

The CD40/CD40 ligand system is expressed in the cutaneous lesions of erythema multiforme and Stevens–Johnson syndrome/toxic epidermal necrolysis spectrum

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Summary

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None declared.

Background Erythema multiforme (EM) and Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) are determined by a dysregulation of cellular immunity.

Objectives To evaluate the effector role of cellular immunity and the involvement of the CD40/CD40 ligand (CD40L) system in the pathogenesis of EM and SJS/TEN.

Methods Biopsy specimens from eight patients with EM and six with SJS/TEN were stained for immunohistochemical examination using the alkaline phosphatase/antialkaline phosphatase method. The monoclonal antibodies used included those to CD1a, CD4, CD8, CD40, CD40L, CD68, Fas, Fas ligand (FasL) and myeloperoxidase.

Results The cellular infiltrate in both EM and SJS/TEN lesions was composed mainly of T lymphocytes and CD68+ macrophages. We also detected large amounts of neutrophils. Fas and FasL were very highly expressed in SJS and TEN, but weakly in EM. CD40 staining was strong in all tissue sections; there were numerous CD40L+ cells in SJS/TEN but much fewer in EM.

Conclusions Activated T lymphocytes and macrophages, but also neutrophils, are presumably the main triggers of mucocutaneous damage in the SJS/TEN disease spectrum. The Fas/FasL system is significantly expressed in SJS/TEN lesions, but not in EM, where this apoptotic pathway presumably does not play a pivotal role in the epidermal damage. We suggest that the CD40/CD40L system may represent an important pathway of induction of SJS/TEN lesions, while in EM it would contribute to the immunoinflammation only as a second-line mechanism.

Erythema multiforme (EM) and Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) spectrum are characterized by polymorphous lesions affecting skin and/or mucosae with variable amounts of necrosis of basal keratinocytes and/or mucosal epithelial cells. Currently, EM and SJS/TEN are considered distinct entities on the basis of clinical, histopathological, epidemiological and aetiopathogenetic differences.^{1–3}

EM is presumed to be determined by dysregulation of cell-mediated immunity resulting from either genetic predisposition⁴ or exogenous triggering factors, including herpes

simplex virus (80% of cases),⁵ other microorganisms⁶ and, rarely, drugs.⁷ CD4+ T lymphocytes that infiltrate perivascular and junctional dermis are probably the main effector cells in EM lesions.⁸ These cells are activated, polarized towards a T-helper (Th) 1 pattern and react specifically with viral or pharmacological antigens exposed on basal and suprabasal keratinocytes of lesional epidermis.^{9,10} Furthermore, interferon (IFN)- γ , secreted by Th1 lymphocytes, would play a key role by either activation of macrophages and keratinocytes or induction of adhesion molecule expression on endothelial

cells, with subsequent diapedesis of macrophages and other lymphocytes. CD8+ T cells presumably also trigger cytotoxicity on sparse keratinocytes exposing herpetic antigens.¹¹

On the other hand, in SJS/TEN drug intake would induce, in genetically predisposed individuals,¹² the development of an immune response against keratinocytes and mucosal epithelial cells. Drug-specific CD8+ T lymphocytes are presumably the main triggering agents of the massive necrolysis (and subsequent dermoepidermal split)¹³ by secretion of perforin, granzyme B and cytokines such as tumour necrosis factor (TNF)- α .^{14–16} Moreover, the interaction between Fas ligand (FasL) and Fas is another well-documented mechanism of keratinocyte apoptosis. FasL is expressed by both T lymphocytes¹⁷ and keratinocytes,¹⁸ and its soluble form is secreted mainly by peripheral blood mononuclear cells.¹⁹ In contrast, Fas is constitutively expressed by basal keratinocytes and further induced by TNF- α and IFN- γ .^{20,21} The binding of FasL to Fas+ cells triggers intracellular signals that eventually result in activation of caspases that cause nuclear fragmentation, thus initiating programmed cell death.

