TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL

Gavin R. Screaton, Juthathip Mongkolsapaya, Xiao-Ning Xu, Alison E. Cowper, Andrew J. McMichael and John I. Bell

A subset of the tumour necrosis factor (TNF) receptor family contain a conserved intracellular motif, the death domain. Engagement of these receptors by their respective ligands initiates a signalling cascade that rapidly leads to cell death by apoptosis. We have cloned a new member of this family, TRICK2, the TRAIL (TNF-related apoptosis-inducing ligand) receptor inducer of cell killing 2. TRICK2 is expressed in a number of cell types, and to particularly high levels in lymphocytes and spleen. Two isoforms of the TRICK2 mRNA are generated by alternative pre-mRNA splicing and differ by a 29 amino-acid extension to the extracellular domain. Overexpression of TRICK2 rapidly induced apoptosis in 293T cells; this induction was dependent upon the presence of the death domain of TRICK2. Using a soluble molecule containing the TRICK2 extracellular domain, we demonstrated that TRICK2, like DR4 [1], is a receptor for TRAIL/APO-2L [2,3] and could inhibit TRAIL-induced killing of lymphocyte lines, such as the Jurkat T-cell line. TRAIL is upregulated upon lymphocyte activation, as is the intensively studied ligand for Fas, FasL [4]. TRAIL and its receptors might therefore provide another system for the regulation of lymphocyte selection and proliferation, as well as providing an additional weapon in the armoury of cytotoxic lymphocytes.

Address: Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK.

Correspondence: Gavin R Screaton

Received: **2 July 1997**
Revised: **1 August 1997**
Accepted: **1 August 1997**

Current Biology 1997, 7:693–696
http://biomednet.com/elecref/0960982200700693

Results and discussion

Cloning of TRICK2

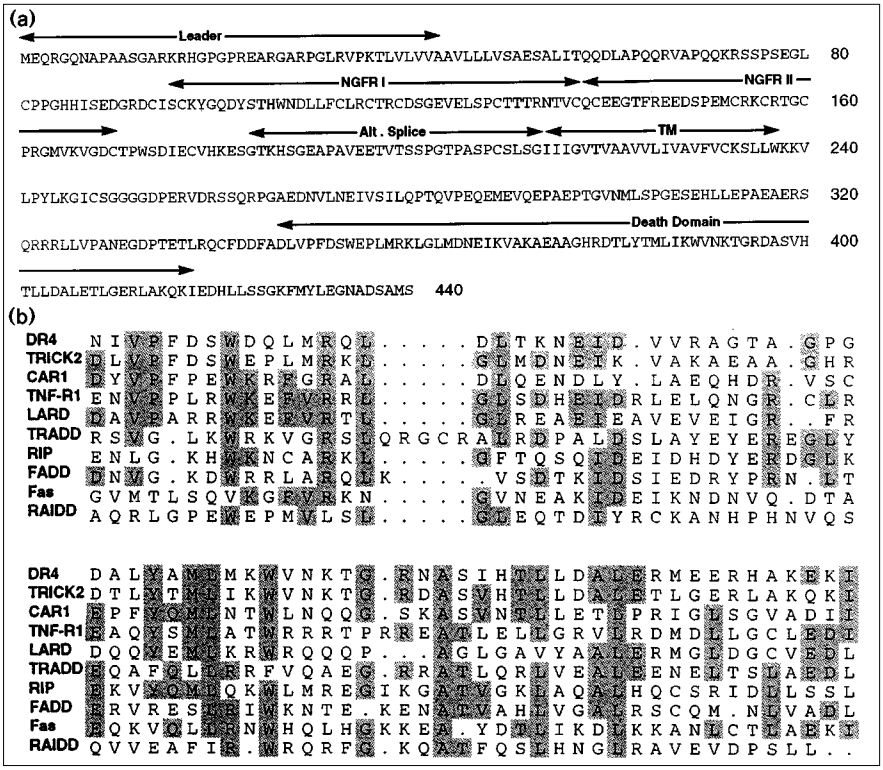
The expressed sequence tag subset of the National Centre for Biotechnological Information database was screened for homology to the cysteine-rich extracellular domain of the TNF receptor family and also for a degenerate death-domain sequence. Several novel sequences were identified, allowing oligonucleotide primers to be designed so as to amplify the coding region of TRICK2. The predicted amino-acid sequence of TRICK2 is shown in Figure 1a. TRICK2 is a type I membrane protein with a 43 amino-acid signal sequence and the mature protein has a predicted molecular mass of 43.4 kDa. The extracellular domain has 58% amino-acid identity to DR4 and encodes one partial and two complete nerve growth factor receptor (NGFR) repeats. Following a 27 amino-acid transmembrane domain, TRICK2 has a 203 amino-acid intracellular domain. A clear carboxy-terminal death domain was identified in TRICK2 and is aligned with the death domains of other proteins in Figure 1b. The death domain of TRICK2 has 30, 19, 31 and 65% identity to the death domains of the TNF receptor TNF-R1, Fas, LARD (lymphocyte-associated receptor of death) and DR4, respectively.

