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## ORIGINAL ARTICLE

# Chemokine receptor expression in HIV-positive persons with oropharyngeal candidiasis

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**OBJECTIVE: In HIV+ persons with reduced CD4$^+$ T cells, oropharyngeal candidiasis (OPC) is often associated with the accumulation of CD8$^+$ T cells at the epithelial/lamina propria interface within the lesion together with increased tissue-associated cytokines and chemokines. Despite this reactivity, a dysfunction in the ability of the CD8$^+$ cells to reach the organism at the outer epithelium is postulated. The purpose of this study was to examine chemokine receptors present in the OPC lesions for a potential role in susceptibility to infection.**

**METHODS: Biopsies taken from buccal mucosa of HIV− persons, healthy mucosa of HIV+ OPC− persons, and OPC lesions were processed for protein immunohistochemical staining or RNA analysis by real-time PCR and Superarray.**

**RESULTS: There was little change in expression of chemokine receptors at the protein or RNA level between OPC+ and OPC− tissue. At the protein level, increases occurred in OPC+ persons only if associated with CD8 cells. In the Superarray, of the 22 chemokine receptor mRNAs expressed, c. 90% remained unchanged (<1.0-fold change) between HIV− and HIV+ tissue and between HIV+ OPC− and HIV+ OPC+ tissue.**

**CONCLUSION: Tissue-associated chemokine receptor expression does not appear to contribute to the dysfunction in cellular migration associated with susceptibility to OPC.**

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**Keywords:** HIV; oropharyngeal candidiasis; chemokine receptors

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## Introduction

Oropharyngeal candidiasis (OPC), caused by *Candida albicans*, is the most common oral infection in HIV+ persons (Klein *et al*, 1984; Macher, 1988; Greenspan *et al*, 1992; Laskaris *et al*, 1992). *Candida albicans* is a commensal organism normally found in the gastrointestinal and reproductive tracts (Calderone, 2002). Most people are exposed to *C. albicans* early in life, which results in protective *Candida*-specific immunity. Under immunocompromised conditions, however, *C. albicans* can convert from commensal to pathogen and cause symptomatic disease (Knight and Fletcher, 1971; Clift, 1984; Klein *et al*, 1984; Macher, 1988; Fichtenbaum *et al*, 2000; Calderone, 2002). OPC presents as red erythematous patches or white curdlike lesions (thrush), both of which occur on the hard and soft palate, buccal mucosa (BM), tongue, and floor of the mouth (Dodd *et al*, 1991). OPC occurs most frequently in HIV+ persons when blood CD4$^+$ T cells are reduced below 200 cells $\mu l^{-1}$. Infections can be acute or recurrent.

Cell-mediated immunity by Th1-type CD4$^+$ T cells is considered the predominant host defense mechanism against mucosal *C. albicans* infections (Klein *et al*, 1984; Greenspan *et al*, 1992; Leigh *et al*, 2001). However, a recent study that attempted to identify a deficiency in *Candida*-specific systemic T cell responses in subjects with OPC found little to no deficiency of Th1-type responsiveness compared to those without OPC (Leigh *et al*, 2001). Instead, it appears that a threshold number of CD4$^+$ T cells (*c*. 200 cells $\mu l^{-1}$) are required to protect the oral mucosa. Below this threshold local or other systemic immune mechanisms are required for protection.

At the local level, oral epithelial cell anti-*Candida* activity was identified and also found to be reduced in subjects with OPC, indicating a potential innate protective mechanism against OPC (Steele *et al*, 2000). In contrast, *Candida*-specific antibodies in saliva were comparable in subjects with and without OPC, supporting the lack of a role for humoral immunity in susceptibility to OPC (Wozniak *et al*, 2002). But the most intriguing observation made was the presence and distinct

