

# Disturbed Patterns of Immunocompetent Cells in Usual-Type Vulvar Intraepithelial Neoplasia

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## Abstract

**Genital infection with human papillomavirus (HPV) is usually transient, as the immune system is capable of eliminating the virus. When immunity “fails” and the infection persists, vulvar intraepithelial neoplasia (VIN) may develop. In this study, we examined the distribution of inflammatory cells in 51 patients with HPV-associated usual-type VIN and in 19 healthy controls. Frozen vulvar tissue samples were tested for the presence of HPV-DNA, and immunohistochemical staining for the markers CD1a, CD207, CD208, CD123/CD11c, CD94, CD4, CD8, and CD25/HLA-DR was performed. Cells were counted in both the epidermis and dermis over at least 2 mm of basal membrane length. In the epidermis of VIN patients, CD1a<sup>+</sup> and CD207<sup>+</sup> (Langerin) dendritic cells (DC) and CD8<sup>+</sup> T cells were significantly lower than in controls, whereas the number of CD123<sup>+</sup>/CD11c<sup>-</sup> plasmacytoid DCs (pDC) was significantly increased. No significant changes were observed for CD208<sup>+</sup> DCs, CD94<sup>+</sup> natural killer (NK) cells, CD4<sup>+</sup> T cells, and CD25<sup>+</sup>/HLA-DR<sup>+</sup> regulatory T cells. In the dermis of VIN patients, elevated numbers of CD208<sup>+</sup>, CD123<sup>+</sup>/CD11c<sup>-</sup>, CD94<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD25<sup>+</sup>/HLA-DR<sup>+</sup> cells were observed when compared with healthy controls. The numbers of CD1a<sup>+</sup> and CD207<sup>+</sup> DCs were not different between groups. In summary, high-risk HPV-related usual-type VIN lesions are characterized by an immunosuppressive state in the epidermis, showing a reduction of immature myeloid DCs (mDC) and CD8<sup>+</sup> T cells. In the dermis, inflammatory activation is reflected by the influx of mature mDCs and pDCs, NK cells, and T cells, suggesting that the cellular immune response on viral HPV infection occurs in the dermis of VIN patients. [Cancer Res 2008;68(16):6617–22]**

## Introduction

Genital infection with human papillomavirus (HPV) is very common, especially among sexually active young adults. The lifetime risk of becoming infected with HPV is estimated at 80% to 85% (1). Most infections proceed asymptotically and cure spontaneously as the immune system is capable of eliminating the virus (2). Persistence of HPV infection, on the other hand, can result in neoplastic changes of the anogenital tract, presented in this study as vulvar intraepithelial neoplasia (VIN).

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There is evidence that cell-mediated immune responses of the host are important determinants in the course of infection, illustrated by an increased incidence of HPV-induced diseases in T-cell immunodeficient individuals (3). The immune response to invading HPV is regulated by cells of both the innate and adaptive immune systems. During the innate immune response, viral antigens are recognized, bound, and processed by antigen-presenting cells with dendritic cells (DC) as important representatives. DCs can be divided into immature or mature myeloid DCs (mDC) and plasmacytoid DCs (pDC). mDCs play a major role in the regulation of antibacterial and antifungal responses, whereas pDCs are receptive for viral interactions. pDCs produce in response large amounts of IFN- $\alpha$  that has direct antiviral effects and stimulates cytotoxicity of macrophages and natural killer (NK) cells. Influenced by NK cells, DCs mature and transport “processed” antigens to secondary lymphoid organs, where naive T cells are primed to mature into CD4<sup>+</sup> T-helper cells, CD8<sup>+</sup> cytotoxic T cells, or regulatory T cells (Treg cells), all functional members of the adaptive immune response (4, 5).

Studies describing the distribution of immunocompetent cells in VIN lesions are scarce (6–11). Limitations of these studies include absence of or inappropriate control groups, an undefined HPV status of samples, or incompletely described qualitative or semiquantitative cell counting. Thus far, only a few cell markers have been analyzed. Detailed insight into the influence of VIN on the distribution of immunocompetent cells in vulvar skin might be helpful to explain clinical and immunologic changes observed after treatment of VIN with topical imiquimod (12).

