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A Unique Population of Extrathymically Derived \( \alpha \beta \text{TCR}^+ \text{CD4}^- \text{CD8}^- \) T Cells with Regulatory Functions Dominates the Mouse Female Genital Tract

Martina Johansson\(^2\) and Nils Lycke

A better understanding of the regulatory role of genital tract T cells is much needed. In this study, we have analyzed the phenotype, distribution, and function of T lymphocytes in the female genital tract of naive, pregnant, or \( Chlamydia trachomatis \)-infected C57BL/6 mice. Unexpectedly, we found that the dominant lymphocyte population (70–90%) in the genital tract was of \( \text{CD3}^+ \alpha \beta \text{TCR}^{\text{int}} \text{CD4}^- \text{CD8}^- \) cells. Moreover, these cells were \( \text{CD90}^{\text{low}} \) but negative for the classical T cell markers CD2 and CD5. The \( \text{CD3}^+ \text{B220}^{\text{low}} \) cells were NK1.1 negative and found in nude mice as well as in mice deficient for MHC class II, \( \beta_2 \)-microglobulin, and CD1, indicating extrathymic origin. They dominated the KJ126\(^+ \)V\( \beta_8 \.)^2 \) population in the genital tract of DO11.10 OVA TCR-transgenic mice, further supporting the idea that the \( \text{CD3}^+ \text{B220}^{\text{low}} \) cells are truly T cells. The function of these T cells appeared not to be associated with immune protection, because only CD4\(^+ \) and CD8\(^+ \) T cells increased in the genital tract following chlamydial infection. Notwithstanding this, the infected, as well as the uninfected and the pregnant, uterus was dominated by a high level of the \( \text{CD3}^+ \text{CD4}^- \text{CD8}^- \text{B220}^{\text{low}} \) cells. Following in vitro Ag or polyclonal stimulation of the \( \text{CD3}^+ \text{CD4}^- \text{CD8}^- \text{B220}^{\text{low}} \) cells, poor proliferative responses were observed. However, these cells strongly impaired splenic T cell proliferation in a cell density-dependent manner. A large fraction of the cells expressed CD25 and produced IFN-\( \gamma \) upon anti-CD3 plus anti-CD28 stimulation, arguing for a strong regulatory role of this novel T cell population in the mouse female genital tract. The Journal of Immunology, 2003, 170: 1659–1666.

Despite much interest in the mechanisms of immune protection against sexually transmitted diseases (STD),\(^3\) few detailed studies have been performed of the phenotype, distribution, and function of lymphocytes found in the genital tract mucosa. In particular, a better knowledge about genital tract T cells is needed to understand not only host protection against STD, but also how tolerance is established in pregnancy to avoid rejection of the fetus. It is now well established that Th1 responses are required for protection against most bacterial or viral STD (1–4). By contrast, for successful pregnancy, it is thought that Th1 activity must be suppressed, because Th1 responses have been associated with miscarriage (5). Moreover, the female genital tract is under hormonal control, and this has been shown to influence both the local distribution of lymphocytes and the presence of Ig in genital tract secretions (6–8). Susceptibility to STD and responsiveness to immunization are also affected by the menstrual cycle (6, 9).

Immunohistochemical studies have indicated that the female genital tract of healthy individuals host few immunocompetent cells and lymphoid nodules (8, 10). Thus, the genital tract mucosa does not appear to share the same immune functions as, for example, the intestinal mucosa with its M cells and Peyer’s patches. Investigations conducted in humans, mice, and Rhesus macaques have demonstrated the presence of dendritic, Langerhans-like cells and T lymphocytes in the epithelium of the vagina (7, 8, 10). The majority of the \( \text{CD3}^- \) T cells were found to express CD4 and the \( \alpha \beta \) TCR, while ~20% expressed the \( \gamma \delta \) TCR (11). In the uterine endometrium, intraepithelial lymphocytes (IEL) were found both at the surface epithelium and at the glandular epithelium (reviewed in Ref. 8). Although it has been shown that these IEL T cells may secrete TGF-\( \beta \), IL-2, and IFN-\( \gamma \) (5), our knowledge about the presence, distribution, and function of lymphocytes in the endometrium is still sparse.

A large proportion of the leukocytes in the endometrium are macrophages, which appear to be independent of hormonal control (8). These macrophages and dendritic cells can be potential APC, either locally in the mucosa (12, 13) or in the draining lymph nodes as they may traffic from the vaginal mucosa to the draining iliac lymph nodes (14). The cells that dominate the human uterine mucosa at the time for implantation are uterine NK (uNK) cells. These cells express the common leukocyte Ag CD45, as well as CD56, and appear to be under strong hormonal control. In the mouse, a similar type of cells has been found in the metrial gland (15).

