Electrophilic stress induced by itaconate and its derivatives regulates ATF3-IκBζ inflammatory axis independently of Nrf2 induction.

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Abstract

Metabolic regulation recently emerged as a novel powerful principle guiding immune responses. Natural metabolite itaconate and its membrane permeable derivative dimethyl itaconate (DI) were recently shown to selectively inhibit a subset of cytokines during macrophage activation (e.g. IL-1β, IL-6, IL-12 but not TNF-α), yet the precise mechanism of this effect remained unclear. We show here that itaconate/DI react with glutathione and subsequently induce both Nrf2-dependent and Nrf2-independent stress responses. We find that striking selectivity of DI action stems from the inhibitory effects of electrophilic stress on IκBζ protein translation, leading to inhibition of only secondary wave of NF-κB signaling. We find that IκBζ regulation occurs in an Nrf2-independent manner, and identify ATF3 as a key mediator of the immunosuppression. This inhibitory effect is conserved across species and cell types, as evident from inhibition of IκBζ production in activating human monocytes and IL-17 stimulated keratinocytes of both human and mice. Finally, DI administration *in vivo* ameliorated IL-17/IκBζ-driven skin pathology in the mouse model of psoriasis, highlighting therapeutic potential of this regulatory pathway.

Recent discoveries in the field of immunometabolism highlighted the importance of the fundamental metabolic principles in immune regulation[1,2,3]. One striking example of novel immunoregulatory metabolite was described in our recent work: itaconate, produced by inflammatory macrophages, regulates their metabolic rewiring, and affects cytokine production[4,5,6]. Using mice lacking natural itaconate due to *Irg1* deficiency, as well as the cell membrane permeable derivative of itaconate, dimethyl itaconate (DI), we showed that itaconate plays an anti-inflammatory role. Itaconate/DI specifically affected a subset of inflammatory cytokines such IL-1β, IL-6, IL-12, while having no effect on cytokines such as TNF-α[6].

The major effects of itaconate on cellular metabolism has been mainly accounted for inhibition of succinate dehydrogenase (SDH)[6,7,8]. However, SDH inhibition alone is not sufficient for the striking immunoregulatory effects observed with DI: utilization of classical SDH inhibitor dimethylmalonate does not yield comparable levels of cytokine suppression[6,9]. Additionally, specific targets within the inflammatory signaling cascade that are responsible for such selective effects of itaconate/DI on inflammatory program have not been identified. Here, we utilize DI as a powerful tool to understand the selectivity of metabolic regulation of the inflammatory program (e.g. effects on IL-6 vs TNF-α production).

To that end, we first utilized our previously published transcriptional analysis of DI-treated bone-marrow derived macrophages (BMDMs)[6]. Differential gene expression showed enrichment for electrophilic/xenobiotic stress response pathways (see Fig. S1B in Lampropoulou et al[6]). Specifically, genes such as *Hmox1*, *Nqo1*, *Gclc* etc. (Fig. 1a) were significantly upregulated upon DI treatment. These genes are classical transcriptional markers of activation of Nrf2-mediated response to oxidative and electrophilic stress[10]. Indeed, DI is an α,β-unsaturated carboxylic ester that can readily act as an electrophile in Michael reaction (Fig. 1b). Accordingly, the Kelch-like ECH-associated protein 1 (Keap1)-Nrf2-associated signature was apparent in transcriptional profiles of DI-treated BMDMs, which was absent from the profiles of Keap1 conditional KO BMDMs, recently described by Kobayashi et al[11] (Fig. 1c). Indeed, we observed that Nrf2 protein

levels as well as protein levels of typical Nrf2 target genes such as NQO1 and HO-1 progressively increased during 12 hour treatment (Fig. 1d). To evaluate whether endogenous itaconate is also able to induce Keap1-Nrf2 response, we compared the levels of Nrf2 protein between *Irg1*$^{-/-}$ and WT macrophages upon LPS activation. Indeed, in the absence of endogenous itaconate, Nrf2 induction was strongly decreased, indicating ability of natural itaconate to induce electrophilic stress response (Fig. 1e).

Such electrophilic stress induced by α,β-unsaturated compounds is commonly controlled by the cells via glutathione reactivity. For instance, covalent conjugation with GSH was described for fumarate, natural unsaturated dicarboxylic acid typically accumulated in cells with mutated fumarate hydratase[12,13]. Thus, we investigated whether DI or natural itaconate can form covalent adducts with GSH yielding diester or acid form of methylsuccinated glutathione (Fig. 1f). Indeed, GCMS and LCMS analysis of the cell media from DI-treated macrophages confirmed that DI was uptaken from media (Extended Data Fig1 a) and revealed a significant peak ($p<0.001$) at retention time 14.4 min with the m/z of 464.1334 that was accumulating extracellularly over the incubation time with DI, absent in cell free media (Fig. 1g, Extended Data Fig. 1c), tentatively corresponding to diester of methylsuccinated glutathione. A standard of the proposed DI-GSH conjugate, was synthesized and used to confirm the proposed metabolite; the retention time of the synthesized standard matched the species observed in cell media (Extended Data Fig. 1b). Furthermore, identity of the peak was confirmed using synthesized $^{13}C_5$-labeled DI for cell treatment (Extended Data Fig. 1d). This conjugate was not observed in a cell-free environment, indicating that DI-GSH is covalently formed intracellularly and then excreted into the extracellular space. Importantly, the reactivity for glutathione was also observed for natural itaconate: we detected methylsuccinated glutathione (Ita-GSH, Fig. 1f) in LPS-stimulated macrophages (Fig .1h). In fact, levels of Ita-GSH conjugate correlated with itaconate production and were absent in *Irg1*$^{-/-}$ cells (Fig. 1h). We confirmed the identity of this metabolite by LCMS retention time with a synthesized standard (Extended Data Fig. 1e).

The reactivity of DI with GSH translated into substantial drop of cellular GSH concentration and associated increase in ROS generation (Fig 1i, Extended Data Fig. 1f). To analyze the contribution of oxidative stress on cytokine production, we tested a panel of antioxidants/ROS scavengers by administering them simultaneously with DI. Neither α-tocopherol nor the mitochondrial ROS scavenger MitoTEMPO were able to reverse the effect of DI treatment on cytokine production. However, the effect of DI was reversed by 1 mM *N*-acetylcysteine (NAC) or cell permeable GSH (EtGSH) co-treatment suggesting that DI acted preferentially by modulating the cellular pool of thiol-containing molecules (Fig. 1j, Extended Data Fig. 1g). To see whether selective effect on the cytokine expression can be attributed also to other electrophilic compounds, we treated cells with dimethyl fumarate (DMF). DMF treatment triggered Nrf2 and showed similar selective effect on IL-6 production (Fig. 1k, Extended Data Fig. 1h, i). Notably, selectivity of DI on IL-6 production was also detected in human blood monocytes (Extended Data Fig. 1j). These data suggested that there is a distinct regulatory pathway that connects electrophilic stress response with selective cytokine regulation during macrophage activation.

