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Antiviral Immune Responses in CTLA4 Transgenic Mice

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The role of B7 binding CD28 in the regulation of T- and B-cell responses against viral antigens was assessed in transgenic mice expressing soluble CTLA4-Hγ1 (CTLA4-Ig tg mice) that blocks B7-CD28 interactions. The results indicate that transgenic soluble CTLA4 does not significantly alter cytotoxic T-cell responses against replicating lymphocytic choriomeningitis virus (LCMV) or vaccinia virus but drastically impairs the induction of cytotoxic T-cell responses against abortively replicating vesicular stomatitis virus (VSV). While the T-independent neutralizing immunoglobulin M (IgM) responses were within normal ranges, the switch to IgG was reduced 4- to 16-fold after immunization with abortively replicating VSV and more than 30-fold after immunization with an inert VSV glycoprotein antigen in transgenic mice. IgG antibody responses to LCMV, as detected by enzyme-linked immunosorbent assay and by neutralizing action, were reduced about 3- to 20-fold and more than 50-fold, respectively. These results suggest that responses in CTLA4-Ig tg mice are mounted according to their independence of T help. While immune responses to nonreplicating or poorly replicating antigens are in general most dependent on T help and B7-CD28 interactions, they are most impaired in CTLA4-Ig tg mice. The results of the present experiments also indicate that highly replicating viruses, because of greater quantities of available antigens and by inducing as-yet-undefined factors and/or cell surface changes, are capable of compensating for the decrease in T help caused by the blocking effects of soluble CTLA4.

Specific immune responses are the result of antigen stimulation and complex lymphocyte interactions guided by complex lymphoid-organ structures (37, 46, 61). Multiple interactions and signal transmissions permit a relatively tight control of both antibody and T-cell responses. Originally a two-signal concept was proposed to describe available experimental observations (9, 56). Accordingly, induction either of antibody production by B cells or of effector T cells required antigen (signal 1) plus a second signal in the form of factors and/or accessory molecules. This proposal has furthered the search for such second signals in transplantation and tumor- and antigen-specific immune reactions (2, 27, 40, 50). In several situations, both in vitro and in vivo, defined cytokines and accessory molecules have been shown to function as signal 2 (12, 22, 34, 56, 57).

The role of accessory molecules such as CD28 and B7.1 as well as GP39 and CD40 in lymphocyte interactions has been analyzed in several model systems in vivo and in vitro using blocking monoclonal antibodies (MAb) (60) or soluble ligands (13, 30, 33, 35) or in gene knockout mice (15, 58, 59, 62). There is convincing evidence that soluble factors and cellular signal-transmitting ligand-receptor interactions are critical under certain conditions. The question is open as to how these different factors and receptor-ligand interactions on cell membranes act alone or in concert in vivo and how limiting each one alone is under conditions in which humoral or cell-mediated immune responses are essential for the survival of the host.

Several gene knockout mice lacking T-cell subsets, transplantation antigens, interleukins, or cell interaction molecules on cell membranes have been evaluated with respect to their capacity to mount antiviral $CD8^+$ and $CD4^+$ T-helper-cell responses or immunoglobulin M (IgM) and IgG B-cell responses in vivo. In particular, the roles of B7.1 and B7.2 on antigen-presenting cells (APC) and B cells interacting with CD28 and CTLA4 have been analyzed in mice lacking CD28 or CTLA4 (18, 59, 62, 66). Alternatively, mice expressing soluble CTLA4 that can block cell-cell interactions or mice treated with specific antibodies against ligands or their receptors have been analyzed. The results indicated that cell interactions via B7.1 and CD28 as well as B7.1 or B7.2 and CTLA4 are often limiting in vitro. In vivo studies suggested that antibody responses were down modulated (29) and that allogeneic organ rejection was impaired (33, 35, 63), whereas T-helper cells or protective cytotoxic T-lymphocyte (CTL) responses against viruses still were induced (26, 54, 59).

The present study evaluated antiviral CTL and antibody responses in mice transgenic for CTLA4-Hγ1 (29, 54). The results revealed that CD28-B.7 interactions were blocked, but mice were still able to respond efficiently with CTL and some IgG responses against widely replicating viruses; however, they responded only marginally with CTLs against a poorly replicating virus and mounted only T-independent IgM responses against nonreplicating viral antigens. These findings indicated that the amount of infectious antigens and/or factors caused by productively replicating infections can compensate for limiting amounts of T help.

MATERIALS AND METHODS

Mice. C57BL/6 mice were obtained from the breeding colony of the Institut für Zuchthygiene, Zürich, Switzerland. The generation of the mice transgenic for CTLA4-Hγ1 (called CTLA4-Ig tg mice here) has been described previously (29, 30). Mice were bred under specific-pathogen-free conditions and tested in a conventional mouse facility.

