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16. Nuclei were prepared by dounce homogenization of transgenic pre-B cells in nuclei isolation buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM tris-HCl (pH 7.4), 1 mM dithiothreitol] containing 0.15% NP-40, incubated with DNase I, and processed (*39*).
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33. The functionally rearranged genomic μ gene, μwt, has been described previously (*11*). For construction of ΔMAR, the 220-bp Hinf I fragment was modified by the addition of Xba I linkers and was inserted into Xba I–digested p1-27 (*35*) to create ΔMARμ-1. This substitution results in the removal of 344 bp and 426 bp from the 5′ end (X_1-H) and 3′ end (H-X_2) of the 1-kb, Eμ-containing X_{1-2} fragment, respectively. ΔMARμ-1 DNA was digested with Nde I (at −1) and Hpa I (at +2586), and the internal fragment was inserted into Nde I– and Hpa I–digested μwt to produce ΔMAR. For construction of μΔ4, the Hinf I sites flanking the 5′ and 3′ ends of the 220-bp enhancer core were converted to Not I and Eag I sites, respectively, to produce pμNE. After digestion of pμNE with Eag I, self-ligation of the large fragment yields μΔ4. For microinjection, plasmids were digested with Sal I and Xho I, and inserts were either excised from low melt agarose as described (*11*) or electroeluted in an Elutrap chamber (S & S), concentrated by butanol extraction, extracted with phenol, precipitated, and resuspended in 10 mM tris-HCl (pH 7.4) and 0.1 mM EDTA.
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35. For the detection of enhancer-proximal, Iμ transcripts, a single-stranded DNA probe 5′ end-labeled with ^{32}P was prepared by extending an oligonucleotide primer (BF-16, 5′-CAGAAGCCACAACCATACATTCCCA) that is complementary to a sequence 220 bp 3′ to X_2 with a Pst I–digested p1-27 template (containing a μwt fragment that extends from −18 to +2603): A linear polymerase chain reaction was performed by mixing 2.5 μl of heat-inactivated kinase reaction mixture, which contained 40 ng of the BF-16 oligonucleotide, with 2 μg of digested p1-27, 100 μM each of nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 5 units of Taq DNA polymerase (Promega), and 2 μl of 10× Taq buffer (supplied by manufacturer; containing 15 mM $MgCl_2$) in a final volume of 20 μl, and subjecting the mixture to 20 rounds of thermal cycling for 1 min each at 94°C, 55°C, and 72°C. Samples were applied to a 5% polyacrylamide gel containing 50% (w/v) urea. The appropriate gel slice was excised and the probe was purified by electroelution (Elutrap). mb-1 transcripts were detected with a single-stranded antisense DNA probe, which was synthesized in a linear polymerase chain reaction from Asp718-digested $pMB1_{T3}$ (which contains the mb-1 cDNA beginning with the AUG codon at +27) and a ^{32}P-labeled oligonucleotide primer, mb1-1 (5′-CACCGTCAGGGATGGTGGACC, extending from +127 to +147) [A. Travis, J. Hagman, R. Grosschedl, *Mol. Cell. Biol.* **11**, 5756 (1991)]. S1 reactions were performed as described (*11*).
36. Results shown in Fig. 1B, together with additional lanes containing 30 μg of the same RNA samples processed in parallel, were analyzed with a PhosphorImager (Molecular Dynamics). Relative levels of expression were determined by taking raw values corresponding to the amount of correctly initiated, transgene-specific mRNA, subtracting background, and dividing by the total level of expression in μwt line 4-1-4.
37. M12 B cells (2×10^7) [K. J. Kim *et al.*, *J. Immunol.* **122**, 549 (1979)] were subjected to electroporation (Bio-Rad Gene Pulser; 0.25 kV, 960 μF in 0.4-cm cuvettes) at room temperature in phosphate-buffered saline containing both 20 μg of plasmid DNA linearized at a unique Xho I site at the 3′ end of the gene and 1 μg of Eco RI–linearized SV2neo. After 24 hours, cells were diluted to a density of 10^5/ml with complete RPMI medium containing Geneticin (2 mg/ml) (Gibco), and 1-ml aliquots were seeded into 24-well plates. Clones were picked 10 to 14 days later and maintained in nonselective medium.
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Treatment of Murine Lupus with CTLA4Ig

