

Expression of α -defensin 1–3 in T cells from severe cutaneous drug-induced hypersensitivity reactions

E. Morel¹, L. Álvarez¹, R. Cabañas², A. Fiandor², R. Díaz³, S. Escamochero¹, N. Prior², M. Blanca⁴ & T. Bellón¹

¹Research Unit, Hospital Universitario La Paz-IdiPAZ, Madrid; ²Allergy Department, Hospital La Paz, Madrid; ³Dermatology Department, Hospital Infanta Sofía, Madrid; ⁴Allergy Department, Hospital Carlos Haya, Málaga, Spain

To cite this article: Morel E, Álvarez L, Cabañas R, Fiandor A, Díaz R, Escamochero S, Prior N, Blanca M, Bellón T. Expression of α -defensin 1–3 in T cells from severe cutaneous drug-induced hypersensitivity reactions. *Allergy* 2010; DOI:10.1111/j.1398-9995.2010.02484.x

Keywords

basic mechanisms; drug allergy; innate immunity; T cells.

Correspondence

Teresa Bellón, Research Unit, Hospital Universitario La Paz-IdiPAZ, Pº Castellana 261, 28046 Madrid, Spain.
Tel.: (+34) 912071511
Fax: (+34) 912071512
E-mail: tbellon.hulp@salud.madrid.org

Accepted for publication 13 August 2010

DOI:10.1111/j.1398-9995.2010.02484.x

Edited by: Pascal Demoly

Abstract

Background: Cytotoxic T cells seem to be the main effector cells in Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). However, recent data support a role of the innate immune system in the etiopathology of drug-induced cutaneous reactions. In this study, we analyzed the expression of α -defensins 1–3 in mononuclear cells from patients with SJS/TEN, drug-induced maculopapular exanthema (MPE), and healthy donors.

Methods: DEFA1A3 gene expression was analyzed by quantitative and end-point RT-PCR. Intracellular flow cytometry, immunofluorescence and immunohistochemistry were carried out to verify α -defensin 1–3 protein expression in mononuclear cells from peripheral blood and skin infiltrates. α -Defensin 1–3 concentration was evaluated in plasma and blister fluid samples by ELISA.

Results: We herein describe DEFA1A3 gene expression in peripheral blood mononuclear cells (PBMCs) from patients with drug-induced cutaneous diseases. Gene expression analysis unveiled transcription in CD4 and CD8 peripheral blood T cells. Protein expression was confirmed by intracellular flow cytometry in mononuclear cells from the patients, including monocytes, NK cells, and T cells from peripheral blood and blister fluid. Further analysis of protein content by flow cytometry revealed higher protein levels in CD56⁺ CD3⁺ lymphocytes from patients with SJS/TEN when compared to MPE and healthy donors. Immunohistological analysis was used to confirm expression in dermal infiltrates. α -Defensin levels were estimated by ELISA to be 3- to 175-fold higher in blister fluid when compared to simultaneously drawn plasma samples.

Conclusion: Upregulation of innate immune molecules such as α -defensins 1–3 in T cells from patients with SJS/TEN may be involved in the etiopathology of these life-threatening diseases induced by medications.

Immune-mediated adverse drug reactions, also known as allergic drug reactions or drug hypersensitivity reactions, account for about one-seventh of adverse reactions to medications (1). They can be produced by any of the four immunological mechanisms proposed by Gell and Coombs. Of these, type IV reactions are mediated primarily by T cells and are

also known as delayed-type hypersensitivity reactions. These reactions have proven to be more complex than first perceived, and different type IV reactions can now be categorized according to the cytokine pattern and preferential activation of different immunocytes (2). Delayed hypersensitivity reactions to drugs comprise a wide variety of clinical entities spanning from benign diseases such as generalized maculopapular exanthemas (MPE) to life-threatening severe reactions such as Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Erythema multiforme (EM) is a less common drug-induced disease with mucocutaneous involvement (3). Although several organs may be involved, the skin is the main target tissue in most of these diseases. SJS and TEN are

Abbreviations

EM, erythema multiforme; FDE, fixed drug eruption; HNP 1–3, human neutrophil peptides 1–3; MPE, maculopapular exanthema; PMNs, polymorphonuclear cells; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; qRT-PCR, quantitative real-time RT-PCR.

the most severe forms of hypersensitivity reactions affecting the skin. Both are characterized by blister formation followed by epidermal detachment that often affects mucous membranes. Death occurs in about 1–5% of patients with SJS and above 30% of patients with TEN (3, 4). Nowadays SJS and TEN are best considered as severity variants of a single disease, on the basis of similar pathology and frequent progression from SJS to TEN (5). Several mechanisms including soluble factors such as FasL and granulysin have been proposed to explain the massive apoptosis of keratinocytes that leads to epidermal necrosis. On the other hand, CD8⁺ cytotoxic lymphocytes seem to be the main effectors of severe bullous reactions (6), and the blister fluid is rich in CD8⁺ CD56⁺ CLA⁺ cytotoxic T cells (7, 8). Furthermore, a relationship between the expression of cytotoxicity-related molecules and the severity of the reaction has been observed (9).

Evidence is accumulating that tissue damage is recognized by the immune system at the cell level via the receptor-mediated detection of intracellular proteins released in response to pathogen challenge or cell death. The term 'alarmin' has been proposed to categorize those endogenous molecules that signal tissue and cell damage (10). Effector cells of innate and adaptive immunity can secrete alarmins when they are activated by Pathogen-associated molecular patterns (PAMPs) or by other alarmins. Endogenous alarmins and exogenous PAMPs convey a similar message, and some authors consider them subgroups of a larger set: the damage-associated molecular patterns (DAMPs) (11). Among the common characteristics of alarmins are their production by immune cells that release them without dying, generally by using specialized secretion systems, and their ability to recruit and activate receptor-expressing cells from the innate immune system. Alarmins may also promote the reconstruction of tissue (11). Alpha defensins have been included in the alarmin family because, in addition to their microbicidal activity, these peptides have an immunomodulatory function and have been involved in the recruitment of immune cells (11, 12).

