cell cycle and abolished the second mitotic wave. The arrested cells were not forced to differentiate along any single pathway. Rather, they could respond to physiological differentiation signals and could adopt a variety of cell fates as they were sequentially recruited to the developing ommatidia. Hence, the expression of p21 does not drive differentiation, but rather maintains precursor cells in a state that permits differentiation. In cultured mammalian cells and in mouse embryos, p21 is expressed in cells as they cease to proliferate and begin to differentiate (*14*). Thus, p21 is likely to function in the developmental switch from proliferation to differentiation.

Although p21 homologs have not yet been found in nonmammalian species, homologs of the cyclins and cyclin-dependent kinases have been found in *Drosophila* (*15*). The ability of human p21 to inhibit *Drosophila* cells from entering S phase suggests that the mechanisms of inhibition of the cyclin-dependent kinases are general and conserved across species. Finally, the ability of human p21 to function in diverse species indicates its utility as an experimental tool for arresting the division of specific populations of cells in vivo.

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Lymphoproliferative Disorders with Early Lethality in Mice Deficient in *Ctla-4*

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The role of the cell-surface molecule CTLA-4 in the regulation of T cell activation has been controversial. Here, lymph nodes and spleens of CTLA-4–deficient mice accumulated T cell blasts with up-regulated activation markers. These blast cells also infiltrated liver, heart, lung, and pancreas tissue, and amounts of serum immunoglobulin were elevated. The mice invariably became moribund by 3 to 4 weeks of age. Although CTLA-4–deficient T cells proliferated spontaneously and strongly when stimulated through the T cell receptor, they were sensitive to cell death induced by cross-linking of the Fas receptor and by gamma irradiation. Thus, CTLA-4 acts as a negative regulator of T cell activation and is vital for the control of lymphocyte homeostasis.

The CTLA-4 receptor has been postulated to play a regulatory role in T cell activation largely on the basis of its similarity to the costimulatory receptor CD28 (*1*). Mouse *Ctla-4* complementary DNA (*2*) is 76% identical in sequence to CD28, and both are located in close proximity on chromosome 1 (*3*). CD28 is found on essentially all resting T cells and can augment the response of antigen-activated T cells (*1*). In contrast, CTLA-4 is not expressed on resting T cells but is detectable on activated T cells after antigen activation (*4*). Although a role for CD28 in augmenting T cell–dependent responses is well established, antibody blocking or cross-linking of CTLA-4 has yielded contradictory results (*4*, *5*). Interpretation of these studies is complicated by the fact that CD28 and CTLA-4 bind to the same ligands B7-1 (CD80) and B7-2 (CD86) (*6*). The shared ligands and the complex expression patterns of both receptors and ligands make it difficult to address definitively the role of CTLA-4. Here, we show that gene-targeted mice lacking CTLA-4 progressively accumulate T cell blasts, which indicates that CTLA-4 is a negative regulator.

A gene-targeting construct (Fig. 1A) was generated (*7*). The construct was electroporated into E14 embryonic stem (ES) cells, the targeted ES clones (Fig. 1B) injected into C57BL/6 blastocysts, and the resulting chimeras bred to C57BL/6 females. Tail DNA of agouti pups showed transmission of the targeted allele (Fig. 1B). T cells were double-stained for αβ T cell receptor (TCRαβ) and either CTLA-4 or one of the activation markers CD25, CD44, or CD69. Activated T cells from *Ctla-4*$^{-/-}$ mice do not express *Ctla-4* but had high levels of expression of these activation markers (Fig. 1C).

Mice heterozygous for the *Ctla-4* mutation appeared normal. Homozygous mice were born at the expected Mendelian frequency and appeared healthy at birth. However, by 2 weeks of age they became sick and became moribund at 3 to 4 weeks of age. The spleen and all lymph nodes were 5 to 10 times the normal size, reflected by an increase in the number of lymphocytes (Table 1). Histology revealed extensive accumulation of activated lymphocytes within lymph nodes, thymus, and the splenic white pulp, which obscured the cortico-medullary com-

