PACLITAXEL-INDUCED MACROPHAGE ACTIVITIES IN THE TUMOR-BEARING HOST: IMMUNOLOGIC IMPLICATIONS AND THERAPEUTIC APPLICATIONS

David Warren Mullins

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Klaus D. Elgert, Chair
S. Ansar Ahmed
Carol J. Burger
Muriel Lederman
Gerhardt G. Schurig

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(ABSTRACT)

Tumors induce immune dysfunction through the production of soluble factors that subvert macrophage (Mϕ) function to favor tumor growth. Previous studies suggested that tumor-induced immune cell dysfunction may be reversible through regimens that disrupt tumor cell suppressor mechanisms and concurrently promote tumoricidal activities. Because the antineoplastic agent paclitaxel (TAXOL™) activates Mϕ function, we studied mechanisms of paclitaxel-mediated cytotoxic and immunostimulatory responses by tumor-induced Mϕs. Although tumor-derived factors, including interleukin-10 and transforming growth factor-β_1, modulate Mϕ response to activation signals, paclitaxel partly reverses tumor-induced Mϕ-mediated suppression of T-cell reactivity through enhanced production of the immunostimulatory cytokine interleukin-12 (IL-12). Concurrently, paclitaxel induces Mϕ cytotoxic and proinflammatory molecule production, including tumor necrosis factor-α and interleukin-1β. In contrast to its apparent immunotherapeutic effect on Mϕ populations, paclitaxel's cytostatic mechanisms suppress lymphocyte proliferation and function. We showed that IL-12 can reverse paclitaxel-mediated suppression of T-cell responses *in vitro*, establishing the foundation for a novel antitumor therapy using paclitaxel in combination with IL-12. We show that the administration of paclitaxel as a chemotherapeutic agent, followed by IL-12 as an immunotherapeutic agent to alleviate paclitaxel-mediated immunosuppression, prolongs survival, reduces tumor progression, and activates immune effector populations in a murine tumor model. These results are the first experimental evidence to suggest that paclitaxel and IL-12 are an effective antitumor modality. Collectively, these studies show that paclitaxel induces multiple antitumor mechanisms that can be enhanced with proper ancillary administration of immunotherapeutic cytokines.

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INTRODUCTION

The objectives of my studies were to enhance our understanding of the mechanisms by which tumor cells dysregulate macrophage (Mϕ; see Appendix A for Abbreviations) antitumor activities and to determine the efficacy of several therapeutic approaches to not only kill the tumor but reverse tumor-induced immune dysfunction. Studies from our laboratory have established several mechanisms by which tumor cells mediate immunosuppression. By understanding *how* tumors dysregulate immunity, we have been able to identify potential therapeutic measures that may counteract immunosuppression and restore the antitumor immune responses, thus inhibiting or eliminating the tumor. Reversal of immune dysfunction may require combinations of targeted chemotherapies and immunotherapies, synergistically acting to achieve higher therapeutic success against cancer. My studies were the first in our laboratory to apply our knowledge of the mechanisms of immunosuppression in search of therapeutic approaches -- including chemotherapeutic, immunotherapeutic, and combined therapies -- to reverse tumor-induced immune dysfunction and impede tumor growth.

The field of tumor immunology began with the inceptive observation, over three-hundred years ago, that certain cancer patients who developed concurrent bacterial infections would demonstrate remissions of their malignancies (320). Yet, it was not until 1892 that this phenomenon was investigated and applied in a therapeutic setting by William B. Coley, an eccentric New York surgeon (453). Coley ascribed a therapeutic activity to some unidentified bacterial product (89), which came to be referred to *Coley's toxin*. Coley's application of the exudate from the infected lesions of cancer patients (which he referred to as the "laudible pus") to treat the tumors marked the humble beginnings of tumor immunotherapy. It would take another 51 years to identify lipopolysaccharide (LPS) as the immunostimulatory agent in Coley's extracts, and 35 more years to identify tumor necrosis factor-α (TNF-α) as the probable effector molecule responsible for tumor regression in Coley's crude therapeutic approach (180).

Since the 1970's, immunologists have described a vast and complex array of immune system cells, molecules, and signal pathways that collectively regulate the immune response. Cancerous tumors, however, use a variety of mechanisms to evade the immune system (176,416,431), including the release of chemical messenger molecules normally associated with immune cells, known as *cytokines* (31). Cytokines act as the intercellular messengers of the immune system, and they are essential for regulating and coordinating immune responses (30,309,397). Regulation of the immune response requires a delicate balance between stimulatory and suppressor cytokines; too much or too little of a particular cytokine opens the possibility for imbalance -- and dysfunction -- in the system. Taking advantage of this precarious balance, tumor cells favor their own growth by producing inhibitory cytokines that upset the normal balance of the immune system, leading to altered immune cell function and immunosuppression

(9,14,16,18,26,123,200,212,444,469). Consequently, an individual's ability to raise a formidable antitumor response is compromised during tumor growth (400).

Currently, cytokine research is enhancing our understanding the processes of immune cell regulation -- and dysregulation -- during tumor growth. If we discover the mechanisms of dysregulation, we can correct cytokine imbalances in immunosuppressed individuals, enhancing both the effectiveness of existing cancer treatments and boosting the patient's ability to mount an effective immunologic response. Highly pleiotropic, cytokines simultaneously act on many cell types throughout the body (335,397); this broad range of activities necessitates careful study of the steady-state levels in healthy individuals. Overdosing with cytokines may cause further dysregulation of cellular and molecular interactions (229), leading to increased immunosuppression (250), harmful side effects (359,380), and lack of tumor regression (75,258). Cytokine steady-state levels are being assessed (344), followed by administration of various cytokine-cytokine or cytokine-drug combinations to patients in an attempt to safely reverse tumor progression (47,150,434). Cytokine therapy is directed at correcting a tumor-induced immune system irregularity; like insulin therapy for a diabetic replaces a missing enzyme but does not correct the underlying disease state, cytokine therapy is aimed at restoring normal immune balance rather than directly assaulting the tumor. Because cytokines are naturally-occurring compounds, proper administration could cause fewer harmful side effects than surgery, radiation therapy, or chemotherapy. Cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ) are used to stimulate antitumor immune functions in immunosuppressed cancer patients (253,383), and cytokines such as TNF-α, which can directly kill tumor cells, are used in anticancer therapy (324,327,367). Another promising immunotherapeutic agent is the Mϕ-derived cytokine interleukin-12 (IL-12).

Administration of recombinant IL-12 has reversed tumor-induced immunosuppression and partially regressed tumors in some cancer patients (319,483).

The anticancer efficacy of cytokines may be enhanced when combined with chemotherapeutic or immunotherapeutic drugs (305,323,432); several pharmaceuticals have such activity (185,390), including the anticancer molecule paclitaxel (TAXOL™) (49,53,262,267,364). Isolated from the leaves of the western yew *Taxus brevifolia* (215,217), paclitaxel acts directly upon tumor cells, inhibiting growth and mediating cell lysis (55,67,114). Paclitaxel also has cell cycle-independent activities, including the induction of cytotoxic antitumor factors by Mϕs (49,53,267). When I joined the laboratory in 1994, very little was know about the immune system activities of paclitaxel. Most notably, I was astonished that, following my first presentation to the American Cancer Society's Virginia Branch meeting at the University of Virginia, I was approached by several clinicians involved in phase III trials of paclitaxel. *They were amazed to learn that paclitaxel activates Mϕ-mediated immune responses!* Paclitaxel had proven its effectiveness as an antitumor chemotherapeutic, and several investigators had described paclitaxel's LPS-mimetic capacity in normal host Mϕs (49,267). However, no studies had described the effects of tumor growth on paclitaxel-mediated activation of Mϕs or investigated the possibility that paclitaxel's immunotherapeutic activity may partially account for its success in treating several human cancers.

Tumors dysregulate immune responses and subvert Mϕs in suppressor cells (123), suggesting that effective antitumor therapies should target both tumor growth and immune dysfunction. While paclitaxel alone is a moderately effective antitumor treatment, its antitumor efficacy may be greatly enhanced when combined with immunotherapeutic regimens to simultaneously restore cytokine balance to the immune

system. We speculated that the combined administration of the anticancer chemotherapeutic paclitaxel and the immune activating cytokine IL-12 might represent a potent antitumor approach, offering several advantages over traditional antitumor modalities (surgery, radiation, and chemotherapy). A combination therapy would likely reduced the effective therapeutic doses of chemotherapeutic drugs, decrease the need for antitumor agents in favor of naturally-produced cytokines, and induce fewer and less severe side effects than traditional chemotherapy. Collectively, a combined therapy may more fully restore balance to the immune system while facilitating a strong anticancer response.

Extending the findings of previous researchers from our group, my studies were aimed at defining immunologic parameters and activities of the anticancer drug paclitaxel by testing the hypotheses: *The anticancer drug paclitaxel reduces tumor-induced cytokine imbalance and Mϕ dysfunction, and restoration of cytokine balance through paclitaxel and IL-12 may be a potent antitumor immunotherapy*. Specifically, my research addressed the following aims:

- *Define paclitaxel-induced cytotoxic functions of Mϕs:* Paclitaxel-mediated production of cytotoxic effector molecules was evaluated in normal host and tumor-bearing host (TBH) Mϕs to determine whether paclitaxel's LPS-mimetic activities are differentially regulated during tumor growth.

- *Determine whether paclitaxel can reverse tumor-induced Mϕ-mediated immunosuppression:* Paclitaxel-pretreated M ϕs were assessed for their capacity to enhance or suppress T-cell alloreactivity in order to determine whether paclitaxel's Mϕ-activating functions have immunotherapeutic efficacy.

- *Determine whether IL-12 can reverse paclitaxel-mediated immunosuppression:* Because paclitaxel can activate Mϕs and simultaneously exacerbate tumor-induced suppression of T-cell proliferative functions, was assessed the capacity of the T-cell-stimulatory cytokine IL-12 to reverse paclitaxel-mediated suppression of T-cell reactivity.

- *Determine the antitumor efficacy of paclitaxel and IL-12 combination therapy:* To determine whether paclitaxel and IL-12 combined therapy can reduce tumor-induced immunosuppression and impede tumor progression, combinations of paclitaxel and IL-12 will be administered *in vivo* and immune cell function analyzed.

These studies will show that paclitaxel induces Mϕ cytotoxic and immunostimulatory functions, partially reverses tumor-induced Mϕ-mediated suppression of T-cell function, and corrects an aberration in cytokine production by TBH Mϕs. Further, these data will suggest a novel mechanism that could partly explain the clinical success of paclitaxel and suggest a potential combined chemotherapeutic and immunotherapeutic approach for the treatment of cancer.

Many experimental approaches were used to accomplish these aims. Throughout this work, the Meth-KDE murine fibrosarcoma model (15,126) was used. This nonmetastatic tumor model allowed us to study how tumor-distal immune cells respond to immunotherapeutic treatments. We employed classic approaches, including enzyme-linked immunosorbent assays (ELISA) and proliferation assays, and we supplemented our studies with cutting-edge approaches to more fully and thoroughly characterize immunologic responses to paclitaxel and IL-12. Ribonuclease protection assay (RPA)

analysis was used to define the molecular basis of a lesion in TBH Mϕ function. Signal transduction pathways were investigated using a Mϕ cell line transfected with a novel reporter gene, and Annexin-V-based flow cytometric analyses were performed to assess paclitaxel-induced apoptotic cell death.

This dissertation is divided into four sections that describe several mechanisms of paclitaxel-mediated immune activity. The first section is a review of the relevant literature, including a synopsis of known mechanisms of tumor-induced immune cell dysfunction and overview of the properties of paclitaxel, IL-12, and key Mϕ-derived antitumor effector molecules. The remaining three sections contain my published or submitted findings that describe immune responses to paclitaxel during tumor growth -- including several mechanisms of paclitaxel-mediated immunotherapeutic activity -- and the efficacy of combined application of paclitaxel and IL-12.

The first section, the *Literature Review*, consists of three subsections. First, the cellular and molecular mediators of antitumor immune responses – Mϕs, T-cells, and cytokine – are introduced. Next, mechanisms by which tumors disrupt immune activities and subvert Mϕ function to favor tumor growth are presented. Finally, several therapeutic approaches for reversing tumor-induced immune dysfunction are discussed, including an extensive review of current literature on paclitaxel and IL-12.

The second section, *Paclitaxel-induced Cytotoxic Functions of Mϕs*, contains three chapters that detail the response of normal host and TBH Mϕs to paclitaxel treatment *in vitro*. Chapter I describes the paclitaxel-induced Mϕ production of the cytotoxic effector molecules nitric oxide (NO) and TNF-α by normal host and TBH Mϕs. Chapter II characterizes interleukin-1β (IL-1β) production in response to paclitaxel-

induced Mϕ activation. Chapter III presents data that demonstrate paclitaxel's capacity to induce Mϕ-mediated tumor cell cytotoxicity.

The third section, *Paclitaxel Reverses Tumor-induced Mϕ-mediated Immunosuppression*, consists of three chapters. In Chapter IV, data are presented from studies using a Mϕ cell line with a luciferase reporter gene behind a nuclear factor-κB (NF-κB)-responsive promoter to characterize Mϕ activation and factor production in the absence or presence of tumor-derived factors. Further, we present the first data to suggest a tumor-induced defect in the IFN-γ signaling response; this lesion may partially explain the modulated response of *in situ* Mϕs to activating agents. Chapter V discusses the capacity of paclitaxel treatment to reverse TBH Mϕ-mediated immunosuppression, establishing an immunotherapeutic component to paclitaxel's antitumor activities. Mechanisms of paclitaxel-mediated immune activation are characterized in Chapter VI, including a suggested mechanism by which paclitaxel induces Mϕ production of IL-12 through an autocrine signaling pathway involving NO.

The fourth section*, Antitumor Efficacy of Paclitaxel, IL-12, and Combination Therapies*, contains four chapters describing the effects of single agents or combined therapies on tumor progression and immune cell function. Chapter VII presents *in vitro* evidence that paclitaxel directly inhibits tumor cell proliferation and induces apoptotic tumor cell death in our model system. Chapter VIII contains the first experimental evidence that chemotherapeutic paclitaxel administration differentially regulates Mϕ functions in the TBH. Chapter IX presents data that establish IL-12's role as an effective immune activating cytokine that can overcome the paclitaxel's suppressive functions. These *in vitro* results lay the foundation for the *in vivo* studies detailed in subsequent chapters. Finally, Chapter X characterizes the efficacy of paclitaxel and IL-12 combined

therapies in a murine tumor model system. These results are the first experimental evidence to suggest that paclitaxel and IL-12 are an effective antitumor modality.

The *Conclusions* offer a summary of the results described in Sections II, III, IV, and V. Furthermore, an updated model of paclitaxel- and IL-12-mediated immunotherapeutic activity is proposed. Although the data in this dissertation extensively characterize the immune activities of paclitaxel and establish a novel mechanism of antitumor therapy, substantial studies will be required in order to characterize the molecular networks involved in tumor-induced immunosuppression and paclitaxel-mediated immune activation. Therefore, the *Conclusions* include suggested approaches for future investigations with the hope that these studies will establish a foundation for future research into improved immune-based anticancer therapies.

SECTION I: LITERATURE REVIEW

The *Literature Review* consists of three subsections. The first subsection, *Cellular and Molecular Effectors of Antitumor Immune Responses,* introduces the mediators of antitumor immune responses – Mϕs, T-cells, and cytokines. The second subsection, *Mechanisms of Tumor-induced Immune Dysfunction*, describes several known mechanisms by which tumors disrupt immune activities and subvert Mϕ function to favor tumor growth. The third subsection, *Immunotherapeutic Approaches to Cancer*, discusses therapeutic approaches for reversing tumor-induced immune dysfunction, including reviews of the current literature on paclitaxel and IL-12.

Cellular and Molecular Effectors of Antitumor Immune Responses

Cancer is a disorder of cellular division, arising from the transformation of normal cells into immortalized cells with uncontrolled growth. Unlike their nontransformed counterparts, cancer cells abandon normal functions in order to devote most of their energy to proliferation. What makes cancer deadly is its malignancy, characterized by loss of normal structural characteristics, sustained cell division, and migration (metastasis). Multiple factors lead to the development of cancer, including chemical carcinogens, environmental factors, radiation, or viruses. Underlying these apparent

causes, however, are fundamental changes in the function of genes. All cancer types are immortal because two sets of growth-regulatory genes (the proto-oncogenes and anti-oncogenes, also called tumor suppressor genes) are aberrantly expressed. Proto-oncogenes are primarily responsible for regulation of cellular metabolism, growth, and differentiation through a variety of protein kinases. In contrast, tumor suppressor genes negatively regulate cellular proliferation, communication, and angiogenesis. Aberrations in proto-oncogene and tumor suppressor gene function lead to unchecked cell cycle progression and rapid cellular proliferation.

The body's first-line response to cancer is mediated through multiple immune cell types that collectively combat neoplasia by direct and indirect mechanisms. Natural killer (NK) cells eliminate tumor cells through major histocompatibility complex (MHC)-independent mechanisms and antibody (Ab)-dependent cell-mediated cytotoxicity. Although NK cells impart significant antitumor efficacy, the remaining discussions will be limited to Mϕs and T cells (the focus of this dissertation). Mϕs, once scorned as the "immunologic trash collectors," are vital effectors of innate immunity and provide a first-line defense through their capacity to mediate direct antitumor cytotoxicity. More significant, Mϕs serve as a link between innate and acquired immune responses. Through the production of soluble signal molecules (*cytokines*), Mϕs "sound the alarm" that elicits the powerful and targeted responses of T-cells. Mediators of the specific immune responses, T-cells are divided into two major subsets that serve a variety of support and effector functions in antitumor immunity. The $CD8^+$, or cytotoxic T-cells, can interact directly with cell surface targets and destroy tumor cells. The $CD4^+$, or helper T-cells, act as regulatory cells that direct the activities of other immune effector populations, including Mϕs, B-cells, and $CD8^+$ T-cells.

Like any organization composed of temporally- and spatially-disparate members, immune system cells require a sophisticated communications network in order to maintain a focussed effort. Cytokines, the molecular messengers of the immune response, coordinate the direct tumor cell killing activities of Mϕs, $CD4^+$ T cells, and $CD8^+$ T-cells. The presence and persistence of tumors, however, suggests a failure in the immune system's ability to suppress their growth. *How do tumors accomplish this?* Substantial data support the hypothesis that tumors induce immunosuppression by dysregulating cytokine networks, thus disrupting interactions between immune populations and impeding antitumor immune function. To understand the mechanisms of tumor-induced immune dysfunction (discussed in subsequent sections of this *Literature Review*), it is necessary to first understand the normal activities and functions of the cellular and molecular components of the immune response to tumors.

Macrophages

Mϕs were originally described over 100 years ago by Metchnikoff, at approximately the same time that Coley began to study the antitumor responses induced by bacterial extracts (328,453). Coley could not have imagined that these immune effector cells were responding to his crude treatments, thereby mediating systemic immune activation and tumor regression.

Mϕs (29,196,254,358) represent the host's "front line" defense against infection and tumor challenge. As part of the mononuclear phagocyte system, Mϕs perform both phagocytic and antigen presenting functions (because of the latter function, they are also called antigen-presenting cells [APCs]). Unlike their T-cell counterparts, Mϕs are neither clonally restricted nor antigen-specific. Mϕs are a phenotypically and functionally

heterogeneous population (290) of highly versatile cells that participate in accessory, secretory, effector, and regulatory activities.

Mϕs develop through a tightly regulated process called *myelopoiesis* (35,117). The life of a Mϕ begins as a pluripotent stem cell, which gives rise to myeloid bone marrow precursors and eventually mature monocytes. Each developmental step requires particular cytokines and growth factors (117). Mature monocytes leave the bone marrow and enter circulation, where they migrate for several days. Eventually, monocytes establish residency in tissues, where they are then referred to as Mϕs. In the tissue microenvironments, Mϕ maturation and differentiation is completed. Although the notion of the Mϕ as a terminally differentiated cells persists, undifferentiated Mϕ have been demonstrated within tissues, and these cells can give rise to several distinct Mϕ subpopulations under the correct conditions (448).

Although Mϕs are primarily recognized as phagocytic cells, they also play important accessory roles in antigen processing and presentation to T-cells, and these functions are vital for antitumor immune responses. During tumor challenge, Mϕs display tumor antigens to antigen-specific $CD4^+$ T cells in the context of MHC class II molecules. In conjunction with the appropriate costimulatory molecules, these interactions activate $CD4^+$ T cells to proliferate and release cytokines. These T-cell-derived cytokines, in turn, activate tumor antigen-specific cytotoxic T cells and MHC-unrestricted NK cells (33). Mϕs play a dual role by stimulating T-cells in response to challenge and modulating T-cell reactivity once the challenge is cleared. The Mϕ-derived cytokines responsible for T-cell activation include IL-1 and granulocyte macrophage-colony stimulating factor (GM-CSF); in contrast, the Mϕ-derived molecules transforming growth factor-β_1 (TGF-β_1), interleukin-10 (IL-10), and prostaglandin E_2

(PGE_2) downregulate T cell responses. Thus, tumors may escape immune responses by dysregulating Mϕ APC function, altering the expression of costimulatory molecules, or inducing Mϕs to produce suppressor cytokines.

On exposure to stimulatory agents such as IFN-γ or bacterial LPS, Mϕs demonstrate limited functional specialization and are referred to as *activated* Mϕs (72,181). Activated Mϕs mediate inflammatory (235) and cytotoxic processes (223,377), and they can secrete over 100 known cytokines and effector molecules (72,307). Resting Mϕs are activated only upon stimulation with both priming and activation signals (4,181), and only activated Mϕs mediate cytotoxic effector functions. Although the priming signal can precede or appear concomitantly with the activation signal, Mϕ activation will not occur with only the priming or only the activating signal. Activation will not be achieved if the activating signal precedes the priming signal (181). Priming signals include cytokines, adherence, and surface molecule stimulation, and these signals trigger mRNA synthesis (1,2,72). Activation signals stimulate or enhance translation of preformed messages, leading to rapid production of mediator molecules (including NO, IL-1β, interleukin-6 [IL-6], and soluble TNF-α) or expression of surface molecules (such as membrane-bound TNF-α and MHC class II molecules) (72).

Mϕ cytostatic and cytotoxic activities against tumor cells contribute significantly to tumor rejection (135,209,223). Through secreted molecules such as NO (393) and TNF-α (136), activated Mϕs directly inhibit or kill tumor cells (although tumors demonstrate variable resistance to Mϕ cytotoxic activities). Tumors may escape Mϕ cytotoxic activities by producing immunomodulatory molecules that inhibit production of cytotoxic effectors *in situ* while simultaneously promoting cytotoxic molecule production by tumor-distal Mϕs. Molecules such as NO fail to mediate antitumor activity when

released in tumor-distal compartments but may compromise T-cell function. Interestingly, TBH Mϕs outwardly appear to be unactivated, but they are actually primed for tumoricidal activity (65,118,269) and production of cytokines (9,11,13), NO (15,296), and prostaglandins (9,11,13,312,334). TBH Mϕs are considered primed because they constitutively express mRNA for TNF-α (9,13,36) and other cytokines. After activation, these TBH Mϕs demonstrate superior capacity to kill tumors and to produce larger amounts of factors, as compared with normal host Mϕs (52,65,137) – *yet tumors survive!*

T Cells

The mediators of specific immune responses, T cells are antigen-specific, MHC-restricted lymphocytes that regulate immune cell activities through surface-associated molecules and cytokines. Like Mϕs, T cells originate from pluripotent bone marrow stem cells; however, T-cells diverge into lymphoid precursors through carefully regulated growth factor cascades. The majority of T-cells migrate to the thymus for further maturation, including most $CD4^+$ and $CD8^+$ T cells bearing αβ TCR. A minor proportion of T cells mature through extrathymic pathways. Upon maturation, antigen-specific self MHC-restricted T cells enter the periphery where they circulate in the blood or lymph or reside in lymphoid tissues.

T cells are phenotypically separated into two subsets, each population possessing discrete functional characteristics. The two subsets are classified by distinct expression of either CD4 or CD8. These molecules serve as convenient phenotypic markers; more importantly, CD4 and CD8 stabilize interactions between TCRs and MHC molecules. CD4 molecules facilitate interactions between TCRs and MHC class II molecules (found

on professional APC, such as Mϕs or B cells), and CD8 molecules promote interactions between TCRs and MHC class I molecules (found on almost all nucleated cells).

$CD4^+$, or helper, T cells mediate homeostatic, autoreactive, and cytotoxic functions. $CD4^+$ T cells can be further partitioned into two functional subsets -- the T_H1 cells and T_H2 cells -- based on the cytokines these cells produce upon activation (293,354,403). T_H1 cells produce IL-2 and IFN-γ, cytokines that enhance the cell-mediated immune response. In contrast, T_H2 cells produce interleukin-4 (IL-4), interleukin-5 (IL-5), IL-6, IL-10, and interleukin-13 (IL-13) – molecules that promote humoral, or Ab-mediated, responses. Two key T-cell-derived cytokines cross-regulate these subsets; the T_H1-predominant cytokine IFN-γ suppresses T_H2 proliferation but not cytokine synthesis (166, 168); coordinately, the T_H2-predominant cytokine IL-10 suppresses T_H1 cytokine synthesis, including IL-2 and IFN-γ. Differentiation into T_H1 or T_H2 subsets is determined by the cytokine status in the T_H-precursor microenvironment; high levels of the Mϕ-derived cytokine IL-12, which drives IFN-γ production, will strongly promote T_H1 development, while T_H2 development is driven by IL-4, and to a lesser extent by IL-10 (402,403). In contrast to the helper function of $CD4^+$ T cells, $CD8^+$ (or cytotoxic) T cells specifically identify and destroy tumor cells and virally-infected cells. $CD8^+$ T cells regulate T_H cell development through their cytokine products, including IFN-γ and IL-4.

Host antitumor immunity is mediated by tumor-infiltrating $CD4^+$ and $CD8^+$ T cells (144,351). Both phenotypic subsets mediate cytotoxic activities *in situ*, and neither population demonstrates a predominance in terms of mediating tumor regression. Tumor infiltrates usually contain both $CD4^+$ and $CD8^+$ T cells, although these cells demonstrate defective signal transduction mechanisms, aberrant cytokine production, and decreased

cytotoxic activities (22,252,277). TBH T cell dysfunction is a result of suppressor activities of both tumor cells and tumor-induced *in situ* Mϕs. Tumor-induced Mϕ-mediated immune suppression is discussed later in the section entitled *Mechanisms of Tumor-induced Immune Dysfunction*.

Cytokines

Cytokines are soluble communication molecules that facilitate interaction between cell types through autocrine, paracrine, and endocrine mechanisms (30,31,229,285,309). Pleiotropic in nature, cytokines are produced by numerous cell types and have multiple targets, both within and outside the immune system. With the exception of TGF-β_1, which is secreted in a latent form and can travel systemically to distal target sites, most cytokines have short half-lives and mediate biological effects only within the surrounding microenvironment.

Cytokines are closely associated with tumor cell growth, angiogenesis, and metastasis. In the normal host, cytokines such as IL-1, IL-2, IL-4, IL-6, IL-12, GM-CSF, and tumor necrosis factor-β (TNF-β) significantly promote Mϕ and T-cell activation, whereas TGF-β_1 and IL-10 downregulate immune cell responses. Tumors, however, may promote their own growth through overproduction of cytokines that modulate immune responses, including TGF-β_1 (15,18), IL-10 (14,15,17,444), and PGE_2 (11,13,101,260). Our laboratory has identified several pathways (reviewed in 123) through which cancer cells and tumor-induced suppressor immune cells disrupt cellular responsiveness to, or alter production of, cytokines. These mechanisms are discussed in the following subsection.

Mechanisms of Tumor-induced Immune Dysfunction

Although Mϕs mediate tumor cytotoxicity, display tumor-associated antigens, and stimulate antitumor lymphocytes, cancer cells routinely circumvent these immune activities, rendering the host incapable of mounting a successful antitumor immune response. Evidence supporting a direct causal relationship between cancer and immune dysfunction suggests that the presence of neoplastic tissue leads to immunologic degeneration and adversely alters Mϕ function and phenotype. This section of the *Literature Review* focuses on the evidence that tumor-derived molecules redirect Mϕ activities to promote tumor development (123).

The observation that tumor growth activates immune defense mechanisms, yet neoplastic tissue eludes these mechanisms, defines the paradox of tumor immunology. Our studies (9,15,18,126,131,444,445,476) show a direct causal relationship between tumor presence and immune dysfunction, suggesting that the proximity of neoplastic tissue leads to immunologic degeneration. While the importance of Mϕs in tumor-induced immune dysfunction is controversial, recent research (122,269) suggests that the cellular basis for suppression includes the generation of immunoregulatory Mϕs that inhibit T-cell responses and that are tumoricidally-dysfunctional.

Various investigators have established that Mϕs can serve as both positive and negative mediators of the immune system (90-92,261,284,372) (Figure 1). As positive effector cells, Mϕs mediate direct antitumor cytotoxicity or presentation of tumor-associated antigens (TAAs), which are effective strategies for the eradication of tumors (46,52,137,169,189). In contrast, tumor-induced Mϕs demonstrate tumor cell growth-

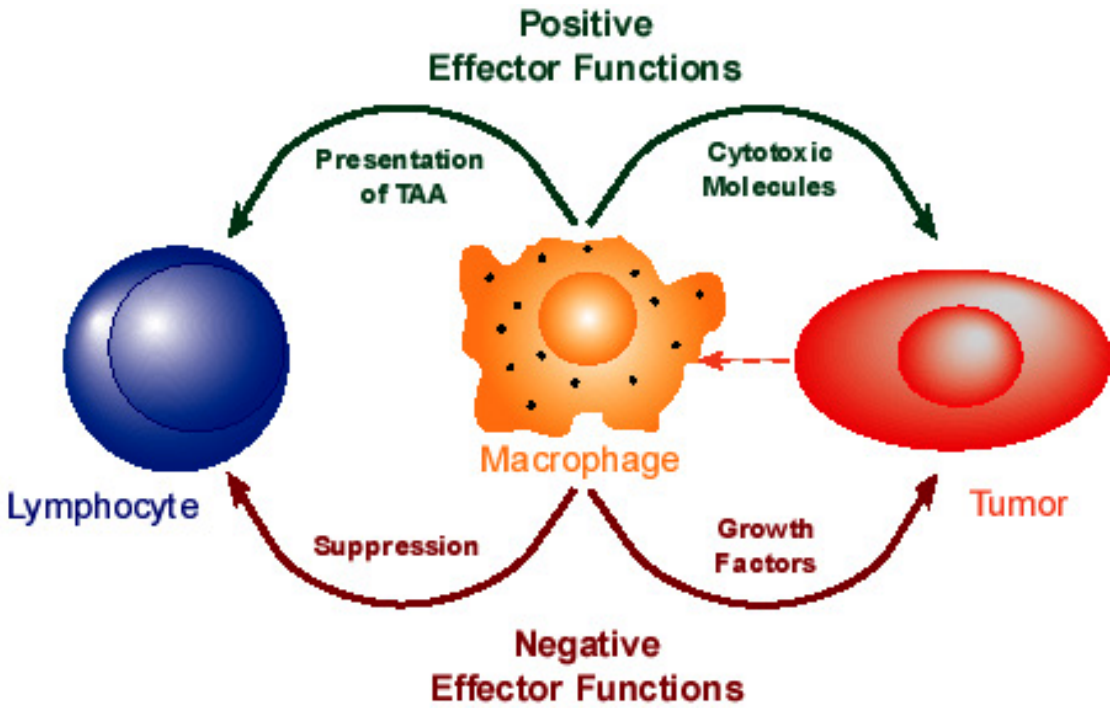


Figure 1. The duality of Mϕ function during tumor growth.
Depending upon their *in vivo* context, Mϕs can serve either as positive (green lines) or negative (red lines) effector functions during tumor growth. As positive effector cells, Mϕs can mediate direct antitumor cytotoxicity and presentation of TAA. In contrast, tumor-derived signals (dashed line) induce negative effector functions, including the production of tumor growth factors and suppression of lymphocyte responsiveness.

promoting abilities (128,269,333,401) that aid in tumor growth, and these Mϕs also directly suppress many T-cell (15,21,125) and NK cell (27) antitumor responses. This dualism begs the question: *what tips the balance toward one-or-the other Mϕ function during tumor assault?* An understanding of the regulatory mechanisms that control Mϕ functions during tumor growth is therefore critical in our attempts to shift the balance between health and disease.

Whether Mϕs serve a positive or a negative function, Mϕ-derived cytokines and proinflammatory substances are critical regulators of Mϕ activities. The discovery that tumors also produce pro- and anti-inflammatory cytokines and other regulatory molecules provides clues to how tumors may subvert Mϕ antitumor functions to favor tumor growth. To further our understanding of this rapidly-evolving field, we will focus on the evidence that suggests tumor-derived molecules can redirect Mϕ activities to promote tumor development.

Mϕs Promote Tumor Growth

Although Mϕs were first investigated for their role in antitumor immune responses, later studies revealed a dark side to TBH Mϕs; cancer actually induces Mϕ functions that *promote* tumor growth (128,129,269,401). These findings revealed the dual nature of Mϕs, which depending on their *in vivo* context can impart diametrically-opposed activities. For example, although they are vital effectors of innate immunity, Mϕs may contribute up to or more than half of a tumor's mass (401), and Mϕs are *required* for tumor survival (128,135,269,401). To ensure survival, tumors actively recruit monocytes by producing chemotactic agents -- including TGF-β_1 (442) and Mϕ chemotactic protein-1 (MCP-1) (270) -- which promote Mϕ infiltration (269,270).

The seemingly paradoxical activity by tumors to promote Mϕ infiltration actually benefits the tumor through a number of mechanisms. For example, Mϕs produce growth factors such as L-arginine-derived polyamines (284,307). L-Arginine is the substrate for Mϕ biosynthesis of the cytotoxic molecule NO through the activity of the inducible form of nitric oxide synthase (iNOS). However, some tumors markedly limit intratumoral Mϕ production of NO (106) by shunting L-arginine metabolism to favor the biosynthesis of ornithine (284), a precursor for polyamine tumor growth factors required for cell replication. Using this mechanism, tumors cause Mϕs to bypass NO synthesis, thus decreasing Mϕ cytotoxic activity while concurrently increasing Mϕ growth-promoting activity at the tumor site.

In addition to promoting tumor growth, Mϕs are potent promoters of angiogenesis (238,401). Through the production of growth factors and cytokines (including GM-CSF, TGF-β, IL-1, IL-6, and prostaglandins), activated Mϕs can potentially influence every phase of angiogenic progression (reviewed in 401). Furthermore, Mϕ presence correlates with tumor growth and metastasis (239); without Mϕs to stimulate the growth and development of tumor-nourishing blood vessels, solid tumors rapidly die (401). One Mϕ-derived molecule that promotes blood vessel growth in normal tissue but destroys blood vessels within solid tumor masses is TNF-α (435). However, TNF-α production by tumor-associated Mϕs (TAMs) is inhibited (36), suggesting that Mϕ-derived angiogenic molecules other than TNF-α affect blood vessel development in tumors (401).

Mϕs also may promote tumor growth through involvement in the metastatic process (433). Formation of aggregates between human breast carcinoma tumor cells and Mϕs, in which the Mϕs partly or completely surround the tumor cells, could impart to the tumor cells many of the properties necessary for tissue invasion, a normal Mϕ function.

Further, such an association would provide the tumor cells with Mϕ-derived growth factors and camouflaging from other cytotoxic immune effector cells.

Tumors Suppress Mϕ Antitumor Activity

Tumors use a variety of mechanisms to evade detection and destruction by the immune system, including the release of cytokines and effector molecules and alterations in Fas-Fas ligand (FasL) interactions normally associated with immune cells (83). Tumor cells release elevated levels of inhibitory cytokines that upset the normal balance of the immune system, leading to altered Mϕ function and immunosuppression (9,14,212,473). The resulting tumor-cell derived cytokine expression and cellular response leads to many changes in immune function, including suppression of host antitumor immune responses.

Tumor Cells Control Mϕ Production of Effector Molecules

Tumors produce substances that both downregulate (15,68,87,104,165,428) and upregulate (130,161,206,480) Mϕ cytotoxic and effector molecule production. These Mϕ-derived cytotoxic molecules include TNF-α (52,137,234,435), NO (259,287), H_2O_2 (226), reactive oxygen intermediates (ROI) (40,137,194), IL-1 (52), and specific proteases (248,307). Tumors can stimulate Mϕs to produce cytotoxic molecules through soluble or membrane-bound TAAs (137,183,206,480), extracellular matrix proteins (ECM) (191), or receptor-mediated binding of Fc portions of Ab (98,99,137) attached to tumor cells (208,449). Although Mϕ production of TNF-α, NO, and ROI imparts cytotoxic and suppressor activities, tumor growth also increases Mϕ production of the noncytotoxic suppressor molecules PGE_2, TGF-β_1, and IL-10. The dual *in vitro* cytotoxic and suppressor functions of TNF-α, NO, and ROI are misleading when

considering the *in vivo* functions of these molecules in tumor-burdened animals. The *in vivo* existence of well-established tumors suggests that production of TNF-α, NO, and ROI does not necessarily lead to tumoricidal activity, but may instead lead to suppression of antitumor lymphocytes.

Tumor-derived Molecules Differentially Regulate Mϕ Functions in Different Compartments

Despite an onslaught of infiltrating Mϕs into a tumor mass and the propensity of tumors to activate Mϕs, tumor cells escape Mϕ antitumor activities by subverting Mϕ functions to minimize antitumor effector function and favor tumor progression. Through the production of cytokines and effector molecules, tumors use the immune system's own communications network to undermine the host's antitumor responses. Depending on the Mϕ's resident tissue and proximity to the neoplasm, tumor-derived cytokines can impart differential effects in various *in vivo* compartments by either the priming of resting Mϕs or the suppression of activated Mϕ cytotoxic molecule production. For example, mRNA expression of several cytotoxic and suppressor molecules is increased in tumor-distal Mϕ populations, including splenic or peritoneal Mϕs (15). Current studies suggest that tumor-derived factors induce increased translocation of the multifunctional transcription factor NF-κB in splenic Mϕ populations, leading to expression of various cytotoxic effector molecules. Simultaneously in the same host, TAM cytotoxic molecule production is largely abrogated (106,394).

Tumors Circumvent TAM-mediated Cytotoxicity

By differentially controlling expression of Mϕ-derived molecules in different *in vivo* locations, tumors exploit the suppressor activity of cytotoxic effectors such TNF-α, NO, and ROI while escaping their cytotoxicity. Tumor-derived TGF-β, IL-4, IL-6, Mϕ colony-stimulating factor (M-CSF), and GM-CSF may activate tumor-peripheral resting Mϕs and downregulate *in situ* activated Mϕs. Circulating tumor-derived cytokines may prime resting tumor-distal Mϕs to produce cytotoxic and suppressor molecules such as TNF-α, NO, H_2O_2, and PGE_2 (10,11,13,15,140,296). During migration to the tumor site, tumor-primed Mϕs encounter increasing concentrations of activation signals such as TAAs and disrupted ECM proteins and become activated. However, as the activated Mϕs enter the tumor microenvironment, tumor-derived cytokines inhibit Mϕ production of cytotoxic molecules. Additionally, these activation molecules may convert the Mϕ-stimulatory action of tumor-derived cytokines to a Mϕ-deactivating action. Therefore, tumor growth downregulates cytotoxic molecule production by monocytes as they migrate to the tumor site. *In vitro*, tumor-derived molecules induce Mϕ production of noncytotoxic suppressor molecules such as PGE_2 (18), suggesting that tumor-proximal Mϕs could remain suppressive, but not cytotoxic.

Although *in vitro* investigations have established that Mϕs can kill tumor cells while leaving normal cells unharmed (2,137,223), Mϕ-derived cytotoxic molecules may not be effective during *in vivo* tumor growth (137,222,223,243,417). This finding suggests that tumors somehow interfere with the activity or production of cytotoxic molecules, leading to the reduction of Mϕ cytotoxic activity (52,87,104,284). Many tumor-derived molecules -- such as IL-4, IL-6, IL-10, Mϕ-deactivating factor (MDF), TGF-β, PGE_2, and M-CSF -- deactivate or suppress activated-Mϕ cytotoxic activity.

Although TBH Mϕs normally are primed for enhanced cytotoxicity, tumor supernatants suppress activated Mϕ tumoricidal activity (9,13-15,65,69,118,130,161,204,206,480), and tumor-derived cytokines and chemotactic molecules fail to stimulate Mϕ TNF-α and NO production (112,435). In our own tumor model, LPS activation of isolated TAMs failed to induce NO production *in vitro*. Furthermore, many tumors (especially spontaneously-arising neoplasms) have mechanisms to resist toxicity from one or more Mϕ-derived cytotoxic molecules (137,223,449). The simultaneous action of several Mϕ-derived molecules is therefore required for lysis of many spontaneous tumors, suggesting that the inhibition of one type of cytotoxic molecule may be sufficient for the tumor to escape lysis (449).

Tumors Activate Distal Mϕ Populations

After cell-cell contact, certain tumor cell membrane constituents directly induce Mϕ TNF-α (183,206) and NO (480) production, and circulating tumor-cell membrane debris may activate distal Mϕs. Tumor supernatants suppress Mϕ TNF-α and NO production (15) but also induce Mϕs to suppress lymphocyte proliferation, partly by stimulating PGE_2 production (18). Membrane preparations of these tumor cell cultures stimulate production of cytotoxic TNF-α and NO. The secretion of monocyte chemotactic substances (269,270), along with the generation of soluble ECM proteins by neoplasms (248), may activate tumor-distal Mϕs (191,248). Mϕ binding of these proteins to adhesion receptors may induce monocyte and Mϕ TNF-α secretion (191,452). Tumor growth may trigger tumor-distal Mϕ TNF-α production and other suppressor molecules by ECM protein binding to Mϕs. Some tumor-derived cytokines may enhance the effects of ECM proteins by increasing expression of tumor-distal Mϕ adhesion molecules that bind soluble ECM proteins (396).

Tumors Induce Mϕ Suppressor Activities

During tumor growth, Mϕs can adversely affect host antitumor responses and mediate lymphocyte suppression (48,153,288,334,415,426,475). In spite of considerable research into Mϕ-mediated immune suppression (122,123,269,372), controversy still exists about the nature and function of "suppressor" Mϕs in immune-dysfunctional cancer hosts. Although the complex interactions of immune cells initially obscured the true identity of the cell that was causing or mediating the suppressive phenomenon, direct evidence that Mϕs suppressed the immune reactivity during tumor growth was reported as early as 1974. Splenic adherent cells from murine sarcoma virus (MSV)-induced hosts suppressed mitogen- and viral antigen-induced blastogenesis of normal lymphocytes (218,219). Even more convincing evidence was provided by follow-up studies with MSV tumor-bearing athymic mice, where suppression was linked to cells with Mϕ-like characteristics (220), and the same group (166) showed Mϕ-like cells with suppressor functions in a rat tumor model. Others (120) showed that Mϕ-like suppressor cells from tumor-bearing mice were capable of blocking *in vitro* immunization against transplantation antigens. Soon thereafter, Mϕs were implicated as suppressor cells in a transplantable fibrosarcoma model (341), and rat splenic Mϕs were shown to inhibit lymphoproliferation in response to TAAs and lymphoma cell lines (322). Subsequently, evidence accumulated to suggest that more than one suppressor cell type could operate during tumor growth. Our investigations into the dualistic cellular (126,131) and molecular (132,133) mechanisms of tumor-induced suppression were immediately corroborated (340), and this Mϕ aspect is discussed throughout.

Tumor Growth Affects Immune Cell Regulation

Mϕs can up- or down-regulate lymphocyte (Figure 2) and other immune cell responses (430). For example, antitumor lymphocytes can be stimulated by TAA presentation, although the survival of tumors may depend on a decline in TAA presentation to antitumor T cells (169). At the same time, tumor-induced Mϕs suppress many T-cell (15,122,126,149,151,153,162,194,207,334,415,471,474) and NK cell (27,334) responses. Altered antigen presentation by TBH Mϕs may further subvert antitumor host responses through altered antigen presentation (451).

During tumor growth, the numbers of TBH splenic suppressor Mϕs, which are usually MHC class II^-, are increased (312,477). PGE_2 is the main inhibitory molecule produced by class II^- Mϕs during tumor growth (11,13,312), and these Mϕs probably are the main PGE_2-producing subpopulation of Mϕs (11,13). Increased numbers and the suppressor activity of TBH class II^- Mϕs downregulate TAA presentation by class II^+ Mϕs to T cells in the spleen (447,482) and perhaps in other compartments (169).

Tumor-derived Cytokines Inhibit T_H1-type Immune Responses

Cell-mediated responses tend to be optimal in the eradication of tumors (42,137). Tumor-derived IL-4, IL-10, TGF-β (83,273), and PGE_2 (41) suppress the necessary cell-mediated responses supported by T_H1-type cells during cancer, while supporting the largely ineffective humoral responses maintained by T_H2-type cells. Tumors may create environments that inhibit Mϕ cytotoxicity by either directly suppressing it or by inhibiting T_H1-type cells that induce Mϕ tumoricidal actions.

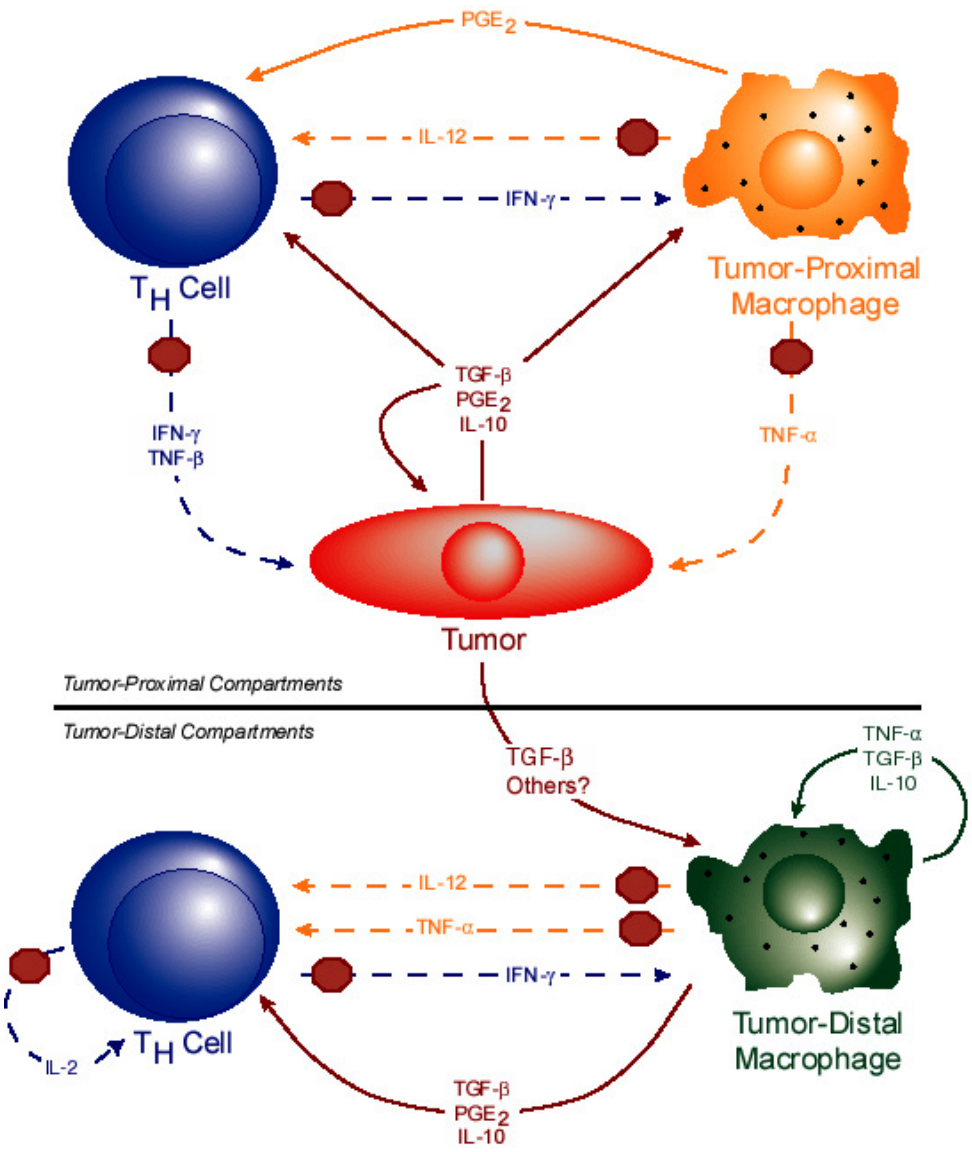


Figure 2. Macrophage activities vary with their proximity to the tumor. Depending on the Mϕ's resident tissue and proximity to the neoplasm, tumor-derived cytokines may either suppress (red stop sign) activated Mϕ cytotoxic molecule production (top) or prime resting Mϕs for enhanced release of proinflammatory mediators (bottom). Tumor-proximal Mϕs are rendered incapable of producing cytotoxic molecules (i.e., tumors are not killed) but still suppress lymphocyte function through PGE_2 production. Tumor-distal Mϕs, in contrast, produce cytotoxic molecules that fail to impart antitumor activity but further suppress lymphocyte function. Dashed lines represent suppressed or abrogated production of the indicated factor.

Tumor-derived IL-10 blocks tumor-induced Mϕ-mediated suppression of lymphocyte proliferation (14), unlike TGF-β_1 and other tumor-derived cytokines that subvert Mϕs into suppressor cells. Therefore, tumor-derived IL-10 blocks Mϕ cytotoxicity at the tumor site (15,289,342) and does not induce Mϕ suppressor activity. At the same time, IL-10 can directly suppress proliferation of alloantigen- or mitogen-stimulated T_H cells (14,289). Studies show that IL-10 preferentially downregulates T_H1-type cell activity by inhibiting Mϕ accessory (costimulatory) activity, which is necessary for T_H1-type cell activation (289). T_H1-type cells promote Mϕ activation by producing IFN-γ, whereas T_H2-type cells suppress Mϕ accessory and cytotoxic activities by producing IL-4 and IL-10 (342). IL-10 hinders Mϕ-mediated suppressor activity by decreasing Mϕ NO, TNF-α (14), and PGE_2 production (317) and may also reduce IFN-α and IL-12 production, which is unfavorable for the generation of T_H1-type cells (355). Collectively, these data suggest that Mϕ-derived IL-10 may play a significant role in controlling T-cell and Mϕ activities during tumor-induced immunosuppression.

Tumor-induced Mϕs Suppress Lymphoproliferation through Production of Proinflammatory and Cytotoxic Factors

Mϕs suppress lymphocyte and NK cell functions mainly by secreting the inhibitory molecules lipocortin (370), PGE_2 (11,13-15,260,418), NO (15,18,241,283,418), H_2O_2 (226), TGF-β (474), IL-10 (387), and the ROIs (278). Although these molecules directly restrain T-cell proliferation, only the inhibition of lipocortin, PGE_2, ROI, and NO production blocks Mϕ-mediated suppression in the TBH. The increased Mϕ output of autoinhibitory TGF-β_1 and IL-10 during tumor growth controls the production of other Mϕ suppressor molecules; PGE_2, NO, and TNF-α (14,18) and enhancing molecules such as GM-CSF (444) are triggered through autocrine

induction. For example, normal host Mϕs slightly curb T-cell proliferation to maintain homeostasis, and antibody-mediated neutralization of TGF-β_1 and IL-10 relieves normal host Mϕ-mediated suppression of T-cell proliferation (14,18). In contrast, TBH Mϕs remain strongly suppressive even after Ab-mediated TGF-β_1 and IL-10 neutralization because of their high output of other Mϕ-derived suppressor molecules.

Activated, but not resting, Mϕs synthesize TGF-β_1 and IL-10 (14,28), allowing these molecules to autocrinely control the production of other Mϕ-derived suppressor. When tumor growth dysregulates Mϕ suppressor molecule production, increased concentrations of TGF-β_1 and IL-10 cannot control Mϕ suppressor activities (14,18). Activated tumor-proximal Mϕ cytotoxic activity is downregulated by tumor- or Mϕ-derived TGF-β_1 and IL-10 (and perhaps PGE_2), but tumor-proximal Mϕs retain their suppressor activity. In addition, tumor growth enhances Mϕproduction of TGF-β_1, IL-10, and PGE_2 (14,18,474), cytokines that strongly inhibit T-lymphocyte proliferation (50,273,289,406). Although levels are boosted, the cytokines have pleiotropic effects during immunosuppression, and can downregulate tumor-induced Mϕ suppressor molecule production (14,18). Perhaps the immune system, through feedback mechanisms, is trying to return itself to basal levels in the presence of continual tumor antigen exposure. This effort is not surprising, because the control of harmful Mϕ reactions by immunosuppressive anti-inflammatory cytokines such as IL-10 and TGF-β_1 limits the pathologic consequences of immune responses, as evidenced by IL-10- and TGF-β_1-deficient mice that develop uncontrolled leukocyte activation and tissue injury (232,385).

In tumor-distal compartments, Mϕ-derived TNF-α actually suppresses T-cell proliferation by autocrinely inducing Mϕ PGE_2 and NO production (9,15). TNF-α

induces peritoneal Mϕs to mediate suppression but causes splenic Mϕs to upregulate T-cell proliferation (13). This TNF-α-induced regulation is hindered in the TBH by increased class II$^-$ splenic Mϕ PGE_2 production (13). In fact, tumor-induced splenic Mϕ-derived TNF-α autocrinely induces Mϕ suppression of autoreactivity through PGE_2 synthesis (12). TNF-α may play a suppressive role *in vivo* because peritoneal Mϕs are the strongest TNF-α producers (37,296,435), and TNF-α would always be produced in the microenvironment of these Mϕs. This suppressive role is confirmed by the administration of TNF-α into normal murine hosts, which causes Mϕ-mediated suppression of lymphocyte proliferation (167). The changes in splenic immune cell content after TNF-α injection into normal mice (167) are similar to the tumor-induced changes observed in a TBH (125,126). In our nonmetastatic fibrosarcoma model, Mϕ TNF-α production occurs even in tissues far-removed from tumor growth (such as by peritoneal and splenic Mϕ populations), an observation that supports the possible *in vivo* suppressor role of TNF-α.

Tumors Limit Mϕ Production of Immunostimulatory Molecules

As we have seen, many reports have documented a tumor-induced increase in Mϕ production of immunosuppressive factors. However, suppression during tumor growth also may arise from a *decrease* in production of a Mϕ-derived *stimulatory* molecule that affects other *in situ* immune cells. One candidate molecule could be IL-12, which promotes T-cell and lymphokine-activated killer cell proliferation and cytotoxicity (58) and favors the generation of T_H1-type cells (355). Although IL-12 does not directly affect tumor growth, IL-12 reduces the metastatic potential of many tumor types by promoting immune cell infiltration of tumors (58,59,63,408,424). Direct *in situ* administration of IL-12 increases the number of infiltrating tumoricidal Mϕs and T cells

(424), suggesting that IL-12 promotes antitumor responses and that tumor growth may compromise IL-12 production. Exogenous IL-12 restores TBH immunocompetence; therefore, tumor-induced Mϕ dysfunction may be manifested in reduced expression of IL-12. (Another candidate molecule may be the IL-12-like cytokine interleukin-18 (IL-18), a Mϕ-derived cytokine that induces IFN-γ production and promotes lymphocyte-mediated immune responses [279]. This possibility will be addressed in the *Conclusions*.)

Dysregulation of IL-12 occurs among both tumor-proximal (182) and -distal (298) immune cell populations. Both fibrosarcoma cells and suppressive Mϕs produce significant amounts of IL-10 and TGF-β_1 (15,444), which may directly or indirectly block IL-12 synthesis. For example, NO induces Mϕ IL-12 p40 gene expression (360). However, tumor-derived factors -- such as TGF-β_1 -- reduce Mϕ NO production by inhibiting iNOS activity (438), and NO production by *in situ* (106) and tumor-distal (151) Mϕ populations is compromised. In the absence of autocrine stimulatory signals, which are blocked by tumor-derived factors, Mϕs may be unable to produce IL-12. As a result of the IL-12 deficiency, $CD4^+$ T cells may be less responsive to activation cytokines. Furthermore, neutralization studies suggest that the inhibition of Mϕ IL-10, TGF-β_1, and NO production significantly reverses Mϕ suppressor activity against T cells (15). This decrease in Mϕ suppressor activity may permit increased expression of IL-12, which would select for the growth of important antitumor T_H1-type cells.

Tumors Induce Mϕ Hyporesponsiveness to Induction Signals

Because of the possibility that Mϕs proliferate in resident tissues, they may increase cell numbers and cytokine concentrations during immune challenge (38). Tumor

growth may partly decrease immunocompetence through Mϕ hyporesponsiveness to proliferation signals, including GM-CSF. An upregulatory molecule produced and used by Mϕs and T cells during immunogenic challenge (315), GM-CSF activities during cancer are not well defined. GM-CSF normally enhances Mϕ activation (52) and accessory function (213) and increases MHC class II molecule expression (139); however, these activities are inhibited by tumor growth (395,443,444,446). In the TBH, Mϕs produce lower levels of, and are hyporesponsive to, GM-CSF (444). GM-CSF increases normal host, but not TBH, splenic Mϕ MHC class II expression. Class II^- TBH Mϕs become more suppressive in the presence of GM-CSF; in contrast, GM-CSF partly reverses suppression mediated by class II^- normal host Mϕs. Because GM-CSF increases Mϕ accessory cell functions and class II molecule expression, tumor-induced decreases in GM-CSF production may account partly for decreased Mϕ accessory functions and reduced class II molecule expression. However, activation of splenic TBH class II^- Mϕs with a high concentration of LPS induces GM-CSF synthesis at concentrations comparable to normal host class II^- Mϕs (444). The class II^- Mϕ subpopulation is significant because it is a potent suppressor population during tumor growth (445,475). GM-CSF increases T-cell reactivity in the presence of normal host class II^- Mϕs, whereas it further suppresses T-cell reactivity in the presence of TBH class II^- Mϕs (446). Although GM-CSF synthesis is unaltered, TBH class II^- Mϕ synthesis of the inhibitory molecule PGE_2 is increased significantly (475,476). This activity, in turn, significantly suppresses T-cell function. Stimulation of TBH Mϕs with GM-CSF does not induce proliferation that is comparable to normal host Mϕ populations (444).

IL-10 contributes to the changes in tumor-induced Mϕ synthesis of, and responsiveness to, GM-CSF. Tumor growth heightens Mϕ susceptibility to IL-10-mediated inhibition of GM-CSF synthesis (444). Low IL-10 concentrations significantly

decrease the production of IL-1α, IL-6, IFN-γ, and TNF-α (51,384). IL-10 also decreases Mϕ synthesis of GM-CSF (444), although the suppression is not as great as that observed with TNF-α synthesis (51). TBH Mϕs are highly susceptible to inhibition of GM-CSF-induced proliferation by IL-10. Although IL-10 significantly inhibits the synthesis of Mϕ-derived cytokines and ROI (51,384), its importance as an inhibitor of Mϕ proliferation is unclear. Even though we did not screen for tumor-induced changes in other potential Mϕ growth factors, our data support the conclusion that tumor growth disrupts GM-CSF activities and that these changes compromise immune cell activities. Altered GM-CSF-mediated functions are compounded by tumor-induced increases to susceptibility to IL-10 suppression. The latter findings suggest IL-10 acts to deactivate suppressor activity during tumor growth. To our knowledge, no studies other than ours (14,444) have found that IL-10 may serve as an inhibitory signal of tumor-induced Mϕ-mediated immunosuppression. Further investigations are required to clarify the *in vivo* relevance of IL-10 during tumor-induced dysfunction.

Collectively, the data described in this subsection show that tumor-mediated regulation of Mϕ activities favor tumor growth. The evidence presented suggests that tumor-derived molecules deactivate tumor-proximal Mϕ populations while activating tumor-distal Mϕ populations. The current challenge is to determine how tumor-derived molecules specifically select Mϕ functions that benefit tumor growth. For example, studies should determine whether cancer cells produce cytokines that inhibit Mϕ cytotoxicity while inducing suppressor activity, and whether tumor growth induces Mϕ production of molecules that are both cytotoxic and suppressive, as reported here for NO and TNF-α. Cytokine cDNA transfection studies may be most useful in assessing the action of certain tumor-derived cytokines. Because different tumor cells expressing the same transfected cytokine elicit varying immune responses, different tumor types may

produce unique factors to manipulate immune responses. More attention should be given to the factors that tumors produce so an association can be made between the production of cytokines and particular tumor types. cDNA transfection of tumor-derived factors or gene-deletion in tumor cells could characterize the importance of various tumor molecules on Mϕ activities. The use of humanized antitumor-derived factor antibody may be effective in interfering with tumor signals that disrupt Mϕ cytotoxic and antigen-presenting activities. In fact, transfected phage-displayed anti-cytokine antibodies may provide a blocking tool. These kinds of studies will provide valuable insights into what changes occur in Mϕs during tumor growth. More importantly, these studies will suggest mechanisms for how tumor growth changes Mϕs. Without the underlying knowledge of how tumor growth alters Mϕ function, we can only describe a phenomenon. As we learn more about tumor growth, we can determine the molecular and cellular origins of tumor-induced changes in the immune system. Understanding this impressive array of biological effects will open a vast number of opportunities for therapeutic intervention, as discussed in the next subsection.

Immunotherapeutic Approaches to Cancer

The incidence, persistence, and mortality of cancer point to the need for novel therapeutic approaches. Although many drugs have been developed to inhibit tumor cell metabolism or proliferation (170,185), this discussion is restricted to therapeutic approaches that target immune cell dysfunction. Beginning with Coley's administration of bacterial exudates, four major lines of immunotherapy have been advanced (Figure 3) (328). Nonspecific immunotherapies strive for a general activation of the immune response in hopes of enhancing and supporting the host's antitumor immune responses.

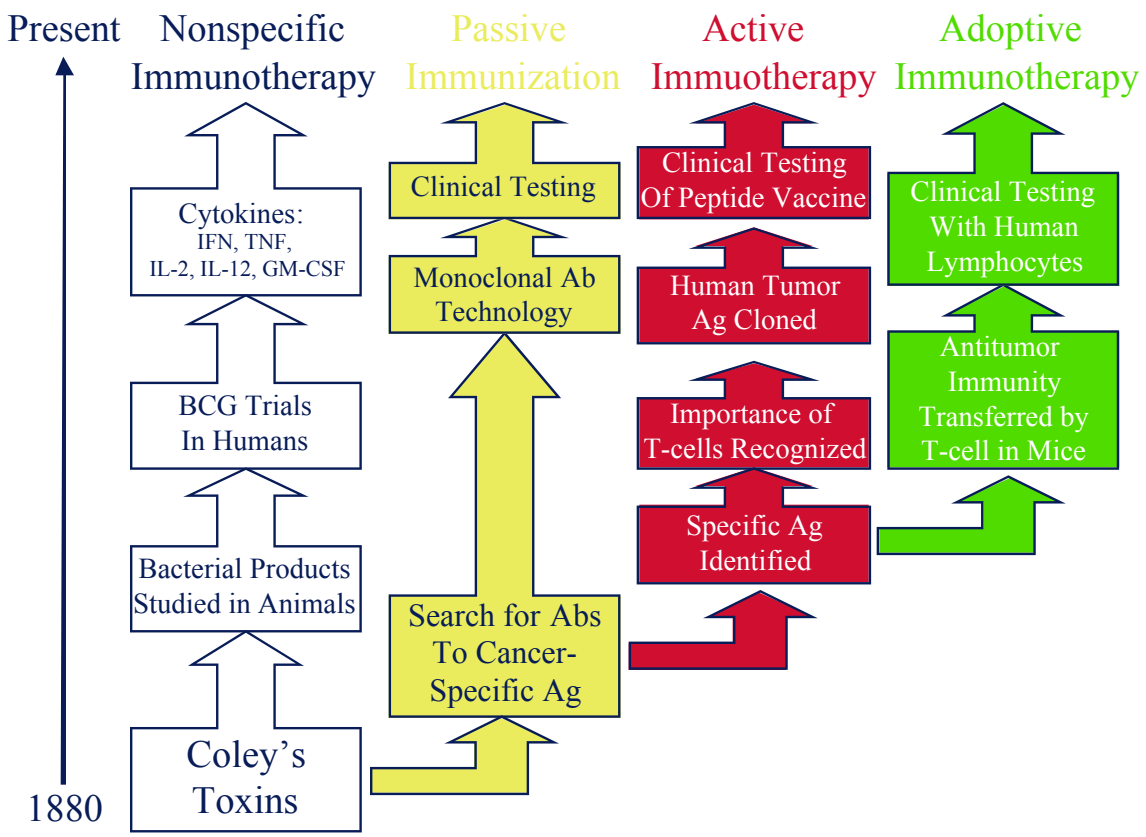


Figure 3. Origins of current approaches to tumor immunotherapy.

Examples include the administration of immunostimulatory cytokines, including IL-2 (75,357), IL-12 (407,481), GM-CSF (291,392), and TNF (138,184,318).

Although nonspecific immunotherapies have achieved limited success, they are not tumor-specific and may have severe side-effects. Additionally, cytokine treatment alone rarely achieves total tumor regression. Passive immunization, referred to as Ab-based therapies, became possible with the advent of monoclonal Ab production. Tumor-specific Abs are being studied as target-specific delivery vectors for antitumor effectors, including radioisotopes, chemotherapeutic drugs, antitumor enzymes, and proinflammatory molecules. Active immunization, or cancer vaccines, are intended to induce specific T-cell-mediated antitumor activity against malignant tissues; inactivated whole cancer cells, tumor-derived peptides, and neoplastic-specific heat shock proteins are under investigation as possible inducers of anticancer immune activity. The introduction of tumor-specific T-cells, or adoptive immunotherapy, is undergoing human clinical trials (328,356). Recently, efforts have turned towards identifying and attacking tumors at the molecular level, including anti-angiogenesis agents, and using combined approaches to enhance the efficacy of existing therapeutic agents. This dissertation will describe the results of combined administration of the chemotherapeutic agent paclitaxel and the immunotherapeutic cytokine IL-12, which are discussed in the following subsections.

Paclitaxel

Paclitaxel (sold under the trade name TAXOL™ by Bristol-Myers Squibb) is a plant-derived diterpenoid (215) and the first molecule containing a taxene ring demonstrated to possess antineoplastic activity (361). Paclitaxel is an organic product

isolated from the bark and leaves of the western yew tree, *Taxus brevifolia* (215), and useful precursors and analogs have been isolated from other *Taxus* species. Total laboratory synthesis was achieved (316), although large-scale pharmaceutical production of synthetic paclitaxel is not economically practical.

Biological Activities

Paclitaxel, which demonstrates antineoplastic activities that are 100-1000 times more cytotoxic than other cancer therapeutics (214), has significant promise against several human tumor types (115,142,178,246,262,361) and has become the chemotherapeutic agent of choice for first-line treatment of metastatic breast cancers (459). The success of therapies involving paclitaxel may be attributed to its multiple mechanisms of activity. Paclitaxel's primary mechanism of antineoplastic activity rests in its ability to irreversibly polymerize α/β tubulin, thereby disrupting cellular microtubule networks (97,373,374) and cell cycling (114,362). Additionally, paclitaxel suppresses protein synthesis (263), increases radiosensitivity (245,282), induces apoptosis (44), and causes cytolysis by TNF-α (457). Although paclitaxel's tumoristatic and tumoricidal activities have been extensively described, few studies (49,112,267) have evaluated its significance for immune cell activation.

While physically dissimilar from all described Mϕ activation agents, paclitaxel functionally resembles LPS (70,112,188,266,440). Because it functionally resembles LPS but lacks the toxicity associated with endotoxin, paclitaxel can be administered *in vivo* for cancer therapy. Mϕ activation occurs because both compounds interact with microtubule-associated protein kinases (112,113). Considerable data show that both paclitaxel and LPS can act synergistically with IFN-γ to trigger Mϕ transcription of IL-1

(8,49,321), TNF-α (49,110,457), and iNOS (267,296), and both paclitaxel and LPS induce tyrosine phosphorylation of microtubule-associated protein kinases (112). Paclitaxel-induced TNF-β and IL-1 mRNA transcription mirrors the mRNA levels induced by LPS (49). We (296) and others (5) have shown that paclitaxel stimulates Mϕ tumoricidal activity, and that paclitaxel-induced, Mϕ-derived cytolytic molecules can mediate significant tumor cell killing (457). Paclitaxel may promote antitumor activities by *in situ* tumor-associated Mϕs by inducing expression and production of cytolytic molecules (8,49,53,66,266).

Paclitaxel binds tubulin with high affinity and preferentially binds to microtubules (373,375), the ubiquitous cellular constituents involved in mitotic function, maintenance of cell shape, cellular motility, and intracellular transport (361). Paclitaxel induces microtubule polymerization by shifting the tubulin dimer-polymer equilibrium in favor of polymer assembly (316). The stoichiometry of binding approaches 1 mole of paclitaxel per 1 mole of polymerized tubulin dimer, and the coupling is reversible with a binding constant of 0.9 μmol/L (198). Clinically relevant concentrations of paclitaxel (corresponding to approximately 0.1-10.0 μM) lead to formation of disorganized arrays of microtubules, often in parallel bundles, during all phases of the cell cycle (361); during mitosis, abnormal spindle asters, which do not require centrioles for enucleation, form in excessive numbers (97,362). Paclitaxel inhibits the normal process of dynamic reorganization by microtubules and leads to arrest in the G_2 or M phases of the cell cycle (263).

Phase I clinical trials, which are small-scale trials designed to evaluate gradually escalating doses of a compound to determine toxicity and maximum dose, were initiated in the early 1980s to assess the safety of paclitaxel administration in human patients.

Because paclitaxel is exceptionally hydrophobic, special diluents are required for *in vivo* administration. Early studies were jeopardized by side effects, including acute severe hypersensitivity reactions; such reactions result from administration of the delivery vehicle (Cremophor EL) alone (361) (a more suitable delivery vehicle is not currently available; anti-allergic pre-medication, such as diphenhydramine, is used to alleviate this problem). Hematological toxicity, due to myelosuppression, consists mainly of neutropenia and is the major dose-limiting factor (57). Paclitaxel inhibits neutrophil locomotion and bacterial killing (352). Paclitaxel is mildly neurotoxic, inducing peripheral sensory neuropathy in a dose-dependent manner; symptoms appear 5-7 days post-treatment and are cumulative and progressive (249). Reversible total or near-total hair loss (alopecia) occurs in virtually all patients. In a limited number of patients, cardiac arrhythmia, gastrointestinal mucositis, and fatigue are reported (316).

Phase I trials led to initial recommendations for dosage and delivery by the National Cancer Institute. A safe administration of paclitaxel may be achieved by intravenous infusion (accompanied by anti-allergic medication) over a 24-hr period every 21 days; paclitaxel may be administered within a dose range of 170-250 mg/m^2 body surface area. Phase II trials, which assessed the efficacy of paclitaxel in small sample groups, have been conducted in patients with ovarian, breast, lung, colon, cervical, and prostate cancers, and renal cell carcinoma (reviewed in 316,361). The most promising results are observed in advanced ovarian cancer and breast cancer (195). Objective response is reported in 20%-50% of patients (121,274) who had received extensive previous chemotherapies. In randomized phase III studies in breast cancer patients, 22% and 29% of patients responded to 135 and 175 mg/m^2 body surface area of paclitaxel, respectively (303). Phase II studies in metastatic melanoma patients revealed an overall response rate of just 14%, and no objective response was noted in patients with renal cell

carcinoma (121). Phase I and phase II combination chemotherapeutic trials, using paclitaxel and the anticancer chemotherapeutics cisplatin or doxorubicin, are underway and initially appear to show enhanced success (316).

While clinical trials show that paclitaxel causes tumor regression *in vivo* (54,146,240,281,336), mechanisms of direct cytotoxicity against tumor cells are poorly characterized. Clinically relevant concentrations (0.1-10.0 μM) lead to formation of disorganized arrays of polymerized microtubules during all phases of the cell cycle, leading to cell-cycle arrest in the G_2 or M phase. Our recent studies evaluated fibrosarcoma cell sensitivity to paclitaxel. *In vitro*, physiologically-relevant concentrations of paclitaxel significantly suppress methylcholanthrene-induced nonmetastatic murine fibrosarcoma (Meth-KDE) cell proliferation and decrease Meth-KDE cell viability. These changes occur during both continuous exposure or 4-hr paclitaxel pretreatment. Data suggest that paclitaxel may promote tumor regression *in vivo* through direct cytostatic and cytotoxic activities (197,349,362) and increased tumor cell susceptibility to Mϕ-derived lytic molecules (457).

