Quantitative and functional defects of dendritic cells in classic Kaposi’s sarcoma

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Abstract In this study, we investigated whether dendritic cells (DCs) are altered in classic Kaposi’s sarcoma (cKS), a lympho-angioproliferative disorder associated with human herpesvirus-8 (HHV-8) infection. By direct analysis of peripheral blood DCs (PBDCs), we demonstrated that cKS patients have lower frequency of myeloid and plasmacytoid DCs than controls. This reduction was greater in patients with advanced stages of disease. PBDCs from cKS patients also showed up-regulated expression of the scavenger receptor CD91 and impaired IL-12 expression. PB monocytes that represent DC precursors in vivo and in vitro showed the same alterations; accordingly, DCs differentiated in vitro from cKS monocytes were similarly affected. The same alterations were induced by addition of cKS plasma during DC differentiation from control monocytes. These results indicate that PBDCs and their precursors are altered in cKS and suggest that soluble circulating factors participate in this process. The study may provide new insights into the pathogenesis of cKS.

Introduction

Dendritic cells (DCs) are widespread distributed specialized antigen-presenting cells with profound influence over the immune system. They induce, coordinate, and regulate adaptive immune responses [1]. Immature DCs residing in peripheral tissues are phagocytic cells that mature into...
migratory and highly efficient stimulators of naïve T cells in response to danger signals [2]. The capacity of DCs to transfer antigenic peptides from outside the cell onto the endogenous class I pathway, together with their high expression of costimulatory molecules, and their production of regulatory cytokines, gives to DCs the unique ability to stimulate naïve CD8+ T lymphocytes and to initiate antigen-specific cytotoxic immune responses [3,4]. For these properties, DCs are critical elements in the induction of antiviral and antitumor immunity.

DCs are present at very low frequency in the peripheral blood (PBDCs), where they can be divided into two subpopulations on the basis of expression of the β-integrin CD11c. CD11c+ myeloid DCs (mDCs) express myeloid markers, intercept invading pathogens in the periphery, and then migrate to the secondary lymphoid tissue where they present pathogen-derived peptides to antigen-specific T cells; they secrete interleukin-12 (IL-12), which drives type 1 helper T cell responses associated with generation of cellular immunity [5]. CD11c– plasmacytoid DCs (pDCs) lack myeloid markers, express the IL-3 receptor α-chain (IL-3Rα) CD123 on their surface, and migrate directly from blood to secondary lymphoid tissues; they secrete lower amounts of IL-12 but are potent producers of interferon-α (IFN-α) [6,7]. Both subpopulations may play therefore an important role in controlling viral infections and tumor growth.

Kaposi’s sarcoma (KS) is a lympho-angioproliferative disorder characterized by angiomatous nodules and plaques that mainly affect the skin but may also spread to internal organs. Four clinical variants of KS have been identified that share identical histologic features but are differentiated on the basis of epidemiological, clinical, and prognostic criteria: classic or Mediterranean (cKS), African or endemic, associated with iatrogenic immunosuppression, and epidemic or AIDS related. Regardless of the clinical variant, KS is consistently associated with human herpesvirus-8 (HHV-8) infection. Because of their role in the activation of antiviral and antitumor immune responses, the possibility that DCs may be involved in this virus-related malignancy deserves consideration.

The aim of the present study was therefore to investigate DCs in KS. To avoid the influence of other concomitant viral infections or immunosuppressive agents, only patients with cKS were studied. PBDCs from 76 cKS patients compared to 72 healthy controls were analyzed for frequency, immunophenotype, and production of regulatory cytokines. CD34+ progenitor cells and CD14+ monocytes that represent DC precursors both in vivo and in vitro were also evaluated. To investigate the contribution of cells and soluble factors to the differentiation process of DCs in cKS patients, DCs differentiated in vitro either from cKS monocytes in the presence of normal human plasma (KSmoDC/Cpl) or from control monocytes in the presence of cKS plasma (CmoDC/KSpl) were studied.

Materials and methods

Patient selection

Seventy-six cKS patients were included in the study, 57 males and 19 females, mean age 71 years (range 33–93). All patients had histologically confirmed diagnosis of KS, were positive for anti-HHV-8 antibody and negative for HIV. Patients in systemic chemotherapy were excluded. Staging was performed in accordance with our classification that takes into account the prevalent type of lesions, localization, clinical behavior, evolutive pattern, and presence of complications [8]. DC evaluation was performed at a single time point on fresh peripheral blood samples; staging at this time is reported in Table 1. Seventy-two age- and sex-matched healthy subjects were included as controls. Ethics approval was obtained from the local Institutional Review Committee, and a signed informed consent was obtained from all participants.

Virological methods

Samples of plasma were tested for HHV-8 antibodies by latent immunofluorescence assay (IFA) using BCBL-1 lines as the target, as previously reported [9]. The same samples were tested for HHV-8-DNA sequences by PCR and nested PCR amplification using primers specific for HHV-8 and human β-globin, as described elsewhere [10]. Briefly, 200 μl of plasma was subject to DNA extraction using the Quiamp DNA Blood Mini Kit (Quiagen, Milan, Italy) and resuspended in 50 μl of ultra-pure water. 10 μl of extracted DNA was loaded onto each PCR reaction. Quantitation by the PCR of KSHV genomes was performed using the limiting-dilution technique [11]. Twenty replicates for each sample were made around the end-point dilution using a KSHV-specific, single-copy sensitivity nested PCR assay. The concentration of the viral target was calculated according to the Poisson distribution. Strict measures were followed throughout the extraction and amplification processes in order to monitor for the presence of PCR false-positive and/or false-negative results. In particular, each plasma sample was preceded and followed by samples containing ultra-pure water (i.e., no DNA templates) that were used as negative controls. Furthermore, for those plasma samples that gave negative KSHV PCR results, the presence of PCR inhibitors was excluded by repeating the nested PCR assay after adding to the template DNA 500 copies of a KSHV synthetic positive-control.