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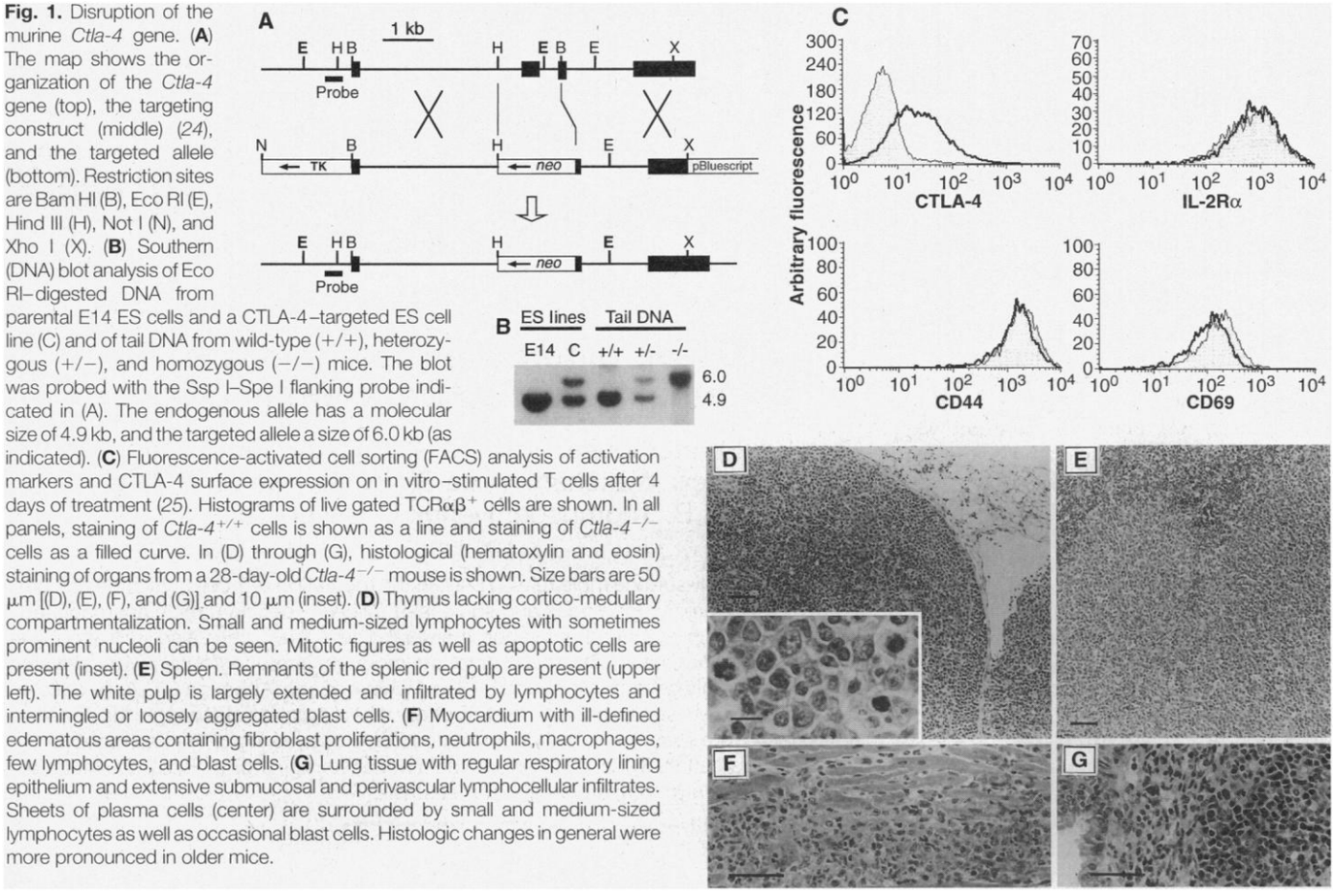