Immunotherapeutic Activities

The success of chemotherapies involving paclitaxel may be associated with changes in TBH Mϕ suppressor activities. Paclitaxel exhibits profound cell cycle-independent effects on murine Mϕ (53,70,203,266,267,296,299,301). Paclitaxel induces normal host Mϕ responses similar to those generated by bacterial LPS (266,440), including enhanced NO (367,296), TNF-α (110), IL-1β (49), and superoxide anion (331) production and induction of NF-κB expression (203). Through increased TNF-α and NO production, paclitaxel enhances *in vitro* tumor cell cytotoxicity (267). Paclitaxel induces

acute internalization of TNF-α receptors and retards induction of TNF-α protein expression (110,111), properties shared by LPS. Paclitaxel stimulates normal host Mϕ responses identical to those induced by LPS and induces expression in all LPS-inducible genes (110). Paclitaxel-induced activity is restricted to Mϕs from mice carrying the wild-type *Lps*^{n} gene, suggesting a genetic link (110). Like paclitaxel, LPS binds β-tubulin with high affinity, suggesting a mechanism by which paclitaxel and LPS could induce similar mechanisms (112). In activated normal host Mϕ, paclitaxel enhances production of NO through increased expression of iNOS (266,267,296) and significantly enhances Mϕ-mediated cytotoxicity toward tumor cells *in vitro* (440). The mediator molecules NO and TNF-α are discussed later in this review.

Because paclitaxel stimulates Mϕ activities (53,70,203,266,267) and tumor growth induces several phenotypic and functional changes among Mϕ populations (9,10,13-15,24-26), we studied whether murine fibrosarcoma growth alters paclitaxel-mediated regulation of Mϕ activities (296). In this dissertation, I will present evidence that paclitaxel pretreatment increases NO production by normal host Mϕs, and that tumor growth modulates paclitaxel-induced Mϕ activation (296,300,301). Because paclitaxel partly reverses tumor-induced immune suppression, and exogenous IL-12 restores TBH immunocompetence, we speculated that paclitaxel's mechanism of action may involve reconstitution of TBH Mϕ IL-12 expression.

A drawback of paclitaxel chemotherapy is its antimitotic effects on bone marrow precursors and lymphoid cells. In contrast to paclitaxel's cell cycle-independent effects on Mϕs, T-cells are susceptible to the same cytostatic activities that impart paclitaxel's antineoplastic functions. Paclitaxel treatment *in vitro* decreases T-cell proliferation and compromises responsiveness to IL-2 (56,86,301); patients experience transient

neutropenia during paclitaxel therapy, but this effect disappears when treatment ends (most patients receive antibiotics during paclitaxel treatments to reduce the risks of secondary infections) (347). The question remains whether transient paclitaxel treatment *in vivo*, such as lymphocyte populations would encounter during chemotherapeutic treatments, will impart similar inhibitory effects on lymphocytes. Furthermore, tumor growth significantly dysregulates T-cell functions, including responsiveness to alloantigen activation (124,444,475) and IFN-γ production (182,300), giving rise to the idea that paclitaxel treatment may exacerbate immune dysfunction in a cancer patient. These findings suggest that immunotherapies used to promote T-cell activation must precede paclitaxel therapies designed to promote direct tumor cell killing and Mϕ cytotoxic activities, as discussed in *Section IV*.

Nitric Oxide

Because of its importance in Mϕ-mediated antitumor activity, and because it is strongly induced by paclitaxel, the reactive intermediate NO merits discussion in this review. NO is a small, reactive nitrogen intermediate produced by a wide variety of mammalian cells (reviewed in 308). NO has the lowest molecular weight of any known mammalian cell-derived bioactive molecule. NO's high chemical reactivity leads to a short half-life and minimal specificity in its interactions. Surprisingly, this simple and fleeting molecule performs a diverse spectrum of roles, including critical functions in homeostasis, host defense, and regulation of cytokine expression (348).

Activated mammalian Mϕs secrete high levels of NO through the activity of iNOS. Murine iNOS efficiently catalyzes a five electron oxidation of the guanidino nitrogen of L-arginine, leading to the production of NO through what is referred to as the

L-arginine-NO pathway (reviewed in 286). This pathway requires a number of co-substrates (molecular oxygen and NADPH) and co-factors (FAD, FMN, heme, and tetrahydrobiopterin). In contrast to constitutive NOS (cNOS) expressed by neuronal or endothelial cells, Mϕ-derived iNOS is calcium-independent but calmodulin-dependent; murine iNOS protein coprecipitates with calmodulin, and the active iNOS unit is likely a tetramer (2 iNOS + 2 calmodulin). Murine (468) and human (75) iNOS genes have been cloned and the iNOS proteins extensively characterized (399).

Murine Mϕ iNOS expression and NO synthesis can be activated by IFN-γ or bacterial LPS (259). These compounds can act in a synergistic manner with other cytokines to induce or enhance NO production. IFN-γ in synergy with LPS, TNF-α, TNF-β, or IL-1 induces expression of iNOS; LPS also will act in a synergistic manner with IFN-α and IFN-β. Recent evidence indicates that TGF-β_1 may control Mϕ expression of NO (15,109). At the transcriptional level, NO expression is controlled by NF-κB (467).

NO-mediated cytotoxicity derives from the reactive nitrogen intermediate's actions on a number of cellular targets, including several enzyme complexes responsible for cellular respiration and DNA replication. The prominent action of Mϕ-derived NO on tumor cells is the disruption of DNA synthesis through inhibition of ribonucleotide reductase (143); NO also may be oxidized to a mutagen that deaminates DNA (413,458). Recent studies from our laboratory have shown that tumor growth dysregulates Mϕ NO production, thus influencing Mϕ cytotoxic and suppressor activities (15,296). Because the cytotoxic activities of Mϕ derive largely from iNOS-derived NO, characterization of differential TBH Mϕ iNOS regulation is vital to understanding tumor-induced Mϕ dysfunction.

Tumor Necrosis Factors

TNF-α, also know as cachectin (71), and TNF-β, also known as lymphotoxin (43), are closely-related proteins, demonstrating approximately 30% amino acid homology. These factors bind to the same cell surface receptors and produce a variety of similar but not identical effects, including the ability to directly kill certain tumor cells (thus, the derivation of the names *tumor necrosis factor* and *lymphotoxin*).

The TNFs (171,437) are generally classified as proinflammatory proteins due to their capacity to initiate, along with IL-1, a cascade of other cytokines and factors that comprise the immune system's response to infection; that same cascade also may be useful in responding to cancer. Such a reaction by the immune system is critical to the successful resolution of infection and metastatic diseases; however, in an uncontrolled manner, these responses can be harmful to the host. Administration of high levels of TNF-α or TNF-β can lead to virtually all of the symptoms of septic shock, and overproduction of TNF has been associated with cachexia (329), autoimmune disorders (343), and septicemia (441). TNFs are highly pleiotropic, due to their ubiquitous receptors, ability to activate multiple signal transduction pathways, and capacity to induce or suppress a vast number of genes (437).

TNF-α is produced by Mϕ, neutrophils, activated lymphocytes, NK cells, lymphokine activated killer (LAK) cells, astrocytes, smooth muscle cells, and some transformed cells (437). Mature human TNF-α is a 157 amino acid residue polypeptide (murine is 156 amino acids) with an apparent molecular mass of 17 kDa under denaturing conditions; biologically-active TNF-α is a non-covalently linked trimer (437).

The TNF-α polypeptide has a long precursor sequence with both hydrophilic and hydrophobic domains and apparently can occur as a membrane-bound protein from which the soluble 17 kDa factor is derived by cleavage of the extracellular domain. The membrane-anchored form of TNF exists on the surface of Mϕ and monocytes; it serves as a reservoir for release of soluble TNF-α, has cytotoxic activity, and may have intercellular communication functions (437).

Two distinct tumor necrosis factor receptor (TNFR) types, TNFR-I (Type B 55 kDa receptor) and TNFR-II (Type A 75 kDa receptor), have been characterized. At least one of these receptor classes has been found on virtually every cell type studied, and both receptor types show affinity for either TNF-α or TNF-β (437). Although immunologically-distinct, the receptors share similar motif structures in their extracellular domains. These receptor bind within the seams between the subunits of a TNF trimer; consequently, each TNF trimer is capable of binding three receptor subunits, and signaling occurs when two or three identical receptor subunits are brought into close proximity. There is evidence that the 55 kDa TNFR-I mediates lytic activity and the 75 kDa TNFR-II is responsible for generating proliferative responses and regulatory signals in lymphocytes (175). Both receptors may participate in a cooperative effort to induce apoptosis; using a ligand passing mechanism, the 75 kDa TNFR-II may transiently bind TNF, then pass it to the 55 kDa TNFR-I through which the signal for apoptosis is delivered (437).

Interleukin-12

IL-12 is a unique heterodimeric cytokine the imparts a variety of biological activities (comprehensively reviewed in 422). IL-12 was independently described by two

groups. In 1989, a group led by Trinchieri (225) described a molecule produced by Epstein-Barr Virus (EBV)-transformed human B lymphoblastoid lines; this molecule, called natural killer cell stimulatory factor (NKSF), was capable of augmenting NK cell cytotoxicity and IFN-γ production (225). A short time later, a second group under the direction of Gately (398) described a cytokine called cytotoxic lymphocyte maturation factor (CLMF) that synergized with IL-2 in the induction of cytotoxic T lymphocyte (CTL) activity and IFN-γ production by LAK cells. Characterization of NKSF and CLMF (177,376,462) lead to the conclusion that a single cytokine was responsible for the observed biologic activities, and the cytokine was subsequently termed IL-12 (462). Since its characterization, there have been significant data that indicate IL-12 represents the link between innate and acquired immune responses (78,461).

Since its characterization in 1989, there have been significant data that indicate IL-12 plays a key role in immune responses (424). In addition to regulating IFN-γ production and augmenting CTL and NK cell cytotoxicity, IL-12 promotes the development of T_H1-type populations of $CD4^+$ T helper cells (379,388,419). Because IL-12 is secreted by professional antigen-presenting cells (B cells, monocytes, and Mϕ) (96) and IL-12 receptors are expressed selectively on activated T-lymphocytes and NK cells (479), IL-12 represents the link between innate resistance and adaptive immunity (78,420,424). Furthermore, IL-12 may be well suited to drive the expansion and maturation of two prevalent antitumor effector cells localized within tumor lesions (T cells and NK cells) (154), making IL-12 an ideal candidate for use in immunotherapy (479) or in concert with bioactive chemotherapeutics such as paclitaxel. Synergism between IL-12 and IL-2 (100,237,332) further enhance the potential for a successful combination immunotherapy.

Structurally unique among interleukins, biologically-active IL-12 is a heterodimer of two dissimilar subunits. The active molecule consists of a 40-kDa (p40) and a 35-kDa (p35) subunit, linked by a disulfide bond (398,463). The p40 subunit has 306 amino acid residues with 10 cysteine residues and 4 potential N-linked glycosylation sites; the p35 subunit has 197 amino acid residues with 7 cysteine residues and 3 potential N-linked glycosylation sites (177). Mapping studies show that human p35 and p40 genes are located on different chromosomes; the p40 gene is on chromosome 5 (5q31-q33), and the p35 gene is on chromosome 3 (3p12-3q13.2) (386). The disulfide-linked heterodimeric structure is required for full biological activity of IL-12 (177,462). Neither the purified subunits nor mixtures of the uncomplexed subunits are active (82,339), although there is some indication that high concentrations of the subunits alone or combined in monomeric form may have limited biological activities or the capacity to competitively inhibit functional IL-12 (419).

The p40 and p35 subunits share no sequence homology (398). Several cytokines, including GM-CSF and IL-6, show relatedness to the p35 subunit (276); the p40 subunit does not relate to any known cytokine, but shares homology with the IL-6 receptor extracellular domain (160). This homology suggests that IL-12 arose from a covalently-linked cytokine/cytokine receptor unit (160,272). Murine IL-12 gene subunits have been cloned and expressed; the p40 subunit and p35 subunit share 70 percent and 60 percent identity with human counterparts (376). Recombinant murine IL-12 has complementary activities with human IL-12, both demonstrating biologic activity on human cells; human IL-12, however, is inactive on murine cells (376). A combination of murine p35 and human p40 was active, but human p35 combined with murine p40 was inactive on murine cells (376), suggesting that human p35 is unable to initiate signal transduction through the murine IL-12 receptor.

The IL-12 receptor is expressed on phytohemagglutin (PHA)-activated lymphoblasts (81) but not on resting peripheral blood mononuclear cells (PBMC) (105). A single high-affinity 110-kDa protein with 1000-9000 sites per lymphoblast (105), the receptor is expressed on mitogen- and IL-2-activated T cells and NK cells but not on resting or activated B cells (105). The disulfide-linked p40 subunit is likely responsible for receptor recognition, as a disulfide-linked p40 homodimer is capable of high-affinity binding to the IL-12 receptor and can inhibit native IL-12 activity (247). IL-12 receptor expression is regulated by several cytokines, including IL-12 itself. IL-12 alone upregulates the IL-12 receptor, and IL-4 acts synergistically with IL-12 to further upregulate receptor expression (105).

Sources of IL-12

Monocytes and Mϕs are the primary IL-12-producing cell types (96). Several EBV-transformed human B cell lines constitutively produce IL-12 (96). Stimulation with phorbol ester upregulates IL-12 expression; resting or phorbol ester-stimulated T cells, myeloid leukemic cell lines, and solid tumor cell lines do not produce measurable IL-12 (96). B cells produce a significant amount of free p40, but no free p35 (96,339). In contrast, many cell lines that do not produce measurable IL-12 heterodimer -- such as T cells and tumor cell lines -- constitutively express p35 mRNA (460), leading some to speculate on the possibility of additional biologic functions for the p40 and p35 (58). Human PBMC constitutively produce both free p40 and heterodimeric IL-12 at low levels (96). Incubation with LPS or bacteria increases both p40 and heterodimeric IL-12 production (96). Many cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IFN-γ, TNF-α, TNF-β, and GM-CSF, do not upregulate IL-12 production in PBMC (96). Severe combined immunodeficiency (SCID) mice splenocytes (which lack both T and B cells)

produce IL-12 when cultured with *Listeria monocytogenes* (425). *L. monocytogenes* stimulates IL-12 production by murine peritoneal Mϕ (199).

Biologic Activities

Like most cytokines, IL-12 activity is highly pleiotropic. IL-12 induces activation of cytolytic activity in cytotoxic NK and LAK cells (225). Recombinant IL-12 promotes cytolytic activity in both human (77,79,158,244,310,353,479) and murine (157) NK cell populations. Enhanced lytic activity occurs in both $CD56^{dim}$ and $CD56^{bright}$ NK cells (353). At low concentrations, IL-2 synergizes with IL-12 to enhance LAK cell induction, but IL-2 becomes inhibitory at high concentrations (244). Induction of cytolytic activity is independent of IL-2 (79,353), IFN-α, IFN-β, IFN-γ, or TNF-α (79). LAK activity derives primarily from $CD56^{+}$ NK cells and is inhibited by TNF-α (158,310), suggesting that endogenous induction of some cytokines, especially TNF-α, may be necessary for induction of LAK activity by IL-12.

IL-12 induces specific human CTL responses to weakly allogeneic tumor cells (158). IL-12 in combination with irradiated melanoma cells induces cytolytic $CD4^{+}$ T cells, which lyse allogeneic target cells. IL-2 induces a similar response; however, ten-fold less IL-12 (pM basis) is required to induce half-maximal response as compared to IL-2 (158). Administration of IL-12 into mice enhances their *in vivo* specific CTL responses (157). IL-12 fails to induce cytolytic activity by Mϕ *in vitro*, but cytolytic activity occurs in peritoneal Mϕ *in vivo* and is apparently IFN-γ-mediated (61). Proliferation of lymphocytes activated by phorbol esters, lectins, IL-2, allogeneic stimulators, or anti–CD3 antibodies is induced by IL-12, although IL-12 fails to induce

proliferation in resting PBMC (20,39,155,338). NK cell proliferation is induced, apparently by direct stimulation of $CD56^+$ NK cells (310).

Generally, IL-12 is a poor inducer of cytokine production, with the exception of IFN-γ (a highly pleiotropic cytokine). IL-12 appears to drive IFN-γ production by resting or activated human peripheral blood lymphocytes (PBL) (73,74,155,177,311,462,464), including T and NK cells, and murine IL-12 induces IFN-γ production by spleen cells (159,376,425). This IL-12-induced IFN-γ may, in turn, promote many additional activities in the immune response. IL-12 also induces small quantities of TNF-α from alloactivated (338) or resting (310) NK cells, and murine IL-10 production by spleen cells increases with IL-12 administration, possibly a regulatory response to IL-12 challenge.

Given that IL-12 is a powerful inducer of IFN-γ production and acts in synergy with IL-2, it is not surprising that IL-12 is a key regulatory cytokine in the induction of T_H1 cells (79,199,355,412,419). Hiseh *et al*. (199) have shown that IL-12 combined with antigen preferentially induce the development of T_H1 cells from naive T cells; conversely, IL-4 induces T_H2 cells in the same situation. In this model, IL-10 inhibits T_H1 development mediated by stimulated Mϕ but not that induced by exogenous IL-12, suggesting that IL-10 may regulate Mϕ production of IL-12 (423). IL-12 in combination with IL-2 can induce proliferation, production of IFN-γ, and promote cellular aggregation in murine T_H1 but not T_H2 cell lines (163). Additional biological activities include upregulation of IL-2 (and other) cytokine cell surface receptors (CD25) on NK and T cells (310,353,464) and synergism with colony stimulating factors and stem cell factor to promote proliferation of primitive hematopoietic stem cells (205).

Antitumor Activities

IL-12 shows significant promise as an anticancer immunotherapeutic agent (reviewed in 32,154). IL-12 reduces the metastatic potential of many tumor types by promoting immune cell infiltration of tumors (59,63,64), and direct *in situ* administration of IL-12 increases the number of infiltrating Mϕs and T cells that can mediate cytotoxicity against tumor cells (231,408). Several *in vivo* murine studies involving IL-12 immunotherapies have shown significant antitumor successes (64,295,319), and gene therapy approaches for delivery of IL-12 as an antitumor immunotherapeutic are currently in progress (350,407-409).

IL-12 has potent antimetastatic and antitumor activity in several established murine tumor models, including metastasis models (such as pulmonary metastases) and subcutaneous (s.c.) tumor models (such as renal cell carcinoma) (63). In models using established s.c. tumors, antitumor activity is achieved with systemic or intratumor injections (306), and intraperitoneal (i.p.) administration of IL-12 leads to a dose-dependent inhibition of tumor growth. IL-12 treatment results in substantial growth inhibition, prolonged survival, and in limited cases total regression (63). With several tumors, IL-12 systemic treatment was effective even when initiated up to two weeks after tumor transfer (63,483). However, in many more cases, IL-12 induced only temporary tumor regression, and tumor growth resumed when treatment was interrupted (63,483). The depletion of $CD8^+$ T cells or both CD8+ and $CD4^+$ T cells was required to suppress the antitumor effect of IL-12; depletion of $CD4^+$ T cells or NK cells had no significant effect (306). The antitumor effect of IL-12 was in part mediated by IFN-γ (61,306,410).

To achieve effective antitumor activity, high doses of recombinant IL-12 are generally required over an extended period of time (422). The half-life of injected recombinant IL-12 is approximately 3.5 hr in mice (465), 18 hr in rhesus monkeys (304), and 5 to 10 hr in humans (251). A common murine protocol would involve five daily injections of IL-12, followed by a 2-day rest; this approach did not induce pulmonary edema, which is observed in mice treated without interruption. Most mouse strains, including BALB/c, tolerate repeated injections of up to 1 μg/day (93). Toxicities observed in normal mice treated with IL-12 included hematological alterations, hepatotoxicity, and skeletal muscle degeneration (60,127). Splenomegaly, largely caused by extramedullar hematopoiesis of erythroid, myeloid, and megakaryocytic lineages, occurred rapidly and in all treated mice (60,127).

Phase I clinical trials of IL-12 were initiated in 1994 in cancer patients; the maximum tolerated dose with reversible side effects was 500 ng/kg (251). Complications included fever, anemia, neutropenia, and fatigue. Biological effects included elevations in serum IFN-γ, which peaked in the first 3 to 4 days and then decreasing despite continuing dosing (251). Phase II trials, which evaluated the efficacy of IL-12 in small non-competitive studies, were initiated in 17 patients with daily 500-ng/kg injections but were halted after two treatment-related deaths, attributed to IL-12-induced multiple organ failure (346). Toxicity was attributed to the fact that phase I trials used daily treatments initiated after 2 weeks a single predose; in phase II trials, daily injections were started immediately, without pre-dosing (346). It was subsequently shown that a single predose (one week before initiation of daily treatment) effectively protected the animals from IL-12 toxicity and allowed for treatment with higher doses (422).

Immunotherapeutic Activities

Because cytokine therapies may induce toxicities (357), clinical chemistry and hematologic parameters of IL-12 administration were evaluated in a murine model (156). At therapeutically effective doses (1μg/injection, 5 times per week), few toxicities are induced by IL-12 treatment. The major IL-12-induced side-effects were leucopenia, increased hepatic transaminases, and moderate anemia in some animals (58).

Because exogenous IL-12 partially restores TBH immunocompetence (408), tumor-induced Mϕ dysfunction may involve decreased expression of IL-12. Mϕs are the predominant source of IL-12 *in vivo* (96), and Mϕ IL-12 production is autocrinely regulated by IL-10 (423,424). TGF-β_1 also regulates IL-12-induced cytotoxic activities (389,423). Thus, dysregulation of Mϕ IL-12 production may occur among both proximal and distal immune cell populations in the tumor-bearing animal if tumor growth development enhances IL-10 or TGF-β_1 production. Both fibrosarcoma cells and suppressor Mϕs produce significant amounts of IL-10 (15,444), which may block IL-12 synthesis by tumor-distal Mϕs. Previous data (16) suggest that $CD4^+$ T cell responses to activation cytokines are poor; and these low responses may reflect an IL-12 deficiency preventing early T-cell activation or commitment. Neutralization studies suggest that inhibition of Mϕ IL-10, TGF-β_1 and NO production significantly reverses suppressor Mϕ activity against T cells (444); suggesting the possibility that blocking these downregulatory signals reconstitutes Mϕ IL-12 expression, which in turn may stimulate T-cell responses. Immune cell populations at the tumor site may demonstrate hyporesponsiveness to IL-12 because of the high concentrations of tumor-derived TGF-β_1 (15,18). These possibilities may explain why exogenous IL-12 shows significant success as an *in vivo* immunotherapeutic agent.

Combined Therapies

Although anticancer therapeutic approaches are constantly evolving and rapidly improving, the persistence of neoplastic disease is evidence of the shortcomings of traditional chemotherapeutic regimens. Clinically successful cancer treatments must disrupt tumor cell activities and reverse tumor-induced immune cell dysfunction. Although tumor-induced Mϕ and T cell dysfunction occurs across a wide spectrum of interlacing signal pathways and molecular targets, many of these defects may be correctable through reconstitution of immunostimulatory cytokines. A promising approach involves the use of cytokines to induce nonspecific antitumor immune responses as a supplement of existing chemotherapies. Specifically, cytokines can be used to correct immune system imbalances, enhancing the effectiveness of existing treatments and the patient's ability to mount an effective immunologic response.

Because the anticancer agent paclitaxel induces not only tumor cell regression but also severe T-cell cytostasis, overall antitumor efficacy may be enhanced through the use of T cell-stimulatory cytokines to reverse paclitaxel-induced immune suppression. Based on our *in vitro* results (300,301), we speculated that the combinatorial administration of the anticancer chemotherapeutic paclitaxel and the immune activating cytokine IL-12 may represent a potent antitumor combination, offering several advantages over traditional antitumor approaches. Paclitaxel will disrupt tumor cell proliferation, viability, autocrine growth factor regulation, and suppressor molecule synthesis – rendering the tumor even more sensitive to immunologic destruction. Simultaneously, paclitaxel will activate Mϕ-mediate cytotoxic activity, and IL-12 will reverse both paclitaxel- and tumor-induced suppression of T-cell populations. This approach will be discussed in Section IV.

SECTION II

MACROPHAGE CYTOTOXIC RESPONSES TO PACLITAXEL

This Section contains three chapters that describe the response of normal host and, in a more clinically-relevant scenario, TBH Mϕs to paclitaxel treatment. Chapter I describes the LPS-mimetic activity of paclitaxel, including its capacity to induced cytotoxic effector molecule production by both normal host and TBH Mϕs; this chapter also presents the first experimental evidence that tumor growth differentially regulates Mϕ responsiveness to paclitaxel treatment *in vitro*. Chapter II characterizes paclitaxel-induced Mϕ production of the important proinflammatory and signaling cytokine IL-1β. Finally, the data in Chapter III demonstrate paclitaxel's capacity to induce Mϕ-mediated tumor cell cytotoxicity, even in the face of tumor-derived immunomodulatory cytokines.

CHAPTER I

PACLITAXEL, A MICROTUBULE-STABILIZING ANTINEOPLASTIC AGENT, DIFFERENTIALLY REGULATES NORMAL AND TBH Mϕ NO PRODUCTION

ABSTRACT

Paclitaxel, a potent antitumor chemotherapeutic, mimics the Mϕ-activating functions of LPS; like LPS, paclitaxel promotes *in vitro* cytotoxic antitumor activities by normal host Mϕs. Because tumor growth induces functional changes among Mϕ populations, we determined whether fibrosarcoma growth (Meth-KDE) modified Mϕ responsiveness to the activating agent paclitaxel. Tumors induce tumor-distal Mϕ populations to become immune suppressor cells, partially through overproduction of the cytotoxic and proinflammatory molecules NO and TNF-α. Beneficial to the TBH when released by tumor-proximal Mϕs, NO and TNF-α suppress lymphoproliferation and fail to impart antitumor activity when expressed in distal compartments. We report that paclitaxel differentially regulated normal host and TBH Mϕ production of the immunosuppressive molecule NO by tumor-distal Mϕ populations. In response to IFN-γ-priming and paclitaxel triggering, TBH Mϕs increase their production of NO as compared to resting Mϕs; however, unlike normal host Mϕs, paclitaxel-induced TBH Mϕ NO production was significantly suboptimal. Modulation of TBH Mϕ NO production in tumor-distal compartments may lead to a partial reversal of tumor-induced suppression and alleviate tumor-induced suppression of T-cell proliferative responses, yet promote sufficient NO production by tumor-associated Mϕs to mediate enhanced cytotoxicity. This novel mechanism of immunotherapeutic activity may partly explain the clinical success of paclitaxel as an anticancer agent.

INTRODUCTION

The anticancer agent paclitaxel is a taxane diterpenoid extracted from the bark and leaves of the western yew *Taxus brevifolia* (450). Paclitaxel has demonstrated significant efficacy as an antitumor chemotherapeutic in human clinical trials (364) with 20-50% objective response (274); the most notable benefits have been observed in patients with advanced ovarian and breast cancers (195). The therapeutic efficacy of paclitaxel is realized through multiple cytostatic and cytotoxic mechanisms of antineoplastic action, including suppression of protein synthesis (262), enhancement of tumor-cell radiosensitivity (245), induction of apoptotic cell death (44), and enhancement of TNF-α-mediated cytolysis (457). Paclitaxel's primary antitumor mechanism derives from its unique action on microtubules (97,373). Unlike other antimicrotubule factors, paclitaxel promotes assembly of microtubule bundles by preventing the depolymerization of α/β tubulin polymers into free tubulin (264); the resultant stabilization of the cellular microtubule array forestalls dynamic polymerization/depolymerization of the microtubule network, preventing progression of the cell cycle through metaphase (362). Paclitaxel thus disrupts cell cycling (114), leading to neoplastic cell death and inhibition of tumor progression (148).

In addition to its well-characterized chemotherapeutic activities, paclitaxel exhibits profound cell cycle-independent effects on murine normal host Mϕs (53,203). Although structurally divergent, paclitaxel functionally resembles bacterial LPS (70,112,188,266,440); paclitaxel stimulates *in vitro* normal host Mϕ responses similar to those induced by LPS and activates expression of all characterized LPS-inducible genes (110). Paclitaxel-induced activity is restricted to Mϕs from mice carrying the wild-type *Lps*^{n} gene, supporting the concept of a genetic link (110). Like paclitaxel, LPS binds β-

tubulin with high affinity, suggesting a mechanism by which paclitaxel and LPS could induce similar signal mechanisms (113).

The most notable activity of paclitaxel on Mϕ populations, from a clinical standpoint, may be its ability to activate normal host Mϕs for enhanced *in vitro* tumor cell cytotoxicity (267), mimicking LPS (2). Considerable data show that both paclitaxel and LPS can act synergistically with IFN-γ, a cytokine which primes Mϕs for enhanced production of cytotoxic and regulatory molecules (294), to trigger transcription of IL-1 (49), TNF-α (457), and iNOS (267). Paclitaxel enhances IFN-γ-primed normal host Mϕ cytotoxicity toward tumor cells *in vitro* (440), primarily through nitric oxide (NO), produced through increased expression of iNOS (266,267). The effects of paclitaxel on tumor-induced Mϕs are unknown.

Mϕ activities during cancer are predominantly nonspecific, including cytotoxicity of tumor cells (137) and suppression of T-cell (152) and NK cell responses (472). Both cytotoxic and immunosuppressive Mϕ functions are partially mediated through production of reactive nitrogen and oxygen intermediates, including NO. Although these Mϕ-derived molecules exert significant tumor cell cytotoxicity, they also directly suppress immune cell function (192). Reactive moieties such as NO may be beneficial to the TBH when expressed by tumor-infiltrating (*in situ*) Mϕs, yet the suppressor activities of these molecules may prevent necessary antitumor responses by tumor-distal lymphocyte populations. Classic studies show that tumor-infiltrating Mϕs have decreased cytotoxic activity as compared to inflammatory Mϕs (270), and Mϕ production of NO and TNF-α is downregulated at the tumor site (36,284). However, we have shown that murine fibrosarcoma growth induces tumor-distal Mϕ populations to produce elevated levels of NO and TNF-α (15). Through overexpression of downregulatory

cytokines, including TGF-β_1 and IL-10, tumors prime Mϕs for enhanced production of proinflammatory and cytotoxic molecules upon LPS exposure (14,456). Mϕ production of cytotoxic and proinflammatory molecules therefore varies, depending upon the Mϕ's resident *in vivo* compartment. Thus, to achieve an optimal Mϕ-mediated antitumor response, Mϕ cytotoxic molecule production must be differentially regulated in tumor-proximal and tumor-distal compartments.

Because paclitaxel stimulates Mϕ activities and tumor growth induces multiple phenotypic and functional changes among Mϕ populations (9,10,15,26), we studied whether murine fibrosarcoma growth alters paclitaxel-mediated regulation of Mϕ activities in tumor-distal compartments. Although paclitaxel shares the activation properties of LPS in normal host Mϕs, priming by tumors may change the Mϕ cytotoxic response to activating drugs such as paclitaxel; no studies have addressed this possibility. Here we report that paclitaxel, like LPS, enhanced IFN-γ-induced NO production by normal host murine Mϕs. Unlike LPS, paclitaxel modulated NO production by TBH Mϕs, limiting NO to significantly suboptimal levels. Thus, paclitaxel may activate Mϕs for direct antitumor cytotoxicity while controlling Mϕ production of proinflammatory and cytotoxic molecules in tumor-distal compartments during paclitaxel therapy. Through its capacity to activate Mϕs, paclitaxel may impart immunotherapeutic activities not previously described, and the success of paclitaxel as anticancer agent may derive partially from these undefined functions.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice (Harlan Sprague-Dawley, Madison, WI) were used. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma, designated Meth-KDE, was used as described (126). The Meth-KDE fibrosarcoma induces significant systemic immunosuppression through the production of the soluble suppressor cytokines IL-10, TGF-β_1, and PGE_2, as described (15). The use of a nonmetastatic tumor facilitates the study of tumor-induced distal Mϕ populations. Tumors were induced by intramuscular (i.m.) injection of 4 x 10^5 transplanted Meth-KDE cells and palpable tumors developed within 10 days. TBH BALB/c mice were used 21 days after tumor induction because tumor-induced Mϕ suppressor activity is maximal at this time, without cachexia or necrosis. All procedures were approved by the Virginia Tech Animal Care Committee.

Media

Mϕs were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma Cell Culture, St. Louis, MO). L-M cells (clone CCL 1.2, American Type Culture Collection [ATCC], Rockville, MD) for TNF-α bioassay were maintained in M199 medium containing 0.5% peptone (Sigma). All media contained 50 mg/L gentamicin sulfate (Tri-Bio Laboratories, State College, PA), 25 mM sodium bicarbonate ($NaHCO_3$), and 25 mM HEPES buffer (Sigma). RPMI-1640 medium was endotoxin-free (<10 pg/ml) as assessed by the *Limulus* Amebocyte Lysate assay (Sigma).

Reagents

Reagents included recombinant murine TNF-α (specific activity 1.2 x 10^7 U/ml; endotoxin content <10 pg/ml) and recombinant murine IFN-γ (specific activity 1.0 x 10^5 U/ml; endotoxin content <10 pg/ml) (generous gifts from Genentech, Inc., San Francisco, CA); rat anti-mouse TNF-α monoclonal antibody (mAb, clone G281-2626) and rat IgG_1 (clone R3-34) isotype control mAb (Pharmingen, Inc., San Diego, CA); and LPS (*Escherichia coli* serotype 026:B6). Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% dimethyl sulfoxide (DMSO, Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution and stored at -80°C. Paclitaxel was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%.

Mϕ Culture and Collection

Normal host and TBH BALB/c splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL), washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium by scraping with a rubber policeman. BALB/c peritoneal Mϕs were collected from 4-day thioglycollate-elicited peritoneal exudate cells by plating pooled cells for 2 h in the same manner. The final Mϕ preparations contained cells that were >95% viable and >96% esterase positive, and flow cytometric analysis with M1/70 and F4/80 mAb (ATCC) showed them to be >80% Mac-1^+ and F4/80^+, respectively.

Mϕ Nitrite Production

Either four x 10^5 peritoneal or 8 x 10^5 splenic normal host or TBH Mϕs (optimal cell numbers) were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY). Each well contained a total volume of 200 μL serum-free RPMI-1640 medium with various concentrations of IFN-γ. LPS, paclitaxel, TNF-α, anti-TNF-α mAb, or isotype control mAb were added to Mϕ cultures at the start of incubation. Supernatants for nitrite assessment were collected at 48 h for peritoneal Mϕs and 72 h for splenic Mϕs (optimal culture times) following centrifugation (400 x g, 5 min). Paclitaxel doses up to 50 μM did not decrease Mϕ viability because the MTT assay (292) verified >95% Mϕ viability throughout the culture periods (not shown).

Because secreted NO quickly reacts with oxygen to yield a stable nitrite endproduct (287), nitrite levels in culture supernatants were measured using the Griess reagent as described elsewhere (172). Briefly, 100 μL Mϕ supernatants were added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3P0_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm (MR 600 microplate absorbance reader; Dynatech Laboratories, Alexandria, VA). A sodium nitrite (Sigma) standard curve was used to calculate nitrite content in supernatants. Nitrite was not detected in RPMI-1640 medium alone or in Mϕ cultures lacking IFN-γ (not shown). DMSO in culture did not affect Mϕ nitrite production (not shown).

Western Analysis of iNOS

For Western analysis of iNOS expression, cells were lysed in 50 mM Tris-Cl pH 7.6 containing 10 μg/ml of leupeptin and aprotinin (Sigma) and 300 mM NaCl. Membranes were pelleted by centrifugation (12,000 x *g*), and protein determined using the Sigma Lowry protein assay kit. Protein (15 μg) was denatured by boiling in β-mercaptoethanol (2-ME, 5%) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% ProtoGel (National Diagnostics, Atlanta, GA) vertical gel, transferred to nitrocellulose, and blocked using 5% non-fat milk. iNOS was labeled using polyclonal rabbit anti-mouse primary Ab (Transduction Laboratories, Lexington, KY) at 1:5000 dilution and (HRP)-conjugated goat-anti-rabbit IgG secondary Ab (Transduction Laboratories) at 1:2000 dilution. Bound iNOS was detected with Luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

TNF-α Quantification

TNF-α production was induced by culturing Mϕs as described for NO production. The MTT assay verified >95% Mϕ viability throughout the culture period. Culture supernatants were collected at 8 to 12 h (optimal incubation time) following centrifugation (400 x g, 5 min) and stored at -80°C. DMSO in culture did not affect Mϕ TNF-α production (not shown).

TNF-α activity was determined by the L-M cell bioassay as described elsewhere (228). Briefly, L-M fibroblasts were added to 96-well flat-bottom tissue culture plates at 4 x 10^4 cells per well in serum-free RPMI-1640. TNF-α-containing supernatants were added, incubated for 4 h at 37°C and 5% CO_2, and supernatants removed. Cytolysis was

measured by staining viable L-M fibroblasts with crystal violet (20% v/v in methanol) and reading absorbance at 570 nm on a MR 600 microplate absorbance reader. One unit of TNF-α was defined as the amount of TNF-α that caused 50% lysis of 4 x 10^4 L-M fibroblasts.

TNF-α protein concentration in Mϕ-derived supernatants, prepared as previously described, was measured by a TNF-α-specific ELISA (Quantikine™ M, R&D Systems, Minneapolis, MN) following the manufacturer's provided protocol. The minimum detection limit was 5.1 pg/ml.

Statistics and Calculations

Cells from six to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for nitrite in the Griess reagent tests and for TNF-α protein concentration using the cytokine-specific ELISA. All experiments were repeated at least three times; data are means ± standard error of the mean (SEM) of triplicate independent determinations. 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

Differential NO Production by Paclitaxel and IFN-γ-primed Normal Host and TBH Mϕs

Paclitaxel's capacity to mimic LPS as a second signal for the induction of NO production by IFN-γ-primed normal host Mϕs has been extensively characterized (267). To determine whether tumor-induced functional and phenotypic changes in *distal* Mϕ populations alter responsiveness to the activating agents, we examined the ability of LPS and paclitaxel to induce Mϕ NO production. First, normal host or TBH peritoneal Mϕs (4×10^5 cells) were primed with 100 U/ml IFN-γ, cultured with different doses of LPS, and nitrite production measured (Figure 4). LPS doses were chosen to achieve optimal NO production; in a dose-dependent manner, LPS enhanced IFN-γ-primed NO production by both normal host and TBH peritoneal Mϕs. LPS doses as low as 0.1 μg/ml significantly enhanced Mϕ NO output ($p<0.05$), and 10 μg/ml induced maximal NO, defining "optimal" Mϕ NO production capacities in this model system (LPS doses greater than 10 μg/ml, up to 1000 μg/ml, failed to further increase Mϕ NO production, not shown). Tumor-induced Mϕs were significantly ($p < 0.01$) more responsive to LPS triggering, producing 66 percent more NO than similarly activated normal host Mϕs at optimal dose levels (10 μg/ml LPS). In the absence of IFN-γ, NO production was not detected (not shown). IFN-γ doses from 1 to 100 U/ml enhanced LPS-triggered NO production in a dose-dependent manner by both normal host and TBH Mϕs (leading to similar NO production profiles), although higher IFN-γ doses ($>10^3$ U/ml) did not further increase Mϕ NO production (not shown).

To determine whether tumor-induced Mϕs respond differently than normal host Mϕs to paclitaxel, normal host or TBH peritoneal Mϕs (4×10^5 cells) were primed with

100 U/ml IFN-γ, cultured with different doses of paclitaxel, and nitrite production measured (Figure 5). Because paclitaxel has already entered human antitumor trials as a chemotherapeutic antitumor agent, paclitaxel doses (ranging from 0 to 50 μM) were chosen to parallel systemic levels achieved during human chemotherapy regimens. All doses of paclitaxel enhanced IFN-γ-primed NO production by normal host Mϕs, leading to NO production profiles similar to LPS triggering (Figure 4). In contrast, increasing doses of paclitaxel decreased NO production by IFN-γ-primed TBH peritoneal Mϕs in a dose-dependent manner; at all doses, paclitaxel-induced TBH Mϕ NO production was significantly ($p < 0.01$) lower than optimal NO levels (LPS-triggered IFN-γ-primed TBH Mϕs). Decreased NO production did not result from loss of Mϕ viability as >95% of cells remained viable throughout the culture period (not shown); paclitaxel doses greater than 50 μM became toxic to the Mϕs and were not used.

To determine whether paclitaxel induced expression of iNOS, the enzyme that mediates Mϕ NO production, TBH peritoneal Mϕs (4×10^5) were cultured for 24 h in 1 ml of serum-free medium without or with IFN-γ (10 U/ml) and paclitaxel (10 μM). Western analysis using polyclonal anti-iNOS Ab revealed that paclitaxel could induce iNOS, although maximal paclitaxel-mediated expression was lower than that achieved with LPS (Figure 6). Densitometric analysis of iNOS expression (Figure 6 B) reveals a correlation between the level of iNOS protein and the production of nitrogen intermediates as measured by chemical methods (see Figure 5).

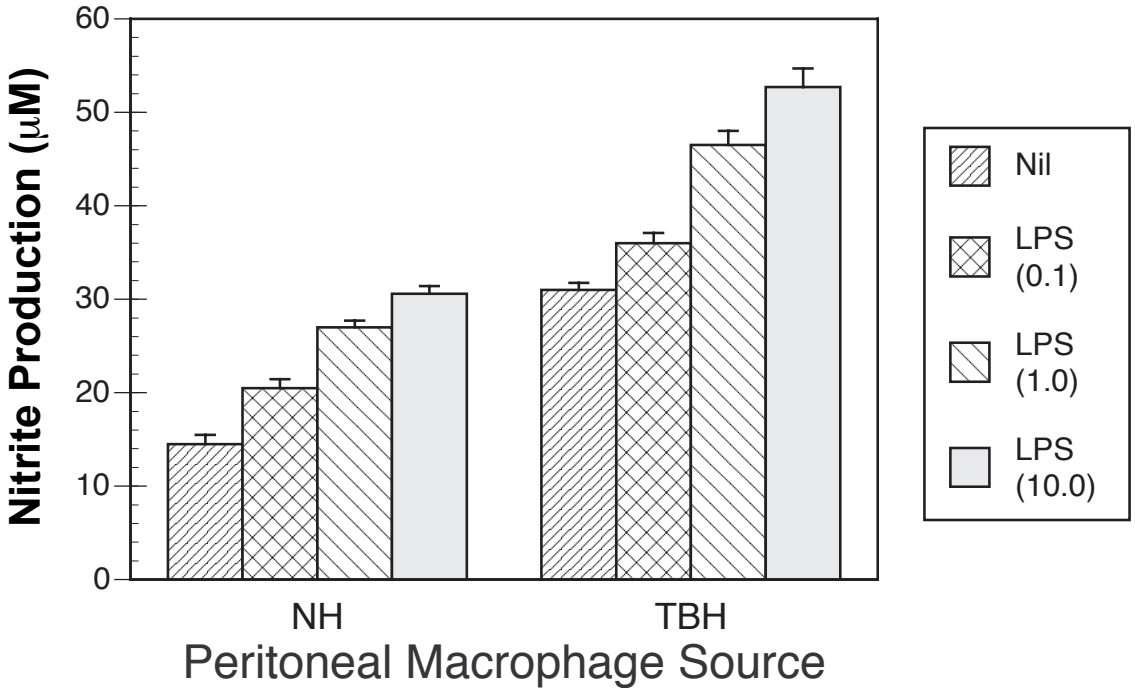


Figure 4. LPS induces peritoneal Mϕ NO production during tumor growth. Four x10^5 thioglycollate-elicited BALB/c normal host or TBH peritoneal Mϕ were cultured in 200 μl of serum-free medium and primed with 100 U/ml of IFN-γ. Various doses of LPS were added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment using the Griess reagent test. Nitrite production was not detected in cultures lacking IFN-γ. Data are means ± SEM of triplicate independent determinations from one of four experiments that had similar results.

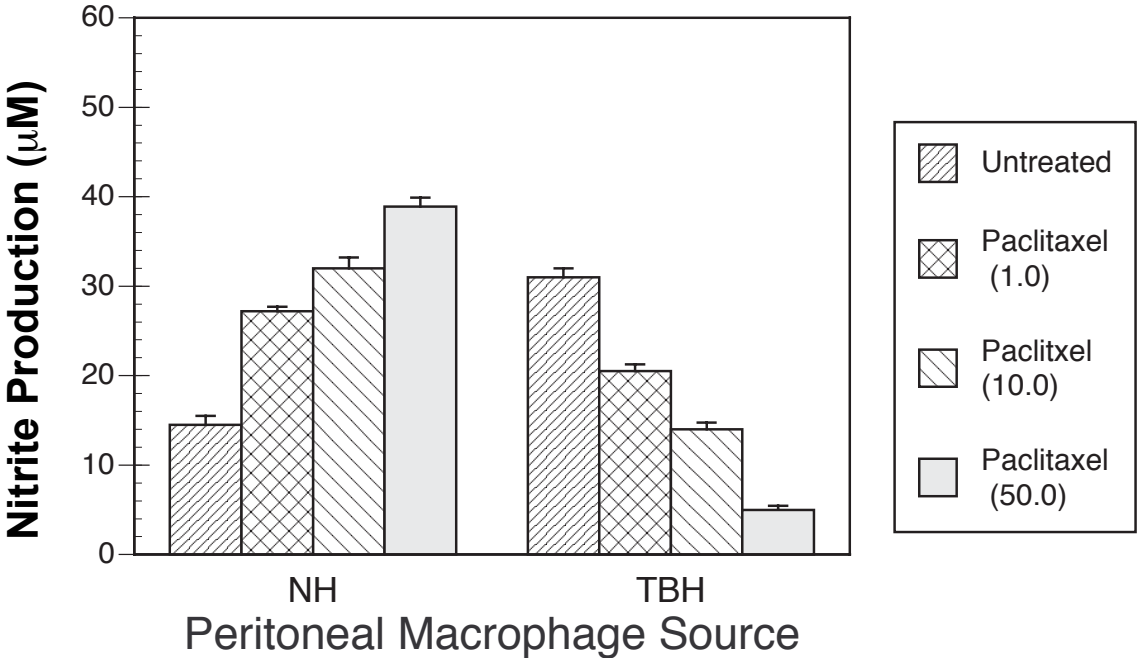


Figure 5. Paclitaxel differentially regulate peritoneal Mϕ NO production during tumor growth. Four x10^5 thioglycollate-elicited BALB/c normal host or TBH peritoneal Mϕ were cultured in 200 μl of serum-free medium and primed with 100 U/ml of IFN-γ. Various doses of paclitaxel were added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment using the Griess reagent test. Nitrite production was not detected in cultures lacking IFN-γ. Data are means ± SEM of triplicate independent determinations from one of four experiments that had similar results.

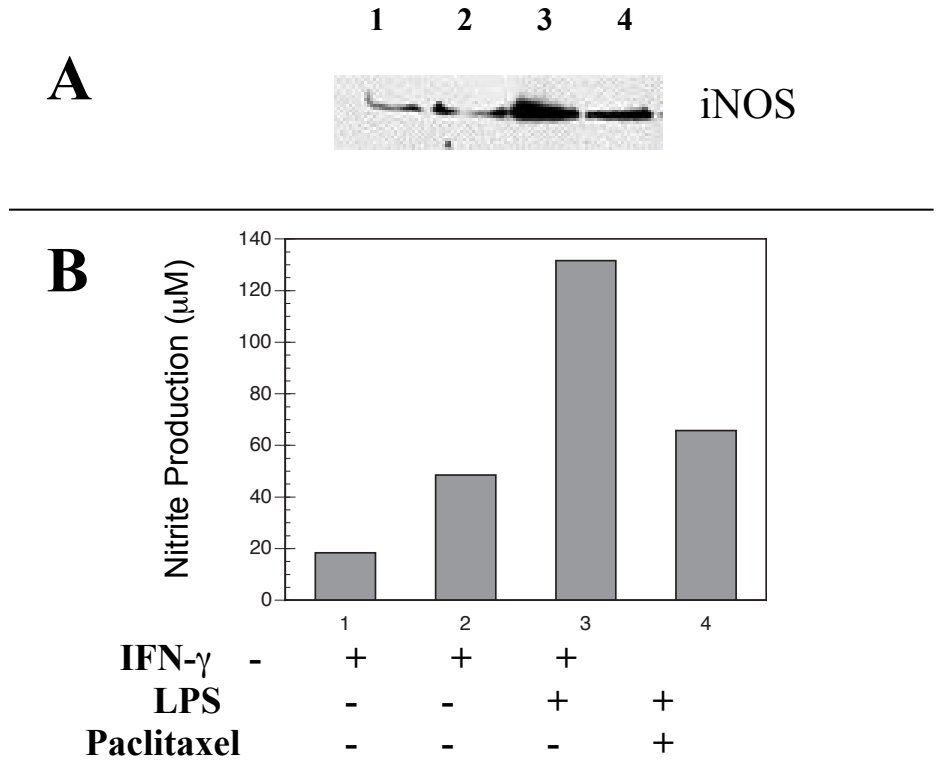


Figure 6. Paclitaxel induces TBH Mϕ iNOS expression and NO production. TBH peritoneal Mϕs (4×10^5) were cultured for 24 h in 1 ml of serum-free medium without or with IFN-γ (10 U/ml) and paclitaxel (10 μM). **(A)** Western analysis of iNOS using polyclonal anti-iNOS Ab. Data are representative results from one of three similar experiments. **(B)** Nitrite content of cell-free supernatants were determined by Griess reaction assay. NH Mϕ cultures produced similar profiles (not shown). Data are averages and SEM of triplicate independent determinations from one of five similar experiments.

To determine further whether tumor-distal Mϕs are differentially responsive to paclitaxel, normal host and TBH splenic Mϕs were primed with 100 U/ml IFN-γ and cultured (Figures 7-8). Because splenic Mϕs produce far less NO than their peritoneal counterparts (4 x 10^5 IFN-γ-primed normal host or TBH splenic Mϕs failed to produce measurable NO at 48 h), optimal cell numbers (8 x 10^5) and culture periods (72 h) for splenic Mϕs were determined and used. Normal host splenic Mϕs, whether stimulated with LPS or paclitaxel, produced only low levels of NO (Figure 7); changes in NO production were beyond the limit of detection in our assay system. In contrast, TBH Mϕs produced appreciable levels of NO, and LPS significantly enhanced NO production by splenic TBH Mϕs (Figure 8). As for peritoneal Mϕs, paclitaxel differentially regulated IFN-γ-stimulated normal host and TBH splenic Mϕ NO production, decreasing TBH Mϕ NO production in a dose-dependent manner. Further results for splenic Mϕ NO production followed the profiles established by peritoneal Mϕ cultures and are not shown.

Induction of Mϕ TNF-α Production by LPS and Paclitaxel

Both paclitaxel and LPS have been shown to induce normal host Mϕ production of TNF-α (49). To determine whether fibrosarcoma growth alters Mϕ TNF-α production, normal host or TBH peritoneal Mϕs were cultured with the maximum doses of paclitaxel (50 μM) or LPS (10.0 μg/ml) either without or with IFN-γ (100 U/ml). Supernatants were collected and assessed for TNF-α activity (Table 1) and protein concentration (Table 2). Paclitaxel alone induced similar amounts of TNF-α production by normal host and TBH peritoneal Mϕs, and paclitaxel synergized with IFN-γ to induce significantly more TNF-α production by TBH peritoneal Mϕs as compared to similarly

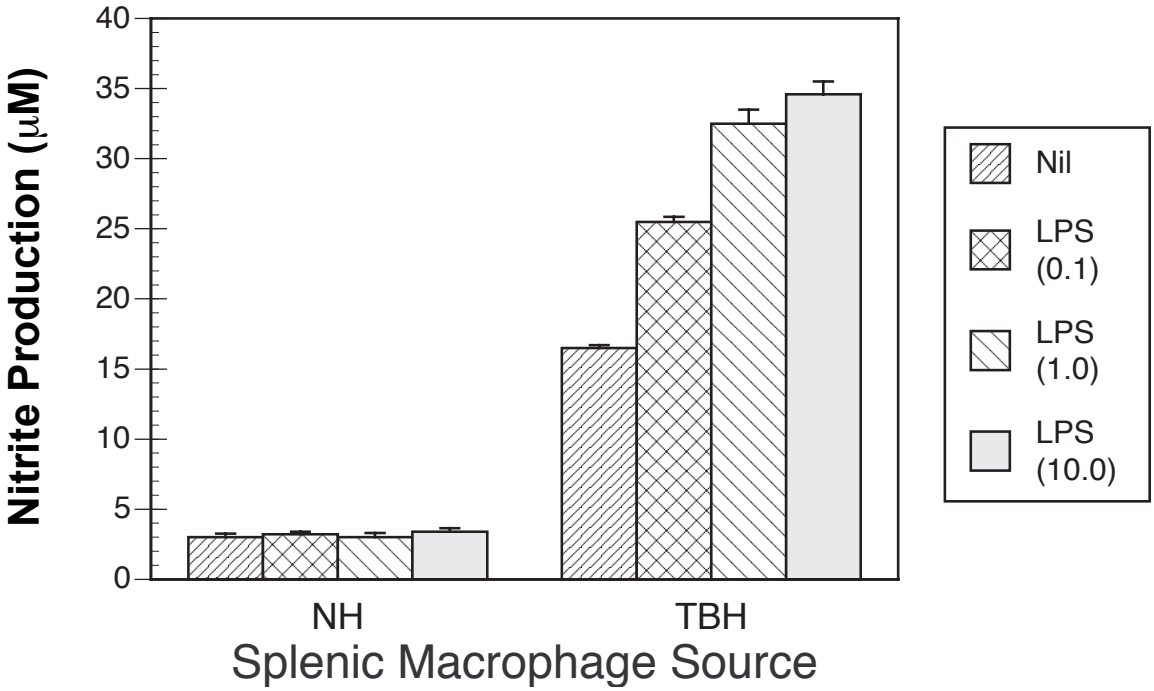


Figure 7. LPS induces splenic Mϕ NO production during tumor growth. Eight x 10^5 BALB/c normal host or TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 100 U/ml of IFN-γ. Twice as many splenic as peritoneal Mϕ (8 x 10^5 and 4 x 10^5, respectively) were cultured for a longer period (72 h versus 48 h) to achieve comparable levels of NO production. Three doses of LPS were added at the start of incubation and supernatants were collected after 72 h (optimal incubation time) for nitrite assessment. Nitrite production was not detected in cultures lacking IFN-γ. Data are means ± SEM of triplicate independent determinations from one of five experiments that had similar results.

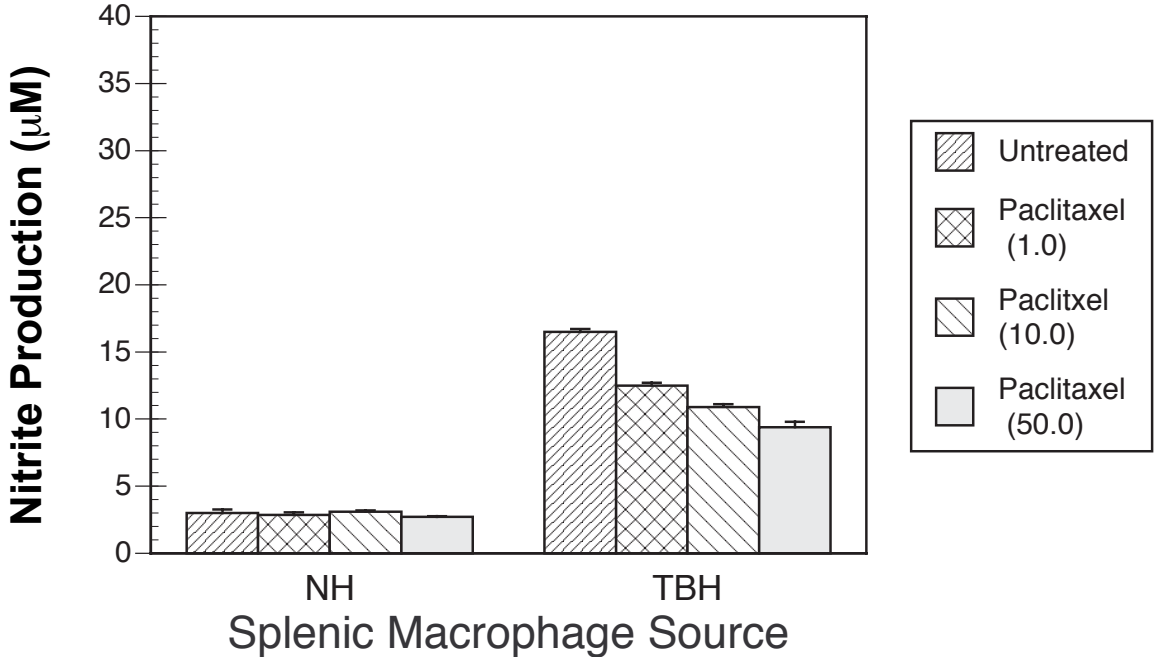


Figure 8. Paclitaxel differentially regulates splenic Mϕ NO production during tumor growth. Eight x 10^5 BALB/c normal host or TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 100 U/ml of IFN-γ. Twice as many splenic as peritoneal Mϕ (8 x 10^5 and 4 x 10^5, respectively) were cultured for a longer period (72 h versus 48 h) to achieve comparable levels of NO production. Three doses of paclitaxel were added at the start of incubation and supernatants were collected after 72 h (optimal incubation time) for nitrite assessment. Nitrite production was not detected in cultures lacking IFN-γ. Data are means ± SEM of triplicate independent determinations from one of five experiments that had similar results.

Table 1. Paclitaxel Induces Mϕ Expression of TNF-α Protein Activity.

TNF-α Protein Activity (U/ml)[1]

Mϕ Culture Additives[2]					
Mϕ Type	**Nil**	**LPS**	**LPS + IFN-γ**	**Paclitaxel**	**Paclitaxel + IFN-γ**
NH PEM	35 ± 4	704 ± 30	1002 ± 104	110 ± 16	190 ± 40
TBH PEM	80 ± 8	1650 ± 151	1700 ± 123	105 ± 10	300 ± 40
NH Splenic	10 ± 2	357 ± 12	475 ± 72	60 ± 20	75 ± 10
TBH Splenic	41 ± 3	1100 ± 98	1308 ± 80	70 ± 10	260 ± 45

[1]One unit of TNF-α is the amount of TNF-α that causes 50% cell lysis of 4 x 10^4 L-M cells. Each result is the mean and SEM of duplicate cultures.

[2]Either 4 x 10^5 peritoneal exudate (PEM) or 8 x 10^5 splenic Mϕ from BALB/c NH and TBH were cultured in 200 μL of medium with paclitaxel (50 μM), paclitaxel and IFN-γ (100 U/ml), LPS (10.0 μg/ml), or LPS and IFN-γ (100 U/ml). Peritoneal Mϕ supernatants were collected after 8 h and splenic Mϕ supernatants were collected after 12 h (optimal incubation times) to measure TNF-α production using the L-M cell bioassay. Lower doses of stimulants induced lowered amounts of TNF-α following typical titration profiles (not shown).

Table 2. Paclitaxel Induces Expression Mϕ Production TNF-α.

TNF-α Protein Concentration (pg/ml)[3]

Mϕ Culture Additives					
Mϕ Type	**Nil**	**LPS**	**LPS + IFN-γ**	**Paclitaxel**	**Paclitaxel + IFN-γ**
NH PEM	ND[4]	105	312	82	174
TBH PEM	29	234	1049	89	363
NH Splenic	ND	658	759	ND	39
TBH Splenic	10	1910	2208	14	62

[3]Macrophage were cultured as indicated for the bioassay and supernatants collected. TNF-α production (pg/ml) was determined using a specific ELISA and calculated using a TNF-α standard curve. Each result is the mean of duplicate cultures, in all cases, SEM was <10%.

[4]ND: Not detected; TNF-α was below the level of detection of the assay system.

treated normal host Mϕs. LPS, however, was 5- to 15-fold more potent than was paclitaxel either without or with IFN-γ in stimulating normal host and TBH peritoneal and splenic Mϕ TNF-α production.

Paclitaxel Induces TBH Mϕ NO Production through TNF-α

TNF-α has been shown to upregulate normal host Mϕ NO production (173,308). To determine whether tumor-induced Mϕs respond differently than normal host Mϕs to TNF-α, exogenous TNF-α was added to IFN-γ-primed normal host and TBH peritoneal Mϕs either without or with LPS (Figure 9). Supernatants were collected at optimal culture time (48 h) and NO production was measured. TNF-α (100 U/ml) increased IFN-γ-primed normal host ($p < 0.10$) and TBH ($p < 0.05$) peritoneal Mϕ NO production, although TNF-α did not significantly enhance NO production by unprimed Mϕs (not shown). Doses of TNF-α as low as 10 U/ml enhanced IFN-γ-primed Mϕ NO production, although higher doses of TNF-α (1000 U/ml) did not further increase NO production (not shown), suggesting that high concentrations of TNF-α are inhibitory. The highest doses of TNF-α did not kill Mϕs because Mϕ viability, assessed by the MTT assay, remained at greater than 95% (not shown).

To determine whether Mϕ-derived TNF-α imparts autocrine control over LPS or paclitaxel regulation of IFN-γ-activated Mϕ NO production, normal host and TBH peritoneal Mϕs were cultured with IFN-γ (100 U/ml) either without or with anti-TNF-α mAb (2 μg/ml) or rat-anti-mouse IgG_1 isotype control (Figure 10-11). LPS (1 μg/ml, Figure 10) or paclitaxel (10 μM, Figure 11) were added to these cultures and nitrite production assessed at optimal culture times. TNF-α neutralization slightly but

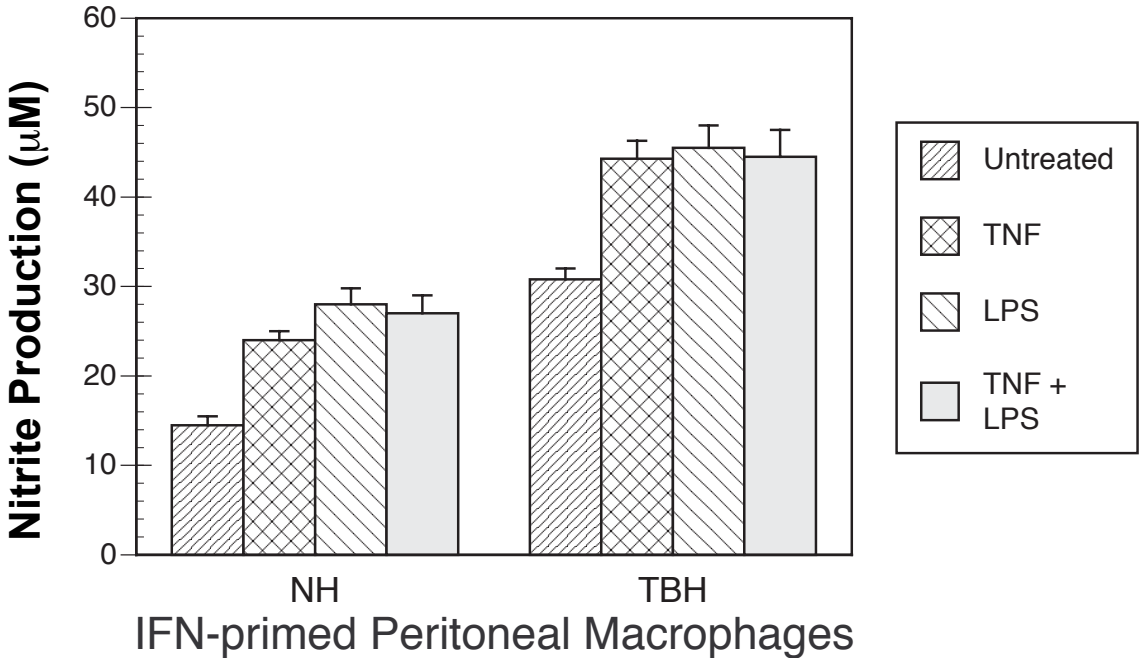


Figure 9. TNF-α induced IFN-γ-primed peritoneal Mϕ NO production during tumor growth. Either 4 x 10^5 normal host or TBH peritoneal Mϕ were cultured in 200 μl of serum-free medium with IFN-γ (100 U/ml) alone or with LPS (1.0 μg/ml). TNF-α (100 U/ml) was added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment. TNF-α doses as low as 10 U/ml gave similar results. Data are means ± SEM of triplicate independent determinations from one of three experiments that had similar results.

consistently enhanced ($p < 0.1$) IFN-γ-primed (100 U/ml) normal host Mϕ NO production triggered by 1μg/ml LPS doses, and similarly treated TBH Mϕs maintained constant levels of NO production (Figure 10). In contrast, TNF-α neutralization significantly ($p < 0.05$) decreased normal host and TBH peritoneal Mϕ NO production induced by 10 μM paclitaxel (Figure 11), suggesting that TNF-α is necessary for paclitaxel to induce TBH Mϕ production of reactive nitrogen intermediates. The modulatory effect of TNF-α neutralization on TBH Mϕ NO production was titratable, and isotype control mAb did not alter NO production (not shown).

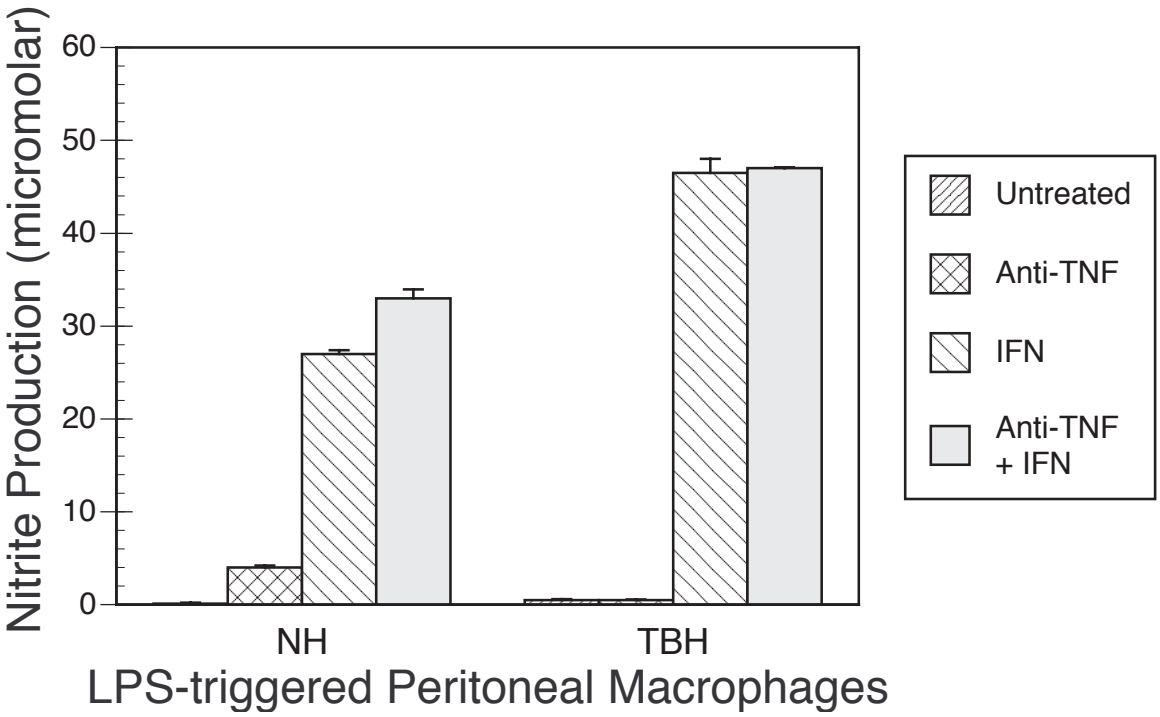


Figure 10. LPS-induced NO production does not require TNF-α. Either 4 x 10^5 normal host or TBH peritoneal Mϕ were cultured in 200 μI of serum-free medium with anti-TNF-α mAb (2.0 μg/ml) and IFN-γ (100 U/ml). Various doses of LPS were added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment. Non-specific isotype mAb did not influence NO production (not shown). Data are means ± SEM of triplicate independent determinations from one of four experiments that had similar results.

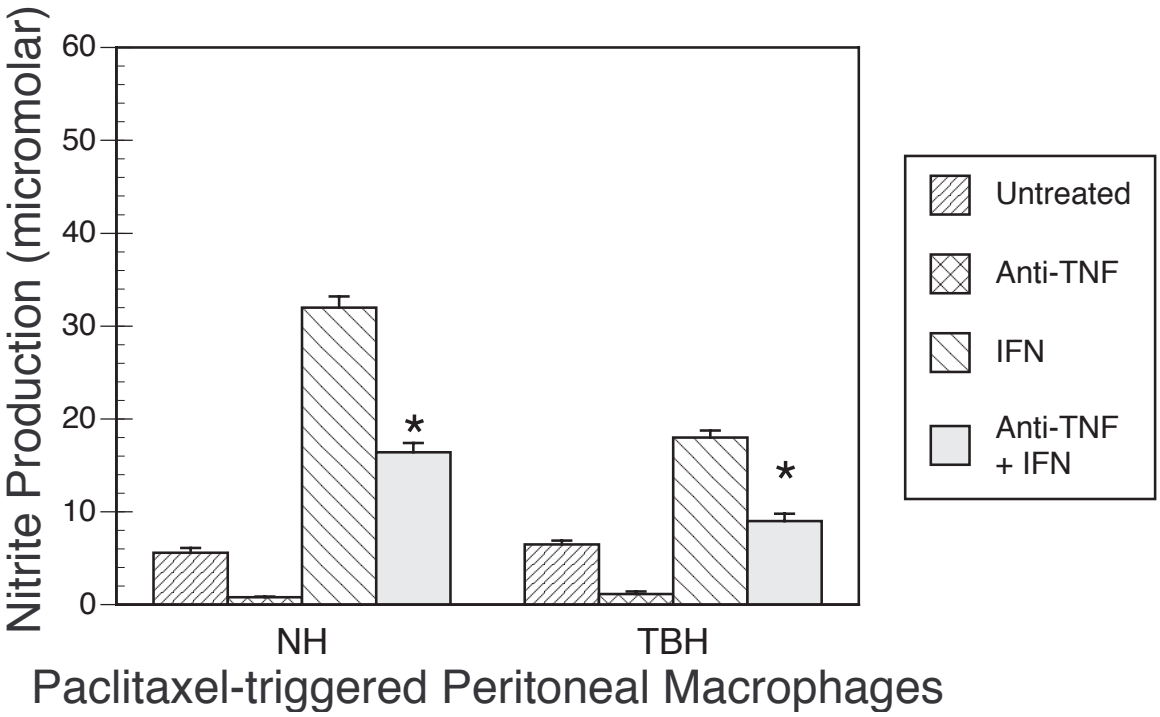


Figure 11. TNF-α neutralization modulates paclitaxel-mediated NO production. Either 4 x 10^5 normal host or TBH peritoneal Mϕ were cultured in 200 μl of serum-free medium with anti-TNF-α mAb (2.0 μg/ml) and IFN-γ (100 U/ml). Various doses of paclitaxel, ranging from 0 to 50 μM, were added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment. NO production by IFN-γ-primed Mϕ was significantly ($p < 0.05$) diminished by TNF-α neutralization (*, as compared to IFN-γ-primed Mϕs). Non-specific isotype mAb did not influence NO production (not shown). Data are means ± SEM of triplicate independent determinations from one of four experiments that had similar results.

