

In Vivo CD40-gp39 Interactions Are Essential for Thymus-dependent Humoral Immunity. II. Prolonged Suppression of the Humoral Immune Response by an Antibody to the Ligand for CD40, gp39

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Summary

The ligand for CD40 has been recently identified as a 39-kd protein, gp39, expressed on the surface of activated CD4⁺ T helper cells (Th). In vitro, soluble CD40 and anti-gp39 have been shown to block the ability of Th to activate B cells, suggesting that gp39-CD40 interactions are important to T cell-dependent B cell activation. Here it is shown that in vivo administration of anti-gp39 dramatically reduced both primary and secondary humoral immune responses to erythrocytes and soluble protein antigens without altering responses to the T-independent type II antigen, trinitrophenyl-Ficoll. Treatment of mice for 4 d with anti-gp39 inhibited the anti-sheep red blood cell (SRBC) response for at least 3 wk and inhibited the expression of all immunoglobulin isotypes in secondary responses to the protein antigen, keyhole limpet hemocyanin. To examine the direct effect of anti-gp39 on Th function, SRBC-immune Th cells from anti-gp39-treated mice were adoptively transferred and shown to be fully capable of providing help. These results suggest that anti-gp39 treatment does not cause Th deletion or anergy. Anti-gp39 may mediate its profound immunosuppressive effects on humoral immunity by blocking gp39-CD40 interactions. Moreover, these studies establish gp39-CD40 as an important receptor-ligand pair for the targeting of therapeutic antibodies to control thymus-dependent humoral responses.

Thymus-dependent (TD)¹ humoral immunity requires the participation of CD4⁺ Th, and constitutes an essential arm of host immune defense to disease. Studies by Mitchison, Claman, Benacerraf, and Raff suggested that interactions between Th and B cells in vivo are essential in the development of humoral immunity. Moreover, in vitro studies have demonstrated that T cell-dependent antibody responses require activation of T cells by dendritic cells followed by an interaction between activated Th cells and B cells (1, 2). The requirement for physical contact between Th and B cells in the humoral immune response cannot be replaced by lymphokines, as all combinations of lymphokines have proven ineffective at inducing resting B cell growth and differentiation. Therefore, a unique cell contact-dependent signal transpires as a consequence of the physical interactions between Th and B cells which induces B cell activation. This contact-

dependent signal is believed to be transduced as a result of the binding of gp39 on activated Th to its receptor, CD40, on B cells (3).

CD40, a mitogenic receptor expressed on all mature B lymphocytes (4, 5), is a member of the nerve growth factor receptor (NGFR) family of receptors (6). The ligand for CD40, gp39, is a type II membrane protein that is homologous to TNF- α and - β (7, 8), other NGFR family ligands. Evidence that CD40 is an important mitogenic receptor on B cells is derived from studies that show highly efficient triggering of human B cells by anti-CD40 and cofactors such as anti-CD20, anti-Ig, and lymphokines (7-12). In the presence of these cofactors, anti-CD40 has been shown to initiate both B cell growth and differentiation. Similar to anti-CD40, gp39, expressed as a recombinant membrane or soluble protein, also activates B cells in the presence of costimulators (7, 13).

gp39 is transiently expressed on activated CD4⁺ Th in vitro (14) and is induced in vivo on CD4⁺ T cells as a result of antigen administration (15). The CD4⁺ T cell population expressing gp39 in vivo has been localized in situ juxtaposed to B cells producing antibodies to the immunizing antigen (15). In vitro and in vivo data suggest that during

¹ Abbreviations used in this paper: Chi-L6, chimeric L6; HIg, hamster Ig; HIM, hyper IgM syndrome; TD, thymus dependent; TI, thymus independent.

the course of cognate Th-B interaction, transient expression of gp39 by CD4⁺ T cells is the result of antigen presentation (16). Once expressed, gp39 binds to CD40 and reciprocally triggers B cell activation. The ability of a mAb specific for gp39, MR1, to block the capacity of gp39-bearing Th to activate B cells in vitro has implicated gp39 as an important molecule in T cell-dependent B cell activation (17).

Further evidence implicating gp39-CD40 involvement in humoral immune responses has recently been provided by several groups demonstrating that mutations in the gene encoding gp39 result in the inability of humans to respond to TD antigens (18-21). An immunodeficiency characterized by failure to mount TD humoral immune responses, hyper-IgM syndrome (HIM), results in the expression of a defective gp39 molecule that lacks CD40 binding capacity. Although the B lymphocytes from these patients are reported to be normal (18, 19, 21), mutations in the gp39 molecule interrupt B cell triggering through CD40 and subsequent B cell activation and Ig production.

The present study examines the ability of a mAb specific for gp39 to neutralize the function of gp39 in vivo. In vivo administration of anti-gp39 reduced primary as well as secondary antibody responses to exogenous TD antigens, but not the T-independent (TI)-type II antigen, TNP-Ficoll. Furthermore, short-term treatment with anti-gp39 produced prolonged suppression of humoral immune responses. Th cells from anti-gp39-treated mice were capable of providing help upon adoptive transfer, suggesting that anti-gp39 treatment did not result in deletion or anergy of responding Th in vivo. Evidence is presented supporting the hypothesis that anti-gp39 exerts its profound immunosuppressive effects by directly blocking gp39-CD40 interactions in vivo.

Materials and Methods

Animals

Female, 6-8-wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used for the in vivo experiments presented in this study. Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School.

T Helper Cell Clones (Th1)

D1.6, an I-A^d-restricted, rabbit Ig-specific Th1 clone (22) was obtained from Dr. David Parker (University of Massachusetts, Worcester, MA). In this paper, D1.6 will be referred to as Th1.

