Excessive generation of oxidants by immune cells results in acute tissue damage. One mechanism by which oxidant exposure could have long-term effects is modulation of epigenetic pathways. We hypothesized that methylation of newly synthesized DNA in proliferating cells can be altered by oxidants that target DNA methyltransferase activity or deplete its substrate, the methyl donor SAM. To this end, we investigated the effect of two oxidants produced by neutrophils, H$_2$O$_2$ and glycine chloramine, on maintenance DNA methylation in Jurkat T lymphoma cells. Using cell synchronization and MS-based analysis, we measured heavy deoxycytidine isotope incorporation into newly synthesized DNA and observed that a sublethal bolus of glycine chloramine, but not H$_2$O$_2$, significantly inhibited DNA methylation. Both oxidants inhibited DNA methyltransferase 1 activity, but only chloramine depleted SAM, suggesting that removal of substrate was the most effective means of inhibiting DNA methylation. These results indicate that immune cell-derived oxidants generated during inflammation have the potential to affect the epigenome of neighboring cells.

Epigenetic processes, including DNA methylation and post-translational modification of histones, are recognized to play a central role in determining gene expression profiles in cells (1, 2). Cues from the external environment play a role in transition between epigenetic states (3, 4), with the potential for these changes to be inherited (5, 6). However, our understanding of the mechanisms through which environmental factors influence the epigenome is not well-characterized. Methylation patterns are maintained in proliferating cells by DNA methyltransferase 1 (DNMT1), which transfers a methyl group onto cytidine residues incorporated into the new strand during DNA synthesis. This is done in accordance with the pattern of methylated cytosine in the complementary parental DNA strand and contrasts with de novo methylation, which is mediated by DNMT3 isoforms during embryo development and differentiation. DNMTs use a nucleophilic cysteine residue to covalently bind a target cytosine and activate the C-5 carbon toward nucleophilic attack of the methyl group from the donor molecule SAM. Reactive cysteine and methionine residues in other proteins are susceptible to oxidative inactivation (7, 8), making DNMT a possible target for mediating epigenetic changes via redox regulation. Also, methionine oxidation could compromise DNMT activity through depletion of SAM.

In this study, we investigated the ability of oxidants derived from activated neutrophils to influence genomic DNA methylation. There is considerable interest in the role of cancer-associated immune cells, including infiltrating neutrophils and monocytes, in tumorigenesis (9–11). Methylation profiles of cancer cells are known to change during tumor progression, and it has been speculated that oxidants produced by immune cells could perturb epigenetic pathways of neighboring cells (9, 12). However, this has not been investigated in any detail.

NOX2 on phagocytic cells converts large amounts of oxygen to superoxide, which rapidly dismutates to produce hydrogen peroxide (13). Myeloperoxidase uses this hydrogen peroxide to oxidize halide ions to hypohalous acids, including the antibacterial agent hypochlorous acid (13). Hypochlorous acid is highly reactive and rapidly consumed by cells, generating a range of secondary products, including chloramines (14). Importantly, hydrogen peroxide and chloramines are highly selective for reactions with different thiols, making them prime candidates for testing our hypothesis (7, 15). Furthermore, hydrogen peroxide and small chloramines such as glycine chloramine are cell-permeable (16). Neutrophils usually protect neighboring host cells from damage by confining production of oxidants to the intracellular phagosome in which microbes are engulfed. However, extracellular release of myeloperoxidase, generation of longer-lived secondary oxidants such as chloramines, and diffusion of these oxidants all increase the likelihood of affecting redox homeostasis within neighboring cells.

To be of physiological significance, redox regulation of DNA methylation needs to occur at sublethal levels of oxidative stress, ensuring continued replication of modified cells. Total cysteine and 5-methylcytosine content in genomic DNA is a direct measure of global DNA methylation. However, it is difficult to use this for detecting inhibition of DNA methylation in proliferating cells under oxidative stress. First, there is no way of distinguishing the parental strand, which will not change via passive demethylation during DNA synthesis. Also, any sublethal oxidative stress will be short-lived, and only a subset of
cells in a population will be undergoing active DNA synthesis at any given time. Therefore, in this study, a new method was developed to measure cytosine methylation of newly replicated DNA using cell synchronization, heavy isotope labeling, and MS. This method was then used to assess the effects of hydrogen peroxide and glycine chloramine on methylation in actively dividing Jurkat T lymphoma cells. The results show that glycine chloramine is able to inhibit methylation of newly replicated DNA via inhibition of DNMT1 and decreasing levels of SAM at doses that do not interfere with cell proliferation.

