

Published in final edited form as:

J Immunol. 2013 October 1; 191(7): . doi:10.4049/jimmunol.1301409.

IFN- α Inhibits Telomerase in Human CD8⁺ T Cells by Both hTERT Downregulation and Induction of p38 MAPkinase Signalling

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Abstract

The cytokine IFN- α is secreted during viral infections and has been shown to inhibit telomerase activity and accelerate T cell differentiation *in vivo*. However the mechanism for this inhibition is not clear. In this study we show that IFN- α inhibits both the transcription and translation of hTERT, the catalytic component of telomerase, in activated CD8⁺ T cells. This was associated with increased activity of the repressor of hTERT transcription E2F and decreased activation of NF- κ B that promotes hTERT transcription. However IFN- α did not affect the translocation of hTERT from the cytoplasm to the nucleus. IFN- α also inhibits AKT kinase activation but increases p38 MAP kinase activity and both these events have been shown previously to inhibit telomerase activity. Addition of BIRB796, an inhibitor of p38 activity, to IFN- α treated cells reversed in part, the inhibition of telomerase by this cytokine. Therefore IFN- α can inhibit the enzyme telomerase in CD8⁺ T cells by transcriptional and post-translational mechanisms. Furthermore, the addition of IFN- α to CD8⁺CD27⁺CD28⁺ T cells accelerates the loss of both these co-stimulatory molecules. This suggests that persistent viral infections may contribute to the accumulation of highly-differentiated/senescent CD8⁺CD27⁻CD28⁻ T cells during ageing by promoting IFN- α secretion during repeated episodes of viral re-activation.

Keywords

CD8; IFN, T cells; telomerase; hTERT

Introduction

Interferon alpha (IFN- α) is a cytokine that modulates innate and specific antiviral immunity that is released during chronic viral infections by plasmacytoid dendritic cells (pDCs) (1-3).

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We showed previously that IFN- γ can induce the loss of the co-stimulatory receptors CD27 and CD28, inhibit the activity of the enzyme telomerase and thus accelerate human T cell differentiation both *in vitro* (4, 5) and *in vivo* (6). In addition, more rapid disease progression occurs during HIV-1 infection when there is sustained IFN- γ production possibly through induction of T cell differentiation towards an end-stage (7, 8). However the mechanism by which this cytokine inhibits telomerase activity in human T cells is not known.

Telomeres are repeating hexameric sequences of nucleotides at the ends of chromosomes, which provide genomic protection and stability. In normal somatic cells telomere length shortens with each cell replication (9). When telomere length becomes critically short cells stop dividing and enter replicative senescence (10). However replicative senescence can be delayed by the activation of the enzyme telomerase, a RNA-dependent DNA polymerase that can replenish telomeric DNA at the 3' ends of eukaryotic chromosomes (11). Although telomerase activity is repressed in most adult somatic cells, human T lymphocytes are able to re-activate this enzyme which maintains telomeres and extends their proliferative lifespan after repeated antigenic stimulation (12). However as T cells progressively differentiate, they lose the capacity to up-regulate telomerase, which leads to telomere erosion and loss of proliferative capacity (13, 14).

Although IFN- γ can inhibit telomerase activity in hematopoietic cell lines (15, 16) and also primary T cells (4-6), the mechanism by which this occurs is not known. The transcriptional down-regulation of the catalytic subunit hTERT is one possible mechanism for telomerase inhibition (9, 15-17). However post-translational mechanisms such as the activation of hTERT by AKT (PKB) (14, 18, 19), inhibition of enzymatic activity by p38 MAPK signalling (20), changes in NF- κ B activity, that affects both transcriptional activation and nuclear import of hTERT (21, 22) and also alterations in activity of the enzyme protein phosphatase 2A (PP2A) that inhibits hTERT activation by dephosphorylating either AKT and hTERT (23, 24) may also be involved.

In this study we show that IFN- γ may regulate telomerase activity in human CD8⁺ T cells by multiple mechanisms. Firstly this cytokine inhibits the transcription of hTERT, that is associated with increased activity of the transcriptional repressor of hTERT transcription E2F (25) and also decreased activation of NF- κ B and AKT. Secondly IFN- γ induces p38 mitogen-activated protein kinase (p38 MAPK) signalling that induces reversible inhibition of telomerase activity. The multifaceted nature of the effects of IFN- γ on telomerase activity highlights the importance of the control of this enzyme during persistent viral infections. This may be a mechanism that prevents the over proliferation of T cells as a result of repeated antigenic challenge.

Materials and methods

Preparation of CD8⁺ T cells from human peripheral blood

Written informed consent was obtained and whole blood was collected in standard heparinised tubes from healthy volunteers. Unless stated, donors tested were <40 yrs of age. The study was approved by the Local Research Ethics Committee of the Royal Free and University College Medical School. Donors did not have any co-morbidity, were not on any immunosuppressive drugs, and retained physical mobility and lifestyle independence. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat samples obtained from healthy donors, by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and resuspended in RPMI-1640 medium, (GIBCO, Paisley, Scotland, UK), supplemented with 10% foetal calf serum (FCS, GIBCO), 2 mM glutamine (Flow Laboratories, McLean, VA, USA) and 100 IU/ml penicillin/streptomycin at 37°C in a

humidified 5% CO₂ incubator. CD8⁺ T cells (95% pure at FACS analysis) were obtained from PBMC by magnetic bead isolation using Miltenyi columns (Miltenyi Biotec Auburn, CA). Purified CD8⁺ T cells were activated in the presence of anti CD3 Ab (purified OKT3, 0,5 µg/mL) plus rhIL-2 (20 IU/mL, R&D systems). In some experiments, the p38 MAPK inhibitor BIRB796 (BIRB) was added to the culture at a final concentration of 500 nM (20). Cells were pre-treated with the inhibitor for 30 min. A solution of 0.1% DMSO was used as control.