The unique features of the genital tract lymphocytes, involved in protection against infection as well as in allowing conception, suggest that the local immune system in the female genital tract hosts unique cell populations not seen at other mucosal sites. Most studies have documented that the normal, healthy genital tract has few immunocompetent cells and, in the virgin naive mouse, T cells are sparsely distributed in the endometrium and B cells and plasma cells are seen only occasionally (8). However, a coherent understanding of the mucosal immune system in the female genital tract is just emerging. In this study, we have analyzed the distribution of lymphocytes in female virgin or pregnant naive mice or mice recovering from a genital tract infection with \( Chlamydia trachomatis \).
We have identified a novel population of small CD3⁺CD20⁻low CD4⁻CD8⁻ T cells in the mouse female genital tract that differs from other known T cell populations in the body. These cells exhibited impaired proliferative responses, but produced cytokine and expressed high levels of CD25 and were strongly down-regulatory on splenic T cell proliferation. The CD3⁺B220⁻CD4⁻CD8⁻ T cells were found in the genital tract of nude, MHC class II⁻/, β₂-microglobulin (β₂-m)⁻/⁻, and CD1⁻/⁻ mice, suggesting that they constitute a unique population of extrathymically derived regulatory T cells (Tr cells).

Materials and Methods

Animals

Female mice (8–10 wk old) were used at the onset of each experiment. MHC class II-deficient (16), β₂-m-deficient (17), and CD1-deficient mice (18) were kindly provided by Dr. S. Cardell (University of Lund, Lund, Sweden). C57BL/6 and nude mice were purchased from B&K Universal (Sollentuna, Sweden). OVA TCR-transgenic (Tg) mice (19) were bred in ventilated cages under pathogen-free conditions at the animal facility of Department of Medical Microbiology and Immunology (University of Göteborg).

Chlamydial bacteria

A human genital tract isolate of C. trachomatis serovar D was propagated in buffalo green monkey kidney cells by conventional techniques (20) and stored frozen at −70°C.

Bacterial inoculation of the female genital tract

The mice were inoculated as previously described (21, 22). Briefly, the mice were given s.c. injections with 2.5 mg/dose of Depo-Provera (medroxyprogesterone; Upjohn, Puurs, Belgium) 7 days before the intravaginal inoculation with live chlamydial infectious elementary body. The infectious dose of C. trachomatis serovar D used throughout the study for primary and challenge infections was 100 ID₅₀, corresponding to 6 × 10⁴ infectious elementary body determined by culture in buffalo green monkey kidney cells. Before evaluating host protection in immune mice following resolution of the primary infection, all mice were tested negative for bacterial shedding.

Detection of genital tract infection

Mice were screened for chlamydial shedding using both conventional culture techniques and a commercial kit, the Syva MikroTrak, EIA, and immunofluorescence kit (Syva, San Jose, CA), according to the manufacturer’s instructions, as previously described (21, 23).

Pregnancy

For matings, naturally cycling virgin C57BL/6 females were caged with C57BL/6 males. The day of detection of a vaginal plug was designated day 1 of pregnancy. On day 4 of pregnancy, the uterus was excised, as described below in the following section.

Isolation of genital tract lymphocytes and intestinal IELs (iIELs)

The iIELs were isolated as previously described (24). This method was modified for the isolation of genital tract lymphocytes. Briefly, the uterus and the cervix were excised, and all surrounding fat was removed. The tissue was cut in small pieces. The pieces were treated with collagenase (type C.2139, Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C with stirring. The supernatant was collected and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in Iscove complete medium and kept on ice until staining. The remaining tissue pieces were once again treated with collagenase as before. This procedure was repeated twice. After three incubations, the collected cells were washed once, diluted in Iscove complete medium, and then counted.

FACS analysis

The cells were suspended in 0.1% BSA/PBS to a concentration of 10⁷ cells/ml and kept on ice. A volume of 100 μl was aliquoted in tubes. The cells were treated with anti-FcγRIIR Ab at 1/100 for 5 min. Thereafter, FITC- and/or PE-labeled Abs from BD Pharmingen (San Diego, CA) were added to the cells and incubated for 30 min. Three corresponding isotype control Abs were used: hamster IgG1 (Fig. 1F), rat IgG1 (Fig. 7A), and rat IgG2a (Fig. 1K) labeled with FITC and/or PE (BD Pharmingen), respectively.

