

# UC Irvine

## UC Irvine Previously Published Works

### Title

Molecular changes associated with increased TNF- $\alpha$ -induced apoptosis in naïve (TN) and central memory (TCM) CD8+ T cells in aged humans

### Permalink

<https://escholarship.org/uc/item/9102h5d5>

### Journal

Immunity & Ageing, 15(1)

### ISSN

1742-4933

### Authors

Gupta, Sudhir  
Su, Houfen  
Agrawal, Sudhanshu  
[et al.](#)

### Publication Date

2018-12-01

### DOI

10.1186/s12979-017-0109-0

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

RESEARCH

Open Access



# Molecular changes associated with increased TNF- $\alpha$ -induced apoptosis in naïve ( $T_N$ ) and central memory ( $T_{CM}$ ) CD8+ T cells in aged humans

Sudhir Gupta<sup>1,2\*</sup>, Houfen Su<sup>1</sup>, Sudhanshu Agrawal<sup>1</sup> and Sastry Gollapudi<sup>1</sup>

## Abstract

**Background:** Progressive T cell decline in aged humans is associated with a deficiency of naïve ( $T_N$ ) and central memory ( $T_{CM}$ ) T cells. We have previously reported increased Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced apoptosis in  $T_N$  and  $T_{CM}$  T cells in aged humans; however, the molecular basis of increased apoptosis remains to be defined. Since expression of TNF receptors (TNFRs) was reported to be comparable in young and aged, we investigated signaling events downstream of TNFRs to understand the molecular basis of increased TNF- $\alpha$ -induced apoptosis in aged  $T_N$  and  $T_{CM}$  CD8+ cells.

**Results:** The expression of TRAF-2 and RIP, phosphorylation of JNK, IKK $\alpha$ / $\beta$ , and I $\kappa$ B $\alpha$ , and activation of NF- $\kappa$ B activation were significantly decreased in  $T_N$  and  $T_{CM}$  CD8+ cells from aged subjects as compared to young controls. Furthermore, expression of A20, Bcl-x<sub>L</sub>, cIAP1, and FLIP-L and FLIP-S was significantly decreased in  $T_N$  and  $T_{CM}$  CD8+ cells from aged subjects.

**Conclusions:** These data demonstrate that an impaired expression/function of molecules downstream TNFR signaling pathway that confer survival signals contribute to increased apoptosis of  $T_N$  and  $T_{CM}$  CD8+ cells in aged humans.

**Keywords:** TNF- $\alpha$ , A20, TRAF-2, RIP, cFLIP, NF- $\kappa$ B

## Background

Aging is associated with a progressive decline in immune responses including impaired proliferative and effector responses, impaired T cell signaling, and increased frequency of infections [1–12]. However, molecular mechanisms for immune dysfunction with age are poorly understood. Following antigenic stimulation naïve CD8+ T cells ( $T_N$ ) undergo activation and clonal expansion to generate effector CD8+ T cells. After clearance of antigen, majority of effector cells undergo apoptosis, and a subpopulation of effectors cells is retained as long-term memory cells [13]. Based upon their homing properties, and expression of adhesion molecules and chemokine

receptors, memory T cells are classified into central memory ( $T_{CM}$ ) and effector memory ( $T_{EM}$ ) CD8+ T cells [14–22]. We, and others have reported their characteristics with regard to proliferative response, cytokine production, effector properties, and sensitivity to apoptosis via death receptors, mitochondrial, and endoplasmic reticulum stress signaling pathways [21, 23–25].

TNF- $\alpha$  is a pleiotropic cytokine that activates T cells via both TNF-RI and TNF-RII and mediates both apoptotic and survival signals [26–35]. TNF $\alpha$ -mediates its biological functions predominantly via TNFR-I. Following binding of TNF- $\alpha$  to TNFR-I, the TNFR-associated death domain (TRADD) is recruited to TNFR-I forming a platform for downstream signaling. TNRF-associated factor 2 (TRAF2) and receptor-interacting protein kinase 1 (RIPK1) are recruited to TRADD forming a signaling complex. TRADD also recruits fas-associated death

\* Correspondence: sgupta@uci.edu

<sup>1</sup>Program in Primary Immunodeficiency and Aging, Division of Basic and Clinical Immunology, University of California, Irvine, USA

<sup>2</sup>Division of Basic and Clinical Immunology, Medical Sci. I, C-240, University of California at Irvine, Irvine, CA 92697, USA

domain (FADD), which initiates activation of apical caspases resulting in activation of effector caspases, and apoptosis. Both RIPK1 and TRAF2 recruit IKK $\alpha$  and IKK $\beta$  to the signaling complex resulting in NF- $\kappa$ B activation [36, 37]. NF- $\kappa$ B translocates to the nucleus, binds to the promoter, and induces a number of anti-apoptotic genes, including FLIP, IAPs, A20, Bcl-x<sub>L</sub> [32–34]. TRAF2 also activates MAP kinase/JNK pathway; prolonged JNK activation may result in apoptosis [38].

In human aging, TNF- $\alpha$  production is increased [9–12]. A number of investigators have reported increased sensitivity of T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and their subsets to death receptors (CD95 and TNF-) mediated apoptosis [25, 39–41]. In aging humans, there is a deficiency in T<sub>N</sub>, which in part appears to be associated with increased sensitivity to death-receptor-induced apoptosis [42–47]. In addition, we have reported a deficiency of T<sub>CM</sub> CD8<sup>+</sup> T cells in aging [22]. Furthermore, we have reported that the expression of TNF receptors is comparable between young and aged subjects [23, 48], therefore suggesting that mechanism(s) for increased sensitivity of T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> cells to apoptosis in aging must lie in signaling pathway downstream of TNFRs. In contrast, effector memory CD8<sup>+</sup> T cells (T<sub>EM</sub> and T<sub>EMRA</sub>) are resistant to apoptosis, and there is no significant difference in TNF- $\alpha$ -induced apoptosis in these subsets between young and aged subjects [48].

In this study we present molecular mechanisms of increased sensitivity of purified T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> T in aged humans to TNF- $\alpha$ -induced apoptosis by investigating signaling downstream of TNFRs. Our data show that increased apoptosis in T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> cells from aged subjects is due to decreased expression/function of molecules involved in the signaling pathway involved in cell survival.

## Methods

### Subjects

Peripheral blood was obtained from 15 healthy young (age 21–35 years with a mean age of 34 years; 9 female and 6 male) and 15 aged (age 65–88 years with a mean age of 72 years, 9 female and 6 male) subjects. Aging subjects belong to middle-class social status and living independently in senior community of Laguna Woods, California. Aging subjects were required to discontinue any and all nutritional supplements at least one week prior to blood draw, to avoid any effect of anti-oxidants, which are commonly used by aging population.

### Reagents and monoclonal antibodies

Directly conjugated monoclonal antibodies against CD8 and CD45RA and their isotypes and unconjugated CD8 antibodies were obtained from BD Biosciences (San Diego, CA). Anti-CCR7 and isotypes were purchased

from R & D systems, Minneapolis, MN, and anti-CD3/CD28 was Life Technology, Camarillo, CA. TNF- $\alpha$  was obtained from Laguna Scientific, Laguna Niguel, CA. Antibodies to FLIP and IAP were purchased from Transduction Laboratories, San Diego, CA, and antibodies to phospho IKK $\alpha$ / $\beta$ , phospho I $\kappa$ B, phospho JNK, phospho TAK1 TAK1, and TAB2 were purchased from Cell Signaling Technologies, Inc. Beverly, MA. Antibodies to A20, TRAF2 and RIPK1 were obtained from Santa Cruz Biotechnology, Dallas, TX. In Situ Cell Death Detection Kit was purchased from Boehringer-Manheim, Indianapolis, IN.

