

Original article:

**TCRV β CLONOTYPIC ANALYSIS OF VAGINAL T LYMPHOCYTES
DURING EXPERIMENTAL VAGINAL CANDIDIASIS IN THE
MURINE SYSTEM**

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ABSTRACT

To further understand the role of localized T cells in protection against vaginal candidiasis (VC), vaginal T lymphocyte TCRV β repertoire was evaluated during experimental VC. RNA was extracted from lymphocytes of naïve and estrogen-treated *Candida albicans*-infected mice and RT-PCRred using TCRV β primers corresponding to 24 common TCRV β genes. All TCRV β rearrangements were detected in vaginal T cells and thymocytes of naive mice. Although, the full complement of TCRV β repertoire was detectable in vaginal T cells at days 14 and 21 post-infection, there was overrepresentation of TCRV β s 1, 6, 10 and 15 at day 14 and TCRV β s 1, 4, 6, 8.3, 10, 13, 14 and 18 at day 21. Expression of all TCRV β rearrangements in vaginal T cells raises the possibility of an extrathymic developmental origin. Time-dependent overrepresentation of specific TCRV β s during VC may indicate infection-related specific T cell clonal expansion.

Keywords: *Candida albicans*, extrathymic T cell development, TCRV β , vaginal candidiasis, vaginal T lymphocytes

INTRODUCTION

Vaginal candidiasis (VC) represents a real health problem to women of childbearing age worldwide (Ferrer, 2000; Enoch et al., 2006; Pfaller et al., 2007). *C. albicans*, a commensal of the genitourinary tract, is encountered in the greater majority of VC cases (Pfaller et al., 2007). The occurrence of VC and recurrent vulvovaginal candidiasis (RVVC) has been attributed to compromised immunity and increased levels of estrogen in the reproductive tract milieu (Larsen et al., 1984; Hamad et al., 2004). A significant percentage of RVVC cases also occurs in pre-menopausal women with competent immunity (Giraldo et al., 2000; Fidel, 2002; Cassone et al., 2007). Although T cell-mediated immunity (CMI), specifically Th1-type response, is consid-

ered to be the major defense mechanism against VC, systemic CMI plays a minor role (Cassone et al., 2007; Fidel et al., 1995a; Fidel, 2002). Systemic T cell responses generated following the induction of VC failed to provide significant protection against subsequent localized *C. albicans* infection in mice with experimental VC (Fidel, 2002 and references therein). Deletion of systemic CD4⁺ or CD8⁺ T cells did not significantly influence the progression of VC in mice (Fidel et al., 1995b). Furthermore, vaginal, but not peripheral, T cells undergo significant kinetic changes during experimental VC in mice (Ghaleb et al., 2003).

Vaginal T cells exhibit a phenotypic profile distinct from that of peripheral T cells (Hamad, 2008; Fidel et al., 1996; Johanson & Lycke, 2003; Ibraghimov et al.,

1995; Fidel et al., 1999). The majority (>80 %) of vaginal T lymphocytes are of the CD3⁺CD4⁺ phenotype. Unlike peripheral T cells, the majority of CD4⁺ vaginal T cells react with the 2B6 anti-CD4 but not the GK1.5 anti-CD4 antibody (Fidel et al., 1999). The percentage of CD8⁺ T cells was reported at 1 % (Cassone et al., 2007; Hamad, 2008). This notwithstanding other findings that have indicated that CD8⁺ T cells predominate the vaginal mucosa during VC (Ghaleb et al., 2003). A wealth of literature seems to suggest that vaginal T cells exhibit phenotypic and developmental properties similar to that of intestinal intraepithelial lymphocytes (IELs) and other localized T cell subsets (Hamad, 2008; Ishikawa et al., 2007). For example, reminiscent of the IEL T cell population, vaginal T cells contain a significant percentage of TCR- $\gamma\delta$ ⁺ T cells (Hamad, 2008; Fidel et al., 1996; Johanson & Lycke, 2003; Ibragimov et al., 1995; Fidel et al., 1999). Few CD4⁺CD8⁺ cells were reported to populate the vaginal mucosa (Ghaleb et al., 2003; Hamad, 2008 and some references therein). A unique population of TCR $\alpha\beta$ ⁺CD3⁺B220^{low}CD4⁻CD8⁻NK1.1⁻ T cells was detected in the vaginal mucosa of nude mice and mice deficient for MHC-II, β_2 -microglobulin or CD1 (Johanson & Lycke, 2003). Furthermore, evidence in support of lymphocyte trafficking between the periphery and the vaginal mucosa are yet to be sufficiently established (Cassone et al., 2007; Rakasz et al., 1998).

