Oral dimethyl fumarate induces changes within the peripheral neutrophil compartment of patients with psoriasis that are linked with skin improvement

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Summary

Background Dimethyl fumarate (DMF) is a treatment for moderate-to-severe psoriasis and multiple sclerosis. DMF therapy typically improves skin inflammation within the first 3 months of treatment. DMF is a prodrug that generates the hydroxycarboxylic acid receptor 2 (HCA2) agonist, monomethyl fumarate (MMF). Despite widespread clinical use, DMF’s mechanism of action is not fully understood.

Objectives We wished to characterize the changes induced by DMF in peripheral neutrophils within the first 3 months of treatment to better understand its early antipsoriatic effects.

Methods Flow cytometry was used to assess T-cell and neutrophil frequencies, apoptosis and activation phenotype. In vitro culture of neutrophils with DMF and MMF was used to evaluate apoptosis and HCA2 internalization. Serum levels of neutrophil degranulation products were measured by enzyme-linked immunosorbent assay.

Results Patients with psoriasis had significantly higher leucocyte counts at baseline compared with controls, with a large population of pro-inflammatory CD62Llo CD11bbright neutrophils. Analysis revealed that DMF treatment reduced the frequency of CD62Llo CD11bbright neutrophils and serum levels of neutrophil activation markers. This reduction was not linked to increased apoptosis.

Conclusions Our results reveal a novel in vivo effect of DMF therapy on pro-inflammatory neutrophils that likely contributes to this treatment’s antipsoriatic efficacy.

What is already known about this topic?

- Oral dimethyl fumarate (DMF) improves psoriasis within the first 3 months of treatment but its mechanism of action has not been fully elucidated.
- It is known that DMF is a prodrug for monomethyl fumarate, which itself is a hydroxycarboxylic acid receptor 2 (HCA2) agonist.
- Treatment with oral DMF is linked to the loss of peripheral T cells (which do not express HCA2), while changes in neutrophils (which are HCA2-positive) have not been widely reported.

What does this study add?

- This study describes that patients with untreated psoriasis have elevated numbers of pro-inflammatory CD62Llo CD11bbright neutrophils.
- DMF therapy reduces both the number of pro-inflammatory neutrophils and the serum concentrations of markers of neutrophil activation within the first 3 months of treatment.
- DMF treatment does not promote neutrophil apoptosis in patients with psoriasis.
Drugs containing dimethyl fumarate (DMF) are important treatments for psoriasis and multiple sclerosis (MS). The primary DMF formulations used for plaque psoriasis are marketed as Fumaderm® and Skilarence® and both induce significant clinical improvement by month 3 of treatment. Although DMF is efficacious in psoriasis and MS, its mechanism of action is poorly understood. DMF is a prodrug that is rapidly and completely metabolized into monomethyl fumarate (MMF) before entering the circulation. MMF is an agonist of the G1/G0 protein-coupled receptor hydroxy-carboxylic acid receptor 2 (HCA2, also named GPR109A). HCA2 is expressed by numerous haematopoietic and non-haematopoietic cells, including neutrophils, monocytes, macrophages, Langerhans cells, microglia, adipocytes, intestinal epithelial cells and keratinocytes. MMF activation of HCA2 on keratinocytes and Langerhans cells causes flushing, a common side-effect of DMF therapy. HCA2 is also required for the protective effect of DMF in mouse models of MS and bullous pemphigoid. However, it is not known if HCA2–MMF interactions play a role in the antipsoriatic effects of DMF.

Multiple groups have shown that DMF treatment of patients with psoriasis and MS has numerous effects on the immune system. Most striking is the development of severe lymphopenia (< 500 lymphocytes μL⁻¹) in approximately 10% of patients treated with DMF. While lymphopenia can lead to treatment cessation, more moderate T-cell losses are widely observed in patients treated with DMF. Both CD4⁺ and CD8⁺ T cells are reduced during DMF therapy with CD8⁺ T cells more strongly affected. Moreover, memory T cells seem particularly sensitive to depletion. DMF treatment also reduces numbers of T-helper (Th) 1 cells and increases frequencies of Th2 cells. As T cells do not appear to express HCA2, these changes potentially occur by indirect mechanisms via other HCA2-expressing cell types such as monocytes and dendritic cells or through HCA2-independent pathways.

