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A Robust Mucosal Immune Response is Present in the Cervix with High-Grade Dysplasia
and is Altered in the HIV-positive Population

by

Akiko Kobayashi, R.N. Ph.D.(c)

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of
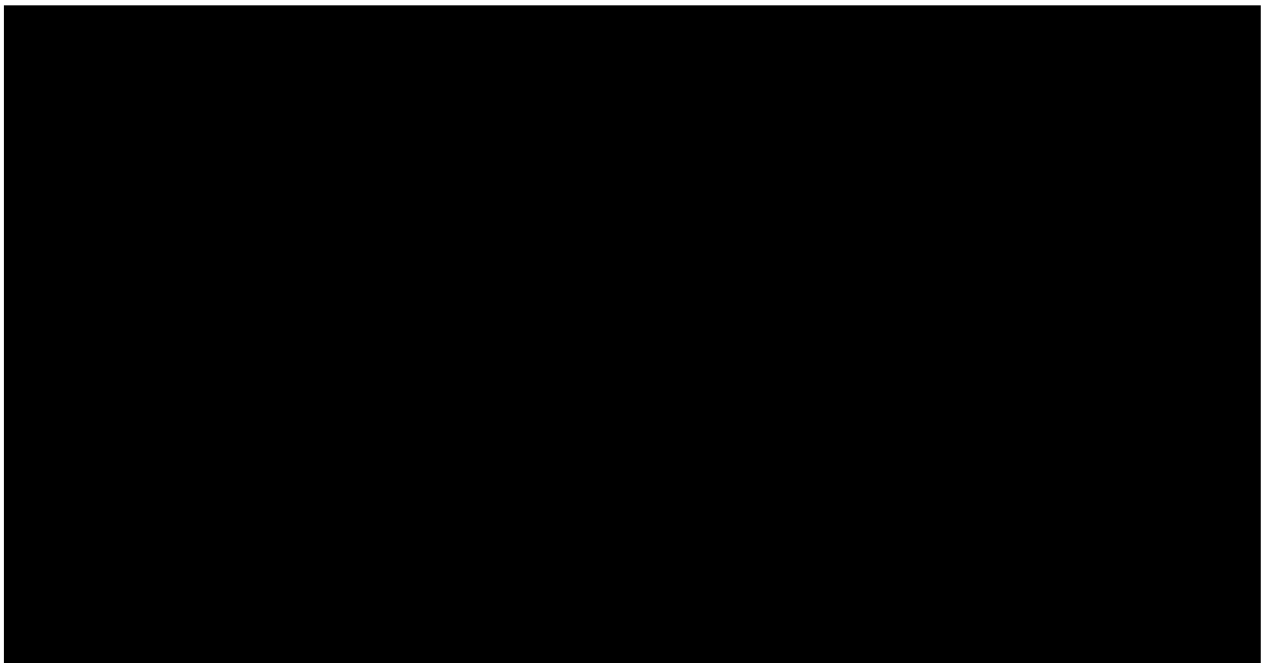
DOCTOR OF PHILOSOPHY

in

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The text of Chapter 1 in this dissertation is a reprint of the material as it appears in Oncology Nursing Forum 27(4), 2000. Permission to use the article for my dissertation was granted by Publishing Division, Oncology Nursing Society on August 3, 2001.

# Abstract

The exact role of the mucosal immune response in the pathogenesis of human pappilomavirus (HPV)-related premalignant and malignant diseases of the genital tract is poorly understood. Using immunohistochemistry, we characterized immune cells in the normal cervix, HIV-negative and HIV-positive high-grade dysplasia. Classical germinal centers were present in 4.7% of normal cervical tissue, 33% of high-grade lesions from HIV-negative women, and 3.3% of high-grade lesions from HIV-positive women (p=0.003). HPV16 E7 antigen was detected in a subset of germinal centers, indicating that the secondary immune response was directed in part against HPV. Lymphoid follicles were present in 9.5% of normal cervical tissue, 57% of HIV-negative high-grade dysplasia, and 50% of HIV-positive high-grade dysplasia (p=0.001 normal versus high-grade). A novel type of lymphoid aggregate, consisting predominantly of CD8+ T cells, was detected in 4.8% of normal cervical tissue, 0% of HIV-negative high-grade dysplasia, and 40% of HIV-positive high-grade dyplasia (p<0.001). In addition, the recurrence rate of high-grade dysplasia within one year was significantly higher in women with such CD8+ T cell-dominant aggregates (p=0.02). The results of quantification of immune cells in the cervical tissue indicated that the cell counts of stroma, namely CD4+ and CD8+ T cells, B cells, macrophages, and mast cells, were significantly elevated in both HIV-negative and HIV-positive high-grade dysplasia groups compared with the group of normal cervical tissue. Furthermore, the macrophage counts in the epithelium in both high-grade dysplasia groups were also elevated compared with the normal group. In the

comparison between HIV-negative and HIV-positive high-grade dysplasia groups, CD4+ T cell counts in the stroma and macrophage counts in the epithelium of HIV-positive group were significantly decreased compared with HIV-negative group. However, in case of mast cells, the stromal mast cell counts in HIV-positive group were significantly increased compared with HIV-negative group. Additional studies on the functional characteristics of immune cells infiltrated in the dysplastic cervix especially in women with HIV disease are needed to determine how HIV infection effects the immune cells responding to dysplasia in the cervix.

(326 words)

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**Introduction**

This dissertation contains three papers. The first paper, titled "Recent developments in understanding the immune response to human papillomavirus (HPV) infection and cervical neoplasia," provides an overview of the immune system and describes the systemic and local immune response to HPV infection in the cervix and to the development of cervical dysplasia [Oncology Nursing Forum 27(4), 2000]. The second paper, titled "Lymphoid follicles are generated in high-grade cervical dysplasia and have differing characteristics depending on HIV status," characterized germinal centers in the cervical mucosa which were associated with high-grade squamous intraepithelial lesions (SIL) and identified CD8+ T cell-dominant aggregates which were unique to the cohort of HIV-positive high-grade SIL (submitted). In addition, HPV 16 E7 protein was detected in some of the germinal centers in cervical tissue with high-grade SIL. Related to the study presented in the second paper, the third paper, titled "Mucosal immune cell numbers are increased in the cervix of high-grade dysplasia but are altered in HIV-positive cohort," reports significantly elevated numbers of mucosal lymphocytes and inflammatory cells in the dysplastic cervical tissue compared with normal cervix. In this study, quantification of immune cells excluded areas with any types of aggregates. The increased numbers of immune cells, namely CD4+ T cells and macrophages, in dysplastic cervical tissue from HIV-positive women were attenuated compared to HIV-negative women. However, the number of mast cells in the stroma of dysplastic cervix in the HIV-positive group

was increased significantly compared to the HIV-negative group, and the number of B cells in the dysplastic epithelium of HIV-positive cohort was significantly elevated compared with the cohort with normal cervix. These findings lead us to further investigate the functional status of these mucosal immune cells in the dysplastic cervix particularly in HIV-positive cohort. Further knowledge of the mucosal immune response in the cervix of high-grade SIL will help identify better prevention and treatment for cervical dysplasia particularly among the HIV-positive population.

# Recent Developments in Understanding the Immune Response to HPV Infection and to Cervical Neoplasia

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**Abstract**

Purpose/Objective: To provide an overview of the immune system and describe the systematic and local immune response to human papillomavirus (HPV) infection in the cervix and to the development of cervical dysplasia.

Data Source: Historical and current medical and nursing literature, current gynecologic oncology texts.

Data Synthesis: The immune system provides protection against a wide variety of pathogens and plays a role in response of the body to neoplasitic cells. The immune system orchestrates the functions of various immune cells and proteins to fight against invading pathogens. Mucosal immunity is one of the key functions of the immune system and has unique features. Humoral and cytotoxic responses in the cervix and in the peripheral blood are seen during clearance of an HPV infection.

Conclusions: The functional status of the immune system is associated closely with the development of cervical dysplasia and cancer in women with HPV infection. Clinicians must assist women in maintaining proper functioning of immune responses.

Implications for Nursing Practice: A better understanding of local and systemic immune responses to HPV infection may help nurses who provide care to women with cervical disease and women at high risk for cervical cancer to deliver better care and clearer information to patients.

**Introduction**

Human Papilloma Virus (HPV) infection is currently the most prevalent sexually transmitted disease in the United States, and over 90% of cervical cancer cases are thought to be associated with HPV (Lorincz, Reid, Jenson, Greenberg, Lancaster, & Kurman, 1992; Schiffman, 1994; Tindle, 1997). About 11% of HPV infections with genital warts or mild HPV infection progress to cervical dysplasia, and only one percent of the cases of mild dysplasia progress to invasive cancer (Osteor, 1993). However, more than 12% of the cases of severe dysplasia progress to invasive cancer if a woman does not receive an adequate intervention. Among the various HPV genotypes, HPV16 and 18 are identified as the predominant types associated with the development of cervical cancer (Yee, Krishnan-Hewlett, Baker, Schlegel, & Howley, 1985; Schwarz, Freese, Gissmann, Mayer, Roggenbuck, Stremlau, et al., 1985; Deurst, Kleinheinz, Hotz, & Gissman, 1985; Deurst, Gissmann, Ikenberg, & zur Hausen, 1983). Human papillomavirus type 16 is more commonly detected in squamous cell carcinoma of the cervix than type 18, and HPV 18 is more frequently associated with the development of adenocarcinoma of the cervix (Arends, Donaldson, Duvall, Wyllie, & Bird, 1993; Sobti, Capalash, Sehgal, Gupta, & Sharma, 1996; Stoler et al., 1992).

Many women who are infected with HPV have latent infection with no clinical or cytological manifestations. Because no viral proteins are produced, women in the latent phase are not infectious. A small proportion of women with

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HPV infection develop condyloma (warts) which can produce infectious viral particles. In most cases, this clinical stage regresses spontaneously (Schneider & Koutsky, 1992). When morphologically abnormal, immature basal-type cells develop above the germinal layer of the epithelium, the infection is considered to have progressed to the next stage of abnormal growth in the cervix, which is called cervical dysplasia (Morrison, 1994). The term dysplasia is used widely to describe abnormal tissue development. Generally, cervical dysplasia is defined as a disturbance in the differentiation of the squamous epithelial lining that has the potential to develop into a malignancy (Wright, Kurman, & Ferenczy, 1994). Cervical dysplasia is divided into three grades based on the presence of specific histological abnormalities: mild (immature basal cells over the lower third of the epithelium), moderate (about one-half to two-thirds of the epithelium), or severe (entire thickness of the epithelium) (Figure 1). Dysplasia may regress without medical or surgical interventions. However, when the lesion is untreated, a woman has a greater chance of developing invasive cervical carcinoma from dysplasia than from condyloma (Osteor, 1993).

Infection with HPV is a major risk factor for the development of cervical cancer, but other factors are also implicated with cervical disease. Cigarette smoking (Ho, Kadish, Burk, Masu, Palan, et al., 1998; Olsen, Dillner, Gjoen, Suer, Orstavic, et al., 1996; Becker, Wheeler, McGough, Parmenter, Stidley, et al., 1994; Ngelangel, Munoz, Bosch, Limson, Festin, et al., 1998; Schiffman, Bauer, Hoover, Glass, Cadell, et al., 1993) and certain sexual behaviors, such as multiple sexual partners (Ho, et al., 1998; Olsen, et al., 1996) are associated with

increased risk for cervical dysplasia. Smokers have up to a 4.3-fold higher risk of cervical cancer than nonsmokers, and a dose-response relationship between cigarette smoking and the development of cervical cancer has been reported (Winkelstein, 1990; Ho, et al., 1998; Greenberg, Vessey, McPherson, & Yeates, 1985). In the United States, cervical cancer is the third most common cancer among Hispanic women (Page, & Asire, 1985), and the incidence and mortality rates of cervical cancer among African-Americans are about twice those reported for Caucasian women (Kosary, Ries, Miller, Hankey, Harris, et al., 1995; Wingo, Bolden, Tong, Parker, & Martin, 1996).

An additional key factor that affects the rate of progression from an HPV infection to neoplastic transformation is the functional status of the immune system of the host. Currently, only limited and conflicting data are available on the link between the local immune response to HPV infection in the cervix and the development of cervical dysplasia. A better understanding of the relationship between the immune response to HPV-associated cervical dysplasia may help clinicians provide clearer information and support to patients and family members. In addition, defining the role of mucosal immunity in disease prevention will allow the development of effective vaccines and new therapeutic strategies. The purpose of this paper is to provide an overview of the immune system and to describe the current state of knowledge regarding the systemic and local immune response in the cervix to HPV infection.

**Overview of the Immune System**

Multiple micro-organisms surrounding us in our everyday lives invade our bodies and cause illness. However, the same pathogenic organism may cause a serious illness in some but may not affect the health of other people. Multiple factors are involved in the variable human responses to pathogens, and one of the major factors is the immune system of the individual. In general, the immune system integrates two activities, the body's recognition of antigens and the response to these antigens. An antigen is a molecule that binds to an antibody or receptors on immune cells. In order for the immune system to respond to invading pathogens or abnormal cell growth, the immune system initially needs to recognize these pathogens or the abnormal cell growth as targets for immune activities. This recognition of pathogens leads to an immune response in one or multiple locations within the host.

<u>Innate Immunity</u>

Various white blood cells including lymphocytes are key components of the immune system. The immune system is characterized by two major arms: innate and acquired immunity (Figure 2). The innate and acquired immune systems are interrelated. The innate immune system is the key player in the initial events of the immune response and it ultimately shapes the outcome of the acquired immune response. The cells of the innate immune system non-specifically recognize invading pathogens. Some examples of innate immune activities include: phagocytosis by macrophages, endocytosis by neutrophils, the

killing effect of natural killer (NK) cells, the killing of large parasites by eosinophils, and the destruction of cells by one of the complement pathways.

Acquired Immunity

The acquired immune system is pathogen-specific. Antigen-specific T cells and B cells, and antigen-presenting cells (APCs), such as dendritic cells and macrophages, are the key components of acquired immunity. Macrophages phagocytose antigens nonspecifically but present portions of phagocytosed antigens to T cells of the acquired immune system. Acquired immunity has two functional branches: humoral and cell-mediated immunity. Antibodies secreted by activated B cells are called immunoglobulins (Ig) and are the major component of humoral immunity. Cytotoxic T cells play a central role in cell-mediated immunity.

Upon invasion into the host, a pathogen is usually captured by an APC of the immune system. A captured antigen is processed within an APC and a small portion of the antigen (peptide) is presented to other cells of the acquired immune system as a complex with a class II major histocompatibility complex (MHC) molecule on the surface of the APC. This complex, made up of an antigen peptide and a class II MHC molecule, is called a class II MHC-peptide complex.

Pathogen-specific acquired immunity has several critical functional characteristics. One of them is the production of memory T cells and B cells which remain in the system for a long period of time. This memory function makes the response of the immune system to re-exposure to the same or related

pathogens more efficient, as the response is more rapid and more intensified during the second exposure. The acquired immune system also has the capability of generating diversity in its recognition molecules, which allows specific recognition to an extremely large number of unique antigen structures.

One of the key components of the acquired immune system is the CD4+ T cell. T cells express T cell receptors (TCRs), which recognize an antigen only when the antigen is presented in an MHC-peptide complex. T cells are divided into two major subgroups based on their distinct cell surface markers, namely CD4 (on helper T cells) and CD8 (on cytotoxic T cells) molecules. Class I and II MHC molecules on an APC participate in the acquired immune system by presenting the antigen to CD8+ and CD4+ T cells, respectively. Once CD4+ T cells recognize the class II MHC-peptide complex and receive additional signals by APCs, they are activated, proliferate, and become functional helper T cells. CD4+ T cells are called helper T cells because, as they become activated, they secrete specific cytokines that induce the differentiation of other cell populations, such as B-cells into antibody-secreting plasma cells, and CD8+ T cells into cytotoxic T lymphocytes (CTLs). Cytokines are protein substances secreted by lymphocytes that can serve as paracrine mediators (i.e., participating in cell-to-cell communications between the immune cells). Certain cytokines also function as autocrine mediators (i.e., communicating with the same cell from which the cytokine was secreted). An example of an autocrine mediator is interleukin (IL)-2, a cytokine secreted by activated helper T cells. Interleukin 2 participates in the induction of differentiation of helper T cells.

## Humoral Immunity

Plasma cells mediate humoral immunity. When B cells encounter antigens, they are activated, differentiate into plasma cells, and secrete antibodies. Antibodies that neutralize invading extracellular pathogens, such as bacteria and parasites, are one of the key features of humoral immunity.

Prior to differentiation to plasma cells, B cells express membrane-bound antibodies on their cell surface. When the membrane-bound antibody binds specifically with high affinity to an antigen and receives appropriate signals from APCs and helper T cells, the B cell is activated, proliferates, and differentiates into antibody-producing plasma cells. Antibodies are classified as IgM, IgG, IgE, and IgA, according to their unique structures and functions, and all are antigen-specific. The secreted antibodies may neutralize the pathogen that expresses a specific antigenic epitope (a region within an antigen, that actually binds to T cell and B cell receptors). A single B cell may express different classes of antibodies on its cell surface, but all antibodies made by one B cell have the same antigen specificity. B cells may switch production of antibodies from one class to another during the response to antigens.

The primary function of all types of antibodies is to bind to antigens, but each class of antibodies also has unique functions (Table 1). For example, IgG is the most abundant class in serum and can activate the complement cascade. The complement system is a major effector of humoral immunity and participates in an enzymatic cascade which destroys pathogens. IgM is the first immunoglobulin class produced in a primary response to an antigen and can also

initiate the complement cascade. IgA is the predominant immunoglobulin in external secretions such as breast milk and mucus and is an essential component of mucosal immunity. IgE is recognized by mast cells in tissues and mediates immediate type hypersensitivity reactions.

Cell-Mediated Immunity

Activated helper T cells also assist CD8+ T cells to become CTLs. Immune function that is mediated predominantly by activated CD8+ T cells is a critical component of cell-mediated immunity, which controls invading intracellular pathogens such as viruses and some bacteria. Since all nucleated cells express class I MHC molecules on their cell surface, all have the potential to be recognized by TCRs on CD8+ T cells after being infected by these intracellular pathogens. Infected cells are called altered self cells because they express foreign antigens as well as self-peptide in class I MHC on their surface. In certain circumstances, transformed (malignant) cells may also express non-self peptide, be recognized as altered-self cells, and become targets of cell-mediated immunity.

Mucosal Immunity

One of the important anatomic barriers against foreign invaders is the mucous membrane. The mucosal immune system present in the mucous membrane protects the host from environmental pathogens and antigens. The functional sites of mucosal immunity are in the respiratory, gastrointestinal, and genitourinary tracts. Although most immune cells cross-circulate between the systemic immune system and the mucosal immune system, the mucosal immune

system is generally thought to be independent of the systemic immune system (Conley & Delacroix, 1987).

One of the key players in mucosal immunity is a specific type of T cell, called the intraepithelial lymphocyte (IEL). These T cells also express TCRs and are localized mainly in the epithelial layer of the mucosa. Most of these IEL cells express CD8 molecules and possess cytotoxic functions.

Secretory IgA (sIgA) primarily mediate mucosal immune responses (Brandtzaeg, 1974a; 1974b; Stokes, Soothill, & Turner, 1975). Columnar epithelial cells express constitutively a group of molecules called secretory component (SC). These molecules translocate sIgA, produced by plasma cells present within the structure of the mucosa, to the surface of the mucous membranes where sIgA can interact with the external environment to neutralize pathogens. Secretory IgA together with SC molecules are important elements of mucosal immunity along with IEL.

Another important characteristic of mucosal immunity is the presence of mucosal associated lymphoid tissues (MALT). Examples of MALT are the tonsils, appendix, and Peyer,s patches. However, MALT-like structures may also exist in the mucosal stroma as loose clusters of lymphoid cells with little organization. The presence of MALT is important in mucosal immunity because the interactions between APCs and T helper cells take place in MALT. In these lymphoid follicles, APCs express a portion of antigen in the MHC complex so that the antigen is recognized by immune cells. Successful antigen-recognition activity may induce immune responses such as activation of CD8+ T cells and B

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cells.  Mucosa without organized lymphoid follicles is likely to be less effective at antigen recognition and hence an attenuated local response.


**Mucosal Immunity of the Lower Female Reproductive Tract**

In order to understand the mucosal immune system in the lower female reproductive tract, particularly in the cervix, knowledge of the anatomy of this area is critical.  Therefore, a brief overview of the anatomy and the mucosal structure of the cervix is presented in the following section.

<u>The Cervix</u>

The cervix of the human uterus is the lowest portion of the uterus and protrudes into the upper vagina.  The cervix has two anatomically distinct areas: the endocervix and the ectocervix also called the exocervix (Figure 3-a).  The endocervix is the canal between the uterine corpus and the external os and is lined with columnar epithelial cells.  These cells secrete mucin into the lower reproductive tract.  The ectocervix is named for the area called the uterine portio which protrudes into the vagina and is covered by stratified squamous epithelial cells.  Beneath the columnar and squamous epithelium throughout the endo- and ectocervix is the stroma, which is composed of connective tissue.  The area where the stratified squamous epithelium of the ectocervix and the mucin-secreting columnar epithelium of the endocervix meet is called the transformation zone (Figure 3-b).

