Optimal treatment of localized prostate cancer remains controversial. Standard therapy with curative intent includes either surgical prostatectomy or external beam radiation therapy (EBRT) with or without androgen-deprivation therapy (ADT). Despite advances in radiation therapy approaches and surgical technique, a significant proportion of patients still relapse, often due to occult metastatic disease (1–3). The addition of ADT to EBRT has been shown to prolong survival in patients with high-risk disease (4–8). Similarly, the addition of ADT after radical prostatectomy in patients with micrometastatic lymph node involvement may improve overall survival (9). Whether other adjuvant therapies can decrease recurrence of localized prostate cancer remains a significant clinical problem and a focus of ongoing studies. We postulated that a well-tolerated systemic therapy, such as vaccination, could target micrometastatic disease when given with radiation therapy. Furthermore, radiation has the potential to alter the tumor phenotype, making irradiated tumor more amenable to immune-mediated killing. In addition, the combination of vaccine and radiation therapy has shown synergistic preclinical antitumor activity (10, 11). Thus, the addition of an effective vaccine could target the primary tumor, as well as occult metastatic disease.

We initially evaluated whether a combination approach using a vaccine targeting prostate-specific antigen (PSA; ref. 12), safely combined with EBRT in patients with localized or locally advanced prostate cancer, could induce immune responses specific to the vaccine. In our original study (13), patients were randomized to receive either EBRT with PSA vaccine or EBRT alone. ADT was allowed in either arm if clinically appropriate. The primary

**Abstract**

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**Experimental Design:** Eighteen patients with localized prostate cancer were treated in a single-arm trial using previously established doses of vaccine and radiation therapy. The vaccine used was a recombinant vaccinia virus engineered to encode PSA admixed with a recombinant vaccinia encoding the costimulatory molecule B7.1, followed by booster vaccinations with a recombinant fowlpox vector expressing PSA. Patients received a total of eight planned vaccination cycles, once every 4 weeks, with granulocyte-macrophage colony-stimulating factor given on days 1 to 4 and interleukin 2 (IL-2) at a dose of 0.6 MIU/M² given from days 8 to 21 after each vaccination. Definitive external beam radiation therapy was initiated after the third vaccination cycle. Patients were evaluated for safety and immunologic response. Toxicity and immunologic activity were compared with the previously reported regimen containing a higher dose of IL-2.

**Results:** Seventeen of 18 patients received all eight cycles of vaccine with IL-2. Five of eight HLA-A2 + patients evaluated had an increase in PSA-specific T cells of ≥3-fold. Toxicities were generally mild, with only seven vaccination cycles of 140 given resulting in grade 3 toxicities possibly attributable to IL-2.

**Conclusions:** Metronomic-dose IL-2 in combination with vaccine and radiation therapy is safe, can induce prostate-specific immune responses, and has immunologic activity similar to low-dose IL-2, with markedly reduced toxicities.

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*Experimental Design:* A recombinant fowlpox vector expressing PSA was used as an adjuvant. Radiation therapy was initiated after the third vaccination cycle. Patients were evaluated for safety and immunologic response. Toxicity and immunologic activity were compared with the previously reported regimen containing a higher dose of IL-2.

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end point of that study was immunologic response specific to the vaccine. Patients treated with EBRT and vaccine, but not those treated with EBRT alone, had a significant increase in PSA-specific T-cell responses (13). This trial showed the safety and feasibility of coadministration of a PSA-specific therapeutic cancer vaccine with radiation therapy, but was not powered to show a benefit in overall survival or time to progression.

In the trial described above, however, significant toxicities were associated with the administration of adjuvant interleukin-2 (IL-2). IL-2 is a cytokine that has pleiotropic effects on T-cell function, depending on the context in which it is given (14). IL-2 is approved for use in the United States as monotherapy for metastatic renal cell carcinoma and metastatic melanoma. The doses used for these indications—600 to 25–30 MIU/M\(^2\) given every 8 h for up to 15 days—are associated with significant toxicity (15). Even at the much lower dose of 4 MIU/M\(^2\) used in our study (designated metronomic adjuvant dose or M-IL-2), there were considerable symptomatic toxicities attributable to IL-2 (13).

