TUMOR-BEARING HOST MACROPHAGE DYSFUNCTION: ROLE OF CD40/CD40L INTERACTIONS

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(ABSTRACT)

A functional immune system is a potential barrier to tumor growth and progression. Cancer is caused, in part, by the loss of immune surveillance leading to the inability of the immune system to destroy the cancer cells. Macrophages (Mϕs) are essential cellular components of the immune system; they influence immune responses in diverse and fundamental ways. As a consequence, Mϕs present targets for tumors to evade, thereby enhancing tumor survival and growth. An interaction between CD40 on Mϕs and CD40L on T cells is required for cell-mediated inflammatory responses. The CD40/CD40L interaction is bi-directional; suppressed expression of either protein by the tumor will prevent activation of both Mϕs and T cells. We showed that tumor growth suppresses T-cell CD40L expression. Decreased CD40L expression disrupted Mϕ activation pathways, leading to impaired production of immunostimulatory cytokines, interleukin (IL)-12 and IL-18 by tumor-bearing host (TBH) Mϕs. Disruption of CD40L expression, via dysregulation of IL-12 and IL-18 production, impeded T-cell interferon (IFN)-γ production, which in turn exacerbated Mϕ dysfunction. We showed that IFN-γ induced interferon consensus sequence binding protein (ICSBP) expression is impaired in TBH Mϕs due to tumor cell-derived TGF-β and, to a lesser extent, IL-10. ICSBP induces CD40L, IL-12, and IL-18 expression. Disruption of the CD40/CD40L interaction via lowered CD40L expression generates an immunosuppressive loop that may be a strategy for tumor survival and growth. This was demonstrated by impaired cytotoxicity; via impaired tumor necrosis factor (TNF)-α and nitric oxide (NO) production by TBH Mϕs against Meth-KDE tumor cells. Collectively, these studies show that multiple antitumor mechanisms could be enhanced by restoration of CD40L expression.

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INTRODUCTION

The objective of my study was to determine whether the Meth-KDE tumor disrupts the interaction between macrophages (Mϕs) and T cells through the CD40/CD40L interaction (due to its being the pivotal costimulatory interaction between Mϕs and T cells) and short-circuits the immune response. Our long-term research objective is to understand the cellular and molecular events involved in tumor-induced Mϕ dysfunction. Our laboratory group published several studies defining mechanisms by which tumor cells mediate immunosuppression (21,22,83,204-209,295,296). The demonstration of tumor-induced suppression of pathways involved in the initiation of natural and acquired immunity, such as CD40/CD40L interactions, the involvement of Mϕs and the role of cytokines could have a major impact on the development of successful anticancer immunotherapy. Discovering these pathways could lead to the development of therapeutic measures to restore the normal immune response and could possibly lead to elimination of the tumor. This study demonstrates an immunosuppressive loop (see proposed model in Figure 10) and how this loop relates to the overall immune response of the TBH.

Over the past century, excitement waxed and waned over the possibility that the extraordinary disease-fighting prowess of the immune system might be enlisted to destroy cancers. Today doubts have vanished, and countless investigators are working to

translate the notion into potent new biological therapies. Clinical support for the idea that the immune system might restrain the development of cancer emerged in the early 1800s, when physicians noted that tumors sometimes regressed in cancer patients who contracted bacterial infections. In the early 1900s, William B. Coley, a surgeon at Memorial Hospital in New York City, was dedicated to creating therapies based on this observation. He made deliberate attempts to infect cancer patients with bacteria and later devised a vaccine consisting of killed bacteria that induced a tumor-killing response. These treatments tried to elicit the body's own defenses -- immunotherapy. Despite the unpredictable results of Coley's experiments, the link between immunity and cancer remained firmly fixed in the minds of some physicians. In the 1960s, the immunosurveillance model was put forth by Lewis Thomas and MacFarlane Burnett. The theory stated that the immune system constantly searches out and destroys cancer cells. Tumors arise when this policing mechanism fails. During the past decade, the science of immunology has undergone revolutionary changes. Researchers discovered and isolated the cells and chemicals that enable the immune system to defend the body against attack. This helped immunologists gain a deeper understanding of the normal immune system's working and the molecules by which it may someday control cancer (228).