Viruses. The lymphocytic choriomeningitis virus (LCMV) WE isolate was originally provided by F. Lehmann-Grube, Hamburg, Germany, and grown on L929 cells with a low multiplicity of infection. Vesicular stomatitis virus (VSV) Indiana (VSV_{IND}) (Mudd-Summers isolate) was originally obtained from D. Kolakofsky, University of Geneva. The recombinant Vacc-IND N was generously provided by B. Moss (36), Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Md. Recombinant baculoviruses expressing the glycoprotein of VSV_{IND} (Baculo-IND G) or the nucleoprotein of LCMV (Baculo-LCMV-

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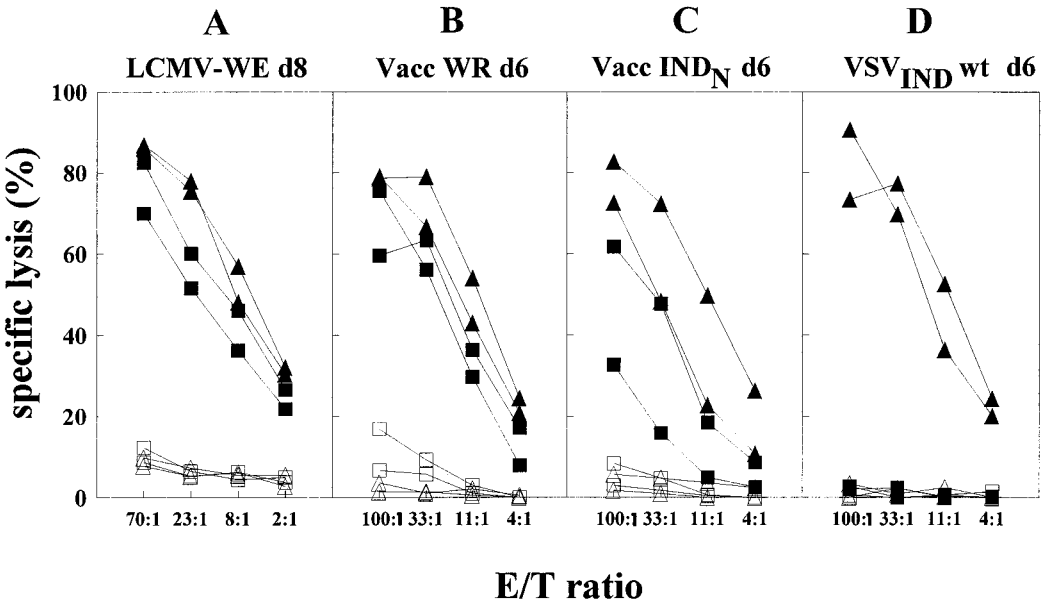

FIG. 1. Cytolytic activity after virus infection. CTLA4-Ig tg mice (squares) and nontransgenic littermates (triangles) were acutely infected with different types of virus. Spleen cells of infected mice were isolated and tested in a ^{51}Cr release assay with target cells expressing virus-specific peptide (closed symbols) and control target cells (open symbols). (A) Cytolytic activity after infection with 200 PFU of LCMV WE was determined on ^{51}Cr-labelled EL-4 cells loaded with GP33 or an unrelated adenovirus peptide as a negative control. Spleen cells of mice infected with wild-type vaccinia virus WR (Vacc WR) (B) or recombinant Vacc-IND N (Vacc IND$_N$) (C) were tested by using vaccinia virus WR-infected or uninfected MC57G cells as target cells. (D) After infection of mice with wild-type (wt) VSV$_{IND}$, a ^{51}Cr release assay was performed with EL-4 cells transfected with the nucleoprotein (N) of VSV or with mock-transfected cells. Data for individual mice are shown and are representative of at least two independent experiments. In all cases, spontaneous release was below 20% during the 5-h assay period. E/T ratio, effector-to-target ratio; d8, day 8; d6, day 6.

NP) were generous gifts of D. H. L. Bishop, NERC Institute of Virology, Oxford, United Kingdom. Recombinant proteins were produced as previously described (3, 39).

Local immunopathology (footpad swelling reaction). Mice were treated in both footpads of the hind legs with 30 μl of virus as described elsewhere (43). Footpad thickness was measured daily with a spring-loaded caliper. The results were expressed as the percent increase compared with preinfection values. Data are the means of at least four footpads. Standard deviations were within 10%.

^{51}Cr release assay. (i) Vaccinia virus. Vaccinia virus WR (2×10^6 PFU per mouse) was injected intravenously (i.v.). Spleen cells were tested 6 days after infection in a standard cytotoxicity assay (5). The test was performed with round-bottom 96-well plates for 5 h at 37°C in a 5% CO_2 incubator. MC57G target cells (10^4; infected with vaccinia virus at a multiplicity of infection of 5 for 2 h) were mixed with prediluted effector cells at effector-to-target ratios of 100:1, 33:1, 11:1, and 4:1, and the plates were spun for 4 min before incubation. Supernatant aliquots (70 ml) were analyzed in a gamma counter (Packard, Zürich, Switzerland).

(ii) LCMV. Eight days after i.v. infection of mice with 200 PFU of LCMV WE, effector spleen cells were tested on GP33-loaded EL-4 cells at effector-to-target ratios of 70:1, 23:1, 8:1, and 3:1 (5).

(iii) VSV. Six days after i.v. infection of mice with VSV$_{IND}$ (2×10^6 PFU), spleen cells were tested on EL-4 cells transfected with the nucleoprotein of VSV or mock transfected (24, 47, 53) in order to monitor VSV nucleoprotein-specific CTL activity.

For CD4$^+$ T-cell depletion, mice were treated with a rat anti-mouse CD4 (YTS 191) as described before (11, 31).

Detection of antiviral CTLp cells in the spleens of mice infected with VSV. Antiviral CTL precursor (CTLp) activity was determined under limiting-dilution conditions as described previously with minor modifications (42). Limiting numbers of responder cells were cultivated in 96-well round-bottom plates with irradiated (30-Gy) VSV-infected spleen cells as stimulators (10^5 cells per well) in Iscove's modified Dulbecco's medium conditioned with interleukins (supernatant from concanavalin A-stimulated rat spleen cells). After 7 days of incubation, the CTL activities in microcultures were determined on EL-4 cells transfected with the nucleoprotein of VSV or mock transfected.