At higher doses, IL-2 is not only more toxic but may be associated with negative regulation of immune response. Examination of T-cell subsets after administration of high-dose IL-2 for melanoma and renal carcinoma revealed an increase in T regulatory cells (Treg) and a decrease in natural killer (NK) cells (16). Trials of lower-dose IL-2 (~1-2 MIU/d) in patients with AIDS have shown a beneficial effect on expansion of the T-cell compartment with reduced toxicity (17–19). Because patients with AIDS have shown a beneficial effect on expansion of the T-cell compartment, we sought to determine if nearly continuous, very low-dose IL-2 (designated metronomic adjuvant-dose or M-IL-2) could serve as a useful vaccine adjuvant in the same clinical setting.

**Patients and Methods**

*Patient selection and trial design.* Entry and exclusion criteria and design for this trial were the same as previously described (13); an exception in the trial reported here was that not all patients receiving M-IL-2 were required to be HLA-A2*+. Briefly, patients had biopsy-proven prostatic adenocarcinoma and were considered candidates for definitive EBRT. All patients in the current study were vaccinated on a 4-wk cycle with a priming dose of vaccinia PSA admixed with vaccinia B7.1, followed by boosts with fowlpox PSA for a total of eight planned cycles. Granulocyte-macrophage colony-stimulating factor was given at 100 ng/d at the vaccination site on days 1 to 4 of each cycle, and IL-2 was given at 0.6 MIU/M\(^2\) on days 8 to 21 as an s.c. injection. Since ~1 wk is needed after poxviral vector vaccination to induce a response, it was believed that starting the low-dose systemic IL-2 administration 8 d postvaccination would preferentially boost the expansion of new antigen-specific effector cells. By dosing through day 21, the total amount of IL-2 given was similar to that given in the standard dose of IL-2 arm. Stopping at day 21 allowed for some resting of the T cells before the next vaccine (day 29). This dose was designed to provide saturation of high-affinity IL-2 receptors for 10 h/d (20, 21). All patients received EBRT and 14 patients received ADT at the discretion of their treating physicians. All protocols were approved by the Institutional Review Board of the National Cancer Institute.

**Immunologic assays.** Collection of mononuclear cells by apheresis, ELISPOT assays and serologic analysis were done as previously described (13).

**Flow cytometry analysis.** Three-color flow cytometry analysis was done on peripheral blood mononuclear cells (PBMC) for phenotypic characterization of Tregs. Cells were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4°C with PerCP-Cy5.5–conjugated anti-CD4 and phycoerythrin-conjugated anti-CD25 (both from BD Biosciences). FoxP3 intracellular staining was done on the cells stained with anti-CD4 and anti-CD25. Cells were fixed and permeabilized using a fix/perm kit (eBioscience) according to the manufacturer's instructions and then labeled with FITC-conjugated anti-FoxP3 antibody (PCH101 clone) or its isotype control antibody (eBioscience) as a negative control. Flow cytometry was done on a Becton Dickinson LSRII (BD Biosciences); 1 × 10\(^5\) cells were acquired, and data were analyzed using FlowJo software (BD Biosciences). To determine the percentage of Tregs, lymphocytes were gated by plotting forward versus side scatter, followed by gating of the CD4\(^+\) population. Then the CD25\(^{high}\) and FoxP3\(^+\) populations were gated. The CD25\(^{high}\) population was separated from the CD25\(^{low}\) population on the basis of the level of CD25 expression in CD4\(^+\) T cells, as previously described.

**Table 1. Patient demographics**

<table>
<thead>
<tr>
<th>Vaccine + S-IL-2*</th>
<th>Vaccine + M-IL-2 †</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (range)</td>
<td>%</td>
</tr>
<tr>
<td>Patients enrolled</td>
<td>19</td>
</tr>
<tr>
<td>Median age</td>
<td>59 (50–77)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>15</td>
</tr>
<tr>
<td>African American</td>
<td>2</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Median PSA (ng/mL) at diagnosis</td>
<td>14.15 (3.84–206)</td>
</tr>
<tr>
<td>Mean (SD) PSA (ng/mL) at diagnosis</td>
<td>35.0 (50.7)</td>
</tr>
<tr>
<td>Median PSA (ng/mL) on study</td>
<td>9.86 (0.17–122.26)</td>
</tr>
</tbody>
</table>

*Adjuvant IL-2 given at 4.0 MIU/M\(^2\) on days 8 to 12 of each cycle.
†Adjuvant IL-2 given at 0.6 MIU/M\(^2\) on days 8 to 21 of each cycle.
A similar procedure was used for flow cytometry analysis of NK cells. PBMCs were stained with FITC-conjugated anti-CD3 and phycoerythrin-conjugated anti-CD56 (BD Biosciences) and analyzed as described above (22, 23).

