TUMOR-INDUCED MACROPHAGE AND T CELL DYSFUNCTION

by

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(ABSTRACT)

Macrophages (Mϕ) and T cells mediate helper, effector, and cytotoxic activities. Tumor growth changes the phenotypic and functional characteristics of Mϕ and T cells and shifts them toward suppressor phenotypes and functional activities. Tumor growth changed Mϕ DNA synthesis when activated through Mac-1 and Mac-3 surface molecules, which suggests that specific receptor-ligand interactions modulate Mϕ cell-cycle kinetics differently in the tumor-bearing host (TBH). Tumor growth changed Mϕ responsiveness to Mϕ colony-stimulating factor (M-CSF). M-CSF did not reverse decreases in autorecognition caused by TBH Mϕ, and increased TBH Mϕ suppression during T-cell alloreactivity. TBH suppressor activities were associated predominantly with MHC class II$^-$ Mϕ. TBH class II$^-$ Mϕ quantitatively and qualitatively suppressed T-cell autoreactivity partly by dysregulation of interferon-gamma (IFN-γ), interleukin-4 (IL-4), and prostaglandin E_2 (PGE_2) production. TBH Mϕ had aberrant regulation of granulocyte-macrophage colony-stimulating factor (GM-CSF). TBH Mϕ produced less GM-CSF than normal host (NH) Mϕ. GM-CSF failed to increase class II molecule expression on TBH Mϕ, and TBH class II$^-$ Mϕ became more suppressive when cultured with GM-CSF. TBH Mϕ GM-CSF dysregulation involved PGE_2 and interleukin-10 (IL-10).

Tumor growth also affected $CD4^+$ and $CD8^+$ T cell phenotype and function. The *in vivo* percentage of $CD8^+$ T cells significantly increased during tumor growth and these cells significantly suppressed T-cell allorecognition and autorecognition. TBH

$CD8^+$ T-cell suppression was mediated partly through dysregulation of IFN-γ, IL-4, and PGE_2 production. TBH $CD4^+$ T cells produced less GM-CSF than NH $CD4^+$ T cells, and GM-CSF dysregulation was linked partly to increased sensitivity to IL-10 and transforming growth factor-β_1 (TGF-β_1). Tumor growth changed $CD4^+$ T cell responsiveness to cytokines associated with T cell activation. TBH $CD4^+$ T cell proliferation was suppressed significantly by taxol.

This research also suggests that taxol can promote tumor regression. Taxol disrupted tumor cell growth through cytostatic and cytotoxic mechanisms, and increased tumor cell susceptibility to taxol-induced, Mϕ-derived lytic molecules. Taxol also disrupted autocrine regulation of TGF-β_1 and stimulated apoptosis.

Collectively, these studies suggest that tumor-associated changes in Mϕ and T cells involve cytokine dysregulation. Immunotherapeutic approaches may partly or completely reverse suppressor immune cell activities during tumor growth.

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INTRODUCTION

The goal of my research was to determine how tumor cells reduce the immunocompetence of macrophages (Mϕ) and T cells. Although most studies from our laboratory have focused on immunosuppression mediated by tumor-bearing host (TBH) Mϕ, my findings suggest that immune cell dysfunction involves both Mϕ and T cells. Tumor growth significantly increases suppressor activities associated with MHC class II$^-$ Mϕ and CD8$^+$ T cells. CD4$^+$ T cells are less responsive to activation cytokines and demonstrate heightened sensitivity to suppressor molecules. The combination of increased suppressor activities and altered cytokine responsiveness most likely accounts for decreased immunocompetence *in vivo*. Reversal of immune dysfunction may require combinations of targeted chemotherapies and immunotherapies. Such reversal would lead to higher therapeutic success against cancer.

Although benign cancers pose little risk to the host, malignant cancers are highly invasive. Cancer cells from malignant tumors divide rapidly and uncontrollably, and can infiltrate tissues proximal and distal to the primary tumor site through a process called metastasis. The vasculature that feeds the secondary tumors is established

quickly through a process called angiogenesis. As the secondary tumors flourish, additional metastasis can occur. If left untreated, the host succumbs to the cancer and dies.

The host is not defenseless against cancer. Immune cell populations including T cells, natural killer (NK) cells, Mϕ, and B cells can mediate an attack against cancer cells. Because cancer cells express altered self antigens, they can be recognized by immune cells. These elaborate "seek and destroy" mechanisms require considerable immune cell communication and appropriate cytokine regulation. Some cytokines produced by activated immune cells promote tumoricidal activities whereas others mediate direct cytolysis. However, suppression during tumor growth does not arise strictly from the cancer itself. When tumoricidal activities of the immune system are disrupted, cancers have the potential to evade immune defenses. Thus, cancers inundate and kill their hosts partly by causing immune cell dysfunction.

Our laboratory uses a murine cancer model to examine how immune cells contribute to immunosuppression during tumor growth. By using a nonmetastatic cancer, we showed that tumor-distal immune cell populations are immunologically compromised. Because the cancer cells used in our model cannot metastasize and infiltrate tumor-distal areas, suppression is not caused by tumor-specific paracrine activities. Decreased immunocompetence arises from either tumor-derived suppressor molecules that work in an endocrine fashion or from immune cells that possess heightened suppressor activities. Although tumor-derived molecules such as transforming growth factor-β_1 (TGF-β_1) can downregulate immune cell activities systemically, our *in vitro* approaches showed that several tumor-distal sites contain highly suppressive immune cell populations. These populations differ from their normal host (NH) counterparts both phenotypically and functionally. By characterizing the physical attributes and biological activities of tumor-induced suppressor im-

mune cells, chemotherapies and immunotherapies can be devised that disrupt tumor cell growth and correct immune cell dysfunctions without toxic side effects.

Our laboratory has identified several relevant mechanisms associated with tumor-induced immunosuppression. Most studies have characterized the profound impact that TBH Mϕ have toward immunocompetence. Mϕ contribute to both T-cell and B-cell immunity and serve accessory, effector, and regulatory functions. These functions are controlled by the surface-associated molecules expressed on activated Mϕ and by the monokines secreted by these cells. Tumor growth changes both Mϕ surface molecule expression and monokine secretion. Consequently, immune cell activities such as proliferation, cytokine production, and responsiveness to activation signals are compromised.

My studies were built upon the foundations established by previous investigators in our laboratory, but I extended my investigations to include other populations (such as T cells and tumor cells) that are involved in tumor-induced immune cell dysfunction. I evaluated recently characterized cytokines that were unknown or incompletely characterized during earlier studies. I identified specific signals associated with tumor-induced Mϕ dysfunction, and showed that molecules associated with activation and downregulation were involved mutually in these changes. I identified tumor-induced changes in T cell cytokine production and responsiveness. Cytotoxic and helper T cell activities are critical during immunosurveillance and reactivity against tumor cells. The omission of TBH $CD4^+$ and $CD8^+$ T cells from previous investigations describing TBH suppressor mechanisms left gaps in the description of our cancer model. My studies also evaluated the significance of specific signals on tumor cells. Although previous work suggested that taxol partly reversed suppressor Mϕ activity, no data existed to suggest whether taxol had any efficacy against

METH-KDE cells or whether taxol increased tumor cell sensitivity to Mϕ cytotoxic activities. My investigations were developed from the following research aims:

1. ***Define the mechanisms of Mϕ dysfunction during tumor growth.*** Phenotypic and functional changes among TBH Mϕ were evaluated to clarify how alterations in responsiveness to activation and downregulatory signals contribute to suppressor activities.

2. ***Define the mechanisms of T cell dysfunction during tumor growth.*** Phenotypic and functional changes among TBH $CD4^+$ and $CD8^+$ T cells were evaluated to determine whether TBH $CD8^+$ T cells contribute to immunosuppression and whether $CD4^+$ T cells were more sensitive to tumor-cell derived and TBH Mϕ-derived suppressor molecules.

3. ***Determine whether taxol can reverse tumor-induced immunosuppression.*** Several approaches were used to determine whether taxol mediates potent cytostatic and cytotoxic activities against the tumor cells used in our model and whether taxol increases tumor cell sensitivity to the Mϕ-derived cytotoxic molecule tumor necrosis factor-alpha (TNF-α).

Many experimental approaches were used to accomplish these aims. Flow cytometry permitted me to determine whether tumor growth changed immune cell surface molecule expression or whether responsiveness to activation signals disrupted DNA synthesis. Several variations of proliferation assays were used to demonstrate whether certain populations of immune cells possessed suppressor activities. These assays also allowed me to identify whether specific populations had heightened sensitivity to suppressor cytokines or increased responsiveness to

stimulatory cytokines. Enzyme-linked immunosorbent assays and cytokine-specific bioassays were employed to determine whether tumor-induced immune cell dysfunction altered the production of several cytokines. Nonradioisotope assays were developed to assess METH-KDE cell sensitivity to cytotoxic molecules.

When I joined the laboratory in 1989, very few cytokines were characterized. Earlier investigations primarily evaluated phenotypic changes in Mϕ surface molecule expression and showed that Mϕ produced specific suppressive substances. Indirect evidence from studies using recombinant molecules and monoclonal antibodies suggested that many cytokines were discoordinately regulated during tumor development. Most of these substances were undefined, and many contemporary investigations by other laboratories were founded on "chicken soupology", or the ability of uncharacterized molecules in culture supernatants to reverse or promote immunosuppression. Rather than describe vague phenomena of immunosuppression, I used recombinant molecules to show how TGF-β_1 and interleukin-10 (IL-10) compromised T cell activation and responsiveness to costimulatory signals. These signals are produced in restricted quantities by Mϕ and T cells 24-48 h after activation, but tumor cells promote TBH Mϕ and T-cell overproduction of these molecules during early stages of activation. Furthermore, tumor cells constitutively produce TGF-β_1 and IL-10. Consequently, tumor-proximal immune cells are suppressed in their ability to mediate cytotoxic activities against the tumor, and tumor-distal immune cells significantly compromise T cell reactivity through their increased suppressor activities.

Immune cell dysfunction involves more than constant cellular bombardment by suppressor molecules or increased sensitivity to downregulatory signals. Tumor-induced immune cell dysfunction also involves hyporesponsiveness or inadequate expression of signals that promote immunocompetence. Two molecules produced

by Mϕ that carefully regulate Mϕ and T-cell activation are TNF-α and granulocyte-Mϕ colony-stimulating factor (GM-CSF). Because these two signals are predominantly produced by Mϕ, our laboratory speculated that tumor-induced Mϕ suppressor activities partly involve dysregulation of TNF-α and GM-CSF. The biological functions of TNF-α and GM-CSF hint that tumor-induced alterations in the expression of these signals contributes to Mϕ suppressor activities. Our recent investigations describe how tumor cells disrupt the regulation of these molecules. TNF-α and GM-CSF promoted appropriate Mϕ and T cell responses during activation in NH, but tumor-induced changes in the regulation of these signals altered several cytokine cascades and immune cell activities. As a result, TNF-α and GM-CSF suppressed several T-cell responses in the presence of TBH Mϕ rather than promote T-cell responses. Both aberrant networks involve prostaglandin E_2 (PGE_2), IL-10, and TGF-β_1. Because our work describes the specific molecules associated with tumor-induced immune dysfunction, our laboratory is closer to reversing these tumor-induced changes through careful manipulations and counter-balances in cytokine regulation.

Our recent studies evaluated whether the anti-cancer drug taxol could appropriately counterbalance tumor-induced immune dysfunction. Collectively, our studies suggest that immunotherapeutic approaches using taxol may reverse most suppressor Mϕ activities and promote tumor regression. Taxol could reverse TBH Mϕ suppressor activities and promote cytotoxic molecule production. My investigations showed that taxol decreases tumor cell proliferation and viability through multiple cytostatic and cytotoxic mechanisms. Furthermore, taxol increases tumor cell sensitivity to TNF-α. The amounts of TNF-α produced by taxol-activated TBH Mϕ corresponded to the amounts of TNF-α that kill taxol-pretreated tumor cells in culture. Taxol-induced TBH Mϕ production of TNF-α occurs independent of T cells, and Mϕ

alone can mediate tumor cell killing. Although tumor growth increases T cell sensitivity to taxol as an antimitotic compound, this caveat is overshadowed by the possibility that Mϕ alone may mediate significant tumor cell killing *in situ*. I hope that future investigators in our laboratory will explore this possibility, and I am optimistic that such studies will confirm the proposed hypotheses discussed in the *Conclusion*.

This dissertation is divided into four sections that describe the mechanisms associated with tumor-induced Mϕ and T cell dysfunction. The first section is a review of the relevant literature describing tumor-induced immune cell dysfunction, whereas the remaining three sections contain my published or submitted findings that describe tumor-induced changes in Mϕ and T cell phenotype and function.

The first section, the *Literature Review*, consists of three subsections. The first subsection explains the significance of Mϕ and T cells during responses against tumors. Anticancer responses mediated by Mϕ and T cells and the cytokines required for appropriate Mϕ-T cell interactions are discussed. The second subsection explains how tumor cells disrupt tumor-proximal and tumor-distal immune cell activities. The cytokines associated with tumor development are described, and *in vivo* mechanisms of suppression are proposed. Furthermore, the changes in immunocompetence that are mediated by both cancer cells and tumor-induced suppressor immune cells are presented. The third subsection describes approaches that might correct tumor-induced immune dysfunction. Immunotherapies and chemotherapies that potentially reverse immunosuppression are defined within the context of animal cancer models and human clinical trials.

The second section, *Macrophage Dysfunction During Tumor Growth*, contains five chapters that characterize several changes in Mϕ phenotype and function during tumor growth. Many of these changes contribute significantly to immunosuppression. Chapter I explains that tumor-induced changes in Mϕ cell cycle kinetics during acti-

vation partly account for TBH Mϕ suppressor activities. Flow cytometry was used to show that Mϕ activation through specific surface molecules is altered during tumor development. Chapter II shows that tumor growth changes Mϕ responsiveness to Mϕ-CSF (M-CSF), and that these changes partly account for Mϕ suppressor activities during T-cell responses to allogeneic and syngeneic MHC class II molecules. Chapter III expands our previous findings that show MHC class II$^-$ Mϕ are a potent suppressor Mϕ population during tumor development. Cytokine networks that involve PGE_2, interferon-gamma (IFN-γ), and interleukin-4 (IL-4) contribute to the suppressor Mϕ activities. Mϕ dysregulation of GM-CSF is described in Chapter IV and Chapter V. Chapter IV describes how Mϕ regulation of GM-CSF is altered during T-cell allorecognition, and Chapter V links GM-CSF dysregulation to Mϕ suppressor activity during T-cell autorecognition.

The third section, *T Cell Dysfunction During Tumor Growth*, is composed of three chapters that discuss the phenotypic and functional changes associated with TBH T cells. Dysfunction among CD4$^+$ and CD8$^+$ T cells is presented. Chapter VI shows that CD8$^+$ T cells are potent suppressor cells that act in concert with MHC class II$^-$ Mϕ during tumor growth. Suppressor molecules involved in these networks are addressed. Chapter VII explains that CD4$^+$ T cell and Mϕ dysregulation of GM-CSF partly invoives heightened sensitivity to IL-10. Chapter VIII builds on these findings and shows that CD4$^+$ T cells are not suppressor cells but demonstrate lower responsiveness to activation and costimulatory cytokines and an increased sensitivity to suppressor molecules.

The fourth section, *Taxol as a Means to Reverse Tumor-Induced Immunosuppression*, contains one chapter (Chapter IX) that explains how taxol disrupts tumor cell growth through both cytostatic and cytotoxic mechanisms. Because taxol increases tumor cell sensitivity to TNF-α, *in situ* taxol-activated TBH Mϕ that

produce TNF-α may demonstrate cytotoxic activities against tumor cells and promote significant tumor regression. The studies described in this section serve as the foundation for future investigations that will clarify the *in vivo* significance of taxol as an immunotherapeutic drug.

The *Conclusion* summarizes the results described in Sections II, III, and IV, and modifies the pre-existing TBH models to include these findings (Figure 64). The *Conclusion* also suggests hypotheses and approaches for future investigations. Some of the possible networks involved during tumor-induced immune cell dysfunction are based on speculation because the cytokines described in these networks were characterized after the studies described herein were published. I am optimistic that this dissertation, like the ones from our laboratory that preceded it, will stimulate and extend the intellectual curiosity of others beyond the scope of this work.

SECTION I: LITERATURE REVIEW

Our laboratory has identified several mechanisms through which tumor cells convert Mϕ and T cells that demonstrate potent antitumor functions into suppressor cells or compromised effector cells. These suppressor cells were characterized phenotypically and functionally. By elucidating the changes in Mϕ surface marker expression or cytokine secretion profile, we can explain how Mϕ compromise T-cell responsiveness to activation signals and how some suppressor Mϕ activities may contribute to tumor growth. $CD8^+$ T cells also decrease immunocompetence through suppressor molecule secretion. In contrast, $CD4^+$ T cells are compromised effector cells that are less responsive to activation signals and more sensitive to suppressor signals than their NH counterparts. Section I describes how these three populations provide protection against tumor challenge, how tumor cells can compromise their functions, and how these changes may be corrected. The first subsection, *What Is the Significance of Mϕ and T Cells During Immunosurveillance Against Tumors?*, describes the functions of Mϕ and T cells and identifies surface-associated and soluble molecules involved with their activation and downregulation. The second subsection, *How Do Tumor Cells Disrupt Immune Cell Activities?*, describes the physical and

functional attributes of cancer cells and provides an overview of our findings regarding tumor cell-derived suppressor activities and immune cell dysfunction. The third subsection, *How Can Tumor-Induced Immune Cell Dysfunctions Be Corrected?*, describes current and predicted immunotherapies and chemotherapies that may reverse tumor-induced immune cell dysfunction.

What Is the Significance of Mϕ and T Cells During Immunosurveillance Against Tumors?

Although several cell types are associated with immunosurveillance against tumor cells through direct and indirect mechanisms, this subsection restricts the discussion to Mϕ and T cells. Mϕ, $CD4^+$ T cells, and $CD8^+$ T cells can mediate direct tumor cell killing, and tumor recognition and rejection requires interactions between these populations. Mϕ can mediate direct tumor cell lysis through soluble mediator molecules such as nitric oxide (NO) and TNF-α, but tumor-specific immunity requires T cells. $CD8^+$ T cells mediate significant lysis through their tumoricidal products, but their activation is dependent upon signals derived from $CD4^+$ T cells. $CD4^+$ T cells can direct tumor-specific immunity and provide cytokines to Mϕ and $CD8^+$ T cells, but require interactions with surface molecules expressed on antigen-presenting cells (APCs) and binding of mediator molecules (such as IL-1 and IL-12) derived from APCs. When these interrelated networks, that involve both surface-associated and secreted molecules, are changed during tumor growth, immunocompetence decreases. This subsection is divided into three topics, *What are Mϕ?*, *What are T*

Cells?, and *What Are the Communication Molecules Used by Mϕ and T Cells?*. The first two topics discuss the phenotype and function of Mϕ and T cells (both $CD4^+$ and $CD8^+$). The third topic is divided into two subtopics that discuss specific surface-associated and secreted molecules that regulate immune cell activation and down-regulation.

What are Mϕ?

Mϕ are ubiquitously distributed mononuclear cells that possess phagocytic and antigen presentation abilities. Mϕ are phenotypically and functionally heterogeneous and are associated with accessory, secretory, effector, and regulatory activities. Mϕ represent a significant cellular line of defense against bacterial, viral, protozoal, and tumor challenge because they are neither clonally restricted nor antigen-specific. However, Mϕ significantly contribute to antigen-specific immunity through their antigen presentation abilities and secreted molecules.

Mϕ develop from pluripotential stem cells to myeloid bone marrow precursors to mature monocytes through myelopoiesis. This process requires growth factors that are expressed through a tightly regulated cascade (118). Once monocytes leave the bone marrow and enter circulation, they migrate for several days until they establish residency in tissues where the tissue-specific microenvironments promote Mϕ maturation and differentiation. Although previous dogmas suggested that Mϕ were terminally differentiated cells and were replenished or replaced only by an influx of monocytes, recent studies confirm that undifferentiated Mϕ are present within

tissues and can proliferate and differentiate into several distinct Mϕ subpopulations (451). The microenvironment of both resident tissues and the bone marrow can change rapidly during immunologic challenge from "steady-state" hematopoiesis to "induced" hematopoiesis. These changes affect the cytokines detectable in serum and the signal transduction pathways associated with Mϕ progenitors (330, 358).

Mϕ demonstrate some functional specialization depending on the location of the resident tissue and the diversity and activation states of other immune cells within the tissue. Mϕ can secrete over 100 different molecules (326a) and direct several homeostatic, inflammatory, and cytotoxic processes (3, 406). Resting Mϕ are activated and mediate their effector functions after receiving both priming and activation signals (4). Although the priming signal can precede or appear simultaneously with the activation signal, Mϕ activation will not occur if only the priming signal is received or if the activation signal precedes the priming signal. Priming signals from cytokines, adherence, and surface molecule stimulation trigger mRNA synthesis whereas activation signals such as lipopolysaccharide (LPS) stimulate or enhance translation of secreted mediator molecules or expression of surface molecules. For example, interferon-gamma (IFN-γ)-primed Mϕ express transcription factors and proto-oncogene products that in turn promote synthesis of inflammatory or cytotoxic molecules during LPS activation.

Mϕ activities such as antigen presentation and cytotoxicity contribute significantly to tumor rejection. Antigen presentation by Mϕ during tumor challenge involves a display of tumor antigens in the context of MHC class II molecules to tumor antigen-specific $CD4^+$ T cells and appropriate interactions of costimulatory molecules on both Mϕ and T cells. These interactions provide activation signals for $CD4^+$ T cells to proliferate and release cytokines that in turn activate tumor antigen-specific cytotoxic T cells and MHC-unrestricted NK cells (reviewed in 3). Mϕ-derived

cytokines such as IL-1 and GM-CSF are required for activation of T cells at the onset of immunogenic challenge whereas signals such as TGF-β_1, IL-10, and PGE_2 down-regulate T cell responses ~~responses~~ once the challenge is cleared. Because APC function is required for tumor rejection, tumor-induced changes in the expression of surface molecules and cytokines that regulate APC function can compromise tumor rejection processes. Mϕ also directly mediate cytostatic and cytotoxic activities against tumor cells through secreted molecules such as NO and TNF-α. Tumors demonstrate variable resistance to Mϕ cytotoxic activities, and Mϕ cytotoxic activities that are stimulated *in vitro* can be suppressed *in vivo* due to tumor-derived downregulatory molecules. Both tumor-proximal and tumor-distal Mϕ activities change during tumor development. Tumor-proximal Mϕ demonstrate lower cytotoxicity whereas tumor-distal Mϕ are suppressive partly through overproduction of cytotoxic molecules. Thus, tumor cells may disrupt both the expression of activation and downregulatory cytokines and the surface expression of cytokine receptors. Tumor-induced Mϕ dysfunctions are discussed in the subsection entitled, *How Do Tumor Cells Disrupt Immune Cell Activities?*.

What are T Cells?

T cells are antigen-specific, MHC-restricted lymphocytes that regulate immune cell activities through surface-associated molecules and cytokines. T cells are partitioned phenotypically into two sets that provide distinct functional properties with some overlap. Both surface molecules, CD4 and CD8, stabilize interactions between

T cell receptors (TCR) and MHC molecules. Whereas CD4 stabilizes interactions between TCR and MHC class II molecules, CD8 stabilizes interactions between TCR and MHC class I molecules. $CD4^+$ T cells are referred to as helper T cells because of their "helper" functions toward other immune cell populations, but $CD4^+$ T cells also mediate homeostatic, autoreactive, and cytotoxic functions. $CD4^+$ T cells can be partitioned into two functional subsets known as T_H1 and T_H2 based on the cytokines these cells use for autocrine growth regulation and cross-regulation (384). T_H1 cells produce IL-2, IFN-γ, and lymphotoxin, whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. Both subsets produce GM-CSF, TNF-α, and IL-3. IFN-γ serves as a cross-regulatory cytokine that suppresses T_H2 proliferation but not cytokine synthesis (166, 168). The T_H2 correlate, IL-10, suppresses synthesis of IL-2 and IFN-γ by T_H1 cells. $CD8^+$ T cells are called cytotoxic T cells because they can identify and destroy virally-infected cells or neoplastic cells. However, $CD8^+$ T cells can mediate limited helper activities and contribute significantly toward T-cell homeostasis through downregulatory pathways (117). $CD8^+$ T cells also regulate T_H cell development through their cytokine products such as IFN-γ and IL-4 (245a).

Adult T cell development occurs primarily within the bone marrow and thymus, although maturation is restricted to the thymus. Some T cell populations mature and differentiate through alternative extrathymic pathways, but most $CD4^+$ and $CD8^+$ T cells bearing $\alpha\beta$ TCR develop through intrathymic pathways. T cells originate from pluripotent bone marrow stem cells and develop into lymphoid precursors through carefully regulated growth factor cascades. Nonfunctional pre-T cells leave the bone marrow as prothymocytes and migrate to the thymus using homing mechanisms that are incompletely characterized. T cells mature in the thymus, and their maturation and differentiation can be tracked by monitoring their surface molecule expression. Upon completion of positive and negative selection processes, mature antigen-

specific, self MHC-restricted T cells that express either CD4 or CD8 enter the periphery. Unlike Mϕ, T cells do not establish residency within tissues.

T cell functions are diverse, and both $CD4^+$ and $CD8^+$ T cells contribute to specific recognition and reactivity against tumor antigens. Many tumor antigen-specific T cells are non-pathological autoreactive cells. Autoreactive T cells that are not eliminated during thymic selection remain in the periphery and provide antigen-specific immunosurveillance against altered self proteins or re-expressed fetal antigens on the surfaces of tumor cells (339, 431). Activation of T cells through their TCR/CD3 complexes by specific peptides presented with the appropriate MHC molecule also requires T cell engagement of either CD28 or CTLA-4 by B7 (CD80) or B70/B7-2 (CD86) molecules on the surface of APCs (23, 64, 271). This second set of cellular interactions is required to promote the transcription of cytokine and cytokine receptor genes. Although both CD80 and CD86 equally bind to either CD28 or CTLA-4, CD86 only appears on APC surfaces as a transient costimulatory signal during early stages of activation. CTLA-4 is expressed on $CD8^+$ T cells and CD28 is expressed on $CD4^+$ and $CD8^+$ T cells. CTLA-4 demonstrates a significantly higher affinity than CD28 for either CD80 or CD86, but CD28 is expressed at higher densities on T cell surfaces than CTLA-4. Studies using the soluble fusion protein CTLA-4Ig suggest that inhibition of the CD28/CD80 or CTLA-4/CD80 interaction during T-cell activation triggers anergy among $CD4^+$ and $CD8^+$ T cells. This anergy is reversible only by multiple activation attempts with cells bearing both MHC/peptide complexes and CD80 in the presence of high amounts of IL-2. MHC class II-restricted $CD4^+$ T cells can recognize tumor antigens that are presented by tumor-associated APCs such as Mϕ and dendritic cells (DC) or by tumor cells themselves if they are MHC class II^+. MHC class I-restricted $CD8^+$ also can recognize tumor cells that express tumor-specific proteins or re-express fetal antigens. However, T-cell recognition of tumor antigens

expressed in the context of MHC molecules is compromised because tumor cells often lack costimulatory molecules such as CD80 that are required for T-cell activation. Furthermore, tumor cells produce molecules such as IL-10, PGE_2, and TGF-β_1 that suppress T-cell activation pathways. Tumor cell recognition by T cells can be disrupted through tumor-derived suppressor mechanisms and decreased tumor cell expression of MHC molecules.

Antineoplastic activity is mediated significantly by tumor-infiltrating $CD4^+$ and $CD8^+$ T cells (reviewed in 464a). Both populations direct cytotoxic activities, and no correlations exist to suggest that the predominance of one population over another assures tumor killing or regression. Although certain tumor types restrict T-cell heterogeneity to some degree, most infiltrates contain both $CD4^+$ and $CD8^+$ T cells that express antigens associated with activation such as CD11a, CD25, and CD71. Tumor-infiltrating T cells demonstrate defective signal transduction mechanisms, aberrant cytokine production, and decreased cytotoxic activities. T-cell anergy varies depending on the model system evaluated. Although T cells from TBH are functionally compromised as compared to NH T cells, suppression also is derived from tumor cells and tumor-associated Mϕ. Tumor-induced Mϕ-derived suppression occurs through several mechanisms that both deprive T cells of required activation signals and growth factors and directly suppress T cell responsiveness to tumor antigens. Tumor-induced T-cell dysfunction is discussed in the subsection entitled, *How Do Tumor Cells Disrupt Immune Cell Activities?*.

What Are the Communication Molecules Used by Mϕ and T Cells?

Surface-Associated Communication Molecules

Mϕ and T cells express surface-associated communication molecules that contribute to tumor rejection. As mentioned previously, TCR-MHC molecule interactions are required for specific responsiveness against tumor antigens. CD4 molecules stabilize TCR-MHC class II molecule interactions and CD8 molecules stabilize TCR-MHC class I molecule interactions. T-cell activation also requires signalling through CD28 or CTLA-4 by costimulatory surface molecules on APCs or tumor cells (23, 64, 271). Although many surface-associated molecules are involved during Mϕ and T-cell activation, a discussion of these molecules is beyond the scope of this dissertation. We previously have evaluated how tumor development alters Mϕ heterogeneity by measuring Mϕ expression of four important surface molecules, and only these surface molecules (Mac-1, Mac-2, Mac-3, and Ia) will be discussed.

Mϕ functional diversity occurs because these cells are heterogeneous and exist as distinct subpopulations that are affected by the microenvironments within the bone marrow and resident tissues (58, 108, 406, 452, 453). Tissue-specific Mϕ subpopulations can proliferate and differentiate from both circulating monocytes (108) and precursors residing in resident tissues (100, 387, 406, 451, 454). Although Mϕ subpopulations can be characterized by their buoyancy (69, 321), enzymatic activities (such as peroxidase and esterase) (184, 301, 305, 415), phagocytic and opsonization abilities, and cytokine production (124, 247), our previous studies suggest that Mϕ

surface molecule expression can correlated to the maturation status and suppressor activities of Mϕ during tumor growth (17, 171, 172, 173, 483). These investigations evaluated TBH Mϕ expression of Mac-1, Mac-2, Mac-3, and Ia surface molecules, and correlated specific phenotypes with functional activities. Mac-1 (CD11b/CD18) functions as the C3bi receptor (30, 109), and contributes to Mϕ adherence, chemotaxis, and phagocytosis (244, 399). Peritoneal Mϕ that express Mac-1 regulate PGE_2 synthesis (173). Mac-1 can be used as a early differentiation and activation marker (213). In contrast, Mac-2 may represent a late differentiation and activation marker (212, 451). It is expressed as a cytoplasmic and surface-associated molecule by elicited Mϕ and DC and may be involved in Mϕ antiparasitic responses (78, 474) and proliferation (269, 319). We have shown that Mac-2^+ Mϕ are the primary source of PGE_2 (17). Mac-3 is expressed on mature Mϕ and DC, but its functional relevance is incompletely characterized. We have shown that Mac-3^+ Mϕ demonstrate significant suppressor cell activities (173). Mac-3 may represent a late differentiation surface molecule (214). Ia antigens are the MHC class II molecules and are required for antigen presentation (430). Although MHC class II molecule expression is regulated partly by activation signals such as IFN-γ and GM-CSF, MHC class II^+ and MHC class II^- Mϕ can arise from distinct progenitor cells (455). MHC class II^- Mϕ are associated with suppressor activities (81, 316, 448, 450). By correlating phenotype with suppressor activities, we identified phenotypic and functional shifts among Mϕ subpopulations during tumor development. These changes contribute to tumor-induced immune cell dysfunction and are discussed in the subsection: *How Does Tumor-Induced Immune Cell Dysfunction Disrupt the Immune System?*

Secreted Communication Molecules

Cytokines are soluble polypeptides that permit communication between cell types through autocrine, paracrine, and endocrine mechanisms. They are produced by numerous cell types and have multiple targets both within and outside the immune system. Cytokine expression, cell activation, and downregulation ar controlled by overlapping cytokine cascades. Most cytokines demonstrate transient half-lives and mediate their biological effects only within the surrounding microenvironment. An exception to this general restriction is TGF-β_1, which is secreted in a latent form and can travel systemically to distal target sites. Although many cytokines are associated with tumor cell growth, angiogenesis, and metastasis, this subtopic will restrict discussion to cytokines that are dysregulated by immune cells during tumor growth. The biological activities of these cytokines are discussed in Sections II, III, and IV.

In the NH, cytokines such as IL-1, IL-2, IL-4, IL-6, IL-12, GM-CSF, and TNF-α significantly promote Mϕ and T-cell activation, whereas TGF-β_1 and IL-10 downregulate immune cell responses. A significant portion of my research evaluated how tumor growth alters the regulation of GM-CSF. GM-CSF is a glycoprotein responsible for the proliferation, differentiation, and activation of precursor and mature Mϕ and granulocytes (359). It increases Mϕ and dendritic antigen-presenting and accessory functions (47, 153, 154), phagocytosis (280), and cytotoxicity (195). GM-CSF also regulates the transcription and release of the mediator molecules IL-1 (394), TNF-α (206), PGE_2 (206), IL-1 receptor antagonist (228, 293), and lymphotoxin (104, 296), and the expression of several cell surface molecules such as membrane-bound IL-1 (153, 314), MHC class II molecules (153, 394), Fc receptors (90), and c-*fms* (178, 183). GM-CSF is produced by several cell types, including monocytes, Mϕ, T cells, and

fibroblasts and endothelial cells (188, 303). GM-CSF is not constitutively synthesized but is inducible through the nuclear transcriptional activator NF-κB (381). IL-10 (107), TNF-α (242), lymphotoxin (242), IFN-γ (466), PGE_2 (354, 466) and IL-1 (157) can regulate GM-CSF expression. Many investigations show that GM-CSF is used as a positive regulator of immune cell function during bacterial (57, 282), fungal (457), and parasitic (360, 361, 396, 465) infections. However, some tumor types express GM-CSF receptors (306) and produce and use GM-CSF as an autocrine growth factor (33, 163, 270). GM-CSF may signal suppressor activities among specific immune cell populations during tumor development. Because tumor growth changes T cell and Mϕ responsiveness to cytokines, I evaluated tumor-induced alterations in T cell and Mϕ production of GM-CSF.

We have identified several pathways through which cancer cells and tumor-induced suppressor immune cells disrupt cellular responsiveness to cytokines or alter cytokine production. As a result, cytokine networks among both tumor-proximal and tumor-distal immune cell populations are disrupted. In some cases, TBH suppressor Mϕ respond aberrantly to cytokines (such as GM-CSF and TNF-α) and mediate suppression rather than help upon exposure to these signals. Tumor-induced alterations in cytokine regulation will be discussed in the subsection: *How Does Tumor-Induced Immune Cell Dysfunction Disrupt the Immune System?*

How Do Tumor Cells Disrupt Immune Cell Activities?

Although Mϕ and T cells can interact to eradicate tumors, cancer cells can disrupt or abrogate antitumor defenses. The first topic of this section, *Cancer Cells Are Functionally and Immunologically Distinct*, explains the phenotypic and functional characteristics of cancer cells. The second topic, *Cancer Cells Suppress Antitumor Responses Through Multiple Mechanisms*, is partitioned into two subtopics. The first subtopic, *How Do Tumor Cells "Turn Off" Components of the Immune System?*, addresses how cancers disrupt immunosurveillance or alter direct antitumor activities. The second subtopic, *How Does Tumor-Induced Immune Cell Dysfunction Disrupt the Immune System?*, discusses how specific Mϕ and T cell populations contribute to decreases in immunocompetence.

Cancer Cells Are Functionally and Immunologically Distinct

Cancer can be defined simply as a collection of nearly 100 diseases that all fundamentally involve the transformation of normal cells into immortalized cells with uncontrolled growth. Cancer cells fail to function like their normal counterparts and devote most of their energy to proliferation. Although the causes of many cancers are undetermined, all cancer types are immortal because two sets of growth-regulatory genes (the proto-oncogenes and tumor suppressor genes) are aberrantly expressed. Proto-oncogenes primarily encode protein kinases that are associated with cellular metabolism, growth, and differentiation, whereas the tumor suppressor genes encode proteins that negatively regulate cellular proliferation, communication, and angiogenesis. Additionally, cancer cells express new phenotypic characteristics that identify them as "altered self" cells. These phenotypic changes are significant because they provide an immunological basis for distinguishing neoplastic cells from normal cells. The phenotypic attributes that distinguish cancer cells from normal cells are peptides derived from either altered self proteins or nonmutated, self proteins that are encoded by silent genes. Because these peptides are expressed in the context of MHC class I molecules (and occasionally by MHC class II molecules), they can be recognized by components of the immune system (reviewed in 368).

Several types of tumor antigens have been described. Many tumors express tumor-associated surface antigens. In some cases, these markers can be used as therapeutic targets if they are not expressed by the individual's noncancerous tissues. Human tumor-associated surface antigens include the oncofetal antigens

(which are associated with hepatomas) and the carcinoembryonic antigens (which are associated with colon carcinomas) (reviewed in 368). Some tumor-associated surface antigens are normal proteins that are encoded by "silent" genes and are not expressed in normal cells. Transformation activates the uncontrolled expression of these genes, and the resulting proteins are potentially immunogenic to cytotoxic T cells. Silent gene products (such as P1A and P1B from the P815 mastocytoma model) are expressed by immunogenic tumor cells but not by nonimmunogenic tumor cells (432). These genes are not expressed in cell lines and are transcribed at very low levels in adult human tissues. Because individuals can mount antitumor responses but fail to show autoimmune responses to these molecules, P1A and P1B antigens may represent embryonic molecules that are expressed and lost before self tolerance is established. Another overexpressed gene product is a transmembrane protein designated neu/erbB2. This molecule is related to the epidermal growth factor receptor (379), and overexpression of this gene is associated with 20% of ovarian and breast carcinomas (11, 174, 392). Many individuals with breast cancer have tumor-infiltrating lymphocytes that recognize neu/erbB2 peptides, but the normal unresponsiveness to this trace antigen may be mediated through anergy or suppression. Although preliminary studies do not suggest that antitumor responses against silent gene products lead to autoimmune responses, the possibility exists that the use of silent gene products as therapeutic targets may stimulate immune reactivity against specific precursor cells that bear these antigens.

In contrast to tumor-associated surface antigens, tumor-specific transplantation antigens are tumor antigens that promote tumor rejection and are unique to cancer cells. These antigens were first characterized in a variety of murine tumor models as distinct, stably expressed surface epitopes that conferred tumor immunogenicity. Immunity could be transferred to naive hosts from immunized hosts through memory

T cells. Identified tumor-specific transplantation antigens are mutated MHC antigens (such as K^{216}) (403), viral antigens (such as from polyoma and papillomaviruses) (73, 176, 272, 302, 357), or hsp-related antigens (the hsp70 and hsp90 families) (429, 480). Tumor-specific antigens are the best therapeutic targets for antitumor responses because unidentified precursor cells or cells that express low levels of embryonic antigens are not inappropriately targeted. Some tumor rejection antigens are derived from structurally abnormal proteins that associate with MHC class I molecules. Proteins encoded by defective oncogenes or tumor-suppressor genes are unique to specific types of cancer and can associate with MHC class I molecules (217). Point mutations in the p53 tumor suppressor gene are the most common genetic alterations identified with human cancers and are associated with many hereditary cancers. Approximately 70% of colon cancers, 50% of breast cancers, 50% of lung cancers, and 100% of small-cell carcinomas of the lung have mutations in the p53 gene (217). Approximately 95% of chronic myelogenic leukemias and 25% of acute lymphoblastic leukemias are associated with a chromosomal translocation event called t(9,22)(q34,q11), which creates a fusion gene from the c-*abl* proto-oncogene on chromosome 9 and breakpoint cluster region (bcr) on chromosome 22 (209, 389). This mutant gene encodes a defective chimeric tyrosine kinase that is immunologically distinct and expressed only by malignant cells. Point mutations in the ras proto-oncogene create defective but immunogenic kinases that are associated with 90% of pancreatic carcinomas, 50% of colorectal carcinomas, and 25% of acute myelogenic leukemias (12, 140, 162). Both $CD4^+$ T cell-specific and $CD8^+$ T cell-specific immunity against mutated p53, bcr/abl, and ras oncogene products have been characterized (235, 342). These tumor-specific proteins may serve as important therapeutic targets because of their uniqueness to cancer cells.

Numerous glycoproteins and glycolipids are associated with transformation, and many individuals with melanomas and neuroblastomas initiate weak antibody responses against gangliosides, which are glycolipid residues associated with differentiation (218). Glycoproteins known as mucins can be expressed by normal cells, but they are preferentially expressed on cancer cells (182). MUC-1 antigens on cancer cells differ from those expressed on normal cells based on the exceptional degree of glycosylation, and many breast and pancreatic carcinomas express MUC-1. Transfection studies confirm that mucin-specific cytotoxic T lymphocytes can react against against MUC-1 (54, 231), and some investigators have evaluated whether mucins can confer protective immunity (1, 113).

Collectively, these data show that tumor cells undergo both functional and phenotypic alterations. Functionally, transformed cells shift their metabolic activities partially or completely from normal function to growth and proliferation without the capacity for differentiation. These functional shifts toward cellular growth occur because proto-oncogene and tumor suppressor gene expression is disrupted. Some changes, such as mutations in p53, inhibit or decrease apoptotic activities that help eliminate cancer cells (16). Phenotypically, transformed cells express surface molecules distinct from their normal counterparts. These antigens are either altered self peptides that are encoded by mutated genes or are self peptides that are encoded by normal "silent" or embryonic genes. Although these surface molecules are immunological markers that are not associated with tolerance and would presumably stimulate immunity, most cancers are weakly immunogenic or nonimmunogenic. The next topic provides an explanation for tumor evasion from the immune system.

Cancer Cells Suppress Antitumor Responses Through Multiple Mechanisms

Although individuals possess an armory of immune cells that are activated by tumor antigens, many tumors are poorly immunogenic. This paradoxical situation arises from a number of interrelated mechanisms that cumulatively suppress or completely abrogate antitumor immunity. These mechanisms involve both changes in the responsiveness of the immune system and the immunogenicity of the tumor.

How Do Tumor Cells "Turn Off" Components of the Immune System?

Many neoplasias such as leukemias, lymphomas, and solid tumors demonstrate decreased expression of MHC class I molecules (51, 307, 382). The downregulation of MHC class I molecules correlates significantly with decreased antitumor immunity because tumor-associated or tumor-specific peptides are presented to cytotoxic T cells with decreased frequency. These observations have been confirmed experimentally using murine tumor models. Nonimmunogenic mouse tumor cells that express very low levels of MHC class I molecules and quickly inundate their murine hosts become highly immunogenic and fail to produce tumors if they are transfected with MHC class I genes (222, 416). Conversely, transfection of anti-sense DNA as a means of disrupting endogenous MHC class I molecule expression significantly promotes tumor growth (223). Decreased MHC class I molecule expression may be associated with changes in the expression of transporter proteins associated with MHC

molecules (362), altered regulation or binding abilities of transcription factors for MHC class I genes (37), and mutations in the MHC molecules (458). Others suggest that low MHC class I molecule expression by specific tumor types may be selected nonimmunologically because it confers better growth characteristics (200). Cancers like Burkitt's lymphoma express low levels of MHC molecules because of their differentiation state (13). Some oncogenes, such as c-*myc*, control MHC molecule expression, and alterations in proto-oncogene expression may contribute to downregulation of MHC molecule expression (382). Colorectal cancers show a selection toward specific MHC alleles rather a general loss in expression (51, 308). These shifts may permit unchecked tumor growth. Some MHC alleles appear to predispose individuals to virally-induced tumors, and associations exist between MHC allele expression and virally-induced tumors for several types of cancer including cervical cancers (250, 459).

Although decreased tumor cell expression of MHC class I molecules contributes to the tumorigenicity and metastatic potential of several cancers, additional evidence suggests that an absence of MHC class II molecules expression contributes to the malignancy of specific cancers (222, 456). Tumor cells that express MHC class II molecules can interact with helper T (T_H) cells which, in turn, promote cytotoxic T cell and NK cell activities through their cytokines (337). Transfection studies suggest that tumor cells must express both MHC class II molecules and the costimulatory surface molecule B7 (CD80) to stimulate significant antitumor immunity (26, 27). CD80 can associate with either CTLA-4 (expressed on $CD8^+$ T cells) or CD28 (expressed on $CD4^+$ and $CD8^+$ T cells) (23, 64, 271), but CD80-associated responses require tumor cell coexpression of MHC class II molecules because transfected $CD80^+$, MHC class I^+, MHC class II^- tumor cells are as immunogenic as $CD80^-$, MHC class I^+, MHC class II^- tumor cells (27). In contrast, tumor cells cotransfected with MHC class II mole-

cules and CD80 were significantly more immunogenic than single transfectant tumor cells or mock transfectant controls (27). These data show that tumor cell expression of MHC class II molecules can stimulate T cell specific antitumor immunity and that MHC class II molecule transfection or induction through cytokines may increase tumor cell immunogenicity.

Some cancers demonstrate alterations in the expression of molecules that facilitate immune cell-tumor cell interactions or enhance metastatic potential. When specific adhesion molecules and integrins are either downregulated or absent, cell-contact dependent mechanisms of cytolysis are compromised or abrogated. A well-characterized mechanism involves the adhesion molecule CD54. Melanoma cells are resistant to lysis by monocytes if they express low levels of CD54, but transfection of CD54 into these melanoma cells permits significant monocyte-mediated cytolysis (233). Adhesion molecules on tumor cells may promote metastasis and increase tumor cell adherence to laminin and collagen (68, 315). These findings suggest that surface-associated adhesion molecules expressed on the tumor cell dictate whether immune cell-tumor cell interactions lead to cytolysis and whether metastatic tumor cells can successfully establish satellite tumors. Other studies suggest that the absence of costimulatory surface molecules such as CD80 on tumors cells triggers a state of unresponsiveness among tumor-specific T cells (75). Many human cancers are derived from tissues that do not express the surface-associated costimulatory molecule CD80, and activation of tumor antigen-specific cytotoxic T cells by CD80-deficient tumors triggers anergy rather than cytolysis (75). Transfection of the B7 gene into murine tumor cells significantly increases tumor cell immunogenicity, and similar immunotherapeutic approaches may bolster immunity against human B7-deficient tumors (76, 425).

Many cancer cells produce potent suppressor molecules that compromise immunocompetence and promote tumor growth. These cytokines are distinct from "tumor-associated" cytokines that are produced by tumor-induced suppressor Mϕ and $CD8^+$ T cells. Tumor cell-derived TGF-β suppresses T cell division and inhibits NK cell activation (409). Both cytokine and cytokine receptor expression are disrupted by TGF-β, and many tumor types such as glioblastomas, breast cancers, and colorectal cancers produce high amounts of TGF-β (40, 48, 87, 105, 210, 251, 391, 409). Transfection of TGF-β into highly immunogenic murine tumor cells reduces tumorigenicity and promotes tumor growth (424). Because TGF-β is secreted in a latent form (292), it can travel through the bloodstream and lymphatics and compromise the activities of distal myeloid and lymphoid populations. TGF-β also can be activated locally by the acidic microenvironment or by tumor-associated and Mϕ-associated extracellular proteases (221, 234). Under these circumstances TGF-β significantly disrupts the antitumor activity of tumor-associated Mϕ and tumor-infiltrating lymphocytes. Localized overexpression of TGF-β promotes angiogenesis and may function as an autocrine growth factor (295). Although we (446) and others (229, 344, 404, 464) show that TGF-β_1 serves as an autocrine growth factor for tumor cells and suppresses tumor-proximal and tumor-distal immune cell responses, one report suggests that TGF-β_1 negatively regulates tumor expansion through an uncharacterized autocrine mechanism (329). IL-10 also serves as a potent tumor-derived immunosuppressive molecule that may promote tumor development. IL-10 is produced by several tumor types and is present in high amounts in the serum and peritoneal fluid of individuals with ovarian and intraperitoneal cancers (10, 175, 187, 262). Overexpression of IL-10 by tumor cells blocks cytokine synthesis by T_H1 cells, inhibits IL-12 production, suppresses NK cell proliferation, and downregulates Mϕ expression of MHC class II molecules (99, 311).

Tumors also use invasion to avoid immunosurveillance. This mechanism is controlled partly by tumor-associated Mϕ, which constitute a significant percentage of the total cellularity within most solid tumors (411). Many monocytes/Mϕ are elicited to the tumor site by tumor-derived chemoattractants such as TGF-β_1 (442) and Mϕ chemotactic protein-1 (MCP-1) (291). In some circumstances, tumor cells actually "fuse" with Mϕ (434). This unusual scenario promotes tumor cell growth because the immortalized cancer cells are camouflaged from other effector cells and simultaneously receive Mϕ-derived growth factors that promote tumor cell growth. Other tumor models have shown that tumor-associated Mϕ provide numerous angiogenic signals and growth factors. Invasion and angiogenesis are linked processes because growing tumors are constantly synthesizing elaborate vasculature. As tumors cells invade surrounding tissues, they produce numerous proteases including a group of well-characterized enzymes called the metalloproteinases (278). Metalloproteinases are produced by several highly invasive tumor cell types including breast carcinoma cells. These enzymes are counterbalanced by normal tissue-derived inhibitors of metalloproteinases (TIMPs). The relative amounts of these two substances at the tumor site influences whether tumor metastasis occurs (204). Some of these proteases may activate TGF-β_1 at the tumor site. Under these circumstances, TGF-β_1 can suppress cytotoxic T cell activities, promote tumor cell growth, and increase angiogenesis. Mϕ also produce significant amounts of extracellular proteases which can activate latent TGF-β_1.

Tumor-induced immunosuppression can be mediated through T-cell anergy. Because T-cell proliferation requires two signals, T cells fail to produce cytokines or proliferate in response to activation signals if specific surface-associated costimulatory molecules are not expressed or discoordinately expressed. Data from murine tumor models strongly suggest this mechanism may contribute significantly

to tumor-induced immunosuppression in humans. CD80-deficient tumor cells can negate selective antitumor responses by tumor antigen-specific T cells, and a similar mechanism may involve TBH APCs such as Mϕ and DC. Using our tumor model, we were unable to show that decreased TBH Mϕ expression of CD80 contributes to $CD4^+$ T cell dysfunction during autorecognition, but we speculate that both CD80 and CD28 expression are altered.

How Does Tumor-Induced Immune Cell Dysfunction Disrupt the Immune System?

In investigating how tumor-induced suppressor immune cells decrease immunocompetence, we focused our attention on immune populations that are located in regions distal to the tumor site (the spleen and peritoneum). Our studies use a nonmetastatic murine fibrosarcoma cell line designated METH-KDE that is perpetuated by intramuscular injection into the left hind leg of BALB/c mice. Palpable tumors form 10-14 days post-inoculum and death occurs 28-35 days post-inoculum. Because the tumor is nonmetastatic, we can evaluate how cancer cells restricted at a specific location within the host can convert effector immune cells located distal to the tumor site into suppressor cells. We can determine how these suppressor immune cell populations disrupt the ability of other TBH immune cells to respond during challenge. These alterations are assessed by measuring surface marker expression, cytokine production, accessory cell activities, and responsiveness to activation signals. Tumor-induced alterations in immune cell activities are evaluated at 21 days

post-tumor initiation because maximum immunosuppression without cachexia is observed at this time.

Previous investigations showed that TBH Mϕ are distinct phenotypically and functionally from NH Mϕ. These changes are tissue-specific (splenic versus peritoneal) and indicate that phenotypic heterogeneity among Mϕ subpopulations shifts toward cells that possess suppressor functions. Splenic Mϕ expression of Mac-1, Mac-2, and Mac-3 increases during tumor development (483). Because Mac-2$^+$ and Mac-3$^+$ Mϕ are suppressor cells, increases in the relative percentages of these subpopulations partly accounts for tumor-induced Mϕ suppression. However, Mϕ that mediate the greatest levels of suppression do not express MHC class II molecules. We have shown that TBH Mϕ express lower levels of MHC class II molecules than their NH counterparts (172, 483) and MHC class II-deficient TBH Mϕ are associated with significant suppressor activity (172, 448, 449, 450). Functionally, TBH Mϕ are less responsive to activation signals such as LPS (485), although peritoneal TBH Mϕ have heightened responsiveness to stimulation through Mac-3 surface molecules (447). These findings suggest that tumor growth may increase the activation potential of specific suppressor subpopulations.

A significant mechanism of tumor-induced immunosuppression involves overexpression of downregulatory signals such as IL-10, TGF-β_1, and PGE_2 (10, 17). Both suppressor Mϕ and fibrosarcoma cells produce significant amounts of these molecules. When these signals are produced by fibrosarcoma cells, they act locally to inhibit lymphocyte reactivity against the tumor. Tumor-derived TGF-β_1 also compromises the functions of distal immune cell populations. Neutralization studies using monoclonal antibodies (mAb) suggest that tumor-derived TGF-β_1 and PGE_2 can serve as autocrine growth factors (446). Tumor-distal Mϕ significantly compromise T-cell

responsiveness during activation through overexpression of these signals and nitric oxide (NO) (446).

Tumor growth can disrupts the regulation of GM-CSF (443, 444, 448, 449). Tumor growth significantly decreases the production of GM-CSF by both activated Mϕ and T cells. IL-1, IL-2, and IL-6 significantly increase GM-CSF production by activated NH $CD4^+$ T cells whereas only IL-2 increases GM-CSF production by activated TBH $CD4^+$ T cells (445). Although GM-CSF normally enhances Mϕ and T-cell activation, in comparison to their NH counterparts, TBH T cells and Mϕ are hyporesponsive to GM-CSF (443, 448, 448, 449). GM-CSF dysregulation partly involves IL-10 (444). GM-CSF generally increases NH T cell activation, but it does not increase TBH $CD4^+$ T cell activation by mitogens, alloantigens, and autoantigens. GM-CSF increases NH splenic Mϕ MHC class II expression (as measured by relative mean fluorescence) but does not increase TBH Mϕ MHC class II expression (443). Many of these alterations in responsiveness to GM-CSF may correlate with defects in the expression of functional GM-CSF receptors. Although these possibilities have not been confirmed, Mϕ and T cell hyporesponsiveness to GM-CSF may correlate with either decreased expression of the GM-CSF-specific α subunit or a decreased ability of the α subunit to form a functional complex with the β subunit. These possibilities do not explain why MHC class II^- TBH Mϕ become more suppressive in the presence of GM-CSF; in contrast, suppression mediated by MHC class II^- NH Mϕ is partly reversed by GM-CSF. Nonetheless, these data suggest that tumor growth changes the production of and responsiveness to GM-CSF and that these alterations compromise immune cell activities.

Tumor growth also disrupts the regulation of the cytotoxic molecules TNF-α and NO (7, 10). TBH Mϕ produce high amounts of TNF-α immediately following activation. This early overexpression of TNF-α suppresses rather than enhances T cell re-

sponses because TNF-α autocrinely regulates the expression of PGE_2. TBH Mϕ disrupt T cell activities through this positive feedback loop. Although TNF-α and NO production by TBH Mϕ seems superficially advantageous, these signals are differentially regulated at sites proximal and distal to the tumor. Whereas tumor-associated Mϕ produce concentrations of TNF-α and NO that are too low to mediate significant cytotoxicity, overexpression of these two signals by splenic and peritoneal Mϕ suppresses T cell activities at distal sites. Excessive Mϕ NO expression may induce apoptosis among T cells. Thus, fibrosarcoma cells induce suppressor activities among distal Mϕ populations by increasing Mϕ production of TNF-α and NO. Tumor cells also downregulate cytotoxic activities of *in situ* tumor-associated Mϕ by decreasing expression of these molecules.

Defective T-cell responses to regulatory cytokines significantly contribute to immune dysfunction during tumor growth (444, 445, 449). $CD4^+$ and $CD8^+$ T cells represent significant effector components of the immune system, and their activities are dictated by their responsiveness to cytokines. $CD8^+$ T cells have increased suppressor activity during tumor growth (449). Blocking studies with neutralizing antibodies suggest that IL-4 and IFN-γ are discoordinately regulated by these cells and that $CD8^+$ T cell-mediated suppression is increased in the presence of MHC class II^- Mϕ. Additive suppression caused by $CD8^+$ T cells and MHC class II^- Mϕ is not reversed with indomethacin. Suppression partly involves TGF-β_1 because TBH $CD8^+$ T cells produce more TGF-β_1 than NH $CD8^+$ T cells. Tumor growth also significantly changes helper T cell responsiveness to several cytokines involved in T-cell activation and downregulation. TBH $CD4^+$ T cells are more sensitive than NH $CD4^+$ T cells to the downregulatory cytokines IL-10 and TGF-β_1 (445). Specific signals associated with T-cell activation such as IL-2, IFN-γ, and anti-IL-4 mAb could partly restore TBH T-cell proliferation to levels comparable to NH T cell levels, but this effect was

blocked by IL-10 and TGF-β_1. TBH $CD4^+$ T cells were unresponsive to the costimulatory cytokines IL-1 and IL-6 and produced lower concentrations of upregulatory cytokines during activation. TBH T_H cells also demonstrated a higher sensitivity to the antimitotic compound taxol than their NH counterparts. This sensitivity was greater for TBH $CD4^+$ T cells even if 4-h taxol pretreatment occurred 24 h before or after T cell activation. Furthermore, taxol compromised TBH $CD4^+$ T cell responsiveness to IL-2 and increased TBH $CD4^+$ T cell sensitivity to TGF-β_1. These data suggest that tumor growth changes helper T cell responsiveness to specific regulatory signals associated with T-cell activation and downregulation. Furthermore, the data suggest a novel mechanism of T-cell suppression by taxol during tumor growth and may partly explain the limited success of taxol as an effective chemotherapy for cancer patients. Other data suggest that suggest taxol compromises the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T cells.

Tumor development also may disrupt Mϕ IL-12 production. IL-12 is a Mϕ-derived and B cell-derived cytokine that promotes the development of T_H1 cells and increases cytotoxic activities of $CD8^+$ T cells and NK cells (reviewed in 52). IL-12 also reduces the metastatic potential of several tumor types by promoting immune cell infiltration. IL-4, IL-10, and TGF-β_1 negatively regulate IL-12 expression, and these suppressor immune cell-derived and tumor cell-derived signals may reduce or inhibit Mϕ production of IL-12 *in situ*. Several studies involving murine tumor models show that exogenous IL-12 promotes tumor rejection, which suggests that Mϕ production of IL-12 rather than T-cell responsiveness to IL-12 is compromised during tumor growth. Future stuides will determine whether tumor growth decreases tumor-proximal and tumor-distal Mϕ IL-12 production.

These data suggest that cancer cells abrogate antitumor immune responses and perpetuate their own growth through the expression of TGF-β_1 and PGE_2. TGF-β_1 can

act distally to manipulate the activities of other immune cell populations. Tumor-induced suppressor Mϕ and T cells mediate additional suppression through the molecules they produce and through alterations in responsiveness to specific cytokines. Although TBH $CD4^+$ T cells do not seem to be suppressor cells, they are hyporesponsive to activation cytokines and hyperresponsive to inhibitory cytokines. These changes in immune cell activities collectively contribute to the decreased immunocompetence that occurs during tumor growth.

How Can Tumor-Induced Immune Cell Dysfunctions Be Corrected?

Although many drugs have been identified that target tumor cell metabolism or proliferation, this discussion is restricted to modifications and therapies that target cancer activities specifically associated with immune cell dysfunction. This subsection is divided into two topics, *Novel Immunotherapies* and *Novel Chemotherapies*. In the first topic, *Novel Immunotherapies*, several approaches are described that increase tumor cell immunogenicity or reverse suppressor molecule activities using immunologic molecules and biological response modifiers. The first subtopic, *Gene Targeting*, describes how surface molecule genes or cytokine genes that are introduced by transfection or transduction increase tumor cell recognition and immune cell activation. The second subtopic, *LAK Cell and APC Therapies*, describes how immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, and NK cells can be activated with exogenous cytokines to mediate cytotoxic activities against tumor cells. A possible therapeutic approach involving *in vitro* priming of APCs such as DC or Mϕ with tumor antigens and cytokines is described as a means of generating tu-

mor immunity. The third subtopic, *Cytokine Antagonists*, describes how modulations in soluble receptor expression or autoantibody production can promote antitumor immunity or reduce tumor-derived suppression. In the second topic, *Novel Chemotherapies*, agents are discussed that promote cytotoxic responses against tumor cells. Many of these activities involve Mϕ. The first subtopic, *Group B Streptococcus toxin*, describes how fetal antigens expressed on tumor endothelial cells can bind GBS exotoxin which induces tumor regression. The second subtopic, *Lipid A Analogs*, describes how molecules that mimic LPS promote Mϕ tumoricidal activities without the toxicity associated with endotoxin. The third subtopic, *Taxol and Taxol Analogs*, shows that natural and synthetic diterpenoids can kill tumor cells and promote Mϕ tumoricidal activities. Data from our laboratory that suggest taxol may promote tumor regression through Mϕ activities also will be discussed.

Novel Immunotherapies

Gene Targeting

Many novel immunotherapeutic approaches promote constitutive tumor cell expression of surface molecules or cytokines. These techniques introduce genes associated with strong promoters into tumor cells to create genetically engineered tumor cells that either enhance tumor antigen presentation or promote T-cell activation (337). The development of genetically engineered human tumor cells for clin-

ical trials has been arduous. Many human tumor types are difficult to maintain as cell lines, and the composition and frequency of tumor-specific surface antigens changes during extended *in vitro* culture (337). Furthermore, human tumor cells are difficult to transfect. Defective retrovirus vectors that yield high transfection efficiencies (227), may facilitate tumor transfection studies considerably.

Murine transfection studies show that tumor cell expression of MHC and costimulatory surface molecules increases immunogenicity. Absence of MHC or costimulatory surface molecules may account for significant T cell tolerance to tumor antigens *in vivo*. Cotransfection of MHC class II molecules and CD80 significantly increases tumor cell immunogenicity (27). This mechanism favors antigen presentation to T_H cells that provide cytokines to cytotoxic T cell and NK cells. Tumor cell coexpression of MHC class II molecules and CD80 averts anergy among tumor antigen-specific T cells that occurs when T cells engage APCs lacking costimulatory molecules. However, human MHC class I and II molecules are highly polymorphic, and "tailored" gene therapies for cancer patients by this approach would be impractical. Cytokine therapies that increase endogenous MHC molecule or CD80 expression may represent the most practical approach for clinical trials. Recently, CD80-transfected, MHC class I^- tumor cells and CD80-transfected, MHC class I^+ tumor cells were used to show that bone marrow-derived APCs, rather than the tumor cells themselves, were the required MHC-expressing cell type during MHC class I-restricted tumor immunity (220). These findings suggest that cytokine therapies designed to promote the development and maturation of natural APCs may improve responsiveness against tumors.

Cytokine gene-transfected and gene-transduced tumors in murine models appear to promote strong antitumor responses *in vivo* (91). Localized secretion of specific cytokines more accurately targets the biological activity of these molecules to

tumor-infiltrating immune cells at the tumor site than can be accomplished by systemic cytokine administration. Many cytokines promote tumor antigen presentation or T cell activation in response to tumor antigens. Although results vary among models based on tumor cell doses, levels of *in situ* cytokine production, and challenge site, several cytokine transfection and transduction models stimulate partial or complete tumor-specific immunity. T cells are involved in all models evaluated, but Mϕ and DC contribute to tumor rejection in many systems.

Successful gene therapies promote tumor cell destruction and enhance APC function. These aims were accomplished using tumor cells transfected with genes encoding IL-2 and GM-CSF (120). The tumor cells were non-immunogenic, and neither mock-transfected nor X-irradiated tumor cells induced specific immunity. Although tumor-specific immunity was not generated by the IL-2 gene-transfected models, IL-2-expressing tumors lost their tumorigenicity. In contrast, double-transfected tumor cells that constitutively expressed IL-2 and GM-CSF promoted tumor cell rejection and conferred tumor-specific immunity. This mechanism relied significantly on APC activation, because tumor cells transfected only with the GM-CSF gene also promoted long-term systemic immunity that involved both $CD4^+$ and $CD8^+$ T cells. The authors speculated that GM-CSF enhanced T-cell activation and promoted the development of APCs such as DC (120). Because GM-CSF-producing tumor cells were MHC class II^-, GM-CSF increased the antigen-presenting capacity of tumor-associated Mϕ and DC rather than the ability of the tumor cells to function as APCs. These results underscore the significance of cytokines that promote APC activation. GM-CSF enhances tumor antigen presentation (189, 192) and may correct tumor-proximal TBH Mϕ dysfunction.

Although our data suggest tumor-distal T cells and Mϕ respond poorly to GM-CSF *in vitro* (444, 448), we do not have data to suggest that tumor-proximal Mϕ

respond poorly to GM-CSF. The cytokine profiles of activated tumor-proximal and tumor-distal Mϕ vary considerably, and GM-CSF may promote immune cell activities *in situ* that are inhibited by suppressor Mϕ in tumor-distal tissues. TNF-α (193) and IL-10 (190) inhibit the ability of GM-CSF to enhance APC functions during antitumor responses. Our work indicates that both TNF-α and IL-10 are expressed aberrantly by activated Mϕ from tumor-distal sites (105) and that these signals compromise TBH Mϕ and T cell responsiveness to GM-CSF (444). Because Mϕ cytotoxic activities are suppressed at the tumor site (partly through decreased TNF-α production), tumor-proximal Mϕ may respond more readily to GM-CSF than tumor-distal Mϕ. The cytokine milieu established at the tumor site as a result of transfection may provide sufficiently high levels of GM-CSF to overcome decreased cytokine responsiveness. GM-CSF administration through biodegradable microspheres also promotes antitumor immunity (186). Enhanced tumor-proximal Mϕ activation by GM-CSF may reduce tumor cell production of TGF-β_1. TGF-β_1 can reach tumor-distal sites and compromise tumor-distal Mϕ responsiveness to GM-CSF. Thus, constitutive GM-CSF expression at the tumor site may indirectly correct defects in GM-CSF responsiveness associated with tumor-distal immune cells.

In other models, IL-2-transduced and -transfected tumor cells promote systemic antitumor responses and significant tumor regression through $CD8^+$ T cells and NK cells (65, 94, 144, 169, 239, 276, 351, 374). Depletion studies show that $CD4^+$ T cells are not involved in the rejection process. IL-4-transduced or -transfected tumor cells promote tumor infiltration by Mϕ and eosinophils. Mϕ present tumor antigens to $CD4^+$ T cells which in turn provide cytokines that promote $CD8^+$ T cell activities critical for tumor regression (185, 347, 419). IL-6-transduced and -transfected tumor cells promote tumor responses through different cell populations depending on the model tested (350). In one IL-6-transfected murine sarcoma model, $CD8^+$ T cells were re-

sponsible for tumoricidal responses (320), whereas another model (410) shows that Mϕ and neutrophils rather than T cells mediated tumor regression. IL-7-transduced tumor cells stimulate antitumor immunity through $CD4^+$ T cells and Mϕ, and immunity occurs independent of $CD8^+$ T cells (215, 294). Although IFN-γ and TNF-α are known to promote antitumor immunity, variable results have been acquired from transfection models using these cytokines. IFN-γ increases MHC class I and II molecule expression in most murine models evaluated. However, systemic immunity and tumor rejection are model-dependent, and cotransfection studies in which the IFN-γ gene is introduced with other cytokine genes causes an inhibitory effect on systemic immunity (94, 130, 170, 279, 349, 363, 461). TNF-α-transduced tumor cells grow slowly *in vitro* and are rejected *in vivo*, but the rejection process may involve direct cytolysis by TNF-α rather than activation by tumor-associated Mϕ. TNF-α-transduced tumors promote the same level of systemic immunity as nontranduced, irradiated control tumors (15, 38, 238, 239, 418). IL-1 transfection studies have not identified the immune cell population responsible for tumor inhibition (119). IL-5-transfected tumor cells do not promote tumor immunity (261).

Gene targeting approaches offer promising therapeutic advantages over chemotherapies because natural communication signals rather than potentially toxic metabolic poisons are used. These studies have highlighted the significance of several cytokines during tumor rejection processes, but they also show that the types of antitumor responses triggered and even the cell types involved are dependent on the model system. These studies also show that *in situ* cytokine production may mediate tumor rejection through activation of APCs rather than through mechanisms that exclusively involve T cells and NK cells.

LAK Cell and APC Therapies

LAK cells can be activated *in vitro* or *in vivo* with specific cytokines to enhance their antineoplastic activities. Depending on the model described, LAK cells are phenotypically NK cells, $CD4^+$ T cells, or $CD8^+$ T cells. IL-2 (102, 197, 281, 355), IL-4 (143, 181, 281, 397), IL-6 (254, 380), IL-7 (43, 341), IL-12, (52, 67, 367, 486), and IFN-γ (36, 390) have been evaluated, and the cytokine combinations IL-2 plus IL-12 or IL-2 plus IFN-γ were shown to promote optimal LAK cell activation. Variable responses may be attributed to tumor-derived molecules that disrupt activation or to aberrant costimulatory molecule expression.

Because APCs are required during $CD4^+$ T cell-mediated tumor-specific immunity, APCs that are activated *in vitro* with tumor antigens may generate tumor immunity *in vivo*. DC are ideal cells because they are efficient APCs (191, 225) and can be cultured and expanded *in vitro* (375). A plausible immunotherapy would involve acquisition of DC from cancer patients, expansion and priming *in vitro* with growth factors and tumor antigens, and reintroduction into the patients. Concomitant administration of GM-CSF may enhance *in vivo* responses. Crude membrane preparations from tumor biopsies provide suitable tumor antigens (191), which avoids the necessity to identify specific proteins on tumor cells that induce immunity. This type of tumor-specific approach may prove successful only if the tumor cells possess some degree of immunogenicity.

Cytokine Antagonists

Natural and synthetic cytokine antagonists can regulate cytokine secretion, activity, and clearance (101). These functions can occur both locally and systemically. Soluble receptors and autoantibodies may have therapeutic potential as cytokine antagonists that can reverse tumor-induced immune cell dysfunction. Some soluble receptors and autoantibodies may function as cytokine agonists to increase the persistence of specific molecules or to chaperone specific cytokines. Although applications of these substances in cancer therapy has not been described, this subsection speculates on potential therapeutic approaches using cytokine antagonists.

Soluble cytokine receptors can serve as either natural, highly specific inhibitors or agonists. Few soluble cytokine receptors have been identified or characterized. Because most soluble receptors lack amino acid residues associated with signal transduction pathways, secreted forms of cytokine receptors appear to clear cytokines from circulation or prevent their extravascular escape. Thus, the biological function of soluble receptors mirrors the biological function of cytokine autoantibodies.

Although autoantibodies directed against cell surface molecules such as hormone receptors or intercellular adhesion molecules can cause pathologic damage, autoantibodies against circulating molecules rarely cause tissue damage (326). However, such molecules can significantly alter cellular communication and lead to harmful consequences for the host (such as anti-insulin antibody production by diabetics). Similarly, soluble cytokine receptor production may trigger adverse immunological responses during tumor development. Rather than cause pathological damage, dysregulation of soluble cytokine receptors may compromise immune cell

activation or antitumor activities. Tumor-induced increases in the production of specific soluble cytokine receptors could promote anergy or unresponsiveness among T cells. Patient responsiveness to therapies against aggressive non-Hodgkin's lymphomas can be predicted partly by determining the levels of soluble IL-2 receptors (sIL-2R) in serum (401). Patients who have high levels of sIL-2R rarely respond favorably to treatment, and those with high levels of serum sIL-2R and IL-10 have the weakest response to therapy (402). These findings suggest that soluble cytokine receptors may inhibit immune cell responsiveness to specific growth factors in certain cancer types, and that other signals may contribute to cellular dysregulation.

No direct evidence exists to suggest that IL-10 promotes sIL-2R production, but our METH-KDE tumor model supports the possibility that TBH Mϕ suppress T cell responsiveness to IL-2 through sIL-2R-associated pathways. Previous investigations show that METH-KDE cells produce IL-10 constitutively and that TBH Mϕ produce higher levels of IL-10 than their NH counterparts (10). IL-10 can downregulate expression of CD80, CD86, and MHC class II molecules, which suggests tumor-induced IL-10 production disrupts Mϕ and T cell costimulatory pathways. IL-10 also can act directly on T cells and inhibit IL-2 production by T_H1 cells. TBH $CD4^+$ T cell proliferation can be restored by IL-2, but exogenous IL-2 cannot completely restore TBH T cell proliferation in the presence of TBH Mϕ. Neutralization studies showed that tumor cell-derived and TBH Mϕ-derived IL-10, TGF-β_1, PGE_2, and NO account for significant levels of immunosuppression. These molecules also may regulate sIL-2R production by T cells (10). Although low levels of sIL-2R expression are associated with tumor rejection in some murine and human studies, these studies do not indicate whether signals such as IL-10 or PGE_2 are associated with immunosuppression

(355). Patients with advanced colorectal cancers that respond poorly to IL-2 immunotherapies have high serum levels of PGE_2 (102).

