Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice

Gabrielle L. Goldberg, Jarrod A. Dudakov, Jessica J. Reiseger, Natalie Seach, Tomoo Ueno, Katerina Vlahos, Maree V. Hammett, Lauren F. Young, Tracy S. P. Heng, Richard L. Boyd and Ann P. Chidgey

*J Immunol* 2010; 184:6014-6024; Prepublished online 5 May 2010;
doi: 10.4049/jimmunol.0802445
http://www.jimmunol.org/content/184/11/6014

**References** This article cites 67 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/184/11/6014.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Sex Steroid Ablation Enhances Immune Reconstitution Following Cytotoxic Antineoplastic Therapy in Young Mice

Gabrielle L. Goldberg,1 Jarrod A. Dudakov,1,2 Jessica J. Reiseger, Natalie Seach, Tomoo Ueno, Katerina Vlahos, Maree V. Hammett, Lauren F. Young, Tracy S. P. Heng, Richard L. Boyd,3 and Ann P. Chidgey3

Cytotoxic antineoplastic therapy is used to treat malignant disease but results in long-term immunosuppression in postpubertal and adult individuals, leading to increased incidence and severity of opportunistic infections. We have previously shown that sex steroid ablation (SSA) reverses immunodeficiencies associated with age and hematopoietic stem cell transplantation in both autologous and allogeneic settings. In this study, we have assessed the effects of SSA by surgical castration on T cell recovery of young male mice following cyclophosphamide treatment as a model for the impact of chemotherapy. SSA increased thymic cellularity, involving all of the thymocyte subsets and early T lineage progenitors. It also induced early repair of damage to the thymic stromal microenvironment, which is crucial to the recovery of a fully functional T cell-based immune system. These functional changes in thymic stromal subsets included enhanced production of growth factors and chemokines important for thymopoiesis, which preceded increases in both thymocyte and stromal cellularity. These effects collectively translated to an increase in peripheral and splenic naive T cells. In conclusion, SSA enhances T cell recovery following cyclophosphamide treatment of mice, at the level of the thymocytes and their stromal niches. This provides a new approach to immune reconstitution following antineoplastic therapy.


Received for publication July 28, 2008. Accepted for publication March 25, 2010.

This work was supported by grants from Norwood Immunology, the Australian Stem Cell Centre, and the National Health and Medical Research Council. J.A.D. was supported by a fellowship from the Cancer Council of Victoria.

Address correspondence and reprint requests to Dr. Gabrielle L. Goldberg at the current address: Baylor Institute of Immunology Research, 3434 Live Oak Street, Dallas, TX 75204, and Dr. Ann P. Chidgey, Monash Immunology and Stem Cell Laboratories, Monash University, Wellington Road, Clayton 3800, Australia. E-mail addresses: gabbygoldberg@gmail.com and ann.chidgey@med.monash.edu.au

Abbreviations used in this paper: CLP, common lymphoid progenitor; cTEC, cortical thymic epithelial cell; Cy, cyclophosphamide; DP, double positive; ETP, early T-lineage progenitor; Fgf, fibroblast growth factor; LHRH, luteinizing hormone-releasing hormone; mtTEC, medullary thymic epithelial cell; SP, single positive; SSA, sex steroid ablation; TEC, thymic epithelial cell; TN, triple negative; Treg, regulatory T cell; UEA-1, Ulex europaeus agglutinin 1.
and chemokines by thymic epithelial cells (TECs) and an increase in TEC-specific growth factors by CD45– nontumoral stromal cells (non-TECs)—apparent in both SSA-treated and sham control mice. Thymic stromal cells in the SSA-treated group, however, showed an enhanced increase in the production of TEC-related growth factors, such as fibroblast growth factor (Fgf) 7 and Fgf10, and chemokines important for thymocyte trafficking through the thymus, facilitating the kinetics of thymocyte and epithelial cell recovery above that of the sham SSA-treated group. SSA as a treatment is readily applicable in the clinic, through the use of luteinizing hormone-releasing hormone (LHRH)/gonadotropin-releasing hormone to chemically and reversibly reduce sex steroids in a number of clinical conditions. LHRH agonist treatment has been standard of care for many millions of patients with sex steroid-exacerbated conditions, such as prostrate and some breast cancers, endometriosis, and precocious puberty. If successful, then SSA should reduce the morbidity and mortality due to infection and even recurrence of malignant disease, thereby improving patient survival following antineoplastic therapy.