Reagents and Antibodies

MR1, hamster anti-murine gp39 mAb (17), was purified by DEAE HPLC from ascites fluid. Hamster Ig (HIg), used as a control antibody, was purified similarly from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY). RG7/7.6.HL, a mouse anti-rat κ chain (strongly cross-reactive with hamster κ chain) antibody, (RG7), (23) was conjugated with horseradish peroxidase or FITC and used as a secondary reagent to detect MR1 and HIg. Affinity-purified goat anti-mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Birmingham, AL) were used as detection antibodies in the antigen-specific ELISAs, as well as in the total IgM and IgG1 ELISAs. EM95 (kindly provided by Dr. T. Waldschmidt, University of Iowa, Iowa City, IA) a mono-

clonal anti-murine IgE, was used as the detection antibody for the IgE anti-KLH ELISA. Chimeric L6 (Chi-L6), a humanized IgG1 specific for the tumor antigen L6 (24), was kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute. Anti-CD4, GK 1.5 (25) was prepared by HPLC purification of ascites fluid. SRBC were purchased from Colorado Serum Co. (Denver, CO). Sea plaque agarose for use in anti-SRBC plaque assay was obtained from FMC Corp. BioProducts (Rockland, MA). Baby rabbit complement was purchased from Cedarlane Laboratories Ltd. (Hornby, ON, Canada). KLH (from *Megathura crenulata*) was purchased from Calbiochem-Novabiochem (La Jolla, CA). CFA for immunizations was obtained from Sigma Chemical Co. (St. Louis, MO). TNP-SRBC, TNP-KLH, and TNP-BSA were prepared as previously described (26).

Immunizations for Generation of In Vivo Primary and Secondary Antibody Responses

Primary Immune Responses. For eliciting primary antibody responses to SRBC or TNP-SRBC, mice were immunized with 200 μ l of 1% SRBC or TNP-SRBC suspension (i.v.). The IgM, anti-SRBC response was assayed 5 d after administration of antigen using a modification of the Jerne plaque assay (27). IgM anti-TNP responses were measured by ELISA on day 6. Primary responses to the heterologous Ig Chi-L6 were generated by intraperitoneal immunization of 100 μ g Chi-L6 on alum per mouse. The serum IgM anti-Chi-L6 antibody response was measured after 7 d. Primary responses to TNP-Ficoll were generated by immunization with 25 μ g of TNP-Ficoll, i.p. The IgM anti-TNP response was measured on day 6 by ELISA.

Secondary Immune Responses. For generation of secondary humoral responses to KLH, animals were immunized with KLH in CFA (50 μ g, i.p.). Mice were subsequently challenged with 10 μ g of soluble KLH (i.p.) 3 mo later. The anti-KLH antibody response was measured on day 7 from the serum of immune mice utilizing isotype-specific ELISAs. Secondary antibody responses to Chi-L6 were generated by challenging Chi-L6 immune mice with 10 μ g soluble Chi-L6, i.p. The serum IgG1 anti-Chi-L6 antibody response was measured after 7 d.

Anti-gp39 Treatment

Sterile, HPLC-purified anti-gp39 (MR1) or HIg (as an antibody control) was administered intraperitoneally on days 0, 2, and 4 after immunization or challenge as indicated for each experiment.

Antigen-specific ELISAs

The antigen-specific IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE antibody titers were determined using isotype-specific ELISAs. Briefly, antigen, (1 mg/ml of KLH, Chi-L6, TNP₁₆-BSA, or TNP₂-BSA in PBS) was adsorbed onto flexible polyvinyl microtiter dishes, overnight at 4°C. Plates were washed and blocked with PBS-1% FCS-sodium-azide. Diluted serum samples were incubated for 2 h at 37°C. Samples were washed and the antigen-specific antibody titers determined with one of the following alkaline-phosphatase-conjugated detection antibodies: goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates). The IgE-specific ELISA was detected using biotin-conjugated EM95 followed by alkaline-phosphatase avidin (Zymed, South San Francisco, CA). All ELISAs were developed by reaction of alkaline-phosphatase with phosphatase substrate (Sigma Chemical Co.). Plates were analyzed on an ELISA reader (model MR700; Dynatech

Laboratories Inc., Chantilly, VA) at 410 nm. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000, and the titer ascertained based on multiple point analysis. The levels of anti-KLH, anti-Chi-L6, and anti-TNP antibodies in unchallenged controls were below detection.

Detection of Serum Anti-gp39

Quantitation of Intact Anti-gp39 in the Serum of Anti-gp39-treated Mice. Serum from mice receiving 750 μ g anti-gp39 (250 μ g on days 0, 2, and 4) was obtained on days 7, 14, and 21 after initiation of anti-gp39 treatment. The serum was run on a 7.5% SDS gel under nonreducing conditions, transferred to nitrocellulose, and blotted with HRPO-conjugated RG7. After chemiluminescent detection, areas of the blot corresponding to 150–165 kD were scanned and digitized using an Apple Scanner and the Image 4.1 software program (Apple Computer, Inc., Cupertino, CA).

Analysis for Biologically Active Anti-gp39 in the Serum of Treated Mice. Anti-CD3-activated Th1, which express gp39, were stained with dilutions of serum from mice receiving 750 μ g anti-gp39 (250 μ g on days 0, 2, and 4) to determine the amount of biologically active gp39 remaining in the serum. Titrations of serum containing anti-gp39 were incubated with activated Th1 cell clones for 30 min at 4°C, followed by washing and subsequent incubation with FITC-RG7 for 30 min at 4°C. A standard curve of mean fluorescence intensity vs anti-gp39 concentration was generated using purified anti-gp39. Samples were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) and the percent anti-gp39 remaining in the serum was deduced based on the anti-gp39 standard curve. The level of anti-gp39 present in the serum at d7 was set at 100%.

Adoptive Transfer of Th Cells

Mice were immunized intravenously with 200 μ l of 1% SRBC, and administered anti-gp39 or HIg (250 μ g on days 0, 2, and 4). On day 7 the splenocytes from nonimmune or SRBC-immune mice were removed, erythrocyte depleted, washed, and transferred intravenously (50×10^6 /mouse) into irradiated recipients (600 rad) with or without 50×10^6 spleen cells from TNP-KLH primed (TNP-KLH-CFA, 50 μ g, i.p.) mice as a source of immune B cells. At the time of transfer, mice were immunized intravenously with 200 μ l of 1% TNP-SRBC. Serum IgG1 anti-TNP titers were ascertained on day 6 after transfer.

