Reactive Oxygen Species Dictate the Apoptotic Response of Melanoma Cells to TH588

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The effect of MTH1 inhibition on cancer cell survival has been elusive. Here we report that although silencing of MTH1 does not affect survival of melanoma cells, TH588, one of the first-in-class MTH1 inhibitors, kills melanoma cells through apoptosis independently of its inhibitory effect on MTH1. Induction of apoptosis by TH588 was not alleviated by MTH1 overexpression or introduction of the bacterial homolog of MTH1 that has 8-oxodGTPase activity but cannot be inhibited by TH588, indicating that MTH1 inhibition is not the cause of TH588-induced killing of melanoma cells. Although knockdown of MTH1 did not impinge on the viability of melanoma cells, it rendered melanoma cells sensitive to apoptosis induced by the oxidative stress inducer elesclomol. Of note, treatment with elesclomol also enhanced TH588-induced apoptosis, whereas a reactive oxygen species scavenger or an antioxidant attenuated the apoptosis triggered by TH588. Indeed, the sensitivity of melanoma cells to TH588 was correlated with endogenous levels of reactive oxygen species. Collectively, these results indicate that the cytotoxicity of TH588 toward melanoma cells is not associated with its inhibitory effect on MTH1, although it is mediated by cellular production of ROS.


INTRODUCTION

Targeting BRAF and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase using specific inhibitors have become the standard of care for patients with late-stage mutant BRAF melanomas (Long et al., 2014). However, the benefits are often of limited duration because of rapid development of resistance (Robert et al., 2015). The antibodies against immune checkpoints, including CTLA4, PD-1, and PD-L1, can result in long-lasting melanoma regression, but they are effective in only a subset of patients (Sharma and Allison, 2015). Although considerable efforts have been made to improve the efficacy of these existing therapeutic agents, an alternative approach that has recently gained resurgence in the treatment of cancer is targeting more generic anomalies of cancer cells (Alexandrov et al., 2013).

Reactive oxygen species (ROS) are by-products of oxidative phosphorylation, which regulates a variety of signaling pathways important for cell survival and proliferation (Hoidal, 2001). Cancer cells commonly contain elevated levels of ROS resulting from oncogenic stimulation (Liou and Storz, 2010). In particular, the increased production of ROS is a major mechanism of melanomagenesis caused by UV radiation (Anna et al., 2007). On one hand, ROS promote cancer cell survival, proliferation, and metastasis (Gius et al., 2006). On the other, high levels of ROS suppress tumor growth through inhibition of proliferation and induction of apoptosis and senescence via damage to DNA (Ramsey et al., 2006; Takahashi et al., 2006; Zhang et al., 2011). Incorporation of oxidized deoxyribonucleotide-triphosphates such as 8-oxo-deoxy-guanine (8-oxo-dGTP) and 2-OH-deoxy-adenosine into genomic DNA plays an important role in apoptosis induced by ROS (Ichikawa et al., 2008). Nevertheless, it seems that cancer cells have adapted to ROS-mediated tumor suppression without eliminating ROS production and their oncogenic functions (Rai, 2012).

One of the mechanisms that protects cancer cells from the cytotoxic effect of high levels of ROS is the expression of MutT homolog 1 (MTH1), which sanitizes oxidized deoxyribonucleotide-triphosphates pools through converting 8-oxo-dGTP and 2-OH-deoxy-adenosine into monophosphates, thus preventing their incorporation into genomic DNA (Ichikawa et al., 2008). Although MTH1 is increased in many types of cancers (Giribaldi et al., 2015; Obtulowicz et al., 2010), it is dispensable for normal cells (Tsuzuki, 2001). This has led to the development of MTH1 inhibitors in the treatment of cancer (Gad et al., 2014; Huber et al., 2014). Among them is TH588, which kills cancer cells, leading to impressive therapeutic responses in various human cancer
Supplementary Figure S1b and c). TH588 did not induce significant apoptosis in Mel-FH, HEMn-MP, and HEMn-DP (Figure 1a, and see Supplementary Table S1 online). The variation was not correlated with MTH1 expression levels, nor was it associated with the mutational status of BRAF and NRAS (see Supplementary Figures S1a and S1b online). There was a wide variation in the sensitivity of melanoma cells to TH588, with the half-maximum inhibitory concentration value ranging from 8.6 μmol/L in Mel-RM to greater than 1,800 μmol/L in Mel-FH cells (see Supplementary Figure S1a and Supplementary Table S1 online). The variation was not correlated with MTH1 expression levels, nor was it associated with the mutational status of BRAF and NRAS (see Supplementary Figure S1b and c). TH588 did not induce significant cell death in any of the three melanocyte lines, HEMa-LP, HEMn-MP, and HEMn-DP (Figure 1a, and see Supplementary Figure S2a and b online). The general caspase inhibitor z-VAD-fmk abolished TH588-induced killing, indicating that cell death was caused by apoptosis (Figure 1b). TH588 caused caspase-3 activation and cleavage of poly (adenosine diphosphate-ribose) polymerase in sensitive (Mel-RM and IgR3) but not resistant (Mel-FH) melanoma cells (Figure 1c).