Table 1 Staging of classic Kaposi’s sarcoma patients

<table>
<thead>
<tr>
<th>Stage</th>
<th>Evolution and complications</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (maculo-nodular)</td>
<td>A (slow)</td>
<td>B (rapid)</td>
</tr>
<tr>
<td>II (infiltrative)</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>III (florid)</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>IV (disseminated)</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Complete remission</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
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</tbody>
</table>

A = slow evolution; B = rapid evolution; with rapid being defined as an increase in the total number of nodules/plaques or in the total area plaques in the 3 months following an examination. c = complications; objective complications include ulcers, bleeding, lymphedema, lymphorrhea; subjective complications include pain, functional grip, ambulatory impotence. Visceral involvement was presented by one patient in stage IVBc.
Immunophenotypic analysis and counting of peripheral blood cells

Whole peripheral blood samples were analyzed by flow cytometry, as previously described [12]. Briefly, PBDCs were identified as positive for anti-HLA-DR PerCP (Becton Dickinson, San Jose, CA) and negative for a mixture of FITC-conjugated monoclonal antibodies (mAbs) (Caltag Laboratories, Burlingame, CA) specific for lineage markers (CD3, CD19, CD20, CD16, CD14). Anti-CD11c PE (Caltag Laboratories) or anti-CD123 PE (Pharmlingen, San Diego, CA) was used for identification of mDCs and pDCs, respectively. Three-color analysis was performed using a FACScan flow cytometer (Becton Dickinson) using CellQuest software (Macintosh). Because PBDCs are characterized by forward scatter (FSc) similar to monocytes and side scatter (SSc) similar to lymphocytes [13], an acquisition gate was established based on FSc and SSc that included both the lymphocyte and monocyte populations (mononuclear cells) but excluded most granulocytes and debris. 50,000 events were routinely collected to visualize and gate on this population. PE-conjugated anti-CD80, CD83, CD86 (Pharmingen) were used to evaluate the activation and maturation states of Lin-/HLA-DR+ DCs. PE-conjugated anti-CD49c (Becton Dickinson) and anti-CD91 (Biomac, Leipzig, Germany) were also used to characterize PBDCs. Positive staining for each marker was determined by comparison with appropriate isotype-matched negative controls. The frequency of DC precursors in the peripheral blood was determined by direct immunofluorescent staining with PE-conjugated anti CD34 (Beckman-Coulter Immuneotech, Marseille, France) and with FITC-conjugated anti CD14 antibodies (Caltag Labaratoires).

Cytoplasmic cytokine expression

The expression of IL-12 and IL-10 by PBDCs and monocytes was determined by flow cytometry, as previously described [12]. Briefly, whole-blood samples were diluted v/v in RPMI 1640 medium (Euroclone, Wetherby, West York, UK) and incubated for 5 h with or without LPS (100 ng/ml; from Escherichia coli, serotype 055:B5; Sigma). The protein transport inhibitor brefeldin A (BFA) (10 μg/ml; Sigma, St. Louis, MO) was added during the last 4 h, to allow intracellular accumulation of cytokines. At the end of the incubation period, samples were aliquoted and labeled with appropriate combinations of mAbs for staining of surface markers. Cells were then fixed, permeabilized, and stained with cytokine-directed mAbs anti-IL-12 PE and anti-IL-10 PE (Caltag Labaratoires), using the Fix and Perm reagent (Caltag Labaratoires). Cells labeled with isotype control mAbs were included to determine background fluorescence. Evaluation of cytokine production was based on the percentage of cytokine expressing cells.

Generation of DCs from monocyte precursors (moDCs)

MoDCs were generated from monocytes of cKS patients and controls as previously described [14]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll density gradient centrifugation (Cedarlane, Hornby, Canada) and allowed to adhere to plastic dishes for 2 h. The adherent cells (~75% CD14+ as assessed by flow cytometry) were cultured in RPMI 1640 with rhGM-CSF (800 U/ml; Novartis Farma, Origgio, Italy) and rIL-4 (10 ng/ml; Peprotech, London, UK). Because of the experimental design, the cultures were performed in the presence of 10% adult human plasma instead of fetal calf serum (FCS). Plasma samples were obtained under sterile conditions from single donors. Sixteen cKS patients in stages I to IV were compared with matched healthy donors. To preserve thermolabile factors, plasma samples were not heat inactivated. Preliminary experiments aimed to validate this substitution showed that, apart from lower expression of CD1a, moDCs generated in human plasma were similar to moDCs generated in FCS. When indicated, IL-6 (25 pg/ml to 25 ng/ml; Peprotech) was added at the beginning of the culture. After 5 days, cells were collected and used for subsequent analysis. The cell morphology was assessed on cytopsin preparations after May-Grünwald-Giemsa or acridine orange (AO) staining.

