**Title Page:**

# The anti-psoriatic agent monomethylfumarate has anti-proliferative, pro-differentiative and anti-inflammatory effects on keratinocytes

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**MMF-induced effects in keratinocytes**

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## Abbreviations:

DMF: Dimethylfumarate

IL-6: Interleukin-6

IL-1α: Interleukin-1α

K10: Keratin 10

MMF: Monomethylfumarate

TGase: Transglutaminase

TPA: 12-O-tetradecanoylphorbol-13-acetate

TNFα: Tumor necrosis factor-alpha

## **Abstract**:

Monomethylfumarate (MMF) is thought to be the bioactive ingredient of the drug Fumaderm®, licensed in Germany since 1994 for the treatment of moderate to severe psoriasis. Psoriasis is a common inflammatory hyperproliferative skin disorder that involves cross-talk between different cell types including immune cells and keratinocytes. Psoriatic lesions are characterized by hyperproliferation, aberrant differentiation and inflammation, with the psoriatic cytokine network maintained by communication between immune cells and keratinocytes. Recently, there is increasing evidence regarding the pivotal role of keratinocytes in mediating the disease process, and these cells can be regarded as safe therapeutic targets. From the data available on human subjects treated with Fumaderm®, MMF is an effective anti-psoriatic agent with known effects on immune cells. However, little is known about its direct effects on keratinocytes. We hypothesized that MMF has direct anti-proliferative, pro-differentiative and anti-inflammatory effects on keratinocytes. Indeed, MMF dose-dependently inhibited [$^3$H]thymidine incorporation into DNA, indicating a direct anti-proliferative action on keratinocytes. MMF significantly increased the protein level of the early keratinocyte differentiation marker, keratin 10 and the activity of transglutaminase, a late differentiation marker. These results are consistent with an ability of MMF to promote keratinocyte differentiation and inhibit proliferation thereby improving psoriatic lesions. In 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced keratinocytes, MMF significantly inhibited the expression of the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNFα), interleukin-6 and interleukin-1α as well as the production of TNFα. Our results support the notion that MMF has direct anti-proliferative, pro-differentiative and anti-inflammatory effects on keratinocytes, highlighting its potential use as a multifactorial anti-psoriatic agent.

## Introduction:

Fumaric acid esters (FAE) have been known as anti-psoriatic agents since 1959; however, their exact effects on different cell types as well as their mechanism of action is as yet unresolved. Fumaderm® is a drug that has been licensed in Germany since 1994 and consists of a mixture of fumaric acid esters including dimethylfumarate (DMF) and three salts of monoethylfumarate (MEF) (Rostami Yazdi and Mrowietz, 2008). Recently, FAE have garnered interest in the United States, especially with various multicenter studies revealing the drug's efficacy and limited safety concerns in most patients (Hodges et al., 2012). Although DMF is the main ingredient of the drug, DMF does not seem to be the active ingredient as it cannot be detected in the blood of patients, unlike monomethylfumarate (MMF), which has been detected in the blood for up to 36 hours after administration (Mrowietz et al., 1999).

Data have been published regarding the effects of FAE (including MMF) on endothelial as well as immune cells; however, little is known about their effects on keratinocytes (Nibbering et al., 1993; Thio et al., 1994; Litjens et al., 2004b; Wallbrecht et al., 2011; Dehmel et al., 2014). Thus, MMF has known effects on immune cells such as dendritic cells and granulocytes, which could possibly account in part for its anti-psoriatic actions (Nibbering et al., 1993; Litjens et al., 2004b). In addition, FAE (including MMF) exhibit inhibitory effects on the proliferation, differentiation, migration and tube formation of endothelial cells (Arbiser, 2011; Garcia-Caballero et al., 2011). However, the known effects of MMF on immune cells and endothelial cells may not adequately explain the superior results with Fumaderm® in treating moderate to severe psoriasis and accompanied by limited and non-serious side effects in comparison with other immune system-targeting drugs such as biological drugs (Mrowietz et al., 1998; Menter et al., 2008).

The skin maintains its barrier function through tight regulation of the proliferation and differentiation of keratinocytes, the primary cellular component of the epidermis. As keratinocytes begin to differentiate, they express early markers of keratinocyte differentiation, such as keratin 1 (K1) and keratin 10 (K10). Expression of K1 and K10, along with growth arrest, is considered the fundamental indicator of the switch from a proliferative basal phenotype to the post-mitotic phenotype. As keratinocytes differentiate and express various differentiation-associated proteins, these proteins are cross-linked by the enzyme transglutaminase (TGase), which catalyzes the formation of the isopeptide bonds that maintain the insolubility of the stratum corneum and hence barrier integrity (Candi et al., 2005). In psoriasis, this differentiation process is dysregulated, and keratinocytes exhibit hyperproliferation and aberrant differentiation, which are two key hallmarks of psoriatic lesions (Roberson and Bowcock, 2010).

Keratinocytes are also considered a major source of a wide spectrum of pro-inflammatory mediators (Alappatt et al., 2000; Grone, 2002), including cytokines such as interleukin (IL)-1, 6, 7, 8, 10, 12, 15, 18 and 20 as well as tumor necrosis factor-α (TNFα) (Grone, 2002), and chemokines such as CXCL8 through CXCL11 and CCL20 (Nestle et al., 2009). Cytokines and chemotactic factors produced by keratinocytes can initiate an inflammatory process in response to local irritation or injury. Thus, agents that have direct anti-proliferative, pro-differentiative and anti-inflammatory effects on keratinocytes could serve as ideal anti-psoriatic agents. In addition, keratinocytes possess cytokine receptors, so they are also targets for some cytokines. Indeed, certain cytokines can activate keratinocytes and lead to hyperproliferation and dysregulated differentiation and further induce the production of additional cytokines by these activated keratinocytes (Grone, 2002). Accordingly, since there is little known about the actions of MMF on keratinocytes, we hypothesized that MMF exerts direct anti-proliferative, pro-differentiative and anti-inflammatory effects on epidermal keratinocytes. These effects may provide MMF an advantage over other current anti-psoriatic agents that may have no or minimal effects on the regulation of keratinocyte growth and differentiation.

