compensate for the absence of BDNF. However, the nodose–petrosal complex as a whole requires both BDNF and NT4, acting in a largely non-compensatory and non-redundant fashion; large-scale compensation or redundancy between these factors is unlikely because reduction of the complex in mice lacking both BDNF and NT4 was not more severe than the sum of the reductions seen in mice lacking either of these neurotrophins individually. The fact that BDNF and NT4 are differentially expressed in different viscera[26] supports the possibility that the subset of visceral afferent innervation remaining intact in *nt4*$^{-/-}$ mice may be quite different from that remaining in *bdnf*$^{-/-}$ mice. The importance of some nodose and petrosal neurons in cardiopulmonary homeostasis has led to the suggestion that loss of these neurons is involved in the early death of *trkb*$^{-/-}$ and *bdnf*$^{-/-}$ mice[7,9,25]. Although it is perhaps surprising that *bdnf*$^{-/-}$*nt4*$^{-/-}$ mice do not succumb more quickly than *bdnf*$^{-/-}$ mice, it may be that the subset of visceral afferents supported by NT4 innervate different targets that are not as critical for survival as those dependent upon BDNF.

Motor neurons display a pattern of neurotrophin requirement unlike that of any of the sensory populations examined. Although motor neurons were reported to be drastically reduced in mice lacking TrkB[7], they are not reduced in *bdnf*$^{-/-}$*nt4*$^{-/-}$ mice. This result, together with the reported early death of TrkB mutant mice[7] as compared with mice lacking both BDNF and NT4, suggests that yet another ligand acts via TrkB *in vivo*; however, it must also be considered that the *trkb*$^{-/-}$ and *bdnf*$^{-/-}$*nt4*$^{-/-}$ mice were generated on different genetic backgrounds and in different laboratory environments. NT3 acting on TrkB may explain differences between *trkb*$^{-/-}$ and *bdnf*$^{-/-}$*nt4*$^{-/-}$ mice: high concentrations of NT3 can activate TrkB *in vitro*[5], and it has already been suggested that NT3 is present at sufficient levels during normal development to act via TrkB[5,27,28]. Because skeletal motor neurons are not reduced in number in NT3 mutant mice[13,14] or in *bdnf*$^{-/-}$*nt4*$^{-/-}$ mice, current data could only be explained if these three factors all act on motor neurons via TrkB in a compensatory or redundant fashion, with none of these factors normally limiting; if this were the case, the loss of motor neurons that normally occurs during embryonic development would then be due to limiting amounts of other target-derived factors.

Finally, the relatively mild deficits thus far associated with lack of NT4 during normal development and early life suggest that NT4 may have a potential role in injury responses or in long-term maintenance of the nervous system. □

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Sensory but not motor neuron deficits in mice lacking NT4 and BDNF

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NEUROTROPHINS play important roles in neuronal survival during vertebrate development[1–3]. Neurotrophin-4 (NT4), alone or in combination with brain-derived neurotrophic factor (BDNF), has been suggested to be necessary for the survival of peripheral sensory neurons and central nervous system (CNS) neurons, including motor neurons[4–16]. To define the role of NT4 *in vivo*, we generated mice lacking NT4 by gene targeting. NT4-deficient mice were viable but exhibited a loss of sensory neurons in the nodose–petrosal and geniculate ganglia. In contrast, motor neurons of the facial nucleus and sympathetic neurons of the superior cervical ganglion were unaffected, and there was no obvious loss of dopaminergic neurons in the substantia nigra. In mice lacking both NT4 and BDNF[14,15], facial motor neurons remained unaffected, whereas the loss of sensory neurons was more severe than with either mutation alone. Thus NT4 is required during development for the survival of some peripheral sensory neurons but not sympathetic or motor neurons.