Most reported data regarding the drug-induced immune response during cutaneous hypersensitivity reactions are related to adaptive T-cell responses. However, in a previous study, we analyzed the gene expression profiles of PBMCs from patients with nonimmediate drug hypersensitivity reactions and found that several alarmin-coding genes were over-expressed during the acute phase of severe reactions. Strikingly, a marked upregulation of *DEFA1A3* was detected in the acute phase (mean fold *DEFA1A3* induction during the acute phase 96 ± 156 in SJS/TEN vs 5.5 ± 11.9 in nonbullous reactions, and 2.5×10^3 -fold increased expression levels in patients with SJS/TEN vs exposed controls) (13). *DEFA1* and *DEFA3* genes appear to be interchangeable variant cassettes within tandem gene arrays, so that the locus was revisited to *DEFA1A3* (14). These genes code the human neutrophil peptides (HPN1–3) also known as α -defensins 1–3. Mature HNP-1 and HNP-3 peptides differ only in their N-terminal aminoacid because of a single nucleotide difference and are major components of azurophilic granules in human neutrophils (15). In addition, α -defensin 1–3 has been detected in peripheral blood monocytes (16), T cells (16, 17) and NK cells (16, 18).

In this work, we explored the expression of *DEFA1A3* at the gene and protein levels in peripheral blood mononuclear subpopulations from patients with SJS/TEN. Enhanced transcription and peptide production was found in peripheral T cells from patients with SJS/TEN and in affected tissue.

Materials and methods

Patients' samples

A total of 30 patients with drug-induced delayed hypersensitivity reactions with cutaneous involvement (14 MPE, 2 EM and 14 SJS/TEN) participated in this study (Table S1). Samples were obtained from the Allergy and Dermatology Departments of Hospital La Paz (Madrid, Spain). In all cases, the patients developed classical cutaneous eruptions at 24 h or later after drug intake, which resolved upon drug discontinuation. Blood was taken during the period of the reaction. From some of these, patients a second sample was obtained at least 1 month after the complete remission of symptoms. Skin biopsy specimens were taken from acute skin eruptions. Fluid was obtained from tense blisters by puncture aspiration into a syringe and cells were collected by centrifugation. The patients had received no systemic corticoids prior to the study. Patients were classified according to the classification proposed by Roujeau (3). 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 or from their legal representatives.

Magnetic separation and cell sorting of CD3⁺, CD4⁺, CD8⁺, and CD14⁺ subpopulations

For CD8⁺ and CD4⁺ lymphocyte purification, PBMCs were depleted of CD16⁺ and CD14⁺ cells by incubation with anti-human CD16 and anti-human CD14 monoclonal antibodies followed by anti-mouse IgG magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The remaining population was then incubated with magnetic beads coated with anti-human CD8 (Miltenyi Biotec). All magnetic separations were performed using the AutoMACS system according to the manufacturer's instructions (Miltenyi Biotec). The resulting fractions were checked by flow cytometry, obtaining >90% pure CD4⁺ and CD8⁺ T-cell subsets. PBMCs from patient SJS 5 and EM 2 in the acute phase were sorted in a FACS-Vantage cell sorter cytometer (BD) to obtain CD4⁺ and CD8⁺ T-cell subsets with a purity >95%. CD3⁺ lymphocytes and CD14⁺ cells were purified from the mononuclear fraction by positive selection using anti-human CD3 or CD14 microbeads respectively (Miltenyi Biotec). Purity of fractions was at least 95%, as assessed by flow cytometry.

TaqMan® Gene Expression Assay-based real-time quantitative PCR (qRT-PCR)

Expression of transcripts was measured in CD14⁺ and CD14[−] PBMCs drawn from patient SJS 2 during acute disease by real-time PCR using the TaqMan® Gene Express-

sion Assays on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described (13).

RT-PCR

Total cellular RNA was isolated using the High Pure RNA Isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and 1 μ g was reverse transcribed with random hexanucleotides and AMV reverse transcriptase (Promega) in a final volume of 20 μ l for 1 h at 42°C. One micro liter of cDNA was PCR amplified in a total volume of 25 μ l following standard procedures. Primers used were as follows: *DEFA1A3* (383 pb) 5'-CTA GCT AGA GGA TCT GTG-3' and 5'-GGT ACA GGA GTA ATA GCA ATT CC-3'; *B2M* (300 pb) 5'-CCA GCA GAG AAT GGA AGG TC-3' and 5'-GAT GCT GCT TAC ATG TCT CG-3'.

Flow cytometry analysis

PBMCs were stained with the following antibodies: CD3-PerCP, CD4-FITC, CD8-FITC, CD14-FITC, CD56-APC and isotype control mouse immunoglobulin IgG1/IgG2-FITC, PerCP, APC (BD Biosciences, San Jose, CA, USA), according to standard protocols. For analysis of α -defensins 1–3, upon staining of extracellular antigens, cells were washed, fixed and permeabilized with FACSTM permeabilizing solution 2 (BD Biosciences). Defensin expression was analyzed with a biotin-conjugated anti-human α -defensin 1–3 MAb (D21; HyCult Biotechnology, Uden, the Netherlands) followed by fluorescent labeling with R-Phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Analysis was performed in a FACS-Calibur cytometer with PROQUEST software (BD Biosciences).

Immunohistochemistry

Skin biopsy specimens were fixed in formalin and paraffin embedded. For α -defensin staining, 5- μ m sections were deparaffinized and rehydrated prior to antigen unmasking by boiling in 10 mM sodium citrate pH 6.0 for 3 min. Sections were blocked in normal serum and stained with mouse anti-human α -defensin 1–3 MAb (HyCult Biotechnologies). Secondary antibody staining was performed using the VECTASTAIN ABC kit (Vector laboratories) and detected with 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin prior to visualization.

ELISA

Alpha defensins 1–3 were quantified in plasma and blister fluids using a commercially available ELISA kit (HyCult Biotechnology) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean $\pm$ SEM of the data obtained from the number of patients described in each figure. Statisti-

cal significance was determined by nonparametric tests using GRAPHPAD software, and *P*-values < 0.05 were considered statistically significant.

The procedures for confocal microscopy are presented as supporting information.

Results

DEFA1A3 expression is upregulated in CD14⁺ PBMCs from patients with SJS

We have previously reported a strong upregulation of several genes encoding endogenous DAMPs or alarmins in PBMCs from patients with SJS/TEN during acute disease (13). Most of these genes are reported to be expressed in cells of myelomonocytic origin. As neutrophils were not present in the blood mononuclear fraction used to perform the gene expression analysis, we explored the expression of alarmin-encoding genes in peripheral blood monocytes in SJS/TEN. CD14⁺ and CD14⁺ cells were isolated from patient SJS 2 during the acute phase (Fig. S1), and gene expression levels were analyzed by qRT-PCR. *TLR4* was included as a positive control. While *TLR4* and other alarmin-encoding genes were mainly expressed by CD14⁺ cells, *DEFA1A3* and *DEFA4* gene expression was mainly detected within the CD14⁺ fraction (Fig. 1).

