Cutaneous distribution of plasmacytoid dendritic cells in lupus erythematosus. Selective tropism at the site of epithelial apoptotic damage

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Abstract

Recent evidences suggest a significant role of Plasmacytoid dendritic cells (PDC) role in the pathogenesis of lupus erythematosus (LE) via production of type I IFN. Taking advantage on the availability of multiple reagents (CD123, BDCA2, and CD2ap) specifically recognizing PDC on fixed tissues, we investigated the occurrence of PDC in a cohort of 74 LE patients. The large majority of LE biopsies (67/74; 90.5%) showed cutaneous infiltration of PDC. PDC were more frequently observed (96.4 vs 72.2) and numerous in cutaneous LE compared to systemic LE (SLE) and correlated with the density of the inflammatory infiltrate ($r = 0.40; p < 0.001$). PDC reduction in SLE might be related to a broader tissue distribution of this cellular population, as indicated by their occurrence in kidneys in 11 out of 24 (45.8%) cases studied. The distribution of cutaneous PDC showed two distinct patterns. More commonly, PDC were observed within perivascular inflammatory nodules in the dermis, associated with CD208+ mature DC and T-bet+ cells [D-PDC]. A second component was observed along the dermal-epithelial junction [J-PDC], in association with cytotoxic T-cells in areas of severe epithelial damage. Notably, chemerin reactivity was observed in 64% of LE biopsies on endothelial cells and in the granular layer keratinocytes. Cutaneous PDC in LE strongly produced type I IFN, as indicated by the diffuse MxA expression, and the cytotoxic molecule granzyme B.

This study confirms cutaneous PDC infiltration as hallmark of LE. The topographical segregation in D-PDC and J-PDC suggests a novel view of the role of these cells in skin autoimmunity.

Keywords: Plasmacytoid dendritic cell; Lupus erythematosus; Interferon; Cutaneous; Apoptosis

Introduction

Plasmacytoid dendritic cells (PDC) has been extensively characterized in the last decades (Facchetti et al. 2003; Colonna et al. 2004; Liu 2005) and shown to correspond to a specialized cell population capable of...
producing large amounts on type I interferons (I-IFN) and differentiate into dendritic cells (Grouard et al. 1997; Cella et al. 1999, 2000). Animal models supporting a definitive role of PDC in pathology are still lacking, however, numerous studies have suggested their potential role in a variety of human diseases (Facchetti et al. 2003; Colonna et al. 2004). In particular, large amount of type I IFN produced by PDC seems to be relevant in the pathogenesis of lupus erythematosus [LE] (Ronnblom and Alm 2001; Banchereau and Pascual 2006; Ronnblom and Pascual 2008), a complex disorder showing a broad spectrum of clinical manifestations, ranging from pure cutaneous form to a severe and progressive systemic disease [SLE]. The hallmark of LE pathogenesis is represented by loss of tolerance to variable nuclear antigen which leads to tissue deposition of immune complex followed by widespread inflammation and tissue damage. In this scenario, alterations in T and B lymphocyte function are retained the main culprit.

Recently, evidences pointed to a potential role of PDC in the pathogenesis of LE. PDC are the major producers of type I IFN, a key protein in the pathogenesis of this disease (Pascual et al. 2006; Gillet et al. 2008). IFN therapy in cancer and viral infections induce autoantibodies formations in addition to other clinical features mimicking SLE (Ronnblom et al. 1991; Stewart 2003). Accordingly, patients with SLE display elevated level of type I IFN (Preble et al. 1982), which correlate with disease activity, and a type I IFN molecular signature in their peripheral blood cells (Blanco et al. 2001; Bennett et al. 2003; Baechler et al. 2004). This has been recently attributed to the presence of type I IFN inducers in the serum of LE patients (Bave et al. 2000, 2003; Lovgren et al. 2004; Barrat et al. 2005; Means et al. 2005). Notably, type I IFN production by PDC in vitro can be induced by SLE serum among a plethora of other stimuli (Dziencek et al. 2001). Surprisingly, PDC blood content is decreased in SLE patients (Cederblad et al. 1998); however, these cells have been found to infiltrate extra-lymphoid lesional tissues (Blomberg et al. 2001; Farkas et al. 2001) and locally produce type I IFN (Blomberg et al. 2001; Farkas et al. 2001; Wenzel et al. 2005). Skin homing of PDC can be driven in addition to other classical PDC-attracting chemokines (Penna et al. 2001; Vanbervliet et al. 2003), by anaphylotoxins C3a and C5a (Gutzmer et al. 2006), and by the recently identified chemotactic molecules Chemerin that binds its putative receptor ChemR23 expressed on lesional PDC (Verme et al. 2005). Based on this large body of evidences, a major attention is now directed toward the understanding of the role of the tissue counterpart of PDC in the pathogenesis of the disease.