Results

Anti-gp39 Inhibits the Generation of Primary Antibody Responses to Erythrocyte Antigens. The impaired TD immunity observed in patients with HIM, as well as the potent inhibitory effects of anti-gp39 and CD40-Ig on Th-dependent B cell activation *in vitro*, provided the basis for the study of the potential immunosuppressive effects of anti-gp39 on humoral-mediated immunity *in vivo*. To investigate the role of gp39-CD40 interactions in primary TD humoral immune responses, the effect of *in vivo* administration of anti-gp39 on the primary antibody response to SRBC was determined. Animals were immunized with SRBC and administered anti-gp39 mAb (or control HIg) over the course of 4 d. On day 5, the primary anti-SRBC antibody response of anti-gp39-treated, HIg-treated, and control mice was ascertained. The

IgM anti-SRBC plaque-forming cell (PFC) response of mice that received a total of 1.5 mg of anti-gp39 (500 μ g/mouse on days 0, 2, and 4) was reduced 99% when compared to the anti-SRBC PFC response from control or HIg-treated mice (Fig. 1 A). In addition, administration of as little as

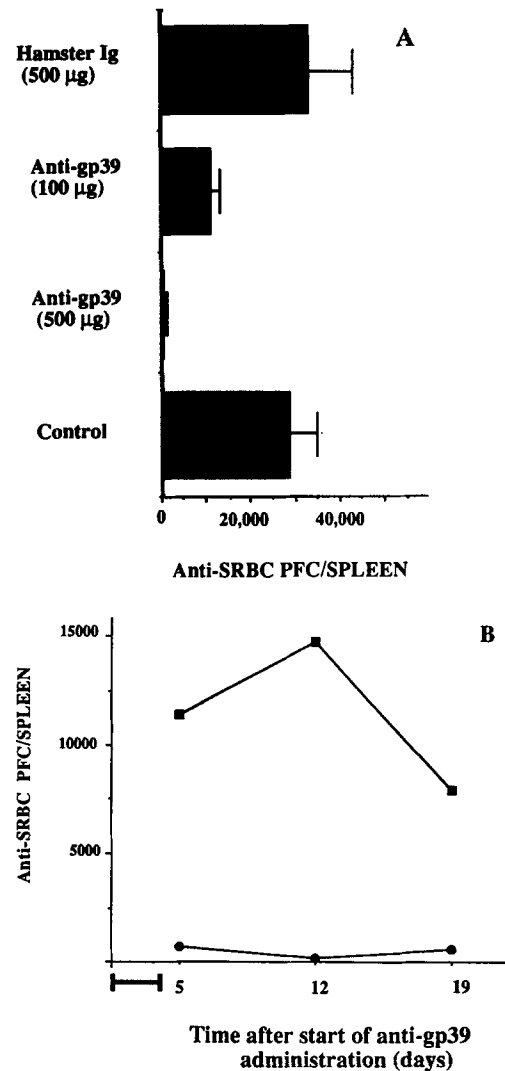


Figure 1. (A) Anti-gp39 inhibits the generation of primary anti-SRBC PFC. Mice (three per group) were administered 200 μ l of 1.0% SRBC, *i.v.*, on day 0. On days 0, 2, and 4 mice were given either 100 or 500 μ g of purified MR1 (hamster anti-murine gp39, purified from ascites by DEAE HPLC) or 500 μ g of purified hamster Ig, *i.p.* The control group consists of mice receiving the immunization, but no antibody treatment. Spleens were removed from the mice on day 5 and the number of direct (IgM) anti-SRBC PFC was determined by a modification of the Jerne plaque assay. The data is representative of three such experiments. (B) Prolonged immune suppression of primary anti-SRBC responses is induced by the administration of anti-gp39. Mice (three per group) were immunized with SRBC (200 μ l of 1.0% SRBC, *i.v.*) and on day 0, 2, and 4, received 250 μ g of anti-gp39 (\bullet) or 250 μ g hamster Ig (\blacksquare), *i.p.* (Black bar) The time of antibody administration. The anti-SRBC PFC response was determined on day 5 after immunization. Additional mice were challenged with antigen (200 μ l of 1.0% SRBC *i.v.*) 7 or 14 d after initial antigen immunization and anti-gp39 administration. The anti-SRBC PFC was then assayed 5 d later. The results are representative of three similar experiments.

300 $\mu\text{g}/\text{mouse}$ (100 $\mu\text{g}/\text{mouse}$ on days 0, 2, and 4) of anti-gp39, reduced the anti-SRBC primary immune response by 66%. Results from these experiments demonstrate that anti-gp39 treatment ablates primary antibody responses *in vivo*.

The duration of the immunosuppressive effects of anti-gp39 on the primary humoral immune response to SRBC was subsequently examined. Mice immunized with SRBC were treated with anti-gp39 for 4 d and assayed at various later time points for the capacity to mount a primary anti-SRBC response. In this set of experiments, all animals were immunized with SRBC on day 0 and administered anti-gp39 or HIg on days 0, 2, and 4. The IgM anti-SRBC PFC response was measured for one group on day 5. Additional SRBC-immune groups were challenged with SRBC on day 7 or 14. 5 d after each antigenic challenge (days 12 and 19, respectively), the IgM anti-SRBC PFC response was measured. The results of one such experiment are depicted in Fig. 1 B. As in Fig. 1 A, the primary anti-SRBC responses were inhibited 80–90% 5 d after anti-gp39 administration was begun. In addition, the primary anti-SRBC responses 12 and 19 d after anti-gp39 treatment were also inhibited >90%. These results demonstrate that brief anti-gp39 treat-

ment results in a prolonged inhibition of primary antibody responses.

Anti-gp39 Inhibits the Generation of Secondary Anti-KLH Antibody Responses. Experiments examining primary antibody responses suggest that gp39–CD40 interactions play a critical role in the initiation of primary humoral immunity. However, these experiments do not address whether gp39-dependent CD40 signaling is required for the generation of secondary antibody responses. Therefore, the effects of anti-gp39 administration on the secondary immune response to soluble challenge with KLH was determined in KLH-immune mice.