The cells were washed with 0.1% BSA/PBS twice and finally suspended in 500 μl of 0.1% BSA/PBS and analyzed in a FACScan (BD Biosciences, San Jose, CA). Lymphocytes were analyzed by using forward and side scatter to exclude other cells and dead cells. The gates were set using splenocytes, and compensation of the FL1 and FL2 channels was performed to place all unstained cells in the first quadrant of the dot plot.

Immunohistochemistry

The cervix, uterus, tubes, and ovaries were removed, snap frozen, and processed, as previously described. To block endogenous peroxidase activity, slides were treated with 0.3% H₂O₂ for 5 min and then stained with the following unlabeled or labeled Abs: anti-CD3 complex, FITC-anti-CD45R, and anti-CD45RB (BD Pharmingen). For CD3 and CD45R, a biotin-conjugated rabbit anti-rat IgG (Vector, Burlingame, CA), diluted 1/100, was used as a secondary Ab. Thereafter, a peroxidase-conjugated avidin (ABCComplex; DAKO, Glostrup, Denmark) or a Texas red-labeled streptavidin was added to each slide and incubated for an additional hour at room temperature. The staining was developed using a peroxidase 3-amine-9-ethylcarbazole substrate kit (Vector), according to the manufacturer’s instructions. Before inspection in the microscope, the tissue was washed and counterstained with HTX (Histolab Products, Via Frömlunda, Sweden) and mounted in Aquamount (BDH Laboratory Supplies, Poole, U.K.). Sections were evaluated and photographed using a Zeiss Axioskop microscope (Zeiss, Cambridge, U.K.). The labeling was highly specific; no staining was observed with an isotype-matched irrelevant control Ab as the primary Ab, and no staining was observed with the second-step anti-rat IgG (Vector) Ab used alone without a primary Ab.

Purification of B220⁺ cells

Streptavidin–coated beads from the CELLection kit (Dynal, Oslo, Norway) were precoated with biotinylated anti-CD45RB (BD Pharmingen). The positive selection was conducted according to the manufacturer’s instructions. Briefly, a single-cell suspension of genital tract cells was incubated on ice for 15 min with the appropriate amount of precoated beads allowing the beads to attach to the B220⁺ cells. The attached cells were sorted out using a magnet to collect the magnetic beads. The bead-cell complexes were washed twice and then diluted in 1% FCS/RPMI 1640. To release the cells from the beads, an accompanying releasing buffer was added. The mixture was incubated for 15 min at room temperature. Thereafter, the magnetic beads were separated from the selected cells using the magnet. The cells were washed, counted, and diluted in Iscove’s complete medium until further use.

In vitro culture

In a 96-well tray, single-cell suspensions from the spleen and the genital tract and positively selected B220⁺ cells were stimulated to assess proliferation and cytokine production. Spleen cells from the same animals were used as controls. For the B220⁺ cells, T cell-depleted spleen cells, irradiated with 25 Gy, were used as APCs at a concentration of 1 × 10⁵ cells/well for both cell types. For uterine lymphocyte (UL) and spleen bulk cultures, 2 × 10⁵ cells/well were used, or as indicated in Fig. 7C. The cells were stimulated in triplicate wells with the following additions: medium, 20% anti-CD3, 20% anti-CD3 plus 1 μg/ml anti-CD28, 1 μg/ml Con A, 1 mg/ml OVA, and 1 μg/ml p523. The cultures were incubated for 72 h at 37°C and 5% CO₂ with the addition of [³H]thymidine for the last 6 h. Thereafter, the plates were stored at −20°C until further analysis. For cytokines, the cultures were incubated for 96 h. Thereafter, the supernatants were collected and analyzed using ELISA.

IFN-γ ELISA

Ninety-six-well, flat-bottom plates (Nunc, Roskilde, Denmark) were coated with 20 μl/well of rat anti-mouse IFN-γ (Rat BD Pharmingen) in PBS at 4°C overnight. After washing and blocking, serial dilutions of supernatants and IFN-γ (PanData, Rockville, MD) were added to the plates and incubated overnight. After washing, the plates were incubated with polyclonal rabbit anti-mouse IFN-γ serum at a 1/300 dilution followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology, Birmingham, AL) at a 1/300 dilution. After washing a phosphatase substrate, p-nitrophenyl phosphate substrate tablets (Sigma-Aldrich) were added. The immunoenzymatic reaction was determined as absorbance in each well using a TiterTek Multiscan spectrophotometer (Flow; Huntsville, AL) at 405 nm. The concentrations were expressed in ng/ml as calculated from the plotted standard curves of serial dilutions of the recombinant cytokine. The sensitivity of detection was 1 ng/ml.

