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# Response of T Lymphocyte Populations in Prostate Cancer Patients Undergoing Radiotherapy: Influence of Neoajuvant Total Androgen Suppression

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**Abstract.** *Background: This study sought to better define the immunological impact of combining neoadjuvant total androgen suppression (TAS) with radiotherapy (xRT) in treating prostate cancer. Materials and Methods: Subjects selected (n=37) were stage I-II prostate cancer patients meeting the eligibility requirements for RTOG protocols 94-08 or 94-13. Flow cytometric monitoring of circulating T helper ($T_h$), T suppressor/cytotoxic ($T_s$), natural killer (NK) and B lymphocytes was performed weekly. Results: Significant reduction of all lymphocyte subsets occurred as a result of xRT. Comparison between treatment groups demonstrated that the B lymphocyte and NK lymphocyte radioresponse was not influenced by TAS, but the $T_h$ and $T_s$ lymphocyte response was, with addition of TAS leading to less radiation-induced decline. Conclusion: The basis for this T cell response is unclear, but may involve a TAS-induced reduction of testosterone's immunomodulation of T cell proliferation and apoptosis and/or a direct, TAS-induced thymic stimulation. Our data suggest that addition of TAS to xRT appears to have no detrimental effects on lymphocyte subsets, and, indeed, may have favorable effects on T cells.*

During the past two decades, radiotherapy (xRT) has played an increasingly important role in the management of prostate cancer. Nevertheless, a significant number of prostate cancer patients who appear to be in complete remission eventually demonstrate an increasing or abnormal level of PSA, suggestive of recurrent disease or treatment failure (1, 2). To address this concern, the use of androgen suppression therapy in combination with xRT has become a popular recourse (3, 4). Unfortunately, the use of antiandrogen therapy in conjunction with xRT has engendered controversy, since additional therapy brings with it the potential for additional negative sequelae (5, 6).

In this regard, the immunological consequences of androgen suppressive therapy are important. Historically, for example, combining xRT with antiandrogen regimens using estrogenic compounds such as diethylstilbestrol and diethylstilbestrol diphosphate caused concern because of these compounds' reported ability to reduce natural killer lymphocyte (NK cell) activity and tumor-associated immunity (7-9). Such effects could add to, or even synergize with, radiation's potential immunotoxic actions to produce serious clinical ramifications. Fortunately, use of the newer luteinizing hormone-releasing hormone (LHRH) analogs in combination with antiandrogenic compounds such as flutamide has been postulated to achieve androgen suppression with less deleterious immunological effects than the above-mentioned synthetic estrogens (7, 9). However, precise definition of the immunological consequences of combining xRT and the newer regimens of antiandrogen therapy is not yet fully delineated. To this end, this investigation, monitoring peripheral blood lymphocyte populations in stage I-II prostate cancer patients undergoing a regimen of either xRT alone or xRT plus total androgen suppression (TAS), was undertaken.

## Materials and Methods

*Patients.* Thirty-seven prostate cancer patients receiving wide-field pelvic (WFP) and prostate boost (PB) xRT were entered into this study. Eighteen patients received xRT alone, while 19 received neoadjuvant TAS + xRT. Eligibility was based on the following:

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*Key Words:* Prostate cancer, radiotherapy, antiandrogen therapy, lymphocytes, flow cytometry.

Table I. *Characteristics of control and patient populations.*

| Parameter | Data[a]/ patient range | |
|---|---|---|
| Healthy controls | | |
| Sample size | 15 subjects | |
| Age (years) | 63.4±6.2 (53-79) | |
| Patient groups | xRT | xRT+TAS |
| Sample size | 18 patients | 19 patients |
| Age (years) | 69.2±7.4 (58-79) | 71.5±5.9 (60-78) |
| Clinicals stage | | |
| $T_{1b\text{-}c}N_0M_0$ | 6 patients | 7 patients |
| $T_{2a\text{-}b}N_0M_0$ | 12 patients | 12 patients |
| PSA (ng/ml) | 14.3±7.2 (3.5-20.2) | 11.3±4.4 (2.8-13.6) |
| Gleasons | 6.07±0.47 (4.13-6.51) | 5.53±0.69 (3.97-5.89) |

[a]Data provided as the mean±SD. Values in parentheses represent the range of patient data for each parameter.

