Impaired Plasmacytoid Dendritic Cell Innate Immune Responses in Patients with Herpes Virus-Associated Acute Retinal Necrosis

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Impaired Plasmacytoid Dendritic Cell Innate Immune Responses in Patients with Herpes Virus-Associated Acute Retinal Necrosis

Nicolai A. Kittan, Antonio Bergua, Sabrina Haupt, Norbert Donhauser, Philipp Schuster, Klaus Korn, Thomas Harrer, and Barbara Schmidt

Plasmacytoid dendritic cells (PDC), the main producers of type I IFNs in the blood, are important for the recognition and control of viral and bacterial infections. Because several viruses induce IFN-α production, severe courses of herpes virus infections in nonimmunocompromised patients may be related to numerical or functional PDC deficits. To evaluate this hypothesis, PBMC and PDC were repeatedly isolated from nine patients with acute retinal necrosis (ARN), caused by herpes simplex or varicella zoster virus. The patients experienced meningitis/encephalitis and frequent infections in childhood (n = 2), recurrent herpes virus infections at unusual localizations (n = 2), ocular surgery (n = 1), infections (n = 4), and stress around ARN (n = 6). The median percentage of isolated PDC was significantly lower in patients compared with 18 age-matched healthy controls (p < 0.001), confirmed by FACS analysis using peripheral blood, and was extremely low during acute disease. PDC counts dropped in five controls suffering from respiratory infections or diarrhea. IFN-α production in PDC and PBMC exposed to different stimuli was significantly lower in patients than in controls (p < 0.05). Anergy to these stimuli was observed on four occasions, in particular during acute disease. PDC of patients showed up-regulated IFN regulatory factor-7 mRNA levels and evidence of in vivo activation (CD80) and maturation (CD83) (p < 0.05). CD8+ cell responses were significantly lower in patients vs controls (p = 0.04). These data support a risk factor model in which numerical and functional deficits in PDC-mediated innate immune responses contribute to an impaired control of latent herpes virus infections and subsequent development of ARN. The Journal of Immunology, 2007, 179: 4219 – 4230.

Acute retinal necrosis (ARN), first described in 1971 (1), is a rare inflammatory necrotic process affecting one or, in some cases, both retinas in immunocompetent as well as immunocompromised patients (2). The patients present with unspecific inflammatory symptoms such as a red eye and ocular pain accompanied by blurred vision. Clinical signs are focal, well-demarcated areas of necrosis in the peripheral retina, rapid centripetal progression, occlusive vasculopathy, and inflammatory responses in the vitreous body and anterior chamber (3). The sequelae are irreversible retinal damage and severely reduced vision or blindness due to necrosis of the retina, which often occurs despite antiviral therapy and vitrectomy with silicon oil instillation. The most frequent causes are varicella zoster virus (VZV), predominantly occurring in elderly individuals, and HSV 1 and 2, associated with a history of encephalitis and meningitis in patients older and younger than 25 years, respectively (4, 5). Triggering events such as periorbital trauma, neurosurgery, and high-dose corticoids have been reported (6).

A pioneering insight into the pathogenesis of ARN was provided by an early animal model in which HSV inoculation into the anterior chamber of rabbits was followed by retinal necrosis of the uninoculated eye (7), later confirmed in mice (8). The virus spreads through synaptically connected nuclei and neurons to the contralateral, but not ipsilateral, optical nerve and retina. In T cell- and NK-cell-depleted mice, however, the virus spreads to both retinas and from the anterior chamber to the ipsilateral retina, respectively, confirming a role for both cells types in the control of virus infection (9–11). Notably, T lymphocyte infiltration of the brain and cytokine production cannot be detected until 1–2 days after virus infection (12). The necrotic process seems to be driven by CD4+ cells, macrophages, polymorphonuclear cells, B cells, and the inflammatory cytokines TNF-α and IFN-γ (13, 14). HSV-1 tegument proteins have been characterized as major targets for T cells within the vitreous fluid (15). In addition, VZV-specific delayed hypersensitivity was absent in a subset of patients with ARN (16).

Recently, plasmacytoid dendritic cells (PDC) have been identified as major producers of type I IFNs in the blood (17, 18). Together with myeloid dendritic cells (MDC), they play a crucial role in innate immune defenses against microbial pathogens (19), including viruses such as HSV (20, 21). Besides a broad antiviral activity, type I IFNs regulate early immune activation toward a cellular-based response,
thus bridging innate and adaptive immunity (22). Furthermore, PDC activate NK cells with subsequent lysis of infected cells (23, 24). PDC are recruited to varicella skin lesions (25) and to human cerebrospinal fluid under neuroinflammatory conditions (26). In addition, their activity in lymph nodes induces strong anti-HSV CTL (27), further emphasizing the role of CD8+ cells in maintaining HSV latency (28, 29). Because it is still unclear why only selected individuals in a large population of HSV- and VZV-seropositive individuals are so severely affected by these viruses, we hypothesized that PDC or related populations such as MDC or NK cells play a role in the onset and pathogenesis of virus-induced ARN.