To examine the role of the innate and cellular local immune responses in patients with high-risk HPV (hrHPV)-associated VIN, we investigated in this study the presence of selected DCs, NKs, and T cells by immunohistochemical staining in the vulvar skin of 51 patients with usual-type VIN and compared results with data obtained from 19 healthy controls. Our results suggest a disturbed distribution pattern of immunocompetent cells in VIN-affected skin.

## Materials and Methods

### Study Population

Fifty-one immunocompetent women (median age, 43 y; range, 22–71 y) with clinically and histologically proven multifocal usual-type VIN participated in this study. On the average, patients were diagnosed as having VIN 5.3 y before enrollment in our study (range, 1 mo to 20 y). In all cases, the histologic diagnosis was confirmed at study entrance. Thirty-seven of these 51 patients had undergone previous treatment at least 3 mo before enrollment (surgical excision, laser therapy, and local chemotherapy), whereas 14 had not been treated before. Nineteen healthy women undergoing elective vulvar cosmetic surgery (reduction of labia minora) served as healthy controls (median age, 40 y; range, 19–56 y). Histologic examination of the removed vulvar tissue revealed no abnormalities.

The Medical Ethical Committees approved our study design and all women voluntarily gave written informed consent.

### Clinical Material

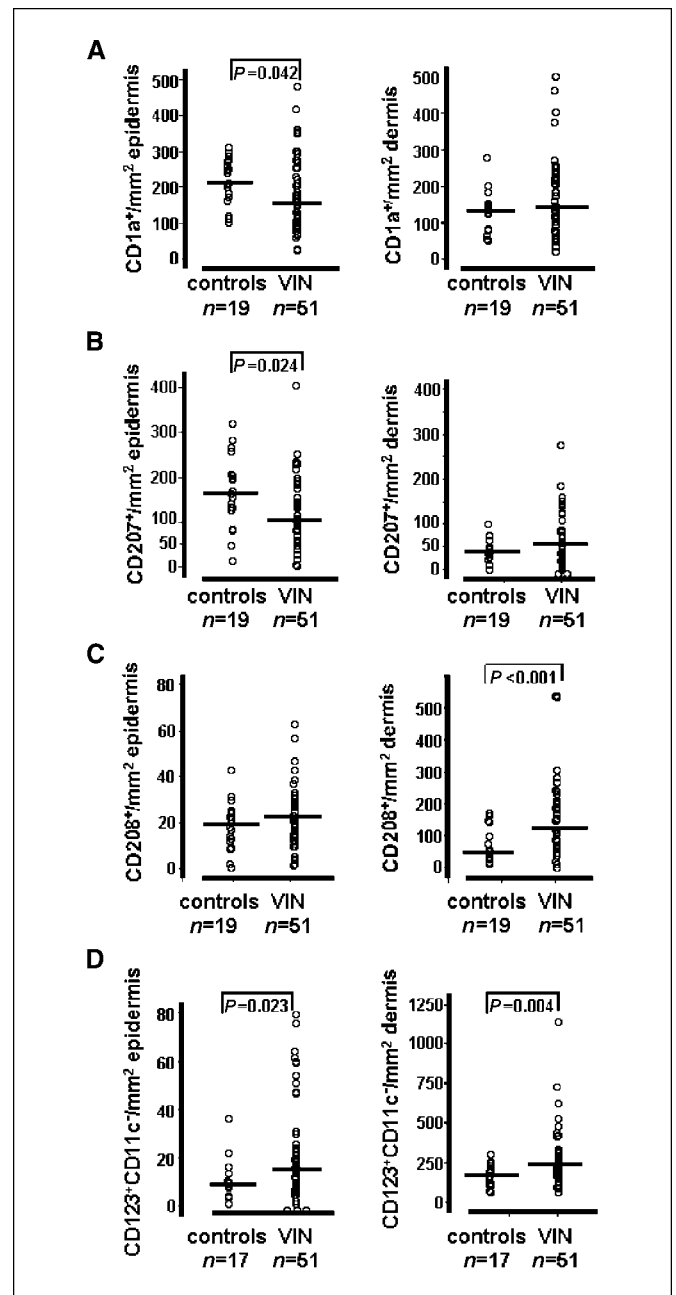
Punch biopsies (4 mm) taken from patients with usual-type VIN and from excised vulvar tissue in healthy controls were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis. Anatomically, biopsies were taken from the same tissue as used for histologic diagnosis.