Fig. 1. Disruption of the murine *Ctla-4* gene. (**A**) The map shows the organization of the *Ctla-4* gene (top), the targeting construct (middle) (*24*), and the targeted allele (bottom). Restriction sites are Bam HI (B), Eco RI (E), Hind III (H), Not I (N), and Xho I (X). (**B**) Southern (DNA) blot analysis of Eco RI–digested DNA from parental E14 ES cells and a CTLA-4–targeted ES cell line (C) and of tail DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. The blot was probed with the Ssp I–Spe I flanking probe indicated in (A). The endogenous allele has a molecular size of 4.9 kb, and the targeted allele a size of 6.0 kb (as indicated). (**C**) Fluorescence-activated cell sorting (FACS) analysis of activation markers and CTLA-4 surface expression on in vitro–stimulated T cells after 4 days of treatment (*25*). Histograms of live gated TCRαβ$^+$ cells are shown. In all panels, staining of *Ctla-4*$^{+/+}$ cells is shown as a line and staining of *Ctla-4*$^{-/-}$ cells as a filled curve. In (D) through (G), histological (hematoxylin and eosin) staining of organs from a 28-day-old *Ctla-4*$^{-/-}$ mouse is shown. Size bars are 50 μm [(D), (E), (F), and (G)] and 10 μm (inset). (**D**) Thymus lacking cortico-medullary compartmentalization. Small and medium-sized lymphocytes with sometimes prominent nucleoli can be seen. Mitotic figures as well as apoptotic cells are present (inset). (**E**) Spleen. Remnants of the splenic red pulp are present (upper left). The white pulp is largely extended and infiltrated by lymphocytes and intermingled or loosely aggregated blast cells. (**F**) Myocardium with ill-defined edematous areas containing fibroblast proliferations, neutrophils, macrophages, few lymphocytes, and blast cells. (**G**) Lung tissue with regular respiratory lining epithelium and extensive submucosal and perivascular lymphocellular infiltrates. Sheets of plasma cells (center) are surrounded by small and medium-sized lymphocytes as well as occasional blast cells. Histologic changes in general were more pronounced in older mice.

partmentalization of the thymus and zonal distribution of T cell– and B cell–dependent areas in the spleen and lymph nodes (Fig. 1, D and E). Diffuse and focal lymphocyte infiltration was prominent in heart, lung, bone marrow, liver, and pancreas tissue but not in the kidneys (Fig. 1, F and G) (*8*). Fresh and older myocardial infarctions were prominent, with granular tissue formation. These mice probably die of myocardial failure as a result of these lesions.

We analyzed the phenotypes of thymocytes (9) and peripheral T cells. The surface markers on TCRαβ T cells isolated from enlarged lymph nodes showed up-regulation of the activation markers CD44 and CD25 [interleukin-2 receptor α (IL-2Rα)] and down-regulation of MEL-14 (Fig. 2A). Increased expression levels were also seen for the CD5, CD28, and CD69 molecules on *Ctla-4*$^{-/-}$ T cells in lymph node and spleen (*10*). Both CD4 and CD8 populations contained activated cells and showed no change in the CD4/CD8 ratio (*10*). The TCRαβ profiles in the bone marrow of *Ctla-4*$^{-/-}$ mice showed massive expansion of activated T cells (Fig. 2B). The relative percentages of V_β6-, V_β8-, V_β14-, and V_α2-positive T cells in *Ctla-4*–deficient animals was similar to that in littermates (*10*), which suggests that the increase in lymphocytes was not a clonal expansion of a small portion of the T cell repertoire.

B cells from lymph node and spleen also showed in vivo activation with up-regulation of B7-2, but not B7-1, molecules (Fig. 3A) (*6*, *11*). The Fas antigen and CD5 were up-regulated on B cells from *Ctla-4*$^{-/-}$ mice (*12*, *13*). An expanded population of activated B cells is also consistent with higher basal serum immunoglobulin (Ig) levels seen in *Ctla-4*$^{-/-}$ mice (Fig. 3B). All of the Ig subtypes measured showed higher serum levels in *Ctla-4*$^{-/-}$ mice, ranging from 10-fold for IgG2a, IgG2b, IgG3, and IgM, to 100-fold for IgG1 and IgA, to several thousandfold for IgE.

