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Short communication

Fumaric acid esters prevent the NLRP3 inflammasome-mediated and ATP-triggered pyroptosis of differentiated THP-1 cells

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ABSTRACT

Fumaric acid esters (FAEs) exert therapeutic effects in patients with psoriasis and multiple sclerosis, however their mode of action remains elusive. Pyroptosis is a caspase-1-dependent pro-inflammatory form of programmed cell death, mediated by the activation of inflammasomes. To understand the pharmacological basis of the therapeutic effects of FAEs, the anti-pyroptotic activity of dimethyl fumarate (DMF) and its hydrolysis metabolite monomethyl fumarate (MMF) was studied in a model of NLRP3 inflammasome-mediated pyroptosis of human macrophages. Phorbol myristate acetate-differentiated THP-1 cells were exposed to lipopolysaccharide (5 μg/ml; 4 h), then pulsed with ATP (5 mM; 1 h). MMF, DMF, or parthenolide (positive control) were added 1 h before the ATP pulse. The pyroptotic cell death was evaluated by morphological examination and quantified by measuring the lactate dehydrogenase leakage. The ATP-triggered death of THP-1 cells (60.4 ± 4.0%) was significantly ($P < 0.01$) prevented by DMF, in a time- and concentration-dependent manner (pIC_{50} and maximal effect were 6.6 and 67.6 ± 1.2%, respectively). MMF was less efficacious than DMF. These effects were accompanied by a decreased intracellular activation of caspase-1 and interleukin-1β release from ATP-treated cells, thus suggesting that FAEs antagonise the effects of ATP by preventing the activation of the pyroptotic molecular cascade leading to cell death. These results indicate that FAEs are endowed with anti-pyroptotic activity, which may contribute to their therapeutic effects.

1. Introduction

The first use in medicine of fumaric acid (FA) derivatives was described by Schweckendiek W., who suffered himself from psoriasis. He raised the hypothesis that disturbances in the Krebs cycle might be implicated in the pathogenesis of this disease, and postulated that exogenous supplementation of FA, which is an intermediate of the Krebs cycle, might reverse the pathological process. Schweckendiek tried to prove his hypothesis by treating himself with FA derivatives and reported on the successful treatment in a self-experiment [1]. A role for an abnormal metabolism of FA in psoriasis was never proven, nevertheless, the effectiveness combined with a favourable long-term safety profile of drug products containing fumaric acid esters (FAEs) has been demonstrated in several clinical trials [2,3]. In 1994, Fumaderm®, a drug product consisting of dimethyl fumarate (DMF), calcium-, magnesium-, and zinc-salts of monoethyl fumarate, was approved in Germany as an oral treatment of *psoriasis vulgaris*. Over the last 15 years, compelling data on the effects exerted by FAEs have been accumulated. In particular, the evidence that BG-12, which in contrast to Fumaderm® consists of only DMF, exerts therapeutic effects in patients with relapsing–remitting multiple sclerosis (RRMS) [4–6] has received a lot of attention. In 2013, BG-12/Tecfidera® was approved in many countries as an oral treatment of RRMS. Despite the accumulated data, the mode of action of FAEs remains a matter of debate.

Pyroptosis is a caspase-1-dependent and pro-inflammatory form of programmed cell death. It can be triggered in myeloid and non-myeloid cell types both by bacterial products and non-bacterial stimuli, including viruses, microparticles, as well as host factors (i.e., misfolded proteins) [7]. Pyroptosis is mediated by the activation of inflammasomes, and is accompanied by release of pro-inflammatory signals, including interleukin (IL)-1β and ATP [7,8]. To date, several inflammasomes have been identified, and the NLRP3 inflammasome is the most studied. The activation of NLRP3 inflammasome is commonly described as a process requiring two stimuli. Many Toll-like receptor ligands (i.e., lipopolysaccharide, LPS; *priming stimulus*) prime cells by inducing the NF-κB-dependent expression of the NLRP3 protein. A further stimulus (i.e., ATP; *activating stimulus*) triggers the formation of the inflammasome complex, that leads to the activation of caspase-1, and eventually pyroptosis [7,8]. As pyroptosis is thought to contribute to the pathogenesis of a broad spectrum of disease, characterized by excessive/uncontrolled cell death and inflammation, compounds endowed with anti-pyroptotic activity are of

