

Fibrosarcoma-induced Dysregulation of Interleukin (IL)-1 β and IL-18 Activities and their Modulation by Paclitaxel

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(ABSTRACT)

Cancer remains an elusive killer due, in part, to the suppression of normal immunologic antitumor responses. The tumor-compromised immune system is unable to recognize or destroy aberrant cells. Normal host (NH) macrophage (M ϕ) populations have tumoricidal effects such as tumor antigen phagocytosis and presentation, and cytokine production. Tumor-infiltrating M ϕ s may evade these activities by dysregulating or inhibiting production of immunostimulatory cytokines (including Interleukin [IL]-1 β , IL-18, and tumor necrosis factor- α [TNF- α]), by production of immunosuppressive factors like IL-10 and tumor growth factor- β (TGF- β), or by other mechanisms. The restoration of IL-1 β , IL-18, and TNF- α production by M ϕ s could re-establish antitumor host immune responses. Previous work in our laboratory suggests that tumor distal (TD) M ϕ s produce more IL-1 β than NH M ϕ s when stimulated with IFN- γ and lipopolysaccharide (LPS). We hypothesize that the presence of immunomodulatory factors like IL-10 and TGF- β dysregulate IL-1 β production in tumor proximal (TP) M ϕ s. Indeed, IL-1 β production was downregulated among *in situ* TP M ϕ s. We have proposed that IL-18, a structural homologue to IL-1 β was similarly dysregulated in TD and TP M ϕ s. IL-18 was enhanced in both distal and proximal M ϕ s. Differences in the functions of these cytokines could account for this dissimilarity. TNF- α , another proinflammatory cytokine, followed the dysregulation pattern of IL-1 β in our tumor-burdened hosts (TBH), likely because of the similar functions of these cytokines. Because it is a potential vehicle for immunotherapeutic treatment, paclitaxel's action on the immune response (TAXOLTM) was investigated. Paclitaxel is a potent M ϕ activator that upregulates a variety of cytokines in an LPS-like manner. Paclitaxel enhanced TD M ϕ production of IL-1 β , IL-18, and TNF- α in an LPS-like manner. Production of IL-1 β and TNF- α was reduced in TP M ϕ s when treated with paclitaxel; however, IL-18 production was enhanced. This difference could be due to the different functions of IL-1 β and IL-18. To determine whether production of these cytokines translates into downstream expression of transcription products, IL-12 and nitric oxide (NO) were assayed. NO was enhanced distally, but paclitaxel treatment failed to enhance NO production. When treated with paclitaxel, IL-12 was produced by NH and TD M ϕ s. Collectively, these studies suggest that tumor-induced cytokine imbalances compromise antitumor immunity and paclitaxel may reverse this activity.

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INTRODUCTION

The first objective of this study was to determine whether tumor-derived factors produced by the Meth-KDE tumor [5] contribute to immune dysfunction by inhibiting tumor-infiltrating and/or tumor-distal macrophage (M ϕ) production of the proinflammatory cytokines Interleukin [IL]-1 β and IL-18. The second objective was to determine whether paclitaxel could modulate tumor-induced dysregulation, possibly through production of NO or IL-12. Understanding how the Meth-KDE tumor mediates M ϕ dysfunction will further our understanding of how modulation of cellular events, such as signal transduction, contributes to this activity. M ϕ populations possess antitumor properties, such as tumor antigen presentation, phagocytosis, and production of proinflammatory cytokines, all which aid in host antitumor defense. Because paclitaxel activates M ϕ s, it was an ideal chemotherapeutic to use for treatment. Identifying the role of paclitaxel in stimulating proinflammatory cytokine production can elucidate new therapies for overcoming tumor dysregulation.

The proinflammatory cytokines IL-1 β and IL-18 are two chemical messengers of antitumor defense. These cytokines activate transcription factors leading to the production of other proinflammatory effector molecules such as tumor necrosis factor- α (TNF- α) [106, 130]. Antitumor defenses are not always successful because the tumor-compromised immune system is often unable to recognize or destroy aberrant cells due to immunosuppressive factors. This lack of recognition and destruction is partly due to alterations in M ϕ function and leads to production of inhibitory polarized M2 M ϕ s *in situ* [130]. M2 M ϕ s can downregulate cytokine production by tumor-infiltrating M ϕ s which, in turn, reduces lymphocyte reactivity against the tumor. A proposed method of tumor-induced immune suppression involves dysregulation of IL-1 β and IL-18. This

dysregulation can be overcome through treatment with the chemotherapeutic agent paclitaxel (TAXOL™), a known stimulator of Mφ-induced proinflammatory gene expression [152].

We aimed to determine whether tumor-derived factors contribute to immune dysfunction by inhibiting IL-1β and IL-18 production by tumor-infiltrating and/or tumor-distal (TD) Mφs. If tumor dysregulation of IL-1β and IL-18 occurs, the host immune system could be further impacted. IL-1β and IL-18 initiate NF-κB translocation, and the downstream products include TNF-α, IL-12, and inducible nitric oxide synthase (iNOS) [107]. TNF-α and IL-12 are potent antitumor molecules that promote T_H1 cell-type responses. Their dysregulation would promote immune escape. If iNOS is dysregulated, induction of NO, a cytotoxic free radical gas, would also be affected and Mφ activation would be reduced [Bogdan, 2001. #7320]. Dysregulation of IL-1β and IL-18 may lead to dysregulation of these transcription products in the tumor-burdened host (TBH) would reduce efficacy of the host immune response.

Previously, we have shown that tumor-bearing host (TBH) splenic and peritoneal Mφs produce more IL-1β than their equivalent normal host counterparts (unpublished data); however tumor destruction does not occur. We proposed that during tumor growth, Mφs located at the tumor site have decreased IL-1β production. In our tumor model microenvironment, immunomodulatory cytokines like IL-10 and TGF-β likely suppress production of proinflammatory responses [6, 155]. Because IL-18 is structurally similar to IL-1β, tumor growth may have similar effects both *in situ* and distally. Production of these immunomodulatory molecules may cause further dysregulation of the cytokines IL-12 and TNF-α production, translocation of NF-κB, and production of the effector

molecule NO. As will be described in Section II, enzyme-linked immunosorbant assays (ELISAs) were used to analyze production of the cytokines IL-1 β , IL-18, TNF- α , and NF- κ B. Greiss reagent was used to determine NO levels. Western blotting (SDS) analysis measured IL-12 expression. The connection between tumor-derived cytokine dysregulation and induction by paclitaxel was also examined.

Smith, Thornton, and Allen [Σμττη, 1995 #5595] reported that paclitaxel enhances IL-1 β production by human monocytes and that paclitaxel alone can enhance human monocyte IL-1 β expression. IL-18, an IL-1 β structural homologue and M ϕ -derived cytokine, was also assessed following paclitaxel treatment. If IL-1 β and/or IL-18 are dysregulated in tumor-associated M ϕ s, restoration of *in situ* production may effectively stimulate the immune system and may offer immunotherapeutic options for treatment. Because paclitaxel has been shown to mimic lipopolysaccharide (LPS) in M ϕ activation [3], we hypothesized that paclitaxel may contribute to decreased tumor growth and reduced tumor activity through restoration of *in situ* immune cell production of IL-1 β , IL-18, and TNF- α .

Paclitaxel is a known stimulator of M ϕ production of IL-12, TNF- α , and inducible NO synthase (iNOS), all of which are important in the host's ability to mount an effective antitumor immune response [152]. If paclitaxel-induced modulation of TBH M ϕ dysfunction was related to one of the above factors, paclitaxel could enhance TNF- α , IL-12, and/or NO production and alter signal transduction through enhanced NF- κ B translocation. Work by Cvetkovic, Miljkovic, Vuckovic, Harhaji, Nikolic, Trajkovic, Mostarica, and Stojkovic [44] showed that paclitaxel does induce iNOS through NF- κ B

activation. Paclitaxel might modulate tumor M ϕ production of IL-1 β and IL-18 in M ϕ s both distally and proximally to the tumor. The second part of this study used ELISAs to test for the presence of the cytokines IL-1 β , IL-18, and TNF- α , and for the translocation of the transcription factor NF- κ B. Western blot analysis was used to test for IL-12 expression, and Griess reagent tested for NO production. If paclitaxel can upregulate IL-1 β and IL-18 production, we will have identified a novel activity of paclitaxel and provided new insight into its immunotherapeutic application for cancer.

Many studies [23, 75, 137, 192, 229, 234], including those conducted in our laboratory [152, 153, 155], offer insight into the role of paclitaxel in ameliorating tumor-induced immune dysregulation. Recent studies suggest that paclitaxel enhances antitumor efficacy when administered to tumor-burdened mice [60]. Paclitaxel is a known stimulator of apoptosis in cancer cells. It works by binding to β -tubulin in microtubules, inhibiting the disassembly of microtubules, and further strengthening microtubules present in the cell [90]. Paclitaxel increases the stability of microtubules formed in its presence by binding to the N-terminal amino acids of the β -tubulin subunit in place of the binding of normal tubulin dimers [121, 124, 178]. Both of these functions disrupt the normal dynamics of the cell, causing its death [181-183].

Paclitaxel's ability to bind β -tubulin makes rapidly dividing cells particularly susceptible to its action and is an ideal and well-established agent for treatment of metastatic breast cancer and ovarian cancer [62]. Treatment of ovarian cancer with taxanes like paclitaxel is often administered for many years due to the nature of the cancer and its antitumor defenses. Despite the efficacy of paclitaxel, it has numerous toxic side-effects including the killing of T-cells[154]. The need for alternative

immunotherapies with fewer side-effects has led to the discovery of novel taxanes with more favorable toxicity profiles and equivalent antitumor activities. One such taxane, CT-2103, is a conjugate of paclitaxel and a water-soluble polymer of glutamic acid [131]. Recent studies show this conjugate to be easily solublized, less toxic, and equally efficient in antitumor activity. Other taxane conjugates such as docosahexaenoic acid-paclitaxel show similar efficacy [225]. Immunotherapies like these with reduced side effects will be beneficial in treating conditions such as ovarian and breast cancer. It is likely that these conjugates enhance production of proinflammatory cytokines and reduce production of immunomodulatory molecules.

While chemotherapeutic agents such as paclitaxel are often used in cancer treatment, administration of cytokines in conjunction is another promising area of immunotherapy. The use of cytokines as immunotherapeutic agents for cancer treatment must be carefully regulated since cytokine overdosing can cause further immunosuppression thus enhancing tumor growth [42]. Our studies [155, 156] outline pathways of cytokine dysregulation, suggesting potential therapies to counteract tumor-induced immune suppression. More specifically, this project examined whether tumor-derived factors contributed to immune dysfunction by inhibiting IL-1 β and IL-18 production by tumor-infiltrating and/or tumor-distal M ϕ s, either through dysregulation of NF- κ B activity and/or NO production. The connection between cytokine dysregulation by tumors and restoration of the immune response by paclitaxel was also examined. This project had the following aims:

Aim 1: Assess whether tumor growth modulates the production of the immunoregulatory, proinflammatory cytokines IL-1 β and IL-18 by tumor-

distal/proximal M ϕ s, and evaluate whether modulated cytokine production is associated with diminished NF- κ B translocation. This was specifically accomplished by:

- determining whether tumor growth modulates the production of M ϕ -derived IL-1 β and/or IL-18.
- determining whether tumor-induced downregulation of IL-1 β or IL-18 is associated with changes in NF- κ B translocation.

Aim 2: Determine whether the M ϕ -activating chemotherapeutic paclitaxel induces IL-1 β and IL-18 production and NF- κ B translocation during tumor growth. The objectives of the aim were to:

- determining whether paclitaxel activates TBH M ϕ production of IL-1 β and IL-18.
- determining whether paclitaxel mediates regulation of M ϕ cytokine production through enhanced production of IL-12 or NO, or changes in NF- κ B translocation.

A variety of experimental approaches were used to accomplish these research aims including ELISA, western blotting, Griess reagent to test for NO, and flow cytometry. Throughout these studies, the Meth-KDE murine fibrosarcoma was used. Because IL-18 was not downregulated in our TBH M ϕ s, IL-18R expression was not tested. No NF- κ B data are available as all experiments produced inconclusive data.

This thesis is divided into two sections. First a review of relevant literature is presented. Next, the mode of tumor-induced M ϕ dysfunction is discussed followed by how paclitaxel modulates the effects of tumor dysregulation in applicable *Results* and *Discussion* sections. These are summarized and explained in *Conclusions*, which also contains a proposed model of tumor-induced M ϕ dysfunction. Finally, *Suggested Future Investigations* are presented.

SECTION I: LITERATURE REVIEW

The *Literature Review* consists of six subsections. *The Macrophage During Tumor Growth* discusses this monocyte-derived cell and its involvement in antitumor responses. The second subsection, *Proinflammatory Cytokines*, describes the chemical messengers IL-1 β , IL-18, IL-12, and IFN- γ . The third subsection, *Signaling Pathways*, discusses the effect of tumor-dysregulation and paclitaxel-modulation on the ubiquitous cellular signaling molecule NF- κ B. The fourth section, entitled *Cytotoxic Mediators* discusses NO and TNF- α as mediators of cytotoxicity; followed by methods of *Tumor Evasion and Progression*. In particular, the role of TGF- β , IL-10, and PGE₂ in tumor-induced immune dysregulation. The sixth section, entitled *Paclitaxel*, discusses how this M ϕ activator and chemotherapeutic drug affects not only production of proinflammatory cytokines but also modulates tumor-induced dysregulation of these cytokines.

THE MACROPHAGE DURING TUMOR GROWTH

Role and Activation

M ϕ s are ubiquitous cells derived from monocytes that mediate both innate and adaptive immunity. The hallmark of M ϕ activity is its ability to engulf foreign pathogens [147]. This function has led to the idea that M ϕ s are ancient cells, because related cell types are found in haemolymph of primitive multicellular organisms [24]. M ϕ s mainly aid in phagocytosis, degradation of apoptotic cells and microbes, secretion of cytokines and other mediators, and the presentation of foreign peptide to T lymphocytes. Although some of these functions are constitutively expressed, most require an endogenous signal to activate the M ϕ s, which is often delivered by cytokines. For instance, tumor cell

killing requires an exogenous signal either relayed by cytokines or through interaction with T cells [24]. This process activates M ϕ s and allows for presentation of major histocompatibility complex (MHC) class II molecules. It was once suggested that these M ϕ s were more phagocytic than resting cells, but it is now known to be untrue [148]. M ϕ s are often defined as phagocytizing cells, but their function as antigen-presenting cells (APCs) is also an important characteristic. M ϕ s cannot become APCs until they are activated. Because M ϕ activities are potentially life-threatening, they are tightly controlled. When this control is lost, M ϕ s can mediate or initiate numerous inflammatory diseases [147].

M ϕ s are remarkable in the way they recognize and engulf a genetically diverse number of infectious pathogens and foreign antigens [147]. The presence of many nonopsonic receptors facilitates this function, which also allows for the removal of dying cells. M ϕ s bind to phosphatidyl serine (on cells undergoing apoptosis) and other markers by their mannose receptor and CD14 [24].

