

Telomere homolog oligonucleotides induce apoptosis in malignant but not in normal lymphoid cells: Mechanism and therapeutic potential

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Human B- or T-cell lymphoma lines and primary murine lymphomas were treated with DNA oligonucleotides homologous to the telomere (TTAGGG repeat; “T-oligo”), either alone or in combination with standard, widely-used anticancer chemotherapeutic agents. T-oligo induces cell cycle arrest and apoptosis in cultured human or murine B or T-lymphoma cell lines and primary tumor cells, but exerts no detectable toxicity on normal human or murine primary lymphocytes. Exposure to T-oligo is hypothesized to mimic exposure of the 3′ telomere repeat sequence, activating the ataxia telangiectasia mutated kinase, which phosphorylates downstream effectors such as p53, but effects are not dependent solely on functional p53. T-oligo causes early S-phase arrest and cooperates well with G₂- or M-phase-specific anticancer agents; when combined at 1/10th of the conventional dose, vincristine and T-oligo produce greater-than-additive killing of human or murine lymphoma cells (78% of cells undergoing apoptosis after 6 hr vs. 5% of control cells). In mice, 1/10th of the conventional dose of a standard combination of cyclophosphamide, adriamycin, vincristine and prednisone is twice as effective when used in combination with low dose T-oligo. Thus, T-oligo sensitizes tumors to traditional anticancer agents and represents a potentially important new addition to the therapeutic arsenal for aggressive lymphomas.

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Telomeres are tandem repeats of a 6-nucleotide sequence (TTAGGG) that protect the ends of linear chromosomes from being recognized as double-stranded DNA breaks. In eukaryotic DNA replication, the DNA at the ends of telomeres is not completely replicated and, therefore, telomeres shorten by 50–100 bp with each mitotic cycle. In somatic cells, when the shortened telomeres have been reduced beyond a critical minimal length, the telomere structure is thought to become unstable, thereby activating DNA damage responses that lead to cell cycle exit and irreversible replicative senescence.^{1–3} This permanent limitation on growth likely protects organisms from the cancer risks associated with unlimited replicative potential.⁴

Treatment of cultured cells with DNA oligonucleotides that are homologous to the TTAGGG repeat sequence of the chromosomal telomere, termed “T-oligos,” induces differentiation, apoptosis or senescence.^{1,3,5} We and others have shown that T-oligo treatment induces DNA damage-like responses: phosphorylation of p53, histone H2AX (γH2AX), p95/Nbs1, Chk1 and Chk2 [downstream effectors of the ataxia telangiectasia mutated (ATM) and ATM-related protein kinases], inhibition of DNA synthesis, a senescent phenotype in fibroblasts and apoptosis or senescence in multiple transformed cells.^{1–3,5–9} Specifically, in normal fibroblasts, prolonged T-oligo treatment induces irreversible cell cycle arrest, flat cell morphology, induction of p16, p21, p53 and p95/Nbs1 and increased expression of senescence-associated β-galactosidase,² indistinguishable from late-passage *in vitro* replicative senescence.^{2,10,11} This phenotype has been suggested to arise because T-oligo treatment may mimic the exposure of single-stranded DNA during telomere crisis that follows telomere shortening; indeed, telomere loop disruption by dominant negative (DN) TRF2 produces a similar senescent phenotype in these cells.²

However, T-oligos induce these responses independently of telomerase expression and without shortening endogenous telomeres, loss of the telomere 3′ G-rich overhang or disrupting telomere structure.^{2,5–7,12} T-oligo treatment does not inhibit telomerase⁵ and its effects are specific to the telomeric DNA sequence, because control scrambled, unrelated or complementary oligonucleotides of the same length are ineffective.^{1–3,5} Remarkably, T-oligos cause apoptosis in many malignant cell types, instead of cell cycle exit and senescence,^{2,3,13} again mimicking experimental telomere loop disruption by DN TRF2.¹⁴ By an unknown mechanism, T-oligos rapidly concentrate in the nucleus,^{1,2,13} where such oligos appear to have a half life of at least several days.¹⁵ Within 24 hr, T-oligos induce S-phase cell cycle arrest, H2AX phosphorylation and cause apoptosis in breast, pancreatic and ovarian carcinoma and melanoma cell lines, including lines that lack p53 and/or p16 and harbor a variety of other abnormalities in key regulatory signaling pathways.^{1,3,13,16} For example, cultures of malignant melanoma (MM-AN) cells³ or breast cancer (MCF-7) cells¹³ show dramatically increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and sub-G₁ DNA content upon T-oligo exposure. Furthermore, these responses occur selectively in malignant cells and not in their nontransformed, normal counterparts.^{3,6,13} This difference between the responses of normal and malignant cultured cells has suggested that T-oligos may have therapeutic potential as anticancer agents.

T-oligos have been tested as an anticancer therapy *in vivo* in preclinical models in mice. T-oligo administered by intralesional, intravenous (i.v.) or intraperitoneal (i.p.) injection in severe combined immunodeficiency mice bearing human MM-AN melanoma or MCF-7 breast cancer xenografts reduced primary tumor volumes and metastases by 85–90%.^{3,13} These reductions in tumor burden were achieved at least in part through T-oligo-specific apoptosis as assayed by TUNEL staining.^{3,13} Yet, under these conditions, no toxicity to normal tissue was apparent by histologic examination at autopsy, including intestinal mucosa, hair follicles, bone marrow, liver, jejunum, brain, lung or kidney,^{3,13} confirming efficacy with no detectable toxicity.

Additional Supporting Information may be found in the online version of this article.

Conflict of Interest: M.S.E. and B.A.G. declare that they are co-inventors for patents related to T-oligos that are licensed to SemaCo, a for-profit company in which they hold equity and from which they have received income.

The first two authors equally contributed to this work

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These data suggested that, in addition to the solitary solid tumors studied to date, T-oligos might also cause apoptosis effectively in lymphoid malignancies. Indeed, at least *in vitro*, human Jurkat T-leukemic cells undergo T-oligo-specific apoptosis.¹ We, therefore, asked whether diverse human and murine T-lymphoid and B-lymphoid tumor cell lines might also exhibit T-oligo-specific apoptosis. We then expanded the studies to a recently developed mouse model with systemic, aggressive B-cell lymphoma/leukemia.

Material and methods

Lymphoma cells

Human CEM¹⁷ and MOLT-4¹⁸ T-leukemic cells, and RL¹⁹ and Toledo²⁰ B-lymphoma cells (American Type Culture Collection, Manassas, VA), were cultured in RPMI1640 (GIBCO/Invitrogen, Grand Island, NY) + 5% fetal bovine serum (FBS; Hyclone, Logan, UT), supplemented with 50 μ M 2-mercaptoethanol, glutamine, penicillin and streptomycin without mitogens except 10% FBS. Unless otherwise specified, all reagents were from Sigma Aldrich (St. Louis, MO). In previously published work, we showed that transgenic mice with E μ -driven constitutive expression of the oncogene Brd2 develop a non-Hodgkin's-like lymphoma (NHL) most similar to the "activated B cell" form of human diffuse large B-cell lymphoma (DLCL).^{21,22} For *in vivo* studies, these lymphomas were propagated in syngeneic mice by adoptive transfer. Primary B cells were isolated from normal mouse spleens by anti-CD43 negative selection with magnetic beads (21; Miltenyi Biotec, Auburn, CA) and stimulated with anti-immunoglobulin M (IgM) (Jackson ImmunoResearch, West Grove, PA) and anti-CD40 antibodies (BD Pharmingen, San Diego, CA) and interleukin-4 (eBioscience, San Diego, CA). Peripheral blood lymphocytes (PBLs) were isolated from normal human blood (with informed consent) by venipuncture, heparin anticoagulation, then centrifugation with Ficoll Paque PLUS (GE Healthcare, Piscataway, NJ); then cultured in RPMI1640 and low-endotoxin FBS (Whittaker Bioproducts, Walkersville, MD) and stimulated with phytohemagglutinin (PHA). Viability was determined by trypan blue exclusion.