Statistical analysis

We used Student’s t test for unmatched data for analysis of significance.
Results

CD3+ B220low T cells dominate the mouse female genital tract

T cells in the mucosal immune system of the genital tract are known to play a key role in host protection against infectious diseases. Despite this, few studies have analyzed in detail the distribution and phenotype of T cells in the genital tract mucosa. To this end, we adapted and modified a method previously used to obtain lymphocytes from gut lamina propria to isolate cells from the genital tract of female mice (24). The uterus and the upper part of the cervix were excised from normal C57BL/6 mice. The tissue was cut into small pieces, which were treated with collagenase to give single-cell suspensions of UL. As assessed by FACS, only 10–20% of the cells were lymphocytes according to the preset forward- and side-scatter gates (using conventional spleen lymphocytes), and most of these cells labeled with anti-CD3 mAb. The collagenase treatment per se did not alter the expression of surface markers as confirmed by FACS analysis of splenocytes before or after collagenase treatment (not shown). Using preset forward- and side-scatter gates, which were based on the identification of splenic lymphocytes, we found that only 10–20% of the genital tract cells were lymphocytes, and most of these cells labeled with anti-CD3 mAb. The CD3+ lymphocytes were dominated by a population of cells that was also B220low (70–90%), a marker found most commonly on B lymphocytes (Fig. 1A). Virtually all CD3+ B220low cells expressed the αβ TCR, which indicated that they were T cells (Fig. 1B). Less than 2% of the UL expressed the γδ TCR (Fig. 1B). By comparison, splenic CD3+ cells did not express B220, whereas a minor population (≤20%) of gut intraepithelial CD3+ cells expressed low levels of B220 (Fig. 1, D and G). Most CD3+ cells from both spleen and IEL expressed higher levels of αβ TCR compared with the UL (Fig. 1, E and H). Furthermore, the UL were CD4+ and CD8+ (Fig. 1C), in contrast to CD3+ cells from the spleen and IEL, of which a majority were either CD4+ or CD8+ cells (Fig. 1, F, I, and L). Thus, the UL appeared to constitute a phenotypically novel and unique T cell population in the body.

Using immunohistochemistry on frozen tissue sections of the uterus, we could verify the presence of T cells in the genital tract mucosa. CD3+ T cells (Fig. 2A) and B220low cells (Fig. 2B) were found in the endometrium of the uterus. Using double-staining, the presence of CD3+ B220low T cells (Fig. 2C) could also be demonstrated throughout the genital tract mucosa, in the uterus as well as the cervix.

Extending the analysis, we positively selected ULs on the basis of B220 expression. Nearly all of the positively selected cells expressed CD3 as well as the αβ TCR (Fig. 3A), strongly suggesting that these cells were truly T cells. Only 1% of the B220low cells expressed CD19 (Fig. 3B), excluding the possibility that these cells could be B cells. Moreover, of other surface markers normally expressed on conventional T cells, we found that our cell population was CD2−, CD5−, and CD90low (Thy1) (Fig. 3, C and D), while splenic CD3+ cells were CD2+, CD5+, and CD90high (not shown). The NK1.1, associated with NKT cells, a subset of CD3+ T cells, was expressed by only a small fraction of the UL (Fig. 3E).

FIGURE 1. A population of CD3+ B220low T cells dominates the female genital tract. UL were isolated from naive female mice using collagenase. The cells were stained with FITC- or PE-conjugated Abs and analyzed in a FACScan. The UL were compared with lymphocytes from the spleen (SP) and IEL from the same animals. UL: A, B220 (CD45R)-FITC and CD3-PE; B, TCR αβ-FITC and TCR γδ-PE; and C, CD4-FITC and CD8-PE. SP: D, B220-FITC and CD3-PE; E, TCR αβ-FITC and TCR γδ-PE; and F, CD4-FITC and CD8-PE. IEL: G, B220-FITC and CD3-PE; H, TCR αβ-FITC and TCR γδ-PE; I, CD4-FITC and CD4-PE; J, UL stained with isotype control hamster IgG1; K, UL stained with isotype control rat IgG2a; and L, CD3-FITC and CD8-PE on iIEL. The results are representative of at least 20 experiments.