Expression of α -defensin 1–3 peptides in peripheral blood mononuclear cells from patients with drug-induced delayed hypersensitivity reactions

We used intracellular flow cytometry analysis to explore the expression of α -defensin 1–3 peptides in subpopulations of mononuclear cells from patients with drug-induced hypersensitivity reactions and in healthy donors. While we did not find substantial amounts of α -defensins in peripheral blood T cells from healthy individuals, we detected intracellular

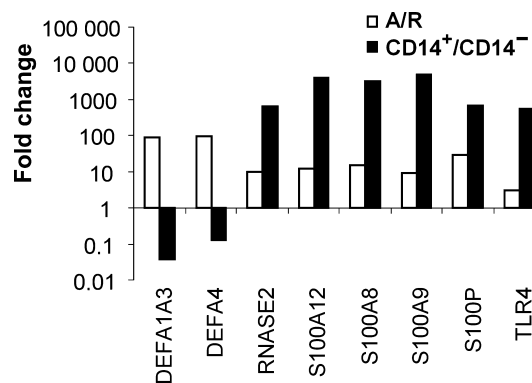


Figure 1 Quantitative RT-PCR analysis of gene expression. PBMCs were isolated from patient Stevens–Johnson syndrome (SJS) 2 during the acute reaction (A) and upon resolution of clinical symptoms (R) and the expression levels of several alarmin-encoding genes were analyzed. The ratio between expression levels in acute and resolution samples is shown (white bars). CD14⁺ and CD14⁺ cells were isolated from PBMCs during acute disease and gene expression was analyzed as above (black bars).

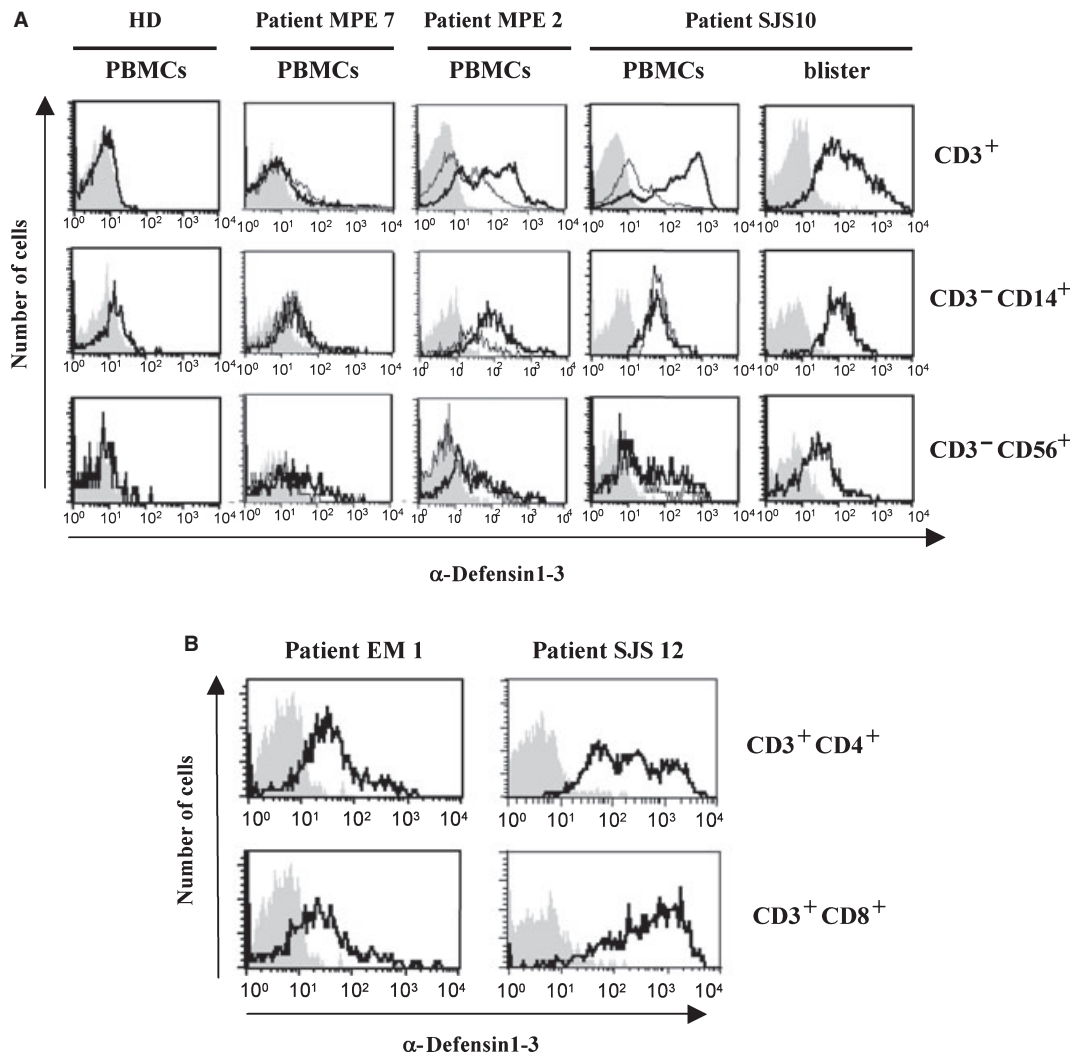


Figure 2 Expression of α -defensin 1–3 in mononuclear cells from patients. Intracellular flow cytometry was performed to analyze the production of α -defensin 1–3 peptides in PBMCs and blister fluid mononuclear cells. (A) Production of α -defensin peptides in T cells ($CD3^+$), monocytes ($CD3^- CD14^+$), and NK cells ($CD3^- CD56^+$) from a healthy donor (HD), two MPE patients and from blister fluid cells

and PBMCs from a representative Stevens–Johnson syndrome patient during the acute phase (thick line) and upon resolution of the disease (thin line). (B) Intracellular α -defensin staining of $CD4^+$ and $CD8^+$ T lymphocytes from patients during the acute phase is shown. Filled gray histograms represent background streptavidin-PE staining in the absence of a primary antibody.

α -defensin 1–3 peptides in peripheral blood T cells from all patients with SJS/TEN analyzed, as well as in peripheral blood monocytes and NK cells (Figs 2A and 3A). Alpha-defensin 1–3 peptides were also detected in T cells from MPE patients at significantly lower levels (Figs 3B and 2A, MPE7), although a few patients showed protein levels comparable to those observed in SJS/TEN (Fig. 2A, MPE 2). Protein levels were downregulated upon resolution of the disease in T lymphocytes and NK cells, but remained elevated for at least 1 month in monocytes from some patients (Fig. 2A). Tense blisters in patients with SJS/TEN contain T lymphocytes that are thought to be the effector cells of the cutaneous reaction (8). Moreover, NK cells (19) and monocytes are found in

blister fluid (7). Mononuclear cells in blister fluids also produced α -defensin 1–3 peptides (Fig. 2A).

