4. TNF-induced apoptosis in aging

INTRODUCTION

TNF-α is a 17-kDa pleiotropic cytokine that is produced by a variety of cells and mediate a wide variety of inflammatory and immunological responses (274). In vivo, it acts as an antiviral agent because cells infected with a number of viruses are susceptible to TNF (275-278) and in most cells, TNF-mediated killing resembles apoptosis characterized by membrane blebbing and DNA fragmentation (279). TNF exerts its effects by binding to two cell surface receptors (TNFRI and TNFRII). Both receptors are present on a large variety of cell types (280, 281).

The cytotoxicity of TNF is regulated by two kinds of opposing activities (i) protein synthesis-independent cytotoxic mechanisms and (ii) protein synthesis-dependent cell protective mechanisms. The balance between these destructive and protective effects allows TNF to act selectively destroying diseased and transformed cells without affecting the viability of normal cells. The protein synthesis-independent cytotoxic pathway involves interaction of death-domain (DD) containing adaptor molecules and caspases leading to apoptosis, whereas, the protein synthesis-dependent protective pathway involves activation of transcription factors, including NF-κB (reviewed in 282).

Recently, we have shown that in T cells from aging, there is an increased susceptibility to Fas-mediated apoptosis as compared to young subjects (283). Because the downstream signaling pathways in Fas-induced apoptosis and TNF-induced apoptosis are similar and in aging, serum levels of TNF-α are elevated (284-286), we studied the role of TNF-α in T cell apoptosis in aging humans. We observed an increased TNF-induced apoptosis in aging T cells which is associated with increased expression of TNFRI and TRADD and a decreased expression of TNFRII and TRAF-2. Furthermore,
an increased and early activation of caspase-8 and caspase-3 was observed in aging lymphocytes as compared to young controls.
RESULTS

4.1 TNF receptor expression

4.1.1 TNFRI and TNFRII protein expression

The expression of TNF receptors (I and II) on T cell subsets from aging and young subjects was determined at the basal level, using dual color flow cytometry. Freshly isolated lymphocytes from aging and young subjects were stained with monoclonal antibodies against TNFRI, TNFRII, CD4 or CD8. Data are expressed in MFC# as a reflection of change in density of the receptors. Data (mean±SD) from ten aging and ten young subjects in CD4+ and CD8+ T cell subsets are shown in Figure 4.1. There was a significantly higher expression (P<0.002) of TNFRI in aging (CD4+: 235±25; CD8+: 212±22) as compared to young controls (CD4+: 144±18; CD8+: 122±21) in both CD4+ and CD8+ T cell subsets. Furthermore, there was a significant decrease (P<0.002) in TNFRII expression in both CD4+ and CD8+ T cell subsets in aging (CD4+: 220±12; CD8+: 350±23) as compared to young controls (CD4+: 316±15; CD8+: 462±24). A representative FACS plot for TNFRI and TNFRII staining from one young and one aging subject is shown in Figure 4.2.