Materials and Methods

Recruitment of patients and controls

From 1996 to 2004, eight patients were diagnosed with ARN and evidence of HSV-DNA or VZV-DNA in the vitreous body and/or cerebrospinal fluid at the Department of Ophthalmology (University Hospital, Erlangen, Germany). Between November 2004 and April 2006, five of these individuals agreed to participate and were enrolled in our study (further referred to as P01, P03–P05, and P07, see Table I). Four additional patients (P02, P06, P08, P09) suffered from ARN during the observation period. The controls were age-matched healthy volunteers in and around the Institute of Clinical and Molecular Virology (University Hospital, Erlangen, Germany). A questionnaire asked for specific circumstances, in particular infections, preceding or occurring around ARN, signs of acute or recurrent infection with HSV or VZV, and clinical symptoms of other viral infections, e.g., warts, lymphadenopathy, exanthema, seizures. Patients and controls were also asked about bacterial infections (common cold, sinusitis, pneumonia, meningitis, children’s diseases, and response to vaccinations. Information was also collected about the susceptibility of family members to bacterial or viral infections, hereditary or autoimmune diseases, and unclear causes of death. This study was approved by the Ethical Committee of the Medical Faculty, University of Erlangen-Nürnberg (No. 3299), and informed consent was obtained from all participants.

Isolation and stimulation of PBMC and PDC

PBMC were obtained from EDTA-containing blood using standard Ficoll gradient centrifugation (Biochrom). PDC were purified from PBMC in a two-step LS/MS column isolation procedure using the BDCA4 (=CD304) cell isolation kit (Miltenyi Biotec) as described previously (30). Purity of isolated PDC was checked in selected donors using FITC-conjugated Abs against the PDC-specific lectin BDCA2 (31) (Miltenyi Biotec) and anti-CD4-PE (BD Biosciences). The viability of isolated PDC was above 85% as revealed by trypan blue staining. PBMC and PDC were cultured in RPMI 1640 medium containing 10% heat-inactivated (56°C, 60 min) FCS (Invitrogen Life Technologies), supplemented with 50 mg/ml glutamine, 200 U/ml penicillin, 90 U/ml streptomycin, and 20 ng/ml IL-3 (R&D Systems). PDC and PBMC were plated at a density of 10⁴ cells/200 μl and 10⁶ cells/500 μl in 96- and 24-well flat-bottom plates, respectively. PDC were stimulated immediately after isolation and PBMC were stimulated the following day, using UV-irradiated supernatant from a clinical HSV-1 isolate stimulated immediately after isolation and PBMC were stimulated the following day, using UV-irradiated supernatant from a clinical HSV-1 isolate (10⁶ PFU/ml), Cpg-A (ODN 2336, 1 μM; Sigma-Aldrich), a synthetic TLR7 agonist (S-27609, 5 μM; purchased from Coley Pharmaceutical Group), a synthetic TLR7 agonist (S-27609, 5 μM; provided by 3M Pharmaceuticals), and LPS (1 μM; Sigma-Aldrich). PDC were stimulated in triplicates whenever sufficient cells were isolated. PBMC supernatants were harvested at 6 and 12 h after stimulation; cells were stored at −80°C after snap-freezing in liquid nitrogen. PDC supernatants were harvested after 24 h of stimulation and stored at −20°C.

Cytokine assays

PBMC and PDC supernatants were analyzed for IFN-α or IL-2 using an ELISA module set (Bender Medsystems) according to the manufacturer’s recommendations. In general, 10 μl of cell culture supernatants were analyzed unless values above the linear range required further dilution.

Flow cytometry

All FACS determinations were performed on EDTA-anticoagulated blood within 4 h after collection. PDC and MDC counts were determined as described previously (32). In brief, 2 ml of whole blood was washed with Dulbecco’s PBS supplemented with 1% FCS and 0.5 mM EDTA (Sigma-Aldrich), followed by incubation with 100 μl of FC-R blocking reagent (Miltenyi Biotec) at 4°C for 10 min to reduce nonspecific staining. Aliquots (100 μl) of this cell suspension were incubated with a

<table>
<thead>
<tr>
<th>Table</th>
<th>Other primers for the generation of standards and real-time PCR primers for mRNA quantification of the housekeeping gene GUS, IRF7, and CCR7, TLR9, and CXCR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Reference Sequence</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GUS</td>
<td>M15182</td>
</tr>
<tr>
<td>IRF7</td>
<td>U73036</td>
</tr>
<tr>
<td>CCR7</td>
<td>NM_002163</td>
</tr>
<tr>
<td>CXCR3</td>
<td>BC035343</td>
</tr>
</tbody>
</table>