Recently published studies have documented the cutaneous accumulation of type I IFN producing PDC either in (Blomberg et al. 2001; Farkas et al. 2001; Wenzel et al. 2007a; McNiff and Kaplan 2008) a limited number of LE patients. Taking advantage on the availability of reagents recognizing PDC on fixed tissue, we studied a cohort of 74 patients and confirmed that cutaneous PDC infiltration represents the hallmark of LE. In particular, this population shows a dual compartmentalization in lesional skin. A dermal subset of PDC (D-PDC) is mainly found surrounding dermal vessels within nodules of inflammatory cells; D-PDC colocalize with DCLAMP+ myeloid DC and Th1-biased T-cells. A second subset of PDC is located at the dermo-epidermal junction (J-PDC) and colocalize with cytotoxic T-lymphocytes at the site apoptotic epithelial damage and immunocomplex deposition. We envisage that this topographical segregation of skin PDC provides direct proof to current models of LE pathogenesis (Pascual et al. 2006) and introduces a novel view of the role of these cells in autoimmunity.

Material and methods

Patients and tissues

The occurrence and distribution of PDC was studied on formalin-fixed paraffin embedded tissues from seventy-four skin biopsies and thirty renal biopsies from patients affected by LE (from the archive of the Department of Pathology, Spedali Civili di Brescia and Department of Dermatology, Medical University of Graz). Clinical data were provided for each case (Department of Dermatology, Rheumatology and Clinical Immunology, Spedali Civili di Brescia, Italy; Department of Dermatology, Medical University of Graz) and patients were classified as cutaneous LE [CLE] and systemic LE [SLE]. Normal skin obtained from plastic surgery was included as control tissues (seven cases). Frozen material from skin biopsies was available from twenty-four patients.

Reagents

Four micron tissue sections were used for immunohistochemical staining using primary antibodies to the following antigens CD123 (Mouse IgG2a, Clone 7G3, Dilution 1:50 overnight incubation, BD Biosciences, San Jose, CA, USA), BDCA2 (Mouse IgG1, Clone AC144, Dilution 1:5, Miltenyi Biotech, Bergisch Gladbach, Germany, EU), BDCA2 (Mouse IgG1, Clone 124B3.13, Dilution 1:50, Dendritics, Lyon, France, EU), MxA (Mouse IgG2a, Dilution 1:500, kindly provided by Dr. O.Haller), DC-LAMP/CD208 (Mouse IgG1, Clone 104.G4, Dilution 1:100, Immunotech, Marseille Cedex, France, EU), Chemerin (Mouse
IgG1, Clone 14G10, Dilution 1:250, kindly provided by Dr. S. Sozzani), Granzyme B (Mouse IgG2a, Clone 11F1, Dilution 1:300, Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK, EU), CD3 (Rabbit, Clone SP7, Dilution 1:100, Thermo Scientific, Fremont, CA, USA), CD20 (Mouse IgG2a, Clone L26, Dilution 1:250, Dako, Glostrup, Denmark, EU), CD2AP (Mouse IgG1, Clone B-4, Dilution 1:1400, Santa Cruz Biotechnology, Santa Cruz, CA, USA), T-bet (Mouse IgG1, Clone 4B10, Dilution 1:30 overnight incubation, Santa Cruz Biotechnology) and Foxp3 (Rat IgG2a, Clone PCH101, Dilution 1:200, eBioscience, Inc., San Diego, CA, USA).

Upon appropriate antigen retrieval, reactivity was revealed using Real EnVision Mouse/Rabbit-HRP (Dako) or Super Sensitive IHC Detection System (BioGenex, San Ramon, CA, USA), or Biotinylated anti-Rat (Dilution 1:200, Vector Laboratories, Inc., Burlingame, CA, USA) followed by DAB.