VSV-neutralizing antibody titers. Neutralizing titers of sera against VSV were determined as previously described (10). Titers are indicated as $\log_2$ of 40-fold prediluted sera. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M 2-mercaptoethanol in saline.

LCMV-specific antibodies. Antibodies were measured with an enzyme-linked immunosorbent assay (ELISA) as described previously (6). Briefly, ELISA plates were coated overnight with 10 ng of Baculo-LCMV-NP in carbonate buffer (pH 9.6). Sera and peroxidase-labelled anti-mouse IgG (PharMingen) were diluted in 1% bovine serum albumin in phosphate-buffered saline. Mice were bled at different times after infection. The results were expressed as the dilutions required to obtain half-maximum readings. All sera were tested in the same assay, and serum samples from individual mice were tested under each experimental condition. LCMV neutralization in vitro was determined with an infectious focus-forming assay as described below. In brief, serial twofold dilutions of antibody were incubated with virus for 90 min at 37°C in a 96-well plate. Then MC57G mouse fibroblasts were added. After adsorption of virus, cells were overlaid with 1% methylcellulose in Dulbecco's modified Eagle's medium. Forty hours later, cell monolayers were fixed, permeabilized with 0.5% Triton X-100, and stained with the LCMV nucleoprotein-specific rat MAb VL4 (6) as the first antibody and a peroxidase-labelled goat anti-rat antibody (A-9037; Sigma) as the second antibody.

RESULTS

Antiviral cytotoxic T-cell responses. CTL responses against replicating LCMV WE and replicating vaccinia virus WR were always lower in CTLA4-Ig tg mice by a factor of about 2 or 3 compared with those of controls (Fig. 1A and B). Differences were also seen when lytic activity per spleen was computed by using as an arbitrary unit the number of spleen cells necessary to lyse 30% of the target cells in a standard assay (not shown). These results were confirmed by the primary CD8$^+$ T-cell-mediated footpad swelling reaction after local infection in the footpad with LCMV (Fig. 2). The overall kinetics of controls and CTLA4-Ig tg mice were comparable, although somewhat reduced in CTLA4-Ig tg mice.

CTL responses to a recombinant vaccinia virus (Fig. 1C) which is attenuated compared to wild-type vaccinia virus WR were reduced three-to fivefold in CTLA4-Ig tg mice.

CTL response to VSV. The CTL response against VSV assayed on day 6 after infection with 2×10^6 PFU i.v. was not measurable (Fig. 1D and 3) in CTLA4-Ig tg mice. Similarly, controls exhibited a 20- to 50-fold-greater response when di-

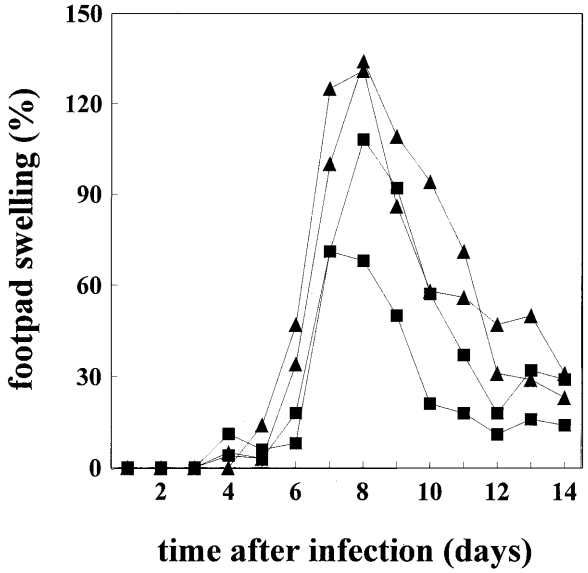

FIG. 2. Footpad swelling reaction after infection with LCMV. CTLA4-Ig tg mice (■) and nontransgenic littermates (▲) were infected with 3×10^3 PFU of LCMV WE in both footpads. Footpad thickness was measured daily. Mean percent increases in the thickness of both footpads of individual mice compared to values determined before infection are shown. The results of one of two comparable experiments are shown.

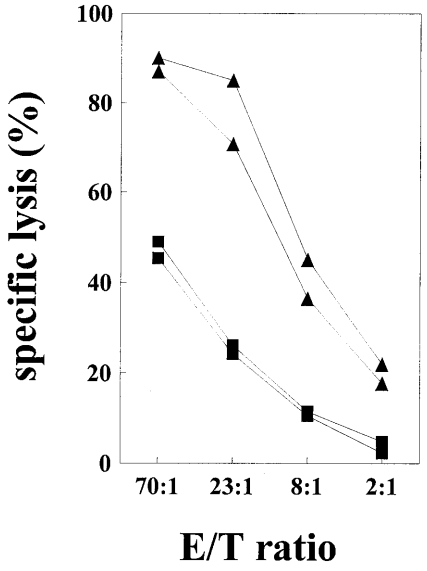

FIG. 4. C57BL/6 mice were treated with an anti-CD4 MAb. C57BL/6 mice were treated with an anti-CD4 MAb on days −3 and −1 to deplete $CD4^+$ T cells (■) or left untreated as controls (▲). On day 0, they were infected with 2×10^6 PFU of wild-type VSV_{IND} i.v. A chromium release assay was performed on day 6 after infection. Nonspecific lysis was below 10%. E/T ratio, effector-to-target ratio.

rect or total lytic activity per spleen was calculated (detailed calculations not shown). Comparable differences were found when the numbers of CTLp cells were determined. About 1,000 to 2,500 CTLp cells per spleen in CTLA4-Ig tg mice were found on day 6 versus 30,000 to 150,000 in controls. By day 20 after priming, CTLp frequencies had dropped to the usually found memory level of 10^{-4} to 10^{-5} spleen cells or 3,000 to 8,000 CTLp cells per spleen in controls. CTLA4-Ig tg mice exhibited similar frequencies, with ranges of 4,000 to 7,000 CTLp cells per spleen. The difference between day 6 and day 20 results suggested that VSV-specific CTLs were induced in CTLA4-Ig tg mice, but proliferation of primed CTLs during the acute phase of the response was apparently hampered.