To further understand the development and function of vaginal T cells, TCRV β repertoire was examined during experimental vaginal candidiasis. Kinetics of TCRV β ⁺ T cell subsets were evaluated in *C. albicans* infected mice at different time points post-infection. The developmental pathway of vaginal T cells was addressed by comparing the patterns of TCRV β gene usage between vagina- and thymus-derived T cells.

MATERIALS AND METHODS

Mice and microorganisms

12-14 week-old female Balb/c mice raised at the Hashemite University vivarium were used in this study. Handling of experimental animals was in full compliance with relevant animal welfare policies and procedures as specified in the 1996 ILAR Guide for the care and use of laboratory animals. ATCC *C. albicans* strain 36082 is a kind gift from Dr. MA Ghannoum (Mycology Reference Laboratory, University Hospital of Cleveland, OH, USA). The fungus was maintained on Sabouraud Dextrose Agar (SDA) (Difco, MI; USA), stored at 4°C and subcultured at 3 months intervals. For inoculation, overnight cultures of *C. albicans* were grown at 37 °C in SDA broth as described previously (Abu-Elteen et al., 1997). Prior to use, cells were harvested and washed twice in sterile physiological saline (SPS). Estrogen (estradiol valerate; Schering AG, Germany) was administered subcutaneously at 0.5 mg per 0.1 ml sesame oil 72 hrs prior to *C. albicans* inoculation and at weekly intervals thereafter. Vaginal *C. albicans* inoculate consisted of 50 μ l containing 2×10^7 viable stationary-phase blastoconidia.

Evaluation of C. albicans colonization

Mice were sacrificed at days 7, 14 and 21 post-infection; vaginal tissues were isolated, examined for the presence of white lesions characteristic of *C. albicans* infection, trimmed and homogenized in 10ml SPS in a sterile glass homogenizer (Ystral GmbH, Göttingen, Germany). Serial 10-fold dilutions were prepared from the homogenate; 1ml aliquots of the appropriate dilution were added into culture plates containing 10 ml SDA and chloramphenicol at 50 mg/L, plates were left to solidify and then incubated at 37 °C; each sample dilution was cultured in triplicate. Yeast colonies were counted 48 hrs after plating and colonization results were expressed as the mean colony-forming unit (CFU) per mouse based on data from three animals per group.

Isolation and flow cytometric analysis of vaginal T lymphocytes

Isolation and immunostaining of vaginal T cells was done according to previously published procedures (Ghaleb et al, 2003). Briefly, 5-6 mice were sacrificed per group per time point, vaginas were isolated and flushed with normal saline, opened up longitudinally and cut into 2 mm pieces. Tissue pieces were placed in 50 ml of warm RPMI-1640 (Sigma Chemicals, MO; USA) solution containing 10 mM EDTA plus 1 mM DTT and stirred for 30 min at 37 °C. The suspension was filtered through a 1 gm nylon wool column moisturized with warm HBSS (Sigma), the filtrate was then centrifuged and the cell pellet was suspended in 1 ml HBSS. Lymphocytes were counted manually; a total of 10^4 - 10^5 cells in 100 µl HBSS were used per sample for flow cytometric analysis. Biotin-labeled anti-CD3 (KT3), phycoerythrin (PE)-labeled anti-CD4 (YTS191.1) and fluorescein isothiocyanate (FITC)-labeled anti-CD8 (KT15) antibodies (Serotec Ltd., Oxford, UK) were used for T cell phenotypic characterization. For single-color analysis, biotin-labeled anti-CD3 was added at 1 µl per sample, left to react for 30 minutes on ice, cells were then centrifuged, washed with 100 µl HBSS, cell pellet was then resuspended in 100 µl HBSS and reacted with PE-labeled streptavidin. For dual-color analysis, each sample was reacted with 2 µl FITC-labeled anti-CD8 and 1 µl PE-labeled anti-CD4 for 30 minutes on ice prior to washing. Flow cytometric analysis was performed on a PARTEC-PAS flow cytometer (Partec, Münster, Germany); 10^4 - 10^5 cells were analyzed per sample and data were collected and analyzed using flowmax software (Partec).

