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Impaired Negative Selection of T Cells in Hodgkin's Disease Antigen CD30–Deficient Mice

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Summary

CD30 is found on Reed–Sternberg cells of Hodgkin's disease and on a variety of non-Hodgkin's lymphoma cells and is up-regulated on cells after Epstein–Barr virus, human T cell leukemia virus, and HIV infections. We report here that the thymus in CD30-deficient mice contains elevated numbers of thymocytes. Activation-induced death of thymocytes after CD3 cross-linking is impaired both in vitro and in vivo. Breeding the CD30 mutation separately into αβTCR- or γδTCR-transgenic mice revealed a gross defect in negative but not positive selection. Thus, like TNF-receptors and Fas/Apo-1, the CD30 receptor is involved in cell death signaling. It is also an important coreceptor that participates in thymic deletion.

Introduction

CD30 was originally identified on mononucleated Hodgkin's (H) and multinucleated Reed–Sternberg (RS) cells in Hodgkin's disease (HD) (Schwab et al., 1982; Stein et al., 1982). Over the past decade, CD30 has also been found on malignant cells from numerous non-HD lymphomas and other malignancies. It is also expressed on some virus-infected cells. The presence of CD30 under this broad range of pathological conditions has thus suggested a role for CD30 in disease.

CD30 is a 120 kDa type I cell surface glycoprotein that also exists in some situations in the plasma as a soluble 84 kDa protein (Froese et al., 1987; Nawrocki et al., 1988). The extracellular domain of CD30 shares sequence similarity with other tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily members, including p55 and p75 TNF receptors, the low affinity NGF receptor, 4-1BB, OX40, CD27, CD40, and the Fas/Apo-1 antigen (Dürkop et al., 1992). All family members are transmembrane proteins that contain extracellular cysteine-rich repeats (Dürkop et al., 1992). However, there is no sequence similarity in the cytoplasmic region between CD30 and other TNF superfamily members, and the cytoplasmic domain of CD30 does not appear to contain the death domain of Fas or other TNF receptor (TNFR) molecules (Cleveland and Ihle, 1995; Gribskov et al., 1987). The CD30 gene is also found on the human chromosome 1p36 in close proximity to other TNFR superfamily members, such as TNFR2 and OX40 (Fonatsch et al., 1992; Kemper et al., 1991; Latza et al., 1994).

CD30 is expressed on virtually all H and RS cells and on anaplastic large cell lymphomas (ALCLs) and is also expressed on some peripheral T cell lymphomas, adult T cell leukemias/lymphomas, immunoblastic lymphomas, and other non-HD lymphomas (Pallesen, 1990; Stein et al., 1985; Suchi et al., 1987; Piris et al., 1990). CD30 expression is not limited to cells of hematopoietic origin. It is also found on various embryonal carcinomas, non-embryonal carcinomas, malignant melanomas, and mesenchymal tumors (Pallesen and Hamilton-Dutoit, 1988; Pallesen, 1990; Mechtersheimer and Moller, 1990). In addition, CD30 has been detected on $CD8^+$ and $CD4^+$ T cells from patients with human immunodeficiency virus (HIV) infection and on $CD4^+$ T cells from allergic patients (Manetti et al., 1994; Maggi et al., 1994; Pizzolo et al., 1994; Del Prete et al., 1995). In vitro, CD30 is up-regulated on T and B lymphocytes after activation with mitogen, on Epstein–Barr virus (EBV)-transformed B cells, and on human T cell leukemia virus I (HTLV-I)- and HTLV-II-transformed lymphocytes (Andreesen et al., 1984; Stein et al., 1985; Schwarting et al., 1989; Ellis et al., 1993).

Histochemical staining of healthy tissues has shown that CD30 expression is largely restricted to lymphoid compartments, predominantly around B cell follicles and at the edge of germinal centers in lymph nodes and spleen (Stein et al., 1985). CD30 is also found in the thymic medulla (Stein et al., 1985; Schuurman et al., 1989). CD30 is not detected on cells of the peripheral blood or on resting lymphocytes (Ellis et al., 1993). A ligand for CD30 (CD30L) has been cloned and found to share sequence similarities with other members of the emerging TNF/NGF cytokine family (Smith et al., 1993). There are few reports on tissue distribution of CD30L, although it appears to be expressed on activated T cells and macrophages (Smith et al., 1993; Gruss et al., 1994).

Defective ligand–receptor systems involving the TNFR family of molecules are known to result in diseases. For example, mutations in the CD40L gene in humans results in an X-linked immunodeficiency hyper–immunoglobulin M (IgM) syndrome (Aruffo et al., 1993; Korthauer et al., 1993; DiSanto et al., 1993; Allen et al., 1993), and defects in the Fas–Fas ligand system are implicated in autoimmune disease in mice (Watanabe-Fukunaga et al., 1992; Lynch et al., 1994; Takahashi et al., 1994).

In vitro studies have shown that CD30 can mediate a variety of activation and differentiation signals, a capacity that varies with cell type and origin. Engagement of CD30 on cell lines has been shown to induce immunoglobulin secretion in EBV-transformed lymphoblastoid cells, proliferation in T cell–like HD-derived cells, null response in B cell–like HD-derived cells, or cell death in ALCL cells (Smith et al., 1993; Gruss et al., 1994). In addition, different anti-CD30 antibodies with both agonist and antagonist effects on $CD30^+$ cells have been

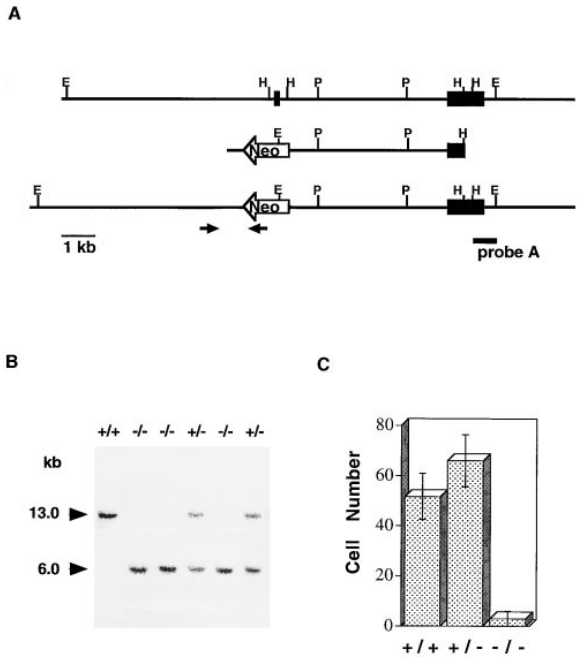

Figure 1. Generation of CD30$^{-/-}$ Mice

(A) Map of the partial CD30 locus (top), the targeting construct (middle), and the targeted locus (bottom). A 7.2 kb genomic fragment was used to construct the targeting vector. The 0.4 kb HindIII fragment containing the second-last exon was replaced by a *neo* gene. Primers specific for the *neo* gene and specific for an intron sequence 5′ of the targeting vector were used for PCR screening of embryonic stem cell clones (arrows). CD30 flanking probe (probe A) was used to confirm the homologous recombination event.

(B) Southern blot analysis of representative mouse tail DNA. Genomic DNA was digested with EcoRI and hybridized with probe A. Sizes of fragments are indicated.

(C) Dish binding assay using mouse CD30L fused to the Fc portion of human IgG. Spleen cells stimulated with anti-CD3 antibody (clone 145 2C11) for 72 hr were cultured on a dish coated with mouse CD30L fused to the Fc portion of human IgG using rabbit anti–human IgG antibody. After 1 hr, the dishes were washed with PBS, and the number of cells bound to immobilized CD30L was counted under six microscopic fields (400×). Mean values (± SD) are shown for one representative assay of three separate experiments. In addition, CD30 surface expression was not detectable on CD30$^{-/-}$ T cells after 5 days of in vitro activation.

described. The observations that CD30 has pleiotropic effects on different cells in vitro has made it difficult to suggest a function for CD30 in vivo. Here we show that mice deficient for CD30 expression after gene targeting manifest a defect in thymocyte negative selection. Thus, our data indicate that CD30 functions similarly to Fas and TNFR in mediating cell death signals. While Fas and TNFR operate mainly in mature T cells, CD30 appears to be the primary death-signaling molecule in thymocytes.

