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Taxol-mediated changes in fibrosarcoma-induced immune cell function: modulation of antitumor activities

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Abstract The anticancer drug taxol (paclitaxel) inhibits tumors through multiple cytotoxic and cytostatic mechanisms. Independently of these mechanisms, taxol induces distinct immunological efficacy when it acts as a second signal for activation of tumoricidal activity by interferon-γ(IFNγ)-primed murine normal host macrophages. We reported that tumor-distal macrophages, which mediate immunosuppression through dysregulated nitric oxide (NO) and tumor necrosis factor α (TNFα) production, are differentially regulated by taxol. Because taxol influences tumor cell growth dynamics and activates immune cell populations, we assessed the ex vivo immunosuppressive and antitumor activities of taxol-treated normal host and tumor-bearing host (TBH) macrophages. Pretreatment of such cells with taxol partly reconstituted T cell alloantigen reactivity, suggesting that taxol mediates a limited reversal of TBH macrophage immunosuppressive activity. Taxol-treated TBH macrophages significantly suppressed the growth of fibrosarcoma cells (Meth-KDE) through soluble effector molecules and promoted direct cell-mediated cytotoxicity, indicating that taxol enhanced tumor-induced macrophage antitumor activities. Tumor-induced helper T cells, however, showed a higher sensitivity to direct taxol-induced suppression. These data demonstrate that taxol exerts pleiotropic effects on antitumor immune responses with the capacity to abate the immunosuppressive activities of macrophages and promote macrophage-mediated antitumor activities simultaneously, but also directly modulating T cell reactivity. Collectively, these studies suggest that the antineoplastic drug taxol may impart antitumor activity through an immunotherapeutic capacity.

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Introduction

The antineoplastic agent taxol (designated paclitaxel by the World Health Organization) is a naturally occurring taxane diterpenoid, extracted from the bark and leaves of the western yew *Taxus brevifolia* [25]. Taxol has demonstrated significant efficacy as an antitumor chemotherapeutic in human clinical trials (reviewed in [21]) with 20%–50% objective response [33]. Taxol displays activity against a wide range of human cancers, notably breast cancers [23] and refractory ovarian tumors [38]. The chemotherapeutic capacity of taxol is realized through multiple mechanisms of antineoplastic action, including suppression of protein synthesis [28], enhancement of tumor cell radiosensitivity [27], induction of apoptotic cell death [5], and enhancement of tumor-necrosis-factor-α(TNFα)-mediated cytolysis [46]. Taxol's primary antitumor mechanism derives from its unique polymerizing action on microtubules [42], which prevents depolymerization of α/β tubulin polymers [29] and halts cell-cycle progression [39]. Taxol-mediated disruption of cell cycling [13] leads to neoplastic cell death and inhibition of tumor progression [18].

In addition to its well-characterized chemotherapeutic activities, taxol has profound cell cycle-independent effects on murine macrophages [7, 24]. Clinically, the most notable activity of taxol on macrophage populations may be its ability to activate normal host macrophages for enhanced in vitro tumor cell cytotoxicity [31]. Taxol stimulates in vitro normal host macrophage responses similar to those induced by bacterial lipopolysaccharide (LPS) [30, 43], activating expression of all characterized LPS-inducible genes [12]. Considerable data show that both taxol and LPS can act synergistically with interferon γ (IFNγ), which primes macrophages for enhanced production of cytotoxic and regulatory molecules, to trigger transcription of interleukin-1 (IL-1) [6], TNFα [6, 46], and inducible nitric oxide

synthase [26, 31]. Taxol enhances IFNγ-primed normal host macrophage cytotoxicity toward tumor cells in vitro [43], primarily through nitric oxide (NO) [30, 31]. Tumor growth, however, induces multiple phenotypic and functional changes among macrophage populations [3, 15]. We recently reported that fibrosarcoma growth altered macrophage responsiveness to taxol, modulating tumor-bearing host (TBH) macrophage NO production in tumor-distal compartments [37]. Because taxol stimulates immune cell activities, we have evaluated taxol's activity on normal host and TBH macrophage and T cell populations.

In this study, we extend our previous findings that suggest taxol may activate macrophages for direct antitumor cytotoxicity while controlling tumor-distal macrophage production of systemic proinflammatory and cytotoxic molecules. Here we report that taxol decreased TBH-macrophage-mediated suppression of alloantigen-activated T cell responsiveness and concurrently enhanced the antitumor activities of macrophages in vitro. Conversely, tumor growth increased $CD4^+$ T cell sensitivity to taxol-mediated modulation of reactivity.

These data collectively suggest that taxol has differential actions on the immune system that vary with cell phenotype and compartment. Through its capacity to activate immune cell cytotoxic activities and control tumor-distal macrophage production of immunosuppressive molecules simultaneously, taxol may impart immunotherapeutic antitumor activities not previously described.

Materials and methods

Murine tumor model

BALB/c ($H\text{-}2^d$) male mice (Harlan Sprague-Dawley, Madison, Wis.) 8–12 weeks old, were used. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (Meth-KDE) was used as described [17]. Briefly, the Meth-KDE fibrosarcoma induces significant systemic immunosuppression through the production of the soluble suppressor molecules interleukin-10 (IL-10), transforming growth factor-β_1 ($TGF\beta_1$), and prostaglandin E_2 (PGE_2) [3]. The use of a nonmetastatic tumor facilitates the study of tumor-induced distal immune cell populations without contaminating tumor cells. Tumors were induced by intramuscular injection of 4×10^5 transplanted Meth-KDE cells. Palpable tumors developed within 10 days, and TBH immune cells were harvested 21 days after tumor induction because tumor-induced macrophage suppressor activity is maximal at this time, without cachexia or necrosis. All procedures followed NIH principles on laboratory animal care and were approved by the Virginia Tech Animal Care Committee.