* Reference sequence and Linear Range (copies/reaction) were provided by Izaguirre et al. (Ref. 34).
FITC-conjugated Ab mixture against lineage (lin) markers CD3, CD14, CD16, and CD20, anti-CD4-PE, and anti-CD11c-PE-Cy5. All mAbs were purchased from BD Biosciences except for anti-BDCA2 and anti-BDCA4 (Miltenyi Biotec). The respective mouse IgG Abs were used as isotype controls. After staining for 20 min at 4°C, red cells were lysed in a buffer containing 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA. Pelleted white cells were resuspended in the FACS buffer described above, and 200,000 events were acquired using a three-color FACSCalibur with CellQuest 3.3 software (BD Biosciences). PDC and MDC were discriminated by CD11c expression (Fig. 1a). Confirmatory staining of PDC was performed using anti-BDCA2-FITC, anti-BDCA4-PE, and anti-CD14-PE-Cy5 (Immunotools) (Fig. 1a). CD4+ and CD8+ T cells were identified by staining with anti-CD3-FITC and anti-CD4-PE or anti-CD8-PE. NK cells were stained with anti-CD16-FITC and anti-CD11c-PE (Fig. 1a). Absolute differential white cell counts were provided by the Department of Transfusion Medicine and Hemostaseology (University Hospital, Erlangen, Germany). Cell counts were expressed as percentages (percent of PBMC) and absolute numbers (cells per microliter) by multiplying percentages with fusion Medicine and Hemostaseology (University Hospital, Erlangen, Germany), anti-CD80-FITC (Immunotools), and anti-CD83-FITC (Becton Dickinson). In this approach, PDC were identified as BDCA4-PE-positive. Confirmatory staining of PDC was performed using anti-BDCA2-FITC, anti-BDCA4-PE, and anti-CD14-PE-Cy5 (Immunotools) (Fig. 1a). Up-regulation of CD83 on PDC, which were identified as BDCA4+ cells after subtracting CD11c+CD14+ cells, was evaluated after exposure of PBMC to HSV-1 for 20 h immediately after cell isolation. In this procedure, MDC were identified as described above, whereas PDC staining additionally excluded CD11c+ cells to adjust for BDCA4 up-regulation on stimulated monocytes and MDC (33) (Fig. 1b).

Real-time PCR amplification of mRNAs

RNA was isolated from PBMC using the RNeasy Mini kit (Qiagen). Genomic DNA was removed using QIAshredder and an on-column DNA digestion (Qiagen), followed by incubation of the eluted RNA with RNase-free DNase I (Roche Diagnostics). After ammonium chloride precipitation, RNA was reverse-transcribed using random primers with and without SuperScript II (both Invitrogen Life Technologies) at 25°C for 10 min, 42°C for 50 min, and 75°C for 15 min. A 1/30 dilution of cDNA was subjected to real-time PCR using the ABI Prism 7500 detection system (Applied Biosystems). The 50-μl reaction contained 0.5× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.1× SYBRGreen 1 Nucleic Acid Gel Stain (Invitrogen Life Technologies), and 100 nM of the respective real-time PCR primers (Table I). Except for IFN regulatory factor 7 (IRF7; Ref. 34), real-time PCR primers were searched using Primer Express, version 2.0 (Applied Biosystems). Amplifications were conducted at 50°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis. For the generation of standard curves, larger PCR products from each gene were generated by conventional PCR with outer primers (also Table I) and photometrically quantified after QIAquick PCR purification or QIAquick gel extraction (both Qiagen). All samples were analyzed in triplicates with a coefficient of variation below 30%. Values of the specific genes were normalized with respect to the housekeeping gene β-glucuronidase (GUS) (35).

Serologic testing

Abs for HSV and VZV were tested using the HSV-1/2-IgG/IgM ELISA (DiaSorin) and VZV-IgG/-IgM ELISA (Institut Virion/Serion), respectively. The plasma samples were diluted until values within the linear range of the assay were obtained. Type-specific Abs against HSV-1 and HSV-2 were discriminated using the HerpeSelect 1 and 2 Immunoblot IgG (Focus Diagnostics; distributed by Mikrogen).

