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Immunopathology and Infectious Disease

Tonsil Epithelial Factors May Influence Oropharyngeal Human Immunodeficiency Virus Transmission

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Tonsil epithelium has been implicated in human immunodeficiency virus (HIV) pathogenesis, but its role in oral transmission remains controversial. To study characteristics of this tissue, which may influence susceptibility or resistance to HIV, we performed microarray analysis of the tonsil epithelium. Our data revealed that genes related to immune functions such as antibody production and antigen processing were increasingly expressed in tonsil compared with the epithelium of another oropharyngeal site, the gingival epithelium. Importantly, tonsil epithelium highly expressed genes associated with HIV entrapment and/or transmission, including the HIV co-receptor CXCR4 and the potential HIV-binding molecules FcRγIII, complement receptor 2, and various complement components. Immunohistochemical staining confirmed the increased presence of CXCR4 in the tonsil epithelium compared with multiple oral epithelial sites, particularly in basal and parabasal layers. This increased expression of molecules involved in viral recognition, binding, and entry may favor virus-epithelium interactions in an environment with reduced innate antiviral mechanisms. Specifically, secretory leukocyte protease inhibitor, an innate molecule with anti-HIV activity, was minimal in the tonsil epithelium, in contrast to oral mucosa. Collectively, our data suggest that increased expression of molecules associated with HIV binding and entry coupled with decreased innate antiviral factors may render the tonsil a potential site for oral transmission. ***(Am J Pathol 2007, 171:571–579; DOI: 10.2353/ajpath.2007.061006)***

The predominant mode of transmission for human immunodeficiency virus (HIV) is through the mucosal route,[1,2] particularly genital and gastrointestinal tract mucosae. To establish infection, HIV must cross the epithelial barrier of the recipient, associate with dendritic cells to infect resident lymphocytes and macrophages, and then spread systemically. The oral cavity is considered a relatively protected mucosal site where the innate host defense molecules of saliva are capable of neutralizing HIV and the epithelium itself is not receptive for transmission.[3–8] Orogenital transmission has a very low per-contact risk of acquiring infection, with estimates that 4 of 10,000 contacts result in infection compared with heterosexual or homosexual genital contact (1 of 200 to 1000).[9–12] Vertical transmission from mother to infant during breast feeding also occurs with varying rates but may be as high as 15% when breast-feeding is prolonged.[13,14]

The most plausible portal for HIV entry in oropharyngeal transmission is thought to be the tonsil. In a primate model, when the surface of macaque palatine tonsils was exposed atraumatically with cell-free simian immunodeficiency virus (SIV), infected cells were first detected within the reticulated epithelium lining the tonsillar crypts.[15,16] This unique anatomical compartment, the tonsil crypt epithelium, is specialized to participate in antigen sampling and immune surveillance. Structurally, the surface epithelium of the palatine and lingual tonsils is a continuation of the stratified squamous epithelium of the oral mucosa, but the epithelium of the crypts becomes reticulated and highly infiltrated with lymphocytes, which together with dendritic cells and M cells facilitate transepithelial access of antigens.[17] Multiple bacteria, viruses, and their products are known to enter the body through this route,[18] including SIV in primates. Nevertheless, in humans although the tonsil is a reservoir and replication site for HIV,[19] it still remains unclear whether oral transmission occurs here, because only secondary infection

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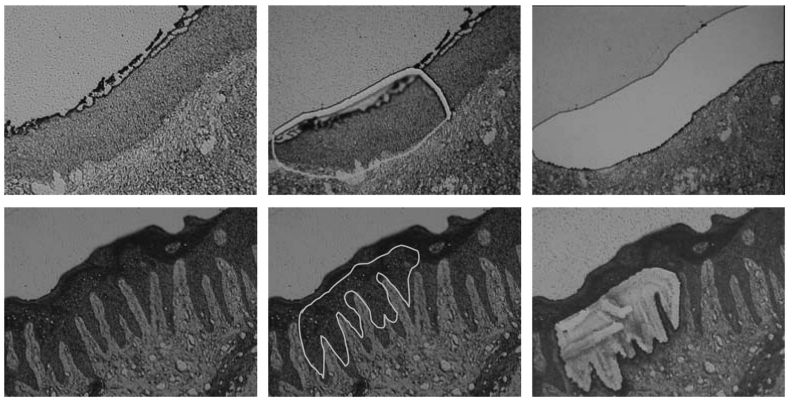
A



B

Shared highly expressed genes in tonsil and gingival epithelium

Gene Title	Gene symbol
• S100 calcium binding protein A8/A9	S100A8/A9
• Keratin 6A	KRT6A
• Keratin 14	KRT14
• Keratin 13	KRT13
• Stratifin	SFN
• Cystatin A	CSTA
• Cystatin B	CSTB
• Aquaporin 3	AQP3
• Annexin A1	ANXA1
• Immunoglobulin heavy locus	IGH

Figure 1. LCM and differential and shared gene expression between tonsil and gingival epithelium. **A:** The epithelium area was outlined for both the tonsil surface epithelium and gingival epithelium and subsequently isolated by LCM. Sections are shown before, during, and after epithelium removal. **B:** Top 10 expressed genes (absolute intensity) in both tonsil and gingival epithelium.

and viral shedding[2,20,21] have been clearly documented within the tonsil epithelium.

