production from Activated Macrophages—Considering that MRP1 and GSTs form an integrated NO transport and storage system (23, 62, 63) and that both are positively regulated by NRF2 (Fig. 5, *A* and *B*) (41, 42), it was important to examine the role of silencing *Nrf2* on cellular ^{59}Fe release in activated RAW 264.7 and J774 macrophages. In macrophages with suppressed NRF2 expression, the extent of ^{59}Fe efflux was significantly ($p < 0.001–0.01$) reduced after incubations with LPS and IFNγ from 4 to 24 h compared with control siRNA-treated cells (Fig. 6, *A* and *B*). This effect occurred despite the marked increase in iNOS levels in these activated RAW 264.7 and J774 macrophages (Fig. 5, *A* and *B*) and concomitant increase in nitrite production (Fig. 6, *C* and *D*). The decrease in ^{59}Fe efflux after *Nrf2* silencing (Fig. 6, *A* and *B*) is probably due to the reduction in MRP1 expression (Fig. 5, *A* and *B*) as this transporter is involved in NO-mediated iron release (22, 23, 70, 72), and silencing its expression or inhibiting its activity blocks NO-induced iron efflux (Fig. 1, *C, D, I,* and *J*). Moreover, the marked decrease in cellular ^{59}Fe release after *Mrp1* silencing occurred (Fig. 1, *C* and *D*) despite the pronounced up-regulation of FPN1 expression (Fig. 2, *A* and *B*), further suggesting that the iron exporter, FPN1, was not involved in NO-mediated ^{59}Fe release.

Inhibition of iNOS Suppresses Effects of LPS and IFNγ on MRP1, NRF2, FPN1, and GSTP1 in Macrophages—The studies above (Figs. 1- 3 and 5) demonstrated there was a time-dependent increase in the expression of MRP1, iNOS, NRF2, FPN1, and GSTP1 in control macrophages activated with LPS and IFNγ. To ascertain the role of iNOS activation and subsequent NO production in the expression of these proteins, studies were initiated using a well characterized NOS inhibitor, namely L-NAME (73). Notably, inhibition of iNOS activity by L-NAME (4 mM) resulted in a significant ($p < 0.001–0.05$) reduction in the time-dependent increase in MRP1, NRF2, FPN1, and GSTP1 expressions in both activated RAW 264.7 and J774 macrophages (Fig. 7, *A* and *B*). These studies showed that NO generation in these cell types plays a major role in the regulation of these proteins after incubation with LPS and IFNγ. However, it is notable that L-NAME did not significantly ($p > 0.05$) affect iNOS expression relative to the control (Fig. 7, *A* and *B*). This observation was in good agreement with other studies using rodent macrophages where inhibition of iNOS using L-NAME or N^G-monomethyl-L-arginine had no effect on its expression (74). Collectively, the results in Fig. 7, *A* and *B,* demonstrate an important role of iNOS activation and subsequent NO production by activated macrophages in the regulation of the integrated GSTP1/MRP1 NO storage and transport system.

Fpn1 Silencing Leads to Increased MRP1, GSTP1, and NRF2 Expression—As described earlier, apart from MRP1 and GSTP1, NRF2 is also known to regulate FPN1, a well known iron exporter (67, 68) that is up-regulated in the presence of NO (69). FPN1 is the major iron exporter (70), and in contrast, MRP1 has been implicated specifically in NO-induced iron efflux from tumor cells (22, 23) and herein from activated

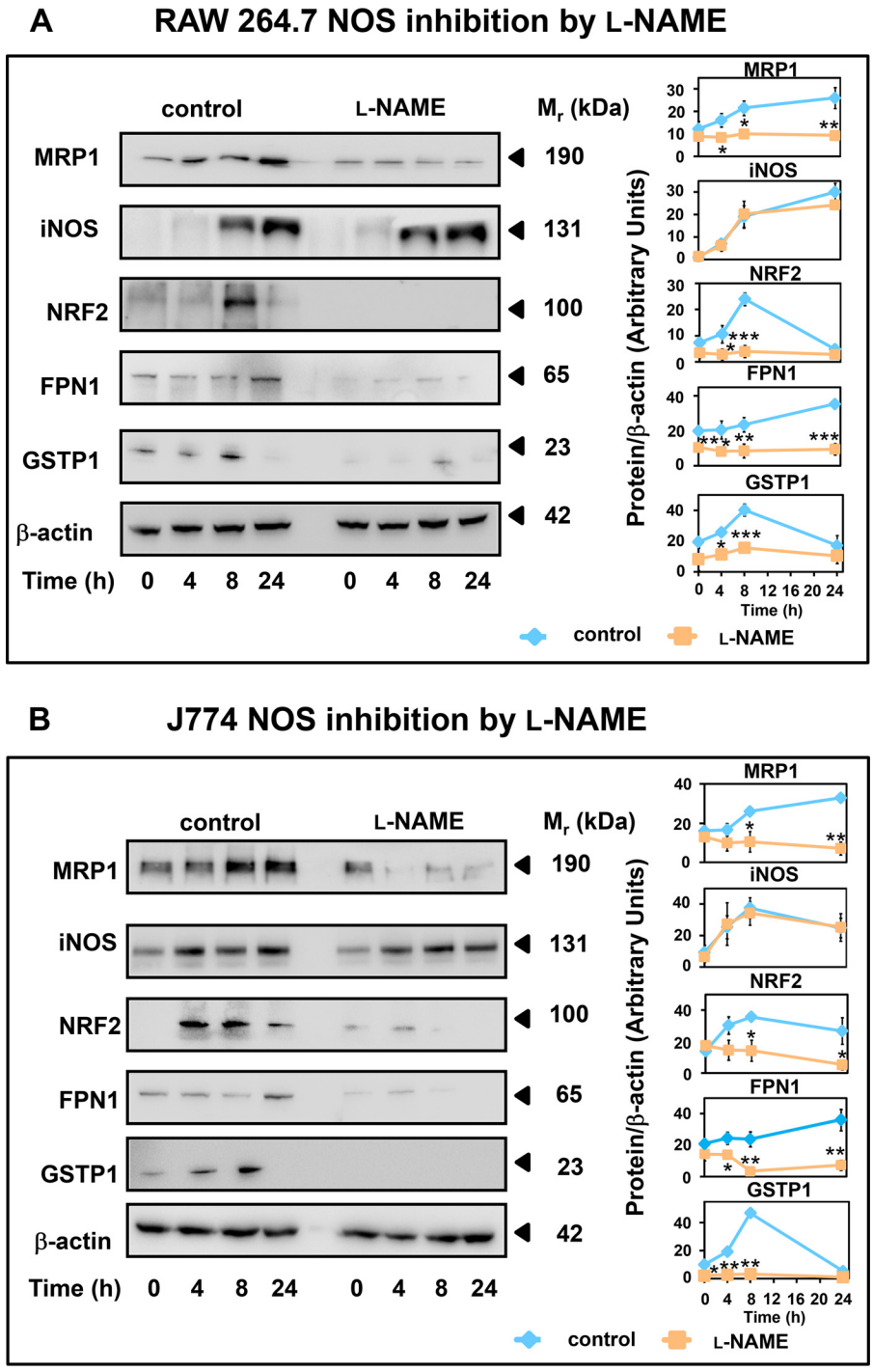


FIGURE 7. **Incubation of activated macrophages with the NOS inhibitor, L-NAME, decreases expression of MRP1, NRF2, FPN1, and GSTP1.** RAW 264.7 cells (*A*) and J774 cells (*B*) were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C in the presence or absence of L-NAME (4 mM). Western blotting analyses were performed to assess the expression of MRP1, iNOS, NRF2, FPN1, and GSTP1. Blots are typical of three experiments, and densitometry is mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control (no L-NAME) at the same time point.

macrophages (Fig. 1, *C, D, I,* and *J*). Hence, it was important to further dissect the relative roles of FPN1 and MRP1 in iron transport from LPS and IFNγ-activated macrophages. Furthermore, the effect of *Fpn1* silencing on the expression levels of GSTP1, NRF2, and iNOS was also examined (Fig. 8, *A* and *B*).

