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Mucosal Immunology of HIV Infection

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Summary

Recent advances in the immunology, pathogenesis, and prevention of human immunodeficiency virus (HIV) infection continue to reveal clues to the mechanisms involved in the progressive immunodeficiency attributed to infection but more importantly have shed light on the correlates of immunity to infection and disease progression. HIV selectively infects, eliminates, and/or dysregulates several key cells of the human immune system, thwarting multiple arms of the host immune response, and inflicting severe damage to mucosal barriers, resulting in tissue infiltration of ‘symbiotic’ intestinal bacteria and viruses that essentially become opportunistic infections promoting systemic immune activation. This leads to activation and recruitment of more target cells for perpetuating HIV infection, resulting in persistent, high level viral replication in lymphoid tissues, rapid evolution of resistant strains, and continued evasion of immune responses. However, vaccine studies and studies of spontaneous controllers are finally providing correlates of immunity from protection and disease progression, including virus-specific CD4⁺ T-cell responses, binding antibodies, innate immune responses, and generation of antibodies with potent antibody-dependent cell-mediated cytotoxicity activity. Emerging correlates of immunity indicate that prevention of HIV infection may be possible through effective vaccine strategies that protect and stimulate key regulatory cells and immune responses in susceptible hosts. Further, immune therapies specifically directed towards boosting specific aspects of the immune system may eventually lead to a cure for HIV-infected patients.

Keywords

HIV; SIV; mucosal immunology; T cell

Introduction

More than 30 years since the first descriptions of human immunodeficiency virus (HIV) infection in humans, the acquired immunodeficiency syndrome (AIDS) epidemic continues relatively unabated into the 21st century. Although tremendous progress has been made in developed countries where anti-retroviral therapy (ART) is available and prevention of mother to child transmission has become standardized, approximately 50,000 people in the United States are still newly infected with HIV each year (according to the Centers for Disease Control) and the epidemic continues to spread in underdeveloped nations. Of those living with HIV, about 70% live in sub-Saharan Africa, most are unaware of their infection, and many cannot access ART (1). Although HIV no longer means a death sentence for those who can afford treatment, we have made little headway in producing a highly effective

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vaccine, and we are no closer to a cure. Education, counseling, and promoting condom use currently remain our best weapons against HIV infection.

We have made great strides towards understanding the pathogenesis and immunology of HIV infection, however, and recent prevention trials in humans and emerging studies in nonhuman primate models of HIV infection suggest effective vaccine and prevention methods are possible. Male circumcision and pre-exposure prophylaxis have shown promise in preventing HIV infection in clinical trials (2), and the results of the RV144 phase 3 vaccine trial in Thailand demonstrated 31.2% efficacy in prevention of infection (3,4). Further, immunology and vaccine studies continue to expand our understanding of the immune deficits and possible correlates of protection from infection, which may lead to more effective prevention strategies in the near future. Understanding the cellular and molecular mechanisms involved in HIV transmission, immunology, and the development of AIDS will be critical for designing new treatment strategies that may eventually lead to a cure for HIV infection.

Mucosal HIV transmission

The vast majority of HIV infections result from mucosal transmission. Vaginal and rectal transmission account for most adult infections, but pediatric HIV infections are usually the result of oral ingestion of maternal fluids (5, 6). Whether pediatric HIV transmission occurs through the tonsils, oral mucosa, or intestinal tract is uncertain, but pediatric macaque studies have demonstrated oral simian immunodeficiency virus (SIV) transmission to infants is more efficient than vaginal or rectal transmission (7). Although oral transmission is considered rare in adults, newborn infants have a neutral gastric pH (8), and conceivably HIV could enter through the intestinal tract in infants. Nonetheless, studies in macaque and explant models in which virus is atraumatically applied to mucosal tissues indicate SIV is readily transmitted across the intact rectal, vaginal, cervical, oral, and (to a lesser extent) penile mucosal surfaces (7,9,10). Since most studies examining the early events in mucosal transmission involve vaginal inoculation models, we focus on what is known regarding the early immunologic events associated with exposure and infection of the female reproductive tract (FRT). However, many of the early immunologic events occurring in other mucosal sites of transmission are likely similar.

Although frequently referred to as ‘vaginal transmission’, the actual anatomical sites where initial HIV infection crosses mucosal barriers and establishes infection in the FRT are somewhat debated. The distal or lower FRT (ectocervix and vagina) of women and macaques is protected by numerous layers of stratified squamous epithelium, which until recently was thought to be an impenetrable barrier to viruses and other microbes. In contrast, the endocervix lacks this thick barrier and is lined only by a single layer of cuboidal epithelial cells, which many believe is a more susceptible site for HIV penetration and transmission. However, infection clearly occurs across the vaginal mucosa, as hysterectomized women, women lacking a cervix, and hysterectomized macaques lacking a cervix can be infected with HIV/SIV (reviewed in 11). Further, administering progesterone or progestins (Depo-provera) to macaques markedly increases vaginal transmission rates, most likely due to thinning of the vaginal and ectocervical epithelium (12, 13). Similarly, the use of hormonal contraceptives in women has been recently shown to increase vaginal HIV transmission rates by almost twofold (14). Hormone-induced vaginal epithelial thinning, which brings luminal antigens in closer proximity to underlying target cells in the vagina, may partially account for this increased susceptibility to HIV infection. However, hormonal influences associated with menstrual cycles, pregnancy, menopause, etc., may also affect the production of antimicrobials and/or early mucosal inflammatory responses to exposure, which could influence HIV transmission rates (15).

The endocervix is also a major site of SIV transmission, as studies in vaginally SIV-challenged macaques have demonstrated foci of infected cells in the cervix within a few days of exposure (16). Conceivably, more proximal sites of the FRT tract like the uterus, fallopian tubes, and ovaries may also be susceptible to infection, but it is simply not known whether initial infections are established in the vaginal, cervical, or other tissues in the FRT. Since most transmission studies have focused on cervicovaginal tissues, we focus on what is known regarding the early immunologic events that occur in these sites following HIV/SIV exposure.

Although the vast majority of HIV infections occur from heterosexual intercourse, The FRT is one of the least efficient sites for HIV transmission. Recent estimates suggest that only 1 in 900 heterosexual exposures result in vaginal HIV transmission (17). This number is consistent with early studies of SIV vaginal transmission in macaques demonstrating that 100 to >1000 times more virus is required to establish infection after intravaginal inoculation compared with intravenous inoculation (9). However, even when large doses of virus are ‘randomly’ applied to the vagina of macaques (with no consideration to age or the menstrual cycle), only a fraction of animals become infected. Therefore, large numbers of animals are usually necessary to demonstrate statistical significance in studies examining rates of vaginal transmission or protection, and the low infection rate also complicates investigations into the early events in transmission.

The reasons why vaginal transmission is so inefficient is unclear. The vaginal environment is far from static, and there are numerous continuously changing variables that may be involved. First, vaginal transmission is clearly associated with higher viral loads in semen, so the ‘dose’ of exposure is clearly important (17). Second, the vaginal environment is defended by a number of innate mucosal barriers and immune mechanisms that likely impart significant protection from infection. For example, the cervicovaginal surface is coated with mucus, which varies in consistency and volume throughout the menstrual cycle. Recent studies have shown cervicovaginal mucus (CVM) impairs the mobility of HIV particles (18). Thus, the consistency, volume, and quality of CVM may play an important role as the first line of defense of the FRT. In addition, in cycling women and nonhuman primates, the vaginal epithelium varies in thickness and cell number throughout the menstrual cycle, which may provide a formidable barrier to transmission, particularly during the follicular or ovulatory stage of the cycle, when the vaginal epithelium is thickest (19, 20). Although the number and distribution of immune cells appears constant in the vagina throughout the cycle (21, 22), thinning of the epithelium during the luteal phase may result in virions reaching the underlying target cells involved in transmission (see below). In support of this, multiple studies have demonstrated macaques are more susceptible to vaginal transmission during the luteal phase of the menstrual cycle (23, 24). Further, years ago, we demonstrated that administering progesterone to macaques resulted in thinning of the vaginal epithelium and a marked increase in vaginal transmission rates to macaques (25). However, it remains unclear whether this is solely attributed to changes in vaginal thickness and integrity, mucus secretion and quality, and/or cyclic changes in the immunologic responses of the FRT in response to hormones. Others have shown the production of antimicrobial substances and immunoglobulin secretion is markedly affected by changes in hormone levels, indicating cyclic changes in the immune response to antigens may be involved in the susceptibility to vaginal HIV transmission (15, 26, 27). Nonetheless, we have refined the hormone model of vaginal transmission and are currently using Depo-provera-treated macaques for examining cellular and viral events in vaginal transmission, as well as for testing preventatives. Using this model, we and others (23,25,28-30) have shown that macaques can consistently be infected with much smaller doses of virus following atraumatic intravaginal inoculation a few weeks after administration of progestins. Definitely identifying the first cells infected

after vaginal and rectal transmission would certainly be helpful for designing new HIV vaccine and prevention strategies.