As anticipated, TH588 at 8 μmol/L triggered accumulation of 8-oxodGTP in DNA and increased nuclear 53BP1 foci in Mel-RM and IgR3 but not Mel-FH cells, indicating that TH588 inhibited MTH1 activity and caused DNA double-strand breaks in sensitive but not resistant melanoma cells (Figure 1d) (Schultz et al., 2000). Consistently, TH588 triggered ATM-dependent phosphorylation of p53 (pSer15-p53) and induction of the p53 target p21 in Mel-RM and IgR3 but not Mel-FH cells (Figure 1e) (Jabbur et al., 2002).

The variation in the sensitivity of melanoma cells to TH588 was reflected by long-term survival of Mel-RM, IgR3, and Mel-FH cells, as shown in clonogenic assays (Figure 1f). Similarly, it was mirrored in cells grown in three-dimensional cultures (Figure 1g). TH588 induced varying degrees of apoptosis in a panel of fresh melanoma isolates, which was similarly not associated with the expression levels of MTH1 (see Supplementary Figure S3a—c online).

**RESULTS**

**The sensitivity of melanoma cells to TH588 is not associated with MTH1 expression levels**

We characterized the cell death-inducing potential of TH588 in a panel of melanoma cell lines, which carried wild-type MTH1, as determined by sequencing of all four exons (including the intron/exon boundaries) of its gene NUDT1, and had varying status of the most common mutations in BRAF (BRAFV600E) and NRAS (NRASQ61K) (Figure 1a). There was a wide variation in the sensitivity of melanoma cells to TH588, with the half-maximum inhibitory concentration value ranging from 8.6 μmol/L in Mel-RM to greater than 1,800 μmol/L in Mel-FH cells (see Supplementary Figure S1a and Supplementary Table S1 online). The variation was not correlated with MTH1 expression levels, nor was it associated with the mutational status of BRAF and NRAS (see Supplementary Figure S1b and c). TH588 did not induce significant cell death in any of the three melanocyte lines, HEMa-LP, HEMn-MP, and HEMn-DP (Figure 1a, and see Supplementary Figure S2a and b online). The general caspase inhibitor z-VAD-fmk abolished TH588-induced killing, indicating that cell death was caused by apoptosis (Figure 1b). TH588 caused caspase-3 activation and cleavage of poly (adenosine diphosphate-ribose) polymerase in sensitive (Mel-RM and IgR3) but not resistant (Mel-FH) melanoma cells (Figure 1c).

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**Knockdown of MTH1 does not affect survival of melanoma cells**

We knocked down MTH1 in melanoma cells using two individual small hairpin RNAs (shRNAs), both of which resulted in reduction in MTH1 protein expression levels by more than 80% in Mel-RM, IgR3, and Mel-FH cells (Figure 2a). Although shRNA knockdown of MTH1 reduced its activity, it did not impinge on cell viability measured on day 2 or day 6 after transduction of shRNAs, even in Mel-RM and IgR3 cells that were sensitive to TH588 (Figures 2b—d, and see Supplementary Figures S4a—c online) (Gad et al., 2014). Similar to TH588, MTH1 knockdown did not trigger signs of DNA double-strand breaks in Mel-FH cells (Figure 2b and c, and see Supplementary Figure S4a and b).

