A Role for DHX32 in Regulating T-cell Apoptosis

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Abstract. *DHX32 is a novel putative RNA helicase with an activation-dependent pattern of expression in T-cells. To gain insight into the role of DHX32, Jurkat-DHX32 cells, a stable Jurkat T-cell line with constitutive DHX32 expression, were generated by retroviral gene transfer. There were no significant differences between control and Jurkat-DHX32 cells in terms of proliferation and response to several chemotherapeutic agents. There was an altered response of Jurkat-DHX32 cells to Fas signaling associated with down-regulation of the anti-apoptotic protein c-FLIP short. In normal peripheral blood lymphocytes, a correlation between DHX32 and c-FLIP short expression was detected in response to different T-cell specific and non-specific activation stimuli. Our results suggest that DHX32 might be involved in regulating T-cell response to certain apoptotic stimuli.*

RNA helicases are a large family of essential enzymes that participate in all aspects of RNA metabolism (1, 2). Several RNA helicases are involved in differentiation (3), whereas several others are dysregulated in various types of cancer (4, 5). These findings suggest that RNA helicases are involved in regulating gene expression. DHX32 was identified by our group as a novel putative RNA helicase, with dysregulated expression in lymphoid malignancies (6, 7). DHX32 has structural homology to helicases involved in mRNA splicing (6). The expression of DHX32 has been previously shown to be dramatically modulated in T-cells upon co-stimulation of CD3 and CD28 (8), suggesting its possible role in regulating T-cell responses. To gain insight into the role of DHX32, a Jurkat T-cell line that constitutively expressed DHX32, was established. In this report, the impact of constitutive expression of DHX32 on Jurkat cell proliferation and response to apoptotic stimuli was studied.

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Materials and Methods

Over-expression of DHX32 in Jurkat cells. Jurkat T-cells were obtained from ATCC and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. DHX32 cDNA Clone 30343301 (GenBank accession number BC068471) was obtained from the I.M.A.G.E. consortium in pPBLUSCRIPT R vector. The insert is 2898 bp long and contains full length coding sequence of DHX32 (489-2720). The plasmid containing DHX32 was digested with *Sac*I (position 474) and blunted, leaving a 19 bp 5' UTR upstream of the initiation ATG codon. The fragment was gel purified and subsequently digested with *Bam*HI at 162 bp in the 3' UTR of DHX32 ORF. The pFB-Neo retroviral vector (Stratagene, La Jolla, CA, USA) was digested with *Eco*RI and blunted, gel purified and digested with *Bam*H1 and ligated with the purified DHX32 insert to produce the pFB-DHX32 recombinant construct. The integrity of the recombinant vector was confirmed by sequencing. The recombinant construct and empty vector were used to produce recombinant and control viral particles, respectively, as described previously (9). Retrovirus-containing supernatants were harvested at 48 and 72 h after transfection, filtered through 0.45 μm filters and stored at –80˚C. Retroviral cell transduction was performed by using 8 μg DNA of recombinant and control constructs with the Polyfect reagent (Qiagen) according to the manufacturer's instructions. Transduced cells were grown in selection medium (800 μg of Neomycin) for more than 1 month.

Flow Cytometry and measurement of apoptosis. Control and Jurkat-DHX32 were permeabilized using Intraprep (Beckman/Coulter) according to the manufacturer's instructions. Blocking was performed by using 3% BSA in TBS buffer for 1 h followed by staining with previously described anti-DHX32 Ab (7) (1 in 200 dilution) for 1 h. The secondary antibody was an FITC-conjugated goat anti-rabbit antibody (Sigma, Oakville, ON, Canada). Cells were run on Facscaliber flow cytomer (BD Bioscience, Oakville, Canada) and analyzed by using Cell Quest Pro software. For Annexin V exposure, control and stimulated cells were incubated with Annexin V (Sigma) according to manufacturer's instructions followed by flow cytometric analysis. The morphological assessment of apoptosis was performed by staining various cellular cytospin preparations with Giemsa and scoring of apoptotic nuclei. Three to five hundred cells were counted and the percentage of apoptotic nuclei was determined.