**Results**

**Measuring changes in DNA methylation on newly synthesized DNA**

We utilized heavy isotope MS to specifically monitor the methylation status of \(^{15}\)N\(_3\)-deoxycytidine (\(^{15}\)N\(_3\)-dC) that had been newly incorporated into DNA (summarized in Fig. 1a). To optimize cellular uptake and utilization of \(^{15}\)N\(_3\)-dC for DNA synthesis, endogenous cytidine levels were decreased by pretreating cells with excess thymidine (17). This treatment had the additional advantage of synchronizing entry of the cell population into S phase upon introduction of \(^{15}\)N\(_3\)-dC. \(^{15}\)N\(_3\)-dC and its methylated derivative (\(^{15}\)N\(_3\)-mC) were detected in DNA harvested from actively proliferating Jurkat T lymphoma cells but not in DNA from cells supplemented with unlabeled dC (Fig. S1, a and b). This confirmed intracellular conversion of \(^{15}\)N\(_3\)-dC to the triphosphate nucleotide, incorporation into DNA, and subsequent methylation by endogenous DNMT activity. Jurkat cells were treated with excess thymidine for 18 h and then supplemented with 50 \(\mu\)M \(^{15}\)N\(_3\)-dC in fresh medium to promote enrichment of new DNA with the isotope. Flow cytometry revealed a time-dependent increase in DNA content, consistent with initiation of cell cycle progression upon replenishment of cytidine pools (Fig. 1b). Cells not supplemented with any form of dC after thymidine block were not able to re-enter S-phase (Fig. S1c). In contrast, cells that were supplemented showed time-dependent incorporation of \(^{15}\)N\(_3\)-dC (Fig. 1c, closed circles) and concomitant methylation to form \(^{15}\)N\(_3\)-mC (Fig. 1d, closed circles).

We then validated the effectiveness of our method to assess methylation of newly synthesized DNA using the DNMT inhibitor 5-aza-2′-deoxycytidine (Aza-dC). Thymidine-blocked Jurkat cells were treated with 5 \(\mu\)M Aza-dC in fresh medium while supplementing with 50 \(\mu\)M \(^{15}\)N\(_3\)-dC. The inhibitor had no effect on cell cycle progression (Fig. 1b) and no effect on uptake or incorporation of the \(^{15}\)N\(_3\)-dC probe (Fig. 1c, open circles). Although only a small amount of Aza-dC is incorporated into newly synthesized DNA, it is sufficient to cause irreversible inactivation of DNMT. By 2 h, conversion of \(^{15}\)N\(_3\)-dC to \(^{15}\)N\(_3\)-mC was inhibited by Aza-dC (Fig. 1d, open circles). To compare the extent to which methylation was inhibited by various treatments, the ratio of \(^{15}\)N\(_3\)-mC to \(^{15}\)N\(_3\)-dC was expressed as a percentage of control, i.e. in the absence of an inhibitor (see Fig. 1f for equations). The use of this ratio controls for variations because of decreased \(^{15}\)N\(_3\)-dC uptake as well as interexperiment and sample loading variability. Aza-dC inhibited methylation of newly synthesized DNA by 45% at 2 h and more than 70% at 6 h (Fig. 1e, open circles). The effectiveness of our method is further demonstrated by comparing the effect of Aza-dC on new methylation versus total methylation. If the peak areas of dC and mC are included in the calculations in addition to their heavy counterparts, then the effect of Aza-dC is not detectable at 2 h (cf. 45% inhibition in new DNA) and decreasing total methylation by only 20% at 6 h (Fig. 1e, closed squares). Thus, by focusing on the heavy labeled species as a measure of methylation on newly synthesized DNA, we have a more sensitive assay to measure the effect of oxidative stress.

**Methylation of replicated DNA is inhibited by glycine chloramine**

We selected two oxidants that can be generated as a result of neutrophil activation: hydrogen peroxide and glycine chloramine. This was based on their selective chemistry, cell permeability, and relatively long half-life compared with other reactive oxygen species (7, 8, 13, 18). Jurkat cells treated in medium with 200 or 500 \(\mu\)M glycine chloramine continued to progress through S phase of the cell cycle albeit slower than control cells (Fig. 2b). With 200 \(\mu\)M glycine chloramine, there was no effect on DNA synthesis (Fig. 2a). With 500 \(\mu\)M glycine chloramine, there was mild inhibition with ~15% less incorporation of \(^{15}\)N\(_3\)-dC at earlier times (Fig. 2a). Strong inhibition of DNA methylation was observed at both concentrations (Fig. 2, c and d). On average, 25% inhibition of DNA methylation was observed in cells treated with 200 \(\mu\)M glycine chloramine and up to 50% inhibition of methylation with 500 \(\mu\)M (Fig. 2d). There was no loss of cell viability 24 h after exposure to 200 \(\mu\)M glycine chloramine as measured by PI staining and FACS analysis. However, cells treated with the higher dose were less viable at 24 h (76.7% ± 0.1%, \(n = 2\)), compared with control cells.

Cells were also treated with 50 or 100 \(\mu\)M hydrogen peroxide in fresh medium supplemented with 50 \(\mu\)M \(^{15}\)N\(_3\)-dC. At the higher concentration of oxidant, cell cycle progression was visibly impaired, and the number of apoptotic cells (sub-G0) also increased (Fig. 2f). Incorporation of the \(^{15}\)N\(_3\)-dC probe in the DNA of hydrogen peroxide-treated cells was decreased with 50 and 100 \(\mu\)M hydrogen peroxide, indicating some inhibition of proliferation (Fig. 2e). However, methylation of newly synthesized DNA was not inhibited in hydrogen peroxide–treated cells (Fig. 2h).

**Inhibition of DNMT1 activity by hydrogen peroxide and glycine chloramine**

One potential mechanism by which glycine chloramine inhibits methylation of newly synthesized DNA is direct inhibition of DNMT1, the mammalian enzyme responsible for maintenance DNA methylation in somatic cells. We developed a method to capture active DNMT1 using DNA enriched with Aza-dC (Fig. S2). Briefly, nuclear protein extracts were prepared from Jurkat cells incubated for selected times in the presence or absence of Aza-dC, and the amount of soluble DNMT1 was measured by Western blotting. Active DNMT1 binds irreversibly to Aza-dC in DNA and is removed during preparation of the extracts (Fig. S3a). Use of synchronized cells increases the sensitivity of this method (Fig. S3b). However, DNMT1 that has been oxidized can no longer bind to Aza-dC in DNA and
will be discarded, leaving only active enzyme in the extract. Cells treated with 50 μM hydrogen peroxide showed inhibition of DNMT1 (Fig. 3a), consistent with an earlier report (19). Treatment of cells with glycine chloramine also caused marked retention of DNMT1 protein in soluble nuclear extracts (Fig. 3b).

