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Up-regulation of CCL17, CCL22 and CCR4 in drug-induced maculopapular exanthema

B. Tapia*, E. Morel*, M.-Á. Martín-Díaz†, R. Díaz†, J. Alves-Ferreira‡, P. Rubio‡, A. Padial§ and T. Bellón*

*Research Unit, Hospital Universitario "La Paz", Madrid, Spain, †Dermatology Department, Hospital Universitario "La Paz", Madrid, Spain, ‡Pathology Department, Hospital Universitario "La Paz", Madrid, Spain and §Allergy Department, Hospital Universitario "La Paz", Madrid, Spain

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Summary

Background Maculopapular exanthema has been reported to be the most frequently drug-induced cutaneous reaction. Although T lymphocytes are involved in the pathomechanism of this disease, little is known about the recruitment of these cells to the skin.

Objective The aim of this work is to study the role of the chemokines TARC/CCL17 and MDC/CCL22 in the lymphocyte trafficking to affected skin in drug-induced exanthemas.

Methods Real-time PCR was performed to quantify gene expression levels of CCL17, CCL22 and their receptor CCR4 in lesional skin biopsies and in peripheral blood mononuclear cells from patients. CCL17 and CCL22 proteins were detected in the skin by immunochemistry.

Protein expression of CCR4 was determined by flow cytometry in peripheral blood lymphocytes. Functional migration assays to CCL17 and CCL22 were assessed to compare the migratory responses of peripheral blood lymphocytes from patients and healthy subjects.

Results CCL17 and CCL22 were up-regulated in maculopapular exanthema-affected skin. CCR4 mRNA levels and protein expression were increased in peripheral blood mononuclear cells during the acute phase of the disease. The increased expression of the receptor was consistent with a higher response of peripheral blood lymphocytes to CCL17 and CCL22 compared with the migratory response in healthy donors.

Conclusion TARC/CCL17 and MDC/CCL22 might cooperate in attracting T lymphocytes to skin in drug-induced maculopapular exanthemas.

Keywords CCL17, CCL22, chemokines, drug allergy, lymphocyte migration, skin

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Correspondence:

Dr Teresa Bellón, Research Unit,
Hospital Universitario "La Paz", Pº
Castellana 261, 28046 Madrid, Spain.
E-mail: tbellon.hulp@salud.madrid.org

Introduction

Delayed hypersensitivity reactions to drugs comprise a wide variety of clinical entities spanning from mild urticaria and maculo-papular exanthema (MPE) to severe reactions such as the Stevens–Johnson syndrome and toxic epidermal necrolysis [1].

MPE has been reported to be the most frequent drug-induced cutaneous reaction. The clinical presentation consists of erythematous maculae and/or papules that typically begin on the trunk and often spread to the extremities in a symmetrical fashion [2].

Cumulative evidence indicates that T cells play a pivotal role in the pathomechanism of delayed hypersensitivity reactions to drugs. Specific TCRs to some drugs have been described, and mononuclear cells from peripheral blood are stimulated by drugs involved in the reactions showing immunological memory [3]. In addition, an increased

number of peripheral blood T cells expressing the skin-homing receptor cutaneous lymphocyte antigen (CLA) has been reported in these diseases [4].

Histopathologic analysis of MPE shows a mild to moderate mononuclear infiltrate with few eosinophils. Microscopically, affected skin may appear even normal. Immunohistochemical studies show that the mononuclear infiltrate is characterized by an accumulation of CD3⁺ T cells (40–70%) with a clear predominance of CD4⁺ T cells containing cytotoxic granule proteins (granzyme B and perforin) [5, 6]. Therefore, a selective migration of T lymphocytes from blood to the inflamed skin is suspected in these cutaneous diseases.

Chemokines, a protein superfamily of structurally related small secreted proteins, have been shown to regulate leucocyte trafficking into peripheral tissues through the interaction with specific receptors. The chemokine family has gained increasing interest with reference to

understanding the mechanisms of leucocyte recruitment in allergic cutaneous inflammation. A high expression of a subset of chemokines including CCL27, CCL17, CCL22, CCL18, CCL11, CCL13 and CCL1 has been described in atopic dermatitis (AD) and allergic contact dermatitis (ACD) [7–10], and CCR10-, CCR4- and CCR8-expressing T cells have been found in AD-affected skin [11–13].

Concerning drug-induced cutaneous allergic reactions, IL-5 and eotaxin/CCL-11 have been involved in eosinophil recruitment to the skin in MPE [14], and IL-8/CXCL8 has been involved in neutrophil recruitment in acute generalized exanthematous pustulosis [15]. CCL27–CCR10 interactions have been associated with drug-induced cutaneous reactions [16]; however, little is known about lymphocyte migration to the skin in drug allergies.

The chemokine receptor CCR4 is expressed by the majority of CLA⁺ T cells. This receptor, together with CCR10, which is expressed by a subset of CLA⁺CCR4⁺ lymphocytes, and their respective ligands thymus activation and regulated chemokine (TARC)/CCL17 and cutaneous T cell-attracting chemokine (CTACK)/CCL27 have been involved in cutaneous immune surveillance and in lymphocyte recruitment to inflamed skin [17–19]. CCL27 is produced exclusively by keratinocytes of the basal layer of the epidermis in healthy skin and its expression is extended to the suprabasal layers in inflamed tissue [11, 16]. CCL17 is constitutively expressed in the thymus [20] and is produced by dendritic cells [21] and endothelial cells of venules in chronically inflamed skin [22]. In addition to CCL17, CCR4 interacts with macrophage-derived chemokine (MDC)/CCL22 [22, 23]. CCL22 is expressed by monocytes and dendritic cells [21, 24]. *In vitro* studies have shown that both CCL17 and CCL22 are produced by keratinocytes stimulated by IFN- γ [25]. CCL17–CCR4 interactions have been reported to be involved in cutaneous diseases such as AD [12, 26], psoriasis vulgaris [27] and cutaneous lupus erythematosus [28]. CCL22 has also been associated to AD [29].

