Patterns of Expression of Vaginal T-Cell Activation Markers during Estrogen-Maintained Vaginal Candidiasis

Ameera Al-Sadeq, MSc, Mawieh Hamad, PhD, and Khaled Abu-Elteen, PhD

The immunosuppressive activity of estrogen was further investigated by assessing the pattern of expression of CD25, CD28, CD69, and CD152 on vaginal T cells during estrogen-maintained vaginal candidiasis. A precipitous and significant decrease in vaginal fungal burden toward the end of week 3 postinfection was concurrent with a significant increase in vaginal lymphocyte numbers. During this period, the percentage of $CD3^+$, $CD3^+CD4^+$, $CD152^+$, and $CD28^+$ vaginal T cells gradually and significantly increased. The percentage of $CD3^+$ and $CD3^+CD4^+$ cells increased from 43% and 15% at day 0 to 77% and 40% at day 28 postinfection. Compared with 29% $CD152^+$ vaginal T cells in naive mice, > 70% of vaginal T cells were $CD152^+$ at day 28 postinfection. In conclusion, estrogen-maintained vaginal candidiasis results in postinfection time-dependent changes in the pattern of expression of CD152, CD28, and other T-cell markers, suggesting that T cells are subject to mixed suppression and activation signals.

Key words: *CD28, CD152, estrogen, vaginal candidiasis, vaginal T lymphocytes*

Vaginal candidiasis (VC) is now recognized as a major health problem for women of childbearing age worldwide.[1,2] The majority of genitourinary tract fungal infections are caused by *Candida albicans*[3]; VC cases owing to *C. glabrata, C. tropicalis,* and *C. kruzi* are also on the rise.[4,5] The majority of women who experience sporadic episodes of VC are otherwise healthy. However, around 10% of women are at increased risk of VC owing to compromised immunity, antibiotic overuse, and increased estrogen concentration in the reproductive tract environment.[1–3,6] Estrogen predisposes to VC by several proposed mechanisms, including the enhancement of the pathogenic potential of *Candida* species and the suppression of host immunity.[7–11] Induction of a pseudoestrous state by estrogen is routinely used to establish experimental persistent *C. albicans* vaginal infection in rodents.[10,12–14] Furthermore, estrogen, administered on a weekly basis, can induce persistent VC in naive non–germ-free Balb/c mice.[13]

It is well established now that both innate and acquired immune responses converge to protect the host against fungal infections (reviewed in Romani[15]). Intact epithelia and endothelia, microbial antagonism, and antimicrobial peptides provide the very first line of defense against fungal infections. Additionally, professional phagocytic cells (neutrophils, monocytes, macrophages, and dendritic cells [DCs]) reduce fungal burden by inducing oxidative and non-oxidative killing of fungi and by restricting fungal growth and infectivity. Nonetheless, localized T cell–mediated immunity (CMI), specifically T-helper (Th)1-mediated responses, remains the major defense mechanism against VC.[16,17] Systemic T-cell responses generated following the induction of VC[18–20] fail to provide significant protection against subsequent localized *C. albicans* infection in mice with experimental VC.[17] Deletion of systemic $CD4^+$ or $CD8^+$ T cells does not significantly influence the kinetics of VC in mice.[18] Furthermore, absolute numbers of vaginal, but not peripheral, T cells undergo significant changes during experimental VC in mice.[12] Previously, it has been shown that while $CD3^+$ T cells from draining lymph node and vaginal mucosa undergo minor activation, expression of T-cell activation markers α4-β7, αM290-β7, and α4-β1 drops during primary or secondary estrogen-maintained VC.[19] In contrast, the expression of mucosal and vascular cell adhesion molecule 1 on vaginal tissue cells is upregulated.[19] These findings suggest that despite upregulated expression of several T-cell activation markers during VC, lack of expression of corresponding ligands limits the capacity of CMI to deal with *C. albicans* vaginal infection. Numerous studies have suggested that CD28/B7 (CD80 or CD86)-dependent T-cell costimulation is essential for induction and mainte-