**Glycine chloramine, but not hydrogen peroxide, decreases methionine and SAM levels**

Methionine is a precursor of the methyl donor SAM and therefore a central metabolite in DNA methylation. Oxidation of methionine by glycine chloramine would limit SAM synthesis. Intracel-
Figure 2. Effect of glycine chloramine and hydrogen peroxide on cell cycle progression and methylation of newly synthesized DNA. a–d, thymidine-blocked Jurkat cells (1 × 10^6/ml) were treated with glycine chloramine in fresh medium supplemented with ^15^N_3-dC. Genomic DNA was extracted for analysis by MS, and incorporation of ^15^N_3-dC (a) or methylation to ^15^N_3-MedC (c) was assessed for control cells (●) and cells treated with 200 μM (▲) or 500 μM (◆) glycine chloramine. Cells treated as described above were fixed and stained with propidium iodide for analysis by flow cytometry to monitor cell cycle progression (b). Representative histograms from control and glycine chloramine-treated cells are shown. The DNA methylation ratio in oxidant-treated cells was calculated as described in the text and Fig. 1 and expressed as a percentage of that in control cells (d). Data in a, c, and d are mean ± S.E. from nine independent experiments. Statistically significant differences from time-matched controls are marked: *, p < 0.05; #, p < 0.01; one-sample t test. e–h, as for a–d, except cells were treated with 50 μM (▲) or 100 μM (◆) hydrogen peroxide and compared with control cells (●). Data in e, g, and h are mean ± S.E. from three independent experiments.
Oxidation of intracellular thiols by glycine chloramine

The rate of glycine chloramine consumption was tested in our system. In the presence of RPMI (10% FCS) and cells, the concentration of reactive chloramine remaining dropped rapidly to less than 5% after 30 min (Fig. S5). The rate of consumption was similar in RPMI (10% FCS) without cells as well as RPMI (no FCS).

The effect of glycine chloramine on intracellular thiols was measured at the doses required to inhibit DNA methylation. The level of reduced GSH, the major intracellular low-molecular-weight thiol, was measured every 15 min over 1 h and did not change when cells were treated with 500 μM glycine chloramine (data not shown). However, the oxidant-sensitive enzyme GAPDH was significantly and rapidly inhibited before recovering to 80% of control levels after 90 min (Fig. 5). These results indicate selectivity for redox-sensitive proteins without major changes in free GSH thiol status.

Discussion

The possibility that the oxidants produced by immune cells could perturb epigenetic pathways in neighboring cells has been proposed (9, 12), but direct evidence has not been reported. Part of the technical challenge stems from the short-lived nature of oxidant species and the percentage of cells undergoing replication at any time. Here we developed a method to detect changes in maintenance DNA methylation that combines cell cycle synchronization, heavy deoxycytidine labeling, and MS. Although others have used heavy isotope labeling to explore effects on DNA methylation (20), the advantage of combining cell synchronization is that it greatly amplifies the signal for changes in methylation of newly synthesized DNA. Using this method, we investigated the effect of cell-permeable oxidants that are generated by neutrophils at sites of inflammation; namely, glycine chloramine and hydrogen peroxide. We have shown that glycine chloramine significantly impaired methylation of newly replicated DNA (summarized in Fig. 6).

Inhibition of methylation occurred under conditions where the oxidant did not compromise DNA synthesis and proliferation or result in significant cell death. This indicates that methylation is particularly sensitive to oxidative stress. Indeed, under identical conditions, there was no significant perturbation of the global antioxidant status of the cell as measured by GSH levels. Although the concentration of glycine chloramine added to cells in medium was higher than hydrogen peroxide, the effective dose is likely to be less. We have shown previously that, when cells are treated with chloramines in medium, the
Redox regulation of DNA methylation

Figure 4. Cells treated with glycine chloramine have decreased intracellular methionine and SAM. a and b, thymidine-blocked Jurkat cells (1 x 10⁶/ml) were resuspended in fresh RPMI 1640 medium (+ 10% FCS) supplemented with 50 μM dC and treated with 50 μM hydrogen peroxide (●) or μM 500 glycine chloramine (○). At the indicated times, cells were harvested for analysis of intracellular l-methionine (a, mean ± range of two experiments) and SAM (b, mean ± S.E. of four experiments), all expressed as a percentage of time-matched controls. Control cells contained 214 ± 51 pmol methionine/10⁶ cells and 176 ± 27 pmol SAM/10⁶ cells.

Figure 5. Cells treated with glycine chloramine have decreased GAPDH activity. Thymidine-blocked Jurkat cells (1 x 10⁶/ml) were resuspended in fresh RPMI 1640 medium (+ 10% FCS) supplemented with 50 μM dC and treated with 200 μM glycine chloramine (●) or μM 500 glycine chloramine (○). At the indicated times, cells were harvested for analysis of GAPDH activity (mean ± S.E. of three to four experiments) expressed as a percentage of time-matched controls.

effect may be limited. It would be of interest to test the effect of sustained hydrogen peroxide generation on DNA methylation.