M ϕ s are important cytokine producers [147]. By initiating transcription, they induce proinflammatory cytokines (IL-1), cytokines that mediate activation of T- and NK cells (IL-12), cytokines that provide feedback and killing activity (e.g., TNF- α), and anti-inflammatory cytokines (IL-10) that downregulate M ϕ s [6, 24].

M ϕ Polarization

M ϕ s that possess proinflammatory functions are called M1 or classical monocytes. M1 M ϕ s produce IL-12 and TNF- α , which drive T_H1 cell development.

There are two other M ϕ subsets that differ from the M1 subset. Type 2 M ϕ s possess anti-inflammatory functions and drive T_H2 cell development [144]. They have increased secretion of IL-10 and have enhanced MHC class II molecule expression. Finally, there is an alternative activation program that produces M ϕ s with distinct and different functional properties than those of the M1 and type 2 M ϕ s [71, 148]. These M ϕ s are called alternatively activated polarized M2 mononuclear M ϕ s and are often induced by cytokines associated with type II responses (IL-3, IL-4, and IL-10). M ϕ s of the M2 lineage produce cytokines such as IL-10 and IL-1 receptor antagonist (IL-1Ra). The M2 M ϕ s have immunosuppressive functions, are involved in tissue repair, can promote tumor progression, and are often called tumor-associated M ϕ s (TAMs) [71, 129, 148]. These monocytes are recruited from blood to the tumor site by specific attractants such as colony stimulating factor-1 (CSF-1) and granulocyte-M ϕ -colony-stimulating factor (GM-CSF) [10, 19]. TAMs are poor antigen presenters and suppress T-cell activation and proliferation by producing IL-10, prostaglandins, and TGF- β [13, 127].

Tumor Cell Destruction

Activated M ϕ s can recognize and destroy tumor cells, distinguishing between tumorigenic and non-tumorigenic cells by their cell membrane composition. There are two mechanisms of M ϕ -mediated tumor destruction: M ϕ -mediated tumor cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). In M ϕ -mediated tumor cytotoxicity, tumor-killing is a slow, contact-dependent mechanism that relies on an intercellular adhesion molecule-1 (ICAM-1), which differs among tumor cells [103]. A direct correlation between enhanced ICAM-1 expression and increased antitumor responses by M ϕ s has been noted [46, 89, 222]. When antibodies against ICAM-1 are added, a

substantial inhibition of M ϕ -mediated tumoricidal activity occurs [18]. In ADCC, M ϕ s lyse tumor cells by the Fc binding of antibody-coated tumor cells to effector cells. After the Fc regions are ligated to effector cells, mediators involved in tumor cell killing are secreted [103].

PROINFLAMMATORY CYTOKINES

M ϕ s produce proinflammatory cytokines such as T_H1-inducing IL-1 β , IL-18, and IL-12. These cytokines show a broad range of effects that include modulation of immune responses and direct regulation of cellular growth and differentiation [43, 112, 197, 228, 230]. M ϕ activation by stimulants such as LPS and paclitaxel can upregulate the production of these antitumor cytokines [152, 155, 224]. This can lead to the induction of antitumor effector molecules, such as IFN- γ by T_H1 cells and NO by M ϕ s [50-52] which can reverse tumor growth by recruiting proinflammatory cytokines. These characteristics suggest that IL-1 β , IL-18, and IL-12 may have potential as immunotherapeutic agents for cancer treatment [43].

Interleukin-1 β

IL-1 β is a 17-kD multifunctional cytokine produced by all nucleated cells except T-cells [228, 230]. Activated monocytes in particular are producers of IL-1 β , a molecule produced intracellularly and activated by caspase 1 processing [21, 197]. Caspase 1 also activates proIL-18 [21, 52, 54, 55, 197]. IL-1 β is an activator of NF- κ B, working in conjunction with TNF receptor-associated factor 6 (TRAF6) and immune activation gene 1 (Act1) and can affect *in vivo* expression of a variety of other molecules by activation or

downregulation of transcription or proform processing [14, 15, 232]. Also, IL-1 β induces iNOS, which mediates the production of NO, a key tumoricidal molecule [133].

IL-1 β acts as an intracellular regulator of apoptosis. It is found in lysosomes, is associated with microtubules, and is secreted throughout the mammalian body [50]. Unlike IL-1 α , another autocrine growth factor present in the M ϕ cytosol, IL-1 β can be found in blood and body fluids [74]. IL-1 β affects *in vivo* expression of a variety of other molecules by activation or downregulation of transcription or proform processing. It can downregulate tumor cell expression of TNF- α receptors, which reduces cell susceptibility to killing by TNF- α [28, 85].

The processing steps involved with IL-1 β activation suggest this cytokine may be vulnerable to tumor-induced dysregulation. Mature IL-1 β cannot be produced without the cleavage and export of pro-IL-1 β . The cleavage products of proIL-1 β include an IL-1 β propiece and mature IL-1 β . This step is linked to processing at the aspartic acid-alanine residue by IL-1 β converting enzyme (ICE or caspase-1) [50, 69]. This cleavage takes place in the cell cytoplasm and allows the export of IL-1 β . ProIL-1 β cannot be exported outside the cell and requires ICE-driven cleavage (Figure 1).

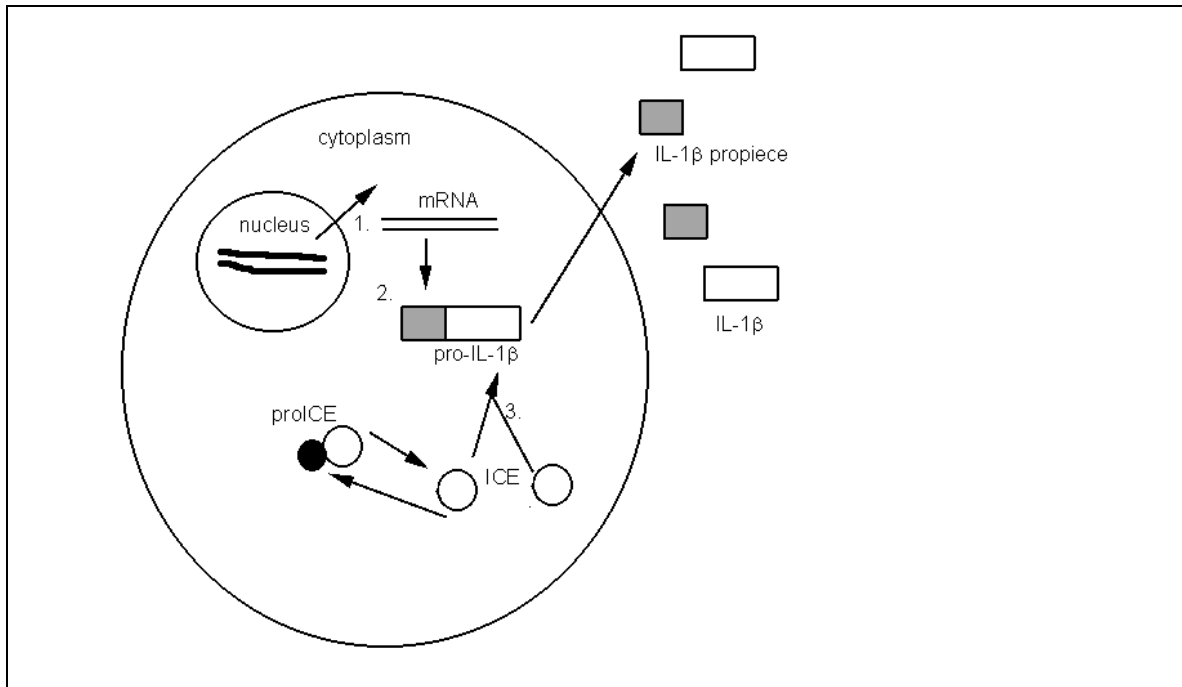


Figure 1: IL-1 β Processing Steps. 1. Transcription of mRNA encoding IL-1 β . 2. The mRNA is translated and pro IL-1 β is expressed in the cytoplasm. 3. proICE is converted to ICE. Two ICE proteins cleave proIL-1 β , allowing for its export outside the cell [52, 56].

Interleukin-18

IL-18 is a member of the T_H1-inducing family of cytokines [54, 196]. It was isolated during endotoxemia in mice preconditioned with an infection of *Propionibacterium acnes* [161, 162]. Originally, IL-18 was called IFN- γ -inducing factor due to its ability to stimulate IFN- γ production by T and NK cells [223]. Researchers were initially perplexed over what to name this molecule. The name IL-1 γ was proposed because it is functionally similar to IL-1, but this was rejected because IL-18 fails to bind the common IL-1R chain [51, 54, 100]. Later, it was found to have additional biological effects, such as enhancing antibody production, revealing that IL-18 was more than just an IFN- γ inducer. It then became officially known as IL-18 [163].

Monocytes, Mφs, keratinocytes, and DCs produce IL-18. Both IL-18 and proIL-18, the IL-18 precursor, are found in human peripheral blood mononuclear cells and are produced constitutively in the epidermal cells of certain mouse strains [51, 54]. Mφs can be induced to produce IL-18 by many known Mφ stimulators, such as LPS, exotoxins, and other microbial products [24, 51, 54].

Besides the structural similarity IL-18 shares with IL-1β, it also requires caspase-1 cleavage to become active. Without this trimming event, the signal peptide sequences of both IL-18 and IL-1β are incapable of biologic activity [21, 74]. This structural resemblance may account for other similarities between IL-18 and IL-1β including the way both are activated and the similarities in their receptors. Research by Gaggero, Ambrosio, Mezzanazana, Piazza, and Rubartelli [66] suggests that tumor-induced immune suppression is linked to dysregulation of caspase-1 processing of IL-18. Further relationships between the two molecules include their receptors: the IL-18R is a member of the IL-1R family and is related to the IL-1R accessory protein [195, 199]. The IL-18R and IL-1R share common intracellular signal pathways [69, 74].

While structurally similar to IL-1β, IL-18 is functionally similar to IL-12; it stimulates IFN-γ production by NK and T cells [228]. IL-18 also manages many activities including induction of T_H1 cells, NK cell activity, translocation of NF-κB, and the production of specific chemokines [21, 132]. The ability of IL-18 to stimulate IFN-γ in synergy with IL-12 makes it a prime candidate for application in cancer immunotherapy, due to the ability of IFN-γ to induce the production of other tumoricidal cytokines [22, 143, 209]. Research by Hikosaka, Hara, Miyake, Hara, and Kamidono [82] suggests that

these cytokines possess antitumor effects when combined in synergy. Regulation of IL-18R, the IL-18 receptor, could account for tumor-induced dysregulation as this receptor is necessary for signal transduction involving NF- κ B, the pivotal transcription factor [100, 116, 232]. More specifically, IL-12 increases gene expression and surface expression of the IL18R α receptor chain [100, 228]. IL-18 suppresses IL-10 and IgE production [51, 54].

Interleukin-12

IL-12 is a proinflammatory cytokine that indirectly contributes to tumor cell destruction [9, 200, 201, 203-206, 208]. It is a heterodimeric protein with two subunits linked by a disulfide bond that mediates its activity through the IL-12R [142]. IL-12 responsiveness relies on the activation of the T cell receptor (TCR). Resting T cells cannot respond to IL-12 because they do not express IL-12R. IL-12 induction occurs only after the TCR is activated through a series of immune responses leading to IL-12 and IL-18 activation [209]. A number of cells, including monocytes, M ϕ s, B cells, dendritic cells, and connective tissue-type mast cells are implicated in the secretion of IL-12, as well as LPS-stimulated human polymorphonuclear cells (PMNs) [45, 149, 205]. The level of IL-12 secretion depends on the stimuli; optimal M ϕ IL-12 levels are achieved in response to bacterial antigen presentation, and by stimulators such as LPS, paclitaxel, and *Mycobacterium tuberculosis* in combination with IFN- γ stimulation [151, 154, 166, 231]. IL-12 is produced by NK cells, CD4⁺ T cells, and CD8⁺ T cells [138, 169].

IL-12 has multiple biological activities and potent antitumor effects [79, 80, 231]. It promotes the development of T_H1-type cells, which enhance the immune responses of the TBH [68, 209]. It can act alone to stimulate low levels of IFN- γ production and in synergy with IL-18 to stimulate high levels of IFN- γ production [156, 166, 202, 207]. Immunosuppressive cytokines, such as IL-10, suppress IL-12 activity when acting in an autocrine fashion [190]. IL-12 inhibits antibody production and suppresses IgE production, leading to decreased humoral immunity [101]. The antitumor effects of IL-12 are well characterized in both human and murine tumor models and, unlike IL-1 β , IL-12 has mild side effects when used as a treatment in the TBH [31-37, 83, 84, 209]. In murine tumor models, either direct intra-tumor injection or systemic administration of IL-12 induces potent antitumor and antimetastatic effects [195, 198]; this antitumor capacity is reproduced, to varying degrees, by the proinflammatory cytokines IL-1 and IL-18. We showed that tumor activity downregulates IL-12 p70 production through selective downregulation of IL-12 p40 [151]. We further found that this downregulation was ameliorated by the chemotherapeutic paclitaxel [152].

Interferon- γ

Interferon- γ is a dimeric protein composed of four exons ranging from 70 to 160 kDa that binds to multiple receptors. Receptors for IFN- γ are expressed on all types of human cells with the exception of erythrocytes. Activated T-cells (CD4⁺ and CD8⁺) and NK cells are the main producers of IFN- γ . These cells are activated by antigens, mitogens, or alloantigens as well as APC-derived cytokines. The molecules IL-2, basic fibroblast growth factor (β FGF), and epidermal growth factor (EGF) induce the synthesis of IFN- γ while dexamethasone and cyclosporine A (CsA) inhibit its synthesis [78].

Interferon- γ is an inducer of T_H1 -driven immune pathways, is an inhibitor of normal and transformed cells, and has multiple antiparasitic and antiviral activities, often due to its synergy with TNF- α and TNF- β . It stimulates CD4 and MHC Class II molecule expression, upregulates NK cell production, and specifically induces transcription of many genes. It enhances M ϕ activity (particularly the induction of IL-1 and TNF- α and antitumor responses) and the expression of MHC class I and II molecules. IL-2 produced by T_H cells acts synergistically with IFN- γ and requires expression of the IL-2 receptor [179]. As mentioned earlier, IL-12 and IL-18 both induce production of IFN- γ , acting both alone and in synergy with one another [102].

The antiproliferative effect of IFN- γ on tumor cells is well established [93] and recent literature has dealt with enhancing this effect *in vitro* [57]. Molecular profiling is also being used to define IFN- γ -induced mechanisms involved in hepatocarcinoma regulation[30]. Despite its role as an inhibitor of tumor cell growth, IFN- γ delivered within the tumor often cannot work because of immunosuppressive molecules like IL-10 produced by the tumor [156]. New methods such as convection-enhanced delivery may overcome this problem, and new studies have discovered that infusion with IL-1 β and IFN- γ via this method induce tumor invasion with M ϕ s and lymphocytes [65].

