Functional Attributes of Mucosal Immunity in Cervical Intraepithelial Neoplasia and Effects of HIV Infection

Aiko Kobayashi,1,4 Ruth M. Greenblatt,2,5 Kathryn Anastos,6 Howard Minkoff,7 Leslie S. Massad,8 Mary Young,9 Alexandra M. Levine,10 Teresa M. Darragh,3 Vivian Weinberg,1 and Karen K. Smith-McCune1,4

1The Cancer Research Institute and Department of Obstetrics, Gynecology, and Reproductive Sciences, 2the Department of Medicine, 3the Department of Pathology, and 4the Comprehensive Cancer Center at University of California at San Francisco, San Francisco, California; 5the Connie Wofsy Study Consortium of Northern California, University of California, San Francisco; 6New York City/Bronx Consortium, Bronx-Lebanon Hospital/Montefiore Medical Center, Bronx, New York; 7Maimonides Medical Center, Brooklyn, New York; 8Southern Illinois University School of Medicine, Springfield, Illinois; 9Washington DC Metropolitan Consortium, Washington DC; 10University of Southern California Consortium, Los Angeles, California

ABSTRACT

The role of mucosal immunity in human papillomavirus (HPV)-related cervical diseases is poorly understood. To characterize the local immune microenvironment in cervical intraepithelial neoplasia (CIN) 2/3 and determine the effects of HIV infection, we compared samples from three groups: normal cervix, CIN 2/3 from immunocompetent women (HIV− CIN 2/3), and CIN 2/3 from HPV seropositive women (HIV+ CIN 2/3). CIN 2/3 lesions contained increased numbers of immune cells from both the acquired and innate arms of the immune response in strata [CD4+ and CD8+ T cells, macrophages, mast cells, B cells, neutrophils, and natural killer (NK) cells] and dysplastic epithelium (CD4+ T cells, macrophages, and NK cells). Immune cells in CIN 2/3 expressed activation markers, as measured by interleukin-2 receptor (II-2R) and transcription factor T bet. Interferon-γ production was significantly up-regulated in CIN lesions and was expressed by CD4+ and CD8+ T cells and NK cells, indicating the activation of immune cells. Abundant presence of transforming growth factor-β+ CD25+ cells in the infiltrates associated with CIN lesions, and of immature CD1a+ dendritic cells expressing IL-10 and transforming growth factor-β, indicate that CIN is associated with an influx of immune cells that produce a mixture of proinflammatory and regulatory cytokines. In HIV+ CIN, immune cell densities (CD4+ T cells, macrophages, neutrophils, and NK cells) and expression of interferon-γ were significantly decreased compared with HIV− CIN. Regulatory cytokines were also down-regulated in this group. Therefore, both pro- and anti-inflammatory responses present in CIN 2/3 lesions are suppressed in HIV-seropositive women.

INTRODUCTION

Cervical cancer is preceded by well-defined changes in the epithelium known as CIN, changes that are associated with genital infection with human papillomavirus (HPV). The majority of genital HPV infections are clinically undetectable and do not result in CIN or cancer. CIN 1 reflects active HPV replication and is rarely precancerous, most often resolving spontaneously. In contrast, CIN 2 and 3 represent potential cancer precursors; ≥12% of CIN 3 lesions will progress to cancer if left untreated (1, 2). More than 90% of CIN 2/3 and cervical cancers are associated with a few high-risk HPV types, most commonly HPV 16 (3–5).

Host immune response to HPV appears to be critical in determining the outcome of infection, because HPV infection is detected more frequently, the incidence of CIN is higher, and the risk of CIN recurrence after treatment is higher among immunocompromised women (6–9). Recent and promising results with an HPV 16-specific vaccine indicate that protection against HPV infection is possible; vaccinated women mounted a systemic humoral response to HPV 16 and were resistant to infection with this HPV type for at least 1 year (10). Cytotoxic immune responses are likely to be important in determining the sequelae of HPV infection once it is established. Women who clear HPV infection are more likely to have detectable levels of interferon-γ (IFN-γ) in cervical cytobrush samples than women whose infection persists (11). In addition, the correlation between presence of HPV protein E6-specific CTLs and the absence of CIN in women with HPV16 infection again indicates that CTL responses may be protective (12). Overall, however, the detection and measurement of potentially protective immune response to HPV has been difficult, raising the possibility that HPV does not generate a strong immune response and may in fact induce immunotolerance (13, 14).