### Isolation of T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> T cells and culture conditions

Purified T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> T were separated from healthy young and aged subjects to determine age-related changes rather than simple differences between young and aged subjects. Peripheral blood mononuclear cells (MNCs) were activated with anti-CD3/CD28 monoclonal for 48 h. Cells are washed and used for purification of T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> T cells (cells are activated because freshly isolated human T cells are resistant to all types of death receptor induced apoptosis). First, CD8<sup>+</sup> T cells were isolated by negative selection with EasySep CD8<sup>+</sup> enrichment cocktail and magnetic nanoparticles (Stem cell Technologies, Vancouver, BC, Canada). Briefly, unwanted cells were specifically-labelled with bispecific tetrameric antibody complexes that recognize unwanted cells and dextran. Dextran-coated magnetic nanoparticles were added and magnetically labeled cells were then separated from unlabeled target cells (CD8<sup>+</sup> T cells) using a magnet. Cells obtained are more than 98% CD8<sup>+</sup>. T<sub>N</sub> (CD8<sup>+</sup>, CD45RA<sup>+</sup> CCR7<sup>+</sup>) and T<sub>CM</sub> T-cells (CD8<sup>+</sup> CD45RA<sup>-</sup> CCR7<sup>+</sup>) were purified to more than 95% by a two-step procedure. First, CD8<sup>+</sup> T cells are separated into CD45RA<sup>+</sup> and CD45RA<sup>-</sup> subpopulations by anti-CD45 RA antibody coated Petri dishes. In the second step, CCR7<sup>+</sup> T cells are isolated by positive selection using EasySep PE selection kit (Stem cell Technologies). Briefly, CD45RA<sup>+</sup> and CD45RA<sup>-</sup> T cells are labeled with phycoerythrin (PE)-conjugated anti-CCR7 antibody. The labeled cells are then incubated with bispecific tetrameric antibody complexes that recognize PE labeled cells and dextran. After 15 min incubation at room temperature, dextran-coated magnetic nanoparticles are added and magnetically labeled cells are separated from unlabeled cells using a magnet. Positively enriched cells are labeled with APC conjugated anti-CD45 and PerCp-conjugated anti-CD8 and the purity of isolated populations are determined by multicolor analysis using FACSCalibur. Purified T<sub>N</sub> and T<sub>CM</sub> CD8<sup>+</sup> T cells were activated with TNF- $\alpha$  to study phosphorylation of signaling molecules by Western blotting.

TNF- $\alpha$ -induced apoptosis was assayed in activated  $T_N$  and  $T_{CM}$  CD8+ because ex-vivo freshly isolated T cell subsets are resistant to TNF- $\alpha$ -induced apoptosis. Furthermore, phenotypes of  $T_N$  cells and  $T_{CM}$  were largely maintained following 48 h of anti-CD3/CD28 stimulation of MNCs.

### Apoptosis

Purified  $T_N$  and  $T_{CM}$  CD8+ T cells were stimulated with TNF- $\alpha$  for 48 h to assay for apoptosis. Apoptosis was measured by TUNEL assay (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP- nick end labeling). Briefly, TNF-activated purified  $T_N$  and  $T_{CM}$  CD8+ cells were fixed with 2% formaldehyde for 30 min at room temperature, washed with phosphate buffer saline (PBS), and permeabilized with sodium citrate buffer containing 0.1% Triton X-100 for 2 min on ice. Following washing, cells were incubated with FITC-conjugated dUTP in the presence of TdT enzyme solution containing 1 M potassium cacodylate and 125 mM Tris-HCl, Ph 6.6 for an hour at 37 °C. Following incubation, cells were washed with PBS, and 10,000 cells were acquired and analyzed by multicolor flow cytometry using FACSCalibur.

### Flow cytometry

MNCs activated with anti-CD3/CD28 for 48 h and, then exposed to TNF- $\alpha$  for 10 min. Cells were first surface stained by CCR7 FITC, CD45RA APC, CD8PerCP antibodies and isotype controls. Stained cells were then fixed by 2% paraformaldehyde for 10 min at room temperature, washed and permeabilized by 90% methanol for 15 min on ice. Cells were washed and kept in PBS/2% FBS for 60 min for rehydration and then stained with purified antibodies to cIAP1 and A20 and isotype controls. Cells were washed and stained with secondary PE conjugated goat anti-rabbit antibody. First cells were gated for CD8+ T cells, and then gated for  $T_N$  (CD8+, CD45RA+ CCR7+) and  $T_{CM}$  T-cells (CD8+ CD45RA- CCR7+) cells. These gated cells were then analyzed for the expression of cIAP1 and A20. Ten thousand cells were acquired, and were enumerated using FACSCalibur. Data were analyzed by Flow jo software.

### Western blotting

Purified  $T_N$  and  $T_{CM}$  cells activated with TNF- $\alpha$  were lysed with lysis buffer (Cell Signaling). Aliquots of cell lysates containing 50  $\mu$ g of total protein were resolved by SDS-PAGE and transferred onto membranes (Millipore, Bedford, MA) by electro blotting. The membranes were blocked for 1 h at room temperature in TBS-T buffer with 5% nonfat dried milk and incubated with 1  $\mu$ g/ml primary antibodies listed above in reagents and anti- $\beta$

actin antibody as loading control used dilution 1:5000 overnight at 4°C. The blots were washed three times for 20 min with TBS-T buffer and then incubated with HRP-conjugated secondary antibodies (1:5000–1:10,000 dilution) for 1 h at room temperature. After washing three times for 20 min in TBS-T buffer, blots were developed using enhanced chemiluminescence reagents (ECL, Thermo Scientific Pierce Biotech, Rockford, IL) and exposed to Clear Blue X-Ray Film. Blots were scanned with densitometer.

### ELISA for NF- $\kappa$ B activity

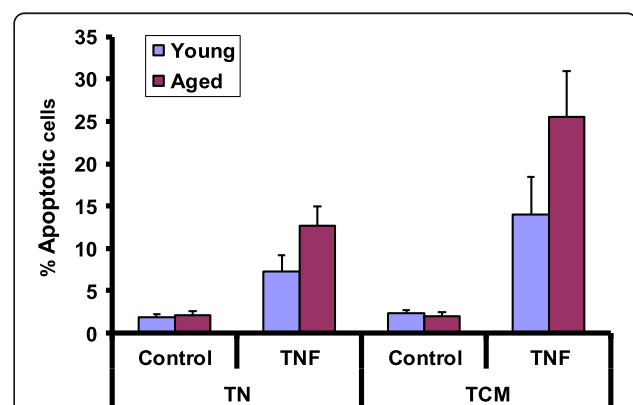
DNA-binding activity of NF- $\kappa$ B was measured using an ELISA kit for NF- $\kappa$ B p65 according to manufacturer's protocol (Active Motif, San Diego, CA). The 96-well plates were coated with the oligonucleotide specific for NF- $\kappa$ B binding and the bound NF- $\kappa$ B was measured using anti-NF- $\kappa$ B p65 antibody as described (23). This method provides advantage over traditional EMSA assay in that it is a sensitive assay without using radioactivity, and a large number of samples with smaller number of cells can be analyzed simultaneously.

Statistical analysis was performed by student t test.