To define further parameters of the tonsil epithelium that may influence its potential for HIV transmission, we isolated the epithelium by laser capture microdissection (LCM) for analysis of its gene expression profile relative to another oral site, the gingiva. The genes most highly expressed in the tonsil compared with gingiva were related to immune functions, such as antibody production and antigen presentation. Among the differentially expressed genes in the tonsil were also the viral co-receptor CXCR4 and factors with the potential to facilitate viral entrapment such as FcRIII, complement receptor 2, and complement components. Protein expression, as demonstrated by immunohistochemical staining of oral tissues, confirmed increased expression of CXCR4 in the tonsil epithelium but a decrease in expression of innate defense molecules, such as secretory leukocyte protease inhibitor (SLPI). This differential expression of factors that may influence HIV may render the tonsil more susceptible for oropharyngeal HIV transmission.

Materials and Methods

Specimen Collection

Human palatine tonsils were obtained from routine therapeutic tonsillectomies (sleep apnea and nontonsillitis) performed on otherwise healthy adults at the George Washington University Hospital with informed consent (institutional review board no. 099920). Gingival tissues were collected from healthy sites with probing depths <3 mm, with no clinical evidence of inflammation during routine therapeutic periodontal surgery at the Department of Periodontics, University of Maryland, with informed consent (institutional review board no. 1201211). Tissues were rinsed in sterile saline, divided into two pieces, and immediately either snap-frozen for the microarray studies or fixed in 4% paraformaldehyde for immunohistochemistry. Archived, formalin-fixed, oral epithelium samples (Department of Diagnostic Sciences and Pathology, University of Maryland) included intact, normal epithelium of the buccal mucosa and floor of mouth with a final diagnosis of a subepithelial pathosis, ie, fibroma and ranula (institutional review board no. H27950).

LCM

Five- to 10-μm frozen tissue serial sections were obtained using an RNase-treated blade at -20°C, mounted onto PEN foil slides (Leica Microsystems, Bannock Burn, IL) and stored at -80°C (maximum of 1 week). To minimize RNA degradation the tissues were thawed at room temperature (30 seconds), ethanol-fixed (30 seconds), stained with RNase-free Mayer's hematoxylin (2 minutes), diethylpyrocarbonate-treated water rinsed (5 seconds), eosin (20 seconds), and dehydrated in graded RNase-free alcohols (95 and 100%, 30 seconds each). Finally, slides were air-dried under a hood for 5 minutes and LCM performed immediately at $\times 10$ magnification, using a Leica AS LMD system (Leica Microsystems). Areas unequivocally identified as epithelium were outlined (Figure 1A), laser-dissected, and captured onto RNase-free 200-μl polymerase chain reaction (PCR) tube caps containing RNA lysis buffer (Qiagen Inc., Valencia, CA). Multiple sections containing epithelial cells were independently captured into individual tubes within 30 minutes from the time of tissue thaw and immediately placed on dry ice.

RNA Isolation, Quality Verification, Microarray, and Real-Time PCR

Total RNA was extracted from the microdissected samples using the Qiagen RNeasy micro kit (Qiagen), quantitated with a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE), and RNA integrity assessed using the 2100 Bioanalyzer (Agilent, Foster City, CA). Preparation of biotin-labeled cRNA, hybridization, and scanning were performed according to the manufacturer's two-cycle protocol (Affymetrix, Santa Clara, CA). In brief, 100 ng of total RNA per sample was used to gen-

erate double-stranded cDNA using a two-cycle cDNA synthesis kit and the oligo(dT)$_{24}$ primer (Affymetrix) containing a 3′ T7 RNA polymerase promoter site. Biotin-labeled cRNA probes were produced from cDNA using the IVT labeling kit (Affymetrix). The probes were purified, fragmented, and hybridized to Affymetrix Plus 2.0 microarray chips that display >47,000 transcripts for 16 hours. Chips were washed and stained using the Affymetrix Fluidics Station 450. Fluorescence intensity was measured using the Affymetrix GeneChip scanner and GeneChip Operating Software (GCOS; Affymetrix).

For RT-PCR, 1 μg of amplified cRNA was reverse-transcribed using oligodeoxythymidylic acid primer (Invitrogen Inc., Carlsbad, CA), and the resulting cDNA was amplified by real-time PCR on an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). Amplification was performed with TaqMan expression assays for GAPDH (assay ID: Hs9999905m1) and for CXCR4 (Hs00607978_s1).

Microarray Data Processing and Statistical Analysis

Affymetrix GCOS version 1.2 software was used to calculate signal and present call values that were stored in the NIH-LIMS, a database for storage and retrieval of chip data maintained at the National Institutes of Health. Data were statistically analyzed using the MSCL analyst's toolbox,[22] (available for download at *http://abs.cit.nih.gov/MSCLtoolbox/*), and the JMP statistical software package (SAS, Inc., Cary, NC). The results for 10 chips were retrieved, and the signal values were subjected to an adaptive variance-stabilizing, quantile-normalizing transformation termed "S10" (Munson, P.J., Gene Logic Workshop of Low Level Analysis of Affymetrix GeneChip Data, 2001). This transform both normalizes between chips over the full data range and makes the variance of replicates nearly uniform over expression level. A major advantage of this approach over the ordinary log-ratio is that changes in S10-transformed values have a uniform variance over the full expression scale. Visualization of the global results and detection of possible outliers among the 10 samples were facilitated by principal component analysis (data not shown) of the transformed data and presentation in bivariate plots of low-order principal components. Results from two separate types of tissues and from the five and seven independent donors were clearly separated in the first and second principal component and are uploaded in the Gene Expression Omnibus Repository (GEO accession no. GSE7224). No outlying chips were detected.