Results

Generation of Gene-Deficient Mice

To examine the physiological function of the HD-associated molecule CD30, we generated a mouse line that lacks CD30 expression by means of gene targeting (Figure 1). The CD30 targeting vector (Figure 1A) was constructed from a 7.2 kb DNA fragment derived from a genomic clone isolated from a 129/J library. The 0.4 kb HindIII fragment containing the second-last exon of the CD30 gene was replaced by a neomycin (*neo*) gene cassette containing a poly(A) termination signal (pMC1neo-polA), placed in an antisense orientation. The linearized construct was introduced into E14K embryonic stem cells by electroporation, and G418-resistant embryonic stem colonies were screened for homologous recombination by polymerase chain reaction (PCR). Targeted clones obtained by PCR were confirmed by genomic Southern blot analysis. The average frequency of homologous recombination was about one in 200 G418-resistant cells or one in 10^7 electroporated cells. Four clones were chosen for injection into blastocysts of C57BL/6 mice to generate mice carrying this CD30$^{-/-}$ mutation (Figure 1B). In an in vitro dish binding assay, anti-CD3 monoclonal antibody (MAb)–activated T lymphocytes from CD30$^{-/-}$ mice did not bind mouse CD30Ls immobilized on a culture dish, indicating the absence of CD30 receptors in CD30$^{-/-}$ mice (Figure 1C). The CD30$^{-/-}$ mice had no overt histological changes in liver, kidney, spleen, lymph nodes, heart, or intestine.

Table 1. Lymphocyte Numbers in Thymus, Spleen, and Lymph Nodes from CD30$^{+/-}$ and CD30$^{-/-}$ Mice

	Total Number of Lymphocytes ($\times 10^8$)	
Lymphatic Organ	CD30$^{+/-}$	CD30$^{-/-}$
Thymus (n = 8)	1.33 ± 0.54	2.25 ± 0.54 ($p < 0.01$)
Spleen (n = 6)	0.78 ± 0.15	0.79 ± 0.11
Lymph nodes (n = 4)	0.19 ± 0.03	0.18 ± 0.03

Total thymocytes, total spleen cells, and total mesenteric lymph nodes cells were isolated and counted. Thymocyte numbers in CD30$^{-/-}$ mice were significantly higher than those of CD30$^{+/-}$ mice. Statistical analysis was done by Student's t test. The difference in numbers of thymocytes was statistically significant ($p < 0.01$). All mice used were 6- to 10-week-old littermates.

Peripheral T and B Cell Function

Lymph node, spleen, and bone marrow contained normal absolute numbers of cells (Table 1). Spleen and lymph nodes also contained normal ratios of Thy-1.2$^+$ and B220$^+$ cells, including expected numbers of CD4$^+$ and CD8$^+$ T cell subpopulations (Table 2). Thus, CD30 deficiency does not result in overt defects in homeostasis in either B or T cell lineages. However, CD30 is upregulated on T cells after activation. We examined proliferative responses of enriched T cells from lymph nodes of CD30 mutant mice, which were similar to controls after activation with anti-CD3 MAb, phorbol myristate acetate (PMA)–calcium ionophore, or staphylococcal enterotoxin B (SEB) (Figure 2). The CD30 mutation also did not have any effect on apoptotic cell death of peripheral T lymphocytes in activation assays or after in vivo stimulation with SEB (data not shown). In mature T cells, CD30 does not appear to be required for either activation or SEB-induced death signals through the T cell receptor (TCR).

The finding that a portion of CD45RO T cells expresses CD30 raised the possibility that CD30 might play a role in T cell memory (Ellis et al., 1993). We therefore assessed CD8$^+$ T cell memory in CD30-deficient mice. CD30$^{-/-}$ and

Table 2. Lymphocytic Subsets in $CD30^{-/-}$ Mice

Subsets	Percentage of Lymphocytes per Total Cells		
	$CD30^{+/+}$	$CD30^{+/-}$	$CD30^{-/-}$
Thymus			
$CD4^+CD8^+$	81.6 ± 4.0	80.8 ± 6.2	78.9 ± 4.7
$CD4^+CD8^-$	11.8 ± 2.5	10.8 ± 1.7	12.1 ± 1.5
$CD4^-CD8^+$	3.3 ± 0.7	3.0 ± 1.1	3.4 ± 0.9
Lymph nodes			
Thy-1.2^+ $B220^-$	79.9 ± 5.9	82.1 ± 1.9	82.4 ± 4.3
Thy-1.2^- $B220^+$	10.6 ± 3.5	9.3 ± 1.2	9.8 ± 0.9
$CD4^+CD8^-$	51.5 ± 4.3	53.3 ± 5.5	55.2 ± 5.8
$CD4^-CD8^+$	21.7 ± 0.9	22.1 ± 1.5	23.5 ± 2.2
Spleen			
Thy-1.2^+ $B220^-$	23.0 ± 3.4	21.1 ± 2.5	21.7 ± 3.1
Thy-1.2^- $B220^+$	63.2 ± 8.0	60.0 ± 2.9	61.3 ± 7.7
$CD4^+CD8^-$	20.2 ± 3.2	17.7 ± 0.9	18.1 ± 2.9
$CD4^-CD8^+$	8.0 ± 0.9	6.9 ± 1.1	7.3 ± 1.3

Cells from thymi, mesenteric lymph nodes, and spleens were collected and stained with anti-CD4 (PE) and anti-CD8 (FITC) or with anti-Thy-1.2 (PE) and anti-B220 (FITC) and analyzed on a FACScan. Three mice were included in each group.

$CD30^{+/-}$ mice were injected intravenously with vesicular stomatitis virus (VSV). The predominant epitope for $CD8^+$ cytotoxic T lymphocyte (CTL) in $H\text{-}2^b$ mice lies within the nucleoprotein of VSV (Kundig et al., 1993b; Puddington et al., 1986). Spleen cells were stimulated in vitro 10 and 40 days after injection of virus with VSV nucleoprotein (VSV-NP)-transfected antigen-presenting cells (Kundig et al., 1993a). In VSV-immune animals, such restimulation generates $CD8^+$ VSV-NP-specific CTL. Spleen cells from $CD30^{+/-}$ and $CD30^{-/-}$ mice generated similar cytotoxicity after restimulation (Figure 3A). The biological relevance of this in vitro assay for memory was confirmed in vivo, where mice were intracerebrally challenged at the same timepoints with vaccinia VSV-NP recombinant virus (Binder and Kundig, 1991). VSV-specific $CD8^+$ memory CTL is required to protect mice against this challenge infection (Bachmann and Kundig, 1994). VSV-immunized $CD30^{+/-}$ and $CD30^{-/-}$ mice were resistant to challenge infection, whereas nonimmunized controls succumbed to vaccinia-induced meningitis (data not shown). Thus, both in vitro and in vivo assays indicated that $CD8^+$ CTL memory was unaltered in the absence of CD30.

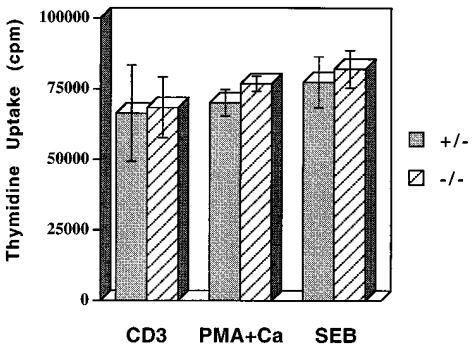

Figure 2. CD30-Deficient T Cells Proliferate Normally

T cells were isolated from spleens using T cell enrichment columns. Enriched T cells were plated in 96-well flat-bottomed plates at 1×10^5 cells per well and stimulated with PMA (15 ng/ml) plus calcium ionophore A23617 (250 ng/ml), SEB (2.5 μg/ml), and anti-CD3 antibody (145 2C11) immobilized by goat anti–hamster immunoglobulin. Syngeneic irradiated spleen cells were seeded as feeder cells (5×10^5). After 3 days, cells were pulsed with 1 μCi of [^{3}H]thymidine per well for 16 hr. Background counts without any stimulation were <1000 cpm. The data presented are from triplicate cultures (± SD) and show one representative example of three experiments.

