It has become evident that naturally occurring CD25+ regulatory T cells (Treg cells) not only influence self-antigen specific immune response but also dampen foreign antigen specific immunity. This report extends our previous findings by demonstrating that immunity to certain herpes simplex virus (HSV) vaccines is significantly elevated and more effective if Treg cell response is curtailed during either primary or recall immunization. The data presented here show that removal of CD25+ Treg cells prior to SSIEFARL-CpG or gB-DNA immunization significantly enhanced the resultant CD8 T-cell response to the immunodominant SSIEFARL peptide. The enhanced CD8 T-cell reactivity in Treg cell-depleted animals was between two- and threefold and evident in both acute and memory stages. Interestingly, removal of CD25+ Treg cells during the memory recall response to plasmid immunization resulted in a twofold increase in CD8 T-cell memory pool. Moreover, in the challenge experiments, memory CD8 T cells generated with plasmid DNA in the absence of Treg cells cleared the virus more effectively compared with control groups. We conclude that CD25+ Treg cells quantitatively as well as qualitatively affect the memory CD8 T-cell response generated by gB-DNA vaccination against HSV. However, it remains to be seen if all types of vaccines against HSV are similarly affected by CD25+ Treg cells and if it is possible to devise means of limiting Treg cell activity to enhance vaccine efficacy.

MATERIALS AND METHODS

Animals, virus, and DNA vaccine preparation. Female C57BL/6 mice, 5 to 6 weeks of age, were purchased from Harlan Sprague Dawley (Indianapolis, Ind.). Animals were used in compliance with institutional animal health and care regulations, and all procedures used in the experiments with animals were approved by the local Institutional Animal Care and Use Committee. HSV-1 KOS (American Type Culture Collection, Manassas, Va.) and vaccinia virus encoding glycoprotein B (gB) of HSV-1 were grown and plaque titrated on Vero cells and kept at 80°C until use. Plasmid DNA was prepared as described previously (8, 24).

Depletion of CD4+ CD25+ T cells. Before immunization, mice were depleted of CD4+ CD25+ regulatory T cells by intraperitoneal administration of anti-CD25 monoclonal antibody clone PC61 (American Type Culture Collection, Manassas, Va.). The antibody was used as the ammonium sulfate precipitate of hybridoma culture supernatant or as ascites produced from PC61 hybridoma in nu/nu mice and purified by Prosep G immunoglobulin purification kit (Millipore, Bedford, Mass.). The depletion capability of these monoclonal antibody preparations did not differ significantly. Depletion efficiency was checked by staining with anti-CD25 antibody clone 7D4 (BD Bioscience, Pharmingen, San Diego, Calif.) and flow cytometry. Results (Fig. 1) show that a high level (≥80%) of depletion was reached by day 4 after intraperitoneal injection of 1.2 mg of depleting anti-CD25 clone PC61.

Immunization. C57BL/6 mice 5 to 6 weeks old were injected with anti-CD25 antibody or normal rat immunoglobulin 4 days earlier to deplete CD4+ CD25+ T cells and then injected with 50 μg of plasmid DNA encoding glycoprotein B of...
HSV-1 (gB-DNA) intramuscularly or SSIEFARL peptide (HSV gB<sub>Bm-s30</sub>) combined with bioactive CpG1826 (SS-CpG) (Coley Pharmaceutical Group) in the footpad. Vaccination was repeated after 3 weeks. Another group of mice were also depleted or not and infected with 10<sup>6</sup> PFU of HSV-1 KO5 in the footpad. HSV infection was used here as a positive control. Primary assessment of immune response was performed after 7 days for mice infected with HSV-1 or immunized with SS-CpG and 12 days for mice vaccinated with gB-DNA. Memory responses were assessed at 60 days post-secondary immunization. Control mice for the gB-DNA-immunized group were injected with 50 μg of plasmid DNA encoding β-galactosidase (β-galactosidase DNA), and control groups for SS-CpG were given nonbioactive CpG1982 or CpG2138.