Determination of donor CMV status

The CMV status of donors was obtained by the overnight stimulation of fresh PBMCs with CMV viral lysate and identification of IFN γ production by CD4⁺ T cells as previously described (5). There was total concordance between IFN γ responses and seropositivity obtained from IgG serology obtained from the diagnostic laboratory of University College London Hospital.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat samples obtained from healthy donors, by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and resuspended in RPMI-1640 medium, (GIBCO, Paisley, Scotland, UK), supplemented with 10% foetal calf serum (FCS, GIBCO), 2 mM glutamine (Flow Laboratories, McLean, VA, USA) and 100 IU/ml penicillin/streptomycin at 37°C in a humidified 5% CO incubator. CD8⁺ T cells (95% pure at FACS analysis) were obtained from PBMC by magnetic bead isolation using Miltenyi columns (Miltenyi Biotec Auburn, CA). Purified CD8⁺ T cells were activated in the presence of anti CD3 Ab (purified OKT3, 0,5 µg/mL) plus rhIL-2 (20 IU/mL, R&D systems). In some experiments, the p38 MAPK inhibitor BIRB796 (BIRB) was added to the culture at a final concentration of 500 nM (20). Cells were pre-treated with the inhibitor for 30 min. A solution of 0.1% DMSO was used as control.

Measurement of telomerase activity

Telomerase activity was determined using the TRAPeze telomerase detection kit (Chemicon Europe) or using the TeloTAGGG telomerase ELISA kit from Roche according to the manufacturer's instructions from extracts of 2×10^3 viable CD8⁺ T cells. Nuclear and cytoplasmic extracts were obtained as described by Andrews and Faller (26). Band intensity was quantified by bi-dimensional densitometry (Bio-Rad, Richmond, CA, USA). The absolute numbers of CD8⁺ T cells were enumerated using Trypan blue (Sigma) and Ki67 analysis (BD Pharmingen).

Real Time PCR

Briefly 1×10^6 viable CD8⁺ T cells stimulated with anti CD3 Ab plus rhIL-2 and treated or not with IFN γ were harvested after 24 h incubation. Samples were resuspended in Trizol (Ambion) and RNA extracted according to the manufacturer's instructions. For cDNA synthesis, 1 µg total RNA/sample was retro transcribed using M-MuLV Reverse Transcriptase (Invitrogen) and random primers. hTERT mRNA was quantitatively detected by real-time reverse transcription polymerase chain reaction (RT-PCR). For quantitative real time RT-PCR 1 µl of cDNA/sample was amplified according to the manufacturer's instructions (Applied Biosystems) on a ABI-PRISM 7500. Levels of hTERT were normalized against ACTB housekeeping expression. hTERT (Hs00972656_m1) and ACTB (Hs01060665_g1) RT primers were purchased from Applied Biosystems. All real-time RT-PCR reactions were performed in triplicate. Normalized TERT expression (TERT/ ACTB) was calculated using the $\Delta\Delta C_t$ method according to the supplier's protocol.

Electrophoretic Mobility Shift Assay (EMSA)

Transcription of hTERT has been shown to be inhibited by the repressor E2F (27), while NF- κ B promotes the transcription of the catalytic subunit of telomerase (21). However, as no NF- κ B specific consensus binding site has been found and p65 was not detected on the hTERT promoter (21), the effect mediated by NF- κ B may be indirect. IFN- γ mediated modulation of E2F and NF- κ B binding to their specific DNA binding-sites was analyzed by EMSA. Nuclear extracts were obtained as previously described (26). Five micrograms of nuclear proteins/reaction were incubated with 30 000 cpm of 32 P-ATP (Amersham) end-labeled oligonucleotides. Extracts were incubated with the sequence corresponding to -184 to -161 bp of the hTERT promoter containing the putative E2F site (5'-CGCCCAGGACCGCGCTCCCCACGT-3')(27) and with a commercially available oligonucleotide containing NF- κ B consensus sequence (obtained from Promega) respectively. Reactions were performed in a 10- μ l volume for 20 min at room temperature in a buffer consisting of 5mg/ml poly (dI-dC), 10mMTris-HCl, 50mMNaCl, 0.5mMDDT, 0.5mM EDTA, 1 mM MgCl₂, 4% glycerol, pH 7.5 (Promega). Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis (PAGE) at 4°C. Dried gels were exposed to X-Ray film (Amersham) at -70°C for 12 h.

Western Blot analysis

For Western Blot analysis of whole cell extracts, purified CD8⁺ T cells were activated in the presence of anti CD3 Ab plus rhIL-2 and lysates obtained by sonicating cells in 50 mM Tris-HCl pH 7.5, 2 mM EGTA, 0.1 % triton X-100 buffer. Cytoplasm and nuclear extracts were prepared as previously described (26). Lysates from 2×10^6 cells for whole cell extracts and 5×10^6 cells for fractionated extracts were separated on 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then probed with anti pAKT1/2/3 (Ser 473), pAKT (Thr 308), hTERT, PP2A, pPP2A (Tyr 307) Abs (all from Santa Cruz Biotech Inc.) and anti AKT1, p38 MAPK and pp38 MAPK (Thr180/Tyr182) Abs (from Cell Signalling) following the instructions provided by the manufacturers. All filters were probed with anti α -actin Ab (Santa Cruz) as loading control. Quality of nuclear extracts was analyzed using anti Histone H1 Ab (Upstate, Lake Placid, NY, USA). Analysis was performed using the ECL Plus Western detection kit (Amersham Pharmacia Biotech).

