Sci Rep. 2016; 6: 31159. PMCID: PMC4976367
Published online 2016 Aug 8. doi: 10.1038/srep31159

# Dimethyl fumarate blocks pro-inflammatory cytokine production via inhibition of TLR induced M1 and K63 ubiquitin chain formation

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Received 2015 Oct 19; Accepted 2016 Jul 15.



## Abstract

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Dimethyl fumarate (DMF) possesses anti-inflammatory properties and is approved for the treatment of psoriasis and multiple sclerosis. While clinically effective, its molecular target has remained elusive - although it is known to activate anti-oxidant pathways. We find that DMF inhibits pro-inflammatory cytokine production in response to TLR agonists independently of the Nrf2-Keap1 anti-oxidant pathway. Instead we show that DMF can inhibit the E2 conjugating enzymes involved in K63 and M1 polyubiquitin chain formation both *in vitro* and in cells. The formation of K63 and M1 chains is required to link TLR activation to downstream signaling, and consistent with the block in K63 and/or M1 chain formation, DMF inhibits NFκB and ERK1/2 activation, resulting in a loss of pro-inflammatory cytokine production. Together these results reveal a new molecular target for DMF and show that a clinically approved drug inhibits M1 and K63 chain formation in TLR induced signaling complexes. Selective targeting of E2s may therefore be a viable strategy for autoimmunity.

Autoimmune disorders represent a diverse range of conditions that remain challenging to treat. The advent of biological drugs, such as anti-TNF agents, provided a significant advance in the treatment of these conditions[1], however they have the disadvantages of not being orally available and that a proportion of patients do not respond. The development of new orally available small molecule drugs for autoimmunity is therefore desirable. Several breakthroughs in this area have recently been made, such as the development of Jak inhibitors and S1P receptor modulating agents, which illustrate the potential of this approach[2,3,4].

Dimethyl fumarate (DMF) is a methyl ester known to have immuno-modulatory properties. In combination with

other fumaric acid esters, DMF has been in use for many years as a treatment for moderate and severe psoriasis[5]. The first report of its use was in 1959[6], although it did not gain widespread acceptance until some time later following the publication of the first clinical trials demonstrating its efficacy in 1990[7]. Subsequently, DMF in combination with three salts of ethylhydrogenfumarate was licensed for use in psoriasis in Germany in 1994[8,9]. More recently, a slow release formulation of DMF has been approved for the treatment of multiple sclerosis[10].

The molecular target of DMF that accounts for its ability to modulate the immune system has been elusive. Amongst the possible explanations for its action, DMF has been shown to reduce T cell numbers, inhibit NFκB mediated transcription and activate the Nrf2 pathway (reviewed in[11,12]). In addition, DMF has been found to modulate cytokine production in a number of immune cell types: cytokine production is regulated by several intracellular signaling systems including NFκB and the ERK1/2 and p38 MAPK pathways, and DMF has been suggested to modulate these pathways. For example, DMF has been shown to prevent the induction of NFκB dependent transcription in LPS stimulated dendritic cells as well as TNF stimulated Human Umbilical Vein Endothelial Cells (HUVEC) or airway smooth muscle cells (ASMC)[13,14,15].

The reported effects of DMF on MAPK signaling are less clear. While some studies have shown that DMF could decrease ERK1/2 activation in cells, others have found it to have no effect[14,16,17]. For p38, DMF has been reported to either have no effect on activation or to result in an increase in p38 phosphorylation[14,18]. MAPKs can, in part, mediate their cellular effects via the activation of downstream kinases. For example, p38α activates the downstream kinases MK2 and MK3 to promote the production of TNF[19]. In addition, p38α can also activate the kinases MSK1 and MSK2[20]. These two kinases, which can also be activated by ERK1/2[20], have been found to have anti-inflammatory functions in macrophages and are required for the maximal induction of IL-10 by macrophages and dendritic cells[21,22]. The ERK1/2 pathway can also activate RSK[23], however the role that this kinase plays in the regulation of cytokine production is less well established. DMF has been shown to affect the activation of both MSKs and RSKs[14,16,17]. For instance, in keratinocytes DMF selectively blocked MSK1 phosphorylation but not ERK1/2 or p38α activation in response to IL-1 stimulation[16]. Similarly DMF also blocked MSK1 and RSK activation in MIF (Macrophage Inhibitory Factor) stimulated keratinocytes and prevented the induction of Cox2[17], a known MSK target gene[24]. DMF has also been reported to inhibit MSK1 phosphorylation in LPS stimulated dendritic cells, however in contrast to the data in keratinocytes, in dendritic cells DMF was able to reduce LPS induced ERK1/2, although not p38 or JNK, phosphorylation[14].