Although substantial quantities of α -defensin peptides were detected in nearly 100% of $CD3^+$ cells in all patients with SJS/TEN analyzed, heterogeneous expression was detected in $CD3^+$ lymphocytes from some patients, suggesting a different expression in $CD4^+$ and $CD8^+$ T-cell subpopulations. Nonetheless, α -defensin 1–3 intracellular staining was detected in both $CD4^+$ and $CD8^+$ T cells from the patients (Fig. 2B). Transcription of *DEFA1A3* was confirmed in T cells by conventional RT-PCR. Fig. 4A shows that *DEFA1A3* specific transcripts were absent in mature neutrophils (PMNs). Similarly, α -defensin 1–3 transcripts were

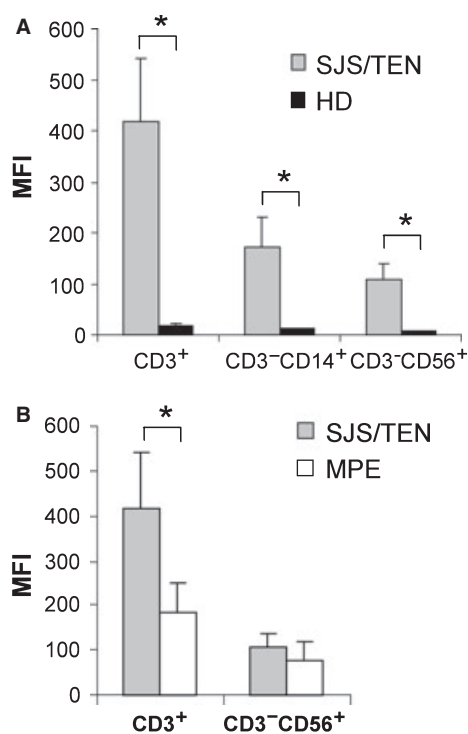


Figure 3 Expression of α -defensin 1–3 in patients and healthy donors. Flow cytometry data from different patients are expressed as mean fluorescence intensity (MFI) of intracellular α -defensin 1–3 staining in selected subpopulations. (A) Mean values $\pm$ SEM from patients with SJS/TEN ($N = 9$) and healthy donors (HD) ($N = 3$) (B) α -defensin expression in patients SJS/TEN ($N = 9$) and MPE ($N = 11$). * $P < 0.05$ (Mann–Whitney U -test). TEN, toxic epidermal necrolysis.

not detected in CD14⁺ cells isolated from patients SJS 2 and SJS 10 as previously reported in healthy donors (15, 16), although protein expression was detected in CD14⁺ cells from both patients (Figs 2 and S2). On the other hand, specific α -defensin 1–3 mRNA was amplified in the CD14⁺ subpopulation, as well as blister fluid cells from the same patients and in purified CD3⁺ lymphocytes from two different patients with EM and MPE (Fig. 4A). Transcription in both CD4⁺ and CD8⁺ T-cell subpopulations was confirmed in three independent patients with MPE, SJS, and EM (Fig. 4B,C).

Immunofluorescence and confocal microscopy analysis showed α -defensin 1–3 staining in the cytoplasm of CD4 and CD8 lymphocytes (Fig. S3).

Differential expression of α -defensin 1–3 peptides in CD3⁺ CD56⁺ and CD3⁺ CD56⁺ peripheral blood T cells from patients with drug-induced delayed hypersensitivity reactions

Expression and secretion of α -defensin 1–3 has been previously described in the CD56⁺ subpopulation of CD3⁺ T cells in healthy subjects (18). Flow cytometry analysis of

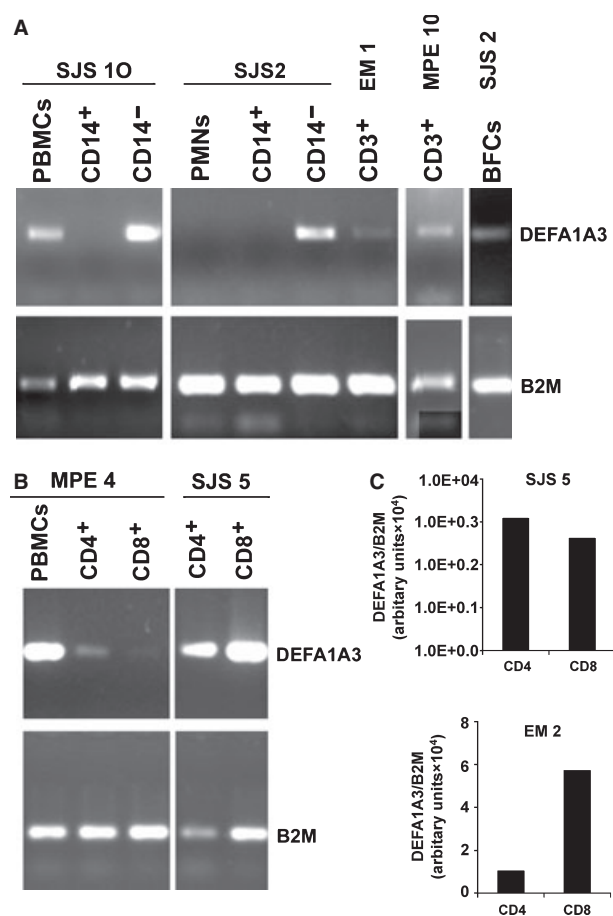


Figure 4 Gene expression of *DEFA1A3* in T cells from patients during acute reactions. (A) RT-PCR analysis of *DEFA1A3* expression in neutrophils (PMNs) and mononuclear cells from patients with drug-induced hypersensitivity reactions CD14⁺, CD14⁺ subpopulations and blister fluid cells were isolated from patients Stevens–Johnson syndrome (SJS) 10 and SJS 2. CD3⁺ lymphocytes were purified from patients EM1 and MPE10 (B) RT-PCR analysis of *DEFA1A3* expression in CD4⁺ and CD8⁺ T cells from patients MPE 4 and SJS 5. An analysis of *B2M* was included as a positive control. (C) Quantitative RT-PCR analysis of *DEFA1A3* in cell sorted CD4 and CD8 T cells from patients SJS 5 and EM2.

the CD3⁺ CD56⁺ subpopulation in SJS/TEN, MPE and control PBMCs showed that there is a predominant expression of α -defensin peptides in this subpopulation during the acute phase of drug-induced cutaneous diseases when compared to CD3⁺ CD56⁺ lymphocytes in the same patients; however, expression levels were found to be considerably higher in bullous diseases (Fig. 5A,B). Blister fluid cells from some patients also showed increased α -defensin 1–3 peptide levels in the CD3⁺ CD56⁺ subpopulation (Fig. 5A). In parallel with the rest of the CD3⁺ cells, α -defensin levels in CD3⁺ CD56⁺ cells decreased dramatically upon resolution of the clinical symptoms although they remained above the mean expression levels found in CD3⁺ CD56⁺ lymphocytes from control healthy donors (not shown).