Nuclear Pore Complex (NPC) analysis

Isolated CD8⁺ T cells in phosphate buffered saline (PBS) at ~2,000 cells/ μ l were allowed to settle in the centre of a previously Poly-L-Lysine (Sigma Aldrich P8920) coated and washed coverslip in a humidified chamber at room temperature (RT) for 10 min. Cells were fixed with 2% Formaldehyde in PBS for 20 min., permeabilized with 0.5% TX-100 in PBS for 5 min. and then incubated with anti Nup358 or anti-NPC 414 Abs (Covance) for 45min. in a humidified chamber, then with secondary Abs conjugated to Alexa 488 or Alexa 647 (Life Technologies). Cells were washed with PBS, 0.1% TX-100, in PBS plus 1 μ g/ml Hoechst 33342. The coverslips were then mounted on glass slides with 2 μ l of Prolong Gold (Life Technologies P36934). The images were collected on an inverted confocal microscope (Olympus) with a 60X objective (N.A. 1.45) with a SIM scanner using the Fluoview software. The fluorescence within a region of interest surrounding the nuclear envelope in four mid-plane sections was measured using ImageJ (Wayne Rasband, NIMH USA).

p38 MAPK blockade

2×10^5 human CD8⁺ T cells were stimulated in the presence of anti CD3 Ab pre-coated plates plus rhIL-2 (5 ng/mL). Where due, p38 blockade was achieved by pre incubating the cells with the chemical inhibitor BIRB796 (500nM) at 37 degrees for 30 as previously

described (20). After 3 days in culture, telomerase activity was measured using the TeloTAGGG telomerase ELISA kit from Roche according to the manufacturer's instructions. 2×10^3 viable CD8⁺ cells were used for the assay.

Statistical analysis

Statistical significance was evaluated using a paired one-way Student's *t*-test. Differences were considered significant when *p* was < 0.05.

Results

The inhibition of hTERT transcription regulation in CD8⁺ T cells by IFN- α

We first confirmed that telomerase activity in CD8⁺ T cells activated by anti-CD3 Ab and rhIL-2 was inhibited in a dose-dependent manner by IFN- α (representative data Fig. 1A, pooled data, Fig. 1B). This inhibition was significant at the highest concentration of IFN- α used (500IU/mL, *p*<0.05) which did not affect cell viability (trypan blue dye exclusion assay, data not shown).

A key mechanism for regulating telomerase activity in human cells is the transcriptional control of the telomerase catalytic subunit gene hTERT (9, 15-17). We therefore examined hTERT mRNA levels in IFN- α treated CD8⁺ T cells activated by anti CD3 Ab plus rhIL-2 for 24 hrs by quantitative real time RT-PCR analysis. IFN- α significantly reduced the hTERT mRNA levels normalized against ACTB housekeeping gene expression (Fig.1C). We next investigated whether the inhibition of telomerase activity was due to down-regulation of hTERT protein expression by Western Blot analysis. Time course studies indicated that hTERT protein was expressed optimally at 36 hrs. (data not shown) when IFN- α induced a significant decrease in hTERT protein expression (representative result in Fig. 1D, pooled data, Fig. 1E). Therefore, the IFN- α induced decline of telomerase activity in activated CD8⁺ T cells is associated with the decrease in hTERT transcription and translation.

IFN- α reduces both cytoplasmic and nuclear levels of hTERT and telomerase activity in activated CD8⁺ T cells

Nuclear localization of hTERT is essential for telomerase function (17, 21). We therefore investigated whether the decreased levels of total cell telomerase activity was accompanied by a reduction in its cytoplasmic and/or nuclear localization. We showed that telomerase activity was detected in both cytoplasmic and nuclear extracts of activated CD8⁺ T cells and that there was a significant reduction of telomerase activity induced by IFN- α in both compartments (Fig. 2A, 2B). This was associated with the decreased expression of cytoplasmic and nuclear hTERT protein (Fig. 2C, 2D).

The significant inhibition of telomerase activity and hTERT levels observed in the nuclei of IFN- α treated CD8⁺ T cells prompted us to analyse whether this was a consequence of a interference with the nuclear import machinery. The trafficking of macromolecules across the nuclear envelope (NE) occurs exclusively through large multi-subunit assemblies called nuclear pore complexes (NPCs). The core of the NPC consists of a luminal "spoke-ring" complex sandwiched between peripheral "rings" (28-30). This scaffold encloses a central channel for signal-mediated transport (30). We next investigated the distribution and composition of NPCs in CD8⁺ T cells from three different donors by indirect immunofluorescence after 24 hours stimulation. A representative example of NPC staining of CD8⁺ T cells within unfractionated PBMC is shown in Fig. 2E. Using anti-Nup358 or anti-414 which targets a panel of FG repeat containing nucleoporins (Nup358, Nup214, Nup153 and p62) no qualitative differences in NPC numbers or staining intensity were

observed in control and IFN- γ treated cells (see supplementary Figure). Collectively these data indicate that the inhibition of telomerase activity by IFN- γ is unlikely to be due to alterations of machinery responsible for the transport from the cytoplasm to the nucleus.