The main purpose of this study was to investigate the functional attributes of the local immune microenvironment in normal cervix, in CIN 2/3 lesions from immunocompetent women, and in CIN 2/3 from HIV+ women. The cytokine profiles in these cohorts indicated that CIN lesions were characterized by both proinflammatory, potentially cytotoxic responses as well as anti-inflammatory potentially immunosuppressive responses. We also found significant differences in the functional properties of the immune response in HIV+ CIN 2/3, most notably failure to generate CTLs despite the abundance of CD8+ T cells. In addition, the production of regulatory cytokines [i.e., transforming growth factor (TGF)-β and IL-10] was reduced in HIV+ CIN 2/3. These findings have important ramifications for understanding of the nature of the immune response in women with CIN 2/3 and the effects of vaccines and therapies that target local immune responses.

MATERIALS AND METHODS

Clinical Specimens. Specimens for the normal cervix group were obtained as paraffin-embedded blocks from the Department of Pathology Archives at University of California, San Francisco (UCSF) from women who underwent hysterectomies for benign uterine disease with no cervical abnormalities and with no evidence of immunocompromise as stated on the pathology report (n = 21, mean age 51 range 39–81). Specimens and data for the immunocompetent (HIV−) CIN 2/3 group were obtained as paraffin-embedded sections of cone and loop excisions from two sources: the UCSF Department of Pathology Archives and the Women’s Interagency HIV Study (WHIS; n = 19, mean age 33, range 19–84). The samples obtained from WHIS (n = 5) were...
from women who were HIV seronegative whereas HIV serostatus was not available for most of the samples from the UCSF Department of Pathology Archives (n = 14). However, the women contributing samples to this group were clinically immunocompetent.

Specimens and data for the HIV+ CIN 2/3 cohort in this study were obtained in collaboration with WIHS, which is a longitudinal multi-site cohort study of women with or at risk for HIV infection (n = 28, mean age 32, range 21–46). The mean CD4 count was 381 (range 1–1277). All samples obtained from the WIHS cohort were selected from women who had HIV 16 detected in cervicovaginal lavage specimens. WIHS sites included consortia based in Bronx and Brooklyn New York, Chicago, Southern California, Washington DC, and the San Francisco Bay area. Detailed methods for this study were published previously (15). All study procedures and consent materials were reviewed and approved by human subject protection committees at each of the collaborating institutions. Verification of HIV infections and HPV testing was described previously (15, 16). Loop and cone biopsies were performed for standard indications, and tissues archived as paraffin blocks after pathological evaluation were accessed for this study. The histologic diagnosis of CIN 2/3 was determined at the originating institution and confirmed after receipt at UCSF. Because of limited availability, we did not include a cohort of normal cervical samples from HIV+ women. For each assay, sample selection was non-biased and was based on availability of appropriate specimens from consecutively accrued women.

**Immunohistochemistry and Immunofluorescence.** Immunohistochemistry (IHC) was performed with primary antibodies against CD4+ T cells with monoclonal antibody to CD45R0 (clone OP4D4), CD8+ T cells with monoclonal antibody to CD8 (clone C8/144B), B cells with CD20 (clone L26), tissue macrophages with CD68 (clone KP1), myeloid-derived dendritic cells (DCs) with CD1a (clone D1a), and tissue mast cells with mast cell tryptase (clone AA1), purchased from DAKO (Carpenteria, CA). Monoclonal antibody against CD15 (clone MY-1) for neutrophils, monoclonal antibody against CD25 (clone IL2R-1), and monoclonal antibody to CD56 (clone 123C3.D5) for natural killer (NK) cells were purchased from ID Labs (London, Ontario, Canada). Polyclonal anti-IFN-γ antibody was purchased from R&D Systems (Minnepolis, MN). Each primary antibody was diluted in PBS with 1% BSA as follows: 1:500 (CD45R0), 1:200 (CD8), 1:1200 (CD20), 1:500 (CD68), 1:100 (CD1a), 1:2000 (tryptase), 1:300 (CD15), 1:400 (CD25), 1:25 (CD56), and 1:2400 (IFN-γ). Routine IHC was performed after manufacturer’s guidelines (Innogenex, San Ramon, CA). Before the inactivation of endogenous peroxidase with 0.1% hydrogen peroxide, all tissue slides except for IFN-γ, CD25, and CD56 staining were digested with 0.25% trypsin for 10 minutes at 37°C. IFN-γ and CD56 antibodies were performed by boiling the slides in a 1.25 kW microwave for 2 minutes in 10 mmol/L Sorensen’s citric buffer (pH 6) and cooling in citric buffer for 30 minutes. Antigen retrieval for primary antibodies against IFN-γ and CD56 was performed by boiling both IFN-γ and CD56 slides in the same microwave for 10 minutes (5 minutes × 2) in 1 mmol/L EDTA solution (pH 8), and then the slides were immediately transferred to PBS at room temperature (RT). Antibodies against CD4, CD8, CD20, CD68, and tryptase were incubated on tissue sections for 1 hour at RT. Antibodies against CD15, CD25, CD56, CD1a, and IFN-γ were incubated over night at 4°C. A 1:3 dilution of hematoxylin gill 1 was used for counter staining. The chromogen used to detect secondary antibodies was 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA).