Oligonucleotides

DNA oligonucleotides with phosphodiester linkage were obtained from the Midland Reagent Company (Midland, TX). A 16-base 100% telomere homolog GTTAGGGTTAGGGTTA (T-oligo) and an 11-base unrelated control sequence available in the laboratory, GTACGTACGTA (c-oligo), were stored as 2 mM stock solutions at -20° C and diluted to 20 μ M in medium for use in cell culture experiments. For *in vivo* experiments, oligos were diluted in sterile buffered saline.

Mice

FVB female mice (6 to 8 wks old; Taconic Farms, Germantown, NY) were sublethally irradiated (6 Gy) and malignant splenic B cells were propagated by adoptive transfer *via* i.p. injection with 2.0×10^7 malignant B cells.²¹ Irradiation and adoptive transfer of cells was defined as Day 1 for all experiments. Mice were maintained on antibiotic (trimethoprim-sulfamethoxazole) water, monitored for the development of lymphoma or leukemia and handled humanely, in compliance with Federal and Boston University IACUC requirements.

Combination chemotherapeutic regimen

The maximum tolerated dose (MTD) of CHOP in mice has been previously established (CHOP: cyclophosphamide 40 mg/kg i.v., adriamycin 3.3 mg/kg i.v., vincristine 0.5 mg/kg i.v. and prednisone 0.2 mg/kg p.o.;²³). In preliminary experiments, we optimized dosage regimens of CHOP and T-oligo separately so that the combination (T-CHOP) gave better-than-additive reductions in tumor burden *in vivo* (data not shown). We defined this optimal

dose to be 0.1 MTD of CHOP/day over 6 days and 30 nmol (15 mg/kg) of T-oligo (Midland Certified Reagent Company, Midland, TX; 16-mer 5'-GTTAGGGTTAGGGTTA-3')/day over 6 days. The complementary oligo was 5'-CTAACCCCTAAC-3' and the "scrambled" oligo was 5'-GATCGATCGAT-3'; Midland³). In the "untreated" control arm, mice were injected with sterile PBS i.p. on treatment days. All formulations were prepared in sterile PBS and injected i.p. in 0.2 ml volume. Lack of T-oligo toxicity at these doses has been previously reported.^{3,13} Treatment began on Day 2 and continued until Day 8 when the mice were euthanized for analysis.

Flow cytometry

Human PBLs were stained for T lineage and activation using anti-CD3-fluorescein isothiocyanate (FITC) (mouse IgG_{2a,k}) and anti-CD69-phycoerythrin (PE) (mouse IgG_{1,k}), respectively (eBioscience), in deficient RPMI1640 medium. Murine lymphocytes were prepared from spleen, lymph node or bone marrow in Hank's buffered salt solution (GIBCO/Invitrogen) and, after erythrocyte lysis, immunophenotyped in the presence of Fc γ receptor blocking antibody (clone 2.4G2, eBioscience) with mouse anti-CD19-FITC (rat IgG_{2a,k}), anti-B220-Pacific Blue (rat IgG_{2a,k}) antibodies and rat isotype controls (eBioscience). Annexin V-PE antibody was from BD Pharmingen. Signals were detected on the FL1 and FL2 channels of a FACScalibur or LSR II flow cytometry system (Becton Dickinson, San Jose, CA). For DNA content, cells were first fixed overnight in 70% ethanol in serum-free RPMI1640 medium, then RNA was digested with RNase and DNA stained with propidium iodide. Doublet discrimination was used to collect 40,000 events. Cell cycle kinetics were measured with bromodeoxyuridine (BrdU) incorporation detected with anti-BrdU-FITC antibody; DNA content was determined with 7-aminoactinomycin D (Becton Dickinson). Analyses were performed with Cell Quest software.

Caspase-3 assay

Caspase-3 activity was generally measured 4–12 hr after exposure to drugs, using a fluorimetric assay kit (Promega, Madison, WI) for enzymatic cleavage of a pro-fluorescent substrate (rhodamine 110, bis-(N-carbobenzoxy-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid (DEVD) amide)), which cleaves the DEVD peptide from the fluorescent product. Detection of substrate conversion was linear through 60,000 relative fluorescent units and 40,000 cells, but we found we could reliably detect this marker of apoptosis using fewer than 1,000 cells. Assays were normalized to 60,000 units typically observed per maximal response in each experiment.

Immunoblot

Levels of p53, phospho-53 (S15), Chk2 and phospho-Chk2 (T68) after chemotherapeutic treatments were determined by immunoblot with polyclonal antibodies (p53, full length 1-393, sc-6243; pp53, sc-11764; Chk2, sc-9064; pChk2, sc-16297; Santa Cruz Biotechnology, Santa Cruz, CA). Cyclophilin was detected by immunoblot with rabbit polyclonal antibody (ab3565, AbCam, Cambridge, MA) to confirm equal loading.

Histology

Tissue sections were fixed overnight in 10% formalin buffered with PBS (Fisher Scientific, Pittsburgh, PA), embedded in paraffin, mounted and stained with hematoxylin and eosin (H&E).

Results

T-oligo reduces yields of NCI-60 cell lines

T-oligo was submitted to the National Cancer Institute's Developmental Therapeutics Program for *in vitro* testing of concentration-dependent reduction in yield (Day 5) of human cancer cells in the standard battery of NCI cell lines (<http://dtp.nci.nih.gov/>)