In this study we examine the mechanism by which DMF blocks cytokine induction in primary macrophages and demonstrate that it affects signaling by inhibiting the formation of M1/K63 hybrid polyubiquitin chains.

## Results

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### DMF inhibits the transcription of cytokines independently of Nrf2

To test the ability of DMF to block cytokine production in response to TLR agonists, BMDMs were incubated with various concentrations of DMF for 4 h (Fig. 1A). The cells were then stimulated with the TLR4 agonist LPS for a further 8 h and cytokine release determined. LPS promoted the secretion of TNF, IL-6, IL-10, IL-13 and GM-CSF; this was blocked by 50 μM DMF (Fig. 1A). To ensure this was not due to a loss of cell viability, cells were incubated with 50 μM DMF and viability determined by FACS. DMF did cause some cell death that increased over time, however the majority of cells were still alive following 12 h of DMF treatment (Fig. 1B). Cells were then treated with or without DMF in the presence of brefeldin A and monensin to block cytokine secretion. TNF levels were then measured at a single cell level by flow cytometry following gating on the live cell population. This showed that DMF blocked LPS stimulated TNF production in the live cells (Fig. 1C). In line with the loss of cytokine secretion (Fig. 1A), DMF also repressed the induction of various cytokine mRNAs, including TNF, IL-6, IL-10, GM-CSF, IL-12p40, IL-23p19 and IFNβ, in response to 1 h of LPS stimulation (

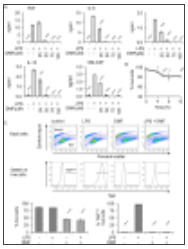


Figure 1

**Inhibition of LPS stimulated cytokine induction by DMF.**

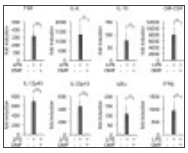


Figure 2

**DMF inhibits LPS induced gene transcription.**

DMF has previously been suggested to act via targeting cysteine residues in Keap1 and activating the Nrf2 anti-oxidant pathway[25,26,27]. To test the potential involvement of this pathway in the regulation of cytokine transcription, Nrf2−/− BMDMs were stimulated with LPS in the presence or absence of DMF and cytokine mRNA levels measured at 1 h. Nrf2 regulates the transcription of several genes including HO-1[28,29]. While LPS alone did not induce the mRNA for HO-1, this mRNA was induced in DMF treated wild type but not Nrf2 knockout BMDMs, indicating that DMF was able to activate Nrf2 in macrophages (Fig. 3). Nrf2 knockout did not affect the induction of TNF, IL-6, IL-12p40, IL-23p19 or IκBα mRNAs in response to LPS relative to wild type cells. There was a small but significant increase ($p < 0.05$, Students unpaired two tailed t-test) in LPS stimulated IL-10 and GM-CSF mRNA induction in Nrf2 knockout cells compared to wild type BMDMs (Fig. 3). This increase in IL-10 mRNA induction is consistent with a recent report showing increased IL-10 production in Nrf2 knockout dendritic cells[30]. DMF was able to inhibit cytokine and IκBα mRNA induction in response to LPS in both wild type and Nrf2−/− cells (Fig. 3). These results suggest that the effects of DMF on LPS induced cytokine induction are largely independent of any effects on the Nrf2 pathway.

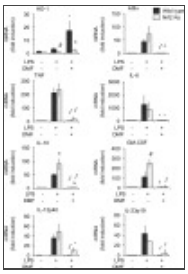


Figure 3

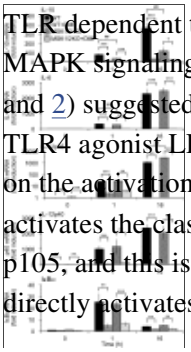
**Effect of DMF on LPS stimulated mRNA induction in Nrf2 knockout BMDMs.**

DMF has been proposed to inhibit the action of MSK1 in dendritic cells[14]. However, the reduced cytokine production caused by DMF in Fig. 1 is inconsistent with the decreased IL-10 production but increased pro-inflammatory cytokine production previously reported in MSK1/2 double knockout macrophages[22], suggesting that DMF must have MSK independent effects. To confirm this, wild type and MSK1/2 knockout BMDCs were tested. As expected MSK1/2 knockout BMDCs exhibited a lower induction of the MSK1/2 target gene IL-10 relative to wild type cells (Fig. 4). Induction of IL-12p35 mRNA was increased in the MSK1/2 knockout BMDCs while IL-6, IL-12p40 and IκBα mRNA induction was not greatly changed. As in macrophages, DMF blocked the LPS induced expression of both IκBα and the cytokine mRNAs tested. This inhibition was comparable in wild type and MSK1/2 knockout cells (Fig. 4).