The major hurdle that remains is the generation of effective anti-tumor immune responses in the face of tumor evasion mechanisms whereby tumors present themselves

as “quick-change artists.” Finding ways of preventing tumor escape will be the main challenge for immunotherapy in the future (231).

Although the promise of tumor immunotherapy has yet to be fulfilled, several of the likely mechanisms by which tumors escape immunesurveillance have been unraveled at a fundamental molecular level (224). Future approaches to tumor immunotherapy will need to be reconciled with mechanisms of immune escape. Tumors have multiple mechanisms for evading the immune system that range from a passive failure to express costimulatory molecules (*e.g.*, CD40 and CD40L) to active strategies such as the production of immunosuppressive cytokines (*e.g.,* TGF-β, IL-10, and PGE_2) (15,32,53,83,189,225).

Clinical trials of active and adoptive immunotherapy are showing the effectiveness of specific biological treatments for cancer (228). There are now definite cases where an initially effective therapy lost efficacy because tumors developed resistance to immunological attack (232). There are probably many more instances where clinical efficacy was never seen because tumors developed resistance even before a therapeutic effect could be recorded. Tumor escape from the immune response may be considered as the greatest impediment to the application of effective immunotherapy (231).

A number of studies, both in our lab (83,84,208) and others (141,157,287,307), provided insights into the role of Mϕs as important antigen-presenting cells (APCs) in the initiation of immune responses. The role of Mϕs in generating effective antitumor immune responses has proved to be the driving force for my project. The observation that tumor growth activates immune defense mechanisms, yet the tumor cells elude these mechanisms, defines the paradox of tumor immunology. Our studies showed a direct causal relationship between tumor presence and immune dysfunction, suggesting that the proximity of neoplastic tissue leads to immunologic degeneration (83,204,205,208).

Tumors may decrease Mϕ production of immunostimulatory cytokines or suppress the expression of co-stimulatory molecules leading to inefficient stimulation by tumor-reactive T cells. Tumors may also prevent the synthesis of transcription factors essential for the production of integral cytokines. I propose tumors can evade host immune surveillance by altering the:

1. expression of co-stimulatory molecules on T cells and Mϕs,
2. production of immunostimulatory cytokines by Mϕs, and
3. synthesis of transcription factors essential for the production of cytokines.

This study provides information about the mechanisms of tumor-induced immunosuppression. This work offers insight into selective immunotherapies.

This study was aimed at understanding and characterizing the immunosuppressive loop in the TBH by demonstrating the role of CD40/CD40L interactions between Mϕs and T cells and how impairment of the interaction is connected to decreased cytotoxicity of TBH Mϕs. This was achieved by testing the hypothesis: *Dysregulation of CD40/CD40L interactions between TBH Mϕs and T cells may lead to impaired Mϕ cytotoxicity via lowered IL-12, IL-18, IFN-γ and ICSBP production and ultimately persistence and survival of the tumor.* Specifically, my research had the following aims:

- *Determine whether tumor growth dysregulates CD40/CD40L interactions between Mϕs and T cells, respectively:* Because the CD40/CD40L interaction is an essential step in the activation of both Mϕs and T cells, we assessed the expression of CD40 and CD40L on the surfaces of Mϕs and T cells, respectively.

- *Determine whether dysregulation of the CD40/CD40L interaction is manifested in the form of reduced cytotoxicity and cytotoxic effector molecule (TNF-α and NO) production in TBH Mϕs:* Normal host and TBH Mϕ-mediated cytotoxicity as well as TNF-α and NO production were assessed to determine whether dysregulation of the CD40/CD40L interaction led to impaired cytotoxicity.