Abbreviations: Ac-YVAD-CMK, Ac-Tyr-Val-Ala-Asp-chloromethylketone; Ac-YVAD-pNA, Ac-Tyr-Val-Ala-Asp-p-nitroaniline; DMF, dimethyl fumarate; FA, fumaric acid; FAEs, fumaric acid esters; GSH, glutathione; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MMF, monoethyl fumarate; PMA, phorbol myristate acetate; RRMS, relapsing–remitting multiple sclerosis.

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interest due to their therapeutic potential [8]. A group of structurally unrelated small molecules, including parthenolide and Bay 11-7082 [9], resatorvid/TAK-242, 5Z-7-oxozeaneol, and bromoxone [10], compound 9 and related derivatives [11] (Fig. S1A) have been shown to prevent pyroptosis in *in vitro* experiments. The effects of FAEs on pyroptotic cell death have never been studied. The aim of this study was to investigate the anti-pyroptotic activity of DMF and its hydrolysis metabolite monomethyl fumarate (MMF; Fig. S1B) in an *in vitro* model of NLRP3 inflammasome-mediated and ATP-triggered pyroptosis of human macrophages.

2. Materials and methods

2.1. Cell cultures

Human myelomonocytic THP-1 cells were cultured in RPMI 1640 medium (purchased from Lonza, Basel, Switzerland), supplemented with foetal bovine serum (10%; Lonza), L-glutamine (2 mM; Lonza), penicillin (100 IU/ml; Lonza), and streptomycin (100 mg/ml; Lonza). Cell culture medium was replaced every 2–3 days, and the cultures were maintained at 37 °C, 95% air/5% CO_2 in a fully humidified incubator. The day before each experiment, cells were plated in 48-well culture plates (7.5×10^4 or 1.5×10^5 cells/well for the measurement of pyroptosis and IL-1β release, respectively) and were differentiated into monocytes/macrophages by treatment with phorbol myristate acetate (PMA; Sigma-Aldrich, Saint Luis, MO, USA; 50 nM, 24 h).

2.2. An in vitro model of pyroptosis

The effects of DMF and MMF were studied in a model of NLRP3 inflammasome-mediated pyroptosis of human macrophages, as described by Gong et al. [10]. Briefly, PMA-differentiated THP-1 cells were primed with LPS (from *Escherichia coli* 055:B5; 5 μg/ml; 4 h; Sigma-Aldrich) in serum-free medium. Pyroptosis was triggered with ATP (5 mM; 60 min; Sigma-Aldrich). The CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, MI, USA), a colorimetric assay based on the measurement of the lactate dehydrogenase (LDH) activity in the collected supernatants, was used to determine the percentage of cell death, according to the manufacturer's instruction.

2.3. Measurement of caspase-1 activity

Caspase-1 activity was measured by a spectrophotometric assay as previously described [11]. Reactions were carried out in 96-well plates in 100 μl final volume at 37 °C by diluting enzyme (50 U) in the reaction buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, pH 7.5, 10 mM DTT) and monitoring liberation of the chromophore from a specific labelled substrate [Ac-Tyr-Val-Ala-Asp-p-nitroaniline (Ac-YVAD-pNA; 200 μM final concentration); Vinci-Biochem; Vinci, Italy] in a Victor X4 (PerkinElmer, Waltham, MA, USA) at a wavelength of 405 nm. Absorbance was monitored at 5-min intervals for 90 min. The caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK; Vinci-Biochem) was employed as positive control.