Like the Fas/FasL system, the CD40/CD40 ligand (CD40L) costimulatory system belongs to the TNF/TNF receptor superfamily. CD40 is ubiquitously expressed on the surface of immune cells, including B cells, monocytes, macrophages and dendritic cells, as well as on nonimmune cells, such as epithelial, endothelial and mesenchymal cells, and platelets, whereas CD40L is expressed preferentially by activated CD4+ T cells and activated platelets. Interactions between CD40 and CD40L represent a major costimulatory system that amplifies the immune response and can promote inflammation.²² The main biological effects include the switch in recombination and synthesis of immunoglobulins by B cells, stimulation of dendritic cells to increase the cell surface expression of other costimulatory molecules such as CD54 and CD86, upregulation of cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, and production of various cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8, IL-12, TNF- α , macrophage inflammatory protein (MIP)-1 α and MIP-1 β , monocyte chemoattractant protein (MCP)-1 and RANTES (regulated on activation, normal T cell expressed and secreted).²²

The aim of our study was to evaluate further the inflammatory response in the skin of subjects with EM and SJS/TEN by immunophenotypic characterization of the cell infiltrate, with particular attention to the possible involvement of the CD40/CD40L system in the pathogenesis of EM and SJS/TEN spectrum.

Materials and methods

Patients and skin samples

Eight patients with EM (four men and four women; mean age 43 years, range 19–63) and six with SJS/TEN spectrum (one man and five women; mean age 57 years, range 30–93) were studied. In each patient the diagnosis was made on the basis

of clinical, histological and aetiological criteria.^{1,2} In particular, we diagnosed seven cases of EM minor, one of EM major, one of SJS, one of SJS/TEN overlap and four of TEN. Four EM cases were herpes-associated, two-drug associated (oral contraceptives in one case, acetylsalicylic acid or macrolides in the other), while in the remaining two no triggering agents could be found. All patients with SJS/TEN were taking medication at the time of the eruption, i.e. vancomycin, meropenem (or ceftriaxone), trimethoprim-sulphamethoxazole (or norfloxacin), chloroquine, cyclophosphamide and taxol.

After informed consent, lesional skin biopsies were obtained from the dorsum of the hands, palms or forearms of the patients with EM, and from the glutei or legs of the patients with SJS/TEN.

Furthermore, with informed consent, control specimens of normal-appearing skin from three men (mean age 45 years, range 35–50) were kindly supplied by the surgical team of our hospital. The skin specimens were immediately frozen at -80°C in liquid nitrogen.

Immunohistochemistry

Tissue specimens were cut into 5- μm thick sections, and stained immunohistochemically. The monoclonal antibodies included those to CD1a (1 : 50; Dako, Copenhagen, Denmark), CD4 (1 : 50; Dako), CD8 (1 : 20; Dako), CD40 (1 : 50; Cymbus Biotechnology, Chandlers Ford, U.K.), CD40L (1 : 50; Cymbus Biotechnology), CD68 (1 : 100; Dako), myeloperoxidase (MPO) (1 : 100; Dako), Fas (1 : 60; Novocastra, Newcastle upon Tyne, U.K.) and FasL (1 : 50; Novocastra). Before staining, frozen sections were air-dried and fixed in acetone (5 min). Immunolabelling was performed using rabbit anti-mouse bridging antibodies conjugated with alkaline phosphatase (1 : 10 dilution, 30 min; Dako) followed by incubation with murine alkaline phosphatase/antialkaline phosphatase complexes (1 : 30 dilution, 30 min; Dako).²³ Negative control sections were incubated with nonimmune mouse sera.

Two independent 'blind' observers evaluated serial sections. For quantitative analysis, the stained cells were counted in three consecutive microscopic fields ($\times 250$) and the mean was calculated. Statistical significance ($P < 0.05$) was assessed by Student's *t*-test. Also, the staining was quantified using the following nomenclature to indicate the cell numbers per field: weak, between 0 and 15; moderate, between 15 and 30; and strong (or intense), > 30 .