accumulation of $CD8^+$ T cells at the epithelium–lamina propria interface, a considerable distance from *Candida* at the outer epithelium, in those with OPC and reduced $CD4^+$ T cell numbers (Myers *et al*, 2003). This was accompanied by increased oral tissue-associated chemokines, RANTES, MCP-1, and IP-10, in OPC+ persons when compared with OPC− persons as well as several cytokines, including CD8 cell-associated cytokines (IL-2, IL-15) (Lilly *et al*, 2004). These results suggested some role for $CD8^+$ T cells against *C. albicans* at the oral mucosa with susceptibility to OPC involving a putative dysfunction in their effector function and/or their ability to migrate toward the organism. Indeed the most recent studies show a putative dysfunction in the microenvironment whereby the adhesion molecule that allows lymphocytes to migrate through mucosa (E-cadherin) is reduced in those with OPC (McNulty *et al*, 2005).

The purpose of the present study was to examine chemokine receptor expression in OPC lesions, including those ligands for RANTES, MCP-1, and IP-10, at the protein level by immunohistochemistry and at the mRNA level by real-time PCR and Superarray to evaluate their role, if any, in susceptibility to infection.

## Materials and methods

*Subjects*

Subjects were recruited and evaluated at the Louisiana State University Health Sciences Center (LSUHSC) HIV Outpatient Clinic associated with the HIV Outpatient Program (HOP) of the Medical Center of Louisiana at New Orleans and the Charity Hospital Dental Clinic. Informed consent was obtained from all patients and all procedures were followed in the conduct of clinical research in accordance with the Institutional Review Board at Louisiana State University Health Sciences Center. Subjects were part of an established cohort ($n = 473$) consisting of HIV-uninfected persons and HIV-infected persons with and without OPC. A subset of this cohort was chosen for this analysis, including HIV− ($n = 3$) and HIV+ OPC− ($n = 25$) persons, both positive for oral asymptomatic *Candida* colonization (described below), and HIV+ OPC+ ($n = 25$) persons. Of these, 16 HIV+ OPC− and 19 HIV+ OPC+ individuals had $<200$ CD4 cells $\mu l^{-1}$. Consistent with our previous observations (Slavinsky *et al*, 2002), a high percentage of those with OPC were smokers (48%). Sixty-eight percent of HIV+ persons in the subset were receiving highly active antiretroviral therapy (HAART). HAART was defined as receiving three or more antiretroviral drugs, while monotherapy or dual therapy without a protease inhibitor was classified as non-HAART.

*Diagnosis of oropharyngeal candidiasis and detection of asymptomatic oral yeast colonization*

The diagnosis of OPC was made on the clinical appearance of white curd-like plaques (pseudomembranous) and/or red, atrophic areas (erythematous) in the oral cavity. Oral swabs from both infected (diseased) and uninfected (clinically healthy) BM were plated on Sabouraud-dextrose agar (SAB; Becton Dickinson Biosciences, San Jose, CA, USA) and Chromagar (CHROMagar, Paris, France) and incubated for 48 h at 34 and 37°C, respectively. Diagnosis of OPC was confirmed by hyphae present on a smear made from the swab with potassium hydroxide (KOH) and a positive swab culture with characteristic colony morphology. Asymptomatic colonization was assigned based on the presence of colonies following plate culture, but without signs of disease or positive KOH smear. Initial speciation was assigned according to colony color on Chromagar. Green colonies were processed for germ tube formation (incubation in fetal bovine serum for 2 h at 37°C), with those forming germ tubes classified as *C. albicans*. Non-green colonies were speciated using API biochemical tests (API 20 AUX: BioMerieux, Hazelwood, MO, USA).

*Sample collection and processing*

*Blood*

Venous blood (10 ml) was collected from each subject. HIV status was verified in serum by enzyme-linked immunosorbent assay, followed by Western blot through the Clinical Immunology Laboratory at the LSU Health Sciences Center. CD4 lymphocyte counts were quantified by flow cytometry. Peripheral blood lymphocytes (PBL) from healthy individuals were isolated by Ficoll Paque (Amersham Biosciences, Piscataway, NJ, USA), adjusted to $4 \times 10^6$ cells $\mu l^{-1}$, and incubated with Phytohemagglutinin (PHA) ($1000 \mu g\ ml^{-1}$) for 24 h at 37°C in AIM-V Medium (GIBCO, Grand Island, NY, USA). The cells were harvested and RNA extracted for use in a standard curve for real-time PCR.