DISCUSSION

Successful anticancer modalities must impart both direct cytotoxicity as well as indirect antitumor activities through a bolstering of the host's own immune responses against the tumor. Conventional therapies -- including surgery, radiotherapy, and chemotherapy -- have shown some clinical successes; however, none of these approaches correct the fundamental defects in the immune response caused by tumor-induced changes among Mϕ populations. Immunotherapies use either cytokines or pharmaceuticals to stimulate cytokine production. Cytokines provide the necessary signals for Mϕ, T-cell, and NK cell activation, leading to enhanced antitumor immune responses and tumor immunity. Many immunotherapies that restore immunocompetence have limited efficacy because the physiologically-relevant cytokine doses may not be attainable in tumor microenvironments using existing techniques. Direct administration of cytokines to achieve the desired *in situ* dose may induce severe systemic toxicity. Thus, ideal human immunotherapies may require compounds that directly affect Mϕ expression of costimulatory molecules and secretion of cytokines necessary for tumor rejection and immune cell activation. The chemotherapeutic drug paclitaxel may provide both tumoricidal activities against cancer cells and immunomodulatory signals for Mϕ populations.

Like LPS, paclitaxel induces Mϕ production of the tumoricidal molecules TNF-α, IL-1, and NO (2,49,110,112,113,268). We showed that tumor growth primed Mϕs for increased NO production following IFN-γ priming. Interestingly, tumor growth changed paclitaxel-mediated -- but not LPS-mediated -- regulation of Mϕ NO production. LPS enhanced IFN-γ-primed NO production by TBH Mϕs (Figures 4 and 7), while paclitaxel modulated NO production by similarly cultured TBH Mϕs (Figures 5 and 8). This study

and others (49,267) suggest that paclitaxel therapy activates normal host Mϕ cytotoxic activity *in vitro* through enhanced NO production. In a more biologically-relevant situation, the question remains if activated TBH Mϕs respond similarly. We demonstrate that paclitaxel differentially regulates Mϕ activities in normal and tumor-burdened hosts, leading to diminished production of the immunosuppressive molecule NO by Mϕs in compartments distal to the tumor.

We have shown that tumor growth induces functional changes in Mϕ responsiveness to the antineoplastic molecule paclitaxel. The proinflammatory Mϕ-derived molecule NO is highly advantageous to the host when produced *in situ* to the tumor but fails to impart antitumor efficacy when produced in tumor-distal sites (435). This suggests that paclitaxel-mediated control NO or other reactive intermediates may partially counteract tumor-induced Mϕ suppressive activities. Mϕs must be proximal to tumors in order to mediate cytolytic activity (224, reviewed in 449). NO, which has a circulatory half-life of only a few seconds, cannot target or infiltrate a tumor mass when expressed in tumor-distal compartments. However, distally-expressed NO is still highly reactive and can severely dysregulate T-cell proliferation and influence blood flow dynamics (118). Although it seems counterintuitive, decreases in NO expression may actually benefit the TBH by reversing Mϕ-mediated suppressor activities in tumor-distal compartments. Furthermore, tumors may escape the cytotoxic effects of tumor-proximal Mϕs by producing Mϕ-deactivating cytokines that inhibit NO and TNF-α production (15). Paclitaxel, which directly inhibits tumor cell division and interrupts protein synthesis, may block tumor production of Mϕ-deactivating molecules while concurrently activating tumor-infiltrating Mϕ production of *in situ* NO and TNF-α, leading to enhanced tumoricidal activity. Thus, in addition to its direct cytotoxic and cytostatic actions, paclitaxel may expand Mϕ-mediated antitumor activities by modulating the

suppressive activities of tumor-distal populations while simultaneously enhancing the antitumor efficacy of tumor-proximal Mϕs.

The modulatory effect of paclitaxel on TBH Mϕ NO production may be explained by tumor-induced priming for increased Mϕ TNF-α production. In agreement with other studies (102,173,211,308), we showed that low doses of TNF-α (10 - 100 U/ml) enhanced NO production by IFN-γ-primed Mϕs (Figure 9). We here report that neutralization of TNF-α activity decreased paclitaxel-triggered TBH Mϕ NO production (Figure 11), confirming the role of TNF-α in paclitaxel-mediated regulation of tumor-distal Mϕ NO production. These observations suggest that paclitaxel, which induces TNF-α production (Table 1), may stimulate low levels of NO production by IFN-γ-primed Mϕ in an autocrine manner through TNF-α. Paclitaxel-induced NO production may be modulated in the TBH through tumor-induced dysfunctions which dysregulate Mϕ responsiveness to activating agents. Tumor-derived cytokines such as IL-10 or TGF-β_1 may compromise Mϕ response to paclitaxel or induce Mϕ-derived molecules which suppress NO. Additionally, paclitaxel may stimulate Mϕ production of suppressive molecules which modulate NO production in an autocrine manner during tumor growth. For example, paclitaxel -- but not LPS -- may induce abnormally high levels of PGE_2, TGF-β, or IL-10 that may autocrinely control Mϕ NO production (9) or play a synergistic role with other paclitaxel-induced molecules to control tumor-induced Mϕ NO production. Because tumor-induced Mϕ TNF-α production increases Mϕ PGE_2 production (9,13) and high PGE_2 levels can suppress Mϕ NO production (15), TNF-α may regulate high levels of Mϕ NO production through PGE_2 synthesis. We previously showed that exogenous PGE_2 addition slightly downregulated Mϕ NO production (15), suggesting that PGE_2 or other arachidonic acid metabolites may be involved in TNF-α-induced suppression of NO production.

The relevance of these results to human oncology patients has been uncertain, owing to the heated debate regarding human monocyte/Mϕ production of NO (103). Recent evidence strongly suggests that human peripheral blood mononuclear cells can produce moderate amounts of NO *in vivo* (119). Human Mϕs possess the gene and functional protein for NO production (454) and NO production has been demonstrated *in vitro* (119,454), suggesting that Mϕ responsiveness to paclitaxel in human cancer patients warrants investigation.

In conclusion, paclitaxel may be a potent immunotherapeutic agent, capable of optimizing host response to tumors while directly inhibiting tumor growth, and the immunotherapeutic activity of paclitaxel may be partially responsible for its successes in human trials. Our ongoing studies will investigate the effects of chemotherapeutically safe paclitaxel doses on tumor cell production of Mϕ-deactivating cytokines, address the ability of paclitaxel to reverse Mϕ-mediated immunosuppressive activities, and assess paclitaxel-mediated TBH Mϕ cytotoxicity at the tumor site.

CHAPTER II

PACLITAXEL ENHANCES BUT DOES NOT INDUCE TBH Mϕ PRODUCTION OF IL-1β

ABSTRACT

Tumor cells escape the immune system, in part, by dysregulating Mϕ production of cytotoxic and proinflammatory cytokines in both tumor-proximal and -distal compartments. Because tumor growth enhances NO production, and the proinflammatory cytokine IL-1β has been shown to induce iNOS expression, we assessed the capacity for tumor growth to induce Mϕ IL-1β production. Splenic macrophages from normal host or TBH mice were cultured without or with IFN-γ and LPS, and IL-1β production was determined by specific ELISA. TBH Mϕ production of IL-1β was significantly enhanced in comparison to similarly treated normal host Mϕs. Because the cell's microtubule system influences inflammatory responses, and the anticancer drug paclitaxel acts directly to irreversibly polymerize microtubules, we determined whether paclitaxel altered Mϕ production of IL-1β. Paclitaxel (0.1 - 10 μM) failed to enhance TBH Mϕ production of IL-1β, although a higher dose of paclitaxel (25 μM) slightly enhanced IL-1β production; paclitaxel failed to induce substantial IL-1β production by normal host Mϕs. Following treatment of TBH Mϕs with LPS, paclitaxel significantly increased TBH Mϕ IL-1β production, suggesting that paclitaxel may enhance Mϕ IL-1β production following activation. Following activation with optimal doses of LPS and paclitaxel, recombinant molecules TGF-β_1 and IL-10 suppressed TBH Mϕ IL-1β production.

INTRODUCTION

The proinflammatory cytokine IL-1β (107), like TNF-α, is an important mediator of sepsis and multiple organ dysfunction syndrome. IL-1β, primarily derived from activated Mϕs, is secreted in a pro-form that requires enzymatic cleavage to become active. In addition to Mϕs, IL-1β is produced by B-cells and select nonimmune tissues, including fibroblasts (230). Like many cytokines, IL-1β is highly pleiotropic, targeting T-cells, B-cells, Mϕs, and endothelium. Biologic functions of IL-1β include lymphocyte activation, Mϕ stimulation, induction of acute phase proteins, and enhancement of leukocyte adhesion (107). IL-1β may be important for cell-mediated antitumor immune responses because it plays a role in IL-12-mediated IFN-γ production by NK cell populations (201), suggesting that IL-1β may be crucial for early T-cell-independent antitumor responses. Further, IL-1β may enhance cytotoxic responses through the induction of iNOS expression (427).

Recent literature suggests that the microtubule system may be involved in a cell's inflammatory response and specifically the production of IL-1β and TNF-α (113). Endotoxin both enhances microtubule formation and induces IL-1β. Therefore, several groups investigated whether paclitaxel, which induces the irreversible polymerization of α/β tubulin and functionally mimics LPS, could induce or enhance Mϕ IL-1β production. Manthey *et al*. (266) show by Northern analysis that paclitaxel induces Mϕ IL-1β production in C3H/HeJ mice, with optimal induction at a dose of 10 μM paclitaxel. Bogdan and Ding (49) showed that 10 mM paclitaxel induced IL-1β expression at 2 h, with maximal expression at 4-6 h. In contrast, Allen *et al.* (8) reported that paclitaxel could enhance, but not induce, IL-1β production; these data suggest that paclitaxel could enhance LPS-induced IL-1β, but that paclitaxel alone failed to induce IL-1β. In terms of

relevance to human oncology, Smith *et al*. (391) reported that paclitaxel enhances IL-1β production by human monocytes, and White *et al.* (455) have shown that paclitaxel alone can enhance human monocytes expression of IL-1β. Because no studies have addressed the effects of tumor growth on Mϕ IL-1β production, we determined TBH Mϕ IL-1β production capacity; further, because tumor growth alters Mϕ responsiveness to paclitaxel, we investigated paclitaxel-induced IL-1β production by tumor-induced Mϕs.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice were used. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma, designated Meth-KDE (126), was used as described in Chapter I.

Media and Reagents

Mϕs were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma Cell Culture, St. Louis, MO), prepared as descrivbed in Chapter I. Reagents included LPS (*Escherichia coli* serotype 026:B6) and recombinant murine IFN-γ (specific activity 1.0 x 10^5 U/ml; endotoxin content <10 pg/ml) (generous gifts from Genentech, Inc., San Francisco, CA). Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution and stored at -80°C. Paclitaxel was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%.

Mϕ Culture and Collection

Normal host and TBH BALB/c splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL), washing away nonadherent cells with warm RPMI-1640 medium, and collecting

adherent Mϕs in cold medium by scraping with a rubber policeman. The final Mϕ preparations contained cells that were >95% viable.

Either eight x 10^5 normal host or TBH splenic Mϕs (optimal cell numbers) were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY). Each well contained a total volume of 200 μL serum-free RPMI-1640 medium without or with IFN-γ, LPS, or paclitaxel. Exogenous recombinant TGF-β_1 (10 ng/ml), IL-10 (5 U/ml), or TGF-β_1 and IL-10 were added to some cultures. Supernatants for nitrite assessment were collected at 18 h (optimal culture times) following centrifugation (400 x *g*, 5 min). Paclitaxel doses up to 25 μM did not decrease Mϕ viability because the Alamar blue™ assay (292) verified >95% Mϕ viability throughout the culture periods (not shown).

IL-1β Quantification

IL-1β production measured in culture supernatant using a specific ELISA (InterTest™-1βX, Genzyme Diagnostics, Cambridge, MA) following the manufacturer's provided protocol. The minimum detection limit was 10 pg/ml.

Statistics and Calculations

Cells from six to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for IL-1β protein concentration using the cytokine-specific ELISA. All experiments were repeated at least three times; data are means ± SEM of triplicate independent determinations. 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

LPS Induces Splenic Mϕ IL-1β Production During Tumor Growth.

To determine whether tumor growth modulated Mϕ production of the proinflammatory and cytotoxic effector molecule IL-1β, 8 x 10^5 normal host or TBH splenic Mϕs were primed with 50 U/ml of IFN-γ and cultured with various doses of LPS (0 – 10 μg/ml). After 18 h (optimal incubation time), supernatants were collected for IL-1β assessment. Tumor-induced Mϕs produced substantial quantities of IL-1β, even without LPS activation (Figure 12). Even at low doses (0.1 μg/ml), LPS significantly ($p < 0.05$) enhanced IL-1β production by both normal host and TBH IFN-γ-primed Mϕs, and LPS-induced increases were more substantial in the TBH Mϕs and compared to similarly-treated normal host cultures. In the absence of IFN-γ priming, neither population of Mϕs produced significant amounts of IL-1β, regardless of LPS triggering (not shown).

Paclitaxel Fails to Induce Mϕ IL-1β Production During Tumor Growth.

To determine whether the antineoplastic agent paclitaxel, an LPS-mimetic, induces IL-1β production, 8 x 10^5 normal host or TBH splenic Mϕs were primed with 50 U/ml of IFN-γ and cultured with various doses of paclitaxel (0 – 25 μM). Supernatants were collected after 18 h and IL-1β assessed by specific ELISA. Paclitaxel (0.1 – 10.0 μM) failed to induce IL-1β production by normal host or TBH Mϕs (Figure 13). At the highest paclitaxel dose tested (25 μM), TBH Mϕ IL-1β production was slightly but significantly ($p < 0.05$) enhanced as compared to untreated TBH cultures. Paclitaxel was not tested at levels beyond 25 μM because these doses are toxic to Mϕs (not shown).

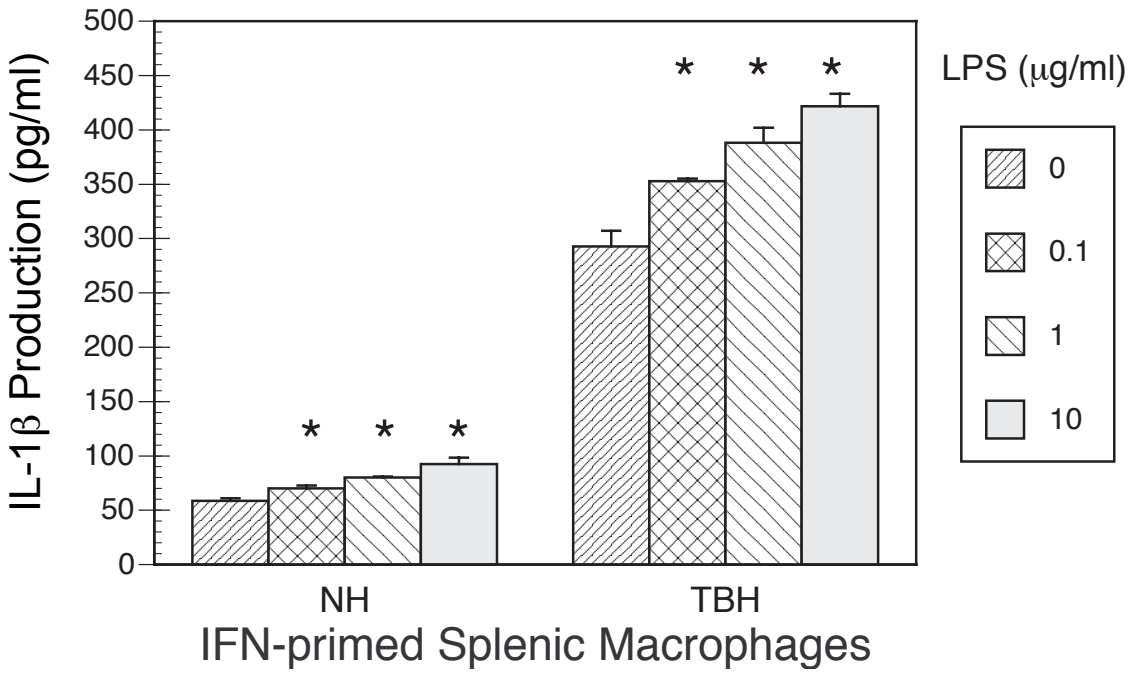


Figure 12. LPS induces splenic Mϕ IL-1β production during tumor growth. Eight x 10^5 BALB/c normal host or TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 50 U/ml of IFN-γ. Various doses of LPS were added at the start of incubation and supernatants were collected after 18 h (optimal incubation time) for IL-1β assessment by specific ELISA. Data are means ± SEM of triplicate independent determinations from one of three experiments that had similar results. *, $p < 0.05$ compared to unstimulated cultures.

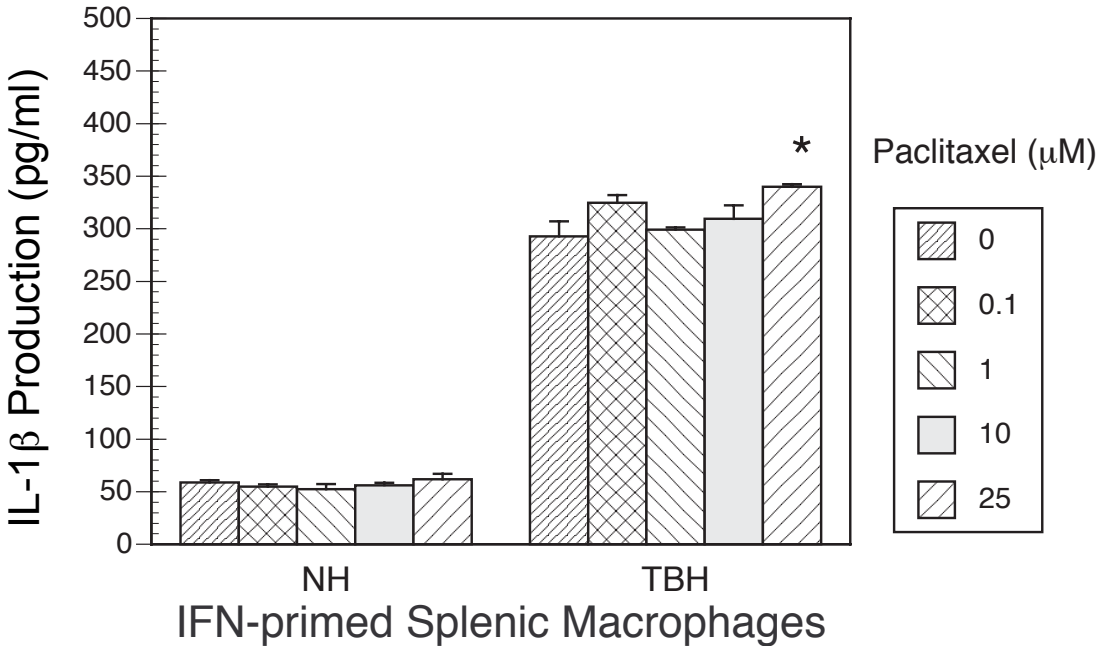


Figure 13. Paclitaxel fails to induce Mϕ IL-1β production during tumor growth. Eight x 10^5 normal host or TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 50 U/ml of IFN-γ. Various doses of paclitaxel were added at the start of incubation and supernatants were collected after 18 h (optimal incubation time) for IL-1β assessment by specific ELISA. Data are means ± SEM of triplicate independent determinations from one of three experiments that had similar results. *, $p < 0.05$ compared to unstimulated TBH Mϕs.

Paclitaxel Induces TBH Mϕ IL-1β Production Following LPS Triggering.

Although paclitaxel alone failed to induce IL-1β production, Smith *et al*. (391) reported that paclitaxel could enhance IL-1β production following activation of normal host Mϕs. To determine whether paclitaxel could enhance TBH Mϕ IL-1β production following IFN-γ-priming and LPS, we cultured TBH splenic Mϕs with IFN-γ (50 U/ml), LPS (10 μg/ml), and various doses of paclitaxel. Supernatants were collected after 18 h IL-1β assessment, as described. In this regimen, the clinically-relevant dose of 10 μM paclitaxel significantly ($p < 0.05$) increased IL-1β production by TBH Mϕs (Figure 14).

Tumor-derived Factors Modulate Paclitaxel's Capacity to Enhance IL-1β Production

The Meth-KDE fibrosarcoma produces high levels of immunosuppressive factors that systemically alter Mϕ phenotype and function (15,126). To determine whether paclitaxel-mediated enhancement of IL-1β is modulated by these factors, splenic Mϕs were primed with IFN-γ (50 U/ml) either without or 10 μg/ml LPS and 10 μM paclitaxel. Some cultures were treated with TGF-β_1 (10 ng/ml), IL-10 (5 U/ml), or TGF-β_1 and IL-10. IL-1β production was assessed after 18 h (optimal incubation time), as described. Interestingly, both TGF-β_1 and IL-10 suppressed TBH Mϕ IL-1β production, even after triggering with LPS and paclitaxel (Figure 15). The combination of TGF-β_1 and IL-10 suppressed TBH Mϕ IL-1β production to levels below that of untriggered TBH Mϕs, demonstrating that these molecules are inhibitors of paclitaxel-mediated triggering of Mϕ IL-1β production.

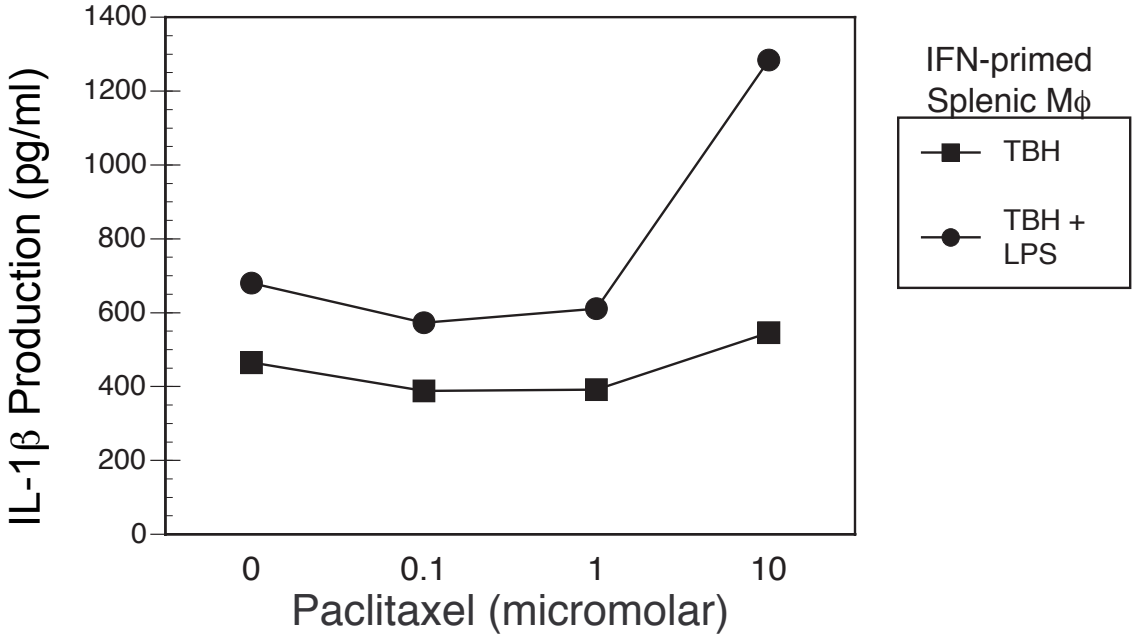


Figure 14. Paclitaxel induces TBH Mϕ IL-1β production following LPS triggering. Eight x 10^5 TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 50 U/ml of IFN-γ and 10 μg/ml LPS. Various doses of paclitaxel were added at the start of incubation and supernatants were collected after 18 h (optimal incubation time) for IL-1β assessment by specific ELISA. Data are means ± SEM of triplicate independent determinations from one of three experiments that had similar results.

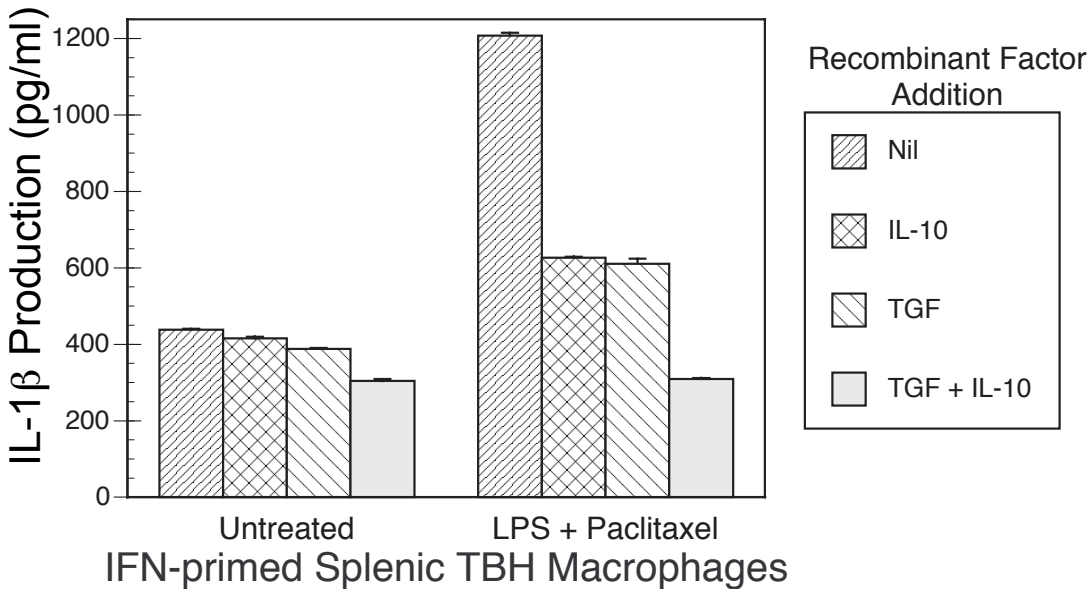


Figure 15. Tumor-derived immunosuppressive molecules modulate paclitaxel's capacity to enhance TBH Mϕ IL-1β production following LPS triggering. Eight x 10^5 normal host or TBH splenic Mϕ were cultured in 200 μl of serum-free medium and primed with 50 U/ml of IFN-γ, either without or with 10 μg/ml LPS and 10 μM paclitaxel. Some cultures were treated with TGF-β_1 (10 ng/ml), IL-10 (5 U/ml), or TGF-β_1 + IL-10. Supernatants were collected after 18 h (optimal incubation time) for IL-1β assessment by specific ELISA. Data are means ± SEM of triplicate independent determinations from one of three experiments that had similar results.

DISCUSSION

A thorough examination of tumor-induced immune dysfunction should not overlook the importance of IL-1β. Functionally, IL-1β resembles TNF-α in terms of biologic activities. Both IL-1β and TNF-α are monocyte/Mϕ-derived proinflammatory cytokines that mediate sepsis, adult respiratory distress syndrome, and multiple organ dysfunction syndrome (107). Unlike TNF-α, IL-1β exists as an intracellular precursor (pro-IL-1β), which is transported to the cell membrane and cleaved by IL-1β-converting enzyme (ICE) (414) to release active IL-1β. Further, several studies (8,265) suggest that microtubule-disrupting agents may induce IL-1β production. Because paclitaxel has potent microtube-polymerizing activity, and tumor growth induces differential Mϕ responsiveness to paclitaxel, we studied whether tumor growth primes Mϕs for enhanced IL-1β production and enhanced responsiveness to paclitaxel triggering.

When splenic Mϕs were primed with IFN-γ and cultured with various doses of LPS (0 - 10 μg/ml), TBH Mϕs produced substantial levels of IL-1β, even without LPS activation (Figure 12). LPS is a potent activator of Mϕ IL-1β production because even low doses (0.1 μg/ml) significantly ($p < 0.05$) enhanced IL-1β production by both normal host and TBH IFN-γ-primed Mϕs. While addition of LPS increased normal host IL-1β production, LPS-induced levels of IL-1β were substantially greater in the TBH Mϕ cultures. These response patterns agree with our previous observations of LPS-induced TNF-α production by normal host and TBH Mϕs (see Table 2). These data support the concept that tumor growth primes distal Mϕ populations for enhanced production of cytotoxic and proinflammatory molecules on activation, as compared to similarly-treated normal host cultures. Furthermore, these data suggest a possible mechanism of enhanced tumor-distal NO production in the TBH (see Figures 4 and 5), because IL-1β has been

associated with the induction of iNOS activity (427). The paucity of normal host Mϕ IL-1β production may be explained by the source of the immune cell populations. We have typically found that normal host splenic Mϕs are significantly less responsive to LPS or paclitaxel, as compared to peritoneal exudate Mϕs. A second possible explanation is offered by O'Brien *et al*. (321), who report that paclitaxel increases pro-IL-1β but not IL-1β secretion in normal host Mϕs. Regardless, our data demonstrate a significant difference in the release and regulation of IL-1β in the TBH.

Because IL-1β production has been associated with microtubule-stabilizing agents, we next determined whether paclitaxel could induce Mϕ IL-1β production. When normal host or TBH splenic Mϕs were primed with IFN-γ and cultured with clinically-relevant doses of paclitaxel (0.1 – 10 μM), IL-1β production was not significantly different from unactivated Mϕs (Figure 13). These data confirm the observations of others (8) that paclitaxel does not induce IL-1β production by normal host Mϕs and further suggest that paclitaxel and LPS induce Mϕ production of immune molecules through multiple divergent signal transduction pathways. We extend these findings by demonstrating that clinically-relevant doses of paclitaxel (0 – 10 μM) fail to induce IL-1β in TBH Mϕs, despite the enhanced response of these tumor-induced Mϕs to LPS triggering. IL-1β production was induced only by treatment of IFN-γ-primed TBH Mϕ with the exceedingly high dose of 25 μM paclitaxel, and the resultant increase in Mϕ IL-1β production was only 16% (as compared to the 19.5% to 58% increases induced by 0.1 or 10 μg/ml LPS, respectively). Because paclitaxel enhances LPS-induced Mϕ activities (391), we next determined whether paclitaxel could enhance IL-1β production following IFN-γ-priming and LPS activation. Following culture with LPS and the clinically-relevant dose of 10 μM paclitaxel, IL-1β production by TBH Mϕs was significantly ($p < 0.05$) enhanced.

Although high doses of paclitaxel alone did increase Mϕ IL-1β production (340 pg/ml IL-1β, as compared to 292 pg/ml IL-1β from untreated TBH Mϕs), and paclitaxel could stimulate IL-1β following LPS priming, the biologic significance of these activities may be irrelevant to the tumor-burdened host. First, current chemotherapeutic regimens do not achieve microenvironment concentrations of paclitaxel in excess of 10 μM, too low to induce IL-1β in this model system. Second, doses of paclitaxel equal to or exceeding 25 μM are highly toxic to lymphocytes; at this dose level, any therapeutic advantage gained by enhanced IL-1β production would be dramatically overshadowed by paclitaxel-mediated abrogation of T-cell-mediated antitumor responses. Finally, it is impractical to consider LPS-activation of Mϕs *in vivo*, considering that very small doses of LPS rapidly induce endotoxemia and death.

Because tumor-derived cytokines such as IL-10 or TGF-β_1 may compromise Mϕ response to paclitaxel (296,301), and the Meth-KDE fibrosarcoma produces high levels of these immunosuppressive factors (15), we determined whether paclitaxel-mediated enhancement of IL-1β is modulated by these factors. When IFN-γ-primed splenic TBH Mϕs were activated with LPS and paclitaxel, either TGF-β_1 or IL-10 suppressed IL-1β production. These data suggest that tumor-distal Mϕs are primed for enhanced IL-1β production on activation, but high concentrations of tumor-derived factors (such as within a solid tumor mass) functionally deactivate Mϕ proinflammatory and cytotoxic molecule production. Therefore, production of IL-1β by *in situ* tumor-infiltrating Mϕs is likely abrogated by high local concentrations of TGF-β_1 and IL-10, regardless of priming or activation signals.

CHAPTER III

PACLITAXEL ENHANCES Mϕ-MEDIATED ANTITUMOR CYTOTOXICITY

ABSTRACT

Paclitaxel inhibits tumors through multiple cytotoxic and cytostatic mechanisms. Independent of these mechanisms, paclitaxel induces distinct immunologic efficacy when it acts as a second signal for activation of cytotoxic activity by IFN-γ-primed normal host murine Mϕs. We reported that tumor-distal Mϕs, which mediate immunosuppression through dysregulated NO and TNF-α production, are differentially regulated by paclitaxel. Because paclitaxel influences tumor cell growth dynamics and activates immune cell populations, we assessed the antitumor activities of paclitaxel-treated normal host and TBH Mϕs. Paclitaxel-treated TBH Mϕs significantly suppressed the growth of fibrosarcoma cells (Meth-KDE) through soluble effector molecules and promoted direct cell-mediated cytotoxicity, indicating that paclitaxel enhanced tumor-induced Mϕ antitumor activities. These data demonstrate that paclitaxel has the capacity to promote Mϕ-mediated antitumor activities.

INTRODUCTION

The antineoplastic agent paclitaxel has demonstrated significant efficacy as an antitumor chemotherapeutic in human clinical trials (reviewed in 178), with activity against a wide range of human cancers. In addition to its well-characterized chemotherapeutic activities, paclitaxel has profound cell cycle-independent effects on murine Mϕs (53,203). A notable activity of paclitaxel may be its ability to activate Mϕs for enhanced *in vitro* tumor cell cytotoxicity (267). Paclitaxel stimulates *in vitro* normal host Mϕ responses similar to those induced by bacterial LPS (266,440), activating expression of all characterized LPS-inducible genes (110). Considerable data show that both paclitaxel and LPS can act synergistically with IFN-γ, which primes Mϕs for enhanced production of cytotoxic and regulatory molecules, to trigger transcription of IL-1 (49), TNF-α (49,457), and iNOS (221,267). Paclitaxel enhances IFN-γ-primed normal host Mϕ cytotoxicity toward tumor cells *in vitro* (440), primarily through NO (266,267). Tumor growth, however, induces multiple phenotypic and functional changes among Mϕ populations (15,122). We reported that fibrosarcoma growth altered Mϕ responsiveness to paclitaxel, modulating TBH Mϕ NO production in tumor-distal compartments (296). Because paclitaxel stimulates immune cell activities, we have evaluated paclitaxel's activity on normal host and TBH Mϕ and T-cell populations.

In this study, we report that paclitaxel enhances Mϕ antitumor activities *in vitro*. These data collectively suggest that paclitaxel has differential immune system actions that vary with cell phenotype and compartment. Through its capacity to activate immune cell cytotoxic activities, paclitaxel may impart immunotherapeutic activities not previously described.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice were used. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (Meth-KDE) (15,126) was used as described in Chapter I.

Media and Reagents

Mϕs were cultured in serum-free RPMI-1640, prepared as described in Chapter I. Meth-KDE fibrosarcoma cells and P815 murine mastocytoma cells (clone TIB 64, [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). Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution and stored at -80°C. Paclitaxel was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%. LPS (*Escherichia coli* serotype 026:B6) was purchased from Sigma. IFN-γ (specific activity 1.0 x 10^5 U/ml; endotoxin content <10 pg/ml) was generously provided by Genentech, Inc. (San Francisco, CA).

Mϕ Collection and Pretreatment

Normal host and TBH BALB/c splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm plastic plates; Lux/Miles Scientific, Naperville,

IL). Nonadherent cells were washed away with warm RPMI-1640 medium, and adherent Mϕs were collected in cold medium by scraping with a rubber policeman.

To prevent paclitaxel carryover into proliferation and cytotoxicity assays, Mϕs were pretreated in 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY) 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 x *g*, 10 minutes) and media removed. Cells were washed once and fresh media added to the final volume of 200 μl.

Tumor Cell Proliferation Assays

Meth-KDE fibrosarcoma cells were seeded into 96-well flat-bottom tissue culture plates at final concentration of 1 x 10^5 cells in a volume of 100 μL. Following 24-h incubation, Mϕ supernatants (100 μl) were added and incubation continued for an additional 24 h. Supernatants were obtained from 72-h cultures of 2 x 10^5 normal host or TBH Mϕs pretreated (4 h) with various concentrations of IFN-γ, LPS, paclitaxel, or combinations. Supernatants contained only Mϕ-derived effector molecules but did not contain pretreatment reagents. Twelve h before harvest, tumor cell cultures were pulsed with 1 μCi/well [^{3}H]-TdR (tritiated thymidine, specific activity 6.7 Ci/mmol, DuPont-NEN, Wilmington, DE). Cells were harvested and sample activities determined using a Beckman LS 6000SC scintillation counter.

Mϕ-mediated Cytotoxicity Assays

To assess the cytotoxic activity of paclitaxel-activated Mϕs against Meth-KDE tumor cells, either pretreated (4 h) normal host or TBH Mϕs (2 x 10^5 cells) were added to 1 x 10^5 tumor cells. Cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay (6). 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 24 h. Pretreated Mϕs 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 Mϕs, 20 μl (10% of the well volume) of Alamar blue™ indicator dye (BioSource International, Camarillo, CA) was added to each well. In the presence of viable cells, Alamar blue™ dye is reduced to a colored product detectable at 580 nm. Four h after addition of the indicator dye, tumor cell viability was assessed using a Molecular Devices Dynamax plate reader. In this system, cytotoxicity is inversely proportional to absorbance.

Cytotoxicity also was assessed using the ^{51}Cr (chromium) release assay (368) with P815 nonadherent murine mastocytoma target cells. Either paclitaxel-pretreated (4 h) normal host or TBH Mϕs (2 x 10^5 cells) were added to ^{51}Cr-labeled tumor targets (1 x 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] x 100.

Statistics and Calculations

Cells from 6 to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for all proliferation and cytotoxicity assays. Data are averages ± 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

Paclitaxel Enhances TBH Mϕ-mediated Tumor Cell Cytostasis

While the direct antitumor activities of paclitaxel are well characterized, the tumoricidal activities of paclitaxel-treated TBH Mϕs have not been described. Paclitaxel has been shown to enhance normal host Mϕ-mediated cytotoxicity (267). Because paclitaxel modulates TBH Mϕ NO production following pretreatment or long-term exposure (296), we investigated the antitumor efficacy of paclitaxel-treated TBH Mϕs against the purified tumor cells used in our model system (Meth-KDE). To assess the capacity for Mϕ-derived effector molecules to suppress tumor cell proliferation, supernatants from paclitaxel-pretreated normal host (Figure 16) or TBH (Figure 17) Mϕs were added to purified tumor cell and tumor cell proliferation measured after 24 h culture. Because Mϕs were pretreated, no paclitaxel was present in the supernatants, and suppression of proliferation was not due to direct paclitaxel-mediated cytostatic activity on the tumor cells. Pretreatment with paclitaxel, either with or without IFN-γ priming, significantly enhanced the ability of both normal host (Figure 16) and TBH Mϕs (Figure 17) to suppress tumor cell proliferation. Paclitaxel-mediated (10 μM) Mϕ antitumor activity was enhanced by IFN-γ priming (10 U/ml). Paclitaxel-pretreated IFN-γ primed TBH Mϕ affected greater suppression of tumor cell proliferation similarly treated normal host Mϕs, leading to a greater than 50% decrease in tumor cell proliferation relative to control cultures.

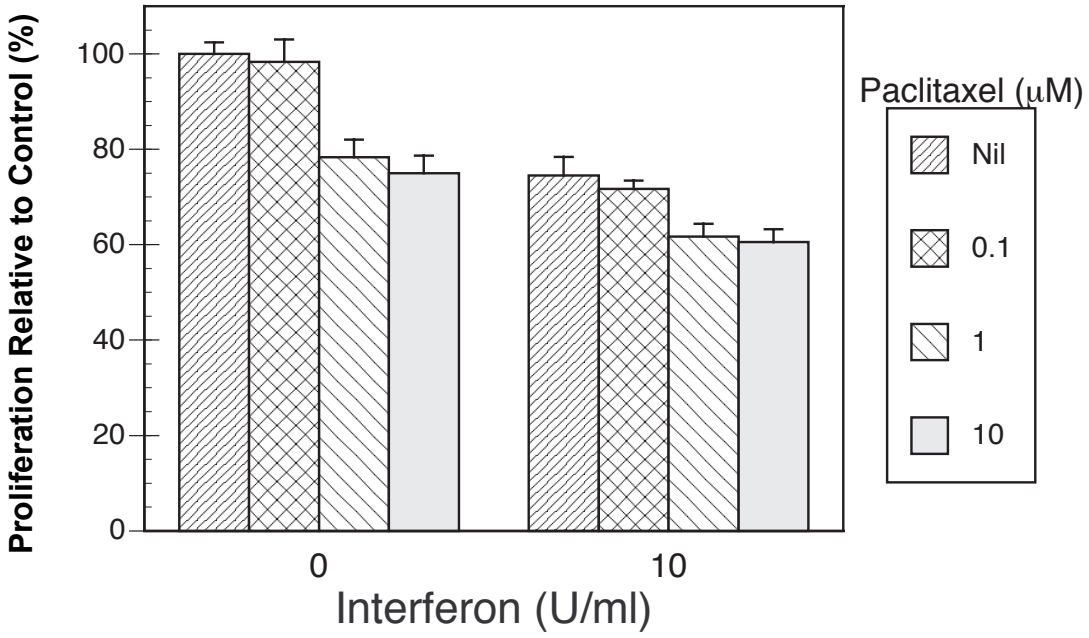


Figure 16. Paclitaxel induces normal host Mϕproduction of soluble antitumor factors. Supernatants (72 h) from 2 x 10^5 control or paclitaxel pretreated (4 h) normal host Mϕs were added to 1 x 10^5 actively growing Meth-KDE tumor cells and culture continued for 24 h. Twelve h before harvest, plates were pulsed with 1 μCi/well [^{3}H]-TdR; cells were harvested and sample activities determined. No paclitaxel was present in the supernatants, and suppression of proliferation was not due to direct paclitaxel-mediated cytostatic activity on the tumor cells. Data are magnitude of response relative to proliferation of untreated tumor cells (control CPM was 96240) and are reported as averages ± SEM of triplicate independent determinations from one of four similar experiments.

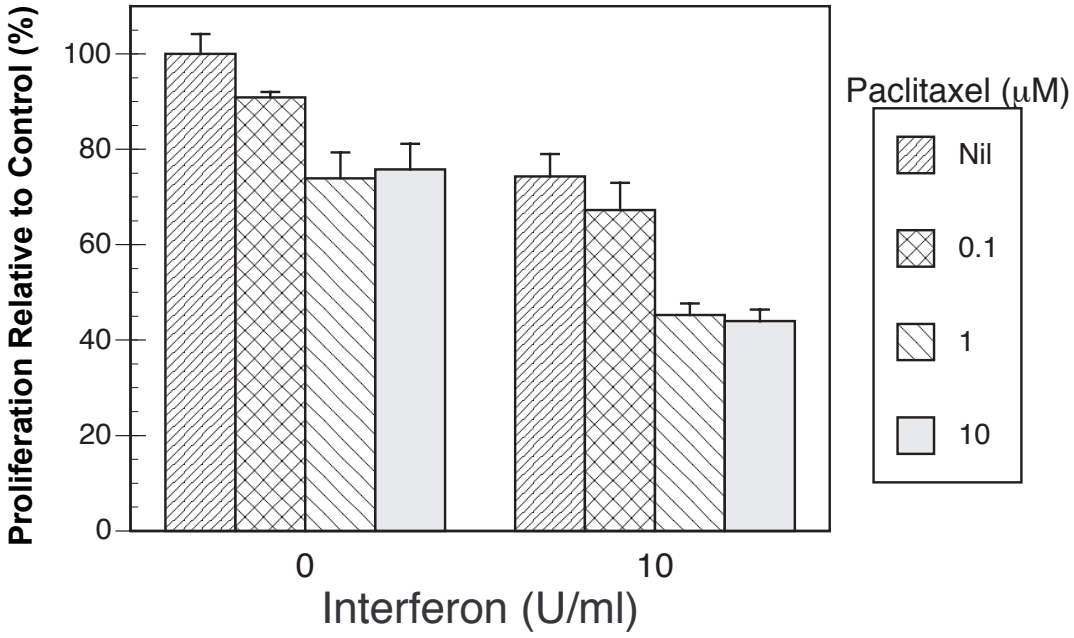


Figure 17. Paclitaxel induces TBH Mϕ production of soluble antitumor factors. Supernatants (72 h) from 2 x 10^5 control or paclitaxel pretreated (4 h) TBH Mϕs were added to 1 x 10^5 actively growing Meth-KDE tumor cells and tumor cell proliferative response determined, as described. Data are magnitude of response relative to proliferation of untreated tumor cells and are reported as averages ± SEM of triplicate independent determinations from one of four similar experiments.

Paclitaxel Enhances Mϕ Cytotoxic Activity

To determine whether paclitaxel differentially regulated TBH Mϕ antitumor cytotoxicity, we tested the cytotoxic capacities of paclitaxel-pretreated Mϕs against two tumor cell lines using two different assay systems. Paclitaxel-pretreated Mϕs were added to growing Meth-KDE tumor cells and cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay. Paclitaxel-pretreated, IFN-γ-primed Mϕs demonstrated increasing cytotoxicity in a dose-dependent manner against the purified adherent Meth-KDE tumor cell line (Figure 18). Normal host and TBH Mϕ-mediated cytotoxicity were enhanced by IFN-γ priming and paclitaxel pretreatment. TBH Mϕs demonstrated cytotoxic activity comparable to similarly treated normal host Mϕs.

To confirm the cytotoxicity results, a ^{51}Cr-release assay was used to measure Mϕ-mediated cytotoxicity against a second tumor cell line. Paclitaxel-pretreated, IFN-γ-primed TBH Mϕs had enhanced cytotoxicity (78.1%) against murine P815 mastocytoma cells (Figure 19), as compared to 40.7% cytotoxicity by IFN-γ-primed Mϕs and 65.1% cytotoxicity by IFN-γ-primed LPS-triggered Mϕs.

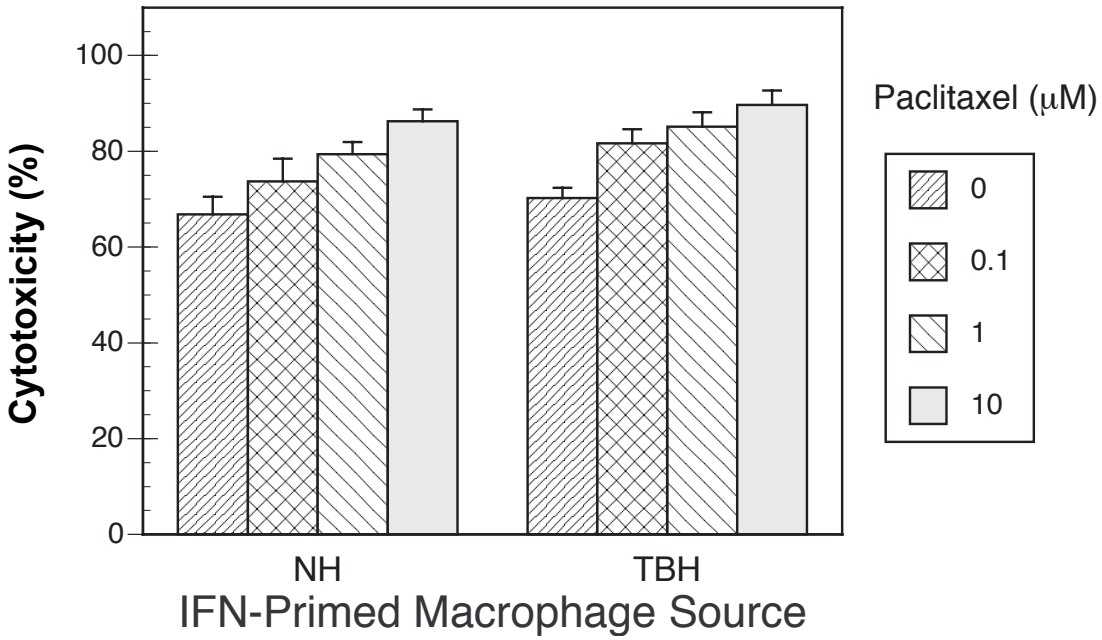


Figure 18. Paclitaxel enhances Mϕ cytotoxic activity. Pretreated (4 h) normal host or TBH Mϕs (2 x 10^5 cells) were added to Meth-KDE fibrosarcoma target cells tumor cell cultures (1 x 10^5 cells per well) and cytotoxicity measured using a modification of the Alamar blue™ colorimetric viability assay. Mϕs were pretreated without or with IFN-γ (10 U/ml) and paclitaxel (0.1, 1.0, or 10.0 μM) for 4 h. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

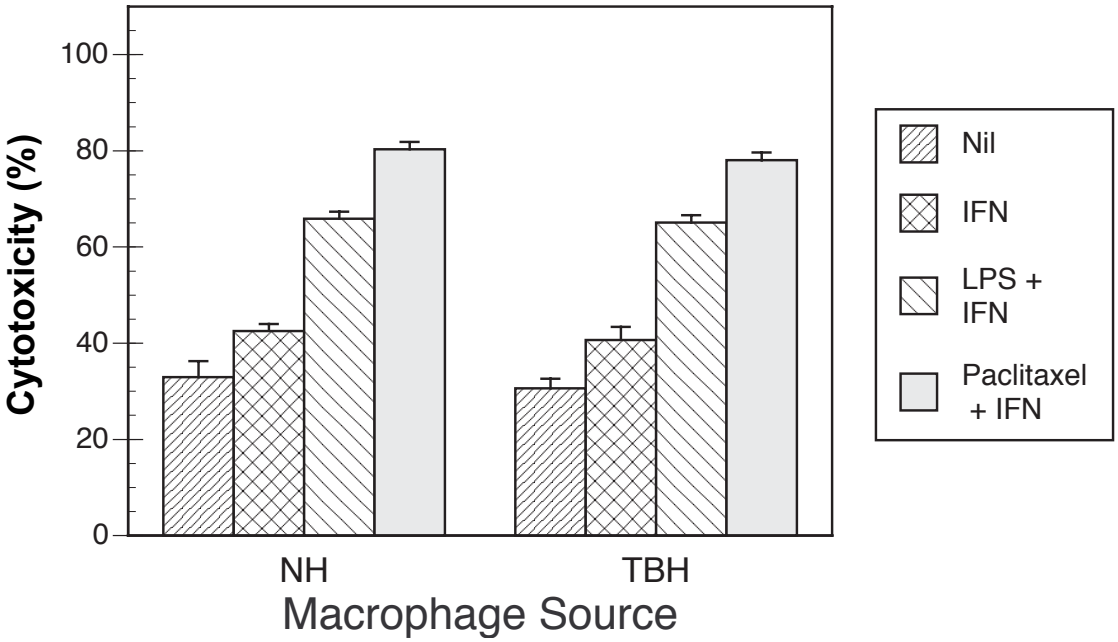


Figure 19. Paclitaxel enhances direct Mϕ-mediated cytotoxic activity. Pretreated (4 h) normal host or TBH Mϕs (2 x 10^5 cells) were added to viable ^{51}Cr-labelled P815 mastocytoma target cells (1 x 10^5 cells per well) and cytotoxicity measured using a ^{51}Cr release assay. Mϕs were pretreated without or with IFN-γ (10 U/ml), LPS (1.0 mg/ml), and paclitaxel (10.0 μM). Lower paclitaxel concentrations (0.1 or 1.0 μM) gave similar profiles (not shown). Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

DISCUSSION

In this study, we demonstrated that the antineoplastic agent paclitaxel promotes antitumor activities by TBH Mϕs. We extend our previous findings (296) by demonstrating that short, transient paclitaxel administration (4 h) can activate *in vitro* Mϕ cytotoxic effector mechanisms. While we and others (266,440) have shown that paclitaxel enhances IFN-γ-activated normal host Mϕ cytotoxicity toward tumor cells *in vitro*, no studies have addressed the effects of tumor growth on paclitaxel's capacity to induce Mϕ-mediated antitumor cytotoxicity. Although paclitaxel modulated NO production by TBH Mϕs (see Chapter I) and has been shown to modulate NK cell-mediated cytotoxicity (85), we determined that paclitaxel treatment enhanced TBH Mϕ antitumor activities. Through soluble antitumor molecules, paclitaxel-triggered Mϕs suppressed tumor cell proliferation (Figures 16 and 17); these enhanced antitumor activities may derive from increased Mϕ production of TNF-α (15).

Paclitaxel also increased direct Mϕ-mediated cytotoxicity of both Meth-KDE and P815 tumor cells (Figures 18 and 19), demonstrating that paclitaxel-induced Mϕ-mediated cytotoxic activity was not restricted to the tumor cell used in our nonmetastatic fibrosarcoma model. These results demonstrate that a significant antitumor activity of paclitaxel. Significantly, although tumor growth modulates paclitaxel-mediated Mϕ NO production capacity, the presence of neoplastic tissue does not abrogate paclitaxel-mediated induction of Mϕ antitumor cytotoxicity. Furthermore, tumors may escape tumor-proximal Mϕ-mediated cytotoxicity by overproducing Mϕ-deactivating cytokines that inhibit local NO and TNF-α production. Paclitaxel, which directly inhibits tumor cell division and interrupts protein synthesis, may compromise tumor production of Mϕ deactivating molecules while concurrently activating tumor-infiltrating Mϕs NO and

TNF-α production, leading to limited tumoricidal activity by tumor-proximal Mϕs. This could further enhance paclitaxel-mediated antitumor activity, and this possibility is currently being investigated.

SECTION III

PACLITAXEL REVERSES TUMOR-INDUCED Mϕ-MEDIATED IMMUNOSUPPRESSION

This Section consists of three chapters that describe the mechanisms by which the anticancer agent paclitaxel mediates immunotherapeutic activity. In Chapter IV, the effect of tumor-derived factors on Mϕ activation and factor production are studied using a Mϕ cell line transfected with a luciferase reporter gene behind a NF-κB-responsive promoter. The tumor-derived molecules TGF-β_1 and IL-10 are shown to affect paclitaxel's ability to induce Mϕ activation. Chapter IV also reports that tumor-derived factors modulate ICSBP expression, suggesting yet another lesion in the Mϕ-mediated immune response during tumor growth. Next, Chapter V discusses the capacity of paclitaxel to reverse tumor-induced Mϕ-mediated immunosuppression, establishing an immunotherapeutic component to paclitaxel's antitumor activities. A proposed mechanism for paclitaxel's immune activity is described in Chapter VI; data are presented to suggest that paclitaxel induces apparent immunotherapeutic responses through the induction of Mϕ IL-12; further, paclitaxel induces IL-12 production through an autocrine signaling pathway involving NO.

CHAPTER IV

TUMOR-DERIVED FACTORS DIFFERENTIALLY REGULATE PACLITAXEL-INDUCED Mϕ ACTIVATION

ABSTRACT

The anticancer agent paclitaxel activates expression of a variety of genes in Mϕs, including TNF-α, iNOS, and IL-12. However, paclitaxel-mediated production of these molecules is compromised in primary Mϕs from TBHs. To determine which tumor-derived factors inhibit paclitaxel-mediated Mϕ activation, we used the RAW264.7 Mϕ cell line transfected with the NF-κB-responsive HIV-1 LTR, which drives a luciferase reporter gene. When RAW Mϕs were continuously cultured with fibrosarcoma (Meth-KDE) cell supernatants, paclitaxel-induced luciferase activity and Mϕ production of NO and IL-12 was significantly diminished. IL-10 and TGF-β were responsible for this inhibition because Ab-mediated absorption of these cytokines from tumor cell supernatants restored luciferase activity and factor production. In contrast, paclitaxel-mediated luciferase activity and factor production were enhanced following pretreatment (4h) of RAW Mϕs with tumor cell supernatant, suggesting that tumors may differentially regulate paclitaxel-mediated Mϕ function, depending upon spatial proximity. Because iNOS and IL-12 signaling involve NF-κB, and the HIV-1 LTR is NF-κB-responsive, tumor growth may regulate paclitaxel-mediated Mϕ cytotoxic and immunostimulatory products through differential regulation of NF-κB. We also determined that tumor growth inhibits Mϕ expression of interferon consensus sequence binding protein (ICSBP) *in vitro*, suggesting another mechanism of tumor-induced dysregulation of immune response during tumor growth.

INTRODUCTION

Tumors evade immunosurveillance, in part, through the production and release of substances that both downregulate (15,123) and upregulate (130,161,206,480) Mϕ cytotoxic and effector molecule production. Mϕ production of the cytotoxic molecules, including TNF-α (52,137,234,435), NO (259,287), H_2O_2 (226), ROI (40,137,194), and IL-1 (52), is enhanced by tumor growth. Although Mϕ production of TNF-α, NO, and ROI may impart cytotoxic functions *in situ*, their capacity to suppress T-cell responses when expressed in tumor-distal compartments should be considered in tumor-burdened animals. The *in vivo* existence of well-established tumors suggests that production of TNF-α, NO, and ROI does not necessarily lead to tumoricidal activity, but may instead lead to suppression of antitumor lymphocytes. Further, simultaneous with induction of cytotoxic molecules, tumor growth increases Mϕ production of the noncytotoxic suppressor molecules PGE_2, TGF-β_1, and IL-10 (15).

Our studies have demonstrated that paclitaxel induces distinct immunologic efficacy when it acts as a second signal for activation of tumoricidal activity by IFN-γ-primed murine Mϕs. However, paclitaxel differentially regulates tumor-distal Mϕs (296,299,301), which mediate immunosuppression through dysregulated NO and TNF-α production (123). Because tumors influence immune cell responses to paclitaxel through the production of soluble effector molecules, we assessed paclitaxel's ability to activate Mϕ cytotoxic and effector functions in the presence of tumor-derived cytokines.

In this study, we used a Mϕ cell line (RAW 264.7) with a luciferase reporter gene under the transcriptional control of a NF-κB-responsive promoter to characterize Mϕ activation and factor production in the absence or presence of tumor-derived factors.

This system allowed for rapid analysis of the effects of various tumor-derived factors on Mϕ response to paclitaxel. Further, corollary data describing tumor-induced alterations in Mϕ production of NF-κB-mediated cytotoxic and immunostimulatory molecules are presented. Collectively, these data suggest that tumor growth may regulate paclitaxel-mediated Mϕ functions through differential regulation of NF-κB.

Although the regulation of NF-κB is certainly involved in the regulation of Mϕ function by tumors, another possible mechanism by which tumors may modulate Mϕ responsiveness involves the control of IFN-γ responsiveness. Because tumor growth modulates IFN-γ-induced Mϕ production of IL-12, and interferon consensus sequence binding protein (ICSBP) has been implicated in the control of IL-12 p40 expression, we assessed whether tumor-derived factors dysregulate the IFN-γ signaling pathway through differential regulation of ICSBP. We report that tumor growth inhibits Mϕ expression of ICSBP *in vitro*, suggesting a possible lesion in the immune response during tumor growth.

MATERIALS AND METHODS

Murine Tumor Model

The BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (Meth-KDE) (15,126) was used to generate tumor-derived supernatants. Meth-KDE cells were purified by a 2-h plating of homogenized tumor mass, collecting the nonadherent cells, and treating them with anti-CD4 (ATCC clone GK1.5), anti-CD8 (ATCC; clone 3.155), anti-IA^d (ATCC; clone MK-D6), and B cell and anti-immature T cell (ATCC; clone J11.D). Unwanted cells were removed by treatment with Low-Tox-M rabbit complement (Accurate Chemical Company, Westbury, NY). The remaining Meth-KDE tumor cells were cultured for at least 30 days in complete medium, then maintained by diluting 1:10 in fresh medium every fifth day.

Mϕ Cell Lines

The RAW264.7 Mϕ cell line (TIB 71), originally derived from the ascites of a Abelson leukemia virus tumor-induced male mouse (345), was acquired from ATCC (Bethesda, MD). The RAW264.7 Mϕ cell line transfected with a luciferase reporter gene behind a NF-κB-responsive promoter (a4 cells) (404) was acquired from Dr. Matthew Sweet (Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, Australia). Briefly, the cell line was established by electroporation of RAW264.7 Mϕs with the pMC1NeoPolyA neomycin phosphotransferase expression plasmid (Stratagene) containing HIV-CAT promoter driving the pGL-2Basic luciferase reporter gene (Promega). Stable transfectants, subsequently referred to as the a4 cell line, were

selected by incubation with 200 μg/ml G418 for 15 days. RAW264.7 Mϕs and a4 Mϕs were maintained by diluting 1:10 in fresh medium every fifth day.

Media and Reagents

Both RAW264.7 Mϕs and a4 Mϕs were cultured in RPMI-1640 (Sigma, St Louis, MO), prepared as described in Chapter I. Cells cultures were supplemented with 5% FBS (Atlanta Biologicals, Norcross, GA). Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution and stored at -80°C. Paclitaxel was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%. LPS (*Escherichia coli* serotype 026:B6) was purchased from Sigma. IFN-γ (specific activity 2.98 x 10^6 U/ml; endotoxin content <10 pg/ml) was purchased from Genzyme , Inc. (Cambridge, MA). Human rTGF-β_1 (100 μg/ml) and rabbit anti-human TGF-β_1 polyclonal Ab (6.5 mg/ml; endotoxin content <10 pg/ml), were generous gifts from Genentech, Inc. (South San Francisco, CA). IL-10 (specific activity 7000 U/ml) was acquired from DNAX (Palo Alto, CA), and anti-murine IL-10 mAbs were obtained from SXC-1 (ATCC; clone HB 10739) hybridoma supernatants. PGE_2 and indomethacin were obtained from Sigma.

Tumor Cell Culture and Supernatant Preparation

Meth-KDE tumor cells (4 x 10^6 cells/ml) were cultured (37°C, 5% CO_2) in 24-well plates (Corning Cell Wells, Corning, NY) in a total volume of 1 ml complete medium. Supernatants were collected 72 h (optimal time) after centrifugation (400 x *g*, 5 min). Fresh Meth-KDE supernatants were depleted of IL-10 or TGFβ_1 by incubating 1

ml of supernatant in 24-well plates that were coated with anti-IL-10 or anti-TGF-β_1 Ab. Plates were coated with Ab by adding concentrated reparations to wells, incubating overnight at 4°C, and washing three times with PBS. Wells were then blocked with sterile PBS containing 5% FBS for 2 h at room temperature and washed three times with PBS. Meth-KDE supernatants incubated on non-Ab coated serum-treated wells were used as controls for nonspecific cytokine binding.

Luciferase Assays

For luciferase assays (see Appendix C for details), a4 cells (1 x 10^6 cells/well) were cultured overnight in 24 well tissue culture plates (Corning Cell Wells) in total volume of 1 ml complete medium. Cells were washed with fresh RPMI-1640, then recultured in a total volume of 1 ml with indicated treatment reagents in pure complete medium either without or with Meth-KDE tumor supernatants (1:2 dilution). Culture was continued for 2 h, then cells were lysed with 20 μL Promega Cell Lysis Solution (Promega, Madison, WI). Cellular debris was removed by centrifugation (12,000 x *g*) for 5 sec, and cellular extracts were assayed for total protein by a variation of the Lowry method using Sigma reagents (see Appendix B for details).

Luciferase activity in cellular extracts was determined using luciferin (Sigma) substrate in a $MgCl_2$ buffer supplemented with 0.1 M ATP. Luciferin substrate mix (100 μL) was added to 10 μL of room-temperature cellular extract, and luciferase activity recorded as relative light units using a Berthold luminometer. Per the manufacturer's recommendation, luciferase activity was assessed for 10 sec, beginning 10 sec after mixing of the substrate and cellular extract because luciferase-mediated light production

is both stable and optimal during this period. Activity is adjusted to the concentration of protein in the cell extract and reported as relative light units per μg of total protein.

Mϕ Nitrite Production

RAW264.7 Mϕs (2 x 10^5 cells) were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells). Each well contained a total volume of 200 μL serum-free RPMI-1640 medium with indicated treatment reagents added at the start of culture. Supernatants for nitrite assessment were collected at 24 h (optimal time) following centrifugation (400 x *g*, 5 min). Paclitaxel doses up to 50 μM did not decrease Mϕ viability because the MTT assay (292) verified >95% Mϕ viability throughout the culture periods (not shown). Nitrite levels in culture supernatants were measured using the Griess reagent (172) as described in Chapter I. Briefly, 100 μL Mϕ supernatants were added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3P0_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm (MR 600 microplate absorbance reader; Dynatech Laboratories, Alexandria, VA).

For Western analysis of iNOS expression (see Appendix B for details), Mϕs were cultured as described for 12 h. Cells were lysed in 50 mM Tris-Cl pH 7.6 containing 10 μg/ml of leupeptin and aprotinin (Sigma) and 300 mM NaCl. Membranes were pelleted by centrifugation (12,000 x *g*), and protein determined using the Lowry microtiter method (see Apppendix B). Protein (15 μg) was denatured by boiling in 2-ME (5%) and separated by SDS-PAGE using a 10% ProtoGel (National Diagnostics, Atlanta, GA) vertical gel, transferred to nitrocellulose, and blocked using 5% non-fat milk. A polyclonal rabbit anti-mouse iNOS Ab (Transduction Laboratories, Lexington, KY) was

used at 1:5000 dilution and HRP-conjugated goat-anti-rabbit IgG secondary Ab (Transduction Laboratories) was used at 1:2000 dilution. Bound iNOS was detected with Luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

IL-12 Protein Measurement

IL-12 production was induced by culturing Mϕs as described for NO production, After 18 h, supernatants were collected and immediately assayed for IL-12 using a p70-specific ELISA (IL-12 DuoSet®; R&D Systems, Minneapolis, MN) per the manufacturer's protocol. The ELISA consisted of anti-mouse IL-12 p70 capture antibody adhered to high-affinity protein binding plates (Nunc MaxiSorp™ ELISA plates), biotinylated secondary antibody, and HRP-conjugated strepavidin detection reagent. ELISA were developed with Sigma tetramethylbenzidine (TMB) liquid substrate system, and absorbance was determined at 450 nm using a MR-600 microplate reader (Dynex, Alexandria, VA). The limit of detection in our hands was approximately 10 pg/ml.

Western Analysis of ICSBP

For Western analysis of Mϕ ICSBP, Mϕs were cultured and cellular proteins prepared as described for iNOS analysis. Protein (20 μg) was denatured by boiling in 2-ME (5%) and separated by SDS-PAGE using a 10% ProtoGel (National Diagnostics) vertical gel, transferred to nitrocellulose, and blocked using 5% non-fat milk. A polyclonal rabbit anti-mouse ICSBP Ab (Zymed Laboratories, Inc., South San Francisco, CA) was used at 2 μg/ml, per the manufacturer's recommendation. HRP-conjugated goat-anti-rabbit IgG secondary Ab (Transduction Laboratories) was used at 1:2000 dilution.

Bound ICSBP was detected with Luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistics and Calculations

Triplicate cultures were tested for nitrite and IL-12. Data for NO and IL-12 are averages and SEM of triplicate independent determinations; data for luciferase activity are mean of duplicate determinations. All experiments were repeated at least three times and representative experiments are shown.

RESULTS

LPS and Paclitaxel Induce Luciferase Activity in A4 Cells

The a4 cell line, a RAW264.7 Mϕ line transfected with an HIV-1 LTR promoter and luciferase reporter gene, is responsive to LPS-mediated activation (404). To verify that a4 cell luciferase activity is LPS-dependent in our hands, a4 cells (1.0 x 10^6 cells) were cultured in 1 ml of complete medium with various doses of LPS (0.1 - 10 μg/ml) for 2 h (Figure 20). Luciferase activity in cell lysates increased in a dose-dependent manner in response to LPS (Figure 20).

To establish that a4 cells respond to paclitaxel-mediated activation, a4 cells were cultured as described with clinically- and physiologically-relevant doses of paclitaxel (0.1 - 10 μM) for 2 h. In a profile mimicking LPS-mediated activation, paclitaxel increased a4 luciferase activity in a dose-dependent manner (Figure 20).

Tumor-derived Factors Inhibit LPS- and Paclitaxel-induced Activation of HIV-1 LTR-mediated Luciferase Activity.

Because Meth-KDE fibrosarcoma cells produce soluble factors that modulate antitumor immune activity, we assessed the effect of tumor cell-derived factors on LPS- and paclitaxel-mediated induction of a4 cell luciferase activity (Figure 21). A4 cells were cultured, as described, either without or with supernatants (1:2 dilution) from 72 h cultures of Meth-KDE cells (4 x 10^6 cells). A4 cells were activated with either LPS (1.0 or 10 μg/ml) or paclitaxel (1.0 or 10 μM) for 2 h and luciferase activity determined. Tumor supernatant was strongly inhibitory of a4 cell activation, suppressing LPS-(1.0 or

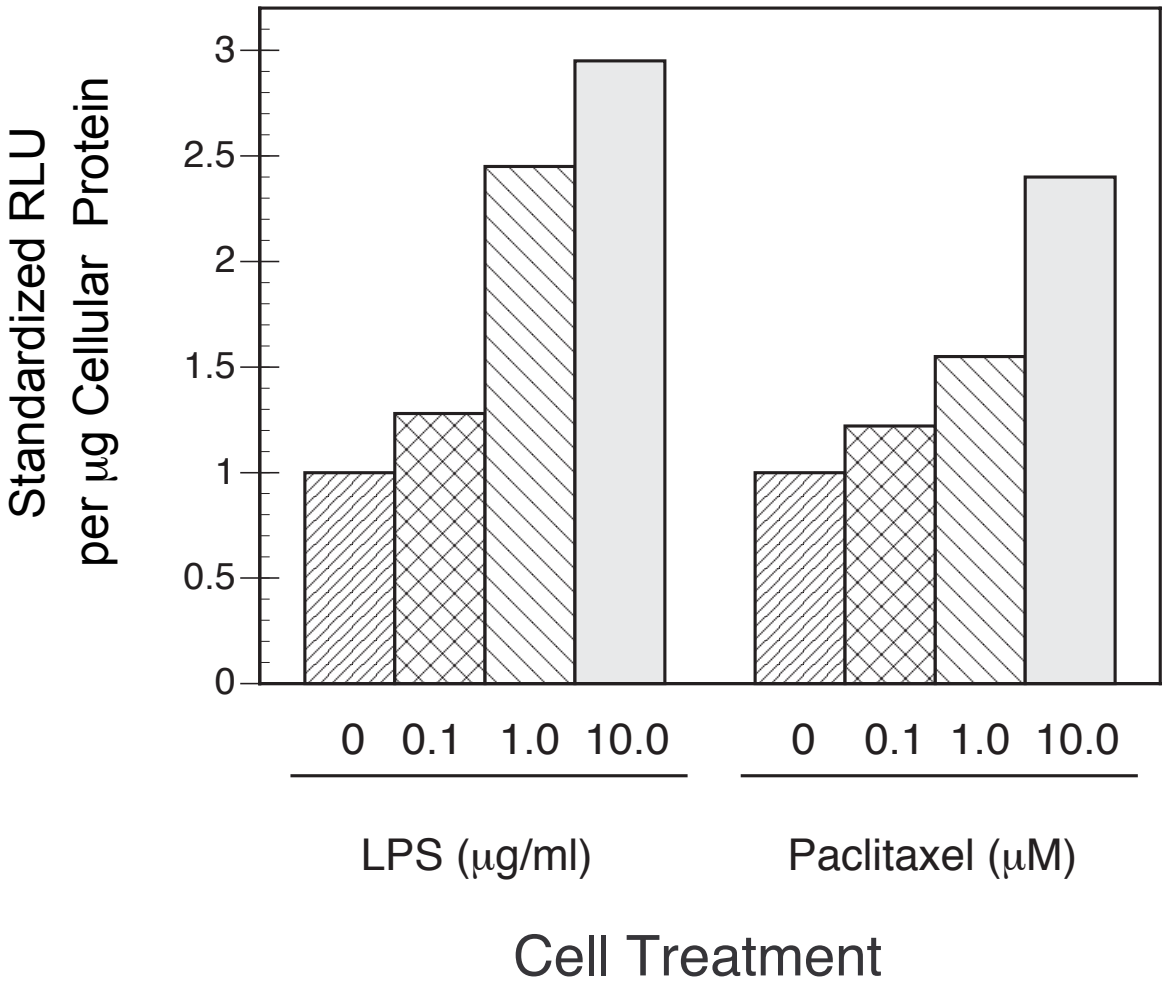


Figure 20. LPS and paclitaxel induce luciferase activity in a4 cells. A4 cells (RAW264.7 Mϕs transfected with an HIV-1 LTR promoter driving expression of a luciferase reporter gene) were cultured in 1 ml of complete medium (1.0 x 10^6 cells) with various doses of LPS (0.1 - 10 μg/ml) or paclitaxel (0.1 - 10 μM) for 2 h. Lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

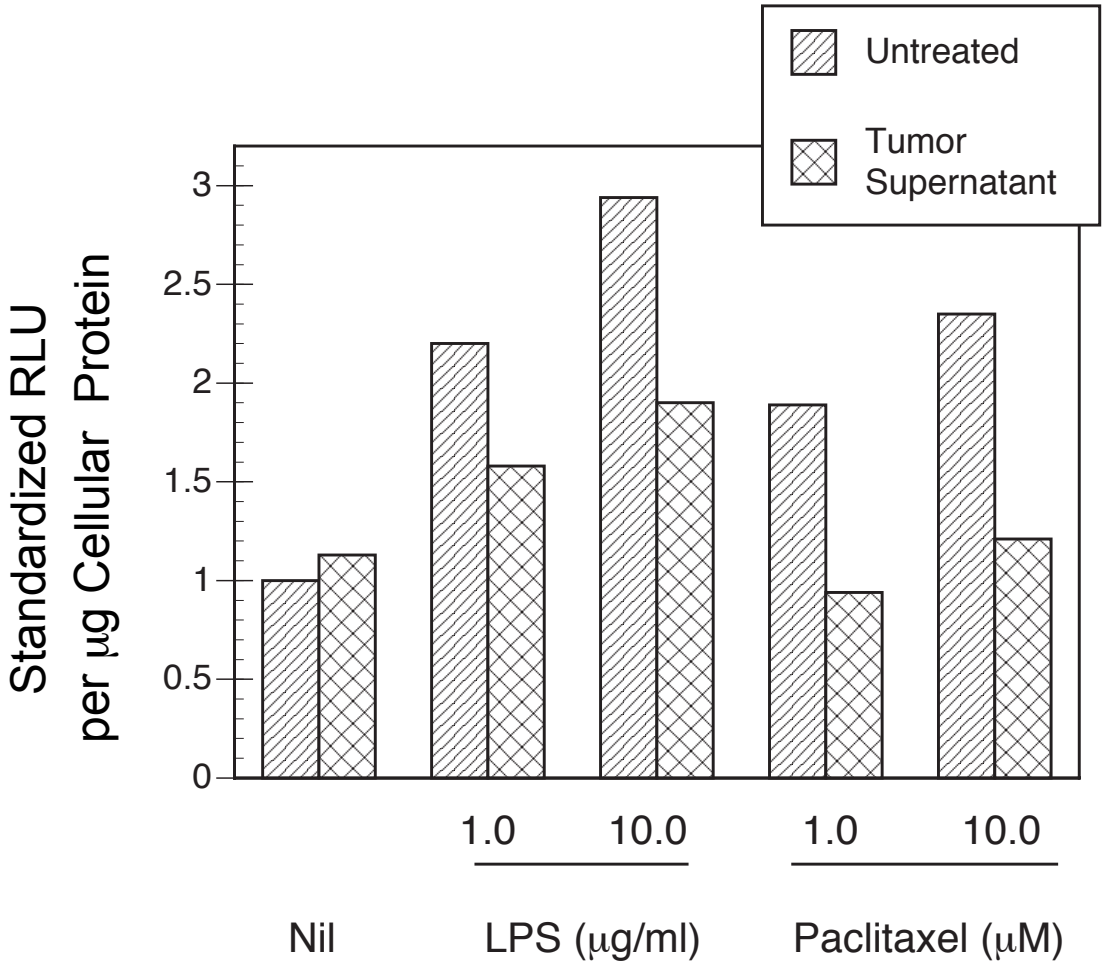


Figure 21. Tumor-derived factors inhibit LPS- and paclitaxel-induced activation of HIV-1 LTR-mediated luciferase activity. A4 cells (1.0×10^6) were cultured either without or with 72 h Meth-KDE supernatants (1:2 dilution). Some cultures were activated with either LPS (1.0 or 10 μg/ml) or paclitaxel (1.0 or 10 μM) for 2 h. Lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

10.0 μg/ml)-induced luciferase activity by approximately 32-36% and paclitaxel (1.0 or 10.0 μM)-induced luciferase activity by 51-52%, respectively. In fact, paclitaxel failed to induce significant activation of a4 cells in the presence of tumor supernatants. Paclitaxel at the optimal dose (10 μM) mediated an 89% increase in luciferase activity; in contrast, tumor-supernatant modulated paclitaxel's capacity to activate a4 cells, led to luciferase activity only 7% greater than unactivated cells.