In the present study, we explored the putative involvement of the CCL17–CCR4, and CCL22–CCR4 axis in MPE. We found that CCL17 and CCL22 gene expression is up-regulated in inflamed skin affected by drug-induced exanthemas and that mRNA levels and protein expression of their receptor CCR4 are increased in the skin infiltrate and in peripheral blood mononuclear cells during the acute phase of the reaction. These results were consistent with the increment detected in peripheral blood lymphocyte migration to both chemokines. Our data suggest an involvement of CCL17 and CCL22 in lymphocyte recruitment to the skin in MPE.

Materials and methods

Subjects, peripheral blood and skin biopsies

A total of 29 patients with drug-induced MPE participated in this study. Skin biopsies and peripheral blood samples

from patients were obtained from the Allergy and Dermatology Departments of Hospital 'La Paz', Madrid. In all cases, the patients developed classical cutaneous eruptions at 24 h or later after drug intake, which resolved upon drug discontinuation. The first blood sample was obtained within a few days after initiation of the reaction, and a second sample was taken at least 1 month later, when the patient had totally recovered. Punch biopsy specimens were taken from acute skin eruptions. The patients had received no systemic corticoids before the study.

Peripheral blood mononuclear cells (PBMCs) from anonymous healthy donors were obtained from the hospital blood bank. Normal skin was obtained from patients undergoing plastic reconstructive surgery.

The study was conducted according to the declaration of Helsinki principles and was approved by the Ethical Committee of Hospital 'La Paz'. Informed consent was obtained from all patients.

RNA extraction and real-time reverse transcription-polymerase chain reaction

PBMCs were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Total RNA was extracted in TriReagent (Molecular Research Center Inc.) according to the manufacturer's instructions. After treatment with DNase I, 1 μ g of total RNA was reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN, USA) for 1 h at 42 °C. Total RNA was extracted from fresh skin biopsies with TriReagent and cDNA was synthesized as described above. Quantitative analysis of human CCL17, CCL22 and CCR4 gene expression was performed by PCR amplification of the resulting cDNAs in a Light Cycler with FastStart DNA Master SYBR Green I (Roche). Primers used were as follows: CCL17 (133 bp) 5'-AGG GAC CTG CAC ACA GAG AC-3' and 5'-CTC GAG CTG CGT GGA TGT GC-3', CCL22 (114 bp) 5'-ATG GCT CGC CTA CAG ACT GCA CTC-3' and 5'-CAC GGC AGC AGA CGC TGT CTT CCA -3', CXCL12 (179 bp) 5'-ATG AAC GCC AAG GTC GTG GTC-3' and 5'-ACA ATC TGA AGG GCA CAG TTT G-3', CCR4 (96 bp) 5'-CAG AAA AGC AAG AAG CTG CTT CTG-3' and 5'-CGA GGG TGG TAT CTG CTA TAT C-3', CXCR4 (270 bp) 5'-ATG GAG GGG ATC AGT ATA TAC AC-3' and 5'-GCG TGA TGA CAA AGA GGA GGT C-3'; β -2-microglobuline (300 bp) 5'-CCA GCA GAG AAT GGA AGG TC-3' and 5'-GAT GCT GCT TAC ATG TCT CG-3'. Standard curves for target mRNA expression were generated using 10-fold serial dilutions of known quantities of plasmids containing the cDNA sequence of CCL17 and CCR4, or quantified amounts of CXCR4-, CCL22- and CXCL12-specific PCR products. Quantification of target gene expression was obtained using Light Cycler system software (Roche). RNA

molecules estimated from the quantification were normalized to β_2 -microglobuline gene expression by calculating the ratio between specific mRNA molecules/ μ L cDNA and molecules of β_2 microglobuline/ μ L cDNA.

Immunohistochemistry

Biopsy specimens were snap frozen and cryopreserved until used. 10 μ m cryostat sections were fixed in cold acetone and stained with anti-human CCL17 (MAB364, IgG1, R&D Systems Inc., Minneapolis, MN, USA) or anti-human-CCL22 (MAB3361, IgG2b, R&D Systems Inc.) monoclonal antibodies. Appropriate isotype-matched negative controls were included. The binding of mouse IgG was detected using an using a HRP-based detection technique and EnVisionTM+system (Dako, Glostrup, Denmark).

Flow cytometry analysis

Mononuclear cells isolated from peripheral blood, and cells that migrated in response to the chemokines were collected and analysed by flow cytometry. Cells were stained with the following antibodies: PerCP-conjugated mouse anti-hCD3, fluorescein isothiocyanate (FITC)-conjugated mouse anti-hCD4 PE-conjugated mouse anti-hCD8 and isotype control mouse immunoglobulin IgG1/IgG2-FITC, PerCP, PE (BD Biosciences, San Jose, CA, USA) according to standard protocols. CCR4 expression was analysed with an anti-hCCR4 monoclonal antibody (R&D Systems), followed by FITC-conjugated rabbit anti-mouse antibody (Dako). Analysis was performed using five parameters (FSC, SSC and three colours PerCP, PE and FITC) in a FACSCalibur cytometer with ProQuest software (Becton-Dickinson, San Jose, CA, USA).