PCR-spectratype analysis

Total cellular RNA was extracted from lymphocytes using the SV Total RNA Isolation System Kit (Promega, WA, USA). Synthesis of cDNA from extracted/purified RNA was performed using the Reverse Transcription System Kit (Promega). Gene segments of rearranged TCRV β were PCR

amplified across the VDJ junctional site using V β and C β primers (Pannetier et al., 1993). Amplification reactions were separately carried out each in a total reaction volume of 100 µl including 10 µl of each synthesized cDNA, 50 µl PCR Master Mix (50 U/ml Taq DNA polymerase, 3 mM MgCl₂, and 400 µM of each dNTP; pH 8.5) (Promega) and 1 µl C β primer. The mixture was equally aliquoted into 24 tubes, each containing 1 µl of V β -specific primer solution. The volume was brought up to 100 µl by adding 38 µl nuclease-free water per tube. PCR amplification was conducted using MyCycler Thermal Cycler (BioRad Laboratories Inc., Life Science Group, California, USA) following the protocol: denaturation for 1min at 94 °C followed by 40 cycles consisting of 70 sec at 94 °C, 1min at 60 °C, and 4 min at 72 °C, followed by 10 min incubation at 72 °C to complete product extension. PCR amplification products were separated on a 3 % agarose gel in TBE buffer (Promega). 12 µl of the PCR product of each sample was mixed with 2 µl of Blue/Orange 6X loading dye (Promega), prior to loading. 1.5 µl of loading dye was mixed with 1.5 µl of the 25 Kb DNA ladder (Promega) consisting of 12 bands ranging in size from in 25-300 bp as a control. Gels were run at 50 volts for 3hrs, destained and photographed using a 312 nm UV transilluminator (Uvitec, EEC) equipped with a gel documentation System (UVIsave, EEC). Amplification of cDNA samples prepared from RNA of *C. albicans* cells was carried out as negative controls of the PCR reaction.

RESULTS

Persistent vaginal candidiasis was evident in estrogen-treated *C. albicans*-infected mice throughout the infection period as compared with the minimal *C. albicans* CFU counts detected in naïve mice (Figure 1A). Vaginal fungal burden in mice infected with *C. albicans* without receiving estrogen declined to background levels within 1 week post-infection (data not shown). Numbers of lymphocytes isolated

from the vaginal tract epithelium of estrogen-treated *C. albicans*-infected mice at days 7, 14 and 21 following the inoculation of *C. albicans* are shown in Figure 1B. Overall, the number of vaginal lymphocytes was significantly higher in experimental mice than that in control mice ($P < 0.05$). The peak value of vaginal lymphocyte number (23×10^6 cells) was reached at day 14 post-infection; which represents an increase of 9-fold as compared with that in control mice.