Determination of IFN-γ production

PBMC were plated in duplicates in 24-well flat-bottom plates at a density of 1×10^6 cells/500 μl. Cells were immediately stimulated after isolation using the UV-irradiated HSV-1 isolate described above and harvested after 20 h. The IFN-γ production was analyzed using the IFN-γ secretion assay detection kit (PE, human) according to the manufacturer’s recommendations (Miltenyi Biotec). In brief, cells were stained with FITC-conjugated CD4+lin- cells (R2), display of CD4+lin- PBMC on a CD11c/CD4 scatter plot with CD11c- cells identified as PDC (R3) and CD11c+ cells identified as MDC (R4) as described previously (32). BDCA2/BDCA4 scatter plot with gating on double-positive PDC (R5) and CD16/CD161 scatter plot with gating on double-positive NK cells (R6). b. Up-regulation of CD83 on PDC, which were identified as BDCA4+ cells after subtracting CD11c+CD14+ cells. c. Expression of IFN-γ on unstimulated (left panels) and HSV-1-stimulated (right panels) CD4+ and CD8+ T lymphocytes and CD16+ NK cells using a tight lymphocyte gate (R7).
<table>
<thead>
<tr>
<th>No.</th>
<th>Age at ARN (Age at Blood Draw, in Years)</th>
<th>Diagnosis of Herpes Virus Infection</th>
<th>Circumstances around ARN</th>
<th>Clinical Symptoms in Childhood</th>
<th>Herpes Virus Affections in Adulthood</th>
<th>Family Members</th>
<th>Number of Visits</th>
</tr>
</thead>
<tbody>
<tr>
<td>P01</td>
<td>18 (21)</td>
<td>HSV-DNA&lt;sup&gt;b&lt;/sup&gt; (VB&lt;sup&gt;+&lt;/sup&gt;, CSF&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Severe respiratory infection immediately before ARN, treated with antibiotics</td>
<td>Severe meningitis at 7 days (hospitalization for 2 mo); plantar warts, sinusitis, tonsillitis, aphthosis</td>
<td>Frequent HSV affections (one time per month) at unusual localizations (finger, chest), but never herpes labialis</td>
<td>Sister with frequent herpes labialis</td>
<td>4</td>
</tr>
<tr>
<td>P02&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>25</td>
<td>HSV-2-DNA&lt;sup&gt;b&lt;/sup&gt; (VB&lt;sup&gt;+&lt;/sup&gt;, CSF nd)</td>
<td>Uneventful</td>
<td>Aphthosis</td>
<td>Frequent herpes labialis</td>
<td>na</td>
<td>1</td>
</tr>
<tr>
<td>P03</td>
<td>38 (44)</td>
<td>HSV-DNA&lt;sup&gt;b&lt;/sup&gt; (VB&lt;sup&gt;+&lt;/sup&gt;, CSF&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Stress</td>
<td>Severe meningitis at 18 mo (hospitalization for 3 mo); frequent sinusitis and common cold</td>
<td>Pneumonia at 20 years; frequent herpes labialis (one time per month); severe bronchitis at 35 years (local corticoids for 2 years); HSV encephalitis at 37 years</td>
<td>Brother corneal HSV ulcer, sister HSV infection at eyelid and herpes zoster, two other siblings healthy</td>
<td>4</td>
</tr>
<tr>
<td>P04</td>
<td>38 (39)</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Stress, common cold 3 wk prior to ARN</td>
<td>Uneventful</td>
<td>Healthy</td>
<td>Healthy</td>
<td>4</td>
</tr>
<tr>
<td>P05</td>
<td>49 (51)</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Stress, common cold 3 wk prior to ARN</td>
<td>Plantar warts, common cold and sinusitis</td>
<td>Frequent herpes labialis (six times per year), arthritis for many years</td>
<td>Daughter frequent HSV affections at unusual localizations (eyelid, cheek)</td>
<td>4</td>
</tr>
<tr>
<td>P06&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>65</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Weight loss and neck pain 3 wk prior to ARN</td>
<td>Uneventful</td>
<td>Herpes zoster at 20 years</td>
<td>Healthy</td>
<td>4</td>
</tr>
<tr>
<td>P07</td>
<td>70 (73)</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF nd)</td>
<td>Cataract surgery one year prior to ARN</td>
<td>Uneventful</td>
<td>Healthy</td>
<td>Healthy</td>
<td>3</td>
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<tr>
<td>P08&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>72</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF nd)</td>
<td>Pneumonia 1 mo prior to ARN, treated with antibiotics</td>
<td>Uneventful</td>
<td>Healthy</td>
<td>Healthy</td>
<td>2</td>
</tr>
<tr>
<td>P09&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>73</td>
<td>VZV-DNA (VB&lt;sup&gt;+&lt;/sup&gt;, CSF nd)</td>
<td>Uneventful</td>
<td>Uneventful</td>
<td>Healthy</td>
<td>Healthy</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> VB, Vitreous body; CSF, cerebrospinal fluid; nd, not done; na, not available.
<sup>b</sup> Serologic evidence of infection with HSV-1 (P01, P03) and HSV-1/2 (P02).
<sup>‡</sup> These patients suffered from ARN during the observation period.
Abs to CD4, CD8, and CD16, PE-conjugated anti-IFN-γ/H9253, and counterstained with CD14-PE-Cy5. IFN-γ production was evaluated using a tight lymphocyte gate (Fig. 1c).

Statistics

Whenever more than one sample was analyzed from patients or controls, median values were used for statistical calculations. The Mann-Whitney U test was used for comparisons between two independent groups, the Spearman rank correlation coefficient for correlations between continuous variables, and the χ² test for categorical variables. All statistical calculations assumed a two-sided significance at p values ≤0.05.