Jurkat T-cell stimulation. The cells (2×10^4) (control or Jurkat-DHX32) were incubated in 1 ml tissue culture medium in 24-well

tissue plates for the specified times. To assess spontaneous proliferation, 10% (V/V) AlamarBlue™ (Biosource International, Camarillo, CA, USA, Cat.# DAL 1025) culture medium was added at the beginning of the culture. At the indicated time intervals, proliferation was determined by measuring the fluorescence of reduced Alamar blue, as previously described (10), using a Spectra Max microplate spectrofluorophotometer (Molecular Devices, Sunnyvale, CA, USA). PHA stimulation was performed by incubation with 2.5 μg/ml. For apoptosis induction, cells were grown as above and treated with Actinomycin D (5 μg/ml), Etoposide (10 μg/ml), Dexamethazone or anti-CD95 Ab. Means and standard deviations were calculated using Microsoft Excel Software.

Peripheral blood lymphocyte (PBL) preparation and T-cell activation. PBL were prepared by Ficoll (Sigma) separation of mononuclear cells from healthy laboratory volunteers followed by overnight culture on plastic tissue culture dishes to remove adherent cells. To activate PBL, cells were stimulated with phytohemaglutinin (PHA) (2.5 mg/ml for 24-48 h). Subsequent TCR simulation of PHA-activated PBL was performed by using control Ab, anti-CD3 and anti-CD28 Ab, as described previously (8).

Immunoblot analysis. Whole cell lysate from control and Jurkat-DHX32 was prepared as follows: cells were lysed at 4˚C with 1X cell lysis buffer (Cell Signaling Technology) supplemented with 1mM PMSF, 1% SDS, 10 mM DTT, 5% deoxycholic acid. In addition, one complete Mini Protease Inhibitor Cocktail Tablet per 10 mL of 1X lysis buffer (Roche Diagnostics, Laval, Quebec, Canada) was added. The lysate was sonicated 3 times (5 sec each time, 1 min on ice) at intensity 30 using a Biosonik III sonicator (Bronwill Scientific, Rochester, NY, USA). The protein concentration was determined by Bradford's method (Bio-Rad Laboratories, Mississauga, ON, Canada). After boiling in sample loading buffer, the samples were sonicated again, if viscous, at intensity 100 for 5 sec. Equal amounts of protein were subjected to SDS-PAGE on 12% resolving gel, transferred to nitrocellulose membrane. Blocking was performed in Tris Base Saline (10 mM Tris pH 7.4, 150 mM NaCl) containing 5% non-fat milk, followed by immunoblotting with primary Ab which was diluted 1 in 500 (anti-DHX32 Ab which has been previously described, or anti-Actin Ab (Sigma), or anti-FLIP$_{S/L}$ Ab (Santa Cruz Biotechnolgy, USA). The secondary Ab (Alkaline phosphatase conjugated anti-rabbit IgG Ab (Sigma) was then allowed to bind and was detected using NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (Sigma) as suggested by the manufacturer.

Reverse transcription-PCR (RT-PCR). The expression of various gene transcripts was detected by RT-PCR experiments using total RNA from control Jurkat cells, Jurkat cells over-expressing DHX32 (Jurkat-DHX32) cells, and PBL subjected to different activation stimuli. Complementary DNA was prepared using the Ready-To-Go RT-PCR Kit (Amersham Biosciences, Canada) according to the manufacturer's recommendations. All PCR reactions were conducted using 3 μL of the RT reaction as cDNA. The following primers were used: DHX32 forward 5'-CTATCTTAGCGT CCTGTG-3', DHX32 reverse 5'-GAGCCAAAAGCAGGTTCT-3'. c-FLIP short forward primer 5'-TCTCCAAGCAGCAATCCA-3', c-FLIP short reverse primer: 5'-
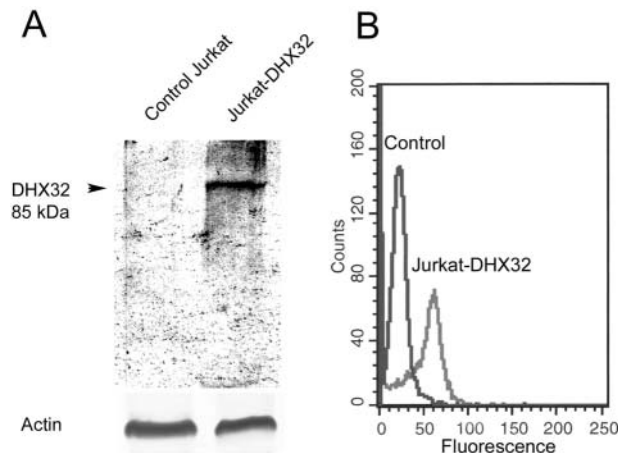