Thus, we thought to identify inflammatory mediators responsible for the interplay between the electrophilic stress response and the selective inhibition of IL-6 but not TNF-α. In fact, *Tnfa* is a transcript produced within the primary transcriptional response to TLR stimulation, whereas the *Il6* transcript is a product of the second wave of transcriptional responses as well as a number of other genes affected by DI (IL-12, Ccl2, etc.)[14,15]. One major transcription factor that was reported to selectively regulate secondary transcriptional response to TLR activation is IκBζ, encoded by the *Nfkbiz* gene[16]. IκBζ is a co-transcription factor that forms ternary complex with DNA binding p50 homodimers[17] and serves as a major driver for the second wave of NF-κB-mediated transcription. Indeed, using *Nfkbiz*-deficient BMDMs, we confirmed the selective downregulation of *Il6* mRNA in response to LPS stimulation, whereas *Tnfa* mRNA production peaked early and was not affected by the absence of IκBζ (Fig. 2a and Extended Data 2a, b). Strikingly, in BMDMs treated with DI and activated with LPS, the IκBζ protein induction was almost completely

abolished in a dose dependent manner (Fig. 2b, Extended Data Fig 2c). Similarly, DI inhibited LPS-mediated IκBζ induction in human blood monocytes (Extended Data Fig 2d). This suggested that observed specificity of DI action on IL-6 may stem from selective inhibition of the secondary wave of transcriptional response to TLR activation. Thus, we explicitly evaluated the effect of DI on the first wave of NF-κB signaling. Indeed, DI did not inhibit IκBα degradation in response to LPS (Extended Data Fig. 2e) nor did it prevent LPS-mediated p65 nuclear translocation, as shown by confocal microscopy (Fig. 2c). Similarly, IKK phosphorylation and IRAK1 expression were unaffected (Extended Data Fig. 2f). Altogether, these results confirmed that the second transcriptional wave of the TLR response is selectively downregulated by DI. Since we have shown that NAC and EtGSH can rescue the inhibitory effect of DI on IL-6, we tested whether this was associated with recovery of IκBζ protein induction. Indeed, co-treatment of BMDMs with DI and NAC (or EtGSH), but not α-tocopherol, restored LPS-mediated IκBζ induction in BMDMs and human blood monocytes (Fig. 2d, Extended Data Fig 2g, h). To further substantiate the role of IκBζ in the regulation of IL-6 production by DI, we confirmed that NAC itself is not able to elevate IL-6 levels on untreated or DI-treated *Nfkbiz*$^{-/-}$ background BMDMs (Extended Data Fig. 2g). Altogether, our data indicate that IκBζ is central inflammatory mediator of selective action of DI on cytokine production.

Consistent with the notion that primary NF-κB-mediated response is not affected by DI, *Nfkbiz* mRNA levels were not affected up to concentrations of ~300 μM (Fig. 2e, Extended Data Fig. 3a). At high concentrations (300 μM and more), DI induces overwhewlming electrophilic and oxidative stress that was previously reported to inhibit NF-κB-signaling unselectively[18] as also evident from the TNF-α production (Extended Data Fig. 1i). However, at the intermediate concentrations of DI, graded electrophilic stress response affected only IκBζ protein induction at the post-transcriptional level. To analyze the mechanism by which DI downregulated IκBζ protein, we tested the effect of various protein degradation inhibitors. Neither proteasomal inhibitor MG132, nor autophagosome-lysosome fusion inhibitor bafilomycin A restored IκBζ protein levels

upon DI treatment (Extended Data Fig. 3b) indicating that the reduction in IκBζ levels is not due to ubiquitination or protein degradation. Since IκBζ has been shown to be post-transcriptionally regulated by mechanisms involving the 3'UTR of *Nfkbiz* mRNA[19], we tested effect of DI treatment on the mouse microglial cell line BV2 transduced with either *Nfkbiz*-3'UTR-GFP reporter system or empty GFP expressing vector. We did not detect changes in GFP intensity in DI-treated cells compared to untreated cells (Extended Data Fig. 3c) suggesting that DI does not interfere with *Nfkbiz* mRNA stability or protein translation through its 3'UTR elements. Finally, cellular stress had been shown to regulate protein translation via phosphorylation-driven inactivation of the Eukaryotic Initiation Factor 2 (eIF2α)[20]. Indeed, we detected a marked increase in macrophage eIF2α phosphorylation in response to DI (Fig. 2f) suggesting, that the inhibitory effect of DI on IκBζ protein levels is exerted via eIF2α-driven repression of IκBζ translation. Yet, this suppression was very specific to IκBζ, and possibly few other proteins, since metabolic labeling of nascent proteins in DI treated and LPS stimulated macrophages showed that the total level of protein synthesis is not affected by DI (Extended Data Fig. 3d, e). Furthermore, proteomic profiling of DI treated macrophages revealed no global suppression of protein levels based on sampling of ~4000 proteins (Extended Data Fig. 3f, g). These results show that the electrophilic stress exerted by DI specifically interferes with the second wave of transcriptional response to TLR activation via post-transcriptional regulation of IκBζ protein levels (Fig. 2g).

To deeper understand this regulation, we set up a panel of DI-derivatives with graded level of electrophilicity and included there DMF as prototypical electrophile (Fig. 2h). We find that DMF, like DI, was also able to downregulate IκBζ (Fig. 2i) and that effect of itaconate derivatives on IκBζ correlated with their electrophilic strength (Fig. 2j) – 3MI was still able to downregulate the response while MI did not affect IκBζ, while still inducing Nrf2-mediated response. We hypothesized that such Nrf2-mediated feedback that upregulates glutathione synthesis in response to electrophilic compounds might mask immunoregulatory effects of MI. For this purpose we tested combination of DI and MI with buthionyl sulfoximide (BSO), non-electrophilic

glutathione synthase inhibitor, which itself did not affect cytokine production and did not trigger Nrf2 response (Extended Data Fig. 4a, b). Strikingly, BSO strenghtened inhibitory effect of DI on IκBζ/IL-6 (Fig 2k, Extended Data Fig. 4c) and unlocked the effect of MI on IκBζ/IL-6 (Fig. 2l, Extended Data Fig. 4d).

Understanding of the interplay between glutathione buffering and immunomodulatory effects of electrophilic stress enabled us to approach the question of physiological relevance natural itaconate (weak electrophile) and IκBζ-regulatory axis. In fact, temporal dynamics of IκBζ downregulation is reciprocal to induction of itaconate: IκBζ is peaking around 1h and is already downregulated significantly at ~4h, while itaconate is only getting induced at ~2h and platoes after ~12 h of LPS stimulation (Fig. 2m). This, indeed, suggests that itaconate might play a role in downregulating IκBζ response, albeit it is not likely to be primary mechanism since at 1-2 h (when IκBζ starts going down) there is not yet sufficient itaconate levels within the cell. Thus, to address the physiological relevance of endogenous itaconate to IκBζ regulation, we designed an experiment where both natural itaconate and IκBζ are present in the cells at the same time. Specifically, we have looked at the in vitro macrophage tolerization, when cells are first stimulated with LPS then re-challenged with LPS in about 16 hours. This design ensures that endogenous itaconate is produced in sufficient amounts and LPS restimulation triggers IκBζ induction once again. We saw that in the presence of BSO, there was striking difference in IκBζ protein levels upon restimulation between Irg1 KO and WT cells (Fig. 2n, Extended Data Fig. 4e), confirming the regulatory effects of natural itaconate on IκBζ.

We next aimed to identify major regulatory hubs connecting the electrophilic stress response to the blockade of IκBζ induction. First, we tested whether Nrf2 is involved in the DI inhibition of IκBζ synthesis. To that end, we measured IκBζ protein levels and cytokine production in Nrf2-deficient BMDMs activated with LPS in the presence and absence of DI. Absence of Nrf2 did not alleviate DI-mediated IκBζ inhibition at various time points tested (Fig. 3a) and over a range of DI concentrations (Extended Data Fig. 3a). DI-exposed cells also showed similar ability

to inhibit IL-6 in *Nrf2*$^{-/-}$ vs. WT cells (Fig. 3b). These results established that DI inhibits IκBζ via Nrf2-independent mechanism. Beside the Nrf2, Keap1 is also known to cooperate with p62/SQSTM1 (hereafter p62), an ubiquitin-binding protein involved in cell signaling, oxidative stress, and autophagy[21,22]. DI was a strong inducer of p62 expression in BMDMs, which was partially independent of Nrf2 induction (Extended Data Fig. 3b). Therefore, we tested the effect of DI on IκBζ in p62-deficient BMDMs. p62 deficiency did not result in restored IκBζ levels in DI-exposed cells, excluding p62 as a possible mediator of the effect of DI on the IκBζ/IL-6 axis (Extended Data Fig. 3c). Finally, we considered *Hmox1*, a prototypical Nrf2 target gene encoding HO-1 that can be induced independent of Nrf2[23]. HO-1 was reported to interfere with inflammatory responses[24,25] and, thus, could be potential mediator of the action of DI on IκBζ. Indeed, we detected HO-1 induction by DI in Nrf2-deficient macrophages (Extended Data Fig. 3d). However, IκBζ was inhibited in *Hmox1*-deficient cells to a similar extend as in WT BMDMs (Extended Data Fig. 3e), showing that HO-1 does not have direct effect on IκBζ upon DI treatment. Together these data indicate that DI inhibits the IκBζ/IL-6 axis in macrophages independently of Nrf2 induction and independently of the stress response genes p62 and HO-1.