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The interaction of B7-related molecules on antigen-presenting cells with CD28 or CTLA-4 antigens on T cells provides a second signal for T cell activation. Selective inhibition of the B7-CD28 or B7–CTLA-4 interactions produces antigen-specific T cell unresponsiveness in vitro and suppresses immune function in vivo. To determine whether selective inhibition of the B7-CD28 or B7–CTLA-4 interactions could suppress spontaneous autoimmune disease, a B7-binding protein was generated by genetic fusion of the extracellular domain of murine CTLA-4 to the Fc portion of a mouse immunoglobulin G2a monoclonal antibody (muCTLA4Ig). In lupus-prone NZB/NZW filial generation (F_1) mice, treatment with muCTLA4Ig blocked autoantibody production and prolonged life, even when treatment was delayed until the most advanced stage of clinical illness. These findings suggest a possible role for human CTLA4Ig in the treatment of autoimmune diseases in humans.

Systemic lupus erythematosus (SLE) is a life-threatening autoimmune disease that is characterized by the production of diverse autoantibodies (*1*). Some of these autoantibodies cause damage directly as a consequence of their specificity (for example, autoimmune hemolytic anemia is caused by antibodies to red blood cells), whereas other autoantibodies cause damage indirectly as a consequence of the formation and deposition of immune complexes (for example, immune complex glomerulonephritis is caused by antibodies to nuclear antigens). Other studies have shown that, both in humans with SLE and in murine models for SLE, the production of pathologic autoantibodies by B cells is dependent on stimulatory influences from $CD4^+$ T cells (*1*, *2*).

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NZB/NZW F_1 (B/W) mice spontaneously develop an autoimmune disease that closely resembles SLE in humans (*1*, *2*). In the past, in an effort to suppress T cell help for autoantibody production, we treated lupus-prone B/W mice with monoclonal antibodies (mAbs) to CD4 (*2*). Although this strategy is effective in murine lupus, trials in individuals with rheumatoid arthritis indicated that even brief therapy with mAbs to CD4 may cause long-lasting depletion of $CD4^+$ T cells in humans (*3*). An alternative strategy was suggested by studies that indicated T cell activation requires at least two signals (*4*–*9*). The first signal is provided by the interaction between the T cell receptor and antigenic peptides in the context of class II major histocompatibility complex antigens (MHC II) (*10*). This signal is augmented by the interaction between CD4 and MHC II, and it can be blocked by mAb to CD4 (*11*). The second signal is provided by other receptor-ligand pairs on T cells and antigen-presenting cells (APCs). In particular, the interactions between CD28 on T cells and B7 on activated B cells and other APCs are capable of providing this second signal (*5*–*9*). We undertook this study to determine whether selective inhibition of this interaction could suppress murine lupus in B/W mice.

Studies have shown that the CD28-B7 interaction can be inhibited by mAb to either CD28 or B7 (*12*). Under appropriate in vitro conditions, this approach can induce antigen-specific T cell anergy (*9*, *12*). Attempts to extend this observation to in vivo systems have focused on a somewhat different strategy that takes advantage of the homology between CD28 and CTLA-4 (*13*, *14*). Like CD28, CTLA-4 is a member of the immunoglobulin (Ig) superfamily (*13*). It is expressed on activated T cells, and it binds B7 with higher avidity than does CD28 (*14*). A protein encoded by genetic fusion of the extracellular domain of human CTLA-4 to an immunoglobulin Cγ1 chain (CTLA4Ig) binds to B7 (*12*) and to a molecule similar to B7 (*15*–*17*). CTLA4Ig has been used successfully in vivo to prolong acceptance of tissue allografts, to inhibit B cell differentiation into Ig-secreting cells, and to suppress antibody responses to T-dependent antigens (*18*–*20*). Despite the success of CTLA4Ig in blocking primary T-dependent immune responses, studies have suggested that it would not prevent secondary immune responses (*20*). When treatment was delayed for as little as 3 days after immunization, immune responses were not blocked (*20*).