Media

Macrophages were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma Cell Culture, St. Louis, Mo.). Normal host and TBH T cells, Meth-KDE fibrosarcoma cells, and P815 murine mastocytoma cells [clone TIB 64, American Type Culture Collection (ATCC), Rockville, Md.), used for proliferation and cytotoxicity assays, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, Ga.). T cell cultures contained 40 μM 2-mercaptoethanol (Sigma). RPMI-1640 medium contained 50 mg/l gentamicin sulfate (Tri-Bio Laboratories, State College, Pa.), 25 mM sodium bicarbonate ($NaHCO_3$), and 25 mM HEPES buffer (Sigma) and was endotoxin-free (<10 pg/ml) as assessed by the *Limulus* amebocyte lysate assay (Sigma).

Reagents

Taxol (Calbiochem, La Jolla, Calif.) was dissolved in 100% dimethyl sulfoxide (Mallinckrodt Chemical, Paris, Ky.) to 4 mM stock solution and stored at –80 °C. Taxol was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of dimethyl sulfoxide in cultures was less than 1%. LPS (*Escherichia coli* serotype 026:B6), sodium nitrite, and N^G-monomethyl-L-arginine (MeArg) were purchased from Sigma. Genentech Inc. (San Francisco, Calif.) generously provided IFNγ. Recombinant murine interleukin-2 (IL-2, specific activity 2×10^5 U/ml) was provided by Dr. R.S. Selvan (Duke University, Durham, N.C.).

Immune cell collection

Normal host and TBH BALB/c splenic macrophages were collected by plating pooled whole spleen cells for 2 h (150 × 15-mm plastic plates; Lux/Miles Scientific, Naperville, Ill.), washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent macrophages in cold medium by scraping with a rubber policeman. The final macrophage preparations contained cells that were more than 95% viable and more than 96% esterase-positive. Flow-cytometric analysis with M1/70 and F4/80 monoclonal antibodies (mAb, ATCC) showed them to be more than 80% $Mac\text{-}1^+$ and $F4/80^+$, respectively. To prevent taxol carryover into proliferation and cytotoxicity assays, macrophages were pretreated in 96-well flat-bottom tissue-culture plates (Corning Cell Wells, Corning, N.Y.) in RPMI-1640 medium. Cells were seeded into plates at known concentrations and pretreatment reagents added; following a 4-h incubation, plates were centrifuged (500 *g*, 10 min) and medium removed. Cells were washed once and fresh medium added to the final volume of 200 μl.

Purified $CD4^+$ T cells were collected by plating whole spleen cells for 2 h in plastic plates, collecting the nonadherent cell fraction, and treating with anti-CD8 (ATCC; clone 3.155), anti-IA^d (ATCC; clone MK-D6), and anti-(B cell) and anti-(immature T cell) (ATCC; clone J11d) mAb and complement (Accurate Chemical Co., Westbury, N.Y.). Red blood cells were lysed by 0.83% ammonium chloride treatment.

Nitrite production by macrophages

Either normal host or TBH splenic macrophages (8×10^5, optimal cell numbers) were cultured in 96-well flat-bottom tissue-culture plates. Each well contained a total volume of 200 μl serum-free RPMI-1640 medium with various concentrations of IFNγ, LPS, or taxol, which were added to the macrophage cultures at the start of incubation. Parallel cultures were treated for 4 h, washed, and recultured in fresh medium. Supernatants for nitrite assessment were collected at 72 h (optimal culture times) following centrifugation (400 *g*, 5 min). Taxol did not decrease macrophage viability because the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [36] verified more than 95% macrophage viability throughout the culture periods (not shown).

Because secreted NO quickly reacts with oxygen to yield a stable nitrite end-product [35], nitrite levels in culture supernatants were measured using the Griess reagent as described elsewhere [19]. Briefly, 100 μl macrophage supernatant was added to 100 μl Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3P0_4; Sigma) and incubated at room temperature for 10 min; the absorbance was then read at 570 nm (MR 600 microplate absorbance reader; Dynatech Laboratories, Alexandria, Va.). A sodium nitrite standard curve was used to calculate nitrite content in supernatants. Nitrite was not detected in RPMI-1640 medium alone or in macrophage cultures lacking IFNγ (not shown). Dimethyl sulfoxide in culture did not affect macrophage nitrite production (not shown).