Statistical considerations. The toxicity profile of metronomic dosing of IL-2 was evaluated using a trial design based on a method for estimating the size of a single-stage, single-arm trial (24). Assuming that the probability of avoiding dose reduction in the S-IL-2 arm was no greater than 10%, we wished to rule out 10% without reductions in favor of a 50% chance of avoiding a dose reduction. With $\alpha = 0.05$ and $\beta = 0.1$, it was considered unacceptable if $V^c$ of the first 12 patients enrolled in the M-IL-2 arm did not require dose reductions and acceptable if 4 to 12 patients avoided dose reductions. The lower bound of a 95% one-sided confidence interval about 4 of 12 is $\hat{p} = 12\%$, thus demonstrating superiority to previously published results of studies using S-IL-2. Because only 3 of the first 12 patients enrolled were HLA-A2+ and 9 was the intended minimum number for evaluation, up to seven additional HLA-A2+ patients were allowed to enroll in the M-IL-2 arm to obtain sufficient immunologic data.

Results

A comparison of baseline characteristics of patients receiving M-IL-2 and patients receiving S-IL-2 (Table 1) reveals that the two treatment arms were similar in age, ethnicity, Gleason score or disease stage, on-study PSA, and use of ADT. PSA at the time of diagnosis did not differ significantly between the two groups ($P = 0.001$ by Student’s two-sided $t$ test), and disease stage was also similar (not shown). Of the 19 patients in the S-IL-2 arm, 17 completed the course of eight vaccinations. One patient withdrew from the study after a single vaccine cycle to receive immediate EBRT, and one patient was diagnosed with invasive bladder cancer after three cycles and was taken off the study. Thus, a total of 140 vaccinations were given to patients in the S-IL-2 arm. In the M-IL-2 arm, one patient withdrew after four cycles due to persistent lymphopenia attributed to EBRT; 17 completed all eight vaccine cycles for a total of 140 cycles.

No grade 4 toxicities were reported in either arm. The majority of vaccinations (75.7%) were associated with grade 2 or lower injection-site reactions (Table 2), but as in the previously reported trial, no toxicities above grade 2 were directly attributable to the vaccine. One patient experienced grade 2 dyspnea:

<table>
<thead>
<tr>
<th>Table 2. On-study adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S-IL-2</strong> (140 cycles given)</td>
</tr>
<tr>
<td><strong>Grade 2, n (%)</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>GM-CSF + vaccine–related</strong></td>
</tr>
<tr>
<td>Injection-site reaction</td>
</tr>
<tr>
<td>Dyspnea</td>
</tr>
<tr>
<td>Arthralgias</td>
</tr>
<tr>
<td><strong>IL-2–related</strong></td>
</tr>
<tr>
<td>Constitutional</td>
</tr>
<tr>
<td>Fatigue</td>
</tr>
<tr>
<td>Fever</td>
</tr>
<tr>
<td>Arthralgias</td>
</tr>
<tr>
<td>Metabolic</td>
</tr>
<tr>
<td>Hyperglycemia</td>
</tr>
<tr>
<td>Blood</td>
</tr>
<tr>
<td>Lymphopenia</td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
</tr>
<tr>
<td>Dehydration/anorexia</td>
</tr>
<tr>
<td>Diarrhea</td>
</tr>
<tr>
<td><strong>Pulmonary</strong></td>
</tr>
<tr>
<td>Dyspnea</td>
</tr>
</tbody>
</table>

Abbreviation: GM-CSF, granulocyte-macrophage colony-stimulating factor.

*Adjuvant IL-2 given at 4.0 MIU/M² on days 8-12 of each cycle.

†Adjuvant IL-2 given at 0.6 MIU/M² on days 8-21 of each cycle.

