The Relative Contribution of the CD28 and gp39 Costimulatory Pathways in the Clonal Expansion and Pathogenic Acquisition of Self-reactive T Cells

By Nathan D. Griggs,* Sally S. Agersborg,* Randolf J. Noelle,‡ Jeffrey A. Ledbetter,§ Peter S. Linsley,§ and Kenneth S.K. Tung*

From the *Department of Pathology, University of Virginia, Charlottesville, Virginia 22908; and ‡Department of Microbiology, Dartmouth Medical College, Hanover, New Hampshire 03756; and §Department of Autoimmunity and Transplantation, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121

Summary

The zona pellucida (ZP), an ovarian extracellular structure, contains three major glycoproteins: ZP1, ZP2, and ZP3. A ZP3 peptide contains both an autoimmune oophoritis–inducing T cell epitope and a B cell epitope that induces autoantibody to ZP. This study investigates two major T cell costimulation pathways in this disease model. Herein we show that blockage of glycoprotein (gp)39 and CD40 interaction with gp39 monoclonal antibody (mAb) results in the failure to induce both autoimmune oophoritis and autoantibody production. Inhibition of ligand binding to the CD28 receptor with the fusion protein, murine CTLA4–immunoglobulin (Ig), also results in failure to generate antibody to ZP and significantly reduces disease severity and prevalence. Surprisingly, the frequencies of antigen-specific T cells in anti–gp39 mAb–treated mice, CTLA4-Ig treated mice, and in mice given control hamster IgG or control fusion protein L6, were equivalent as determined by limiting dilution analysis ($\approx$1:5,000). These T cells, which produced comparable amounts of interleukin 4 and interferon γ in vitro, were able to transfer oophoritis to normal recipients. When anti–gp39 mAb and CTLA4-Ig were given together, the effect was additive, leading to inhibition of T cell activation as determined by in vitro proliferation and limiting dilution analysis ($\approx$1:190,000); disease and antibody responses were absent in these mice. By studying these two costimulatory pathways in parallel, we have shown that autoimmune disease and autoantibody production are inhibitable by blocking either the gp39 or the CD28 pathway, whereas inhibition of clonal expansion of the effector T cell population occurs only when both pathways are blocked.

T cell activation by APCs is known to require multiple intercellular ligand interactions. The precise mechanisms involved in this process are only beginning to be unraveled. Whereas specificity is maintained through the TCR complex (1), many studies have shown that engagement of this receptor alone is insufficient to drive full T cell activation (2–4). T cell costimulatory molecules and soluble factors like IL-4 and IFN-γ contribute to the process of converting naive T cells into a population of clonally expanded, effector T cells (5). The best characterized of the costimulatory molecules is CD28 and its receptors on APCs, CD80 (B7.1) and CD86 (B7.2) (for a review see reference 6). CD28 engagement transmits a signaling cascade different from that of the TCR, possibly involving PI 3-kinase activity (6). A number of in vitro studies have suggested that blockage of this important pathway results in T

cell anergy (7–9). In CD28 knockout mice, normal thymocyte development occurs but peripheral T cell proliferative responses to the mitogen Con A are impaired (10). Humoral immune responses in these knockout mice were also impaired with much reduced IgG production as compared to control animals (10).

CTLA-4 is a marker of activated T cells that was originally identified by screening a murine cytolytic T cell cDNA library (11). CTLA-4 can bind the same receptor as CD28 but does so with 20-fold higher affinity (12). The function of CTLA-4 is unclear but recent work by Walunas et al. (13) has suggested a potential role for CTLA-4 as a negative regulator of T cell activation. The binding of CTLA-4 to CD80 was first demonstrated through the use of a soluble fusion protein consisting of the extracellular domain of CTLA-4 and the Fc portion of a human IgG1 (12). Use of

this reagent has been effective in blocking in vitro proliferation of alloreactive cells (14), preventing xenograft rejection (15) and prolonging allograft rejection (16).

A second costimulatory pathway is regulated by glycoprotein (gp)[1]39 (CD40L, T-BAM), a type II membrane protein expressed on activated T cells. The gp39 receptor, CD40, is expressed on B cells and other APCs and is critical for the maturation and differentiation of B cells in response to thymus-dependent antigens (17). Mice in which the gp39 molecule has been disrupted exhibit a decreased IgM response to thymus-dependent antigens and fail to produce germinal centers, suggesting an inability to develop memory B cell responses (18, 19). The importance of this pathway in normal human immune function is underscored by patients with hyper-IgM syndrome that results from a mutated gp39 molecule (20). These patients have normal or elevated concentrations of polyclonal IgM and undetectable or markedly reduced levels of IgA, IgE, and IgG. Several studies have shown that inhibition of the gp39 pathway results in autoimmune disease prevention. Allospecific CTL responses and anti-DNA autoantibodies in graft-vs.-host disease were prevented by the in vivo administration of anti-gp39 mAb (21). Collagen-induced arthritis was similarly blocked by the in vivo administration of anti-gp39 mAb (22). Recently, Mohan et al. (23) have shown that anti-gp39 mAb was able to suppress the development of murine lupus nephritis and delay the onset of spontaneous autoantibody formation in lupus-prone mice.