## The Transformation Zone

The transformation zone, also called the squamocolumnar junction, moves constantly towards the uterine corpus as a woman ages. Squamous metaplasia is the process by which columnar epithelial cells are replaced by stratified squamous cells. The transformation zone is the area of the cervix where cervical dysplasia and cervical cancers most often develop.

## Mucosa of the Ectocervix

The squamous epithelium of the ectocervix is divided into four layers: the basal, parabasal, intermediate, and superficial (Figure 4). During transition from the basal layer to the surface of the epithelium, cells differentiate into mature squamous cells. The layer of cells attached to the basal lamina overlying the stroma is called the germinal cell layer and is composed of basal cells and parabasal cells. A single basal-cell layer on the basal lamina is the source of epithelial cell regeneration. A few layers of small round-shaped parabasal cells with large nuclei lie above the row of basal cells. Above the parabasal-cell layer are intermediate cells which are more differentiated. The intermediate layer dominates the epithelium. The outer most surface of the ectocervical mucosa is the superficial layer composed of flattened, highly differentiated, non-keratinized cells with a high cytoplasmic to nuclear ratio.

In the stroma below the epithelium, a well-developed capillary network exists to supply nutrients and oxygen to the epithelial cells. In addition to capillaries, subepithelial stroma contains connective tissue fibers, loose accumulations of lymphocytes, and occasional nerve fibers. In the

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transformation zone, glands lined with mucin-secreting columnar epithelial cells, are also present in the stroma (Ferenczy & Wright, 1994).

Mucosal Immunity in the Lower Female Reproductive Tract

Although no MALT-like organized follicles are thought to be present in the lower female reproductive tract, studies demonstrate loose accumulations of lymphoid cells in the cervix and in the transformation zone. CD4+ and CD8+ T cells have been observed either as individual cells or in loose accumulations along with scattered macrophages and dendritic cells in the squamous epithelium and the stroma of the cervical and vaginal mucosa (White, Yeaman, Givan, & Wira, 1997b). Small numbers of B cells have been found in the mucosa of the cervix and the vagina. Variable numbers of Langerhans cells (a type of antigen presenting cell) and accumulations of a large number of intraepithelial lymphocytes (predominantly CD8+ T cells) were also observed in the cervix, vagina, and vulva, and in the transformation zone. Since the transformation zone contains aggregates of lymphocytes, researchers have proposed that the transformation zone might be immunologically as effective as other sites where MALT or organized follicles are present (Edwards & Morris, 1985). The key elements of mucosal immunity in the cervix are depicted in Figure 5.

Intraepithelial Lymphocytes in the Cervical Mucosa

Intraepithelial lymphocytes (IELs) are present in the ectocervical epithelium, particularly in the transformation zone. Most IELs are CD8+ T cells, but CD4+ T cells are also seen in the epithelium with less frequency (Miller, McChesney, & Moore, 1992; Roncalli, Sideri, Giáe, & Servida, 1988; White et al.,

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1997a; White et al., 1997b).  CD8+ T cells are less abundant in the stroma of the ectocervix than in the epithelium.  Conversely, CD4+ T cells are more abundant in the stroma than in the epithelium, especially inside the lymphoid accumulations in the transformation zone.  Natural killer (NK) cells are normally not present but have been observed in the setting of infections (Roncalli et al., 1988).  The predominant presence of CD8+ T cells in the epithelium of the transformation zone, the ectocervix, and the vagina suggests that cell-mediated cytotoxic immune responses against viral and bacterial infections occurs in the lower female reproductive tract.

Immunoglobulins in the Cervical Mucosa

IgG and IgA are the major antibodies found in cervico-vaginal secretions and may originate from the peripheral circulation or be produced locally by plasma cells (Kutteh & Mestecky, 1994).  The most predominant immunoglobulin class in the mucosa, IgA, is produced by plasma cells distributed in the subepithelial mucosa, and is translocated across epithelial cells into external secretions by secretory component (SC).  These SC molecules are expressed mainly on columnar epithelial cells in the endocervical mucosa and in the Fallopian tubes (Kutteh, Hatch, Blackwell, & Mestecky, 1988).  This finding suggests that IgA present in the cervico-vaginal secretions originates predominantly from the endocervix.

## Hormonal Changes in the Ectocervix and its Influence on Cervical Mucosal Immunity

Epithelial and stromal fibroblast-like cells of the cervix have estrogen and progesterone receptors. Estrogen receptors are expressed on intermediate, parabasal, and basal cells, especially during the follicular phase of the menstrual cycle when the maturation process of the epithelium is accelerated (Konishi et al., 1991; Nonogaki et al., 1990; Press, Nousek-Goebl, Bur, & Greene, 1986) (Fig. 6 for menstrual cycle). However, compared with the endocervix, squamous epithelial cells of the ectocervix and the vagina have relatively limited cyclic variation in estrogen receptor expression (Press et al., 1986). Progesterone receptors may be expressed during the luteal phase in the parabasal cells. Stromal cells express both estrogen and progesterone receptors throughout the menstrual cycle (Press et al., 1986).

Along with the cyclic variations in the expression of estrogen and/or progesterone receptors, structural changes in the mucosa of the lower female reproductive tract occur in association with changes in the circulating levels of sex hormones (Konishi et al., 1991; Nonogaki et al., 1990; Press et al., 1986). For example, the maturation process of epithelial cells in the endometrium of the uterus is accelerated during the follicular phase of the menstrual cycle. However, little data are available on the effects of cyclic hormonal variations on the functional aspects of mucosal immunity in the lower reproductive tract.

In humans, the most vulnerable time period for infections of the female lower reproductive tract is during the luteal phase of the cycle and during

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pregnancy when estrogen receptor expression is reduced and progesterone receptor expression is increased in the squamous epithelium of the cervix (Konishi et al., 1991). During these periods of time, the local production of IgA in the lower reproductive tract is decreased, compared to levels during the follicular phase (Kutteh, Moldoveanu, & Mestecky, 1998; Kutteh, Prince, Hammond, Kutteh, & Mestecky, 1996), leaving the host susceptible to infection. Findings from one study indicated that in humans, *in vitro* cytolytic activity by CD8+ T cells existing within squamous epithelial cells or in the stroma of the ectocervix persisted throughout the menstrual cycle and following menopause (White et al., 1997b). In contrast, the *in vitro* cytotoxic activity by CD8+ T cells isolated from the endometrium of the uterus was lost during the secretory phase of the menstrual cycle (White et al., 1997a). Reduced mucosal immune responses in the female reproductive tract during pregnancy may serve to diminish maternal anti-fetal responses, thereby making spontaneous abortion less likely (White et al., 1997a).

**Immune Responses to HPV Infection and to Cervical Neoplasia**

<u>Systemic Immune Response to HPV Infection</u>

Only a limited number of studies are available on the immune responses to HPV infection and to cervical neoplasia, and the findings are not always consistent. Therefore, contrasting findings and implications for future studies are presented in this section. In one study on immune cells derived from the peripheral blood, CTL activity by CD8+ T cells against HPV antigen was more

prevalent in HPV-seropositive women without cervical dysplasia than HPV-seropositive women with dysplasia (Nakagawa et al., 1997). However, in other studies, CTL activity against HPV antigen in the peripheral blood was detected in women with cervical cancer (Evans, Man, Evans, & Borysiewicz, 1997) and women with high-grade cervical dysplasia (Nimako, Fiander, Wilkinson, Borysiewicz, & Man, 1997). Although these studies used similar CTL assays and HPV antigen stimulation, there are variations in the cell preparation process which may have produced differences in the results.

Antibody levels against HPV in the peripheral blood are elevated during persistent HPV infection, and women with persistent HPV infection are more likely to develop high grade cervical dysplasia or cancer (Carter et al., 1996; de Gruijl et al., 1997; Lehtinen et al., 1996; Nonnenmacher et al., 1995; Shah, Viscidi, Alberg, Helzlsouer, & Comstock, 1997; Wideroff et al., 1995). Therefore, while antibody production and CTL activity in the peripheral blood are present in HPV infection, the role of these parameters in viral clearance is unclear.

Mucosal Immune Responses to HPV Infection in the Cervix

Most studies on immune responses to HPV infection and cervical dysplasia use immune cells derived from the peripheral blood. Currently, the relationship between the systemic immune response detected in the peripheral blood and the actual functioning of the local immune system in the cervix remains unclear. The results of a limited number of studies on mucosal immune responses to HPV infection in the cervix are summarized in the following section.

T cell recruitment to sites of HPV infection and cervical dysplasia has been observed (Bell et al., 1995; Edwards et al., 1995; Evans et al., 1997; Takehara, 1996). However, the number of T cells at the site of cervical cancer are not consistent across studies. CD4+ T cells are abundant in the cervical stroma below the area of dysplasia, whereas CD8+ T cells are predominant within dysplastic epithelium (Bell et al., 1995; Takehara, 1996). CD8+ tumor infiltrating lymphocytes (TILs) have been observed in cervical cancer (Bell et al., 1995; Edwards et al., 1995; Evans et al., 1997). Once a tumor develops, the numbers of TILs may continue to increase in some cases, but presumably they are not effective in inhibiting the existing neoplasia from further growth.

Mast cells are known to have anti-tumor activity (Young, Liu, Butler, Cohn, & Galli, 1987). Higher numbers of mast cells accumulate in the area of high-grade cervical dysplasia than in the area of invasive cancer (Jing, Xue, Zhang, Yao, & Dong, 1993). Possibly, the anti-tumor activity of mast cells is initiated in the early stage of tumorigenesis, but the activity becomes ineffective after the tumor grows to a certain size.

A study to determine immunoglobulin isotypes associated with HPV demonstrated that IgG-secreting plasma cells (activated B cells) are elevated in cervical mucosa with HPV infections and low grade dysplasia. However, the numbers of these cells decreased as cervical disease progressed. This finding suggests that the local production of antibodies is recruited during an active HPV infection but is lost in the neoplastic stage of cervical disease (Edwards et al., 1996).

**Implications for Future Research**

Because of the paucity of research in this area, numerous studies are needed to characterize the systemic and local mucosal immune responses to HPV infection and cervical dysplasia. Immunological studies of the cervix of women with HPV infection with or without cervical dysplasia, utilizing in situ hybridization techniques, need to be done to characterize the activities of immune cells identified and localized in cervical biopsy samples by immunohistochemistry. In addition, the technique of flow cytometry can be utilized to characterize immune cells at an individual cell level in women with HPV infection with or without cervical dysplasia. These types of studies may lead to the development of specific interventions at the systemic or local level to prevent the development of cervical dysplasia.

**Clinical Implications**

Currently, it remains unclear why the immune system in some women can successfully control HPV infections whereas the immune system in others is not so successful. Many factors may affect a response to HPV infection, including compromised immunity. It is undoubtedly important for healthcare workers to assist women, particularly those at high risk, to maintain proper functioning of local as well as systemic immune responses. In order to have an adequate level of immune functioning, the foci of educational programs for women need to include the following areas. The importance of routine physical examinations

with Papanicolaou smears and, when indicated, colposcopy needs to be emphasized to monitor the condition of the cervix and to detect cervical disease at an early stage. The role of HPV typing in women with persistent or recurrent HPV infection is an area of active investigation. The American College of Obstetrics and Gynecology does not recommend routine HPV typing since HPV testing lacks the specificity necessary to be a useful screening test for cervical cancer or precursors, and because high-risk HPV types (i.e., 16 and 18) can also be detected in many patients with low grade cervical dysplasia or no detectable abnormalities (ACOG Technical Bulletin, 1993). Maintaining proper nutrition including modifying alcohol intake is recommended for optimizing immune functioning (Friedman, 1998; Szabo, 1998; Keusch, 1998; Pedersen, Ostrowski, Rohde, & Bruunsgaard, 1998). An increased risk of cervical cancer is observed in smokers, and a dose-response relationship between cigarette smoking and cervical cancer have been reported (Winkelstein, et al., 1990; Greenberg, et al., 1985). In addition, smoking cessation may result in a reduction in the size of low-grade lesions in the cervix (Szarewski, Jarvis, Sasieni, Anderson, Edwards, et al., 1996). Smoking cessation and refraining from drug use are proposed as behavioral approaches to enhance immune function and prevent cervical diseases (Ho et al., 1998; Roteli-Martins et al., 1998).

HPV-infected individuals need education about safe sexual practices because a decreased frequency of high-grade cervical dysplasia was reported with increased frequency of condom use (Ho et al., 1998; Muanoz et al., 1996). These epidemiological studies suggest that intervention studies are warranted to

evaluate the effects of various approaches to behavioral modification on prevention of cervical cancer and the enhancement of the immune system.

The negative effects of stress on immune system are documented in a number of clinical and research papers (Ader & Cohen, 1993; Brosschot et al., 1994; Brosschot et al., 1998; Dantzer, 1997; Kay, Tarcic, Poltyrev, & Weinstock, 1998; Stein, 1989). Stress reduction activities may prove beneficial in maintaining optimal functioning of the immune system (Kang, Coe, Karaszewski, & McCarthy, 1998; Whitehouse et al., 1996). However, more research is needed on the effects of stress on the immune system and whether or not stress reduction techniques prove to be beneficial.

The information presented in this paper, particularly the research findings on mucosal immunity, may not be immediately applicable to clinical practice. More research on systemic and mucosal immune responses to HPV infection and cervical cancer are needed before definitive preventive strategies or treatment techniques are implemented in the clinic. However, oncology nurses need to have a working knowledge of the functioning of the immune system at the systemic and local level in order to better understand the complex interactions between HPV infection and neoplastic transformation. In addition, women should be taught strategies to enhance the optimal functioning of the immune system and reduce the risk of developing cervical cancer.

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Table 1. Characteristics of antibodies

| Antibody class | Characteristics |
|---|---|
| IgM | The first immunoglobulin (Ig) class in a primary response; initiates the complement cascade; the best in agglutinating |
| IgG | The most abundant class in serum; initiates the complement cascade; the only Ig class that passes through the placenta and confers maternal immunity on the fetus |
| IgA | Predominant Ig class in external secretions; an essential component of mucosal immunity |
| IgE | Recognized by mast cells; mediates the immediate hypersensitivity response; important Ig class in parasitic infections |

**Figure legends**


Fig 1. Schematic representation of cervical cancer precursors and different

terminologies that have been used to refer to them.

Mild dysplasia is defined as immature basal cells over the lower third of the

epithelium, moderate dysplasia is about one-half to two-thirds of the epithelium,

and severe dysplasia indicates immature cells over the entire thickness of the

epithelium. CIN: cervical intraepithelial neoplasia; SIL: squamous intraepithelial

lesion.


Fig 2. Summary of the immune system


Fig 3. Schematic diagram of the human uterus.

a.  General view of uterus and vagina.

b.  b. Enlarged view of the cervix. Squamous mataplasia of the transformation

    zone moves constantly toward the uterine corpus as a woman ages.

BM: basement membrane.


Fig 4. Histological diagram of the normal ectocervix.

During the transaction from the basal layer to the surface of the epithelium, cell

differentiate into mature squamous cells.

Fig 5. Mucosal immune cells in the ectocervix.

CD8 T cells are abundant in the epithelium. A phenotype of dendritic cells (Langerhans cells) is present in the basal cell layer. Dendritic cells and macrophages are scattered in the epithelium and the stroma. A loose association of lymphocytes is seen in the stroma. Lower numbers of B cells are present. Natural killer cells generally are not present unless infection occurs.

Fig 6. Plasma hormone levels throughout the menstrual cycle.

# Fig.1

| NORMAL | LOW GRADE SIL | | HIGH GRADE SIL | | | INVASIVE CANCER |
|---|---|---|---|---|---|---|
| | | CIN 1 | CIN 2 | CIN 3 | | |
| | CONDY-LOMA | MILD DYSPLASIA | MODERATE DYSPLASIA | SEVERE DYSPLASIA | IN SITU CARCINOMA | |



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Fig. 2

Immune System
- Innate: Macrophage, Neutrophil, NK Cell, Eosinophil
- Acquired: APC and CD4+ T cell
  - Humoral: B cell
  - Cell-mediated: CD8+ T cell

# Fig. 3

a.

UTERINE CORPUS

ENDOCERVIX

ENLARGED WITH DETAIL IN b.

ECTOCERVIX

VAGINA

b.

STRATIFIED SQUAMOUS EPITHELIUM

BM

COLUMNAR EPITHELIUM

GLANDS OF COLUMNAR EPITHELIUM

CERVIX

VAGINA

SQUAMOUS METAPLASIA

TRANSFORMATION ZONE

**40**

# FIG. 4



SUPERFICIAL CELL

INTERMEDIATE CELL

PARABASAL CELL
BASAL CELL
} GERMINAL

CAPILLARY NETWORK

EPITHELIUM

BM

STROMA

41

# FIG. 5



SUPERFICIAL CELL

INTERMEDIATE CELL

PARABASAL CELL
BASAL CELL
} GERMINAL

EPITHELIUM

BM

STROMA

CD 8 T CELL

CD 4 T CELL

B CELL, Plasma CELL

MACROPHAGE

CAPILLARY NETWORK

DENDRITIC CELL

BASEMENT MEMBRANE

42

Fig. 6

MENSES   FOLLICULAR   OVULATORY   LUTEAL   MENSUS

PROGESTERONE ——
ESTRADIOL ——

43

# Lymphoid follicles are generated in high grade cervical dysplasia and have differing characteristics depending on HIV status

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## Abstract

The exact role of the mucosal immune response in the pathogenesis of human papillomavirus (HPV)-related premalignant and malignant diseases of the genital tract is poorly understood. We used immunohistochemical analysis to characterize immune cells in normal cervical tissue (N=21), HIV-negative high-grade dysplasia (N=21), and HIV-positive high-grade dysplasia (N=30). Classical germinal centers were present in 4.7% of normal cervical tissue, 33% of high-grade lesions from HIV-negative women and 3.3% of high-grade lesions from HIV-positive women (p=0.003). HPV16 E7 antigen was detected in a subset of germinal centers, indicating that the secondary immune response was directed in part against HPV. Lymphoid follicles were present in 9.5% of normal cervical tissue, 57% of HIV-negative high grade SIL, and 50% of HIV-positive high-grade dysplasia (p=0.001 normal versus high grade). A novel type of lymphoid aggregate, consisting predominantly of CD8+ T cells, was detected in 4.8% of normal cervical tissue, 0% of HIV-negative high-grade dysplasia, and 40% of HIV-positive high-grade dysplasia (p<0.001). The recurrence rate of high-grade dysplasia within one year was significantly higher in women with such CD8+ T cell-dominant aggregates (p=0.02). In summary, lymphoid follicles were present in the cervix of high-grade dysplasia and the types of lymphoid follicles in HIV-positive women were significantly different than those from HIV-negative women.

## Introduction

Human papillomavirus (HPV) infections of the cervix can result in a series of changes in the epithelium known as cervical dysplasia. The early changes (i.e., low-grade squamous intraepithelial lesion [SIL]) reflect active viral replication and are clinically innocuous. The more advanced forms of dysplasia (i.e., moderate and severe dysplasia and carcinoma in situ; high grade SIL) represent potential cancer precursors; ≥ 12% of severe dysplasia will progress to cancer if left untreated (1). HPV from high-risk viral types can be detected in >90% of high grade SIL and cervical cancers, strongly implicating the virus as an etiological agent in cervical carcinogenesis (2-4). However, a large percentage of HPV infections are clinically undetectable and do not result in dysplasia or cancer. Multiple epidemiological studies have reported that HPV infection is detected more frequently, and that the incidence and severity of genital neoplasia are higher among immunocompromised women, including those with human immunodeficiency virus (HIV) infection (5-10). These data suggest that the host immune response to HPV is critical in determining the outcome of HPV infection, and in 1992, invasive cervical cancer was included as an AIDS-defining illness in HIV-positive women by the Centers for Disease Control and Prevention (11).

The exact relationship between altered immune function and the development of HPV-related cervical cancers has not been elucidated. The purpose of this study was to test the hypothesis that high-grade SIL of women with HIV infection is characterized by attenuation of the numbers and function of

infiltrating mucosal lymphocytes and inflammatory cells compared to lesions from uninfected women. Since low-grade SIL is unlikely to progress to cancer and has a high likelihood of spontaneous regression, we focused this study on high-grade SIL, a true cancer precursor. We performed immunohistochemical analyses to characterize the types and organization of inflammatory cells in normal cervical tissue and samples of high-grade SIL from HIV-positive and HIV-negative women. We find that lymphoid follicles are a common feature of high-grade SIL. In addition, classical germinal centers are found in a subset of lesions from HIV-negative women but are uncommon in HIV-positive women, and a novel type of aggregate is abundant in lesions from HIV-positive women.

## Materials and methods

Specimens and data for the HIV-positive participants in this study were obtained in collaboration with the Women's Interagency HIV Study (WIHS), a longitudinal multi-site cohort study of women with or at risk for HIV infection. Study sites included consortia based at the Bronx Lebanon Hospital; State University of New York, Brooklyn; Georgetown University; Cook County Hospital; University of California, San Francisco; and the University of Southern California. Detailed methods for this study were published previously (12). All study procedures and consent materials were reviewed and approved by human subjects protection committees at each collaborating institution and by the WIHS executive committee. HIV infections were verified by EIA serology with Western blot confirmation. CD4 cell counts of peripheral blood were performed by flow

cytometry in laboratories participating in the FACS quality assurance program.