2.4. Western blot analyses

Western blot analyses were performed as previously described [10]. Caspase-1 [both the precursor peptide = procasp-1 (45 kDa) and the p10 subunit] and IL-1β [both the precursor peptide = proIL-1β (37 kDa) and the mature IL-1β (17 kDa)] were detected with rabbit polyclonal antibodies (sc-515 and sc-7884, respectively; Santa Cruz Biotechnology, Dallas, TX, USA). To confirm the homogeneity of the proteins loaded, the membranes were stripped, and incubated with an anti-β-actin monoclonal antibody (Sigma-Aldrich). The membranes were overlaid with Western Lightning Chemiluminescence Reagent *Plus* (PerkinElmer Life Science, Norwalk, CT, USA), and exposed to Hyperfilm ECL film (Amersham Biosciences, Piscataway, NJ, USA).

2.5. IL-1β release

PMA-differentiated THP-1 cells were primed with LPS (5 μg/ml; 4 h) and pulsed with ATP (5 mM; 60 min). Supernatants were collected, centrifuged to discharge cellular debris and assayed for IL-1β by ELISA (eBioscence, Inc., San Diego, CA, USA).

2.6. Statistical and data analysis

Data were fitted and analysed by using the software Origin version 6.0 (Microcal Software, Northampton, MA). Statistical significance was evaluated by one-way ANOVA followed by the *post hoc* Bonferroni's correction; differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. NLRP3 inflammasome-mediated pyroptosis of differentiated THP-1 cells

Cells dying by pyroptosis undergo a measurable size increase, cell swelling and lysis [7]. Morphological changes consistent with the pyroptotic cell death were observed in PMA- and LPS-treated THP-1 cell cultures exposed to ATP (5 mM; Fig. 1A). Many cells appeared with marked size increase and underwent lysis. Moreover, compared to vehicle alone, a significantly higher ($60.4 \pm 4.0\%$; $P < 0.01$) LDH activity was measured in the supernatant collected from cell cultures exposed to ATP (5 mM, 60 min), indicating that most cells underwent lysis after exposure to this recognized pyroptotic stimulus (Fig. 1B). No significant increase in the LDH activity was instead observed in the supernatant from cell cultures exposed to either LPS or ATP alone.

These results indicate that differentiated and primed THP-1 cells pulsed with ATP are a suitable model to study pyroptosis.

3.2. Effects of FAEs on the ATP-triggered pyroptosis of differentiated THP-1 cells

To assess the anti-pyroptotic activity of FAEs, the effects exerted by MMF and DMF on the NLRP3 inflammasome-mediated and ATP-triggered pyroptosis were studied and compared to those exerted by parthenolide, which is endowed with anti-pyroptotic activity [9]. None of these compounds (10 μM, 1 h) inhibited LDH activity (data not shown), indicating that measurement of this parameter allows to study their anti-pyroptotic effects.

First, primed THP-1 cells were pre-treated (15–60 min) with either MMF, DMF, or parthenolide (all at 10 μM), and then pulsed with ATP (5 mM, 60 min). The ATP-triggered cell death was significantly prevented ($P < 0.05$) by these compounds in a time-dependent manner (Fig. 1C). Compared to MMF, DMF exerted larger effects. As expected, the nucleotide-triggered cell death was also prevented by parthenolide.

Then, cells were pre-treated (60 min) with increasing concentrations (0.001–10 μM) of MMF, DMF, or parthenolide, and then exposed to ATP (5 mM, 1 h). The ATP-triggered cell death was prevented by these compounds in a concentration-dependent manner (Fig. 1D); pIC_{50}s were 6.6, and 7.2, for DMF and parthenolide, respectively. Both DMF and MMF were significantly less efficacious than parthenolide ($P < 0.01$ for both comparisons), although DMF was significantly more efficacious than MMF ($P < 0.01$).

Collectively these results indicate that FAEs are endowed with anti-pyroptotic activity.