Significant changes in T cells during DMF treatment are typically noticeable after 4–6 months, while improvements in psoriasis are observable within 2–3 months. Thus, it is possible that other cell types important for psoriatic disease are affected earlier by DMF treatment. Neutrophils express HCA2 and represent one of the initial immune cells to infiltrate the skin during plaque formation and likely contribute significantly to tissue inflammation by producing reactive oxygen species (ROS), degranulation, neutrophil extracellular trap (NET) formation and pro-inflammatory cytokine expression. Neutrophils are also an important source of proteases (e.g. cathepsin G, proteinase E and elastase) that cleave interleukin (IL)-36 family cytokines into their active pro-inflammatory forms. Extravasation and tissue infiltration by neutrophils involves multiple molecules, for example, CD62L (L-selectin) on neutrophils binds ligands on endothelial cells. As CD62L is shed during neutrophil extravasation and activation, low levels of neutrophil CD62L reported in patients with psoriasis likely represent enhanced activation and reduced tissue migration. Because neutrophils express HCA2 and are potentially important in psoriasis pathogenesis, we postulated that during early DMF treatment neutrophil activity is modulated by MMF–HCA2 interaction. To gain insights into this hypothesis we have analysed changes in neutrophils from patients with psoriasis during the first 3 months of DMF treatment.

### What is the translational message?

- Pro-inflammatory CD62L⁻ neutrophil numbers correlate with psoriasis severity during DMF treatment.
- The reduction in CD62L⁻ neutrophils during treatment may represent both a mechanism by which DMF improves psoriasis and a biomarker of treatment efficacy.

### Methods

#### Patients

Patients with psoriasis and healthy controls were enrolled at the Psoriasis Center of the Department of Dermatology at the University Medical Centre Schleswig-Holstein, Kiel campus. This was a single-centre cohort study. Approval was obtained from the local ethics committee (AZ: D443/15). Data were collected between September 2015 and July 2019. All study participants provided informed written consent. Patients were over 18 years of age and diagnosed with plaque-type psoriasis. Only DMF-naïve patients were included. DMF dosing followed the indicated guidelines for Fumaderm® and Skilarence® and was modified based on patient tolerance. Peripheral blood was taken by venipuncture prior to DMF treatment (month 0) and at every month until month 3. Psoriasis Area Severity Index (PASI) was determined at every visit. Healthy controls and patients were matched as closely as possible in terms of age and sex (Table 1).

#### Cell isolation

Leucocytes were isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood by hypertonic red blood cell lysis and centrifugation. Isolated total leucocytes were then counted using dye exclusion with trypan blue. Leucocytes were diluted to a concentration of 10 × 10⁶ mL⁻¹ in phosphate-buffered saline before staining for flow cytometry. Neutrophils were isolated from EDTA-anticoagulated blood by magnetic negative-selection with MACSxpress Whole Blood
Neutrophil isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions to yield untouched neutrophils for in vitro studies. Neutrophils were diluted in Dulbecco’s Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Darmstadt, Germany) with 10% inactivated fetal calf serum (FCS; Merck, Munich, Germany) and 1X HEPES (Merck) to a concentration of $10^6$ mL$^{-1}$. Only neutrophils from healthy volunteers were used for in vitro experiments.

**Flow cytometry**

For analysis of T lymphocytes, blood leucocytes were stained with antihuman CD45 Brilliant Violet 510, CD62L Brilliant Violet 605, CD4 PerCP-Cy5.5, CD8 PE-Cy7, CD3 Alexa Fluor 700 and Zombie NIR live/dead dye. Neutrophils were labelled with antihuman CD45 Brilliant Violet 510, CD62L Brilliant Violet 605, CD11b PerCP-Cy5.5, CD16 PE-Cy7 and Zombie NIR live/dead dye. Neutrophil apoptosis was analysed by staining in Annexin V binding buffer with CD45 Brilliant Violet 605, CD11b PerCP-Cy5.5, CD16 PE-Cy7, Annexin V fluorescein isothiocyanate and Zombie NIR live/dead dye. HCA2 staining was performed with antihuman HCA2 Alexa Fluor 647 and rat IgG2B Alexa Fluor 647 isotype control in combination with T-cell or neutrophil markers plus live/dead staining. All antibodies and dyes were purchased from BioLegend (Koblenz, Germany), apart from the antihuman HCA2 and isotype control antibodies (R&D Systems, Wiesbaden-Nordenstadt, Germany). Cytometry was conducted on a CytoFLEX flow cytometer using CytExpert software (Beckman Coulter, Krefeld, Germany). The resulting data were analysed using FlowJo v10 software (Becton, Dickinson & Company, Heidelberg, Germany).