Mucosal target cells involved in early HIV/SIV transmission

Regardless of the anatomical site of FRT infection, timed inoculation and viral inactivation studies have demonstrated SIV crosses the mucosal barrier and establishes infection within 60 minutes of vaginal inoculation (31). Possible mechanisms for transmission across an intact squamous epithelium of the vagina involve the interaction of virus with dendritic cells (DCs) and/or CD4⁺CCR5⁺ T cells in the superficial vaginal mucosa. Multiple studies suggest vaginal epithelial DCs termed Langerhans cells (LCs) may rapidly capture and transport virus to underlying susceptible cells in the initial minutes/hours of infection (12, 31, 32). Langerhans' cells are abundant in the cervix and vaginal epithelium of humans (33-36) and rhesus macaques (22, 32, 37). Although vaginal LCs do not express CD4 or CCR5 (22), they do express HLA-DR, CD1a (Fig. 1) and a number of mannose dependent C-type lectin receptors (MCLRs) that can function as highly efficient viral attachment factors (36, 38-41). Further, these cells have dendritic cytoplasmic processes that extend through the epithelium to the vaginal lumen, where they are involved in sampling luminal antigens (32). In mice, it has been demonstrated that antigens are readily adsorbed through LCs of the vagina (42). Thus, a plausible mechanism of mucosal HIV-1 infection involves the capture of viral particles on the vaginal luminal surface by DC processes expressing one or more MCLRs. Once the virus has been captured, the immature DCs then migrate and present the virus to the underlying *lamina propria* that contains abundant CD4⁺ and CCR5⁺ T cells, macrophages, and other cells that may support viral amplification. However, we have been unsuccessful in preventing transmission with topical applications of various DC/LC receptor blockers (authors' unpublished observations). Whether CD4⁺ T cells are directly infected by HIV or by HIV presented by LCs in the cervicovaginal mucosa is still unclear, since the dynamics of these interactions are difficult to extrapolate from tissue sections representing single 'snap shots' in time. Detection of the first cell(s) infected after vaginal exposure is difficult to detect by current technologies, indirect, yet emerging, and converging evidence suggest CD4⁺CCR5⁺ memory T cells may actually be the primary targets for direct infection and viral replication in the earliest stages of infection.

Abundant CD4⁺CCR5⁺ T cells reside in the normal vaginal mucosa, and these are among the early targets for SIV infection and CD4⁺ T-cell destruction (22,43). Although these cells are normally found in the deeper layers of the vaginal epithelium (21, 22), breaks, inflammation, and/or hormone-induced physiologic epithelial thinning accompanied by infiltrating T cells may all bring these target cells closer to viruses penetrating the superficial vaginal epithelium. For example, thinning or less mucus production may allow more contact of the epithelial cells with luminal antigens, promoting local lymphokine production and promoting local inflammatory responses. Further, emerging evidence indicates SIV/HIV alone may trigger inflammatory responses facilitating transmission (44).

Essentially all vaginally transmitted HIV strains utilize CD4 and CCR5 as their receptors for attachment and entry into cells (45). We now know initial HIV and SIV infection is usually acquired from a single virus genotype (or infected cell) in the vast majority of cases, and essentially all of early transmitted founder viruses (TFV) utilize CD4 and CCR5 as their primary receptors (46, 47). In early SIV infection, CD4⁺ T cells are almost exclusively infected, and viruses produced later in infection show higher affinity for other myeloid cell types (48, 49). The established virus probably arises from a single TFV and nidus of infection, as the detection of a single small cluster of 40–50 cells positive for SIV RNA 3 and 4 days post vaginal exposure suggests small infected founder cell populations form at the initial portal of entry (44).

The upper layers of the vaginal epithelium lack tight junctions and are permeable to large-molecules and viruses (50). Thus, virions can penetrate the upper layers of the vaginal epithelium freely and reach the deeper, parabasal layers of epithelium where CD4⁺ T cells reside, without requiring capture and transport by LCs (Tom Hope, personal communication). Moreover, epithelial cells respond to external antigens by producing an array of innate immune mediators including chemokines and cytokines that trigger cell recruitment and homing of target cells to the initial site of infection. Thus, repeated HIV exposure to the epithelium itself may result in inflammation and recruitment of CD4⁺CCR5⁺ T cells into the more superficial layers of the epithelium, where they can directly contact virus. Emerging evidence from macaque models suggests HIV may even rely on this early innate inflammatory response to the virus itself to facilitate transmission (see below).

In some experimental models, the probability of mucosal infection is directly proportional to the availability of CD4⁺CCR5⁺ T cells in mucosal tissues (51). Further, HIV TFVs replicate well in CD4⁺ T-cell cultures but not in monocyte-derived macrophage cultures (48,52). Moreover, human *ex vivo* explant models have shown CD4⁺CCR5⁺ T cells are productively infected without requiring LCs (53). Finally, macaque studies have consistently shown that vaginal transmission of CCR5-using (R5) SHIVs can be completely prevented by topical application of substances that block viral attachment/fusion to either the CD4 binding site of HIV gp120 or CCR5 (13, 29, 54-59). Although this does not explain how HIV crosses epithelial barriers nor does it necessarily prove CD4⁺CCR5⁺ T cells are the first cells infected, it does imply that infection and amplification within CD4⁺CCR5⁺ T cells is critical to successful vaginal SIV/HIV transmission and the establishment of infection.

Although the earliest events in HIV infection are difficult to examine, the results are certainly consistent with the small-founder-population concept and host tropisms observed in the SIV rhesus macaque model (44). Thus, it is currently thought that HIV infection results from a single nidus of infection in the FRT, most likely from infection of CD4⁺CCR5⁺ T cells, which instigates an inflammatory reaction, resulting in recruitment and activation of additional CD4⁺ target cells to the site and an expanding nidus of infection (44). Expansion of these foci may either be through recruitment of additional target cells or simply growth of the existing foci, but nonetheless, the nidus continues to grow until infected cells exit the mucosa and enter the afferent lymphatics of the draining lymph nodes, where viral infected cells can be detected within 48 h of vaginal exposure (16,44).

Infections with other sexually transmitted diseases (STDs) can also increase susceptibility to HIV infection, likely from the inflammation and recruitment of CD4⁺CCR5⁺ target cells (60-62). For example, marked increases in CD4⁺, CXCR4⁺ and CCR5⁺ T cells have been demonstrated in the endocervix of women vaginally infected with *Chlamydia* when compared to uninfected women (63). Ulcerative STDs, such as syphilis, Herpes simplex type 2 (HSV-2), and chancroid also result in epithelial damage and loss of mucosal integrity. Further, inflammatory STDs, such as *Neisseria gonorrhea*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* also result in the recruitment of inflammatory cells, including activated CD4⁺ T cells (62). These cells, recruited to control vaginal infections, apparently serve as additional 'fuel' for HIV infection and further local replication, which rapidly results in dissemination to draining lymph nodes and the systemic circulation. In other words, emerging evidence indicates mucosal inflammation, driven by infection, trauma, or innate immune responses to HIV exposure alone, results in recruitment of additional CD4⁺CCR5⁺ target cells that establishes a local, expanding site of infection in mucosal tissues, which eventually reaches the lymphatics and systemic circulation.

Early vaginal immune responses to SIV

Although the normal FRT of women and female macaques does not contain organized or 'inductive' lymphoid tissues, there are resident populations of DCs, LCs, T and B cells, and macrophages in the *lamina propria* of the vagina and cervix that may be involved in the early response to HIV infection. The distribution and phenotype of immune cells and molecules has been characterized in both macaque (21, 22, 32, 64) and human (64, 65) vaginal tissues by flow cytometry and immunohistochemistry.

Vaginal and/or cervical epithelial cells may play a key, early role in the early innate immune responses to HIV. Generally the innate immune system provides the first line of host defense against invading mucosal pathogens. Innate immunity is triggered when host pattern recognition receptors (PRRs) on innate cells detect microbe-associated molecular patterns (MAMPs) within microbial molecules, such as lipopolysaccharide (LPS), flagellin, peptidoglycan, or danger-associated molecular patterns (DAMPs) released from damaged endogenous host cells including ATP and host DNA (66,67). The most studied PRRs are the Toll-like receptors (TLRs), which are transmembrane or cytoplasmic proteins that recognize evolutionarily conserved patterns present in microorganisms, including bacteria, fungi, protozoa, and viruses. To date, 10 different TLRs have been identified in humans; TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface and mainly recognize hydrophobic molecules unique to microbes, whereas TLR3, TLR7, TLR8, and TLR9 are located in endosomal compartments and are specialized in recognition of microbial nucleic acids (68).

In early microbial exposure, vaginal epithelial cells as well as DCs recognize pathogens via TLRs and respond to this stimulation by producing antimicrobial peptides as well as chemokines to recruit neutrophils to the site of infection. For example, stimulation of TLRs triggers secretion of the chemokine IL-8, which in turn recruits neutrophils to the vaginal mucosa to combat infection (69, 70) and production of antimicrobial peptides such as human beta defensin-2 (hBD-2), which can directly inhibit bacteria (71). Further, in response to recognition of infectious pathogens, the vaginal mucosa mobilizes a variety of other defenses. Antimicrobial proteins, including lysozyme, lactoferrin, and small antimicrobial peptides such as human β -defensins (HBDs) are secreted in response to HIV infection (72). HIV mucosal exposure also results in rapid upregulation of cytokines and chemokines and upregulation of chemokine receptors, which result in activation and recruitment of additional plasmacytoid DCs (pDCs) and T cells. Infiltrating cells have been shown to secrete IFN- α , β , and the CCR5⁺ chemoattractant chemokines MIP-1 α , MIP-1 β , and CCL5 (RANTES), which recruit additional CD4⁺CCR5⁺ T cells to the site, which may also be infected by HIV, expanding the nidus of infected cells (16,44,73).