ELISPOT for IFN-γ. ELISPOT plates (MultiScreen HA sterile plates, Millipore, Bedford, Mass.) were coated with capture anti-gamma interferon (IFN-γ) antibody in carbonate buffer, pH 9.6, overnight (BD Biosciences Pharmingen, San Diego, Calif.). Plates were then blocked with RPMI 1640 (Sigma, St. Louis, Mo.) supplemented with 10% fetal bovine serum. Responder cells from spleens or lymph nodes of immunized and control mice and stimulator cells prepared from naive mouse spleens pulsed with HSV-gB<sub>Bm-s30</sub> peptide and x-irradiated were added to coated plates and incubated at 37°C for 48 to 72 h and thereafter developed, and spots were counted as fully described elsewhere (24).

Intracellular cytokine staining for IFN-γ. We stimulated 10<sup>6</sup> spleen or lymph node cells per well with SSIEFARL in the presence of GolgiPlug (BD Biosciences Pharmingen, San Diego, Calif.) and 50 U of interleukin-2 (Hemagen). Responders were added to plates and incubated at 37°C for 5 days with gB-DNA or SS-CpG. Responses were measured 5 days later. Figures 4A and C show memory recall responses of T<sub>reg</sub>-depleted mice showed twofold increases in depleted versus nondepleted mice in lymph nodes and spleen, respectively. Similarly, vaccination with SS-CpG showed 2- to 4-fold increases in depleted versus nondepleted mice in lymph nodes and spleen, respectively.

Thus, the pattern of response observed with the two vaccine preparations in T<sub>reg</sub>-cell-depleted mice, although less in extent, was similar in profile to that of T<sub>reg</sub>-cell-depleted virus-infected mice (Fig. 2A). Taken together, these results indicated that eliminating the influence of the T<sub>reg</sub> cell population before vaccination with gB-DNA or SS-CpG peptide enhanced the primary CD8<sup>+</sup> T-cell response.

Depletion of CD4<sup>+</sup> CD25<sup>+</sup> T cells prior to primary vaccination improves the memory pool of CD8<sup>+</sup> T cells. We examined the influence of CD4<sup>+</sup> CD25<sup>+</sup> T-cell depletion on systemic T-cell memory generated with gB-DNA or SS-CpG vaccination. In the first instance, mice were depleted of T<sub>reg</sub> cells prior to primary vaccination. Restimulation was performed on day 21, and memory responses were measured 60 days later. Figure 3 shows IFN-γ-producing memory CD8<sup>+</sup> T cells in a representative experiment. Memory CD8<sup>+</sup> T-cell response in gB-DNA- or SS-CpG-immunized mice decreased two- and fourfold when measured at 60 days post-secondary immunization, respectively. T<sub>reg</sub>-cell-depleted animals had approximately twofold higher responses than nondepleted mice with both types of vaccines. Higher responses dominated in the spleens than the lymph nodes. Compared to the two vaccine preparations, HSV infection (Fig. 3A) had more responding CD8<sup>+</sup> T cells than gB-DNA or SS-CpG immunization. These results show that removal of T<sub>reg</sub> cells prior to primary immunization positively influenced the magnitude of the memory CD8<sup>+</sup> T cells of animals vaccinated with gB-DNA or SS-CpG.

We were curious to know if depletion of T<sub>reg</sub> cells in the memory phase equally enhanced the T-cell response. Therefore, in the second instance mice were infected with HSV or vaccinated with gB-DNA or SS-CpG. Sixty days later the animals were depleted of CD4<sup>+</sup> CD25<sup>+</sup> T cells and boosted after 5 days with gB-DNA or SS-CpG. Responses were measured 5 days later. Figures 4A and C show memory recall responses of CD8<sup>+</sup> T cells that produced IFN-γ upon restimulation in vitro. An approximately twofold increase in the number of IFN-γ-producing spleen CD8<sup>+</sup> T cells was observed between depleted DNA-vaccinated mice and nondepleted mice. HSV-infected mice had a similar pattern of response (Fig. 4A). Finally, depleting T<sub>reg</sub> cells at both the primary and memory phases did not produce further enhancement of the immune response to gB-DNA immunization or HSV infection (Fig. 5A). There was no statistically significant difference (P ≥ 0.05) between doubly depleted or singly depleted mice. This indicated that reactivation of the residual memory CD8<sup>+</sup> T-cell pool was also subject
to regulation by T<sub>reg</sub> cells and was affected by the removal of these cells. These results also indicated that T<sub>reg</sub> cells control the reactivity of memory T cells and that inhibiting the function of T<sub>reg</sub> cells even when memory is established allows the memory T cells to reactivate to a higher frequency.