To confirm the results with shRNA knockdown of MTH1, we carried out small interfering (siRNA) knockdown experiments using two siRNAs with published sequences (Gad et al., 2014), both of which similarly caused reduction in MTH1 protein expression levels by more than 80% in Mel-RM, IgR3, and Mel-FH cells (see Supplementary Figure S5a online). However, they triggered DNA double-strand breaks in only Mel-RM and IgR3 but not in Mel-FH cells (see Supplementary Figure S5b). Similar to shRNA knockdown, siRNA knockdown of MTH1 did not affect survival of melanoma cells measured on day 2 or day 6 after siRNA transfection (see Supplementary Figure S5c) (Gad et al., 2014). The magnitude of DNA damage caused by MTH1 knockdown using shRNA or siRNA is less than that resulting from TH588 treatment in Mel-RM and IgR3 cells (Figure 1d and 2b, and see Supplementary Figures S4b and S5b).

We also carried out clonogenic assays using Mel-RM and IgR3 cells with or without MTH1 knocked down by shRNA or siRNA, as previously reported (Gad et al., 2014). The results showed that knockdown of MTH1 did not affect the clonogenic potential of Mel-RM and IgR3 cells (Figure 2e, and see Supplementary Figure S5d).

To test the possibility that the remaining MTH1 protein after shRNA or siRNA knockdown is sufficient to limit DNA damage and thus protect cells from apoptosis, we established a Mel-RM subline that carried an inducible MTH1 shRNA system in response to tetracycline. The addition of tetracycline achieved visually complete inhibition of MTH1 protein expression and caused DNA damage (Figure 2f and g). Nevertheless, it did not impinge on Mel-RM cell survival on day 2 or day 6 after treatment (Figure 2h). Similarly, it did not affect the clonogenic activity of Mel-RM cells (Figure 2i).

When Mel-RM, IgR3, and Mel-FH cells with MTH1 knocked down by shRNA or siRNA were exposed to TH588, it was found that the combination of MTH1 knockdown and TH588 further enhanced DNA damage and apoptosis in melanoma cells (Figure 2j, and see Supplementary Figures S5e and S6 online).

**TH588 induces apoptosis of melanoma cells independently of MTH1**

We stably introduced a construct with increasing concentrations of wild-type MTH1 cDNA into Mel-RM and IgR3 cells (Figure 3a). Overexpression of MTH1 at even the highest

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Figure 1. TH588 induces apoptosis of melanoma cells. (a) Various sensitivities of melanoma cell and melanocyte lines to TH588. (b) Killing of melanoma cells by TH588 was inhibited by z-VAD-fmk. (c) TH588 activated caspase-3 and cleaved PARP in IgR3 and Mel-RM cells. (d) TH588 triggered nuclear accumulation of 8-oxo-dGTP and 53BP1 foci in Mel-RM and IgR3 cells. Green, 8-oxo-dGTP; red, 53BP1; blue, DAPI (nuclei). Numbers represent relative staining intensity. Scale bar = 25 μm. (e) TH588 induced p53pS15 and p21 in Mel-RM and IgR3 cells. (f) TH588 inhibited the clonogenic potential in IgR3 and Mel-RM. Scale bar = 1 cm. (g) TH588 kills IgR3 and Mel-RM cells grown in three-dimensional culture. Green, living cells; red, dead cells. Scale bar = 25 μm. n = 3, mean ± standard error of the mean or representative. *P < 0.05, Student t-test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, hours; hrs, hours; M, mutant; M, mol/L; PARP, poly (adenosine diphosphate-ribose) polymerase; W, wild-type.
Figure 2. MTH1 knockdown does not affect melanoma cell survival. (a) shRNA knockdown of MTH1 (b) triggered nuclear accumulation of 8-oxo-dGTP and 53BP1 foci, (c) induced p53S315 and p21, but did not affect (d) melanoma cell survival or (e) the clonogenic potential. (f) Tetracycline-triggered MTH1 silencing (g) caused DNA damage, but did not affect (h) survival or (i) the clonogenic potential of Mel-RM cells carrying an inducible MTH1 shRNA system. (j) Melanoma cells with MTH1 knocked down by shRNA were more sensitive to TH588. n = 3, mean ± standard error of the mean or representative; *P < 0.05, Student t test. (b, g) Scale bar = 25 μm. (e, i) Scale bar = 1 cm. Numbers represent either relative levels MTH1 or staining intensity. 8-oxo-dGTP, 8-oxo-deoxy-guanine; Ctrl, control; shRNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; small hairpin RNA; Tet, tetracycline; UT, untreated.
levels achieved did not significantly inhibit TH588-induced apoptosis (Figure 3b). Similarly, it only modestly inhibited TH588-triggered DNA damage (Figure 3c). These results suggest that mechanisms other than inhibition of MTH1 play a major role in DNA damage and apoptosis induced by TH588 in melanoma cells.

