Regulation of the tonsil cytokine milieu favors HIV susceptibility

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Abstract: Mucosal associated lymphoid tissues are major targets of HIV during early infection and disease progression but can also provide a viral safe haven during highly active antiretroviral therapy. Among these tissues, the tonsils remain enigmatic regarding their status as primary and/or secondary sites of retroviral infection. To dissect the mechanisms underlying susceptibility to HIV in this compartment, isolated tonsil cells were studied for phenotypic and functional characteristics, which may account for their permissiveness to infection. For this, tonsil cells and PBMC were infected in parallel with HIV, and viral replication was monitored by p24 ELISA. Our results demonstrate that unstimulated tonsil cells were more readily infected than PBMC with HIV. Phenotypic characterization of the tonsil cells revealed heterogeneous lymphoid populations but with increased expression of early activation markers and the viral co-receptor CXCR4, relative to PBMC, all of which may contribute to viral susceptibility. Furthermore, the cytokine microenvironment appeared to be key in facilitating HIV infection and tonsil-secreted products enhanced HIV infection in PBMC. Of the cytokines detected in the tonsil supernatants, TH2 cytokines, particularly IL-4, promoted HIV infection and replication. Interestingly, this TH2 profile appeared to dominate, even in the presence of the TH1 cytokine IFNγ and the anti-viral factor IFNα, likely due to the enhanced expression of suppressor of cytokine signaling (SOCS) proteins, which may disengage IFN signaling. These and other local environmental factors may render tonsil cells increasingly susceptible to HIV infection. J. Leukoc. Biol. 80: 000–000; 2006.

Key Words: mucosal associated lymphoid tissue · TH2 · IL-4 · IFN · SOCS

INTRODUCTION

The tonsil mucosal associated lymphoid tissues are implicated in all stages of HIV pathogenesis. In presymptomatic stages of infection, a stable pool of virions may be present on the surfaces of dendritic cells (DC), together with a smaller pool of infected lymphocytes [1]. As the disease progresses, the tonsils can become heavily infected, especially in the germinal center and in the lymphoepithelium where characteristic multinucleated giant cells can be identified [2–4]. While there is no doubt that tonsils are a secondary site of infection, uncertainty continues regarding primary infection via the oral mucosa. In primate models, infection has been shown to occur through the surface mucosa of the tonsil [5, 6], where transmission of the virus is speculated to involve specialized M cells and DC capable of transporting HIV to the interior of the tonsil. However, in humans, oral transmission occurs rarely [7, 8] and HIV binding to tonsil epithelium exhibits limited progression to primary infection [9]. Mucosal innate host defense molecules including, but not limited to, lysozyme, thrombospondin, defensins, and secretory leukocyte protease inhibitor (SLPI) [9–13] may provide protection, given that the viral inoculum does not exceed the anti-viral capacity of saliva. Once infected, either initially or secondarily, the tonsil, which is a site of constant immune stimulation, favors viral host cell recruitment [4] and HIV replication [3, 14, 15]. Since viral reservoirs within the tonsil lymphoid tissue during highly active antiretroviral treatment have also been documented [16] [17], understanding the mechanisms of infection and persistence of the virus within this tissue is crucial for the development of therapies that target protected compartments.

In spite of the ongoing interest in the tonsil as a target and potential reservoir, the cellular and molecular mechanisms associated with HIV infection in this tissue are not fully understood. To date, in a tonsil histoculture system, a preferential X4 co-receptor utilization has been demonstrated [18] and associated with severe depletion of CD4+ T cells, suppressed humoral responses, and macrophage infection [3, 19, 20]. However, recent evidence indicates that R5 HIV variants also infect tonsil lymphoid tissues ex vivo. Interestingly, the R5 HIV prefers a CD4+ HLADR– CD62L– CCR5+ population, whereas X4 variants target HLADR+ CD4+ CD62L+ CXCR4+ cells [20, 21].
To further characterize HIV-susceptible tonsil cell populations and to identify potential unique permissive factors for HIV infection and replication that might exist in this milieu, we have isolated tonsil lymphoid cells for ex vivo infection in comparison with PBMC. The differential infection kinetics, susceptibility, and virus replication in these two target populations led us to compare the cellular characteristics and cytokine profiles, which may underlie their unique resistance and susceptibility patterns. We demonstrate that increased tonsil cell susceptibility to infection may reflect, in part, the increased expression of the viral co-receptor CXCR4 and a heightened immune activation of the tonsil cell population. Additionally, the cytokine milieu contributes to increased HIV infection/replication, in that tonsil TH2 cytokines, notably IL-4, enhance HIV replication despite the presence of the TH1 anti-viral cytokine IFNγ and IFNα. Increased expression of cytokine regulators such as the suppressors of cytokine signaling (SOCS) may account for a local disengagement of the IFN signaling, favoring TH2 enrichment. Defining the factors that facilitate HIV infection in this and other susceptible compartments may provide insight for designing novel strategies for prevention and therapeutic intervention.