*Biopsy*

Elliptical biopsies were taken from the BM of HIV− and HIV+ OPC− individuals and from infected (diseased) sites of HIV+ OPC+ persons. All OPC+ persons evaluated had pseudomembranous candidiasis with *C. albicans* identified as the causative agent in the lesion. For immunohistochemical analysis, excised tissue was oriented for cross-sectional analysis and snap-frozen in Tissue-Tek cryomolds (Miles Corp, Elkhart, IN, USA) using optimal cutting temperature medium (Sakura Finetek USA, Inc., Torrance, CA, USA) and stored at −80°C. For RNA extraction, tissue was placed in 4 ml of Hanks Balanced Salt Solution (GIBCO) for transport. Once in the laboratory, frozen tissue was sectioned (5 $\mu$m), collected on glass slides, fixed in acetone (5 min), and stored at −20°C for immunohistochemical analysis. For RNA extraction, tissue was homogenized in 500 $\mu$l of Ultraspec RNA Isolation Reagent (Biotecx Laboratories, Houston, TX, USA). RNA extracted from tissue and cultured PBL was stored in frozen isopropanol until precipitated and resuspended in nuclease-free $H_2O$ for cDNA synthesis.

*Immunohistochemistry*

*Hematoxylin and eosin*

Buccal mucosa tissue sections were stained with hematoxylin and eosin (Biochemical Sciences, Swedesboro,

NJ, USA) according to manufacturer's instructions, in order to confirm tissue orientation.

*Chromophore staining of cell surface antigen*
Immunohistochemical staining of BM using chromogen has been previously described (Myers *et al*, 2003). Briefly, all steps were performed at 4°C using Anti-Mouse Cell and Tissue Staining Kit, HRP-AEC (R&D Systems, Minneapolis, MN, USA). Serial sections were warmed 5 min at room temperature, re-hydrated in phosphate-buffered saline, blocked with 3% hydrogen peroxide, mouse serum, avidin, and biotin, then incubated overnight with primary antibodies (CD8, CCR2, CCR5, CXCR3) (1–10 $\mu$g ml$^{-1}$). Treated slides were washed and incubated with anti-mouse (R & D Systems, Minneapolis, MN, USA) biotinylated IgG secondary antibody (5 $\mu$g ml$^{-1}$) for 1 h. Washed slides were then incubated for 30 minutes with high sensitivity streptavidin–horseradish peroxidase conjugate (HSP-HRP) (R & D Systems), washed, and incubated with the substrate 3-amino-9-ethylcarbazole (AEC) chromogen (R & D Systems) for 10 min. Mayer's hematoxylin (Fisher Diagnostics, Fair Lawn, NJ, USA) was used to counterstain. Slides were preserved by using Aqueous Mounting Medium solution (R&D Systems).

*Real-time PCR*
Real-time PCR was performed as described previously (Lilly *et al*, 2004) using HIV+ OPC− and HIV+ OPC+ tissue mRNA. Predeveloped primers (for amplification) and probes (for detection of product) (Applied Biosystems, Foster City, CA, USA) for each chemokine receptor (CCR2, CCR3, CCR5) were combined with TaqMan Universal PCR Master Mix (Applied Biosystems) for each reaction. cDNA was synthesized from total RNA using the TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR was performed in the iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Two fluorescent probes, FAM-490 (target gene) and VIC-530 (housekeeping gene), were detected for each cDNA sample. PCR product was measured in consecutive cycles (real time) through a total of 40 cycles during each reaction using the iCycler iQ Multi-Color Real-Time PCR Detection System Software. Human cyclophilin was used as the housekeeping gene. Unknown cDNA samples were quantified using the standard curve generated from PHA-stimulated PBL and normalized to cyclophilin cDNA. cDNA from unstimulated PBL of a healthy volunteer was also quantified according to the standard curve and used as a calibrator to control for interassay variability. Finally, a ratio of the unknown sample to the calibrator was computed and data for each chemokine receptor was expressed as a relative amount.