Figure 1. *Constitutive expression of DHX32 in Jurkat cells. Full length DHX32 was cloned in pFB neo-retroviral vector (Stratagene) and used to transfect Jurkat cells. A) The immunoblot of whole cell lysate using anti-DHX32 Ab directed against the C-terminus 15 aa peptide. Expression of an 85-kDa band corresponding to full length DHX32 was noted in Jurkat-DHX32 cells. B) The uniform expression of DHX32 in Jurkat-DHX32 cells a determined by flow cytometric analysis after permeabilization and staining with anti-DHX32 primary Ab and FITC-conjugated anti-rabbit Ab.*

GTAGAGGCAGTTC CATGT-3'. Primers c-FLIP short forward and c-FLIP short results in a 115-bp product corresponding to c-FLIP short transcript (Genbank accession # U97075). Actin: forward: 5'-TGGACATCCGCAAAGA CCTGTACGC-3', reverse: 5'-TGTCACCTTCA CCG TTCCAGTT-3'. The PCR conditions were as follows: an initial 95˚C for 5 min followed by 35 cycles of 95˚C for 30 sec, 48˚C for 30 sec and 72˚C for 3 min and a final 72˚C for 10 min.

Results

Constitutive expression of DHX32 alters Jurkat response to Fas signaling and c-FLIP short expression. The expression of DHX32 in Jurkat cells is modulated by co-stimulation of the CD3 chain of the TCR and CD28, suggesting a physiologically relevant role during the immune response (8). To gain insight into this role, a stable Jurkat cell line that constitutively expressed DHX32 through retroviral gene transfer was generated. Expression of DHX32 in Jurkat-DHX32, but not in control cells (transfected with empty vector), was documented by Western blot and flow cytometry analyses (Figure 1). DHX32 overexpression did not result in significant changes in morphology, cell cycle phases or the expression of CD3/CD4/CD8 cell surface molecules (data not shown). There were no significant differences between the two cell lines in proliferation (Figure 2 A and B). Consistent with this finding, no significant differences between the two cell lines in their IL-2 production (spontaneous or in response to PHA stimulation, data not shown) were detected.
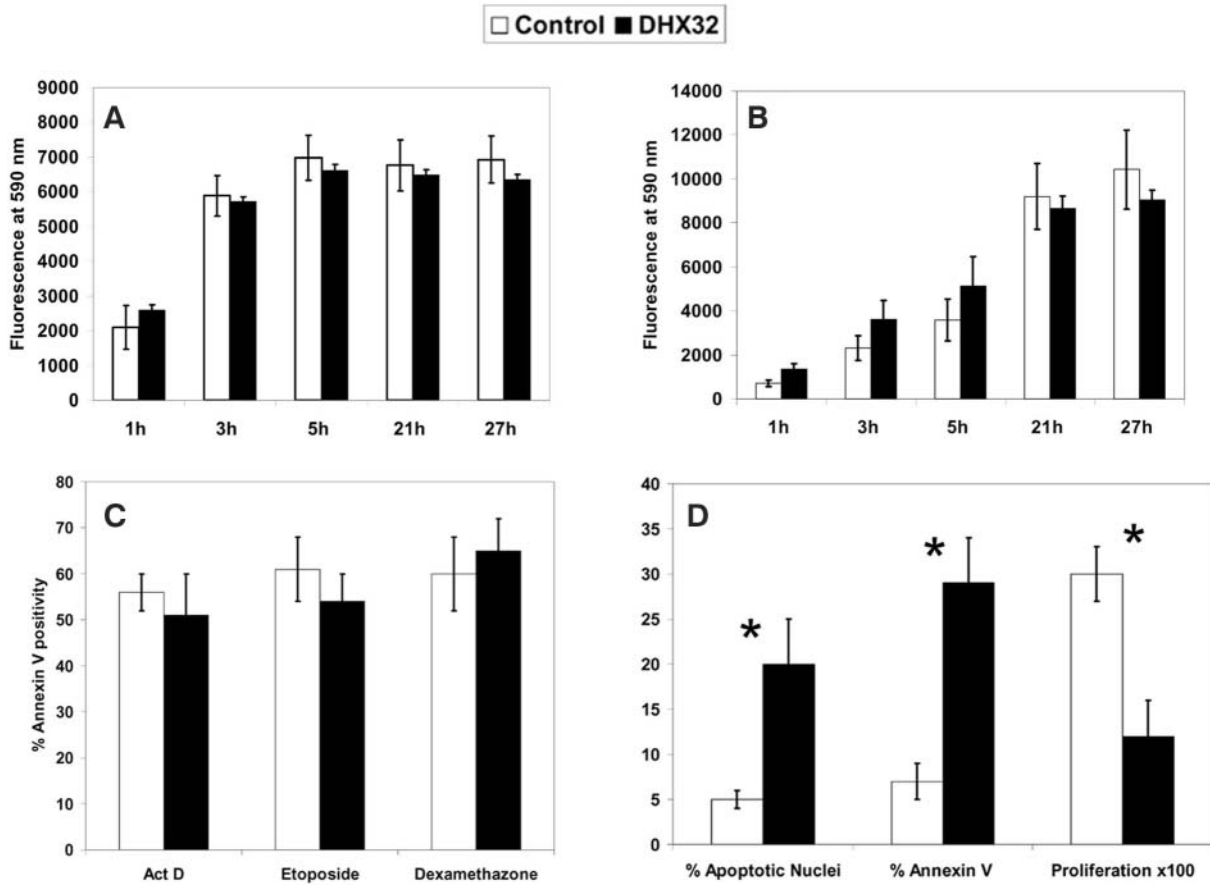
Figure 2. *Response of control and Jurkat-DHX32 to proliferation and apoptosis. No significant differences in spontaneous proliferation (A) or proliferation in response to PHA stimulation (B) of control and Jurkat-DHX32 cells as determined by measurement of fluorescence of reduced Alamar Blue dye. C) No significant differences in spontaneous apoptosis or in response to Actinomycin D (Act D), Etoposide, and Dexamethazone as measured by flow cytometric determination of surface exposure of Annexin V after 16 h of incubation were observed. D) Significant differences between control and Jurkat-DHX32 cells in response to Fas stimulation by using anti-Fas Ab (1 µg/ml for 16 h). The percentage of apoptotic nucleic was determined by morphological examination; Annexin V exposure was determined by flow cytometry; proliferation was determined by fluorescence of reduced Alamar Blue dye. The results represent the mean ± SEM, statistical significance was determined by Student's t-test *(p<0.05).*
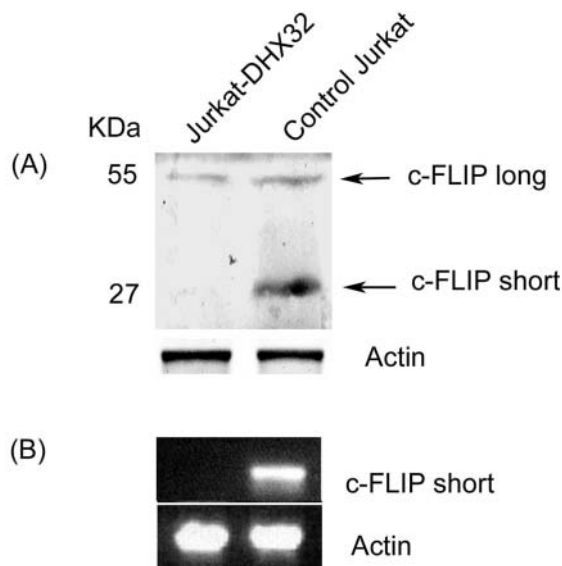