Cysteine-rich NGFR repeats are common to all TNF receptor family members, but the number of repeats varies, there being four in TNF-R1 and LARD/DR3/Wsl1/APO-3/TRAMP [5–9], three in Fas and only two in DR4, TRICK2 and the chicken death receptor CAR1 [10]. The TRICK2/DR4 NGFR repeats show homology to repeats 2 and 3 of the TNF receptor, consistent with the crystal structure of the TNFβ–TNF-R1 complex which revealed that the interaction surface on TNF-R1 contains residues in repeats 2 and 3 [11]. The occurrence and functional integrity of these two-domain receptors raises the question as to the function of the additional repeats found in other members of the TNF receptor family. It has been proposed that the first repeat of TNF-R1 could promote dimerisation of the receptor, which might not initiate a signalling pathway, and would therefore protect the cell from apoptosis in the absence of TNF [12].

Expression and alternative splicing of TRICK2

Northern blot analysis using TRICK2 cDNA as a probe revealed that a 4.5 kb mRNA was expressed in most tissues (thymus, prostate, testis, ovary, small intestine, colon, heart, placenta, lung, liver, skeletal muscle and kidney). Expression was particularly high in peripheral blood lymphocytes (PBLs), pancreas and heart, but was absent from brain (data not shown). After a longer exposure of the blot, an additional band at around 6.5 kb was visualised in many of the samples. As TRICK2 is expressed at high levels in PBLs and spleen, we examined its expression following lymphocyte activation. After 3 days' treatment of PBLs with phytohaemagglutinin (PHA), the expression of TRICK2 did not appear to change, but notably the expression of TRAIL increased markedly from almost undetectable levels in the resting state (Figure 2a). The upregulation of TRAIL mimics the upregulation of Fas ligand (FasL) seen after lymphocyte activation and might have consequences

Figure 1

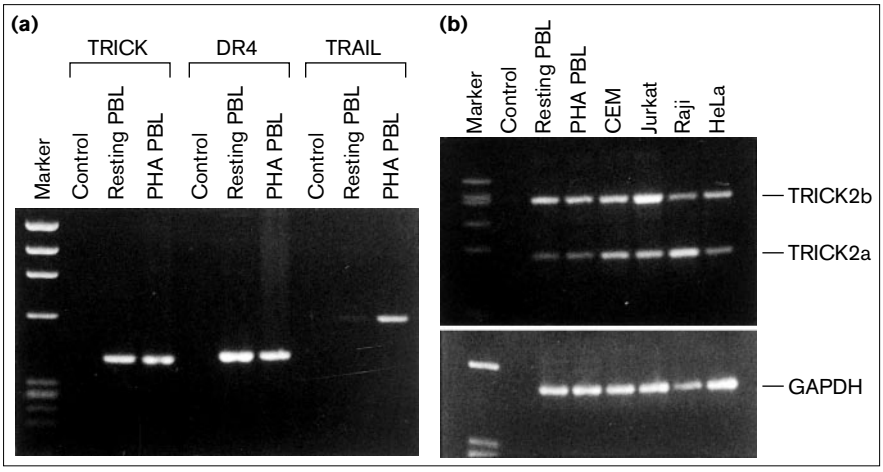


(a) Predicted amino-acid sequence of TRICK2b showing the predicted leader sequence (Leader), NGFR repeats (NGFRI and II), the region absent in the alternatively spliced clone TRICK2a (Alt. Splice), transmembrane domain (TM) and death domain. Genbank accession numbers: TRICK2a AF018657; TRICK2b AF018658. **(b)** Alignment of the death-domain sequences of a variety of death-domain-containing proteins. Shaded letters indicate conserved amino-acid residues.