Basal serum levels for various subclasses of immunoglobulin (IgM, IgG1, IgG2a, IgG2b, and IgG3) were comparable for mutant and control mice (data not shown). In addition, we assessed humoral immune responses after infection with VSV. This infection normally induces a rapid and T help–independent neutralizing IgM response, followed by a T help–dependent IgG class switch (Bachmann and Kundig, 1994). Both IgM responses and class switching to IgG was similarly present in $CD30^{-/-}$ and $CD30^{+/-}$ mice (Figure 3B). These data indicate that, in the absence of CD30, B cell function and responses are normal and T help for B cells is unimpaired.

Thymus Is Enlarged in Mutant Mice

CD30 is expressed on small numbers of cells in the thymic medulla (Stein et al., 1985), a thymic microenvironment where negative selection of thymocytes is known to occur (Surh and Sprent, 1994; Sprent and Webb, 1995). In addition, a recent study found CD30 mRNA expression to be abundant in the thymus (E. R. Podack, personal communication). We therefore searched for a defect in the thymus of $CD30^{-/-}$ mutant mice. The thymi of $CD30^{-/-}$ mice showed normal T cell subpopulations as defined by CD4 and CD8 expression (Table 2). However, the total number of thymocytes in mutant animals was elevated compared with littermates (Table 1). To examine the possible impact of CD30 deficiency on thymocyte survival, we cultured thymocytes in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). Cells from mutant animals showed similar death rates up to 4 days. We then evaluated responses of thymocytes to dexamethasone and γ-irradiation, both

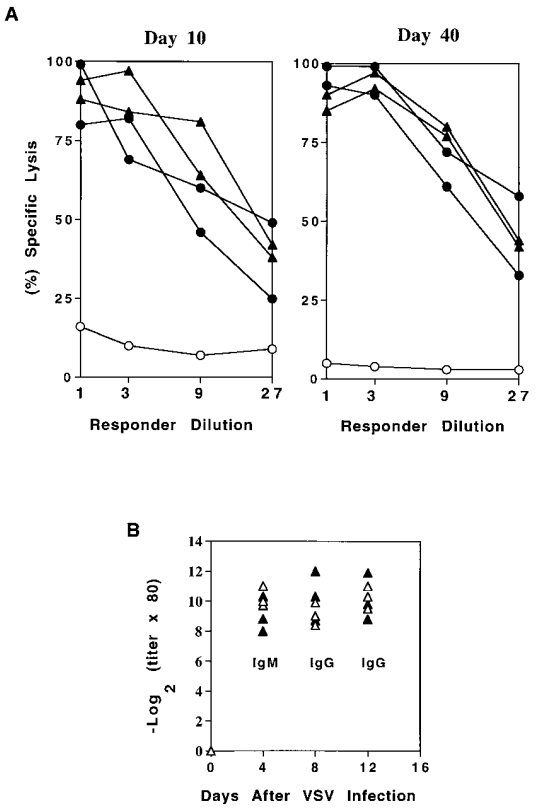

Figure 3. Peripheral Immune Responses Are Unaltered in CD30-Deficient Mice

(A) Secondary in vitro $CD8^+$ CTL responses are unaltered in the absence of CD30. $CD30^{+/-}$ (closed circles) and $CD30^{-/-}$ (closed triangles) mice were intravenously immunized with VSV or left unprimed (open circles). After 10 days (left) and after 40 days (right), spleen cells were restimulated in vitro with viral NP, and NP-specific cytotoxicity was assessed on MC57 target cells infected with VSV. Specific lysis of uninfected MC57 was less than 16% for all effectors shown.

(B) Humoral immune response against VSV is unaltered in $CD30^{-/-}$ mice. $CD30^{+/-}$ (closed triangles) and $CD30^{-/-}$ (open triangles) mice were intravenously immunized with VSV. Neutralizing serum IgM titers were determined 4 days after immunization. Neutralizing serum IgG was measured after 8 and 12 days. Titers in unprimed control sera are not detectable.

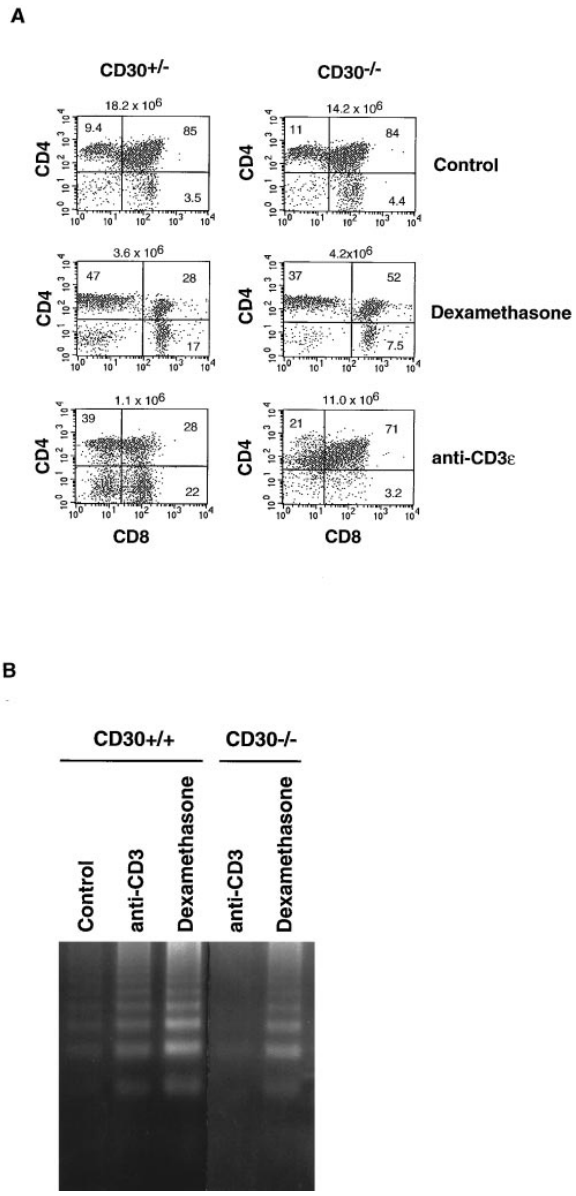

Figure 4. Defect in Activation-Induced Death of Thymocytes

(A) Thymocytes (2×10^7) isolated from $CD30^{+/-}$ and $CD30^{-/-}$ mice were cultured in RPMI 1640 media containing 5% FCS (control), 1 μM dexamethasone, or immobilized anti-CD3 antibody (10 μg/ml) for 24 hr. As a control, thymocytes were cultured without any stimulations for 24 hr. Viable cells were then counted using trypan blue exclusion and double stained with anti-CD8–FITC and anti-CD4–PE MAbs. Viable cells (10,000) were analyzed using FACScan. Numbers inside panels indicate relative percentages of positively stained cells in each quadrant. Numbers above panels show total numbers of viable cells after 24 hr of each treatment (Wallace et al., 1992).

(B) DNA fragmentation of thymocytes following in vitro treatment with dexamethasone (1 μM) or anti-CD3 (10 μg/ml) for 24 hr. Each lane represents DNA from 1×10^6 thymocytes. Control indicates DNA from thymocytes cultured for 24 hr in RPMI 1640 media containing 5% FCS.

of which are known to cause rapid apoptotic death of cortical double-positive (DP) ($CD4^+CD8^+$) thymocytes (Sellins and Cohen, 1987; Wyllie, 1980; Scollay et al., 1984), and found no difference in cell death between mutant and control mice (Figure 4A). We further investigated thymocyte responses to cross-linking with anti-CD3 antibodies, which are known to activate mature T cells but kill immature DP ($CD4^+CD8^+$) thymocytes in vitro (Smith et al., 1989) and in vivo (Shi et al., 1991). By 24 hr after CD3 cross-linking in vitro, there were about 10-fold greater numbers of live thymocytes and an approximately 25-fold increase of DP cells in mutant mice as compared with controls (Figure 4A). A similar phenomenon was seen 20 hr after anti-CD3 treatment in vivo, where mutant animals showed 4-fold greater numbers of DP thymocytes compared with treated littermates (data not shown). In agreement with these findings, DNA of thymocytes from control and $CD30^{-/-}$ mice after anti-CD3 treatment in vitro were different in their nucleosomal fragmentation pattern, which is characteristic of apoptosis (Figure 4B). Thus, it appears that DP thymocytes from $CD30^{-/-}$ mice are less susceptible to anti-CD3-mediated apoptosis.