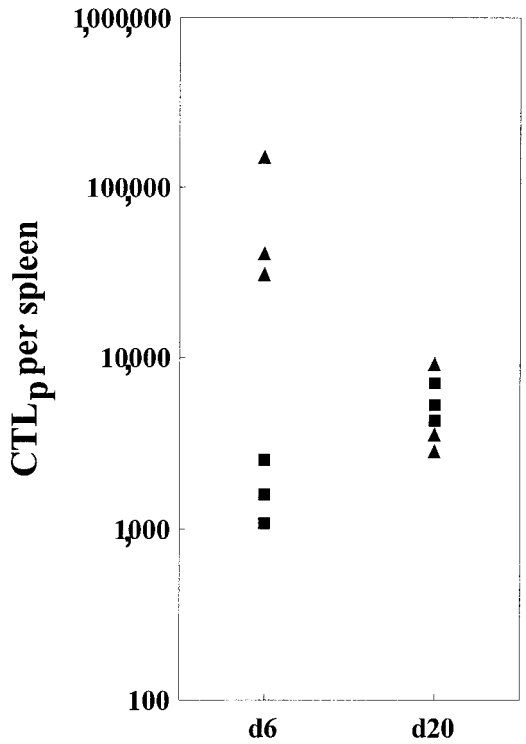

FIG. 3. CTL response after infection with wild-type VSV_{IND}. CTLA4-Ig tg mice (■) and nontransgenic littermates (▲) were infected with 2×10^6 PFU of wild-type VSV_{IND} i.v.; at 6 and 20 days (d6 and d20, respectively) after infection, the numbers of CTLp cells in the spleens of mice were determined by limiting-dilution analysis.

Since $CD4^+$ T-helper cells are usually not measurably involved in anti-LCMV CTL responses (1, 31, 32) but enhance CTL responses against poorly replicating viruses, such as vaccinia viruses, or abortively replicating VSV (26, 32), we tested the effect of $CD4^+$ T-cell depletion on anti-VSV CTL responses in C57BL/6 mice (Fig. 4). In this experiment, the primary CTL response against VSV on day 6 was reduced about 10-fold in CD4-depleted control C57BL/6 mice, to about the level found in CTLA4-Ig tg mice (reduction compared to controls, about 20- to 50-fold). Thus, the anti-VSV CTL responses in CTLA4-Ig tg mice were even more reduced than those of T-help-depleted control C57BL/6 mice.

Antibody responses against infectious virus. The levels of nonneutralizing anti-LCMV nucleoprotein IgG antibodies, as measured by ELISA, were 3- to 20-fold lower in CTLA4-Ig tg mice compared to those in controls (Fig. 5A and B). The levels of neutralizing anti-LCMV-GP1 antibodies are usually low in controls, with titers of 640 to 1,280. CTLA4-Ig tg mice failed to exhibit measurable neutralizing antibodies (Fig. 5C). Neutralizing IgM responses against VSV_{IND}, which are largely $CD4^+$ T-cell independent, were 4- to 16-fold lower in CTLA4-Ig tg mice than in control mice. Neutralizing IgG antibody responses, which are strictly $CD4^+$ T-help dependent, were also reduced about 16-fold. Nevertheless, transgenic mice exhibited titers of about 500 to 2,000, which, however, varied more than did those in controls (Fig. 5D).