*Superarray*
cDNA synthesized from biopsy mRNA was labeled with a [$^{32}$P]-dCTP probe during a linear polymerase reaction using the Ampolabeling LPR kit (Superarray Bioscience Corp, Frederick, MD, USA) The [$^{32}$P]-labeled cDNA was then hybridized overnight onto a GEArray Q Series membrane from Superarray Bioscience Corporation consisting of genes for 25 chemokine receptors and 71 other receptors and ligands. The membranes were exposed on a storage phosphor screen for 24 h and scanned using a Typhoon Trio phosphorimager (Amersham Biosciences). Using ImageQuant TL analysis software (Amersham Biosciences), all raw densitometric signals were corrected by subtracting out the background of the negative controls (blanks). All densitometric values were normalized to the housekeeping gene, cyclophilin. A ratio of experimental to control groups (HIV− to HIV+ OPC−, HIV+ OPC− to HIV+ OPC+) was used to determine specific changes for each receptor.

*Statistics*
For real-time PCR, differences in relative chemokine receptor expression between each patient group were determined by the Mann–Whitney *U*-test. All statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

## Results

*Chemokine receptors in OPC lesions*
Biopsy tissue sections of lesions from OPC+ persons ($n = 11$) and healthy tissue from OPC− persons ($n = 13$) were chromogen stained for CD8 and the chemokine receptors CCR2, CCR5, and CXCR3. Representative images are shown in Figure 1. Both CCR2 (frame A) and CCR5 (frame B) were not CD8 cell-associated (staining not matched to CD8) and were equally present in OPC− and OPC+ tissue, whereas CXCR3 that was CD8 cell-associated CXCR3 (frame C) (matched to CD8) was increased as CD8$^+$ T cells were increased in the lesion.

As another measure of chemokine receptor expression, tissue from OPC− ($n = 15$) and OPC+ ($n = 17$) persons was evaluated for chemokine receptor mRNA expression. Results in Figure 2 show that CCR2 (frame A), CCR3 (frame B), and CCR5 (frame C) mRNA was detected in tissue of most HIV+ persons, but no differences were detected between those with and without OPC.

In addition, chemokine receptor mRNA expression was detected by Superarray. Figure 3a shows a representative image of an array membrane. To initially determine the effect of HIV, receptor mRNA expression in HIV− persons ($n = 3$) was compared with that from HIV+ OPC− individuals ($n = 10$). Results showed that mRNA for 22 of the possible 25 chemokine receptors was expressed in both HIV− and HIV+ persons. Of the 22 chemokine receptor mRNAs expressed, 41% were increased, 42% were decreased, and 17% were unchanged. Of those with changes, 67% were $<0.5$-fold difference, and only two mRNAs showed $>1.0$-fold difference (CCR2, 1.5-fold increase; IL-8R$\beta$, 2.1-fold increase). Figure 3b shows the median fold change (0.69), 25th and 75th quartiles, range (0–1.5) and outliers. In OPC+ persons ($n = 10$), 18% of receptor mRNAs were increased, 36% were decreased and 46%