- *Determine whether dysregulated CD40/CD40L interactions, and as a result impaired cytotoxicity, are mediated by tumor-induced changes in IL-12, IL-18, and IFN-γ*

production: IL-12, IL-18, and IFN-γ production was measured in normal host and TBH Mϕs. IL-12 and IL-18 induce IFN-γ that regulates a variety of important immunologic pathways. IFN-γ is an important regulator in host defenses, in both innate and acquired immunity.

- *Determine whether tumor growth suppresses ICSBP production and if the tumor-derived factors IL-10, TGF-β, and PGE_2 are responsible:* ICSBP production was determined to assess the effect of tumor-derived factors on ICSBP production. This helped define the suggested immunosuppressive loop in the TBH because ICSBP regulates IL-12p40, IL-18, and CD40L expression.

Studies to determine the levels of NO, TNF-α, IL-12, IL-18, IFN-γ, and ICSBP production in normal and TBH mice will show that the tumor, by dysregulating CD40/CD40L interactions, initiates a cascade of events eventually leading to the inability of Mϕs to eliminate the tumor. These studies will suggest mechanisms employed by the tumor to manage to proliferate and survive but, more importantly, they will provide an opportunity for therapies (*e.g.,* restoration of CD40/CD40L interactions in TBH mice) to be tested. A variety of experimental approaches were used to accomplish these aims. Throughout these studies, the Meth-KDE murine fibrosarcoma model was used (16,85). Techniques like western blotting and approaches like enzyme-linked immunosorbent assays and cytotoxicity assays were used.

My thesis is divided into two sections. The first section is a review of relevant literature. The second section contains my submitted findings that describe and provide data for an immunosuppressive loop initiated by the tumor involving dysregulation of the CD40/CD40L interaction. The *Conclusions* section offers a summary of the results described in Section II. Furthermore an updated model of tumor-induced Mϕ dysfunction via dysregulation of CD40/CD40L interactions is proposed. The *Conclusions* section suggests approaches for future investigations.

SECTION I: LITERATURE REVIEW

The *Literature Review* consists of five subsections. *The Essentials of an Antitumor Immune Response* introduces some of the immune cells that constitute an integral part of an antitumor response - Mϕs and T cells, and their role in this response. *Costimulatory Interactions -- The role of the CD40/CD40L Interaction* reviews co-stimulatory molecule interactions and why they are essential for the initiation of an immune response with a special emphasis on CD40/CD40L interactions. *Cytokines and the transcription factor ICSBP* discusses the cytokines IL-12, IL-18, IFN-γ, and the transcription factor ICSBP. *Cytotoxic Effector Molecules* discusses NO and TNF-α as molecular mediators of cytotoxicity. *Evasion Strategies of Tumors* reviews some relevant mechanisms of immune escape employed by tumors.

Essentials of an Antitumor Immune Response

Macrophages

For many years Mϕs were viewed as scavenger cells and considered to be mainly involved in phagocytic functions and inflammatory reactions (55,60,211,258,269,277). During the last two decades, it has become apparent that Mϕs influence immune responses in diverse and fundamental ways (6,73,131,163,306). Capabilities such as

phagocytosis, antigen presentation, production of cytokines capable of modulating the responses of other immune cells and of themselves as well, and bactericidal/tumoricidal activities contribute to the role of the Mϕ in host defense (110,268,287).