Results

Characteristics of patients and controls

Five subjects suffered from ARN 0.5–6 years before study enrollment and sampling, four patients (P02, P06, P08, P09) were included with acute disease (Table II). Subjects with ARN due to

Table III. Absolute counts

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Patients</th>
<th>Controls</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4^lin^CD11c^- PDC</td>
<td>5.51 (3.76–6.88)</td>
<td>8.49 (7.53–11.13)</td>
<td>0.007</td>
</tr>
<tr>
<td>CD4^lin^CD11c^- MDC</td>
<td>3.96 (3.48–7.07)</td>
<td>6.75 (5.56–8.42)</td>
<td>0.08 (NS)</td>
</tr>
<tr>
<td>CD16^-CD161^- NK cells</td>
<td>46 (30–53)</td>
<td>39 (22–47)</td>
<td>0.96 (NS)</td>
</tr>
<tr>
<td>CD4^+ T lymphocytes</td>
<td>777 (495–1025)</td>
<td>932 (773–1110)</td>
<td>0.21 (NS)</td>
</tr>
<tr>
<td>CD8^- T lymphocytes</td>
<td>504 (317–694)</td>
<td>710 (517–852)</td>
<td>0.12 (NS)</td>
</tr>
<tr>
<td>Total lymphocytes</td>
<td>2370 (1750–2500)</td>
<td>2280 (1960–2690)</td>
<td>0.34 (NS)</td>
</tr>
<tr>
<td>Total granulocytes</td>
<td>3870 (3460–4840)</td>
<td>3720 (2540–4360)</td>
<td>0.33 (NS)</td>
</tr>
</tbody>
</table>

a Absolute counts of PDC, MDC, CD16^-CD161^- NK cells, CD4^+ and CD8^- T lymphocytes as well as total lymphocyte and granulocyte counts in patients and controls. NS, Not significant.
HSV (P01, P02, P03) were younger than those with VZV-associated retinopathy (P04–P09). Personal interviews revealed specific circumstances around ARN in six patients, namely stress (P03, P04, P05), severe respiratory infection treated by antibiotics (P01, P08), common cold (P05), weight loss, and neck pain (P06). Four patients also had a medical history of severe and/or recurrent infections. These included severe meningitis/encephalitis in early childhood (P01, P03), frequent respiratory infections in childhood and recurrent herpes simplex...

**FIGURE 3.** Comparison of IFN-α production in PDC (a) and PBMC (b) obtained from patients and healthy controls. PDC and PBMC were exposed to different stimuli for 24 and 12 h, respectively. Dots represent values from individual subjects, box plots indicate median and interquartile ranges, whiskers show 10th and 90th percentiles. Values of p were obtained using the Mann-Whitney U test. Mock unstimulated cells; n.s., not significant.

**FIGURE 4.** Cell-type analyses in the peripheral blood and vitreous body of one patient with acute VZV-associated retinopathy (P08) (a) and another patient with subacute HSV-2-associated ARN (P02) (b). Forward/side scatter plots with gating on mononuclear cells (R1) (left panels), CD4/CD8 scatter plots (middle left panels), CD4/lin/CD11c scatter plots with gating on PDC (R3) and MDC (R4) (middle right panels), and CD19/CD20 scatter plots (right panels). Influx of NK cells into the vitreous body was not observed in any of the patients (data not shown).
infections in adulthood (P01, P02, P03, P05), herpes simplex encephalitis (P03) (36), and herpes zoster at a young age (P06) (Table II). In the remaining two patients (P07, P09), there was no apparent trigger for ARN and the medical history was uneventful with respect to infectious diseases; the only notable event was cataract surgery 1 year before ARN in patient P07. Three patients (P01, P03, P05) reported recurrent HSV infections in family members, in two cases at unusual localizations (eyelid, cheek).

None of the 18 controls reported serious infections similar to the patients, and only 3 controls (C03, C04, C017) reported recurrent herpes labialis, albeit at a low frequency (once or twice a year). Infrequent herpes labialis among family members was reported by three patients and controls were matched for age (median, 51 years vs 42 years; range, 21–73 years vs 25–74 years; interquartile range, 39–70 years vs 29–58 years; p = 0.61, NS) and gender (percentage of females, 33 vs 44%; p = 0.92).

Numerical PDC deficit in patients with ARN
BDCA4+ cells were isolated in 19 and 56 separate experiments from patients and controls, respectively. Patients were examined on 2–3 occasions, controls on 1–10 occasions. For all subjects with three or more isolation procedures (n = 12), the median coefficient of variation was 25.4% (IQR, 20.1–36.1%), similarly observed in patients and controls. The purity of isolated cells, determined as median percentage of BDCA2+ CD4+ cells in six controls, was 97.1% (IQR, 95.9–97.4%). On 14 of 19 patient visits, percentages of isolated BDCA4+ cells were below 0.20%, compared with only 1 of 56 visits in controls (p < 0.001) (Fig. 2a). Percentages of isolated BDCA4+ cells were significantly lower in patients compared with controls (median, 0.15 vs 0.32%; IQR, 0.11–0.19% vs 0.25–0.40%; p < 0.001). When controls suffered from bacterial or viral infections, e.g., common cold, diarrhea, cough, and sore throat, percentages of isolated BDCA4+ cells dropped below the lowest value observed when they were healthy on 5 of 6 occasions. FACS analyses of the peripheral blood were performed in parallel to the isolation of BDCA4+ cells on 39 occasions; they revealed a significant correlation of CD4+lin−CD11c− cells and isolated BDCA4+ cells (r = 0.54, p < 0.001) (Fig. 2b). Only one patient (P03) had consistently higher percentages of CD4+lin−CD11c− cells compared with isolated BDCA4+ cells. The percentage of isolated BDCA4+ cells significantly correlated with the percentage of BDCA2+CD4+CD14+ cells obtained by FACS analyses of the peripheral blood (n = 14; r = 0.42; p = 0.005). The latter also significantly correlated with the percentage of CD4+lin−CD11c− cells (n = 22; r2 = 0.75; p < 0.001) (data not shown).