Materials and Methods

Mice and Cy treatment
Male C57BL/6J 8- to 12-wk-old male mice were obtained from the Baker Institute Precinct Animal Centre (Melbourne, Victoria, Australia), University of Adelaide (Adelaide, South Australia, Australia), or the Walter and Eliza Hall Institute (Melbourne, Victoria, Australia). Mice were administered with a total of 200 mg/kg Cyclophosphamide (Pharmacia, Sydney, Australia) (Cy) via i.p. injection over 2 d.

Surgical castration
Mice were anesthetized and a small scrotal incision was made to reveal the testes. These were tied off and removed along with surrounding fatty tissue. The wound was closed using surgical staples. Sham SSA was performed using the same surgical procedure, without removal of the testes. SSA was performed on the second day of Cy treatment.

Flow cytometric analysis of leukocytes
Splenocytes or thymocytes were washed in FACS buffer (PBS/1% v/v FCS/0.1% w/v azide), and 1–3 × 10^6 cells were incubated for 30 min at 4°C with primary Abs (and anti-FcR) then washed twice with FACS buffer. Where necessary, cells were incubated with appropriate streptavidin conjugates for a further 30 min at 4°C. Stained cells were resuspended in FACS buffer and analyzed on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) with CellQuest software.

Flow cytometry reagents
The following fluorochrome-labeled or biotinylated Abs against murine Ags (as well as the appropriate isotype controls) were obtained from BD Pharmingen (San Diego, CA) unless otherwise stated: CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8β2, (clone 53-5.8), TCRβ (clone H57-597), NK1.1 (clone PK136), CD45R/B220 (clone RA3-6b2), CD11b (clone M1/70), Ly-6G/Ly-6c (Gr-1) (clone RB6-8C5), CD117 (c-kit) (clone 2B8), Sca-1 (clone D7), CD11c (clone HL3), MHC-II IA/IE (clone M1/70), Ly-6G/Ly-6c (Gr-1) (clone RB6-8C5), CD25 (clone PC61), CD44 (clone IM7). Triple negative (TN) thymocyte lineage mixture consisted of CD3, CD4, CD8β2, CD45R/B220, CD11b, and Gr-1. Early T cell progenitor lineage mixture consisted of CD3, CD8β2, CD45R/B220, CD11b, and Gr-1. Early T cell progenitor lineage mixture consisted of CD3, CD8β2, CD45R/B220, CD11b, and Gr-1. Early T cell progenitor lineage mixture consisted of CD3, CD8β2, CD45R/B220, CD11b, CD11c, Gr-1, NK1.1, CD127, and CD25. Common lymphoid progenitor (CLP)-2 lineage mixture consisted of CD3, CD4, CD8β2, CD45R/B220, CD11b, CD11c, Gr-1, NK1.1, CD127, and CD25. Common lymphoid progenitor (CLP)-2 lineage mixture consisted of CD3, CD4, CD8β2, CD45R/B220, CD11b, CD11c, Gr-1, and CD127. Streptavidin-FITC, PE, PerCP, and allophycocyanin also were obtained from BD Pharmingen. Anti-IL-7Ra (A7R34) Ab was obtained from Chemicon International (Temecula, CA). Anti-murine FcR block (2.4G2) was originally obtained from the Shortman laboratory at the Walter and Eliza Hall Institute. Stromal cell Abs are biotinylated anti-EpCAM (G8.8a; a gift from Dr. A. Farr, University of Washington, Seattle, WA), PE-conjugated anti-Ly51 (6C3), PE-conjugated anti-I-A/I-E (M5/14.15.2), and allophycocyanin-conjugated anti-CD45 (30-F11). Secondary reagent is streptavidin–allophycocyanin. The lectin Ulex europaeus agglutinin 1 (UEA-1), conjugated to FITC, was purchased from Vector Laboratories (Burlingame, CA). Vβ TCR usage was assessed using TCR Vβ Screening Panel (BD Pharmingen). Intracellular FoxP3 staining was performed using the PE-conjugated anti-FoxP3 (FJK-16) staining kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Individual thymic stromal cell isolation by enzymatic digestion
This protocol was performed as previously described in Gray et al. (41). Briefly, individual thymus were digested in collagenase/dispase/DNase (Roche, Basel, Switzerland), and the resulting suspensions were passed through a 100-μm mesh to remove debris. All of the fractions from each thymus were pooled and counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). For flow cytometric analysis, 5 × 10^4 cells from each individual thymus were stained with appropriate Abs.