Immunophenotypic analysis of moDCs

MoDCs were analyzed by flow cytometry, as previously described [15]. Briefly, moDCs were incubated with different FITC- or PE-conjugated monoclonal antibodies (mAbs) for 30 min at 4°C in the dark. The following mAbs were used: anti-CD14 (Caltag Labarotoires), -CD1a, -CD40, -CD49c, -CD80, -CD83, -CD86 (Becton Dickinson); -CD11c (Caltag, Burlingame, CA); -CD91 (Biomac, Leipzig, Germany); -ILT-2, -ILT-3, -ILT-4 (VII International Workshop on Leukocyte Typing) [16]. Negative controls were isotype-matched irrelevant mAbs. Cells were electronically gated according to light scatter properties to exclude cell debris.

Allogenic T cell proliferation assay

To test their allostimulatory activity, moDCs (15 × 10^3) were cocultured in 96-well plates with 3 × 10^5 allogeneic, monocyte-depleted PBMCs in triplicate for 5 days, as previously described [14]. 5-Bromo-2′-deoxyuridine (BrdU, 20 μM; Sigma) was added in each well during the last 6 h of culture, and lymphocyte proliferation was assessed by flow cytometry as BrdU incorporation by CD4+ lymphocytes, as described by Toba et al. [17]. Results were expressed as percentage of proliferating (BrdU+) lymphocytes.

Immunoenzymatic cytokine measurements

Cytokines were measured in plasma samples and in the supernatants of unstimulated moDC cultures by specific enzyme-linked immunosorbent assay (ELISA). IL-1β, IL-6, IL-12, TNF-α, TGF-β1, and VEGF were measured with commercial kits (R&D Systems, Minneapolis, MN), used according to the manufacturers’ instructions. IL-10 was measured by use of commercially available pairs of mAbs (Endogen, Woburn, MA).

Statistical analysis

Statistical analysis was performed with Openstat 3 software (open source statistics package by Bill Miller, Iowa)
State University). Comparisons of samples to establish the statistical significance of difference were determined by the two-tailed Mann—Whitney rank sum test for independent samples. The paired Wilcoxon test was also used when indicated. Correlations were performed by Spearman rank test. Values of $P < 0.05$ were considered significant.

**Results**

**Frequency of PBDCs and DC precursors**

The frequency of PBDCs, identified as lin-/HLA-DR+ cells, in the mononuclear cell population was significantly lower in the PB of cKS patients (median 0.80%) compared to healthy controls (1.10%; $P < 0.001$). As shown in Fig. 1A, the decrease of PBDCs was not restricted to either cell subset but involved both mDCs and pDCs: the median frequency of mDCs in cKS was 0.47%, compared to 0.65% ($P = 0.002$) in controls; the median frequency of pDCs was 0.23% in cKS, compared to 0.31% ($P < 0.001$) in controls. Within the group of cKS patients, the decrease of PBDCs was more severe in patients with more advanced stages of disease (stages III—IV vs. stages I—II: 0.64% vs. 0.91%, $P = 0.018$); as shown in Fig. 1B, this correlation with the stage of disease involved both DC subpopulations (mDCs: 0.41% vs. 0.50%, $P = ns$; pDCs: 0.19% vs. 0.27%, $P = 0.029$). To confirm the influence of the stage of disease on DC subpopulations, six cKS patients in complete remission had PBDC frequency similar to healthy controls (1.05%). Moreover, six patients who presented a stable stage of disease over a period of 6 months were analyzed for PBDCs at a second time point (3 patients in stages I—II, and 3 in stages III—IV) and showed the persistence of the observed DC defects (median PBDCs: 0.86 and 0.84; mDCs: 0.50 and 0.52; pDCs: 0.23 and 0.25). Finally, the repeated analysis of PBDCs in two patients who presented modification of the clinical stage during the study revealed a partial increase of PBDC frequency concomitant with a partial regression of cKS lesions (patient 1 from stage IIIb to IA: PBDCs from 0.69 to 1.00, mDCs from 0.42 to 0.66, pDCs from 0.20 to 0.31; patient 2 from stage IIIb to IA: PBDCs from 0.90 to 1.20, mDCs from 0.60 to 0.84, pDCs from 0.21 to 0.26). The frequency of monocytes in the overall population of leukocytes was also significantly lower in cKS patients (5.86%) compared to controls (7.15%, $P < 0.001$) (Fig. 1C). On the contrary, the frequency of CD34+ cells was significantly higher in the PB of cKS patients (0.11%) than in the PB of healthy controls (0.05%, $P < 0.001$). Neither the frequency of monocytes nor that of CD34+ cells was correlated to the stage of disease or with the presence of plasmatic HHV-8-DNA.