Tumoricidal activity is often considered the ultimate state of Mϕ activation for which two signals, a priming signal (IFN-γ) and a trigger signal (LPS), are required (287). Mϕs serve vital roles in host defense against tumors (8,122,314), including tumor cytotoxicity (273,314) and stimulation of antitumor lymphocytes (251). Through secreted molecules such as NO and TNF-α, activated Mϕs directly inhibit or kill tumor cells (273). Studies from several investigators (251,311) and of our own (83,84) suggest that Mϕs play important effector and regulatory roles in the development of tumors. During tumor challenge, Mϕs display tumor antigens to antigen specific $CD4^+$ T cells in the context of MHC class II molecules (21). In conjunction with the appropriate co-stimulatory molecules such as CD40, CD40L, CD28, and B7, these interactions activate $CD4^+$ T cells to proliferate and release cytokines like IFN-γ. These T cell-derived cytokines activate tumor antigen-specific cytotoxic T cells (242) and MHC-unrestricted NK cells (25). Tumors may escape immune responses by dysregulating Mϕ APC function (312) or altering the expression of co-stimulatory molecules on both T cells and Mϕs (301).

Tumors can circumvent host-mediated immune activities through the production and release of immunomodulatory factors such as TGF-β_1, IL-10, and PGE_2 that adversely alter Mϕ function (16) and phenotype (12,13). These tumor-derived factors generate Mϕs that are tumoricidally-dysfunctional.

Resting tumor-distal Mϕs produce cytotoxic and suppresor molecules such as TNF-α, NO, H_2O_2, and PGE_2 when primed by circulating tumor-derived cytokines (11-13,16,94,203). Tumor-primed Mϕs encounter increasing concentrations of activation signals such as TAAs and disrupted ECM proteins during migration to the tumor site and become activated. However, tumor-derived cytokines inhibit Mϕ production of cytotoxic molecules as the activated Mϕs enter the tumor microenvironment. Therefore, cytotoxic molecule production by monocytes as they migrate to the tumor site is downregulated by tumor growth.

Tumor-associated Mϕs (TAMs) originate in the circulation and are recruited to the tumor site by speific tumor-derived attractants (183,185). TAMs bind to tumor cells and unlike Mϕs that are involved in inflammation, TAMs proliferate at the tumor site. Although the TAMs can induce tumor cytotoxicity and stimulate antitumor lymphocytes, tumor cells can not only block this but also benefit from the activities of the TAMs. Tumor-derived molecules can actually redirect TAM activities to promote tumor survival and growth. Among the tumor-derived factors, IL-4, IL-6, IL-10, TGF-β_1, and PGE_2

reduce the cytotoxic activity of the TAMs (18,83). Thus, TAMs can directly or indirectly contribute to tumor survival, growth, and metastasis (293). Identification of the molecular and cellular origins of tumor-induced changes in Mϕ function (especially via the secretion of circulating tumor-derived cytokines) will increase opportunities for immunotherapeutic intervention by identifying key targets.

T Cells

There are two well-defined subpopulations of T cells: T helper (Th) and T cytotoxic (Tc) cells. T helper and Tc cells can be distinguished from one another by the presence of the membrane glycoproteins CD4 or CD8, respectively. T cells displaying CD4 generally function as Th cells whereas those displaying CD8 generally function as Tc cells.

$CD4^+$ Th cells are divided into two subpopulations which are characterized by the panel of cytokines they secrete (202,245,275). The Th1 subset is distinguished by its secretion of IL-2 and IFN-γ. The Th1 subset is responsible for driving cell-mediated functions, such as delayed-type hypersensitivity and the activation of Tc cells. The Th2 subset secretes IL-4, IL-5, IL-6, and IL-10 and functions more effectively as a helper for B-cell activation (88,95,202,275).

Host antitumor immunity is mediated by tumor-infiltrating $CD4^+$ (98,244) and $CD8^+$ T cells (244). Both cell types can mediate cytotoxic activities *in situ*, and neither population demonstrates a predominance in terms of mediating tumor regression. Tumor infiltrates usually contain both $CD4^+$ and $CD8^+$ T cells, although these cells demonstrate defective signal transduction mechanisms, aberrant cytokine production, and decreased cytotoxic activities (20,170,193). TBH T-cell dysfunction is a result of suppressor activities of both tumor cells and tumor-associated Mϕs (14,84,85).