Negative Selection in αβTCR-Transgenic CD30$^{-/-}$ Mice Is Defective

To investigate the role of CD30 in thymocyte apoptosis, we bred the CD30$^{-/-}$ mutation into TCR-transgenic animals and examined selection processes in the thymus. Treatment of normal thymocytes with anti-CD3 is thought to mimic activation-induced cell death similar to that observed with thymic deletion. Thus, our results above pointed toward a possible defect in negative selection. We hypothesized that defects in negative selection in CD30-deficient mice might only be subtly visible owing to the small fraction of thymocytes that actually undergoes negative selection processes (Table 1). To increase the fraction of thymocytes subjected to negative selection, we bred the CD30 mutation into TCR-transgenic mice with receptors specific for self-antigen.

An αβTCR-transgenic mouse line specific for the male antigen (H-Y) was bred with the CD30$^{-/-}$ mutant strain. Thymocytes expressing the H-Y-specific transgenic αβTCRs are positively selected in female H-2^b mice, negatively selected in male H-2^b mice, and nonselected in H-2^d mice (Teh et al., 1988; von Boehmer, 1990). Thymocyte phenotypes were similar in positive-selecting CD30$^{-/-}$ and CD30$^{+/-}$ mice, as defined by anti-CD4 and anti-CD8 antibodies and staining for the transgenic TCR Vβ8.2 chain (Figure 5A). Thymocytes from these animals also showed similar expression of heat-stable antigen (HSA), CD69, Pgp-1, and the transgenic TCR Vα chain (data not shown). In nonselecting transgenic mice, there were also no differences in thymocyte profiles between CD30$^{-/-}$ and CD30$^{+/-}$ animals (data not shown).

Conversely, in negative-selecting male H-Y-transgenic mice, we observed a dramatic phenotype in CD30$^{-/-}$ animals (Figure 5B). The negative-selecting mice normally show a marked, absolute reduction of thymocyte numbers as well as a proportionate decrease in DP cells (Teh et al., 1988). In negatively selecting male mice with CD30$^{-/-}$ mutations, the thymus contained an approximately 20-fold increase in DP cells compared with CD30$^{+/-}$ mice (Figure 5B; Table 3). However, there is still about a 3- to 5-fold reduction in thymocyte numbers in CD30$^{-/-}$ mice compared with the thymi of positively selecting (H-2^b) female mice (Table 3). Thus, these data indicate that H-Y-transgenic CD30$^{-/-}$ mice have only a partial, but clearly significant, defect in negative selection in the thymus.

On the other hand, lymph nodes and spleen contained similar numbers of lymphocytes in negatively selecting CD30$^{-/-}$ and CD30$^{+/-}$ mice (Table 3). The number of T cells expressing the transgenic TCR Vα chain detected by the clonotypic MAb in these organs was also comparable for both groups of mice (Table 3). To determine whether transgenic T cells from male CD30$^{-/-}$ mice were autoreactive, we performed a syngeneic mixed lymphocyte reaction (Figure 5C). Whereas transgenic T cells from female mice responded to the antigen, T cells from lymph nodes of CD30$^{-/-}$ male mice did not proliferate

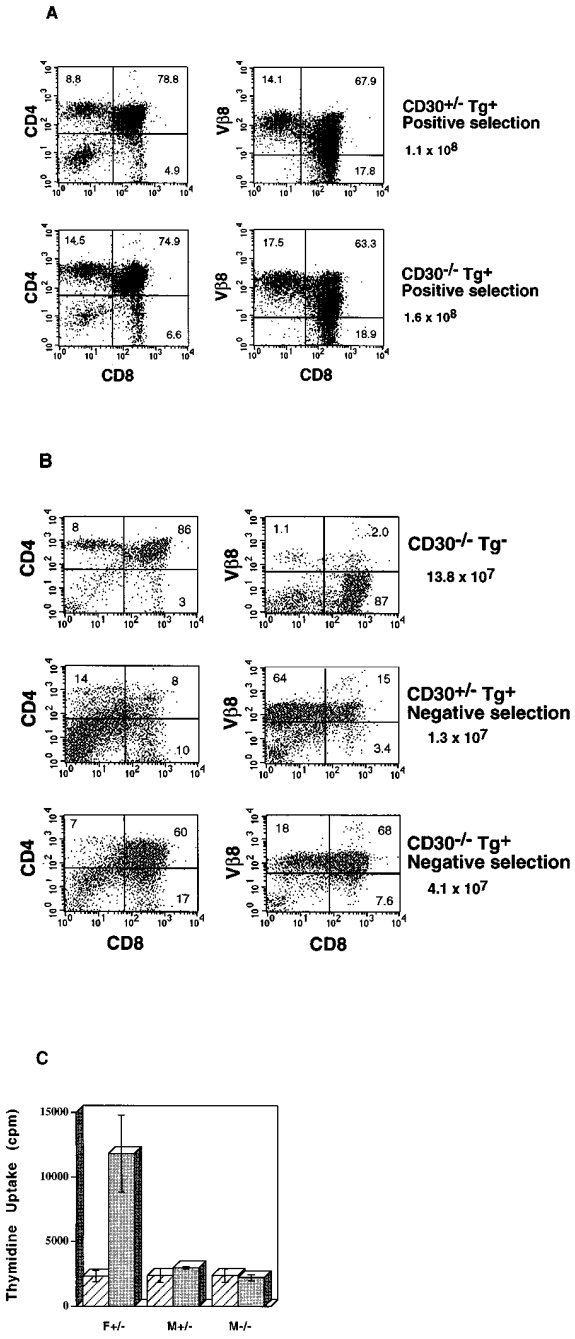

Figure 5. Clonal Deletion of H-Y TCR–Transgenic T Cells Is Defective in CD30$^{-/-}$ Mice

(A) Thymocytes from CD30$^{+/-}$ and CD30$^{-/-}$ H-Y TCR–transgenic mice in a positive-selecting background (H-2^b female) were double stained with anti-CD8–FITC and anti-CD4–PE or anti-CD8–FITC and anti-Vβ8–PE MAbs. Numbers indicate relative percentages of positive cells within a quadrant. Total numbers of thymocytes are also shown.

(B) Thymocytes from CD30$^{-/-}$ control mice and CD30$^{+/-}$ and CD30$^{-/-}$ H-Y TCR–transgenic mice in a negative-selecting background (H-2^b male) were double stained with anti-CD8–FITC and anti-CD4–PE or anti-CD8–FITC and anti-Vβ8–PE MAbs. Numbers indicate percentages of positive cells within a quadrant. Total numbers of thymocytes are also shown.

(C) Mixed lymphocyte culture of transgenic lymph node cells. Lymph node cells (5 × 10^5) from CD30$^{+/-}$ H-Y TCR–transgenic mice in a positive-selecting background (H-2^b female, F$^{+/-}$), CD30$^{+/-}$ H-Y TCR–transgenic mice in a negative-selecting background (H-2^b male, M$^{+/-}$), and CD30$^{-/-}$ H-Y TCR–transgenic mice in a negative-selecting background (H-2^b male, M$^{-/-}$) were incubated with 5 × 10^5 irradiated (2000 rads) spleen cells from syngeneic male mice. Hatched and stippled bars indicate proliferation of responder cells with or without stimulator cells, respectively.