Double-staining immunofluorescence (IF) was performed to detect IL-10 or TGF-β producing cells in the cervical tissue. IL-10 antibody (PE-conjugated rat IgG2a, clone: JES5-19F1, BD Bioscience, San Jose, CA) or TGF-β that was detected by antibody against TGF-β1 latency-associated peptide (goat IgG latency-associated peptide antibody, R&D Systems) was combined with CD4, CD8, CD56, CD1a, and CD20 primary antibodies (clone MY-1) for neutrophils, monoclonal antibody to CD56 (clone 123C3.D5), or anti-digoxigenin antibody (1:800 dilution) for 1 hour at RT. Specificity of the secondary antibody was confirmed by omission of the primary antibody. Slides were incubated over night at 4°C with primary antibody, slides were blocked with 1× Power Block (BioGenex, San Ramon, CA), and each corresponding secondary antibody was incubated for 1 hour at RT after the primary antibody incubation. Slides then were incubated with 1:200 4′,6-diamidino-2-phenylindole (D9542, Sigma-Aldrich, St. Louis, MO) for 5 minutes at RT and mounted with Prolong Anti-fade kit (Molecular Probes). Between each step, slides were washed thoroughly in PBS with 0.05% Tween 20. All steps involving fluorescent dyes were processed in the dark.

**Quantification of Immune Cell Numbers.** The numbers of immunophenotyped cells in cervical tissues were determined using image analysis software. In each photographic image of a microscopic field (40× objective), Stromal and epithelial areas were separately measured in mm² (Openlab 2.2.4, Improvision, Lexington, Massachusetts), and the stained cells were counted by hand after visual inspection. Blood vessels and endocervical glands in the stroma that were larger than 500 μm² were subtracted from the area. The number of cells was then divided by area to determine cell density (cell no. per mm²). Intraobserver variability of cell counts was <10% as determined by triplicate counts of 10 fields. For counts of stromal cells, 10 microscopic fields within 600 μm² of the epithelial basement membrane were randomly selected from each slide. Stromal areas containing organized aggregates described previously (17) were excluded from this analysis. The average densities from 10 fields from each sample were used for statistical comparisons. Ten microscopic fields for epithelial cell counts were not always available because dysplastic epithelium was partially denuded in some samples. Cell densities from five 40× objective microscopic fields with CIN 2/3 were averaged for each sample for statistical comparison. To avoid counting pseudopodia when counting macrophages, we focused on stained areas larger than 200 pixels. Tryptase, secreted by mast cells into surrounding stroma, was often diffusely present surrounding mast cells. Therefore, to be counted as a mast cell, the stained area must have included a nucleus.

For quantification of cells stained by IFN-γ antibody, the stromal and epithelial areas are combined to obtain generalized density in the cervical tissue. Five 40× microscopic fields that contained both stroma and epithelium were randomly selected from each slide, and the cell counts per mm² from five fields were averaged and compared statistically. Because IL-10–positive cells were widely distributed in the stroma, a slide scanner (Aperio Technologies, Vista, CA) was used to determine the number of IL-10–positive cells. Five stromal areas not limited to 600 μm² from the basement membrane were randomly selected from each sample of normal cervix (n = 4), HIV+ CIN 2/3 (n = 4), and HIV+ CIN 2/3 (n = 9), and the density of IL-10–positive cells was measured as stated above.