In this study we have examined the contributions of the CD28 and gp39 costimulatory pathways in the generation of autoreactive T cells and autoantibody production in a murine model of autoimmune oophoritis. CTLA4-Ig was used to dissect the involvement of the CD28 pathway whereas anti-gp39 mAb was used to examine the contribution of the gp39 pathway. Previous studies have reported the efficacy of these reagents in inhibiting their respective costimulatory pathways (14–16, 21–23). We now demonstrate that inhibition of either pathway is not sufficient to block clonal expansion of the antigen-specific T cell population, yet blockage of both pathways results in failure of T cells to clonally expand. In contrast, inhibition of either pathway alone blocks T cell effector function, including disease induction and T cell help. This study therefore provides new insight into the relative contribution of the CD28 and gp39 costimulatory pathways in T cell activation and autoimmune disease pathogenesis, and suggests an important function for the gp39 pathway in T cell activation and clonal expression.

Materials and Methods

Mice. (C57BL/6 × A/J) F_1 (B6AF$_1$) female mice, obtained from the Jackson Laboratory (Bar Harbor, ME) were used ranging in age from 6 to 9 wk.

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[1]*Abbreviations used in this paper:* gp, glycoprotein; IF, immunofluorescence; ZP, zona pellucida.

Antibodies and Reagents. The hamster anti-gp39 mAb, MR-1, and murine CTLA4-Ig and mAb L6 have been previously described (17, 24). Control hamster IgG was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-purified anti-gp39 mAb or hamster IgG was administered (250 μg/injection, i.p.) on days 0, 4, 8, and 12 after immunization. CTLA4-Ig (200 μg/injection) was administered on days 0, 2, 4, and 10 after immunization. L6 is a control mouse IgG2a mAb against a human carcinoma antigen.

Histology and Immunohistochemistry. Ovaries were fixed in Bouin's solution and embedded in paraffin. Approximately 50 serial step sections per ovary, 5 μm thick, were stained with hematoxylin and eosin. Histopathology of oophoritis was evaluated as coded specimens, with severity graded from 1 to 4: 1, focal inflammation in interstitial space; 2 and 3, increasing multifocal inflammation and/or granuloma between and within ovarian follicles; and 4, loss of ovarian follicles and ovarian atrophy.

Peptide Synthesis and Purification. Zona pellucida (ZP)3(330-342) and ZP3(330-340) were synthesized with an automated peptide synthesizer (model AMS422; Gilson Medical Electronics, Inc., Middleton, WI) using fluorenyl-methoxycarbonyl (Fmoc) chemistry. Peptides were cleaved from resins by using TFA/ethandithiol/thioanisole/anisole at a ratio of 90:3:5:2 (vol/vol). Peptides were purified by HPLC on a C18 reverse phase column (Waters, Milford, MA). All peptides exceeded 95% purity. ZP3(330-342) and ZP3(330-340) exhibit similar immunogenicity and oophoritogenicity and were used interchangeably in this study.

Immunization. Peptides dissolved in milli-Q water and sterilized by ultrafiltration were emulsified in an equal volume of CFA (Sigma Chemical Co.). Mice received 0.1 ml (50 nmol) of the mixture in one footpad and at the base of the tail. Animals were killed 14 d later and serum, lymph nodes, and ovaries were collected.

Detection of Antibody to ZP3 Peptides or the ZP by Solid Phase ELISA. Each well of a 96-well flat bottom plate (Corning Glass Inc., Corning, NY) was coated with 100 μl of a 10 μM ZP3 peptide solution overnight and blocked with 3% BSA, fraction V (Sigma Chemical Co.) in PBS the following day. Sera were diluted and added to the wells in duplicate. After 60 min at 20°C, the plates were washed and further incubated with goat anti–mouse IgG antibody conjugated with peroxidase (Southern Biotechnology Associates, Birmingham, AL) (1:2,500) for 40 min at 20°C. After the plates were washed, a substrate mixture of O-phenylenediamine and hydrogen peroxide (Sigma Chemical Co.) was added. Color intensity of the mixture was determined at 490 nm on an ELISA reader (Molecular Devices, Corp., Menlo Park, CA).

Immunofluorescence. Ovaries were snapfrozen in liquid nitrogen and embedded in OCT compound, and 5-μm-thick sections were cut in a cryostat. After the sections were fixed in 90% ethanol for 15 min, they were rinsed in PBS for direct or indirect immunofluorescence (IF). For indirect IF, the normal mouse ovarian sections were incubated with serum diluted in PBS containing 3% BSA for 30 min. After rinsing in PBS, the sections were stained with FITC-conjugated goat antibody to mouse IgG or to mouse IgM (Cappel Inc., Malvern, PA). Sections were mounted in glycerol with 10% PBS. As a standard, a group of slides stained by twofold dilutions of a pooled antiserum to ZP3(330-342) were studied in parallel. For direct IF, the sections were incubated with FITC-conjugated goat anti–mouse IgG antibody.

Limiting Dilution Analysis. Lymph node cells were seeded into 96-well, round-bottomed microtiter plates at decreasing concentrations from 3 × 10⁴ to 117 cells/well, in doubling dilutions. The cells were seeded in 200 μl of DME supplemented with 2-ME (5 × 10⁻⁵ M), L-glutamine (2 mM), penicillin (100 U/

ml), streptomycin (100 μg/ml), and 10% FCS (vol/vol). Irradiated splenocytes (2,500 rad) were added as feeder cells (10^{-5} cells/well). Cultures were maintained in the absence or presence (20 μM) of antigen, Con A (3 μg/ml), or irradiated feeder cells. 24 wells were seeded at each cell concentration: 18 with antigen, 3 with Con A, and 3 without antigen. Plates were incubated for 5 d in 5% CO_2 at which time 100 μl of supernatant was transferred into a 96-well flat-bottomed plate. 50 HT-2 indicator cells were then added and allowed to incubate for an additional 3 d at which time 10 U of recombinant human IL-2 (a kind gift from M. Gately, Hoffmann La Roche, Nutley, NJ) was added to each well. Cultures were incubated for an additional 3 d. Uptake of ^{3}[H]thymidine was measured in the last 18 h of culture. A positive well was scored by thymidine uptake exceeding the mean plus 2 SD of the control wells. Percent negative wells were plotted against cell concentration on a semilog plot. The cell number corresponding to 37% negative wells was considered to represent the frequency of lymph node cells responding to the antigen.