Plasma HIV levels were determined using the Organon Teknica NASBA test in

laboratories participating in the NIH virology laboratory quality assurance

program. Cervical infections with Chlamydia trachomatis (C. trachomatis) and

Neisseria gonorrheae (N. gonorrheae) were identified by PACE-2 DNA probe

tests (GenProbe, San Diego CA). Bacterial vaginosis was identified by gram

stain as previously described (13). Candidal and trichomonas vaginitis were

identified by wet mount with saline and 10% KOH. Cervicovaginal lavage with

10ml saline was performed at each visit, and the resulting material aliquoted and

frozen within 6 hours of collection. HPV testing was performed using polymerase

chain reaction (PCR) with LI consensus primers as described previously (14).

Pap smears were obtained from each woman, and colposcopy was performed for

those women with abnormal results. Loop and cone biopsies were performed for

standard indications and tissues were archived as paraffin blocks after

pathological evaluation at the originating institution.


Specimens for the normal cervix group were obtained as paraffin-

embedded blocks from the Department of Pathology archives at UCSF from

women who underwent hysterectomies for benign uterine disease with no

cervical abnormalities. Specimens and data for the HIV-negative high-grade SIL

group were obtained as paraffin-embedded sections of cone and loop excisions

from the UCSF Department of Pathology Archives and WIHS. All samples

obtained from the WIHS cohort were selected from women who had HPV 16 detected on cervicovaginal lavage specimens.

Immunohistochemistry (IHC) was performed with primary antibodies against CD4+ T cells with monoclonal antibody (mAb) to CD45R0 (clone OPD4), CD8+ T cells with mAb to CD8 (clone C8/144B), B cells with mAb to CD20 (clone L26) and tissue macrophages with mAb to CD68 (clone KP1) purchased from DAKO, Carpenteria, California. Antibody against perforin (clone Delta G9) was purchased from Endogen (Woburn, Massachusetts), against Ki 67 (clone MIB 1) from Immunotech (Miami, Florida), against BCA-1 (clone 53610.11) from R & D Systems (Minneapolis, Minnesota), and against HPV 16 E7 (TVG710Y) from Santa Cruz Biotechnology (Santa Cruz, California). Each primary antibody was diluted in phosphate buffered saline with 1% bovine serum albumin as follows: 1:500 (CD45R0), 1:200 (CD8), 1:1200 (CD20), 1:500 (CD68), 1:400 (perforin), 1:300 (HPV 16 E7), 1:200 (Ki 67), and 1:20 (BCA-1). Routine IHC was performed following manufacturer's guidelines (Innogenex, San Ramon, California). Prior to the inactivation of endogenous peroxidase with 0.1% hydrogen peroxide, tissue slides were digested with 0.025% trypsin for 10 minutes at 37°C. Antigen retrieval for all primary antibodies with the exception of BCA-1 was performed by heating the slides in a 1.25kW microwave for 2 minutes in 10mM Sorensen's citric buffer (pH 6) and cooling in citric buffer for 30 minutes. Antigen retrieval for BCA-1 was performed by heating the slides three times in a 1.25kW microwave for 2 minutes in 100 mM Tris buffer with 5% urea (pH 9). All antibodies with the

exceptions of BCA-1 and Ki 67 were incubated on tissue sections for one hour at room temperature. Antibodies against BCA-1 and Ki 67 were incubated overnight at 4°C. A 1:3 dilution of hematoxylin gill #1 was used for counter staining. 3, 3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, California) and 3-amino-9-ethylcarbozole (AEC) (BioGenex, San Ramon, California) were used as chromogens.

The E7 open reading frame was subcloned from the HPV16 E7 viral genome (from Dr. J. Palefsky, University of California San Francisco) into the pGEX plasmid (Amrad Corp), to produce a GST-E7 fusion protein. Bacterial cultures in log phase growth were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) 0.1mM, for 3 hours at 37°C. Frozen bacterial pellets were resuspended in 5 volumes of 0.025M Tris at pH 8, 0.025M NaCl, 10% sucrose containing protease inhibitors (1mM pefablock, 0.1mg/ml aprotinin, 0.1 mg/ml leupeptin, and 5ug/ml pepstatin), and sonicated on ice. KCl (0.25M) and DTT (10mM) were added and the lysates were centrifuged at 17,400g for 60 minutes. Supernatents were mixed with 1ml of a 50% slurry of glutathione-agarose beads (Sigma) and incubated for 2 hours at 4°C. The beads were washed four times with ice-cold PBS prior to elution with 50mM Tris pH 8.1 containing 0.25 KCl and 5 mM reduced glutathione (Sigma) for 1 hour at 4°C. The supernatant was dialyzed against 50mM Hepes pH 7.6, 50 mM KCl at 4°C over night. The size and purity of GST and GST-E7 proteins were verified by polyacrylamide gel elecrophoresis. Prior to IHC, the antibody was incubated with

a 10-fold molar excess of GST-E7 or GST at 4°C for 2 hours prior to adding the antibody to the slides.

DNA fragmentation as a result of apoptosis was measured by end-labeling DNA with digoxigenin-labeled dUTP and terminal dioxytransferase, and detection with peroxidase-conjugated anti-digoxigenin secondary antibodies (Apoptag Kit, Oncor, Gaithersburg, Maryland). Size determinations of lymphocyte aggregates were determined using an AcCell apparatus on an Olympus microscope (Ampersand Medical Corp).

The presence of C. trachomatis infection was determined in non-WIHS samples using DNA isolated from paraffin sections in the ligase chain reaction (LCR) (Abbott, Chicago, IL), in the laboratory of Dr. Julius Schacter, San Francisco General Hospital. Briefly, each slide was treated with 0.1% trypsin at room temperature for 5 minutes. DNA was extracted by adding 4μl of LCx urine resuspension buffer (Abbott) to each slide, which was then placed in a heating block stabilized at 97°C for 15 minutes to release the DNA into the buffer. DNA was then added to LCR reaction mixture, and the presence of C. trachomatis was determined following manufacturer's instructions (Abbott).

The five-micron sections stained for each tissue sample in this study were selected randomly, determined by the position of the tissue at the edge of the paraffin blocks at the time of sectioning. If any section from an individual patient

had a specific phenotype of aggregate present, the patient was scored as being positive for that type of aggregate. To determine whether such sampling of the paraffin blocks was representative of the entire sample, we sectioned completely through the paraffin blocks from four different surgical cases of high-grade SIL. Immunohistochemical analysis of every 15th section was then performed with antibodies against CD8 and CD20.

Univariate comparisons were carried out using a chi square or Fisher's exact test for categorical data (e.g. lymphoid aggregates) and analysis of variance methods for continuous variables (e.g. age). For analysis, patients with any lymphoid follicles or germinal centers were combined into one category and the patients with CD8+ T cell-dominant aggregates alone were placed in another. For some of the analyses, any sample with both lymphoid follicles or germinal centers and CD8+ T cell-dominant aggregates were placed in a third group. Subsets of samples stained for E7, Ki67, BCA1 or perforin were selected based upon availability of additional slides or tissue on the block as well as the presence of lymphocyte aggregates on nearby sections.

## Results

The clinical characteristics of the women who contributed tissue samples to this study are summarized in Table 1. The mean age of the normal group (50.1 years) was significantly older than the two groups who had high-grade SIL (32.4 for the HIV-negative and 32.6 for the HIV-positive cohort) (p<0.0001). This

difference in age is likely attributable to the fact that the women in the group with normal cervix were undergoing hysterectomies, and therefore were more likely to be peri- or postmenopausal. Data on smoking was not available for many cases among the normal and HIV-negative high-grade SIL group (31% missing). The reported rate of current smoking among the HIV-positive women was 16 of 28 (57%), 5 of 12 in the HIV-negative group (42%) and 5 of 10 in the normal group (50%).

Organized lymphoid structures were visible in the stroma of many of the high-grade SIL samples as determined by immunostaining for CD4, CD20, CD8 and CD68 (Fig 1 a-d respectively). These dense cellular accumulations had a distinct morphology: the centers of the aggregates were characterized by a predominance of B cells with widely distributed CD4+ helper T cells and macrophages. CD8+ T cells were scattered at the periphery of the aggregates. These accumulations resemble germinal centers (GCs) that are present in secondary lymphoid organs such as tonsils and lymph nodes. Apoptotic bodies were present in the macrophages in cervical GCs as detected by morphology (tingible body macrophages, Fig 1e) and by DNA fragmentation analysis (Fig 1f). The mean diameter of GCs was 286.2 microns (range 78 - 591 microns, N = 16). As shown in Table 2, we observed well formed GCs in 1/21 samples from normal cervix (4.7%), 7/21 samples of high-grade SIL from HIV-negative women (33.3%) and 1/30 samples of high-grade SIL from HIV-positive women (3.3%); differences between the 3 groups are statistically significant (p=0.003). The difference in the

frequency of GCs between high-grade SIL from HIV-negative women versus high-grade SIL from HIV-positive women is also statistically significant (p=0.006).

Germinal centers have been described in the cervix in the context of chronic and follicular cervicitis (15, 16). In our study, the diagnosis of follicular cervicitis was reported on the pathology report of only 2 of the 8 samples with immunophenotypically defined GCs, and there was no mention of GCs on the other pathology reports. These data indicate that the presence of GCs is a common finding in samples of high-grade SIL from HIV-negative women, and their presence would not necessarily be appreciated by standard histological analysis.

Several studies have shown an association between GCs and chlamydia infection (17-19), and chlamydia infection in turn is associated with the presence of SIL (18, 20-22) and cervical cancer (23). To determine whether the GCs observed in the high-grade samples were associated with the dysplastic process itself, or a reflection of a concurrent sexually transmitted disease or vaginitis, we reviewed the WIHS database of patients contributing samples to the study. Previously published work has demonstrated that chlamydial infections were uncommon (<1%) amongst the HIV+ women in the WIHS cohort (13). All WIHS patients are screened for chlamydia, gonorrhea, and other vaginal infections (candida, trichomonas, and bacterial vaginosis) at enrollment to the study and at follow-up or treatment visits thereafter as necessary. None of the WIHS patients

was reported to have concurrent chlamydia, gonorrhea, trichomonas, or bacterial vaginosis at the time of biopsy sampling. Two patients had concurrent yeast vaginal infection. All non-WIHS GC-positive samples with tissue remaining in the paraffin block were tested for C. trachomatis by ligase chain reaction (5 HIV-negative and 1 HIV-positive). All tested samples were C. trachomatis negative.

In many samples, lymphoid follicle-like (LF-like) structures were observed that were not as well defined structurally as the GCs described above (Fig 2). Aggregates were scored as LF-like if they contained a dense core of CD20+ cells interspersed with CD68+, CD4+ and CD8+ cells, and lacked the distinct structure and tingible-body macrophages of GCs. Lymphoid follicles, with or without GCs, were seen in 2 of 21 samples from normal cervical tissue (9.5%), 12 of 21 (57%) samples of high-grade SIL from HIV-negative patients, and 15 of 30 (50%) samples of high-grade SIL from HIV-positive patients (Table 2). An increased frequency of GCs and LF-like structures was observed among high-grade SIL patients (p=0.001). The mean diameter of LF-like structures was 243.5 microns (range 96 - 484 microns, N = 17). There was no significant difference in the size of aggregates from HIV-negative and HIV-positive patients.

Another functional property of GCs is proliferation of B cells that bind to a specific antigen displayed in the follicle, allowing for clonal expansion of selective B cells. To characterize the functional status of cervical lymphoid aggregates, 10 samples known to have GC or LF-like structures from both the

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HIV-negative and HIV-positive patients (5 from each cohort) were selected for proliferation analysis as determined by Ki 67 staining. Abundant proliferation was detected within GCs and LF-like structures independent of HIV status (Fig 3 a and b), indicating functional capacity of cells within cervical follicles to undergo clonal expansion.

A complex interplay of chemokines such as B lymphocyte chemoattractant or B cell-attracting chemokine (BCA-1), secondary lymphoid tissue chemokine (SLC), lymphotoxin $\alpha 1\beta 2$, and others are implicated in the recruitment, organization and maturation of T and B cells required for the development of specific immune responses [reviewed in (24, 25)]. Recently, BCA-1 has been implicated as a critical initiating factor in the formation of lymphoid aggregates (26). We investigated BCA-1 expression in cervical stroma of 3 samples of normal cervical tissue and 13 samples of high-grade SIL (5 from the HIV-negative and 8 from the HIV-positive cohorts) by immunodetection. BCA-1 expression was detected in cells surrounding GC or LF-like structures but not within the aggregates themselves (Fig 3 c and d). This finding was observed in samples from both HIV-negative and HIV-positive women. Samples of normal cervical tissue had no positively-stained cells.

In order to investigate the nature of the antigens displayed in the follicles found in cervix, we used IHC to determine whether epitopes of HPV16 protein E7 or HIV protein p24 could be detected in GCs. HIV p24 was not detected in any of

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the samples tested. However, HPV 16 E7 antigen was detected within the GCs

and LFs of samples from both HIV-positive (2 of 4 sample tested) and HIV-

negative patients (3 of 6 samples tested) (Fig 4-a, b). The specificity of the E7

antibody was tested by blocking anti-E7 antibody with synthetic GST-E7 fusion

protein. Preincubation of the antibody with GST-E7 resulted in loss of

immunohistochemical staining, whereas preincubation with GST alone had no

effect on immunostaining (Fig 4-c, d, e, f). Specificity for the E7 immunoreactivity

was also indicated by: 1) positive staining of dysplastic epithelium (Fig 4-h); 2)

absent or low staining of adjacent normal tissue (data not shown) and of

subepithelial stroma (Fig 4-a, e, h); and 3) absent staining of a GC (Fig 4-g) on

the same section of a sample with positive staining in an adjacent GC (Fig 4-a).

Another type of lymphoid aggregate was commonly observed in

cervical stroma of samples from HIV-positive women with high-grade SIL. These

aggregates contained primarily CD8+ T cells, were interspersed with CD4+ T

cells, and had few, if any, CD20+ or CD68+ cells (Fig 5). The majority of CD8+ T

cell-dominant aggregates were present in high-grade SIL samples from HIV-

positive women who did not have co-existing LFs or GCs (Table 2). CD8+ T cell-

dominant aggregates without co-existing LFs were seen in 1 of 21 normal

samples (4.8%), 0 of 21 high-grade SILs from HIV-negative patients and 12 of 30

(40%) high-grade SILs from HIV-positive patients. Thus, the occurrence of CD8+

T cell-dominant aggregates was associated with HIV infection (p≤0.001). Among

HIV-positive patients, there was no association between the presence of CD8+ T

cell-dominant aggregates and CD4 count depletion below 400 cells/mm$^3$. Given the high frequency (90%) of either CD8+ T cell-dominant or LF-like aggregates in HIV-positive patients, there was a highly statistically significant association between HIV infection and the presence of lymphoid aggregates in the cervix (p<0.001).

To assess a possible relationship between disease outcome and the types of aggregates, the recurrence of high-grade SIL within one year following the biopsy sampling was compared between the groups with and without CD8+ T cell-dominant aggregates. Data on follow-up at one year after the initial treatment were available in 34 women. Eight of 18 women who had CD8+ T cell-dominant aggregates developed high-grade SIL in one-year follow-up period and 1 of 16 women lacking CD8+ T cell-dominant aggregates developed high-grade SIL (p=0.02), indicating an association between the presence of CD8+ T cell-dominant aggregates and worse disease outcome.

To assess the possibility that the small fraction of the paraffin block examined in this study produced sampling bias, we performed complete analyses of blocks from 4 surgical cases as described in Material and Methods. Each series contained several more examples of the same type of lymphoid aggregate scored on the initial slides. In one sample of high-grade SIL from an HIV-positive patient initially scored as having CD8+ T cell-dominant aggregates, there were 3 additional distinct CD8+ T cell-dominant aggregates and no LFs or GCs in 340

microns of tissue. In a sample of high-grade SIL from an HIV-negative patient that was scored as having both types of aggregates (LF-like and CD8+ T cell-dominant) on the original sections, there were 11 CD8+ T cell-dominant aggregates and 8 LF-like structures in 235 microns of tissue. Therefore, sections from a fraction of the tissue blocks appeared to accurately represent the aggregates present in the cervix.

The functional status of CD8+ T cells in the cervical tissues was assessed using a primary antibody against perforin. Perforin staining was performed on 7 random samples of normal cervix, 8 samples from HIV-negative patients with high-grade SIL, and 8 samples of HIV-positive patients with high-grade SIL (selected for samples with CD8+ T cell-dominant aggregates). Regardless of HIV status, or presence or absence of CD8+ T cell-dominant aggregates, it was unusual to see any cells that contained perforin. In the few exceptions, 1-2 cells per field (40x objective) did stain with perforin (data not shown). Tonsillar tissue that was processed in parallel with the cervical samples was used as a positive control for perforin staining; abundant perforin-staining cells were present in tonsils. These results indicate that the presence of perforin is rare in cervical mucosa, and presumably reflects the transient nature of perforin expression in CTLs when analyzed on fixed tissue sections. Thus, we are unable to make meaningful comparisons of functional status of CTLs with respect to HIV status based on perforin staining.

Several possible confounding variables may affect the presence and types of lymphoid aggregates in these specimens. It would be unlikely for age to result in the increased number of CD8+ T cell-dominant aggregates seen in the HIV-positive group, since the mean ages of the HIV-positive and HIV-negative high-grade SIL groups were almost identical. However, the ages of women who contributed the normal samples are significantly higher than those of either high-grade SIL groups and only 14% of the normal group contained any aggregates. Aggregates were observed more often among younger than older women (p=0.0001). GC or LF-like aggregates were observed in 25 of 45 women (55.6%) < 40 years of age and in 4 of 27 (14.8%) ≥ 40 (p=0.001). CD8+ T cell-dominant aggregates were seen in 9 of 45 of women (20%) <40 and in 4 of 27 (14.8%) ≥40 years. When a similar comparison was made among women with high-grade SIL, no difference was seen, with 34 of 44 (77.3%) of samples from women <40 and 5 of 7 (71%) of samples from women ≥40 having aggregates.

Another possible confounding variable that may have affected the presence and types of lymphoid aggregates present is cigarette smoking. There was no statistically significant association between smoking and the type of lymphoid aggregates in the cohorts studied. However, data about smoking were available in only a subset of the patients in the normal and HIV-negative high-grade SIL cohorts (Table 1), which constrained this analysis.

Due to reports that levels of secretory antibodies and cytokine profiles in the female lower reproductive tract are influenced by the hormonal fluctuations of the female menstrual cycle (27-29), we compared presence and types of lymphoid follicles to phase of the menstrual cycle. Data on the last menstrual period (LMP) was available for 69% of women in the high-grade SIL cohorts (HIV-positive and HIV-negative combined). We assigned patients to follicular phase if they were 0-15 days since their LMP at the time that surgery was performed, and luteal phase if they were $\geq 16$ days from their LMP. No apparent association was found between type of aggregate and phase of the menstrual cycle (Table 3).

## Discussion

This study is the first report describing classical germinal centers and lymphoid follicles as a common feature in cervical samples from women with high-grade SIL and the detection of HPV 16 E7 protein in a subset of these germinal centers. In addition, we characterized a unique CD8+ T cell-dominant aggregate in HIV-positive women.

Germinal centers are a feature of the secondary immune response commonly found in lymph nodes and mucosal-associated lymphoid tissue (MALT). In GCs, antigen is displayed on follicular dendritic cells; those B cells that bind antigen proliferate and undergo genetic rearrangement of the immunoglobulin genes to produce cellular clones with higher affinity for antigen

binding (somatic hypermutation). B cells that do not have high affinity to specific antigens undergo programmed cell death and are ingested by macrophages. B cells in the cervical GCs described here are presumably recruited by BCA-1 expressed by the stromal cells in the surrounding area, indicating that similar mechanisms govern GC formation in the cervix as are found in other secondary lymphoid tissues. In addition, our data demonstrate that cervical GCs have functional properties of mature GCs found in other secondary lymphoid tissue, including cellular proliferation, apoptosis, and tingible body macrophages in the B-cell rich centers.

Cervical GC formation was previously described in association with cervicitis. In one study by Crum et al., GCs were found in 9 of 102 (8.8%) of cervical biopsies selected due to the presence of a chronic inflammatory infiltrate (17). In another study by Roberts et al., lymphoid follicles were present in 2.4 % of 450 consecutive hysterectomy specimens (15). These rates of GC detection are similar to those found in our samples of benign cervix (4.7%) and indicate that GC formation can occur in cervical tissue under a variety of situations other than the presence of SIL.

Several reports have documented a relationship between cervical GCs and C. trachomatis infection (17-19). However, it is unlikely that the GCs and LFs described here can be attributed entirely to the presence of C. trachomatis because LCR assays performed on 6 of the 8 study samples with well-formed GCs were negative for C. trachomatis. In addition, women in the WIHS cohort were well-screened clinically, had C. trachomatis and gonorrhea cultures, wet

mounts and gram stains performed at entry into the study, and had these tests repeated if obvious cervicitis or vaginitis was detected at follow-up visits. The overall rate of STDs was low in this cohort (13), and none of the patients had known C. trachomatis or gonorrhea at the time of the procedure that generated the study sample.

The high incidence of GCs in samples of high-grade SIL from HIV-negative women (33%) are likely to reflect a mucosal response to the dysplastic process itself, and specifically to HPV antigens present in the lesions. This proposition is supported by the presence of HPV 16 E7 antigen in a subset of GCs. Staining occurred mainly in the intercellular spaces, presumably due to antigen binding to interdigitating follicular dendritic cells.


