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INVESTIGATIVE REPORT

Expression of CCR2 on Monocytes and Macrophages in Chronically Inflamed Skin in Atopic Dermatitis and Psoriasis

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Monocytes form a significant component of the inflammatory reaction taking place in the skin of atopic dermatitis and psoriasis. Chemokines are pivotal in mediating the attraction of leucocytes to sites of inflammation. The CC-chemokine, monocyte chemotactic protein 1 (MCP-1/CCL2), is expressed by keratinocytes in both atopic dermatitis and psoriasis. MCP-1 binds to the chemokine receptor CCR2 which is known to be expressed on monocytes and macrophages. We examined the expression of CCR2 on peripheral blood monocytes from patients with psoriasis ($n = 8$) and atopic dermatitis ($n = 7$) and found it to be expressed on approximately 90% of the cells, whereas monocytes from healthy donors had a significantly lower CCR2 expression ($p < 0.05$). Skin biopsies from patients suffering from atopic dermatitis and psoriasis revealed that CCR2-positive cells expressed CD163, a marker for monocytes/macrophages. However, not all CD163-positive cells expressed CCR2, which could be interpreted as a mechanism for retaining the macrophages in the skin. Furthermore, we found that keratinocytes are able to express MCP-1, when stimulated with tumour necrosis factor-α and/or interferon-γ in a dose-dependent manner. Thus MCP-1 and CCR2 interaction is likely of importance for the monocyte/macrophage trafficking of inflammatory skin disorders. *Key words: atopic dermatitis; monocytes; chemokines; CCR2; CCL2.*

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Monocytes are a significant component in the skin inflammation of atopic dermatitis (AD) and psoriasis, even though both diseases are dominated by T cells and neutrophil granulocytes (1, 2). The monocytes invade the skin and differentiate into macrophages, which can act as antigen-presenting cells.

The chemokines are a group of small proteins which mediate activation and attraction of leucocytes. They are divided into four groups according to the relative position of two cysteine residues in their amino acid sequence, CC-, CXC-, CX_3C-, and C-chemokines, where C denotes cysteine in the one letter code and X denotes any other amino acid. The chemokine receptors are seven transmembrane G-protein coupled receptors which are classified as CCR, CXCR, CX_3CR and CR according to which chemokines they bind. The receptors may bind several different chemokines, but only within the same family, resulting in a high degree of redundancy (3).

The chemokine receptor CCR2 which binds monocyte chemotactic protein 1 (MCP-1; CCL2 according to the systematic nomenclature) (4) is expressed on circulating monocytes (5) and mediates attraction of monocytes to inflamed tissue. Indeed decreased invasion of monocytes is seen in sites of allogenic implants in CCR2-/- animals (6). CCR2 has also been described as pivotal for monocyte invasion in atherosclerotic plaques (7, 8), and it is also expressed on monocytes in the synovium of rheumatoid arthritis (9).

MCP-1 is produced by keratinocytes in the skin of patients suffering from psoriasis and AD (10–12). The serum concentration of MCP-1 is increased in AD (13). The MCP-1 production of keratinocytes is synergistically augmented by interferon (IFN)-γ and tumour necrosis factor (TNF)-α (14).

We investigated if the expression of CCR2 on peripheral monocytes in psoriasis and AD differed from that of healthy controls and if macrophages in the skin lesions of psoriasis and AD express CCR2. We also investigated the ability of keratinocytes in culture to produce MCP-1 on stimulation with TNF-α and IFN-γ and if this was dose-dependent.

MATERIALS AND METHODS

Immunohistochemistry

Punch biopsies (4 mm) from involved and uninvolved skin were taken from seven patients suffering from chronic AD and eight patients suffering from chronic plaque psoriasis after signed consent. None of the patients received systemic therapy at the time when the biopsy was taken. The biopsies were snap-frozen in liquid nitrogen, cryosected, mounted on slides, and stored at $-80°C$ until further use. The staining procedure was performed as follows; the sections were allowed to dry at room temperature overnight, after which they were immersed in acetone for 10 min. The slides were