The assay we used for assessing DNMT1 activity means that inhibition was due to direct oxidation of its active site cysteine or impaired targeting of DNMT1 to DNA. Of interest, one group demonstrated that treatment with hydrogen peroxide actually results in increased targeting of DNMT to DNA (21), suggesting that inhibition with this oxidant is via the catalytic site. Further studies are required to determine whether glycine chloramine acts in the same way.

Although we observed oxidative inhibition of DNMT1 and SAM depletion, previous work has looked at the effect of direct oxidation of nucleic acids. Cells exposed to hypochlorous acid are reported to have elevated levels of chlorinated nucleobases, of which the most abundant is 5-chlorocytosine (22, 23). 5-Chlorocytosine has been shown to bind MeCP2 (24) and alter the site specificity of DNMTs (25), leading to altered DNA methylation patterns. However, 5-chlorocytosine was found at ~3 bases per 10⁶ (26) and is therefore likely to have a greater effect on mutagenicity than global DNA methylation (27). Oxidation of guanosine at CpG sites to 8-oxo-2’-deoxyguanosine (8-oxo-dG) also directly interferes with methylation, but the extent of oxidation is reported to be one 8-oxo-dG per 10⁸-10⁹ deoxyguanosines (28). Furthermore, mouse models of chronic inflammation (29) as well as colonic samples from a patient cohort with inflammatory bowel disease (26) did not find increased levels of 8-oxo-dG or other classical oxidative DNA lesions compared with control samples. Therefore, sublethal oxidative stress is unlikely to have significant effects on global DNA methylation via these mechanisms.

An important epigenetic phenomenon is the capacity of cells to produce consistent phenotypic outcomes despite being challenged by variable conditions (6, 30, 31). This epigenetic “buffering” capacity of cells can be altered by mutation or by specific environmental cues during critical stages of differentiation. With respect to inhibition of DNA methylation by glycine chloramine, this could result in decreased promoter methylation and increased expression of genes that favor death or survival and proliferation. Given that the mechanisms we explored are not DNA sequence–specific, we would expect inhibition of methylation to be heterogeneous with respect to genomic location across a population of cells. However, this could result in a
proportion of cells where changes in gene expression provide a survival advantage. Over time, and in conjunction with muta-
tions, this could result in transition to cancer or in cancer cells becoming metastatic (32, 33). On the other hand, if demethyl-
ation of tumor suppressor promotors or retrotransposons occurs, then this could lead to senescence or cell death. Going beyond tumor suppressors and oncogenes, changes in methyl-
ation have the capacity to alter the chromatin structure and, therefore, cell phenotype, via downstream changes in histone methylation and acetylation.

In conclusion, we designed and validated a method to screen metabolites and chemicals for their effect on global methyla-
tion of newly synthesized DNA. Our results reveal a mechanism by which neutrophil-derived oxidants could inhibit mainte-
nance methylation and influence the course of disease (34–36).

**Experimental procedures**

**Jurkat cell culture**

The Jurkat T lymphoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were
grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humified incubator with 5% CO₂. A log phase of growth was maintained by subculturing the cells every 2–3 days.

**Reagent preparation**

Crystals of Aza-dC were dissolved in PBS, and the concentra-
tion was determined spectrophotometrically (ε₂₉₉ = 8.2 mm⁻¹ cm⁻¹). Glycine chloramine was freshly prepared by adding hypochlorous acid dropwise to a five molar excess of glycine in an equivalent volume of PBS while vortexing. Glycine chloramine concentration was determined spectrophotometrically (ε₂₅₂ = 415 M⁻¹ cm⁻¹) (16). Absorption spectra from 200–400 nm were monitored to ensure the absence of dichloramine (λₘₐₓ, 300 nm) and any unreacted hypochlorous acid (λₘₐₓ, 290 nm). Hydrogen peroxide was diluted into PBS, and the concentration was determined spectrophotometrically (ε₂₄₀ = 43.6 M⁻¹ cm⁻¹). Jurkat cells were treated using 100-fold dilutions of reagent stock solutions.

**Incorporation of the stable ¹⁵N₃-2'-deoxycytidine isotope into DNA of cultured cells**

Depletion of intracellular cytidine pools was achieved by thy-
midine block. 1 M 2'-deoxythymidine (thymidine) was dis-
solved in 1 M NaOH and diluted 10-fold in PBS to give a 100 mM stock solution. Jurkat cells were resuspended in fresh RPMI 1640 medium at a density of 1 × 10⁶/ml and treated with thy-
midine (final concentration of 1 mM). A pipette aid was used to gently mix the cell suspension before leaving it to incubate for 18 h at 37 °C. Subsequently, thymidine-blocked Jurkat cells were pelleted (1000 × g for 5 min) and resuspended in RPMI 1640 medium. Cells were then treated with Aza-dC or oxidants and simultaneously initiated into S phase by supplementing with 50 μM of isotopically labeled ¹⁵N₃-dC or dC (Cambridge Isotope Labs).
Redox regulation of DNA methylation

Genomic DNA isolation

DNA extraction was carried out using the AxyPrep™ Multisource Genomic DNA Miniprep kit. Purification was carried out as described by the manufacturer (Axygen Biosciences).