Co-stimulatory Interactions – The role of CD40/CD40L interactions

Induction of an immune response requires several complex interactions between Th cells and various APCs that lead to the expansion of a small number of antigen-specific cells, including B cells and T cells, induction of various regulatory molecules on the surface of these and other cells, and, ultimately, the secretion of cytokines that generally govern the outcome of the immune reaction (93,192,300). The initial activation and expansion of T cells appears to be critical for generating a successful and long-lasting response against most protein antigens, and the lack of appropriate signals early in the response may lead to a period where the cells are refractory to further stimulation (143). Evidence accumulated over the last few years suggests that the interaction between Th cells and APCs determines whether a successful T-cell response is initiated; the extent and nature of the continuing response is determined by subsequent interactions (41,65,299).

Accessory molecules expressed on the surface of APCs are responsible for a MHC-bearing cell being able to present an antigenic stimulus efficiently whereas the T-cell response relies on an integration of signals through the T-cell receptor (TCR) with co-stimulatory signals from accessory molecules (41,65,299). Certain facets of T-cell activation may be controlled solely by TCR signals but still rely on the presence of accessory molecules. The ability of the T cell to receive signals from the APC is altered after activation, with differentiation from a naïve state to either a memory or effector state. Accessory molecule interactions may dictate the cytokine patterns elicited by responding T cells (41,65,174).

After T cells receive appropriate co-stimulatory signals, a period of expansion occurs, essential for an effective immune response, during which the cells differentiate into a so-called 'effector' state, in which they are capable of interacting efficiently with other immune cells, and inducing or directing the overall response (274). Effectors are defined as activated immune cells that have much of the machinery for response in partial readiness. These cells still require recognition of antigen and, as detailed below, additional stimulatory events through the TCR to perform their function (78). After an initial expansion, these primary effectors undergo a period during which their numbers diminish. This may involve programmed cell death or active negative regulatory signals from molecules such as FasL and CTLA4 (65).

Many molecules implicated in playing roles during T-cell activation and inducing co-signals act in concert with TCR signals (128,198,256). It has become clear that virtually all accessory molecules that bind counter-receptors on the T cell induce co-stimulatory signals (65,198,256). Most molecules, at least to some extent, also promote adhesion between T cells and APCs, an effect that allows amplification of signals through the TCR (43,65). After a Mϕ presents an antigen to a T cell receptor the first costimulatory interactions are between B7/CD28, which is followed by the most important costimulatory interaction CD40/CD40L between the Mϕ and T cell, respectively. These interactions are essential for Mϕ and T cell rescue from apoptosis and ultimately Mϕ cytokine production. Co-stimulation is generally assessed by measuring the ability of T cells to secrete IL-2 and to proliferate (78). CD4 and CD8, which are intimately associated with the TCR, play critical roles in T cell responses (57).

Co-stimulation was originally defined as the capacity to generate intracellular signals that were different from those induced through the TCR (49). Recent studies highlighted the lack of co-stimulatory molecules, in particular CD40L, as a key reason for the inefficient stimulation of tumor-reactive T cells (130,212). Efficient elicitation of T cell-mediated immune responses requires the TCR-mediated signal upon antigen recognition and a co-stimulatory signal provided by contact with APCs (212). Among the co-stimulatory signals, those mediated by the interaction of CD40L on T cells with CD40 on Mϕs are significant (35,61,116,190,221,288).