## Results

### Increased sensitivity to TNF- $\alpha$ -induced apoptosis in $T_N$ and $T_{CM}$ CD8 cells in aged subjects

Purified activated  $T_N$  and  $T_{CM}$  CD8+ cells from young and aged subjects were incubated in the absence or presence of TNF- $\alpha$  for 48 h. Apoptosis was measured by TUNEL assay. Fig. 1 shows data from 10 young and 10 aged subjects. No significant difference was observed in



**Fig. 1** TNF- $\alpha$ -induced apoptosis in  $T_N$  and  $T_{CM}$  CD8+ T cells. Activated purified  $T_N$  and  $T_{CM}$  CD8+ T cells subsets were incubated in the absence or presence of TNF- $\alpha$  for 48 h and apoptosis was measured by TUNEL assay using FACSCalibur. Ten thousand cells were acquired. Data for spontaneous and TNF- $\alpha$ -induced apoptosis from 10 young and 10 aged subjects are presented as percent of TUNEL positive cells. A significantly increased ( $P < 0.001$ ) apoptosis was observed in aged subjects. No significant difference was observed in spontaneous apoptosis between young and aged subjects

spontaneous apoptosis between young and aged group. However, a significantly higher ( $P < 0.001$ ) TNF- $\alpha$ -induced apoptosis was observed in both  $T_N$  and  $T_{CM}$  CD8+ T cells from aged as compared to young controls. This is in agreement with previous reports [23, 48].

#### TRAF-2 and RIP expression is decreased in aged $T_N$ and $T_{CM}$

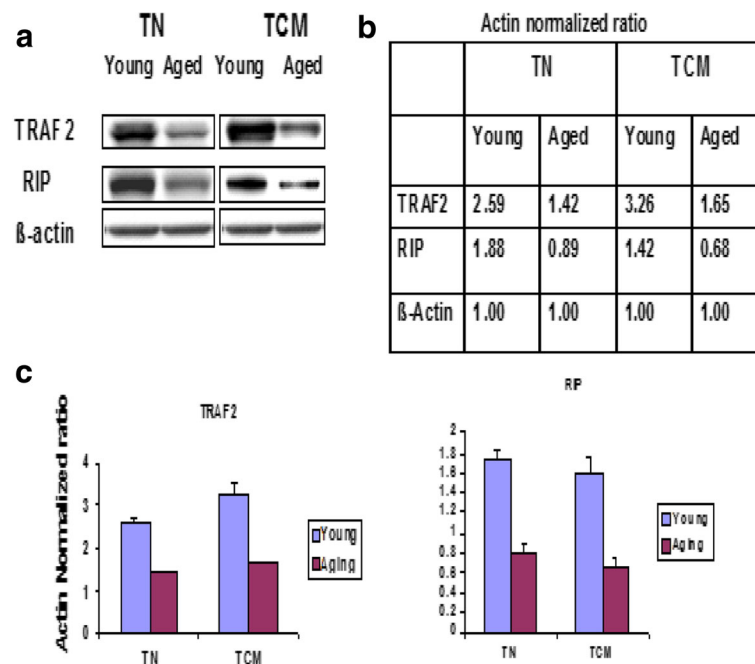
Since TNFRI and TNFRII expression on aged  $T_N$  and  $T_{CM}$  CD8+ cells is comparable to young subjects [23, 48] we reasoned that an impaired expression/function of adapter molecule TRAF2 may play an important role in increased sensitivity of  $T_N$  and  $T_{CM}$  CD8+ T cells in aged humans, via decreased activation of RIP and TAK1 resulting in decreased NF- $\kappa$ B activity and an impaired induction of NF- $\kappa$ B target anti-apoptotic genes. Therefore, first we examined the expression of TRAF-2 and RIPK1 in  $T_N$  and  $T_{CM}$  CD8+ T cells. Proteins were extracted from purified subsets from young and aged subjects and the expression of these molecules was analyzed by Western blotting with specific antibodies and analyzed by densitometry. Actin was used as a loading internal control. Fig. 2a shows a representative Western blots and Fig. 2b shows data from densitometry of Western blot normalized for actin loading control. Figures 2c shows cumulative densitometry data of Western blot of  $T_N$  and  $T_{CM}$  CD8+ T cells from five young and five aged subjects normalized for actin

loading control. The expression of TRAF-2 and RIPK1 was significantly decreased ( $P < 0.001$ ) in  $T_N$  and  $T_{CM}$  CD8+ T cells from aged subjects.

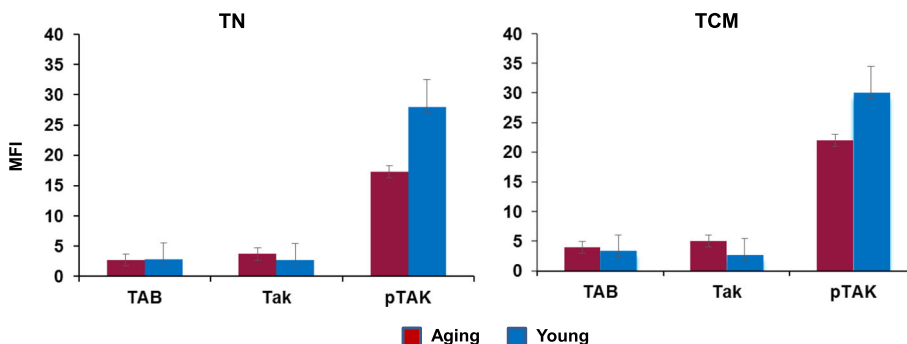
#### Phosphorylated TAK1 is decreased in aged $T_N$ and $T_{CM}$ CD8 cells

RIPK1 activates TAK-1 via recruitment of TAK-1 complex and interaction of K<sup>63</sup>ubiquitin chains to TAB2 and subsequent phosphorylation of TAK1 [49]. Since RIPK1 levels are decreased in  $T_N$  and  $T_{CM}$  CD8+ T cells in aged, we examine the expression of TAB2 and TNF- $\alpha$ -induced phosphorylation of TAK-1 in  $T_N$  and  $T_{CM}$  CD8+ T cells. Purified  $T_N$  and  $T_{CM}$  CD8+ T cells were incubated in the presence or absence of TNF- $\alpha$ , and the expression of TAB2 and TAK1, and phosphorylation of TAK-1 was measured with specific antibodies and flow cytometry. Isotype antibodies were used as background control. Figure 3 represents cumulative data of mean fluorescence intensity (density of molecules) from five each young and aged subjects. TAB2 and TAK1 expression was comparable; however, phosphorylated TAK-1 was significantly decreased ( $P < 0.004$ ) in aged cells.

**TNF- $\alpha$ -induced phosphorylation of IKK $\alpha/\beta$ , I $\kappa$ B $\alpha$  and activation of NF- $\kappa$ B is impaired in aged  $T_N$  +  $T_{CM}$  CD8 cells**  
TAK1 phosphorylates IKK $\beta$ , which in turn phosphorylates I $\kappa$ B $\alpha$ , resulting in the release and activation of NF- $\kappa$ B (p65/p50), providing a survival signal [50]. In



**Fig. 2** TRAF-2 and RIP expression in  $T_N$  and  $T_{CM}$  CD8+ T cells. Protein extracted from purified  $T_N$  and  $T_{CM}$  CD8+ T cells from aged and young subjects was analyzed by Western blotting using specific antibodies. **[a]** Western blots from a representative experiment, **[b]** densitometry data, and **[c]** cumulative densitometry data from five each young and aged subject. Both TRAF-2 and RIP expression was significantly increased ( $P < 0.001$ ) in aged subjects