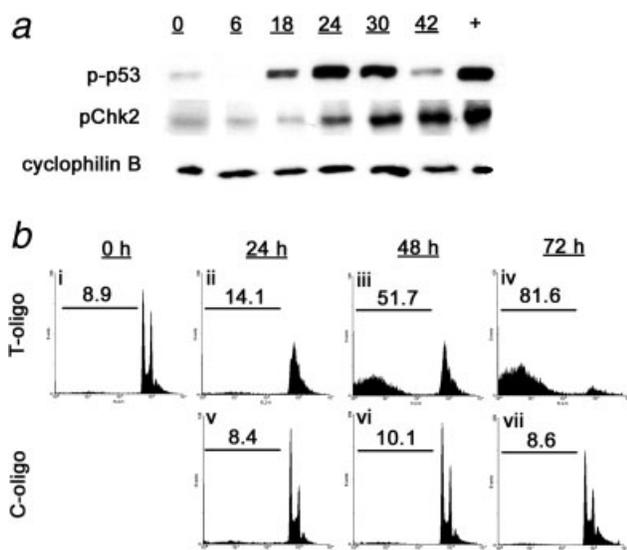


FIGURE 1 – T-oligo exposure induces phospho-p53 (S15) and phospho-Chk2 (T68) in CEM cells. (a) Extracts were prepared from human CEM T leukemic cells treated with 20 μ M T-oligo for the indicated times in hours (h). Extracted proteins were separated by SDS-PAGE, then immunoblotted with polyclonal antibody against p-p53 (S15), pChk2 (T68) or cyclophilin B as a loading control. The positive control (+) was exposure to 0.25 μ M adriamycin for 6 hr. (b) Cell cycle profiles of CEM cells exposed to 20 μ M T-oligo as above, then fixed with ethanol, stained with propidium iodide and analyzed by flow cytometry. Data were collected on the FL2 channel with the detector set on logarithmic scale for visualization of the apoptotic population. Percentages indicated ungated populations of cells with apoptotic, sub-G₁ DNA content. Results are representative of 3 independent experiments. T-oligo DNA (histograms i–iv) is compared to C-oligo DNA (complementary DNA sequence (1-3, 5-7; histograms v–vii), both at 20 μ M.

screening.html). All cell lines showed dose-dependent reductions in percentage growth; the cell line that showed the least growth inhibition at the highest concentration of T-oligo was the ovarian carcinoma cell line IGR-OV1, whereas the cell line that showed the greatest growth inhibition was the T-cell leukemia line CCRF-CEM. Full results of the NCI-60 screen are included as Supporting Figure 1. These results prompted us to study CCRF-CEM cells in detail; we hypothesized that the observed GI₅₀ of 7.58×10^{-7} M for reduction in cell yield was largely due to T-oligo-specific apoptosis.

T-oligo treatment induces both Chk2 and p53 phosphorylation in CEM cells, followed by apoptosis

DNA damage characteristically causes phosphorylation of p53, a key mediator of cell cycle arrest and apoptosis, on serine-15 (S15).^{24,25} T-oligo treatment has also been shown to induce this response in U2OS osteosarcoma cells¹² and MCF-7 breast cancer cells,¹³ as well as in normal human fibroblasts,⁷ although cell cycle arrest is not dependent on wild type p53 function.^{1,3,7,13} We, therefore, tested whether T-oligo treatment induced S15 phosphorylation of p53 and also phosphorylation of its upstream effector, the ATM substrate Chk2 on threonine-68 (T68), in cultured CEM cells. CEM cells were treated with 20 μ M T-oligo in a 42 hr time course experiment. We observed strong pp53 (S15) signals at 24 and 30 hr, and strong pChk2 (T68) signals at 30 and 42 hr (Fig. 1a). These events were followed by the appearance of a population of cells with sub-G₁ DNA content, as measured by propidium iodide stain and flow cytometry analysis, consistent with apoptosis (Fig. 1b). The increase in the fraction of sub-G₁ events was time-dependent, until most cells were dead by 72 hr. In the case of complementary oligo treatment, the percentage of cells with sub-G₁

DNA content did not increase above 10.1% over the time course, values expected for these cells under basal conditions, confirming T-oligo sequence-specific apoptosis.^{1,3,5-7}

T-oligo treatment causes apoptosis in malignant B-lymphoid cells but not normal primary B and T cells

These results prompted us to test if this T-oligo-dependent cell death is widely shared among lymphoid tumor cell lines, or if it is unique to T-cell leukemias (Jurkat¹ and CEM). We, therefore, examined several human large B-cell lymphoma lines, including RL and Toledo cells. T-oligo treatment of RL cells caused a very early S-phase arrest apparent at 24 hr, (compare histograms i and ii of Fig. 2a) that continued through 72 hr (Fig. 2a, histogram iv). Cells with sub-G₁ DNA content did not comprise a significant fraction of the population until 48 hr (Fig. 2a, histogram vii), when 41% of the cells were observed to be undergoing DNA fragmentation. T-oligo treatment caused a further significant increase in the number of sub-G₁ events by 72 hr (67%, Fig. 2a, histogram viii). Similar results were observed for the human DLCL cell lines Toledo and Farage (results not shown). As a positive control for cell death upon DNA damage, RL cells treated with adriamycin (0.25 μ M) demonstrated a prominent S-phase arrest, followed by accumulation at the mitotic checkpoint after only 24 hr (Fig. 2a, panel ix) and eventual apoptosis. As expected, adriamycin exposure caused significant death in all cell types by 60 hr (results not shown).

Induction of p53 initiates apoptosis in response to many anti-cancer agent treatments of diverse tumor cell types; immunoblot of T-oligo-treated RL cells showed significant induction of p53 protein levels by 4 hr, which continued at an elevated level through at least 24 hr (Fig. 2b). In contrast to necrosis and other forms of cell death, the intrinsic cell death pathway in response to DNA damage is characterized by the induction of caspase-3 activity upon p53 stabilization and phosphorylation. To establish that T-oligo-induced cell death of lymphoid tumors shared this characteristic, we measured caspase-3 activity as a function of T-oligo concentration at 4 hr, and observed a dose-dependent increase in activated caspase-3 (Fig. 2c). As a control for p53 dependence, p53 null MOLT-4 T cells were also exposed to T-oligo. Caspase-3 activity was induced in both cell types. Both RL and MOLT-4 cells were killed by T-oligo treatment, but MOLT-4 cells were less sensitive than RL cells, consistent with previous results that p53 contributes to, but is not required for, T-oligo-dependent apoptosis.^{1,16}

We have shown previously that the apoptotic effects of T-oligo are observed only in malignant cells, and not in the corresponding normal cell types even when the cells are proliferating at a comparable rate.^{3,13} To determine if this is also true of normal primary lymphoid cells, we exposed nonmalignant, mitogenically-stimulated, primary conventional B-2 cells from mouse spleen to T-oligo under the same *in vitro* conditions as above. Upon isolation from wild-type mice, normal, conventional B-2 cells are not in cell cycle²⁶ and are unresponsive to T-oligo (Fig. 3a, histograms i–iii). Therefore, we mitogenically activated the cells to observe the effect of T-oligo on proliferating cells. Under these conditions, the normal (control) sub-G₁ content of mitogenically-stimulated B cells was 16.6% after 72 hr (Fig. 3a, histogram iv). However, when treated with T-oligo for the same length of time, after 72 hr, no increase in the number of sub-G₁ events was detected in these cells (Fig. 3a; histogram v). Exposure to scrambled, nontelomere-based DNA oligonucleotide likewise did not induce apoptosis compared with saline control (Fig. 3a, histogram vi). Hence, in agreement with prior findings in normal cells of other lineages, exposure to T-oligo does not induce apoptosis in either resting or proliferating normal primary B lymphocytes.