Percentages of CD4+lin−CD11c− cells in the peripheral blood were significantly lower in patients vs controls (median, 0.23 vs 0.43%; IQR, 0.19–0.32% vs 0.31–0.55%; p = 0.005), respectively (Fig. 2c). This was also true for the percentages of BDCA2+CD4+CD14+ cells (median, 0.20 vs 0.49%; IQR, 0.17–0.29% vs 0.34–0.62%; p = 0.014; Fig. 2d) and CD4+lin−CD11c− MDC (median, 0.20 vs 0.32%; IQR, 0.17–0.24% vs 0.24–0.39%; p = 0.03, Fig. 2e). In contrast, no significant differences were found for CD16+CD161+ NK cells (p = 0.56, Fig. 2f), CD3+CD4+ T
lymphocytes \( (p = 0.59, \text{Fig. 2g}) \), and CD3\(^+\)CD8\(^+\) T lymphocytes \( (p = 0.56, \text{Fig. 2h}) \). These data were confirmed using absolute cell counts (for details see Table III), except for CD4\(^+\)lin\(^-\)CD11c\(^+\)MDC \( (p = 0.08, \text{NS}) \). In addition, no significant differences were found for absolute lymphocyte and granulocyte counts between patients and controls (Table III).

**Functional abnormalities in IFN-\(\alpha\) production in patients with ARN**

The intra- and interassay variability of the IFN-\(\alpha\) ELISA was determined using a low (520 pg/ml) and a high positive control (4222 pg/ml). Respective coefficients of variation were 4.9 and 7.1\% in quadruplicate analyses and 27.0 and 23.0\% in seven consecutive runs. IFN-\(\alpha\) production was determined for PDC and PBMC obtained from patients and controls in 19 and 20 as well as in 16 and 26 separate experiments, respectively. IFN-\(\alpha\) production was significantly lower in patients compared with controls after exposure of PDC to HSV-1 \( (p = 0.009) \), CpG-A \( (p = 0.016) \), and the TLR7 agonist \( (p = 0.001) \) (Fig. 3a). Significant differences were also observed after exposure of PBMC to these stimuli for 6 h (data not shown) and 12 h \( (p = 0.005, p = 0.01, \text{and } p = 0.008) \) (Fig. 3b). No IFN-\(\alpha\) production was noticed when PBMC were exposed to LPS, consistent with the finding that PDC do not express TLR4 (38). On four occasions, cells isolated from patient P01, P08, and P09 did not respond to any of the stimuli with IFN-\(\alpha\) levels above 100 pg/ml.

**PDC depletion and functional anergy in acute VZV-associated retinopathy**

Four patients were admitted to the Department of Ophthalmology after suffering from blurred vision for one to several days. Patients P06 and P08 underwent vitrectomy within 1 day of admission and diagnosis was confirmed by detection of VZV DNA in the vitreous body. FACS analyses of peripheral blood and vitreous humor in both patients showed undetectable to very low PDC counts in both compartments and an influx of CD8\(^+\) T lymphocytes into the vitreous body (Fig. 4a). PDC and PBMC of both patients were anergic toward all stimuli at this occasion (data not shown). Patient P09 received systemic corticoids for 10 days before the diagnosis of VZV-associated ARN was finally confirmed by surgery, which may explain the relatively high PDC count on this occasion. After corticoid therapy was stopped, the PDC count decreased considerably (Fig. 2a). Patient P02 was treated with oral aciclovir for 6 wk because peripheral necrosis without infiltration of the vitreous body did not require immediate surgery. After deterioration of the clinical situation, the patient finally received a diagnostic vitrectomy, revealing the presence of HSV-2 DNA. FACS analysis of this subacute infection no longer revealed an influx of CD8\(^+\) cells but PDC and B cells, suggesting a Th2-type reaction.

