Bcl-xL inhibits T-cell apoptosis induced by expression of SARS coronavirus E protein in the absence of growth factors

Yu YANG*[1], Zeyu XIONG*[1], Sheng ZHANG*, Yan YAN*, Justin NGUYEN*, Bernard NG*, Huifang LU*, John BRENDESE*, Fan YANG*, Hong WANG*† and Xiao-Feng YANG*‡[2]

*Department of Medicine, Laboratory of Immunopathology, Biology of Inflammation Center, Baylor College of Medicine, Houston, TX 77030, U.S.A., †Section of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine, Houston, TX 77030, U.S.A., and ‡Department of Immunology, Baylor College of Medicine, Houston, TX 77030, U.S.A.

One of the hallmark findings in patients suffering from SARS (severe acute respiratory syndrome) is lymphopenia, which is the result of massive lymphocyte death. SARS-CoV (SARS coronavirus), a novel coronavirus that has been etiologically associated with SARS cases, is homologous with MHV (murine hepatitis coronavirus), and MHV small envelope E protein is capable of inducing apoptosis. We hypothesized that SARS-CoV encodes a small envelope E protein that is homologous with MHV E protein, thus inducing T-cell apoptosis. To test this hypothesis, a cDNA encoding SARS-CoV E protein was created using whole gene synthesis. Our results showed that SARS-CoV E protein induced apoptosis in the transfected Jurkat T-cells, which was amplified to higher apoptosis rates in the absence of growth factors. However, apoptosis was inhibited by overexpressed antiapoptotic protein Bcl-xL. Moreover, we found that SARS-CoV E protein interacted with Bcl-xL *in vitro* and endogenous Bcl-xL *in vivo* and that Bcl-xL interaction with SARS-CoV E protein was mediated by BH3 (Bcl-2 homology domain 3) of Bcl-xL. Finally, we identified a novel BH3-like region located in the C-terminal cytosolic domain of SARS-CoV E protein, which mediates its binding to Bcl-xL. These results demonstrate, for the first time, a novel molecular mechanism of T-cell apoptosis that contributes to the SARS-CoV-induced lymphopenia observed in most SARS patients.

Key words: Bcl-xL, lymphopenia, protein–protein interaction, severe acute respiratory syndrome (SARS) virus, SARS-CoV E protein, T-cell apoptosis.

INTRODUCTION

During the 2002–2003 worldwide outbreak, SARS (severe acute respiratory syndrome) was identified as a new life-threatening pneumonia [1]. One of the hallmark findings in patients suffering from SARS was lymphopenia – observed in up to 100 % of these patients [2,3] – which occurs as a consequence of massive lymphocyte death [4]. At 700–1300 cells/μl [2], the lymphocyte counts in patients with lymphopenia are significantly less than the normal range of 1500–4000 cells/μl. Notwithstanding, the mechanism by which SARS-CoV (SARS coronavirus) induces the death of immune cells remains poorly defined.

A novel coronavirus (SARS-CoV) has been etiologically associated with cases of SARS [5], fulfilling Koch's postulates on causative agents, as modified by Rivers for viral diseases. Although homologous with each of the existing three groups of coronaviruses, SARS-CoV is most similar to the group 2 coronaviruses, which includes MHV (murine hepatitis virus) [6]. The positive-stranded RNA genome of SARS-CoV is approx. 30 kb in length, and its organization is similar to that of other coronaviruses [7]. Coronavirus encodes five virus-specific proteins, S, M, N, HE and E proteins [8]. E protein (i.e. small envelope protein) is present in infected cells, as well as in the viral envelope, and plays a significant role in virus replication [9]. Importantly, E protein is also conserved among all the coronaviruses [Pfam (protein family database): pfam02723.8, NS3_envE]. SARS-CoV E protein is an integral membrane protein with 76 amino acids (GenBank® accession no. NP_828854) [10] and it adapts a membrane topology with its C-terminal tail extended towards the cytosol [10,11]. In addition, recent reports confirmed that SARS-CoV E protein is indeed expressed in the SARS-CoV-infected cells [12,13], which lays a physiopathological foundation for further studies on the function of SARS-CoV E protein. The E protein from MHV can induce apoptosis that can be inhibited by overexpression of the antiapoptotic protein Bcl-2 [14]. Bcl-2 is a Bcl-xL homologue and the founding member of the Bcl-2/Bcl-xL apoptosis regulatory protein family [15]. It was the high structural homology between SARS-CoV E protein and MHV E protein that first suggested that the apoptosis induction feature of the SARS-CoV E protein might be conserved.

Cytotoxic and helper T-cells are essential to the direct clearance of coronavirus infection, and they enhance humoral immune responses to SARS-CoV [16]. It was observed that as the condition of SARS patients improved, T lymphocyte counts gradually returned to normal ranges [3]; this fact supported the idea that lymphopenia is temporally associated with disease severity. In addition, pathological observations [4] suggested that lymphocyte apoptosis directly induced by SARS-CoV might be the major cause of lymphopenia [14,17]. Recent progresses support this possibility that lymphocytes might be directly infected by SARS-CoV. Two new receptors for SARS-CoV infection have been identified, which are ACE2 (angiotensin-converting enzyme 2)

Abbreviations used: ACE2, angiotensin-converting enzyme 2; BH domain, Bcl-2 homology domain; ER, endoplasmic reticulum; FBS, fetal bovine serum; GST, glutathione S-transferase; MHV, murine hepatitis coronavirus; NP40, Nonidet P40; PBMC, peripheral blood mononuclear cells; Pfam, protein family database; RTN, reticulon; SARS, severe acute respiratory syndrome; SARS-CoV, SARS coronavirus; TCTP, translationally controlled tumour protein; TNT, transcription and translation.

[1] These authors have contributed equally to this work.

[2] To whom correspondence should be addressed, at Department of Medicine, Biology of Inflammation Center, Baylor College of Medicine (email xyang1@bcm.tmc.edu).

[18] and CD209L (also called L-SIGN, DC-SIGNR and DC-SIGN2) [19]. ACE2 is expressed in tonsil lymphocytes [20] and CD209L is expressed in Jurkat T-cells and lymph nodes [21], suggesting that T-cells could be directly infected by SARS-CoV via ACE2 and CD209L. Elucidation of the molecular mechanism for lymphocyte apoptosis induced by SARS-encoded proteins may lead to the development of SARS-CoV E-peptide-based inhibitors that will specifically prevent apoptosis in T-cells and enhance anti-SARS-CoV T-cell immunity.

The Bcl-2/Bcl-xL protein family plays a central role in the regulation of apoptosis [15] and the Bcl-2/Bcl-xL family members share sequence homology in one or more of the four BH (Bcl-2 homology) domains, including BH1–BH4. The Bcl-2/Bcl-xL family consists of more than 20 proteins that can be subdivided into three groups: (i) antiapoptotic proteins (Bcl-2 and Bcl-xL), (ii) pro-apoptotic proteins (Bax and Bak), and (iii) a large group of BH3-only death proteins [15]. We, and others, have previously demonstrated that antiapoptotic isoforms of the Bcl-x, Bcl-xL and Bcl-xγ genes contribute to the survival of T-cells [22–24]. Most recently, we identified a novel Bcl-xL interaction protein, TCTP (translationally controlled tumour protein), and we found that the interaction of Bcl-xL and TCTP in cytosol and mitochondria promotes T-cell survival [25]. A previous report showed that apoptosis induced by coronavirus [14] and other viruses can be commonly inhibited by antiapoptotic Bcl-xL [26]. However, an important question remained regarding whether SARS-CoV E protein-induced T-cell apoptosis could specifically be inhibited by Bcl-xL.

In the present study, we hypothesized that Bcl-xL inhibits T-cell apoptosis promoted by SARS-CoV E protein. We found that SARS-CoV E protein promoted apoptosis of transfected Jurkat T-cells induced by serum withdrawal. In addition, our results showed that SARS-CoV E protein interacted *in vitro* and *in vivo* with the antiapoptotic protein Bcl-xL via the Bcl-xL BH3 domain and a BH3-like domain of SARS-CoV E protein. This discovery revealed a novel molecular mechanism of T-cell apoptosis underlying SARS-CoV-induced lymphopenia.