To confirm that T-oligo treatment also did not induce apoptosis in normal primary human T cells, unfractionated PBLs, the majority of which are T cells, were stimulated in mixed culture with the T-cell mitogen PHA. The activated T cells were doubly stained

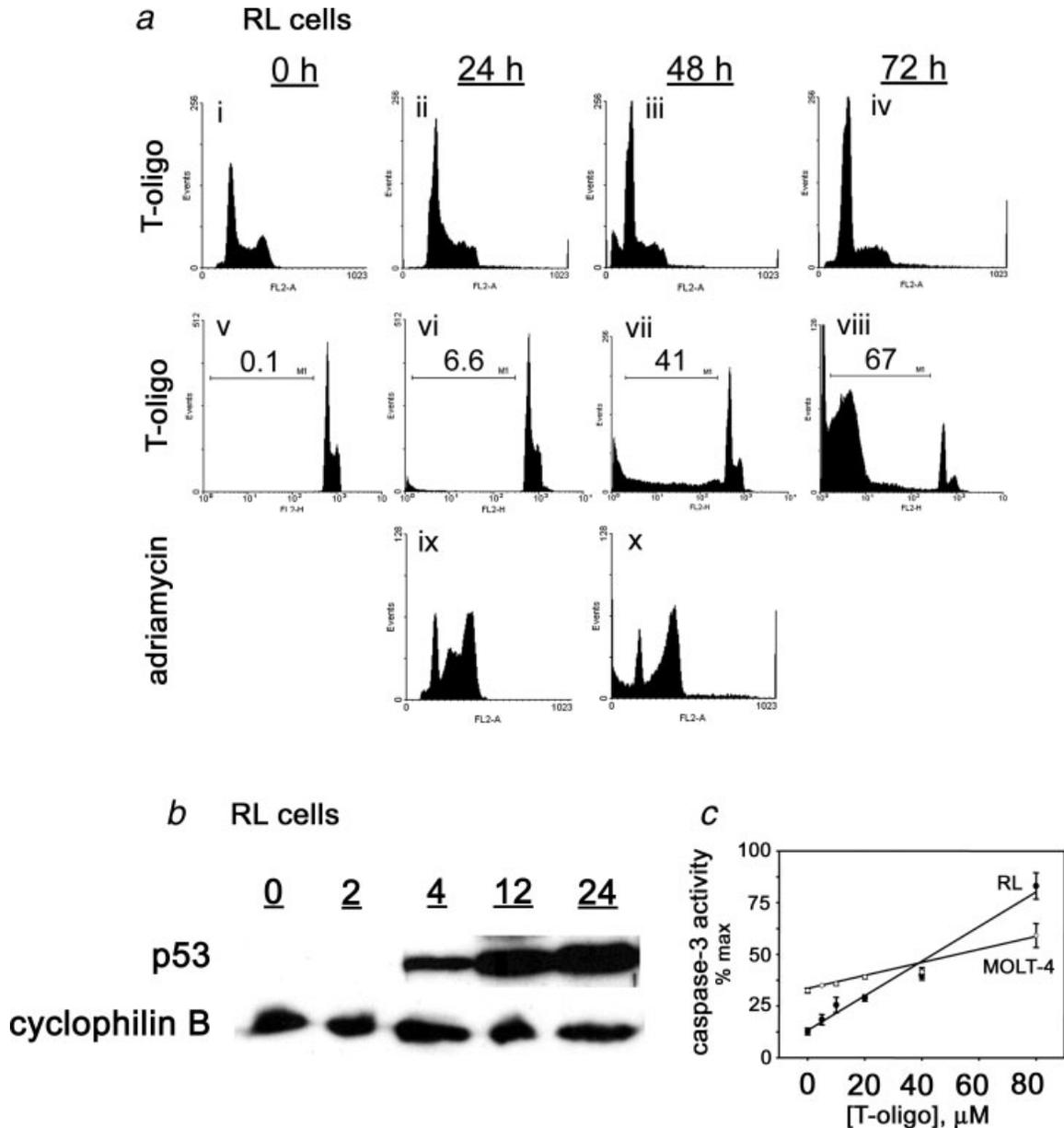


FIGURE 2 – T-oligo causes apoptosis in B cell lymphoma lines. (a) Human RL B cell lymphoma cells were treated with T-oligo and analyzed as in Fig. 1b. Results are representative of 3 independent experiments. (i–iv, ix and x, FL2 detector was in linear mode; v–viii, FL2 detector was in logarithmic mode). For positive control (ix, x), RL cells were treated with 0.25 μ M adriamycin. DNA content at 0 hr (i, v), 24 hr (ii, vi, ix), 48 hr (iii, vii, x) and 72 hr (iv, viii) after exposure is shown. Percentages of ungated sub-G₁ events are indicated (v–viii). (b) RL cell extracts were immunoblotted for total p53 at the indicated times after exposure to T-oligo. (c) T-oligo dose-response assay of caspase-3 activity in RL cells (●) and p53-null MOLT-4 cells (○) treated with T-oligo for 4 hr ($n = 3$). Fluorescence values are shown as relative fluorescence units.

with CD3-FITC antibody to identify T lineage and with Annexin V-PE antibody, an early cell surface marker of apoptosis. After 6 hr T-oligo exposure, cells were analyzed by flow cytometry. No significant T cell-associated Annexin V-PE staining was detected in response to T-oligo treatment (Fig. 3b, dot plot ii), unlike the strong signal seen in the positive control experiment, in which we exposed proliferating T cells to 0.25 μ M adriamycin (Fig. 3b, dot plot iii). Immunophenotyping with CD69 confirmed T-cell activation (Fig. 3c, dot plot ii), compared with unstimulated control (Fig. 3c, dot plot i). Flow cytometry of cell surface activation markers (for B cells, CD25, B7-1, B7-2, which increase upon activation; and IgD, which decreases) confirmed the efficacy of T and B-cell mitogenic stimuli (Suppl. Fig. 2c). The

lack of induction of Annexin V staining (Fig. 3b, dot plot ii) correlated with the absence of apoptosis as measured by propidium iodide stain and flow cytometry. The normal (control) sub-G₁ content of mitogenically stimulated PBLs was 5.0% (± 1.3 , $n = 3$) (Fig. 3c, histogram iii). No significant increase in the number of sub-G₁ events was detected 72 hr after T-oligo treatment (5.7% ± 0.4 , $n = 3$) (Fig. 3c, histogram iv). We then repeated the experiment of Figure 1a with these PHA-stimulated, primary, normal human T cells and assayed pp53 (S15) and pChk2 (T68) induction upon exposure to 20 μ M T-oligo across a 42 hr time course (Fig. 3d). T-oligo treatment minimally induced these DNA damage responses, compared with an adriamycin-positive control.