Based on these observations, some tumor-induced immune cell dysfunctions could be corrected through therapies that simultaneously suppress the production of sIL-2R and neutralize tumor-derived suppressor signals. These therapies could involve *in vivo* administration of soluble receptors or mAb specific for suppressor signals. Murine tumor models show that anti-TGF-β_1 mAb can retard tumor growth and partly decrease tumor-derived immunosuppression. Although soluble receptor therapy is untested, this approach is superior to therapies involving anticytokine antibodies. Anticytokine antibodies must be of the IgG_4 subclass to avoid complement activation, and cytokine-immunoglobulin molecules can still function as inflammatory complexes. Genetic engineering has led to the production of "humanized" rodent antibodies or antibody fragments against cytokines (TNF-α) and cytokine receptors (IL-2R). Because these molecules can neutralize specific signalling pathways without possessing a short serum half-life or triggering serum sickness (472), they could be used to neutralize specific tumor-derived suppressor signals.

Natural anticytokine autoantibodies have been identified that block the biological activities of IL-1, IL-6, IFN-α, and IL-10 (31, 101). Other serum antibodies have been identified that can bind but not neutralize TNF-α, IL-2, IL-4, and IL-8 activity (101). Tumor-induced immune cell dysfunctions may be corrected partly if such antibodies can be regulated successfully during tumor growth. Induction of anti-IL-10 and anti-TGF-β_1 autoantibodies would significantly reduce suppression within microenvironments at the tumor site and tumor-distal lymphoid tissues.

New Chemotherapies

Group B Streptococcus Toxin

Phase I clinical trials have evaluated the tumoricidal efficacy of a potent exotoxin derived from group B *Streptococci* (GBS) (208, 421). GBS was initially characterized as the causative agent of early onset disease which affects neonates and triggers life-threatening sepsis and respiratory distress. The susceptibility to early onset disease is restricted to infants less than four days old and targets only lung endothelial cells. Neonatal pulmonary endothelial cells express surface antigens that bind GBS. GBS-binding elicits strong inflammatory responses that include accumulations of granulocytes and macrophages, localized production of inflammatory cytokines, infiltrations of T lymphocytes, extensive lesion formation and necrosis, and capillary thrombosis. Several tumor types including human lung cell carcinomas possess vasculature that express antigens identical to those present on neonatal pulmonary endothelium. GBS inhibits tumor growth in TBH mice through predominantly inflammatory mechanisms. Toxicity is restricted to the tumor site and pathological damage has not been observed in tumor-distal sites. Although GBS does not appear to mediate cytotoxicity against tumor cells by inducing tumor-specific cytotoxic T cell or NK cell activities, GBS increases the number of these cells that accumulate at the tumor site. Because GBS-induced antitumor responses are restricted to the tumor site and do not occur in healthy individuals, GBS therapies may be a possible alternative for patients who respond poorly to immunotherapies that promote only tumor antigen-specific T cell responses. Significant antitumor re-

sponses may reflect the ability of GBS to promote cytotoxic activities and enhance antigen presentation by Mϕ.

Lipid A Analogs

Lipid A analogs have numerous immunopharmacological benefits including promotion of Mϕ antitumor activities (432, 268, 313, 388). Although these compounds stimulate Mϕ production of TNF-α and NO, many are significantly toxic. The synthetic lipid A analog designated ONO-4007 demonstrates 0.01% the toxicity of LPS associated with *Escherichia coli*, promotes significant *in vivo* activity against several tumor types in murine models, and is water-soluble. ONO-4007 stimulates tumoricidal activities by tumor-associated Mϕ (476). Phase I clinical trials are confirming whether ONO-4007 stimulates tumor-associated Mϕ to direct potent tumoricidal activities through a TNF-α-mediated mechanism. Lipid A analogs may reverse tumor-induced immunosuppression by promoting Mϕ cytotoxic activities.

Taxol and Taxol Analogs

Phase I clinical trials are evaluating the efficacy of potent anticancer drugs called paclitaxel (taxol) and docetaxel (taxotere). Taxol is an organic extract from the bark and needles of the Western Yew (*Taxus brevifolia*) (248), whereas taxotere is a semi-synthetic molecule that is produced from a precursor extracted from the needles of

Taxus baccata (287). Taxol and taxotere block progression through the G_2/M phase of the cell cycle by promoting stable polymerization of microtubules and inducing the formation of large dysfunctional mitotic asters (286, 369). Both drugs demonstrate significant antineoplastic activity against several tumor cell types and are 100-1000 times more cytotoxic than other cancer therapeutics (245). Although several clinical studies show that taxol can promote tumor regression, preliminary studies indicate that taxotere has more tumoricidal activity than taxol and demonstrates efficacy without prolonged drug usage (438). Taxol and taxotere are exceptionally hydrophobic and require special diluents for *in vivo* administration. Both drugs possess low toxicity and adverse reactions during therapies are attributed to the diluents used. Taxol and taxotere mediate several tumoristatic and tumoricidal activities, but few studies have evaluated the significance of these compounds for immune cell activation and proliferation. This subsection will discuss how taxol and taxotere alter immune cell activities.

Taxol is dissimilar physically from all Mϕ activation agents, but it functionally resembles LPS. Both taxol and LPS stimulate Mϕ production of TNF-α and expression of TNF-α receptors (112). Taxol-induced TNF-α and IL-1 mRNA transcription mirrors the mRNA levels induced by LPS (41). Taxol also promotes LPS-inducible gene expression and protein phosphorylation (288), and this activity can be inhibited with LPS antagonists (289). Mϕ activation occurs because both compounds interact with microtubule-associated protein kinases (110, 111). In addition to these activities, taxol stimulates Mϕ production of IL-2 (46) and increases GM-CSF mRNA stability (348). However, taxol and LPS initiate signal transduction through similar but nonidentical mechanisms. LPS interaction with CD14 is required but not completely sufficient for LPS-induced Mϕ gene activation (289). In contrast, taxol induces Mϕ gene transcription through both CD14-dependent and -independent pathways (249). Taxol

circumvention of LPS surface receptor-dependent pathways and entry into Mϕ probably occurs because of its extreme hydrophobicity.

Because taxol functionally resembles LPS but lacks the toxicity associated with endotoxin, taxol can be administered *in vivo* for cancer therapy. Unlike other cancer drugs that exclusively arrest tumor cell growth or disrupt metabolism (440), taxol probably promotes tumor regression by compromising tumor cell activities and stimulating tumor-associated Mϕ cytotoxic activities. This dualistic mechanism may account for taxol's success as a chemotherapeutic drug. Despite taxotere's ability to associate with Mϕ microtubules or mediate direct cytotoxicity against tumor cells, it does not stimulate Mϕ gene transcription (289).

We (7) and others (290) have shown that taxol stimulates Mϕ tumoricidal activity, and that taxol-induced, Mϕ-derived cytolytic molecules can mediate significant tumor cell killing (446, 467). Our data also suggest that taxol partly reverses tumor-distal Mϕ suppressor activities. Previous studies show that peritoneal TBH Mϕ overexpress inflammatory and cytotoxic molecules such as TNF-α and NO when activated with IFN-γ or LPS (7). NO expression is highest when peritoneal TBH Mϕ are primed with IFN-γ before LPS activation. Mϕ overexpression of TNF-α and NO suppresses T-cell responses because TNF-α autoregulates Mϕ production of PGE_2 and NO induces T-cell apoptosis. Taxol downregulates IFN-γ-primed peritoneal TBH Mϕ production of NO, and taxol-activated TBH Mϕ produce less TNF-α than TBH Mϕ activated with LPS. However, the level of TNF-α is significantly greater than the amount produced by taxol-activated NH Mϕ. Because the levels of TNF-α and NO are within a range associated with cytotoxic activity rather than suppressor activity, our data suggest that tumor-distal Mϕ dysfunctions are reversible with taxol. Although we do not have data to suggest that taxol enhances tumor-proximal TBH Mϕ cytotoxic activity, we have shown that taxol significantly increases tumor cell sensitivity to

TNF-α (446). Complete TNF-α-mediated cytolysis of METH-KDE cells occurs only when the tumor cells are cultured with taxol or preincubated with taxol for 4 h. The amounts of TNF-α correlate with the amounts produced by taxol-activated TBH Mϕ. Future studies should be performed to confirm whether taxol-activated tumor-proximal TBH Mϕ can lyse METH-KDE cells *in vitro* and *in vivo* because tumor cell-derived suppressor factors may block TBH Mϕ activation by taxol. Taxol-induced Mϕ gene transcription is inhibited by IL-10 (41) and signals such as TGF-β_1 and PGE_2 that are produced by tumor cells may disrupt taxol-triggered Mϕ activities. Tumor cell-derived TGF-β_1 most likely inhibits tumor-proximal TBH Mϕ NO production *in vivo* because TGF-β_1 promotes iNOS mRNA and protein degradation and inhibits iNOS translation (66). These events may be prevented if tumor cell exposure to taxol compromises suppressor molecule production. Taxol disrupts protein synthesis by displacing endoplasmic reticulum-associated ribosomes and fragmenting Golgi complexes responsible for post-translational modifications and protein export (422). By disrupting growth factor regulation and suppressor molecule release in addition to cell division, taxol may compromise tumor cell viability such that tumor-proximal Mϕ can mediate significant tumor regression.

A drawback of taxol chemotherapy is its antimitotic effects on bone marrow precursors and lymphoid cells. Patients experience transient neutropenia during taxol therapy, but this effect disappears when treatment ends. Most patients receive antibiotics during taxol treatments to reduce the risks of secondary infections. Significant alterations in the proliferation or cytotoxic activities of T cells and NK cells have been identified by us (445) and others (50, 83, 84, 97, 253, 370, 371). We have shown that tumor growth increases T_H cell sensitivity to taxol because TBH proliferation is suppressed more than NH $CD4^+$ T cell proliferation. Taxol-mediated suppression is significant even when taxol is administered 48 h after T cell activation. Furthermore, IL-2

and TGF-β_1 increase TBH $CD4^+$ T cell sensitivity to taxol. Others show that T cells and NK cells lose responsiveness to IL-2 through a mechanism that inhibits IL-2 receptor expression (83, 84, 370). Cytotoxicity against tumor targets significantly decreases (83, 84, 253), although limited cytotoxic activity occurs even when microtubule and Golgi complex functions are compromised by taxol (63). These data suggest that T cell and NK cell cytolytic functions are suppressed by taxol. However, these changes may be circumvented. Although IL-2 preactivation of T cells and NK cells cannot prevent taxol-mediated inhibition of proliferation, it can abrogate taxol-mediated suppression of cytotoxic activity (83, 84). These findings suggest that immunotherapies used to promote T-cell activation should precede taxol therapies designed to promote direct tumor cell killing and Mϕ cytotoxic activities. Furthermore, taxol may disrupt suppressive tumor cell activities *in vivo*. By eliminating tumor cell-derived suppression, T cells may be less compromised by taxol. We have confirmed that taxol demonstrates significant cytostatic and cytotoxic activity against the tumor cells in our murine cancer model (446)

No data have been reported to indicate whether taxol alters B cell activities. Because taxol and LPS are functionally similar, taxol may significantly affect B-cell proliferation and differentiation. Although the humoral response does not play a primary role in tumor rejection, antibodies against tumor specific antigens can facilitate antibody-dependent cell mediated cytotoxicity and enhance Mϕ opsonization during challenge. Furthermore, no data have been reported to suggest whether taxotere alters T cell or NK cell proliferation or cytotoxicity.

Summary

Based on the findings presented in this review, clinically successful cancer treatments must disrupt tumor cell activities and reverse tumor-induced immune cell dysfunction. Because humans are an outbred population that can acquire cancer at any age, optimal cancer treatments must promote tumor regression regardless of tumor antigen specificity or MHC restriction and must work independent of hormone influence. Most importantly, successful cancer therapies must correct defects associated with immune cell activation or suppression.

Although tumor-induced Mϕ and T cell dysfunction occurs through several interdigitating parameters, many of these defects may be correctable using agents such as taxol. Taxol disrupts tumor cell activities such as proliferation, viability, autocrine growth factor regulation, and suppressor molecule synthesis. Taxol also increases tumor cell sensitivity to radiation and cytolytic molecules. Taxol reverses some suppressor Mϕ activities and shifts Mϕ effector functions toward cytotoxic pathways. Taxol may stimulate Mϕ production of IL-12 which promotes T_H1 responses and augments cytotoxic T cell and NK cell responses. Taxol may be a chemotherapeutic wonder drug because it disrupts tumor cell activities and activates

Mϕ tumoricidal activities. By reversing suppressor Mϕ activities and inhibiting tumor cell suppressor molecule production, taxol may indirectly correct $CD4^+$ T cell defects that are induced partly through TBH Mϕ and tumor cells. Although taxol demonstrates limited toxicity toward lymphoid cells, this toxicity may be averted if taxol is administered using liposomes. Mϕ readily phagocytose liposomes, and taxol-containing liposomes may activate Mϕ and prevent toxicity against T cells and NK cells.

The next three sections describe my published and submitted findings on tumor-induced Mϕ and T cell dysfunction. Although these results clarify the mechanisms associated with tumor-induced immunosuppression, many questions remain. The *Conclusion* includes a model that describes known and speculative pathways of dysfunction and suggests several hypotheses for future investigations.

SECTION II: MACROPHAGE DYSFUNCTION DURING TUMOR GROWTH

The five chapters in this section describe how TBH Mϕ suppress T cell activities and show altered responsiveness to activation or downregulatory factors. Chapter I shows that TBH Mϕ exhibit differences in DNA synthesis when activated through important surface-associated molecules such as Mac-1 and Mac-3. Because Mϕ intracellular and extracellular activities are associated with the cell cycle, these findings suggest that specific receptor-ligand interactions modulate Mϕ cell-cycle kinetics differently in the TBH. Chapter II explains that tumor growth changes Mϕ responsiveness to M-CSF. Although this cytokine promotes NH Mϕ accessory activities during T-cell recognition of self MHC class II molecules, M-CSF does not reverse decreases in autorecognition caused by TBH Mϕ. Furthermore, M-CSF increases TBH Mϕ suppression during T-cell reactivity against alloantigens. Chapter III explains that TBH Mϕ suppressor activities are associated predominantly with MHC class II$^-$ cells. TBH MHC class II$^-$ Mϕ quantitatively and qualitatively suppress T-cell autoreactivity partly by dysregulation of IFN-γ, IL-4, and PGE_2. Chapters IV and V il-

lustrate that TBH Mϕ show aberrant regulation of the stimulatory cytokine GM-CSF. For example, TBH Mϕ produce less GM-CSF than NH Mϕ. GM-CSF fails to increase MHC class II molecule expression on TBH Mϕ, and TBH MHC class II$^-$ become more suppressive when cultured with GM-CSF. GM-CSF dysregulation by Mϕ during tumor growth involves PGE_2 and IL-10. These changes in TBH Mϕ GM-CSF regulation also contribute to decreases in T-cell allorecognition and autorecognition. These data suggest that changes in TBH Mϕ might be corrected through specific cytokine immunotherapies.

CHAPTER I

MACROPHAGES STIMULATED BY RECEPTOR-LIGAND INTERACTIONS EXHIBIT DIFFERENCES IN CELL-CYCLE KINETICS DURING TUMOR GROWTH: STIMULATION AT MAC-1 AND MAC-3 RECEPTORS ALTERS DNA SYNTHESIS

ABSTRACT

Phenotypic and functional changes associated with TBH Mϕ are partly responsible for immunosuppression during tumor growth. Flow cytofluorometric analyses revealed differences in cell-cycle kinetics between NH and TBH Mϕ that were stimulated at specific receptors. Receptor-ligand interactions were induced by antibodies against Mac-1, -2, -3, and Ia receptors and changes in DNA synthesis were measured over a 12-hr time course by incorporation of propidium iodide. TBH Mϕ showed higher DNA synthesis than NH Mϕ over this time course irrespective of the receptor induced. NH Mϕ stimulated at the Mac-1 receptor demonstrated higher DNA synthesis than control NH Mϕ although TBH Mϕ stimulated at this receptor and control TBH Mϕ failed to show any differences. Both NH and TBH Mϕ exhibited small, short-term decreases in DNA synthesis when stimulated at the Mac-2 receptor. TBH Mϕ that were stimulated at the Mac-3 receptor demonstrated higher DNA synthesis than their control counterparts while NH Mϕ stimulated at this receptor and control NH Mϕ showed identical levels of DNA synthesis. No differences in DNA synthesis were present among normal or TBH Mϕ that were stimulated through Ia. Differences in DNA synthesis did not appear to be attributable to differences in receptor expression. Further analysis of Mac-1 and Mac-3 stimulated cells revealed that DNA synthesis in NH Mϕ stimulated at the Mac-1 receptor returned to control levels at 48 hr. However, levels of DNA synthesis for TBH Mϕ stimulated through the Mac-3 receptor were higher than control levels until 72 hr. In addition, these differences could be reproduced upon restimulation with antibodies after 96 hr. Because Mϕ intra- and extracellular activities can be associated with specific kinetics of the cell cycle, the

data suggest that receptor-ligand interactions modulate Mϕ cell-cycle kinetics differently in the TBH.

INTRODUCTION

Mϕ possess a diverse array of functional capacities such phagocytosis, chemotaxis, monokine production and secretion, antigen processing and presentation, and tumor cytotoxicity (3, 406). However, tumors induce immunosuppression and inhibit or reduce the normal functions of Mϕ by over-stimulating or suppressing the release of soluble factors which regulate Mϕ development and activation (8, 55, 484). In addition, TBH Mϕ can alter the normal activities of other immune cells such as T cells by significantly reducing their ability to proliferate in allogeneic and syngeneic mixed lymphocyte cultures (450, 482). Recently several tumor types were identified that produce and release Mϕ mediator molecules such as M-CSF, GM-CSF, TNF-α, and PGE_2, which suggests tumor cells may have direct control of Mϕ functions through soluble factors (45, 426). Tumors also shed large amounts of antigens into circulation which bind to Mϕ and literally exhaust them so that they are ineffective in their abilities to bind or respond to the primary tumor. Collectively, these tumor cell activities seem to control Mϕ abilities through specific receptor-ligand interactions. Our data suggest TBH Mϕ exhibit differences in DNA synthesis through induced receptor-ligand interactions.

Mϕ possess more than fifty distinct surface markers (203); four of these receptors are evaluated in our system. The Mac-1 (CD11b) surface marker is a multivalent receptor which binds the C3bi component of the complement cascade (109) and performs a critical role with lymphocyte function antigen-1 (LFA-1) and p150/95 in cell-cell adhesion (30, 400). This marker is most abundant on activated cells and is constantly expressed (95). In contrast, the Mac-2 receptor is expressed only on elicited Mϕ in response to certain signals (78). In the TBH, Mac-2^+ Mϕ are associated

with suppressor activity (173). Like Mac-1 antigens, Mac-3 receptors are expressed on both resident and thioglycollate-elicited peritoneal Mϕ (214), but their functional role is unclear. Ia markers are MHC class II molecules that regulate foreign antigen recognition and presentation by Mϕ to T cells (3).

The relationship between receptor expression and function changes during tumor growth, and some of the these changes may directly relate to alterations in the kinetics of the cell cycle. Activated Mϕ exhibit increased adherence *in vitro* and a higher degree of phagocytosis involving C3b-coated particles. Mϕ size increases as does cellular ATP, oxygen consumption, PGE_2 release, and superoxide release (3). Many characteristics associated with activated Mϕ correlate with the receptors they express, and stimulation of those receptors often has an impact on cellular activities. The results support work in our laboratory concerning the alterations in Mϕ phenotype and function during tumor growth and suggest that DNA synthesis is uniquely affected in TBH Mϕ when receptor-ligand interactions are induced at the Mac-1 and Mac-3 receptors.

MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Harlan Sprague-Dawley, Madison, WI) were used. A single-cell suspension of 8 x 10^5 cells from a methylcholanthrene-induced nonmetastatic transplantable fibrosarcoma was injected into the left hind leg muscle of the TBH 3 weeks before use. Palpable tumors form by days 10-14, and death occurs by days 28-35 (123). Three weeks of tumor growth significantly suppresses immune cell reactivity, but immunosuppression is not caused by a generalized inflammatory response (173, 484, 485).

Medium

All cells were grown in RPMI-1640 (Hazelton, Denver, PA) medium with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO). All media contained 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$ and 25 *mM* HEPES (Research Organics, Cleveland, OH)

Monoclonal Antibody Purification

Hybridoma cell lines M1/70, M3/38, M3/84, and MK-D6 (American Type Culture Collection [ATCC], Rockville, MD), which produce anti-Mac-1, -2, -3, and anti-Ia monoclonal antibodies (mAb), respectively, were cultured in two-liter roller bottles with complete RPMI medium. Supernatants were harvested every three days and saturated with a pH 7.4 ammonium sulfate solution (485). After standing at 4°C overnight, precipitates were collected by centrifugation (10,000 × g, 30 min), resuspended as 20X concentrates in phosphate buffered saline (PBS), dialyzed against PBS for 48 hr, and applied to an affinity column. The column was washed with PBS to remove any unbound protein. The mAb fraction was eluted with a glycine buffer and immediately dialyzed against PBS. Column fractions were assayed for mAb using goat-anti-rat antibody, heavy- and light-chain specific (Cappel, Malvern, PA) in an Ouchterlony immunodiffusion test. Positive fractions were pooled, assayed for antibody concentration, diluted to 1.0 mg/ml, sterile filtered, and stored at -70°C.

Cell Harvest and Culture

Four days before sacrifice, normal and TBH mice received an intra-peritoneal injection of 2.0 ml fluid thioglycollate. Mice were sacrificed by cervical dislocation and Mϕ were lavaged from the peritoneal cavity with 25-30 ml ice-cold RPMI 1640 medium. Cell suspensions were centrifuged (5 min, 150 × g) and resuspended in RPMI 1640 complete medium. Cells were incubated for 2 hr at 37°C and 5% CO_2 on tissue-treated culture plates (150 × 15 mm plastic plates; Lux/Miles Scientific,

Naperville, IL). The plates were washed with warm RPMI 1640 medium to remove nonadherent cells, after which the adherent cells were removed by scraping using rubber policeman and ice-cold RPMI 1640 medium. The adherent cells were centrifuged and resuspended in RPMI 1640 complete medium. Cell suspensions were adjusted to 4×10^6 cells/ml and viability (> 95%) was determined by trypan blue exclusion. Cell suspensions were aliquoted into 2.0 ml V-bottom centrifugation tubes (Skatron, Sterling, VA) and treated with appropriate mAb during the course of incubation (37°C, 5% CO_2). Untreated Mϕ and isotype-control treated normal rat immunoglobulin-G (NRIgG) (Cappel, Malvern, PA) Mϕ controls were run in parallel to the treatment groups. Untreated controls received no mAb during incubation and thus did not synthesize DNA because of induced receptor-ligand interactions. Isotype-control treated Mϕ that were exposed to NRIgG had nonspecific binding at the Fc receptor which did not affect DNA synthesis.

Colorimetric MTT Assay

Survival and viability of Mϕ during the course of the mAb treatments were confirmed by trypan blue exclusion and the colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (317). Briefly, 10 μl MTT (Sigma) solution (5 mg/ml in PBS) was added to 96 well flat-bottom plates containing Mϕ (4×10^6 cells/ml) in 100 μl volume 4 hr before harvest time. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to the wells at the end of the incubation. Plates were read 15 min later on an ELISA reader using test and reference wavelengths of 600 nm and a calibration setting of 1.99.

Determination of DNA Synthesis

After incubation with mAb, Mϕ were pelleted by centrifugation. Supernatants were removed and cells were fixed using 0.5 ml cold PBS and 1.0 ml 95% cold ethanol. During the 20 min fixation, cells were kept on ice. Cells were pelleted and washed twice with cold PBS. The samples were resuspended in 1.0 ml of 3.8 *mM* $Na_2C_6H_3O_7$ buffered solution containing 0.5 mg propidium iodide (Sigma, St. Louis, MO) and 1.0 mg RNase A (Sigma, St. Louis, MO) (258, 284). The cells were incubated at 4°C overnight on ice and were analyzed by flow cytometry.

Fluorescent Antibody Labelling

Purified Mϕ were incubated 37°C and 5% CO_2 with either anti-Mac-1, -2, -3, or -la^d-specific mAb for 45 min at 4°C (this time and temperature allows effective Ab binding and maintains cell viability while minimizing membrane turnover and Ab internalization). Normal rat immunoglobulin G (NRIgG) and mouse immunoglobulin G (NMIgG) served as the isotype controls. Cells were washed three times and treated with affinity purified goat-anti-rat $F(ab')_2$ fragment FITC-labelled Ab for anti-Mac-1, -2, or -3 and NRIgG mAbs or goat-anti-mouse $F(ab')_2$ fragment FITC-labelled Ab for NMIgG and anti-la^d (Cappel Organon-Teknika, Malvern, PA) mAb

for 45 min at 4°C. Cells were washed three times and resuspended in 1.0 ml RPMI-1640 and analyzed for receptor expression using flow cytometry.

Flow Cytofluorometric Analysis

Flow cytofluorometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics, Hialeah, FL) laser flow cytometer and cell sorter. This instrument was calibrated with fluorescent 10-micron-sized microsphere standards before analysis of the Mϕ samples. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc., Palo Alto, CA). All data collection was done with the multiparameter data acquisition and display system (MDADS, Coulter Electronics).

Statistics/Calculation of Results

Pooled cells from four normal and four TBH mice were used for each duplicate experiment. For the flow cytometry data, all percentages were determined by the MDADS computer and represent the percentage of 10,000 cells that were considered positively labeled after the negative control was subtracted. All numbers in tables and data points on graphs were tested for significance by Student's *t* test.

RESULTS

Mϕ DNA synthesis is altered during tumor growth

To determine if Mϕ stimulated through induced receptor-ligand interactions demonstrate altered DNA synthesis during tumor growth, mAb-stimulated peritoneal Mϕ were analyzed using flow cytofluorometric analyses. Unstimulated and isotype-control stimulated (NRIgG) Mϕ were run in parallel as controls. Results for the isotype-control stimulated Mϕ closely resembled the results of unstimulated Mϕ (data not shown). TBH Mϕ demonstrated higher levels of DNA synthesis than their normal counterparts. However, levels of DNA synthesis by control NH and TBH Mϕ were comparable by 48 hr. Upon stimulation with mAb against Mac-1, -2, -3, and Ia receptors, TBH Mϕ again had altered levels of DNA synthesis when compared to NH Mϕ (Figure 1). NH Mϕ stimulated at the Mac-1 receptor showed higher levels of DNA synthesis whereas TBH Mϕ stimulated at this receptor did not change (Figure 1A). Stimulation of NH and TBH Mϕ at the Mac-2 receptor induced small decreases in levels of DNA synthesis at 3 hr, but these differences were absent at all other time points measured (Figure 1B). Stimulation at the Mac-3 receptor did not alter levels of DNA synthesis in NH Mϕ, but TBH Mϕ exhibited a significant increase in DNA synthesis when stimulated at this receptor (Figure 1C). NH and TBH Mϕ stimulated through their Ia receptors showed no differences in DNA synthesis when compared to their control counterparts (Figure 1D). Thus, alterations in Mϕ DNA synthesis during tumor growth occurred when Mac-1 and Mac-3 receptor-ligand interactions were induced.

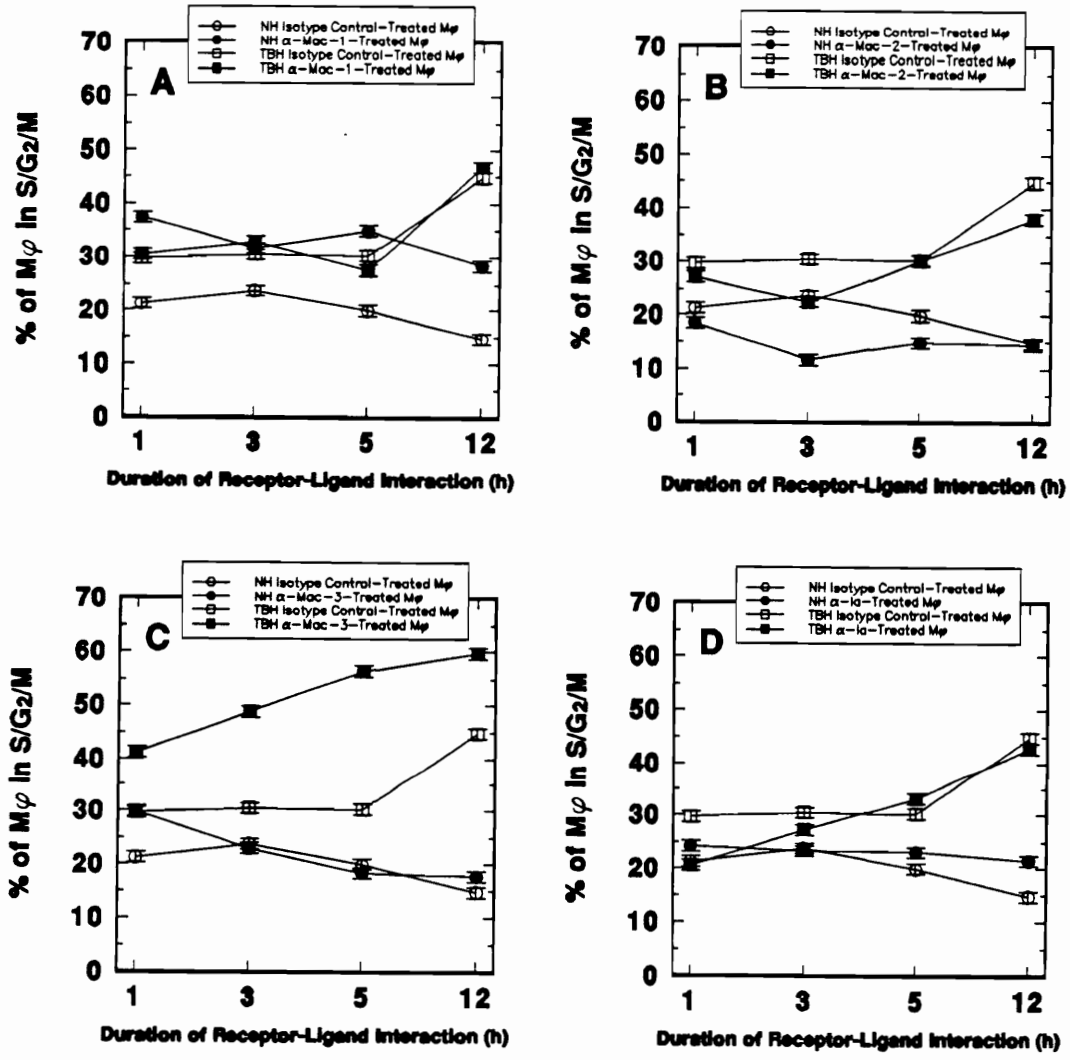

Figure 1. DNA synthesis by Mϕ stimulated through receptor-ligand interactions at the Mac-1, Mac-2, Mac-3, and Ia receptor: Peritoneal Mϕ were incubated for 1, 3, 5, and 12 hr with mAb against the receptor before fixation with ethanol and treatment with propidium iodide. The X-axis represents the time of incubation and the Y-axis represents the percentage of cells in the $S/G_2/M$ phase of the cell cycle.

Because these alterations may have been due to or at least partly associated with differences in levels of Mac and Ia Ag expression, we also analyzed tumor-induced differences in Mϕ receptor expression (Figure 2). NH and TBH Mϕ expressed similar levels of Mac-1, while NH Mϕ significantly expressed higher levels of Mac-2, -3, and Ia receptors. The differences in Mϕ expression of these Ag are not consistent with the differences in DNA synthesis incurred after ligand stimulation, and such results suggest alterations in DNA synthesis are due to TBH Mϕ responsiveness to receptor-ligand interaction and not changes in marker expression.

Alterations in Mϕ DNA synthesis are long term

To identify if the alterations in DNA synthesis returned to control levels over time, Mϕ were treated with mAb against Mac-1, -2, -3, and Ia receptors and analyzed using flow cytofluorometric analyses at 24, 48, 72, and 96 hr after modulation. NH Mϕ stimulated at the Mac-1 surface receptor showed higher levels of DNA synthesis until 48 hr, whereas TBH Mϕ continued to show no differences in DNA synthesis over time (Table 1). NH Mϕ stimulated at the Mac-3 receptor did not show any differences in DNA synthesis over time, but TBH Mϕ stimulated through receptor-ligand interactions at this surface marker showed higher levels of DNA synthesis above control levels until 72 hr after stimulation. The percentages of cells in the $S/G_2/M$ phase of the cell cycle at 96 hr after modulation were comparable to control levels (data not shown).

Because changes in DNA synthesis may have reflected changes in cell survival or viability over time, we confirmed culture stability by trypan blue exclusion and the colorimetric MTT assay. The concentration of cells recovered after incubation were

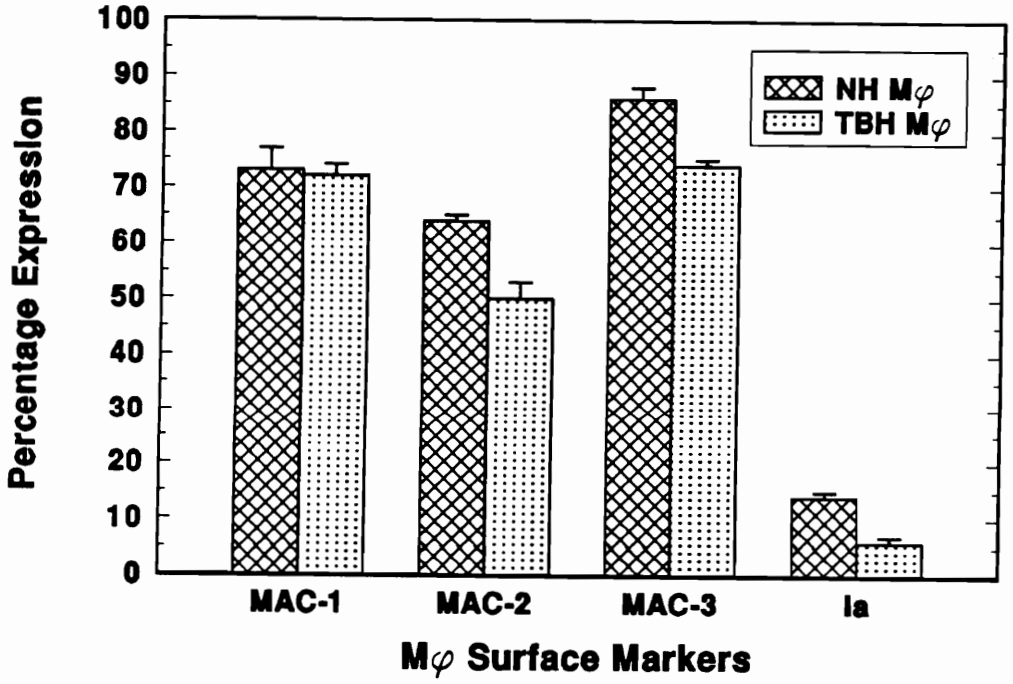

Figure 2. Mac-1, -2, -3, and Ia surface marker expression on NH and TBH Mϕ: Expression was determined using fluorescently-labelled mAb against these markers. The X-axis represents the Mϕ surface marker and the Y-axis represents the percentage expression.

Table 1. Peritoneal Mϕ DNA synthesis induced by receptor-ligand interactions

Mϕ[a]	Time of Receptor-Ligand Interaction (hr)	Isotype Control[b]	Antibodies Against Mac-1	Mac-2	Mac-3	Ia
NH	24	23 ± 1[c]	28 ± 1	23 ± 1	19 ± 1	16 ± 1
	48	23 ± 1	16 ± 1	26 ± 1	26 ± 1	25 ± 1
	72	16 ± 1	21 ± 1	17 ± 1	21 ± 1	25 ± 1
TBH	24	19 ± 1	21 ± 1	21 ± 1	41 ± 3	14 ± 1
	48	16 ± 1	21 ± 1	17 ± 1	31 ± 2	17 ± 1
	72	16 ± 1	17 ± 1	17 ± 1	20 ± 1	20 ± 1

[a]Mϕ were collected from NH and TBH as stated in the Materials and Methods.

[b]Isotype controls consisted of Mϕ that were treated with NRIgG.

[c]Values represent percentage of Mϕ in the $S/G_2/M$ phase of the cell cycle as determined by the MDADS cell cycle software. Cells were labeled with propidium iodide as stated in the Materials and Methods.

similar to starting concentrations, and levels of formazan production by treated Mϕ were identical to or slightly higher than control counterparts (data not shown).

Altered DNA synthesis through stimulation at Mac-1 and Mac-3 surface markers is reversible

Significant up-regulation in DNA synthesis occurred when normal host Mϕ were stimulated at the Mac-1 receptor and when TBH Mϕ were stimulated at the Mac-3 receptor. Increased DNA synthesis was detectable in NH Mϕ during Mac-1 stimulation from one to 24 hr and returned to levels of the controls at 48 hr. In contrast, TBH Mϕ DNA synthesis remained above control levels for 48 hr and returned to levels of the control by 72 hr. To determine if the up-regulation of DNA synthesis that was induced by receptor-ligand interactions was reproducible, cells were re-exposed to anti-Mac-1, -2, -3, or anti-Ia mAb 12 hr prior to analyses at 96 hr after initial stimulation (Table 2). At 96 hr after initial stimulation, NH and TBH Mϕ DNA synthesis levels were identical to levels of the isotype-stimulated controls. However, among Mϕ treatment groups that were restimulated with mAb, only the NH Mϕ stimulated through the Mac-1 receptor and the TBH Mϕ stimulated through the Mac-3 receptor showed significant increases in DNA synthesis 12 hr after restimulation. Additionally, these differences were similar to the levels of DNA synthesis incurred at 12 hr after initial stimulation.

Table 2. Peritoneal Mϕ DNA synthesis after restimulation with mAb

Mϕ[a]	Time of Receptor-Ligand Interaction (hr)	Isotype Control[b]	Antibodies Against Mac-1	Antibodies Against Mac-3
NH	96	15 ± 1[c]	13 ± 1	17 ± 1
	96*[d]	18 ± 1	28 ± 1	13 ± 1
TBH	96	16 ± 1	17 ± 1	27 ± 1
	96*	20 ± 1	15 ± 1	44 ± 4

[a]Mϕ were collected from NH and TBH as stated in the Materials and Methods.

[b]Isotype controls consisted of Mϕ that were treated with NRIgG.

[c]Values represent percentage of Mϕ in the $S/G_2/M$ phase of the cell cycle as determined by the MDADS cell cycle software. Cells were labeled with propidium iodide as stated in the Materials and Methods.

[d]Asterisk (*) indicates Mϕ that were re-exposed to mAb 12 hr prior to cell fixation.

DISCUSSION

Tumor growth significantly alters the surface marker expression and diverse functional capacities of various tissue Mϕ (171, 483, 485), and the data presented in this report provide another route by which tumor cells exert a modulatory effect. In both the spleen and the peritoneum, Mϕ phenotypes shift toward suppressive types that produce high levels of inhibitory molecules which down-regulate Mϕ functions (284). TBH Mϕ also exhibit lowered responsiveness to factors such as lipopolysaccharide (18, 481), interferon-γ, and GM-CSF (unpublished data), which are considered potent Mϕ activators in the NH. In addition, TBH Mϕ affect T cells by significantly reducing their proliferative capacities in allogeneic and syngeneic mixed lymphocyte cultures (450, 482).

We suggest that cell-cycle kinetics are disrupted in TBH Mϕ, and that receptor-ligand interactions induced at Mac-1 and Mac-3 receptors by specific mAb modulate DNA synthesis. Flow cytofluorometric analyses showed that TBH peritoneal Mϕ consistently synthesized higher amounts of DNA than NH Mϕ irrespective of the receptor induced. TBH Mϕ stimulated through their Mac-1 surface receptor synthesized levels of DNA comparable to control levels while NH Mϕ stimulated at this receptor demonstrated an increase in DNA synthesis above control levels for 48 hr. In contrast, TBH Mϕ stimulated through their Mac-3 receptors exhibited DNA synthesis above control levels for 72 hr while stimulated NH Mϕ did not show any change in DNA synthesis. Further analysis of anti-Mac-1 and anti-Mac-3 treatments revealed that the altered DNA synthesis levels observed during the initial receptor-ligand interactions could be reproduced upon restimulation. Stimulation of Mac-2 receptors caused a small, short-term decrease in DNA synthesis by both normal and TBH Mϕ,

but these differences were absent after 5 hr of incubation. Modulation of Ia surface markers failed to induce changes in DNA synthesis by either NH or TBH Mϕ.

The actual synthesis of DNA by Mϕ and the implications of subsequent cellular division suggest that Mϕ *in vivo* possess the ability to proliferate in various body tissues. The conventional dogma describes Mϕ as terminal cells that are incapable of cellular division and further implicates blood monocytes as the source of replacement cells in the steady-state (29). However, the constant influx of blood monocytes fails to account for a large portion of the DNA synthesis and cell division identified among Mϕ populations (441). A current and more accepted rationale suggests that Mϕ sustain and replace their numbers in areas such as the spleen and peritoneal cavity through a combination of recruitment from bone marrow cells and monocytes and from the proliferation of local Mϕ populations (100, 300, 433). Melnicoff *et al.* (299) confirmed this hypothesis through two-color flow cytometry analyses and reported that Mϕ from the peritoneal cavity, in the absence of an inflammatory stimulus, were replaced by locally proliferating cells rather than recruited Mϕ even up to 49 days after *in situ* labeling. Chimera studies on pulmonary alveolar Mϕ also identified self-renewal as the major source of replenishment for Mϕ (417). Others identify two sources of replacement for many tissue Mϕ populations (6). Even tumor tissues, where up to 50% of the cells in the tumor mass can be Mϕ (405), have been shown to be rich in Mϕ that are synthesizing DNA and actively dividing (45, 131).

Because considerable evidence attributes part of the DNA synthesis and proliferation in tissues such as the spleen and peritoneal cavity to local Mϕ populations, analysis of Mϕ DNA synthesis during tumor growth may shed light on tumor-induced alterations that occur to Mϕ in these tissues. Phenotypic and functional analyses by us (483, 485) and others (451) have defined specific Mϕ subpopulations that possess different regulatory and secretory mechanisms. Tumor growth significantly alters the

Mac-antigen expression and accessory cell capacities of these subpopulations (171), and the data presented here indicate levels of DNA synthesis are also altered. Our previous studies confirm that DNA synthesis within the spleen and peritoneal cavity occurs at a much higher rate in the TBH than in the NH (485). Tumor-induced changes in surface marker expression do not account for tumor-induced changes in DNA synthesis, and stimulation by ligand did not appear to hinder cell viability or survival. Walker and Beelen (454) describe peritoneal Mϕ DNA synthesis and proliferation and point out that Mϕ in different phases of the cell cycle express different surface markers and demonstrate different functional abilities. In our model, tumors induce alterations in the responsiveness of Mϕ to ligand stimulations and subsequently induce alterations in the cell cycle. As a result, proliferative rates among Mϕ subpopulations are altered and different functional capacities such as factor release and gene induction are incurred. The lack of DNA synthesis by TBH Mϕ stimulated at the Mac-1 receptor may reflect a decreased ability of these cells to perform in Mϕ/tumor target interactions. The ligation of anti-Mac-1 antibody to the Mac-1 receptor has been reported to increase Ia expression, enhance peroxidase activity, and elicit Mϕ activation signals (109), but many of these properties, especially Ia expression, appear to be decreased in TBH Mϕ (259, 323). Interestingly, Mac-1^+ Mϕ appear to be regulatory cells of PGE_2 in the NH, but this Mac-1^+ Mϕ-mediated control is lost in the TBH (173). Stimulation of Mac-2 receptors through receptor-ligand interactions did not appear to affect DNA synthesis in TBH Mϕ. The Mac-2 receptor is associated with IgE binding, and in the TBH, Mac-2^+ Mϕ are implicated with suppressive activity and PGE_2 production. Both NH and TBH Mϕ experienced slight short-term decreases in DNA synthesis upon ligand stimulation, but their differences paralleled their respective controls. These results suggest that the level of DNA synthesis in TBH Mϕ is not altered during receptor-ligand interactions at Mac-2

receptors. However, Mac-3 receptor stimulation did alter DNA synthesis. Previous work (103) has shown that TBH Mϕ are inexhaustible producers of PGE_2 and that this factor is a major contributor to the suppression demonstrated during tumor growth. Because Mac-3^+ Mϕ are associated with suppressive activity and the release of PGE_2 in the TBH (173), the increase in DNA synthesis by TBH Mϕ when stimulated at the Mac-3 receptor may reflect the receptor's role in PGE_2 regulation. In the NH, where PGE_2 regulation is more tightly controlled, stimulation of the Mac-3 receptor did not change DNA synthesis. The expanded population of suppressive Mac-3^+ TBH Mϕ would certainly account for the high levels of PGE_2 present in TBH. Lastly, DNA synthesis was unaltered in the TBH with respect to Ia surface marker binding because of the poor expression of this receptor on peritoneal Mϕ (485). Only 10% of NH peritoneal Mϕ actually express Ia, while only 2% of TBH Mϕ express Ia receptors.

From the data presented, Mϕ that are stimulated by receptor-ligand interactions demonstrate differences in cell-cycle kinetics during tumor growth. Mac-1 and Mac-3 receptors appear to be significant in the regulation of DNA synthesis in TBH Mϕ because their binding by mAb dramatically altered DNA synthesis. Proliferation by Mϕ is minuscule when compared to the degree of proliferation displayed by activated T cells in the presence of interleukin-2, but the increased number of suppressor Mϕ that result from such proliferation during tumor growth plays an overwhelming role in the immunosuppressed host. Determining the degree of proliferation by Mϕ stimulated at Mac-1 and Mac-3 receptors will provide further insight into the actual relevance of altered DNA synthesis in the TBH.

CHAPTER II

TUMOR GROWTH ALTERS MACROPHAGE RESPONSIVENESS TO MACROPHAGE COLONY-STIMULATING FACTOR DURING REACTIVITY AGAINST ALLOGENEIC AND SYNGENEIC MHC CLASS II MOLECULES

ABSTRACT

Tumor-induced changes in Mϕ accessory activities significantly suppress T-cell recognition of allogeneic and syngeneic MHC class II molecules. Because these changes are often associated with altered responses to stimulatory and inhibitory cytokines, we investigated the possibility that tumor growth alters the contribution of a macrophage regulatory cytokine, M-CSF, during reactivity against allogeneic and syngeneic MHC class II molecules. T-cell reactivity against allogeneic MHC class II molecules was significantly suppressed by TBH Mϕ in the presence of M-CSF. M-CSF-induced suppression was independent of TBH Mϕ PGE_2 synthesis. T-cell reactivity against syngeneic MHC class II molecules increased in the presence of M-CSF when NH Mϕ served as the source of syngeneic molecules. However, T-cell reactivity against syngeneic MHC class II molecules in the presence of M-CSF did not change when TBH Mϕ served as stimulator/accessory cells. Although T-cell reactivity against NH syngeneic MHC class II molecules was additively increased by M-CSF and indomethacin (a PGE_2 synthesis inhibitor) treatment, reactivity against TBH syngeneic MHC class II molecules increased solely through PGE_2 synthesis inhibition. Admixtures of both NH and TBH Mϕ in the absence or presence of M-CSF suggest that tumor-induced suppression was not strictly due to decreased expression of MHC class II molecules. Collectively, these data suggest that TBH Mϕ are partly suppressive through altered responsiveness to M-CSF.

INTRODUCTION

Previous investigations suggest both phenotypic and functional alterations among Mϕ populations contribute significantly to decreased immune responses during tumor growth. Phenotypically, TBH Mϕ express fewer MHC class II molecules on their surfaces (171, 483) and are unresponsive to stimuli such as GM-CSF, which induce MHC class II molecule expression (443). Functionally, TBH Mϕ demonstrate altered responsiveness to numerous cytokines such as GM-CSF (448), TNF-α (9), IFN-γ (450), TGF-β (unpublished observations), and IL-10 (unpublished observations). Tumor-induced suppressor Mϕ activities significantly decrease T-cell responses during mitogenic and antigenic challenge (93, 123, 141), and MHC class II$^-$ TBH Mϕ are the subpopulation primarily associated with these suppressor activities (449). Tumor-induced production of Mϕ-derived PGE_2 is partly responsible for decreased T-cell responsiveness (284). Because tumor growth disrupts Mϕ expression of MHC class II molecules and alters Mϕ responsiveness to cytokines, we hypothesized that tumor-induced suppression through TBH Mϕ may be caused by the Mϕ regulatory cytokine, M-CSF. M-CSF down-regulates Mϕ MHC class II molecule expression (469) and stimulates production of Mϕ-derived PGE_2 (356). Our findings show tumor growth alters Mϕ responsiveness to M-CSF during T-cell reactivity against allogeneic and syngeneic MHC class II molecules through a mechanism that is independent of PGE_2 synthesis and MHC class II molecule expression.

MATERIALS AND METHODS

Animals

The 8 to 12 week-old male BALB/c mice (Harlan-Sprague-Dawley, Madison, WI) used were LDH virus negative (Microbiological Associates, Bethesda, MD). A single-cell suspension of 4 x 10^5 methylcholanthrene-induced, nonmetastatic, fibrosarcoma cells was injected i.m. into each mouse's left hind leg. Palpable tumors form by days 10-14 and kill the TBH by days 28-35 (123). Splenic immune cells from 21-day TBH mice were used throughout. Immunosuppression was not caused by a general inflammatory response (484).

Medium

NH and TBH cells were cultured in complete RPMI-1640 (JRH Scientific, Denver, PA) medium with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO), 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$, and 25 *mM* HEPES buffer (Research Organics, Cleveland, OH).

Chemicals

Indomethacin (Sigma) was resuspended in 70% ethanol at 10^{-2} *M* and stored at 4°C. Unless specified, indomethacin was added to cultures at a final concentration of 10^{-7} *M*. This concentration inhibits the arachidonic acid pathway for PGE_2 synthesis and is nontoxic to immune cells (284).

Cytokines

Recombinant murine M-CSF was generously provided by Genetics Institute (Cambridge, MA). M-CSF activity (1.65×10^6 U/ml) was determined by half maximal colony formation in the murine bone marrow colony assay. Aliquots were stored at -70°C until use. Several concentrations of M-CSF were tested, ranging from 1 to 300 U/ml, and 100 U/ml is a representative dose of the changes induced by M-CSF.

Mϕ and T Lymphocyte Preparation

Murine spleens were pulped using a stomacher (Tekmar, Cincinati, OH) and the resulting cell suspensions were centrifuged and resuspended in RPMI-1640 medium. Erythrocytes were lysed with cold 0.83% NH_4Cl for 5 min. Cells were washed and incubated for 2 hr at 37°C and 5% CO_2 on tissue culture plates (150 x 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL). To collect $CD4^+$ T lymphocytes, nonad-

herent cells were eluted from the plates and further depleted of B cells, Mϕ, and other contaminating cells by adding monoclonal Abs anti-Lyt-2 (ATCC clone 3.155), anti-IA^d (ATCC clone MK-D6), anti-immature T lymphocyte and B cell (ATCC clone J11d), and anti-IE^d (ATCC clone 14-4-4), and a 1:12 dilution of complement (Cedarlane Laboratories, Ontario, Canada). Purified $CD4^+$ T lymphocytes (>99% $CD4^+$ T cells as determined by fluorescence-activated cell sorter [FACS] analysis) were washed at least three times to remove residual complement, and the viable cells were resuspended to 4×10^6 cells/ml in complete medium. Mϕ (>96% esterase positive) were collected by removing nonadherent cells and scraping using a rubber policeman and ice-cold RPMI-1640. Mϕ were resuspended to 8×10^6 cells/ml in complete medium and were X-irradiated with 2000 rads (TFI Minishot II X-irradiator, New Haven, CT). Nonadherent C3H allogeneic stimulator cells were collected from plates, resuspended to 8×10^6 cells/ml in complete medium, and X-irradiated with 2000 rads.

Allogeneic and Syngeneic Mixed Lymphocyte Reactions (MLR)

For the allogeneic MLR, BALB/c ($H\text{-}2^d$) $CD4^+$ T cells (2×10^5 responder cells) and C3H ($H\text{-}2^k$) splenic lymphocytes (4×10^5 stimulator cells) were added per well to "U" bottom 96-well tissue cultures plates (Nunc, Roskilde, Denmark). For the syngeneic MLR, BALB/c $CD4^+$ T cells (4×10^5 responder cells) and X-irradiated BALB/c Mϕ (4×10^5 stimulator cells) were seeded. These base numbers served as control level alloresponses and autoresponses, respectively. When Mϕ were added to the allogeneic and syngeneic MLR, the percentages of Mϕ added (4×10^4 [10%], 2×10^5 [50%], or 4×10^5 [100%] cells/well) were respective to the number of stimulator cells

present and represented low, medium, and high doses. Cultures were incubated for 4 days at 37°C and 5% CO_2. Six hr before cell harvest, each culture was pulsed with tritiated thymidine (^{3}H-TdR: specific activity 6.7 Ci/*mM*, Dupont NEN Research Products, Boston, MA). Cells were harvested onto filters (Whatman 934-AH, Thomas Scientific, Phila., PA) using a MASH harvester. Samples were placed into counting vials (Skatron, Sterling, VA) with Scintilene (Thomas Scientific, Swedesboro, NJ) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

Statistics/Calculation of Results

Pooled cells from 3 to 6 mice were used for each experiment. Three replicate wells were run for each test, and each experiment was repeated three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p<0.05$) and presented as mean$\pm$standard error.

RESULTS

TBH Mϕ suppress T-cell reactivity against alloantigen through M-CSF

Previous data show that TBH Mϕ significantly suppress T-cell reactivity against allogeneic MHC class II molecules (allorecognition) (18, 485). To determine if M-CSF contributes to TBH Mϕ suppression of T-cell alloreactivity, T cells and allogeneic stimulator cells were cultured with either NH or TBH Mϕ in the presence or absence of M-CSF (Figure 3). M-CSF did not affect T-cell reactivity mediated by high NH Mϕ doses, but alloreactivity mediated by high TBH Mϕ doses was further suppressed (25,580 ± 785 to 17,547 ± 926, $p < 0.005$, 31.4% decrease). These data suggest that M-CSF acts as a suppressor molecule for TBH Mϕ during T-cell allorecognition.

M-CSF-induced suppression of allorecognition occurs through TBH Mϕ by a pathway that is independent of PGE_2

TBH Mϕ suppress allorecognition partly through the overproduction of PGE_2 (284). To determine if M-CSF suppresses allorecognition through TBH Mϕ by stimulating PGE_2 synthesis, T cells and allogeneic stimulator cells were cultured with M-CSF, the PGE_2 synthesis inhibitor indomethacin, and either NH or TBH Mϕ. (Figure 4). Inhibition of PGE_2 synthesis by NH Mϕ did not significantly change T-cell reactivity against alloantigens in the presence of M-CSF. In contrast, inhibition of PGE_2 synthesis by TBH Mϕ significantly decreased T-cell reactivity against

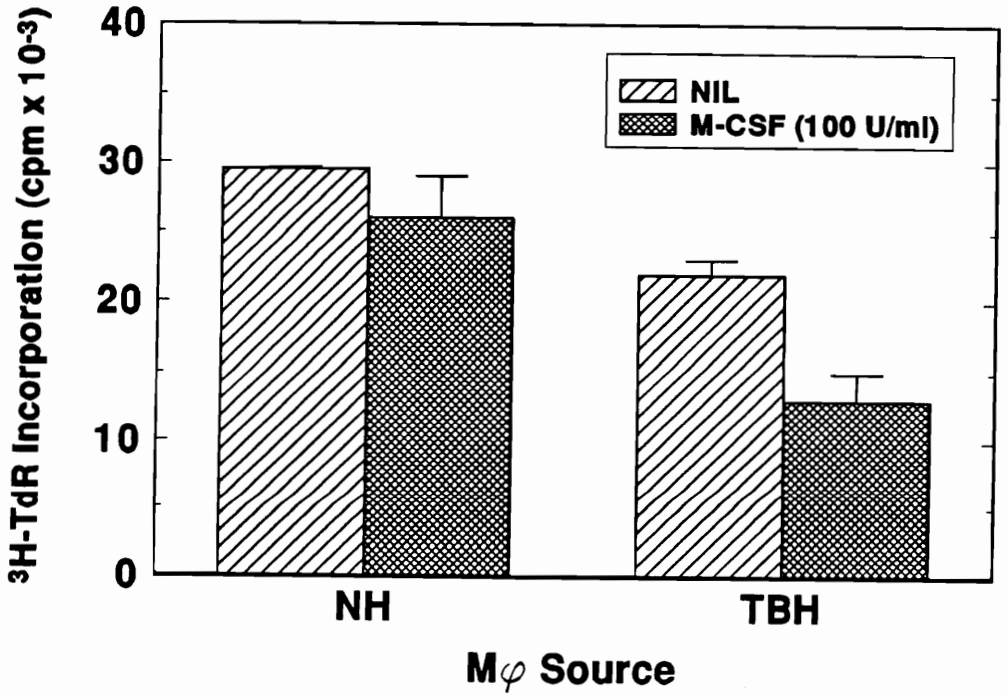

Figure 3. TBH Mϕ suppress T-cell reactivity against alloantigen through M-CSF: Two x 10^5 NH $CD4^+$ T cells (BALB/c) were stimulated with 4 x 10^5 X-irradiated lymphocytes (C3H) in the presence of NH or TBH Mϕ, and cultured for 4 days. M-CSF was added at a final concentration of 100 U/ml. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the Mϕ used, and the Y-axis shows the ^{3}H-TdR incorporation in proliferating alloreactive T cells. Background cpm for T cells or Mϕ alone were <802 cpm.

alloantigens in the presence of M-CSF. This suppression was most significant when high concentrations of TBH Mϕ were present (33,412 ± 412 to 16,648 ± 1,578, $p < 0.005$, 50.2% decrease). These data suggest M-CSF causes additional TBH Mϕ-induced suppression through mechanisms independent of PGE_2 synthesis.

Tumor growth inhibits Mϕ responsiveness to M-CSF during autorecognition

Tumor growth also compromises T-cell reactivity against syngeneic MHC class II molecules (autorecognition). We have previously shown that autorecognition is severely impaired when TBH Mϕ are used as the stimulator/accessory population (18, 484). To determine if M-CSF is involved in TBH Mϕ suppression of autorecognition, T cells were cultured with either NH or TBH Mϕ in the absence or presence of M-CSF (Figure 5). T-cell reactivity against syngeneic MHC class II molecules on NH Mϕ significantly increased (42%) in the presence of M-CSF (25,012 ± 1,003 vs. 35,517 ± 888, $p < 0.005$). However, T-cell reactivity against autoantigens on TBH Mϕ was unchanged (3% increase) in the presence of M-CSF (10,361 ± 344 vs. 10,672 ± 705). Inhibition of PGE_2 synthesis significantly increased T-cell autorecognition when either NH (43,614 ± 1,224) or TBH (15,001 ± 933) Mϕ were the stimulator/accessory population. When both M-CSF and indomethacin were present, T-cell autorecognition stimulated by NH Mϕ additively increased (51,988 ± 1,422). In contrast, T-cell autorecognition stimulated by TBH Mϕ was similar to levels obtained with indomethacin alone (14,926 ± 843). These findings suggest that tumor growth abrogates the stimulatory effect of M-CSF during T-cell recognition of syngeneic antigens.

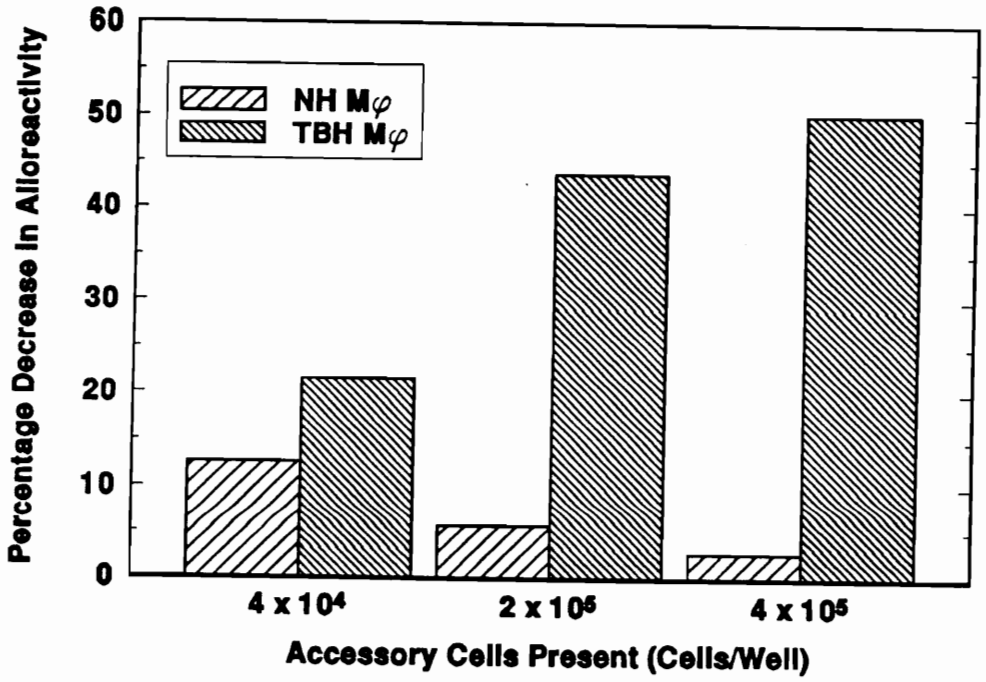

Figure 4. M-CSF-induced suppression of allorecognition occurs through TBH Mϕ by a pathway that is independent of PGE_2: Two x 10^5 NH $CD4^+$ T cells (BALB/c) were stimulated with 4 x 10^5 X-irradiated lymphocytes (C3H) in the presence of NH or TBH Mϕ, and cultured for 4 days. M-CSF was added at a final concentration of 100 U/ml. Indomethacin was added to wells at a final concentration of 10^{-7} *M*. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the concentration of Mϕ used, and the Y-axis shows the percentage decrease in alloreactivity as measured by ^{3}H-TdR incorporation. Background cpm for T cells or Mϕ alone were <758 cpm.

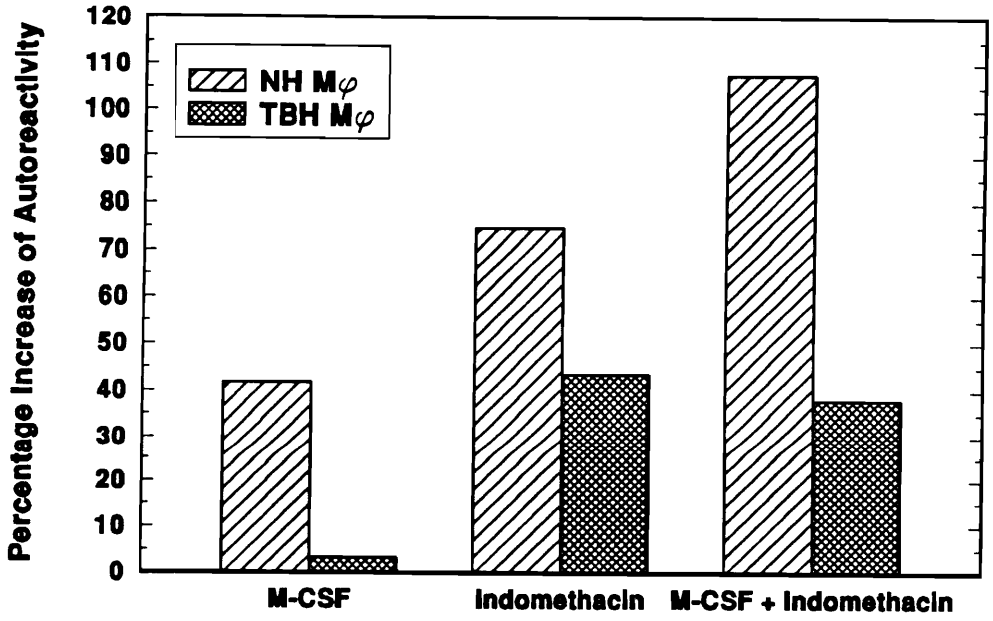

Figure 5. Tumor growth inhibits Mϕ responsiveness to M-CSF during autorecognition: Four x 10^5 NH $CD4^+$ T cells were stimulated with 4 x 10^5 X-irradiated NH or TBH Mϕ, and cultured for 4 days. M-CSF was added to wells at a final concentration of 100 U/ml. Indomethacin was added to wells at a final concentration of 10^{-7} *M*. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the treatment groups, Mϕ used, and the Y-axis shows the percentage increase in autoreactivity as measured by ^{3}H-TdR incorporation. Background cpm for T cells or Mϕ alone were <991 cpm.

TBH Mϕ suppression of autorecognition is not strictly due to decreased MHC class II molecule expression

Previous data show that TBH Mϕ suppress autorecognition through quantitative reductions in MHC class II marker expression and qualitative shifts in cytokine production (172, 443, 481, 483). Although autoreactivity is stimulated by Mϕ expression of MHC class II molecules, TBH Mϕ that express low amounts of class II molecules on their surfaces generate suppression though additional pathways because even low supplemental doses of TBH Mϕ significantly reduce reactivity in syngeneic cultures containing NH T cells and NH Mϕ. To observe if M-CSF has a role in the suppression caused by TBH Mϕ, T-cell reactivity against syngeneic antigens was assessed through admixture cultures containing NH T cells and NH Mϕ supplemented with low concentrations of either NH or TBH Mϕ (Figure 6). Supplemental Mϕ from NH did not significantly alter T-cell reactivity against syngeneic MHC class II molecules (25,783 ± 690 versus 25,130 ± 1,102; not significant). M-CSF slightly increased T-cell reactivity against syngeneic MHC class II molecules in the presence of supplemental NH Mϕ (28,955 ± 228). In contrast, supplemental TBH Mϕ significantly suppressed T-cell reactivity against syngeneic MHC class II molecules (25,783 ± 690 versus 13,092 ± 612; $p < 0.005$). M-CSF further suppressed autorecognition in the presence of supplemental TBH Mϕ (8,697 ± 644). These findings suggest TBH Mϕ suppression of autorecognition is not strictly due to decreased MHC class II molecule expression and that tumor growth alters Mϕ responsiveness to M-CSF.

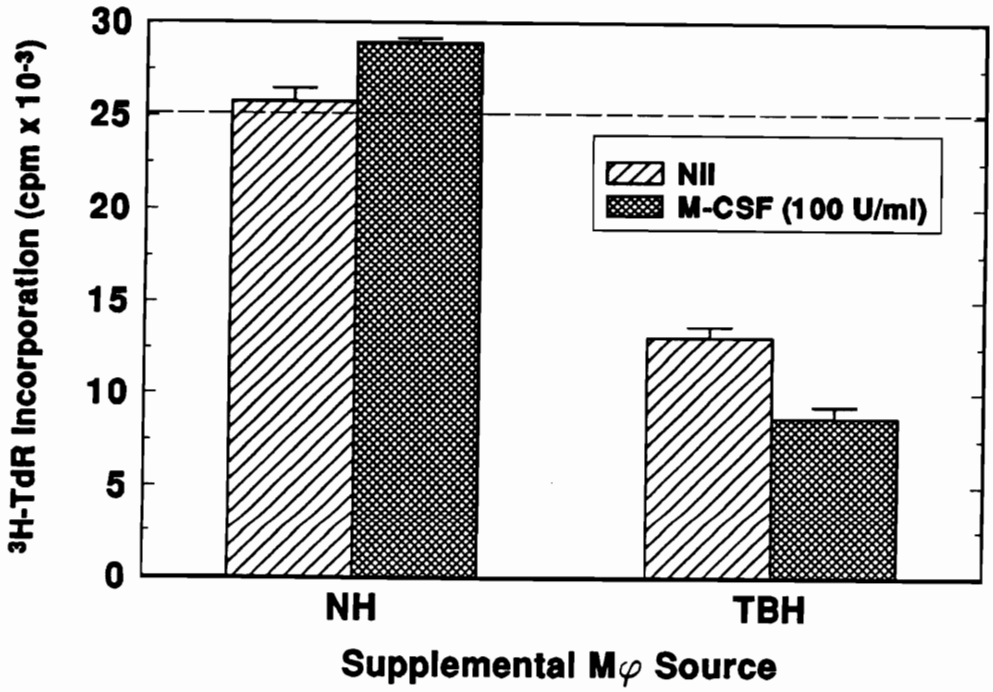

Figure 6. TBH Mϕ suppression of autorecognition is not strictly due to decreased MHC class II molecule expression: Four x 10^5 NH $CD4^+$ T cells and 4 x 10^5 X-irradiated NH Mϕ were co-cultured with supplemental Mϕ (either 4 x 10^4 NH Mϕ or 4 x 10^4 TBH Mϕ) for 4 days. M-CSF was added to wells at a final concentration of 100 U/ml. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the supplemental Mϕ source used, and the Y-axis shows T-cell reactivity against syngeneic antigen as measured by ^{3}H-TdR incorporation. The dashed line indicates T-cell reactivity against syngeneic antigens in the absence of supplemental Mϕ. Background cpm for T cells or Mϕ alone were <1,126 cpm.

DISCUSSION

Tumor growth triggers significant phenotypic and functional changes in Mϕ populations, and these changes directly suppress T-cell activities. In the present study, we addressed the significance of M-CSF during TBH Mϕ suppression of T-cell allorecognition and autorecognition. Because T cells that respond to allogeneic and syngeneic antigens are associated with helper, cytotoxic, and suppressor functions, they are considered to be integral components of a normal immune system (147, 273). Mϕ serve as accessory cells during allorecognition and autorecognition by providing regulatory cytokines. During autorecognition, Mϕ additionally serve as a source of syngeneic MHC class II molecules. Although T-cells that recognize allogeneic and syngeneic MHC class II molecules contribute to immunological homeostasis within the host, several immunocompromised states including tumor growth disrupt allorecognition and autorecognition. Mϕ are the primary suppressor cells of allorecognition and autorecognition, but the mechanism of suppression is not completely defined.

Previous data show that Mϕ MHC class II molecule expression decreases during tumor growth and that MHC class II-deficient TBH Mϕ are the predominant suppressor population. Furthermore, TBH Mϕ demonstrate altered responsiveness to cytokines and increased production of the inhibitory molecule PGE_2. We investigated the significance of M-CSF during TBH Mϕ suppression of T-cell allorecognition and autorecognition because M-CSF regulates Mϕ MHC class II molecule expression and PGE_2 synthesis. Mϕ suppressor functions elicited through M-CSF have been identified previously because Mϕ pre-treated with M-CSF significantly suppress mitogen- and antigen-stimulated lymphocyte proliferation *in vitro* (471). Additionally,

T-cell responses are significantly decreased during acute GvHR due to Mϕ production of and receptor expression for M-CSF (353). The current findings suggest M-CSF also elicits TBH Mϕ suppressor activity during T-cell allorecognition and autorecognition.

Although M-CSF did not change T-cell reactivity against allogeneic MHC class II molecules in the presence of NH Mϕ, M-CSF further decreased T-cell reactivity against syngeneic MHC class II molecules in the presence of TBH Mϕ. The change in Mϕ responsiveness to M-CSF may be associated with changes in constitutive M-CSF expression. Certain fibrosarcomas transcribe M-CSF and M-CSF receptors constitutively (133), and this continuous M-CSF saturation may initiate several suppressive activities in TBH Mϕ. Irrespective of this possibility, the data suggest that tumor growth alters Mϕ responsiveness to M-CSF. We are currently investigating M-CSF production in our fibrosarcoma model.

To determine if M-CSF elicited Mϕ suppressor activity through increased PGE_2 synthesis, cultures were treated with the PGE_2 synthesis inhibitor indomethacin in the presence of M-CSF. Although blocking TBH Mϕ PGE_2 synthesis restores T-cell allorecognition to control levels, indomethacin failed to reverse TBH Mϕ suppression when M-CSF was present. In fact, inhibition of TBH Mϕ PGE_2 synthesis during allorecognition led to further suppression. These findings confirm that TBH Mϕ have altered responsiveness to M-CSF and that M-CSF does not trigger Mϕ suppression through PGE_2 synthesis. Other investigations show that Mϕ responsiveness to TNF-α, GM-CSF, IFN-γ, IL-4, and IL-10 is also disrupted. TBH Mϕ production of TNF-α is associated with significant suppression of allorecognition and autorecognition (9). Because M-CSF induces TNF-α gene expression and protein synthesis (376), we are currently determining if tumor growth alters M-CSF-induced Mϕ production of TNF-α.