EXPERIMENTAL

Synthesis of cDNA encoding SARS coronavirus E protein

A full-length cDNA encoding SARS-CoV E protein (GenBank® accession no. AY323975) [9,10] was fused on to the expression tag 3 × FLAG™ (Sigma–Aldrich, St. Louis, MO, U.S.A.); it was synthesized at DNA 2.0 Inc., a whole-gene synthesis company (Menlo Park, CA, U.S.A.), cloned into the pDrive Cloning Vector (Qiagen, Valencia, CA, U.S.A.) and verified by DNA sequencing from both strands at SeqWright (Houston, TX, U.S.A.).

Sequence analyses

Sequence analyses were performed using the GenBank® databases at the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov/), the NCBI SAGEmap database at http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd=tagsearch&org=Mm&tag=ACGAGCTGTT&anchor=NLAIII, the PROSITE database at the ExPASy Proteomics Tools website (http://us.expasy.org/tools/#proteome) and the Pfam at http://pfam.wustl.edu/.

Site-directed mutagenesis of SARS-CoV BH3-like region

A SARS-CoV BH3-like region mutant was constructed by mutating the BH3-like region encoding sequence directly on the wild-type SARS-CoV E cDNA pDrive vector and the wild-type SARS-CoV E cDNA pW120 vector, using a QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) with three pairs of mutagenic primers. The first pair of primers included the sense mutagenic primer SM1 (5′-ATTGTTAACGTGAGTGCAGTAGCACCAACGGCTTACGCCTACTCGGCTGTTAAAAATCTGAAC-3′) and the antisense mutagenic primer SM2 (5′-GTTCAGATTTTTAACAGCCGAGTAGGCGTAAGCCGTTGGTGCTACTGCACTCACGTTAACAAT-3′). The second pair of primers included the sense primer SM3a (5′-ATTGTTAACGTGAGTGCAGTAAAACCAACGGCTTACGTCTACTCGCGTGTTAAAAATCTGAAC-3′) and the antisense primer SM3b (5′-GTTCAGATTTTTAACACGCGAGTAGACGTAAGCCGTTGGTTTTACTGCACTCACGTTAACAAT-3′). The third pair of primers included the sense primer SM4a (5′-ATTGTTAACGTGAGTTTAGTAAAACCAACGGCTTACGCCTACTCGGCTGTTAAAAATCTGAAC3-′) and the antisense primer SM4b (5′-GTTCAGATTTTTAACAGCCGAGTAGGCGTAAGCCGTTGGTTTTACTAAACTCACGTTAACAAT-3′). The underlined sequences were designed for mutations of the BH3-like region of SARS-CoV, located from amino acids 51 to 61. The cDNAs encoding SARS-CoV E protein mutants were confirmed by DNA sequencing.

In vitro TNT (transcription and translation)

pDrive empty vector (Qiagen), pDrive vector containing cDNA encoding SARS-CoV E protein and pDrive vector SARS-CoV E protein BH3-like region mutant were used, respectively, in the *in vitro* TNT with a TNT® T7-coupled system (Promega, Madison, WI, U.S.A.) [23].

Preparation of GST (glutathione S-transferase), Bcl-xL and Bcl-xγ fusion proteins

Full-length Bcl-xL and full-length Bcl-xγ were fused to the C-terminus of GST in frame by subcloning Bcl-xL and Bcl-xγ cDNA fragments respectively into the GST vector pGEX-3X (GE Healthcare/Amersham Biosciences, Piscataway, NJ, U.S.A.) [23] and confirmed by DNA sequencing. The GST and GST fusion proteins were purified as described previously (GE Healthcare/Amersham Biosciences) [27].

GST pull-down assay

GST pull-down experiments were performed as described previously [25]. Briefly, ^{35}S-labelled recombinant proteins synthesized by TNT were confirmed on SDS/PAGE followed by autoradiography. TNT-prepared proteins (10 μl) were mixed with either 10 μg of GST protein, 10 μg of the GST fusion proteins GST–Bcl-xL or GST–Bcl-xγ respectively, together with 50 μl of glutathione–Sepharose® 4B bead slurry each, and 500 μl of ice-cold EBC-immunoprecipitation buffer with 0.5 % NP40 (Nonidet P40) [25] (Roche Applied Science, Indianapolis, IN, U.S.A.) [0.5 % NP40, 50 mM Tris/HCl, pH 8.0, and 120 mM NaCl, supplemented with the complete proteinase inhibitor cocktail (Roche Applied Science)]. After incubation and washing, interaction proteins bound to GST proteins and GST fusion proteins were eluted and subjected to separation by gradient SDS/PAGE (Invitrogen, Carlsbad, CA, U.S.A.), followed by detection with autoradiography (Eastman Kodak, Rochester, NY, U.S.A.).

Peptide competitive inhibition of Bcl-xL–SARS-CoV E protein interaction with Bcl-xL BH3 peptide

A peptide competitive inhibition assay with Bcl-xL BH3 domain peptide (*N*-MAAVKQALREAGDEFELRYRR-*C*) from Abgent (San Diego, CA, U.S.A.) was performed as reported previously

[25]. In brief, 5 μg of purified GST–Bcl-xL was incubated with 10 μl of [^{35}S]methionine-labelled *in vitro* translated SARS-CoV E protein, in the absence or presence of increasing concentrations of the peptide. After incubation, the mixtures were subsequently incubated with 50 μl of glutathione–Sepharose beads and the remaining GST pull-down procedures were completed [25].

T-cell apoptosis induced by SARS-CoV

Jurkat T-cells were cultured in RPMI 1640 and supplemented with 10 % (v/v) FBS (fetal bovine serum) at 37°C. The cDNA of SARS-CoV E protein was subcloned in the pCMV-10-FLAG (where CMV refers to the immediate early promoter of cytomegalovirus) expression vector under the direction of CMV promoter (Sigma–Aldrich). The Bcl-xL cDNA was subcloned into the c-Myc expression vector under the direction of CMV promoter (Invitrogen). Four groups of cDNAs, including (i) the pCMV-10-FLAG empty vector, (ii) pCMV-10-FLAG-SARS-CoV E, (iii) pc-Myc-Bcl-xL and (iv) pCMV-10-FLAG-SARS-CoV E plus pc-Myc-Bcl-xL, were transfected into Jurkat T-cells (A.T.C.C., Manassas, VA, U.S.A.) with the transfection reagent FuGENE 6 (Roche Applied Science). The Jurkat T-cells were selected with an antibiotic neomycin analogue (800 μg/ml G418) 24 h after transfection. Stably transfected Jurkat T-cells were cultured, 2 weeks after selection, at 37°C for 48 h, either in the G418-free RPMI 1640 medium supplemented with 10 % FBS (untreated control) or in the G418-free RPMI 1640 medium without FBS (growth-factor withdrawal). At 48 h after growth-factor withdrawal, apoptosis rates of the transfected Jurkat T-cells were measured by flow cytometry with the Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences/PharMingen, San Diego, CA, U.S.A.). The flow cytometry was performed on the Beckman-Coulter EPICS XL-MCL™ flow cytometer (Beckman Coulter, Miami, FL, U.S.A.) at Baylor Flow Cytometry Core Laboratories.

Co-immunoprecipitation and Western blotting

Co-immunoprecipitation was performed, as described previously [25]. Briefly, cells were lysed in EBC-immunoprecipitation buffer with 0.5 % NP40 (Roche Applied Science) [25]. After incubation with Protein G–Sepharose 4 Fast Flow beads (GE Healthcare/Amersham Biosciences) and an antibody followed by washing, the immunoprecipitates were eluted, loaded on gradient SDS/PAGE (Invitrogen), analysed by Western blotting with anti-FLAG M2 (Sigma–Aldrich) and revealed by chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) after exposure on an X-ray film (Kodak).