Migration assays

Lymphocyte migration was evaluated through 5 μ m pore polycarbonate filters in 24-well TranswellTM chambers (Corning, Costar, Corning, NY, USA). Briefly, mononuclear cells isolated from peripheral blood were added to the top chamber resuspended in RPMI medium plus 0.5% bovine serum albumin at 5×10^6 cells/mL in a final volume of 100 μ L. Recombinant hCCL17/TARC (R&D Systems), hCCL22/MDC (Preprotech EC, London, UK) or hSDF1 α /CXCL12 (Preprotech EC, London, UK) were added to the bottom chamber of the transwell in a volume of 600 μ L. After 1 h of incubation at 37 °C in 5% CO₂ atmosphere, cells that transmigrated to the lower chamber were collected and lymphocytes (defined according to FSC and SSC parameters) were counted with a FACSCalibur flow cytometer (Becton-Dickinson) at a constant low rate for 1 min as described [30, 31]. Results are shown as the net migration that represents the difference between number of cells per minute migrated in response to the chemokine

and number of cells per minute migrated in medium alone. Preliminary experiments with peripheral blood lymphocytes from healthy donors and patients showed optimal concentrations of 100 nM for hTARC/CCL17 and 10 nM for hMDC/CCL22. The specificity of the response was assessed by incubation with blocking monoclonal antibodies (R&D Systems). SDF1- α /CXCL12 was used at 10 nM as described previously [32].

Statistical analysis

The results are expressed as the mean $\pm$ SD of the data obtained from a number of patients or healthy subjects described in each figure. Kolmogorov-Smirnov test was applied to verify the normal distribution of the data. Statistical significance was determined by Student's *t*-test, and *P*-values of <0.05 were considered to be statistically significant.

Results

Gene expression of TARC/CCL17 and MDC/CCL22 is up-regulated in skin affected of drug-induced maculo-papular exanthema

In order to investigate whether the chemokines CCL17, CCL22 and their receptor CCR4 are involved in lymphocyte recruitment to skin in drug-induced MPE, we first quantified CCL17- and CCL22-specific mRNA levels in affected skin. We used real-time RT-PCR to study the gene expression levels of the chemokines CCL17 and CCL22 in lesional skin biopsies from a total of 12 patients with MPE and compared them with healthy skin samples from 10 control donors undergoing plastic surgery. As shown in Fig. 1, quantitative RT-PCR showed that CCL17 and CCL22 genes are expressed at variable levels in healthy skin and that the specific mRNA levels of both chemokines were higher in all the MPE samples tested. Statistical analysis showed significant differences between the mean expression values in healthy and MPE-affected skin.

CCL17 and CCL22 proteins are expressed in maculo-papular exanthema-affected skin

To analyse the protein production and to localize the expression of CCL17 and CCL22 in the skin, frozen sections from MPE lesions were examined using immunohistochemical staining for CCL17 and CCL22, respectively. The results are summarized in Fig. 2. CCL22 immunostaining was localized to the mononuclear infiltrate mainly in the dermal compartment, and to a few cells invading the epidermis. (Figs 2a and b). Detailed observation of positive cells showed that in addition to small round cells (probably lymphocytes), large cytoplasm dendritic or

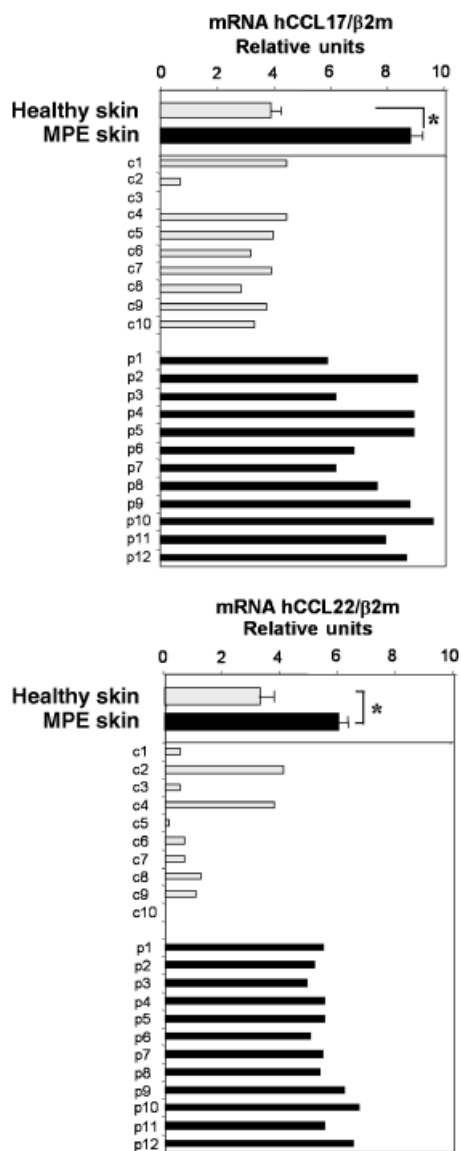


Fig. 1. Quantitative PCR analysis of CCL17 and CCL22 gene expression in maculo-papular exanthema (MPE)-affected skin. Total RNA was extracted from affected skin of patients with MPE (p1–p12, black bars) and from skin of healthy donors (c1–c10, grey bars). Real-time RT-PCR was performed to quantify CCL17 and CCL22 gene expression levels. Results are shown as relative units of specific mRNA molecules normalized to β 2-microglobulin mRNA. Mean values $\pm$ SD of mRNA levels in MPE skin and in healthy skin are shown at the top of the figure. Statistical analysis was performed by *t*-test to compare CCL17 and CCL22 expression levels in affected and healthy skin samples. *Statistically significant ($P < 0.005$).