Our data demonstrate differences in the properties of lymphoid aggregates in high-grade SIL from HIV-positive women. Specifically, women with HIV infection and high-grade SIL had a significantly lower frequency of well-formed GCs than women without HIV infection. Given the known correlation between HIV infection and higher rates of SIL and of recurrence after treatment (5, 6, 8-10), our data suggest that failure to generate GCs may explain in part this difference in outcomes in HIV-infected individuals.

A second profound difference in the mucosal immune response in SIL samples from HIV-positive women is the finding of a distinct type of accumulation, consisting primary of densely clustered CD8+ T cells, almost entirely restricted to samples from HIV-positive women. There are several recent

observations of increased numbers or clusters of CD8+ T cells in the dysplastic or cancerous cervix (30-32), including samples from HIV-positive women (33, 34). Our data are the first to demonstrate an association between the presence of CD8+ T cell-dominant aggregates and recurrence of high-grade SIL within one year after therapy. One explanation for this apparently counter-intuitive association is that the CD8+ T cells in the aggregates are not functioning effectively as CTLs, consistent with published data about CD8+ T cells from HIV-positive individuals (35-38).

Recent data from model systems of progressive carcinogenesis have raised questions about the appropriateness of increased immune responses during cancer development. In a transgenic mouse model of HPV-induced squamous carcinogenesis, production of a matrix metalloproteinase (MMP9) is necessary for the development of SIL and cancer (39). The same finding was reported in a transgenic animal model of pancreatic cancer of the $\beta$-cell islets (40). Interestingly, MMP9 was not produced by the neoplastic cells in either model, but was instead contributed by infiltrating cells adjacent to the neoplastic lesion in one case (40) and from bone-marrow derived mast cells and neutrophils in another (39). The pivotal role of MMP9 in carcinogenesis was attributed in part to its role in releasing active growth factors otherwise sequestered in the extracellular matrix (40). Therefore, by analogy, proteases and other factors secreted by cells in cervical CD8+ T cell-dominant aggregates may contribute to the maintenance, persistence, or progression of HPV infection in women. If so, these findings would have significant implications for therapeutic and protective

vaccine trials currently underway. Further investigation of the functional properties of lymphocytes and inflammatory cells in high-grade SIL will contribute to our understanding of both the protective and the potentially permissive effects of the elevated immune response in HPV-induced cervical neoplasia.

## Acknowledgement

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**Table 1. Participants' demographic information**

| HIV Status | Pt's ID | Age | Dominant Aggregate Types | Smoking Status | CD4 Count |
|---|---|---|---|---|---|
| Normal cervix<br><br>**N = 21**<br><br>Mean age :<br>50.1 | 1 | 44 | No | Current | |
| | 2 | 44 | No | | |
| | 3 | 49 | No | No | |
| | 4 | 42 | No | Current | |
| | 5 | 42 | No | No | |
| | 6 | 49 | GC | | |
| | 7 | 43 | No | | |
| | 8 | 47 | No | No | |
| | 9 | 42 | No | | |
| | 10 | 48 | No | Current | |
| | 11 | 41 | No | No | |
| | 12 | 43 | No | Current | |
| | 13 | 40 | No | No | |
| | 14 | 81 | No | Current | |
| | 15 | 49 | No | | |
| | 16 | 60 | No | | |
| | 17 | 62 | No | | |
| | 18 | 52 | CD8 | | |
| | 19 | 85 | LF-like | | |
| | 20 | 51 | No | | |
| | 21 | 39 | No | | |
| high grade<br><br>HIV-<br><br>**N = 21**<br><br>Mean age :<br>32.4 | 22 | 29 | No | No | |
| | 23 | 39 | Both[A] | Current | |
| | 24 | 23 | GC | No | |
| | 25 | 30 | No | | |
| | 26 | 84 | No | No | |
| | 27 | 47 | No | | |

| | | | | |
|---|---|---|---|---|
| | 28 | 22 | GC | Current | |
| | 29 | 35 | LF-like | Current | |
| | 30 | 38 | No | | |
| | 31 | 30 | No | No | |
| | 32 | 23 | No | No | |
| | 33 | 34 | No | No | |
| | 34 | 26 | LF-like | | |
| | 35 | 24 | GC | | |
| | 36 | 24 | LF-like | Current | |
| | 37 | 23 | GC | | |
| | 38 | 20 | Both | | |
| | 39 | 39 | No | | |
| | 40 | 27 | GC | No | |
| | 41 | 38 | GC | Current | |
| | 42 | 26 | GC | | |
| high grade HIV+ **N = 30** Mean age : 32.6 | 43 | 40 | CD8 | Current | 366 |
| | 44 | 29 | LF-like | Current | 807 |
| | 45 | 30 | LF-like | No | 544 |
| | 46 | 40 | Both | Current | 452 |
| | 47 | 43 | CD8 | Current | 687 |
| | 48 | 46 | LF-like | No | 80 |
| | 49 | 34 | Both | No | 883 |
| | 50 | 37 | CD8 | Current | 12 |
| | 51 | 27 | No | Current | 1 |
| | 52 | 24 | CD8 | No | 68 |
| | 53 | 46 | CD8 | Current | 108 |
| | 54 | 33 | LF-like | No | 106 |
| | 55 | 38 | Both | Current | 350 |
| | 56 | 24 | CD8 | Current | 520 |
| | 57 | 32 | CD8 | Current | 429 |

| | | | | |
|---|---|---|---|---|
| 58 | 30 | LF-like | No | 30 |
| 59 | 28 | No | Current | 118 |
| 60 | 39 | Both | No | 1109 |
| 61 | 21 | CD8 | No | 429 |
| 62 | 28 | Both | No | 199 |
| 63 | 32 | CD8 | Current | 223 |
| 64 | 22 | LF-like | Current | 704 |
| 65 | 24 | CD8 | No | 23 |
| 66 | 27 | LF-like | Current | 1277 |
| 67 | 25 | CD8 | Current | 536 |
| 68 | 33 | CD8 | No | 280 |
| 69 | 39 | LF-like | No | 169 |
| 70 | 35 | GC | Current | 151 |
| 71 | 35 | Both | | Missing |
| 72 | 36 | No | | 200 |

[A] Both refers to the presence of LF-like and CD8+ T cell dominant aggregates in the same sample.

**Table 2. Distribution of lymphoid aggregates by clinical status**

| | GC | LF-like | CD8 | Both (LF-like & CD8) | None | Total |
|---|---|---|---|---|---|---|
| **Normal** | 1 | 1 | 1 | 0 | 18 | 21 |
| **HGSIL[A] HIV -** | 7 | 3 | 0 | 2 | 9 | 21 |
| **HGSIL[A] HIV +** | 1 | 8 | 12 | 6 | 3 | 30 |

[A] HGSIL is high grade SIL

**Table 3. Distribution of lymphoid aggregates by phase of the menstrual cycle**

|  | GC and LF-like N (%) | CD8 N (%) | None N (%) | Total number |
|---|---|---|---|---|
| **Follicular** | 7 (50%) | 4 (28.6%) | 3 (21.4%) | 14 |
| **Luteal** | 11 (52.4%) | 7 (33.3%) | 3 (14.3%) | 21 |

# Figure legends

## Figure 1. Lymphoid follicles with germinal centers are present in cervix from women with high grade SIL.

Immunohistochemistry on serial sections from a parrafin-embedded sample of high grade SIL (HIV-negative) was performed with antibody against CD4 (a), CD20 (b), CD8 (c), CD68 (d); positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Serial but not adjacent sections were stained with antibodies against CD68 (e) and for DNA fragmentation (f). Photos taken with 10x (a-d) and 40x (e and f) objective. E: epithelium; S: stroma; G: endocervical gland.

## Figure 2. Lymphoid follicles without germinal centers are present in cervix from women with high grade SIL

Immunohistochemistry on serial sections from a paraffin-embedded sample of high grade SIL (HIV-negative) was performed with antibodies against CD4 (a), CD20 (b), CD8 (c), and CD68 (d); positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 10X objective. E: epithelium; S: stroma; G: endocervical gland.

## Figure 3. Functional characterization of cervical GCs.

Immunohistochemistry on paraffin-embedded sections of high grade SIL with GC and LF-like structures was performed with antibody against Ki 67 (a and b) and

BCA-1 (c and d). The stromal-epithelial junction is marked with a dashed line. Photos were taken with the 10x (a and c) or 40x objective (b, and d). Photos b and d are the areas outlined in a and c, respectively. Immunoreactivity towards mouse IgG1 (isotype control) was negligible (data not shown). E: epithelium; S: stroma; G: endocervical gland; LF: lymphoid follicle.

**Figure 4. HPV16 E7 antigen in cervical GCs**

Immunohistochemistry on paraffin-embedded sections of high-grade SIL with GCs was performed with antibody against HPV16 E7 antigen (a and b), antibody pre-incubated with GST-E7 (c and d), and pre-incubated with GST (e and f). Panel g shows another GC on the same slide as panel a, but was negative for E7 immunostaining. Panel h shows E7 immunostaining of high-grade SIL but not of cervical stroma. The bar in panels b and c represent 50 µm. E: epithelium; S: stroma; GC: germinal center.

**Figure 5. Aggregates of CD8+ T cells are present in high grade SIL from HIV+ women.**

Immunohistochemistry on serial sections from high grade SIL (HIV-positive patient) was performed with antibodies against CD4 (a), CD20 (b), CD8 (c), and CD68 (d). The stromal-epithelial junction is marked with a dashed line. Photos taken at 10X objective magnification. E: epithelium; S: stroma.

**Fig 1**
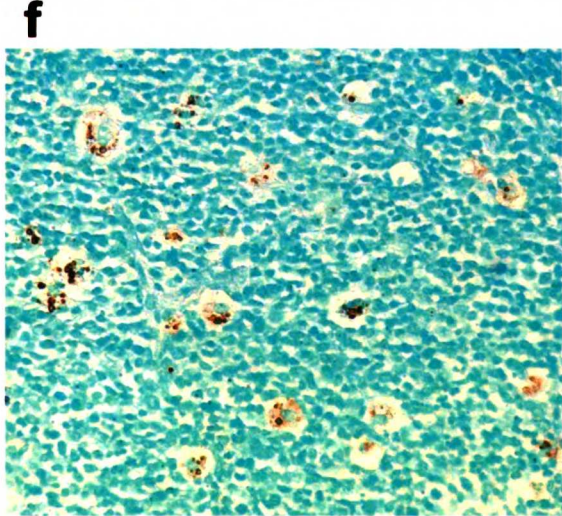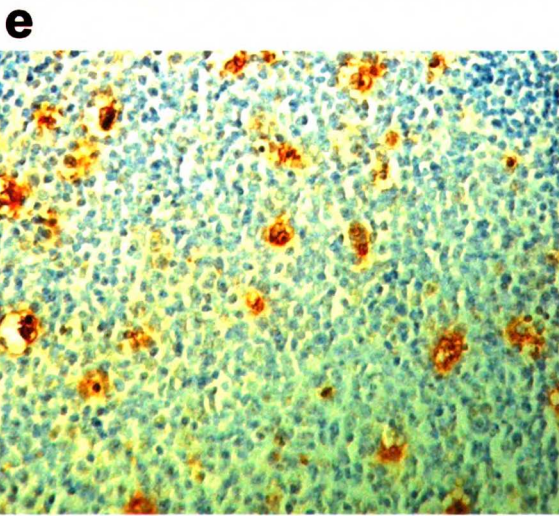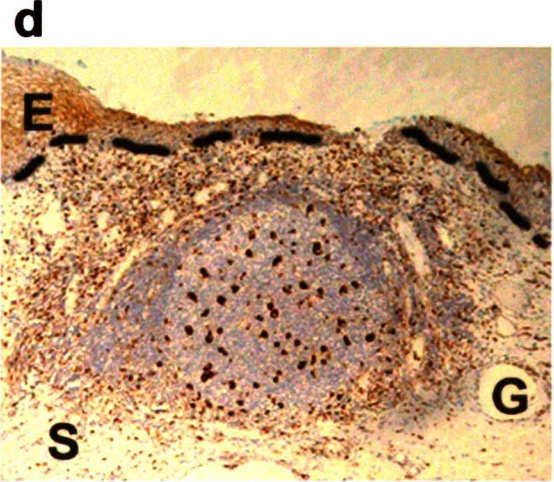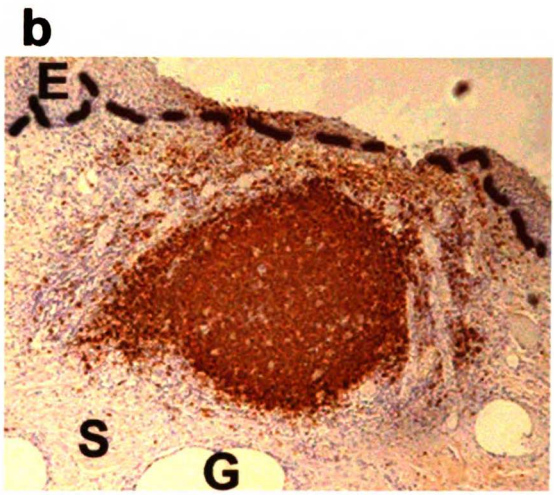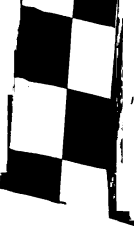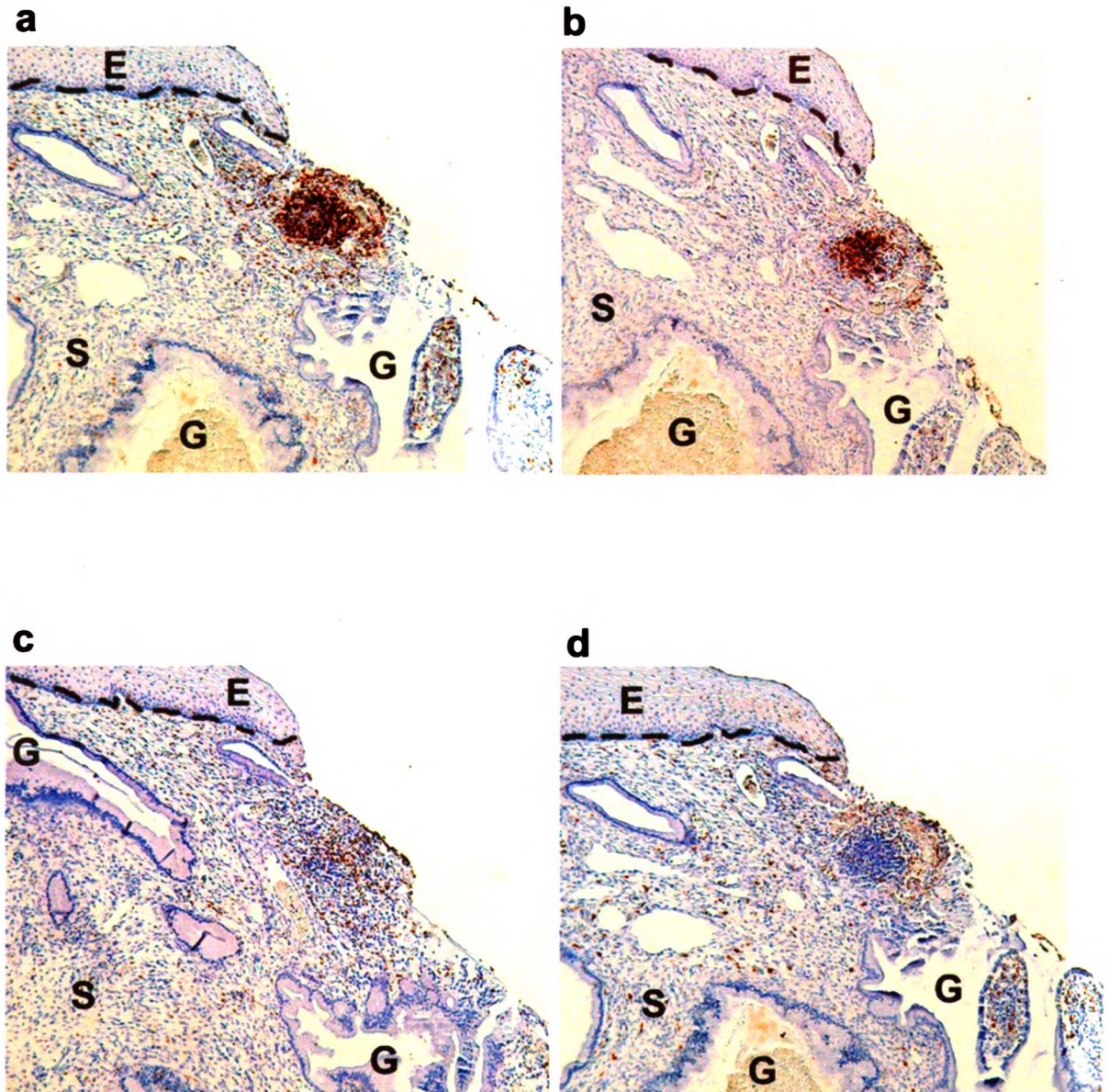
# Fig 2