Mϕ responsiveness to M-CSF during autorecognition was also altered by tumor growth. Although M-CSF-exposed TBH Mϕ were not suppressive to autorecognition, they were much less responsive than their NH counterparts. Autoreactivity stimulated by NH Mϕ increased when M-CSF was present, whereas autoreactivity stimulated by TBH Mϕ was unaffected. Autorecognition is dependent on the expression of MHC class II molecules, and previous work has shown MHC class II molecule expression significantly decreases during tumor growth (483). Furthermore, MHC class II^- TBH Mϕ are highly suppressive (448). Although M-CSF suppresses basal levels of MHC class II molecule expression and inhibits class II molecule induction on bone marrow-derived Mϕ (469), M-CSF does not appear to affect MHC class II molecule expression among splenic Mϕ. Instead, M-CSF enhanced autorecognition and additively increased autorecognition in the presence of indomethacin. Because TBH Mϕ responses to indomethacin treatment alone were very similar to the responses for M-CSF and indomethacin treatment, the data suggest that M-CSF did not significantly alter TBH Mϕ activity during autorecognition and that the lack of reactivity to M-CSF during autorecognition is independent of PGE_2 synthesis. We hope to determine if TBH Mϕ fail to respond like their NH Mϕ counterparts because of abnormal IL-1-α production. Others have shown IL-1-α is a key immunoregulatory cytokine induced by M-CSF during tumor growth (133). Although IL-1 or IL-1 with IL-2 enhance autorecognition mediated by NH Mϕ, IL-1 has no effect on TBH Mϕ-mediated autoreactivity (18). This lack of responsiveness is significant because TBH Mϕ produce IL-1 transcripts in the absence of activation signals (David Askew, personal communication).

Although autorecognition is dependent on T-cell recognition of MHC class II molecules, we ruled out the possibility that tumor-induced suppression was caused strictly by decreased MHC class II molecule expression. T cells were stimulated with

NH Mϕ in the presence of supplemental Mϕ from either NH or TBH. We determined that T-cell reactivity against syngeneic antigens in the presence of supplemental TBH Mϕ was significantly lower than reactivity in the presence of supplemental NH Mϕ. However, this suppression cannot be due to low MHC class II molecule expression on the supplemental TBH Mϕ because sufficient amounts of syngeneic MHC class II molecules were expressed on the surfaces of the NH Mϕ stimulator cells. These data suggest that low concentrations of suppressor Mϕ can significantly alter Mϕ-T cell interactions involving M-CSF. This scenario may be especially significant *in vivo* when tumor growth initiates phenotypic changes among various Mϕ populations. Although the data suggest TBH Mϕ suppression of autorecognition is not solely due to low MHC class II molecule expression, we hope to determine using flow cytometry if M-CSF decreases TBH Mϕ MHC class II molecule expression.

In summary, the data suggest TBH Mϕ suppression of allorecognition and autorecognition is partly controlled by changes in Mϕ responsiveness to M-CSF. Identification of such changes in cytokine regulation will hopefully improve immunotherapies and provide a better understanding of cytokine signalling during immunocompromised disease states.

CHAPTER III

IA^- MACROPHAGES AND CYTOKINE NETWORKS CONTRIBUTE TO TUMOR-INDUCED SUPPRESSION OF $CD4^+$ AUTOREACTIVE T CELLS

ABSTRACT

Tumor growth changes the functions and phenotypes of Mϕ and T cells. Suppression of $CD4^+$ T cell autoresponses during tumor growth was contributed primarily by Mϕ. Tumor-induced alterations in the abilities of these cells to mediate autorecognition were assessed through syngeneic mixed lymphocyte reaction (SMLR) assays. TBH Mϕ were significantly more suppressive (60-90%) than normal host (NH) Mϕ, and this suppression was caused partly by reduced Ia expression. TBH Ia^- Mϕ were significantly more suppressive (50-80%) than their NH counterparts. The suppression mechanism was controlled partly by PGE_2, because treating cultures with indomethacin and titrated NH and TBH Ia^- Mϕ led to increased T-cell responsiveness, although responsiveness never reached levels of assays containing unseparated Mϕ. Blocking studies using anti-IFN-γ monoclonal antibodies (mAb), anti-interleukin 4 (anti-IL-4) mAb, and indomethacin suggested that IFN-γ, IL-4, and PGE_2 contributed to tumor-induced Mϕ-mediated suppression. Our results suggested that a quantitative shift in Mϕ phenotype and a qualitative shift in Mϕ function in addition to differences in cytokine-directed accessory activities are partly responsible for tumor-induced suppression $CD4^+$ T cell autoresponses.

INTRODUCTION

Mϕ are accessory cells with major histocompatibility complex markers encoded by the class II region genes (called Ia) and release soluble modulators that either stimulate or suppress T cells. In the SMLR, an *in vitro* measure of autoreactivity, Mϕ are the chief stimulatory/regulatory cells because of their Ia expression and release of soluble modulators (28, 85, 324, 336). Autoreactive T cells do not respond to foreign antigen such as fetal bovine serum in the culture medium (324) but are responsive to self Ia molecules (28, 85, 324, 336). This reaction to self is important because the host must recognize self before it can respond to nonself (230). Mϕ Ia presentation to autoreactive T cells is the first signal in autoreactive T cell regulation (336) because blocking mAb prevent T cell proliferation (324). Autoreactive T cells are L3T4$^+$ (CD4$^+$), Thy-1$^+$ (CD5$^+$), Ia$^-$, and Lyt-2$^-$ (CD8$^-$) (28, 106), and their immunoregulatory functions include help (28, 85, 324, 336), suppression (85, 260), and cytotoxicity (264, 423, 428). Autoreactive T cells also can stimulate naive CD4$^+$ T cells and participate in T-T cell interactions (246, 325). These functions suggest that autoreactive T cells are part of the body's immunoregulatory network and play a role in normal immune responsiveness.

Others have shown autoreactivity is defective in diseased hosts (128, 201, 257, 484), and our work supports this conclusion. We have shown that TBH Mϕ suppress autoreactive T cell responses and that this suppression is partly due to reduced phenotypic expression of Ia and increased PGE_2 secretion (484). TBH autoreactive T cells are less responsive to Mϕ stimulation and to the lymphokines IL-1, IL-2, and IL-4 (482). These findings suggest that tumor growth affects both Mϕ accessory function and autoreactive T cell activity.

Recently, we discovered that tumor-induced shifts in Ia$^-$ Mϕ are responsible for reduced CD4$^+$ T cell alloresponses to foreign class II major histocompatibility complex antigens[3]. The present study evaluated the mechanisms of tumor-mediated suppression during autorecognition. We examined the suppressive contribution of Ia$^-$ Mϕ to CD4$^+$ T cell autoresponses and the cytokine networks associated with Ia$^-$ Mϕ-mediated suppression of autorecognition. We showed that tumor growth leads to changes in Ia$^-$ Mϕ functions and that this cell type partly mediates CD4$^+$ T cell autoreactivity suppression through disrupted cytokine balances.

MATERIALS AND METHODS

Animals

The 8 to 12 week-old male BALB/c mice (Harlan-Sprague Dawley, Madison, WI) used were LDH virus negative (Microbiological Associates, Bethesda, MD). A single-cell suspension of 8 x 10^5 methylcholanthrene-induced, nonmetastatic fibrosarcoma cells was injected i.m. into the left hind leg of mice. Palpable tumors formed by days 10-14 which would kill the hosts by days 28-35 (123). Splenic immune cells from 21-day TBH mice were used throughout. Immunosuppression was not caused by a general inflammatory response, because normal BALB/c mice injected with 8 to 12 week-old C3H mouse muscle tissue (rather than tumor cells) had normal autoreactive responses (data not shown; 482, 484).

Medium

All cells were cultured in complete medium: RPMI-1640 (Hazelton, Denver, PA) medium with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO), 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$, and 25 *mM* HEPES (Research Organics, Cleveland, OH).

Flow Cytometry

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics, Hialeah, FL) laser flow cytometer and cell sorter. This instrument was calibrated with fluorescent 10-micron-sized microsphere standards. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc., Palo Alto, CA). Data were collected with the multiparameter data acquisition and display system (MDADS, Coulter Electronics). Fluorescein isothiocyanate- (FITC) labeled monoclonal antibodies (mAb) fluorescence was measured as the log of the green fluorescence.

Mϕ and CD4$^+$ T Cell Preparation

Briefly, BALB/c splenic Mϕ (>96% esterase positive) were collected after washing the plated spleen cells to remove nonadherent cells and scraping with a rubber policeman to remove the adherent cells (123, 482, 484). Treatment of Mϕ suspensions with anti-mouse dendritic cell mAb (American Type Culture Collection [ATCC], Rockville, MD; ATCC clone 33D1) did not significantly change autoreactivity (data not shown). To prepare Ia$^-$ Mϕ, Mϕ were treated with anti-IA^d mAb (ATCC clone MK-D6) and anti-IE^d mAb (ATCC clone 14-4-4) plus a 1:12 dilution of complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). The purity of the Ia$^-$ Mϕ population was confirmed by flow cytometric analysis showing >96% Ia$^-$ Mϕ. Mϕ (unseparated or Ia$^-$) were counted and resuspended to 8 x 10^6 cells/ml in complete medium. Mϕ were X-irradiated with 2000 rads (TFI Minishot II X-irradiator, New Haven, CT).

To collect $CD4^+$ T cells, nonadherent cells were eluted from plastic culture plates after a 2 hr incubation and depleted of Ia^+ B cells, Mϕ, and other contaminating cells by mixing with anti-Lyt-2 mAb (ATCC clone 3.155), anti-IA^d mAb (ATCC clone MK-D6), anti-immature T cell and B cell mAb (ATCC clone J11d), and anti-IE^d mAb (ATCC clone 14-4-4), and a 1:12 dilution of complement (>99% $CD4^+$ T cells as determined by fluorescence-activated cell sorter (FACS) analysis). The viable cells were resuspended to 8 x 10^6 cells/ml in complete medium.

SMLR

Four x 10^5 $CD4^+$ T cells/well were added to each well of a "U" bottom 96-well plate (Flow Laboratories, McLean, VA). Four x 10^5 unseparated Mϕ stimulator cells were also added per well. This combination comprised the "base" SMLR and was required for control level autoreactive T cell responsiveness (28, 85, 324, 336). Four x 10^4 (10%), 2 x 10^5 (50%), or 4 x 10^5 (100%) Mϕ/well were added to the SMLR. The percentage of Mϕ added to some SMLR represents the cell number compared to the number of normal host (NH) or TBH Mϕ stimulator cells added. Indomethacin was added to some SMLR cultures at a final concentration of 10^{-7} *M*. This concentration inhibits the arachidonic acid pathway for PGE_2 synthesis among all concentrations of SMLR cultures examined and is nontoxic to immune cells (284). Fifty μl of a saturating dose of anti-mouse IFN-γ mAb (ATCC clone R4-6A2) or anti-mouse IL-4 mAb (ATCC clone 11B11) were added to some of the SMLR cultures at the start of the 4-day incubation. The isotype controls (rat IgG1) for anti-IFN-γ mAb and anti-IL-4 mAb showed no effect on T cell autoresponses. Anti-IFN-γ mAb and anti-IL-4 mAb

specificity controls were done using 10 U of recombinant murine IFN-γ (4.5-9 x 10^6 units/mg @$\geq$22 μg/ml; Genzyme, Cambridge, MA) and 20 units of recombinant murine IL-4 (10^8 units/mg; Genzyme); both activities were inhibited by their appropriate mAb. SMLR cultures were incubated for 4 days at 37°C. Six hr before cell harvest, each culture was pulsed with tritiated thymidine (^{3}H-TdR: specific activity 6.7 Ci/*mM*, Dupont NEN Research Products, Boston, MA). Cells were harvested onto filters (Whatman 934-AH, Thomas Scientific, Phila., PA) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

Statistics/Calculation of Results

Pooled cells from 3 to 6 mice were used for each experiment. For the SMLR assays, three replicate wells were run for each test, and each experiment was repeated three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p<0.05$) and presented as mean$\pm$standard error.

RESULTS

Suppressed autorecognition is mainly caused by tumor-induced changes in Mϕ

To determine whether $CD4^+$ T cell autoresponses are suppressed during tumor growth by Mϕ and/or T cells, four different cell combinations were examined (Figure 7). First, NH $CD4^+$ T cells stimulated by NH Mϕ represented the standard SMLR reaction and served as an *in vitro* correlate of NH syngeneic responses. Second, NH $CD4^+$ T cell autoresponses stimulated by TBH Mϕ was roughly 90% less than $CD4^+$ T cells stimulated by NH Mϕ. Third, $CD4^+$ T cells from TBH had only a 29% decrease in autoreactivity when stimulated by NH Mϕ. Fourth, TBH $CD4^+$ T cell autoresponses to TBH Mϕ (the *in vivo* situation during tumor growth) had the lowest level of autoreactivity. These results suggest that suppressed autorecognition during tumor growth is mediated primarily by Mϕ (>60% of the suppression). An examination of TBH $CD4^+$ T cell-mediated suppression will be communicated separately.

Tumor-induced suppression of T cell autoresponses is partly due to decreased Ia expression on Mϕ

Because tumor growth can lead to reduced Ia expression on TBH Mϕ (117, 328, 482, 484), we wanted to determine if this defect altered autoreactivity (Figure 8). Splenic Mϕ Ia Ag expression decreased 58% during tumor development and gener-

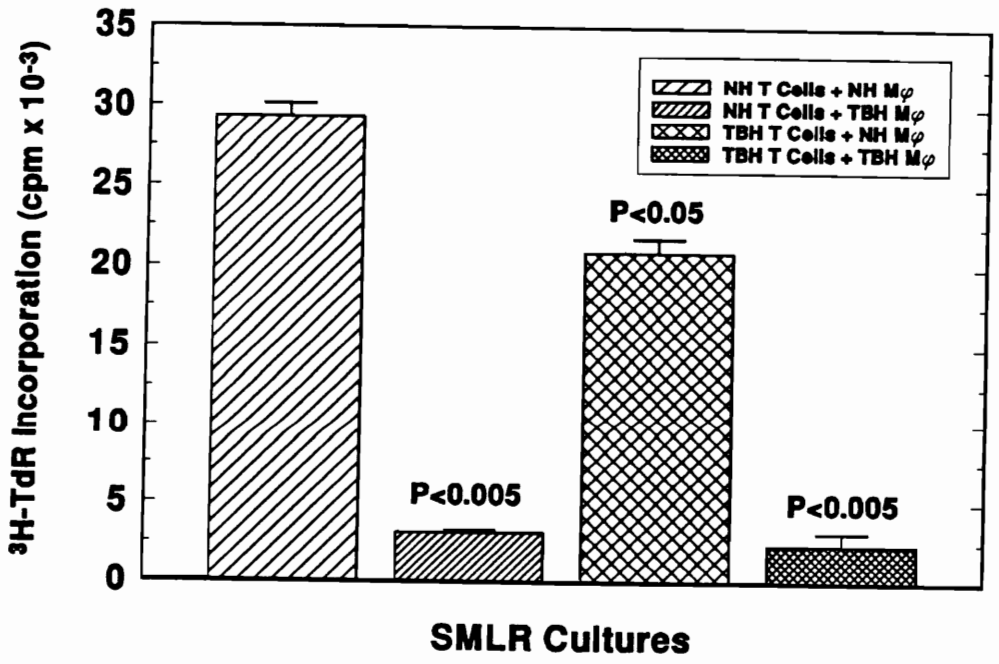

Figure 7. Autoreactivity during tumor growth is suppressed mainly through Mϕ: Four x 10^5 NH or TBH T cells were stimulated with either 4 x 10^5 NH or TBH Mϕ, and cultured for 4 days. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the Mϕ used, and the Y-axis shows the ^{3}H-TdR incorporation by proliferating autoreactive T cells. Background cpm for T cells or Mϕ alone were <650 cpm.

ated an influx of Ia$^-$ Mϕ with respect to the total population (44 $\pm$ 1% NH Ia$^-$ Mϕ versus 60 $\pm$ 5% TBH Ia$^-$ Mϕ, $p < 0.05$; data not shown). Autoreactivity stimulated by unseparated TBH Mϕ was significantly less (62% as reactive) than autoreactivity stimulated by unseparated NH Mϕ. Addition of NH or TBH Ia$^-$ Mϕ reduced CD4$^+$ T cell autoresponses to 3% and 4%, respectively, of CD4$^+$ T cell autoresponses stimulated by unseparated NH or TBH Mϕ. This result confirms the requirement for Mϕ Ia expression autoreactive CD4$^+$ T lymphocytes are unable to proliferate in the absence of this marker. The requirement for Ia activation of helper T cells was shown further by blocking Ia with specific antibody. T cell proliferation was inhibited when these cells were co-cultured with Mϕ and anti-IA^d and anti-IE^d mAb (data not shown).

NH and TBH Ia$^-$ Mϕ suppress autoreactive CD4$^+$ T cells

To investigate the importance of Ia$^-$ Mϕ to CD4$^+$ T cell autorecognition, NH and TBH Ia$^-$ Mϕ were added to SMLR cultures containing NH CD4$^+$ T cells and unseparated Mϕ (Figure 9). NH or TBH unseparated Mϕ additions to SMLR cultures served as crowding controls. Low, medium, and high doses of Mϕ were used because Mϕ activity is dose-dependent (92). The addition of NH or TBH Ia$^-$ Mϕ significantly ($p < 0.005$) suppressed CD4$^+$ T cell responsiveness in a dose-dependent manner (Figure 9A). TBH Ia$^-$ Mϕ were significantly ($p < 0.005$) more suppressive than their NH counterparts. A low dose (10%) of either NH or TBH Ia$^-$ Mϕ reduced CD4$^+$ T cell autoresponses by 4% and 52%, respectively, compared with 0% and 25%, respectively, when unseparated NH and TBH Mϕ (controls) were added. Similar patterns of suppression occurred when medium (50%) and high (100%) doses of NH or TBH Ia$^-$ Mϕ or medium and high doses of NH and TBH unseparated Mϕ were used. High

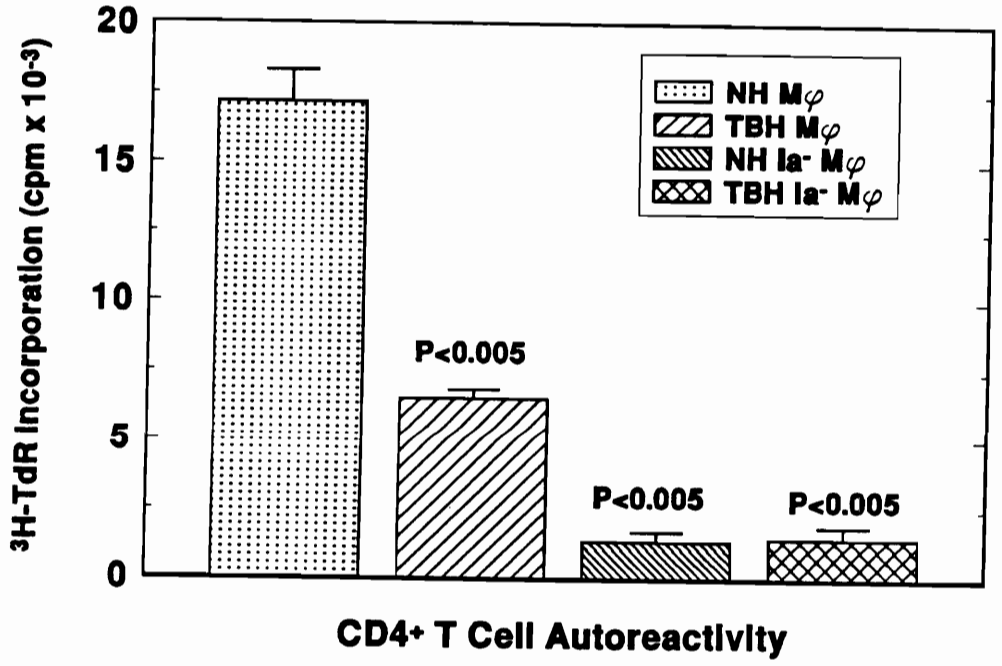

Figure 8. Ia expression is required for stimulation of CD4$^+$ autoreactive T cells: Four x 10^5 NH T cells were stimulated by 4 x 10^5 NH unseparated, TBH unseparated, NH Ia$^-$, or TBH Ia$^-$ Mϕ. The X-axis shows the Mϕ used, and the Y-axis shows the ^{3}H-TdR incorporation by proliferating autoreactive T cells. Background cpm for T cells or Mϕ alone were <650 cpm.

doses of NH and TBH Ia$^-$ Mϕ suppressed autorecognition by 69% and 97%, respectively, while NH and TBH unseparated Mϕ only suppressed autorecognition by 20% and 72%, respectively. Previous work showed that the observed Mϕ-mediated suppression was not caused by The above results were consistent with other studies and suggest that: (i) Ia$^-$ Mϕ are suppressor Mϕ (81), and (ii) tumor growth significantly increases Ia$^-$ Mϕ activity[3]. Suppression of autoreactive CD4$^+$ T cells by unseparated Mϕ populations was dose-dependent and consistent with previous data (92). The suppression is probably due to the Ia$^-$ Mϕ present in the unseparated groups.

PGE_2 is not the primary agent responsible for Ia$^-$ Mϕ-mediated suppression of autoreactivity

Because Mϕ-produced PGE_2 suppresses T cell proliferation and tumor growth increases PGE_2 secretion (103, 179, 285, 332), we examined the possibility that NH or TBH Ia$^-$ Mϕ-mediated suppression was due to PGE_2. When compared to untreated cultures (Figure 9A), SMLR cultures treated with indomethacin demonstrated increased T cell autoresponses (Figure 9B). Indomethacin-restored SMLR cultures with NH or TBH Ia$^-$ Mϕ were significantly ($p < 0.005$) less reactive than SMLR cultures containing additional NH or TBH unseparated Mϕ populations. Furthermore, indomethacin-treated SMLR cultures containing TBH Ia$^-$ Mϕ were significantly ($p < 0.005$) less reactive than indomethacin-treated SMLR cultures containing NH Ia$^-$ Mϕ. Because indomethacin had little or no effect on Ia$^-$ Mϕ function, PGE_2 may not be the primary agent responsible for Ia$^-$ Mϕ-mediated suppression of autoreactivity.

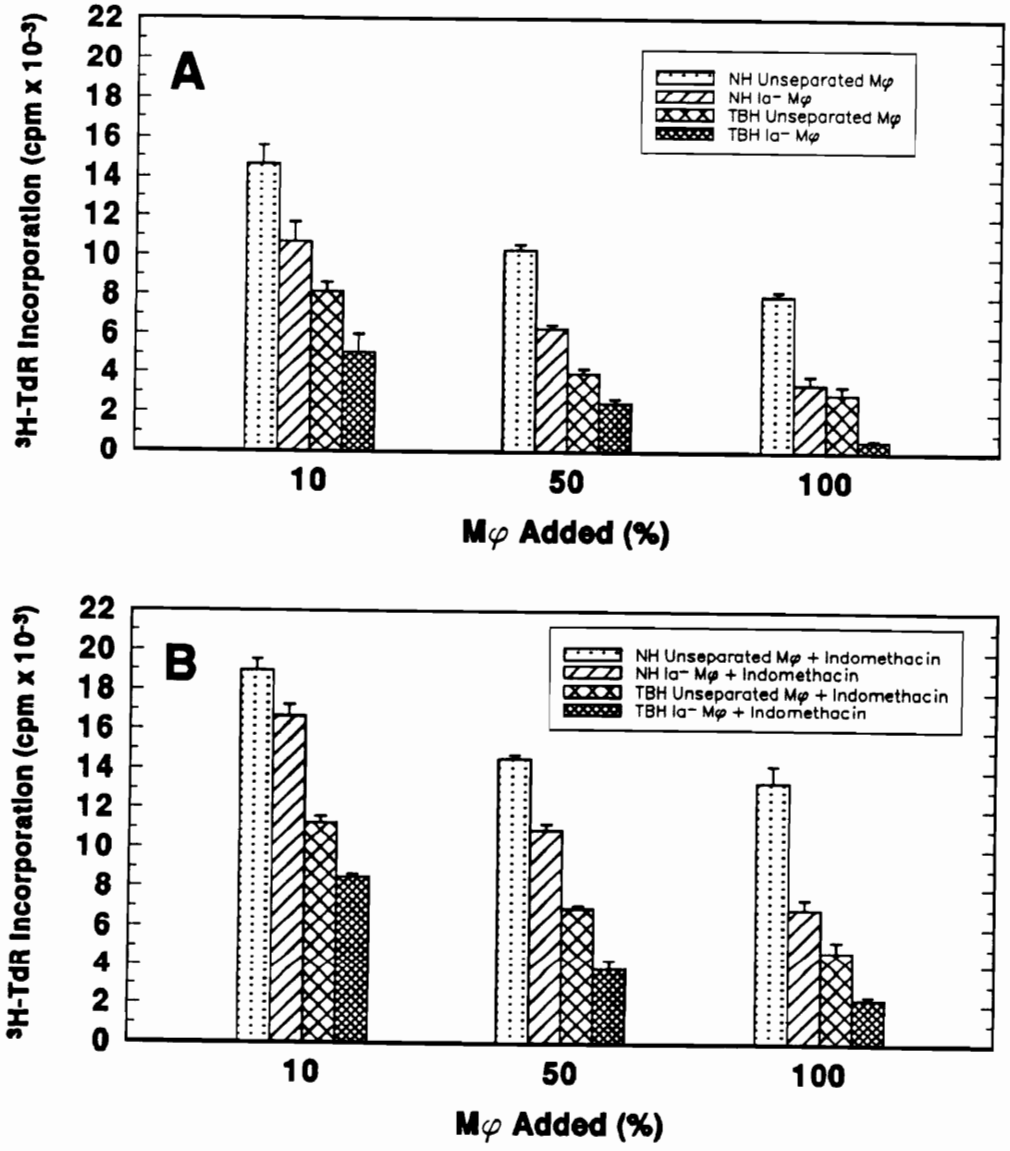

Figure 9. Ia$^-$ Mϕ quantitatively and qualitatively suppress autoreactivity: "Base" SMLRs containing 4 x 10^5 NH T cells and 4 x 10^5 NH unseparated Mϕ were supplemented with 4 x 10^4 (10% dose), 2 x 10^5 (50% dose), or 4 x 10^5 (100% dose) of NH unseparated, TBH unseparated, NH Ia$^-$, or TBH Ia$^-$ Mϕ. Panel A shows autoreactivity in the absence of indomethacin, and Panel B shows autoreactivity in the presence of 1 x 10^{-7} *M* indomethacin. The X-axis shows the percentage of additional Mϕ added, and the Y-axis shows the ^{3}H-TdR incorporation by proliferating CD4$^+$ autoreactive T cells. Control autoreactivity without additional Mϕ was 11,344 ± 710 cpm and 18,291 ± 1936 cpm for Panels A and B, respectively. Background cpm for T cells or Mϕ alone were <1230 cpm and <1470 cpm for Panels A and B, respectively.

Specific cytokines (PGE_2, IFN-γ, and IL-4) make different contributions to TBH Mϕ-mediated suppression of autoreactivity

In contrast to Ia^- Mϕ, unseparated Mϕ suppress autorecognition by releasing PGE_2. Partitioning $CD4^+$ T cells into T_H1 and T_H2 subpopulations shows that T_H1 cells synthesize IFN-γ (also produced by Mϕ) and T_H2 cells synthesize IL-4. To learn if tumor-induced changes in the synthesis and contribution of specific cytokines (PGE_2, IFN-γ, and IL-4) might mediate suppression of autoreactivity, the SMLR cell combinations of NH $CD4^+$ T cells and either NH or TBH Mϕ were cultured in the presence or absence of blocking agents against PGE_2, IFN-γ, and IL-4 (Figure 10). Indomethacin blocked endogenous PGE_2, and mAb specific for murine IFN-γ and IL-4 blocked endogenously-produced IFN-γ and IL-4. Indomethacin treatment of TBH Mϕ caused an almost 2-fold increase (3119$\pm$175 cpm to 8763$\pm$392 cpm) in autoreactivity; however, this increase represented approximately 30% and 15% of T cell autoresponses stimulated by untreated and treated NH Mϕ (Figure 10A). Blocking IFN-γ by specific mAb showed that IFN-γ plays a major role (roughly a 5-fold increase) in TBH Mϕ-stimulated T cell autoresponses when compared to their untreated counterparts (Figure 10B). However, the TBH Mϕ-stimulated autoresponses did not return to levels comparable to either untreated or antibody-treated NH Mϕ-stimulated T cell autoresponses. These results suggest IFN-γ may suppress T cell autoreactivity cooperatively with another mediator. However, anti-IL-4 mAb treatment decreased autoreactivity induced by NH Mϕ but did not decrease TBH Mϕ-induced autoreactivity (Figure 10C). Although IL-4 may be necessary for autorecognition in the NH system, the results suggest IL-4 has only a marginal involvement in the TBH system.

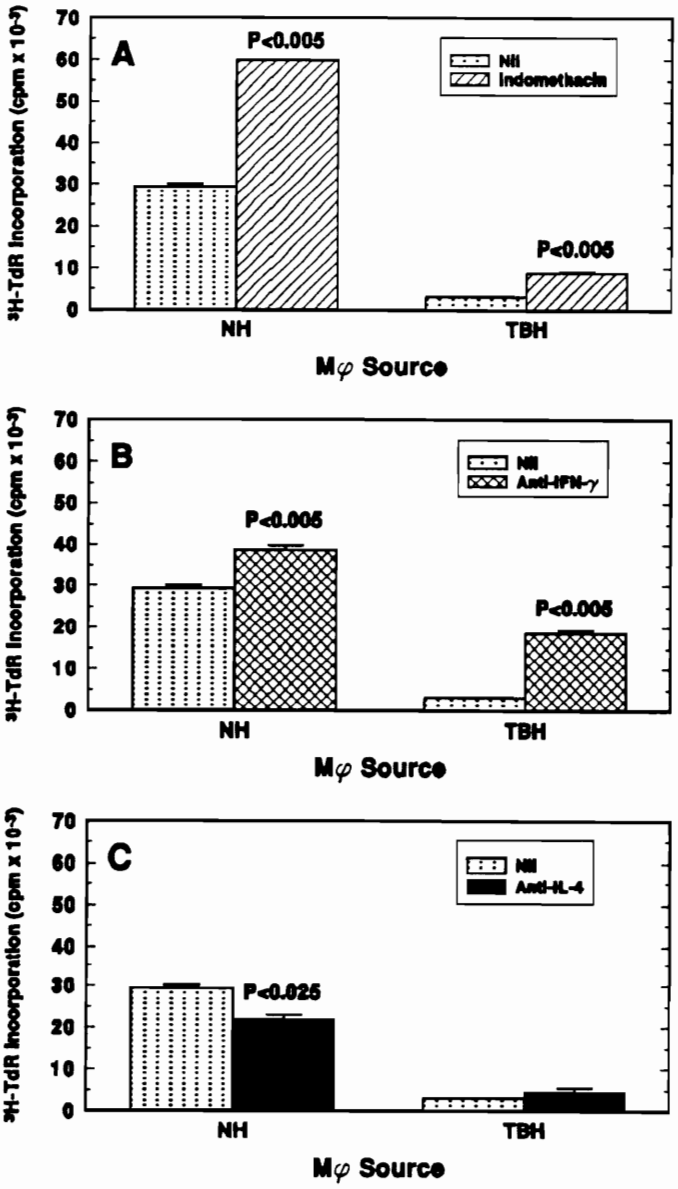

Figure 10. PGE_2, IFN-γ, and IL-4 offer different contributions to TBH Mϕ-mediated suppression of T cell autoresponses: Four x 10^5 NH T cells were stimulated by 4 x 10^5 NH or TBH unseparated Mϕ in the absence or presence of 10^{-7} *M* indomethacin (Panel A), 10 neutralizing units anti-IFN-γ mAb (Panel B), or 20 neutralizing units anti-IL-4 mAb (Panel C). In all panels, the X-axis shows the Mϕ used, and the Y-axis shows the ^{3}H-TdR incorporation in proliferating autoreactive T cells. Background cpm for T cells or Mϕ alone were <1547 cpm.

Disrupted cytokine networks involving PGE_2, IFN-γ, IL-4 affect TBH Mϕ-mediated suppression of autoreactivity

The results of the preceding blocking experiments led us to examine whether interplays between PGE_2, IFN-γ, and IL-4 also contribute to tumor-induced alterations of autoresponses. SMLR cultures were treated with four combinations of blocking agents (Figure 11): (i) indomethacin and anti-IFN-γ mAb, (ii) indomethacin and anti-IL-4 mAb, (iii) anti-IFN-γ and anti-IL-4 mAb, and (iv) indomethacin plus anti-IFN-γ and anti-IL-4 mAb. Three of the four treatments significantly increased autoresponses in the presence of NH or TBH Mϕ (Figure 11, panels A, B, and D). In all groups, the percentage of increased autoreactivity caused by TBH Mϕ was significantly higher than their untreated counterparts. This finding suggests that PGE_2, IFN-γ, and IL-4 have altered roles in TBH. Only the group treated with anti-IFN-γ mAb and anti-IL-4 mAb (Figure 11C) had reduced $CD4^+$ T cell autoresponses. Comparing the latter treatment group to either the anti-IFN-γ mAb or anti-IL-4 mAb alone (Figure 10, panels B and C), suggests that IL-4 is a required chemical signal in autoreactivity caused by NH Mϕ but may play no role in the suppressed autoreactivity caused by TBH Mϕ. Only the combination of indomethacin plus anti-IFN-γ and anti-IL-4 mAb restored TBH Mϕ-stimulated T cell autoresponses to untreated NH Mϕ-stimulated T cell autoresponses, but not to the levels of their treated NH counterparts. Other comparisons between combination and single treatments help explain the participation of and the interactions among PGE_2, IFN-γ, and IL-4 in tumor-induced Mϕ-mediated suppression of autorecognition. Comparisons of cultures with indomethacin alone (Figure 10A) or indomethacin plus anti-IFN-γ mAb (Figure 11A) show that blocking IFN-γ activity decreases autorecognition when NH Mϕ are used

but enhances it when TBH Mϕ are used. The interplay between PGE_2 synthesis and IL-4 activity is important because the addition of indomethacin (Figure 11B) reverses the blocking of IL-4 activity (Figure 10C). In fact, the addition of anti-IL-4 mAb with indomethacin restores NH Mϕ-stimulated $CD4^+$ T cell autoresponses to indomethacin alone levels (Figure 10A) and TBH Mϕ-stimulated $CD4^+$ T cell autoresponses to NH untreated levels (Figure 11B).

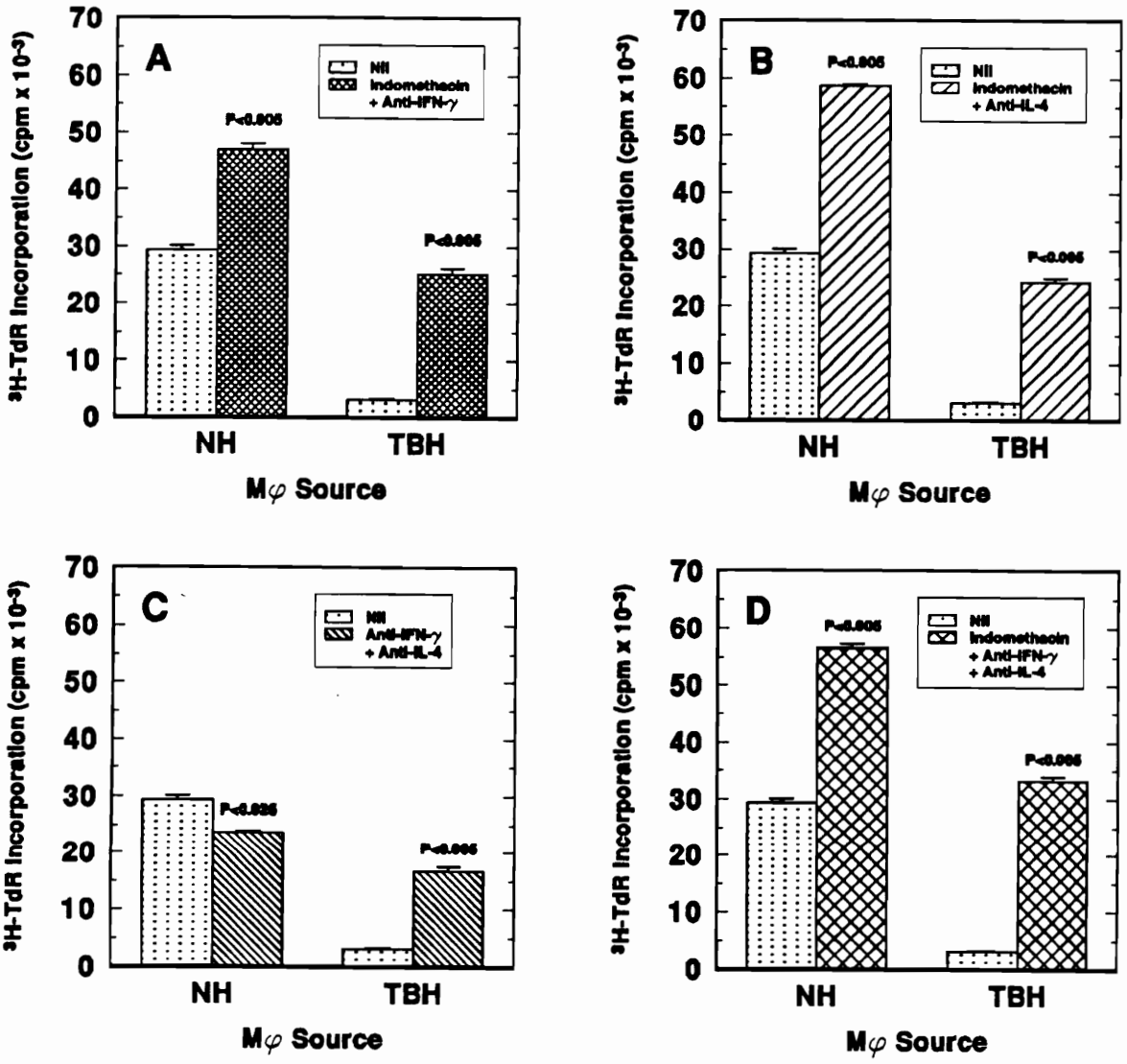

Figure 11. PGE_2, IFN-γ, and IL-4 interact during tumor-induced Mϕ-mediated suppression of T cell autoresponses: Four x 10^5 NH T cells were stimulated by 4 x 10^5 NH or TBH unseparated Mϕ in the absence or presence of 10^{-7} *M* indomethacin and 10 neutralizing units anti-IFN-γ mAb (Panel A), indomethacin and 20 neutralizing units anti-IL-4 mAb (Panel B), anti-IFN-γ mAb and anti-IL-4 mAb (Panel C), or indomethacin, anti-IFN-γ mAb and anti-IL-4 mAb (Panel D). In all panels, the X-axis shows the Mϕ used, and the Y-axis shows the ^{3}H-TdR incorporation in proliferating autoreactive T cells. Background cpm for T cells or Mϕ alone were <1547 cpm.

DISCUSSION

In this work, we examined the importance of TBH Ia$^-$ Mϕ in suppressing autoreactive CD4$^+$ T cells and analyzed suppression mediated through changes in some relevant chemical messengers. Although tumor growth can affect antigenic expression and activities of tumor-associated macrophages, tumors also induce phenotypic and functional changes in Mϕ derived from several tissues away from the tumor site such as the spleen, peritoneum, lungs, and liver. In the present study we examined helper T cell autoresponses using four different splenic T cell-Mϕ combinations. We found that >60% of the tumor-induced suppression of autoreactivity is mediated by Mϕ. Autorecognition is driven by self-Ia molecules (as expressed on syngeneic Mϕ) and tumor growth causes decreased Ia expression in Mϕ (172, 328, 482, 484). Decreased T cell autoresponses are caused by a lack of Ia molecules to drive the response, but data from our laboratory suggest decreased Ia expression is only partly responsible for reduced autorecognition (484). We presented data here to confirm this notion. Others have shown that Ia$^-$ Mϕ in the NH can act as the final suppressor cell in suppression of cytotoxic T-cell reactivity (81), and that tumor growth can initiate shifts among Mϕ from an Ia$^+$ to Ia$^-$ phenotype (327, 328). This shift changes Mϕ from an enhancing to a suppressive class of cells. Our earlier studies support these findings when we described a mechanism for tumor-induced suppression of CD4$^+$ T cells to foreign class II antigens[3]. We also showed a quantitative and qualitative shift in TBH Ia$^-$ Mϕ; that is, there is a two- to threefold increase in *in vivo* TBH Ia$^-$ Mϕ numbers and an increase in TBH Ia$^-$ Mϕ-mediated suppression of alloreactivity. In this current study, we showed that Ia$^-$ Mϕ significantly suppressed autoreactive T cell responsiveness and that tumor growth induced this significant

shift toward suppressor Mϕ function. Although direct comparisons between Ia$^+$ and Ia$^-$ Mϕ were not performed due to poor yields acquired from cell sorting, the use of unseparated Mϕ (a mixture which contained Mϕ bearing Ia antigens) clearly showed that Ia expression is necessary for optimum reactivity.

The quantitative loss of Mϕ Ia markers may be caused by PGE_2 (327, 328, 395), transforming growth factor-β (98), and/or macrophage colony-stimulating factors (M-CSF) (469). These cytokines can down-regulate Ia expression and are produced in excess in the TBH by Mϕ and tumor cells. In our experimental system, however, Ia$^-$ Mϕ were not acting alone as accessory cells, because they were being titrated into cultures containing Ia$^+$ Mϕ. If a defect in Ia expression was the only difference between Ia$^+$ and Ia$^-$ Mϕ, then T cell autoresponses would not be suppressed by Ia$^-$ Mϕ because the Ia$^+$ Mϕ would be able to express and present Ia to autoreactive T cells. Even the lowest dose of Ia$^-$ Mϕ suppressed autoreactivity, which suggests soluble suppressor factors are also involved. Although excess PGE_2 may have quantitatively reduced Ia expression during tumor growth, it is not the primary agent responsible for Ia$^-$ Mϕ-mediated suppression of autoreactivity. The relationship between altered Mϕ phenotype and cytokine regulation that occurs during tumor growth is not completely understood, but molecular analyses of Ia expression currently is being conducted in our laboratory and should provide further insight into this relationship.

We and others have shown that PGE_2 does contribute to suppression induced by unseparated TBH Mϕ. Mϕ-produced PGE_2 can control T cell reactivity by down-regulating IL-1, IL-2, and IL-4 responsiveness (436, 482, 488), reducing IL-2 receptor (436) and Ia expression (327),

and inhibiting IL-1 production (266). Indomethacin treatment significantly enhances all groups of cell combinations; however, indomethacin addition to cultures with TBH

Mϕ did not increase T cell autoresponses to NH levels, probably because TBH Mϕ secrete other suppressor factors besides PGE_2 (20, 81, 103, 284). Mϕ products such as IFN-γ and M-CSF can shift Mϕ phenotypes and/or functions during tumor growth (469). Although such molecules can act directly on Mϕ and T cells, they can modulate autorecognition indirectly by altering the contribution of accessory cells to the $CD4^+$ T cells. $CD4^+$ T cells have two subpopulations: T_H1 cells produce IFN-γ while T_H2 cells produce IL-4 (79). Although exceptions to this secretory pattern exist (340), these two molecules appear to act antagonistically through the regulation of IL-1, a potent activator of monocytes and Mϕ (116). During tumor growth, IFN-γ and IL-4 offered different contributions to autorecognition. IFN-γ alone was suppressive and was associated with PGE_2-induced suppression, but IL-4 appeared to be a required cytokine for optimal autoregulation because autoresponses decreased when anti-IL-4 mAb was used with NH Mϕ and $CD4^+$ T cells. However, IL-4 offered no direct contribution to decreased TBH Mϕ-mediated autoresponses. Blocking of all three molecules (PGE_2, IFN-γ, and IL-4) returned TBH responses to levels comparable to NH responses, which suggests these molecules interact through a partially characterized network. IL-4 can suppress production of IFN-γ (343). Therefore, the absence or decreased production of IL-4 by TBH Mϕ may allow for the dysregulation of IFN-γ and its induced agent, PGE_2. Both IFN-γ and IL-4 can up-regulate MHC class II antigens (96, 116, 146, 255, 352), but tumor growth may disrupt these properties as well.

Although we did not investigate changes in the autoreactive T cells themselves, previous data showed that autoreactive T cells are less responsive to interleukins (482) and may suppress immune responsiveness against tumors (264). In addition, we currently are assessing the contribution of TBH-derived Ia^+ Mϕ. In the experimental system presented here, base cultures consisted of Ia^+ and Ia^- Mϕ, but only the Ia^- Mϕ were considered to function as modulators of activity. Although Ia^+ Mϕ

serve as helper Mϕ, the unseparated Mϕ population is contaminated with a significant number of Ia$^-$ Mϕ. As a result, Ia$^+$ helper Mϕ functions are masked by the overriding suppression mediated by Ia$^-$ suppressor Mϕ. The question is not whether Ia$^+$ Mϕ are helper Mϕ, which indeed they are for autoreactive T cells (336), but does tumor growth also change TBH Ia$^+$ Mϕ function? This question is now under study using sorted Ia$^+$ Mϕ.

The results of this study lead us to conclude that tumor growth alters the contribution of Mϕ to autoreactivity. TBH Ia$^-$ Mϕ were significantly more suppressive than their NH counterparts and this fact, coupled with increased numbers *in vivo*, suggests a potential cause of suppression of autoreactivity. In summary, these findings suggest that tumor growth leads to at least two distinct but not mutually exclusive mechanisms of attack on autoreactive T cells. Questions remain. What are the molecular mechanisms controlling the changes in Ia$^-$ Mϕ? Can the changes be reversed or prevented? We are now doing experiments at the molecular level to try to answer these questions. Further understanding of tumor-induced suppression mechanisms will help direct the development of new therapeutic strategies aimed at interrupting this destructive cycle of immune system suppression and tumor growth.

CHAPTER IV

TUMOR GROWTH CHANGES THE CONTRIBUTION OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR DURING MACROPHAGE-MEDIATED SUPPRESSION OF ALLORECOGNITION

ABSTRACT

TBH Mϕ suppress T-cell alloresponses, and this study suggests GM-CSF, a molecule associated with suppressive Mϕ activity during tumor growth, signals more immunosuppression. In the absence of Mϕ, GM-CSF increased T-cell proliferation in response to alloantigen. However, TBH Mϕ-mediated suppression of allorecognition was further induced by GM-CSF. Allogeneic mixed lymphocyte reaction (MLR) cultures, containing NH Mϕ, were either unaffected or enhanced. PGE_2, a highly suppressive monokine that decreases alloreactivity, did not seem to be involved in the suppression caused by the TBH Mϕ/GM-CSF interaction. M-CSF addition to cultures did not reverse the suppression caused by TBH Mϕ and GM-CSF, and inhibition of PGE_2 synthesis did not change the response to M-CSF. TBH Ia^- Mϕ, a suppressor population that predominates among splenic Mϕ during tumor growth, demonstrated significantly lower reactivity in the presence of GM-CSF. In contrast, alloresponses suppressed by NH Ia^- Mϕ demonstrated higher reactivity in the presence of GM-CSF. The data collectively suggest that TBH Mϕ respond differently to GM-CSF, and that tumor-induced changes in GM-CSF responsiveness affect Mϕ accessory ability.

INTRODUCTION

GM-CSF is a regulatory hemopoietic glycoprotein produced by many cell types such as Mϕ, T lymphocytes, B lymphocytes, and fibroblasts (188, 241). Besides stimulating the development of Mϕ and granulocyte precursors, GM-CSF can induce the proliferation and activation of mature immune cells. GM-CSF can trigger the proliferation of T cells and Mϕ (74, 80, 267), the synthesis of IL-1 (226, 314, 394), TNF-α (206, 226), and PGE_2 (206), and the induction of tumoricidal activity (195). GM-CSF also increases antigen presentation capacity and class II marker expression on monocytes and Mϕ (153, 394, 469). GM-CSF is not constitutively synthesized, but can be induced by many substances such as LPS (34, 420), TNF-α (385), IL-1 (385), PGE_2 (354), and fetal bovine serum (FBS) (188, 420). Because GM-CSF may be associated with Mϕ suppressor activities, we investigated the possibility that tumor growth amplifies GM-CSF-induced suppressor activity.

We have shown tumor growth significantly disrupts Mϕ accessory activity during T-cell allorecognition (17). Allorecognition is the ability of $CD4^+CD8^-$ T cells to respond to foreign MHC surface molecules. Mϕ serve as accessory cells to T cells during alloresponses by secreting both enhancer (IL-1) and suppressor (PGE_2) cytokines. Tumor-induced Mϕ-mediated suppression of allorecognition is facilitated by several mechanisms. Tumor growth changes Mϕ responsiveness to specific stimuli. Splenic TBH Mϕ become significantly more suppressive when they are pre-exposed to LPS (485). Accessory activity also is disrupted through altered synthesis and/or release of PGE_2, a negative regulator of class II marker expression and Mϕ function (164, 395). TBH Mϕ-derived PGE_2 causes allogeneic hyporesponsiveness by reducing monocyte/Mϕ MHC class II antigen expression and inhibiting T-cell prolif-

eration (172, 173, 284). In addition to affecting Mϕ and T cells, PGE_2 can promote further tumor growth *in vivo* (481). Tumor-induced phenotypic changes in Mϕ size and antigen expression (481) also are attributable to Mϕ-mediated suppression. Reduced Mϕ Ia antigen expression correlates with suppressive activity (172, 328, 482, 484), and data suggest that Ia^- Mϕ are a primary cell type responsible for promoting tumor growth (237).

Several human and murine tumors respond to and/or produce GM-CSF without stimulation (14, 33, 378, 408, 427, 479), and some investigators correlate the production of GM-CSF by these tumors with the suppressive activities of monocytes and Mϕ (426). The present study supports the correlation between GM-CSF and Mϕ suppressor activity and demonstrates that TBH Mϕ are highly suppressive to allorecognition in the presence of GM-CSF. Because of this change in Mϕ activity, we hypothesize that tumors may evade immune cell defense mechanisms by disrupting the accessory and effector activities induced by GM-CSF.

MATERIALS AND METHODS

Animals

The 8 to 12 week-old male BALB/c mice (Harlan-Sprague-Dawley, Madison, WI) used were LDH virus negative (Microbiological Associates, Bethesda, MD). A single-cell suspension of 4 x 10^5 methylcholanthrene-induced, nonmetastatic, fibrosarcoma cells was injected i.m. into each mouse's left hind leg. Palpable tumors form by days 10-14 and kill the TBH by days 28-35 (123). Splenic immune cells from 21-day TBH mice were used throughout. Immunosuppression was not caused by a general inflammatory response (172, 173). Irradiated splenic lymphocytes from 8 to 12 week-old male C3H mice (Harlan-Sprague-Dawley) were used as the source of stimulator cells for allogeneic MLR cultures.

Medium

NH and TBH cells were cultured in complete RPMI-1640 (JRH Scientific, Denver, PA) medium with 10% heat-inactivated FBS (Gibco, Grand Island, NY), 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO), 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$, and 25 *mM* HEPES buffer (Research Organics, Cleveland, OH).

Chemicals and Monoclonal Antibodies

Indomethacin (Sigma) was resuspended in 70% ethanol at 10^{-2} *M* and stored at 4°C. Unless specified, indomethacin was added to cultures at a final concentration of 10^{-7} *M*. This concentration inhibits the arachidonic acid pathway for PGE_2 synthesis and is nontoxic to immune cells (284). The following hybridoma cell lines (American Type Culture Collection [ATCC], Rockville, MD) were used to produce monoclonal antibodies (mAb): anti-IA^d (ATCC clone MK-D6), anti-IE^d (ATCC clone 14-4-4), anti-immature T cell and B cell (ATCC clone J11d), and anti-Lyt-2 (ATCC clone 3.155). All mAb were used as ammonium sulfate-precipitated fractions of culture supernatants.

Cytokines

Recombinant murine GM-CSF and M-CSF were generously provided by Genetics Institute (Cambridge, MA). GM-CSF was purified from Cos cells transfected with a plasmid expressing murine GM-CSF. GM-CSF activity (1×10^6 U/ml) and M-CSF activity (1.65×10^6 U/ml) were determined by half maximal colony formation in the murine bone marrow colony assay. Aliquots were stored at -70°C until use.

Mϕ and T Lymphocyte Preparation

Murine spleens were pulped using a stomacher (Tekmar, Cincinati, OH) and the resulting cell suspensions were centrifuged and resuspended in RPMI-1640 medium. Erythrocytes were lysed with cold 0.83% NH_4Cl for 5 min. Cells were washed and incubated for 2 hr at 37°C and 5% CO_2 on tissue culture plates (150 × 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL). To collect $CD4^+$ T lymphocytes, nonadherent cells were eluted from the plates and further depleted of B cells, Mϕ, and other contaminating cells by adding anti-Lyt-2, anti-IA^d, anti-immature T lymphocyte and B cell, and anti-IE^d mAb, and a 1:12 dilution of complement (Cedarlane Laboratories, Ontario, Canada). Purified $CD4^+$ T lymphocytes (>99% $CD4^+$ T cells as determined by fluorescence-activated cell sorter [FACS] analysis) were washed at least three times to remove residual complement, and the viable cells were resuspended to 4 x 10^6 cells/ml in complete medium. Mϕ (>96% esterase positive) were collected (after washing to remove nonadherent cells) by scraping using a rubber policeman and ice-cold RPMI-1640. Ia^- Mϕ were prepared by treating the cells with anti-IA^d plus a 1:12 dilution of complement. The purity of the Ia^- Mϕ population was confirmed by flow cytometric analysis showing >96% Ia^- Mϕ. Mϕ (unseparated or Ia^-) were counted and resuspended to 8 x 10^6 cells/ml in complete medium. Mϕ were X-irradiated with 2000 rads (TFI Minishot II X-irradiator, New Haven, CT). Nonadherent C3H allogeneic stimulator cells were collected from plates, resuspended to 8 x 10^6 cells/ml in complete medium, and X-irradiated with 2000 rads.

Allogeneic MLR

BALB/c (H-2^d) $CD4^+$ T cells (2 x 10^5 responder cells) and C3H (H-2^k) splenic lymphocytes (4 x 10^5 stimulator cells) were added per well to "U" bottom 96-well tissue culture plates (Nunc, Roskilde, Denmark). This base number served as a control level alloresponse. When Mϕ (either unseparated or Ia^-) were added as accessory cells to the MLR, the percentages of Mϕ added (4 x 10^4 [10%], 2 x 10^5 [50%], or 4 x 10^5 [100%] cells/well) were respective to the number of C3H stimulator cells present and represented low, medium, and high doses. Cultures were incubated for 4 days at 37°C and 5% CO_2. Six hr before cell harvest, each culture was pulsed with tritiated thymidine (^{3}H-TdR: specific activity 6.7 Ci/*mM*, Dupont NEN Research Products, Boston, MA). Cells were harvested onto filters (Whatman 934-AH, Thomas Scientific, Philadelphia, PA) using a MASH harvester. Samples were placed into counting vials (Skatron, Sterling, VA) with Scintilene (Thomas Scientific, Swedesboro, NJ) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

Statistics/Calculation of Results

Pooled cells from 3 to 6 mice were used for each experiment. Three replicate wells were run for each test, and each experiment was repeated at least three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p<0.05$) and presented as mean$\pm$standard error.

RESULTS

GM-CSF contributes to tumor-induced Mϕ-mediated suppression of allorecognition

Previous findings (172, 173, 485) suggest that TBH Mϕ suppress T-cell proliferation during allorecognition. This suppression is greatest when high percentages of Mϕ are added to the cultures. We wanted to determine if GM-CSF, a molecule associated with suppressive Mϕ activity, could trigger more suppression during TBH Mϕ-mediated suppression of alloresponses. Allogeneic MLR cultures were supplemented with low, medium, and high doses of NH and TBH Mϕ in the presence of GM-CSF (100 U/ml) (Table 3). GM-CSF increased alloreactivity in the absence of Mϕ, which confirms that GM-CSF augments T-cell proliferation. GM-CSF did not significantly change alloresponses when low doses of NH or TBH Mϕ served as accessory cells, although activity was still lower than alloresponses without accessory cells. GM-CSF further suppressed alloresponses when medium doses of TBH Mϕ were present, whereas alloresponses with NH Mϕ accessory cells were unaffected by GM-CSF addition. A high dose of TBH Mϕ significantly suppressed T-cell alloresponses, but GM-CSF did not change the suppression caused by this Mϕ dose. In contrast, GM-CSF significantly increased alloresponses when a high dose of NH Mϕ was present. However, reactivity did not return to levels comparable to alloresponses in the absence of Mϕ. Thus, GM-CSF appears to signal further TBH Mϕ-mediated suppression of allorecognition, and at high doses TBH Mϕ are unresponsive.

Table 3. GM-CSF Contributes to tumor-induced Mϕ-mediated suppression of allorecognition

Mϕ Source	Treatment	Accessory Cells Present (cells/well)[a]			
		0	4×10^4	2×10^3	4×10^3
-----	-----	67.55 ± 2.81[b]	-----	-----	-----
-----	GM-CSF[c]	83.04 ± 2.64	-----	-----	-----
NH	-----	-----	76.44 ± 2.85	63.52 ± 2.77	36.71 ± 0.06
NH	GM-CSF	-----	62.09 ± 2.97	63.29 ± 4.58	47.91 ± 2.33
TBH	-----	-----	65.66 ± 2.28	52.63 ± 2.44	33.72 ± 2.21
TBH	GM-CSF	-----	71.07 ± 2.63	42.92 ± 2.82	34.46 ± 2.47

[a]Allogeneic MLR cultures contained 2×10^5 BALB/c $CD4^+$ splenic T cells stimulated by 4×10^5 C3H splenic lymphocytes. Cultures were incubated 4 days.

[b]Values represent cpm x 10^{-3} as determined by ^{3}H-TdR incorporation.

[c]GM-CSF was added at a final concentration of 100 U/ml per well. GM-CSF was generously provided by the Genetics Institute, Cambridge, MA.

The contribution of GM-CSF to tumor-induced Mϕ-mediated suppression of allorecognition is not reversed by inhibiting PGE_2 synthesis

Increased production of PGE_2 by Mϕ accounts for a significant percentage of observed suppression during allorecognition. We tested the possibility that PGE_2 was responsible for the GM-CSF-induced changes in TBH Mϕ-mediated suppression of allorecognition. Indomethacin was added to allogeneic MLR cultures containing GM-CSF and either NH or TBH Mϕ. GM-CSF or indomethacin alone significantly increased alloresponses in the absence of Mϕ (Table 4). The combination of GM-CSF plus indomethacin increased alloreactivity more than each alone. When accessory cells were present, the observed trends in alloresponses were similar to those seen in the absence of indomethacin. Previous findings show that low doses of NH and TBH Mϕ plus indomethacin increase allorecognition and autorecognition (T-cell responsiveness to syngeneic MHC surface molecules) above control levels (482). GM-CSF plus indomethacin increased allorecognition with NH accessory Mϕ. However, GM-CSF plus indomethacin decreased allorecognition with TBH accessory Mϕ. The defect in TBH Mϕ responsiveness to GM-CSF appears to be independent of the tumor-induced changes in Mϕ production of PGE_2.

M-CSF does not reverse the alloresponse suppression caused by TBH Mϕ and GM-CSF

Because splenic TBH Mϕ appear developmentally immature as determined by their activities and surface marker expression (18, 485), we hypothesized that expos-

Table 4. Inhibition of PGE_2 synthesis does not reverse the contribution of GM-CSF to tumor-induced Mϕ-mediated suppression of allorecognition

Mϕ Source	Treatment	Accessory Cells Present (cells/well)[a]			
		0	4×10^4	2×10^5	4×10^5
-----	Indomethacin[b]	83.16 ± 1.81[d]	-----	-----	-----
-----	GM-CSF[c] + Indomethacin	153.76 ± 2.45	-----	-----	-----
NH	Indomethacin	-----	111.56 ± 2.17	85.28 ± 8.18	54.27 ± 3.28
NH	GM-CSF + Indomethacin	-----	135.58 ± 1.82	107.03 ± 4.45	87.45 ± 8.57
TBH	Indomethacin	-----	125.46 ± 1.71	72.63 ± 1.08	57.61 ± 4.59
TBH	GM-CSF + Indomethacin	-----	92.48 ± 1.83	56.10 ± 1.11	38.17 ± 0.09

[a]Allogeneic MLR cultures contained 2×10^5 BALB/c $CD4^+$ splenic T cells stimulated by 4×10^5 C3H splenic lymphocytes. Cultures were incubated 4 days.

[b]Indomethacin was added per well at a final concentration of 10^{-7} M.

[c]GM-CSF was added at a final concentration of 100 U/ml per well. GM-CSF was generously provided by the Genetics Institute, Cambridge, MA.

[d]Values represent cpm x 10^{-3} as determined by ^{3}H-TdR incorporation.

ing these Mϕ to a monokine which promotes proliferation and differentiation of immature Mϕ would alleviate some of the tumor-induced suppression that occurs during allorecognition. M-CSF, which promotes the survival and development of myeloid lineage cells, was selected because it does not stimulate the proliferation or differentiation of mature Mϕ (24). We predicted that the combination of GM-CSF and M-CSF could partly reverse Mϕ-mediated suppression by signalling proliferation and/or differentiation events among the immature suppressor Mϕ (Figure 12). M-CSF suppresses certain T-cell activities and caused more suppression during T-cell allorecognition in the absence of NH Mϕ accessory cells in a dose-dependent manner (Figure 12A). The suppression was more pronounced when increasing concentrations of NH accessory Mϕ were added to the cultures. In contrast, no M-CSF doses affected TBH Mϕ-mediated suppression (Figure 12B), although reactivity was significantly lower than allorecognition in the absence or presence of Mϕ and GM-CSF. These data suggested that M-CSF increased Mϕ-mediated suppression of allorecognition, and that it does not reverse the suppression caused by TBH Mϕ and GM-CSF.

M-CSF does not prevent TBH Mϕ-mediated suppression of allorecognition when PGE_2 production is inhibited

Although PGE_2 does not appear to be involved in the TBH Mϕ/GM-CSF suppressive interaction that decreases alloresponsiveness, we wanted to determine if the responsiveness of TBH Mϕ to M-CSF could be affected by inhibiting PGE_2 synthesis (Figure 13). Although alloreactivity was higher when PGE_2 synthesis was inhibited, M-CSF caused more suppression during T-cell allorecognition in the absence

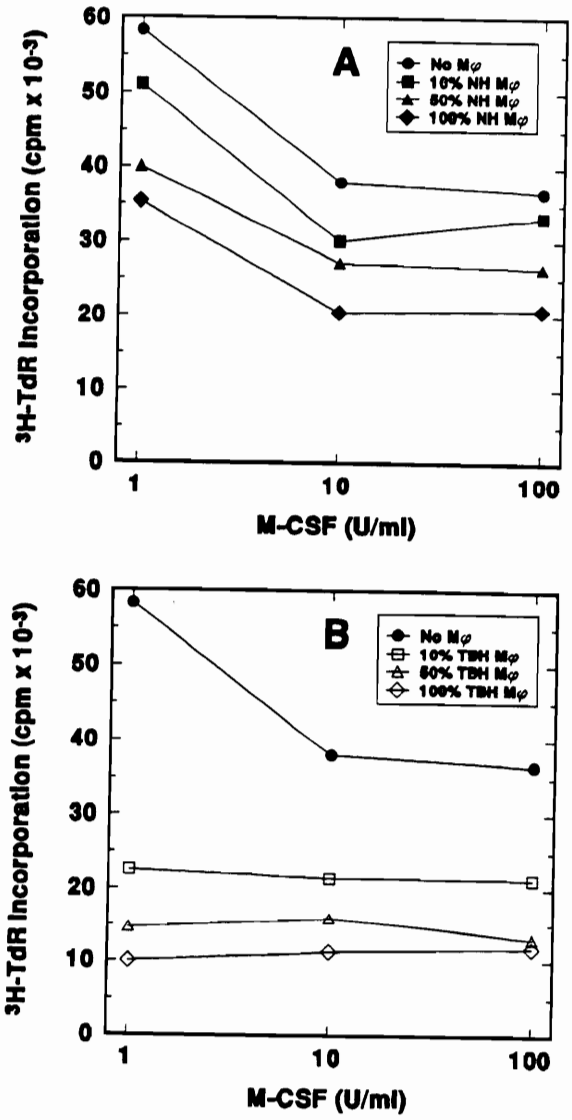

Figure 12. M-CSF does not reverse the alloresponse suppression caused by TBH Mϕ and GM-CSF: Allogeneic MLR cultures contained 2 x 10^5 BALB/c $CD4^+$ T cells (responder cells) and 4 x 10^5 C3H splenic lymphocytes (stimulator cells). Recombinant murine GM-CSF was added to cultures at 30 U/ml. Recombinant murine M-CSF was added to cultures at 1, 10, and 100 U/ml (X-axis). Some cultures were supplemeneted with accessory Mϕ (4 x 10^4 [10%], 2 x 10^5 [50%], or 4 x 10^5 [100%] cells/well). Cultures were incubated for 4 days and pulsed with ^{3}H-TdR 6 hr before cell harvest. ^{3}H-TdR incorporation is shown on the Y-axis.

of NH accessory Mϕ (Figure 13A). At all doses, M-CSF did not affect TBH Mϕ-mediated suppression (Figure 13B), and reactivity was significantly lower than allorecognition in the absence or presence of Mϕ and GM-CSF. These data suggested that inhibition of PGE_2 synthesis does not change TBH Mϕ-mediated suppression of allorecognition.

GM-CSF signals more suppression during allorecognition supplemented with TBH Ia$^-$ accessory Mϕ

Based on the above findings, we considered the possibility that a specific subpopulation of TBH Mϕ was responsible for most of the suppression observed during alloresponses affected by Mϕ/GM-CSF interactions. Mac-1$^+$2$^+$ Mϕ have been shown to be highly suppressive during tumor growth (426), but we and others (328, 481, 484) have observed that Ia$^-$ Mϕ are mediators of suppression to allorecognition and that TBH-derived Ia$^-$ Mϕ are significantly more suppressive than their NH counterparts. Furthermore, the percentage of Ia$^-$ Mϕ in the splenic population increases from 30-40% to 60-70%. Because unseparated splenic TBH Mϕ appear more inhibitory in the presence of GM-CSF, we hypothesized that GM-CSF also altered TBH Ia$^-$ Mϕ-mediated suppression of allorecognition (Figure 14). NH and TBH Ia$^-$ Mϕ were negatively selected for by mAb and complement and added in equal concentrations to allogeneic MLR cultures. High doses of NH Ia$^-$ Mϕ significantly decreased alloresponses, but this suppression was partly reversed by GM-CSF. In contrast, all doses of TBH Ia$^-$ Mϕ became more suppressive on addition of GM-CSF. This effect was observed irrespective of GM-CSF dose (data not shown). Therefore, these

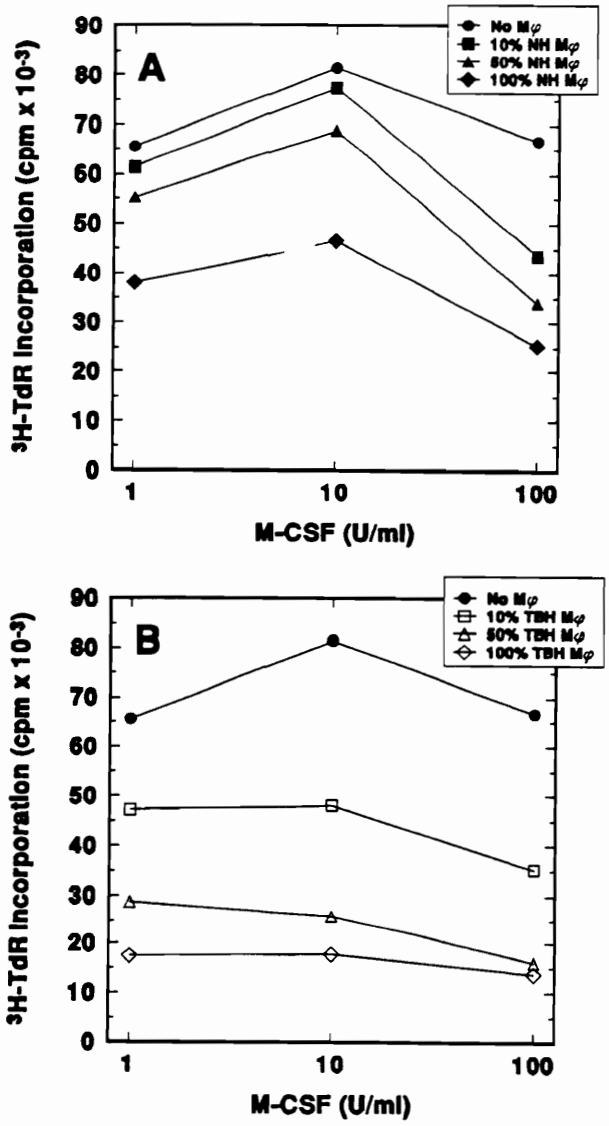

Figure 13. M-CSF does not prevent TBH Mϕ-mediated suppression of allorecognition when PGE_2 production is inhibited: Allogeneic MLR cultures contained 2 x 10^5 BALB/c $CD4^+$ T cells (responder cells) and 4 x 10^5 C3H splenic lymphocytes (stimulator cells). Indomethacin was added to cultures at 10^{-7} *M*. Recombinant murine GM-CSF was added to cultures at 30 U/ml. Recombinant murine M-CSF was added to cultures at 1, 10, and 100 U/ml (X-axis). Some cultures were supplemeneted with accessory Mϕ (4 x 10^4 [10%], 2 x 10^5 [50%], or 4 x 10^5 [100%] cells/well). Cultures were incubated for 4 days and pulsed with ^{3}H-TdR 6 hr before cell harvest. ^{3}H-TdR incorporation is shown on the Y-axis.

findings suggest a significant part of the suppression observed during allorecognition is due to tumor-induced changes in Ia^- Mϕ responsiveness to GM-CSF.

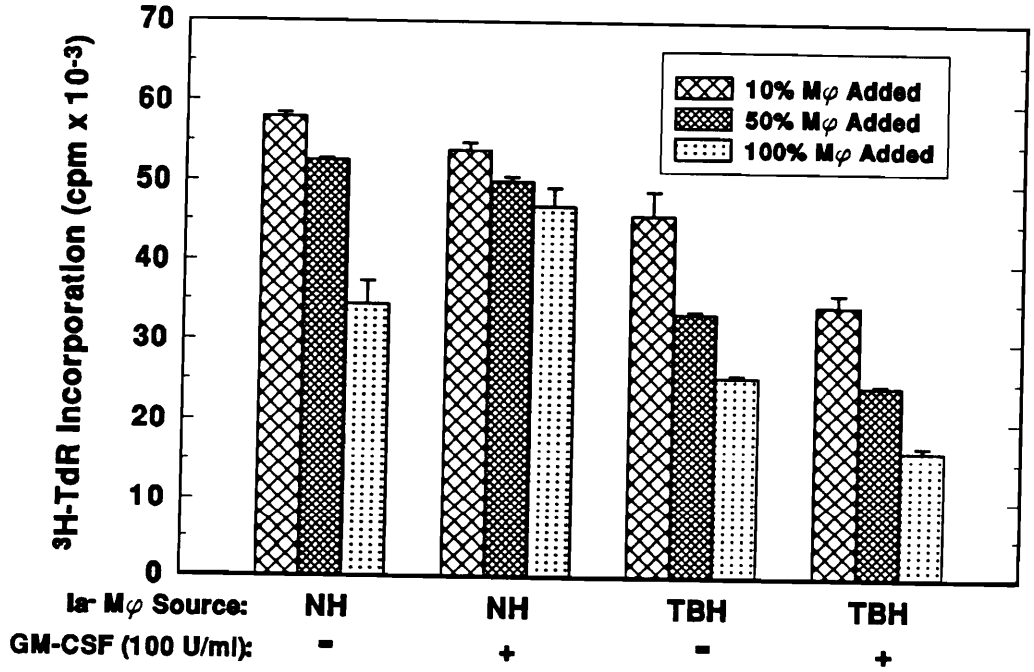

Figure 14. GM-CSF signals more suppression during allorecognition supplemented with TBH Ia$^-$ accessory Mϕ: Ia$^-$ Mϕ (4 x 10^4 [10%], 2 x 10^5 [50%], or 4 x 10^5 [100%] cells/well) were added to allogeneic MLR cultures and represent low, medium, and high doses. Ia$^-$ Mϕ were enriched by negative selection using anti-IA^d mAb (ATCC clone MK-D6) plus a 1:12 dilution of complement. The purity of the Ia$^-$ Mϕ population was confirmed by flow cytometric analysis showing >96% Ia$^-$ Mϕ. Recombinant murine GM-CSF was added to cultures at 100 U/ml. The source of Mϕ and the addition of GM-CSF is indicated on the X-axis. Cultures were incubated for 4 days and pulsed with ^{3}H-TdR 6 hr before cell harvest, and the amount of ^{3}H-TdR incorporation is shown on the Y-axis.