incubated with 2% mouse serum in saline for 30 min at room temperature, followed by thorough washing in Tris-buffered saline (TBS). The slides were then incubated with the first antibody, goat anti-human CCR2 (Calbiochem, cat. no. 227001) diluted 1:200, for 2 h. The slides were washed in TBS and the result was visualized using a LSAB+ kit (new fuchsin) (DAKO, cat. no. K 0678) according to the manufacturer's instructions. The slides were then incubated with mouse anti-human CD163 (DAKO, cat. no. M 0794, clone Ber-MAC3) diluted 1:100, for 2 h at room temperature. The result was visualized using Envision+ (DAB) (DAKO, cat. no. K4008). The slides were finally counterstained with Meyers haematoxylin. Non-specific isotype antibodies were used for control.

Flow cytometry

Blood samples were taken from five healthy controls, seven patients with chronic AD and six patients with active plaque psoriasis after signed consent (not the same as above). None of the patients received systemic therapy at the time of taking the biopsies. Mononuclear cells were isolated using Lympho-prep (Nycomed Pharma, Norway) according to the manufacturer's instructions. The cells were washed and resuspended to a concentration of 1×10^6 in PBS containing 2% fetal calf serum (FCS) after which they were incubated with PE-conjugated monoclonal IgG mouse anti-CCR2 antibodies (R&D Systems, cat. no. FAB151P, clone no. 48607) for 25 min. The cells were washed and incubated with FITC-conjugated monoclonal IgG mouse anti-CD14 antibodies (DAKO, cat. no. F0844, clone TÜK4) for 25 min. The cells were washed and resuspended in Isotone, and analysed on an EPICS® XL-MCL flow cytometer using EXPO™ ADC 32 software.

Cell cultures

Keratinocytes were isolated by trypsinization of skin samples from adult healthy persons undergoing reduction plastic surgery. The keratinocytes were cultured as described previously (15). Briefly, the keratinocytes (2×10^5) were cultured at 37°C and 5% CO_2 in 2 ml serum-free keratinocyte medium with 0.09% Ca^{2+} (Invitrogen, Denmark) and added 5 ng/ml human recombinant epidermal growth factor, 50 μg/ml bovine pituary extract and 5 μg/ml gentamicin. The cells were cultured for 4–5 days until 60–70% confluence (app. 1.5×10^6) after which they were changed to medium without human recombinant growth factor and bovine pituary extract, and cultured for 24 h before stimulation. The cell cultures were then stimulated with IFN-γ (R&D Systems, cat. no. 285-IF) and/or TNF-α (R&D Systems, cat. no. 210 TA) in various concentrations. The viability of the cells in the cultures was assessed by light microscopy. The supernatants were collected after 24 h of stimulation and stored at −20°C until further analysis.

ELISA

The supernatants were carefully thawed and mixed in a 1:1 solution with 1% bovine serum albumin in PBS. The ELISA kit used was a sandwich ELISA from R&D Systems (cat. no. DY 279) which was used according to the manufacturer's instructions. Briefly, 96-well Maxisorp trays (Nunc, Denmark) were incubated with the capture antibody (1 μS) for 24 h at room temperature. Next, standards and samples were added to the wells and incubated for 2 h at room temperature, followed by incubation with a biotinylated detection antibody

(100 ng/ml in 1% BSA in PBS) for 2 h. Lastly, horseradish peroxidase (HRP)-conjugated streptavidin was added and incubated for 20 min. The reaction was visualized using substrate solution (R&D Systems, cat. no. DY999) and measured in a Labsystems iEMS reader MF at 450 nm at room temperature.

Statistics

Data were analysed using Students' t-test after being checked for normal distribution by QQ-plots.

RESULTS

CCR2-positive cells in the skin are CD163-positive

Immunohistochemistry was used to double-stain skin biopsies from patients with AD and psoriasis for CCR2 and CD163. This showed a large number of CD163-positive cells (monocytes/macrophages) in the dermis in both types of patients (Fig. 1A and B). Some of the CD163-positive cells also stained positive for CCR2 especially around the dermal vessels, whereas the CD163-positive cells lying more peripherally did not express CCR2 (Fig. 1E and F). Skin biopsies from uninvolved skin showed few CD163-positive cells and only occasional CCR2-CD163 positive cells (Fig. 1C and D).