Quantitative PCR analysis

T cell proliferation assay
Splenocytes (5 × 10^6 cells per well) were stimulated with plate-bound αCD3 (clone 145-2c11) at concentrations of 10, 5, 2.5, and 1.25 μg/ml and αCD28 (clone 37.51) at a concentration of 5 μg/ml. In the assay using purified T cells, T cells were isolated using CD5 microbeads (Miltenyi Biotec, Auburn, CA) and were ≥90% pure. Cells were cultured for 4 d and pulsed with 1 μCi per well [^3H]thymidine for the last 18 h. Cells were harvested on a TopCount harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Statistical analysis
All of the values are expressed as mean ± SEM. Statistical analysis was performed by testing with the nonparametric, unpaired Mann-Whitney U test using Prism (GraphPad, San Diego, CA) or SPSS software (SPSS, Chicago, IL). A p value of <0.05 was considered statistically significant.

Results

Young mice were surgically castrated and treated with a common clinically therapeutic dose of Cy (200 mg/kg) to assess the effects of SSA on immune reconstitution following cytotoxic antineoplastic therapy. Mice 8- to 12-wk-old were chosen as representative of postpubertal/young adult patient groups.

SSA enhances thymic recovery following Cy treatment of young mice
Thymic cellularity was greatly decreased following Cy treatment of young mice, as expected, but as early as 7 d after SSA and Cy treatment, it had returned to pretreatment levels, whereas the sham SSA (i.e., full anesthetic and surgery but no removal of gonads) mice remained significantly lower than both untreated and Cy-treated, SSA mice (Fig. 1A). By day 14, the thymic cellularity of Cy-treated, sham SSA mice had also returned to untreated levels, reflecting the young age (and hence strong residual thymic function) of the mice. Thymic cellularity of SSA mice at the same time point was significantly higher than those of both untreated and Cy-treated, sham SSA mice and remained so for up to 42 d after treatment (Fig. 1A). We further examined thymic reconstitution by analyzing thymocyte subsets. Double-negative (CD4–CD8–) thymocytes are significantly increased in the SSA group 14 and 28 d after Cy treatment (Fig. 1B). Double positive (DP) (Fig. 1C), CD4 single positive (SP) (Fig. 1D), and CD8 SP (Fig. 1E) thymocytes were significantly increased in the Cy-treated SSA group at all of the time points when compared with those of the Cy-treated sham SSA controls.
To address whether reconstitution is instigated at the level of intrathymic progenitor cells, we analyzed the CD3^+CD4^-CD8^- (TN) subsets. TN1 cells (CD44^+CD25^-) were significantly increased in the Cy-treated SSA mice at 14 and 28 d after treatment compared with those in sham SSA controls (Fig. 2A). The earliest thymic T cell progenitor identified to date is the Lin^IL-7R^+CD25^+CD44^-CD117^- early T-lineage progenitor (ETP) (43). ETPs were significantly increased, as a proportion of TN1 cells, at days 7, 14, and 42, and numerically from day 14 after Cy treatment (Fig. 2B, 2C). ETP numbers were also significantly higher than those of untreated controls 14 d after SSA/Cy treatment. The SSA-induced increase in thymic cellularity and its maintenance may be thus due, in part, to an increase in ETP immigration or proliferation. However, the initial delay in ETPs indicates that it may not be a key triggering event. CLP-2 cells are defined as Lin^IL-7R^+B220^+cKit^- preTee (44). A population of intrathymic progenitors identified as Lin^IL-7R^+B220^+cKit^-, a subset containing the CLP-2 cell, were significantly increased in SSA mice 7, 28, and 42 d after Cy treatment compared with that in sham SSA mice (Fig. 2D). Transitional, TN1–2 cells (Fig. 2E) and TN4 cells (Fig. 2H) were also increased significantly in the SSA group 7, 14, and 28 d after Cy treatment, whereas TN2 (Fig. 2F) and TN3 (Fig. 2G) cells were increased at all of the time points following treatment in the SSA group.