The number of samples contributing data for analyses and P values are indicated in Table 1 unless otherwise stated in the text and reflects the numbers of available appropriate specimens, which in some cases are less than the numbers stated above attributable to depletion of the sample reservoir over time.

For the intensity measurement of IL-10 and TGF-β staining, a confocal microscopy (Carl Zeiss, Inc, Thornwood, NY) was used. For IL-10 intensity, five randomly selected stained cells from each sample of HIV+ CIN 2/3 (n = 4) and of HIV− CIN 2/3 (n = 4) were analyzed. For TGF-β intensity, five randomly selected stained cells from each sample of HIV+ CIN 2/3 (n = 3) and HIV− CIN 2/3 (n = 2) were analyzed. The z-stack images from each cell were projected for maximum value after background thresholding and a mean intensity score of each projection as calculated by Zeiss LSM software was recorded.

**In situ Hybridization.** The T bet clone, kindly provided by Dr. L. Glimcher, was inserted into pcDNA3 vector (Invitrogen, Carlsbad, CA). Plasmid DNA used to produce antisense and sense probes was linearized with the restriction enzymes NotI and BamHI. One microgram of linearized plasmid was used as a template for in vitro transcription and labeling using a digoxigenin RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Paraffin-embedded cervical tissue sections were deparaffinized, rehydrated, and antigen retrieval was performed with 10 μg/ml proteinase K (The Diagnostics, Indianapolis, IN) for 30 minutes at 42°C. After denaturation of probes at 95°C for 5 minutes, the tissue sections were hybridized with the RNA antisense or sense probe (3 ng/μl in 2× standard saline citrate, 1× Denhardt’s solution, 10% dextran sulfate, 0.02% SDS, and 50% formamide) at 62°C for 16 hours. Subsequently the slides were vigorously washed twice in 2× standard saline citrate with 1 mmol/L EDTA, in 0.1 standard saline citrate and 1 mmol/L EDTA at 62°C for 2 hours), and twice in 0.5× standard saline citrate. Slides were incubated with anti-digoxigenin antibody (1:800 dilution) for 1 hour at RT and color development was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution at RT in the dark.
MUCOSAL IMMUNITY IN CERVICAL INTRAEPITHELIAL NEOPLASIA

RESULTS

A Distinctive Immune Microenvironment Is Present in CIN 2/3.

To investigate the nature of the mucosal immune response in CIN 2/3 lesions, we performed IHC on cervical samples from women with normal cervix or with CIN 2/3, half of whom were immunosuppressed secondary to HIV infection. The sources of samples for each of these groups are described in Materials and Methods. Staining patterns revealed that infiltrates in the stroma were localized to areas subtending the CIN lesions, suggesting localized responses to events within the epithelium. Examples of IHC for CD4+ T cells and macrophages are shown in Fig. 1A.

Cell densities were quantitated using image analysis as described in Materials and Methods, and statistical comparisons of the mean cell densities for each group were performed. Cells involved in the acquired immune response (CD4+ and CD8+ T cells and B cells) were significantly increased in the stroma among HIV− CIN 2/3 samples compared with normal cervix. In addition, the numbers of CD4+ T cells in the epithelium were significantly elevated in HIV− CIN 2/3 compared with normal cervical epithelium (Table 1).

The cells in the innate immune response (macrophages, NK cells, and neutrophils) in CIN 2/3 were also studied. Neutrophils were essentially restricted to the stroma and were significantly increased in HIV− CIN 2/3 samples compared with normal cervix (Table 1). Densities of macrophages and NK cells in the stroma and the epithelium were both elevated significantly in CIN 2/3 compared with normal cervix (Table 1).

When HIV+ CIN 2/3 samples were compared with normal cervix, significant increases were found in CD4+ and CD8+ T cells and mast cells in the stroma, and macrophages in the epithelium of CIN 2/3 samples (Table 1). The vast majority of CD8+ cells also express CD3 (17), indicating that the majority of CD8+ cells are indeed T cells rather than NK cells.