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nance of protective immunity against fungal infections.[20–22] Additionally, CD152 engagement with B7 (CD80 or CD86) on antigen presenting cells (APCs) has been shown to subdue localized Th1-mediated immune response.[20,23] To further investigate this issue, the pattern of expression of several T-cell activation markers (CD25, CD28, CD69, and CTLA-4 or CD152) was evaluated on vaginal and peripheral T cells at several time points during estrogen-maintained experimental VC in mice. The pattern of expression of these markers, at each time point, was correlated with tissue fungal burden and lymphocyte numbers.

Materials and Methods

Mice and Microorganisms

Adult 12- to 14-week-old non-pregnant Balb/c female mice raised under clean but non–germ-free conditions at the Hashemite University vivarium were used throughout the study.[13] Animal handling was in accordance with institutionally drafted guidelines. American Type Culture Collection *C. albicans* 36083 strain, kindly provided by Dr. Mahmoud Ghannoum (Center for Medical Mycology Laboratory, University Hospital of Cleveland, OH), was used throughout the study. The fungus was maintained on Sabouraud dextrose agar (SDA) (HiMedia, Mumbai, India) slants supplemented with chloramphenicol at 50 mg/L at 4°C and subcultured at 3-month intervals.

Induction of Experimental VC

Methods of induction of estrogen-dependent experimental VC are published elsewhere.[12] Briefly, mice were injected subcutaneously with 0.5 mg estradiol valerate diluted in 0.1 mL sesame oil (Schering AG, Germany) 3 days prior to *C. albicans* inoculation and at weekly intervals thereafter. Each mouse received a single 100 μL intravaginal inoculum of 2×10^7 viable stationary-phase blastoconidia grown overnight in trypton soya broth (ADSA Micro, Spain). Age- and sex-matched mice that received either a single intravaginal injection of 0.1 mL autoclaved phosphate-buffered saline (PBS) or a single 100 μL intravaginal inoculum of 2×10^7 viable stationary-phase blastoconidia were used as controls.

Evaluation of Tissue (Vagina and Spleen) Fungal Burden

Five to six mice per group were sacrificed by cervical dislocation at different time points post–*C. albicans* inoculation. Vaginas were isolated, examined for the presence of white lesions characteristic of candidiasis, pooled, and trimmed into about 5 mm pieces. About one-fifth of trimmed tissue was homogenized in 10 mL PBS in a sterile glass homogenizer; the rest was saved for isolation of vaginal lymphocytes. Spleen and draining lymph node homogenates were prepared by pressing the tissue through a sterile stainless steel mesh screen into 10 mL PBS under aseptic conditions. Separate serial 10-fold dilutions (10^{-1}, 10^{-2}, 10^{-3}) of homogenates were prepared and aliquots of 1 mL/dilution were poured into separate culture plates containing 10 mL premelted SDA supplemented with chloramphenicol; each sample dilution was cultured in triplicate. Plates were left to solidify at room temperature and then cultured for 48 hours at 37°C; colonies were counted and expressed as the mean colony-forming unit (CFU) per mouse ± standard deviation.

Isolation of Lymphocytes

Isolation of vaginal lymphocytes was performed as described previously.[12] Briefly, five to six mice were sacrificed per group per time point. Vaginas were isolated, flushed with RPMI-1640 media (Sigma Chemicals, St. Louis, MO), opened up longitudinally, and cut into 5 mm pieces. Tissue pieces were placed in 50 mL warm PBS containing 1 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol (DTT). The mixture was stirred for 30 minutes at 37°C; cell suspensions were centrifuged for 5 minutes at 250*g*. Splenocytes were prepared by pressing intact spleens through sterile stainless steel mesh screens into 5 mL RPMI-1640; cells were then collected by centrifugation. Pellets were washed once and resuspended in RPMI-1640. Lymphocytes were counted using a hemocytometer chamber (Superior, Germany), and viability was determined by trypan blue exclusion.