**Fig 3**
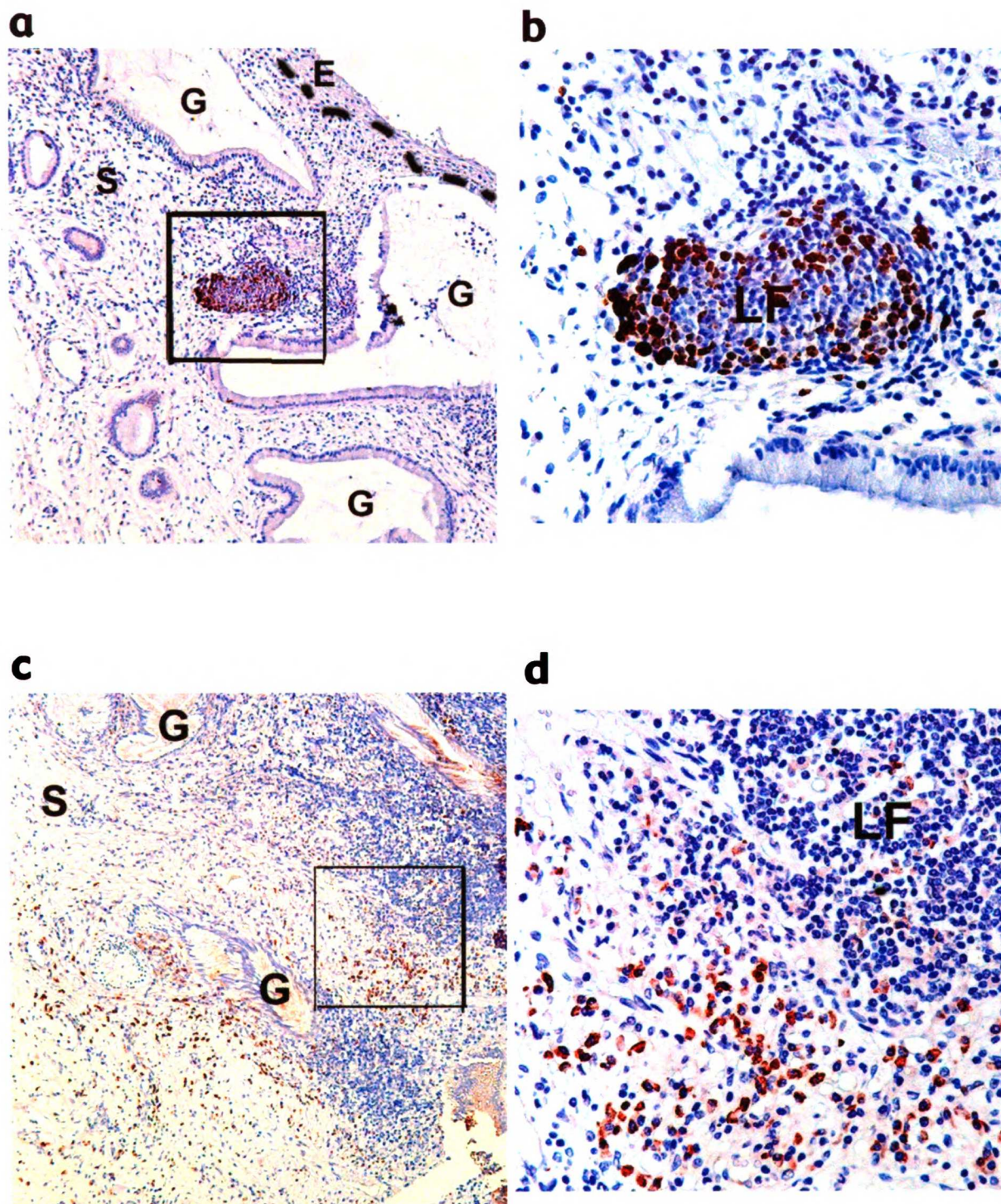
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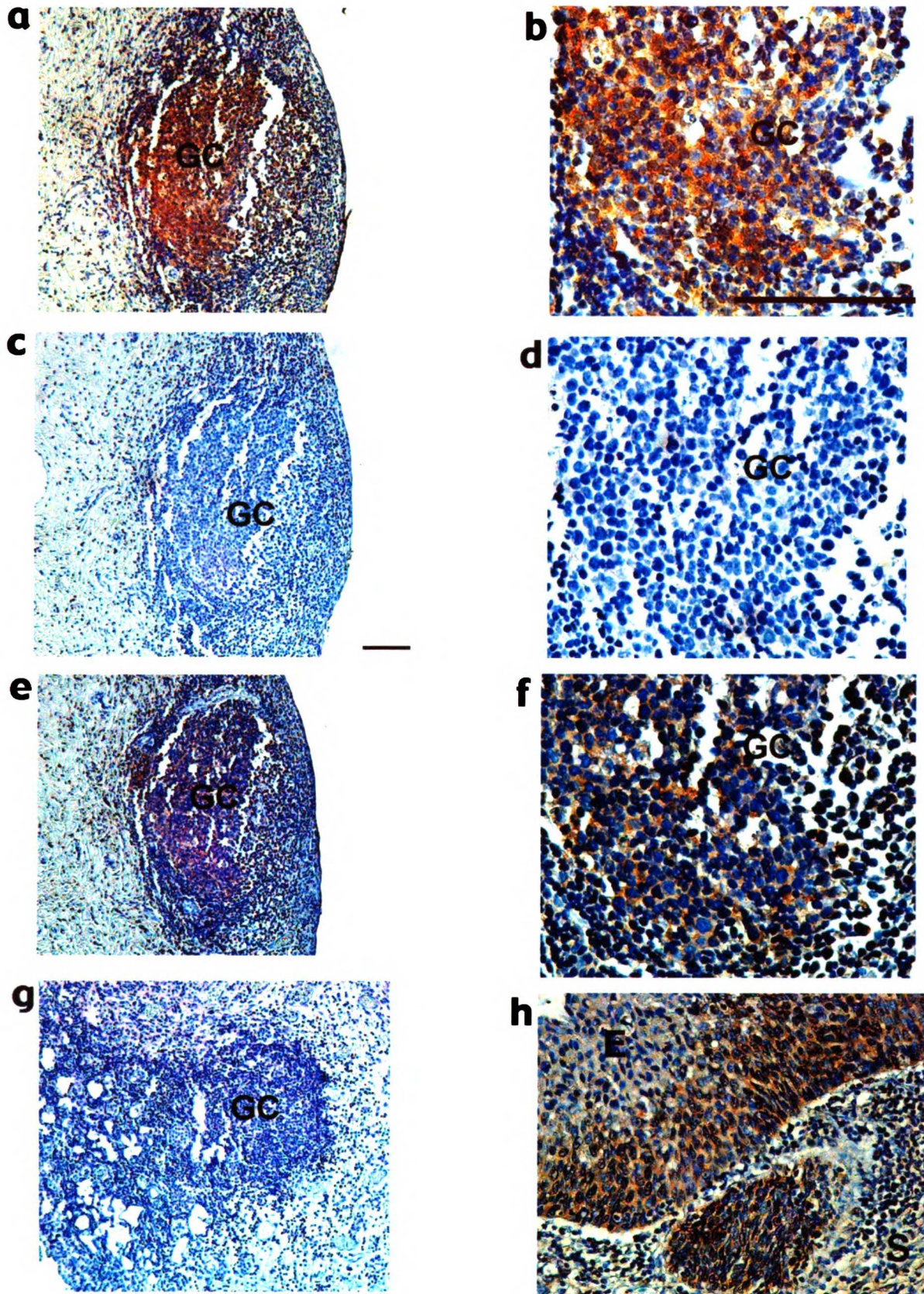
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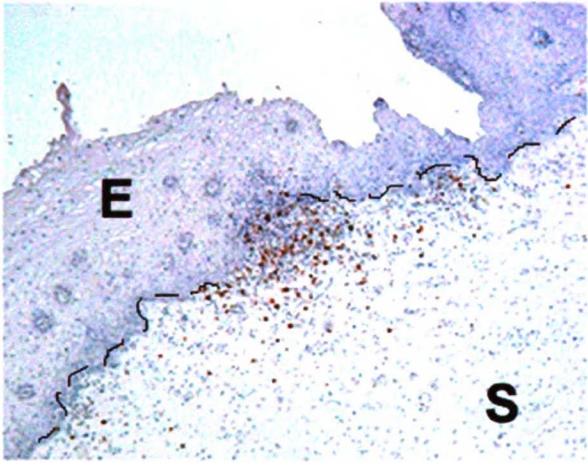


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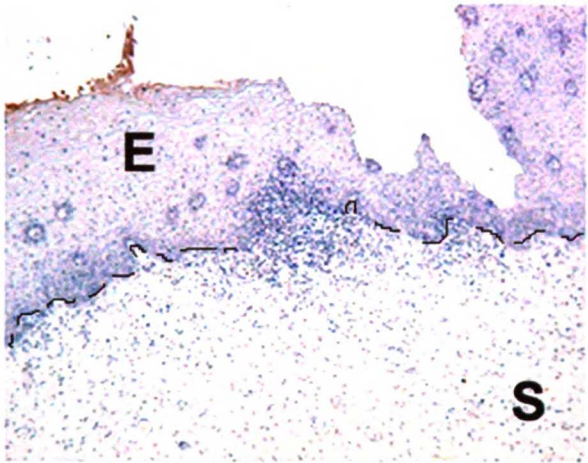


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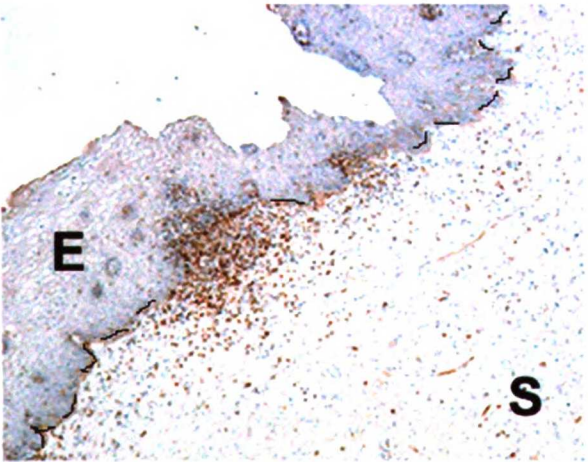


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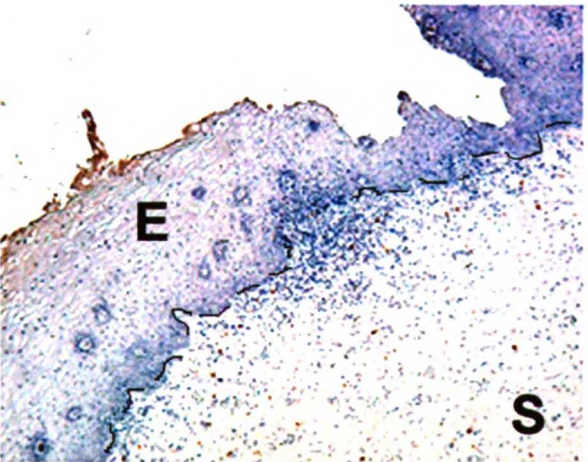


CD68

# Mucosal immune cell numbers are increased in the cervix of high-grade dysplasia but are altered in HIV-positive cohort

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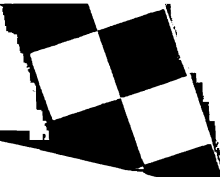
## Abstract

The mucosal immune response in the cervix to neoplastic development is not clearly understood. Following our previous reports indicating the presence of lymphoid follicles including classic germinal centers in the cervix associated with high-grade dysplasia, we hypothesized that samples of high-grade dysplasia would contain higher numbers of mucosal lymphocytes and inflammatory cells in the dysplastic cervical mucosa compared with the normal cervix and that the infiltration would be attenuated in high-grade dysplasia from HIV-positive women compared with uninfected women. We performed immunohistochemical analyses to characterize the distributions of inflammatory cells in normal cervical tissue and in samples of high-grade dysplasia from HIV-positive and HIV-negative women. The results indicated that the cell counts of stromal immune cells, namely CD4+ T cells, CD8+ T cells, CD20+ B cells, CD68+ macrophages, and tryptase+ mast cells, were all significantly elevated in both HIV-negative and HIV-positive high-grade dysplasia groups compared with the group of normal cervical tissue. Furthermore, the macrophage counts in the epithelium in both high-grade dysplasia groups were also elevated compared with the normal group. In the comparison between HIV-negative and HIV-positive high-grade dysplasia groups, CD4+ T cell counts in the stroma of HIV-positive group was significantly decreased compared with HIV-negative group, and epithelial macrophage counts in HIV-positive group was also significantly decreased compared with HIV-negative group. However, in the case of mast cells, the stromal mast cell counts in HIV-positive group were significantly increased compared with HIV-negative
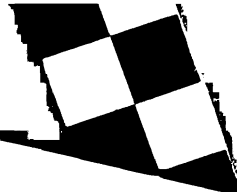
86

groups. Additional studies on the functional characteristics of immune cells infiltrated in the dysplastic cervix especially in women with HIV disease are needed to determine how HIV infection effects the immune cells responding to dysplasia in the cervix.
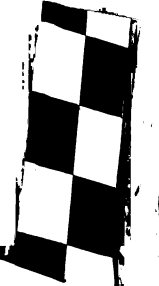
**Introduction**

Human papillomavirus (HPV) infections of the cervix can result in a series

of changes in the epithelium known as cervical dysplasia. The early changes

(low-grade squamous intraepithelial lesion [SIL]) reflect active viral replication

and are clinically innocuous. The more advanced forms of dysplasia (i.e.,

moderate and severe dysplasia and carcinoma in situ; high-grade SIL) represent

potential cancer precursors; $\geq$ 12% of severe dysplasia will progress to cancer if

left untreated (Ostor 1993). HPV from high-risk viral types can be detected in

>90% of high grade SIL and cervical cancers, strongly implicating the virus as an

etiological agent in cervical carcinogenesis (Lorincz et al. 1992; Schiffman et al.

1993; Walboomers and Meijer 1997). However, a much larger percentage of

HPV infections are clinically undetectable and do not result in dysplasia or

cancer.

Multiple epidemiological studies have reported that HPV infection is

detected more frequently, and that the recurrence rate and severity of genital

neoplasia are higher among immunocompromised women, including those with

HIV infection (Ahdieh et al. 2000; Chopra and Tyring 1997; Fowler et al. 1997;

Maiman 1998; Penn 1986; Vernon et al. 1995). These data imply that the host

immune response to HPV is critical in determining the outcome of an HPV

infection. In 1992, invasive cervical cancer was included as an AIDS-defining

illness in HIV-positive women by the Centers for Disease Control and Prevention

(CDC, 1993). Aberrations of the immune system associated with HIV infection

are thought to play a significant role in the pathogenesis of HPV-related cancers.

Previously we reported that lymphoid follicles, some containing germinal centers, were found more commonly in high-grade SIL samples from HIV-negative and HIV-positive women and were rare in samples from normal cervical tissue (manuscript submitted). HPV16 E7 protein was detected in a subset of these germinal centers. In addition, CD8+ T cell dominant aggregates, with a paucity of B cells, were detected specifically in HIV-positive women with high-grade dysplasia. We observed marked infiltration of immune cells in the dysplastic cervical mucosa even in samples which did not contain aggregates suggesting that other mechanisms were present for generating immune responses in the dysplastic cervix.

Therefore, we hypothesized that samples of high-grade SIL would contain higher numbers of mucosal lymphocytes and inflammatory cells in the dysplastic cervical mucosa compared with the normal cervix and that the infiltration would be attenuated in high-grade SIL from HIV-positive women compared with uninfected women. We performed immunohistochemical analyses to characterize the distribution of inflammatory cells in normal cervical tissue and in samples of high-grade SIL from HIV-positive and HIV-negative women.

**Materials and methods**

Specimens and data for the HIV-positive participants in this study were obtained in collaboration with the Women's Interagency HIV Study (WIHS), which is a longitudinal multi-site cohort study of women with or at risk for HIV infection. Study sites included consortia based at the Bronx Lebanon Hospital; State University of New York, Brooklyn; Georgetown University; Cook County Hospital;

University of California, San Francisco; and the University of Southern California. Detailed methods for this study were published previously (Barkan et al. 1998). All study procedures and consent materials were reviewed and approved by Human Subjects Protection Committees at each of the collaborating institutions and by the WIHS executive committee. HIV infections were verified by EIA serology with Western blot confirmation. CD4+ T cell counts of peripheral blood were performed by flow cytometry in laboratories participating in the FAS quality assurance program. Plasma HIV levels were determined using the Organon Teknica NASBA test in laboratories participating in the NIH virology laboratory quality assurance program. Cervico-vaginal lavage with 10ml saline was performed at each visit, and the resulting material aliquoted and frozen within 6 hours of collection. HPV testing was performed using polymerase chain reaction (PCR) with LI consensus primers as described previously (Greenblatt et al. 1999). Pap smears were obtained from each woman, and colposcopy was performed for those women with abnormal results. Loop and cone biopsies were performed for standard indications and tissues were archived as paraffin blocks after pathological evaluation at the originating institution.

Specimens for the normal cervix group were obtained as paraffin-embedded blocks from the Department of Pathology archives at UCSF from women who underwent hysterectomies for benign uterine disease with no cervical abnormalities. Specimens and data for the HIV-negative high-grade SIL group were obtained as paraffin-embedded sections of cone and loop excisions

from two sources: the UCSF Department of Pathology Archives and WIHS. All samples obtained from the WIHS cohort were selected from women who had HPV 16 detected in cervicovaginal lavage specimens. Due to limited availability, we did not include a cohort of normal cervical samples from HIV-positive women.

Immunohistochemistry (IHC) was performed with primary antibodies against CD4+ T cells with monoclonal antibody (mAb) to CD45R0 (clone OPD4), CD8+ T cells with mAb to CD8 (clone C8/144B), B cells with mAb to CD20 (clone L26), tissue macrophages with mAb to CD68 (clone KP1), and tissue mast cells with mAb to mast cell tryptase (clone AA1), purchased from DAKO, Carpenteria, California. Each primary antibody was diluted in phosphate buffered saline with 1% bovine serum albumin as follows: 1:500 (CD45R0), 1:200 (CD8), 1:1200 (CD20), 1:500 (CD68), and 1:2000 (mast cell tryptase). Routine IHC was performed following manufacturer's guidelines (Innogenex, San Ramon, California). Prior to the inactivation of endogenous peroxidase with 0.1% hydrogen peroxide, tissue slides were digested with 0.025% trypsin for 10 minutes at 37°C. Antigen retrieval for all primary antibodies was performed by boiling the slides in a 1.25kW microwave for 2 minutes in 10mM Sorensen's citric buffer (pH 6) and cooling in citric buffer for 30 minutes. All antibodies were incubated on tissue sections for one hour at room temperature. A 1:3 dilution of hematoxylin gill #1 was used for counter staining. 3, 3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, California) and 3-amino-9-ethylcarbozole (AEC) (BioGenex, San Ramon, California) were used as chromogens.

The numbers of immunophenotyped cells present in cervical tissues were determined using image analysis software. In each photographic image of a microscopic field (40x objective), stromal and epithelial areas were separately measured in $mm^2$ [Openlab 2.2.4 (Improvision, Lexington, Massachusetts)] and the stained cells within each measured area were counted visually. The cell number was then divided by the area to normalize the value. In order to determine the intra-observer variability in cell counts done by this method, cell counts in 10 images of randomly selected 40x objective microscopic fields were repeated three times on three different days. The variability in the counted numbers of cells/$mm^2$ at three different times was <10% for all 10 images. For cell counts in the stroma, 10 microscopic fields within 400μm of the high-grade epithelium were randomly selected from each slide. Stromal areas with aggregates were excluded from the analysis. The cell counts per $mm^2$ from 10 fields were then averaged. Ten microscopic fields of high-grade epithelium were not available in every sample since dysplastic epithelium was not always intact. Therefore, five 40x-objective microscopic fields with high-grade SIL were selected randomly from each slide for epithelial cell counts and the normalized cell counts from five fields were averaged.

For counting cells that have unique morphologies or staining patterns, we set criteria for these cell types in order to be consistent in counting. For macrophages, we focused on stained areas larger than 200 pixels since many macrophages extend pseudopodia from cell bodies, which may or may not be

counted as additional cells. For mast cells, since tryptase staining often includes surrounding areas of the cells, only a stained area containing a nucleus was counted as one cell.

**Results**

The clinical characteristics of the women who contributed tissue samples to this study are summarized in Table 1. There were nine samples of normal cervical tissue, 11 samples of high-grade SILs with no HIV infection, and 20 samples of high-grade SILs with known HIV infection. The mean age of the normal group (49.3 years; range 39 - 62) was significantly older than the two groups who had high-grade SIL (27.9 years; range 20 - 39 for the HIV-negative and 31.2 years; range 21 - 46 for the HIV-positive cohort; $p < 0.0002$). This difference in age is likely attributable to the fact that the women in the group with normal cervical tissue were undergoing hysterectomies, and therefore were more likely to be peri- or post-menopausal.

CD4+ T Cell Counts

CD4+ T cells in the stroma of the normal cervix were spread out (Fig 1-a), and CD4+ T cells in the epithelium were localized near the basement membrane if they were at all present (Fig 1-b). Infiltrations of CD4+ T cells in the stroma in high-grade SIL samples were localized near endocervical glands or at the stromal-epithelial junction (Fig 1-c and d). CD4+ T cells in the dysplasitic epithelium were often detected near the basement membrane (Fig 1-d). In general, CD4+ T cells were less frequently observed in the epithelium than in the

stroma of all three groups (groups with normal cervix, with HIV-negative high-grade SIL, and with HIV-positive high-grade SIL) (Fig 6-a).

As shown in Table 2 and Figure 6-a, the average CD4+ T cell counts in the stroma of HIV-negative high-grade SIL group was 2720 cells/mm$^2$, significantly elevated from the count in the normal cervix (530 cells/mm$^2$; p<0.0001). CD4+ T cell counts in the stroma of HIV-positive high-grade SIL group was 1134 cells/mm$^2$, also significantly increased compared with the normal cervix (p<0.02). A decrease in the number of stromal CD4+ T cells in the HIV-positive high-grade SIL group compared with HIV-negative group was significant (p<0.001). The average CD4+ T cell count in the epithelium was 101 cells/mm$^2$ in normal cervical tissue, and 227 cells/mm$^2$ in HIV- negative and 227 cells/mm$^2$ in HIV-positive groups (Table 2), and the differences in epithelial CD4+ T cell counts in both high-grade SIL groups were not significant compared with normal cervical tissue (Fig 6-a).

CD8+ T Cell Counts

A small number of CD8+ T cells in the stroma of normal cervical tissue was localized near the stromal-epithelial junction (Fig 2-a), and most CD8+ T cells in the epithelium of the normal cervical tissue were localized near the basal and parabasal layers but some cells were also found in the intermediate epithelial layers (Fig 2-b). CD8+ T cells in the stroma of high-grade SIL groups were spread out (Fig 2-c) but many cells were also detected along the basement membrane and endocervical glands where SIL was present (Fig 2-d).

As shown in Figure 6-b and Table 2, the average CD8+ T cell counts in the stroma of normal cervical tissue was 577 cells/mm$^2$, and the counts were elevated in both high-grade groups; 1925 cells/mm$^2$ in HIV-negative, and 1754 cells/mm$^2$ in HIV-positive (p<0.003, p<0.002, respectively). On the other hand, the CD8+ T cell counts in the epithelium of all three groups were within a similar range (553 cells/mm$^2$ in normal cervix, 453 cells/mm$^2$ in HIV-negative high-grade SIL, and 707 cells/mm$^2$ in HIV-positive high-grade SIL).

CD20+ B Cell Counts

CD20+ B cells were generally rare in the stroma as well as in the epithelium of normal cervical tissue (Fig 3-a and b) and the dysplastic cervix (3-c and d). The increases in the number of stromal B cells in both high-grade SIL groups were significant compared with normal cervix; 53 cells/mm$^2$ in normal stroma, 418 cells/mm$^2$ in HIV- negative high-grade SIL (p<0.01), and 219 cells/mm$^2$ in HIV-positive high-grade SIL (p<0.002). B cells in the epithelium were 2 cells/mm$^2$ in normal cervix, 12 cells/mm$^2$ in HIV-negative high-grade SIL, and 19 cells/mm$^2$ in HIV-positive high-grade SIL, and the difference between the normal and HIV-positive high-grade SIL groups was significant (p<0.05)(Table 2 and Fig 6-c).

CD68+ Macrophage Counts

Macrophages were localized throughout the stroma and epithelium of all three groups, and not concentrated at the stromal-epithelial junction as were the lymphocytes (Fig 4-a through d). As shown in figures 4-c and d, macrophages

appeared to change their morphology in dysplastic tissue, and they often expressed more distinct pseudopodia.

The number of macrophages in the stroma increased in both high-grade SIL groups, 860 cells/mm$^2$ in HIV-negative, and 852 cells/mm$^2$ in HIV-positive group, compared with 462 cells/mm$^2$ in normal cervical tissue (Table 2), and the increases in the stroma of both high-grade SIL groups were significant (both were $p<0.05$). The increases in the number of macrophages in the epithelium in both high-grade SIL groups were also significant compared with the normal cervical tissue: 715 cells/mm$^2$ in HIV-negative group ($p<0.005$); 336 cells/mm$^2$ in HIV-positive group ($p<0.01$); and 111 cells/mm$^2$ in normal cervical tissue (Fig 5-d). The decrease in epithelial macrophage counts in HIV-positive high-grade SIL group compared with HIV-negative high-grade SIL was significant ($p<0.03$).