FIGURE 2. CD3+ B220+ T cells are found throughout the genital tract mucosa. Frozen tissue sections of the uterus taken from naive mice were labeled with rat anti-CD3 complex or rat anti-B220 Abs followed by biotin-conjugated rabbit anti-rat IgG and developed using peroxidase and FITC- and PE-labeled streptavidin. Sections from naive mice exhibited no staining. A, CD19-FITC and isotype control mouse IgG1; B, CD19-FITC and rat anti-CD3 complex; C, CD19-FITC and rat anti-B220 Abs. Sections from naive mice exhibited no staining. A, CD19-FITC and isotype control mouse IgG1; B, CD19-FITC and rat anti-CD3 complex; C, CD19-FITC and rat anti-B220 Abs. Sections from naive mice exhibited no staining.
**Extrathymic origin of the dominant CD3⁺ B220⁻⁺ T cell population in the genital tract**

Many of the iIELs are known to develop extrathymically and can be found in the intestine of nude mice, which lack the thymus. To evaluate whether the CD3⁺ B220⁻⁺ UL were independent of the thymus, and whether they required MHC class I, class II, or CD1 for development, we investigated genital tract T cells from nude, MHC class II⁻, β₂-m⁻, and CD1-deficient mice. Unexpectedly, the CD3⁺ B220⁻⁺ UL were found in all four mouse strains to the same extent as in normal wild-type mice (Fig. 4). Importantly, there was no significant difference in the absolute cell numbers between gene-knockout mice and wild-type controls. This strongly suggested that the CD3⁺ B220⁻⁺ UL were extrathymically derived and could develop in the absence of MHC class I, class II, or CD1.

**TCR oligoclonality and idiotypic-specificity in genital tract CD3⁺ B220⁻⁺ T cells from wild-type and TCR-Tg mice, respectively**

To strengthen the notion that the CD3⁺ B220⁻⁺ UL were indeed of T cell origin, we asked whether the UL were oligoclonal with regard to the TCR usage and whether epitope-specific TCR could be found in the CD3⁺ B220⁻⁺ population in the genital tract of TCR-Tg mice. In a normal C57BL/6 mouse spleen, a multiplicity of Vβ chains were used, with Vβ 8.1 and 8.2 representing roughly 18% of the total CD3⁺ T cells (Table I). Also among the UL, we observed a high frequency (33%) of Vβ 8.1- and 8.2-positive cells (Table I). Of the 15 Vβ chains that we screened for, we detected 13 in splenic T cells, while 7 were detectable in UL, indicating that the CD3⁺ B220⁻⁺ UL were of oligoclonal origin (Table I). Furthermore, similar to splenic T cells in DO11.10 OVA TCR-Tg mice (Fig. 5, B and D), we found that the genital tract CD3⁺ B220⁻⁺ UL expressed the Vβ 8.2 TCR (Fig. 5A) and labeled with the clonotype-specific mAb KJ126 (Fig. 5C), whereas Vβ 8.2 was expressed only on a fraction of these cells in a wild-type mouse. Thus, because the Tg TCR is expressed under a T cell promoter (19), these results provided further evidence that the CD3⁺ B220⁻⁺ UL were truly T cells.

**CD3⁺ B220⁻⁺ T cells may be involved in pregnancy but appear not to affect host protection against a genital tract infection**

We and others have previously shown that CD4⁺ T cells are critical in the clearance of a genital tract infection with *C. trachomatis* (3, 25, 26). Very few CD4⁺ T cells were found in the genital tract of naive mice (Fig. 6B). However, at 8 days following infection, we observed an increase in the CD4⁺ and CD8⁺ single-positive populations with a subsequent decrease in the frequency of CD3⁺ B220⁻⁺ cells (Fig. 6C). However, in the infected uterus, more cells accumulated, and therefore the decrease in frequency was not accompanied by a drop in absolute numbers of CD3⁺ B220⁻⁺ cells. After infection, at 20 days, we observed 10% CD4⁺ (Fig. 6D) and 8% CD8⁺ T cells (Fig. 6D) in the uterus. The fact that expression of CD2, CD5, and CD90 also increased following infection (not shown) suggests that the CD4⁺ and CD8⁺ T cells were recruited into the uterus and that the CD3⁺ B220⁻⁺ population took no active part in host protection against *C. trachomatis* disease.

| Table I. Vβ chain distribution |

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<th>Vβ Chain on T Cells</th>
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An alternative function of these cells could be linked to pregnancy. Therefore, we pooled ULs from day-4 pregnant mice. No apparent difference in absolute numbers of CD3⁺B220low UL was observed during early pregnancy (not shown). Neither did we detect any phenotypical difference in the CD3⁺B220low population in pregnant mice compared with naive controls (Fig. 6, E and F). Furthermore, using intracellular staining of cytokines in situ, in tissue sections, we could detect increased production of neither Th1 (IFN-γ) nor Th2 (IL-4 and IL-10) cytokines (not shown).