Embryonic stem cells were transfected with a targeting vector containing a NT4 gene genomic DNA fragment in which the entire coding sequence is deleted (Fig. 1*a*). Targeted embryonic stem cell clones were identified by Southern blot analysis and used to derive NT4 mutant mice (Fig. 1*b*). Homozygous mutant mice were obtained from two independently targeted embryonic stem cell clones and showed no overt phenotype. To examine the role of NT4 in neuronal survival, serial sections of the heads from day 18.5 embryos and dorsal root ganglia from adults were prepared, and neurons in the facial motor nucleus and in peripheral sensory and sympathetic ganglia were counted. Although no difference in the number of facial motor neurons or superior cervical ganglion neurons was seen, homozygous mutant mice displayed a significant loss of sensory neurons. Approximately half of the neurons were lost in the nodose–petrosal and the geniculate ganglia (Table 1). To determine whether the neuronal deficiency correlated with the loss of a particular phenotypic subpopulation of nodose–petrosal neurons, antibodies against calcitonin gene-related peptide, neuropeptide Y, substance P, tyrosine hydroxylase, somatostatin and the NT4 receptor, TrkB[8], were used for immunohistochemistry of sections prepared from these ganglia. No obvious differences

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were detected between the mutant and control mice (data not shown).

Because NT4 is a highly potent survival factor for embryonic central dopaminergic neurons in culture[12], and is expressed in the midbrain[7], neurons in the substantia nigra were examined in adult mutant mice using anti-tyrosine hydroxylase immunohistochemistry. Figure 2 shows that there is no obvious loss of central dopaminergic neurons in NT4 mutant mice (Fig. 2*b*) as compared with age-matched controls (Fig. 2*a*). We suggest that NT4 may not be required for the survival of the midbrain dopaminergic neurons, although we cannot exclude subtle changes which would require a rigorous quantitative comparison of mutant and wild-type mice.

Because NT4 and BDNF signal through the same receptor, TrkB[6,8,17], the mild phenotype seen in NT4 mutant mice could be explained if BDNF compensated for the lack of NT4. To study whether NT4 and BDNF can functionally substitute for each other, we crossed NT4 with BDNF mutant mice. Mice homozygous for the NT4 mutation and heterozygous for the BDNF mutation were viable and fertile. Most mice homozygous for both mutations, however, died within 48 hours of birth, and only a few survived for up to 10 days. The double-mutant mice were about the same size as their wild-type littermates at birth but failed to grow. They showed defective coordination of movement and balance, which is characteristic for BDNF mutant mice[14,15]. To examine whether additional neuronal loss occurred in the double-mutant mice, the heads of day 18.5 embryos of NT4 and BDNF double-mutant, BDNF single-mutant, and wild-type embryos were sectioned and neurons counted. In BDNF mutant embryos, the neuronal loss in nodose–petrosal and geniculate ganglia (Table 1) were similar to that previously reported at postnatal day 0 (P0) (ref. 15) or P14 (ref. 14). Interestingly, no statistically significant loss of facial motor neurons was detected in the BDNF–NT4 double mutants (Table 1). However, only $10 \pm 1\%$ and $6 \pm 1\%$ of the neurons were left in the nodose–petrosal and the geniculate ganglia of the double-mutant embryos, respectively, which by far exceeds that seen in the single-mutant mice (Table 1). The number of neurons in other ganglia was also significantly reduced, with $70 \pm 8\%$ remaining in the trigeminal ganglion and $18 \pm 3\%$ in the vestibular ganglion, similar to BDNF mutant mice. We conclude that lack of both NT4 and BDNF appears not to affect motor-neuron survival but leads to an additive neuronal loss in the nodose–petrosal and geniculate ganglia.

The main defect in mice lacking NT4 was a significant loss of nodose–petrosal and geniculate ganglion neurons. These *in vivo* results are consistent with previous studies showing that NT4 supports the survival of cultured nodose–petrosal neurons[9]. The finding that the loss of sensory neurons in the double mutants is to a large extent additive suggests that the two factors act mostly on distinct and non-overlapping populations of sensory neurons. Because BDNF and NT4 are ligands for the same receptor, our results are consistent with the possibility that different groups of nodose–petrosal or geniculate neurons innervate distinct targets which may selectively express only one or the other factor and thus result in differential survival of neurons in the respective mutant. Another possibility is that different expression of TrkB receptor isoforms[18,19] in neurons of nodose–petrosal and geniculate ganglia leads to different responses of the neurons towards BDNF or NT4. It is also of interest that mice deficient for NT3 display a neuronal loss of about 50% in nodose–petrosal ganglia[20,21]. Because virtually all nodose–petrosal neurons are lost in the BDNF–NT4 double-mutant mice, it appears possible that NT3 and BDNF–NT4 may act at different developmental stages to support neuronal survival in nodose–petrosal ganglia, as has been shown previously for other neurons[22,23]. Alternatively, a simultaneous action of NT3 with BDNF and/or NT4 may be required for the survival of some of the nodose–petrosal neurons.