histologically confirmed, locally confined adenocarcinoma of the prostate, with or without significant risk for lymph node involvement based on a clinical stage of T1-T2, a grade of $T_{1\text{-}2c}N_0M_0$ and PSA and Gleasons scores of ≤21 and ≤6.5, respectively. Eighteen of the 37 patients were also entered into either RTOG 94-08 or RTOG 94-13. The 19 patients who were not on protocol were treated according to procedures outlined in RTOG 94-13. For our normal, control group, 15 consenting healthy male adults were enrolled, ranging from 53-79 years with no history of previous cancer or antiandrogen therapy. Blood was obtained from controls only once during the study. This study was approved by the University Medical Center Institutional Review Board (UMCIRB). Signed informed consent was obtained from all subjects prior to entrance into the study.

*Endocrine therapy.* TAS therapy consisted of administering the drugs flutamide and zoladex. Flutamide (NSC#147834), a nonsteroidal antiandrogen that is metabolized into a hydroxylated derivative which competes with hydrotestosterone for androgen receptor sites, was supplied as 125 mg capsules and administered orally at a dose of 250 mg 3 times a day. The drug was started 2 months prior to xRT and continued until xRT was completed or an overall treatment time of 112 days was achieved. Zoladex (NSC#606864), a luteinizing hormone-releasing hormone (LHRH) analog with substitutions for the L-amino acid glycine in positions 6 and 10, that result in an analog with 50-100 times the potency and longer duration of action than the naturally occurring peptide, was supplied in a disposable syringe containing 3.6 mg of drug. The drug was injected into a pocket of subcutaneous fat every 4 weeks beginning 2 months before xRT for an overall time of 112 days.

*Radiation therapy.* Patients were treated in a supine position, using isocentric technique with curative intent. The patients had a planning computed tomography (CT) scan and a computer-generated isodose distribution evaluation to assist in target localization and beam arrangement. The xRT employed a 4-field box technique, consisting of opposed anterior-posterior and lateral fields, using a 20-MV X-ray beam, and 4-5 fractions per week at 1.8 Gy per fraction were given. Pelvic xRT to 46.8 Gy (RTOG 94-08) or 50.4 Gy (RTOG 94-13) to the regional lymphatics was followed by a 19.8 Gy boost to the prostate, resulting in a cumulative tumor dose of 66.6 Gy (#94-08) or 70.2 Gy (#94-13).

*Calculation of equivalent body dose.* Fractional integral doses were calculated and added together to derive a total integral dose. Briefly, the integral radiation dose is defined as the total energy absorbed after a beam of radiation enters the body and is derived from the equation.

$$D_{\text{int}} = \int_0^t \rho\, A\, D(x)\, dx$$

*$D_{int}$=integral dose; ϱ=tissue density; A=field area; x=depth along the beam direction; D(x)=dose as a function of depth along the beam; and t=patient thickness*

The equivalent whole body dose (EQ dose) was calculated by dividing the integral dose by the patient's kilogram body weight.

*Flow cytometry.* EDTA-blood samples were obtained by venipuncture from all patients prior to the start of xRT (pre-xRT) and weekly during xRT. Nucleated blood cell counts were determined by Coulter Counter. Using a Becton-Dickinsen FACScan flow cytometer, monocyte and granulocyte populations were delineated on the basis of light scattering characteristics, while lymphocyte subsets were determined by analysis of cell surface markers using the Simultest IMK Plus kit (Becton-Dickinson), following the manufacturer's directions. This kit contains fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies specific for the following cell surface antigens – CD19 (B lymphocytes), CD16/CD56 (NK lymphocytes), CD3 (T lymphocytes), CD4 (T helper lymphocytes) and CD8 (T suppressor lymphocytes). Briefly, an aliquot of freshly drawn blood was incubated with fluorescently-conjugated antibodies for 20 minutes, red cells were lysed using FACS Lysing Solution (Becton-Dickinson), washed twice with phosphate-buffered saline (PBS), and fixed with PBS containing 1% paraformaldehyde. All samples were analyzed within 24 hours, and both positive (CD45-FITC/CD14-PE) and negative ($\gamma_1$-FITC/$\gamma_1$-PE) controls were assessed. CellQuest software was used to derive subset percentages.