RESULTS

Overexpression of SARS-CoV E protein in T-cells promoted apoptosis

The E protein from MHV, a close homologue of SARS-CoV, can induce apoptosis [14]. Bioinformatic analyses have shown that SARS-CoV E protein has both the amino acid sequence (53 %) and the secondary structure homologous with the E protein of murine hepatitis virus (GenBank® accession no. P06591). In addition, recent reports showed that SARS-CoV E protein is expressed in the SARS-CoV infected cells [12,13]. These results suggest that the feature of apoptosis promotion by SARS-CoV E protein might be conserved. Thus we hypothesized that SARS CoV E protein (Figure 1A) could induce apoptosis in T-cells. We synthesized, via whole gene synthesis approach, the full-length cDNA encoding SARS-CoV E protein [28]. In view of technical difficulties related to the low efficiency of transient transfection

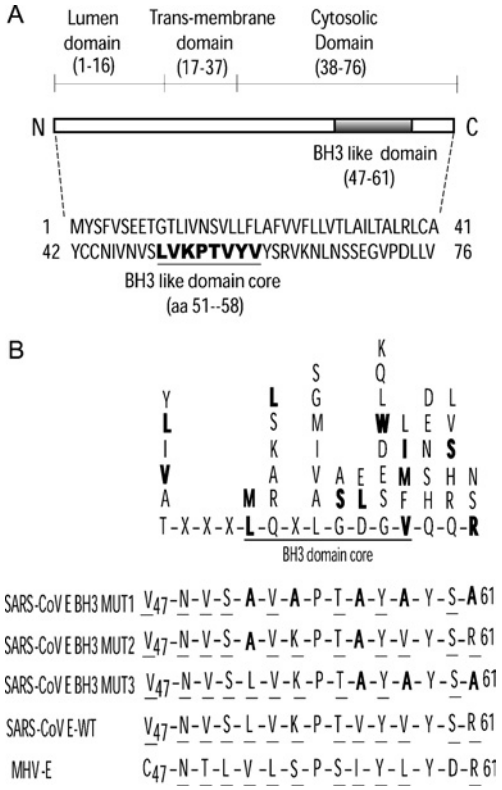

Figure 1 Structure of SARS-CoV E protein

(**A**) Schematic structure and sequence of SARS-CoV E protein. There are three protein domains in SARS-CoV E protein: the N-terminal lumen domain (N-terminal 16 amino acids), the transmembrane domain (amino acids 17–37) and the C-terminal cytosolic domain (from amino acid 38 to the C-terminus). The lower part of (**A**) shows the SARS-CoV E protein sequence (GenBank® accession no. AY323975). A BH3-like region located from amino acid 51 to 58 of the SARS-CoV E protein is shown in boldface and underlined. (**B**) BH3-like region of SARS-CoV E protein. The upper panel shows the updated BH3 domain consensus sequence (also see PROSITE accession no. PS01259). The lower panel shows the alignment of the sequences of three SARS-CoV E protein BH3-like region mutants, the sequence of the SARS-CoV E protein BH3-like region and the sequence of the homologous region in murine hepatitis virus with the BH3 domain consensus sequence. The identical/homologous amino acids in the BH3 domain consensus are shown in boldface. The mutant amino acids in SARS-CoV E protein BH3-like domain mutant are also in boldface. The homologous or identical residues in five sequences – including three SARS-CoV E BH3 mutants, SARS-CoV E protein (wild-type) and MHV E protein – are underlined.

in T-cells and the constant removal of dead cells during stable transfection against G418 selection, serum withdrawal (growth factor withdrawal) was widely documented as an apoptosis amplification approach [29] and used for SARS-CoV E-induced T-cell apoptosis. As depicted in Figures 2(A)–2(C), our results showed that the overexpression of SARS-CoV E protein resulted in increased apoptosis in transfected Jurkat T-cells in the absence of growth factors, from 20.7 % (in the medium with 10 % FBS) to 79.67 % (in the medium without FBS). In contrast, in the vector-transfected control cells, apoptosis rates were only increased from 11.4 % (in the medium with 10 % FBS) to 21.55 % (in the medium without FBS) (Figure 2B), which was similar to that of non-transfected cell controls (21.20 %) (Figure 2B). Similar apoptosis rates were also obtained in these samples by measuring with propidium iodide staining, followed by FACS analyses (results not shown), as described previously [23]. These results suggest that expression of SARS-CoV E protein in T-cells induces and sensitizes apoptosis independent of serum withdrawal. To consolidate this finding, we used a documented approach [30]

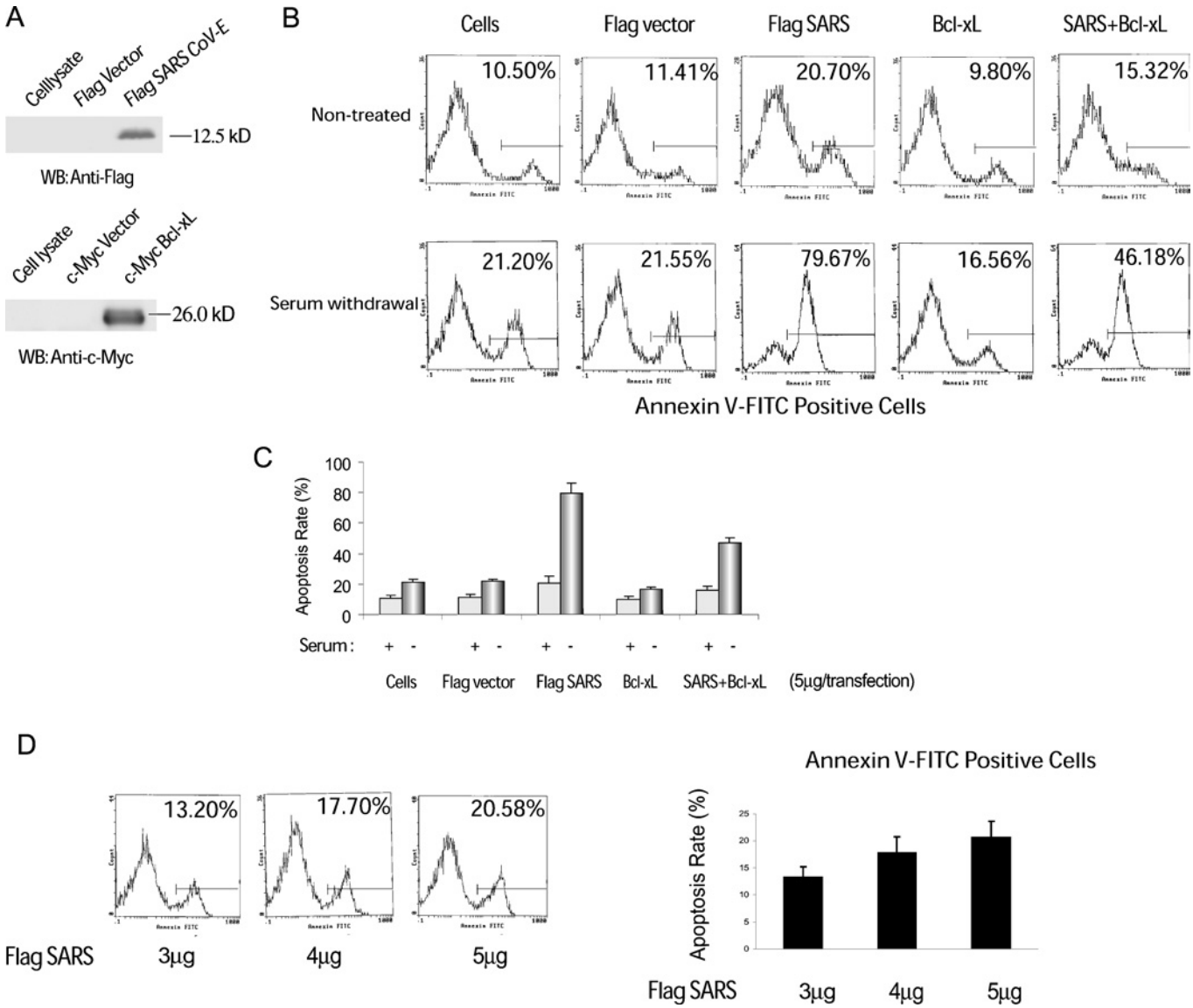

Figure 2 Induction of Jurkat T-cell apoptosis by expression of SARS-CoV E protein in the absence of growth factors