DISCUSSION

Because tumor-induced changes in Mϕ functions and phenotypes directly and indirectly disrupt T-cell alloresponses, we investigated the significance of GM-CSF to Mϕ accessory activity during tumor growth. GM-CSF is produced by Mϕ in response to several stimuli, but it is not constitutively expressed (188). Specific tumors exist that either excessively respond to or constitutively produce GM-CSF; we hypothesized that the regulation of this cytokine also may be disrupted among Mϕ during tumor growth. We assessed Mϕ accessory activity through the allogeneic MLR assay, an *in vitro* correlate to Mϕ-T cell interactions during allograft rejection. GM-CSF encourages Mϕ secretion and membrane-bound expression of IL-1, a cytokine that enhances T-cell responsiveness to specific stimuli (314). GM-CSF also increases Ia antigen expression in certain Mϕ populations (137, 138, 469), although some reports suggest Mϕ Ia antigen expression does not increase following GM-CSF exposure either *in vitro* or *in vivo* (90, 314). Several investigators have confirmed the ability of resident or tissue-specific Mϕ to proliferate (100, 131, 405), and GM-CSF stimulates the proliferation of tissue-derived Mϕ (74). We noted that GM-CSF addition (100 U/ml) to allorecognition cultures in the presence of a high NH Mϕ dose enhanced reactivity. Both NH and TBH Mϕ suppress alloreactivity in a dose-dependent manner, but GM-CSF partly reverses NH Mϕ-mediated suppression. At a high TBH Mϕ dose, GM-CSF appeared to have no effect, which suggests this cytokine's abilities to stimulate IL-1 production, Ia expression, or normal proliferation are disrupted or lost during tumor growth. These speculations are confirmed by our observation that TBH Mϕ stimulated with IFN-γ express abnormal levels of IL-1-α mRNA. Also, splenic TBH Mϕ do not express higher levels of Ia antigen in response to class II-inducing agents

such as LPS, TNF-α, or GM-CSF (submitted for publication). *In situ* proliferation seems to account for part of the progressive increase in Mϕ numbers during tumor growth (131, 405), but flow cytofluorometric data suggest this activity is disrupted at sites away from the tumor such as the spleen and peritoneal cavity (18, 485).

The inhibitory effects of TBH Mϕ-derived PGE_2 were abrogated by supplementing the cultures with indomethacin. Besides its modulatory effects, PGE_2 can act synergistically with IL-2 to induce GM-CSF synthesis among specific T_H1 clones (354). However, PGE_2 inhibits IL-2 production and decreases the responsiveness of cultures supplemented with accessory Mϕ to exogenous IL-1, IL-2, and IL-4 (482). Because exogenous GM-CSF addition could stimulate PGE_2 synthesis, we evaluated the ability of GM-CSF-primed NH and TBH Mϕ to produce PGE_2. We observed that PGE_2 synthesis inhibition did increase alloreactivity, but the responsiveness to GM-CSF of cultures with accessory Mϕ remained the same. Cultures supplemented with NH accessory Mϕ (medium and high Mϕ doses) were more reactive when GM-CSF (100 U/ml) was present, whereas cultures supplemented with the same doses of TBH Mϕ were unresponsive or further suppressed. PGE_2 did not appear to be a contributing factor in the altered TBH Mϕ responsiveness to GM-CSF, but other implicated monokines and lymphokines may offer down regulatory signals. TNF-α, a functionally pleiotropic cytokine, both enhances and inhibits Mϕ Ia antigen expression (71, 216, 487). TNF-α transcription is induced by GM-CSF (206), and we have noted TNF-α also suppresses alloresponses in the presence of TBH Mϕ (submitted for publication). We are evaluating the interaction between GM-CSF and TNF-α, and preliminary evidence suggests TNF-α contributes significantly to TBH Mϕ suppression.

Because a significant percentage of TBH Mϕ appear developmentally immature and demonstrate abnormal cell-cycle activities (18, 485), we added M-CSF to cultures containing Mϕ that were supplemented with GM-CSF. M-CSF does not stimulate the

proliferation or differentiation of mature Mϕ (24) but does promotes the proliferation and differentiation of immature Mϕ and Mϕ precursors. GM-CSF enhances Mϕ responsiveness to M-CSF (74), and we anticipated NH Mϕ accessory activity would be unchanged after exposure to both cytokines but their TBH counterparts would become less suppressive because they would receive appropriate proliferatory/differential signals. Instead, M-CSF increased TBH Mϕ-mediated suppression of allorecognition in the presence of GM-CSF. We feel this result may have occurred because M-CSF is suppressive during certain T-cell activities in the presence of accessory cells (353). Addition of indomethacin slightly increased alloreactivity, but M-CSF still generated higher suppression as more M-CSF was added. These findings suggest that TBH Mϕ-mediated suppression in the presence of GM-CSF and M-CSF is not through PGE_2. We feel this suppression may be attributable to another down-regulatory cytokine not reported here. TNF-α activity during tumor growth appears to affect Ia antigen expression, GM-CSF protein release, and PGE_2-suppressed T-cell proliferation. TBH Mϕ dysregulation of this second molecule may cascade suppression through a unique mechanism under investigation.

To address the possibility that a specific tumor-induced phenotype could be responsible for GM-CSF dysregulation, we examined the ability of GM-CSF to modulate alloresponses suppressed by Ia^- Mϕ. Ia^- Mϕ are part of the normal splenic Mϕ repertoire, but tumor growth causes significant reductions in Ia antigen expression and density. The resulting Ia^- subpopulation in the TBH makes up approximately 65% of the total Mϕ population. These Ia antigen reductions cause qualitative changes in the abilities of these Mϕ to regulate allorecognition and autorecognition (328, 481, 483, 484). NH Ia^- Mϕ are suppressive, but TBH Ia^- Mϕ are qualitatively more suppressive in alloreactivity assays. This heightened suppression is especially evident when admixtures of TBH Mϕ are incorporated into syngeneic MLR cultures

stimulated by NH Mϕ. Because NH Mϕ express sufficient levels of Ia antigen to drive autoreactivity, the observed suppression is primarily attributable to improper monokine production and responsiveness. We demonstrated in this report that GM-CSF regulation also is disrupted during Ia$^-$ TBH Mϕ-mediated suppression of allorecognition. NH Mϕ can increase Ia antigen expression in response to GM-CSF (137, 138, 469) and as a result reactivity mediated by a high dose of Ia$^-$ Mϕ was significantly increased. In contrast, Ia$^-$ TBH Mϕ were more suppressive at all doses when GM-CSF was present.

In summary, the data collectively suggest that tumor growth changes the responsiveness of Mϕ to GM-CSF during alloreactivity. Identification of such changes is important in light of therapeutic interventions, which may use GM-CSF as a restorative agent during myeloid depletion caused by radiation treatment. GM-CSF can offer significant help medically in cancer and disease scenarios, and our findings provide further illumination into the functional significance of this molecule.

CHAPTER V

TUMOR GROWTH CHANGES RESPONSIVENESS TO AND PRODUCTION OF GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR DURING RECOGNITION OF SELF MHC CLASS II MOLECULES

ABSTRACT

Tumor growth decreases T-cell recognition of self MHC class II molecules by inducing changes in splenic Mϕ phenotype and function. The current investigation shows tumor-induced alterations in autorecognition also are associated with changes in responsiveness to and production of GM-CSF. In contrast to NH Mϕ, TBH Mϕ failed to express higher MHC class II molecule density after exposure to GM-CSF. Autoreactive T cells stimulated by either NH or TBH Mϕ were suppressed by GM-CSF. Inhibition of PGE_2 synthesis reversed GM-CSF-induced suppression of autoreactivity to NH Mϕ and, to a lesser extent, to TBH Mϕ. When TBH autoreactive T cells were stimulated by TBH Mϕ, autoreactivity increased when GM-CSF was added and PGE_2 synthesis was inhibited. Although M-CSF can contribute to tumor-induced suppression, it did not affect the contribution of GM-CSF during autorecognition. Increased GM-CSF production was responsible, at least in part, for the TBH Mϕ-mediated suppression. Low concentrations of GM-CSF were produced endogenously by tumor isolates, and GM-CSF production was significantly increased when isolates were stimulated with LPS. Autoreactive T cells stimulated solely by TBH Mϕ produced more GM-CSF than autoreactive T cells stimulated by NH Mϕ. Cultures supplemented with several concentrations of NH or TBH Mϕ produced similar amounts of GM-CSF in a dose-dependent manner. Inhibition of PGE_2 synthesis by NH and TBH Mϕ reduced GM-CSF production equally. Collectively, these results suggest that during tumor growth responsiveness to and production of GM-CSF alters recognition of self MHC class II molecules.

INTRODUCTION

Autoreactive T cells are a unique population of $CD4^+/CD8^-$ T cells that fail to recognize foreign cell surface antigens but do respond to cells bearing self MHC class II molecules (28, 147, 393, 463). Activated or unactivated Mϕ and B cells are potent stimulators for autoreactive T cells, although recognition of MHC class II molecules is the only signal required for autoreactive T cell proliferation (86, 149). Because autoreactive T cells provide help, regulate cytotoxicity, and mediate suppression (28, 147, 393, 463). they are considered part of the normal immune repertoire. Several autoimmune disease states such as encephalomyelitis (435), myasthenia gravis (165), multiple sclerosis (199), systemic lupus erythematosus (257, 414), and diabetes melitis (487) cause significant changes in autoreactivity, and we have shown that tumor development also changes autoreactive T-cell responsiveness (283, 482, 484). Autorecognition is affected by accessory cells such as $CD8^+$ T cells (53, 148) and Mϕ (482, 484) and their cytokines. In the present study, we demonstrate that autorecognition is altered by tumor-induced changes in the response to and release of GM-CSF.

GM-CSF is a well characterized hemopoietic growth factor produced by T cells, Mϕ, fibroblasts, and endothelial cells (303, 330) in response to activating stimuli such as LPS, TNF-α, and IL-1 (241, 242, 420). In addition to stimulating the maturation of neutrophils and Mϕ (303, 310), GM-CSF can induce tumoricidal (136, 195), accessory (154), and anti-parasitic (282) activities in mature Mϕ and T cells. GM-CSF increases MHC class II molecule expression on bone marrow-derived and resident Mϕ (153, 154). Interestingly, several human and murine tumors produce GM-CSF *in vitro* and *in vivo* (33, 408, 427, 479), and this activity may account for TBH immune cell

dysfunctions. Because our previous work (482, 484) shows that tumor growth causes significant reductions in MHC class II molecule expression on peritoneal and splenic Mϕ and that several TBH Mϕ-derived products can suppress immune cell activities, we investigated the significance of GM-CSF to autorecognition during tumor growth. The results suggest that tumor growth disrupts Mϕ-mediated autorecognition by changing immune cell production of and responsiveness to GM-CSF. These changes appear to be a mechanism by which immunity is compromised during tumor growth.

MATERIALS AND METHODS

Animals

The 8 to 12 week-old male BALB/c mice (Harlan-Sprague-Dawley, Madison, WI) used were LDH virus negative (Microbiological Associates, Bethesda, MD). A single-cell suspension of 4 x 10^5 methylcholanthrene-induced, nonmetastatic, fibrosarcoma cells was injected i.m. Palpable tumors form by days 10-14 and kill the hosts by days 28-35 (123). Splenic immune cells from 21-day TBH mice were used throughout. Immunosuppression was not caused by a general inflammatory response, because normal BALB/c mice injected with C3H muscle tissue 3 weeks before had normal autoreactive responses (484).

Medium

NH and TBH cells were cultured in complete medium: RPMI-1640 (Hazelton, Denver, PA) medium with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO), 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$, and 25 *mM* HEPES buffer (Research Organics, Cleveland, OH). The GM-CSF-responsive cell line DA3 was maintained in Iscove's Modified Dulbecco's Medium (Gibco) with 10% heat inactivated FBS, 4 × 10^{-5}M 2-mercaptoethanol, 50 mg/L gentamicin, 3.024 g/L $NaHCO_3$, and 25 *mM* HEPES buffer.

Chemicals and Monoclonal Antibodies

Unless specified, indomethacin (Sigma) was added to cultures at a final concentration of 10^{-7} *M*. This concentration inhibits the arachidonic acid pathway for PGE_2 synthesis and is nontoxic to immune cells (284). The following hybridoma cell lines (American Type Culture Collection [ATCC], Rockville, MD) were used to produce monoclonal antibodies (mAb): anti-IA^d (ATCC clone MK-D6), anti-IE^d (ATCC clone 14-4-4), anti-immature T cell and B cell (ATCC clone J11d), and anti-Lyt-2 (ATCC clone 3.155). All mAb were used as ammonium sulfate-precipitated fractions of culture supernatants.

Cytokines

Recombinant murine M-CSF was generously provided by Genetics Institute (Cambridge, MA). M-CSF activity (1.65×10^6 U/ml) was determined by half maximal colony formation in the murine bone marrow colony assay. Aliquots were stored at -70°C until use. Recombinant murine GM-CSF generously provided by Immunex (Seattle, WA) was used to maintain the DA3 cell line. GM-CSF activity (4×10^7 U/ml) was determined by half maximal colony formation in the murine bone marrow colony assay. Aliquots were stored at -70°C until use.

Mϕ and CD4$^+$ T Cell Preparation

Mice were sacrificed by cervical dislocation. Murine spleens were pulped using a stomacher (Tekmar, Cincinnati, OH) and the resulting cell suspensions were centrifuged and resuspended in RPMI-1640 medium. Erythrocytes were lysed with cold 0.83% NH_4Cl for 5 min. Cells were washed and incubated for 2 hr at 37°C and 5% CO_2 on tissue culture plates (150 × 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL). To collect CD4$^+$ T lymphocytes, splenic nonadherent cells were eluted from the plates and further depleted of B cells, Mϕ, and other contaminating cells by adding anti-Lyt-2, anti-IA^d, anti-immature T lymphocyte and B cell, and anti-IE^d mAb, and a 1:12 dilution of complement (Cedarlane Laboratories, Ontario, Canada). Purified CD4$^+$ T cells (>99% CD4$^+$ T cells as determined by cytofluorometric analysis) were washed at least three times to remove residual complement, and the viable cells were resuspended to 4 x 10^6 cells/ml in complete medium. Mϕ (>96% esterase positive) were collected (after washing to remove nonadherent cells) by scraping using a rubber policeman and ice-cold RPMI-1640 medium. Mϕ were resuspended to 8 x 10^6 cells/ml in complete medium. For syngenic mixed lymphocyte reactions (SMLR), Mϕ were X-irradiated with 2000 rads (TFI Minishot II X-irradiator, New Haven, CT).

Whole Tumor Isolate Preparation

Tumors were excised aseptically from the left hind leg of TBH and seeded as a single-cell suspension (8 x 10^5 cells/well) in complete medium.

Fluorescent Antibody Labelling

Purified Mϕ were seeded (8×10^6 cells/ml) with GM-CSF in flat-bottom 24-well tissue-treated plates (Flow Laboratories, McLean, VA) and incubated at 37°C and 5% CO_2. Cells were collected by washing with ice-cold RPMI-1640 medium and concentrated to 1.0 ml. Cells were incubated with the IA^d-specific mAb MK-D6 or IE^d-specific mAb 14.4.4 for 45 min at 4°C (this time and temperature allowed effective Ab binding and maintained cell viability while minimizing membrane turnover and Ab internalization). Normal mouse immunoglobulin G (NMIgG) served as the isotype control. Cells were washed three times and treated with affinity purified goat-anti-mouse $F(ab')_2$ fragment FITC-labeled Ab (Cappel Organon-Teknika, Malvern, PA) for 45 min at 4°C. Cells were washed three times and resuspended in 1.0 ml RPMI-1640 medium and analyzed for MHC class II molecule expression using flow cytometry.

Flow Cytometry

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics, Hialeah, FL) laser flow cytometer and cell sorter. This instrument was calibrated with fluorescent 10-micron-sized microsphere standards before analysis of the Mϕ samples. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc., Palo Alto, CA). Data were collected with the multiparameter data ac-

quisition and display system (MDADS, Coulter Electronics). Fluorescein isothiocyanate- (FITC) labeled mAb fluorescence was measured as the log of the green fluorescence.

SMLR

$CD4^+$ T cells (4×10^5 H-2^d-restricted responder cells) and Mϕ (4×10^5 H-2^d stimulator cells) were were added per well to "U" bottom 96-well tissue-treated plates (Nunc, Roskilde, Denmark). This base number was required for control level autoreactive T-cell responses. In some cases, additional Mϕ (4×10^4, 2×10^5, or 4×10^5 cells/well) were added to the SMLR. SMLR cultures were incubated for 4 days at 37°C and 5% CO_2. Six hr before cell harvest, each culture was pulsed with tritiated thymidine (^{3}H-TdR: specific activity 6.7 Ci/*mM*, Dupont NEN Research Products, Boston, MA). Cells were harvested onto filters (Whatman 934-AH, Thomas Scientific, Phila., PA) using a MASH harvester. Samples were placed into counting vials (Skatron, Sterling, VA) with Scintilene (Thomas Scientific, Swedesboro, NJ) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

DA3 Cell Line

The GM-CSF-responsive cell line DA3 was obtained from Drs. Giovanni Rovera and Brent Kreider (Wistar Institute, Philadelphia, PA) and maintained by biweekly passage into fresh medium containing 20 U/ml GM-CSF. To determine the presence of GM-CSF in supernatants, 100 μl (2.5×10^4 cells/well) were seeded with 100 μl supernatant in flat-bottom 96-well tissue-treated plates (Nunc). Cultures were incubated for 3 days at 37°C and 5% CO_2, and proliferation was measured by ^{3}H-TdR incorporation as described under *SMLR*.

Supernatant Collection

Supernatants were collected from cultures by centrifugation and were either added directly into the GM-CSF activity assay or stored at -70°C until use.

Statistics/Calculation of Results

Pooled cells from 4 to 8 mice were used for each experiment. For the SMLR and GM-CSF bioactivity assays, three replicate wells were run for each test, and each experiment was repeated three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p < 0.05$) and presented as mean $\pm$ standard error. In the GM-CSF production experiments, the amounts of

GM-CSF present in the samples were extrapolated from activity curves generated by DA3 cells stimulated with recombinant murine GM-CSF. For the flow cytometry data, all percentages were determined by the MDADS computer and represent the percentage of 10,000 cells that were considered positively labeled after the negative control was subtracted.

RESULTS

TBH Mϕ are not responsive to class II molecule induction by GM-CSF

Previous flow cytometric analyses revealed that MHC class II molecule expression is significantly reduced on TBH Mϕ (172, 483, 484). Whereas splenic NH Mϕ are 60-70% class II^+, TBH splenic Mϕ are only 25-30% class II^+. This tumor-induced reduction in class II molecule expression significantly decreases Mϕ abilities to present foreign antigens and recognize autologous antigens. TBH class II^- Mϕ are qualitatively more suppressive than their NH counterparts because of changes in cytokine production. We hypothesized that tumor-induced changes in class II molecule expression may be reversible if TBH Mϕ received an appropriate class II molecule-inducing signal. GM-CSF induces class II molecule expression in bone marrow-derived Mϕ (469), but at several time points tested, GM-CSF failed to increase the percentage of splenic Mϕ positive for class II molecules regardless of host source (not shown). However, class II molecule density (as determined by mean fluorescence) was increased by GM-CSF (Figure 15). NH Mϕ showed a dose-dependent increase in class II molecule expression per cell whereas TBH Mϕ class II molecule density did not change significantly. This increase was restricted to molecules encoded by the IA subregion; GM-CSF did not alter NH or TBH Mϕ expression of class II molecules encoded by the IE subregion. With respect to IE^d expression, NH Mϕ in the absence or presence of GM-CSF were approximately 28% class II^+ (mean fluorescence 156 ± 2) and TBH Mϕ in the absence or presence of

GM-CSF were approximately 17% class II^+ (mean fluorescence 161 ± 2). These data suggest TBH Mϕ are not responsive to class II molecule induction by GM-CSF.

Tumor growth changes Mϕ regulation of GM-CSF and PGE_2 responsiveness

Because TBH Mϕ were unresponsiveness to class II molecule induction by GM-CSF, we hypothesized that this defect may disrupt functional Mϕ-T cell interactions. The SMLR illustrates changes in Mϕ accessory activity because this reaction is class II molecule-restricted and requires direct cellular associations between Mϕ and T cells. Although TBH T cells have some reductions in responsiveness to autoantigens, TBH Mϕ are the primary suppressor cells of the reaction. Tumor-induced changes in GM-CSF responsiveness were investigated using NH T cells stimulated by NH or TBH Mϕ (Table 5). GM-CSF significantly ($p < 0.05$) decreased autorecognition stimulated by either NH (29%) or TBH (22%) Mϕ. Mϕ production of and responsiveness to GM-CSF is controlled partly through PGE_2, a potent inhibitory molecule (285, 345). PGE_2 is over-produced by TBH Mϕ and accounts for part of the observed suppression during autorecognition. Because tumor-induced increases in PGE_2 synthesis may affect responsiveness to GM-CSF, autoreactive T cells stimulated by NH or TBH Mϕ were treated with indomethacin. Inhibition of PGE_2 synthesis significantly ($p < 0.05$) increased TBH Mϕ-stimulated autorecognition (51%) above control levels to a greater extent than NH Mϕ-stimulated autorecognition (22%). However, the reverse trend was observed when indomethacin and GM-CSF were added together. These results suggest that inhibition of PGE_2 synthesis pre-

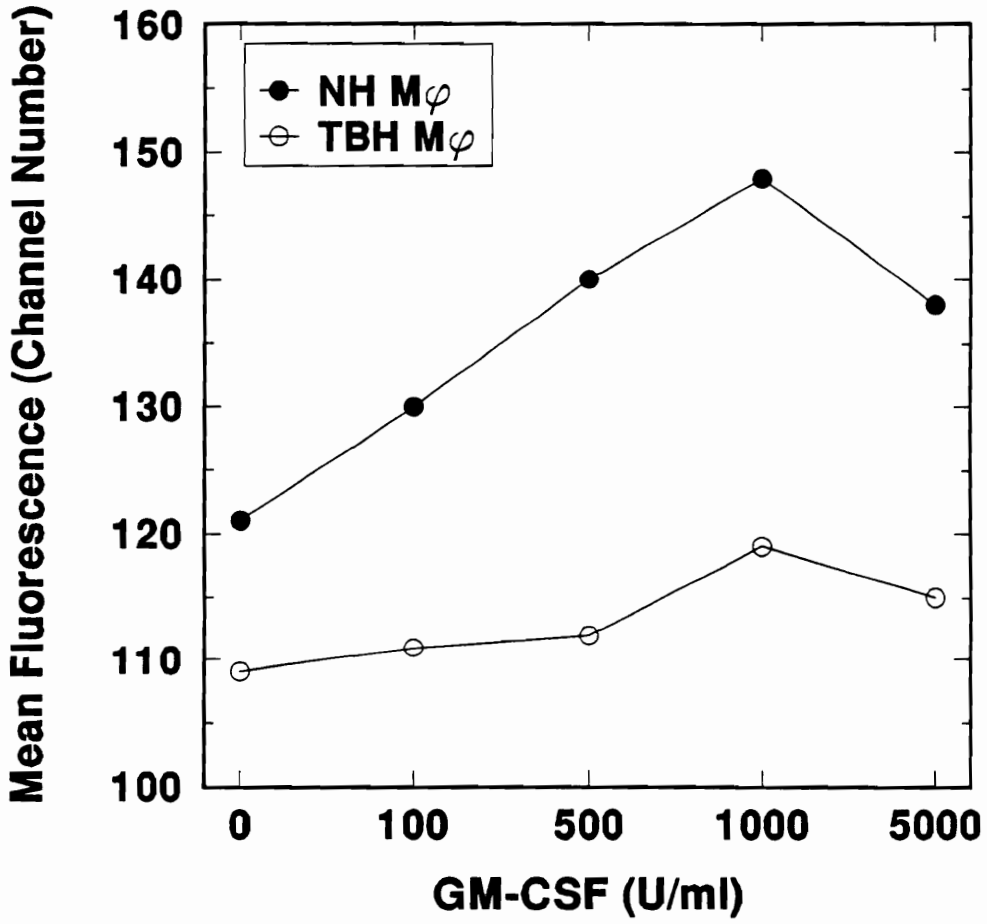

Figure 15. TBH Mϕ are not responsive to class II molecule induction by GM-CSF: Eight x 10^6 Mϕ/ml were cultured with GM-CSF in flat-bottom 24-well tissue-treated plates for 72 hr. Mϕ were labelled using the IA^d-specific mAb MK-D6 or IE^d-specific mAb 14.4.4 and FITC-labelled goat-anti-mouse $F(ab')_2$ fragment.

vents the induction of suppressor Mϕ activities by GM-CSF, but that tumor growth disrupts the pathway.

Although TBH T-cell autorecognition is only slightly but significantly ($p < 0.05$) lower (18%) than NH T-cell autorecognition, we investigated the possibility that GM-CSF may be an important regulatory cytokine to TBH T cells during autorecognition. TBH T cells stimulated by NH Mϕ showed no change in autorecognition when GM-CSF was present (Table 5), although TBH T cells stimulated by TBH Mϕ demonstrated a significant ($p < 0.05$) increase in reactivity (25%). Inhibiting PGE_2 synthesis in the presence of GM-CSF significantly ($p < 0.05$) increased autorecognition stimulated by TBH (75%) Mϕ, but NH Mϕ-stimulated autoreactive T cells demonstrated only half the percentage increase (38%) in reactivity as TBH Mϕ-stimulated T cells. This finding is consistent with previous reports which show TBH Mϕ suppression is mediated through increased PGE_2 synthesis. These results suggest that GM-CSF increases of TBH T cell autorecognition, which is augmented by inhibition of PGE_2 synthesis.

M-CSF does not affect Mϕ responses to GM-CSF

M-CSF regulates several Mϕ activities including accessory function (353) and class II molecule expression (353). To determine if M-CSF affected the contribution of GM-CSF to TBH Mϕ-mediated suppression of autorecognition, autoreactive T cells were supplemented with both GM-CSF and M-CSF (Table 6). GM-CSF used at a 30 U/ml dose in conjunction with M-CSF offered optimal reactivity in our assays. Autorecognition stimulated by either NH or TBH Mϕ decreased in the presence of

Table 5. The contribution of GM-CSF and PGE_2 during TBH Mϕ-mediated suppression of autorecognition

Treatment	T Cell Source[a]	Autoreactivity With NH Mϕ	Change in Autoreactivity (%)	Autoreactivity With TBH Mϕ	Change in Autoreactivity (%)
-----	NH	38.33 ± 1.51[b]	-----	11.85 ± 0.32	-----
GM-CSF[c]	NH	27.20 ± 0.86	29.0 ↓	9.16 ± 0.19	22.7 ↓
Indomethacin[d]	NH	46.82 ± 0.89	22.2 ↑	17.92 ± 0.47	51.2 ↑
GM-CSF + Indomethacin	NH	58.59 ± 0.65	52.9 ↑	15.81 ± 0.52	33.4 ↑
-----	TBH	22.71 ± 1.76[b]	-----	10.02 ± 1.01	-----
GM-CSF[c]	TBH	22.89 ± 1.98	0.8 ↑	12.56 ± 0.76	25.3 ↑
Indomethacin[d]	TBH	28.14 ± 2.23	23.9 ↑	17.23 ± 0.90	72.0↑
GM-CSF + Indomethacin	TBH	31.52 ± 2.44	38.8 ↑	17.55 ± 1.28	75.2 ↑

[a]T cells (4×10^5 cells/well) were stimulated with either irradiated NH or TBH Mϕ (4×10^5 cells/well).

[b]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

[c]GM-CSF (100 U/ml) was generously provided by Immunex, Seattle, WA.

[d]Indomethacin, a prostaglandin synthetase inhibitor, at 10^{-7} *M* suppressed PGE_2 synthesis and was nontoxic to immune cells.

both cytokines. When PGE_2 synthesis was inhibited, autorecognition in TBH Mϕ-stimulated cultures was significantly increased. These data suggest M-CSF does not alter Mϕ responsiveness to GM-CSF during Mϕ-mediated suppression of autorecognition.

GM-CSF-induced suppression is not strictly due to reduced MHC class II molecule expression

To determine if GM-CSF unresponsiveness was due solely to low class II molecule expression or involved suppressor activity through soluble mediators, NH T cells stimulated with NH Mϕ were supplemented with additional Mϕ from either NH or TBH (Figure 16). GM-CSF addition to autoreactive T cells with NH or TBH Mϕ lead to suppression at all Mϕ concentrations tested (Figure 16A). However, inhibition of PGE_2 synthesis reversed the suppression caused by NH Mϕ and GM-CSF at all doses (Figure 16B). In contrast, autoreactive T cells supplemented with TBH Mϕ showed no change in reactivity. These data suggest TBH Mϕ are unresponsive to GM-CSF during autorecognition and that suppression is mediated through mechanisms besides MHC class II molecule expression.

Tumor isolates produce GM-CSF

Tumor cell production of GM-CSF can enhance or suppress local and systemic immune responses. Because several fibrosarcomas synthesize and release GM-CSF, we determined if tumor isolates from our fibrosarcoma model produce

Table 6. M-CSF does not change the contribution of GM-CSF to autorecognition

Treatment[a]	NH Mϕ	Change in Autoreactivity (%)	TBH Mϕ	Change in Autoreactivity (%)
-----	46.33 ± 2.52[b]	-----	11.65 ± 1.31	-----
GM-CSF[c]	33.01 ± 0.82	28.8 ↓	11.39 ± 0.51	2.2 ↓
M-CSF[d]	31.59 ± 1.13	31.8 ↓	10.78 ± 0.01	7.5 ↓
Indomethacin[e]	50.61 ± 0.05	9.2 ↑	15.80 ± 0.7	35.6 ↑
GM-CSF + Indomethacin	52.82 ± 0.89	14.0 ↑	17.09 ± 1.54	46.7 ↑
M-CSF + Indomethacin	53.10 ± 2.69	14.6 ↑	15.73 ± 0.45	35.0 ↑
GM-CSF + M-CSF	28.42 ± 1.62	38.7 ↓	9.75 ± 0.55	16.3 ↓
GM-CSF + M-CSF + Indomethacin	48.92 ± 1.39	5.6 ↑	19.30 ± 0.72	65.7 ↑

[a]NH T cells (4×10^5 cells/well) were stimulated with either irradiated NH or TBH Mϕ (4×10^5 cells/well).

[b]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

[c]GM-CSF (30 U/ml) was generously provided by Immunex, Seattle, WA.

[d]M-CSF (100 U/ml) was generously provided by the Genetics Institute, Cambridge, MA.

[e]Indomethacin, a prostaglandin synthetase inhibitor, at 10^{-7} *M* suppressed PGE_2 synthesis and was nontoxic to immune cells.

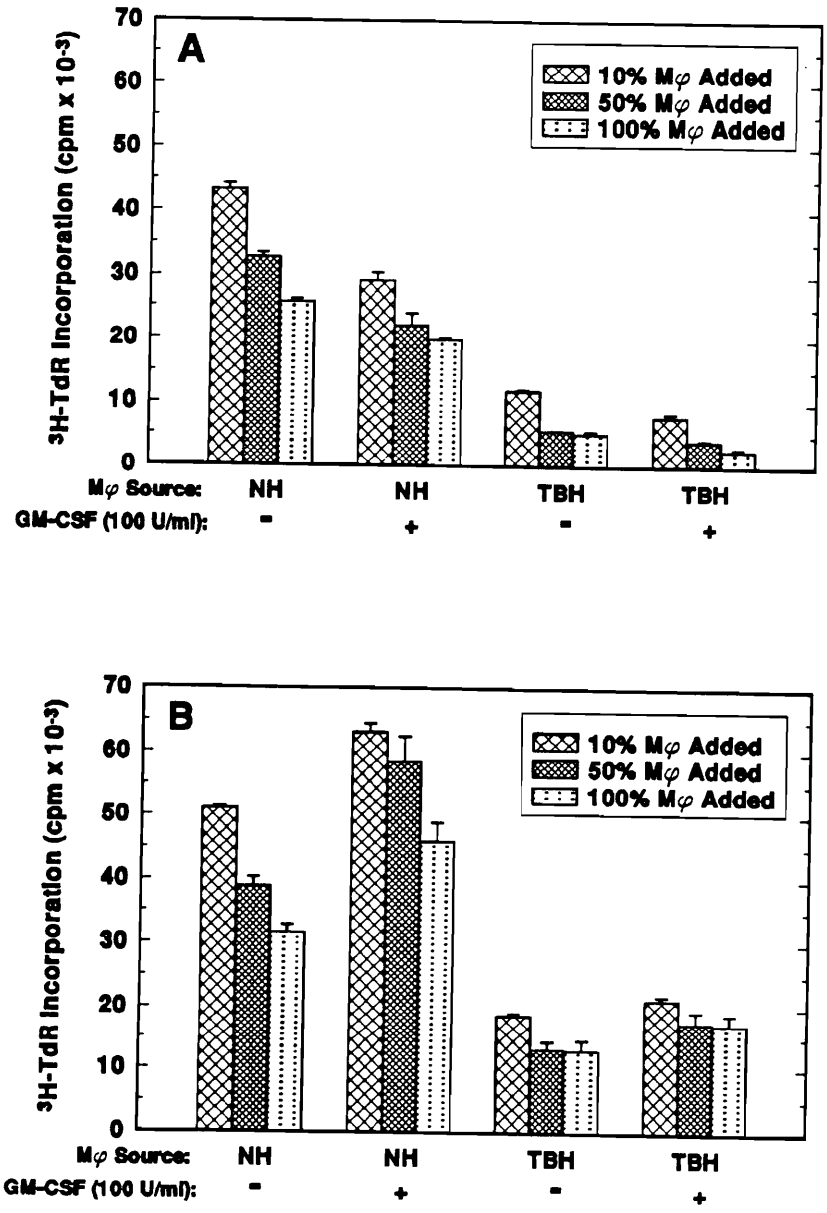

Figure 16. Tumor-induced changes in GM-CSF responsiveness are not limited to Mϕ MHC class II molecule expression: Four x 10^5 NH T cells were cultured with 4 x 10^5 NH Mϕ. Different concentrations of supplemental NH and TBH Mϕ were added to the SMLR. The percentage of supplemental Mϕ is with respect to the stimulator population. GM-CSF (100 U/ml) was added to some of the cultures. Panel A shows autoreactivity in the absence of indomethacin, and Panel B shows autoreactivity in the presence of indomethacin (10^{-7} *M*). Cultures were incubated for 4 days. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the Mϕ source, and the Y-axis shows the ^{3}H-TdR incorporation in proliferating autoreactive T cells. Background cpm for T cells or Mϕ alone were <946 cpm and 206 cpm, respectively.

GM-CSF. Tumor isolates cultured in the absence of activating agents produced low but detectable levels of GM-CSF (Figure 17). GM-CSF production significantly increased when whole tumor isolates were stimulated with LPS. Concanavalin A did not stimulate GM-CSF production by tumor isolates. These data collectively suggest that tumor isolates containing fibrosarcoma cells and tumor-associated Mϕ can produce GM-CSF.

Tumor growth increases GM-CSF production during autorecognition

Because the data suggested tumor growth changes Mϕ responsiveness to GM-CSF, we wanted to determine whether tumor growth also alters Mϕ production of GM-CSF. Using the GM-CSF-restricted cell line DA3 (224), supernatants from cultures of autoreactive T cells stimulated by TBH Mϕ had higher GM-CSF levels than cultures stimulated by NH Mϕ (Figure 18). Inhibition of PGE_2 synthesis reduced GM-CSF production by cultures stimulated by either Mϕ type. These data suggest that tumor growth increases GM-CSF production during autorecognition.

Tumor-induced changes in GM-CSF responsiveness are independent of GM-CSF production

Data suggested that TBH Mϕ suppression was through mechanisms besides low class II molecule expression because NH Mϕ-stimulated autoreactive T cells admixed with TBH Mϕ were unresponsive to GM-CSF. To determine if the lack of responsiveness to GM-CSF was due to changes in the production of GM-CSF, admixture

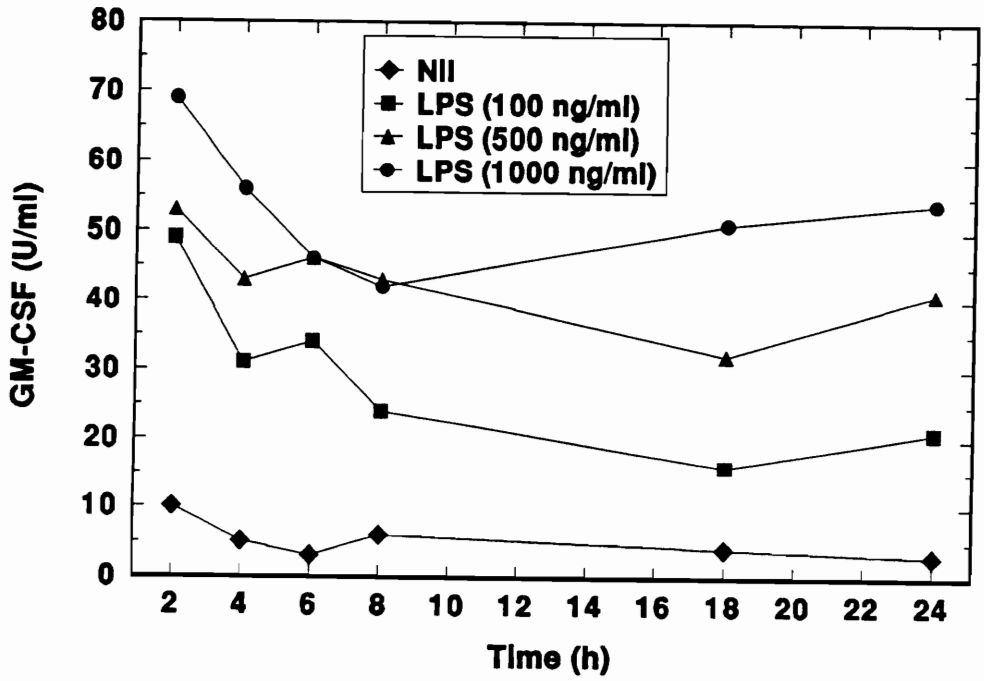

Figure 17. Tumor isolates produce GM-CSF: GM-CSF production by tumor isolates (8 x 10^5/well) was assessed using the DA3 cell line. DA3 cells (2.5 x 10^4 cells/well) were co-cultured with 100 μl spent medium for 3 days. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the Mϕ source used to stimulate the autoreactive T cells, and the Y-axis shows the GM-CSF activity (U/ml) as extrapolated from an activity curve using purified GM-CSF. Background cpm for DA3 cells in the absence of detectable GM-CSF were < 55 cpm.

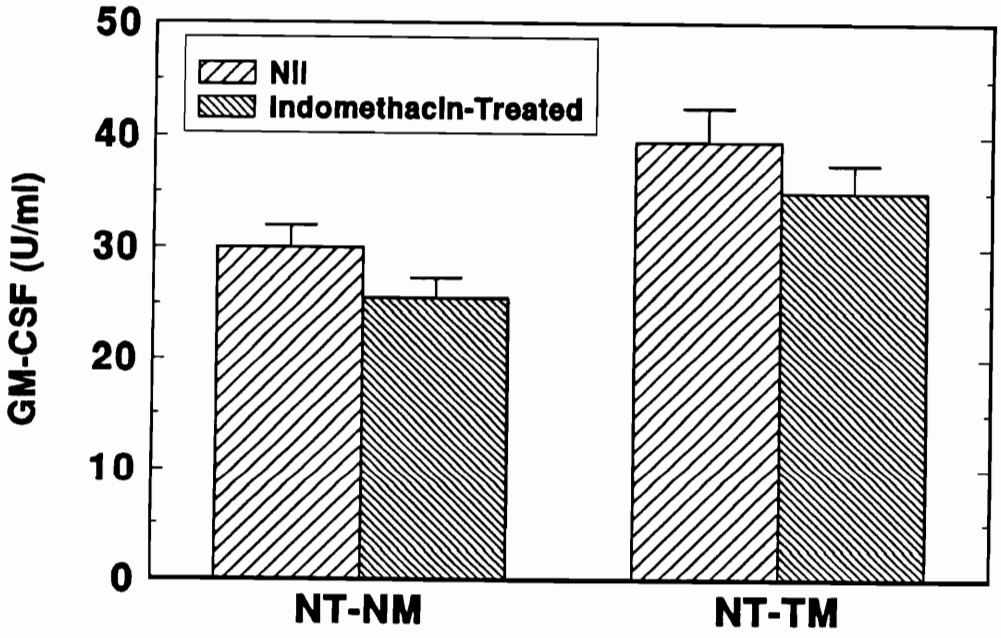

Figure 18. Tumor growth increases the production of GM-CSF during autorecognition: GM-CSF production in SMLR cultures was assessed using the DA3 cell line. DA3 cells (2.5 x 10^4 cells/well) were co-cultured with 100 μl spent medium for 3 days. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the Mϕ source used to stimulate the autoreactive T cells, and the Y-axis shows the GM-CSF activity (U/ml) as extrapolated from an activity curve using purified GM-CSF. Background cpm for DA3 cells in the absence of detectable GM-CSF were <32 cpm.

culture supernatants were tested. Both NH and TBH admixture supernatants had similar GM-CSF activity at all Mϕ concentrations tested (Figure 19). Supernatants showed lower GM-CSF activity when PGE_2 synthesis was inhibited except for high admixture concentrations at which no differences in GM-CSF activity were observed. These data suggest that tumor-induced alterations in GM-CSF responsiveness are not associated with changes in GM-CSF production during autorecognition.

DISCUSSION

Mϕ make significant contributions during immunological challenge through accessory activities. Accessory function, such as during Mϕ-T cell interactions, dictates the levels of T-cell activation and proliferation that occur in response to self antigens. Recent reports have established that Mϕ production of and responsiveness to specific cytokines and expression of MHC class II molecules are significantly altered by tumor growth. We investigated the importance of GM-CSF to Mϕ accessory activity because it can modulate Mϕ and T-cell functions and regulate class II molecule expression. Accessory activity was assessed through the self MLR because the reaction depends on Mϕ-T cell interactions and requires MHC class II molecule expression. Previous data show that tumor growth significantly reduces autorecognition and that suppression is primarily mediated through Mϕ.

We first analyzed class II molecule induction by GM-CSF on NH and TBH Mϕ. Autorecognition is dependent on T-cell identification of self MHC molecules (463), and without significant expression of these molecules, autorecognition cannot occur. Tumor growth reduces Mϕ class II molecule expression and induces class II^- sub-

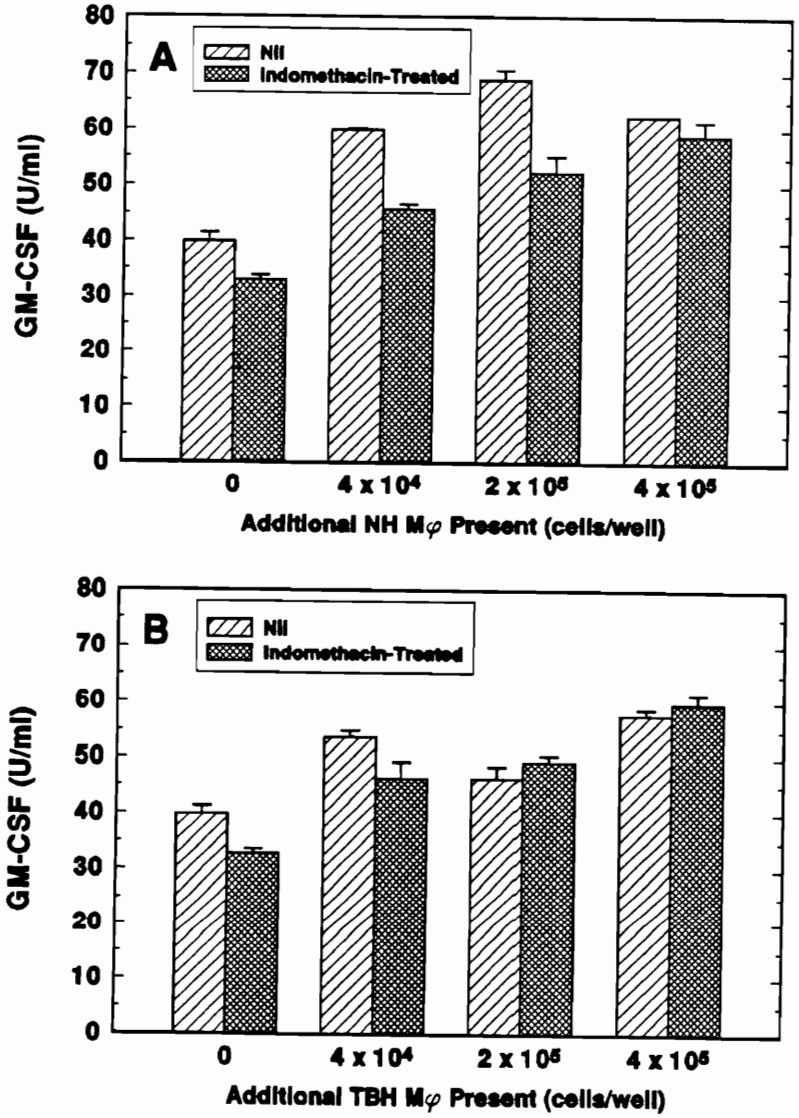

Figure 19. Tumor-induced changes in responsiveness to GM-CSF are not associated with changes in production of GM-CSF: Spent medium from SMLR cultures containing admixtures of NH (panel A) and TBH (panel B) Mϕ in the presence or absence of indomethacin (10^{-7} *M*) were tested for GM-CSF activity using the DA3 cell line. DA3 cells (2.5 x 10^4 cells/well) were co-cultured with 100 μl spent medium for 3 days. The cultures were pulsed with ^{3}H-TdR 6 hr before termination of the assay. The X-axis shows the percentage of additional Mϕ added, and the Y-axis shows the GM-CSF activity (U/ml) as extrapolated from an activity curve using purified GM-CSF. Background cpm for DA3 cells in the absence of detectable GM-CSF were < 82 cpm.

populations. This phenotypic shift is important during tumor-induced suppression because the class II^+ Mϕ demonstrate significant tumoricidal activity at the tumor site and are the primary cell type causing tumor regression (132). As tumor growth progresses, however, class II^- Mϕ become predominant both at the tumor site and in various resident tissues, and they mediate significant levels of immunosuppression (81, 172, 328, 481, 484). GM-CSF regulates the expression of several monocyte and Mϕ surface molecules including MHC class II molecules (153, 154, 172, 178), and we hypothesized that TBH Mϕ-mediated suppression of autorecognition may be reversible if a class II molecule-inducing agent was administered to the cells. Both NH and TBH Mϕ MHC class II molecule expression were unchanged by GM-CSF exposure, but we believe this result can be attributed to the selective responsiveness of Mϕ subpopulations to GM-CSF. Although bone marrow-derived Mϕ increase class II molecule expression on GM-CSF exposure (153, 154, 469), GM-CSF does not increase class II molecule synthesis or expression on peritoneal Mϕ (90, 314). The data suggest splenic Mϕ respond to GM-CSF like peritoneal Mϕ with respect to MHC class II molecule induction. However, we did observe NH splenic Mϕ class II density increased in response to GM-CSF in a dose-dependent manner. In contrast, TBH splenic Mϕ showed no significant change in class II density. This finding suggests that the inability to respond to GM-CSF may limit the rate at which class II molecule-dependent reactions occur. Other Mϕ molecules associated with GM-CSF may be affected during tumor growth. Tumor-induced suppressor Mϕ that synthesize GM-CSF are Mac 1^+2^+ (426) and GM-CSF transgenic mice express extremely high levels of Mac 2 and Mac 3 (125). Because tumor growth shifts the expression of these molecules (171), we are currently investigating the contribution of GM-CSF to tumor-induced changes in Mac antigen expression.

We next determined if tumor-induced changes in Mϕ responsiveness to GM-CSF could be detected during Mϕ accessory activities. Previous investigations revealed that TBH Mϕ-stimulated autoreactive cultures are significantly less responsive to IL-1, IL-2, and IL-4 then their NH counterparts (482). We observed that GM-CSF further suppressed both NH and TBH Mϕ accessory activity. GM-CSF increases certain Mϕ accessory functions (154, 426, 448), but appears to serve as a suppressor molecule during autorecognition. This suppression may occur because GM-CSF triggers the synthesis and secretion of the suppressor molecule PGE_2. PGE_2 is synthesized in significant quantities during tumor growth and may regulate Mϕ-T cell interactions by "damping" potential overshoot activities (179). Because high levels of GM-CSF are detectable during pathologic immunological challenge (303), adding GM-CSF during autorecognition may trigger high PGE_2 synthesis which in turn suppresses T-cell proliferation. Others showed that GM-CSF-induced PGE_2 production eventually limits GM-CSF-induced activation and proliferation (17, 241, 354). Our results suggested that this mechanism is likely because NH Mϕ-stimulated autoreactive T cells demonstrated increased reactivity in the presence of GM-CSF only when PGE_2 synthesis was inhibited. TBH Mϕ-stimulated autoreactive T cells also showed an increase in reactivity in the presence of GM-CSF when PGE_2 synthesis was inhibited, but not to the same degree as their NH counterparts. This finding further confirms that Mϕ responsiveness to its own GM-CSF is affected by tumor growth.

We also wanted to determine if tumor growth changed autoreactive T-cell responsiveness to GM-CSF. Autoreactive T cells deviate from normal function during several disease states (199, 257, 414), and many autoreactive T cells appear to arise from splenic precursors (106). Tumor growth changes TBH T-cell responsiveness to several Mϕ-derived cytokines (283), and many T-cell lines have been identified that respond differently to GM-CSF. Sometimes, GM-CSF serves as a T-cell growth factor

and can induce tumorigenicity if the GM-CSF gene is self-regulated (270). In our system, GM-CSF increased TBH T-cell autorecognition whether or not PGE_2 synthesis was blocked. Although these findings contrast data from NH T cells, this may be due to changes in TBH T-cell responsiveness to GM-CSF. Because GM-CSF can stimulate the growth of T cells and both myeloid and nonhemopoietic malignant cell lines (33, 188), tumor-associated changes in T-cell proliferation may override the expected decrease in reactivity caused by Mϕ-derived PGE_2. This tumor-associated change probably involves the expression of GM-CSF receptors because certain leukemias (188) and tumor cell lines derived from T cells (33, 338) either express high levels of GM-CSF receptors or demonstrate constitutive activation of the receptors. We are currently evaluating GM-CSF receptor expression on TBH Mϕ and T cells (unstimulated and activated) to determine if this scenario is correct.

We also investigated the importance of M-CSF during autorecognition. GM-CSF and M-CSF can regulate Mϕ-T cell interactions antagonistically or synergistically (469), and M-CSF alone induces several Mϕ activities (470, 471). M-CSF may change TBH Mϕ responsiveness to GM-CSF because TBH Mϕ react differently to M-CSF during allo- and autorecognition (unpublished observations). However, both NH and TBH Mϕ-stimulated autoreactive T cells responded similarly in the presence of both cytokines with or without indomethacin. M-CSF induces suppression during several Mϕ-mediated activities (471), but does not seem to play a significant role during autorecognition with GM-CSF. This result may occur because GM-CSF-induced activities "mask" the suppressor effect noted with M-CSF alone.

Admixture experiments verified that the defect in Mϕ responsiveness to GM-CSF was not limited to an inability to increase class II density. Autoreactive T cells supplemented with TBH Mϕ did not show a significant increase in activity when PGE_2 synthesis was blocked. This finding is intriguing because the disrupted

GM-CSF/PGE_2 pathway should be restricted to the TBH Mϕ. NH Mϕ are present in the cultures with TBH Mϕ at ratios of 10:1, 2:1, and 1:1, but cultures containing even low TBH Mϕ concentrations had low reactivity. Because the NH Mϕ express normal or higher numbers of class II molecules in response to GM-CSF and NH T cells proliferate to a greater extent when PGE_2 synthesis is inhibited, the suppression in admixture cultures supplemented with TBH Mϕ must be attributable to an additional molecule. IL-10 (151), TGF-β (98), and TNF-α (466) may individually or additively account for the change in Mϕ responsiveness because these molecules have been associated with suppressor activity.

We investigated the possibility that tumor cell-derived GM-CSF promoted immunosuppression because many tumors produce and use GM-CSF as a growth factor. Whole tumor isolates which contained tumor cells and any additional tumor-associated Mϕ or tumor-infiltrating lymphocytes were used because they closely mimicked the *in situ* microenvironment. Tumor isolates produced low but detectable levels of of GM-CSF; this production may result from either interactions among immune cells or between immune cells and tumor cells. When tumor isolates were stimulated with the Mϕ activation agent LPS, GM-CSF production significantly increased. Tumor isolates stimulated with the T-cell mitogen concanavalin A did not produce GM-CSF. These findings suggest tumor-infiltrating lymphocytes are unable to synthesize GM-CSF but that tumor-associated Mϕ can produce GM-CSF. LPS-induced synthesis of GM-CSF by fibrosarcoma cells has not been ruled out but is not likely because LPS does not directly stimulate fibroblast synthesis of GM-CSF and because LPS does not alter the ability of other cytokines to modulate fibroblast synthesis of GM-CSF (385). Because tumor isolates contained small percentages of tumor-infiltrating lymphocytes and tumor-associated Mϕ, we expected to detect low endogenous levels of GM-CSF derived from these cell types. Our results suggest

fibrosarcoma cells may induce cellular anergy among T cells and Mϕ within the tumor. GM-CSF inhibits the proliferation of certain tumor cell types and increases tumoricidal activities among immune cell populations. Inhibition of GM-CSF production within the tumor mass may be advantageous to fibrosarcoma cells because T-cell and Mϕ tumoricidal activities and cytotoxic effector mechanisms which are dependent on GM-CSF would be unavailable.

We lastly considered the possibility that TBH Mϕ admixtures synthesized abnormal levels of GM-CSF. Using a GM-CSF-restricted cell line, we determined that GM-CSF production during TBH Mϕ-stimulated autorecognition is higher than NH Mϕ-stimulated autorecognition. Inhibition of PGE_2 synthesis decreased the GM-CSF activity in cultures stimulated by TBH Mϕ, but not to the same level observed with NH Mϕ. The tumor-induced increase in GM-CSF activity suggests that the GM-CSF/PGE_2 network is affected in TBH Mϕ because cultures with the lowest GM-CSF activity demonstrated the highest levels of autorecognition.

Collectively, these data suggest that tumor growth alters the response to and production of GM-CSF during autorecognition. GM-CSF has shown clinical promise in therapies designed to reconstitute compromised immune systems. Patients undergoing chemotherapy, irradiation, autologous bone marrow transplant, AIDS therapy, and burn recovery (49, 198, 303) have a rapid response to GM-CSF because it significantly induces progenitors of Mϕ and neutrophils to proliferate (135, 263, 310). GM-CSF's ability to induce tumoricidal activity have promising clinical applications (195, 383). However, some researchers suggest that GM-CSF can have a therapeutic advantage only in those possessing the appropriate progenitor cells, and that sometimes such cells may be lost during therapy (310). Because many cancers constitutively produce GM-CSF or overly respond to GM-CSF (33, 408, 427, 479), GM-CSF therapies must administer the proper dose to avoid stimulating the growth

of tumor tissue or initiating a cascade of oncogene-associated events in pre-leukemic cells (188). Investigations into the biological significance of GM-CSF during tumor growth, even among cells away from the tumor site, will prove valuable in defining effective immunotherapies against cancer.

SECTION III: T CELL DYSFUNCTION DURING TUMOR GROWTH

Although tumor-induced changes in Mϕ are associated with immunosuppression, the three chapters in this section underscore the significance of tumor-induced alterations in T cell phenotype and function. Both $CD4^+$ and $CD8^+$ T cells are involved with tumor-induced immunosuppression. Chapter VI shows that the number of $CD8^+$ T cells *in vivo* significantly increased during tumor growth and that $CD8^+$ T cells significantly suppress T-cell allorecognition and autorecognition. Similar to TBH MHC class II^- Mϕ, TBH $CD8^+$ T cells are more suppressive than their NH counterparts and suppression is mediated partly through dysregulation of IFN-γ, IL-4, and PGE_2 production. The greatest suppression of allorecognition occurs when both TBH $CD8^+$ T cells and TBH Ia^- Mϕ were present as accessory cells. In contrast, chapters VII and VIII explain that TBH $CD4^+$ T cells are not as suppressive as TBH $CD8^+$ T cells. Nonetheless, these cells contribute to decreased immunocompetence. These chapters show that TBH $CD4^+$ T cells produce less GM-CSF than NH $CD4^+$ T cells, and that GM-CSF dysregulation is linked partly to increased sensitivity to tumor-derived and

TBH Mϕ-derived molecules such as IL-10 and TGF-β_1. Tumor growth also changes $CD4^+$ T-cell responsiveness to cytokines associated with T_H1 and T_H2 responses, which excludes the possibility that tumor growth triggers functional shifts among helper T cells. TBH $CD4^+$ T cells also are more sensitive to the antimitotic activity of taxol, which may explain why taxol therapies show limited success in clinical trials.

CHAPTER VI

CYTOKINES AND SUPPRESSOR MACROPHAGES CAUSE TUMOR-BEARING HOST CD8$^+$ T CELLS TO SUPPRESS RECOGNITION OF ALLOGENEIC AND SYNGENEIC MHC CLASS II MOLECULES

ABSTRACT

Quantitative and qualitative tumor-associated changes in T-cell phenotype and function were identified in $CD8^+$ T cells. Tumor growth changed splenic $CD4^+/CD8^+$ T-cell ratios and induced the appearance of more cells with the $CD8^+$ phenotype. In comparison to equal concentrations of normal host (NH) counterparts, tumor-bearing host (TBH) $CD8^+$ T cells were highly suppressive to allorecognition and autorecognition. Suppression was not due to quantitative reductions in $CD4^+$ T cells although minor qualitative differences were observed. Suppression appeared to be mediated partly by prostaglandin E_2 (PGE_2). Interferon-γ (IFN-γ) and interleukin-4 (IL-4) contributed to TBH $CD8^+$ T cell-mediated suppression. Blocking studies using monoclonal antibodies (mAb) in conjunction with indomethacin suggested that cytokine networks involving IFN-γ, IL-4, and PGE_2 were disrupted during tumor growth and promoted TBH $CD8^+$ T-cell suppression. Alloresponses and autoresponses were significantly suppressed when TBH $CD8^+$ T cells mediated these reactions simultaneously with TBH Ia^- macrophages. Inhibition of PGE_2 production was unable to reverse the additive suppression caused by these two cell types. These results collectively suggest tumor-induced changes in $CD8^+$ T cells lead to suppressed allorecognition and autorecognition through both soluble mediator molecules and cellular interactions.

INTRODUCTION

Tumor growth initiates a myriad of phenotypic and functional alterations among several cell types such as Mϕ and T cells (482). These changes cause extreme immunosuppression to the host, which can lead to heightened susceptibility to infection and progression of the tumor. Our previous work shows that TBH Mϕ-mediated suppression can be demonstrated *in vitro* through allogeneic and syngeneic MLR (172, 484). The allogeneic MLR measures $CD4^+$ T-cell responsiveness to foreign MHC class II antigens (273), while the syngeneic MLR measures $CD4^+$ T-cell responsiveness to self MHC class II antigens (28, 463). Whereas our earlier investigations showed TBH Mϕ significantly suppress both foreign and self H-2^d-restricted $CD4^+$ T-cell proliferation through soluble mediator molecules, the present study showed that TBH $CD8^+$ T cells are highly suppressive because of specific cytokine signals and Mϕ interactions.

Although phenotypically $CD8^+$ T cells appear virtually homogeneous, functionally they represent a population of lymphoid lineage cells that offer both help and cytotoxicity during immune challenge (322). $CD8^+$ T cells are responsible for surveillance against certain tumors (460) and can mediate autologous tumor regression through the production of GM-CSF, TNF-α, and IFN-γ (383). The rejection of Ia^- syngeneic tumors can be accomplished by $CD8^+$ T cells in the absence of $CD4^+$ T cells. These activities verify that the $CD8^+$ population can offer both help and effector activities during tumoricidal responses (139).

However, $CD8^+$ T cells also are associated with numerous suppressor functions (21), and our work suggests these suppressive activities are amplified during progressed tumor development. The CD8 molecule is a ligand that can induce sup-

pression (202), and cells bearing this marker may accomplish suppression in the absence of cytolytic activity by inhibiting signal transduction between the TCR and CD3 (211). Monocytes and Mϕ can serve as alloantigen-presenting cells (allo-APC) and trigger unprimed $CD8^+$ T cells to respond to MHC class I allogeneic antigen challenge through a mechanism independent of $CD4^+$ helper T cells (297). Mϕ and monocytes stimulate $CD8^+$ T-cell proliferation by regulating IL-2 and IL-2 receptor induction (297). Investigators have suggested that Mϕ and monocytes regulate a suppressive interaction with $CD8^+$ T cells through PGE_2 production. PGE_2 is a monocyte and Mϕ-derived signal that works sequentially with IFN-γ to induce $CD8^+$ T-cell differentiation (127). PGE_2 also appears to work with IFN-γ to reduce $CD4^+$ T-cell proliferation and decrease immunoglobulin production (127). IFN-γ further elicits certain APC that induce suppressive $CD8^+$ T cells (333). Ia^- Mϕ especially are important in suppressive Mϕ-T cell interactions and significantly inhibit CTL development (81).

Using our tumor model, we investigated the significance of $CD8^+$ T cells and Ia^- Mϕ to tumor-induced suppression of allorecognition and autorecognition. Although phenotypic variation between cytotoxic and suppressive $CD8^+$ T cells is somewhat limited (256), we identified a significant quantitative increase in the percentage of $CD8^+$ T cells present during tumor growth. Furthermore, we showed that TBH $CD8^+$ T cells were qualitatively highly suppressive when compared to equal concentrations of NH counterparts. We demonstrated that the mediator molecules PGE_2 and IFN-γ, which act during development and induction of $CD8^+$ T cells with suppressor characteristics in the NH, were important to TBH $CD8^+$ T cell-mediated suppression. IL-4 appeared to be a required signal during both NH and TBH allorecognition. Lastly, we showed that tumor growth amplifies the suppressive interactions between $CD8^+$ T cells and Ia^- Mϕ. Collectively, our data implicate $CD8^+$ T cells as a significant

contributor to suppression in the TBH because of cytokine dysregulation and Mϕ interactions.

MATERIALS AND METHODS

Animals

The 8 to 12 week-old male BALB/c mice (Harlan-Sprague-Dawley, Madison, WI) used were LDH virus negative (Microbiological Associates, Bethesda, MD). A single-cell suspension of 8 x 10^5 methylcholanthrene-induced, nonmetastatic fibrosarcoma cells was injected i.m. into the left hind leg of mice. Palpable tumors formed by days 10-14, which would kill the hosts by days 28-35 (123). Splenic immune cells from 21-day TBH mice were used throughout. Immunosuppression was not caused by a general inflammatory response, because normal BALB/c mice injected with C3H muscle tissue 3 weeks before had normal autoresponses (484). Irradiated splenic lymphocytes from 8 to 12 week-old male C3H mice were used as the source of stimulator cells for allogeneic MLR cultures.

Medium

All cells were cultured in complete medium: RPMI-1640 medium with 10% heat-inactivated fetal bovine serum, 4 x 10^{-5} *M* 2-ME, 50 mg/l gentamicin, 2 g/l $NaHCO_3$, and 25 *mM* HEPES.

Chemicals and mAb

Indomethacin was added to cultures at a final concentration of 10^{-7} *M*. This concentration inhibits the arachidonic acid pathway for PGE_2 synthesis and is nontoxic to immune cells (284). The following hybridoma cell lines (American Type Culture Collection [ATCC], Rockville, MD) were used to produce mAb: anti-IA^d (ATCC clone MK-D6), anti-IE^d (ATCC clone 14-4-4), anti-immature T cell and B cell (ATCC clone J11d), anti-Lyt-2 (ATCC clone 3.155), anti-L3T4 (ATCC clone GK1.5), anti-mouse IFN-γ (ATCC clone R4-6A2), and anti-mouse IL-4 (ATCC clone 11B11). All mAb were used as ammonium sulfate-precipitated fractions of culture supernatants. The isotype controls (rat IgG1) for anti-IFN-γ mAb and anti-IL-4 mAb showed no affect on T-cell autoresponses. Anti-IFN-γ mAb and anti-IL-4 mAb specificity controls were done using 10 U of recombinant murine IFN-γ (4.5-9 x 10^6 units/mg @$\geq$22 μg/ml; Genzyme, Cambridge, MA) and 20 units of recombinant murine IL-4 (10^8 units/mg; Genzyme); both activities were inhibited by their appropriate mAb.

Flow Cytometry

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics, Hialeah, FL) laser flow cytometer and cell sorter. This instrument was calibrated with fluorescent 10-micron-sized microsphere standards. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc., Palo Alto, CA). Data were collected with the multiparameter data acquisition and display system (MDADS,

Coulter Electronics). FITC-labeled mAb fluorescence was measured as the log of the green fluorescence.

T Cell and Mϕ Preparation

Mice were sacrificed by cervical dislocation and spleens were pulped. Erythrocytes were removed by treating suspensions with cold 0.83% NH_4Cl for 5 min. Cells were washed and incubated for 2 hr at 37°C and 5% CO_2 on 150 × 15 mm tissue culture plates. To collect $CD4^+$ and $CD8^+$ T cells, nonadherent cells were eluted from the plates and further depleted of Ia^+ B cells, Mϕ, and other contaminating cells by mixing with anti-IA^d, anti-IE^d, anti-immature T cell and B cell, and either anti-Lyt-2 (to remove $CD8^+$ T cells) or anti-L3T4 mAb (to remove $CD4^+$ T cells) in conjunction with a 1:12 dilution of complement. Purified $CD4^+$ and $CD8^+$ T cells were washed at least three times to remove residual complement and the viable cells were resuspended to 4 x 10^6 and 8 x 10^6 cells/ml, respectively, in complete medium. Fluorescence-activated cell sorter (FACS) analyses determined this procedure yields >99% $CD4^+$ or $CD8^+$ T cells. $CD8^+$ T cells were X-irradiated with 2000 rads (TFI Minishot II X-irradiator, New Haven, CT).

Mϕ (>96% esterase positive) were collected by washing (to remove nonadherent cells) and scraping with a rubber policeman and ice-cold RPMI-1640. To prepare Ia^- Mϕ, Mϕ were treated with anti-IA^d and anti-IE^d plus a 1:12 dilution of complement. The purity of the Ia^- Mϕ population (>96% Ia^- Mϕ) was confirmed by flow cytometric analyses. Mϕ (whole population [WP] or Ia^--enriched [Ia^-]) were counted and re-suspended to 8 x 10^6 cells/ml in complete medium and were X-irradiated with 2000

rads. Studies using Ia$^+$ Mϕ were not performed because only low cell quantities could be obtained through positive cell sorting. However, WP Mϕ from both NH and TBH contained a significant number of cells with an Ia$^+$ phenotype and demonstrated significant functional differences from negatively-selected Ia$^-$ Mϕ.

Allogeneic and Syngeneic MLR

For the allogeneic MLR, BALB/c (H-2^d-restricted) CD4$^+$ T cells (2 x 10^5 cells/well) and irradiated C3H (H-2^k-restricted) stimulator cells (4 x 10^5 cells/well) were seeded in "U" bottom 96-well plates. This number was required for a control level allorecognition and served as the "base" allogeneic MLR. For the syngeneic MLR, H-2^d-restricted CD4$^+$ T cells (4 x 10^5 cells/well) and irradiated H-2^d-restricted stimulator Mϕ (4 x 10^5 cells/well) were used. This number was required for control level autoreactive T-cell responsiveness and served as the "base" syngeneic MLR. The necessity for presentation of autologous MHC class II molecules in the syngeneic assay was verified by addition of mAb against IA^d and IE^d; these mAb completely abrogated autoreactivity when added to syngeneic MLR cultures. Four x 10^4 (10%), 1 x 10^5 (25%), 2 x 10^5 (50%), or 4 x 10^5 (100%) CD8$^+$ T cells/well or Mϕ/well (unseparated or Ia$^-$) were added to the assays as indicated. The percentage of Mϕ or CD8$^+$ T cells added to cultures represents the cell number compared to the number of stimulator cells added. Indomethacin, anti-IFN-γ mAb, and anti-IL-4 mAb were added to wells at the start of the 4-day incubation. Six hr before cell harvest, each well was pulsed with tritiated thymidine (^{3}H-TdR: specific activity 6.7 Ci/*mM*, Dupont NEN Re-

search Products, Boston, MA). Cells were harvested onto filters and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

Statistics/Calculation of Results

Cells from 3 to 6 mice were pooled for each experiment. For the allogeneic and syngeneic MLR assays, three replicate wells were run for each test, and each experiment was repeated three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p<0.05$) and presented as mean $\pm$ standard error.

RESULTS

Tumor growth induces shifts in T cell populations

Because earlier observations suggested phenotypic shifts among T cell populations were associated with tumor-induced immunosuppression, we measured CD4 and CD8 marker expression on splenic T cells from NH and TBH (Figure 20). Flow cytometric analyses detected a decrease in the percentage of $CD4^+$ T cells (64% to 49%; $p < 0.05$) and an increase in the percentage of $CD8^+$ T cells (36% to 51%; $p < 0.05$) during tumor growth. The expression of CD4 or CD8 per T cell did not change (no change in mean or peak fluorescence; data not shown). These data suggest tumor growth induces changes in the $CD4^+/CD8^+$ T cell ratios.

$CD8^+$ T cells from TBH suppress allorecognition and autorecognition

The significant ($p < 0.05$) increase in the percentage of $CD8^+$ T cells associated with tumor growth led us to hypothesize that the tumor-induced T cell population shift toward the $CD8^+$ phenotype affected alloreactivity. Equal concentrations of NH and TBH $CD8^+$ T cells suppressed alloreactivity, but TBH $CD8^+$ T cells were significantly ($p < 0.05$) more suppressive than NH $CD8^+$ T cells (Figure 21). Low and high concentrations of NH $CD8^+$ T cells, respectively, reduced alloreactivity to 85.1% and 69.0% of control levels, whereas TBH $CD8^+$ T cells reduced alloreactivity to 56.4% and 45.5% of control levels. These qualitative differences in allorecognition appear independent of the $CD4^+$ helper T cell response to alloantigen challenge because

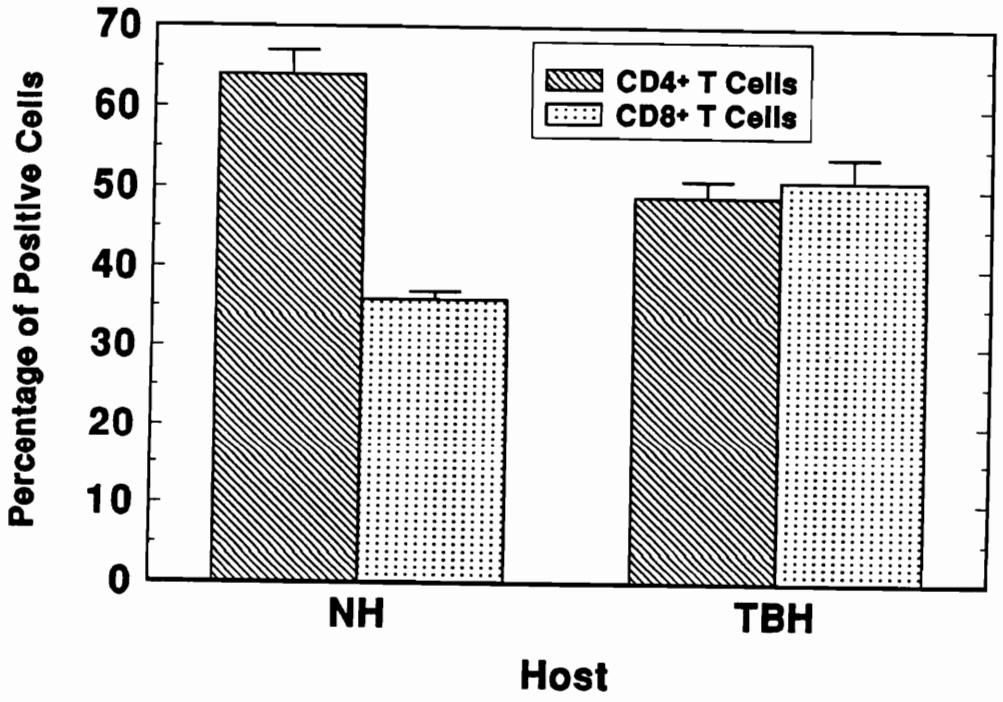

Figure 20. Tumor growth induces shifts in T cell populations: T cells were collected as stated in Materials and Methods. TBH represent mice with 21 days of tumor growth. T cells were labeled with anti-L3T4 ($CD4^+$ T cells) or anti-Lyt-2 ($CD8^+$ T cells) antibody, washed, incubated with the appropriate secondary FITC-tagged antibodies, and assessed by flow cytometry, as stated in Materials and Methods.

reactivity was only slightly reduced (18% decrease) when helper T cells from TBH were used (not shown). However, TBH $CD4^+$ T-cell responsiveness to alloantigen was statistically similar to NH $CD4^+$ T-cell responsiveness when TBH $CD8^+$ T cells mediated either reaction. Addition of anti-CD8 mAb to cultures lacking $CD8^+$ T cells had no affect, but did reverse the observed suppression when added to cultures containing $CD8^+$ T cells (not shown). These data suggest that tumor growth amplifies suppression mediated by $CD8^+$ T cells.