MATERIALS AND METHODS

Specimen collection

Human palatine tonsils were obtained from routine therapeutic tonsillectomies (sleep apnea and/or tonsillitis) performed on HIV seropositive and seronegative adults at the George Washington University Hospital with informed consent (IRB # 099920). Tissues were either snap-frozen, fixed in 4% paraformaldehyde, or placed in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY), supplemented with antibiotics, and kept on ice until processed.

Immunohistochemistry

Formaldehyde-fixed tissues were dehydrated, embedded in paraffin, and sectioned for immunohistochemical staining. An antibody against SOCS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody for indirect immunostaining with a tyramide signal amplification kit (NEN, Boston, MA) [22].

Isolation and culture of tonsil cells, PBMC, and T cells

Tissues were processed with an automated mechanical disaggregation system (Medimachine, Becton Dickinson, San Jose, CA) to obtain single-cell suspensions with >97% viability. Mononuclear cells were separated by Ficoll-LSM (Lymphocyte Separation Medium, ICN Biomedicals, Aurora, OH) gradient centrifugation, and PBMC were obtained by leukapheresis from healthy volunteers (NHB Blood Bank, Department of Transfusion Medicine, Bethesda, MD) and separated by Ficoll gradient centrifugation [12]. T cells were separated by elutriation. For culture, tonsil cells, PBMC, and T cells were re-suspended in RPMI 1640 medium with 2 mM of L-glutamine, 10 μg/ml gentamycin, and 10% FCS (Life Technologies, Gaithersburg, MD) at 2–3 × 10^6 cells/ml in 15 ml tubes (Becton Dickinson, Franklin Lakes, NJ).

Flow cytometry

The phenotype of tonsil cells and PBMC was compared by flow cytometry using the following antibodies: CD3, CD4, CD8, CD20, CD68, HLADR, CD25, CD69, CXCR4,CCR5, CD40L, CD1A (Becton Dickinson, San Jose, CA), CD40 (BioSource, Camarillo, CA), TNFR and TNFRII (R&D Systems, Minneapolis, MN) and p24 (Coulter Clone, Miami, FL). Antibodies were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Cells were analyzed with a FACScalibur system (Becton Dickinson). For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit according to the manufacturer’s specifications (Becton Dickinson).

HIV infection and p24 ELISA

Tonsil cells, PBMC, and T cells were infected in parallel with R5 HIVIIIB, X4 HIVHXB (Advanced Biosciences, Columbia, MD) and dual-tropic X4/R5 HIV89.6 (generously provided by Dr. Carl Wild, Panac600 Pharmaceuticals Inc., Gaithersburg, MD) at TCID50 = 10^5/ml [23], washed and cultured at 37°C for 5 to 10 days. Culture supernatants were collected every 1–3 days and tested for HIV p24 antigen by ELISA (Perkin-Elmer, Boston, MA). Cells were fixed in 2.5% glutaraldehyde, processed for transmission electron microscopy [3] and viewed in a Zeiss EM10 microscope (LEO Electron Microscopy, Thornwood, NY).

Cytokine, chemokine, and transcription factor ELISA

Culture supernatant levels of IL-4, IL-6, IL-10, IFNα, and IFNγ from tonsil cells and PBMC were measured by ELISA (BioSource) at indicated intervals after infection with dual-tropic HIV 89.6. SDF-1 α levels were measured by ELISA (R&D Systems), and a multiplex ELISA was used for the detection of chemokines, MIP-1α, MIP-1β, and RANTES (BioSource).

Signal transducer and activator of transcription 1 (STAT-1) activation was measured with an ELISA-based kit (Active Motif, Carlsbad, CA) performed on nuclear extracts of tonsil cells and PBMC [23].