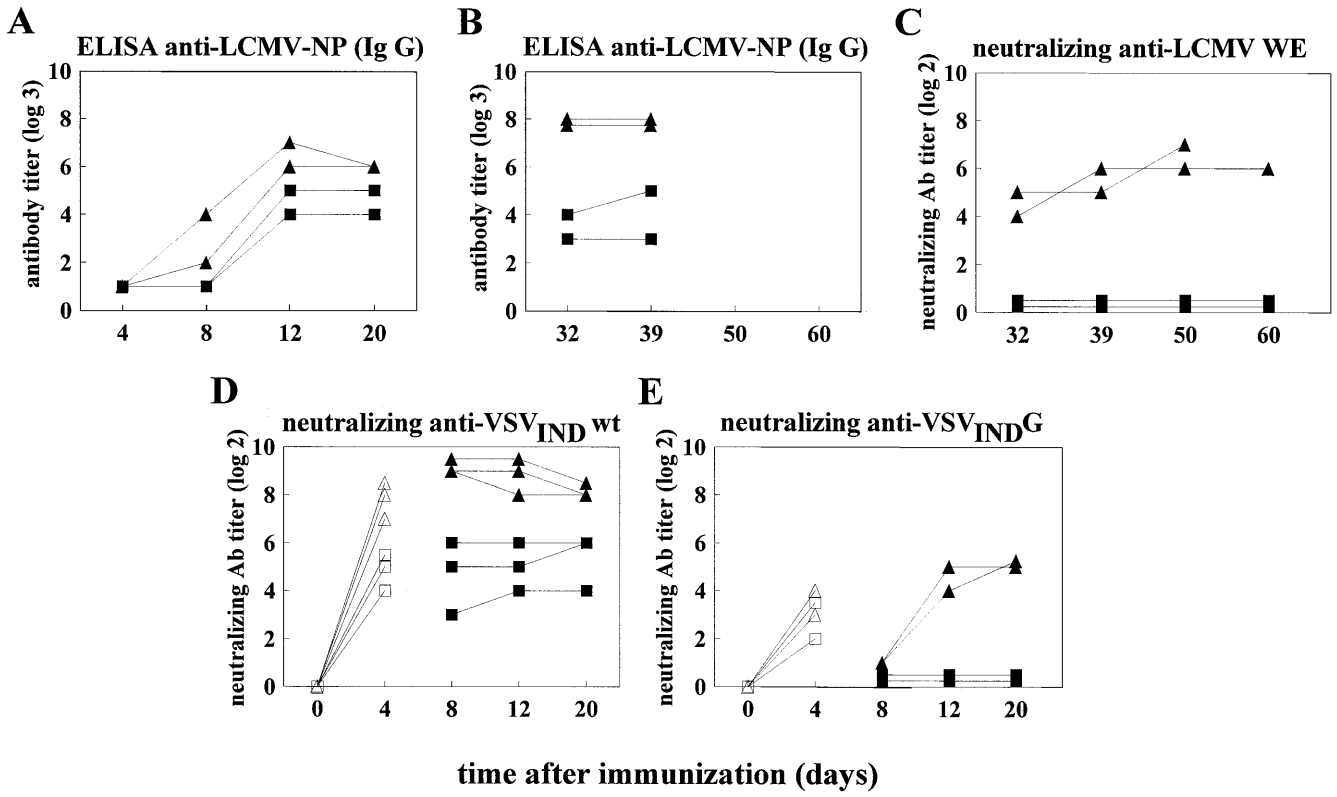

FIG. 5. Antibody formation after infection with LCMV or VSV or priming with recombinant viral protein. After infection of CTLA4-Ig tg mice (squares) and nontransgenic littermates (triangles) with 2×10^6 PFU of LCMV WE, sera were taken at the indicated times and were tested by ELISA for antibodies (Ab) binding to Baculo-LCMV-NP (A and B) or by a neutralization assay for LCMV-neutralizing antibodies (C). To test for neutralizing antibodies against VSV, mice were infected with 2×10^6 PFU of wild-type (wt) VSV_{IND} i.v. (D) or primed with 2 μg of recombinant Baculo-IND G (E). Titer steps of virus-neutralizing IgM (open symbols) and IgG (closed symbols) are shown. Neutralizing activity was determined in sera taken at the indicated times. Sera were prediluted 1:40 for VSV and 1:10 for LCMV neutralization assays and diluted further in steps of two. For anti-LCMV-NP ELISA, sera were prediluted 1:30 and further diluted in steps of three. Data for individual mice from one of two similar experiments are shown.

Antibody response against noninfectious VSV glycoprotein. Recombinant VSV glycoprotein (2 μg) injected i.v. induced comparable titers of neutralizing IgM antibodies in all mice (Fig. 5E). This response was earlier shown to be largely of T-help-independent type 2. Neutralizing IgG antibody responses, which are strictly $CD4^+$ T-helper dependent, reached 1,200 in controls from day 12 to day 20, but no IgG antibodies were found in CTLA4-Ig tg mice up to day 20 (Fig. 5E).

DISCUSSION

The capacity of CTLA4-Ig tg mice to mount CTL or IgG responses against viruses and viral antigens was inversely related to the T-help dependence of these responses. Highly replicating viruses in general induced more potent immune responses than abortively replicating viruses and inert viral antigens. Whether these differences in T-help dependence are caused by differences in antigen quantities alone or also reflect differences in factors (inflammation, cytokines, etc.) induced considerably more extensively by widely replicating viruses than by inert antigens is unclear.

The question of whether and how infectious agents influence immune responses compared to those influenced by inert antigens has been discussed from distinct points of view (26, 40, 68, 70). First, infectious agents and toxins, but not noninfectious agents, cause stress to infected cells and APC; therefore, they activate APC and probably increase second signals. To achieve this with an inert antigen, a depot plus local inflammation is needed, as suggested by the empirical use of granuloma-forming agents as adjuvants (16, 17, 20). Second, destruction of cells by replicating agents force APC to pick up antigen and to migrate to local lymph nodes or the spleen (55). Such antigens, foreign or peripheral self antigens, have to reach organized lymphoid organs in a concentration gradient and during a minimal period (usually for 3 to 7 days) for a T-cell immune response to be induced. Third, the findings in this study and parallel results with CD28 gene knockout mice (26) suggest that CD28 and/or CTLA4-B7.1 and -B7.2 interactions, reflecting dependence on T help, are much more necessary for T-cell and IgG responses against innocuous antigens than for those against infectious and replicating ones. The results of present experiments do not help in deciding whether peripheral events or interactions in lymphoid organs are limited. They do suggest, however, that the sum of immune reactions caused by replicating virus possibly creates a milieu that is optimal for T lymphocytes to thrive. In its simplest form, this bystander effect may be the result of a great multiplicity of antigenic stimuli causing upregulation of the major histocompatibility complex (MHC), interleukins, and interferons. Such a milieu is provided either by $CD4^+$ T cells, by many $CD8^+$ T cells, or via as-yet-undefined virulence factors (e.g., lipopolysaccharide superantigens) provided or triggered early by infectious agents themselves, particularly if replicating.