Using schedules of anti-gp39 administration that reduced the primary anti-SRBC PFC response, experiments were designed to evaluate the effects of anti-gp39 treatment on the secondary antibody responses. In these experiments, KLH-immune mice (immunized 3 mo before with CFA and KLH) were challenged with soluble KLH (10 $\mu\text{g}/\text{mouse}/\text{i.p.}$). On the day of antigen challenge (day 0), mice were also given 250 μg of anti-gp39 or HIg, followed by anti-gp39 or HIg on days 2 and 4. At days 7 (Fig. 2 A) and 14 (Fig. 2 B) after challenge with KLH, the mice were bled and the titers of

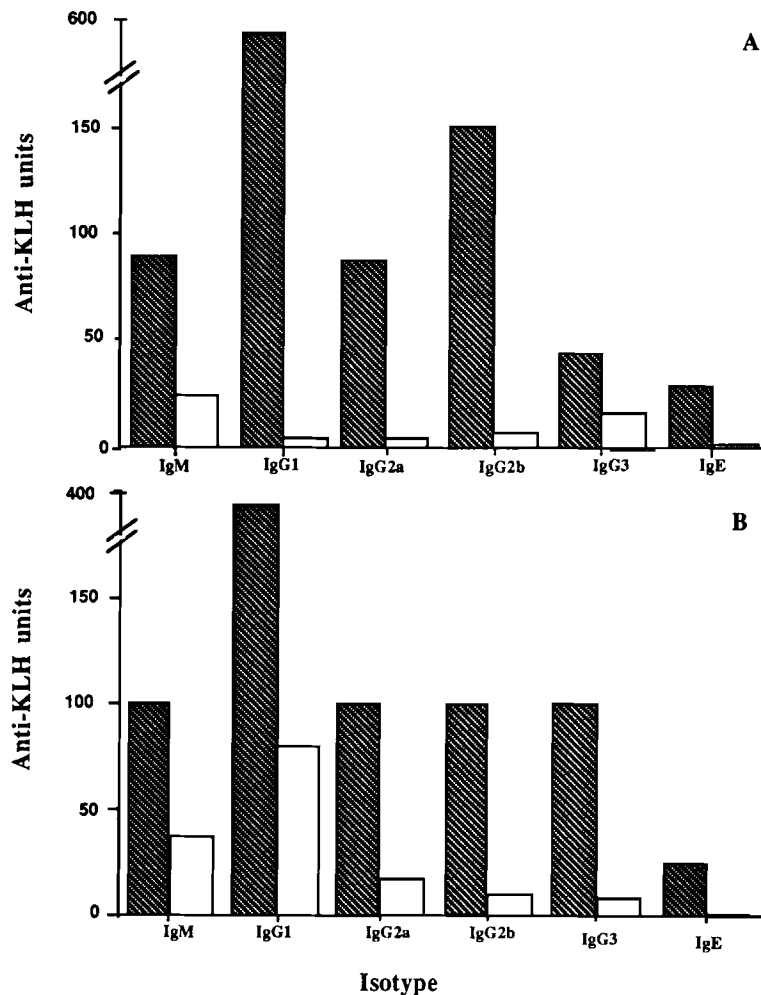


Figure 2. Anti-gp39 inhibits the generation of secondary anti-KLH antibody responses. Mice (three per group) were immunized with KLH in CFA (50 $\mu\text{g}/\text{mouse}$, *i.p.*) 3 mo after immunization, mice were given a soluble boost with 10 μg of KLH (*i.p.*). On days 0, 2, and 4, immune mice received 250 μg of anti-gp39, *i.p.* (open bars) or 250 μg HIg (hatched bars). Serum from individual mice was collected on day 7 (A) or 14 (B) after antigenic challenge, pooled, and levels of anti-KLH antibodies were determined using isotype-specific ELISAs. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The levels of anti-KLH antibodies in unchallenged controls were below detection. The SE within each group were always <10%. These results are representative of three such experiments.

IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE anti-KLH antibodies were determined. The results demonstrate several points: (a) challenge with soluble KLH induced an enduring secondary immune response that persisted for up to 14 d; (b) the administration of anti-gp39 significantly reduced the secondary anti-KLH response of the isotypes measured when compared to the administration of equal quantities of HIg; and (c) the immunosuppressive effects of anti-gp39 appeared to be sustained for at least 14 d after the initiation of anti-gp39 treatment. Taken together, results from these experiments demonstrate that similar to primary humoral immune responses, the generation of secondary humoral immune responses were also blocked by anti-gp39.

Anti-gp39 Inhibits the Generation of Antibody Responses to Heterologous Ig. Experiments depicted in Fig. 1 demonstrate the immunosuppressive activity of anti-gp39 during a primary response to a strongly immunogenic particulate antigen, SRBC. The cellular nature of erythrocytes makes them unique in their capacity to elicit strong immune responses. Heterologous Ig molecules share this characteristic of being highly immunogenic, and therefore provide an additional model antigen system with which to examine the effects of anti-gp39 treatment on the generation of primary and secondary antibody responses. Animals were immunized with a heterologous Ig molecule, Chi-L6, a humanized mouse antitumor cell mAb, and treated with anti-gp39 or control HIg. After 7 d, sera was collected and assayed for the production of IgM anti-Chi-L6 antibodies. In addition, mice were challenged with Chi-L6 14 d after initial immunization and anti-gp39 treatment, and assayed for IgG1 anti-Chi-L6 antibody production on day 21. Fig. 3 depicts the results of one such experi-

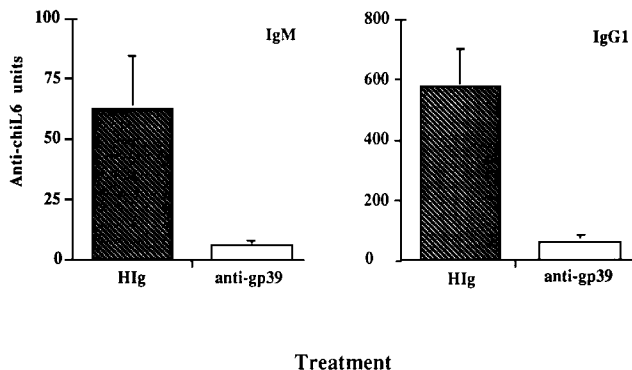


Figure 3. Anti-gp39 inhibits the generation of primary and secondary antibody responses to heterologous Igs. Mice (three per group) were immunized intraperitoneally with 100 μ g Chi-L6 absorbed on alum. On days 0, 2, and 4, immune mice received 250 μ g of anti-gp39, i.p. (open bars) or 250 μ g HIg (hatched bars). Serum from individual mice was collected on day 7 after initial immunization (for IgM) or antigenic challenge (for IgG1). The levels of anti-Chi-L6 IgM and IgG1 antibodies were determined using antigen-specific ELISAs. Units represent arbitrary values based on the titration curve or a standard immune serum. All experimental groups were titrated from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The levels of anti-Chi-L6 antibodies in unchallenged controls were below detection. The results are representative of two separate experiments.

ment. The primary antibody response to Chi-L6 in mice treated with anti-gp39 is inhibited by >90% when compared to HIg-treated mice. Moreover, the secondary, IgG1 response to Chi-L6 is similarly inhibited. These results demonstrate that anti-gp39 treatment ablates primary and secondary antibody responses to a second type of TD antigen, heterologous Ig, as effectively as it suppresses responses to erythrocyte and soluble protein antigens.