Tumor-derived Factors Downregulate RAW264.7 Mϕ NO Production and iNOS Expression.

Because tumor-derived factors, including TGF-β_1, IL-10, and PGE_2, can inhibit NO production by primary (splenic and peritoneal exudate) Mϕs (15), we assessed the effect of tumor-derived factors on LPS- and paclitaxel-induced RAW264.7 Mϕ NO production. Priming and activation with IFN-γ (10 U/ml) and LPS (10 μg/ml) or paclitaxel (10 μM) for 24 h enhanced RAW264.7 Mϕ NO production, but the addition of tumor-derived supernatant to culture suppressed NO production regardless of priming and activation signals (Figure 22). Kinetic analyses, in which Mϕs were cultured with activating agents in the presence or absence of tumor supernatants (1:2), revealed that tumor-derived factors modulated production of NO throughout the course of a 48 h culture with LPS (Figure 23 A) or paclitaxel (Figure 23 B).

NO production requires Mϕ expression and translation of iNOS, the enzyme that mediates the conversion of L-arginine into ornithine and free NO (466). Therefore, NO production can be affected at the level of transcription, translation, or enzymatic activity. To determine whether tumor-derived factors dysregulate Mϕ production of the iNOS

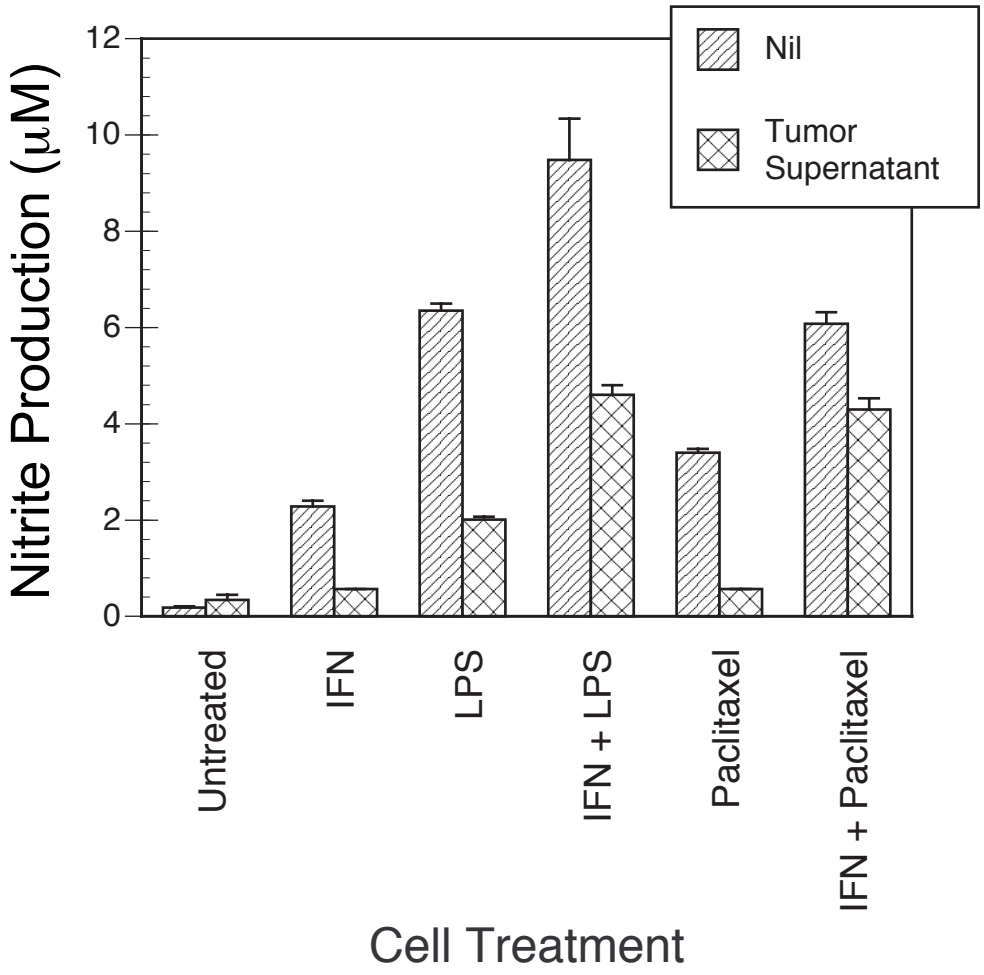


Figure 22. Tumor-derived factors downregulate LPS- and paclitaxel-induced RAW264.7 Mϕ NO production. RAW264.7 Mϕs (2 x 10^5 cells) were cultured without or with 72 h Meth-KDE supernatants (1:2 dilution). Tumor-derived factors modulated NO production in response to priming and activation with IFN-γ (10 U/ml) and LPS (10 μg/ml) or paclitaxel (10 μM), respectively, for 24 h.

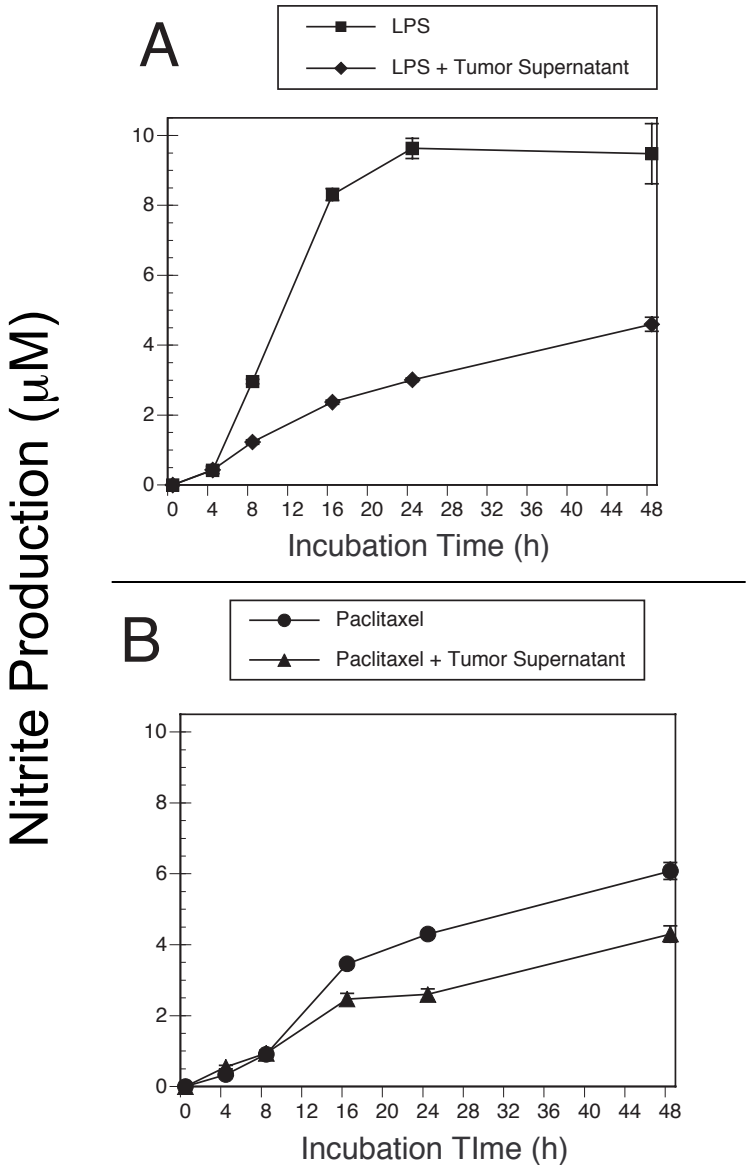


Figure 23. Tumor-derived factors prevent the induction of NO production by LPS- and paclitaxel-activated RAW264.7 Mϕs. RAW264.7 Mϕs (2 x 10^5 cells) were cultured without or with 72 h Meth-KDE supernatants (1:2 dilution). Tumor-derived factors modulated NO production in response to priming and activation with IFN-γ (10 U/ml) and LPS (10 μg/ml, Panel A) or paclitaxel (10 μM, Panel B) over a period of 48 h.

enzyme, RAW264.7 Mϕs were primed and activated with IFN-γ (10 U/ml), LPS (10 μg/ml), and paclitaxel (10 μM) either without (Figure 24) or with (Figure 25) 72 h Meth-KDE supernatants (1:2 dilution) for 12 h. Total cellular proteins were harvested, separated on 10% polyacrylamide gels, electroblotted to nitrocellulose, and probed with rabbit-anti-murine iNOS polyclonal antisera. Parallel cultures were tested for NO production by Griess reaction. Western analysis revealed that priming and activation enhanced iNOS expression, and the level of iNOS paralleled the production of NO (Figure 24). In agreement with our previous results in primary Mϕs (see Figure 6), iNOS expression is closely linked to the level of NO production. In the presence of tumor-derived factors, iNOS expression was inhibited (Figure 25), and LPS (10 mg/ml) or paclitaxel (10 mM) induced only minimal iNOS expression. In the presence of tumor-derived factors, paclitaxel-mediated iNOS expression was no different than untreated Mϕs, as revealed by densitometric analysis.

Tumor-derived Factors Downregulate RAW264.7 Mϕ IL-12 Production.

Because IL-12 is an important Mϕ-derived mediator of host antitumor immune responses, and tumor growth dysregulates IL-12 production by primary Mϕs (182,298) (to be discussed in Chapter VI), we cultured RAW264.7 Mϕs (2×10^5 cells) without or with 72 h Meth-KDE supernatants (1:2 dilution) for 18 h and measured IL-12 production using a p70-specific ELISA (Figure 26). IL-12 production was strongly inhibited, regardless of activation, by tumor-derived factors. Notably, tumor supernatant significantly decreased paclitaxel's capacity to induce IL-12 production to only 25.5% of the level achieved in the absence of tumor-derived immunomodulatory factors.

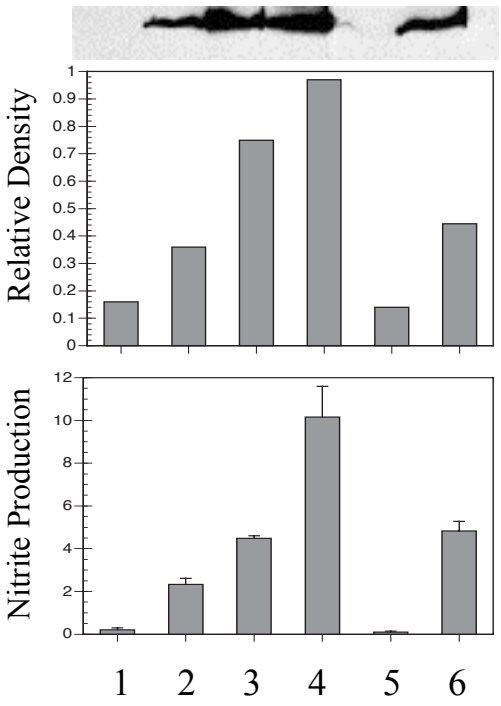


Figure 24. LPS or paclitaxel induce RAW264.7 Mϕ NO production and iNOS expression. RAW264.7 Mϕs were cultured for 12 h (Western analysis of iNOS) or 24 h (nitrite determination). IFN-γ (10 U/ml, lanes 2, 4, and 6), LPS (10 μg/ml, lanes 3 and 4), and paclitaxel (10 μM, lanes 5 and 6) were added at the start of culture. Densitometric analysis was performed using NIH-Image software for Macintosh and represents the relative density of each band.

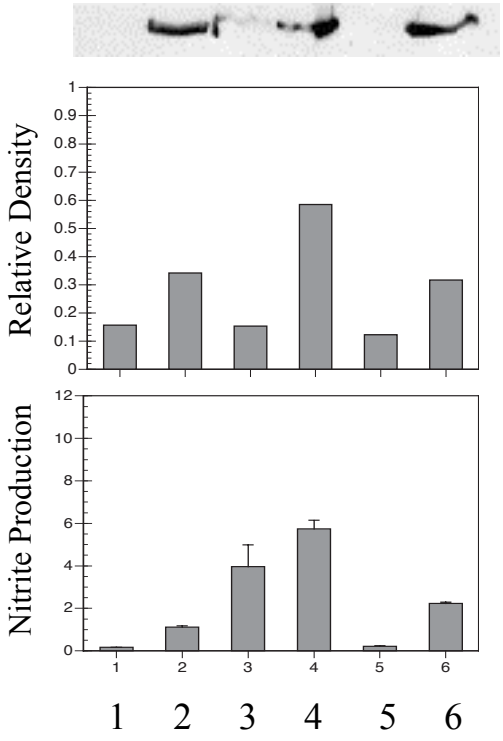


Figure 25. Tumor-derived factors downregulate RAW264.7 Mϕ NO production and iNOS expression. RAW264.7 Mϕs were cultured either without or with 72 h Meth-KDE supernatants (1:2 dilution) for 12 h (Western analysis of iNOS) or 24 h (nitrite determination). IFN-γ (10 U/ml, lanes 2, 4, and 6), LPS (10 μg/ml, lanes 3 and 4), and paclitaxel (10 μM, lanes 5 and 6) were added at the start of culture. Densitometric analysis was performed using NIH-Image software for Macintosh and represents the relative density of each band.

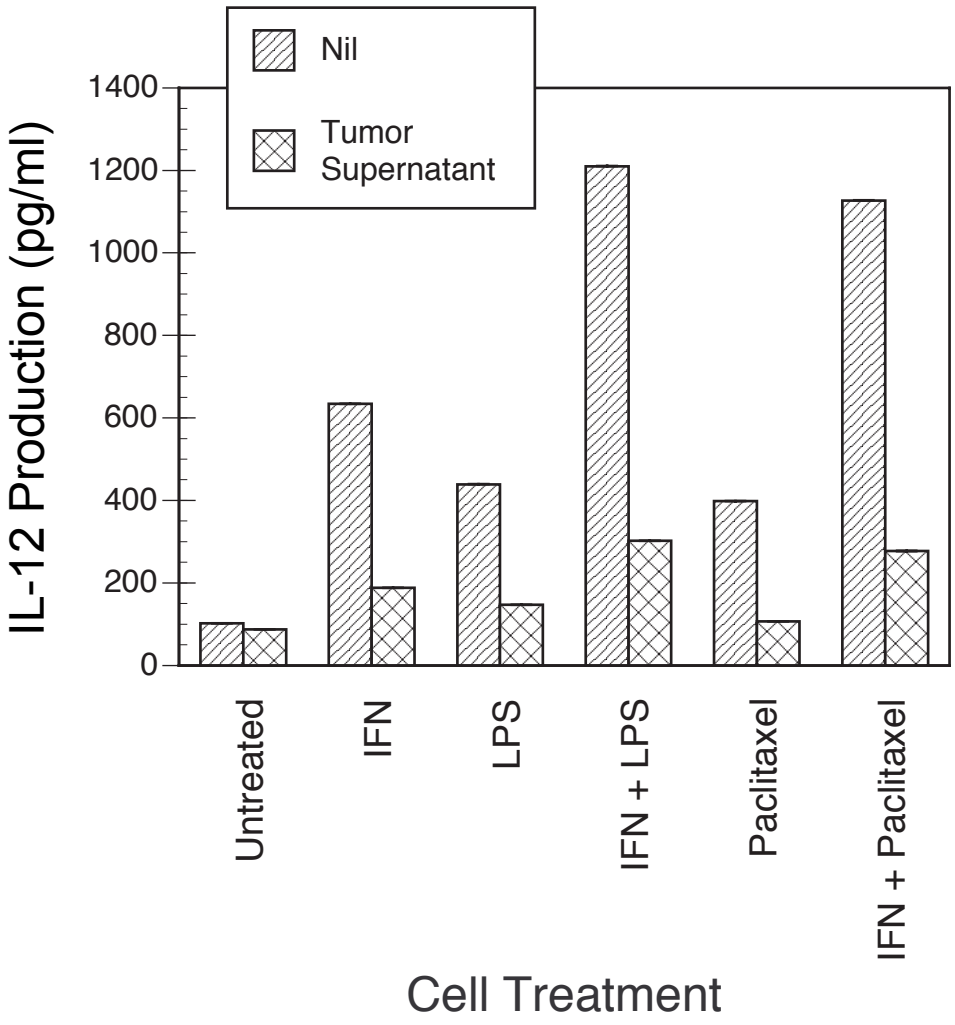


Figure 26. Tumor-derived factors downregulate RAW264.7 Mϕ IL-12 production. RAW264.7 Mϕs (2 x 10^5 cells) were cultured without or with 72 h Meth-KDE supernatants (1:2 dilution) for 18 h. Some cultures were primed with IFN-γ (10 U/ml) and activated with either LPS (10 μg/ml) or paclitaxel (10 μM). Tumor supernatant modulated IL-12 production regardless of activation.

Tumor-derived TGF-β_1 and IL-10 Inhibit LPS- and Paclitaxel-induced A of A4 Mϕs.

The Meth-KDE fibrosarcoma produces several immunomodulatory factors, including TGF-β_1, IL-10, and PGE_2 (15,123), all of which could potentially contribute to the modulation of paclitaxel-mediated activation of Mϕs. In order to determine the role of various factors in the tumor-induced modulation of paclitaxel-mediated Mϕ activation, Meth-KDE supernatants were depleted of specific molecules, either by Ab absorption (TGF-β_1- and IL-10-depleted supernatants) or the use of inhibitors to block production (PGE_2-lacking supernatants). A4 cells (1.0×10^6) were cultured without or with LPS (10 μg/ml) or paclitaxel (10 μM) and supplemented with either fresh or factor-depleted Meth-KDE supernatant (1:2 dilution). Parallel cultures were treated with recombinant cytokines or PGE_2. After 2 h, lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations. Meth-KDE supernatant downregulated a4 luciferase activity in all cultures, but depletion of TGF-β_1 (Figure 27) or IL-10 (Figure 28) partly reversed the inhibitory effects of the tumor-derived factors. Addition of physiologic levels (15) of recombinant TGF-β_1 (10.0 ng/ml) or IL-10 (3.0 U/ml) also modulated a4 luciferase activity. In contrast, supernatant from indomethacin (10^{-7} M)-treated Meth-KDE cells retained the capacity to inhibit a4 luciferase activity (Figure 29), and exogenous PGE_2 (25.0 ng/ml) was only moderately suppressive of a4 activation as compared with TGF-β_1 or IL-10. These data suggest that Meth-KDE-derived TGF-β_1 (Figure 27) or IL-10 (Figure 28), in part, are primary mediators of tumor-induced suppression of paclitaxel-mediated Mϕ activation.

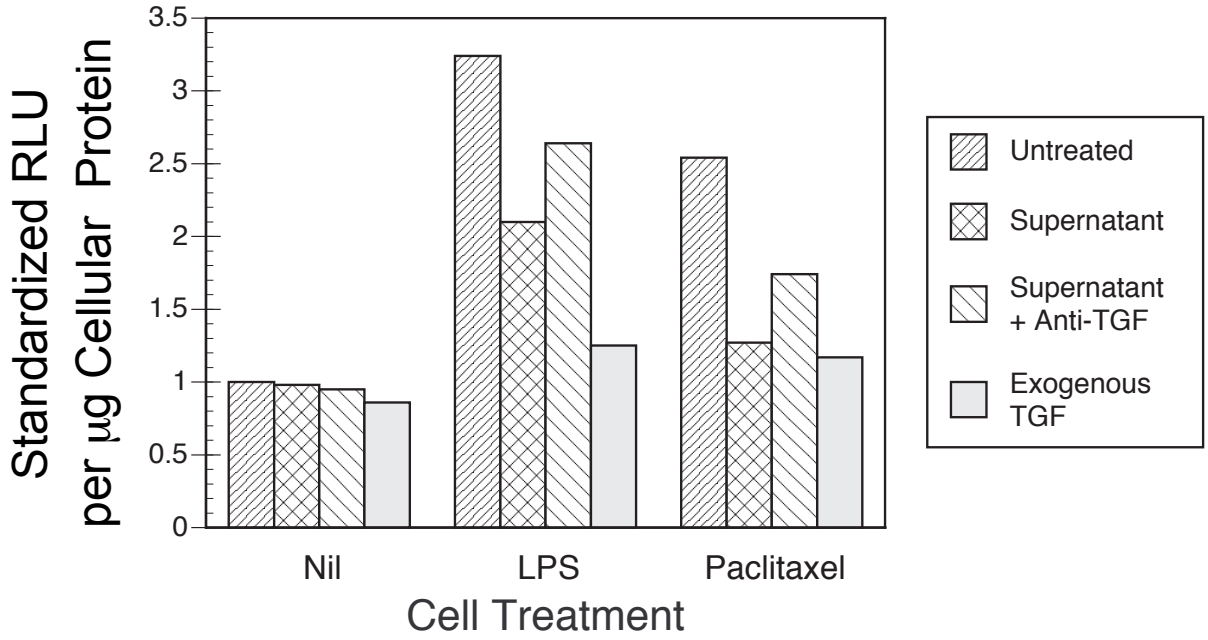


Figure 27. Tumor-derived TGF-β_1 inhibit LPS- and paclitaxel-induced activation of a4 Mϕs. A4 cells (1.0 x 10^6) were cultured without or with LPS (10 μg/ml) or paclitaxel (10 μM). Some cultures were supplemented with either fresh or TGF-β_1-depleted Meth-KDE supernatant (1:2 dilution); parallel cultures were treated with rTGF-β_1 (10 ng/ml). After 2 h, lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

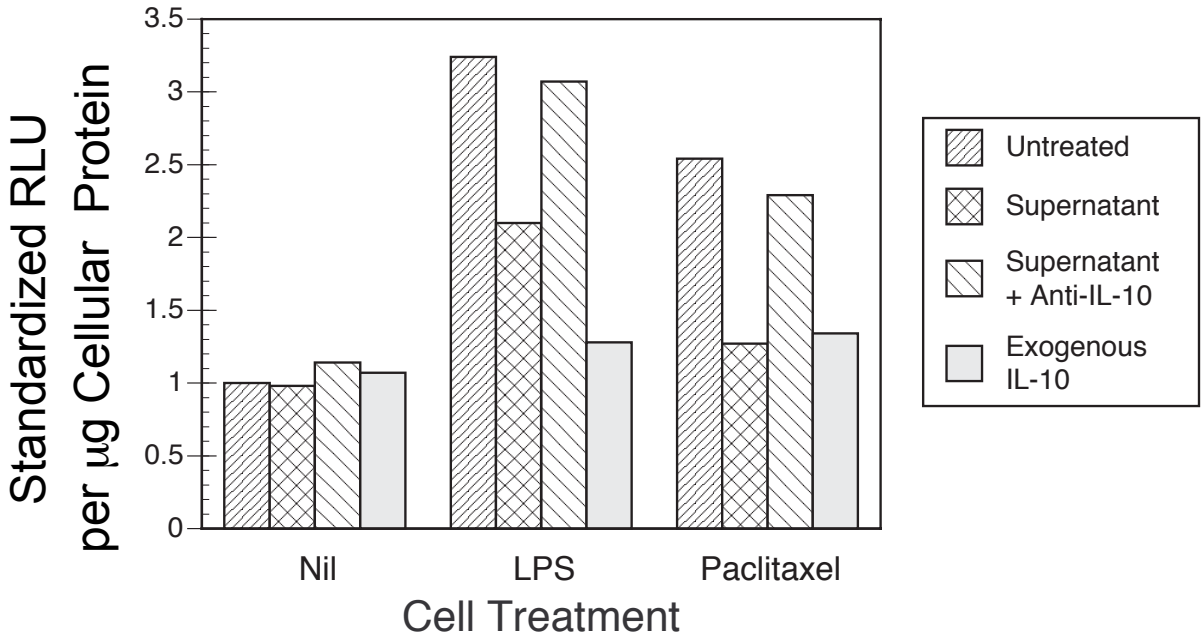


Figure 28. Tumor-derived IL-10 inhibits LPS- and paclitaxel-induced activation of a4 Mϕs. A4 cells (1.0 x 10^6) were cultured without or with LPS (10 μg/ml) or paclitaxel (10 μM). Some cultures were supplemented with either fresh or IL-10-depleted Meth-KDE supernatant (1:2 dilution); parallel cultures were treated with IL-10 (3.0 U/ml). After 2 h, lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

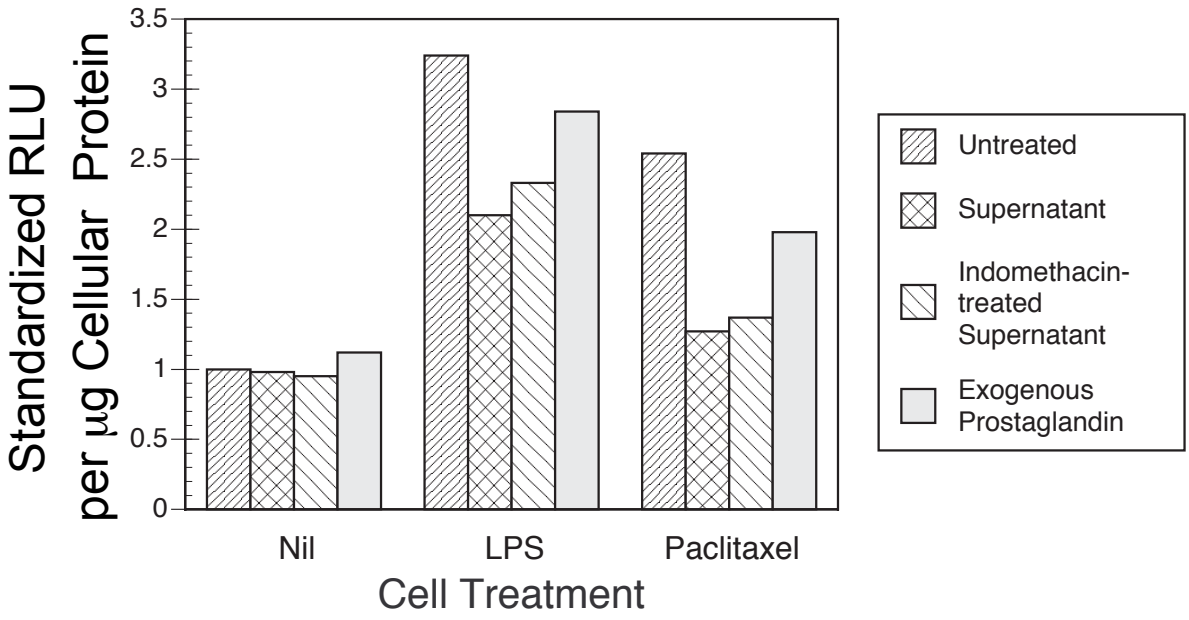


Figure 29. Tumor-derived PGE_2 is not a significant inhibitor of LPS- and paclitaxel-induced activation of a4 Mϕs. A4 cells (1.0×10^6) were cultured without or with LPS (10 μg/ml) or paclitaxel (10 μM). Some cultures were supplemented with fresh Meth-KDE supernatant (1:2 dilution). Parallel cultures were supplemented with supernatant from indomethacin (10^{-7} M)-treated Meth-KDE cells (1:2 dilution) or exogenous PGE_2 (25.0 ng/ml). After 2 h, lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

Pretreatment with Tumor-derived Factors Primes A4 Mϕs for Enhanced Responsiveness to LPS- and Paclitaxel-induced Activation.

To determine whether a4 cell responses to LPS and paclitaxel are differentially regulated by continuous versus transient exposure to tumor-derived factors, a4 cells were either co-treated or pre-treated with activating agents and tumor supernatant (Figure 30). For pre-treatment, a4 cells (1 x 10^6) were cultured with tumor supernatant for 4 h, washed twice in fresh medium, and recultured in fresh medium without or with LPS (10 μg/ml) or paclitaxel (10 μM) for an additional 2 h. For co-treatment cultures, tumor supernatants and activating agents were added simultaneously at the start of the 2-h culture period. In contrast to co-treatment cultures, in which tumor-derived factors suppressed LPS- and paclitaxel-induced luciferase activity, pre-treatment primed the a4 cells for enhanced response to activation, as indicated by increased luciferase activity (Figure 30).

Tumor-derived Factors Downregulate RAW264.7 Mϕ ICSBP Expression

Because tumor-derived factors can inhibit Mϕ production of IFN-γ-inducible factors, such as IL-12, we assessed the effect of tumor-derived factors on expression of the important signal transduction molecule ICSBP. Mϕs were primed with IFN-γ (10 – 100 U/ml) and activated with LPS (10 μg/ml) or paclitaxel (10 μM) for 24 h. In the absence of tumor-derived factors, RAW264.7 Mϕ ISCBP expression was increased in a dose-dependent manner by IFN-γ (Figure 31), but LPS or paclitaxel did not induce ICSBP expression. When Mϕs were cultured in 72 h Meth-KDE supernatants (1:2 dilution), IFN-γ-induced ICSBP expression was downregulated (Figure 32). Tumor-derived factors inhibited ICSBP expression, even when Mϕs were primed with high doses of IFN-γ (100 U/ml).

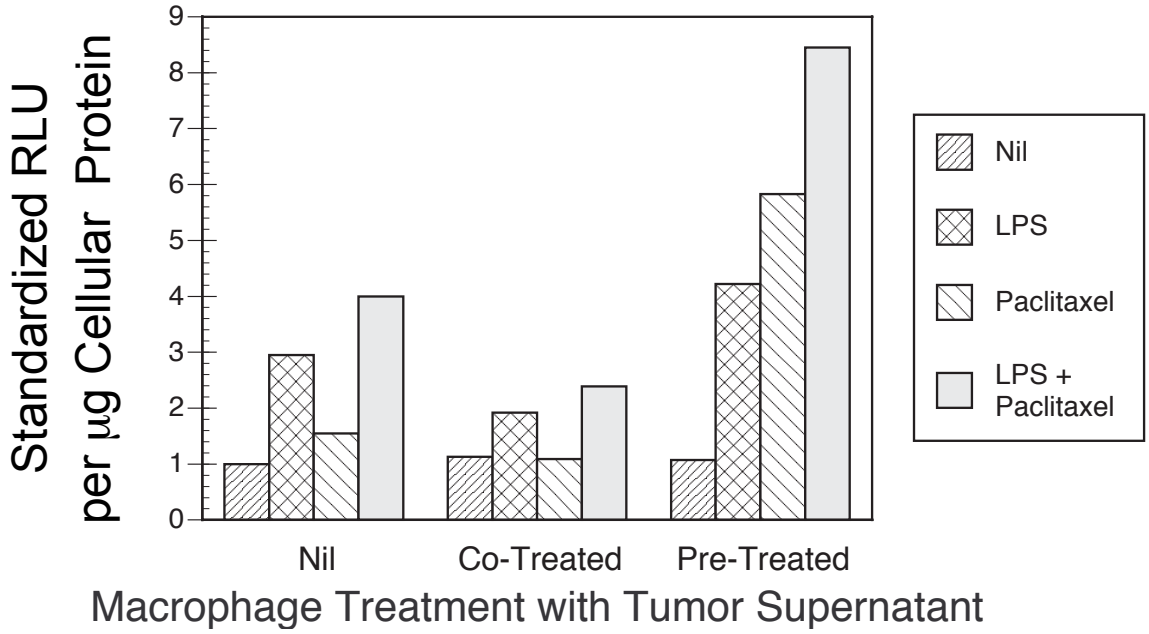


Figure 30. Pretreatment with tumor-derived factors primes a4 Mϕs for enhanced responsiveness to LPS- and paclitaxel-induced activation. A4 cells (1.0×10^6) were cultured without or with 72 h Meth-KDE supernatant (1:2 dilution). For pre-treatment cultures, a4 cells were cultured with tumor supernatant for 4 h, washed twice in fresh medium, and recultured in fresh medium without or with LPS (10 μg/ml) or paclitaxel (10 μM) for an additional 2 h. For co-treatment cultures, tumor supernatants and activating agents were added simultaneously at the start of the 2 h culture period. Lysates were prepared and assayed for luciferase activity. Data points are the average of duplicate determinations.

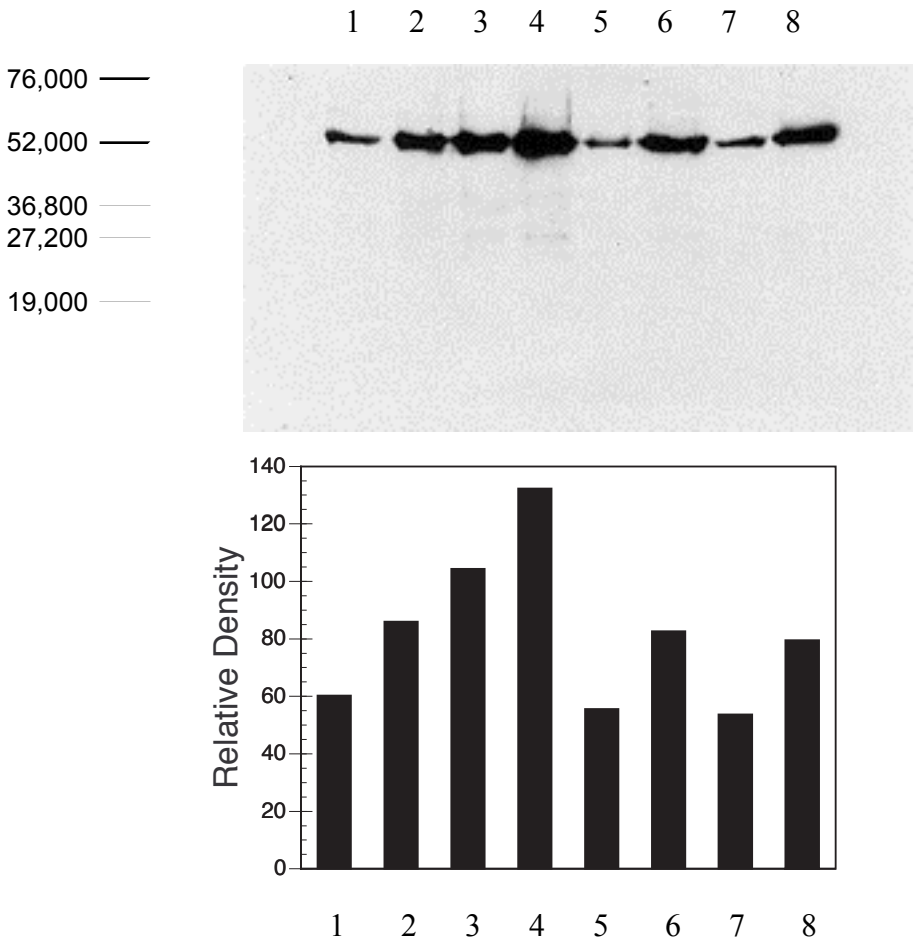


Figure 31. IFN-γ induces RAW264.7 Mϕ ICSBP expression. RAW264.7 Mϕs were cultured in fresh medium (lane 1), primed with IFN-γ (10 – 100 U/ml, lanes 2-4), LPS (10 μg/ml, lane 5), IFN-γ (10 U/ml) + LPS (lane 6), paclitaxel (10 μM, lane 7), or IFN-γ + paclitaxel (lane 8) for 12 h Densitometric analysis was performed using NIH-Image software for Macintosh and represents the relative density of each band.

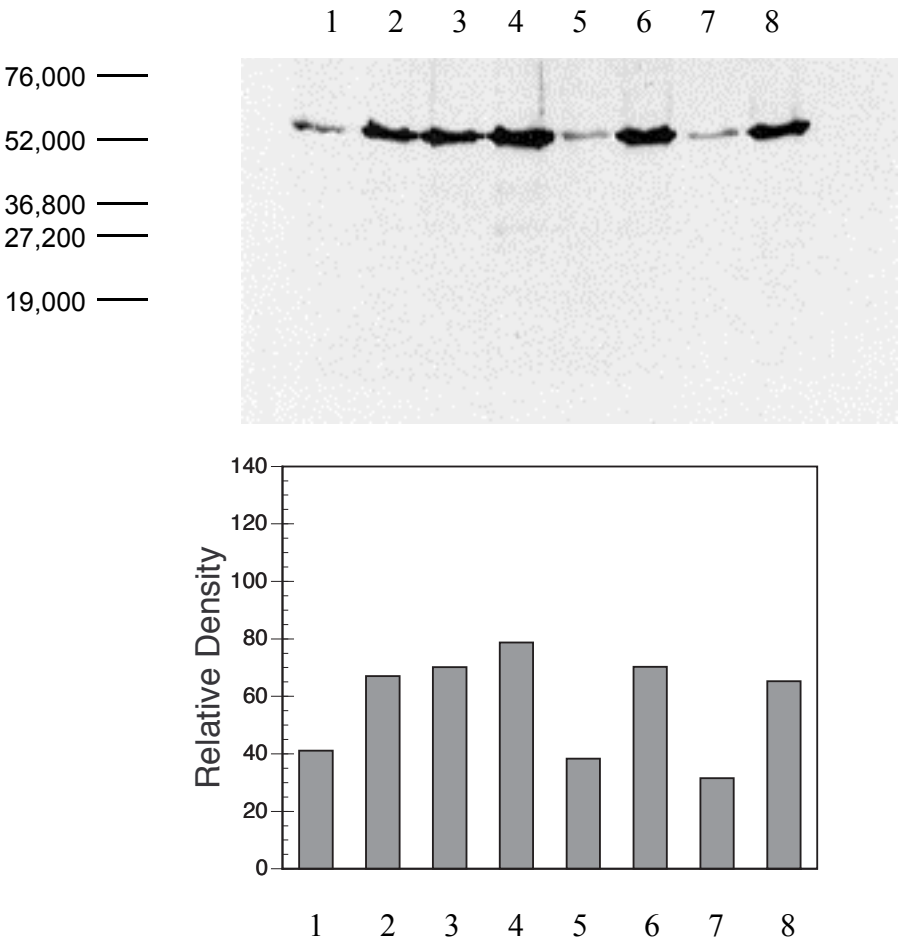


Figure 32. Tumor-derived factors downregulate RAW264.7 Mϕ ICSBP expression. RAW264.7 Mϕs cultured in fresh medium (lane 1), primed with IFN-γ (10 – 100 U/ml, lanes 2-4), LPS (10 μg/ml, lane 5), IFN-γ (10 U/ml) + LPS (lane 6), paclitaxel (10 μM, lane 7), or IFN-γ + paclitaxel (lane 8) for 12 h in the presence of 72 h Meth-KDE supernatant (1:2 dilution). Densitometric analysis was performed using NIH-Image software for Macintosh and represents the relative density of each band.

DISCUSSION

Activity of the HIV-1 LTR has been reported to be almost completely dependent upon LPS or LPS-like stimuli (404), making the a4 cells useful for the study of tumor-induced effects on Mϕ activation. The luciferase reported gene provides a simple, inexpensive, and rapid readout system for NF-κB-mediated signaling, and luciferase activity in a4 cell lysates increased in a dose-dependent manner in response to LPS activation (Figure 20).

Taking advantage of this readout system, we assessed the effects of the major suppressor molecules (TGF-β_1, IL-10, and PGE_2) produced by the Meth-KDE fibrosarcoma cells used in our tumor model (15) on Mϕ activation. Further, because we (296) and others (267) have demonstrated that paclitaxel differentially regulates tumor-induced Mϕ populations (296,299,301), we used the a4 cell line to characterize paclitaxel-mediated Mϕ activation and factor production in the absence or presence of tumor-derived factors. The a4 cell has the specificity and appropriate functionality for these experiments because the cells respond to paclitaxel-mediated activation, as assessed by measuring luciferase activity, in a dose-dependent manner (Figure 20). When treated with Meth-KDE-derived tumor supernatant, LPS- or paclitaxel-activated a4 cell activation is suppressed by approximately 30% to 50%, respectively. Notably, paclitaxel failed to induce significant activation of a4 cells in the presence of tumor supernatants, even when used at the optimal dose level (10 μM).

The luciferase activity in a4 cells is driven by an NF-κB-responsive promoter element; therefore, we also assessed the effects tumor-derived factors on the NF-κB-responsive molecules iNOS and IL-12 using RAW264.7 Mϕs (the non-transfected

parental line of the a4 cells). Priming with IFN-γ (10 U/ml) and activation with LPS (10 μg/ml) or paclitaxel (10 μM) for 24 h enhanced NO production (Figure 22), but the addition of tumor-derived factors downregulated NO production, even when activated with the optimal dose of LPS (Figure 23). Likewise, priming and activation enhanced iNOS expression (in a manner that paralleled the production of NO, Figure 24), but tumor-derived factors modulated both iNOS expression and NO production (Figure 25). Strikingly, even LPS (10 mg/ml) induced only minimal iNOS expression, suggesting that tumors dysregulate Mϕ expression of genes required to express cytotoxic activity. IFN-γ, however, did induce a modest increase in iNOS expression and NO production, suggesting that IFN-γ may be an important cytokine for mediating Mϕ priming and activation for cytotoxic antitumor responses *in situ*. Given that tumors modulate IFN-γ production (182,300), these data may explain why tumors appear to evade Mϕ-mediated antitumor cytotoxic *in vivo*. Loss of IFN-γ production may either result from or lead to compromised IL-12 production, and tumor-derived factors dysregulate Mϕ production of IL-12 (Figure 26). The dramatic inhibition of IL-12 production by tumor-derived supernatants suggests that IL-12 production *in vivo* may be strongly inhibited, effectively breaking the link between Mϕ-mediated innate immunity and T-cell-mediated acquired immunity. Because specific T-cell antitumor cytotoxic responses are necessary to achieve significant regression of neoplasia, tumor-induced dysregulation of IL-12 may account for system immunosuppression in the TBH.

Because the Meth-KDE fibrosarcoma produces several immunomodulatory factors, including TGF-β_1, IL-10, and PGE_2 (15,123), we next assessed the effects of individual factors on the suppression of paclitaxel-mediated Mϕ activation. Meth-KDE supernatant downregulated a4 Mϕ activation in response to LPS or paclitaxel, and depletion of TGF-β_1 (Figure 27) or IL-10 (Figure 28) partly reversed the inhibitory

effects of the tumor-derived factors. Corollary experiments, in which physiologically-relevant levels (15) of recombinant TGF-β_1 or IL-10 were added to a4 cells, demonstrated that these factors individually modulate a4 luciferase activity (Figures 27-28), suggesting that tumor-derived TGF-β_1 and IL-10 may significantly downregulate Mϕ activation *in situ*. Supernatants from indomethacin-treated Meth-KDE cells, which should be free of PGE_2, inhibited a4 luciferase activity (Figure 29), and exogenous PGE_2 only moderately suppressed a4 activation. Collectively, these data suggest that the tumor-derived factors TGF-β_1 or IL-10 are the primary mediators of tumor-induced suppression of paclitaxel-mediated Mϕ activation, and that PGE_2 plays only a minor role in this model system.

In vivo, Mϕs exist in both tumor-proximal (*in situ*) and tumor-distal compartments, and these populations encounter different types and levels of inhibitory molecules produced by tumors. Unlike *in situ* populations, which are constantly exposed to high levels of tumor-derived immunomodulatory factors, distal population may experience only low-level or transient exposure to these factors. We have reported (15,296,301) that tumor-distal Mϕs produced elevated levels of NO and TNF-α on activation with LPS or paclitaxel, suggesting that these populations are primed by tumor growth for enhanced cytotoxic and effector molecule production (see Figures 4-5 and Tables 1-2). These data contrast with our *in vitro* results using a4 cells, in which tumor-derived factors modulate activation in response to LPS or paclitaxel. Therefore, we determined whether a4 cell responses to LPS and paclitaxel are differentially regulated by continuous versus transient exposure to tumor-derived factors. Pre-treatment of a4 cells with tumor supernatant for 4 h led to a priming effect, and these cells demonstrated substantial levels of luciferase activity on activation. These data suggest that transient exposure to tumor-derived factors may enhance Mϕ response to activation, and this

scenario may reflect the *in vivo* response of tumor-distal immune cells. Cells continuously treated with tumor-derived factors demonstrated suppressed LPS- and paclitaxel-induced luciferase activity, suggesting that continuous exposure to these factors prevents a4 cell activation regardless of the stimulus. These data complement our work in primary Mϕs, suggesting that tumor growth primes distal populations for enhanced cytotoxic and immunosuppressive molecule production while concurrently suppressing *in situ* Mϕ antitumor activity. Continuous stimulation with certain tumor-derived factors may suppress Mϕ activation while transient treatment induces a priming effect. Alternatively, tumors may produce distinct priming and suppressing factors; in this scenario, pretreatment (or transient, low-level exposure in tumor-distal compartments) lead to priming. Simultaneously, continuous culture (or chronic high level exposure *in situ*) overpower the priming signals and prevent Mϕ activation, and our *in vitro* data (15,123,296,299,301) support this model. These data also underscore the importance of IFN-γ in antitumor activity. In isolation, these data may suggest that restoration of IFN-γ production *in vivo* could enhance the activity of Mϕ-activating agents such as paclitaxel; however, tumor-derived factors modulate ICSBP expression (Figure 30), suggesting that tumors induce a lesion in IFN-γ-mediated signaling. Further studies will investigate the role of ICSBP in tumor-induced Mϕ dysfunction.

CHAPTER V

PACLITAXEL REVERSES TUMOR-INDUCED Mϕ-MEDIATED IMMUNOSUPPRESSION

ABSTRACT

The anticancer drug paclitaxel, which inhibits tumors through multiple cytotoxic and cytostatic mechanisms, induces distinct immunologic efficacy when it acts as a second signal for activation of *in vitro* cytotoxic activity by IFN-γ-primed murine normal host Mϕs. Because paclitaxel influences tumor cell growth dynamics and activates immune cell populations, we assessed the *ex vivo* immunosuppressive activities of paclitaxel-treated normal host and TBH Mϕs. Pretreatment of TBH Mϕs with paclitaxel partly reconstituted T-cell alloantigen reactivity, suggesting that paclitaxel mediates a partial reversal of TBH Mϕ immunosuppressive activity. Tumor-induced helper T cells, however, showed a higher sensitivity to direct paclitaxel-induced suppression. These data demonstrate that paclitaxel exerts pleiotropic effects on antitumor immune responses with the capacity to partly abate the immunosuppressive activities of Mϕs but simultaneously inhibit T-cell proliferative reactivity. Collectively, these studies suggest that the antineoplastic drug paclitaxel, which may have direct immunosuppressive activities against T-cell populations, may enhance antitumor immune activity through a correction of tumor-induced Mϕ dysfunction.

INTRODUCTION

The antineoplastic agent paclitaxel is a naturally-occurring taxane diterpenoid extracted from the bark and leaves of the western yew *Taxus brevifolia* (215). Paclitaxel has demonstrated significant efficacy as an antitumor chemotherapeutic in human clinical trials (reviewed in 178) with 20-50% objective response (274). Paclitaxel displays activity against a wide range of human cancers, notably breast cancers (195) and refractory ovarian tumors (330). The chemotherapeutic capacity of paclitaxel is realized through multiple mechanisms of antineoplastic action, including suppression of protein synthesis (263), enhancement of tumor-cell radiosensitivity (245), induction of apoptotic cell death (44), and enhancement of TNF-α-mediated cytolysis (457). Paclitaxel's primary antitumor mechanism derives from its unique polymerizing action on microtubules (373), which prevents depolymerization of α/β tubulin polymers (264) and halts cell cycle progression (362). Paclitaxel-mediated disruption of cell cycling (114) leads to neoplastic cell death and inhibition of tumor progression (148).

In addition to its well-characterized chemotherapeutic activities, paclitaxel cell cycle-independent responses in murine Mϕs (53,203). Tumor growth, however, induces multiple phenotypic and functional changes among Mϕ populations (15,122). We reported that fibrosarcoma growth altered Mϕ responsiveness to paclitaxel, modulating TBH Mϕ NO production in tumor-distal compartments (296). Because paclitaxel stimulates immune cell activities, we have evaluated paclitaxel's activity on normal host and TBH Mϕ and T-cell populations.

In this study, we extend our previous findings that suggest paclitaxel may activate Mϕs for direct antitumor cytotoxicity while controlling tumor-distal Mϕ production of

systemic proinflammatory and cytotoxic molecules. Here we report that paclitaxel decreased TBH Mϕ-mediated suppression of alloantigen-activated T-cell responsiveness. Conversely, tumor growth increased $CD4^+$ T-cell sensitivity to paclitaxel-mediated modulation of reactivity. These data collectively suggest that paclitaxel has differential immune system actions; although paclitaxel may directly suppress T-cell proliferation, it simultaneously enhances antitumor immune activity through a correction of tumor-induced Mϕ dysfunction.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice were used. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (Meth-KDE) (15,126) was used as described in Chapter I.

Media and Reagents

Mϕs were cultured in serum-free RPMI-1640 medium, prepared as described in Chapter I. Normal host and TBH T cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). T cell cultures contained 4 x 10^{-5} M 2-ME (Sigma). Paclitaxel (Calbiochem, La Jolla, CA) was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution and stored at -80°C. Paclitaxel was diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%. LPS (*Escherichia coli* serotype 026:B6), sodium nitrite, and NMMA were purchased from Sigma. Genentech, Inc. (San Francisco, CA) generously provided IFN-γ. Recombinant murine IL-2 (specific activity 2 x 10^5 U/ml) was provided by Dr. R.S. Selvan (Duke University, Durham, NC).

Immune Cell Collection

Normal host and TBH BALB/c splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm plastic plates; Lux/Miles Scientific, Naperville,

IL), washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium by scraping with a rubber policeman. The final Mϕ preparations contained cells that were >95% viable and >96% esterase positive. Flow cytometric analysis with M1/70 and F4/80 mAb (ATCC) showed them to be >80% Mac-1^+ and F4/80^+, respectively. To prevent paclitaxel carryover into proliferation assays, Mϕs were pretreated in 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY) 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 x *g*, 10 minutes) and media removed. Cells were washed once and fresh media 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 immature T-cell (ATCC; clone J11d) mAb and complement (Accurate Chemical Co., Westbury, NY). Red blood cells were lysed by 0.83% ammonium chloride treatment.

Mϕ Nitrite Production

Either normal host or TBH splenic Mϕs (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 IFN-γ, LPS, or paclitaxel that were added to Mϕ 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 x

g, 5 min). Paclitaxel did not decrease Mϕ viability because the MTT assay (292) verified >95% Mϕ viability throughout the culture periods (not shown).

Because secreted NO quickly reacts with oxygen to yield a stable nitrite endproduct (287), nitrite levels in culture supernatants were measured using the Griess reagent as described elsewhere (172). Briefly, 100 μL Mϕ supernatants were added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3P0_4; Sigma), incubated at room temperature for 10 min, and absorbance 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 Mϕ cultures lacking IFN-γ (not shown). DMSO in culture did not affect Mϕ nitrite production (not shown).

Alloantigen-activated T-cell Proliferation Assay

Either normal host or TBH Mϕs (4×10^5 cells) were pretreated without or with combinations of LPS, IFN-γ, or paclitaxel and added to normal host BALB/c $CD4^+$ T-cell cultures (2×10^5 cells). The NO inhibitor NMMA (0.5 mM) was added to parallel cultures. Irradiated (2000 R) whole splenic cell preparations (4×10^5 cells) from C3H ($H\text{-}2^k$) mice were used as allogeneic stimulator cells. Cultures were incubated in U-bottom 96-well microtiter plates (Corning) for 96 h. Eighteen h before harvest, cultures were pulsed with 1 μCi per well [^{3}H]-TdR (specific activity 6.7 Ci/mM; DuPont-NEN Research Products, Boston, MA). 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 x 10^5 cells/ml) were activated with Concanavalin-A (Con-A, 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]-TdR incorporation.

Statistics and Calculations

Cells from 6 to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested in all nitrite and proliferation assays. Data are averages ± 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 Paclitaxel Treatment Regulates TBH Mϕ NO Production

Tumor-distal Mϕs are immunosuppressive, partly because of tumor-distal over-production of NO (15). We recently reported that paclitaxel-triggered Mϕ NO production in the normal host and the TBH is differentially regulated following continuous exposure to paclitaxel (296). During chemotherapeutic regimens, however, exposure to high concentrations of paclitaxel occurs in a transient manner. In a clinically more relevant scenario, Mϕs were pretreated with relevant doses of paclitaxel (corresponding to systemic concentrations during human therapeutic antitumor treatment) for 4 h, washed, and cultured in fresh medium. Paclitaxel triggered normal host splenic Mϕ production of NO (Figure 33). In a synergistic manner, paclitaxel further enhanced NO production by IFN-γ-primed normal host Mϕs. In contrast, paclitaxel pretreatment modulated TBH Mϕ NO production (Figure 33). Although paclitaxel enhanced IFN-γ-primed TBH Mϕ NO production (as compared to unstimulated Mϕs), the level of NO was significantly reduced as compared to maximal levels achieved after treatment with the Mϕ-triggering agent LPS.

Paclitaxel Partially Reverses TBH Mϕ Immunosuppressive Activities

Because NO produced by tumor-distal Mϕs is immunosuppressive, paclitaxel-mediated modulation of TBH Mϕ NO production may lessen tumor-induced Mϕ suppressor activities. Therefore, we investigated the immunologic implications of the differential response of tumor-induced distal Mϕs to paclitaxel. Alloantigen-activated

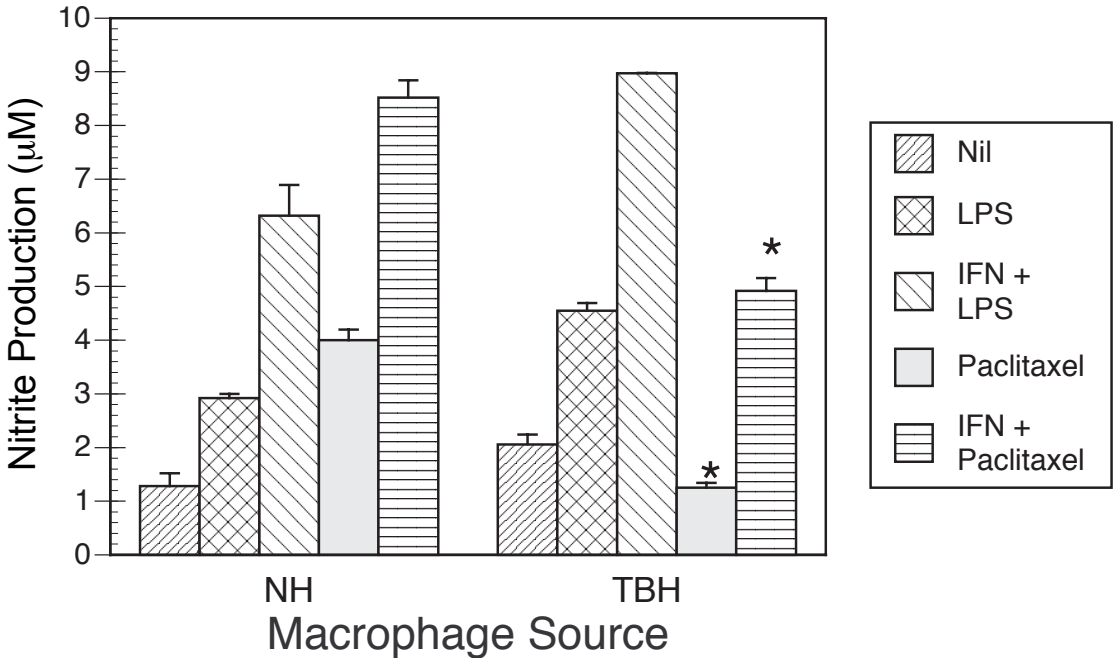


Figure 33. Paclitaxel pretreatment modulates TBH Mϕ NO production. Either 4 x 10^6 normal host or TBH splenic Mϕs were cultured in 200 μl of serum-free medium. Some cultures were primed with IFN-γ (10 U/ml). LPS (1 μg/ml) or paclitaxel (0.1, 1.0, or 10.0 μM) were added to some samples at the start of culture. Following four h of culture, media was removed, cells washed, media replenished, and culture continued. Paclitaxel-induced NO production by IFN-γ-primed TBH Mϕs was significantly different (*, $p < 0.05$) from LPS-triggered NO production. Similar results were obtained using 0.1 or 1.0 μM paclitaxel (data not shown); doses greater than 25 μM became toxic to Mϕs (data not shown). Data are averages ± SEM of triplicate independent determinations from one of four similar experiments.

proliferation of normal host T-cells was assessed in a mixed lymphocyte reaction containing untreated or pretreated normal host or TBH Mϕs (Figure 34). Paclitaxel pretreatment (4 h) of TBH Mϕ led to a modest but consistent and statistically-significant reconstitution of T-cell proliferation as compared to LPS-treated or untreated TBH Mϕs ($p < 0.05$). Untreated TBH Mϕs decreased T-cell alloreactivity by greater than 66% as compared to untreated normal host Mϕs. Paclitaxel-pretreated TBH Mϕ suppressed proliferative response by only 21.8%, suggesting that paclitaxel mediates a partial reversal of Mϕ-mediated immunosuppression in tumor-distal compartments. Modulation of Mϕ NO production may partially explain the reversal of immunosuppressive activity by paclitaxel-pretreated TBH Mϕs because abrogation of Mϕ NO production with NMMA (0.5 mM) partly reconstituted alloreactivity.

Tumor Growth Increases $CD4^+$ T-cell Sensitivity to Paclitaxel

While paclitaxel imparts immune activating functions on Mϕ populations, paclitaxel's direct cytotoxic and cytostatic activity are most effective on rapidly-dividing cell populations. Because paclitaxel may decrease T-cell proliferation and compromise responsiveness to IL-2 (56,86) and tumor growth significantly dysregulates T-cell responsiveness to alloantigen activation (124,444,475), we determined whether paclitaxel exacerbates tumor-induced changes in $CD4^+$ T-cell responsiveness. Paclitaxel significantly suppressed mitogen-induced (8 μg/ml Con-A) proliferation by both normal host and TBH T-cell populations (Table 3), suggesting factors which enhance proliferative response also enhance sensitivity to paclitaxel-mediated cytotoxicity. Interestingly, the T-cell stimulatory cytokine IL-2 (100 U/ml, Figure 35) increased T-cell

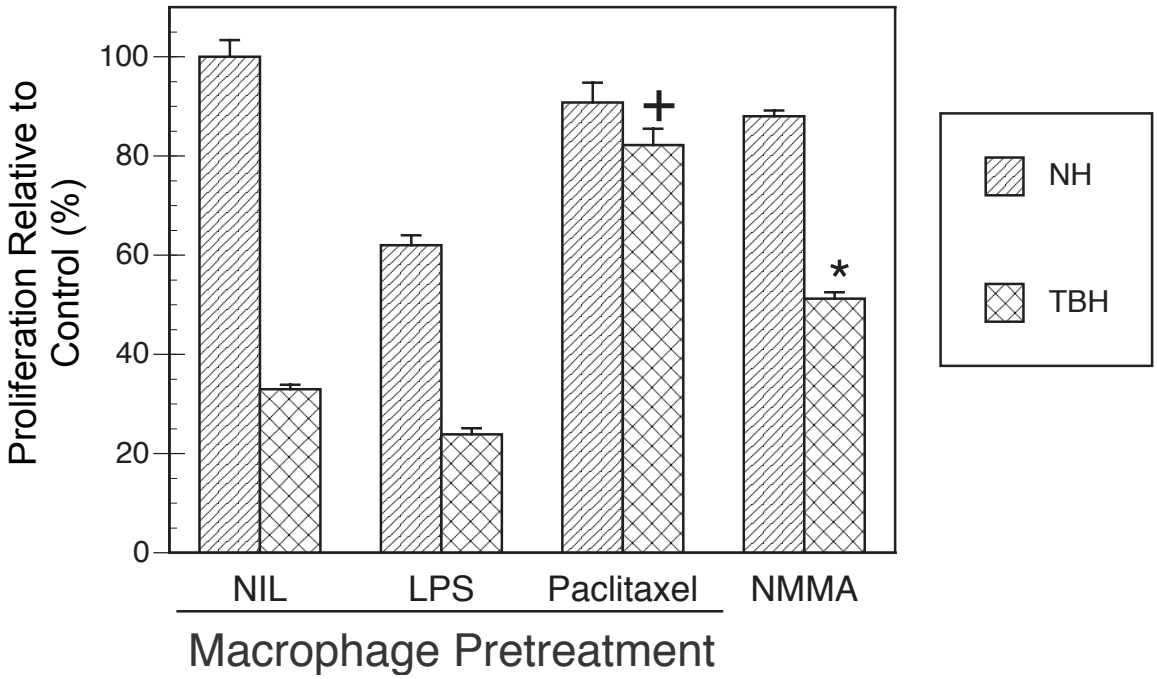


Figure 34. Paclitaxel-pretreated TBH Mϕs are less immunosuppressive of T-cell proliferation. Eight x 10^6 BALB/c normal host or TBH Mϕs were pretreated with paclitaxel and added to 4 x 10^6 normal host BALB/c $CD4^+$ T cells and 8 x 10^6 X-irradiated (2000 R) C3H whole spleen cells in a mixed lymphocyte reaction. Mϕs were pretreated in 200 μl of serum-free without or with 1 μg/ml LPS or 10 μM paclitaxel. Following 4 h of culture, Mϕs were washed and added to the T-cell cultures. NMMA (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 of ^{3}[H]-TdR and proliferation determined. Data are magnitude of response relative to proliferation in the presence of untreated normal host Mϕs (control CPM was 62690). Proliferation of normal host responder cells or X-irradiated stimulators cells alone were less than five percent of control. Paclitaxel-treated Mϕs (+, $p < 0.01$) were significantly less suppressive ($p < 0.05$) as compared to untreated or LPS-triggered Mϕs. Lower doses of paclitaxel (0.1 and 1.0 μM) yielded similar results (not shown). NMMA partially reversed TBH Mϕ-mediated suppression (*, $p < 0.05$) as compared to untreated TBH Mϕs. Data are averages ± SEM of triplicate independent determinations from one of four similar experiments.

Table 3. Tumor Growth Increases T-cell Sensitivity to Paclitaxel.

Paclitaxel[1] Addition (h)	NH T-cell Proliferation[2] (cpm)	Change in Proliferation[3] (%)	TBH T-cell Proliferation (cpm)	Change in Proliferation (%)
Nil	136.09 ± 3.42[4]	--	82.60 ± 2.19	--
0	96.82 ± 2.37	29 ↓	34.44 ± 0.52	61 ↓
24	79.74 ± 2.00	41 ↓	26.85 ± 1.02	67 ↓
48	123.81 ± 5.76	9 ↓	60.84 ± 2.11	26 ↓

[1]Paclitaxel was used at a final concentration of 10 μM and was added at the time of T-cell activation (0 h) or post-activation (24 or 48 h). Proliferation was assessed 72 h post-activation. Similar data were acquired using 0.1 μM and 1.0 μM paclitaxel (not shown).
[2]Normal host and TBH $CD4^+$ T cells (4×10^6 cells/ml) were stimulated with Con-A (8 μg/ml).
[3]Percent change in proliferative response as compared to untreated controls.
[4]Values are expressed as CPM x 10^{-3} ± SE as determined by [^{3}H]-TdR incorporation.

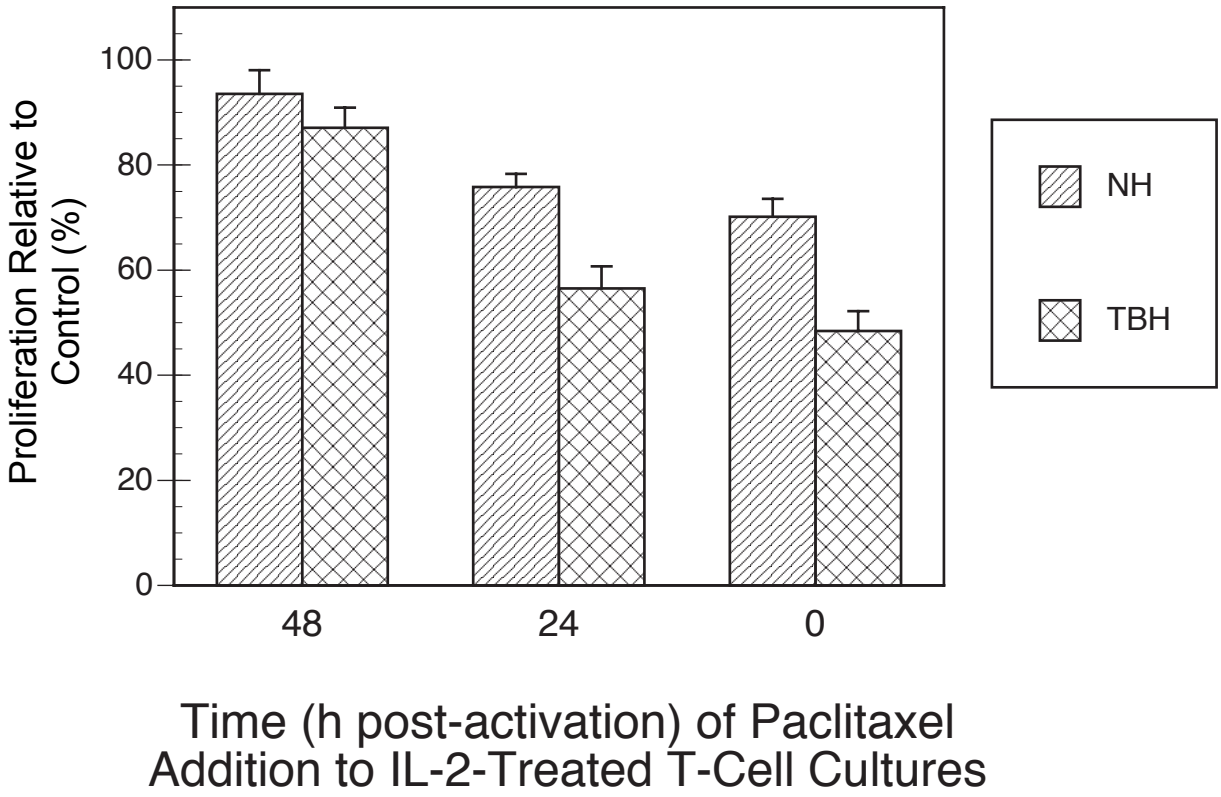


Figure 35. IL-2 increases TBH CD4$^+$ T-cell sensitivity to paclitaxel. Normal host and TBH CD4$^+$ T cells were stimulated with Con-A (8 μg/ml) and IL-2 (100 U/ml). Paclitaxel (10 μM) was added to cultures 48 or 24 h after activation or at the time of activation (0 h). Normal host and TBH T-cell proliferation were assessed after 72 hours by [^{3}H]-TdR incorporation. Similar data were acquired using 1 μM or 0.1 μM paclitaxel and 50 U/ml or 200 U/ml IL-2 (not shown). Data are magnitude of response relative to T-cell proliferation in the absence of IL-2 and paclitaxel (dashed line, control CPM was 136090) from triplicate independent determinations in one of three similar experiments.

sensitivity to paclitaxel. TBH T cells were more sensitive to paclitaxel-mediated suppression than normal host T-cells, even when paclitaxel was added 24 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 paclitaxel (4 h), washed, and cultured. The T-cell mitogen Con-A (8 μg/ml) was added at 0, 12, or 24 h post-culture (Figure 36). Paclitaxel pretreatment of TBH T cells significantly ($p < 0.05$) compromised Con-A-stimulated T-cell proliferation (Figure 36 A), and TBH T-cell proliferation was suppressed more than normal host T-cell proliferation even when cells were pretreated with paclitaxel 12 h before activation (Figure 36 B). However, both normal host and TBH $CD4^+$ T cells recovered from paclitaxel pretreatment if they were pretreated (24 h) prior to activation (Figure 36 C). Collectively, these data suggest that paclitaxel directly contributes to tumor-induced T-cell dysfunctions and that the activation status of the T cells influences the degree of sensitivity to paclitaxel-mediated inhibition.