**Number of cycles with adverse events and percentage of total number of cycles given.

<p>| Table 3. Induction of PSA-specific T-cell responses in five patients after vaccination and administration of metronomic adjuvant dose IL-2 |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Patient</strong></th>
<th><strong>Sample</strong></th>
<th><strong>PSA3 peptide</strong></th>
<th><strong>Flu peptide</strong></th>
<th><strong>HIV peptide</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>31 Pre</td>
<td>&lt;1/200,000</td>
<td>1/35,294</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3</td>
<td>&lt;1/200,000</td>
<td>1/17,143</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3 + 2</td>
<td>1/45,455</td>
<td>1/17,857</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 8</td>
<td>1/60,000</td>
<td>1/46,154</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>32 Pre</td>
<td>1/120,000</td>
<td>1/11,111</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3</td>
<td>1/17,391</td>
<td>1/12,121</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3 + 2</td>
<td>&lt;1/200,000</td>
<td>1/14,634</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 8</td>
<td>&lt;1/200,000</td>
<td>1/15,385</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>34 Pre</td>
<td>&lt;1/200,000</td>
<td>1/13,636</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3</td>
<td>1/46,154</td>
<td>1/14,634</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 3 + 3</td>
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<td>1/13,636</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 8</td>
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<td>1/17,143</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>37 Pre</td>
<td>1/150,000</td>
<td>1/125,000</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 2</td>
<td>&lt;1/200,000</td>
<td>&lt;1/200,000</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 5</td>
<td>1/12,000</td>
<td>1/9,234</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>38 Pre</td>
<td>&lt;1/200,000</td>
<td>1/25,000</td>
<td>&lt;1/200,000</td>
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</tr>
<tr>
<td>Post 3</td>
<td>1/65,714</td>
<td>1/10,169</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
<tr>
<td>Post 8</td>
<td>1/38,462</td>
<td>1/15,385</td>
<td>&lt;1/200,000</td>
<td></td>
</tr>
</tbody>
</table>

*Samples were obtained after indicated vaccine cycle (i.e., post 3 + 2 = 2 mo after cycle 3).
attributable to granulocyte-macrophage colony-stimulating factor, the other immune adjuvant used in this trial. These toxicities did not differ appreciably from those previously described in patients receiving higher-dose IL-2. However, toxicities attributable to IL-2 differed markedly between the two arms (Table 2). In the S-IL-2 arm (IL-2 at 4 MIU/M²), there were 129 cycles with adverse events attributed to IL-2 (103 grade 2, 26 grade 3). In the M-IL-2 arm (IL-2 at 0.6 MIU/M²), the number of cycles leading to grade 2 and grade 3 toxicities was 55 (48 grade 2, 7 grade 3). In particular, there were no grade 3 constitutional symptoms in the M-IL-2 arm, and only 4.4% of cycles were associated with grade 2 fatigue possibly due to IL-2. There were no grade 3 metabolic abnormalities in the M-IL-2 arm, and the proportion of grade 2 hyperglycemia was the same in both arms. The percentage of cycles reporting grade 2 lymphopenia (S-IL-2, 16%; M-IL-2, 20.4%) and grade 3 lymphopenia (S-IL-2, 6%; M-IL-2, 3%) was roughly the same for both arms. Interestingly, a higher percentage of cycles was associated with grade 2 injection site reactions in the M-IL-2 arm (75.7%) than in the S-IL-2 arm (45%). The clinical significance of this finding is not known.

In the S-IL-2 arm, 16 patients (84.2%) had a dose reduction of IL-2, 5 patients (26.3%) had IL-2 held for one or more cycles, and 2 patients (11.8%) had IL-2 discontinued for toxicities. In the M-IL-2 arm, four patients (22.2%) had dose reductions, four patients (22.2%) had dose reductions, four patients (22.2%) had IL-2 held for one or more cycles for IL-2-related toxicities, but no patient had IL-2 discontinued for toxicities attributable to IL-2. Thus, M-IL-2 met the criteria we had prospectively defined (see Patients and Methods) for demonstrating superiority to S-IL-2.