These early mucosal front-line defenses are designed to prevent bacterial and viral infections of the FRT, by secretion of multiple inhibitory molecules which usually prevent pathogen replication, including SDF-1, MIP-1 α / β , and RANTES, and the chemokine macrophage inflammatory protein 3 α /CC chemokine ligand 20 (CCL20), which is also known to recruit LC precursors (74,75). However, many if not all of these antimicrobial substances, designed to resist a variety of mucosal infections, may create an inflammatory milieu in the vaginal mucosa, which may actually assist HIV in establishing infection. The early innate mucosal inflammatory response generally facilitates transmission of SIV and HIV-1 by compromising the integrity of the mucosal barrier and increasing target cell availability, where they may also create conditions for highly efficient cell-to-cell spread of infection.

Additional evidence that the inflammatory response facilitates HIV transmission comes from experimental prevention trials. Innate immune agonists applied topically facilitated,

rather than inhibited, SIV vaginal transmission (76). Further, TLR stimulation increases susceptibility of cells to HIV infection (77). Thus, the early cytokine response is dominated by the induction of pro-inflammatory cytokines, which contribute to local recruitment of additional target cells and viral amplification, whereas the induction of cytokines inducing antiviral activity occur too late to prevent virus replication and dissemination.

Early mucosal inflammation is a key and possibly necessary feature for successful vaginal HIV transmission. Once target cells (CD4⁺CCR5⁺ T cells) in the FRT *lamina propria* are infected with the primary founder viruses, a cytokine/chemokine ‘storm’ is released consisting mainly of proinflammatory mediators (78), which recruit additional target cells to the site, perpetuating local infection, until viral-infected cells reach draining lymph nodes, and then the gut, which contains the majority of the hosts viral target cells for HIV replication, amplification, and establishment of irreversible infection. Precisely how the virus or viral infected cells travel from the draining lymph nodes to the gut in the earliest days of transmission remain a mystery and a focus of intense investigation in our laboratory, as it may provide keys to the design of an effective vaccine.

The gastrointestinal tract and HIV

The gastrointestinal tract is arguably the most important organ in the pathogenesis of SIV and HIV infection (79). Regardless of the site of infection, the intestinal mucosa essentially serves as the ‘feeding ground’ for HIV amplification in early infection. Further, in chronic infection, the gut-associated lymphoid tissues (GALT) are among the largest and most important sites of persistence for virus in SIV-infected macaques (80) and, by inference, HIV-infected patients.

The small intestine is the longest organ in the human body, measuring roughly 16 feet with an approximate diameter of 1 inch. Lying just beneath the single layer of intestinal epithelial cells, the *lamina propria* contains abundant activated (CD69⁺), memory CD4⁺ T cells engaged in either promoting [T-helper 17 (Th17) cells] or inhibiting [T-regulatory (Treg) cells] immune responses to foreign and dietary antigens encountered through absorption through the intestinal epithelial cells. Although there is little organized lymphoid tissue in the upper small intestine, the number of T cells in the epithelium and *lamina propria* of this ‘effector site’ alone has been estimated to exceed the number of T cells in the rest of the body. Thus, the majority of HIV target cells necessary for viral expansion and persistence lie in the intestinal tract, making this tissue a primary focus of immunologic investigations (79, 81). Regardless of the route of transmission, numerous studies in macaques and humans have shown that within days of infection, there is massive infection and depletion of activated, memory, CD4⁺CCR5⁺ T cells in the intestine of SIV-infected macaques (82, 83) and HIV-infected humans (84-86). Early peak viral replication in plasma likely corresponds to this massive infection and amplification in intestinal tissues. Tremendous viral diversification occurs during this burst of replication, and subsequently, multiple escape mutants are generated and selected under the pressure of ensuing adaptive immune responses (87).

Similar to the vaginal mucosa, TLRs are expressed on intestinal epithelial cells, macrophages, dendritic cells, B cells, T cells and stromal cells, and are critical for intestinal homeostasis. Binding of TLRs activates the innate immune response characterized by NF- κ B activation, cytokine production, and chemokine-mediated recruitment of acute inflammatory cells. Normally, TLR signaling in the intestine is involved in epithelial cell proliferation, IgA production, maintenance of tight junctions and antimicrobial peptide expression (88-90). Nucleotide-binding oligomerization domain receptors (NOD proteins) are another class of innate receptors that recognize PAMPs. NOD1 is expressed by intestinal

epithelial cells, and is required for recognition of invasive Gram-negative bacteria (91). Interestingly, the formation of gut-associated lymphoid tissue (GALT) is also induced by the presence of Gram-negative commensal bacteria through NOD1 signaling (92). Thus, the intestinal tract is unique in that it is the only organ in the body that actually requires constant interaction with a complex community of resident microbes to develop and function properly. It is now known that commensal bacteria in the intestine modulate the differentiation and maturation of the mucosal immune system, and are essential for mammalian health. Communication between the microbiota and the host establishes and maintains immune homeostasis, enabling protective immune responses against pathogens while preventing adverse inflammatory responses to harmless commensal microbes. The density of bacteria increases from the proximal small intestine to the colon, and these microbes aid the host by breaking down food into absorbable products, preventing the outgrowth of pathogenic organisms, and shaping the immune compartments, in exchange for an environment with a constant influx of nutrients necessary for their survival (93,94). Thus, the microbial communities that inhabit the mammalian intestine, termed the intestinal microbiota, maintain a symbiotic relationship with their host. However, microbial products breaching the intestinal epithelium and reaching the *lamina propria* may activate the innate immune response. Innate pattern-recognition receptors such as TLRs, NLRs, and CLRs play a crucial role in the host recognition of probiotics and other microorganisms. Signaling via these receptors directly influences the chemokine and cytokine response of dendritic cells as well as the crosstalk between the epithelium and the immune cells in the *lamina propria* for orchestrating proper immune responses during host defense, which can influence the population of effector and regulatory T-cell subsets in the mucosa. For example, colonization of the gut with certain bacteria elicits IL-17 and IL-22-producing Th17 cells in the *lamina propria* (95,96).

Loss of intestinal immune regulation leading to aberrant immune responses to commensal microbiota are believed to precipitate inflammation observed in the gastrointestinal tract of patients with inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis (97). Innate immune receptors that recognize conserved components derived from the microbiota are widely expressed by both epithelial cells and leukocytes of the gastrointestinal tract and play a key role in host protection from infectious pathogens. Abnormal innate immune activation may also drive intestinal pathology, as patients with IBD exhibit extensive infiltration of innate immune cells to the inflamed intestine. Thus, a balanced interaction between the microbiota and innate immune activation is required to maintain a healthy mutualistic relationship between the microbiota and the host. Disruption of this fine-tuned regulation results in alterations in intestinal mucosal integrity, which may actually play a causative role in the systemic immune activation typical of SIV/HIV infection.

The intestinal epithelium and immune system interact with dietary antigens and enteric microbiota in a highly regulated manner to maintain mucosal homeostasis, by simultaneously preventing immune responses to dietary antigens, while promoting inflammatory responses to potential pathogens. Several subsets of T cells in the intestine are involved in this regulation including CD4⁺ T-helper 17 cells (Th-17), T-regulatory (Treg) cells, $\gamma\delta$ T cells, CD8⁺ T cells, natural killer (NK), and NK-T cells. These cells contribute to the mucosal response to pathogens by secreting a variety of key regulatory cytokines including interleukin-17A (IL-17A), IL-17F, IL-22, and IL-26 (98). Importantly, intestinal CD4⁺ T cells play major regulatory roles in intestinal mucosal homeostasis and defense against microbial invasion. Disruption of this immune homeostasis resulting from HIV infection and massive intestinal CD4⁺ T-cell loss can thus result in alterations in the immune responses to antigens, as well as changes in the commensal intestinal bacterial microflora.

In the distal small intestine and throughout the large intestine, inductive immune tissues organized lymphoid tissues including Peyer's patches (terminal ileum) and isolated lymphoid follicles are distributed throughout the *lamina propria*, and all of these are covered by a specialized follicle-associated epithelium (FAE) containing M cells, which pinocytose and sample antigens in the lumen (99). These inductive lymphoid tissues have a structure resembling lymph nodes, with B-cell follicles surrounded by mantle zones primarily consisting of resting CD4⁺ and CD8⁺ T cells (Fig. 2). Antigens encountered through the FAE are presented to T and B cells, which recirculate and home preferentially to the diffuse *lamina propria* of the intestine as activated, effector T cells or antibody secreting plasma cells. Thus, virtually all of the CD4⁺ T cells in the diffuse *lamina propria* (an immune effector site) of the intestine are highly activated CD4⁺ T cells (Fig. 3), most of which express the CCR5 coreceptor, and these are the major targets for early SIV/HIV infection and amplification (85,86,100,101). In fact, early peak viral replication coincides with intestinal infection and viral amplification within these intestinal CD4⁺CCR5⁺ T cells. Although the intestine contains abundant CD4⁺ T cells, recent studies have shown that CD4⁺ T cells in the intestinal tract are very heterogeneous, having a variety of phenotypes and regulatory functions. Thus, infection and depletion of intestinal CD4⁺ T cells has dire consequences on intestinal integrity and immune functions.