Circulating monocytes express CCR2

We stained peripheral blood monocyte cells (PBMCs) from healthy controls with CD14 and CCR2, and using flow cytometry we measured the percentage of CCR2-positive monocytes (Fig. 2B). There was no significant difference in the percentage of monocytes isolated in the blood samples (14.94 ± 3.07 in AD, 13.38 ± 2.30 in psoriasis and 15.54 ± 3.55 in healthy controls). This revealed that 83.92% ($\pm 4.83\%$) of the CD14-positive cells expressed CCR2 in healthy controls (Fig. 2A). However, the number of CD14-positive cells expressing CCR2 was significantly increased ($p < 0.05$) in patients with AD and psoriasis: 89.82% ($\pm 3.63\%$) and 89.92% ($\pm 2.67\%$) compared with the healthy controls. Back-gating the CCR2-positive cells from the flow cytometry results showed that virtually all were CD14-positive and therefore monocytes.

TNF-α and IFN-γ induce MCP-1 production in a dose-dependent manner

Keratinocytes were cultured until 70% confluence and stimulated with TNF-α (0, 1, 10 and 100 ng/ml) and/or IFN-γ (0, 1, 10 and 100 ng/ml), after which the production of MCP-1 was measured (Fig. 3). During stimulation the cells in the cultures were deemed to be viable as based on the microscopic appearance. Stimulation with IFN-γ showed a dose-dependent response; however, stimulation with very
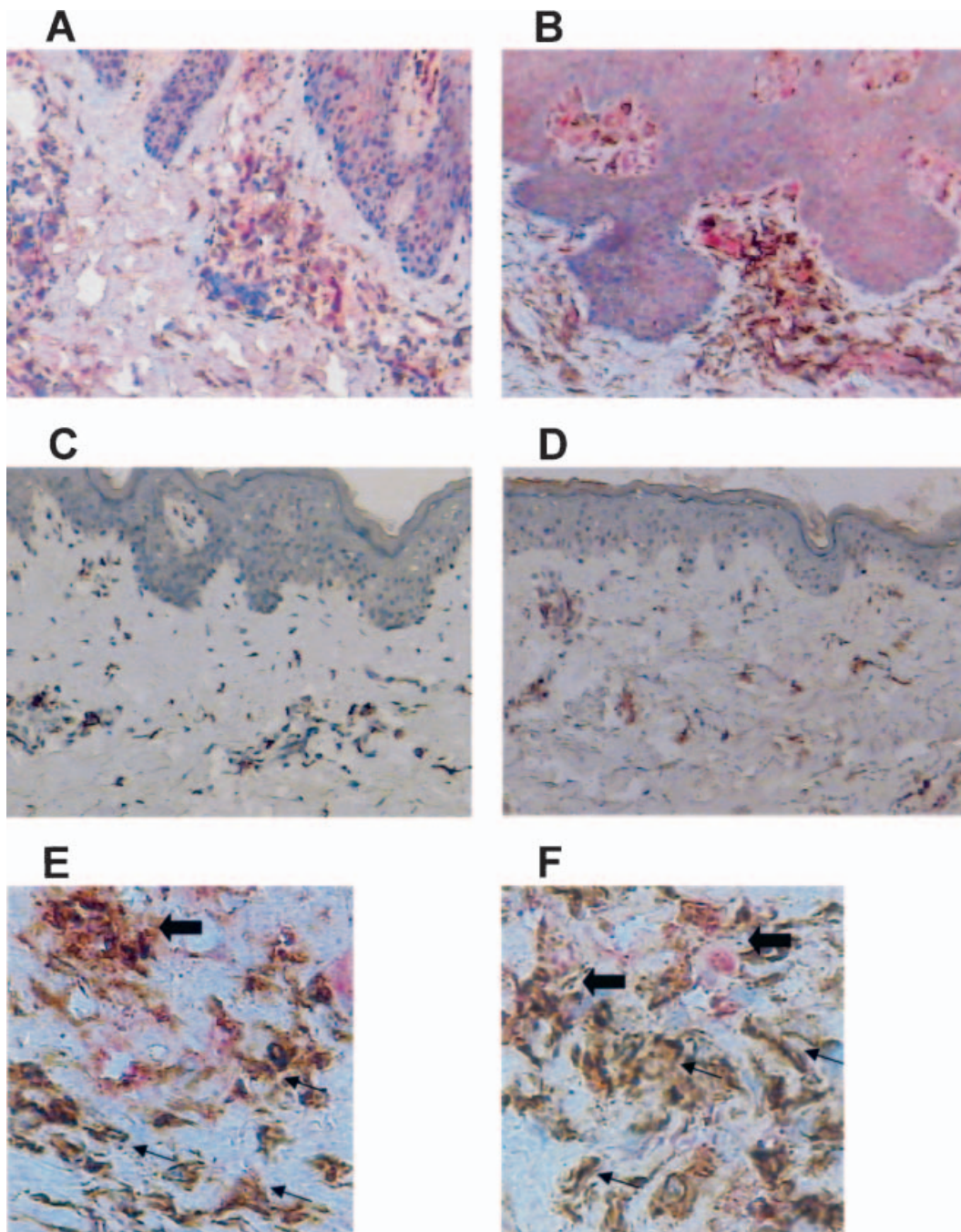
Fig. 1. Biopsies from atopic dermatitis (AD) and psoriasis patients stained with anti-CD163 (brown) and anti-CCR2 (red). (A) Typical staining of skin from an AD lesion. The CD163/CCR2 double-positive cells are typically located around the dermal complexes, whereas the CD163 single-positive cells are located more peripherally in the dermis. (B) Typical staining of psoriasis skin showing the same staining pattern as in AD. (C) and (D) CD163/CCR2 staining of uninvolved skin from AD and psoriasis patients respectively. (E) AD and (F) psoriasis, show a large magnification of the cells surrounding the dermal vessel complexes. The thick arrows indicate the dermal vessels and the double-positive cells (CCR2 and CD163) and the thin arrows indicate the single-positive (CD163) cells lying at some distance from the vessels.