Ex vivo lymphocytes from *Ctla-4*$^{-/-}$ mice proliferated in amounts equal to those in littermate controls in response to treatment with concanavalin A (Con A). T cells from *Ctla-4*$^{-/-}$ mice, however, exhibited consistently higher proliferation rates in response to soluble antibody to CD3 (anti-CD3) or soluble anti-TCRαβ (Fig. 4A). Purified T cells from *Ctla-4*$^{-/-}$ mice also displayed spontaneous proliferation in medium alone (Fig. 4A, inset), which declined after 24 hours (*14*). Thus, ex vivo spontanous proliferation reflects in vivo lymphoproliferation in *Ctla-4*$^{-/-}$ mice. These data imply that *Ctla-4*$^{-/-}$ T cells have a less stringent requirement for proliferation in response to stimuli that either activate or mimic T cell receptor signals.

Activated T cells are susceptible to Fas receptor–induced apoptosis (*15*). We analyzed the susceptibility of freshly isolated lymph node T cells to Fas cross-linking. Our results show that ex vivo T cells from *Ctla-4*$^{-/-}$ mice were still susceptible to Fas-mediated cell death in the presence or

Table 1. Increase in size and lymphocyte numbers of lymph nodes and spleens of *Ctla-4*$^{-/-}$ mice. The organs were isolated from 4-week-old littermates and pooled and the weight and lymphocyte number per animal determined (n = 4, *Ctla-4*$^{+/+}$; n = 6, *Ctla-4*$^{+/-}$). The weights and cell numbers for two individual *Ctla-4*$^{-/-}$ mice are shown.

Genotype	Wet weight (mg)		Lymphocytes (10^7)	
	Lymph nodes	Spleen	Lymph nodes	Spleen
Ctla-4$^{+/+}$	71	69	1.3	3.1
Ctla-4$^{+/-}$	97	77	1.7	3.1
Ctla-4$^{-/-}$	540	145	28.0	7.7
Ctla-4$^{-/-}$	380	501	12.0	16.5

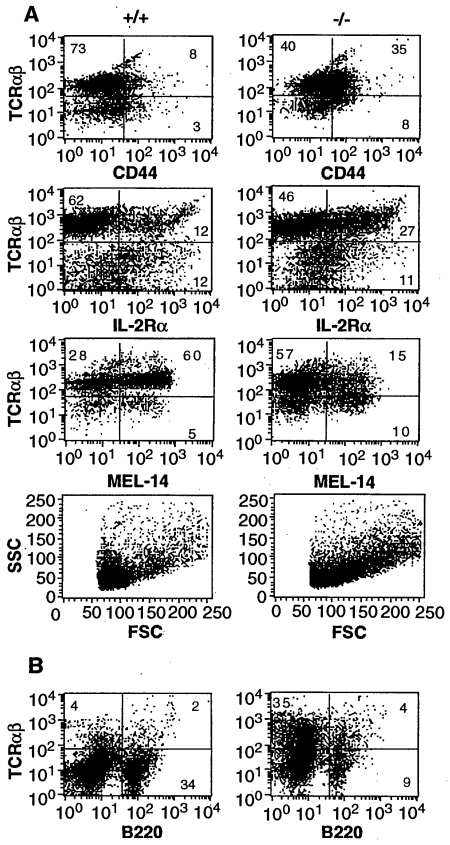

Fig. 2. FACS analysis of surface markers on T lymphocytes from the lymph nodes of 24-day-old *Ctla-4*$^{-/-}$ (−/−) and wild-type (+/+) littermates. (**A**) Lymph node cells were double-stained with anti-TCRαβ (FITC) and either anti-CD44 (PE), anti–MEL-14 (PE), or anti–IL-2Rα (biotin) (*10*). Staining combinations are indicated; cells analyzed are shown in side (SSC) and forward (FSC) scatter profiles. These profiles also show the increased proportion of blast cells in *Ctla-4*$^{-/-}$ mice. (**B**) Staining profile of lymphocytes in the bone marrow of 24-day-old mice. Cells were double-stained with anti-TCRαβ (PE) and anti-B220 (FITC) (*10*). The percent of analyzed cells is shown in each quadrant.