Table II. *Comparison of blood subpopulations among healthy controls and prostate cancer patients undergoing xRT or xRT+TAS*

| Blood subpopulation | Healthy controls | xRT patients | | xRT+TAS Patients | | |
|---|---|---|---|---|---|---|
| | | Pre xRT | Post xRT | Pre TAS | Pre xRT | Post xRT |
| WBCs (#/μl) | 6394±2890 | 6291±1432 | 4672±1709[a] | 6370±1276 | 6185±1060 | 4837±2398[b] |
| Granulocytes (#/μl) | 4406±653 | 4258±712 | 3737±752 | 4339±805 | 4096±689 | 3805±771 |
| Monocytes (#/μl) | 348±58 | 379±82 | 401±154 | 394±107 | 413±98 | 357±64 |
| NK Lymphocytes (#/μl) | 229±209 | 240±151 | 108±42[a] | 261±148 | 276±160 | 116±59[b] |
| B Lymphocytes (#/μl) | 142±44 | 161±72 | 24±12[a] | 129±105 | 157±99 | 27±13[b] |
| Th Lymphocytes (#/μl) | 596±162 | 625±241 | 201±117[ac] | 604±224 | 636±173 | 282±176[bc] |
| Ts Lymphocytes (#/μl) | 675±268 | 628±207 | 202±110[ac] | 643±282 | 607±127 | 251±145[bc] |

Values represent the means±the standard deviations (SD) for 15 healthy controls, 18 patients receiving xRT alone, and 19 patients receiving xRT+TAS.

[a] Post xRT value significantly different from pre xRT value

[b] Post xRT value significantly different from pre TAS and pre xRT value

[c] Post xRT value significantly different between treatment groups

Blood population concentrations were determined by multiplying total nucleated cells by the percentages obtained from the Cell Quest analysis.

*Statistical analysis.* Statistical Analysis Systems (SAS) Procedure Mixed was used for statistical analyses. This procedure allows for missing observations within a repeated measures design and computes regression coefficients under that design. For estimation of regression model parameters, a second degree, polynomial regression (linear-quadratic) model with an autoregressive [AR(1)] error structure was fit within each group and cell type. Analysis of variance (ANOVA) was used for assessment of group differences in age, disease stage, PSA levels, Gleasons scores, and pre- and post-treatment blood subpoulation parameters. The level of statistical significance was set at $p<0.05$.

## Results

*Characteristics of control and patient populations.* The clinical characteristics of the patient population contributing to this study are summarized in Table I. Patients selected for this study presented without positive nodes or any evidence of metastatic disease and, based on clinical stage, the distribution of patients in both treatment groups favored the T2 over the T1 stage by a ratio of approximately 2:1. Analysis of variance revealed no evidence of statistical differences between the two treatment groups for either PSA levels or Gleasons scores, and the mean age of subjects receiving xRT *vs* xRT+TAS was not statistically different from each other or from the healthy controls.

Comparisons of blood population parameters are presented in Table II. Before initiation of any treatment, mean values within the healthy controls, xRT and xRT+TAS groups fell within published normal ranges (10), and intergroup comparisons of compartment sizes revealed no statistical differences. Following xRT, however, both the xRT and xRT+TAS groups demonstrated significant declines (~25%) in white blood cell (WBC) numbers which appeared to be due primarily to a reduction in lymphocyte subsets, since monocytes appeared to be relatively unaffected by xRT, and only a modest, statistically insignificant decline in granulocyte number was seen. Because changes in granulocyte and monocyte numbers were not substantial, further studies focused on characterization of lymphocyte response to xRT or xRT+TAS.

*Kinetics of lymphocyte response in patients receiving xRT alone.* As can be seen in Table II, significant interindividual variation in absolute blood compartment numbers was observed. To facilitate comparisons among patients, therefore, all patient lymphocyte data collected were normalized using preirradiaton control values (obtained directly prior to the start of xRT) and presented as a percent of control. In Figure 1, these normalized data have been plotted as a function of equivalent total body dose (EQ dose) received, with a mean dose response curve obtained from a second-order, least-squares polynomial fit of the dose-response data from all patients in the group added to each graph. Additionally, the mean dose response curves for all four lymphocyte subpopulations are plotted together in Figure 3a to facilitate direct comparison of the subpopulations. From these two figures, it can be seen that the radiation-induced decline in lymphocyte compartments was most marked among B lymphocytes (B cells), which were characterized by a rapid and severe decline in numbers, reaching a nadir of ~14% of the prexRT control. Radiation-induced decline was least significant for NK cells, which only fell to ~45% of the prexRT control by treatment end, and $T_h$ and $T_s$ cell populations were intermediate in their radioresponse, each declining to