high concentration (100 ng/ml) decreased the MCP-1-inducing effect. The same pattern was observed when the cells were stimulated with TNF-α. When the cells where stimulated with both cytokines a synergistic effect on the production of MCP-1 could be observed, although very high concentrations of one or the other cytokines decreased the production of MCP-1.

DISCUSSION

MCP-1 has long been recognized as a potent attractor for monocytes produced by keratinocytes (16). MCP-1 is induced by IFN-γ (16) and TNF-α in a synergistic manner (14). Keratinocytes from patients suffering from psoriasis have been shown to respond more potently to IFN-γ than keratinocytes from patients
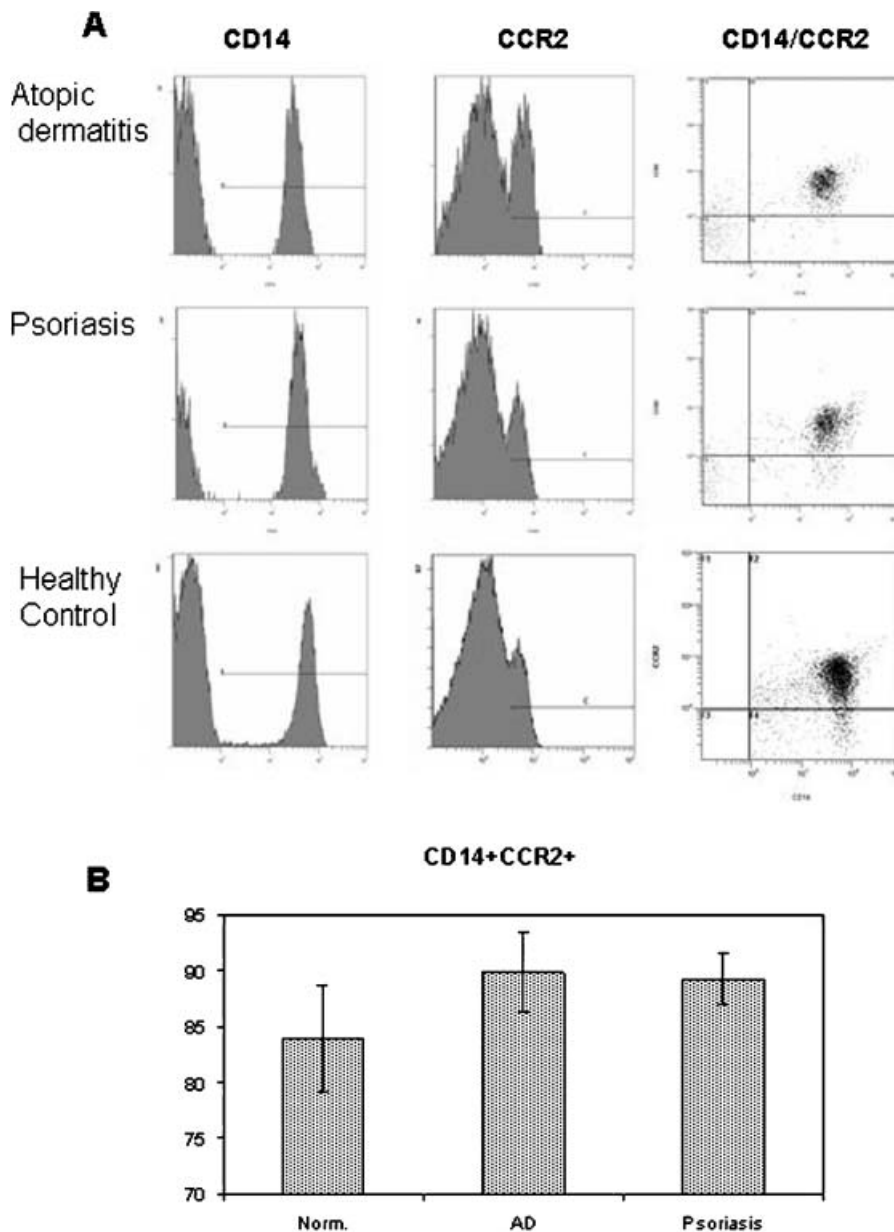
Fig. 2. (A) Flow cytometric results from patients with atopic dermatitis (AD) or psoriasis and from healthy controls. The first column shows the typical CD14 staining of peripheral blood, and the second column shows the typical result when staining with CCR2. The third column shows a dot-plot with CD14 intensity on the X-axis and CCR2 on the Y-axis. (B) The mean percentage of CD14-positive cells expressing CCR2 in healthy controls and patients with AD or psoriasis. The bars indicate the standard deviation.

with AD (10) and the production of MCP-1 has been demonstrated *in situ* in biopsies from patients suffering from psoriasis (11). We confirm that MCP-1 can be induced in keratinocytes by IFN-γ and TNF-α, and we show that the induction is dose-dependent, suggesting that the degree of the triggering stimuli also determines the degree of invasion of the skin by monocytes. This supports the proposition that activated T cells via IFN-γ release induce keratinocytes to participate in the final inflammatory outcome.

CCR2, which is the receptor for MCP-1 (4), plays a vital role in the recruitment of monocytes to sites of inflammation (17–19). Lack of CCR2 receptors impairs the ability of the monocytes for firm adhesion to endothelium (20) and the ability to clear certain intracellular bacterial infections (*Listeria monocytogenes*) (18). Lack of CCR2 also leads to a decreased Th1 response, which is likely to be due to a decreased ability of Langerhans' cells to relocate to draining lymph nodes (17, 21) and a decreased ability of the monocytes/macrophages to migrate to sites of inflammation and act as antigen-presenting cells.