SIGNALING PATHWAYS

The above mentioned proinflammatory cytokine activities are initiated through signal transduction pathways. IFN- γ and IL-12 both act through the JAK/STAT4 signaling transduction pathway, while IL-1 β and IL-18 act by the NF- κ B signaling

pathway (Figure 2). Translocation of NF- κ B leads to production of IL-12, which can then activate the JAK/STAT4 pathway [48].

NF- κ B

The transcriptional factor NF- κ B is involved in regulating anti-apoptotic molecules, inducible enzymes, proinflammatory cytokines, and surface cell activation and adhesion molecules [16, 95, 97, 134]. Cytoplasmic NF- κ B is a collection of dimers consisting of five identical proteins with a conserved N-terminal region called the Rel-homology domain (RHD) [47]. The RHD contains two different regions: a DNA-binding site and nuclear localization signal regions. When NF- κ B is bound to I κ B it is inactive and is present in the cytoplasm of unstimulated cells [94]. The most common stimulated form is a heterodimer composed of a 50- and a 65-kDa subunit that consists of a processed form of the NF- κ B1 protein and the Rel A protein respectively [214]. The activated form is present only when all seven inhibitory factors (I κ B's) are inactivated [97, 134, 187].

I κ B α and I κ B β are the two most important inhibitors of NF- κ B. Inactivation occurs by binding to the Rel homology domain [12, 115]. I κ B β is constitutively expressed; its phosphorylation allows the activation of NF- κ B, which, in turn, induces the expression of I κ B α and the inactivation of NF- κ B [95-97]. NF- κ B is activated most effectively by TNF- α and IL-1 as shown in Figure 2 [134].

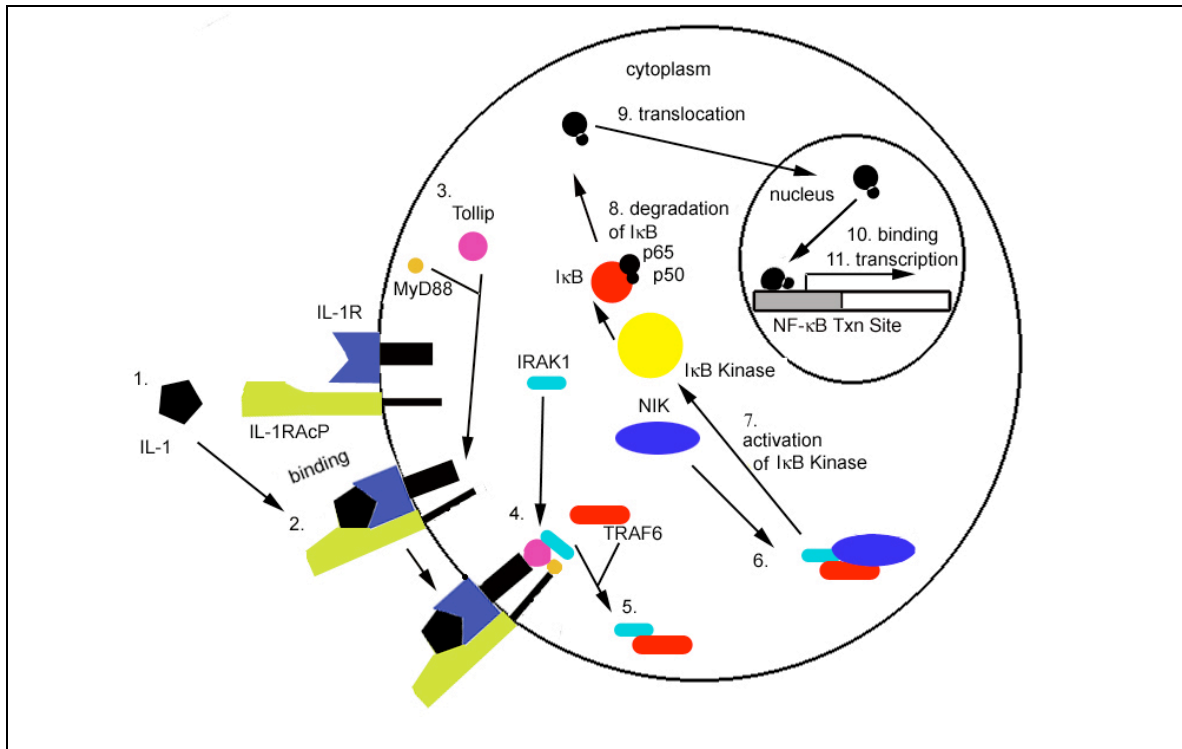


Figure 2: IL-1-induced NF-κB translocation. 1-2. IL-1 binds to IL-1R and IL-1RAcP. 3. Tollip and MyD88 are activated and bind to the complex. 4. IL-1 receptor-associated kinase 1 (IRAK1) binds to the complex and is then released to 5. bind with tumor necrosis factor receptor-associated factor 6 (TRAF6). 6. NF-κB-inducing kinase (NIK) is recruited to the complex and 7. IκB kinase is activated. 8. This kinase phosphorylates IκB and the p65 and p50 regions of NF-κB are released. 9. NF-κB translocates into the nucleus and binds to the NF-κB transcription site. 10. Transcription is initiated. IL-18 can also activate translocation by binding to IL-18R

CYTOTOXIC MEDIATORS

Mφs produce the cytotoxic molecules NO and TNF-α when activated. These molecules are highly effective against tumors and are critical to host immunosurveillance [25, 104, 105, 173, 215]. However, tumors can evade host immune mechanisms by producing immunomodulatory molecules like IL-10 [151, 153, 155] that directly alter

these cytotoxic molecules [17]. Recent studies show that tumor cells can selectively deactivate monocytes by blocking molecules involved with these cytotoxic mediators such as CD44 expression on monocytes, inhibiting their antitumor response [158]. Decreases in CD44 expression are linked to decreases in TNF- α secretion [108].

Nitric Oxide

Nitric oxide (NO), a soluble L-arginine cytotoxic molecule, is an important molecule involved in proinflammatory responses. Nitric oxide is an inorganic free radical gas that is soluble in lipids and water. It can react with oxygen to yield radicals, stable anions, oxides, and peroxides [119]. Three genes encode enzymes that mediate NO production: NOS1, NOS2 (iNOS), and NOS3 [Bogdan, 2001. #7320;Xie, 1992 #6499]. NOS1 and NOS3 are responsible for the constitutive expression of NO, which is important in cardiovascular and nervous system signaling in the body of a healthy host. The inducible form of NOS (iNOS) is produced by M ϕ s only when the host is experiencing periods of infection and inflammation [119] leading to M ϕ activation. M ϕ s produce NO in sufficient concentration to mediate localized cytostatic and cytotoxic effects on tumor cells [88, 226].

Tumor-induced dysregulation of M ϕ NO production is well-established [212]. LPS activation failed to induce TAM NO production in our Meth-KDE fibrosarcoma model [155], and distal M ϕ production of NO by our tumor model was dysregulated [157]. M ϕ induction of NO production can occur using a variety of molecules like LPS. Most cytokines, such as IL-1 β , and many microbial products can stimulate NO production

[Bogdan, 2001. #7320;Xie, 1992 #6499]. In certain cell lines, IFN- γ and LPS can individually stimulate expression ; however, IFN- γ and LPS synergize to induce NO expression in most M ϕ cells lines, leading to more potent antitumor responses [88]. The combination of paclitaxel (substituted for LPS) and IFN- γ can stimulate TBH M ϕ production of NO [153]. As mentioned, NO is a potent antitumor molecule; researching the role of serum L-arginine and whether it affects NO production could further elucidate our understanding of how NO affects antitumor responses.

Recent studies of M ϕ -derived NO have focused on its role in the aging process [87]. A correlation has been noted between aging and cancer progression. The decline in immune cell function and reductions in serum L-arginine are linked to the aging process. The role of L-arginine in many immunological processes is well-established. A study by Izgut-Uysal, Ozkaya, Ozdemir, Yargicoglu, and Agar [87] focused on how treatment with L-arginine prevented age-related changes in phagocytic function of peritoneal M ϕ s. This and future studies offer a possible understanding to the connection between aging and cancer.

TNF- α

The proinflammatory cytokine TNF- α has a wide range of biological activities such as *in vivo* and *in vitro* cytostasis of tumors, endothelium alterations, neutrophil attraction and production of IL-1 and PGE₂ [2]. It is an important molecule in cell-mediated immunity against bacteria. Stimulation with LPS induces production of TNF- α by monocytes, M ϕ s, neutrophils, T-cells, and NK-cells [111]. The receptor soluble

tumor necrosis factor- α (sTNFR1) promotes tumor growth and reduces host immunity in both our Meth-KDE fibrosarcoma [unpublished work] and in other models [Sugano, 2004 #7355]. This receptor induces apoptosis in immune disorders through the immunomodulatory TGF- β 1 [219] and can inactivate receptors for vascular endothelial growth factor (VEGF) [195].

TUMOR EVASION AND PROGRESSION

Tumors evade host defenses by multiple mechanisms at both induction and effector phases [235]. Alterations of M ϕ function, altering/shedding their antigens, downregulating MHC class I molecules, and production of cytokines all aid tumors in eluding host immune surveillance as will be discussed below. The Meth-KDE fibrosarcoma also has strategies to evade host defenses.

The Meth-KDE Fibrosarcoma

The Meth-KDE fibrosarcoma suppresses immunity through the production of IL-10, TGF- β , and prostaglandin E₂ (PGE₂) [6]. The Meth-KDE fibrosarcoma is a nonmetastatic murine methylcholanthrene-induced fibrosarcoma that is perpetuated by intramuscular injections into the hind legs of Balb/c (H-2^d) mice. Ten to 14 days post inoculum, palpable tumors form; at 21 days mice are immune suppressed, and death occurs between 28-35 days post-inoculum due to the load of the tumor. It has been found that the Meth-KDE fibrosarcoma expresses production of the markers monocyte chemoattractant protein-1 (CCL2) and α sTNFr1 and induces production of VEGF-A [unpublished data]. These markers will be discussed in detail below

Alteration of M ϕ Function

As mentioned earlier, TAMs are M ϕ s of the M2 type that promote tumor progression through immunosuppression [71, 129, 148]. Tumor-derived chemotactic factors attract these monocytes to the tumor site where they can participate in angiogenesis or secrete tumor cytotoxic mediators [10, 103]. Typically, TAMs suppress T-cell activation and proliferation by inducing production of IL-10, prostaglandins, and TGF- β as is discussed later [13, 58, 127]. M ϕ -derived activities such as NO production and TNF are also dysregulated [157].

Tumors Disguise Themselves to Evade Host Immunity

Tumors often disguise themselves so the host immune system cannot recognize them as foreign. This can be accomplished in many ways: by shedding its cell-surface antigen, altering its cell-surface antigen, downregulating MHC class I molecules, or epigenetic gene regulation [118, 180]. Tumor-associated antigens can be shed, often into lymph fluid, causing B and T cell tolerance to the antigen. This tolerance allows the tumor to escape undetected, and an immune response is not triggered [109]. This well-documented phenomenon is the target of many immunotherapies designed to enhance tumor recognition. Alteration of cell surface markers, or antigenic modulation, also allows tumors to evade host immune responses [170]. One such human marker is HLA-DRB, which is known to be differentially expressed in tumor cells from breast carcinoma

[164]. Downregulation of MHC class I molecules reduces antitumor defenses because MHC class I molecules draw in CD8⁺ T cells, which can lyse and destroy tumor targets. Downregulating these molecules reduces antitumor cytotoxicity and allow the tumor to remain undetected and proliferate [11]. Tumors can evade host immunity by inducing production of immunosuppressive chemokines as discussed below [165]. Immune therapies such as taxane conjugates designed to reverse these antitumor evasion responses could boost host immunity and cause tumor regression.

Immunosuppression and Tumor Progression via TGF- β , IL-10, PGE₂ and VEGF-A and the Markers CCL2 and α sTNFR1

The immunosuppressive factors IL-10, TGF- β , and PGE₂ are critical players in tumor evasion [6, 58]. The cytokine TGF- β exists in five isoforms and has an entire family of glycoprotein receptors [72]. The transduction mechanisms of TGF- β are largely unknown. Recent studies have linked its production to the action of SMAD proteins [86]. Specifically, TGF- β inhibits M ϕ production of the proinflammatory cytokine IL-12 and modulates surface receptors required for cell activation and growth [49, 112]. T-lymphocytes are inhibited by TGF- β production largely through downregulating IL-2 mediated signals. TGF- β inhibits proliferation of IL-1-induced lymphocytes as well as TNF- α and IFN- γ production [155]. *In vivo* antitumor activity is reduced through these signals, and synthesis of granulocyte M ϕ -colony stimulating factor (GM-CSF) is inhibited. TGF- β is possibly linked with tumor metastasis because it modulates interactions with tumor cells and the cellular matrix [77]. TGF- β is known to regulate genes involved in late tumor progression [86].

IL-10 is an important immunomodulatory cytokine produced by T_H2 cells following lectin stimulation [194]. Ly-1 B cells expressing CD5 and CD11 also produce IL-10, which reduces antigen presentation and inhibits T_H1 T-cells and T cell activation [70]. IL-10 inhibits proinflammatory cytokines such as IFN- γ , TNF- α , and IL-2 and suppresses IL-12 synthesis via accessory cells [73]. IL-10 can reduce proinflammatory responses produced by treatment with LPS [146]. Its anti-inflammatory role has made IL-10 the focus of many clinical trials to treat chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease [73]. This cytokine also inhibits M ϕ activation and induces production of alternatively activated M ϕ s [127, 155, 227]. It is hypothesized that IL-10 is responsible for tumor-associated M ϕ production of the mediator CCL18. This chemokine is activated by dendritic cells (DCs) [218], Gaucher cells [27], and alternatively polarized M ϕ s [186] that is involved in immunoregulatory pathways. IL-10 is responsible for attracting naïve T-cells in the tumor periphery dominated by Type II M ϕ s that may act in an immunosuppressive manner [127]. IL-10 contributes to the development of non-Hodgkin's B cell lymphoma associated with acquired immunodeficiency syndrome (AIDS). It has been suggested that IL-10 may stimulate proliferation of these malignant lymphoma cells in an autocrine manner [29]. IL-10 is linked to the T_H2-inducing primary cutaneous lymphomas (CLs). It is believed that IL-10 contributes to CL progression by enhancing HLA-G protein upregulation [210].

PGE₂ is a prostaglandin synthesized by prostaglandin synthetase from arachidonic acid [76]. This prostaglandin is produced by cancer cells and systemically blocks antitumor immunosurveillance while promoting tumor growth [227]. It has pro- and anti-inflammatory effects. PGE₂ is produced by M ϕ s to downregulate immune responses. Such activities include inhibition of antibody production and lymphocyte

mitogenesis. PGE₂ contributes to immunosuppression often through cyclooxygenase-2 (COX-2). COX-2 is often overexpressed in cancer, particularly in breast cancer. Pockaj, Basu, Pathangey, Gray, Hernandez, Gendler, and Mukherjee [172] found that DC and T-cell functions were reduced in patients with breast cancer through PGE₂ secretion related to COX-2 overexpression. The mechanism of PGE₂ immunosuppression induced by COX-2 tumor expression is unclear although COX-2 is a regulator of prostaglandin PGE₂ production [4].