Given the Nrf2-independent nature of DI action, we decided to explore global transcriptome changes independent of Nrf2 response. Thus, we performed RNA-Seq analysis in *Nrf2*$^{-/-}$ and WT BMDMs and analyzed the genes that were differentially expressed upon DI treatment (Fig. 3c). A number of pathways were differentially regulated upon DI addition in Nrf2 independent manner (Extended Data Fig. 3f). Most notably, DI upregulated ER stress response pathways (including genes for amino acyl tRNA synthetases, *Atf3*, *Atf4* and *Eif2ak3* (PERK) *etc.*), while downregulating interferon response pathway (*Ifit2, Ifit3, Isg15* etc.) (Fig. 3d). Among the transcription factors induced by DI, ATF3 is known to regulate basal interferon gene expression and also acts as a gatekeeper of secondary transcriptional response in LPS-stimulated macrophages[26,27]. Focusing on ATF3 as potential candidate, we compared our Nrf2-independent transcriptional signature of DI treatment to the publicly available dataset that profiled WT and *Atf3*$^{-}$

224 $^{-/-}$ macrophages at their basal states[26]. Strikingly, we found highly statistically significant overlap
225 between genes regulated by ATF3 and genes regulated by DI (Fig. 3e), suggesting that DI action
226 is mediated by ATF3. Indeed, ATF3 protein was upregulated on *Nrf2*$^{-/-}$ background by DI treatment
227 (Fig. 3f). Strikingly, *Atf3*$^{-/-}$ cells restored IκBζ protein levels upon DI treatment (Fig. 3g). Consistent
228 with restored IκBζ levels, we found significantly increased IL-6 production in DI-treated *Atf3*$^{-/-}$ cells
229 compared to WT (Fig. 3h). Of note, DI was still inducing Nrf2 response in *Atf3*$^{-/-}$ cells (Extended
230 Data Fig. 3g). Furthermore, in the absence of ATF3, DI failed to increase eIF2α phosphorylation
231 (Fig. 3i), translational hallmark of integrated stress response associated with posttranscriptional
232 regulation of IκBζ (see Fig. 2f). DI-mediated ATF3 expression was efficiently decreased by co-
233 treatement with NAC or EtGSH in both mouse macrophages and human monocytes (Fig 3j, 3k,
234 Extended Data Fig. 5h). We also tested ATF3 expression in WT and *Irg1*$^{-/-}$ BMDMs tolerized in
235 the presence of BSO. ATF3 was strongly induced in WT cells but not in the *Irg1*$^{-/-}$ (Fig. 3l) providing
236 strong evidence that Irg1/itaconate induction modulate ATF3 expression in physiological settings.
237 Overall, our data dissect the role of electrophilic stress in regulation of ATF3-IκBζ inflammatory
238 axis in Nrf2-independent manner.

239 Intriguingly, IκBζ also plays major role outside the macrophage context[28]: it is induced
240 upon IL-17 treatment of epithelial cells and orchestrates downstream inflammatory responses[29,30].
241 In fact, *Nfkbiz* polymorphisms have been associated with a number of immune related
242 conditions[31], including psoriasis. Thus, we first tested the *in vitro* effect of DI pretreatment on IκBζ
243 induction in IL-17-stimulated mouse and human primary keratinocytes. Induction of IκBζ was
244 inhibited by DI pre-treatment in a dose-dependent manner in primary keratinocytes from both
245 species (Fig. 4a, b) without affecting cell viability (Extended Data Fig. 6a). To further examine the
246 DI inhibitory effect on IκBζ induction mediated by IL-17, we analyzed expression of the well
247 characterized IκBζ target genes such as *Defb4, S100a7a, Lcn2* and *S100a9* in mouse and human
248 keratinocytes. As expected, expression of these genes was significantly downregulated by DI in
249 a dose-dependent manner (Fig. 4c, d) that correlated with IκBζ protein levels. These data suggest

that in addition to its effects on IκBζ in macrophages, DI can also modulate induction of IκBζ in multiple immune contexts.

Thus, we next explored the ability of DI to interfere with IκBζ signaling *in vivo*. We used a mouse model of psoriasis induced by the TLR7/8 agonist imiquimod (IMQ)[32]. In this model, psoriasis-like skin inflammation is induced by topical application of IMQ cream on mouse ears and resembles human psoriatic pathology. As expected, daily topical application of IMQ to the mouse ear skin for 7 days led to significant scaling and edema of the skin in control animals, as assessed by histological analysis of ear skin (Fig. 4e, f, g). In contrast, in mice that received daily i.p. injections of DI in addition to IMQ these psoriasis-like skin changes were barely detectable. Likewise, quantitative analysis of ear skin-derived mRNA showed significant induction of the IκBζ target genes *Defb4, S100a9, S100a7a*, and *Lcn2* after IMQ application (Fig. 4h) whereas their expression was markedly reduced in the skin of mice treated with DI. Daily administration of DI did not significantly affect SDH activity in heart and liver (Extended Data Fig 4b, c). These data demonstrate that DI can act as an IκBζ inhibitor *in vivo* and could provide a targeted approach for treating psoriasis.

In summary, we have shown that DI and itaconate exerts electrophilic stress leading to both Nrf2-dependent and Nrf2-independent responses, such as cellular GSH depletion and subsequent induction of integrated stress response pathways. The ATF3-dependent stress response pathway inhibited protein synthesis of IκBζ leading to inhibition of secondary transcriptional response to TLR activation. Inhibitory effect of DI on IκBζ is conserved in human cells and across the cellular types. We translated these findings into *in vivo* model of psoriasis, where DI suppressed disease associated pathology and IκBζ-mediated gene transcription. Thus, targeting the DI-IκBζ regulatory axis can serve as an important new strategy for the treatment of IκBζ-mediated autoimmune disease.

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Methods

Experimental animals

C57BL/6N WT from Charles River Laboratories. *Nrf2*$^{-/-}$ mice (Cat. No. 017009) and control mice (Cat. No. 000664) were purchased from Jackson Laboratory. *Nfkbiz*$^{-/-}$ mice described[16] were kindly provided by Prof. Shizuo Akira (IFReC, Osaka University, Japan). p62 deficient mice[33] were kindly provided by Prof. Herbert W. Virgin (Department of Pathology and Immunology, Washington University School of Medicine, USA). Mice were maintained at Washington University under specific pathogen-free conditions in accordance with Federal and University guidelines and protocols approved by the Animal Studies Committee of Washington University. Femurs and tibias from *Hmox1*$^{lox/-}$ and control *LyzM*$^{cre/cre}$*Hmox1*$^{lox/-}$ described[34] were kindly provided by Dr. Miguel P. Soares (Instituto Gulbenkian de Ciência, Portugal). Femurs and tibias from *Atf3*$^{-/-}$ mice as described[35] were kindly provided by Dr. Tsonwin Hai (Department of Biological Chemistry and Pharmacology, Ohio State University, USA).