In contrast to previous studies which showed that NT4 promotes the survival of central dopaminergic and facial motor neurons in culture or in lesion experiments[12,13], no defect was found in facial motor nucleus and no obvious loss of dopaminergic neurons was detected in the animals lacking NT4, although our data do not exclude subtle losses in dopaminergic neurons. Our data suggest that *in vitro* results or manipulations in postnatal animals either may not reflect the physiological function of the protein *in vivo*, or that the loss of NT4 is compensated for by other neurotrophic factors. Indeed, the loss of motor neurons in TrkB mutant mice and the lack of a motor-neuron defect in BDNF mutant mice had suggested that NT4 might sustain normal motor-neuron development in BDNF mutant

TABLE 1 Neuronal cell counts in sensory and sympathetic ganglia and facial motor nuclei of wild-type and mutant mice

	Wild-type		*nt4*$^{-/-}$		*bdnf*$^{-/-}$		*bdnf*$^{-/-}$/*nt4*$^{-/-}$	
Ganglion	Mean no. of neurons	% of control	Mean no. of neurons	% of control	Mean no. of neurons	% of control	Mean no. of neurons	% of control
Trigeminal	30,112 ± 1,920 (n = 3)	100 ± 6	29,704 ± 2,312 (n = 3)	98 ± 8			21,168 ± 2,264 (n = 3)	70 ± 8*
Vestibular	2,988 ± 240 (n = 3)	100 ± 8	2,364 ± 152 (n = 3)	79 ± 5	720 ± 96 (n = 4)	24 ± 3**	536 ± 100 (n = 4)	18 ± 3**
Superior cervical	15,564 ± 1,084 (n = 3)	100 ± 7	17,432 ± 1,208 (n = 3)	112 ± 8			14,908 ± 644 (n = 3)	96 ± 4
Nodose–petrosal	4,368 ± 368 (n = 4)	100 ± 8	1,768 ± 96 (n = 4)	41 ± 2**	1,876 ± 220 (n = 4)	43 ± 5**	416 ± 40 (n = 6)	10 ± 1***
Geniculate	912 ± 8 (n = 3)	100 ± 1	452 ± 48 (n = 3)	50 ± 5**	476 ± 108 (n = 4)	52 ± 12**	54 ± 12 (n = 6)	6 ± 1***
Dorsal root (L4)	6,744 ± 272 (n = 3)	100 ± 5	5,828 ± 124 (n = 4)	86 ± 2				
Facial motor neurons	2,788 ± 296 (n = 4)	100 ± 10	2,580 ± 308 (n = 4)	92 ± 11	2,864 ± 312 (n = 4)	102 ± 11	2,480 ± 216 (n = 4)	89 ± 7

Heads from embryos at day 18.5 of gestation were fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin and sectioned at 5 μm thickness. Sections were stained with cresylviolet. Neurons with a clear nucleus and nucleoli were counted in every eighth section. Dorsal root ganglia were dissected from two-month-old mice perfused with 4% paraformaldehyde and postfixed in 4% paraformaldehyde for 16 hours, equilibrated with 30% sucrose and sectioned at 7 μm thickness. Every fourth section was counted. Counts are displayed as number ±s.e.m.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