VEGF-A is a molecule produced by tumor cells that can exert systemic effects on host immunity. DC differentiation, maturation, migration, and function are all affected by tumor cell production of VEGF-A [Yang, 2004 #7349; Sugano, 2004 #7355]. VEGF-A can be activated by expression of α sTNFr1, an antagonist for TNF- α expressed by tumor cells. The marker CCL2 is also expressed by tumor cells and can promote immune escape [189].

PACLITAXEL

Paclitaxel (registered trade name TAXOL[™]) was first isolated from the bark of the western yew tree, *Taxus brevifolia* [221]. It is a plant-derived diterpenoid that binds to β -tubulin in microtubules, which inhibits the disassembly of microtubules thus increasing the number of microtubules present in the cell [90-92]. It increases the stability of microtubule formed in its presence, due to paclitaxel binding to the N-terminal amino acids of the β -tubulin subunit instead of the binding of tubulin dimers [121-124, 177]. Both of these functions disrupt the normal dynamics of the cell causing its death [181-184]. Paclitaxel acts on any dividing cell, but its effects are more dramatic on those

rapidly dividing because they are rapidly dividing, making paclitaxel an ideal agent for treatment of metastatic breast cancer and ovarian cancer [62, 155].

Paclitaxel is a potent M ϕ activator through elements within the LPS receptor/signaling complex [39, 150, 154, 157, 216, 217]. M ϕ activation requires two signals: the first to “prime” the M ϕ and the second to “trigger” the M ϕ ’s tumoricidal activities [140, 141, 185]. The priming signal, typically IFN- γ , can occur either before or concurrent with the “trigger” signal, typically LPS or paclitaxel [167]. Paclitaxel activates M ϕ s through elements within a LPS receptor/signaling complex [81]. Paclitaxel and LPS share the same pathway for microtubule based signaling, shown by suppression due to anti-mitotic inhibitors; however, LPS and paclitaxel have different activation pathways [20, 176]. The pathway for M ϕ activation is separate from paclitaxel’s ability to bind β -tubulin [159]. The major paclitaxel-binding protein for murine M ϕ activation is CD18 [20]; however, other unidentified proteins may be involved [62, 90, 124, 178, 183]. Once activated, M ϕ responses include TNF- α secretion, translocation of NF- κ B, and upregulation of IL-1 β and IL-18 production [224]. Cvetkovic *et al.* [44, 150] show that paclitaxel is a potent activator of iNOS, the NO precursor. Released NO has antitumor effects [98, 99]. Paclitaxel has adverse immunologic effects including compromising T cell proliferation. Paclitaxel-induced T-cell suppression can be overcome with IL-12 [153]. It is precisely paclitaxel’s involvement with stimulation of TNF- α , NF- κ B, and NO that makes paclitaxel an exciting chemotherapeutic for our study. We believe that all these factors will be upregulated by paclitaxel, and we further hypothesize that paclitaxel will stimulate IL-18 production. This cytokine is structurally and functionally similar to IL-1 β and IL-12, both of which paclitaxel modulates [191]. If we can elucidate a role for

paclitaxel in overcoming putative tumor suppression of IL-18, we will have identified another critical use for the drug.

The cytokines IL-1 β , IL-12, TNF- α , and IL-18 are all immunotherapeutic agents which could ameliorate host immunity in tumor-burdened subjects. Because tumor cells secrete immunosuppressive molecules, such as TGF- β , PGE₂, IL-10, and others, a normal immune response is prevented, leading to a decrease in proinflammatory cytokine production [6, 152]. Treatment with paclitaxel can cause subsequent increase in the production of specific immunostimulatory cytokines, such as IL-1 β , IL-12, TNF- α , and IL-18, and can lead to increased production of many other tumoricidal molecules, such as NO [224]. An increase in the production of IL-1 β and IL-18 could lead to increased NF- κ B translocation and IFN- γ levels, which can help mount an efficient immune response against the tumor cells through induction of proinflammatory molecules like NO and TNF- α and other cancer-fighting molecules [134]. Mechanisms that decrease cytokine production by tumor cells are known and include production of immunosuppressive molecules like IL-10 [6, 113]; however, many questions still remain. For instance, how do these molecules directly affect the immune system? Do they target production of proinflammatory cytokines or do they block their action? Does upregulation of proinflammatory cytokines translate into increased antitumor response? Once these questions are answered, we will have a greater insight into mechanisms that can regulate cytokine levels to mount an effective immune response. This project will suggest ways to correct cytokine imbalances in immunosuppressed cancer patients and allow cytokine therapy to become a more realistic alternative.

SECTION II: Fibrosarcoma-induced Dysregulation of Interleukin (IL)-1 β and IL-18 Activities and their Modulation by Paclitaxel

ABSTRACT

Antitumor activities expressed by M ϕ populations such as tumor-antigen presentation, phagocytosis, and production of the proinflammatory cytokines Interleukin [IL]-1 β , IL-18, and TNF- α aid in host defense. The tumor-compromised immune system often cannot mount an immune response because immunosuppressive molecules, such as TGF- β , PGE₂ and IL-10, are produced by the tumor. Amelioration of proinflammatory cytokine production boosts the host immune system and enhances survival. In this study, we researched the proinflammatory cytokines IL-1 β , IL-18, and TNF- α . These cytokines were assessed because they are all initiators of NF- κ B translocation. Activation of NF- κ B leads to increased production of many other cancer fighting molecules, such as nitric oxide (NO) and TNF- α . Because tumor cells produce immunomodulatory factors, we predicted that tumor distal (TD) M ϕ s would produce different amounts of proinflammatory cytokines than tumor proximal (TP) M ϕ s. We found that IL-1 β and TNF- α production were enhanced in TD M ϕ s and diminished in TP M ϕ s. IL-18 was present in TD and TP M ϕ s, suggesting that this cytokine is functionally different from the structurally similar IL-1 β . To determine whether cytokine dysregulation translates into enhanced downstream production of NF- κ B transcription products, IL-12 and NO were assayed. NO production was enhanced distally, although IL-12 was not produced by any TBH M ϕ population. Amelioration of IL-1 β , TNF- α , and NO production in our model could convey enhanced immunity and survival. A potential vehicle for immunotherapeutic treatment is paclitaxel (registered trade name TAXOL[™]), a potent M ϕ activator that upregulates a variety of cytokines in a lipopolysaccharide (LPS)-like manner. We found that paclitaxel enhanced TD M ϕ production of IL-1 β , IL-18, and TNF- α in an LPS-like manner. Paclitaxel failed to modulate production of IL-1 β and TNF- α *in situ* and overcome its immunosuppressive

nature. Production of IL-1 β and TNF- α was reduced in TP M ϕ s when treated with paclitaxel; however, IL-18 production was unexpectedly enhanced *in situ*. To determine whether TD enhancement of these cytokines translates into enhanced downstream production of transcription products, IL-12 and NO were assayed. TD M ϕ s produced much NO when stimulated with LPS but paclitaxel treatment failed to enhance NO production. When treated with paclitaxel, IL-12 production occurred in normal host (NH) and TD M ϕ s. Differences in the activation pathways of LPS and paclitaxel could account for these differences. Collectively, these studies the nature of cytokine imbalances in our tumor model and suggest the role of paclitaxel in this process.

INTRODUCTION

Mφs are responsible for direct tumor cytotoxicity and act indirectly as antigen-presenting cells that stimulate not only antitumor lymphocyte activation and differentiation but also the production of proinflammatory cytokines such as IL-1β and IL-18 [52, 54]. IL-1β is a Mφ-derived cytokine that activates T cells, intracellularly regulates apoptosis, and induces iNOS, leading to production of NO [133, 143, 228]. Although IL-18 is related structurally to IL-1β, it is functionally similar to IL-12 in its ability to induce IFN-γ production from NK cells and T cells [106, 223]. Because our previous work revealed that tumor production of suppressor molecules such as TGF-β, IL-10 and PGE₂ [6] impeded host antitumor immunity through dysregulation of macrophage (Mφ) proinflammatory cytokine production, we focused on the quantification of tumor production of the cytokines.

We proposed that during tumor growth, Mφs located distally from the tumor have enhanced production of IL-1β and IL-18 likely due to the presence of T_H1 cells but at the tumor site, levels are significantly lower, due to both immunomodulatory factors present *in situ* and the possibility that these *in situ* Mφs are inappropriately polarized. Tumor-induced dysregulation of these cytokines *in situ* would functionally abrogate host immune responses to the tumor and promote its growth. We chose to analyze these cytokines via ELISA in supernatants taken from TD Mφs as well as *in situ* TP Mφs. Because the cytokines IL-1β and IL-18, along with TNF-α, initiate activation of the transcription factor NF-κB [97, 107, 187], and because activated NF-κB can transcribe genes such as the proinflammatory molecules IL-12, iNOS, and TNF-α, we measured the production of these substances. IL-12 was assessed for by western blot and TNF-α

by ELISA. Griess reagent was used to detect the presence of NO. This study reports that TD Mφs display enhanced NF-κB initiation molecules, NF-κB activation, and NO production, a product of NF-κB activation. Further, TP Mφs display enhanced IL-18 production, but fail to produce the studied products of NF-κB translocation (NO, IL-12).

The cell's microtubule system influences inflammatory responses; in particular it has been shown to increase TNF-α-induced endothelial cell permeability via p38 mitogen-activated protein kinase activation [171]. Because the anticancer drug paclitaxel irreversibly polymerizes microtubules, we determined whether paclitaxel would influence proinflammatory responses by altering Mφ production of IL-1β. Paclitaxel functionally mimics LPS by inducing the irreversible polymerization of α/β tubulin, which could induce or enhance Mφ IL-1β production [159]. Since IL-1β is an initiator of NF-κB translocation, paclitaxel could further influence this transduction cascade in Mφs located both proximally and distally to the tumor. Smith *et al.* [Σμτη, 1995 #5595] report paclitaxel enhances IL-1β production by human monocytes, and that paclitaxel alone enhances IL-1β expression/production in human monocytes. Further, White *et al.* [224] show that paclitaxel alone enhances steady-state levels of IL-1β mRNA in unprimed human monocytes and in breast cancer cell lines. In our study, we found the immunotherapeutic drug paclitaxel ameliorates IL-1β production in TD Mφs. We find that paclitaxel enhances TD Mφ production of the NF-κB initiator IL-18 as well as production of the NF-κB-derived product IL-12. Further, TP Mφ production of IL-18 was enhanced by paclitaxel. These results collectively demonstrate the ability of paclitaxel to combat tumor-derived immune dysfunction through enhancement of proinflammatory cytokines.

MATERIALS AND METHODS

Murine Tumor Line

Eight to 12 week-old BALB/c (H-2_d) tumor-bearing (TBH) male mice (Jackson Laboratories, Bar Harbor, ME) were used as the source of TBH Mφs. Meth-KDE, a nonmetastatic, methylcholanthrene-induced transplantable fibrosarcoma administered intramuscularly was used, as previously described [6, 7, 150, 157]. Tumors were induced in a population of age-matched animals. Palpable tumors form 10-14 days post-inoculum and death occurs at 28-35 days.. All animal protocols were consistent with accepted National Institute of Health guidelines for the care and use of laboratory rodents, and the Virginia Tech Institutional Animal Care and Use Committee approved all procedures.

Media and Reagents

Immune cells were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma Cell Culture, St. Louis, MO). All media contained 50 mg/L gentamicin sulfate (Sigma). LPS (*Escherichia coli* serotype 026:B6, 1 µg/mL) and recombinant murine IFN-γ (50 U/mL; endotoxin content <10 pg/ml) (generous gifts from Genentech, Inc., San Francisco, CA) were used. Paclitaxel (TAXOL™, 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 Collection and Culture

Normal host and TD Mφs from 21-day TBH mice were elicited by intraperitoneal injection of sterile thioglycollate and collected 4 days post induction. Purified Mφs were obtained by plating pooled 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. TP Mφs were collected by using the Easy-Sep™ positive selection system (Stem Cell Technologies, Vancouver, BC) (Appendix B). Briefly, tumors were dissociated on a screen and trypsinized. The resulting mixture was centrifuged on a ficoll gradient using lympholyte-M (Cedarlane Laboratories, Ontario, Canada) and the lymphocyte fraction was collected and incubated with phycoerythrin (PE)-tagged antibody (Ab) against Mφ marker CD11b. A PE-specific tetramer was added followed by a magnetic nanoparticle binding to the tetramer. The mixture was then placed in the Easy-Sep™ magnet and the resulting CD11b⁺ cells collected. The final Mφ preparations were ≥ 90% pure when analyzed using flow cytometry and cells were >95% viable via alamar blue assay. Either 5 x 10⁶ NH, TD, or TP Mφs were cultured in 24-well flat-bottom tissue culture plates containing a total volume of 1 mL serum-free RPMI-1640 medium with either IFN-γ and LPS, IFN-γ and various concentrations of paclitaxel, or paclitaxel alone as done previously (unpublished data). Supernatants were collected at the optimal time for each assay (18, 24, or 48 h) and either stored at -80°C until use or assayed immediately.

IL-1 β , IL-18, and TNF- α Quantification

Cells from 5 to 10 mice were collected for each experiment. Triplicate culture supernatants were tested for IL-1 β , IL-18, and TNF- α protein concentration using cytokine-specific ELISAs (InterTest™-1X, Genzyme Diagnostics, Cambridge, MA, IL-1 β Quantikine ELISA, IL-18 ELISA, R&D Systems, Minneapolis, MN, and eBiosciences, San Diego, CA) following the manufacturer's protocol.

Measurement of NO

NO was detected by combining 1 part M ϕ supernatant with 1 part Greiss reagent (Sigma) in a clean 96-well flat-bottom tissue-culture plate. Sodium nitrite (Sigma) was used as a standard. The standard was run on a separate plate, skipping every other well. Plates were incubated at room temperature for 10 minutes and read at 570 nm on a microplate absorbance reader. Freezing supernatants reduced nitrite potential, so samples were used directly after collection.

IL-12 Detection

IL-12 was detected by western blotting. Samples were analyzed by SDS-page using 1:1000 diluted biotinylated anti-IL-12 (p35) (eBiosciences) and 1:250 diluted Streptavidin-HRP (BD Pharmingen, Franklin Lakes, NJ). The Pierce SuperSignal® West Dura Extended Duration substrate was used and the sample was developed for 5 minutes.