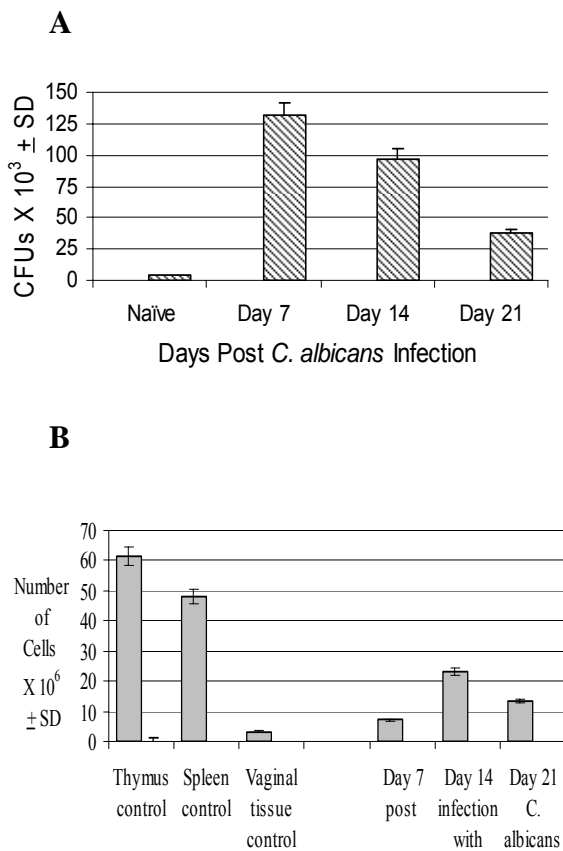


Figure 1: (A) Vaginal *C. albicans* burden during experimental vaginal candidiasis. Levels of *C. albicans* colonization in naïve untreated mice, untreated infected mice and treated infected mice were assessed at days 7, 14 and 21 post-infection. Mean *C. albicans* CFU count per vagina + SD was calculated based on three separate experiments per group. (B) Kinetics of vaginal lymphocytes during experimental vaginal candidiasis. Relative numbers of total vaginal lymphocytes isolated from the naïve untreated mice and estrogen-treated *C. albicans*-infected were evaluated at days 7, 14 and 21 post-infection. At each time point, 5-6 mice per group were sacrificed, pooled cell preparations were enumerated and the average number of cells per vagina was calculated. Data shown is the mean ± SD of three separate experiments.

To establish the general phenotypic profile of this cell population, flow cytometric analysis was carried out on vaginal cell isolates from naïve mice and experimental mice at different time points during the infection (Table 1 and Figure 2). Consistent with previous studies (Ghaleb et al., 2003; Fidel et al., 1996), the percentage of CD3⁺ T cells in vaginal cell isolates ranged between 20 % at the start of the infection period to about 45 % at day 21 post-infection; possibly indicative of T cell proliferation as part of the incessant anti-fungal immune response. Although the predominance of CD8 in the vaginal T cell pool was evident, a significant percentage of CD4⁺ T cells was present at all time points. Interestingly, a minor T cell population (<5 % of total vaginal T cells) expressing both CD4 and CD8 was also detected.

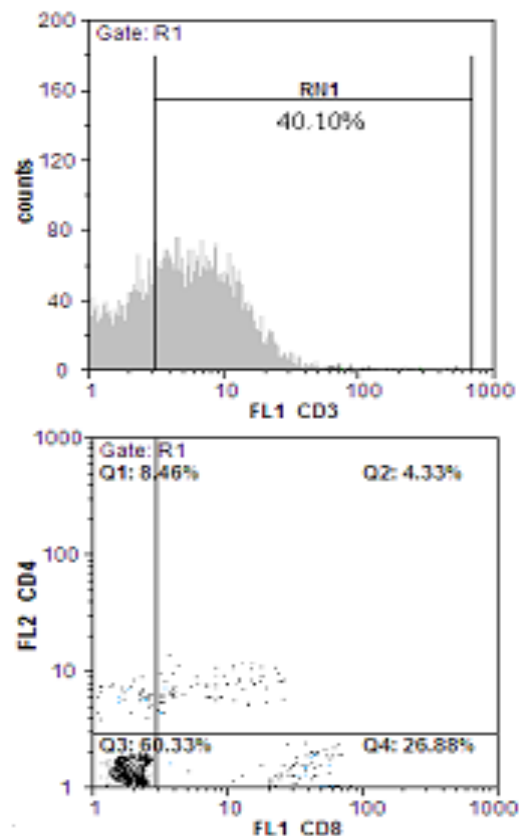


Figure 2: Expression of CD3, CD4 and CD8 T cell markers on vaginal T cells during experimental vaginal candidiasis. Vaginal T cells were isolated from *C. albicans* infected – estrogen-treated mice at week 3 post-infection, were pooled from 5-6 mice per group and reacted with anti-CD3 or with anti-CD8 and anti-CD4. Data presented are representative of three separate experiments.