Because hysterectomies are usually performed in older women, the mean age of the group contributing samples of normal tissue (51 years) was significantly greater than the HIV− and HIV+ CIN 2/3 groups (31 and 32 years, respectively). Therefore we explored whether age and cell densities were correlated among women with normal cervical tissue. Strong correlations existed between age and numbers of epithelial CD4+ T cells (r = 0.83, P = 0.002), stromal B cells (r = 0.92, P = 0.0002), and stromal macrophages (r = 0.72, P = 0.03) with older age having higher cell counts. Given the younger mean ages of the CIN 2/3 groups, age is unlikely to contribute to the higher densities of these cell types observed in CIN 2/3. There was no apparent correlation between age and epithelial cell counts of CD8+ T cells, B cells, macrophages or mast cells.

HIV Infection Results in Alterations of the Immune Microenvironment in CIN 2/3.
The mean densities of CD4+ T cells in the stroma and epithelium and the macrophage density in the epithelium in HIV− CIN 2/3 samples were significantly lower than HIV− CIN 2/3 samples. In addition, significant elevations of stromal B cells and macrophages seen in HIV− CIN 2/3 were not observed in HIV+ CIN 2/3. Densities of neutrophils and NK cells were also attenuated in HIV+ CIN 2/3 compared with HIV− CIN 2/3 (Table 1). There was no apparent correlation between cervical CD4+ T cell densities (in stroma, epithelium, or both combined) and peripheral CD4 counts (at the visit proximate to the surgical procedure or nadir).

IFN-γ Production of Lymphocytes Infiltrating CIN 2/3 Lesions.
To determine whether the cells in the CIN 2/3 lesions were committed to a Th1 pathway, we examined expression of cytokines and cytokine receptors in the clinical samples. Expression of the IL-2R-α, which is necessary for lymphocyte proliferation and differentiation involved in the Th1 response, was abundantly expressed in the infiltrates associated with CIN 2/3, and no differences were apparent between HIV− and HIV+ women (data not shown). IFN-γ–positive cells were abundantly distributed in the stroma near dysplastic epithelium from HIV− women but were dramatically decreased in samples from HIV+ women with CIN 2/3 (Fig. 1B), even in CD8+ cell aggregates (Fig. 1C). Quantification of IFN-γ–positive cells (Fig. 1D) indicated that the mean density of IFN-γ–positive cells in the HIV+ CIN 2/3 group was significantly lower than what we observed in HIV− CIN 2/3 and was similar to the density in normal cervix. Hence, despite abundant numbers of T cells in CIN 2/3 from HIV+ women, IFN-γ production in these cells is significantly impaired.

IFN-γ expression in HIV− CIN 2/3 was localized to both CD4+ (Fig. 2A) and CD8+ (Fig. 2B) T cells by double staining IF, indicating the presence of Th1 responses in CIN 2/3 lesions of immunocompetent women. In addition, NK cells also expressed IFN-γ (data not shown). A. Kobayashi and K. K. Smith-McCune, unpublished data.

---

Table 1. Summary of cell densities among the three clinical groups.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Normal cervix</th>
<th>HIVneg CIN</th>
<th>HIVpos CIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>N = 11</td>
<td>N = 14</td>
<td>N = 17</td>
</tr>
<tr>
<td>Stroma</td>
<td>62 ± 51††</td>
<td>167 ± 161‡‡</td>
<td>85 ± 85¶</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>N = 11</td>
<td>N = 13</td>
<td>N = 17</td>
</tr>
<tr>
<td>Stroma</td>
<td>215 ± 157§</td>
<td>1083 ± 689¶¶</td>
<td>665 ± 488‖</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>N = 11</td>
<td>N = 13</td>
<td>N = 17</td>
</tr>
<tr>
<td>Stroma</td>
<td>408 ± 560</td>
<td>434 ± 321</td>
<td>585 ± 445</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>N = 11</td>
<td>N = 14</td>
<td>N = 20</td>
</tr>
<tr>
<td>Stroma</td>
<td>330 ± 264‡‡‡</td>
<td>850 ± 577¶¶</td>
<td>1045 ± 704‖</td>
</tr>
<tr>
<td>NK cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>N = 9</td>
<td>N = 14</td>
<td>N = 20</td>
</tr>
<tr>
<td>Stroma</td>
<td>277 ± 204‡‡‡</td>
<td>550 ± 273¶¶</td>
<td>503 ± 361</td>
</tr>
</tbody>
</table>

* Cell densities are expressed by cell number/mm² ± SD.
† P = 0.01.
‡ P = 0.02.
§ P = 0.03.
¶ P = 0.04.
†† P = 0.01.
‡‡ P = 0.001.
§§ P = 0.002.
‖‖ P = 0.003.
¶¶ P < 0.001.
‖‖‖ P < 0.001.
shown), although given that their cumulative density (219 cells/mm²) is much lower than CD8+ (1284 cells/mm²) or CD4+ (1250 cells/mm²) T cells (Table 1), the predominant source of IFN-γ is likely to be from T cells.