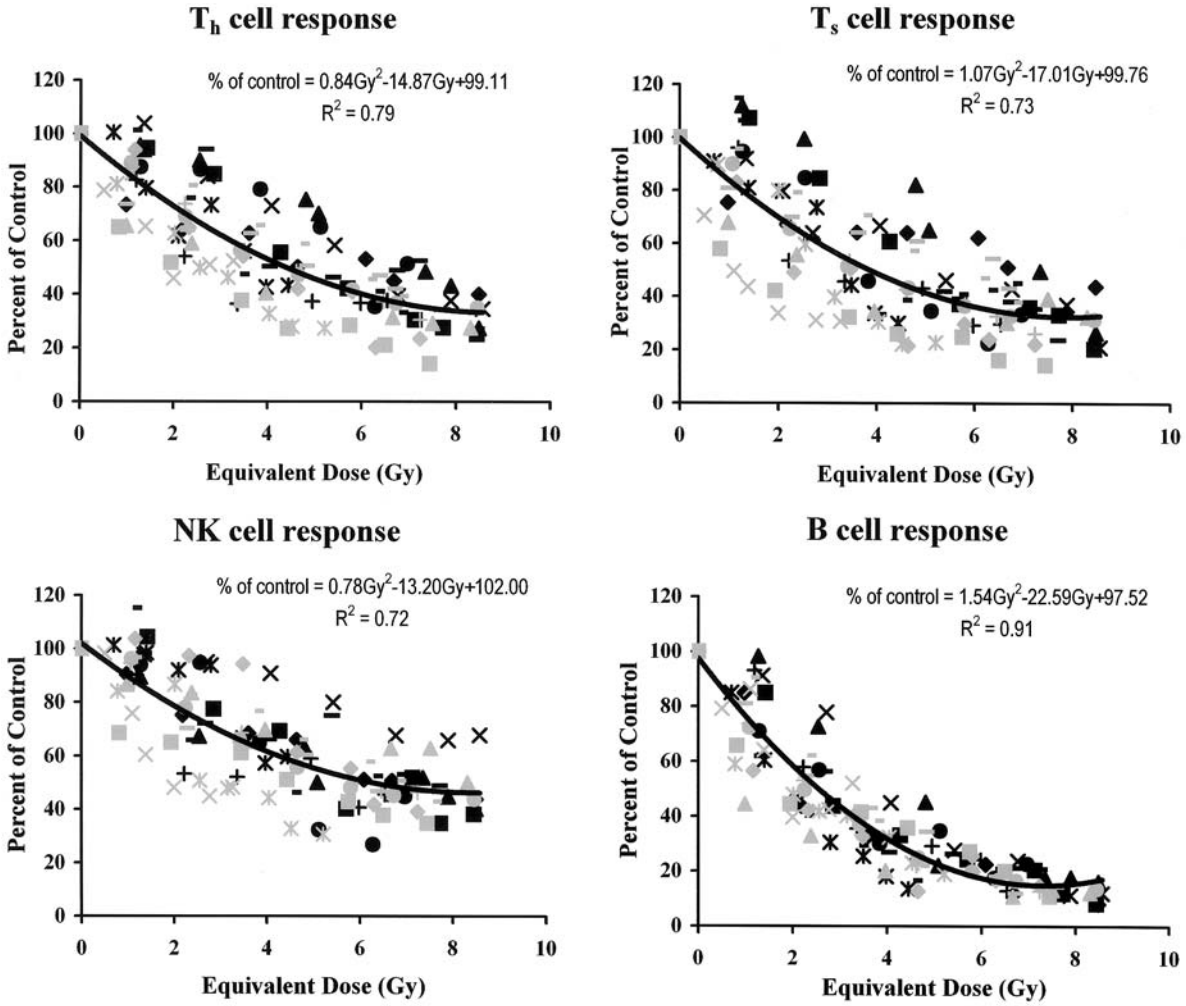


Figure 1. *Response of the different lymphocyte subsets during xRT alone. Patient results (n=18) are normalized to prexRT values, expressed as % of control, and plotted against equivalent body dose received. Individual patient values are indicated using different symbols. Mean dose response curves are derived from a second degree, least squares polynomial fit of all patient data. In all subsets, the radioresponse fits a linear-quadratic model of decline with statistical significance ($p<0.05$).*

~32% of the prexRT control. Both $T_h$ and $T_s$ cell subsets displayed a similar pattern of decline, resulting in a $T_h/T_s$ ratio which was essentially unchanged during the xRT.