**Table 1** Characteristics of patients with psoriasis and healthy controls

|                        | Patients with psoriasis, $n=25$ | Healthy controls, $n=14$ |
|------------------------|---------------------------------|--------------------------|
| Female (%)             | 41-7                            | 57-1                     |
| Age (years)            | 45-1 ± 16-6                     | 41-6 ± 15-2              |
| BMI                    | 29-0 ± 8-0                      | 25-3 ± 4-9               |
| Psoriasis duration (years) | 16-3 ± 12-6                    | NA                       |
| Mean PASI              |                                 |                          |
| Month 0                | 10-7 ± 4-3                      | NA                       |
| Month 1                | 7-9 ± 4-5                       | NA                       |
| Month 2                | 6-7 ± 4-1                       | NA                       |
| Month 3                | 5-5 ± 3-8                       | NA                       |
| NLR                    | 5-69 ± 3-84                     | 1-91 ± 0-59              |
| Other conditions       | None, 15; T2D, 2; HT, 6; CHD, 1; RA, 1 | None, 14                |
| Previous systemic psoriasis treatments | 1; ET, 1                       | NA                       |

BMI, body mass index; PASI, Psoriasis Area and Severity Index; NLR, neutrophil–lymphocyte ratio (prior to dimethyl fumarate treatment); T2D, type 2 diabetes; HT, hypertension; CHD, coronary heart disease; RA, rheumatoid arthritis; CS, corticosteroids; MTX, methotrexate; ET, etanercept; NA, not applicable.

Negative selected neutrophils were cultured in 96-well flat-bottomed plates (Sarstedt, Nümbrecht, Germany) at $10 \times 10^6$ mL$^{-1}$ in DMEM + 10% FCS and 1X HEPES. For analysis of HCA2 downregulation, neutrophils were stimulated for 1 h at 37°C with 100 μmol L$^{-1}$ MMF, 100 μmol L$^{-1}$ DMF or 100 μmol L$^{-1}$ nicotinic acid (all from Merck). Dimethyl sulfoxide solvent (DMSO)-treated neutrophils were included as controls. Thereafter, cells were stained for flow cytometry and analysed. For analysis of fumarate-induced apoptosis, isolated neutrophils were cultured for 4 h at 37°C with 25, 50 or 100 μmol L$^{-1}$ of MMF or DMF. Neutrophils exposed to DMSO were used as controls. Stimulated neutrophils were then stained with Annexin V and analysed by flow cytometry.

**Enzyme-linked immunosorbent assay**

Serum was isolated from blood and stored at −80°C before analysis. Myeloperoxidase, neutrophil elastase and soluble CD62L levels were measured by enzyme-linked immunosorbent assay. Validated kits were purchased from R&D Systems and used according to the manufacturer’s instructions.

**Statistics**

All data were analysed with Graphpad Prism 7 software (San Diego, CA, USA). For the analysis of multiple groups, one-way ANOVA (Bonferroni) was utilized. Unpaired t-tests were used to compare two groups. Correlation of DMF dose with neutrophils and PASI with the number of CD62L$^{lo}$ neutrophils was analysed using repeated measures correlation (rmcorr).$^{28}$ Values of $P < 0.05$ were considered significant.

**Results**

**Dimethyl fumarate therapy improves psoriasis within 3 months of treatment**

In this cohort study, 42 patients with psoriasis were enrolled and assessed before commencement of DMF therapy, and then at months 1, 2 and 3 of treatment. Of 42 patients enrolled, 25 completed the study. Twelve patients dropped out due to flushing and/or gastrointestinal complaints, four were removed due to failure to attend the clinic at the required timepoints and one patient became pregnant. Eleven of the 25 patients were treated with Fumaderm$^{22}$ and 14 with Skilarence$^{38}$. Fourteen healthy volunteers were included as controls (Table 1). The average daily DMF dose during month 1 was 155 mg, 434 mg in month 2 and 474 mg at month 3. When analysed in all 25 patients, PASI fell from a baseline mean of 10-7 to 7-9 at month 1, 6-7 at month 2 and 5-5 at month 3 (Table 1 and Figure 1a). When converted to PASI 50 response (50% reduction in the PASI score), 34-6% of the patients treated with DMF obtained PASI 50 at month 2 and 57-7% at month 3 (Figure 1b). There was no significant
difference in clinical outcomes between patients treated with Fumaderm® and Skilarence® (not shown). Blood counts revealed that at baseline patients with psoriasis had elevated leucocyte numbers compared with healthy controls (Figure 1c). After commencement of DMF treatment the number of leucocytes in the patients with psoriasis began to fall progressively until month 3 (Figure 1c). The significant difference in leucocytes between controls and patients was abolished by month 2 ($P = 0.217$). DMF treatment diminished the leucocyte count to 75.1% of baseline by month 3 (Figure 1c). Therefore, DMF causes a progressive improvement in psoriasis during the first 3 months of treatment with a concurrent reduction in peripheral leucocytes.