for the survival of activated cells. In this respect, it has been shown that APO-2L/TRAIL causes apoptosis in interleukin-2-stimulated T lymphocytes [13].

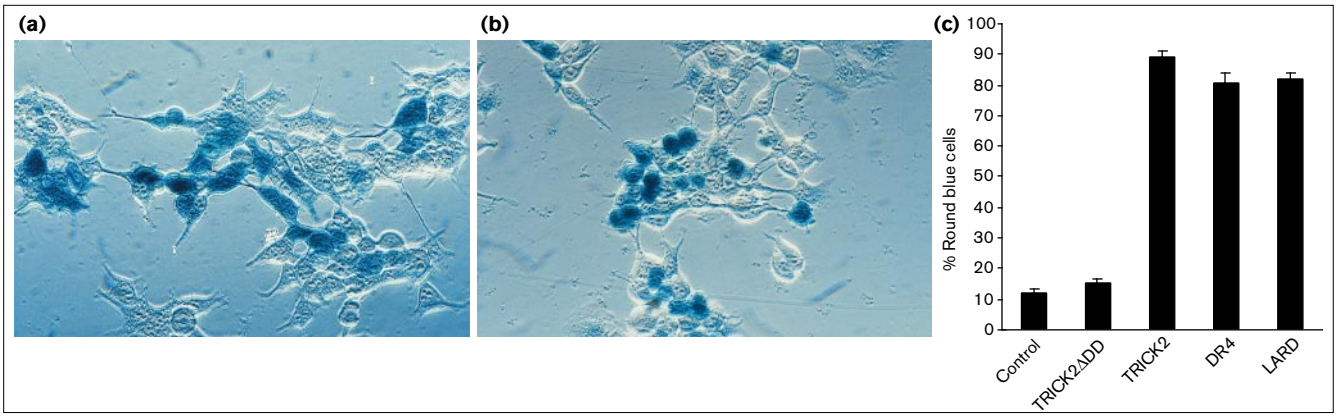
We identified two TRICK2 isoforms, TRICK2a and TRICK2b, which are expressed in a variety of tissues. TRICK2b has an 87 base-pair insertion compared with TRICK2a, resulting in an in-frame insertion of 29 amino acids. Alignment of the TRICK2 isoforms with DR4 revealed that DR4 does not contain this inserted sequence and is therefore similar in structure to TRICK2a. The inserted sequence may represent a retained intron as it has flanking sequences that match consensus 5′ and 3′ splice sites. In contrast to LARD [5], the ratio of TRICK2a : TRICK2b did not change upon T-cell activation, although a difference in the ratios of these two forms was found in a survey of lymphocyte tumour cell lines (Figure 2b, compare CEM with Jurkat). The expression of two isoforms of TRICK2 that have different spacings between the transmembrane domain and the TRAIL-binding domain might regulate the response to TRAIL. The reason for the apparent functional redundancy in the TRAIL system, in which there are two receptors for one ligand, is not clear. TNF has two receptors, but only one of these, TNF-R1 has a death domain. It will be interesting to study whether a complex formed by the binding of two or three different receptors — DR4, TRICK2a and TRICK2b — to a TRAIL trimer can signal death.