T Cell Proliferation Assay. Lymph node cells from peptide-immunized mice were stimulated in vitro with varying concentrations of peptide in 200 μl complete medium in a 96-well flat-bottomed plate. The cells were allowed to incubate for 4 d at 37°C in 5% CO_2 and air. 0.5 μCi of [^{3}H]thymidine was added the final day of culture, and cell-associated radioactivity was determined in a beta counter. Data were expressed as delta counts per minute (Δcpm; cpm in T cells cultured with antigenic peptide − cpm in T cells cultured without antigenic peptide).

Cytokine Quantitation. Lymph node cells from peptide-immunized mice were bulk stimulated in vitro with peptide for 7 d at 3 × 10^5 cells/ml. After the 7-d incubation, 10^6 cells were added to each well of a 24-well plate coated with 0.5 μg anti-CD3 antibody (Accurate Chemical & Scientific Corp., Westbury, NY) at 4°C overnight. Supernatants were collected 2 d later and used to stimulate indicator cell lines. IFN-γ was detected using the macrophage cell line RAW 264 which produces nitrite in the presence of IFN-γ and LPS (25). IL-4 was detected using the IL-4–
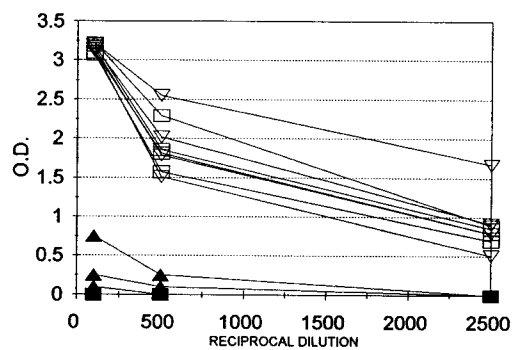

Figure 1. Serum antibody levels are markedly decreased in mice receiving anti-gp39 mAb and/or CTLA4-Ig. Sera were collected from mice 14 d after peptide immunization from treated control hamster IgG (□), control L6 (∇), anti-gp39 mAb (▲), CTLA4-Ig (●), and combined anti-gp39 mAb plus CTLA4-Ig (■) animals. Standard ELISA data are presented as optical density vs. reciprocal dilution of sera. A representative of five independent experiments is shown with four mice per group. Note that the CTLA4-Ig–treated group and combined gp39 plus CTLA4-Ig–treated groups (hidden) elicited no detectable antibody response.

dependent cell line CT.4S (a kind gift from Dr. William Paul, National Institutes of Health, Bethesda, MD). Cytokine amounts were standardized against recombinant IFN-γ and IL-4 in each bioassay (R&D Systems, Inc., Minneapolis, MN).

Adoptive Transfer of Autoimmune Oophoritis. Cell donors were immunized with peptide in CFA, with or without CTLA4-Ig or anti-gp39 mAb treatment regimens. Regional lymph node cells obtained 14 d later were stimulated in vitro with 30 mM peptide for 4 d; viable T cells obtained on Ficoll-Hypaque gradients were injected, intraperitoneally into normal syngeneic recipients at 10^7 cells per mouse. Ovarian pathology was determined histologically
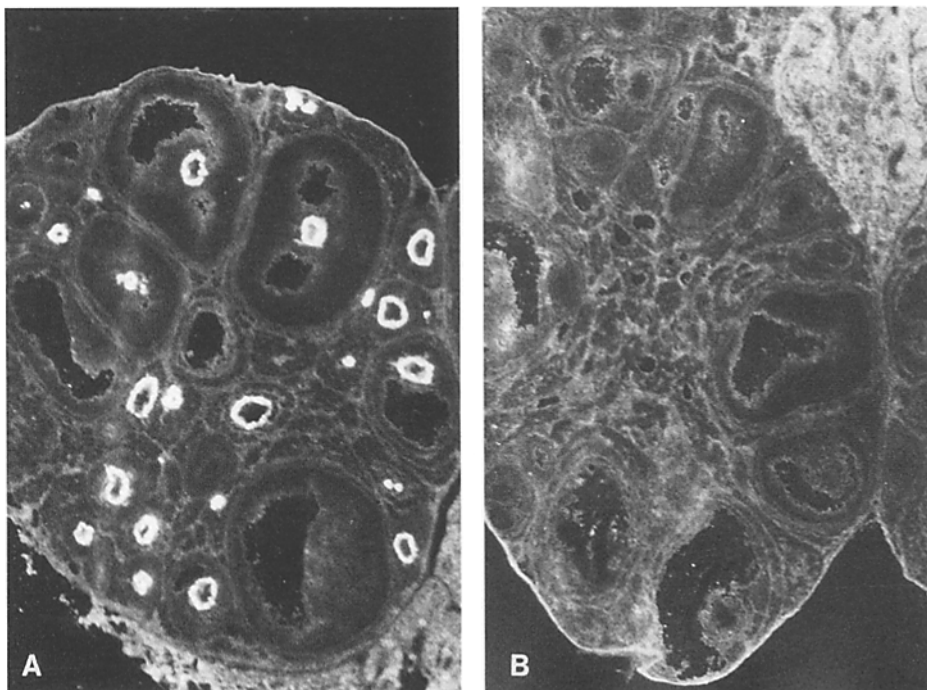

Figure 2. IgG bound to the ZP is detected by direct IF in ovaries of peptide-immunized mice receiving control L6 (*A*), but not in ovaries of peptide-immunized mice receiving CTLA4-Ig (*B*). ×100.