Epithelial (TEC) and nonepithelial (non-TEC) thymic stromal cells provide essential signals to developing thymocytes. Conversely, thymocytes are essential for proper stromal cell development and differentiation; this dual reliance is often referred to as “cross talk” (45). We therefore analyzed the impact of Cy on thymic stromal cells and whether SSA could expedite reconstitution.

Treatment with Cy depletes thymic stromal cells and SSA promotes their regeneration

In addition to having a profound effect on thymocytes, Cy treatment also caused a major loss in TECs, gated as CD45^+MHC class II^ (Fig. 3A). UEA-1^+ medullary TECs (mTECs), in particular, were more sensitive than Ly51^+ cortical TECs (cTECs) (Fig. 3A); the ratio of mTECs to cTECs decreased from ~2.5:1–1:1 at day 3 after Cy treatment (Fig. 3B). Interestingly, at day 28, and reducing slightly at day 42, the mTEC-to-cTEC ratio increased above the control levels; this is consistent with a homeostatic response of the SSA group also caused a major loss in TECs, gated as CD45^+MHC class II^ (Fig. 3A). UEA-1^+ medullary TECs (mTECs), in particular, were more sensitive than Ly51^+ cortical TECs (cTECs) (Fig. 3A); the ratio of mTECs to cTECs decreased from ~2.5:1–1:1 at day 3 after Cy treatment (Fig. 3B). Interestingly, at day 28, and reducing slightly at day 42, the mTEC-to-cTEC ratio increased above the control levels; this is consistent with a homeostatic response of the TECs to the hypertrophy of total thymocytes (Fig. 3B). Consistent with the effects of Cy on cycling cells (given its usage as an anticancer therapy), it caused a major loss of Ki67^+ mTECs, cTECs, and non-TEC stromal cells. All of these subsets had spontaneously recovered their proliferative capacity to untreated levels by day 7, but this was significantly increased with SSA (Fig. 3C).

Numerically, TECs as a population were significantly depleted by Cy, but the SSA group showed hypertrophy at days 14 and 28. Of these TECs, mTECs were the most severely depleted, barely recovering to the control untreated level by day 42. With SSA, however, TEC numbers were significantly increased by day 10, reaching untreated levels by day 14 and surpassed by day 28 (Fig. 3C). cTEC numbers were also depleted but recovered by day 7 in both control and SSA-treated animals, although the SSA group was significantly greater at days 10, 14, and 28 (Fig. 3C).