CD40L is a 33 kDa, Type II membrane protein belonging to the TNF family. Use of specific monoclonal antibodies demonstrated the expression of CD40L on human and mouse T cells (114). It also was demonstrated that T cell-mediated Mϕ activation in CD40L-deficient mice is impaired (234). CD40L binds to its counter-receptor CD40, a 45-50 kDa glycoprotein that is a member of the TNF receptor superfamily expressed on the surface of APCs (114). Crosslinking the CD40 molecules with CD40L on APCs induces functional changes that contribute to cell-mediated inflammatory responses, including induction of cytokine synthesis and upregulation of the expression of co-stimulatory and adhesion molecules (80,114,187,234). On antigen stimulation, CD40L is rapidly transduced; it binds to CD40 on APCs and induces or enhances the expression of B7-1 (CD80) and B7-2 (CD86) and the secretion of IL-12 (57,142,234,316) and possibly IL-18 (Martins and Elgert, unpublished observations). B7 enhances CD40L expression on activated T cells. The CD40L transduced co-stimulation of T cells can further upregulate T-cell activation (114). Prolonged B7/CD28 interaction amplifies T-cell responses (78) while IL-12 and perhaps IL-18 regulate T-cell differentiation and cytokine secretion (156). The interaction between CD40 and CD40L is bi-directional and promotes the activation of both APCs and T cells (289). CD40/CD40L interactions are important for cross-priming of $CD8^+$ T cells and the rescue of Mϕs from apoptosis (190,234).

The CD40/CD40L interaction plays a critical role in the induction of antitumor immunity, especially the essential role in the induction of Tc cells (279). Several reports

show that CD40 cross-linking induces monocyte and Mϕ production of NO, TNF-α, and IL-12 (133,145,220,278). Mackey *et al*. showed that CD40-deficient mice have impaired antitumor responses (175). Imaizumi *et al*. showed that tumoricidal activity of alveolar Mϕs was inhibited by the addition of a NO synthesis inhibitor or an anti-TNF-α antibody, suggesting the important role NO and TNF-α play in the generation of Mϕ tumoricidal activities through CD40/CD40L interactions (133).

CD40/CD40L interactions lead to potent activation and survival stimuli that result in the production of various cytokines and upregulation of adhesion and co-stimulatory molecules on Mϕs and T cells (128,271). CD40L is transiently expressed on the surface of $CD4^+$ and $CD8^+$ lymphocytes, mast cells, NK cells and γδ T lymphocytes. CD40 delivers potent co-stimulatory signals to effector cells through CD40L and is responsible for the initiation of immune responses (9).

When compared to their wild type counterparts, activated T cells from CD40L knockout mice are deficient in their ability to induce Mϕ TNF-α production (270). A direct role for CD40/CD40L interactions in the induction of Mϕ NO production is suggested both by the ability of anti-CD40L antibodies to inhibit the induction of NO by fixed activated lymphocytes and by the observation that T cells from CD40L knockout mice are impaired in their ability to induce Mϕ NO production (270).

A significant reduction in both IL-4 and IFN-γ production was observed in CD40L-deficient mice, suggesting that CD40L is required *in vivo* for clonal expansion of antigen-specific T cells (115,220,270). CD40L expression on T cells is regulated by IL-12 and IFN-γ. This was substantiated by experiments that showed *in vivo* administration of anti-IL-12 and anti-IFN-γ antibodies causes inhibition of CD40L expression on T cells and suppression of cytotoxic activity (137). Peng *et al.* showed that IL-12 enhances functional CD40L expression on T cells (235). A signal delivered to T cells via CD40L triggering can upregulate T-cell activation and synergize with IL-12 to induce IFN-γ production (235). IL-12 has potent antitumor activity in several established murine tumor models, and Mϕ-derived IL-12 promotes cell-mediated antitumor responses during tumor growth (45,100).

Cytokines and the Transcription Factor ICSBP

Cytokines are soluble communication molecules that facilitate interactions between immune cell types through autocrine, paracrine, and endocrine mechanisms (23,24,154,197,215,229). Cytokines are produced by numerous cell types and have multiple targets, both within and outside the immune system. Cytokines are closely associated with tumor cell growth, angiogenesis, and metastasis (158,161,300). Tumors may promote their own growth through overproduction of cytokines that modulate

immune responses, including TGF-β_1 (16,18,257), IL-10 (15-17,138,257,294), and PGE_2 (12,13,72,180). We have identified several pathways through which cancer cells disrupt cellular responsiveness to, or alter production of, cytokines (83,204,208).