Figure 3. *Decreased expression of c-FLIP short in Jurkat-DHX32 cells. A) Immunoblot analysis using total cell lysates from control and Jurkat-DHX32 cells stained with anti-c-FLIP$_{S/L}$ Ab that detects c-FLIP long and c-FLIP short isoforms. Similar levels of the 55 kDa c-FLIP long isoform were seen, whereas a marked reduction of the 27 kDa c-FLIP short was noted in Jurkat-DHX32 cells. B) The results of RT-PCR using c-FLIP short mRNA transcript-specific primers indicates lower levels of c-FLIP short and in Jurkat-DHX32 cells. Actin controls for loading are shown.*
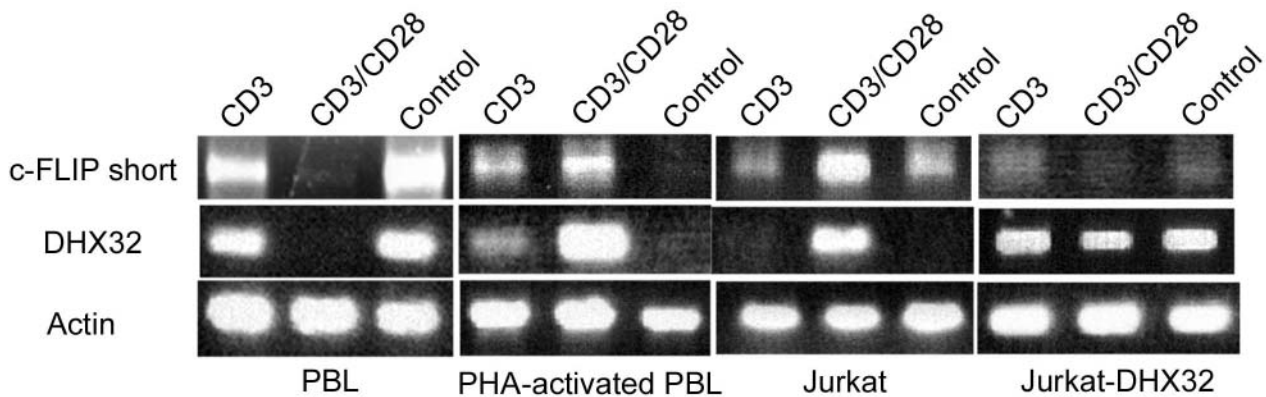
Figure 4. *Correlation between DHX32 and c-FLIP expression. RT-PCR analyses using primers specific for DHX32 and c-FLIP short transcripts showed a correlation between their expression in normal peripheral blood lymphocytes (PBL) and PHA-activated PBL subjected to ligation of CD3 and CD3/CD28 antigen. In the control Jurkat cells, there was a correlation between the two transcripts in response to CD3/CD28 stimulation. In the Jurkat-DHX32 cells with persistent expression of DHX32, there was no up-regulation of c-FLIP short in response to CD3/CD28 stimulation.*