Cytokine treatment and inhibition experiments

PBMC of tonsil cells were cultured for 18–72 h with IL-4, IL-6, IL-10, IFNα, or IFNγ at 1, 10, or 100 ng/ml (NCI-FCRDC, Frederick, MD), or with 72 h culture supernatants from unstimulated tonsil cells and PBMC. For cytokine inhibition experiments, antibodies to IL-4, IL-6, IL-10 and IFNγ (e Bioscience, San Diego, CA) were used at concentrations corresponding to 50–100× the detected cytokine concentrations in the tonsil supernatants.

Western blot analysis

Equal amounts of protein (1 mg) from whole cell lysates [23] were immunoprecipitated using SOCS-3 antibody (Fusion Antibodies, Belfast, Northern Ireland). The immunoprecipitates were electrophoresed using Tris-Glycine 4–20% gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membranes, and immunoblotted with anti-SOCS-3 antibody (Santa Cruz Biotechnology). Immunoblots were developed by enhanced chemiluminescence with Super Signal substrate (Pierce, Rockford, IL).

RNA isolation, cDNA expression array, RT-PCR, and RNase protection assay (RPA)

Total cellular RNA was isolated from tonsil tissues (n=4) and from PBMC (10–20×10^6 cells) (n=4) within 1–2 h after isolation, using the standard TRIZOL (Invitrogen) procedure for Atlas cDNA Expression array (Clontech, Palo Alto, CA) RT-PCR and RPA. For RT-PCR, 1 μg of total RNA was reverse transcribed using oligo(dT)20 primer (Invitrogen), and the resulting cDNA was amplified by PCR. The primer sets for SOCS-1 were 5′-TCCCGTTCCAGATTGACCGG-3′ (forward) and 5′-AAGAGTGAAGAGGCAGGATTCG-3′ (reverse), for SOCS-3 5′-TCCCCCAGAGAGCTAT TAATG-3′ (forward) and 5′-TCCGGACAGATGCTGAAGTG-3′ (reverse) and for GAPDH the primer sets were 5′-GAAGTGTAAGCTGCGATGC-3′ (forward) and 5′-GAAGTGTAAGCTGCGATGC-3′ (reverse). For RPA, 5 μg of total RNA was used with the human SOCS multi-probe set (Becton Dickinson) and the RNA levels were normalized to the expression of GAPDH.

Real-time PCR

For RT-PCR, 1 μg of total RNA was reverse-transcribed using oligo(dT)20 primer (Invitrogen); the resulting cDNA was amplified by real-time PCR, on an ABI Prism 7500 Sequence Detector. Amplification was performed with TaqMan expression assays for HPRT (Assay ID: Hs09999909m1), GAPDH (Assay ID: Hs00164014_m1), IFNα (Assay ID: Hs00265382_s1), IFNγ (Assay ID: Hs00174413_m1), SOCS1 (Assay ID: Hs00174413_m1), and SOCS3 (Assay ID: Hs00174413_m1).
Hs00705164_s1), and SOCS3 (Assay ID: Hs00269575_s1, Applied Biosystems). HPRT and GAPDH housekeeping genes were used for normalization controls. Amplification conditions and parameters were set by the manufacturer. Data were analyzed using the 2^-ΔΔCT method [24] and results were reported as fold change.

Statistical analysis
Means, standard deviations and standard errors were calculated and student t tests performed to define significant differences (P<0.05).

RESULTS
Tonsil cells exhibit increased susceptibility to HIV infection in vitro
Tonsils are recognized as a site of active HIV replication in vivo [1], and this enhanced susceptibility of tonsil lymphoid cells to HIV infection appears to be retained ex vivo. Exposure of equivalent numbers of tonsil cells, isolated from HIV sero-negative individuals and unstimulated PBMC to HIV in parallel culture conditions, yielded marked differences in infection. Measurement of p24 levels in the supernatants was consistent with significantly increased HIV production by the tonsil cells, compared with PBMC, irrespective of virus R5, X4, or R5X4 tropism (Fig. 1A, P<0.05, day 5 shown). The increased supernatant p24 levels in the tonsil corresponded to a modest increase in the percentage of HIV-infected CD4+ target cells in the tonsil compared with the PBMC (8±3% vs. 2±3%, Fig. 1A, insert), consistent with increased viral replication. Moreover, the kinetics of infection in the tonsil cell cultures revealed rapid viral replication evident by elevated p24 levels within 3 days and peak infection around day 5, declining at later time points (Fig. 1B), which was likely due to viral-induced cell death as previously reported [18].