Table 3. Profile of H-Y TCR Transgenic Model

		Male			Female	
	Tg^- $CD30^{+/-}$	Tg^+ $CD30^{+/-}$	Tg^+ $CD30^{-/-}$	TG^+ $CD30^{-/-}$	Tg^+ $CD30^{+/-}$	Tg^+ $CD30^{-/-}$
Thymus ($\times 10^6$)	101	12.6	39.2	41.4	110	160
$CD4^+CD8^+$ (%)	86.0	8.0	60.0	56.0	78.0	74.9
$CD4^+CD8^+$ ($\times 10^6$)	86.9	1.0	23.5	23.2	86.9	120
Mesenteric lymph node ($\times 10^6$)	14.4	23.4	16.2	19.8	—	—
$T3.70^+$ cells (%)	2.81	20.4	15.6	15.8	—	—
Spleen ($\times 10^6$)	24.0	25.8	47.5	25.8	—	—
$T3.70^+$ cells (%)	1.73	6.93	3.23	7.03	—	—

Total thymocytes, mesenteric lymph node cells, and spleen cells were isolated from a $CD30^{+/-}$ αβTCR transgenic mouse in a negative-selecting background (male, H-2^b), and a control $CD30^{+/-}$ mouse. Total thymocytes were also isolated from $CD30^{+/-}$ and $CD30^{-/-}$ αβTCR transgenic mice with positively selecting backgrounds (female, H-2^b). All mice were age matched. Cells were stained simultaneously with anti-CD8 (FITC) and anti-CD4 (PE) or with anti-Thy-1.2 (PE) and clonotypic T3.70 antibody (rat IgG, supernatant), which recognizes the transgenic TCR Vα chain. T3.70 antibody was visualized using FITC-labeled goat anti–rat IgG (Southern Biotechnology Associates). In double-staining experiments using T3.70, nonspecific staining due to remaining anti–rat immunoglobulin sites was blocked using 2 μg per 100 μl of rat IgG (Sigma). Samples were analyzed using a FACScan. Results were expressed as a percentage of $T3.70^+$ cells among $Thy\text{-}1.2^+$ cells.

in response to γ-irradiated spleen cells from male mice bearing the H-Y antigen. This suggested that the transgenic T cells in the lymph nodes of $CD30^{-/-}$ mice were not functionally autoreactive as determined by this experimental approach.

Defective Negative Selection in γδTCR-Transgenic $CD30^{-/-}$ Mutant Mice

To evaluate further whether CD30 is required for thymic negative selection of γδ T cells, we bred a transgenic γδTCR (Vγ2 Vα11.3) (Dent et al., 1990) mouse strain with $CD30^{-/-}$ mice. The γδTCR genes were originally cloned from a BALB/c (H-$2^{d/d}$) nu/nu-derived T cell line with alloreactivity against major histocompatibility complex class I Tla molecules of H-$2^{b/b}$ mice (Bluestone et al., 1988). In the H-$2^{d/d}$ mice, thymocytes undergo positive selection mediated by H-$2^{d/d}$ class I molecules. In the negative-selecting H-$2^{b/b}$ mice, transgenic γδ thymocytes are deleted, and fewer than 2% of thymocytes express a γδTCR (Dent et al., 1990).

Thymus phenotypes were similar in positively selecting mice with $CD30^{-/-}$ and $CD30^{+/+}$ backgrounds (Figure 6A). The numbers of γδ T cells in spleen and lymph nodes were also comparable in positive-selecting mice (data not shown). Thus, positive selection appeared normal in γδTCR $CD30^{-/-}$ mice.

Negative selection of transgenic γδTCR thymocytes, however, was impaired in $CD30^{-/-}$ mice (Figure 6B). The γδTCR-transgenic H-$2^{b/b}$ mice in $CD30^{+/+}$ backgrounds contained a small ($\sim 3 \times 10^5$) residual population of γδTCR T cells in the thymus. The negative-selecting mice with $CD30^{-/-}$ mutations, on the other hand, had about 15-fold increased numbers ($\sim 5 \times 10^6$) of γδTCR thymocytes. In these $CD30^{-/-}$ mice, over 95% of the $\gamma\delta TCR^+$ thymocytes also expressed the activation marker Pgp-1 (Figure 6B) (Skeen and Ziegler, 1993; Tatsumi et al., 1993). Taken together, the data from both γδ- and αβTCR-transgenic mice demonstrate that in the absence of CD30 negative selection of thymocytes is partially impaired.

Although lymph nodes and spleens contained similar numbers of lymphocytes in negatively selecting $CD30^{-/-}$ and $CD30^{+/+}$ mice, the thymi of $CD30^{-/-}$ mice were consistently severalfold larger. Moreover, the $CD30^{-/-}$ mice showed increased numbers of γδ T cells in these organs (Table 4). To examine the autoreactivity potential of the γδ T cells in $CD30^{-/-}$ mice, a mixed lymphocyte reaction was carried out (Figure 6C). Proliferative responses of transgenic γδ peripheral T cells isolated from positive-selecting (H-$2^{d/d}$) $CD30^{+/+}$ and $CD30^{-/-}$ mice after activation with spleen cells from negative-selecting $CD30^{-/-}$ mice (H-$2^{b/b}$) were similar. In addition, stimulation with allogeneic H-$2^{b/b}$ cells from negative-selecting $CD30^{-/-}$ mice showed no proliferative responses for T cells derived from spleen of negatively selecting $CD30^{-/-}$ and $CD30^{+/+}$ mice, suggesting that transgenic γδ T cells in the spleen of $CD30^{-/-}$ mice were not autoreactive.

Deletion of Endogenous Superantigen Mls-2^a–Responsive T Cells in $CD30^{-/-}$ Mice Is Not Impaired

To assess whether clonal deletion of thymocytes reactive to endogenous viral superantigens is also impaired in $CD30^{-/-}$ mice, we crossed $CD30^{-/-}$ mice with an A/J mouse strain (H-$2^{k/k}$) that expresses an endogenous viral superantigen, Mls-2^a. $V\beta3^+CD4^+$ and $V\beta11^+CD4^+$ cells, both of which are reactive to Mls-2^a, were efficiently deleted in the thymus (data not shown) and the lymph nodes (Table 5) of both $CD30^{-/-}$ and $CD30^{+/-}$ mice. On the other hand, $CD4^+$ cells carrying $V\beta6^+$ or $V\beta14^+$ receptors, which do not recognize Mls-2^a, were not deleted in these organs of $CD30^{-/-}$ and $CD30^{+/-}$ mice. These data indicate that CD30 is not essential for clonal deletion of T cells reactive to this endogenous viral superantigen.

Discussion

Despite intensive investigation and the association of CD30 with HD and other lymphomas, the cellular function of the CD30 molecule in vivo has remained a mystery. Preactivated peripheral T cells and certain HD-derived cell lines (expressing T cell markers) have been reported to show enhanced proliferation upon CD30 engagement (Smith et al., 1993; Gruss et al., 1994). We found enriched T cells from CD30 mutant mice to have normal proliferative responses after anti-CD3 cross-linking or treatment with mitogens. Spleen cells from mutant