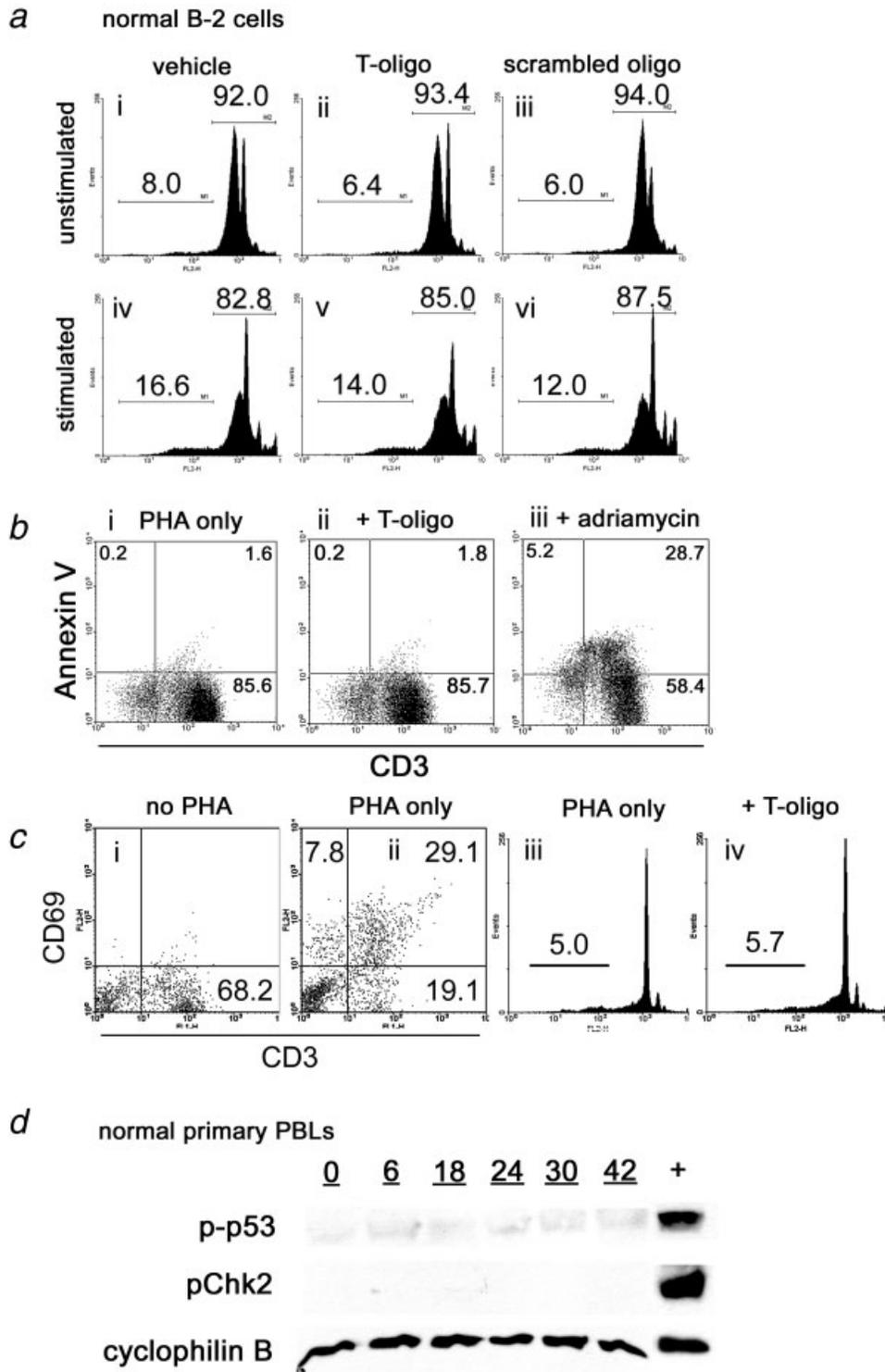


FIGURE 3 – T-oligo treatment does not cause apoptosis in normal primary B or T cells. (a) Primary B-2 cells were purified from mouse spleens, cultured and stimulated with 1 $\mu\text{g/ml}$ goat anti-mouse IgM F(ab')₂ antibody (μ chain specific), 1 $\mu\text{g/ml}$ rat anti-mouse CD40 and 10 ng/ml murine interleukin-4 (iv–vi), or left unstimulated as controls (i–iii). The proliferating cells after 48 hr mitogenic stimulation (iv–vi), and resting cells (i–iii) immediately after plating, were exposed for 72 hr to 20 μM T-oligo (ii, v); saline vehicle (i, iv) or 20 μM of a telomere-unrelated (scrambled) oligo (iii, vi) as negative controls, then harvested, fixed and analyzed for DNA content by propidium iodide staining and flow cytometry as in Fig. 1b. Percentages of ungated cells are shown. (b) Human primary PBLs were mitogenically stimulated for 48 hr with 10 $\mu\text{g/ml}$ PHA (dot plot i), then exposed to T-oligo (dot plot ii) and assayed for Annexin V induction after an additional 6 hr. Annexin V was detected with a PE conjugated reagent; data were collected on the FL2 channel with the detector in logarithmic mode. T lineage was assayed by anti-CD3-FITC (FL1 channel, logarithmic mode). As a positive control, cells were mitogenically stimulated, then exposed to 0.25 μM adriamycin (dot plot iii). Percentages of gated lymphocytes in each quadrant are shown. (c) As a control, human primary PBLs were assayed for T-cell activation by anti-CD69-PE (FL2 channel, logarithmic mode; dot plot ii) compared to unstimulated control (dot plot i). T lineage was assayed by anti-CD3 as in (b). Percentages of gated lymphocytes in each quadrant are shown. Stimulated cells were then exposed to 20 μM T-oligo (iv) or left unexposed as control (iii) and assayed for DNA content at 72 hr as in panel A. Percentages of ungated sub-G₁ events are indicated. (d) PHA-stimulated human primary lymphocytes were exposed to 20 μM T-oligo and assayed by immunoblot for induction of pp53 (S15) and pChk2 (T68) for the indicated times (hr) as in Fig. 1a. Adriamycin once again was the positive control for DNA damage response (+) and cyclophilin B was the loading control.

T-oligo treatment sensitizes lymphoid tumor cells to anticancer chemotherapeutic agents

The multiagent “CHOP” chemotherapy regimen has been the standard of care for NHL for decades and newer therapeutic agents are now used in conjunction with CHOP. We, therefore, explored whether T-oligo would enhance the activity of CHOP *in vitro* and/or allow for dose reduction. We tested a single CHOP component, vincristine, because cooperativity between T-oligo and vincristine was observed in preliminary experiments (data not shown). Cooperativity between T-oligo and adriamycin was not observed, therefore we did not explore this combination further (data not shown). Caspase-3 activity was assayed as a marker of activation of both intrinsic and extrinsic apoptotic pathways.^{27,28} For RL and Toledo cell lines, exposure to submaximal doses of vincristine and T-oligo in combination resulted in about a 2.5-fold increase in caspase-3 activity in cell extracts, unlike treatment with either agent alone (Fig. 4a).

These results suggested that T-oligo treatment might be effective in systemic lymphoid malignancy, including peripheral leukemias. We, therefore, examined an established, preclinical murine model with histological and molecular characteristics of DLCL.^{21,22} The gross pathology of E μ -BRD2 mice has been described.²¹ A typical, severely enlarged and involved spleen is shown in Supporting Figure 3. The chemotherapeutic treatments described below reduced spleen size from enlarged (Suppl. Fig. 3, panel ii) to close to normal (Suppl. Fig. 3, panel i). Thus, spleen weight (described below) was a good indicator of responsiveness to CHOP, to which this model of NHL is very sensitive.