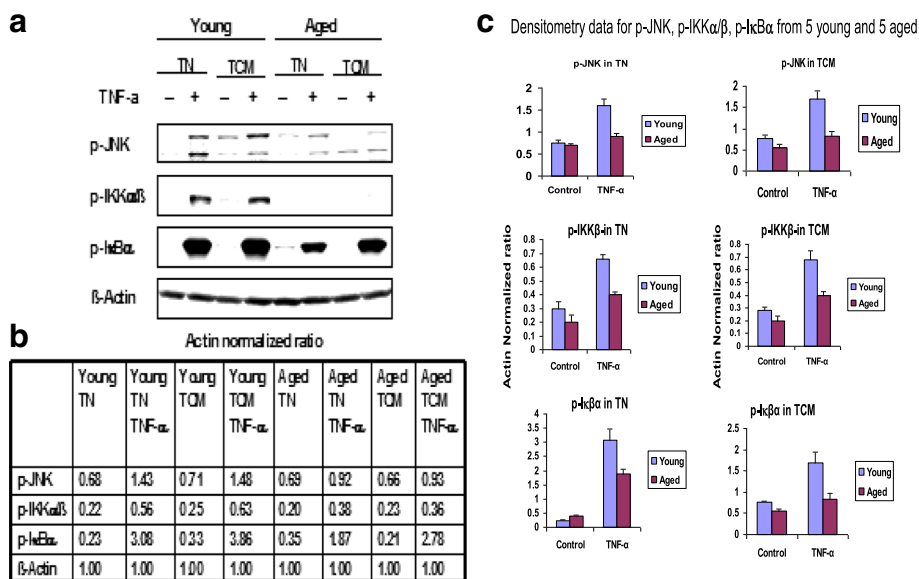
**Fig. 3** Expression of TAB2, TAK1 and phospho TAK1. Purified T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells were incubated in the absence or presence of TNF- $\alpha$  for 10 min, and the expression of TAB2 and TAK1, and phosphorylation of TAK-1 were measured with specific antibodies and flow cytometry. Isotype antibodies were used as background control. Data were analyzed for fluorescence intensity (MFI) as an indicator of density of molecules. Cumulative data from 5 young and 5 aged subjects show a significantly decreased ( $P < 0.004$ ) pTAK1 in aged subjects as compared to young subjects

contrast, TAK1 activates JNK that promotes apoptosis [51]. Therefore, we examined TNF- $\alpha$ -induced phosphorylation of JNK, IKK $\alpha/\beta$ , I $\kappa$ B $\alpha$ , and activation of NF- $\kappa$ B in young and aged subjects.

Purified T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells were activated with TNF- $\alpha$  for 10 min, and the protein was extracted and analyzed by Western blotting, using specific antibodies against phospho IKK $\alpha/\beta$ , phospho I $\kappa$ B $\alpha$ , and phospho JNK. A representative Western blot is shown in Fig. 4a and the densitometry data from these blots normalized for actin loading control are shown in Fig. 4b. Fig. 4c shows cumulative densitometry data of Western blots from five aged subjects and five young subjects. The levels of phospho JNK, IKK $\alpha/\beta$ , and I $\kappa$ B $\alpha$  in T<sub>N</sub> and

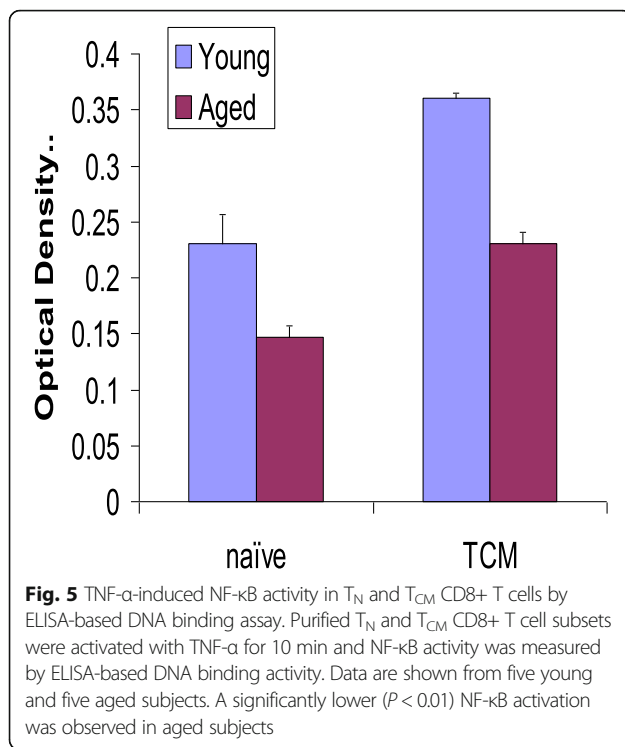
T<sub>CM</sub> CD8+ T cells from aged subjects were significantly decreased ( $P < 0.05$ - $< 0.01$ ) as compared to young subjects. No difference was observed in the expression of NEMO (data not shown). While JNK signaling can contribute to TNF-induced apoptosis, it is unlikely that decreased JNK activation contributes to increased apoptosis in aged subjects under these experimental conditions.

We also compared NF- $\kappa$ B activity in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells in young and aged subjects. Purified subsets were activated with TNF- $\alpha$  for 10 min, and NF- $\kappa$ B activity was measured by ELISA-based DNA binding activity. Data from five young and five aged subjects are shown in Fig. 5. Both T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells from aged



**Fig. 4** Effect of TNF- $\alpha$  on the phosphorylation of JNK, IKK $\beta$ , I $\kappa$ B in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells. Purified T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells from young and aged subjects were stimulated with TNF- $\alpha$  for 10 min and then analyzed for expression of phospho JNK, IKK $\beta$ , I $\kappa$ B, using specific antibodies.  $\beta$ -actin was used as a loading control. **[a]** Western blot from one such experiment, **[b]** densitometry data, and **[c]** shows densitometry data from 5 each young and aged subjects. A significant decrease in pJNK, pIKK $\beta$ , and pI $\kappa$ B ( $P < 0.05$ ,  $P < 0.01$ ) was observed in aged subjects as compared to young subjects





subjects show significantly lower NF- $\kappa$ B activity following TNF- $\alpha$  activation ( $P < 0.01$ ) as compared to young subjects.

#### Bcl- $X_L$ , FLIP $_L$ , FLIP $_S$ , A20 and cIAP expression is decreased in $T_N$ + $T_{CM}$ CD8 cells from aged humans

Since NF- $\kappa$ B activates a number of anti-apoptotic genes [52–60], next we examined the expression of cIAP, A20, FLIP and Bcl- $x_L$  in purified  $T_N$  and  $T_{CM}$  CD8+ T cells in aged and young subjects. Protein extracted from purified  $T_N$  and  $T_{CM}$  CD8+ T cells from aged and young subjects was analyzed by Western blotting using specific antibodies. A representative Western blot for A20, Bcl- $X_L$ , and FLIP $_L$ , FLIP $_S$  expression in  $T_N$  and  $T_{CM}$  CD8+ T cells is shown in Fig. 6a and densitometry data from these blots are shown in Fig. 6b. Fig. 6c shows cumulative densitometry data (mean  $\pm$  sd) of Western blots from five young and five aged subjects. The expression of A20, FLIP $_L$  and FLIP $_S$ , and Bcl- $x_L$  was significantly ( $P < 0.05$ – $< 0.001$ ) decreased in aged subjects.