**HPV-DNA testing.** Frozen tissue samples were analyzed for the presence of HPV-DNA by using a standard GP5<sup>+</sup>/6<sup>+</sup> PCR enzyme immunoassay followed by reverse line blot analysis, as described previously (13).

**Immunohistochemical staining.** Frozen tissue specimens were cut into serial 6- $\mu\text{m}$ -thick sections on a Micronic, Adamas cryostat, transferred to poly-L-lysine-coated microscope slides (Menzel-Glaser, Omnilabo), dried, and restored at  $-80^{\circ}\text{C}$ . The following markers and their primary antibodies were selected for immunohistochemical staining: CD1a, classic marker for immature mDCs, in the skin known as Langerhans cells (Okt-6 Orthoclone, Ortho Biotech); CD207, marker for immature mDCs expressing Langerin (DCGM4; Beckman Coulter); CD208, DC-Lamp, marker for mature mDCs (104.G4; Beckman Coulter); CD94, marker for NKs (HP.3b1; Beckman Coulter); CD4, marker for T-helper cells (MT.310; Dako); CD8, marker for cytotoxic T cells (DK25; Dako); CD25/HLA-DR, marker for Treg cells (AKT-1; Dako/1E5; Sanquin); and Foxp3, marker for Treg cells (PHC 101; Bioscience). For pDCs, characterized by the presence of CD123 and the absence of CD11c, antibodies for both markers were used [anti-CD123 (9F5; Becton Dickinson) and anti-CD11c (SHCL-3; Becton Dickinson)]. Antibodies in staining procedures were applied in optimal concentrations varying from 0.1 to 1.0  $\mu\text{g}/\text{mL}$ .

**Single staining (CD1a, CD207, CD208, CD94, CD4, CD8, and Foxp3).** Sections were defroze, fixed in acetone for 10 min, and rinsed with PBS (pH 7.8) for 5 min. The staining procedure was then continued in a half automatic stainer (Sequenza, Shandon Scientific), where the slides were incubated with 10% normal goat serum (NGS; Sanquin) for 10 min and subsequently for 60 min with mouse anti-human antibodies against CD1a, CD207, CD208, CD94, CD4, and CD8, respectively, and rat anti-human antibody against Foxp3. All antibodies had been diluted in 1% block buffer (blocking reagent in PBS; Roche Diagnostics GmbH). During the whole staining process, incubation steps were always followed by rinsing with PBS for 5 min. All following incubations with antibodies were in the presence of 10% normal human serum (NHS; Sanquin). After incubation with primary antibodies, sections were rinsed and, with the exception of Foxp3, incubated with biotinylated goat anti-mouse antibodies as secondary antibodies (BioGenex HK325-UM; Klinipath) for 30 min followed by incubation with alkaline phosphatase-conjugated streptavidin (BioGenex HK321-UK; Klinipath) for another 30 min. In the case of Foxp3, the secondary antibody was alkaline phosphatase-conjugated goat anti-rat antibody; this incubation was followed by incubation with rat alkaline phosphatase anti-alkaline phosphatase (Dako). Slides were rinsed with both PBS and substrate Tris buffer (0.1 mol/L Tris-HCl, pH 8.5) and then incubated for 30 min with a new fuchsin substrate (Chroma). Finally, the sections were washed again, counterstained with Gill's hematoxylin (Merck) for 30 s, rinsed with tap water, dried, and embedded in VectaMount (Vector). Control staining was performed according to the same procedure using isotype controls.