In parallel cultures, ultrastructural analysis demonstrated the presence of productively infected tonsil cells with abundant budding and mature HIV virions on their surfaces (Fig. 1C). Quantitation of infected cells at the ultra structural level confirmed the increased presence of infected cells (Fig. 1E).
Tubuloreticular inclusions (TRI), which have been associated with IFNα production [25], were frequently present in these infected tonsil lymphocytes (Fig. 1C, arrow); and numerous multinucleated giant cells (MNGC) (Fig. 1D) were also evident in the infected tonsil cultures as reported in vivo [3].

Increased expression of early activation markers and CXCR4 on tonsil cells
To identify potential unique features of the tonsil lymphoid cells that may render them more susceptible to HIV infection, a phenotypic comparison of isolated tonsil cells and PBMC was performed by flow cytometry. Despite their relative ease of infection, FACS analysis revealed a significantly reduced percentage of CD4+ potential targets in the tonsil (30±7.3) compared with PBMC (50±10) (Fig. 2A, P<0.05). Reduced numbers of CD4+ T cells, as well as total T cells (CD3), reflect the increased proportion of B cells (CD20+, P<0.05) in the tonsil population. The tonsil lymphoid cells also appeared to be slightly enriched in CD1A+ dendritic cells and in CD68+ macrophages.

The expression of activation markers was additionally monitored as an indication of functional or maturational differences, which might account for enhanced viral production by the tonsil cells. The percentage of cells expressing the classical marker of T cell activation IL2Rα (CD25) was not significantly higher in the total tonsil cell population compared with PBMC (Fig. 2A, n=13), consistent with the reduced percentage of T cells and the lack of evidence of active proliferation (data not shown). Nevertheless, the increased presence of the early activation marker CD69 in the tonsil (Fig. 2A, P<0.05), and particularly in the CD4+-susceptible population (Fig. 2B), indicated a basal level of activation [26], as did increased HLA-DR (P=0.03) and the CD40 co-stimulatory molecule (Fig. 2A, P<0.05). Enhanced CD40L expression, although not significant, was also indicative of heightened immune responsiveness.

To further define parameters related to the underlying enhanced viral susceptibility, we examined the tonsil cells for differential expression of viral co-receptors, which might facilitate viral attachment and/or entry. The T cell tropic co-receptor CXCR4 was expressed on a significantly higher percentage of tonsil cells compared with PBMC (60%±10 vs. 20%±5, P<0.05, n=13) and was present on more than 90% of the CD4+ cells in the tonsil (Fig. 2B). The M-tropic co-receptor, not as pronounced in the tonsil cells or the PBMC (Fig. 2A), was detected on the majority of the tonsil CD68 macrophages (Fig. 2B). Furthermore, the predominant co-receptor CXCR4 was highly co-expressed with activation markers such as CD69 and HLA-DR (Fig. 2B), favoring enhanced susceptibility. Many of these susceptibility markers present in the tonsil population remain high following in vitro exposure to HIV and during peak viral replication (day 5, data not shown).

Tonsil chemokine profile
Having identified surface markers in the tonsil population that may be associated with the susceptibility of this population, we proceeded to investigate the differential secretion of soluble mediators that may also influence infection, such as chemokines that bind to HIV co-receptors to intercept viral entry [27]. For this purpose, we measured levels of the CCR5 binding chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and the CXCR4 binding chemokine SDF-1α in supernatants of tonsil and PBMC (infected and uninfected) cultures (day 5, n=3, Fig. 3A–C). Decreased, albeit not significant, levels of MIP-1α and MIP-1β were detected in tonsil cultures (data not shown). A significant decrease was seen in RANTES (P=0.03, Fig. 3A), which was almost undetectable in the tonsil cultures, especially after infection. By comparison, secretion of the X4 chemokine SDF-1α, minimally detectable in PBMC cultures, was elevated in the tonsil cultures (Fig. 3B), which may influence differential R5/X4 susceptibility.