IFN- α inhibits hTERT transcription by modulating NF- κ B and E2F activation

Transcription of hTERT mRNA has been shown to be inhibited by the repressor E2F (27), while NF- κ B can promote the transcription of the catalytic subunit of telomerase (21). Since NF- κ B specific consensus binding site has been identified in the proximal hTERT promoter, the effect of this transcription factor on telomerase has been suggested to be indirect (22). EMSA-based experiments were performed to analyze whether there was evidence for the presence of proteins in activated CD8 $^{+}$ T cells that bind to the E2F and NF- κ B specific DNA sequences. Nuclear proteins were isolated from CD8 $^{+}$ T cells that were activated in the presence or absence IFN- γ (Fig. 3A, 3B). These nuclear protein extracts were incubated with the sequence corresponding to -184 to -161 bp of the hTERT promoter containing the putative E2F site (27) and with the standard oligonucleotide containing NF- κ B consensus sequence respectively. We found that IFN- γ up-regulated the binding of proteins (presumably E2F) to the E2F specific sequence of the hTERT promoter and decreased the binding activity of proteins (presumably NF- κ B) to its specific consensus sequence (representative experiment in Fig. 3A, pooled data from 3 different experiments in Fig. 3B). These observations suggest that the IFN- γ mediated decrease of hTERT mRNA transcription is related to increased activation of the E2F transcription factor and a decline of NF- κ B activity.

IFN- α mediates down regulation of AKT signalling

AKT signalling is crucial for the regulation of telomerase activity by activating hTERT and promoting its nuclear translocation (17, 19, 31) and defective AKT phosphorylation at both p-AKT^{Ser473} and p-AKT^{Thr308} sites is a molecular feature of highly differentiated CD8 $^{+}$ T cells (14, 18, 32). We therefore investigated whether the down-regulation of telomerase activity in activated CD8 $^{+}$ T cells by IFN- γ was related to AKT expression or phosphorylation. We found that the addition of this cytokine was able to decrease AKT phosphorylation in CD8 $^{+}$ T cells at both Ser⁴⁷³ and Thr³⁰⁸ sites while total AKT expression was not significantly affected (representative result in Fig. 4A). In addition, the mean of the ratio of the densitometric values of Ser⁴⁷³-AKT and Thr³⁰⁸-AKT values to total AKT showed a substantial reduction of AKT phosphorylation at both sites which reflects a significant kinase inactivation 24 hours following IFN- γ treatment (Fig. 4B). We next investigated whether IFN- γ had any effect on proteins upstream of AKT activation. PP2A phosphatase has been known to negatively regulate AKT activity by dephosphorylation (24). We therefore investigated whether the addition of IFN- γ to activated CD8 $^{+}$ T cells had any effect on the expression of PP2A. We found that addition of this cytokine increased the total levels PP2A and at the same time decreased the inactive form of the enzyme that is phosphorylated on Tyr³⁰⁷ (Fig. 4A, 4B) (33). Therefore, the inhibition of telomerase by IFN- γ is associated with the increased synthesis and activity of PP2A that inhibits AKT, which is essential for telomerase activity in human CD8 $^{+}$ T cells (13, 14). Housekeeping actin was used as loading control in these experiments (Fig. 4A).

IFN- α activates p38 MAPK signalling that inhibits telomerase activity

Active p38 MAPK signalling pathway has recently been demonstrated to be involved in the decline of telomerase activity in highly differentiated CD4 $^{+}$ T cells (20). Upon activation, in response to both inflammatory cytokines and stress, p38 MAPK is phosphorylated on Thr180 and Tyr182 (34). We found that activated p-p38^{Thr180/Tyr182} MAPK was found to be up-regulated in activated CD8 $^{+}$ T cells by IFN- γ , while total p38 expression was not affected (Fig. 5A). The ratio of the densitometric values of p-p38^{180/182} to total p38 showed

a strong increase of p38 phosphorylation after 24 hour exposure to IFN- γ (Fig. 5B) which reflects a significant activation of the kinase. In all these experiments housekeeping β -actin was used as loading control (Fig. 5A).

To confirm a direct role of p38 MAPK signalling in the IFN- γ mediated impairment of telomerase activity in CD8 $^{+}$ T cells we blocked p38 signalling, in IFN- γ treated cells, by the addition of BIRB796, an inhibitor that blocks the activation of all four of the isoforms of p38 (35). The addition of BIRB796 reversed the down-regulation of telomerase activity that was induced by this cytokine (Fig. 5C,D). However we have not yet determined whether p38 blockade also reverses the inhibition of hTERT transcription or the inactivation of AKT that is also induced by IFN- γ .

IFN- α accelerates the loss of CD27 and CD28 after activation of CD27 $^{+}$ CD28 $^{+}$ T cells

CD8 $^{+}$ T cells can be subdivided on the basis of co-stimulatory receptor expression into those that are CD27 $^{+}$ CD28 $^{+}$ that have relatively long telomeres, CD27 $^{+}$ CD28 $^{-}$ cells that have intermediate telomere length and CD27 $^{-}$ CD28 $^{-}$ cells that have the shortest telomeres and have characteristics of highly differentiated T cells (14). We found that the CD27 $^{+}$ CD28 $^{+}$ T cells decrease while the CD27 $^{-}$ CD28 $^{-}$ T cells increase significantly with age (Fig. 6A,B). Furthermore we found that persistent infection with cytomegalovirus (CMV) induces a further decrease of CD27 $^{+}$ CD28 $^{+}$ and increase of CD27 $^{-}$ CD28 $^{-}$ T cells independently of age (Fig. 6B). We found that the CD27 $^{-}$ CD28 $^{-}$ T cells expressed significantly lower telomerase activity compared to the other two subsets after activation (Fig. 6C). Furthermore, in a previous study we also showed that these cells have decreased hTERT transcription and Akt^{Ser 473} phosphorylation after activation therefore showing close resemblance to IFN- γ treated cells (14). Therefore the effects of IFN- γ on reducing telomerase activity are likely to reflect the effects of this cytokine on the CD27 $^{+}$ CD28 $^{+}$ and CD27 $^{+}$ CD28 $^{-}$ T cells rather than on the CD27 $^{-}$ CD28 $^{-}$ population that already exhibit these features.