macrophage-appearing cells were expressing CCL22 (Fig. 2c–f). CCL17 immunoreactivity was also found mainly within the dermal compartment. In addition to being expressed by inflammatory cells, we found strong staining in blood vessels localized in the deep dermis in some patients (Fig. 2l).

CCR4 expression is increased in skin and in peripheral blood lymphocytes from patients with drug-induced maculo-papular exanthema

As we found increased expression of CCL17 and CCL22 in affected skin, and both chemokines attract CCR4-expressing cells, we next measured CCR4 mRNA levels in skin biopsies as an estimate of CCR4⁺ leucocytes that migrated to the affected skin. We found a higher number of CCR4-specific mRNA molecules in all lesional skin samples tested compared with healthy skin (Fig. 3a). CD3 and CCR4 membrane expression analysed by two-colour flow cytometry in lymphocytes purified from skin biopsies revealed that CCR4 was expressed by over 60% of the infiltrating T cells (not shown). Chemokine receptor expression is as important as chemokine production for regulation of the chemokine system. To further investigate the role of CCL17 and CCL22 in lymphocyte recruitment to skin in drug-induced MPE, we analysed the expression of their specific receptor CCR4 in circulating mononuclear cells (PBMCs) from 12 patients. For each patient CCR4 expression was analysed in PBMCs from a first blood sample obtained during the acute phase, and in PBMCs from a second blood sample drawn from the same individual upon resolution of the clinical symptoms (2–3 months after the cutaneous lesions disappeared). The ratio between these two values is summarized in Fig. 3b. The results show a significant increase in CCR4 gene expression during the acute phase of MPE in PBMCs from every patient studied. In contrast, expression of CXCR4, which was analysed as a control chemokine receptor gene expressed by all leucocytes, showed no differences in the same samples.

To further confirm whether the increment in CCR4 mRNA levels correlated with an increase in cell membrane protein expression, we performed flow cytometry analysis in peripheral blood lymphocytes from patients with drug-induced MPE. During the acute phase of MPE, the percentage of T cells-expressing CCR4 was augmented compared with the percentage of lymphocytes expressing the receptor upon resolution of the disease, which was very similar to the percentage of CD3⁺CCR4⁺ cells in healthy subjects. Figure 3c shows data from a representative patient and a healthy donor control sample. Figure 3d shows the mean value of the percentage of peripheral blood CD3⁺CCR4⁺ lymphocytes from a total of 27 patients with MPE during the acute phase, which was higher than the percentage of CD3⁺CCR4⁺ cells found in 10 healthy donors analysed.

Peripheral blood lymphocytes from patients with maculo-papular exanthema show higher chemotactic response to CCL17 and CCL22

As we detected an increase in CCR4 gene and protein expression in PBMCs, we next investigated whether