Patients in this study who received M-IL-2 were able to mount specific T-cell immunologic responses with a frequency similar to that of patients who received S-IL-2. Eight patients with HLA-A2 haplotype in the M-IL-2 arm were evaluated for PSA-specific immune responses by ELISPOT assay (Table 3). Of these eight patients, five developed T-cell-specific immune responses to PSA at some point during the course of the trial. Of the patients who developed PSA-specific responses, three developed at least a 3-fold increase in specific T-cell response after the third vaccine cycle, but this immune response diminished in two of these individuals. The third patient maintained this level of immune response beyond the eighth cycle, which was the last time titers were measured. One patient showed an immune response after the fifth cycle that was maintained until the eighth cycle, and one developed an immune response after the fifth cycle but was not evaluated further. Seventeen patients with HLA-A2 haplotype who were treated with S-IL-2 were similarly evaluated for generation of PSA-specific responses (13). In this cohort, 13 of 17 patients developed specific immune responses to PSA. Interestingly, 4 of the 17 patients already had PSA-specific T-cell precursor frequencies of >1:100,000 before starting the protocol, whereas

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>% NK cells</th>
<th>Patient</th>
<th>Sample</th>
<th>% NK cells</th>
</tr>
</thead>
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<tr>
<td>5</td>
<td>Pre</td>
<td>15.26</td>
<td>35</td>
<td>Pre</td>
<td>7.88</td>
</tr>
<tr>
<td></td>
<td>Post 3</td>
<td>12.64</td>
<td></td>
<td>Post 3</td>
<td>10.38</td>
</tr>
<tr>
<td></td>
<td>Post 8</td>
<td>20.87</td>
<td></td>
<td>Post 8</td>
<td>9.71</td>
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<tr>
<td>10</td>
<td>Pre</td>
<td>5.05</td>
<td>36</td>
<td>Pre</td>
<td>12.52</td>
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<tr>
<td></td>
<td>Post 3</td>
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<td></td>
<td>Post 3</td>
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<td></td>
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<td>11.19</td>
<td></td>
<td>Post 8</td>
<td>14.86</td>
</tr>
<tr>
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<td>Pre</td>
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<td>Pre</td>
<td>19.19</td>
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<td>Post 3</td>
<td>20.01</td>
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<td></td>
<td>Post 8</td>
<td>31.42</td>
</tr>
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<td>10.99</td>
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<tr>
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<td>Post 3</td>
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<td>Post 3</td>
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<tr>
<td></td>
<td>Post 8</td>
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<td></td>
<td>Post 8</td>
<td>21.60</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>Pre</td>
<td>10.95 (5.49)</td>
<td>Mean (SD)</td>
<td>Pre</td>
<td>12.64 (4.77)</td>
</tr>
<tr>
<td></td>
<td>Post 3</td>
<td>9.73 (1.97)</td>
<td></td>
<td>Post 3</td>
<td>14.97 (4.15)</td>
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<tr>
<td></td>
<td>Post 8</td>
<td>17.10 (4.22)</td>
<td></td>
<td>Post 8</td>
<td>19.39 (9.37)</td>
</tr>
</tbody>
</table>

*Adjuvant IL-2 given at 4.0 MIU/M² on days 8 to 12 of each cycle.
*Adjuvant IL-2 given at 0.6 MIU/M² on days 8 to 21 of each cycle.
*Samples were obtained after indicated vaccine cycle.
*Percentage of CD3⁻/CD56⁺ PBMCs.

Table 4. Antigen cascade in three patients after vaccination against PSA with administration of metronomic adjuvant dose of IL-2

Table 5. NK cells in a sample of patients immunized against PSA
only one patient in the M-IL-2 arm had such high titers. Eight of 17 patients evaluated who developed substantial increases in PSA-specific T cells with S-IL-2 maintained these increases until after the eighth cycle of therapy, whereas three of eight patients in the M-IL-2 arm retained immune responses beyond the eighth cycle of therapy. Based on these limited data, M-IL-2 seems to be as effective as S-IL-2 in inducing long-lasting T-cell responses after vaccination with a PSA-containing poxviral-based vaccine.

The previous trial using S-IL-2 (13) showed that the combination of radiation therapy and vaccine could induce immunoreactive T cells specific to a broad range of antigens other than PSA (so-called epitope spreading or antigen cascade). To determine if M-IL-2 would induce an antigen cascade, PBMCs from three patients with HLA-A2 haplotype were evaluated for immune response to other antigens in the ELISPOT assay using specific peptides. As seen in Table 4, two patients developed immunoreactivity to XAGE-1 and a third developed immunoactivity to PAGE-4, both members of the PAGE/GAGE gene family of antigens that are expressed on prostate carcinoma cells (25). An additional five patients were evaluated for response to MUC-1 only. Of these five patients, two developed T-cell responses specific to MUC-1. Of note, all patients were HIV+ before enrollment and had no T-cell response to HIV either before or after vaccination, further demonstrating the specificity of this immune response. These data suggest that the combination of vaccine and radiotherapy can induce immunity to a range of tumor-associated antigens beyond those present in the vaccine, which may have positive implications for the efficacy of immunotherapy in this setting.