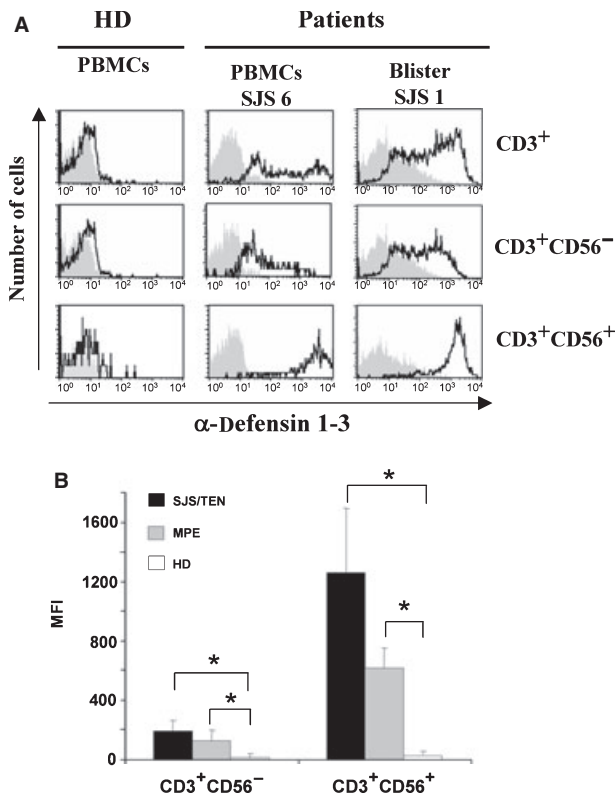


Figure 5 Flow cytometry analysis of α -defensin 1–3 peptides in CD56⁺ lymphocytes from patients. (A) Intracellular flow cytometry analysis of α -defensin 1–3 peptide content in T cells (CD3⁺), and in CD3⁺ CD56⁻ and CD3⁺ CD56⁺ subpopulations of T cells from a healthy donor (HD), patient Stevens–Johnson syndrome (SJS) 6 during the acute phase, and blister fluid cells from patient SJS 2. Filled gray histograms represent background streptavidin-PE staining in the absence of a primary antibody. (B) Data from PBMCs are shown as mean fluorescence intensity (MFI) of α -defensin intracellular staining in selected CD3⁺ CD56⁻ and CD3⁺ CD56⁺ subpopulations. Mean values $\pm$ SEM from SJS/TEN patients ($N = 8$), MPE patients ($N = 5$), and HD ($N = 3$) are shown. * $P < 0.05$ (Mann–Whitney U -test). TEN, toxic epidermal necrolysis.

Detection of α -defensin 1–3 in affected skin from patients with drug-induced delayed hypersensitivity reactions

In order to determine whether α -defensin-expressing cells were also present within the cutaneous mononuclear infiltrate, we performed immunohistochemical analyses on paraffin-embedded sections of skin biopsies from patients with MPE and SJS. While no specific staining was detected in healthy skin samples (not shown), α -defensin 1–3 protein was detected within the dermal infiltrate in affected skin from both clinical entities (Fig. 6A). Secreted α -defensin 1–3 levels were measured in the blister fluid and compared to plasma levels in patients with SJS/TEN. Elevated concentrations of soluble α -defensin 1–3 were found in plasma

from patients with SJS/TEN when compared to healthy donors (Fig. 6B). Moreover, the concentration of α -defensin 1–3 was further increased in blister fluids when compared to plasma samples simultaneously obtained from the patients (Fig. 6C).

Discussion

We have previously reported that several alarmins or endogenous DAMPs are upregulated during the acute phase of bullous drug-induced reactions. Among them, expression of *DEFA1A3* and *DEFA4*, coding for α -defensins 1–3 and α -defensin four antimicrobial peptides, increased dramatically during the acute phase of severe bullous diseases (13). In this study, we confirmed the expression of *DEFA1A3* in mononuclear cells from patients with SJS/TEN during the acute disease at the mRNA and protein levels. We performed intracellular flow cytometry studies to verify α -defensin 1–3 protein expression in T lymphocytes, NK cells, and monocytes during the acute phase in the patients with drug-induced, delayed hypersensitivity reactions. Lower quantities of intracellular peptides were detected upon resolution of the disease, which suggests a transient upregulation of gene expression in lymphocytes associated with the pathology. In this regard, we did not detect α -defensin 1–3 mRNA in CD14⁺ monocytes from two patients with SJS, in agreement with previous data from healthy donors reporting the absence of gene transcription in mature phagocytes cells (15, 16). However, specific mRNAs were found in CD4⁺ and CD8⁺ T cells from patients with MPE and SJS as well as in blister fluid cells, in agreement with flow cytometry data. Although HNPs have been detected in lymphocyte nuclei (20) immunofluorescence analysis of CD4 and CD8 lymphocytes shows preferential localization within the cytoplasm. Expression and secretion of α -defensin peptides has been reported in CD3⁻CD56⁺ NK cells and in the CD3⁺ CD56⁺ subpopulation of T lymphocytes from healthy donors in response to PAMP recognition (18). Along these lines, we detected a higher intensity of α -defensin intracellular staining in CD3⁺ CD56⁺ PBMCs from patients during the acute phase when compared to the mean fluorescence intensity of the total CD3⁺ population or their CD3⁺ CD56⁻ counterparts. Higher expression levels in life-threatening cutaneous reactions when compared to benign exanthemas suggest that α -defensin expression levels in this particular subpopulation might be related to the severity of the disease. Blister fluid cells from most patients with SJS/TEN are enriched in the CD3⁺ CD56⁺ subpopulation (21). Along these lines, the identification by mass analysis of HNP-3 as a marker in peripheral blood lymphocytes of patients with cutaneous T-cell lymphomas is of particular interest (22). It is tempting to speculate that in certain diseases, upregulation of *DEFA1A3* gene expression may occur in T cells with cutaneous tropism. On the other hand, α -defensin 1–3 peptide production has been detected in CD8⁺ T cells from HIV⁺ donors (17, 23). In this sense, it is of note that patients who are HIV⁺ are at higher risk of developing SJS/TEN (24).