**Double staining CD25/HLA-DR and CD123/CD11c.** After fixation in acetone and washing with PBS, endogenous peroxidase was blocked with 0.1% sodium azide and 0.03% hydrogen peroxide in PBS for 30 min. Sections were rinsed and incubated with 10% NGS and 10% normal rabbit serum (Sanquin) followed by incubation with mouse anti-human antibodies against CD25 for 60 min at room temperature. The sections were then rinsed, incubated with biotinylated goat anti-mouse antibodies and 10% NHS for 30 min, rinsed, incubated with alkaline phosphatase-conjugated streptavidin and 10% NHS for 30 min, and rinsed again. Thereafter, the slides were incubated with 10% normal mouse serum (Sanquin) for 10 min followed by FITC-conjugated mouse anti-human antibodies against HLA-DR for 60 min. Rinsed again, incubation with horseradish peroxidase-conjugated rabbit anti-FITC antibodies followed. After rinsing with PBS and substrate Tris buffer, slides were incubated for 30 min in Fast Blue substrate



**Figure 1.** CD1a<sup>+</sup>, CD207<sup>+</sup>, CD208<sup>+</sup>, and CD123<sup>+</sup>/CD11c<sup>-</sup> DCs in the epidermis and dermis of VIN lesions and normal vulvar skin. Horizontal lines, median values.

(Sigma). Finally, sections were rinsed and incubated with peroxidase Nova Red substrate (Vector) for 10 min, rinsed with PBS, and embedded in VectaMount.

In a similar procedure as described above for double staining CD25/HLA-DR, sections were incubated with 10% NGS. Primary antibodies were substituted with mouse anti-human CD11c antibodies and with phycoerythrin-labeled mouse anti-human CD123 antibodies. Secondary antibodies were biotinylated goat anti-mouse antibodies with alkaline phosphatase-conjugated streptavidin for CD11c and rabbit anti-phycoerythrin (AbD Serotec) and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) for CD123. Staining was performed with Fast Blue substrate for CD123 and with aminoethylcarbazol substrate (Sigma) for CD11c.

**Light microscopic evaluation.** Light microscopic evaluation was performed in a blinded session. Stained cells were counted throughout

the entire epidermal thickness and 100  $\mu\text{m}$  deep into the dermis of each biopsy specimen following at least 2 mm of basal membrane length (range, 2–5 mm). After measuring the total area of both the epidermis and dermis by using the Leica Image Analysis System, the number of cells per square millimeter was calculated for each layer separately.

### Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences 15.0 software for Windows.

Preliminary, Kolmogorov-Smirnov tests showed a nonnormal distribution for some cell types. Accordingly, the nonparametric Mann-Whitney test was used for evaluation of differences in cell counts between two independent groups (VIN patients versus healthy controls). The possible influence of different previous treatments on cell counts in VIN patients was investigated by means of the nonparametric Kruskal-Wallis test. Spearman's correlations were used to investigate possible relations between cell counts and duration of the disease or age of the patients. A two-tailed  $P = 0.05$  was chosen to represent statistical significance.

## Results

### Patients

Spearman's correlations between cell counts for all investigated cell types and the duration of VIN or age of the patients at study entrance were not significant (data not shown). There was also no statistically significant difference in cell counts between previously untreated patients ( $n = 14$ ), patients treated with surgical excision ( $n = 18$ ), or patients undergoing laser treatment ( $n = 12$ ). Groups for other treatment modalities ( $n = 7$ ) were too small for statistical evaluation.

### HPV-DNA Testing

Forty-nine of 51 patients tested positive for hrHPV-DNA. HPV types detected were HPV-16 ( $n = 40$ ), HPV-33 ( $n = 8$ ), and HPV-18 ( $n = 1$ ). All healthy controls were HPV-DNA negative.

### Analysis of Inflammatory Cells in VIN Lesions and Normal Vulvar Skin

**Dendritic cells.** Immunohistochemical analysis of epidermis and dermis showed that the majority of immature mDCs were

located in the epidermis. Staining for CD1a and CD207 identified immature DCs that spread over the whole epidermis, including the basal layer. There were no DCs in the superficial layers of the epidermis. In the dermis, the majority of DCs were situated in focal infiltrates. Compared with healthy controls, a significant decrease in CD1a<sup>+</sup> and CD207<sup>+</sup> cells in the epidermis of VIN patients was observed, but in the dermis, the numbers of these mDCs were not different for the two groups. Data are summarized in Fig. 1A and B. The number of mature CD208<sup>+</sup> cells in epidermis was low compared with the numbers of immature mDCs. In the epidermis, there was no difference in numbers between healthy controls and VIN-affected skin, but at least twice as many cells were observed in the dermis of VIN patients when compared with controls (Fig. 1C; Table 1).