Figure 4

**Effect of DMF on LPS stimulated mRNA induction in MSK1/2 knockout BMDCs.**

## DMF inhibits NFκB and ERK1/2 activation in response to TLR agonists



TLR dependent transcription and secretion of cytokines is regulated by the NFκB as well as the ERK1/2 and p38 MAPK signaling pathways[31]. The ability of DMF to block the induction of multiple LPS induced genes (Figs 1 and 2) suggested that it might have a suppressive effect on both MAPK and NFκB activation in response to the TLR4 agonist LPS. TLR4 signals via both MyD88 and Trif dependent pathways, which are thought to converge on the activation of Tak1[31,32,33,34]. Tak1 in turn activates a complex of IKKβ, IKKα and NEMO. IKKβ then activates the classical NFκB pathway via the phosphorylation of IκBα. In addition IKKβ also phosphorylates p105, and this is required for the activation of Tpl2, which is then able to activate the ERK1/2 pathway. Tak1 also directly activates the MKKs required for the activation of the p38 and JNK pathways (Fig. 5A)[31,32,33,34].

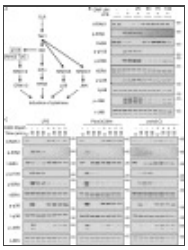


Figure 5

**DMF inhibits multiple signals downstream of TLR4.**

DMF was able to inhibit the activation of the classical NFκB pathway, as 50 μM of DMF was sufficient to block the degradation of IκBα in response to 30 min stimulation with LPS (Fig. 5B). In addition, 50 μM DMF blocked the phosphorylation of IKKβ on Ser177 and 181 (Fig. 5B), sites that correlate with its activation[35]. In agreement with the loss of IKKβ activity, DMF also blocked the phosphorylation of the IKKβ substrate p105 and inhibited the activation of ERK1/2, as judged by phosphorylation on its TXY activation motif. DMF treatment also resulted in a partial inhibition of JNK activation, that was maximal at 75 μM DMF. In contrast, DMF did not inhibit the activation of p38 at any of the concentrations tested. In the MyD88 pathway, IRAKs are involved in propagating the signal from MyD88 to Traf6 and then Tak1. During this process IRAK1 becomes modified with K63 polyubiquitin chains, resulting in the disappearance of the IRAK1 band at its predicted molecular weight on immunoblots that can be reversed by treatment of the lysates with deubiquitinating enzymes[36,37]. Interestingly, the loss of the IRAK1 band was partially reversed by 25 μM DMF and completely reversed by 50 μM DMF suggesting that DMF may interfere with IRAK1 ubiquitination (Fig. 5B).

To examine this process in more detail, cells were incubated in 50 μM DMF and then a time course of LPS stimulation carried out (Fig. 5C). As in the previous experiment, DMF inhibited the loss of IRAK1 and IκBα, blocked the phosphorylation of IKKβ and p105 and greatly reduced the phosphorylation of ERK1/2. The effect on JNK phosphorylation was more complex. DMF reduced the phosphorylation of JNK at 30 min. In the absence of DMF, JNK phosphorylation was transient and not observed at 60 or 90 min. In contrast, JNK phosphorylation was still observed at 60 and 90 min of LPS stimulation in the presence of DMF.

LPS has the ability to signal via both the MyD88 and Trif adaptors. To determine if the effects of DMF on signaling were common to pathways downstream of both adaptors, cells were stimulated with either $Pam_3CSK_4$ or poly(I:C). $Pam_3CSK_4$ stimulates TLR1/2 and acts via MyD88 and not Trif. The effects of DMF on $Pam_3CSK_4$ induced signaling mirrored the results seen with LPS (Fig. 5C). Poly(I:C) activates TLR3 and this utilizes Trif and not MyD88 for downstream signaling. Consistent with this poly(I:C) did not strongly promote the ubiquitination of IRAK1. Poly(I:C) was able to induce both IKKβ and p105 phosphorylation and this was blocked by DMF. In addition DMF blocked poly(I:C) induced ERK1/2 activation, however, as for LPS and $Pam_3CSK_4$ there was little effect on p38 activation (Fig. 5C). Poly(I:C) was only a weak activator of JNK, and this activation was slightly increased in the presence of DMF.