Selection of Significant Genes

To quantify the significance of gene expression differences, a one-way, two-level analysis of variance was applied comparing tonsil (n = 5) to gingival (n = 7) tissues. The P value for differences between the two tissues was collected for each probe set. To ameliorate the multiple comparison problems, the false discovery rate (FDR)[23] was controlled. Log-fold changes were computed as the difference between average values for the two groups. Probe sets with a greater than twofold change in either direction, with FDR less than 10% and with a present call in greater than 50% of samples in at least one group (four of seven and three of five) were selected for further analysis.

Immunohistochemistry and Image Analysis

Paraffin-embedded tissues (tonsil, n = 5; gingiva, n = 3; buccal mucosa, n = 3; and floor of mouth, n = 3) were cut into 5-μm sections, deparaffinized, and rehydrated, followed by heat-induced epitope retrieval. Methanol containing 3% hydrogen peroxide was used to block the endogenous peroxidase for 15 minutes. Sections were blocked with the corresponding preimmune serum for 30 minutes and incubated overnight at 4°C with primary antibodies to CXCR4, CCR5, CD19, CD3, defensin-β1, defensin-β4 (Abcam, Cambridge, MA), SLPI (R&D Systems, Minneapolis, MN), ICAM-3 (Novocastra, Newcastle, UK), CD4 (Invitrogen), and GalCer (Chemicon, Temecula, CA). After washing with phosphate-buffered saline three times, immunolabeling was detected using a biotinylated secondary antibody followed by visualization with an avidin-biotin horseradish peroxidase labeling kit (Invitrogen) and diaminobenzidine staining. Finally, the specimens were counterstained with Mayer's hematoxylin and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Negative controls were performed by replacing primary antibody with preimmune serum.

Snap-frozen tissues were sectioned (5 μm), fixed in 95% ethanol for 45 minutes, washed, incubated with 100 mmol/L glycine (MP Biomedicals, Illrich, France) for 10 minutes, and blocked with 5% bovine albumin (Sigma-Aldrich, St. Louis, MO) or with preimmune serum for 30 minutes before staining. Sections were incubated overnight with antibodies to CCR5, CXCR4, CD32, and pan-cytokeratin (Abcam) followed by visualization with an avidin-biotin horseradish peroxidase labeling kit (Invitrogen) or staining with secondary antibodies conjugated to Texas Red and fluorescein and mounted with Vectashield containing 4,6-diamidino-2-phenylindole for fluorescence microscopy (all from Vector Laboratories, Burlingame, CA).

Staining was evaluated by two independent investigators (N.M., N.N.). To evaluate levels of expression in each sample, sequential electronic images from 20× fields were taken for the length of the epithelium for an average of 20 images per tonsil sample and 10 images per oral sample (smaller biopsy). The images were evaluated for the presence, localization, and intensity of staining (weak, moderate, and strong) and percentage of positively stained cells. For the latter, epithelial cells in each epithelial compartment (basal, parabasal, spinal, and keratin) were evaluated and the positive cells counted. Means and SDs of percent positive cells were calculated per sample type and a Wilcoxon signed-rank test was used to compare expression between groups.

Results

Comparison of Gene Expression Profiles between Tonsil and Gingival Epithelium

The squamous epithelia lining the oropharyngeal area share functions necessary to protect underlying tissues from injury and invasion but also exhibit location-dependent unique characteristics associated with their specific functions. Accordingly, the epithelium of the gingiva is increasingly keratinized to withstand masticatory forces, whereas the tonsil epithelium is specialized to traffic antigens to the underlying lymphoid compartment. To differentiate potential features that may influence susceptibility to HIV, we isolated the respective epithelia by LCM (Figure 1A) and subjected them to microarray gene expression analysis. Multiple probe sets showed similar expression patterns between the two groups. Based on absolute expression intensities, many of the genes most highly expressed in both tonsil and gingival epithelium were common (Figure 1B). Among them were keratin genes (6A, 13, 14), the water channel protein aquaporin-3, the keratinocyte factor stratifin, cystatins A and B, and S100 binding Ca^{+2} binding proteins, all of which are associated with tissues of epithelial origin.[24–27] Combined with our selective dissection of the epithelium, these results confirm the epithelial cell specificity of the dissected specimens.

Further analysis of the variability of gene expression in the tonsil and gingival data set revealed a clear separation between the two sample groups, indicating that each tissue type has a distinct gene expression profile. Statistical analysis of the microarray data yielded 660 probe sets that were significantly differentially expressed between the two groups. These data demonstrate that surface epithelia from different locations exhibit both distinct and shared characteristics.