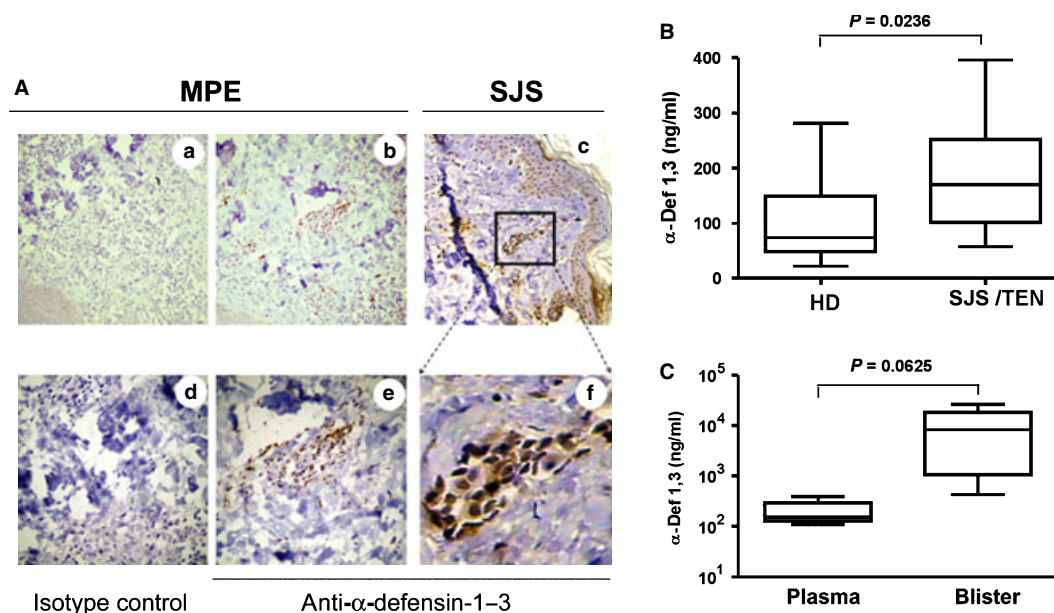


Figure 6 (A) Immunostaining of MPE-affected skin from patient MPE 3 (a, b, d, e) and Stevens–Johnson syndrome (SJS) 11 (c, f). Specific α -defensin 1–3 staining is shown (b, c, e, f). Original magnification 200 $\times$ (a–c) and 400 $\times$ (d–f). (B) Concentration of α -defensin

1–3 was measured by ELISA in plasma from healthy donors (HD) ($N = 13$) and SJS patients ($N = 10$). $P = 0.0236$; Mann–Whitney U -test. (C). Concentration of α -defensin 1–3 in plasma and blister fluid. $P = 0.0625$, Mann–Whitney U -test.

In addition to their antimicrobial activity, recent studies have implicated α -defensins in the regulation of inflammatory and immunologic processes including cytotoxicity, chemotaxis of T cells and monocytes, induction of epithelial cytokine release, and wound repair (15). However, α -defensins can be cytotoxic to mammalian cells in a dose- and time-dependent manner (25, 26). Alternatively, alarmins are characterized by their *in vivo* immunostimulatory activities (10), which may exacerbate the immune response in SJS/TEN. The high α -defensin levels found in serum and blister fluid could contribute to the pathology in both ways. Although soluble peptides could stem from neutrophil damage or degranulation, defensin mRNA analyses suggest that neutrophils are not the sole factor leading to elevated defensin levels in the sera of patients. Moreover, increased concentrations in blisters suggest a contribution of the mononuclear infiltrate to the local quantity of peptides. In this sense, HNP 1–3 have been identified by mass spectrometry from T- and NK-cell culture supernatants (16) and by immunofluorescence in granules of NK cells (18). The same authors reported increased expression and secretion of α -defensins upon stimulation with PAMP ligands in the presence of cytokines. We have not been able to detect the release of defensins to cell culture media of PBMCs from patients in the presence or in the absence of the suspected drugs. It has been suggested that alarmins use special nonclassical secretory pathways and/or are released upon cell death. As acute lymphopenia has been described in SJS/TEN, it is possible that defensins are released by dying lymphocytes.

Although HNP 1–3 were detected in the affected skin of patients with MPE and SJS/TEN, assessment of α -defensin

1–3 protein expression levels in peripheral blood mononuclear cells from patients with benign and severe diseases confirmed a relationship between α -defensin 1–3 upregulation and severity of the disease. Increased transcription and expression of defensins in CD4 and CD8 T cells from the patients indicates that in severe reactions a massive activation of T cells occurs. The elevated levels found in a few patients with MPE may reflect a predisposition to develop a more severe reaction, as in some patients MPE may evolve to SJS.

Altogether, the data show that in severe cutaneous reactions induced by drugs such as SJS/TEN, T lymphocytes may acquire functions of innate immune cells such as the production of α -defensin peptides. The data suggest an involvement of the innate immune system and in particular of α -defensin peptides, among other alarmins, in the pathogenesis of life-threatening bullous cutaneous diseases induced by drugs. Further research is needed to test whether α -defensin 1–3 expression levels could serve as a tool for differential diagnosis of cutaneous bullous diseases, to unravel the mechanisms responsible for increased expression of DEFA1A3 in severe reactions and to elucidate the precise contribution of T cells to the increased serum and blister fluid levels of α -defensins.

Funding sources

This work was supported by grants from the Ministerio de Sanidad/ISCIII, Spain (PI 02/0484 to L. Álvarez, PI 06/0441 to T. Bellón, and RETICS Red Investigación Alergenos y Reacciones Adversas a Fármacos 07/0064, RIRAAF to M Blanca). E. Morel and S. Escamochero are supported by

Fundación de Investigación Biomédica Hospital La Paz (FIBHULP).

Supporting information

Additional supporting information may be found in the online version of this article:

Data S1. Methods for confocal microscopy.

Figure S1. Isolation of CD14⁻ and CD14⁺ PBMCs from patient SJS 2.

Figure S2. Intracellular flow cytometry analysis of α -defensin 1–3 in CD14⁺ cells from patient SJS 2.

Figure S3. Confocal microscopy analysis of α -defensin 1–3 in CD4 and CD8⁺ cells from patient MPE 11.