Table 1: Percentage expression of CD3, CD4 and CD8 on murine vaginal T lymphocytes during experimental vaginal candidiasis

Animal group	Mean % positive cells \pm SD*			
	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ CD8 ⁺
Naïve Controls	22.6 \pm 2.3	6.6 \pm 0.9	15.1 \pm 1.9	2.3 \pm 0.7
Day 7	30.1 \pm 3.0	12.4 \pm 1.5	16.7 \pm 2.4	1.8 \pm 0.5
Day 14	44.5 \pm 3.6	20.3 \pm 3.8	21.0 \pm 2.1	3.2 \pm 0.6
Day 21	41.3 \pm 4.2	8.7 \pm 2.1	27.3 \pm 1.8	4.7 \pm 0.7

* Mean percentages of vaginal T cells expressing the specific marker were calculated based on three separate experiments

The patterns of TCRV β rearrangements in vaginal and thymus-derived T cells isolated from naïve mice were evaluated by RT-PCR clonotypic analysis (Figure 3). As shown in the top panel of Figure 3, thymocytes contained all TCRV β rearrangements except for TCRV β s 19 and 20 which were either absent or very faint. As for peripheral T cells (spleen and lymph nodes), TCRV β s 1, 4, 10, 11, 13, 15, 16 and 17 were strongly represented perhaps indicative of increased relative cell numbers expressing these rearrangements. In cells from both tissues, TCRV β s 3.1, 5.1, 5.3, 8.1, 8.2, 8.3, 9, 19 and 20 were consistently low or absent (data not shown). With regard to vaginal T cells in naïve mice, the expression of TCRV β s 5.3 and 20 was consistently weak

while that of TCRV β s 4, 6, 7, 8.1, 8.2, 13, 14 and 15 was very prominent perhaps reflecting increased relative cell number of clones expressing the corresponding rearrangements (lower panel of Figure 3). Therefore, the pattern of TCRV β gene usage in vaginal T lymphocytes seems to be similar, though not completely identical, to that observed in thymocytes. Amplification reactions using the same primers set and PCR conditions were carried out on cDNA samples prepared from *C. albicans* cells as negative controls. As expected, no PCR products were observed in this series of experiments indicating that the PCR products detected are lymphocyte-specific (data not shown).