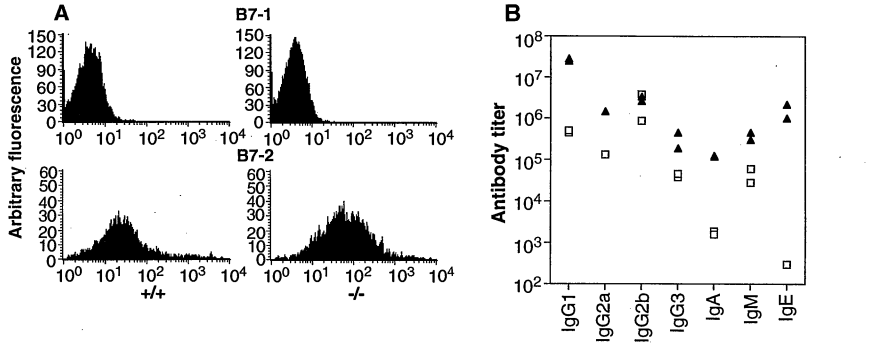

Fig. 3. Activation of B cells. (**A**) Cells isolated from lymph node were stained with anti-B220 (FITC) and antibodies to either B7-1 or B7-2 (PE) (*10*). Histograms of B220^{+} cells are shown. (**B**) Elevated basal levels of Igs in *Ctla-4*$^{-/-}$ mice. Specific Ig levels in sera from individual 4-week-old littermates were determined as described (*7*). Two mice from each group were analyzed in duplicate. Triangles, *Ctla-4*$^{-/-}$ mice; squares, *Ctla-4*$^{+/+}$ mice.

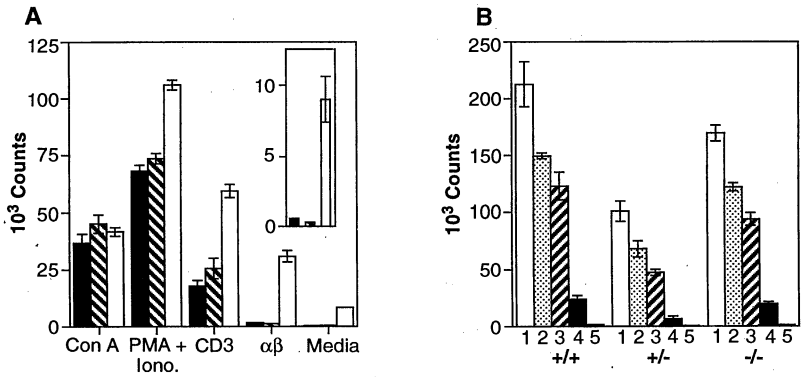

Fig. 4. In vitro proliferative responses. (**A**) Lymphocytes isolated from lymph nodes of CTLA-4$^{+/+}$ (solid bars), CTLA-4$^{+/-}$ (hatched bars), or CTLA-4$^{-/-}$ (open bars) littermates were cultured for 48 hours in medium alone or in medium containing anti-TCRαβ (1 μg/ml), anti-CD3 (1 μg/ml), Con A (2.5 μg/ml), or phorbol 12-myristate 13-acetate (PMA) (12 ng/ml) and calcium ionophore (Iono.) A23617 (50 ng/ml). Cells were pulsed with 1 μCi of [^{3}H]thymidine for 8 hours. (**Inset**) Proliferation of ex vivo CTLA-4$^{-/-}$ T cells. T cells from the lymph nodes of 3-week-old CTLA-4$^{-/-}$, CTLA-4$^{+/-}$, or CTLA-4$^{+/+}$ littermates were cultured in medium alone (4 hours, 37°C) and then pulsed with [^{3}H]thymidine. (**B**) Responses of CTLA-4$^{-/-}$ T cells to gamma irradiation. T cells from CTLA-4$^{-/-}$ mice or wild-type (+/+) or heterozygous (+/−) littermates were irradiated with 0 Gy (1); 1 Gy (2); 2 Gy (3); 5 Gy (4); or 10 Gy (5) and cultured for 72 hours with anti-CD3 (1 μg/ml) and IL-2 (20 U/ml). Cells were pulsed with [^{3}H]thymidine.

absence of anti-CD3 plus IL-2 (*16*). In vitro–stimulated T cells from *Ctla-4*$^{+/+}$ and *Ctla-4*$^{-/-}$ mice also displayed the same kinetics of survival when cultured in media alone (*17*). In addition, 1 to 10 Gy (gray) of gamma irradiation (*18*) produced similar decreases in mitogen-induced proliferation of T cells in mutant, heterozygote, and wild-type littermates (Fig. 4B). These results indicate that CTLA-4 has no obligatory function in cell death induced by either Fas or DNA damage.