The loss of CD27 and CD28 in CMV infected individuals has been suggested to be a consequence of CMV-induced IFN- γ secretion (5). We show that the addition of IFN- γ during the activation of isolated CD27 $^{+}$ CD28 $^{+}$ T cells accelerates the loss of both CD27 and CD28 on these cells (Fig. 6C representative experiment shown) that confirms reports in CD4 $^{+}$ T cells (5) and previous data showing that IFN- γ induces the loss of CD28 on activated CD8 $^{+}$ T cells (4). Therefore multiple strands of evidence suggest indirectly that persistent viral infections may induce the secretion of pro-inflammatory cytokines such as IFN- γ that may inhibit telomerase activity as well as induce phenotypic differentiation of CD8 $^{+}$ T cells.

Discussion

Since thymic involution occurs early in life (36), long-term immunity requires the expansion and contraction of specific T cell populations upon re-encounter with antigen. However, there is a limit, imposed by telomere erosion, to the extent that T cells can proliferate suggesting that repeated activation may eventually lead to loss of specific cells (37). The reactivation of telomerase in T cells upon antigenic stimulation compensates for telomere loss but highly differentiated T cells, induced by repeated stimulation, lose the ability to up-regulate this enzyme (5, 11, 14). The broad implication for this is the potential loss of memory T cells as a result of repeated antigenic challenge that may lead to the impairment of immunity during ageing (37). Understanding why telomerase is down-regulated in highly differentiated T cells may identify signalling pathways that can be manipulated to enhance the persistence of functional T cells during ageing. The proof of principle that restoration of telomerase activity promotes T cell persistence comes from studies showing that the

transduction of highly differentiated T cells with hTERT considerably extends their proliferative lifespan *in vitro* (38-40).

We showed previously that highly differentiated T cells have reduced hTERT transcription and that there is a defect in AKT phosphorylation at the Ser473 site in these cells (14). We also showed that p38 MAPK signalling, that is increased in highly differentiated T cells, is involved in telomerase down-regulation and that blocking p38 activity with a specific inhibitor can restore telomerase activity in these cells (20). It appears therefore that telomerase inhibition in highly differentiated T cells occurs by both translational and post-translational mechanisms. The key observation of the present study is that IFN- γ inhibits telomerase by mechanisms that are identical to those responsible for inhibition of this enzyme in naturally occurring highly differentiated CD8⁺ T cells. This includes the decreased transcription and translation of hTERT, the decrease in AKT phosphorylation and the increase of p38 MAPK activity. Furthermore, highly differentiated T cells lose the expression of the co-stimulatory molecules CD28 and CD27 (5, 13, 14) that can also be induced in CD4 and CD8 T cells by treatment with IFN- γ (4, 5). Therefore IFN- γ accelerates the phenotypic and functional differentiation of activated T cells. This raises the question of whether the increase in highly differentiated T cells that have low telomerase activity in older humans (5, 38, 41-43) is actually a secondary manifestation of increased viral infection that induces secretion of this cytokine. In this regard, persistent infection with cytomegalovirus (CMV) induces large numbers of highly differentiated T cells (44) and this may be associated with the release of high concentrations of IFN- γ by pDCs, that are activated with CMV antigens (5, 45).

A previous study by Weng et al (2001) highlighted the importance of phosphorylation and translocation of hTERT to the nucleus of activated human T cells (46). In this study we showed that IFN- γ reduced both telomerase activity and hTERT expression in both the cytoplasmic and nuclear compartments. This in conjunction with the observation that nuclear pore complex (47) numbers were not altered by IFN- γ treatment, suggests that this cytokine is unlikely to inhibit telomerase activity in CD8⁺ T cells by altering nuclear translocation of hTERT. Instead, we found that the decreased AKT activation by IFN- γ was linked to an upstream event, namely the up-regulation of the activity of the enzyme PP2A, which deactivates AKT and possibly also hTERT (23, 24). In addition, IFN- γ induced p38 MAPK signalling which we showed was directly involved with inhibition of telomerase activity. However we do not know at present how these two post transcriptional events that inhibit telomerase activity are linked. Furthermore, other pro-inflammatory cytokines such as TNF- α can also induce p38 expression and telomerase down-regulation in T cells (20). It is not clear whether this and other cytokines can inhibit telomerase by the same or different mechanisms but it does highlight the impact that excessive inflammation induced by persistent infections or autoimmune diseases may have on driving T cells towards senescence. In this context, the correlations that have been observed between excessive telomere erosion in leucocytes and certain forms of ill health (48-50) are likely to be a secondary consequence of the deleterious effects of inflammation on different tissues.

IFN- γ therapy has been approved for the treatment of hepatitis C, viral infections and cancer (51-54) and is currently used as adjuvant in vaccination protocols in melanoma patients (55). The present study has critical clinical implications in this regard. Sustained treatment with IFN- γ has been shown to increase telomere loss in naive T cells, while inhibiting the accumulation of T cell memory expansions (56). Furthermore, the presence of IFN- γ during a cutaneous immune response in humans *in vivo* has also been linked with accelerated local T cell differentiation (6). These studies collectively confirm our current observations and highlight the previously unappreciated role of this cytokine in regulating end-stage T cell differentiation. The fact that multiple mechanisms are engaged by this cytokine in the

inhibition of telomerase in T cells underscores the importance of this process *in vivo*. Our results lead to the question of whether IFN- γ and other pro-inflammatory cytokines can regulate telomerase activity in proliferative non-lymphoid tissues and if this is detrimental to their function. This may explain the link between excessive leucocyte differentiation, inflammation and ill health that is found in certain individuals (57-59).