4.1.2 TNFRI and TNFRII mRNA expression

To determine whether the changes in TNFRI and TNFRII in aging are regulated at transcriptional or translational level, we compared the expression of TNF receptors (TNFRI and TNFRII) in aging and young subjects at the mRNA level, using quantitative PCR. Total cellular RNA was prepared from freshly isolated lymphocytes from aging and young subjects. 200 ng of total cellular RNA was used to synthesize cDNA using RT and random hexamers. Graded amounts of cDNA (1 and 2 μl) were used to perform quantitative PCR using primers for TNFRI, TNFRII and β-actin and PCR was carried out...
Figure 4.1 TNF receptor (TNFRI and TNFRII) expression in T cell subsets.
Freshly isolated mononuclear cells from aging and young subjects were stained using TNFRI, TNFRII, CD4 or CD8 monoclonal antibodies. CD4+ and CD8+ T cells were gated and percent cells expressing TNFRI or TNFRII and their mean fluorescence channel intensities determined using FL1 channel. Mean±SD data (in MFC#) from ten aging and ten young subjects are shown.
Figure 4.2 TNF receptor (TNFRI) expression in T cell subsets
Freshly isolated mononuclear cells from aging and young subjects were stained using TNFRI, CD4 or CD8 monoclonal antibodies. CD4+ and CD8+ T cells were gated and percent cells expressing TNFRI and their mean fluorescence channel intensities determined using FL1 channel. Representative FACS plot from one aging and one young subject is shown.
Figure 4.2 TNF receptor (TNFRII) expression in T cell subsets
Freshly isolated mononuclear cells from aging and young subjects were stained using TNFRII, CD4 or CD8 monoclonal antibodies. CD4⁺ and CD8⁺ T cells were gated and percent cells expressing TNFRII and their mean fluorescence channel intensities determined using FL1 channel. Representative FACS plot from one aging and one young subject is shown.
for 25 cycles. The mean±SD data (in O.D ratio) from three subjects using 1 and 2 μl cDNA is shown in Figure 4.3. A representative gel photograph (from three experiments) for quantitative PCR from one aging and one young subject (in triplicates) is shown in Figure 4.4. An increased TNFRI mRNA and a decreased TNFRII mRNA expression was observed in lymphocytes from aging as compared to young controls.

4.2 TNF-induced apoptosis

Next, we compared the susceptibility of T cells from aging and young subjects to undergo TNF-α-induced apoptosis. The cells were activated for 48 hours in the presence of anti-CD3, followed by culture in IL-2 containing medium for another four days. The cells were then treated with TNF-α (1μg/ml) for an additional 48 hours and the percent apoptotic cells determined by TUNEL assay. In order to determine the percent apoptotic cells in CD4⁺ and CD8⁺ T cell subsets, cells were stained with PE-labeled CD4 or CD8 monoclonal antibodies. Data (mean±SD) data from ten aging and ten young subjects are shown in Figure 4.5. There was an increased susceptibility of T cells from aging (CD4⁺TUNEL⁺: 22±5; CD8⁺ TUNEL⁺: 40±10) to undergo TNF-α-induced apoptosis (P<0.01) as compared to young controls (CD4⁺ TUNEL⁺: 14±4; CD8⁺ TUNEL⁺: 26±7). A representative TUNEL graph from one aging and one young subject is shown in Figure 4.6.

Next, DNA was extracted from the lymphocytes activated as above and treated with TNF-α for 48 hours. DNA was electrophoresed on a 2% agarose gel and DNA ladder analyzed. In aging, there was an increased DNA fragmentation as compared to young controls. Representative gel photographs from two aging and two young subjects are shown in Figure 4.7.
Figure 4.3 TNFRI and TNFRII mRNA expression in lymphocytes

2 µl cDNA was obtained following RT using 100 ng of total cellular RNA and PCR carried out in triplicates for 25 cycles using TNFRI, TNFRII and β-actin primers. PCR products were electrophoresed and exposed to X-ray film for 4 hours. Mean±SD data (in O.D.) from three aging and three young subjects are shown.
Figure 4.4 *TNFRI* and *TNFRII* mRNA expression in lymphocytes

2 μl cDNA was obtained following RT using 100 ng of total cellular RNA and PCR carried out in triplicates for 25 cycles using *TNFRI*, *TNFRII* and β-actin primers. PCR products were electrophoresed and exposed to X-ray film for 4 hours. Representative gel photograph from one aging and one young subject is shown.
TNF-induced apoptosis in T cell subsets

Figure 4.5 TNF-induced apoptosis in T cell subsets from aging
Following culture of freshly isolated MNC, cells were treated with TNF-α for 48 hours. Nicked DNA was stained with FITC-labeled dUTPs using TUNEL assay and PE-labeled CD4 and CD8 antibodies. CD4+ and CD8+ cells were gated, and percentage of cells undergoing apoptosis was determined using FL1 channel. Mean±SD data (in % TUNEL+ cells) from ten aging and ten young subjects are shown.
Figure 4.6 Increased TNF-induced apoptosis in T cell subsets from aging