A transcription factor, T bet, is required in the expression of IFN-γ in the Th1 response (18). We analyzed T bet expression by in situ hybridization to determine whether the low expression of IFN-γ in HIV+ samples was attributable to the absence of this necessary transcription factor (Fig. 2C). T bet RNA was detected in six of eight HIV+ CIN 2/3 samples and in eight of nine HIV+ CIN 2/3 samples. Hybridization with sense probe showed no staining (data not shown). All 6 T bet-positive samples from HIV+ CIN 2/3 also expressed IFN-γ. However, in HIV+ CIN 2/3, three of eight T bet-positive samples failed to produce any IFN-γ, indicating that despite the
expression of a key transcription factor, IFN-γ was not produced in this group.

Regulatory Cytokines in CIN 2/3. Recent studies have shown that IFN-γ production in the cytotoxic (Th1) response is down-regulated by TGF-β and IL-10, secreted by regulatory cells including Tr1 and Th3 (reviewed in ref. 19). We therefore tested whether regulatory cytokines might also be involved in the local immune microenvironment in CIN 2/3. In normal cervix, TGF-β cells were scattered throughout the stroma in a pattern similar to that of IL-10 staining cells described below. In CIN 2/3 lesions, TGF-β staining was detected in CD25 cells (Fig. 3A), macrophages, CD1a+ cells, and CD4+ T cells and was not detected in cervical keratinocytes (data not shown). Compared with normal cervix, there was an increased density of stromal TGF-β cells in the infiltrates adjacent to the CIN 2/3 lesions, particularly in HIV− samples, although less intensively stained TGF-β cells were also detected in the HIV+ group (Fig. 3B). Although the overall TGF-β cell density comparison did not show a marked difference as a result of HIV infection, the apparent difference in staining intensity was further investigated by immunofluorescence (Fig. 3C). The mean fluorescent intensity score of TGF-β cells for HIV− CIN 2/3 was 62.5 (±13.4) while mean intensity score for HIV+ CIN 2/3 was 36.8 (±8; Table 2). Although statistical comparisons are not applicable because of the small number of patient samples in each group, the data indicate that the overall expression of TGF-β is reduced in HIV+ samples.

IL-10 Expressing Immature Dendritic Cells in CIN 2/3. We also investigated the expression of another regulatory cytokine, IL-10, attributable to its role in the regulation of Th1 responses (reviewed in ref. 19). Whereas all other immune cells were concentrated in regions proximate to the dysplastic epithelium, IL-10–positive cells had a distinctive distribution throughout the cervical stroma (Fig. 4A and B). IL-10 staining did not co-localize with cervical keratinocytes, CD4+ cells, CD25+ cells, macrophages, or mast cells (data not shown) but did co-localize with CD1a+ cells (Fig. 4D). CD1a is used as a marker for myeloid-derived DCs. Epithelial tissues contain specialized antigen presenting cells known as Langerhans cells; however, cervical CD1a+ DCs did not co-localize with S100, a Langerhans cell marker. S100-positive cells were distributed primarily in the dysplastic epithelium and had a dendritic morphology (data not shown), whereas CD1a+ cells were detected exclusively in the stroma. As shown in Fig. 4B-D, CD1a+ IL-10+ S100− cells lack mature DC morphology. Triple color IF showed IL-10 and TGF-β co-localized with CD1a in cervical stroma, indicating that a portion of CD1a+ DCs produces both regulatory cytokines (Fig. 4D). These immature cervical DCs demonstrated differences in the number
and intensity of IL-10 expression depending on HIV status. The density of IL-10+ cells were increased in HIV− CIN 2/3 (69 cells/mm² ± 40) compared with normal cervical samples (23 cells/mm² ± 22) or HIV+ CIN 2/3 (15 cells/mm² ± 15). In addition, the mean intensity of IL-10 staining in positively stained cells from HIV− CIN 2/3 (44.7 ± 9.9) was markedly higher than in HIV+ CIN 2/3 (29.4 ± 3.3; Fig. 4C; Table 2). Although statistical comparison was not applied because of small sample sizes, the data indicate a difference in the levels of IL-10 expression as an alteration in DC function in HIV infection.