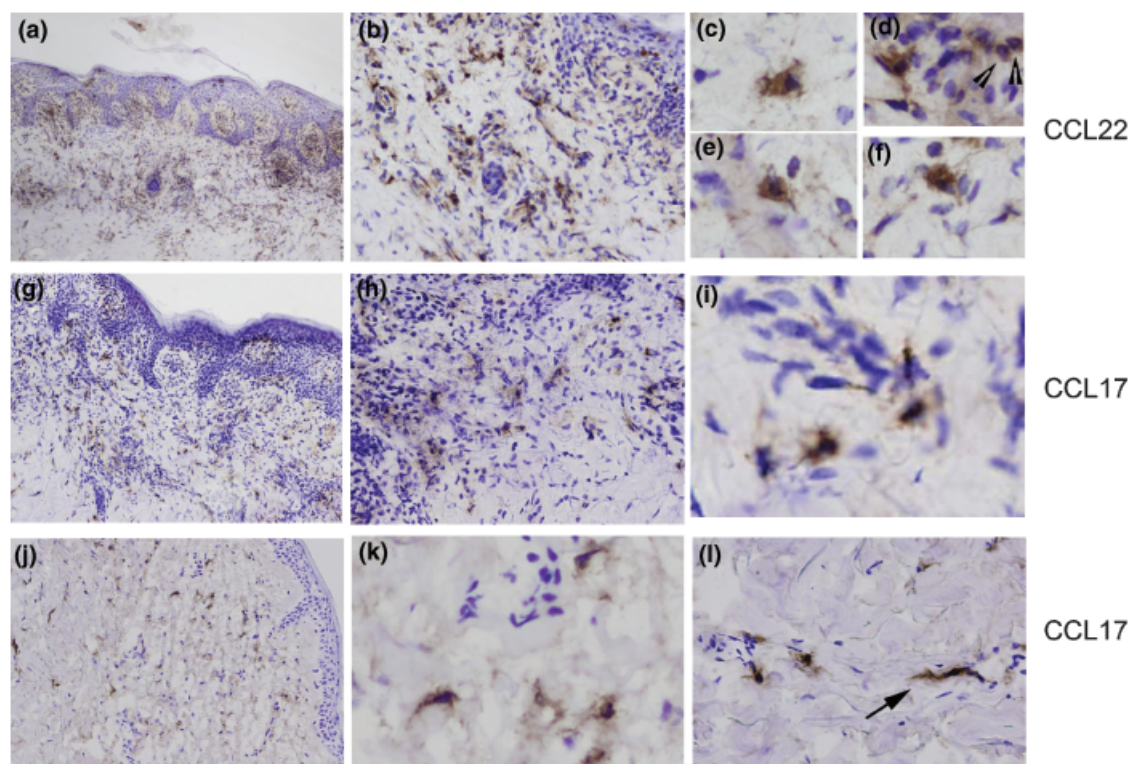


Fig. 2. Immunohistochemical analysis of CCL17 and CCL22 expression in maculo-papular exanthema (MPE) affected skin. CCL22 immunostaining of MPE-affected skin from patient p5 (a–f). CCL17 immunostaining of MPE-affected skin from patient p5 (g–i) and patient p6 (j–l). Arrowheads show small round cells stained with anti-CCL22. The arrow shows CCL17 staining in a blood vessel. Original magnification 100 $\times$ (a, g, j), 200 $\times$ (b, h) and 400 $\times$ (c–f, i, k, l).

lymphocytes from patients with MPE could show an increased functional response to its specific ligands CCL17 and CCL22. Preliminary titration experiments defined 100 nM CCL17 and 10 nM CCL22 as the optimal concentrations to detect migration under the conditions tested. Data from a representative donor are shown in Fig. 4a. Therefore, PBMCs obtained from patients with drug-induced MPE during the acute reaction and upon resolution of the cutaneous lesions were challenged with 100 nM rh-TARC/CCL17 and 10 nM rh-MDC/CCL22 in 5 μ m TranswellTM chambers to perform migration assays. As a control of the functional capabilities of the cells obtained at different time-points, migration assays to the homeostatic chemokine CXCL12 were included in the experimental design. In all the cases studied, we consistently found a better response to CCL17 and CCL22 in peripheral blood lymphocytes (PBLs) drawn from of patients in the acute phase compared with the response of cells obtained upon resolution of the cutaneous lesions. Data from a representative patient are shown in Fig. 4b. We next analysed the responsiveness to CCL17, CCL22 and CXCL12 of PBLs from patients with drug-induced MPE ($n = 12$), and compared it with the response in healthy subjects ($n = 7$). We found that PBLs from patients in the acute phase migrated more efficiently to both CCL17 and CCL22 than PBLs from

healthy donors (Fig. 4c), consistent with the observed up-regulation in CCR4 receptor mRNA and protein levels. Finally, we were interested in studying which T cell subset was being attracted to CCL17 and CCL22. To that purpose, cells that migrated in response to the chemokines were collected from the lower chambers of the transwells and analysed by flow cytometry. Peripheral blood CD4⁺ T cells were preferentially attracted to both CCR4-specific ligands, although a percentage of CD8⁺ lymphocytes were also responding to both chemokines (Fig. 4d).

Discussion

MPE is the most frequently observed drug-induced delayed hypersensitivity reaction. A large body of evidence obtained from immunohistochemical studies and the generation of drug-specific T cell clones from allergic patients indicates that T cells are involved in the pathogenesis of these cutaneous diseases. The mononuclear infiltrate in the cutaneous lesions is composed primarily by CLA⁺CD4⁺ T cells of the memory phenotype, as determined by immunohistochemical analysis ([5, 6], and our own unpublished results). The skin being the main target organ, a selective recruitment of lymphocytes to the skin is suspected. Several publications report TARC/CCL17 as