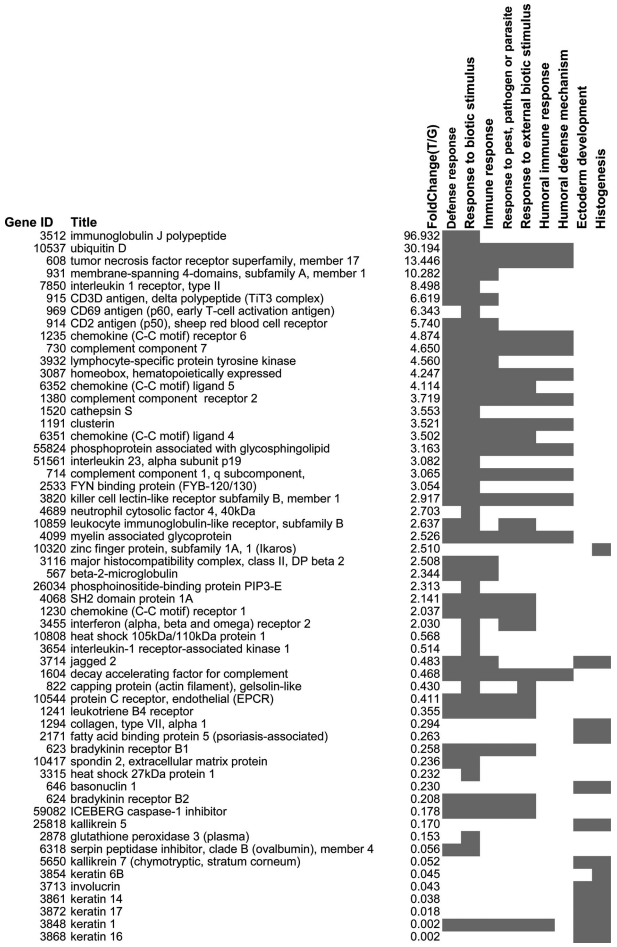


Figure 2. GO scan analysis of differential gene expression between tonsil and gingival epithelium. Affymetrix probe set IDs were linked to gene identifiers and functional Gene Ontology annotations using the Netaffx website at *http://www.affymetrix.com/analysis/index.affx*. Statistically differentially expressed genes were organized into functional categories using the Gene Ontology (GO) database (*http://www.geneontology.org*) and the GO-Significant Collections of Annotations (GOSCAN) program (*http://abs.cit.nih.gov/goscan/*). Ratios of tonsil to gingival epithelium gene expression is reported as tonsil/gingiva and colored areas indicate the functional category(ies) that each gene belongs to.

The Gene Expression Profile of the Tonsil Surface Epithelium Reflects Immunological Functions

We next focused on the significantly differentially expressed genes and their functions. With GO-scan analysis, a computerized process that links probe set annotations with their listed function, we categorized the differentially expressed genes by their known functions. As evident in Figure 2, in the tonsil the genes most highly expressed were categorized as defense response, response to biotic stimuli, immune response, and response to pathogens, consistent with an immunological role for this compartment. Among these were genes associated with antibody production such as the J chain of dimeric IgJ (up-regulated 96-fold) and the Ig κ light chain (29-fold), genes linked to major histocompatibility complex antigen presentation such as MHCII and cathepsin S[28] (2.5- to 3.5-fold), and genes related to the activation of the complement cascade (CR2, 4.6; C7, 3.7; and C1q, 3) (Figure 2). Consistent with this pattern, lymphocyte and particularly T- and B-cell-related genes were also pronounced in the tonsil epithelium as seen by the significant up-regulation of antibody components and B-cell markers (CD19, CD79) as well as T-cell markers (CD3, CD2, CD69) (Figures 2 and 3A) and by immunohistochemical staining for CD19 and CD3 (Figure 3, D and E; and Table 1).

In the gingiva the more highly expressed genes belonged to the categories of organ development and cytoskeleton (Figure 2). Genes of the keratin family dominated, including keratins 6, 14, 16, and 17, and cytokeratin 2 (up-regulated up to 500-fold), reflecting the increased potential for keratinization in the gingival sites, although the highest layers of keratin were not included in our dissection. Although the majority of highly expressed genes were not linked to immune functions, some genes encoded factors that may also participate in the immune response such as the kallikrein proteases 5, 6, and 7 (5- to 20-fold), which may be involved in matrix reorganization during inflammation,[29] and spondin 2 (fivefold), a