It has been difficult to determine conclusively whether CTLA-4 functions as a costimulatory molecule or a negative regulator of T cell activation (*1*–*6*). Here, T cells from *Ctla-4* mutant animals were activated and proliferated in vivo. Thus, CTLA-4 plays an important role in regulating T cells that are continuously activated as well as in lymphocyte expansion. These data, together with the finding that CD28-deficient animals are nonresponsive to certain antigen stimulation (*7*), clearly demonstrate that although CD28 augments immune responses, its structurally related counterpart CTLA-4 acts as a negative regulator of T cells.

Our data do not preclude a role for CTLA-4 in thymic selection. CD28 can provide a costimulatory signal for TCRαβ-mediated apoptosis of CD4^{+}CD8^{+} immature thymocytes (*19*), and expression of B7 ligands on thymic epithelial cells is correlated with the functional ability of these cells to mediate positive or negative selection (*20*). However, the development of thymocytes is normal in CD28$^{-/-}$ (*7*) and B7-1$^{-/-}$ mice (*11*), and T cell selection could not be blocked by inhibiting CD28-B7 costimulatory signals in normal mice (*21*). In addition, expression of CTLA-4 is not detectable in thymocytes (*22*).

The marked infiltration of lymphocytes into various organs of *Ctla-4*$^{-/-}$ mice is reminiscent of lymphoproliferative disease. A similar but less severe lymphoproliferation can be observed in mice with a mutation in Fas (*12*). Interestingly, we found no obligatory involvement of CTLA-4 in cell death induced by Fas or irradiation. The recent report that TGFβ$^{-/-}$ (transforming growth factor β) mice also develop lymphoproliferative disorders (*23*) indicates that there are multiple mechanisms that coordinately work to negatively regulate lymphocyte expansion. Our data show that CTLA-4 is a key molecule in the regulation of lymphocyte homeostasis.