Statistics and Calculations

Cells from 6 to 10 NH or 21-day TBH mice were pooled for each experiment. Triplicate cultures were tested using cytokine-specific ELISAs. All experiments were repeated at least once; data are means $\pm$ 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

TD Mφs Display Enhanced Production of IL-1β Regardless of LPS or Paclitaxel Stimulation

Because our previous work suggested that IL-1β production was dysregulated in TD Mφs (unpublished data), we tested for IL-1β, IL-18, and TNF-α production. These cytokines were assayed in supernatants taken from NH, TD, and TP Mφ cultures to determine whether tumor presence or Mφ location alters the type or amount of molecules produced. We considered that the *in situ* tumor environment would likely contain greater amounts of immunosuppressive molecules and more polarized Mφs than the tumor distal environment because of localized tumor immune evasion activities. Further, we predicted that TD Mφs located distally from the tumor would display enhanced production of these proinflammatory cytokines because of their anti-tumor roles. IL-1β production was assessed when NH, TP, or TD Mφs were cultured with either LPS (1 μg/mL) or paclitaxel (10 μM) alone or primed with IFN-γ (50 U/mL) and incubated for 48 h (optimal time). Supernatants were collected and assayed for IL-1β via ELISA.

Treatment with paclitaxel significantly enhanced production of IL-1β in NH and TD Mφs in an LPS-like manner, but not in untreated cells, suggesting that both LPS and paclitaxel stimulate proinflammatory cytokine in significant and similar ways, likely by similar pathways (Figure 3). Treatment with LPS or paclitaxel failed to significantly enhance TP Mφ production of IL-1β over the baseline untreated population production. Notably, IL-1β was not produced in TP Mφs to the level produced in NH or TD Mφs. In

the absence of IFN- γ priming, neither M ϕ population produced significant amounts of IL-1 β , regardless of LPS (data not shown) or paclitaxel (Figure 3) triggering.

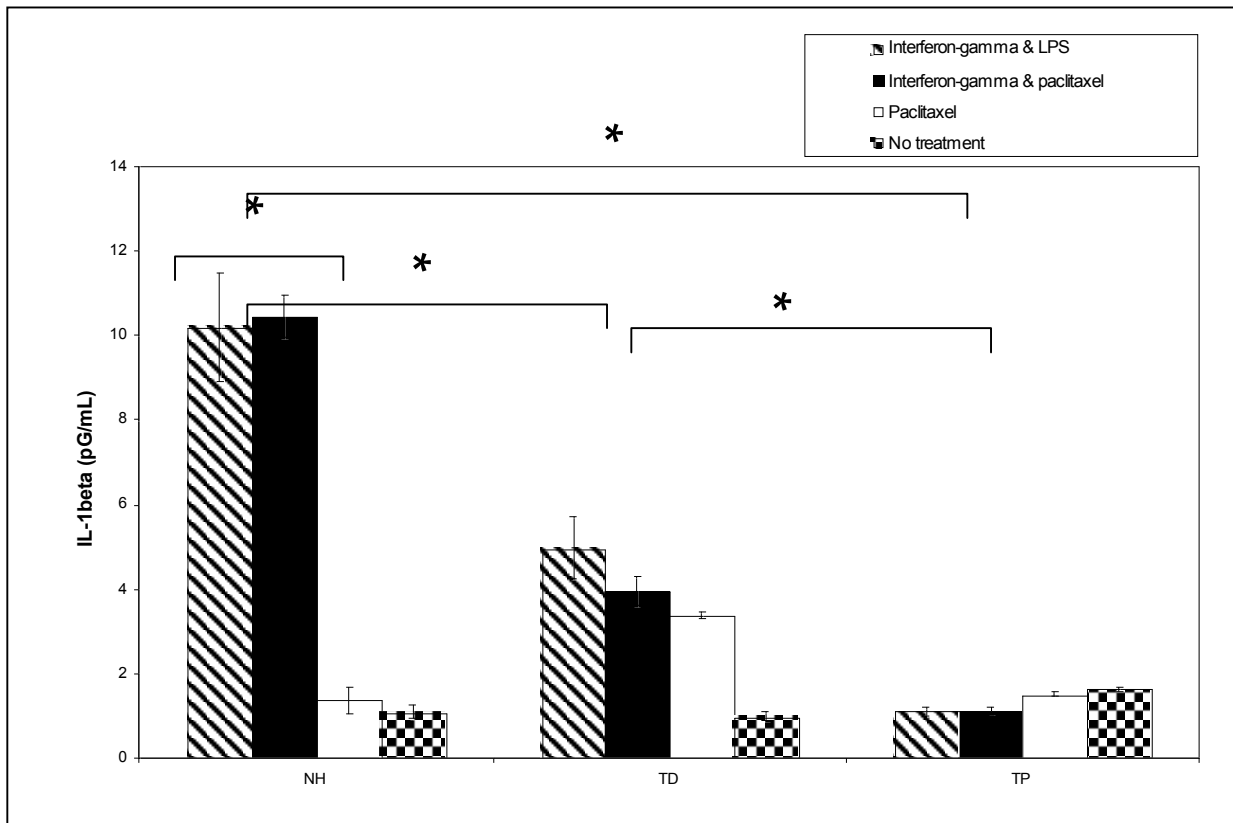


Figure 3: LPS and Paclitaxel Enhance Distal M ϕ Production of IL-1 β .

NH, TBH –distal (TD), or TBH –proximal (TP) M ϕ s (5×10^6) primed with IFN- γ (50 U/mL) and cultured with either LPS (1 μ g/mL) or paclitaxel (10 μ M or 1 μ M) for 48 h. Supernatants were collected and assayed for IL-1 β by ELISA. Data are averages $\pm$ SEM of triplicate independent determinations from one of three similar experiments. *, $p < 0.05$.

Paclitaxel Stimulates TBH M ϕ IL-18 Production.

Because IL-18 is a proinflammatory cytokine with anti-tumor effects and it is similar to IL-1 β in activation and structure, we chose to analyze IL-18. The similarities between IL-1 β and IL-18 prompted us to hypothesize that IL-18 production would be enhanced distally and diminished proximally. NH, TP, and TD M ϕ s were cultured for 18 hours as described. Supernatants were collected and assayed for IL-18 by ELISA. Treatment with LPS stimulated production of IL-18 in NH and TD M ϕ s (Figure 4). This effect was diminished significantly in TP M ϕ s, which produced slightly less IL-18 than their NH and TD counterparts, suggesting an antagonist tumor microenvironment. Paclitaxel treatment significantly enhanced production of IL-18 in NH M ϕ s, whereas TD M ϕ s produced less. Enhanced TP M ϕ production of IL-18 in paclitaxel-treated versus LPS-treated cells suggests that paclitaxel was able to overcome, to a degree, the immunomodulatory factors present *in situ*. The results for this assay were dissimilar to those obtained in Figure 3. Differences in the functions of these cytokines could account for this dissimilarity as IL-18 resembles IL-12 in its activity rather than IL-1 β .

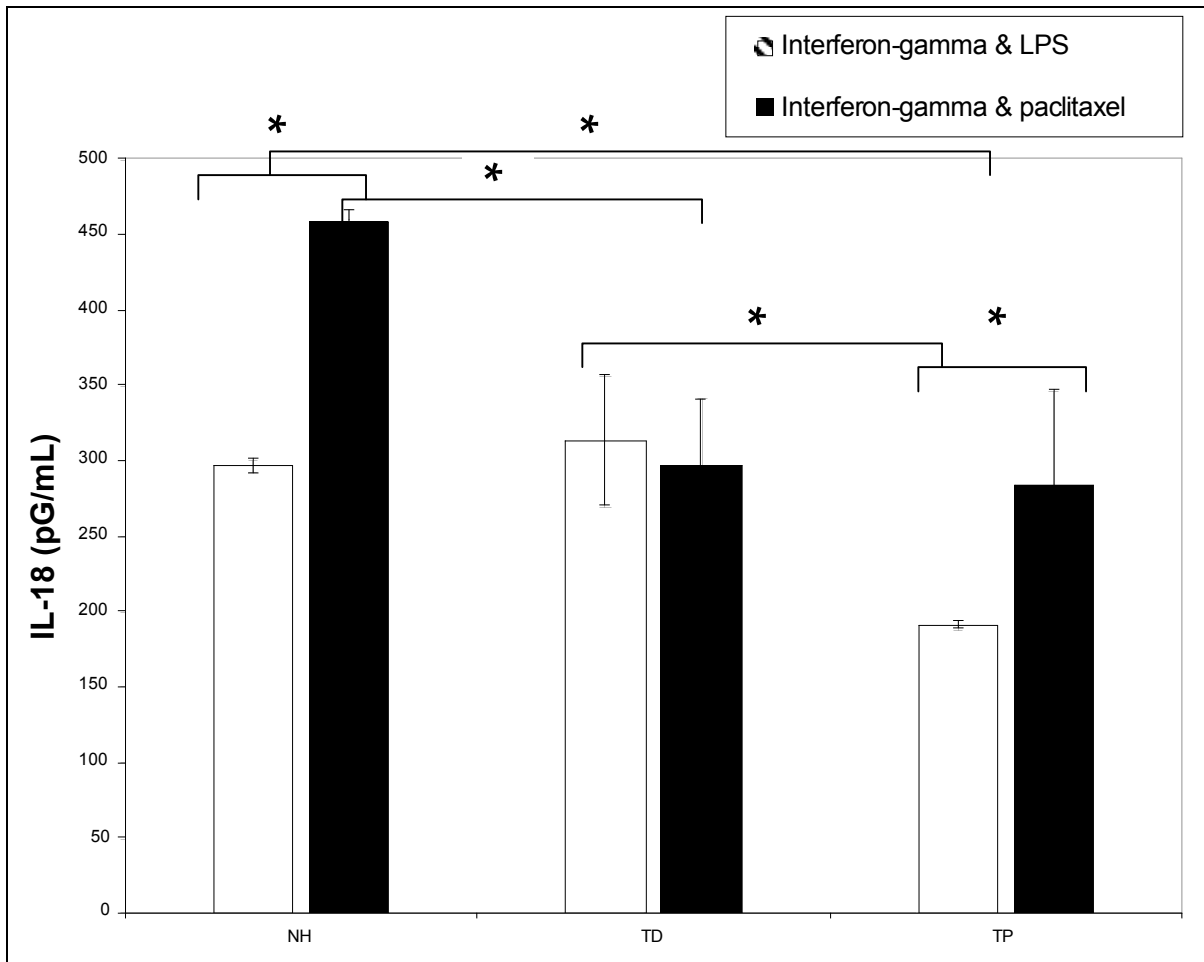


Figure 4: Paclitaxel Stimulates Tumor-Derived M ϕ IL-18 Production. NH, TBH – distal (TD), or TBH–proximal (TP) M ϕ s (5×10^6) primed with IFN- γ (50 U/mL) and cultured with either LPS (1 μ g/mL) or Paclitaxel (10 μ M) for 18 h. Supernatants were collected and assayed for IL-18 via ELISA. Data are averages and SEM of triplicate independent determinations from one experiment. *, $p < 0.05$.

TNF- α Production is Enhanced in TD M ϕ s.

Because TNF- α is a proinflammatory cytokine that we show to be dysregulated in TD M ϕ s [6], we investigated the role of this cytokine in our tumor model. NH, TP, and TD M ϕ s were cultured for 48 hours as described. Supernatants were collected and assayed for TNF- α by ELISA. Treatment with IFN- γ and LPS caused significant enhancement of TNF- α production in both NH and TD M ϕ s compared to the untreated controls (Figure 5). A similar significant but diminished effect was seen in NH and TD M ϕ s treated with IFN- γ and paclitaxel. TP M ϕ s, however, displayed no significant difference in TNF- α production when either untreated or treated with IFN- γ and either LPS or paclitaxel. The dysregulation pattern of TNF- α , then, is similar to that of IL-1 β as it is enhanced in TD M ϕ s and diminished in TP M ϕ s.

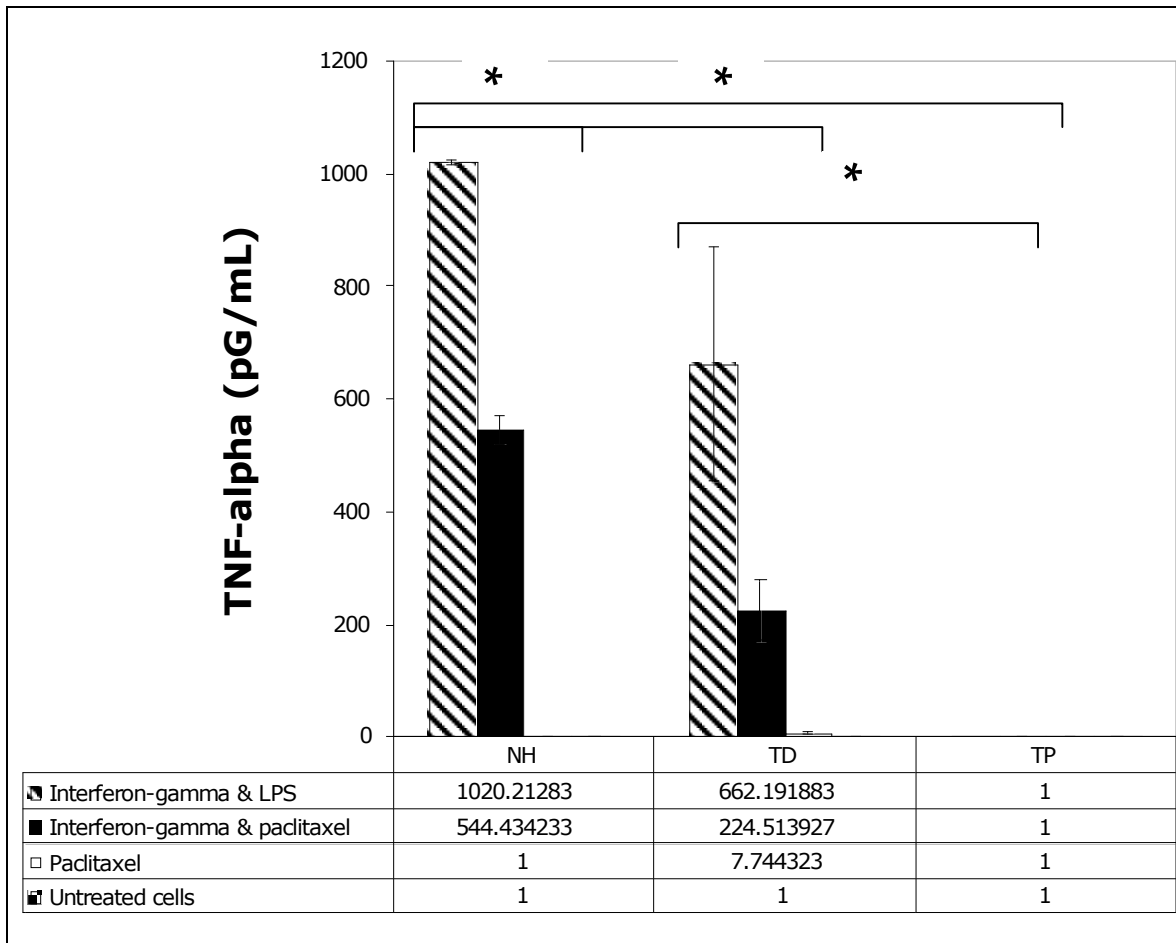


Figure 5: *TNF- α* Production is Enhanced in TD M ϕ s. To determine whether enhanced tumor M ϕ production of IL-1 β and IL-18 would lead to downstream enhancement of TNF- α , NH, TBH-proximal (TP), or TBH-distal (TD) M ϕ s (5×10^6) primed with IFN- γ (50 U/mL) and cultured with either LPS (1 μ g/mL) or Paclitaxel (10 μ M) for 48 h (optimal time). Supernatants were collected and assayed for TNF- α via ELISA. Data are averages $\pm$ SEM of triplicate independent determinations from one of three similar experiments. *, $p < 0.05$.