We next evaluated the effect of M-IL-2 on levels of NK cells, which are also thought to potentially play a role in cell-mediated immunity induced by therapeutic tumor vaccines. In preclinical studies using poxviral vaccines, NK depletion was associated with decreased survival. To determine if M-IL-2 would impair the generation of NK cells in this setting, we determined the percentage of NK cells in patients in the M-IL-2 arm before, during, and after vaccination. As seen in Table 5, S-IL-2 induced an increase in the percentage of NK cells in samples from four of four patients studied (average, 80%; range, 7-155%). Similarly, all four patients in the M-IL-2 arm showed increases in the percentage of NK cells (average, 51%; range, 19-97%). A representative flow cytometry result for both NK and Treg cells (see below) is shown in Fig. 1. These data suggest that M-IL-2 has approximately the same effect on the generation of NK cells as the previously evaluated S-IL-2 regimen, but without the associated toxicity seen with S-IL-2.

The effect of S-IL-2 on Treg generation was evaluated by flow cytometry at baseline and at multiple points after vaccination. For patients treated with both M-IL-2 and S-IL-2, the percentage of CD4+CD25high/FoxP3+ cells was determined at baseline just after three and eight cycles of vaccination and 3 months after the last vaccination. At baseline, the percentage of Tregs in both cohorts was not significantly different from that of normal donors (data not shown). As seen in Table 6, the percentage of CD25high/FoxP3+ cells (as a percentage of total CD4+ cells) increased from baseline to maximum at cycle 8, then decreased to near baseline at 3 months after the last vaccination. In the M-IL-2 cohort, a similar increase in the percentage of Tregs was noted, with a slightly earlier rise in some patients but an average return to baseline in all patients at 3 months after the last vaccination, similar to the S-IL-2 arm. The earlier rise seen in several patients in the M-IL-2 arm may have been due to the proximity of IL-2 administration. In the S-IL-2 arm, the last dose of IL-2 was given 12 days before analysis of Treg subsets, whereas in the M-IL-2 arm, the last dose of IL-2 was given only 7 days before analysis of Treg populations. It is notable that both cohorts returned to pretreatment levels by 3 months after the last vaccination. Therefore, unlike high-dose IL-2, which has been reported to increase Tregs, neither S-IL-2 nor M-IL-2, did so in this trial.

**Discussion**

The uncertainty concerning the optimum adjuvant treatment for localized prostate cancer prompted us to evaluate the safety and immunologic response of a viral-based vaccine in patients with high-risk localized prostate cancer. In the study reported previously (13), the number and severity of toxicities attributable to IL-2 were significant. We report now on a subsequently enrolled cohort of patients who were treated with M-IL-2 to compare the safety and immunologic responses of low and metronomic dosing. In the original trial, the S-IL-2 arm...
received IL-2 at a dose of 4 MIU/M² on days 8 to 12 of each cycle; in the subsequently enrolled M-IL-2 arm, 0.6 MIU/M² of IL-2 was given daily on days 8 to 21 of each 28-day cycle. Both treatment arms received vaccine on day 2. As our analysis shows, M-IL-2 enhanced patient safety, resulted in fewer adverse events, and did not seem to significantly alter vaccine-induced T-cell responses. In this trial, IL-2 was used as an immunologic adjuvant to boost T-cell function and vaccine efficacy, not as a stimulus to induce immune rejection of the tumor, as in therapy for renal cell carcinoma.

Continuous low-dose or metronomic therapy has received increasing interest in recent years (26). When applied to standard cytotoxic therapies, metronomic therapy has been used to potentiate theoretical antiangiogenic activity (27) through inhibition of endothelial proliferation. This method of administration limits not only endothelial regrowth through constant low-grade growth suppression but also the toxicities associated with high doses of cytotoxic agents (26). In the context of immunotherapy, similar principles apply. Generating effective immune responses from current vaccine modalities requires administration of adjuvant therapies. IL-2 is commonly used in these circumstances for its effects on T-cell proliferation and activation. Bolus administration of S-IL-2 has several disadvantages, the primary one being the relative toxicity observed. Metronomic dosing in this context is superior, as shown in this study. In the M-IL-2 arm, there were fewer grade 2 toxicities or higher and more patients completed vaccine therapy. Based solely on safety and feasibility, M-IL-2 seems superior to standard dosing and administration.