Table 1. *Inhibition of Autoimmune Oophoritis by Blockage of the CD28/B7 and/or gp39/CD40 Costimulatory Pathways*

Treatment	Incidence	1	2	3	4
				Oophoritis	
CTLA4-Ig	5/13	2	3	0	0
L6 or PBS	17/18	3	4	10	0
Anti-gp39	1/19	0	1	0	0
Hamster IgG or PBS	24/28	7	8	9	0
CTLA4-Ig + anti-gp39	0/9	0	0	0	0
L6 + hamster IgG	9/9	1	4	4	0

Peptide-immunized mice were treated with control reagents, CTLA4-Ig, anti-gp39 antibody, or both CTLA4-Ig and anti-gp39 antibody. Ovaries were harvested 14 d after immunization and disease was scored on a scale from 1 to 4 as outlined in Materials and Methods.

14 d later.

Statistical Analysis. A chi-squared analysis was used to determine the significance of difference in disease incidence among experimental groups.

Results

The Effect of Costimulatory Molecule Inhibition on Autoantibody Production. IgG autoantibody production in immu-

nized mice treated with anti-gp39 mAb, murine CTLA4-Ig, or anti-gp39 mAb plus CTLA4-Ig was assessed (Fig. 1). Mice that received control reagents (PBS, L6, hamster Ig) had significantly higher titers of antipeptide antibody at all dilutions tested compared with mice receiving anti-gp39 mAb, CTLA4-Ig, or both reagents. Only at the lowest dilution of sera were any antipeptide antibodies detected in the anti-gp39 mAb-treated group. Both the CTLA4-Ig and combined anti-gp39 mAb plus CTLA4-Ig group failed to elicit a detectable autoantibody. Similar results were obtained for IgM autoantibody and antibodies detected by indirect IF (data not shown).

Direct IF of antibody bound to ZP in vivo revealed a pattern of responsiveness similar to the ELISA antibody finding. Those immunized mice that received control reagents had intense staining of the ZP, whereas mice treated with anti-gp39 mAb, CTLA4-Ig, or both reagents had no detectable zona-bound IgG (Fig. 2).

Effect of Costimulatory Molecule Inhibition on Ovarian Pathology. 2 wk after immunization with an oophoritogenic peptide, mice were killed and the ovaries collected. Ovarian histopathology from control and treated animals were scored from 1 to 4. Mice that received control reagents (PBS, L6, hamster Ig, L6 plus hamster Ig) had a high incidence and severity of disease (Table 1). Those mice that received anti-gp39 mAb had virtually no disease with only 1 mouse in 19 developing low grade inflammation after 14 d (Fig. 3). Mice were also studied for disease induction up to 40 d and they remained free of any ovarian pathology.
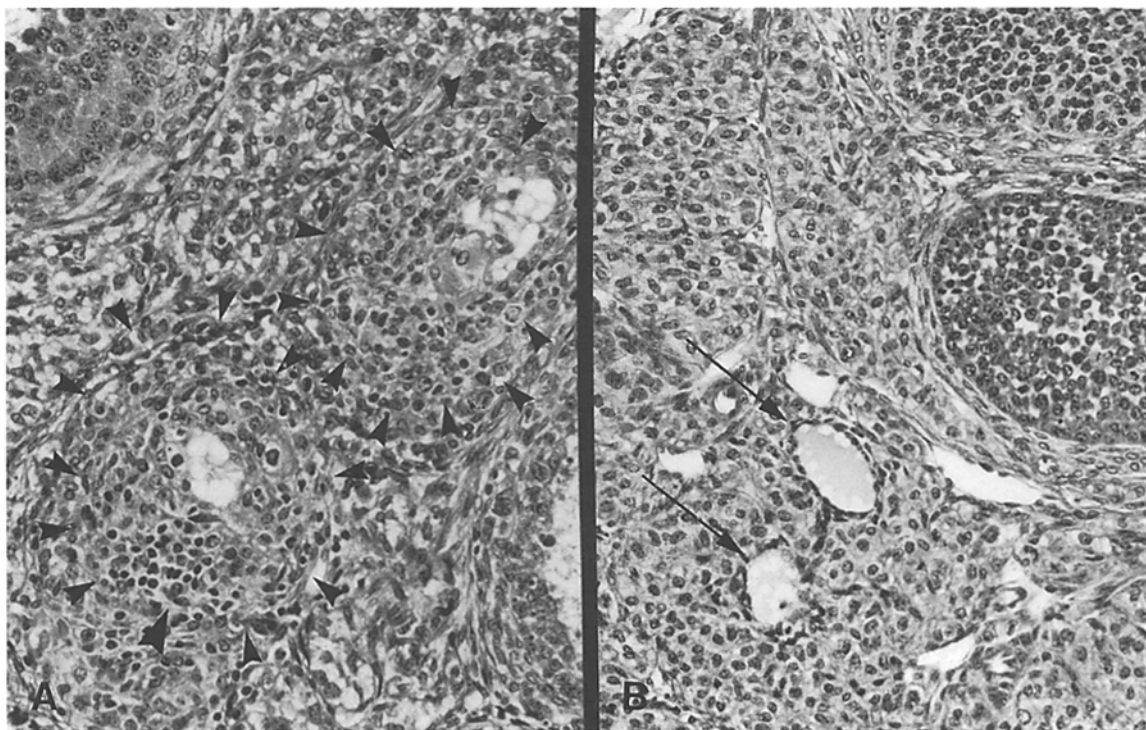

Figure 3. Ovarian inflammation affecting atretic follicles (*arrowheads, A*) is detected in peptide-immunized mice receiving control hamster IgG, but not in mice receiving anti-gp39 mAb (*B*). (*Arrows, B*) Normal atretic follicles free of inflammation (hematoxylin and eosin. ×200).