### Dimethyl fumarate treatment does not initially result in significant changes to peripheral CD4$^+$ and CD8$^+$ T-cell numbers

Next, we analysed the frequencies of CD4$^+$ and CD8$^+$ T cells to determine if changes in these populations accounted for the loss of leucocytes. Examination of the number of total CD3$^+$ T cells plus CD4$^+$ and CD8$^+$ subpopulations prior to DMF treatment showed no significant difference between patients with psoriasis and healthy controls (Figure 2a–c). DMF did not result in significant decreases in total CD3$^+$ T cells or CD4$^+$ and CD8$^+$ subpopulations during the first 3 months of treatment (Figure 2a–c). All patients remained above the 500 lymphocytes µL$^{-1}$ threshold for severe lymphopenia.

**The number of peripheral neutrophils is reduced by dimethyl fumarate treatment**

Because lymphocytes did not account for the loss of leucocytes, we subsequently analysed neutrophils in our patients. Before DMF treatment, patients with psoriasis had significantly higher numbers of neutrophils compared with controls (Figure 3). After 1 month of treatment the number of neutrophils began to fall and continued to decrease until month 3. This decrease equated to a fall to 71.6% of the baseline neutrophil number by month 3 (Figure 3). There was also a weak negative association between DMF dose and number of neutrophils ($r_{cv} = -0.271$, $P = 0.049$). Therefore, the decrease in leucocytes we observed during DMF treatment is primarily due to loss of neutrophils compared with the pre-DMF baseline.

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**Figure 1** Dimethyl fumarate (DMF) treatment improves psoriasis within the first 3 months of treatment and is associated with a reduction in peripheral leucocytes. (a) Mean absolute Psoriasis Area and Severity Index (PASI) of patients with psoriasis at baseline (month 0) and at months 1, 2 and 3 of DMF treatment. The significance of differences between months 1, 2 and 3 compared with month 0 were determined by one-way ANOVA, $n = 25$. (b) PASI 50 (50% reduction in PASI score) response at months 1, 2 and 3 of DMF therapy. (c) Left panel: total leucocyte counts (per µL of blood) of healthy controls (HC) and patients with psoriasis at month 0 and months 1, 2 and 3 of DMF treatment. Right panel: percentage of total leucocytes at months 1, 2 and 3 of DMF treatment relative to month 0. Horizontal lines and bars indicate mean. Error bars show SEM. Significant differences were assessed by one-way ANOVA. **$P < 0.01$, ***$P < 0.001$.**
Monomethyl fumarate does not induce neutrophil apoptosis

One explanation for the reduced neutrophil number during DMF treatment is increased apoptosis. MMF is a high-affinity agonist of HCA2, a receptor expressed by human neutrophils but not CD4+ and CD8+ T cells (Figure 4a). HCA2 expression did not vary in these cell types between controls and patients with psoriasis (not shown). Exposure to MMF caused a significant downregulation of surface HCA2 expression on freshly isolated neutrophils, similar to nicotinic acid (the cognate HCA2 agonist). DMF failed to induce a significant change in neutrophil HCA2 expression (Figure 4b). Stimulation of neutrophils from healthy volunteers with MMF or DMF for 4 h did not change the frequency of Annexin V+ cells compared with vehicle controls (Figure 4c). Analysis of treated patients