Cytokines, like IL-12 and IL-18, are messenger molecules that stimulate the immune system directly by interacting with specific receptors on cell membranes and target effector cells such as Tc cells and NK cells (125,213,252,285,321). These cells can be stimulated to generate specific or nonrestricted cytolytic responses and are potentially effective in the control of cancer growth and its metastasis (124). Such immunologically mediated antitumor responses can sometimes induce immunological memory whereas conventional chemotherapy alone, rather than stimulating the immune system, is usually immunosuppressive and can even lead to infection by opportunistic microorganisms (112). Since it was characterized in 1989, there has been significant data that indicate IL-12 plays a key role in immune responses (265,282). In addition to regulating IFN-γ production, IL-12 promotes the development of $CD4^+$ Th cells (263). Because IL-12 is secreted by professional APCs (68) and IL-12 receptors are expressed selectively on activated T lymphocytes and NK cells, IL-12 may represent the link between innate and adaptive immunity (50,281).

Interleukin-12

IL-12 is a heterodimeric cytokine produced mainly by phagocytic cells and APCs (68,126,173,264,282). Mapping studies show that the p35 and p40 genes are located on different chromosomes; the p40 gene is on chromosome 5 (5q31-q33), and the p35 gene is on chromosome 3 (3p12-3q13.2) (120). IL-12 is best characterized by its ability to induce IFN-γ secretion from T cells and NK cells (101,151), thereby facilitating Th1 responses (181) and regulating the balance of Th1 and Th2 cells (237). Studies using anti-IL-12 Ab or IL-12 knockout mice demonstrate that increased resistance to a number of infectious agents requires production of endogenous IL-12 (106). IL-12 plays a unique role in linking innate and acquired immune responses, allowing phagocytic cells to facilitate the development of cell-mediated immunity (59,310).

Bioactive IL-12 p70 is a heterodimer comprised of two disulfide-linked subunits, designated p35 and p40, which are regulated independently (254,315). Whereas IL-12 p40 is secreted in monomeric and homodimeric forms (166), secretion of IL-12 p35 has not been reported to date (68). Typically IL-12 p70 production *in vitro* and *in vivo* is accompanied by excess production of the p40 subunit (104,106) which functions as an antagonist of bioactive IL-12 p70 (166). Among the cytokines, IFN-γ augments IL-12 production whereas IL-4, IL-10, IL-13 and TGF-β suppress IL-12 production (67,266). T cells or NK cells infiltrating tumors in IL-12-treated mice secrete IFN-γ, which in turn

enhances the functions of Mϕs or T cells present at the tumor site. IL-12 induces high levels of IFN-γ, which can mediate a variety of antitumor effects (120).

IFN-γ exerts a potent priming effect on IL-12 p40 induction in cultured Mϕs stimulated with LPS (261). Cytolytic peritoneal Mϕs were obtained from mice injected with IL-12, mediated in part by the induction of IFN-γ (120). IL-12 p40 is also induced by a T cell-dependent mechanism through the CD40/CD40L interaction (261).

A more recent area of investigation is the role of Mϕs in mediating the antitumor effects of IL-12. Mϕs were found to infiltrate Renca (44,105) and melanoma (276) tumors in IL-12-treated mice. Cytolytic peritoneal Mϕs were obtained from mice injected with IL-12, mediated in part by the induction of IFN-γ (120). IL-12 treatment also induced NO production *in vivo* and IFN-γ also promotes NO production by Mϕs (64).

Interleukin-18

IL-18 was originally described as an IFN-γ-inducing factor in mice primed with *Propionibacterium acnes* and challenged by LPS (227). The murine IL-18 gene maps to chromosome 9 (249) and the human IL-18 gene is located on chromosome 11q22 (222).