LPS treatment Leads to Downstream TD M ϕ Production of TNF- α and NO While Paclitaxel Treatment Leads to TD M ϕ TNF- α and IL-12 Production.

M ϕ s were incubated as described earlier with IFN- γ and either LPS or paclitaxel and assayed for the presence of NO, and IL-12, possible endproducts of IL-1 β , IL-18, and/or TNF- α -induced NF- κ B translocation. NH, TP, and TD M ϕ s were cultured as previously described. Aliquots of supernatant were removed at 12 and 18 hours for immediate testing of NO production by Griess reagent. Twelve, 18, and 24 hour samples were frozen at -80 °C and later tested for the presence of IL-12 via western blot. Forty-eight hour samples were collected and assayed for TNF- α by ELISA as per the manufacturer's recommendations. Baseline NO production was seen at 12 hour incubation across all samples (unpublished data); however at 18 hours incubation great differences ($p < 0.05$) were seen among the samples (Figure 6), suggesting that NO production was not catalyzed until 18 hours, possibly because NF- κ B activation was required to produce iNOS, which eventually would induce NO production. When treated with LPS, TD M ϕ s displayed significantly ($p < 0.05$) more NO production than either NH or TP M ϕ s. When treated with paclitaxel, NH, TD, and TP M ϕ s displayed significant NO production similar to that of untreated cells, suggesting that paclitaxel failed to enhance NO production (Figure 6).

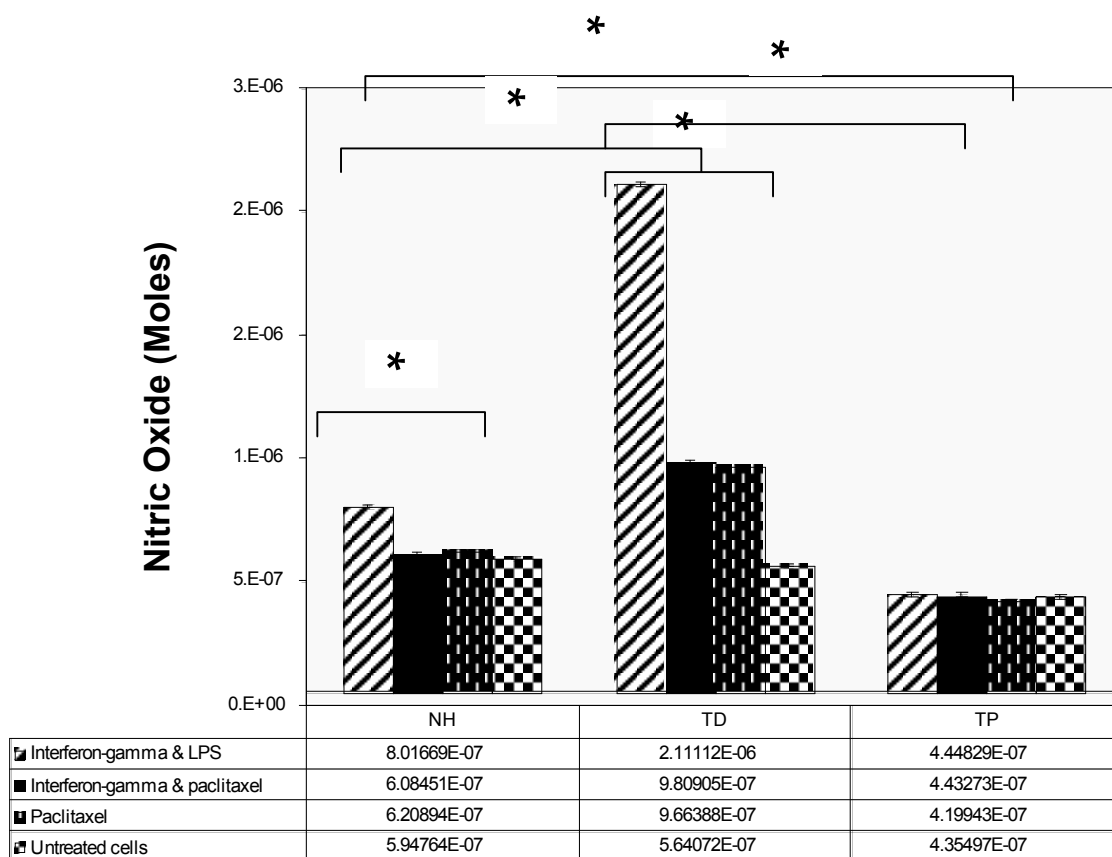


Figure 6: Paclitaxel Does Not Mediate Regulation of Mφ Cytokine Production Through NO. NO production was assayed in NH, TP, or TD Mφs (5×10^6) primed with IFN- γ (50 U/mL) and cultured with either LPS (1 μ g/mL) or paclitaxel (10 μ M) for 18 h (optimal time). Supernatants were collected and assayed for NO using Greiss reagent. Data are averages $\pm$ SEM of triplicate independent determinations from one of three similar experiments. *, $p < 0.05$.

IL-12 was not produced in any M ϕ population incubated with either LPS or paclitaxel for 12 or 18 hours, likely because these were not optimal timepoints (data not shown). At 24 hours, IL-12 was not expressed in any LPS-treated populations, perhaps because LPS treatment does not lead to production of this cytokine (Figure 7) or because levels of this cytokine were produced below the detection-limit of the assay. Because the western blot is a qualitative assay, quantitative analysis such as an ELISA procedure could be performed to detect any IL-12 production by M ϕ s stimulated with paclitaxel. Paclitaxel treatment for 24 hours of NH and TD M ϕ s did show enhanced IL-12 production; however, no IL-12 was detected in TP M ϕ s and only baseline production of NO was seen in this population (Figure 7).

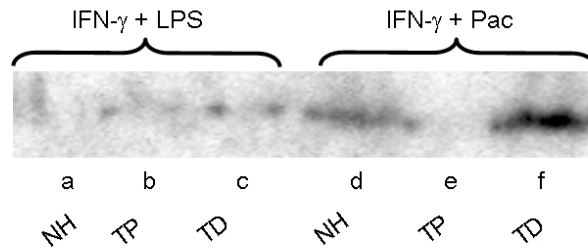


Figure 7: Paclitaxel Enhances IL-12 Production in NH and TD Mφs. IL-12 production was assayed in NH, TP, or TD Mφs (5×10^6) primed with IFN- γ (50 U/mL) and cultured with either LPS (1 μ g/mL) or paclitaxel (10 μ M) for 24 h (optimal time). Western blot was performed on Mφ supernatants using 1:1000 dilution of anti-IL-12 p35. Equal protein concentrations were applied to each lane. Molecular weight standards were run (not shown).

DISCUSSION

This study demonstrates how the Meth-KDE fibrosarcoma evades the tumor-compromised host immune system through dysregulation of the proinflammatory cytokines IL-1 β , TNF- α , and IL-12 produced by both TD and TP M ϕ s. Normal host M ϕ populations have various tumoricidal activities including tumor antigen phagocytosis and presentation and cytokine production [151]. The proinflammatory cytokines such as IL-1 β , IL-18, and TNF- α , by binding to their receptors, are important to antitumor host defenses because they initiate activation of the transcription factor NF- κ B [95, 97, 107, 187]. This initiates a cascade involving many proteins ultimately releasing NF- κ B to start transcription of the intended target gene, which leads to production of the proinflammatory molecules IL-12, iNOS, IL-6, TNF- α , and IL-1 [25, 26, 43].

We first determined the levels of the proinflammatory cytokines IL-1 β , IL-18 and TNF- α in NH M ϕ s. We found that when stimulated with IFN- γ and LPS, NH M ϕ s produced significantly elevated amounts of IL-1 β and TNF- α over untreated cell populations (Figures 3 and 5). IL-18 production by NH M ϕ s was noted although comparison with untreated controls was not possible (Figure 4). Because these cytokines can initiate NF- κ B and ultimately lead to production of other proinflammatory molecules such as IL-12 and NO, we tested for the production of these molecules by NH M ϕ s. We found NO to be slightly enhanced over untreated controls whereas IL-12 was not enhanced (Figures 6 and 7). This suggests that NH M ϕ s produce only baseline amounts of NO when stimulated with LPS, and involvement of NF- κ B in stimulating NO

is probably minimal. LPS stimulation failed to activate the pathway necessary to produce IL-12.

Is cytokine dysregulated in our TBH Mφs? We predicted that Mφs located in the immunosuppressive tumor microenvironment would produce different cytokines than Mφs isolated from the TD environment. We proposed that during tumor growth, Mφs located distally from the tumor have enhanced cytokine production because these Mφs are activated by the tumor. However, at the tumor site, these levels would be inhibited because of immunosuppressive molecules like IL-10, TGF-β, or PGE₂ [155]. These predictions were true for IL-1β, TNF-α, NO, and IL-12, which were inhibited in TP Mφs when stimulated with IFN-γ and LPS (Figures 3, 5, 6, and 7); however, similarly stimulated TP Mφs produced significantly enhanced amounts of IL-18 in comparison with NH Mφs (Figure 4). Work by Shen *et al.* [188] reveals that IL-1β and TNF-α block growth of breast cancer cells by inhibiting growth factors. It is likely that growth factors are elevated in the TD environment of our tumor model and that these factors aid in host immunity. Induction of the NF-κB signal cascade is unlikely to occur in TP Mφs through IL-1β as production of this cytokine is significantly ($p < 0.05$) reduced *in situ* although it could occur through IL-18 production. Interestingly, Lee *et al.* [110] report that whereas IL-1β induced the expression of a NF-κB reporter gene by degrading its inhibitor and was suppressed by competitive inhibition of NF-κB binding, IL-18 responses were weak or absent. IL-18 failed to degrade the inhibitor bound to NF-κB. As mentioned, differences in IL-1β and IL-18 function could have been responsible for these results. It is also possible that IL-18 was not affected by the immunomodulatory molecules produced by the tumor, which would account for its production by TP Mφs (Figure 4). The presence of IL-18 in the TP environment suggests that it is produced by Mφs

associated with the Meth-KDE tumor and could cause NF- κ B activation and subsequently lead to production of an immunosuppressive subset of cytokines, adhesion molecules, and chemokines. It is also possible that IL-18 is not compromised during tumor growth. These results suggest that the immunomodulatory molecules such as IL-10, TGF- β , or PGE₂ may not affect IL-18 [112], at least at the levels that they are present in the tumor. Future studies involving treatment of TP M ϕ s with these molecules will elucidate whether IL-18 production is ever affected.

The TD environment promoted proinflammatory responses. The cytokines IL-1 β and TNF- α and NO were enhanced over untreated controls in TD M ϕ s when stimulated with IFN- γ and LPS. TD M ϕ production of NO was significantly ($p < 0.05$) greater than NH M ϕ production, indicating that this effector molecule is likely used in host antitumor responses or possibly not affected by immunomodulatory molecules produced by the tumor circulating in the TD compartment. When treated with LPS, the cytokines IL-1 β and TNF- α , while produced in greater amounts than untreated cells, were produced in amounts lower than NH M ϕ s similarly treated. This suggests that these cytokines were either restricted by immunosuppressive molecules produced by the tumor or that they were unnecessary in host immune activity. TD M ϕ s produced approximately the same amount of IL-18 as NH M ϕ s, suggesting that host defenses do not recruit this cytokine more than baseline NH production. IL-18 likely does not have a great antitumor role in our cancer model or is possibly produced by the tumor. LI *et al.* [112] report that IL-18 is expressed in the tumor microenvironment in patients with non-small cell lung cancer although not to the level of expression of the immunosuppressive molecules present. Further, Zheng *et al.* [232] reveal that IL-18 is expressed in the cytoplasm of leukemia cells. LPS failed to stimulate IL-12 production in TD M ϕ s which agrees with our work

[152], suggesting that LPS does not activate this cytokine *in situ*. Work by Butcher *et al.* [38] shows that LPS triggering fails to activate IL-12 in murine Mφs when infected by *Toxoplasma gondii*. Suppression of Mφ proinflammatory cytokine production is apparently notable in both TBH environments as well as those dealing with *Toxoplasma gondii* infection.

Because tumor growth alters Mφ responsiveness to paclitaxel [150, 152, 153], we investigated the role of paclitaxel-induced cytokine production by Mφs in our tumor model. Our previous work demonstrated the ability of paclitaxel to enhance IL-12 production through production of NO [152], molecules which are dysregulated in the TP microenvironment (see Figures 6 and 7). Restoration of production of not only IL-12 and NO but the proinflammatory cytokines IL-1β and TNF-α by Mφs could re-establish antitumor immune responses and promote host immunity. Therefore, the role of paclitaxel treatment after IFN-γ priming was determined.

Paclitaxel acts in an LPS-like manner by stimulating NH Mφ production of IL-1β. TNF-α production in NH Mφs treated with paclitaxel was significantly ($p < 0.05$) elevated but not to the degree of LPS stimulation. This could have occurred because LPS and paclitaxel act on different cell receptors, thus leading to differential transcription of downstream NF-κB target genes [20, 176]. LPS and paclitaxel share the common TLR4-CD14 receptor and binding by either to this receptor leads to transcription of certain target genes. LPS, however, can also bind to the CD11/18-CXCR4-CDF5 receptor, which can activate a different internal pathway [168]. The difference in the extracellular binding to these receptors could have led to the selection of different intracellular cascade events over others, culminating in different products, accounting for

the difference in how LPS and paclitaxel influence cytokine production. Differential action of LPS and paclitaxel was observed in NH M ϕ s for production of the molecules IL-18, IL-12, and NO. Paclitaxel enhanced production of IL-18 and IL-12 in a manner greater than LPS but failed to upregulate NO in NH M ϕ s. IL-18 and IL-12 share similar functions [82, 209, 213], and it is expected that they might be similarly stimulated by paclitaxel treatment. The effect of paclitaxel on IL-1 β and IL-18 was dissimilar despite the shared structure and activation pathway because these cytokines are very different in function. The failure of paclitaxel to upregulate NO suggests that other cells or molecules are necessary for its production.