Double staining

Double immunohistochemistry was performed on formalin fixed tissue sections. After completing the first immune reaction (see above), the second immune reaction was visualized using Mach 4 MR-AP (Biocare Medical, Concord, CA, USA), followed by Ferangi Blue (Biocare Medical) as chromogen.

Double immunofluorescence was performed on five micron frozen tissue sections -air dried overnight and fixed in acetone for ten minutes using a combination of primary antibodies including anti-CD123 (Dilution 1:40), anti-BDCA2 (Miltenyi Biotech), anti-DCLAMP (Dilution 1:200). Appropriate Texas Red- and FITC-conjugated isotype-specific secondary antibodies (SouthernBiotech, Birmingham, AL, USA) were used to reveal the primary antibodies.

Quantitative immunohistochemistry

For quantitative evaluation immunostained sections were examined and scored by two pathologists (WV and AS). The density of the inflammatory infiltrate was evaluated on hematoxylin and eosin stained sections and scored as 1 (scarce), 2 (moderate) and 3 (dense). PDC content was scored on formalin fixed paraffin-embedded tissue sections stained for CD123 and BDCA2 and reported as percentage of the total mononuclear infiltrate: 0 (very rare positive cells), 1 (1–10% of positive cells), 2 (10–50% of positive cells), 3 (>50% of positive cells). Absolute counts for CD123, BDCA2, DC-LAMP were performed in at least 5 HPF and value expressed as mean ± SEM/HPF (0.037 mm²). Digital images taken using the Olympus BX60 microscope were captured using a DP-70 Olympus digital camera and processed using Analysis Image Processing software (Olympus).

Apoptotic assays

Two different techniques were used for the identification of apoptotic cells. Formalin-fixed tissue sections were stained for Active Caspase-3 (Rabbit polyclonal, Dilution 1:200, Trevigen, Gaithersburg, MD, USA) and anti–single-stranded DNA (ssDNA; Bender Med-Systems, Vienna, Austria). For each case, the number of stained cells was counted in at least 5 high power fields. Positive control samples for immunostains were represented by sections from cases Burkitt lymphoma.

Statistical analysis

Statistical analysis was performed by using the Mann-Whitney test (differences in PDC and inflammatory scores between CLE and SLE groups) and the Chi-square test (differences in J-PDC and D-PDC frequency in CLE vs SLE). The Spearman correlation coefficient was calculated to determine the correlation between the number of PDC and CD208+ mature dendritic cells. Statistical significance was defined as a P value of .05 or less.

Results

Cutaneous accumulation of PDC in lupus erythematosus

The occurrence of PDC was tested in 74 LE skin biopsies and seven normal control skin. PDC were identified based on their plasmacytoid morphology and expression of CD123, CD2ap (Marafioti et al. 2008) and BDCA-2 (Fig. 1). All these markers were suitable for the PDC identification on fixed tissues, although CD123+ cells were always slightly more numerous as revealed by double immunofluorescence staining (not shown). The large majority of LE biopsies (67/74; 90.5%) showed cutaneous infiltration of PDC, whereas no PDC were observed in normal skin (not shown). Cutaneous PDC infiltration was more frequent in CLE compared to SLE (96.4 vs 72.2) and, based on the PDC score (Table 1), the PDC content was significantly higher in CLE compared to SLE (p = 0.014). These data were confirmed on frozen sections from 24 LE cases using anti-CD123 stain and evaluating the absolute PDC number (CLE: mean 8.32/HPF; SLE: 1/HPF; p = 0.018). A higher density of the inflammatory infiltrate was observed in DLE compared to SLE (p = 0.006) and the PDC score correlated with the density of the inflammatory infiltrate (r = 0.40;
p < 0.001), suggesting that the degree of inflammation can be regulated by PDC via production of Type I IFN (Wenzel et al. 2007a, b; Wenzel and Tuting 2007) or inflammatory chemokines (Penna et al. 2002a, b).

In analogy with previous studies (Farkas et al. 2001) we found that the occurrence of systemic manifestations represented a predictor of lower PDC score in LE. In our patients this difference was not dependent on the administration of an immunosuppressive treatment. We
tested the hypothesis that the difference might be related to variability in the expression of the PDC chemoattractant chemerin, that we previously reported to be expressed in LE skin biopsies (Vermi et al. 2005).