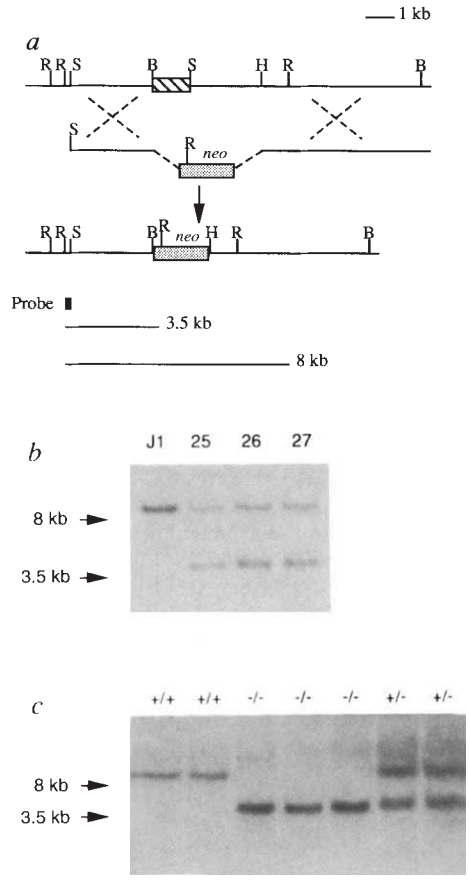

FIG. 1 Targeted deletion of the NT4 gene (*nt4*) in mouse embryonic stem cells and generation of mutant mice. *a*, Schematic diagram of the strategy used to target *nt4*. Top, restriction map of the *nt4* genomic DNA fragment; boxed area covers the entire coding sequences. Middle, the 11-kb replacement type targeting vector carrying a *neo* gene driven by a phosphoglycerate kinase promoter, which replaced the *Bgl*II–*Hin*dIII DNA fragment of *nt4*. Bottom, the structure of the targeted allele in which the entire coding sequences of *nt4* were deleted by the homologous recombination; the genomic probe external to the 5′ homologous arm is indicated that hybridizes to a 3.5-kb and an 8-kb DNA fragment from mutant and wild-type alleles, respectively. R, *Eco*RV; H, *Hin*dIII; B, *Bgl*II; S, *Sph*I. *b*, Southern blot of embryonic stem cell clones. DNA from parental J1 embryonic stem cells and independently cloned G418 resistant embryonic stem cell clones (number 25, 26, 27) were digested with *Eco*RV and hybridized to the external probe shown in *a*. Clones containing the expected 3.5-kb *Eco* RV fragment diagnostic for homologous recombinant were obtained at a frequency of 1 in 7. The blot was rehybridized to a *neo* probe to verify a single integration. The blot was also rehybridized to a NT4 cDNA probe to verify further the homologous recombinants in which the intensity of the 8-kb band was reduced to half that from J1 embryonic stem cells. Two independent recombinant embryonic stem cell clones contributed to the germ line of recipient embryos after blastocyst injection. *c*, Southern blot of offspring from a $nt4^{+/-} \times nt4^{+/-}$ cross. Tail DNA was extracted and digested with *Eco*RV and analysed as described in *b*.

METHODS. The J1 embryonic stem cells were obtained and cultured as described[29]. Following electroporation of embryonic stem cells with 25 μg ml^{-1} of linearized targeting vector at setting of 400 V, 25 μg (Bio-Rad gene pulsar), cells were selected in 0.4 mg ml^{-1} G418. Resident colonies were trypsinized individually. Half of the cells were expanded for freezing, and the other half for genotyping. Mice were generated as described[29].

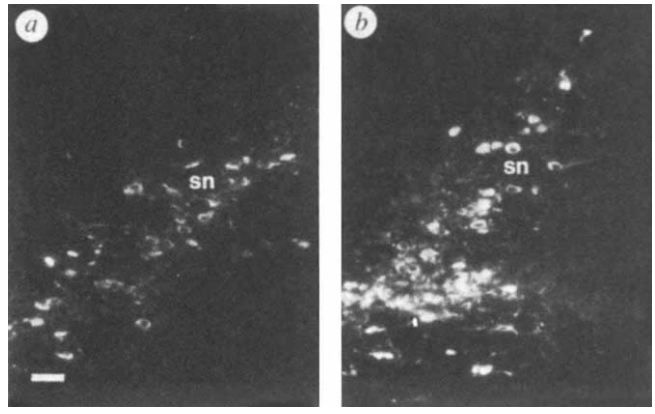

FIG. 2 Tyrosine hydroxylase immunohistochemistry of sections through the midbrain of *a*, wild-type, and *b*, NT4 mutant mice; sn, substantia nigra; scale bar, 10 μm.