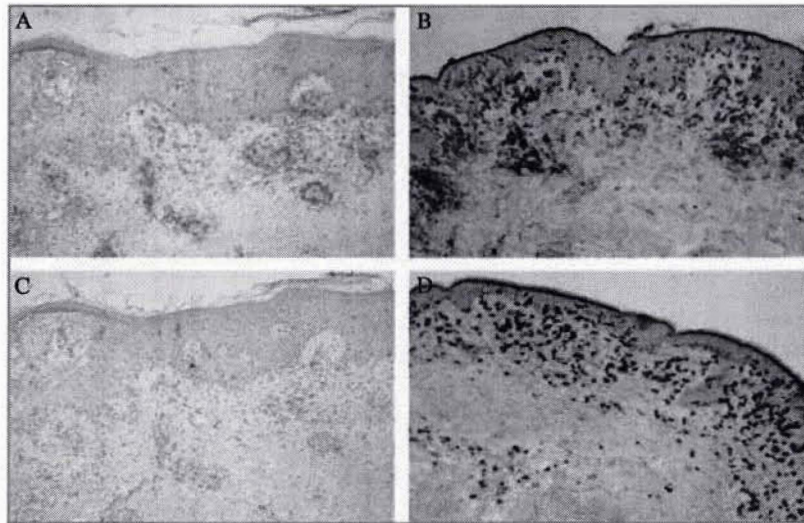
The project was approved by the Ethics Committees of our hospitals.

Results

Cell populations

CD1a+ Langerhans cells were moderately represented in the lower epidermis and in the perivascular superficial dermis of the EM and SJS/TEN skin, and the distribution and number were similar to those found in healthy control skin.

Fig 1. (A) CD4+ T cells are distributed in the perivascular and junctional dermis of erythema multiforme (EM) lesional skin; some elements also infiltrate the lower epidermis. (B) CD4+ T cells infiltrating the perivascular/junctional dermis and the epidermis of a Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) overlap skin lesion. (C) CD8+ T lymphocytes infiltrating the perivascular and junctional dermis of an EM specimen; some elements infiltrate the lower epidermis. (D) CD8+ T lymphocytes infiltrating the dermoepidermal junction and the epidermis in an SJS/TEN overlap lesion. Original magnification $\times 200$.



In EM the CD4+ and CD8+ cell populations were similarly represented (T ratio = 1.13), whereas in SJS/TEN CD4+ cells outnumbered the CD8+ cells (T ratio = 1.62). The CD4+ lymphocytes were distributed in the superficial perivascular and junctional dermis in all lesional specimens (Fig. 1A,B). In SJS/TEN lesions many CD4+ lymphocytes also infiltrated the epidermis. There were more CD4+ lymphocytes in SJS/TEN than in EM ($P = 0.0006$). The CD8+ lymphocytes were similarly distributed in all specimens in the perivascular superficial dermis with evidence of epidermal exocytosis varying from focal in EM (Fig. 1C) to evident in SJS/TEN lesions (Fig. 1D).

CD68+ cells were the most abundant infiltrating population in all the specimens, with perivascular and interstitial distribution, and in one TEN specimen they also infiltrated the epidermis. There were more macrophages in SJS/TEN than in EM, but the difference in their number was not statistically significant.

MPO+ cells, i.e. neutrophil granulocytes, were strongly detectable in all lesional specimens with a mostly perivascular distribution. Few cells were also detectable within the epidermis in some specimens. However, there was no statistical difference between EM and SJS/TEN.

Lymphocytes, macrophages and neutrophils were very slightly detectable in the superficial dermis of the healthy control skin, in significantly smaller numbers than in EM and SJS/TEN ($P < 0.0001$ in all cases).