Surprisingly, depletion of T<sub>reg</sub> cells in the memory phase of SS-CpG-immunized mice did not lead to significant expansion of the responding CD8<sup>+</sup> T cells (Fig. 4B and C). Both depleted and nondepleted mice from the SS-CpG-immunized group responded similarly, and the magnitude of response was severalfold lower than that observed in gB-DNA- and SS-CpG-vaccinated mice. The percentage shown in each cytogram represents the mean of IFN-γ-producing CD8<sup>+</sup> T cells obtained from each of four mice per group in three separate experiments. *, statistically significant (P < 0.05) compared to isotype immunoglobulin- and CpG1982-treated groups.

FIG. 2. CD8<sup>+</sup> T-cell primary response to vaccination after depletion of CD4<sup>+</sup> T cells. C57BL/6 mice were either depleted of CD25<sup>+</sup> T cells or injected with isotype immunoglobulin. Five days later, the animals were vaccinated with gB-DNA intramuscularly or SS-CpG in the footpad. Control mice were injected with either β-galactosidase DNA or nonbioactive CpG1982 or -2138. HSV infection of depleted and nondepleted mice was used as a positive control. Spleen and draining lymph nodes were collected for analysis on either day 7 postimmunization for SS-CpG or day 12 for gB-DNA vaccination. IFN-γ ELISPOT and intracellular staining were performed as described in Materials and Methods. (A) IFN-γ ELISPOT for gB-DNA vaccination and HSV infection, (B) ELISPOT for SS-CpG vaccination, (C) intracellular staining for IFN-γ gB-DNA- and SS-CpG-vaccinated mice. The percentage shown in each cytogram represents the mean of IFN-γ-producing CD8<sup>+</sup> T cells obtained from each of four mice per group in three separate experiments. *, statistically significant (P < 0.05) compared to isotype immunoglobulin- and CpG1982-treated groups.

depletion of T<sub>reg</sub> cells before SS-CpG immunization did not contribute to the magnitude of the memory response.

Cytotoxic T lymphocytes generated by vaccination following T<sub>reg</sub> cell depletion efficiently lyse their targets. We assessed whether the CD8<sup>+</sup> T cells generated in CD4<sup>+</sup> CD25<sup>+</sup> depleted mice after vaccination were functional CTLs. A standard chromium release assay was performed on splenocytes after expansion for 5 days in vitro. Figures 6A and B show that in the acute phase, incubation of effector T cells from gB-DNA- and SS-CpG-vaccinated T<sub>reg</sub> cell-depleted mice with target cells showed increased lysis of targets pulsed with gB<sub>308-505</sub> peptide. CTL activity of cells isolated from depleted mice was higher than that of nondepleted animals. Similarly, CTL activity of cells from T<sub>reg</sub> cell-depleted SS-CpG-vaccinated mice was higher than that of nondepleted mice. Memory (Fig. 6C and D) CTL activity of the gB-DNA-vaccinated group showed even
larger differences between depleted and nondepleted mice. SS-CpG immunization resulted in a poor memory CTL response, as effector cells from such mice did not lyse the targets efficiently. When these two forms of immunization were compared to Treg cells from depleted virus-infected mice, the latter had more potent CTLs (data not shown) than gB-DNA- and SS-CpG-vaccinated mice at both primary and memory phases.

The difference between the depleted and nondepleted groups at the memory phase suggested that removal of Treg cell control allowed generation of a high frequency of CTLs that efficiently lysed their target, which accounted for a better memory response in terms of efficacy as described subsequently. Control lysis assays with major histocompatibility complex class I mismatched target cells or MC38 cells pulsed with an irrelevant peptide from ovalbumin showed that lysis was specific to the HSV antigen-sensitized targets (data not shown).