To examine the likelihood that TH588 at the concentration used also inhibited exogenously introduced MTH1, we expressed the bacterial homologue of MTH1 (MutT) that has 8-oxo-dGTPase activity but cannot be inhibited by TH588 in melanoma cells as described previously (Figure 3d) (Gad et al., 2014). Expression of mitochondrial MutT or coexpression of mitochondrial and nuclear MutT did not rescue melanoma cells from cell death caused by TH588 (Figure 3e), further confirming that TH588 kills melanoma cells through mechanisms that are not associated with inhibition of MTH1.

Oxidative stress determines the sensitivity of melanoma cells to TH588

Because inhibition of MTH1 causes DNA damage by ROS (Gad et al., 2014), we examined whether induction of ROS affects the potential cytotoxicity of MTH1 knockdown toward melanoma cells. Although the oxidative stress inducer elesclomol did not affect survival of Mel-RM and IgR3 cells transduced with the control shRNA, it induced apoptosis of the cells with MTH1 knocked down (Figures 2a and 4a) (Qu et al., 2010). Moreover, it triggered apoptosis, albeit moderately, in Mel-FH cells with MTH1 knocked down (Figure 4a).

We also examined whether TH588-induced apoptosis of melanoma cells is caused by oxidative stress. Indeed, elesclomol enhanced apoptosis induced by TH588 (Figure 4b). In contrast, the ROS scavenger N-acetyl-L-cysteine or the antioxidant glutathione attenuated apoptosis induced by TH588 (Figure 4c). These results show that the
apoptosis-inducing potential of both MTH1 inhibition and TH588 is associated with the severity of oxidative stress and suggest that TH588 kills melanoma cells by interfering with a mechanism(s), which, similar to MTH1, protects melanoma cells from oxidative stress-induced killing.

Sensitivity of melanoma cells to TH588 is correlated with the levels of endogenous ROS

We quantitated ROS production in melanoma cell lines by staining with the fluorescent ROS probe DHR123 (Lee et al., 2015). As anticipated, the levels of ROS were commonly higher in melanoma cell lines than melanocyte lines, which were positively correlated with the sensitivity of melanoma cells to apoptosis induced by TH588 (Figure 5a and b). The levels of carbonylated proteins that reflect ROS levels were similarly correlated with TH588-induced apoptosis (Figure 5c and d) (Dalle-Donne et al., 2003). The relationship between the levels of ROS and the sensitivity of melanoma cells to TH588 was consolidated in the panel of fresh melanoma isolates (Figure 5e, and see Supplementary Figures S3a and S7 online).

Activation of BAD and down-regulation of BCL-2 and MCL-1 mediate apoptosis of melanoma cells induced by TH588

TH588 activated the mitochondrial apoptotic pathway, as shown by induction of caspase-9 activation, reduction in the mitochondrial membrane potential, and mitochondrial release of apoptogenic proteins in Mel-RM and IgR3 cells (see Supplementary Figure S8a and b online) (Kroemer et al., 2007). However, it did not affect the expression of PUMA and NOXA, which are known to be responsive to DNA damage (see Supplementary Figure S8c) (Zinkel et al., 2006). Instead, it caused dephosphorylation (activation) of BAD and down-regulation of BCL-2 and MCL-1 in Mel-RM and IgR3 but
not in Mel-FH cells (Figure 6a). Although MTH1 knockdown did not cause any of these changes, it enabled elesclomol to activate BAD and down-regulate BCL-2 and MCL-1 (Figure 6b, and see Supplementary Figure S8d and). The role of BAD in apoptosis induced by TH588 or by elesclomol along with MTH1 knockdown was confirmed by shRNA knockdown of BAD (Figures 6c-e). On the other hand, overexpression of MCL-1 or BCL-2 protected Mel-RM and IgR3 cells from apoptosis induced by TH588 or elesclomol in combination with MTH1 knockdown (Figures 6f-h).

