JOURNAL OF VIROLOGY, Dec. 2004, p. 13082–13089 Vol. 78, No. 23
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.23.13082–13089.2004

$CD4^+$ $CD25^+$ T Cells Regulate Vaccine-Generated Primary and Memory $CD8^+$ T-Cell Responses against Herpes Simplex Virus Type 1

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Received 27 May 2004/Accepted 28 July 2004

It has become evident that naturally occurring $CD25^+$ regulatory T cells (T_{reg} 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 T_{reg} cell response is curtailed during either primary or recall immunization. The data presented here show that removal of $CD25^+$ T_{reg} 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 T_{reg} cell-depleted animals was between two- and threefold and evident in both acute and memory stages. Interestingly, removal of $CD25^+$ T_{reg} 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 T_{reg} cells cleared the virus more effectively compared with control groups. We conclude that $CD25^+$ T_{reg} 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 $CD25^+$ T_{reg} cells and if it is possible to devise means of limiting T_{reg} cell activity to enhance vaccine efficacy.

It has been clear that the outcome of several in vivo immunological events is influenced by T cells that suppress the function of other cells involved in immunity. Recent focus has been on regulatory T cells (T_{reg} cells) initially recognized to prevent genetically susceptible mice from developing certain autoimmune diseases (2, 19, 20). Subsequently, T_{reg} cells were shown to influence transplantation immunity as well as immune and inflammatory reactions to infectious agents (3, 4, 12, 17, 22). Of particular interest, T_{reg} cells had a pivotal influence on the outcome of chronic parasitic infections (3). Additionally, it was shown that animals depleted of T_{reg} cells showed markedly superior acute and memory $CD8^+$ T-cell responses to infection with herpes simplex virus (HSV) (22). Animals lacking T_{reg} cells in fact generated more effective protective immunity. Subsequently, T_{reg} cell depletion was also shown to result in more severe immunopathological responses to virus infection.

The fact that immunity to HSV was superior and more sustained when infection occurred in T_{reg} cell-depleted animals was considered to impact upon vaccine design. Thus, HSV is one of those clinically important agents for which there is currently no effective vaccine. Conceivably, the inhibiting activity of T_{reg} cells could help explain the difficulty in achieving effective immunity.

In the present report, we measured the influence of T_{reg} cells on the immune response of mice to DNA and peptide vaccine preparations against HSV. Our results show that immunity to DNA and peptide vaccines measured systemically were compromised by the presence of T_{reg} cells. Most strikingly, if T_{reg} cells were inhibited prior to recall in the memory phase, responses to DNA vaccination were elevated twofold and animals showed notably increased resistance to challenge. Our results are discussed in terms of selecting vaccine approaches that are least affected by T_{reg} cell response as well as the need to identify procedures that minimize the T_{reg} cell response during vaccination. We also comment about mechanistic events that could account for T_{reg} cell interference.

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, Manasas, 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

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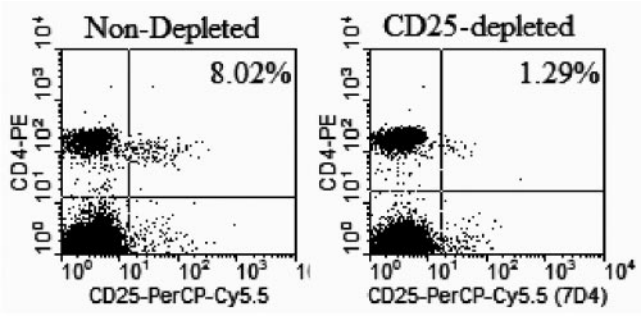

FIG. 1. Depletion of $CD4^+$ $CD25^+$ T cells in C57BL/6 mice. Mice were injected intraperitoneally with 1.2 mg of anti-CD25 monoclonal antibody clone PC61. Four or 5 days later, peripheral blood mononuclear cells were stained with $CD25^+$-peridinin chlorophyll protein Cy5.5 clone 7D4 and $CD4^+$-phycoerythrin. Flow cytometry analysis was done in FACScan (Becton Dickinson), and data were analyzed with CellQuest software.