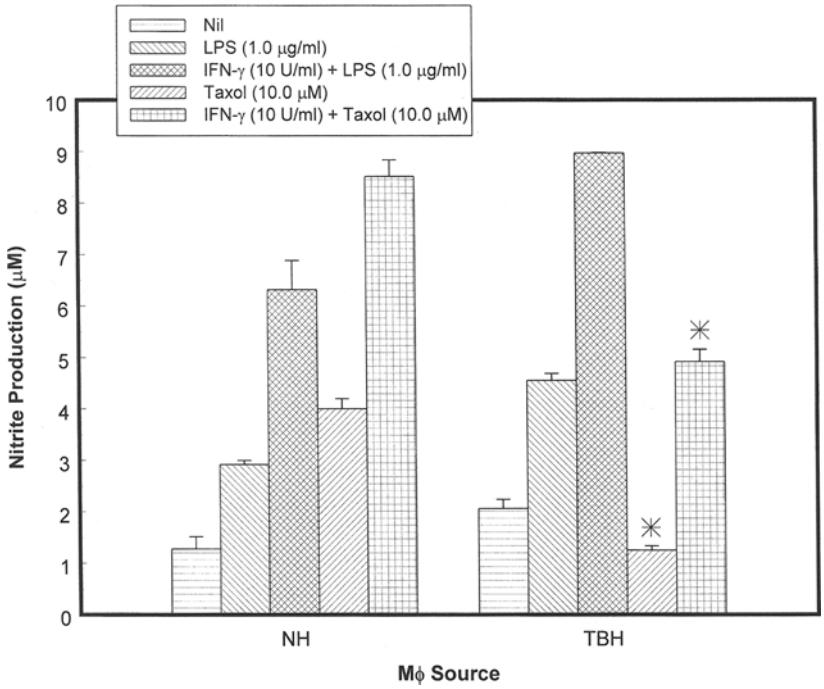

Fig. 1 Taxol modulates tumor-bearing host macrophage (*TBH Mϕ*) NO production. Either 4×10^6 normal host or TBH splenic macrophages were cultured in 200 μl serum-free medium. Some cultures were primed with interferon γ (IFNγ; 10 U/ml). Lipopolysaccharide (*LPS*; 1 μg/ml) or taxol (0.1, 1.0, or 10.0 μM) was added to some samples at the start of culture. Following 4 h of culture, medium was removed, cells washed, medium replenished, and culture continued. Taxol-induced NO production by TBH macrophages was significantly different (* $P < 0.05$) from LPS-triggered NO production, either without or with IFNγ priming. Similar results were obtained using 0.1 μM or 1.0 μM taxol (data not shown); doses greater than 25 μM became toxic to the macrophages (data not shown). Data are averages and standard errors of the mean (SEM) of triplicate independent determinations from one of four similar experiments

Alloantigen-activated T cell proliferation assay

Either normal host or TBH macrophages (4×10^5 cells) were pretreated without or with combinations of LPS, IFNγ, or taxol and added to normal host BALB/c CD4+ T cell cultures (2×10^5 cells). The NO inhibitor MeArg (0.5 mM) was added to parallel cultures. Irradiated (20 Gy) whole splenic cell preparations (4×10^5 cells) from C3H (H-2^k) mice were used as allogeneic stimulator cells. Cultures were incubated in U-bottom 96-well microtiter plates (Corning) for 96 h. Cultures were pulsed with 1 μCi/well tritiated thymidine ([^{3}H]dT, specific activity 6.7 Ci/mM; DuPont-NEN Research Products, Boston, Mass.) 18 h before harvest. Cells were harvested and sample activities determined using a Beckman LS 6000SC scintillation counter.

Mitogen-activated T cell proliferation assay

T cell proliferation as a result of activation was used to evaluate immune cell responsiveness during tumor burden. T cells (4×10^6/ml) were activated with concanavalin A (ConA, 8 μg/ml) in the absence or presence of mediators that promote or suppress T cell activation. All cultures were maintained in sterile 96-well flat-bottom microtiter plates with a final culture volume of 200 μl. T cell proliferation was assessed after 72 h by [^{3}H]dT incorporation.

Tumor cell proliferation assays

Meth-KDE fibrosarcoma cells were seeded into 96-well flat-bottom tissue-culture plates at a final concentration of 1×10^5 cells in a volume of 100 μl. Following 24 h incubation, macrophage supernatants (100 μl) were added and incubation continued for an additional 24 h. Supernatants were obtained from 72-h cultures of 2×10^5 normal host or TBH macrophages pretreated (4 h) with various concentrations of IFNγ, LPS, taxol, or their combinations. Supernatants contained only macrophage-derived effector molecules but did not contain pretreatment reagents. Tumor cell cultures were pulsed with 1 μCi/well [^{3}H]dT 12 h before harvest. Cells were harvested and sample activities determined using a Beckman LS 6000SC scintillation counter.

Macrophage-mediated cytotoxicity assays

To assess the cytotoxic activity of taxol-activated macrophages against Meth-KDE tumor cells, either pretreated (4 h) normal host or TBH macrophages (2×10^5 cells) were added to 1×10^5 tumor cells. Cytotoxicity was measured using a modification of the Alamar blue colorimetric viability assay (1). Briefly, Meth-KDE cells were seeded into 96-well flat-bottom tissue-culture plates in 100 μl RPMI-1640 medium supplemented with 10% FBS and incubated for 24 h. Pretreated macrophages were added and culture continued for 24 h in the presence of 10 nM actinomycin D (Sigma), an antiproliferative agent used to prevent unwanted tumor cell proliferation. Following 20 h of culture with pretreated macrophages, 20 μl (10% of the well volume) of Alamar blue indicator dye (BioSource International, Camarillo, Calif.) was added to each well. In the presence of viable cells, Alamar blue dye is reduced to a colored product detectable at 580 nm. Tumor cell visibility was assessed 4 h after addition of the indicator dye, using a Molecular Devices Dynamax plate reader. In this system, cytotoxicity is inversely proportional to absorbance.

Cytotoxicity was also assessed using the ^{51}Cr-release assay [41] with P815 nonadherent murine mastocytoma target cells. Either taxol-pretreated (4 h) normal host or TBH macrophages (2×10^5 cells) were added to ^{51}Cr-labeled tumor targets (1×10^5 cells) and incubated for 18 h. Supernatants were harvested and ^{51}Cr release determined. Cytotoxicity was calculated using the formula [(control –test) ÷ test] × 100.