Based on the suppressed alloreactivity mediated by TBH $CD8^+$ T cells, we hypothesized that TBH $CD8^+$ T cells would also significantly suppress autorecognition (Figure 22). During NH Mϕ-stimulated autorecognition, TBH $CD8^+$ T cells were significantly ($p < 0.05$) more suppressive than NH $CD8^+$ T cells at all doses (Figure 22A). X-irradiated NH $CD4^+$ T cells served as a crowding control. Whereas low, medium, and high doses of NH $CD8^+$ T cells suppressed autoreactivity 10%, 30%, and 52%, respectively, TBH $CD8^+$ T cells suppressed autoreactivity 41%, 56%, and 70%. These results suggest that tumor growth increases the suppressive accessory activity of $CD8^+$ T cells during autorecognition.

We have previously shown that the Mϕ-derived suppressor molecule PGE_2 contributes to decreased reactivity during Mϕ-mediated autoreactivity (482). To identify the significance of PGE_2 during $CD8^+$ T cell-modulated autorecognition, indomethacin was added to syngeneic MLR cultures containing various doses of NH or TBH $CD8^+$ T cells. Cultures containing TBH $CD8^+$ T cells were 2 to 3 times less reactive than cultures containing NH $CD8^+$ T cells (Figure 22B). In comparison to untreated controls (Figure 22A), inhibition of PGE_2 synthesis had no affect when high concentrations of TBH $CD8^+$ T cells were used. TBH Mϕ-stimulated syngeneic MLR cultures supplemented with TBH $CD8^+$ T cells demonstrated the lowest autoreactivity and were the least responsive to indomethacin (not shown). Addition of exogenous PGE_2 to

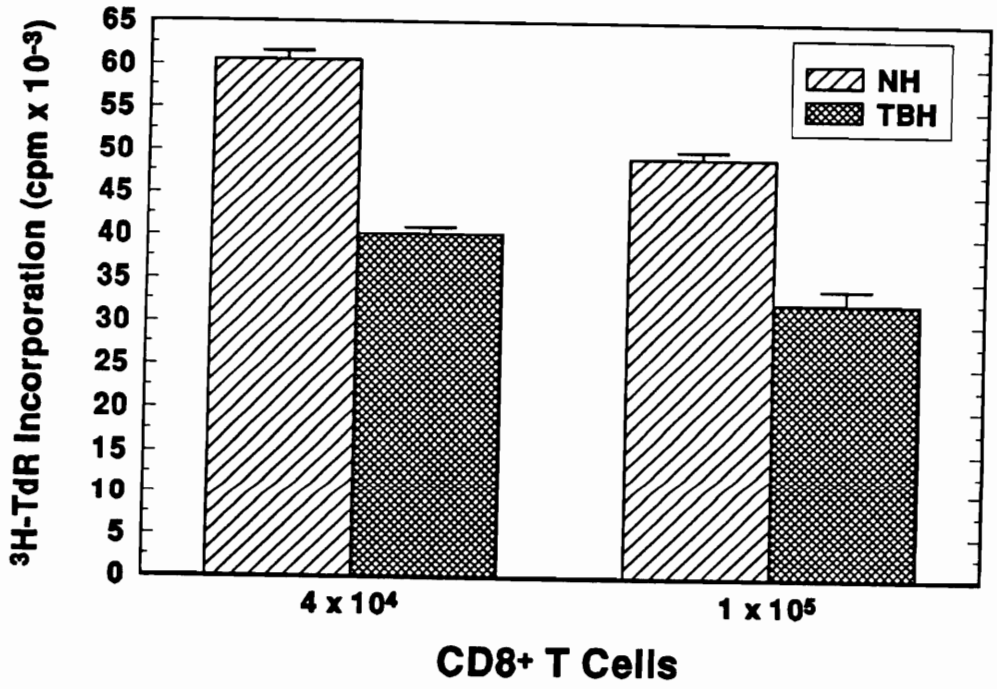

Figure 21. TBH $CD8^+$ T cells suppress allorecognition: Two × 10^5 cells/well BALB/c (H-2^d-restricted) $CD4^+$ T cells were stimulated with 4 × 10^5 cells/well X-irradiated C3H (H-2^K-restricted) lymphocytes. $CD8^+$ T cells (4 × 10^4 or 1 × 10^5 cells/well) from NH or TBH were added to the assays. Alloreactivity was measured by ^{3}H-TdR incorporation. Alloreactivity in the absence of $CD8^+$ T cells was 71,264 ± 2,299 cpm. Background proliferation for T cells alone was 910 cpm.

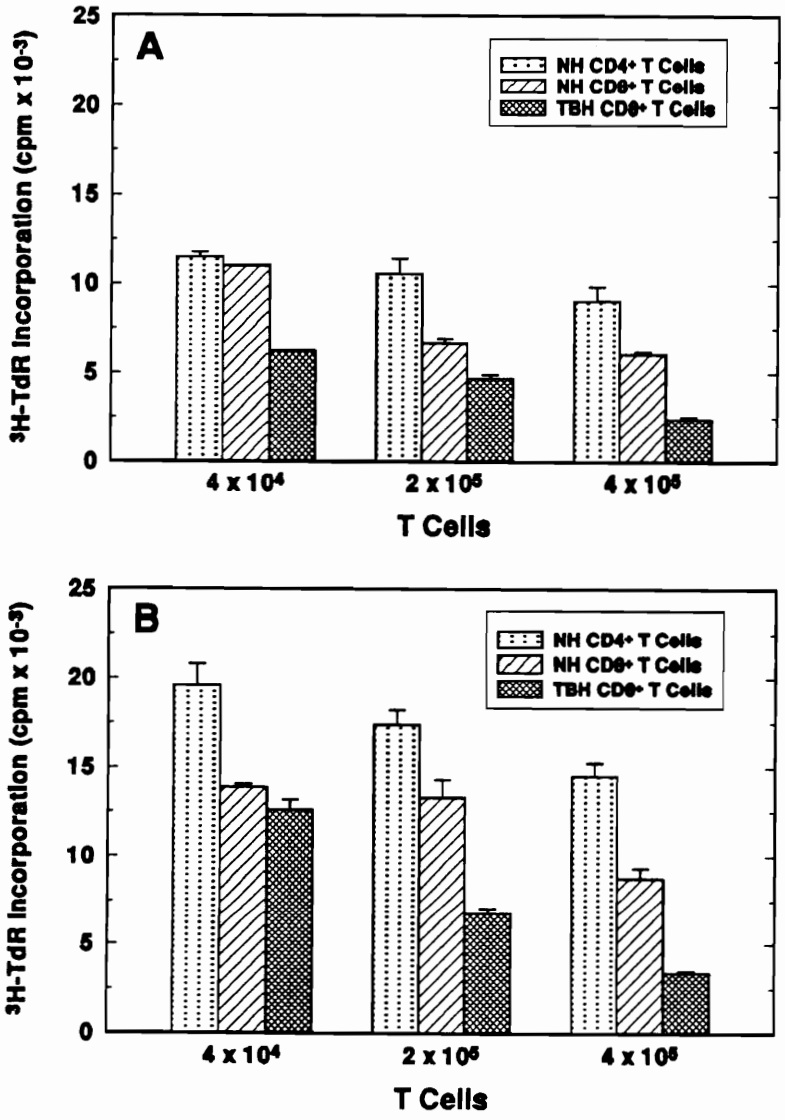

Figure 22. TBH $CD8^+$ T cells significantly suppress autorecognition: Four x 10^5 cells/well NH $CD4^+$ BALB/c $CD4^+$ T cells were stimulated with 4 x 10^5 cells/well X-irradiated NH Mϕ. Low (4 x 10^4 cells/well), medium (2 x 10^5 cells/well), or high (4 x 10^5 cells/well) concentrations of NH or TBH $CD8^+$ T cells were added to cultures as accessory cells. NH $CD4^+$ T cells served as crowding controls. Autoreactivity was measured by ^{3}H-TdR incorporation (panel A). Autoreactivity in the absence of $CD8^+$ T cells was 12,190 ± 500 cpm. Background proliferation for T cells alone was 882 cpm. PGE_2 synthesis was inhibited by indomethacin addition at a final concentration of 10^{-7} *M* (panel B). Autoreactivity in the absence of $CD8^+$ T cells was 20,382 ± 382 cpm.

cultures lacking TBH $CD8^+$ T cells partly mimics the observed suppression (not shown). Although indomethacin-treated syngeneic MLR cultures did not return to control levels, they lost approximately 50% of their suppressive activity. These findings suggest other molecules in addition to PGE_2 must affect $CD8^+$ T cell-mediated suppression of autoreactivity.

Cytokines associated with alloreactive and autoreactive T cells are involved in TBH $CD8^+$ T cell-mediated suppression

After determining that tumor growth was associated with quantitative and qualitative changes in $CD8^+$ T cell phenotype and accessory activity, we sought to determine if defective T-cell alloresponses and autoresponses were caused by disrupted cytokine pathways. Because $CD4^+$ alloreactive and autoreactive T cells can be partitioned into T_H1 and T_H2 subpopulations (79, 318, 366), we selected two cytokines that are uniquely synthesized by each T_H group. IFN-γ, which is produced by T_H1 cells, regulates several T-cell activities and can inhibit proliferation. IFN-γ additionally is produced by and targets $CD8^+$ T cells and triggers suppression. IL-4, which is produced by T_H2 cells, primarily targets B cells and monocytes, but unlike IFN-γ, can stimulate T-cell proliferation (194). Previous investigations showed that tumor growth causes alterations in the secretory patterns of IFN-γ and IL-4 during autoresponses with accessory Mϕ (450). We hypothesized that $CD8^+$ T-cell accessory activities could be influenced by these two cytokines.

Addition of anti-IFN-γ mAb to alloreactive cultures containing TBH $CD8^+$ T cells (all doses) restored activity to levels identical to allorecognition mediated by low

concentrations of NH $CD8^+$ T cells (Figure 23). The opposite effect (decreased allorecognition) was observed when exogenous IFN-γ (10 U/ml) was added to allogeneic MLR cultures. These findings suggested that most of the suppression observed during TBH $CD8^+$ T cell-mediated allorecognition may be due to IFN-γ. However, syngeneic MLR cultures treated with anti-IFN-γ mAb did not reverse the activity (Figure 24). Although mAb-treated syngeneic MLR cultures containing TBH $CD8^+$ T cells showed a significant ($p < 0.05$) increase in autoreactivity (Figure 24B), the responsiveness did not approach mAb-treated cultures containing NH $CD8^+$ T cells (Figure 24A). Autoreactivity mediated by NH $CD8^+$ T cells and anti-IFN-γ mAb was restored to control levels at all concentrations tested, whereas only autoreactivity mediated by low concentrations of TBH $CD8^+$ T cells and anti-IFN-γ mAb was restored to control levels. When anti-IFN-γ mAb and indomethacin were added together, autoresponses mediated by TBH $CD8^+$ T cells were significantly higher than syngeneic cultures mediated by TBH $CD8^+$ T cells treated with anti-IFN-γ mAb alone. However, suppression caused by high doses of TBH $CD8^+$ T cells was not completely reversed in the presence of both blocking agents. These data suggest IFN-γ and PGE_2 additively suppress allorecognition and autorecognition supplemented with TBH $CD8^+$ T cells.

Addition of anti-IL-4 mAb significantly ($p < 0.05$) reduced alloreactivity (Table 7); addition of exogenous IL-4 (20 U/ml) caused a corresponding increase in allorecognition (data not shown). When anti-IL-4 mAb was added to alloreactive cultures supplemented with $CD8^+$ T cells, TBH $CD8^+$ T cell-mediated alloreactivity significantly ($p < 0.05$) decreased at all doses whereas NH $CD8^+$ T cell-mediated alloresponses were unaffected. When anti-IL-4 mAb was used with indomethacin, both NH and TBH $CD8^+$ T cell-mediated alloresponses were significantly ($p < 0.05$) higher than the groups treated with anti-IL-4 mAb alone but were lower than cultures

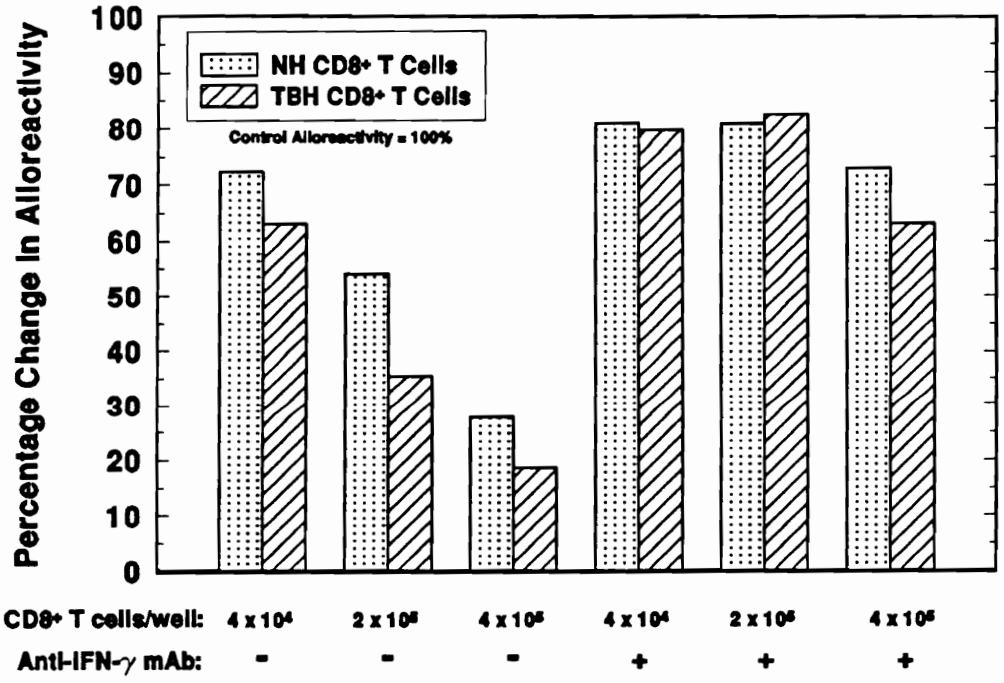

Figure 23. IFN-γ contributes significant levels of suppression to TBH CD8$^+$ T cell-mediated allorecognition: Allogeneic MLR cultures were supplemented with low, medium, and high concentrations of NH or TBH CD8$^+$ T cells (as described in Figure 22) in the absence or presence of anti-IFN-γ mAb (R4-6A2). The change in alloreactivity was calculated from controls that were not supplemented with CD8$^+$ T cells.

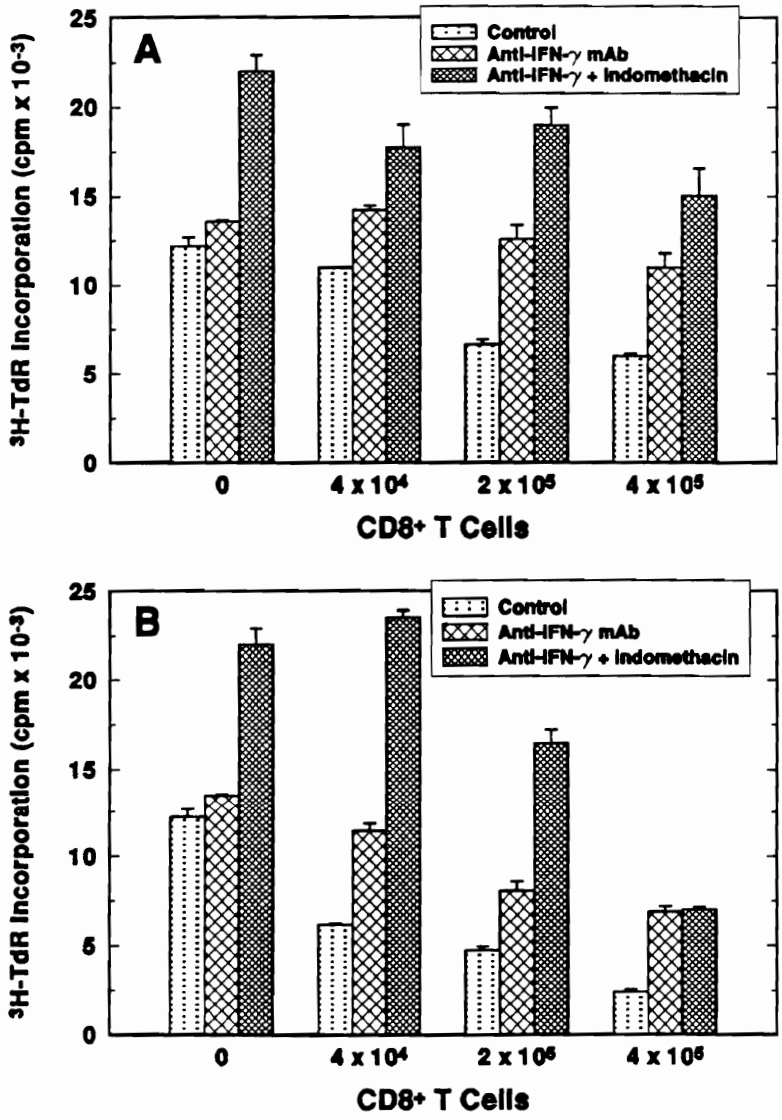

Figure 24. **IFN-γ and PGE_2 additively suppress TBH $CD8^+$ T cell-mediated autorecognition:** Syngeneic MLR cultures were supplemented with low, medium, and high concentrations of NH (panel A) or TBH (panel B) $CD8^+$ T cells (as described in Figure 22) in the absence or presence of anti-IFN-γ mAb and indomethacin.

treated with indomethacin alone. These results suggest that IL-4 is required during NH T-cell allorecognition and that tumor growth heightens the necessity of this cytokine during challenge with alloantigen. Previous results suggest IL-4 has marginal activity during autorecognition (450) and was not further tested here.

Ia$^-$ Mϕ interact with TBH CD8$^+$ T cells to suppress alloreactivity and autoreactivity

Previous investigations show that Mϕ lacking MHC class II antigens (Ia$^-$ Mϕ) contribute to the immunosuppression seen during tumor growth (172, 450). The percentage of splenic TBH Mϕ deficient in Ia antigen expression increases significantly during tumor growth (44 ± 1% NH Ia$^-$ Mϕ vs. 60 ± 5% TBH Ia$^-$ Mϕ, $p < 0.05$; data not shown), and TBH Ia$^-$ Mϕ are responsible for a majority of the suppression observed during allorecognition and autorecognition. Because tumor growth shifts the phenotype and accessory activities of both Mϕ and T cells, we wanted to determine if Ia$^-$ Mϕ and CD8$^+$ T cells interact to suppress allorecognition and autorecognition. Because preliminary investigations suggested alloresponses and autoresponses mediated simultaneously by CD8$^+$ T cells and Mϕ were dose and cell ratio dependent, CD8$^+$ T cells were used at 4×10^4 cells/well and 1×10^5 cells/well while Mϕ were used throughout at 4×10^4 and 2×10^5 cells/well (Figure 25).

We first determined that TBH Mϕ significantly ($p < 0.05$) suppressed allorecognition (Figure 25A). Low concentrations of NH Mϕ, both whole population (WP) and Ia$^-$-enriched (Ia$^-$), increased alloreactivity, whereas low concentrations of TBH Mϕ were suppressive. Low concentrations of TBH Ia$^-$ Mϕ were more suppressive than TBH WP Mϕ. High concentrations of Mϕ, irrespective of the host,

Table 7. Interaction between IL-4 and PGE_2 during $CD8^+$ T cell-mediated allorecognition

T Cell Source[a]	Treatment	$CD8^+$ T Cells Present (cells/well)			
		0	4×10^4	2×10^5	4×10^5
-----	-----	36.36 ± 1.56[b]	-----	-----	-----
-----	Anti-IL-4 mAb	21.67 ± 1.71[c]	-----	-----	-----
-----	Indomethacin[d]	83.47 ± 1.63	-----	-----	-----
-----	Anti-IL-4 mAb + Indomethacin	58.04 ± 0.85	-----	-----	-----
NH	-----	-----	32.51 ± 3.57	25.76 ± 2.15	25.97 ± 2.37
NH	Anti-IL-4 mAb	-----	24.26 ± 1.64	22.73 ± 1.43	24.59 ± 0.50
NH	Indomethacin	-----	89.38 ± 3.30	69.15 ± 3.47	46.74 ± 6.14
NH	Anti-IL-4 mAb + Indomethacin	-----	47.87 ± 4.46	46.72 ± 4.45	37.70 ± 0.03
TBH	-----	-----	28.[illegible] ± 0.42	20.06 ± 2.14	18.00 ± 1.14
TBH	Anti-IL-4 mAb	-----	14.[illegible] ± 0.96	8.36 ± 0.98	10.78 ± 2.14
TBH	Indomethacin	-----	64.69 ± 7.82	41.37 ± 3.31	29.29 ± 3.44
TBH	Anti-IL-4 mAb + Indomethacin	-----	51.40 ± 7.36	20.99 ± 2.91	25.73 ± 2.51

[a]Allogeneic MLR cultures contained 2×10^5 BALB/c $CD4^+$ splenic T cells stimulated by 4×10^5 C3H splenic lymphocytes.

[b]Values represent cpm x 10^{-3} as determined by ^{3}H-TdR incorporation.

[c]Anti-IL-4 mAb (ATCC clone 11B11) was added per well at a final concentration which inhibited the activity of 20 U/ml IL-4 (Genzyme, Cambridge, MA).

[d]Indomethacin was added at a final concentration of 10^{-7} M per well.

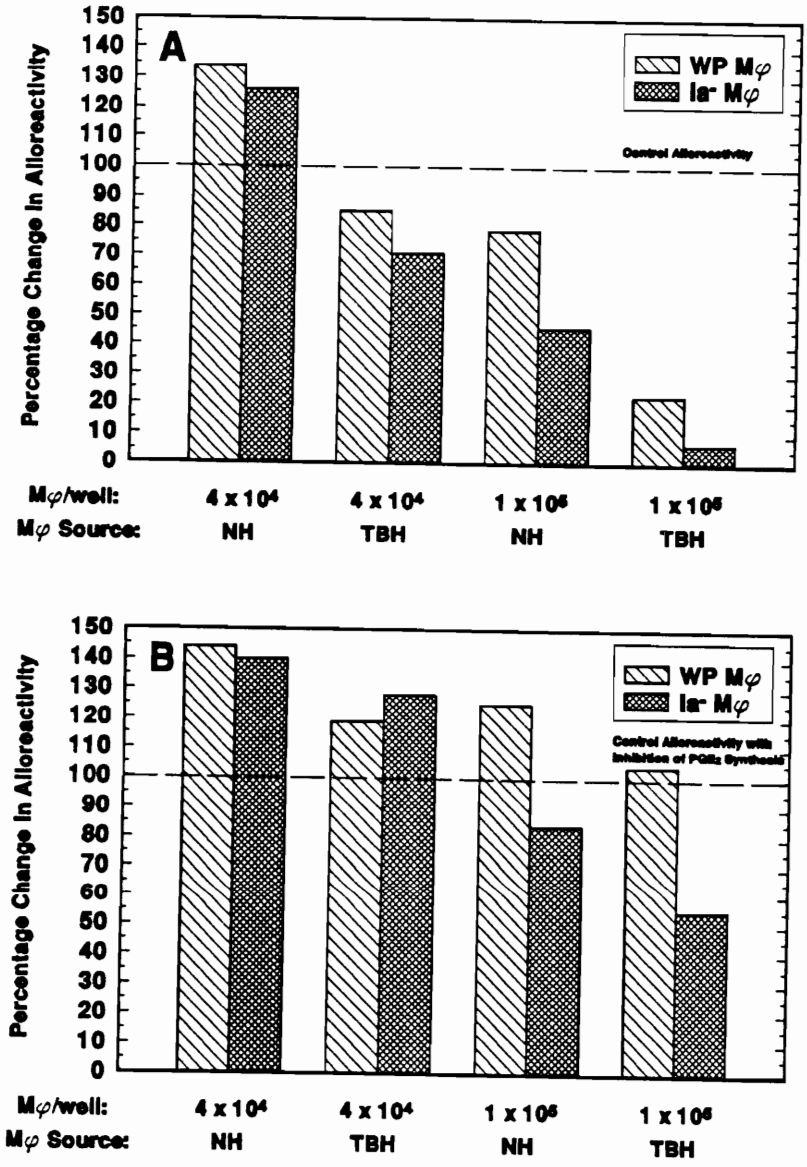

Figure 25. TBH Ia⁻ Mϕ significantly suppress allorecognition: Allogeneic MLR cultures were supplemented with 4×10^4 or 2×10^5 WP or Ia⁻ Mϕ from NH or TBH. The change in alloreactivity was calculated from controls that were not supplemented with Mϕ (panel A). PGE_2 synthesis was inhibited by indomethacin addition at a final concentration of 10^{-7} *M* (panel B).

suppressed alloreactivity, and Ia$^-$ Mϕ were the most suppressive. High concentrations of TBH Ia$^-$ Mϕ generated the greatest suppression. These results suggest Ia$^-$ Mϕ are significantly more suppressive to allorecognition than WP Mϕ, and that tumor growth magnifies this suppression.

To determine if Ia$^-$ Mϕ-mediated suppression was due to PGE_2 production, cultures were supplemented with indomethacin (Figure 25B). All cultures mediated by Mϕ except those mediated by high concentrations of TBH Ia$^-$ Mϕ demonstrated activity at or above alloresponsive cultures lacking Mϕ. Tumor growth increases PGE_2 synthesis, which may affect other cell types that are responsive to PGE_2. These data suggested PGE_2 contributed to suppression mediated by both WP and Ia$^-$ Mϕ, and that PGE_2 derived from Mϕ may be a key signal for CD8$^+$ T-cell suppressor activity during tumor growth.

To test this hypothesis, cultures containing CD8$^+$ T cells were admixed with WP or Ia$^-$ Mϕ to mimic the *in vivo* scenario (Figure 26). With only one exception, admixtures of NH Mϕ did not induce significant suppression of allorecognition (Figure 26A). However, when TBH allogeneic MLR cultures containing CD8$^+$ T cells and admixtures of Mϕ are compared to control cultures lacking Mϕ (Figure 26B), the data suggest Mϕ in the TBH offer additional suppression and signal TBH CD8$^+$ T cells to become more suppressive. This signalling was confirmed by 24 and 48 hr preincubation experiments of CD8$^+$ T cells with the appropriate Mϕ supernatants alone. High concentrations of TBH Mϕ, both WP and Ia$^-$, significantly ($p < 0.05$) increased suppressor activity by CD8$^+$ T cells. The combination of high concentrations of TBH CD8$^+$ T cells and TBH Ia$^-$ Mϕ caused the greatest suppression. These data verify that TBH Mϕ are a rich source of PGE_2, which serves as a signal for TBH CD8$^+$ T-cell suppressor activity. Inhibition of PGE_2 synthesis verifies the significance of PGE_2 to allorecognition mediated by Mϕ and CD8$^+$ T cells (Table 8). Although PGE_2 synthesis

inhibition reversed part of the suppression in all cultures tested, allorecognition was consistently less responsive when mediated by TBH accessory cells. This result further confirms that the suppressive interaction between TBH Ia$^-$ Mϕ and TBH CD8$^+$ T cells is through soluble mediators such as PGE_2.

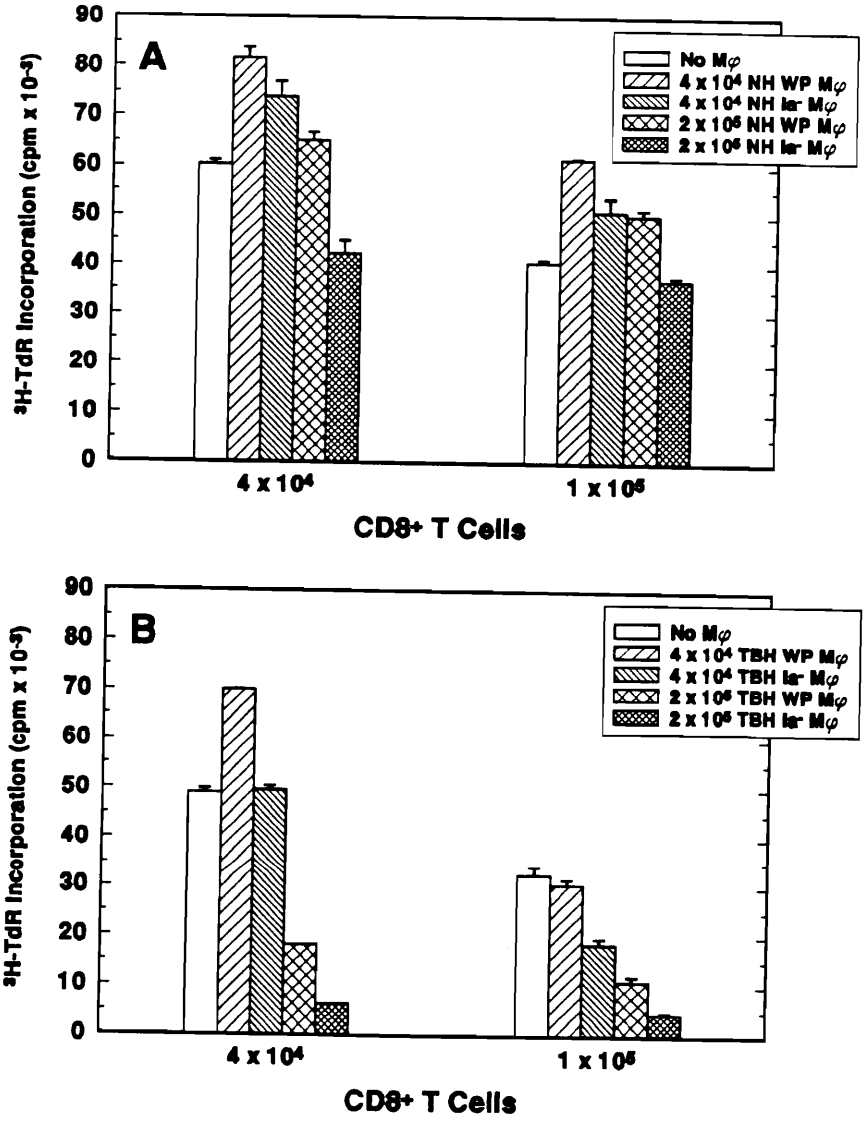

Figure 26. TBH Ia⁻ Mϕ interact with CD8⁺ T cells to mediate high levels of suppression during allorecognition: Allogeneic MLR cultures were supplemented with 4 x 10^4 or 2 x 10^5 WP or Ia⁻ Mϕ from NH or TBH and 4 x 10^4 or 1 x 10^5 NH (panel A) or TBH (panel B) CD8⁺ T cells. Control cultures not containing Mϕ as accessory cells are indicated by the open bar in each group.

Table 8. Contribution of PGE_2 to allorecognition mediated by Mϕ and $CD8^+$ T cells

Cell Source[a]	Mϕ/well	$CD8^+$ T cells/well	Indomethacin Absent	Indomethacin Present[c]
-----	-----	-----	49.14 ± 1.72[b]	62.86 ± 2.52
NH	4×10^4	4×10^4	52.00 ± 2.86	90.29 ± 2.91
TBH	4×10^4	4×10^4	38.86 ± 1.43	78.86 ± 0.44
NH	4×10^4	1×10^3	46.29 ± 1.64	74.71 ± 1.15
TBH	4×10^4	1×10^3	30.28 ± 0.28	65.14 ± 0.61
NH	2×10^3	4×10^4	42.77 ± 1.02	88.56 ± 1.71
TBH	2×10^3	4×10^4	23.43 ± 0.57	52.56 ± 1.11
NH	2×10^3	1×10^3	33.72 ± 1.91	80.56 ± 0.60
TBH	2×10^3	1×10^3	12.59 ± 1.71	34.86 ± 1.60

[a]Allogeneic MLR cultures contained 2×10^5 BALB/c $CD4^+$ splenic T cells stimulated by 4×10^5 C3H splenic lymphocytes. The number of accessory cells (Mϕ and $CD8^+$ T cells) added to the assays are indicated.

[b]Values represent cpm $\times 10^{-3}$ as determined by ^{3}H-TdR incorporation.

[c]Indomethacin was added at a final concentration of 10^{-7} M per well.

DISCUSSION

The presented data strongly suggest that tumor growth induces phenotypic and functional changes in $CD8^+$ T cells and that this population mediates significant levels of suppression in the TBH. TBH $CD4^+$ T cells are less reactive to alloantigen and autoantigen challenge but the suppression mediated by $CD8^+$ T cells is significantly greater. Although $CD8^+$ T cells are generally associated with cytolytic activity during immune challenge by tumor cells, $CD8^+$ T cells are also potent suppressor cells *in vitro* and *in vivo* (202, 211, 333). Efforts to phenotypically fractionate $CD8^+$ T cells into cytotoxic and suppressor subsets have largely been unsuccessful, although $CD8^+$ T cell-derived clones suggest cytolytic activity is associated with a $CD11b^-/CD28^+$ phenotype, and suppressive activity is exclusively found with a $CD11b^+/CD28^-$ phenotype (256, 475). Although the significance of such partitioning is not fully resolved, such data suggest that either two stable subpopulations of $CD8^+$ T cells exist *in vivo* or that certain events within the host induce alterations in phenotypic expression which correlate to unique effector functions. Furthermore, recent evidence suggests subsets of $CD8^+$ T cells exist (based on their effector functions and cytokine production/responsiveness) both *in vivo* and *in vitro* (39). Our flow cytometric data suggested that tumor growth induced a significant shift toward the $CD8^+$ phenotype but that the antigen density per cell was unaffected. Whether the $CD8^+$ T cells also possessed the suppressor $CD11b^+/CD28^-$ phenotype has not been tested, but our functional data suggest that the $CD8^+$ T cells present in the TBH are predominantly suppressor cells. The suppression caused by this cell type appears to be amplified by soluble mediators such as IFN-γ and PGE_2 and by interactions with Mϕ, whereas the presence of IL-4 appears required for both NH and TBH responsiveness. The

necessity of IL-4 and antagonistic regulation of responsiveness by IFN-γ suggest that the predominant $CD8^+$ T cell population present during tumor growth is a suppressor phenotype, which

$CD8^+$ T-cell suppressor activity appears to be independent of cytotoxic activity (211). This predominant suppressor function is also apparent when TBH $CD8^+$ T cells serve as the sole responder population during alloreactivity (283). In the NH, $CD8^+$ T cells suppress mitogen-stimulated $CD4^+$ T-cell proliferation and B-cell immunoglobulin production (127). In addition, $CD8^+$ T cells suppress alloresponsive (202) and autoresponsive (53) $CD4^+$ T cells. Addition of anti-CD8 mAb to cultures containing $CD8^+$ T cells reversed suppression, and our findings are consistent with others that show both *in vitro* and *in vivo* administration of mAb can functionally downregulate $CD8^+$ T cells and increase T-cell proliferation (53). Our data reflect the suppressive capacity of $CD8^+$ T cells to alloreactivity and autoreactivity and further suggest that $CD8^+$ T cells are significantly more suppressive during tumor growth. Mϕ also are significantly more suppressive during tumor growth. Both NH and TBH Mϕ mediate a significant portion of their suppression through PGE_2 production, and we have previously shown TBH Mϕ disrupt NH T-cell activities such as recognition of foreign and self MHC class II antigens through increased PGE_2 synthesis (284, 484). In an attempt to reverse $CD8^+$ T cell-suppression during autorecognition, we added indomethacin to $CD8^+$ T cell-mediated autoreactive cultures. Cultures containing TBH $CD8^+$ T cells were the least responsive to indomethacin treatment, which suggests that other agents in addition to PGE_2 account for $CD8^+$ T cell-mediated suppression. In the assays, PGE_2 was generated primarily by the syngeneic stimulator cells. PGE_2 is primarily a monocyte and Mϕ-derived molecule, and it is possible that its modulatory effect occurs *in vivo* where TBH Mϕ secrete PGE_2 in overabundance. The over-production of PGE_2 by TBH Mϕ not only suppresses $CD4^+$ T-cell responses but

may account for the increased $CD8^+$ T cells present in the spleen because PGE_2 is a $CD8^+$ T-cell differentiation signal (126). *In vivo* administration of indomethacin or other cyclooxygenase inhibitors during tumor growth may partly reverse $CD8^+$ T-cell suppression by reducing Mϕ PGE_2 production and $CD8^+$ T-cell differentiation. Although cyclosporin significantly prolongs graft survival by inhibiting $CD4^+$ and $CD8^+$ T-cell functions (19), such therapy may prove detrimental to TBH whose immune system is already compromised.

Because significant mediator molecules are associated with tumor-induced suppression of allorecognition and autorecognition, we sought to determine if these same cytokines were implicated during TBH $CD8^+$ T cell-induced suppression. IFN-γ and IL-4 are representative cytokines from $CD4^+$ T_H1 and T_H2 cell secretory patterns, and a separate report shows that these two cytokines are dysregulated during tumor growth (450). The suppressor functions inducible by IFN-γ are well characterized. IFN-γ induces several suppressor activities in $CD8^+$ T cells (127, 333) and suppress B-cell and T-cell responses (167, 168). IFN-γ is produced by $CD8^+$ T cells during tumor growth (383), and during NH T-cell activation through cross-linkage of CD3 and CD8 (377). Furthermore, many $CD8^+$ T cell clones have the T_H1 cytokine secretory pattern (159). Blocking both of these cytokines with mAb restored alloreactivity and autoreactivity modulated by TBH $CD8^+$ T cells, but other molecules may be involved because $CD4^+$ T-cell reactivity to alloantigens and autoantigens was never restored to control levels. Other investigators propose that a partially characterized molecule called T suppressor cell differentiation factor (TsDF) is partly responsible for $CD8^+$ T-cell suppression during allorecognition (82). The TCR α chain on $CD8^+$ T cells may also be part of an antigen-specific soluble suppressor molecule (265). We suspect two cytokines, TGF-β and IL-10, not investigated in this report, may contribute to $CD8^+$ T cell-mediated suppression. TGF-β is a chemotactic factor for $CD8^+$ T cells (2) and

suppresses both alloreactive and autoreactive T-cell proliferation during tumor growth (412). Although IL-10 increases cytotoxic effector functions (77), it is not clear if this molecule increases IL-2 responsiveness among all $CD8^+$ T cells or just those demonstrating cytolytic activity. IL-10 significantly inhibits cytokine synthesis by T_H1 cells and suppresses several Mϕ functions (152), and preliminary evidence suggests tumor growth disrupts Mϕ synthesis of IL-10 (unpublished data). We are currently determining if these two molecules are responsible for the remaining suppression not accounted for during $CD8^+$ T cell-mediated allorecognition and autorecognition.

We also investigated the possibility that Mϕ and $CD8^+$ T cells interacted to mediate suppression. Mϕ can serve as allo-APC to $CD8^+$ T cells (297, 398) and facilitate their development and induction *in vivo* (126, 127). Ia^- Mϕ are a suppressor population (328) and can interact through soluble mediators with $CD8^+$ T cells to decrease development (81). The Ia^- Mϕ population increases during tumor development just as the $CD8^+$ T cell population does, and we showed these two suppressive cell types contribute to suppression. Indomethacin treatment during alloreactivity mediated by both $CD8^+$ T cells and Mϕ significantly increased reactivity in all groups tested and confirmed Mϕ-derived PGE_2 is a major suppressive molecule during tumor growth. Although reactivity did not reach control values, alloreactivity mediated by high concentrations of TBH Mϕ and TBH $CD8^+$ T cells increased 176% after indomethacin treatment. We speculate that *in vivo* administration of indomethacin or other PGE_2 inhibitors may alleviate some of the observed suppression by preventing the differentiation of suppressive $CD8^+$ T cells and by subsequent induction of suppressor activities.

Based on our findings, $CD8^+$ T cells represent a significant suppressor population during tumor growth. Although phenotypic differentiation between $CD8^+$ T cells exhibiting suppressor activities and cytolytic activities appears restricted and may not

relate to unique functional abilities, *in vitro* data suggest suppressor functions may be reversible through immunotherapies which target soluble mediator molecules such as IFN-γ and PGE_2. *In vivo* administration of mAb against $CD8^+$ T cell-inducing agents and cell products or inhibitory drugs against suppressor $CD8^+$ T cell function may prove beneficial for cancer patients.

CHAPTER VII

TUMOR GROWTH ALTERS T CELL AND MACROPHAGE PRODUCTION OF AND RESPONSIVENESS TO GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR: PARTIAL DYSREGULATION THROUGH INTERLEUKIN-10

ABSTRACT

Tumor growth induces phenotypic and functional changes among splenic T cells and Mϕ that contribute to the immunosuppression observed in TBH. These changes partly arise through alterations in immune cell production of and responsiveness to cytokines. GM-CSF is an important T cell- and Mϕ-derived cytokine that is produced during normal host immunogenic challenge, but it's involvement during cancer is poorly defined. In contrast, IL-10 is an inhibitory cytokine that is produced by immune cells as a deactivation factor. IL-10 can disrupt GM-CSF synthesis and may be associated with tumor-induced changes in cytokine synthesis. We determined if tumor growth alters T cell and Mϕ synthesis of and responsiveness to GM-CSF, and if these alterations occur because tumor growth heightens immune cell sensitivity to IL-10. Tumor growth significantly decreased T-cell synthesis of GM-CSF during activation by concanavalin A, and TBH T cells were more susceptible to GM-CSF synthesis inhibition by IL-10 than their NH counterparts. This suppression was observed using both unseparated splenic lymphocyte preparations and purified $CD4^+$ and $CD8^+$ T cells. Similarly, TBH Mϕ (both splenic and peritoneal) produced less GM-CSF than NH Mϕ during activation by LPS. Tumor growth also altered MHC class II^- Mϕ GM-CSF synthesis. TBH Mϕ were more susceptible to GM-CSF synthesis inhibition by IL-10 than their NH counterparts. Although TBH T cells demonstrate less proliferation than NH T cells during activation, tumor growth did not compromise T-cell responsiveness to GM-CSF. However, tumor growth did increase TBH T-cell susceptibility to inhibition of proliferation by IL-10. Tumor growth suppressed Mϕ responsiveness to GM-CSF, and IL-10 further decreased Mϕ responsiveness to GM-CSF. Collectively, these results suggest T cell and Mϕ production of and re-

sponsiveness to GM-CSF is disrupted during tumor growth, and that TBH T cells and Mϕ are more susceptible to the suppressor activity of IL-10 than their NH counterparts.

INTRODUCTION

Tumor growth initiates a myriad of functional and phenotypic changes in T cells and Mϕ. Tumor growth functionally decreases T-cell proliferation in response to mitogens (123, 141), antigens (93), allogeneic MHC molecules (122, 449), and syngeneic MHC molecules (482, 484), and phenotypically reduces the percentage of $CD4^+$ T cells (449). Tumor growth also alters Mϕ accessory functions during T-cell proliferation (448, 482, 485) and decreases Mϕ expression of the MHC class II molecule Ia (171, 481). Many functional changes are associated with tumor-induced changes in cytokine synthesis and responsiveness. The current investigations evaluated tumor-induced changes in immune cell production of and responsiveness to GM-CSF.

GM-CSF is a glycoprotein responsible for the proliferation, differentiation, and activation of precursor and mature Mϕ and granulocytes (359). GM-CSF increases Mϕ and dendritic antigen-presenting and accessory functions (47, 153, 154), and induces endothelial cell migration and proliferation (59). GM-CSF increases pinocytosis (252), phagocytosis (280), and cytotoxicity (195, 280). GM-CSF also regulates the transcription and release of several mediator molecules such as IL-1 (394), TNF-α (206), PGE_2 (206), IL-1 receptor antagonist (228, 293), and lymphotoxin (104, 296), and the expression of several cell surface molecules such as membrane-bound IL-1 (153, 314), MHC class II molecules (153, 394), Fc receptors (90), and c-*fms* (178, 183). GM-CSF is produced by several cell types, including Mϕ, monocytes, T cells, and fibroblasts and endothelial cells (188, 303). GM-CSF is not constitutively synthesized but is inducible through the nuclear transcriptional activator NF-κB (381). Many cytokines such as IL-10 (107), TNF-α (242), lymphotoxin (242), IFN-γ (466), PGE_2 (354,

466), and IL-1 (157) regulate the expression of GM-CSF. Mitogenic stimuli such as concanavalin A (240) and LPS (420) also affect GM-CSF synthesis.

Many investigations show that GM-CSF is utilized as a positive regulator of immune cell function during bacterial (57, 282), fungal (457), and parasitic (360, 361, 396, 465) infections. However, the expression of GM-CSF during tumor challenge is unknown. Several tumor types express GM-CSF receptors (306) or produce and use GM-CSF as an autocrine growth factor (33, 163, 270). Because tumor growth compromises both T cell and Mϕ function, this study evaluated tumor-induced alterations in T cell and Mϕ production of GM-CSF. We also investigated if tumor growth increases T cell and Mϕ susceptibility to the suppressor cytokine IL-10 because IL-10 downregulates the production of GM-CSF by T cells and Mϕ (107, 150). IL-10 is a recently characterized suppressor cytokine that can inhibit T-cell proliferation and cytokine synthesis. IL-10 is dysregulated during specific parasitic (177, 180, 207) and viral infections (219, 312, 439), but the significance of IL-10 during cancer is unknown. Our results suggest that tumor growth alters T cell and Mϕ synthesis of and responsiveness to GM-CSF, and that IL-10 contributes to these changes.

MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Harlan-Sprague-Dawley, Madison, WI) were used in all experiments. A single-cell suspension of 4 x 10^5 methylcholanthrene-induced, nonmetastatic, fibrosarcoma cells was injected i.m. into the left hind leg of hosts. Palpable tumors form by days 10-14 and kill the hosts by days 28-35. Splenic immune cells from TBH mice 21 days post-tumor induction were used throughout. Immunosuppression was not caused by a general inflammatory response because normal BALB/c mice injected with C3H muscle tissue 3 weeks before had normal T cell responses (data not shown). Immunosuppression was not caused by viral infection because all TBH mice tested negative for LDH virus (Microbiological Associates, Bethesda, MD).

Medium

Unless specified, NH and TBH cells were cultured in complete medium: RPMI-1640 medium (Hazelton, Denver, PA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and 4 x 10^{-5} *M* 2-mercaptoethanol (Sigma, St. Louis, MO), 50 mg/l gentamicin (Gibco), 2 g/l $NaHCO_3$, and 25 *mM* HEPES buffer (Research Organics, Cleveland, OH).

Cytokines and Reagents

Recombinant murine GM-CSF was generously provided by Immunex (Seattle, WA). GM-CSF activity (4×10^7 U/ml) was determined by half maximal colony formation in the murine bone marrow colony assay. Recombinant murine IL-10 was generously provided by Dr. Kevin W. Moore (DNAX, Palo Alto, CA). IL-10 activity (7×10^3 U/ml) was determined using the MC/9 cell assay. All cytokines, concanavalin A (Difco Laboratories, Detroit, MI), and LPS (Sigma) were stored at -70°C.

Mϕ and T-Cell Preparation

Mice were sacrificed by cervical dislocation. Murine spleens were pulped using a stomacher (Tekmar, Cincinati, OH), and the resulting cell suspensions were centrifuged and resuspended in RPMI-1640 medium. Erythrocytes were lysed with cold 0.83% NH_4Cl for 5 min. Cells were washed and incubated for 2 hr at 37°C and 5% CO_2 on tissue culture plates (150 × 15 mm plastic plates; Lux/Miles Scientific, Naperville, IL). Although contaminating B cells were kept in unseparated lymphocyte preparations, data suggest that IL-10 does not mediate its suppressive action on T cells through B cells (151, 152). Flow cytometric analyses indicated that splenic lymphocyte preparations were >70% Thy 1.2^+. Purified $CD4^+$ and $CD8^+$ T cells were collected using complement and anti-immature B and T cell (American Type Culture Collection [ATCC] clone J11d; Rockville, MD), anti-IA^d (ATCC clone MK-D6), and either anti-CD8 (ATCC clone 3.155) or anti-CD4 (ATCC clone GK1.5). Nonadherent cells were resuspended to 8×10^6 cells/ml in complete medium. Splenic and peritoneal

Mϕ (>96% esterase positive) were collected (after washing to remove nonadherent cells) by scraping using a rubber policeman and ice-cold RPMI-1640 medium. Mϕ viability after scraping was >95%. Peritoneal Mϕ were elicited by an intra-peritoneal injection (0.5 ml) of thioglycollate and aspirated 4 days post-injection by lavage. Splenic class II$^-$ Mϕ were prepared by treating the cells with anti-IA^d plus a 1:12 dilution of complement. Flow cytometric analyses suggested that splenic Mϕ preparations were >80% MAC-1 positive. Mϕ were resuspended to 8 x 10^6 cells/ml in complete medium.

T-Cell and Mϕ Proliferation Assays

Lymphocytes, purified T cells, or Mϕ were seeded at various concentrations into flat-bottom 96-well microtiter plates in the absence or presence of concanavalin A, GM-CSF, or IL-10 to yield a final well volume of 0.2 ml. T cell and Mϕ assays were incubated three days and four days, respectively, in a humidified, water-jacketed incubator (37°C, 5% CO_2). Six to 18 h before harvest, all wells were pulsed with 1 μCi/ml tritiated thymidine (^{3}H-TdR; specific activity 6.7 Ci/mM, DuPont NEN Research Products, Boston, MA). Cells were harvested onto glass fiber filters using a Basic96 cell harvester (Skatron, Sterling, VA). Activity was counted using a 6895 Betatrac liquid scintillation counter (TM Analytic, Elk Grove Village, IL).

GM-CSF Bioassay

The GM-CSF-responsive cell line DA3 was obtained from Drs. Giovanni Rovera and Brent Kreider (Wistar Institute, Philadelphia, PA) and maintained by biweekly passage into fresh medium containing 20 U/ml GM-CSF. To determine the presence of GM-CSF in supernatants, 50 μl (2.5×10^4 cells/well) were seeded with 50 μl supernatant in flat-bottom 96-well tissue-treated plates (Nunc). Cultures were incubated for 3 days at 37°C and 5% CO_2, and proliferation was measured using the MTT assay (317). DA3 cells were unaffected by direct addition of concanavalin A, LPS, or IL-10 (data not shown).

Supernatant Collection

Supernatants were collected from cultures by centrifugation and were either added directly into the GM-CSF bioassay or stored at -70°C until use. Analyses of T-cell supernatants from several time points suggested maximum GM-CSF synthesis occurred 8 h post-stimulation (data not shown). Unless specified, Mϕ supernatants were collected 18 h post-stimulation.

Statistics/Calculation of Results

Pooled cells from 4 to 8 mice were used for each experiment. All tests in the T-cell and Mϕ proliferation assays and all tests in the GM-CSF bioassay were run in triplicate unless stated otherwise. Each experiment was repeated at least three times. All numbers in tables and data points on graphs were tested for significance by Student's *t* test ($p < 0.05$) and presented as mean $\pm$ standard error. In the GM-CSF production experiments, the amounts of GM-CSF present in the samples were extrapolated from activity curves generated by DA3 cells stimulated with recombinant murine GM-CSF. Fifty units were defined by half-maximal proliferation of DA3 cells.

RESULTS

Tumor growth decreases T-cell production of GM-CSF

Tumor growth alters the production of several cytokines; the dysregulation of cytokines such as IL-1, IL-6, and TGF-β by host immune cells or tumor cells significantly disrupts immune cell interactions and responses (134, 407, 412). To determine if tumor growth changes T-cell production of GM-CSF, supernatants from concanavalin A-stimulated lymphocytes (Table 9) and T cells (Figure 27) were screened using the GM-CSF-responsive cell line DA3. GM-CSF synthesis by TBH T cells was lower than that of NH T cells. These data suggest that tumor growth significantly decreases T cell production of GM-CSF.

T-cell GM-CSF synthesis is suppressed during tumor growth through IL-10

Because tumor growth increases T-cell susceptibility to suppression caused by IL-10, we hypothesized that TBH T cells may also be more susceptible to cytokine synthesis inhibition caused by IL-10. During activation with the T cell-specific mitogen concanavalin A, TBH lymphocyte (Table 10) and T-cell (Figure 28) production of GM-CSF decreased in the presence of IL-10. In contrast, NH lymphocyte and T-cell synthesis of GM-CSF was either unaffected or suppressed to a lesser ex-

Table 9. Tumor growth decreases concanavalin A-stimulated lymphocyte production of GM-CSF

Mitogen (μg/ml)[c]	GM-CSF (U/ml)[a]							
	8×10^6/ml[b]		4×10^6/ml		2×10^6/ml		1×10^6/ml	
	NH	TBH	NH	TBH	NH	TBH	NH	TBH
16	73	40	24	13	13	5	2	0
8	41	36	29	17	5	5	1	0
4	52	19	10	11	3	2	1	0
0	3	2	0	1	0	0	0	0

[a]GM-CSF concentrations were determined using the GM-CSF bioassay as described in the *Materials and Methods*. All data represent the average of two replicate wells. Each experiment was performed three times. A representative experiment is shown.

[b]NH and TBH lymphocytes were seeded in 24 well tissue culture plates in 1.0 ml complete medium and incubated at 37°C. Supernatants were collected 8 h post-stimulation. Analyses of lymphocyte supernatants from several time points suggested maximum GM-CSF synthesis occurred 8 h post-stimulation (data not shown).

[c]Concanavalin A was added from a 10 mg/ml stock solution.

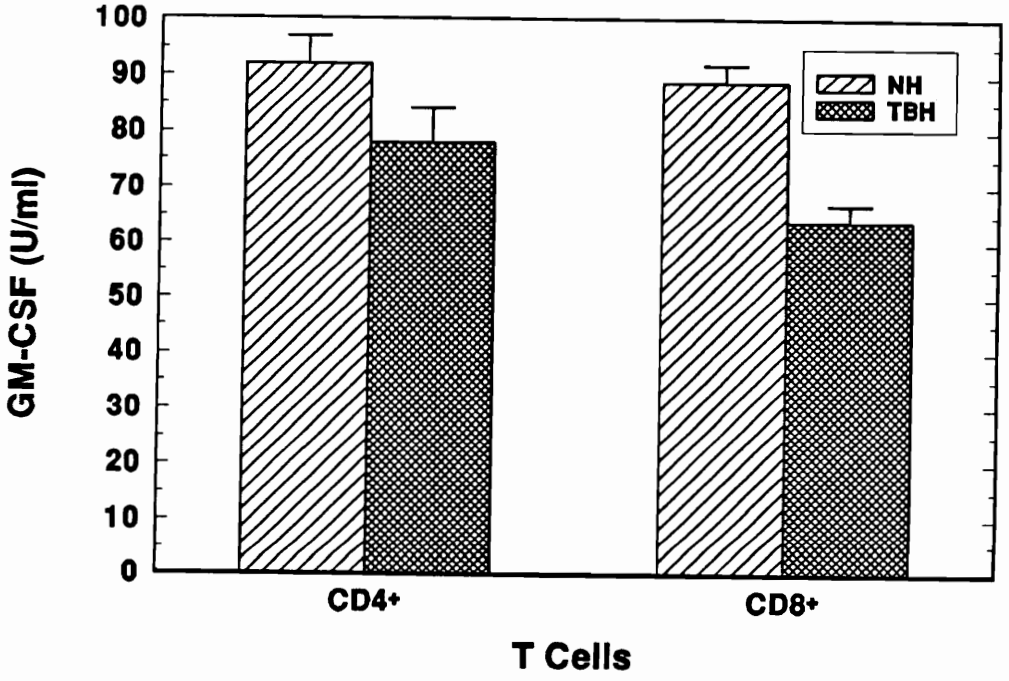

Figure 27. Tumor growth decreases T cell synthesis of GM-CSF: NH and TBH splenic $CD4^+$ and $CD8^+$ T cells (8 x 10^5/well) were seeded into flat-bottom 96-well microtiter plates and stimulated with concanavalin A (8 μg/ml) to yield a final well volume of 0.2 ml. Supernatants were collected by centrifugation and GM-CSF production was measured using the GM-CSF bioassay. Briefly, 2.5 x 10^4 DA3 cells in a 50 μl volume were seeded in flat-bottom 96-well microtiter plates with 50 μl supernatant and incubated for 3 days. Proliferation of DA3 cells was assessed using the MTT assay. The concentration of GM-CSF present in each sample is expressed as units/ml (Y-axis), and was extrapolated from an activity curve generated from culturing DA3 cells in the presence of several dilutions of GM-CSF. Fifty units is defined as the amount of GM-CSF required to induced half-maximal proliferation of DA3 cells.

tent than TBH T cells by IL-10. These findings suggest that tumor growth increases T cell susceptibility to GM-CSF synthesis inhibition mediated by IL-10.

Tumor growth decreases Mϕ production of GM-CSF

To determine if tumor growth alters Mϕ production of GM-CSF, splenic (Figure 29) and peritoneal (Figure 30) Mϕ were stimulated with the Mϕ activation agent LPS and supernatants were screened for the presence of GM-CSF. Although splenic Mϕ produced more GM-CSF than peritoneal Mϕ, both splenic and peritoneal NH Mϕ synthesized more GM-CSF than their TBH counterparts. In the absence of LPS, NH and TBH Mϕ did not synthesize GM-CSF (data not shown). However, Mϕ adherence is a sufficient signal to initiate GM-CSF gene transcription (420), and the high GM-CSF concentrations that are detected 2 h post-stimulation with LPS may be the result of a synergistic effect between adherence and LPS. Therefore, subsequent measurements of Mϕ GM-CSF production were made 18 h post-stimulation.

Tumor-induced alterations in splenic Mϕ GM-CSF synthesis were dose-dependent, because GM-CSF concentrations from NH and TBH Mϕ were nearly identical when low concentrations of LPS (100 ng/ml) were used as stimuli (Figure 31). When higher concentrations of LPS (500 and 1000 ng/ml) were used, TBH Mϕ synthesized less GM-CSF. This dose-dependent response was reversed when NH and TBH MHC class II$^-$ Mϕ GM-CSF synthesis was compared (Figure 32). Previous phenotypic and functional studies show that the percentage of MHC class II$^-$ Mϕ increases during tumor growth and that this subpopulation significantly suppresses several T cell activities (482). Peritoneal Mϕ were not evaluated because both NH and TBH peritoneal Mϕ express low levels of MHC class II molecules. Al-

Table 10. Concanavalin A-stimulated lymphocyte GM-CSF synthesis is further suppressed by IL-10 during tumor growth

	GM-CSF (U/ml)[a]							
	8 x 10^6 NH cells/ml[b]		8 x 10^6 TBH cells/ml		4 x 10^6 NH cells/ml		4 x 10^6 TBH cells/ml	
Mitogen (μg/ml)[c]	Control	IL-10 (10 U/ml)[d]	Control	IL-10 (10 U/ml)	Control	IL-10 (10 U/ml)	Control	IL-10 (10 U/ml)
16	73	70	40	23	24	16	13	7
8	41	42	36	28	29	20	17	7
4	52	30	19	13	10	10	11	4
0	3	0	2	0	0	0	1	0

[a]GM-CSF concentrations were determined using the GM-CSF bioassay as described in the *Materials and Methods*. All data points represent the average of two replicate wells. Each experiment was performed three times. A representative experiment is shown.

[b]NH and TBH lymphocytes were seeded in 24 well tissue culture plates in 1.0 ml complete medium and incubated at 37°C. Supernatants were collected 8 h post-stimulation. Analyses of lymphocyte supernatants from several time points suggested maximum GM-CSF synthesis occurred 8 h post-stimulation (data not shown).

[c]Concanavalin A was added from a 10 mg/ml stock solution.

[d]IL-10 was generously provided by DNAX, Palo Alto, CA.

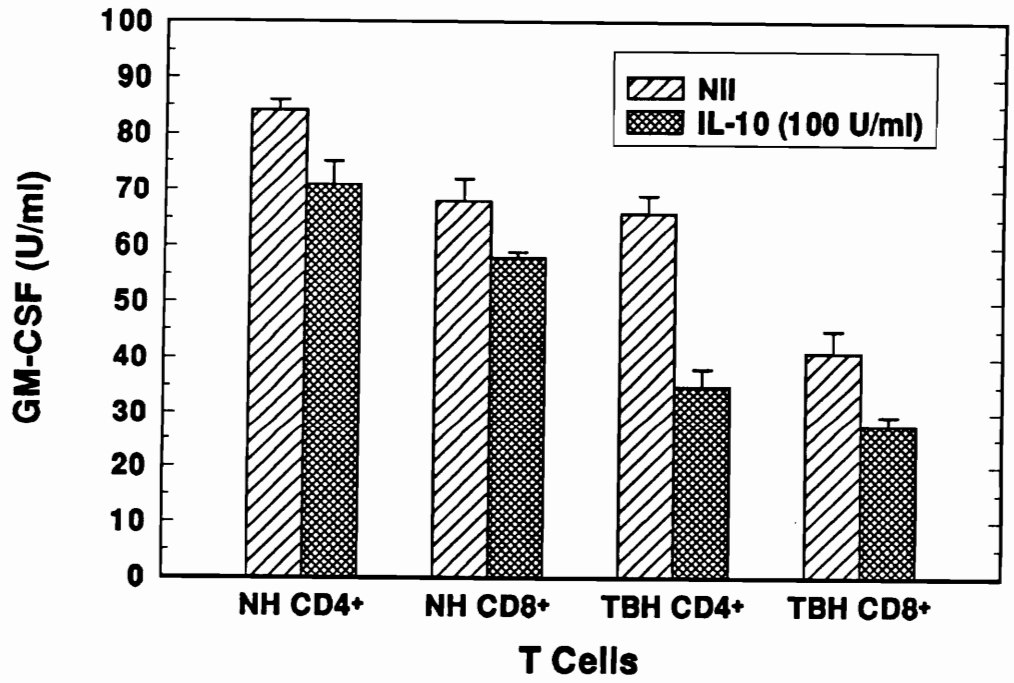

Figure 28. IL-10 suppresses T cell synthesis of GM-CSF during tumor growth: NH and TBH splenic $CD4^+$ and $CD8^+$ T cells (8×10^5/well; X-axis) were stimulated with concanavalin A (8 μg/ml) in the absence or presence of IL-10 (100 U/ml). GM-CSF production was measured 8 h post-stimulation using the GM-CSF bioassay. GM-CSF concentrations are expressed as units/ml (Y-axis).

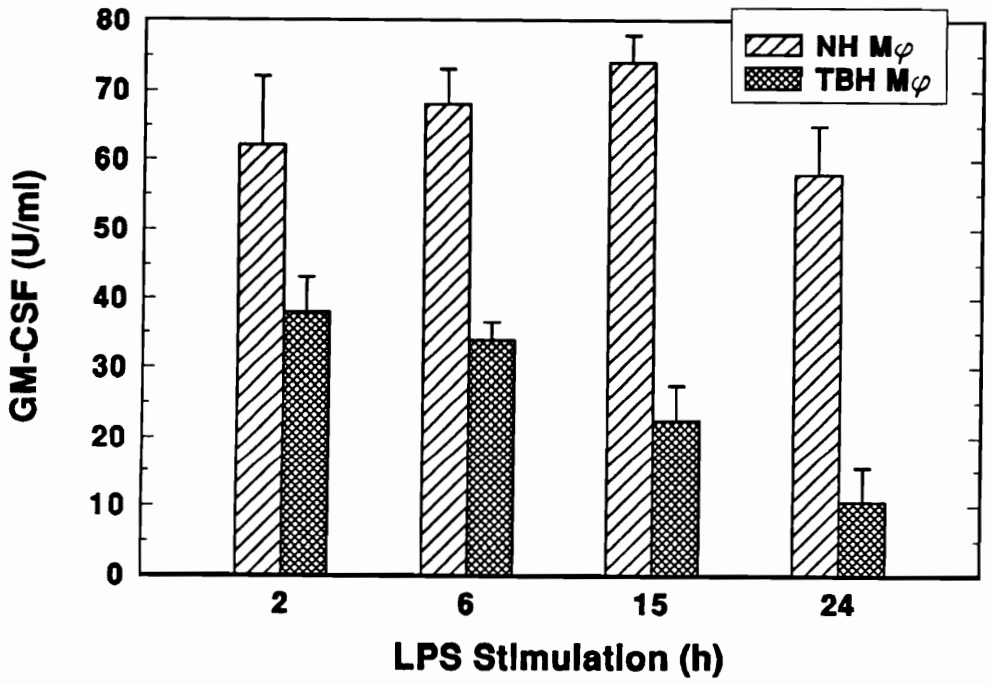

Figure 29. Tumor growth decreases splenic Mϕ GM-CSF synthesis: Splenic NH or TBH Mϕ (4 x 10^5 cells/well) were seeded into flat-bottom 96-well microtiter plates and stimulated with LPS (500 ng/ml) to yield a final well volume of 0.2 ml. Supernatants were collected by centrifugation and GM-CSF production was measured kinetically (X-axis) using the GM-CSF bioassay as described in Figure 27. The concentration of GM-CSF present in each sample is expressed as units/ml (Y-axis).

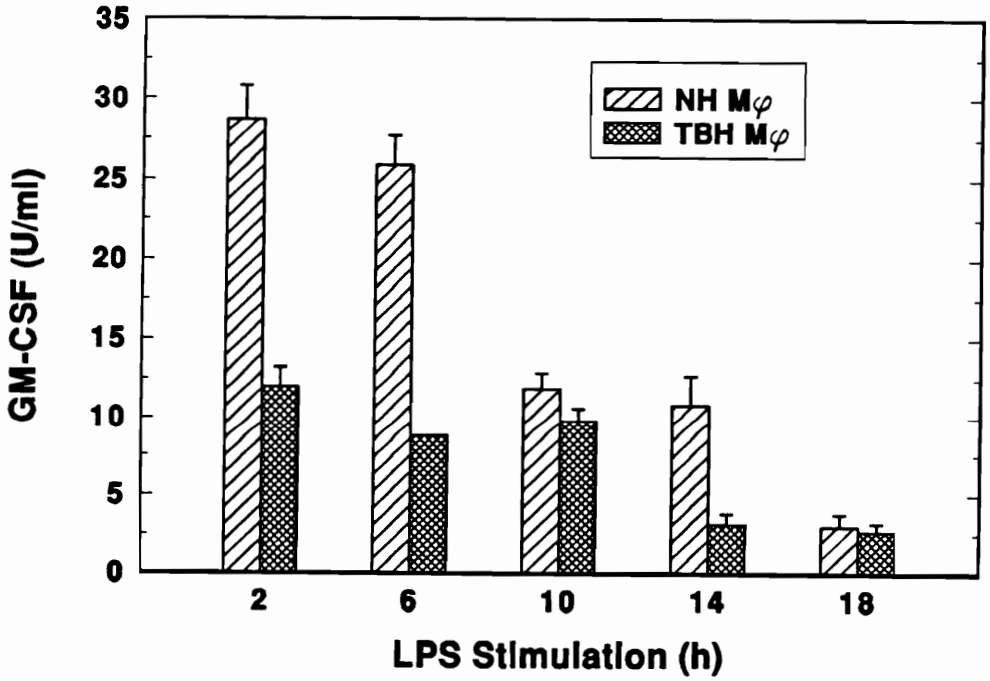

Figure 30. Tumor growth decreases peritoneal Mϕ GM-CSF synthesis: Peritoneal NH or TBH Mϕ (8 x 10^5 cells/well) were seeded into flat-bottom 96-well microtiter plates and stimulated with LPS (1000 ng/ml) to yield a final well volume of 0.2 ml. GM-CSF production was measured kinetically (X-axis) using the GM-CSF bioassay as described in Figure 27. GM-CSF concentrations are expressed as units/ml (Y-axis).

though TBH MHC class II$^-$ Mϕ produced less GM-CSF than their NH counterparts when stimulated with low concentrations of LPS, GM-CSF synthesis by these two subpopulations was not significantly different when they were stimulated with high concentrations of LPS. These finding collectively suggest that tumor growth decreases Mϕ GM-CSF synthesis, and that these changes occur among Mϕ populations in different tissues/regions and among different subpopulations.

IL-10 suppresses Mϕ synthesis of GM-CSF

We also determined if IL-10 suppressed Mϕ production of GM-CSF. Although IL-10 significantly ($p < 0.05$) decreased both NH and TBH Mϕ synthesis of GM-CSF (Figure 33), IL-10 decreased TBH Mϕ GM-CSF synthesis more as determined by percentage decrease (72% versus 52%). TBH Mϕ are more susceptible to GM-CSF synthesis inhibition by IL-10.

Tumor growth does not affect T-cell responsiveness to GM-CSF

Tumor growth decreases several T cell functions during immune challenge such as proliferation and cytokine synthesis. To determine if tumor growth compromises T-cell responsiveness to GM-CSF during activation, unseparated lymphocytes and purified T cells were activated with the T cell-specific mitogen concanavalin A and proliferation was assessed in the presence or absence of GM-CSF (Table 11). Even though GM-CSF increased both NH and TBH T cell proliferation, TBH T cell prolifer-

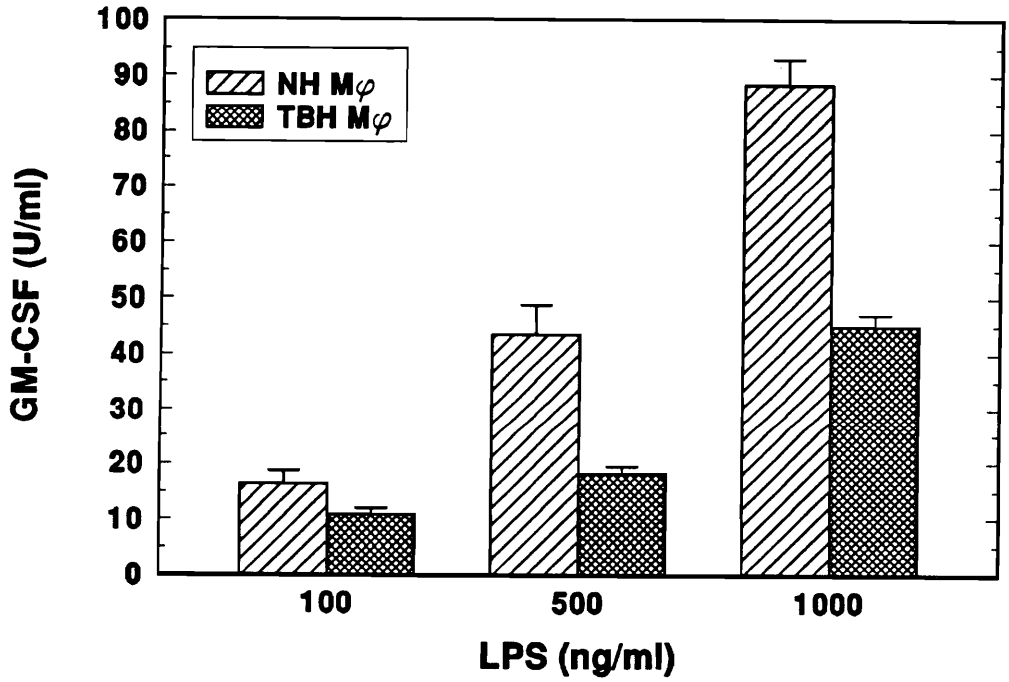

Figure 31. Tumor-induced alterations in Mϕ GM-CSF synthesis are dose-dependent: Splenic NH and TBH Mϕ (8 x 10^5 cells/well) were seeded into flat-bottom 96-well microtiter plates and stimulated with various concentrations of LPS (X-axis) to yield a final well volume of 0.2 ml. GM-CSF production was measured 18 h post-stimulation using the GM-CSF bioassay. GM-CSF concentrations are expressed as units/ml (Y-axis).

ation was not restored to the levels observed with NH T cells. These findings suggest that tumor growth does not interfere with GM-CSF-enhanced T-cell proliferation.

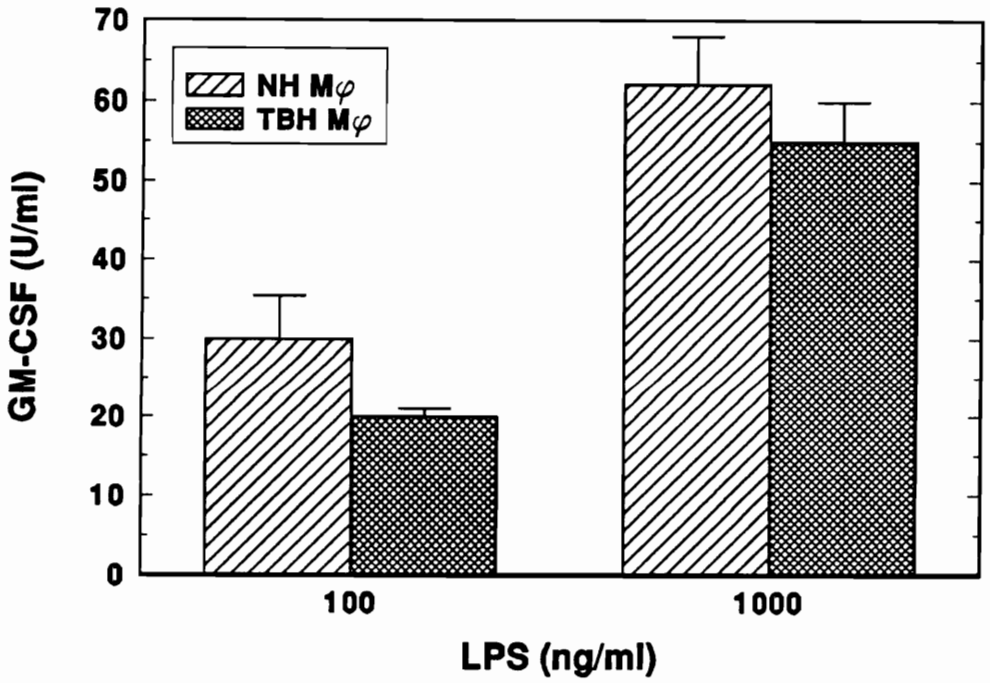

Figure 32. Tumor-induced alterations in GM-CSF production by splenic class II⁻ Mϕ are dose-dependent: Splenic class II⁻ NH and TBH Mϕ (8 x 10^5 cells/well) were stimulated with LPS (X-axis) and GM-CSF production was measured 18 h post-stimulation using the GM-CSF bioassay. GM-CSF concentrations are expressed as units/ml (Y-axis).