Figure 2



(a) RT-PCR amplification of TRICK2, DR4 and TRAIL from RNA from resting PBLs and PHA blast PBLs; the control samples contained no cDNA in the PCR step. **(b)** RNA from a range of cell lines was subjected to RT-PCR analysis across the alternatively spliced region of TRICK2, yielding products of 262 bp for TRICK2b and 175 bp for TRICK2a. The lower panel shows amplification using control GAPDH primers. RNA (10 μg) was used in an oligo-dT-primed cDNA synthesis reaction driven by avian myeloblastosis virus reverse transcriptase: 10% of this reaction was then amplified by PCR for 30 cycles with primers specific for the extracellular domains of TRICK2, DR4 and TRAIL.

Figure 3

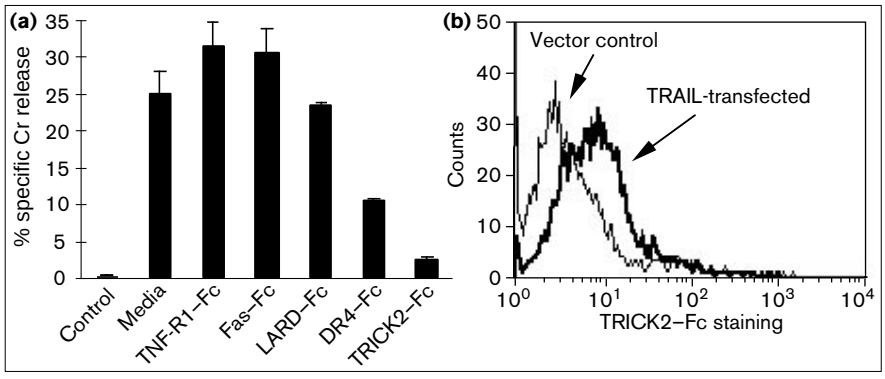


Effect of TRICK2 upon cell viability. COS cells cotransfected with a β-galactosidase plasmid plus **(a)** control plasmid (pCDNA3-Invitrogen), or **(b)** full-length TRICK2. The TRICK2-expressing blue cells show the typical features of apoptosis and round up compared to the cells transfected with the control plasmid. **(c)** Cells were counted 24 h after transfection and round blue cells were expressed as a percentage of total blue cells [15]. This figure is representative of five separate experiments in COS-1 or 293T cells, which gave equivalent results. TRICK2b was amplified using *Pfu* polymerase (Stratagene) and the primers F TRICK2 *Kpn* AGCTCGGTACCCCAACAAGACCT AGCTCCCCAG and R TRICK2 *Not* GAGTCCGCGGCCGCACTTTC CTACTGACTGGAGTCC, and following digestion with *Kpn*I and *Not*I TRICK2b was cloned into the expression vector pCD33L which contains the CD33 leader sequence [5]. Primer pair F TRICK2 *Kpn* (above) and R TRICK2 *Eco* GCAGAATTCTTACTTCCACAGTAAAGA CTTGCAAAC were used to clone the extracellular and transmembrane domains of TRICK2 into pCD33L. COS-1 and 293T cells were grown to 25% confluence in 6-well tissue culture plates and cotransfected with 0.25 μg pcDNA 3.1Myc-His/lacZ (Invitrogen) and 2 μg of the indicated second plasmid using calcium phosphate precipitation. Cells were fixed 24 h after transfection, stained with X-Gal and 100 cells from each well were counted.

Overexpression of TRICK2 leads to apoptosis

Overexpression of death-domain-containing molecules leads to apoptosis in the absence of their cognate ligands [14]. This effect presumably represents inappropriate aggregation of the overexpressed receptor which then recruits the intracellular death adaptor molecules leading to activation of the death pathway. TRICK2 was co-transfected with a β-galactosidase reporter plasmid into COS cells, and apoptosis was scored on the basis of cellular morphology: healthy cells correspond to those with several long pseudopodia whereas cells undergoing apoptosis round up and subsequently detach from the