Non-TECs were also depleted by Cy treatment but to a much lesser extent and had recovered by day 10 (Fig. 3C). SSA again induced elevated levels of these cells, consistent with the increase in TECs and thymocytes, thereby maintaining the normal ratio of these cells relative to the other thymus subsets. With Ki67 as a measure of proliferation, mTECs, cTECs, and non-TEC stromal cells from SSA mice were all proliferating significantly more than those of sham SSA mice 10 d after Cy treatment (Fig. 3B). Return to untreated mTEC-to-cTEC ratios is therefore likely to be influenced by both the increase in SP mature thymocytes, evident from day 7 after SSA and the increased proliferation of mTECs from day 10.

### Table I. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL19</td>
<td>GCTAATGATCGGAGGAAGACTG</td>
<td>ACTCACTGAGCATCTTTCAG</td>
</tr>
<tr>
<td>CCL21</td>
<td>GACGTTGAGGAGGGGTTTACG</td>
<td>CGGGTGAGAGAACAGATGTG</td>
</tr>
<tr>
<td>CCL25</td>
<td>TGAACATTGCGCTTTTGGCC</td>
<td>GTGAAGATTTCTTCAG</td>
</tr>
<tr>
<td>CXCL12</td>
<td>GCTCTGCATCAAGCTAGGCAGTA</td>
<td>TGCTGCTGCTGCTGCTGCTGAC</td>
</tr>
<tr>
<td>IL-6</td>
<td>GGTAGTAGTATGAGGGTTGAGG</td>
<td>TGGCTGAGGCTGGTTG</td>
</tr>
<tr>
<td>SCF</td>
<td>AGAAGACAGACGACTAGAAC</td>
<td>CAGATTCCAGCAGAAAGAGAC</td>
</tr>
<tr>
<td>FGF7</td>
<td>GCCACATGGAAGACTAGCAGACTG</td>
<td>TGACGATGTTGAGAGGAGGACTG</td>
</tr>
<tr>
<td>FGFI0</td>
<td>GACACAGAGCAGAGAGACTG</td>
<td>TGGATGAGGCTGAGGAGGAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCATGTAGGTGAGGGTCAATGAAG</td>
<td>GGTTGAGGCTGAGGAGGAC</td>
</tr>
</tbody>
</table>

Growth factor and chemokine production by stromal cells after SSA

cTECs, mTECs, and non-TEC stromal cell-derived chemokines and growth factors (Table I) were analyzed at various early time points after chemotherapy in SSA and sham SSA control mice to determine their impact on thymic regrowth (Fig. 4). Chemotherapy induced an early increase in T cell growth factors, such as IL-7, up to a 10-fold increase by day 2, and an increase in chemokines involved in thymocyte trafficking through the functionally distinct areas of the thymus, such as CXCL12, CCL25, CCL21, and CCL19. The majority of growth factors tested were not significantly different when comparing sham SSA and SSA groups of cTECs and mTECs throughout regeneration following Cy treatment. However, SSA enhanced the early increases in Fgf7 (KGF), CCL19, and CXCL12 compared with those in the sham controls. cTEC IL-6 expression was also significantly increased in SSA mice at day 28 (Fig. 4A).

**SSA increases splenic cellularity and T cell numbers following Cy treatment**

Previous studies that have shown that thymic export is proportional to thymic size (46). This would predict that SSA following Cy treatment should increase thymic export, leading to more rapid peripheral T cell reconstitution. After Cy treatment, there was evidence of splenomegaly after 7 d, within both SSA and sham SSA control groups (Fig. 5A). This no doubt reflects a global nonlymphoid response to the cytotoxicity. From day 14 onwards, the SSA group continued to have an increased splenic cellularity compared with that of the sham SSA group (Fig. 5A). Analysis of the subsets demonstrated that this involved splenic increases in CD4^+ and CD8^+ T cells in SSA mice 28 and 42 d after Cy

Downloaded from http://www.jimmunol.org/ by guest on April 13, 2017
treatment (Fig. 5B). In particular, naive (CD62L+CD44−) and memory (CD62L−CD44+) CD4+ and CD8+ T cells were both significantly increased at 14 and 28 d following SSA and Cy treatment. Interestingly, the naive-to-memory T cell ratio was significantly decreased following Cy treatment. SSA reversed this in both CD4+ and CD8+ T cells as early as 14 d after treatment.