CD123<sup>+</sup> pDCs were found evenly distributed through the whole thickness of both epidermis and dermis. The numbers were significantly increased in VIN-affected skin when compared with controls (Fig. 1D).

**NK cells.** CD94<sup>+</sup> NK cells were sporadically found in the epidermis, preferentially in the basal layer just above the basal membrane. In the dermis, NK cells were situated mainly in infiltrates. The number of CD94<sup>+</sup> NK cells was not different in the epidermis but was more than doubled in the dermis of VIN-patients when compared with healthy women (Fig. 2).

**T cells.** Predominantly, CD4<sup>+</sup> T cells were located in the dermis just beneath the basal membrane. In the epidermis, the numbers did not differ between VIN patients and healthy controls, but in VIN-affected dermis, the numbers were significantly increased when compared with healthy skin (Fig. 3A). There were significantly less CD8<sup>+</sup> cells in the epidermis and more CD8<sup>+</sup> cells in the dermis of VIN-affected skin than in healthy controls (Fig. 3B).

Treg cells were analyzed by a staining procedure targeting CD25 and HLA-DR expression. The number of dermal CD25<sup>+</sup>/HLA-DR<sup>+</sup> cells was higher in VIN patients than in controls. In the epidermis, no differences between patients and healthy controls were observed (Fig. 3C). The results for CD25<sup>+</sup>/HLA-DR<sup>+</sup> Treg cells were controlled in 20 biopsies by staining sequential sections with Treg

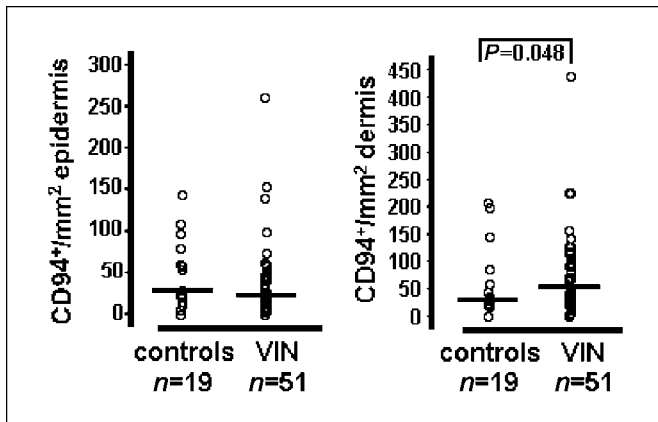
**Table 1.** Immunocompetent cells in the epidermis and dermis of VIN lesions compared with samples from healthy women

	Epidermis			Dermis		
	All patients ( $n = 51$ ), median (range)	Controls ( $n = 19$ ), median (range)	$P$	All patients ( $n = 51$ ), median (range)	Controls ( $n = 19$ ), median (range)	$P$
CD1a <sup>+</sup>	157 (23–478)	212 (104–311)	0.042	142 (17–498)	133 (51–277)	0.2
CD207 <sup>+</sup>	106 (2–407)	166 (14–319)	0.024	54 (0–278)	41 (0–103)	0.1
CD208 <sup>+</sup>	21 (2–148)	19 (1–43)	0.3	124 (3–539)	45 (15–172)	<0.001
CD123 <sup>+</sup> /CD11c <sup>-</sup>	14 (0–78)	8* (0–35)	0.023	234 (56–1,140)	160* (57–302)	0.004
CD94 <sup>+</sup>	22 (0–261)	28 (0–144)	0.3	52 (0–438)	30 (0–209)	0.048
CD4 <sup>+</sup>	279 (31–925)	269 (89–591)	0.6	1,162 (237–2,909)	748 (406–1,060)	<0.001
CD8 <sup>+</sup>	83 (2–357)	130 (51–526)	0.011	354 (45–2,430)	181 (76–491)	0.001
CD25/HLA-DR <sup>+</sup>	0 (0–7)	0 <sup>†</sup> (0–3)	0.5	53 (0–231)	26 <sup>†</sup> (5–118)	0.007

NOTE: CD1a, classic marker for Langerhans cells; CD207, marker for immature DCs expressing Langerin; CD208, DC-Lamp, marker for mature DCs; CD123/CD11c, marker for pDCs; CD94, marker for NK cells; CD4, marker for T-helper cells; CD8, marker for cytotoxic T cells; CD25/HLA-DR, marker for Treg cells.

