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In the Absence of a CD40 Signal, B Cells Are Tolerogenic

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Summary

When B cells are deprived of signaling through CD40, they exhibit the ability to induce T cell tolerance. The in vivo administration of anti-gp39 and allogeneic B cells diminished the ability of mice to mount an allogeneic response. Tolerance induction was specific for the haplotype expressed on the allogeneic B cells. Selective allospecific unresponsiveness was induced in the CD8 and CD4 compartments by the administration of anti-gp39 and class II–deficient B cells or class I–deficient B cells, respectively. As predicted by studies with anti-gp39 treatment, diminished allospecific responsiveness was induced by the administration of B cells to mice genetically deficient in gp39. Taken together, these data are consistent with the premise that deprivation of CD40 signaling engenders B cells with enhanced tolerogenicity. These studies provide insights into the tolerogenic capacity of resting B cells and outlines a practical approach to exploit this function.

Introduction

The activation of naive T cells exclusively through the T cell antigen receptor (TCR) without engagement of other T helper (Th) costimulatory molecules results in T cell unresponsiveness (Schwartz, 1992; Linsley and Ledbetter, 1993). Costimulatory molecules, like B7-1 and B7-2, through binding to their counterreceptors on T cells (CD28, CTLA4), heighten lymphokine production, promote T cell expansion, and prevent the induction of T cell anergy (Harding et al., 1992; Schwartz, 1990; Koulova et al., 1991). The capacity of antigen-presenting cells (APCs) to induce T cell activation proficiently hinges upon the density of the costimulatory molecules that are expressed. Although some professional APCs may constitutively express high densities of costimulatory molecules (Liu et al., 1992; Freedman et al., 1991), resting B cells do not and this is believed to be the primary reason for their incompetent APC function (Kreiger et al., 1985; Finkelman et al., 1992; Parker, 1993). While it is clear that resting B cells are ineffective at positively triggering T cell responses, only a limited number of studies have demonstrated that resting B cells induce T cell tolerance. In vitro, it has been shown that B cells can render T cell clones unresponsive to subsequent antigenic challenge (Gilbert and Weigle, 1994; Hori et al, 1989). In vivo, the tolerogenic capacity of B cells has been demonstrated in only two instances. First, it has been shown that the administration of male antigen-bearing (HY) B cells to female mice induced cytotoxic T lymphocyte (CTL) unresponsiveness to HY (Fuchs and Matzinger, 1992). In another case, it was shown that a monomeric rabbit anti-δ, preferentially targeted to resting B cells, induced T cell tolerance to the heterologous rabbit immunoglobulin (Eynon and Parker, 1992). In spite of these successes, repeated attempts to broaden the use of resting B cells to induce tolerance to a greater diversity of antigens have been unsuccessful. As studied here, for example, the administration of resting allogeneic B cells does not induce allospecific tolerance in the host.

One limitation for the use of B cells to induce tolerance in vivo may be the ease with which they acquire "professional" APC capacities upon transfer, and therefore lose their tolerogenic potential. It is anticipated that upon entry into the host, antigen-bearing B cells are acted upon by the immune system of the host and triggered to upregulate APC activities. Although it is not clear which host mediators elicited by administration of B cells can enhance their APC function in vivo, a plethora of activities can alter B cell costimulatory molecule expression and the APC function of B cells in vitro. In vitro B cell APC function is dramatically enhanced by mitogenic stimulation by lipopolysaccharide (LPS), soluble gp39 (Kennedy et al., 1994; Roy et al., 1994), and anti-immunoglobulin (Lenschow et al., 1994). For the most part, the transition to a competent APC is accompanied by an increase in the density of B7-2. However, in vivo it is not clear what signals are critical for inducing the transition of resting B cells from an incompetent APC to a competent APC.

The studies in this report focus on the role of CD40 in triggering resting B cells to become professional APCs. The system developed to investigate this issue measures the ability of allogeneic B cells to elicit alloreactive $CD4^+$ and $CD8^+$ T cells when the allogeneic B cells are deprived

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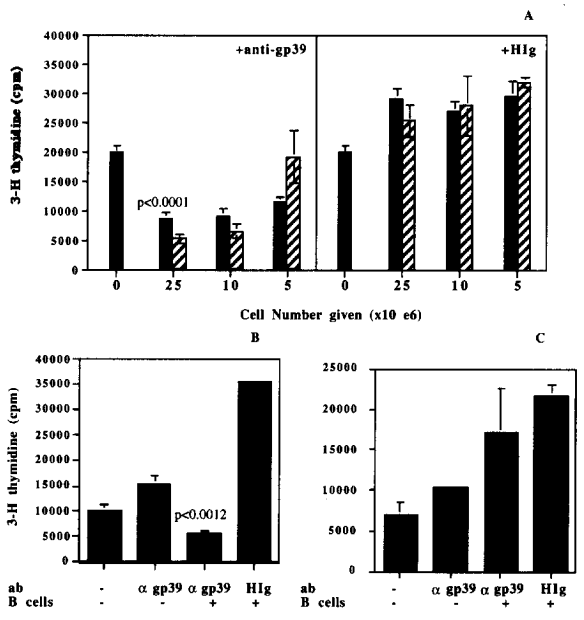

Figure 1. Administration of Allogeneic B Cells and Anti-gp39 Inhibits the Response of CD4 T Cells in an MLR

Allogeneic splenic B cells from C57BL/6 (H-2^b) mice were injected (intravenously) into recipient BALB/c (H-2^d) mice, which were treated (intraperitoneally) with either anti-gp39 or Hlg every 2 days for 10 days. On day 12, spleen cells were isolated and used as responders in a 2:1 ratio to stimulators in an MLR assay. In (A), B cells were separated either by Percoll or Ficoll gradient. The groups are as follows: closed bars, naive control; shaded bars, resting Percoll-fractionated B cells; striped bars, Ficolled B cells. Responders were stimulated with spleen cells from either (A, B) C57BL/6 mice or (C) B10.BR mice, H-2^k. Results are representative of pooled spleens from 2 mice in each group and eight experiments. p values were determined by Student's t test using the WormStat (version 2.0) program.