Measurement of DNA methylation levels in newly synthesized DNA

Conditions for separation and detection of dC, \(^{15}\)N\(_3\)-dC, mC, and \(^{15}\)N\(_3\)-mC were established based on a previous method (37). For LC, a Phenomenex C-18 column (150 \(\times\) 2.0 mm) held at 25 °C was used, and the mobile phases were as follows: solvent A, 7 mM ammonium acetate (pH 6.7) containing 5% (v/v) methanol; solvent B, 100% (v/v) methanol. A linear gradient was applied in which solvent A (0–15 min) was used to elute the analytes and solvent B (16–19 min) was used to wash the column of impurities before re-equilibrating with solvent A (20–37 min). The flow rate was 0.2 ml/min, and 50 \(\mu\)l of hydrolyzed DNA sample or standard solution was injected onto the column.

Eluant from the column was introduced into the mass spectrometer for analysis without splitting. A Thermo Finnigan LCQ Deca XP Plus Ion Trap instrument (San Jose, CA) equipped with an electrospray ionization (ESI) source was used. The electrospray needle was held at 5 kV, nitrogen sheath gas was set at 50 units, and the temperature of the heated capillary was 300 °C. This method causes loss of the deoxyribose moiety from cytosine deoxynucleoside species; therefore, no collision energy was required to promote fragmentation of protonated species (37). Cytosine base ions derived from fragmentation of the four deoxycytosines (\(^{15}\)N\(_3\)-dC, endogenous dC, \(^{15}\)N\(_3\)-mC, and endogenous mC) in the ESI source were produced and detected in positive ion mode. The ion trap range was set to detect endogenous dC \(\rightarrow\) cytosine (\(m/z\) 111.5–112.5) and \(^{15}\)N\(_3\)-dC \(\rightarrow\) \(^{15}\)N\(_3\)-cytosine (\(m/z\) of 114.5–115.5) in the first time window from 0–6 min. Spectra for endogenous mC \(\rightarrow\) methylcytosine (\(m/z\) 125.5–126.5) and \(^{15}\)N\(_3\)-mC \(\rightarrow\) \(^{15}\)N\(_3\)-methylcytosine (\(m/z\) 128.5–129.5) were collected in the second time window from 6–12 min.

Cell cycle analysis

Cells were prepared for cell cycle analysis as published previously (38). Cells (0.5–1 \(\times\) \(10^6\)) were pelleted, washed once in PBS, resuspended in 100 \(\mu\)l of PBS, and fixed by dropwise addition of 900 \(\mu\)l of 70% (v/v) ethanol. Fixed cells were kept at −20 °C for 15 min or stored for a few days. Cells were centrifuged at 1000 \(\times\) g for 5 min, washed once with 1 ml of PBS, and then resuspended in 500 \(\mu\)l of PBS to which 800 \(\mu\)l of DNA extraction buffer (1.92 mM Na\(_2\)HPO\(_4\) and 0.8 mM citric acid adjusted to pH 7.8) was added. After 5-min incubation at room temperature, cells were pelleted and resuspended in 250 \(\mu\)l of PBS containing 5 \(\mu\)l of DNA staining solution (1 mg/ml PI and 2 mg/ml RNase A). After 30-min incubation in darkness, DNA content for 10,000 cells/sample was determined using a FC500 MPL flow cytometer (Beckman Coulter Inc.), and single-parameter DNA histograms were compared using CXP software to visualize the progression of cells through the cell cycle.

Cell viability

Plasma membrane integrity was monitored by addition of 1.25 \(\mu\)g of PI to 0.25 \(\times\) \(10^6\) cells in medium, and cell fluorescence was measured by flow cytometry. PI-positive cells (non-viable) were expressed as the proportion of total cells analyzed (10,000 cells).

Determination of intracellular methionine levels

Intracellular levels of methionine and methionine sulfoxide in control or oxidant-treated Jurkat cells were determined by MS. Following treatment, 1 \(\times\) \(10^6\) cells were centrifuged for 2 min at 2000 \(\times\) g. The cell pellet was washed in 1 ml of PBS and centrifuged again. MilliQ water (50 \(\mu\)l) was added, and cells were frozen at −80 °C. Prior to analysis, cells were subjected to three freeze–thaw cycles between a 37 °C water bath and −80 °C freezer before adding 140 \(\mu\)l of acetonitrile and 10 \(\mu\)l of \(\_\)methionine-d\(_3\) (1 \(\mu\)M final concentration internal standard). The mixture was vortexed vigorously and clarified by centrifugation. Supernatant (50 \(\mu\)l) was injected onto a Cosmosil HILIC column (4.6 \(\times\) 150 mm, Nacalai Tesque Inc., Kyoto, Japan), and isocratic elution was carried out with 70% (v/v) acetonitrile/30% (v/v) 10 mM ammonium acetate (pH 6.8) at a flow rate of 0.5 ml/min. A Thermo Finnigan LCQ Deca XP Plus instrument (San Jose, CA) equipped with an ESI source was used to detect methionine (room temperature, 6.7 min) and methionine sulf oxide (room temperature, 10.4 min) in positive ion mode. The electrospray needle was held at 5 kV with nitrogen sheath gas set at 58 units and the temperature of the heated capillary at 260 °C. Selective reaction monitoring was used to detect total ion fragmentation of methionine of \(m/z\) 150 → 104, 133 and internal standard \(\_\)methionine-d\(_3\). \(m/z\) 153 → 107, 136 in two scan segments in the first time window (0–8 min) and methionine sulfoxide \(m/z\) 166 → 75, 149 in the second time segment (8–12 min). The collision energy applied for fragmentation of these three species was 28%, 28%, and 26%, respectively. The amount of methionine and methionine sulfoxide in cell lysates was quantified by comparing MS peak areas with external standards of methionine and methionine sulfoxide. Samples and standards were normalized to the signal intensity of a fixed concentration of \(\_\)methionine-d\(_3\) internal standard.