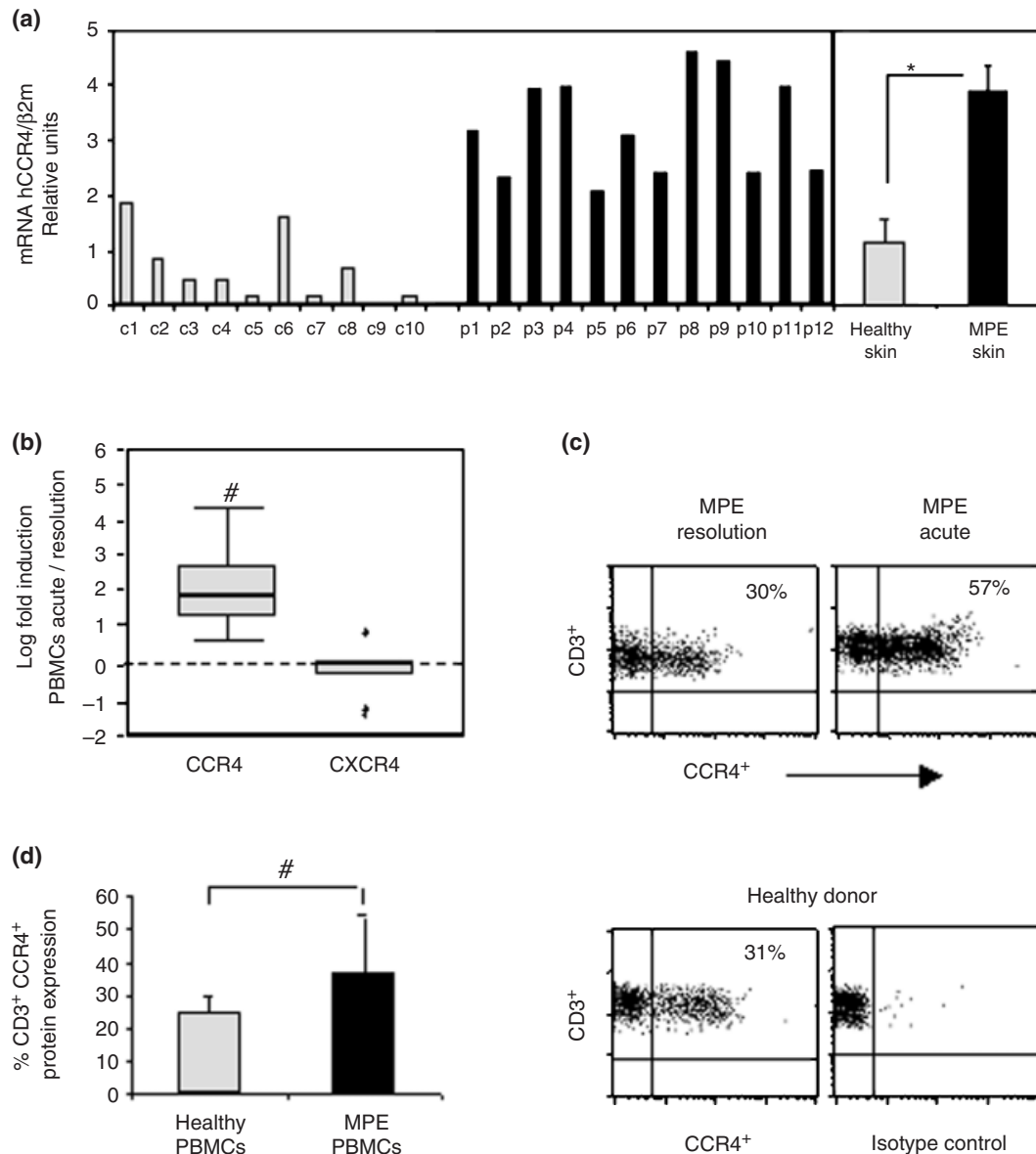


Fig. 3. CCR4 expression in drug-induced maculo-papular exanthema (MPE). (a) Total RNA was extracted from affected skin of patients with MPE (p1–p12, black bars) and from skin of healthy donors (c1–c10, grey bars). Real-time RT-PCR was performed to quantify CCR4 gene expression levels. Results are shown as relative units of specific mRNA molecules normalized to β 2-microglobulin mRNA. Mean values $\pm$ SD of mRNA levels in MPE skin and in healthy skin are shown at the right of the figure. Statistical analysis was performed by *t*-test to compare CCR4 expression levels in affected and healthy skin samples. *Statistically significant ($P < 0.005$). (b) Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) obtained from patients with drug-induced MPE during the acute phase and from the same patients upon resolution of the disease ($n = 10$). Real-time RT-PCR was performed to quantify CCR4 gene expression. CXCR4 expression levels were analysed in the same samples for comparative purposes. Data were normalized to β 2-microglobulin mRNA molecules in the same samples. Fold induction was calculated for each patient as normalized mRNA molecules in the acute phase/normalized mRNA molecules upon resolution of the cutaneous reaction. Box plots represent log fold induction for each gene analysed. The horizontal middle line represents the median, the top and bottom lines represent the quartile values, and the T bars represent the maximum and minimal values. Asterisks represent outliers. Statistical analysis was performed by *t*-test to compare the mean value of CCR4 gene expression in PBMCs during the acute phase and upon resolution of the disease. #Statistically significant ($P < 0.05$) increase of CCR4 gene expression levels in PBMCs obtained during the acute phase. (c) CCR4 membrane expression in total peripheral blood CD3⁺ T cells during the acute and resolution phases of a representative patient with drug-induced MPE, and in PBMCs from a representative healthy donor. The percentage of CD3⁺ CCR4⁺ cells is shown in each case. (d) Percentage of CD3⁺ CCR4⁺ expression in PBMCs from patients with drug-induced MPE ($n = 27$) and from healthy subjects ($n = 10$). #Statistically significant ($P < 0.05$) by *t*-test.