Finally, it has been shown recently that while the secretion of type 1 IFN (that includes IFN- α) during early infection with lymphocytic choriomeningitis virus (LCMV) is essential for viral clearance, the prolonged exposure to this group of cytokines can suppress the immune system and prevent the control of virus replication (60, 61). It has been suggested that this may be a control mechanism that limits immunopathology that may arise from chronic stimulation in tissues (6, 60, 61). The therapeutic inhibition of IFN- γ signalling led to an increase in virus-specific T cell responses and re-establishes control of the virus (60, 61). Our observations suggest that the detrimental effects of these cytokines may be mediated in part by the induction of end-stage differentiation of reactive T cells that has been shown to occur in both *in vitro* (5) and *in vivo* (6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support This work was supported by “IV Progetto AIDS” (Istituto Superiore di Sanità) to OF and “Alleanza Contro il Cancro” (Istituto superiore di Sanità) to OF, from the Medical Research Council to AL and the British Biological and Biotechnological Sciences Research Council to ANA. JS is supported by a grant from Dermatrust to ANA and MHAR.

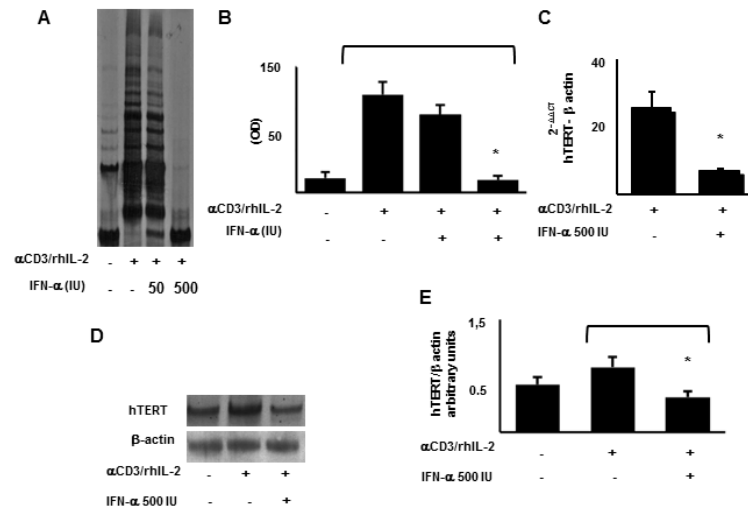
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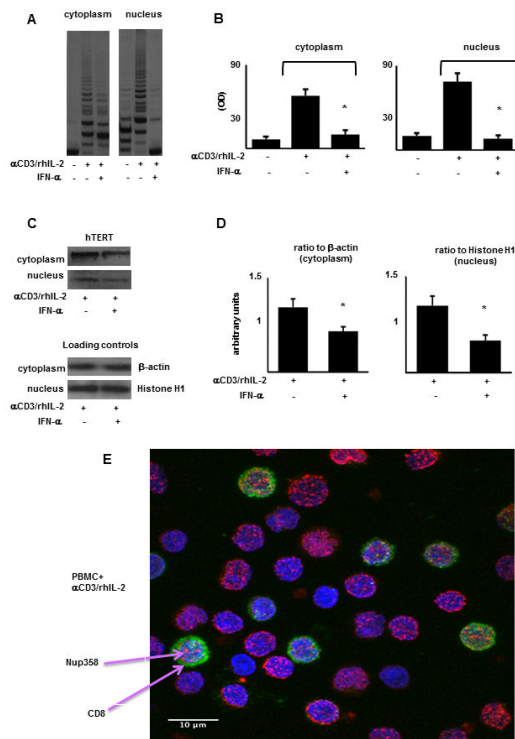
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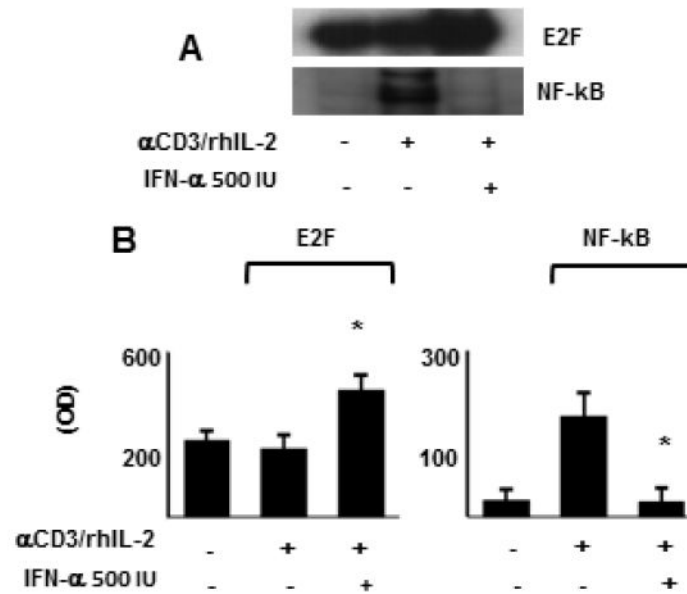
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**FIGURE 1.**

IFN- γ down-regulates telomerase activity in CD8 $^{+}$ T cells. A representative blot of telomerase activity (TRAP Assay, see Material and Methods) of whole cell extracts from 5×10^3 viable CD8 $^{+}$ T cells determined 72 hours following activation with anti CD3 Ab plus rhIL-2 is shown in A. Graph showing pooled results of the effect of IFN- γ on telomerase activity from 3 separate donors is shown in B. The effect of IFN- γ (500 IU/ml) on hTERT mRNA expression of CD8 $^{+}$ T lymphocytes 24 hours following activation with anti CD3 Ab plus rhIL-2 analyzed by quantitative real-time RT-PCR is shown in C. Levels of hTERT are normalized against ACTB housekeeping gene expression. The graph shows the difference in terms of gene expression working out the Delta Delta CT algorithm between TERT and the housekeeping ACTB. Data shown are representative of 3 independent experiments. The effect of IFN- γ on hTERT expression tested on whole cell extracts of 2×10^6 viable CD8 $^{+}$ T lymphocytes 36 hours following activation with or without anti CD3 Ab plus rhIL-2 was analyzed by Western Blot. A representative Blot is shown in D. IFN- γ reduces total cell expression of hTERT in CD8 $^{+}$ T cells. Gel loading control was based on β -actin expression. Graph showing the mean \pm SD of the ratio protein/ β -actin band intensity values for 3 donors is shown in E. All p values were calculated using one-way paired Student's *t*-test. Asterisk indicates $p < 0.05$.