As shown in Figs. 2, 3, 5, and 7, FPN1 expression increased as a function of time after incubation with control siRNA in activated RAW 264.7 and J774 cells, whereas *Fpn1* silencing markedly and significantly ($p < 0.001–0.05$) reduced FPN1 expression from 0 to 24 h (Fig. 8, *A* and *B*). Interestingly, *Fpn1* silencing resulted in a significant ($p < 0.001–0.05$) increase in both MRP1 and GSTP1 levels at incubation times from 0 to 24 h (Fig. 8, *A* and *B*). In addition, NRF2 expression was also significantly ($p < 0.01–0.05$) up-regulated by *Fpn1* siRNA from 0 to 24 h relative to control siRNA-treated macrophages (Fig. 8, *A*

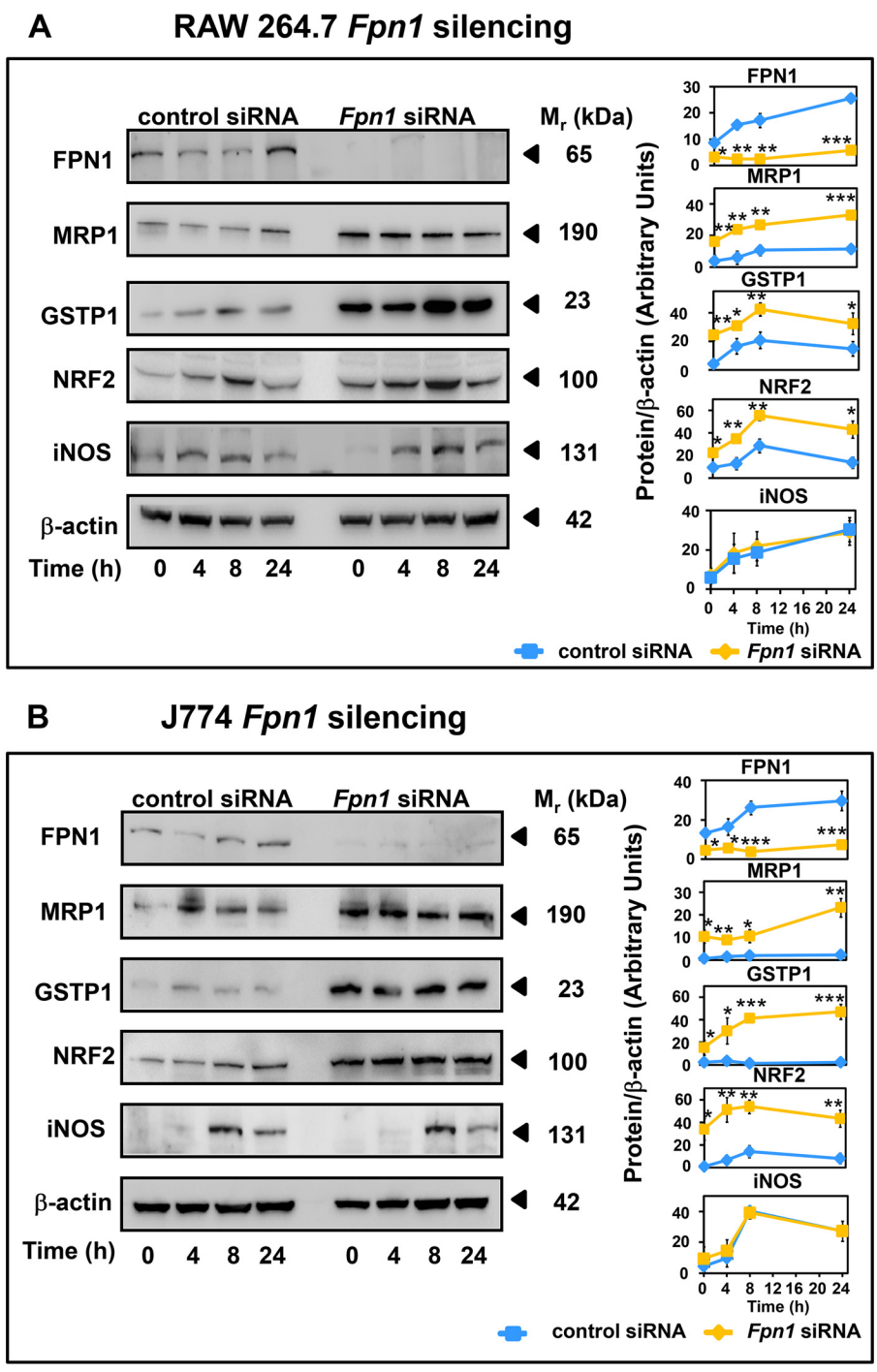


FIGURE 8. ***Fpn1* silencing in activated macrophages results in increased in MRP1, GSTP1, and NRF2 expression.** RAW 264.7 cells and J774 cells were transiently transfected with siRNA specific for *Fpn1* or control siRNA. The cells were then stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C before being harvested. Western blotting analyses were done for RAW 264.7 (*A*) and J774 (*B*) cells for the assessment of FPN1, MRP1, GSTP1, NRF2, and iNOS expression. Blots are typical of three experiments, and densitometry is mean ± S.D. (three experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the control siRNA at the same time point.

and *B*). In contrast, there was no significant ($p > 0.05$) alteration in iNOS expression between control and *Fpn1* siRNA-treated cells. In summary, *Fpn1* silencing suggested an inverse relationship between its expression and the levels of MRP1 and GSTP1.

FPN1 Is Not Involved in NO-induced ^{59}Fe Release from Activated Macrophages—There was no significant ($p > 0.05$) effect on cellular ^{59}Fe efflux after silencing *Fpn1* relative to control siRNA in activated RAW 264.7 and J774 macrophages (Fig. 9, *A* and *B*). Together with Fig. 1, *C*, *D*, *I*, and *J*, these data indicate that FPN1 is not responsible for NO-induced ^{59}Fe release from these activated macrophages. Critically, by assessing Fig. 8, *A* and *B*, it could be suggested that *Fpn1* silencing should lead to an increase in cellular ^{59}Fe release due to the increase in MRP1 expression. However, it is notable that *Fpn1* silencing also leads to marked up-regulation of GSTP1 (Fig. 8, *A* and *B*), and it is