Whereas no reactivity can be found in normal skin (Fig. 2A), chemerin expression was observed in 64% (16/25) cases; this reactivity was restricted to endothelial cells of dermal blood vessels and in the granular layer keratinocytes (Fig. 2B). However, no correlation between chemerin expression and PDC content was observed. Finally, we considered the possibility that lower PDC content in SLE could also be due to a

<table>
<thead>
<tr>
<th>Disease type (number of cases)</th>
<th>Frequency of PDC infiltration</th>
<th>PDC score</th>
<th>Inflammation score</th>
<th>PDC location</th>
</tr>
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<tbody>
<tr>
<td>SLE (18)</td>
<td>13 (72.2)</td>
<td>4 (22.2)</td>
<td>13 (72.2)</td>
<td>11/13 (84.6)</td>
</tr>
<tr>
<td>CLE (56)</td>
<td>54 (96.4)</td>
<td>3 (5.3)</td>
<td>20 (35.7)</td>
<td>41/54 (75.9)</td>
</tr>
<tr>
<td>Total (74)</td>
<td>67 (91.5)</td>
<td>7 (9.4)</td>
<td>33 (44.6)</td>
<td>52/67 (77.6)</td>
</tr>
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Fig. 2. Cutaneous expression of chemerin and MxA in lupus erythematosus. Sections obtained from skin (A, B, D, E and F) and kidney (C) are immunostained for chemerin (A and B), MxA (D and E) and CD123 (C and F). Cytoplasmic chemerin is observed in the granular layer of keratinocytes and in endothelial cells of dermal vessels (B and inset). Serial sections (E and F) from a LE case containing numerous PDC (F) shows a strong MxA expression in the cytoplasm of keratinocytes, dermal inflammatory cells and vessels (E). No reactivity for chemerin and MxA is observed in the control skin (A and D). Numerous CD123 + PDC are present in the kidney interstitium from a SLE patient (C). Sections are counterstained with Meyer’s haematoxylin. Original magnification: 100 × (A, C–E) and 200 × (B) and 400 × (inset in B).
two components. Related differences were observed in the frequency of therapy. In DLE compared to SLE (PDC [J-PDC]). D-PDC were more commonly observed in the dermis or subcutaneous tissue (from here referred as junctional epithelial junction (from here referred as junctional epithelium) and inflammatory cells (Fig. 2C). Altogether these findings indicate that J-PDC may contribute together with cytotoxic T-cells to the generation of apoptotic skin damage, via the GrB-Perforin pathway.

D-PDC co-localize with mature myeloid DC and induce a local Th1 skewed T-cell response

Via their secretory products, PDC can regulate innate and adaptive immune cells. In LE, activation of PDC leads to generation and mature myeloid dendritic cells (Blanco et al. 2001) from peripheral blood monocytes and differentiation of B-lymphocytes in antibody secreting plasma cells (Jego et al. 2003, 2005). We tested the hypothesis that cell-to-cell interactions might occur in the skin of LE patients. CD208 (DC-LAMP) expression was tested in twenty LE cases. Of note, CD208+ mature DC were commonly observed in PDC-containing LE cases (14/20; 70%) and were located in close proximity to clusters of D-PDC (Fig. 3G). In addition, CD208+ cell number correlated with PDC content (r = 0.39; p = 0.043). Interestingly, similarly to other inflammatory dermatoses (Santoro et al. 2005), cutaneous PDC do not express CD208.

Jego et al. (Jego et al. 2003) have reported that type I IFN and IL-6 produced by PDC can drive B-cell differentiation toward antibody-producing plasma cells. However, in our study the large majority of lymphoid cells infiltrating LE skin were represented by CD3+ T-lymphocytes with only rare CD20+ lymphoid B-cells (not shown). In addition, CD138+ plasma cells were very rare or absent (not shown), suggesting that B-cell instruction by PDC might take place in other tissues.
High level of type I IFN produced in LE skin induce a Th1-biased inflammatory immune response with recruitment of CXCR3+ T-lymphocytes via production of IP10/CXCL10 by lesional keratinocytes (Blomberg et al. 2001; Farkas et al. 2001; Wenzel et al. 2005). To further extend these data, we analyzed by...
immunohistochemistry the expression of T-bet and Foxp3 in 10 PDC-rich cases. Notably, T-bet+ cells represented the dominant T-cell subset in all cases, always outnumbering Foxp3+ regulatory T-cells (Fig. 3H).