\*Controls ( $n = 17$ ).

† Controls ( $n = 16$ ).



**Figure 2.** CD94<sup>+</sup> NK cells in the epidermis and dermis of VIN lesions and normal vulvar skin. Horizontal lines, median values.

cell markers Foxp3 and CD25/HLA-DR, respectively. Staining results with both markers were fully comparable (data not shown).

Cell distribution for different inflammatory cells is shown in Fig. 4.

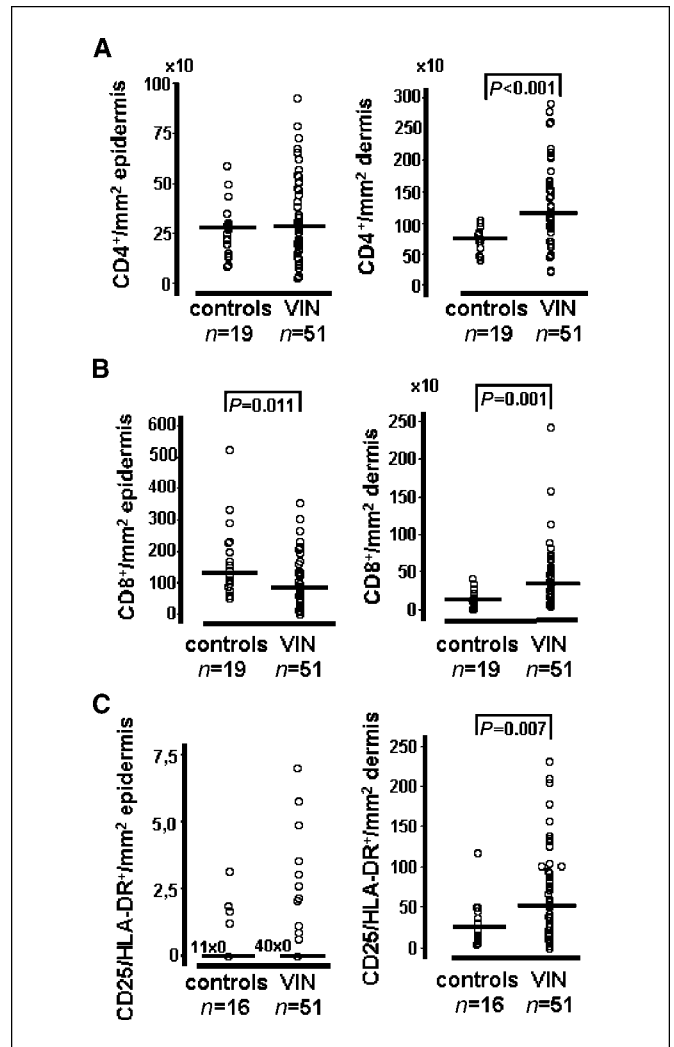
**Discussion**

To our knowledge, this is the first study characterizing the distribution of a broad spectrum of immunocompetent cells in epidermis and dermis of vulvar skin from patients with hrHPV-associated usual-type VIN and from HPV-negative healthy controls. There are only a few studies investigating immunocompetent cells in VIN-affected skin, mostly dealing with CD4<sup>+</sup> and CD8<sup>+</sup> T cells and/or CD1a<sup>+</sup> DCs (6, 10, 11). Study designs vary significantly, especially when it comes to the presence of hrHPV but also in the choice of VIN-inflicted vulvar layers, in cell counting procedures, and, most important, in the choice of control groups. In our study, vulvar material from healthy HPV-negative women was used; this is in contrast to other studies where normal tissue was isolated from resection margins of vulvar specimens taken from patients undergoing surgery for carcinoma or for benign vulvar diseases (6, 10, 11).