**SSA enhances splenic T cell proliferation without perturbing Vβ usage**

Splenic T cell proliferation was measured using thymidine incorporation following αCD3/αCD28 stimulation (Fig. 6A, 6B). Fourteen days after Cy treatment, there was no significant difference in the proliferation of T cells from SSA and sham SSA control mice (Fig. 6A). By day 42, T cells from SSA mice proliferated significantly more than those from the sham SSA group, appearing functionally similar to those of untreated mice (Fig. 6A). To further confirm the functionality of T cells following SSA and Cy treatment, T cells were isolated by CD5 purification from spleens of SSA and sham SSA control mice at day 42. On a per T cell basis, after isolation of T cells, there was no significant difference in T cell proliferation (Fig. 6B).

To ensure that the increased proliferation observed was not due to peripheral clonal expansion of T cells, the Vβ repertoires of untreated, Cy-treated/SSA, and Cy-treated/sham SSA were assessed by flow cytometry (Fig. 6C). Although there were slight (albeit significant) differences observed among the three groups, overall there were no major perturbations in Vβ usage.

**SSA enhances splenic regulatory T cell and NKT cell reconstitution after Cy treatment**

Splenic CD4+CD25+FoxP3+ regulatory T cells (Tregs) were significantly increased in SSA mice 28 and 42 d after Cy treatment when compared with those of sham SSA Cy-treated mice (Fig. 6D). CD4+CD8− NKT cells were significantly increased 28 and 42 d after Cy treatment in SSA mice, whereas CD4+ NKT cells were only increased at day 42 (Fig. 6E).

**Discussion**

The present study shows that inhibition of sex steroids can significantly enhance thymic reconstitution following Cy treatment. Clinically, improved restoration of the immune system could be expected to decrease the incidence of infections and reduce the
chance of malignant relapse, leading to enhanced overall survival following antineoplastic therapy. Re-establishment of normal T cell function is a necessary component of the restoration of immune responsiveness following cytotoxic antineoplastic therapy. For this to occur, thymic production of naive T cells of a varied TCR repertoire is essential (47–49). Age-related thymic atrophy, which becomes most pronounced after puberty, and the subsequent decline in thymic output severely retard this process (6, 50). This does not only affect older patients; impaired T cell reconstitution has been observed in children as young as 10 y of age (51), and T cell reconstitution takes significantly longer in 18- to 24-y-old patients compared with those under 18 y of age (12). We have found that SSA, following Cy treatment, significantly enhances thymic regeneration and T cell reconstitution. Thymic cellularity was increased, as were all of the thymocyte subsets (TN, DP, and SP). We also showed that within the stromal niche Cy treatment caused a selective loss of mTECs and that SSA enhanced early production of growth factors that contribute to both thymocyte and stromal cell regeneration.

Previous studies from our laboratory showed that Cy treatment preferentially depletes ETPs (38). In this study, we have shown that SSA following Cy treatment led to a striking increase in ETPs as early as 14 d after treatment. The effect appears to be prolonged with ETP numbers significantly elevated as late as 42 d after treatment. This may have implications for the described defects in ETP number, proliferation, and survival observed with age (52). Accordingly, Min et al. (52) suggested that age-related thymic atrophy is at least in part due to a decline in these early thymic progenitors with age. Indeed, the changes observed in the thymic microenvironment may be triggered by the age-related decrease in mature thymocytes. If this is the case, then the increase in ETP numbers observed with SSA may play an important role in triggering global thymic reconstitution. CLP-2 cells are a subset of lymphoid precursors that have been shown to efficiently seed the thymus but lack a robust T cell potential and may be involved in noncanonical T cell development pathways in a damaged model (53) In this study, we show that the CLP-2 population also significantly increased following Cy treatment and SSA. Less is