METHODS. Four-month-old mice were perfused with 4% paraformaldehyde. Brains were dissected, postfixed for 16 hours, equilibrated with 30% sucrose and sectioned at 7 μm thickness. Sections were preincubated in dilution buffer (0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3, 3% bovine serum albumin and 0.3% Triton X-100) for 1 h followed by overnight incubation with rabbit anti-tyrosine hydroxylase antiserum (Peel-Freeze) diluted at 1:200 in dilution buffer containing 5% goat serum. After 4 washes in PBS, sections were incubated for 2 h with a rhodamine-conjugated goat anti-rabbit secondary antiserum (Cappel) (1:300 dilution).

mice by substituting for BDNF's function[14,16]. The observation that NT4–BDNF double-mutant mice have no significant loss of facial motor neurons suggests that BDNF and NT4 are not essential for motor-neuron survival, but does not exclude other potential involvement of these two neurotrophins in motor-neuron function. It is also possible that other unidentified factors may activate TrkB. For example, unrelated neurotrophic factors such as GDNF, which has been shown to support motor-neuron survival *in vitro*[24], and rescue motor neurons as well as mesancephalic dopaminergic neurons from degeneration *in vivo*[25–28], could replace some of the functions of BDNF and NT4 in embryonic development. Because of the potential therapeutic value of neurotrophins in the treatment of neurodegenerative diseases, it will be crucial to elucidate their role in the prenatal and postnatal nervous system. □

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Spontaneous resistance to acute T-cell leukaemias in TCRVγ1.1Jγ4Cγ4 transgenic mice

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THE concept of tumour surveillance implies that specific and non-specific components of the immune system eliminate tumours in the early phase of malignancy[1,2]. The immunological mechanisms that control growth of preneoplastic cells are, however, not known. T cells expressing γδ T-cell receptors (TCR) were first described as lymphocytes with reactivity against various tumour cells, which suggests that γδ T cells could mediate tumour surveillance[3–6]. Here we show that TCRVγ1.1Jγ4Cγ4 transgenic mice[7] are spontaneously resistant to acute T-cell leukaemias but cannot reject non-haematopoietic tumours. TCRVγ1.1Jγ4Cγ4$^+$ hybridomas isolated from these mice react *in vitro* against almost all haematopoietic tumour cell lines tested. Recognition of tumour cells depends on the γδ TCR but is independent of major histocompatibility complex (MHC) class I, MHC class II, or TAP-2 peptide transporter expression. Ligand recognition is influenced by the murine *Nramp* gene, which confers resistance or susceptibility to tuberculosis, lepra and leishmaniasis[8,9]. These data indicate that TCRVγ1.1$^+$ T cells confer spontaneous immunity against haematopoietic tumours *in vivo* and link innate resistance to bacterial infections with tissue-specific tumour surveillance by γδ$^+$ T cells.

To determine the function of circulating γδ T cells, we injected TCRVγ1.1Jγ4Cγ4 transgenic mice[7] with two T-cell leukaemia cell lines, ALC and RBL-5 (Fig. 1). The TCRVγ1.1 chain is the most prominent TCRγ chain expressed on T cells in the spleen, lymph nodes and peripheral blood of mice, but is only sparsely found in other tissues[3–6]. Injection of ALC or RBL-5 leukaemia cells leads to tumour growth in C57Bl/6 syngenic mice, defined by ascites and massive infiltration of the kidney, intestinum, liver, spleen and lymph nodes. TCRVγ1.1 transgenic mice were spontaneously resistant against both tumours as compared with TCRVγ1.1 non-transgenic littermates and C57Bl/6 mice (Fig. 1). We tested three TCRVγ1.1 transgenic mouse lines containing different copy numbers and transgenic integration sites. In two mouse strains containing high and intermediate copy numbers