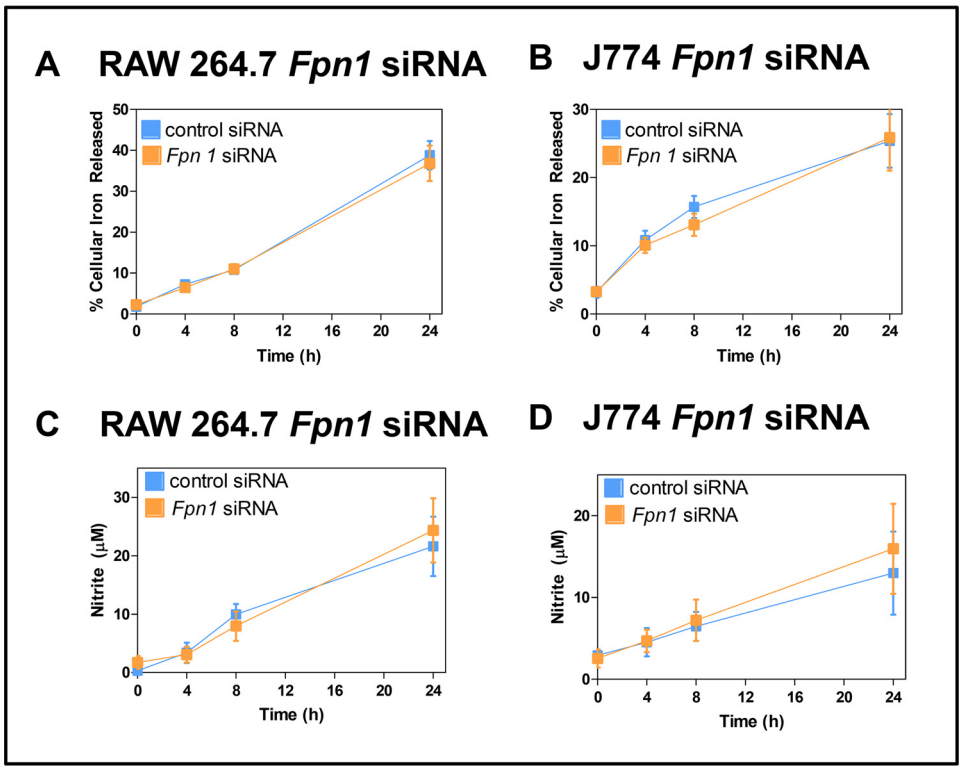


FIGURE 9. ***Fpn1* silencing in activated macrophages does not affect ^{59}Fe efflux or nitrite production.** RAW 264.7 cells (*A*) and J774 cells (*B*) were transiently transfected with siRNA specific for *Fpn1* or control siRNA. The cells were then labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and re-incubated with control media containing LPS (50 ng/ml) and IFNγ for 0, 4, 8, and 24 h at 37 °C, and cellular ^{59}Fe efflux into the overlying media was assessed. Results are mean ± S.D. (three experiments). RAW 264.7 cells (*C*) and J774 cells (*D*) transfected with *Fpn1* siRNA or control siRNA were stimulated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C, and the presence of nitrite in the incubation media was determined. Results are mean ± S.D. (three experiments).

known that GSTP1 expression inhibits NO-mediated ^{59}Fe efflux via MRP1 (Fig. 4, *A* and *B*) through the direct binding of DNICs (23, 37). Hence, the lack of an increase in cellular ^{59}Fe efflux after *Fpn1* silencing, despite the increase in MRP1 expression, could be due to the marked up-regulation of GSTP1 expression.

Notably, there were no significant ($p > 0.05$) differences in nitrite generation between cells treated with control siRNA or *Fpn1* siRNA (Fig. 9, *C* and *D*). These data are in agreement with the comparable iNOS expression between control siRNA and the *Fpn1* siRNA-treated RAW 264.7 and J774 macrophages in Fig. 8, *A* and *B*. Collectively, these data indicate that NO-induced ^{59}Fe efflux from macrophages is not via the iron transporter FPN1.

Mrp1 and Gstp1 Silencing Decrease Cellular Viability of Activated Macrophages—Considering the studies above demonstrating the crucial role of GSTP1 and MRP1 in NO storage and transport in activated RAW 264.7 and J774 macrophages, we then examined the effects of silencing their expression on cellular viability after activation (Fig. 10, *A–D*). Both activated RAW 264.7 and J774 cells transfected with siRNA specific for *Mrp1* displayed a significant ($p < 0.001$) 15–42% decrease in cellular viability after incubations of 4–24 h relative to cells incubated with control siRNA (Fig. 10, *A* and *B*).

A similar significant ($p < 0.001$) decrease in viability was observed after 4–24 h when both activated RAW 264.7 and J774 cells were transfected with siRNA specific for *Gstp1* relative to control siRNA (Fig. 10, *C* and *D*). Importantly, and in marked contrast, transfection of non-activated RAW 264.7 and J774 macrophages with *Mrp1* or *Gstp1* siRNA led to no significant ($p > 0.05$) change in cellular viability (results not shown), suggesting the role of NO in the cytotoxicity observed. Furthermore, co-incubation of activated macrophages with the NOS inhibitor, L-NAME, significantly ($p < 0.001–0.05$) abrogated the cytotoxic effect of silencing *Mrp1* or *Gstp1* (Fig. 10, *A–D*). In contrast, the addition of L-NAME to cells transfected with control siRNA had no significant ($p > 0.05$) effect on the viability of macrophages (data not shown).

Collectively, these results indicate the important role of MRP1 and GSTP1 in protecting activated macrophages against NO-mediated cytotoxicity. These data are also consistent with our previous investigation using human MCF7 breast cancer cells hyper-expressing GSTP1 and MRP1 that were resistant to exogenous NO relative to MCF7 cells expressing basal levels of these proteins (23).

Nrf2 Silencing Decreases Cellular Proliferation and Survival in Activated Macrophages—The results in Fig. 10, *A–D*, indicate MRP1 and GSTP1 play vital cyto-protective roles in activated RAW 264.7 and J774 macrophages. Considering these proteins were up-regulated upon LPS and IFNγ activation (Figs. 2, *A* and *B*, 3, *A* and *B*) and that NRF2 is known to transcriptionally induce MRP1 and GSTP1 (41, 42), studies then investigated the effect of *Nrf2* silencing on the survival of activated macrophages (Fig. 10, *E* and *F*). The silencing of NRF2 expression in activated RAW 264.7 and J774 macrophages led to a significantly ($p < 0.001$) lower percentage