FIGURE 2. Reconstitution of early thymocyte progenitors from treatment with Cy is enhanced by SSA. Untreated, age-matched control mice (white), Cy-treated sham SSA mice (gray), and Cy-treated SSA mice (black). TN1 numbers (A), percentage ETPs of TN1s (B), ETP numbers (C), CLP-2s (D), TN1–2 numbers (E), TN2 numbers (F), TN3 numbers (G), and TN4 numbers (H). Results are expressed as the combined mean ± SEM of 10–15 mice for each group representing at least two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001, represent significant differences when comparing sham SSA and SSA treatment groups. ^ or #p < 0.05; ^^ or ##p < 0.01; ^^^ or ###p < 0.001, represent significant differences when comparing either treatment group to the untreated, age-matched control.
FIGURE 3. Withdrawal of sex steroids in Cy-treated mice enhances thymic stromal cell recovery. Untreated, age-matched control mice (white), Cy-treated sham SSA mice (gray), and Cy-treated SSA mice (black). Representative dot plots of thymic epithelial subsets and UEA-1+ mTECs and Ly51+ cTECs (A), mTEC-to-cTEC ratios and stromal cell proliferation as measured by Ki67 expression (B), and stromal cell numbers (C). Results are expressed as the combined mean ± SEM of 5–10 mice for each treatment group representing one or more independent experiments. *p < 0.05; **p < 0.01, represent significant differences when comparing sham SSA and SSA treatment groups. ^ or #p, 0.05; ^^ or ##p, 0.01, represent significant differences when comparing either treatment group to the untreated, age-matched control.
known about these cells in the context of aging and thymic atrophy, but again an increase in these cells may, in part, be responsible for the enhanced thymic regeneration following Cy treatment and SSA.

We have shown that thymic stromal cells are sensitive to Cy treatment, with mTECs appearing to be the most susceptible. This has important clinical implications, such as compromised presentation of peripheral Ags, leading to the potential escape of autoreactive clones. By day 42, mTEC numbers in both SSA and sham SSA control mice had returned to untreated levels. Interestingly, the significant increase in Ki67 staining in cTECs and non-TEC stromal cells at day 7 preceded that seen in mTECs at day 10.

Earlier transplant studies, using androgen-resistant testicular feminization mice (tfm) mice, suggest that thymic involution is predominantly due to the interaction of sex steroids with androgen receptors on stromal cells (54). To date, it remains unclear how SSA acts through stromal cell factors or those produced by the hematopoietic compartment. Expression of growth factors and cytokines increased early after Cy treatment regardless of the sex steroid levels, suggesting that it was the lymphopenic state that induced these gene expression changes. SSA did not appear to have any major impact on the TEC subsets, other than a late increase in IL-6 expression. Although fibroblasts produce IL-6 to a greater extent than TECs (55), cTEC production of IL-6 was substantially increased at day 28 in the SSA group. There are contradicting reports as to the function of IL-6 within the thymus. Early studies showed that IL-6 expression promotes the survival and proliferation of developing thymocytes (56, 57) and a critical role in the development and homeostasis of both epithelial cell meshwork and lymphoid lineage, thus regulating the maintenance of thymic architecture (58). Although Sempowski et al. (59) showed that i.v. injection of IL-6 led to thymic atrophy, this may be a reflection of the impact of IL-6 in the different thymic compartments and stages of thymocyte differentiation. Furthermore, the