of a CD40 signal. The hypothesis set forth in this study suggests that if the gp39 function of the host is either blocked by antibody (anti-gp39) or abolished by targeted deletion of the gene, then allogeneic B cells will be unable to up-regulate the expression of costimulatory molecules. This lack of costimulation hinders allospecific responses and will induce host-allospecific T cell unresponsiveness. This hypothesis is based on the premise that gp39 is the primary regulator of B cell APC function in vivo and that other agonists cannot replace this function. Data presented show that the coadministration of anti-gp39 and allogeneic B cells, or the administration of allogeneic B cells alone to gp39-deficient mice, diminishes allospecific CD4 responsiveness, CD8 responsiveness, or both. Tolerance induced by anti-gp39 is not observed if allogeneic tumor cells or thymocytes are used as immunogens, indicating that the unresponsiveness is dependent upon the administration of nonactivated B cells. Using allogeneic B cells from mice deficient in class I or class II major histocompatibility complex (MHC) expression, it is shown that the induction of CD4 and CD8 hyporesponsiveness is dependent on expression of the relevant MHC antigen on the B cell and that the induction of hyporesponsiveness in each compartment is independent of the other MHC molecule. Results also indicate that when animals are pretolerized to F1 alloantigens, spleen cells from these mice are unable to induce graft-versus-host disease (GVHD) upon adoptive transfer. These studies present a novel approach to exploit the tolerogenic potential of resting B cells to induce allospecific tolerance and underscore the important and active contribution of the host in regulating the APC function of donor cells via gp39–CD40 interactions.

Results

Administration of Anti-gp39 with Allogeneic B Cells Inhibits the Mixed Lymphocyte Response

The premise being tested in this investigation asserts that if resting allogeneic B cells are kept in a quiescent state by blocking CD40-mediated activation, allospecific unresponsiveness will be induced. To test this hypothesis, allogeneic B cells from H-2^b (C57BL/6) mice were administered to allogeneic H-2^d recipients (BALB/c) in the presence of anti-gp39 or hamster immunoglobulin (Hlg). After 12 days, the allospecific mixed lymphocyte response (MLR) was measured. Mice administered allogeneic B cells (H-2^b) and control Hlg had a slightly elevated MLR to the relevant alloantigen, H-2^b. In contrast, the combined administration of allogeneic B cells (H-2^b) and anti-gp39 reduced responses in the MLR to levels below that of the naive MLR (Figure 1B). The responses of cells from animals receiving B cells alone were identical to control Hlg-treated animals (data not shown). The in vivo administration of anti-gp39 alone had no impact on the responses in the MLR to H-2^b. Furthermore, the diminished MLR imposed by the coadministration of H-2^b B cells and anti-gp39 was specific for the H-2^b alloantigen, since the MLR of these cells to H-2^k-bearing stimulators was unaltered (Figure 1C). These studies show that the MLR to specific alloantigens can be reduced by the coadministration of alloantigen-bearing B cells and anti-gp39.

Experiments were performed to evaluate the respective efficacy of T-depleted spleen cell preparations and highly purified resting B cell preparations for the induction of T cell unresponsiveness when coadministered with anti-gp39. First, the in vivo administration of neither B cells nor resting B cell preparations caused a diminished in vitro MLR (Figure 1A). Second, when coadministered with anti-gp39, resting B cell populations were only marginally more effective than the T-depleted cell populations at low numbers of cells (5 × 10^6/mouse) at inducing diminished MLR.

Anti-gp39 Prevents the Induction of Allospecific CTL by Allogeneic B Cells

A panel of allogeneic B cells from H-2^b (C57BL/6), H-2^d (BALB/c), and H-2^k (B10.BR) were administered to allogeneic recipients, either BALB/c or C57BL/6, in the presence or absence of anti-gp39. The CTL response to donor alloantigen was measured 5 days after immunization by a standard ^{51}Cr release assay. Responses to H-2^b, H-2^d, and H-2^k were induced by the administration of the respective allogeneic B cells in the absence of anti-gp39. In contrast, mice that received anti-gp39 and allogeneic cells did not generate CTL in vivo in response to allogeneic B cells (Figure 2). As presented above, no differences were ob-

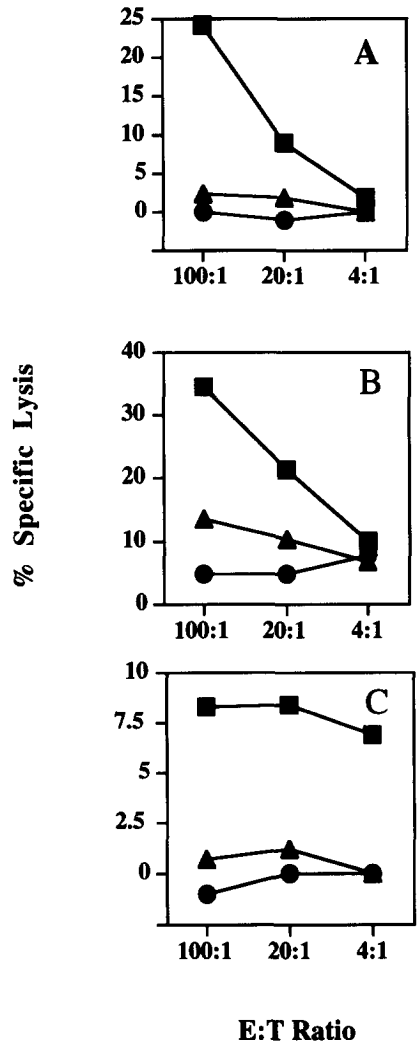

Figure 2. Anti-gp39 Prevents the Induction of Allospecific CTL by Allogeneic B Cells

Allogeneic splenic B cells from C57BL/6 (A), BALB/c (B) and B10.BR (C) were injected (intravenously) into BALB/c (A and C) and C57BL/6 (B) recipients and administered either Hlg or anti-gp39 (250 μg/mouse intraperitoneally on days 0, 2, and 4). On day 6, spleens were removed and used as effector cells to determine CTL responses against H-2^b (A), H-2^d (B), and H-2^k (C). Targets used in these assays were P815 (H-2^d), E female K1 (H-2^b), and SL8 (H-2^k). Groups represented are untreated (closed square), anti-gp39 treated (closed triangle), and naive mice (closed circle). Results are representative of 3 mice in each group and the experiment presented is representative of five experiments.

served between tolerogenic capacity of T-depleted spleen cells and high density Percoll-fractionated B cells (data not shown).