HSV-1 (gB-DNA) intramuscularly or SSIEFARL peptide (HSV $gB_{498-505}$) 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 10^6 PFU of HSV-1 KOS 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 naïve mouse spleens pulsed with HSV-$gB_{498-505}$ 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 10^6 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) per ml for 5 h at 37°C. The cells were processed further as described by Kumaraguru and Rouse (11). The fluorescently labeled antibodies used were purchased from BD Biosciences Pharmingen, San Diego, Calif..

CTL assay. A standard 4-h ^{51}Cr release assay was performed to assess cytolytic activity of the $CD8^+$ T cells isolated from immunized and control mice as described elsewhere (9, 24). Data were corrected by the formula ([experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Challenge and virus titration. Mice were challenged at 60 days following initial immunization. A vaccinia virus challenge model, where intraperitoneal injection of vaccinia virus causes initial replication in the ovaries, was used to test efficacy of virus clearance in vaccinated animals. Female C57BL/6 mice were injected intraperitoneally with a recombinant vaccinia virus encoding gB of HSV-1 at a low dose (10^5 PFU) and a high dose (10^7 PFU). Ovaries were collected on days 3, 5, and 7, homogenized, clarified in phosphate-buffered saline, and used for virus titration. A conventional viral plaque assay was used.

Statistics. Where appropriate, significant differences were calculated with Student's t test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of $CD25^+$ depletion prior to DNA or SS-CpG vaccination on $CD8^+$ T-cell response. C57BL/6 mice were either depleted 4 days previously with anti-CD25 antibody clone PC61 or given normal rat immunoglobulin and exposed to infectious HSV, gB-DNA, or SS-CpG vaccine formulations. At 7 or 12 days postimmunization, both spleens and lymph nodes were collected to quantify $CD8^+$ T-cell responses to the immunodominant peptide SSIEFARL by ELISPOT or intracellular cytokine staining for IFN-γ. The results of representative experiments are shown in Fig. 2.

As is evident, these acute-phase responses were elevated in mice that were depleted of $CD25^+$ T_{reg} cells prior to antigen exposure. This supports previous observations that depleted mice exposed to infectious virus showed increase of immune response over nondepleted animals. DNA vaccination of T_{reg} cell-depleted mice showed two- and threefold increase over nondepleted mice in lymph nodes and spleen, respectively. Similarly, vaccination with SS-CpG showed 2- and 2.8-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_{reg} cell-depleted mice, although less in extent, was similar in profile to that of T_{reg} cell-depleted virus-infected mice (Fig. 2A). Taken together, these results indicated that eliminating the influence of the T_{reg} cell population before vaccination with gB-DNA or SS-CpG peptide enhanced the primary $CD8^+$ T-cell response.

Depletion of $CD4^+$ $CD25^+$ T cells prior to primary vaccination improves the memory pool of $CD8^+$ T cells. We examined the influence of $CD4^+$ $CD25^+$ 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_{reg} 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 $CD8^+$ T cells in a representative experiment. Memory $CD8^+$ 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_{reg} 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 $CD8^+$ T cells than gB-DNA or SS-CpG immunization. These results show that removal of T_{reg} cells prior to primary immunization positively influenced the magnitude of the memory $CD8^+$ T cells of animals vaccinated with gB-DNA or SS-CpG.