In chronic infection, systemic immune activation is a hallmark of HIV/SIV infection (102), and a better predictor of disease progression than either plasma viral load or peripheral blood CD4⁺ T-cell counts (103,104). Both HIV and SIV infection (in susceptible hosts) results in marked and persistent immune activation, leading to the development of AIDS. However, SIV infection of nonprogressing hosts (that do not develop AIDS) does not result in persistent immune activation. In fact, chronic activation is a crucial factor that distinguishes pathogenic from nonpathogenic SIV infection in non-human primates (105-108). Thus, similar to the immunologic events described above that promote early mucosal infection and transmission, chronic systemic immune activation may be at least be partly responsible for the progressive immunologic decline and the development of AIDS in susceptible hosts.

Systemic activation in SIV/HIV patients is characterized by (i) high - cell turnover and proliferation of T-cell targets supportive of continued viral replication (109-111), (ii) high levels of immune activation and exhaustion of B and T-cell clones (112-114), (iii) increased frequencies of apoptotic T cells (115), and (iv) increased production of pro-inflammatory and pro-apoptotic cytokines known as the 'cytokine storm' (78,116,117). Increasing evidence suggests much if not most of the systemic activation characteristic of HIV infection may be the result of increased damage of lymphoid and intestinal mucosal tissues leading to microbial translocation (118,119).

Microbial translocation

Chronic immune activation of HIV/SIV infection may result from intestinal mucosal damage and leakage of bacterial products from the intestinal lumen into the systemic circulation, in a process termed microbial translocation (118,120,121). Pathogenic SIV infection results in disruption of the intestinal barrier in GALT, and the translocation of commensal microbial products from the intestinal lumen into the systemic circulation, resulting in systemic immune activation (118,122). These bacterial and fungal products may include peptidoglycan, lipoteichoic acid, lipopolysaccharide (LPS), flagellin, ribosomal DNA (rDNA) and unmethylated CpG-containing DNA. These products activate a number of immune receptors including nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, as well as Toll-like receptor 2 (TLR2), TLR4, TLR5, TLR6, and TLR9. Innate immune cells such as monocytes, macrophages and dendritic cells, binding of these

receptors to microbial products, activates a signaling cascade, eliciting the production of pro-inflammatory cytokines including interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor (TNF), and type I interferons (IFN- α and IFN- β). Although these responses may be beneficial, if not essential, to the host in response to most infections, pro-inflammatory cytokines can induce tissue damage, increasing the proliferation of local immune cells. This provides SIV/HIV with a continuous supply of activated CD4⁺ T cells to which are continually depleted by viral lysis and extensive apoptosis (116,123,124).

HIV/SIV-induced immunodeficiency is characterized by progressive functional immune impairment, loss of CD4⁺ T cells, and severe disruption of gut-associated lymphoid tissues, which limits immune reconstitution, even after initiation of anti-retroviral therapy (ART). Twenty percent of HIV-1-infected patients have no significant increase in their peripheral blood CD4⁺ T-cell count after initiation of HAART, and few reconstitute CD4⁺ T-cell counts to normal levels even after years of ART. Even with partial normalization of peripheral blood CD4⁺ T-cell counts, populations of CD4⁺ T cells remain depleted by as much as 50% in secondary lymph nodes (LNs) and gut associated lymphoid tissue (GALT) (84,125,126), suggesting perpetual damage of lymphoid structures. Intestinal epithelial damage, including loss of intestinal epithelial cells (enterocytes) and disruption of tight junctions between the cells, may lead to increased villous atrophy, malabsorption of nutrients and diarrhea, intestinal barrier permeability, microbial translocation, and changes in the intestinal microflora and virome. Loss of enterocytes may be directly attributed virus production or bystander effects (127). Tight junction loss occurs within 14 days post-infection. Of these, villous atrophy is accompanied with crypt hyperplasia and resulting in reduction of immunoglobulin A (IgA)-producing plasma cells, which is consistent with underlying B cell dysfunction and decreased IgA concentrations in the intestinal lumen in SIV-infected animals and HIV-infected individuals. Intestinal damage precipitated by HIV/SIV infection leads to microbial translocation, which in turn is associated with systemic immune activation, loss of Th17 cells, and disease progression.

A chief characteristic of the immune system is the ability to rapidly expand rapidly the number of antigen-specific lymphocytes to combat infection; however, HIV preferentially infects and destroys HIV-specific CD4⁺ T cells (128), and evidence in NHP models indicates SIV rapidly and selectively infects and kills dividing CD4⁺ T cells, regardless of their specificity (124), making it difficult to fully quantify the actual dynamics of the cellular immune response to infection (129). However, converging evidence suggests SIV/HIV selectively infects and eliminates specific types of CD4⁺ T cells, which results in dampening of the immune response to HIV infection, while simultaneously inducing nonspecific immune stimulation through microbial translocation, resulting in a continuous supply of target cells to support viral replication and persistence throughout the life of the host. Moreover, persistent depletion of specific CD4⁺ T-cell subsets likely induces marked immunodeficiencies in cellular and humoral responses, and locally, marked alterations in the intestinal flora, as recently indicated by the discovery of marked expansion of the intestinal virome in SIV-infected macaques (130). Thus, previously unsuspected, opportunistic viral infections of intestinal mucosal cells may be contributing to the destruction of intestinal epithelia and loss of barrier function.

Key CD4⁺ T cell subsets involved in HIV pathogenesis

Massive and early depletion of activated, memory CD4⁺ T cells is a hallmark of HIV/SIV infection (85,86,100,101,131-133). Since most activated memory T cells co-express CCR5 and are transcriptionally active, logically these cells are a target for direct HIV infection and lysis. However, advances in phenotyping and functional analyses have shown that there are several distinct subsets of CD4⁺ T cells that play key roles in immune regulation and

homeostasis, particularly in mucosal tissues. Although the reasons are not entirely clear, it is increasingly apparent that certain subsets of CD4⁺ T cells are selectively infected and eliminated in HIV infection, whereas others are relatively spared, resulting in marked changes in immune homeostasis, and the inflammatory response to HIV, and other pathogens. Although difficult to examine in humans, correlates between this marked change in immune homeostasis most likely occurs first in the intestinal tract, where it is evident from direct and indirect evidence that loss of regulatory T cells in the gut results in rapid changes in immune phenotype, function, barrier permeability, and changes in the intestinal flora, which are apparently all regulated by key mucosal CD4⁺ T-cell subsets.

The Th1/Th2 paradigm

Originally, effector CD4⁺ T cells were defined based on their cytokine secretion patterns, as either pro-inflammatory responses (Th1) producing IFN- γ , IL-2, lymphotoxin- α , and/or TGF- β which drive cell mediated immune responses to control intracellular pathogens, or Th2 responses, producing IL-4, IL-5, IL-6, IL-10, and IL13, which dampen cellular responses, and promote humoral responses to control extracellular pathogens (134-136). However, this paradigm is continuously evolving, as overlap exists with some of the functions and cytokines produced by these cells, and as new Th subsets are identified that do not fit into these defined categories (i.e. Th17 cells, innate lymphoid cells, see below). Nonetheless, in early HIV and SIV infection, the cytokine storm described above consists largely of pro-inflammatory cytokine responses, consistent with a Th1 response. Some of these cytokines have antiviral activity, such as IFN γ , IL-15, and IL-18 which enhance innate and adaptive cellular immune responses (137). However, in the case of HIV infection, the intense early cytokine response may promote viral replication and immunopathology (137). Although there is marked patient variation and some discrepancy in the literature, it appears that as HIV disease progresses, the profile appears to shift from a Th1 to Th2 response, as evidenced by decreased production of IFN- γ , IL-2, and IL-12, and an increase in production of IL-4, IL-5, IL-6, and IL-10 (138,139), which are associated with more rapid disease progression in HIV patients (140) and SIV-infected macaques (139,141). Excessive activation of CD4⁺ T cells and preferential replication of HIV-1 in Th1 cells may eventually result in a shift from inflammatory to a suppressive response, which may be associated with the progression to AIDS (142). In support of this, HIV preferentially infects Th1 type cells *ex vivo* (142,143). However, this Th1/Th2 paradigm is continuously evolving, as several other key regulatory cells have been identified that do not clearly follow this paradigm.

Th17 cells

Another major CD4⁺ T-cell subset that is targeted and eliminated early in SIV/HIV infection are Th17 cells (121). Th17 cells represent a distinct lineage from Th1 and Th2 cells, and are characterized by the production of IL-17A, IL-17F, IL-22, and IL-26 (144). Th17 cell differentiation is directed by the transcription factor ROR γ t, which appears specific for the Th17 lineage. The pro-inflammatory cytokines IL-6 and TGF- β appear to drive Th17 differentiation, at least in the mouse model, while IL-23 appears to be indispensable for the protective effect of the Th17 response against mucosal pathogens like *C. rodentium*, *Klebsiella pneumoniae*, and *S. typhimurium* (98,144).