DISCUSSION

Although a number of MTH1 inhibitors, including TH588 and SCH51344, have been shown to kill cancer cells potently, leading to impressive therapeutic responses in various human cancer xenografts (Gad et al., 2014; Huber et al., 2014), recent studies have cast doubt on the potential of MTH1 as a therapeutic target (Kawamura et al., 2016; Kettle et al., 2016; Petrocchi et al., 2016). This is because, unlike reported first-in-class MTH1 inhibitors, some newly synthesized specific MTH1 inhibitors did not have any inhibitory effect on cell survival (Kawamura et al., 2016; Kettle et al., 2016). Moreover, knockdown of MTH1 or its knockout did not impinge on survival of cancer cells (Kawamura et al., 2016; Kettle et al., 2016). Although the reason(s) leading to these inconsistent observations remains unclear, the experimental conditions used by different investigators varied considerably (Brautigam et al., 2016; Gad et al., 2014; Gao et al., 2015; Huber et al., 2014; Kawamura et al., 2016). Regardless, because dysfunctional redox regulation is of particular importance in the pathogenesis of melanoma (Hall et al., 2013), we were prompted to examine the effect of MTH1 inhibition on survival of melanoma cells.

Our results indicate that TH588 induces apoptosis of melanoma cells independently of its inhibitory effect on MTH1 (Kawamura et al., 2016; Kettle et al., 2016). This is primarily for two reasons. First, although TH588 induced apoptosis of melanoma cells, MTH1 knockdown using multiple shRNAs and siRNAs did not affect the viability of melanoma cells that were sensitive to TH588-induced apoptosis, which was tested in diverse experimental settings at various time points after introduction of shRNAs or siRNAs (Gad et al., 2014). Second, overexpression of wild-type MTH1 or introduction of MutT that has 8-oxo-dGTPase activity but cannot be inhibited by TH588 did not rescue melanoma cells from killing by TH588.
Figure 6. BAD activation and BCL-2 and MCL-1 down-regulation mediate TH588-induced apoptosis of melanoma cells. (a) TH588 activated BAD and down-regulated BCL-2 and MCL-1 in IgR3 and Mel-RM but not Mel-FH cells. (b) Elesclomol triggered BAD activation and BCL-2 and MCL-1 up-regulation in melanoma cells with MTH1 knocked down. (c) shRNA knockdown of BAD inhibited (d) TH588-induced killing of IgR3 and Mel-RM cells and (e) elesclomol-triggered killing in IgR3 and Mel-RM cells with MTH1 knocked down. (f) Overexpression of MCL-1 or BCL-2 protected (g) IgR3 and Mel-RM cells from TH588-induced killing and (h) elesclomol-triggered killing in IgR3 and Mel-RM cells with MTH1 knocked down. n = 3, mean ± standard error of the mean or representative. *P < 0.05, Student t test. Ctrl, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, hours; hrs, hours; M, mol/L; shRNA, small hairpin RNA.
Although MTH1 knockdown, similar to treatment with TH588, resulted in DNA damage in melanoma cells that were sensitive, but not in those resistant, to TH588-induced apoptosis, the magnitude of DNA damage caused by MTH1 knockdown is less than that resulting from TH588. Given our results showing that knockdown of MTH1 does not affect melanoma cell survival, these observations suggest that, despite its inhibitory effect on MTH1, TH588 also triggers DNA damage and apoptosis in melanoma cells through inhibiting targets that share similar roles with MTH1 in protecting melanoma cells from oxidative stress (Brautigam et al., 2016). In support of this, inhibition of MTH1 sensitized melanoma cells to TH588-induced apoptosis. Moreover, although the oxidative stress inducer esclomol rendered melanoma cells sensitive to MTH1 knockdown, it also enhanced apoptosis induced by TH588. On the other hand, a ROS scavenger or an antioxidant attenuated melanoma cell death caused by TH588.