Since antibodies to A20 and cIAP for flow cytometry became available, and flow cytometry is experimentally less cumbersome and less time consuming than Western blotting, we analyzed the expression of A20 and cIAP in  $T_N$  and  $T_{CM}$  CD8+ T cells from aged and young subjects by flow cytometry. Data in Fig. 7a is a representative FACS plots, and data in Fig. 7b is cumulative from 4 young and 4 aged subjects (mean  $\pm$  sd). The expression of A20 (similar to Western blot data in Figure 6) and

cIAP1 is significantly decreased ( $P < 0.001$ ) in  $T_N$  and  $T_{CM}$  CD8+ T cells from aging as compared to young subjects. These data show that flow cytometry for the analysis of these molecules is a reliable technique, and has advantage over Western blotting in that [a] analysis can be performed on small number of cells, [b] there is no requirement of purification of  $T_N$  and  $T_{CM}$  CD8+ T cells, and [c] provide better quantitative analysis.

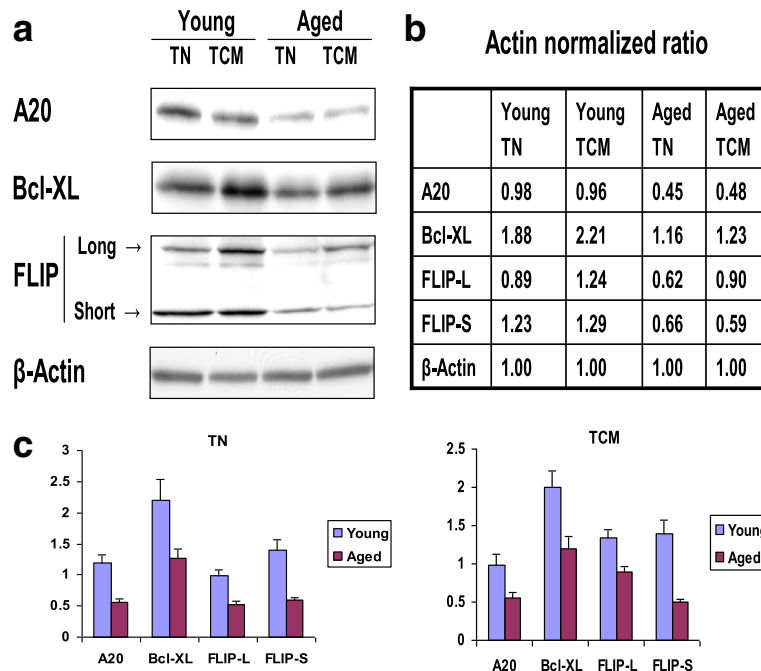
#### Discussion

Following virus infection or antigen stimulation, naïve T cells undergo a series of proliferative and differentiation steps resulting in the development of effector and memory cells [3]. The differential expression of adhesion molecule (CD62L) and chemokine receptor (CCR7) on memory T cells results in their homing either to lymph nodes ( $T_{CM}$ ) or to extra nodal sites such as liver and lung ( $T_{EM}$ ) [14–21]. Both our group, and others have reported decreased in  $T_N$  and  $T_{CM}$  T cells in aged humans [22, 42–47]. Although a role of thymus in decreased output of naïve T cells is well-established, we and others have also shown that an increased apoptosis may also contributes to decreased  $T_N$  cells in aging [42, 47]. Previously we have reported that  $T_N$  and  $T_{CM}$  CD8+ T cells are more sensitive to both TNF- $\alpha$ -and CD95-induced apoptosis via activation of caspases as compared to  $T_{EM}$  and  $T_{EMRA}$  CD8+ T cells [23, 42, 48]; however, expression of TNFRs is comparable to young subjects [23, 48]. Our present data also show increased TNF- $\alpha$ -induced apoptosis in both  $T_N$  and  $T_{CM}$  CD8+ from aged subjects, which may contribute to their deficiency in aged humans.

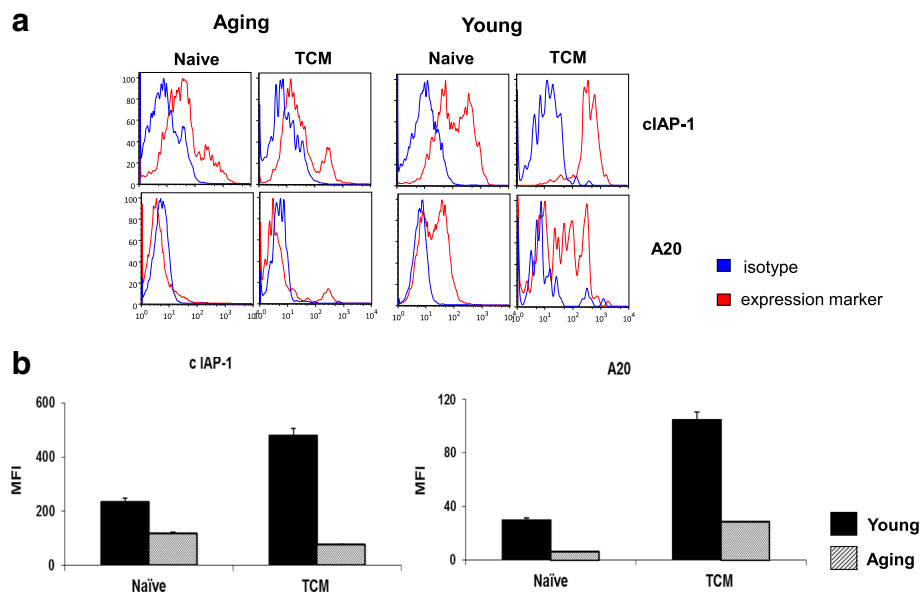
TNF- $\alpha$  is a proinflammatory molecule that plays an important role in diverse cellular events including induction of cytokines, cellular proliferation, differentiation, survival and apoptosis [26, 35]. TNF- $\alpha$ -mediates these processes via TNFR-I and/or TNFR-II, apoptosis is predominantly mediated via TNFR-I.

Previously, we have shown that expression of TNFR-I and TNFR-II is comparable in all four subsets of CD8+ T cells ( $T_N$ ,  $T_{CM}$ ,  $T_{EM}$ ,  $T_{EMRA}$ ); however,  $T_{EM}$  and  $T_{EMRA}$  CD8+ T cells are resistant to TNF- $\alpha$ -induced apoptosis [48]. Furthermore, in aging, apoptosis and activation of caspase 3 and caspase 8 are increased only in  $T_N$  and  $T_{CM}$  CD8+ T cells [48]. Therefore, these data suggest that the differences in TNF- $\alpha$ -induced apoptosis in aged  $T_N$  and  $T_{CM}$  are due to differences in signaling pathway downstream of TNFRs.

The interaction and binding of TNF- $\alpha$  to TNFR-I leads to trimerization of TNFR-I and via death domain and by protein-protein interaction recruits TRADD, which acts as a platform to recruit other proteins including FADD, TRAF2, and RIPK1, forming a signaling complex that activates NF- $\kappa$ B, which induces anti-apoptotic genes. We



**Fig. 6** Expression of A20, Bcl-X<sub>L</sub>, and FLIP<sub>L</sub> and FLIP<sub>S</sub> in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells. Protein extracted from purified T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells from aged and young subjects was analyzed by Western blotting using specific antibodies. **[a]** shows a representative Western blot for A20, Bcl-X<sub>L</sub>, and FLIP expression in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells **[b]** shows densitometry data from these blots, **[c]** shows cumulative densitometry data (mean ± sd) of Western blots from five young and five aged subjects. T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells subsets from aged subjects display significantly decreased expression of A20 ( $P < 0.05$ ), Bcl-X<sub>L</sub>, ( $P < 0.01$ ), FLIP<sub>L</sub> ( $P < 0.05$ ) and FLIP<sub>S</sub> ( $P < 0.05$ )