*Kinetics of lymphocyte response in patients receiving xRT + TAS.* Figure 2 summarizes the changes in lymphocyte subsets observed in patients receiving xRT in conjunction with total androgen suppression (xRT + TAS). As in Figure 1, all data have been normalized to preirradiaton control values, presented as a percent of control, plotted against the EQ dose received, and a mean dose response curve (obtained from a second-order, least-squares polynomial fit of data) added to each graph. Additionally, mean dose response curves for all four lymphocyte subpopulations monitored in this treatment group are presented together in Figure 3b to facilitate comparison among the subsets. Similar to the xRT alone treatment group, B cells within this group exhibited marked radiosensitivity, falling to ~17% of the prexRT control, and NK cells demonstrated relative radioresistance, declining to ~42% of the prexRT control. However, $T_h$ and $T_s$ cells within this group were similar to NK cells in their radioresponse, falling to only ~44% and ~41% of the prexRT control, respectively. No significant change in the $T_h/T_s$ ratio was seen.

*Intergroup comparison of lymphocyte subpopulation radioresponse.* To allow intergroup comparison between the two treatment groups, the mean dose response curves for each lymphocyte subset are presented in Figure 4. As stated above, the magnitude of decline for circulating B cells and NK cells for the two treatment regimens was similar, with