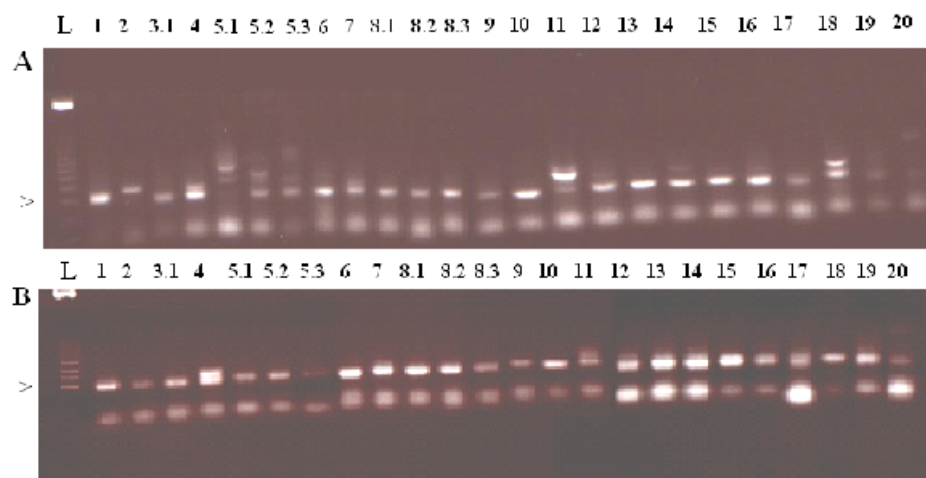


Figure 3: PCR-spectraype analysis of V β gene rearrangements in T cells isolated from the thymus (A) and vaginal tract T cells (B) of naïve control mice. Numbers above panels indicate V β gene number and the 25-bp DNA ladder is indicated by L on top of the first lane from left. Data shown are representative of three separate experiments.

Vaginal T lymphocytes isolated from estrogen-treated *C. albicans*-infected mice exhibited an interesting TCRV β repertoire (Figure 4). Clear shifts in the TCRV β gene usage seems to occur between the naïve state versus the early stages of infection and between the early stages of infection versus those when the infection was well established. At day 7 post-infection (Figure 4, top panel), TCRV β s 2, 3, 6 and 7 were missing while TCRV β s 5.1 and 10 were strongly represented. At day 14 post-infection (Figure 4, middle panel), the whole TCRV β gene complement reappeared with TCRV β s 1, 6, 10 and 15 being overrepresented in comparison with the rest of the TCRV β complement. At day 21 post-infection (Figure 4, lower panel) extensive rearrangement of TCRV β s 1, 4, 6, 8.3, 10, 13, 14 and 18 was evident. TCRV β s 5.1, 8.1 and 8.3 were well represented in vaginal T cells of experimental mice at day 14 and day 21 post-infection as compared with that in vaginal T cells of naïve mice. These findings were consistently observed in 3 separate experiments.

DISCUSSION

Findings presented here should help in further understanding the development and function of vaginal T cells. The important role of vaginal T cells in protection against vaginal candidiasis is discernable from several findings. First, there was a significant increase in the number of vaginal lymphocytes during the infection which is consistent with previous studies (Ghaleb et al., 2003; Fidel et al., 1996; Al-Sadeq et al., 2008). In agreement with previous reports (Ghaleb et al., 2003; Al-Sadeq et al., 2008), the majority of T cells that undergo expansion during the course of VC infection are of the CD8⁺ phenotype (Figure 2). Second, there was consistent overrepresentation of certain TCRV β s (6 and 10 in particular) during vaginal candidiasis. A likely explanation of this finding is that T cell clones expressing these rearrangements may be directly involved in responding to *C. albicans* antigens.