Blockage of the CD28 pathway with the CTLA4-Ig reagent also resulted in reduction of disease. Although 5 of 13 mice developed oophoritis, the disease was uniformly mild and of low incidence compared to control animals ($p < 0.005$). When both costimulatory pathways were inhibited, none of the mice developed disease.

Thus, inhibition of CD28 and/or gp39 costimulatory pathways resulted in the reduction of disease incidence and severity and inhibition of IgG autoantibody production. We next investigated whether disease and antibody inhibi-

tion were associated with a reduction of effector T cell generation.

Effect of Costimulatory Molecule Inhibition on In Vitro Proliferation of Lymph Node Cells. Lymph node cells from control and treated animals were stimulated in vitro with various concentrations of the immunizing peptide (Fig. 4). At all of the peptide concentrations examined, no statistical difference in the proliferative capacity was noted between anti-gp39 mAb-treated and control animals (Fig. 4 *A*). Similarly, no statistical difference was noted between the CTLA4-Ig–treated group and control animals (Fig. 4 *B*). However, when both the gp39 and CD28 costimulatory pathways were blocked, proliferation was not seen at any peptide concentration tested (Fig. 4 *C*). These results suggest that blockage of either the gp39 or CD28 costimulatory pathway does not render the antigen-specific T cells unresponsive to a second in vitro challenge with the immunizing peptide.

Frequency of Responding Antigen-specific T Cells Determined by Limiting Dilution Analysis. The frequency of ZP3 peptide–specific responding T cells was determined in mice of all treatment groups 2 wk after immunization. Frequency analysis of the antigen–specific T cell population failed to detect a significant difference between the anti-gp39 mAb-treated and control animals (Fig. 5 *A*). A calculated frequency of 1 in 4,971 was determined for the anti-gp39 mAb-treated animals whereas the control animals displayed a frequency of 1 in 6,096 cells being antigen specific. Likewise, clonal expansion of antigen-specific T cells occurred in CTLA4-Ig–treated animals at the same frequency as that seen in control animals (1 in 5,914 vs. 1 in 5,849, respectively; Fig. 5 *B*).

When both the gp39 and CD28 pathways were blocked, T cell frequency analysis revealed a marked reduction in the number of responding antigen-specific T cells as compared to control animals (1 in 184,894 vs. 1 in 5,551) (Fig. 5 *C*). Moreover, the gross size of the regional lymph nodes in control animals and those animals that received either anti-gp39 mAb or CTLA4-Ig alone were enlarged, whereas lymph nodes from mice that received both anti-gp39 mAb and CTLA4-Ig were similar to those of unimmunized mice (data not shown). These results suggest that clonal expansion of autoreactive T cells does not depend exclusively on a single pathway, but rather, depends on multiple complementary pathways.

It is interesting that mice treated with anti-gp39 mAb alone or with CTLA4-Ig alone had proliferative T cell responses to the immunizing peptide without oophoritis or anti-ZP antibody production. We next determined whether the cytokines produced by these T cells had altered in the presence of these inhibitors. In addition, we determined the ability of the clonally expanded T cells to adoptively transfer oophoritis to normal recipients.

Cytokine Production by Clonally Expanded T Cells in Mice Treated with CTLA4-Ig or Anti-gp39 mAb. When activated in vitro with the immunizing peptide, T cells from mice treated with CTLA4-Ig or anti-gp39 mAb produced amounts of IFN-γ and IL-4 comparable with control mice (Table
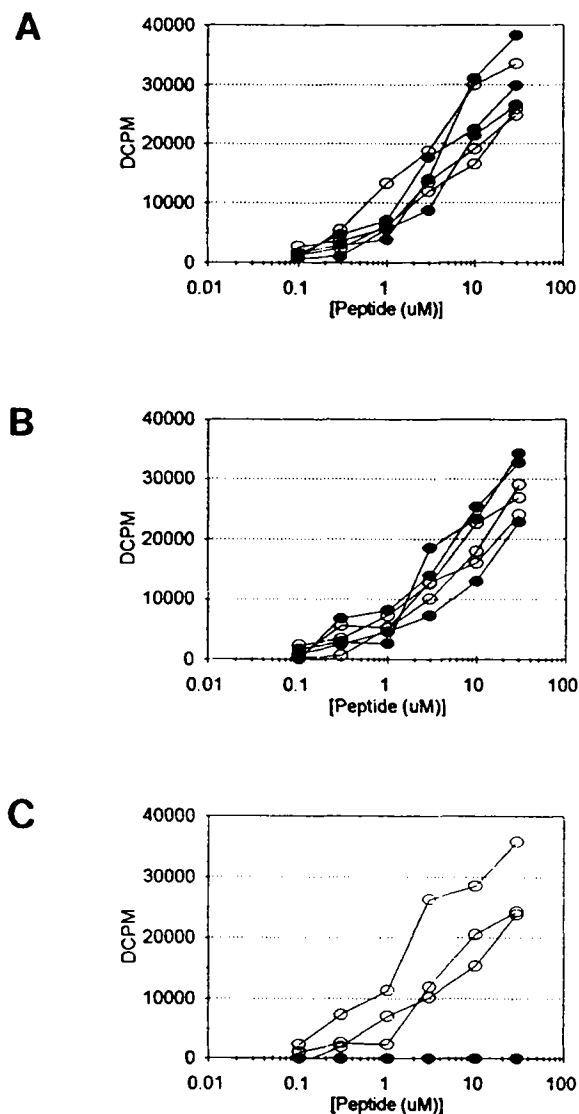