Markers of cellular activation

Expression of Fas was observed in a remarkable proportion of keratinocytes and many dermal infiltrating cells in SJS/TEN specimens (Fig. 2B), and significantly more than in EM skin specimens ($P = 0.028$) where Fas staining was moderately distributed in the superficial dermis and diffusely in the lower epidermis (Fig. 2A). In SJS/TEN lesional skin, FasL+ cells strongly infiltrated the perivascular and junctional dermis as well as the lower epidermis (Fig. 2D) in numbers significantly

greater than in EM, where the FasL staining was very weak and detectable in only a few perivascular cells (Fig. 2C). Fas and FasL stainings were completely negative in the healthy skin specimens.

Anti-CD40 monoclonal antibodies intensely stained the dermal infiltrating cells and the lower epidermal layers in all lesional specimens (Fig. 3A,B). CD40+ cells were similarly represented in EM and SJS/TEN. However, CD40L+ cells were present only focally around dermal vessels in EM (Fig. 3C), whereas in SJS and TEN specimens they were strongly represented in the perivascular and subjunctional dermis. Moreover, in SJS/TEN some CD40L+ cells infiltrated the basal and suprabasal epidermis, where some keratinocytes were FasL+ as well (Fig. 3D). Both CD40 and CD40L stainings were practically negative in the control specimens.

Discussion

EM and SJS/TEN spectrum are currently considered as cell-mediated reactions aimed at the destruction of keratinocytes and/or mucosal epitheliocytes expressing herpes- or drug-related antigens, respectively.

In the present study we wished to characterize better the cellular infiltrate in the lesional skin of subjects with EM and SJS/TEN; in particular, we evaluated the most relevant effector immune cells linked to the CD40/CD40L system in order to assess the pathogenic relevance of this system in these diseases.

Langerhans cells were present in moderate numbers in both the epidermis and the dermis of EM whereas in a previous study only few CD1a+ cells were demonstrated in both of these compartments.²⁴ In SJS/TEN, factor XIIIa+ dendrocytes were found to be increased in the perivascular dermis during the early phases²⁵ and then diminished.²⁶ In our SJS/TEN cases, CD1a+ Langerhans cells were distributed in both the epidermis and perivascular dermis with distribution and numbers similar to those seen in healthy control skin. Such

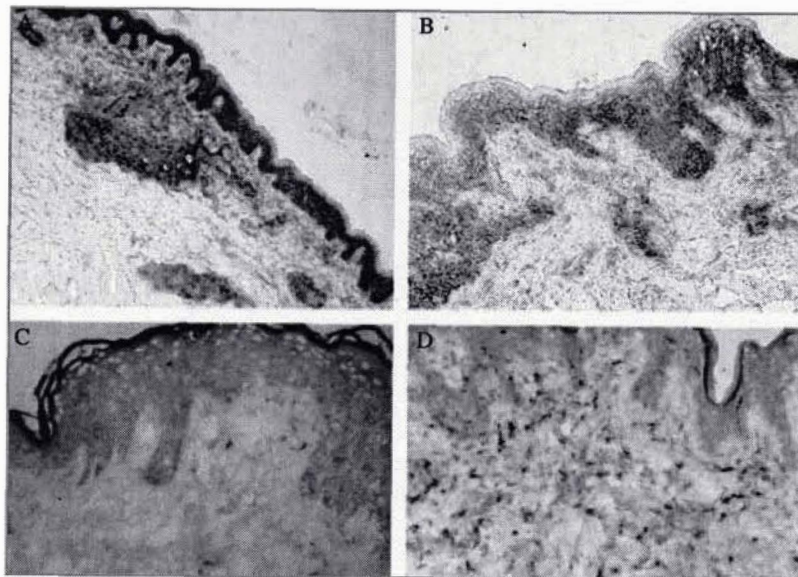


Fig 2. (A) Diffuse expression of Fas in the epidermis and in the perivascular dermis in a specimen of erythema multiforme (EM). (B) Most keratinocytes express Fas and many Fas+ cells infiltrate the perivascular dermis of a Stevens-Johnson syndrome (SJS) lesion. (C) Only Fas ligand (FasL)+ cells infiltrate the papillary dermis in EM. (D) FasL+ cells strongly infiltrating the perivascular and junctional dermis as well as the lower epidermis in an SJS specimen. Original magnification $\times 200$.