Tonsil cytokine profile
Further, to explore potential molecular factors that may bias tonsil cell infectibility, we compared gene expression profiles between tonsil cells and PBMC using cDNA expression arrays (Moutsopoulos, N. et al., manuscript in preparation). Among the differentially expressed genes between these two popula-
tions, we focused on cytokine genes that might frame the micromilieu of the tonsil. A distinct pattern emerged in that the cytokine genes most up-regulated in the tonsil population included IL-4, IL-5, IL-6, IL-10, and IFN-γ, whereas neither IL-2 and IL-12 nor TNF-α were overrepresented in the tonsil global gene expression analysis, suggesting a TH2 bias.

When supernatants from cultured HIV infected and uninfected populations of blood and tonsil lymphoid cells were collected at different time points after infection and analyzed by ELISA (Fig. 4), the pattern of cytokine secretion reflected the gene expression. The TH2-related cytokines IL-4, IL-6, and IL-10 were detectable in the supernatants of unstimulated and uninfected tonsil cells, at levels significantly ($P<0.05$) exceeding those present in parallel PBMC cultures (Fig. 4; 5 days in culture shown). Following infection with HIV, significant increases were observed for both IL-10 and IL-4, compared with PBMC. Despite this prevalence of TH2 type cytokines, the TH1 cytokine IFN-γ was also detected before and after in vitro exposure to HIV (Fig. 4A). Given the tonsil cell susceptibility to viral infection, the constitutive presence of IFN-γ, as well as IFN-α, a potent anti-viral cytokine, in uninfected tonsil cell cultures compared with PBMC ($P<0.05$) and
following infection ($P < 0.05$) was intriguing (Fig. 4B, 5 days post infection).

**Tonsil secreted products enhance HIV infection in blood PBMC**

Based on the enriched cytokine milieu of the tonsil cell cultures, we investigated the potential role of tonsil-released cytokines in influencing HIV susceptibility, in a less-responsive population of cells. For these studies, we pretreated (18 h) freshly isolated PBMC with culture supernatants from 72 h cultures of uninfected tonsil cells or PBMC, prior to exposure to either HIV$_{Bal}$ or HIV$_{IIIB}$. This exposure to soluble products from tonsil cell cultures effected more than a twofold increase in X4 infection of PBMC (from $67/1006$ to $147/1006$ ng/ml), with a smaller increase in R5 infection (from $14/1006$ to $22/1006$ ng/ml). This increase was statistically significant for X4 infection compared with the increase effected by culture supernatants derived from PBMC ($P < 0.05$), indicating that tonsil cells in culture, and likely in situ, release molecules that can influence HIV binding-entry and/or replication. To determine which, if any, of the tonsil-derived cytokines might account for the enhanced susceptibility, we subsequently added neutralizing antibodies to the tonsil supernatants, prior to evaluating their influence on HIV infection of PBMC with an X4 virus. Of the cytokine antibodies tested, inhibition of IL-4 significantly abrogated the tonsil supernatant-enhancing effect in X4 infection (from $147/1001$ to $96/1001$ ng/ml, $P < 0.05$), indicating that this cytokine may facilitate infection in the tonsil milieu (Fig. 5A).

In a direct assessment of these cytokines, we added recombinant cytokines to unstimulated PBMC, prior to exposure to HIV$_{Bal}$/HIB (Fig. 5B). IL-4 independently augmented HIV$_{HIB}$ and HIV$_{Bal}$ replication, whereas the opposite effect occurred in the presence of IFN$_{a}$ and to a lesser extent with IFN$_{y}$. Correlated with this altered permissiveness to HIV infection, treatment with IL-4 or tonsil supernatants increased the percentage of PBMC expressing CXCR4 and CD69 but did not influence the expression of CCR5 or HLADR (Fig. 5C). Thus, the tonsil cytokine milieu, and particularly IL-4, appear to be associated with cellular and molecular pathways favoring infection, whereas IFN$_{y}$, as well as IFN$_{a}$, although present, appear to be ineffective at combating infection.