Following culture of freshly isolated MNC, cells were treated with TNF-α for 48 hours. Nicked DNA was stained with FITC-labeled dUTPs using TUNEL assay kit and PE-labeled CD4 and CD8 antibodies. CD4+ and CD8+ cells were gated, and percentage of cells undergoing apoptosis was determined using FL1 channel. Representative histograms from one aging and one young subject are shown.
Following TNF-\(\alpha\) treatment of cultured lymphocytes from aging and young, cells were lysed and DNA extracted and electrophoresed on 2\% gel. Representative gels from two aging and two young subjects are shown. Increased number of DNA ladder pattern is seen in aging (A) as compared to young (Y).
4.3 Expression of TRADD

TRADD is an adaptor protein, which is recruited to the cytoplasmic domain of the TNFRI following receptor trimerization. Interaction of TRADD with TNFRI leads to the activation of apoptotic pathway. In contrast, interaction of TRADD with TRAF-2 leads to the activation of NF-kB pathway, which is involved in cell proliferation. Therefore, we compared the expression of TRADD in lymphocytes from aging and young both at the protein and the mRNA level.

4.3.1 Expression of TRADD protein

The expression of TRADD at the protein level was determined on lymphocytes from aging and young using Western blotting. Cell lysates were prepared from freshly isolated lymphocytes, equal amounts of proteins were loaded, and the expression of TRADD was determined with monoclonal antibody and Western blotting and chemiluminiscent detection method. In aging, there was an increased expression of TRADD as compared to young controls. Representative gel photograph from two aging and two young subjects are shown in Figure 4.8.

4.3.2 Expression of TRADD mRNA

In order to determine that if the expression of TRADD protein is regulated at transcriptional level, we determined the expression of TRADD at the mRNA level using Northern blotting. Total cellular RNA was extracted from lymphocytes from aging and young subjects and 200 μg was electrophoresed in a denaturing gel. The expression of TRADD mRNA was quantitated using [α-32P]dATP labeled probe. The probe for TRADD was prepared by extracting the TRADD cDNA from the plasmid encoding full length TRADD transcript (pRK5-myc-TRADD). The plasmid map and the purified insert are shown in Figure 4.9. In aging, there was an increased constitutive expression of
Figure 4.8 TRADD expression in lymphocytes from aging
Cell lysates were prepared from freshly isolated lymphocytes from aging and young subjects and TRADD expression at the protein level was determined by Western blotting. Data from two subjects in each group are shown. An increased TRADD expression is seen in aging subjects as compared to young.
**TRADD Pasmid Map**

Figure 4.9 Full length coding region for TRADD
Plasmid map encoding TRADD coding region (pRK5-myc-TRADD) is shown on the left. The insert was extracted and purified from the expression vector used as a probe for Northern blotting. The insert DNA (1.5 kb) and the vector (4 kb) are shown on the right.

**TRADD insert**

1: TRADD plasmid
2: linearized plasmid
3: purified insert

**Figure 4.10 TRADD mRNA expression in lymphocytes from aging**
Total cellular RNA was prepared from freshly isolated lymphocytes from aging and young subjects and TRADD mRNA expression was determined using Northern blotting. Data from two aging and two young subjects are shown. Increased TRADD mRNA expression is observed in aging as compared to young controls.
TRADD mRNA as compared to young controls. Representative Northern blot gels from two aging and two young subjects are shown in Figure 4.10.

4.4. Expression of TRAF-2

The TRAF-2 is another adaptor protein which plays a role in signaling via TNF-α. TRAF-2 does not possess the death domain, but possesses another region of homology, ‘TRAF homology domain’ which is shared by other TRAF protein family members. TRAF-2 directly interacts with TNFRII via TRAF-homology domain and results in the activation of transcription factor NF-κB. Therefore, next, we compared the expression of TRAF-2 in the lymphocytes from aging and young.

4.4.1 Expression of TRAF-2 protein

The expression of TRAF-2 at the protein level was determined in lymphocytes from aging and young using Western blotting. Cell lysates were prepared from freshly isolated lymphocytes, equal amounts of proteins loaded, and the expression of TRAF-2 was determined with monoclonal antibody by Western blotting and chemiluminiscent detection method. In aging, there was a decreased expression of TRAF-2 as compared to young controls. Representative gel photographs from two aging and two young subjects are shown in Figure 4.11.