**Strong down-regulation of T cell responses by the CD3⁺B220low ULs**

Tr cells may exert their function by cell-cell contact-dependent mechanisms rather than by the production of cytokines (27). Expression of CD25 may indicate a regulatory role of T cells. We found that a majority of the CD3⁺B220low population was expressing CD25 on their surface (Fig. 7A), comparable to the levels found on CD25⁺ spleen cells. Although only 7% of the spleen cells express CD25 (Fig. 7A), >30% of the ULs were CD25high. These findings could be indicative of a regulatory role for the CD3⁺B220low ULs.

Next, we assessed the responsiveness of this novel T cell population to polyclonal activation. We found that the CD3⁺B220low T cell population failed to proliferate in response to anti-CD3 or even anti-CD3 plus anti-CD28 mAb stimulation. Despite extended efforts, using even highly enriched CD3⁺B220low cells, we could not detect proliferation at any time. Importantly, the poor proliferation did not correlate with an enhanced frequency of cells undergoing apoptosis following anti-CD3 plus anti-CD28 mAb stimulation as confirmed by TUNEL staining (not shown). Rather, the fact that CD3⁺B220low cells, selected on the basis of B220 expression using beads (>90% purity), produced IFN-γ following activation, suggested a regulatory role for the CD3⁺B220low cells in the genital tract (Fig. 7B). To test this hypothesis, we took advantage of our observation that CD3⁺B220low TCR-Tg T cells were found in the uterus of DO11.10 mice. The responsiveness of the CD3⁺B220low TCR-Tg ULs to anti-CD3, OVA, or specific peptide p323 (not shown), was dramatically impaired (Fig. 7C). More importantly, though, when cocultured with splenocytes from the DO11.10 TCR-Tg mice, the proliferation of the splenocytes was markedly decreased in comparison to that of splenocytes cultured alone (Fig. 7C). The inhibitory effect was not cell-density dependent, but a result of down-regulation of the splenic T cell responsiveness due to presence of graded numbers of inhibitory CD3⁺B220low T cells from the genital tract (Fig. 7C). The down-regulatory effect was roughly 85% with the highest density of CD3⁺B220low cells added to the splenocyte cultures. In these cultures, IFN-γ was detected but not IL-10 or soluble TGF-β (not shown). Taken together, a majority of the CD3⁺B220low cells were CD25⁺ with striking ability to down-regulate splenic T cell responses.

**FIGURE 5.** The CD3⁺B220low T cells in the genital tract express common Vβ TCR chains. Genital tract lymphocytes were isolated from naive female mice. Different T cell populations have been shown to differ in their usage of Vβ-chains. In a normal naive mouse spleen, ~20% of the T cells express the Vβ 8 chain: TCR Vβ 8.1 8.2-FITC and CD3-PE in spleen (B); although the expression was not that defined in the UL, an increase was found: TCR Vβ 8.1 8.2-FITC and CD3-PE in UL (A). In OVA TCR-Tg mice, all CD3⁺ T cells both in the genital tract (C) and in the spleen (D) expressed the Vβ 8.2 chain. Most CD3⁺ T cells could also be detected with the Ab against KJ126, specific for the Tg TCR, in both genital tract (E) and the spleen (F).

**FIGURE 6.** Following infection with C. trachomatis, CD4⁺ and CD8⁺ T cells are found in the genital tract. Genital tract lymphocytes were isolated from infected female mice on day 20 following infection using collagenase. The cells were stained with FITC- or PE-conjugated Abs and analyzed in a FACSscan. In naive mice, the CD3⁺B220low population dominates (A) and no CD4⁺ or CD8⁺ T cells can be found (B). However, 20 days following the onset of chlamydial infection, single-positive CD4⁺ and CD8⁺ T cells (D) can be detected. The results are representative of at least seven experiments. No effect on the CD3⁺B220low population could be seen on day 4 of pregnancy (E), and neither did we find any increase in CD4⁺ or CD8⁺ T cells (F).
responses to Ag and polyclonal activation. These data strongly suggest that the CD3+CD25low T cells are a unique and novel mucosal Tr cell population located in the female genital tract.