Tryptase+ Mast Cell Counts

Mast cells in the normal cervical tissue were distributed throughout the stroma (Fig 5-a), but rarely found in the epithelium (Fig 5-b). Stromal mast cells in the high-grade SIL groups appeared to be detected more frequently than in normal cervical tissue but the distribution pattern remained unchanged (Fig 5-c). Some mast cells were detected in the dysplastic epithelium but they remained within the basal layer of the epithelium (Fig 5-d). Mast cells were detected by an antibody against its enzyme, mast cell tryptase, which is present in cytoplasmic granules as well as secreted from a mast cell into its surrounding area, and as a result the mast cell staining showed a distinctive pattern with shades around mast cells (Fig. 5-a and 5-c).

As shown in Table 2 and Figure 6-e, the number of mast cells in the stroma from the HIV-negative high-grade SIL group was 235 cells/mm$^2$, which was over twice as many as in normal cervix group (100 cells/mm$^2$; p<0.0003). The stromal cell counts in the HIV-positive high-grade SIL group was 352 cells/mm$^2$, and was significantly increased compared with normal cervical tissue (p<0.0002). Furthermore, the increase in the number of stromal mast cells in HIV-positive high-grade SIL was also significant compared with the HIV-negative high-grade SIL group (p<0.03). This is the first report of a significant increase in mast cell counts in the stroma of the cervix with high-grade SIL from HIV-positive cohort in comparison with HIV-negative high-grade SIL. Mast cell counts in the epithelium were 20 cells/mm$^2$ in normal group and 74 cells/mm$^2$ in HIV- negative and 57 cells/mm$^2$ in HIV-positive groups, and these differences were not significant.

CD4/CD8 T Cell Ratio

The CD4/CD8 T cell ratio provides a relative value of CD4+ T cells in addition to its direct counts. As shown in Table 3, the average ratio in the stroma in the group with normal cervical tissue was 1.18 (range: 0.58-3.94), and elevated to 1.60 (range: 0.86- 3.72) in HIV-negative high-grade SIL group, but decreased to 0.70 (range: 0.10-1.62) in HIV-positive high-grade SIL group. The increase in the stromal CD4/CD8 ratio in the HIV-negative group compared with the normal group was not significant but the difference in the stromal CD4/CD8 ratios between HIV-negative and HIV-positive groups was significant (p<0.004). This significant decrease in the stromal CD4/CD8 ratio was due to a significantly

lower number of stromal CD4+ T cell counts in HIV-positive group; CD4+ T cell counts in HIV-negative group was 2720 (range: 1359-5710 cells/mm$^2$) and CD4+ T cell counts in HIV-positive group was 1134 (range: 224-3154 cells/mm$^2$; $p<0.001$). The difference in stromal CD8+ T cell counts between HIV-positive and HIV-negative groups was not significant. This finding indicates that CD4+ T cell counts but not CD8+ T cell counts in the stroma were affected by HIV infection. The decrease in the CD4/CD8 T cell ratio among HIV-positive women with high-grade SIL is congruent with previous reports (Bell et al. 2000; Olaitan et al. 1996). No significant difference was found in CD4/CD8 ratios in the epithelium among the three groups; 0.52 in the epithelium of normal cervix, 0.63 in HIV-negative high-grade SIL group, and 0.57 in HIV- positive high-grade SIL group (Table 3).

**Discussion**

This report enhances the findings from our previous study on germinal centers (GCs) and CD8+ T cell aggregates associated with high-grade SIL in cervical samples (submitted). Our previous data demonstrated that GCs were associated with high-grade SIL and that a distinctive type of accumulation that consisted primarily of CD8+ T cells was seen commonly in the HIV-positive women with high-grade SIL.

Our current data on cell counts confirm one of our initial hypotheses that samples of high-grade SIL would contain higher numbers of mucosal lymphocytes and inflammatory cells compared with samples of normal cervical tissue. The stromal cell counts of immune cells that we stained for this study,

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namely CD4+ T cells, CD8+ T cells, CD20+ B cells, CD68+ macrophages, and tryptase+ mast cells, were all significantly elevated in both high-grade SIL groups compared with normal cervical tissue. Furthermore, the cell counts in the epithelium demonstrated that the macrophage counts in both high-grade SIL groups were also significantly elevated compared with normal cervical tissue. This finding suggests that macrophages may have migrated to the epithelium from the stroma in response to antigens in the dysplastic epithelium.

Although CD8+ T cell counts in both high-grade SIL groups were significantly elevated compared with the counts in the normal group, the CD8+ T cell counts in the HIV-positive group may have been even greater than reflected by the data in Table 2 because we excluded the stromal areas with aggregates from cell counting. Since CD8+ T cell aggregates were unique to the HIV-positive group, the exclusion of CD8+ T cell-dominant aggregates may have affected the CD8+ T cell counts disproportionally in the HIV-positive group. Although the areas with GCs were also excluded from counting, GCs and lymphoid follicles (LFs) were present in both HIV-negative and HIV-positive groups. Therefore, the exclusion of GCs/LFs from the cell counts would have affected both HIV-negative and HIV-positive groups similarly. Further study is required to determine whether or not the difference in the stromal CD8+ T cell counts between HIV-negative and HIV-positive groups is in fact significant.

Our data partially support our second hypothesis that the mucosal immunce cell numbers would be attenuated in high-grade SIL from HIV-positive women compared with uninfected women. CD4+ T cell counts in the stroma of

the HIV-positive high-grade SIL group were significantly decreased compared with HIV-negative group, and epithelial macrophage counts in HIV-positive cohort were also significantly decreased compared with HIV-negative group. However, in the case of tryptase+ mast cells, the stromal mast cell counts in HIV-positive group were increased significantly compared with HIV-negative groups. In addition, epithelial B-cell counts were significantly elevated in the HIV-positive group compared to the normal cervical group.

Possible explanations for the decrease in CD4+ T cell and macrophage counts in HIV-positive group include the fact that both CD4+ T cells and macrophages are known to express CD4 molecules and specific coreceptors required for HIV entry into cells (Alkhatib et al. 1996; Dragic et al. 1996; Oberlin et al. 1996). Therefore, they are the primary targets of HIV infection and may subsequently be destroyed by HIV. In addition, these cell types may become target cells for HIV-specific cytotoxic T lymphocyte (CTL)-mediated killing if they are in an initial phase of HIV infection (Reimann et al. 1994; Safrit et al. 1994; Walker and Plata 1990).

However, the decrease in CD4 T cell counts occurred in the stroma whereas the decrease in macrophages occurred in the epithelium. The different patterns of reduction of these two cell types in HIV infection may be associated with the unique functions of these cell types in the immune response. Macrophages detected in the epithelium may have migrated into the epithelium responding to antigens in the epithelium and phagocytosed these antigens using multiple extended pseudopodia as seen in Figure 4. If macrophages present in

the epithelium were latently infected with HIV, their response to antigens may have stimulated HIV gene transcription and facilitated viral replication (Reviewed by Pantaleo and Fauci 1995), leading to possible cell destruction and resulting in depletion in the epithelial but not in the stromal compartment. It is also possible that in HIV infection, fewer macrophages migrate to the epithelium than in the non-infected cases.

On the other hand, in the presence of functional CD8+ T cells, HIV replication in macrophages and CD4+ T cells would be suppressed by soluble factors released by CD8+ T cells (Barker et al. 1998; Chun et al. 2001; Walker et al. 1991; Walker and Levy 1989; Walker et al. 1986; Wasik et al. 2000). As seen in Figure 7-a, the major cell type present in the epithelium of HIV-positive high-grade SIL was CD8+ T cells (>50%), and if these CD8+ T cells in HIV-positive high-grade SIL group maintained their functional properties including soluble factors, macrophages in the epithelium should have been protected from being depleted by HIV infection. On the contrary, there was a report that indicated that soluble factors secreted by CD8+ T cells enhanced HIV replication in macrophages (Copeland et al. 1998). This report may explain and support our finding of macrophage depletion in the dysplastic epithelium from the HIV-positive group due to an enhanced viral replication within epithelial macrophages leading to destruction.

CD4+ T cell counts in the HIV-positive high-grade SIL group were decreased significantly in the stroma compared with the HIV-negative group. CD4+ T cells, that function as helper cells, are presumably recruited to areas

where they interact with and activate other immune cells to facilitate immune responses. It is known that the initiation of integrated HIV gene transcription in CD4+ T cells is associated with the activation of HIV-infected T cells by certain cytokines, including IL-2. Therefore, as more CD4+ T cells are activated as functional helper cells expressing IL-2 and other cytokines, more HIV are produced, leading to destruction of these cells and resulting in depletion of CD4+ T cells in the stroma (reviewed by Pantaleo and Fauci 1995).

The role of CD8+ T cells in association with depletion of CD4+ T cells and macrophages is not yet clear, and contradictory findings are reported (Barker et al. 1998; Chun et al. 2001; Copeland et al. 1998; Walker et al. 1991; Walker and Levy 1989). Multiple mechanisms may be involved in attenuation of CD4+ T cell and macrophage counts in HIV infection. Our findings of depletion of CD4+ T cells in the stroma and macrophage depletion in the epithelium lead us to further questions regarding the functional status of immune cells, particularly CD8+ T cell interactions with other immune cells, that are present in the cervical tissue of the HIV-positive group.

The significant increase in the stromal tryptase+ mast cell counts in HIV-positive high-grade SIL group compared with HIV-negative group suggests a possible association between mast cells and neoplastic development in HIV infection. As we discussed in our previous report (submitted), it is possible that mast cells secrete factors that may facilitate neoplastic changes in the cervix (Coussens et al. 2000). On the other hand, the increase in mast cell counts in HIV-positive women may support controversial reports of a type 2 immune

response associated with HIV infection (Barcellini et al. 1994; Clerici et al. 1997; Clerici and Shearer 1993; Leigh et al. 1998; Wasik et al. 2000). However, without studying the functional characteristics of mast cells in HIV-positive group, it is difficult to hypothesize a possible relationship between elevated mast cell counts and the development of cervical neoplasia in HIV-positive group.

An epithelial B cell increase in HIV-positive high-grade SIL cohort compared to the group with normal cervix is also a new finding. Detection of B cells in our study was done by using antibody against CD20 and from this staining the functional status of B cells in the epithelium is not clear. It is necessary to further investigate the functional characteristics of B cells in the epithelium of HIV-negative and HIV-positive groups in order to understand the possible relationship between the epithelial B cell increase and the altered mucosal immune response in the cervix of the HIV-positive cohort.

In summary, the quantification of various immune cell types in the cervix confirmed our initial hypotheses that the numbers of immune cells in the cervix were elevated in high-grade SIL groups and that these immune cell numbers were attenuated in HIV-positive cohort. Additional studies on the functional characteristics of immune cells in the dysplastic cervix are now required to determine the effect of HIV infection on the local immune response in the cervix. Further knowledge on the host immune response to neoplastic lesions in the cervix will contribute to our understanding of the pathophysiology of HPV-induced epithelial neoplasia, and may help to explain the different clinical outcomes of HPV infection in immunocompromised versus immunocompetent populations.

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**Table 1.** Participants' demographic information

| HIV Status | Pt's ID | Age | Smoking Status | CD4 Count |
|---|---|---|---|---|
| Normal cervix N = 9 Mean age : 49.3 ± 7.0 | 1 | 44 | Current | |
| | 2 | 44 | | |
| | 3 | 42 | No | |
| | 4 | 60 | | |
| | 5 | 62 | | |
| | 6 | 52 | | |
| | 7 | 51 | | |
| | 8 | 39 | | |
| high grade HIV- N = 11 Mean age : 27.9 ± 5.9 | 9 | 39 | Current | |
| | 10 | 23 | No | |
| | 11 | 22 | Current | |
| | 12 | 26 | | |
| | 13 | 24 | | |
| | 14 | 23 | | |
| | 15 | 20 | | |
| | 16 | 27 | No | |
| | 17 | 38 | Current | |
| | 18 | 26 | | |
| | 19 | 39 | Current | |
| high grade HIV+ N = 20 Mean age : 31.2 ± 6.1 | 20 | 29 | Current | 807 |
| | 21 | 46 | No | 80 |
| | 22 | 34 | No | 883 |
| | 23 | 37 | Current | 12 |
| | 24 | 27 | Current | 1 |
| | 25 | 24 | No | 68 |
| | 26 | 46 | Current | 108 |
| | 27 | 24 | Current | 520 |
| | 28 | 32 | Current | 429 |
| | 29 | 21 | No | 429 |
| | 30 | 28 | No | 199 |
| | 31 | 22 | Current | 704 |
| | 32 | 24 | No | 23 |
| | 33 | 27 | Current | 1277 |
| | 34 | 25 | Current | 536 |
| | 35 | 33 | No | 280 |
| | 36 | 39 | No | 169 |
| | 37 | 35 | Current | 151 |
| | 38 | 35 | | Missing |
| | 39 | 36 | | 200 |

Table 2. Average numbers of cells (cells/mm$^2$)

| | WNL[1] | | | HG-SIL[2] HIV-negative | | | HG-SIL HIV-positive | | |
|---|---|---|---|---|---|---|---|---|---|
| | Epithelium | Stroma | Total (av. dev)[3] | Epithelium | Stroma | Total (av. dev) | Epithelium | Stroma | Total (av. dev) |
| CD4+ T | 101 ±62 | 530 ±326 | 630 ±255 | 227 ±153 | 2720 ±853 | 2947 ±1141 | 227 ±225 | 1134 ±653 | 1361 ±939 |
| CD8+ T | 553 ±510 | 577 ±403 | 1131 ±824 | 453 ±229 | 1925 ±801 | 2378 ±1146 | 707 ±468 | 1754 ±910 | 2460 ±1028 |
| CD20 | 2 ±3 | 53 ±66 | 54 ±53 | 12 ±17 | 418 ±350 | 430 ±295 | 19 ±25 | 219 ±159 | 238 ±181 |
| CD68 | 111 ±92 | 462 ±266 | 572 ±36 | 678 ±715 | 808 ±860 | 1576 ±724 | 336 ±225 | 852 ±506 | 1188 ±398 |
| Mast cell | 20 ±29 | 100 ±25 | 120 ±36 | 74 ±62 | 235 ±44 | 309 ±31 | 56 ±44 | 352 ±180 | 409 ±180 |

[1] WNL: cervix within normal limit
[2] HG-SIL: cervix with high grade SIL
[3] av. dev: average deviation

**Table 3.** CD4/CD8 ratio

|  | Stroma | Epithelium | Total |
|---|---|---|---|
| Normal cervix | 1.18 | 0.52 | 0.87 |
| ST. Deviation | ±0.62 | ±0.49 | ±0.59 |
| HG-SIL HIV − | 1.61 | 0.63 | 1.17 |
| ST. Deviation | ±0.51 | ±0.46 | ±0.60 |
| HG-SIL HIV + | 0.70* | 0.57 | 0.64** |
| ST. Deviation | ±0.60 | ±0.33 | ±0.51 |

\* $p < 0.004$: HIV+ vs HIV- stromal CD4/CD8 ratio
\*\* $p < 0.03$: HIV+ vs HIV- total CD4/CD8 ratio

**Figure legends**

Figure 1. Localization of CD4+ T cells.

Immunohistochemistry on serial sections from a paraffin-embedded sample of normal cervix was performed with antibodies against CD4 (a: normal stroma; b: normal epithelium; c: stroma beneath high-grade SIL; and d: epithelium with hig-grade SIL). Positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 40X objective. E: epithelium; S: stroma; c: capillary vessel, G: endocervical gland.

Figure 2. Localization of CD8+ T cells.

Immunohistochemistry on serial sections from a paraffin-embedded sample of normal cervix was performed with antibodies against CD8 (a: normal stroma; b: normal epithelium; c: stroma beneath high-grade SIL; and d: epithelium with hig-grade SIL). Positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 40X objective. E: epithelium; S: stroma; c: capillary vessel, G: endocervical gland.

Figure 3. Localization of CD20+ B cells.

Immunohistochemistry on serial sections from a paraffin-embedded sample of normal cervix was performed with antibodies against CD20 (a: normal stroma; b: normal epithelium; c: stroma beneath high-grade SIL; and d: epithelium with hig-grade SIL). Positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 40X objective. E: epithelium;

S: stroma; c: capillary vessel, G: endocervical gland.

Figure 4. Localization of CD68+ macrophages.

Immunohistochemistry on serial sections from a paraffin-embedded sample of normal cervix was performed with antibodies against CD68 (a: normal stroma; b: normal epithelium; c: stroma beneath high-grade SIL; and d: epithelium with hig-grade SIL). Positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 40X objective. E: epithelium; S: stroma; c: capillary vessel, G: endocervical gland.

Figure 5. Localization of mast cell tryptase+ cells.

Immunohistochemistry on serial sections from a paraffin-embedded sample of normal cervix was performed with antibodies against mast cell tryptase (a: normal stroma; b: normal epithelium; c: stroma beneath high-grade SIL; and d: epithelium with hig-grade SIL). Positively stained cells appear brown. The stromal-epithelial junction is marked with a dashed line. Photos taken with 40X objective. E: epithelium; S: stroma; c: capillary vessel, G: endocervical gland.

Figure 6. Graphic comparisons of cell counts/mm$^3$ by HIV-status.

a; quantification of CD4+ T cells, b; quantification of CD8+ T cells, c; quantification of CD20+ B cells, d; quantification of CD68+ macrophages, and e; quantification of mast cells.

Figure 7. Comparisons of the relative distribution of immune cells by HIV-status.

a; relative distribution of cells in the stroma, b; in the epithelium, and c; the

relative distribution of the total number of cells.

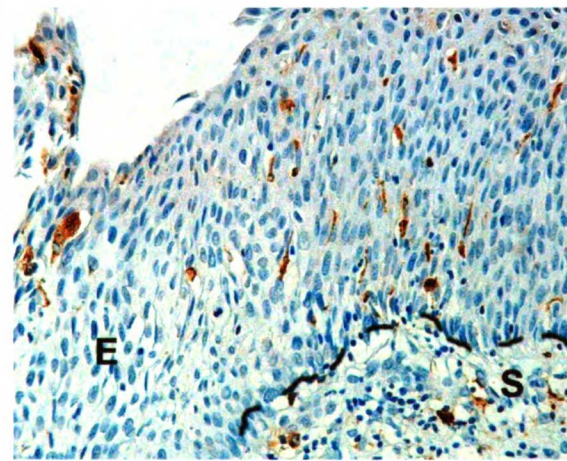# Fig 1 CD4 T Cell Staining
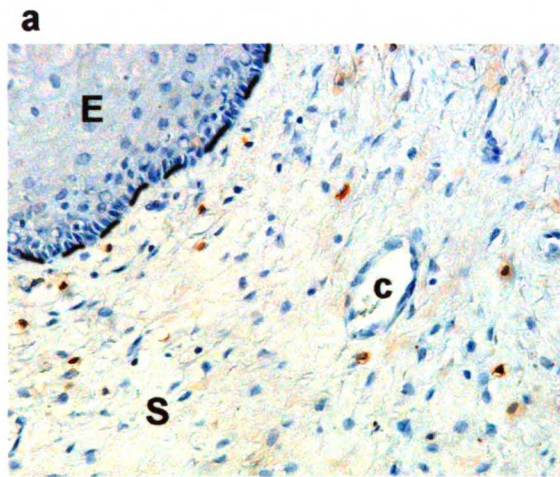