(**A**) Expression of SARS-CoV E protein and Bcl-xL in transfected Jurkat T-cells. The upper panel shows expressed SARS-CoV E protein fused to FLAG tag in the transfected Jurkat cells, detected by Western blotting (WB) with anti-FLAG antibodies. The lower panel shows expressed Bcl-xL fused to c-Myc tag in transfected Jurkat cells, detected by Western blotting with anti-c-Myc antibodies. (**B**) Bcl-xL inhibition of SARS-CoV E protein-induced apoptosis in Jurkat T-cells. Apoptosis was induced by expressed SARS-CoV E protein in T-cells (in the upper panels), which was amplified in the absence of growth factors (in the lower panels). The apoptosis rates of the following groups of Jurkat T-cells were measured by Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences/PharMingen): (i) Jurkat T-cell control; (ii) Jurkat T-cells transfected by FLAG vector; (iii) Jurkat T-cells transfected with FLAG–SARS-CoV E; (iv) Jurkat T-cells transfected with Bcl-xL; and (v) Jurkat T-cells co-transfected with FLAG–SARS-CoV E and Bcl-xL. Before measurement, the cells were either cultured in a serum-containing medium or cultured in a medium without serum (growth-factor withdrawal) for 48 h (untreated control). Percentages of Annexin V-FITC positive cells are shown. (**C**) Summary of the Annexin V-FITC flow cytometry data from three independent experiments. The mean percentage of apoptotic cells ± S.D. in each group is presented. (**D**) The left panel shows the positive correlation of increased apoptosis rates with increasing amounts of transfected SARS-CoV E protein cDNA. The right panel presents a summary of the Annexin V-FITC flow cytometry data from three independent experiments. The mean percentage of apoptotic cells ± S.D. in each group is presented.

to examine further the possibility that the apoptosis-inducing effect of SARS-CoV E protein is positively correlated with increasing doses of SARS-CoV E protein cDNA used for transfection. As shown in Figure 2(D), the apoptosis rates in SARS-CoV E protein cDNA-transfected cells were increased from 13.20 to 20.58 % as the SARS-CoV E protein cDNA used for transfection was increased from 3 to 5 μg/transfection. The positive correlation of increased T-cell apoptosis with increased amount of SARS-CoV E protein cDNA used for transfection suggests that expression of SARS-CoV E protein in T-cells indeed induces apoptosis.

SARS-CoV E protein induced apoptosis, which was inhibited by the antiapoptotic protein, Bcl-xL

Previous reports have shown that apoptosis induced by E protein from MHV, a SARS-CoV E homologous protein, can be inhibited by overexpression of the antiapoptotic protein Bcl-2, a Bcl-xL homologue [14]. To explore the potential mechanism and pathway whereby SARS-CoV E protein induces T-cell apoptosis, we expressed Jurkat T-cells with SARS-CoV E and Bcl-xL respectively (Figure 2A). As depicted in Figure 2(B), the results showed that co-transfected Bcl-xL inhibited T-cell apoptosis