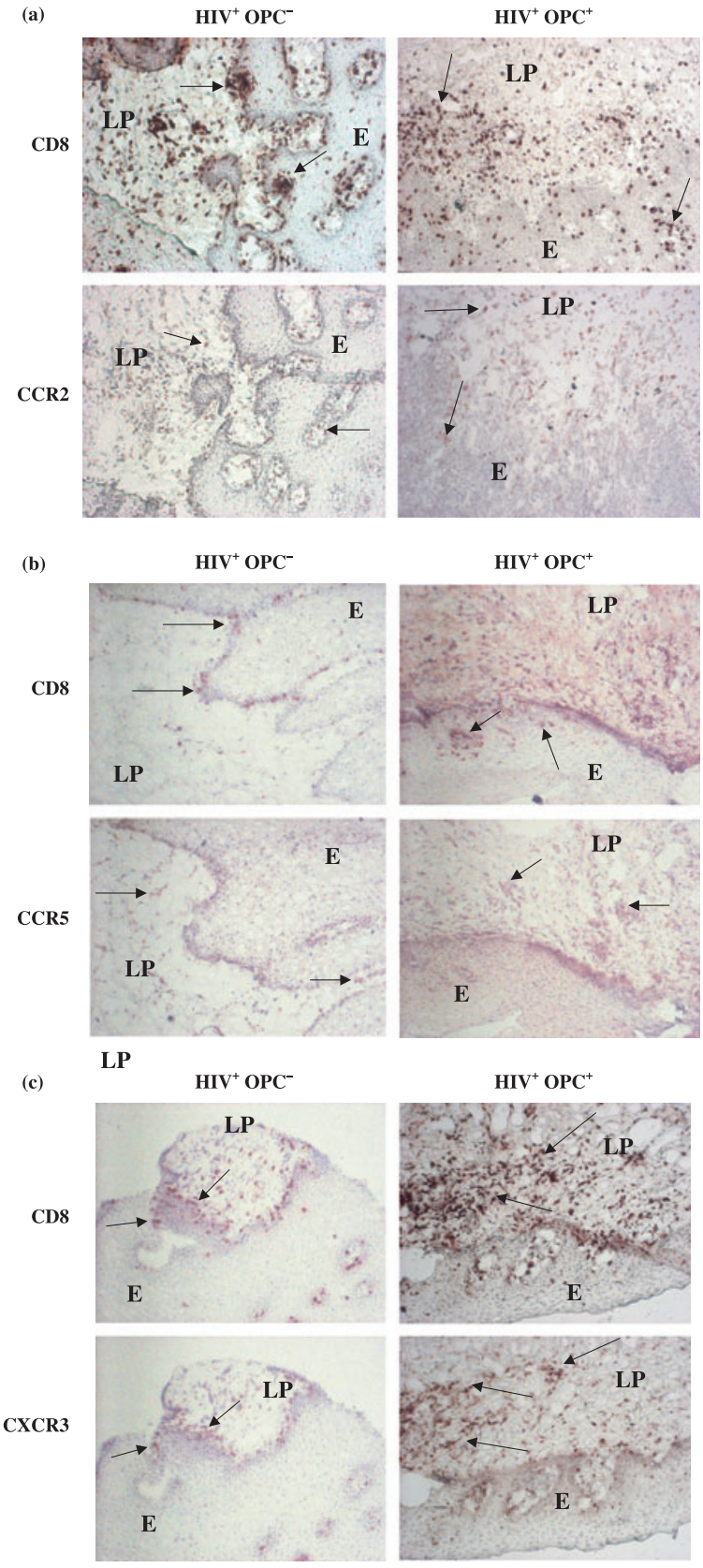


**Figure 1** Chemokine receptors in OPC lesions. Representative tissue images from HIV+ OPC− ($n = 13$) and HIV+ OPC+ ($n = 11$) persons are shown for (**a**) CCR2, (**b**) CCR5, and (**c**) CXCR3 staining compared to CD8 cell staining. Arrows denote representative positively stained cells revealing differences (**a** and **b**) or similarities (**c**) between the receptor and CD8 cells. Magnification ×10. LP, lamina propria; E, epithelium

showed no change compared with tissue from OPC− persons. Of those with changes, 75% were $<0.5$-fold difference, and only one receptor mRNA showed $>1.0$-fold difference (CCXCR1, 1.2-fold increase). Figure 3c shows the median fold change (0.48), quartiles, and range (0–1.2).