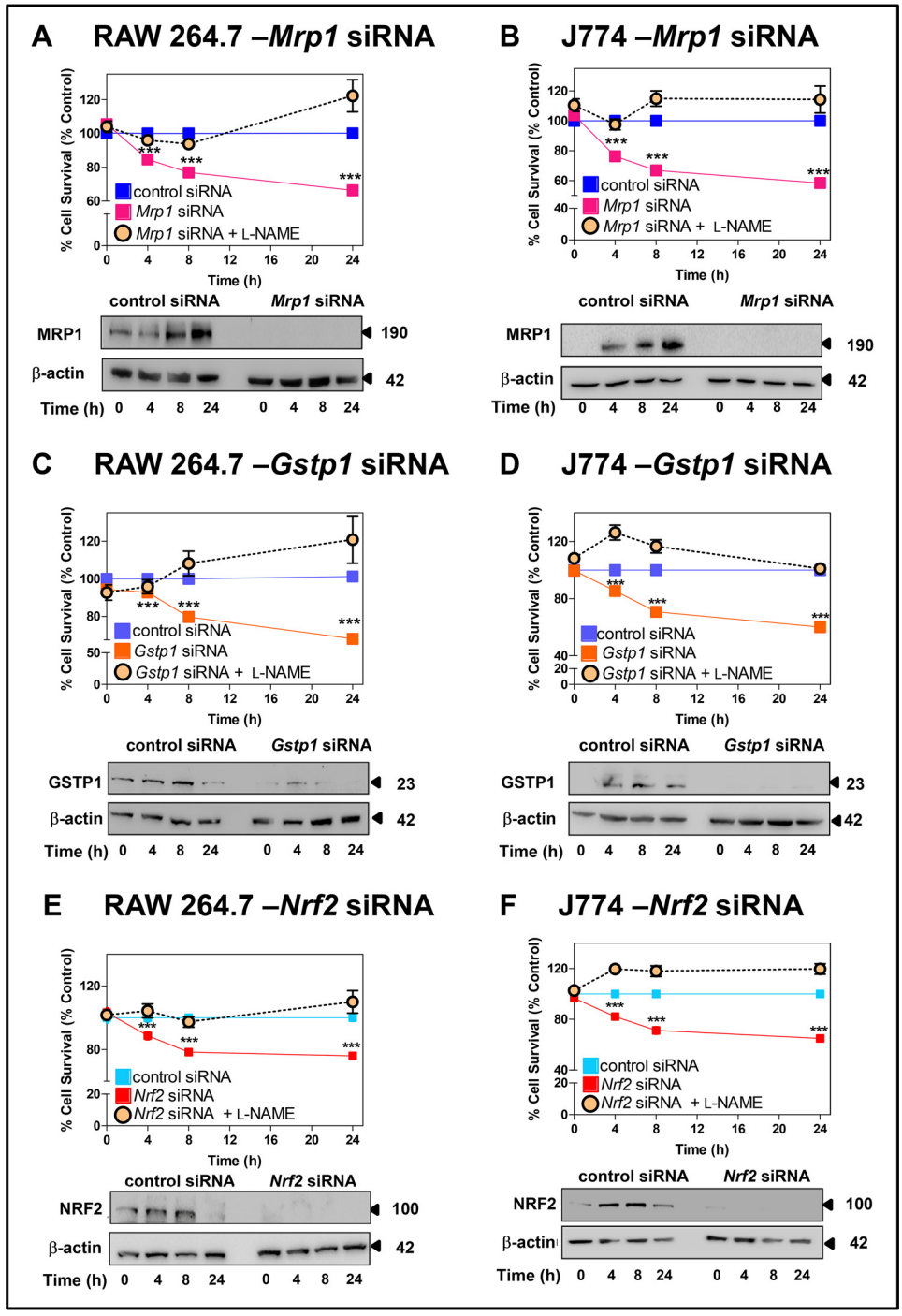


FIGURE 10. **Silencing of *Mrp1*, *Gstp1*, or *Nrf2* decreases activated macrophage viability.** RAW 264.7 (*A*) and J774 (*B*) cells were transiently transfected with control siRNA or *Mrp1* siRNA. Control siRNA or *Gstp1* siRNA was transiently transfected into RAW 264.7(*C*) and J774 (*D*) cells, although RAW 264.7 (*E*) and J774 (*F*) macrophages were transfected with *Nrf2* siRNA or control siRNA. The transfected cells were then incubated with media containing LPS (100 ng/ml) and IFNγ (50 units/ml) in the presence or absence of the NOS inhibitor L-NAME (4 mM) for 0, 4, 8, and 24 h at 37 °C. Cell survival was assessed using MTT assays validated by viable cell counts. Results are mean ± S.D. (three experiments). ***, $p < 0.001$ relative to the control siRNA at the same time point.

(60 – 80% of the control) of cellular viability after a 4 to 24-h incubation. Furthermore, the cytotoxic effect of silencing *Nrf2* in activated macrophages was ablated by the NOS inhibitor, L-NAME (Fig. 10, *E* and *F*), suggesting the cytotoxic role of NO in the decreased survival observed. Again, the addition of L-NAME to cells transfected with control siRNA had no significant ($p > 0.05$) effect on macrophage viability (data not shown).

These results demonstrate that NRF2 up-regulation in activated macrophages (Fig. 5) is part of the machinery to ensure cell survival. This pro-survival effect of NRF2 may be mediated, at least in part, via its role in up-regulating the downstream targets GSTP1 and MRP1 (Fig. 5). Taken together, these results in Fig. 10, *A–F,* demonstrate that NRF2 and its downstream targets, MRP1 and GSTP1, are important for macrophage survival after cellular activation.

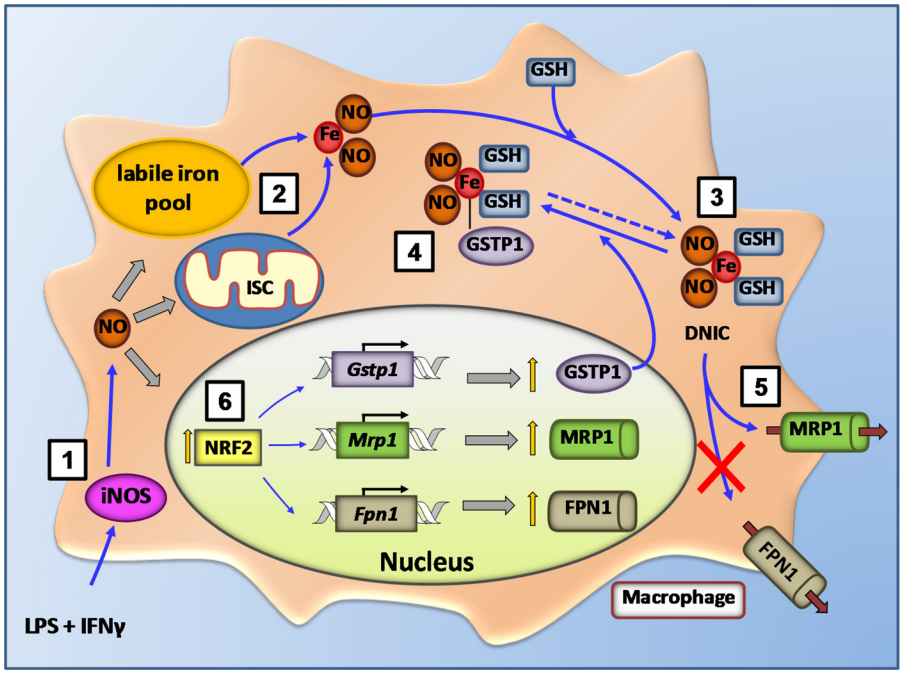


FIGURE 11. **Schematic illustrating the respective roles of NRF2, MRP1, and GSTP1 in cellular protection of activated macrophages against endogenous NO cytotoxicity.** *Step 1,* stimulation of macrophages by LPS and IFNγ leads to activation of iNOS, resulting in NO production. *Step 2,* because of the high affinity of NO for iron, it then binds iron from iron-sulfur cluster-containing proteins, or the labile iron pool, to form a coordination complex with GSH (*i.e.* DNIC; *step 3*). *Step 4*, notably, DNICs can be sequestered by GSTP1 and/or effluxed out of the cell via MRP1. *Step 5,* similarly, as found for cytotoxic drugs (26–28), GSTP1 and MRP1 form a coordinated unit in macrophages to prevent the cytotoxicity of NO as DNICs. *Step 6,* notably, NO can also activate *NRF2* expression (43), which in turn increases GSTP1 and MRP1 expression (41, 42) to inhibit NO cytotoxicity by binding DNICs or transporting them out of the macrophage, respectively.

Discussion

We have previously described a novel integrated NO storage and transport mechanism facilitated by GSTP1 and MRP1 that renders human neoplastic cells more resistant to the cytotoxic effects of NO (23). For the first time, we report the existence of this integrated NO storage and transport system in activated macrophages and its novel role in protecting these "professional killer" cells from endogenously generated NO (Fig. 11). Indeed, Hickok *et al.* (20) demonstrate that DNICs form the largest proportion of all NO adducts. Thus, appropriate mechanisms such those mediated by GSTP1 and MRP1 are critical to regulate intracellular DNIC levels and also prevent their cytotoxicity.

The function of this system in NO metabolism parallels the role of GSTs and MRP1 in acting as a coordinate unit that prevents the cytotoxicity of chemotherapeutics such as doxorubicin, etc., in cancer cells (27, 28). In this case, GSTs act to catalyze conjugation of GSH to xenobiotic substrates to effect detoxification (26–28, 34). The GSH conjugates can then be effectively transported out of the cell via MRP1 to prevent cytotoxicity (40). In terms of the integrated NO storage and transport system, GSTP1 can directly bind DNICs (composed of a complex of iron, NO, and GSH) via Tyr-7, which coordinates to the iron atom through its phenolate group by displacing a GSH ligand (37). Moreover, GSTP1 expression leads to the accumulation of intracellular DNICs that can prevent NO-mediated iron release via MRP1 (23).

It is well known that FPN1 plays a role in the release of iron from cells (70), but our current studies using macrophages and previous investigations implementing neoplastic cell types indicate that NO-induced iron release is different and occurs via MRP1 (22, 23). Indeed, evidence for a role of MRP1 in the integrated NO storage and transport system in activated macrophages includes the following: 1) increased MRP1 expression upon macrophage activation (Fig. 1, *A* and *B*); 2) the inhibition of NO-induced ^{59}Fe release by *Mrp1* siRNA or the specific MRP1 inhibitor MK571 (Fig. 1, *C, D, I,* and *J*); and 3) the accumulation of intracellular DNICs in activated macrophages upon silencing *Mrp1* (Fig. 1, *K* and *L*). It is also notable that DNICs accumulate intracellularly, and iron release is suppressed after *Mrp1* silencing despite marked up-regulation of the iron transporter FPN1 (Fig. 2, *A* and *B*), suggesting that FPN1 is not involved in NO-mediated iron release. Furthermore, silencing *Fpn1* in activated macrophages does not affect NO-mediated ^{59}Fe release (Fig. 9, *A* and *B*), demonstrating that this transporter is not involved in NO-mediated ^{59}Fe efflux from these cells.