Figure 4



Soluble TRICK2 inhibits TRAIL-mediated killing of Jurkat cells **(a)** The entire coding region of TRAIL was amplified from PHA blast PBL cDNA and cloned into pcDNA3. Then, 293T cells were cotransfected by calcium-phosphate precipitation with pcDNA3 TRAIL (40 μg per 15 cm plate) and a second plasmid containing the CD8α cDNA (10 μg per plate). Cells were lifted with PBS containing 3 mM EDTA 48 h after transfection, and cotransfected cells were isolated using anti-CD8 coated magnetic beads (DYNAL). Cells (10^5 per well) were seeded into 96-well plates and then mixed with ^{51}Cr-labelled Jurkat T cells (10^4) in the presence or absence of 20 μg/ml soluble fusion proteins comprising the extracellular domains of the receptors fused to the constant Fc domain of the IgG1 heavy chain. Background release was calculated by culturing the Jurkat cells in medium alone, RPMI supplemented with 10% foetal calf serum, or with CD8-purified 293T cells that had not been cotransfected with TRAIL. Supernatants were harvested 12–16 h later and ^{51}Cr release counted by scintillation on a beta-plate counter. The extracellular domains of Fas (amino acids 17–173), LARD (amino acids 25–200), TNF-R1 (amino acids 24–210), DR4 (amino acids 104–217) and TRICK2 (amino acids 57–183) were cloned to create a fusion with the CD5 leader and the Fc domain of IgG1. Recombinant protein was produced in 293T cells and purified using protein A–sepharose. **(b)** Soluble TRICK2 binds to TRAIL-transfected 293T cells.The cells were cotransfected with CD8 plus either TRAIL or an empty vector control plasmid. Cells were then incubated with a biotinylated TRICK2–Fc fusion protein and counterstained with phycoerythrin-conjugated streptavidin. Fluoroscein isothiocyanate-conjugated anti-CD8 antibody was used to detect and set a gate for the cotransfected cells as shown in the histogram.

monolayer [15]. Most cells transfected with the full-length receptor manifested the characteristic morphological changes associated with apoptosis (Figure 3a,b); the quantification of these results is shown in Figure 3c. Transfection with the empty vector, or with a deletion mutant of TRICK2 lacking the cytoplasmic domain (TRICK2ΔDD) did not lead to apoptosis; however, transfection with LARD or DR4 gave comparable results to TRICK2.

TRICK2 is a receptor for TRAIL

To examine whether TRICK2 is a receptor for TRAIL, a bioassay using TRAIL-mediated cytotoxicity was developed. This assay is similar to that commonly used to study FasL activity [16]. The targets in this assay were ^{51}Cr-labelled Jurkat T cells; by RT-PCR analysis, Jurkat cells were found to express both TRICK2 and DR4 (data not shown). The labelled cells were incubated for 12 hours with 293T cells that had been transfected with TRAIL and ^{51}Cr release was monitored in the supernatants by scintillation. We produced soluble fusion proteins comprising the extracellular domains of the death receptors TNF-R1, Fas, LARD, DR4 and TRICK2 and the immunoglobulin G1 Fc constant regions. The ability of these proteins to block TRAIL-mediated cytotoxicity was then tested in the bioassay. As expected, soluble TNF-R1, Fas and LARD had no inhibitory effect (Figure 4a). We observed a partial block to TRAIL cytotoxicity by DR4 and almost 100% inhibition by the TRICK2–Fc fusion protein, demonstrating that TRICK2 is a receptor for TRAIL. To confirm that TRICK2 is a TRAIL receptor, we stained cells with biotinylated TRICK2–Fc. TRICK2 stained TRAIL-transfected but not control cells (Figure 4b).

FasL has assumed a prominent role in the function and control of the immune system. The induction of FasL upon lymphocyte activation has two opposing effects: the augmentation of cytotoxic T-cell activity and the exposure of activated cells to the danger of apoptosis [4]. Mice deficient in both perforin and Fas expression still show some cytotoxic T-cell activity at 20 hours implying that other cytotoxic pathways, possibly including those induced by TNF, remain intact [17]. TRAIL is expressed at low levels on T cells and we have shown here that it is induced upon lymphocyte activation. This expression is similar to that of FasL and suggests that the TRAIL/DR4/TRICK2 system has the potential to play an auxiliary role to that of FasL/Fas in lymphocyte cytotoxicity and homeostasis.