**T**<sub>reg</sub> **cell-depleted and vaccinated mice clear challenge virus efficiently.** To show that the enhanced immune response following removal of regulatory T cells affected the outcome of a challenge by virus, we measured the clearance of a recombinant vaccinia virus encoding gB of HSV. This challenge model utilized the fact that vaccinia virus initially replicates in the ovaries of mice, which could provide a good measure of systemic responses against challenge by virus. Separate groups of gB-DNA- or SS-CpG-vaccinated mice were intraperitoneally infected with two different doses of vaccinia virus gB, a low dose, 10<sup>7</sup> PFU, and high dose, 10<sup>7</sup> PFU per mouse, and followed for 7 days. Table 1 shows titers of vaccinia virus gB titrated on Vero cells from homogenized ovaries of mice challenged with a low dose of virus. Challenge virus was detected in all groups of mice vaccinated with peptide, either Treg-depleted or nondepleted, although titers were only modest. Virus replication could be detected in these mice through day 7 of observation. In contrast, the depleted and nondepleted gB-DNA-vaccinated groups showed that on day 3 replication ensued and an approximately 2 log difference in viral titer was observed between depleted and nondepleted animals. For the
depleted group, the virus could only be titrated on day 3 and
was not detected on days 5 and 7.

Challenge with high-dose vaccinia virus gB showed that virus
replicated in the ovaries of all mice irrespective of depletion
status and vaccination (Table 2). All SS-CpG-vaccinated
groups were not protected from the high virus dose challenge,
and titers reached 4 logs of magnitude and could be detected
throughout the observation period. Threefold difference in

FIG. 4. Effect of CD4+ CD25+ T-cell depletion on memory recall responses. Mice were immunized without prior depletion of Treg cells. At 60 days
after initial immunization, the mice were depleted of Treg cells with anti-CD25 monoclonal antibody and 5 days later boosted with gB-DNA or SS-CpG. Control mice were injected with either β-galactosidase DNA or nonbioactive CpG1982 or -2138. HSV infection of depleted and nondepleted mice was
used as a positive control. Responses were analyzed 5 days later with IFN-γ ELISPOT and intracellular assays as described in Materials and Methods.
(A) IFN-γ ELISPOT for gB-DNA vaccination and HSV infection, (B) ELISPOT for SS-CpG vaccination, (C) intracellular staining for gB-DNA- and SS-CpG-vaccinated mice. The percentage shown in each cytogram represents the mean of IFN-γ-producing CD8+ T cells obtained from each of four
mice per group in two separate experiments. *, statistically significant (P ≤ 0.05) compared to isotype immunoglobulin- and CpG1982-treated groups.

FIG. 5. Influence of double depletion of CD4+ CD25+ T cells on memory recall responses. Mice were depleted of Treg cells prior to vaccination and
and depleted again at 60 days post-initial immunization. Control mice were injected with either β-galactosidase DNA or nonbioactive CpG1982 or -2138. ELISPOT and intracellular staining for IFN-γ were performed 5 days boosting with gB-DNA or SS-CpG. (A) ELISPOT for gB-DNA
vaccination and HSV infection, (B) ELISPOT for SS-CpG vaccination.
viral titers was shown between nondepleted and depleted gB-DNA-vaccinated mice on both days 3 and 5, and the virus could still be detected on day 7 in nondepleted gB-DNA-vaccinated group. Control groups, SS-CpG1982- and β-galactosidase-treated mice, had the highest viral titers after both a low dose and a high dose challenge, which shows evidence of virus replication in this challenge model. Moreover, in these control animals the ovaries were hyperemic and largely edematous by day 7.

This challenge model indicated that depletion of CD25+ Treg cells led to induction of CTLs or other mechanisms that contributed to efficient virus clearance. Although virus replication still occurred, the time of clearance was reduced to at least 5 days in DNA-vaccinated Treg cell-depleted mice and 3 days in HSV-infected Treg cell-depleted mice at the high virus challenge dose. Overall, depletion of Treg cells influenced the efficacy of DNA vaccination.