We attempted to suppress murine lupus with injections three times per week of the human CTLA4Ig fusion protein. In these experiments, mice had an immune response to the fusion protein, and there was no beneficial effect on autoimmunity. Therefore, to circumvent the problem of host immunity to therapy, we produced a murine CTLA4Ig fusion protein composed of the extracellular domain of murine CTLA-4 linked to a murine Ig Cγ2a chain (muCTLA4Ig). The purified recombinant protein bound murine B7 with high avidity, blocked binding of CD28 to B7, and inhibited T cell activation in vitro (*21*).

We first treated groups of 15 5-month-old female B/W mice with either muCTLA4Ig [50 μg, intraperitoneally (IP) three times per week for 4 months] or L6, a control mouse IgG2a mAb against a human carcinoma antigen (*22*). Concentrations of muCTLA4Ig in sera were determined by a $B7^+$ CHO cell binding assay (*7*). This assay established that the dosing regimen maintained serum concentrations of ≥10 μg/ml. Treatment with muCTLA4Ig retarded the progression of murine lupus. Mice treated with muCTLA4Ig did not make antibodies to double-stranded DNA (dsDNA) at any time during the experiment, including the 3 months of observation after treatment was stopped (Fig. 1A). In contrast, control mice produced antibodies to dsDNA beginning at age 6 months. The geometric mean titers of antibodies to dsDNA in the two groups differed significantly from age 6 months to age 10 months ($P < 0.01$, by t test). After age 10 months, there were too few mice remaining in the control group for statistical comparison (Fig. 1).

Suppression of autoantibody production was accompanied by a significant reduction in the severity of lupus nephritis. When therapy was completed at age 9 months, the frequency of significant renal disease, defined as proteinuria ≥100 mg/dl, was 13% in mice treated with muCTLA4Ig, as compared with 87% in the control mice ($P < 0.01$, by χ^2 test). This improvement coincided with a significant improvement in survival (Fig. 1B). At 9 months of age, 93% of mice treated with muCTLA4Ig were still alive, as compared with only 40% of mice treated with L6 ($P < 0.01$, by χ^2 test). The benefit of muCTLA4Ig use persisted even after treatment was discontinued at age 9 months. During the ensuing 3 months after cessation of therapy, none of the mice that had received muCTLA4Ig died, whereas all but one of the mice in the control group died. Serum concentrations of muCTLA4Ig declined to <0.2 μg/ml within 2 months after treatment. Thus, the beneficial effects of muCTLA4Ig persisted despite declining serum concentrations.

To establish that our findings represented

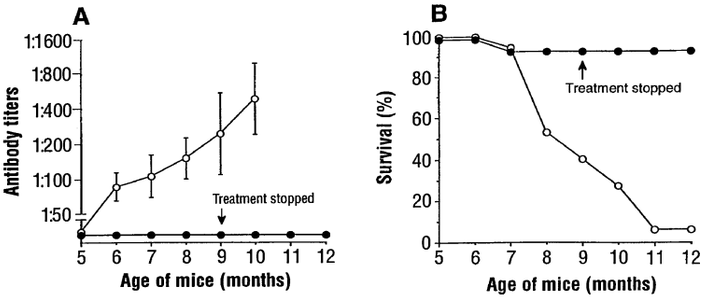

Fig. 1. (**A**) Effect of treatment with muCTLA4Ig on the titer (geometric mean ± SEM) of antibodies to dsDNA. Two groups of 15 female B/W mice were housed at the San Francisco VA Medical Center. The mice were treated with either muCTLA4Ig (closed circles) or L6 (open circles) from age 5 months to age 9 months (50 μg, IP three times per week). We analyzed sera from individual mice monthly during treatment and for 3 months thereafter, using an enzyme-linked immunosorbent assay (ELISA) as described (*2*). In this assay, the titer of antibodies to dsDNA in normal mouse serum was <1:50. (**B**) Effect of treatment with muCTLA4Ig on survival. Mice treated with muCTLA4Ig (closed circles); mice treated with L6 (open circles).