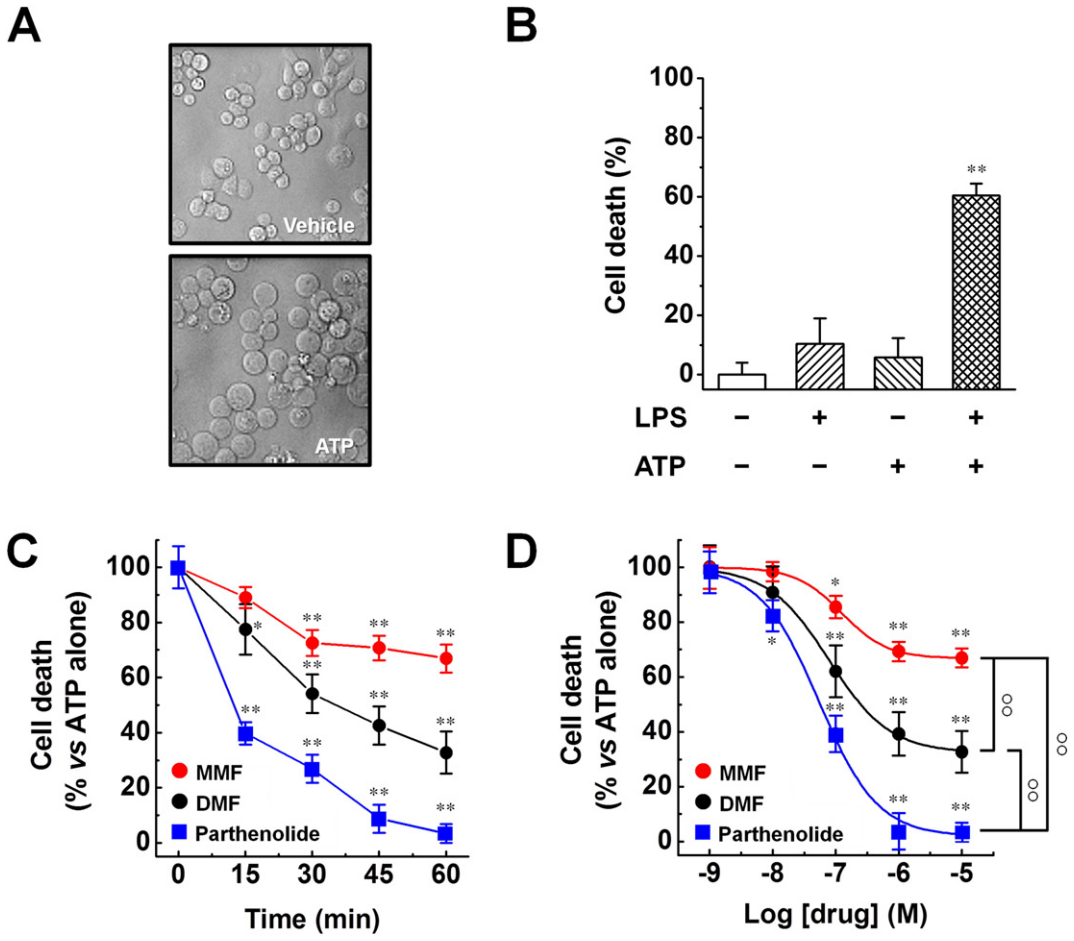

Fig. 1. An *in vitro* model of NLRP3 inflammasome-mediated and ATP-triggered pyroptotic cell death and effects of FAEs. PMA-differentiated THP-1 cells were primed with LPS (5 μg/ml; 4 h), then exposed to either vehicle alone or ATP (5 mM, 60 min). (A) Cells exposed to ATP displayed enlarged size and underwent lysis. (B) LDH activity was measured in the collected supernatants to determine the percentage of cell death. (C) Cells were pre-treated (60–15 min) with MMF, DMF, or parthenolide (all at 10 μM), and then exposed to ATP (5 mM, 1 h). (D) Cells were pre-treated (60 min) with increasing concentrations (0.001–10 μM) of DMF, MMF, or parthenolide, and then exposed to ATP (5 mM, 1 h). The ATP-triggered cell death was evaluated by measuring LDH activity in the collected supernatants. Data are the mean ± s.e.m. of six experiments run in triplicate. **P < 0.01 *vs* vehicle alone; ○○ P < 0.01 for the indicated comparisons (ANOVA plus Bonferroni's correction).