Paclitaxel failed to ameliorate production of IL-1 β , TNF- α , NO, and IL-12 in TP M ϕ s likely because immunomodulatory molecules such as IL-10, TGF- β , or PGE₂ inhibited production of these cytokines. In TP M ϕ s, paclitaxel significantly ($p < 0.05$) enhanced IL-18 production in an LPS-like manner but not to the level that NH M ϕ s were stimulated. Since both paclitaxel and LPS destabilize β tubulin [63], they can have similar functions. As was suggested earlier, paclitaxel and LPS-induced IL-18 production by TP M ϕ s are likely not affected by immunosuppressive factors in the tumor environment. However, the total lack of production of IL-12, NO, and TNF- α by TP M ϕ s suggests that these molecules are affected by these factors. The mechanism needed for the production of IL-12, NO, and TNF- α may be blocked by TP M ϕ production of molecules like IL-10, TGF- β , or PGE₂ which may either block or alternatively activate the NF- κ B signal transduction cascade, a known transcription factor for the proinflammatory cytokines. The failure of paclitaxel to ameliorate conditions in TP M ϕ s suggest that it is alone a poor modulator of immunosuppressive conditions in our tumor model alone and

likely requires combined therapy with other molecules such as IL-12 to stimulate antitumor responses *in situ*.

Our previous work suggested that paclitaxel would induce production of IL-1 β in TD M ϕ s. Indeed, paclitaxel treatment induced TD M ϕ s to produce significantly ($p < 0.05$) elevated levels of the cytokines IL-1 β and TNF- α over their untreated counterparts. TD M ϕ production of IL-1 β occurred regardless of IFN- γ priming, indicating that paclitaxel was not only enhancing but inducing production of this cytokine. Paclitaxel-induced production of IL-18 (as with IL-1 β) occurred in an LPS-like fashion; however, paclitaxel-induced M ϕ production of TNF- α was lower than that stimulated by LPS. This again suggests the different roles and receptors of these molecules within the immune system. Paclitaxel slightly but significantly ($p < 0.05$) upregulated production of NO and IL-12 in TD M ϕ s, possibly occurring through the NF- κ B signal transduction cascade, a known transcription factor for IL-12 and NO. These data collectively reveal the nature of cytokine dysfunction in our tumor model and suggest the efficacy of paclitaxel in ameliorating dysregulation of the proinflammatory cytokines in the TD compartments. Proinflammatory cytokines were not upregulated in TP compartments because antagonistic molecules were present. Further research is needed to find therapies able to combat the immunosuppressive nature of our *in situ* tumor environment.

CONCLUSION

This study reinforces and extends our understanding of how the Meth-KDE fibrosarcoma evades antitumor immunity and causes host death by dysregulating production of proinflammatory cytokines. We demonstrated that TD M ϕ s produce increased amounts of IL-1 β , IL-18, TNF- α , and the cytotoxic effector, NO (see Figures 3, 4, 5, and 6); however, these proinflammatory molecules are not causing tumor regression. TP M ϕ s produced reduced amounts of IL-1 β , TNF- α , IL-12, and NO (see Figures 3, 5, 6, and 7). This environment is clearly immunosuppressive and promotes immune escape.

Current literature suggests that the cytokines IL-1 β and TNF- α are dysregulated in several different types of cancer [61, 108, 112, 114, 117, 157, 158, 160, 219]. The role of IL-1 β as a proinflammatory cytokine is well-established: it is an activator of NF- κ B [50, 52] and a transcription factor for the target genes include iNOS, IL-12, TNF- α , and IL-6 [233]. IL-1 β induces iNOS, mediating production of NO, a key tumoricidal molecule [133]. IL-1 β impairs the growth of breast cancer cells by reducing the ability of insulin-like growth factor-I (IGF-I) to promote cancer cell DNA synthesis [188]. Recent studies have demonstrated that IL-1 β gene polymorphism promote cancer progression. Garza-Gonzalez, Bosques-Padilla, El-Omar, Hold, Tijerina-Menchaca, Maldonado-Garza, and Perez-Perez [67] found that the presence of polymorphic IL-1 β -31 increased the risk of distal gastric cancer. Similar findings by Chang, Jang, Kim, Lee, Lee, Jung, Dong, Kim, Kim, Lee, and Chang [40] point to IL-1 β polymorphisms as promoting tumor growth. The antitumor role of the cytokine TNF- α is well-established:

TNF- α not only induces tumor cytostasis both *in vitro* and *in vivo* but also causes production of IL-1, another proinflammatory cytokine [2]. Cessation of TNF- α activity can promote tumorigenicity and genetic polymorphisms in TNF- α can enable tumor pathogenesis [117]. Our studies found that both of these cytokines were enhanced in TD M ϕ s (5 pG/ mL of IL-1 β and 600 pG/mL of TNF- α) and diminished in TP M ϕ s (1 pG/mL of IL-1 β and 0 pg/mL of TNF- α) (see Figures 3 and 5), a clear pattern suggesting the immunosuppressive nature of the *in situ* tumor environment. It is possible that in this environment, alterations to the genetic makeup of these cytokines aid in enhancing tumorigenicity. Because IL-1 β requires processing by caspase-1 to become active [174], this step could be altered by tumors to promote their survival. Wang, Gaggero, Rubartelli, Rosso, Miotti, Mezzanzanica, Canevari, and Ferrini [220] found this step to be altered in ovarian cancer cell lines causing reduced production of IL-18. As IL-18 and IL-1 β share this processing step [52, 53, 220], it is likely that it is altered in our tumor model. Inhibition of caspase-1 production would prevent release of mature active IL-1 β and could account for the lack of its presence in TP M ϕ s.

Because IL-18 is structurally similar to IL-1 β and they both require caspase-1 processing to become active [52, 53, 220], we initially hypothesized that the pattern of IL-18 production would be similar to that of IL-1 β (see Figure 3). This proved to be incorrect (see Figure 4) as IL-18 was enhanced in both TD and TP M ϕ s. It is unlikely that defective caspase-1 processing of IL-18 occurs in our tumor model because comparable amounts of the cytokine were produced in both TD and TP M ϕ s. This suggests that IL-18 was either not affected by immunosuppressive molecules *in situ* or that the tumor itself produced IL-18. Binay, Thiounn, De Pinieux, Viellefond, Debre, Bonnefoy, Fridman, and Pages [22] demonstrate that human prostate cancer cells

produce IL-18 in response to interferons, and IL-18 production aided in the clinical outcome of the patient. These prostate tumor cells secreted IL-18 in response to IFN- γ in the tumor microenvironment, and IL-18 acted as a autocrine/paracrine factor for the tumor. More experiments are needed to determine if this is occurring in our tumor model. Alternatively, IL-18 produced by the tumor could aid in tumorigenicity by producing immunosuppressive molecules. IL-18 stimulates NF- κ B translocation [110, 132, 136, 223], a pathway that activates both T_H1 and T_H2 responses [1, 41, 96, 115, 135, 145, 193]. One target gene of NF- κ B activation is COX2. COX2 is often overexpressed in cancer and can reduce DC and T-cell functions [172]. It also induces PGE₂ secretion, an immunosuppressive molecule produced by M ϕ s that can downregulate immune responses [227]. It is possible that IL-18 is stimulating NF- κ B to induce production of COX2, which is inducing production of immunomodulatory molecules like PGE₂. These molecules could be measured using ELISAs. Investigations into the nature of the immunosuppressive *in situ* environment will produce a finer understanding of this connection in our tumor model.

Because previous work suggested the ability of paclitaxel to ameliorate IL-12 production in our tumor model [152], we chose to further investigate this chemotherapeutic drug. The drug paclitaxel is a known activator of M ϕ responses, including TNF- α and NO secretion [152, 153]. We found that paclitaxel enhances TD M ϕ production of IL-1 β , TNF- α , NO, and IL-12 (see Figures 3, 5, 6, and 7). The ability of paclitaxel to enhance NO in TD peritoneal M ϕ s supports our earlier work [152]. We had not previously characterized the activity of paclitaxel in our *in situ* TP M ϕ s, and this was next endeavored. Paclitaxel failed to ameliorate production of these cytokines in TP M ϕ s, suggesting that the drug is unable to overcome immunosuppressive factors present *in*

situ. Paclitaxel itself can cause immune suppression by downregulating T-cells, which could also hinder host immunity. Using paclitaxel in combination with other therapies may overcome suppression of immunity; however, there is no perfect immune therapy to overcome cancer. McLaughlin, Jaglowski, Verderame, Stack, Leure-Dupree, and Zagon [137] found that using paclitaxel in conjunction with opiod growth factor reduced growth of human squamous cell carcinoma of the head and neck between 48% and 69% in 48 hours. Using chemotherapeutics in combination with antioxidants, in particular selenium, vitamins C and E, and beta carotene, is effective in reducing tumor growth [120, 175, 211]. As mentioned, the proinflammatory cytokine IL-12 possesses antitumor effects. Work by Melero, Mazzolini, Narvaiza, Qian, Chen, and Prieto [139] suggests the efficacy of combination treatments with IL-12 gene therapy. Although not discussed here, it is possible that combining IL-12 gene therapy with paclitaxel treatment could boost antitumor host immunity to levels higher than when using those therapies alone.

In summary, this study demonstrates, in part, how the Meth-KDE fibrosarcoma evades the tumor-compromised host immune system through downregulation of the proinflammatory cytokines IL-1 β , TNF- α , and IL-12 produced by TP M ϕ s. We predict that this occurs due to immunosuppressive molecules present *in situ*. Because IL-18 is structurally similar to IL-1 β and because they both require caspase-1 processing to become active, we tested TP M ϕ s for IL-18 and found it to be produced in levels similar to those from NH and TD M ϕ s. TD M ϕ s produced elevated levels of IL-1 β , TNF- α , NO and IL-12 because these molecules are all products of activated M ϕ s. Paclitaxel enhanced TD M ϕ production of IL-1 β , TNF- α , NO, and IL-12 but failed to upregulate production of these cytokines in TP M ϕ s. IL-18 was produced regardless of LPS or paclitaxel stimulus. These experiments reveal the nature of cytokine dysfunction in our

tumor model and suggest the efficacy of paclitaxel in ameliorating dysregulation of proinflammatory cytokines in the TD compartments; however, these proinflammatory cytokines are not reaching the tumor site and are therefore ineffective. The inability of paclitaxel to ameliorate production of proinflammatory cytokines in the tumor environment suggests that this chemotherapeutic drug cannot overcome immunosuppressive factors present. Future work is needed to reveal therapies which can ameliorate host immunity.

MODEL OF TUMOR DYSREGULATION

Immunity defense mechanisms are activated by tumor growth; however, tumors circumvent these defenses by producing immunomodulatory molecules and by dysregulating or inhibiting production of certain immunostimulatory cytokines by tumor-infiltrating Mφs [64]. The tumor-compromised immune system is unable to recognize or destroy aberrant cells. This lack of recognition and destruction is partly due to alterations in Mφs. The hallmark of Mφ activity is its ability to engulf foreign pathogens and they are involved in both innate and adaptive immunity [147]. Mφs also aid in degradation of apoptotic cells and microbes, secretion of cytokines and other mediators, and the presentation of foreign peptide to T lymphocytes. Tumor cells thrive when the immune system fails to recognize them as foreign, allowing uninhibited growth. This is due, in part, to the tumor cells' ability to suppress immunity by secreting molecules such as TGF-β₁, IL-10, PGE₂ [6, 59, 113], and others (unpublished). This secretion can be achieved through polarized M2 Mφs produced by the tumor [125-128]. These inhibitory molecules can downregulate cytokine production by tumor-infiltrating Mφs, which, in turn, reduces lymphocyte reactivity against the tumor [8]. For this study we chose to analyze TD and TP Mφs. TD Mφs are those found in the host but not inside the tumor. TP Mφs are those from inside the tumor. We hypothesized that these two types of Mφs would produce unique subsets of cytokines because of their locations.

Cytokines affected by TGF-β₁, IL-10, and PGE₂ include IL-1β and TNF-α, which are downregulated *in situ*. Dysregulation of these proinflammatory cytokines leads to further dysregulation of the transcription factor NF-κB, which is unable to translocate to the nucleus. This leads to further dysregulation of proinflammatory responses through

the downregulation of IL-12 and the cytotoxic effector molecule NO. The cytokine IL-18 was not downregulated *in situ* and thus could initiate NF- κ B translocation. It is possible that IL-18 is leading to transcription and translation of COX2 (a product of NF- κ B translocation), which is inducing production of immunomodulatory molecules like PGE₂ and further leading to immunosuppression of host immunity.

M ϕ s from the TD environment, however, showed an opposite trend in regards to the production of immunosuppressive molecules. TD M ϕ s were involved in proinflammatory responses. We found the cytokines IL-1 β and TNF- α and the chemokine NO to be significantly enhanced over untreated controls in TD M ϕ s when stimulated with IFN- γ and LPS. TD M ϕ production of NO was significantly greater ($p < 0.05$) than NH M ϕ production, suggesting that this chemokine is likely employed in host antitumor responses or possibly not affected by immunomodulatory molecules produced by the tumor circulating in the TD compartment. Enhanced levels of these cytokines could suggest that NF- κ B translocation is also unregulated, which would translate to further production of proinflammatory cytokines.

Activation of M ϕ s by stimulants such as paclitaxel can upregulate the production of antitumor cytokines, such as IL-1 β , and IL-12 [152, 155, 224], which can lead to a reversal of tumor growth through the induction of antitumor effector molecules, such as IFN- γ and NO [50, 152, 155, 166]. We found paclitaxel to be effective in TD M ϕ s in enhancing production of the cytokines IL-1 β , IL-12 and TNF- α as well as the cytotoxic effector molecule NO. Paclitaxel failed to stimulate these molecules in TP M ϕ s likely because its NF- κ B translocation pathway was blocked; however, IL-18 was produced in TD and TP M ϕ s amounts equivocal to that stimulated by LPS production. These results

suggest that paclitaxel fails to activate pathways involved in promotion of proinflammatory cytokines *in situ* likely because the chemotherapeutic cannot overcome the immunosuppressive molecules present. In TD Mφs, paclitaxel acted in an LPS-like manner to modulate the proinflammatory cytokines as described in Figure 8.