Data from this study demonstrate that MMF dose-dependently inhibited keratinocyte proliferation. We showed for the first time that MMF had pro-differentiative effects, enhancing the protein expression of K10 and TGase enzyme activity. Furthermore, we showed that MMF significantly inhibited the mRNA expression of TNFα, IL-6 and IL-1α in 12-O-tetradecanolylphorbol 13-acetate (TPA)-induced keratinocytes as well as TNFα secretion from these cells. These direct effects of MMF on keratinocytes, together with its known effects on immune cells and endothelial cells, may explain its superior clinical results and suggest that MMF is likely a multifactorial anti-psoriatic agent.

## Methods:

### Materials

Commercial keratinocyte serum-free medium (K-SFM) and the appropriate supplements were obtained from Gibco® Invitrogen (Grand Island, NY). Keratinocyte Basal Medium (KBM-Gold) was from Lonza, Inc. (Walkersville,MD). Monomethylfumarate (MMF) was from Sigma-Aldrich (St. Louis, MO). [$^3$H]Thymidine was from Moravek Biochemicals, Inc. (Brea, CA) and Ecolite scintillation fluid from MP Biomedicals (Solon, OH). Protein assay products and mini-protean TGX precast gels were obtained from Bio-Rad (Hercules, CA). Total RNA was reverse transcribed to cDNA using iScript Reverse Transcription Supermix from Bio-Rad Life Sciences (Hercules, CA), and RNA purification kits were from 5 PRIME (Gaithersburg, MD). IL-1α, IL-6 and TNFα Taqman probes were from Life Technologies (Grand Island, NY). Primary rabbit anti-keratin 10 antibody was from Covance (Princeton, NJ). Primary monoclonal mouse anti-β-actin antibody was from Sigma-Aldrich (St Louis, MO). Secondary antibodies used for western blot analysis were: AlexaFluor IRDye 800-conjugated goat anti-rabbit IgG and IRDye 680-conjugated goat anti-mouse IgG secondary antibody from LI-COR (Lincoln, NE).

### Primary mouse keratinocyte culture

Mouse epidermal keratinocytes were isolated from newborn male and female ICR CD1 outbred mice and cultured as previously described (Griner et al., 1999). Briefly, skins were harvested and floated overnight on 0.25% trypsin at 4°C overnight prior to a brief incubation at 37°C. The epidermis and dermis were then mechanically separated and the keratinocytes scraped from the underside of the epidermis. Cells were collected by centrifugation and seeded overnight in dialyzed serum-containing plating medium as described previously (Dodd et al., 2005). After incubation overnight, the medium was then replaced with K-SFM (50 µM $CaCl_2$). The medium was replaced every other day and the cells were cultured until they reached the desired confluence for experimentation.

## Normal human epidermal keratinocyte culture

Adult normal human male and female epidermal keratinocytes (NHEK Catalog# 192627) were obtained from Lonza, Inc. (Walkersville, MD) and were sub-cultured according to the supplier's instructions. Cells were plated in 6-well plates and cultured in commercially available Keratinocyte Basal Medium (KBM-Gold medium) from Lonza. This medium contains a calcium concentration of 14.7mg/l (99.986µM) in the form of calcium chloride dihydrate and is supplemented with hydrocortisone, transferrin, epinephrine, gentamicin sulfate, amphotericin-B, bovine pituitary extract, recombinant human epidermal growth factor and insulin. Supplements were supplied separately in quantities specified by the manufacturer and were added to the fresh medium just before use. The medium was replaced every other day, and the cells were cultured until they reached the desired confluence for experimentation.

## Measurement of cell proliferation by [$^3$H]thymidine incorporation assay

Primary cultures of mouse keratinocytes or normal human keratinocytes (60-70% confluent) were treated with the indicated concentrations of MMF in K-SFM or complete KBM-Gold medium, respectively, for 24 hours. Control samples contained no MMF. Cells were then labelled with 1 µCi/ml [$^3$H]thymidine for an additional 1 hour. Cells were washed twice with phosphate-buffered saline (PBS) lacking divalent cations and the reactions terminated with ice-cold 5% trichloroacetic acid. Cells were washed sequentially with 5% trichloroacetic acid followed by deionized water and were solubilized in 0.3M NaOH. An aliquot of this NaOH extract was counted in Ecolite scintillation fluid using a scintillation spectrometer (Beckman Coulter, Inc., Brea, CA).

## Measurement of cellular transglutaminase activity

Keratinocytes (80-90% confluent) were treated with the indicated concentrations of MMF prepared in K-SFM or complete KBM-Gold medium. Control samples contained no MMF. Cells were harvested in homogenization buffer containing 0.1M Tris-acetate (pH 8.5), 0.2mM EDTA, 20µM AEBSF, 2µg/ml aprotonin, 2µM leupeptin and 1µM pepstatin, and transglutaminase activity was assayed according to the technique of Folk and Chung (Folk and Chung, 1985) with minor modifications as described in our previous publication (Griner et al., 1999).

## Western blot analysis

After treatment cultured keratinocytes were solubilized with 330 µL warm lysis buffer containing 0.1875M Tris (pH 8.5), 3% sodium dodecyl sulfate (SDS), 1.5mM EDTA and water. Sample buffer containing 30% glycerol, 1% bromophenol blue, 15% β-mercaptoethanol and 54% water was added to each cell lysate to constitute Laemmli buffer (Laemmli, 1970). Lysates were then briefly boiled and stored at -20°C. Equal volumes of protein were separated on 8-10% SDS gels and transferred to Immobilon-P membranes followed by blocking for 1 hour and incubation with primary antibodies overnight at $4^{o}$C. Membranes were then washed with Tris-buffered saline with 0.1% Tween and incubated with secondary antibodies for an hour at room temperature. Membranes were visualized using an infrared Odyssey imaging system (LI-COR, Biosciences, Lincoln, NE) and immunoreactive bands quantified with the Odyssey software.