In the synovial fluid of rheumatoid arthritis 24% of the monocytes are CCR2-positive, whereas 84% of monocytes in the peripheral blood are CCR2-positive (9). A possible explanation for this down-regulation could be the ability of IFN-γ to down-regulate CCR2 on monocytes and thereby retain them at the inflammatory sites (22). We show that in patients with AD or psoriasis and healthy controls almost all circulating
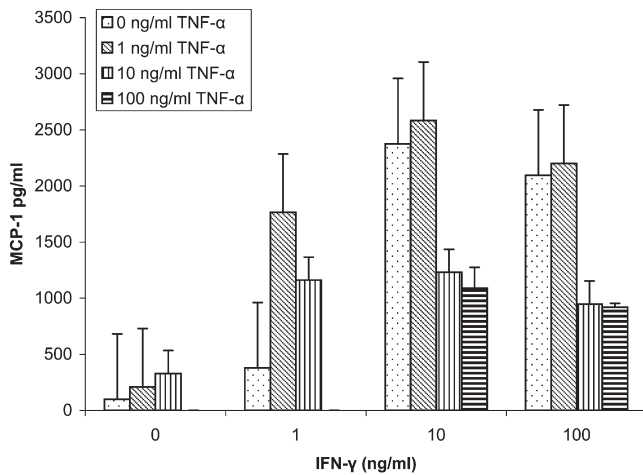
Fig. 3. MCP-1 production by keratinocytes after stimulation with various concentrations of TNF-α and/or IFN-γ. Stimulation with 100 ng/ml TNF-α and 0 ng/ml or 1 ng/ml IFN-γ did not yield any MCP-1 production. Bars indicate the standard deviation.

CD14+ cells (monocytes) are CCR2-positive as described by others (5), although we found a slight but significant overexpression of CCR2 on monocytes from patients with AD and psoriasis. CD163 is a marker for monocytes and macrophages, and the level of expression has been shown to be upregulated by IL-10, a cytokine which is produced in AD skin but not psoriasis skin. However CD163 is expressed on macrophages which have not been stimulated with IL-10, although to a lesser degree (23, 24). When examining the immunohistochemical staining of AD and psoriasis skin, CCR2 is expressed on CD163-positive cells around the dermal complexes, but not on CD163-positive cells more peripherally in the dermis. This could be explained in the same manner as for the monocytes in the synovial fluid of rheumatoid arthritis by the ability of IFN-γ to down-regulate CCR2 expression and thus retaining the monocytes/macrophages in the inflamed tissue. However, the keratinocytes from AD and psoriasis patients have different responses to IFN-γ with regard to CCL2 production. Keratinocytes from psoriasis produce more CCL2 in response to IFN-γ than keratinocytes from AD patients (10). However, as shown here, IFN-γ has a general capacity to induce CCL2 production in keratinocytes.

Thus IFN-γ has a dual role: 1) attraction of monocytes through the induction of MCP-1 production in keratinocytes and 2) retention of the monocytes in the skin through down-regulation of CCR2. The IFN-γ level in serum of psoriasis is elevated compared with normal controls (25), whereas it is low or not detectable in AD (26), which seems to contradict the hypothesis of IFN-γ as a down-regulator of CCR2 expression. However, CCR2 expression is found on monocytes in the peripheral blood of psoriasis patients as shown here. This suggests that another factor, which remains

to be elucidated, in the skin and synovium of rheumatoid arthritis may be involved in the down-regulation of CCR2. However, as CCR2 is expressed on macrophages in several different inflammatory conditions, and as the pathogeneses of AD and psoriasis are very different – although MCP-1 (10) and CCR2 are expressed in both conditions – it is likely that CCR2–CCL2 interaction is a basic inflammatory mechanism for attracting monocytes/macrophages. In non-lesional skin of AD and psoriasis some CD163-positive cells express CCR2, which could indicate that CCR2 is also involved in the normal cell trafficking, although there is evidence that CCR2 does not play an essential role in this process (20).

In conclusion we confirm that MCP-1 can be induced in a synergistic manner in keratinocytes cultured *in vitro*, which offers an explanation of the production of MCP-1 in the inflammatory skin diseases AD and psoriasis. Furthermore, we show that circulating CD14-positive monocytes express CCR2. This is also the case for CD163-positive monocytes/macrophages. Based on their location around the dermal complexes, it may be speculated that they recently have invaded the skin and as they move further into the skin have shed the CCR2 receptor. As described above it has been shown that IFN-γ is able to down-regulate CCR2 on macrophages (22); however, s-IFN-γ is increased in psoriasis creating a contradiction as regards the mechanism of CCR2 regulation which remains to be elucidated. Also, other factors such as IL-1α and -β up-regulate CCR2 expression (23), indicating that the regulation of CCR2 expression is complex and depends on multiple factors in the microenvironment. Thus the CCR2–CCL2 axis is likely to be a central, albeit common, inflammatory mechanism for attracting monocytes to sites of inflammation.

ACKNOWLEDGEMENT

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