Bone marrow-derived macrophages (BMDMs) and mouse cell cultures

BMDM were prepared from 6- to 8-week-old mice as described[4] and seeded at concentration 10^6 cells/mL in tissue-culture plates of various formats in RMPI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin and mouse recombinant M-CSF (20 µg/mL, Peprotech). Cells were treated with various concentrations of DI (Cat. No. 592498, Sigma), DMF (Cat. No. 242926, Sigma), 3-(ethoxycarbonyl)but-3-enoic acid (3MI, Aris Pharmaceuticals Inc.), 4-ethoxy-2-methylene-4-oxobutanoic acid (MI, Aris Pharmaceuticals Inc.) for indicated times and activated as shown with LPS (100 ng/mL; Sigma, *E. coli* 0111:B4). In some experiments cells were treated with α-tocopherol (10 µM), MitoTEMPO (500 µM), *N*-acetylcysteine (1 mM), bafilomycin A (100 nM), all obtained from Sigma, or ethylester of glutathione (EtGSH; 1 mM, Santa Cruz) or MG132 (10 µM, Selleckchem) as indicated. BV2

microglial cell line was a kind gift from Prof. Herbert W. Virgin (Department of Pathology and Immunology, Washington University School of Medicine, USA).

RNA-Seq Analysis

mRNA was extracted with oligo-dT beads (Invitrogen), and libraries were prepared and quantified as described[36]. Raw and processed data was deposited to Gene Expression Omnibus with access number GSE102190. Pre-ranked gene set enrichment analysis was done using fgsea R package[37]. For analysis of WT and *Nrf2*$^{-/-}$ BMDMs genes were ranked according signal to noise statistic, only top 10 000 genes ordered by mean expression were considered. MSigDB C2 and H gene set collections were used. Heatmaps were generated using Phantasus web-service (https://artyomovlab.wustl.edu/phantasus/).

Western Blotting

Cells were lysed in RIPA Lysis Buffer System (Santa Cruz) and heat-denatured at 95 °C for 5 min in reducing sample buffer (BioRad). Proteins were separated on 4%–20% polyacrylamide gradient gels (BioRad) and transferred onto PVDF membranes. Non-specific binding was blocked with 5% skim milk or 5% BSA, and membranes were probed with primary antibodies specific to Nrf2 (#12721), HO-1 (#70081), IκBζ (mouse specific, #93726), IκBζ (#9244), ATF3 (#D2Y5W) IRAK1 (#4504), phospho-IKK (Ser176/180, #2697), p62/SQSTM1 (#5114), phospho-eIF2α (Ser51, #9721), eIF2α (#5324) from Cell Signaling; GAPDH (sc-25778), IκBα (sc-1643), ATF3 (sc-188), SDHA (sc-166909) from Santa Cruz; NQO1 (ab28947) from Abcam, followed by incubation with anti-rabbit-HRP (1:10,000; sc-2030) or anti-mouse-HRP (1:10,000; sc-2031) from Santa Cruz and Clarity western ECL substrate (Bio-Rad).

Gas-Chromatography Mass Spectrometry (GCMS)

For DI measurement media was collected from cells at various time points of incubation with 250 µM DI and placed on ice. An equal volume of ethyl acetate (Sigma) was added and the samples were vortexed at 4 °C for 1 min. After centrifugation at 14,000 *g* for 2 min at 4 °C, the organic phase was collected and approx. 20-30 mg of sodium sulfate (Sigma) was added. The samples were vortexed prior to analysis. GCMS analysis was performed using a Thermo Trace 1300 GC equipped with a 30m DB-35MS capillary column connected to a Thermo TSQ Quantum MS operating under electron impact (El) ionization at 70 eV. One µL of sample was injected in splitless mode at 270 °C, using helium as the carrier gas at a flow rate of 1 mL min^{-1}. The GC oven temperature was held at 100 °C for 3 min and increased to 240 °C at 3.5° min^{-1}. The MS source and quadrupole were held at 230 °C and 280 °C, respectively, and the detector recorded ion abundance in the range of 30 – 800 *m/z*.

Metabolite profiling – LCMS

Bone marrow-derived macrophages were seeded in 96-well plates at 10^5 cell per well for all analyses. After treatment, media was removed from the wells and the cells were washed 3 x with PBS (37 °C) and immediately placed on dry ice. The frozen samples were kept on dry ice or stored at -80°C until extraction. Cell extracts were prepared by adding 180 uL of 70/30 Ethanol/ H_2O solution at 70 °C with 300 ng/mL $^{13}C_5$ $^{15}N_1$ d5-Glutamate as the internal standard. After rigorous mixing, the supernatant was collected after centrifugation (4000 rpm for 10 min at 4 °C) and transferred to another 96-well plate and the solvent was evaporated under reduced pressure (Genevac). Prior to injection, dried extracts were reconstituted in LCMS grade water. The extracted samples were analyzed by high-resolution accurate mass (HRAM) liquid-chromatography–mass spectrometry. LC separation was achieved by reverse-phase ion-pairing chromatography. The UHPLC system consisted of a Vanquish (Thermo Fisher Scientific, San Jose, USA) pumping system, coupled to an autosampler and degasser. Chromatographic separation was performed using a Synergy Hydro-RP column (100 mm×2 mm, 2.5 µm particle

size, Phenomenex, Torrance, CA). The elution gradient was carried out with a binary solvent system as described previously[38]. HRAM data was acquired using a QExactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific), which was equipped with a heated electrospray ionization source (HESI-II), operated in negative electrospray mode. Ionization source working parameters were optimized; the heater temperature was set to 300 °C, ion spray voltage was set to 3500 V. An m/z scan range from 70 to 700 was chosen and the resolution was set at 70,000. The automatic gain control target was set at 1e6 and the maximum injection time was 250 ms. Instrument control and acquisition was carried out by Xcalibur 2.2 software (Thermo Fisher Scientific). All data analysis was conducted using MAVEN software[39].

Synthesis of DI-GSH adduct (N^5-(1-((carboxymethyl)amino)-3-((4-methoxy-2-(methoxycarbonyl)-4-oxobutyl)thio)-1-oxopropan-2-yl)glutamine)

To a vial charged with dimethyl 2-methylenesuccinate (0.158 g, 1.0 mmol) was added ethanol (1.000 mL), triethylamine (0.167 mL, 1.200 mmol) and the mixture cooled in an ice water bath prior to the addition of glutathione (0.369 g, 1.200 mmol). The resulting suspension was stirred overnight and allowed to slowly warm to room temperature (ice melt) affording a light yellow solution. The mixture was dried under reduced pressure and purified by RP-HPLC: Instrumentation: Agilent Automated Purification System w/Single Quad MS and DAD; Waters AcQuity UPLC I-Class with QDa and UV. Method: XSelect CSH Prep C18 OBD 5um 19x100 column. Solvents A and B are water w/ 0.1% formic Acid and acetonitrile, respectively. 10 minute method time with a gradient from 10% B to 40% B over 5 minutes. Samples were loaded at 10% B. The flow rate during the loading was 25 mL/min and it was raised to 40 mL/min during separation affording N5-(1-((carboxymethyl)amino)-3-((4-methoxy-2-(methoxycarbonyl)-4-oxobutyl)thio)-1-oxopropan-2-yl)glutamine as a white solid (314 mg, 67.4%) ESI m/z (M+H)+= 466.0 1H NMR (400 MHz, DMSO-d6) δ 8.70 (t, J = 6.6 Hz, 1H), 8.32 (d, J = 8.6 Hz, 1H), 8.13 (d,

J = 1.2 Hz, 1H), 4.43 – 4.33 (m, 1H), 3.67 (m, 1H), 3.60 (s, 3H), 3.58 (s, 3H), 3.25 (m, 3H, under water in DMSO), 3.00 – 2.70 (m, 3H), 2.70 – 2.56 (m, 3H), 2.28 (m, 2H), 1.95 – 1.75 (m, 2H).