We were curious to know if depletion of T_{reg} 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 $CD4^+$ $CD25^+$ 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 $CD8^+$ T cells that produced IFN-γ upon restimulation in vitro. An approximately twofold increase in the number of IFN-γ-producing spleen $CD8^+$ 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_{reg} 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 ($P \geq 0.05$) between doubly depleted or singly depleted mice. This indicated that reactivation of the residual memory $CD8^+$ T-cell pool was also subject

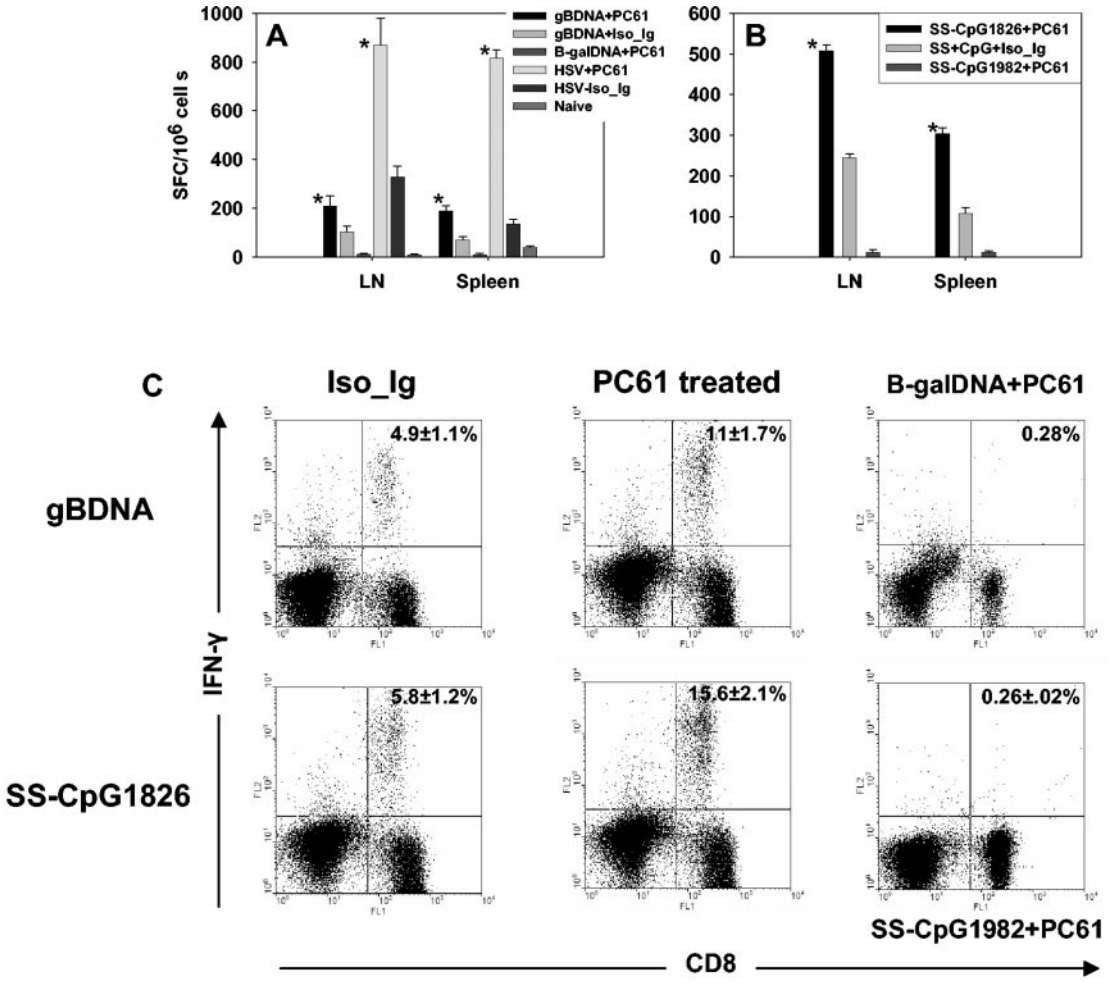

FIG. 2. $CD8^+$ T-cell primary response to vaccination after depletion of $CD4^+$ $CD25^+$ T cells. C57BL/6 mice were either depleted of $CD25^+$ T cells or injected with isotype immunoglobulin. Five days later, the animals were vaccinated with gB-DNA intramuscularly or SS-CpG in the footpad. Control 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 $CD8^+$ T cells obtained from each of four mice per group in three separate experiments. ∗, statistically significant ($P \leq 0.05$) compared to isotype immunoglobulin- and CpG1982-treated groups.

to regulation by T_{reg} cells and was affected by the removal of these cells. These results also indicated that T_{reg} cells control the reactivity of memory T cells and that inhibiting the function of T_{reg} cells even when memory is established allows the memory T cells to rapidly reactivate to a higher frequency.