3.3. Effects of FAEs on caspase-1 activity and IL-1β production

To explore the mechanism underlying the anti-pyroptotic activity of FAEs, the effects of MMF, DMF and parthenolide on both caspase-1 activity and caspase-1 intracellular activation were studied. Caspase-1 activity was measured by a cell-free system assay, as previously described [11]. As shown in Fig. 2A, caspase-1 activity was significantly ($P < 0.01$) inhibited by the caspase-1 inhibitor Ac-YVAD-CMH (positive control; 100 μM), and parthenolide, but not by DMF and MMF (all at 10 μM). These finding indicate that the anti-pyroptotic activity of FAEs does not depend on a direct inhibition of caspase-1. By contrast, the ATP (5 mM; 1 h)-triggered caspase-1 intracellular activation in differentiated and LPS-primed THP-1 was prevented by MMF, DMF, and parthenolide (all at 10 μM; added 1 h before the ATP pulse; Fig. 2B). To confirm this finding, the release of IL-1β in the extracellular space was evaluated as a marker of intracellular caspase-1 activation. Compared to cell cultures exposed to vehicle alone, a significantly higher cytokine level was measured in the supernatant collected from the primed cultures exposed to ATP (5 mM, 60 min; 10.4 ± 29.7 *vs* 752.8 ± 111.4 pg/ml, respectively; $P < 0.01$). As shown in Fig. 2C, the ATP-triggered IL-1β release was significantly ($P < 0.05$) and in a concentration-dependent manner prevented by MMF, DMF, and parthenolide (0.01–10 μM; added 1 h before the ATP pulse): pIC_{50}s were 5.8, and 6.8 for DMF and parthenolide, respectively. Both DMF and MMF were significantly less efficacious than parthenolide ($P < 0.01$ for both comparisons), although DMF was significantly more efficacious than MMF ($P < 0.01$). Of note, comparable levels of proIL-1β and mature IL-1β were measured in extracts prepared from primed cells exposed to MMF, DMF, and parthenolide (all at 10 μM; 1 h; Fig. 2D), thus indicating that the effects of these compounds on the extracellular IL-1β level were due to the prevention of the ATP-triggered, caspase-1-mediated IL-1β processing, instead of IL-1β expression.

Collectively these results indicate that FAEs exert anti-pyroptotic effects by preventing the intracellular caspase-1 activation.

4. Discussion

Compounds endowed with anti-pyroptotic activity may be novel agents to counteract disorders characterized by excessive/uncontrolled cell death and inflammation [8]. In addition, evaluation of the anti-pyroptotic actions of drugs may be helpful to better understand their therapeutic effects. In this study the first evidence on the anti-pyroptotic activity of FAEs is provided. The effects of DMF and MMF have been evaluated in a well-established cellular model to study the NLRP3 inflammasome-mediated pyroptosis of human macrophages [10]. In our experiments, the ATP-triggered death of LPS-primed and PMA-differentiated THP-1 cells was prevented by DMF and MMF when they were added just before ATP, used to trigger cell death. Moreover, it was accompanied by a decreased intracellular activation of caspase-1. Thereby, FAEs antagonise the effects of ATP by preventing