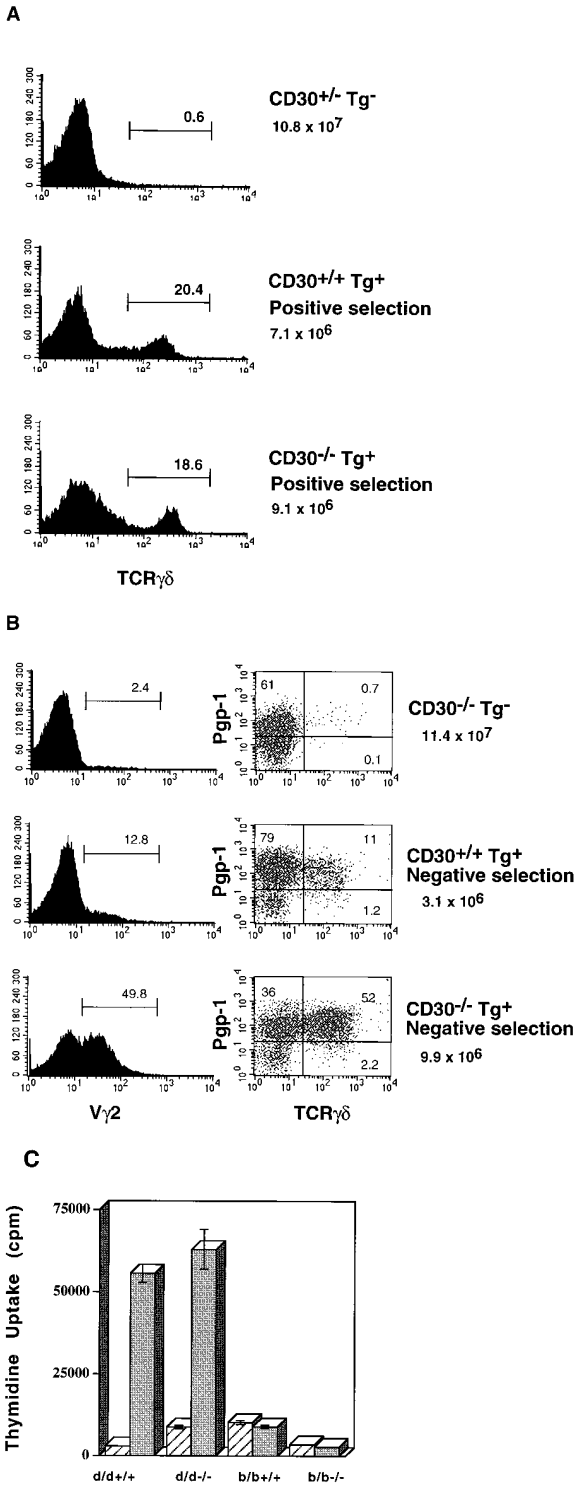

Figure 6. Clonal Deletion of Transgenic γδ T Cells Is Impaired in CD30$^{-/-}$ Mice

(A) Thymocytes from CD30$^{+/-}$ control mice and CD30$^{+/-}$ and CD30$^{-/-}$ γδTCR-transgenic mice in a positive-selecting background (H-2$^{d/d}$) were stained with anti-γδ–PE MAb. Numbers within histogram indicate percentages of γδTCR-positive cells. Total numbers of thymocytes are shown.

(B) Thymocytes from CD30$^{-/-}$ control mice and CD30$^{+/+}$ and CD30$^{-/-}$ γδTCR-transgenic mice in a negative-selecting background (H-2$^{b/b}$) were stained with anti–TCR Vγ2–FITC MAb or anti–pan γδTCR–FITC and anti-Pgp-1–PE MAbs. Numbers in histogram indicate percentages of TCR Vγ2–positive cells. Numbers in panels indicate percentages of positive cells in each quadrant. Total numbers of thymocytes are shown.

(C) Proliferative responses of spleen cells from γδTCR-transgenic mice were measured by [^{3}H]thymidine incorporation after 3 days of culture in a mixed lymphocyte reaction. Responder cells (5×10^5) from CD30$^{+/+}$ (d/d$^{+/+}$) or CD30$^{-/-}$ (d/d$^{-/-}$) γδTCR-transgenic mice in a positive-selecting background (H-2$^{d/d}$) and CD30$^{+/+}$ (b/b$^{+/+}$) or CD30$^{-/-}$ (b/b$^{-/-}$) γδTCR-transgenic mice in a negative-selecting background (H-2$^{b/b}$) were cultured with 5×10^5 irradiated (2000 rads) spleen cells (H-2$^{b/b}$) from CD30$^{-/-}$ γδTCR-transgenic mice in a negatively selecting background (stippled bars). As controls, responder cells were cultured without stimulators (hatched bars).

mice immunized with VSV also generated normal cytotoxic responses in vitro, suggesting that proliferation and effector functions of T cells were unimpaired. Thus, although CD30 may have the ability to modulate T cell proliferation in vitro, our data indicate that CD30 deficiency does not cause a discernible defect in proliferation and effector functions of T lymphocytes.

EBV-immortalized B cell lines have been shown to have enhanced immunoglobulin production and secretion after cross-linking with anti-CD30 antibodies (Gruss et al., 1994). CD30 has also been found on T helper cell subsets that reportedly exhibit potent helper function for immunoglobulin production (Alzona et al., 1994). In CD30 mutant mice, basal serum immunoglobulin levels and T helper–dependent IgG class switching against VSV infection were normal, as were T helper–independent IgM responses. As a result, there is no evidence that CD30 is required in either maturation or class switching of normal B cells. This does not preclude a role for CD30, however, in malignant or transformed B cells.

CD30 is expressed on a subset of T cells expressing the isoform CD45RO (Ellis et al., 1993), considered to be a marker of memory T cells (Merkenschlager and Beverley, 1989). Our finding that both primary and secondary T cell responses were normal in CD30 mutant mice demonstrates that CD30 is not essential in either the production or maintenance (assessed up to 40 days) of memory T cells.

Our findings in this report indicate that CD30 is a surface receptor that influences the outcome of selection events in the thymus. This observation is consistent with the finding that CD30 mRNA expression is mainly found in the thymus (E. R. Podack, personal communication). It is interesting to note that, although we observed a prominent role for CD30 in negative selection of thymocytes, its expression in the thymus does not appear to be extensive. It was reported that CD30 is present on only small numbers of the so-called large cells in the thymic medulla (Stein et al., 1985). A recent report also shows that apoptotic cell death due to negative selection can be seen in only very few cells in the thymus (Surh and Sprent, 1994). Thus, it is possible that CD30 is transiently expressed on thymocytes that are destined to die.

Significant numbers of male antigen–specific H-Y-transgenic T cells are found in the periphery of negatively selecting male mice with either CD30$^{+/+}$ or CD30$^{+/-}$

Table 4. Profile of γδTCR Transgenic Model (Negative Selection)

Mice	Thymus			Mesenteric Lymph Nodes	
	Total Cell Number ($\times 10^6$)	γδTCR$^+$ Cells (%)	γδTCR$^+$ Cells ($\times 10^6$)	Total Cell Number ($\times 10^6$)	γδTCR$^+$/ Thy-1.2$^+$ (%)
Litter 1					
CD30$^{+/-}$ Tg$^-$	114.0	2.4	2.7	41.0	0.9
CD30$^{+/-}$ Tg$^+$	3.1	12.8	0.4	2.5	5.8
CD30$^{-/-}$ Tg$^+$	9.9	49.8	4.9	4.1	42.0
Litter 2					
CD30$^{+/-}$ Tg$^+$	5.0	10.5	0.50	7.2	6.4
CD30$^{+/-}$ Tg$^+$	16.0	53.5	8.6	4.0	69.0

Total thymocytes and mesenteric lymph node cells were isolated from CD30$^{+/-}$ and CD30$^{-/-}$ γδTCR transgenic (Tg) mice in negative-selecting backgrounds (H-2^b) and a control CD30$^{+/-}$ mouse (H-2^b). Cells were stained with anti-γδTCR (PE) and anti-Thy-1.2 (FITC) antibodies and analyzed on a FACScan. Two separate experiments are shown.

backgrounds. CD30$^{-/-}$ mice, however, showed no additional elevation in the numbers of H-Y-specific T cells in the periphery. When the Tla^b-specific γδTCR mice were examined, the numbers of transgenic TCR lymphocytes in the thymus and lymph nodes were elevated in CD30$^{-/-}$ mice compared with CD30$^{+/+}$ littermates. These data suggested that the γδ T cells were more likely to escape deletion in the CD30$^{-/-}$ mice. It should be noted that, in this model, few transgenic γδTCR T cells are normally found in the periphery of mice in the negative-selecting background (Dent et al., 1990). For both the negative-selecting H-Y- and γδTCR-transgenic models, transgenic T cells from lymph nodes of CD30$^{-/-}$ mice were shown to be functionally nonresponsive, indicating that, in addition to deletional mechanisms, other forms of tolerance induction are operational in CD30$^{-/-}$ mice.

Curiously, while CD30 is important for clonal deletion of thymocytes reactive to antigens such as male H-Y and Tla^b antigens, CD30 is not required for clonal deletion of thymocytes reactive to the endogenous viral superantigen, Mls-2^a. In this context, it is interesting to note that, in the absence of gp39–CD40 interactions, negative selection of Mls-reactive thymocytes is impaired (Foy et al., 1995). Thus, it is likely that the process of negative selection may involve multiple, and to some extent redundant, costimulation signals.