Surprisingly, depletion of T_{reg} cells in the memory phase of SS-CpG-immunized mice did not lead to significant expansion of the responding $CD8^+$ 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-immunized or HSV-infected mice (Fig. 4A and C). This observation indicates that even though there is enhancement of primary immune response in the absence of T_{reg} cells, the effect had less impact on the memory response if the primary $CD8^+$ T cells were generated in the absence of $CD4^+$ T-cell help. Therefore, depletion of T_{reg} cells before SS-CpG immunization did not contribute to the magnitude of the memory response.

Cytotoxic T lymphocytes generated by vaccination following T_{reg} cell depletion efficiently lyse their targets. We assessed whether the $CD8^+$ T cells generated in $CD4^+$ $CD25^+$ 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_{reg} cell-depleted mice with target cells showed increased lysis of targets pulsed with $gB_{498-505}$ peptide. CTL activity of cells isolated from depleted mice was higher than that of nondepleted animals. Similarly, CTL activity of cells from T_{reg} 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

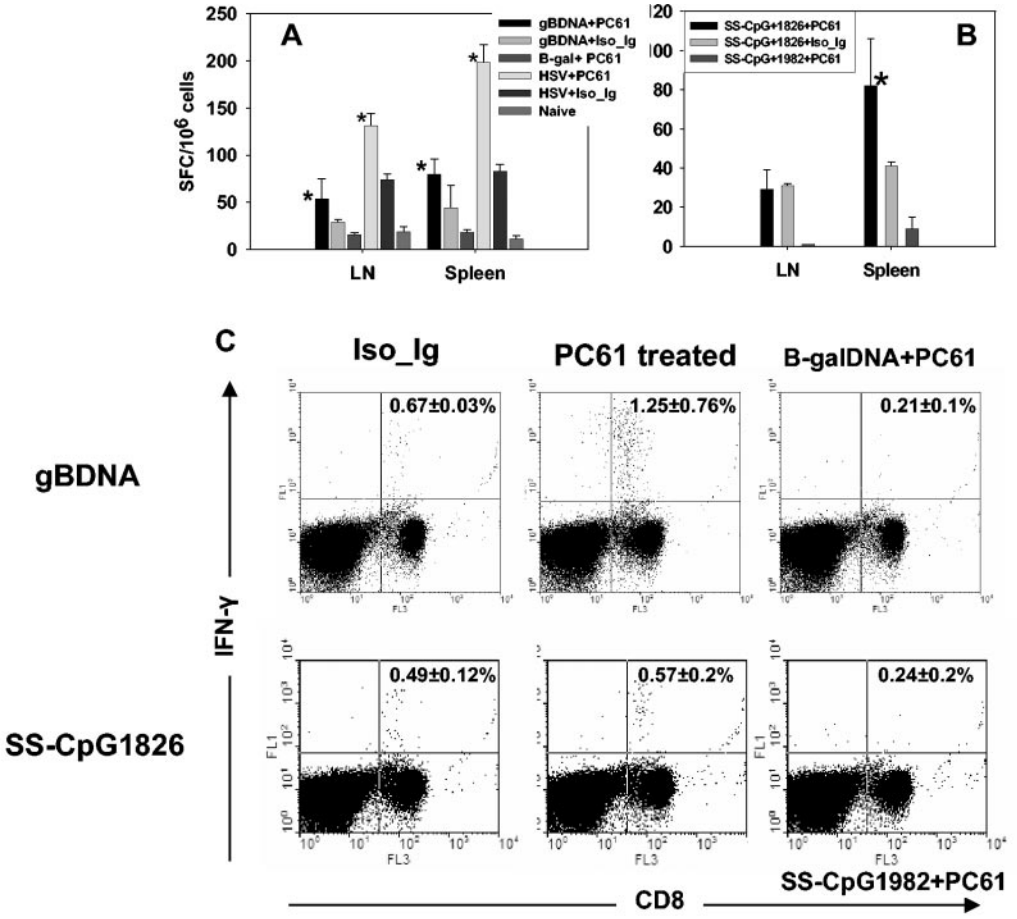

FIG. 3. $CD8^+$ T-cell memory response to vaccination following depletion of $CD4^+$ $CD25^+$ T cells. C57BL/6 mice were either depleted of $CD25^+$ T cells or injected with isotype immunoglobulin. Five days later the animals were immunized with gB-DNA intramuscularly or SS-CpG in the footpad. Control mice were injected with either β-galactosidase DNA or nonbioactive CpG 1982 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 day 60 postimmunization. IFN-γ ELISPOT and intracellular staining were performed as described in Materials and Methods. (A) 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 experiments performed. ∗, statistically significant ($P \leq 0.05$) compared to isotype immunoglobulin- and CpG1982-treated groups.

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 T_{reg} 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 T_{reg} 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_{reg} 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, 10^5 PFU, and high dose, 10^7 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 T_{reg} 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