Antibodies and Flow Cytometric Analysis

Antibodies used in this study included fluorescein isothiocyanate (FITC)-labelled rat antimouse CD3 (clone KT3), phycoerythrin (PE)-labelled rat antimouse CD4 (clone YTS191.1); FITC-labelled rat antimouse CD8α (clone KT15); PE-labelled hamster antimouse CD28 (clone 37.51.1); PE-Cy5-labelled hamster antimouse CD69 (clone H1.2F3); and PE-labelled rat antimouse CD25 (clone PC61.5.3). These reagents and the Ig isotype-matched controls were all purchased from Serotec Ltd. (Oxford, UK). An FITC-labelled rat antimouse CTLA-4 (CD152) (clone 63828) antibody was purchased from R&D Systems (Emeryville, CA). About 10^6 viable cells in 100 μL PBS were reacted with titrated concentrations of FITC-labelled CD3 and PE-labelled CD4 for dual-colour analysis or with PE-labelled CD25, CD28, or CD69 or FITC-labelled

CD8 or CD152 for single-colour analysis. Reaction tubes were kept on ice for 20 to 25 minutes before fixation with 1 mL of 2% paraformaldehyde per sample tube. Flow cytometric analysis was done on a Partec PAS flow cytometer (Partec, Münster, Germany) using *Flowmax* software (Partec) for data acquisition and analysis. Gating of the target population was performed based on lymphocyte physical properties and percentage expression of CD3. Cursors were set based on preruns of cell samples stained with isotype-matched control antibodies. On average, 50,000 events were collected for single-colour analysis and 70,000 events for double-colour analysis. Percentage positive staining was computed to the 99% confidence level at a logarithmic scale of three decades.

Statistical Analysis

One-way analysis of variance was employed to determine levels of significance within experimental groups, and the Fisher least significant difference test was used to determine the presence of significant differences between different means.

Results

Consistent with previous studies,[12–14] estrogen was able to induce persistent VC in treated *C. albicans*–infected mice throughout the study period (Figure 1). CFU counts/vagina in treated infected mice were consistently and significantly ($p < .05$) higher than those in naive control mice ($\approx 2 \times 10^3$ CFU/vagina). Vaginal fungal burden in experimental mice peaked at day 21 postinfection, reaching 80×10^3 CFU/vagina, and then precipitously dropped to about 17×10^3 CFU/vagina (see Figure 1). Splenic fungal burden in experimental mice, which was detectable only during the first 2 weeks, was only slightly higher than that in the spleens (see Figure 1) of control groups. No detectable fungal burden was detected in draining lymph node homogenates prepared from experimental and control mice (data not shown). The mortality rate in the experimental group was insignificantly higher than that in the control groups (data not shown). The number of vaginal lymphocytes gradually and significantly increased from about 1.5×10^6 cells/vagina in naive control mice to $> 14 \times 10^6$ cells/vagina in experimental mice at day 35 postinfection (Figure 2A). The number of spleen lymphocytes also increased from about 60×10^6 cells/spleen in naive mice to $> 95 \times 10^6$ cells/spleen at day 14 postinfection, which then precipitously dropped to background levels at days 28 and 35 postinfection (Figure 2B).

The percentage of $CD3^+$ vaginal T cells isolated from control mice was > 20%, 25% of which were $CD4^+$ (Figure 3). The percentage of $CD28^+$ and $CD152^+$ vaginal T cells in these mice was 11% and 29%, respectively; the proportion of vaginal T cells expressing CD25 or CD69 was negligible (< 2%). Following the induction of VC, however, the percentage of $CD3^+$ vaginal T cells significantly increased to reach about 80% at