Anti-gp39 Does Not Inhibit the Generation of Primary Antibody Responses to the T1-Type II Antigen, TNP-Ficoll. Although the previous experiments demonstrate that anti-gp39 effectively blocks the generation of primary and secondary antibody responses to TD antigens in vivo, it is unclear whether gp39-CD40 interactions play a role in the initiation of humoral responses to T1 antigens. Data presented in the accompanying paper (15) demonstrate that immunization with the T1-type II antigen, TNP-Ficoll, results in gp39 expression by Th cells in vivo. To address whether gp39-CD40 interactions are necessary for the generation of antibody responses to this T1 antigen, the effect of anti-gp39 treatment on mice immunized with TNP-Ficoll, was assessed. Mice immunized with TNP-Ficoll or TNP-SRBC were treated with anti-gp39 or HIg and the IgM anti-TNP antibody response determined after 6 d. Fig. 4A demonstrates that animals immunized with the TD antigen TNP-SRBC elicit significant anti-TNP serum antibody responses. As predicted from the previously described experiments, anti-gp39 treatment dramatically inhibits the primary anti-TNP response generated in these mice. In contrast, mice immunized with TNP-Ficoll

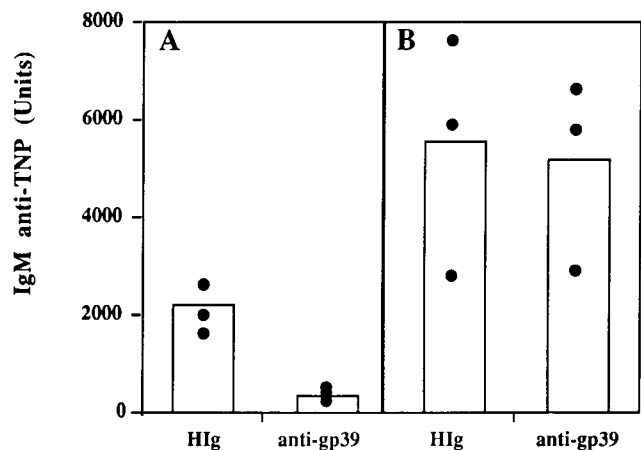


Figure 4. Anti-gp39 administration does not inhibit the generation of primary antibody responses to TNP-Ficoll. (A) Mice (three per group) were immunized with 200 μ l 1% TNP-SRBC, i.v. On days 0, 2, and 4 mice received 250 μ g anti-gp39 or HIg. On day 6, mice were bled and the IgM anti-TNP antibody titers determined by TNP₁₆-BSA ELISA. (B) Mice (three per group) were immunized with 25 μ g TNP-Ficoll, i.v. On days 0, 2, and 4 mice received 250 μ g anti-gp39 or HIg. On day 6 mice were bled and the IgM anti-TNP antibody titers determined by TNP₁₆-BSA ELISA. Units represent arbitrary values based on the titration curve or a standard immune serum. All experimental groups were titrated from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The anti-TNP titer of nonimmune mice was 390 U. The results are representative of two separate experiments.

mount a higher titered anti-TNP antibody response (Fig. 4 B); however, treatment with anti-gp39 does not inhibit the antibody response to TNP-Ficoll. Results from these experiments demonstrate that, unlike responses to TD antigens, anti-gp39 does not block the generation of humoral responses to TNP-Ficoll, suggesting that responses to TI antigens may be gp39 independent.

Anti-gp39 Administration Does Not Functionally Delete SRBC-specific Th. From the previous experiments, it is known that anti-gp39 interferes with the development of TD humoral immunity. However, the mechanism by which anti-gp39 treatment suppresses humoral responses is not clear. Immune suppression by anti-gp39 could be mediated by: (a) the negative signaling of gp39-bearing T cells causing Th anergy; (b) mAb-mediated cytotoxic deletion of anti-gp39 bearing CD4⁺ T cells; and/or (c) the blocking of gp39 binding to CD40. A series of experiments were performed to gain insight into which of these mechanisms may be operative in the protracted immune suppression observed with anti-gp39 therapy. To explore the possibility that antigen-specific Th were deleted or anergized by anti-gp39 therapy, antigen-specific Th function from gp39-treated mice was measured by adoptive transfer. Briefly, mice were immunized with SRBC (to prime SRBC-specific Th) and administered anti-gp39 or HIg (250 µg/mouse on days 0, 2, and 4). After 7 d, spleen cells from unimmunized mice or SRBC-immune spleen cells from HIg- or anti-gp39-treated mice were adoptively transferred into recipient mice with TNP-immune spleen cells as a source of TNP-primed B cells. Mice were simultaneously challenged with TNP-SRBC, and the IgG1 anti-TNP titer ascertained on day 5. SRBC-primed Th cells are required to elicit a secondary anti-TNP response in the recipient mice as demonstrated by the fact that recipients that received spleen cells from nonimmune donors produced substantially lower IgG1 anti-TNP compared to those mice that received spleen cells from SRBC-primed animals (Fig. 5). More importantly, results of these experiments revealed that the SRBC helper activity from HIg- and anti-gp39-treated mice was similar, indicating that anti-gp39 treatment did not alter Th function or block the priming of Th. Moreover, antigen-responsive Th were not deleted or anergized as a result of anti-gp39 treatment, as they provided helper-effector function upon transfer.