4.4.2 Expression of TRAF-2 mRNA

In order to determine that if the expression of TRAF-2 protein is regulated at transcriptional level, we determined the expression of TRAF-2 at the mRNA level using Northern blotting. Total cellular RNA was extracted from lymphocytes from aging and young subjects and 200 μg was electrophoresed in a denaturing gel. The expression of TRAF-2 mRNA was quantitated using [α-32P]dATP labeled probe. The probe for TRAF-2 was prepared by extracting the TRAF-2 cDNA from the plasmid encoding full length...
Figure 4.11 TRAF-2 expression in lymphocytes from aging
Cell lysates were prepared from freshly isolated lymphocytes from aging and young subjects and TRAF-2 expression at the protein level was determined by Western blotting. Data from two subjects in each group are shown. A decreased TRAF-2 expression is seen in aging subjects as compared to young.
TRAF-2 transcript. The plasmid map and the purified insert are shown in Figure 4.12. In aging, constitutive expression of TRAF-2 mRNA was decreased as compared to young controls. Representative Northern blot gels from two aging and two young subjects are shown in Figure 4.13.

4.5 Expression of RIP

The RIP is an adaptor protein which interacts indirectly with FADD and TRADD, however, the precise role of RIP is still unknown. The RIP protein contains a kinase domain and has been shown to play a role in apoptosis upon TNF-α treatment.

4.5.1 Expression of RIP protein

The expression of RIP at the protein level was determined on lymphocytes from aging and young using Western blotting. Cell lysates were prepared from freshly isolated lymphocytes, equal amounts of proteins loaded, and the expression of RIP was determined with monoclonal antibody by Western blotting and chemiluminiscent detection method. There was a comparable expression of RIP between aging and young samples. Representative gel photographs from two aging and two young subjects are shown in Figure 4.14.

4.5.2 Expression of RIP mRNA

In order to determine whether the expression of RIP protein is regulated at transcriptional level, we determined the expression of RIP mRNA using Northern blotting. Total cellular RNA was extracted from lymphocytes from aging and young subjects and 200 μg was electrophoresed in a denaturing gel. The expression of RIP mRNA was quantitated using [α-32P]dATP labeled probe. The probe for RIP was prepared by extracting RIP cDNA from the plasmid encoding full length RIP transcript (pRK5-Flag-RIP). The plasmid map and purified insert are shown in Figure 4.15. There
Figure 4.12 Full length coding region for TRAF-2
Plasmid map encoding TRAF-2 coding region (pcDNA3 AU1 FADD) is shown on the left. The insert was extracted and purified from the expression vector used as a probe for Northern blotting. The insert DNA (700 bp) and the vector (5.4 kb) are shown on the right.

Figure 4.13 TRAF-2 mRNA expression in lymphocytes from aging
Total cellular RNA was prepared from freshly isolated lymphocytes from aging and young subjects and TRAF-2 mRNA expression was determined using Northern blotting. Data from two aging and two young subjects are shown. Decreased TRAF-2 mRNA expression is observed in aging as compared to young controls.
Figure 4.14 RIP expression in lymphocytes from aging
Cell lysates were prepared from freshly isolated lymphocytes from aging and young subjects and RIP expression at the protein level was determined by Western blotting. Data from two subjects in each group are shown. No significant difference in RIP expression was observed between aging and young.
Figure 4.15 Full length coding region for RIP
Plasmid map encoding RIP coding region (pRK5-Flag-RIP) is shown on the left. The insert was extracted and purified from the expression vector used as a probe for Northern blotting. The insert DNA (1.5 kb approx.) and the vector (4 kb approx.) are shown on the right.

Figure 4.16 RIP mRNA expression in lymphocytes from aging
Total cellular RNA was prepared from freshly isolated lymphocytes from aging and young subjects and RIP mRNA expression was determined using Northern blotting. Data from two aging and two young subjects are shown. No significant difference in RIP mRNA expression is observed between aging and young.
was no significant difference in the constitutive expression of RIP mRNA between aging and young. Representative Northern blot gels from two aging and two young subjects are shown in Figure 4.16.