### DISCUSSION

It is evident now that naturally occurring CD25+ Treg cells not only influence self-antigen specific immune response (15, 18, 20) but also dampen foreign antigen specific immunity (3, 4, 22). Our initial observation with herpes simplex virus infection showed that the magnitude of CD8+ T-cell response was tightly regulated by CD25+ Treg cells. This report extends the previous findings by demonstrating that immunity to certain HSV vaccines is significantly enhanced and more effective if the Treg cell response is curtailed during primary or recall immunization. The data presented here show that removal of CD25+ Treg cells prior to SSIEFARL-CpG or gB-DNA immunization significantly enhanced the resultant CD8+ T-cell response to the immunodominant SSIEFARL peptide. This was shown by different in vitro assays, ELISPOT, CTL assay and intracellular IFN-γ staining that measured the CD8+ T-cell reactivity to SSIEFARL epitope.

The enhanced CD8+ T-cell reactivity in Treg cell-depleted animals was between two- and threefold and was evident in

### TABLE 1. Mean titers of virus in the ovaries collected at days 3, 5, and 7 following challenge with 10^5 PFU/mouse of vaccinia virus gB

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean log_{10} titer ± SD(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>HSV + PC61</td>
<td>0</td>
</tr>
<tr>
<td>HSV + isotype Ig</td>
<td>1.28 ± 0.31</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>3.08 ± 1.1</td>
</tr>
<tr>
<td>SS-CpG1826 + PC61</td>
<td>1.21 ± 0.40</td>
</tr>
<tr>
<td>SS-CpG1826 + isotype Ig</td>
<td>1.92 ± 0.14</td>
</tr>
<tr>
<td>SS-CpG2138 + PC61</td>
<td>4.81 ± 1.3</td>
</tr>
<tr>
<td>gB-DNA + PC61</td>
<td>1.28 ± 0.62</td>
</tr>
<tr>
<td>gB-DNA + isotype Ig</td>
<td>3.00 ± 1.78</td>
</tr>
<tr>
<td>β-Galactosidase DNA</td>
<td>4.5 ± 0.1</td>
</tr>
</tbody>
</table>

\(a\) Values represent means for four mice per group in two separate experiments.

### TABLE 2. Mean titers of virus in the ovaries collected at days 3, 5, and 7 following challenge with 10^7 PFU/mouse of vaccinia virus gB

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean log_{10} titer ± SD(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>HSV + PC61</td>
<td>1.12 ± 0.16</td>
</tr>
<tr>
<td>HSV + isotype Ig</td>
<td>2.79 ± 0.21</td>
</tr>
<tr>
<td>Phosphate buffer saline</td>
<td>4.11 ± 1.5</td>
</tr>
<tr>
<td>SS-CpG1826 + PC61</td>
<td>4.12 ± 0.16</td>
</tr>
<tr>
<td>SS-CpG1826 + isotype Ig</td>
<td>4.790 ± 0.26</td>
</tr>
<tr>
<td>SS-CpG2138 + PC61</td>
<td>4.11 ± 1.5</td>
</tr>
<tr>
<td>gB-DNA PC61</td>
<td>1.21 ± 0.65</td>
</tr>
<tr>
<td>gB-DNA + isotype Ig</td>
<td>3.99 ± 1.18</td>
</tr>
<tr>
<td>β-Galactosidase DNA + PC61</td>
<td>4.9 ± 0.13</td>
</tr>
</tbody>
</table>