Table S1. Clinical data of patients.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

- Gomes ER, Demoly P. Epidemiology of hypersensitivity drug reactions. *Curr Opin Allergy Clin Immunol* 2005;**5**:309–316.
- Pichler W, Yawalkar N, Schmid S, Helbling A. Pathogenesis of drug-induced exanthems. *Allergy* 2002;**57**:884–893.
- Roujeau JC. Clinical heterogeneity of drug hypersensitivity. *Toxicology* 2005;**209**:123–129.
- Pereira FA, Mudgil AV, Rosmarin DM. Toxic epidermal necrolysis. *J Am Acad Dermatol* 2007;**56**:181–200.
- Allanore L, Roujeau JC. Clinic and pathogenesis of severe bullous skin reactions: Stevens-Johnson syndrome, toxic epidermal necrolysis. In: Pichler WJ, editor. *Drug Hypersensitivity*. Basel: Karger, 2007; pp. 267–277.
- Hari Y, Frutig-Schnyder K, Hurni M, Yawalkar N, Zanni MP, Schnyder B et al. T cell involvement in cutaneous drug eruptions. *Clin Exp Allergy* 2001;**31**:1398–1408.
- Le Cleach L, Delaire S, Boumsell L, Bagot M, Bourgault-Villada I, Bensussan A et al. Blister fluid T lymphocytes during toxic epidermal necrolysis are functional cytotoxic cells which express human natural killer (NK) inhibitory receptors. *Clin Exp Immunol* 2000;**119**:225–230.
- Nassif A, Bensussan A, Boumsell L, Deniaud A, Moslehi H, Wolkenstein P et al. Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J Allergy Clin Immunol* 2004;**114**:1209–1215.
- Posadas SJ, Padial A, Torres MJ, Mayorga C, Leyva L, Sanchez E et al. Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity. *J Allergy Clin Immunol* 2002; **109**: 155–161.
- Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* 2005;**17**:359–365.
- Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 2007;**81**:1–5.
- Presicce P, Giannelli S, Taddeo A, Villa ML, Della BS. Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. *J Leukoc Biol* 2009;**86**:941–948.
- Bellon T, Alvarez L, Mayorga C, Morel E, Torres MJ, Martin-Diaz MA et al. Differential gene expression in drug hypersensitivity reactions: induction of alarmins in severe bullous diseases. *Br J Dermatol* 2010; **162**:1014–1022.
- Aldred PM, Hollox EJ, Armour JA. Copy number polymorphism and expression level variation of the human alpha-defensin genes DEFA1 and DEFA3. *Hum Mol Genet* 2005; **14**:2045–2052.
- Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003;**3**:710–720.
- Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood* 2000;**96**:3086–3093.
- Trabattoni D, Caputo SL, Maffei G, Vichi F, Biasin M, Pierotti P et al. Human alpha defensin in HIV-exposed but uninfected individuals. *J Acquir Immune Defic Syndr* 2004;**35**:455–463.
- Chalifour A, Jeannin P, Gauchat JF, Blaecke A, Malissard M, N'Guyen T et al. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* 2004;**104**:1778–1783.
- Chung WH, Hung SI, Yang JY, Su SC, Huang SP, Wei CY et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med* 2008; **14**:1343–1350.
- Blomqvist M, Bergqvist J, Westman A, Hakansson K, Hakansson P, Fredman P, Ekman R. Identification of defensins in human lymphocyte nuclei. *Eur J Biochem* 1999;**263**:312–318.
- Nassif A, Bensussan A, Dorothee G, Mami-Chouaib F, Bachot N, Bagot M et al. Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol* 2002;**118**:728–733.
- Escher N, Spies-Weissart B, Kaatz M, Melle C, Bleul A, Driesch D et al. Identification of HNP3 as a tumour marker in CD4⁺ and CD4- lymphocytes of patients with cutaneous T-cell lymphoma. *Eur J Cancer* 2006;**42**:249–255.
- D'Agostino C, Lichtner M, Mastroianni CM, Ceccarelli G, Iannetta M, Antonucci S et al. *In vivo* release of alpha-defensins in plasma, neutrophils and CD8 T-lymphocytes of patients with HIV infection. *Curr HIV Res* 2009;**7**:650–655.
- Revuz JE, Roujeau JC. Advances in toxic epidermal necrolysis. *Semin Cutan Med Surg* 1996;**15**:258–266.
- Lehrer RI. Multispecific myeloid defensins. *Curr Opin Hematol* 2007;**14**:16–21.
- Liu CY, Lin HC, Yu CT, Lin SM, Lee KY, Chen HC et al. The concentration-dependent chemokine release and pro-apoptotic effects of neutrophil-derived alpha-defensin-1 on human bronchial and alveolar epithelial cells. *Life Sci* 2007;**80**:749–758.

Supplementary materials

Expression of α -defensin 1-3 in T cells from severe cutaneous drug-induced hypersensitivity reactions

SHORT TITLE: Overexpression of α -defensins in T cells during SJS/TEN

Authors: Esther Morel¹, Luis Álvarez¹, Rosario Cabañas³, Ana Fiandor³, Rosa Díaz⁴, Salvador Escamochero¹, Nieves Prior³, Miguel Blanca², Teresa Bellón¹.

Affiliations:

¹Research Unit. Hospital Universitario La Paz-IdiPAZ, Madrid, Spain.

²Allergy Department. Hospital Carlos Haya, Málaga, Spain.

³Allergy Department. Hospital La Paz, Madrid, Spain.

⁴Dermatology Department. Hospital Infanta Sofía, Madrid, Spain.

Corresponding author: Teresa Bellón. Research Unit. Hospital Universitario La Paz-IdiPAZ, Pº Castellana 261, 28046 Madrid, Spain. Phone: (+34) 912071511; Fax: (+34) 912071512; E-mail: tbellon.hulp@salud.madrid.org

Methods

Immunofluorescence staining and confocal microscopy analysis of α -defensins 1-3

Purified PBMCs from a MPE patient with high levels of α -defensins expression as assessed by intracellular flow cytometry were affixed onto microscope slides using a cytocentrifuge. Cells were first fixed with 4% paraformaldehyde for a minimum of 15 minutes. Then the slides were washed three times with PBS. After washing, cells were blocked with PBS, 5% goat serum, 5% human γ -globulin, 1% BSA and 2% saponin, and stained for α -defensins using anti-human-defensin monoclonal antibody (D21 from HyCult Biotechnology) at 5 μ g/ml overnight, followed by washing three times with PBS. Control slides without antibody were also prepared. Alexa Fluor 594 Goat Anti-mouse SFX Kit was used for fluorescence labelling at 10 μ g/ml for 1 hour at RT, followed by washing. The blocking solution: PBS, 5% human γ -globulin, 0.5% BSA and 10% mouse serum, was used before surface labelling. CD4-FITC and CD8-FITC (Miltenyi Biotec) were used for surface labelling at 10 μ g/ml for 1 hour at RT. After washing, cells were counterstained using Hoechst 33258 (1 μ g/ml). Slides were analyzed using Leica UV/VIS SPE DMI 4000 CSQ V-VIS confocal microscope and Leica LAS AF SPE software.