**FIGURE 2.**

IFN- γ down-regulates telomerase activity in the cytoplasm and the nucleus of CD8⁺ T cells. A representative gel showing telomerase activity (TRAP Assay, see Material and Methods) of cytoplasmic and nuclear extracts from 5×10^3 viable CD8⁺ T cells tested 72 hours following activation with anti CD3 Ab plus rhIL-2 is shown in A. Graph showing pooled results of the effect of IFN- γ on telomerase activity of fractionated extracts from 3 separate donors is shown in B. A representative experiment showing the effect of IFN- γ on hTERT expression, tested on cytoplasmic and nuclear extracts from 5×10^6 viable CD8⁺ T lymphocytes 36 hours following activation by Western Blot is shown in C. Quality of extracts was tested using anti β -actin and Histone H1 Ab respectively. The graph in the panel D shows the mean \pm SD of the ratio protein/ β -actin and Histone H1 for cytoplasmic and nuclear compartments respectively, for 3 donors. The effect of IFN- γ on the distribution and composition of NPCs was tested on CD8⁺ T cells in terms of changes in the expression levels of Nup358 in three different donors by indirect immunofluorescence following 24 hours stimulation with anti CD3 Ab plus rhIL-2 with or without IFN- γ . No qualitative differences in NPC numbers or staining intensity were observed (See supplementary Figure). In panel E a representative image of the staining is shown. The images were collected on an inverted confocal microscope (Olympus) with a 60X objective (N.A. 1.45) with a SIM scanner using the Fluoview software. The fluorescence within a region of interest surrounding the nuclear envelope in twelve mid-plane sections was measured. All p values were calculated using one-way paired Student's *t*-test. Asterisk indicates $p < 0.05$.

**FIGURE 3.**

Role of E2F and NF-κB in IFN- γ mediated control of telomerase in CD8⁺ T cells. A representative gel of the binding of nuclear extracts from CD8⁺ T cells to the sequence corresponding to -184 to -161 bp of the hTERT promoter containing the putative E2F site (5'-CGCCCAGGACCGCGCTCCCCACGT-3') and to commercially available NF-κB consensus oligonucleotide, 24 hours following activation with anti CD3 plus rhIL-2, determined using EMSA is shown in A. IFN- γ treatment up-regulates the binding of nuclear proteins to the E2F sequence of the proximal hTERT promoter. Binding of factors obtained from the nuclei of CD8⁺ T cells treated with IFN- γ to the specific NF-κB sequence is reduced. Graphs of Panel B show the mean \pm SD for 3 donors. Statistical significance was evaluated using one-way paired Student *t*-test analysis. Asterisk indicates $p < 0.05$.

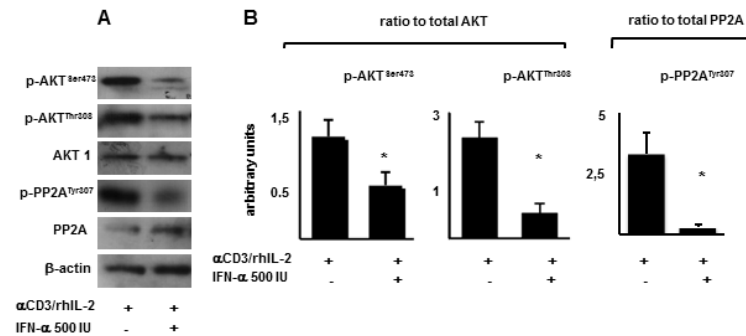
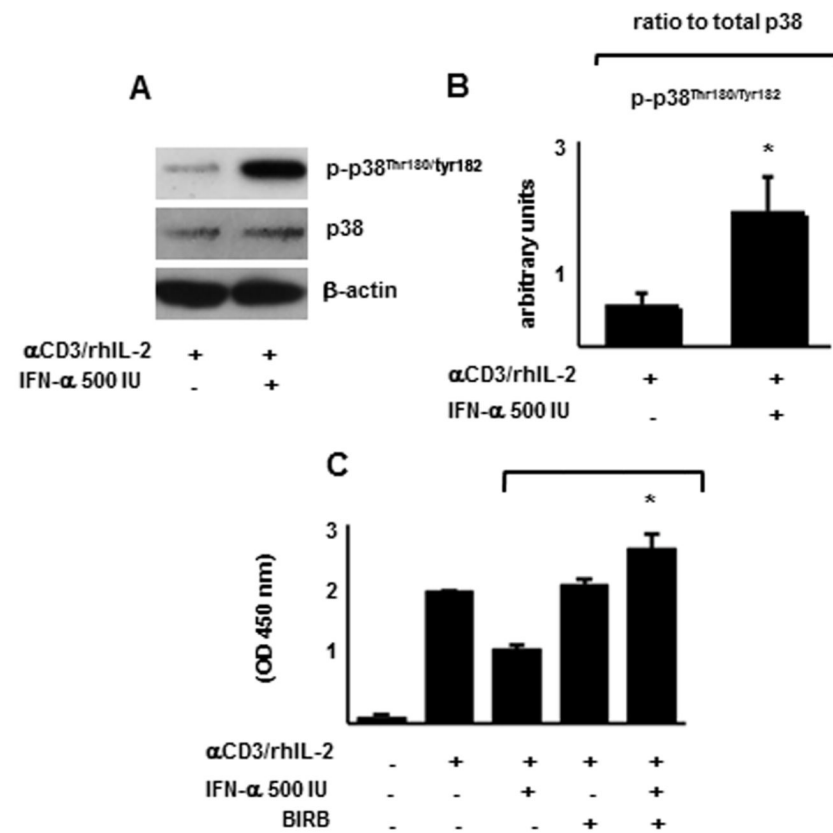
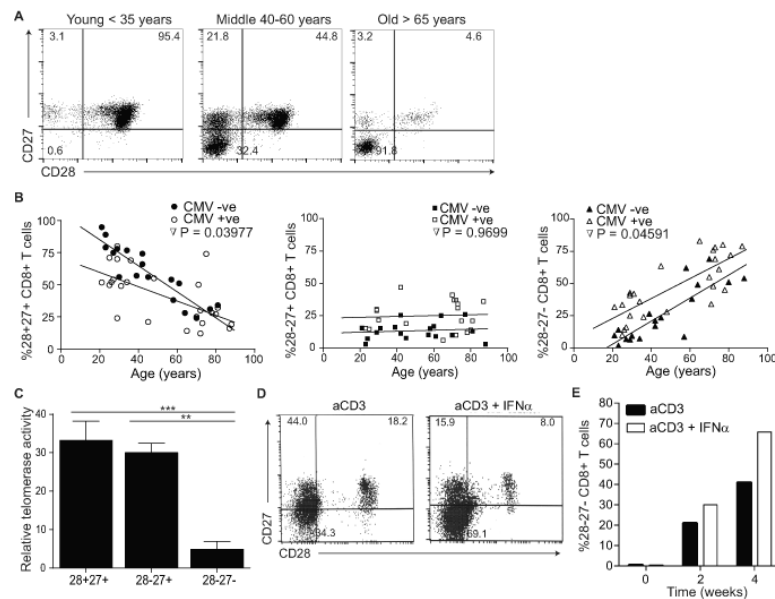