\(a\) Values are means for four mice per group in two separate experiments.
both the acute and memory stages. Interestingly, removal of CD25+ T<sub>reg</sub> cells resulted in a twofold increase in effector cells, and virus-challenged animals cleared infection more effectively. A boost of such immunity by T<sub>reg</sub> cell depletion was not noted in CpG peptide-immunized mice. We conclude that CD25+ T<sub>reg</sub> cells quantitatively as well as qualitatively affect the CD8<sup>+</sup> T-cell immune response generated by gB-DNA vaccination against HSV. However, it remains to be seen if all types of vaccines against HSV are similarly affected by CD25+ T<sub>reg</sub> cells and if it is possible to devise means of limiting T<sub>reg</sub> cell activity to enhance vaccine efficacy. The renaissance of T<sub>reg</sub> cells emphasized their role in limiting the expression of AIDS. More recently, however, it became evident that T<sub>reg</sub> cells influence the immune response to exogenous antigens, especially those expressed by pathogens. Our observation that the CD8<sup>+</sup> and later the CD4<sup>+</sup> response to HSV was limited if T<sub>reg</sub> cells were present during primary infection raised several questions. Among these was whether the response was unique to a replicating virus and if the phenomenon might serve to limit the efficacy of certain vaccines. Our observation that the magnitude of the CD8<sup>+</sup> T-cell response to a DNA vaccine as well as an adjuvanted peptide vaccine was elevated approximately the same as the virus when the response of T<sub>reg</sub> cell-depleted or nondepleted animals were compared was surprising. Accordingly, we had expected that the activation of T<sub>reg</sub> cells was a combination of recognition by viral antigen-specific T<sub>reg</sub> cells and activation, perhaps nonspecific, by components of the virus or stress molecules generated by dying infected cells. However, the responses to both the DNA vaccine encoding gB and the CpG peptide vaccine were equally subject to T<sub>reg</sub> cell control, as was the response to HSV.

CD4<sup>+</sup> CD25<sup>+</sup> T cells were reported to influence mostly CD4<sup>+</sup> cells (1, 14). Here additional evidence shows that murine CD4<sup>+</sup> CD25<sup>+</sup> T cells can also regulate the responses of CD8<sup>+</sup> cells. The fact that there was a marked difference between depleted and nondepleted groups of mice indicated that clonal expansion of CD8<sup>+</sup> T cells was inhibited in mice not depleted of T<sub>reg</sub> cells. The mechanisms involved in the regulation of antigen-specific CD8<sup>+</sup> T cells were not directly studied, but a recent report (6) showed substantial inhibition of interleukin-2 transcription and interleukin-2 production which coincided with equally marked inhibition of interleukin-2 receptor α expression. Additionally, the same report suggested that poor performance of the CD8<sup>+</sup> T cells under the influence of T<sub>reg</sub> cells was due to limited transcription and production of IFN-γ and other molecules such as perforin and granzyme B, responsible for the cytolytic activity of CD8<sup>+</sup> T cells. The gB-DNA vaccine could be recognized by CD8<sup>+</sup> and CD4<sup>+</sup> T cells, including T<sub>reg</sub> cells, but at present we do not have positive evidence for the latter. The peptide vaccine should only be recognized by CD8<sup>+</sup> T cells but was regulated by T<sub>reg</sub> cells, and hence it needs to be explained how the T<sub>reg</sub> cell function is expressed in this instance. The observation that there was a regulatory mechanism imposed on CpG peptide vaccination which inhibited the immune responses indicated that the mechanism may involve nonspecific activation of regulatory T cells. Although there is no evidence of nonspecific activation of T<sub>reg</sub> cells, the use of CpG, a ligand for Toll-like receptor 9 expressed by dendritic cells in the vaccine preparation may have induced a cytokine-chemokine milieu conducive for activation of T<sub>reg</sub> cells, since there are reports demonstrating that T<sub>reg</sub> cells are particularly sensitive to inflammatory cytokines/chemokines (5, 10).

Direct interaction of CpG and T<sub>reg</sub> cells can be ruled out because murine T<sub>reg</sub> cells do not express Toll-like receptor 9. However, the finding by Caramalho et al. (7) that seven out of nine murine Toll-like receptors are expressed by T<sub>reg</sub> cells suggests that a rather wide spectrum of inflammation-associated endogenous and pathogen-specific molecules might directly influence their activation. This possible line of evidence for nonspecific T<sub>reg</sub> cell activation is also seen in the study by Moser et al. (16) in which CpG-treated dendritic cells were first pulsed with OT-I peptide and injected into mice previously adoptively transferred with OT-I cells. Reponses in mice depleted of T<sub>reg</sub> cells were greatly enhanced compared to nondepleted mice. However, examination of this effect in Toll-like receptor 9-deficient cells and animals might give insight into the mechanism of T<sub>reg</sub> cell activation in the case of immunization with a major histocompatibility complex class I-restricted peptide and CpG.