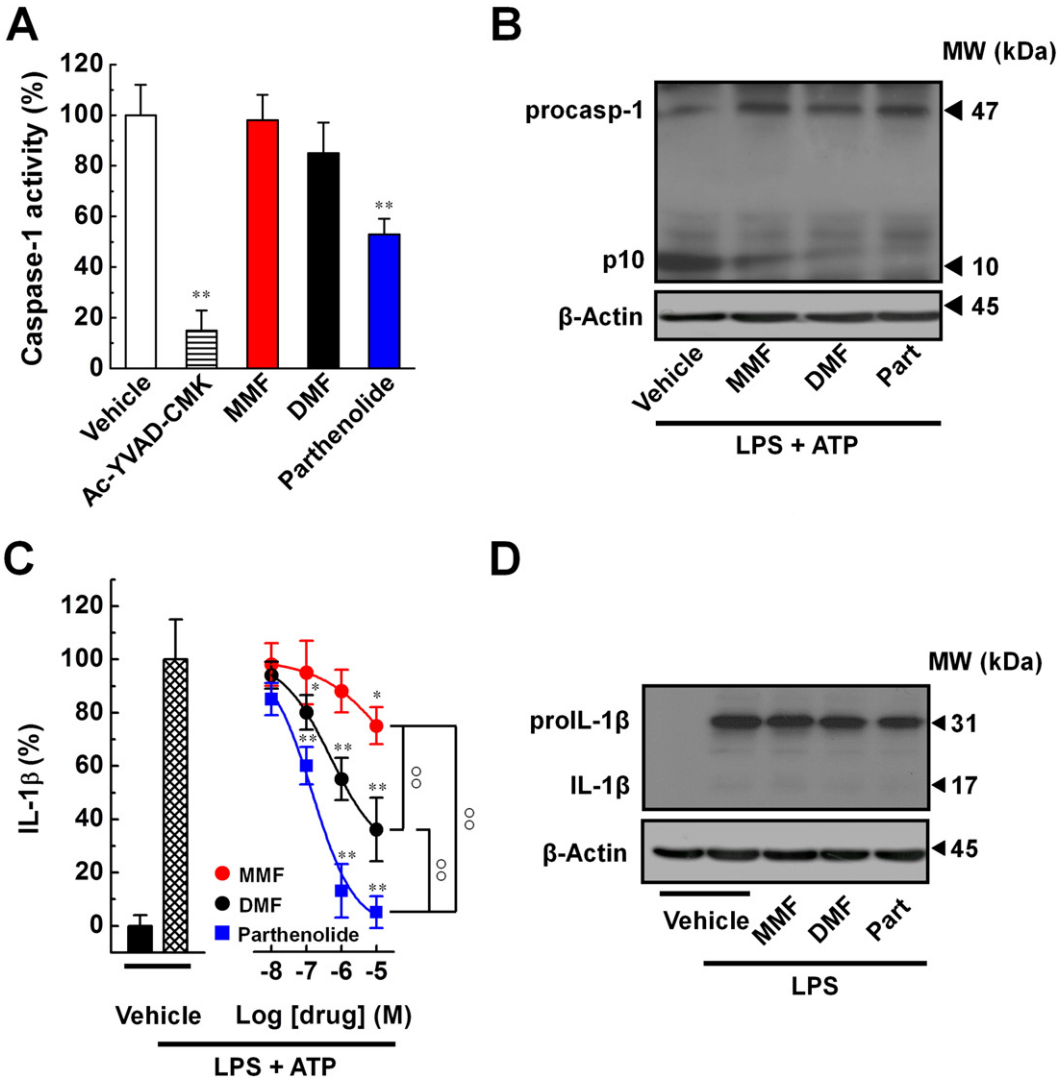

Fig. 2. Effects of FAEs on caspase-1 activation and IL-1β release. (A) Caspase-1 (50 U) from THP-1 lysates was incubated at 37 °C in the presence of either vehicle alone (DMSO 0.1%), Ac-YVAD-CMK (positive control, 100 μM), MMF, DMF, or parthenolide (Part; all at 10 μM) for 90 min. Caspase-1 activity was measured by monitoring (at 405 nm) liberation of the chromophore from the labelled substrate Ac-YVAD-pNA. (B) Differentiated and primed THP-1 cells were pre-treated with either vehicle alone (DMSO 0.1%), MMF, DMF, or parthenolide (all at 10 μM; 60 min), and then exposed to ATP (5 mM, 1 h). Caspase-1 activation was evaluated by detecting the precursor (procasp-1; 47 kDa) and the cleaved subunit p10 by western blot analysis. (C) Differentiated and LPS (5 μg/ml; 4 h)-primed THP-1 cells were pre-treated (60 min) with either vehicle alone (DMSO 0.1%) or increasing concentrations (0.01–10 μM) of MMF, DMF, or parthenolide, and then exposed to ATP (5 mM, 1 h). IL-1β release was evaluated by measuring the cytokine concentration in the collected supernatants by ELISA. (D) THP-1 cells were pre-treated (60 min) with either vehicle alone (DMSO 0.1%), MMF, DMF, or parthenolide (all at 10 μM) and the intracellular level of both proIL-1β (31 kDa) and mature IL-1β (17 kDa) were evaluated by western blot analysis. Data are the mean ± s.e.m. of three experiments run in triplicate. $^{**}P < 0.01$ *vs* vehicle alone; ○○ $P < 0.01$ for the indicated comparisons (ANOVA plus Bonferroni's correction). Images are representative of three experiments.