The differential expression of CD30L on thymic cells might determine whether positive or negative selection occurs during thymocyte development. Positive selection is thought to occur on the epithelial cells of the thymus, while the interaction of bone marrow–derived dendritic cells with autoreactive thymocytes probably mediates deletion (Sprent et al., 1988; Fowlkes and Pardoll, 1989; Ramsdell and Fowlkes, 1990; Robey and Fowlkes, 1994; Schwartz, 1989). Thus, expression of CD30L on thymic dendritic cells might provide a means of mediating deletion of autoreactive thymocytes. It is noteworthy that bone marrow–derived macrophages express CD30L (Smith et al., 1993). Alternatively, negative selection through CD30–CD30L binding might occur as a result of interactions between thymocytes. In this context, it is interesting that peripheral T cells activated with anti-CD3 antibodies or mitogens up-regulate both CD30 and CD30L (Smith et al., 1993).

Since CD30 is a TNFR family member, it was not surprising that the molecule can be involved in mediating death signals. It is intriguing, however, that direct sequence comparison showed no significant similarities between the CD30 cytoplasmic region and the newly defined TNFR family death domain, which is present in TNFR p55, Fas antigen, the low affinity NGF receptor, ankyrin, MORT1, TRADD, MORT1/FADD, and RIP (Cleveland and Ihle, 1995). Although these TNFR molecules all contain death domain homology, they appear to mediate death signals with varying kinetics and through different mechanisms (Clement and Stamenkovic, 1994). In light of this, it is likely that CD30, with its highly unique cytoplasmic domain, might mediate death signals through interactions with a novel protein(s).

Fas plays a critical role in the deletion of mature peripheral T cells, but has no known role in negative selection in the thymus (Singer and Abbas, 1994). TNFR p55– and TNFR p75–deficient mice also have normal thymic development (Pfeffer et al., 1993; Erickson et al., 1994; Rothe et al., 1993), while p75 has been found to mediate death of mature CD8$^+$ T cells (Zheng et al., 1995). In our CD30-deficient mice, quantitative analysis of T and B cells in peripheral lymph nodes and spleen, as well as examination in vivo of activation-induced cell death in response to SEB treatments, indirectly suggests that peripheral tolerizing (selection) mechanisms are functional. This is also consistent with the lack of overt disease in these mice. By whatever mechanism CD30 induces deletion, it appears to have a unique function in death signaling during thymocyte ontogeny. Thus, different TNFR superfamily members appear to be delegated unique responsibilities in cell death signaling.

Table 5. TCR Vβ Expression on Lymph Node Cells from CD30$^{+/-}$ and CD30$^{-/-}$ Mice

CD30	Mls-2	Vβ3	Vβ11	Bβ6	Vβ14
+/−	a	0.2	0.2	9.4	9.2
+/−	a	0.1	0.2	8.3	10.7
+/−	b	3.6	8.2	7.3	6.9
−/−	a	0.1	0.1	9.8	5.0
−/−	a	0.3	0.1	9.0	6.2
−/−	b	3.1	6.7	8.8	7.2

CD30$^{-/-}$ mice (H-2$^{b/b}$, Mls-2^b) were back-crossed into the A/J mouse strain (H-2$^{k/k}$, Mls-2^a). Lymph node cells and thymocytes from 6-week-old mice were stained simultaneously with FITC- or PE-conjugated antibodies to variable regions of the TCR and with PE- or FITC-conjugated anti-CD4 MAbs. Results are expressed as a percentage of Vβ$^+$ cells among CD4-positive T cells.

Although CD30 was initially described as a marker for HD lymphomas, observations in CD30 mutant mice have so far provided no obvious explanations of the role of CD30 in HD, ALCL, or other non-HD lymphomas and malignancies. Our findings in CD30 mutant mice provide no evidence that CD30 is required for proliferative signals. Rather, the results of this study indicate that, at least in the context of the thymus, CD30 is a cell death–inducing molecule. Thus, CD30 may not be involved in tumor formation, or may even retard lymphoma development. This latter suggestion might be consistent with the observation that HD is a relatively slow-growing lymphoma. On the other hand, one may speculate that mutations in the CD30 gene or alteration of its expression (or other proteins participating in its signaling pathway) might interfere with cell death, conferring growth advantage to premalignant cells, perhaps leading to tumor progression. Thus, it is of interest that patients with HD can exhibit elevated levels of soluble CD30 in their plasma (Pizzolo et al., 1990; Gause et al., 1991).

Finally, CD30 cross-linking has recently been found to induce activation of the NF-κB transcription factor (McDonald et al., 1995), which is recognized as a transactivator involved in myriad signaling pathways, including those regulating growth and proliferation. It is quite possible that CD30 can provide either death or activation signals depending on the cell type and developmental stage of the cell.

Experimental Procedures

Cells

E14K embryonic stem cells from 129/Ola mice were maintained on a layer of mitomycin C–treated embryonic fibroblasts in culture medium (Dulbecco's modified Eagle's medium) supplemented wih leukemia inhibitory factor, 15% FCS, L-glutamine, and β-mercaptoethanol.

Generation of CD30$^{-/-}$ Mutant Mice

Murine cDNA for CD30 was cloned from a mouse T cell cDNA library in λZapII (Stratagene) using the human CD30 cDNA (Dürkop et al., 1992) (provided by Dr. H. Stein). The CD30 cDNA clone was partially sequenced and used for screening a mouse genomic library (partial MboI digest of 129/J genomic DNA in λDASH phage vectors). Genomic clones were mapped and partially sequenced. A 7.2 kb genomic fragment (gCD30) containing two exons of the CD30 gene was used to construct the targeting vector. A 0.4 kb genomic HindIII fragment containing the second-last exon was replaced with a *neo* resistance gene cassette, pMC1neo-polA (Thomas and Capecchi, 1987) to obtain the targeting vector TV30neoA. The *neo* resistance gene was inserted in antisense orientation to the CD30 transcriptional orientation and contained a polyadenylation signal (Figure 1A). E14 embryonic stem cells (5×10^6) were electroporated with 20 μg of linearized targeting vector TV30neoA DNA (340 V, 250 μF; Bio-Rad Gene Pulser). G418 (300 μg/ml) selection was started 48 hr after transfection, and G418-resistant colonies were obtained after 10 days. PCR screening for homologous recombination was carried out as described previously (Fung-Leung et al., 1991). A primer specific for the *neo* resistance gene (5′-TATCAGGACATAGCGTTGGC-3′) and an outside primer specific for the CD30 gene (5′-CAACCCTGGCTGA GTTACTCTACCC-3′) upstream of the construct were used in PCR. Homologous recombination was subsequently confirmed by EcoRI digestion of genomic DNA and hybridization with probe A (Figure 1A). Chimeric mice were produced by injection of embryonic cells into 3.5-day-old blastocysts as described previously (Caligariscappio et al., 1995; Thomas and Capecchi, 1987; Fung-Leung et al., 1991; Bradley et al., 1984; Thompson et al., 1989). The contribution of embryonic stem cells to the germline of chimeric mice was assessed by breeding with C57BL/6 mice and screening for agouti offspring. Germline transmission of the CD30 mutation was confirmed by Southern blot analysis of tail DNA, and mice heterozygous for the mutant gene were interbred to homozygosity. All mice used for experiments were 6–10 weeks of age.

Mice

CD30$^{-/-}$ (H-2^b) male mice were crossed with H-Y TCR (Vα3 Vβ8.2) transgenic female mice (H-2^d, CD30$^{+/+}$) (Teh et al., 1988; von Boehmer, 1990) or γδTCR (Vα11.3Jδ1Cδ/Vγ2Jγ1Cγ1) transgenic female mice (H-2^d, CD30$^{+/+}$) (Dent et al., 1990; Bluestone et al., 1988). F1 mice (H-2$^{b/d}$, CD30$^{+/-}$) carrying either H-Y- or γδ-transgenic TCRs were inter-crossed to obtain the H-Y- or γδTCR-transgenic mice in appropriate backgrounds. Typing of mice for TCR transgenes was carried out by staining peripheral blood lymphocytes with anti-Vβ8 MAb (phycoerythrin [PE] conjugated) and anti-Thy-1.2 MAb (fluorescein isothiocyanate [FITC] labeled) for H-Y-transgenic mice or with anti-γδTCR MAb (PE conjugated) and anti-Thy-1.2 MAb (FITC labeled) for γδTCR-transgenic mice. All mice were bred in the animal facilities of the Ontario Cancer Institute. Care of animals was in accordance with guidelines of the Medical Research Council of Canada.