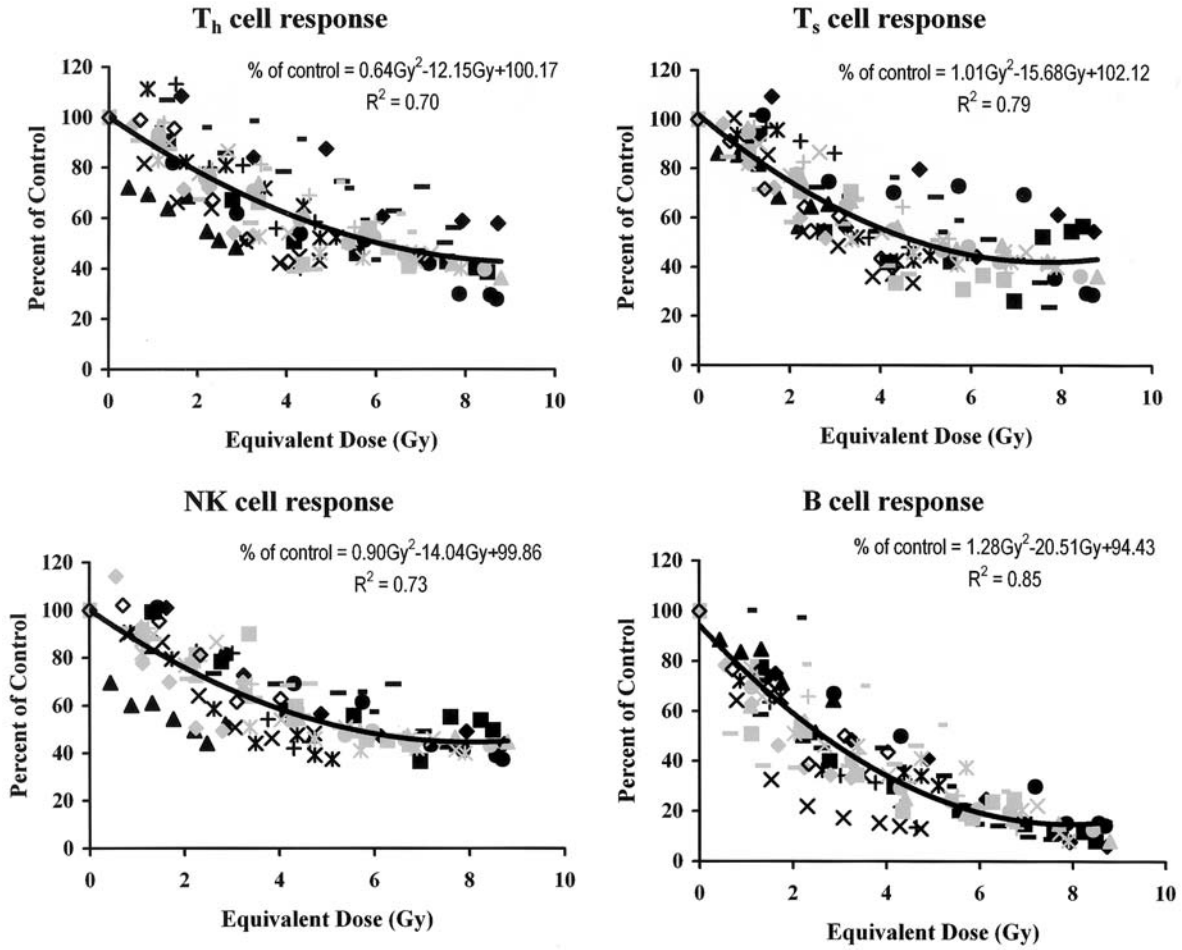


Figure 2. *Response of the different lymphocyte subsets during xRT + TAS. Patient results (n=19) have been normalized to prexRT values, expressed as % of control, and plotted against equivalent body dose received. Individual patient values are indicated using different symbols. Mean dose response curves are derived from a second degree, least squares polynomial fit of all patient data. In all subsets, the radioresponse fits a linear-quadratic model of decline with statistical significance ($p<0.05$).*

no statistical differences being noted. However, for $T_h$ cells and $T_s$ cells, the pattern of decline was significantly different. Specifically, both $T_h$ and $T_s$ compartments were significantly larger ($p<0.02$ & $p<0.01$, respectively) in the xRT+TAS group than in the xRT alone group.

## Discussion

Despite numerous studies, the precise nature of the lymphocyte changes occurring during xRT remains elusive. Some of this uncertainty stems from differences among individual patients with respect to their intrinsic radiosensitivity profiles and/or from variances with the xRT regimen itself. However, an additional factor obscuring the precise definition of lymphocyte response to radiation is whether or not the xRT is administered in combination with other therapeutic modalities. In the case of prostate cancer, for example, xRT is frequently administered in conjunction with androgen suppression therapy. However, knowledge of the effects that combined xRT and androgen suppression therapy have upon lymphocyte response is limited. The purpose of this study, therefore, was to compare circulating peripheral blood lymphocyte subsets in patients being treated for primary adenocarcinoma of the prostate receiving xRT alone to those receiving neoadjuvant TAS plus xRT.

In general, the results demonstrated that both treatment groups exhibited a significant, and similar, radiation-induced decline in overall WBC numbers which can largely be attributed to reductions within the lymphocyte subsets. Given the well-published radiosensitivity of lymphocyte populations, these observations are not totally surprising.

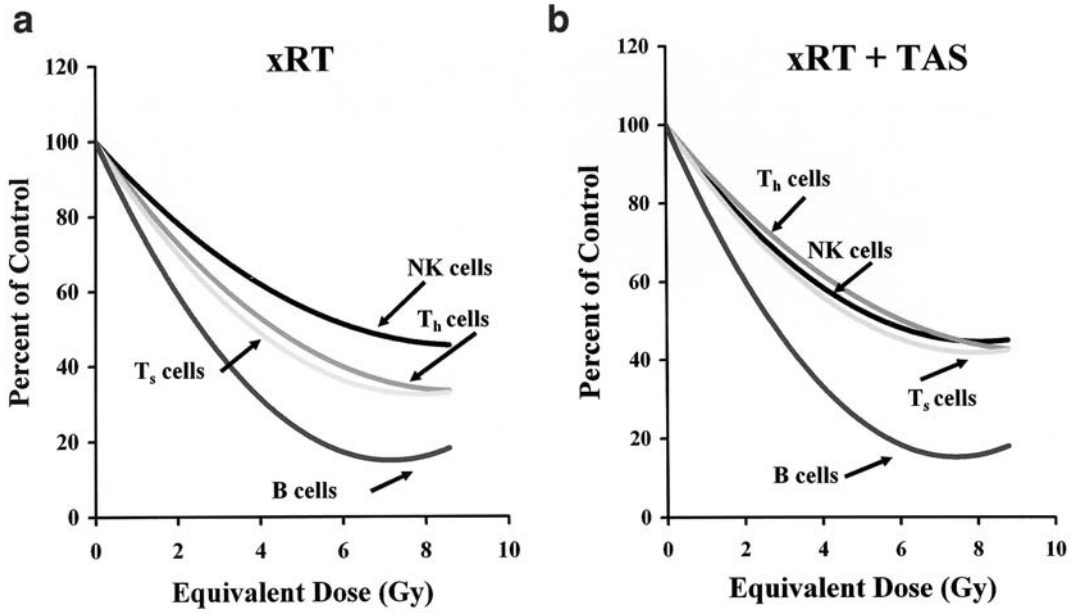


Figure 3. *Intragroup comparison of the individual lymphocyte subsets in prostate cancer patients receiving either xRT alone or xRT + TAS. For ease of comparison, only the mean dose response curves for the different lymphocyte subsets are presented. Plots demonstrate the marked variability in radioresponse of the individual lymphocyte subpopulations.*