Figure 4. Mice receiving both anti-gp39 mAb and CTLA4-Ig have no in vitro proliferative response to the immunizing peptide. Draining lymph node cells were collected from mice 14 d after peptide immunization from (*A*) anti-gp39 mAb and hamster IgG, (*B*) CTLA4-Ig and L6, and (*C*) combined anti-gp39 mAb/CTLA4-Ig and hamster IgG/L6 treated groups. (*Open symbols*) Control animals; (*filled symbols*) experimental animals. Data are presented as Δcpm vs. concentration of stimulating peptide. Results of one of three independent experiments are shown with each line representing the response of a single mouse and three mice contained within each group.
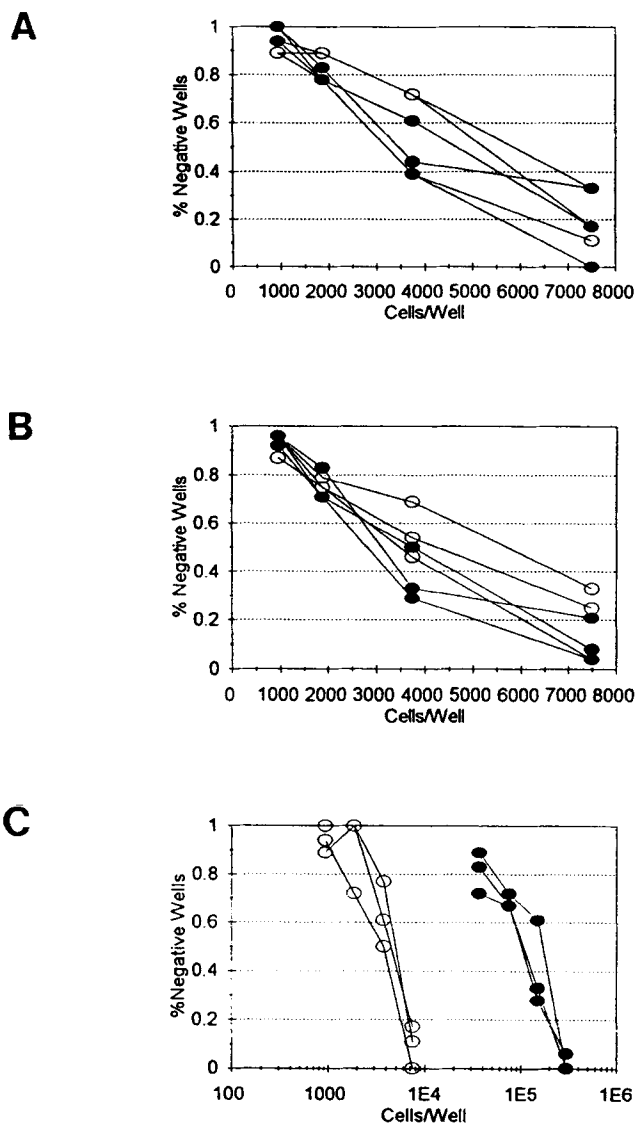
A

B

C

Figure 5. Mice receiving both anti-gp39 mAb and CTLA4-Ig have a marked reduction in the clonal expansion of antigen-specific T cells. Draining lymph node cells were collected 14 d after peptide immunization from (A) anti-gp39 mAb and hamster IgG, (B) CTLA4-Ig and L6, (C) combined anti-gp39 mAb/CTLA4-Ig and hamster IgG/L6 treated groups. (*Open symbols*) Control animals; (*filled symbols*) experimental animals. Data are presented as percent negative wells vs. number of cells per well. Results of one of three independent experiments are shown.

2). Therefore, a change in cytokine profile or T helper cell subset is not associated with the reduction in the incidence of autoimmune oophoritis in the treated groups.

Adoptive Transfer of Autoimmune Oophoritis by Lymph Node Cells from Peptide-immunized Mice Treated with CTLA4-Ig or Anti-gp39 mAb. Lymph node cells from peptide-immunized mice transferred comparable incidence and severity of oophoritis to normal recipients regardless of whether the donors had received CTLA4-Ig (3/4), anti-gp39 mAb (6/8), or control reagents (5/7) (Fig. 6). Whereas clonally expanded T cells do not elicit significant oophoritis in the donors,

Table 2. *Inhibition of the CD28/B7 or gp39/CD40 Costimulatory Pathways Does not Alter the Cytokine Phenotype of Lymph Node Cells*

Treatment	Cytokine	
	IFN-γ	IL-4
	U	pg
CTLA4-Ig	104 ± 34	29 ± 27
Anti-gp39	106 ± 27	42 ± 50
Control	106 ± 70	58 ± 51

Peptide-immunized mice were treated with control reagents, CTLA4-Ig, or anti-gp39 Ab. Lymph node cells were harvested 14 d after immunization and cytokine production assessed as outlined in Materials and Methods. The results from six mice per group are shown.

these cells, upon activation in vitro, are pathogenic. Also, Con A–stimulated spleen cells from animals treated with both CTLA4-Ig and anti-gp39 mAb failed to transfer disease (0/6). Lymph node cells from animals treated with both CTLA4-Ig and anti-gp39 mAb failed to generate enough cells for adoptive transfer.