The molecular target(s) responsible for TH588-induced killing of melanoma cells remains undefined, but it is known that OGG1 and MUTYH are important in repair of 8-oxo-dGTP and 2-OH-deoxy-adenosine lesions in DNA (Nakabeppu, 2014). However, overexpression of OGG1 or MUTYH did protect cells from TH588-induced cell death (Gad et al., 2014). Regardless, the potential of TH588 as an antimelanoma drug warrants further investigations, because TH588 did not have significant toxicity toward melanocytes and other types of normal cells (Gad et al., 2014). The finding that the sensitivity of melanoma cells to TH588 was not related to the most common mutations in BRAF and NRAS suggests that these oncogenic drivers do not pose a barrier for application of TH588 in the treatment of melanoma. Because melanoma cells, similar to many other types of cancer cells, contain elevated levels of ROS (Liou and Storz, 2010; Myant et al., 2013; Nogueira and Hay, 2013), it is plausible that the sensitivity of melanoma cells to TH588 is correlated with the endogenous levels of ROS. This not only suggests that the constitutive oxidative stress determines the potency of TH588 but also implies that ROS levels may be a biomarker for prediction of the response of melanomas to the compound.

Our results showed that TH588-induced apoptosis of melanoma cells was mediated by activation of BAD and down-regulation of BCL-2 and MCL-1. This may be associated with high levels of ROS that inhibit protein kinase B activation and promote activation of c-Jun N-terminal kinase and p38, which in turn contribute to BAD activation and down-regulation of BCL-2 and MCL-1 caused by TH588 (Zhang et al., 2011). Moreover, inhibition of NF-kB by high levels of ROS may also be involved (Xu et al., 2015). Elesclomol-induced apoptosis in melanoma cells with MTH1 knockdown was also mediated by BAD, consistent with the finding that inhibition of MTH1 sensitizes melanoma cells to oxidative stress.

The results from this study bear several implications about the potential usefulness of TH588 and MTH1 inhibition in the treatment of melanoma: (i) TH588-induced apoptosis of melanoma cells is not associated with its inhibitory effect on MTH1, (ii) TH588 remains a promising candidate for the treatment of melanoma, (iii) MTH1 inhibition in combination with oxidative stress inducers may be a useful approach in melanoma treatment, and (iv) the endogenous levels of ROS are a potential biomarker for predicting the response of melanomas to TH588 and MTH1 inhibition in combination with oxidative stress inducers.

**MATERIALS AND METHODS**

**Cell culture**

Human melanoma cell lines and human melanocyte lines were obtained and cultured as described previously (Jiang et al., 2010). Human fresh melanoma isolates were prepared from surgical specimens according to the published method (Nguyen et al., 2001). Studies using human tissues were approved by the Human Research Ethics Committee of The University of Newcastle, Australia (X11-0023&HREC/11/RPA) and are in agreement with the guidelines set forth by Declaration of Helsinki. All participants provided written informed consent.

**Three-dimensional culture**

Three-dimensional culture was performed using the hanging drop technique, as previously described (Jiang et al., 2015). Cells were stained with calcein AM (living cell stain) and ethidium homodimer-1 (dead cell stain) (Life Technologies, Scoresby, Victoria, Australia) for 24 hours, followed by treatment.

**Antibodies and reagents**

Antibodies and reagents used are listed in Supplementary Tables S1, S2, and S3 online.

**Western blotting**

Western blotting was carried out as described previously (Jiang et al., 2011). The intensity of Western blot bands was quantitated with ImageReader LAS-4000 (Fujifilm, Tokyo, Japan).

**Preparation of mitochondrial and cytosolic fractions**

Methods used for subcellular fraction were similar to the methods previously described (Wang et al., 2016).

**Dihydrorhodamine 123 staining assays**

ROS were measured by detecting the fluorescent intensity of oxidant-sensitive probe dihydrohodamine 123 (Lee et al., 2015), which was recorded using a Synergy 2 multidetection microplate reader (BioTek, Winooski, VT) at excitation of 485 nm and emission of 538 nm.

**Protein carbonyl assays**

Protein carbonyls were measured from homogenates using 2,4-dinitrophenylhydrazine (DNPH) derivatization with a Protein Carbonyl Assay Kit (Abcam, Cambridge, MA) according to the manufacturer’s instructions (Dalle-Donne et al., 2003).

**Immunofluorescence**

Immunofluorescence staining was carried out as described previously (Zhang et al., 2000). Quantification was done by manual counting of positive cells under a fluorescence microscope (Carl Zeiss, Sydney, Australia).

**Statistics**

Statistical analysis was performed using JMP Statistics Made Visual software (SAS, Cary, NC). Student t test was used to assess differences between different groups. A P-value less than 0.05 was considered statistically significant.
CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1111/jid.2016.06.625.

REFERENCES


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