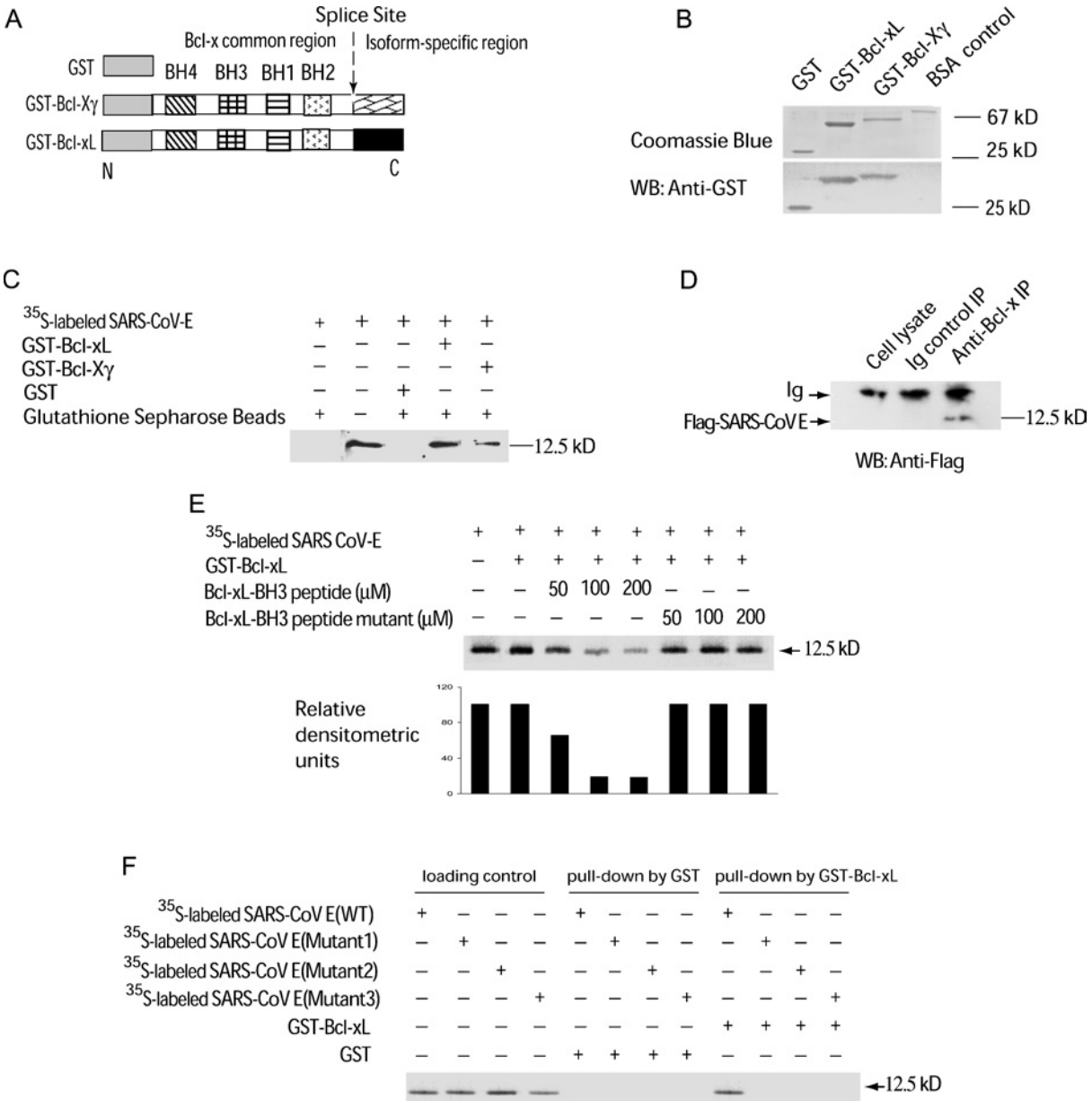

Figure 3 Interaction of SARS-CoV E protein and the antiapoptotic protein Bcl-xL

(**A**) Schematic representation of GST, GST–Bcl-xL and GST–Bcl-xγ fusion proteins. The N-terminal common region and the C-terminal isoform-specific region of Bcl-x isoforms are shown. BH domains 1–4 are located in the N-terminal common region, and shared by these two antiapoptotic Bcl-x isoforms Bcl-xL and Bcl-xγ. (**B**) Verification of purified GST and GST fusion proteins with Coomassie Blue staining, and Western blotting with anti-GST antibodies, which also serve as the protein loading controls for (**C**). (**C**) Interaction of SARS-CoV E protein with the N-terminal common region of Bcl-xL and that of Bcl-xγ. ^{35}S-labelled SARS-CoV E protein prepared by *in vitro* TNT was precipitated by GST–Bcl-xL and GST–Bcl-xγ, but not by an equivalent amount of GST. (**D**) Interaction of SARS-CoV E protein with endogenous Bcl-xL protein. The co-immunoprecipitation (IP) was performed with anti-Bcl-xL antibodies followed by Western blotting with anti-FLAG antibodies. (**E**) Interaction of SARS-CoV E protein with Bcl-xL via Bcl-xL BH3 domain. The precipitation of ^{35}S-labelled SARS-CoV E protein by GST–Bcl-xL was inhibited by increased concentrations (50, 100 and 200 μM) of Bcl-xL BH3 domain peptide. (**F**) Interaction of SARS-CoV E protein with Bcl-xL via the BH3-like region of SARS-CoV E protein. GST–Bcl-xL, but not GST, precipitated ^{35}S-labelled wild-type SARS-CoV E protein, but GST–Bcl-xL did not precipitate any of three ^{35}S-labelled SARS-CoV E BH3-like region mutants (mutants 1–3).

promoted by SARS-CoV E protein – from 79.67 % in SARS-CoV E alone transfected T-cells to 46.18 % in the T-cells co-transfected with SARS-CoV E and Bcl-xL cDNAs. This result implied that SARS-CoV E protein might induce T-cell apoptosis via a pathway antagonistic to the mitochondrion-dependent mechanism of Bcl-xL [25]. In our further efforts to examine SARS-CoV E protein induction of apoptosis in T-cells through direct interaction with the antiapoptotic protein Bcl-xL, we applied the GST pull-down assay [25]. As shown in Figure 3(B), equal amounts and similar purities of purified GST and GST fusion proteins were used in the GST pull-down assay (Figure 3C). We found that GST–Bcl-xL (Figures 3A and 3B) was able to precipitate ^{35}S-labelled SARS-CoV E protein (Figure 3C). In contrast, using equal amounts of control GST protein and glutathione–Sepharose

beads control, the SARS-CoV E protein could not be precipitated (Figure 3C). These results suggested that SARS-CoV E protein interacted directly with Bcl-xL.

Subsequently, we examined whether the Bcl-xL N-terminal common region or the Bcl-xL C-terminal isoform-specific region mediates SARS-CoV E–Bcl-xL interaction. When using GST–Bcl-xγ fusion protein in the GST pull-down assay, we found that, similar to GST–Bcl-xL, GST–Bcl-xγ (Figures 3A and 3B) could also precipitate SARS-CoV E protein (Figure 3C). Since Bcl-xγ and Bcl-xL are alternative spliced isoforms of Bcl-x transcripts (Figure 3A) and are known to share a long N-terminal common region (amino acids 1–188) [23], these results suggested that the N-terminal common region shared by these two Bcl-x isoforms is responsible for mediating the interaction of SARS-CoV E protein with Bcl-xL [25]. Finally, we transiently transfected HeLa cells with FLAG–SARS-CoV E cDNA. Then, we performed co-immunoprecipitation of endogenous Bcl-xL in HeLa cells using anti-Bcl-xL antibodies and antibody Ig control, followed by Western blotting with anti-FLAG antibodies. As shown in Figure 3(D), endogenous Bcl-xL interacted with SARS-CoV E protein. SARS-CoV E protein is a viral protein, but not an endogenous cellular protein, which prevented us from performing similar co-immunoprecipitation experiments with anti-SARS-CoV E protein antibodies, followed by Western-blot detection with anti-Bcl-xL antibodies. Notwithstanding, our findings showed that, in contrast with the result obtained using anti-Bcl-xL antibodies, non-specific IgG control could not co-precipitate SARS-CoV E protein (Figure 3D); this verified the specificity of the *in vivo* interaction between SARS-CoV E protein and Bcl-xL inside the cells.

The interaction of SARS-CoV E–Bcl-xL was mediated by Bcl-xL BH3 domain and a BH3-like region of SARS-CoV E protein

We then examined whether Bcl-xL interacts with SARS-CoV E protein via Bcl-xL BH3 domain in the N-terminal region by performing a peptide competition assay [25], which used a peptide that comprised the wild-type Bcl-xL BH3 domain (L^{90}REAGDEF^{97}). As shown in Figure 3(E), increased concentrations of the peptide bearing the wild-type Bcl-xL BH3 domain sequence (0, 50, 100 and 200 μM) significantly attenuated the binding of ^{35}S-labelled SARS-CoV E protein to GST–Bcl-xL, in comparison with that without peptide competition. It is noteworthy that positive correlation between the enhanced inhibition of SARS-CoV E protein binding to Bcl-xL and the increased concentrations of Bcl-xL BH3 peptide suggested the high specificity of peptide competition. A reduction of SARS-CoV E–Bcl-xL interaction at 200 μM of the Bcl-xL BH3 peptide was similar to that at 100 μM, suggesting that the assay had reached a plateau. In contrast, the mutated BH3 peptide of Bcl-xL in an equivalent concentration did not inhibit the interaction of SARS-CoV E with Bcl-xL (Figure 3E), suggesting that the integrity of the Bcl-xL BH3 domain is required for the interaction of Bcl-xL with SARS-CoV E protein. It is noteworthy that we recently demonstrated the reliability of the BH3 peptide competition assay in identifying the interaction of Bcl-xL and TCTP mediated by the BH3 domain of Bcl-xL and the BH3-like N-terminal region of TCTP [25]. Thus our findings suggested that Bcl-xL interaction with SARS-CoV E protein was specifically mediated by the Bcl-xL BH3 domain.