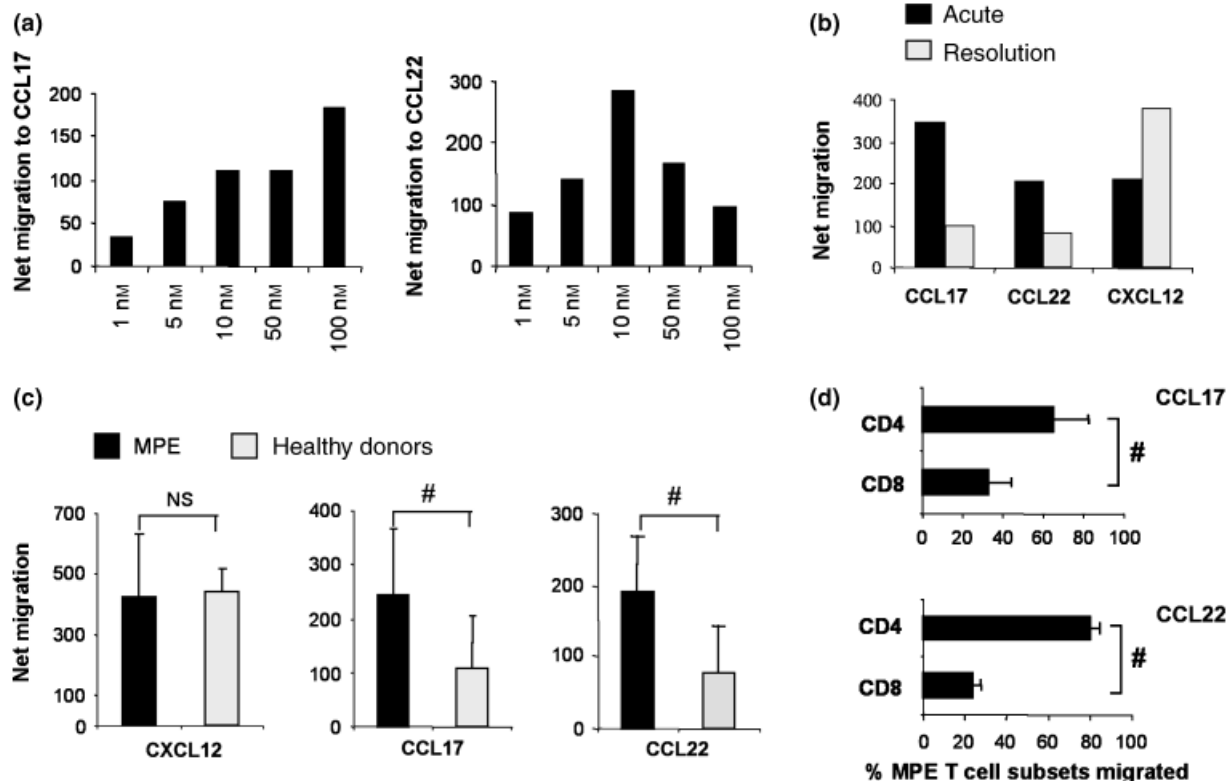


Fig. 4. Migration to CCL17 and CCL22 of peripheral blood lymphocytes from patients with drug-induced maculo-papular exanthema (MPE). (a) Migration assays to increasing concentrations (1, 5, 10, 50 and 100 nM) of recombinant human chemokines CCL17 and CCL22 were performed in 5 μ m TranswellTM chambers with peripheral blood mononuclear cells (PBMCs) obtained from healthy donors. Cells that migrated to the lower chamber of the transwell were collected and lymphocytes (gated according to forward and side light scatter criteria) were counted during 1 min at constant flow rate in a flow cytometer. Data are shown as net migration calculated as the ratio between cells migrated in the presence of the chemokine and cells migrated in medium alone. (b) Migration assays to 100 nM rh-thymus activation and regulated chemokine (TARC)/CCL17, 10 nM rh-macrophage-derived chemokine (MDC)/CCL22, and 10 nM rh-SDF-1 α /CXCL12 were performed as described above with PBMCs obtained during the acute and resolution phases of patients with MPE. Data from one representative patient with MPE are shown as net migration. (c) Migration assays to rh-TARC/CCL17 (100 nM), rh-MDC/CCL22 (10 nM) and rh-SDF-1 α /CXCL12 (10 nM) were performed as described above with PBMCs from patients during the acute phase of MPE ($n = 12$) and from healthy subjects ($n = 7$). (d) Cells that migrated in response to the chemokines CCL17 and CCL22 (assays shown in c) were collected and stained with PerCP-anti hCD3, fluorescein isothiocyanate-anti hCD4 and PE-anti hCD8. CD3⁺ T cells were gated and analysed for the expression of CD4 and CD8. Data are shown as percentage of positive CD4⁺CD3⁺ and CD8⁺CD3⁺ cells (mean values $\pm$ SD). NS, no significant differences. #Statistically significant ($P < 0.05$) by t -test.

being the major chemokine responsible for lymphocyte migration to skin in cutaneous inflammation as its specific receptor, CCR4, is expressed by the majority of CLA⁺ skin homing lymphocytes [12, 18, 19, 22]. CCL17 shares its specific receptor CCR4 with the chemokine MDC/CCL22, which was primarily identified as a chemoattractant for monocytes, dendritic cells and natural killer cells [24]. However, MDC/CCL22 has also recently been shown to attract memory T cells [22] and has been associated with the lymphocyte recruitment to skin in AD in co-operation with TARC/CCL17 [29]. In this work, we sought to investigate the role of the CCR4 ligands TARC/CCL17 and MDC/CCL22 in T cell migration to the skin in drug-induced MPE.

We found increased mRNA expression of CCL17 and CCL22 in lesional skin in all the cases studied when compared with healthy skin from different donors. Several

authors report the absence of CCL17 protein production in healthy skin [12, 15, 26]. However, TARC/CCL17 expression has been described in several cell types, such as keratinocytes, PBMCs, dermal fibroblasts, dermal dendritic cells, Langerhans cells and cutaneous microvasculature in inflamed skin [9, 10, 12, 20–22, 25]. We identified TARC immunoreactivity in dermal cells, as well as in blood vessels in MPE-affected skin, but we did not find staining of CCL17 within the epidermis. Some authors described CCL17 expression in keratinocytes in AD and contact dermatitis [25]. In contrast, keratinocytes did not express CCL17 in inflamed skin from positive epicutaneous tests with different allergens [10]. Although we have not found CCL17 expression in keratinocytes in the patients studied, its expression in endothelial cells suggests that it may contribute to the recruitment of leucocytes to inflamed skin. CCL22, the other known CCR4