Dish Binding Assay

Lymph node cells collected from CD30$^{+/+}$, CD30$^{+/-}$, and CD30$^{-/-}$ mice were stimulated in HL-1 medium (Hycor Biomedical) containing 2% FCS with anti-CD3 antibody (145 2C11; Pharmingen) immobilized on the culture dishes using goat anti–hamster IgG antibody (Jackson Immunoresearch). After 72 hr, cells were washed three times with phosphate-buffered saline (PBS) and then used for dish binding assay. Center portions of plates (1008; Falcon) were coated with 70 μl of rabbit anti–human IgG antibody (10 μg/ml; Southern Biotechnology Associates) for 2 hr at room temperature. Plates were washed three times with PBS and then incubated with 3 ml of PBS containing 1% bovine serum albumin (BSA) for 14 hr at 4°C. Plates were washed once with PBS and incubated for 1 hr at room temperature with 400 μl of HL-1 medium containing 2% FCS and 2.5 μg/ml of mouse CD30L fused to the Fc portion of human IgG (CD30L–Fc; provided by Dr. C. Smith). For controls, plates were incubated in HL-1, 2% FCS medium without CD30L–Fc. Plates were then washed twice with PBS. Lymph node cells (2×10^6) activated with anti-CD3 antibody as described above were cultured in these plates in HL-1 medium containing 2% FCS at 37°C for 1 hr. Plates were finally washed three times with PBS, and the number of cells bound to the center portions coated with CD30L–Fc was counted for three different microscopic fields (400×).

Flow Cytometric Analysis

The following MAbs (all obtained from Pharmingen) were used: anti-CD4 (FITC labeled or PE conjugated), anti-CD8 (FITC or PE labeled), anti-B220 (FITC labeled), anti-Thy-1.2 (FITC or PE labeled), anti-γδ (PE conjugated), anti-Vβ3 (PE conjugated), anti-Vβ6 (FITC labeled), anti-Vβ8 (PE conjugated), anti-Vβ11(FITC labeled), anti-Vβ14 (FITC labeled), anti-Vγ2 (FITC labeled), anti-H-2K^b (FITC labeled), anti-H-2K^d (PE conjugated), and anti-Pgp-1 (PE conjugated). Blood samples (20 μl) were collected in heparinized capillary tubes and washed once in immunofluorescence staining buffer (PBS, 1% BSA, 0.1% NaN_3). Single cell suspensions from thymocytes, spleen cells, lymph node cells, and bone marrow cells from 6- to 10-week-old mice were prepared as described previously (Wallace et al., 1992), resuspended in PBS, and incubated with appropriate MAbs for 30 min at 4°C. Stained cells were analyzed using a FACScan flow cytometer (Becton–Dickinson).

T Cell Stimulation Assay

T cells were isolated from spleens and lymph nodes of CD30$^{+/-}$ and CD30$^{-/-}$ mice using columns to negatively enrich for T cells (R&D Systems). Purified T cells (1×10^5) were placed into flat-bottomed 96-well plates containing 5×10^5 irradiated (2000 rads) splenic feeder cells and freshly prepared Iscove's modified Dulbecco's medium (10% FCS, 10^{-5} M β-mercaptoethanol). Optimal concentrations of PMA–calcium ionophore (250 ng/ml calcium ionophore and 12.5

ng/ml PMA) and SEB (10 μg/ml) were added. For TCR–CD3 cross-linking, plates were coated overnight at 4°C with 10 μg per well of goat anti–hamster immunoglobulin and subsequently with anti–mouse CD3ε (145 2C11 hamster IgG; Pharmingen) at 37°C for 2 hr. To remove unbound antibodies, plates were washed with PBS before incubation of T cells. T cells were harvested at day 3 after a 16 hr pulse with 1 μCi of [^{3}H]thymidine per well. [^{3}H]thymidine uptake was counted using a gas-phase scintillation counter.

Assays for Secondary Cytotoxicity

Mice were immunized with VSV (2×10^6 pfu). On day 40, spleen cells from immunized mice were restimulated with γ-irradiated (4000 rads) EL-4 cells transfected with VSV-NP (N_1) (Kundig et al., 1993a). Spleen cells (4×10^6) were restimulated with 1×10^4 N_1 cells for 5 days, harvested, and tested for VSV-specific cytotoxicity on VSV-infected and uninfected MC57 (H-2^b) cells.

Determination of Serum Antibody Titers against VSV

Mice were intravenously infected with VSV (2×10^6 pfu). After 4, 8, and 12 days, sera were collected, and neutralizing antibody titers were determined as described previously (Binder and Kundig, 1991). In brief, 1:2 dilutions of 40-fold prediluted serum is incubated with VSV for 90 min and then tested for the presence of remaining infectious virus by incubating this virus–serum mixture on fibroblasts for another 24 hr. The serum dilution that reduced the number of viral plaques by 50% is taken as the titer. On days 8 and 12, IgG is determined by preincubating the sera with 2-β-mercaptoethanol, which reduces IgM levels.

Mixed Lymphocyte Culture

Lymph node cells were isolated from female CD30$^{+/-}$ H-Y TCR–transgenic mice (H-2^b), male CD30$^{+/-}$ H-Y TCR mice (H-2^b), and male CD30$^{-/-}$ H-Y TCR mice (H-2^b). Lymph node cells (5×10^5) were placed into flat-bottomed 96-well plates containing 5×10^5 irradiated (2000 rads) splenic stimulator cells from male CD30$^{+/-}$ H-Y TCR (H-2^b) or male CD30$^{-/-}$ H-Y TCR (H-2^b) mice in freshly prepared Iscove's modified Dulbecco's medium (10% FCS, 10^{-5} M β-mercaptoethanol).

Spleen cells (5×10^5) from CD30$^{+/+}$ γδTCR-transgenic mice (H-2$^{d/d}$), CD30$^{-/-}$ γδTCR-transgenic mice (H-2$^{d/d}$), CD30$^{+/+}$ γδTCR-transgenic mice (H-2$^{b/b}$), and CD30$^{-/-}$ γδTCR-transgenic mice (H-2$^{b/b}$) were cultured with 5×10^5 irradiated (2000 rads) splenic stimulator cells from CD30$^{-/-}$ γδTCR-transgenic mice (H-2$^{b/b}$) in the same conditions as described above for H-Y TCR–transgenic mice. Responder cells were also cultured without stimulators to determine background proliferation. After 3 days, responder lymph nodes and spleen cells were harvested after a 16 hr pulse with 1 μCi of [^{3}H]thymidine per well. [^{3}H]thymidine uptake was counted using a gas-phase scintillation counter.

Anti-CD3- and Dexamethasone-Induced DNA Fragmentation

Purified anti-CD3 MAbs (10 mg/ml, 145 2C11, hamster IgG, sodium azide free; Pharmingen) were placed on a plate and incubated at 37°C for 2 hr. The plates were washed three times with PBS. Thymocytes (5×10^6) from CD30$^{+/+}$ or CD30$^{-/-}$ mice were treated with dexamethasone (1 μM) or coated anti-CD3 antibodies for 24 hr. Cells were collected by centrifugation at 1000 rpm at 4°C for 10 min. Cells were resuspended in 0.5 ml of TTE solution (10 mM EDTA, 50 mM Tris [pH 8.0], 0.5% Triton X-100) with 0.5 mg of proteinase K and incubated for 2 hr at 50°C. DNA samples were extracted twice with phenol–chloroform and precipitated with isopropanol. The DNA was collected by centrifugation at 13,000 × g at 4°C for 10 min. DNA pellets were washed in 70% ethanol. DNA samples (20 μl) were electrophoresed on a 1% agarose gel containing 0.05 μg/ml ethidium bromide.

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