FIGURE 4.

IFN- γ mediates down regulation of AKT signalling. A representative experiment showing the effect of IFN- γ on, p-Ser⁴⁷³-AKT, p-Thr³⁰⁸-AKT, total AKT, p-Tyr³⁰⁷-PP2A, total PP2A expression tested on whole cell extracts of 2×10^6 viable CD8⁺ T lymphocytes 24 hours following activation with anti CD3 Ab plus rhIL-2 by Western Blot is shown in A. IFN- γ reduces phosphorylation of AKT on both Ser⁴⁷³ and Thr³⁰⁸ sites, while total AKT is not affected. IFN- γ treatment up-regulates total PP2A and decreases the inactive form Tyr³⁰⁷-PP2A. Gel loading control was performed analyzing β -actin expression. Graphs of the mean \pm SD of the ratio of band intensity values of Ser⁴⁷³-AKT and Thr³⁰⁸-AKT to total AKT and pTyr³⁰⁷-PP2A to total PP2A respectively for 3 donors are shown in B. All *p* values were calculated using one-way paired Student's *t*-test. Asterisk indicates *p* < 0.05.

**FIGURE 5.**

IFN- γ activates MAPK p38 signalling. A representative experiment showing the effect of IFN- γ on p-38^{Thr180/182} MAPK and total p38 MAPK expression tested on whole cell extracts of 2×10^6 viable CD8⁺ T lymphocytes 24 hours following activation with anti CD3 Ab plus rhIL-2 by Western Blot is shown in A. Gel loading control was performed analyzing actin expression. IFN- γ treatment induces activation of p38 through phosphorylation of Thr180/Tyr182. Panel B shows the mean of the ratio \pm SD of p-38^{Thr180/182} to total p38 for 3 donors. The role of p38 in IFN- γ mediated inhibition of telomerase activity is evaluated by a p38 inhibition experiment by blocking p38 signalling in stimulated T cells by the addition of BIRB796 (BIRB), an inhibitor that blocks the activation of all four of the isoforms of p38. Telomerase activity measured by ELISA is shown as proportional to the OD at 450 nm after due normalization against a negative control (heat-inactivated telomerase sample). p38 blockade enhances cell telomerase activity in CD8⁺ T cells (C). All *p* values were calculated using one-way paired Student's *t*-test. Asterisk indicates *p* < 0.05.

**FIGURE 6.**

IFN- γ accelerates the loss of CD27 and CD28 on activated CD8⁺ T cells. The expression of CD27 and CD28 was investigated in CD8⁺ T cells from healthy individuals of different ages (A; representative profile for young, middle aged and old subjects). The pooled results from donors of different ages who were stratified on the basis of whether they were CMV⁺ (closed circles) or CMV⁻ (open circles are shown in (B)). The significance of the difference between the CMV⁺ and CMV⁻ subjects are shown. The telomerase activity of CD8⁺ T cells divided on the basis of their expression of CD27 or CD28 (C; n=4). These cells were activated for 72 hours with anti-CD3 antibody and irradiated autologous APCs. We activated CD8⁺CD27⁺CD28⁺ T cells as above in the presence or absence of IFN- γ (100 IU/ml). Before culture >97% of the cells expressed both CD27⁺CD28⁺ (not shown) and these cells were maintained by 10 day cycles of activation followed by culture in IL-2 (2ng/ml) and IFN- γ was replenished when at each point of re-stimulation. We measured the expression of these markers at 2 and 4 weeks after culture (D,E; results after 4 weeks of culture are shown in D). The results from one of two representative experiments are shown.