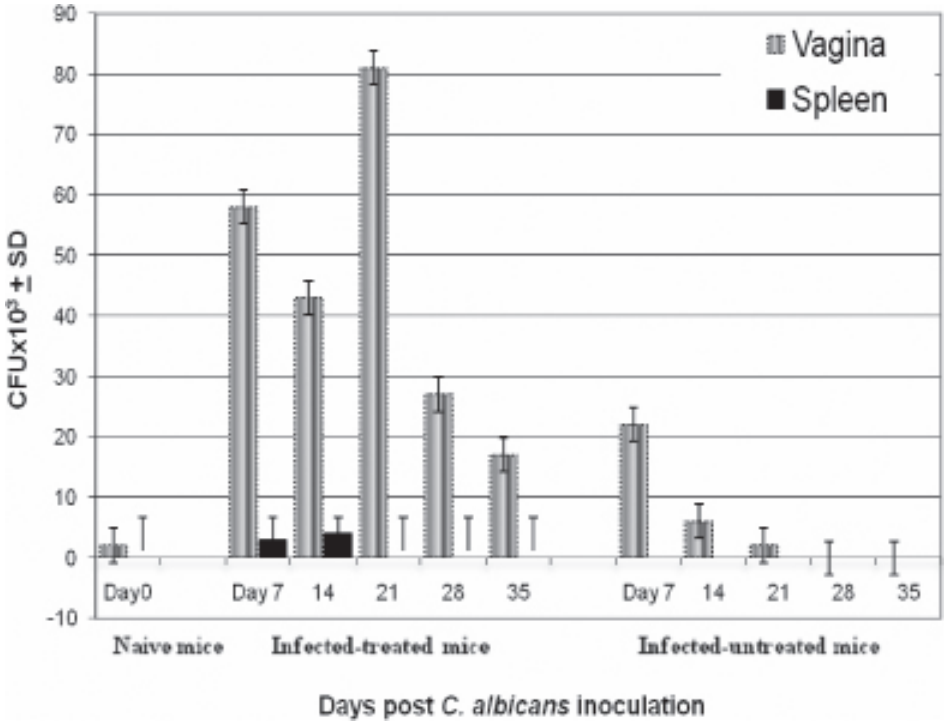

Figure 1. Vaginal and splenic fungal burdens were evaluated in phosphate-buffered saline–treated control mice, estrogen-treated *Candida albicans*–infected experimental mice, and untreated *C. albicans*–infected control mice at days 7, 14, 21, 28, and 35 postinfection. The data shown represent mean CFU/tissue ± SD as calculated from three separate experiments using five to six mice/time point/experiment.

day 28 postinfection (see Figure 3). At this time point, about 50% of $CD3^+$ vaginal T cells were $CD4^+$. The proportion of $CD28^+$ vaginal T cells isolated from experimental mice significantly increased to > 55% at day 28 postinfection (Figure 4A). The percentage of $CD152^+$ vaginal T cells jumped from 29% in naive mice to 72% in experimental mice at day 28 postinfection ($p < .001$). Although the percentage of vaginal T cells positive for CD25 or CD69 did not significantly change during the first 3 weeks postinfection compared with that in control mice (see Figure 4A), a significant ($p < .076$) increase in the percentage of vaginal T cells expressing CD25 was noted at week 4 postinfection.

As shown in Figure 4, the greatest week-to-week jump in the level of expression of CD28 and CD152 on both vaginal and splenic T cells occurred between weeks 3 and 4. Compared with 19% $CD28^+$ vaginal T cells at day 21 postinfection, $CD28^+$ cells represented > 55% at day 28 postinfection. As for $CD152^+$ vaginal T cells, their percentage jumped from 36% at day 21 to > 72% at day 28 postinfection. During this phase, the proportion of $CD3^+$ and $CD3^+CD4^+$ vaginal T cells was about 80% and 40%, respectively (Figure 3). Interestingly, these significant changes were concomitant with the precipitous decrease in vaginal fungal burden, as was noted earlier (see Figure 1). Furthermore, changes in CD28 and CD152 levels of expression corresponded with significant and incremental increases in vaginal lymphocyte numbers (Figure 2A). It is worth noting that although the percentage of cells expressing CD152 was significantly ($p < .05$) higher than that of cells expressing CD28 in the vaginal mucosa and the spleen, disparities in the level of expression of both markers were more pronounced in the case of vaginal T cells.