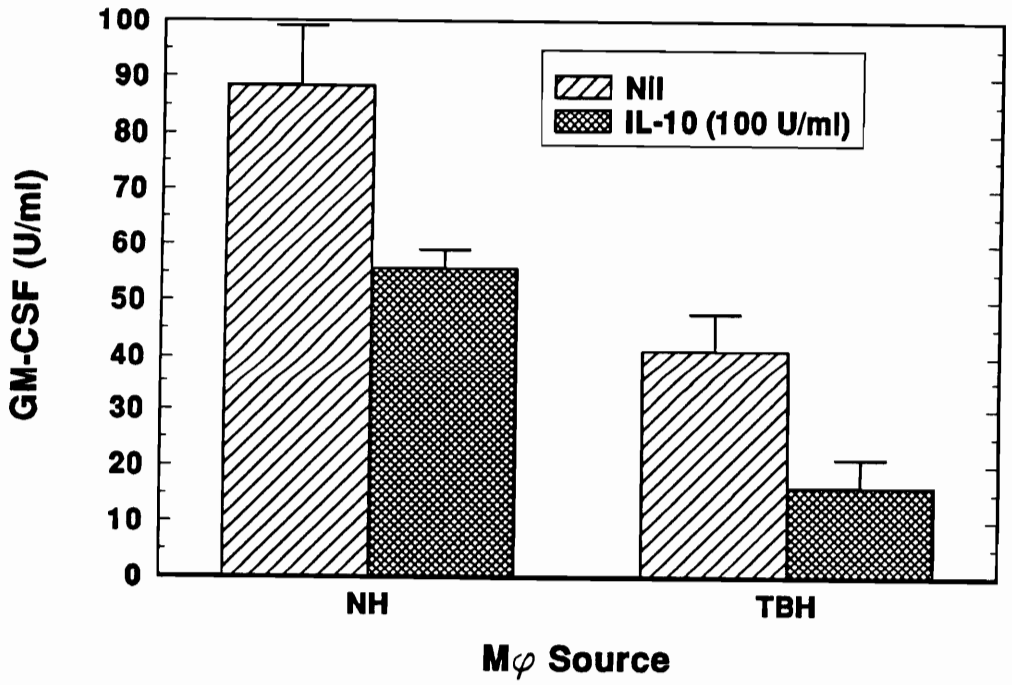

Figure 33. IL-10 suppresses Mϕ synthesis of GM-CSF during tumor growth: Splenic NH and TBH Mϕ (8 x 10^5/well; X-axis) were stimulated with LPS (1000 ng/ml) in the absence or presence of IL-10. GM-CSF production was measured 18 h post-stimulation using the GM-CSF bioassay. GM-CSF concentrations are expressed as units/ml (Y-axis).

Table 11. Tumor growth does not affect enhancement of lymphocyte proliferation by GM-CSF

		NH Lymphocytes		TBH Lymphocytes	
Cell Conc.	Mitogen (μg/ml)[a]	Control (No GM-CSF)	GM-CSF (100 U/ml)[b]	Control (No GM-CSF)	GM-CSF (100 U/ml)[b]
8 x 10^3/well	0	0.35 ± 0.01[c]	2.78 ± 0.45	1.59 ±0.24	1.59 ± 0.74
8 x 10^3/well	4	120.35 ± 4.26	159.78 ± 6.21	32.66 ± 0.67	45.31 ± 0.61
8 x 10^3/well	8	188.27 ± 5.83	196.75 ± 2.31	56.34 ± 0.02	64.63 ± 1.86
8 x 10^3/well	16	119.12 ± 10.05	135.04 ± 1.0	98.4 ± 3.61	114.46 ± 1.33
4 x 10^3/well	0	0.28 ± 0.01	1.44 ± 0.28	0.81 ± 0.01	1.01 ± 0.28
4 x 10^3/well	4	170.31 ± 2.9	180.94 ± 10.20	113.78 ± 4.28	119.81 ± 0.47
4 x 10^3/well	8	158.52 ± 8.34	180.05 ± 7.56	108.78 ± 2.37	120.43 ± 1.90
4 x 10^3/well	16	45.63 ± 3.42	42.41 ± 8.02	41.22 ± 0.67	59.76 ± 5.16

[a]Concanavalin A was added from a 10 mg/ml stock solution.

[b]GM-CSF was generously provided by Immunex, Seattle, WA.

[c]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

Tumor growth increases T-cell susceptibility to suppression caused by IL-10

Although tumor growth did not affect T-cell proliferation by blocking signals induced through GM-CSF, we considered the possibility that tumor growth alters T-cell responsiveness during challenge through suppressor cytokines. Although the significance of IL-10 activity during cancer is unknown, IL-10 inhibits T-cell proliferation and cytokine synthesis and appears to be expressed during specific parasitic and viral infections. We hypothesized that tumor growth may decrease T-cell responsiveness during challenge by increasing T-cell sensitivity to suppressor cytokines such as IL-10. IL-10 was suppressive to both NH and TBH unseparated lymphocyte (Table 12) and T-cell (Figure 34) proliferation, but IL-10 was more suppressive to TBH lymphocytes and T cells. Based on these findings, TBH T cells are more susceptible than NH T cells to suppression caused by IL-10.

Tumor growth suppresses Mϕ responsiveness to GM-CSF

Previous findings from our laboratory suggest that Mϕ activities are significantly changed during tumor growth. TBH Mϕ demonstrate altered surface molecule expression and suppressive accessory cell activity. Although TBH possess a higher concentrations of Mϕ in resident tissues such as the spleen, a majority of these cells appear phenotypically and functionally immature (17, 483). We hypothesized that tumor growth may suppress Mϕ proliferation stimulated by GM-CSF, which can serve as a developmental signal for immature Mϕ. After stimulation by GM-CSF, TBH Mϕ

Table 12. Tumor growth increases lymphocyte susceptibility to IL-10-mediated suppression

IL-10 (units/ml)[a]	NH T Cells[b]	Change in Proliferation (%)	TBH T Cells[b]	Change in Proliferation (%)
0	186.51 ± 0.02[c]	-----	134.37 ± 0.01[c]	-----
0.1	165.00 ± 0.59	11.5 ↓	105.77 ± 2.20	21.2 ↓
1	155.42 ± 7.8	16.7 ↓	94.25 ± 2.70	29.8 ↓
10	134.77 ± 5.53	27.7 ↓	73.20 ± 7.86	45.5 ↓

[a]IL-10 was generously provided by DNAX, Palo Alto, CA.

[b]NH and TBH lymphocytes (4×10^5 cells/well) were stimulated with concanavalin A (8 μg/ml).

[c]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

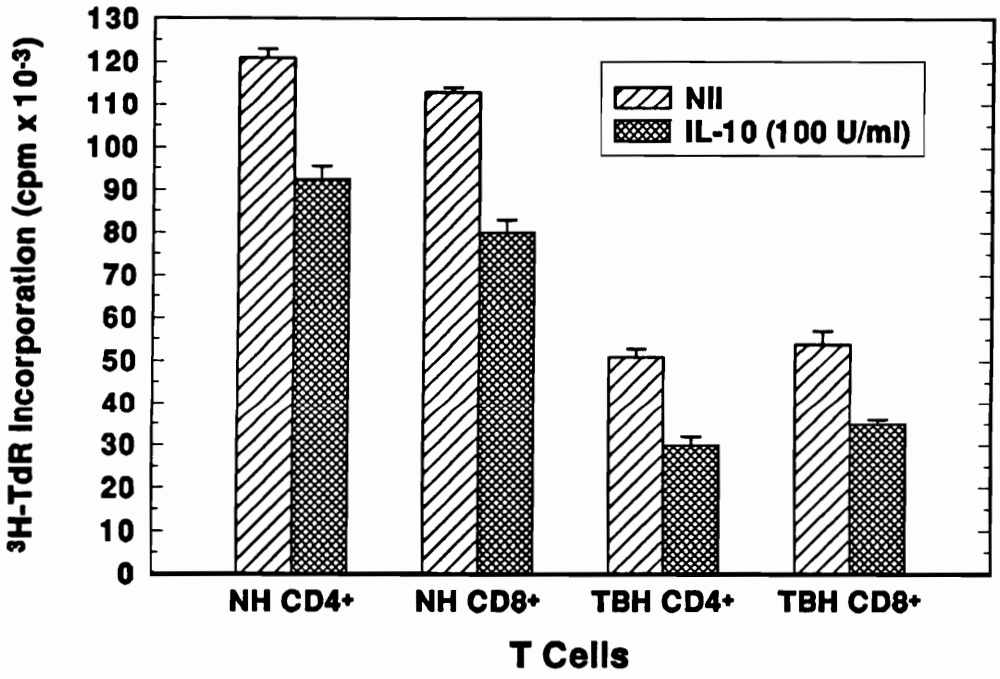

Figure 34. Tumor growth increases T cell susceptibility to suppression caused by IL-10: NH or TBH splenic $CD4^+$ and $CD8^+$ T cells (8 x 10^5/well) were seeded into flat-bottom 96-well microtiter plates in the absence or presence of IL-10 (100 U/ml). Cultures were incubated 4 days and pulsed with ^{3}H-TdR 6 h before cell harvest. Proliferation was assessed by ^{3}H-TdR incorporation (Y-axis).

proliferated less than NH Mϕ (Figure 35). These data suggest that tumor growth compromises Mϕ responsiveness to GM-CSF.

IL-10 further suppresses Mϕ responsiveness to GM-CSF during tumor growth

Because tumor growth suppresses T-cell proliferation partly through increased sensitivity to IL-10, we hypothesized a similar mechanism may occur in Mϕ. To determine if IL-10 compromises Mϕ proliferation, NH and TBH splenic Mϕ were stimulated with GM-CSF in the presence or absence of IL-10 (Table 13). IL-10 decreased both NH and TBH Mϕ responsiveness to GM-CSF, but IL-10 mediated suppression of NH Mϕ responsiveness was significant ($p < 0.025$) only at one concentration of GM-CSF tested whereas TBH Mϕ responsiveness was significantly ($p < 0.05$) suppressed by IL-10 at all concentrations of GM-CSF tested. Thus, IL-10 further suppresses TBH Mϕ responsiveness to GM-CSF.

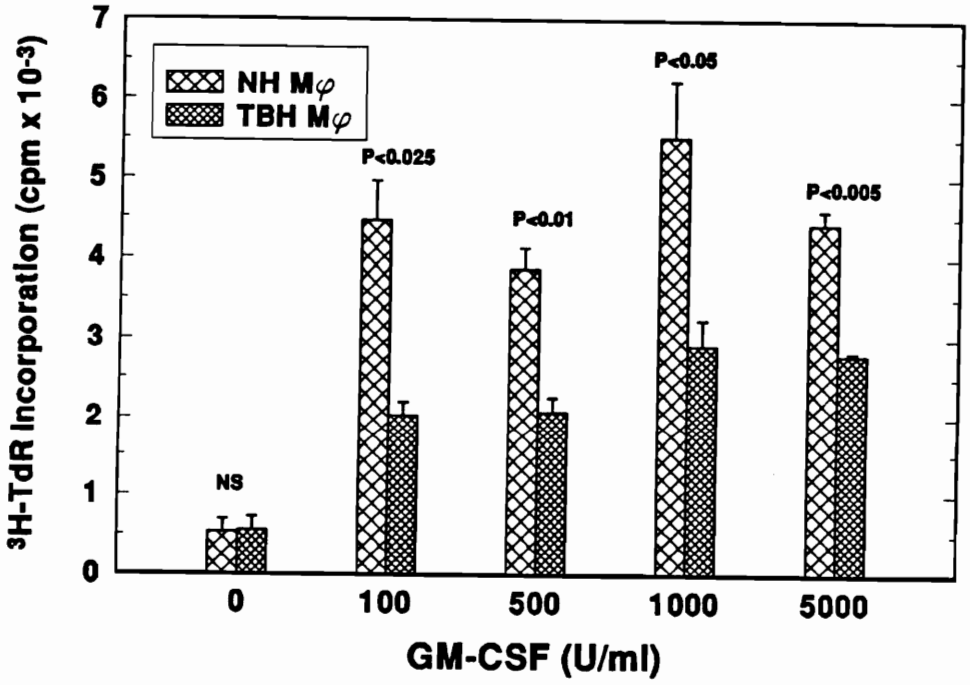

Figure 35. Tumor growth suppresses Mϕ responsiveness to GM-CSF: Splenic NH or TBH Mϕ (8 x 10^6) were seeded into flat-bottom 96-well microtiter plates in the absence or presence of various concentrations of GM-CSF (X-axis) to yield a final well volume of 0.2 ml. Cultures were incubated 4 days and pulsed with ^{3}H-TdR 6 h before cell harvest. Proliferation was assessed by ^{3}H-TdR incorporation (Y-axis).

Table 13. IL-10 further suppresses Mϕ responsiveness to GM-CSF during tumor growth

Host	IL-10 (100 U/ml)[b]	GM-CSF (U/ml)[a] 0	100	500	1000	5000
NH	–	0.31 ± 0.08[c]	4.32 ± 1.15	5.35 ± 1.98	5.42 ± 0.72[d]	4.64 ± 1.78
NH	+	1.02 ± 0.48	4.35 ± 1.63	2.40 ± 0.41	1.97 ± 0.31[d]	2.03 ± 0.14
TBH	–	1.07 ± 0.36	4.77 ± 0.69[d]	4.99 ± 0.68[e]	4.69 ± 1.37[f]	4.19 ± 0.41[e]
TBH	+	0.21 ± 0.05	1.48 ± 0.36[d]	1.46 ± 0.17[e]	1.28 ± 0.01[f]	1.77 ± 0.27[e]

[a]GM-CSF was generously provided by Immunex, Seattle, WA.

[b]IL-10 was generously provided by DNAX, Palo Alto, CA.

[c]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

[d]Denotes $p < 0.025$ during comparison between Mϕ cultured in the absence or presence of IL-10.

[e]Denotes $p < 0.01$ during comparison between Mϕ cultured in the absence or presence of IL-10.

[f]Denotes $p < 0.05$ during comparison between Mϕ cultured in the absence or presence of IL-10.

DISCUSSION

GM-CSF is an up-regulatory molecule produced and utilized by T cells and Mϕ during immunogenic challenge, but the involvement of GM-CSF during cancer is poorly defined. Our study suggests that cancers may inhibit immune cell activities through reductions in immune cell synthesis of and responsiveness to GM-CSF. These changes are compounded by tumor-induced increases in susceptibility to suppression by IL-10.

Tumor growth significantly decreased T-cell GM-CSF synthesis. Tumor-induced suppression was demonstrated using both unseparated lymphocyte preparations and purified $CD4^+$ and $CD8^+$ T cells. Others have shown that increasing age also disrupts immunocompetence and leads to decreased T-cell reactivity and GM-CSF synthesis (62). Our data suggest that tumor growth similarly compromises T cell reactivity and GM-CSF production. The inability of TBH T cells to synthesize adequate quantities of GM-CSF following stimulation suggests that an *in vivo* challenge may lead to sub-optimal production of GM-CSF. Although metastasis may contribute to tumor-induced suppression of GM-CSF synthesis during certain cancers, we have ruled out this possibility in our model by using a non-metastatic tumor.

TBH T-cell GM-CSF synthesis was decreased further by IL-10. This finding was confirmed using both unseparated lymphocytes and purified T cells. Others show that IL-10 either weakly suppresses or does not suppress GM-CSF synthesis by T_H1 cell clones; suppression appears to be dependent on the type of clone used (150). The use of mitogen-stimulated lymphocytes and $CD4^+$ T cells, which consist of both T_H1 and T_H2 types, may account for the mixed suppression of GM-CSF synthesis demonstrated by NH T cells. Nonetheless, TBH T cells synthesized significantly less

GM-CSF in the presence of IL-10 than their NH counterparts. IL-10 significantly down-regulates T_H1 cytokine synthesis *in vivo* during parasitic infections (368), and this inhibition correlates with decreased immunocompetence. During tumor-induced immunosuppression, IL-10 may similarly down-regulate GM-CSF synthesis by T_H1 cells. We are now determining if tumor growth decreases the expression of T_H1-specific cytokines.

After identifying tumor-induced changes in T-cell production of GM-CSF, we determined that tumor growth inhibits TBH Mϕ synthesis of GM-CSF. Both splenic and peritoneal Mϕ that were activated with LPS produced less GM-CSF than their NH counterparts. These differences were most pronounced among splenic Mϕ when high concentrations of stimuli were used. Because GM-CSF increases Mϕ accessory cell functions and class II molecule expression, tumor-induced decreases in GM-CSF production may account partly for decreased Mϕ accessory functions and reduced class II molecule expression. However, activation of splenic TBH class II$^-$ Mϕ with a high concentration of LPS induced GM-CSF synthesis at concentrations comparable to NH class II$^-$ Mϕ. The class II$^-$ Mϕ subpopulation is significant because it is a potent suppressor population during tumor growth (449, 482). Previous data show that GM-CSF increases T-cell reactivity in the presence of NH class II$^-$ Mϕ whereas it further suppresses T-cell reactivity in the presence of TBH class II$^-$ Mϕ (448). Although GM-CSF synthesis is unaltered, TBH class II$^-$ Mϕ synthesis of the inhibitory molecule PGE_2 is significantly increased (482, 484). This activity, in turn, significantly suppresses T cell function.

We also determined that tumor growth heightens Mϕ susceptibility to inhibition of GM-CSF synthesis by IL-10. Low IL-10 concentrations significantly decrease the production of IL-1α, IL-6, IFN-γ, and TNF-α (42, 151). Our data suggest IL-10 also decreases Mϕ synthesis of GM-CSF although the sensitivity is not as great as observed

with TNF-α synthesis (42). This difference may exist because IL-10 sensitivity in our studies was measured using splenic Mϕ instead of peritoneal Mϕ.

After identifying tumor-induced alterations in immune cell synthesis of GM-CSF, we determined if tumor growth alters immune cell responsiveness to GM-CSF. Using unseparated lymphocytes and purified T cells, the data suggest that tumor growth did not affect enhancement of T-cell proliferation caused by GM-CSF, although TBH T-cell proliferation as a result of mitogen activation was significantly lower than the response by NH T cells. Previous studies also show that GM-CSF up-regulates T-cell reactivity against alloantigen (448). Mϕ appear to be the primary cell type altered by tumor growth in our system; TBH Mϕ suppress T-cell reactivity in the presence of GM-CSF (448), whereas NH Mϕ increase T-cell reactivity in the presence of GM-CSF (153, 240).

However, tumor growth did increase T-cell susceptibility to inhibition caused by IL-10. Although initial reports suggested that IL-10 does not directly target T cells and that its suppressor activities are mediated only through Mϕ (152), other studies show IL-10, in the absence of Mϕ, significantly inhibits T-cell proliferation in response to mitogen (413). Our current data using purified $CD4^+$ and $CD8^+$ T cells confirm that IL-10 can act directly on T cells. IL-10 suppressed both NH and TBH T-cell proliferation, but TBH T cells were more susceptible to IL-10 as determined by percentage decrease in proliferation. Because IL-10-mediated inhibition of proliferation occurs through decreased IL-2 synthesis (413), the higher TBH immune cell susceptibility to IL-10 may correlate with a lower ability to synthesize IL-2. We have recently confirmed this hypothesis (unpublished observations) and are evaluating if additional signals such as IL-4 or TGF-β contribute to the decrease in IL-2 synthesis. Previous findings also suggest that TBH $CD8^+$ T cells are highly suppressive (449) and that depletion of this phenotype increases TBH T-cell IL-2 production (56). Based on these

facts, the higher TBH T-cell susceptibility to IL-10 may be abrogated through depletion of $CD8^+$ T cells. Using purified $CD4^+$ T cells, we show that elimination of $CD8^+$ suppressor T cells does not alter helper T cell sensitivity to IL-10. This finding supports the possibility that phenotypic shifts in T_H1 and T_H2 subsets account for T cell susceptibility to IL-10.

Because tumor growth also significantly alters Mϕ activities, we evaluated tumor-induced alterations in Mϕ responsiveness to GM-CSF. Although GM-CSF enhances Mϕ accessory function (240) and increases MHC class II molecule expression (153), these activities are inhibited by tumor growth (443, 448). TBH Mϕ are immature phenotypically and functionally, and stimulation of these cells with GM-CSF, a differentiation and proliferation signal for Mϕ, did not induce proliferation that was comparable to NH Mϕ proliferation. Because Mϕ proliferation in resident tissues replenishes the cell population and increases cell numbers and cytokine concentrations during immune challenge (32), tumor growth may partly decrease immunocompetence through Mϕ hyporesponsiveness to proliferation signals.

TBH Mϕ also were highly susceptible to inhibition of GM-CSF-induced proliferation by IL-10. Although IL-10 significantly inhibits the synthesis of Mϕ-derived cytokines and reactive oxygen intermediates (42, 151), its significance as an inhibitor of Mϕ proliferation is unclear. The susceptibility to inhibition mirrors the effect observed with T cells; tumor growth may disrupt the synthesis of a Mϕ growth factor. Although we did not screen for tumor-induced alterations in all potential Mϕ growth factors, our data support the conclusion that tumor growth disrupts synthesis of and responsiveness to GM-CSF.

Collectively, our data suggest that tumor growth decreases T cell and Mϕ responsiveness to and production of GM-CSF. The reduced ability to respond to and synthesize GM-CSF may account partly for decreased immunocompetence during

tumor challenge. Immunotherapies which introduce GM-CSF may provide the host with immune signals that stimulate hemopoietic precursor expansion and raise *in vivo* concentrations of GM-CSF to levels required for optimal immune responsiveness. Furthermore, these findings suggest the possible involvement of IL-10 as a suppressor molecule during tumor growth. To our knowledge, no previous studies have suggested that IL-10 may serve as an inhibitory signal during tumor-induced immunosuppression. Although TGF-β is an important inhibitory cytokine that is expressed during tumor growth (412), further investigations are required to clarify the *in vivo* relevance of IL-10 during tumor-induced dysfunction. Future studies will determine if tumor growth correlates with increased production of IL-10 by immune cells and if the fibrosarcoma cells used in our model produce IL-10.

CHAPTER VIII

FIBROSARCOMA GROWTH ALTERS CD4$^+$ T CELL RESPONSIVENESS TO COSTIMULATORY CYTOKINES AND INCREASES SENSITIVITY TO TRANSFORMING GROWTH FACTOR-β_1 AND THE ANTICANCER DRUG TAXOL

ABSTRACT

Tumor growth induces several functional changes among $CD4^+$ T cells that include alterations in responsiveness to cytokines. The current investigation evaluated whether tumor growth changes $CD4^+$ T cell responsiveness to cytokines that promote or suppress T-cell activation and to the anticancer drug taxol. T-cell responsiveness to specific signals was assessed by comparing NH and TBH mitogen-activated T-cell proliferation. TBH helper T cells were more sensitive than NH helper T cells to the downregulatory cytokines IL-10 and TGF-β_1. Specific signals associated with T-cell activation such as IL-2, IFN-γ, and anti-interleukin-4 monoclonal antibodies (anti-IL-4 mAb) partly restored TBH T-cell proliferation to NH levels. IL-10 and TGF-β_1 suppressed reconstitution of T-cell activity by IFN-γ and anti-IL-4 mAb. Previous studies show that IL-10 and TGF-β_1 are produced by suppressor immune cells and cancer cells. TBH helper T cells were unresponsive to the costimulatory cytokines IL-1 and IL-6. TBH helper T cells showed a higher sensitivity to the antimitotic compound taxol. TBH T-cell sensitivity to taxol significantly increased when TGF-β_1 was present. These data collectively suggest that tumor growth changes helper T cell responsiveness to specific regulatory signals associated with T-cell activation and downregulation. Furthermore, our data suggest a novel mechanism of T-cell suppression by taxol during tumor growth and may partly explain the limited success of taxol as an effective immunotherapy for cancer patients.

INTRODUCTION

$CD4^+$ helper T cells initiate and terminate responses against pathogens and cancer cells by producing cytokines. These molecules serve as communication signals between immune cells and can act both locally and systemically. Cytokines control T cell differentiation and cross-regulate T cell subsets (384). However, cancers significantly disrupt immune cell activities by changing both immune cell production of and responsiveness to specific cytokines. Tumor cell-derived cytokines mediate tumor cell communication, promote angiogenesis, enhance metastatic activities, increase tumor cell proliferation, and suppress anticancer immune cell activities (274). Our current investigations are determining which specific cytokines are dysregulated during tumor growth.

Tumor growth alters immune cell phenotype and function (9, 443, 444, 447, 448, 449, 450, 481, 482). Tumor-induced alterations are so dramatic that immune cells located far from the tumor site demonstrate significant decreases in their ability to respond during challenge, and *in situ* immune cells are unable to kill or inhibit the growth of tumor cells. Using a nonmetastatic fibrosarcoma, we showed that immune cell responsiveness to IL-1, IL-2, IL-4, GM-CSF, M-CSF, and TNF-α is disrupted during tumor growth (7, 10, 444, 484). Dysregulation partly arises through Mϕ production of suppressor cytokines and prostaglandins.

In this study, we extend our previous findings that suggest Mϕ contribute to tumor-induced immune dysfunction. The present investigations examine whether tumor growth disrupts cytokine responses by purified $CD4^+$ T-cells in the absence of suppressor Mϕ. We used T-cell proliferation and cytokine production as indicators of tumor-induced suppression. GM-CSF synthesis was measured because it con-

tributes to T-cell activation and is dysregulated by T cells and Mϕ during tumor burden (444). Furthermore, GM-CSF is produced by both T_H1 and T_H2 cells. We hypothesized that IL-10 and TGF-β_1 suppress TBH $CD4^+$ T cell activities. These signals normally downregulate immune responses (292, 311) but are expressed during tumor development by suppressor immune cells and fibrosarcoma cells (10, 424). We also determined whether specific signals associated with $CD4^+$ T-cell activation could restore TBH T cell proliferation to NH levels. We hypothesized that tumor-induced changes affect T_H1 cells. T_H1 cells are significant because they promote anticancer activities that are mediated by NK cells, cytotoxic T cells, and Mϕ. We evaluated whether TBH $CD4^+$ T cell proliferation could be restored by IL-2, a T_H1 autocrine growth factor, and by either IFN-γ or anti-IL-4 mAb. IFN-γ indirectly promote T_H1 cell activities by suppressing T_H2 cell activities. Anti-IL-4 mAb neutralize IL-4 which crossregulates T_H1 cell activities. We also determined whether IL-10, a T_H2-derived inhibitor of T_H1 activity, mediated TBH T cell suppression. To determine whether tumor growth disrupts T_H2 cell activities, we investigated whether tumor growth compromises T_H2 cell responsiveness to the costimulatory cytokines IL-1 and IL-6.

We also evaluated TBH $CD4^+$ T cell sensitivity to the anti-mitotic cancer drug taxol. Taxol is used during certain therapies to inhibit tumor cell proliferation and potentially may promote tumoricidal activities by Mϕ *in vivo* (290, 467). Taxol enters cells non-specifically because of its exceptional hydrophobicity and mediates significant cytostatic and cytotoxic activities against cells committed within the cell cycle. We speculated that the limited success of taxol therapies may be associated with increased T-cell sensitivity to taxol during tumor burden.

Our data suggest that tumor development increases $CD4^+$ T cell sensitivity to suppressor cytokines and that both T_H1 and T_H2 cell populations are dysfunctional.

Furthermore, tumor growth increases $CD4^+$ T-cell sensitivity to taxol and TGF-β_1 increases this sensitivity. These data collectively suggest that changes in T-cell responsiveness to specific cytokines associated with activation and suppression contribute to decreased immunocompetence during tumor growth. Furthermore, to our knowledge, we are the first to report that tumor growth increases T cell sensitivity to taxol. We have provided explanations for this mechanism and believe that this differential sensitivity may explain the reduced success of taxol as a therapeutic agent. The identification of these changes can lead to better immunotherapies in cancer treatment.

MATERIALS AND METHODS

Animals

All studies used a murine tumor that has been previously described (123). Eight to 12 week old male BALB/c mice were used as the source of splenic immune cells. Tumor growth was initiated by injecting tumor cells from a methylcholanthrene-induced, non-metastatic fibrosarcoma into the animal's left hind leg. Palpable tumors form by 10-14 days and kill the host by days 28-35. Purified $CD4^+$ splenic T cells from 21-day TBH mice were used throughout because TBH demonstrate maximum immunosuppression at this time.

Cytokines and Reagents

Recombinant IL-10 was generously provided by Anne O'Garra (DNAX, Palo Alto, CA). Recombinant TGF-β_1 and IFN-γ was generously provided by Genentech (San Francisco, CA). Recombinant IL-2 was generously provided by Dr. R.S. Selvan (Duke University, Durham, NC). IL-1 and IL-6 were acquired from R & D Systems (Minneapolis, MN). Taxol was generously provided by Dr. David G. Kingston and Dr. Richard A. Walker (Virginia Tech, Blacksburg, VA) and acquired from Calbiochem (Santa Clara, CA).

T-Cell Proliferation Assays

T-cell proliferation as a result of activation was used to evaluate immune cell responsiveness during tumor burden. T cells (4 x 10^6/ml) were activated with the T-cell specific mitogen concanavalin A (8 μg/ml) in the absence or presence of mediators that promote or suppress T-cell activation. All cultures were maintained in sterile 96-well flat-bottom microtiter plates with a final culture volume of 0.2 ml. T-cell proliferation was assessed after 72 h (37°C, 5% CO_2) by tritiated thymidine (^{3}H-TdR) incorporation.

Supernatant Collection

Supernatants were collected from T-cell cultures (4 x 10^6 cells /ml activated with 8 μg/ml concanavalin A) by centrifugation and were either added directly into the GM-CSF bioassay or stored at -70°C until use. Analyses of T-cell supernatants from several time points suggested maximum GM-CSF synthesis occurred 8 h post-stimulation (data not shown). Unless specified, Mϕ supernatants were collected 18 h post-stimulation.

GM-CSF Bioassay

The GM-CSF-responsive cell line DA3 was obtained from Drs. Giovanni Rovera and Brent Kreider (Wistar Institute, Philadelphia, PA) and maintained by biweekly passage into fresh medium containing 20 U/ml GM-CSF. To determine the presence of GM-CSF in supernatants, 50 μl (2.5×10^4 cells/well) were seeded with 50 μl supernatant in flat-bottom 96-well tissue-treated plates (Nunc). Cultures were incubated for 3 days at 37°C and 5% CO_2, and viability was assessed using Alamar Blue.

Analyses of IL-10 and TGF-β_1 production

IL-10 and TGF-β_1 production was assessed using murine-specific ELISA (Endogen, Inc., Boston, MA). ELISAs were performed using specific protocols that were provided. TGF-β_1 and IL-10 production by T cells (4×10^6 cells/ml) was assessed after 24 h activation with Concanavalin A (8 μg/ml).

Statistics and Calculations of Results

All samples were run in triplicate and analyzed for significance using the Student's *t* test ($p < 0.05$). All data are presented as means ± standard errors. All experiments were performed two to five times; representative experiments are shown.

RESULTS

Tumor growth increases $CD4^+$ T cell sensitivity to the suppressor cytokines IL-10 and TGF-β_1

Previous investigations show that fibrosarcoma cells and suppressor macrophages produce significant amounts of IL-10 and TGF-β_1 (10). These cytokines significantly decrease T-cell proliferation. To determine whether tumor growth alters T-cell responsiveness to these downregulatory molecules, NH and TBH $CD4^+$ T cells were activated in the absence or presence of IL-10 (Figure 36) and TGF-β_1 (Figure 37). Both IL-10 and TGF-β_1 compromised TBH $CD4^+$ T cell proliferation to a greater extent than NH $CD4^+$ T cell proliferation. Furthermore, both IL-10 and TGF-β_1 significantly suppressed GM-CSF synthesis (Figure 38). An autocrine mechanism of suppression may occur because activated TBH $CD4^+$ T cells produce more TGF-β_1 than NH $CD4^+$ T cells (data not shown). IL-10 synthesis by NH and TBH $CD4^+$ T cells was similar. These data suggest that tumor growth increases $CD4^+$ T cell sensitivity to the suppressor cytokines IL-10 and TGF-β_1 and that autocrine production of TGF-β_1 may contribute to these tumor-induced changes.

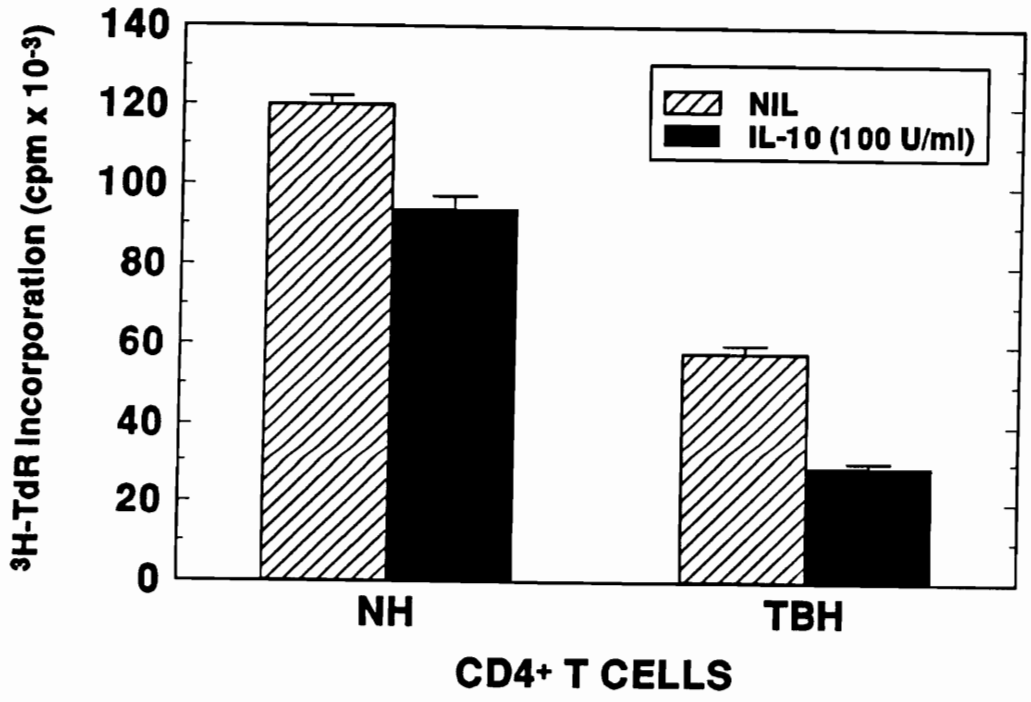

Figure 36. Tumor growth increases CD4$^+$ T-cell sensitivity to the suppressor cytokine IL-10: NH or TBH CD4$^+$ T cells were stimulated with the T-cell mitogen concanavalin A in the absence or presence of IL-10. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Similar data were acquired using 10, 50, and 200 units/ml IL-10 (data not shown). NH and TBH CD4$^+$ T cell proliferation were suppressed equally by $\geq$ 400 U/ml IL-10.

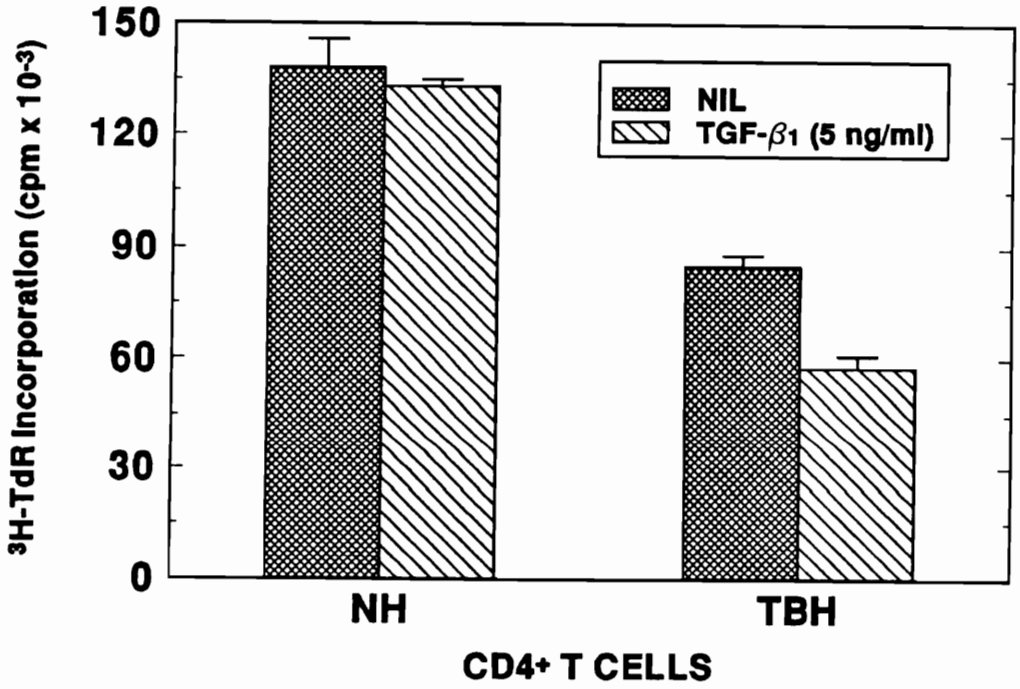

Figure 37. Tumor growth increases CD4$^+$ T-cell sensitivity to the suppressor cytokine TGF-β_1: NH or TBH CD4$^+$ T cells were stimulated with concanavalin A in the absence or presence of TGF-β_1. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Similar data were acquired using 2.5 and 10 ng/ml TGF-β_1 (data not shown). NH and TBH CD4$^+$ T cell proliferation were suppressed equally by $\geq$ 25 ng/ml TGF-β_1.

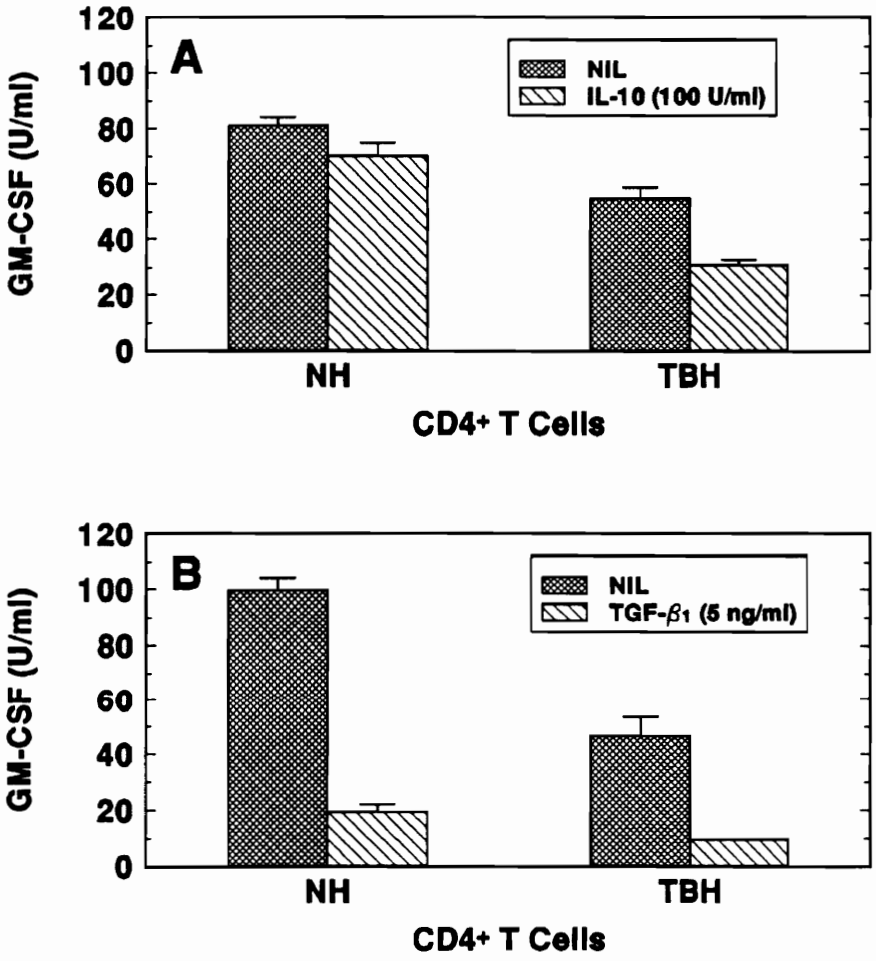

Figure 38. IL-10 and TGF-β_1 further suppress GM-CSF synthesis by TBH CD4$^+$ T cells: NH and TBH CD4$^+$ T cells were seeded into flat-bottom 96-well microtiter plates and stimulated with concanavalin A in the absence or presence of IL-10 (panel A) or TGF-β_1 (panel B). Supernatants were collected by centrifugation and GM-CSF production was measured using the GM-CSF bioassay.

Signals that promote T_H1 cell responses partly reverse tumor-induced immune suppression

We hypothesized that tumor growth may suppress $CD4^+$ T cell activation by altering T_H1 cell cytokine production of or responsiveness to autocrine growth factors. To determine whether exogenous T_H1-promoting signals could partly or completely restore $CD4^+$ T cell activity to normal levels, NH and TBH $CD4^+$ T cells were activated in the absence or presence of IL-2 (Figure 39), IFN-γ (Figure 40), or anti-IL-4 mAb (Figure 41). Exogenous IL-2 was used to promote T_H1 cell expansion. Exogenous IFN-γ was administered to inhibit the production of cross-regulatory cytokines (such as IL-10 and TGF-β_1) by T_H2 cells. Anti-IL-4 mAb was used to bind endogenous IL-4 and restrict the expansion of activated T_H2 cells. Tumor-induced decreases in $CD4^+$ T cell proliferation were completely reversed in the presence of IL-2 and only partly reversed in the presence of either IFN-γ or anti-IL-4 mAb. Addition of anti-IL-10 mAb to TBH $CD4^+$ T cell cultures did not reverse tumor-induced suppression (data not shown). When combinations of these signals were provided (Figure 42), complete reconstitution occurred when both T_H1 cell activity was promoted using IL-2 and T_H2 cell activity was downregulated using either IFN-γ or anti-IL-4 mAb. We also determined whether signals that promote T_H1 activity could enhance GM-CSF synthesis by activated TBH $CD4^+$ T cells. Although IL-2 restored TBH $CD4^+$ T cell proliferation, tumor-induced suppression of GM-CSF synthesis was not reversed (Figure 43). IFN-γ, anti-IL-4 mAb, and anti-IL-10 mAb did not enhance GM-CSF production by NH or TBH $CD4^+$ T cells (data not shown). Collectively, these data suggest that tumor-induced decreases in T-cell responsiveness during activation partly involve

dysregulation of T_H1 cell cytokine expression. These data also suggest that T_H2 cells in culture do not suppress T_H1 activities through IL-10 synthesis.

Restoration of T cell activities through T_H1-promoting signals is not compromised by IL-10 and TGF-β_1

Although signals that promoted T_H1 cell activities or suppressed T_H2 cell cytokine synthesis significantly increased TBH $CD4^+$ T cell proliferation, we hypothesized that tumor-induced increases in T cell sensitivity to IL-10 and TGF-β_1 may supercede reconstitution by T_H1 cell-promoting costimulatory signals. To test this possibility, activated NH and TBH $CD4^+$ T cells were cultured with IL-2, IFN-γ, and anti-IL-4 in the absence or presence of IL-10 or TGF-β_1 (Figure 44). IL-10 and TGF-β_1 blocked the ability of IFN-γ and anti-IL-4 mAb to reverse tumor-induced immunosuppression. In contrast, IL-10 and TGF-β_1 did not alter reconstitution of TBH $CD4^+$ T cell proliferation by IL-2. Thus, IL-10 and TGF-β_1 abrogate restoration of T cell proliferation through T_H2-suppressing signals but do not alter reconstitution of T cell activity by the T_H1-promoting cytokine IL-2.

Tumor growth inhibits $CD4^+$ T cell responsiveness to IL-1 and IL-6

To determine whether tumor-induced suppression is restricted to T_H1 cells, we evaluated TBH $CD4^+$ T cell responses to cytokines that promote T_H2 cell activities. IL-1 and IL-6 are Mϕ-derived costimulatory signals that enhance activated T cell proliferation and cytokine expression. To determine whether T cell responsiveness to

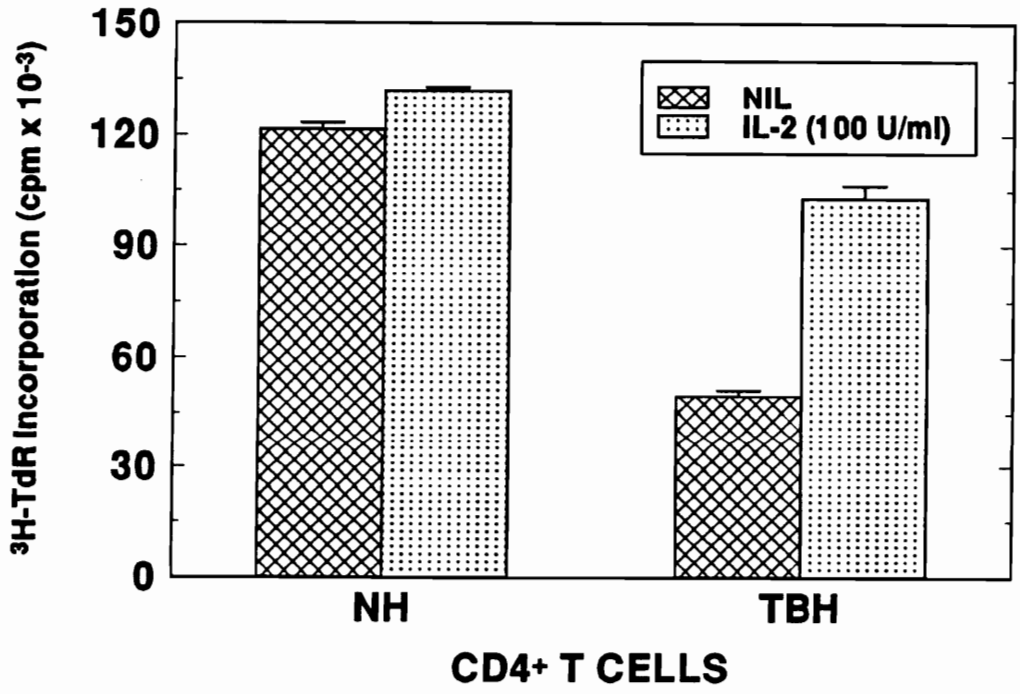

Figure 39. IL-2 restores TBH CD4$^+$ T-cell proliferation: NH and TBH CD4$^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-2. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Similar data were acquired using 50 and 200 units/ml IL-2 (data not shown).

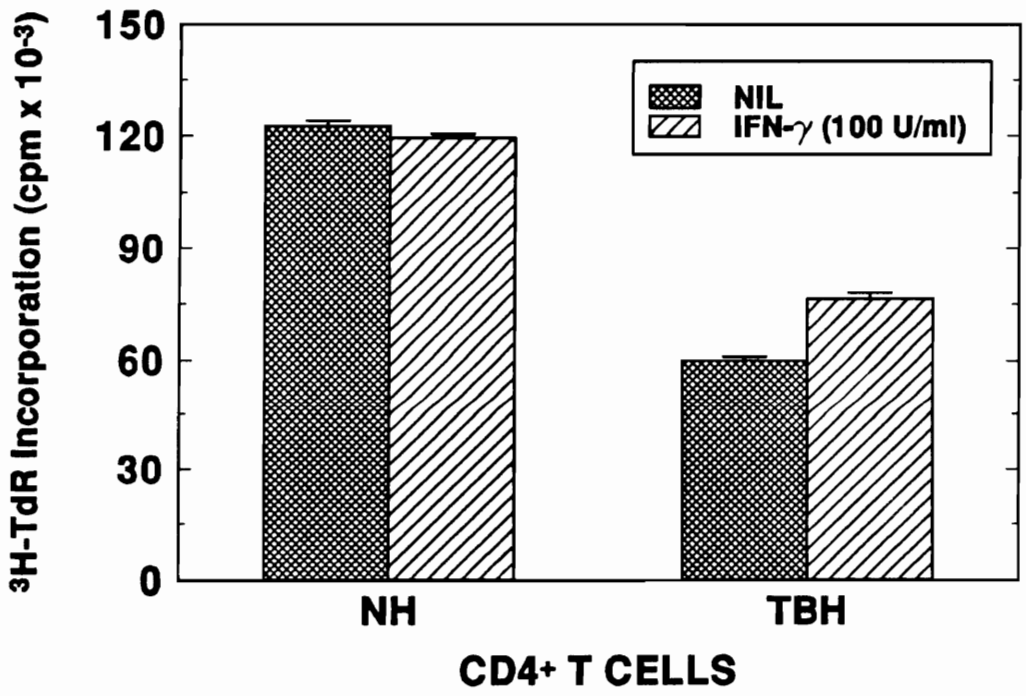

Figure 40. IFN-γ partly restores TBH CD4⁺ T-cell proliferation: NH and TBH CD4⁺ T cells were stimulated with concanavalin A in the absence or presence of IFN-γ. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Lower doses of IFN-γ (10 U/ml) did not increase TBH CD4⁺ T cell proliferation (data not shown).

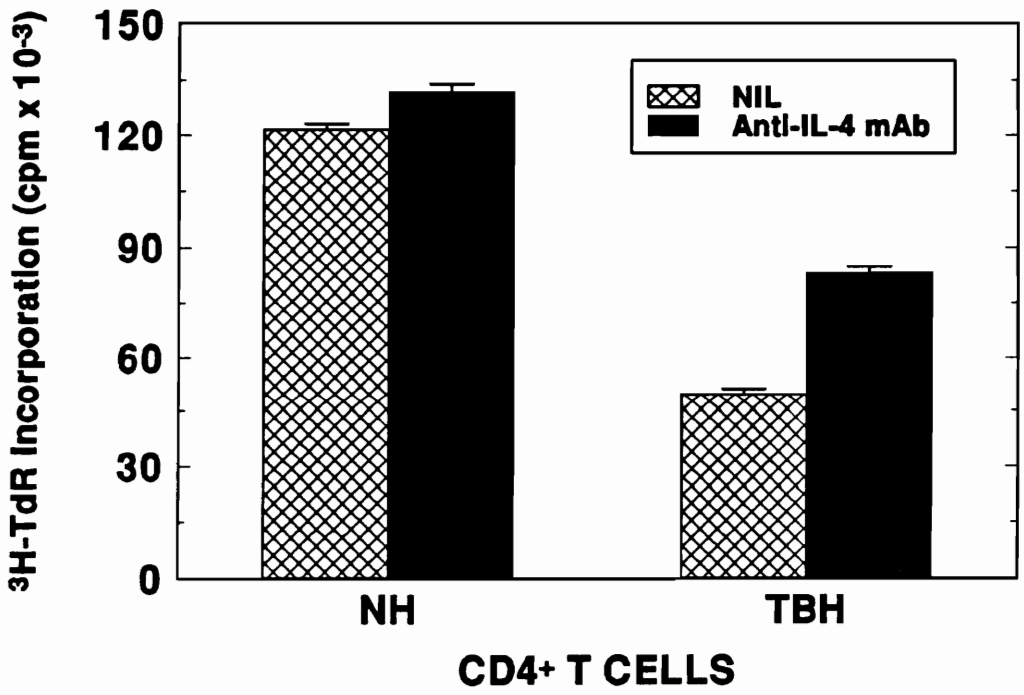

Figure 41. Inhibition of endogenously produced IL-4 partly restore TBH $CD4^+$ T-cell proliferation: NH and TBH $CD4^+$ T cells were stimulated with concanavalin A in the absence or presence of mAb against IL-4 (0.25 neutralizing units/ml). NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation.

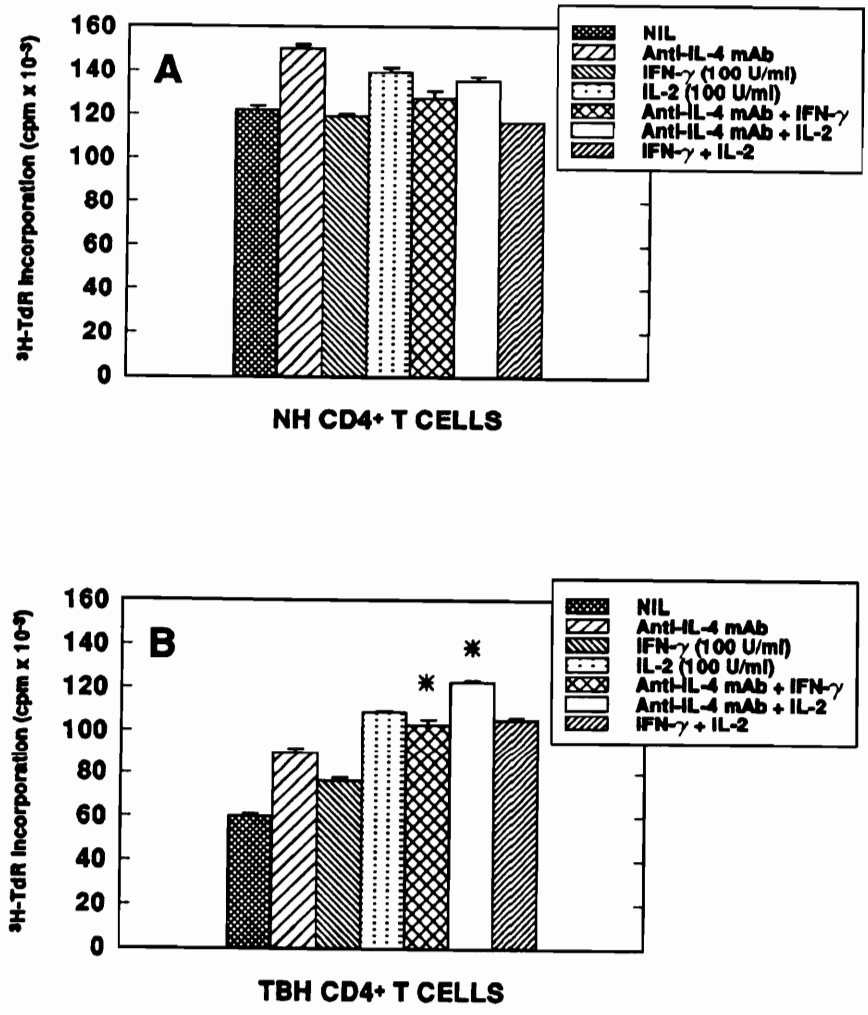

Figure 42. Administration of multiple activation signals additively restores TBH CD4$^+$ T-cell proliferation: NH (panel A) and TBH (panel B) CD4$^+$ T cells were stimulated with concanavalin A in the absence or presence of anti-IL-4 mAb, IFN-γ, and IL-2. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Asterisks (*) indicate that cultures demonstrated an additive increase above controls in their level of activation.

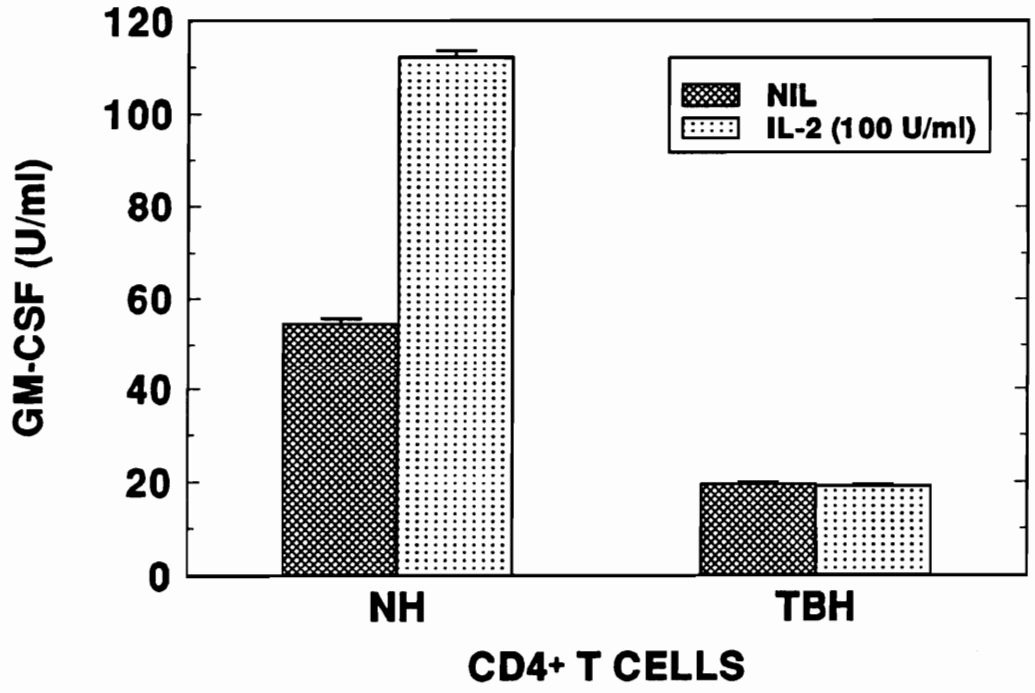

Figure 43. IL-2 does not enhance GM-CSF synthesis by TBH CD4$^+$ T cells: NH and TBH CD4$^+$ T cells were seeded into flat-bottom 96-well microtiter plates and stimulated with concanavalin A in the absence or presence of IL-2 (100 U/ml). Supernatants were collected by centrifugation and GM-CSF production was measured using the GM-CSF bioassay.

IL-1 and IL-6 are compromised during tumor growth, T cell proliferation and GM-CSF production were measured. Tumor growth significantly inhibited $CD4^+$ T cell re-

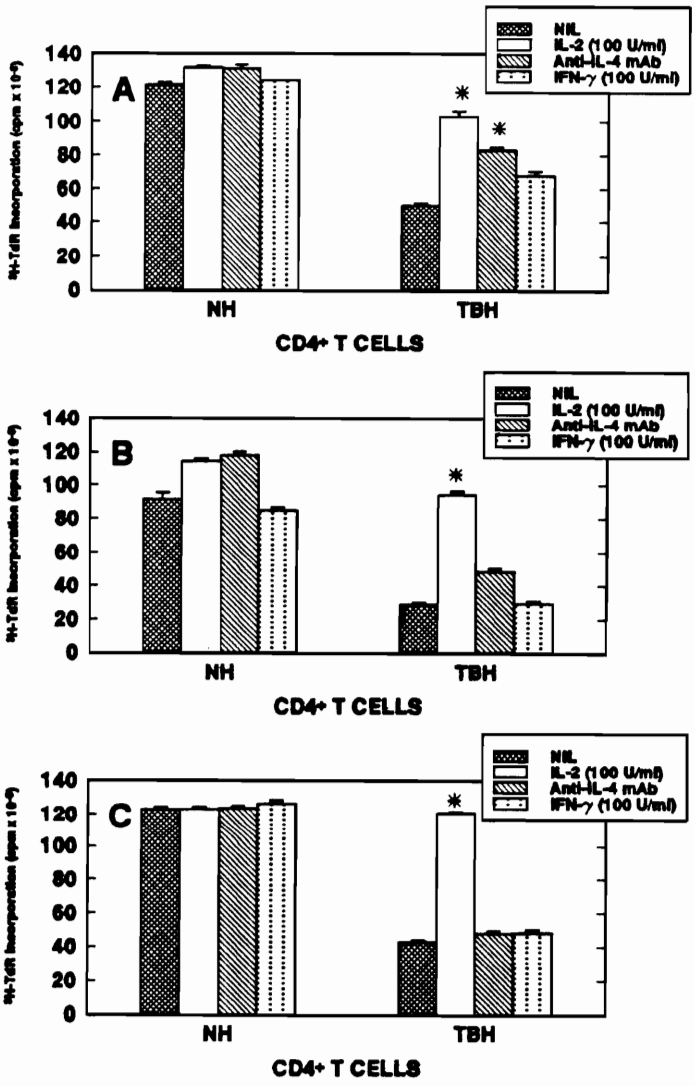

Figure 44. IL-10 and TGF-β_1 inhibit TBH $CD4^+$ T-cell proliferation in the presence of IFN-γ and anti-IL-4 mAb: NH and TBH $CD4^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-2, anti-IL-4 mAb, IFN-γ, or IL-2 (panel A). Test cultures were also activated in the presence of 100 U/ml IL-10 (panel B) or 5 ng/ml TGF-β_1 (panel C). NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Asterisks (*) indicate that cultures demonstrated an additive increase above controls in their level of activation.

sponsiveness to both IL-1 (Figure 45) and IL-6 (Figure 46). IL-1 in combination with either IL-2 or IL-4 did not enhance TBH $CD4^+$ T cell proliferation. Tumor growth also blocked IL-1-induced and IL-6-induced GM-CSF synthesis by activated $CD4^+$ T cells (Figure 47). These findings suggest that tumor growth disrupts T_H2 cell responsiveness to costimulatory cytokines.

Tumor growth increases $CD4^+$ T cell sensitivity to the anticancer drug taxol

We recently initiated several studies to determine whether the anticancer drug taxol affects tumor-induced immune cell dysfunctions. Others suggest that taxol decreases T cell proliferation by polymerizing microtubules and compromising responsiveness to IL-2 (50, 83). Because taxol decreases T cell proliferation, we wanted to determine whether taxol exacerbates tumor-induced changes in T cell functions. Taxol significantly suppressed both NH and TBH T cell proliferation, but taxol-mediated suppression was greater with TBH T cells than with NH T cells (Table 14). Taxol significantly compromised TBH T cell proliferation to a greater extent than NH T cell proliferation even when it was added 24 or 48 h post-activation. Both IL-2 (Figure 48) and TGF-β_1 (Figure 49) increased T cell sensitivity to taxol. Taxol pretreatment of TBH T cells also significantly compromised T cell proliferation (Figure 50). TBH T cell proliferation was suppressed more than NH T cell proliferation even when cells were pretreated with taxol 12 h before activation. However, both NH and TBH $CD4^+$ T cells recovered from taxol pretreatment if they were pretreated 24 h before activation. Collectively, these data suggest that taxol contributes to tumor-induced T cell dysfunctions.

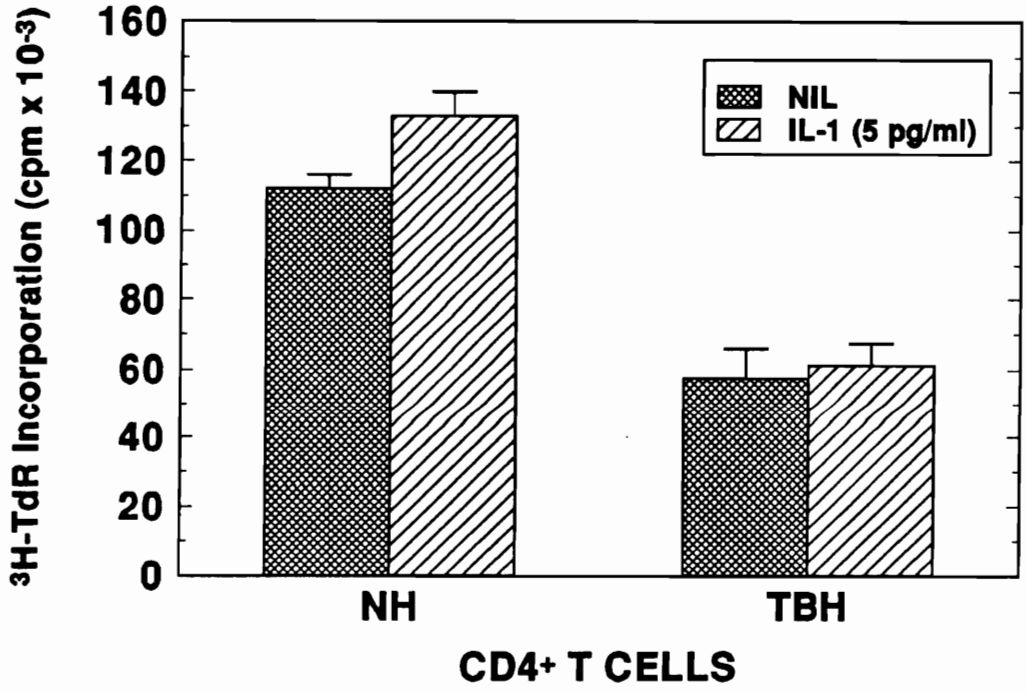

Figure 45. Tumor growth inhibits enhancement of $CD4^+$ T cell proliferation by IL-1: NH and TBH $CD4^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-1. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Similar data were acquired using 2.5 and 10 pg/ml IL-1 (data not shown).

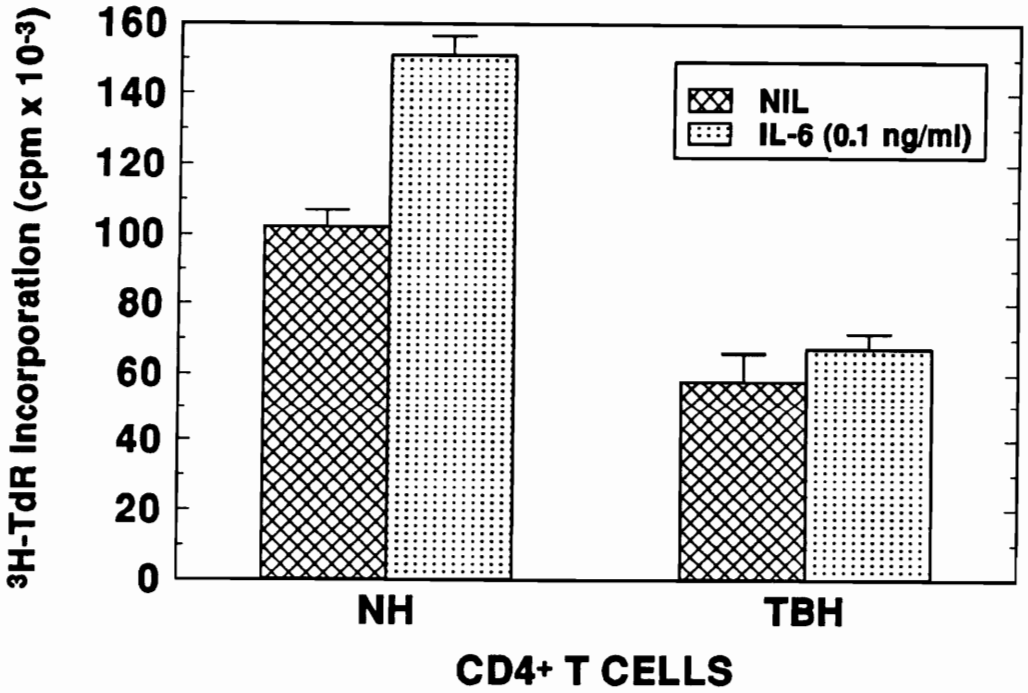

Figure 46. Tumor growth inhibits enhancement of $CD4^+$ T cell proliferation by IL-6: NH and TBH $CD4^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-6. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Similar data were acquired using 0.05 and 0.2 ng/ml IL-6 (data not shown).

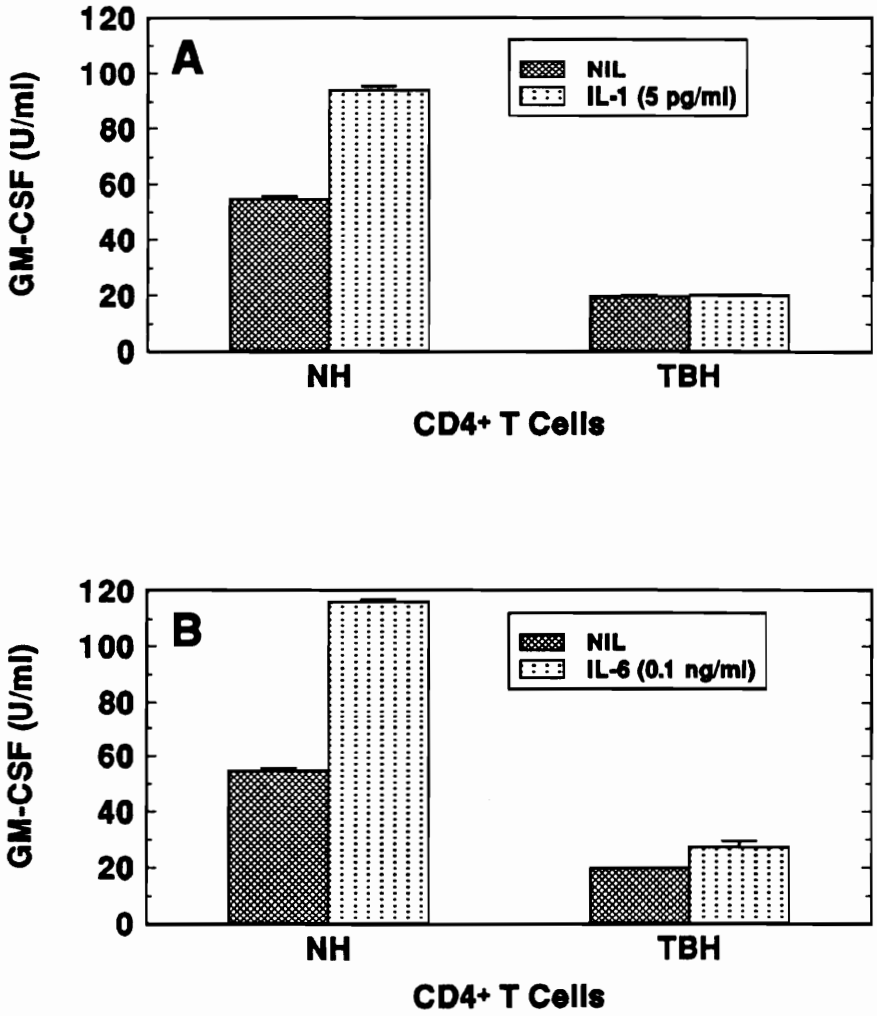

Figure 47. Tumor growth inhibits enhancement of $CD4^+$ T cell GM-CSF production by IL-1 and IL-6: NH and TBH $CD4^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-1 (panel A) or IL-6 (panel B). GM-CSF production was assessed using the GM-CSF bioasssay.

Table 14. Tumor growth increases T-cell sensitivity to taxol

Taxol Addition (h)[a]	NH T Cells[b]	Change in Proliferation (%)	TBH T Cells[b]	Change in Proliferation (%)
NIL	136.09 ± 3.42[c]	-----	82.6 ± 2.19[c]	-----
0	96.82 ± 2.37	29 ↓	32.44 ± 0.52	61 ↓
24	79.74 ± 2.0	41 ↓	26.85 ± 1.01	67 ↓
48	123.81 ± 5.76	9 ↓	60.84 ± 2.11	26 ↓

[a]Taxol was generously provided by Dr. David G. Kingston (Dept. of Chemistry) and Dr. Richard A. Walker (Dept. of Biology), Virginia Tech. Taxol was used at a final concentration of 10 μM and was added at the time of T-cell activation (0 h) or post-activation (24 h or 48 h). Proliferation was assessed 72 h post-activation. Similar data were acquired using 1 μM and 0.1 μM taxol (not shown).

[b]NH and TBH $CD4^+$ T cells (4×10^6 cells/ml) were stimulated with concanavalin A (8 μg/ml).

[c]Values are expressed as cpm x 10^{-3} ± SE as determined by ^{3}H-TdR incorporation.