Despite increased patient safety, however, there is still a concern that M-IL-2 therapy may not effectively induce appropriate immune responses. IL-2 causes expansion of activated T cells, but in the context of antigen presentation, IL-2 can potentiate antigen-induced cell death (14). There are, however, significant nonclinical and clinical data supporting the use of M-IL-2 as an adjuvant for vaccine therapy. High concentrations of IL-2 can cause cell death by inducing cell-cycle entry and facilitating active apoptosis through Fas ligand and tumor necrosis factor-α (28). In HIV therapy, low-dose IL-2 has been shown to be superior to high-dose IL-2 for stimulating immune cell function. The addition of low-dose IL-2 (1 MIU/d × 5 days every other week) to highly active antiretroviral therapy caused a statistically significant increase in CD4 and CD8 T cells, as well as a decrease in apoptosis, compared with highly active antiretroviral therapy alone in HIV+ patients (17).

### Table 6. Treg cells during treatment

<table>
<thead>
<tr>
<th>Patient</th>
<th>S-IL-2*</th>
<th>M-IL-2†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pre 1</td>
<td>Post 3</td>
</tr>
<tr>
<td>1</td>
<td>2.08 (1:17.92)‡</td>
<td>2.64 (1:15.54)</td>
</tr>
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<td>2</td>
<td>1.39 (1:12.06)</td>
<td>6.21 (1:5.71)</td>
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<tr>
<td>5</td>
<td>1.99 (1:17.17)</td>
<td>3.65 (1:13.84)</td>
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<tr>
<td>7</td>
<td>3.97 (1:8.61)</td>
<td>5.21 (1:6.54)</td>
</tr>
<tr>
<td>9</td>
<td>2.24 (1:16.93)</td>
<td>2.84 (1:15.59)</td>
</tr>
<tr>
<td>10</td>
<td>2.42 (1:18.91)</td>
<td>2.98 (1:14.66)</td>
</tr>
<tr>
<td>11</td>
<td>3.87 (1:12.14)</td>
<td>6.26 (1:7.60)</td>
</tr>
<tr>
<td>12</td>
<td>4.38 (1:5.58)</td>
<td>5.10 (1:10.54)</td>
</tr>
<tr>
<td>13</td>
<td>0.89 (1:53.85)</td>
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</tr>
<tr>
<td>14</td>
<td>3.47 (1:13.04)</td>
<td>5.81 (1:8.18)</td>
</tr>
<tr>
<td>15</td>
<td>1.9 (1:27.6)</td>
<td>2.32 (1:23.92)</td>
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<tr>
<td>17</td>
<td>4.62 (1:9.83)</td>
<td>4.59 (1:10.24)</td>
</tr>
<tr>
<td>Mean</td>
<td>2.77 (1:17.80)</td>
<td>4.34 (1:12.10)</td>
</tr>
<tr>
<td>31</td>
<td>1.51 (1:36.22)</td>
<td>3.26 (1:18.21)</td>
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<tr>
<td>32</td>
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<td>6.53 (1:6.39)</td>
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<tr>
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<tr>
<td>Mean</td>
<td>3.69 (1:13.51)</td>
<td>6.38 (1:7.41)</td>
</tr>
</tbody>
</table>

*Adjuvant IL-2 given at 4.0 MIU/M² on days 8 to 12 of each cycle.
†Samples were obtained after indicated vaccine cycle (post 8 + 3 = 3 mo after cycle 8).
‡Percentage of CD25high/FoxP3+ cells (as percentage of total CD4+ cells) and, in parentheses, ratio of CD4+/CD25high/FoxP3+CD4+/FoxP3+ cells. For healthy donors, 5.24% = 95th percentile.
§Adjuvant IL-2 given at 0.6 MIU/M² on days 8 to 21 of each cycle.
CD95 (Fas) on CD8+ cells. In a similar study in patients with AIDS and AIDS-associated malignancies, Khatri et al. showed that IL-2 given daily at 1.2 MIU/M2 for 3 months significantly increased IFN-γ gene expression in vivo, with normalization of a profound deficit of IFN-γ production upon in vitro stimulation (29). The source of IFN-γ seemed to be NK cells and CD8+ T cells. In another study (30), a higher concentration of IL-2 (7.5 MIU/d s.c. × 5 days) caused an increase in spontaneous apoptosis of both CD4+ and CD8+ cells.