Figure 2 Dimethyl fumarate (DMF) does not significantly decrease the number of peripheral T cells in the first 3 months of treatment. (a) Left panel: number of blood CD3+ lymphocytes per μL in healthy controls (HC) and patients with psoriasis treated with DMF at month 0 and months 1, 2 and 3. Right panel: percentages of CD3+ lymphocytes after months 1, 2 and 3 of DMF in relation to month 0. (b) Left panel: number of CD4+ T cells (CD3+CD4+CD8−) in HC and patients with psoriasis at month 0 and months 1, 2 and 3 of DMF therapy. Right panel: percentages of CD4+ T cells after 1, 2 and 3 months of DMF therapy relative to month 0. (c) Left panel: number of CD8+ T cells (CD3+CD4−CD8+) in HC and patients with psoriasis treated with DMF at baseline and months 1, 2 and 3. Right panel: percentages of CD8+ T cells between 1 and 3 months of DMF treatment compared with baseline. Horizontal lines show mean. HC, n = 14; patients, n = 25. Differences were evaluated by one-way ANOVA.
DMF treatment reduces pro-inflammatory neutrophils in patients with psoriasis, P.J. Morrison et al.

Figure 3 Oral dimethyl fumarate (DMF) treatment induces a significant reduction in the number of circulating neutrophils. Left panel: absolute number of blood neutrophils (defined by forward/side scatter and CD16 expression) in healthy controls (HC) and patients with psoriasis prior to DMF therapy and at months 1, 2 and 3 of DMF treatment. Right panel: percentages of neutrophils at 1, 2 and 3 months of DMF treatment relative to the pretreatment baseline. Horizontal lines indicate mean. HC, n = 14; patients, n = 25. Differences were assessed by one-way ANOVA. ***p < 0.01, ****p < 0.001.

showed a downward trend in the percentage of Annexin V+ neutrophils and a significantly reduced number of Annexin V+ neutrophils by month 3 (Figure 4d). These data indicate that while neutrophils are able to respond to MMF, fumarates do not significantly induce neutrophil apoptosis.

Dimethyl fumarate treatment reduces the expression of neutrophil activation markers

Because DMF treatment reduced the number of circulating neutrophils without inducing apoptosis, we investigated whether DMF therapy had an impact on other factors that may affect neutrophil homeostasis. CD62L is expressed at high levels by neutrophils and is shed during activation and plays a role in extravasation. Analysis revealed that patients with untreated psoriasis had significantly higher frequencies of CD62Llo neutrophils compared with controls. Moreover, during DMF therapy the proportion of CD62Llo neutrophils fell progressively to near healthy control levels (Figure 5a,b). Conversely, CD62L expression by CD4+ and CD8+ T cells did not change significantly during treatment (not shown). Given that the loss of CD62Llo neutrophils may reflect reduced activation, we also assessed neutrophil CD11b expression during DMF therapy. High CD11b expression is considered a marker of neutrophil activation. We found that during treatment the mean fluorescence intensity of neutrophil CD11b was significantly decreased by month 3 (Figure 5c). Furthermore, before DMF treatment patients with psoriasis had elevated serum levels of myeloperoxidase, neutrophil elastase and soluble CD62L (Table 2). During DMF therapy the concentration of these markers marginally decreased. Finally, examination of the correlation between PASI and the number of CD62Llo neutrophils suggested that there is a moderate positive association between the severity of psoriasis and the number of these cells (Figure 5d). Together, these results suggest that DMF treatment improves psoriasis in part by downregulating neutrophil activation.

Discussion

In this study we have analysed a cohort of patients with psoriasis at baseline and during the initial 3 months of DMF treatment and have demonstrated that DMF causes a loss of pro-inflammatory CD62Llo neutrophils. These findings highlight a novel mechanism of action for DMF that may contribute to its clinical effectiveness.

The clinical use of DMF is associated with decreases in circulating lymphocytes, rather than significant changes in neutrophils. This loss of T cells has been linked to the anti-inflammatory effects of DMF treatment. Due to the early timepoints analysed in this study significant lymphocyte losses were not detected; however, all patients who continued DMF therapy were routinely monitored and five patients reduced dosage or ceased treatment due to falling lymphocyte counts.