When T<sub>reg</sub> cells in gB-DNA-immunized mice were depleted in the memory phase and boosted with antigen, more CD8<sup>+</sup> T cells were recalled and increased twofold in comparison to nondepleted animals. The control of memory T cells by T<sub>reg</sub> cells has also been reported by Kursar et al. (12). In their studies on Listeria monocytogenes, when DNA-immunized mice were depleted of T<sub>reg</sub> cells in the memory phase and then later restimulated, a 10-fold increase in the responding CD8<sup>+</sup> T cells was observed. In a recent study the same authors showed a similar effect of memory depletion of T<sub>reg</sub> cells on CD8<sup>+</sup> T cells during vaccination with nonviable Listeria monocytogenes (13). Although we used a different antigen-vector combination, we obtained a somewhat inferior response at recall compared to that of Kursar et al. This difference in increase could result from a less restricted activation of Listeria-specific memory T cells (12) compared to HSV-specific cells, especially those generated by DNA encoding gB, which generally gives a weak immune response. What is not known in both cases is whether the T<sub>reg</sub> cells exert direct control on the memory CD8<sup>+</sup> T cells or through other means. It is also clear that T<sub>reg</sub> cells control the generation of effector CD8<sup>+</sup> T cells as well as the expansion of the T-cell memory pool of CD8<sup>+</sup> upon reexposure to antigen, but what is not known is whether T<sub>reg</sub> cells play a role in the contraction and maintenance of CD8<sup>+</sup> T-cell memory.

Importantly, depletion of regulatory T cells notably affected the level of memory response generated after gB-DNA vaccination. In contrast, depletion of T<sub>reg</sub> cells did not improve memory to peptide or CpG immunization. The poor performance of CD8<sup>+</sup> T cells generated by peptide vaccination could result from the events occurring at the priming stage. It has been reported earlier that priming CD8<sup>+</sup> T-cell response in the absence of helper T cells impairs the memory response of those CD8<sup>+</sup> T cells (25). Evidently, removal of T<sub>reg</sub> did not alter the programming of the CD8<sup>+</sup> T cells to mimic that which occurs during priming in the presence of CD4<sup>+</sup> T-cell help.

From the immunization standpoint, it is important to understand what other consequences may apply to the T<sub>reg</sub> cell manipulation approach to vaccination against microbes. Tagu-
chi and Takahashi (23) reported that injection of anti-CD25 antibodies into normal animals induced localized autoimmune disease. However, no such side effect was observed in the present study following administration of the depleting antibody. In Sutmuller’s (21) studies on tumor vaccination involving removal of Treg cells, autoimmunity developed only when CTLA-4 was used in combination to exclude the suppressive function of CD25

Treg cells. Thus, this point is critical in understanding how to carefully manipulate such vital cells so as to benefit vaccination against viral infections. Likely, a vaccination protocol to include manipulation of Treg cells would mean applying a reagent in a single dose followed shortly by the vaccine, since the immune-enhancing effect of depleting CD25+ Treg cells was observed only after a few days of depletion. Such a procedure would eliminate repeated depletion of Treg cells.

In summary, the data reported here suggest that the level of immune response observed in intact animals to DNA vaccination may be a result of a higher threshold of T-cell activation imposed by CD4+ CD25+ Treg cells. Consequently, vaccination against infectious agents may be enhanced by altering the regulatory pathway involving Treg cells, which may improve vaccine efficacy. Indeed, depletion of CD4+ CD25+ T cells improves DNA vaccine efficacy, which implies that the rational design of vaccines against viruses should consider means of circumventing the suppressive function of the regulatory T cells in inducing primary immune response or secondary responses during boosting of existing immunity. However, it remains to precisely define the strategy that could allow achievement of careful and successful manipulation of regulatory T cells, either a low-dose immunologic approach, which is a less likely approach, a chemical approach, or other means yet to be described.

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