Discussion

Experimental murine autoimmune oophoritis is induced by immunization with a 10- or 11-mer synthetic peptide corresponding to mouse ZP3 proteins 330-341 and 330-342 (26–28). The ovarian inflammation is transferrable by a CD4+ clone of the Th1 phenotype (27). Animals injected with the ZP3 peptide also develop autoantibodies to the ZP, and the response is driven, in part, by endogenous ZP antigens (26). However, induction of high ZP antibody titers, without concomitant T cell response to the peptide, does not cause ovarian pathology (29). Thus, like experimental autoimmune encephalomyelitis, oophoritis is primarily an inflammatory CD4+ T cell–mediated autoimmune disease. Based on this model, we have evaluated, in parallel, the participation of the gp39 and the CD28 pathways in autoantibody induction, ovarian disease, and clonal expansion of ZP3 peptide–specific T cells.

Our results indicate that blockage of each of the pathways was associated with inhibition or significant reduction in the prevalence and severity of ovarian pathology. The ZP antibody response was simultaneously ablated. Somewhat surprisingly, these changes were not associated with reduction in clonal expansion of the ZP3 peptide–specific T cells as measured by limiting dilution analysis. Moreover, T cells from mice treated with anti-gp39 mAb or CTLA4-Ig produced amounts of IFN-γ and IL-4 similar to that of untreated mice, and they were able to transfer severe oophoritis to normal recipients.

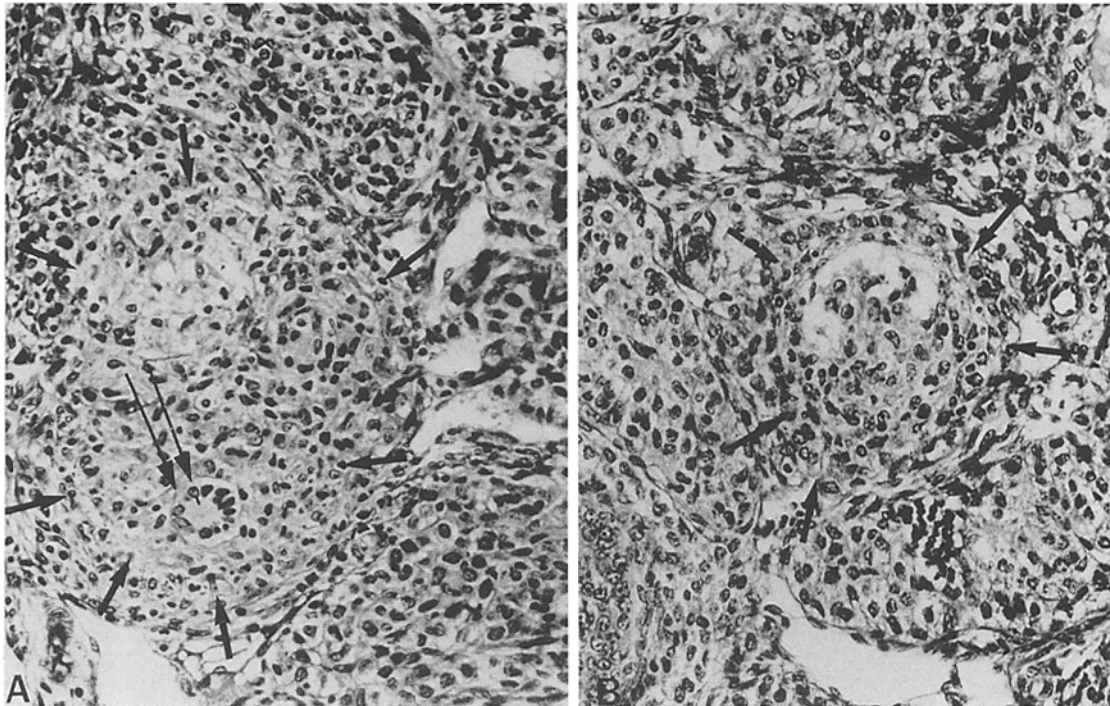In contrast, when both pathways were blocked, T cell responses, ovarian pathology, and antibody induction were

Figure 6. Adoptive transfer of in vitro–stimulated lymph node cells from peptide-immunized and anti-gp39 mAb (*A*) or CTLA4-Ig (*B*) treated mice results in ovarian pathology. (*A*) The focus of granulomatous inflammation is outlined by thick arrows; long arrows point to a multinuclear giant cell. (*B*) Arrows point to an inflammed atretic follicle that contains lymphocytes and macrophages. (hematoxylin and eosin, ×400).

completely inhibited. This state of unresponsiveness was temporary and showed no evidence of long-term tolerance. Thus 1 mo later, when CTLA4-Ig and anti-gp39 mAb levels had declined, these mice were fully capable of developing both T cell responsiveness and autoimmune oophoritis when rechallenged with ZP3 peptide in CFA (data not shown).

The interaction of gp39 and CD40 is critical in the formation of isotype-switched antibody as well as germinal center formation within secondary lymphoid tissues (17–19). In vivo administration of anti-gp39 mAb dramatically reduced both primary and secondary humoral immune responses without altering responses to T-independent antigens (30). Likewise, blockage of the CD28 pathway inhibits autoantibody production. Milich et al. (24) using a hepatitis B e-Ag transgenic system found CTLA4-Ig effective in suppressing autoantibody production. Murine lupus, a disease mediated via autoantibody production, was also effectively treated with CTLA4-Ig (31). Thus, it is not surprising that either anti-gp39 mAb or CTLA4-Ig also inhibited antibody production in our model of autoimmunity, reinforcing the potential application of anti-gp39 mAb and CTLA4-Ig in the treatment of antibody-mediated autoimmune diseases.