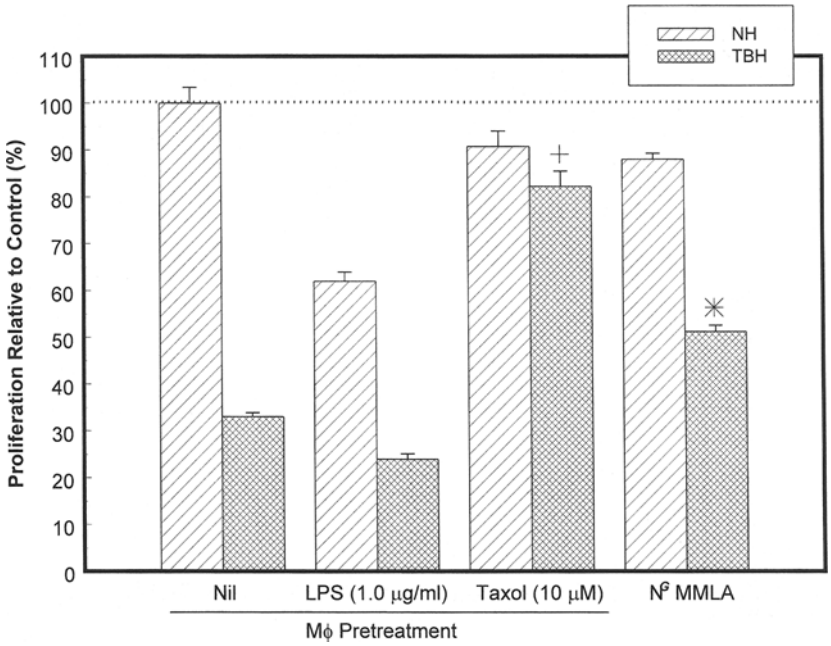

Fig. 2 Taxol-treated TBH macrophages are less immunosuppressive of T cell proliferation. Samples containing 8×10^6 BALB/c normal host or TBH macrophages were pretreated with taxol and added to 4×10^6 normal host (NH) BALB/c CD4+ T cells and 8×10^6 X-irradiated (20 Gy) C3H whole spleen cells in a mixed lymphocyte reaction. Macrophages were pretreated in 200 μ serum-free medium without or with 1 μg/ml LPS or 10 μM taxol. Following 4 h of culture, macrophages were washed and added to the T cell cultures. N^G-monomethyl-L-arginine (N^GMMLA; 0.5 mM) was added to parallel cultures. Mixed lymphocyte cultures were incubated for 96 h; 18 h prior to harvest, cells were pulsed with 1 μCi ^{3}[H]dT and proliferation determined. Data are the magnitude of response relative to proliferation in the presence of untreated normal host macrophages (control reactivity was 62690 cpm). Proliferation of unstimulated normal host responder or X-irradiated stimulator cells was less than 5% of control. Taxol-treated macrophages (+ $P < 0.01$) were significantly less suppressive as compared to untreated or LPS-triggered cells. Lower doses of taxol (0.1 μM and 1.0 μM) yielded similar results (not shown). N^G-monomethyl-L-arginine partially reversed TBH-macrophage-mediated suppression (* $P < 0.05$) as compared to untreated TBH macrophages. Data are averages and standard error of the mean (SEM) of triplicate independent determinations from one of four similar experiments

Statistics and calculations

Cells from six to ten normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for nitrite in the Griess reagent tests and were used for all proliferation and cytotoxicity assays. Data are averages with standard errors of the mean (SEM) of triplicate independent determinations. All experiments were repeated at least three times and representative experiments are shown. All data points on graphs were tested for significance by Student's *t*-test, and all comparisons are significant at the $P < 0.05$ level, unless otherwise stated.

Results

Transient taxol treatment regulates TBH macrophage NO production

Tumor-distal macrophages are immunosuppressive, partly because of tumor-distal over-production of NO [3]. We recently reported that taxol-triggered NO production by macrophages in the normal host and in the TBH is differentially regulated following continuous exposure to taxol [37]. During chemotherapeutic regimens, however, exposure to high concentrations of taxol occurs in a transient manner. In a clinically more relevant scenario, splenic macrophages were pretreated with stimuli for 4 h, washed, and cultured in fresh medium. Pretreatment with LPS (1 μg/ml) significantly increased NO production by normal host and TBH macrophages, and IFNγ (10 U/ml) enhanced NO production in a synergistic manner (Fig. 1). Pretreatment of macrophages with relevant doses of taxol (10 μM, corresponding to systemic concentrations during human therapeutic antitumor treatment) triggered normal host macrophage production of NO, and IFNγ priming synergistically enhanced taxol-induced NO production. In contrast, taxol pretreatment modulated NO production by TBH macrophages. Although taxol enhanced NO production by IFNγ-primed TBH macrophages (as compared to unstimulated TBH macrophages), the level of NO was significantly reduced as compared to maximal levels achieved after treatment with the macrophage-triggering agent LPS, either without or with IFNγ priming.

Taxol partially reverses TBH macrophage immunosuppressive activities

Because NO produced by tumor-distal macrophages is immunosuppressive, taxol-mediated modulation of NO production by TBH macrophages may lessen tumor-induced macrophage suppressor activities. Therefore, we investigated the immunological implications of the differential response of tumor-induced distal macrophages to