4.6 Caspase activity

We had previously shown that an increased susceptibility of T cell subsets from aging to undergo Fas-mediated apoptosis and this increased apoptosis is associated with an early activation of caspases and increased caspase (caspase-8 and caspase-3) activity. Therefore, we compared whether the increased TNF-α-induced apoptosis in T cells from aging is also associated increased caspase activity.

4.6.1 Caspase-8 activity

Freshly isolated lymphocytes from aging and young subjects were activated in the presence of anti-CD3 for 48 hours followed by culture in IL-2 containing medium for another 4 days. Following incubation, the cells were treated with TNF-α for 0, 24 or 48 hours. Cell lysates were prepared and the caspase-8 activity was determined using Western blotting and chemiluminescent detection system. Upon activation, the pro-form of caspase-8 is cleaved into its enzymatically active form (p20) and is detected by anti-p20 antibody. In aging, there was an early detection of the cleaved form of caspase-8 (within 24 hours of TNF-α treatment) as compared to young controls (48 hours following TNF-α treatment). A representative gel photograph from three aging and three young subjects is shown in Figure 4.17.

4.6.2 Caspase-3 activity

Freshly isolated lymphocytes from aging and young subjects were activated in the presence of anti-CD3 for 48 hours followed by culture in IL-2 containing medium for another 4 days. Following incubation, the cells were treated with TNF-α for 0, 24
Figure 4.17 Caspase-8 activity in lymphocytes from aging and young. Activated lymphocytes (as described in cell culture) from aging and young subjects were treated with TNF-α for 0, 24 or 48 hours. Cell extracts were prepared and Western blotting was performed using antibody against p20 (cleaved form of caspase-8) fragment of caspase-8. Data from two aging and one young subjects are shown.

1, 2, and 3 are time points 0, 24, and 48 hours respectively.
or 48 hours. Caspase-3 activity was determined using following criteria: (i) its ability to cleave a peptide substrate (DEVD-pNA) to release a colorometric substrate, pNA and (ii) its ability to cleave its 116 kDa substrate PARP to release a 85 kDa fragment. Cells were lysed and caspase-3 activity was quantitated by colorometric detection method using ApoAlert™ kit. Mean±SD data (in O.D.) from five aging and five young subjects are shown in Figure 4.18. In aging, there was an increased caspase-3 activity following TNF-α treatment (within 24 hours of TNF-α treatment) as compared to young controls (within 48 hours of TNF-α treatment). Furthermore, caspase-3 activity was significantly higher (P<0.005) in aging as compared to young controls at 48 hours of TNF-α treatment.

In order to detect the PARP cleavage activity of caspase-3 upon TNF-α treatment, cell lysates were prepared from lymphocytes from aging and young samples. Equal amounts of proteins were loaded and the PARP cleavage analyzed using Western blotting and chemiluminiscent detection system. In aging, the cleaved form of PARP (p85) was detected at 24 hours following TNF-α treatment as compared to 48 hours in young controls. A representative gel photograph from three aging and three young subjects is shown in Figure 4.19.