The Coadministration of Anti-gp39 and Allogeneic B Cells In Vivo Reduces the Secondary CTL Response In Vitro

Secondary in vitro CTL responses were measured from naive mice and mice immunized with allogeneic B cells and treated with anti-gp39 or Hlg. After 12 days, spleen cells from treated mice were stimulated in vitro with H-2^b

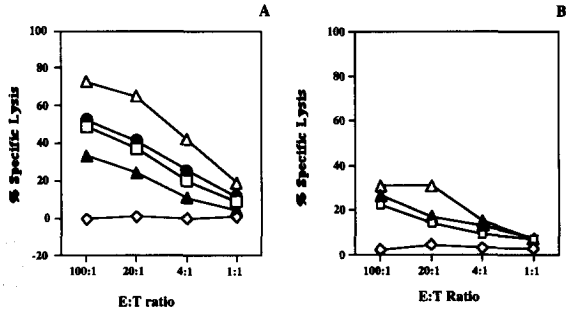

Figure 3. The Coadministration of Anti-gp39 and Allogeneic B Cells In Vivo Prevents the Secondary Anti-Allospecific CTL Response In Vitro

(A) Specific alloresponses in vitro of mice treated with allogeneic B cells. BALB/c mice were primed in vivo with H-2^b-bearing B cells, as described in Figure 1. Spleens from the anti-gp39 and Hlg-treated mice were cultured for 5 days with H-2^b or H-2^d stimulators. After 5 days, the responder cells were assayed for CTL activity against P815 (H-2^d; syngeneic control, data not shown) and E female K1 (H-2^b). Groups are unstimulated (open diamond), naive (open square), anti-gp39 alone (closed circle), B cells and anti-gp39 (closed triangle), and B cells and Hlg (open triangle). Results are representative of eight experiments with pooled spleens from 2 mice in each group.

(B) Anti- H-2^k CTL responses (third party) from mice treated with allogeneic B cells. Spleen cell cultures as in (A) were analyzed for anti-H-2^k allogeneic responses using B10.BR (H-2^k) spleen cells as the stimulators and SL8 (SL8, H-2^k) as the targets in the CTL assay. Groups are unstimulated (open diamond), naive (open square), B cells and anti-gp39 (closed triangle), and B cells and Hlg (open triangle).

B cells for 6 days and CTL responses determined. As anticipated, in vivo immunization of mice with H-2^b B cells and Hlg resulted in a high titered secondary anti-H-2^b CTL response in vitro (Figure 3A). The heightened secondary anti-H-2^b CTL response was not observed using lymphocytes derived from mice immunized with allogeneic B cells and anti-gp39 in vivo. The response of mice given anti-gp39 and H-2^b-bearing B cells was lower than that of naive mouse cells cultured in vitro. The lowered CTL response is dependent on alloantigen and anti-gp39, since the response of mice treated with anti-gp39 alone is indistinguishable from the response of naive cells cultured in vitro.

To assess whether the administration of anti-gp39 and allogeneic B cells altered the CTL response to a third party alloantigen, lymphocytes from mice treated with H-2^b B cells with or without anti-gp39 were stimulated in vitro with H-2^k-bearing cells and assayed against an H-2^k-bearing target (Figure 3B). Results show that the CTL response to a third party alloantigen-bearing target was unaltered by the administration of anti-gp39 and allogeneic B cells. Taken together, these data show that the administration of alloantigen-bearing B cells and anti-gp39 can diminish CTL reactivity to specific alloantigens.

Alteration of the Tolerogenic Capacity of B Cells by Stimulation with LPS

The minimal APC function of B cells is believed to contribute to the tolerogenic effects observed in the presence of anti-gp39. Stimulation of B cells with LPS has been shown (Kennedy et al., 1994) to augment their APC function.

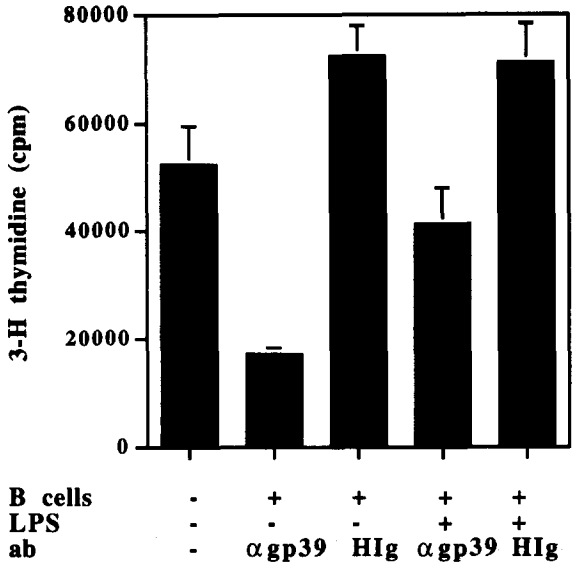

Figure 4. LPS-Activated B Cells Can Not Induce Tolerance in CD4 T Cells When Administered with Anti-gp39

Unstimulated B cells or 2-day-activated LPS (50 μg/ml) blasts were injected (intravenously) and treated with antibody as described in Figure 1. On day 12, spleens were removed and the cells were used as responders in an MLR. Results shown are representative of three experiments with pooled spleens from 2 mice in each group.