In Vivo Clearance of Hamster Anti-gp39. Previous studies have established that anti-gp39 (MR1) blocks the binding of gp39 to CD40 (16) and thus support the hypothesis that the in vivo immunosuppressive effects of anti-gp39 are due to the blocking of gp39-CD40 interactions. If one assumes this hypothesis to be correct, the long-term immune suppression observed with anti-gp39 administration requires the persistence of anti-gp39 in the host. To determine if anti-gp39 could be detected for the period of time that immune suppression was evident, the in vivo clearance rate of anti-gp39 from serum was determined. Mice were given a regime of antibody (3 × 250 µg anti-gp39) over the course of 4 d and assayed for the levels of serum anti-gp39 at 7, 14, and 21 d after the initiation of antibody administration. Western blot analysis for nonreduced MR1 (160 kD) indicated that

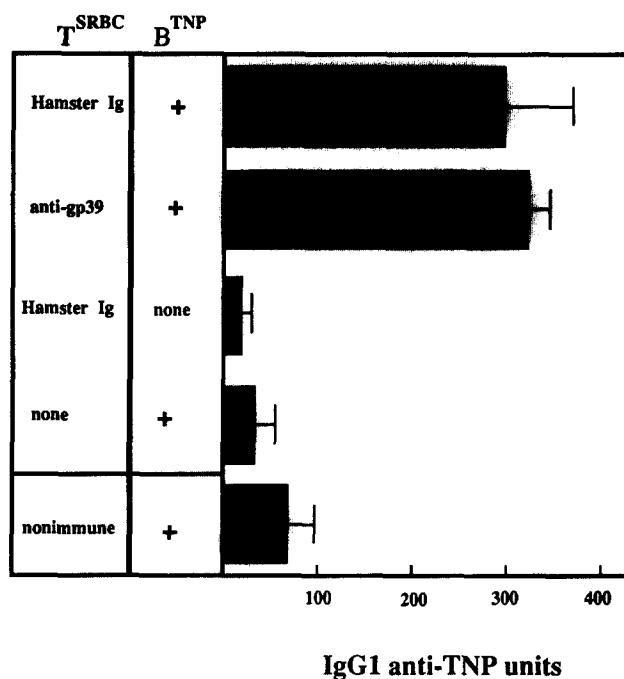


Figure 5. Anti-gp39 administration does not functionally delete SRBC-specific Th. Mice (three per group) were immunized with SRBC (200 µl of 1.0% SRBC, i.v.) and administered anti-gp39 or HIg (on days 0, 2, and 4; 250 µg/d). On day 7, the spleens from nonimmune or SRBC-immune mice were removed and transferred (i.v., 50 × 10⁶/mouse) into irradiated recipients (600 rad) with/without 50 × 10⁶ spleen cells from TNP-KLH primed (KLH/CFA 50 µg, i.v.) mice as a source of immune B cells. At the time of transfer, mice were also immunized with TNP-SRBC (200 µl of 1.0% TNP/SRBC). Serum IgG1 anti-TNP titers were ascertained on day 6 after transfer using a TNP₂-BSA ELISA. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The data are representative of two such experiments.

intact, serum anti-gp39 could be detected for at least 21 d after the initiation of antibody treatment (Fig. 6 A). The serum concentration of anti-gp39 in animals at 21 d was ~5% (based on scanning densitometry), when compared to the signals derived from serum of animals analyzed 7 d after initiation of antibody therapy.

Although it was determined that intact anti-gp39 was present in serum, it was also important to ascertain that the anti-gp39 was biologically active. Therefore, sera from mice that received 3 × 250 µg of anti-gp39 over the course of 4 d were used to stain gp39-bearing Th (Fig. 6 B). The level of serum anti-gp39 3 d after the last injection (7 d after initiation of antibody treatment) was set at 100%. 14 d after the initiation of antibody therapy, ~10–15% of the biologically active anti-gp39 mAb was detected in the serum. 21 d after initiation of therapy, 2–3% of anti-gp39 remained in the serum. Therefore, both the determination of intact gp39 by Western blotting and of biologically active anti-gp39 revealed that ~5% of the anti-gp39 was present 21 d after beginning anti-gp39 therapy. These results demonstrate the half-life of

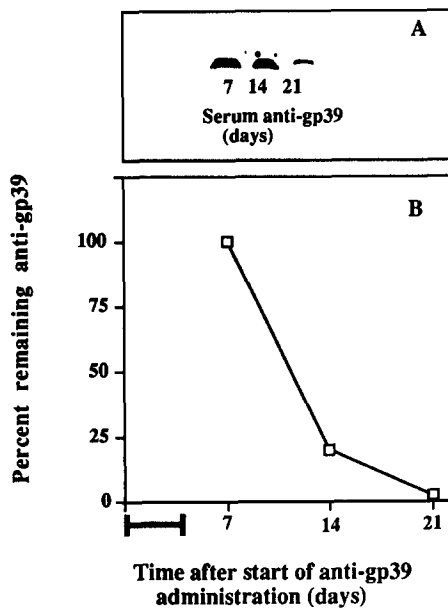


Figure 6. In vivo clearance of hamster anti-gp39. Mice were administered $3 \times 250 \mu\text{g}$ of anti-gp39 on days 0, 2, and 4. On days 7, 14, and 21, the amount of remaining anti-gp39 was determined as follows: (A) serum ($1.5 \mu\text{l}$) was electrophoresed under nonreducing conditions, transferred to nitrocellulose, and blotted with HRP-conjugated RG7 (mouse anti-rat κ chain), followed by chemiluminescent detection. Areas of blot corresponding to 150–165 kD were scanned and digitized. (B) Titrations of serum were used to stain activated Th1 to determine the amount of biologically active anti-gp39 present in the serum. Activated Th1 were stained with titrations of serum followed by FITC-anti-rat κ chain (RG7). The percent anti-gp39 remaining in serum was deduced based on a standard curve of mean fluorescence intensity vs serum concentration, using day 7 as 100%. (Black bar) The time of antibody administration. The results are representative of two such experiments.

anti-gp39 to be approximately 12 d and offer evidence consistent with the hypothesis that prolonged suppression of humoral immune responses by anti-gp39 is due to persistent blocking of Th function.

Discussion

The present study demonstrates that in vivo administration of an anti-gp39 antibody which blocks gp39–CD40 interactions in vitro, results in profound inhibition of both primary and secondary humoral immune responses to TD antigens, but not TI-type II antigens. In addition, this study demonstrates that anti-gp39 treatment does not block the priming of antigen-primed Th cells. Therefore, the gp39–CD40 ligand-receptor pair can be used as a target for the therapeutic manipulation of the humoral immune response.