4.7 DNA fragmentation

Finally, we analyzed the kinetics of DNA fragmentation following TNF-α treatment in lymphocytes from aging and young using TUNEL assay. Freshly isolated lymphocytes from aging and young subjects were activated with anti-CD3 as described before and treated with TNF-α for 0, 24 and 48 hours. There was no significant difference in the percent cells undergoing apoptosis (%TUNEL + cells) between aging and young at 24 hours following TNF-α treatment. In contrast, at 48 hours,
higher proportions of lymphocytes from aging were TUNEL positive as compared to young controls. A representative histogram for TUNEL assay from one aging and one young subject is shown in Figure 4.20.
Figure 4.18 TNF-induced caspase-3 activity in aging
Lymphocytes from aging and young subjects were activated and treated with TNF-α for 0, 24, or 48 hours. Caspase-3 activity was determined by colorometric assay using ApoAlert kit. Mean±SD data (in O.D.) from six aging and six young subjects are shown.
Figure 4.19 Cleavage of PARP in lymphocytes from aging and young
Activated lymphocytes (as described in cell culture) from young and aging
subjects were treated with TNF-α for 0, 24 or 48 hours. Following incubation,
the protein was extracted and PARP cleavage examined by Western blotting.
Cleaved form of PARP (p85) was seen at 24 hours in aging (A1, A2 and A3) as
compared to 48 hours in young (Y1, Y2, and Y3).
Figure 4.20 Increased DNA fragmentation in lymphocytes from aging
Activated lymphocytes were treated with TNF-α for 0, 24 or 48 hours. Following incubation, cells were harvested and percent apoptotic cells determined using TUNEL assay. Representative graphs from one aging and one subject are shown. A significantly increased TUNEL⁺ cells are seen in lymphocytes from aging following 24 and 48 hours of TNF-α treatment as compared to young controls.
DISCUSSION

In the present study, we have shown that in aging, there is an increased susceptibility of CD4^+ and CD8^+ T cell subsets to undergo TNF-α-induced apoptosis. Furthermore, the increased apoptosis in aging was associated with increased TNFRI expression and a decreased TNFRII expression as compared to young controls. Finally, we have shown that the constitutive expression of TRADD was increased and that of TRAF-2 decreased in lymphocytes from aging. An increased and early activation of caspases (caspase-8 and caspase-3) involved in the TNF/TNFR pathway was observed in aging lymphocytes.

TNFRI mediates most of the biological properties of TNF-α including programmed cell death and activation of transcription factor NF-κB (287, 288). Upon oligomerization, TNFRI binds to and recruits TRADD and indirectly binds to FADD via interaction between death domain of FADD and TRADD. FADD, in turn, binds to caspase-8 through death effector domain (DED) and when activated, is cleaved into enzymatically active form (p20) leading to the activation of downstream caspase cascade, including caspase-3 resulting in apoptosis (139, 288). TRADD also interacts with TRAF-2 suggesting that TRADD may function as an adaptor to recruit other signaling proteins to TNFRI upon stimulation (140). TNFRII lacks death domain and interacts directly with TRAF-2 via ‘TRAF homology domain” (142, 143, 289a, 289b). TRAF-2 is required for the recruitment of cellular inhibitor of apoptosis protein (cIAP) family to the TNFRII signaling complex and antagonizes T cell apoptosis (290). TRAF-2 activates both NF-κB and JNK and mediates it anti-apoptotic effect (291-293). Therefore, TNFRII is involved in anti-apoptotic effect of TNF; whereas TNFRI is involved in both apoptotic and anti-apoptotic signaling.
We also observed that T cells from aging have increased susceptibility to undergo apoptosis as compared to young controls. We suggest that the increased TNFRI expression may play a role in increased T cell apoptosis in aging. TNFRI overexpression has been shown to trigger cell death (294).

In this study, we have observed an increased TNFRI expression in T cells from aging as compared to young controls. The increase was observed in the percent cells expressing TNFRI as well as in the mean fluorescence channel intensity (MFC#), the latter suggesting increased expression of TNFRI molecules. Ware et al. (295) have shown that fresh lymphocytes lack TNFRI expression and following activation, there is an upregulation of TNFRI expression and downregulation of TNFRII. We determined the TNF receptor expression on freshly isolated lymphocytes from aging and young controls. An increased TNFRI expression and a decreased TNFRII expression in aging as compared to young may suggest an increased in vivo activation of T cell subsets in aging as compared to young controls.

We also observed an increased TRADD expression and a decreased TRAF-2 expression in lymphocytes from aging as compared to young controls. Overexpression of TRADD results in apoptotic cell death in many cell lines which was similar to TNF-induced cell death (296).