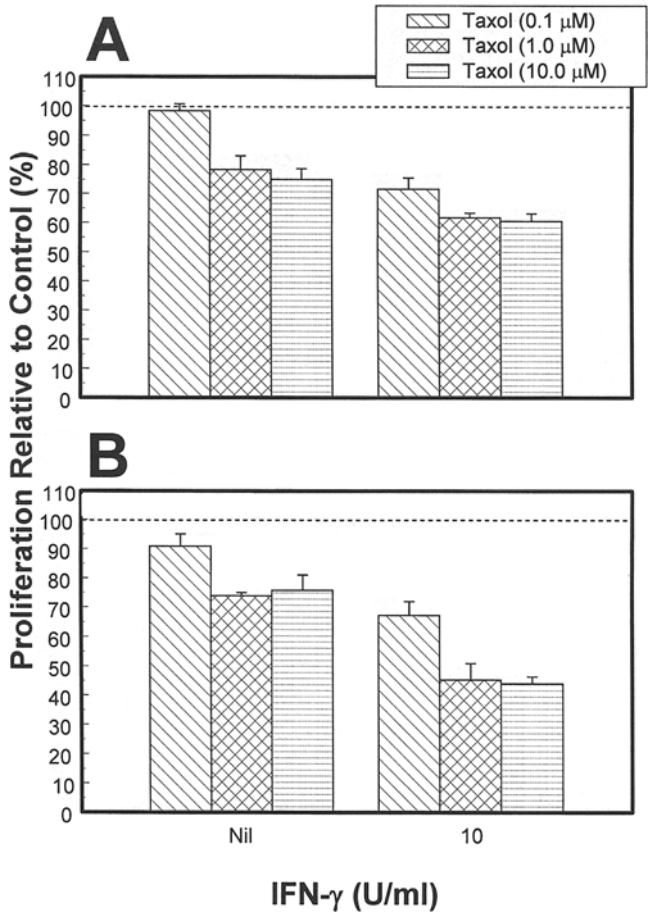

Fig. 3A, B Taxol-pretreated macrophages strongly suppress tumor cell proliferation. Supernatants (72 h) from 2×10^5 control or taxol pretreated (4 h) normal host (**A**) and TBH (**B**) macrophages were added to 1×10^5 actively growing Meth-KDE tumor cells and culture was continued for 24 h. Cultures were pulsed with 1 μCi/well [^{3}H]dT 12 h before harvest; cells were harvested and sample activities determined. Because the macrophages were pretreated, no taxol was present in the supernatants, and suppression of proliferation was not due to direct taxol-mediated cytostatic activity on the tumor cells. Data are the magnitude of response relative to proliferation of untreated tumor cells (control reactivity was 96240 cpm) and are reported as averages and standard errors of the mean (SEM) of triplicate independent determinations from one of four similar experiments

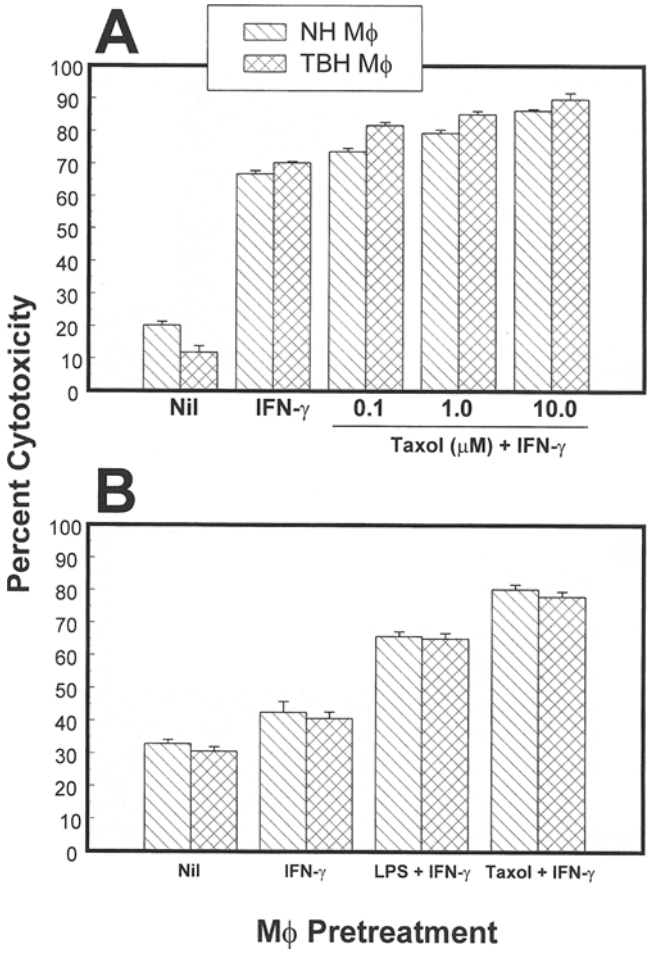

Fig. 4A, B Taxol enhances macrophage cytotoxic activity. Pretreated (4 h) normal host or TBH macrophages (2×10^5 cells) were added to viable tumor cell cultures (1×10^5 cells/well) and cytotoxicity measured using a modification of the Alamar blue colorimetric viability assay (Meth-KDE fibrosarcoma target cells, **A**) or a ^{51}Cr-release assay (P815 mastocytoma target cells, **B**). Macrophages were pretreated with IFNγ (10 U/ml) and taxol (0.1, 1.0, or 10.0 μM) (**A**), or IFNγ, LPS (1.0 mg/ml), and taxol (10.0 μM) (**B**). Similar results were obtained in the ^{51}Cr-release assay using lower taxol concentrations (0.1 μM or 1.0 μM). Data are averages and standard errors of the mean (SEM) of triplicate independent determinations from one of three similar experiments

taxol. Alloantigen-activated proliferation of normal host T cells was assessed in a mixed lymphocyte reaction containing untreated or pretreated normal host or TBH macrophages (Fig. 2). Taxol pretreatment (4 h) of TBH macrophages led to a modest but consistent reconstitution of T cell proliferation as compared to LPS-treated or untreated TBH macrophages ($P < 0.05$). Untreated TBH macrophages decreased T cell alloreactivity by more than 66% as compared to untreated normal host macrophages. Taxol-pretreated TBH macrophages suppressed the proliferative response by only 21.8%, suggesting that taxol mediates a partial reversal of macrophage-mediated immunosuppression in tumor-distal compartments. Modulation of NO production by macrophages may partially explain the reversal of immunosuppressive activity by taxol-pretreated TBH macrophages because abrogation of the NO production with MeArg (0.5 mM) partly reconstituted alloreactivity (Fig. 2).