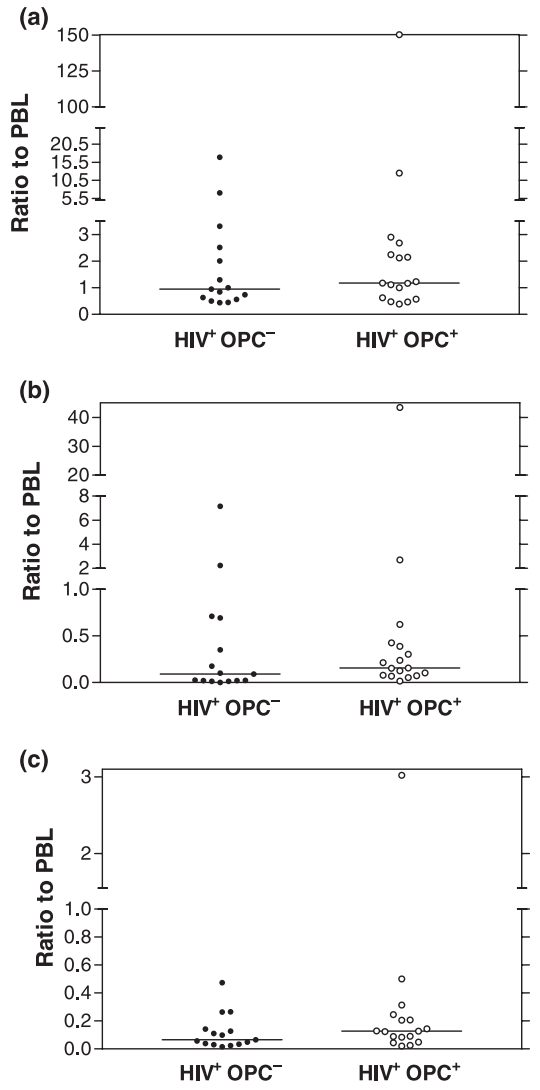


**Figure 2** Tissue-associated chemokine receptor mRNA expression in OPC lesions detected by real-time PCR. Chemokine receptor mRNA expression was evaluated in HIV+ OPC− ($n = 15$) and HIV+ OPC+ ($n = 17$) persons. Results are expressed as a ratio to unstimulated peripheral blood lymphocytes with medians illustrated. (**a**) CCR2, (**b**) CCR3, and (**c**) CCR5

## Discussion

Besides reduced $CD4^+$ T cells, the factors associated with susceptibility to OPC in HIV+ persons remain elusive, although a recent focus on $CD8^+$ T cells and epithelial cells have shed some light. To this end, a protective role for oral epithelial cells has been suggested by the reduced epithelial cell anti-*Candida* activity in those with OPC (Steele *et al*, 2000). For $CD8^+$ T cells, their increased presence in OPC lesions is suggestive of a putative response to infection, although their accumulation at a distance from *C. albicans* at the outer epithelium indicates a potential factor in susceptibility to the infection (Myers *et al*, 2003). Indeed, we recently identified a putative dysfunction in the microenvironment of the lesion in which the adhesion molecule that allows lymphocytes to migrate through the mucosa, E-cadherin, is reduced in those with OPC (McNulty *et al*, 2005).

The accumulation of $CD8^+$ T cells in the lesion is also accompanied by an increase in cytokine and chemokine mRNA as additional evidence of a response toward the infection. The increased chemokine mRNA expression prompted the analysis of chemokine receptor expression. We chose commercially available reagents for chemokine receptors that were CD8 or T cell-associated, and ligands for the chemokines shown to be increased (RANTES, IP-10, and MCP-1) in OPC+ tissue. Although reagents were limiting to some degree, the Superarray enabled the detection of 25 chemokine receptors.

On protein detection, CXCR3 (receptor for IP-10), known to be expressed on effector T cells such as cytotoxic lymphocytes (Moser *et al*, 2003), was cell-associated and paralleled the accumulation of $CD8^+$ cells at the lamina propria-epithelial interface in OPC lesions as well as the increase in IP-10 mRNA in the tissue (Lilly *et al*, 2004). Thus, there was no evidence for any deficiency in CXCR3 during OPC that would affect susceptibility to infection. In contrast, CCR2 (receptor for MCP-1) and CCR5 (receptor for RANTES), while present, were not CD8 cell-associated, and thus had no proportional changes to CD8 cells. In each case, however, no differences were detected in these chemokine receptors between OPC− and OPC+ tissue. Results for CCR2 and CCR5 mRNA expression were similar to the protein results showing no differences between OPC− and OPC+ tissues. The same was true for CCR3 that was not evaluated at the protein level (reagents unavailable).