Figure 4 Monomethyl fumarate (MMF) does not induce neutrophil apoptosis. (a) Representative histograms showing hydroxycarboxylic acid receptor 2 (HCA2) expression by total CD3+ lymphocytes, CD4+ T cells, CD8+ T cells and neutrophils from a healthy volunteer. (b) Mean fluorescence intensity (MFI) of HCA2 on neutrophils after 1 h of in vitro stimulation with 100 μmol L−1 MMF, DMF or nicotinic acid (NA). Neutrophils were isolated from healthy individuals, n = 8. (c) Frequency of Annexin V+ neutrophils after 4 h of stimulation with indicated concentrations (μmol L−1) of MMF or DMF. Neutrophils were isolated from healthy volunteers, n = 4. (d) Left panel: frequency of Annexin V+ cells within the neutrophil population of patients with psoriasis before and after 1, 2 and 3 months of DMF treatment. Right panel: number of Annexin V+ neutrophils prior to and during the first 3 months of DMF treatment, n = 12. Bars indicate means (SEM). Significant differences were evaluated by one-way ANOVA. *p < 0.05, **p < 0.01.
between months 4 and 6. Indeed, most published studies show that drops in T cells become statistically significant between months 3 and 6.\textsuperscript{13,14,33,34} Losses of T cells typically coincide with the maximal clinical outcomes of DMF treatment and, therefore, it is likely that oral DMF causes multiple overlapping effects on HCA2-expressing cells and via HCA2-
DMF treatment reduces pro-inflammatory neutrophils in patients with psoriasis, P.J. Morrison et al.

**Figure 5** Dimethyl fumarate (DMF) treatment reduces the frequency of CD62L\textsuperscript{lo} neutrophils and neutrophil expression of CD11b in patients with psoriasis. (a) Representative histograms illustrating the proportion of CD62L\textsuperscript{lo} cells within the neutrophil population of a single patient with psoriasis during the first 3 months of DMF. (b) Left panel: percentage of CD62L\textsuperscript{lo} cells in the neutrophil population of healthy controls (HC) and patients treated with DMF. Right panel: number of CD62L\textsuperscript{lo} neutrophils in HC and patients at months 0, 1, 2 and 3 of DMF therapy. (c) Mean fluorescence intensity (MFI) of CD11b on neutrophils from patients with psoriasis prior to and during the initial 3 months of DMF treatment, n = 14. (d) Correlation between Psoriasis Area and Severity Index (PASI) and number of CD62L\textsuperscript{lo} neutrophils in all patients. Bars and horizontal lines indicate means. Error bars show SEM. Multiple groups were compared by one-way ANOVA. CD11b mean fluorescence intensity was assessed by t-test. Correlation was examined with repeated measures correlation (rmcorr).\textsuperscript{28} *P < 0.05, **P < 0.01, ***P < 0.001.
DMF treatment reduces pro-inflammatory neutrophils in patients with psoriasis, P.J. Morrison et al.

Table 2 Serum levels of neutrophil activation markers during DMF treatment: serum concentrations of myeloperoxidase (MPO), neutrophil elastase (ELA2) and soluble CD62L in healthy controls (HC) and DMF-treated patients with psoriasis at baseline and during the first 3 months of DMF treatment. All numbers indicate mean concentrations ± SD

| Month of treatment | ng mL⁻¹ |   |
|--------------------|---------|---|
|                    | HC      | 0 | 1 | 2 | 3 |
| MPO                | 408.6 ± 179.5 | 780.2 ± 454.0 | 696.0 ± 369.1 | 657.6 ± 371.0 | 686.1 ± 295.5 |
| ELA2               | 269.6 ± 117.1 | 509.6 ± 380.8 | 540.6 ± 333.3 | 459.6 ± 277.7 | 479.9 ± 331.8 |
| CD62L              | 1469.4 ± 325.6 | 1752.3 ± 564.3 | 1502.1 ± 388.7 | 1471.3 ± 501.3 | 1660 ± 423.4 |

independent pathways over differing timescales.³ The pleiotropic nature of DMF is demonstrated by work in MS showing the antioxidative stress and cytotoxic protective properties of DMF treatment mediated by HCA2-independent activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway in neural cells.⁴⁻⁶ Thus, while changes in neutrophils during early timepoints correlated with skin improvements it is distinctly possible that numerous effects occur in vivo, including changes in T cells, which lead to the full antipsoriatic effects of DMF treatment.

At baseline our patients with psoriasis displayed higher leucocyte counts compared with healthy controls, in agreement with other published studies.³⁷⁻⁹ Further analysis showed that significantly elevated neutrophils in circulating neutrophils were responsible for this increase. While we saw no association between body mass index and leucocyte number, we cannot rule out that the higher average body mass of patients with psoriasis affected observed cell counts. During DMF treatment the number of neutrophils fell significantly, leading to a commensurate decrease in leucocyte count. Neutrophil counts remained within the standard clinical reference range in all patients. DMF treatment-induced apoptosis does not appear to account for this loss of neutrophils as we failed to find increased levels of Annexin V during treatment; nor were MMF or DMF able to promote neutrophil apoptosis in vitro.⁴⁰ These observations suggest that DMF treatment alters the set of conditions favouring high blood neutrophil counts in patients with psoriasis rather than depleting neutrophils directly.