Therefore, it was of interest to determine whether prior activation with LPS would alter the tolerogenic capacity of allogeneic B cells administered to mice together with anti-gp39. Allogeneic B cells were activated in vitro with LPS for 2 days and then administered to mice with or without anti-gp39. The administration of allogeneic B cells or allogeneic LPS blasts "primed" the MLR equally, causing an approximate 50% increase in the MLR (Figure 4). The coadministration of anti-gp39 resulted in a diminished MLR with either of the B cell sources, but B cells caused a greater reduction in the response than LPS blasts. Solely based on proliferative responses, it appeared that in the presence of anti-gp39, the B cells tolerized the MLR and the LPS blasts failed to prime the MLR. Therefore, the preactivation of allogeneic B cells does reduce their ability to mediate allospecific T tolerance in the presence of anti-gp39.

Anti-gp39 Does Not Inhibit the Allospecific CTL Response When Allogeneic T Cells or Allogeneic Tumor Cells Are Used as Immunogens

To evaluate whether anti-gp39 was interfering broadly with the generation of allospecific CTL, other alloantigen-bearing cells were used as immunogens. Allogeneic thymocytes and P815 (H-2^d) tumor cells were administered to mice with or without anti-gp39 and the allospecific CTL response measured. Figure 5 shows that anti-gp39 did not diminish the primary in vivo CTL response elicited by either allogeneic thymocytes (Figure 5A) or allogeneic tumor cells (Figure 5B); however, treatment with B cells and anti-gp39 did reduce by >80% the CTL response to allogeneic B cells. Although the magnitude of the CTL response to allogeneic thymocytes was low (10% lysis), in multiple

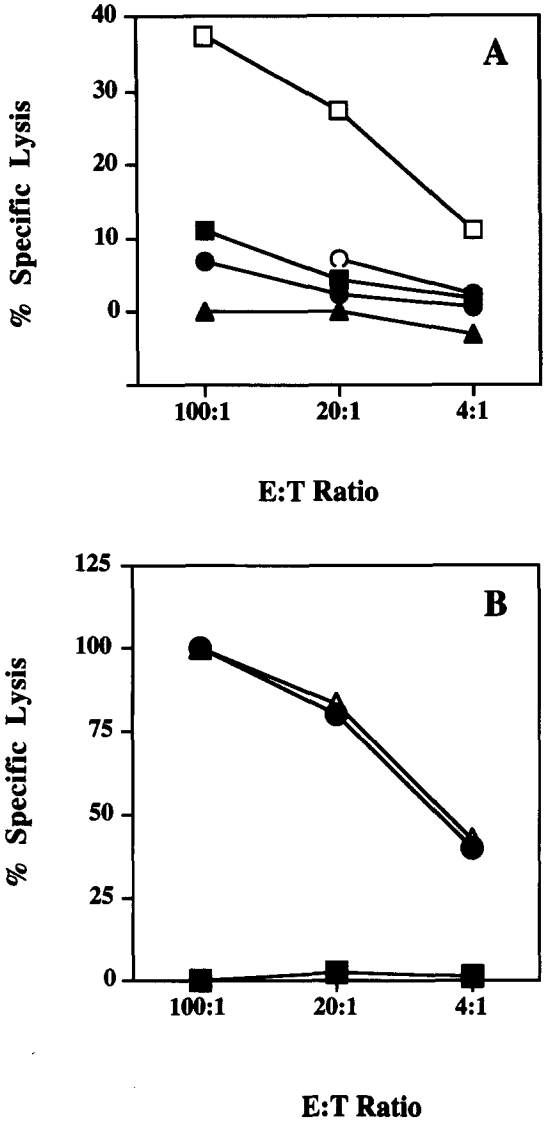

Figure 5. Anti-gp39 Has No Effect on the In Vivo Priming of Mice with Allogeneic Thymocytes or Tumor Cells

(A) Response to allogeneic thymocytes. BALB/c mice (H-2^d) were immunized with thymocytes (50 × 10^6/recipient) or B cells (50 × 10^6/recipient) from C57BL/6 mice (H-2^b) and then either HIg treated or anti-gp39 treated (250 μg/mouse intraperitoneally on days 0, 2, and 4). On day 6, CTL responses were measured. Groups are the following: thymocytes and HIg (closed square) or anti-gp39 (closed circle); B cells and HIg (open square) or anti-gp39 (open circle); no immunization (closed triangle). Target cells used in this assay were E female K1(H-2^b). The results are representative of three such experiments.

(B) Response to allogeneic tumor cells. C57BL/6 mice (H-2^b) were immunized intraperitoneally with P815 tumor cells (50 × 10^6/recipient; H-2^d) in the presence or absence of anti-gp39 (administered on days 0, 2, 4, 6, 8, and 10). On day 12, mice were sacrificed and CTL responses measured against H-2^d-bearing target (P815). Groups are nonimmune (closed square), P815 cells (closed circle), P815 and anti-gp39 (open triangle). Results are representative of two experiments with pooled spleens from 3 mice in each group.

trials (3), no diminution of the CTL response was observed by the administration of anti-gp39. Therefore, it appears that the inhibitory effect of anti-gp39 was limited by the nature of the cell type used (B cells) for immunization.