Th17 cells are important mediators of immune defenses in the gut and are induced by the microbiota in the intestinal *lamina propria* (145). Th17 cells produce IL-17 and IL-22 in response to stimulation, which promote recruitment of neutrophils to sites of bacterial infection, induce enterocyte proliferation, maintain mucosal epithelial integrity, and induce production of antibacterial defensins, and thus play an important role in antibacterial immunity (121). Th17-dependent innate response mechanisms, including neutrophil activation and epithelial cell antimicrobial peptide secretion, appear essential for proper host

defense against extracellular and intracellular bacterial pathogens in the gut. Expansion of Th17 cells can also be negatively regulated by intestinal epithelial cells, which produce IL-25 following microbial recognition (146). IL-17A is constitutively expressed in GALT and epithelial cells of the small intestine, where it plays a major role in the defense against intestinal bacterial pathogens (147). In addition, IL-17A regulates the expression of β -defensin3 in the intestinal epithelium, which can effectively kill bacteria prior to invasion of the mucosa (148).

In pathogenic SIV and HIV infections, Th17 cells are rapidly depleted in the intestine (121,149-152). Since Th17/IL-17 are clearly involved in maintenance of intestinal integrity and resistance to bacterial invasion of the gut, the selective depletion of Th17, and innate lymphoid cells (ILCs) that also produce IL-17 (153) may play a key role in the loss of intestinal mucosal integrity, resulting in microbial translocation. In contrast, gut resident Th17 cells are preserved in natural hosts of SIV, which may contribute to the lack of microbial translocation and chronic immune activation in SIV-infected, nonprogressing hosts (121,154). Normal levels of Th17 cells have also been reported in HIV infected elite controllers (155) and long-term nonprogressors (156), which is also consistent with a role for these cells in protection against HIV disease progression.

Treg cells

Treg cells are a subgroup of T lymphocytes with immunosuppressive activities. Classical Tregs are immunophenotypically defined as CD25^{hi}, CD127^{lo}, and they intracellularly express the Forkhead box P3 protein (FoxP3)(157) and are abundant in the intestine (Fig. 4). During progressive HIV-1 infection, the relative frequency of classical Tregs is increased, while their absolute counts are reduced as a consequence of lower total CD4⁺ T-cell counts (158). This observation suggests Tregs decline at a slower rate than conventional CD4⁺ T cells during in HIV-1 infection, indicating an imbalance of these cells may play an important role in the immune pathogenesis of HIV-1 infection. Increased frequencies of Treg cells have been documented in the GI tract of macaques with progressive pathogenic SIV infection, where their frequency is positively correlated with viral load (159). In HIV-infected humans, Treg cells suppress virus-specific T-cell responses, and it has been proposed that chronic infection leads to induction of Treg responses that suppress the antiviral immune response to HIV, as another means of immune escape (160).

Proliferating CD4⁺ T cells

Evidence indicates recently dividing CD4⁺ T cells are rapidly and selectively eliminated in early SIV infection, indicating SIV targets not only memory CD4⁺ T cells but also cells proliferating in response to non-specific antigenic stimulation (124). Although selective infection and destruction of HIV-specific CD4⁺ T cells has been described (128), emerging evidence suggests that most of the proliferating CD4⁺ T cells are susceptible to destruction, possibly due to their co-expression of CCR5, and state of activation (124,129). Thus, following SIV/HIV infection, proliferative responses to new antigens or pathogens may be thwarted by the selective infection and elimination of the earliest responding CD4⁺ T cells, thus impairing primary responses to invading pathogens. Since CD4⁺ T-cell help is necessary for the development of essentially all aspects of the adaptive immune response, particularly primary responses, selective targeting of proliferating CD4⁺ T cells may be a fundamental mechanism involved in dampening the immune response to HIV, and the development of opportunistic infections. Moreover, since CD4⁺ T cells are well known for their role in providing critical signals during priming of cytotoxic CD8⁺ T-lymphocyte (CTL) responses *in vivo* (161-163), loss of proliferating or regulatory CD4⁺ T cells may also be involved in the host failure to generate effective CD8⁺ CTL responses in HIV. In

summary, the severe depletion of activated, memory, and dividing CD4⁺ T cells in early HIV/SIV infection likely play a central role in the immunopathogenesis of AIDS.

T-follicular helper cells

In the first steps of the humoral immune response to a new antigen, the antigen is captured by an antigen-presenting cell (APC) and transported to organized lymphoid tissues in lymph nodes, spleen, or GALT, through the mantle T-cell zone into the B-cell follicles, which initiates the activation and interaction of resident T and B cells, resulting in the germinal center (GC) reaction. GCs are discrete structures within the B-cell follicles of secondary lymphoid tissues in which the processes of somatic hypermutation of antibodies, immunoglobulin class switching, and affinity maturation of activated B cells occurs, resulting in the production of mature memory B cells, and antibody-secreting plasma cells (164,165). For GCs to develop, B cells must first receive cognate help from specific CD4⁺ T helper cells named T-follicular helper (Tfh) cells, which reside within these follicles and are central to the development and regulation of primary humoral immune responses. Tfh cells are thus the crucial T-helper cell necessary for initiation and maintenance of GC responses that generate memory B cells and long-lived plasma cells (166-168). Tfh cells in humans and mice have been reported to express and produce regulatory molecules that facilitate their functional interactions with antigen-specific B cells, including the co-stimulatory molecule programmed death-1 (PD-1), inducible T-cell costimulator (ICOS), CXCR5, transcriptional factor B-cell lymphoma 6 (Bcl-6), and the cytokine IL-21, which promotes growth, differentiation, and class switching of B cells (169). In resistance to HIV infection and AIDS, binding antibodies, not just neutralizing antibodies, are now believed to be very important for protection from HIV infection, but the early effects of HIV infection on Tfh-dependent humoral immune responses and the mechanisms behind the failure to develop adequate antibody responses are unknown. However, studies have demonstrated Tfh cells are expanded in lymph nodes in both HIV (170) and SIV (171,172) infection, and the recent finding of HIV DNA in Tfh of HIV infected humans, suggest TFH may be the major persistent reservoir for virus in HIV patients (173). Moreover, persistent infection of TFH may result in disruptions of B-cell development and antibody production, which may be still another mechanism by which HIV subverts the adaptive immune response.

Virus-specific CD8⁺ T-cell and CTL responses in HIV

CTLs are critical for protective immunity against many intracellular pathogens. In pathogenic HIV and SIV infection, the first CTL responses coincide with peak viremia, yet peak T-cell responses do not develop until 1–2 weeks after peak viremia (137,174-176), suggesting the responses is ‘too little and too late’ to eliminate viral replication and to prevent systemic dissemination (177). Nonetheless, it is generally accepted that virus-specific CD8⁺ T lymphocytes play a central role in controlling HIV and SIV replication. Transient depletion of CD8⁺ lymphocytes in macaques *in vivo*, either on the day of challenge or in chronic infection, results in increased viral replication (178-180), confirming a critical role for CD8⁺ T cells in protection from SIV replication. During early HIV/SIV infection, CTL responses may provide more protection than antibodies, as neutralizing antibodies are produced much later. However, natural HIV infection escapes immune control in human and experimental models, despite variable, yet often strong CTL responses.

In SIV-infected macaques, virus-specific CTL responses are detectable as early as 12 days after experimental infection (181), but even at this early stage, virus has disseminated throughout the circulation and is well established in intestinal and lymphoid tissue reservoirs, and the immune system is unable to clear the infection (182). Virus-specific CTLs emerge in the intestine at the same time as in blood in SIV infection (181), and in

chronic infection, percentages of CTLs are frequently higher in mucosal tissues compared to blood (183), suggesting most of the struggle for viral control occurs in the intestinal tract throughout the course of infection. Mucosal CTLs may play a role in limiting infection in mucosal tissues in HIV and SIV infection (184). However, they largely fail to control viral replication, as evidenced by persistence of virus and loss of target CD4⁺ T cells in GALT. HIV-specific CTLs have also been identified in cervical cytobrush cells from women who are repeatedly exposed to HIV, yet remain seronegative and apparently uninfected (185). Although highly controversial, this finding has been interpreted to suggest a protective role for mucosal CTLs in highly exposed, seronegative individuals (186).

The magnitude, function, and quality of CTL responses are likely to be important factors in the effectiveness of the cellular response. The ability of CD8⁺ T cells to contain infection may be associated with various factors, including intrinsic (i.e. TCR repertoire, polyfunctionality, regulatory molecules) and extrinsic (inflammatory microenvironment) factors associated with infection. The quality (polyfunctionality) of CD8⁺ T-cell responses (measured by IFN- γ , IL-2, TNF- α , MIP-1 β and CD107a) rather than the quantity has been shown to better correlate with control of viral replication in HIV nonprogressors and elite controllers (187). Following peak CTL responses in blood, viral sequences change dramatically with rapid selection of mutations. The earliest T-cell responses are often specific for env, tat, and nef epitopes, whereas responses to other viral proteins, including more conserved Gag and Pol proteins, tend to arise during later waves of T-cell responses and may help maintain viral set point (188-191).