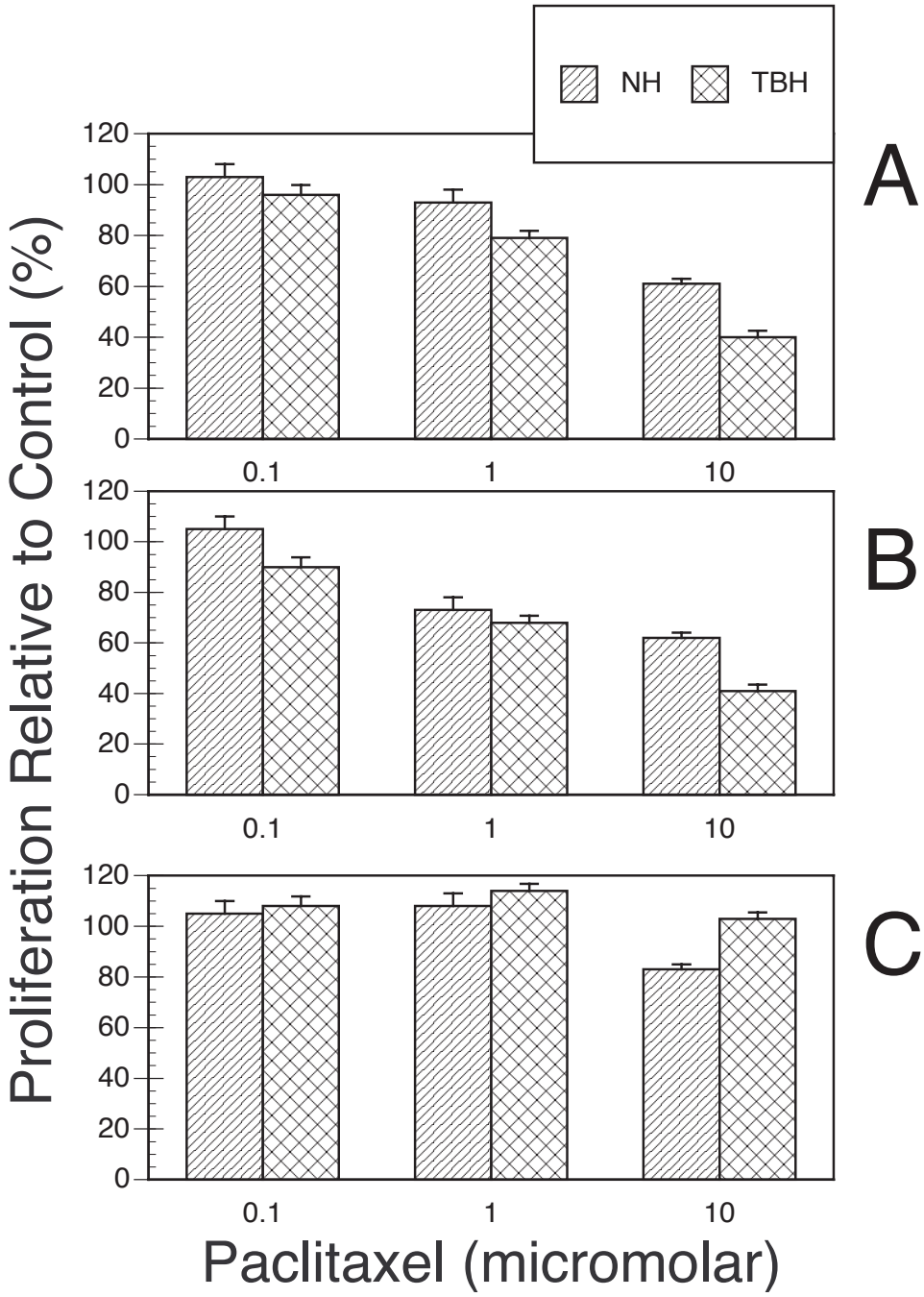


Figure 36. Tumor growth compromises $CD4^+$ T-cell recovery from paclitaxel pretreatment. Normal host and TBH $CD4^+$ T cells were cultured for 4 h with paclitaxel. Cells were washed twice and subsequently activated with Con-A (8 μg/ml) at 0 (panel A), 12 (panel B), or 24 h (panel C) after paclitaxel pretreatment. Normal host and TBH T-cell proliferation were assessed after 72 hours by [^{3}H]-TdR incorporation. Data are expressed as percentage proliferation relative to normal host control from triplicate independent determinations in one of three similar experiments.

DISCUSSION

In this study, we demonstrated that the antineoplastic agent paclitaxel partially reverses tumor-induced immunosuppression. We extend our previous findings (296) by demonstrating that short, transient paclitaxel administration (4 h) can regulate *in vitro* Mϕ NO production. Paclitaxel's control of the Mϕ proinflammatory molecules such as NO (Figure 33) may reverse the tumor-induced immunosuppression that is characteristic of tumor-distal Mϕs, and this effect is apparent *in vitro* (Figure 34).

A notable immunologic activity of paclitaxel may be its ability to activate Mϕ to enhance T-cell responses. Because Mϕs must be proximal to tumors for direct cytolytic activity, it begs the question of cytotoxic molecule production by tumor-distal Mϕs. Studies show that tumor-infiltrating Mϕs have decreased cytotoxic molecule production as compared to inflammatory Mϕs (270) and that Mϕ production of NO and TNF-α is downregulated at the tumor site (36,284). In contrast, tumor-distal Mϕ populations produce elevated levels of NO and TNF-α (15,296). Thus, Mϕ production of cytotoxic and proinflammatory molecules varies, depending upon the Mϕs resident *in vivo* compartment. Although enhanced TBH Mϕ production of cytotoxic effectors such as TNF-α and NO outwardly seems advantageous, overexpression of these signals by tumor-distal Mϕs may merely suppress T-cell activities (7,174,192) yet fail to impart antitumor effector activity. Paclitaxel-mediated modulation of tumor-distal Mϕ inflammatory and cytotoxic responses may benefit the TBH by limiting Mϕ-mediated suppression of T-cell responsiveness.

Because paclitaxel inhibits proliferation of many cell types, including lymphocytes, we also investigated whether tumor growth increases T-cell sensitivity to

paclitaxel. Paclitaxel significantly suppressed mitogen-induced proliferation (8 μg/ml Con-A) proliferation by both normal host and TBH T-cell populations (Table 3), and TBH $CD4^+$ T-cell proliferation was suppressed to a greater extent than the normal host counterpart (Table 3) even when paclitaxel was administered 48 h after T-cell activation. Furthermore, IL-2 (Figure 35) increased TBH $CD4^+$ T-cell sensitivity to paclitaxel. These data suggest that IL-2 immunotherapies used in conjunction with paclitaxel could adversely affect *in vivo* T cell activities. Heightened sensitivity may occur because IL-2 significantly restores TBH $CD4^+$ T-cell proliferation to normal host levels, committing T-cells within the cell cycle where cell populations are most sensitive to paclitaxel. Alternatively, paclitaxel may compromise IL-2 production or suppress expression of functional IL-2 receptors because proliferation of paclitaxel-treated lymphocytes cannot be restored by addition of exogenous IL-2 (365). Our data support this possibility because TBH $CD4^+$ T cells are less responsive to IL-2 in the presence of paclitaxel than their normal host counterparts (Figure 36 A). Although others have shown that paclitaxel can suppress the proliferation and cytotoxic activities of activated T cells (56,86), we are the first to report that tumor growth increases T-cell sensitivity to paclitaxel (Figure 35). Tumor growth, however, does not eradicate the ability of $CD4^+$ T cells to recover from transient paclitaxel treatment (Figure 36 C), lessening the potential insult to T-cell antitumor responses during paclitaxel-based antitumor therapies.

These results suggest that paclitaxel may control TBH Mϕ immunosuppressive activities in tumor-distal compartments, mediating a partial reversal of tumor-induced immunosuppression. Considering that similarly-treated Mϕs mediated significant antitumor cytotoxicity *in vitro* (see Figures 18 and 19), these data indicate that reversal of immunosuppression can be achieved without compromising Mϕ-mediated cytotoxic capacity. In addition to its direct cytostatic and cytotoxic functions on neoplastic cells,

paclitaxel may compromise tumor cell resistance to immune attack and enhance the efficacy of other chemotherapeutic or immunotherapeutic approaches. The antineoplastic agent paclitaxel, a powerful chemotherapeutic agent, also imparts immunotherapeutic activities in the TBH (296,299,301). Through its dual capacity to directly inhibit tumor growth and to activate certain immune populations, paclitaxel may prove a powerful immunotherapeutic agent either alone or in combination therapies. Specifically, we speculate that paclitaxel's immune activities may derive from a capacity to induce Mϕ IL-12 production, and this possibility is discussed in Chapter VI. Collectively, these studies enhance our understanding of the antitumor functions of paclitaxel and suggest that paclitaxel could be a beneficial agent in combination immunotherapies (including exogenous IL-12, as discussed in Chapter X).

CHAPTER VI

PACLITAXEL INDUCES Mϕ PRODUCTION OF IL-12 THROUGH AN AUTOCRINE INDUCTION MECHANISM INVOLVING NO

ABSTRACT

Tumor-induced Mϕs mediate immunosuppression, in part, through increased production of factors that suppress T-cell responsiveness and underproduction of positive regulatory cytokines. Pretreatment of TBH Mϕs with the anticancer agent paclitaxel partially reverses tumor-induced Mϕ suppressor activity, suggesting that paclitaxel may restore TBH Mϕ expression and production of proimmune factors. Because paclitaxel demonstrates LPS-mimetic capabilities, and increased production of the LPS-induced immunostimulatory cytokine IL-12 could account for enhanced T-cell responsiveness, we investigated whether paclitaxel can induce TBH Mϕ IL-12 production. Tumor growth significantly downregulated Mϕ production of IL-12 p70 heterodimer, and ribonuclease protection analysis revealed that tumor growth selectively dysregulates expression of the IL-12 p40 subunit. LPS stimulation failed to overcome tumor-induced dysregulation of p40 expression. In contrast, paclitaxel significantly enhanced both normal host and tumor-bearing host Mϕ IL-12 p70 production *in vitro*, although TBH Mϕ IL-12 production was lower than that of similarly-treated normal host Mϕs. Paclitaxel enhanced p40 expression in a dose-dependent manner. Through reconstituted Mϕ IL-12 expression, paclitaxel pretreatment relieved tumor-induced Mϕ suppression of T-cell alloreactivity. Blocking Mϕ NO suppressed paclitaxel's ability to induce IL-12 production. This suggests that paclitaxel-induced activities may involve a NO-mediated autocrine induction pathway. Collectively, these data demonstrate that paclitaxel restores IL-12 production in the TBH Mϕ and ascribe a novel immunotherapeutic component to the pleiotropic activities of NO. Through its capacity to induce IL-12 production, paclitaxel may contribute to the correction of tumor-induced immune dysfunction.

INTRODUCTION

Mϕs serve vital roles in host defense against tumors, including tumor cytotoxicity and stimulation of antitumor lymphocytes. However, tumors circumvent these host-mediated immune activities through the production and release of immunomodulatory factors such as IL-10, PGE_2, and TGF-β_1 that adversely alter Mϕ function and phenotype. These tumor-derived factors generate immunoregulatory Mϕs that inhibit T-cell responses and that are tumoricidally-dysfunctional (reviewed in reference 123). Identification of the molecular and cellular origins of tumor-induced changes in Mϕ function will increase opportunities for immunotherapeutic intervention. Recent studies (296,299,301) ascribed immune activating properties to paclitaxel (TAXOL™), suggesting that chemotherapeutic applications of paclitaxel may impart novel immunotherapeutic activities on tumor-induced Mϕs.

A plant-derived diterpenoid (215), paclitaxel first gained recognition as a potent inhibitor of cell cycle progression, leading to neoplastic cell death and arrest of tumor progression (114,148,362). Paclitaxel demonstrated significant antitumor efficacy in human clinical trials (178) and became the chemotherapeutic agent of choice for first-line treatment of metastatic breast cancers (459). Paclitaxel's primary mechanism of antineoplastic activity rests in its ability to irreversibly polymerize α/β tubulin, thereby disrupting cellular microtubule networks (373). Additionally, paclitaxel enhances tumor cell radioresponse (282) and sensitivity to immune effectors such as TNF-α (457).

In addition to its well-characterized chemotherapeutic activities, paclitaxel has profound cell cycle-independent effects on murine Mϕs (53,296,299,301). Paclitaxel induces normal host Mϕ responses similar to those generated by bacterial LPS (266,440),

including enhanced NO (267,296), TNF-α (110), IL-1β (49), and superoxide anion (331) production and induction of NF-κB expression (203). Through increased TNF-α and NO production, paclitaxel enhances *in vitro* tumor cell cytotoxicity (267).

Paclitaxel's most significant immune activity, from a therapeutic standpoint, may be its capacity to reverse tumor-induced Mϕ-mediated immune suppressor activities. Paclitaxel pretreatment of TBH-derived Mϕs modulated suppression of alloantigen-activated T-cell responsiveness (301) and concurrently enhanced Mϕ antitumor cytotoxicity *in vitro* (301). Paradoxically, both the cytotoxic (267,301) and immunosuppressive (192) functions of TBH Mϕs are mediated partially through the overproduction of reactive nitrogen and oxygen intermediates, including NO, and proinflammatory cytokines such as TNF-α. However, ablation of Mϕ-derived suppressor molecules NO and TNF-α failed to fully overcome the suppressive effects induced by tumor growth in our model system (Mullins and Elgert, unpublished observation), leading to speculation that TBH Mϕs may be deficient in immunostimulatory factor production. The capacity of the Mϕ-derived cytokine IL-12 to promote cell-mediated antitumor immune responses during tumor growth (251,407) and the LPS-mimetic activities of paclitaxel (70,112,147,188,266,440) suggested to us that paclitaxel may reverse tumor-induced suppressor activities through reconstitution of Mϕ IL-12 production in the TBH. We report that fibrosarcoma growth dysregulates Mϕ production of IL-12, compromising antitumor activity, and that paclitaxel induces IL-12 expression through an NO-dependent autocrine induction mechanism. Paclitaxel activated Mϕs co-express cytotoxic and immunostimulatory activities that collectively may impart significant antitumor immunotherapeutic functions.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice (Harlan Sprague-Dawley, Madison, WI) were used as source for normal host and TBH lymphocytes. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma, designated Meth-KDE, was used as described in Chapter I (15,126). TBH BALB/c mice were used 21 days after tumor passage because tumor-induced Mϕ suppressor activity is maximal at this time (124), without cachexia or necrosis. Eight to 12 week-old male C3H-HeJ (H-2^k) mice were source of alloantigen stimulator cells.

Medium and Reagents

Lymphocytes were cultured in serum-free RPMI-1640 medium, prepared as described in Chapter I. T-cell cultures were supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA). Paclitaxel was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to a 4 mM stock solution, stored at -80°C, and diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%. Bacterial LPS (*Escherichia coli* serotype 026:B6) and the specific NO inhibitor NMMA (Sigma) were reconstituted in RPMI-1640 and diluted to assay concentrations immediately before use. Other reagents included recombinant murine IFN-γ (2.98 x 10^5 U/ml, endotoxin content <0.1 ng/ml; Genzyme, Cambridge, MA), recombinant murine IL-12 (specific activity 2.5 x 10^6 U/ml, endotoxin content <10 pg/ml; Genetics Institute, Cambridge, MA), and paclitaxel (Calbiochem, La Jolla, CA). Neutralizing anti-IL-12 mAb (465) was purified by ammonium sulfate

fractionation (187) of spent medium from C17.8 hybridoma cells (G. Trinchieri, Wistar Institute, Philadelphia, PA) grown for 4 days in RPMI-1640 supplemented with 10% FBS. The mAb fraction was concentrated by dialysis against fresh medium. Neutralizing anti-IL-2 mAb (S4B6) and rat IgG_{2a} isotype control mAb were acquired from PharMingen (LaJolla, CA).

Immune Cell Collection and Culture

Normal host and TBH peritoneal exudate Mϕs were collected by lavage with cold RPMI-1640 medium, four days after i.p. injection with 2 ml of 3% sterile thioglycollate (Difco, Detroit, MI). Mϕs were purified by plating peritoneal exudate cells for 2 h (37°C, 5% CO_2) in glass plates, washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium by scraping with a rubber policeman. Splenic Mϕs were collected by plating pooled whole spleen cells for 2 h, washing away nonadherent cells with warm medium, and collecting adherent Mϕs. The final Mϕ preparations contained cells that were >95% viable and >96% esterase positive, and flow cytometric analysis with M1/70 and F4/80 monoclonal antibodies (ATCC, Rockville, MD) showed them to be >80% Mac-1^+ and F4/80^+, respectively.

T-cells were collected by plating normal host-derived whole spleen cells for 2 h in glass plates and collecting the nonadherent cell fraction. Purified $CD4^+$ T cells were obtained by treatment with anti-CD8 (ATCC clone 3.155), anti-IA^d (ATCC clone MK-D6), and anti-B cell and immature T-cell (ATCC clone J11d) antibodies and complement (Low-Tox™ rabbit complement, Accurate Chemical Co., Westbury, NY). Red blood cells were lysed with 0.83% ammonium chloride (Sigma). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

IL-12 Protein Measurement

Either normal host or TBH Mϕs (4 x 10^5 cells/well) were cultured with the indicated reagents in 96-well flat-bottom plates. Supernatants were collected and immediately assayed for IL-12 using a p70-specific ELISA (IL-12 DuoSet®; R&D Systems, Minneapolis, MN) per the manufacturer's protocol. The ELISA consisted of anti-mouse IL-12 p70 capture antibody adhered to high-affinity protein binding plates (Nunc MaxiSorp™ ELISA plates), biotinylated secondary antibody, and HRP-conjugated strepavidin detection reagent. ELISA were developed with Sigma tetramethylbenzidine liquid substrate system, and absorbance was determined at 450 nm using a MR-600 microplate reader (Dynex, Alexandria, VA). The limit of detection in our hands was approximately 10 pg/ml.

Ribonuclease Protection Assay Analysis of IL-12 Subunit Expression

Either normal host or TBH Mϕs (5.0 x 10^6 cells) were cultured in serum-free medium with the indicated reagents. Mϕs were cultured for 2h in 24-well flat-bottom plates (Corning Cell Wells, Corning, NY), collected, and stored in liquid nitrogen for subsequent analysis. Total mRNA was extracted using the RNeasy™ total RNA kit following cell disruption by passage through a QIAshredder™ (Qiagen, Chatsworth, CA), per the manufacturer's protocol (for procedural details, see Appendix F).

Antisense riboprobes were constructed from specific IL-12 cDNA templates (PharMingen) using the MAXIscript™ T-7 *in vitro* transcription kit (Ambion, Austin, TX) and labeled using the BrightStar™ psoralin-biotin system (Ambion). RPA was performed using the Ambion RPA-II™ system, per the manufacturer's protocol. RPA

products were separated on a 5% polyacrylamide gel containing 8M urea, transferred to nylon membrane, and visualized with the Ambion BioDetect™ non-isotopic detection system on Kodak BioMax™ MR-1 autoradiography film. Images were digitized and densitometric analyses performed using NIH-Image software for Macintosh.

PCR Analysis of IL-12 Subunit Expression

Mϕs were cultured as described, then pelleted in liquid nitrogen for subsequent analysis. mRNA was extracted using the MicroFast Track mRNA Isolation kit (Invitrogen, San Diego, CA) and converted to cDNA with 1 μg of random primers and 200 U of reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) for 1 h at 37°C. Each DNA sample was digested with 1 μg RNase for 15 min. The oligonucleotide primer sequences (see legend, Figures 39 and 40) were chosen with IntelliGenetics PRIMER software (Mountain View, CA) and prepared on an Applied Biosystems automated synthesizer in the St. Jude Children's Research Hospital Molecular Resource Center (Memphis, TN). Taq DNA polymerase (1.25 U; Promega, Madison, WI) was used to amplify 15% of the cDNA under the following conditions: 1 sec, 94°C; 1 sec, 55°C; and 15 sec, 72°C for 35 cycles in an air thermocycler (Model 1605, Idaho Technology, Idaho Falls, ID) in 50 mM Tris buffer (pH 8.5) containing 20 mM KCl, 500 μM dNTP, 500 μg/ml BSA, and 5 mM $MgCl_2$. PCR products were visualized by electrophoresis in 1.2% agarose gel containing ethidium bromide. PCR product was analyzed by gel densitometry using NIH-Image software.

T-cell Alloreactivity Assays

Either normal host or TBH Mϕs (2 x 10^5 cells) were added to normal host T-cell cultures (2 x 10^5 cells) in 96-well U-bottom tissue culture plates (Nunc). Some Mϕs were pretreated with paclitaxel or LPS for 4h before addition to T-cell cultures. Irradiated (2000 R) whole splenic cell preparations (4 x 10^5 cells) from C3H (H-2^k) mice were used as allogeneic stimulator cells. Neutralizing anti-IL-12 mAb (C17.8, 10 μg/ml) or rat IgG_{2a} isotype control mAb (10 μg/ml) was added to some cultures. Eighteen h before harvest, cultures were pulsed with 1 μCi per well [^{3}H]-TdR, (specific activity 6.7 Ci/mM; DuPont-NEN Research Products, Boston, MA). Cells were harvested with a Skatron (Chantilly, VA) automated cell harvester and sample activities determined using a Beckman LS 6000SC scintillation counter.

Mϕ Nitrite Production

Following incubation, supernatants (100 μL) were collected from alloantigen-activated T-cell proliferation cultures. NO was analyzed by measuring total nitrite levels in culture supernatants using the Griess reagent (172), as described in Chapter I. Briefly, 100 μL Mϕ supernatants were added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3P0_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm. A sodium nitrite (Sigma) standard curve was used to calculate nitrite content in supernatants. Nitrite was not detected in RPMI-1640 medium alone.

Statistics and Calculations

Cells from 6 to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested in the specific ELISA, Griess reagent test, and proliferation assays. Data are presented as means ± SEM of triplicate independent determinations. All experiments were repeated at least three times; representative experiments are shown. All comparisons were tested for significance by Student's t test, and all comparisons are significant at the $p < 0.05$ level, unless otherwise stated.

RESULTS

Tumor Growth Dysregulates Mϕ Production of IL-12 p70 Heterodimer

In the presence of tumor-induced Mϕs, T-cell proliferative responses are suppressed, suggesting that neoplastic tissues subvert Mϕ function to favor tumor growth (123). In our model system, tumor-derived factors such as PGE_2, TGF-β_1, and IL-10 induce systemic dysregulation of Mϕ functions (15). Therefore, we determined whether tumor growth compromises IL-12 production by Mϕs in tumor-distal compartments. Normal host or TBH peritoneal Mϕs (4×10^5 cells) were IFN-γ-primed (10 U/ml) and cultured without or with LPS (1 μg/ml, optimal dose in our hands) for 24 h. Supernatants were collected and assayed for IL-12 heterodimer by p70-specific ELISA (Figure 37). IL-12 production in the absence of IFN-γ priming was minimal (not shown), consistent with the findings that the IL-12 p40 gene promoter is primed by IFN-γ (256). Tumor growth significantly ($p < 0.05$) inhibited IFN-γ-induced IL-12 production (74% decrease compared to normal host Mϕs). LPS triggering stimulated normal host Mϕ IL-12 production, leading to greater than a 120% increase in IL-12. LPS enhanced IFN-γ-primed TBH Mϕ production of IL-12 by 115% as compared to untriggered Mϕs; however, the level of IL-12 production was significantly ($p < 0.005$) less than similarly-treated normal host Mϕs. TBH Mϕ IL-12 production never exceeded that of untriggered normal host Mϕs, demonstrating a serious lesion in the response of TBH Mϕ populations. Splenic Mϕ cultures produced similar profiles (not shown).

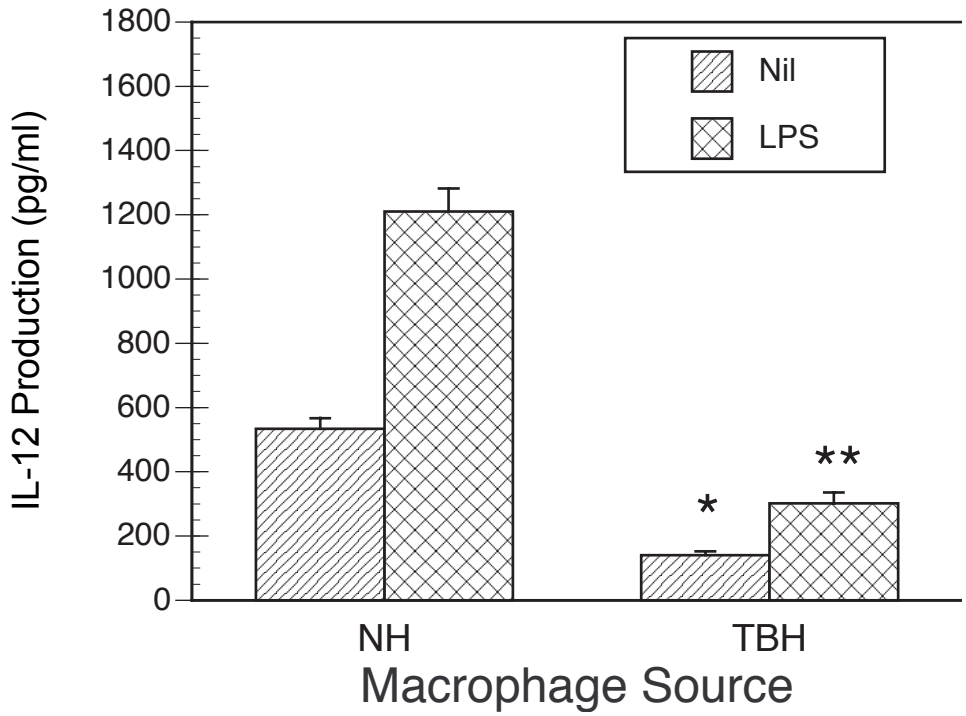


Figure 37. Tumor growth dysregulates Mϕ production of IL-12 p70 heterodimer. Either 4 x 10^5 normal host (NH) or TBH peritoneal Mϕs were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml). LPS (1 μg/ml) was added to some wells at the start of culture. After 24 h, supernatants were collected and assayed for IL-12 heterodimer by p70-specific ELISA. Data are averages and SEM of triplicate independent determinations from one of five similar experiments. *, $p < 0.05$ compared with similarly-activated normal host Mϕs; **, $p < 0.005$ compared with similarly-activated normal host Mϕs.

Tumor Growth Dysregulates Mϕ IL-12 p40 Expression

The bioactive IL-12 heterodimer is composed of the constitutively-expressed p35 subunit and the inducible p40 subunit (255). Because the p70 heterodimer is necessary for biologic activity, and p70 production is regulated by the level of available p40, we determined whether tumor growth dysregulates Mϕ p40 expression in our murine fibrosarcoma model. Either normal host or TBH peritoneal Mϕs (5 x 10^6 cells) were IFN-γ-primed (10 U/ml) and cultured for 4 h (optimal time) without or with LPS (1 μg/ml). Total RNA was collected and p40 expression measured by RPA (Figure 38 A). LPS failed to enhance p40 expression in tumor-induced Mϕs, further defining the lesion in TBH Mϕ function (LPS did induce p40 expression in normal host cells, consistent with the reports of others [186]). Tumor growth dysregulates IL-12 heterodimer production through abrogated activation response of the inducible p40 subunit. Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (mGADPH) was consistent throughout all samples (Figure 38 B). RT-PCR analysis confirmed that tumor growth dysregulates IL-12 p40 expression (Figure 39), but not p35 expression (Figure 40).

Paclitaxel Reconstitutes Mϕ IL-12 Production

Paclitaxel has Mϕ-activating functions *in vitro* (267,296) and *in vivo* (301), and paclitaxel pretreatment of TBH Mϕs reverses tumor-induced Mϕ suppression of T-cell proliferation (301). Because administration of exogenous IL-12 can promote antitumor immune function and paclitaxel can activate expression of several LPS-inducible genes, we hypothesized that reconstituted Mϕ IL-12 production may account for restored T-cell

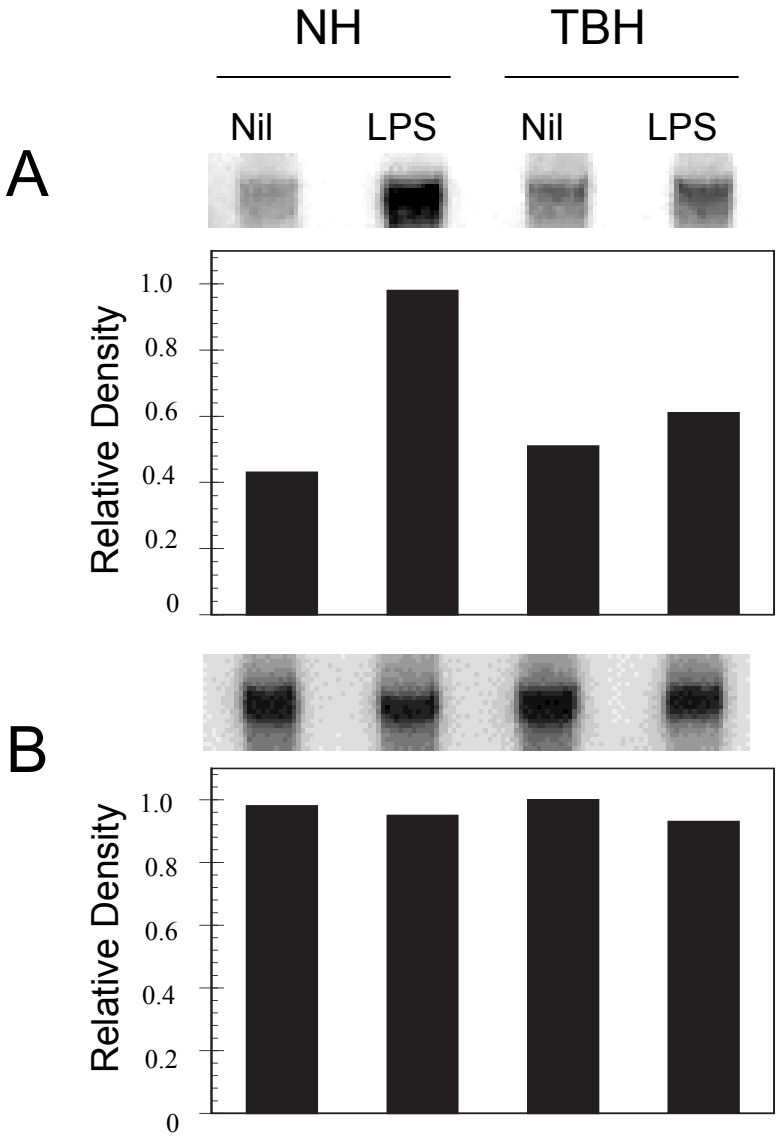


Figure 38. Tumor growth dysregulates Mϕ IL-12 p40 expression. Either 5 x 10^6 NH or TBH peritoneal Mϕs were cultured as described for 4 h and total RNA collected. LPS (1 μg/ml) was added to some wells at the start of culture. A, RPA analysis of IL-12 p40 expression using p40-specific primers, with relative densitometric units as determined using NIH-Image. B, Expression of the housekeeping gene mGADPH, with relative densitometric units. Data are representative results from one of three similar experiments.

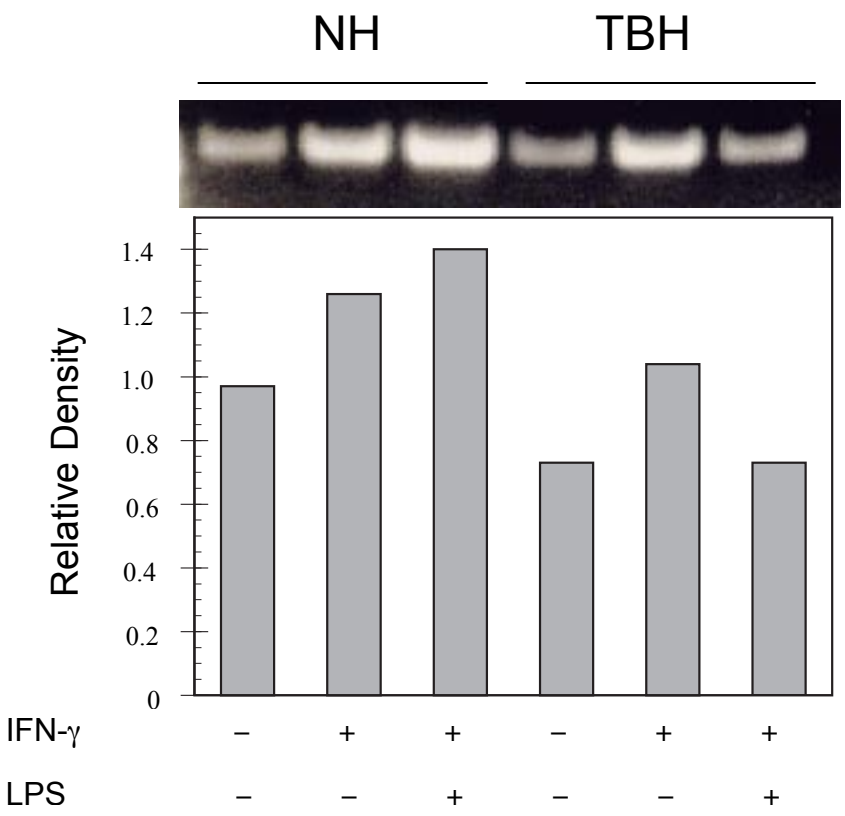


Figure 39. Tumor growth dysregulates expression of the p40 subunit of IL-12. To determine whether the IL-12 subunits were differentially affected by tumor-induced dysfunction, subunit mRNA was isolated following 45 min culture with the indicated reagents and amplified by semi-quantitative RT-PCR using the following primers:

IL12p40 A	ATGTTGTAGAGGTGGACTGG
IL12p40 S	TGCTACTGCTCTTGATGTTG

Similar results were obtained when following longer Mϕ incubation times, and no bands were detected in negative control lanes.

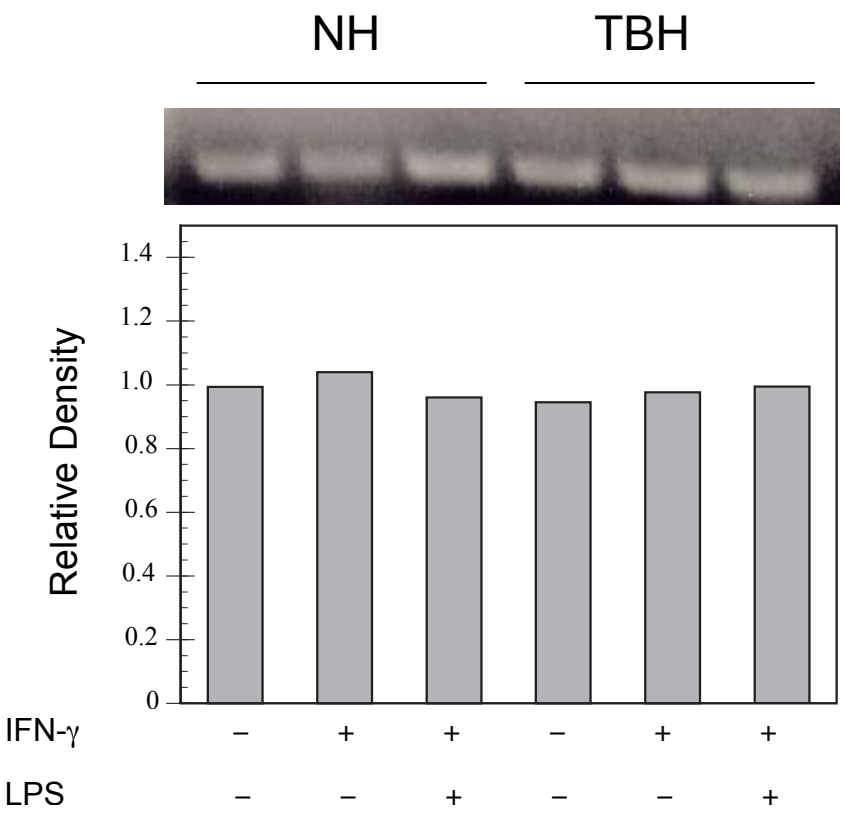


Figure 40. Expression of the p35 subunit of IL-12 is not dysregulated by tumor growth. Subunit mRNA was isolated as described following 45 min and amplified by RT-PCR using the following primers:

IL12p35 A	GCCTTGGTAGCATCTATGAG
IL12p35 S	TCACTCTGTAAGGGTCTGCT

Similar results were obtained when following longer Mϕ incubation times, and no bands were detected in negative control lanes.

proliferative responses. Therefore, we determined whether paclitaxel can induce Mϕ IL-12 production. Normal host or TBH (4×10^5) peritoneal Mϕs were primed with IFN-γ (10 U/ml) and cultured without or with paclitaxel (10 μM) for 24 h. Supernatants were collected and assayed for IL-12 heterodimer as described. Paclitaxel significantly ($p < 0.05$) enhanced both normal host and TBH Mϕ IL-12 production (Figure 41), increasing IL-12 levels by 64% and 112%, respectively. Splenic Mϕ cultures produced similar profiles, although total IL-12 production by all samples was approximately 50% less than comparably-treated peritoneal Mϕ cultures (not shown). Paclitaxel doses as low as 0.1 μM enhanced IL-12 production, although doses in excess of 10 μM did not further enhance IL-12 (not shown). The induction of IL-12 was not an artifact of endotoxin contamination because boiling paclitaxel for 60 sec abrogated its capacity to induce IL-12 (not shown).

Paclitaxel Overcomes Tumor-induced Suppression of Mϕ IL-12 p40 Expression

Abrogation of TBH Mϕ p40 expression (see Figures 38 and 39) and paclitaxel-mediated reconstitution of TBH Mϕ IL-12 heterodimer production (see Figure 41) suggest that paclitaxel may induce IL-12 p40 expression. To test this hypothesis, TBH peritoneal Mϕs (5×10^6 cells) were IFN-γ-primed, cultured for 4 h without or with various doses of paclitaxel (0.1 - 10 μM), and p40 expression assessed by RPA (Figure 42 A). Paclitaxel induced IL-12 p40 expression in a dose-dependent manner in both normal host (not shown) and TBH Mϕs. Paclitaxel doses in excess of 10 μM did not further enhance p40 expression (not shown). Expression of mGADPH was consistent throughout all samples (Figure 42 B).

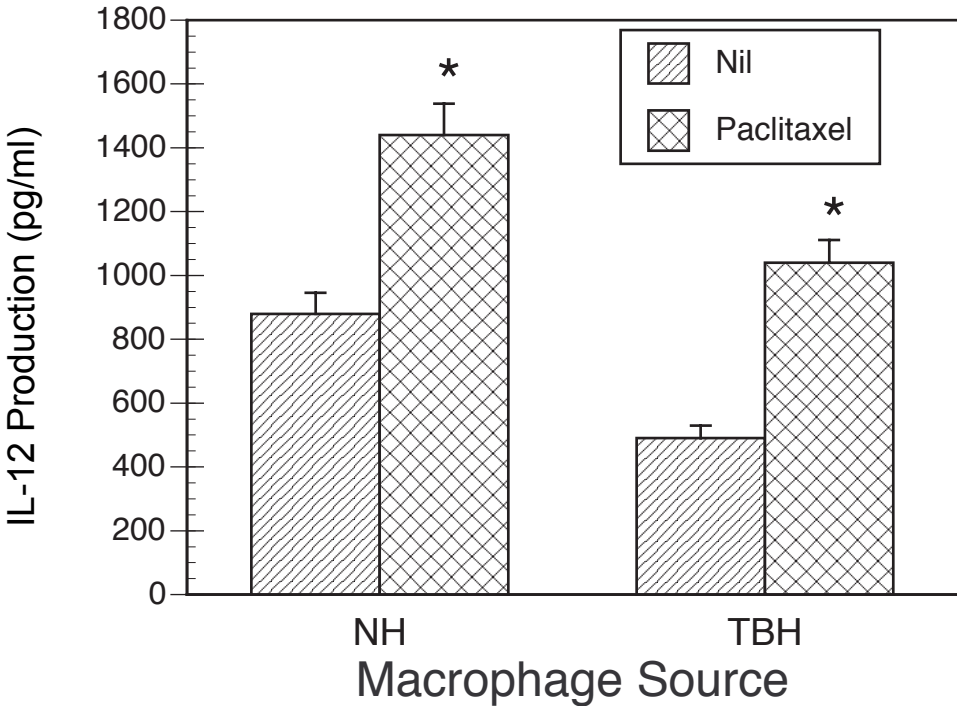


Figure 41. Paclitaxel reconstitutes Mϕ IL-12 production. Either 4 x 10^5 NH or TBH peritoneal Mϕs were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml). Paclitaxel (10 μM) was added to some wells at the start of culture. After 24 h, supernatants were collected and assayed for IL-12 heterodimer by specific ELISA. Data are averages and SEM of triplicate independent determinations from one of five similar experiments. *, $p < 0.05$ compared with unactivated Mϕs.

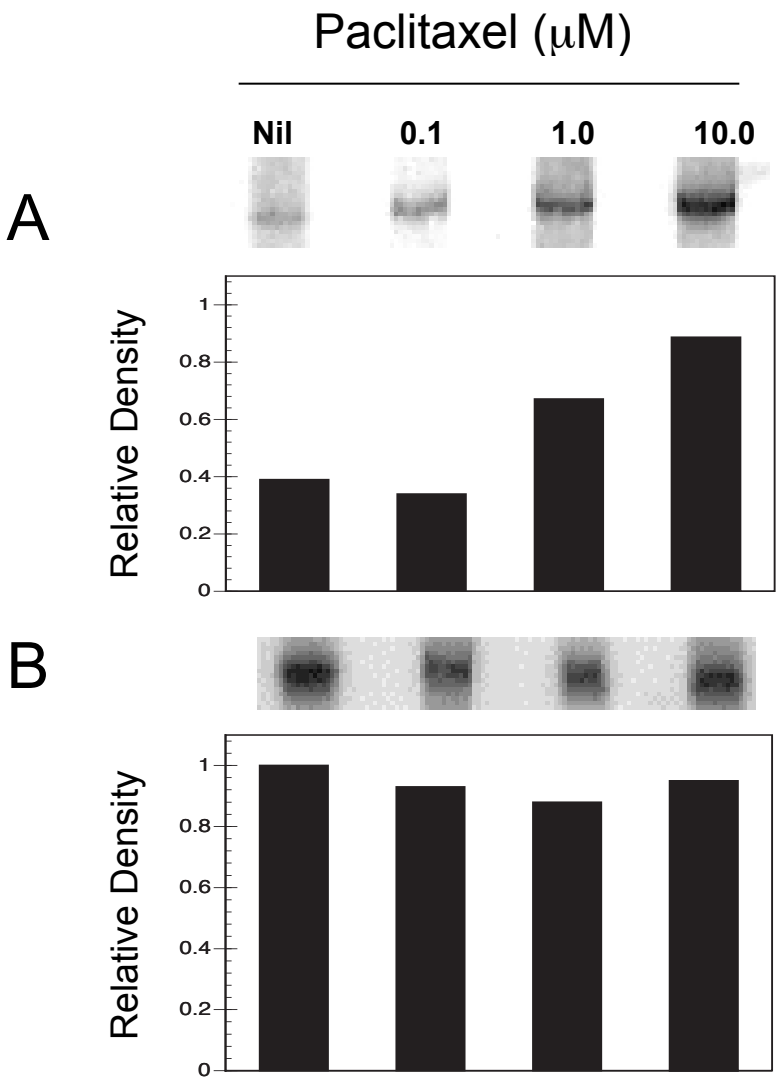


Figure 42. Paclitaxel overcomes tumor-induced suppression of Mϕ IL-12 p40 expression. TBH peritoneal Mϕs (5 x 10^6) were cultured for 4h, as described, and total RNA collected after 4 h. Paclitaxel was added to some wells at the start of culture. A, RPA analysis of IL-12 p40 expression using p40-specific primers, with relative densitometric units as determined using NIH-Image. B, Expression of the housekeeping gene mGADPH, with relative densitometric units. Higher doses of paclitaxel (35 μM) did not enhance p40 expression (not shown). Data are representative results from one of three similar experiments.

Paclitaxel Induces Mϕ IL-12 Production through NO

We (296,301) and others (267) have shown that paclitaxel stimulates Mϕ production of the pleiotropic effector molecule NO. Because NO regulates IL-12 gene expression in the RAW264.7 Mϕ cell line (360), we determined whether paclitaxel-induced NO stimulates murine Mϕ IL-12 production (Figure 43). IFN-γ-primed (10 U/ml) normal host or TBH peritoneal Mϕs were cultured for 24 h without or with paclitaxel (10 μM) and the specific NO-inhibitor NMMA (0.1 mM, sufficient to eliminate measurable NO production without compromising Mϕ viability). Paclitaxel significantly ($p < 0.05$) enhanced normal host Mϕ IL-12 production, but paclitaxel's capacity to induce IL-12 was blocked with the addition of NMMA. Although tumor growth negatively modulates paclitaxel-induced Mϕ NO production (299,301), paclitaxel significantly ($p < 0.05$) enhanced TBH Mϕ IL-12 production, and paclitaxel-mediated IL-12 production was NO-dependent (Figure 43). In the absence of NO production, paclitaxel-induced normal host or TBH Mϕ IL-12 production was not significantly different from basal levels.

Paclitaxel-induced IL-12 Reverses Tumor-induced Suppression of T-cell Alloreactivity

Tumor growth induces Mϕ suppressor activities (123), compromising T-cell proliferative responses in alloantigen-activated MLR cultures (11,23,446). We reported that paclitaxel pretreatment of TBH Mϕs partially reversed tumor-induced suppressor functions (301). Because paclitaxel induces Mϕ IL-12 production, and IL-12 can enhance T-cell proliferative responses (225,271,300), we assessed whether paclitaxel-induced IL-12 was responsible for the reversal of TBH Mϕ-mediated immune suppression by measuring lymphocyte responsiveness in culture with paclitaxel-

pretreated Mϕ in the absence or presence of IL-12. Normal host BALB/c (H-2^d) $CD4^+$ T-cell (2 x 10^5 cells) responsiveness to alloantigen stimulation (4 x 10^5 X-irradiated C3H [H-2^k] splenocytes) was assessed in MLR with TBH Mϕs (2 x 10^5 cells). Mϕs were pretreated (4 h, optimal time) without or with paclitaxel. Alloreactivity was assessed in the absence or presence of neutralizing anti-IL-12 mAb (C17.8, 10 μg/ml), anti-IL-2 (S4B6), or rat IgG_{2a} isotype control mAb (10 μg/ml) (Figure 44). Data are expressed as magnitude of response relative to proliferation in the presence of unactivated TBH Mϕs (control CPM was 42,020). Paclitaxel pretreatment relieves tumor-induced Mϕ suppressor activity, significantly ($p < 0.005$) enhancing alloantigen-activated T-cell proliferation (Figure 44) in the presence of treated TBH Mϕs. Neutralization of IL-12 activity in culture with anti-IL-12 mAb did not significantly impact T-cell proliferation in the presence of untreated Mϕs. In contrast, neutralization of IL-12 abrogated paclitaxel-mediated reversal of tumor-induced suppression. Neither rat IgG_{2a} isotype control nor anti-IL-2 mAb affected lymphocyte reactivity (not shown), suggesting that paclitaxel-induced IL-12 is the principle factor responsible for reconstitution of T-cell reactivity.

Because Mϕ-derived NO is required for paclitaxel-induced IL-12 expression (see Figure 43), we assessed whether paclitaxel-induced NO production plays a role in the reversal of tumor-induced Mϕ suppressor functions. $CD4^+$ normal host T-cell alloantigen reactivity was assessed, as described, in the absence or presence of the NO-inhibitor NMMA (Figure 45). NO production was 10.1 ± 0.3 μM and 37.4 ± 2.8 μM in cultures containing untreated or paclitaxel-treated TBH Mϕs, respectively. NO production in cultures containing NMMA was beyond the limit of detection of our assay system. In this system, paclitaxel pretreatment failed to relieve tumor-induced Mϕ suppressor activity in the presence of the NO inhibitor, demonstrating the Mϕ NO production is involved in the mechanism of enhanced T-cell alloreactivity. Addition of exogenous IL-

12 (1000 pg/ml, approximately equal to paclitaxel-induced [10 μM] IL-12 production by TBH Mϕs, see Figure 41) to NMMA-containing cultures reconstituted T-cell proliferative response (not shown).

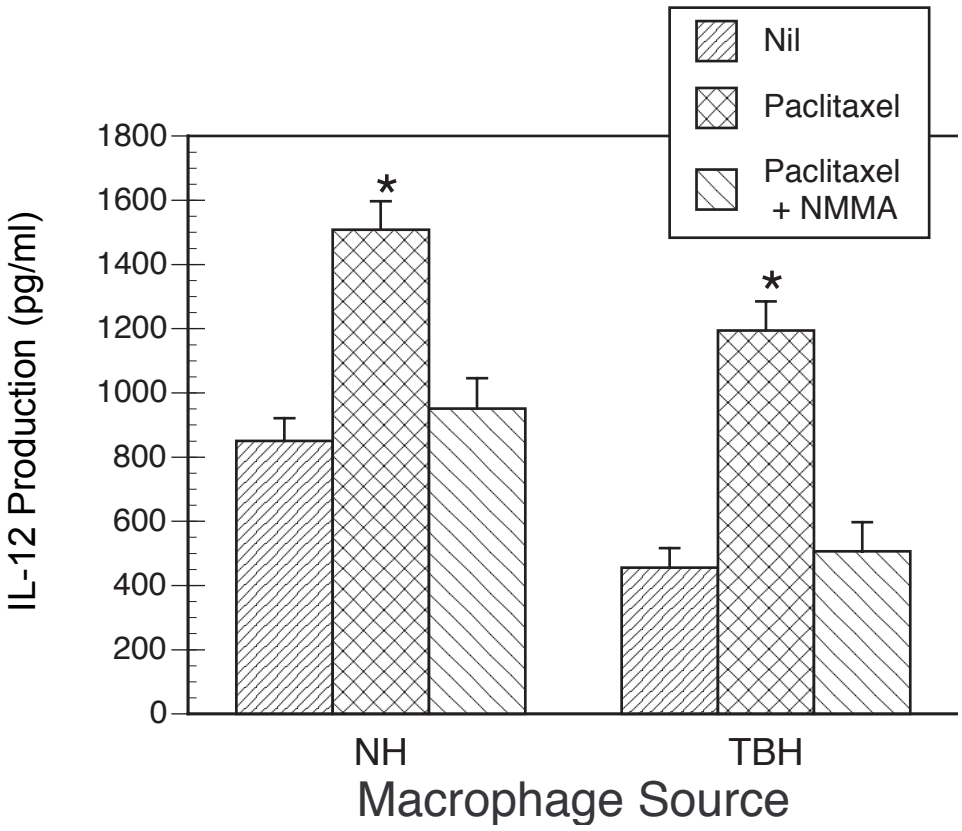


Figure 43. Paclitaxel induces Mϕ IL-12 production through NO. Normal host or TBH peritoneal Mϕs (4 x 10^5) were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml) and either without or with the NO-inhibitor NMMA (0.1 mM). Paclitaxel (10 μM) was added to some wells at the start of culture. After 24 h, supernatants were collected and assayed for IL-12 heterodimer by specific ELISA. NMMA did not reduce Mϕ viability (not shown). Data are averages and SEM of triplicate independent determinations from one of three similar experiments. *, $p < 0.05$ compared with unactivated Mϕs.

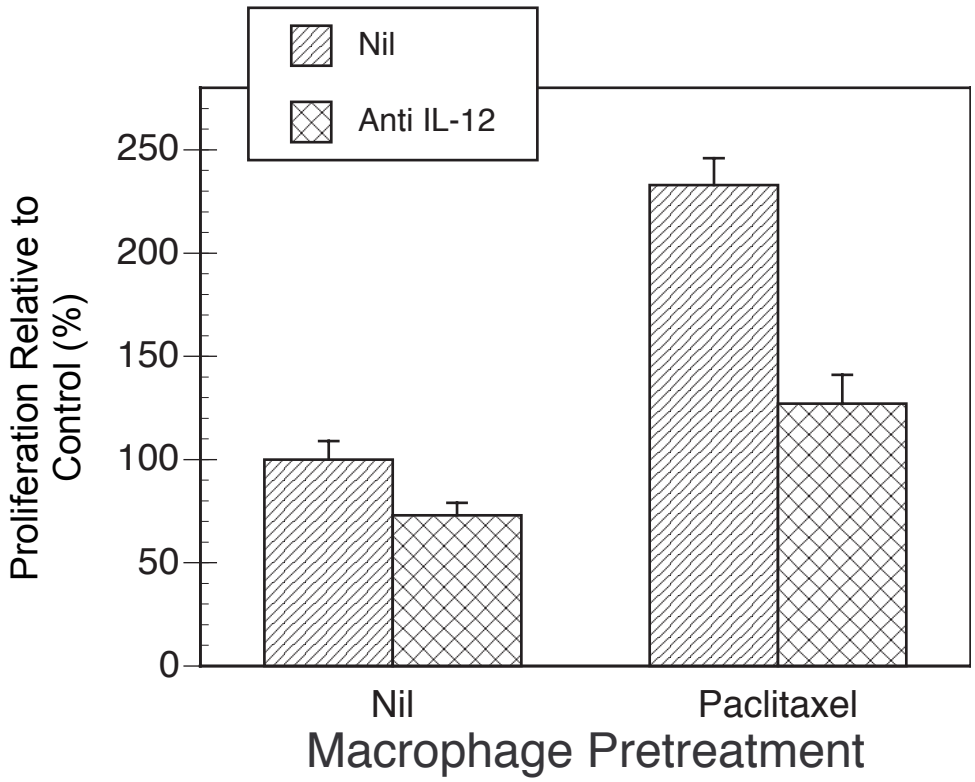


Figure 44. Paclitaxel-induced IL-12 reverses tumor-induced suppression of T-cell alloreactivity. NH and TBH peritoneal Mϕs were pretreated for 4 h in 200 μl of serum-free medium without or with paclitaxel (10 μM), then washed twice in fresh medium. Two x 10^5 Mϕs were added to 2 x 10^5 NH $CD4^+$ T cells and 4 x 10^5 X-irradiated (2000 R) C3H whole spleen cells in a total volume of 200 μl of serum supplemented with 10% FBS and cultured for 96 h. Neutralizing anti-IL-12 mAb (C17.8, 10 μg/ml), rat IgG_{2a} isotype control mAb (10 μg/ml) or neutralizing anti-IL-2 mAb (10 μg/ml) was added to some cultures. Eighteen h prior to harvest, cells were pulsed with 1 μCi of ^{3}[H]-TdR and proliferation determined. Data are magnitude of response relative to proliferation in the presence of untreated Mϕs (control CPM was 42,020). Proliferation of normal host responder cells or X-irradiated stimulators cells alone were less than five percent of control. Data are averages and SEM of triplicate independent determinations from one of three similar experiments. *, $p < 0.05$ compared with control.

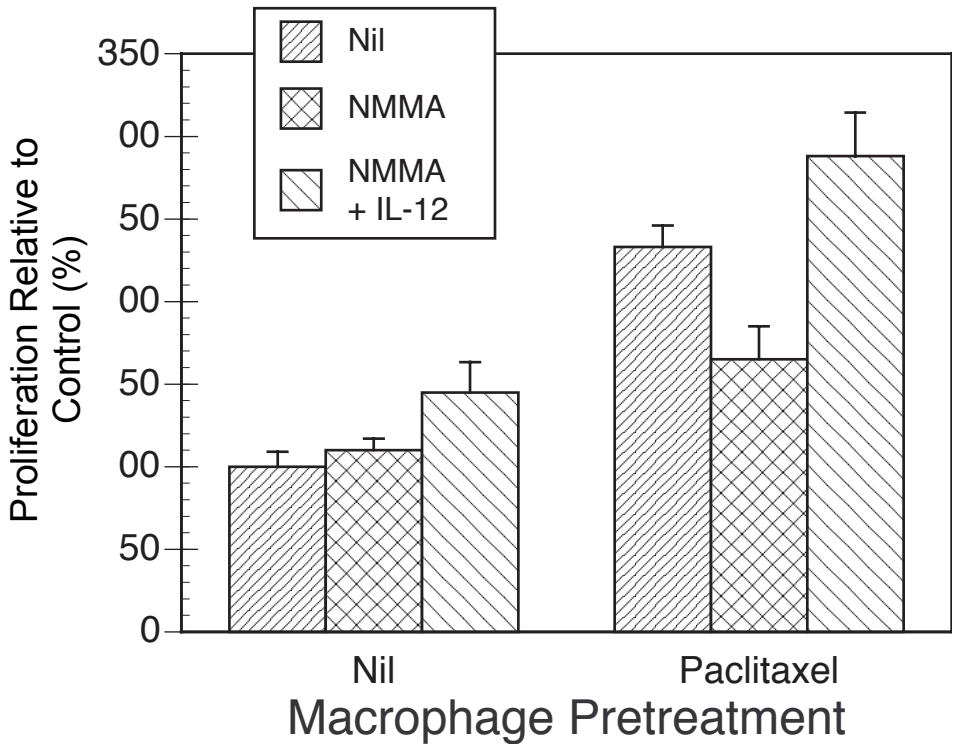


Figure 45. NO-induced IL-12 production reverses Mϕ-mediated suppression of T-cell alloreactivity during tumor growth. TBH peritoneal Mϕs were pretreated for 4 h in 200 μl of serum-free medium without or with paclitaxel (10 μM), then washed twice in fresh medium. Two x 10^5 Mϕs were added to 2 x 10^5 NH $CD4^+$ T cells and 4 x 10^5 X-irradiated (2000 R) C3H whole spleen cells in a total volume of 200 μl of medium supplemented with 10% FBS and cultured for 96 h. The NO-inhibitor NMMA (0.1 mM) and exogenous IL-12 (1000 pg/ml) was added to some wells at the start of culture. Eighteen h prior to harvest, cells were pulsed with 1 μCi of ^{3}[H]-TdR and proliferation determined. Data are magnitude of response relative to proliferation in the presence of untreated Mϕs (control CPM was 39,220). Proliferation of normal host responder cells or X-irradiated stimulators cells alone was less than five percent of control. Proliferation data are averages and SEM of triplicate independent determinations from one of three similar experiments.

DISCUSSION

Tumor growth significantly alters Mϕ phenotype and function; much research has focused on defining how tumors induce Mϕ production of immunomodulatory signals (reviewed in 123), demonstrating that these factors are important mediators of immunosuppression during cancer. However, our recent studies on the immune activities of the anticancer drug paclitaxel led us to address tumor-induced immune suppression from a different point of view. Perhaps in addition to overexpression of inhibitory cytokines, host antitumor response may be compromised by the lack of a necessary priming, activating, or costimulatory signal(s), preventing the induction of immune responses to tumors. An ideal candidate for study was Mϕ-derived IL-12, a single factor that possessed the potential to correct many TBH immune dysfunctions.

IL-12 (reviewed in 422) is a novel heterodimeric cytokine that enhances antitumor cytotoxic responses (157,275). Produced primarily by phagocytes and APCs, including Mϕs, monocytes, and dendritic cells (233,420), IL-12 is structurally unique among interleukins. Biologically active IL-12 is a heterodimer of dissimilar subunits; the bioactive molecule consists of the inducible p40 subunit and the constitutive p35 subunit, linked by a disulfide bond. Pleiotropic in its activities, IL-12 induces T-cell (381,382) and natural killer (NK) cell (73,74) IFN-γ production and promotes T-cell development along the T_H1 pathway (257,388,461,470). These functions led to speculation that IL-12 could be useful as an anticancer immunotherapeutic agent (156,479), and clinical trials using direct administration (306,407) and gene therapy approaches (251,409,481) have yielded promising results.

The capacity of exogenous IL-12 to partially restore TBH immunocompetence suggests that tumor-induced Mϕ dysfunction may involve decreased production of IL-12. Dysregulation of Mϕ IL-12 may occur among both tumor-proximal and -distal immune cell populations if the tumor produces or induces the production of immunomodulatory factors such as IL-10 and TGF-β_1 (252), as occurs in our tumor model (15,123). Handel-Fernandez *et al.* (182) reported that a nonmetastatic mammary adenocarcinoma (D1-DMBA-3) dysregulated peritoneal exudate Mϕ p40 expression through PGE_2 and phosphatidyl serine. Unlike these studies, we used a p70-specific ELISA to avoid the potential interference of excess free p40 or p40 homodimers. Using this system, we found that murine fibrosarcoma growth significantly ($p < 0.05$) inhibited IFN-γ-induced IL-12 p70 production by 74% (Figure 37); although LPS enhanced IFN-γ-primed TBH Mϕ IL-12, the level of production remained significantly ($p < 0.005$) less than similarly-treated normal host Mϕs. Most striking, optimal triggering of TBH Mϕs with LPS could not induce IL-12 production even to the levels of IFN-γ-primed normal host Mϕs. Tumor-induced dysregulation of IL-12 occurs at the level of transcription, as p40 expression was largely abrogated in tumor-induced Mϕs regardless of priming or triggering with IFN-γ and LPS, respectively (Figures 38 and 39). Because IL-12 drives the induction of the T_H1-type immune response -- the most effective antitumor response -- the dysregulation of IL-12 production could effectively disable this arm of the immune response and allow tumors to grow unhindered.

Recognizing that tumor growth negatively regulates Mϕ production of immunostimulatory factors, perhaps therapeutic agents that reconstitute immune activity through indirect action could be inducing the production of stimulatory cytokines. We reported that pretreatment of TBH Mϕs with the anticancer chemotherapeutic agent paclitaxel partially reversed Mϕ-mediated suppressor activity (301). This suggested that

paclitaxel, which has demonstrated Mϕ-activating functions *in vitro* (267,296) and *in vivo* (301), may enhance T-cell reactivity by activating Mϕ IL-12 production. Analyzing for IL-12 p70 showed that paclitaxel significantly ($p < 0.05$) enhanced IFN-γ-primed Mϕ IL-12 production regardless of tumor growth (Figure 41). After finding that tumor growth dysregulates Mϕ p40 expression (Figures 38 and 39) -- but not p35 expression (Figure 40) -- and paclitaxel mediates reconstitution of IL-12 p70 heterodimer (Figure 41), we investigated whether paclitaxel directly induces IL-12 p40 expression. RPA analysis showed that paclitaxel activates p40 expression in a dose-dependent manner (Figure 42).

Although our data strongly suggest that paclitaxel induces IL-12 production and p40 expression, the mechanisms remained to be clarified. One possibility involved paclitaxel-mediated induction of Mϕ NO production. NO regulates IL-12 gene expression in the RAW264.7 Mϕ cell line (360), and paclitaxel stimulates Mϕ production of NO (267,296,301). We determined that NO is a key regulator of paclitaxel-mediated IL-12 production (Figure 43) in our model system. Paclitaxel significantly ($p < 0.05$) enhanced both normal host and TBH Mϕ IL-12 production, but paclitaxel's capacity to induce IL-12 was substantially blocked with the abrogation of NO production. These data suggest that a primary mechanism of IL-12 induction by paclitaxel may involve autocrine signaling through Mϕ-derived NO. Another mechanism is suggested by studies demonstrating that paclitaxel induces NF-κB translocation in Mϕs (203,337). The IL-12 p40 promoter element contains an NF-κB half-site that binds the NF-κB p50/p65 and p/50/c-Rel dimers in Mϕs activated by LPS (302). Further, low levels of NO enhance NF-κB activation in TNF-α-primed endothelial cells (429), raising the possibility that NO induces similar effects in IFN-γ-primed Mϕs. Collectively, these observations suggest that NF-κB may play a pivotal role in paclitaxel-mediated signaling, and we are currently investigating this possibility.

Although tumors mediate functional and phenotypic changes in Mϕ populations (123), T-cells may not be functionally altered, opening the possibility that restoration of Mϕ IL-12 production through chemotherapeutics may be beneficial to the TBH. Because paclitaxel pretreatment of TBH Mϕs partially reversed tumor-induced suppressor functions (301), we determined whether paclitaxel-mediated Mϕ IL-12 production was responsible for the reversal of immune suppression (Figure 44). Neutralization of IL-12 activity in T-cell alloreactivity cultures abrogated paclitaxel-mediated reversal of tumor-induced suppression, suggesting that paclitaxel's apparent immunotherapeutic activity is achieved through the induction of Mϕ IL-12 production. This suggests that the TBH immune system may be fully capable of responding to cancer if provided with appropriate stimulatory signals, and it emphasizes the importance of determining the mechanism of tumor-induced IL-12 dysregulation.

Interestingly, paclitaxel-induced Mϕ NO production plays a role in the restoration of immunocompetence. In the presence of the NO inhibitor NMMA, paclitaxel pretreatment failed to relieve tumor-induced Mϕ suppressor activity (Figure 45). These results provide an intriguing link between IL-12 and NO, molecules with disparate function in tumor immunity. In terms of signaling mechanisms, Schwacha and Eisenstein (378) reported that IL-12 was necessary for the induction of NO production in an infectious disease models, and Wigginton *et al.* (456) showed that IL-12 primed TBH Mϕs for enhanced NO production. These studies suggest that IL-12, through its capacity to induce IFN-γ production, leads to priming of Mϕ populations for enhanced NO production (IL-12 has no direct effect on normal host or TBH Mϕ NO production; Mullins and Elgert, unpublished observation). This report suggests another regulatory pathway by demonstrating that NO can induce Mϕ IL-12 production through an autocrine

induction mechanism involving NO, thus perpetuating the cycle of activation and enhancing the potential for strong cell-mediated immune responses.

In terms of T-cell reactivity, reconstitution of alloantigen-activated proliferative response in the presence of NO and loss of response in the absence of NO production may seem counterintuitive. NO is a potent antagonist of lymphocyte proliferative responses and can severely dysregulate T-cell reactivity (7,174,473). In our model system, we observed that LPS will drive enhanced Mϕ NO production, leading to drastic decreases in T-cell alloreactivity and blocking NO with specific inhibitor relieves suppression. In the TBH, Mϕs are primed for enhanced NO production on LPS signaling, but these same cells are incapable of substantial IL-12 production (Figure 41). When LPS-activated TBH Mϕs are added to normal $CD4^+$ T-cells activated by alloantigens, T-cell proliferation remains severely compromised, partly as a result of high levels of Mϕ NO. In contrast, paclitaxel pretreatment induces TBH Mϕ production of NO at lower levels, which may have direct inhibitory effects of the T cells; however, that Mϕ-derived NO also acts in an autocrine manner to induce Mϕ IL-12 production, which in turn enhances T-cell proliferative responses (Figure 44). Administration of exogenous IL-12 enhances IFN-γ in TBH (202), which in turn can enhance Mϕ iNOS expression and NO production *in situ*, leading to an amplification of antitumor responses. Collectively, these results suggest that the restoration of IL-12 has significant positive implications for the TBH, and that the stimulatory effects of the IL-12 on T-cell reactivity outweigh the suppressor effects of NO.

Controversy surrounds human monocyte/Mϕ production of NO (103), leading to questions about the relevancy of murine data to human oncology studies. Human Mϕs possess the gene and functional protein for NO production (454), and NO production

occurs *in vitro* (119,454). Recent data indicate that human peripheral blood mononuclear cells can produce moderate amounts of NO *in vivo* (119). From the opposing view, it could be argued that lack of NO production by human Mϕs may contribute to tumor-induced immunosuppression by the loss of IL-12 and IFN-γ induction mechanisms. Regardless of human Mϕ NO production, further investigation of Mϕ responsiveness to paclitaxel in human cancer patients is warranted.

These results demonstrate that the antineoplastic agent paclitaxel restores IL-12 production in the TBH, a novel immunotherapeutic activity that may help correct tumor-induced immune dysfunction. Further, these data ascribe a novel signaling component to the pleiotropic activities of NO. We suggest that the immune activities of paclitaxel should be considered in a clinical context, because recognition of paclitaxel's immune activating properties may lead to optimization of current chemotherapeutic treatment regimens.

SECTION IV

ANTITUMOR EFFICACY OF PACLITAXEL, IL-12, AND COMBINATION THERAPIES

This Section contains four chapters describing the effects of single agents or combination therapies on tumor progression and immune cell function. Chapter VII presents experimental evidence that paclitaxel treatment *in vitro* directly inhibits tumor cell proliferation and induces apoptotic tumor cell death in our model system. These are the first data to confirm that paclitaxel induces apoptosis in the Meth-KDE tumor cell line. Chapter VIII contains the first experimental evidence that chemotherapeutic paclitaxel administration differentially regulates Mϕ functions in the TBH, and Chapter IX presents data to support IL-12's role as an effective immune-activating cytokine. The data in Chapter IX suggest that IL-12 may be useful in modulating paclitaxel's immunosuppressive side-effects during chemotherapeutic administration, and these *in vitro* results lay the foundation for subsequent *in vivo* studies. Finally, Chapter X characterizes the efficacy of paclitaxel and IL-12 combinatorial therapies in a murine tumor model system. These results are the first experimental evidence to suggest that paclitaxel and IL-12 are an effective antitumor modality.

CHAPTER VII

PACLITAXEL DIRECTLY INHIBITS FIBROSARCOMA PROLIFERATION AND INDUCES APOPTOTIC CELL DEATH

ABSTRACT

Although paclitaxel demonstrates multiple antineoplastic activities, its mode antitumor action in a murine fibrosarcoma model is not fully described. Our model, which uses a methylcholanthrene-induced nonmetastatic murine fibrosarcoma designated Meth-KDE, has shown resistance to immune antitumor cytokines, including TNF-α. However, 4-h pretreatment of Meth-KDE cells with clinically relevant concentrations of paclitaxel significantly decreased tumor cell proliferation and viability. Paclitaxel treatment also increased Meth-KDE cell sensitivity to TNF-α. Flow cytometric analysis with Annexin V revealed that paclitaxel induces apoptosis in Meth-KDE cells, suggesting that the cytostatic and cytotoxic actions of paclitaxel against fibrosarcoma cells culminate in apoptosis.

INTRODUCTION

Paclitaxel, an organic product isolated from the bark and leaves of the western yew tree *Taxus brevifolia* (215), demonstrates antineoplastic activities that are 100-1000 times more cytotoxic than other cancer therapeutics (214). The success of therapies involving paclitaxel may be attributed to its multiple mechanisms of activity. Paclitaxel's primary mechanism of antineoplastic activity rests in its ability to irreversibly polymerize α/β tubulin, thereby disrupting cellular microtubule networks (97,373,374) and cell cycling (114,362). Additionally, paclitaxel suppresses protein synthesis (263), increases radiosensitivity (245,282), and causes cytolysis by TNF-α (457). Although paclitaxel's tumoristatic and tumoricidal activities have been extensively described, no studies have evaluated its significance in a fibrosarcoma model, in which we have described several mechanisms through which fibrosarcoma cells convert Mϕ and T cells into suppressor cells (123). Therefore, we determined whether the Meth-KDE fibrosarcoma cells used in our tumor model were sensitive to the cytostatic or cytotoxic activities of paclitaxel. Furthermore, we determined whether the Mϕ-derived cytokine TNF-α, the production of which is induced paclitaxel, contributes to fibrosarcoma cell sensitivity.

Because apoptosis (88) is an important cellular mechanism for the removal of deleterious from the host, and pharmaceutical agents that induce tumor cell apoptosis represent a promising therapeutic approach to the treatment of cancer, we assessed the capacity of paclitaxel to induce apoptosis in the Meth-KDE cell line. The classic hallmarks of apoptotic cell death include nuclear chromatin, activation of endogenous nuclease activity, and changes in cell size and shape (88). We chose to evaluate an additional change in the early phases of apoptotic cell death – the loss of membrane phosopholipid asymmetry, leading to exposure of phosphatidylserine (PS) on the outer

membrane. Using Annexin V (227), a protein that rapidly and specifically binds outer membrane PS, we determined that paclitaxel induces apoptotic tumor cell death, suggesting that paclitaxel imparts both tumoristatic and tumoricidal activities against Meth-KDE fibrosarcoma cells.

MATERIAL AND METHODS

Tumor Cell Lines

The murine fibrosarcoma cell line Meth-KDE (15,124) and the P815 mastocytoma cell line were propagated in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO), prepared as described in Chapter I. Tumor cell cultures were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 50 mg/L gentamicin (Tri Bio Labs, State College, PA).

Reagents and Cytokines

Paclitaxel was acquired from Calbiochem (La Jolla, CA); stock solutions (4 mM) were prepared in 100% DMSO and stored at -80°C until use. Bacterial LPS (*Escherichia coli* serotype 026-B6) was purchased from Sigma (St. Louis, MO). Recombinant murine TNF-α (specific activity 1.2 x 10^7 U/ml, endotoxin content <10 pg/ml) and IFN-γ (specific activity 1.0 x 10^5 U/ml; endotoxin content <10 pg/ml) was provided by Genentech, Inc. (San Francisco, CA).