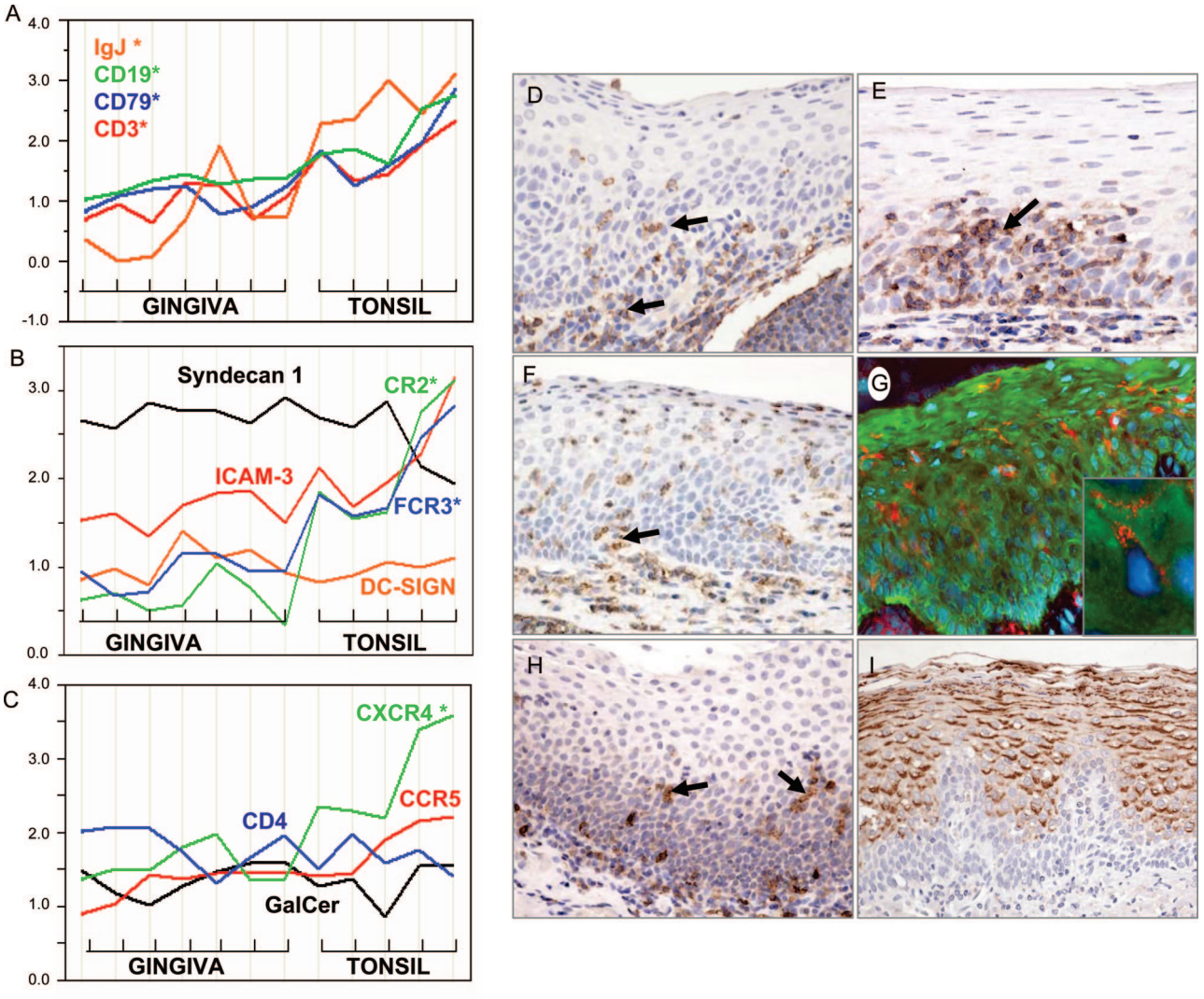


Figure 3. Differential expression of HIV-related genes. **A:** Parallel plot showing gene expression intensity for B-cell markers (CD19, CD79), antibody-related genes (Ig J polypeptide), and T-cell marker (CD3) in gingival ($n = 7$) and tonsil ($n = 5$) samples. **B:** Parallel plot showing expression levels/intensity for genes associated with HIV entrapment/transmission in the tonsil ($n = 5$) and gingival ($n = 7$) epithelium. **C:** Parallel plot showing gene expression intensity for the HIV receptor CD4 and co-receptors (GalCer, CXCR4, and CCR5) in the tonsil and gingival epithelium, *FDR $< 10\%$ and $P < 0.05$ of differential gene expression between sample groups. Immunohistochemical staining for CD19 in the tonsil (**D**) and CD3 (**E**). Immunohistochemical staining for ICAM-3 (**F**), for CD4 (**H**), and GalCer (**I**) in the tonsil epithelium. Immunofluorescence FcR-CD32 (red), pan-cytokeratin (green) and 4,6-diamidino-2-phenylindole (**G**). **Arrows** indicate positive staining. Original magnifications: ×20 (**D–F**, **H**, and **I**); ×63 (**G**).

pattern recognition molecule for microbial pathogens that participates in innate defenses.[30]

Gene Expression Profile of the Tonsil Includes HIV Trafficking Molecules

We next investigated whether the differential gene expression patterns between the two tissue types might influence HIV susceptibility. In this regard, the increased keratinization of the gingival tissue, reflected by enhanced regulation of keratin genes, may contribute to a barrier against HIV. Conversely, an increased presence of immune cells in the tonsil epithelium may favor HIV entry and access to target cells.[26] Within the differentially overexpressed tonsil genes were Fcγ receptor III,[31] complement receptor CR2, and various complement components[26] (FDR <10%). Other key HIV-binding molecules such as the adhesion molecule ICAM-3,[32] the dendritic cell-specific C-type lectin DC-SIGN,[33] and syndecan-1[34] were not significantly differentially expressed (Figure 3B). Gene expression for the viral co-receptor CXCR4, al-

Table 1. Semiquantitative Evaluation for Immunological Markers

Markers	Tonsil epithelium	Oral epithelium
CD19	+	−
CD3	+	−
ICAM-3	+	−
FcR	+	−
CD4	+	−
GalCer	++	++