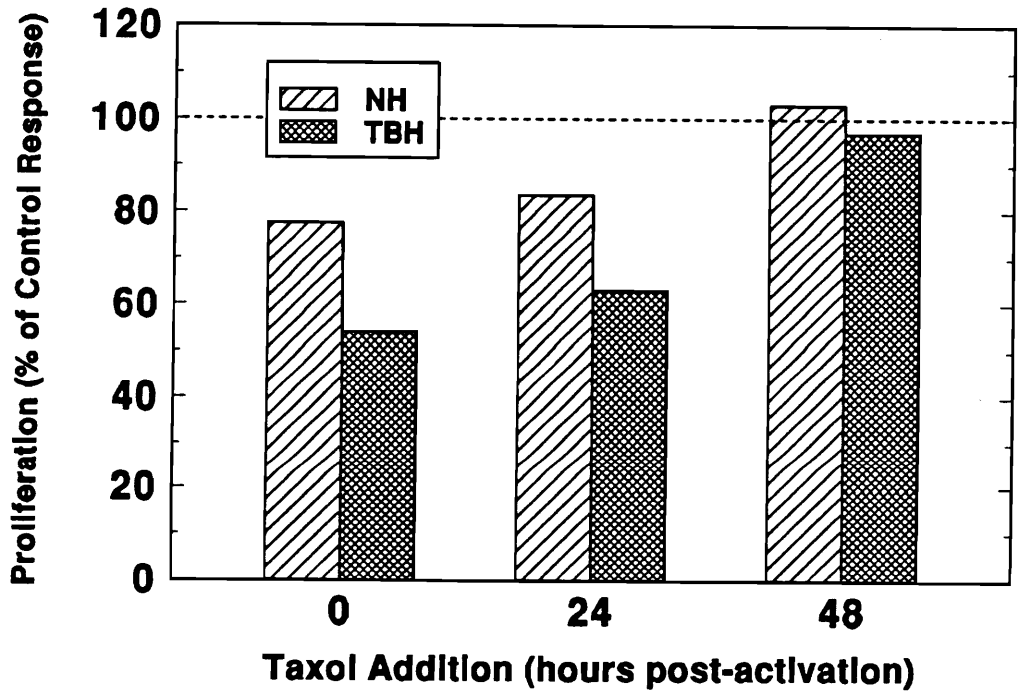

Figure 48. IL-2 increases TBH CD4$^+$ T cell sensitivity to taxol: NH and TBH CD4$^+$ T cells were stimulated with concanavalin A in the absence or presence of IL-2 (100 U/ml). Taxol (10 μM) was added to cultures 0, 24, or 48 h after activation. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Data are expressed as percentage proliferation relative to control (dotted line). Similar data were acquired using 1 μM and 0.1 μM taxol (data not shown).

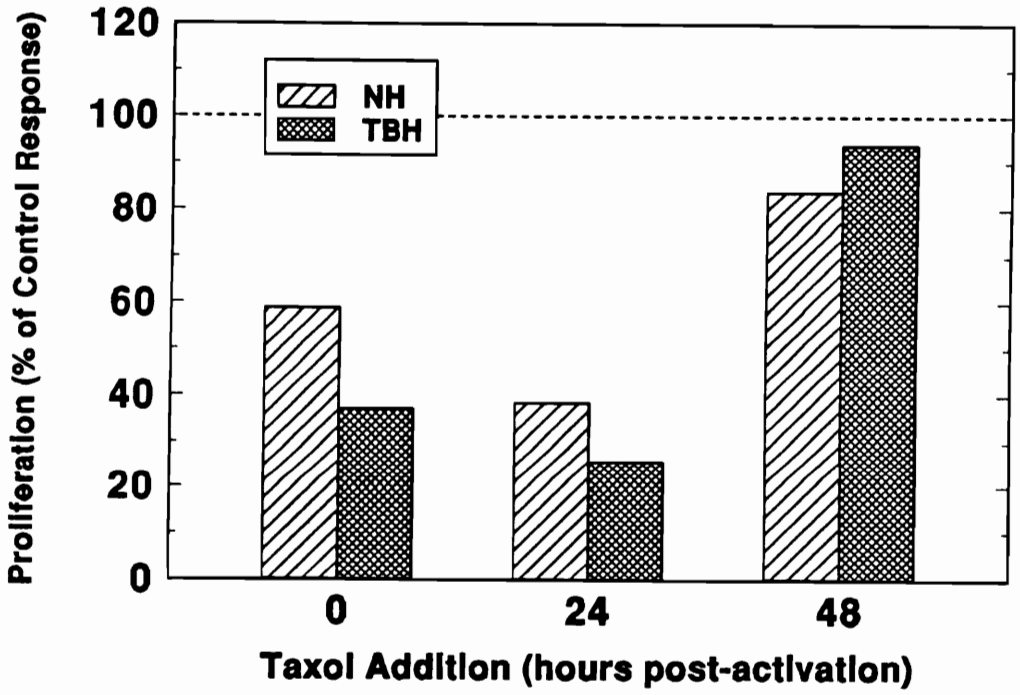

Figure 49. **TGF-β_1 increases TBH CD4$^+$ T cell sensitivity to taxol:** NH and TBH CD4$^+$ T cells were stimulated with concanavalin A in the absence or presence of TGF-β_1 (5 ng/ml). Taxol (10 μM) was added to cultures 0, 24, or 48 h after activation. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Data are expressed as percentage proliferation relative to control (dotted line). Similar data were acquired using 1 μM and 0.1 μM taxol (data not shown).

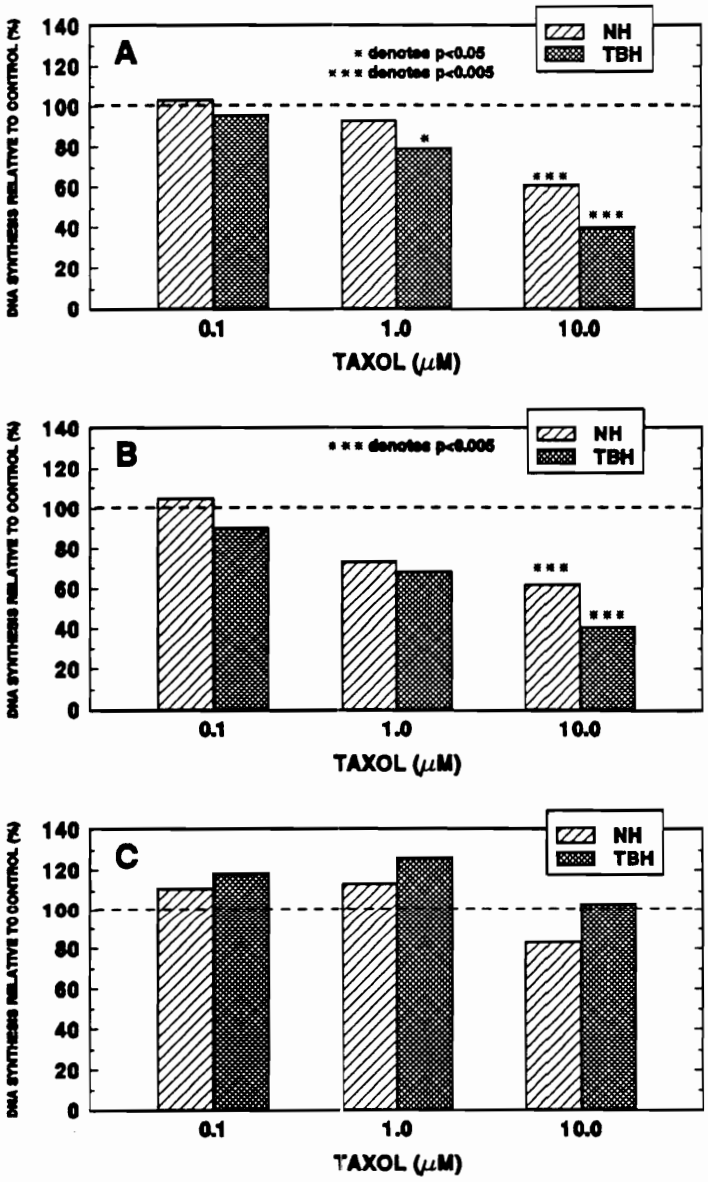

Figure 50. Tumor growth does not compromise the ability of CD4$^+$ T cells to recover from taxol pretreatment: NH and TBH CD4$^+$ T cells were cultured for 4 h with taxol. Cells were washed twice and subsequently activated with concanavalin A either 0 (panel A), 12 (panel B), or 24 h (panel C) after taxol pretreatment. NH and TBH T-cell proliferation were assessed after 72 hours by ^{3}H-TdR incorporation. Data are expressed as percentage proliferation relative to control (dotted line).

DISCUSSION

In this study, we show that tumor development disrupts $CD4^+$ T cell cytokine production and responsiveness, which contributes to tumor-induced immune dysfunction. Tumor growth increases TBH $CD4^+$ T cell sensitivity to suppressor signals such as IL-10 and TGF-β_1, and decreases $CD4^+$ T cell responsiveness to specific autocrine growth factors. Tumor growth also increases $CD4^+$ T-cell sensitivity to the anticancer drug taxol. These findings extend our previous studies that indicate tumor growth increases Mϕ suppressor activities. Our data also suggest that tumor-induced immunosuppression, derived from suppressor immune cells and tumor cells, can targets $CD4^+$ T cells.

We first determined that IL-10 significantly suppresses TBH $CD4^+$ T cell proliferation (Figure 36). IL-10 is produced constitutively by the fibrosarcoma cells used in our murine cancer model and mediates significant immunosuppression (10). We (444) and others (413) have shown that IL-10 significantly inhibits T-cell proliferation in the absence of antigen-presenting cells. Increased sensitivity to IL-10 is not due to the presence of TBH $CD8^+$ T cells which suppress $CD4^+$ T cell activation (449) Because IL-10-mediated inhibition of proliferation may occur partly through decreased IL-2 synthesis (413), TBH $CD4^+$ T cell susceptibility to IL-10 may correlate with a lower ability to synthesize IL-2. Although we did not directly measure IL-2 production by TBH $CD4^+$ T cells, we indirectly confirmed this hypothesis because exogenous IL-2 almost completely restores TBH $CD4^+$ T cell proliferation to NH T cell levels in the presence of IL-10 (Figure 42).

We also showed that TGF-β_1 significantly suppresses TBH $CD4^+$ T cell proliferation (Figure 37). Like IL-10, TGF-β_1 is produced by the tumor cells in our cancer

model (10). However, TGF-β_1 can cause both local and systemic immunosuppression, depending on whether the molecule is activated at the tumor site or retained in its latent form. Tumor cell-derived TGF-β_1 appears to serve as an autocrine growth factor and as a potent immunosuppressant against anticancer activities (unpublished observations). TGF-β_1 suppresses T cell activation and proliferation (243, 373), and TGF-β_1 may disrupt TBH $CD4^+$ T cell helper functions that are required for effective cytotoxic T lymphocyte actions against tumor targets (22). We determined that TGF-β_1 inhibits the synthesis of GM-CSF, which we previously showed was an important regulatory cytokine (444). Others report that TGF-β_1 can compromise T-cell production of other cytokines (129). *In vivo*, TGF-β_1-mediated suppression may occur because it is overproduced by both tumor cells and immune cells. TBH $CD4^+$ T cells and TBH Mϕ produce more TGF-β_1 during activation than their NH counterparts, and TGF-β_1 protein is detected earlier in TBH cell supernatants than in NH supernatants. During other specific disease states, $CD4^+$ T cells produce elevated amounts TGF-β_1 and display several suppressor activities. Our findings suggest that inappropriate TGF-β_1 regulation through aberrant tumor cell and immune cell production may shift the physiological signalling associated with this cytokine from proinflammatory to immunosuppressive (295).

We next determined that cytokines associated with T_H1 responses partly reverse tumor-induced suppression of $CD4^+$ T cell proliferation. T_H1 cells regulate delayed-type hypersensitivity responses and promote anticancer effector activities by cytotoxic T cells, NK cells, and Mϕ. Although T_H1 and T_H2 cells are phenotypically indistinguishable and appear to differentiate from a common precursor (364), they can be distinguished by their functional traits. T_H1 cells produce IL-2 and IFN-γ whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (384). IFN-γ serves as a cross-regulatory cytokine that suppresses T_H2 proliferation but not T_H2 cytokine synthesis

(166, 168). IFN-γ-mediated T_H2 suppression indirectly promotes T_H1 activity, and anti-IL-4 mAb (which neutralizes endogenous IL-4) can mimic this activity. T_H2-derived IL-10 suppresses synthesis of IL-2 and IFN-γ by T_H1 cells. Activated T_H1 cells respond to IL-2 alone whereas activated T_H2 cells respond to IL-2 or IL-4 only in the presence of IL-1 (72, 145, 166, 462). Based on these observations, we hypothesized that TBH $CD4^+$ T cell proliferation may be compromised due to decreased cytokine production. Exogenous IL-2 restored TBH $CD4^+$ T cell proliferation to NH levels (Figure 39) and these data suggest that TBH $CD4^+$ T cell production of IL-2 is compromised. Decreased IL-2 production does not appear to be mediated through T_H2 cells because IFN-γ (Figure 40) and anti-IL-4 mAb (Figure 41) did not restore proliferation to NH levels. Only combinations of these signals with IL-2 could restore TBH T cell proliferation (Figure 42). Furthermore, addition of anti-IL-10 mAb to TBH $CD4^+$ T cell cultures did not reverse tumor-induced suppression (data not shown), which suggests that T_H2 cells in culture are not suppressing T_H1 activity through IL-10 synthesis. When either exogenous IL-10 or TGF-β_1 were present, TBH $CD4^+$ T cell proliferation was not restored by anti-IL-4 mAb and IFN-γ (Figure 44). This finding suggests that tumor growth compromises T_H1 cytokine production through increased sensitivity to the suppressor signals rather than through increased T_H2 activity. These data also exclude the possibility that T_H1 cells are anergic (156), because TBH $CD4^+$ T cell proliferation is restored to NH levels by IL-2. Tumor growth compromises cytokine transcriptional pathways that are regulated partly (but not exclusively) by IL-2 (Figure 43). Tumor cell-derived suppressor signals probably suppress T_H1 responses *in vivo*. Future studies will investigate this possibility and will determine if tumor growth decreases the expression of IL-12, a potent regulatory cytokine that significantly promotes T_H1 activities and anticancer responses.

Tumor growth disrupts $CD4^+$ T cell responsiveness to IL-1 and IL-6. Both of these signals enhance T cell proliferation and cytokine synthesis but only T_H2 cells express IL-1 receptors and produce IL-6 (318). T_H2 cells require both IL-1 and IL-4 during autocrine growth maintenance, and T_H2 cells respond to IL-2 and IL-4 only when these cytokines are administered with IL-1 (72, 145, 166). Because TBH $CD4^+$ T cell proliferation (Figures 45 and 46) and GM-CSF synthesis (Figure 47) were not enhanced by IL-1 and IL-6, we conclude that tumor growth compromises T_H2 cell activities. Tumor-induced suppression of T_H2 cells involves a mechanism distinct from the one that disrupts T_H1 activities. Whereas exogenous T_H1-specific growth factors restored T_H1 cell activity, combinations of cytokines that promote T_H2 expansion failed to enhance T cell proliferation or cytokine synthesis. Because T-cell anergy targets only IL-2-producing populations (156), unresponsiveness to IL-1 may occur because T_H2 cells do not express functional IL-1 receptors. Although several disease states involve cascades that deplete or hinder the development of mature T_H2 cells, these scenarios involve T_H1 activities which appear to be disrupted or inoperative during during tumor growth. Thus, tumor growth compromises $CD4^+$ T cell responses to cytokines that promote T_H2 activities through a mechanism that does not involve phenotypic shifts in T_H1 and T_H2 cells.

We showed that tumor growth increases T cell sensitivity to the anticancer drug taxol. TBH $CD4^+$ T cell proliferation is suppressed to a greater extent than the NH counterpart (Table 14), and taxol-mediated suppression is significant even when taxol is administered 48 h after T cell activation. Furthermore, IL-2 (Figure 48) and TGF-β_1 (Figure 49) increase TBH $CD4^+$ T cell sensitivity to taxol. These data suggest that either IL-2 immunotherapies used in conjunction with taxol or administration of taxol to patients with TGF-β_1-producing cancers could adversely affect *in vivo* T cell activities. Heightened sensitivity may occur because IL-2 significantly restores TBH $CD4^+$

T cell proliferation to NH levels. This increase in proliferation may commit T cells within the cell cycle where cell populations are most sensitive to taxol. In contrast, TGF-β_1 may increase TBH $CD4^+$ T cell sensitivity to taxol by disrupting the synthesis and export of autocrine growth factors. Although others have shown that taxol can suppress the proliferation and cytotoxic activities of activated T cells (50, 83), we are the first to report that tumor growth increases T cell sensitivity to taxol. Tumor growth does not alter the ability of $CD4^+$ T cells to recover from 24-h pre-treatment with taxol (Figure 50).

Several parameters may contribute to heightened T cell sensitivity to taxol during tumor burden. First, taxol may compromise IL-2 production. Taxol can interact with rough endoplasmic reticulum (ER) and displace ER-associated ribosomes (422). Taxol also can fragment Golgi complexes and significantly disrupt the post-translational modification and export of proteins (422). Second, taxol may suppress expression of functional IL-2 receptors. Roy et al. (370) reported that proliferation of taxol-treated lymphocytes cannot be restored by addition of exogenous IL-2. Our data support this possibility because TBH $CD4^+$ T cells are less responsive to IL-2 in the presence of taxol than their NH counterparts (Figure 48). Data has not been reported to suggest whether an IL-2 receptor defect exists that is caused by decreased receptor subunit transcription, assembly and transport, or surface expression. Taxol may compromise subunit association into a functional, high affinity IL-2 receptor after expression. Other costimulatory molecules such as CD28 and VTLA-4 similarly may be restricted in their surface expression. Third, taxol may differentially compromise TBH T cell proliferation because these cells contain lower concentrations of tubulin. TBH T cells are smaller morphologically than their NH counterparts and lower tubulin content would explain the significant differences in sensitivity between NH and TBH T cells. Fourth, heightened $CD4^+$ T cell sensitivity to taxol may occur through

apoptotic pathways. Taxol induces apoptosis among cells committed within the cell cycle, and future studies will address whether TBH T cells undergo programmed cell death more quickly than NH T cells. Apoptosis may involve increased T-cell susceptibility to oxidative stress (60). All of these possibilities merit additional attention in future studies.

Collectively, our data suggest that tumor growth increases $CD4^+$ T cell sensitivity to the suppressor signals IL-10 and TGF-β_1. Tumor growth compromises the activities of both T_H1 and T_H2 cells, but the mechanisms of suppression are distinct. T_H1 cell activities are suppressed because autocrine growth factor (IL-2) production is decreased. In contrast, T_H2 cell activities appear to be compromised due to decreased responsiveness to autocrine growth factors. Decreased responsiveness probably involves decreased IL-1 receptor expression. Furthermore, tumor growth increases $CD4^+$ T cell sensitivity to the anticancer drug taxol. TBH $CD4^+$ T cells were less responsive to IL-2 and more sensitive to TGF-β_1 in the presence of taxol. Knowledge of these changes during tumor growth can be used to increase our understanding of cancer-mediated suppression and improve the efficacy of immunotherapies.

SECTION IV: TAXOL AS A MEANS TO REVERSE TUMOR-INDUCED IMMUNOSUPPRESSION

This last section of the dissertation describes recent studies that suggest taxol can promote tumor regression *in vivo* by acting as an immunotherapeutic drug. Recent studies from our laboratory show that taxol reverses TBH Mϕ suppressor activities and stimulates Mϕ tumoricidal activities. Chapter IX shows that taxol disrupts tumor cell growth directly through both cytostatic and cytotoxic mechanisms, and increases tumor cell susceptibility to Mϕ-derived lytic molecules that are induced by taxol. Taxol also disrupts autocrine regulation of TGF-β_1 and stimulates apoptosis. By understanding the mechanisms of cytotoxicity associated with taxol treatments, immunotherapies can be developed that target tumor cells and activate tumoricidal Mϕ while avoiding the adverse side effects on T cells.

CHAPTER IX

TAXOL DISRUPTS MURINE FIBROSARCOMA CELL GROWTH THROUGH MULTIPLE CYTOSTATIC AND CYTOTOXIC MECHANISMS

ABSTRACT

Taxol demonstrates several significant anti-neoplastic activities but the mechanisms of action against tumor cells are incompletely characterized. The present study evaluated fibrosarcoma cell sensitivity to taxol. Our cancer model uses a non-metastatic, methylcholanthrene-induced, murine fibrosarcoma designated METH-KDE. *In vitro*, continuous exposure and 4-h pre-treatment of METH-KDE cells with physiologically relevant concentrations of taxol significantly decreased proliferation and viability. Taxol increased METH-KDE cell radiosensitivity and disrupted autocrine regulation of transforming growth factor-beta$_1$ (TGF-β_1). Taxol and TGF-β_1 significantly increased METH-KDE cell sensitivity to tumor necrosis factor-alpha (TNF-α). Taxol-mediated cytotoxicity was restricted to specific stages of the cell cycle. Taxol induced METH-KDE cell apoptosis based on membrane blebbing, and apoptosis occurred through a nitric oxide-independent mechanism. Apoptosis was most significant in the presence of both taxol and TNF-α. Reconstitution studies suggest that METH-KDE cells may recover partly from taxol exposure through an efflux mechanism. Collectively, these data suggest that taxol changes fibrosarcoma cell proliferation, viability, TGF-β_1 regulation, and TNF-α sensitivity. Furthermore, these data suggest that the cytostatic and cytotoxic actions of taxol against fibrosarcoma cells culminate in apoptosis. Taxol-mediated tumor regression *in vivo* may occur through 1) direct cytostatic and cytotoxic activities against fibrosarcoma cells and 2) increased tumor cell sensitivity to taxol-induced, macrophage-derived TNF-α.

INTRODUCTION

The anti-cancer drug taxol and its natural analogs are diterpenoid compounds extracted from the bark and leaves of yew trees and related plants in the genus *Taxus* (248). Recently, taxol and some related analogs were synthesized *in vitro* (331). Although some cancers demonstrate variable resistance to taxol (248, 334), many cancers are sensitive to taxol and show partial or complete remission during taxol chemotherapy (35, 115, 298, 437, 477). Taxol resembles a group of anti-cancer drugs called the vinca alkaloids (vincristine and vinblastine) because it is exceptionally hydrophobic, permeates cell membranes non-specifically, and targets intracellular microtubules (286). Unlike these compounds which disrupt microtubule assembly, taxol promotes the assembly of tubulin dimers into stable microtubule complexes and inhibits the depolymerization of the resulting microtubule structures. Taxol mediates cytostatic and cytotoxic activities against cancer cells when the drug associates with microtubules during specific stages of the cell cycle (114, 369, 468). Taxol increases tumor cell sensitivity to radiotherapy (277, 304) and cytolysis by TNF-α (467), and it promotes tumoricidal activities among macrophages (Mϕ) (41, 110, 288, 290).

Using a murine tumor model, we have described several mechanisms through which fibrosarcoma cells convert Mϕ and T cells into suppressor cells (10, 444, 449). We currently are determining whether taxol reverses tumor-induced suppressor immune cell activities by disrupting tumor growth and promoting Mϕ effector functions. The purpose of this study was to determine if the METH-KDE fibrosarcoma cells used in our tumor model were sensitive to the cytostatic or cytotoxic activities of taxol. Although taxol disrupts tumor cell growth through several mechanisms (114, 277, 304, 369, 467, 468), no studies to date have determined if multiple taxol activities can tar-

get the same tumor cell line. We evaluated whether many of the of these independent activities affect METH-KDE cells. Furthermore, we determined whether specific cytokines associated with taxol therapies and tumor growth contributed to fibrosarcoma cell sensitivity. Our results suggest that taxol has both tumoristatic and tumoricidal activities against METH-KDE fibrosarcoma cells.

MATERIALS AND METHODS

METH-KDE Fibrosarcoma Cells

The murine fibrosarcoma cell line METH-KDE (10) was propagated in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 50 mg/L gentamicin (Tri Bio Labs, State College, PA). Single-cell suspensions of fibrosarcoma cells (4 x 10^5/host) injected i.m. into the left hind leg of BALB/c mice induce palpable tumors within 10-14 days and kill the hosts within 28-35 days. Macrophages and T cells from fibrosarcoma-bearing mice 21 days post-tumor induction demonstrate several significant immunosuppressive activities (444, 449).

Reagents and Cytokines

Taxol was provided by Drs. David G.I. Kingston (Department of Chemistry, Virginia Tech) and Richard A. Walker (Department of Biology, Virginia Tech) and was acquired from Calbiochem (La Jolla, CA). Taxol stock solutions (4 mM) were prepared in 100% DMSO and stored at -80°C until use. Taxol was used at a final concentration of 0.1, 1.0, or 10.0 μM. Anti-TGF-β_1 mAb was provided by Dr. James Dasch (Celtrix Pharmaceuticals, Inc., Santa Clara, CA) and used at a final concentration of 5 μg/ml. LPS (derived from *Escherichia coli* serotype 026-B6) was purchased from Sigma (St. Louis, MO). Recombinant murine TNF-α (specific activity 1.2 x 10^7 U/ml)

was provided by Genentech, Inc. (San Francisco, CA) and was acquired from Sigma (specific activity 3 x 10^6 U/ml). TNF-α was used at a final concentration of 1 to 1600 U/ml. TGF-β_1 was provided by Genentech, Inc. (San Francisco, CA). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; specific activity 4 x 10^7 U/ml) was provided by Immunex (Seattle, WA). Recombinant murine interleukin-1 (IL-1) and interleukin-6 (IL-6) were acquired from R & D Systems (Minneapolis, MN). Recombinant murine interleukin-2 (IL-2; specific activity 2 x 10^5 U/ml) was provided by Dr. R.S. Selvan (Duke University, Durham, NC). Indomethacin (Sigma) stock solutions were reconstituted in 70% ethanol and used at a final concentration of 0.1 μM. Sodium nitroprusside (Sigma) was used at a final concentration of 5.0 mM.

METH-KDE Cell Proliferation Assays

METH-KDE cells were seeded in 96-well flat-bottom microculture plates (Corning Glass, Corning, NY) at a final concentration of 5 x 10^5 cells/ml. Final well volumes were 0.2 ml. Plates were incubated, and 18 h Eighteen h before harvest, all wells were pulsed with 1 μCi/well tritiated thymidine (^{3}H-TdR; specific activity 6.7 Ci/mM; Dupont NEN Research Products, Wilmington, DE). Cells were harvested onto glass fiber filters using a Basic96 cell harvester (Skatron, Sterlin, VA), and sample activities were determined using a Beckman LS 6000SC scintillation counter (Beckman, Fullerton, CA).

METH-KDE Cell Viability Assays

METH-KDE cells were seeded in 96-well flat-bottom or round-bottom microculture plates (Corning Glass, Corning, NY) at a final concentration of either 5 x 10^5 or 2.5 x 10^5 cells/ml. Final well volumes were 0.2 ml. Plates were incubated, and 4 h before harvest, all wells were pulsed with 0.02 ml Alamar Blue (Alamar, Sacramento, CA). Changes in sample absorbance were determined in a MicroELISA titer plate reader (Molecular Devices, Menlo Park, CA) using a 570 nm test wavelength and a 600 nm reference wavelength.

METH-KDE Cell Cytotoxicity Assays

METH-KDE cells were cultured as described during viability assays except that METH-KDE cells were seeded in the presence of 10 nM actinomycin D. Unless specified, cytotoxicity was assessed 48 h after the initiation of the assay.

X-irradiation of METH-KDE Cells

METH-KDE cells were X-irradiated using a TFI Minishot II tabletop X-irradiator (TFI, New Haven, CT).

Morphological Characterization of METH-KDE Cells

All photographs were taken using an Olympus OM-2 35mm camera and an Olympus IMT-2 inverted research microscope. Unless specified, magnification was 600x.

Nitric Oxide Assays

Because nitric oxide (NO) has a very short half-life and quickly reacts with oxygen-yielding nitrite (309), nitrite levels in culture supernatants were measured using the Griess reagent (196). Briefly, METH-KDE cells were seeded in 96-well flat-bottom microculture plates at a final concentration of 5 x 10^5 cells/ml. Final well volumes were 0.2 ml. Plates were incubated until supernatants were collected by centrifugation. Supernatant samples (0.1 ml) were mixed with 0.1 ml Griess reagent (0.1% naphthylenediamine dihydrochloride; 1% sulfanilamide; 2.5% H_3PO_4) in 96-well flat-bottom microculture plates. Plates were incubated at room temperature for 10 min and A_{570} was read on an MR 600 microplate absorbance reader (Dynatech Laboratories, Alexandria, VA). Nitrite concentration was determined by comparison to a 0.1 M $NaNO_2$ calibration curve.

Statistics and Calculations of Results

All samples were run in triplicate and analyzed for significance using the Student's *t* test ($p < 0.05$). All data are presented as means ± standard errors. All experiments were performed three to five times; representative experiments are shown.

RESULTS

Taxol decreases METH-KDE cell proliferation

Our initial studies determined that taxol compromised METH-KDE cell proliferation. METH-KDE cells were cultured in the absence or presence of taxol and proliferation was assessed kinetically by ^{3}H-TdR incorporation (Figure 51). Lipopolysaccharide (LPS) was used as a negative control in these experiments because LPS can bind to β-tubulin but does not promote irreversible polymerization of microtubules. At all doses and time points, taxol significantly suppressed METH-KDE proliferation (Figure 51A). In contrast, LPS significantly promoted tumor cell division (Figure 51B). These data show that taxol acts cytostatically against METH-KDE cells.

Taxol-mediated suppression of METH-KDE cell proliferation is not dependent upon exposure time

Although taxol can suppress cell proliferation, some immortalized cell lines have variable resistance to taxol. To verify that the long-term suppressive activity of taxol was not an artifact of continuous culture either by the oversaturation of cultures or by the release of taxol by efflux or cell death, we repeated these experiments using METH-KDE cells that were pre-treated with taxol for 4 h. Excess taxol was removed from the cells by two washes, and the resulting cell preparations were cultured under conditions identical to our continuous exposure experiments (Figure 52). These data

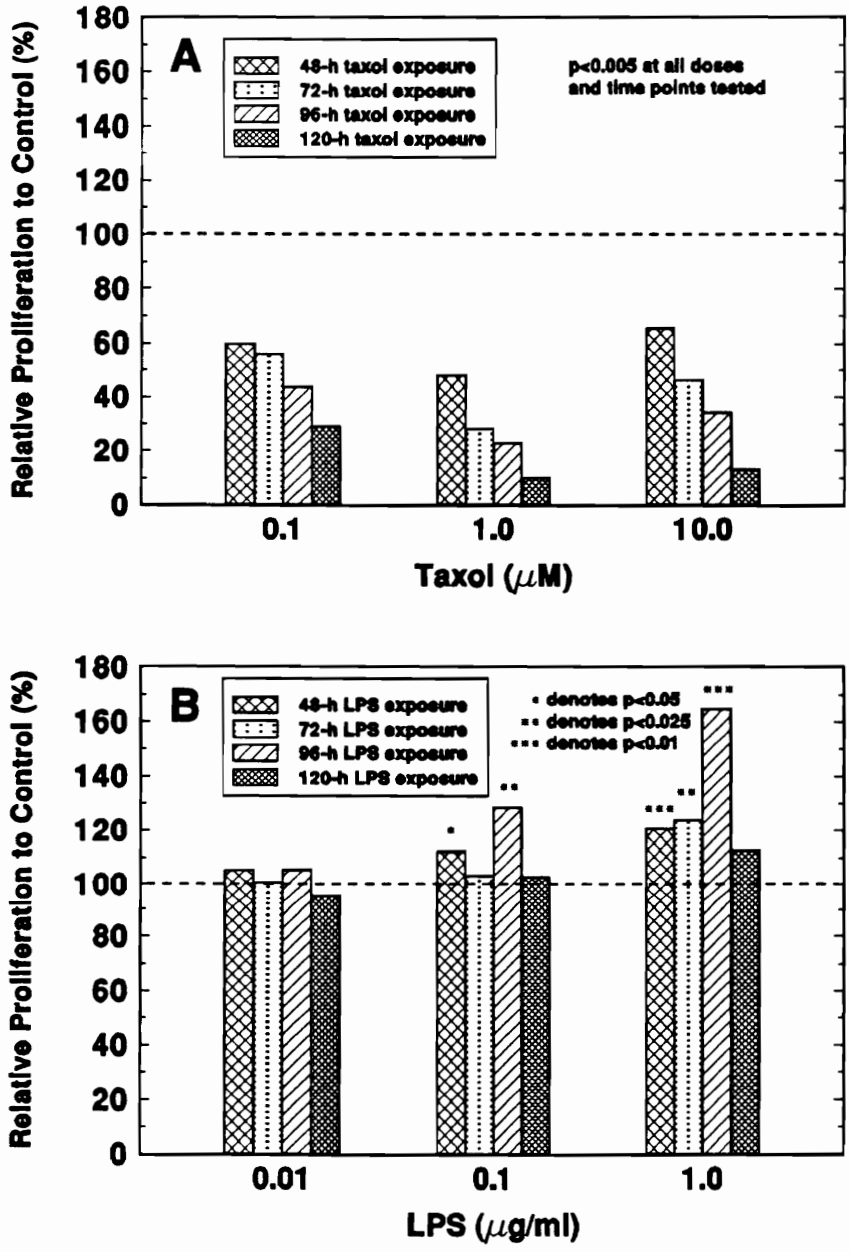

Figure 51. Taxol suppresses METH-KDE cell proliferation: METH-KDE cells (5 x 10^5/ml) were cultured in the absence or presence of 0.1, 1.0, and 10.0 μM taxol (panel A) or 0.01, 0.1, and 1.0 μg/ml LPS (panel B). Proliferation was assessed by ^{3}H-TdR incorporation. Data are expressed as percentage proliferation relative to control (dotted line).

parallel the continuous exposure data and suggest that temporary 4-h pre-treatment of METH-KDE cells mimics the suppressive activity of taxol under continuous exposure conditions.

Taxol decreases METH-KDE cell viability

To determine whether taxol alters METH-KDE cell viability during culture, cells were cultured in the absence or presence of taxol and viability was assessed kinetically using the indicator dye Alamar Blue. When Alamar Blue was added 3 h post-culture initiation, all samples had equal absorbances at 24 and 48 h (data not shown). These observations confirmed that equal concentrations of METH-KDE cells were present at the initiation of the assay and that the viabilities of control METH-KDE groups and taxol-treated METH-KDE groups were equal soon after taxol exposure. Changes in viability over time were masked because the METH-KDE cells reduced the Alamar Blue before significant cell death occurred. Differences in cell viability that occurred later in culture were obscured by the initial irreversible redox reaction. To circumvent this problem, Alamar Blue was added to parallel cultures 4 h before analyses (24 and 48 h). When this approach was used, significant dose-dependent decreases in absorbance for taxol-treated groups were observed (Figure 53). These data suggest that taxol decreases METH-KDE cell viability and that taxol may be directly cytotoxic to tumor cells.

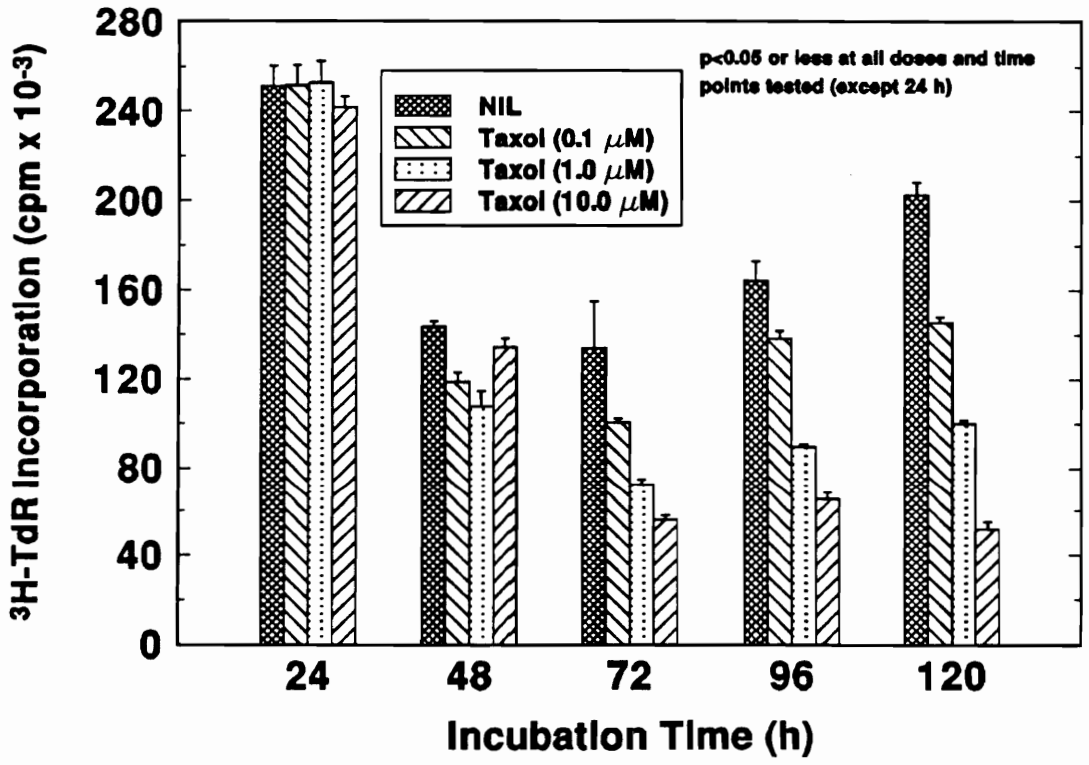

Figure 52. Taxol-mediated suppression of METH-KDE cell proliferation does not require continuous exposure: METH-KDE cells (5 x 10^5/ml) were pre-cultured for 4 h in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and cells were washed twice to remove unincorporated taxol. Proliferation was assessed by ^{3}H-TdR incorporation.

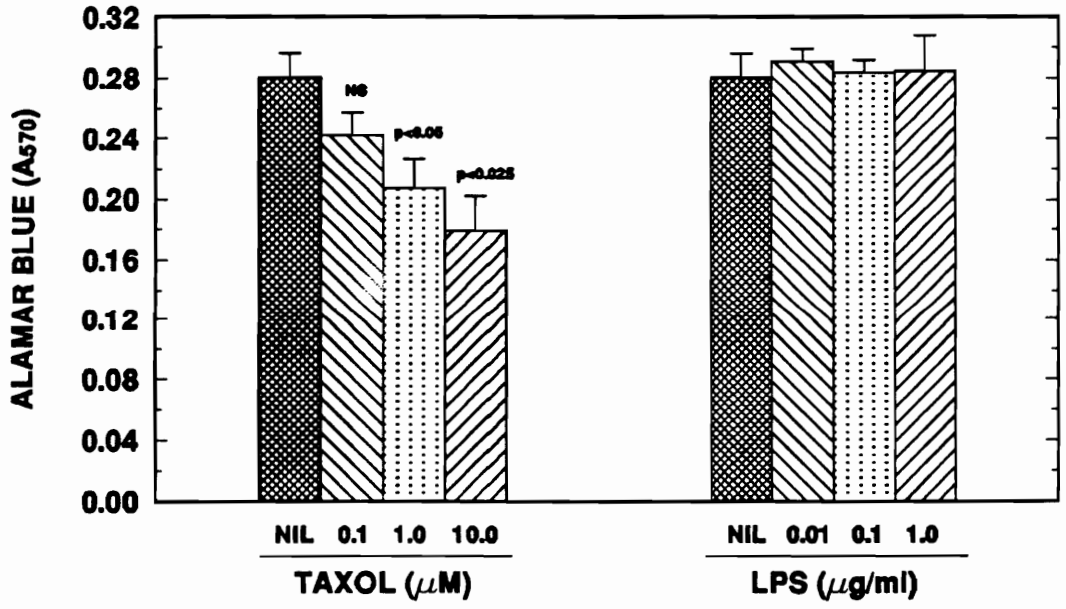

Figure 53. Taxol decreases METH-KDE cell viability: METH-KDE cells (2.5 x 10^5/ml) were cultured in the absence or presence of 0.1, 1.0, and 10.0 μM taxol or 0.01, 0.1, and 1.0 μg/ml LPS. Viability was assessed after 48 h by Alamar Blue reduction. Similar data was acquired at 72 and 96 h (not shown). Statistics represent comparisons of treatment groups to untreated controls.

Taxol increases METH-KDE cell radiosensitivity

Others show that taxol acts as a radiosensitizing agent on some tumor cell types (277, 304). To determine whether taxol increases METH-KDE cell sensitivity to X-irradiation, METH-KDE cells were cultured for 24 h in the absence or presence of taxol and subsequently exposed to various doses of radiation (Table 15). Proliferation was measursed 96 h post-X-irradiation. At all doses tested, taxol significantly increased the radiosensitivity of METH-KDE cells. These data suggest that taxol potentiates the anti-proliferative effects of X-irradiation on METH-KDE cells.

Taxol alters METH-KDE cell regulation of TGF-β_1

Previous work shows that METH-KDE cells synthesize high amounts of PGE_2 and TGF-β_1 (10). After 4 days in culture, the medium is acidic and contains high concentrations of TGF-β_1 (8.5 $\pm$ 0.5 ng/ml) and PGE_2 (27.3 $\pm$ 2.1 ng/ml). These factors autocrinely regulate tumor cell growth and proliferation. We hypothesized that taxol-specific cytotoxicity against METH-KDE cells may involve alterations in the autocrine pathways associated with PGE_2 and TGF-β_1. To investigate this possibility, METH-KDE cells were cultured in the absence or presence of taxol and either indomethacin (an arachidonic acid pathway inhibitor) or neutralizing mAb against TGF-β_1. Interestingly, neither indomethacin (Figure 54) nor anti-TGF-β_1 mAb (Figure 55) treatment alone significantly altered METH-KDE proliferation. Anti-TGF-β_1 mAb suppressed METH-KDE cell proliferation only when the cells were pre-treated with high concentrations of taxol (10 μM). Exogenous TGF-β_1 significantly increased METH-KDE cell proliferation but significantly suppressed METH-KDE cell

Table 15. Taxol acts as a radiosensitization agent for METH-KDE cells

	RAD exposure			
Taxol[a]	0	500	1000	2000
Nil	118.08 ± 4.88[b]	20.8 ± 1.66	13.4 ± 1.07	11.16 ± 0.58
0.1	95.86 ± 3.77 (81.2)[c]	4.74 ± 0.29 (22.7)	2.72 ± 0.12 (20.1)	2.13 ± 0.04 (19.0)
1.0	34.04 ± 0.8 (28.8)	4.05 ± 0.09 (19.3)	2.93 ± 0.06 (21.7)	2.08 ± 0.13 (18.5)
10.0	5.01 ± 0.23 (4.2)	1.32 ± 0.0 (6.3)	1.46 ± 0.12 (10.7)	1.59 ± 0.07 (14.2)

[a]METH-KDE cells (5 x 10^5 cells/ml) were cultured in the absence or presence of taxol (0.1, 1.0, or 10.0 μM) for 24 h, washed twice, and X-irradiated. Cells were seeded at 5 x10^5 cells/ml and proliferation was assessed after 96 h by ^{3}H-TdR incorporation.

[b]Values are expressed as cpm x 10^{-3} ± SE. $P < 0.005$ at all taxol doses tested in comparison to non-taxol pre-treatment controls.

[c]Numbers in parentheses represent relative percentage decrease in proliferation with respect to non-taxol pre-treated controls.

proliferation in the presence of taxol (Figure 56). To determine if taxol disrupts regulation of other potential autocrine growth factors, METH-KDE cells were cultured in the absence or presence of GM-CSF, IL-1, IL-2, and IL-6. Several tumor cell types have been identified that produce and use these cytokines as autocrine growth factors (121, 134, 160, 205, 346, 365, 426). However, both METH-KDE cell proliferation (Table 16) and viability (Table 17) were unaffected by these cytokines and these signals did not significantly alter METH-KDE cell sensitivity to taxol. Collectively, these data suggest that taxol disrupts METH-KDE cell autocrine regulation of TGF-β_1.

Taxol increases METH-KDE cell sensitivity to TNF-α

Data from our laboratory and others suggest that taxol-mediated tumor regression may occur through two linked mechanisms in which: (i) taxol induces Mϕ expression of the lytic cytokine TNF-α (290) and (ii) taxol increases tumor cell susceptibility to lysis by TNF-α. To elucidate whether taxol increases or decreases METH-KDE sensitivity to lysis by TNF-α, METH-KDE cells were cultured in the absence or presence of TNF-α and proliferation was assessed by ^{3}H-TdR incorporation (Figure 57). Initial studies using ^{3}H-TdR uptake suggested that TNF-α-mediated lysis was partly or completely masked if METH-KDE cells were permitted to proliferate during TNF-α exposure (Figure 57A). Therefore, cytotoxicity was assessed using a modification of the Alamar Blue reduction assay. METH-KDE cells were cultured with 10 nM actinomycin D which completely inhibited proliferation but did not compromise viability during the 48-h assay. Under these experimental conditions, TNF-α mediated significant dose-dependent decreases in viability (Figure 57B). Decreased viability was confirmed further by microscopic inspection of the cultures (Figure 58). No sig-

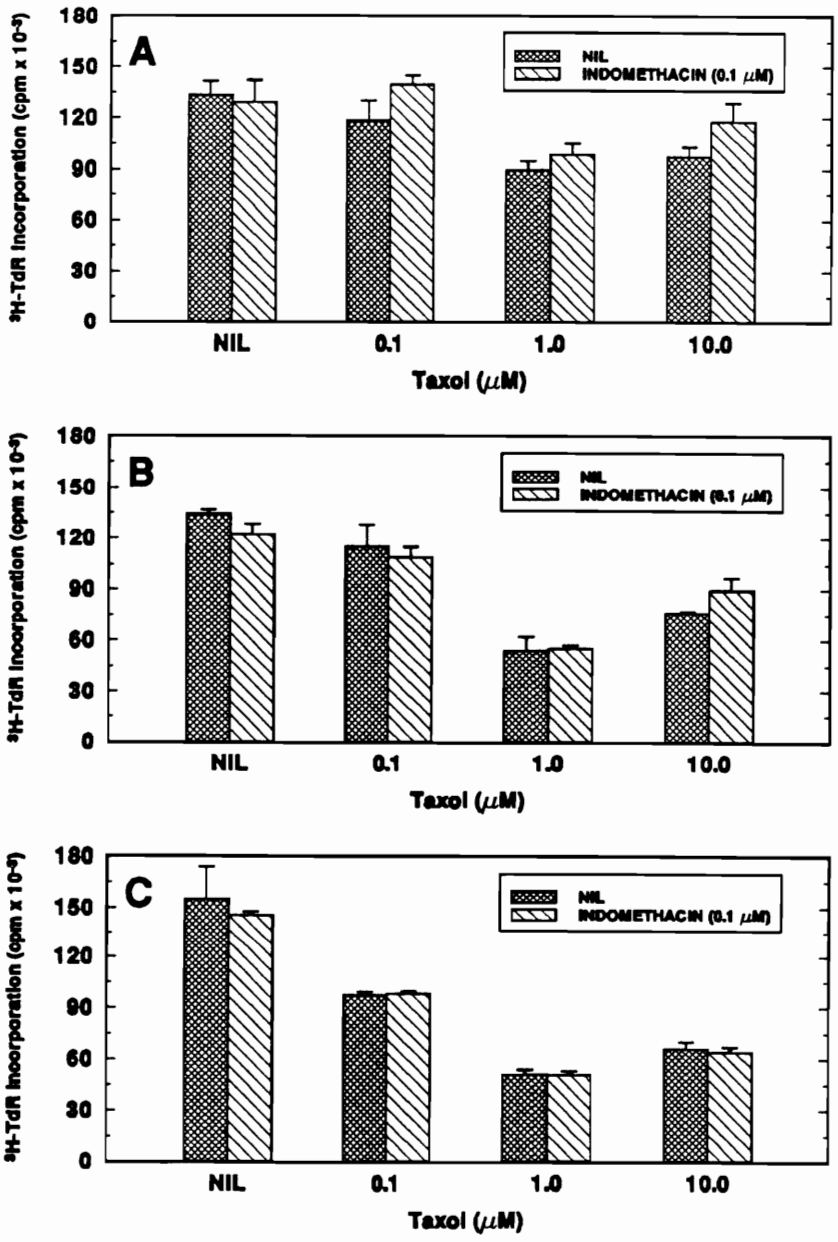

Figure 54. PGE_2 is not required as an autocrine factor for METH-KDE cells and is not involved during taxol-mediated suppression: METH-KDE cells (5 x 10^5/ml) were cultured in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and in the absence or presence of the arachidonic acid pathway inhibitor indomethacin (0.1 μM) for 48 h (panel A), 72 h (panel B), and 96 h (panel C). Proliferation was assessed kinetically by ^{3}H-TdR incorporation.

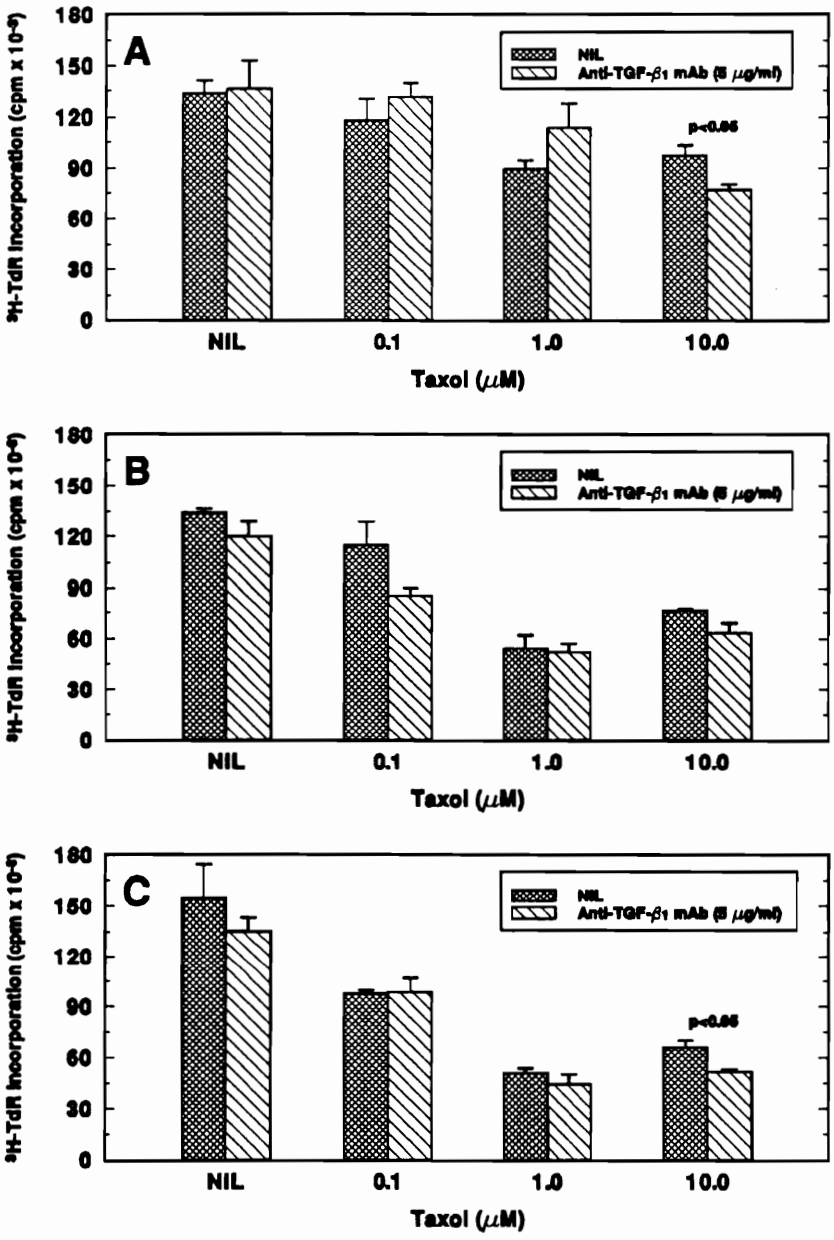

Figure 55. TGF-β, may be required as an autocrine factor for METH-KDE cells: METH-KDE cells (5 x 10^5/ml) were cultured in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and in the absence or presence of anti-TGF-β, mAb (5 μg/ml) for 48 h (panel A), 72 h (panel B), and 96 h (panel C). Proliferation was assessed kinetically by ^{3}H-TdR incorporation. Anti-TGF-β, mAb was generously provided by Celtrix.

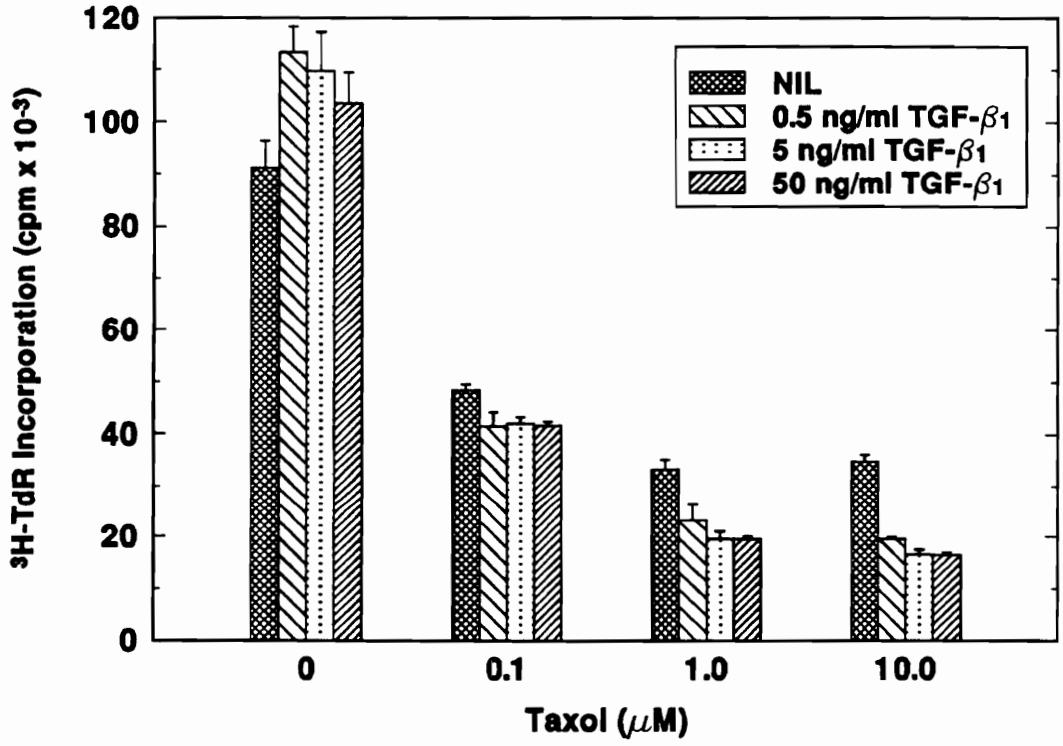

Figure 56. TGF-β_1 is a METH-KDE cell growth factor and responsiveness to TGF-β_1 is disrupted by taxol: METH-KDE cells (5 x 10^5/ml) were cultured in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and in the absence or presence of 0.5, 5.0, or 50.0 ng/ml TGF-β_1. Proliferation was assessed kinetically by ^{3}H-TdR incorporation. TGF-β_1 was generously provided by Genentech, Inc..

Table 16. METH-KDE cell proliferation is not affected by GM-CSF, IL-1, IL-2, or IL-6

Growth Factor[a]	Taxol (μM)			
	Nil	0.1	1.0	10.0
Nil	85.54 ± 3.26[b]	33.86 ± 0.28	9.49 ± 0.49	5.16 ± 0.45
GM-CSF (500 U/ml)	79.49 ± 9.77	37.23 ± 0.68	10.81 ± 0.46	6.54 ± 0.16
IL-1 (200 pg/ml)	101.58 ± 6.33	42.85 ± 0.55	8.00 ± 0.64	5.29 ± 0.51
IL-2 (500 U/ml)	85.64 ± 5.36	37.61 ± 0.73	10.35 ± 0.71	6.7 ± 0.18
IL-6 (4 ng/ml)	85.25 ± 1.71	34.98 ± 2.12	9.07 ± 0.45	5.77 ± 0.23

[a]METH-KDE cells (5 x 10^3 cells/ml) were cultured in the absence or presence of taxol (0.1, 1.0, or 10.0 μM) and growth factors. Proliferation was assessed after 96 h by ^{3}H-TdR incorporation.

[b]Values are expressed as cpm x 10^{-3} ± SE.

Table 17. METH-KDE cell viability is not affected by GM-CSF, IL-1, IL-2, or IL-6

Growth Factor[a]	Taxol (μM)			
	Nil	0.1	1.0	10.0
Nil	0.535 ± 0.005[b]	0.473 ± 0.036	0.32 ± 0.023	0.376 ± 0.009
GM-CSF (500 U/ml)	0.454 ± 0.029	0.505 ± 0.044	0.319 ± 0.012	0.347 ± 0.006
IL-1 (200 pg/ml)	0.485 ± 0.025	0.553 ± 0.083	0.326 ± 0.036	0.277 ± 0.021
IL-2 (500 U/ml)	0.502 ± 0.009	0.544 ± 0.019	0.381 ± 0.02	0.369 ± 0.03
IL-6 (4 ng/ml)	0.531 ± 0.017	0.41 ± 0.019	0.276 ± 0.036	0.358 ± 0.019

[a]METH-KDE cells (5 x 10^3 cells/ml) were cultured in the absence or presence of taxol (0.1, 1.0, or 10.0 μM) and growth factors. Viability was assessed after 96 h by Alamar Blue reduction.

[b]Values are expressed as A_{570} ± SE.

nificant decreases in viability or alterations in cell morphology were observed when METH-KDE cells were exposed to taxol in the presence of actinomycin D. (Figure 59). METH-KDE cells cultured with actinomycin D, TNF-α, and taxol showed significantly lower viability than did METH-KDE cells cultured with actinomycin D and TNF-α. High doses of TNF-α (that are physiologically expected from taxol-activated Mϕ) suppressed proliferation (Figure 59A) and killed METH-KDE cells (Figure 59B) only in the presence of taxol. Furthermore, TGF-β_1 pre-treatment significantly increased METH-KDE cell sensitivity to taxol and TNF-α (Table 18). Collectively, these data suggest that taxol is cytotoxic to cells committed within the cell cycle, but that taxol-mediated increases in METH-KDE cell sensitivity to TNF-α occur irrespectively of events associated with the cell cycle. Furthermore, these findings implicate that taxol-induced METH-KDE cell sensitivity to TNF-α may involve an apoptotic mechanism.

Taxol alters METH-KDE cell morphology

Because our data suggest that taxol mediates both cytostatic and cytolytic activities against METH-KDE cells through an apoptotic mechanism, we speculated that visual inspection of METH-KDE cells might clarify the severity of cytotoxicity (Figure 60). During cell passage, METH-KDE cells are generally large (20-40 μm) and gradually shift in morphology over a few days from fibroblast-like (Figure 60A) to ameboid (Figure 60B) to spherical (Figure 60C). METH-KDE cells proliferate rapidly and form easily discernible blasts that detach from the floor of the culture flask (Figure 60D). Under microculture conditions, METH-KDE cells (2.5×10^5/ml) were cultured in the absence or presence of taxol for 48 h and were photographed (Figure 61).

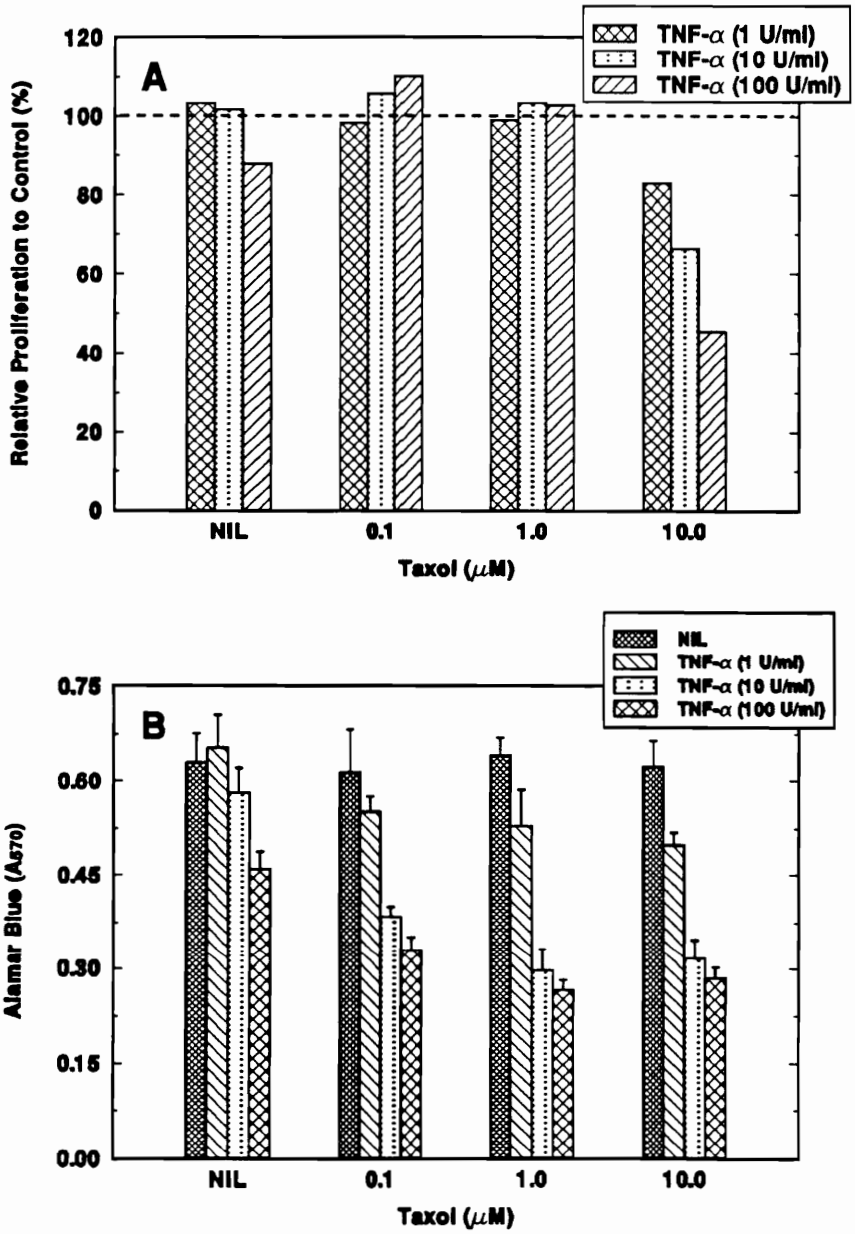

Figure 57. Taxol and TNF-α synergize to kill METH-KDE cells: METH-KDE cells (5 x 10^5/ml) were cultured for 48 h with actinomycin D (10 nM) in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and in the absence or presence of 1, 10, and 100 U/ml TNF-α. Proliferation was assessed by ^{3}H-TdR incorporation (panel A). Data are expressed as percentage proliferation relative to control (dotted line). Viability was assessed by Alamar Blue reduction (panel B).

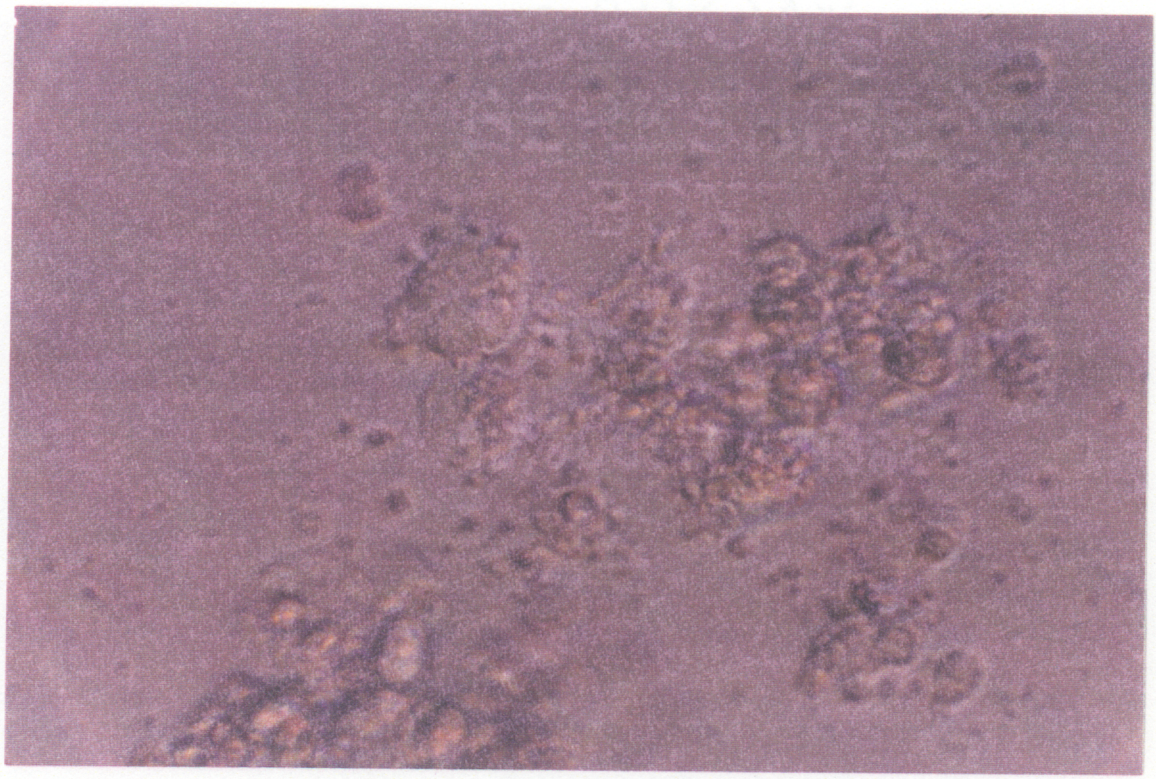

Figure 58. Taxol and TNF-α kill METH-KDE cells: When METH-KDE cells were cultured for 48 h with actinomycin D in the presence of 10.0 μM taxol and 100 U/ml TNF-α, the majority of tumor cells were small and possessed ruffled edges and rough surfaces. Cells with identical morphologies in parallel cultures were non-viable. Significant amounts of cytosolic and membrane debris were present.

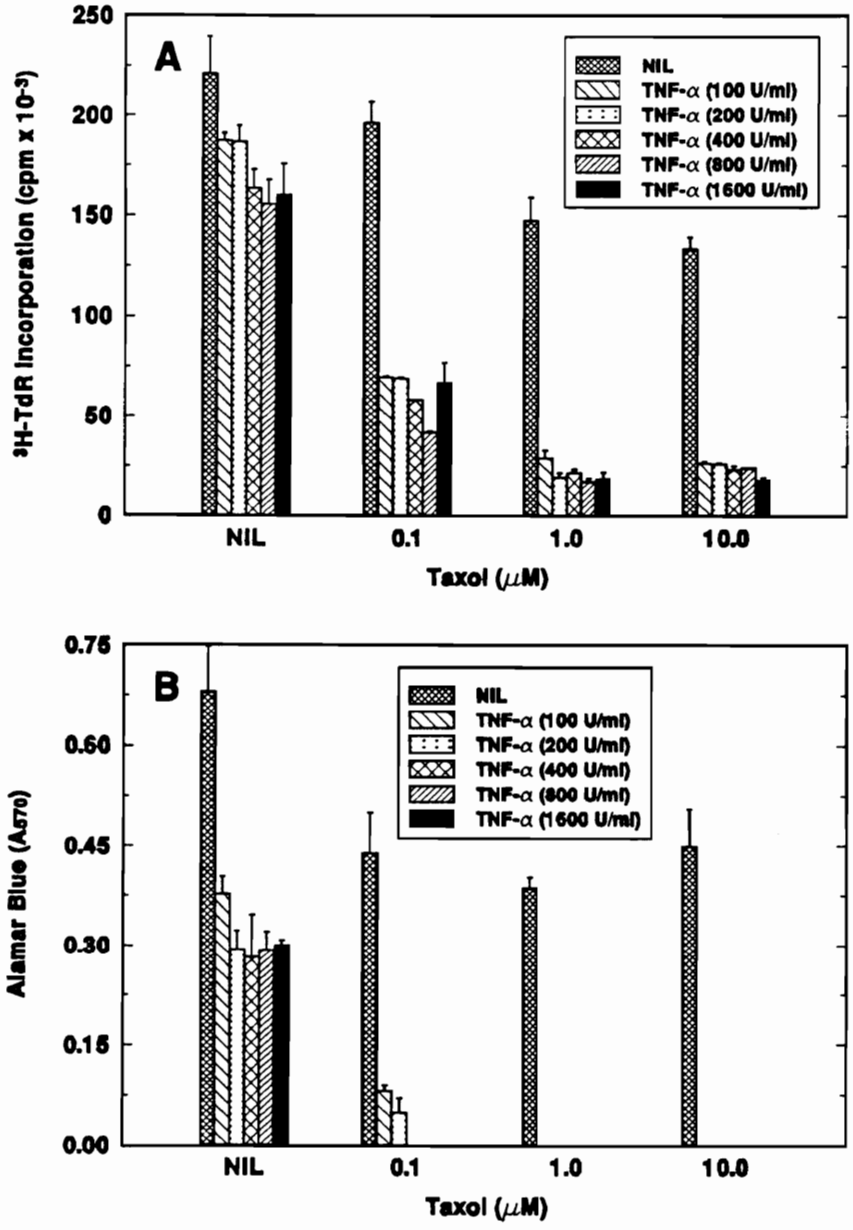

Figure 59. Physiological concentrations of TNF-α kill METH-KDE cells in the presence of taxol: METH-KDE cells (5 x 10^5/ml) were cultured for 48 h with actinomycin D (10 nM) in the absence or presence of 0.1, 1.0, or 10.0 μM taxol and in the absence or presence of 100, 200, 400, 800, or 1600 U/ml TNF-α. Proliferation was assessed kinetically by ^{3}H-TdR incorporation (panel A) and viability was assessed by Alamar Blue reduction (panel B).

Table 18. TGF-β_1 increases METH-KDE cell sensitivity to taxol and TNF-α

Taxol (μM)	TNF-α (U/ml)	TGF-β_1 Pre-Treatment (ng/ml)[a]		
		Nil	0.5	5.0
Nil	Nil	0.442 ± 0.033	0.368 ± 0.017	0.206 ± 0.026[e]
0.1	Nil	0.475 ± 0.034	0.335 ± 0.024[c]	0.132 ±0.022[e]
Nil	100	0.387 ± 0.017	0.234 ± 0.024[d]	0.105 ± 0.028[e]
0.1	100	0.317 ± 0.004	0.094 ± 0.006[e]	0.012 ± 0.011[e]

[a]METH-KDE cells (5 x 10^5 cells/ml) were cultured in the absence or presence of TGF-β_1 for 24 h, washed twice, and reseeded (5 x10^5cells/ml) in the absence or presence of taxol, TNF-α, or both. Viability was assessed after 48 h by Alamar Blue reduction.

[b]Values are expressed as A_{570} ± SE.

[c]Indicates $p < 0.05$ in comparison to non-TGF-β_1 pre-treatment control.

[d]Indicates $p < 0.01$ in comparison to non-TGF-β_1 pre-treatment control.

[e]Indicates $p < 0.005$ in comparison to non-TGF-β_1 pre-treatment control.

Taxol-treated METH-KDE cells (Figure 61A-C) were significantly smaller than untreated control cultures (Figure 61D). These changes were dose-dependent. Many cells appeared to possess ruffled edges and rough surfaces. Some taxol-treated cells demonstrated membrane "blebs" which represent one diagnostic observation of apoptosis. Few blasts were seen. X-irradiated cultures served as anti-proliferation controls (Figure 61E). Although the radiation dose used (2000 rads) is lethal and can completely abrogate cell division, X-irradiated cells do not demonstrate significant degradation until 72 hours after exposure. The number and general morphologies of METH-KDE cells in X-irradiated groups (Figure 61E) differ very little from the beginning to the end of the assay. These morphological data suggest that taxol is cytotoxic to METH-KDE cells and that high concentrations of taxol induce programmed cell death.