Taxol enhances TBH macrophage-mediated tumor cell cytostasis

While the direct antitumor activities of taxol are well-characterized, the tumoricidal activities of taxol-treated TBH macrophages have not been described. Taxol has been shown to enhance normal host macrophage-mediated cytotoxicity [31]. Because taxol modulates TBH macrophage NO production following pretreatment (Fig. 1) or long-term exposure [37], we investigated the antitumor efficacy of taxol-treated TBH macrophages against the

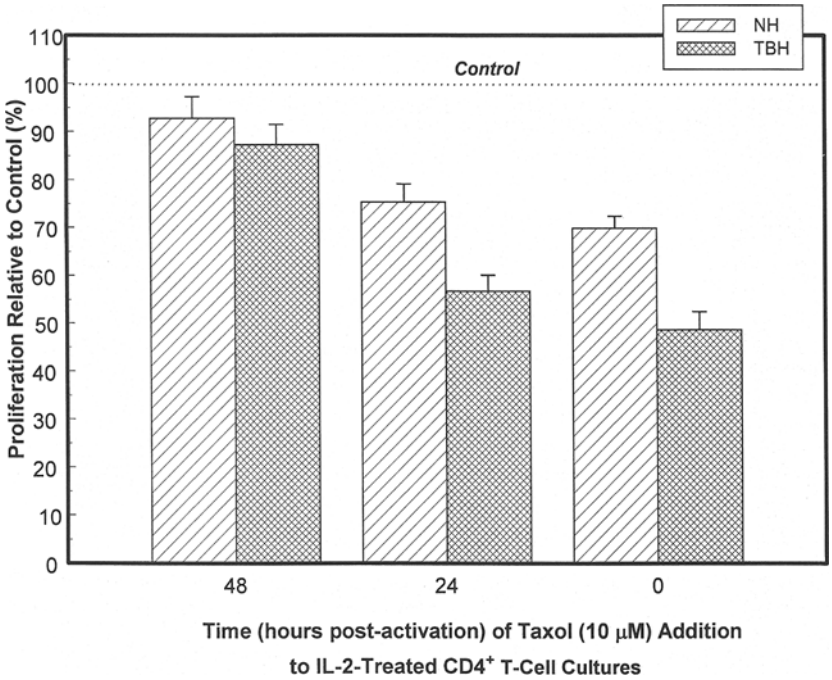

Table 1 Tumor growth increases T cell sensitivity to taxol. Taxol was used at a final concentration of 10 μM and was added at the time of T cell activation (0 h) or after activation (24 h or 48 h). Proliferation was assessed 72 h after activation. Similar data were acquired using 0.1 μM and 1.0 μM taxol (not shown). Normal host and tumor-bearing host (*TBH*) CD4+ T cells (4×10^6 cells/ml) were stimulated with concanavalin A (8 μg/ml). The change in proliferative response is shown as a percentage of the untreated control values. [^{3}H]dT incorporation is expressed ± SEM

Taxol addition (h)	$10^{-3}\times$[^{3}H]dT incorporation by normal host T cells (cpm)	Change in proliferation (%)	$10^{-3}\times$[^{3}H]dT incorporation by TBH T cells (cpm)	Change in proliferation (%)
Nil	136.09 ± 3.42	–	82.6 ± 2.19	–
0	96.82 ± 2.37	29 ↓	32.44 ± 0.52	61 ↓
24	79.74 ± 2.0	41 ↓	26.85 ± 1.02	67 ↓
48	123.81 ± 5.76	9 ↓	60.84 ± 2.11	26 ↓

purified tumor cells used in our model system (Meth-KDE). To assess the capacity for macrophage-derived effector molecules to suppress tumor cell proliferation, supernatants from taxol-pretreated normal host (Fig. 3A) or TBH (Fig. 3B) macrophages were added to purified tumor cells and tumor cell proliferation was measured after 24 h culture. Because the cells were pretreated, no taxol was present in the supernatants, and suppression of proliferation was not due to direct taxol-mediated cytostatic activity on the tumor cells. Pretreatment with taxol, either with or without IFNγ priming, significantly enhanced the ability of both normal host (Fig. 3A) and TBH macrophages (Fig. 3B) to suppress tumor cell proliferation. Taxol-mediated (10 μM) macrophage antitumor activity was enhanced by IFNγ priming (10 U/ml). Taxol-pretreated, IFNγ-primed TBH macrophages effected greater suppression of tumor cell proliferation than did similarly treated normal host macrophages, leading to a greater than 50% decrease in tumor cell proliferation relative to control cultures.

Fig. 5 Interleukin-2 (*IL-2*) increases TBH CD4+ T-cell sensitivity to taxol. Normal host and TBH CD4+ T cells were stimulated with concanavalin A (8 μg/ml) and IL-2 (100 U/ml). Taxol (10 μM) was added to cultures 48 h or 24 h after activation or at the time of activation (0 h). Normal host and TBH T cell proliferation was assessed after 72 h by [^{3}H]dT incorporation. Similar data were acquired using 1 μM or 0.1 μM taxol and 50 U/ml or 200 U/ml IL-2 (not shown). Data are the magnitude of response relative to T-cell proliferation in the absence of taxol and IL-2 (..... line, control reactivity was 136 090 cpm) from triplicate independent determinations in one of three similar experiments.