TRAF-2 has been shown to involved in the regulation of lymphocyte function and growth in vivo. TRAF-2 appears to be required for NF-κB-mediated cell activation via both TNF receptors (139, 140, 142, 143). Recently, it has been shown that NF-κB activation acts as a break for apoptosis (136-138). In addition, TRAF-2 mediates JNK activation via TNFRI (291-293). Therefore, TRAF-2 exerts its anti-apoptotic effects in lymphocytes via both TNF receptors. Lee et al. (297) have shown TRAF-2 induced
NF-κB-independent anti-apoptotic pathways during TNF-induced apoptosis and inhibition of TRAF-2 leads to splenomegaly, lymphoadenopathy and increased number of B cells. Mice deficient in TRAF-2, *traf-2<sup>-/-</sup>*, appear to be normal at birth but become progressively runted and die prematurely. In these mice, thymocytes and other hematopoietic progenitors were shown to be highly sensitive to TNF-α-induced cell death and serum TNF levels were found to be elevated (298). It is interesting to note that in aging humans, serum TNF levels are increased (284-286) and we have observed decreased expression of TRAF-2 and increased sensitivity to TNF-induced apoptosis. It is unclear whether decreased TRAF-2 expression in aging may play a role in increased apoptosis of lymphocytes via NF-κB-dependent or NF-κB-independent pathway or both.

Recently, cells lacking STAT proteins have been shown to be resistant to TNF-α-induced apoptosis (261). The resistance to apoptosis was associated with low constitutive levels of caspases, including caspase-3. We have observed an increased level of constitutive expression of caspase-8 and caspase-3 in lymphocytes from aging as compared to young (Aggarwal and Gupta, manuscript submitted). Furthermore, in the present study, we have observed an early and increased activation of caspase-8 and caspase-3 following TNF-α treatment. Increased activation of caspases in aging could be due to increased number of cells undergoing apoptosis. However, the time kinetics studies show an early activation of caspase-8 (24 hours) and caspase-3 (24 hours) in aging lymphocytes at a time when no apoptosis is observed by TUNEL assay; apoptosis by TUNEL assay was observed at 48 hours. This would suggest that increased activation of caspases at least in part, appears to be due to increase in activation rather than due to increased number of cells undergoing apoptosis. In
addition, increased constitutive expression of caspases in aging could in part, play a role in increased apoptosis. Whether increased expression of constitutive caspase-3 and caspase-8 leads to early and increased activation remains unclear.

In summary, lymphocytes in aging humans are more susceptible to TNF-induced apoptosis which is associated with increased activation of apoptotic pathway and decreased expression of molecules (TNFRII and TRAF-2) involved in anti-apoptotic pathway of TNF-α-mediated signaling. Increased serum levels of TNF and increased susceptibility of T cells to TNF-induced apoptosis may play a role in the pathogenesis of lymphopenia and lymphocyte dysfunction in aging.
SUMMARY

Aging is characterized by increased T cell lymphopenia, T cell dysfunction, and increased serum TNF-levels. In this study, we have examined the role of TNF-induced apoptosis in T cell deficiency in lymphocytes from aging humans. The constitutive expression of TNF receptors (TNFRI and TNFRII) and the adaptor molecules including TRADD, TRAF-2, and RIP were analyzed both at the protein level by flow cytometry or Western blotting and at the mRNA level using Quantitative PCR or Northern blotting in lymphocytes from aging and young subjects. The susceptibility of T cells to undergo TNF-induced apoptosis was analyzed using TUNEL and DNA ladder assays. Caspase (caspase-8 and caspase-3) activation was compared between aging and young subjects using Western blotting and colorometric assays. In lymphocytes from aging humans, there was an increased susceptibility of CD4+ and CD8+ T cells to undergo TNF-α-induced apoptosis as observed by TUNEL assay and DNA fragmentation ladder assay. An increased constitutive expression of TNFRI and TRADD and decreased expression of TNFRII and TRAF-2 was observed in lymphocytes from aging as compared to young controls. In addition, there was an early and increased activation of caspases (caspase-8 and caspase-3) involved in TNFR/TNF signaling pathway as evident by early cleavage of caspase-8, PARP and caspase-3 substrate DEVD-pNA. These data suggest that an increased TNF-α-induced apoptosis may play a role in T cell deficiency associated with human aging.