Lymphopenia following xRT regimens has been reported in several earlier reports (11-15), along with the marked differences in radioresponse among the various individual lymphocyte subsets (*i.e.* the high radiosensitivity of B cells *vs*. the relative radioresistance of NK cells) that we also observed. What is not yet well-defined are the mechanisms that underlie the dissimilarities in radioresponse among the different lymphocyte subsets. On this note, it has recently been suggested that the potential to undergo apoptotic induction may be involved (15) – an idea that gains support from observations which demonstrate that the apoptotic indices of B, T and NK cells observed following 1 and 2 Gy of *in vitro* radiation correlate closely with their *in vivo* radiosensitivity profiles (16-18). As yet, however, it can not be ruled out that other processes such as cell cycle arrest (19) and reproductive cell death (20) may be contributing to B, T and NK cell radioresponse profiles.

With respect to the similarity of response observed between the $T_h$ (CD4+) and $T_s$ (CD8+) subsets in these studies, our data are, once again, in agreement with recent studies (13, 15, 17) that suggest these two populations have nearly identical radiosensitivity profiles, resulting in a relatively unchanged $T_h/T_s$ ratio in the blood. However, our results are in contrast to earlier investigations, which reported a more pronounced decrease of Th cells than $T_s$ cells, resulting in a markedly altered blood $T_h/T_s$ ratio during xRT (21-24). The reasons for these conflicting reports are not known, but they may be due to variability in such experimental parameters as the xRT regimen used, adjuvant treatments employed, time of sampling, and/or body region and volume irradiated.

Perhaps the most interesting result of these studies is the observation that, while the radioresponse of both B cells and NK cells appeared to be unaffected by the addition of TAS, the T cell radioresponse seemed to be significantly influenced by its administration. Specifically, addition of neoadjuvant TAS to the treatment regimen resulted in marked protection of the $T_h$ and $T_s$ compartments during the xRT regimen. Indeed, $T_h$ and $T_s$ cell radioresistance rivaled that seen for NK cells in the xRT + TAS group. The precise mechanisms underlying this apparent TAS-induced T cell response remain unclear, but several investigations have reported that the immune response is sexually dimorphic and can be modulated by sex steroid hormones (25-29). In general, these reports support the concept that female sex hormones (estrogen and progesterone) tend to be immunostimulatory, while male sex hormones (androgens) tend to be immunosuppressive. Both groups appear to act through classic hormone receptors expressed on the cells of the immunohematopoietic system. Moreover, with regard to androgens in particular, McMurray *et al.* (26) reported that exposing T cell lines to testosterone inhibited cell proliferation, induced accumulation of T cells in the S/G2-phases of the cell cycle, and increased apoptosis. Furthermore, they demonstrated that these effects of testosterone were selective for T cells. B cell lines did not demonstrate this response when exposed to testosterone.