Synthesis of Ita-GSH adduct (2-(((2-(4-amino-4-carboxybutanamido)-3-((carboxymethyl)amino)-3-oxopropyl)thio)methyl)succinic acid)

To a vial charged with 2-methylenesuccinic acid (itaconic acid) (0.021 g, 0.163 mmol) was added water (0.651 ml) and glutathione (0.05 g, 0.163 mmol). The resulting susoension was heated at 37^{o}C overnight affording a pale yellow solution which was directly purified by RP-HPLC as follows: XSelect Prep C18 5um 19x100 column. Solvents A and B are water w/ 0.1% formic acid and acetonitrile, respectively with a 10 min method time and a gradient from 5% B to 10% B over 5 minutes. Samples were loaded at 5% B. The flow rate during the loading and the separation was 40 ml/min. Mass spectral data were acquired from 200-1000 amu in electrospray positive mode. Product was successfully isolated, 2-(((2-(4-amino-4-carboxybutanamido)-3-((carboxymethyl)amino)-3-oxopropyl)thio)methyl)succinic acid (28.4 mg, 0.065 mmol, 39.9 % yield) as a pale white solid. ESI m/z (M+H)+= 437.1 1H NMR (400 MHz, Deuterium Oxide) δ 4.48 (ddd, J = 8.7, 5.0, 3.3 Hz, 1H), 3.86 (s, 2H), 3.71 (t, J = 6.3 Hz, 1H), 2.97 (ddt, J = 12.1, 9.1, 5.6 Hz, 2H), 2.87 – 2.52 (m, 5H), 2.43 (td, J = 7.5, 5.4 Hz, 2H), 2.06 (q, J = 7.3 Hz, 2H).

Synthesis of ^{13}C-DI

E. coli ita23 (provided by Klamt Lab, Max Planck Institute)[40] was allowed to grow in ^{12}C-glucose-LB broth (10 mL of 10 g/L bacto-trypsin, 5 g/L yeast extract, 10 g/L NaCl, 0.28 g/L $CaCl_2$, 125 mg/L kanamycin and 0.2% (w/v) of ^{12}C-glucose) overnight at 30 ^{o}C and 210 rpm (until OD_{420}: 2.6). The production of ITA was next initiated by dilution of 100 µL of the above culture into 250 mL of a^{13}C-glucose minimal media (5.0 g/L K_2HPO_4, 3.5 g/L KH_2PO_4, 3.5 g/L $(NH_3)NaHPO_4$, 0.25 g/L

$MgSO_4$, 11.3 mg/L $CaCl_2$, 1.5 g/L glutamic acid, 0.5 mg/L thiamine, 25 mg/L kanamycin, 1 mL trace element solution and 0.4% ^{13}C-glucose). The trace element solution consists of 1.6 g/L $FeCl_3$, 0.2 g/L $CoCl_2$ $6H_2O$, 0.1 g/L $CaCl_2$, 0.2 g/L $ZnCl_2$ $4H_2O$, 0.2 g/L $NaMoO_4$, 0.05 g/L H_3BO_3. The bacteria were allowed to grow for 6 days until the OD_{420} reached 1.9-2.1. The cells were pelleted by centrifugation for 30 min at 14,000 × g and 4°C. The supernatant was collected and lyophilized to afford a white powder (3.78 g). The powder was re-dissolved in distilled H_2O (10 mL) and the pH reduced to 2 using concentrated HCl (ca. 0.5 mL). ^{13}C-labelled ITA was extracted into ethyl acetate (4 × 15 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford ^{13}C-labelled ITA as an off-white solid (283 mg, 1.1 g/L of culture). **^{1}H NMR** (d_6-DMSO, 500 MHz) δ 12.38 (br s, 2H, -COOH), 6.30-5.92 (m, 1H, H-2a), 5.71 (ddd, 1H, $^1J_{HC}$: 158.8 Hz, $^2J_{HC}$: 12.0 Hz, $^3J_{HC}$: 5.9 Hz, H-2b), 3.38-3.03 (m, 2H, H-4); **^{13}C NMR** (d_6-DMSO, 125 MHz) δ 172.0 (dt, $^1J_{CC}$: 55.7 Hz, $^2J_{CC}$: 2.6 Hz, C-1), 167.5 (d, $^1J_{CC}$*:* 68.8 Hz, C-5), 135.3 (tdd, $^1J_{CC}$*:* 69.1 Hz, $^1J_{CC}$: 46.7 Hz, $^2J_{CC}$: 2.8 Hz, C-3), 127.4 (d, $^1J_{CC}$: 70.8 Hz, C-2), 38.1-36.5 (m, C-4) **HR-MS** $^{13}C_5H_6O_4$ ($M+Na^+$) calcd. 158.0326, found 158.0328.

Preparation of ^{13}C-dimethyl itaconate (^{13}C-DI)

The reactant ^{13}C-itaconic acid (0.10 g, 9.5 mmol) was dissolved into methanol (1 mL). To this solution was added 1 drop of concentrated H_2SO_4 and the mixture was refluxed overnight (16 hours). The reaction was quenched with sat. $NaHCO_3$ (1 mL), then extracted into dichloromethane (2 × 2 mL). The combined organic layers were dried over anhydrous Na_2SO_4 to afford a brown liquid. Yield: 97 mg, 80%. **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 6.62-6.00 (m, 1H, H-2a), 6.00-5.36 (m, 1H, H-2b), 3.74 (d, 3H, $^3J_{HC}$: 3.8 Hz, H-6), 3.67 (d, 2H, $^3J_{HC}$: 3.9 Hz, H-7), 3.60-2.99 (m, 2H, H-4); **^{13}C NMR** ($CDCl_3$, 75 MHz) δ 172.0-170.6 (m, C-1), 167.5-166.0 (m, C-5), 133.9 (tdd, $^1J_{CC}$*:* 71.9 Hz, $^2J_{CC}$ 46.5 Hz, $^3J_{CC}$ 3.0 Hz, C-3), 130.0-127.2 (m, C-2), 52.4-52.3 (m, C-6), 52.3-52.2 (m, C-7), 38.7-36.7 (m, C-4) **HR-MS** $C_2{}^{13}C_5H_{10}O_4$ ($M+Na^+$) calcd. 168.0639, found 168.0638.

Protein mass spectrometry

Metabolic labeling of nascent protein synthesis with Click chemistry

BMDMs were grown in 12-well plate, 10^6 cell per well. Treated with DI (250 μM) for 12 h and stimulated with LPS. 2 h before the end of DI treatment cells were washed 3 x with Met deficient media and cells were starved for Met for 1h, in presence of 200 μM DI. After that L-azidohomoalanine (Click-iT® AHA, C10102, Invitrogen) was added directly to cell media to total concentration of 50 μM. In control sample, cells were treated with puromycin 5 μg/mL 2 h before LPS stimulation to block translation. Cells were lysed in 100 μL of lysis (50 mM Tris-HCl, pH 8, 1% SDS supplemented with protease inhibitor cocktail, PMSF and Na_3VO_4 from Santa Cruz. Lysates were incubated on ice for 30 min, sonicated and cleared by centrifugation 13 000 rcf for 5 min at 4 °C. Total protein concentration was determined using RC/DC™ Protein Assay (Biorad). 30 μg of protein was used for downstream reaction with 40 nM biotin-alkyne (B10185, Invitrogen) and reaction was carried out in Click-iT® Protein Reaction Buffer Kit (C10276, Invitrogen) according to manufacturer's protocol. Lysates were separated by SDS PAGE and biotinylated proteins were detected by western blot with streptavidin-HRP conjugate (1:1000, #554066, BD Phagarmingen). Membrane was striped using 0.2 M NaOH and re-probed to detect IκBζ (see Western blotting analysis section).