the activation of the pyroptotic cascade leading to cell death, thus mimicking (at least in part) the effects of not only parthenolide, but also Bay 11-7082 [9], resatorvid, 5Z-7-oxozeaneol, and bromoxone [10], compound 9 and related derivatives [11]. FAEs exerted anti-pyroptotic effects at low micromolar concentrations. Moreover, DMF was more efficacious than MMF in preventing the ATP-triggered pyroptotic cell death as well as IL-1β release from primed THP-1 cells. Differences in the physicochemical properties (i.e., lipophilia) between these two molecules could explain, at least in part, this profile. In addition, a different reactivity toward biological thiols could also contribute to it. The adduction of DMF and MMF to the free thiol group of glutathione (GSH) [12] and in the side chain of certain cysteine residues in proteins [13] has been demonstrated, and it has been shown that DMF is more reactive than MMF toward these functional groups. Interestingly, albeit structurally unrelated, most of the anti-pyroptotic compounds so far identified have electrophilic substructures (Fig. S1A), and Michael-like reactions with free thiol groups in proteins of the pyroptotic cascades have been postulated to underlie their activity [9–11]. FAEs even are Michael acceptors (Fig. S1B), and the adduction to biological targets (i.e., GSH, Keap-1) has been already suggested to mediate their effects [14–16]. Thereby, in keeping with previous findings, the adduction to proteins of the pyroptotic cascade could mediate the anti-pyroptotic effects of FAEs. Our preliminary findings on the reactivity of cysteine residues in proteins of the pyroptotic cascade toward FA support this hypothesis [17].

DMF has been demonstrated to be an essential component of Fumaderm® [18]. Results on BG-12/Tecfidera® are consistent with this data [4–6]. However, pharmacokinetic studies have shown that DMF undergoes to a large pre-systemic conversion to MMF and adduction to GSH [19]. Many *in vitro* data, including our results, indicate that compared to MMF, DMF is significantly more efficacious. This evidence suggests that the therapeutic effects of orally administered DMF could be related to its actions on the gut-intrinsic mechanisms involved in the regulation of the immune response. For example, previous studies [15,16] have shown that DMF inhibits the production by dendritic cells of pro-inflammatory cytokines (i.e., IL-6, IL-12, and IL-23) involved

in the differentiation of T helper 17 cells, which preferentially accumulate in the intestine [20]. Therefore, by altering the local humoral milieu, DMF could affect the differentiation of T helper 17 cell. Our data support this hypothesis. Indeed, as cells undergoing pyroptosis release IL-1β and ATP [7,8], which are involved in the T helper 17 cell differentiation process [20], we would propose that by preventing the NLRP3 inflammasome-dependent pyroptosis of intestinal myeloid cells, orally administered FAEs may exert beneficial effects by interfering with a gut-intrinsic mechanism implicated in the pathogenesis of autoimmune disease, including psoriasis and multiple sclerosis [20]. This hypothesis needs further investigation.

In conclusion, the anti-pyroptotic compounds are an emergent class of drugs; our findings indicate that FAEs belong to this class. Together with previous data our results might contribute to unveil the enigmatic pharmacological basis behind the therapeutic effects of FAEs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2015.06.011.

Conflicts of interest statement

The authors declare no conflict of interests.

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