Despite the fact that only minimal splenic *C. albicans* colonization was noted during the first 2 weeks postinfection in experimental mice, significant changes in the pattern of expression of various splenic T-cell markers were noted during the course of the infection. Whereas about 40% of splenic T cells isolated from control mice were $CD3^+$ T cells and about 14% were $CD3^+CD4^+$, > 80 of splenic T cells isolated from experimental mice were $CD3^+$ and > 40% were $CD3^+CD4^+$ at day 28 postinfection (see Figure 4B). The percentage of $CD28^+$ splenic T cells gradually and significantly increased from 43% in naive control mice to > 70% on cells isolated at days 28 and 35 postinfection (see Figure 4B). Additionally, the percentage of splenic T cells expressing CD152 increased from 53% in naive mice to 88% in experimental mice at day 28 postinfection (see Figure 4B). It is worth noting that the percentage of $CD3^+$, $CD28^+$, and $CD152^+$ cells isolated from experimental mice at weeks 4 to 5 postinfection was extremely high compared with that in naive mice. In other words, the majority of splenocytes of experimental mice were T cells; this is perhaps suggestive

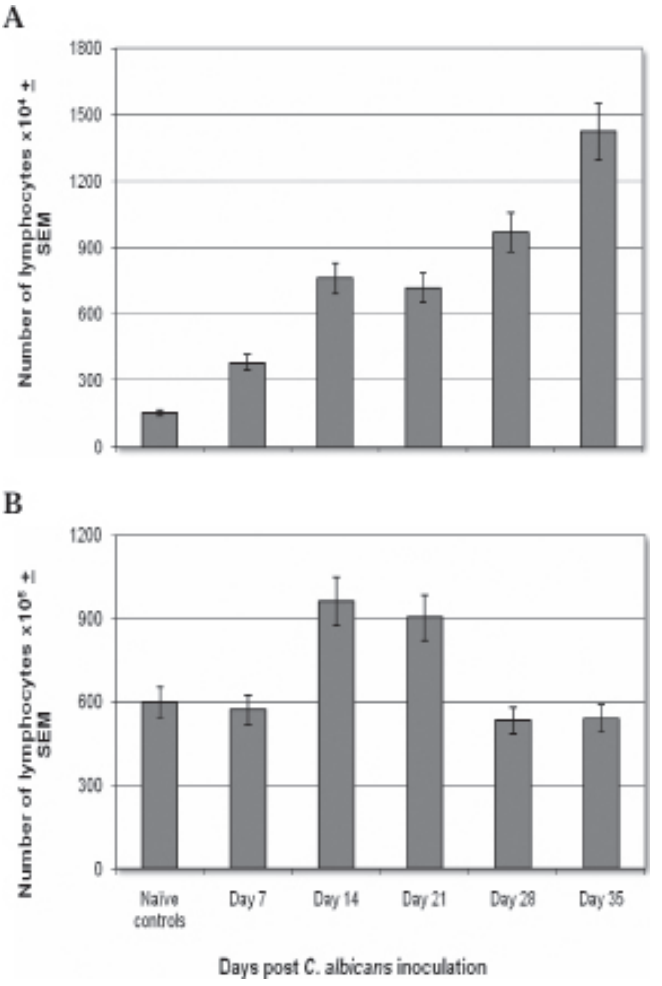

Figure 2. Absolute numbers of vaginal (*A*) and splenic (*B*) lymphocytes isolated from phosphate-buffered saline–treated control mice and estrogen-treated *Candida albicans*–infected experimental mice at days 7, 14, 21, 28, and 35 postinfection. Cell counts were plotted against the time points at which cells were harvested. The mean number of lymphocytes/tissue ± SEM was calculated based on data from three separate experiments, five to six mice/time point/experiment.

of extensive T-cell proliferation or activation in the spleen following *C. albicans* colonization. Compared with splenocytes isolated from naive control mice, a considerable but insignificant ($p = .113$) increase in $CD25^+$ T cells was noted in experimental mice at weeks 4 and 5 postinfection. The pattern of expression of CD69 on splenic T cells was similar to that on vaginal lymphocytes (see Figure 4B).