**SOCS proteins may influence cytokine and infection profiles**

Enigmatic was the presence of both IFN$_{a}$ and IFN$_{y}$ but without evident anti-viral activity. To define this seeming inconsistency, we explored whether inhibitory molecules, such as SOCS negative regulators of cytokine signaling [28], might play a role in counteracting IFN activity. In this regard, we documented that SOCS-1 was constitutively expressed in the tonsil to a greater extent than in PBMC as determined by RT-PCR (Fig. 6A). In addition to SOCS-1, SOCS-3, which is often linked with TH2 cells [28], was higher in the tonsil, both by constitutive gene expression (Fig. 6A, $n=3$–4) and by protein levels (Fig. 6B, $n=2$). By immunohistochemical analysis, SOCS-1 protein was evident in the tonsil tissue, as shown in the germinal centers where HIV was also abundant (Fig. 6C). Analysis of SOCS-1 and SOCS-3 RNA expression in tissues from HIV seronegative and asymptomatic HIV seropositive individuals revealed that both SOCS-1 and -3 were higher in the HIV-infected tonsil tissue than that seen in tonsils from uninfected individuals (Fig. 6D–E). HIV infection of purified T cells from peripheral blood ex vivo also led to an increase in

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**Fig. 2.** Phenotype of tonsil cells. (A) FACS staining for cell surface markers on freshly isolated tonsil cells and PBMC. Data presented are mean ± SD of 13 donors, *, $P \leq 0.05$, between tonsil and PBMC. Staining included antibodies to: CD3, CD4 (T cells), CD20 (B cells), CD68 (macrophages), and CD1A (dendritic cells), markers of cell activation (CD25, CD69, HLADR, CD40, CD40L), and HIV co-receptors (CCR5, CXCR4). (B) Dual color-staining of tonsil cells and PBMC with antibodies as indicated (representative of 3 experiments).
SOCS-1 and SOCS-3 gene expression, coinciding with enhanced IFN (particularly IFN\(\text{\textsuperscript{\textbeta}}\)/H9251, Fig. 6F).

The constitutive expression of SOCS proteins in the tonsil population and their up-regulation following HIV infection led us to investigate functional correlates of SOCS activity downstream of IFN signaling, such as STAT-1 activation and inhibition of HIV infection. In an ELISA that detects active STAT-1, the active form was detected in the tonsil population and was minimally elevated following in vitro stimulation with IFN\(\text{\textbeta}\). By comparison, in resting PBMC treated with IFN\(\text{\textbeta}\), a significant increase in active STAT-1 levels occurred (Fig. 6G, \(P<0.05\)). Reflecting this lack of responsiveness by tonsil cells to IFN\(\text{\textbeta}\) ex vivo, the addition of IFN\(\text{\textbeta}\) did not drive these cells to block HIV infection in cell culture. Consistent with IFN\(\text{\textbeta}\)-inducible STAT-1 activation in PBMC, IFN\(\text{\textbeta}\) suppressed their ability to become infected (Fig. 6H).

DISCUSSION

Critical events in HIV transmission, infection, and AIDS pathogenesis occur in the mucosal and gut associated lymphoid tissues that serve as major viral reservoirs throughout the course of disease. In these sequestered areas, viral-host cell interactions can lead to ongoing viral replication, storage, and persistence [29]. Defining the mechanisms that underlie these events is crucial for the development of therapeutics and vaccines aimed at mucosal targets. In this endeavor, the tonsil histoculture system has been frequently used as an ex vivo model system to study disease parameters of infection and pathogenesis at lymphoid sites [30]. To further characterize unique susceptibility factors of this compartment at the cellular and molecular level, we used isolated tonsil lymphoid cells and demonstrated their enhanced susceptibility to HIV X4 and R5, in vitro compared with PBMC, even in the absence of external stimulation or manipulation. Key features inherent to this population were retained ex vivo, contributing to an increased vulnerability even outside of the unique tissue microenvironment.

While multiple contributing factors may support HIV replication in this compartment, our detailed analysis of the tonsil population revealed that, whereas CXCR4 and/or CCR5 chemokine ligands were not discriminatory [27], the cytokine milieu favors the infection process. In this regard, constitutively secreted products from uninfected tonsil cells converted relatively resistant resting PBMC to a more receptive population, indicating that secreted factors are important in the progression of infection. Initial gene expression analysis on HIV seronegative tonsil tissues revealed that the TH2 cytokines IL-4, IL-5, IL-6, and IL-10 were highly expressed, but that the TH1 cytokine IFN\(\text{\textgamma}\) and IFN\(\text{\textbeta}\) were also represented despite limited expression of other TH1 factors such as IL-12 and IL-2. In our ex vivo tonsil cultures, we detected a TH2 bias in the cytokine profile and report an increase in cytokine production after HIV infection, which reached significance for IL-4 and IL-10, as seen in late-stage AIDS patients [31], while in the tonsil histoculture system no alteration of the cytokine mRNA expression profile was observed after HIV infection [32], although protein production was not measured for IL-4 and IL-10 in these studies.