## Quantitative real-time PCR

Primary mouse keratinocytes (80-90% confluent) were treated with different concentrations of MMF for 24 hours. At 22 hours, keratinocytes were treated for 2 hours with TPA (100 nM) to induce a pro-inflammatory response in keratinocytes (for a total of 24 hours of exposure to MMF). Total RNA was extracted using PerfectPure RNA tissue kits (5 PRIME, Inc., Gaithersburg, MD) as per the manufacturer’s protocol. The quality and quantity of total RNA were assayed using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE). The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) was used to reverse transcribe equal quantities of total RNA (1µg) following the manufacturer’s instructions. qRT-PCR was performed using an ABI Step-One Plus Fast Real-Time PCR system (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions with the recommended parameters. Each cDNA sample (5 µL; 125 ng) was added to 10 µL Fast Reagent Master Mix, 4 µL DNase-RNase free water and 1 µL Taqman for a final volume of 20 µL and loaded into each well of a 96-well plate. Negative controls containing water instead of cDNA were performed to ensure purity of all reagents. Relative gene expression was calculated by the delta-delta Ct (ΔΔCt) method with GAPDH used for normalization. The results were expressed as the fold difference in gene expression relative to the endogenous gene and compared to control samples. Values were then expressed as "fold regulation" as described in Yuan et al. (Yuan et al., 2006).

## TNFα protein expression by ELISA

Mouse and human TNFα ELISA kits from BD Biosciences (San Jose, CA) were used. Near-confluent mouse and human keratinocytes were treated with different concentrations of MMF (300 µM, 500 µM, 750 µM and 1mM) for 24 hours. Primary mouse and normal human keratinocytes were co-treated with or without 100 nM TPA for the final 2 hours or 8 hours, respectively (for a total of 24 hours of exposure to MMF). Cell culture supernatants were collected and stored at -80°C and subjected to a single freeze-thaw cycle. Reagents and standards were prepared according to the manufacturer's instructions. Briefly, an aliquot of 50 µL of each sample was placed in each well. An aliquot of 50 µL ELISA diluent was added, and the plate was incubated for 2 hours at room temperature followed by aspiration of the contents of each well and washing 5 times with at least 300 µL of washing buffer. After the last wash, the plate was blotted on absorbent paper to remove any residual buffer. An aliquot of 100 µL of detection antibody was added to each well and the plate was incubated for 1 hour at room temperature. Aspiration of liquid and washing was performed as above. An aliquot of 100 µL enzyme working reagent (only for the mouse kit) was added to each well and incubated for 30 minutes at room temperature. A final aspiration/washing procedure was performed followed by the addition of 100 µL TMB One-step substrate reagent and incubation for 30 minutes. Finally, an aliquot of 50 µL stop solution was added to each well and the optical density and protein content was detected in each sample (well) within 30 minutes, at a wavelength of 450 nm and corrected at 570 nm.

## Statistical analysis

All experiments were performed independently and repeated a minimum of three times in duplicate. The values were statistically analyzed by one way-ANOVA, with a Student-Newmann-Keuls post-hoc test, using Prism software (Graph Pad Software Inc., San Diego, CA) with statistical significance assigned at $p<0.05$. All quantified data are expressed in the form of bar graphs with values representing means ± standard error (SEM).

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## Results:

### Inhibition of keratinocyte proliferation with different concentrations of MMF

We initially wished to confirm the anti-proliferative effect of MMF, which was previously demonstrated by cell counting (Thio et al., 1994), using an alternative proliferation assay. Keratinocytes were incubated for 24 hours with medium only or medium containing different concentrations of MMF (300 μM, 500 μM, 750 μM and 1mM), and the ability of MMF to inhibit keratinocyte proliferation was examined by monitoring the incorporation of [$^3$H]thymidine into DNA. As shown in Figure 1, MMF significantly inhibited the proliferation of actively growing normal keratinocytes in a dose-dependent manner. Figure 1A shows the anti-proliferative effect of MMF on primary mouse keratinocytes, whereas Figure 1B illustrates a similar effect in adult normal human keratinocytes. This result suggests that MMF inhibits keratinocyte proliferation in both species, an effect that would presumably be beneficial in improving hyperproliferative psoriatic lesions in human patients.

### Induction of keratinocyte differentiation by MMF

Differentiation is another important factor required for epidermal homeostasis, and psoriasis is characterized by abnormal keratinocyte differentiation (Nestle et al., 2009). We next investigated whether the anti-proliferative effect of MMF is accompanied by a pro-differentiative action. We tested for the effect of MMF on two keratinocyte differentiation markers that represent two different stages of the keratinocyte differentiation process, keratin 10 protein levels (K10, an early marker of keratinocyte differentiation) and transglutaminase enzyme activity (TGase, a late marker of keratinocyte differentiation) (Eckert et al., 2005; Helwa et al., 2013). To investigate the ability of MMF to stimulate early differentiation, near-confluent cultures of primary mouse keratinocytes were treated with different concentrations of MMF for 24 hours; then cell lysates were prepared and western blot analysis was used to detect the levels of K10 in MMF-treated and untreated cells. Our data indicate that a 24-hour treatment with MMF significantly increased K10 protein expression, with a maximum effect at a 500µM concentration (Figure 2). The activity of TGase, measured as the incorporation of the radiolabelled substrate [$^3$H]putrescine into casein, was then monitored to investigate the capacity of MMF to induce late differentiation in keratinocytes. Near-confluent cultures of primary mouse keratinocytes and adult normal human keratinocytes were treated with different concentrations of MMF for 24 hours, and transglutaminase enzyme activity assay was monitored as described in the Methods section. MMF induced a significant increase in TGase activity in both mouse and human keratinocytes (Figure 3A and B, respectively) with a maximum elevation at the 1mM concentration. These data suggest that at lower concentrations, MMF triggers early differentiation of keratinocytes and at higher concentrations, MMF induces keratinocytes to terminally differentiate. Thus, MMF has anti-proliferative, pro-differentiative effects on keratinocytes, stimulating both early and late markers of keratinocyte differentiation, a result that suggests that this drug may be able to correct the imbalance between proliferation and differentiation in psoriatic keratinocytes.