Our data showed that patients with untreated psoriasis have a large population of CD62Llo neutrophils. This low expression of CD62L likely indicates enhanced neutrophil activation, as it is shed during this process.⁴¹⁻⁴³ CD62L binds to multiple ligands and mediates transient adhesion to endothelial walls, a critical step in extravasation.³¹⁻⁴³ Thus, loss of CD62L on neutrophils in patients with psoriasis may indicate reduced extravasation⁴³ and contribute to the higher neutrophil counts we observed. Reduced neutrophil extravasation may be antipsoriatic but this does not align with the low CD62L levels in patients with untreated psoriasis with active disease.²⁷ Mouse studies have suggested that reduced neutrophil extravasation leads to an expansion of IL-17A⁺ γδ T cells and nonconventional αβ T cells due to increased macrophage and dendritic-cell production of IL-23.⁴⁴ IL-23 production by these cells is normally regulated by phagocytes of extravasated apoptotic neutrophils.⁴⁵ This phenomenon would not only promote psoriasis but may also increase the rate of granulopoiesis via induction of granulocyte colony-stimulating factor⁴⁶⁻⁴⁸ and aligns with our data of increased peripheral neutrophils and low CD62L levels in the context of untreated psoriasis. Increased neutrophil CD62L levels in treated patients may be due to MMF–HCA2 signalling leading to a cell-intrinsic inhibition of CD62L shedding or extrinsic effects leading to lower neutrophil activation and/or granulopoiesis. Preliminary in vitro experiments whereby neutrophils were pre-treated with MMF (10 μmol L⁻¹ or 100 μmol L⁻¹) followed by activation with phorbol 12-myristate 13-acetate (PMA) or N-formylmethionyl-leucyl-phenylalanine (fMLP) did not show significant inhibition of CD62L shedding by MMF (not shown). These in vitro simulation conditions may not reflect processes occurring in patients that lead to CD62L loss by neutrophils. Thus, the in vivo effects of DMF treatment on neutrophils likely involve multiple extrinsic and intrinsic factors that modulate cellular activation and homeostasis.

We also noted that neutrophil CD11b expression decreased during the first 3 months of DMF therapy. Upregulation of neutrophil CD11b occurs after exposure to lipopolysaccharides, fMLP, tumour necrosis factor-α (among others) and during infection.³⁸ Thus, this reduction in CD11b during treatment supports the notion that DMF inhibits neutrophil activation. Analysis of CD62Llo and CD62Lhi neutrophils revealed that CD62Llo cells had higher levels of CD11b, although this difference was not statistically significant (not shown). These CD62Llo CD11b⁺ neutrophils resemble pro-inflammatory aged neutrophils described in mice and are thought to be the more able to undergo NETosis.⁴⁷,⁴⁸ DMF treatment also reduced the levels of serum myeloperoxidase and neutrophil elastase, suggesting reduced neutrophil activation and degranulation.¹⁸ Together with the positive correlation of CD62Llo neutrophils with PASI, these changes indicate that oral DMF is able to ameliorate psoriasis in part by reducing the pro-inflammatory activity of neutrophils. However, Hoffmann et al. have shown that MMF does not significantly inhibit NETosis and moderately decreases ROS production after PMA stimulation in vitro, indicating that DMF treatment may not strongly affect these terminal neutrophil effector functions.⁴⁹ Interestingly, unlike other systemic antipsoriatic drugs, DMF is not associated with increased rates of bacterial infection despite T-
cell loss, suggesting that the effects of oral DMF on neutrophils do not reduce their ability to protect from infection.50

In conclusion, this study has demonstrated that patients with psoriasis have elevated neutrophil CD62Llo neutrophils, which also express enhanced levels of CD11b. Oral DMF modified this state so that the neutrophil compartment of patients with psoriasis more closely resembled that of healthy controls. Further investigation is required to determine if MMF acts directly on neutrophils in vivo and how neutrophil effector functions are affected during DMF treatment. Overall, modulation of neutrophil activation may represent one of the antipsoriatic effects of DMF and helps to explain its therapeutic effectiveness.

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