In CTLA4-Ig tg mice, B7-CD28 interactions became limiting for IgG responses in all of the instances tested here, less for

those against infectious virus and considerably more for those against inert viral antigens. A similar strong influence on T-cell-dependent antibody responses has also been documented for CD40-CD40L interactions (4, 8, 14, 21, 28, 45, 49, 69). These findings parallel previously repeated findings on the role of B7-CD28 in T help and fit the notion that contact-dependent interactions between T and B cells are important limiting steps for IgG responses.

Why LCMV-neutralizing antibodies are so much more affected by the block of B7-CD28 interaction than are the LCMV nucleoprotein-specific IgG responses is open for discussion. According to the hypothesis illustrated by the works of Planz et al. (52) and Battegay et al. (7), B cells expressing LCMV-neutralizing antibodies are infected by noncytopathic LCMV selectively and become eliminated during the first wave of the antiviral CTL response. However, the late LCMV-neutralizing antibody response is generated by B cells, which presumably are stimulated by persisting viral antigen after the CTL response has decreased. For this stimulation, only very little antigen is available. Therefore, costimulatory signals might be very important to induce the LCMV-neutralizing humoral response; the block of these signals in CTLA4-Ig tg mice might prevent the generation of LCMV-neutralizing antibodies.

The role of T-helper cells in $CD8^+$ T-cell responses is contact independent, at least in mice, where T cells do not express class II MHC antigen (38). $CD4^+$ T-independent help to $CD8^+$ T cells is therefore thought to be mediated via soluble factors released by helper T cells; they include interleukin-2 (IL-2), IL-4, probably gamma interferon, and others (44). Alternatively, T-help-dependent activation of APC can upregulate accessory molecules important for $CD8^+$ T-cell induction and thereby enhance $CD8^+$ T-cell responses (51).

It has been shown earlier that soluble CTLA4 may be injected up to 48 h after immunization with keyhole limpet hemocyanin and still be able to block the response effectively (35). This suggested indirectly that CTLA4 interferes probably mostly with expansion rather than with induction of T cells. Whether the lack of IL-2 is the only limitation remains to be shown (25). Our findings that the numbers of CTLp cells against VSV were comparable on day 20 after infection but were much lower on day 6 in CTLA4-Ig tg mice are compatible with the view that CTLA4-blocked T-helper mechanisms are more important for the expansion of CTLs than for their induction (23, 64). Then the same frequency on day 20 could reflect induction, expansion, and inactivation of T cells in wild-type mice, in which T cells become susceptible to CTLA4-mediated inhibition; this does not occur in CTLA4-Ig tg mice because T cells are not sufficiently activated and/or expanded. B7-CD28 interaction enhances the induction of several immune responses; soluble CTLA4 blocks this interaction because of its about 20-times-greater affinity for B7. Evidence exists for the downregulation of activated T cells expressing CTLA4 by interaction with B7 on APC (41, 58, 62, 65). Although the CTL kinetics against VSV might suggest such a possibility, the comparable CTL responses against LCMV and vaccinia virus WR argue against it.

An increasing number of studies have revealed the well-recognized correlations between virulence and immunogenicity, particularly when a major pathway of lymphocyte interactions does not function (26, 40, 68, 70). These findings fit long-standing observations and recent results that completely inactivated vaccines (particularly after denaturation) are not very immunogenic and often require high and repetitive doses to be protective. Therefore, live vaccines or real infections may induce better immune responses more quickly for at least three reasons. (i) Agents have to reach relevant intracellular compartments for proper loading of class I MHC antigens, sometimes also class II MHC antigens (19, 48, 67); replicating intracellular agents achieve this best. (ii) There is an obvious important quantitive aspect in favor of replicating agents that is, however, difficult to assess. (iii) As also suggested by this study, stimulatory conditions induced by replicating agents directly, the great amounts and multiplicity of various antigens, cell and tissue damage, inflammation, etc., are provided by infections and toxins but not or much less so by inert antigens (1, 26).