## MMF has an inhibitory effect on cytokine production by keratinocytes

Keratinocytes play an active role in the production of inflammatory mediators (cytokines and chemokines) in the psoriatic cytokine network (Steinhoff et al., 2001; Grone, 2002; Nestle et al., 2009). Accordingly, we hypothesized that MMF exerts a unique anti-inflammatory action by inhibiting major inflammatory mediators produced not only by immune cells (Litjens et al., 2004b) but also by keratinocytes. We opted to examine the effect of MMF on the expression of TNFα and IL-1α and IL-6 in keratinocytes treated with TPA, which is known to activate keratinocytes and induce inflammatory mediator release (Kim et al., 2014). Near-confluent primary mouse keratinocytes were treated with different concentrations of MMF for 24 hours. At 22 hours cells were spiked with 100nM TPA (for 2 hours), and mRNA was isolated. Quantitative

RT-PCR results showed that MMF significantly inhibited the TPA-induced expression of TNFα (Figure 4A), IL-6 (Figure 4B) and Il-1α (Figure 4C), thus revealing a specific anti-inflammatory effect of this drug on keratinocytes. Moreover, we confirmed the qRT-PCR results with TNFα using an ELISA assay to monitor the secretion of TNFα from both primary mouse and normal human keratinocytes. Near-confluent primary mouse keratinocytes were treated as above and the supernatants collected for assay by ELISA. The secretion of TNFα by MMF-pretreated cells activated with TPA was significantly inhibited, with levels returned almost to basal levels. For normal human keratinocytes, near-confluent cells were treated with different concentrations of MMF for 16 hours and cells were then co-treated with 100nM TPA for and additional 8 hours, prior to ELISA assay of the supernatants. Our data showed that MMF also inhibited TNFα production in TPA-activated human keratinocytes as compared to keratinocytes treated with TPA only. The inhibition in the case of human cells was so effective that TNFα levels in the supernatants of MMF-pretreated cells were below the limit of detection (2pg/ml) of the ELISA kit.

## Discussion:

Dimethylfumarate (DMF) is considered the essential ingredient of the anti-psoriatic drug, Fumaderm®. However, DMF does not seem to be the bioactive ingredient as it is not detected in patients' blood for a sufficient length of time to be exerting an action on its own (Litjens et al., 2004a). Furthermore, according to previous clinical and experimental studies, most of the DMF is hydrolyzed to MMF, which in turn is thought to act as an anti-psoriatic agent (Rostami Yazdi and Mrowietz, 2008; Rostami-Yazdi et al., 2009). Therefore, in our study we focused on the bioactive ingredient of the drug, MMF, rather than the main ingredient, an apparent prodrug with low bioavailability, using MMF concentrations that have recently been examined in other cell types (Bozard et al., 2012; Ananth et al., 2013; Promsote et al., 2014).

We show for the first time that MMF has direct pro-differentiative and anti-inflammatory effects on keratinocytes. Furthermore, using a reliable proliferation assay (incorporation of [$^3$H]thymidine into DNA), we confirmed previous published results concerning the anti-proliferative effects of MMF (Thio et al., 1994). Our results show that MMF significantly inhibited proliferation with greater than a 50% reduction in DNA synthesis at the highest concentration (1mM; Figure 1). This effect was observed in both primary mouse as well as normal human keratinocytes. However, to date, no studies have investigated the effect of MMF on keratinocyte differentiation. To investigate the effect of MMF on keratinocyte differentiation, we selected two differentiation markers that are relevant to the abnormality in psoriatic skin and that reflect two different stages of the differentiation process. K10 is one of the first proteins expressed by keratinocytes and reflects the fundamental switch from a proliferative basal phenotype to the post-mitotic phenotype; this switch is abnormal in psoriasis, in which diseased keratinocytes maintain their proliferative capacity into suprabasal layers (Candi et al., 2005). K10 can be regarded as the "keratinization marker of keratinocytes" (Moll et al., 2008), and it has been reported that K10 is greatly reduced in psoriatic skin (Iizuka et al., 2004). We observed that MMF significantly induced K10 protein expression at a concentration of 500µM (Figure 2). The biphasic effect of MMF on K10, with concentrations greater than 500μM inducing less of an increase in K10 levels, is likely because higher concentrations of MMF and with the long treatment interval (24 hours) drive keratinocytes to late differentiation. At later stages of differentiation, a decline in K10 expression is expected, accompanied by an increase in TGase activity, a late marker of differentiation, as shown in Figure 3.

TGase is another important protein that facilitates terminal keratinocyte differentiation and the formation of the cornified envelope (stratum corneum) (Eckert et al., 2005). Thus, K10 and TGase delineate the effect of MMF not only on initial keratinocyte differentiation but also on the terminal stage involving stratum corneum formation and hence barrier integrity. Furthermore, psoriatic lesions have previously been shown to exhibit aberrant epidermal TGase distribution (Candi et al., 2002), and inhibition of TGase in mouse skin leads to hyperproliferation and parakeratosis (Harrison et al., 2007). MMF significantly increased TGase enzyme activity at a 1mM concentration by almost 2-fold (200%) in primary mouse keratinocytes and about 1.5-fold (150%) in adult normal human keratinocytes.

We also investigated the effect of MMF on the third major hallmark of psoriasis, inflammation. TNFα, IL-6 and IL-1α are key cytokines in the psoriatic cytokine network and can be secreted by immune cells as well as keratinocytes, thus amplifying their pro-inflammatory potential. These cytokines are also capable of activating keratinocytes (Nestle et al., 2009). Awareness of the role of TNFα in the psoriatic-cytokine network has led investigators to the development of anti-psoriatic biologic drugs that target TNFα, such as Etanercept© (Griffiths, 2010). Moreover, the serum levels of TNFα and IL-6, another pro-inflammatory cytokine, are significantly higher in psoriasis patients as compared to healthy controls (Arican et al., 2005). IL-1α is a known regulator of keratinocyte proliferation and differentiation, as well as immune function (Taniguchi et al., 2014). Indeed, IL-1α overexpression in the basal epidermal layer (in an IL-1α transgenic mouse model) produces a psoriasis-like phenotype with hyperproliferation and immune cell infiltration (Groves et al., 1995; Gudjonsson et al., 2007). Furthermore, with the extensive cross-talk between keratinocytes and immune cells in psoriasis, it is unclear which is the major source of these cytokines, and the role of activated keratinocytes in cytokine production cannot be excluded. In fact, keratinocytes are thought to be the primary producers of IL-1 in the skin (Brotas et al., 2012). In order to stimulate cytokine production by keratinocytes, we used TPA, which is a known inducer of the keratinocyte inflammatory response (Kim et al., 2014). However, one caveat of our study is the use of normal keratinocytes, since exposure of psoriatic keratinocytes to an inflammatory milieu may alter their responses to various agents including possibly MMF. Nevertheless, with the technical difficulty of obtaining sufficient psoriatic keratinocytes to perform all of the reported experiments, the absence of an available psoriatic keratinocyte cell line, and the clinical success of Fumaderm in psoriatic patients, our results can be regarded as a proof of concept regarding the effects of MMF in keratinocytes. Future studies are required to validate these data in psoriatic keratinocytes.