**Immunophenotype of PBDCs and monocytes**

The activation state of PBDCs, assessed as expression of the costimulatory molecules CD80 and CD86 on lin-/HLA-DR+ cells, in the mononuclear cell population was significantly lower in the PB of cKS patients (median 0.80%) compared to healthy controls (1.10%; $P < 0.001$). As shown in Fig. 1A, the decrease of PBDCs was not restricted to either cell subset but involved both mDCs and pDCs: the median frequency of mDCs in cKS was 0.47%, compared to 0.65% ($P = 0.002$) in controls; the median frequency of pDCs was 0.23% in cKS, compared to 0.31% ($P < 0.001$) in controls. Within the group of cKS patients, the decrease of PBDCs was more severe in patients with more advanced stages of disease (stages III—IV vs. stages I—II: 0.64% vs. 0.91%, $P = 0.018$); as shown in Fig. 1B, this correlation with the stage of disease involved both DC subpopulations (mDCs: 0.41% vs. 0.50%, $P = ns$; pDCs: 0.19% vs. 0.27%, $P = 0.029$). To confirm the influence of the stage of disease on DC subpopulations, six cKS patients in complete remission had PBDC frequency similar to healthy controls (1.05%). Moreover, six patients who presented a stable stage of disease over a period of 6 months were analyzed for PBDCs at a second time point (3 patients in stages I—II, and 3 in stages III—IV) and showed the persistence of the observed DC defects (median PBDCs: 0.86 and 0.84; mDCs: 0.50 and 0.52; pDCs: 0.23 and 0.25). Finally, the repeated analysis of PBDCs in two patients who presented modification of the clinical stage during the study revealed a partial increase of PBDC frequency concomitant with a partial regression of cKS lesions (patient 1 from stage IIIb to IA: PBDCs from 0.69 to 1.00, mDCs from 0.42 to 0.66, pDCs from 0.20 to 0.31; patient 2 from stage IIIb to IA: PBDCs from 0.90 to 1.20, mDCs from 0.60 to 0.84, pDCs from 0.21 to 0.26). The frequency of monocytes in the overall population of leukocytes was also significantly lower in cKS patients (5.86%) compared to controls (7.15%, $P < 0.001$) (Fig. 1C). On the contrary, the frequency of CD34+ cells was significantly higher in the PB of cKS patients (0.11%) than in the PB of healthy controls (0.05%, $P < 0.001$). Neither the frequency of monocytes nor that of CD34+ cells was correlated to the stage of disease or with the presence of plasmatic HHV-8-DNA.

**Figure 1** Frequency of PBDCs and DC precursors in cKS patients and healthy controls. (A) A significant decrease in total PBDCs (left axis), myeloid DCs and plasmacytoid DCs (right axis) was observed in cKS patients. Data were obtained by direct cytofluorimetric analysis of peripheral blood samples and were expressed as percentage of DCs in the mononuclear population. (B) Within the group of cKS patients, the decrease of PBDCs (left axis) was more severe in patients with more advanced stages of disease; this correlation with the stage of disease involved both myeloid and plasmacytoid DCs (right axis). (C) The frequency of DC precursors was also altered in the peripheral blood of cKS patients. Monocytes in the overall population of leukocytes (left axis) were significantly decreased, while CD34+ precursors (right axis) were increased. Each symbol represents a single sample. Median values represented by horizontal lines in each series. Comparison between groups performed with the two-tailed Mann—Whitney rank sum test.
DR+ cells, and their maturation state, assessed as expression of CD83, did not differ between patients and controls (percentage of CD80+ DCs: 4.85% vs. 4.10%; CD86+ DCs: 69.38% vs. 72.77%; CD83+ DCs: 3.28% vs. 2.41%). The percentage of PBDCs expressing the HHV-8 receptor CD49c on their surface was significantly higher in cKS patients than in healthy controls (7.31% vs. 4.38%, \( P = 0.022 \)), as it was the percentage of PBDCs expressing CD91 (8.87% vs. 4.87%, \( P < 0.001 \)) (Fig. 2A). Similar results were obtained when mean fluorescence intensity (MFI), rather than the percentage of positive cells, was considered. There was no correlation between DC immunophenotype and stage of disease.

Similarly to PBDCs, the activation state of monocytes was similar in cKS patients and controls. The percentage of monocytes expressing CD49c was higher in cKS than in controls (3.01% vs. 1.57%, \( P = 0.042 \)), as it was the percentage of monocytes expressing CD91 (5.46% vs. 2.43%, \( P < 0.001 \)) (Fig. 2B).

Cytokine production by PBDCs and monocytes

The analysis of intracellular cytokine expression in unseparated Lin-/HLA-DR+ PBDCs showed that in both cKS patients and controls, incubation of PB samples with LPS induced a significant increase in the percentage of DCs expressing either IL-12 or IL-10 (Wilcoxon matched-pairs signed rank test \( P < 0.001 \)). As shown in Fig. 3A, the constitutive expression of IL-12 and IL-10 was similar in the two groups. The percentage of DCs expressing IL-12 upon LPS stimulation was significantly lower in cKS patients (median 23.22%) than in controls (31.87%; \( P < 0.001 \)); on the contrary, the percentage of DCs expressing IL-10 upon LPS stimulation was significantly higher in patients (9.90%) compared to healthy controls (7.42%, \( P = 0.025 \)). Similar results were obtained when MFI for cytokine staining, rather than the percentage of positive cells, was considered. We further evaluated the ability of PBDCs to produce IFN-α, by stimulating PBMCs with CpG and then measuring the cytokine released in the supernatants. DC production of IFN-α did not differ between cKS (median 50.6 pg/ml) and controls (81.3 pg/ml). No correlation was found between cytokine production by PBDCs and stage of disease.