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REFERENCES

1. **Ahmed, R., L. D. Butler, and L. Bhatti.** 1988. $T4^+$ T helper cell function in vivo: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses. J. Virol. **62:**2102–2106.
2. **Allison, J. P.** 1994. CD28-B7 interactions in T-cell activation. Curr. Opin. Immunol. **6:**414–419.
3. **Bailey, M. J., D. A. McLeod, C. Y. Kang, and D. H. Bishop.** 1989. Glycosylation is not required for the fusion activity of the G protein of vesicular stomatitis virus in insect cells. Virology **169:**323–331.
4. **Banchereau, J., F. Bazan, D. Blanchard, F. Brière, J. P. Galizzi, C. van-Kooten, Y. J. Liu, F. Rousset, and S. Saeland.** 1994. The CD40 antigen and its ligand. Annu. Rev. Immunol. **12:**881–922.
5. **Battegay, M., M. F. Bachmann, C. Burhkart, S. Viville, C. Benoist, D. Mathis, H. Hengartner, and R. M. Zinkernagel.** 1996. Antiviral immune responses of mice lacking MHC class II or its associated invariant chain. Cell. Immunol. **167:**115–121.
6. **Battegay, M., S. Cooper, A. Althage, J. Baenziger, H. Hengartner, and R. M. Zinkernagel.** 1991. Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. J. Virol. Methods **33:**191–198.
7. **Battegay, M., D. Moskophidis, H. Waldner, M. A. Brundler, W.-P. Fung-Leung, T. W. Mak, H. Hengartner, and R. M. Zinkernagel.** 1993. Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. J. Immunol. **151:**5408–5415.
8. **Borrow, P., A. Tishon, S. Lee, J. Xu, I. S. Grewal, B. A. Oldstone, and R. A. Flavell.** 1996. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. J. Exp. Med. **183:**2129–2142.
9. **Bretscher, P., and M. Cohn.** 1970. A theory of self-nonself discrimination. Science **169:**1042–1049.
10. **Charan, S., and R. M. Zinkernagel.** 1986. Antibody mediated suppression of secondary IgM response in nude mice against vesicular stomatitis virus. J. Immunol. **136:**3057–3061.
11. **Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann.** 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature **312:**548–551.
12. **Damle, N. K., K. Klussman, P. S. Linsley, and A. Aruffo.** 1992. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. J. Immunol. **148:**1985–1992.
13. **Fanslow, W. C., K. N. Clifford, M. Seaman, M. R. Alderson, M. K. Spriggs, R. J. Armitage, and F. Ramsdell.** 1994. Recombinant CD40 ligand exerts potent biologic effects on T cells. J. Immunol. **152:**4262–4269.
14. **Foy, T. M., X. T. N. Lapik, and R. J. Noelle.** 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J. Exp. Med. **180:**157–163.
15. **Freeman, G. J., F. Borriello, R. J. Hodes, H. Reiser, K. S. Hathcock, G. Laszlo, A. J. McKnight, J. Kim, L. Du, D. B. Lombard, et al.** 1993. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. Science **262:**907–909.
16. **Freund, J., E. R. Stern, and T. M. Pisani.** 1947. Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion. J. Immunol. **57:**179–194.
17. **Frey, J. R., and P. Wenk.** 1957. Experimental studies on the pathogenesis of contact eczema in the guinea-pig. Int. Arch. Allergy **11:**81–100.
18. **Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson.** 1994. Absence of B7-dependent responses in CD28-deficient mice. Immunity **1:**501–508.
19. **Jacobson, S., R. P. Sekaly, C. L. Jacobson, H. F. McFarland, and E. O. Long.** 1989. HLA class II-restricted presentation of cytoplasmic measles virus an-