Memory CD8⁺ T cells are a major component of long-term immunity because of their longevity, and other unique properties including the ability to maintain a high proliferative potential to robustly expand upon secondary infection, when they rapidly re-express cytotoxic proteins and cytokines upon restimulation (192). During acute viral infection, naive CD8⁺ T cells respond to antigen by undergoing a pronounced clonal expansion during which a large number of antigen-specific T cells are generated (193). This initial expansion and acquisition of effector functions is followed by a contraction phase where the majority of the reactive CD8⁺ T cells undergo programmed cell death, leaving behind a small population of surviving effector and/or transitional memory cells, (193,194). These memory subsets increase expression of IL-7R α (IL-7R α hi) and lymphoid homing molecules such as CCR7, CXCR3, and CD62L, and gain the ability to produce IL-2. The enhanced secondary response can be attributed partly to the increased precursor frequency of antigen-specific cells but also to an altered responsiveness of the memory CD8⁺ T cells themselves, a process referred to as ‘memory programming’ (195). This programming depends on CD4⁺ T-cell help and Th1-produced IL-2, because memory CD8⁺ T cells that are generated in the absence of MHC class II or IL-2 signaling are impaired in their ability to respond to subsequent challenge (193). During chronic viral infections, virus-specific CD8⁺ T cells undergo an altered pattern of differentiation and become ‘exhausted’. CD8⁺ T-cell exhaustion is a transcriptionally altered state of T-cell differentiation distinct from functional effector or memory CD8⁺ T cells (196). Exhausted CD8⁺ T cells undergo a hierarchical loss of function, ultimately resulting in virus-specific CD8⁺ T cells with severely compromised effector function, and in some cases these cells are physically deleted (197). Although the phenotypic definition of a protective CTL response is still lacking, considerable evidence indicates a major feature of an exhausted CD8⁺ T-cell response is the sustained expression of multiple inhibitory receptors such as programmed death-1 (PD-1), B lymphocyte induced maturation protein-1 (Blimp-1), basic leucine transcription factor ATF-like (BATF), lymphocyte activation gene 3 (LAG3), 2B4, CD160, CTLA-4, PIR-B, GP49, and Tim-3 (196,198-200). Since CD4⁺ T-cell help is required for generation of primary CTL responses as well as in promoting protective CD8⁺ memory T-cell development (161-163), impaired CD8⁺ T-cell responses may be linked to the rapid depletion of effector CD4⁺ T cells. The

rapid decline of CD8⁺ T-cell responses is consistent with the impaired long-term CD8⁺ T-cell memory that has been observed in CD4⁺ T-cell depleted murine models (163). Thus, continued infection and/or destruction of memory CD4⁺ T-cell help be a major causal factor in the impaired or exhausted CD8⁺ CTL response described in HIV. In support of this idea, CD4⁺ lymphocyte-depleted macaques show similar peaks of viremia, do not manifest a post-peak decline of virus replication, and display rapid disease progression (201), suggesting CD4⁺ T cells play a major role in modulating early CTL responses. Thus, a weakened CD4⁺ T-helper cell repertoire/response may provide suboptimal help for generating effective CTL responses.

Although virus-specific CD8⁺ T cells are clearly associated with some level of immune control, the fact that relatively large numbers of CTLs are detected in animals and humans that protect to AIDS, combined with the observation that CTL responses do not usually correlate with protection in vaccine studies or elite control, indicate other factors are likely to prove more effective in prevention strategies than CTL responses. There is some evidence that at least in blood of SIVmac-infected animals, levels of CD8⁺ T-cell responses directly, rather than indirectly, correlate with viremia (unpublished observations). Further, higher env-specific CTL responses were detected in vaccinated persons who became infected with HIV in the AIDS/VAX vaccine trial (202), also calling into question the value of only testing CD8⁺ T-cell responses in vaccine studies. At least in high-risk populations, it has been suggested that certain immunization strategies may boost pre-existing immune responses, due to pre-infection exposure, resulting in increased HIV susceptibility (202). Although the link between highly HIV exposed, seronegative persons and virus specific immune responses is still vague, it is likely that vaccines or possibly concurrent infections that trigger activation and proliferation of CD4⁺CCR5⁺ cells may abrogate the effects of protection and may increase rates of transmission. Thus, continued and perhaps even more thorough pre-clinical testing of HIV vaccine candidates, in relevant animal models, clearly remains warranted.

Innate lymphoid cells in HIV

Mounting evidence suggests a role for innate immune cells, combined with specific antibody responses, in the control of HIV infection. Originally, non-T non-B lymphocytes (lineage negative) were simply termed natural killer (NK) cells based on their function and lack of receptors associated with other immune cell lineages. The term 'innate lymphoid cells' (ILCs) has now been proposed to include classical NK cells including a heterogeneous population of lineage negative cells in blood and mucosal tissues, which are further classified based upon their ability to produce type 1, 2, and Th17 cell-associated cytokines (136). The non-T, non-B CD127⁺RORγ⁺ populations include the lymphoid tissue inducer (LTi) cells as well as the NK receptor⁺ LTi-like cells, which are distinct from conventional NK cells (203, 204). Interestingly, LTi are now believed to be identical to cells found in the small clusters of cells originally described in the small intestine of mice as 'cryptopatches' containing CD3^{neg}CD4⁺ cells, which are thought to be sites of extrathymic T-cell development (205,206). We now know these cryptopatches contain clusters of CD4⁺CD3^{neg} LTi cells (Fig. 5), which are thought to be important for generation of new lymphoid follicles in the intestine (206).

ILCs as immune effector cells initiate immune responses to pathogens with the capacity to rapidly secrete effector cytokines, mediating interactions between ILCs, B and T cells (207). In addition to cytokine secretion, ILCs may directly modify adaptive B and T-cell responses via direct cell-to-cell interactions. Indeed, RORγ⁺ ILCs coordinate T-cell independent IgA class switching via follicular DCs and plasma cell differentiation via DCs in ILFs (208). Clusters of LTi cells may have acquired additional signaling pathways including LT

signaling, which provides the ability to recruit B cells that can then undergo T-cell-independent class switching and play a key role in the formation of lymphoid follicles in GALT. LT α cells, which coexpress the NK cell activating receptor NKp46 yet lack cytotoxic effectors such as granzymes or perforin, secrete IL-22, which initiate innate immune responses, and are thought to promote B and T-cell memory (209, 210).

In the intestine, ILCs mediate responses between host cells and commensal bacteria and play key roles in inflammation and tissue homeostasis (207, 211). Although ILCs do not express antigen-specific receptors like adaptive lymphocytes, they can produce (upon stimulation) several cytokines, which regulate the balance between protective immunity and destructive inflammation in the gut. Similar to Th17 cells, ILC17 cells are depleted in chronically SIV-infected macaques, which likely contributes to the loss of mucosal integrity (153,212). However, the role of LT α and other ILCs are relatively unexplored in SIV/HIV infection. Due to the primary role of LT α cells in mucosal lymphoid tissue organogenesis, it may be beneficial to explore strategies to promote LT α cells or their function in the restoration of mucosal lymphoid tissue damage in HIV/SIV infection.

Classical NK cells are also involved in resistance to HIV infection and/or disease progression (213). The killer immunoglobulin-like receptors (KIRs) expressed on NK cells regulate the inhibition and activation of NK-cell responses through recognition of MHC class I molecules on target cells. Coexpression of specific MHC ligands and KIR are associated with slower disease progression. Specifically, the NK activating KIR3DS1 and KIR3DL1 receptors are associated with delayed progression to AIDS in individuals with HLA class I allotypes containing the 80Ile variant of the Bw4 motif⁵², which are thought to be ligands for these receptors (213,214). Conceivably, KIR3DS1 mediates specific recognition of HIV-infected cells by NK cells, although the exact nature of the ligand is elusive. Nonetheless, these observations suggest an influence of interactions between KIR receptors and HLA-B alleles on the development and/or function of NK cells which may help to control viremia (137)

Although NK cells have an important role in defense against HIV-1, evasion mechanisms, such as nef-mediated downregulation of HLA-A and B molecules, are used by HIV-1 apparently to prevent T-cell recognition (215). However, the dominant KIR2D ligand, HLA-C is relatively spared, suggesting there is a balance between T and NK cell evasion (213). Thus, downregulation of HLA molecules represents still another mechanism by which HIV has evolved to evade even the ‘innate’ host immune response.

Humoral immune responses to HIV

Converging data from various vaccine studies in humans and nonhuman primates are finally providing hints of what will be required for an effective vaccine capable of preventing HIV infection. Several antibodies have been identified that are capable of neutralizing most circulating HIV strains, including from the 1% of HIV elite controllers (216). However, it is becoming increasingly clear that defective antibody responses are the key to HIV gaining an irrevocable foothold on infection and perhaps persistence. In HIV and SIV infection, lymph nodes and GALT are reservoirs for viral persistence, even in patients on therapy or those controlling infection (217-219). Vigorous follicular hyperplasia in secondary lymphoid tissues (lymph nodes and GALT), are hallmarks of primary HIV/SIV infection, as B cells become activated, resulting in an increase in the size and number of lymphoid follicles, lymphadenopathy and immune activation. However, in chronic infection, there is generalized lymphoid depletion characterized by a reduction in GC size and number, which is eventually accompanied by fibrosis, and follicular involution with nearly complete destruction of the follicular architecture (122, 220, 221). These changes have been shown to

gradually result in an inability to mediate antigen-specific T helper cells and antibody responses in late stages of infection, contributing to AIDS (122,125, 222). This is likely all the result of the early massive depletion of regulating CD4⁺ T-cell subsets in the intestine and follicular networks. Consistently, progressive depletion of proliferating B cells and disruption of the follicular dendritic cell (FDC) network in germinal centers (GC) is evident in lymph nodes as early as 20 days after viral challenge of macaques (125, 223) and, as previously mentioned, HIV-infected humans within weeks of infection (223, 224). Thus, impairment of B cells and follicular T-helper cells in lymph nodes and GALT is consistent with undetectable, weak, and/or transient neutralizing antibody responses over the course of HIV/SIV infection (225-227).