**Elevated levels of IRF7 mRNA in patients with ARN**

To ensure sufficient representation of different RNA transcripts, we excluded samples with \(<100 \text{ copies/reaction of the housekeeping gene GUS}. \) This procedure left 15 samples from 9 patients and 15 samples from 15 controls for subsequent analysis. GUS levels were comparable between patients and controls (copies per reaction; median, 1645 vs 1168; IQR, 1040–1722 vs 783–2073; \( p = 0.55 \)). The mean percentage of contaminating genomic DNA was 0.35\%; 67 of 74 samples had genomic DNA levels below 1\%. We studied five cellular transcripts which we suspected to be involved in the pathogenesis of ARN. In this respect, IRF7 has been characterized.
as key molecule in the induction of IFN production and is constitutively expressed in PDC (34). IRF8 was selected because knockout mice have a lower percentage of PDC compared with wild-type mice (39); CCR7 was selected because it is up-regulated upon PDC stimulation and promotes migration to secondary lymphatic tissue (40). TLR9 was selected because reduced TLR9 transcripts were found in PDC (34). IRF8 was selected because knockout of IRF8 is lethal (39); CCR7 was selected because it is up-regulated upon PDC stimulation (40). Moreover, the migratory capacity of PDC was not significantly different between patients and controls, as evident from the expression of CXCR3 (p = 0.28, Fig. 6d).

No evidence of impaired humoral immunity in patients with ARN

To evaluate humoral immune responses, HSV and VZV IgG and IgM Abs were determined in the plasma of patients and controls. IgM Abs were not detected in any of the control and patient plasma samples, in particular not during the acute episodes of ARN. These data indicate that ARN resulted from herpes viral reactivation and not primary infection in all patients of our study. All patients and controls were seropositive for VZV, whereas all nine patients and 11 of 18 controls were seropositive for HSV. The levels of HSV-IgG and VZV-IgG were not significantly different between patients and controls (p = 0.54 and p = 0.98, respectively; Fig. 7a). The discrimination of HSV-1 and HSV-2 Abs revealed seropositivity for HSV-1 in P01 and P03 and seropositivity for HSV-1 and HSV-2 in P02.

Impaired CD8⁺ cell responses in patients with ARN

To evaluate cellular adaptive immune responses, PBMC of patients (n = 9) and HSV-seropositive controls (n = 11) were exposed to HSV-1 for 20 h and then analyzed for IFN-γ production using FACS analysis. The increase in IFN-γ production in stimulated vs unstimulated cells from patients compared with controls was considerably lower in CD4⁺ T lymphocytes and CD16⁺ NK cells, and significantly reduced in CD8⁺ CTLs of patients (median, 0.22 vs 0.65%; IQR, 0.07–0.41% vs 0.41–1.34%; p = 0.04; Fig. 7b). The stimulation of an HSV-seronegative donor did not
result in IFN-γ expression, indicating that the activity in HSV-seropositive donors was stimulus specific (data not shown).

**Other immunologic tests**

None of the patients exhibited antinuclear Abs on Hep2 cells. One patient (P04) showed a weak reactivity in a *Borrelia* spp. IgG-ELISA, which however could not be confirmed by Western blotting.

**Discussion**

Our data strongly suggest that low PDC counts are associated with severe recurrent herpes virus infections, as evident from significantly lower percentages of isolated BDCA4⁺ cells (Fig. 2a), CD4⁺ lin⁻ CD11c⁻ cells (Fig. 2c), and BDCA2⁺ BDCA4⁺ CD14⁻ cells in the peripheral blood (Fig. 2d) in patients with ARN compared with healthy controls. Notably, the PDC counts of our control group were very similar to recently published data (32). Although limited by the small number of patients with ARN, we could also draw some conclusions on PDC dynamics. Thus, PDC counts showed substantial fluctuations over time in ARN patients as well as in controls. A particularly low percentage of PDC was observed in patients tested during acute disease who had not received systemic corticoid therapy. Notably, low PDC values were observed among the controls especially during episodes of acute infections and with one exception the PDC counts observed during these episodes were the lowest found in the respective individuals (Fig. 2a). Similar data have recently been published for acute dengue virus infection, in which an early decline of circulating PDC was predictive of severe disease (42). Whether low numbers of circulating PDC are predictive of herpes virus-associated ARN or just an associated symptom among others remains to be determined. Additional studies are required to clarify whether low PDC counts occur in bacterial and immunologic uveitis as well.

IFN-α production in response to different stimuli was also reduced in patients compared with controls, both after stimulation of PBMC and PDC (Fig. 3). However, there was no clear functional defect as we originally suspected after complete anergy had been found in the first sample from P01, because all patients with anergic PBMC/PDC on one occasion showed IFN-α production on another occasion. The overall reduced IFN-α production, and in particular the intermittent anergy, may play an important role in the pathogenesis of ARN, because a major role of type I, but not type II IFNs in limiting HSV replication in the cornea and nervous system was shown using mice with knockout mutations in IFN receptors (43).