Our subsequent analyses employing the website-based proteome tools at http://us.expasy.org/tools/#proteome showed that SARS-CoV E protein is an integral membrane protein with three domains (Figure 1A), which are an N-terminal short lumen domain with 16 amino acids, a transmembrane domain with 20 amino acids (amino acids 17–37) and a C-terminal cytosolic domain with 39 amino acids (amino acids 38–76). The domain structure suggested that the C-terminal cytosolic region of SARS-CoV E protein could be spatially accessible for mediating interactions with other cytosolic proteins. In addition, a previous study showed that the C-terminal tail of a coronavirus E protein mediates interaction with the coronavirus M protein [31], indicating that the C-terminal domain of SARS-CoV E protein actually mediates protein–protein interaction(s). To determine whether SARS-CoV E protein contained a BH3-like region (Figure 1B) to potentially mediate the interaction between SARS-CoV E protein and Bcl-xL, we searched the C-terminal domain sequence of SARS-CoV E protein. As shown in Figure 1(B), there was an eight-amino-acid region L^{51}VKPTVYV^{58} that showed high homology – with two identical amino acids and four homologous amino acids – with the updated eight-amino-acid BH3 domain consensus core sequence [32] compiled from the BH3 domains of 20 published Bcl-2/Bcl-xL family members (updated from the deposit of PROSITE ID no. PS01259); such degrees of homology in the BH3 domain core are commonly observed among Bcl-2/Bcl-xL family members [15]. Moreover, the hydrophobicity of the region, predicted by the Kyte–Doolittle method and Eisenberg's method [23], further indicated that this region could function as a BH3-like region in mediating SARS-CoV E–Bcl-xL binding [15]. To determine whether this region was responsible for the interaction, we used site-directed mutagenesis to mutate an extended BH3 core region from the sequence L^{51}VKPTVYVYSR^{61} in the wild-type E protein into the sequence $\underline{A}^{51}$V$\underline{A}$PT$\underline{A}$Y$\underline{A}$YS$\underline{A}^{61}$ in mutant 1, the sequence $\underline{A}^{51}$VKPT$\underline{A}$YVYSR^{61} in mutant 2, and the sequence L^{51}VKPT$\underline{A}$Y$\underline{A}$YS$\underline{A}^{61}$ in mutant 3 (Figure 1B) respectively. In mutant 1, we mutated three hydrophobic residues, Leu^{51}, Val^{56} and Val^{58}, and two charged residues, Lys^{53} and Arg^{61}, into alanine residues. It was a concern that mutations of five residues in the 11 residues of the BH3-like domain region might affect protein folding. Therefore we constructed two additional mutants, including mutants 2 and 3. In mutant 2, we mutated two hydrophobic residues, Leu^{51} and Val^{56}, into alanine residues; and in mutant 3, we mutated two hydrophobic residues, Val^{56} and Val^{58}, and one charged residue, Arg^{61}, into alanine residues. The rationale for these mutations was that the charged-to-alanine mutation might disrupt protein–protein interactions, while causing a minor perturbation in protein structure [25]. In addition, the mutations to change the hydrophobic residues were designed in accordance with mutations previously used in the Bcl-xL BH3 mutant peptide to disrupt hydrophobic interactions between interacting partners [25]. The results showed that all the three SARS-CoV E protein mutants could not be precipitated by GST–Bcl-xL in the GST pull-down assay, suggesting that the region from amino acid 51 to 61 was responsible for the interaction between SARS-CoV E protein and Bcl-xL (Figure 3F). Even though there were differences between mutant 2 and mutant 3 in three amino acid residues, which were the amino acids 51, 58 and 61, these two mutants of SARS-CoV E protein shared one mutant residue Ala^{56}. Our results suggested that the evolutionarily conserved hydrophobic residue Val^{56} in SARS-CoV E protein (Ile^{56} in the MHV E protein) is critical for the interaction of SARS-CoV E protein with Bcl-xL. Our results on the importance of the sixth residue (Val^{56} in SARS-CoV E protein) (Figure 1B) in the BH3 domain core (D/**L**/E in the BH3 domain core; where boldface signifies identical/homologous amino acids in the BH3 domain consensus) also correlated with the previous report on the key residues in the BH3 domain of pro-apoptotic proteins [33]. Of note, the residue Val^{56} in SARS-CoV E protein and the residue Ile^{56} in the MHV E protein are homologous with

the residue Leu in this position of the BH3 domain core. It should also be noted that the high homology of SARS-CoV E protein with the BH3 core consensus sequence was also conserved in a C-terminal region of MHV E protein (GenBank® accession no. NP_068673.1; Figure 1B), which was reported to be capable of inducing apoptosis [14]. The analysis suggested that the E proteins of coronaviruses may share biochemical approach in induction of apoptosis.

DISCUSSION

The high prevalence of primary lymphopenia among SARS patients, upon admission [2], suggests that primary lymphopenia did not result from glucocorticoid therapy [34]. Similarly, cats infected with feline coronavirus and pigs infected with porcine respiratory coronavirus also had lymphopenia [35], suggesting that lymphopenia is a common feature of coronavirus infection. Previous studies showed that lymphopenia results from lymphocyte apoptosis induced by coronavirus proteins [17]. In addition, several other potential mechanisms have been proposed regarding lymphopenia induced by various viral infections [36]. Those mechanisms are effects of cytokines, bystander killing, syncytium formation, syncytium-dependent apoptosis, incapacity of fast thymic output and incapacities of homoeostasis-driven proliferation of peripheral T-cells. Notwithstanding, the mechanism underlying SARS-CoV-induced lymphopenia has been poorly defined. The results presented in this study demonstrate, for the first time, that SARS-CoV E protein induces apoptosis that is inhibited by Bcl-xL via SARS-CoV E–Bcl-xL interaction. More specifically, SARS-CoV E protein–Bcl-xL interaction is mediated by the BH3 domain of Bcl-xL and a BH3-like region in the C-terminal cytosolic domain of SARS-CoV E protein. Through a mitochondrion-dependent pathway, Bcl-xL plays a critical role in the inhibition of type II apoptosis triggered by growth-factor withdrawal, DNA damage and other apoptosis-inducing factors [15]. It is noteworthy that expression of SARS-CoV E protein alone induces T-cell apoptosis independent of serum starvation. SARS-CoV E protein-induced apoptosis is inhibited by overexpressed Bcl-xL, suggesting that the apoptosis induced by SARS-CoV E protein is triggered through the same mitochondrion-dependent pathway as serum starvation. Thus it is appropriate to employ serum starvation as an amplification approach for SARS-CoV E-induced apoptosis, as others reported [29]. Of note, in the cell controls and the vector transfection controls, the serum starvation in the absence of expression of SARS-CoV E protein in T-cells did not induce high rates of apoptosis. Collectively, our results support the theory that T-cell apoptosis induced by SARS-CoV E protein is an important mechanism of lymphopenia observed in patients infected with SARS-CoV.

Two receptors for SARS-CoV have been recently identified: ACE2 [18] and CD209L (also called L-SIGN, DC-SIGNR and DC-SIGN2) [19]. ACE2 is expressed in tonsil lymphocytes [20] and CD209L is expressed in Jurkat T-cells and lymph nodes [21], suggesting that T-cells could be directly infected with SARS-CoV via ACE2 and CD209L. In conjunction with these reports, our bioinformatic analyses with the NCBI SAGEmap database showed that ACE2 is expressed in human lymph nodes at the level of 40 tpm (tags/million), in contrast with 19 and 16 tpm in human colonic epithelium and human breast tissue respectively (NCBI UniGene accession no. Hs.178098); similar studies for the CD209L-specific tag did not yield any entry in the NCBI SAGEmap database (NCBI UniGene accession no. Hs.421437). Moreover, a member of the CEA (carcinoembryonic antigen) family, CEACAM1 (also known as CD66a or biliary glycoprotein), has been identified as the coronavirus receptor [37] expressed in T-cells [38]. T-cell activation can also induce the expression of CD13 (aminopeptidase N), another membrane receptor for coronaviruses [39]. Of note, receptor expression is not the sole determinant of viral tropism [40], suggesting that T-cells could be directly infected by coronaviruses, including SARS-CoV, through either one or all of these three receptors; this was verified by a recent report showing that human PBMC (peripheral blood mononuclear cells), including T-cells, are directly infected with SARS-CoV [41].

Previous studies have identified several viral proteins that induce mitochondrion-dependent apoptosis [17]. Thus our studies did not exclude the possibility that other proteins encoded by the SARS-CoV genome could induce apoptosis. Similarly, two proteins encoded by HIV, Nef and Tat, are known to induce apoptosis [17]. In 2004, several reports showed that SARS-CoV induced apoptosis in Vero E6 cells [42,43] and COS-1 cells [29]. A recent report specifically indicated that SARS-CoV N protein might induce apoptosis in COS-1 cells [29]. In addition, infection of human PBMC with SARS-CoV results in the up-regulation of 13 apoptosis-inducing genes [41]. Our future work proposes to examine the question, 'do SARS-CoV-induced apoptosis genes and SARS-CoV N protein act synergistically with SARS-CoV E protein in the induction of lymphopenia?'