Considering that both MRP1 and FPN1 are involved in iron efflux under different conditions, it is probable that the mechanisms involved in iron efflux by FPN1 and MRP1 are different. For instance, it well known that FPN1 is involved in cellular iron release (70), and recently it has been demonstrated that the chaperone protein, poly(rC)-binding protein 2 (PCBP2), is involved in transporting intracellular iron to FPN1 for release via a direct interaction with its C terminus (75). Moreover, MRP1 is known to transport glutathione conjugates (76), including DNICs composed of glutathione (22, 23). These differences in the mechanism of iron transport may explain the current observations regarding the efflux of DNICs by MRP1 rather than FPN1.

We previously showed that GSTP1 binds and stores DNICs intracellularly in several human tumor cell lines using a variety of techniques (23). Furthermore, silencing GSTP1 expression resulted in increased NO-induced ^{59}Fe release (23). This finding is in good agreement with studies demonstrating that DNICs composed of GSH directly bind to GSTP1 (37). Notably, in this study, silencing *Gstp1* in activated macrophages increased cellular ^{59}Fe efflux (Fig. 4, *A* and *B*), consistent with a role for GSTP1 in DNIC binding/storage in macrophages.

The coordinated role of MRP1 and GSTP1 expression in activated macrophages and other cell types is suggested by the compensatory up-regulation of GSTP1 when MRP1 expression was silenced and vice versa (Figs. 2, *A* and *B*, and 3, *A* and *B*). Furthermore, the importance of MRP1 and GSTP1 in preventing NO-mediated cytotoxicity in activated macrophages is exemplified by the fact that silencing either *Mrp1* or *Gstp1* by siRNA leads to decreased viability of activated macrophages (Fig. 10, *A–D*) but not their non-activated counterparts. The role of NO in inducing cytotoxicity after *Mrp1* or *Gstp1* silencing was demonstrated by using the NOS inhibitor L-NAME (56, 57).

Previous studies have examined the kinetics of DNIC formation in one of the macrophage cell types used in this investigation (RAW 264.7 macrophages) (20). Interestingly, there was an initial increase in intracellular DNIC formation in RAW 264.7 macrophages up to a 10-h incubation followed by a steady decrease up to 24 h (20). These kinetics of cellular DNIC levels are in accordance with our model of the GSTP1/MRP1 NO storage and transport system. In this case, the initial increase in DNICs is consistent with the intracellular reaction of NO with iron pools, although the slow decrease could be due to the efflux of excess DNICs by MRP1.

It is of interest to note that MRP1 and GSTP1 are both positively regulated by NRF2, the master regulator of redox balance in the cell (44–46, 77). Silencing of *Nrf2* prevented the expression of its downstream targets upon macrophage activation, including MRP1, GSTP1, and FPN1 (Fig. 5, *A* and *B*). This appeared to be a crucial cellular response, as silencing *Nrf2* decreased activated macrophage viability probably because of the lack of proteins such as MRP1 and GSTP1 that protect against NO-induced cytotoxicity by transporting and storing DNICs, respectively (Fig. 10, *A–D*) (23).

Silencing *Nrf2* also increased iNOS expression (Fig. 5, *A* and *B*), which is in agreement with studies examining *Nrf2* knock-out mice, which displayed enhanced iNOS production (46, 71). The increased iNOS in the *Nrf2* knockdown model has been attributed to the higher mortality rate in experimental sepsis (71) and hypersensitivity to LPS-induced neuroinflammation (46). These results confirm the importance of NRF2 in regulating cellular NO metabolism in macrophages and other cell types and its crucial role in up-regulating the integrated GSTP1 and MRP1 NO storage and transport system that protects these cells.

This study examining activated macrophages enabled a generalized model of NO and its interaction with iron to be proposed (Fig. 11). Activation of macrophages using LPS and IFNγ results in the induction of iNOS that leads to NO generation. The NO generated has been reported to result in the activation of NRF2 and the transcription of downstream effector targets, such as heme oxygenase 1 (78), NAD(P)H:quinone oxidoreductase 1, and glutamate-cysteine ligase (43). The high affinity of NO for iron centers in proteins such as iron-sulfur cluster-containing proteins (3) and potentially others, such as iron-chaperone proteins, including the poly(rC)-binding proteins, disturbs their normal functions (*e.g.* the delivery of iron from PCBP2 to FPN1 (75)) and results in DNIC formation that can be bound by GSTP1 (23, 37). The binding of DNICs by GSTP1 markedly increases the half-life of NO from less than a second to many hours (38) and could be important for maintaining a pool of bioavailable NO. It is also probable that the storage of DNICs within GSTP1 (37) prevents NO cytotoxicity (Fig. 10, *C* and *D*) (23). Excess DNIC not bound to GSTP1, or in equilibrium with GSTP1, could be released from cells via MRP1 (22, 23). Therefore, the effective storage and transport of DNICs by the integrated GSTP1 and MRP1 system are important in terms of preventing macrophage cytotoxicity (Fig. 10, *A–D*).

In conclusion, this investigation has demonstrated that MRP1 and GSTP1 form an integrated transport and storage system for NO as DNICs in activated macrophages (Fig. 11). This is the first demonstration of the physiological role of this storage and transport unit after endogenous NO generation that protects the macrophage from NO-mediated cytotoxicity.

Experimental Procedures

Tissue Culture—Murine J774.A1 (herein referred to as J774) and RAW 264.7 cells of the macrophage lineage were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All cell lines were maintained at 37 °C, in a humidified atmosphere of 5% CO_2. RAW 264.7 cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640; Life Technologies, Inc.) supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). In contrast, J774 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) with the same growth supplements as described for RAW 264.7 cells.

To activate RAW 264.7 and J774 cells, a standard protocol was implemented utilizing incubations from 4 to 24 h at 37 °C with media containing LPS (100 ng/ml; Sigma) and IFNγ (50 units/ml; Sigma) (48, 49). These incubation conditions have previously been shown to maximally induce iNOS, leading to robust NO generation (48, 49).

MEFs from *Mrp1* knock-out mice and their wild-type littermates were obtained from Prof. P. Borst (NKI-AVL, Amsterdam, Netherlands). MEFs from *Gstp1/2* knock-out mice and their wild-type counterparts were from Prof. K. Tew (Medical University of South Carolina, Charleston). Cells were grown in DMEM with the same supplements as described above for RAW 264.7 and J774 cells.

siRNA Studies—All siRNAs and the negative control siRNA (control siRNA; catalog no. AM4635) were purchased from Life Technologies, Inc. The siRNAs specific for *Mrp1* (catalog no. 4390826, ID S543; catalog no. 4390815, ID S69748), *Gstp1* (catalog no. 4390825, ID S194476), *Nrf2* (catalog no. 4392421, ID S9492), and *Fpn1* (catalog no. 4390815, ID S79377) and the

negative control siRNA (catalog no. AM4635) were used for gene silencing studies in RAW 264.7 macrophages and J774 macrophages. Cells were transiently reverse-transfected with siRNA (100 nM) using Lipofectamine RNAiMax (Life Technologies, Inc.) implementing a 24 to 48-h at 37 °C incubation period. After this, a 4 to 24-h at 37 °C incubation with LPS (100 ng/ml) and IFNγ (50 units/ml) was utilized, and the overlying medium from cell culture experiments was assayed for nitrite production. Relevant protein expression was assessed using Western blotting, as described below.

Western Blotting Analysis—Western blotting analysis was performed (79) using antibodies against GSTP1 (Medical and Biological Laboratories, Nagoya, Japan, catalog no. MBL 231), iNOS (Enzo Life Sciences, New York, catalog no. ADI-KAS-NO001-D), MRP1 (Alexis, San Diego, CA, catalog no. ALX-801-007-C250), NRF2 (Santa Cruz Biotechnology, Dallas, TX, catalog no. sc-722), FPN1 (Alpha Diagnostics, New York, catalog no. MTP11A), and β-actin (Sigma; catalog no. A1978). All primary antibodies were used at 1:1,000 dilutions, apart from β-actin, which was used at 1:5,000.