Unexpectedly in these experiments, incubation in DMF alone induced the phosphorylation of p38 and, to a lesser extent, JNK in the absence of any other stimuli (Fig. 5C, lane 5). To examine this further a time course of DMF

IRAK1 ubiquitination or p105 phosphorylation (Fig. 6A). DMF treatment did however induce phosphorylation of p38, and to a lesser extent JNK, over time. Tyrosine phosphatase inhibitors have previously been found to activate MAPK signaling in cells[38]. Tyrosine phosphatases possess an active site cysteine and may therefore be a target for electrophilic drugs such as DMF[39]. Treatment with the tyrosine phosphatase inhibitor pervanadate induced the phosphorylation of p38 and JNK although, in contrast to DMF, pervanadate also strongly induced ERK1/2 phosphorylation (Fig. 6A). As would be expected pervanadate strongly induced global tyrosine phosphorylation levels. In contrast, DMF had little effect on global tyrosine phosphorylation levels indicating that it does not act as a general tyrosine phosphatase inhibitor in cells (Fig. 6B). MAPKs can be dephosphorylated by DUSPs (Dual Specificity Phosphatases) and inhibition of these enzymes by DMF may account for the p38 and JNK activation. Inhibition of the DUSP would be expected to be specific for the MAPK and not affect the upstream MKK. DMF was found to increase the phosphorylation of MKK3 and 6 suggesting that its effects on p38 and JNK phosphorylation were independent of DUSP inhibition (Fig. 6A).

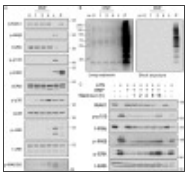


Figure 6

**DMF can activate p38 MAPK and can covalently modify its targets in the cell.**

DMF has the ability to covalently modify cysteine residues in its targets, as is proposed for Keap1. We assessed whether DMF inhibition remained following washout of DMF from the cells. BMDMs were treated for 4 h with 50 μM DMF and then either directly stimulated with LPS or washed extensively and incubated in media without DMF for various times. This showed that signaling was still inhibited by DMF 4 h after DMF being washed off the cells, but that inhibition was lost by 16 h (Fig. 6C). This slow loss of inhibition would be consistent with DMF covalently modifying its target in cells.

*In vivo* DMF can be converted to MMF. We therefore checked if MMF was able to mimic the effects of DMF on cell signaling. Unlike DMF, MMF did not inhibit LPS induced ERK1/2 or p105 phosphorylation, or the ubiquitination of IRAK1 (Supplementary Figure 1).

## DMF inhibits E2 conjugating enzymes *in vitro*

Together these results suggest that DMF may act at an upstream point in the TLR signaling cascade. The activation of Tak1 and the recruitment of the IKK complex involves the formation of K63/M1 hybrid polyubiquitin chains which can be added to several proteins in the MyD88 signaling cascade including MyD88 and IRAKs[34,40]. The formation of polyubiquitin chains requires a cascade of three enzymes; an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase to mediate the transfer of the ubiquitin to the substrate. E2 enzymes use a cysteine in their active site to covalently couple to ubiquitin. DMF is an electrophilic compound and therefore has the potential to react with the SH group in cysteines (Fig. 7A). We therefore investigated if DMF could inhibit Ubc13 or UbcH7, the major E2 enzymes proposed to be involved in the formation of K63 and M1 chains[41,42,43,44,45]. *In vitro*, addition of the E1 UBE1, ubiquitin and Mg-ATP to an E2 is able to promote the loading of ubiquitin onto the E2, which can be resolved as a mobility shift on SDS polyacrylamide gels[45]. Addition of DMF to this reaction inhibited the loading of ubiquitin onto Ubc13 with an IC50 of between 10 and 20 μM (Fig. 7B). In parallel assays, UbcH7 was more strongly inhibited by DMF with an IC50 of less than 10 μM and complete inhibition by 50 μM (Fig. 7B). If DMF were to inhibit Ubc13 or UbcH7 via covalent modification of a cysteine, the molecular mass of the E2 should be increased following DMF treatment. MALDI-TOF mass spectrometry was therefore used to determine the molecular mass of recombinant Ubc13 and UbcH7 before and after treatment with DMF. The recombinant Ubc13 has a theoretical mono-isotopic mass of 17295 Da, which corresponded well with the observed mass of 17301 Da. For Ubc13, an increase in the molecular mass of 144 Da

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