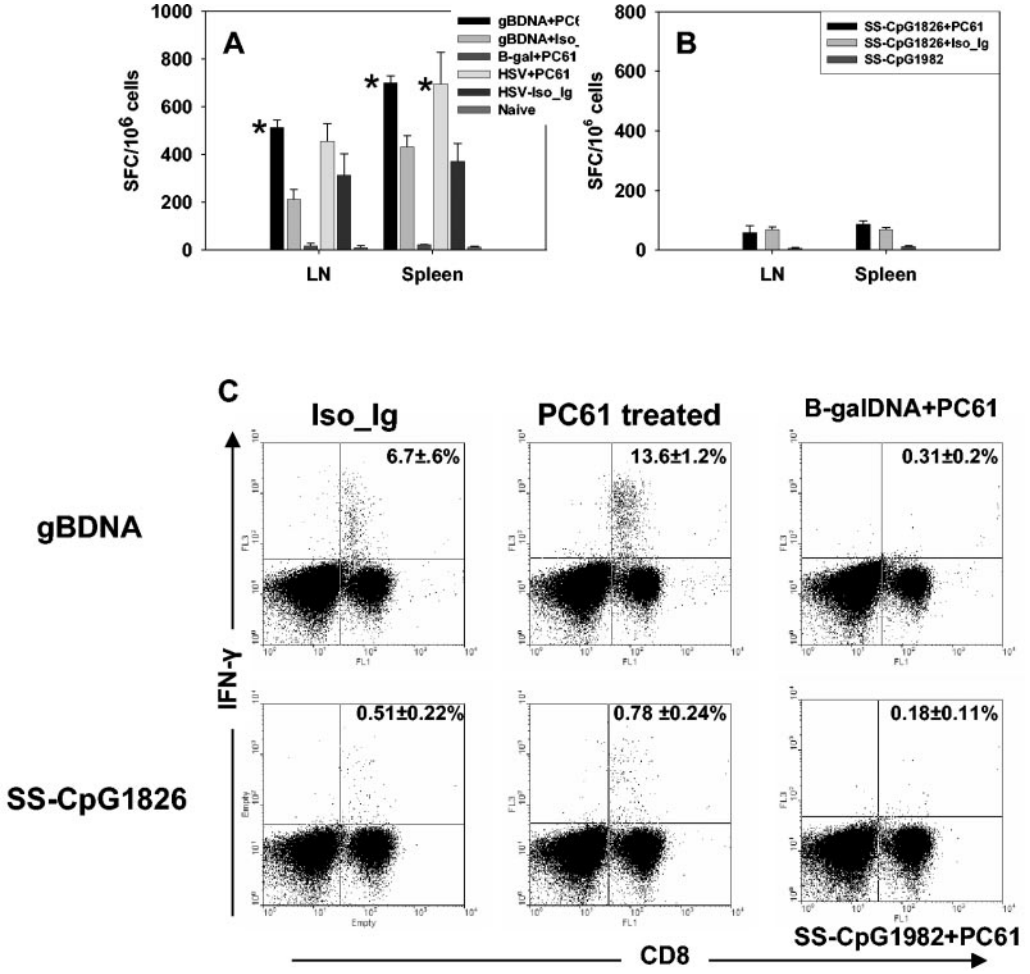

FIG. 4. Effect of $CD4^+$ $CD25^+$ T-cell depletion on memory recall responses. Mice were immunized without prior depletion of T_{reg} cells. At 60 days after initial immunization, the mice were depleted of T_{reg} cells with anti-CD25 monoclonal antibody and 5 days later boosted with gB-DNA or SS-CpG. 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 ($P \leq 0.05$) compared to isotype immunoglobulin- and CpG1982-treated groups.

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

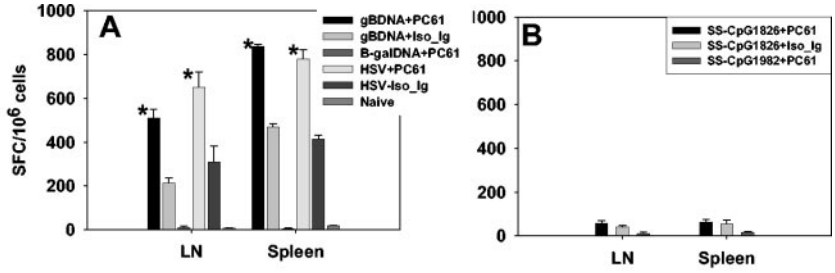

FIG. 5. Influence of double depletion of $CD4^+$ $CD25^+$ T cells on memory recall responses. Mice were depleted of T_{reg} cells