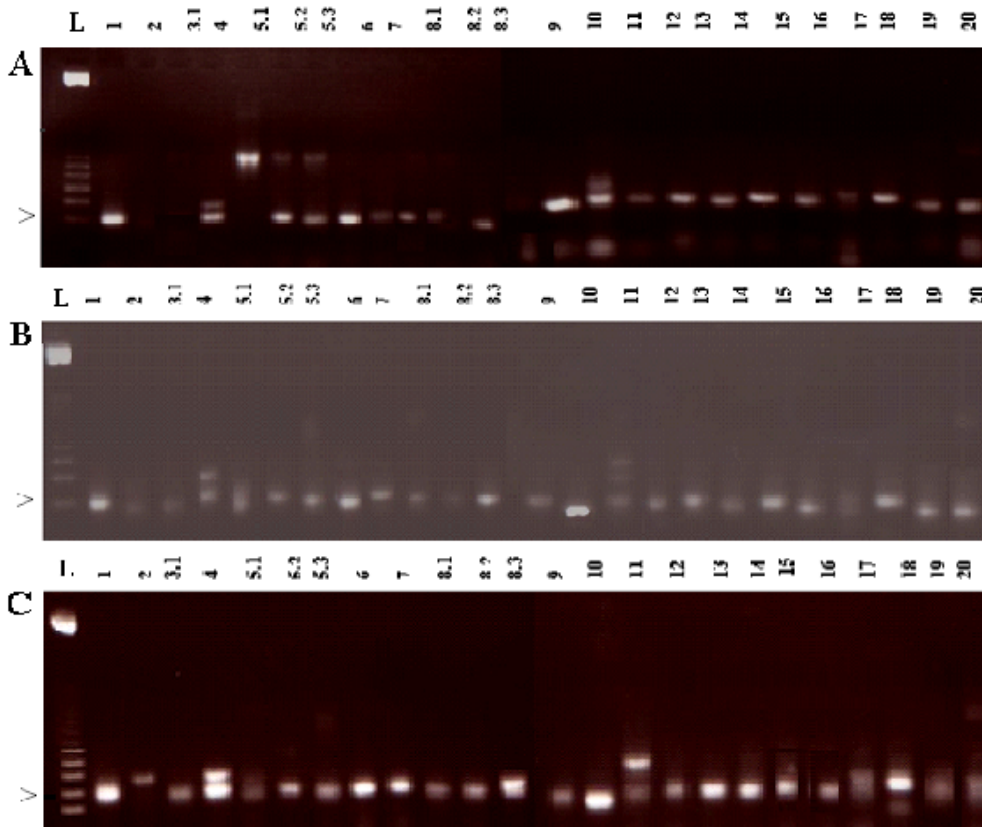


Figure 4: PCR-spectratype analysis of V gene rearrangements in T cells isolated from the vaginal tracts of *C. albicans*-infected mice at days 7 (A), 14 (B) and 21 (C). Numbers above panels indicate V β gene number and the 25-bp DNA ladder is indicated by L on top of the first lane from left. Data shown are representative of three separate experiments.

Third, there were unique time-dependent changes in the pattern of TCRV β rearrangement dominating the vaginal T cell pool during the course of infection. It is possible that the profile of *C. albicans* antigens being presented to vaginal T cells shifts overtime hence the occurrence of concurrent shifts in the dominating subset of T cell clones expressing the appropriate TCRV β rearrangements. Based on this observation, it is postulated that T cell clones positive for TCRV β 1, 6, 10 and 15 may represent early T cell responders (T_{helper}) while those expressing TCRV β s 4, 8.3, 13, 14 and 18 represent late T cell effectors (T_{cytotoxic}) cell subset (Figure 4, middle and lower panels). Should these results hold true, there could be significant therapeutic implications in the form of agonistic monoclonal antibodies to target phase-specific T cells. Refining the rationale of cloning and adoptive T cell transfer into naïve or infected hosts as means of treating fungal infections is a second envisaged therapeutic avenue (Beck et al., 2006).

Evidence for an extrathymic pathway of vaginal T cell development as presented in this article derives from the finding that the pattern of TCRV β gene usage in the vaginal T cell pool is more similar to that in thymocytes than that in peripheral (spleen or lymph node) T cells. The fact that the vaginal tract contained the full complement of TCRV β provides compelling evidence that this site functions as a primary lymphoid organ in addition to its established role as a secondary lymphoid organ. In other words, it is likely that some T cells resident within the vaginal mucosa may extrathymically develop within the vaginal mucosa. This is further supported by previous findings which have indicated that the vaginal mucosa contains a minor subset of immature double positive (CD4⁺CD8⁺) T cell precursors (Ghaleb et al., 2003; Ibraghimov et al., 1995). Furthermore, the vaginal mucosa of nude mice and mice deficient for MHC-II, β_2 -microglobulin or CD1 were all shown to house a unique population of TCR $\alpha\beta$ ⁺CD3⁺B220^{low}CD4⁺CD8⁻NK1.1⁻ T cells (Johanson & Lycke, 2003). Evidence

in support of a possible extrathymic development of similar localized T cell subsets like the IEL subset has been amply furnished (Hamad, 2008; Poussier et al., 1992; Hamad et al., 1995). The close similarities between the small intestinal mucosa and the vaginal mucosa in terms of structure, continuous exposure to pathogens and the need for rapid and effective immunity are to be considered in this regard.

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