It is noteworthy that several cellular BH3-only pro-apoptotic proteins (e.g. Bik, Blk, Hrk, Bim, Bnip3 and Nix) [15] are also integral membrane proteins. The membrane anchorage of the BH3-only proteins suggests that a BH3-like region containing SARS-CoV E protein may also be anchored on the membrane and promote apoptosis in a mode similar to the membrane-anchored BH3-only proteins. Also, it has been reported that coronavirus E protein is located on the membrane of the pre-Golgi compartment [44]. Based on the structural homology between SARS-CoV E protein and other coronavirus E proteins, this would imply that SARS-CoV E protein could be located in the same intracellular compartment, which was verified recently [45]. Our data on SARS-CoV E protein interaction with Bcl-xL, collected via biochemical methods, were consistent with the intracellular co-location of SARS-CoV E protein and Bcl-xL, the latter being located in both cytosol and mitochondria [46] as we verified recently [25]. A current model suggests that antiapoptotic proteins Bcl-xL/Bcl-2 inhibit apoptosis triggered by growth-factor withdrawal, DNA damage, ER (endoplasmic reticulum) stress and a pathway of death receptor signalling via truncated Bid etc. [15,47]. The mechanism underlying Bcl-xL/Bcl-2 function is that Bcl-xL inhibits apoptosis by blocking the activation and translocation of multidomain pro-apoptotic proteins Bax (Bak) and pro-apoptotic BH3-only proteins to mitochondria. Inhibition of Bcl-xL would lead to activation and translocation of BH3-only pro-apoptotic proteins and multidomain pro-apoptotic proteins from cytosol to mitochondria and trigger cytochrome *c* release and activation of caspase cascade [15,47]. On the basis of our own data and the current model of Bcl-xL inhibition of apoptosis, we have proposed a novel working model for the mechanism of SARS-CoV E protein in induction of T-cell apoptosis (Figure 4). Our future work will continue to test this model in which the 'sequestration' of Bcl-xL by SARS-CoV E protein on the pre-Golgi-compartment membranes [45] prevents Bcl-xL from inhibiting the activation of cytosolic pro-apoptotic protein Bax, or BH3-only pro-apoptotic proteins from translocating and docking to mitochondria, and enables pro-apoptotic proteins to trigger downstream apoptotic processes (e.g. cytochrome *c* release and caspase activation) [15]. It has been shown that a pro-apoptotic ER membrane protein, RTN-XS (where RTN stands for reticulon)

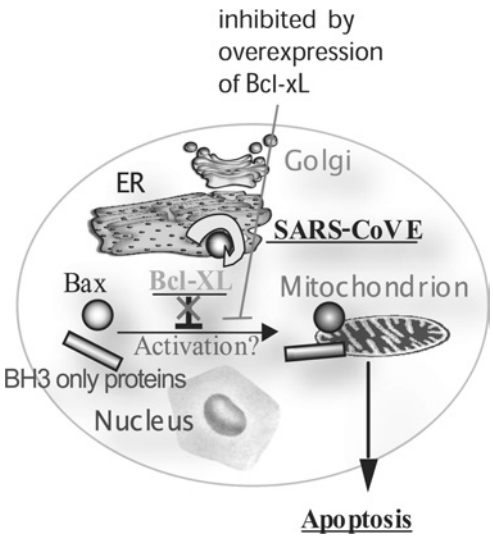

Figure 4 Schematic representation of the working model

SARS-CoV E protein induces apoptosis by 'sequestering' Bcl-xL to the membranes of ER and Golgi, where the SARS-CoV E protein is located. As a consequence, the existing balance between pro-survival protein Bcl-xL and pro-apoptotic proteins, including Bax and BH3-domain-only proteins, is tipped by SARS CoV E protein, so that sequestered Bcl-xL could not fulfil its normal function in inhibition of apoptosis. We have demonstrated that overexpressed Bcl-xL partially rescues the imbalance, and inhibits apoptosis induced by SARS-CoV E protein in T-cells.

[48], interacts with Bcl-xL on the ER membrane and reduces Bcl-xL antiapoptotic activity. Our results suggest that SARS-CoV E protein may promote apoptosis in a mode that is functionally similar to that of RTN-XS.

In summary, we have presented several findings: first, SARS-CoV E protein interacts with Bcl-xL *in vitro* and endogenous Bcl-xL *in vivo*; secondly, SARS-CoV E protein interaction with Bcl-xL is mediated by a novel BH3-like domain of SARS-CoV E protein and BH3 domain of Bcl-xL; finally, we have also identified a novel BH3-like region of SARS-CoV E protein that mediates its binding to Bcl-xL and presumably its pro-apoptotic activity. These results demonstrate, for the first time, a novel molecular mechanism of T-cell apoptosis that contributes to the SARS-CoV-induced lymphopenia observed in most SARS patients. Further elucidation of the mechanism(s) of SARS-CoV-induced lymphopenia may lead to the development of novel therapeutic interventions related to the prevention of lymphopenia and the enhancement of anti-virus immune responses.

We are grateful to Dr D. P. Huston and Dr S. Greenberg of Baylor College of Medicine for their encouragement. In addition, we greatly appreciate the efforts of Dr L. Guo, our Section Administrator, Ms K. Franks and her associates, as well as the editorial assistance of J. E. Young and G. Bennett. This work was supported in part by National Institutes of Health grants AI054514, P30 DK56238 and P20 CA103698; funding was also provided by the American Heart Association (Texas Affiliate), the Leukemia and Lymphoma Society, the Myeloproliferative Disorders Foundation, and T. T. Chao Scholar Award (to X.-F. Y.).

REFERENCES

1 Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H. R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R. A. et al. (2003) Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348**, 1967–1976
2 Booth, C. M., Matukas, L. M., Tomlinson, G. A., Rachlis, A. R., Rose, D. B., Dwosh, H. A., Walmsley, S. L., Mazzulli, T., Avendano, M., Derkach, P. et al. (2003) Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area. JAMA, J. Am. Med. Assoc. **289**, 2801–2809
3 Yang, M., Li, C. K., Li, K., Hon, K. L., Ng, M. H., Chan, P. K. and Fok, T. F. (2004) Hematological findings in SARS patients and possible mechanisms. Int. J. Mol. Med. **14**, 311–315
4 Ding, Y., Wang, H., Shen, H., Li, Z., Geng, J., Han, H., Cai, J., Li, X., Kang, W., Weng, D. et al. (2003) The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. J. Pathol. **200**, 282–289
5 Holmes, K. V. and Enjuanes, L. (2003) Virology. The SARS coronavirus: a postgenomic era. Science **300**, 1377–1378
6 Snijder, E. J., Bredenbeek, P. J., Dobbe, J. C., Thiel, V., Ziebuhr, J., Poon, L. L., Guan, Y., Rozanov, M., Spaan, W. J. and Gorbalenya, A. E. (2003) Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. **331**, 991–1004
7 Lai, M. and Holmes, K. V. (2001) Coronaviridae: the viruses and their replication. In Fundamental Virology (Knipe, D. and Howley, P. M., eds.), pp. 641–663, Lippincott Williams and Wilkins, Philadelphia, PA
8 Thiel, V., Ivanov, K. A., Putics, A., Hertzig, T., Schelle, B., Bayer, S., Weissbrich, B., Snijder, E. J., Rabenau, H., Doerr, H. W. et al. (2003) Mechanisms and enzymes involved in SARS coronavirus genome expression. J. Gen. Virol. **84**, 2305–2315
9 Maeda, J., Repass, J. F., Maeda, A. and Makino, S. (2001) Membrane topology of coronavirus E protein. Virology **281**, 163–169
10 Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A., Butterfield, Y. S., Khattra, J., Asano, J. K., Barber, S. A., Chan, S. Y. et al. (2003) The genome sequence of the SARS-associated coronavirus. Science **300**, 1399–1404
11 Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Penaranda, S., Bankamp, B., Maher, K., Chen, M. H. et al. (2003) Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300**, 1394–1399
12 Chan, W. S., Wu, C., Chow, S. C., Cheung, T., To, K. F., Leung, W. K., Chan, P. K., Lee, K. C., Ng, H. K., Au, D. M. et al. (2005) Coronaviral hypothetical and structural proteins were found in the intestinal surface enterocytes and pneumocytes of severe acute respiratory syndrome (SARS). Mod. Pathol. **18**, 1–8
13 Tripp, R. A., Haynes, L. M., Moore, D., Anderson, B., Tamin, A., Harcourt, B. H., Jones, L. P., Yilla, M., Babcock, G. J., Greenough, T. et al. (2005) Monoclonal antibodies to SARS-associated coronavirus (SARS-CoV): identification of neutralizing and antibodies reactive to S, N, M and E viral proteins. J. Virol. Methods **128**, 21–28
14 An, S., Chen, C. J., Yu, X., Leibowitz, J. L. and Makino, S. (1999) Induction of apoptosis in murine coronavirus-infected cultured cells and demonstration of E protein as an apoptosis inducer. J. Virol. **73**, 7853–7859
15 Opferman, J. T. and Korsmeyer, S. J. (2003) Apoptosis in the development and maintenance of the immune system. Nat. Immunol. **4**, 410–415
16 Yang, Z. Y., Kong, W. P., Huang, Y., Roberts, A., Murphy, B. R., Subbarao, K. and Nabel, G. J. (2004) A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature (London) **428**, 561–564
17 Everett, H. and McFadden, G. (2001) Viruses and apoptosis: meddling with mitochondria. Virology **288**, 1–7
18 Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C. et al. (2003) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature (London) **426**, 450–454
19 Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., Thomas, Jr, W. D., Thackray, L. B., Young, M. D., Mason, R. J. et al. (2004) CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc. Natl. Acad. Sci. U.S.A. **101**, 15748–15753
20 Harmer, D., Gilbert, M., Borman, R. and Clark, K. L. (2002) Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. FEBS Lett. **532**, 107–110
21 Liu, H., Yu, W., Liou, L. Y. and Rice, A. P. (2003) Isolation and characterization of the human DC-SIGN and DC-SIGNR promoters. Gene **313**, 149–159
22 Ma, A., Pena, J. C., Chang, B., Margosian, E., Davidson, L., Alt, F. W. and Thompson, C. B. (1995) Bclx regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci. U.S.A. **92**, 4763–4767
23 Yang, X. F., Weber, G. F. and Cantor, H. (1997) A novel Bcl-x isoform connected to the T cell receptor regulates apoptosis in T cells. Immunity **7**, 629–639
24 Yang, X.-F., Ye, Q., Press, B., Han, R. Z., Bassing, C. H., Sleckman, B. P., Alt, F. W. and Cantor, H. (2002) Analysis of the complex genomic structure of Bcl-x and its relationship to Bcl-xgamma expression after CD28-dependent costimulation. Mol. Immunol. **39**, 45–55
25 Yang, Y., Yang, F., Xiong, Z., Yan, Y., Wang, X., Nishino, M., Mirkovic, D., Nguyen, J., Wang, H. and Yang, X. F. (2005) An N-terminal region of translationally controlled tumor protein is required for its antiapoptotic activity. Oncogene **24**, 4778–4788