To gain insight into how anti-gp39 was exerting its immunosuppressive effect on humoral immunity, the direct effects of anti-gp39 on Th function were addressed. The data indicate that SRBC-immune Th from anti-gp39-treated mice were fully capable of providing help upon adoptive transfer, suggesting that anti-gp39 treatment did not cause Th deletion

or anergy in vivo. These results led to the speculation that anti-gp39 mediates its immunosuppressive effects by blocking gp39 binding to CD40 and not by the inactivation of gp39-bearing Th. In support of this hypothesis, in vitro studies have established that anti-gp39 blocks the binding of CD40 to gp39 (17). Furthermore, biologically active anti-gp39 could be detected in serum for the period of time that immune suppression was apparent. Although only 5% of anti-gp39 was present in serum at a time when immune suppression was evident, it is possible that the local tissue concentrations of anti-gp39 in specific sites of secondary lymphoid organs is higher and clearance rates are slower than that of serum anti-gp39. Further insights are clearly needed to conclusively address the mechanism(s) of action of anti-gp39. Currently, studies are underway examining the effect of Fab and F(ab')_2 anti-gp39 on humoral immune responses so as to allow us to verify that anti-gp39-mediated inhibition is the result of gp39 blockade.

Treatment of mice with anti-gp39 inhibited the primary immune response to SRBC and heterologous Ig $>90\%$ for prolonged periods of time. Assuming that anti-gp39 is mediating the inhibition by blocking gp39 function, these data implicate gp39–CD40 interactions as essential in the development of primary immune responses to TD antigens. Immunohistochemical analysis establish that gp39 is induced as a consequence of immunization with TD antigens and may be of functional significance. The in situ studies of gp39 expression illustrate that the initial site of gp39–CD40 interactions during primary humoral immune responses is in the peripheral aspects of the periarteriolar lymphoid sheaths (PALS) and around the terminal arterioles (TA) of the spleen (15). It is at these sites that conjugates between gp39-expressing Th and antigen-specific B cells were found juxtaposed, suggesting that the outer PALS is a major site of T–B cell interactions during primary humoral immune responses. Therefore, the PALS may be the site at which anti-gp39 interacts with gp39-expressing Th cells to ultimately inhibit T–B interaction and subsequent Ig production. Immunohistochemical analysis of the distribution of anti-gp39 in anti-gp39-treated mice is underway to determine if this is the case.

Similar to primary responses, the secondary humoral immune response of mice primed to KLH in CFA was also shown to be inhibited by the administration of anti-gp39. Consistent with the reduction of anti-SRBC PFC by anti-gp39, reductions in serum antibodies titers to antigenic challenge were also observed. The serum titers of all anti-KLH Ig isotypes measured (IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE) were reduced by the treatment of mice with anti-gp39. The effect of anti-gp39 administration was apparent for at least 14 d after secondary challenge with antigen, establishing a persistent immune suppression by anti-gp39. Anti-gp39-mediated immune suppression of secondary responses to KLH is not unique to KLH, since secondary immune responses to heterologous Ig and heterologous erythrocytes (data not shown) were also inhibited by anti-gp39 therapy. The anatomical distribution of gp39-expressing Th was identical to that observed upon primary immunization, however, the fre-

quency of gp39-expressing Th in immune spleen was increased over that observed during primary immune responses. No gp39-expressing Th were found in the germinal centers or follicles of immune spleen (15). Thus, it appears that B cells are triggered to respond to activated Th cells in the PALS and TA of the spleen and later migrate to the follicles and germinal centers.

Although the *in vivo* studies demonstrate that anti-gp39 effectively blocks the generation of responses to TD antigens, it appears that gp39-CD40 interactions play little if any role in the initiation of humoral responses to antigens that have classically been characterized as T cell independent. Data presented in the accompanying paper demonstrate that gp39-expressing Th cells were found subsequent to immunization with both TD and one TI-type II antigen, TNP-Ficoll. However, based on our supposition that anti-gp39 is working via the blockade of gp39 function, the inability of anti-gp39 treatment to inhibit primary responses to TNP-Ficoll suggests that gp39-CD40 interactions are not required for the generation of humoral responses to TI-type II antigens. There are reports (15) that humoral immune responses to TNP-Ficoll are greatly augmented by Th. Therefore, it appears that the response to TNP-Ficoll requires Th but not gp39 function. A more comprehensive group of TI antigens are now under study to evaluate the gp39 dependence of these antigens for inducing humoral immune responses. Using anti-gp39, one should be able to refine the definition of TI and TD antigens as gp39 dependent or independent.

Recent studies on patients with HIM have provided genetic proof that gp39 is an essential component in TD humoral immunity (18-21). Patients with HIM are characterized by increased susceptibility to bacterial infections, associated with low levels of IgG, IgA, and IgE, a severe reduction in follicles and a complete lack of germinal centers (28). However, normal or increased levels of isohaemagglutinin, antityphoid, and Forssman antibodies are typically observed in HIM patients. In these patients, vaccination often results in normal primary (IgM restricted) antibody responses, yet boosting rarely results in specific IgG responses (29). Given the results presented herein demonstrating that gp39 inactivation prohibits TD immune responses, the question emerges as to how patients with HIM, a genetic inactivation of gp39, mount primary IgM responses. First, many of the IgM responses observed in HIM patients may be due to TI type antigens

eliciting restricted immunoglobulin isotype profiles. Our studies with TI antigens in mice indicate that the IgM responses to TI type II antigens are gp39 independent. Second, some antigens that we consider TD in the mouse, may act as TI antigens in humans. Third, it is not clear that all mutations in gp39 result in complete functional inactivation of the molecule and therefore some HIM patients may express partially functional TD responses. Indeed, it has been reported that activated T cells from at least one HIM patient weakly bound a soluble, recombinant form of CD40 (18). This observation supports the idea that some gp39 mutations may allow for the production of incomplete TD responses. Alternatively, one could suggest that gp39-CD40 interactions *per se* are not essential to the development of primary immune responses. Following this logic, one must then deduce that the immunosuppressive effects of anti-gp39 administration on the primary immune response are due to the deletion of activated, gp39-bearing Th. However, this is inconsistent with the data presented.

The focus of the present study was to demonstrate the potential use of anti-gp39 in the control of TD humoral immunity. Brief treatment regimes with the anti-gp39 resulted in prolonged suppression, an attractive attribute of this therapeutic antibody. Of special interest may be the capacity of anti-gp39 to prevent primary and secondary humoral responses to other heterologous, therapeutic antibodies such as Chi-L6. This would permit the exposure of patients to repeated administrations of heterologous therapeutic antibodies. Inhibitory effects on humoral immunity have been observed with other mAbs, *i.e.*, anti-CD4 (27, 28). Although it is unclear how anti-CD4 mediates immune suppression, extensive deletion of CD4⁺ T cells is correlated with suppressive efficacy (30), a phenomenon not observed with anti-gp39 therapy (data not shown). In addition to anti-CD4, it has been shown that the interference by CTLA-4 of CD28 triggering, a costimulatory molecule on Th cells, also suppresses TD antibody responses (31) and blocks xenogeneic graft rejection (32). Similar to anti-gp39 administration, CTLA-4 induced a state of prolonged immune suppression. Because anti-gp39 and CTLA-4 mediate their immunosuppressive effects at distinct stages of the humoral immune response, coadministration of these two immunosuppressive drugs may provide additive or synergistic immunosuppressive effects on immunity.