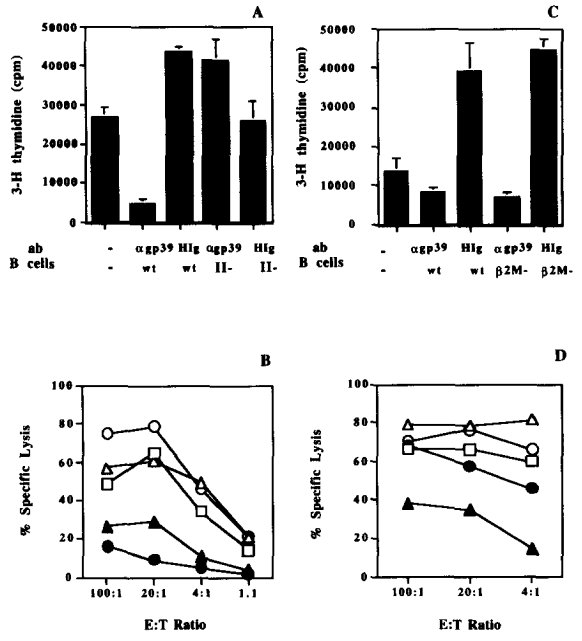

Figure 6. Class II Molecules on B Cells Are Required to Tolerize CD4 T Cells and Class I Molecules on B Cells Are Required to Tolerize CD8 T Cells

Donor B cells were prepared from either class II or β_2m knockout mice. These were used along with wild-type B cells in the immunizing protocol described in Figure 1. After 12 days, spleens were removed and restimulated in vitro with wild-type spleen cells.

(A) Class II knockouts. Mice were immunized with either wild-type or class II$^-$ (II$^-$) B cells; the ability of anti-gp39 and B cells to tolerize CD4 T cells was measured in an MLR.

(B) Recipient spleens were cultured for 6 days in the presence of wild-type allogeneic spleen cells and the ability of donor B cells to tolerize CD8 T cells was measured in a CTL assay directed against E female K1 target cells. Groups refer to the immunizing protocol and are as follows: naive (open square), wild-type B cells plus anti-gp39 (closed triangle), II$^-$ B cells plus anti-gp39 (closed circle), wild-type B cells plus Hlg (open triangle), and II$^-$ B cells plus Hlg (open circle).

(C) β_2m knockouts. Mice were immunized with either wild-type or β_2m knockout (β_2m$^-$) B cells and the ability to tolerize CD4 T cells, in the presence of anti-gp39, was determined in an MLR.

(D) Responders were stimulated and used in a CTL assay as described for (B). Groups represent the immunizing protocol and are as follows: naive (open square), wild-type B cells plus anti-gp39 (closed triangle), β_2m$^-$ B cells plus anti-gp39 (closed circle), wild-type B cells plus Hlg (open triangle) and β_2m$^-$ B cells plus Hlg (open circle). Experiments are representative of pooled spleens from 2 mice per group and three different experiments.

Class II and Class I Molecules Are Required to Tolerize the CD4 and CD8 Alloreactive T Cell Compartments, Respectively

To evaluate whether anti-gp39-mediated tolerance in the CD4 and CD8 compartments was interdependent, we utilized class II and β-2 microglobulin (β_2m) knockout mice as a source for allogeneic tolerogenic B cells. The coadministration of anti-gp39 and allogeneic B cells derived from class II$^-$ mice did not diminish the MLR, (Figure 6A), yet did reduce the class I–restricted CTL response (Figure 6B). The reductions of the CTL response mediated by wild-type and class II$^-$ B cells in the presence of anti-gp39 were indistinguishable.

Using B cells from mice deficient in β_2m, the role of class I MHC molecules in MLR and CTL responses was addressed. When mice deficient in β_2m were used as a source for allogeneic B cells, the MLR was unaffected by the lack of class I on the surface of the allogeneic B cells and the responses were identical to responses generated with wild-type B cells (Figure 6C). However, if class I was absent, coadministration of B cells and anti-gp39 did not induce tolerance in the CTL response (Figure 6D). Together, these data indicate that tolerance in the CD4 and CD8 T cell compartments is induced independently.

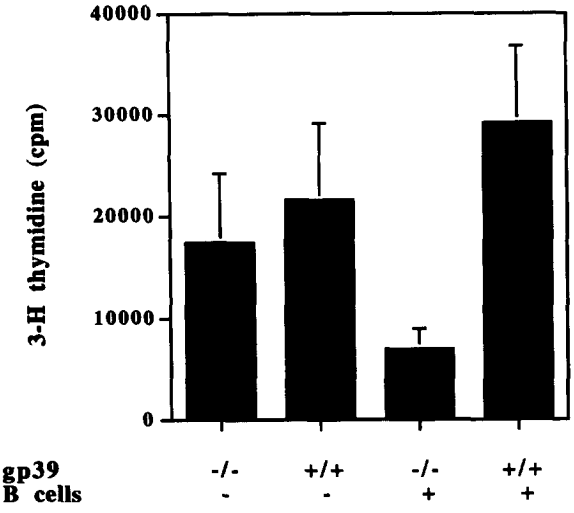

Figure 7. Administration of Allogeneic B Cells to gp39 Knockout Mice Causes Tolerance to In Vitro Challenge with Alloantigen

B cells were prepared from BALB/c mice and injected (intravenously) into either gp39 knockouts (−/−) or wild-type controls (+/+). On day 12, spleens were used as responders in an MLR. A representation of four experiments with a total of 8 mice per group is shown.

Administration of Allogeneic B Cells to gp39 Knockout Mice Leads to Diminished Allogeneic Responses

To dismiss the potential contribution of the anti-gp39 monoclonal antibody to alterations in alloreactive T cell responsiveness, the effect of administration of allogeneic B cells to gp39 knockout mice was evaluated. Allogeneic B cells (H-2^d) were prepared and injected into either gp39 knockout or wild-type control mice. The MLR of gp39 knockout mice was similar to that observed with wild-type recipients (Figure 7). Prior administration of allogeneic B cells to the gp39 knockout mice inhibited the normal response to alloantigen >60%. This inhibition was only seen in the gp39 knockouts and not the wild-type controls.