Semiquantitative analysis of immunohistochemical staining. Symbols shown represent percent positive staining; −, <5%; +, 5 to 25%; ++, 25 to 50%; +++, 50 to 75%; ++++, 75 to 100%.

though variable (2- to 10-fold difference in expression between donors, as seen by microarray and RT-PCR analysis data not shown), was significantly higher in the tonsil. CCR5 and galactosylceramide (GalCer),[35] as well as the primary HIV receptor CD4, were not significantly differentially expressed (Figure 3C). By immunohistochemical staining, ICAM-3 and FcR (CD32) were detected predominantly in the tonsil epithelium (Table 1). Importantly, expression of FcR (CD32) was not exclusive to the infiltrating immune cells but was also present on epithelial cells (Figure 3G). Consistent with the gene expression data, immunohistochemistry demonstrated that GalCer was similarly expressed in tonsil and oral sites (25 to 50%, Table 1), whereas detection of CD4 was low in both areas (11.5 ± 5.7% in the tonsil epithelium and 3.9 ± 0.9% in the oral epithelia) (Figure 3, E–G; Table 1).

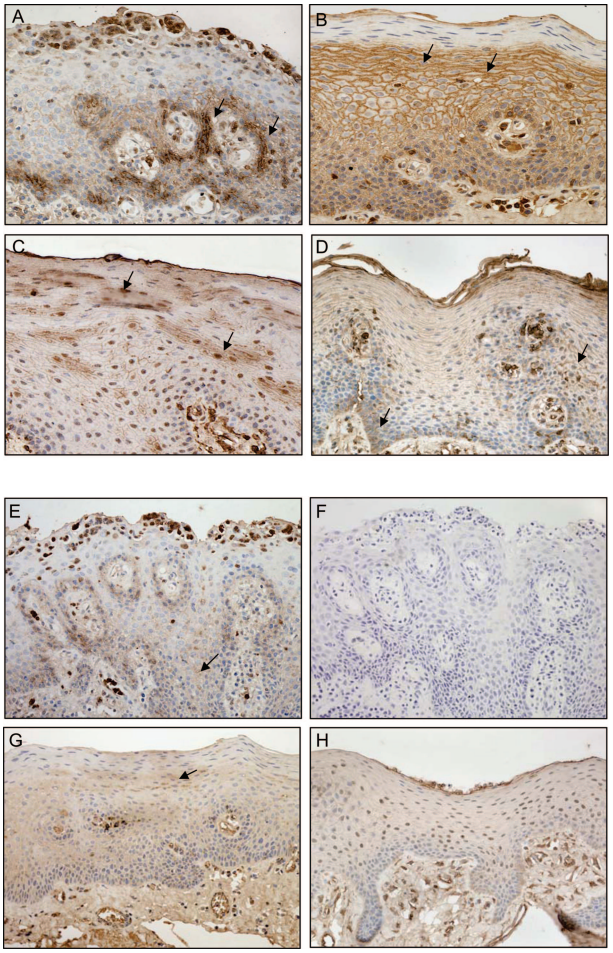


Figure 4. CXCR4 and CCR5 protein expression in the tonsil and oral epithelia. **A** and **B:** Immunohistochemical staining for CXCR4 in the tonsil epithelium. Staining of high intensity (grade 3) is seen in the basal and parabasal cell layers (**A**) and in the spinous layer of select samples (**B**, **arrows** indicate staining of high intensity in **A** and **B**). Weak to moderate CXCR4 staining is also seen in the oral tissues keratinized (**C**) and nonkeratinized (**D**, **arrows** indicate staining of moderate intensity in **C** and **D**). **E:** Immunohistochemical staining for CCR5 in the tonsil epithelium (**E** and **F**, negative control) and in the parakeratinized (**G**) and nonkeratinized (**H**) oral epithelium. Staining is moderate (shown by **arrows**) and weak in most areas. Original magnifications: ×20 (**A–D**, and **F**); ×10 (**E**, **G**, and **H**).

Expression of the HIV Co-Receptors CXCR4 and CCR5 in the Tonsil and Oral Epithelia

Next, we investigated protein expression of the co-receptor CXCR4, which was found to be significant in the tonsil compared with the keratinized gingival epithelium by gene expression analysis. Quantification of staining revealed abundant CXCR4 staining in the majority of fields examined for both the tonsil and oral epithelium (Figure 4, A–D; Table 2, 49 to 65%), but with significantly increased staining intensity in the tonsil. Although most CXCR4-positive cells stained moderately in the tonsil (intensity 2, 81.4%), in the oral epithelia, the majority of cells stained weakly (intensity 1, 82.5 and 75.3%; $P < 0.05$). Strongly stained cells were also present in the tonsil (intensity 3, 15%) but not represented in the oral sites. Regarding CXCR4 localization, the area most frequently stained in the tonsil epithelium was the parabasal layer (85.2%), followed by the basal layer (54.4%) and spinous cell layer (50%, Table 2). In the oral mucosa, staining was more evenly distributed throughout the epithelial layers, with no significant differences between keratinized and nonkeratinized oral epithelia (Table 2). In the case of CCR5, in both oral and tonsil epithelia, staining was weak to moderate (Figure 4, E–H) and the percentage of positive cells was comparable between tissues (Table 2, 49 to 55%). This staining pattern was also demonstrated in frozen tissues (data not shown). CCR5 staining was most evident in the squamous layer, distinct from CXCR4.