Secondary antibodies implemented included the following: horseradish peroxidase (HRP)-conjugated anti-rabbit (Sigma catalog no. A6154), anti-mouse (Sigma catalog no. A4416), and anti-rat (Sigma catalog no. A9037); all were used at 1:10,000. The membranes were developed using chemiluminescent detection (Luminata Crescendo Western HRP Substrate, Millipore, Darmstadt, Germany) and visualized using a Chemidoc (Bio-Rad). Densitometric analysis was performed to quantify the intensities of each band produced using Image Lab Software (Bio-Rad). The relative intensities of target protein bands were normalized to β-actin.

Protein Labeling—Apo-transferrin (apo-Tf; Sigma) was labeled with ^{59}Fe (PerkinElmer Life Sciences) to generate diferric ^{59}Fe-Tf using established methods (80, 81). Cellular ^{59}Fe uptake and release was monitored using a γ-counter (PerkinElmer Life Sciences).

^{59}Fe Efflux Studies and siRNA Treatment—RAW 264.7 cells and J774 cells were transfected with siRNA specific for *Mrp1*, *Gstp1*, *Nrf2*, *Fpn1*, or control siRNA, as described above. The cells were then labeled with ^{59}Fe-Tf ([iron] = 1.5 μM; [Tf] = 0.75 μM) for 24 h at 37 °C. After that, the cells were washed four times on ice and then incubated for 0, 4, 8, and 24 h at 37 °C with LPS (100 ng/ml) and IFNγ (50 units/ml; Sigma) (48). The supernatants and cell pellets were then separated and collected in counting tubes for ^{59}Fe measurement using the γ-counter above.

Determination of Nitrite Concentration—Accumulation of nitrite in culture media was used as an indicator of iNOS activity in activated macrophages (48, 82). Briefly, aliquots of medium from treated cells were collected, and nitrite production was quantified using a modified Griess method (83). Nitrite was also examined by chemiluminescence (Sievers Nitric Oxide Analyzer, NOA 280i; GE Analytical Instruments, Boulder, CO) according to the manufacturer's protocols, as described previously (20, 84). Both methods gave comparable results. As a negative control, cells were also incubated with the NOS inhibitor (L-NAME (56, 57); 4 mM; Sigma) in the presence of LPS (100 ng/ml) and IFNγ (50 units/ml).

^{59}Fe Efflux Studies Using the NOS Inhibitor, L-NAME—RAW 264.7 cells and J774 cells were labeled with ^{59}Fe-Tf (0.75 μM) for 24 h at 37 °C, washed on ice, and then re-incubated with either control media or this medium containing LPS (100 ng/ml) and IFNγ (50 units/ml) in the presence or absence of the NOS inhibitor, L-NAME (4 mM), for 24 h at 37 °C. The release of ^{59}Fe from cells was then determined as described above.

^{59}Fe Efflux Studies Using the MRP1 Inhibitor MK571—RAW 264.7 cells and J774 cells were initially labeled with ^{59}Fe-Tf ([Tf] = 0.75 μM) for 24 h at 37 °C. After this incubation, the cells were washed four times on ice and then incubated with media in the absence or presence of the selective MRP1 inhibitor MK571 (20 μM; Sigma) (61, 85), for 30 min at 37 °C. This was followed by incubation with either control media or this medium containing LPS (100 ng/ml) and IFNγ (50 units/ml) in the absence or presence of MK571 (20 μM) for 0, 4, 8, and 24 h at 37 °C. The supernatants and cells were separated and placed in counting tubes for ^{59}Fe measurement using the γ-counter above.

EPR Spectroscopy—RAW 264.7 and J774 cells previously transfected with siRNA targeting Mrp1 or control siRNA, as described above, were treated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 16 h at 37 °C. Cells (1×10^{10}) were then harvested and analyzed by low temperature EPR using a Bruker EMX spectrometer with 100 kHz modulation at 77 K, as described in Ref. 22.

Cell Survival—Cell survival and proliferation were determined using the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay using standard procedures (86). The RAW 264.7 cells and J774 cells were transfected with siRNA targeting *Mrp1*, *Gstp1*, or *Nrf2* and control siRNA, as described above. These cells were then incubated with LPS (100 ng/ml) and IFNγ (50 units/ml) for 0, 4, 8, and 24 h at 37 °C before the cells were incubated with MTT. Direct cell counts using trypan blue demonstrated that there was a direct correlation between MTT color formation and cell number (86).

Statistics—Results are expressed as mean ± S.D. (number of experiments). Data were compared using Student's *t* test. Results were considered significant when $p < 0.05$.

Author Contributions—H. C. L. conducted most of the experiments, analyzed the results, and wrote the paper. S. S. conducted studies using L-NAME. S. S., P. J. J., and Z. K. wrote the paper with H. C. L. C. L. H. performed analysis using electroparamagnetic resonance spectroscopy. D. R. R. conceived the idea for the project, obtained funding, and wrote the paper with input from all authors.

Acknowledgments—We thank Dr. Michael Huang, Dr. Danuta Kalinowski, Dr. Darius Lane, and Dr. Vera Richardson for their kind assistance with revising and proofreading the manuscript prior to submission.

References

1. Wink, D. A., and Mitchell, J. B. (1998) Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic. Biol. Med.* **25,** 434–456
2. Lancaster, J. R., Jr. (1997) A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* **1,** 18–30

3. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157,** 87–94
4. Lancaster, J. R., Jr., and Hibbs, J. B., Jr. (1990) EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1223–1227
5. de Oliveira, G. A., Cheng, R., Ridnour, L. A., Basudhar, D., Somasundaram, V., McVicar, D. W., Monteiro, H. P., and Wink, D. A. (2016) Inducible nitric oxide synthase (NOS2) in the carcinogenesis of gastrointestinal cancers. *Antioxid. Redox Signal.* 10.1089/ars.2016.6850
6. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9265–9269
7. Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M., and Herman, A. G. (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345,** 346–347
8. Marletta, M. A. (1994) Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78,** 927–930
9. Förstermann, U., and Sessa, W. C. (2012) Nitric oxide synthases: regulation and function. *Eur. Heart J.* **33,** 829–837
10. Marletta, M. A. (1993) Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268,** 12231–12234
11. McLeod, R., and Remington, J. S. (1977) Studies on the specificity of killing of intracellular pathogens by macrophages. *Cell. Immunol.* **34,** 156–174
12. Murray, H. W. (1981) Interaction of *Leishmania* with a macrophage cell line. Correlation between intracellular killing and the generation of oxygen intermediates. *J. Exp. Med.* **153,** 1690–1695
13. Murray, H. W. (1981) Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* **153,** 1302–1315
14. Drapier, J. C., and Hibbs, J. B., Jr. (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest.* **78,** 790–797
15. Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1984) Iron depletion: possible cause of tumor cell cytotoxicity induced by activated macrophages. *Biochem. Biophys. Res. Commun.* **123,** 716–723
16. Nathan, C. F., Arrick, B. A., Murray, H. W., DeSantis, N. M., and Cohn, Z. A. (1981) Tumor cell anti-oxidant defenses. Inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. *J. Exp. Med.* **153,** 766–782
17. Drapier, J. C., Pellat, C., and Henry, Y. (1991) Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. *J. Biol. Chem.* **266,** 10162–10167
18. Bastian, N. R., Yim, C. Y., Hibbs, J. B., Jr., and Samlowski, W. E. (1994) Induction of iron-derived EPR signals in murine cancers by nitric oxide. Evidence for multiple intracellular targets. *J. Biol. Chem.* **269,** 5127–5131
19. Vanin, A. F., Serezhenkov, V. A., Mikoyan, V. D., and Genkin, M. V. (1998) The 2.03 signal as an indicator of dinitrosyl-iron complexes with thiol-containing ligands. *Nitric Oxide* **2,** 224–234
20. Hickok, J. R., Sahni, S., Shen, H., Arvind, A., Antoniou, C., Fung, L. W., and Thomas, D. D. (2011) Dinitrosyliron complexes are the most abundant nitric oxide-derived cellular adduct: biological parameters of assembly and disappearance. *Free Radic. Biol. Med.* **51,** 1558–1566
21. Li, Q., Li, C., Mahtani, H. K., Du, J., Patel, A. R., and Lancaster, J. R., Jr. (2014) Nitrosothiol formation and protection against Fenton chemistry by nitric oxide-induced dinitrosyliron complex formation from anoxia-initiated cellular chelatable iron increase. *J. Biol. Chem.* **289,** 19917–19927
22. Watts, R. N., Hawkins, C., Ponka, P., and Richardson, D. R. (2006) Nitrogen monoxide (NO)-mediated iron release from cells is linked to NO-induced glutathione efflux via multidrug resistance-associated protein 1. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 7670–7675
23. Lok, H. C., Suryo Rahmanto, Y., Hawkins, C. L., Kalinowski, D. S., Morrow, C. S., Townsend, A. J., Ponka, P., and Richardson, D. R. (2012) Nitric oxide storage and transport in cells are mediated by glutathione *S*-transferase P1-1 and multidrug resistance protein 1 via dinitrosyl iron complexes. *J. Biol. Chem.* **287,** 607–618
24. Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., and Deeley, R. G. (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res.* **54,** 5902–5910
25. Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P., and Deeley, R. G. (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res.* **54,** 357–361
26. Morrow, C. S., Diah, S., Smitherman, P. K., Schneider, E., and Townsend, A. J. (1998) Multidrug resistance protein and glutathione *S*-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. *Carcinogenesis* **19,** 109–115
27. Morrow, C. S., Smitherman, P. K., Diah, S. K., Schneider, E., and Townsend, A. J. (1998) Coordinated action of glutathione *S*-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. *J. Biol. Chem.* **273,** 20114–20120
28. Morrow, C. S., Smitherman, P. K., and Townsend, A. J. (1998) Combined expression of multidrug resistance protein (MRP) and glutathione *S*-transferase P1-1 (GSTP1-1) in MCF7 cells and high level resistance to the cytotoxicities of ethacrynic acid but not oxazaphosphorines or cisplatin. *Biochem. Pharmacol.* **56,** 1013–1021
29. Depeille, P., Cuq, P., Mary, S., Passagne, I., Evrard, A., Cupissol, D., and Vian, L. (2004) Glutathione *S*-transferase M1 and multidrug resistance protein 1 act in synergy to protect melanoma cells from vincristine effects. *Mol. Pharmacol.* **65,** 897–905
30. Depeille, P., Cuq, P., Passagne, I., Evrard, A., and Vian, L. (2005) Combined effects of GSTP1 and MRP1 in melanoma drug resistance. *Br. J. Cancer* **93,** 216–223
31. Peklak-Scott, C., Smitherman, P. K., Townsend, A. J., and Morrow, C. S. (2008) Role of glutathione *S*-transferase P1-1 in the cellular detoxification of cisplatin. *Mol. Cancer Ther.* **7,** 3247–3255
32. Sibhatu, M. B., Smitherman, P. K., Townsend, A. J., and Morrow, C. S. (2008) Expression of MRP1 and GSTP1-1 modulate the acute cellular response to treatment with the chemopreventive isothiocyanate, sulforaphane. *Carcinogenesis* **29,** 807–815
33. Paumi, C. M., Ledford, B. G., Smitherman, P. K., Townsend, A. J., and Morrow, C. S. (2001) Role of multidrug resistance protein 1 (MRP1) and glutathione *S*-transferase A1-1 in alkylating agent resistance. Kinetics of glutathione conjugate formation and efflux govern differential cellular sensitivity to chlorambucil versus melphalan toxicity. *J. Biol. Chem.* **276,** 7952–7956
34. Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **45,** 51–88
35. Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* **360,** 1–16
36. De Maria, F., Pedersen, J. Z., Caccuri, A. M., Antonini, G., Turella, P., Stella, L., Lo Bello, M., Federici, G., and Ricci, G. (2003) The specific interaction of dinitrosyl-diglutathionyl-iron complex, a natural NO carrier, with the glutathione transferase superfamily: suggestion for an evolutionary pressure in the direction of the storage of nitric oxide. *J. Biol. Chem.* **278,** 42283–42293
37. Cesareo, E., Parker, L. J., Pedersen, J. Z., Nuccetelli, M., Mazzetti, A. P., Pastore, A., Federici, G., Caccuri, A. M., Ricci, G., Adams, J. J., Parker, M. W., and Lo Bello, M. (2005) Nitrosylation of human glutathione transferase P1-1 with dinitrosyl diglutathionyl iron complex *in vitro* and *in vivo*. *J. Biol. Chem.* **280,** 42172–42180
38. Lo Bello, M., Nuccetelli, M., Caccuri, A. M., Stella, L., Parker, M. W., Rossjohn, J., McKinstry, W. J., Mozzi, A. F., Federici, G., Polizio, F., Pedersen, J. Z., and Ricci, G. (2001) Human glutathione transferase P1-1 and nitric oxide carriers; a new role for an old enzyme. *J. Biol. Chem.* **276,** 42138–42145
39. Pedersen, J. Z., De Maria, F., Turella, P., Federici, G., Mattei, M., Fabrini, R., Dawood, K. F., Massimi, M., Caccuri, A. M., and Ricci, G. (2007) Glutathione transferases sequester toxic dinitrosyl-iron complexes in cells. A protection mechanism against excess nitric oxide. *J. Biol. Chem.* **282,** 6364–6371
40. Cole, S. P., and Deeley, R. G. (2006) Transport of glutathione and glutathione conjugates by MRP1. *Trends Pharmacol. Sci.* **27,** 438–446