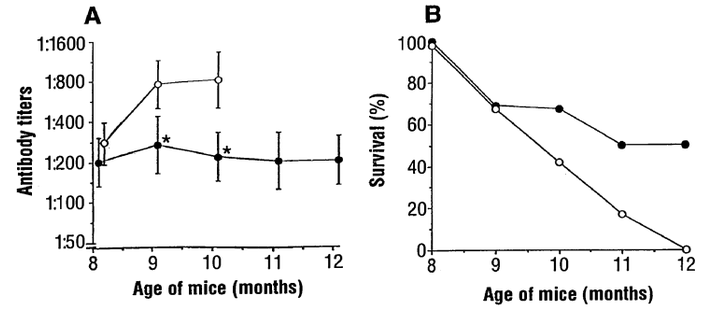

Fig. 2. (**A**) Effect of late treatment with muCTLA4Ig on the titer (geometric mean ± SEM) of antibodies to dsDNA. Forty female B/W mice were monitored until, at age 8 months, 40% of the original cohort had died from murine lupus and 50% of the surviving mice had proteinuria >100 mg/dl. At that time, two groups of the surviving mice (12 per group) began treatment with either muCTLA4Ig (closed circles) or L6 (open circles) for 4 months (50 μg, IP three times per week). Statistically significant differences between groups are denoted by asterisks. After 10 months of age, there were too few surviving control mice for further statistical comparison. Sera were analyzed as in Fig. 1A. (**B**) Effect of muCTLA4Ig on survival after initiation of treatment at age 8 months.

treatment, rather than prevention, of active autoimmunity, we tested muCTLA4Ig in the late stages of clinical illness. A cohort of 40 female B/W mice was monitored until age 8 months, by which time 40% of the mice had died from murine lupus. Then, at this advanced stage of disease, we began treating the remaining mice with either muCTLA4Ig or L6 (50 μg, IP three times per week). At initiation of therapy, the mean titer of antibodies to dsDNA was >1:200 (titer in normal mouse serum was <1:50), and 50% of the mice had proteinuria >100 mg/dl. Despite the severity of the underlying illness, treatment blocked further autoantibody production (Fig. 2A) and prolonged life (Fig. 2B). After 4 months of therapy, all of the control mice had died, whereas 50% of the treated mice were still alive ($P < 0.02$, by χ^2 test). Thus, muCTLA4Ig can suppress an established, pathological immune response. This is the latest stage at which any intervention has been shown to be effective in B/W mice.

Because selective inhibition of the B7-CD28 interaction can cause sustained antigen-specific T cell unresponsiveness in vitro (*9*, *12*), it has been postulated that CTLA4Ig might cause antigen-specific T cell tolerance in vivo. If this were true, it might be possible to achieve sustained inhibition of autoantigen-driven responses, even without specific knowledge of the inciting autoantigens. In our first experiment, production of antibodies to dsDNA was suppressed for 3 months after cessation of therapy (Fig. 1A); it is possible, therefore, that this suppression is evidence for this hypothesis. However, our studies were not designed to establish conclusively whether in vivo tolerance was in fact achieved.