Tumor Cell Proliferation Assays

Meth-KDE cells were seeded in 96-well flat-bottom tissue culture plates (Corning Glass, Corning, NY) at a final concentration of 2.5 x 10^5 cells/well in a final volume of 0.2 ml. Some cultures contained paclitaxel (0.1 – 10 μM) or LPS (0.1 – 10 μg/ml). Eighteen h before harvest, all wells were pulsed with 1 μCi/well [^{3}H]-TdR (specific activity 6.7 Ci/mM; DuPont NEN Research Products, Wilmington, DE). Cells were

harvested onto glass fiber filters using a Basic96 cell harvester (Skatron, Sterling, VA), and sample activities were determined using a Beckman LS 6000SC scintillation counter (Beckman, Fullerton, CA).

Tumor Cell Viability Assays

Meth-KDE cells were seeded in 96-well flat-bottom tissue culture plates (Corning Glass, Corning, NY) at a final concentration of either 2.5 x 10^5 cells/well in a total volume of 0.2 ml. Some cultures contained paclitaxel (0.1 – 10 μM) or LPS (0.1 – 10 μg/ml). Four h before harvest, all wells were pulsed with 0.02 ml (10% well volume) Alamar blue™ (BioSource International, Camarillo, CA). Sample absorbances were determined in Molecular Devices ELISA plate reader using a 570 nm test wavelength and a 600 nm reference wavelength.

Tumor Cell Cytotoxicity Assays

Meth-KDE cells were cultured as described during viability assays. The antiproliferative agent actinomycin D (10 nM) was added at the beginning of culture. Cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay (6), as described in Chapter III.

Analysis of Apoptosis

To determine whether paclitaxel induces apoptosis in our tumor model, Annexin V analysis was performed using the R&D Systems (Minneapolis, MN) Apoptosis Kit (KNX50-020), per the manufacturer's suggested protocol. Briefly, Meth-KDE

fibrosarcoma cells or P815 mastocytoma cells were collected and resuspended to 1 x 10^6 cells/ml in RPMI-1640 + 10% FBS. Tumor cell suspensions (0.5 ml) were added to each well of 24-well tissue culture plates. Some cultures were treated with paclitaxel, TNF-α, or paclitaxel and TNF-α; treatment reagents added in a volume of 20 μl. Cells were incubated at 37°C, 5% CO_2 for various times (0 – 6 h), then resuspended in culture medium, transferred into 1.5 ml sterile microfuge tube, centrifuged for 5 min at 1500 rpm in a DuPont Sorvall microfuge, and washed three times in 1 ml of sterile PBS. Cells were resuspended in 0.5 ml of 1x binding buffer (R&D), then 0.1 ml was transferred to a new microfuge tube. Staining reagents were added, at manufacturer's recommended concentration (10 μl Annexin V, 10 μl Propidium Iodide) and cells gently vortexed. Following 30 min incubation (37°C, 5% CO_2) in the dark, 0.4 ml binding buffer (R&D) was added. Within 30 min, samples were analyzed at the Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Lab using the PI/FITC protocol on the Coulter EPICs flow cytometer.

For DNA laddering analysis, tumor cells are prepared to concentrations of 1 x 10^7 cells per sample. Treatment reagents are added and cells incubated at 37°C, 5% CO_2 for 2 h. Cells were harvested, washed 3 times with ice cold PBS, and resuspended in 200 μl of ice-cold lysis buffer (see Appendix E). Proteins were digested by incubation with Proteinase K (1 mg/ml) at 50°C for 12 hours, and RNA was digested by incubation RNase A (50 mg/ml) at 37°C for 30 min. DNA was extracted by phenol/chloroform extraction and ethanol precipitation. Fragmented DNA separated visualized by electrophoresis in 1% agarose gel (100 V for 2 hours) containing ethidium bromide (1 μg/ml).

Statistics and Calculations

Triplicate cultures were tested in all viability and cytotoxicity assay. Data are presented as means ± SEM of triplicate independent determinations. All experiments were repeated at least three times; representative experiments are shown. All comparisons were tested for significance by Student's t test, and all comparisons are significant at the $p < 0.05$ level, unless otherwise stated.

RESULTS

Paclitaxel Inhibits Meth-KDE Proliferation.

At clinically-relevant doses (0.1 – 10 μM), paclitaxel compromised Meth-KDE cell proliferation in a dose-dependent manner (Figure 46), suggesting that paclitaxel imposes cytostatic activity against Meth-KDE cells. In contrast, LPS (which binds β-tubulin but does not promote microtubule polymerization) failed to suppress tumor cell proliferation (Figure 46). In fact, proliferation was enhanced in LPS-treated Meth-KDE tumor cell cultures.

Paclitaxel Decreases Meth-KDE Cell Viability.

To determine whether paclitaxel alters Meth-KDE cell viability during culture, cells were cultured in the absence or presence of paclitaxel and viability was assessed kinetically using the indicator dye Alamar blue™. Significant dose-dependent decreases in tumor cell viability were observed in paclitaxel-treated groups (Figure 47). Because actinomycin D was added to all cultures, unwanted tumor cell proliferation was halted, and decreases in viability may be associated with paclitaxel-mediated cytotoxic activity (301). Thus, these data suggest that paclitaxel compromises Meth-KDE tumor cell viability through direct cytotoxic activity.

Paclitaxel Increases Meth-KDE Cell Sensitivity to TNF-α.

Paclitaxel's chemotherapeutic efficacy has been associated with its ability to

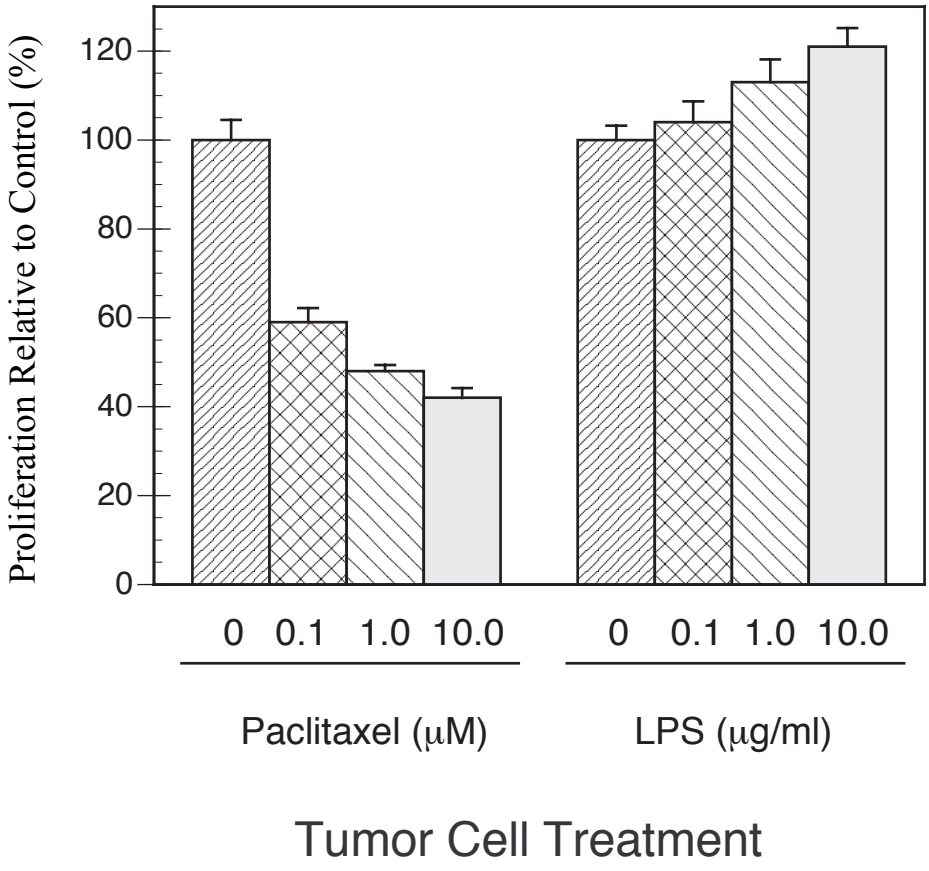


Figure 46. Paclitaxel suppresses Meth-KDE cell proliferation. Meth-KDE cells (2.5 x 10^5) were cultured for 48 h in the absence or presence of paclitaxel (0.1, 1.0, or 10.0 μM) or LPS (0.1, 1.0, or 10.0 μg/ml). Proliferation was assessed by [^{3}H]-TdR incorporation. Data are expressed as percentage proliferation relative to untreated control culture.

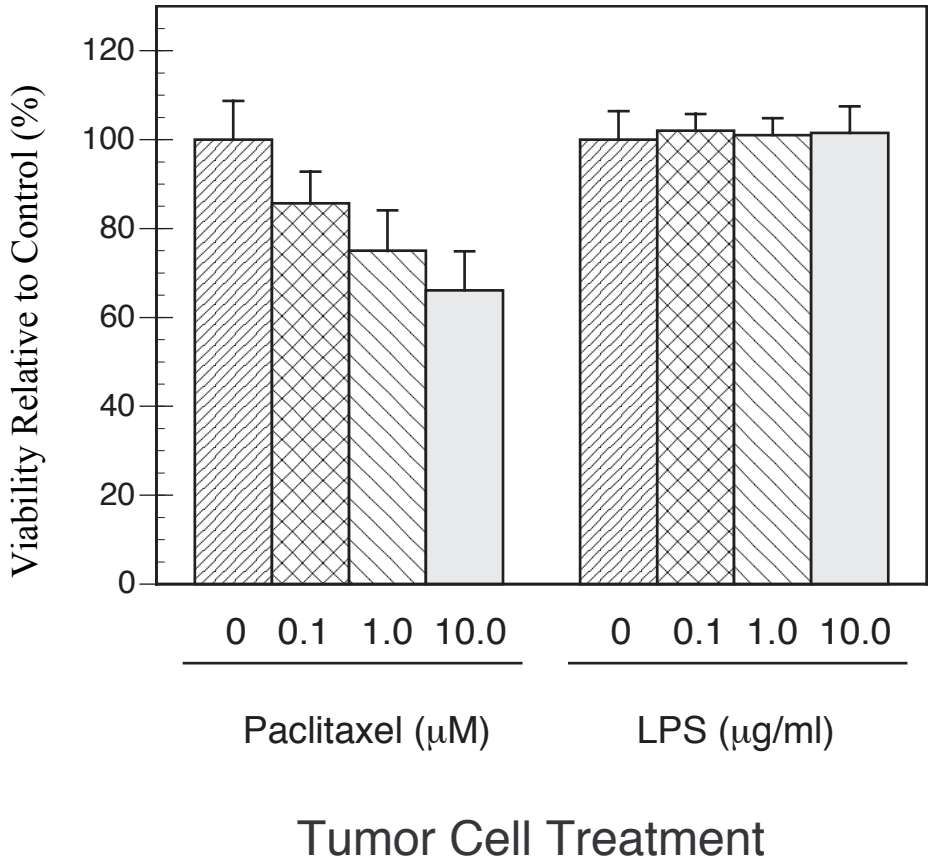


Figure 47. Paclitaxel compromises Meth-KDE tumor cell viability. Meth-KDE cells (2.5×10^5) were cultured in the absence or presence of paclitaxel (0.1, 1.0, or 10.0 μM) or LPS (0.1, 1.0, or 10.0 μg/ml). Viability was assessed after 48 h by Alamar blue ™ assay. Similar data was acquired at 72 and 96 h (not shown). Statistics represent comparisons of treatment groups to untreated controls.

mediate tumor regression through direct cytotoxic and cytostatic mechanisms. Because paclitaxel induces TBH Mϕ production of the lytic cytokine TNF-α (see Chapter I), we determined whether paclitaxel treatment increases tumor cell susceptibility to TNF-α-mediated cytotoxic action. Meth-KDE cells cultured with paclitaxel (10 μM) and actinomycin D, recombinant TNF-α moderately but significantly decreases tumor cell viability in a dose-dependent manner (Figure 48).

Paclitaxel Induces Tumor Cell Apoptosis

We hypothesized that paclitaxel treatment *in vitro* induces apoptotic death in the Meth-KDE tumor cell line. Following exposure to paclitaxel, morphologic changes associated with apoptosis (membrane blebbing, loss of cytoplasmic volume, and formation of apparent apoptotic bodies) were noted in Meth-KDE cells (Walker and Elgert, unpublished observation). Because paclitaxel has been demonstrated to induce apoptotic cell death in other tumor models (179,210), and paclitaxel leads to apoptosis-associated changes in gross morphology in cultured Meth-KDE cells, we investigated paclitaxel's capacity to induce apoptosis in the cells used in our tumor model. To establish the specificity and sensitivity of our methodology (Annexin V labeling of apoptotic cells, see Appendix E), we first assessed apoptosis in the widely-studied P815 murine mastocytoma cell line (Figure 49). Five x 10^5 cells were cultured without or with paclitaxel (10 μM) for 2 h, and apoptosis measured by labeling with Annexin V (horizontal axis) and propidium iodide (vertical axis). All plots represent results for 5,000 events. In agreement with the results of others, paclitaxel (10 μM) induced membrane flipping and exposure of PS on the outer membrane, as indicated by Annexin V staining.

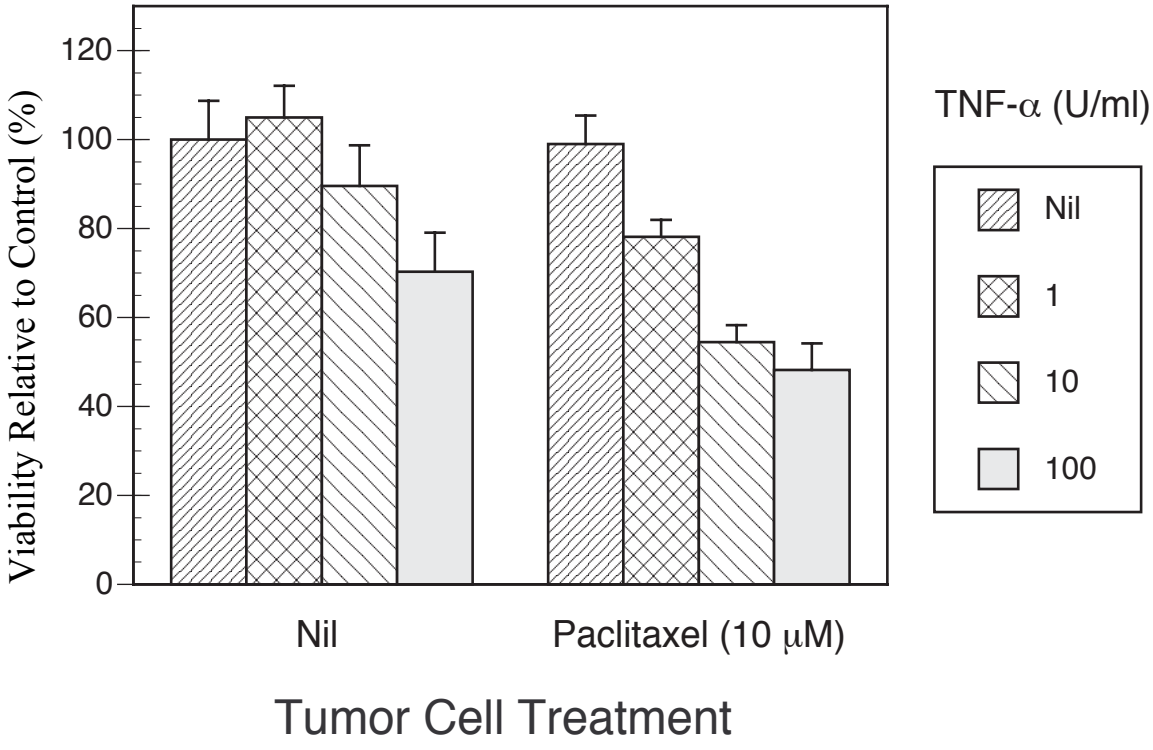


Figure 48. Paclitaxel enhances Meth-KDE tumor cell sensitivity to TNF-α. Meth-KDE cells (2.5×10^5) were cultured for 48 h in the absence or presence of paclitaxel (0.1, 1.0, or 10.0 μM) or TNF-α (1, 10, and 100 U/ml). All cultures contained actinomycin D (10 nM). Viability was assessed using the Alamar blue™ assay.

To determine whether paclitaxel induces apoptotic cell death in our tumor model, Meth-KDE fibrosarcoma cells were cultured without or with paclitaxel (0.1 - 10 μM) and labeled with Annexin V (horizontal axis) and propidium iodide (vertical axis) either immediately (Figure 50) or after 2 h incubation (Figure 51). At the initial timepoint, no Annexin V binding was detected (Figure 50), demonstrating the stability of Meth-KDE cells in the absence of apoptosis-inducing conditions. However, following 2 h incubation with paclitaxel, Annexin V staining was enhanced, indicating that clinically-relevant doses of paclitaxel induce apoptotic tumor cell death *in vitro*. Maximal induction of apoptotic death, as indicated by the percent cells positive for Annexin V, was mediated by 1.0 uM paclitaxel. DNA laddering analysis (Figure 52) reveal that paclitaxel induces DNA fragmentation, a fundamental process of the programmed cell death mechanism.

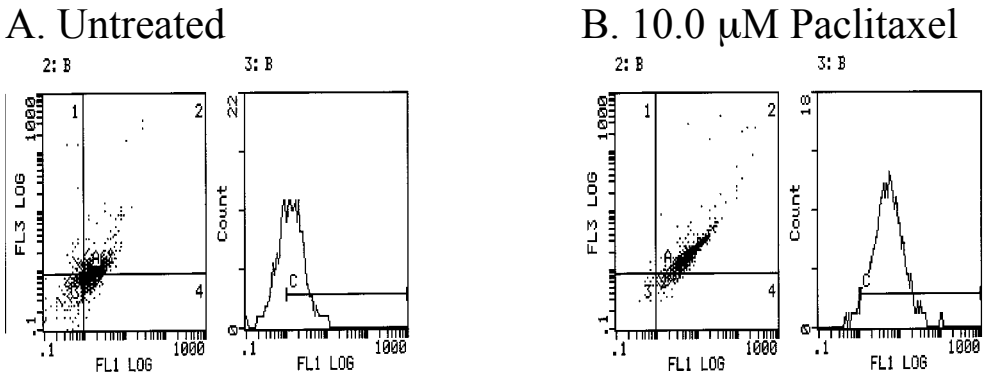
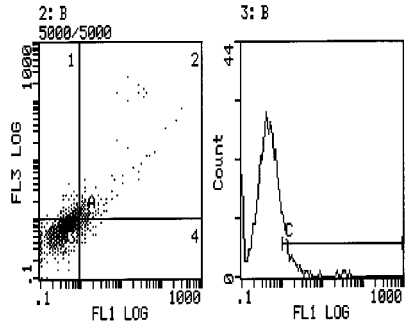
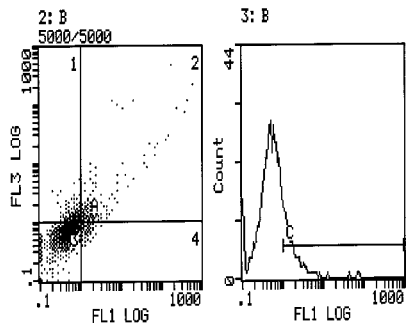


Figure 49. Paclitaxel induces apoptosis in P815 mastocytoma cells. Five x 10^5 P815 murine mastocytoma cells were cultured without or with paclitaxel (10 μM) for 2 h. Following incubation, cells were labeled with Annexin V (horizontal axis) and propidium iodide (vertical axis). Plots represent results for 5,000 events.

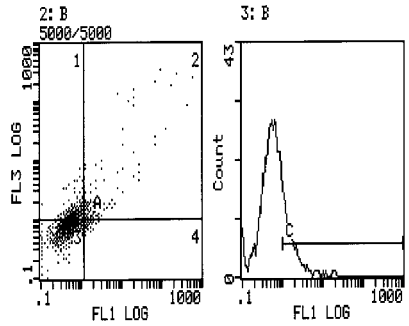
A. Untreated



B. 0.1 μM Paclitaxel



C. 1.0 μM Paclitaxel



D. 10.0 μM Paclitaxel

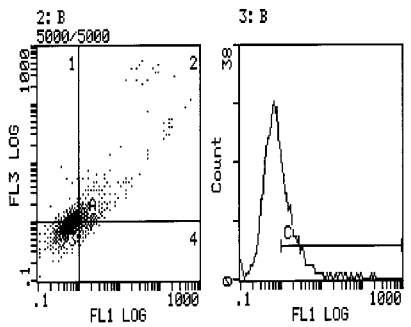


Figure 50. Meth-KDE murine fibrosarcoma cells do not exhibit spontaneous apoptosis. Five x 10^5 Meth-KDE cells were cultured without or with paclitaxel (0.1 - 10 μM) and immediately labeled with Annexin V (horizontal axis) and propidium iodide (vertical axis). Plots represent results for 5,000 events.

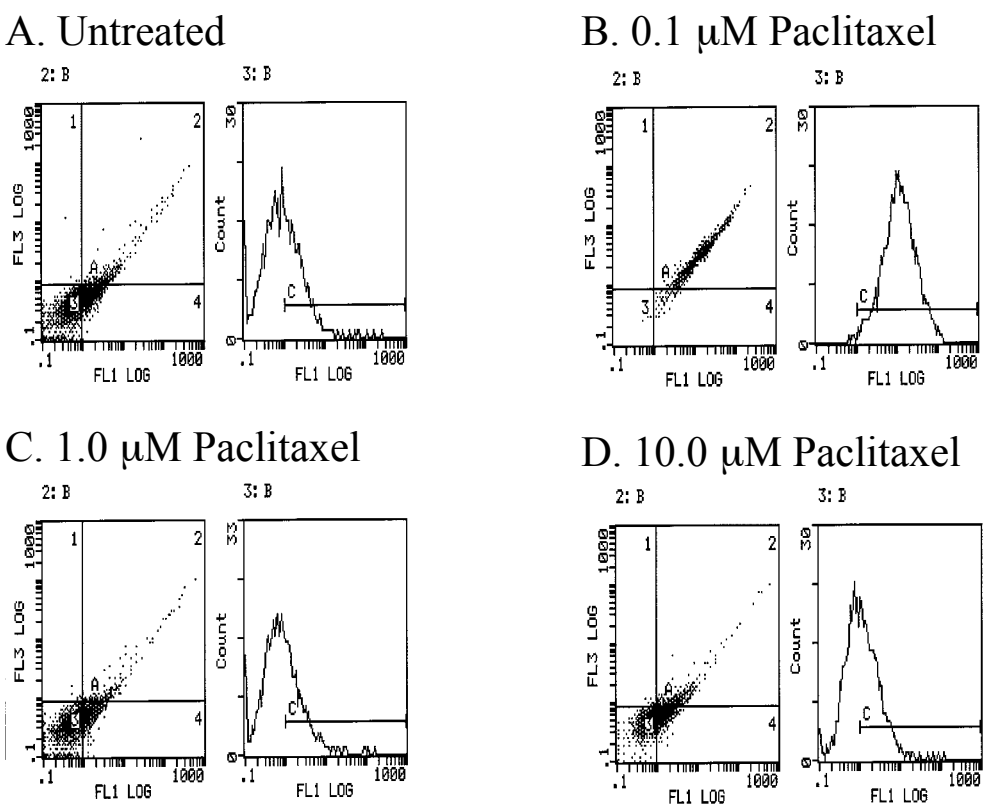


Figure 51. Paclitaxel induces apoptosis in Meth-KDE murine fibrosarcoma cells. Five x 10^5 Meth-KDE cells were cultured without or with paclitaxel (0.1 - 10 μM) for 2 h. Following incubation, cells were labeled with Annexin V (horizontal axis) and propidium iodide (vertical axis). Plots represent results for 5,000 events.

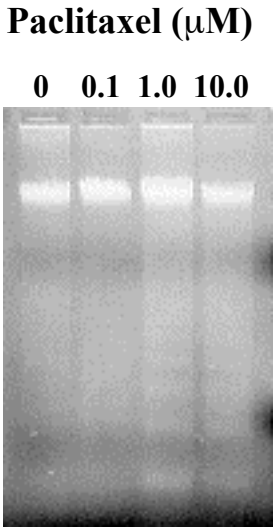


Figure 52. Paclitaxel induces DNA laddering in Meth-KDE murine fibrosarcoma cells. One x 10^7 Meth-KDE cells were cultured without or with paclitaxel (0.1 - 10 μM) for 2 h. Following incubation, cells were lysed and DNA separated on an agarose gel. DNA ladders were visualized using ethidium bromide.

DISCUSSION

While paclitaxel is best known as a natural *chemotherapeutic* drug, our studies have focused on paclitaxel's unique immunotherapeutic activities. However, successful anticancer therapies may involve both the disruption of tumor growth and activation of Mϕ antitumor effector functions. Using a murine cancer model, we have shown that fibrosarcoma growth significantly alters immune cell phenotype and function (123), and tumor-induced Mϕs suppressor significantly compromise immunocompetence. We determined that paclitaxel can reverse tumor-induced suppressor Mϕ activities (see Section III), suggesting that paclitaxel may mediate multiple antitumor effector mechanisms *in vivo*. However, before proceeding with *in vivo* studies of paclitaxel in an immunotherapeutic context, we sought to confirm that paclitaxel has direct cytostatic and cytotoxic activity against the fibrosarcoma cells used in our murine cancer model. In the present study, we evaluated Meth-KDE fibrosarcoma sensitivity to paclitaxel.

Paclitaxel's primary antineoplastic activity derives from its unique capacity to induce stable polymerization of microtubules (373,375), the ubiquitous structural framework of the cell. Microtubules are involved in mitotic function, maintenance of cell shape, cellular motility, and intracellular transport (361). Clinically relevant concentrations of paclitaxel (corresponding to approximately 0.1-10.0 μM) lead to formation of disorganized arrays of microtubules, often in parallel bundles, during all phases of the cell cycle (361). Paclitaxel-mediated polymerization inhibits the normal process of dynamic reorganization by microtubules and leads to arrest in the late (G_2 or M) phases of the cell cycle in murine fibroblasts and HeLa cells. Although cell division is completely blocked, flow cytometric analyses and [^{3}H]-TdR incorporation studies show that paclitaxel does not significantly disrupt events associated with the G_1 or S

phases of the cell cycle (56). In agreement with these observations, paclitaxel (0.1 - 10 μM) treatment of Meth-KDE cells (48 h) significantly inhibited proliferation (Figure 46). LPS, which can bind to β-tubulin and associate with microtubules without causing irreversible polymerization, did not suppress Meth-KDE cell proliferation (Figure 46). Although LPS acts as a growth-promoter in some tumor cell lines, most cell lines are unaffected or respond negatively.

Although decreased proliferative responses suggest that paclitaxel is cytostatic to Meth-KDE cells, these studies fail to determine whether paclitaxel mediates direct antitumor cytotoxicity in this model system. Cytotoxicity was measured using a modification (301) of the Alamar blue™ colorimetric viability assay (6). Cells were cultured with paclitaxel or LPS and the antiproliferative agent actinomycin D; in this system, cytotoxicity is inversely proportional to viability. At clinically-relevant doses, paclitaxel reduced Meth-KDE cell viability dose-dependently, while LPS failed to change Meth-KDE cell viability (Figure 47). Because decreased viability can only occur as a result of paclitaxel-mediated cytotoxic activity, we conclude that paclitaxel mediates direct cytotoxic activity against Meth-KDE fibrosarcoma cells.

We showed that paclitaxel, through its Mϕ-activating function, induces the production of the naturally-produced antitumor cytokine TNF-α (15,296). Because paclitaxel directly induces antitumor cytotoxicity and simultaneously activates Mϕ cytotoxic factor production, we determined whether paclitaxel treatment enhanced Meth-KDE cell sensitivity to TNF-α (Figure 48), as shown in other tumor model systems (457).

A possible mechanism by which paclitaxel may decrease tumor cell viability is induction of tumor cell apoptosis, or programmed cell death. Paclitaxel-mediated apoptosis has been reported in other tumor cell lines (44,179,210,280), and we previously observed morphologic changes characteristic of apoptosis in paclitaxel-treated Meth-KDE cells (Walker and Elgert, unpublished observation). Meth-KDE cells demonstrated membrane blebbing, a qualitative indicator of apoptosis (88,436). To confirm that paclitaxel leads to apoptotic cell death in cultured Meth-KDE cells, we assessed paclitaxel-induced apoptosis by flow cytometric analyses and gel electrophoresis. Annexin V specifically detects outer membrane PS, an indicator of apoptosis. Following 2 h incubation with paclitaxel (0.1 - 10 μM), Annexin V staining was enhanced (Figure 51), indicating that clinically-relevant doses of paclitaxel induce apoptotic Meth-KDE cell death *in vitro*. Further, DNA laddering analysis (Figure 52) confirmed that paclitaxel induced DNA fragmentation; in both systems, optimal induction of apoptosis was mediated by a moderate (1.0 μM) dose of paclitaxel.

Collectively, our studies suggest that Meth-KDE fibrosarcoma cells are sensitive to paclitaxel through multiple mechanisms. Paclitaxel exerts cytostatic and direct cytotoxic effects, enhances tumor cell sensitivity to immune-derived antitumor factors, and induces programmed cell death through the apoptotic pathway. Through its direct antitumor activity and simultaneous immunotherapeutic efficacy (see Section II), paclitaxel may mediate multiple antitumor effector mechanisms. We showed that paclitaxel induces Mϕ TNF-α production *in vitro* (see Chapter I) (296), and that paclitaxel administration primes Mϕs for enhanced TNF-α production on activation (see Chapter VIII) (299). If chemotherapeutic administration of paclitaxel enhances tumor cell sensitivity to TNF-α (as it does *in vitro*, Figure 47), the immunotherapeutic efficacy of paclitaxel-mediated Mϕ cytotoxic molecule production could impart significant

cytotoxic effects *in situ*. Therefore, future studies should evaluate the mechanisms of paclitaxel sensitivity and cytokine signaling *in vivo* to better refine current therapeutic regimens.

CHAPTER VIII

TUMOR GROWTH MODULATES Mϕ NO PRODUCTION FOLLOWING PACLITAXEL ADMINISTRATION

ABSTRACT

The antineoplastic agent paclitaxel mimics bacterial LPS in normal host Mϕs, enhancing antitumor cytotoxicity *in vitro.* Because paclitaxel is used as an antitumor chemotherapeutic agent and tumor growth alters Mϕ phenotype and function, we assessed effector molecule production and cytotoxic activity by normal host and TBH Mϕs following paclitaxel administration. Paclitaxel treatment, duplicating human chemotherapeutic regimens, primed normal host splenic Mϕs for enhanced production of the cytotoxic mediator NO; in contrast, paclitaxel's NO-inducing activity was significantly suppressed in TBHs. In contrast to NO regulation, Mϕ capacity for TNF-α production in both normal hosts and TBHs was enhanced by paclitaxel administration. Although tumor growth modulated paclitaxel-induced Mϕ NO production, paclitaxel administration enhanced both normal host and TBH Mϕ cytotoxic antitumor activity. Blocking NO with a competitive inhibitor abrogated Mϕ cytotoxicity, suggesting paclitaxel-induced TBH Mϕ NO production, although suboptimal, remains sufficient to mediate antitumor activity. These data demonstrate that paclitaxel's *in vivo* immune activities are differentially regulated during tumor burden and suggest that paclitaxel's immunotherapeutic functions may contribute to its success as an anticancer agent.

INTRODUCTION

The antineoplastic agent paclitaxel (TAXOL™, Bristol-Myers Squibb, Princeton, NJ) is a taxane diterpenoid (215) that was originally extracted from the bark and leaves of the western yew *Taxus brevifolia* (450). Once in limited supply, complete laboratory synthesis of paclitaxel has been achieved (316), and paclitaxel is now produced commercially through biochemical modification of related taxane compounds (216). Paclitaxel has undergone extensive clinical development as an antitumor chemotherapeutic (reviewed in 178) and demonstrates considerable activity against a wide range of human malignancies, most notably breast cancers and refractory ovarian tumors. Paclitaxel's primary antitumor mechanism derives from its unique polymerizing action on microtubules; paclitaxel prevents dynamic depolymerization of α/β tubulin polymers (264), halting cell cycle progression (362). Paclitaxel-mediated disruption of cell cycling leads to neoplastic cell death (114) and inhibition of tumor progression (148).

In addition to its well-characterized chemotherapeutic activities, recent studies demonstrated that paclitaxel exerts profound effects on immune cells, including Mϕs. In murine Mϕs, paclitaxel stimulates *in vitro* responses similar to those induced by bacterial LPS, including almost indistinguishable patterns of protein phosphorylation and gene expression (266) and induction of the tumoricidal molecules TNF-α (3) and NO (267). However, fibrosarcoma growth induces phenotypic and functional changes in both tumor-proximal (106) and tumor-distal (15) Mϕ populations, including modulated splenic and peritoneal Mϕ NO production in response to paclitaxel-triggering *in vitro* (296). Although Mϕ NO production in compartments distal to the tumor may not directly influence tumor growth, NO may impart indirect immunosuppressive effects (34,192). To more clearly define the immunologic activities of paclitaxel, which is currently used

as a clinical antitumor chemotherapeutic agent, we assessed normal host and TBH Mϕ effector molecule production and cytotoxic capacity following *in vivo* exposure to paclitaxel. In this study, we extend our previous findings that suggest paclitaxel may activate Mϕs for direct antitumor cytotoxicity while controlling tumor-distal MO production of systemic proinflammatory and cytotoxic molecules (296,301). These data collectively suggest that chemotherapeutic administration of paclitaxel may impart significant immune system actions and immunotherapeutic antitumor activities.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice were used as source for normal host and TBH Mϕs. A BALB/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (Meth-KDE) (15,126), which facilitates the study of tumor-induced distal Mϕ populations, was used as described in Chapter I.

Medium and Reagents

Mϕs were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma, St. Louis, MO), prepared as described in Chapter I. Recombinant murine IFN-γ (specific activity 1.0 x 10^5 U/ml, endotoxin content <10 pg/ml) was a generous gift from Genentech, Inc. (South San Francisco, CA). Bacterial LPS *(Escherichia coli* serotype 026:B6) and NMMA were acquired from Sigma. Neutralizing anti-TNF-α mAb was acquired from PharMingen (San Diego, CA).

Paclitaxel was obtained from Calbiochem (La Jolla, CA). For *in vitro* studies, paclitaxel was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to 4 mM stock solution, stored at -80°C, and diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1 %.

Paclitaxel Administration

For *in vivo* administration, paclitaxel was prepared in 50% polyoxyethylated castor oil (Cremophor EL, Sigma) and 50% dehydrated alcohol at 6 mg/ml, stored at -80°C, and diluted to assay concentrations in sterile PBS immediately before administration. Paclitaxel was administered by i.p. injection (145) at doses of 15-60 mg/kg, approximating human chemotherapeutic regimens. Parallel control animals received an equivalent volume of vehicle only. Splenic Mϕs were collected 24 h post-treatment.

Mϕ Culture and Collection

Normal host and TBH BALB/c splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm Pyrex glass plates), washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium by scraping with a rubber policeman. Red blood cells were lysed by 0.83% ammonium chloride (Sigma) treatment and all cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The final Mϕ preparations contained cells that were >95% viable and >96% esterase positive, and flow cytometric analysis with M1/70 and F4/80 monoclonal antibodies (ATCC, Rockville, MD) showed them to be >80% Mac-1^+ and F4/80^+, respectively.

Mϕ Nitrite Production

Either normal host or TBH Mϕs (4 x 10^5, optimal cell number) were cultured in 96 well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY). Each well

contained a total volume of 200 μL serum-free RPMI-1640 medium supplemented with the indicated concentrations of IFN-γ and LPS. Paclitaxel was added to some cultures for *in vitro* studies. For pretreatment studies, Mϕs were treated with the indicated reagents for 4 h, washed twice, and recultured in fresh medium. Supernatants for nitrite assessment were collected at 96 h (optimal culture time), following centrifugation (400 x *g*, 5 min), and immediately assayed for nitrite.

Because secreted NO quickly reacts with oxygen to yield a stable nitrite endproduct (287), nitrite levels in culture supernatants were measured using the Griess reagent (172), as described in Chapter I. Briefly, 100 μl of Mϕ supernatant was added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3PO_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm (MRX microplate absorbance reader; Dynex, Alexandria, VA). Paclitaxel doses up to 35 μM did not decrease Mϕ viability because the MTT assay (292) verified >95% Mϕ viability throughout the culture periods (not shown).

TNF-α Quantification

TNF-α production was induced by pretreating (4 h) Mϕs as described for NO production. Culture supernatants were collected at 12 h (optimal incubation time) following centrifugation (400 x *g*, 5 min) and stored at -80 °C. TNF-α protein concentration in Mϕ-derived supernatants was measured by a TNF-α-specific ELISA *(Quantikine M,* R&D Systems, Minneapolis, MN) following the manufacturer's provided protocol. The limit of detection was 5.1 pg/ml.

Mϕ-mediated Cytotoxicity Assays

To assess the cytotoxic activity of paclitaxel-activated Mϕs against Meth-KDE tumor cells, Mϕs (2 x 10^5 cells) from untreated or paclitaxel-treated normal hosts or TBHs were added to 1 x 10^5 tumor cells. Cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay (6) as previously described (301). Briefly, Meth-KDE cells were seeded into 96-well flat bottom tissue culture plates in 100 μl RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and incubated 24 h. Normal host or TBH Mϕs 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, 20 μl (10% of the well volume) of Alamar blue™ indicator dye (BioSource International, Camarillo, CA) was added to each well. In the presence of viable cells, Alamar blue™ dye is reduced to a colored product detectable at 580 nm. Four h after addition of the indicator dye, tumor cell viability was assessed by measuring absorbance at 570 nm on a MRX microplate absorbance reader.

Statistics and Calculations

Cells from six to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for nitrite in the Griess reagent tests, TNF-α protein in the specific ELISA, and cytotoxicity in the Alamar blue™ assays. Data are means ± SEM of triplicate 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

Tumor Growth Modulates Paclitaxel's Capacity to Induce NO Production by IFN-γ-primed Mϕs

Paclitaxel has demonstrated LPS-mimetic characteristics, acting as a second signal for the induction of normal host Mϕ cytotoxicity and NO production *in vitro* (267). However, tumors induce functional and phenotypic changes that may alter Mϕ responsiveness to activating agents (15). To more clearly define whether tumor growth modified Mϕ response to paclitaxel, we first examined the capacity of LPS and paclitaxel to induce NO production by IFN-γ-primed splenic Mϕs *in vitro* (Table 4). LPS enhanced IFN-γ-primed NO production by both normal host and TBH splenic Mϕs, and tumor-induced Mϕs were more responsive to LPS triggering. Paclitaxel-induced NO production by IFN-γ-primed normal host Mϕs was comparable to that achieved by LPS triggering. In contrast, tumor growth modulated paclitaxel-mediated Mϕ NO production; although tumor growth primed Mϕs for enhanced LPS responsiveness, paclitaxel-induced TBH Mϕ NO production was significantly ($p < 0.01$) lower than achieved with LPS triggering or IFN-γ treatment alone. Increasing doses of paclitaxel (up to 35 μM) failed to overcome tumor-induced inhibition of paclitaxel-mediated NO production (not shown).

During clinical antitumor chemotherapeutic regimens (reviewed in 178), paclitaxel administration occurs in a discontinuous manner. To achieve a more clinically-relevant representation of Mϕ responsiveness following transient exposures to paclitaxel, IFN-γ-primed normal host or TBH Mϕs were pretreated with LPS or paclitaxel for 4 h, recultured in fresh medium, and NO production assessed (Table 4).

Table 4. Tumor Growth Modulates NO Production following Transient Paclitaxel Treatment *in vitro.*

Activating Agent	Continuous Culture[1]		Four Hour Pretreatment[2]	
	NH Mϕs	TBH Mϕs	NH Mϕs	TBH Mϕs
NIL	3.11 ± 0.14[3]	16.41 ± 0.21	2.04 ± 0.13	3.11 ± 0.17
LPS (1 μg/ml)	6.03 ± 0.27	32.5 ± 0.87	6.32 ± 0.57	8.97 ± 0.29
Paclitaxel (10 μM)	6.48 ± 0.41	9.37 ± 0.41[4]	8.52 ± 0.32	4.92 ± 0.22[5]

[1]Either 4 x 10^5 normal host or TBH Mϕs were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml). LPS or paclitaxel was added at the start of culture. NO was measured following 96 h (optimal culture time). Similar data were acquired using lower and higher doses of treatment reagents (0.1, 1.0, or 35 μM paclitaxel, 0.1 or 10 μg/ml LPS, and 1 or 100 U/ml IFN-γ) (not shown).

[2]Either 4 x 10^5 normal host or TBH Mϕs were cultured as described; after 4 h, cells were washed and recultured in fresh medium. NO was measured following 96 h (optimal culture time). Similar data were acquired using lower and higher doses of treatment reagents (not shown).

[3]Values are expressed as mean nitrite (μM) ± SEM.

[4]NO production following continuous culture with paclitaxel was significantly different as compared to LPS-triggered TBH Mϕs ($p < 0.01$) or paclitaxel-triggered NH Mϕs ($p < 0.05$)

[5]NO production following paclitaxel pretreatment was significantly different as compared to LPS-triggered TBH Mϕs ($p < 0.05$) or paclitaxel-triggered NH Mϕs ($p < 0.05$)

LPS pretreatment enhanced IFN-γ-primed NO production by both normal host and TBH splenic Mϕs, and TBH Mϕs produced more NO in response to LPS triggering than similarly activated normal host Mϕs. NO production by paclitaxel-pretreated IFN-γ-primed TBH Mϕs was significantly ($p < 0.05$) lower than the NO produced in response to LPS triggering. In contrast to TBH Mϕs continuously exposed to paclitaxel, which produced 45% more NO than similarly treated normal host Mϕs, paclitaxel-pretreated TBH Mϕs produced 42% less NO than the corresponding normal host Mϕs.

Mϕ NO Production is Differentially Regulated by Normal Hosts and TBHs following Paclitaxel Administration

Although we (296,301) and others (112,267) have characterized the effects of *in vitro* paclitaxel exposure on Mϕ function, the immune consequences of direct paclitaxel treatment remain unclear, and no studies address the effects of tumor growth on Mϕ responsiveness to paclitaxel exposure *in vivo.* To more definitively determine the regulatory activities of paclitaxel on Mϕ populations during chemotherapeutic application, we assessed the NO production capacity of normal host (Figure 53) and TBH (Figure 54) splenic Mϕs following a chemotherapeutic paclitaxel administration regimen. In a response pattern closely mimicking that achieved after *in vitro* pretreatment (see Table 4), paclitaxel administration (30 mg/kg) primed normal host Mϕs for enhanced NO production upon subsequent signaling with IFN-γ and LPS (Figure 53). While administration of the vehicle (Cremophor EL) alone slightly enhanced Mϕ NO production in response to activation, paclitaxel treatment produced a significantly greater ($p < 0.01$) increase in Mϕ NO production upon culture with IFN-γ and LPS, as compared to the untreated or vehicle-treated populations.

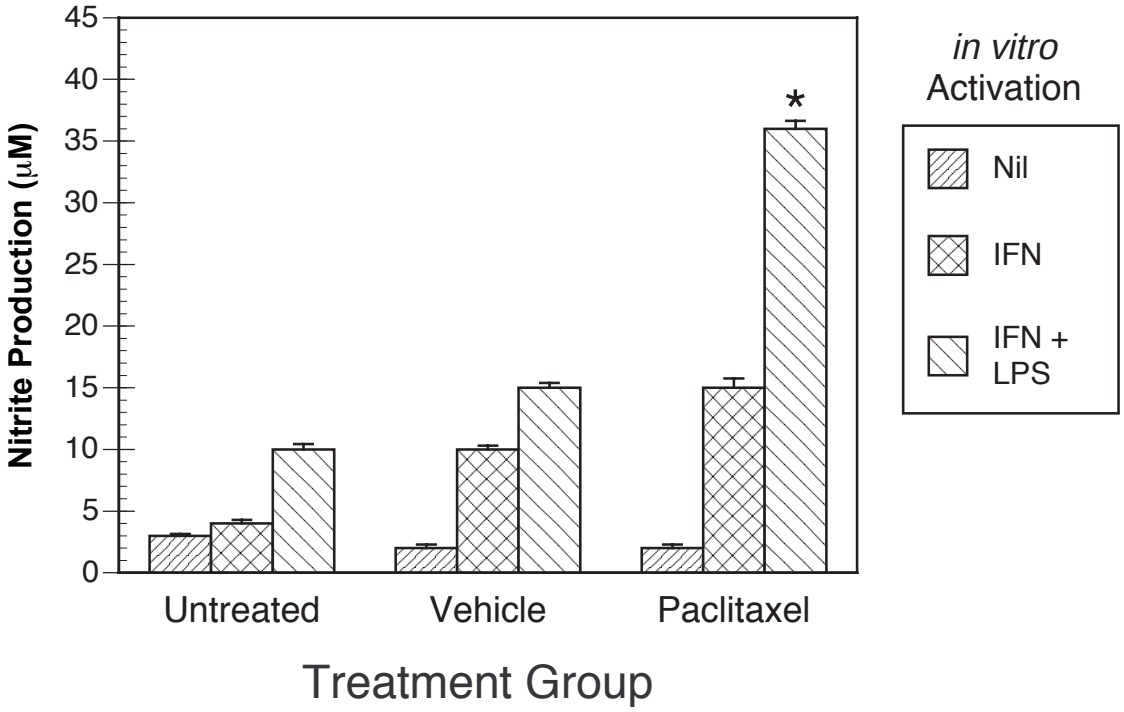


Figure 53. Paclitaxel treatment primes normal host Mϕs for enhanced NO production. Clinically-relevant doses of paclitaxel (30 mg/kg), equivalent volumes of vehicle, or PBS alone were administered i.p. to normal hosts. Twenty-four h post-injection, 4 x 10^5 splenic Mϕs were harvested and cultured in 200 μl of serum-free medium without or with IFN-γ or LPS. Nitrite levels in supernatants were determined at 96 h. Lower and higher doses of paclitaxel (15 mg/kg and 60 mg/kg) produced similar profiles (not shown). Data are averages ± SEM of triplicate independent determinations from one of four similar experiments. *, $p < 0.01$ compared to similarly-activated Mϕs from untreated or vehicle treated normal hosts.

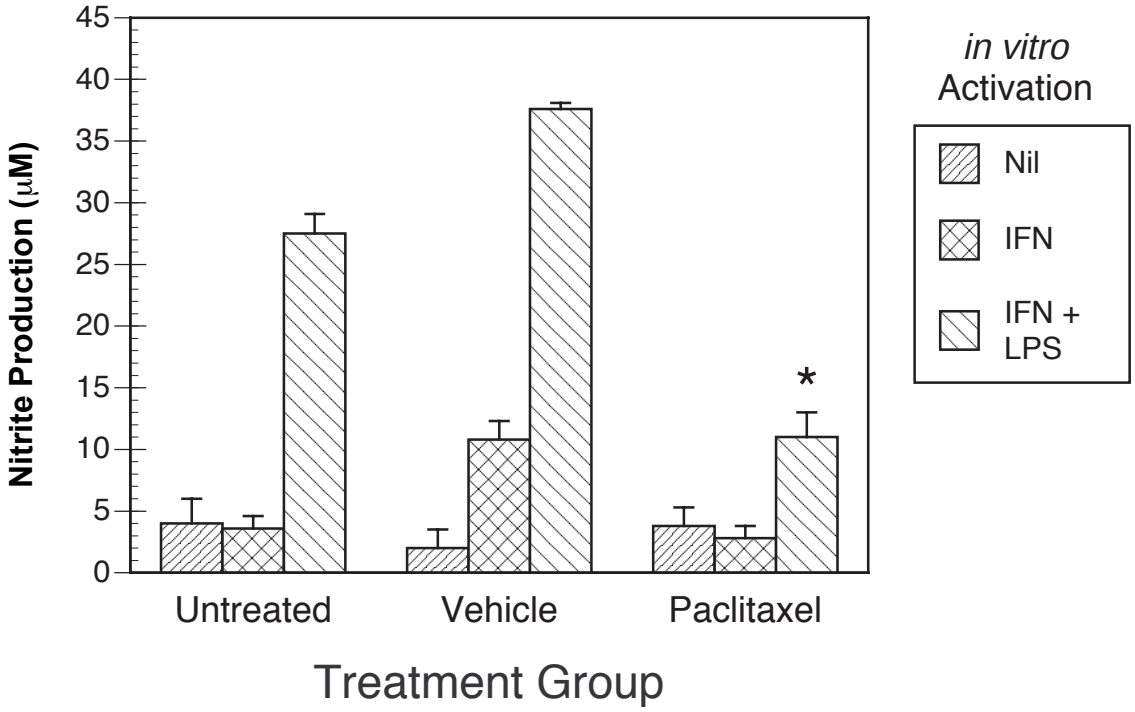


Figure 54. Tumor growth modulates paclitaxel-mediated Mϕ NO production capacity. Following administration of PBS, vehicle, or clinically-relevant doses of paclitaxel (30 mg/kg) to TBH, splenic Mϕs were collected and cultured as described. Nitrite levels in supernatants were determined at 96 h. Lower and higher doses of paclitaxel (15 mg/kg and 60 mg/kg) produced similar profiles (not shown). Higher doses of IFN-γ (100 U/ml) and LPS (10 μg/ml) failed to overcome tumor-induced modulation of paclitaxel-mediated Mϕ NO production. Data are averages ± SEM of triplicate independent determinations from one of four similar experiments. *, $p < 0.01$ compared to similarly-activated Mϕs from untreated or vehicle-treated normal hosts.

Tumor growth altered Mϕ NO production in response to paclitaxel treatment (Figure 54). Paclitaxel-induced NO production by IFN-γ-primed, LPS-triggered TBH Mϕs was significantly ($p < 0.01$) compromised as compared to similarly-treated Mϕs from untreated or vehicle-treated TBHs. In agreement with *in vitro* pretreatment results (see Table 4), Mϕ NO production was differentially regulated in normal hosts and TBHs in response to paclitaxel administration. In both normal hosts and TBHs, different doses of paclitaxel (15 or 60 mg/kg) and varying concentrations of IFN-γ (1 or 100 U/ml) or LPS (0.1 or 10 μg/ml) gave similar profiles (not shown). The highest doses of IFN-γ and LPS failed to overcome tumor-induced inhibition of paclitaxel-triggered Mϕ NO production.

Paclitaxel Administration Enhances Mϕ TNF-α Production

Because tumor growth functionally alters paclitaxel-induced Mϕ NO production, and the regulation of TNF-α is fundamentally coupled to NO, we examined the effect of tumor growth on paclitaxel's capacity to induce Mϕ TNF-α production. For *in vitro* assessment of paclitaxel-induced TNF-α, normal host or TBH splenic Mϕs (4×10^5 cells) were cultured with paclitaxel (10 μM) and IFN-γ (10 U/ml) for 4 h, recultured in fresh media, and supernatants assayed for TNF-α protein (Figure 55). *In vitro* treatment with paclitaxel increased TNF-α production by both IFN-γ-primed normal host and TBH Mϕs. In contrast to the effects of tumor growth on NO production, TBH Mϕs were primed for enhanced TNF-α production by paclitaxel administration.

To determine the effects of paclitaxel administration on Mϕ TNF-α production, normal hosts and TBHs were injected i.p. with paclitaxel, vehicle alone, or PBS alone, as

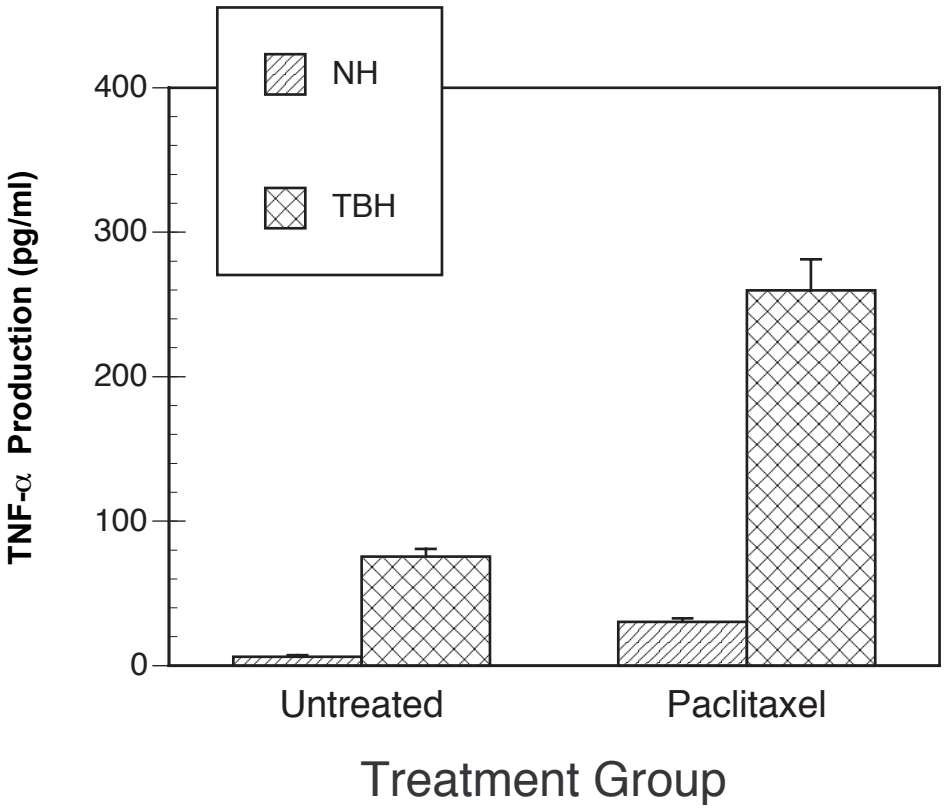


Figure 55. Paclitaxel treatment *in vitro* enhances IFN-γ-primed Mϕ TNF-α production. Mϕ TNF-α production was assessed following *in vitro* exposure to paclitaxel. Splenic Mϕs (4×10^5) were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml). TNF-α protein levels in supernatants were determined at 12 h. Similar data were acquired using lower and higher doses of treatment reagents (1 or 100 U/ml IFN-γ). Data are averages ± SEM of triplicate independent determinations from one of four similar experiments.

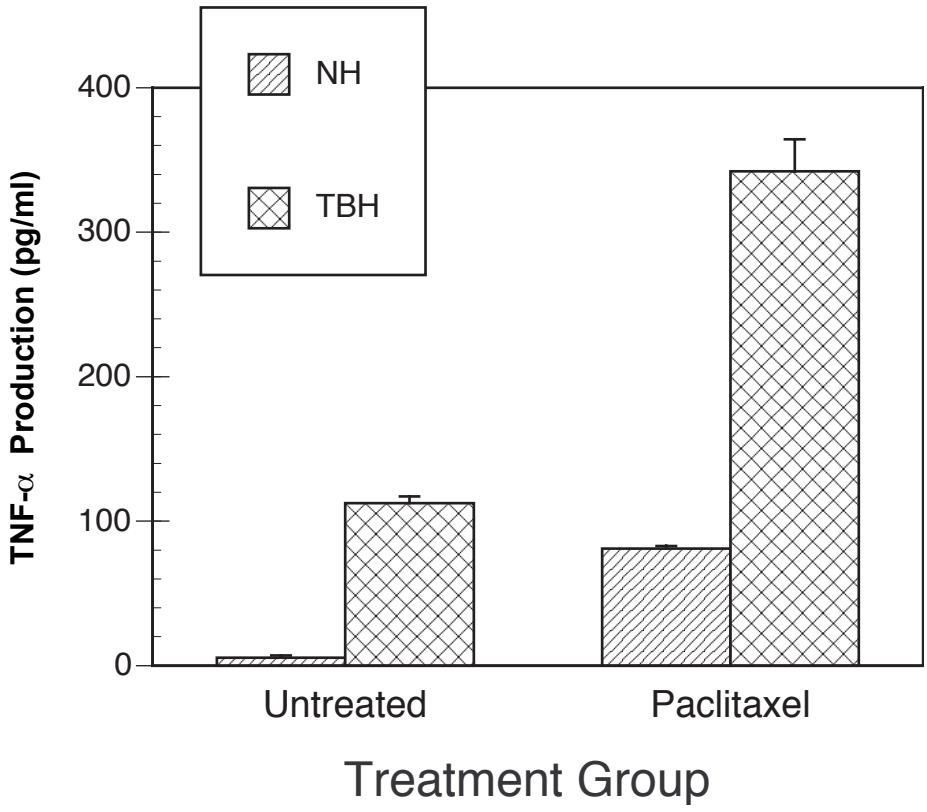


Figure 56. Paclitaxel administration enhances IFN-γ-primed Mϕ TNF-α production. Mϕ TNF-α production was assessed following paclitaxel administration. Splenic Mϕs (4 x 10^5) were cultured in 200 μl of serum-free medium with IFN-γ (10 U/ml). TNF-α protein levels in supernatants were determined at 12 h. Similar data were acquired using lower and higher doses of treatment reagents (1 or 100 U/ml IFN-γ), and lower or higher doses of paclitaxel (15 mg/kg and 60 mg/kg) produced similar profiles (not shown). Administration of vehicle or PBS alone had no effect on Mϕ TNF-α production, and TNF-α was not detected in Mϕ cultures lacking IFN-γ (not shown). Data are averages ± SEM of triplicate independent determinations from one of four similar experiments.

described, and splenic Mϕs harvested 24 h post-injection; Mϕs were cultured (4 x 10^5 cells) with IFN-γ (10 U/ml) for 12 h and supernatants were assayed for TNF-α (Figure 56). Administration of paclitaxel primed both normal host and TBH Mϕs for enhanced production of TNF-α upon subsequent culture with IFN-γ (10 U/ml) (Figure 56), in agreement with our *in vitro* results (see Figure 55). Tumor growth primed Mϕs for enhanced production of TNF-α following paclitaxel stimulation. Administration of vehicle or PBS alone had no effect on Mϕ TNF-α production, and TNF-α was not detected in Mϕ cultures without IFN-γ (not shown).

Paclitaxel Enhances Mϕ-mediated Cytotoxicity

To determine whether paclitaxel administration alters Mϕ antitumor cytotoxic capacity, Mϕs from untreated and paclitaxel-treated (30 mg/kg) normal hosts (Figure 57) or TBHs (Figure 58) were added to growing Meth-KDE tumor cells and cytotoxicity measured. Paclitaxel administration primed Mϕs for enhanced cytotoxicity in a dose-dependent manner against the purified adherent Meth-KDE tumor cell line (Figure 57). Both normal host and TBH Mϕ-mediated cytotoxicity were enhanced by paclitaxel administration, although TBH Mϕs demonstrated enhanced cytotoxic activity as compared to similarly treated normal host Mϕs. To determine whether Mϕ-derived NO was involved in paclitaxel-induced cytotoxic activity, the NO inhibitor NMMA was used to block Mϕ NO production. Addition of 0.5 mM NMMA (sufficient to abrogate Mϕ NO production, not shown) inhibited paclitaxel-mediated cytotoxic activity and limited TBH Mϕ-mediated cytotoxicity to levels of untreated Mϕs, suggesting that NO is the primary effector of cytotoxicity in paclitaxel-stimulated Mϕ cultures. Because we had previously reported that TNF-α induces Mϕ NO production (296), we determined whether paclitaxel-induced TNF-α enhances macrophage-mediated cytotoxicity. Neutralization

of TNF-α with mAb (2 μg/ml) slightly inhibited Mϕ-mediated cytotoxicity against the tumor cells used in our model system (Figures 57 and 58); however, anti-TNF-α mAb had significantly less modulatory effect as compared to the NO-inhibitor NMMA.

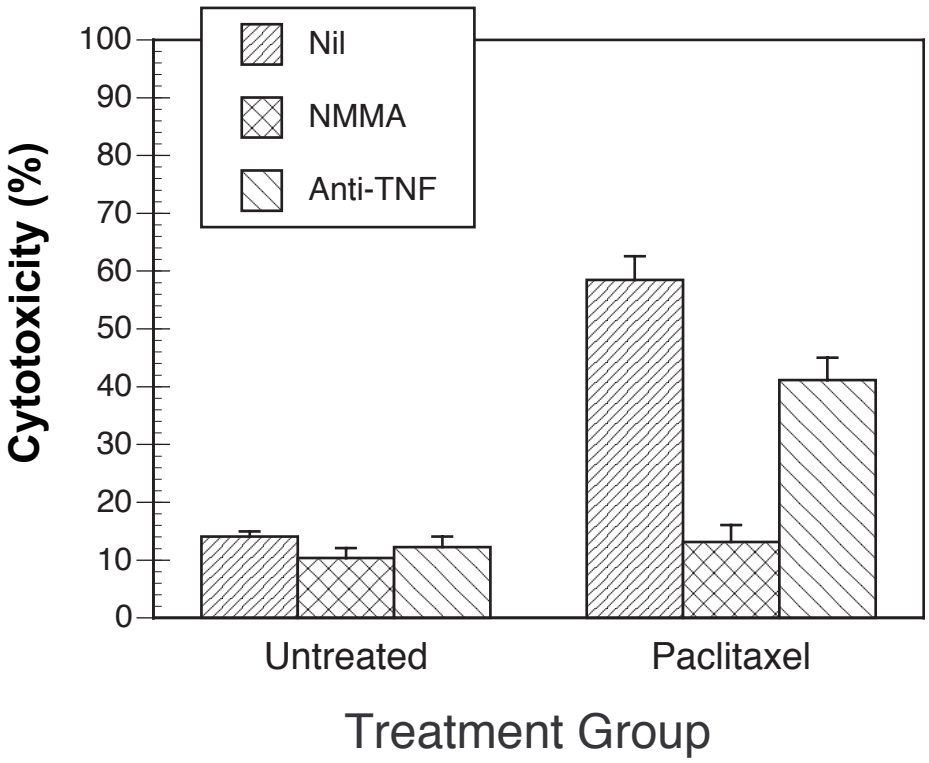


Figure 57. Paclitaxel enhances normal host Mϕ cytotoxic activity through NO. Mϕs (2 x 10^5 cells) from untreated or paclitaxel-treated (30 mg/kg) normal hosts were added to 1 x 10^5 Meth-KDE tumor cells and cultured for 24 h in the presence of 10 nM actinomycin-D. Cytotoxicity measured using a modification of the Alamar blue™ colorimetric viability assay. Percent cytotoxicity was calculated using the formula [1-[(control - test) ÷ test]] x 100. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

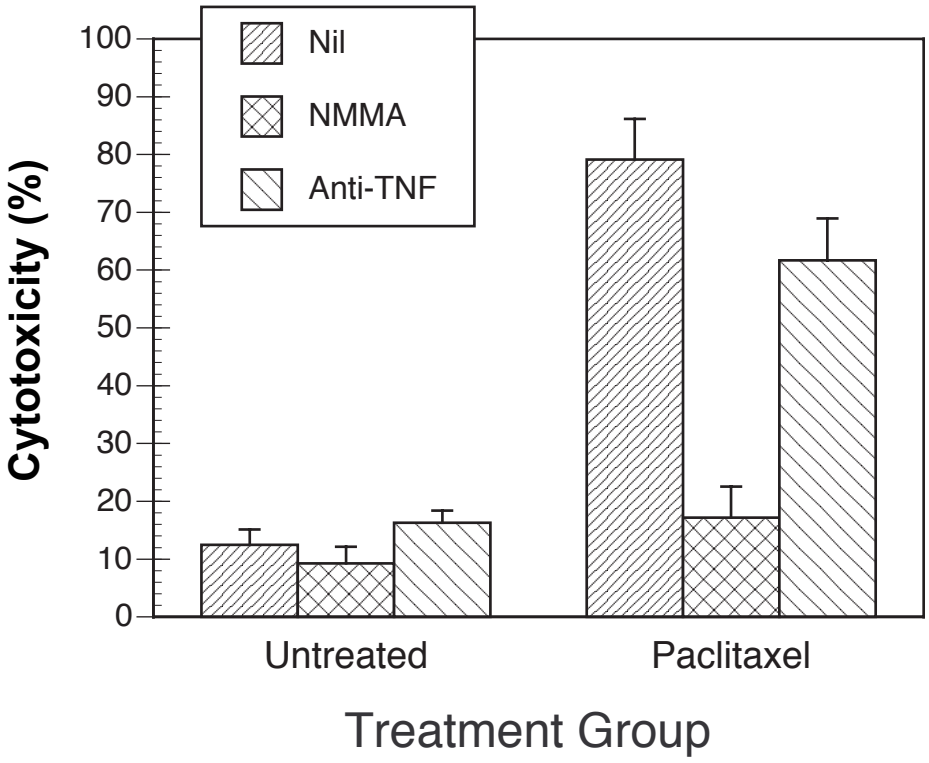


Figure 58. Paclitaxel enhances TBH Mϕ cytotoxic activity through NO. Mϕs (2×10^5 cells) from untreated or paclitaxel-treated (30 mg/kg) TBHs were added to 1×10^5 Meth-KDE tumor cells and cultured for 24 h in the presence of 10 nM actinomycin-D. Cytotoxicity measured using a modification of the Alamar blue™ colorimetric viability assay. Percent cytotoxicity was calculated using the formula [1-[(control - test) ÷ test]] x 100. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

DISCUSSION

Although paclitaxel has been extensively characterized as an antineoplastic chemotherapeutic, paclitaxel's most notable immunologic activity may be its ability to enhance Mϕ-mediated tumor cell cytotoxicity through induction of Mϕ NO production (267). Although enhanced NO production may seem intuitively favorable to the TBH, both the beneficial and pathologic activities of NO must be weighed when considering the use of NO-activating agents. For example, Mϕ-derived reactive nitrogen and oxygen species (including NO) can mediate both cytotoxic (267,301) and immunosuppressive (34,192) functions, depending on the level of production and site of expression. We showed that murine fibrosarcoma growth induces tumor-distal (splenic or peritoneal-exudate) Mϕ populations to produce elevated levels of NO and TNF-α *in vitro* (15). Through over-expression of immunomodulatory cytokines -- including TGF-β_1 and IL-10 -- tumors prime nonresident Mϕs for enhanced production of proinflammatory and cytotoxic molecules upon activation (15). However, NO has a circulatory half-life of only a few seconds and cannot target or infiltrate a tumor mass when expressed in tumor-distal compartments, yet distally expressed Mϕ NO can severely dysregulate T-cell proliferation (7,174,192) and influence blood flow dynamics (118). Simultaneously, high *in situ* concentrations of tumor-derived modulatory factors may inhibit the efficacy of tumor-infiltrating Mϕs by abrogating cytotoxic activity (270) and limiting Mϕ production of NO and TNF-α at the tumor site (36,284). Mϕ production of reactive moieties therefore varies, depending both on the Mϕ's host and resident *in vivo* compartment. Thus, in order to achieve an optimal Mϕ-mediated antitumor response, cytotoxic molecule production must be differentially regulated in tumor-proximal and tumor-distal compartments.

Interestingly, we found that tumor growth suppressed IFN-γ-primed tumor-distal Mϕ NO production following transient paclitaxel exposure *in vitro* (Table 4). LPS, in contrast, strongly stimulated NO production by both normal host and TBH Mϕs. Paclitaxel enhanced TBH Mϕ NO production to levels greater than endogenous NO released from resting cells, but optimal paclitaxel-induced NO was significantly lower than the optimal LPS-triggered levels. In a more biologically-relevant situation, the question remains if transient paclitaxel treatment *in vivo* will elicit similar responses in Mϕs. We found that tumor growth differentially regulated Mϕ responsiveness to chemotherapeutic paclitaxel treatments. Paclitaxel treatment primed normal host Mϕs for enhanced NO production upon subsequent signaling with priming and activating agents (Figure 53). In contrast, tumor growth inhibited paclitaxel-mediated priming, significantly diminishing NO production by TBH Mϕs as compared to Mϕs from untreated TBHs (Figure 54). Even optimal doses of IFN-γ and LPS failed to trigger high levels of NO production following pretreatment.

Because of paclitaxel's extreme hydrophobicity, the drug must be delivered in a special carrier vehicle consisting of a 1:1 (v/v) mixture of ethanol and the polyoxyethylated castor oil derivative Cremophor-EL (reviewed in 116). Following treatment with the vehicle alone, TBH Mϕ demonstrated enhanced NO production in response to IFN-γ and LPS (Figure 54). We speculate that the vehicle may enhance Mϕ activation and response to certain activating agents, including LPS. Tumor growth primes splenic and peritoneal macrophages for enhanced NO production on LPS signaling (15,296), and the carrier vehicle appears to further enhances this responsiveness. Although no studies have reported its effects on NO production, Cremophor induces immune activation, including the induction of hypersensitivity reactions and complement activation (363,405) and may influence Mϕ activation *in vivo.*

These activities, however, do not counteract the downregulated NO production response of TBH Mϕs to paclitaxel administration.

Several mechanisms may account for the modulatory effect of tumor growth on paclitaxel-induced Mϕ NO production. Tumor-derived immunomodulatory factors such as IL-10 or TGF-β_1, which can enter systemic circulation and act on tumor-distal immune cells, may compromise Mϕ responsiveness to paclitaxel. Additionally, paclitaxel may directly or indirectly induce autocrine production of PGE_2, TGF-β_1, or IL-10, which in turn may exert control over Mϕ NO production (9,15). Finally, tumor growth may interfere with paclitaxel-induced signal transduction pathways, including translocation of the pleiotropic activation signal NF-κB, and we are assessing this possibility.

While Mϕ-derived NO is a potent antitumor effector molecule, Mϕs may mediate antineoplastic activity through the production of other proinflammatory and cytotoxic cytokines, including TNF-α. In this study, we found that paclitaxel administration primed both normal host (Figure 55) and TBH Mϕs (Figure 56) for enhanced production of TNF-α. Although the consequences are unclear, induction of Mϕ TNF-α production – even by tumor-distal populations -- may have beneficial consequences for the TBH. Specifically, TNF-α may induce or enhance the production of other cytokines that mediate antitumor immune responses, such as the T-cell-stimulatory cytokine IL-12, both directly (255) and indirectly through increased T-cell production of IFN-γ (313). Furthermore, TNF-α can promote the generation of $CD8^+$ T-cell-mediated antitumor cytotoxicity through reversal of TGF- β_1-induced inhibition (168), and TNF-α-dependent production of reactive nitrogen intermediates can mediate Mϕtumoricidal activity through IFN-γ and IL-2 (94).

In addition to its direct antineoplastic activities, paclitaxel has the capacity to enhance Mϕ-mediated cytotoxicity toward tumor cells *in vitro* (267,301). However, no studies have addressed whether paclitaxel administration has the capacity to induce or enhance Mϕ-mediated antitumor cytotoxicity. In our model, paclitaxel administration increased direct Mϕ–mediated cytotoxicity of Meth-KDE tumor cells by both normal (Figure 57) and TBH Mϕs (Figure 58), demonstrating that paclitaxel has the capacity to prime Mϕs for enhanced antitumor cytotoxicity, regardless of the presence of neoplastic growth. Interestingly, paclitaxel induced greater cytotoxic activity by TBH Mϕs (Figure 58), as compared to normal host Mϕs (Figure 57), even though paclitaxel-treated TBH Mϕs produce less NO. Paradoxically, abrogation of NO production with the competitive inhibitor NMMA expunged paclitaxel-induced cytotoxic activity; however, neutralization of TNF-α only partially abrogated cytotoxicity, suggesting that NO is the primary effector molecule in this model system. The enhanced cytotoxic activity of paclitaxel-activated TBH Mϕs may derive from the combined induction of NO and TNF-α. We have shown that TNF-α acts in an autocrine manner to enhance Mϕ NO production (296). Furthermore, TNF-α may enhance tumor cell sensitivity to NO, rendering tumors susceptible to even modest levels of NO. NO generation by Mϕs is enhanced when effector Mϕs are exposed to tumor targets (242), and Mϕ-derived TNF-α may induce tumor cell autocrine production of NO (134), which would enhance the apparent cytotoxic effectiveness of paclitaxel-activated TBH Mϕs.