prior to vaccination and depleted again at 60 days post-initial immunization. Control mice were injected with either β-galactosidase DNA or nonbioactive CpG1982 or -2138. 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.

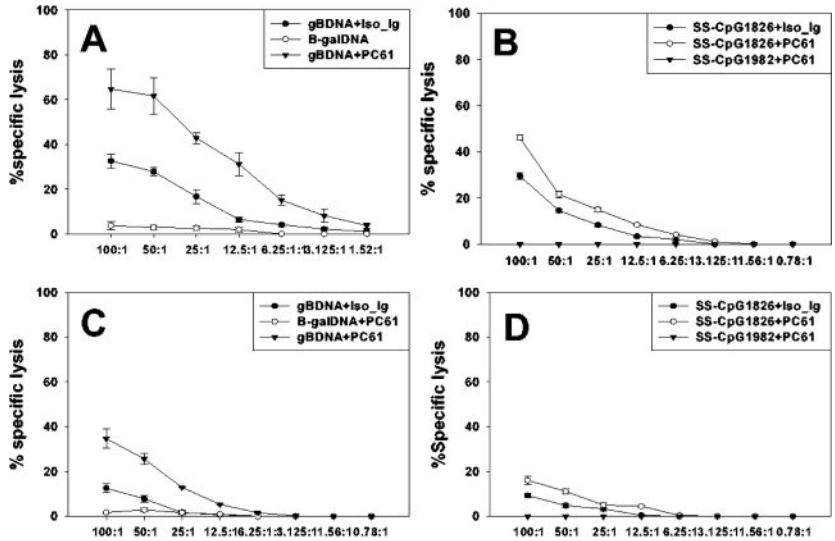

FIG. 6. Cytotoxic T lymphocytes generated in the absence of regulatory T cells efficiently lyse peptide-sensitized targets. Splenocytes were isolated and expanded in vitro for 5 days, and CTL activity was determined as described in Materials and Methods. (A and B) CTL activity measured during the acute phase of immunization for gB-DNA and SS-CpG1826, respectively. (C and D) Memory-phase CTL activity for gB-DNA and SS-CpG1826, respectively.