Taken together, these data indicate that PDC locally participate to myeloid DC activation and maturation and are associated with a TH1 skewed immune response.

Discussion

In the last decade experimental evidences sustained the role of PDC in LE pathogenesis (Ronnblom and Alm 2001; Banchereau and Pascual 2006; Ronnblom and Pascual 2008) and it has been proposed that the interaction of type I IFN producing PDC with other leukocytes is central in the generation of auto-reactive clones and peripheral tissue damage [10]. PDC have been documented in lesional tissues of LE patients, (Blomberg et al. 2001; Farkas et al. 2001; Wenzel et al. 2007a; Fiore et al. 2008; McNiff and Kaplan 2008; Tucci et al. 2008). In this study we validate this finding, documenting the occurrence of two topographically distinct subsets of PDC in LE skin. Dermal PDC [in this study referred as D-PDC] co-localize with mature DC in dermal inflammatory nodules and might coordinate the dermal immune response by inducing a Th1-skewed phenotype in T-cells. In contrast, junctional PDC [J-PDC] express GrB and co-localize with Perforin+ cytotoxic T-cells in areas of epithelial damage. These two populations are similar in morphology and phenotype and likely represent different stages of intra-cutaneous migration.

Cutaneous PDC infiltration was documented by previous studies in a limited number of LE cases (Blomberg et al. 2001; Farkas et al. 2001; Wenzel et al. 2007a; McNiff and Kaplan 2008). Our cohort of 74 patients expands the screened population and establishes PDC infiltration as hallmark of LE cutaneous pathology. PDC content is lower in SLE compared to CLE and this reduction is associated to a global reduction of skin inflammation, suggesting that PDC can drive the cutaneous immigration of other leukocytes via production of Type I IFN (Wenzel et al. 2005, 2007b; Wenzel and Tuting 2007) or inflammatory chemokines (Penna et al. 2001, 2002b). A potential explanation for the reduction of PDC in SLE can be found in their redistribution to other extra-cutaneous tissues (Fiore et al. 2008; Tucci et al. 2008) or variability in cutaneous Chemerin expression. Notably, our study documented PDC infiltration in SLE kidney. Chemerin was significantly induced LE skin (64% vs 0% in control skin) on endothelial and epithelial cells. Even in the absence of a significant correlation with the PDC content, this finding clearly sustains its role as cutaneous PDC chemo-attractant as observed in other PDC-rich dermatoses (Parolini et al. 2007; Albanesi et al. 2009; Skrzeczynska-Moncznik et al. 2009).

By testing cutaneous expression of MxA, this study demonstrates strong type I IFN production by PDC from LE patients. Notably, MxA reactivity was also observed in cases with poor cutaneous PDC infiltration, indicating that high levels of type I IFN in the serum can be maintained by other cell type or by PDC in extra-cutaneous compartments.

PDC have been documented in other skin diseases including psoriasis, allergic contact dermatitis and lichen planus (Nestle et al. 2005; Santoro et al. 2005; Parolini et al. 2007; Albanesi et al. 2009; Skrzeczynska-Moncznik et al. 2009). The main finding of this study was represented by the identification of two PDC subsets based on their distinct intra-cutaneous distribution, namely D-PDC and J-PDC. Notably, the striking accumulation of Granzyme B+ J-PDC along the dermal-epithelial junction was associated with the occurrence of cytotoxic T-cells and apoptotic cells in areas of epithelial damage. A similar PDC component was previously reported by our group in lichen planus (Santoro et al. 2005), an inflammatory dermatoses characterized by epithelial cell death. This similarity highlight the potential contribution of J-PDC to the generation epithelial cell death either indirectly (Wenzel et al. 2005, 2007b) or in a more direct fashion via production of effector molecules such as TRAIL (Gilliet et al. 2004) and GrB (this study).

In summary, this study confirms cutaneous PDC infiltration as hallmark of LE and highlight a topographical segregation of this innate cell population which suggests a novel view of skin autoimmunity in lupus erythematosus.

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