**Discussion**

In the present study, we report on a novel population of CD3+CD4−CD8− lymphocytes expressing the B cell marker B220. The CD3+CD25low cells appear to belong to the T cell lineage, because they express the αβ TCR. Moreover, using TCR-Tg mice, we could demonstrate that clonotype-specific KJ-126 CD3+CD25low cells were present in the genital tract of these mice and expressed the Vβ8 chain used by the Tg TCR+ cells. In normal mice, the CD3+CD25low cells were found to be oligoclonal with regard to Vβ chain usage, expressing 7 of the 15 Vβ-chains that we screened for. The CD3+CD25low cells were found in nu/nu, MHC II-, β2-m-, and CD1-deficient mice, indicating that they constitute an extrathymically derived T cell population.

It is well recognized that, because of the unique ability both to protect against infections and to tolerate the semiallogenic conceptus during pregnancy, the female genital tract mucosa host highly specialized cell populations not found elsewhere. The genital tract mucosa of healthy individuals has relatively few T and B cells. This is in contrast to most other mucosal sites, e.g., the intestine where large numbers of T cells can be found. Furthermore, the genital tract mucosa differs from other mucosal surfaces in that it is under hormonal control, which has been shown to influence the presence and distribution of lymphocytes and other cells in the uterus and vagina (6, 28). Although cells like macrophages and dendritic and NK cells reside in the uterus, the CD3+CD4−CD8− T cell population described in the present study accounted for a majority of the lymphocytes found. These cells did not proliferate to Ag or polyclonal activators but produced cytokine, and a large proportion of them expressed CD25. The presence of specialized cells in the genital tract mucosa is not surprising. Rather it is likely that these T cells play a regulatory role and host unique functions in the genital tract mucosa.

The CD3+CD4−CD8− TCRβμB220low UL found in the present study have, to our knowledge, not been described previously. Although the phenotype may in part resemble other recently reported cell populations, the distribution of cell surface markers differs from those of previously described populations. Whereas the ULs all expressed the αβ TCR, 30−50% of iIELs express the γδ TCR (29) and only a small population of these are double-negative T cells (30, 31). The fact that the CD3+CD4−CD8− T cell population described in the present study accounted for a majority of the lymphocytes found. These cells did not proliferate to Ag or polyclonal activators but produced cytokine, and a large proportion of them expressed CD25. The presence of specialized cells in the genital tract mucosa is not surprising. Rather it is likely that these T cells play a regulatory role and host unique functions in the genital tract mucosa.

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A literature survey finds some resemblance between the CD3+CD25low ULs and other cell populations. The ULs were selected on the basis of their expression of B220, the full-length isofrom of the tyrosine phosphate CD45, which is predominantly found on B cells. Other isoforms of CD45 are found on most
lymphocytes, but recent studies suggest that B220 may be up-regulated on T cells undergoing apoptosis. This has been reported in thymocytes following irradiation (33), as well as in peripheral T cells and CD8+ T cells following activation (34). In the (prf1/gld) mouse (35), Mixter et al. (36) found a large proportion of the CD3+ cells that were also B220+, which accumulated in these animals and were not undergoing apoptosis. Thus, an emerging theory argues that expression of B220 on T cells may be a pre-conditioning stage for Fas-mediated apoptosis (33). However, using TUNEL staining on frozen sections of the uterus, we could not detect increased levels of apoptotic CD3+ cells in the genital tract compared with spleen or gut intestine (M. Johansson, unpublished observation). B220+CD19- cells have previously been noted in the murine female genital tract (37) but not further investigated. Moreover, a phenotypically unusual T cell population in the vaginal tissue was described by Wormley et al. (38), using a special mAb identifying an atypical form of CD4 (11). The CD4+ cells in the vagina were detected using 2B6 but not GK1.5, two epitope-different anti-CD4 Abs that are both recognized by splenic CD4+ T cells. However, not even with this clone (2B6) did we find any CD4+ T cells in the uterus (M. Johansson, unpublished observation). Based on this and the other observations, we would rather suggest that the CD3+B220- cells in the genital tract constitute a novel T cell population with regulatory function.