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 $CD25^+$ T_{reg} 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 T_{reg} cell-depleted mice and 3 days in HSV-infected T_{reg} cell-depleted mice at the high virus challenge dose. Overall, depletion of T_{reg} cells influenced the efficacy of DNA vaccination.

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

Group	Mean $\log_{10}$ titer ± SD[a]		
	Day 3	Day 5	Day 7
HSV + PC61	0	0	0
HSV + isotype Ig	1.28 ± 0.31	0	0
Phosphate-buffered saline	3.08 ± 1.1	4.4 ± 0.19	3.73 ± 0.19
SS-CpG1826 + PC61	1.21 ± 0.40	1.11 ± 0.93	1.0 ± 0.67
SS-CpG1826 + isotype Ig	1.92 ± 0.14	1.01 ± 0.5	1.1 ± 0.41
SS-CpG2138 + PC61	4.81 ± 1.3	4.01 ± 0.29	3.37 ± 0.39
gB-DNA + PC61	1.28 ± 0.62	0	0
gB-DNA + isotype Ig	3.00 ± 1.78	2.51 ± 0.2	0
β-Galactosidase DNA	4.5 ± 0.1	4.54 ± 0.68	3.98 ± 0.67

[a] Values represent means for four mice per group in two separate experiments.

DISCUSSION

It is evident now that naturally occurring $CD25^+$ T_{reg} 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 $CD25^+$ T_{reg} cells. This report extends the previous findings by demonstrating that immunity to certain HSV vaccines is significantly enhanced and more effective if the T_{reg} cell response is curtailed during primary or recall immunization. The data presented here show that removal of $CD25^+$ T_{reg} 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 T_{reg} cell-depleted animals was between two- and threefold and was evident in

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

Group	Mean $\log_{10}$ titer ± SD[a]		
	Day 3	Day 5	Day 7
HSV + PC61	1.12 ± 0.16	0	0
HSV + isotype Ig	2.79 ± 0.21	1.10 ± 0.12	0
Phosphate buffered saline	4.11 ± 1.5	5.56 ± 0.9	3.23 ± 0.32
SS-CpG1826 + PC61	4.12 ± 0.16	4.0 ± 1.01	2.15 ± 0.78
SS-CpG1826 + isotype Ig	4.790 ± 0.29	4.10 ± 0.24	2.29 ± 0.65
SS-CpG2138 + PC61	4.11 ± 1.5	5.56 ± 0.9	3.23 ± 0.32
gB-DNA PC61	1.21 ± 0.65	1.1 ± 0.43	0
gB-DNA + isotype Ig	3.99 ± 1.18	3.21 ± 0.23	2.31 ± 0.21
β-Galactosidase DNA + PC61	4.9 ± 0.13	5.3 ± 1.89	4.18 ± 0.77

[a] Values are means for four mice per group in two separate experiments.

both the acute and memory stages. Interestingly, removal of $CD25^+$ T_{reg} cells during the memory phase prior to plasmid recall immunization resulted in a twofold increase in effector cells, and virus-challenged animals cleared infection more effectively. A boost of such immunity by T_{reg} cell depletion was not noted in CpG peptide-immunized mice. We conclude that $CD25^+$ T_{reg} cells quantitatively as well as qualitatively affect the $CD8^+$ 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_{reg} cells and if it is possible to devise means of limiting T_{reg} cell activity to enhance vaccine efficacy.