Increased Production of SLPI in the Oral Epithelia

To explore further the resistance and susceptibility factors unique to the tonsil epithelium, we studied the expression of several anti-HIV factors that may influence permissiveness, such as SLPI,[36] defensins,[7] lysozyme, and thrombospondin.[4] Interestingly, SLPI, defensins, and thrombospondin were expressed at a lower level in the tonsil epithelium (1.5- to 3-fold), although only SLPI was significantly different between tonsil and gingiva ($P < 0.05$). By comparison, lysozyme, a broad-range anti-viral factor with limited evidence of direct HIV activity,[37] was highly expressed in the tonsil ($P < 0.05$, Figure 5A). Immunohistochemical studies for SLPI revealed a striking difference of expression between tissues (Figure 5B). In the tonsil surface epithelium, SLPI staining intensity was either undetected or weak whereas the detected staining was scarce (10% positive cells, Figure 5, C and D, negative control). On the contrary, all of the oral epithelial sites studied were heavily stained for SLPI, which was expressed by all epithelial layers, with the exception of the keratinized layers (Figure 5, E and F). Studies for defensin-β1 and defensin-β4 expression (which were both expressed above the level of detection in our microarray analyses, present call >50%), indicated that these molecules, unlike SLPI, were not differentially expressed between tissue types (defensin-β1: 36.6 ± 11.7% in tonsil and 31.8 ± 10.3% in oral mucosa; defensin-β4: 28.2 ± 21.4% in tonsil and 29.15 ± 15.6% in oral

Table 2. Quantitation of CXCR4 and CCR5 Staining

	Tonsil, mean % (±SD)	Oral nonkeratinized, mean % (±SD)	Oral keratinized, mean % (±SD)
CXCR4			
Total positive cells	65.3 (32.3)	52.6 (16.6)	49.5 (19.6)
Basal layer	54.4 (34.4)	38 (19.5)	43.7 (22.6)
Parabasal	85.2 (24.7)	50 (16.4)	52.7 (23.3)
Spinous	50 (35.2)	64 (15)	50.3 (14.6)
Keratinized	N/A	N/A	13.2 (3.4)
Total intensity score			
Intensity 1	2.6 (5.6)	82.5 (23.6)	75.3 (15.5)
Intensity 2	81.4* (19.4)	17.5 (23.6)	25.5 (13.3)
Intensity 3	15.6* (19.3)	0 (0)	0 (0)
Cases (*n*)	5	6	3
Total fields counted	120	60	20
CCR5			
Total positive cells	49.2 (15.4)	48.9 (24.4)	55.8 (14.7)
Basal layer	35.5 (15.6)	32.5 (12.3)	41.4 (15.5)
Parabasal	30.2 (12)	20.5 (10.3)	44 (13.7)
Spinous	70 (30.1)	85 (23.4)	75.3 (15.6)
Keratinized	N/A	N/A	5.3 (2.2)
Total intensity score			
Intensity 1	60.6 (13.5)	70.2 (15.5)	65.3 (14.5)
Intensity 2	23.4 (10.5)	35.6 (12.4)	33.2 (11.2)
Intensity 3	0 (0)	0 (0)	0 (0)
Cases (*n*)	5	6	3
Fields counted	120	60	20

N/A, not available.

Total percentage reflects the percent positive cells in all layers, all other percentages are per layer (mean percent positive cells and SD). Intensity grades: 3, strong; 2, moderate; 1, weak, reported for all layers of the epithelium as mean ± SD.

*$P < 0.05$.

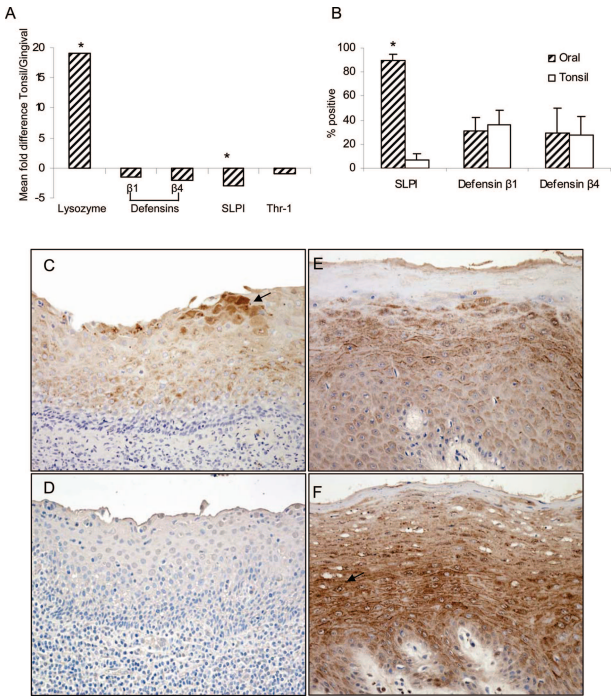


Figure 5. SLPI expression in the tonsil and oral epithelia. **A:** Mean fold change of gene expression between tonsil and gingiva for the innate immune factors lysozyme, defensin-β1, defensin-β4, SLPI, and thrombospondin-1. *$P \leq 0.05$. **B:** Quantitation of percent positive SLPI, defensin-β1, and defensin-β4 cells in the tonsil ($n = 5$) and oral epithelium ($n = 5$). *$P \leq 0.05$. **C:** SLPI staining in the tonsil is weak (grade 1) with isolated cells staining moderately (grade 2, **arrow** showing higher intensity staining). **D:** Negative control. In the oral mucosa, SLPI staining is abundant and of high intensity (grade 2 to 3) for both keratinized (**E**) and nonkeratinized (**F**) oral epithelia (**arrow** shows high-intensity staining). Original magnifications: ×20 (**D–F**).

mucosa; Figure 5B). This differential presence of SLPI and or other innate antiviral molecules may contribute to the relative susceptibility and resistance patterns associated with each mucosal epithelial target.