Both ADT and IL-2 can influence the distribution of T-cell subsets during treatment. ADT increases pre-B-cell levels in mice and thymocyte numbers in both animal models and humans treated with GnRH. This thymic expansion may increase the diversity of the T-cell repertoire, although this has not been proved (31). IL-2 therapy also has an influence on the subsets of lymphocytes that repopulate the T-cell compartment during immune reconstitution after chemotherapy. In sarcoma patients treated with cyclophosphamide and adjuvant IL-2 (at a dose of either 9 × 10^6 IU/M2/d CIIVI 4 d/wk × 3 weeks starting on weeks 6, 12, 18, or 3 × 10^6 IU/M2/dose SQ thrice/wk × 16 weeks starting at week 6) in combination with vaccine, IL-2 caused a preferential expansion of the CD4+/CD25+ Treg compartment (32). In that study, the higher of two doses of IL-2 used seemed to induce a greater increase in Tregs, although the difference did not reach statistical significance. We have previously shown that patients with prostate cancer have peripheral blood levels of Tregs similar to those of healthy donors, but with significantly greater suppressive functionality (33). In the present study, we saw a clear increase in Treg populations during the period of IL-2 administration, with a return to baseline levels 3 months after the last dose. Our data do not indicate a clear improvement in the Treg profile with metronomic dosing, but there was no prolongation of Treg expansion due to the longer period of IL-2 dosing in the M-IL-2 arm. One caveat, however, is that in the S-IL-2 arm the time from last IL-2 administration until measurement of Tregs was 14 days, whereas in the M-IL-2 arm the delay was only 7 days. It is unclear what effect this may have had on Treg levels, but the shorter time to measurement from the date of last IL-2 dose may have increased the apparent number of Tregs in the M-IL-2 arm. Any difference in Treg activity between the two treatment arms has not been determined.

Various doses of IL-2 have also been shown to augment NK-cell populations in humans (16, 18). Because many tumors do not express MHC molecules, which are necessary for CD8-mediated killing, NK cells (which selectively kill cells lacking MHC molecules) are a vital part of the innate immune system in this vaccine strategy. In patients with advanced malignancies, daily administration of low-dose IL-2 (1.25 MIU/M2), with pulse dosing after 4 to 6 weeks with up to 15 MIU/M2/d × 3 days (repeated every 2 weeks), expanded the T-cell population by ~50%, the NK-cell population by ~8-fold, and the subpopulation of CD56bright cells by 32-fold (34). In a study of patients with AIDS and non–Hodgkin’s lymphoma, 1 MIU/M2 IL-2 given daily for 8 weeks caused a statistically significant 1.6-fold increase in the percentage of NK cells (18). IL-2 likely causes expansion of NK-cell populations by inducing maturation of progenitors and inhibiting mature NK-cell apoptosis (35). Interestingly, in a study of renal carcinoma patients treated with IL-2, thalidomide, and radiotherapy, IL-2 increased the percentages of NK cells, but in patients concomitantly treated with radiation therapy, there was no increase (36). In contrast, all patients in our study were treated with both IL-2 and radiation therapy and all patients tested experienced an increase in NK cells.

Our data show that administration of IL-2 in a metronomic dosing schedule (low doses given daily for 14 of 28 days) is safe. In addition, immune responses are similar to those in patients treated with the identical vaccine strategy, but with higher doses of IL-2 given for a shorter period of time. M-IL-2 dosing is well tolerated and should allow for increased use of IL-2 in vaccine protocols. Further study is needed to determine if M-IL-2 has an effect on clinical outcomes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Cancer Therapy: Clinical


Safety and Immunologic Response of a Viral Vaccine to Prostate-Specific Antigen in Combination with Radiation Therapy when Metronomic-Dose Interleukin 2 Is Used as an Adjuvant

Robert J. Lechleider, Philip M. Arlen, Kwong-Yok Tsang, et al.

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