Discussion

The findings reported here clearly show that persistent vaginal *C. albicans* infection results in significant changes in the number, phenotypic profile, and state of activation of vaginal T cells. Based on the temporal kinetics of vaginal fungal bur-

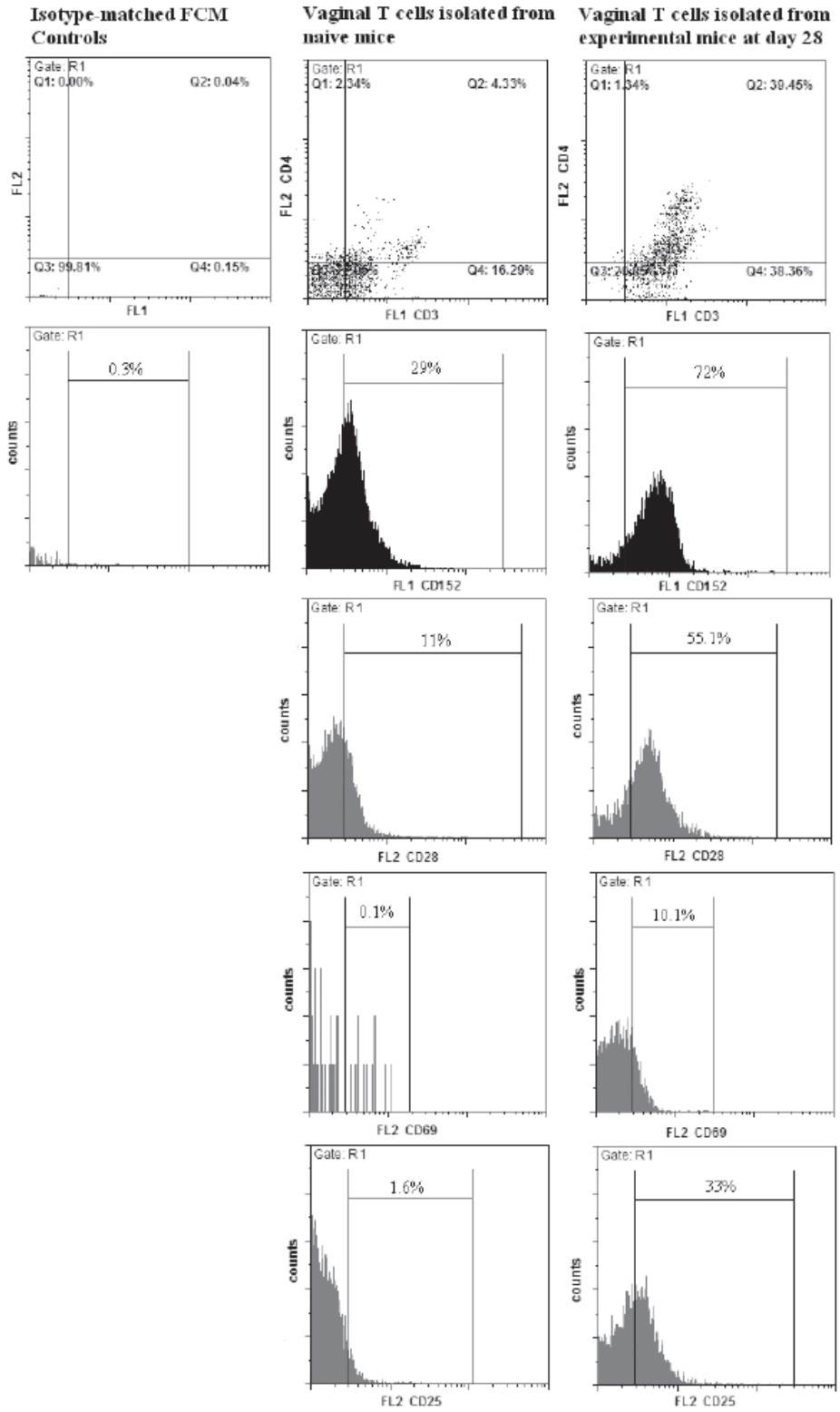

Figure 3. Vaginal lymphocytes isolated from phosphate-buffered saline–treated control mice and from estrogen-treated *Candida albicans*–infected experimental mice at day 28 postinfection were separately pooled from five to six mice and stained with anti-CD3 and anti-CD4 for two-colour flow cytometric (FCM) analysis or with