The renaissance of T_{reg} cells emphasized their role in limiting the expression of AIDS. More recently, however, it became evident that T_{reg} cells influence the immune response to exogenous antigens, especially those expressed by pathogens. Our observation that the $CD8^+$ and later the $CD4^+$ response to HSV was limited if T_{reg} 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 $CD8^+$ 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_{reg} cell-depleted or nondepleted animals were compared was surprising. Accordingly, we had expected that the activation of T_{reg} cells was a combination of recognition by viral antigen-specific T_{reg} 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_{reg} cell control, as was the response to HSV.

$CD4^+$ $CD25^+$ T cells were reported to influence mostly $CD4^+$ cells (1, 14). Here additional evidence shows that murine $CD4^+$ $CD25^+$ T cells can also regulate the responses of $CD8^+$ cells. The fact that there was a marked difference between depleted and nondepleted groups of mice indicated that clonal expansion of $CD8^+$ T cells was inhibited in mice not depleted of T_{reg} cells. The mechanisms involved in the regulation of antigen-specific $CD8^+$ 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 $CD8^+$ T cells under the influence of T_{reg} 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 $CD8^+$ T cells.

The gB-DNA vaccine could be recognized by $CD8^+$ and $CD4^+$ T cells, including T_{reg} cells, but at present we do not have positive evidence for the latter. The peptide vaccine should only be recognized by $CD8^+$ T cells but was regulated by T_{reg} cells, and hence it needs to be explained how the T_{reg} 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_{reg} 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_{reg} cells, since there are reports demonstrating that T_{reg} cells are particularly sensitive to inflammatory cytokines/chemokines (5, 10).

Direct interaction of CpG and T_{reg} cells can be ruled out because murine T_{reg} 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_{reg} 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_{reg} 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_{reg} 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_{reg} cell activation in the case of immunization with a major histocompatibility complex class I-restricted peptide and CpG.

When T_{reg} cells in gB-DNA-immunized mice were depleted in the memory phase and boosted with antigen, more $CD8^+$ T cells were recalled and increased twofold in comparison to nondepleted animals. The control of memory T cells by T_{reg} cells has also been reported by Kursar et al. (12). In their studies on *Listeria monocytogenes*, when DNA-immunized mice were depleted of T_{reg} cells in the memory phase and then later restimulated, a 10-fold increase in the responding $CD8^+$ T cells was observed. In a recent study the same authors showed a similar effect of memory depletion of T_{reg} cells on $CD8^+$ 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_{reg} cells exert direct control on the memory $CD8^+$ T cells or through other means. It is also clear that T_{reg} cells control the generation of effector $CD8^+$ T cells as well as the expansion of the T-cell memory pool of $CD8^+$ upon reexposure to antigen, but what is not known is whether T_{reg} cells play a role in the contraction and maintenance of $CD8^+$ 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_{reg} cells did not improve memory to peptide or CpG immunization. The poor performance of $CD8^+$ T cells generated by peptide vaccination could result from the events occurring at the priming stage. It has been reported earlier that priming $CD8^+$ T-cell response in the absence of helper T cells impairs the memory response of those $CD8^+$ T cells (25). Evidently, removal of T_{reg} did not alter the programming of the $CD8^+$ T cells to mimic that which occurs during priming in the presence of $CD4^+$ T-cell help.

From the immunization standpoint, it is important to understand what other consequences may apply to the T_{reg} 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 T_{reg} cells, autoimmunity developed only when CTLA-4 was used in combination to exclude the suppressive function of $CD25^+$ T_{reg} 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 T_{reg} cells would mean applying a reagent in a single dose followed shortly by the vaccine, since the immune-enhancing effect of depleting $CD25^+$ T_{reg} cells was observed only after a few days of depletion. Such a procedure would eliminate repeated depletion of T_{reg} 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 $CD4^+$ $CD25^+$ T_{reg} cells. Consequently, vaccination against infectious agents may be enhanced by altering the regulatory pathway involving T_{reg} 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.

ACKNOWLEDGMENTS

This work was supported by grants AI 14981 and AI 46462 from the National Institutes of Health.

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