The Coadministration of Anti-gp39 and Allogeneic B Cells Renders CTL Incapable of Mounting a Graft versus Host CTL Response In Vitro or In Vivo

To evaluate more fully the functional capacity of the unresponsive CTL from mice rendered tolerant to alloantigen, the tolerized cells were challenged both in vivo and in vitro. C57BL/6 mice (H-2^b) were administered F1 spleen cells (H-2^{bd}) with or without anti-gp39, with the goal of inducing tolerance to the disparate (H-2^d) alloantigen. To test

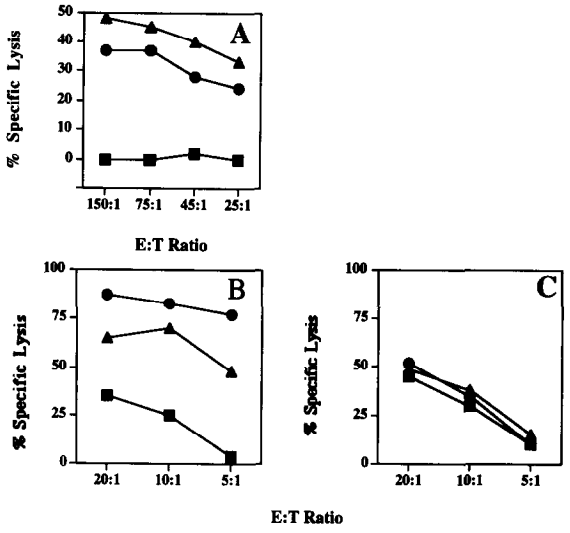

Figure 8. Transfer of F1 Spleen Cells to Anti-gp39-Treated Parental Mice Induces Tolerance as Measured In Vitro and In Vivo

(A) Transfer of pretolerized C57BL/6 cells does not induce acute GVHD when donor animals are treated with anti-gp39. Approximately 80 × 10^6 spleen cells from C57BL/6 mice administered B6D2F1 spleens in the presence or absence of anti-gp39 were transferred into B6D2F1 animals in a model for acute GVHD. H-2^b anti-H-2^d CTL responses were determined using the ^{51}Cr release assay using p815 (H-2^d) as the target cell line. Groups represented are transferred spleens from animals treated with F1 spleen cells plus anti-gp39 (closed square), F1 cells and no antibody (closed circle), or transfer of naive C57BL/6 spleen cells in the normal induction of acute GVHD (closed triangle).

(B) Anti-gp39 reduces the CTL H-2^b anti- H-2^d response when in vivo-primed spleens are rechallenged in vitro with B6D2F1 spleens. C57BL/6 were immunized intravenously with 100 × 10^6 B6D2F1 spleen cells on day 0, and then not treated (closed circle) or treated with anti-gp39 on days 0 and 3 (closed square). On day 14, spleens were removed and CTL responses determined in vitro by 5 day stimulation with mitomycin c-treated B6D2F1 (H-2^{bd}) spleens (5 × 10^6 per 5 × 10^6 responder cells). CTL responses were determined by ^{51}Cr release assay using P815 (H-2^d) cells as the tumor target cell line. All syngeneic responses were not significant. The figure also indicates the response of naive C57BL/6 to in vitro challenge with B6D2F1 spleen cells (closed triangle).

(C) Anti-gp39 does not affect the H-2^b anti-H-2^k CTL response after in vitro challenge with H-2^k-expressing cells. C57BL/6 were immunized intravenously with 100 × 10^6 B6D2F1 spleen cells on day 0, and then untreated (closed circle) or treated with anti-gp39 on days 0 and 3 (closed square). On day 14, spleens were removed and CTL responses determined in vitro by 5 day stimulation with mitomycin c-treated CBA (H-2^k) spleens (5 × 10^6 per 5 × 10^6 responder cells). CTL responses were determined by ^{51}Cr release assay utilizing SL8 (H-2^k) cells as the target cell line. All syngeneic responses were not significant. The figure also indicates the response of naive C57BL/6 to in vitro challenge with B6D2F1 spleen cells (closed triangle).

whether tolerance was induced, spleen cells from the recipient mice were transferred into an F1 (H-2^{bd}), which should induce an in vivo GVH response. Mice then were assayed for the development of anti-H-2^d CTL activity. Results indicated that H-2^b mice that were administered F1 cells and anti-gp39 were unable to generate anti-H-2^d CTL responses in vivo (Figure 8A). In addition, the anti-H-2^d CTL responses of C57BL/6 spleen cells generated in vitro from mice administered F1 cells were greatly reduced compared with the CTL response of naive C57BL/6 spleen cells (Figure 8B). Spleen cells from mice that were administered F1 (H-2^{bd}) spleen cells were rendered unresponsive specifically to the H-2^d alloantigen, since the in vitro CTL response to a third party alloantigen (H-2^k) was unaffected (Figure 8C). Experiments performed using either F1 B cells or F1 spleen cells to tolerize the host to the disparate alloantigen have yielded indistinguishable results.

Discussion

This study shows that the coadministration of anti-gp39 and allogeneic B cells reduced the MLR and CTL response to the alloantigen; the prior activation of the allogeneic B cells with LPS diminished their tolerogenic potential when coadministered with anti-gp39; anti-gp39 has no impact on the induction of CTL to allogeneic tumor cells or thymocytes; alloantigens on the allogeneic B cells independently tolerized alloreactive CD4 and CD8 T cells; the administration of allogeneic B cells (in the absence of anti-gp39) to gp39 knockout mice rendered them hyporesponsive to alloantigen; and the coadministration of allogeneic B cells and anti-gp39 induced a state of profound in vivo CTL unresponsiveness. The extent of unresponsiveness in the CTL compartment that resulted from the administration of alloantigen and anti-gp39 was demonstrated by the inability to induce a GVH CTL response from tolerized mice.