The role of various lymphocyte subtypes in pregnancy is still unclear. In both humans and rodents, nT cells have been demonstrated in the decidua following implantation (15, 39). Like the ULs, the nT cells are present in both male and SCID mice suggesting an extrathymic development (8). However, unlike the ULs, these cells do not express CD3 on their surface, although cytosolic CD3 has been detected (8, 15). The nT cells appear to express some T cell markers, such as CD2, CD7, and CD38, but are deficient in others, such as CD4, CD5, or CD8 (8, 15). By comparison the CD3+B220 of ULs were clearly negative for CD2, CD4, CD5, CD7 (M. Johansson, unpublished observation), and CD8. Furthermore, in the peri-implantation uterus, the presence of large numbers of other NK-like cells, expressing CD3, but not CD4 or CD8, with the same intermediate expression of TCR as our CD3+B220 population was described in pregnancy (35). In contrast to both the NKT and uNK cells, the NK1.1 marker was not expressed on the CD3+B220 ULs. Of note, it is still poorly understood how the induction of tolerance is established in pregnancy. However, it is likely that T cells unique for the genital tract mucosa are involved. An analysis of the uterus from day-4 pregnant mice demonstrated no apparent phenotypical differences in the CD3+B220 population. Neither was the absolute number of these cells increased or decreased following immunization. However, this does not exclude a function for the ULs in pregnancy. Although uNK cells, for example, have been identified in both mice and humans, they show some discrepancies in their surface markers. Whether the CD3+B220 cells described in the present study also are present in the human uterus remains to be investigated.

The expression of CD25 and the inability to proliferate suggest a more regulatory role for the CD3+B220 cells. Some cells with known regulatory function, such as the CD4+CD25+ cells, have been found to exert contact-dependent down-regulatory effects on the microenvironment rather than cytokine-mediated effects. Although the CD3+B220 UL produced low levels of IFN-γ upon stimulation, we hypothesized that they were more likely to act through contact-dependent mechanisms. This was confirmed in the coculture experiments with Ag or polyclonal stimulation of splenic T cells, where the CD3+B220 cells were much more inhibitory compared with the low level of IFN-γ that we could detect. Neither did we detect soluble IL-10 or TGF-β in the cultures. Although we did not attempt to detect cell membrane-bound TGF-β, as was recently documented to play an inhibitory role (40), we speculate that this or some other mechanism for contact-dependent down-regulation is operational in the activities of the CD3+B220 ULs. Such a possible regulatory effect by the CD3+B220 ULs would be difficult to detect in the pregnant mice, which could explain why the causative agent of tolerance in pregnancy is still unknown.

In the present study, we also hypothesized that the CD3+B220 ULs could be involved in protection against STDs. We found that, following infection with C. trachomatis, no apparent change within the CD3+B220 UL population or other evidence for the involvement of these cells in protective immunity was observed. However, following a genital tract infection with C. trachomatis, we did find CD4+ T cells in the uterus. Accumulation of leukocytes and lymphoid aggregates has been reported following infection (41) or in seminiferous (42) in the uterine endometrium, and these aggregates or infiltrates contained T cells that were recruited to the genital tract mucosa as a result of inflammation. We believe that the CD4+ T cells we observed in the genital tract of infected animals were, indeed, recruited into the mucosa, and not derived from the local CD3+B220 T cells. However, this remains to be proven.

Despite several attempts to stimulate the CD3+B220 ULs, we were unable to detect significant proliferation at any time. Following TCR stimulation in the presence of anti-CD28, the positively selected CD3+B220 cells produced some IFN-γ, indicating that these cells were functionally active. In the last few years, much interest has been focused on Tr cells mediating tolerance and suppressing autoimmune diseases. We found that coculturing of UL with splenocytes led to an inhibition of T cell proliferation. This was not due to high density, because cultures of equal density without CD3+B220 ULs showed no inhibition of proliferation. The inhibitory effect was rather a direct effect of the CD3+B220 cell density. Taken together, these results suggest a regulatory function for the CD3+B220 ULs. Although most studies on Tr cells report on CD4+CD25+ cells, some documentation on other populations with regulatory function can be found. A novel CD3+αβTCR+CD4+CD8- T cell population with regulatory function was recently described by Zhang et al. (43, 44). These Tr cells expressed IFN-γ, TGF-β, and TNF-α, and could inhibit T cell function in an Ag-specific manner. The latter Tr cells showed some resemblance to the CD3+B220CD4+CD8-αβTCR int cells, which are described in this study and which expressed CD25 on their surface in support of our hypothesis that cells with this phenotype have a regulatory function. We speculate that the CD3+B220 cells are functionally active and constitute a unique subpopulation with regulatory function in the female genital tract.

References