Our results show that MMF was a potent inhibitor of inflammatory cytokine expression by TPA-activated keratinocytes. As mentioned earlier, in an inflammatory response, keratinocytes are a major source of a spectrum of different cytokines including IL-6 and TNFα (Grone, 2002) (Nestle et al., 2009). By stimulating keratinocytes with TPA we mimicked the initiation phase of the psoriatic process (Helwa et al., 2013), and MMF was capable of efficiently inhibiting this stimulation. This conclusion was reached based on qRT-PCR and ELISA findings. In the case of human keratinocytes, MMF treatment reduced the TPA-induced TNFα secretion to undetectable levels, reflecting the effective anti-inflammatory action of MMF on keratinocytes. Thus, we can conclude that MMF inhibits proliferation, induces differentiation and decreases the expression/production of cytokines by keratinocytes and hence can presumably improve psoriatic lesions by down-regulating the psoriatic cytokine network and restoring keratinocyte homeostasis.

Most available anti-psoriatic drugs target immune cells and/or mediators, and most researchers and dermatologists believe that targeting these cells and molecules is the ultimate goal for an anti-psoriatic agent. However, considering the essential role of keratinocytes in the psoriatic cytokine network, targeting keratinocytes as well will amplify the drug's effect and may not only be effective but also safe, as there will be less compromise of the patient's immune system. Indeed, hyperproliferation, absent or abnormal differentiation and inflammation (inflammatory cytokine production and immune cell infiltration) are the three major hallmarks of psoriasis. A drug with a pleiotropic mode of action that can target all three of these aspects should be considered the ultimate therapeutic strategy to treat psoriasis. Considering the already proven clinical efficacy of Fumaderm® (Mrowietz et al., 1998), studies to determine the effects and mechanism of action of this drug certainly seem warranted.

A major question remains concerning the mechanism of action of MMF, and studies are in progress in our laboratory to investigate the possible signaling pathways that underlie the effects of MMF. Among these possibilities is the G-protein coupled receptor GPR109A (HCA2). In 2008 Tang et al. (Tang et al., 2008) reported that MMF is an agonist of GPR109A. However, little is known about the physiological functions of GPR109A in the skin and the possible mechanisms activated by its binding of MMF. In addition, *in vitro* and animal studies as well as clinical data suggest that MMF up regulates the anti-oxidative transcription factor Nrf2 (Onderdijk et al., 2014; Promsote et al., 2014). Our group is currently attempting to address these questions.

To our knowledge, this is the first report to show a direct pro-differentiative, anti-inflammatory effect of MMF on keratinocytes. Moreover, we also confirmed the anti-proliferative effect of MMF on keratinocytes. These effects seem to account, at least in part, for its action as an anti-psoriatic agent. These findings, in addition to previously published data, suggest that MMF is exerting inhibitory functions on the three major cell-types involved in the pathogenesis of psoriasis (keratinocytes, immune cells, and endothelial cells) (Nibbering et al., 1993; Garcia-Caballero et al., 2011). Together with the clinical data obtained for the anti-psoriatic drug Fumaderm® (Mrowietz et al., 1998; Rostami Yazdi and Mrowietz, 2008), used in Germany since

1994, these results suggest that MMF may be an ideal therapy for treating moderate to severe psoriasis. Our findings may help to pique interest in the anti-psoriatic actions of MMF, which may encourage physicians in other countries (including the US) to investigate the use of the drug for the treatment of psoriasis. These data also suggest that MMF may be effective for treating other hyperproliferative skin disorders. In addition, a drug containing DMF as its major ingredient (Tecfidera®; BG-12) is available on the US market now for the treatment of multiple sclerosis (Nicholas et al., 2012; Salmen and Gold, 2014). Thus, our results suggest the possibility of repurposing this already FDA-approved drug for the benefit of psoriatic patients.

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## **Authorship Contributions**:

Participated in research design (IH, RP, PK, IK-D, VC, WBB), conducted experiments (IH, RP, PK, IK-D, VC), performed data analysis (IH, WBB), wrote or contributed to the writing of the manuscript (IH, I-KD, VC, WBB)

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## **Footnotes**:

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## **Legends for Figures:**

**Figure 1: MMF exerted an anti-proliferative effect on keratinocytes.**

Near-confluent primary mouse keratinocytes or normal human epidermal keratinocytes (panels A and B, respectively) were treated with the indicated concentrations of MMF (or no MMF for the control) in K-SFM or complete KBM-Gold, respectively, for 24 hours. [$^3$H]Thymidine incorporation was measured as in Griner et al. (Griner et al., 1999). Values, expressed as means ± SEM, represent the percentage over the control of 5 separate experiments performed in duplicate; $*p \leq 0.05$, $** p \leq 0.005$, $*** p \leq 0.0005$ versus the control. For primary mouse keratinocytes, the mean (± SEM) value of the control was 14,043 ± 2,042 cpm/well. For adult normal human keratinocytes, the mean control value was 170,478 ± 7,378 cpm/well.

**Figure 2: MMF at a lower concentration induced the protein expression of the early marker of differentiation, keratin 10 (K10).**

Near-confluent primary mouse keratinocytes were treated with 0, 300µM, 500µM, 750µM and 1mM MMF for 24 hours. Cells were then harvested and proteins analyzed by western blotting. Cytokeratin 10 levels were determined using rabbit anti-K10 antibody (Covance) and the LiCor Odyssey system. Shown in panel A is a representative blot. Panel B shows the quantification and statistical analysis of the results of four separate experiments, with values normalized to actin levels and shown as the means ± SEM; $*p \leq 0.05$ versus the control.