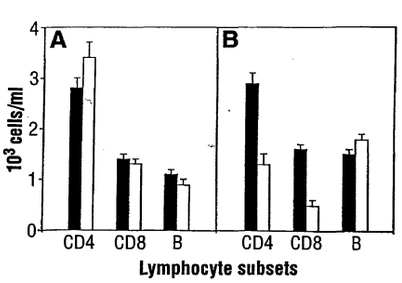

Fig. 3. Effect of treatment with muCTLA4Ig on peripheral blood lymphocyte subsets. Absolute lymphocyte counts from individual mice (12 mice per group) were obtained by means of an automated cell counter (Technicon Instruments, Tarrytown, New York). Peripheral blood lymphocytes subsets were then analyzed as described (*2*): A fluorescein-conjugated mAb to B220 (hybridoma RA3.6B2) detected B cells, mAb to CD4 (hybridoma GK1.5) detected $CD4^+$ T cells, and mAb to CD8 (hybridoma 53-6) detected $CD8^+$ T cells. The data shown here indicate the mean cell counts (± SEM) per milliliter of sera in mice treated with muCTLA4Ig (**A**) and in control mice treated with L6 (**B**) immediately before therapy at age 5 months (dark bars) and at the completion of therapy at age 9 months (white bars).

There are several mechanisms through which muCTLA4Ig might suppress murine lupus. It may exert its beneficial effects by binding to B7-related molecules on activated B cells and other APCs, preventing their interaction with CD28 and thus inhibiting the second signal for T cell activation. Alternatively, muCTLA4Ig may inhibit the function of activated T cells by blocking the interaction between CTLA-4 on activated T cells and B7 or a related ligand, B7-2 (*15–17*).

Beyond its effects on T cell function, muCTLA4Ig may suppress autoimmunity through direct effects on B cells. In particular, because murine IgG2a binds complement, muCTLA4Ig may effectively inhibit autoantibody production by depleting activated B cells. To assess this possibility, we monitored peripheral blood lymphocyte subsets (Fig. 3). These studies demonstrated that treatment with muCTLA4Ig did not significantly alter the absolute number of circulating B cells, suggesting that muCTLA4Ig did not suppress autoimmunity simply by the depletion of B cells. These findings are supported by studies in mice transgenic for CTLA4Ig that also imply that CTLA4Ig may not inhibit immune function by directly effecting B cells or by blocking the induction of T cell responses, but rather by blocking T cell effector functions, such as T cell help for B cells (*23*, *24*).

Treatment with muCTLA4Ig not only spared B cells, but it also prevented the progressive T cell lymphopenia that accompanies murine lupus in B/W mice (*2*). In control mice, there was an age-dependent decline in the absolute number of circulating T cells, which did not occur in mice treated with muCTLA4Ig (Fig. 3). This preservation of a stable lymphocyte profile throughout therapy most likely reflects amelioration of the underlying disease.

Attempts to suppress autoimmunity with biological agents have encountered two principal obstacles. First, treatment may have long-lasting adverse effects on the immune system, as has been seen in trials of antibodies to CD4 that caused prolonged depletion of $CD4^+$ T cells in people with rheumatoid arthritis (*3*). This observation raised questions about the long-term immune competence of treated individuals, as well as the feasibility of repeated courses of therapy. Our findings suggest that CTLA4Ig may circumvent this problem. Because muCTLA4Ig does not deplete lymphocytes, the effects of therapy on overall immune competence may be limited to the duration of therapy. Although this remains to be proven in B/W mice, studies in other strains indicate that, within a short time after cessation of therapy with muCTLA4Ig, the immune system is competent to mount normal immune responses (*20*). This may be particularly important in SLE because SLE is a disease with spontaneous remissions and relapses. Therefore, it is desirable to develop therapeutic strategies that might be used during periods of relapse without causing adverse effects that extend into periods of relative remission.

The second major obstacle to the use of biologic agents for chronic autoimmune diseases stems from the fact that many of these agents, such as mAbs or toxin conjugates, elicit a host immune response that can interfere with efficacy or cause toxicity (*25*). The use of CTLA4Ig may limit this problem. As a fusion product of two autologous proteins, CTLA4Ig appears to be poorly immunogenic. In B/W mice, for example, muCTLA4Ig elicited only a weak immune response that occurred after cessation of therapy, whereas human CTLA4Ig elicited an immediate and potent immune response (*26*). Consistent with these findings, human CTLA4Ig was cleared quickly from B/W mice and was ineffective against murine lupus, whereas muCTLA4Ig maintained high serum concentrations and succeeded in suppressing autoimmune disease.

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