We began with assays for markers of apoptosis in these murine lymphoma cells *in vitro*. Malignant B cells were either left untreated as a control, treated with 1/10th the typical dose of vincristine (25 nM) or T-oligo (2 μ M), or both. After 6 hr, flow cytometry measured Annexin V expression on the cell surface, vs. CD19 as the B-lineage marker (Fig. 4b). Vincristine alone at this low dose produced an Annexin V (+) fraction of 5% (Fig. 4b, dot plot ii), compared with 1% for the control (Fig. 4b, dot plot i). Exposure to low dose T-oligo alone increased Annexin V (+) cells to 55% (Fig. 4b, dot plot iii), but the combination treatment increased the proportion of (+) cells to 78% with a markedly higher average Annexin V expression per cell (Fig. 4b, dot plot iv).

T-oligo reduces tumor burden in mouse models of lymphoma

We followed the logic of this combination approach *in vitro* above and next evaluated the antitumor activity of T-oligo *in vivo* in the murine model, alone and in combination with conventional chemotherapy. For these experiments, i.p. injection of 2.0×10^7 lymphoma cells typically caused clinical signs in 9 days, including failure to nest, inactivity, ruffled fur, squinting, tremors, loss of appetite; and death by 12 days. Postmortem analysis (flow cytometry and histology of organs) confirmed that lymphoid organs (spleen, peritoneal cavity, lymph nodes and bone marrow) were heavily infiltrated with lymphoma cells.²¹

After 6 days of treatment, spleen weights of mice treated with T-oligo alone or CHOP alone were reduced comparably, and reduced by about half in the T-CHOP arm (Fig. 5a). Flow cytometry confirmed that T-oligo or CHOP treatment reduced the tumor burden in the bone marrow comparably, as determined by the proportion of CD19⁺ cells (Fig. 5b). As with the spleen, T-CHOP was more effective in reducing tumor burden. Splenic caspase-3 activity increased substantially more in animals treated with the T-CHOP combination compared with submaximal doses of either agent alone (Fig. 5c), paralleling results obtained with vincristine with or without T-oligo in lymphoma cells *in vitro* (Fig. 3). The fraction of sub-G₁ lymphoma cells in peritoneal cavity at sacrifice increased from 19% in untreated to 35% in T-CHOP-treated mice (Fig. 5d), consistent with selective death of malignant cells by apoptosis. Lastly, we measured the effect of low dose T-oligo single agent treatment on the proliferation of tumor cells in mesenteric

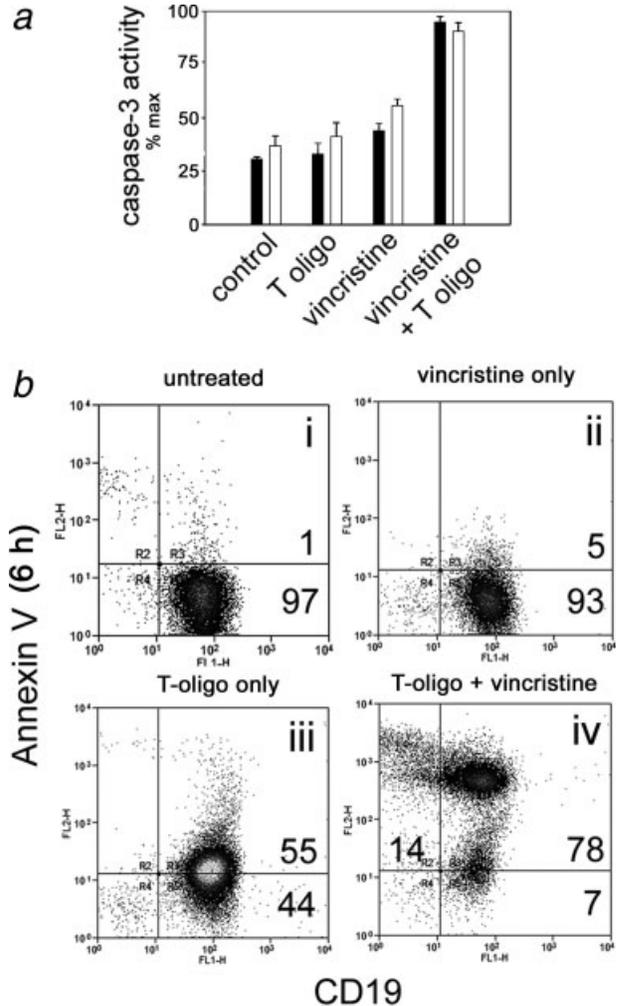


FIGURE 4 – Vincristine cooperates with T-oligo at low doses to induce apoptosis of B cell lymphoma. (a) Assay of caspase-3 activity 12 hr after addition of low dose vincristine (25 nM) and low dose T-oligo (2 μ M), together or separately, to human B cell lymphoma cells *in vitro*. Control was saline alone (SD shown, $n = 3$; filled bars, RL cells; open bars, Toledo cells). ($p < 0.001$; Holm-Sidak pairwise comparison of combination to saline control) (b) Induction of Annexin V after 6 hr exposure to T-oligo and vincristine, as in A, of murine primary DLCL B cells.^{21,22} (i, vehicle control; ii, vincristine alone; iii, T-oligo alone; iv, both agents together). B lineage was identified with anti-CD19-FITC and data were collected on the FL1 channel with the detector in logarithmic mode. Annexin V was detected as in Fig. 3. Percentages of gated malignant lymphocytes are indicated in quadrants. Double positive cells are therefore Annexin V-staining B lymphocytes.

lymph nodes. In a separate experiment, tumor bearing mice were treated with i.p. T-oligo for 6 days as above, *in vivo* labeled by i.p. administration of BrdU, then sacrificed and mesenteric lymph nodes harvested for assay of malignant B cell burden and proliferation. Lymphocytes were assayed for B lineage, BrdU incorporation and DNA content by flow cytometry (Suppl. Fig. 2). Systemic T-oligo treatment reduced BrdU incorporation by 89%, to background levels observed in normal lymph nodes (Fig. 5e).

At the end of the chemotherapeutic regimen, tissues were harvested and images of H&E-stained tissues were examined (Fig. 6). Leukemic infiltrates were observed for all tissues except brain and heart (results not shown); the vasculature represented a source of leukemic infiltration in all target tissues. H&E stain revealed heavy infiltration of lung architecture with large malignant cells²¹;