Taxol enhances macrophage cytotoxic activity

To determine whether taxol differentially regulated TBH macrophage antitumor cytotoxicity, we tested the cytotoxic capacities of taxol-pretreated macrophages against two tumor cell lines using two different assay systems. Taxol-pretreated macrophages were added to growing Meth-KDE tumor cells and cytotoxicity was measured using a modification of the Alamar blue colorimetric viability assay. Taxol-pretreated, IFNγ-primed macrophages demonstrated increasing cytotoxicity in a dose-dependent manner against the purified adherent Meth-KDE tumor cell line (Fig. 4A). Both normal host and TBH macrophage-mediated cytotoxicities were enhanced by IFNγ priming and taxol pretreatment. TBH macrophages demonstrated comparable cytotoxic activity to similarly treated normal host macrophages.

To confirm the cytotoxicity results, a ^{51}Cr-release assay was used to measure macrophage-mediated cytotoxicity against a second tumor cell line. Taxol-pretreated, IFNγ-primed TBH macrophages had enhanced cytotoxicity (78.1%) against murine P815 mastocytoma cells (Fig. 4B), as compared to 40.7% cytotoxicity by IFNγ-primed macrophages and 65.1% cytotoxicity by IFNγ-primed LPS-triggered cells.

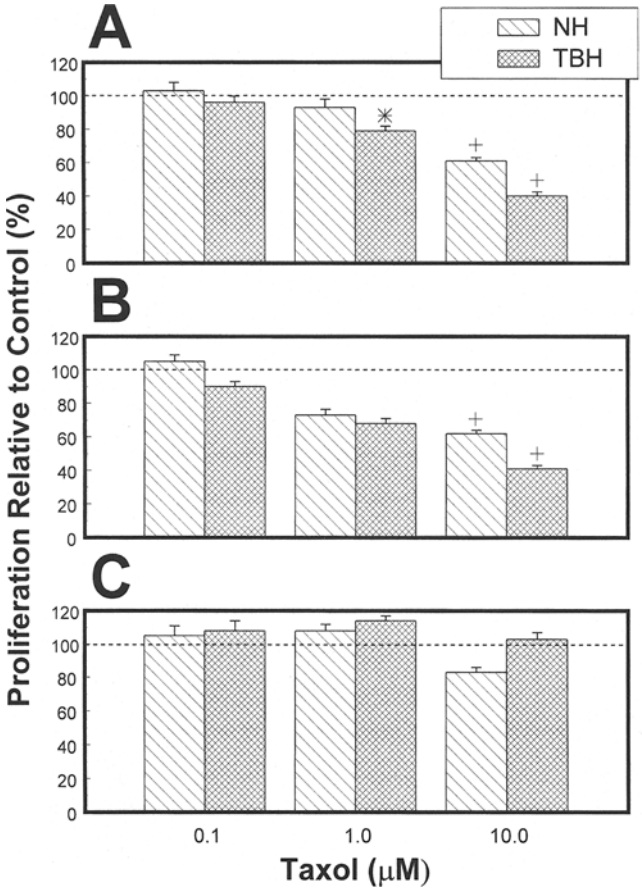

Fig. 6A–C Tumor growth partly compromises CD4+ T cell recovery from taxol pretreatment. Normal host and TBH CD4+ T cells were cultured for 4 h with taxol. Cells were washed twice and subsequently activated with concanavalin A (8 μg/ml) 0 (**A**), 12 h (**B**), or 24 h (**C**) after taxol pretreatment. Normal host and TBH T cell proliferation were assessed after 72 h by [^{3}H]dT incorporation. Data are expressed as the percentage proliferation relative to normal host control from triplicate independent determinations in one of three similar experiments. The results indicated (* $P<0.05$; + $P<0.005$) were significantly lower than control levels

Tumor growth increases CD4+ T cell sensitivity to taxol

While taxol imparts immune-activating functions to macrophage populations, taxol's direct cytotoxic and cytostatic activities are most effective on rapidly dividing cell populations. Because taxol may decrease T cell proliferation and compromise responsiveness to IL-2 [8, 10] and tumor growth significantly dysregulates T cell responsiveness to alloantigen activation [16, 44, 47], we determined whether taxol exacerbates tumor-induced changes in CD4+ T cell responsiveness. Taxol significantly suppressed mitogen-induced (8 μg/ml ConA) proliferation by both normal host and TBH T cell populations (Table 1), suggesting that factors that enhance proliferative response also enhance sensitivity to taxol-mediated cytotoxicity. Interestingly, the T-cell-stimulatory cytokine IL-2 (100 U/ml, Fig. 5) increased T cell sensitivity to taxol. TBH T cells were more sensitive to taxol-mediated suppression than were normal host T cells, even when taxol was added 24 h or 48 h after activation.

To determine whether the activation status of CD4+ T cells contributes to the degree of sensitivity, T cells were pretreated with taxol (4 h), washed, and cultured. The T cell mitogen ConA (8 μg/ml) was added 0, 12, or 24 h after culture (Fig. 6). Taxol pretreatment of TBH T cells significantly compromised ConA-stimulated T cell proliferation (Fig. 6A), and TBH T cell proliferation was suppressed more than normal host T cell proliferation even when cells were pretreated with taxol 12 h before activation (Fig. 6B). However, both normal host and TBH CD4+ T cells recovered from taxol-mediated inhibition if they were pretreated (24 h) prior to activation (Fig. 6C). Collectively, these data suggest that taxol directly contributes to tumor-induced T cell dysfunctions and that the activation status of the T cells influences the degree of sensitivity to taxol-mediated inhibition.

Discussion

In this study, we demonstrated that the antineoplastic agent taxol partially reverses tumor-induced immunosuppression and promotes antitumor activities by TBH macrophages. We extend our previous findings [37] by demonstrating that short, transient taxol administration (4 h) can regulate in vitro macrophage NO production. Taxol's control of macrophage proinflammatory molecules, such as NO (Fig. 1), may reverse the tumor-induced immunosuppression that is characteristic of tumor-distal macrophages, and this effect is apparent in vitro (Fig. 2).