Of the cytokines secreted by tonsil cells, IL-4 appeared to independently support enhanced susceptibility to X4 HIV infection. Exposure of PBMC to IL-4 enhanced X4 infection and conversely, inhibiting IL-4 in the tonsil supernatants reversed their ability to enhance infection, linking this cytokine to regulation of virus susceptibility. No significant changes in R5 HIV replication were observed with exposure to tonsil culture supernatant, anti-IL-4 or IL-4. The effect of IL-4 on X4 HIV replication was seen despite the low detected levels for IL-4 in the tonsil supernatants, which may be a reflection of the cytokine being bound to receptors and/or other mechanisms interfering with detection. IL-4 was also effective in up-regulating the expression of the viral co-receptor CXCR4 in the PBMC, pointing to a mechanism through which IL-4 may enhance infection in the tonsil. Cytokine signals are known to induce CXCR4 expression and, in particular, IL-4 has been previously documented to induce functional CXCR4 in both T cells [33] and macrophages [34]. Consistent with the presence of IL-4, CXCR4 is constitutively highly expressed in the tonsil,

![Fig. 3. Chemokine levels in tonsil and PBMC supernatants. HIV infected and uninfected tonsil cells and PBMC were cultured at 2\times10^6 cells/ml, supernatants collected on day 5 (n=3 donors). Levels of RANTES were measured by multiplex ELISA (A). SDF-1α levels were measured by ELISA (B). Data represent mean ± SD of 3 donors.](http://www.jleukbio.org)
and tonsil-derived supernatants could augment CXCR4 expression in PBMC. The majority of the CD4+ target cells in the tonsil population were documented to express this co-receptor, facilitating a preferential X4 and X4/R5 infection documented in our cultures as well as in the histoculture system [18].

In addition to soluble mediators facilitating infection, tonsil cell characteristics appeared conducive to this vulnerability. Phenotypic analysis of the tonsil population provided evidence of a level of immune activation, which may support the superior viral replication in the tonsil cells. Immune activation is known to increase viral replication in vivo [35] and in vitro [36], and PHA-induced proliferation supports HIV-1 replication in T cells in vitro. The tonsil exhibited a unique profile of immune activation, characterized by the expression of antigen presentation molecule HLA-DR, co-stimulatory factors (CD40, CD40L), and the early activation molecule CD69 [37] but a low expression of the classical activation marker CD25 (IL-2R), typically indicative of proliferative and effector responses [38]. This state of readiness particularly characterized the HIV target CD4 or CXCR4 population. Whether this partial immune activation of the tonsil is confined to the adult population, which we studied exclusively and which may be associated with a diagnosis of tissue hyperplasia is not clear. However, this level of activation is not consistent with acute tonsillitis, which was excluded from our sample collections and is characterized by an increased expression of CD25 (data not shown).

In the non-acutely infected tonsil tissues, immune activation
appears to remain subdued. By what mechanisms the tonsil lymphoid tissue lies in readiness, partially activated and ready to respond to challenge, is unknown, but defining these pathways may provide insight into vulnerability to retroviral infection. For example, recent findings demonstrate that activation-arrested cells appear to be an initial target population of SIV infection in the GALT, capable of supporting viral replication [29] and contributing to the acute phases of infection that ultimately lead to CD4+ T cell depletion and disease dissemination.