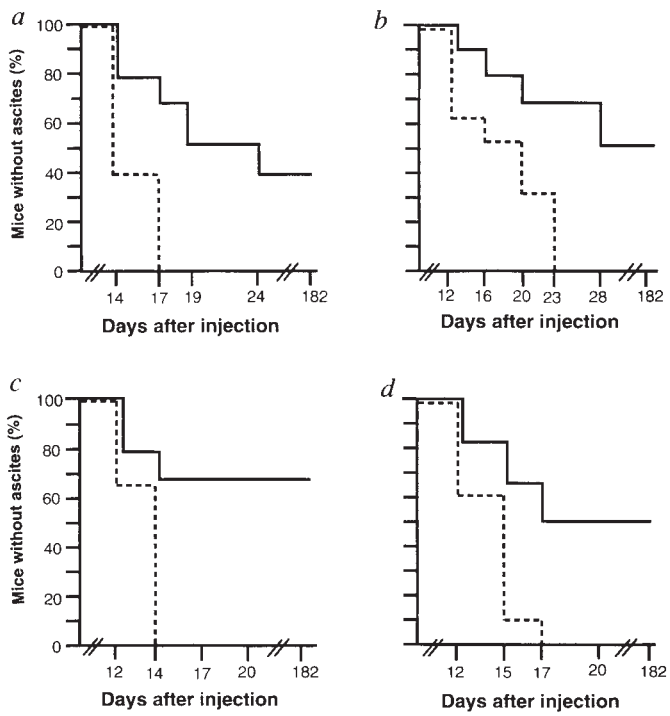

FIG. 1 Spontaneous resistance to RBL-5 (*a* and *b*) and ALC (*c* and *d*) leukaemia cells in TCRVγ1.1Jγ4Cγ4 transgenic mice. *a*, 1000 RBL-5 cells per C57Bl/6 (broken line) or syngenic TCRVγ1.1Jγ4Cγ4 (solid line) mice; 8 mice per group. *b*, 500 RBL-5 cells per TCRVγ1.1Jγ4Cγ4 transgenic-negative (broken line) or TCRVγ1.1Jγ4Cγ4 transgenic-positive (solid line) littermates; 9 mice per group. *c*, 1000 ALC cells per C57Bl/6 (broken line) or TCRVγ1.1Jγ4Cγ4 transgenic (solid line) mice; 9 mice per group. *d*, 500 ALC cells per C57Bl/6 (broken line) or syngenic TCRVγ1.1Jγ4Cγ4 Tg (solid line) mice; 10 mice per C57Bl/6 group and 16 mice per TCRVγ1.1$^+$ group. *a*, *c*, Very high copy number strain (~200 transgenic copies). *b*, *d*, Intermediate copy number strain (~20 transgenic copies). Data from the low copy number strain (~10 copies) are not shown[7]. Mice were followed for 6 months after tumour injection (day 182).
METHODS. Five-week-old TCRVγ1.1$^+$ mice (6th backcross into a C57Bl/6 background) and age-matched C57Bl/6 mice or TCRVγ1.1$^+$ and TCRVγ1.1$^-$ littermates were injected intraperitoneally with ALC or RBL-5 tumour cells, and the development of the tumour (ascites) was followed daily. Spontaneous resistance to tumour cells is dependent on the dose of tumour cells (not shown). Mice with ascites were killed for ethical reasons. To determine whether syngenic TCRVγ1.1$^+$ transgenic mice were immunologically compatible with C57Bl/6 mice, all transgenic mice resistant to RBL-5 or ALC tumours were transplanted with C57Bl/6 skin. We never observed skin rejection. In addition, RBL-5 and ALC tumour cells were not recognized by NK cells (not shown). Expression of the transgene was determined by genomic Southern blotting of *Eco* RI-digested tail DNA using a TCRCγ4 probe (not shown). All experiments conformed to Guidelines of the Canadian Research Council, and were approved by the Animal Care Committee of the Ontario Cancer Institute.

of the transgene the Vγ1.1 transgene conferred spontaneous resistance to ALC and RBL-5 (Fig. 1), whereas the strain containing a low copy number was not resistant against acute T leukaemias. Thus anti-tumour activity of TCRVγ1.1$^+$ cells depends on the expression level of the transgene and is not linked to the transgenic integration site.

ALC tumour cells expressing the clonotypic TCRVβ12 chain could be detected in the blood of TCRVγ1.1 transgenic mice 3 days after inoculation. In parallel, an expansion of γδ$^+$ T cells was detected in the spleen of TCRVγ1.1 transgenic and C57Bl/6 mice after injection of leukaemic T cells (not shown). Transgenic mice expressing the TCRVγ2Jγ1Cγ1 chain did not reject ALC or RBL-5 T cell leukaemias (not shown). In addition, growth kinetics of two C57Bl/6-derived fibrosarcomas (MC57X and