Although we have focused our studies on the activities of paclitaxel in tumor-distal Mϕs, others show that tumor-associated Mϕs are incapable of NO production due to high intratumoral concentrations of Mϕ-deactivating cytokines (106,164). Through its direct inhibition of tumor cell division and protein synthesis, paclitaxel may diminish tumor production of Mϕ-deactivating molecules while concurrently activating tumor-

infiltrating Mϕ production of *in situ* NO and TNF-α, leading to enhanced tumoricidal activity. Thus, in addition to its direct cytotoxic and cytostatic actions, paclitaxel may expand Mϕ-mediated antitumor activities by modulating the suppressive activities of tumor-distal populations while simultaneously enhancing the antitumor efficacy of tumor-proximal Mϕs, and we are currently assessing this possibility.

Regarding human monocyte/Mϕ production of NO (103), the relevance of these results to human oncology patients may be questioned. However, human Mϕs possess the gene and functional protein for NO production (454), and NO production has been demonstrated *in vitro* (119,454). Recent data indicate that human peripheral blood mononuclear cells can produce moderate amounts of NO *in vivo* (119), suggesting that Mϕ responsiveness to paclitaxel in human cancer patients warrants further investigation.

Collectively, these results demonstrate that paclitaxel administration mediates differential immune activities vary with cell phenotype and compartment. These studies, which complement our previously reported *in vitro* results (15,296,301) suggest that paclitaxel may simultaneously control Mϕ–mediated immunosuppressive activities in tumor-distal compartments while facilitating antitumor activities by tumor-proximal Mϕ populations. We suggest that the immune activities of paclitaxel should be considered in a clinical context. Recognition of paclitaxel's immune activating properties may lead to optimization of current chemotherapeutic treatment regimens.

CHAPTER IX

IL-12 REVERSES THE T-CELL INHIBITORY EFFECTS OF PACLITAXEL

ABSTRACT

The antineoplastic agent paclitaxel is a potent inhibitor of tumor cell division and a useful chemotherapeutic for the treatment of refractory ovarian and breast carcinoma. Multiple immune system actions have been ascribed to paclitaxel, including the capacity to induce Mϕ antitumor cytotoxic molecule production. However, T-cells are susceptible to paclitaxel's cytostatic functions, and no studies have investigated the effects of direct paclitaxel administration on lymphocyte function in the TBH. Because paclitaxel is currently used as an antitumor chemotherapeutic agent and tumor growth alters leukocyte functions, we assessed T-cell function following chemotherapeutic-type paclitaxel treatment. Paclitaxel administration significantly compromised the proliferative capacity of both normal host and TBH lymphocytes *in vitro*. Although tumor growth impaired T-cell IFN-γ production, paclitaxel treatment did not alter IFN-γ production. We speculate that the immunostimulatory cytokine IL-12, which promoted T-cell activation and proliferation, was capable of reversing paclitaxel-mediated immunosuppression. Exogenous IL-12 fully reconstituted proliferative reactivity and enhanced IFN-γ production by both normal host and TBH lymphocytes *in vitro*. Collectively, these data suggest that chemotherapeutic paclitaxel regimens impart significant but reversible inhibition of lymphocyte populations, and IL-12 may be a useful ancillary immunotherapeutic to overcome paclitaxel-induced modulation of lymphocyte activities.

INTRODUCTION

The antineoplastic agent paclitaxel has been extensively developed as a clinical antitumor chemotherapeutic (reviewed in 178). A taxane diterpenoid (215) originally extracted from the bark and leaves of the western yew *Taxus brevifolia* (450), paclitaxel demonstrates activity against a wide range of human malignancies, most notably drug-refractory breast (45) and ovarian cancers (141). Paclitaxel's primary antitumor mechanism derives from its unique polymerizing action on microtubules (373), preventing dynamic depolymerization of α/β tubulin polymers (264) and halting cell cycle progression (362). Paclitaxel-mediated disruption of cell cycling leads to neoplastic cell death (114) and inhibition of tumor progression (148). The chemotherapeutic capacity of paclitaxel is augmented through additional mechanisms, including the enhancement of tumor cell sensitivity to TNF-α-mediated cytolysis (457) and to radiation (245), and the induction of apoptotic tumor cell death (280).

Paclitaxel's *in vitro* effects on immune cell populations, including Mϕs (49,267,296,301), NK cells (86), and T-cells (56,95,347,365,366,455), have been extensively documented. In murine Mϕs, paclitaxel stimulates *in vitro* responses similar to those induced by bacterial LPS, including almost indistinguishable patterns of protein phosphorylation and gene expression (70,266) and induction of the cytotoxic mediators NO and TNF-α (15,296). In contrast to paclitaxel's cell cycle-independent effects on Mϕs, T-cells are susceptible to the same cytostatic activities that impart paclitaxel's antineoplastic functions. Paclitaxel treatment *in vitro* decreases T-cell proliferation and compromises responsiveness to IL-2 (56,86,301). The question remains whether transient paclitaxel treatment *in vivo*, such as lymphocyte populations would encounter during chemotherapeutic treatments, will impart similar inhibitory effects on

lymphocytes. Furthermore, tumor growth significantly dysregulates T-cell functions, including responsiveness to alloantigen activation (124,444,475) and IFN-γ production, giving rise to the idea that paclitaxel treatment may exacerbate immune dysfunction in a cancer patient. We report that paclitaxel administration dysregulates T-cell proliferative responsiveness *in vitro*, and TBH T-cells were more severely impacted by paclitaxel.

Recent studies reported the efficacy of IL-12 as an antitumor immunotherapeutic agent (193,251). IL-12 is a potent T-cell stimulatory cytokine, promoting proliferative responsiveness (155) and IFN-γ production (74). We report that exogenous IL-12 fully reconstituted *in vitro* T-cell proliferative responses following paclitaxel treatment, demonstrating that paclitaxel's inhibitory actions on lymphocytes are transient and reversible.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice were used as the source for normal host and TBH lymphocytes. A nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (designated Meth-KDE) (15,126) was used as described in Chapter I.

Medium and Reagents

Lymphocytes were cultured in serum-free RPMI-1640 medium with 2 MM L-glutamine (Sigma, St. Louis, MO), prepared as described in Chapter I. Recombinant murine IL-12 (specific activity 2.5 x 10^6 U/ml, endotoxin content <10 pg/ml) was generously provided by Genetics Institute (Cambridge, MA). Paclitaxel was obtained from Calbiochem (La Jolla, CA). For *in vitro* studies, paclitaxel was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to a 4 mM stock solution, stored at -80°C, and diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%.

Paclitaxel Administration

For *in vivo* administration, paclitaxel was prepared in 50% polyoxyethylated castor oil (Cremophor EL, Sigma) and 50% dehydrated alcohol at 6 mg/ml, stored at -80°C, and diluted to assay concentrations in sterile PBS immediately before administration. Paclitaxel was administered to twenty-day post-induction TBHs by i.p.

injection at doses of 15 or 30 mg/kg (299), approximating human chemotherapeutic regimens (145). Parallel control animals received an equivalent volume of vehicle or physiologic saline only. Immune cells were collected 12 or 24 h post-paclitaxel administration.

Lymphocyte Culture and Collection

Normal host and TBH BALB/c whole spleen cells were collected by homogenizing spleens in RPMI-1640. Red blood cells were removed by lysis with 0.83% ammonium chloride. Purified splenic $CD4^+$ 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 immature T-cell (ATCC clone J11d) monoclonal antibodies and complement (Accurate Chemical Co., Westbury, NY). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Lymphocyte Proliferation Assays

T-cell proliferation as a result of activation was used to evaluate immune cell responsiveness following paclitaxel administration. To assess mitogen-induced lymphocyte reactivity, either normal host or TBH T cells (4×10^5) from paclitaxel-treated, vehicle-treated or control animals were activated with Con-A (8 μg/ml, Sigma) in the absence or presence of mediators that promote or suppress T-cell activation. All cultures were maintained in sterile 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY) with a final culture volume of 200 μl. T-cell proliferation was assessed after 72 h by measuring [^{3}H]-TdR (specific activity 6.7 Ci/mM; DuPont-NEN

Research Products, Boston, MA) incorporation. Twelve h before harvest, cultures were pulsed with 1 μCi per well of [^{3}H]-TdR. Cells were harvested onto glass fiber filters using an automated Basic96 Harvester (Skatron, Sterling, VA) and sample activities determined in ScintiLene cocktail (Fisher Scientific, Pittsburgh, PA) using a Beckman LS 6000SC scintillation counter.

To assess the effects of transient paclitaxel exposure *in vitro*, normal host or TBH lymphocytes were pretreated with paclitaxel for 4 h at 37°C in a humidified atmosphere containing 5% CO_2, washed 3 times with fresh medium, and cultured in 96-well flat-bottom tissue culture plates. Paclitaxel was diluted to assay concentration in FBS-supplemented (10%) RPMI-1640. Proliferation was assessed as described. Lymphocyte viability was >95% following paclitaxel pretreatment, as assessed by the Alamar blue™ (BioSource International, Camarillo, CA) viability assay (6).

IFN-γ Quantification

IFN-γ production was induced by culturing $CD4^+$ normal host or TBH T-cells (4 x 10^5 cells) from paclitaxel-treated, vehicle-treated, or control animals in 24-well flat-bottom plates with Con-A (8 μg/ml), either without or with IL-12 (100 U/ml). Cell-free supernatants were collected at 24 h and assayed for IFN-γ by specific ELISA (Quantikine M, R&D Systems, Minneapolis, MN) per the manufacturer's directions. The limit of detection was 5 pg/ml.

Statistics and Calculations

Cells from six to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for [^{3}H]-TdR incorporation in proliferation assays and for IFN-γ in the specific ELISA. Data are means ± SEM of triplicate determinations. All experiments were repeated at least three times and representative experiments are shown.

RESULTS

Paclitaxel Treatment Compromises T-cell's Ability to Proliferate

To determine whether paclitaxel administration inhibits T-cell proliferative capacity, we assessed the lectin responsiveness of whole spleen cell populations (Figure 59 A) or purified $CD4^+$ T-cell populations (Figure 59 B) from either normal host or TBHs. Tumor growth compromised the responsiveness of whole spleen cell populations to Con-A (Figure 59 A), likely through the activities of tumor-induced suppressor Mϕ activities (9,16). Purified $CD4^+$ TBH T cell cultures, which lack tumor-induced Mϕs suppressor activity, were more responsive to mitogenic activation (Figure 59 B). Twenty-four h after paclitaxel administration (30 mg/kg), the *in vitro* stimulant-induced proliferation of both normal host and TBH whole spleen cell populations (Figure 59 A) and $CD4^+$ T cells (Figure 59 B) was significantly inhibited. In the whole spleen cell cultures, normal host and TBH proliferative responses were diminished by 51% and 76% ($p < 0.05$), respectively, as compared to the response of cells from untreated control animals. Proliferation of $CD4^+$ normal host and TBH T cells was diminished by 71% and 87% ($p < 0.05$), respectively. Administration of vehicle or PBS alone had no effect on mitogen-induced whole spleen cell or T-cell proliferation, and similar results were obtained following administration of lower concentrations (15 mg/kg) of paclitaxel (not shown).

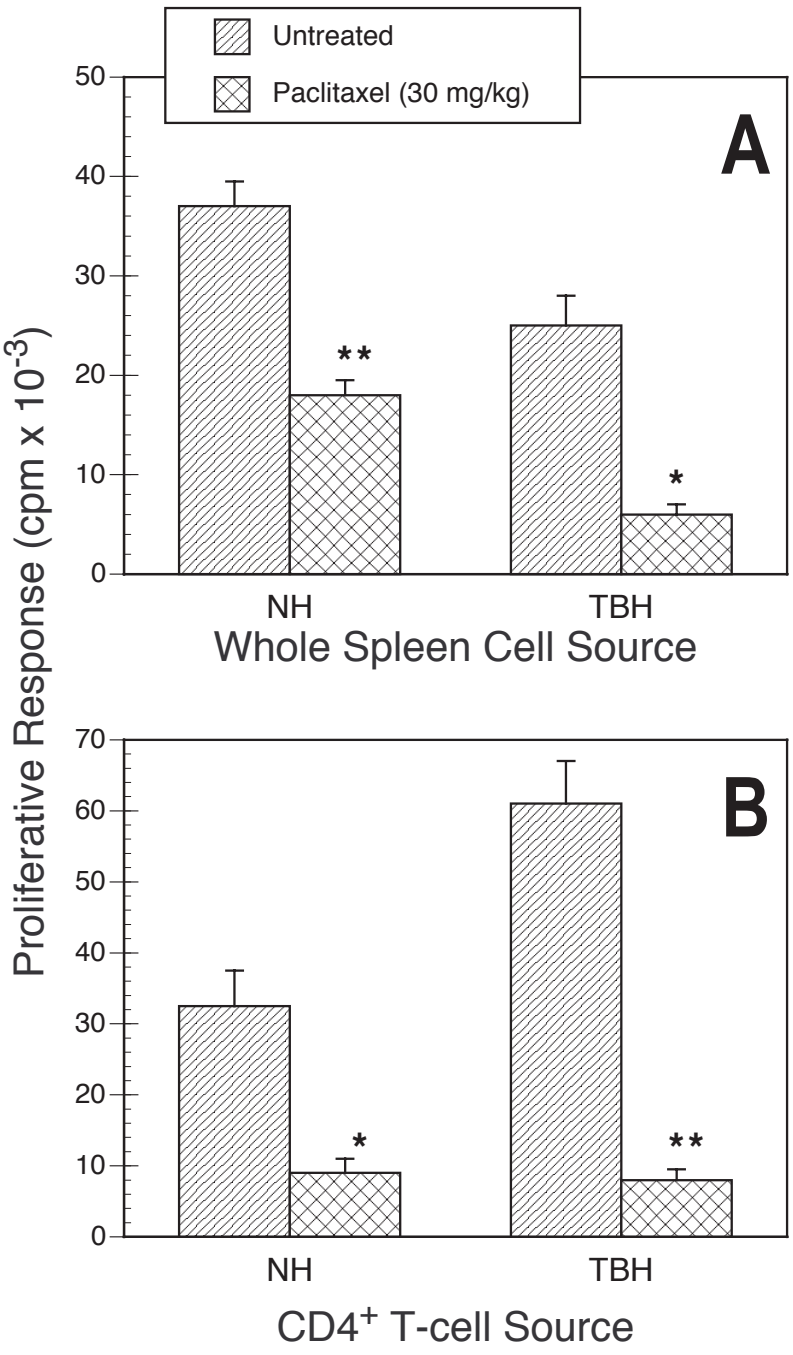


Figure 59. Paclitaxel treatment compromises T-cell proliferation. Twenty-four h after administration of saline alone or clinically-relevant doses of paclitaxel (30 mg/kg), 4 x 10^5 whole spleen cells (Panel A) or purified $CD4^+$ T-cells (Panel B) from normal hosts and TBHs were cultured with Con-A (8 μg/ml, optimal dose). Proliferation was assessed after 72 h by measuring [^{3}H]-TdR incorporation. Similar results were obtained following administration of 15 mg/ml paclitaxel (not shown). Vehicle administration had no effect on proliferative responsiveness (not shown). Data are averages ± SEM of triplicate independent determinations from one of four similar experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ as compared to cultures from untreated hosts.

Paclitaxel Administration Does Not Compromise IFN-γ Production

To determine whether paclitaxel treatment compromises T-cell function, we assessed the production of the T cell-derived cytokine IFN-γ. In our model, tumor growth alone compromised T-cell IFN-γ production (Figure 60), and paclitaxel administration (30 mg/kg) had no significant effect on IFN-γ production (24 h post-administration). Administration of vehicle or saline alone had no effect on IFN-γ production (not shown).

IL-12 Reconstitutes T-cell Proliferation Following Paclitaxel Treatment

Because the Mϕ-derived proimmune cytokine IL-12 promotes T-cell activation and antitumor immune responses, we determined whether exogenous IL-12 could overcome suppression of T-cell proliferative that is mediated by tumor growth and paclitaxel treatment. First, to determine whether tumor-induced dysfunction in our fibrosarcoma model could be reversed by exogenous IL-12, normal host or TBH whole spleen cells or $CD4^+$ T-cells were cultured in the absence or presence of 100 U/ml recombinant IL-12 (Figure 61). In all cultures, exogenous IL-12 enhanced T-cell proliferative responses to mitogen stimulation (8 μg/ml Con-A). $CD4^+$ T-cells showed a dose-dependent proliferative response to IL-12 with maximal response at 100 U/ml, but doses in excess of 100 U/ml did not further enhance response (not shown). Second, to determine whether paclitaxel-induced inhibition of T-cell proliferative capacity could be reversed by exogenous IL-12, normal host (Figure 62 A) or TBH (Figure 62 B) $CD4^+$ T-cells were pretreated for 4 h with paclitaxel (0.1 - 10 μM) *in vitro*, then cultured in the absence or presence of recombinant IL-12 (100 U/ml). In a dose-dependent manner,

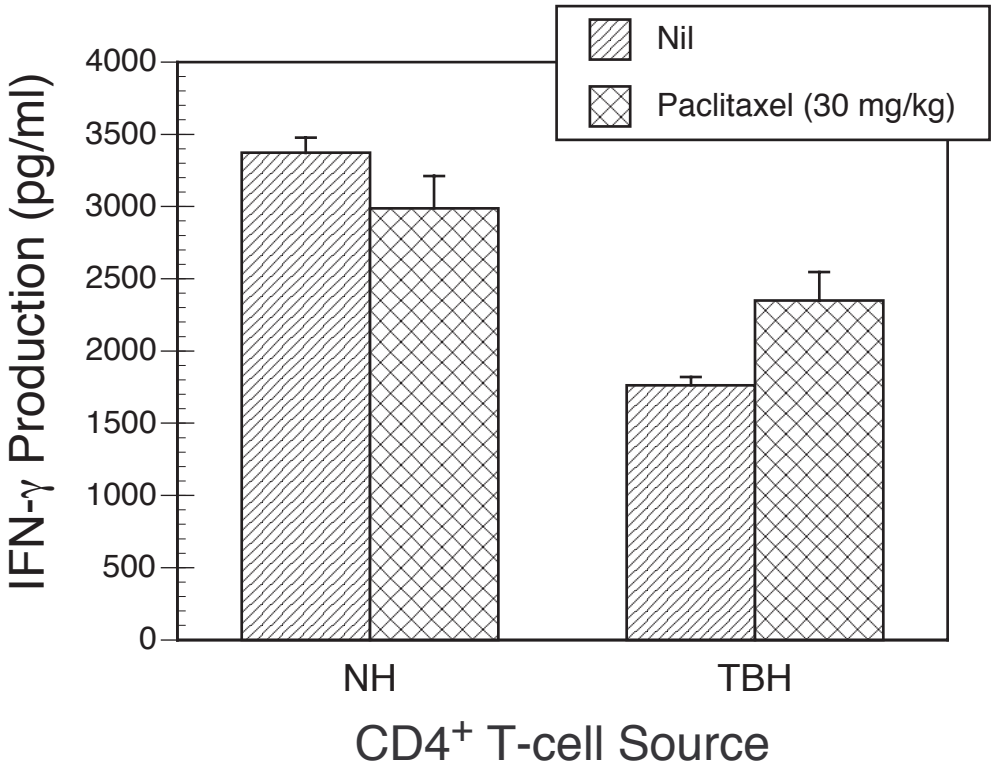


Figure 60. Tumor growth compromises IFN-γproduction. Twenty-four h after administration of saline alone or paclitaxel (30 mg/kg), 4 x 10^5 purified $CD4^+$ T-cells from normal hosts and TBHs were cultured with Con-A (8 μg/ml, optimal dose). IFN-γ production was assessed after 24 h culture using a specific ELISA. Similar results were obtained following administration of 15 mg/ml paclitaxel (not shown). Vehicle administration had no effect on IFN-γ production (not shown). Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

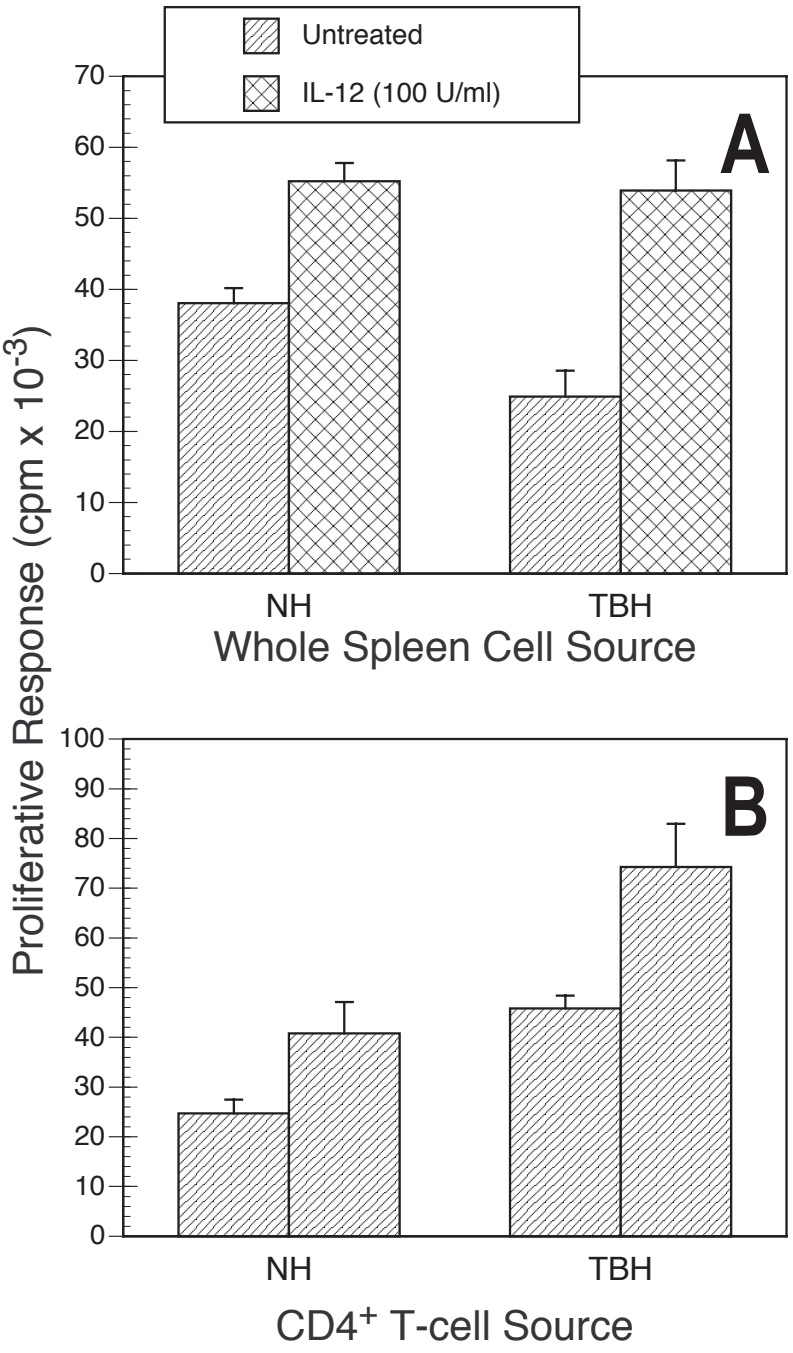


Figure 61. IL-12 enhances T-cell reactivity. Either 4 x 10^5 normal host or TBH whole spleen cells (Panel A) or purified $CD4^+$ T-cells (Panel B) were cultured with Con-A (8 μg/ml) in the absence or presence of 100 U/ml recombinant IL-12 (Figure 3). Proliferation was assessed after 72 h by measuring [^{3}H]-TdR incorporation. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments.

paclitaxel pretreatment significantly ($p < 0.05$) inhibited both normal host and TBH T-cell proliferative responsiveness to stimulation. Addition of IL-12 to culture after paclitaxel pretreatment enhanced T-cell proliferative response by untreated normal host and TBH T-cells. Exogenous IL-12 reconstituted and significantly ($p < 0.05$) enhanced proliferation of normal host T-cells pretreated with 0.1 and 1.0 μM paclitaxel, but not with the highest dose of 10 μM. Likewise, IL-12 significantly ($p < 0.05$) enhanced the TBH T-cell growth following pretreatment with the low dose of paclitaxel (0.1 μM), but IL-12 did not overcome paclitaxel-induced suppression at higher doses (1.0 and 10.0 μM).

To determine whether T-cell proliferative responses following *in vivo* paclitaxel administration could be restored by exogenous IL-12, normal host (Figure 63 A) or TBH (Figure 63 B) $CD4^+$ T-cells were collected from untreated or paclitaxel (30 mg/kg)-treated (24 h post-administration) animals and cultured in the absence or presence of recombinant IL-12 (100 U/ml). Although paclitaxel treatment inhibited both normal host and TBH T-cell proliferation, addition of exogenous IL-12 (100 U/ml) to *in vitro* cultures significantly ($p < 0.05$) enhanced T-cell proliferative responses by both normal host and TBH T-cells from paclitaxel-treated animals. Doses of IL-12 as low as 1U/ml enhanced proliferation, but doses in excess of 100 U/ml did not further enhance the response (not shown).

IL-12 Enhances T-cell IFN-γ Production

The T-cell-stimulatory cytokine IL-12 strongly induces IFN-γ production (73). To determine whether IL-12 can overcome tumor-induced dysregulation of IFN-γ production in our model system, normal host or TBH $CD4^+$ T-cell IFN-γ production was

assessed following a 24-h culture without or with 100 U/ml IL-12 (Figure 64 A). IFN-γ production by $CD4^+$ normal host and TBH T-cells was significantly ($p < 0.05$) enhanced by 48% and 172%, respectively. Paclitaxel administration did not significantly compromise the capacity of IL-12 to induce T-cell IFN-γ production (Figure 64 B). IL-12 (100 U/ml) significantly ($p < 0.05$) enhanced normal host and TBH T-cell IFN-γ production *in vitro* by 49% and 131%, respectively, at 24 h following paclitaxel administration (30 mg/kg). Administration of vehicle or saline alone had no effect on subsequent IL-12-induced IFN-γ production *in vitro* (not shown).

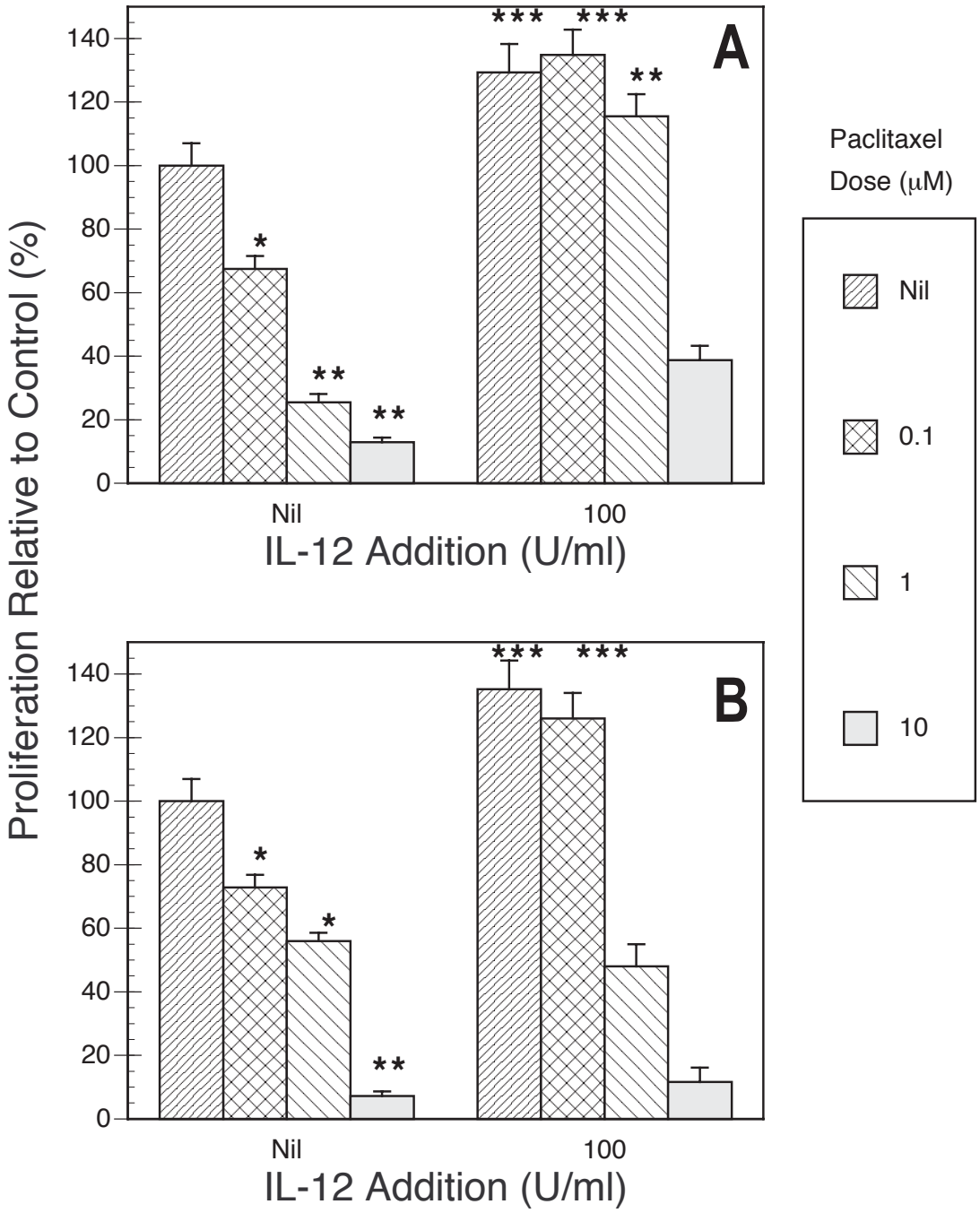


Figure 62. IL-12 reconstitutes T-cell reactivity following paclitaxel treatment *in vitro*. Either 4 x 10^5 normal host (Panel A) or TBH (Panel B) $CD4^+$ T-cells were pretreated for 4 h with paclitaxel (0.1 - 10 μM) *in vitro*, then cultured with Con-A (8 μg/ml) in the absence or presence of recombinant IL-12 (100 U/ml). Proliferation was assessed after 72 h by measuring [^{3}H]-TdR incorporation. Data are averages ± SEM of triplicate independent determinations from one of three similar experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ as compared to untreated cultures lacking IL-12; $^{***}p < 0.05$ as compared to culture treated with equal concentration of paclitaxel lacking IL-12.

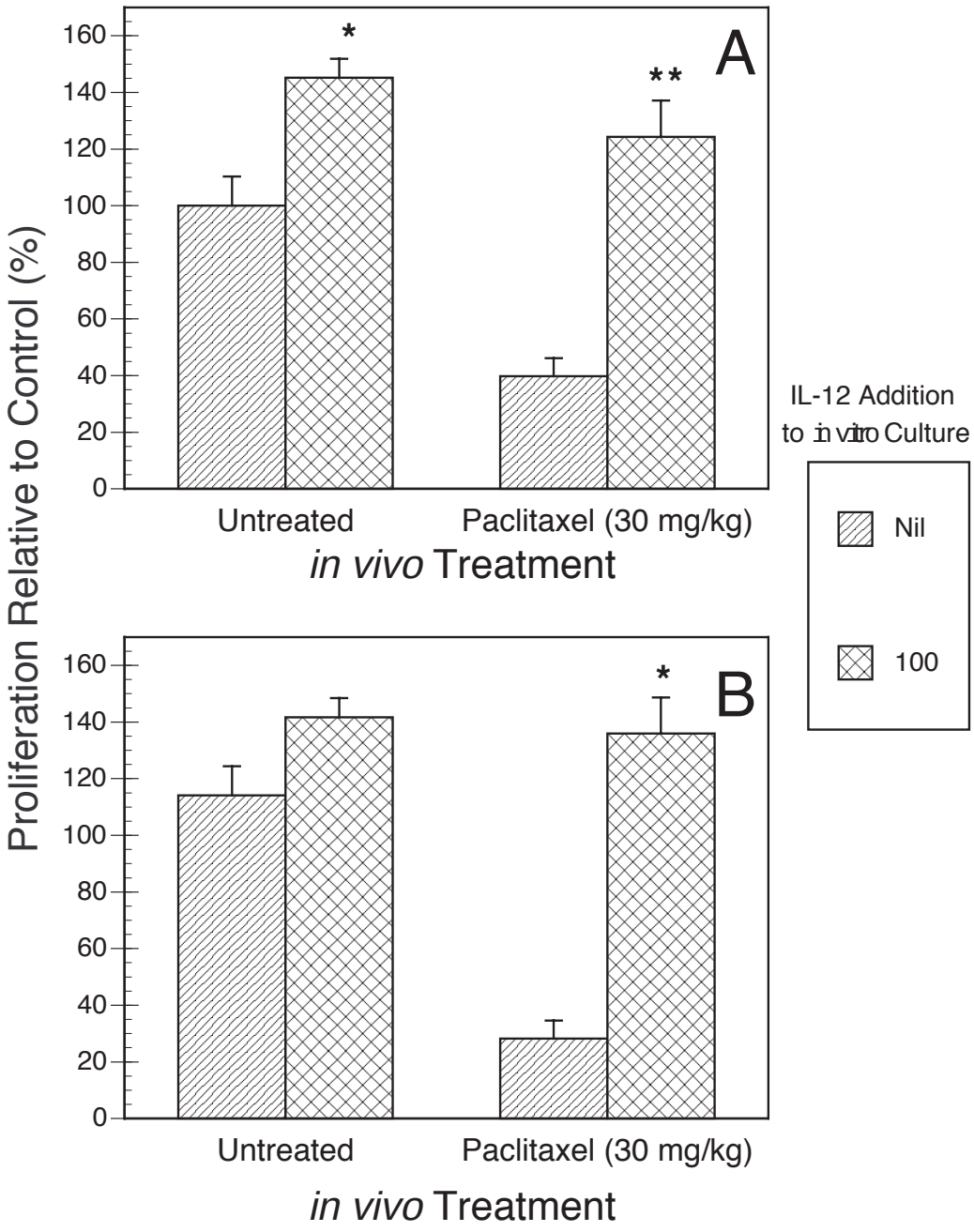


Figure 63. IL-12 reconstitutes T-cell reactivity *in vitro* following paclitaxel treatment *in vivo*. Twenty-four h after administration of saline alone or clinically-relevant doses of paclitaxel (30 mg/kg), 4 x 10^5 purified $CD4^+$ T-cells from normal host (Panel A) or TBHs (Panel B) were cultured with Con-A (8 μg/ml, optimal dose) in the absence or presence of recombinant IL-12 (100 U/ml). Proliferation was assessed after 72 h by measuring [^{3}H]-TdR incorporation. Similar results were obtained following administration of 15 mg/ml paclitaxel (not shown). Vehicle administration had no effect on proliferative responsiveness (not shown). Data are averages ± SEM of triplicate independent determinations from one of three similar experiments. $^*p < 0.05$, $^{**}p < 0.005$ as compared to similarly-treated cultures lacking IL-12.

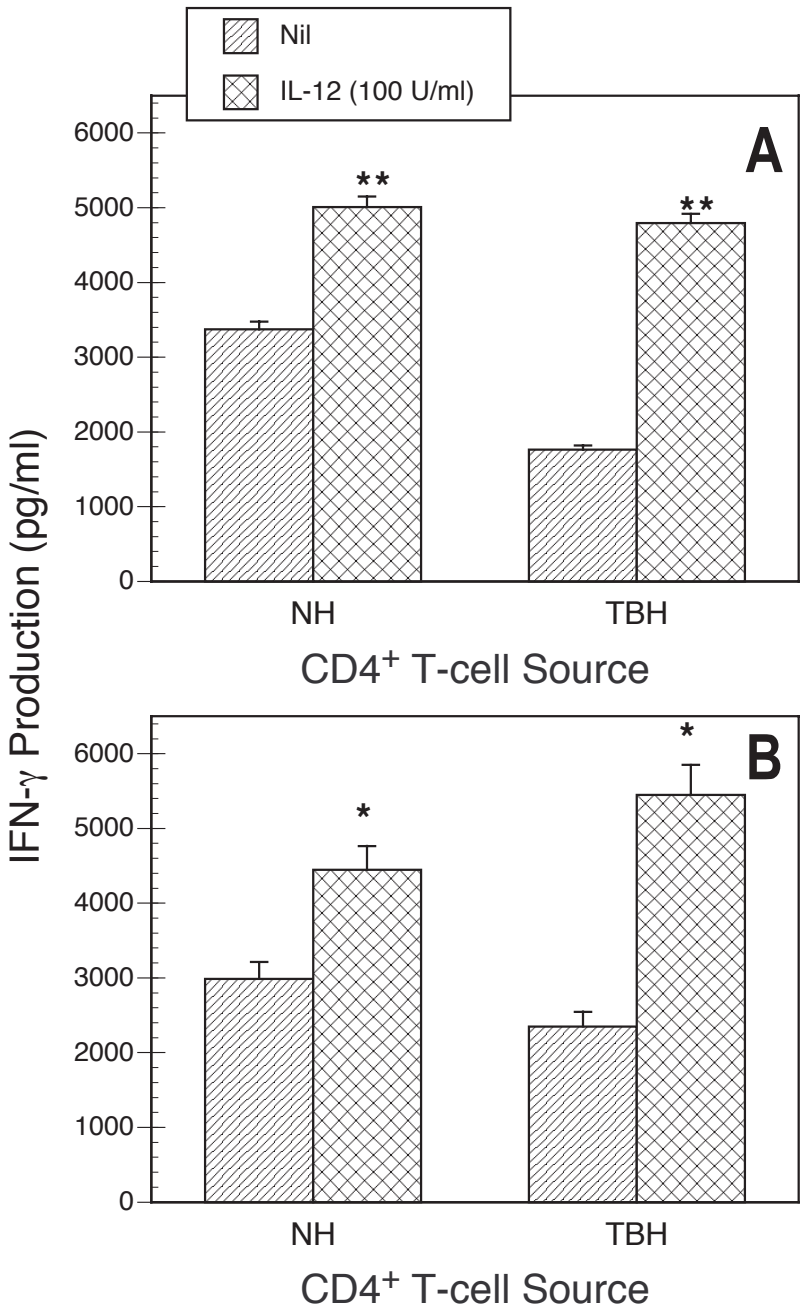


Figure 64. IL-12 enhances T-cell IFN-γ production. Purified $CD4^+$ T-cells (4 x 10^5) from untreated (Panel A) or paclitaxel-treated (30 mg/kg, Panel B) normal hosts and TBHs were cultured with Con-A (8 μg/ml) in the absence or presence of recombinant IL-12 (100 U/ml). IFN-γ production was assessed after 24 h culture using a specific ELISA. Similar results were obtained following administration of 15 mg/ml paclitaxel (not shown). Administration of vehicle or saline alone had no effect on subsequent IL-12-induced IFN-γ production *in vitro* (not shown). Data are averages ± SEM of triplicate independent determinations from one of three similar experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ as compared to similarly-treated cultures lacking IL-12.

DISCUSSION

Paclitaxel's best-defined antitumor functions -- polymerization of microtubule bundles with resulting inhibition of cell cycle progression and cell death – impart the greatest cytotoxic and cytostatic effects on rapidly-dividing cell populations. However, the same mechanisms that give paclitaxel its antineoplastic potency may simultaneously ravage vital immune system responders, particularly the T lymphocytes. Paclitaxel treatment *in vitro* decreases T-cell proliferative responses (56,95,366), limits alloantigen-mediated T-cell responsiveness (365), compromises responsiveness to IL-2 (56,86,301), and impairs cytotoxic activity against tumor cells (86). Because tumor growth diminishes T-cell alloreactivity and responsiveness to activating cytokines, tumor-induced T-cells may be particularly susceptible to the effects of paclitaxel-mediated inhibition. We previously reported that tumor growth increased T-cell sensitivity to paclitaxel co-culture *in vitro* (301). Here we report in an *in vivo* counterpart study that paclitaxel administration, in regimens corresponding to current human chemotherapeutic applications, exacerbates tumor-induced changes in T-cell proliferation.

Paclitaxel treatment compromised proliferation of both whole spleen cells and purified $CD4^+$ T-cells from normal hosts and TBHs (Figure 59). Tumor growth significantly compromises lymphocytes in whole spleen cell populations (Figure 59 A), likely through the action of tumor-induced suppressor Mϕs (9,16), and paclitaxel further suppresses T-cell proliferative capacity. Interestingly, paclitaxel induces murine Mϕ production of the cytotoxic and immunosuppressive factor NO (15,267,296,301). At the tumor site, NO is a potent cytotoxic effector; distal to the tumor, NO can severely inhibit T-cell activities. Exogenous NO inhibits T-cell production of T_H1 and T_H2-type cytokines *in vitro*, including IL-2 (411), IL-4, IL-5, IL-10, and IFN-γ (34), and abrogation

of IL-2 production by exogenous NO donors can inhibit T_H1-type cell proliferation (411). Thus, paclitaxel-mediated inhibition of TBH whole spleen cell proliferation may derive both from direct cytostatic action as well as paclitaxel-induced Mϕ NO production.

The suggested role of tumor-induced Mϕs in mediating suppression of T-cell proliferation in the whole spleen populations is supported by the increased reactivity of untreated TBH $CD4^+$ T cells in comparison to parallel normal host cultures. Purified $CD4^+$ TBH T cells, which lack tumor-induced Mϕ suppressor activity, were more proliferative (Figure 59 B), suggesting that tumor growth indirectly suppresses, but does not qualitatively change, $CD4^+$ T-cells. Paclitaxel strongly inhibited the proliferative responses of both normal host and TBH $CD4^+$ T-cells (Figure 59 B), and tumor-induced T-cells were particularly susceptible to paclitaxel-mediated suppression. This enhanced sensitivity may derive from an advanced activation state. We reported that IL-12 pretreatment, which promotes T-cell growth, enhanced T-cell sensitivity to paclitaxel (301). Tumor growth may drive $CD4^+$ T-cells into an advanced state of activation, rendering them more susceptible to the cytostatic effects of paclitaxel.

Tumor-derived factors dysregulate a multitude of immune cell effector functions, including T-cell production of the critical T_H1-type cytokine IFN-γ (80,369). In our nonmetastatic fibrosarcoma model -- which produces large quantities of the immunomodulatory factors IL-10, PGE_2, and TGF-β_1 (15) -- tumor growth diminished IFN-γ production by $CD4^+$ T-cells (Figure 60). While T-cell production of IFN-γ is induced by cytokines, including IL-12 (74), paclitaxel administration had no significant impact on IFN-γ production. Thus, although tumor growth compromises T-cell functionality, paclitaxel administration does not exacerbate tumor-induced dysfunction.

These data suggest that paclitaxel's microtubule-binding activities, which lead to T-cell cytostasis, do not regulate IFN-γ signaling pathways in T-cells.

Although paclitaxel directly inhibits T-cell proliferative responses, we showed that paclitaxel pretreatment of TBH Mϕs *in vitro* partially reverses Mϕ-mediated suppression of T-cell alloreactivity (301), suggesting that paclitaxel may activate Mϕ production of immunostimulatory factors. One such Mϕ-derived factor that could account for enhanced T-cell alloreactivity is the potent immunostimulatory cytokine IL-12 (39). IL-12 is a cytokine with a unique heterodimeric structure and a variety of biological activities (reviewed in 233). IL-12 regulates IFN-γ production, augments cytotoxic T-cell and NK cell cytotoxicity, and promotes the development of T_H1-type populations of $CD4^+$ T helper cells (379,388,419). Because IL-12 is secreted by professional antigen-presenting cells (B cells, monocytes, and Mϕs) (96) and IL-12 receptors are expressed selectively on activated T-lymphocytes and NK cells (479), IL-12 may represent the link between innate resistance and adaptive immunity (78,420,424). Furthermore, IL-12 may be well suited to drive the expansion and maturation of two prevalent antitumor effector cells localized within tumor lesions (T-cells and NK cells) (154), making IL-12 an ideal candidate for use in immunotherapy. To determine whether exogenous IL-12 could overcome tumor-induced and paclitaxel-mediated suppression of T-cell proliferative reactivity, we cultured normal host and TBH whole spleen cells or $CD4^+$ T-cells in the absence or presence of exogenous recombinant IL-12. Addition of IL-12 enhanced T-cell proliferative responses, and IL-12 reconstituted proliferation of T-cells from TBHs (Figure 61). This suggests that IL-12 would be a useful therapeutic for reversing tumor-induced immune suppression (62,407). These data are particularly interesting given the recent observations that tumor growth dysregulates IL-12 production by both *in situ* (182) and tumor-distal Mϕs (297).

Because we have shown that paclitaxel inhibits T-cell proliferative reactivity, and that IL-12 can enhance T-cell responsiveness to activation agents, we determined whether IL-12 may be a useful immunotherapeutic treatment to reverse immune suppression following paclitaxel administration. First, exogenous IL-12 was added to normal host (Figure 62 A) or TBH (Figure 62 B) $CD4^+$ T-cells that had been pretreated for 4 h with paclitaxel (0.1 - 10 μM) *in vitro*. In this experiment, which mimics the transient paclitaxel exposure lymphocytes may encounter during *in vivo* treatments, IL-12 reconstituted and enhanced proliferation of normal host T-cells pretreated with 0.1 and 1.0 μM paclitaxel and enhanced TBH T-cell proliferative response following pretreatment with the low dose of paclitaxel (0.1 μM). These data suggest that immune activating cytokines, such as IL-12, may be useful for restoring lymphocyte function following transient low-dose paclitaxel exposure, but that IL-12 can not overcome paclitaxel-induced suppression at higher doses (10.0 μM or greater). Although IL-12 restored normal host T-cell proliferation following exposure to paclitaxel concentrations as high as 1.0 μM, IL-12 could only reconstitute proliferation in TBH cell cultures following low-dose paclitaxel exposure (0.1μM). This result suggests that TBH $CD4^+$ T-cells, even when activated after paclitaxel administration, are more sensitive than their normal host counterparts to the negative regulatory effects of paclitaxel. We extended these studies to investigate whether T-cell proliferative responses following paclitaxel administration could be enhanced by exogenous IL-12 (Figure 63). In agreement with our *in vitro* studies (300), addition of IL-12, which drives T-cell activation and promotes antitumor responses, fully restored T-cell proliferation by both normal host (Figure 63 A) and TBH (Figure 63 B) T-cells from paclitaxel-treated animals. This is particularly interesting because we showed that *in vitro* pretreatment of TBH Mϕs with paclitaxel partially reversed the suppressor activities of tumor-induced Mϕs on T-cell alloreactivity (301). This suggests that paclitaxel activates Mϕ production of IL-12 (unpublished

observation), which then increases T-cell proliferation and reverses tumor-induced suppression. These results further demonstrate the potential of IL-12 for use as an adjuvant immunotherapy to paclitaxel treatment.

We demonstrated that tumor growth diminished IFN-γ production by $CD4^+$ T-cells (see Figure 60). Because IL-12 strongly promotes stimulates T-cell production IFN-γ (73), we determined whether IL-12 could overcome tumor-induced dysregulation of IFN-γ production in our model system. Exogenous IL-12 enhanced normal host and TBH T-cell IFN-γ production *in vitro* (Figure 64 A), and paclitaxel administration did not affect IL-12-induced IFN-γ production (Figure 64 B). These data show that IL-12 may be useful in restoring suppressed IFN-γ production in the TBH (182). It is possible that restoration of IL-12, which is dysregulated by tumor growth (182,297), may induce additional Mϕ IL-12 production through the induction of T-cell IFN-γ.

These data, in combination with our previous *in vitro* studies (301) that showed IL-2 activation enhanced paclitaxel-mediated suppression of lymphocyte reactivity, would argue that paclitaxel treatment should precede immunotherapeutic intervention. Paclitaxel could be used as an antitumor chemotherapeutic and delivered in transient, temporally-separated regimens; paclitaxel regimens would be followed by IL-12 therapy, which would both assist in the recovery of lymphocyte populations following paclitaxel, as well as impart distinct and independent antitumor activities. These conclusions contrast with the observations of others who suggest that paclitaxel should follow immune therapy because paclitaxel impedes IL-2-mediated cytotoxicity but not the cytotoxic capacity of IL-2-pretreated human lymphocytes *in vitro* (85). Clearly, *in vivo* studies of immune cell function following various regimens of paclitaxel and IL-12 combination therapy will be needed to conclusively answer these questions.

Collectively, these studies suggest that chemotherapeutic paclitaxel regimens impart significant but reversible inhibition of lymphocyte populations. IL-12, which alone has significant promise as an antitumor therapy (59,193), may be a useful ancillary immunotherapeutic to overcome paclitaxel-induced modulation of lymphocyte activities. Recognition of paclitaxel's immune system activities, both positive and negative, may lead to optimization of current chemotherapeutic treatment regimens. Through the use of antitumor cytokines, such as IL-12, paclitaxel may impart combined antitumor and immunotherapeutic effects, simultaneously assaulting tumors and restoring cytokine balance to the immune system.

CHAPTER X

PACLITAXEL AND IL-12 COMBINATION THERAPY INHIBITS TUMOR PROGRESSION

ABSTRACT

Although the antineoplastic agent paclitaxel is a potent inhibitor of tumor cell division, it simultaneously induces significant suppression of lymphocyte proliferative responses. T-cells are susceptible to paclitaxel's cytostatic functions, and direct paclitaxel administration inhibits lymphocyte function in the TBH. The T-cell stimulatory cytokine IL-12, which promoted T-cell activation and proliferation, has been demonstrated to reverse paclitaxel-mediated immunosuppression *in vitro*. Furthermore, IL-12 has been shown to induce nonspecific antitumor immune responses in several murine tumor models. Therefore, we assessed the efficacy of combined administration of paclitaxel and IL-12 in a nonmetastatic murine fibrosarcoma model. Treatment groups receiving the combined therapy demonstrated enhanced survival, decreased tumor mass, and enhanced immune cell function on activation with LPS *in vitro*, as compared to TBH receiving vehicle alone, paclitaxel alone, or IL-12 alone. Collectively, these data suggest that the chemotherapeutic activities of paclitaxel and the immunotherapeutic efficacy of IL-12 may combine to achieve significant antitumor immune activity and enhanced tumor regression..

INTRODUCTION

Anticancer therapeutic approaches are constantly involving and rapidly improving. However, the persistence of neoplastic disease is evidence of the shortcomings of current therapies. A promising line of research involves the use of cytokines to induce nonspecific antitumor immune responses or supplement existing therapies. Cytokine research (31,47,138,190,309) is enhancing our understanding the processes of immune cell regulation, as well as dysregulation, during tumor growth. As we discover the mechanisms of dysregulation, we can target cytokines to correct imbalances in immunosuppressed individuals, enhancing both the effectiveness of existing cancer treatments and the ability of the patient to mount an effective immunologic response.

Cytokine therapies may be useful for reversing chemotherapy-induced immune suppression. For example, paclitaxel induces severe T-lymphocyte cytostasis, potentially compromising host antitumor immune responses. The Mϕ-derived cytokine IL-12, a promising immunotherapeutic agent, has reversed tumor-induced immunosuppression and partially regressed tumors in some cancer patients (319,483). Furthermore, we demonstrated that IL-12 treatment *in vitro* could reverse the immunosuppressive effects of paclitaxel administration (300) (see Chapter IX). Therefore, while paclitaxel alone is a moderately effective antitumor treatment, therapies that combine antitumor and immunotherapeutic regimens to simultaneously assault the tumor *and* restore cytokine balance to the immune system may have even greater antitumor efficacy.

Based on our *in vitro* results, we speculated that the combined administration of the anticancer chemotherapeutic paclitaxel and the immune activating cytokine IL-12

may represent a potent antitumor combination, offering several advantages over traditional antitumor approaches. Using our well-established murine fibrosarcoma model, we assessed the efficacy of paclitaxel and IL-12 combined therapy on tumor growth and immune cell function. In this study, we demonstrate that the combined administration of paclitaxel and IL-12 impedes tumor progression while activating both Mϕ and T-cell antitumor activities. Further, combined therapy significantly reduces tumor progression and diameter, as compared to vehicle, paclitaxel, or IL-12 alone. These data are the first to demonstrate that paclitaxel can be combined with immunotherapeutic cytokines to achieve enhanced antitumor activity, and we propose that a combined therapeutic approach should be considered in a clinical context.

MATERIALS AND METHODS

Murine Tumor Model

Eight to 12 week-old BALB/c (H-2^d) male mice (Jackson Laboratories, Bar Harbor, ME) were used as the source for normal host and TBH lymphocytes. A nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma (designated Meth-KDE) (15,126) was used as described in Chapter I. Tumors were induced in a population of age-matched animals, which were then coded and randomly divided into treatment groups. Tumor progression was assessed by measuring the diameter of the left hind leg; all animals were coded and measurements taken in a blinded manner.

Medium and Reagents

Lymphocytes were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma, St. Louis, MO), prepared as described in Chapter I. Recombinant murine IL-12 (specific activity 2.5 x 10^6 U/ml, endotoxin content <10 pg/ml) was generously provided by Genetics Institute (Cambridge, MA). Paclitaxel was obtained from Calbiochem (La Jolla, CA). For *in vitro* studies, paclitaxel was dissolved in 100% DMSO (Mallinckrodt Chemical, Paris, KY) to a 4 mM stock solution, stored at -80°C, and diluted to assay concentrations in RPMI-1640 medium immediately before use. The final concentration of DMSO in cultures was less than 1%.

Paclitaxel

Paclitaxel was prepared in the same manner as human chemotherapeutic regimens. Paclitaxel was dissolved in 50% polyoxyethylated castor oil (Cremophor EL, Sigma) and 50% dehydrated alcohol at 6 mg/ml, stored at -80°C, and diluted to assay concentrations in sterile PBS immediately before administration. Paclitaxel was administered by i.p. injection at doses of 20 mg/kg (299). Total injection volume was < 0.2 ml/animal to avoid Cremophor-induced toxicity, and vehicle-treated animals demonstrated no adverse reactions or vehicle-induced death (not shown).

Interleukin-12

IL-12 (specific activity 2.7 x 106 U/ml, endotoxin content < 6 EU/ml) was a generous gift of the Genetics Institute (Cambridge, MA) and was diluted in PBS immediately before administration. IL-12 was administered at a dose of 1 mg/animal (optimal dose [421]) by i.p. injection.

Mϕ Culture and Nitrite Production

Splenic Mϕs were collected by plating pooled whole spleen cells for 2 h (150 x 15 mm Pyrex glass plates), washing away nonadherent cells with warm RPMI-1640 medium, and collecting adherent Mϕs in cold medium by scraping with a rubber policeman. Red blood cells were lysed by 0.83% ammonium chloride (Sigma) treatment and all cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

To assess Mϕ NO production, Mϕs (4 x 10^5, optimal cell number) from treated, vehicle-treated, or control animals were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells, Corning, NY). Each well contained a total volume of 200 μL serum-free RPMI-1640 medium supplemented with the indicated concentrations of IFN-γ and LPS. Nitrite levels in culture supernatants were measured using the Griess reagent (172), as described in Chapter I. Briefly, 100 μl of Mϕ supernatant was added to 100 μL Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3PO_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm (MRX microplate absorbance reader; Dynex, Alexandria, VA). Paclitaxel doses up to 35 μM did not decrease Mϕ viability because the Alamar blue™ assay (6) verified >95% Mϕ viability throughout the culture periods (not shown).

Lymphocyte Culture and IFN-γ Quantification

Purified splenic $CD4^+$ 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 immature T-cell (ATCC clone J11d) monoclonal antibodies and complement. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. IFN-γ production was induced by culturing $CD4^+$ normal host or TBH T-cells (4 x 10^5 cells) from treated, vehicle-treated, or control animals in 24-well flat-bottom plates with Con-A (8 μg/ml). Cell-free supernatants were collected at 24 h and assayed for IFN-γ by specific ELISA (Quantikine M, R&D Systems, Minneapolis, MN) per the manufacturer's directions. The limit of detection was 5 pg/ml.

Statistics and Calculations

Treatment groups contained a minimum of five animals. Cells from five mice were pooled for each *in vitro* experiment. Triplicate cultures were tested for nitrite and IFN-γ. Data are means ± SEM of triplicate determinations. Representative experiments are shown.

RESULTS

Paclitaxel and IL-12 Combined Treatment Enhances Survival in Meth-KDE Fibrosarcoma-burdened Hosts.

Both paclitaxel (45,281) and IL-12 (407,409) have been shown to retard tumor progression; a recent study (478) reported that the simultaneous administration of paclitaxel and IL-12 failed to induce a therapeutic effect, but no studies have assessed the efficacy of paclitaxel treatment followed by IL-12. We induced Meth-KDE nonmetastatic fibrosarcoma by i.m. injection with 4×10^5 transplanted tumor cells. In a more clinically-relevant scenario, treatment was initiated only after primary tumors were detected by palpation (day 10). TBH were treated with paclitaxel (20 mg/kg), IL-12 (1 μg/animal), paclitaxel plus IL-12, or vehicle alone. Paclitaxel was administered at day 10, 15, and 20, and IL-12 was administered at day 11, 16, and 21. Animals were sacrificed before tumors became necrotic. Treatment of TBH with the combination of paclitaxel and IL-12 significantly enhanced survival as compared to untreated TBH ($p < 0.01$) or paclitaxel or IL-12 (not shown) alone ($p < 0.05$) (Figure 65). Vehicle did not influence survival (not shown).

Paclitaxel and IL-12 Combined Treatment Retards the Growth of Established Tumors.

Paclitaxel mediates direct cytotoxic and cytostatic antitumor activity (67,148,246), and IL-12 induces nonspecific immunotherapeutic efficacy that has been shown to retard tumor growth (84,193,423). Because these agents have independent efficacy, and IL-12 reverses paclitaxel-mediated immunosuppressive activities *in vitro*

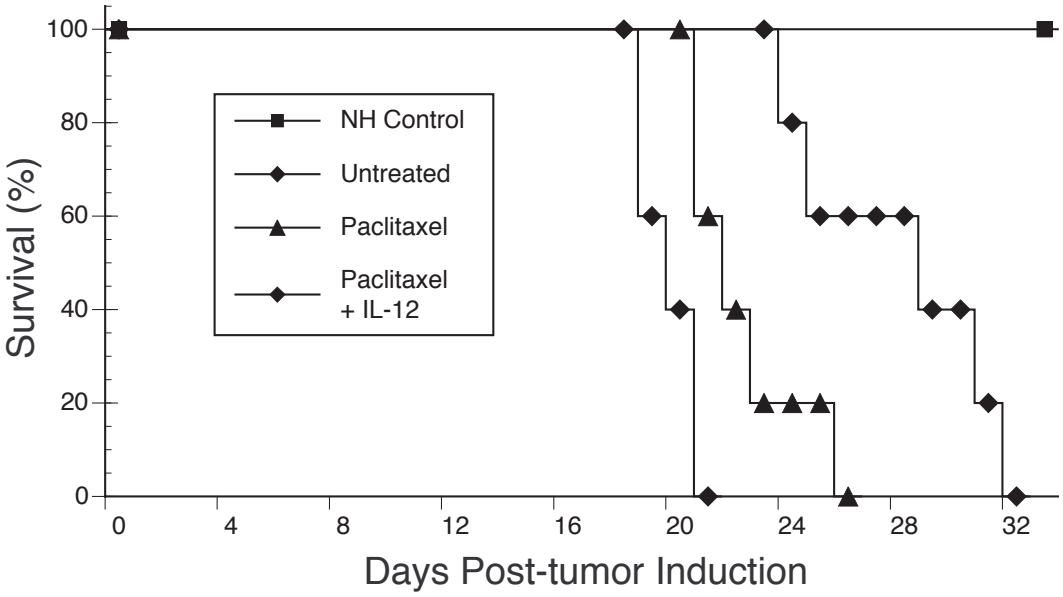


Figure 65. Paclitaxel and IL-12 combined treatment enhances survival in Meth-KDE fibrosarcoma-burdened hosts. Meth-KDE nonmetastatic fibrosarcoma was induced by i.m. injection with 4 x 10^5 transplanted tumor cells. Treatment with paclitaxel (20 mg/kg), paclitaxel plus IL-12 (1 μg/animal), or vehicle alone was initiated at day 10 of tumor growth. Paclitaxel was administered on days 10, 15, and 20. IL-12 was administered on days 11, 16, and 21. Animals were sacrificed when tumors became necrotic. Survival was enhanced by paclitaxel treatment alone ($p < 0.05$, n = 5) and combined treatment ($p < 0.01$, n = 5). as compared to untreated TBH (n = 5). Vehicle did not influence survival (not shown).

(see Chapter IX), we assessed the efficacy of paclitaxel and IL-12 combined therapy on tumor progression. Treatment with paclitaxel (20 mg/kg), IL-12 (1 mg/animal), paclitaxel plus IL-12, or vehicle alone was initiated at day 10 of tumor growth. Paclitaxel was administered at day 10, 15, and 20. IL-12 was administered at day 11, 16, and 21. There was no difference in tumor growth from induction to day 16, regardless of treatment. At day 17, tumor diameter was significantly ($p < 0.01$) less in the group receiving combined treatment as compared to untreated TBH (Figure 66). At day 21, tumor size in combined treatment group was significantly smaller as compared to untreated TBH ($p < 0.05$) or paclitaxel-treated animals ($p < 0.01$) (Figure 67).

In the Meth-KDE tumor model, cachexia is a common side effect of advanced tumor growth (126). At twenty-one days after tumor induction, the mean body mass of untreated TBH is significantly less than untreated normal animals ($p < 0.01$) (Figure 68). However, paclitaxel and IL-12 treatment does not alter body weight. The mean body mass of TBHs treated with paclitaxel or paclitaxel plus IL-12 is not significantly different from untreated TBH (Figure 68), indicating that decreased tumor mass (see Figure 67) is not a nonspecific artifact of decreased body mass.

Combined Paclitaxel and IL-12 Treatment Primes Mϕs for Enhanced NO Production on Activation.

Paclitaxel activates Mϕs for enhanced cytotoxic molecule production *in vitro* (267,296,301), and paclitaxel administration primes Mϕs for NO and TNF-α production on *in vitro* activation (299). To determine whether combined therapy with paclitaxel and IL-12 primes Mϕs for enhanced activity, splenic Mϕs were isolated from TBHs treated

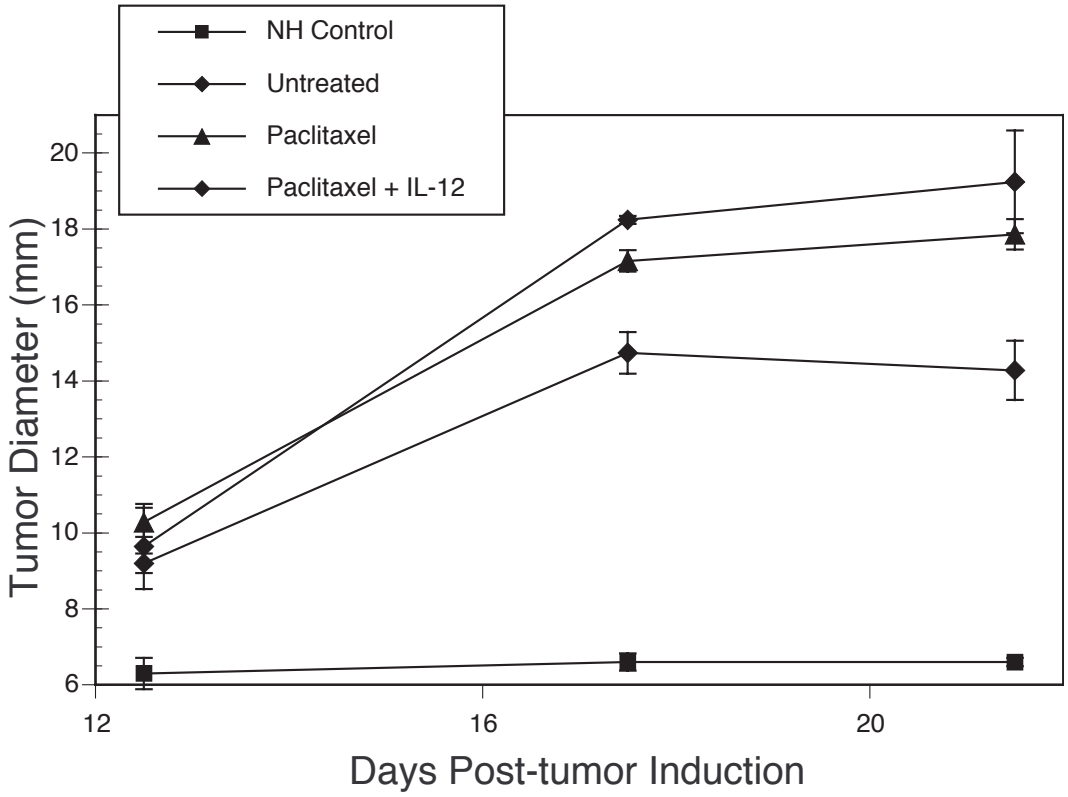


Figure 66. Paclitaxel and IL-12 combined treatment retards the growth of established tumors. Treatment with paclitaxel (20 mg/kg), paclitaxel plus IL-12 (1 mg/animal), or vehicle alone was initiated at day 10 of tumor growth. Paclitaxel was administered on days 10, 15, and 20. IL-12 was administered on days 11, 16, and 21. Tumor growth was assessed by measuring diameter across the lateral axis of the left hind leg (location of the nonmetastatic Meth-KDE tumor) in a blinded manner. Vehicle did not influence tumor growth (not shown).

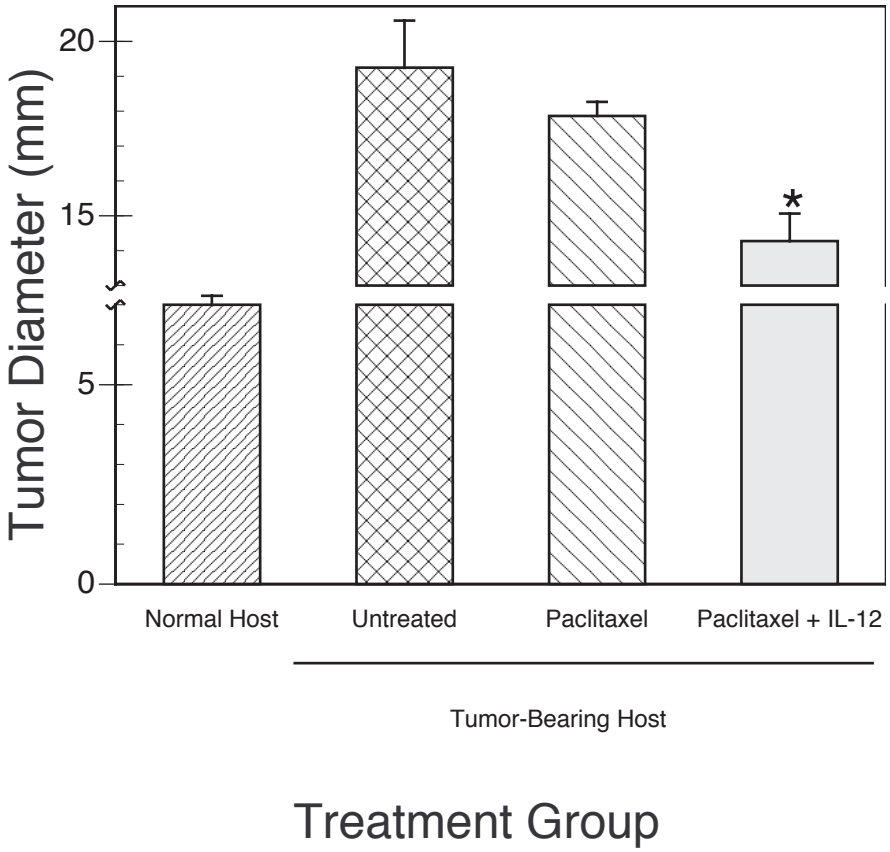


Figure 67. Tumor diameter is significantly smaller in TBH receiving combined paclitaxel and IL-12 treatment. TBHs were treated with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Tumor growth was assessed by measuring diameter across the lateral axis of the left hind leg in a blinded manner. At day twenty-one, tumor size in combined treatment group was significantly smaller as compared to untreated TBH ($p < 0.05$) or paclitaxel-treated animals ($p < 0.01$). Vehicle did not influence tumor growth (not shown).

with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Mϕs cultured with IFN-γ and LPS for 72 h (optimal time). NO production was significantly increased in Mϕs following combined therapy as compared to untreated TBH ($p < 0.01$) (Figure 69).

Combined Paclitaxel and IL-12 Treatment Primes T-cells for Enhanced IFN-γ Production.

IL-12 is a potent activator of T-cell IFN-γ production *in vitro* (19,381,382), and paclitaxel administration enhances T-cell IFN-γ production upon *in vitro* activation through an unspecified mechanism (299). To assess the efficacy of combined therapy on lymphocyte function, IFN-γ production in response to mitogenic stimulation was used as an indicator of T-cell responsiveness. TBHs were treated with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Splenic $CD4^+$ T-cells were collected at 21 days post-tumor induction and were cultured without or with Con-A (8 mg/ml, optimal dose) for 24 h (optimal time). IFN-γ production was significantly increased in T-cells following combined therapy as compared to untreated TBH ($p < 0.01$) (Figure 70). Paclitaxel alone also increased T-cell IFN-γ production, although vehicle did not influence T-cell IFN-γ production (not shown).

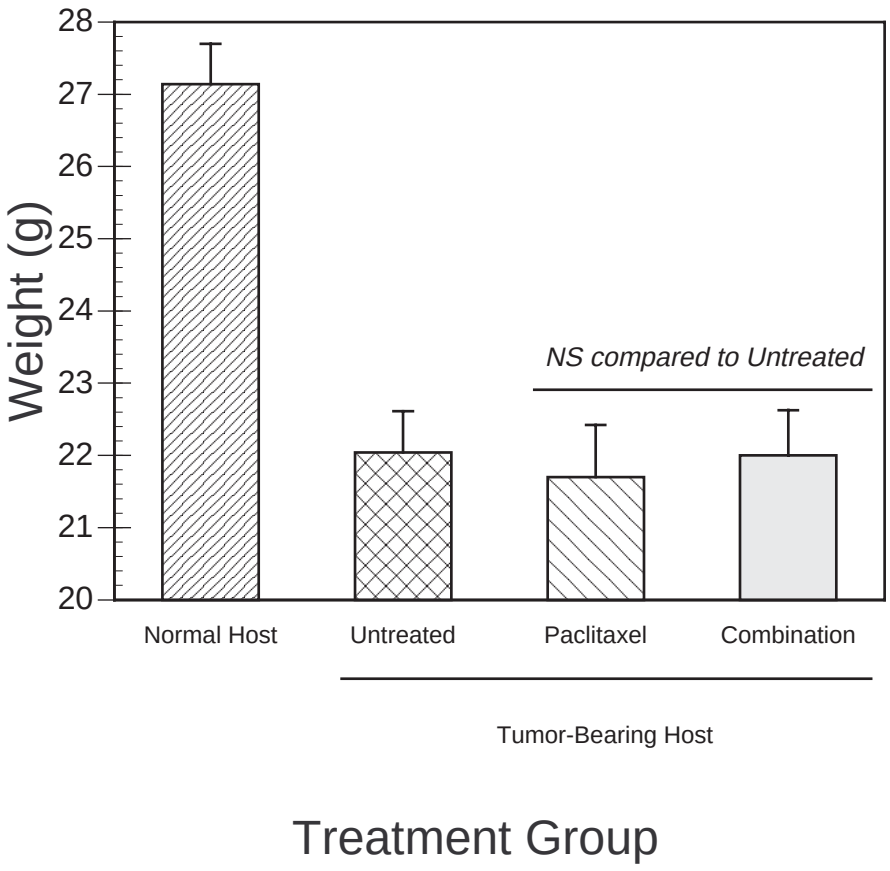


Figure 68. Paclitaxel and IL-12 treatment does not alter body weight or inhibit cachexia. TBHs were treated with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Total body mass was recorded in a blinded manner at day twenty-one. There was no significant difference in mean body mass of TBHs regardless of treatment. Vehicle did not influence body weight (not shown).