Measurement of SAM

Intracellular SAM was measured in Jurkat cells exposed to oxidants. Following oxidant exposure, 1.5 \(\times\) \(10^6\) cells were centrifuged for 2 min at 2000 \(\times\) g. Medium was removed, and 80 \(\mu\)l of 0.1 M sodium acetate buffer (pH 6.0) containing 1% (w/v) CHAPS was added for 5 min at room temperature. Samples were then deproteinized by addition of 20 \(\mu\)l of ice-cold HClO\(_4\) (4 \(\times\)) and left on ice for 20 min. The samples were clarified by centrifugation, and 50 \(\mu\)l of the soluble fraction was injected onto a Phenomenex Luna 5 \(\mu\)m C18 column (250 \(\times\) 4.60 mm) connected to a Waters 2690 HPLC system with UV detection at 254 nm (Waters 996 photodiode array). A 30-min gradient was used starting with mobile phase A (50 mM NaH\(_2\)PO\(_4\), 10 mM heptane sulfonic acid sodium salt, 20% (v/v) methanol (pH 4.35)) for 5 min, followed by mobile phase B (50 mM NaH\(_2\)PO\(_4\), 8.5% (v/v) methanol (pH 4.39)) from 7–20 min and re-equilibration with mobile phase A from 22–30 min. The flow rate was
0.9 ml/min. SAM eluted at 13.4 min and was quantified by comparing peak areas with standard SAM-Cl. Stock SAM-Cl solution (1 nM) was prepared in MilliQ water, and the actual concentration determined spectrophotometrically (ε₂₅₇ = 15,000 M⁻¹ cm⁻¹) (39). Standards of SAM-Cl (0.5–50 µM) were prepared by diluting the stock solution in 0.4 M HClO₄. Chromatograms were analyzed using Chromeleon™ software ( Dionex Corp.).

**Glyceraldehyde 3-phosphate dehydrogenase activity**

Jurkat cells (1 × 10⁶) were harvested and lysed in 100 µl of extract buffer (40 mM Hepes (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.6 mg/ml Complete™ protease inhibitors and 1% CHAPS). GAPDH activity in cell lysates was measured by following the oxidation of NADH at 340 nm via the enzyme-linked reactions of GAPDH and phosphoglycerate kinase. 2.5, or 10 µl of control or oxidant-treated samples were added to a 96-well plate containing 20 µl of Tris/HCl (1 M, pH 8.0) with EDTA (5 mM), 20 µl of MgCl₂ (0.1 M), 10 µl of NADH (2 or 10 mM), 20 µl of phosphoglycerate kinase (50 units/ml), and MilliQ water to a final volume of 240 µl. The plate was incubated at 37 °C, and the reaction was initiated with 100-µl solution of 3-phosphoglycerate (20 mM) and ATP (16 mM, adjusted to pH 7). The loss in absorbance at 340 nm was followed over time. The linear rate of activity in wells containing oxidant-treated samples was compared with that of untreated control wells and expressed as a percentage of GAPDH activity remaining (40).

**Determination of intracellular GSH**

The concentration of reduced GSH in cells after exposure to oxidants was measured using the fluorescent thiol-binding compound monobromobimane (MBB) (41). Following oxidant exposure, 1 × 10⁶ cells were pelleted at 10,000 × g for 1 min, and then 380 µl of 1 mM MBB solution (adjusted to pH 8.0 with 0.1 M KOH) was added. Cells were incubated at room temperature for 20 min in darkness before adding 20 µl of saturated trichloracetic acid solution to precipitate protein. The protein precipitate was pelleted at 10,000 × g for 5 min, and 100 µl of the supernatant was injected onto an HPLC system. The column used was a Brownlee Spheri-5 ODS C18 (100 × 4.6 mm), and the flow rate was 1.5 ml/min. Mobile phases A (9% (v/v) acetonitrile, 0.25% (v/v) acetic acid) and B (75% (v/v) acetonitrile were used in a stepwise gradient of mobile phase A (0–6 min), followed by mobile phase B (8–12 min) and re-equilibration with mobile phase A (14–22 min). Derivatized GSH was monitored at an excitation of 394 nm and emission of 480 nm using a fluorescence detector (Hitachi F-4500, Tokyo, Japan). The retention times and peak areas from the cell extracts were compared with external standard solutions prepared by derivatizing pure GSH (0–20 µM) with MBB as described above.

**Preparation and analysis of nuclear extracts by Western blotting**

Nuclear extracts from 1–5 × 10⁶ Jurkat cells per sample were prepared using a standard protocol (42). Nuclear fractions typically contained 1.2–1.5 µg/µl protein and were used immediately or stored at −80 °C. Discontinuous SDS-PAGE was performed on a Mini-Protean II system (Bio-Rad) according to the Laemmli protocol (43). Blots were analyzed with anti-DNMT1 primary antibody (goat polyclonal from Santa Cruz Biotechnology, 1:500) and horseradish peroxidase–conjugated rabbit anti-goat secondary antibody (1:13,000). Antibodies bound to DNMT1 were detected by ECL and visualized using the ChemiDoc™ XRS system and Quantity One® software (Bio-Rad). Total protein was visualized by staining PVDF membranes with Coomassie Brilliant Blue solution.