Blockage of either the gp39 or CD28 pathway had no effect on T cell clonal expansion, whereas inhibition of both significantly reduced T cell expansion. Thus, either of the pathways can mediate T cell clonal expansion; when one is blocked, the other remains operative, and vice versa. The

results also imply that other costimulatory pathways considered important in T cell activation do not always compensate for the CD28 and gp39 pathways in vivo. In this regard, several receptor/ligand pairs have been shown to interact and costimulate T cell activation in vitro. Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) can efficiently costimulate the proliferation of resting T cells while poorly stimulating antigen-specific T cells (32). Also, CD80/CD86 and LFA-3 have been implicated in the preferential stimulation of antigen-specific T cells. Finally, these and other studies suggest that the requirements for T cell activation may change with the differential stage of the responding T cell (33).

The result of normal clonal expansion of ZP3-specific T cells in the presence of CTLA4-Ig is also unexpected given the importance of the CD28 costimulatory pathway for IL-2 production (34, 35). However, similar results were reported by Ronchese et al. (36), in that mCTLA4-Hg1 transgenic mice were found to have enhanced levels of antigen-specific T cells after secondary or tertiary immunization with KLH. Together, these two studies suggest that alternative surface receptors may be available for clonal expansion and IL-2 production. On the other hand, recent work by Kearney et al. (37) has suggested that antigen-dependent clonal expansion is solely contingent upon CD28 costimulation. They found that blockage of CD28/B7 interaction with CTLA4-Ig results in failure to clonally expand a population of adoptively transferred transgenic T cells to a foreign ovalbumin peptide. The reason for the

discrepancy between their finding of inhibition of clonal expansion and our lack of such a finding is unclear but may be due to differences in immunogenicity between the foreign ovalbumin peptide and a self-peptide that may have primed the immune system previously.

Blockage of either the gp39 or the CD28 pathway did not inhibit clonal expansion of ZP3-specific T cells, yet these T cells failed to cause significant ovarian disease. Thus cooperation between the two pathways is required in functional acquisition of clonally expanded T cells. Indeed, it has been postulated that these pathways may operate sequentially for clonally expanded T cells to differentiate into functionally active effector T cells (38). The fact that T cell clonal expansion occurs without concomitant functional acquisition also supports the in vitro finding that T cell activation occurs in multiple discrete steps each of which requires distinct signals (39).

Why the clonally expanded T cells should fail to elicit oophoritis is an important question. They may lack homing molecules or additional downstream costimulatory molecules. Failure of these cells to produce appropriate cytokines for induction of ovarian pathology is a possibility under current investigation. Alternatively, the circulating anti-gp39 mAb may block T cell recognition of target antigens in the ovary. This is, however, unlikely since anti-gp39 mAb does not inhibit oophoritis induced by adoptive transfer of a pathogenic ZP3-specific T cell line (our unpublished data). Finally, the clonally expanded T cells are not anergic as they responded to the stimulating peptide in vitro without exogenous IL-2. Indeed, adoptive transfer of these in vitro–stimulated cells into naive hosts resulted in ovarian pathology (Fig. 6). Thus, the functional state of these autoreactive T cells would appear to be contingent in some way on the gp39/CD40 interaction. Whether this gp39 help is provided indirectly via soluble mediators from the APC or through an as yet unidentified signaling structure associated with the gp39 molecule is currently being investigated.

A role for gp39 in costimulation of CD4+ T cells is also supported by a recent study (40). These investigators found that of a panel of P815 transfectants, CD40-P815 preferen-

tially costimulated in vitro proliferation of resting T cells in the presence of anti-CD3 mAb whereas CD56, ICAM, VCAM, CD31, CD72, and FAS transfected P815 cells all failed to elicit proliferation. It is interesting that hyper-IgM syndrome patients are particularly susceptible to opportunistic infections that involve the T cell effector arm of the immune system (41). Alderson et al. (42) have demonstrated that in human monocytes, gp39/CD40 interaction promotes the production of TNF-α and IL-6 in the monocyte population. Taken together, there is mounting evidence for direct or indirect signaling to the T cell via the gp39 molecule.

In the context of immunologic tolerance, one potential mechanism is T cell ignorance. this describes the presence of self-reactive T cells being oblivious or ignorant to normal, accessible self-peptides (for a review see reference 43). Our findings suggest that clonally expanded, self-reactive T cells can still remain ignorant.

When mice were treated with both CTLA4-Ig and anti-gp39 antibody, neither clonal expansion nor oophoritis was manifested. Moreover, their splenic T cells, activated with Con A, also did not transfer oophoritis to normal recipients. This is not surprising in view of the low frequency of ZP3-specific T cells detected in this group of animals. A more challenging issue is whether tolerance or some suppression mechanism has been imposed by ZP3 immunization in animals with blockage of both costimulatory pathways. Our preliminary results do not support tolerance induction. At 5 wk after initial immunization, when CTLA4-Ig and anti-gp39 antibody should have disappeared from the mice, two of four animals developed severe oophoritis upon rechallenge with ZP3 peptide in CFA. However, this point needs further investigation.

Inhibition of the gp39 and the CD28 pathways provides potential therapeutic approaches to control autoimmune disease and allograft rejection. It will be important to determine whether inhibition of these pathways also reverses autoimmune oophoritis. Nevertheless, the results of this study suggest that inhibition of both gp39 and CD28 costimulatory pathways will provide much more complete immunosuppression than inhibition of either pathway alone.

Address correspondence to Nathan Griggs, Box 214, Health Sciences Center, Department of Pathology, University of Virginia, Charlottesville, VA 22908.

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