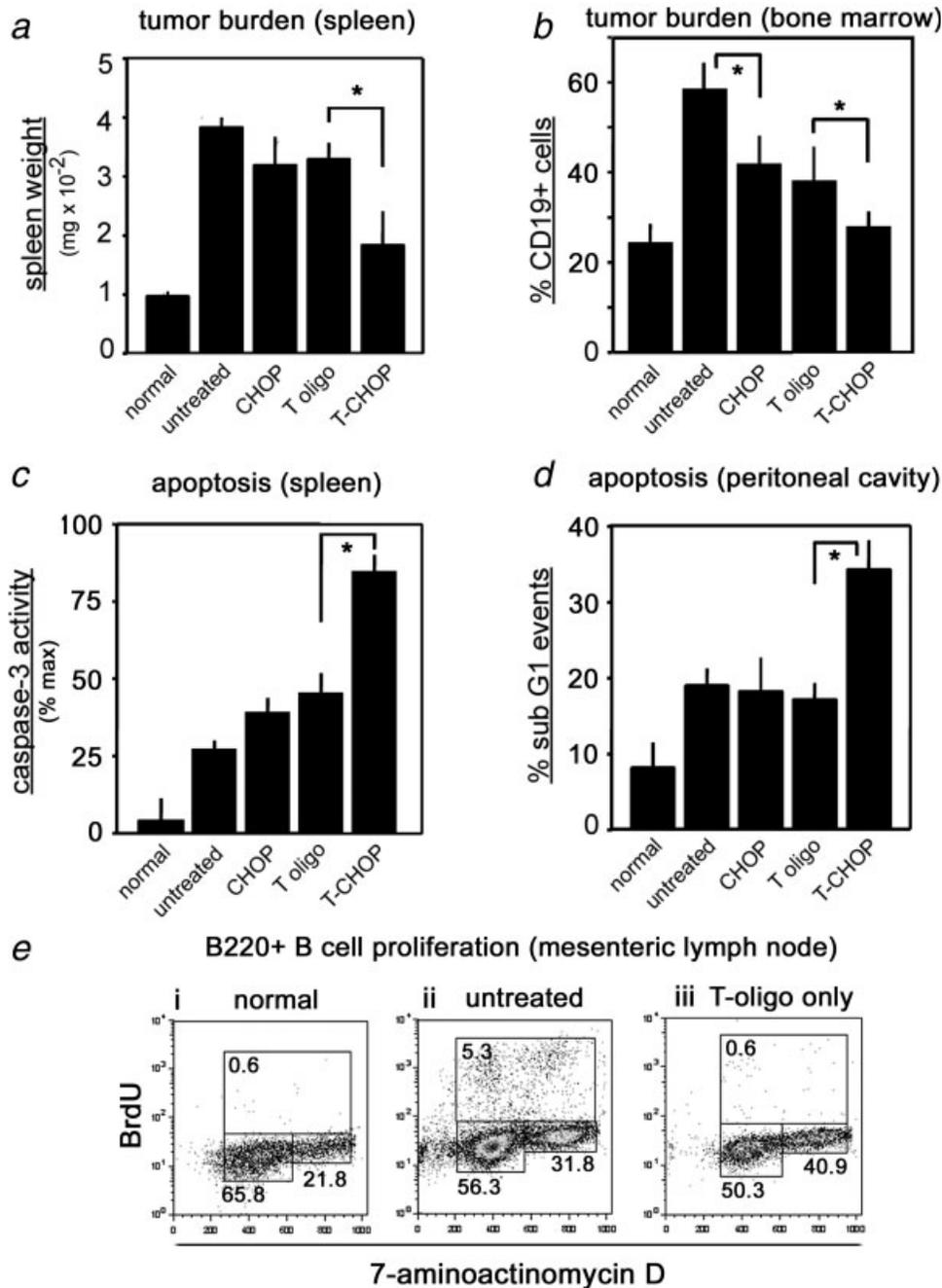


FIGURE 5 – Ability of T-CHOP to reduce tumor burden of lymphoid organs *in vivo*. (a) Spleen weight was measured after completion of *in vivo* combination chemotherapy studies ($n = 6$; SD shown). Dosages of anti-cancer chemotherapeutic drugs were as specified in Material and methods. Normal refers to mice not inoculated with tumor cells. Untreated refers to tumor-bearing mice with PBS injection *i.p.*; CHOP, T oligo or T-CHOP refers to tumor-bearing mice treated *i.p.* with CHOP alone, T-oligo alone or T-oligo and CHOP together. Significant difference between means is indicated (*) ($p < 0.01$). (b) Percentage of CD19⁺ (B lineage) cells in bone marrow, as a function of all cells in bone marrow measured by flow cytometry ($n = 3$; SD shown). (c) Caspase-3 activity in spleen ($n = 3$; SD shown). (d) Percentage of sub-G₁ (apoptotic) events in peritoneal cavity ($n = 5$; SD shown). (e) Proliferation of malignant B cells in mesenteric lymph nodes after *i.p.* treatment with T-oligo alone (dot plot iii) compared to untreated (dot plot ii) and normal (dot plot i). Mice were labelled by *i.p.* injection of BrdU at the end of the course of treatment and sacrificed 1 hr later. Mesenteric lymph nodes were then harvested, immunophenotyped with anti-B220-Pacific Blue (as a pan-B cell marker), fixed, permeabilized, and then doubly stained with anti-BrdU-FITC antibody and 7-aminoactinomycin D (7-AAD) for DNA content. A total of 40,000 events within a double gate for lymphocytes and B220⁺ cells were analyzed; one representative experiment of 3 is shown. Percentages of cells within each stage of the cell cycle are shown within each panel: lower left rectangle defines G₀/G₁-phase population, upper central rectangle defines S-phase population and lower right rectangle defines G₂/M-phase population.

alveolar walls were compromised with infiltrates, dramatically reducing the alveolar surface area available for gas exchange in untreated animals (Fig. 6, panel ii) compared to control, non-lym-

phoma mice (Fig. 6, panel i); mice with heavy infiltrations were dyspneic. After CHOP treatment, we observed an increase in available air spaces in the alveoli and reduced leukemic infiltration

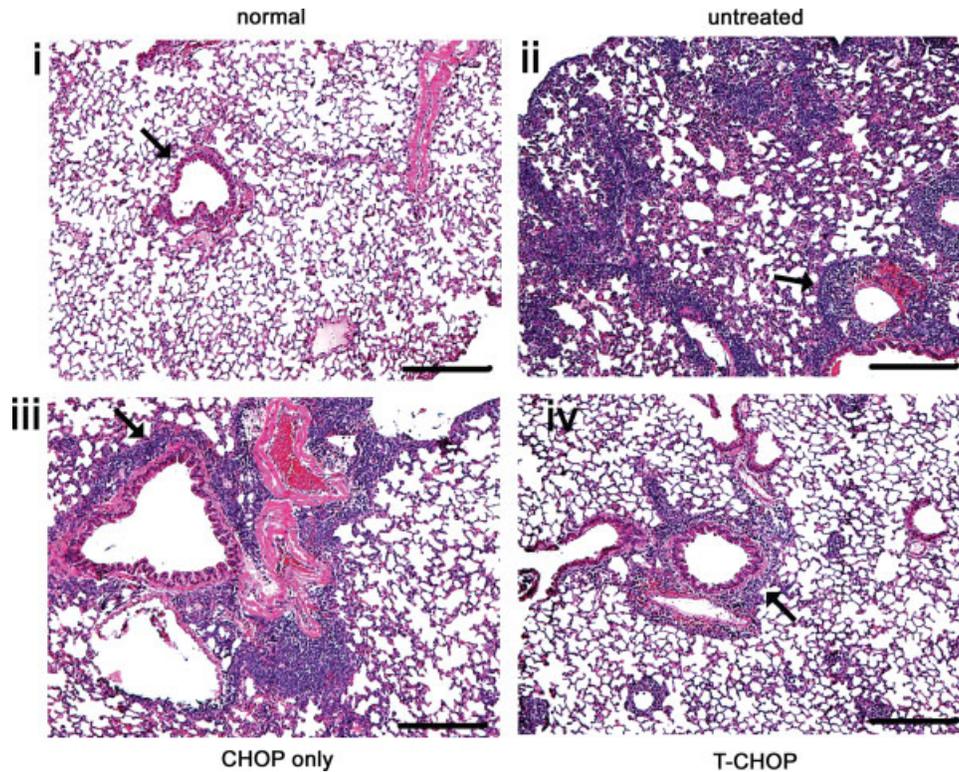


FIGURE 6 – Tumor burden in lung after chemotherapy. Lungs were removed from chemotherapy-treated mice after euthanasia, fixed in formalin and stained with H&E. (i) lung of normal, irradiated mouse (arrow indicates bronchiole); (ii) lung of untreated lymphoma mouse (arrow emphasizes heavy accumulation of malignant cells around a bronchiole); (iii) lung of mouse treated with CHOP only; (iv) lung of mouse treated with T-CHOP combination (arrow emphasizes reduced tumor infiltration at bronchiole) (bar, 100 μ m).