**Fig. 7** Expression of A20 and cIAP1 by flow cytometry. MNCs activated with anti-CD3/CD28 for 48 h and, then exposed to TNF- $\alpha$  for 10 min. Cells were first surface stained by CCR7 FITC, CD45RA APC, CD8PerCP antibodies and isotype controls. Stained cells were then fixed and permeabilized, and then stained with purified antibodies to cIAP1 and A20 and isotype controls. Cells were washed and incubated with secondary PE conjugated goat anti-rabbit antibody. First cells were gated for CD8+ T cells, and then gated for T<sub>N</sub> (CD8+, CD45RA+ CCR7+) and T<sub>CM</sub> T-cells (CD8 + CD45RA- CCR7+) cells. These gated cells were then analyzed for the expression of cIAP1 and A20. **a** is a representative FACS plot. Blue line represents isotype control, and red line is for A20 and cIAP1. **b** shows cumulative data for MFI from 5 young and 5 aged subjects. T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells from aged subjects show significantly decreased ( $P < 0.001$ ) expression of both A20 and cIAP1



have shown that deficiency of FADD plays an important role in an increased apoptosis of lymphocytes from aged humans [61]. FADD expression is increased in lymphocytes from aged subjects, and transfection of aged lymphocytes with FADD dominant negative plasmid significantly reduced TNF-induced apoptosis in aged lymphocytes comparable to young subjects. Furthermore, we demonstrated that an overexpression of FADD in lymphocytes from young subjects with wild-type FADD resulted in an increased apoptosis of young lymphocytes to a level similar to aged subjects.

RIPK1, a multifunctional protein, and TRAF-2 are required for the activation of NF- $\kappa$ B. It has been demonstrated that in TNF-induced apoptosis caspase-8 cleaves RIPK1 [62]. TRAF2 together with ubiquitin conjugating enzyme complex catalyzes the synthesis of a unique polyubiquitin chain K<sup>63</sup> of ubiquitin [63–66]. K<sup>63</sup> polyubiquitination of RIPK1 leads to its activation and recruitment of TAK1 complex and IKK complex [50, 67–70]. This results in the activation of TAK1 kinase complex through interaction between the K<sup>63</sup> polyubiquitin chain and an ubiquitin-binding domain on TAB2 regulatory units of TAK1 complex [50] and of IKK $\gamma$  (NEMO) via interaction with K<sup>63</sup> polyubiquitin chain [69]. TAK1 phosphorylates and activates IKK $\beta$ , resulting in phosphorylation and degradation of I $\kappa$ B $\alpha$ , and activation of NF- $\kappa$ B [50, 71]. In the current study, we observed decreased expression of both TRAF2 and RIP. TAK1 and not TAB1 or TAB2 plays a role in multiple signaling pathways [72]. In this study we did not see any difference in TAB2 expression in T<sub>N</sub> and T<sub>CM</sub> CD8 cells between young and aged; however, we observed decreased phosphorylation of TAK1, IKK $\beta$ , and I $\kappa$ B $\alpha$ , and decreased activation of NF- $\kappa$ B in T<sub>N</sub> and T<sub>CM</sub> CD8 cells. Taken together signaling molecules downstream of TNFR appear to be responsible for increased sensitivity to TNF- $\alpha$ -induced apoptosis in T<sub>N</sub> and T<sub>CM</sub> CD8 cells from aged humans.

The anti-apoptotic genes that are target of NF- $\kappa$ B activation include *cIAP1*, *cIAP2*, *Bcl-x<sub>L</sub>*, *A20* and *FLIP* show decreased expression in aged naïve and T<sub>CM</sub> CD8+ T cells [52–60].

A20 (tumor necrosis factor alpha-induced protein 3), a ring finger ubiquitin-modifying enzyme, is essential for the termination of TNF- $\alpha$ -induced activation of NF- $\kappa$ B and inhibition of TNF-induced apoptosis [56–58]. A20 has dual activity in that it inhibits apoptosis as well as activates NF- $\kappa$ B [56, 73]. Interaction of A20 and cIAP with TRAF2 results in the releases of cIAP from the TRAF2-signaling complex, and allows these proteins to exert their anti-apoptotic effects. Our data show decreased expression of A20 and cIAP in aged T<sub>N</sub> and T<sub>CM</sub> CD8+, which are more sensitive to TNF- $\alpha$ -induced apoptosis as compared to young. Therefore, A20

deficiency in aging may be contributing to both increased apoptosis and inflammation. Our data suggest that in primary human CD8+ T cells A20 may function preferentially as an anti-apoptotic molecule.

IAP family proteins have a key role in the inhibition of apoptosis [55, 74, 75]. The cIAP-1 and cIAP2 are structurally homologous proteins. cIAP1 is recruited to DISC of TNFR-I by TRAF-2. Previously we have reported decreased expression of cIAP in CD4+ and CD8+ T cells in aging [76]. In this study we observed decreased expression of cIAP1 in aged T<sub>N</sub> and T<sub>CM</sub> CD8 cells as compared to young subjects, which may contribute to increased sensitivity to TNF- $\alpha$ -induced apoptosis in aged.

cFLIP, an apoptosis inhibiting molecules is a target of NF- $\kappa$ B [52]. FLIP comes in two alternatively spliced forms, the cFLIP<sub>L</sub> and cFLIP<sub>S</sub>. cFLIP<sub>S</sub> contains two death effector domains (DED) and inhibits procaspase-8 activation, whereas, cFLIP<sub>L</sub> is enzymatically inactive. In addition to its inhibitory effect on procaspase-8 activation, cFLIP by associating with Raf-1 activate MEK1, which subsequently activates ERK. cFLIP associates with TRAF2, resulting in NF- $\kappa$ B activation [53, 54, 77, 78]. cFLIP<sub>L</sub> inhibits the interaction of caspase 8 prodomain with RIP1 death domain, and regulates caspase 8-dependent NF- $\kappa$ B activation [79]. Our data show a significant decreased expression of both cFLIP<sub>L</sub> and cFLIP<sub>S</sub> in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells in aged as compared to young subjects. It remains to determine whether decreased FLIP expression contribute to increased TNF- $\alpha$ -induced activation of caspase-8 and caspase-3 in T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells in aged humans (48).

## Conclusions

Our data demonstrate that an impaired expression of adaptor proteins resulting in decreased activation of IKK pathway and decreased NF- $\kappa$ B activation, and decreased expression of anti-apoptotic molecules that are target of NF- $\kappa$ B might play a role in increased sensitivity of T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells, thus contributing to their deficiency and T cell dysfunction in aged humans. However, data presented are correlative, and in vitro overexpression of these molecules may provide the mechanistic explanation for increased sensitivity of T<sub>N</sub> and T<sub>CM</sub> CD8+ T cells in aged humans.

## Abbreviations

c-FLIP: FLICE-like inhibitory protein; cIAP1: cellular inhibitor of apoptosis 1; FADD: fas-associated death domain; RIPK1: receptor-interacting protein kinase 1 (RIPK1); T<sub>CM</sub>: Central memory T cells; T<sub>N</sub>: Naïve T cells; TNFR-1 and TNFR-II: Tumor necrosis factor I and II; TRADD: TNFR-associated death domain; TRAF2: TNRF-associated factor 2

## Funding

Was provided by unrestricted research funds of the Division of Basic and Clinical Immunology, University of California, Irvine.

## Availability of data and materials

Experimental data are stored in experimental books and research computers.

**Authors' contributions**

SGu conceptualized and designed experiments, interpreted data and wrote the manuscript. HS performed Western blotting, SA was responsible for flow cytometry analysis, and SGo supervised HS, performed apoptosis and NF- $\kappa$ B ELISA assay, compiled all data, and performed statistical analysis. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The study was carried out in accordance with the recommendation of The Institution Review Board Committee (Human) of the University of California, Irvine with approved protocol and informed consent from all subjects. All subjects gave informed consent according to the Declaration of Helsinki. The protocol and consent forms were approved by Institutional Review Board Human Subject Committee.