An important issue raised by these studies is the mechanism of how anti-gp39 interferes with the development of allospecific MLR and CTL responses. The administration of allogeneic B cells alone induced slightly heightened MLR: vigorous primary CTL responses in vivo and enhanced secondary CTL responses in vitro. The coadministration of allogeneic B cells and anti-gp39 reduced the MLR, ablated the in vivo CTL response, and reduced the secondary CTL response below that observed from unprimed mice. All of these data suggest that the coadministration of anti-gp39 and allogeneic B cells inactivated both the CD4 and CD8 alloreactive precursors. Although these experiments do not provide a definitive answer to the mechanism of immunosuppressive action by anti-gp39, it is evident that the inhibitory effect of anti-gp39 is dependent upon the nature of cells used for immunization. Since CTL responses to allogeneic tumor cells and thymocytes were intact in the presence of anti-gp39, the antibody cannot be simply deleting or anergizing alloreactive CTL or alloreactive helper T cells required for the response. Furthermore, the fact that anti-gp39 administration does not interfere with the priming of sheep red blood cell–specific helper T cells (Foy et al., 1993), block the antigen-induced expression of lymphokines in situ (Van den Eertwegh et al., 1993), or skew the pattern of lymphokine expression in situ (Van den Eertwegh et al., 1993) strongly suggests that there are no or minimal direct effects of anti-gp39 on T cells. Furthermore, the fact that mice genetically deficient in the expression of gp39 are rendered hyporesponsive to alloantigen by the administration of allogeneic B cells all supports the hypothesis that in the absence of a CD40 signal, B cells are tolerogenic.

Studies using a panel of allogeneic cells to induce tolerance provided clues to the mechanism of immunosuppression imposed by anti-gp39. The fact that class I and class

II MHC-deficient B cells selectively tolerized the CD4 and CD8 compartments, respectively, suggest that the anti-gp39 was not suppressing allospecific responses by interfering with alloreactive helper T cell function. These data indicate that the tolerance in these two compartments was induced independently. Furthermore, the fact that allogeneic LPS blasts were not as tolerogenic as nonactivated B cells indicated that the activation state of the B cell was critical for it to elicit unresponsiveness. Finally, the hypothesis that anti-gp39 was mediating its effects by simply blocking gp39 function (and not delivering direct signals to the T cell) was supported by the diminished allospecific responsiveness observed in gp39-deficient mice that were administered allogeneic B cells.

The data presented are consistent with the hypothesis that anti-gp39 prevents the up-regulation of costimulatory molecules at a critical moment when cognate interactions between host T cells and the allogeneic B cells are occurring. The first tenet of this hypothesis is that gp39 is critical in the regulation of B7-1/B7-2 during cognate interactions. It has been shown that during T–B cell interactions, antigen recognition via TCR induces the up-regulation of gp39 on the antigen-specific T cell; and, in turn, gp39 induces the up-regulation of B7-1 and B7-2 on the cognate B cell (Roy et al., 1994). Therefore, gp39 blockade prevents the up-regulation of B7-1 and B7-2. Another tenet of this hypothesis is that the level of B7-1/B7-2 expression is a crucial regulator of tolerance versus immunity. Supportive of this idea is that blocking of B7-1/B7-2 function results in T cell tolerance, as reviewed by Linsley and Ledbetter (1993). For example, studies by Turka and coworkers have shown that the coadministration of allogeneic spleen cells and CTLA-4Ig, a soluble receptor that blocks B7-1 and B7-2 binding to their coreceptors, induced a state of prolonged transplantation tolerance and fostered the long-term engraftment of cardiac allografts (Lin et al., 1993; Bolling et al., 1994). Therefore, the tolerance induced by the blockade of B7-1 and B7-2 seems to elicit similar tolerogenic effects on the host as anti-gp39 administration. All of these approaches appear to take advantage of the central role of costimulation to tip the balance of the immune system towards unresponsiveness.

Another issue raised as a result of these studies focuses on the inability of professional APC in the B cell inoculum to prevent the induction of tolerance. The studies reported show that the administration of highly purified Percoll-fractionated resting B cells or allogeneic T-depleted or nondepleted splenocytes induced allospecific unresponsiveness. These data show that low numbers of macrophages and activated B cells (likely 3%–5% of the population) cannot prime in the presence of anti-gp39 and resting B cells. The dominant effect of the resting B cells in the preparation is also consistent with the effect of anti-gp39 on GVHD (Durie et al., 1994). That is, brief administration of anti-gp39 blocks the occurrence of both the acute and chronic forms of GVHD for the length of the experiments (>14 weeks). In this case, tolerance was induced even when all of the host APCs (macrophages, dendritic cells) could potentially present alloantigen. We speculated that the large number of quiescent host B cells (imposed by anti-gp39) tolerized the donor T cells, rendering them tolerant and preventing GVHD. In effect, the resting B cells preempted priming by host APC. An alternative explanation for the tolerogenic effects that anti-gp39 imposed on alloresponses is that dendritic cells and macrophages also require CD40 triggering to up-regulate costimulatory molecule expression and become proficient APCs. Support for a central role of gp39 in the up-regulation of dendritic cell APC function has been recently provided by a series of studies that show that freshly isolated dendritic cells are deficient in APC function or costimulatory molecule expression and that CD40 triggering up-regulated APC function (Caux et al., 1994; Inaba et al., 1994). Therefore, anti-gp39 administration may globally down-regulate APC function.

The practical significance of anti-gp39 and B cell–induced allo-unresponsiveness to the transplantation of allogeneic tissue has been demonstrated by Parker and coworkers (Parker et al., submitted). Mice, which were rendered tolerant to alloantigen by the coadministration of allogeneic B cells and anti-gp39, accepted allogeneic pancreatic β islet cells. Upon histological examination of the site of implantation there was no indication of any inflammatory processes, which was consistent with the long-term engraftment observed. Although anti-gp39 treatment alone did extend the longevity of the transplant in some mice, the combined administration of anti-gp39 and allogeneic B cells permitted the long-term survival of the tissue allografts in virtually all of the mice. Given these data, it is hopeful that the combined administration of anti-gp39 and donor lymphocytes may enhance the success of allogeneic tissue or organ grafts in humans.

Experimental Procedures

Animals

Female 6- to 8-week-old BALB/c, C57BL/6, CB6F1, B10.BR, B6D2F1, and CBA/J, β_2m (Koller et al., 1990) and class II knockout mice (Cosgrove et al., 1991) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School. The gp-39 knockout mice (H-2^b) were produced as previously described (Xu et al., 1994).