Table S1 Clinical data of Patients

Patient Code and Clinical Entity*	Drug(s) involved	Sex	Age	Study
MPE 1	Amoxicillin, Clavulanic acid	M	4	FCM
MPE 2	Cefotaxime, Vancomycin, Metamizole	M	2	FCM
MPE 3	Carbamacepine	M	17	Immunohistochemistry
MPE 4	Phenytoin	F	67	RT-PCR
MPE 5	Vancomycin, levofloxacin	F	73	FCM
MPE 6	Amoxicillin	F	32	FCM
MPE 7	Ibuprofen	F	46	FCM
MPE 8	Pseudoephedrin	F	39	FCM
MPE 9	Ciprofloxacin	M	41	FCM
MPE 10	Amoxicillin, Clavulanic acid	F	35	RT-PCR, FCM
MPE 11	Acetylsalicylic acid	F	57	Immunofluorescence, FCM
MPE 12	trimetoprim-sulfametoxazol	M	46	FCM
MPE 13	Amoxicillin	M	2	FCM
MPE 14	Metamizol	M	53	FCM
EM 1	Diclofenac	M	46	RT-PCR, FCM
EM 2	Allopurinol, levofloxacin, nystatin	M	79	RT-PCR, FCM
SJS 1	Spiramycin, Metronidazole, Ibuprofen	F	27	FCM, ELISA
SJS 2	Undetermined	F	64	Q-PCR, RT-PCR, FCM
SJS 3	Anticonvulsant	F	17	FCM
SJS 4	Allopurinol	F	71	FCM
SJS 5	Isoniazide	M	62	RT-PCR, FCM
SJS 6	Phenytoin,	F	60	FCM, Immunohistochemistry
SJS 7	Anticonvulsant	M	8	FCM, ELISA
SJS 8	Phenytoin	M	67	FCM, ELISA
SJS 9	Amoxicillin	F	74	FCM, ELISA
SJS 10	Amoxicillin, Paracetamol	F	39	FCM, ELISA
SJS 11	Clindamycin	F	42	Immunohistochemistry
SJS 12	Undetermined	F	39	ELISA, FCM
SJS 13	Paracetamol	F	82	ELISA
TEN	Ibuprofen	M	37	FCM, ELISA

MPE: maculopapular exanthema, **FDE:** fixed drug eruption, **DRESS:** drug reaction with eosinophilia and systemic symptoms, **SJS:** Stevens-Johnson síndrome, **TEN:** toxic epidermal necrolysis, **EM,** erythema multiforme, **Q-PCR:** quantitative RT-PCR, **FCM,** flow cytometry

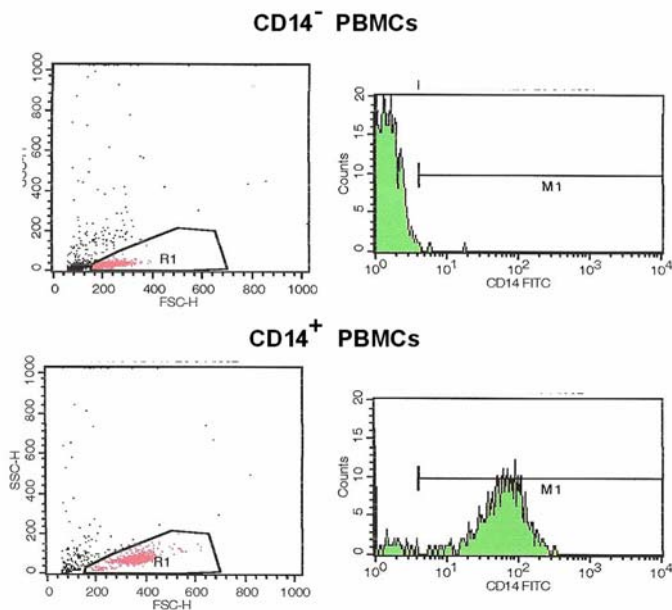


Figure S1: isolation of CD14⁻ and CD14⁺ PBMCs from patient SJS 2. CD14⁺ cells were isolated from PBMCs by positive selection using CD14⁻ magnetic beads. Flow cytometry analysis of CD14 expression in the positive and negative fractions is shown

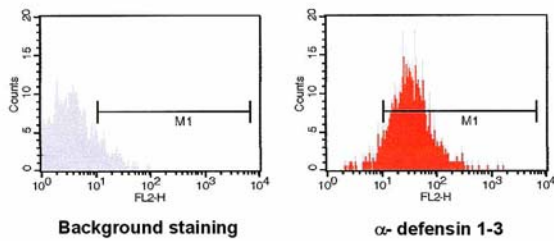


Figure S2: Intracellular flow cytometry analysis of α -defensin 1-3 in CD14⁺ cells from patient SJS 2

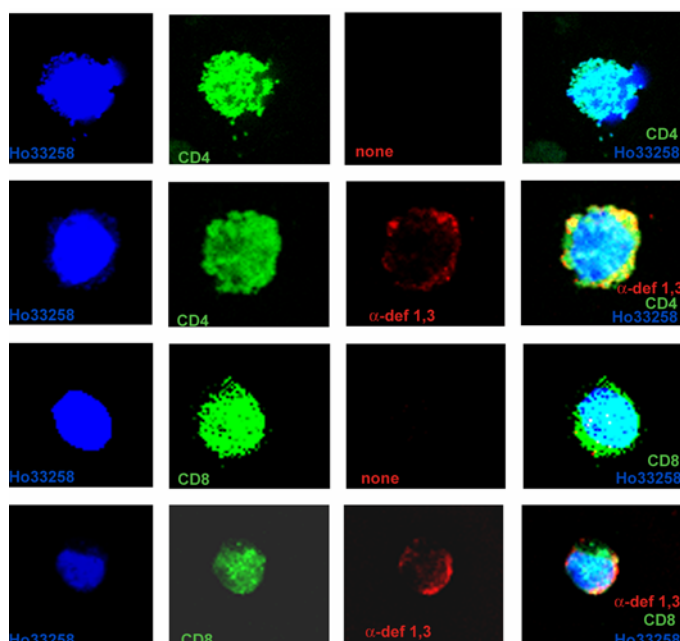


Figure S3: Confocal microscopy analysis of α -defensin 1-3 in CD4 and CD8+ cells from patient MPE 11. Confocal microscopy images showing α -defensin 1-3 staining (red) in CD4 and CD8 lymphocytes (green). Control slides without primary antibody are shown. Nuclei were counterstained with Hoechst in blue. Merge images are shown in the last column.