In the epidermis of hrHPV-positive usual-type VIN, significantly less CD1a<sup>+</sup> and CD207<sup>+</sup> DCs were observed than in epidermal vulvar tissue from control women. These results are supported by Singh and colleagues (9) who describe an inverse correlation between the numbers of intraepithelial CD1a<sup>+</sup> DCs and the stage of VIN. The significant reduction of CD1a<sup>+</sup> and CD207<sup>+</sup> immature mDCs in epidermal hrHPV-positive VIN tissue could be the result of migration into the dermis under the influence of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  and interleukin (IL)-1 $\beta$  (14). These cytokines are produced during antigen-induced DC activation and down-regulate the expression of the adhesion molecule E-cadherin on Langerhans cells (15, 16). E-cadherin mediates contact between keratinocytes and Langerhans cells. Down-regulation of the adhesion molecule not only prevents stimulation of DCs by HPV-infected keratinocytes (17) but also facilitates migration of the DCs. A defect in repopulation of epidermal DCs through suppression of migration of immature Langerhans precursor-like cells by hrHPV-16 E6 and E7 proteins could also contribute to the observed decrease in mDCs (18).

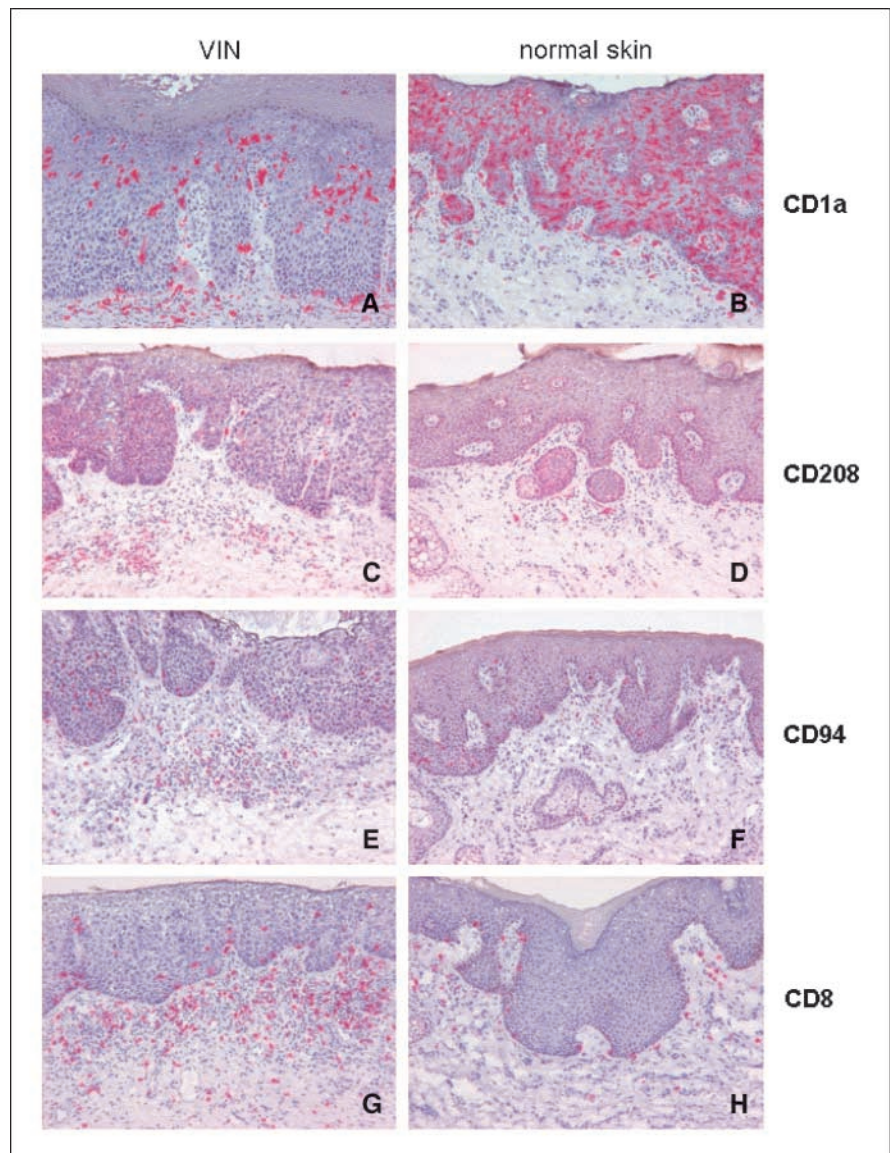
Interestingly, Mulvany and Allen (19) observed increased numbers of CD1a<sup>+</sup> cells compared with the surrounding normal epithelium in differentiated-type VIN, which is not related to HPV and behaves biologically different.