26 Moriishi, K., Koura, M. and Matsuura, Y. (2002) Induction of Bad-mediated apoptosis by Sindbis virus infection: involvement of pro-survival members of the Bcl-2 family. Virology **292**, 258–271

27 Yang, X. F., Wu, C. J., McLaughlin, S., Chillemi, A., Wang, K. S., Canning, C., Alyea, E. P., Kantoff, P., Soiffer, R. J., Dranoff, G. et al. (2001) CML66, a broadly immunogenic tumor antigen, elicits a humoral immune response associated with remission of chronic myelogenous leukemia. Proc. Natl. Acad. Sci. U.S.A. **98**, 7492–7497

28 Wildin, R. S., Wang, H. U., Forbush, K. A. and Perlmutter, R. M. (1995) Functional dissection of the murine lck distal promoter. J. Immunol. **155**, 1286–1295

29 Surjit, M., Liu, B., Jameel, S., Chow, V. T. and Lal, S. K. (2004) The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. Biochem. J. **383**, 13–18

30 Yang, X., Khosravi-Far, R., Chang, H. Y. and Baltimore, D. (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell (Cambridge, Mass.) **89**, 1067–1076

31 Corse, E. and Machamer, C. E. (2003) The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. Virology **312**, 25–34

32 Kelekar, A. and Thompson, C. B. (1998) Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol. **8**, 324–330

33 Day, C. L., Chen, L., Richardson, S. J., Harrison, P. J., Huang, D. C. and Hinds, M. G. (2005) Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. J. Biol. Chem. **280**, 4738–4744

34 Panesar, N. S., Lam, C. W., Chan, M. H., Wong, C. K. and Sung, J. J. (2004) Lymphopenia and neutrophilia in SARS are related to the prevailing serum cortisol. Eur. J. Clin. Invest. **34**, 382–384

35 Jabrane, A., Girard, C. and Elazhary, Y. (1994) Pathogenicity of porcine respiratory coronavirus isolated in Quebec. Can. Vet. J. **35**, 86–92

36 Gougeon, M. L. (2003) Apoptosis as an HIV strategy to escape immune attack. Nat. Rev. Immunol. **3**, 392–404

37 Tan, K., Zelus, B. D., Meijers, R., Liu, J. H., Bergelson, J. M., Duke, N., Zhang, R., Joachimiak, A., Holmes, K. V. and Wang, J. H. (2002) Crystal structure of murine sCEACAM1a[1,4]: a coronavirus receptor in the CEA family. EMBO J. **21**, 2076–2086

38 Nakajima, A., Iijima, H., Neurath, M. F., Nagaishi, T., Nieuwenhuis, E. E., Raychowdhury, R., Glickman, J., Blau, D. M., Russell, S., Holmes, K. V. et al. (2002) Activation-induced expression of carcinoembryonic antigen-cell adhesion molecule 1 regulates mouse T lymphocyte function. J. Immunol. **168**, 1028–1035

39 Lendeckel, U., Arndt, M., Frank, K., Wex, T. and Ansorge, S. (1999) Role of alanyl aminopeptidase in growth and function of human T cells. Int. J. Mol. Med. **4**, 17–27

40 Murray, M. G., Bradley, J., Yang, X. F., Wimmer, E., Moss, E. G. and Racaniello, V. R. (1988) Poliovirus host range is determined by a short amino acid sequence in neutralization antigenic site I. Science **241**, 213–215

41 Ng, L. F., Hibberd, M. L., Ooi, E. E., Tang, K. F., Neo, S. Y., Tan, J., Murthy, K. R., Vega, V. B., Chia, J. M., Liu, E. T. et al. (2004) A human *in vitro* model system for investigating genome-wide host responses to SARS coronavirus infection. BMC Infect. Dis. **4**, 34

42 Mizutani, T., Fukushi, S., Saijo, M., Kurane, I. and Morikawa, S. (2004) Importance of Akt signaling pathway for apoptosis in SARS-CoV-infected Vero E6 cells. Virology **327**, 169–174

43 Yan, H., Xiao, G., Zhang, J., Hu, Y., Yuan, F., Cole, D. K., Zheng, C. and Gao, G. F. (2004) SARS coronavirus induces apoptosis in Vero E6 cells. J. Med. Virol. **73**, 323–331

44 Lim, K. P. and Liu, D. X. (2001) The missing link in coronavirus assembly. Retention of the avian coronavirus infectious bronchitis virus envelope protein in the pre-Golgi compartments and physical interaction between the envelope and membrane proteins. J. Biol. Chem. **276**, 17515–17523

45 Nal, B., Chan, C., Kien, F., Siu, L., Tse, J., Chu, K., Kam, J., Staropoli, I., Crescenzo-Chaigne, B., Escriou, N. et al. (2005) Differential maturation and subcellular localization of severe acute respiratory syndrome coronavirus surface proteins S, M and E. J. Gen. Virol. **86**, 1423–1434

46 Hsu, Y. T., Wolter, K. G. and Youle, R. J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. U.S.A. **94**, 3668–3672

47 Jiang, X. and Wang, X. (2004) Cytochrome C-mediated apoptosis. Annu. Rev. Biochem. **73**, 87–106

48 Tagami, S., Eguchi, Y., Kinoshita, M., Takeda, M. and Tsujimoto, Y. (2000) A novel protein, RTN-XS, interacts with both Bcl-XL and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. Oncogene **19**, 5736–5746

Received 27 April 2005/20 July 2005; accepted 28 July 2005
Published as BJ Immediate Publication 28 July 2005, doi:10.1042/BJ20050698