The GALT is a major yet sometimes overlooked component of the humoral immune system. GALT is a major site for the production of antibodies and generation of antigen-specific CTLs (228). In fact, most of the immunoglobulin-produced daily by the body is IgA generated in the gut (Fig. 2). Locally produced IgA is released into the lumen, providing protection as a first line of defense against mucosal pathogens. Within the intestinal tract, mucosal IgA plays a crucial role in controlling microbial populations and mediating clearance of pathogens. Secretory IgA can neutralize pathogenic bacteria, control commensals and in general, is necessary for maintaining intestinal homeostasis. For example, IgA deficiency increases the risk for chronic giardiasis (229). Further, IgA production is regulated in GALT, which is highly dependent on the microbiota, and changes in the composition of the intestinal flora can alter the pattern of IgA production (230). TLR4 signals in the intestinal epithelium promote IgA production in the small intestine, by increasing B-cell recruitment and promoting class switching of B cells to IgA (89). Unlike IgG production, specific IgA responses lack typical memory characteristics, enabling changes in the specificity of IgA in response to shifts in the dominant intestinal microflora (230). Further, T-cell-independent IgA generation requires Tfh cells (231) and TGF- β (232) generated in isolated lymphoid follicles in gut (232, 233). In summary, antigen captured by antigen-presenting cells in GALT initiates the activation and interactions of Tfh and B cells, the production of germinal centers, affinity maturation of B cells, and formation of antibody-secreting plasma cells (PCs) in the intestine. Although B cells do not appear to be directly infected in HIV/SIV infection, B cells are critically dependent on help from Tfh in GCs of follicles in GALT (and systemic lymphoid tissues), and thus infection or dysfunction of Tfh cells may play a key role in the B-cell dysregulation associated with HIV infection.

In HIV patients, the first detectable B-cell responses in blood occur by 8 days after exposure, and detectable antibodies to env glycoprotein 41 (gp41) do not appear in circulation until 13 days after the appearance of viremia (137). Production of env gp120-specific antibodies is delayed for an additional 14 days, and antibodies that actually neutralize autologous virus *in vitro* develop even slower, arising ~12 weeks or longer after HIV infection (137, 234, 235). However, most patients do not develop broadly neutralizing antibody responses (bnAb) to HIV, and of those that do, many do not develop bnAb until 20-30 months after infection (137, 236). Further, evidence suggests the early, non-neutralizing antibodies may even mask key neutralizing epitopes, resulting in delayed or impaired neutralizing antibody responses (237).

The first HIV-1-specific IgA responses may be detected as early as 3 weeks after infection, and these antibodies are also directed against gp41 (137). Unlike intestinal secretions that contain mostly locally produced secretory IgA (S-IgA), cervicovaginal fluid and semen contain more IgG than IgA, and levels of immunoglobulins in the female genital tract are influenced by hormones (238). Both cervicovaginal secretions and semen contain plasma-derived as well as locally produced immunoglobulins, mainly of the IgG isotype (239, 240).

Studies in the SIV macaque model have clearly shown antibodies play a role in protection against SIV transmission and disease progression. Intravenous and genital inoculation of SIV in rhesus macaques induced similar systemic humoral responses consisting mostly of IgG and detectable but lower levels of IgA and IgM (241). However, anti-SIV response in the vaginal washes consisted mainly of IgG with essentially no IgA or IgG plasma cells, and few IgM plasma cells, suggesting the mucosal immune system of the FRT is markedly impaired in chronic SIV infection (241). Intravenously administered antibodies (passive infusion studies) have been shown to protect macaques against intravenous or mucosal SHIV challenge (242). Topically applied antibodies can also protect macaques against vaginal SHIV challenge (28, 59, 243). Considering their prime role in many successful vaccines in the past, antibody-based vaccines were the first choice in the initial stages of vaccine development.

Abundant evidence indicates that early and marked failures in B-cell development, especially in GALT, contribute to AIDS. In chronic infection and AIDS, B-cell development and responses are clearly impaired due to the collagen deposition and destruction of GCs in lymph nodes and GALT of SIV-infected macaques and HIV-infected humans (120, 126, 223). However, these deficits apparently starts very early in HIV infection, as half of the GC in the gut are lost within weeks of HIV infection (223, 224). Early loss of GCs and dysfunctional follicular CD4⁺ T-helper cells may result in defects in the ability to rapidly generate high-affinity virus-specific binding antibodies and may lead to a delay in the induction of virus-neutralizing antibodies. Consistently, HIV-1 infection is associated with early class switching of polyclonal B cells and is associated with marked increases in the number of blood and tissue naive and memory B cells and plasma cells (137). In GALT, both HIV-1 and SIV is associated with apoptosis and lysis of follicular B cells. A rapid loss of Tfh and/or GC function may be responsible for the delay/inability of patients to develop effective neutralizing antibodies.

Although bnAbs can clearly prevent SIV transmission in passive infusion experiments, the inability to elicit sufficient levels of bnAb with vaccines, and perhaps more importantly, an inability to show correlations between protection and generation of bnAbs in vaccinated patients (see below) has increased attention on the role of binding yet non-neutralizing antibody responses. Antibodies that bind but do not neutralize HIV in tissue cultures may still impart antiviral effects through a variety of mechanisms including direct NK cell killing of infected cells, recruiting activated effector cells, which in turn induce cytolysis or apoptosis of infected cells, and or by mediating antibody-dependent cellular cytotoxicity (ADCC) by forming a complex between the IgG Fab portion of the antibody with the viral protein on the cell surface, and binding of the Fc portion to the receptor (FcγRs) on effector cells. Potential effector cells include NK cells, macrophages, dendritic cells, γδ T cells, and neutrophils. In addition to lysis, binding Fcγ receptors can also lead to release of antiviral cytokines, which appear dependent on the specific receptor (239). Recent studies suggest that ADCC may play a major role in protection from infection or disease progression in vaccine models of HIV (see below), and such antibodies are increasingly emerging as a major correlate of immune protection in HIV infected controllers, and protected vaccinees.

Vaccines and correlates of immunity

Despite tremendous efforts, neither the development of an effective vaccine nor identification of consistent correlate(s) of immunity has been found for HIV. As we have attempted to illustrate throughout this article, this is mostly because, unlike virtually any other known pathogen encountered to date, HIV selectively targets and subverts essentially all components of the host immune system, particularly the major cells that initiate immune

responses, allowing the virus to persist and replicate to high levels throughout the life of susceptible hosts, until the immune system completely collapses, resulting in AIDS.

Early phase 3 HIV vaccine efficacy trials tested recombinant HIV-1 envelope glycoprotein subunit (rgp120) that elicited antibody responses to env proteins, yet these vaccines showed no efficacy in protection from infection (244, 245). Since antibody responses to env provided no protection, the next generations of vaccine candidates focused on generating cellular (T cell) responses. However, the Merck STEP vaccine trial, which involved an adenoviral vector to deliver gag, nef, and pol peptides, also failed to provide protection from infection, despite detectable CTL responses in vaccines and may even have resulted in higher rates of infection in uncircumcised men with pre-existing immunity to the adenoviral vector (246). This reinforced concerns of using viral vaccine vectors in populations where the vectors were prevalent, as pre-existing immunity to the vectors may elicit immune activation/inflammation (247), which again may support rather than prevent HIV transmission/replication. A prior SIV vaccine study using recombinant varicella-zoster virus vaccine vectors expressing SIV env resulted in enhanced viral replication and disease progression in vaccinated macaques after SIVmac challenge (248), but it was not clear from the study precisely what immunologic mechanisms were responsible for the enhanced viral replication. Nonetheless these studies indicate that certain vaccines have the potential to be harmful, so thorough testing in relevant animals models remains a necessity.

A more recent HIV vaccine trial, however, finally provided some positive results, both in protection from infection and for demonstrating some correlates of protection. The Thai Phase III HIV vaccine clinical trial, also known as RV144, tested a 'prime-boost' combination of the two previously failed vaccines. This prime-boost vaccine combination lowered the rate of HIV infection by 31.2 percent in vaccinees compared to placebo controls (3). This was a surprising result, first because neither vaccine had proven effective alone but also because protection did not correlate with levels of HIV-specific CD8⁺ T-cell responses or levels of bnAbs (87,249). Interestingly, the vaccine did elicit proliferative CD4⁺ T-cell responses and the production of binding antibodies to the V1/V2 region of env (3,87), the latter correlating with protection. Furthermore, the vast majority of vaccines demonstrated strong ADCC activity, suggesting the binding antibodies may have been involved in mediating protection through this mechanism (250). However, in patients who became infected after receiving the vaccine, no difference in viremia or levels of viral control was detected, indicating the vaccine had minimal effects on viral replication after transmission. Although the level of efficacy was not sufficient to advance this vaccine to the general population, the results suggest that if sufficient levels of antibodies against specific regions of the HIV envelope can be elicited, protection from a vaccine may be possible (3).