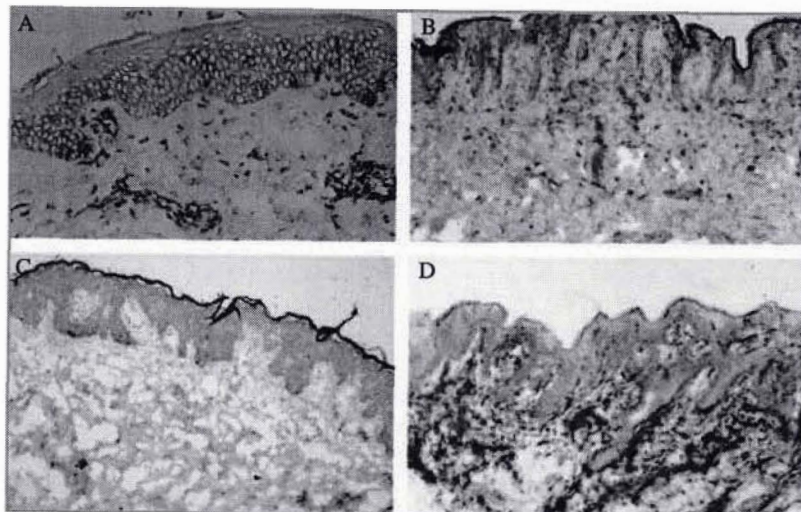


Fig 3. (A) CD40 expression in the lower epidermal layers and dermal infiltrating cells of erythema multiforme (EM) lesional skin. (B) Similar findings in a Stevens-Johnson syndrome (SJS) specimen. (C) Focal expression of CD40 ligand (CD40L) in EM. (D) Many CD40L+ cells infiltrating the perivascular/subjunctional dermis and the lower epidermis in a case of SJS; some CD40L+ keratinocytes are also observed. Original magnification $\times 200$.

discrepancies probably depend on the greatly varied concentrations of such antigen-presenting cells during migration to local lymph nodes.²⁵

Different from the findings of other reports (dermal T ratio around 2),^{8,24,27} we found similar amounts of CD4+ and CD8+ subsets in EM. This may depend on the different composition of the infiltrating lymphocytes in different phases. In agreement with previous studies,²⁸ CD4+ cells were more numerous than CD8+ cells in the lesional dermis of SJS/TEN. We confirmed that the epidermal exocytosis is focal and mainly composed by CD8+ T lymphocytes in EM,²⁴ while in SJS/TEN the lesional epidermis was strongly infiltrated by both CD4+ and CD8+ cells.⁸ On the basis of these findings, we can confirm that Th lymphocytes play a major role in the induction of EM lesions. In contrast, CD8+ T lymphocytes are probably the main trigger of the immunoinflammation in the SJS/TEN spectrum,²⁹ although activated CD4+ T lymphocytes

might be the key effector cells in SJS/TEN lesions, where they were even more numerous than in EM.