Table 2  Cellular IF intensity scores of regulatory cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HIV− CIN2/3 samples</th>
<th>HIV+ CIN2/3 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Mean intensity score</td>
<td>44.7 ± 9.9</td>
<td>29.4 ± 3.3</td>
</tr>
<tr>
<td>TGF-β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Mean intensity score</td>
<td>62.5 ± 13.4</td>
<td>36.8 ± 8.0</td>
</tr>
</tbody>
</table>
DISCUSSION

This report demonstrates that CIN 2/3 lesions are associated with mucosal immune responses characterized by activated CD4+ and CD8+ T cells as well as cells displaying the phenotype of an immunosuppressive regulatory T cell. HIV infection impairs the production of IFN-γ and regulatory cytokines in the lesions. Our data are the first to provide absolute cell densities for B cells, T cells, macrophages, mast cells, neutrophils, NK cells, and IFN-γ-expressing cells in normal cervix, and in CIN 2/3 lesions from both HIV+ and HIV− women. These findings are expressed as cell density (cell number/mm²), which has advantages over other less quantitative methods because of its universal relevance, thereby allowing precise evaluation of interventions such as protective and therapeutic vaccines, and making possible comparisons across other cohorts or tissue samples.

Our results demonstrate that CIN 2/3 lesions are characterized by complex infiltrates of immune cells from both the acquired and the innate arms of the immune response. The finding of increased numbers of stromal CD8+ T cells in CIN 2/3 compared with normal cervix confirms reports from other groups (20, 21). The presence of abundant intraepithelial CD8+ T cells in normal cervical epithelium suggests that the cervix, like the gut, is protected by a specific group of resident intraepithelial lymphocytes (22).

Our results indicate that the local T cells are activated and produce both IFN-γ as well as regulatory cytokines in CIN 2/3. There are conflicting findings of IFN-γ levels associated with CIN possibly attributable to differences in type of sample [cervicovaginal washing (23) versus tissue levels reported here], or techniques used [relative mRNA expression in microdissected paraffin sections (24) versus the
absolute cell density of positive IHC staining reported here]. Our findings are the first to localize IFN-γ and other cytokines to specific cellular compartments and indicate the presence of a robust Th1 response in CIN 2/3 lesions. This response is presumably elicited to contain or eradicate local HPV infection and/or protect against development of HPV-induced neoplasia. The protective role of a localized immune response has been inferred from the correlation of a subset of CD8+ T cells called tumor-infiltrating lymphocytes with improved outcomes in several invasive cancers (25–27). In addition, infiltrating lymphocytes are correlated with regression of genital warts in both humans and animal models (28–31). In the setting of HIV infection, peripheral CD4 counts are inversely correlated with the presence of CIN, indicating a protective role of CD4+ T cells in this population (32). Because our study is a cross-sectional study, the functional role of the lymphocytic infiltrates in CIN lesions cannot be ascertained. Recent insights into cancer biology have indicated that the role of the immune response in cancer contributes both protective and permissive factors (33). For example, inflammatory cells secrete matrix metalloprotease-9, which has been shown to be essential for neovascularization and lesion progression in a model of HPV-induced skin carcinogenesis (34). In human CIN, cells located within germinal centers express high levels of matrix metalloprotease-9 in the human CIN 2/3 lesions.12

Another possible explanation for the presence of CIN 2/3 despite the abundant expression of IFN-γ could be attributable to a competing immunosuppressive response. Recently, subsets of CD25+ regulatory T cells have been described to suppress Th1 response through secretion of IL-10 and TGF-β (35). Similar findings of T-cell activation and increase of IL-10 in CIN 2/3 have been reported by others using different techniques (36, 37). We have identified TGF-β-producing CD4+ and CD25+ cells in stroma of CIN 2/3 samples, suggesting that regulatory T cells are recruited to CIN 2/3 lesions. Regulatory T cells are produced in the context of persistent antigen exposure through a mechanism that triggers DCs to produce IL-10, which then directs differentiation of naïve CD4+ T cells to regulatory T cells (38, 39). HPV typically does not elicit strong local or systemic immune responses, through various mechanisms of immune evasion (reviewed in 13, 14, 40), including the relative paucity of DCs in the transformation zone compared with the exocervix (41), the low expression of CD3 ε chain in T cells in women with CIN and cervical cancer (42, 43) and direct effects of viral genes on suppression of the immune response (reviewed in ref. 14). The presence of TGFβ+/CD25+ cells in CIN lesions described here suggests that putative T regulatory cells are recruited to CIN lesions and adds a possible new mechanism by which HPV evades the immune response. In the context of a CIN 2/3 lesion, the competing influences of cytotoxic and suppressive immune responses may govern the eventual clinical outcomes of regression or persistence.