GSH measurement

Total GSH concentration in cells was determined by GSH/GSSH Ratio Detection Assay Kit (Abcam) according to manufacturer protocol. Briefly, 10^6 BMDMs we lysed in 100ul of 0.5% NP-40 in PBS, pH 6. Samples were deproteinized using trichloroacetic acid and neutralized by addition of 1M $NaHCO_3$ to achieve pH 4-6. Collected extracts were directly used for GSH measurement.

552

553 **Cytokine detection**

554 Cytokines in cell supernatants were analyzed using DuoSet® ELISA kits according to
555 manufacturer protocol (R&D Systems).

556

557 **RNA isolation and revers transcription-quantitative PCR (RT-qPCR)**

558 RNA from cultured cells was isolated using a Total RNA I kit (OMEGA). RNA from mouse ear skin
559 was extracted using RNAeasy mini kit (Qiagen) after tissue disruption with sterile zirconium beads
560 on a MagNA Lyser (Roche). Isolated RNA was reverse transcribed using a Superscript III reverse
561 transcriptase (Agillent) according to the manufacturer's protocol. qPCRs were performed in 96-
562 well plates using a SYBR green I master mix (Roche) using a LightCycler® 96 (Roche
563 Diagnostics). All assays were performed at least in duplicate, and reaction mixtures in 20 µL
564 volumes were processed under the following cycling conditions: initial 3-min denaturation at 95
565 °C, followed by 50 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. A melting-curve
566 analysis was carried out from 72 °C to 97 °C with 0.2 °C increments; threshold cycle (CT) values
567 for each sample were determined by automated threshold analysis. Expression levels of all
568 mRNAs were normalized to reference gene *Actb*; to *Rpl19* in mouse keratinocytes and tissue
569 samples; to *RPLP0* in human keratinocytes. The relative increase in the expression level of a
570 cytokine was normalized to the level of expression in unstimulated control cells in each
571 experiment. Primer pairs used are listed in Extended Data Table 1.

572

573 **Lentiviral transduction**

574 Mouse *Nfkbiz* 3'UTR Lenti-reporter-GFP vector or pLenti-UTR-GFP-Blank vector were purchased
575 from Applied Biological Materials. Lentiviruses for BV2 transduction were prepared by mixing 1.5-
576 mL aliquots of Opti-MEM medium (Invitrogen), 18 µg of psPAX2 (gift from Didier Trono, Addgene
577 plasmid #12260), 13 µg of pCMV-V-SVG (gift from Bob Weinberg, Addgene plasmid #8454)[41], 20

578 µg of lentiviral construct, and 105 µL of polyethylenimine (1 mg/mL; 25 kDa; linear form;
579 Polysciences). The mixture was incubated for 20 min at room temperature before it was added to
580 HEK-293T packaging cells in medium for cultivation of BMDMs in a 150-cm^2 tissue culture flask.
581 48 hours later, virus-containing medium was filtered through 45 µm pore-size cellulose acetate
582 filters and used directly for BV2 infection in the presence of polybrene (8 µg/mL, Millipore) at the
583 day 1. At the day 3, cells were selected with puromycin at 5 µg/mL. Cells were assayed after at
584 least one week in selection.
585
586 **Flow Cytometry**
587 For ROS measurements, cells were treated with DI, loaded with 10 µM CM-H_2DCFDA (Invitrogen)
588 at RT for 30 min in Hank's balanced salt solution (HBSS). After incubation, cell were rinsed with
589 warm HBSS, harvested and analyzed. In some experiments GFP signal was determined in live
590 cells. For analysis of cell viability cells were stained with propidium iodide (1 µg/mL). Cells were
591 acquired on LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo v.9.5.2
592 software (Tree Star).
593
594 **Confocal microscopy**
595 BMDMs were seeded at eight-well multitest microscopy slides (MP Biomedicals). Cells were
596 treated with DI (250 µM, 12 hours) and then stimulated with LPS (100 ng/mL, 30 min). Cells were
597 then fixed with 3% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-
598 100 in PBS for 30 min. After washing with PBS, free binding sites were blocked with 1% BSA and
599 subsequently labeled with 50x diluted p65-specific antibody (#8242, Cell signaling), followed by
600 AF568-conjugated anti-rabbit secondary antibody (#A11011, Thermo Fisher Scientific). After
601 labeling, the cells were washed and mounted in 50% (w/v) glycerol in PBS, pH 8.5 containing
602 DAPI (1 µg/mL, Sigma) to label nuclei. Samples were examined with a confocal laser scanning

603 microscope (Leica TCS SP8) equipped with a 40x (numerical aperture, 1.4) oil immersion
604 objective.
605
606 **Human monocytes isolation and treatment**
607 Blood from healthy donors was provided by the Pheresis Center of Barnes Jewish Hospital.
608 Peripheral blood mononuclear cells (PBMCs) from buffy coats were recovered from the Ficoll
609 interface after a 400 x g centrifugation for 30 min. Monocytes were isolated by adherence on a
610 cell culture dish for 1 hour at 37 ˚C and 5% CO_2 in RPMI containing 1% Human Serum Albumin
611 (HSA, Grifols, Spain). After extensive washes to allow removal of non-adherent cells, monocytes
612 (≥ 95% purity) were harvested, counted and $5x10^5$ cells were plated per well in 24 well plates.
613 Human blood monocytes were then treated with DI at 50, 150 or 250 µM for 12 hours. Monocytes
614 were then challenged with LPS (100 ng/mL) for 1 hour or 4 hours for analysis of protein expression
615 by western blot; 24 hours for cytokine production assays or left unstimulated.
616
617 **Primary mouse and human keratinocytes**
618 Primary keratinocytes were isolated from C57BL/6 WT newborn mice or human foreskins as
619 previously described[42]**.** $2x10^5$ cells in 2 ml of media were plated in 12-well tissue culture plates.
620 After 2-3 days of cultivation, media was exchanged for 1 mL of fresh media and cells were treated
621 with DI for 12 hours. Mouse or human cells were then stimulated with mouse recombinant IL-17A
622 (100 ng/mL; Cat. No. 421-ML, R&D Systems) or human recombinant IL-17A respectively (100
623 ng/mL; Cat. No. 7955-IL, R&D Systems).
624
625 **IMQ-induced psoriasis**
626 To induce experimental psoriasis, Imiquimod (IMQ, Imiquimod Cream 5%, Perrigo. Co.) was
627 applied daily to mice on both ears, (~5 mg per ear) for 7 days. For the DI-treated mice: DI was
628 administered via the intraperitoneal route at 20 mg /500 µL sterile PBS per mouse one day prior

to IMQ application and daily thereafter for 7 days. After 7 days mice were euthanized and ears were used for RNA extraction or histological analysis by performing H&E staining on 7 µm thick sections following paraffin embedding. Average ear thickness in each sample was quantified from images obtained at the same settings using Fiji[43].

SDH activity in mouse heart and liver

Mice were injected with DI dose 20 mg i.p. either once per day for total length of 4 days (DI daily) or every two hours 3 times in total (DI overdose). In daily DI administration, last injection was 6 h before the organ harvest, in DI overdose protocol last injection was 2 h before harvest. Mice were sacrificed and heart (~50 mg) and liver tissue (200 mg) were harvested, washed in PBS and processed for mitochondria isolation with Mitochondria Isolation Kit for Tissue (#89801, Thermo Scientific) according to manufacturer's instruction. Cytoplasmic and mitochondrial fractions were combined with Laemli sample buffer and analyzed by western blotting for SDH and GAPDH presence (see western blot analysis section). SDH activity in isolated mitochondria was analyzed using SDH Activity Colorimetric Kit (#MAK197, Sigma). Mitochondria were directly resuspended in SDH Assay Buffer. Protein concentration in each sample was determined using RC/DC™ Protein Assay (Biorad) and activity was normalized to total protein concentration.

Statistical Analysis

Statistical analyses were performed in Excel (two-tailed Student’s t test) or GraphPad Prism 6 software using statistical tests indicated for each experiment.