**Consent for publication**

Approved and signed consent form lists consent to publish.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 8 October 2017 Accepted: 28 December 2017

Published online: 19 January 2018

**References**

- Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. *J Pathol.* 2007;211:144–56.
- Vallejo AN. Immune aging and challenges for immune protection of the graying population. *Aging Disease.* 2011;2:339–45.
- Sanson P, Vescivini R, Biasini C, Zanni F, Telera A, Lucchini G, Passeri G, Monti G, Frnchesi C, Passeri M. The Immune system in extreme longevity. *Exp Gerontol.* 2008;43:61–5.
- Pawlec G, Larbi A, Derhovanessian E. Senescence of the human immune system. *J Compl Pathol.* 2010;42(suppl 1):S39–44.
- Pawlec G, Hirokawa K, Fulop T. Altered T cell signaling in ageing. *Mech Ageing Dev.* 2001;122:1613–37.
- Ershler WB. Interleukin-6: a cytokine for gerontologists. *J Am Geriatric Soc.* 1993;41:176–81.
- Gupta S. Membrane signal transduction in T cells in aging humans. *Annals of NY Acad Sciences.* 1989;568:277–82.
- Powlec G, Barnett Y, Effros R, Forsey R, Frasca D, Globerson A, Mariani E, McLeod J, Caruso C, Franceschi C, Fulop T, Gupta S, Mocchegiani E, Solana R. T cells and aging. *Front Biosci.* 2002;7:d1058–183.
- Fagiola U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, Monti D, Franceschi C, Paganelli R. Increased cytokine production in mononuclear cells of healthy elderly people. *Eur J Immunol.* 1993;23:2375–8.
- Brunnsgaard H, Andersen-Ranberg K, Hjelmberg JB, Pedersen BK, Jeu B. Elevated tumor necrosis factor alpha and mortality in centenarians. *Amer J Med.* 2003;115:278–83.
- Trzonkowski P, Mysliwska J, Godlewska B, Szmít E, Lukaszuk K, Wieckiewicz J, Brydak L, Machala M, Landowski J, Mysliwski A. Immune consequences of the spontaneous pro-inflammatory status in depressed elderly patients. *Brain Behav Immun.* 2004;18:135–48.
- Penninx BWJH, Kritchevsky SB, Newman AB, Nicklas BJ, Simonsick EM, Rubin S, Nevitt M, Visser M, Harris T, Pahor M. Inflammatory markers and incident mortality limitation in the elderly. *J Amer Gerontol Soc.* 2004;52:1105–13.
- Kaech SM, Ahmed R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nature Immunol.* 2001;2:415–22.
- Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999;401:708–12.
- Masopust D, Vezys V, Marzo AL, Lanzavecchia A. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* 2001;291:2413–7.
- Weninger W, Crowley MA, Manjunath N, von Andriane UH. Migratory properties of naïve, effector, and memory CD8(+) T cells. *J Exp Med.* 2001; 194:953–66.
- Tomiyama H, Matsuda T, Takiguchi M. Differentiation of CD8+ T cells from a memory to memory/effector phenotype. *J Immunol.* 2002;168:5538–50.
- Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood.* 2003;101:4260–6.
- Van Lier RAW, ten Berge IJM, Gamadia LE. Human CD8+ T cell differentiation in response to viruses. *Nat Rev Immunol.* 2003;3:931–8.
- Gupta S, Bi R, Su K, Yel L, Chiplunkar S, Gollapudi S. Characterization of naïve/memory effector subsets of CD8+ T cells: changes in aged humans. *Exp Gerontology.* 2004;20:545–50.
- Gupta S, Gollapudi S. Molecular mechanisms of TNF- $\alpha$ -induced apoptosis in naïve and memory T cell subsets. *Autoimmun Rev.* 2006;5:264–8.
- Gupta S. Molecular mechanisms of TNF- $\alpha$ -induced apoptosis in naïve and memory T cell subsets: effect of age. *Immunol Rev.* 2005;205:114–25.
- Gupta S, Bi R, Gollapudi S. Differential sensitivity of naïve and central and effector memory CD8+ T cells to TNF- $\alpha$ -induced apoptosis. *J Clin Immunol.* 2006;26:193–203.
- Gupta S, Gollapudi S. Central and effector memory CD4+ and CD8+ T cells display differential sensitivity to TNF- $\alpha$ -induced apoptosis. *N Y Acad Sci.* 2005;1050:108–14.
- Salvioli S, Capri M, Scarcella E, Mangherini S, Faranca I, Volterra V, De Ronchi D, Marini M, Bonafe M, Franceschi C, Monti D. Age-dependent changes in the susceptibility to apoptosis of peripheral blood CD4+ and CD8+ T lymphocytes with virgin or memory phenotype. *Mech Ageing Dev.* 2003; 124:409–18.
- Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science.* 2002; 296:1634–5.
- Screaton G, Xu X-N. T cell life and death signaling via TNF-receptor family members. *Curr Opin Immunol.* 2000;12:316–322.
- Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ.* 2003;10:45–65.
- Gupta S. Molecular steps of TNF receptor-mediated apoptosis. *Curr Mol Med.* 2001;1:299–306.
- Hayden MS, Ghosh S. Shared principles in NF- $\kappa$ B signaling. *Cell.* 2008; 132:344–62.
- Vallabhapurapu S, Karin M. Regulation and function of NF- $\kappa$ B transcription factors in the immune system. *Annu Rev Immunol.* 2009;27: 693–733.
- Ghosh S, Karin M. Missing pieces in the NF- $\kappa$ B puzzle. *Cell.* 2002;109:S81–S96.
- Li Q, Verma IM. NF- $\kappa$ B regulation in the immune system. *Nature Rev Immunol.* 2002;2:725–34.
- Li X, Stark GR. NF- $\kappa$ B-dependent signaling pathways. *Exp Hematol.* 2002; 30:285–96.
- Varfolomeev EE, Ashkenazi A. Tumor necrosis factor: an apoptosis JunKie. *Cell.* 2004;116:491–7.
- Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Z-g L. The distinct role of TRAF2 and RIP in IKK activation by TNFR1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity.* 2000;12:419–29.
- Michaev O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell.* 2003;114:181–90.
- Deng Y, Ren X, Yang L, Lin Y, Wu X. A JNK-dependent pathway is required for TNF- $\alpha$ -induced apoptosis. *Cell.* 2003;115:61–70.
- Aggarwal S, Gollapudi S, Gupta S. Increased TNF- $\alpha$ -induced apoptosis in lymphocytes from aged humans: changes in TNF- $\alpha$  receptor expression and activation of caspases. *J Immunol.* 1999;162:2154–61.
- Aggarwal S, Gupta S. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Bcl-2 and Bax. *J Immunol.* 1998;160:1627–37.
- Gupta S, Gollapudi S. Susceptibility of naïve and subsets of memory T cells to apoptosis via multiple signaling pathways. *Autoimmunity Rev.* 2007;6: 476–81.
- Gupta S, Gollapudi S. CD95-mediated apoptosis in naïve, central, and effector memory subsets of CD4+ and CD8+ T cells in aged humans. *Exp Gerontol.* 2008;43:266–74.
- Fagnoni FF, Vescovini R, Passeri G, Bologna G, Pedrazzoni M, Lavagetto G, Casti A, Franceschi C, Passeri M, Sansoni P. Shortage of circulating naïve CD8+ T cells provides new insights on immunodeficiency in aging. *Blood.* 2000;95:2860–8.