ligand, is also reported to be absent in healthy skin [9, 10, 25]. CCL22 was predominantly localized to the upper dermis, in areas of strong leucocyte infiltration, and to some scattered cells infiltrating the epidermis. These findings are similar to those reported in two different models of inflamed skin [9, 10]. We observed immunoreactivity in some small round positive cells (probably lymphocytes) and within the cytoplasm of larger cells with the appearance of macrophages or dendritic cells. CCL22 expression has been shown to be restricted to keratinocytes and dendritic cells in skin lesions of AD [9, 25]. We have not found expression in keratinocytes in drug-induced MPE. However, although we have not been able to perform double staining, the pattern of expression suggests that MDC/CCL22 might also be expressed by some lymphocytes infiltrating the skin. Expression of MDC by *in vitro* stimulated T cells has been reported [33]; therefore, it is possible that activated lymphocytes in the skin produce CCL22. The different localization in the skin of these chemokines might be due to a difference in functionality. CCL17 may act first in the endothelium to trigger lymphocyte extravasation and then, in co-operation with CCL22 could subsequently guide cellular migration.

Besides CCL17 and CCL22, we found increased expression of their common receptor CCR4 in lesional skin in the patients. It is tempting to speculate that the higher number of transcripts found in affected skin must correspond to the presence of a lymphocytic infiltrate specifically bearing CCR4 receptor. In this sense, flow cytometry analysis of lymphocytes isolated from a MPE skin biopsy showed that approximately 60% of CD3⁺ cells were memory cells (CLA⁺ CD45RO⁺) expressing CCR4 (not shown). We also found higher levels of CCR4 mRNA in peripheral blood mononuclear cells during the acute phase compared with the resolution sample in the same patient in every case studied. In addition, when we analysed CCR4 protein expression on the cell surface by flow cytometry, we found that the percentage of PBMCs expressing CCR4 was increased during the acute phase in all the patients studied, according to the increased mRNA levels. Altogether these data support the expression of CCR4 in T cells infiltrating the skin.

Receptor expression is often not the sole determinant of responses to chemokines [34]. Functional migration assays to CCL17 and CCL22 with PBMCs from patients support the hypothesis of an involvement of both chemokines in lymphocyte recruitment to the skin in MPE. Moreover, the increased expression of CCR4 observed in PBMCs from patients with MPE during the acute phase correlates with the higher migratory response of these cells compared with the response of PBMCs from either the same patients upon resolution of the disease or from healthy subjects. Flow cytometry analysis of the migrated cells suggests that CCL17 and CCL22 are attracting CD4⁺ T cells in MPE, according to the prevalence of this

population in MPE skin biopsies ([5, 6], and our own unpublished results). One could suspect that these CD4⁺ T cells exhibit a T-helper type 2 (Th2) profile, as several authors have reported that TARC/CCL17 and MDC/CCL22 attract primarily '*in vitro*' differentiated Th2 cells [35]. However, '*ex vivo*' studies show that while almost all IL-4 producing cells are CCR4⁺, this represents only about 10% of total CCR4⁺ T cells, and about half of the polarized CCR4⁺CD4⁺ lymphocytes in peripheral blood are Th1 cells [36, 37]. Although MPE, as a drug-induced non-immediate hypersensitivity reaction, has been frequently associated with a Th1 cytokine pattern (IFN- γ , IL-2, TNF- α) [38], the idea of a heterogeneous Th1/Th2 cytokine profile is suspected. Drug-specific T cells obtained from patients with MPE can produce Th1 (IFN- γ) and Th2 (IL-4, IL-5) cytokines [39]. Immunohistochemical studies have demonstrated the presence of these cytokines in lesional skin samples [5, 6, 14], consistent with the hypothesis that both Th1 and Th2 cells are recruited to the skin in MPE. According to these previous findings, we could suggest that TARC and MDC might recruit both Th1 and Th2 type CD4⁺ T cells.

A plethora of chemokines namely CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL17, CCL18, CCL20, CCL22, CCL26, CCL27, CXCL9, CXCL10, and CX₃CL1 and their respective receptors, have been associated to the lymphocyte recruitment to inflamed skin in atopic and non-atopic diseases [7–9, 11–13, 16–18, 22, 25–29]. Several chemokines are likely involved in driving T cells to the skin in drug-induced MPE, and their study deserves further investigation. In this sense, a novel mechanism of positive regulation for chemokine-induced leucocyte responses based on the co-operation between CCR4 natural ligands and other non-agonistic chemokines has been proposed [40]. Chemokines and their receptors have attracted considerable interest as new targets for inflammatory diseases [41]. TARC and CCR4 have been proposed as possible future targets for therapy of skin diseases such as AD, bullous penphigoid and mycosis fungoides [42]. In this sense, antagonists of CCR4 are being tested as immunomodulatory agents. CCR4 small molecule antagonists have not yet reached the clinic, but at least one of them has been validated in an *in vivo* model [43].

As we have found simultaneous up-regulation of both CCL17 and CCL22 in the skin and of their receptor CCR4 in the skin and in peripheral blood, and have demonstrated increased functional responses to both chemokines, we would like to propose that CCL17 and CCL22 may co-operate in attracting CLA⁺ T cells to the skin in drug-induced MPE.

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