FIGURE 4. Molecular profile of cTECs, mTECs, and non-TEC stromal cells following SSA and Cy treatment in young mice. Black circles and solid line represent SSA Cy-treated mice. Gray squares and dashed line represent sham SSA Cy-treated mice. Target gene expression levels relative to those of GAPDH were determined using the $2^{-\Delta\Delta CT}$ method. cTECs (A), mTECs (B), non-TECs (C). *$p < 0.05$; **$p < 0.01$, represent significant differences when comparing sham SSA and SSA treatment groups.
FIGURE 5. Splenic cellularity and T cell numbers were increased in young mice following SSA and Cy treatment. Untreated, age-matched control mice (white), Cy-treated sham SSA mice (gray), and Cy-treated SSA mice (black). Splenic cellularity (A) and T cell subsets (B). Total TCRβ+CD4+, total TCRβ+CD8+, naive CD4+ and CD8+, memory CD4+ and CD8+, and naive-to-memory ratios. Results are expressed as the combined mean ± SEM of 10–15 mice for each group representing two or more independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001, represent significant differences when comparing sham SSA and SSA treatment groups. ^ or #p < 0.05; ^^ or ##p < 0.01; ^^^ or ###p < 0.001, represent significant differences when comparing either treatment group to the untreated, age-matched control.
FIGURE 6. Splenic T cell function was enhanced following Cy treatment and SSA. NKT cell and Treg numbers were increased following treatment. Untreated, age-matched control mice (white, open triangle and short dashed line), Cy-treated sham SSA mice (gray, gray square and long dashed line), and Cy-treated SSA mice (black, black circle and solid line). α-CD3/α-CD28 plate-bound proliferation assay 14 and 42 d after (A), α-CD3/α-CD28 plate-bound proliferation assay of CD5 purified T cells at 42 d (B), TCR Vβ repertoire or CD4+ and CD8+ splenic T cells 42 d after treatment (C), FoxP3+ Treg numbers and ratio of Tregs to CD4 T cells (D), and NKT cell numbers (E). Results are expressed as the combined mean ± SEM of 10 mice for each group representing at least two independent experiments. *p < 0.05; **p < 0.01, represent significant differences when comparing sham SSA and SSA treatment groups. ^p < 0.05; ^^p < 0.01, represent significant differences when comparing either treatment group to the untreated, age-matched control.
expansion of IL-7–dependent TN subsets TN2 and TN3 occurs in the absence of an increase in IL-7 levels. These data together suggest that the increases observed in these subsets are IL-7–independent.

Non-TEC stromal cells produce growth factors important to epithelial cell survival and expansion, such as Fgf-7 (KGF) (60) and Fgf-10 (61). Fibroblasts also produce chemokines essential for thymocyte trafficking through the various functional compartments, such as CXCL12, CCL19, and CCL21 (62). In this study, there was an early increase in the production of these growth factors and chemokines by non TECs, slightly greater in SSA groups (Fgf-7, CXCL12, and CCL19). Enhanced production of chemokines by stromal cells after SSA may improve intrathymic trafficking of developing thymocytes, effectively “opening up” niches for continued developmental progression after Cy-induced damage. Long lasting more global thymic effects beyond the return of stromal growth factor production to homeostatic levels are likely sustained by overall increases in niche cellularity. We therefore suggest that SSA acts at several levels, influencing both the hematopoietic and stromal compartments.

Thymocyte development, from the earliest progenitor to mature SP cells, takes 3–4 wk in young adult mice (63, 64). It is therefore not surprising that we do not observe increases in splenic T cell numbers at the early time points. By day 28, however, there are significantly more CD4+ and CD8+ splenic T cells in the SSA group after Cy treatment. CD4+ CD25FoxP3+ Tregs are also significantly more CD4+ and CD8+ splenic T cells in the SSA group compared with untreated levels in the SSA group at day 42. These results indicate that androgens may also have a direct, suppressive effect on peripheral T cells. Although the classic intracellular androgen receptor expressed by peripheral T cells appears to be nonfunctional under normal conditions, the more tracellular androgen receptor expressed by peripheral T cells is found to have a suppressive effect on peripheral T cells. Although the classic intracellular androgen receptor expressed by peripheral T cells appears to be nonfunctional under normal conditions, the more tracellular androgen receptor expressed by peripheral T cells is found to have a suppressive effect on peripheral T cells.


References

Castration Enhances Thymic Recovery from Immunodepletion