**Figure 3: Higher concentrations of MMF increased TGase activity, a late marker of keratinocyte differentiation.**

Near-confluent cultures of primary mouse keratinocytes (panel A) and normal human keratinocytes (panel B) were treated with the indicated concentrations of MMF for 24 hours and TGase activity was assayed. For each experiment, the TGase activity in treated keratinocytes was normalized to protein content and then to the TGase activity of control (untreated) cells. Data are expressed as the percentage of control and represent the means ± SEM of at least three separate experiments in duplicate; $*p \leq 0.05$ versus the control. For primary mouse keratinocytes, the mean value of the control was 3,075 ± 1,171 cpm/µg protein. For adult normal human keratinocytes, the mean control value was 3,523 ± 183 cpm/µg protein. In addition, a positive control (1mM calcium-containing medium) performed with the same cells yielded an approximate 444 ± 48% increase in TGase activity in mouse and 195 ± 34% in human keratinocytes.

**Figure 4: MMF down-regulated the mRNA expression of inflammatory cytokines in keratinocytes treated with TPA.**

Near-confluent cultures of primary mouse keratinocytes were treated with 0, 300µM, 500µM, 750µM or 1mM MMF for 22 hours. Cells were then co-treated with or without 100nM TPA for 2 hours (for a total of 24 hours of exposure to MMF). RNA was isolated and transcribed to cDNA, and qRT-PCR was performed to determine the mRNA levels of TNFα (n=6), IL-6 (n=3) and IL-1α (n=3). Values represent the means ± SEM of 3-6 separate experiments performed in duplicate; $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$ versus TPA alone; $\$p \leq 0.05$ versus control (untreated) cells. Please note that initially, we treated both primary mouse keratinocytes and adult normal human keratinocytes with TPA for the same length of time (2 hours); however, the TNFα levels were undetectable in the control untreated human keratinocytes as well as in the MMF-treated human cells. Therefore, we increased the time of incubation to 8 hours in an unsuccessful attempt to bring the TNFα levels in the control and/or MMF-treated samples into the detectable range.

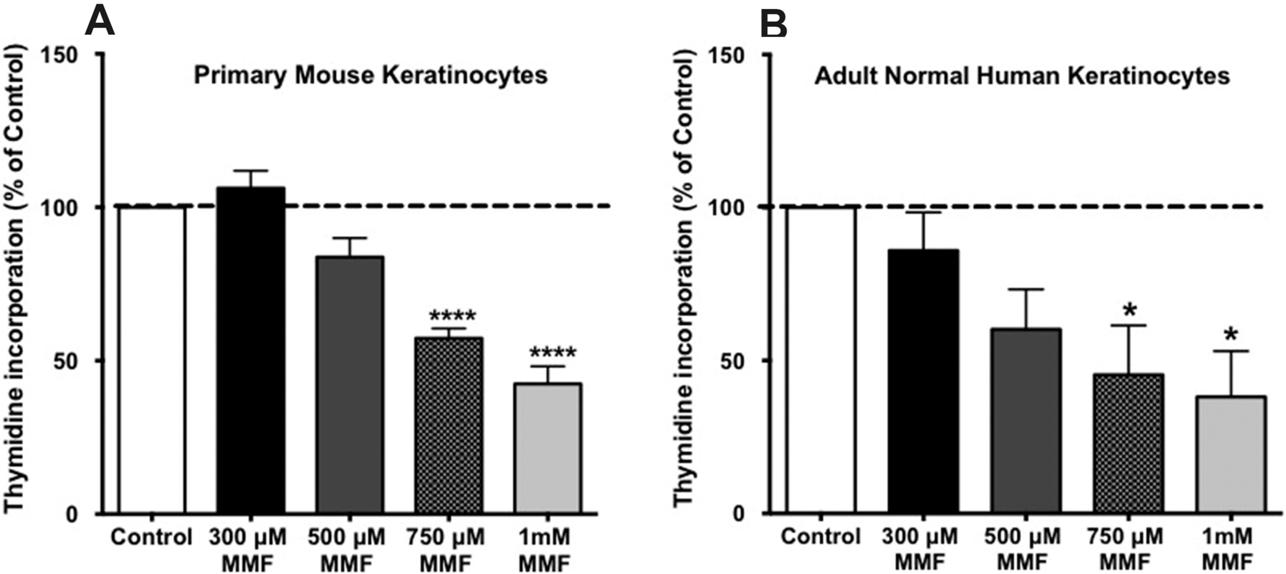
**Figure 5: MMF inhibited the production of TNFα by keratinocytes treated with TPA.**

Near-confluent cultures of primary mouse keratinocytes and normal epidermal keratinocytes were treated with 0, 300µM, 500µM, 750µM or 1mM MMF for 22 and 16 hours, respectively. Cells were then co-treated with or without 100nM TPA for an additional 2 hours and 8 hours, respectively (for a total of 24 hours of exposure to MMF). TNFα ELISA assays were performed and values represent the means ± SEM of 3 separate experiments performed in duplicate; *p≤0.05, **p≤0.005, ***p≤0.0005 versus TPA alone; $p≤0.05 versus control (untreated) cells.

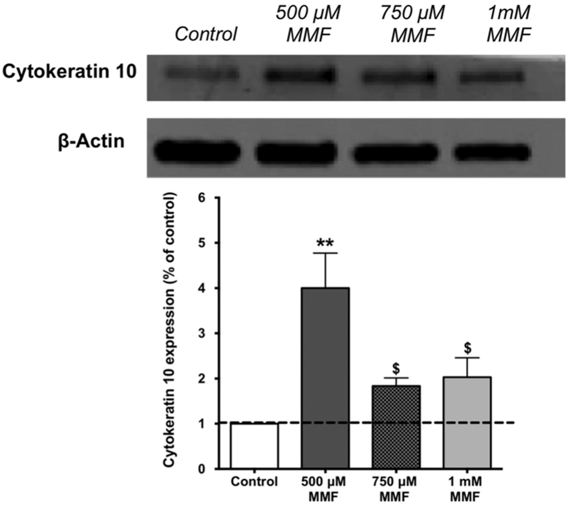
**Figure 6: The role of keratinocytes in the psoriatic-cytokine network and the effect of MMF in ameliorating the disease process.**

This figure illustrates the interactions between keratinocytes and immune cells, which mediate the initiation, progression, and maintenance of the inflammatory process in psoriasis. A stimulus such as trauma or an infection can initiate an inflammatory response in genetically susceptible individuals. Keratinocytes are capable of initiating an immune response through the production of various pro-inflammatory cytokines, which feed back on the keratinocytes, inducing further activation and production of cytokines. Activated keratinocytes exhibit hyperproliferation and abnormal differentiation. MMF is capable of counteracting these effects to restore the balance between proliferation and differentiation as well as exerting an anti-inflammatory effect, all of which would improve psoriatic symptoms.