Stimulation of PDC causes an activation of a downstream signaling cascade resulting in nuclear translocation of NF-κB and type I IFN secretion (44). IRF7 mRNA, which is constitutively expressed in PDC (34), was found significantly elevated in unstimulated patient vs control PBMC (Fig. 5b), which may be interpreted as evidence of a stimulated TLR/IFN pathway in vivo. In this respect, self- and cross-hyporesponsiveness have been reported after TLR stimulation (45–47), suggesting that the intermittent anergy, which we observed in patient PBMC and PDC in vitro, may have been caused by preceding PDC stimulation in vivo. In particular, maturation of PDC has been associated with a reduced capacity of IFN-α production (30). This hypothesis is supported by significantly higher expression of activation and maturation markers on patient PDC (Fig. 6a). PDC stimulation is followed by their homing to lymphatic tissue (40), where they serve as APCs (31). The depletion of CCR7-expressing PDC may explain why neither elevated mRNA levels (Fig. 5d) nor enhanced expression of CCR7 (Fig. 6a) were found in patient PBMC and PDC, respectively.

It remains unclear what caused the activation of PDC in our patients. It is tempting to speculate that the trigger was an ongoing subclinical herpes virus infection, which was not adequately controlled by the adaptive immune system of the patients. Our data clearly show no evidence of impaired humoral immunity (Fig. 7a); Ag presentation by PDC and MDC seems to work sufficiently, as is evident from the up-regulation of costimulatory CD80 and CD83 after exposure to HSV-1 (Fig. 6b, and data not shown). In contrast, significantly lower CD8⁺ T lymphocyte IFN-γ production upon HSV-1 exposure was found in patients compared with controls (Fig. 7b), indicating an impaired cellular immune control of HSV infections. In this respect, the important role of CD8⁺ cells in controlling and maintaining HSV-1 latency has been reported (28, 29). However, alternatively, the low PDC counts in our studies could impair an effect of priming CTLs or NK cells, which has recently been described by others (23, 24). Following this hypothesis, elevated IRF7 levels and increased surface expression of CD80 and CD83 may serve as compensatory mechanism for low PDC counts.

A detailed insight into the pathogenesis of ARN was provided by animal models (7, 8). The neuronal spread of HSV inoculated into the anterior chamber was followed by an infiltration of T lymphocytes (12), which, in concert with other cells and inflammatory cytokines, triggered the elimination of virus-infected cells (13, 14). We observed a similar influx of CD8⁺ T cells into the vitreous body in the two cases of acute VZV-associated ARN (Fig. 4a). The speed of T cell recruitment to the site of infection and proper NK cell function seem to be crucial for limiting virus spread (10, 11), suggesting that low PDC and low NK cell counts may have contributed to the occurrence of ARN in three patients with very low NK cell counts (P01, P06, P09). In this respect, it may be important to study IRF8 knockout mice as a model for HSV reactivation, which have lower PDC counts compared with wild-type mice (48). In the patient with subacute HSV-2-associated ARN (P02), the CD8⁺ cell influx was replaced by an influx of B cells and PDC (Fig. 4b), suggesting a role for different cell populations in the course of ARN (13).

Two patients (P01, P02) reported severe meningitis and/or encephalitis in early childhood (Table II), reminiscent of perinatal HSV-2 infections which were followed by immediate or delayed necrotic retinitis (49, 50). HSV-1 encephalitis preceding ARN was described by our group (36) and others (51–54). Elevated PDC counts in cerebrospinal fluid were detected in neuroinflammatory conditions, suggesting that PDC may contribute to the orchestration of local immune responses in this immune-privileged compartment (26). Our data show lack of PDC infiltration in acute episodes of ARN (Fig. 4a), which may indicate a defective innate immune response contributing to the severity of ARN. However, our data provide evidence for the presence of PDC in vitreous humor in advanced stages of ARN (Fig. 4b).

In conclusion, our data support a multifactorial model for the development of ARN. Impaired control of latent HSV and/or VZV infection due to low PDC counts and function as well as reduced CD8⁺ cell activity may lead to severe herpes virus reactivation like ARN. This finding would be most apparent if acute events like bacterial or viral infections, stress, ocular trauma, or corticosteroid treatment as well as factors in the personal histories of patients, e.g., (neonatal) herpes virus meningitis or encephalitis, accumulate. This risk factor model is supported by evidence of other groups (4–6, 55). The family histories of the patients in our study point to a—presumably genetically determined—defect in the control of latent HSV and/or VZV infection. Notably, a case of HSV-2-associated ARN was described in a patient with systemic lupus erythematosus (56). In this disease, PDC are depleted from...
the peripheral blood into skin lesions (57), similar to PDC depletions to varicella skin lesions (25). Systemic lupus erythematosus patients show a characteristic granulocyte expansion in the peripheral blood (58). This signature was found on 4 of 21 occasions in patients (see Fig. 4b), but on 4 of 32 occasions in controls as well (p = 0.80). Altogether, naturally low numbers of circulating PDC or induced migration of these cells from the peripheral blood to lymphatic tissue or skin may contribute to an impaired first line of defense against infection and reactivation of latent viruses, as we suggest for herpes virus-associated ARN.

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Disclosures

The authors have no financial conflict of interest.

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