tigens to cytotoxic T cells. J. Virol. **63:**1756–1762.
20. **Janeway, C. A.** 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symp. Quant. Biol. **54:**1–13.
21. **Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani.** 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity **1:**167–178.
22. **Koulova, L., E. A. Clark, G. Shu, and B. Dupont.** 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. J. Exp. Med. **173:**759–762.
23. **Krummel, M. F., and J. P. Allison.** 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J. Exp. Med. **183:**2533–2540.
24. **Kündig, T. M., M. F. Bachmann, L. Lefrancois, L. Puddington, H. Hengartner, and R. M. Zinkernagel.** 1993. Non-immunogenic tumor cells may efficiently restimulate tumor antigen-specific cytotoxic T cells. J. Immunol. **150:**4450–4456.
25. **Kündig, T. M., H. Schorle, M. F. Bachmann, H. Hengartner, R. M. Zinkernagel, and I. Horak.** 1993. Immune responses in interleukin-2-deficient mice. Science **262:**1059–1061.
26. **Kündig, T. M., A. Shahinian, K. Kawai, H.-W. Mittrücker, E. Sebzda, M. F. Bachmann, T. W. Mak, and P. S. Ohashi.** 1996. Duration of TCR stimulation determines costimulatory requirements of T cells. Immunity **5:**41–52.
27. **Lafferty, K. J., and A. J. Cunningham.** 1975. A new analysis of allogeneic interactions. Aust. J. Exp. Biol. Med. Sci. **53:**27–42.
28. **Lane, P., T. Brocker, S. Hubele, A. Padovan, A. Lanzavecchia, and F. McConnell.** 1993. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. J. Exp. Med. **177:**1209–1213.
29. **Lane, P., C. Burdet, S. Hubele, D. Scheidegger, U. Muller, F. McConnell, and M. Kosco Vilbois.** 1994. B cell function in mice transgenic for mCTLA4-H gamma 1: lack of germinal centers correlated with poor affinity maturation and class switching despite normal priming of CD4+ T cells. J. Exp. Med. **179:**819–830.
30. **Lane, P., W. Gerhard, S. Hubele, A. Lanzavecchia, and F. McConnell.** 1993. Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human gamma 1. Immunology **80:**56–61.
31. **Leist, T. P., S. P. Cobbold, H. Waldmann, M. Aguet, and R. M. Zinkernagel.** 1987. Functional analysis of T lymphocyte subsets in antiviral host defense. J. Immunol. **138:**2278–2281.
32. **Leist, T. P., M. Kohler, and R. M. Zinkernagel.** 1989. Impaired generation of anti-viral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. Scand. J. Immunol. **30:**679–686.
33. **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 CTLA4Ig. Science **257:**789–792.
34. **Linsley, P. S., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter.** 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. **173:**721–730.
35. **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 **257:**792–795.
36. **Mackett, M., T. Yilma, J. K. Rose, and B. Moss.** 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. Science **227:**433–435.
37. **MacLennan, I. C. M.** 1994. Germinal centers. Annu. Rev. Immunol. **12:**117–139.
38. **Massa, P. T., R. Dorries, H. Wege, and V. ter Meulen.** 1987. Analysis and pathogenetic significance of class II MHC (Ia) antigen induction on astrocytes during JHM coronavirus infection in rats. Adv. Exp. Med. Biol. **218:** 203–217.
39. **Matsuura, Y., R. D. Possee, H. A. Overton, and D. H. Bishop.** 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. **68:**1233–1250.
40. **Matzinger, P.** 1994. Tolerance, danger, and the extended family. Annu. Rev. Immunol. **12:**991–1045.
41. **Mittrücker, H. W., A. Shahinian, D. Bouchard, T. M. Kundig, and T. W. Mak.** 1996. Induction of unresponsiveness and impaired T cell expansion by staphylococcal enterotoxin B in CD28-deficient mice. J. Exp. Med. **183:**2481–2488.
42. **Moskophidis, D., and F. Lehmann-Grube.** 1983. The immune response of the mouse to lymphocytic choriomeningitis virus. III. Differences of numbers of cytotoxic T lymphocytes in spleens of mice of different strains. Cell. Immunol. **77:**279–289.
43. **Moskophidis, D., and F. Lehmann-Grube.** 1989. Virus-induced delayed-type hypersensitivity reaction is sequentially mediated by CD8+ and CD4+ T lymphocytes. Proc. Natl. Acad. Sci. USA **86:**3291–3295.
44. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
45. **Noelle, R. J., M. Roy, and M. V. Alvares.** 1992. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc. Natl. Acad. Sci. USA **89:**6550–6554.
46. **Nossal, G. J.** 1992. Cellular and molecular mechanisms of B lymphocyte tolerance. Adv. Immunol. **52:**283–331.
47. **Oehen, S., H. Waldner, T. M. Kündig, H. Hengartner, and R. M. Zinkernagel.** 1992. Antivirally protective cytotoxic T cell memory to lymphocytic choriomeningitis virus is governed by persisting antigen. J. Exp. Med. **176:** 1273–1281.
48. **Oxenius, A., M. F. Bachmann, P. A. Ashton-Rickardt, S. Tonegawa, R. M. Zinkernagel, and H. Hengartner.** 1995. Presentation of endogenous viral proteins in association with major histocompatibility complex class II: on the role of intracellular compartmentalization, invariant chain and the TAP transporter system. Eur. J. Immunol. **25:**3402–3411.
49. **Oxenius, A., K. A. Campbell, C. R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R. M. Zinkernagel, and M. F. Bachmann.** 1996. CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral CD4+ T cell functions. J. Exp. Med. **183:**2209–2218.
50. **Pardoll, D. M.** 1993. Cancer vaccines. Immunol. Today **14:**310–316.
51. **Peters, J. H., R. Gieseler, B. Thiele, and F. Steinbach.** 1996. Dendritic cells: from ontogenetic orphans to myelomonocytic descendants. Immunol. Today **17:**273–277.
52. **Planz, O., P. Seiler, H. Hengartner, and R. M. Zinkernagel.** 1996. Evidence for cytotoxic T cell-mediated elimination of B cells producing neutralising antibodies after viral infection. Nature **382:**726–729.
53. **Puddington, L., M. J. Bevan, J. K. Rose, and L. Lefrancois.** 1986. N protein is the predominant antigen recognized by vesicular stomatitis virus-specific cytotoxic T cells. J. Virol. **60:**708–717.
54. **Ronchese, F., B. Hausmann, S. Hubele, and P. Lane.** 1994. Mice transgenic for a soluble form of murine CTLA-4 show enhanced expansion of antigen-specific CD4+ T cells and defective antibody production in vivo. J. Exp. Med. **179:**809–817.
55. **Roost, H.-P., S. Charan, and R. M. Zinkernagel.** 1990. Analysis of the kinetics of antiviral memory T help in vivo: characterization of short-lived cross-reactive T help. Eur. J. Immunol. **20:**2547–2554.
56. **Schwartz, R. H.** 1990. A cell culture model for T lymphocyte clonal anergy. Science **248:**1349–1356.
57. **Schwartz, R. H.** 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell **71:**1065–1068.
58. **Sethna, M. P., L. van Parijs, A. H. Sharpe, A. K. Abbas, and G. J. Freeman.** 1994. A negative regulatory function of B7 revealed in B7-1 transgenic mice. Immunity **1:**415–421.
59. **Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak.** 1993. Differential T cell costimulatory requirements in CD28-deficient mice. Science **261:**609–612.
60. **Tan, P., C. Anasetti, J. A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J. A. Ledbetter, and P. S. Linsley.** 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. J. Exp. Med. **177:**165–173.
61. **Tew, J. G., R. M. DiLosa, G. F. Burton, M. H. Kosco, L. I. Kupp, A. Masuda, and A. K. Szakal.** 1992. Germinal centers and antibody production in bone marrow. Immunol. Rev. **126:**99–112.
62. **Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe.** 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity **3:**541–547.
63. **Turka, L. A., P. S. Linsley, H. Lin, W. Brady, J. M. Leiden, R. Q. Wei, M. L. Gibson, X. G. Zheng, S. Myrdal, D. Gordon, et al.** 1992. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. Proc. Natl. Acad. Sci. USA **89:**11102–11105.
64. **Walunas, T. L., C. Y. Bakker, and J. A. Bluestone.** 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. J. Exp. Med. **183:**2541–2550.
65. **Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone.** 1994. CTLA-4 can function as a negative regulator of T cell activation. Immunity **1:**405–413.
66. **Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak.** 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science **270:**985–988.
67. **Weiss, S., and B. Bogen.** 1991. MHC class II-restricted presentation of intracellular antigen. Cell **64:**767–776.
68. **Wu, Y., and Y. Liu.** 1994. Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for CD4+ T-cell help in CD8+ T-cell responses. Curr. Biol. **4:**499–505.
69. **Xu, J., T. M. Foy, E. A. Elliot, J. J. Dunn, T. J. Waldschmidt, J. Elsemore, R. J. Noelle, and R. A. Flavell.** 1994. Mice deficient for the CD40 ligand. Immunity **1:**423–431.
70. **Zinkernagel, R. M.** 1996. Immunology taught by viruses. Science **271:**173–178.