From a clinical standpoint, the most notable immunological activity of taxol may be its ability to activate macrophages for enhanced tumor cell cytotoxicity [31]. Because macrophages must be proximal to tumors for direct cytolytic activity, it begs the question of cytotoxic molecule production by tumor-distal macrophages. Studies show that tumor-infiltrating macrophages have decreased cytotoxic activity as compared to inflammatory macrophages [32] and that macrophage production of NO and the antitumor cytokine TNFα is downregulated at the tumor site [4, 34]. In contrast, tumor-distal macrophage populations produce elevated levels of NO and TNFα [3, 37]. Thus, macrophage production of cytotoxic and proinflammatory molecules varies, depending upon the macrophage's cell compartment in vivo. Although enhanced TBH macrophage production of cytotoxic effectors such as TNFα and NO outwardly seems advantageous, overexpression of these signals by tumor-distal macrophages may merely suppress T cell activities [2, 20, 22] yet fail to impart antitumor cytotoxic activity. Taxol-mediated modulation of tumor-distal macrophage inflammatory and cytotoxic responses may benefit the TBH by limiting macrophage-mediated suppression of T cell responsiveness.

While we and others [30, 43] have shown that taxol enhances IFNγ-activated normal host macrophage cytotoxicity toward tumor cells in vitro, no studies have addressed

the effects of tumor growth on taxol's capacity to induce macrophage-mediated antitumor cytotoxicity. Although taxol modulated NO production by TBH macrophages (Fig. 1) and has been shown to modulate natural-killer(NK)-cell-mediated cytotoxicity [9], we determined that taxol treatment enhanced macrophage antitumor activities (Fig. 3). Through soluble antitumor molecules, taxol-triggered macrophages suppressed tumor cell proliferation; these enhanced antitumor activities may derive from increased macrophage production of TNFα [3]. Taxol also increased direct macrophage-mediated cytotoxicity of both Meth-KDE and P815 tumor cells (Fig. 4), demonstrating that taxol-induced macrophage-mediated cytotoxic activity was not restricted to the tumor cell used in our nonmetastatic fibrosarcoma model. These results demonstrate that a significant immunotherapeutic activity of taxol, the capacity to modulate macrophage NO production in the TBH, does not abrogate taxol-mediated induction of macrophage antitumor cytotoxicity. Furthermore, tumors may escape tumor-proximal macrophage-mediated cytotoxicity by overproducing macrophage-deactivating cytokines that inhibit local NO and TNFα production. Taxol, which directly inhibits tumor cell division and interrupts protein synthesis, may compromise tumor production of macrophage-deactivating molecules while concurrently activating the NO and TNFα production of tumor-infiltrating macrophages, leading to limited tumoricidal activity by tumor-proximal cells. This could further enhance taxol-mediated antitumor activity, and this possibility is currently being investigated.

Because taxol inhibits the proliferation of many cell types, including lymphocytes, we investigated whether tumor growth increases T cell sensitivity to taxol. Taxol significantly suppressed mitogen-induced (8 μg/ml ConA) proliferation by both normal host and TBH T cell populations (Table 1), and TBH CD4+ T cell proliferation was suppressed to a greater extent than that of the normal host counterpart (Table 1) even when taxol was administered 48 h after T cell activation. Furthermore, IL-2 (Fig. 5) increased TBH CD4+ T cell sensitivity to taxol. These data suggest that IL-2 immunotherapies used in conjunction with taxol could adversely affect in vivo T cell activities. Heightened sensitivity may occur because IL-2 significantly restores TBH CD4+ T cell proliferation to the levels in the normal host, committing T cells within the cell cycle where cell populations are most sensitive to taxol. Alternatively, taxol may compromise IL-2 production or suppress expression of functional IL-2 receptors, because proliferation of taxol-treated lymphocytes cannot be restored by addition of exogenous IL-2 [40]. Our data support this possibility because TBH CD4+ T cells are less responsive to IL-2 in the presence of taxol than are their normal host counterparts (Fig. 6A). Although others have shown that taxol can suppress the proliferation and cytotoxic activities of activated T cells [8, 10], we are the first to report that tumor growth increases T cell sensitivity to taxol (Fig. 5). Tumor growth, however, does not eradicate the ability of CD4+ T cells to recover from transient taxol treatment (Fig. 6C), lessening the potential insult to T cell antitumor responses during taxol-based antitumor therapies.

Collectively, these results suggest that taxol may simultaneously control macrophage-mediated immunosuppressive activities in tumor-distal compartments while facilitating antitumor activities by tumor-proximal macrophage populations, indicating that a partial reversal of tumor-induced immunosuppression can be achieved without compromising macrophage-mediated cytotoxic capacity. In addition to its direct cytostatic and cytotoxic functions on neoplastic cells, taxol may compromise tumor cell resistance to immune attack and enhance the efficacy of other chemotherapeutic or immunotherapeutic approaches. While the relevance to human oncology of taxol-mediated control of macrophage NO production has been debated [11], recent evidence strongly suggests that human peripheral blood mononuclear cells can produce moderate amounts of NO both in vitro [14, 45] and in vivo [14], suggesting that macrophage responsiveness to taxol in human cancer patients warrants investigation.

The antineoplastic agent taxol, a powerful chemotherapeutic agent, has been shown to impart immunotherapeutic activities in the TBH. Through its dual capacity to inhibit tumor growth directly and to activate certain immune populations, taxol may prove a powerful immunotherapeutic agent either alone or in combination therapies. These studies enhance our understanding of the antitumor functions of taxol and suggest that taxol could be a beneficial agent in combination immunotherapies.

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