Taxol induces METH-KDE cell apoptosis through a NO-independent mechanism

NO is a degradative reactive nitrogen intermediate molecule produced by some cell types during apoptosis (89). Others suggest that tumor cell sensitivity to TNF-α involves induction of NO synthase, and that optimal expression of NO synthase requires IFN-γ (275). To determine if taxol induced fibrosarcoma cell production of NO, tumor cells were cultured in the absence or presence of taxol and NO production was measured by the Griess reaction (Figure 62). Interestingly, METH-KDE cells treated with taxol, TNF-α, or both did not produce NO (Figure 62A). Parallel cell cultures demonstrated significant decreases in viability when they were treated with both taxol and TNF-α (Figure 62B). METH-KDE cells treated with the combination of taxol,

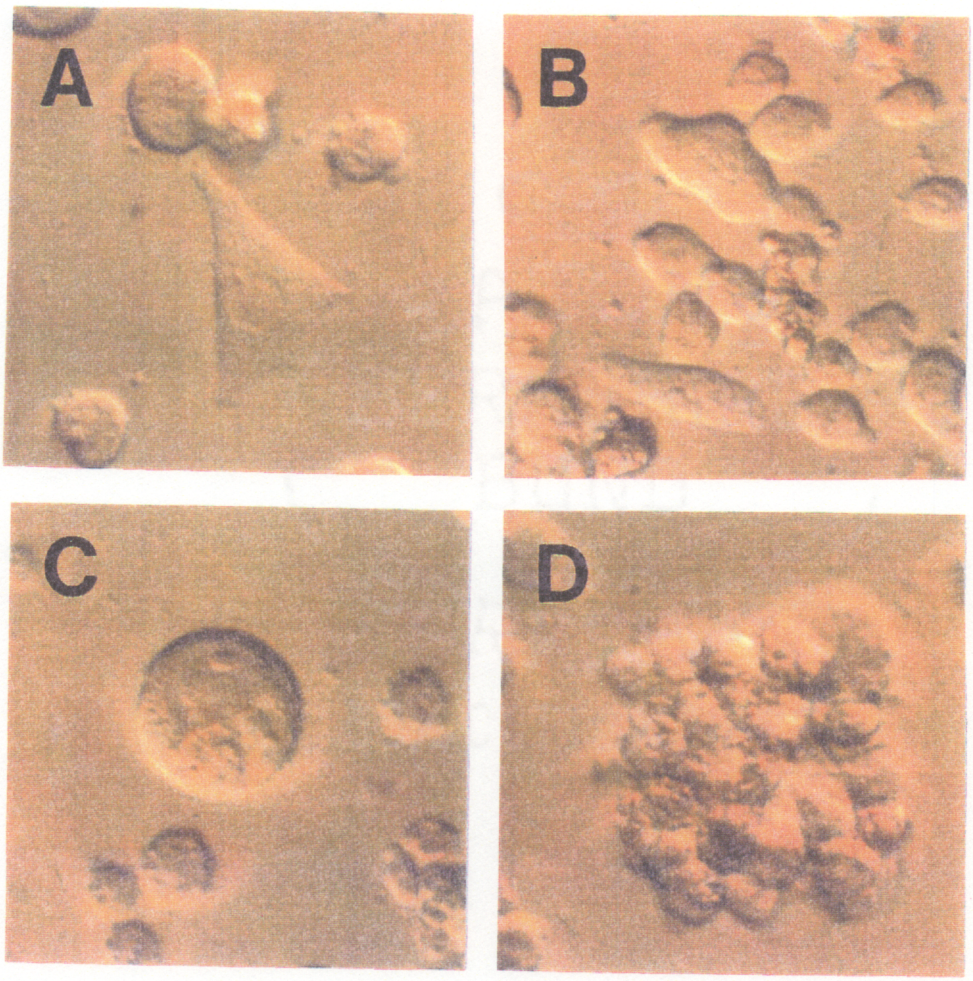

Figure 60. Morphological appearance of METH-KDE fibrosarcoma cells during culture: At the initiation of cultures using low cell densities, the majority of METH-KDE cells were adherent. These cells gradually shifted in morphology over a few days from fibroblast-like (panel A) to ameboid (panel B) to spherical (panel C). METH-KDE cells proliferated rapidly and also formed easily discernible blasts that detached from the floor of the culture well (panel D). The majority of cells were non-adherent and were clustered as large detached colonies. Viabilities of 2, 3, and 4-day cultures were >95%.

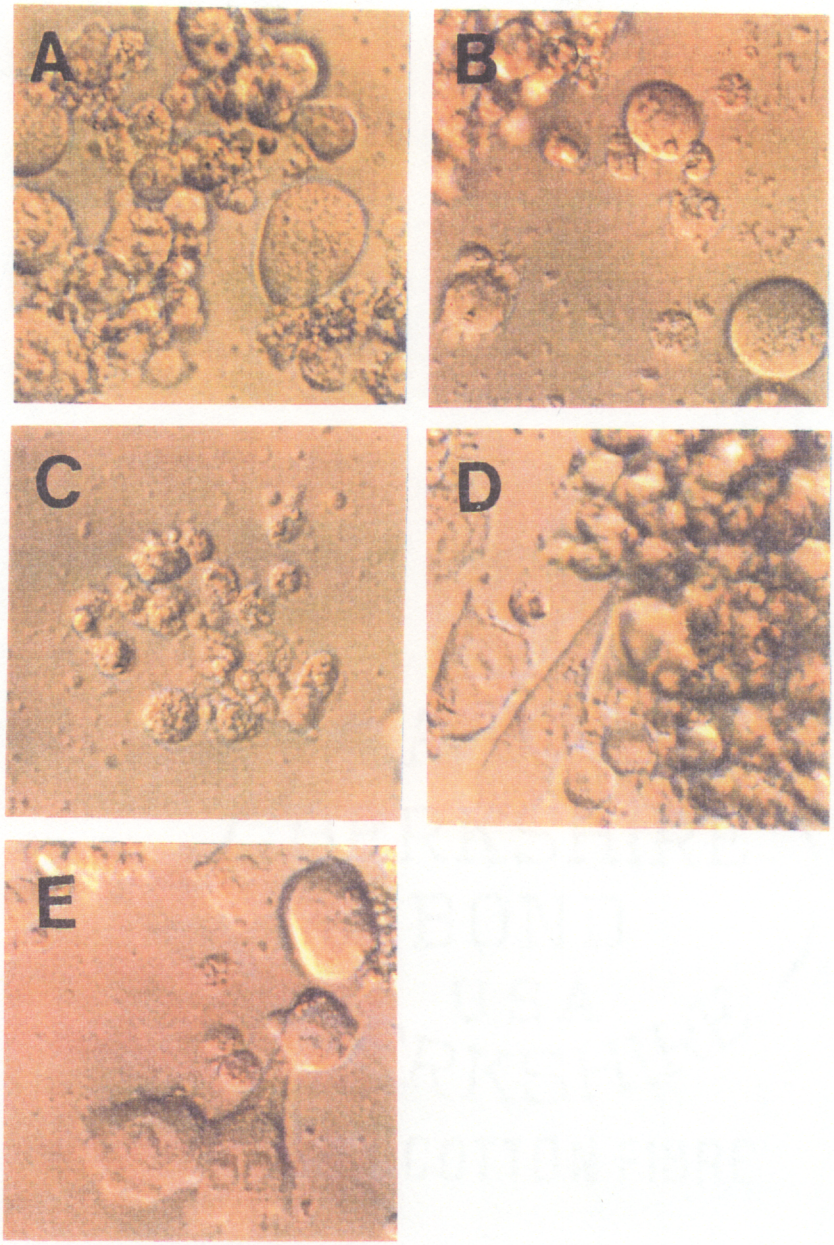

Figure 61. Morphological appearance of METH-KDE fibrosarcoma cells during exposure to taxol: When METH-KDE cells (5 x 10^5 cells/ml) were cultured with 0.1 μM taxol (panel A), most cells appeared large and round but some cells were small and had ruffled edges and rough surfaces. Significant amounts of cytosolic and membrane debris were present. With 1.0 μM taxol (panel B), approximately half of the cells possessed ruffled edges and rough surfaces. Significant amounts of cytosolic and membrane debris were present. Several cells had membrane "blebs" that suggest taxol mediated cytotoxicity through an apoptotic mechanism. In the presence of 10.0 μM taxol (panel C), the majority of METH-KDE cells were small and possessed ruffled edges and rough surfaces. Cells with identical morphologies were non-viable. Significant amounts of cytosolic and membrane debris were present. Control cultures grown in the absence of taxol (panel D) highly viable and healthy. X-irradiated (2000 rads) METH-KDE cells (panel E) served as a negative proliferation control. METH-KDE cells were viable at 72-h post-irradiation but did not proliferate. The general density of 48-h post-irradiation cultures mirrors the appearance of non-X-irradiated cultures at the start of the assay. Most cells were adherent and pleiomorphic. The amount of cellular debris present in X-irradiated cultures was similar to the amount present in non-X-irradiated control cultures.

TNF-α, and IFN-γ produced low amounts of NO. NO expression was greatest when METH-KDE cells were treated with a combination of LPS, TNF-α, and IFN-γ. Parallel cultures had low viabilities. When cultures were supplemented with sodium nitroprusside (an exogenous donator of NO during culture), METH-KDE cells had membrane blebs, decreased viability, and significant DNA fragmentation (data not shown). Although NO production is associated with apoptosis in some cell types, these data suggest that taxol-mediated apoptosis occurs through a NO-independent mechanism.

METH-KDE cells potentially recover from taxol-induced suppression through a cellular efflux mechanism

Data suggest that taxol significantly suppressed METH-KDE cell proliferation irrespective of exposure time (continuous exposure versus 4-h pre-treatment). However, some tumor cell lines can recover from taxol-induced suppression (369). Based on these studies, we speculated that METH-KDE cell efflux of taxol (or even taxol release from dead cells) could artificially create continuous exposure conditions. To remove taxol that might have accumulated in the wells as a result of cellular efflux or cell death, METH-KDE cells that were pre-treated with taxol for 4 h were washed twice and reconstituted in fresh medium 48 h post-taxol treatment (Figure 63). This approach removed excreted or released taxol and eliminated potentially toxic cellular debris and metabolites that accumulated in the wells as a result of taxol-induced cytotoxicity. Cell proliferation (Figure 63A) and viability (Figure 63B) were measured 48 h after reconstitution. At all doses tested, only cultures pre-treated with high concentrations of taxol were suppressed. Similar data were acquired at 96-h post-

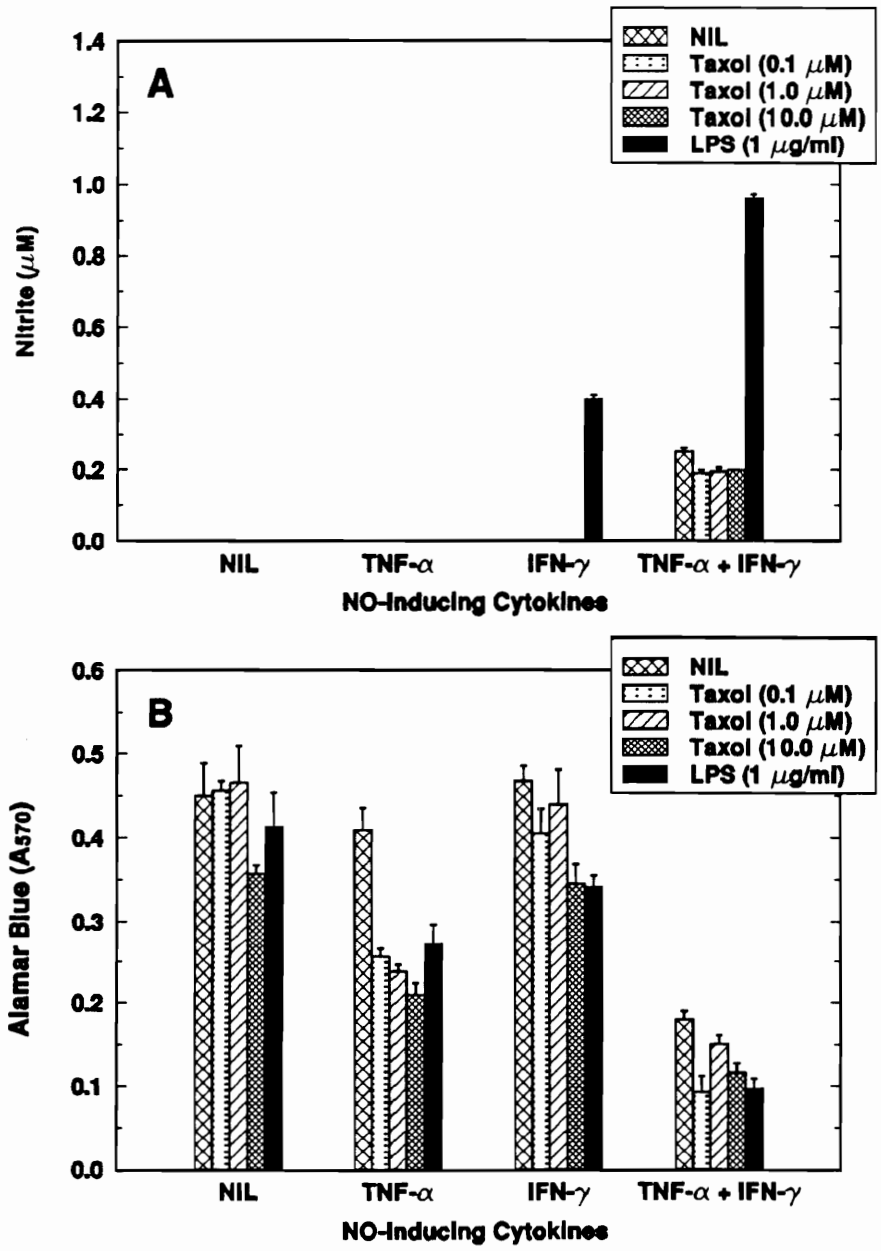

Figure 62. Taxol and TNF-α trigger METH-KDE cell apoptosis through a NO-independent mechanism: METH-KDE cells (5 x 10^5/ml) were cultured for 48 h in the absence or presence of TNF-α (100 U/ml), IFN-γ (250 U/ml), and either taxol (0.1, 1.0, and 10.0 μM) or LPS (1 μg/ml). NO production (panel A) was assessed by the Griess reagent test. Viability of parallel cultures (panel B) was determined by Alamar Blue reduction.

reconstitution (data not shown). These data suggest that partial taxol recovery during culture may occur through a cellular efflux mechanism.

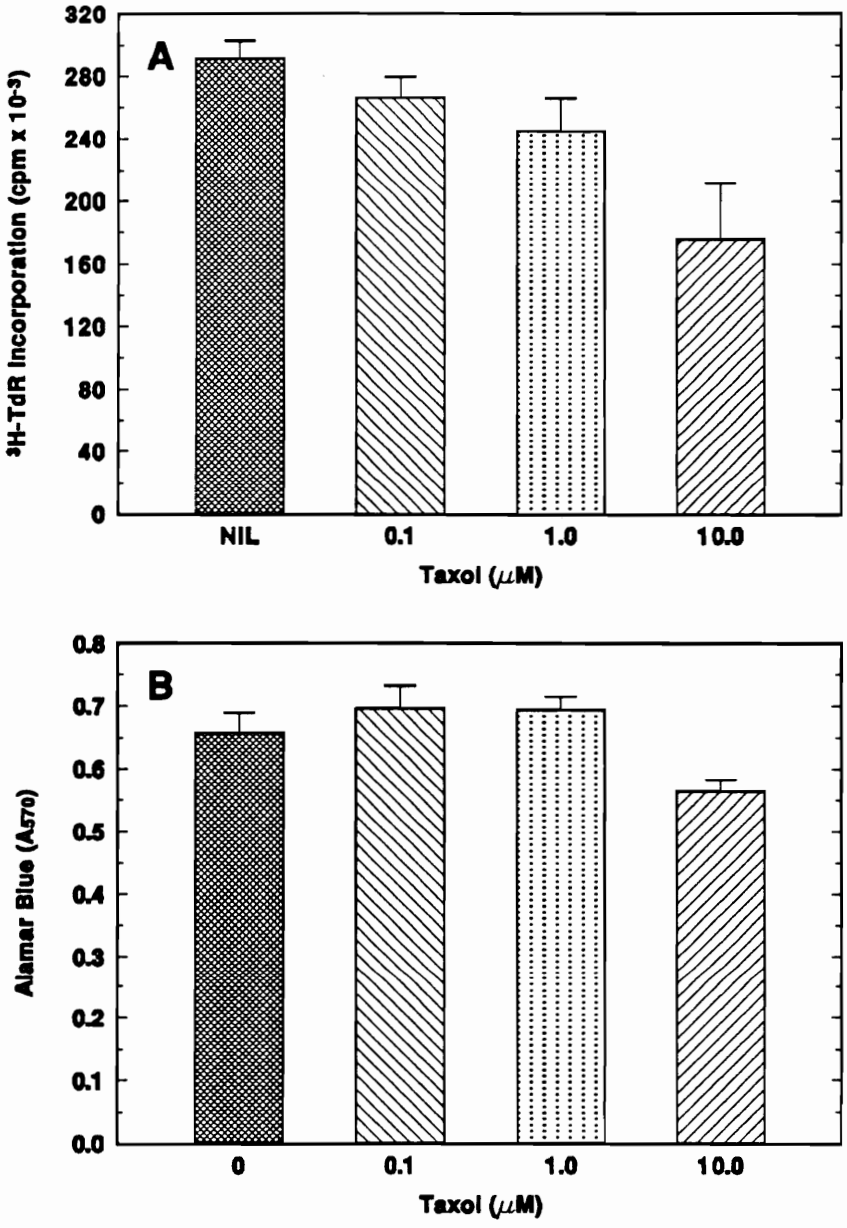

Figure 63. METH-KDE cells partly recover from taxol-mediated suppression: METH-KDE cells (5 x 10^5/ml) were pre-cultured for 4 h in the absence or presence of 0.1, 1.0, or 10.0 μM taxol before the initiation of the assay. Two days later, cultures were washed twice by centrifugation and re-cultured for 48 h in the presence of RPMI-1640 medium supplemented with 10% fetal bovine serum. Proliferation was assessed kinetically by ^{3}H-TdR incorporation (panel A). Tumor cell viability was assessed by Alamar Blue reduction (panel B).

DISCUSSION

Taxol is a diterpenoid compound extracted from the leaves and bark of the western yew (*Taxus brevifolia*) and has been touted by the National Cancer Institute as the most promising natural chemotherapeutic drug. The success of taxol during phase I clinical trials occurred because taxol both disrupts tumor growth and activates Mϕ anti-tumor effector functions. Using a murine cancer model, we have shown that fibrosarcoma growth significantly alters immune cell phenotype and function (10, 444, 449). Tumor-induced suppressor Mϕ populations significantly compromise immunocompetence, and our current investigations are determining whether taxol can reverse tumor-induced suppressor Mϕ activities by promoting Mϕ tumoricidal functions. We first wanted to confirm that taxol has direct cytostatic and cytotoxic activity against the fibrosarcoma cells used in our murine cancer model. The present study evaluated whether METH-KDE fibrosarcoma cells were sensitive to taxol. Our data suggest that METH-KDE fibrosarcoma cells are sensitive to taxol through multiple cytostatic and cytotoxic mechanisms.

Taxol acts cytostatically against numerous tumor cell types. Tumor cell proliferation is suppressed because taxol induces stable polymerization of microtubules (50, 97). Microtubules are required during cell division and undergo extensive restructuring during the cell cycle. Although cell division is completely blocked, flow cytometric analyses and ^{3}H-TdR incorporation studies show that taxol does not significantly disrupt events associated with the G_1 or S phases of the cell cycle (50). Others suggested that taxol-mediated cytotoxicity occurs during late stages of the cell cycle (114). Although taxol-mediated mitotic arrest does not affect DNA synthesis, cells remain locked in the G_2/M phase of the cell cycle (369). As predicted, the pro-

liferation of METH-KDE cells was decreased significantly by both continuous exposure to taxol (Figure 51A) and taxol pre-treatment (Figure 52). In contrast, LPS did not suppress METH-KDE cell proliferation (Figure 51B). LPS was used as a negative control because LPS can associate directly with microtubules without causing irreversible polymerization. Both LPS and taxol can bind to β-tubulin and interact intracellularly with microtubule-associated protein kinases (111), but we have not determined whether LPS induces transcription of fibrosarcoma cell growth factors. LPS acts as a growth-promoter in some tumor cell lines but most cell lines are unaffected or respond negatively.

Taxol reduced METH-KDE cell viability (Figure 53). Although our proliferation data suggested that taxol is tumoristatic, these data did not indicate if the decreases in proliferation were caused by decreases in cell viability. We used an Alamar Blue assay to determine if taxol decreased METH-KDE cell viability. Alamar Blue is a sensitive non-toxic indicator dye that can be used to assess cell viability quantitatively (5, 335). This approach is significantly more sensitive and less subjective than visual evaluation of viability using trypan blue exclusion. Taxol decreased METH-KDE cell viability in a dose-dependent manner whereas LPS did not change METH-KDE cell viability. Although the mechanisms through which taxol decreases cell viability are not clear, taxol-mediated decreases in cell viability probably involve dysregulation of autocrine growth factors and induction of an apoptotic pathway.

Taxol increased the radiosensitivity of METH-KDE cells (Table 15). Milas *et al.* suggest that increased radiosensitivity is related to the time between taxol administration and radiation exposure (304). A 24-h taxol pre-treatment appears to most significantly enhance radioresponses because it induces mitotic arrest. Mitosis is the most radiosensitive phase of the cell cycle, and subsequent radiation exposure kills the cells through apoptotic mechanisms. Although not all tumor cell types become

radiosensitized by taxol (277), our data suggest that taxol potentiates METH-KDE cell radiosensitivity.

Taxol partly disrupted autocrine regulation of TGF-β_1 (Figures 55-56) but did not alter autocrine regulation of PGE_2 (Figure 54). Our previous studies (10) suggest that fibrosarcoma cells synthesize several molecules that autocrinely regulate cell growth and proliferation. We showed that METH-KDE cells produce significantly high amounts of PGE_2 and TGF-β_1 (10). Tumor-derived PGE_2 promotes tumor growth by serving as an intermediate signal in an elaborate cascade of intracellular signals that enhance progression through the cell cycle (155). PGE_2 locally suppresses tumor-infiltrating Mϕ and T-cell activity (155). Tumor-derived TGF-β_1 similarly promotes tumor growth and inhibits immune cell activity but through different mechanisms (292). Although we have only partly identified the regulatory significance of PGE_2 and TGF-β_1, these two signals are important tumor-derived mediators that both promote fibrosarcoma growth and disrupt immune cell function. Interestingly, neither indomethacin (Figure 54) nor anti-TGF-β_1 mAb (Figure 55) treatment alone significantly altered METH-KDE cell proliferation. Although the selected concentrations of indomethacin and anti-TGF-β_1 should have neutralized most endogenous PGE_2 and TGF-β_1, METH-KDE cells may require only small amounts of these molecules. Anti-TGF-β_1 mAb suppressed METH-KDE cell proliferation only when the cells were pre-treated with high concentrations of taxol (10 μM). Whereas anti-TGF-β_1 mAb neutralized endogenous TGF-β_1 that was secreted into the culture medium, taxol may have decreased TGF-β_1 synthesis. Taxol can interact with rough endoplasmic reticulum (ER) and displace ER-associated ribosomes (422). Taxol also can fragment Golgi complexes and significantly disrupt the post-translational modification and export of proteins (422). METH-KDE cell proliferation increased in the presence of exogenous TGF-β_1 (Figure 56) which suggests that TGF-β_1 is a fibrosarcoma growth

factor. Although TGF-β_1 enhanced cell division, cells committed within the cell cycle are extremely sensitive to taxol. METH-KDE cells cultured in the presence of TGF-β_1 became more sensitive to the cytostatic activities of taxol. Although we screened several cytokines potentially associated with autocrine tumor cell growth (GM-CSF, IL-1, IL-2, and IL-6), these signals did not promote METH-KDE cell growth and did not alter METH-KDE cell responsiveness to taxol. Future studies will expand the range of indomethacin concentrations used to confirm that PGE_2 acts as a local inhibitor of immune cell activity rather than as an autocrine tumor cell growth factor.

Taxol significantly increased METH-KDE cell sensitivity to TNF-α (Figures 57-59). These results are supported by Williams *et al.* (467) who used ovarian carcinoma cell lines to demonstrate that taxol modulates tumor cell sensitivity to TNF-α. Whereas taxol increased the sensitivity of some tumor cell lines (Caov-3 and A2780) to lysis by TNF-α, other tumor cell lines (SK-OV-3 and OVCAR-3) developed heightened resistance. Although we do not have data to suggest that taxol increases METH-KDE cell binding or internalization of TNF-α, taxol treatment may alter membrane permeability or surface molecule expression. Internalized TNF-α by taxol-induced alterations in membrane permeability also could trigger apoptosis and may explain why taxol plus TNF-α caused the highest levels of cell death. Either co-expression or independent expression of Fas antigens and TNF-α receptors can lead to apoptosis (473, 478), and taxol may enhance the co-expression of these two molecules or restrict their disassociation. We determined that pre-treatment of METH-KDE cells with TGF-β_1 significantly increased sensitivity to taxol and TNF-α (Table 18). Because exogenous TGF-β_1 promotes tumor cell division, TGF-β_1 most likely increased the percentage of cells engaged in the cell cycle where taxol-mediated cytotoxicity is most significant. Although TGF-β_1 pre-treatment of L929 fibroblasts induces the expression of extracellular proteins that confer resistance to TNF-α (70), our data sug-

gest that this mechanism does not occur in fibrosarcoma cells. While the mechanism is not completely clear, taxol-induced METH-KDE cell sensitivity to TNF-α appears to involve an apoptotic pathway.

We used two observations to determine whether taxol induced apoptosis among METH-KDE cells. First, the normal morphology of METH-KDE cells (Figure 60) changed during taxol exposure. METH-KDE cells possessed ruffled membranes and cytoplasmic blebs (Figure 61A-C) when exposed to taxol. Taxol-induced alterations in METH-KDE cell morphology were dose-dependent. Membrane blebbing is a qualitative indicator of apoptosis and differs visually from cytolysis (88). Non-taxol-treated (Figure 61D) and X-irradiated (Figure 61E) controls did not have apoptotic morphologies. Second, we determined whether taxol-mediated cytotoxicity involved NO production (Figure 62). Although NO partly regulates apoptosis among certain cell types (25), METH-KDE cells did not produce NO when exposed to taxol, TNF-α, or both. IFN-γ is a potent stimulus for NO production and acts synergistically with taxol or LPS. The combination of taxol, TNF-α, and IFN-γ induced weak NO expression by METH-KDE cells. NO expression was greatest when METH-KDE cells were treated with LPS, TNF-α, and IFN-γ. METH-KDE cells treated with LPS, TNF-α, and IFN-γ were the least viable. Although IFN-γ in conjunction with taxol and TNF-α induces NO expression and decreases viability, an NO-mediated mechanism does not account for decreased viability and apoptosis among cultures treated only with taxol and TNF-α. To determine if NO could trigger apoptosis in METH-KDE cells, some cultures were supplemented with sodium nitroprusside, which spontaneously generates NO in solution. Although exogenous NO promoted apoptosis, we conclude that endogenous NO does not contribute to apoptosis among taxol-treated or TNF-α-treated METH-KDE cells. These findings are supported partly by Fast *et al.*

(142) who showed that NO production is not directly involved during TNF-α-mediated cytotoxicity.

Because apoptosis is associated with DNA fragmentation, our future studies will use cytofluorometric analyses to determine whether METH-KDE cells demonstrate mitotic arrest and DNA fragmentation when exposed to taxol. Donaldson *et al.* (114) showed that HeLa cells either arrested in the G_0 to G_1 transition or trapped in the G_2/M phases of the cell cycle were highly susceptible to taxol whereas cells immediately post-mitotic demonstrated low sensitivity to taxol. DNA gel electrophoresis suggested that cells committed within G_0 to G_1 transition or G_2/M had extensive DNA fragmentation when exposed to taxol. Because the amount of relative fluorescence within a a particular cell correlates to the cell's location in the cell cycle, we are using propidium iodide staining to confirm that taxol arrests METH-KDE cells within the G_2/M portion of the cell cycle. Propidium iodide is a fluorescent intercalating stain that readily diffuses across cell membranes and associates with DNA. We also are staining METH-KDE cells with propidium iodide to elucidate whether taxol significantly increases the percentage of METH-KDE cells containing less than diploid DNA. These observations could suggest that taxol-induced mitotic arrest is followed by DNA fragmentation. Taxol-induced DNA fragmentation most likely occurs through alterations in the intracellular expression and distribution of the bcl-2 oncoprotein (468). Inappropriate distribution and localization of bcl-2 permits intracellular expression of nucleases that degrade DNA (468). We also will confirm that DNA fragmentation occurs by using a DNA-specific ELISA. This approach will show sensitively and quantitatively whether taxol-treated cells possess extensively fragmented DNA accumulations in the cytosol. We are currently evaluating taxol-induced DNA fragmentation of METH-KDE cells by gel electrophoresis.

Lastly, we determined whether METH-KDE cells recover from taxol exposure. Although METH-KDE cells demonstrated limited recovery from taxol exposure (Figure 63), our data do not prove that the cells developed taxol resistance. The results may reflect that high concentrations of taxol were still retained within some METH-KDE cells at the time of reconstitution. Once these cells died and released their taxol, the newly-released taxol could potentially re-incorporate into neighboring cells in culture. This scenario is partly confirmed because METH-KDE cells that were pre-treated with low doses of taxol did not show significant decreases in either proliferation or viability.

Three mechanisms of taxol resistance by tumor cell lines have been identified. First, some taxol-resistant cell lines demonstrate reversible bundling of their microtubules in the presence of taxol (369). The cytotoxic action of taxol depends partly on the stability of taxol-induced microtubule bundles during interphase. Cells that recover from taxol-induced microtubule bundling presumably remain responsive to autocrine growth factors and maintain clonogenic potential. Cell lines (such as Daudi and K562) that can reverse microtubule polymerization in the presence of taxol demonstrate higher viability than taxol-sensitive counterparts (such as LC8A and HL-60) which cannot reverse microtubule bundling (369). We predict that taxol promotes irreversible, stable polymerization of microtubule bundles in METH-KDE cells. We hope to confirm this likelihood using indirect immunofluorescence staining. The second mechanism of taxol resistance involves cellular modification of α and β tubulin proteins (61). A human small-cell lung cancer cell line (H69/Tx1) that demonstrates both taxol resistance and taxol dependence also possesses increased acetylation of α tubulin (331). At present, we have not acquired data that suggest METH-KDE cells develop gradual resistance to taxol through increased acetylation of α tubulin. The third mechanism of taxol resistance involves cellular efflux of taxol

from the cytosol through an ATP-dependent transmembrane protein designated P-glycoprotein (372). This protein is encoded by the multi-drug resistance gene (MDR-1), and taxol resistance is associated with enhanced expression of this gene (158, 236). Cells expressing P-glycoprotein are resistant to other hydrophobic antimitotic agents such as vinca alkaloids and can restrict intracellular accumulation of these compounds. We do not have evidence to suggest that METH-KDE cell expression of P-glycoprotein changes during either continuous exposure to taxol or during taxol pre-treatment. Future studies will screen supernatants from METH-KDE cells pre-treated with ^{3}H-labelled taxol to determine if METH-KDE cells efflux taxol during culture. Furthermore, we will screen METH-KDE cell supernatants for taxol using a taxol-specific ELISA.

Collectively, our studies suggest that METH-KDE fibrosarcoma cells are sensitive to taxol through multiple cytostatic and cytotoxic mechanisms. Taxol sensitivity implicates specific cytokines that are expressed during tumor growth and may involve monokines triggered during taxol immunotherapies. Future studies will determine if taxol increases METH-KDE cell sensitivity to lysis by effector Mϕ and whether taxol compromises METH-KDE cell production of the suppressor signals TGF-β_1, PGE_2, and IL-10. If taxol suppresses or inhibits fibrosarcoma synthesis of these signals, these data would suggest that taxol may promote tumor regression by disrupting critical tumor-derived suppressor networks. Furthermore, we will evaluate whether taxol analogs demonstrate similar efficacy and if *in vivo* administration of taxol abrogates tumor-induced immune suppression. By understanding the mechanisms of taxol sensitivity and cytokine signals associated with taxol treatments, clinicians may be able to better refine current therapeutic regimens.

CONCLUSION

This section briefly summarizes the results described in Sections II, III, and IV, and modifies the pre-existing TBH model to include these findings. This section also suggests hypotheses and approaches for future investigations.

DATA SUMMARY

We have shown that tumor growth changes the phenotypic and functional characteristics of Mϕ and T cells. My research expands our previous findings and clarifies the role of molecules such as GM-CSF, M-CSF, PGE_2, IL-1, IL-2, IL-4, IL-6, IL-10, and TGF-β_1 in immune cell dysfunction.

Section II: Macrophage Dysfunction During Tumor Growth

Flow cytometry was used to show that Mϕ activation through specific surface molecules is altered during tumor development (Chapter I; 447). This investigation extended previous studies that showed tumor growth changes Mϕ phenotypic and functional heterogeneity. My data suggest that tumor-induced changes in Mϕ cell cycle kinetics during activation may account partly for TBH Mϕ suppressor activities. We have shown that Mac-3$^+$ Mϕ are associated with suppressive activity and the release of PGE_2 (173). Increased TBH Mϕ DNA synthesis, when stimulated through the Mac-3 surface molecule, may reflect the receptor's role in PGE_2 regulation and Mϕ activation. In the NH, where PGE_2 regulation is more tightly controlled, antibody-directed stimulation of Mac-3 surface molecules does not change DNA synthesis. Tumor-induced changes in Mac-3$^+$ Mϕ activities would account for the high levels of PGE_2 produced during tumor growth.

Tumor growth also changes Mϕ responsiveness to M-CSF, and these changes partly account for Mϕ suppressor activities during T-cell alloreactivity and autoreactivity (Chapter II; 443a). M-CSF downregulates Mϕ MHC class II molecule expression and induces PGE_2 synthesis, and others show that Mϕ suppressor functions can be elicited through M-CSF (353, 471). My findings suggest that M-CSF partly contributes to TBH Mϕ suppressor activity during T-cell allorecognition and autorecognition.

Neutralization studies were used to show that the suppressor activities associated with MHC class II$^-$ TBH Mϕ during T-cell autoreactivity involve changes in PGE_2, IFN-γ, and IL-4 production (Chapter III; 450). We previously showed that tumor growth compromises T-cell autorecognition partly because Mϕ MHC class II mole-

cule expression decreases (484). However, decreased MHC class II molecule expression is responsible only partly for reduced autorecognition. Dysregulation of PGE_2, IL-4, and IFN-γ partly explains why tumor growth increases MHC class II^- Mϕ suppressor activity.

My investigations suggest that GM-CSF triggers additional immunosuppression from TBH Mϕ (Chapter IV; 448). In the absence of Mϕ, GM-CSF increases T-cell proliferation in response to alloantigen. However, TBH Mϕ-mediated suppression of allorecognition was further induced by GM-CSF. Allogeneic MLR cultures containing NH Mϕ are either unaffected or enhanced. This mechanism does not involve PGE_2 or M-CSF. My findings suggest that tumor growth specifically changes MHC class II^- Mϕ responsiveness to GM-CSF because MHC class II^- TBH Mϕ become more suppressive in the presence of GM-CSF during T-cell allorecognition. In contrast, alloresponses suppressed by NH MHC class II^- Mϕ demonstrate higher reactivity in the presence of GM-CSF. The data collectively suggest that TBH Mϕ respond differently to GM-CSF, and that tumor-induced changes in GM-CSF responsiveness affect Mϕ accessory ability.

GM-CSF dysregulation is associated with Mϕ suppressor activity during T-cell autorecognition (Chapter V; 443). Tumor growth reduces Mϕ MHC class II molecule expression and induces MHC class II^- Mϕ that demonstrate suppressor functions. This phenotypic shift is important during tumor-induced suppression because MHC class II^+ Mϕ demonstrate significant tumoricidal activity and are the primary cell type involved during tumor regression (132). Because GM-CSF regulates Mϕ expression of MHC class II molecules (178), I hypothesized that GM-CSF would reverse TBH Mϕ-mediated suppression of autorecognition through increased Mϕ expression of MHC class II molecules. However, tumor growth inhibits GM-CSF-induced MHC class II molecule expression TBH Mϕ. GM-CSF partly reverses TBH Mϕ suppression of

autoreactivity when PGE_2 synthesis is inhibited. Furthermore, autoreactive T cells stimulated solely by TBH Mϕ produce more GM-CSF than autoreactive T cells stimulated by NH Mϕ. These findings suggest that increased T-cell GM-CSF production represents an unsuccessful attempt to correct defective Mϕ activities during autorecognition. Other studies (Chapter VII, 444) indicate that activated TBH Mϕ produce less GM-CSF than activated NH Mϕ.

Section III: T Cell Dysfunction During Tumor Growth

Several tumor-induced suppressor mechanisms associated with $CD8^+$ T cells were elucidated (Chapter VI; 449). My data strongly suggest that tumor growth induces phenotypic and functional changes in $CD8^+$ T cells and that this population mediates significant levels of suppression in the TBH. Tumor growth increases the relative percentage of splenic $CD8^+$ T cells, and these cells significantly suppress $CD4^+$ allorecognition and autorecognition. Neutralization studies suggest that suppression is mediated partly by PGE_2, IFN-γ, and IL-4. Alloresponses and autoresponses are suppressed significantly when TBH $CD8^+$ T cells regulate these reactions simultaneously with TBH MHC class II^- Mϕ. These studies collectively show that tumor-induced changes in $CD8^+$ T cells lead to suppressed allorecognition and autorecognition through mediator molecules and suppressor Mϕ.

$CD4^+$ T cells also are functionally changed during tumor growth (Chapter VII; 444). I showed that tumor growth changes $CD4^+$ T-cell regulation of GM-CSF and characterized how tumor growth disrupts Mϕ regulation of GM-CSF. $CD4^+$ T cell and Mϕ dysregulation of GM-CSF partly involves heightened sensitivity to IL-10. IL-10 is

an inhibitory cytokine that is produced by T_H2 cells and Mϕ. Additional studies show that tumor growth increases Mϕ and T-cell production of IL-10 and that METH-KDE cells constitutively produce IL-10 (10, 445). Tumor growth significantly decreases $CD4^+$ and $CD8^+$ T-cell synthesis of GM-CSF, and TBH T cells are more susceptible to GM-CSF synthesis inhibition by IL-10 than their NH counterparts. Both splenic and peritoneal LPS-activated TBH Mϕ produce less GM-CSF than NH Mϕ, and this tumor-induced decrease in GM-CSF production also occurs with MHC class II^- Mϕ. Furthermore, TBH Mϕ are more susceptible to GM-CSF synthesis inhibition by IL-10 than their NH counterparts. Tumor growth also changes T-cell and Mϕ responsiveness to GM-CSF. Tumor growth increases TBH T-cell susceptibility to inhibition of proliferation by IL-10. Tumor growth suppresses Mϕ responsiveness to GM-CSF, and IL-10 further decreases Mϕ responsiveness to GM-CSF. These results suggest that TBH T-cell and Mϕ production of and responsiveness to GM-CSF may be disrupted *in vivo* by IL-10. Because IL-12 production and IL-12-associated activities also are inhibited by IL-10, future studies should evaluate whether this important Mϕ-derived cytokine is dysregulated during tumor growth by increased immune cell sensitivity to IL-10.

I next demonstrated that $CD4^+$ T cells have lower responsiveness to additional activation and co-stimulatory cytokines and display an increased sensitivity to suppressor molecules associated with tumor development (Chapter VIII; 445). Signals that can promote T_H1 cell T-cell activation such as IL-2, IFN-γ, and anti-IL-4 mAb partly restore TBH $CD4^+$ T-cell proliferation to NH levels, but reconstitution of TBH $CD4^+$ T cell proliferation is blocked by the suppressor cytokines IL-10 and TGF-β_1. These findings suggest that tumor cell-, TBH Mϕ-, and TBH $CD8^+$ T cell-derived IL-10 and TGF-β_1 may compromise immunocompetence *in vivo*. My studies also show that TBH T cells are unresponsive to IL-1 and IL-6, which suggests that

T_H2 cells demonstrate decreased functional responsiveness. I also determined that tumor growth increases $CD4^+$ T-cell sensitivity to anti-cancer drug taxol. Tumor-induced increases in sensitivity to taxol may explain partly the limited efficacy of taxol chemotherapies.

Section IV: Taxol as a Means to Reverse Tumor-Induced Immunosuppression

The studies described in this section (Chapter IX; 446) serve as the foundation for future investigations that will clarify the significance of taxol as a inducing agent for *in situ* Mϕ tumoricidal activities. In collaboration with Lisa Maddox (an undergraduate research associate), we determined that taxol disrupts tumor cell growth through several cytostatic and cytotoxic mechanisms. Taxol significantly decreases METH-KDE cell proliferation and viability, and increases METH-KDE cell radiosensitivity. Taxol disrupts autocrine regulation of TGF-β_1 and increases METH-KDE cell sensitivity to TNF-α. The latter finding suggests that *in situ* taxol-activated TBH Mϕ, which produce TNF-α, may demonstrate cytotoxic activities against tumor cells and may contribute to tumor regression. Thus, taxol-mediated tumor regression *in vivo* may occur through 1) direct cytostatic and cytotoxic activities against fibrosarcoma cells and 2) increased tumor cell sensitivity to taxol-induced, Mϕ-derived TNF-α.

MODEL OF TUMOR-INDUCED Mϕ AND T CELL DYSFUNCTION

Based on the conclusions from my investigations and the information that is presented in the *Literature Review*, a model for tumor-induced Mϕ and T cell dysfunction is proposed (Figure 64). This model incorporates previous observations of how tumor growth compromises immune cell activities both at the tumor site and within distal lymphoid tissues. This model also includes speculations regarding how additional molecules contribute to tumor-induced immune cell dysfunction.

At the tumor site, tumors decrease immunocompetence through multiple mechanisms. Tumors express either tumor-associated surface antigens or tumor-specific transplantation antigens that can be expressed in context of MHC class I molecules (and occasionally MHC class II molecules). However, neoplastic cells downregulate their own MHC molecule expression and decrease MHC class II molecule expression on tumor-associated APCs such as Mϕ and DC. This downregulation is accomplished partly through constitutive production of suppressor molecules such as PGE_2, TGF-β_1, and IL-10. Other molecules such as IL-4, IL-6, or IL-13 also may compromise tumor-associated APC functions, cytolytic activities, or responsiveness to activation cytokines. Tumor types that express MHC molecules but do not express CD80 may induce anergy among tumor antigen-specific T cells. Tumor cell expression of adhesion molecules partly dictates whether cancer cells are resistant to Mϕ cytolytic activities and whether they possess metastatic potential. Specific tumor types that do not express adhesion molecules such as CD54 are resistant to Mϕ-mediated cytolysis, whereas other tumor types which increase the expression of specific ad-

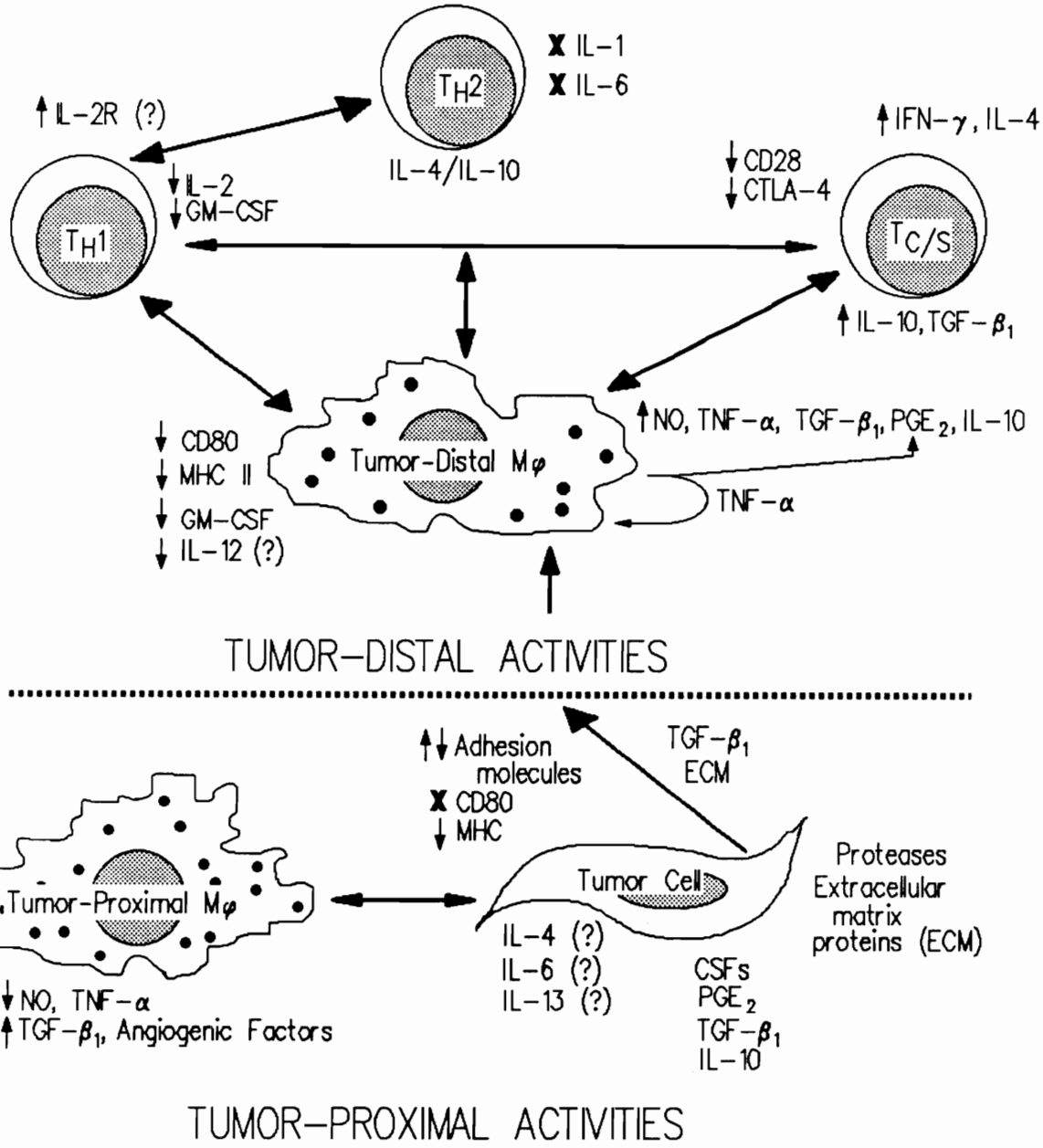

Figure 64. Mechanisms of tumor-induced Mϕ and T cell dysfunction: This model suggests that both tumor-proximal and tumor-distal immune cell activities are compromised during tumor development. Tumor-induced changes disrupt the regulation of cytokines and surface molecules associated with immune cell activation. Both tumor-proximal Mϕ cytotoxic activities and cytolysis mediated by tumor-infiltrating lymphocytes (not shown) decreases. Suppression *in situ* may arise from both tumor cells and Mϕ. In tumor-distal tissues, Mϕ and $CD8^+$ T cells show increased suppressor activities. TBH $CD4^+$ have decreased responses to activation cytokines and increased sensitivity to suppressor molecules. Taxol may reverse tumor-induced immune cell dysfunction by simultaneously disrupting tumor cell suppressor activities and promoting Mϕ cytolytic activities. Upward arrow (↑) indicates increased production or responsiveness, downward arrow (↓) indicates decreased production or responsiveness, and X indicates inhibited or blocked expression.

hesion molecules demonstrate greater metastatic potential and increased adherence to extracellular matrix proteins. Tumor cells also produce numerous proteases that promote metastasis and activate TGF-β_1. Tumor-associated Mϕ may produce proteases that contribute to TGF-β_1 activation. Some tumor cells "fuse" with Mϕ which camouflages them from T cells. Tumor-associated Mϕ also provide growth factors and angiogenic factors for tumor expansion and metastasis. Tumor cell-derived and immune cell-derived factors such as TGF-β_1 may travel through the lymphatics to the resident tissues and bone marrow where they disrupt myeloid developmental pathways. This mechanism partly may explain why Mϕ phenotypic heterogeneity and functional activities change during tumor growth. Thus, tumor-proximal populations are compromised in their abilities to eradicate the tumor and under certain conditions may propagate tumor growth.

Within tumor-distal lymphoid tissues, immunocompetence is compromised primarily by suppressor immune cell activities, although tumor cell-derived TGF-β_1 may also reach tumor-distal regions if it is released in a latent form or if it associates with circulating blood proteins (carrier proteins, soluble receptors, or possibly cytokine-specific autoantibodies). Tumor-distal Mϕ predominantly shift toward suppressor phenotypes. Many tumor-distal Mϕ are MHC class II$^-$ and may express decreased levels of important costimulatory surface molecules such as CD80. Activated tumor-distal Mϕ produce aberrant levels of suppressor molecules (IL-10, TGF-β_1, NO, and PGE_2), which directly suppress T cell activation. This mechanism partly is perpetuated through dysregulation of TNF-α which autocrinely regulates PGE_2 synthesis. IL-10, TGF-β_1, and possibly IL-4 may in turn autoregulate IL-12 production, which also would decrease T-cell and NK cell responsiveness during challenge. Tumor-distal Mϕ also aberrantly regulate GM-CSF. Tumor growth decreases GM-CSF production by both T cells and Mϕ, which compromises T-cell activation and MHC class II mol-

ecule expression. Both T cells and Mϕ demonstrate decreased responsiveness to GM-CSF, and responsiveness is further compromised by IL-10. IFN-γ is involved during tumor-induced immunosuppression because IFN-γ-primed Mϕ produce excessive levels of suppressor molecules when activated. IFN-γ also stimulates suppressor functions by TBH $CD8^+$ T cells. TBH $CD8^+$ T cells produce IL-10 and TGF-β_1 and can mediate significant levels of suppression in concert with MHC class II^- TBH Mϕ. TBH $CD8^+$ T cells mediate suppression through IL-4 (which suppresses IL-12 production by Mϕ) and IFN-γ (which promotes TBH Mϕ suppressor molecule synthesis). Phenotypically, $CD8^+$ suppressor cells may lack CD28 expression. Tumor growth changes $CD4^+$ T cell responsiveness to several autocrine and paracrine growth factors. These changes may be caused by decreased cytokine expression, increased soluble receptor production, or increased sensitivity to suppressor signals. Both T_H1 and T_H2 cells are affected. T_H1 cells produce low amounts of autocrine growth factors such as IL-2 and T_H2 cells responsiveness to IL-1 and IL-6 is inhibited. Because tumor cells increase Mϕ and $CD8^+$ T cell suppressor activities, these cells in turn disrupt T_H cell responsiveness during activation. Collectively, tumor-induced dysfunction among tumor-distal immune cells involves changes in the expression and responsiveness of cytokines and alterations in the expression of surface-associated surface molecules.

Tumor-induced immune cell dysfunctions may be reversible through regimens that simultaneously disrupt tumor cell suppressor mechanisms and promote tumoricidal activities. Taxol has shown significant success as a chemotherapeutic, and taxol's efficacy may reflect its ability to compromise tumor cell growth and stimulate Mϕ cytolytic activities. Taxol decreases tumor cell viability and proliferation and disrupts autocrine growth factor regulation. Taxol also may hinder tumor cell production of PGE_2, IL-10, and TGF-β_1. Taxol stimulates Mϕ cytolytic activity by in-

ducing TNF-α and NO production; *in situ* activation of tumor-associated Mϕ may mediate tumor cell killing. Taxol also may increase tumor cell sensitivity to cytolysis by TNF-α. Although taxol can decrease T cell and NK cell proliferation and cytotoxic functions, these problems may be adverted by using liposome delivery systems. Liposomes are readily phagocytosed by Mϕ, and taxol-based chemotherapies using liposomes may facilitate activation of tumor-associated Mϕ cytotoxic activities. Taxol-containing liposomes could be given with lower doses of i.v.-administered taxol so that both tumor cell suppressor activities and Mϕ cytotoxic functions are targeted. Because taxol functionally resembles LPS, future studies may address whether taxol stimulates Mϕ IL-12 production or induces CD80 expression. Increased IL-12 production would promote T_H1 and NK cell activities and increased CD80 expression would prevent anergy during Mϕ-T cell interactions. Taxol therapies administered in conjunction with cytokines that increase MHC class II molecule expression on tumor-associated APCs may promote tumor regression.

SUGGESTED FUTURE INVESTIGATIONS

My investigations have answered several relevant questions regarding tumor-induced Mϕ and T cell dysfunction. However, they have spurred many research questions that should be addressed by future investigators in our laboratory. The following research questions merit attention during future studies:

- ***Does tumor growth alter TBH Mϕ and T cell expression of surface-associated costimulatory molecules?*** Murine transfection tumor models show that either cytokine-specific activation of *in situ* APCs (120) or tumor cell expression of CD80 (76) contribute to tumor rejection. T-cell activation without costimulation through CD80-CD28 interactions induces anergy. Both $CD80^-$ tumor cells and $CD80^-$ TBH Mϕ could trigger unresponsiveness among antigen-specific T cells. I hypothesize that tumor growth decreases activated Mϕ expression of CD80 and that METH-KDE cells are $CD80^-$. Tumor-induced decreases in Mϕ CD80 expression partly would explain why TBH T cells are hyporesponsive. Furthermore, tumor-induced changes in T-cell CD28 expression should be evaluated. Tumor-induced decreases in CD28 expression also may account for T-cell hyporesponsiveness during activation. Others have shown that $CD28^-$ T cells possess limited cytotoxicity (23a) but are hyporesponsive to activation signals and cannot be costimulated with CD80. Flow cytometric analyses of CD80 and CD28 expression should be performed to verify whether tumor growth modulates the expression of these important surface molecules.

- ***What is the role of IL-13 during tumor growth?*** IL-13 is a recently characterized T_H2-derived cytokine (originally designated P600) that may be dysregulated during tumor growth (489). IL-13 decreases Mϕ NO production. Overexpression of this signal may disrupt both T cell and Mϕ activation and responsiveness during tumor development. Future studies should determine whether tumor growth increases T cell production of IL-13 and whether tumor cells synthesize this cytokine. Recombinant IL-13 and anti-IL-13 neutralizing antibodies could be used in several *in vitro* applications (Mϕ NO and TNF-α production) to determine whether IL-13 suppresses TBH Mϕ cytotoxic responses. No data exists to sug-

gest whether dysregulation of this molecule contributes to tumor-induced immune cell dysfunction.

- ***Do taxol analogs promote Mϕ cytotoxic activities or compromise T cell proliferation?*** Taxol analogs such as taxotere demonstrate greater cytotoxicity than taxol but do not stimulate Mϕ tumoricidal activities (438). Future collaborations may permit our laboratory to acquire three recently isolated taxol-related compounds (David Kingston, personal communication). Because our data suggest that taxol partly reverses TBH Mϕ suppressor activities, future studies should determine whether taxol-related analogs that demonstrate tumoricidal activity can stimulate Mϕ production of NO and TNF-α. Furthermore, T-cell sensitivity to taxol analogs should be evaluated. Our data suggest that taxol adversely affects T cell responsiveness to activation and downregulatory cytokines (445). Future studies may clarify whether the antineoplastic activities of taxol and taxol analogs are restricted toward tumor cells or if they involve immune cells such as Mϕ and T cells.

- ***Does taxol reverse TBH Mϕ suppressor activities against T cells through induction of IL-12?*** Several anticancer mechanisms are associated with Mϕ-derived IL-12 (reviewed in 52). IL-12 increases $CD8^+$ T cell cytotoxicity and proliferation, promotes the development of uncommitted $CD4^+$ T cells toward a T_H1 cell type, and stimulates T cell and NK cell expression of IFN-γ. Many *in vivo* murine studies indicate that IL-12 can treat numerous tumor types such as sarcomas, melanomas, carcinomas, and lymphomas. However, IL-10, TGF-β_1, and IL-4 negatively regulate IL-12 production and IL-12-induced activities. Because these suppressor signals are overexpressed during tumor development, I

speculate that Mϕ production of IL-12 is significantly disrupted during tumor growth. IL-12 may be expressed aberrantly among both proximal and distal immune cell populations. Locally, tumor-associated Mϕ may produce little or no IL-12. We have shown previously that both fibrosarcoma cells and suppressor Mϕ produce significant concentrations of IL-10 (10), which may block IL-12 synthesis. This possibility may explain why exogenous IL-12 shows significant success as an *in vivo* immunotherapeutic agent. Populations at the tumor site may demonstrate hyporesponsiveness to IL-12 because of the high concentrations of TGF-β_1 present in the infiltrate. Among distal Mϕ populations, we believe that IL-12 production also is disrupted. Previous data suggest that $CD4^+$ T cell responses to activation cytokines are poor (445); these low responses may reflect that an IL-12 deficiency is preventing early T-cell activation or commitment. Furthermore, neutralization studies suggest that inhibition of Mϕ IL-4, IL-10, TGF-β_1, and NO production significantly reverses suppressor Mϕ activity against T cells (10, 449, 450). This partial block of suppressor Mϕ activity may permit increased expression of IL-12. Taxol may compromise tumor cell IL-10 and TGF-β_1 production and stimulate Mϕ to produce IL-12. Until a commercial ELISA kit is available, an IL-12-specific bioassay (which measures T_H1. cell IFN-γ production) could be used to determine whether tumor growth compromises Mϕ IL-12 production and whether taxol stimulates IL-12 synthesis. Recombinant IL-12 and anti-IL-12 neutralizing antibodies could be used in our *in vitro* assay systems to determine whether hypoproduction of IL-12 is associated with decreased immunocompetence during tumor growth.

- ***Does taxol disrupt the growth of other tumor cell lines through multiple cytostatic and cytotoxic mechanisms?*** Although we have shown that taxol disrupts several

tumor cell activities (446), future studies should expand the panel of tumors evaluated to confirm that these mechanisms are not unique to METH-KDE fibrosarcoma cells. Additionally, I hope that three current investigations will confirm indisputably that taxol induces apoptosis in METH-KDE cells. We are using a propidium iodide staining technique for detection of hypoploid cells and both an ELISA and DNA gel electrophoresis approach for detection of apoptosis-induced DNA fragmentation.

- ***Will a liposome-engineered delivery system prove effectively "target" taxol to Mϕ and avoid lymphocyte toxicity?*** The limited success of taxol chemotherapies may arise because taxol compromises T-cell and NK cell activities and affects the proliferation of specific myeloid precursors. Furthermore, the diluents used to administer taxol *in vivo* induce discomforting side effects. These adverse responses may be avoidable if cancer therapies involve taxol that is encapsulated within liposomes. Liposomes are rapidly phagocytosed by Mϕ and may serve as a potential vehicle for delivering taxol to the population associated with taxol-induced tumoricidal activity. This approach, if used in conjunction with low doses of systemically delivered taxol, may permit clinicians to lower the concentration of efficacy doses for patients. Lower levels of taxol and diluent in the bloodstream could minimize taxol accumulations in lymphocytes and the bone marrow and decrease the incidence of diluent-associated toxicities. To date, a liposome-based delivery system has not been developed. This approach could be characterized initially using our murine tumor model. Potentially adverse responses such as increased TBH Mϕ production of suppressor (IL-10, NO, PGE_2) should be checked.

Collectively, the studies described herein have helped identify several tumor-induced alterations among Mϕ and T cells. Successful cancer therapies will simultaneously disrupt tumor cell suppressor activities and correct tumor-induced immune cell dysfunction. By abrogating tumor cell suppressor activities and reversing suppressor Mϕ and T cell activities, the war against cancer may be won.

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Appendix A. ABBREVIATIONS

- Ab, antibody;
- APC, antigen-presenting cell;
- ATCC, American Type Culture Collection;
- C, complement;
- CSF, colony-stimulating factor;
- DC, dendritic cell;
- ELISA, enzyme-linked immunosorbent assay;
- FBS, fetal bovine serum;
- FCS, fetal calf serum;
- GM-CSF, granulocyte-macrophage colony-stimulating factor;
- Ig, immunoglobulin;
- IFN-γ, interferon-γ;
- IL, interleukin;
- LAK cells, lymphokine-activated killer cells;
- LPS, lipopolysaccharide;
- Mϕ, macrophages;

- mAb, monoclonal antibodies;
- M-CSF, macrophage colony-stimulating factor;
- METH-KDE, Methylcholanthrene-induced fibrosarcoma;
- MHC, major histocompatibility complex;
- MLR, mixed lymphocyte reaction;
- N^GMMA, N^Gmonomethyl-L-arginine;
- NH, normal host;
- NK cells, natural killer cells;
- NMIgG, normal mouse immunoglobulin G;
- NO, nitric oxide;
- NU, neutralizing units;
- PGE_2, prostaglandin E_2;
- SEM, standard error of the mean;
- TBH, tumor-bearing host;
- T_H, helper T cell;
- ^{3}H-TdR, tritiated thymidine;
- TGF-β_1, transforming growth factor-beta-one;
- TNF-α, tumor necrosis factor-alpha;
- WP, whole population.

Appendix B. CELL STAINING WITH PROPIDIUM IODIDE

Propidium iodide is a fluorescent intercalating stain that readily diffuses across cell membranes and associates with DNA. The amount of fluorescence within a particular cell correlates to the cell's location within the cell cycle. This occurs based on the relative amount of double-stranded DNA present in the cell. For example, G_2/M phase cells with two sets of chromosomes fluoresce significantly brighter than do cells in S phase that contain between one and two sets of chromosomes. Additionally, S phase cells fluoresce significantly brighter than do cells in G_1 phase that possess one set of chromosomes.

Briefly, cells are fixed on ice using 0.5 ml cold PBS and 1.0 ml cold 95% ethanol. Cells are washed twice in PBS and stained in 1.0 ml buffered solution containing 3.8 mM sodium citrate, 0.5 mg/ml propidium iodide (Calbiochem), and 1.0 mg/ml RNase A (Sigma). Cells are incubated on ice overnight, washed three times, and analyzed by flow cytometry.

Appendix C. GM-CSF BIOASSAY

The GM-CSF-dependent myeloid cell line DA3 can be used to determine GM-CSF synthesis by various cell types. Because DA3 cells die in the absence of GM-CSF, any proliferation by starved cells when cultured with test supernatants indicates the presence of GM-CSF. Proliferation can be correlated to specific amounts of biological activity by comparing the test samples to a control curve generated from DA3 cells cultured with several two-fold dilutions of GM-CSF. Proliferation can be measured using either ^{3}H-TdR or MTT. The assay is very reproducible and the cells are easy to grow. **The protocol described below involves proliferation measurement using MTT.**

MAINTENANCE OF DA3 CELL LINE

DA3 is a GM-CSF-responsive, nonadherent, myeloid cell line that grows only in the presence of low concentrations of GM-CSF. DA3 cells are not cross-reactive to other CSFs or other cytokines. These cells can be grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and GM-CSF (20-200 units/ml). Cells are passaged every 3-4 days as a 1:10 or 1:20 dilution.

1. Observe the appearance of cells under phase contrast. Cells should be large and circular. Cells lose cytoplasm and lyse if starved too long. Medium may appear off-orange rather than red.

2. Wash cells 1x with RPMI medium for 10 min / 4°C / 1600 rpm.

3. Resuspend pellet in 10.0 ml RPMI medium.

4. Transfer 1.0 ml cells into 26.0 ml RPMI medium, 3.0 ml FBS, and 30 μl GM-CSF (100 U/μl stock). Thoroughly mix and split between two 25 cm^2 culture flasks (15.0 ml/flask).

TECHNICAL NOTES:

- If the cells appear unusual, problems may be due to bad GM-CSF, contamination, over-dilution, etc.

- Make certain you have FBS (thawed and heat-inactivated) and GM-CSF (thawed) before you begin.

- Aseptic technique is an absolute must!!!

- Gentle mixing of GM-CSF avoids degradation of the protein.

- Abide by the rule: **If it ain't at 37°C, keep it on ice.**

- Return FBS and GM-CSF to appropriate temperatures immediately.

- Label any flasks with DA3, date, dilution, CSF concentration, and your initials.

- Do not over-tighten or under-tighten flask; avoid dribble syndrome.

DA3 ASSAY

1. DA3 cells should be starved 72 h before to the assay. Cells starved for less time produce significant levels of background proliferation. Cells starved for longer than 72 h have low viability and respond poorly in the assay. The contents of the culture flask containing the cells (usually a 83 cm^2 flask w/ 15 ml RPMI medium 1640 + 10% FBS) is aseptically transferred into a sterile tube and spun in the Juoan G4.11 centrifuge

1600 rpm/10 min/4°C. The pellet is then washed again in just RPMI 1640 and re-spun.

2. Resuspend the pellet in 5 ml RPMI 1640 medium w/10% FBS. Count the cells under 400x magnification; final concentration should be adjusted to 5 x 10^5/ml. Because 50 μl of cells are added to each well, the final cell number will be 25,000 cells/well. Keep cells on ice until samples are seeded into the assay.

3. Supernatants for analyses should be completely thawed and mixed before addition into the 96-well flat-bottom microtiter plates.

4. Save one plate for a control curve. The first triplicate (A1-A3) should be seeded with 50 μl RPMI 1640 w/10% FBS. This will serve as the cells alone control. The 2nd triplicate (A4-A6) should be seeded with 100 μl RPMI 1640 w/10% FBS. This will serve as the media control. If the cells are starved appropriately before the assay, these two triplicates should read identically on the ELISA reader.

5. Prepare GM-CSF standards:

   a. Label 20 sterile microcentrifuge tubes. Fill Tube #1 with 380 μl RPMI 1640 medium w/10% FBS and tubes 2-20 with 200 μl RPMI 1640 medium w/10% FBS.

   b. In tube #1, mix 20 μl GM-CSF stock (10^5 U/&ml) into the 380 μl RPMI 1640 medium w/10% FBS. This creates a sample of GM-CSF at 5000

U/ml. (In the assay this sample will have a final concentration of 2500 U/ml because it will be diluted 1:2 when the cells are added.)

c. Using a new pipette tip, transfer 200 μl from tube #1 into tube #2. Thoroughly mix. This creates a sample of GM-CSF at 2500 U/ml. (In the assay this sample will have a final concentration of 1250 U/ml because it will be diluted 1:2 when the cells are added.)

d. Using a new pipette tip, transfer 200 μl from tube #2 into tube #3. Thoroughly mix. Continue this serial two-fold dilution scheme for all twenty tubes. Always remember to change tips between samples.

e. Add 50 μl/well of tube #20 to the third triplicate (A7-A9). Add 50 μl/well of tube #19 to the fourth triplicate (A10-A12). Continue until all the control samples are dispensed. Remember to use a new pipette tip for each sample.

6. Add 50 μl of test supernatants in triplicate into empty wells of the test plates.

7. Thoroughly mix DA3 cells and dispense 50 μl/well using a micro-aliquoter with 3.0 ml syringe and 23-26 gauge needle.

8. Incubate plates 72 h in the 37°C/5% CO_2 humidified water-jacketed incubator. Four prior to the termination of the assay, add 10 μl MTT into each well. Return plates to the incubator.

9. At the end of the assay, remove plates from the incubator and add 100 μl acid/alcohol solution. Mix thoroughly to dissolve the crystals. **Also see TECHNICAL NOTES.**

10. Plates are read on a Dynatech MR580 Microelisa reader using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99. Read the plates within 1 h of adding the acid/alcohol. **Also see TECHNICAL NOTES.**

11. A standard curve is generated by plotting the concentration of GM-CSF against the optical density of the controls. The slope of the plotted data defines the biological activity. The plateaus at the lower and upper ends of the curve are discarded. Fifty units of biological activity for GM-CSF is defined as the concentration at which half-maximal proliferation of 25,000 DA3 cells occurs after 72 h.

TECHNICAL NOTES:

- Avoid using GM-CSF stock solutions or test supernatants that have been repeatedly freeze/thawed.

- Two or three dilutions of GM-CSF are suggested to ensure that the sample will fall within the curve.

- DA3 cells can be used at concentrations of 5 x 10^5/ml (25,000 cells/well) to 2 x 10^6/ml. (10^5 cells/well).

- To completely dissolve blue precipitating crystals that form in the wells during the assay, use the Titertek 8-well pipettor set at 100 μl to thoroughly mix the contents of the wells. DO NOT set the pipettor at 200 μl because the acid/alcohol solution will run up into the pipettor during mixing. You can use the same 8 pipette tips for all samples if you thoroughly wash the tips in a dish containing the acid/alcohol solution in between sample columns. Use new solution for each plate.

- Read the plate at 600 nm (channel 4 on the ELISA reader).

Appendix D. VIABILITY ASSAY USING MTT

MTT is a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma cat. # M2128) that measures the activity of dehydrogenase enzymes in active mitochondria of living cells. MTT can be used as an alternative to radioisotopes such as ^{3}H-TdR to measure cell proliferation. MTT (a yellow solution) is added to all wells 2-4 h before the termination of the assay and is cleaved by mitochondrial enzymes to yield a colored (blue) product. The resulting crystals are solubilized using an acid/alcohol solution. The amount of cell proliferation can be correlated to the intensity of the well color. The assay is very inexpensive and does not require isotopes. Data can be acquired using a scanning multi-well spectrophotometer (ELISA reader).

PREPARATION OF REAGENTS

1. MTT is dissolved in PBS at 5 mg/ml. (I generally make a 20 ml batch which is enough for 20 plates). Thorough vortex this solution and sterile filter it to remove small insoluble particles. Store in a dark container at 4°C. **Also see TECHNICAL NOTES.**

2. Acid/alcohol solution is prepared on a stir plate. Into 1 L of isopropyl alcohol, slowly add 3.3 ml of 12.1 N HCl. This will make a final solution of 0.04 N HCl in isopropanol. Store in a glass container at 4°C.

3. Final volumes for any assay must be 100 μl. Four h before to the termination of the assay, add 10 μl MTT into each well. Return plates to the incubator.

4. At the end of the assay, remove plates from the incubator and add 100 μl acid/alcohol solution. Mix thoroughly to dissolve the crystals. **Also see TECHNICAL NOTES.**

5. Plates are read on a Dynatech MR580 Microelisa reader using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99. Read the plates within 1 h of adding the acid/alcohol. **Also see TECHNICAL NOTES.**

TECHNICAL NOTES:

- All samples should be prepared in triplicate. This assay is not as sensitive as ^{3}H-TdR but is useful for growth factor-dependent assays like the GM-CSF cell line DA3.

- Only 100 μl final volume samples can be analyzed in this assay. Many protocols such as the MLR generate confusing data because of the number of cell populations and reagents in the well and because these assays require round-bottom plates. MTT assays are good for simple mitogen assays and growth factor assays.

- Remember to leave well A1 on every plate as a blank! Blanks should be cells alone (no stimulus) or media alone.

- MTT degrades over time: MTT solutions should not be stored beyond 6 months.

- To completely dissolve blue precipitating crystals that form in the wells during the assay, use the Titertek 8-well pipettor set at 100 μl to thoroughly mix the contents of the wells. DO NOT set the pipettor at 200 μl because the acid/alcohol solution will run up into the pipettor during mixing. You can use the same 8 pipette tips for all samples if you thoroughly wash the tips in a dish containing the acid/alcohol solution in between sample columns. Use new solution for each plate.

- Read the plate at 570 to 600 nm (channel 4 on the ELISA reader).

Appendix E. VIABILITY AND CYTOTOXICITY ASSAYS USING ALAMAR BLUE

Alamar Blue is a nontoxic colorimetric growth indicator that changes irreversibly from blue to red when reduced by excreted cellular metabolites. Thus, the cell viability of a treatment group can be assessed by adding Alamar Blue to cultures shortly after initiation and by monitoring kinetically the absorbance of the sample.

For viability assays, cells are seeded in 96-well flat-bottom or round-bottom microculture plates (Corning Glass, Corning, NY) at a final concentration of either 5×10^5 or 2.5×10^5 cells/ml. Final well volumes were 0.2 ml. Plates are incubated, and 4 h before harvest, all wells were pulsed with 0.02 ml Alamar Blue (Alamar, Sacramento, CA). Alamar Blue is used "as is" (no diluents). Changes in sample absorbance were determined in a MicroELISA titer plate reader (Molecular Devices, Menlo Park, CA) using a 570 nm test wavelength and a 600 nm reference wavelength.

For cytotoxicity assays, cells are cultured as described during viability assays except that cells are seeded in the presence of 10 nM actinomycin D. Unless specified, cytotoxicity are assessed 48 h after the initiation of the assay.

Vita

Thomas Matthew Walker was born May 26, 1967, in Richmond, Virginia. He earned both his B.S. degree (Biology, 1989) and Ph.D. (Microbiology and Immunology, 1995) from Virginia Tech. Matt also received a minor in English. His dissertation, entitled *Tumor-Induced Macrophage and T Cell Dysfunction*, was earned under the direction of Klaus D. Elgert. Matt's first-authored and co-authored work lead to eleven published articles and submitted manuscripts. His work was presented at 30 national, regional, and local meetings for professional scientific societies such as the Federation of American Societies for Experimental Biology, the American Association of Immunologists, the Society for Leukocyte Biology, the Virginia Chapter of the American Cancer Society, the Virginia Branch of the American Society for Microbiology, the Virginia Academy of Sciences, and the Virginia Tech Graduate Student Assembly. He received seven awards for his presentations in student competitions. He also presented five departmental seminars. Matt's research was funded partly by 14 grants that were acquired through Sigma Xi, the Virginia Tech Graduate Student Assembly, the Virginia Academy of Sciences, and

Society for Leukocyte Biology, and the Virginia Tech Department of Biology. Matt also worked with the Virginia Junior Academy of Sciences and served twice as a judge for student competitions. Matt taught numerous lab courses at Virginia Tech including General Biology (5 sections), Principles of Biology (two sections), Microbiology (13 sections), and Immunology (four sections). For his superlative teaching, Matt received two departmental nominations for Virginia Tech teaching awards, two Virginia Tech Outstanding Graduate Teaching Assistantship awards, and a Commonwealth Fellowship. He received 10 partial or complete tuition waivers from the Virginia Tech Biology Department. In addition to his responsibilities at Virginia Tech, Matt also held an adjunct faculty appointment (1993-1995) at the College of Health Sciences in Roanoke, Virginia, where he taught Microbiology lectures (six sections) and laboratories (nine sections). Collectively, Matt taught approximately 660 students at Virginia Tech and 215 students at the College of Health Sciences. He is a student member of the Virginia Academy of Sciences, the American Society of Microbiologists, the American Association for the Advancement of Science, the American Association of Immunologists, the Society for Leukocyte Biology, and the Virginia Branch of the American Society of Microbiologists. He is currently an Assistant Professor of Microbiology and Immunology at the University of Central Arkansas in Conway, AR.