anti-CD28, or anti-CD69 or anti-CD152 for single-colour FCM analysis. The data shown are representative of three separate experiments.

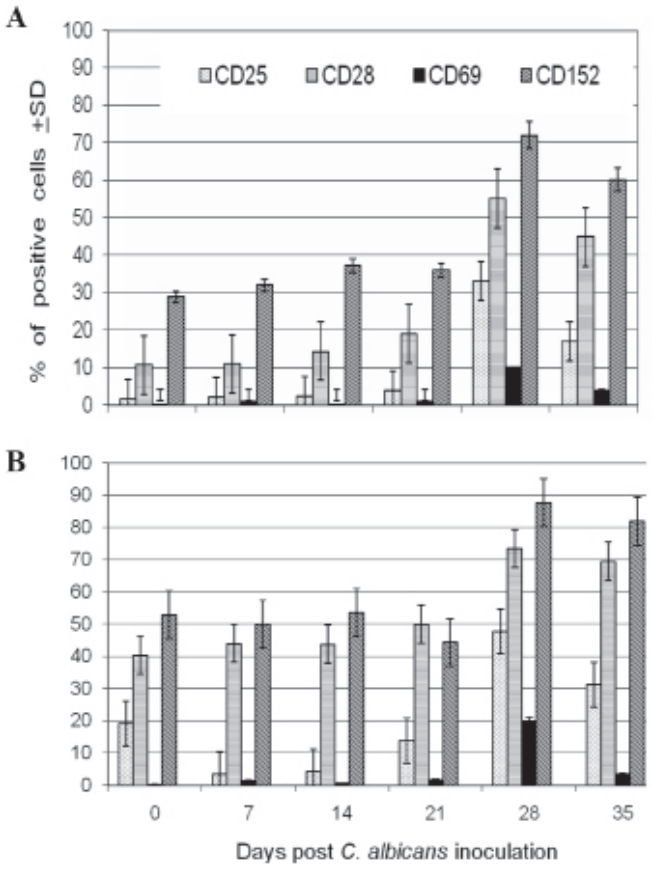

Figure 4. Summary of the mean percentage expression ± SD of CD25, CD28, CD69, and CD152 on (*A*) vaginal and (*B*) splenic T cells isolated from estrogen-treated *Candida albicans*–infected experimental mice at days 0, 7, 14, 21, 28, and 35 postinfection. Means ± SD were calculated based on three separate experiments; at each time point, tissue lymphocytes were isolated from five to six mice and pooled prior to staining and flow cytometric analysis.