Figure legends

Figure 1. DI induces an electrophilic stress response in macrophages. (**a**) Expression of Nrf2 dependent genes in BMDMs treated with DI (250 µM) for 12 h. (**b**) Scheme of DI structure reacting with thiol-containing nucleophile in Michael reaction. (**c**) Transcriptional comparison of Keap1 conditional KO (KeapCKO) and WT BMDMs and enrichment of genes upregulated by DI. (**d**) Western blot analysis of Nrf2 induction and Nrf2 target genes expression (NQO1, HO-1) in BMDMs treated with indicated concentrations of DI for 12 h. (**e**) Western blot analysis of Nrf2 induction in WT and *Irg1*$^{-/-}$ BMDMs stimulated with LPS for indicated times. (**f**) Chemical structure of DI- or itaconate (Ita-) conjugates. (**g**) DI-GSH conjugate levels detected by LCMS in media of BMDMs treated with DI (250 µM) for indicated times. Results represents mean±SD (n=2). (**h**) Levels of Ita-GSH conjugate and itaconate in media of WT and *Irg1*$^{-/-}$ BMDMs stimulated with LPS for 24 h detected by LCMS. (**i**) Relative GSH concentration in BMDMs treated with DI (250 µM) for indicated times. Mean±SE (n=2). (**j**) BMDMs were treated with DI (250 µM) for 12 h in presence of antioxidants: N-acetylcysteine (NAC, 1 mM), MitoTEMPO (MT; 500 µM), α-tocopherol (AT, 10 µM). Cells were then stimulated with LPS for 4 h. (k) BMDMs were treated with DMF (50 µM) for 12 h and stimulated with LPS for 24 h. Cytokine levels in media were determined by ELISA. Mean±SE (n=2). In all western blots GAPDH is used as a loading control and representative results from 3 independent experiments are shown. p values were calculated using two-tailed Student's t test, *$p < 0.05$; **$p < 0.01$.

Figure 2. DI inhibits LPS-mediated IκBζ induction. (**a**) Cytokine mRNA levels detected by RT-qPCR in WT and *Nfkbiz*$^{-/-}$ BMDMs stimulated with LPS for indicated times. (**b**) Western blot analysis of IκBζ induction in BMDMs treated with DI (250 µM) for 12 h and stimulated with LPS for indicated times. (**c**) BMDMs treated with DI (250 µM) for 12 h and stimulated with LPS for 30 min. Cells were fixed and permeabilized and p65 was detected using specific primary antibody followed by secondary antibody conjugated with AF568. Cell nuclei were labeled with DAPI.

Representative images from one experiment are shown. Bars 25 µm. (**d**) BMDMs were treated with DI (250 µM) for 12 h in presence of antioxidants N-acetylcysteine (NAC; 1 mM) or α-tocopherol (AT; 10 µM) and then stimulated with LPS for 1h. IκBζ expression was determined by western blot. (**e**) Densitometric quantification of IκBζ expression (mean±SE, n=3) plotted together with *Nfkbiz* mRNA levels determined by RT-qPCR (mean±SD, n=2) in BMDMs treated with DI (250 uM) for 12 h and stimulated with LPS for 1 h. (**f**) BMDMs were treated with DI (250 µM) for 12 h. Levels of phosphorylated eIF2α and total eIF2α were determined by western blot. The arrow indicates molecular weight corresponding to eIF2α. (g) Scheme of DI action on transcriptional response to LPS stimulation. (**h**) Chemical structures of compounds arranged according to electrophilicity. (**i**) BMDMs were treated with DMF (50 µM) for 12 h and stimulated with LPS for indicated times. IκBζ induction was determined by western blot. (**j**) BMDMs were treated with indicated compounds for 12h (DI at 250 µM; MI and 3MI at 5 mM) and stimulated with LPS for 1 h. IκBζ induction was determined by western blot. (**k**) BMDMs were treated with DI at indicated concentration for 12 h in presence or absence of BSO (500 µM) and stimulated with LPS for 1 h. IκBζ induction was determined by western blot. (**l**) BMDMs were treated with MI (10 mM) for 12 h in presence or absence of (500 µM) and stimulated with LPS for 1h. IκBζ induction was determined by western blot. (**m**) Densitometric quantification of IκBζ expression from b plotted together with quantification of itaconate production determined by mass spectrometry in BMDMs stimulated with LPS for indicated times. (**n**) BMDMs were stimulated with LPS for 18 h in presence or absence of BSO (500 µM).Media was then exchanges and cells were re-stimulated with LPS for 1h. IκBζ expression was determined by western blot. All western blot data are representative results from 3 independent experiments and GAPDH was used as loading control.

Figure 3. DI induced stress response independent of Nrf2, and inhibits IκBζ/IL6 axis via action of ATF3. (**a**) WT of *Nrf2*$^{-/-}$ BMDMs were treated with DI (250 µM) for 12 h and stimulated with LPS for indicated times. IκBζ induction was determined by western blot. (b) WT of *Nrf2*$^{-/-}$

BMDMs were treated as in a and stimulated with LPS for 4 h. Cytokine levels in media were determined by ELISA. Mean±SE (n=3). (c). Transcriptional analysis of WT and *Nrf2*$^{-/-}$ BMDMs treated with DI for 12 h. Panels on the right show GSEA statistics for UPR and IFN pathways. (d) Selected genes from the UPR and IFN pathways. (e) Transcriptional comparison of *Atf3*$^{-/-}$ and WT BMDMs and enrichment of genes downregulated by DI. (f) WT or *Nrf2*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h. Expression of ATF3 was determined by western blot. (g) WT or *Atf3*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for indicated times. IκBζ induction was determined by western blot. (h) WT or *Atf3*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for 24 h. Cytokine production in cell media was determined by ELISA. Mean±SE (n=3). (i) WT or *Atf3*$^{-/-}$ BMDMs were treated with DI (250 μM) and phosphorylated eIF2α and total eIF2α were determined by western blot. (j) BMDMs were treated with DI (250 μM) in presence of NAC (1 mM) and stimulated with LPS. ATF3 expression was determined by western blot. (**k**) Human blood monocytes were treated with DI (125 μM) and EtGSH (1 mM) for 12 h and stimulated with LPS for 1 h. ATF3 expression was determined by western blot. (**l**) BMDMs were stimulated or not with LPS for 18 h in presence or absence of BSO (500 μM). Media was then exchanges and cells were restimulated with LPS for 1h. ATF3 expression was determined by western blot. All western blot data are representative results from 3 independent experiments and GAPDH was used as loading control. p values were calculated using two-tailed Student's t test, *$p < 0.05$; **$p < 0.01$.

Figure 4. DI-IκBζ regulatory axis is conserved between species and cell types. (**a**) Mouse primary keratinocytes were treated with indicated concentrations of DI for 12 h and stimulated with Il-17A (100 ng/mL) for indicated times. IκBζ induction was determined by western blot. Data are representative from 3 independent experiments. (**b**) Human primary keratinocytes were treated with indicated concentrations of DI for 12 h and stimulated with Il-17A (100 ng/mL) for indicated times. IκBζ induction was determined by western blot. Data from one donor are shown.

(**c**) Mouse primary keratinocytes were treated and stimulated as in a. Gene expression was analyzed by RT-qPCR. Mean±SE (n=3). (**d**) Human primary keratinocytes were treated and stimulated as in (b) and gene expression was analyzed with RT-qPCR. Mean±SD (n=2). (**e**) Scheme of DI administration in imiquimod (IMQ)-induced psoriasis. For details see the Methods. Ear skin tissue was collected for histological and RNA analysis. (**f**) Histology analysis of ear thickness of mice treated as in g. 3 animals were used per group and data are representative from 2 independent experiments. Bars = 100 µm. (**g**) Quantification of ear thickness of data in f. Mean±SE (n=2). (**h**) RNA was isolated from ear tissue of mice treated as in h and gene expression analysis was performed by RT-qPCR. Each dot represents data from one mouse. p values were calculated using two-tailed Student's t test, *$p < 0.05$; **$p < 0.01$.