Similarly to PBDCs, the analysis of intracellular cytokine expression in unseparated PB monocytes showed that in cKS patients and controls incubation of PB samples with LPS induced a significant increase in the percentage of monocytes expressing either IL-12 or IL-10 (Wilcoxon matched-paired signed rank test \( P < 0.001 \)). The constitutive expression of IL-12 and IL-10 by monocytes was similar in patients and controls (Fig. 3B). The percentage of monocytes expressing IL-12 upon LPS stimulation was significantly lower in cKS (9.54%) than in controls (16.16%, \( P = 0.005 \)), while the percentage of monocytes expressing IL-10 upon LPS stimulation did not significantly differ between groups (10.27% vs. 8.35%, \( P = \text{ns} \)). As shown in Fig. 3C, both in patients and controls, the expression of cytokines by monocytes and DCs was directly correlated.

Correlation between PBDCs, HHV-8 and plasmatic levels of inflammatory cytokines

Plasmatic HHV-8-DNA was detected in 34.9% of the cKS patients, with slightly higher frequency in patients with more advanced stages of disease (stages III–IV vs. stages I–II: 44.4% vs. 33.3%) and lower frequency in patients in complete remission (16.6%). Detection of viremia appeared to be correlated with the clinical evolutive pattern of KS, HHV-8-DNA being positive in 6.3% of patients with slow evolution (all stages A) compared with 48.8% of those with rapid evolution (all stages B). The presence of viral DNA in the plasma was associated with a more severe alteration of some PBDC parameters. In particular, plasmatic HHV-8-DNA was associated with a greater decrease of PBDC frequencies (HHV-8-DNA positive vs. negative cKS patients: PBDCs 0.77% vs. 0.84%, \( P = 0.048 \); mDCs 0.36% vs. 0.49%, \( P = 0.041 \); pDCs 0.21% vs. 0.26%)

Figure 2  Immunophenotype of PBDCs and monocytes in cKS patients and healthy controls. (A) The immunophenotypic differences between PBDCs from cKS patients and those from control subjects concerned the expression of CD49c and CD91. PBDCs from cKS showed increased expression of CD49c, integrin involved in the cell entry of HHV-8; and increased expression of CD91, scavenger receptor involved in the cross-presentation of antigens uptaken from dying cells. (B) Similarly to PBDCs, monocytes from cKS patients showed increased expression of CD49c and CD91. Each symbol represents a single sample. Median values represented by horizontal lines in each series. Comparison between groups performed with the two-tailed Mann–Whitney rank sum test.
with higher PBDC expression of CD83 (percentage of CD83+ DCs: 6.53% vs. 2.99%; \( P = 0.043 \)), CD49c (12.99% vs. 5.94%; \( P = 0.082 \)); and with slightly higher expression of LPS-induced IL-10 (11.32% vs. 9.73%; \( P = \text{ns} \)). No correlation was observed between viral DNA and DC precursors.
To evaluate whether inflammatory cytokines could also influence PBDCs in cKS patients, the plasmatic levels of IL-6, IL-1β, and TNF-α were measured. Detectable levels of IL-6 were found in 58.2% of the patients (median 15.5 pg/ml) and in none of the healthy controls. Compared with patients with undetectable plasmatic levels, patients with detectable IL-6 showed slightly lower frequencies of PBDCs (PBDCs 0.74% vs. 0.82%; mDCs 0.43% vs. 0.49%; pDCs 0.21% vs. 0.23%; P = ns) and monocytes (5.84% vs. 6.16%; P = ns); lower expression of LPS-induced IL-12 (22.57% vs. 25.0%) and higher expression of LPS-induced IL-10 (10.26% vs. 8.89%; P = ns). No significant correlation was observed between IL-6 levels and plasmatic HHV-8-DNA or clinical stage of KS. The plasmatic levels of IL-1β and TNF-α were below or close to the sensitivity limit of the assays, without differences between patients ad controls. The plasmatic levels of VEGF did not differ between groups (69.7 vs. 38.5 pg/ml; P = ns).

**Generation, phenotype, and functional characterization of moDCs derived from cKS monocytes (KSmDC/Cpl)**

Because DCs can differentiate both in vivo and in vitro from monocytes, we further investigated whether the alterations observed in PBDCs from cKS patients could be observed at least in part in moDCs obtained by in vitro differentiation of cKS monocytes. To this purpose, KSmDC/Cpl were compared to moDCs obtained from control monocytes. Exposure of either monocyte to GM-CSF plus IL-4 led within 5 days to loss of plastic adherence associated with cellular aggregation in large-cell clusters and appearance of typical DC morphology, as assessed by fluorescence microscopy after AO staining. KSmDC/Cpl were morphologically similar to CmoDC/Cpl, with lobulated nuclei and numerous fine cytoplasmic projections, consistent with a DC phenotype. The percentage of cells with DC morphology as well as the percentage of viable cells (trypan blue exclusion test) were calculated after 5 days of culture. Cell viability was >90% in all experiments. As shown in Fig. 4A, the yield of viable KSmDC/Cpl, expressed as percentage of the number of monocytes plated at day 0, was significantly lower (median 51%) than the yield of CmoDC/Cpl (67%, Mann—Whitney rank sum test P = 0.004). The results of immunophenotypic analysis, reported in Table 2, indicated that the activation and maturation state of KSmDC/Cpl were similar to CmoDC/Cpl, while KSmDC/Cpl expressed CD91 on a higher proportion of cells than CmoDC/Cpl (P < 0.001). To evaluate the allostimulatory activity of KSmDC/Cpl, we analyzed the direct pathway of alloantigen presentation, assessed in MLR as BrdU incorporation by allogeneic, analyzed the direct pathway of alloantigen presentation, and similar levels of TNF-α (14 vs. 12 pg/ml), IL-1β (23 vs. 26 pg/ml) and IL-10 (51 vs. 54 pg/ml) (Fig. 4C). HHV-8-DNA was absent in all the samples.