Chemokine receptor mRNA detected by Superarray also showed few differences. Although we detected some increases or decreases between HIV− and HIV+ tissue or between OPC− and OPC+ tissue, 90% of the receptor mRNA showed changes of $< 1.0$ fold and were considered unchanged. Of the remainder, mRNA for two receptors was increased in response to HIV (CCR2, IL-8R$\beta$), while only mRNA for one receptor (CCXCR1) was increased in response to OPC. Although the sample size for the HIV− subjects was relatively low, the results for the three subjects were consistent to one another, and the few changes observed when compared to HIV+ persons were equally consistent to results for OPC− *vs* OPC+ persons. Thus, we feel that the results for the HIV− group are representative.

Both CCR2 and IL-8R$\beta$ had greater than one-fold increases in HIV+ compared with HIV− individuals. Our previous study found that mRNA for MCP-1 was increased in those with HIV (Lilly *et al*, 2004). Thus, it was not surprising to find mRNA for its ligand, CCR2, to be increased as well. On the other hand, the same study failed to show any increase of IL-8 mRNA in those with HIV that would correspond with the increase in IL-8R$\beta$ mRNA in the present study. In OPC+ individuals, we found no increased receptor mRNA that corresponded to the chemokine mRNAs increased in the lesion (RANTES, MCP-1, IP-10), including CCR2.