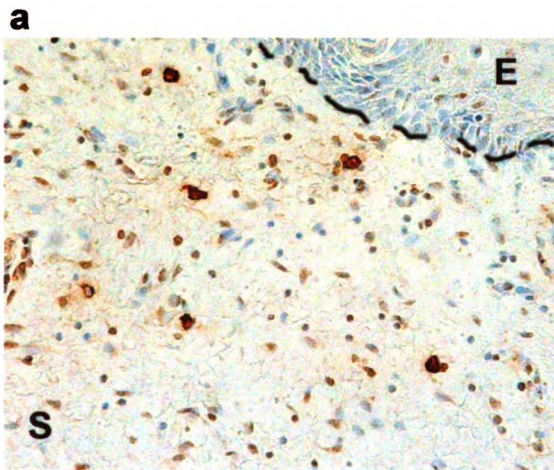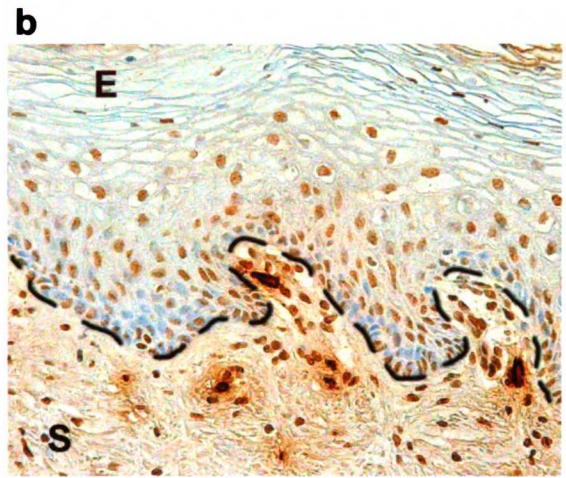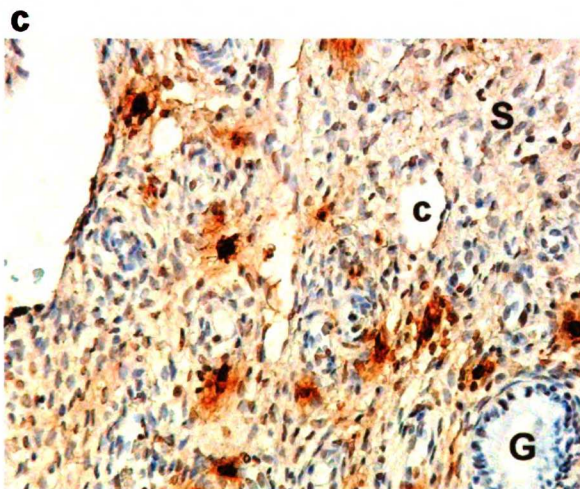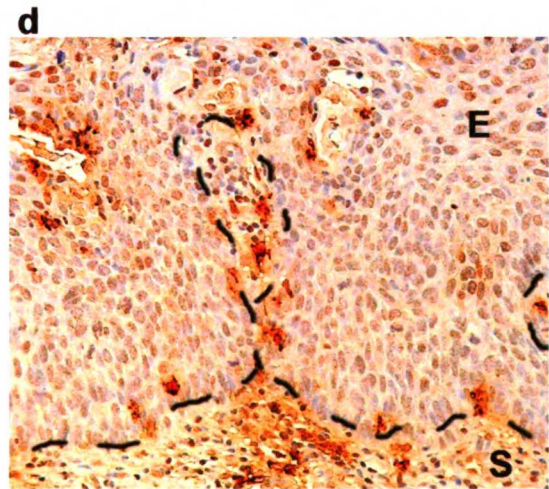Normal (stroma)
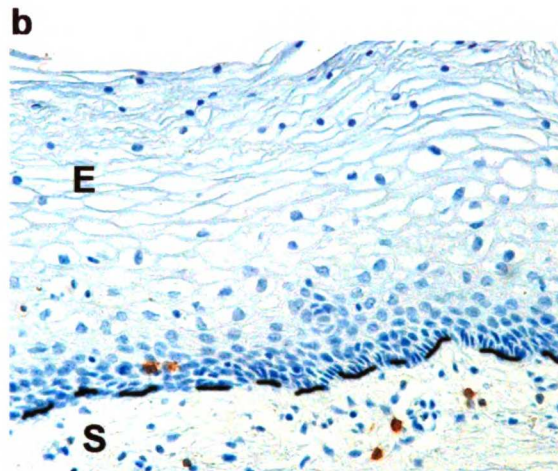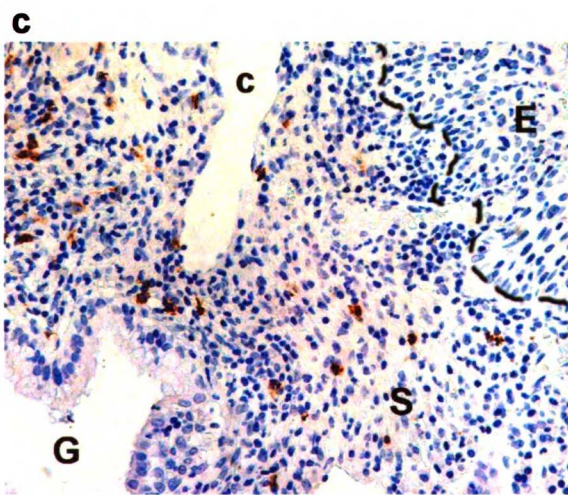


Normal (epithelium)



High-grade SIL (stroma)
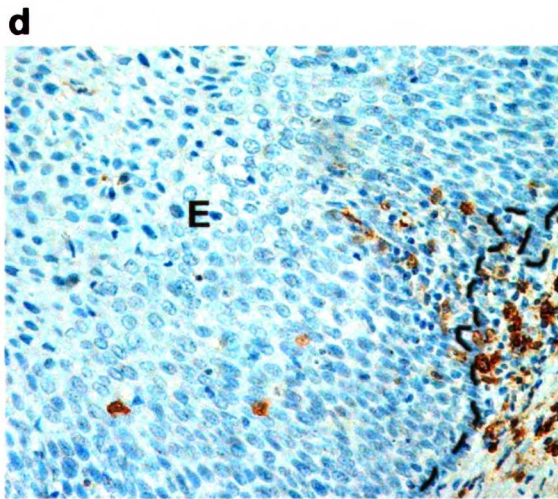


High-grade SIL (epithelium)
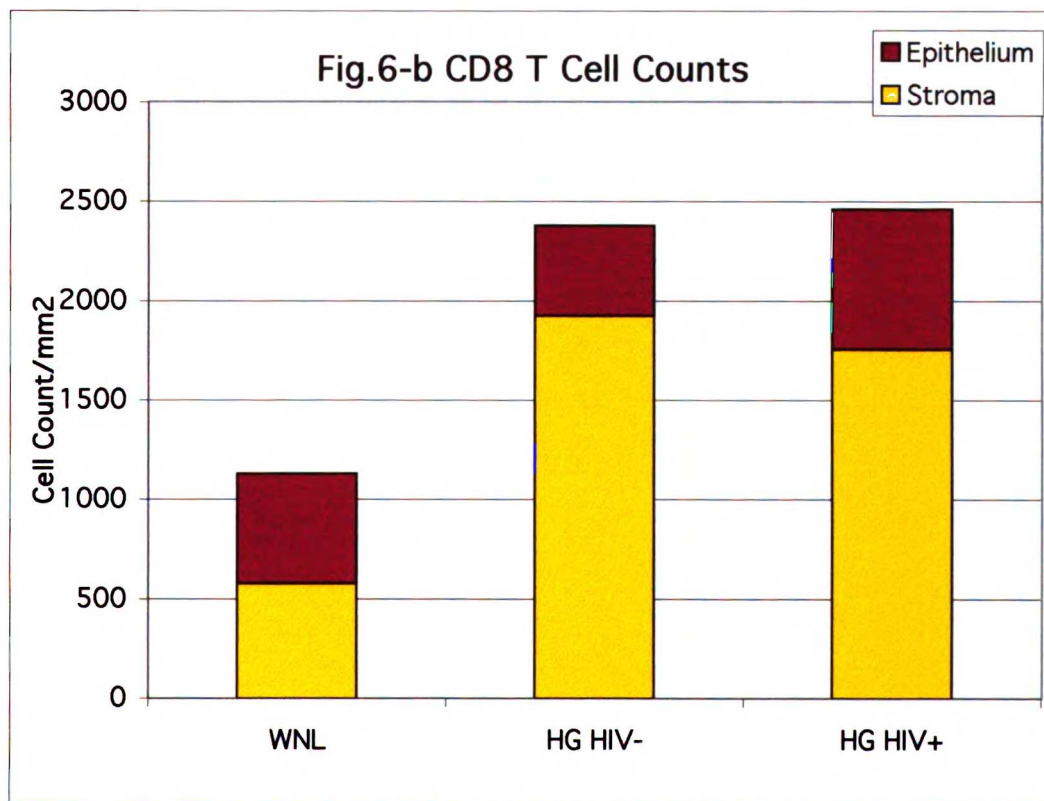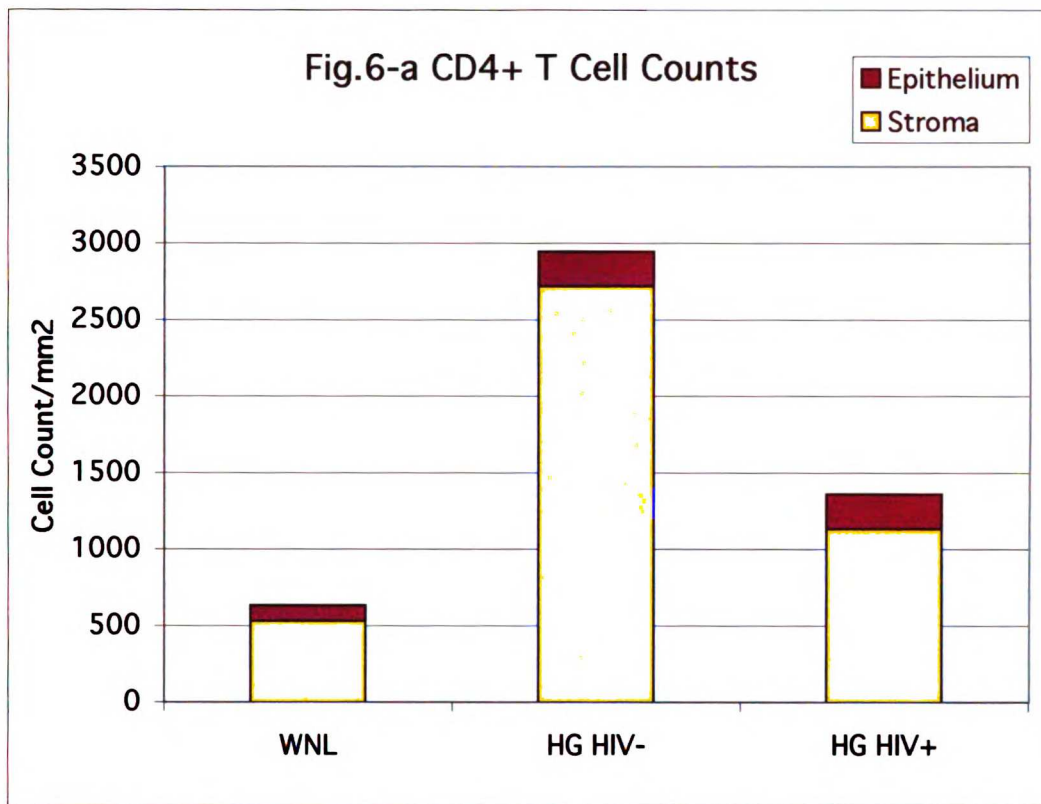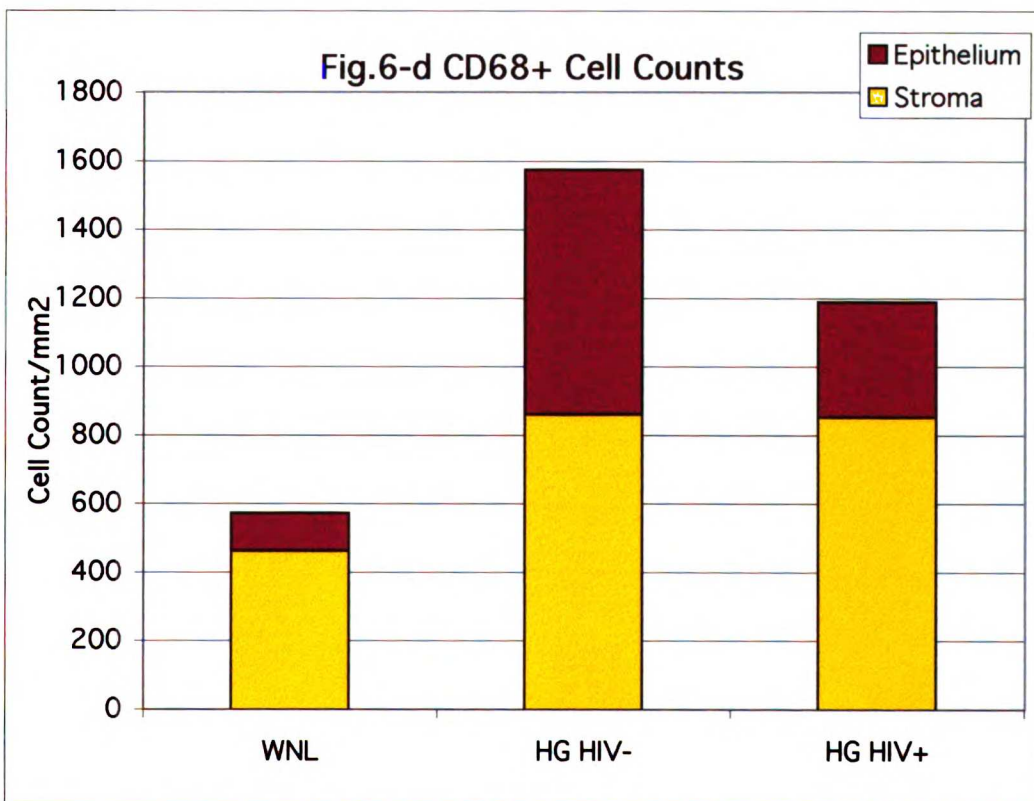
# Fig 2 CD8 T Cell Staining
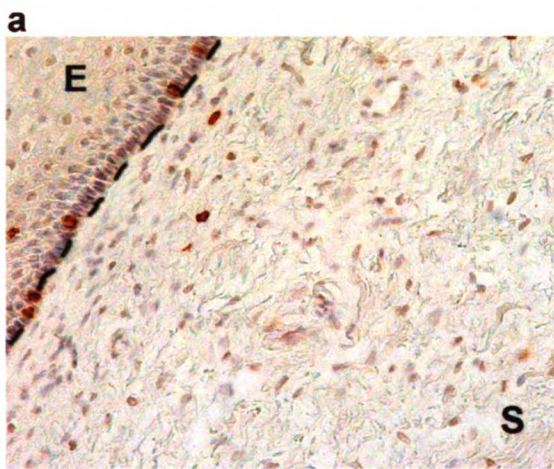


**Normal (stroma)**
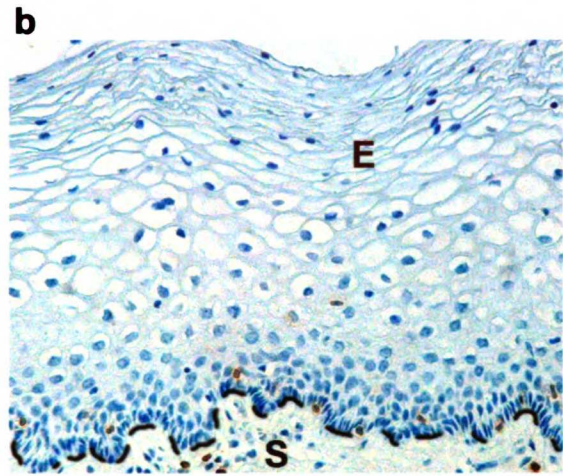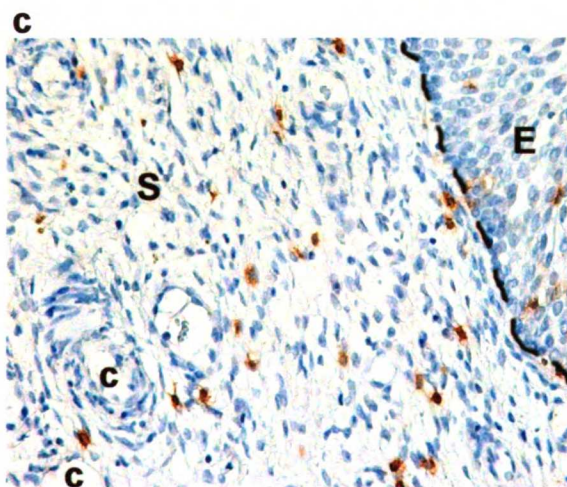
**Normal (epithelium)**

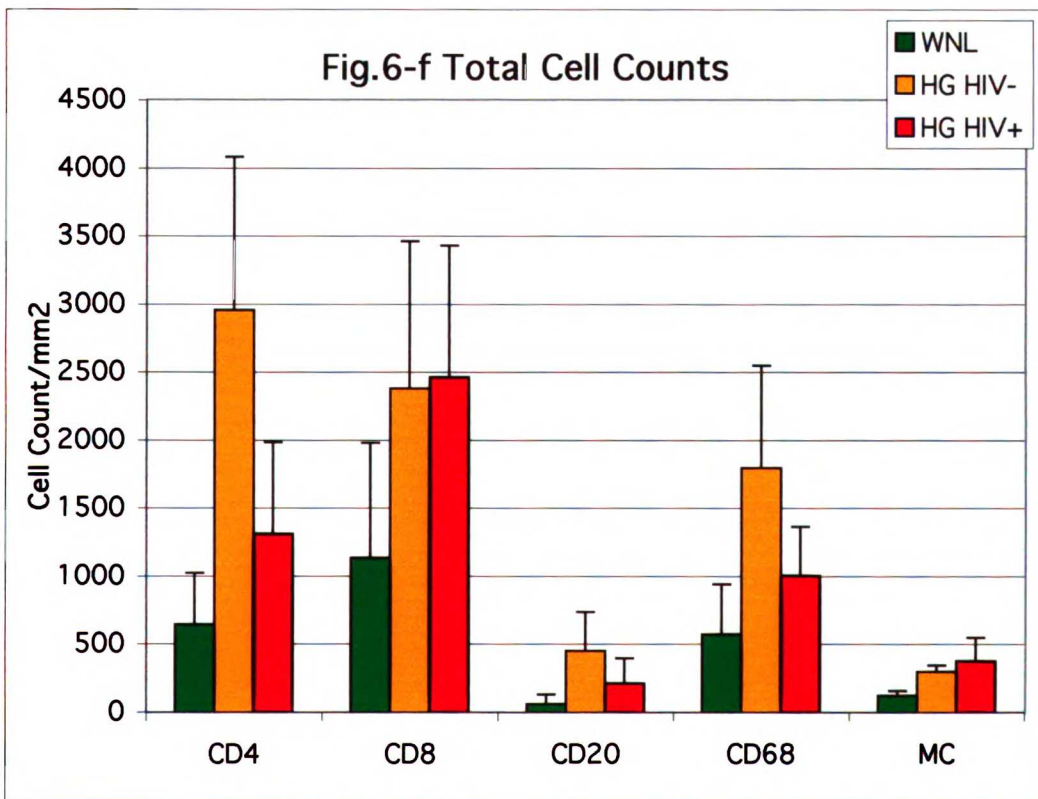**High-grade SIL (stroma)**
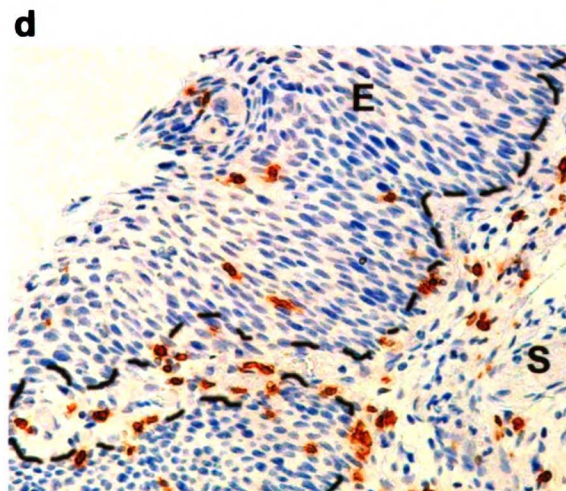
**High-grade SIL (epithelium)**

# Fig 3 CD20 B Cell Staining
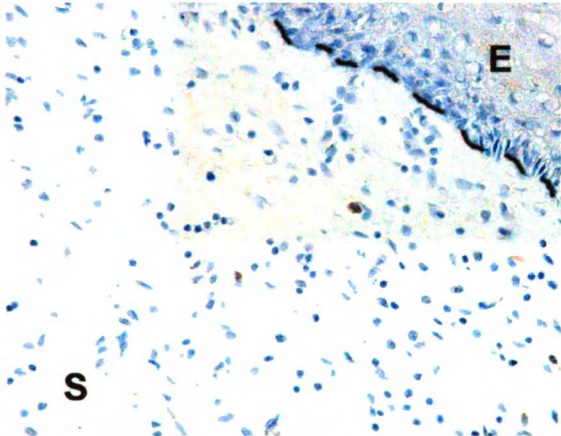


Normal (stroma)
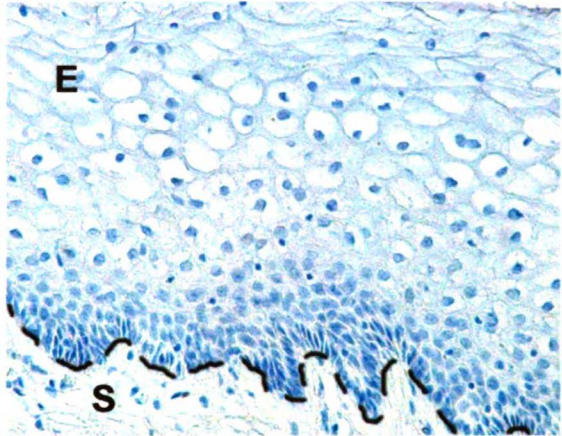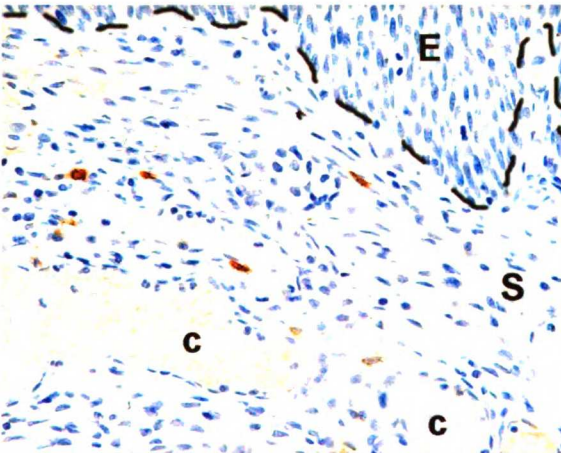
Normal (epithelium)