**Generation, phenotype, and functional characterization of moDCs derived from control monocytes in the presence of cKS plasma (CmoDC/KSpl)**

To investigate the possible influence of soluble factors present in the plasma of cKS patients in the differentiation process of monocytes into DCs, CmoDC/KSpl were compared to CmoDC/Cpl. Experiments were performed as for KSmDC/Cpl. As shown in Fig. 5A, the yield of viable CmoDC/KSpl was significantly lower (58%) compared to the yield of CmoDC/Cpl (67%, P = 0.010). As reported in Table 2, CmoDC/KSpl showed an immunophenotype more activated and mature than CmoDC/Cpl, characterized by lower expression of CD1a, and higher expression of CD80 and CD83. CmoDC/KSpl also displayed higher expression of CD91 than CmoDC/Cpl. As shown in Figs. 5B and C, CmoDC/KSpl presented a lower allostimulatory activity compared with CmoDC/Cpl (percent BrdU+ CD4+ lymphocytes, median of 11 independent experiments: 4.18 vs. 11.87, P = 0.002). The analysis of cytokines in culture supernatants indicated that, similarly to KSmDC/Cpl, CmoDC/KSpl produced lower levels of IL-12 (median 2 vs. 20 pg/ml; Wilcoxon matched-paired signed rank test P < 0.001) and higher levels of IL-6 (161 vs. 47 pg/ml; P < 0.001) than CmoDC/Cpl. According to their lower allostimulatory activity, CmoDC/KSpl also produced higher levels of the immunosuppressive cytokines IL-10 (74 vs. 58 pg/ml; P = 0.043) and TGF-β (9260 vs. 7300 pg/ml; P = 0.007) (Fig. 5D).

Analysis of the plasma samples used to generate CmoDC/KSpl revealed that 10 of 16 samples contained detectable levels of IL-6 (median 15.5 pg/ml) compared with undetectable IL-6 in all the control samples. Other inflammatory cytokines, namely IL-1β and TNF-α, were undetectable in both patients and controls. HHV-8-DNA was detected in only one of the cKS plasma samples. No correlation was observed between CmoDC/KSpl alterations and the clinical stage of plasma donors.

**Generation, phenotype, and functional characterization of moDCs derived from control monocytes in the presence of exogenous IL-6 (IL6-DCs)**

To investigate a possible role for IL-6 in the alterations observed in PBDCs and moDCs from cKS patients, experiments were performed in which the differentiation process of monocytes into DCs was driven in the presence of IL-6. As shown in Fig. 6A, the yield of viable IL6-DCs was lower compared to the yield of control DCs, in a dose-dependent manner. A significant reduction was observed when cells were cultured in the presence of IL-6 at 25 ng/ml (40% vs. 67%; P = 0.039). Furthermore, exogenous IL-6 up-regulated dose-dependently the expression of CD91 on DCs, with a significant increase observed at 2.5 ng/ml (percentage of CD91+ IL6-DCs: 20.1% vs. 10.8%; P = 0.014) and at 25 ng/ml (29.9% vs. 10.8%; P = 0.014) (Fig. 6B). No other immunophenotypic changes were produced by the addition of IL-6. The allostim-
Discussion

Decrease of circulating DCs may be relevant to the immune compromise that characterizes all the clinical variants of KS. In this study, we analyzed PBDCs in patients with the classic variant of the disease, to avoid the confounding effects of HIV co-infection or immunosuppressive therapy that are present in the other clinical variants of KS. We applied flow cytometric methods that allow the enumeration and characterization of DCs directly in whole peripheral blood samples [12,13,18]. With this method, that directly reflects the in vivo situation, we observed that the frequency of lin-/ HLA-DR+ DCs in the peripheral blood of cKS patients is significantly lower compared with controls. The reduction, which involves both mDCs and pDCs, is more severe in patients with more severe stages of disease, and it is reverted by disease regression. Our findings, together with the selective decrease of circulating pDCs described in patients with AIDS-related KS [19], may add HHV-8 to the list of viral infections that are associated with a decrease in PBDCs. In fact, a reduction of mDCs and/or pDCs has been described in HIV-1 infection, chronic C hepatitis, measles, and SARS [20–23]. This may suggest that direct or indirect modifications of DCs may represent a common viral immune evasion strategy shared by small RNA viruses and large DNA viruses; impairment of DCs could play a key role in establishing and maintaining viral persistence. Beyond viral infections, also tumors have been demonstrated to cause a decrease of PBDCs [12,24,25]. Although we cannot rule out the possibility that PBDC reduction in our patients may be related to the presence of KS independently of HHV-8 infection, the finding that individuals with detectable HHV-8-DNA show greater decrease of PBDC frequency may suggest that HHV-8 infection may drive DC impairment in our patients.
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Immunophenotypic pattern of moDCs obtained from cKS monocytes and from control monocytes with cKS plasma