44. Romanyukha AA, Yashin AI. Age-related changes in population of peripheral T cells: towards a model of immunosenescence. *Mech Ageing Dev.* 2003;124:433–43.
45. Effros RB, Boucher N, Porter V, Zhu X, Spaulding C, Walford RL, Kronenberg M, Cohen D, Schachter F. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause of both in vivo and in vitro immunosenescence. *Exp Gerontol.* 1994;29:601–9.
46. Nociari MM, Telford W, Russo C. Postthymic development of CD28-CD8+ T cell subsets: age-associated expansion and shift from naive to memory phenotype. *J Immunol.* 1999;162:3327–35.
47. Brzezinska A, Magalska A, Szybinska A, Sikora E. Proliferation and apoptosis of human CD8+CD28+ and CD8+CD28- lymphocytes during aging. *Exp Gerontol.* 2004;39:539–44.
48. Gupta S, Gollapudi S. TNF- $\alpha$ -induced apoptosis in human naive and memory CD8+ T cells in aged humans. *Exp Gerontol.* 2006;41:69–77.
49. Broglie P, Matsumoto K, Akira S, Brautigan DL, Ninomiya-Tsuji J. Transforming growth factor beta-activated kinase 1 (TAK1) kinase adaptor, TAK1-binding protein 2, plays dual roles in TAK1 signaling by recruiting both an activator and an inhibitor of TAK1 kinase in tumor necrosis factor signaling pathway. *J Biol Chem.* 2010;285:2333–9.
50. Adhikari A, Xu M, Chen ZJ. Ubiquitin-mediated activation of TAK-1 and IKK. *Oncogene.* 2007;26:3214–26.
51. Huang C-H, Omori E, Akira S, Matsumoto K, Ninomiya-Tsuji J. Osmotic stress activates the TAK1-JNK pathway while blocking TAK1-mediated NF- $\kappa$ B activation: TAO2 regulates TAK1. *J Biol Chem.* 2006;281:28802–10.
52. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF- $\kappa$ B signals induce the expression of c-FLIP. *Mol Cell Biol.* 2001;21:5299–305.
53. Imler M, Thome M, Hahne M, Schnieder P, Hoffmann K, Steiner V, Bodmer J-L, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. *Nature.* 1997;388:190–5.
54. Kataoka T, Budd RC, Holler N, Thome M, Martinon F, Imler M, Burns K, Hahne M, Kennedy N, Kovacovics M, Tschopp J. The caspases-8 inhibitor FLIP promotes activation of NF- $\kappa$ B and ERK signaling pathways. *Curr Biol.* 2000;10:640–8.
55. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nature Rev Mol Cell Biol.* 2004;3:401–10.
56. Heyninck K, Beyaert R. A20 inhibits NF- $\kappa$ B activation by dual ubiquitin-editing functions. *Trends Biochem Sci.* 2005;30:1–4.
57. Lin S-C, Chung JY, Lamothe B, Rajashanker K, Lu M, Lo Y-C, Lam AY, Darnay BG, Wu H. Molecular basis for the deubiquitinating activity of the NF- $\kappa$ B inhibitor A20. *J Mol Biol.* 2008;376:526–40.
58. Komander D, Barford DS. Structure of the A20 OUT domain and mechanistic insight into deubiquitination. *Biochem J.* 2008;408:77–85.
59. Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M. Tumor necrosis factor-induces Bcl-2 and Bcl-x expression through NF- $\kappa$ B activation in primary hippocampus neurons. *J Biol Chem.* 1999;274:8531–8.
60. Chen C, Edelstein LC, Gelinas C. The Rel/NF- $\kappa$ B family directly activates expression of the apoptotic inhibitor Bcl-x (L). *Mol Cell Biol.* 2000;20:2687–95.
61. Gupta S, Kim H, Yel L, Gollapudi S. A role of fas-associated death domain (FADD) in increased apoptosis in aged humans. *J Clin Immunol.* 2004;24:24–9.
62. Lin Y, Devin A, Rodriguez Y, Liu ZG. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* 1999;13:2514–26.
63. Xia Z-P, Chen ZJ. TRAF2: a double edge sword? *Science Stke.* 2005;72:1–4.
64. Chung JY, Park YC, Ye H, Wu H. All TRAFs are not created equal: common distinct molecular mechanisms of TRAF-mediated signal transduction. *J Cell Sci.* 2002;115:679–88.
65. Sun L, Chen J. The novel functions of ubiquitination in signaling. *Curr Opin Cell Biol.* 2004;16:119–26.
66. Chen ZJ. Ubiquitination in signaling to and activation of IKK. *Immunol Rev.* 2012;246:95–106.
67. Wu C-J, Conze DB, Li T, Srinivasula SM, Ashwell JD. Sensing of Lys63-linked polyubiquitination by NEMO is a key event in NF- $\kappa$ B activation. *Nature Cell Biol.* 2006;8:398–406.
68. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. Activation of IKK by TNF $\alpha$  requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell.* 2006;22:245–57.
69. Li H, Kobayashi M, Blonska M, You Y, Lin X. Ubiquitination of RIP is required for tumor necrosis factor- $\alpha$ -induced NF- $\kappa$ B activation. *J Biol Chem.* 2006;281:13636–43.
70. Israel A. NF- $\kappa$ B activation. Nondegenerative ubiquitination implicates NEMO. *Trends Immunol.* 2006;27:395–7.
71. Morioka S, Broglie P, Omori E, Ikeda Y, Takaesu G, Matsumoto K, Ninomiya-Tsuji J. TAK1 kinase switches cell fate from apoptosis to necrosis following TNF stimulation. *J Cell Biol.* 2014;204:607–23.
72. Shim J-H, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee K-Y, Bussey C, Steckel M, Tanaka N, Yamada AS, Matsumoto K, Ghosh S. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev.* 2005;19:2668–81.
73. Pujari R, Hunte R, Khan WN, Shembade N. A20-mediated negative regulation of canonical NF- $\kappa$ B signaling pathway. *Immunol Res.* 2013;57:166–71.
74. Deveraux QL, Reed JC. IAP family proteins: suppressor of apoptosis. *Genes Dev.* 1999;13:239–52.
75. Mahoney DJ, Cheung HH, Mrad RL, Plenchette S, Simard C, Enwere E, Arora V, Mak TW, Lacasse EC, Waring J, Korneluk RG. Both cIAP1 and cIAP2 regulate TNF- $\alpha$ -mediated NF- $\kappa$ B activation. *Proc Natl Acad Sci (USA).* 2008;105:11778–83.
76. Gupta S. A role of inhibitor of apoptosis (IAP) proteins in increased TNF- $\alpha$ -induced apoptosis in lymphocytes from aged humans. *Mech Ageing Dev.* 2004;125:99–101.
77. Golks A, Brenner D, Krammer PH, Lavrik IN. The c-FLIP-NH2 terminus (p22-FLIP) induces NF- $\kappa$ B activation. *J Exp Med.* 2006;203:1295–305.
78. Kataoka T, Tschopp J. N-terminal fragment of cFLIP (L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF- $\kappa$ B activation. *Mol Cell Biol.* 2004;24:2627–36.
79. Matsuda I, Matsuo K, Matsushita Y, Haruna Y, Niwa M, Kataoka T. The C-terminal domain of the long form of cellular FLICE-inhibitory protein (c-FLIPL) inhibits the interaction of the caspase 8 prodomain with the receptor-interacting protein 1 (RIP1) death domain and regulates caspase 8-dependent nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation. *J Biol Chem.* 2014;289:3876–87.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)