41. Ji, L., Li, H., Gao, P., Shang, G., Zhang, D. D., Zhang, N., and Jiang, T. (2013) Nrf2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer. *PLoS ONE* **8,** e63404
42. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236,** 313–322
43. Dhakshinamoorthy, S., and Porter, A. G. (2004) Nitric oxide-induced transcriptional up-regulation of protective genes by Nrf2 via the antioxidant response element counteracts apoptosis of neuroblastoma cells. *J. Biol. Chem.* **279,** 20096–20107
44. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem.* **275,** 16023–16029
45. Ishii, Y., Itoh, K., Morishima, Y., Kimura, T., Kiwamoto, T., Iizuka, T., Hegab, A. E., Hosoya, T., Nomura, A., Sakamoto, T., Yamamoto, M., and Sekizawa, K. (2005) Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J. Immunol.* **175,** 6968–6975
46. Innamorato, N. G., Rojo, A. I., García-Yagüe, A. J., Yamamoto, M., de Ceballos, M. L., and Cuadrado, A. (2008) The transcription factor Nrf2 is a therapeutic target against brain inflammation. *J. Immunol.* **181,** 680–689
47. Jain, A., Lamark, T., Sjøttem, E., Larsen, K. B., Awuh, J. A., Øvervatn, A., McMahon, M., Hayes, J. D., and Johansen, T. (2010) p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* **285,** 22576–22591
48. Wardrop, S. L., Watts, R. N., and Richardson, D. R. (2000) Nitrogen monoxide activates iron regulatory protein 1 RNA-binding activity by two possible mechanisms: effect on the [4Fe-4S] cluster and iron mobilization from cells. *Biochemistry* **39,** 2748–2758
49. Wardrop, S. L., and Richardson, D. R. (2000) Interferon-γ and lipopolysaccharide regulate the expression of Nramp2 and increase the uptake of iron from low relative molecular mass complexes by macrophages. *Eur. J. Biochem.* **267,** 6586–6593
50. LaMarre, J., Wolf, B. B., Kittler, E. L., Quesenberry, P. J., and Gonias, S. L. (1993) Regulation of macrophage α2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-γ. *J. Clin. Invest.* **91,** 1219–1224
51. Held, T. K., Weihua, X., Yuan, L., Kalvakolanu, D. V., and Cross, A. S. (1999) γ Interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. *Infect. Immun.* **67,** 206–212
52. Gomez-Flores, R., Rodriguez-Padilla, C., Mehta, R. T., Galan-Wong, L., Mendoza-Gamboa, E., and Tamez-Guerra, R. (1997) Nitric oxide and TNF-α production by murine peritoneal macrophages activated with a novel 20-kDa protein isolated from *Bacillus thuringiensis* var. *thuringiensis* parasporal bodies. *J. Immunol.* **158,** 3796–3799
53. Richardson, D. R., and Ponka, P. (1997) The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim. Biophys. Acta* **1331,** 1–40
54. Dunn, L. L., Suryo Rahmanto, Y., and Richardson, D. R. (2007) Iron uptake and metabolism in the new millennium. *Trends Cell Biol.* **17,** 93–100
55. Silverstein, P. S., Audus, K. L., Qureshi, N., and Kumar, A. (2010) Lipopolysaccharide increases the expression of multidrug resistance-associated protein 1 (MRP1) in RAW 264.7 macrophages. *J. Neuroimmune Pharmacol.* **5,** 516–520
56. Andrade, S. P., Hart, I. R., and Piper, P. J. (1992) Inhibitors of nitric oxide synthase selectively reduce flow in tumor-associated neovasculature. *Br. J. Pharmacol.* **107,** 1092–1095
57. Tozer, G. M., Prise, V. E., and Chaplin, D. J. (1997) Inhibition of nitric oxide synthase induces a selective reduction in tumor blood flow that is reversible with L-arginine. *Cancer Res.* **57,** 948–955
58. Richardson, D. R., and Baker, E. (1991) The release of iron and transferrin from the human melanoma cell. *Biochim. Biophys. Acta* **1091,** 294–302
59. Baker, E., Page, M., and Morgan, E. H. (1985) Transferrin and iron release from rat hepatocytes in culture. *Am. J. Physiol.* **248,** G93–G97
60. Baker, E., Vicary, F. R., and Huehns, E. R. (1981) Iron release from isolated hepatocytes. *Br. J. Haematol.* **47,** 493–504
61. Gekeler, V., Ise, W., Sanders, K. H., Ulrich, W. R., and Beck, J. (1995) The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem. Biophys. Res. Commun.* **208,** 345–352
62. Lok, H. C., Sahni, S., Richardson, V., Kalinowski, D. S., Kovacevic, Z., Lane, D. J., and Richardson, D. R. (2014) Glutathione *S*-transferase and MRP1 form an integrated system involved in the storage and transport of dinitrosyl-dithiolato iron complexes in cells. *Free Radic. Biol. Med.* **75,** 14–29
63. Suryo Rahmanto, Y., Kalinowski, D. S., Lane, D. J., Lok, H. C., Richardson, V., and Richardson, D. R. (2012) Nitrogen monoxide (NO) storage and transport by dinitrosyl-dithiol-iron complexes: long-lived NO that is trafficked by interacting proteins. *J. Biol. Chem.* **287,** 6960–6968
64. Hayashi, A., Suzuki, H., Itoh, K., Yamamoto, M., and Sugiyama, Y. (2003) Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochem. Biophys. Res. Commun.* **310,** 824–829
65. Li, C. Q., Kim, M. Y., Godoy, L. C., Thiantanawat, A., Trudel, L. J., and Wogan, G. N. (2009) Nitric oxide activation of Keap1/Nrf2 signaling in human colon carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 14547–14551
66. Itoh, K., Mochizuki, M., Ishii, Y., Ishii, T., Shibata, T., Kawamoto, Y., Kelly, V., Sekizawa, K., Uchida, K., and Yamamoto, M. (2004) Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Δ(12,14)-prostaglandin J(2). *Mol. Cell. Biol.* **24,** 36–45
67. Marro, S., Chiabrando, D., Messana, E., Stolte, J., Turco, E., Tolosano, E., and Muckenthaler, M. U. (2010) Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position −7007 of the FPN1 promoter. *Haematologica* **95,** 1261–1268
68. Ward, D. M., and Kaplan, J. (2012) Ferroportin-mediated iron transport: expression and regulation. *Biochim. Biophys. Acta* **1823,** 1426–1433
69. Nairz, M., Schleicher, U., Schroll, A., Sonnweber, T., Theurl, I., Ludwiczek, S., Talasz, H., Brandacher, G., Moser, P. L., Muckenthaler, M. U., Fang, F. C., Bogdan, C., and Weiss, G. (2013) Nitric oxide-mediated regulation of ferroportin-1 controls macrophage iron homeostasis and immune function in Salmonella infection. *J. Exp. Med.* **210,** 855–873
70. Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., Paw, B. H., Drejer, A., Barut, B., Zapata, A., Law, T. C., Brugnara, C., Lux, S. E., Pinkus, G. S., Pinkus, J. L., *et al.* (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403,** 776–781
71. Thimmulappa, R. K., Lee, H., Rangasamy, T., Reddy, S. P., Yamamoto, M., Kensler, T. W., and Biswal, S. (2006) Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J. Clin. Invest.* **116,** 984–995
72. Abboud, S., and Haile, D. J. (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J. Biol. Chem.* **275,** 19906–19912
73. Hibbs, J. B., Jr, Taintor, R. R., and Vavrin, Z. (1987) Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235,** 473–476
74. Ruetten, H., and Thiemermann, C. (1996) Prevention of the expression of inducible nitric oxide synthase by aminoguanidine or aminoethyl-isothiourea in macrophages and in the rat. *Biochem. Biophys. Res. Commun.* **225,** 525–530
75. Yanatori, I., Richardson, D. R., Imada, K., and Kishi, F. (2016) Iron export through the transporter ferroportin 1 is modulated by the iron chaperone PCBP2. *J. Biol. Chem.* **291,** 17303–17318
76. Ballatori, N., Hammond, C. L., Cunningham, J. B., Krance, S. M., and Marchan, R. (2005) Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins. *Toxicol. Appl. Pharmacol.* **204,** 238–255
77. MacLeod, A. K., McMahon, M., Plummer, S. M., Higgins, L. G., Penning, T. M., Igarashi, K., and Hayes, J. D. (2009) Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes:

demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds. *Carcinogenesis* **30,** 1571–1580
78. Buckley, B. J., Marshall, Z. M., and Whorton, A. R. (2003) Nitric oxide stimulates Nrf2 nuclear translocation in vascular endothelium. *Biochem. Biophys. Res. Commun.* **307,** 973–979
79. Xu, X., Sutak, R., and Richardson, D. R. (2008) Iron chelation by clinically relevant anthracyclines: alteration in expression of iron-regulated genes and atypical changes in intracellular iron distribution and trafficking. *Mol. Pharmacol.* **73,** 833–844
80. Richardson, D. R., and Baker, E. (1990) The uptake of iron and transferrin by the human malignant melanoma cell. *Biochim. Biophys. Acta* **1053,** 1–12
81. Richardson, D., and Baker, E. (1992) Two mechanisms of iron uptake from transferrin by melanoma cells. The effect of desferrioxamine and ferric ammonium citrate. *J. Biol. Chem.* **267,** 13972–13979
82. Watts, R. N., and Richardson, D. R. (2001) Nitrogen monoxide (NO) and glucose: unexpected links between energy metabolism and no-mediated iron mobilization from cells. *J. Biol. Chem.* **276,** 4724–4732
83. Miranda, K. M., Espey, M. G., and Wink, D. A. (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* **5,** 62–71
84. MacArthur, P. H., Shiva, S., and Gladwin, M. T. (2007) Measurement of circulating nitrite and *S*-nitrosothiols by reductive chemiluminescence. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **851,** 93–105
85. Koley, D., and Bard, A. J. (2012) Inhibition of the MRP1-mediated transport of the menadione-glutathione conjugate (thiodione) in HeLa cells as studied by SECM. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 11522–11527
86. Richardson, D. R., Tran, E. H., and Ponka, P. (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* **86,** 4295–4306