**Protein assay**

Protein concentration in cell lysates was measured with the Bio-Rad DC Protein Assay Kit using BSA as the standard.

**Statistical analysis**

Data are expressed as mean ± S.E. of the mean of n independent experiments or means ± range of two independent experiments. Two-way analysis of variance (ANOVA) was used to compare differences among more than two groups, and one-way ANOVA was performed for comparison of two groups. Significance was determined using the Holm–Sidak method. Statistical differences in data were considered significant when the p value was less than 0.05. All analyses were performed using SigmaStat version 3.10 (Systat Software).

**Data availability**

For access to data not shown, please contact Andrew Das (andrew.das@otago.ac.nz) or Mark Hampton (mark.hampton@otago.ac.nz). All other data are in the manuscript.

**Author contributions**—K. M. O. and A. B. D. data curation; K. M. O. investigation; K. M. O. methodology; K. M. O. writing-original draft; A. B. D. formal analysis; A. B. D., C. C. W., and M. B. H. writing-review and editing; C. C. W. and M. B. H. conceptualization; C. C. W. and M. B. H. supervision; C. C. W. and M. B. H. funding acquisition; M. B. H. project administration.

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**References**

1. Janke, R., Dodson, A. E., and Rine, J. (2015) Metabolism and Epigenetics. *Annu. Rev. Cell Dev. Biol.* 31, 473–496 CrossRef Medline
2. Felsenfeld, G. (2014) A brief history of epigenetics. *Cold Spring Harb. Perspect. Biol.* 6, a18200 CrossRef Medline
3. Fell, R., and Fraga, M. F. (2012) Epigenetics and the environment: emerging patterns and implications. *Nat. Rev. Genet.* 13, 97–109 CrossRef Medline
4. Etchegaray, J. P., and Mostoslavsky, R. (2016) Interplay between metabolism and epigenetics: a nuclear adaptation to environmental changes. *Mol. Cell* 62, 695–711 CrossRef Medline
5. Radford, E. J. (2018) Exploring the extent and scope of epigenetic inheritance. *Nat. Rev. Endocrinol.* 14, 345–355 CrossRef Medline
6. Waddington, C. H. (1953) Genetic assimilation of an acquired character. *Evolution* 7, 118–126 CrossRef
7. Winterbourne, C. C., and Hampton, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.* 45, 549–561 CrossRef Medline
Redox regulation of DNA methylation

8. Midwinter, R. G., Cheah, F. C., Moskovitz, J., Vissers, M. C., and Winterbourn, C. C. (2006) 1xB is a sensitive target for oxidation by cell-permeable chloramines: inhibition of NF-kB activity by glyoxime chloride through methionine oxidation. *Biochem. J.* **396**, 71–78 CrossRef Medline

9. Lysiotis, C. A., and Kimmelman, A. C. (2017) Metabolic interactions in the tumor microenvironment. *Trends Cell Biol.* **27**, 863–875 CrossRef Medline

10. Palucha, A. K., and Coussens, L. M. (2016) The basis of oncoimmunology. *Cell* **164**, 1233–1247 CrossRef Medline

11. Weitzman, S. A., Weitberg, A. B., Clark, E. P., and Stossel, T. P. (1985) Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science* **227**, 1231–1233 CrossRef Medline

12. Cyr, A. R., and Domann, F. E. (2011) The redox basis of epigenetic modifications: from mechanisms to functional consequences. *Antioxid. Redox Signal.* **15**, 551–589 CrossRef Medline

13. Winterbourn, C. C., Kettle, A. J., and Hampton, M. B. (2016) Reactive oxygen species and neutrophil function. *Ann. Rev. Biochem.* **85**, 765–792 CrossRef Medline

14. Grisham, M. B., Jefferson, M. M., Melton, D. F., and Thomas, E. L. (1984) Chlorination of endogenous amines by isolated neutrophils: ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. *J. Biol. Chem.* **259**, 10404–10413 Medline

15. Peskin, A. V., and Winterbourn, C. C. (2001) Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Radic. Biol. Med.* **30**, 572–579 CrossRef Medline

16. Thomas, E. L., Grisham, M. B., and Margaret Jefferson, M. (1986) Preparative and characterization of chloramines. *Methods Enzymol.* **132**, 569–585 CrossRef Medline

17. Wheater, R. F., and Roberts, S. H. (1987) An improved lymphocyte culture technique: deoxycytidine release of a thymidine block and use of a constant humidity chamber for slide making. *J. Med. Genet.* **24**, 113–114 CrossRef Medline

18. Peskin, A. V., Midwinter, R. G., Harwood, D. T., and Winterbourn, C. C. (2005) Chlorine transfer between glyoxine, thione, and histamine: reaction rates and impact on cellular reactivity. *Free Radic. Biol. Med.* **38**, 397–405 CrossRef Medline

19. Kilgore, J. A., Du, X., Melito, L., Wei, S., Wang, C., Chin, H. G., Posner, B., Pradhan, S., Ready, J. M., and Williams, N. S. (2013) Identification of DNMT1 selective antagonists using a novel scintillation proximity assay. *J. Biol. Chem.* **288**, 19673–19684 CrossRef Medline