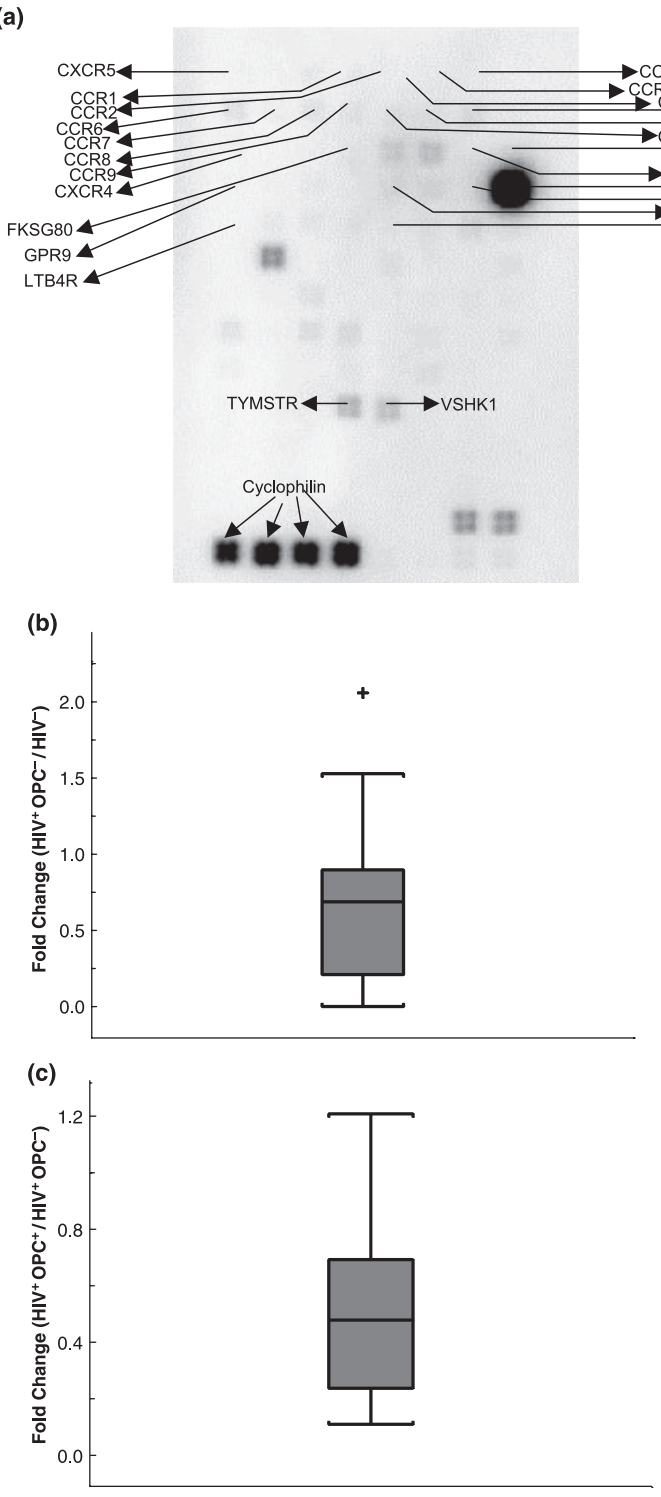


**Figure 3** Chemokine receptor mRNA expression in oropharyngeal candidiasis (OPC) lesions detected by Superarray. Tissue-associated mRNA for 25 chemokine receptors was evaluated in HIV− ($n = 3$), HIV+ OPC− ($n = 10$), and HIV+ OPC+ ($n = 10$) persons. (**a**) Representative image of an array membrane. (**b**) Fold change in HIV+ OPC− persons compared with HIV− individuals. (**c**) Fold change in HIV+ OPC+ persons compared with HIV+ OPC− persons. The median (horizontal line), 25th and 75th quartiles (box), range (T), and outliers (individual points) are illustrated

Thus, no degree of consistency was observed in changes of mRNA for chemokine ligands *vs* their receptors in our cohort.

For those receptors evaluated by both superarray and real-time PCR (CCR2, CCR3, and CCR5), the results were consistent, namely that each was found to be expressed by the respective assay and each was expressed at similar levels for OPC+ *vs* OPC− tissue. However, as the units of measurement were different for each assay, direct quantitative comparisons were not possible. Noteworthy too were the results for CCR5. Previous studies have shown expression of the HIV coreceptor CCR5 to be low in gingival tissue from healthy HIV− individuals, but significantly increased under inflamed conditions that could potentiate HIV infection (Jotwani *et al*, 2004). We found that CCR5

expression was low in the BM as well. However, under the inflammatory condition of OPC, we failed to detect increased CCR5 at both the mRNA and protein levels. Thus, it is possible that some inflammatory conditions will not increase CCR5 and potentiate HIV infection. We recognize, however, that the low levels of detectable CCR5 expression may preclude highly conclusive interpretations.

Overall, very few changes were observed in oral tissue-associated chemokine receptor expression in response to HIV or OPC, as detected by several parameters at both the protein and mRNA level. This does not infer that the receptors were not under some level or degree of change. It is possible that some chemokine receptors are equally being internalized and redistributed on tissue because of high frequency binding, resulting in a net zero change via protein and mRNA between various groups. Although seemingly remote for the small degree of change observed for the 25 receptors, previous studies have shown that cellular responses to chemokines are rapid in onset and fleeting through a process called receptor desensitization in which G protein-receptor kinases phosphorylate chemokine-occupied receptors. This process allows for endocytic uptake of chemokine-receptor complexes and continuous redistribution of chemokine receptors on the cell surface (Ebert *et al*, 2004). Based on the increased chemokine expression and cellular migration in OPC+ individuals compared with OPC− individuals (Lilly *et al*, 2004), modulation in expression of several chemokine receptors was expected. However, if so, it was not detectable compared with those with no uptake and redistribution. For this, radioimmunoassays will need to be conducted to confirm receptor internalization dynamics. Nevertheless, changes in chemokine receptor expression appear quite distinct to chemokine changes detected in OPC. Thus, while the presence of the chemokine receptors supports the migration and presence of $CD8^+$ T cells in OPC lesions, there is no evidence to suggest that chemokine receptor expression plays a role in susceptibility to OPC.

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