Preparation of B Cells

T cell–depleted splenocytes were prepared by treating single cell splenic suspensions with ammonium chloride treatment to lyse erythrocytes. T cells were cytotoxically eliminated by treatment of the spleen cells with anti-Thy1.2 (HO-13-4; American Type Culture Collection [ATCC], Rockville Pike, Maryland) (Marshak-Rothstein et al., 1979), anti-CD4 antibodies (RL172/4) (Mizouchi et al., 1989), and rabbit complement. Viable cells were isolated by flotation on Ficoll–Hypaque (Sigma Chemical Company, St. Louis, Missouri). Cells isolated were typically >95% mouse immunoglobulin positive, had a uniform low degree of near forward light scatter, and were unresponsive to concanavalin A. Resting splenic B cells were prepared by sedimentation on discontinuous Percoll gradients, as described previously (DeFranco et al., 1982). Cells isolated from the 70%–75% (density of 1.087–1.097) Percoll interface were typically >97% mouse immunoglobulin positive, had a uniform low degree of near forward light scatter, and were unresponsive to concanavalin A.

Antibodies

Anti-gp39, a hamster anti-mouse antibody (MR1; Noelle et al., 1992), was produced as ascites in SCID mice and purified by ion exchange high pressure liquid chromatography (HPLC). Hamster serum was obtained from Pel-Freez Biologicals, Rogers, Arkansas, and the immu-

noglobulin G fraction purified by ion exchange HPLC. Anti-Thy1.2 was used directly from ascites prepared from ATCC clone HO13.4 and ATCC clone RL172/4, which were titered for the depletion of thymocytes in the presence of complement.

Target Cell Lines

E female K1 (H-2^b, T cell lymphoma derived from C57BL/6 strain) (Green et al., 1979), P815 (H-2^d, mastocytoma, derived from DBA/2J strain, obtained originally from ATCC), SL8 (H-2^k, a spontaneous T cell leukemia derived from AKR strain) (Nowinski et al., 1977), and EL4 (H-2^b, T cell lymphoma derived from C57BL/6N strain) were used as target cells lines in the ^{51}Cr release assay.

Immunization Protocols

Recipient mice were administered 30–50 × 10^6 allogeneic splenic B cells or allogeneic thymocytes, as a source of T cells. Cells were administered intravenously in balanced salt solution. These mice were untreated or treated with anti-gp39 (250 μg/mouse for 2 days) on days 0, 2, and 4. On day 6, spleens were removed and used as effector cells in the standard ^{51}Cr release assay (Durie et al., 1994). Tumor responses were determined by administering 5 × 10^6 P815 cells intraperitoneally. Recipient (C57BL/6) mice were treated with anti-gp39 or HIg on days 0, 2, 4, 6, 8, and 10. On day 12, H-2^b anti-H-2^d responses were measured in a CTL assay.

MLR

Spleens from in vivo-primed animals treated with either anti-gp39 or HIg were removed and used as responders. For stimulators, spleen cells were prepared from either syngeneic mice or donor allogeneic mice. In the case of third party responses, spleen cells were prepared from B10.BR mice (H-2^k). Stimulator cells were treated with 25 μg/ml mitomycin C (Sigma) for 20 min at 37°C and then washed extensively in balanced salt solution. Final cell preparations were resuspended in MLR media, RPMI 1640 with 25 mM HEPES (Bio Whittaker, Walkersville, Maryland), containing 10% fetal calf serum (JRH Biosciences, Lenexa, Kansas), 500 U/ml penicillin, and 5000 U/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 50 mM 2-mercaptoethanol (BioRad Laboratories, Hercules, California). Responders were resuspended at 4 × 10^6/ml and stimulators at 2 × 10^6/ml and plated in ratios of 2:1 in U-bottomed 96-well plates. Each ratio was set up in triplicate and cultured for 3 days. During the last 6 hr of culture, [^3H]thymidine, 1 μCi/well was added. Cells were harvested and thymidine incorporation was determined by scintillation counting.

In Vitro Culture of CTL and Assessment of CTL Activity

Spleen cells from in vivo-primed animals treated with anti-gp39 or untreated were used as responders (5 × 10^5/ml) for in vitro challenge. In vitro culture of CTL was performed by culturing syngeneic or allogeneic 5 × 10^5/ml splenic B cell (anti-Thy1.2 and complement treatment) stimulators (mitomycin C-treated, 25 μg/ml, 20 min at 37°C) and responder cells groups in complete enriched RPMI 1640 medium (Bio Whittaker) containing 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), 1 mM sodium pyruvate (Bio Whittaker), 1 × nonessential amino acids (Bio Whittaker), 1 × 10^5 M 2-mercaptoethanol (BioRad Laboratories), 2 mM L-glutamine (Sigma), 500 U/ml penicillin, and 5000 U/ml streptomycin (Sigma). After 5 days, the responder cells were harvested, dead cells were removed by centrifugation on a density gradient, and the resulting live cells were used as effectors in a CTL assay against the relevant target cell lines.

In vitro CTL activity was measured by the standard ^{51}Cr release assay as previously described (Durie et al., 1994).

Acute GVHD

C57BL/6 mice were administered 100 × 10^6 spleen cells from B6D2F1 (C57BL/6 × DBA/2)F1, H-$2^{b\times d}$) intravenously on day 0 and either treated with anti-gp39 on days 0 and 3 or untreated. On day 14, spleen cells were removed from these recipient animals and 20 × 10^6 cells were used as responders in an MLC using B6D2F1 or CBA/J mitomycin C-treated spleen cells as the stimulators. The remaining spleen cells (approximately 80 × 10^6 cells) were used to immunize B6D2F1 recipients in a model of acute GVHD. Induction of GVHD was measured by determining the H-2^b anti-H-2^d CTL response characteristic of acute GVHD.

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