Several other vaccine candidates in nonhuman primate models are showing remarkable efficacy in either preventing or resisting vaginal SIV/SHIV transmission to macaques, or in reducing viral loads after infection, and in some cases, possibly clearance of infection through vaccination, suggesting a cure may also some day be possible (251). Decades ago, it was demonstrated that vaccination (or infection) with attenuated, live strains of SIV lacking one or more genes essential for pathogenicity could result in what appears to be sterilizing immunity against homologous challenge with pathogenic SIV (252, 253). However, the possibility of reversion to pathogenicity renders the LAV approach less desirable for a vaccination strategy. Nonetheless, these vaccines have provided important information on the correlates of immunity that a vaccine needs to induce. For example, the LAV SIVΔ-nef, which was originally shown to confer protection against pathogenic challenge, was also recently shown to elicit potent ADCC activity, in the absence of neutralizing antibodies, again suggesting binding antibodies mediating innate immune responses may play a significant role in protection (254). Further studies demonstrated that LAV protection

correlated with virus-specific CD4⁺ and CD8⁺ T-cell responses in lymph nodes rather than blood, likely due to the persistence of LAV (antigenic persistence) in the Tfh of GCs (255).

A vaccination strategy using replicating adenovirus type 5 HIV/SIV recombinants has also shown significant protection against SHIV challenge (256), which was also mediated through NK mediated mechanisms (257) and also correlated with virus specific mucosal IgA responses (258). Further, low levels of neutralizing antibodies and env-specific CD4⁺ T-cell responses have been correlated with protection against SIVsmE660 challenge in DNA prime/adenoviral (259) prime followed by replication-defective lymphocytic choriomeningitis virus (rLCMV) boost strategies (260). Several other vaccine or challenge studies in rhesus macaques have shown that ADCC/ADCVI activity correlates with reduced viral load post challenge (261-264), and thus converging evidence from both human and macaque studies suggest that binding antibodies that elicit ADCC activity may best correlate with protection from HIV infection and disease progression.

Vaccination using SIV protein-encoding vectors based on rhesus cytomegalovirus (RhCMV) specifically to induce durable effector memory T-cell responses demonstrated resistance to SIVmac239 infection and control of infection after repeated rectal challenge (251). Although animals did not show sterilizing immunity, only transient low level blips of virus were detected, and mucosal and memory CD4⁺ T cells were spared, indicating a remarkable level of protection. Further, in many of the vaccinated animals, virus cannot be detected in blood or tissues by current technologies, even in the face of experimental CD8⁺ T-cell depletion, suggesting these animals may have completely cleared the infection (87). Thus, in addition to ADCC activity, preservation of memory CD4⁺ T cells and perhaps virus-specific CD4⁺ T-cell responses are other major correlates of protection that will be required from an effective vaccine.

The case for CD4⁺ T cells in *protection* from HIV

Although to this point we have discussed CD4⁺ T-cell subsets as the major targets for viral replication, it is increasingly apparent that, in addition to the innate responses above, virus specific CD4⁺ T-cell responses are emerging as a major correlate of immunity in vaccinated protected subsets, and elite controllers. Several recent studies have demonstrated CD4⁺ T cells may be the most important component of an effective antiviral response (265-267), and differences in the breadth of CD4⁺ T-cell profiles correlate with control of viremia in HIV patients (268). Expansion of viral specific CD4⁺ T cells has recently been demonstrated to be a much better predictor of control of viremia than CD8⁺ T cells in both hepatitis infection of chimpanzees (269) and HIV infection of humans (266). In fact, expansion of cytolytic CD4⁺ T cells bearing perforin and granzymes best correlates with control of HIV infection. Further, this control was not associated with specific MHC alleles such as HLA-B27 or HLA-B57, which are often associated with spontaneous control of HIV (270). This is particularly exciting, as it suggests an immunologic mechanism that may be manipulated to make 'susceptible' populations resistant to HIV infection.

Numerous studies of HIV and SIV have also shown an association between control of viremia and CD4⁺ T-cell responses, which can kill HIV/SIV-infected macrophages (201,271,272). Thus, we predict that virus-specific CD4⁺ T-cell response will emerge as a much better correlate of protection from infection and possibly even clearance of HIV from infected patients. However, distinguishing the proper mechanism for eliciting the appropriate CD4⁺ T-cell response will be key, as we must first distinguish the elements of a protective response from a CD4⁺ T-cell activation response that actually facilitates infection and disease progression.

One key that suggests a link between the protective CD4⁺ T-cell response and effective ‘innate’ ADCC responses is the importance of proper CD4⁺ T-cell help in B-cell development and ‘programming’. For example, persistence of antigen (and not lytic virus) within GCs and the proper stimulation of Tfh cells to generate, promote, and mature B-cell responses into effective plasma cells secreting appropriately programmed/matured antibodies (referred to as somatic hypermutation) might distinguish protection from progression to disease. Conceivably, the persistence of antigen in proximity to Tfh cells, and appropriate activation of Tfh to provide proper B-cell programming and somatic hypermutation may play key roles in the development of protective immunity. This hypothesis unifies many of the correlates of immunity to date, including the previously demonstrated need for antigenic persistence in lymph nodes (LAV and various vaccine studies), correlations of virus specific CD4⁺ and CD8⁺ T-cell responses in lymph nodes (but not blood) and generation of effective ADCC antibodies in protected vaccines and spontaneous controllers, combined with the findings of preserved CD4⁺ T-cell subsets and ILCs (IL17 etc.) in tissues of nonprogressing hosts. Although much work remains in deciphering how to appropriately stimulate effective CD4⁺ T-cell responses, without eliciting additional target cells for HIV, at least we now have clues for what immunologic responses will be necessary for an effective vaccine or therapy.

Conclusions and future directions

In susceptible hosts (macaques and humans), SIV/HIV selectively infects and destroys activated, memory CD4⁺CCR5⁺ T cells, which are the orchestrating cell of the immune system, which are recruited to early sites of pathogen exposure/infection, to facilitate effective immune responses against most pathogens. Thus, most of the innate and adaptive immune responses simply provide additional fuel for viral persistence and replication. Further, despite being classified as ‘lentiviruses’ (slow viruses), SIV/HIV rapidly stimulates local immune responses that allow local expansion and systemic spread of transmitted founder virus (TFV) to the intestine, where abundant target cells support marked, early, and high levels of viral replication (peak viremia). Since HIV has such a high rate of mutation, this results in rapid and tremendous viral diversity and emergence of viral variants that are already capable of evading the slowly emerging cellular and humoral immune responses developing in response to the TFV. Infection and dysregulation of key mucosal CD4⁺ T-cell subsets leads to intestinal barrier damage and subsequent HIV-specific and nonspecific systemic immune activation stimulate continual production of new effector CD4⁺CCR5⁺ T cells, providing a continual source of target cells to support sustained replication throughout host tissues. Finally, the HIV genome encodes for a variety of proteins that evade immune responses, including those that downregulate key host immunoregulatory molecules on cells and bind or inactivate the effects of host proteins or antimicrobials generated in what was intended as a ‘protective’ response to infection, thus limiting essentially all components of the host immune response to control HIV infection.

Decades of study of the immunology and pathogenesis of HIV infections have taught us more about human immunology and host defense than perhaps any other disease in history, yet with a pathogen so complex and cleverly evolved to thwart all major components of the human immune response, it is no wonder it has been so difficult to produce an effective vaccine for HIV. Nonetheless, after decades of failure, persistence in vaccine research is finally paying off, as we have achieved modest success in preventing HIV infections through a partially effective vaccine, and more importantly, we are finally beginning to understand at least some of the correlates of immunity generated by protected vaccines. In addition, remarkable successes are emerging from new vaccine strategies in nonhuman primate models, which may pave the way for more effective vaccines in the near future. As vaccine research begins to finally show promise rather than failure, and correlates of innate

and adaptive immune responses that may prevent infection, or even purge HIV from infected hosts, we may at least hope that an effective vaccine will finally be forthcoming in the near future.

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Fig. 1. Langerhans cells in the vaginal epithelium distinguished by co-expression of HLA-DR and CD1a (LC appear yellow – see arrows)
Other DCs are evident in the deeper *lamina propria* as HLA-DR⁺ (green), yet HLA-DR alone is not specific for DCs.

Fig. 2. Lymphoid follicles within organized lymphoid tissue
Follicles are within the intestine (A) and lymph node (B) showing distribution of B cells (red) and T-cell zones (blue). Note IgA (green) is readily detected in cells in the intestinal *lamina propria* but not detectable in peripheral lymph nodes.

Fig. 3. T-cell proliferation (Ki-67⁺, red) and activation (CD69⁺, green) in the intestinal *lamina propria* (A) and lymph node (B) of a normal macaque
 Abundant activated (CD69⁺; green) KI-67⁺ T cells are constitutively present in intestinal tissues, but activated cells are rare in normal lymph nodes. Note intestinal crypt epithelial cells are also highly Ki-67⁺ due to intrinsic rapid rate of turnover.

Fig. 4. T-regulatory (Treg) cells in the intestinal *lamina propria* of a normal macaque distinguished by intracellular FoxP3 (red nuclei) and surface CD25 (green) expression

Fig. 5. Comparison of T cells in the intestine and lymph node of macaques

Tissues are stained for CD3 (blue), CD4 (red) and CD8 (green) so CD3⁺CD4⁺ T cells appear 'purple' and CD8⁺ T cells appear 'yellow'. Note both gut (A) and lymph node (C) contain CD4⁺ and CD8⁺ T cells. However, the intestine contains small 'cryptopatches' containing CD3⁻CD4⁺ cells (red;B) consistent with Lti cells, and CD3⁻CD8⁺ ILC (green), which are not detected in lymph nodes (C).