To determine the effect of DHX32 expression on Jurkat apoptosis, control and Jurkat-DHX32 cells responses to various stimuli were compared. No significant differences were noted between the two cell lines in response to Actinomycin D, Etoposide or Dexamethazone (Figure 2C). However, there was a significant increase in apoptosis of Jurkat-DHX32 cells in response to stimulation of Fas through ligation with anti-Fas Ab (Figure 2D).

Fas signaling is a major pathway of activation-induced cell death in T-cells, which is regulated by c-FLIP protein isoforms (11-13). To elucidate the possible mechanism responsible for increased susceptibility of Jurkat-DHX32 cells to Fas signaling, immunoblot analysis was used to detect changes in the expression of major components of the Fas signaling pathway on whole cell lysates. Down-reglution of the anti-apoptotic c-FLIP short isoform was noted. No significant difference in the expression of c-FLIP long was seen. The down-regulation of c-FLIP short was also noted at the RNA level (Figure 3). There were no significant differences in the expression of other components of the Fas signaling pathway (Fas, Fas ligand, and caspase-8, data not shown).

Correlation between DHX32 and c-FLIP short expression in response to T-cell activation in normal peripheral blood lymphocytes (PBL). The expression of c-FLIP short in peripheral T-cells was shown to vary based on the state of activation (14). We have previously shown that the expression of DHX32 was modulated in response to CD3/CD28 co-stimulation in Jurkat cells (8). To determine whether the expression of DHX32 is correlated with that of c-FLIP short in PBL subjected to various activation stimuli,

RT-PCR analysis was employed to test for transcript expression. As shown in Figure 4, PBL control cells and PBL subjected to T-cell receptor stimulation with anti-CD3 showed expression of c-FLIP short and DHX32 transcripts. Co-stimulation of T-cell receptor and costimulatory molecules CD28 using anti-CD3 and anti-CD28 Ab resulted in down-regulation of both the c-FLIP short and DHX32 transcripts.

The activation of PBL with PHA resulted in the down-regualtion of DHX32 and c-FLIP short (similar to the effect of CD3/CD8 co-stimulation). Subsequent co-stimulation of PHA-activated PBL with anti-CD3/CD28 resulted in significant up-regulation of both DHX32 and c-FLIP. The expression of both transcripts was correlated in response to anti-CD3 activation only.

The expressions of DHX32 and c-FLIP short in control Jurkat cells were also correlated. Both transcripts were significantly up-regulated in response to CD3/CD28 stimulation. The correlation of expression between DHX32 and c-FLIP was absent in Jurkat-DHX32 cells.