in perivascular and peribronchiolar areas (Fig. 6, panel iii). Most dramatically, histology revealed a reduction in tumor burden of the lungs of mice that received the T-CHOP combination (Fig. 6, panel iv); alveolar surface area was improved and bronchiolar infiltration significantly reduced. We performed image analysis of Figure 6 panels by converting RGB pixel information to grayscale then sampling the image in 5 independent locations to quantify density of stain of alveolar spaces. The intensity of normal lung alveoli (panel i) was 60.02 (SD 3.42; $n = 5$), untreated (panel ii) was 123.19 (SD 6.99), CHOP-treated (panel iii) was 73.79 (SD 5.45) and T-CHOP-treated (panel iv) was 68.77 (SD 4.19) (Suppl. Fig. 4). Alveolar tumor burden was similar in lungs of CHOP and T-CHOP treated animals, but peribronchiolar tumor burden was lower with T-CHOP treatment than with CHOP treatment alone (compare Fig. 6, panels iii and iv, arrows). This reduction of tumor burden in response to treatment correlated well with other clinical parameters (Fig. 5). These findings indicate cooperativity between low-dose CHOP and low-dose T-oligo in this lymphoma model.

Discussion

Lymphomas, which include NHL, are the fifth most common type of cancer diagnosed and the sixth most common cause of death in the United States. Despite progress in treatment since supplementation of the CHOP regimen with rituximab, relapse rates remain a concern for aggressive disease,^{29,30} which strongly demands the development of new agents and adjuvant protocols. Here, we show that a novel DNA oligonucleotide causes apoptosis in primary malignant lymphoid cells or cell lines and in mouse models, while causing little or no cytopathic effects on normal lymphoid cells of murine or human origin.

Recent work^{1-3,7,8,12,13} suggests that the cell detects exposure of single-stranded DNA, consisting of the telomere repeat sequence TTAGGG, at times of acute DNA damage or telomere dysfunction, which results in DNA damage responses mediated through multiple and redundant signaling pathways to cause apoptosis (as in lymphocytes) or senescence (as in fibroblasts). We hypothesize that under physiologic conditions this mechanism plays an important role in cancer prevention and acts as a fail-safe to eliminate cells that harbor DNA replication errors that could cause cancer after multiple rounds of cell division.³¹ These physiological responses appear to be defective in malignant cells, but can be triggered by providing cells or tissues with T-oligos that exaggerate the physiologic response.^{1,3,5} Proliferating B and T cells express significant amounts of telomerase,³² yet the T-oligo response is unrelated to telomerase inhibition, as it occurs in telomerase negative lines such as U2OS osteosarcoma cells³³; occurs within 3-4 days, rather than over weeks as reported for telomerase inhibitors that act by progressive telomere shortening³⁴; and most definitively, T-oligo treatment does not inhibit telomerase activity over the period of time in which the antiproliferative effects are observed.⁵

T-oligo and adriamycin clearly cause different kinds of cell cycle arrest in the lymphoma cell lines, suggesting that the upstream signaling mechanisms are different. T-oligo induced pChk2 (T68) and pp53 (S15), and then a G₁/S-phase arrest of the cell cycle in lymphoid tumor cells. However, induction of p53 was not an essential part of the pathway for either drug, because caspase-3, a downstream effector of apoptosis, was activated even in p53 null lymphoma cells. Therefore, the early events are likely to vary across cell lines, whereas the later events converge to the same apoptotic outcome. In at least some malignant cell types, as well as in normal fibroblasts, the nuclear sensor for T-oligos is the Werner protein¹² and this interaction leads to auto-phosphoryla-

tion (activation) of ATM,^{7,12,13} known to be an upstream activator of p53 and many other effector proteins involved in apoptosis. As we learn about differences in signal transduction pathways for T-oligos vs. conventional cancer chemotherapeutics, we expect to gain insight into how patients with different lymphoid malignancies might respond to T-oligo and leverage functional checkpoints for optimal responses to combination treatment. Based on the present data, however, we anticipate that T-oligo treatment will prove beneficial even for patients with relapsed, p53-mutated lymphomas.

We found that low doses of T-oligo worked better-than-additively with low doses of vincristine, a component of CHOP, to induce apoptosis. The B cells of mice do not express CD20, therefore, we were unable to model T-CHOP combination therapy with rituximab-like approaches. The anticipated mechanistic differences with T-oligo action were hypothesized to create additive or better-than-additive effects in combination, given the firmly established principle of cancer chemotherapy that multiple, independent modes of drug action are usually more effective than single agents or agents that act in the same pathway. These results were encouraging because low dose vincristine and low dose T-oligo might conceivably work well together while reducing side effects in DLCL patients. Overall, our data suggest that T-oligos represent a potentially important addition to the arsenal of anti-cancer drugs for these types of lymphoma.

In summary, we found that T-oligo treatment causes apoptosis in both malignant B- and T-cell lines and primary tumor cells, but not in normal proliferating primary B or T cells. Caspase-3 activation and immunoblot assays establish that, after a G₁/S-phase cell cycle arrest, treated cells induce p53 and undergo apoptosis over 72 hr after a single dose. The mechanism of T-oligo S-phase cell cycle arrest, attributable to the p95 Nijmegen breakage protein Nbs-1, at least in fibroblasts⁷ and subsequent apoptosis, attributable at least in part to p53 and to p73 in its absence,³ differs from

that of adriamycin, in that the latter follows arrest at the G₂/M checkpoint and occurs about 24 hr earlier. Senescence was not excluded, although T-oligos can induce senescence in addition to apoptosis¹³ or instead of apoptosis⁵ in some malignant cell types. However, senescence is very unlikely to contribute to the reduction in lymphoma cell yields, as cells of lymphocytic origin react to telomere disruption,¹⁴ reduced mitogenic stimulation³⁵ or other stresses³⁶ overwhelmingly by apoptosis.

Of particular relevance to clinical applications, we note reductions in the tumor burden of T-oligo treated animals that bear aggressive NHL tumors. Most interestingly, T-oligo induction of caspase-3 activity cooperated well with vincristine in *in vitro* settings and with CHOP in *in vivo* models. These results raise enthusiasm for the potential of the T-oligo approach as a new chemotherapeutic modality for lymphoid malignancy.

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