# Figure 1


A
Primary Mouse Keratinocytes
Thymidine incorporation (% of Control)
150
100
50
0
****
****
Control
300 μM MMF
500 μM MMF
750 μM MMF
1mM MMF
B
Adult Normal Human Keratinocytes
Thymidine incorporation (% of Control)
150
100
50
0
*
*
Control
300 μM MMF
500 μM MMF
750 μM MMF
1mM MMF

# Figure 2


Control
500 μM MMF
750 μM MMF
1mM MMF
Cytokeratin 10
β-Actin
Cytokeratin 10 expression (% of control)
0
1
2
3
4
5
6
**
$
$
Control
500 μM MMF
750 μM MMF
1 mM MMF

# Figure 3

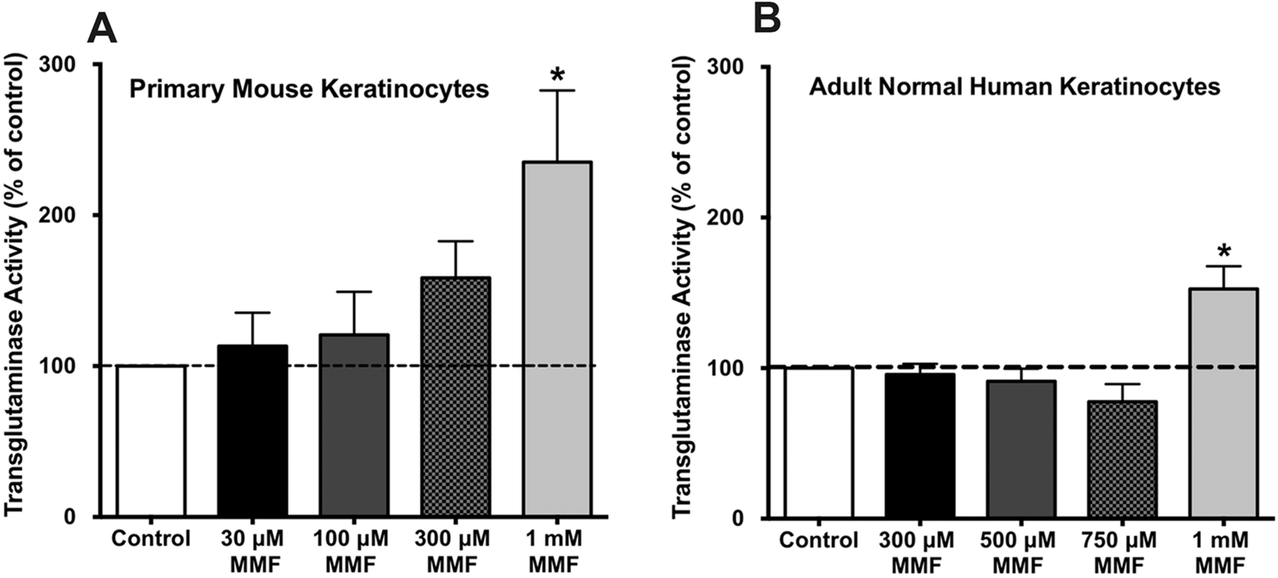

A
Primary Mouse Keratinocytes
Transglutaminase Activity (% of control)
300
200
100
0
*
Control
30 µM MMF
100 µM MMF
300 µM MMF
1 mM MMF
B
Adult Normal Human Keratinocytes
Transglutaminase Activity (% of control)
300
200
100
0
*
Control
300 µM MMF
500 µM MMF
750 µM MMF
1 mM MMF