The significant reduction of immature mDCs in the epidermis of our patients seems to be compensated by a significant increase in the number of pDCs, suggesting involvement of pDCs in the immune response. Our results are supported by a study of Lee and colleagues (20), who report decreased mDCs and increased pDCs in peripheral blood of patients with cervical squamous intraepithelial lesions. The results are also in agreement with the suspected role of pDCs in viral infections. Lenz and colleagues (21) showed in an *in vitro* study that HPV-16 is bound by freshly isolated immature pDCs and CpG matured pDCs, that internalization of the virus preferentially occurs in immature pDCs, and that it induces the production of IFN- $\alpha$  and IL-6, important factors for the production of antibodies. Bontkkes and colleagues (22) showed that pDCs are present in cervical cancer lesions and that HPV-16 virus-like particles are able to activate pDCs. In the dermal layer of VIN-afflicted skin, no differences for cell numbers of immature mDCs



**Figure 3.** CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD25/HLA-DR<sup>+</sup> Treg cells in the epidermis and dermis of VIN lesions and normal vulvar skin. Horizontal lines, median values.

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**Figure 4.** A to H, representative photographs of positive (red stained) immunocompetent cells in VIN lesions and normal vulvar skin. Magnification,  $\times 10$ . A and B, compared with healthy controls, VIN-affected skin showed a decrease of mDCs stained with CD1a in the epidermis. C and D, twice as many CD208<sup>+</sup> (mature) DCs were observed in the dermis of VIN compared with healthy controls. E and F, NK cells stained with antibodies directed against CD94 are more numeric in the dermis of VIN-affected skin than in normal skin. G and H, less CD8<sup>+</sup> cells were observed in the epidermis, whereas significantly more CD8<sup>+</sup> cells were seen in the dermis of VIN-affected skin when compared with normal skin.

were observed, but the more mature CD208<sup>+</sup> (DC-Lamp) DCs were significantly increased. This seems to indicate that persistent HPV infection may indeed lead to maturation and to accumulation of these antigen-presenting DCs, possibly caused by a disturbed migration out of the dermis.

The observed increase of NK cells in the dermis of usual-type VIN lesions is in accordance with activation of the innate immune response and may contribute to initiation of the CD8<sup>+</sup> T-cell response against viral infection, as suggested by Robbins and colleagues (23).

Dermal influx of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in VIN-affected skin has been described previously (10, 11). The observed significant increase of dermal CD4<sup>+</sup> T-helper cells, CD8<sup>+</sup> CTLs, and CD25/HLA-DR<sup>+</sup> Treg cells in our study indicates local activation of the adaptive immune system in HPV-related usual-type VIN. This is in accordance with observations by van Poelgeest and colleagues (24) and Todd and colleagues (25) about systemic activation of cell-mediated immunity by hrHPV infection. van Poelgeest detected HPV-16-specific CD4<sup>+</sup> T-cell immunity in the circulation of

patients with persistent HPV-16-induced VIN. Todd and colleagues showed CD8<sup>+</sup> T-cell reactivity to one or more proteins of the HPV-16 oncoproteins E6 and E7 in the peripheral circulation of patients with high-grade VIN.

It seems that antigen-presenting DCs are the key regulators of immune responses. DCs migrate from the epidermis into draining lymph nodes where they activate naive T cells and initiate cellular immunity. Different studies have shown that this trafficking of DCs is controlled by soluble chemotactic factors known as chemokines (26–28). Recent evidence has shown that chemokines not only direct the trafficking of DCs but also can regulate their maturation status (29). Further studies of DC trafficking and the responsible chemokines in hrHPV-based vulvar lesions will be necessary to provide more insight into the immunologic basis of hrHPV-related usual-type VIN.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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