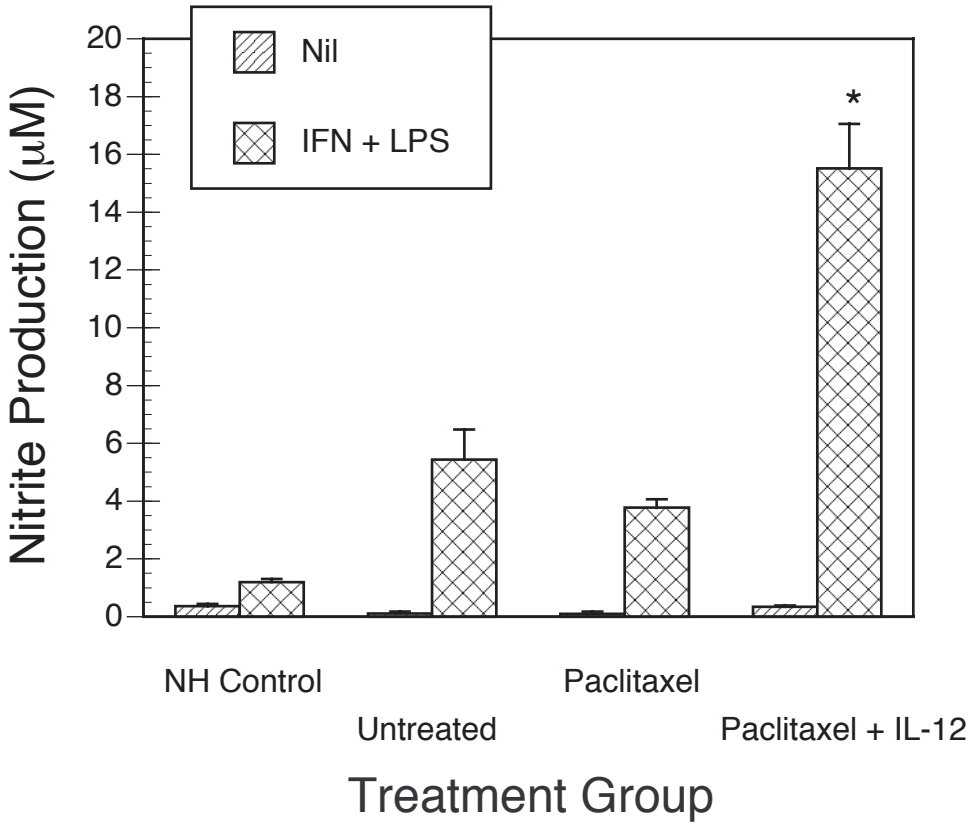


Figure 69. Combined paclitaxel and IL-12 treatment primes Mϕs for enhanced NO production on activation. TBHs were treated with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Animals were sacrificed and splenic Mϕs collected at 21 days post-tumor induction. Mϕs were cultured without or with IFN-γ and LPS and NO assessed at 72 h (optimal time). NO production was significantly increased in Mϕs following combined therapy as compared to untreated TBH ($p < 0.01$). Vehicle did not influence Mϕ activation (not shown).

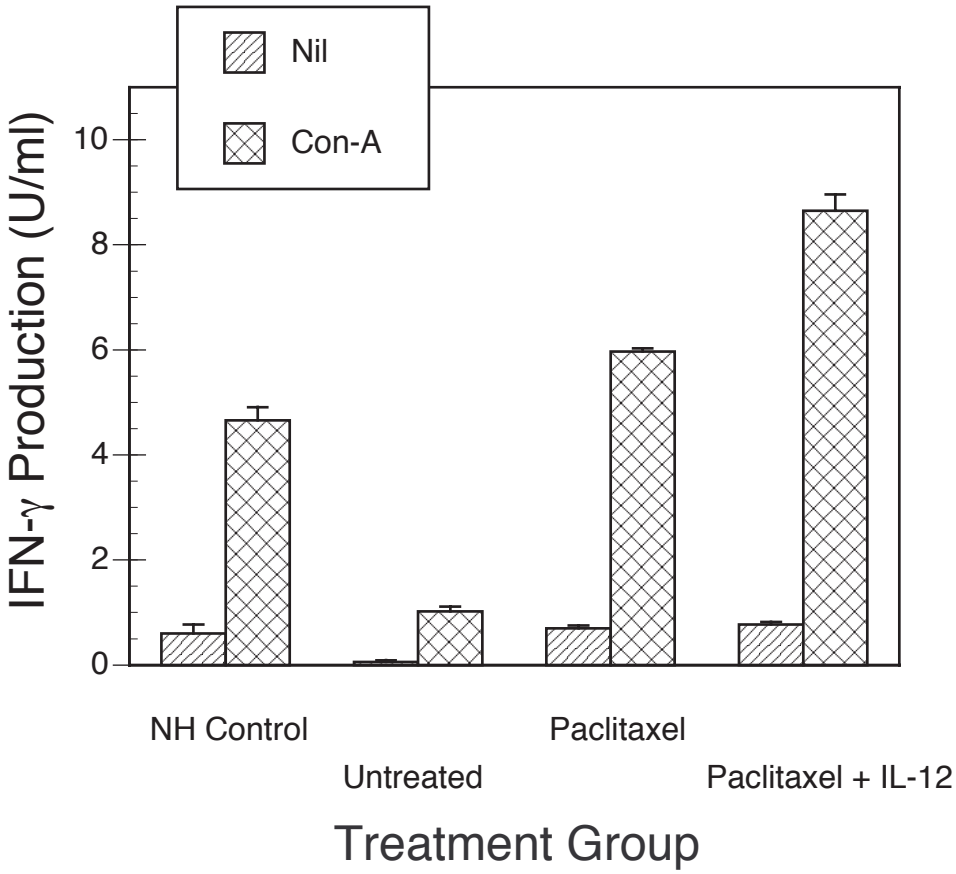


Figure 70. Combined paclitaxel and IL-12 treatment primes T-cells for enhanced IFN-γ production *in vitro*. TBHs were treated with paclitaxel, IL-12, paclitaxel plus IL-12, or vehicle alone as described. Animals were sacrificed and splenic $CD4^+$ T-cells collected at 21 days post-tumor induction. T-cells were cultured without or with Con-A (8 mg/ml, optimal dose) and IFN-γ production assessed at 24 h (optimal time). IFN-γ production was significantly increased in T-cells following combined therapy as compared to untreated TBH ($p < 0.01$). Vehicle did not influence T-cell IFN-γ production (not shown).

DISCUSSION

Paclitaxel is an effective chemotherapeutic that exerts direct cytostatic and cytotoxic antitumor activity (see Chapter VII). Paclitaxel's primary antitumor function -- polymerization of microtubule bundles with resulting inhibition of cell cycle progression and cell death -- imparts the greatest inhibitory effects on rapidly-dividing cell populations. However, the same mechanisms that make paclitaxel a useful antineoplastic agent may simultaneously ravage vital immune system responders, particularly T lymphocytes. Paclitaxel treatment *in vitro* decreases T-cell proliferative responses (56,95,366), limits alloantigen-mediated T-cell responsiveness (365), compromises responsiveness to IL-2 (56,86,301), and impairs cytotoxic activity against tumor cells (86). Because tumor growth diminishes T-cell alloreactivity and responsiveness to activating cytokines, tumor-induced T-cells may be particularly susceptible to the effects of paclitaxel-mediated inhibition. We previously reported that tumor growth increased T-cell sensitivity to paclitaxel co-culture *in vitro* (301), and *in vivo* paclitaxel administration also exacerbates tumor-induced changes in T-cell proliferation (299).

In order to optimize antitumor therapies, the negative immunologic implications of paclitaxel must be addressed. One possibility is combined therapy, in which immunotherapeutic agents are used in conjunction with chemotherapeutic agents in order to reverse immune suppression. A combination therapy would likely reduced the effective therapeutic doses of chemotherapeutic drugs, decrease the need for antitumor agents in favor of naturally-produced cytokines, and induce fewer and less severe side effects than traditional chemotherapy. Collectively, a combined therapy may more fully restore balance to the immune system while facilitating a strong anticancer response. Because paclitaxel inhibits T-cell proliferative reactivity, and IL-12 can reverse

paclitaxel-mediated suppression of T-cell responsiveness *in vitro* (see Chapter IX), we determined whether IL-12 may be a useful immunotherapeutic to reverse immune suppression *in vivo* following paclitaxel administration.

Systemic application of recombinant IL-12 (rIL-12) promotes limited antitumor activities (306), but this approach may not represent the optimal treatment modality for established tumor masses. Generalized activation of the immune responses may be incapable of reversing progression of established tumors or the immunosuppressive activities mediated by such tumors. Further, at the maximal tolerated systemic dose of IL-12, the concentration of IL-12 in the tumor microenvironment may be suboptimal. IL-12 may, however, be useful to support host antitumor responses and supplement chemotherapeutic-based therapies -- such as paclitaxel – that have direct cytotoxic effects on tumor cells.

Others (478) have attempted to use combined therapies consisting of paclitaxel and immune stimulating cytokines with limited success. In contrast to these studies, in which immunotherapy either preceded or was delivered in direct combination with paclitaxel administration (85), we proposed the use of IL-12 following chemotherapy. Our *in vitro* studies (300,301) showed IL-2 activation enhanced paclitaxel-mediated suppression of lymphocyte reactivity, suggesting that paclitaxel treatment should precede immunotherapeutic intervention. Therefore, paclitaxel should be used as an antitumor chemotherapeutic and delivered in transient, temporally-separated regimens followed by IL-12 therapy. In this scenario, the immunostimulatory cytokine should both assist in the recovery of lymphocyte populations following paclitaxel as well as impart distinct and independent antitumor activities.

Because no studies have assessed the efficacy of a combined application of paclitaxel followed by IL-12, we tested this scenario in our Meth-KDE nonmetastatic murine fibrosarcoma model. To achieve a more clinically-relevant result, treatment was initiated only after primary tumors were detected by palpation; this contrasts with many published animal model studies, in which treatment begins immediately after tumor induction. However, this approach is functionally prophylactic, rather than therapeutic. Treatment of TBH with the combination of paclitaxel followed by IL-12 significantly enhanced survival as compared to untreated TBH ($p < 0.01$) or paclitaxel alone ($p < 0.05$) (Figure 65). To our knowledge, this is the first demonstration of an effective antitumor approach using paclitaxel in combination with an immunotherapeutic agent.

Because paclitaxel mediates direct cytotoxic and cytostatic antitumor activity (see Chapter VII), and IL-12 has been shown to mediate tumor regression (319,483), we assessed tumor progression during combined therapy (Figure 66). Early in the treatment regimen (days 10 – 16), there was no difference in tumor growth regardless of treatment group. By day 17, however, tumor diameter was significantly less in the group receiving combined treatment ($p < 0.01$) as compared to untreated TBH (Figure 66). By day twenty-one, following three applications of paclitaxel and IL-12, tumor size in combined treatment group was significantly smaller as compared to untreated TBH ($p < 0.05$) or paclitaxel-treated animals ($p < 0.01$) (Figure 67). To confirm that decreased tumor diameter was not a nonspecific artifact of decreased body mass that accompanies tumor-induced cachexia, body mass was compared. At 21 days post-tumor induction, the mean body mass of untreated TBH is significantly less than untreated normal animals ($p < 0.01$) (Figure 68), in agreement with our past observations in this model system. Treatment with paclitaxel and IL-12, individually or in combination, did not alter body weight. The mean body mass of treated TBHs was not significantly different from

untreated TBH (Figure 68), indicating that decreased tumor mass (see Figure 67) is a specific effect of therapy.

An attractive element of a paclitaxel-IL-12 combined approach is the potential activation of multiple antitumor immune mechanisms. Paclitaxel activates Mϕs for enhanced cytotoxic molecule production *in vitro* (267,296,301), and paclitaxel administration primes Mϕs for NO and TNF-α production on *in vitro* activation (299). IL-12 promotes lymphocyte IFN-γ production and proliferative capacity. In conjunction, these agents may activate both Mϕ- and T-cell-mediated antitumor effector mechanisms. To determine whether combined therapy with paclitaxel and IL-12 has immune activating capacity, we assessed Mϕs NO production and $CD4^+$ T-cell IFN-γ production *in vitro* following therapy. These molecules, which both impart antitumor immune activity and signaling functions, are reliable indicators of Mϕ and T-cell activation, respectively.

Following combined therapy, Mϕs cultured with IFN-γ and LPS produced significantly more NO as compared to untreated TBH ($p < 0.01$). NO was also enhanced in Mϕs from the paclitaxel treatment group (Figure 69), as compared to untreated TBH, suggesting that paclitaxel administration may prime Mϕs for cytotoxic molecule production on activation. The additional NO capacity induced by IL-12 combined therapy may be an indirect result of IL-12-induced IFN-γ production (Figure 70), because IL-12 does not directly induce or prime Mϕs for enhanced NO. Conversely, paclitaxel administration enhances T-cell IFN-γ production upon *in vitro* activation through an unspecified mechanism (299), but our data suggest that paclitaxel-mediated IL-12 production (298) may drive increased IFN-γ production. In the current study, combined therapy with paclitaxel and IL-12 significantly ($p < 0.01$) enhances T-cell IFN-γ production *in vitro*, as compared to untreated TBH (Figure 70). Therefore, combined

therapy may promote Mϕ and T-cell activity through both direct and indirect mechanisms.

Tumor-induced immunosuppression remains a major obstacle to cancer therapy. By defining mechanisms of tumor-induced immune dysfunction, we have identified potential therapeutic measures that may counteract immunosuppression and restore host antitumor immune responses, thus inhibiting or eliminating the tumor. We show that a combination therapy using paclitaxel and IL-12 -- agents currently used individually in Phase III trials or clinical applications -- has both chemotherapeutic and immunotherapeutic effects and reduces tumor progression. We further speculate that the optimal chemo-immunotherapeutic response may not have been achieved in our present study; the efficacy of this therapy derives from its simultaneous assaults on tumors and restoration of cytokine balance to the immune system. Clearly, further *in vivo* studies of immune cell function following various regimens of paclitaxel and IL-12 combination therapy will enhance our understanding of the *in vivo* activities of these molecules and allow for improvement of the dosing regimen to achieve optimal antitumor responses with minimal adverse effects.

CONCLUSIONS

These studies have advanced our understanding of several mechanisms by which tumor-induced dysregulation of Mϕ activity may be reversed. The efficacy of antitumor approaches can be enhanced if the therapy can simultaneously mediate tumor cell cytotoxicity and reverse tumor-induced immune dysfunction. This section summarizes the results described in Sections II, III, and IV, which collectively suggest that successful chemo-immunotherapeutic efficacy can be achieved with the combined use of paclitaxel and IL-12. This section explores the possible therapeutic implications of our research and suggests hypotheses and approaches for future investigations.

DATA SUMMARY

We have described multiple mechanisms of action and Mϕ responses to the anticancer agent paclitaxel. Most significant, we demonstrated that paclitaxel can concurrently induce Mϕ antitumor cytotoxic activities and reverse tumor-induced Mϕ suppressor activities. Based on these initial experiments, we showed that paclitaxel enhances Mϕ production of the proimmune cytokine IL-12, which may contribute to paclitaxel's apparent chemotherapeutic efficacy against certain cancers. Further, we proposed a novel combined therapeutic approach using paclitaxel in combination with

exogenous IL-12 to simultaneously assault tumor cells and activate host antitumor immune responses. We conclude with evidence that the temporal administration of paclitaxel and IL-12 is a promising antitumor approach that slows tumor progression, lengthens survival, and activates immune cell populations in a murine tumor model.

SECTION II: Paclitaxel-induced Cytotoxic Function of Mϕs

In this section, I presented data that describe the response of normal host and -- in a more clinically-relevant scenario -- TBH Mϕs to paclitaxel treatment. Interestingly, although several groups had begun to study paclitaxel-induced Mϕ activation (112,267), no data described the effects of tumor growth on Mϕ responses to paclitaxel. Recognizing that tumor growth dysregulates Mϕ function (123), we used our established murine tumor model to assess the effects of tumor growth on Mϕ (and later T-cell) function following paclitaxel treatment. In Chapter I, the first published studies to describe the LPS-mimetic activity of paclitaxel in TBH Mϕs (296), we show that paclitaxel induces cytotoxic effector molecule production by both normal host and TBH Mϕs, although tumor growth modulates Mϕ responsiveness to paclitaxel treatment *in vitro*. The resulting downregulation of paclitaxel-induced Mϕ NO production in tumor-distal compartments may alleviate tumor-induced Mϕ suppression of T-cell proliferative responses, yet promote sufficient NO production by tumor-associated Mϕs to mediate enhanced cytotoxicity. This novel mechanism of immunotherapeutic activity may partly explain the clinical success of paclitaxel as an anticancer agent. If paclitaxel can induce production of the potent effector molecule NO, even at low levels, a *in situ* Mϕ populations, the NO may contribute to antitumor efficacy by inducing tumor cell apoptosis or enhancing tumor cell sensitivity to paclitaxel or TNF-α.

Because paclitaxel enhances NO production, and the proinflammatory and signaling cytokine IL-1β induces iNOS expression and NO production (236,427), we characterized paclitaxel-induced Mϕ production of IL-1β (Chapter II). Tumor growth significantly enhanced tumor-distal Mϕ IL-1β production. We show that tumor-distal Mϕ populations produce elevated levels of NO and TNF-α in response to IFN-γ priming and LPS activation (15), leading to the hypothesis that tumors prime distal Mϕ populations for enhanced cytotoxic molecule production. Although cytotoxic factor production may seem advantageous to the TBH, tumor-induced Mϕ production of proinflammatory cytokines and cytotoxic effector molecules in spatially-disparate compartments may mediate immunosuppression without significant antitumor cytotoxicity. Elevated IL-1β may contribute to systemic immune dysfunction in the TBH.

In Chapter III, we demonstrate paclitaxel's capacity to induce Mϕ-mediated tumor cell cytotoxicity, even in the presence of tumor-derived immunomodulatory cytokines (301). Paclitaxel-treated TBH Mϕs significantly suppressed the growth of Meth-KDE fibrosarcoma cells through soluble effector molecules and promoted direct cell-mediated cytotoxicity, showing paclitaxel's capacity to enhance tumor-induced Mϕ antitumor activities. These data suggest that paclitaxel-induced Mϕ activation may impart antitumor cytotoxicity, even though tumor growth modulates Mϕ NO production. The potency of NO as an effector of tumor cell death leads to the speculation that even low levels of NO, produced by cells directly adjacent the tumor cells, is sufficient to mediate antitumor efficacy. Paclitaxel-induced NO likely also contributes to Mϕ activation through autocrine induction mechanisms, leading to enhanced Mϕ production of TNF-α. Further, even if expressed at lesser concentrations, NO and TNF-α may render tumor cells highly susceptible to other effectors, including the direct cytotoxic activities of

paclitaxel. Collectively, the data in these Chapters describe several responses of Mϕs to paclitaxel, suggesting that paclitaxel may mediate *in vitro* immunotherapeutic functions in addition to its potent antineoplastic chemotherapeutic activity.

SECTION III: Paclitaxel Reverses Tumor-induced Mϕ-mediated Immunosuppression

Section III describes several mechanisms by which the anticancer agent paclitaxel mediates immunotherapeutic activity and which tumor-derived factors are involved in regulation of TBH Mϕ responses to paclitaxel-mediated activation. In Chapter IV, we extend our studies that suggest tumor growth differentially regulates Mϕ response to activating agents (15,296,301). Using a Mϕ cell line transfected with a luciferase reporter gene downstream of an NF-κB-responsive promoter, we determined the effect of specific tumor-derived factors on Mϕ activation. Using this novel reporter system, the tumor-derived molecules TGF-β_1 and IL-10 are shown to negatively affect paclitaxel's ability to induce Mϕ activation and production or expression of a variety of factors, including TNF-α, iNOS, IL-12. Because the reporter construct in this model is under the transcriptional control of an NF-κB-responsive element, these data suggest tumors may mediate suppressive functions through dysregulation of the pleiotropic signaling factor NF-κB, and this possibility is under investigation in our laboratory. Further, removal of TGF-β_1 and IL-10 from culture reverses the tumor-induced suppression of paclitaxel-induced Mϕ activation, suggesting that abrogation of TGF-β_1 and IL-10 could represent a potent immunotherapeutic co-treatment for paclitaxel.

In Chapter IV, we also present the first evidence that tumors dysregulate immune function through disruption of IFN-γ signaling. We show that tumors induce a lesion in IFN-γ-mediated signaling through the modulation of Mϕ ICSBP expression.

Downregulation of Mϕ ICSBP could impose a damping effect on all IFN-γ-mediated responses of Mϕs. Further, IL-12 p40 expression is ICSBP-dependent, and this mechanism may account for tumor-induced suppression of IL-12 production. Further studies will investigate the role of ICSBP in tumor-induced Mϕ dysfunction.

In Chapter V, we discuss paclitaxel's capacity to reverse tumor-induced Mϕ-mediated immunosuppression, including the first *in vitro* demonstration of paclitaxel's direct immunotherapeutic activities toward T-cells (301). Pretreatment of TBH Mϕs with paclitaxel partly reconstituted T-cell alloantigen reactivity in the presence of Mϕs, suggesting that paclitaxel mediates a limited reversal of TBH Mϕ immunosuppressive activity. Although we earlier reported that tumor growth modulates paclitaxel-mediated NO production *in vitro*, decreased NO cannot alone explain the reversal of immunosuppression. Abrogation of NO production fails to mediate T-cell reactivity, suggesting that paclitaxel may induce Mϕ production of an immunostimulatory cytokine or costimulatory ligand, as discussed in Chapter VI. A dark side emerges, however, with the discovery that tumor growth amplifies T-cell sensitivity to direct paclitaxel-induced suppression. These data demonstrate that paclitaxel exerts pleiotropic effects on antitumor immune responses with the capacity to simultaneously abate the immunosuppressive activities of Mϕs and modulate T-cell reactivity.

Given the finding that paclitaxel pretreatment of TBH Mϕs partially reverses their suppressor activity, we investigated the mechanism of paclitaxel-mediated immune activity. In Chapter VI, we present data suggesting that paclitaxel induces an apparent immunotherapeutic response in T-cells through the induction of Mϕ IL-12 production (298). Tumors perpetuate their own existence, in part, through the dysregulation of Mϕ IL-12, effectively breaking the link between innate and acquired immunity. Further, the

data reveal that paclitaxel induces IL-12 production through an autocrine signaling pathway involving NO, suggesting that paclitaxel-mediated induction of cytotoxic effector molecules has the counter-intuitive effect of stimulating immunostimulatory factors.

SECTION IV: Antitumor Efficacy of Paclitaxel, IL-12, And Combination Therapies

The final data Section contains four chapters describing the effects of paclitaxel (either alone or in combination therapies) on immune cell function and tumor progression. Based on the *in vitro* data presented in Sections II and III, *in vivo* studies were developed and implemented to assess the efficacy of chemotherapeutic paclitaxel regimens in terms of immunologic responsiveness. First, we established the specificity of our tumor model system. In Chapter VII, we show that paclitaxel treatment *in vitro* directly inhibits Meth-KDE tumor cell proliferation and induces apoptotic tumor cell death. These are the first data to confirm that paclitaxel induces apoptosis in the Meth-KDE tumor cell line. We next investigated whether the *in vitro* activating functions of paclitaxel (see Section II) occur during paclitaxel administration. Because paclitaxel is widely administered as an antitumor chemotherapeutic, understanding the responses of tumor-induced Mϕ populations to paclitaxel is critical for the development of effective immunotherapeutic approaches. In Chapter VIII, we present the first experimental evidence that chemotherapeutic paclitaxel administration (in a manner identical to human clinical use) differentially regulates Mϕ functions in the TBH (299). These studies, our first *in vivo* trials with paclitaxel, show that paclitaxel treatment primes Mϕs for enhanced production of cytotoxic mediator NO, but that tumor growth modulates paclitaxel's NO-inducing activity. Concurrently, paclitaxel administration enhanced both normal host and TBH Mϕ cytotoxic antitumor activity and TNF-α production, suggesting that Mϕ-derived

TNF-α is a key mediator of paclitaxel-induced antitumor cytotoxicity. Further, these data suggest that paclitaxel's *in vivo* immune activities may contribute to its success as an anticancer agent by simultaneously enhancing cytotoxic activity and while limiting immunosuppressive tumor-distal NO production.

Although paclitaxel has potent cell-cycle independent effects on Mϕs, it's suppressive effects T-cell proliferation calls into question its usefulness in an immunotherapeutic regimen. Because paclitaxel reverses tumor-induced Mϕ-mediated immune suppression through increased IL-12 production, we next investigated whether exogenous IL-12 could enhance T-cell function and proliferation following paclitaxel treatment. The data in Chapter IX suggest that IL-12 may be useful in modulating paclitaxel's immunosuppressive side-effects during chemotherapeutic administration through its capacity to rescue T-cells from paclitaxel-induced cytostasis. These *in vitro* results lay the foundation for subsequent *in vivo* studies, in which we assess the effects of IL-12 administration in a temporally-spaced regimen with paclitaxel. In Chapter X, we describe a regimen that uses paclitaxel and IL-12. First, paclitaxel is administered in a standard chemotherapeutic regimen; IL-12 is administered one day later, in hopes that IL-12 will then reverse paclitaxel-mediated suppression of antitumor lymphocyte activities. Using this novel approach, we demonstrated that paclitaxel and IL-12 combination therapy prolongs survival, reduces tumor progression, and activates immune effector populations. These results are the first experimental evidence to suggest that paclitaxel and IL-12 are an effective antitumor modality. Both paclitaxel and IL-12 are currently in clinical use and Phase III trials, respectively, and the combination therapy induces both chemotherapeutic and immunotherapeutic effects, leading to suppression of tumor growth. Further *in vivo* studies of immune cell function following various regimens of paclitaxel and IL-12 combination therapy will enhance improve our understanding the *in*

vivo activities of these molecules and likely improve the dosing regimen to achieve optimal antitumor responses with minimal adverse effects.

MODEL OF PACLITAXEL- AND IL-12-MEDIATED REVERSAL OF TUMOR-INDUCED Mϕ AND T CELL DYSFUNCTION

We have described multiple mechanisms by which tumors induce immune dysfunction (see Figure 2) (123), but our previous studies suggested that tumor-induced immune cell dysfunction may be reversible through regimens that disrupt tumor cell suppressor mechanisms and concurrently promote tumoricidal activities. Through these studies, we have demonstrated that the antineoplastic agent paclitaxel and the immunostimulatory cytokine IL-12 can independently or cooperatively reverse the negative effects of tumor growth on antitumor immune function. Furthermore, we are the first to show that tumor-derived factors modulate ICSBP expression (see Chapter IV), suggesting that tumors induce a lesion in IFN-γ-mediated signaling. These findings suggest that ICSBP may contribute to tumor-induced Mϕ dysfunction.

Paclitaxel (Figure 71) has shown significant success as a chemotherapeutic through its capacity to simultaneously compromise tumor cell growth and activate Mϕ cytolytic and effector functions. In our tumor model, paclitaxel mediates direct antitumor cytotoxicity (see Chapter VII) and cell cycle-independent activation of Mϕ functions (see Chapters I-VI). Through its capacity to activate Mϕ function, paclitaxel stimulates TNF-α and NO production, which may enhance tumor cell sensitivity to direct paclitaxel treatment or independently induce tumor cell death. Paclitaxel also reconstitutes Mϕ IL-12 production, thereby promoting T-cell antitumor responses.

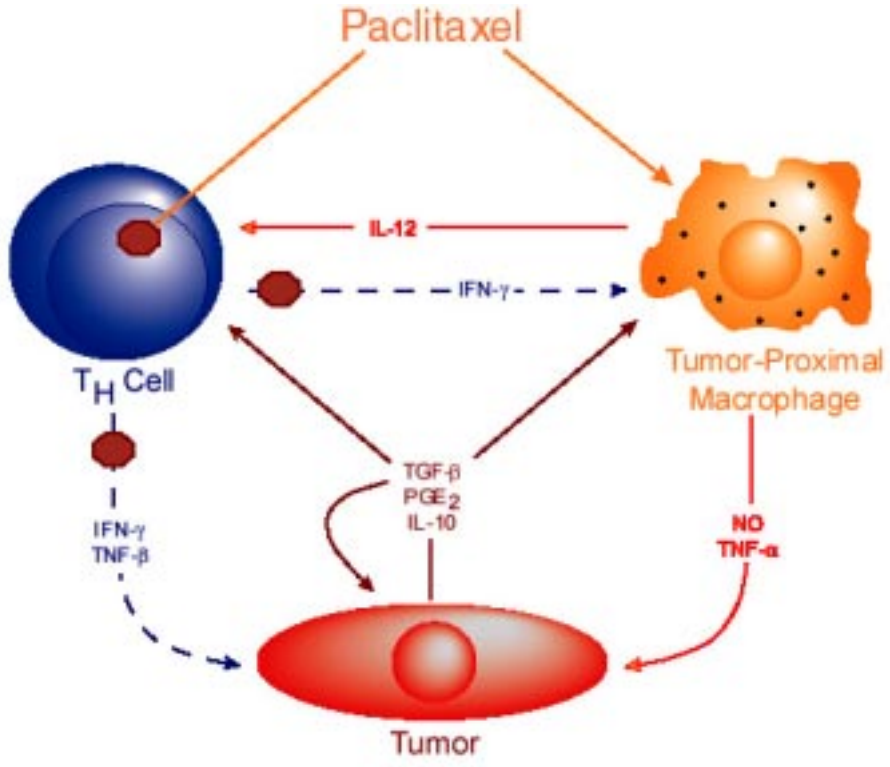


Figure 71. Paclitaxel stimulates Mϕ antitumor functions. Tumor-derived cytokines may suppress activated Mϕ cytotoxic molecule production. Through its LPS-mimetic activities, paclitaxel stimulates (represented by solid orange lines) Mϕs to produce both antitumor molecules (NO and TNF-α) and proimmune cytokines (IL-12). Paclitaxel may also induce the production of other factors, including IL-18.

IL-12 (Figure 72), known for its capacity to drive the development of T_H1-type responses, enhances T-cell IFN-γ production. IFN-γ production will, in turn, promote Mϕ antitumor cytotoxic molecule production and mediate direct antitumor functions against certain IFN-γ-responsive tumors. IL-12 will also promote T_H1-type responses, which are necessary for effective antitumor responses through the acquired immune system. More significantly, IL-12 has shown the capacity to reverse paclitaxel-mediated suppression of T-cell proliferation, suggesting that IL-12 is useful as an immunologic ancillary to complement paclitaxel's antitumor cytotoxic and cytostatic functions.

In the combination therapy (Figure 73), paclitaxel and IL-12 combine to kill tumor cells through the induction of multiple immune effector functions. Paclitaxel mediates direct antitumor cytotoxicity and simultaneously stimulates Mϕ antitumor molecule (NO and TNF-α) and proimmune cytokine (IL-12 and possibly IL-18) production. Mϕ-derived IL-12, in turn, may help stimulate spatially-proximal T-cell populations, although the level of IL-12 is likely too low to mediate significant systemic efficacy. Exogenous IL-12 further stimulates T-cell IFN-γ production and reconstitutes (represented by solid blue lines) T-cell proliferation following paclitaxel administration. IL-12-mediated IFN-γ may then prime Mϕs for enhanced cytotoxic responses on subsequent activation with paclitaxel, leading to enhanced NO, TNF-α, and IL-12. Thus, Mϕs and T-cells begin to support the functions of the other population**.** Collectively, paclitaxel and IL-12 represent a potent antitumor modality through their capacity to mediate a multifaceted *chemotherapeutic* and *immunotherapeutic* attack on the tumor.

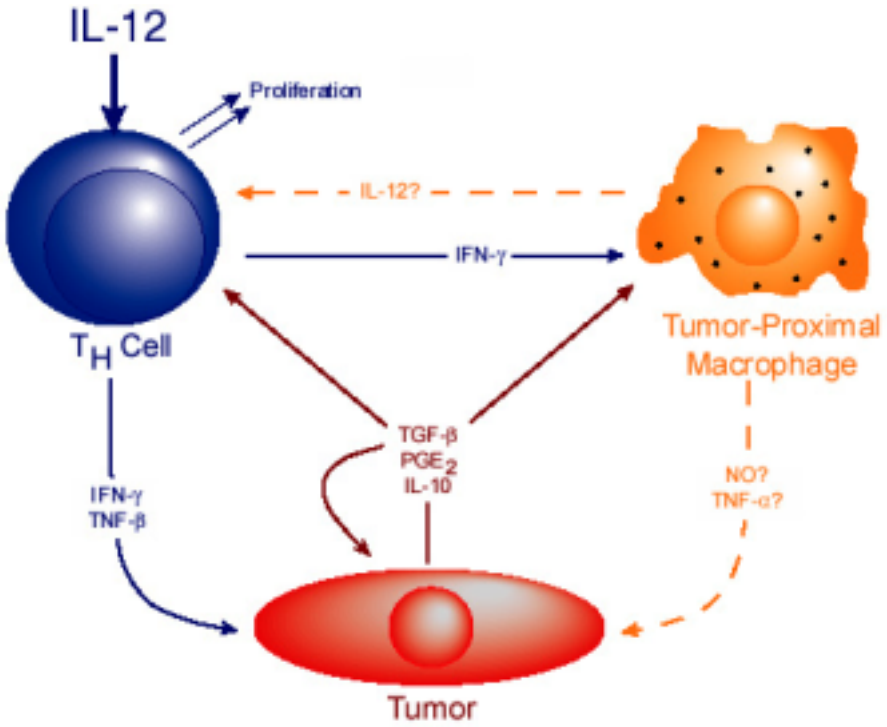


Figure 72. IL-12 stimulates T-cell antitumor functions and reverses paclitaxel-mediated lymphocyte suppression. IL-12, a potent stimulator of T-cell IFN-γ production and proliferative responses, reconstitutes (represented by solid blue lines) T-cell proliferation and factor production even following paclitaxel administration. IL-12-mediated IFN-γ may then prime Mϕs for enhanced cytotoxic responses on subsequent activation with paclitaxel.

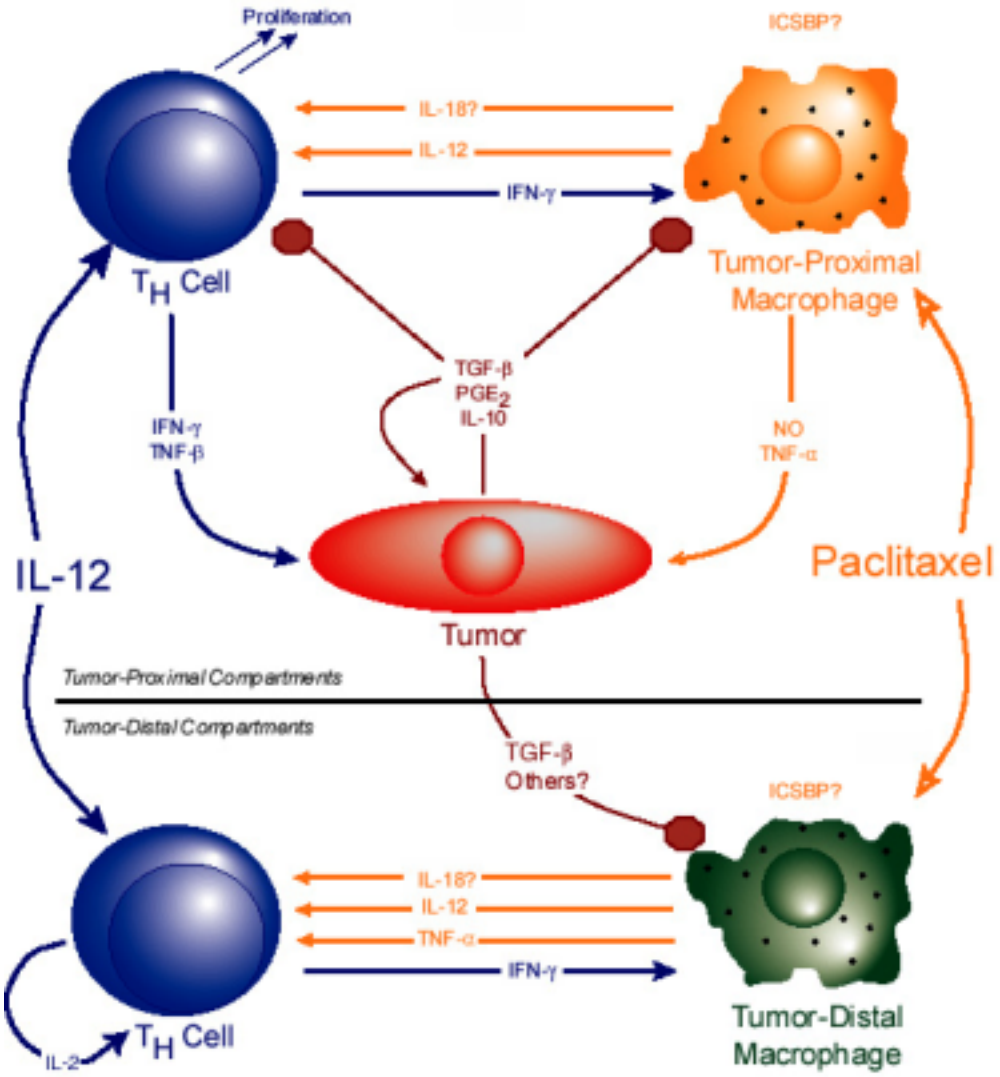


Figure 73. Paclitaxel and IL-12 combine to kill tumor cells through the induction of multiple immune effector functions. Paclitaxel, which mediates direct antitumor cytotoxicity, stimulates (represented by solid orange lines) Mϕs to produce the antitumor molecules NO and TNF-α and proimmune cytokine IL-12. Paclitaxel also may induce the production of other factors, including IL-18. Mϕ-derived IL-12, in turn, helps to stimulate T-cell populations. Exogenous IL-12 further stimulates T-cell IFN-γ production and reconstitutes (represented by solid blue lines) T-cell proliferation following paclitaxel administration. IL-12-mediated IFN-γ may then prime Mϕs for enhanced cytotoxic responses on subsequent activation with paclitaxel, leading to enhanced NO, TNF-α, and IL-12. Thus, Mϕs and T-cells begin to support the functions of the other population.

SUGGESTED FUTURE INVESTIGATIONS

Although my studies have extensively characterized the antitumor and proimmune activities of paclitaxel, many questions remain. It is my hope that this work will provide a foundation on which to build future studies involving paclitaxel, IL-12, and combination therapies. Specifically, the following questions merit investigation:

- ***What are the mechanisms of paclitaxel-mediated antitumor immunotherapy in vivo?*** We show that paclitaxel mediates multiple antitumor mechanisms *in vitro*, and we demonstrate the efficacy of paclitaxel administration as an antitumor agent. The precise mechanisms of paclitaxel-mediated antitumor activity (in particular, immunologic-based efficacy) remain undefined. I propose that paclitaxel-mediated NO is crucial for antitumor efficacy, both through its direct cytotoxic function and its capacity to induce IL-12. Therefore, iNOS knockout mice (available from Jackson Laboratories, Bar Harbor, ME) should be used to determine whether paclitaxel-mediated antitumor functions are NO-dependent. Specifically, iNOS knockout Mϕ IL-12 production in response to IFN-γ and LPS (optimal activating signals) should be determined either without or with paclitaxel administration. I propose that iNOS knockout mice will be particularly susceptible to the Meth-KDE fibrosarcoma, and that paclitaxel will have decreased antitumor efficacy as compared to wild type animals. I further speculate that these mice will exhibit deficient IL-12 production. To further define paclitaxel-mediated immune antitumor function, the role of IL-12 should be studied using IL-12 knockout mice; although iNOS knockout animals may lack paclitaxel-mediated IL-12, any changes in response to tumor challenge *in vivo* or activating agents *in vitro* may not be a specific effect of abrogated IL-12 production. By assessing T-cell function and tumor progression following paclitaxel treatment, the role of paclitaxel-induced Mϕ IL-12 may be more clearly defined.

- ***What is the role of IL-18 during tumor growth?*** IL-18, also known as IFN-γ-inducing factor (326), is a recently characterized cytokine that is a potent activator of T-cell function. IL-18 is up to one-thousand times more potent as a stimulator of IFN-γ, as compared with IL-12 (325). The absence of significant IFN-γ production during the early phases of tumor growth is likely a result of tumor-dysregulated Mϕ IL-12 production (182,298), thus breaking the link between innate and acquired immune responses. However, chronic suppression of IFN-γ in the TBH may be the result of tumor-mediated dysregulation of IL-18 production. Loss of IL-18 production may also explain the lack of NF-κB-mediated responses, because IL-18 drives the expression of NF-κB-responsive genes (108). Future studies should determine whether tumor growth decreases Mϕ production of IL-18; using ELISA or RPA, the effects of tumor growth on LPS- and paclitaxel-activated IL-18 production and expression should be determined. Currently, no data exists to suggest whether dysregulation of this molecule contributes to tumor-induced immune cell dysfunction.

- ***What is the role of ICSBP in tumor-induced dysregulation of immune responsiveness?*** ICSBP, one of the growing family of IFN regulatory factors (314), is dysregulated by tumor-derived factors (see Chapter IV). Tumor-induced dysregulation of ICSBP may seriously comprise the host's ability to respond to tumor growth through the loss of ICSBP-mediated factors. Specifically, IL-12 p40 expression is ICSBP-dependent (371), suggesting another mechanism by which tumors may short-circuit the immune response. Future studies should determine whether tumor growth decreases production of ICSBP in primary Mϕs, and which tumor-derived factors are responsible for loss of ICSBP expression. The role of tumor-derived factors (including but not limited to IL-10, PGE_2, and TGF-β_1) in tumor-induced ICSBP dysregulation should be determined. Using mAb (α-IL-10 and

α-TGF-β_1) or specific blocking agents (indomethacin), tumor-derived supernatants lacking specific molecules can be used to determine which molecules are responsible for dysregulation.

- ***What is the role of NF-kB in paclitaxel-mediated Mϕ IL-12 production?*** We have shown that paclitaxel induces Mϕ IL-12 production through a mechanism involving NO (see Chapter VI). Paclitaxel's LPS-mimetic activity induces NO, which in turn stimulates IL-12. However, the mechanism of NO-mediated IL-12 production remains unclear. Therefore, the signaling mechanism should be investigated. Specifically, Mϕ IL-12 production should be assessed in the absence and presence of paclitaxel, NMMA (specific NO inhibitor), pyrrolidine dithiocarbamate (PDTC, an NO inhibitor that acts through the dysregulation of NF-κB translocation), NMMA plus SNAP (exogenous NO source), and PDTC plus SNAP. If the mechanism of NO-induced IL-12 production does not involve NF-κB, SNAP should reconstitute IL-12 in the presence of PDTC. If, however, NF-κB is required for NO-mediated IL-12 production, PDTC should abrogate IL-12 even in the presence of exogenous NO from SNAP. These studies may define yet another novel mechanism of paclitaxel-mediated immune function.

- ***How is Mϕ IL-12 production regulated throughout the course of tumor development and growth?*** We have shown that IL-12 production is dysregulated by tumor growth at twenty-one days post-induction (298). This time point, however, corresponds to maximal tumor-induced immune suppression in our model system (15,126). The level of IL-12 production should be determined in a kinetic manner at several points throughout the course of tumor development. In a kinetic manner, normal host and TBH Mϕs should be collected throughout the course of tumor

progression and cultured with the priming and activating agents IFN-γ and LPS (to achieve optimal induction of IL-12) and IL-12 p70 production determined by ELISA. Additionally, Mϕ IL-12 expression over the course of tumor growth should be assessed by flow cytometric analysis of cytoplasmic IL-12 levels immediately following Mϕ collection, or *in situ* hybridization using p40-specific probes.

IL-12 is characteristically produced by early in the course of immunologic challenge by cells of the innate immune system (dendritic cells and Mϕs), followed by a decrease in production as the acquired system begins to respond. However, failure to eliminate the offending cells or organisms should result in additional IL-12 production, which seems to be impeded in the TBH. Likely, an initial burst of IL-12 activity occurs early in the course of tumor development, but tumor-derived immunosuppressive cytokines and factors reach inhibitory levels before acquired immunity can clear the tumor; subsequently, IL-12 production is controlled by the tumor. Alternatively, tumor-derived antigen (TAAs) and extracellular matrix may induce a state of "paralysis" in Mϕs, much like endotoxin tolerance that is observed when Mϕs are continuously exposed to LPS (439). This possibility should be assessed *in vitro* by measuring Mϕ IL-12 production following repeated exposure to TAAs or ECM.

- ***Can the efficacy of combined therapies involving paclitaxel and IL-12 be improved with alternative dosing regimens?*** In our initial studies of paclitaxel and IL-12 combined therapy (see Chapter X), we followed a kinetic regimen suggested by our *in vitro* data (298). Because our previous *in vitro* studies (301) that showed IL-2 activation enhanced paclitaxel-mediated suppression of lymphocyte reactivity, we hypothesized that paclitaxel treatment should precede immunotherapeutic

intervention. Paclitaxel was therefore used an antitumor chemotherapeutic and delivered in transient, temporally-separated regimens. Following each paclitaxel treatment (24 h), IL-12 was administered to both assist in the recovery of lymphocyte populations following paclitaxel treatment and impart distinct and independent antitumor activities. This contrasts with the conclusions of others, who report that paclitaxel should follow immune therapy because paclitaxel impedes IL-2-mediated cytotoxicity but not the cytotoxic capacity of IL-2-pretreated human lymphocytes *in vitro* (85). Clearly, *in vivo* studies of immune cell function following various regimens of paclitaxel and IL-12 combination therapy will be needed to conclusively answer these questions. Furthermore, given the modest but significant results of our initial trials with paclitaxel and IL-12 *in vivo*, more studies should address whether alternative dosing regimens can improve efficacy. Specifically, because IL-12 is most effective when delivered in daily doses over a period of several days, the efficacy of a therapy using paclitaxel followed by long-term IL-12 dosing should be investigated.

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APPENDIX A

ABBREVIATIONS

- ATCC, American Type Culture Collection
- Ab, antibody
- APC, antigen presenting cell
- ^{51}Cr, chromium
- Con-A, Concanavalin-A
- cNOS, constitutive nitric oxide synthase
- CLMF, cytotoxic lymphocyte maturation factor
- CTL, cytotoxic T lymphocyte
- DMSO, dimethyl sulfoxide
- EBV, Epstein-Barr virus
- ELISA, enzyme-linked immunosorbent assay
- ECM, extracellular matrix
- FasL, Fas ligand
- FBS, fetal bovine serum
- mGADPH, glyceraldehyde-3-phosphate dehydrogenase
- GM-CSF, granulocyte macrophage-colony stimulating factor

- iNOS, inducible nitric oxide synthase
- ICSBP, interferon consensus sequence binding protein
- IFN-α, interferon-alpha
- IFN-β, interferon-beta
- IFN-γ, interferon-gamma
- IL-18, interleukin eighteen
- IL-5, interleukin five
- IL-4, interleukin four
- IL-1, interleukin one
- IL-6, interleukin six
- IL-10, interleukin ten
- IL-13, interleukin thirteen
- IL-12, interleukin twelve
- IL-2, interleukin two
- i.m., intramuscular
- i.p., intraperitoneal
- LAK, lymphokine activated killer cell
- LPS, lipopolysaccharide
- Mϕ, macrophage
- MCP-1, macrophage chemotactic protein-one
- M-CSF, macrophage-colony stimulating factor
- MDF, macrophage deactivating factor
- MHC, major histocompatibility complex
- 2-ME, β-mercaptoethanol
- Meth-KDE, methylcholanthrene-induced nonmetastatic murine fibrosarcoma
- mAb, monoclonal antibody

- MSV, murine sarcoma virus
- NMMA, N^gmonomethyl-L-arginine
- NK, natural killer cell
- NKSF, natural killer cell stimulatory factor
- NO, nitric oxide
- NH, normal host
- ND, not defined
- NFκB, nuclear factor-κB
- PBL, peripheral blood lymphocyte
- PBMC, peripheral blood mononuclear cell
- PEM, peritoneal exudate macrophages
- PS, phosphatidyl serine
- PHA, phytohemagglutin
- PGE_2, prostaglandin-E_2
- PDTC, pyrrolidine dithiocarbamate
- ROI, reactive oxygen intermediates
- RLU, relative light units
- RT-PCR, reverse transcription-polymerase chain reaction
- RPA, ribonuclease protection assay
- s.c., subcutaneous
- SCID, severe combined immunodeficiency
- SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- SNAP, sodium nitroprusside
- SEM, standard error of the mean
- TCR, T-cell receptor
- TGF-β_1, transforming growth factor-beta one

- TMB, tetramethyl benzidine
- [^{3}H]-TdR, tritiated thymidine
- TAA, tumor-associated antigen
- TAM, tumor-associated macrophage
- TBH, tumor-bearing host
- TNF-α, tumor necrosis factor-alpha
- TNF-β, tumor necrosis factor-beta
- TNFR, tumor necrosis factor-receptor

APPENDIX B

LUCIFERASE ASSAY

The a4 cell line, a RAW264.7 Mϕ line transfected with an HIV-1 LTR promoter and luciferase reporter gene (404), is responsive to LPS-mediated activation and provides a rapid and reliable reporter system for studying Mϕ activation.

General Protocol

1. A4 Mϕ cells (1 x 10^6 cells/well) are cultured (37℃, 5% CO_2) overnight in 24 well tissue culture plates in total volume of 1 ml complete medium (RPMI-1640 + 10% FBS)
2. Wash cells with fresh medium, then reculture for 2 h in a total volume of 1 ml with desired treatment reagents
3. Decant and wash cells in cold PBS
4. Lyse cells with 20 μL Promega Cell Lysis Solution (Promega, Madison, WI) or other lysis solution
5. Remove cellular debris by centrifugation at 12,000 x *g* for 5 sec
6. Assay cellular extracts (see Lowry method, Appendix C) for total protein

7. Measure luciferase activity in cellular extracts using luciferin (Sigma) substrate in reaction buffer (1 M Gly-Gly, 2 M $MgCl_2$) supplemented with 0.1M ATP
8. Add luciferin substrate mix (100 μL at 0.6 mM) to 10 μL of room-temperature cellular extract
9. Record luciferase activity as relative light units (RLU) using a Berthold luminometer (see below)
    - assess luciferase activity for 10 sec, beginning 10 sec after mixing of the substrate and cellular extract
    - luciferase-mediated light production is both stable and optimal during this period
10. Report as relative light units per μg of total protein

Using the Berthold Luminometer

1. Turn on unit, allow 10 min for warm-up
2. Perform a wash-through of the delivery lines
    - place delivery line in clean ddH_2O
    - select *services* #14, press *start* (twice)
    - repeat
3. Place delivery line into luciferin substrate
    - fill delivery line with luciferin by pressing *start* (twice)
4. Program for luciferase activity by keying the following sequence:
    - *Skip*
    - *No*
    - *Measure*

- *Continue*
- *Enter*
- *Raw data*
- *Enter*

5. Add cell extract to tube, place into machine, press *start*
    - repeat for all samples
6. Remove luciferase, repeat wash sequence (step 2)

APPENDIX C

WESTERN BLOTING FOR iNOS PROTEINS

The protein iNOS is an important Mϕ enzyme, produced in response to the priming and activating agents IFN-γ, LPS, and paclitaxel. The function of iNOS is to convert L-arginine into the potent nitrogen intermediate NO. Because paclitaxel mediates cytotoxic and signaling functions through NO, measuring iNOS expression became important to the success of these studies. Therefore, we assessed iNOS production in response to priming and activation by Western blotting. Further, because we were interested in the differential expression of iNOS by normal host and TBH Mϕs, it was important to ensure that each sample contained an equivalent concentration of protein; therefore, we modified the Lowry procedure to create a sensitive and reliable protein assay that that could be performed in microtiter plates. Although the procedure described is specifically designed for iNOS, both the Western blotting and protein assay techniques are applicable to almost any protein of interest.

The procedure for Western blot requires the use of an SDS-denaturing gel in the presence of 2-ME to break disulfide linkages. The use of a stacking gel will make separation of proteins easier, but it is not necessary. After the gel has been run, the proteins are transferred to nitrocellulose where antibodies are used to detect specific

proteins (if antibodies are against conformational antigens, a non denaturing gel lacking SDS or 2-ME) must be used. For some protein analyses, visualization of proteins on gel is sufficient.

Reagents

Cell Lysis Solution

- 300 mM NaCl (1.75 g/100 ml)
- 50 mM Tris-Cl pH 7.6
    - 0.606 g Tris-Cl to 40 ml ddH_2O
    - pH to 7.6, qs to 100 ml
- 0.5% Triton X-100 (0.5 ml/100 ml)
- 10 μg/ml leupeptin (1 mg/ml, Sigma)
- 10 μg/ml aprotinin (1 mg/ml, Sigma)

10% ProtoGel ™ SDS separating gel

- 3.3 ml ProtoGel™ (National Diagnostics EC-890)
- 2.6 ml ProtoGel Buffer (EC-892)
- 3.9 ml dd H20
- 10 μl Temed (Sigma)
- 100 μl 10% ammonium persulfate (Sigma)

4% ProtoGel ™ SDS stacking gel

- 1.3 ml ProtoGel™ (EC-890)
- 2.5 ml ProtoGel™ Buffer (EC-892)
- 6.1 ml dd H20

- 10 μl Temed
- 50 μl 10% ammonium persulfate

Sample Buffer

- 62 mM Tris-HCI pH 6.8
  - 3.75 g Tris-base
  - 35 ml dd H_2O
  - pH 6.8 with HCI
- 37.5 ml glycerol
- 25 ml 2-ME
- 10 g SDS
- 1 mg bromophenol blue (Sigma)
- qs to 100 ml with dd H_20

5X Electrode buffer (1 L, pH of final solution is 8.3 -- do not adjust)

- 15.1 g Tris base
- 72 g glycine (Sigma)
- 5g SDS
- qs to 1 L with ddH_2O

Transfer buffer

- 18.16 g Tris base
- 86.50 g glycine
- 1200 ml methanol (Fisher)
- qs to 6 L with ddH_20
- *KEEP COLD (4°C)*

General Protocol for SDS-PAGE of Protiens

1. Cast gel, allow to solidify (approximately 20 min for resolving gel, 30 min for stacking gel if using BioRad Mini gel)
2. Add equal quantity of protein (approximately 10-15 μg/lane; determined by Lowry assay, as described below) to 5x sample buffer (e.g. 20 μl sample with 5 μl buffer) in locking-cap microfuge tube
3. Boil for 2 min (hot plate, beaker with boiling chips); incubate on ice at least 2 min
4. Deliver sample to gel
5. Run gel at 200 volts until tracking dye reaches the bottom of the gel (approximately 45 min)
6. Use small flat spatula to separate gel from plates and remove gel
7. Prepare electrotransfer apparatus
    - prepare a stack consisting of wet Scotch-Brite Pad, Whatman 3MM filter paper, gel, nitrocellulose membrane, Whatman 3MM filter paper, and Scotch-Brite Pad
8. Run transfer at 200 mA for 1 hour
    - be certain to load gel closest to negative pole and nitrocellulose closest to positive pole
    - run at cold temperature (4°C) to prevent gel from melting
9. Remove gel and prepare nitrocellulose for Western blot

General Protocol for Western Detection of Protein

- note: the membrane *must* remain wet throughout the procedure
    - *do not allow the membrane to dry*

1. Rinse nitrocellulose membrane 3x with 0.l% Tween in TBS(TBS-T)
2. Incubate membrane in blocking buffer (5% skim milk in TBS-T) for 1 h at room temperature or overnight at 4°C
3. React the membrane with a 1:5000 dilution (or at manufacturer's recommendsed concentration) of primary antibody in 5% milk-TBS-T for 1 hour at room temp
4. Rinse membrane 3x with TBS-T
5. React the membrane with a 1:2000 dilution (or at manufacturer's recommended concentration) of horseradish peroxidase -conjugated secondary antibody in TBS-T for 1 hour at room temp
6. Rinse membrane 4x with TBS-T
7. Add 2 ml substrate (Sigma TMB) to membrane for 1 min
8. Shake off excess Chemiluminescent Reagent; wrap in plastic wrap
9. Expose to radiographic film (Kodak MR1) in regular screen cassette at room temperature for 30 sec
10. Develop in automated developer
11. Digitize image for densitometric analysis

Quantify Proteins by Lowry Microtiter Assay

In order to make qualitative and semi-quantitative comparisons between cell preparations, each Western analysis must begin with a uniform amount of total cellular protein. Routinely, we normalize samples by using equal starting cell numbers; this, however, may not guarantee that the total protein preps will have a uniform amount of protein. Using a Sigma Protein Assay Kit (P 5656), based on a modification of the Lowry assay, total protein can be determined. Because we will have only a small volume of protein, we have modified the protocol for microtiter applications.

General Protocol

1. Mix all reagents in a microtiter plate (use a flat-bottom plate with at least 250 μl/well capacity)
2. Make protein standards from 50 – 500 μg/ml using Sigma protein standard solution
3. Add protein samples to water such as to achieve 100 μl/well final
    - 1 μl protein sample to 99 μl water or 2 μl protein sample to 98 μl water is usually a sufficient dilution
    - run in duplicate
4. Add 100 μl of water to at least 3 wells for blanks
5. Add 100 μl of Lowry reagent to all wells and incubate at room temperature for 20 minutes
6. Add 50 μl of Folin and Ciocalteu's Phenol Reagent (*Toxic*, wear gloves) and incubate at room temperature for 30 minutes
7. Read absorbance at 650 nm in microtiter plate reader

8. Plot standard curve and calculate sample protein content (multiply by appropriate dilution factor!)

APPENDIX D

ANNEXIN V ANALYSIS OF APOPTOSIS

Apoptosis (88) is an important cellular mechanism for the negative selection of cells deleterious to the host. Additionally, cytokines or pharmaceutical agents that induce tumor cell apoptosis represent a promising therapeutic approach to the treatment of cancer. Of particular interest is the anticancer agent paclitaxel, which has been shown to induce tumor cell apoptosis in several experimental systems (179,210).

The classic hallmarks of apoptotic cell death include nuclear chromatin, activation of endogenous nuclease activity, and changes in cell size and shape (88). An additional change in the early phases of apoptotic cell death is loss of membrane phosopholipid asymmetry, resulting in exposure of phosphatidylserine (PS) on the outer membrane. Annexin V, a member of the calcium and phosholipid binding proteins, rapidly and specifically binds outer membrane PS, providing a useful method for the identification of apoptotic cells (227).

To determine whether paclitaxel induces apoptosis in our tumor model, Annexin V analysis was performed using the R&D Systems Apoptosis Kit (catalog number KNX50-020), as described below:

1. Meth-KDE fibrosarcoma cells or P815 mastocytoma cells were maintained by 1:5 passage every three day in RPMI-1640 + 5% FBS

2. Tumor cells were collected and resuspended to 1 x 10^6 cells/ml in RPMI-1640 + 10% FBS (cRPMI)

3. 0.5 ml of cell suspension was added to each well of a 24-well tissue culture plate

4. Treatment reagents were added in a volume of 20 μl

5. Cells were incubated at 37°C, 5% CO_2 for various times (0 – 6 h)

6. Cells were resuspended in culture medium and transferred into 1.5 ml sterile microfuge tube and centrifuged for 5 min at 20% power (DuPont Sorvall microfuge)

7. Cells were resuspended and washed three times in 1 ml sterile PBS

8. Cells were resuspended in 0.5 ml of 1x binding buffer (provided in kit); 0.1 ml was transfered to a new microfuge tube

9. Staining reagents were added, at manufacturer's recommended concentration, and cells gently vortexed
   - 10 μl Annexin V
   - 10 μl Propidium Iodide

10. Cells were incubated (37°C, 5% CO_2) in dark for 30 min

11. Binding buffer (0.4 ml, provided in kit) added to each sample

12. Samples analyzed by flow cytometry using PI/FITC protocol

APPENDIX E

DNA LADDERING ANALYSIS OF APOPTOSIS

A classic hallmarks of apoptotic cell death is activation of endogenous nuclease activity, resulting in internecleosomal cleave of cellular DNA. When separated on an agarose gel, the nucleosomal fragments produce a laddering effect, the definitive indicator of apoptotic cell death.

DNA laddering analysis of apoptosis is performed as described:

1. Tumor cells are prepared to concentrations of 1 x 10^7 cells per sample. Treatment reagents are added and cells incubated at 37°C, 5% CO_2 for various times (0 – 6 h).

2. Cells are harvested, washed 3 times with ice cold PBS, and resuspended in 200 μl of ice-cold lysis buffer
    - 20mM Tris.HCI (pH 8)
    - 10mM EDTA
    - 0.2 % Triton X-10 (a detergent which will lyse the cell membranes).

3. Digest proteins by incubating with Proteinase K (1 mg/ml) at 50°C for 12 hours

4. Digest RNA using RNase A (50 mg/ml) at 37°C for 30 min

5. Extract DNA by phenol/chloroform extraction and ethanol precipitation

6. Pellet DNA by ultracentrifugation; dry, then dissolve in Tris-HCl/EDTA buffer

7. Fragmented DNA is visualized by electrophoresis in 1% agarose gel containing ethidium bromide (1 μg/ml)
    - ***Caution! Ethidium bromide (EtBr) is highly toxic! Wear gloves at all times when using EtBr!***
    - Cast gel by pouring tempered agarose (40 ml) into horizontal gel casting apparatus , then allow the gel to solidify for 20 minutes
    - Run at 100 V for approx. 2 hours (or until running dye reaches end of gel)

8. View gel on UV transilluminator

APPENDIX F

RIBONUCLEASE PROTECTION ASSAY

RPA is a sensitive method for the detection and quantitation of RNA. Hybridization is conducted in solution rather than on a membrane, using an excess of an antisense RNA probe. After hybridization, the excess probe is removed by digestion with single-strand specific RNase leaving behind only those probe molecules that were "protected" from digestion by virtue of having formed a duplex with their complementary mRNA target.

To define tumor-induced changes in Mϕ expression of cytokines, normal host and TBH Mϕs were cultured and activated. Total cellular mRNA is isolated and expression of particular mRNAs assessed by RPA. A fluorescent-labeled RNA probe that is complementary to a part of the target mRNA is synthesized. The labeled probe is mixed with sample RNA and incubated under conditions that favor hybridization of complementary transcripts. After hybridization, the mixture is treated with ribonuclease to degrade single-stranded (unhybridized) probe; labeled probe that hybridized to complementary RNA will be protected from ribonuclease digestion, and this species will be separated on a polyacrylamide gel and visualized using a chemiluminescence

detection system. Because the probe will be present in molar excess over the target fragment in the hybridization reaction, the intensity of the protected fragment will be directly proportional to the amount of complementary RNA in the sample mixture (unlike semiquantitative reverse transcription-PCR reactions).

Reagents

- *RNA Isolation System:* Qiagen RNEasy™ Total RNA Isolation Kit
- *Cytokine Templates:* PharMingen RiboQuant™ Template Set mCK-2b (catalog # 45051P)
    - cDNA templates selected to generate complementary mRNA species to selected cytokines
    - multiple cytokines in a single template set (allows for simultaneous analysis of multiple cytokines in a single sample)
- *Transcription Kit:* Ambion MAXIscript™ T-7 (catalog # 1312)
    - For generation of mRNA probes from CDNA templates
- *Non-Isotopic Primer Labeling System:* Ambion BrightStar™ Psoralen-Biotin Kit (Catalog # 1480)
- *Ribonuclease Protection Assay (RPA) Kit:* Ambion RPA-II™ Kit (catalog # 1410)
- *Nonlsotopic Detection System:* Ambion BrightStar™ BioDetect™ (Catalog # 1930)

General Procedure for RPA

1. Synthesize RNA probes from DNA templates using a T polymerase-based transcription system
2. Label the probes with a non-isotopic marker

3. Hybridize the probe to cellular mRNA of interest
4. Treat with ribonuclease to destroy unhybridized RNA
5. Separate on a polyacrylamide gel
6. Electroblot to nylon membrane and crosslink
7. Visualize on autoradiography film
8. Quantify by densitometry

Specific Protocol for RPA (Derived from Ambion's literature and technical bulletins)

Probe Synthesis

1. Assemble the transcription reaction at room temperature in a 1.5 ml microfuge tube
   - nuclease-free H_2O up to 20 µl
   - DNA template 1 µl
   - 10x Transcription Buffer 2 µl
   - 10 mM ATP 1 µl
   - 10 mM CTP 1 µl
   - 10 mM GTP 1 µl
   - 10 mM UTP 1 µl
   - T7 RNA Polymerase 2 µl
2. Incubate at 37°C for 1 h
3. Add 1 µl DNase I (2 U/ml), mix and flash-spin, and incubate at 37°C for 30 min
4. Add 80 µl nuclease-free ddH_20. Extract the mixture with 100 µl phenol:chloroform: isoamyl alcohol (Fisher).

- retain the aqueous layer (*the top layer*), discard tube and lower layer into phenol waste
- note: water is to increase the volume prior to the extraction; if using small volume, may lose probe in phenol layer
- note: goal of phenol:chloroform:isoamyl alcohol extraction is to remove protein, including the DNase I
- *optional:* can perform second extraction with chloroform to remove excess phenol

5. Precipitate with 10 μl of 5M NH_4OAc and 275 μl of 100% ethanol
   - chill at -20°C for 15-30 min
   - recover transcripts by high-speed centrifugation at 4°C
   - resuspend in lx TE buffer
   - proceed to labeling reaction, or store at -70°C
6. Measure RNA concentration and adjust to 50 ng/ml using lx TE buffer

Labeling Reaction

1. Add 1 μl psoralin-biotin reagent to 10 μl of nucleic acid solution in microfuge tube; Mix and transfer to well of uncoated microliter plate.
2. Place a 365 nm UV light source 1-2 cm above microliter place and irradiate for 45 min
3. Dilute to 100 μl using 89 μl of lx TE buffer; transfer to clean microfuge tube
4. Add 200 μl n-butanol; vortex; centrifuge 1 min at 7000 x *g*
5. Pipet off n-butanol (*top layer*) and repeat; remove as much n-butanol as possible
6. Store in 5 μl aliquots at -80°C

Ribonuclease Protection Assay

1. Aliquot 1-20 μl of input RNA into 0.5 ml microfuge tubes
2. Prepare 2 control tubes containing 2 μl (10 μg) yeast RNA (label Cl and C2)
3. Dry samples in speed vac without heat
4. Resuspend samples in 8 μl RPA II hybridization buffer and vortex
5. Add 2 μl of probe to each sample; heat 2-3 min at 95°C
6. Hybridize overnight at 56°C in waterbath or thermocycler
7. Digest ssRNA
    - Dilute RNase A/Tl mix (Ambion solution R) 1:1,000 in RNase dilution buffer (solution Bx)
    - Add 100 μl diluted RNase to all tubes except one tube of the yeast RNA (C2); add 100 μl dilution buffer to tube C2
    - Vortex, pulse-spin, and incubate at 30°C for 45 min
    - Add 150 μl inactivation/precipitation solution (Dx) to each tube
    - Vortex, pulse-spin, and incubate at -20°C for 30 min
    - Centrifuge at high speed, 4°C, for 30 min
    - Aspirate supernatants, air-dry 5-10 min.
    - Resuspend in 4 μl gel loading buffer (E); resuspend the yeast RNA control in 40 μl

Separation of RPA Fragments and Membrane Preparation

1. Prepare a 5% polyacrylamide gel non-denaturing separating gel
    - use 0.75 mm spacers in 11 x l3 vertical gel apparatus
        - 4.2 ml Proto-Gel

- 6.5 ml Proto-Gel buffer
- qs to 25 ml with ddH_2O
- 25 μL Temed
- 200 μL 10% Ammonium Persulfate

2. Run gel at 100 volts for approximately 2-3 h
   - 1Ox TBE gel running buffer
     - 109 g Tris base
     - 55 g boric acid
     - 40 ml 0.5M EDTA pH 8.0
     - qs to 1 L with ddH_2O
3. Electroblot nucleic acid onto a nylon membrane
   - cut a Bright-Star™ nylon membrane to match gel
   - cut 4 pieces of filter paper
   - wet membrane in 0.5 x TBE
   - assemble sandwich with nylon closest to positive pole
   - apply 200 mA for 1 h
   - wrap membrane in Saran wrap, UV crosslink
     - use "optical hybridization" setting on the Shirley lab UVcrosslinker

Detection and Visualization of RPA Fragments

- note: the membrane *must* remain wet throughout the procedure
  - *do not allow the membrane to dry*

1. Wash membrane 2 x 5 min in lx Wash Buffer (1 ml/cm^2)
2. Wash membrane 2 x 5 min in Blocking Buffer (0.5 ml/cm^2)

3. Wash membrane 1 x 30 min in Blocking Buffer (1 ml/cm^2)
4. Wash membrane 1 x 30 min in Conjugate Solution (10 ml Blocking Buffer plus 1 μl Streptavidin Alkaline Phosphatase Conjugate/100 cm^2)
    - note: Streptavidin-Alkaline Phosphatase Conjugate is not stable for storage when diluted in Blocking Buffer. *Prepare only the volume of Conjugate Solution necessary for immediate use.*
5. Wash membrane 1 x 10 min in Blocking Buffer (0.5 ml/cm^2)
6. Wash membrane 3 x 5 minutes in lx Wash Buffer (1 ml/cm^2)
7. Wash membrane 2 x 2 minutes in lx Assay Buffer (0.5 ml/cm^2)
8. Wash membrane 1 x 5 minutes in CDP-Star™ (5 ml/ 10 cm^2)
9. Shake off excess Chemiluminescent Reagent; wrap in plastic wrap
10. Expose to radiographic film (Kodak MR1) in regular screen cassette at room temperature; develop in automated developer
    - note: It takes 2 h for CDP-Star™ to reach peak in light emission, at Which point emission falls to a plateau which persists for several days. Initial short exposures of one to two hours usually yields the desired signal, followed by shorter exposures (five minutes to two hours) the following day to achieve the desired image.
11. Digitize image for densitometric analysis

David Warren Mullins

David Warren Mullins was born in Richlands, Virginia on January 18, 1967. He earned Bachelor of Science degrees in Biology (Microbiology and Immunology Option, 1993) and Biochemistry (Chemistry minor, 1994) from Virginia Tech. During his undergraduate education, David worked as a Research Associate at The Wilmer Eye Institute (Johns Hopkins University Medical Institutions) in Baltimore, Maryland. David also conducted undergraduate research at the Virginia-Maryland Regional College of Veterinary Medicine.

David began graduate studies in 1994 completed his dissertation, entitled "Paclitaxel-Induced Macrophage Activities in the Tumor-Bearing Host: Immunologic Implications and Therapeutic Applications," under the guidance of Dr. Klaus D. Elgert. David's research at Virginia Tech led to seven first-author manuscripts either published or submitted, and he was a co-author on a major review article in the field of tumor immunology. His work was presented at 20 local, state, and national meetings of professional societies, including the American Association of Immunologists, the Society for Leukocyte Biology, the American Association for the Advancement of Science, the Virginia Academy of Science, the Virginia Branch of the American Society for Microbiology, the American Cancer Society's Symposium of Cancer Researchers in Virginia, and the Virginia Tech Graduate Research Symposium. David received seven

awards for his presentations, including *Best Student Paper* from the Virginia Academy of Science, and he was a finalist in the Society for Leukocyte Biology's Presidential Student Awards Competition. David's research was funded, in part, by grants from the Virginia Academy of Science, *Sigma Xi*, the Graduate Research Development Program of Virginia Tech, and the Department of Biology; he was also awarded travel grants by the Society for Leukocyte Biology and the Graduate Student Assembly of Virginia Tech.

While a graduate student at Virginia Tech, David devoted significant time and effort to teaching. He instructed laboratory classes in Soil Microbiology (2 sections), General Microbiology (9 sections), and Immunology (5 sections). Additionally, David supervised undergraduate research projects and mentored students in the Biological Sciences Initiative Teaching Internship program. In total, David instructed over 450 students at Virginia Tech, and he maintained a near-perfect student evaluation rating. For his efforts, David was selected as the university's Outstanding Graduate Teaching Assistant in 1998, and he was honored at the Founder's Day ceremony.

Recognizing the tripartite mission of a land-grant university, David also participated in numerous service projects. Notably, he served for two years as Chief Justice of the Graduate Honor System and was a member of the Commission on Graduate Studies and Policies. David is an active member of several professional societies, including the American Association of Immunologists, the Society for Leukocyte Biology, the American Society for Microbiology, and the Virginia Academy of Science.

David is currently a postdoctoral research associate at the Beirne B. Carter Center for Immunology Research, University of Virginia Health Sciences Center, Charlottesville, Virginia.