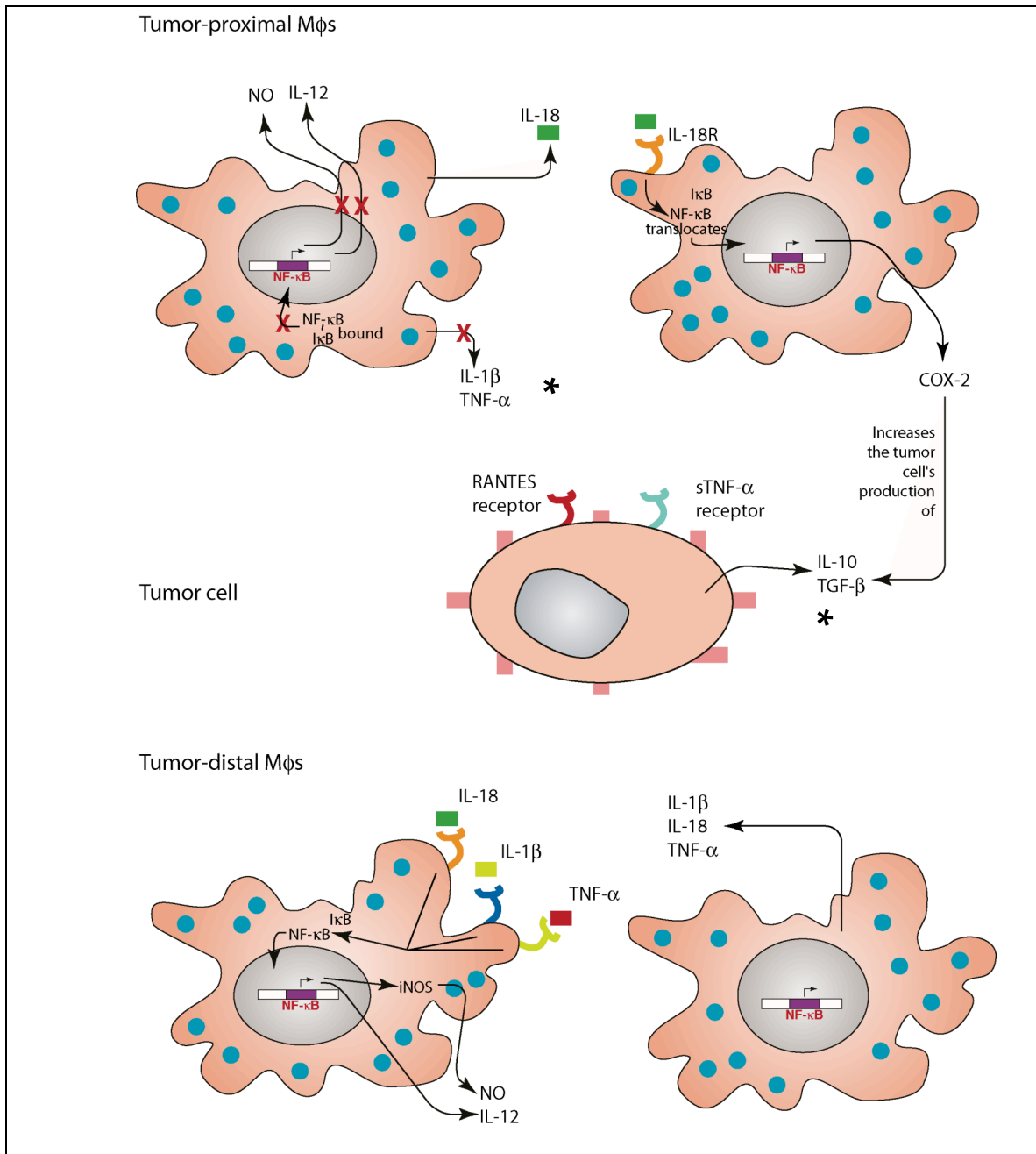


Figure 8: Tumor-Induced Dysregulation Model TP Mφs produce IL-18 which can bind to its receptor and induce translocation of NF-κB. Translation of proteins like COX2 can then occur and increase tumor cell production of IL-18 and TGF-β. TP Mφs failed to produce IL-12 or NO, indicating that NF-κB translocation is not occurring to create these molecules. Our tumor cells express RANTES and sTNFR1. TP Mφs produced IL-1β, IL-18, and TNF-α. These molecules can then bind to their receptors and initiate NF-κB translocation. Production of iNOS can then induce NO production and IL-12 can also be secreted. *Indicates work done previously in our laboratory. x indicates the action is blocked.

FUTURE INVESTIGATIONS

My studies show how the Meth-KDE fibrosarcoma dysregulates the proinflammatory cytokines IL-1 β , IL-18, and TNF- α and illustrates the role paclitaxel plays in modulating this dysregulation. Further investigations as to the nature of tumor dysfunction are:

- **How does our tumor influence M ϕ caspase-1 processing of IL-1 β and IL-18?** An alternative hypothesis for the dysregulation in IL-1 β and IL-18 production is through alterations in their activation pathways. Both IL-1 β and IL-18 require cleavage by caspase-1 to become active (see Figure 1); without this trimming event, the signal peptide sequences of both IL-18 and IL-1 β are incapable of biologic activity [21, 74]. If there is tumor-induced dysregulation of caspase-1 production, the activation of both IL-1 β and IL-18 would be decreased. Wang *et al.* [220] used RT-PCR and immunohistochemistry to show that IL-18 is dysregulated in human ovarian carcinoma lines because of defective processing events involving caspase-1. Similar protocols could be carried out to elucidate the role of caspase-1 processing on both IL-1 β and IL-18 in our tumor model.
- **What are the M ϕ mRNA levels for IL-1 β , IL-18, IL-12, and TNF- α ?** The investigation measured protein levels of the proinflammatory cytokines IL-1 β , IL-18, IL-12, and TNF- α . Studies identifying the levels of mRNA for these cytokines would suggest whether transcription or translation was affected by our fibrosarcoma. This would further elucidate how our tumor acts to counter antitumor defenses.

- **How do IL-12 protein levels compare with IL-18 protein levels?** In these investigations we used the western blot technique to show IL-12 is dysregulated. This method provides qualitative analysis that dysregulation is occurring but does not offer any quantitative data. An ELISA would provide quantitative data and further reveal similarities with the cytokine IL-18. IL-12 and IL-18 are known to have similar functions and an ELISA would provide concrete numbers to reveal the similar natures of these two cytokines.

- **Is there a staining pattern of IL-1 β , IL-18, IL-12, and TNF- α production in our tumor microenvironment?** Immunohistochemistry is a tool that can be used to locate molecules in tissue samples. Tumors from TBH mice could be isolated, sectioned, and stained for IL-1 β , IL-18, IL-12, and TNF- α . This would reveal the locations of these cytokines in our tumor model and a timepoint study could be done to analyze early versus late tumors. Methods like those used by Wang *et al.* [220] would be ideal for completing this experiment. This would reveal how tumor progression affects the production of these cytokines *in situ* and would further refine understanding of our tumor model.

- **How do immunosuppressive cytokines directly affect production of the proinflammatory cytokines IL-1 β , IL-18, IL-12, and TNF- α ?** Previous work in our lab suggests the role of the immunosuppressive cytokines TGF- β and IL-10 in paclitaxel-induced M ϕ activation [155]. Further work in this area could demonstrate how the constitutive expression or addition of these cytokines affects the proinflammatory cytokines IL-1 β , IL-18, IL-12, and TNF- α . This could be done by adding the immunosuppressive molecules in culture to NH,

TD, and TP Mφs. Next, ELISAs like those used in this study could be used to determine the direct effect of these immunomodulatory cytokines and how they tie in with our tumor model. If these immunosuppressive cytokines directly suppress proinflammatory molecules and their suppression ameliorates this dysregulation then we will have identified a novel way to increase the efficacy of the antitumor response in our tumor model.

- **What is the involvement of NF-κB in our tumor model?** The transcription factor NF-κB is an important regulator of many immune and inflammatory responses and is activated by the binding of the cytokines IL-1β, IL-18, IL-12, and TNF-α to their receptors. Previous work in our laboratory using electrophoretic mobility shift assay (EMSA) on nuclear fractions suggests that NF-κB is dysregulated in our tumor model (unpublished data); however, attempts at refining our understanding using ELISAs have been thwarted. NH, TD, and TP Mφs previously were isolated and cytosolic and nuclear fractions collected and NF-κB activation assayed via ELISA; however, results were inconclusive. The collection of samples and ELISA procedure are difficult and multiple steps are involved, which all could lead to inconclusive results. Alternative nuclear and cytosolic fraction techniques could be used as this collection step is difficult to perform. Larger cell numbers than those tried (5×10^6 cells/mL) could also be attempted as using larger cell batches will likely convey greater yields. Other future investigations could involve collecting both nuclear and cytosolic fractions from NH, TD, and TP Mφs and performing EMSA analysis on them since this method has been used before. This would elucidate the role of NF-κB and would further aid in the understanding of the antitumor defenses in our tumor model.

- **Can combination treatments with paclitaxel upregulate proinflammatory cytokine production in TP Mφs?** Paclitaxel alone failed to upregulate the production of proinflammatory cytokines in TP Mφs. We suggest that combining paclitaxel with other therapies such as OGF or antioxidants could ameliorate production of these cytokines *in situ*. This could suggest new immunotherapeutic therapies designed to correct cytokine imbalances and restore immune function in our tumor model and beyond.

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APPENDIX A: ABBREVIATIONS

- Ab, antibody
- Act1, activation gene 1
- ADCC, antibody-dependent cellular cytotoxicity
- AIDS, acquired immunodeficiency syndrome
- APC, antigen presenting cell
- β FGF, basic fibroblast growth factor
- COX-2, cyclooxygenase-2
- CsA, cyclosporine A
- DMSO, dimethyl sulfoxide
- EGF, epidermal growth factor
- ELISA, enzyme-linked immunosorbent assay
- GM-CSF, granulocyte-macrophage colony-stimulating factor
- ICAM1, intercellular adhesion molecule-1
- ICE, IL-1 β converting enzyme
- IFN- γ , interferon-gamma
- IgE, Immunoglobulin E
- I κ B, inhibitory factor- κ B
- IL-1 β , interleukin 1 β
- IL-1R, interleukin 1 receptor
- IL-2, interleukin 2
- IL-10, interleukin 10
- IL-12, interleukin 12
- IL-18, interleukin 18

- IL-18R, interleukin 18 receptor
- i.m., intramuscular
- iNOS, inducible nitric oxide synthase
- i.p., intraperitoneal
- IRAK1, IL-1 receptor associated kinase
- LPS, lipopolysaccharide
- M ϕ , macrophage
- Meth-KDE, methylcholanthrene-induced nonmetastatic murine fibrosarcoma
- MHC, major histocompatibility complex
- NK cell, natural killer cell
- NO, nitric oxide
- NH, normal host
- NF- κ B, nuclear factor-kappa B
- NIK, nuclear factor-kappa B inducing kinase
- PBS, phosphate buffered saline
- PGE2, prostaglandin E2
- PE, phycoerythrin
- RHD, rel-homology domain
- RT-PCR, reverse transcription-polymerase chain reaction
- SEM, standard error of the mean
- sTNFR1, soluble tumor necrosis factor- α receptor 1
- TAM, tumor-associated macrophage
- TBH, tumor-bearing host
- TCR, T-cell receptor
- TD, tumor distal
- TGF- β , transforming growth factor –beta

- Th, T helper cell
- TNF- α , tumor necrosis factor-alpha
- TP, tumor proximal
- TRAF6, tumor necrosis factor receptor-associated factor 6

Appendix B: EasySep™ Procedure

This procedure can be run on ANY cell type (macs, T cells, neutrophils, etc.) and ANY cell tissue (tumor-infiltrated tissue, spleen, thymus, etc.) The appropriate PE-labelled antibody specific to your cell line is all that is needed (i.e. – anti-PE cd11b for macs).

1. Isolate your tissue of interest. I will show two below:
 - a. Tumor Tissue – Use at least 4-5 animals
 - i. Cut out tumor of interest by breaking the physical tumor into small pieces using scissors and place in stomacher bag with RPMI. Roll a pipette over this mixture and stomach the bag for 2 intervals of **30-45 seconds** with a **30 second break** in between intervals.
 - ii. Add 5 mL of RPMI with 10% Trypsin (Sigma) (in fridge-provided by David Mullins). This will aid in dissociation of the tumor. Let this sit for 3-4 minutes MAX. Spin this down at 1500 RPM for **5 minutes**. Remove the supernatant and resuspend in 10% FBS (5 mL FBS, 45 mL RPMI). This will help deplete the effects of trypsin. Re-spin this mixture and pour off supernatant.
 - b. Spleen – Use at least 2 animals
 - i. Isolate spleens from mice and dissociate on screen into 5 mL RPMI. Stomach the mixture for 2 intervals of **30-45 seconds** with a **30 second** break in between intervals.

- ii. Add 45 mL of RPMI to bring the cells up to 50 mL. Spin the cells at 1500 RPM for **5 minutes** and then resuspend in 50 mL RPMI and spin again. Pour off supernatant
2. The next steps are similar for either method. It is advisable to run a ficol-hypaque (CedarLane Labs) gradient if you are worried about dead cell matter (particularly in tumor-infiltrated tissues). Instructions for this technique can be found in the Immunology Methods.
3. Divide your sample into 2 x (the number of mice used). For example, if I used 4 TBH mice, I will divide my pellet into 8 tubes. Resuspend your samples in 2 mL of RPMI per tube. Next, add the appropriate PE-labelled antibody to each tube. This step is light sensitive, and after addition of antibodies, the samples should be promptly relocated to the 4° C fridge. This incubation step lasts **30 minutes**. Halfway through the incubation time, mix samples by tapping tube.
4. Add RPMI to the top of the tube and spin the mixture. Resuspend in 2.5 mL and add 50 uL of clear tetramer (Stem Cell Technologies). Let this sit at room temperature (RT) for **15 minutes**.
5. Add RPMI to the top of the tube and spin the mixture. Resuspend in 2.5 mL and add 100 uL of metallic colloid (colored) (Stem Cell Technologies). Let this sit at RT for **15 minutes**.

6. Put this mixture into magnet tube and hold in magnet (Stem Cell Technologies) for **5 minutes** at RT. Next, invert the tube quickly and return to upright position. Do not shake off any droplets that may hang at the mouth of the tube.

7. Remove the tube from the magnet and add 2.5 mL RPMI.

8. Repeat steps 5 and 6 for a total of 3 (5 minute) separations in the magnet. Note that only one tube can be run at a time

****I had success this past weekend in running 2 tubes at a time. After 2 separations in the magnet, I added tube 2 media and ran 3 more separations, and had good yield****

9. Resuspend your positively selected cells into your desired volume and count under the hemacytometer. You can now use these cells for FACS analysis, measurement of cytokine-proliferation, and other uses.

Elizabeth Paige Falwell

Elizabeth “Beth” Paige Falwell was born in Lynchburg, Virginia, on October 3, 1980. She graduated in 2002 with her undergraduate degrees: a Bachelor of Science in Biology and a Bachelor of Arts in History. She minored in Chemistry. Throughout her undergraduate career, Beth was actively involved in university life. She was an active sister with Chi Delta Alpha service sorority for 2 years and was a member of Circle K organization for 3 years. As an undergraduate, Beth volunteered at many retirement communities, free clinics, and animal shelters. She also worked at a pediatric facility as a filerom clerk during breaks.

Beth began her graduate studies in August 2002 and completed her thesis, “Fibrosarcoma-induced Dysregulation of Interleukin (IL)-1 β and IL-18 Activities and their Modulation by Paclitaxel” under the direction of Dr. Klaus D. Elgert. Beth’s research at Virginia Tech resulted in one manuscript before her graduation. Her research was presented at local, state and national meetings, including the Experimental Biology symposium, the Virginia Academy of Science, and the Biology Department Research Day. Beth’s research was funded, in part, by grants from Sigma Xi, the Virginia Academy of Science, Graduate Research Development Program of Virginia Tech, and the Biology Department of Virginia Tech.

While Beth was completing her master’s degree at Virginia Tech, she enjoyed teaching. She taught laboratory classes in General Biology (2 sections), Principles of Biology for Life Sciences (7 sections), Principles of Biology for Biology majors (2

sections), and Immunology (3 sections). Beth maintained good student evaluation ratings throughout.

Beth will be abroad for a period after graduation and then hopes to pursue a career in medicine or law.