We have described a class of immature CD1a+ DCs resident throughout the cervical stroma expressing IL-10 and TGF-β. Immature stromal DCs have been described previously in the cervix and found to be less abundant in the transformation zone, the site of CIN development, than in the exocervix (41). Submucosal DCs have also been described in the vagina and were shown to be responsible for trafficking of antigen to draining lymph nodes in an animal model of herpes simplex virus infection (44). The presence of IL-10+ CD1a+ cells in the cervix is intriguing in light of recent descriptions of a novel class of DCs that differentiate in the presence of IL-10 and are capable of inducing immunotolerance in mice (45) and humans (46). The function of IL-10+ TGF-β+ immature DCs in cervical stroma is under investigation.

Our study included an HIV+ CIN 2/3 cohort to determine the effects of immunosuppression on the local immune microenvironment. Samples from HIV+ CIN 2/3 demonstrated a robust infiltration of CD8+ T cells, confirming the findings of others (37, 47). The HIV+ CIN 2/3 group had significantly lower numbers of stromal and epithelial CD4+ T cells and NK cells, as well as epithelial macrophages compared with HIV− women. It is worth noting, however, that local CD4+ T cell and macrophage numbers were significantly increased in HIV+ CIN 2/3 compared with normal cervix, indicating a capability for effective extravasation and emigration of lymphocytes to the affected site despite systemic immunosuppression. Therefore the attenuation of the local immune response in HIV+ CIN 2/3 is relative rather than absolute. Macrophages and DCs express CD4 and coreceptors for HIV entry, and this might explain the decreased immune cell densities and consequent decrease of stromal IL-10+ CD1a+ regulatory cells in the HIV− CIN 2/3 group. We also found that the stromal density of mast cells was significantly increased in HIV+ CIN 2/3 samples compared with normal cervix, a finding not present in HIV− CIN 2/3 samples, suggesting a shift in the mucosal immune response from Th1 to Th2 in the HIV− group. In addition, we observed a dramatic diminution of IFN-γ production in CIN 2/3 samples from HIV+ women. Similar functional impairments in peripheral T cells from HIV+ patients have been described by others (48, 49) and are due in part to failure to signal through the STAT pathway (50). The regulatory function of the local cervical immune response is also affected in HIV+ women, as seen in lower per-cell expressions of IL-10 and TGF-β. Our finding of reduced IL-10 production in HIV+ samples is interesting in light of a recent report that HPV-specific CTLs were stimulated rather than inhibited by IL-10 (51), although these findings were generated using peripheral lymphocytes, and hence their relevance to the cervical microenvironment is unknown.

In summary, our data indicate that both the innate and the cellular immune responses are activated in CIN 2/3 lesions. In addition, regulatory T cells presumably contribute to an immunosuppressive microenvironment. Widespread stromal DCs expressing IL-10 and TGF-β may also contribute to immunotolerance against HPV and possibly other cervical pathogens. Systemic HIV infection suppresses multiple functional properties of the cervical immune responses, including IFN-γ and regulatory cytokine production. These findings provide insights into the mechanisms governing both the protective and the potentially immunosuppressive effects of the mucosal immune response in HPV-induced cervical neoplasia and will contribute to further understanding of the effects of vaccines and therapies targeting a local immune response.

REFERENCES


12 A. K. and K. S. M., unpublished data.


Functional Attributes of Mucosal Immunity in Cervical Intraepithelial Neoplasia and Effects of HIV Infection

Akiko Kobayashi, Ruth M. Greenblatt, Kathryn Anastos, et al.

*Cancer Res* 2004;64:6766-6774.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/18/6766

Cited articles
This article cites 46 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/18/6766.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/64/18/6766.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.