Extended Data Figure 1. (**a**) DI content in cell media of BMDMs treated with DI (250 µM) for indicated times determined by GCMS. Data represents mean±SD (n=2). (**b**) Mass spectra of DI-GSH structure detected in DI treated BMDMs compared to synthesized DI-GSH standard. (**c**) DI-GSH conjugate levels detected by LCMS in media of BMDMs treated with DI (250 µM) for indicated times. Results represents mean±SD (n=2). (**d**) DI-GSH conjugate levels in BMDMs treated with $^{13}C_5$-labeled DI (250 µM) for indicated times or in their media detected by LCMS. (**e**) Mass spectra of Ita-GSH structure detected in LPS stimulated BMDMs compared to synthesized Ita-GSH standard. (**f**) ROS detection using CM-H2DCFDA in BV2 cells treated with DI (250 µM) for indicated times determined by flow cytometry. MFI is shown at y axis, mean±SD (n=2). (**g**) BMDMs were treated with DI (250 µM) for 12 h in presence of EtGSH (1 mM) and stimulated with LPS for 4 h. Cytokine levels in media were determined by ELISA. Mean±SE (n=3). (**h**) BMDMs were treated with DMF for 12 h as indicated and HO-1 induction was determined by western blot. GAPDH was used as loading control and representative results from 3 independent experiments are shown. (**i**) BMDMs were treated with DI or DMF for 12 h

as indicated and stimulated with LPS for 4h (DI) of 24h (DMF). Mean Cytokine levels in media were determined by ELISA. Mean±SE (n=2). p values were calculated using two-tailed Student's t test, *p < 0.05; **p < 0.01; ***p < 0.001.

Extended Data Figure 2. (**a**) WT or *Nfkbiz*$^{-/-}$ BMDMs were stimulated with LPS for indicated times. IκBζ expression was determined by western blot. (**b**) WT or *Nfkbiz*-/- BMDM were stimulated with LPS for 4 h. Cytokine levels were determined in media by ELISA. Mean±SE (n=3). (**c**) BMDMs were treated with indicated concentrations of DI for 12 h and stimulated with LPS for 1 h. IκBζ induction was determined by western blot. (**d**) Human blood monocytes were treated with indicated concentration of DI for 12 h and stimulated with LPS as shown. IκBζ induction was determined by western blot. (**e**, **f**) BMDMs were treated with 250 μM DI for 12 h and stimulated with LPS for indicated times. IκBα degradation (**e**) or IRAK1 expression and IKK phosphorylation (**f**) were determined by western blot. Sensitivity of IRAK1 detection diminishes upon IRAK1 K63 ubiquitination[16]. (**g**) BMDMs were treated with DI (250 μM) for 12 h in presence of EtGSH (1 mM) and stimulated with LPS for 1 h. IκBζ expression was determined by western blot. (**h**) Human blood monocytes were treated with DI (125 μM) in presence of EtGSH (1 mM) for 12h and stimulated with LPs for 1 h. IκBζ expression was determined by western blot. (**i**) WT or *Nfkbiz*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h in presence of NAC (1 mM) and stimulated with LPS for 4 h. Mean±SD (n=2).

Extended Data Figure 3. (**a**) Densitometric quantification of IκBζ expression (mean±SE, n=3) plotted together with *Nfkbiz* mRNA levels determined by RT-qPCR (mean±SD, n=2) in cells treated with indicated concentration of DI for 12h and stimulated with LPS for 1 h. (**b**) BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for 1 h. In some samples MG132 (10 μM) or bafilomycin A (BafA; 100 nM) were added 30 min before LPS stimulation. IκBζ induction was determined by western blot. (**c**) BV2 cells were transduced by lentiviruses encoding

Nfkbiz 3'UTR reporter system expressing GFP or GFP only (EMPTY vector). Cells were selected in puromycin, treated with DI (250 μM) for 12 h and stimulated with LPS for 1 h. GFP expression was determined by flow cytometry. (**d**) BMDMs, treated with DI (250 μM) and stimulated with LPS for 1h were feeded with alkyne containing analog of Met 1h before end of treatment period. Nascent proteins were then labeled with biotin-azide using Click chemistry (see methods) and detected with western blot using streptavidin-HRP. Same membrane was reprobed for IκBζ. (**e**) Densitometric quantification of signal in each lane in membrane probed for biotin in d. (**f**) Log fold change of proteomic signal in unstimulated versus LPS stimulated cells. (**g**) Log fold change of transcripts versus protein.

Extended Data Figure 4. (**a**) BMDMs were treated with indicated concentration of BSO or with DI (250 μM) for 12 h. Nrf2 expression was analyzed by western blot. (**b**) BMDMs were treated with BSO (500 μM) for 12 h and stimulated with LPS for indicated time. IL-6 levels were determined by ELISA. Mean±SE (n=3). (**c**) BMDMs were treated with DI (250 μM) and BSO (500 μM) for 12 h and stimulated with LPS for indicated time. IL-6 levels were determined by ELISA. Mean±SE (n=3). (**d**) BMDMs were treated with MI (5 mM) and BSO (500 μM) for 12 h and stimulated with LPS for indicated time. IL-6 levels were determined by ELISA. Mean±SE (n=3). (**e**) BMDMs were stimulated or not with LPS for 18 h in presence or absence of BSO (500 μM). Media was then exchanges and cells were re-stimulated with LPS for 1h. IκBζ expression was determined by western blot. All western blot data are representative results from 3 independent experiments and GAPDH was used as loading control. p values were calculated using two-tailed Student's t test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Extended Data Figure 5. (a) WT or *Nrf2*$^{-/-}$ BMDMs were treated with indicated concentration of DI for 12 h and stimulated with LSP for 1h. Expression of IκBζ was determined by western blot. (b) WT of *Nrf2*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for

indicated times. P62 expression was analyzed by western blot. (c) WT or p62-deficient BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for indicated times. IκBζ and p62 expression were determined by western blot. (d) Cells as in (b) were analyzed for HO-1 expression by western blot. (e) WT or *Hmox1*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h and stimulated with LPS for indicated times. IκBζ and HO-1 expression were determined by western blot. (f) WT or *Nrf*$^{-/-}$ BMDMs were treated with DI (250 μM) for 12 h. RNA-Seq analysis was performed and differentially expressed genes between DI treated and untreated cells were analyzed. Pathways that are differentially regulated upon DI addition in Nrf2 independent manner are shown, together with Gene ranks, normalized enrichment score (NES), p value and adjusted p value (padj). (g) WT or *Atf3*-/- BMDMs were treated with DI (250 μM) for 12 h and Nrf2 expression was analyzed by western blot. (**h**) BMDMs were treated with DI (250 μM) in presence of NAC (1 mM) or EtGSH (1mM) and stimulated with LPS. ATF3 expression was determined by western blot. In all western blot data GAPDH was used as loading control and data in a, g, h are representative from 3 independent experiments, data in b, c, d from 2 independent experiments and data in e were performed in one experiment.

Extended Data Figure 6. (**a**) Mouse and human primary keratinocytes were treated with indicated concentration of DI for 12 h. Viability of cells was determined by propidium iodide staining and flow cytometry. Results from one experiment performed in duplicates. (**b**) Scheme of DI administration for analysis of SDH activity in heart and liver. (**c**) Mice were treated as in b, sacrificed and and heart and liver was harvested. Mitochondria was isolated from heart and liver tissue and enzymatic activity of SDH was determined. (**d**) Mitochondrial and cytoplasmic fractions from heart and liver of mice treated as in b were analyzed for SDH and GAPDH content by western blot.

Extended Data Table 1. Primers used for qPCR gene expression analysis.

Acknowledgements

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