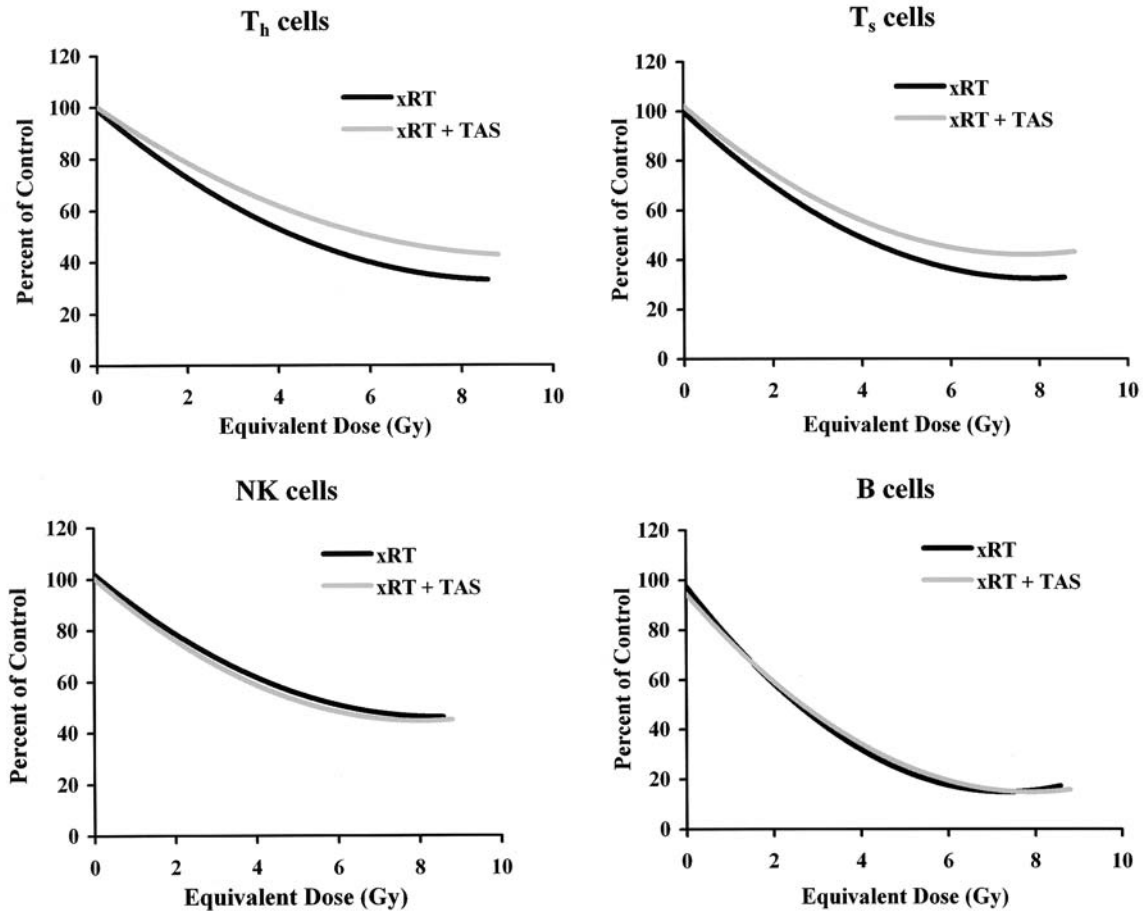


Figure 4. *Intergroup comparison of the individual lymphocyte subsets in prostate cancer patients receiving either xRT alone or xRT + TAS. For ease of comparison, only the mean dose response curves for the different lymphocyte subsets are presented. Statistical analysis revealed that treatment group differences (xRT vs. xRT + TAS) for B cells and NK cells were not significant, but they were highly significant for $T_h$ cells ($p<0.02$) and $T_s$ cells ($p<0.01$).*

Additionally, studies by Olsen *et al.* (27-29) have elegantly demonstrated that androgens exert considerable influence on lymphocytes through modulation of proliferation/apoptosis. Of interest, their studies have documented that androgen's modulating effects do not appear to be on the mature peripheral lymphocytes *per se*, but rather at the level of developmental maturation (*i.e.* the androgen receptor is not found on peripheral blood lymphocytes, but is expressed on such components of the immunohematopoietic system as the bone marrow stroma, thymic epithelial cells and lymphocyte progenitors). Finally, studies by Marchetti *et al.* (25) suggest that LHRH agonists themselves may affect the T cell compartment by direct stimulation of the thymus. Specifically, they found that rat thymus glands contain LHRH receptor binding sites, and that chronic administration of an LHRH agonist in aging rats up-regulated the number of these receptors present, reversed the age-associated decline of thymus weight and significantly increased thymocyte proliferation. In light of these studies, our observations of heightened T cell numbers in the xRT + TAS patients may reflect either a TAS-induced reduction of androgen's reported modulation of T cell development and/or a potential direct thymic stimulation. Clearly, more studies monitoring T cell proliferative and apoptotic indices in response to TAS are in order before definitive conclusions can be drawn, but our results suggest that the addition of TAS to conventional xRT for treatment of prostate cancer appears to have no adverse effects on blood cell population profiles and, for T cells, may even have favorable effects by reducing the magnitude of the xRT-induced decline.

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