Monocytes/macrophages were one of the most numerous populations in the dermal infiltrate;^{30,31} the observation that some CD68+ cells also infiltrate the epidermis in TEN³⁰ was confirmed by our study. Macrophages would be activated by IFN- γ and would enhance antigen-presenting cell function by the upregulation of HLA-DR molecules; moreover, they may release inflammatory cytokines and chemokines able to recruit neutrophils. Thus, in EM, but especially in SJS/TEN, the activated macrophages could contribute to epidermal cell death via the secretion of TNF- α and IL-6.³²

The intense infiltration of MPO+ neutrophil granulocytes in the perivascular dermis and, focally, in the epidermis does not match the findings of previous studies that reported just slight numbers of CD15+ polymorphonuclear cells.^{24,30,31} Thus, our study suggests that neutrophils, recruited in the inflammatory

sites via the endothelial expression of adhesion molecules and local secretion of specific chemotactic molecules, could also contribute to the tissular damage, particularly at the dermo-epidermal junction, by release of radical oxygen species and lysosomal enzymes.

There have been few studies on the role of the Fas/FasL system in EM.^{33,34} In accordance with those authors, the present study shows that the interaction between Fas and FasL is active in EM lesions, as many keratinocytes expressed Fas and some FasL+ T lymphocytes infiltrated the dermis. In contrast, the Fas/FasL system is considered a major mechanism of cell death in SJS/TEN,^{17,19,21} although its overall importance is being re-evaluated. In fact, it has recently been hypothesized that the FasL+ keratinocyte-mediated pathway would represent a defence mechanism against cytotoxic T lymphocytes rather than a way of propagating apoptosis among epidermal cells.¹⁸ Nevertheless, the presence of many Fas+ keratinocytes and the strong dermoepidermal infiltration of FasL+ T lymphocytes in our SJS/TEN specimens confirmed that this mechanism is widely involved in determining the necrolysis.

To date, no data concerning the involvement of the CD40/CD40L system in EM and SJS/TEN have been reported in the literature. In the present study, we have shown that CD40 was strongly expressed by numerous dermal cells and basal keratinocytes in all lesional specimens of EM, but that only a few infiltrating cells expressed its ligand, CD40L. Thus, the interaction between CD40 and CD40L appears not to play a significant role in the induction of the immunoinflammation in EM, although this intense epidermal expression of CD40 remains to be interpreted. We might speculate that the upregulation of the CD40 receptor in the epidermis could be related to the activation of an unknown cellular pathway induced by antigen exposure. In contrast, a CD40L-induced activation of CD40+ cells may represent an important pathway of induction of SJS/TEN lesions. In fact, CD40L+ CD4+ T lymphocytes, distributed around the dermal vessels, just below the dermo-epidermal junction and among basal and suprabasal keratinocytes, might bind to CD40+ perivascular cells (particularly dendrocytes) and to CD40+ junctional macrophages, enhancing their antigen-presenting cell function, and to CD40+ keratinocytes, inducing the secretion of numerous paracrine mediators. In particular, activated CD40+ dendrocytes and macrophages may overexpress VCAM-1, ICAM-1, CD54 and CD86, and produce IL-8, IL-12, TNF- α and MIP-1 α , while CD40+ keratinocytes may secrete IL-1, IL-6, IL-8, MCP-1 and nitric oxide (NO),¹⁷ and upregulate adhesion molecules. Many of these mediators are able to exert chemotactic activity on T lymphocytes and monocytes (particularly MIP-1 α and MCP-1), and neutrophils (particularly IL-8). Finally, IL-1, IL-6, TNF- α and NO, secreted by the inducible form of the NO synthase,³⁵ may contribute to the apoptotic phenomena that are present in the lesional epidermis of SJS/TEN.³⁶

In sum, this study evidences certain additional pathogenetic differences between EM and SJS/TEN spectrum. In EM, the main pathogenetic mechanism is presumably represented by the activity of Th1 lymphocytes, primarily via the secretion of

IFN- γ and other proinflammatory mediators, and secondarily via the expression of FasL and CD40L. In contrast, the SJS/TEN lesions are mainly determined by perforin-secreting CD8+ T lymphocytes, together with the activity of FasL+ CD40L+ T lymphocytes. Macrophages and neutrophil granulocytes could also play an important role in the enhancement of the cutaneous damage in both EM and SJS/TEN.

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