Discussion

The exact cellular and molecular events involved in the initial viral transmission continue to be dissected,[38,39] because information regarding interactions of HIV or HIV-infected cells with epithelial surfaces of the human mucosa comes mostly from *in vitro* experimentation. Our data, derived from gene expression analyses and immunohistochemical studies, demonstrate an increased expression of factors that may foster a receptive environment in the tonsil epithelium compared with other oropharyngeal epithelial sites. The gene expression profile of the tonsil reflects an increased representation of immune cells in this compartment with decreased keratinization compared with the gingiva, making this area potentially more permeable to insulting agents and accessible for antigen sampling. While confirming these recognized features of the tonsil epithelium, we also identified an increased expression of molecules that could conceivably promote HIV binding and entry in this site, such as the FcγRIII, complement receptor 2, complement components, and the co-receptor CXCR4, all of which have previously been linked with HIV and could be expressed by the epithelium or the infiltrating lymphocytes and dendritic cells.[6,26,33] This together with a reduction in innate antiviral mediators, including SLPI, may encour-

age viral-tonsil interactions. To our knowledge, this is the first comparison of differential expression of pro- and anti-HIV molecules within distinct oropharyngeal sites.

In an *ex vivo* model, extensive binding of HIV virions or infected cells by tonsillar epithelium has been documented.[3] Although the molecules responsible for viral capture were not identified in these studies, a number of candidates emerge from our microarray analysis and include FcγRIII, complement components, and complement receptor 2 (CR2). After entrapment, actual viral entry requires fusion of the viral glycoproteins with the cell membrane through CD4 or possibly the alternate epithelial receptor GalCer and co-receptors CXCR4 and CCR5,[40,41] all of which were previously found to be present in the tonsil epithelium of pediatric tonsillitis patients (age 3 to 20 years).[42] In our population of adults with hyperplastic, noninfected tonsils, we document a similar distribution of CD4, GalCer, CXCR4, and CCR5 in the tonsil epithelium and further we compare their expression with that in other oral sites. We document an increased expression of the CXCR4 co-receptor in the tonsil compared with the oral epithelial sites. In the tonsil CXCR4 may synergize with CD4 or GalCer[43,44] to allow for X4 transmission, and/or viral entrapment together with other binding molecules that may facilitate infection of available CCR5 targets. Finally, CXCR4 is expressed in all layers of the epithelium and highest in the parabasal and spinous cell layers, consistent with previous reports in the tonsil[42] and with localization of this co-receptor in other HIV susceptible epithelial sites, including colon[45] and ectocervix.[30]

Gene expression analysis for innate immune molecules with anti-HIV potential such as SLPI,[5,36] β-defensins,[7] and thrombospondin[4] showed a trend for decreased expression in the tonsil epithelium. Of the molecules examined, only lysozyme, a broad-range antimicrobial factor with limited evidence of direct HIV targeting[37] was significantly up-regulated in the tonsil. SLPI, a protein with demonstrated anti-HIV activity *in vivo* and *in vitro*[46] was minimally expressed in the tonsil while abundant in the oral epithelia. SLPI stained with high intensity all layers of the various oral epithelia, with the exception of the highly differentiated keratin layer,[47] which in itself may act as a protective barrier against HIV. In these tissues the presence of a keratin/parakeratin layer that does not express SLPI, may also contribute to lesser differential gene expression. Originally identified as a serine protease inhibitor,[48] SLPI is a 12-kd protein found in mucosal secretions, including saliva, breast milk, seminal fluid, and other mucosal secretions, which originates from acinar cells of submucosal glands and from epithelial cells lining mucosal surfaces where it contributes to innate host defense.[19,49] It may also function as a potent anti-inflammatory mediator in the context of wound healing and infection, exerting its action within tissues.[50] The mechanism through which SLPI exerts its anti-viral action was previously shown to involve a host cell molecule and not to target the virus itself and to occur early in the viral life cycle.[5,6]

Finally, the architectural uniqueness of the crypt epithelium probably also contributes to HIV transmission vulnerability in the tonsil. In the area of the crypt, the epithelium is thinner and has multiple breaks that may provide portals of entry for the virus while creating a less accessible environment to the anti-viral components of saliva, including SLPI.[2,20] These anatomical features may synergize with the cellular composition, as well as the gene and protein expression profile in the tonsil epithelium to influence accessibility to HIV. Continued elucidation of candidate tonsil epithelium-specific factors relevant to HIV susceptibility will further our understanding of vulnerability and resistance in this site.

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