<table>
<thead>
<tr>
<th>Marker</th>
<th>Percentage</th>
<th>MFI</th>
</tr>
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<tbody>
<tr>
<td>CD1a</td>
<td>12.6 (6.3–22.9)</td>
<td>14.5 (6.9–22.9)**</td>
</tr>
<tr>
<td>CD40</td>
<td>36.7 (22.5–70.8)</td>
<td>37.3 (25.9–50.4)</td>
</tr>
<tr>
<td>CD80</td>
<td>37.0 (29.5–48.7)</td>
<td>37.3 (26.8–56.9)</td>
</tr>
<tr>
<td>CD86</td>
<td>11.9 (8.2–15.8)</td>
<td>12.3 (9.2–19.2)</td>
</tr>
<tr>
<td>CD49c</td>
<td>10.7 (4.2–19.7)</td>
<td>13.8 (12.5–21.2)</td>
</tr>
<tr>
<td>ILT-2</td>
<td>82.0 (78.8–84.3)</td>
<td>81.9 (78.8–82.6)</td>
</tr>
<tr>
<td>ILT-3</td>
<td>10.5 (5.3–22.1)</td>
<td>11.9 (5.3–21.2)</td>
</tr>
<tr>
<td>ILT-4</td>
<td>13.7 (6.7–21.1)</td>
<td>14.3 (10.3–21.4)</td>
</tr>
</tbody>
</table>

CD49c (integrin α3β1), expressed on all cell types as a receptor for GM-CSF and IL-4, was also increased in frequency in the peripheral blood of our cKS patients helps to confirm the role of cytokines of DC origin in the control of viral infections and of tumor progression.

Our results further indicate that PBDCs from cKS patients not only are reduced in frequency but also differ from control DCs in some immunophenotypic and functional aspects. In particular, PBDCs from cKS patients express CD49c and CD91 on a higher percentage of cells than control PBDCs. CD49c (integrin α3β1), expressed on all cell types susceptible to HHV-8-infection, is bound specifically by an RDG motif contained in HHV-8 gB allowing therefore HHV-8 entry into target cells [26]; augmented expression of CD49c on DC surface may augment DC susceptibility to HHV-8-infection. CD91 is a scavenger receptor involved in the uptake of material from dying cells which has been demonstrated to endow DCs with the ability to prime the adaptive arm of immune responses by promoting antigen cross-presentation [27]. Its importance in antiviral and antitumor immunity is suggested by the demonstration of its up-regulation on monocytes from HIV-infected long-term nonprogressors and from melanoma slow progressors [28,29].

We also observed that PBDCs from cKS patients differ from control DCs in their production of regulatory cytokines. In particular, upon LPS stimulation cKS PBDCs are lower producers of IL-12 and higher producers of IL-10. IL-12 promotes cell-mediated immune responses that are crucial for the detection and elimination of virus-infected and tumor cells [30]. IL-10 is a suppressive cytokine, whose main function appears to be keeping immune responses in check and preventing dangerous inflammation provoked by pathogens. IL-10 achieves this effect by acting on many different types of cells [31]. A perturbation of DC cytokine production characterized by low levels of IL-12 and high levels of IL-10 has been previously reported by us and other authors in patients with neoplasms or chronic viral infections [12,21,32,33]. The finding of a similar cytokine perturbation in our cKS patients helps to confirm the role of cytokines of DC origin in the control of viral infections and of tumor progression.