Discussion

It was shown that constitutive expression of DHX32 in Jurkat T-cells altered their response to Fas-mediated apoptosis. Control and Jurkat-DHX32 cells have the same morphology, cell cycle and proliferation responses. In addition, both cell types showed similar responses to signals acting through different apoptotic pathways (Actinomycin D, Etoposide, and Dexamethazone). These findings suggest that the altered susceptibility of Jurkat-DHX32 cells to Fas signaling is specific. Down-regulation of the c-FLIP short isoform was found to be associated

with increased susceptibility to Fas-mediated apoptosis (14). The low expression level of the c-FLIP short isoform in Jurkat-DHX32 cells can provide a potential explanation for the increased susceptibility of these cells to Fas-mediated signaling.

The structural similarity of DHX32 to helicases involved in mRNA splicing (6) suggests a role in RNA processing. DHX32, but not other closely related splicing helicases, undergoes a dramatic modulation of expression in response to T-cell activation (7). This finding, together with the fact that several of the genes involved in regulating T-cell responses are regulated at the level of mRNA alternative splicing (reviewed in 15), suggest a role for DHX32 in regulating critical immune response genes. A correlation was found between DHX32 expression and the expression of the c-FLIP short transcript in PBL response to various T-cell-specific (CD3/CD28) and non-specific (PHA) stimuli. The lack of correlation between c-FLIP short expression and DHX32 in Jurkat-DHX32 could result from its dysregulated expression in the form of persistent constitutive expression. This abnormal pattern can lead to disruption of the expression pattern of potential target genes, such as c-FLIP short. The expression of DHX32 is dysregulated in various types of lymphoid malignancies in the form of down-regulation or over-expression (7). Therefore, it can be speculated that dysregulation of DHX32 in lymphoid malignancy might contribute to malignancy by altering the response to apoptotic stimuli, such as Fas signaling.

In summary, our data suggest that DHX32 plays a role in regulating apoptosis in T-cells. Determination of the impact of targeted dysregulated expression of DHX32 in a whole animal context (*e.g.* T-cell-specific transgenic and mutant mice) would contribute to determining the role of DHX32 in T-cell homeostasis.

Acknowledgements

References

1 Tanner NK and Linder P: DExD/H Box RNA helicases: from generic motors to specific dissociation functions. Mol Cell *8*: 251-262, 2001.

2 Abdelhaleem M, Maltais L and Wain HO: The human DDX and DHX families of putative RNA helicases. Genomics *81*: 618-622, 2003.

3 Abdelhaleem M: RNA helicase: regulators of differentiation. Clin Biochem *38*: 499-503, 2005.

4 Abdelhaleem M: Do RNA helicases have a role in cancer? Biochim Biophys Acta *1704*: 37-46, 2004.

5 Abdelhaleem M: Over-expression of RNA helicases in cancer. Anticancer Res *24*: 3951-3954, 2005.

6 Abdelhaleem M: The novel helicase homologue DDX32 is down-regulated in acute lymphoblastic leukemia. Leukemia Res *26*: 945-954, 2002.

7 Alli Z, Ho M and Abdelhaleem M: Expression of DHX32 in lymphoid tissues. Exp Mol Pathol *79*: 219-223, 2005.

8 Alli Z, Nam EH, Beimnet K and Abdelhaleem M: The activation-induced expression of DHX32 in Jurkat T-cells is specific and involves calcium and nuclear factor of activated T-cells. Cell Immunol *237*: 141-146, 2005.

9 Kitamura T, Onishi M, Kinoshita S, Shibuya A, Miyajima A and Nolan GP: Efficient screening of retroviral cDNA expression libraries. Proc Natl Acad Sci USA *92*: 9146-9150, 1995.

10 Ahmed SA, Gogal RM Jr and Walsh JE: A new rapid and non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. J Immunol Meth *170*: 211-224, 1994.

11 Thorburn A: Death receptor-induced cell killing. Cell Signal. *16*: 139-144, 2004.

12 Green DR, Droin N and Pinkoski M: Activation-induced cell death in T-cells. Immunol Rev *193*: 70-81, 2003.

13 Krueger A, Baumann S, Krammer PH and Kirchhoff S: FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol *24*: 8247-8254, 2001.

14 Kirchhoff S, Muller WW, Li-Weber M and Krammer PH: Up-regulation of c-FLIP short and reduction of activation-induced cell death in CD28-costimulated human T-cells. Eur J Immunol *30*: 2765-2774, 2000.

15 Lynch KW: Consequences of regulated pre-mRNA splicing in the immune system. Nat Rev Immunol *4*: 931-940, 2004.