High-grade SIL (stroma)
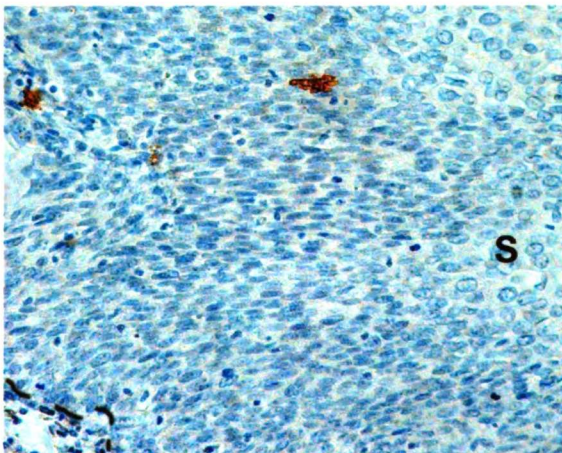
High-grade SIL (epithelium)

# Fig 4 CD68 Macrophage Staining
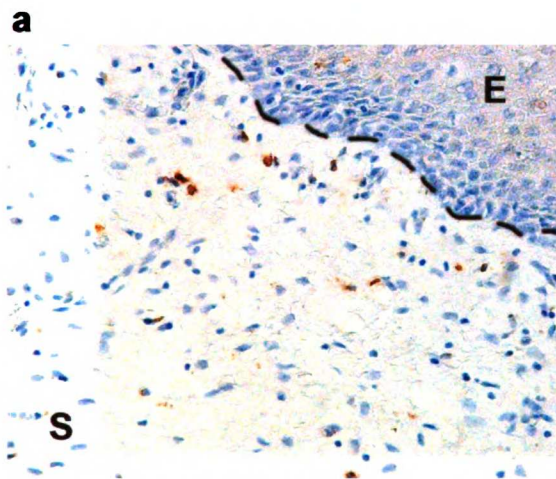


Normal (stroma)
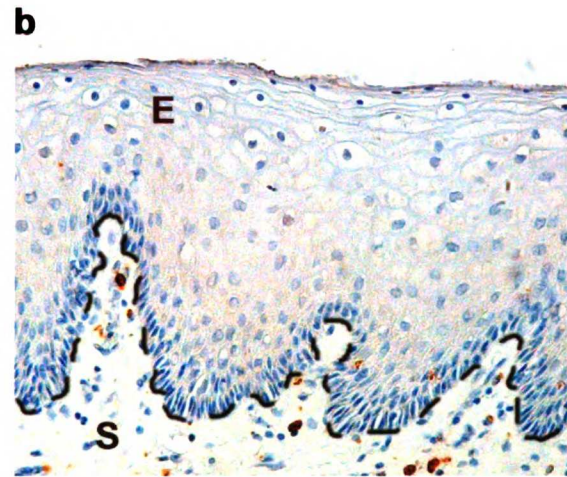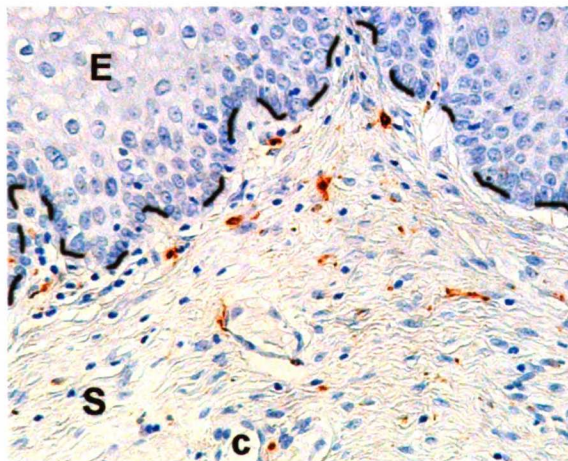
Normal (epithelium)

High-grade SIL (stroma)

High-grade SIL (epithelium)

# Fig 5 Mast Cell Staining



**a**

Normal (stroma)



**b**

Normal (epithelium)



**c**

High-grade SIL (stroma)



**d**

High-grade SIL (epithelium)

Fig.6-a CD4+ T Cell Counts



Fig.6-b CD8 T Cell Counts

Fig.6-c CD20+ Cell Counts



Fig.6-d CD68+ Cell Counts

Fig.6-e mast Cell Counts



Fig.6-f Total Cell Counts

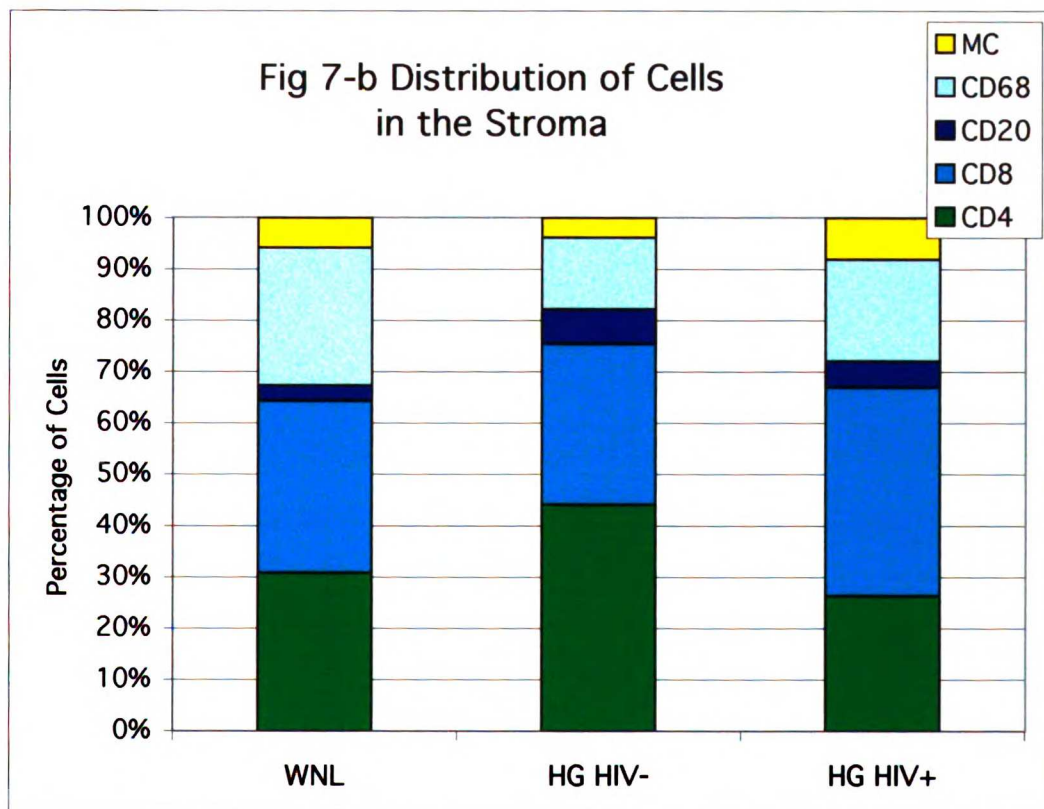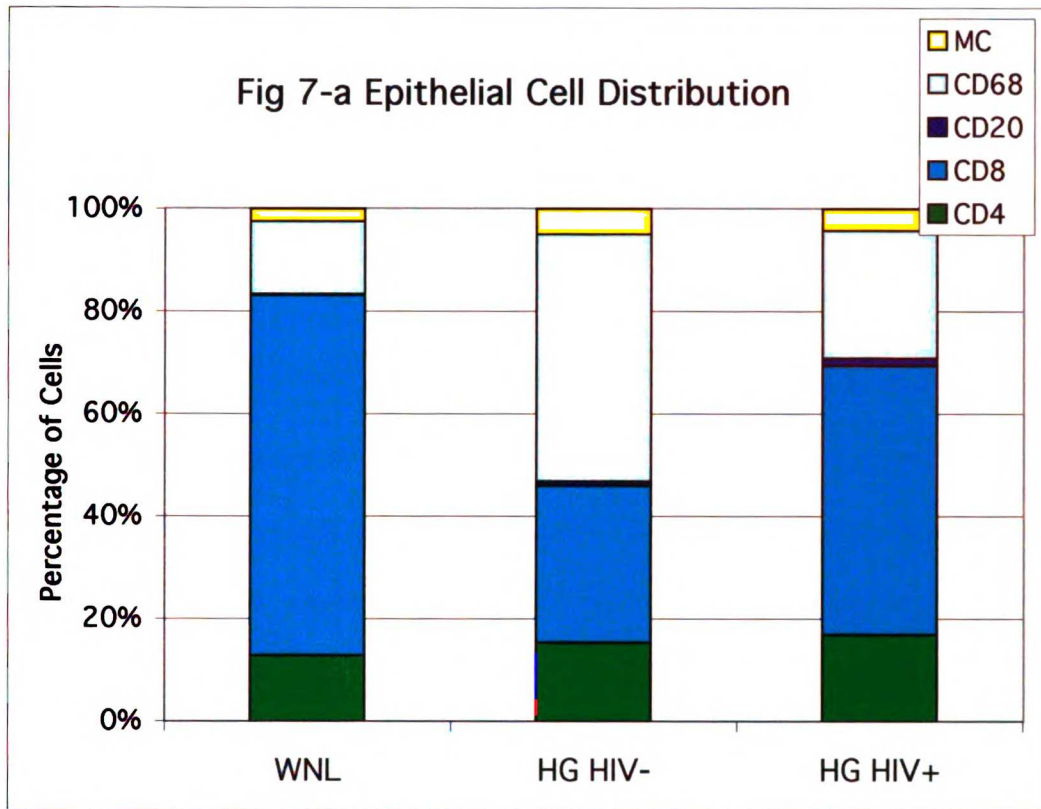Fig 7-a Epithelial Cell Distribution



Fig 7-b Distribution of Cells in the Stroma
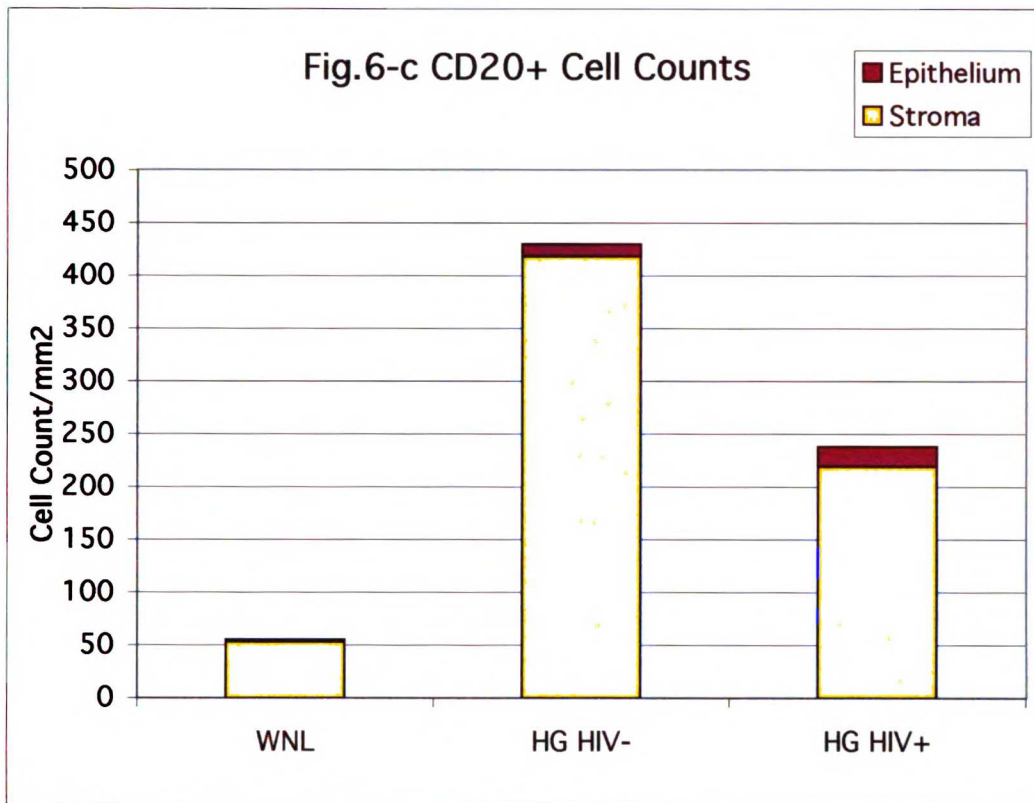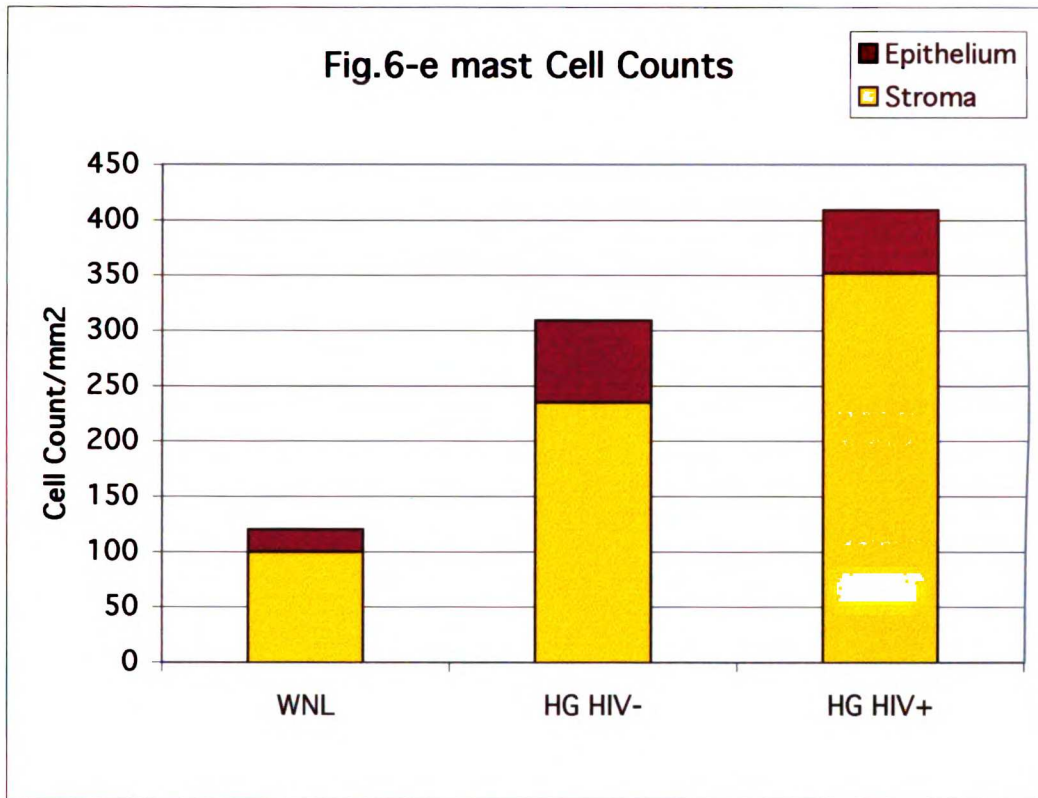
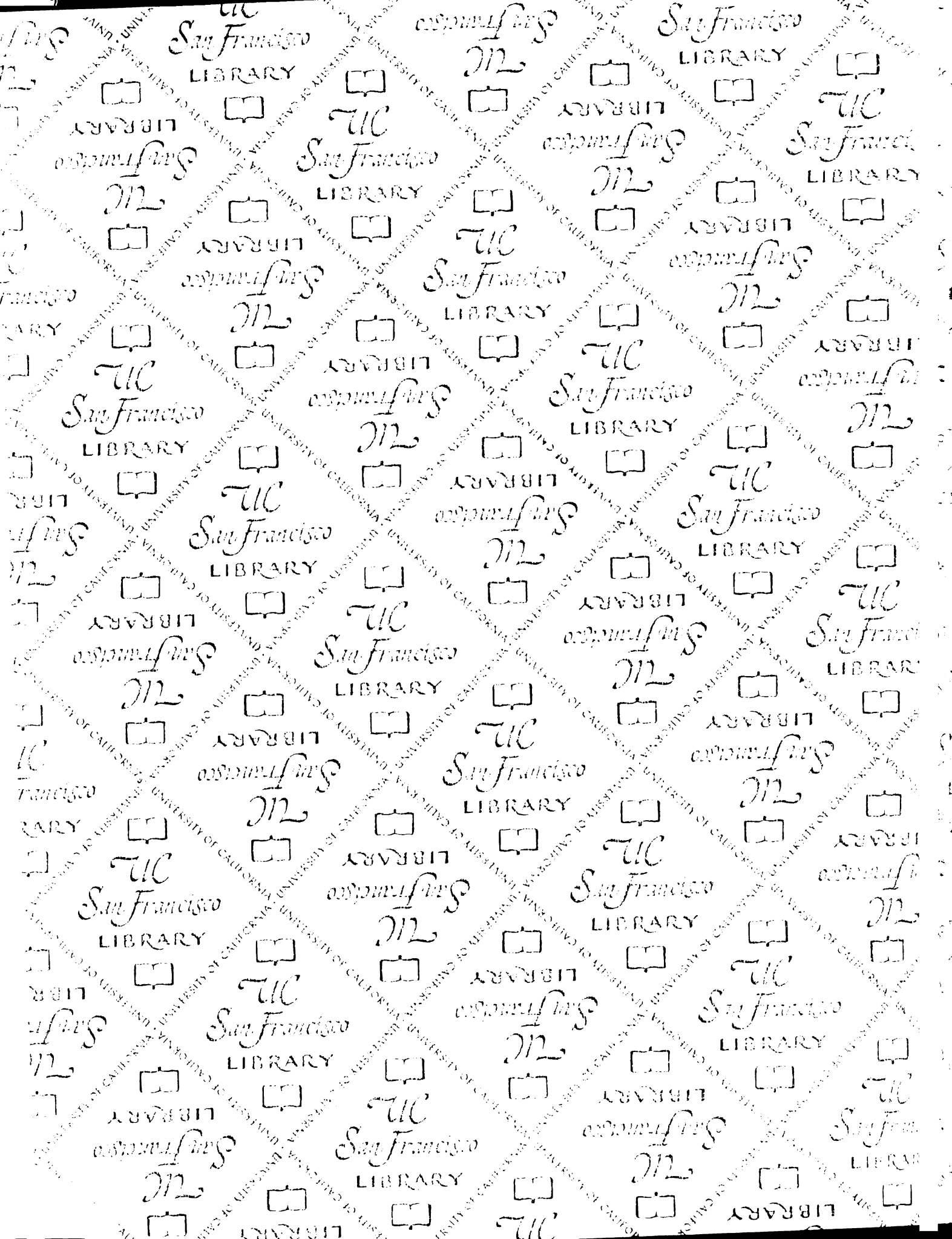## Conclusions and Recommendations for Future Research

The purposes of the studies presented here were to explore and describe the mucosal immune response in the cervix to high-grade dysplasia and to compare the local immune response to dysplasia between the HIV-negative and HIV-positive populations. The results indicated the presence of a robust mucosal immune response in the cervix to dysplasia. In addition, the presence of CD8+ T cell-dominant aggregates was unique to the HIV-positive high-grade SIL population. The immune cell counts are the first report to describe numbers of cells per $mm^2$ of cervical tissue. These descriptive study results lead us to further investigate functional characteristics of immune cells in the cervix. Therefore, future research studies will focus on the functional status of immune cells recruited to areas of high-grade SIL. One approach for functional analysis is detection of cytokine production following antigenic stimulation. CD4+ and CD8+ T cells which can be isolated from cervical tissue will be studied to determine intracellular production of cytokines through ex vivo activation of these cells using HPV antigens and analysis using flow cytometry. Functional analysis may explain the relationship of altered mucosal immune responses in HIV-positive women observed in our study and the high incidence of cervical dysplasia in this population.

In future studies, we plan to compare lymphocytes isolated from peripheral blood and cervical tissue from the same woman simultaneously and detect the intracellular cytokine production in these lymphocytes. These studies will

establish the relationship between local and systemic immune responses to HPV antigens associated with cervical dysplasia.

Since the epithelial CD8+ T cell counts in the normal cervix and both high-grade groups were within a small range (260-520 cells/mm$^2$), CD8+ T cells in the dysplastic epithelium may not have migrated to the epithelium in response to antigens present in or invading through the epithelium. Instead, some of these cells may have resided in the epithelium as intraepithelial lymphocytes (IEL). Therefore, it is also important to identify phenotypes of CD8+ T cells within the epithelium of all three groups, as well as to detect their functional properties.

In addition, studying functional properties and phenotypes of mast cells in the cervix is also important since mast cells increased significantly in the stroma of HIV-positive high-grade SIL group compared with the HIV-negative group. There has never been a report of tryptase+ mast cell increase in the stroma of the dysplastic cervix in the HIV-positive group, and the reason for this mast cell increase is not clear. An increase in B cells in the dysplastic epithelium in HIV-positive cohort is also a new finding. In addition to cytokine study, detection of antibody classes produced by these B cells in response to a specific antigen may also help understand altered immune responses observed in HIV-positive cohort. These future studies will help to develop more effective medical interventions as well as potential vaccine development against HPV and possibly HIV.