Our results further indicate that not only PBDCs, but also their circulating precursors, are affected in KS. In fact, CD34+ cells are increased in frequency in the peripheral blood of our patients. On the other hand, circulating monocytes are reduced and share the same properties of cKS PBDCs, in that they express CD91 on a higher proportion of cells than controls, and they are lower producers of IL-12 upon stimulation. According to these findings, the in vitro differentiation of cKS monocytes into DCs produces cells (KSmDC/Cpl) that again share the same alterations of cKS PBDCs. In particular, KSmDC/Cpl are obtained in lower numbers compared with CmoDC/Cpl, express CD91 on a higher proportion of cells, and produce lower levels of IL-12. Therefore, in KS, the differentiation process of DCs appears to be perturbed with the involvement of different cell types lying along the differentiation lineage of DCs. One possibility is that this perturbation is the result of direct...
Figure 5  Characterization of moDCs derived from control monocytes in the presence of cKS plasma (CmoDC/KSpl). (A) The number of CmoDC/KSpl was significantly lower than the number of DCs obtained from control monocytes in the presence of control plasma (CmoDC/Cpl). Monocytes from control subjects were cultured for 5 days in GM-CSF and IL-4, in the presence of human plasma obtained either from cKS patients or from healthy controls. The number of DCs, characterized by typical DC morphology and CD14 down-regulation, was expressed as percentage of the monocytes plated at day 0. Comparison between groups performed with the paired Wilcoxon test. (B and C) CmoDC/KSpl showed a lower allostimulatory activity than CmoDC/Cpl. Allostimulation was assessed in MLR, as BrdU incorporation by allogeneic, monocyte-depleted PBMCs. In panel B, results are expressed as percentage of BrdU+CD4+ lymphocytes, measured by flow cytometry. Comparison performed with the paired Wilcoxon test. In panel C, a representative cytofluorimetric analysis is shown. Control cultures included monocyte-depleted PBMCs alone (left plot), cocultured with CmoDC/Cpl (middle plot), or cocultured with CmoDC/KSpl (right plot). (D) Production of cytokines by moDCs. Similarly to KSmoDC/Cpl, CmoDC/KSpl (shaded bars) produced lower levels of IL-12 and higher levels of IL-6 than CmoDC/Cpl (open bars). According to their impaired allostimulatory activity, CmoDC/KSpl also produced higher levels of IL-10 and TGF-β than CmoDC/Cpl. Control monocytes were cultured for 5 days in GM-CSF and IL-4 in the presence of plasma from cKS patients or healthy controls, and the released cytokines were measured in the supernatants by ELISA. The median of 16 independent experiments is shown, and comparison between groups performed with the paired Wilcoxon test.
infection by HHV-8 of DC precursors. It has been reported that CD34+ cells can be infected by several viruses, including hepatitis C virus (HCV), herpes simplex virus (HSV), and HHV-8 itself [34–36]. In our patients, we observed indeed that the presence of plasmatic HHV-8-DNA, which likely reflects higher levels of viral load, is associated with more marked PBDC alterations. However, we could not find HHV-8-DNA in KSmoDC/Cpl. Another possibility is that the perturbation of DC differentiation in our patients is the result of soluble factors produced by the virus itself, by tumor cells or by the host in response to the viral infection and/or to the presence of the tumor. Several factors may be involved in this process [23,25]. Our results seem to indicate that IL-6 may be a relevant factor in cKS. IL-6 with other inflammatory cytokines are known to play a central role in the reactivation of HHV-8 infection and in the pathogenesis of KS lesions [37]. In vitro, infection with HHV-8 induces IL-6 expression in different cell types [38,39]; in vivo, exacerbations of HHV-8 are associated with increased human IL-6 [40]. According to this, when we analyzed plasmatic IL-6, we found that it was undetectable in all the samples from controls while it was detected, although at low levels, in high proportion of cKS samples. Moreover, patients with detectable IL-6 showed alterations of PBDCs slightly more marked than patients with undetectable cytokine. The ability of IL-6 to inhibit and affect the differentiation of CD34+ cells into DCs has been demonstrated in other tumors, including renal carcinoma, multiple myeloma, and chronic lymphocytic leukemia [36,41,42]. In the present study, we could demonstrate that exogenous IL-6 added during the differentiation culture of control monocytes drives the generation of moDCs (IL6-DCs) with alterations similar to those observed in cKS-DCs, characterized by low cellular yield and up-regulated surface expression of CD91. Noteworthy, the concentration of exogenous IL-6 required to obtain these alterations was much higher than the concentrations measured in cKS plasma. Moreover, the results obtained by differentiation of moDCs in the presence of cKS plasma (CmoDC/KSpl) showed that alterations similar to those observed in cKS-PBDCs and KSmoDC/Cpl were observed in CmoDC/KSpl also when plasma samples with undetectable human IL-6 were used. Because HHV-8 codes for a viral homologue of human

Figure 6 Characterization of moDCs derived from control monocytes in the presence of exogenous IL-6 (IL6-DCs). (A) Monocytes from control subjects were cultured for 5 days in GM-CSF and IL-4 with control human plasma, in the presence of increasing doses of IL-6. Exposure of monocytes to exogenous IL-6 induced a dose-dependent reduction of the number of DCs obtained at the end of the culture. The number of DCs, characterized by typical DC morphology and CD14 down-regulation, was expressed as percentage of the monocytes plated at day 0. Comparison was performed with the paired Wilcoxon test. (B) Exposure of monocytes to exogenous IL-6 induced a dose-dependent up-regulation of surface expression of CD91 on moDCs. Percentages indicate percentage of cells in the upper right quadrant of each plot. One representative of 6 independent experiments is shown.
IL-6 (vIL-6) which shares with the human cytokine many biologic activities [43], it is plausible that HHV-8-derived viral IL-6, likely elevated in the plasma of patients, may be involved in affecting DCs in KS both in vitro and in vivo.

Compared with KSmoDC/Cpl, CmoDC/KSpl showed additional alterations. In particular, they resulted to be more activated and mature than CmoDC/Cpl, as assessed by higher expression of CD80 and CD83, and lower expression of CD1a. Despite their mature immunophenotype, CmoDC/KSpl resulted to be functionally impaired, with lower allostimulatory activity than CmoDC/Cpl. This impaired allostimulatory activity could be related, at least in part, to higher surface expression of the inhibitory molecule IILT3, and to increased production of the immunosuppressive cytokines IL-10 and TGF-β. We have recently demonstrated that these same mechanisms are involved in impaired stimulatory activity of tolerogenic DCs, generated in a different experimental model [15]. All together, results from CmoDC/KSpl may suggest that several factors are probably present in the plasma of cKS patients responsible for the multifaced alterations in DC differentiation and maturation. IL-6, either of human or viral origin, could likely play a part. Other inflammatory cytokines, in particular IL-1β and TNF-α, and VEGF do not seem to be involved. Further studies are required to investigate other candidate molecules.

In conclusion, in this study, we demonstrate that DCs are affected in KS, independently from interfering factors like HIV co-infection. Numerical, immunophenotypic, and functional alterations are present in DCs and their precursors and are at least in part supported by soluble plasmatic factors that may have a causative role in the perturbation of the process of DC differentiation in cKS patients.

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References

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