den and the pattern of expression of T-cell activation markers, estrogen-maintained persistent VC seems to proceed in two sequential phases. During the first phase (first 3 weeks), the pathogen seems to overcome whatever resistance it faces from the local immune response. This is probably necessary should the pathogen be able to establish a persistent state of infection. During this phase, the number of vaginal T cells and the expression of T-cell activation markers CD25, CD69, and CD28 are all subdued. This phase is also marked by the presence of a dominant T-cell population expressing CD152 but not CD25 or CD28, perhaps indicative of suppressed vaginal T-cell activity. Engagement of CD152-B7 (CD80 or CD86) was reported to mediate inhibitory signals leading to T-cell anergy, apoptosis, or the production of inhibitory Th2 cytokines.[23] Additionally, T cells can instruct DCs to manifest tolerogenic properties via CD152 engagement with B7 on DCs.[20] Consistent with the possible immunosuppressive tolerogenic state noted in our study, draining lymph node DCs during VC were reported to predominantly express an immunoregulation-associated $CD11c^+B220^+$ plasmacytoid phenotype.[24] Furthermore, although draining lymph node $CD3^+$ T cells were slightly activated during primary and secondary *C. albicans* infections, the numbers of $CD3^+$ T cells expressing α4-β7, αM290-β7, and α4-β1 homing markers were reduced.[19]

During the second or resolution phase, which starts toward the end of week 3, a massive and precipitous decrease in vaginal fungal burden occurs concurrently with a significant surge in the number of vaginal lymphocytes and a very significant increase in the percentage of vaginal T cells expressing CD3, CD4, CD25, and CD28. Paradoxically, this phase is also marked by the presence of significant numbers of cells expressing CD152. Increased presence of T cells expressing the stimulatory marker CD28 (and probably those expressing CD25) is suggestive of scaled-down immunosuppression, thus permitting T cells to expand. Once again, engagement of CD28-B7 (CD80 or CD86) mediates a stimulatory signal, leading to T-cell activation and release of cytokines or immune mediators.[21–23] The paradoxical presence of both $CD152^+$ and $CD28^+$ cells during this phase may represent a state of competition between these two populations to interact with CD80/CD86 ligands on vaginal mucosa tissue APCs.

Changes in peripheral (splenic) lymphocyte numbers and phenotypic profiles suggest that responses to VC may involve a systemic aspect preceding or concurrent with the appearance of the localized response. This is consistent with the current understanding that CMI at the mucosa level partially derives from the systemic immune circuit.[17] Persistent upregulation of CD152, subdued number of T-cell subsets expressing CD69, and the gradual increase in $CD25^+$ and $CD28^+$ splenic T cells being similar to that of vaginal T cells are an indication that the spleen, as a peripheral immune compartment, is also subject to estrogen-mediated immunosuppression. As to whether the minimal levels of *C. albicans* colonization noted in the spleen during the early phase of the infection (see Figure 1) was responsible for the noted changes in splenic cell number and phenotype ican not be readily established. However, the capacity of estrogen to alter the immunocompetence of the periphery cannot be overlooked. Several reports have suggested that estrogen suppresses the delayed type hypersensitivity (DTH) response[10] and other innate immune responses.[25,26] Additionally, estrogen was recently shown to reduce the number and potential of APCs to present *C. albicans* antigenic peptides to T cells; it was also shown to be capable of suppressing T-cell activity.[11] Estrogen treatment can reduce the recovery of APCs from the peritoneal cavity and can inhibit the production of interleukin (IL)-12 and interferon-γ but not IL-10.[11] Whereas estrogen receptor α (ER-α) defi-

ciency in macrophages was shown to result in increased stimulation of $CD4^+$ T cells, estradiol-17β acting through ER-α was shown to increase the synthesis of IL-4 and GATA -3 from $CD4^+$ T cells.[25] The selective ER modulators tamoxifen and raloxifene were able to impair DC differentiation and activation.[27] In a Fas-ER fusion protein chimera system, estrogen was able to reduce apoptosis-mediated cytotoxic T lymphocyte activity.[28]

In conclusion, the capacity of estrogen to induce persistent VC seems to depend on its capacity to suppress host immunity, possibly by upregulating the expression of CD152 on vaginal and peripheral T cells. Furthermore, resolution of the infection may depend on the ability of T lymphocytes to counter immunosuppression, possibly by upregulating the expression of T-cell activation markers such as CD28 and CD25.

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