Aberrant FasL expression occurs in a number of disease processes, such as HIV and malignant tumours. The expression of FasL by the infected or neoplastic cells might deliver a death signal to cytotoxic T cells expressing Fas [18,19]. This process would protect the target cells from cytotoxic T lymphocytes and allow their escape from immune surveillance. DR4 and TRICK2 are co-expressed in PBLs and lymphocyte cell lines, which are exquisitely sensitive to TRAIL-mediated cytotoxicity [2]. It is therefore possible that, as for FasL, inappropriate expression of TRAIL could protect cells from immune attack.

Acknowledgements

We thank the Washington University-Merck EST sequencing project for the provision of plasmids. We also thank the Wellcome Trust, MRC, Arthritis and Rheumatism Council, Siriraj Hospital Faculty of Medicine Mahidol University Thailand and Australian Government CARG for the support of the personnel and expenses of this research.

References

1. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, *et al.*: **The receptor for the cytotoxic ligand TRAIL.** *Science* 1997, **276:**111-113.
2. Wiley RR, Schooley K, Smolak PJ, Din WS, Huang C-P, Nicholl JK, *et al.*: **Identification and characterization of a new member of the TNF family that induces apoptosis.** *Immunity* 1995, **3:**673-682.
3. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A: **Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family.** *J Biol Chem* 1996, **271:**12687-12690.
4. Nagata S: **Apoptosis by death factor.** *Cell* 1997, **88:**355-365.
5. Screaton GR, Xu XX, Olsen AL, Cowper AE, Tan R, McMichael AJ, *et al.*: **LARD: A new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing.** *Proc Natl Acad Sci USA* 1997, **94:**4615-4619.
6. Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, *et al.*: **Signal transduction by DR3, a death domain-containing receptor related to TNF-R1 and CD95.** *Science* 1996, **274:**990-992.
7. Kitson J, Raven T, Jiang Y-P, Goeddel DV, Giles KM, Pun K-T, *et al.*: **A death-domain-containing receptor that mediates apoptosis.** *Nature* 1996, **384:**372-375.
8. Marsters SA, Sheridan JP, Donahue CJ, Pitti RM, Gray CL, Goddard AD, *et al.*: **Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-kappa B.** *Curr Biol* 1996, **6:**1669-1676.
9. Bodmer JL, Burns K, Schneider P, Hofmann K, Steiner V, Thome M, *et al.*: **TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and Fas(Apo-1/CD95).** *Immunity* 1997, **6:**79-88.
10. Brojatsch J, Naughton J, Rolls MM, Zingler K, Young JAT: **CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis sarcoma viruses and mediates apoptosis.** *Cell* 1996, **87:**845-855.
11. Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, Broger C, *et al.*: **Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation.** *Cell* **73:**431-445.
12. Naismith JH, Devine TQ, Kohno T, Sprang SR: **Structures of the extracellular domain of the type I tumor necrosis factor receptor.** *Structure* **4:**1251-1262.
13. Marsters SA, Pitti RM, Donahue CJ, Ruppert S, Bauer KD, Ashkenazi A: **Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA.** *Curr Biol* 1996, **6:**750-752.
14. Boldin MP, Mett IL, Varfolomeev EE, Chumakov I, Shemer AY, Camonis JH, *et al.*: **Self-association of the 'death domains' of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects.** *J Biol Chem* 1995, **270:**387-391.
15. Kumar S, Kinoshita M, Noda M, Copeland NG, Jenkins NA: **Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1 beta-converting enzyme.** *Genes Dev* 1994, **8:**1613-1626.
16. Suda T, Takahashi T, Golstein P, Nagata S: **Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family.** *Cell* 1993, **75:**1169-1178.
17. Braun MY, Lowin B, French L, Acha Orbea H, Tschopp J: **Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease.** *J Exp Med* 1996, **183:**657-661.
18. Xu X, Screaton GR, Gotch FM, Dong T, Tan R, Almond N, *et al.*: **Evasion of CTL responses by Nef-dependent induction of Fas ligand expression on simian immunodeficiency virus-infected cells.** *J Exp Med* 1997, **186:**1-10.
19. Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, *et al.*: **Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape.** *Science* 1996, **274:**1363-1366.