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References

1. Inaba, K., M.D. Witmer, and R.M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858.
2. Inaba, K., and R.M. Steinman. 1985. Protein-specific helper T-lymphocyte formation initiated by dendritic cells. *Science (Wash. DC)*. 229:475.
3. Noelle, R.J., and E.C. Snow. 1991. Cognate interactions of helper T cells and B cells. *Immunol. Today*. 11:361.
4. Gordon, J., M.J. Millsum, G.R. Guy, and J.A. Ledbetter. 1987. Synergistic interaction between interleukin 4 and anti-Bp50 (CDw40) revealed in a novel B cell restimulation assay. *Eur. J. Immunol.* 17:1535.
5. Clark, E.A., and J.A. Ledbetter. 1986. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp 50. *Proc. Natl. Acad. Sci. USA*. 83:4494.
6. Stamenkovic, I., E.A. Clark, and B. Seed. 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1403.
7. Hollenbaugh, D., L. Grosmaire, C.D. Kullas, N.J. Chalupny, R.J. Noelle, I. Stamenkovic, J.A. Ledbetter, and A. Aruffo. 1992. The human T cell antigen p39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4313.
8. Armitage, R.J., W.C. Fanslow, L. Strockbine, T.A. Sato, K.N. Clifford, B.M. Macduff, D.M. Anderson, S.D. Gimpel, T. Davis-Smith, C.R. Maliszewski, et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature*. 357:80.
9. Valle, A., C.E. Zuber, T. Defrance, O. Djossou, R.M. De, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur. J. Immunol.* 19:1463.
10. Gordon, J., M.J. Millsum, R.L. Flores, and S. Gillis. 1989. Regulation of resting and cycling human B lymphocytes via surface Ig and the accessory molecules interleukin-4, CD23 and CD40. *Immunology*. 68:526.
11. Jabara, H.H., S.M. Fu, R.S. Geha, and D. Vercelli. 1990. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* 172:1861.
12. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature (Lond.)*. 353:678.
13. Armitage, R.J., T.A. Sato, B.M. Macduff, K.N. Clifford, A.R. Alpert, C.A. Smith, and W.C. Fanslow. 1992. Identification of a source of biologically active CD40 ligand. *Eur. J. Immunol.* 22:2071.
14. Lane, P., A. Traunecker, S. Hubele, S. Inui, A. Lanzavecchia, and D. Gray. 1992. Activated human T cells express a ligand for the human B cell-associated CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur. J. Immunol.* 22:2573.
15. Van den Eertwegh, A., R. Noelle, M. Roy, D. Shepherd, A. Aruffo, J. Ledbetter, W. Boersma, and E. Claassen. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. *J. Exp. Med.* 178:1555.
16. Noelle, R.J., J.A. Ledbetter, and A. Aruffo. 1992. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B cell activation. *Immunol. Today*. 13:431.
17. Noelle, R.J., M. Roy, D.M. Shepherd, I. Stamenkovic, J.A. Ledbetter, and A. Aruffo. 1992. A novel ligand on activated T helper cells binds CD40 and transduces the signal for the cognate activation of B cells. *Proc. Natl. Acad. Sci. USA*. 89:6550.
18. Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, J. Disteche, D.K. Simoneaux, et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM Syndrome. *Science (Wash. DC)*. 259:990.
19. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubauer, et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell*. 72:291.
20. DiSanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, A. Fischer, and G. de Saint Basile. 1993. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:541.
21. Korthauer, U., D. Graf, H.W. Mages, F. Brieres, M. Padayachee, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.L. Levinsky, and A. Kroccek. 1993. Defective Expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature (Lond.)*. 361:539.
22. Kurt-Jones, E., S. Hamberg, J. Ohara, W.E. Paul, and A.K. Abbas. 1987. Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production. *J. Exp. Med.* 166:1774.
23. Springer, T.A., A. Bhattacharya, J.T. Cardoza, and F. Sanchez-Madrid. 1982. Monoclonal antibodies specific for rat IgG1, IgG2a, and IgG2b subclasses, and kappa chain monotypic and allotypic determinants: reagents for use with rat monoclonal antibodies. *Hybrid*. 1:25.
24. Hellstrom, I. 1986. Monoclonal mouse antibodies raised against human lung carcinoma. *Cancer Res.* 46:3917.
25. Wilde, D.B., P. Marrack, J. Kappler, D.P. Dialynas, and F.W. Fitch. 1983. Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK1.5 (anti-L3T4a) blocks class II MHC antigen-specific proliferation, release of lymphokines, and binding by cloned murine helper T lymphocyte lines. *J. Immunol.* 131:2178.
26. Snow, E.C., and R.J. Noelle. 1987. Thymus-dependent antigenic stimulation of hapten-specific B lymphocytes. *Immunol. Rev.* 99:173.
27. Jerne, N.K., C. Henry, A.A. Nordin, H. Fuji, A.M. Koros, and I. Lefkovits. 1974. Plaque forming cells: methodology and theory. *Transplant Rev.* 18:130.
28. Ochs, H.D., and R.J. Wedgewood. 1989. Disorders of the B cell system. In *Immunologic Disorders in Infants and Children*. 3rd ed. E.R. Sreihm, editor. W.B. Saunders, Co., Philadelphia. 226-256.
29. Nartarangelo, L.D., M. Duse, and A.G. Ugazio. 1992. Immunodeficiency with hyper-IgM (HIM). *Immunodef. Rev.* 3:101.
30. Shizuru, J.A., S.A. Alters, and C.G. Fathman. 1992. Anti-CD4 monoclonal antibodies in therapy: creation of nonclassical tolerance in the adult. *Immunol. Rev.* 129:103.
31. Linsley, P.S., P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh, and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science (Wash. DC)*. 257:792.
32. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, and J.A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA41g. *Science (Wash. DC)*. 257:789.