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8. Liver, kidney, and pancreas tissue was analyzed with the use of conventional histology. Focal infiltrations were seen in liver and bone marrow but were inconspicuous in kidney tissue. Lymphocyte infiltration of the pancreas was diffuse or follicular. The β islets were largely destroyed, and the accompanying atrophy and fibrosis of the exocrine parenchyma were reminiscent of chronic pancreatitis.
9. Within the thymuses of *Ctla-4*$^{-/-}$ mice, both CD4 and CD8 single-positive populations were increased fourfold. The proportion of double-positive cells was reduced, resulting in a smaller thymus. The expression of the maturation markers CD69, CD5, and CD44 was similar between *Ctla-4*$^{+/+}$, *Ctla-4*$^{+/-}$, and *Ctla-4*$^{-/-}$ mice. For immunofluorescence staining, see (*10*).
10. Increased mean staining intensity of markers on *Ctla-4*$^{-/-}$ αβ T cells in lymph node and spleen included CD5 (threefold), CD28 (two- to threefold), and CD69 (eightfold). The CD4$^+$/CD8$^+$ ratio remained 3/1 in *Ctla-4*$^{-/-}$ mice, and both CD4$^+$ and CD8$^+$ populations showed up-regulation of CD25, CD44, and CD69. For staining, cells were purified and stained as described [J. Penninger *et al.*, *Science* **260**, 358 (1993)]. For detection of specific TCR V_β and V_α expression, lymph node T cells (from two individual *Ctla-4*$^{-/-}$ mice and four to five pooled *Ctla-4*$^{+/+}$ or *Ctla-4*$^{+/-}$ 4-week-old littermates) were double-stained with anti–Thy-1.2 [phycoerythrin (PE) or fluorescein isothiocyanate (FITC)] and anti-TCR V_β6 (FITC), anti-V_β8 (PE), anti-V_β14 (FITC), or anti-V_α2 (PE). All antibodies were purchased from PharMingen.
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13. The double-positive populations increased for CD5 (threefold) and Fas (fivefold) B220-positive cells. The mean staining intensity of CD5 increased two- to threefold and of Fas increased fivefold on B220-positive cells.
14. Spontanous proliferation of *Ctla-4*$^{-/-}$ T cells decreased to 2000 cpm at 24 hours and to background levels at 48 hours. [^{3}H]Thymidine incorporation in lymph node–derived T cells from a 10-day-old *Ctla-4*$^{-/-}$ mouse was 1300 cpm (12 hours) and background levels at 24 hours.
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16. Purified T cells (2×10^6 cells/ml) from *Ctla-4*$^{-/-}$ mice were cultured with or without anti-CD3 (1 μg/ml) plus IL-2 (20 U/ml) and with or without 1 μg/ml of anti-Fas (*12*). T cells showed a 30% reduction in viable cells (stained with Trypan blue) after 24 hours of culture with anti-Fas in the presence or absence of mitogenic stimulation. Fas expression on ex vivo T cells was the same for *Ctla-4*$^{+/+}$ and *Ctla-4*$^{-/-}$ mice. Four days of mitogenic stimulation is necessary to render T cells sensitive to Fas-triggered death (*15*).
17. T cells were stimulated for 3 days (with 1 μg/ml of anti-CD3 and 20 U/ml of IL-2) and washed, and 4×10^6 viable cells were further cultured in media without mitogenic stimuli. The number of viable cells was then determined by Trypan blue staining after 24 and 48 hours of culture. Cell numbers after 24 hours were as follows: 1.6×10^6 for *Ctla-4*$^{+/+}$ and 1.5×10^6 for *Ctla-4*$^{-/-}$ T cells; after 48 hours: 0.5×10^6 for *Ctla-4*$^{+/+}$ and 0.6×10^6 for *Ctla-4*$^{-/-}$ T cells.
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24. The targeting construct consisted of the neomycin (*neo*) cassette, the 5′ and 3′ CTLA-4 arms, and the thymidine kinase (TK) cassette into pBluescript. The vector was introduced in E14 ES cells by electroporation and cultured in the presence of G418 and ganciclovir (*7*).
25. T cells isolated from 21-day-old *Ctla-4*$^{+/+}$ or *Ctla-4*$^{-/-}$ littermates were cultured in 10-cm plates coated with hamster antibody to mouse CD3ε. Con A supernatant was added after 24 hours. Cells on day 4 were stained with anti-TCRαβ (FITC-labeled) and anti–CTLA-4 (PE-labeled) or antibodies to CD25, CD44, or CD69 (all biotinylated) (*10*).
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Genomic Structure of an Attenuated Quasi Species of HIV-1 from a Blood Transfusion Donor and Recipients

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A blood donor infected with human immunodeficiency virus–type 1 (HIV-1) and a cohort of six blood or blood product recipients infected from this donor remain free of HIV-1–related disease with stable and normal CD4 lymphocyte counts 10 to 14 years after infection. HIV-1 sequences from either virus isolates or patient peripheral blood mononuclear cells had similar deletions in the *nef* gene and in the region of overlap of *nef* and the U3 region of the long terminal repeat (LTR). Full-length sequencing of one isolate genome and amplification of selected HIV-1 genome regions from other cohort members revealed no other abnormalities of obvious functional significance. These data show that survival after HIV infection can be determined by the HIV genome and support the importance of *nef* or the U3 region of the LTR in determining the pathogenicity of HIV-1.

Among people infected with HIV-1 there are some who, even after infection for 10 years or more, remain healthy with no signs of clinical progression to acquired immunodeficiency syndrome (AIDS) and have stable, normal CD4 lymphocyte counts (*1*–*5*). The explanation for the benign course of HIV-1 infection in such long-term nonprogressors (LTNPs) may be stochastic or related to host or viral factors or a combination of both (6).

In 1989, a review of the registry of individuals in New South Wales with blood transfusion–transmitted HIV infection revealed that 6 years after transfusion two infected recipients and a common donor were asymptomatic, with normal CD4 counts. A total of seven HIV-1–infected recipients of HIV-1–infected blood from the same donor (D36, a sexually active homosexual male who became infected between December 1980 and April 1981) have been found (Table 1) (*1*, *7*) among recipients of components of units donated by D36 between 3 February 1981 and 24 July 1984 (8). The donor and recipients (hereafter referred to as the

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