Reflecting the state of activation in the tonsil, IFN-α and IFN-γ were constitutively expressed but clearly ineffective in neutralizing HIV, implicating barriers to IFN activity. Consistent with such a barrier, increased IFN levels in the tonsil or exogenous IFN exposure were not accompanied by evidence of downstream signaling cascades, as reflected by lack of STAT-1 activation, nor did IFN-α promote suppression of HIV replication in the tonsil cultures. Our data support members of the SOCS family, which are key physiological regulators of cytokine-mediated homeostasis in innate and adaptive immunity [28], as one potential impediment to a successful IFN response. SOCS proteins block JAK-STAT and TLR signaling pathways in a classic negative-feedback loop [39]. In the tonsil, a site of constant antigenic challenge, these proteins may function to regulate potential exacerbated immune responses and consequently, SOCS-1, by inhibiting STAT-1 [40], which may suppress the anti-viral activity of IFN-γ and IFN-α [41, 42]. Recently, silencing of SOCS-1 in dendritic cells has been proposed as a promising vaccine strategy, in light of the embellished anti-viral TH1 and antibody responses mounted when SOCS-1 is inhibited [43]. Whether DC, via SOCS-1, are the coordinators of immune control in the tonsil remains to be determined, but our data are not inconsistent with this possibility. Furthermore, SOCS-3, which acts to foster a TH2 environment by multiple mechanisms, including inhibition of IL-12 mediated STAT-4 activation and TH1-related T-box transcription factor t-bet expression [44, 45], may contribute to this conducive milieu. As a result of the TH2 dominance, the secretion of IL-10 may also sustain SOCS-3 gene expression.

![Fig. 5. Effect of tonsil supernatants and cytokines on PBMC phenotype and HIV infection. (A) PBMC (5×10⁶ cells/ml) were cultured for 48 h in medium only or in 72 h supernatants from unstimulated PBMC or tonsil cells and infected with HIV X4 (IIIB) and R5 (Bal). Infection was monitored by p24 ELISA (day 7 post-infection shown). PBMC cultures containing tonsil supernatants (1:1 dilution with media) were pretreated with or without antibodies to IL-4, IL-6, IL-10, and IFN-γ (0.5–1 μg/ml) for 48 h, washed, and then infected with X4 or R5 HIV. Infection was monitored by p24 ELISA (day 7 post-infection shown, n=3). (B) PBMC (n=3 donors) were cultured for 18 h in the presence or absence of recombinant IL-4, IL-6, IL-10, IFN-γ, or IFN-α (100 ng/ml shown) and thereafter infected with X4 or R5 HIV. Infection was monitored by p24 ELISA (day 7 shown, n=3 donors). (C) IL-4 (100 ng/ml) and tonsil supernatant (1:1) were added to PBMC for 18 h, and the expression of surface markers (n=3), measured by flow cytometry. Mean ± SD of percent positive cells from 3 donors is reported, P = NS.](http://www.jleukbio.org)
further blocking IL-12 signaling in a cyclic dampening of TH1 responses [46, 47].

Taken together, our results indicate that mechanisms of immune regulation, which have evolved to protect areas such as the tonsil from constant immune activation, may be recognized by HIV as prime candidates to support its lifestyle. Further dissection of these virally selective activation pathways may offer new insights into pathways of virus blockade.

Fig. 6. Expression of SOCS-1 and SOCS-3 in the tonsil. (A) RT-PCR for SOCS-1, SOCS-3, and GAPDH on cDNA from tonsil tissue (n=4) and freshly isolated PBMC (n=3). (B) Increased SOCS-3 protein expression in tonsil tissue (n=2) compared with freshly isolated PBMC (n=2) as determined by immunoprecipitation and Western blot analysis. (C) Immunohistochemical staining for SOCS-1 protein in formalin-fixed tonsil tissue from a representative HIV seronegative tonsil (n=4). Positive staining in the tonsil germinal center is shown. (D) RNase protection assay for SOCS-1 and SOCS-3 on 5 μg of RNA from HIV+ (n=2) and HIV–tonsil tissue (n=3). (E) Graph represents mean ± s.e expression normalized to GAPDH. (F) Real-time PCR for SOCS-1, SOCS-3, IFNα, and IFNγ on HIV X4 infected and non-infected peripheral blood T cells on days 1, 3, 5, and 7 of culture. Fold changes between infected/uninfected cells at each time point are reported (representative of three experiments shown). (G) ELISA for active STAT-1 on nuclear extracts (20 μg) from freshly isolated PBMC (n=2) and tonsil cells (n=3) with and without IFNα (10 ng/ml). Mean ± s.e shown,*P < 0.05. (H) p24 ELISA on PBMC and tonsil cells infected with HIVIIIB and treated or not with IFNα (1 ng/ml). Mean of percent inhibition by IFNα presented for 3 experiments (*P<0.05).