Figure 4

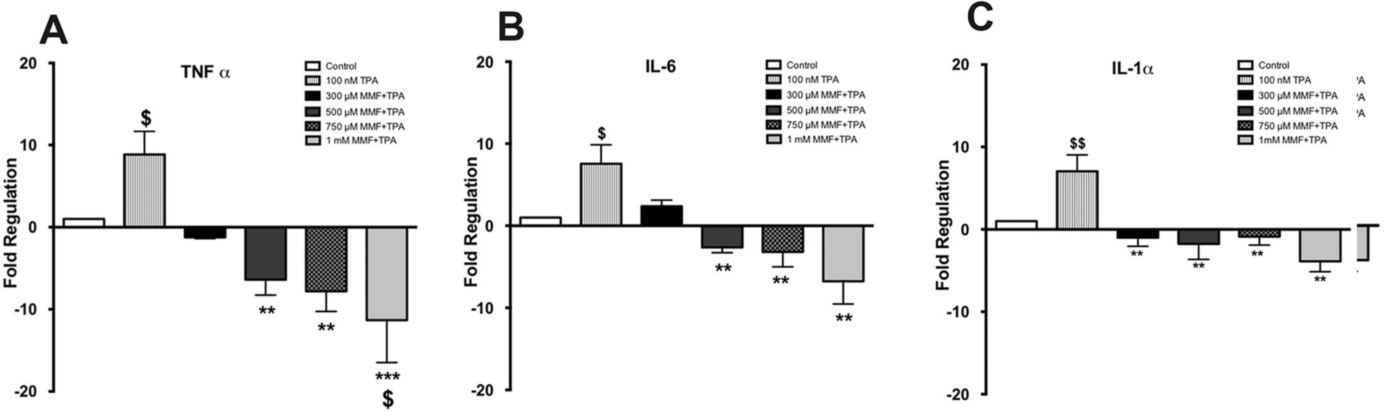
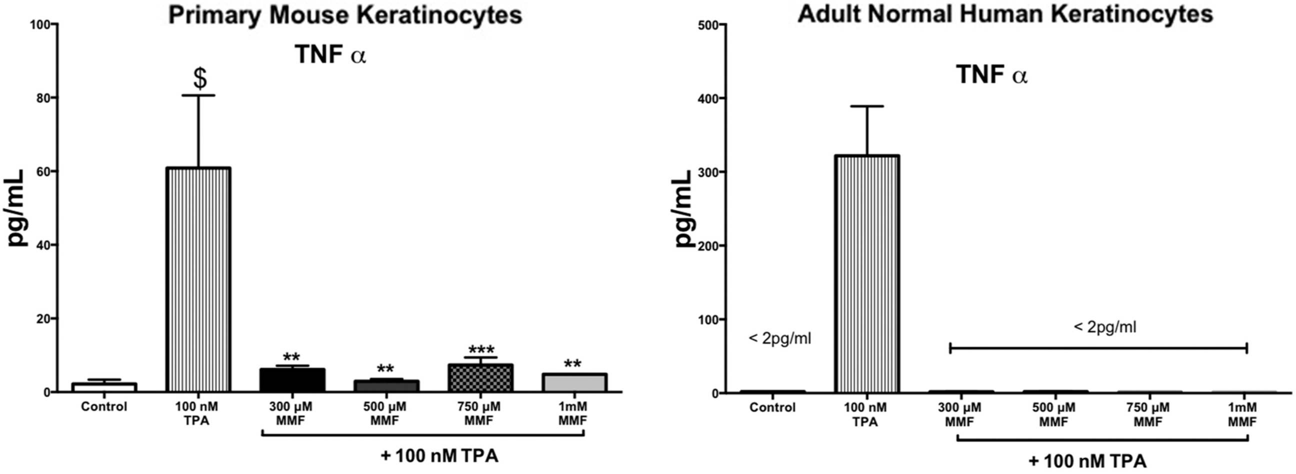
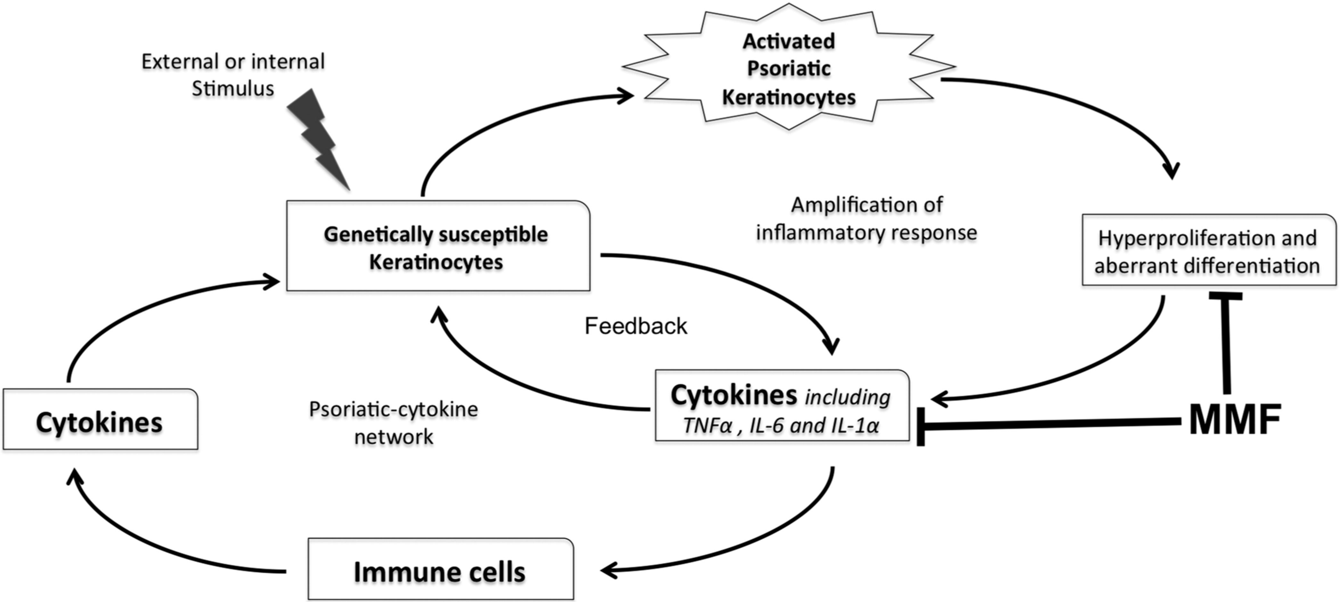

A
TNF α
Control
100 nM TPA
300 μM MMF+TPA
500 μM MMF+TPA
750 μM MMF+TPA
1 mM MMF+TPA
Fold Regulation
20
10
0
-10
-20
$
**
**
***
$
B
IL-6
Control
100 nM TPA
300 μM MMF+TPA
500 μM MMF+TPA
750 μM MMF+TPA
1 mM MMF+TPA
Fold Regulation
20
10
0
-10
-20
$
**
**
**
C
IL-1α
Control
100 nM TPA
300 μM MMF+TPA
500 μM MMF+TPA
750 μM MMF+TPA
1mM MMF+TPA
Fold Regulation
20
10
0
-10
-20
$$
**
**
**
**

**Figure 5**


Primary Mouse Keratinocytes
TNF α
pg/mL
0
20
40
60
80
100
$
**
**
***
**
Control
100 nM TPA
300 µM MMF
500 µM MMF
750 µM MMF
1mM MMF
+ 100 nM TPA
Adult Normal Human Keratinocytes
TNF α
pg/mL
0
100
200
300
400
500
< 2pg/ml
< 2pg/ml
Control
100 nM TPA
300 µM MMF
500 µM MMF
750 µM MMF
1mM MMF
+ 100 nM TPA

# Figure 6


External or internal Stimulus
Activated Psoriatic Keratinocytes
Genetically susceptible Keratinocytes
Amplification of inflammatory response
Hyperproliferation and aberrant differentiation
Feedback
Cytokines
Psoriatic-cytokine network
Cytokines including TNFα , IL-6 and IL-1α
MMF
Immune cells