20. Herring, I. L., Rogstad, D. K., and Sowers, L. C. (2009) Enzymatic methylation of DNA in cultured human cells studied by stable isotope incorporation and mass spectrometry. *Chem. Res. Toxicol.* **22**, 1060–1068 CrossRef Medline

21. O’Hagan, H. M., Wang, W., Sen, S., Destefano Shields, C., Lee, S. S., Zhang, Y. W., Clements, E. G., Cai, Y., Van Neste, L., Easwaran, H., Casero, R. A., Sears, C. L., and Baylin, S. B. (2011) Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and Polycorb members to promoter CpG islands. *Cancer Cell* **20**, 606–619 CrossRef Medline

22. Kang, J. I., and Sowers, L. C. (2008) Examination of hypochlorous acid-induced damage to cytosine residues in a CpG dinucleotide in DNA. *Chem. Res. Toxicol.* **21**, 1211–1218 CrossRef Medline

23. Winterbourn, C. C., and Kettle, A. J. (2000) Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic. Biol. Med.* **29**, 403–409 CrossRef Medline

24. Valinluck, V., Wu, W., Liu, P., Neidigh, J. W., and Sowers, L. C. (2006) Impact of cytosine 5-halogenation on the interaction of DNA with restriction endonucleases and methyltransferase. *Chem. Res. Toxicol.* **19**, 556–562 CrossRef Medline

25. Valinluck, V., and Sowers, L. C. (2007) Inflammation-mediated cytosine damage: a mechanistic link between inflammation and the epigenetic alterations in human cancers. *Cancer Res.* **67**, 5583–5586 CrossRef Medline

26. Knutson, C. G., Mangerich, A., Zeng, Y., Raczyński, A. R., Liberman, R. G., Kang, P., Ye, W., Prestwich, E. G., Lu, K., Wishnok, J. S., Konzenk, J. R., Wogan, G. N., Fox, J. G., Dedon, P. C., and Tannenbaum, S. R. (2013) Chemical and cytokine features of innate immunity characterize serum and tissue profiles in inflammatory bowel disease. *Proc. Natl. Acad. Sci. U.S.A.* **110**, E2332–E2341 CrossRef Medline

27. Fedele, B. I., Freudenthal, B. D., Yau, E., Singh, V., Chang, S. C., Li, D., Delaney, J. C., Wilson, S. H., and Essigmann, J. M. (2015) Intrinsic mutagenic properties of 5-chlorocytosine: a mechanistic connection between chronic inflammation and cancer. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E4571–E4580 CrossRef Medline

28. Fraga, C. G., Shigenaga, M. K., Park, J. W., Degan, P., and Ames, B. N. (1990) Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4533–4537 CrossRef Medline

29. Mangerich, A., Dedon, P. C., Fox, J. G., Tannenbaum, S. R., and Wogan, G. N. (2013) Chemistry meets biology in colitis-associated carcinogenesis. *Free Radi. Res.* **47**, 958–986 CrossRef Medline

30. Waddington, C. H. (1942) Regulation of development and the inheritance of acquired characters. *Nature* **150**, 563–565 CrossRef

31. Pujadas, E., and Feinberg, A. P. (2012) Regulated noise in the epigenetic landscape of development and disease. *Cell* **148**, 1123–1131 CrossRef Medline

32. Shen, H., and Laird, P. W. (2013) Interplay between the cancer genome and epigenome. *Cell* **153**, 38–55 CrossRef Medline

33. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 CrossRef Medline

34. Malech, H. L., and Gallin, J. I. (1987) Neutrophils in human diseases. *N. Engl. J. Med.* **317**, 687–694 CrossRef Medline

35. Kietzmann, T., Petry, A., Shvetsova, A., Gerhold, J. M., and Görlach, A. (2017) The epigenetic landscape related to reactive oxygen species formation in the cardiovascular system. *Br. J. Pharmacol.* **174**, 1533–1554 CrossRef Medline

36. Mikheev, Y., Görlach, A., Knauß, U. G., and Daiber, A. (2015) Redox regulation of genome stability by effects on gene expression, epigenetic pathways and DNA damage/repair. *Redox Biol.* **5**, 275–289 CrossRef Medline

37. Friso, S., Choi, S.-W., Dolnikowski, G. G., and Selhub, J. (2002) A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Anal. Chem.* **74**, 4526–4531 CrossRef Medline

38. Gray, J. W., and Coffino, P. (1979) Cell cycle analysis by flow cytometry. in *Methods Enzymol.* **58**, 233–248 CrossRef

39. Elranta, T. O., Kajander, E. O., and Raina, A. M. (1976) A new method for the assay of tissue: S-adenosylhomocysteine and S-adenosylmethionine: effect of pyridoxine deficiency on the metabolism of S-adenosylhomocysteine, S-adenosylmethionine and polyamines in rat liver. *Biochem. J.* **160**, 287–294 CrossRef Medline

40. Grimes, A. (1985) Red cell metabolism: a manual of biochemical methods, 3rd edition. *Biochem. Soc. Trans.* **13**, 1259 CrossRef

41. Cotgreave, I. A., and Moldëus, P. (1986) Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems. *J. Biochem. Biophys. Methods* **19**, 231–249 CrossRef Medline

42. Staal, F. J., Anderson, M., and Herzenberg, L. A. (1995) Redox regulation of activation of NF-κB transcription factor complex: effects of N-acetylcysteine. *Methods Enzymol.* **252**, 168–174 CrossRef Medline

43. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 CrossRef Medline