Murine and human IL-18 gene sequences encode 192 and 193 amino acid precursors proteins, respectively (286). IL-18 is synthesized as a biologically inactive, 24 kDa precursor molecule lacking a signal peptide. Cleavage into an active mature 18 kDa molecule requires intracellular cysteine protease, IL-1β converting enzyme (ICE), also known as caspase-1 (108,117). Caspase-3, involved in apoptosis, cleaves both precursor and mature forms of IL-18 into biologically inactive degraded products and may constitute a potential downregulator of IL-18 (7).

The IL-18 receptor complex is comprised of a ligand-binding chain designated IL-18Rα that has a low affinity for IL-18 and another non-IL-18-binding chain designated IL-18Rβ. IL-18Rβ binds to the complex formed by IL-18 bound to the IL-18Rα chain, and this heterodimer is called the high affinity complex (75).

IL-18 induces IFN-γ production by activated murine and human T cells, in synergy with IL-12 (4,210). The upregulation of IL-18Rα gene expression by IL-12 may explain its capacity to increase IL-18-induced IFN-γ production by T cells (317). The synergy between IL-12 and IL-18 can be explained by the use of two distinct transduction pathways to trigger IFN-γ gene transcription (26). IL-18 directly induces IFN-γ promoter activity at the AP-1 site, whereas IL-12 induces the occupancy of the STAT4 binding site (26). The combination of both cytokines activates AP-1 and STAT4 binding sites, as demonstrated in human $CD4^+$ T cells (26).

Due, in large part to its ability to induce IFN-γ production, IL-18 is one of the most promising new cytokines with antitumor activity (76). IL-18 administered to mice as an adjuvant for increased tumor immunogenecity caused enhanced NK-cell activation, production of IL-2, and sequentially decreased IL-10 levels (39). IL-18 stimulates primarily NK cell-mediated antitumor effects against interperitoneal growing Meth A sarcoma, induces cytotoxic $CD4^+$ T cells, and evokes immunological memory (195). IL-18 initially stimulates a non-specific arm of the immune response, followed by the development of a specific Tc-mediated immune response (195).

Interferon-γ

IL-12 and IL-18 induce IFN-γ production that regulates a variety of important immunologic pathways. IFN-γ is the predominant cytokine during Th1-dominated immune reactions and participates during antigen presentation (40). IFN-γ regulates host defenses in both innate and acquired immunity. It enhances the activity of Mϕs and NK cells, the expression of MHC class I and II, and Ig secretion by B cells (37). In monocytes and Mϕs, IFN-γ induces IL-1 and TNF-α secretion (127). IFN-γ is secreted mostly from T cells when they are activated by antigens or mitogens (313). IFN-γ was previously called the Mϕ activation factor and, indeed, it plays particularly important roles in Mϕs, which include elicitation of antipathogenic activity and antitumor activity,

stimulation of chemokine/cytokine production, and enhanced antigen presentation (305,314). Activation of the JAK/STAT pathway is the first event in IFN-γ signal transduction (58). Binding of IFN-γ to the receptor leads to activation of JAK1 and JAK2 kinases, which phosphorylate the latent transcription factor STAT1. STAT1 is translocated into the nucleus to bind to the IFN-γ activation site, after which transcriptional induction of IFN-γ responsive genes ensues. STAT1 is required for IFN-γ dependent transcription and for its biological activities (58). IFN-γ has anti-proliferative activity on tumor cells (247).

IFN-γ enhances many antigen-nonspecific immune and nonimmune mechanisms, many of which favor tumor regression. IFN-γ production promotes immune recognition of tumor cells by enhancing MHC molecule expression and stimulates cytotoxicity mediated by NK cells, T lymphocytes, and Mϕs. IFN-γ promotes tumor regression through the inhibition of cell proliferation, direct toxic effect on cells in combination with TNF-α, and induction of iNOS (37).

Effect of IL-12 and IL-18 on Tumors

IL-12 enhances cellular immune mechanisms by favoring the differentiation of $CD4^+$ Th cells towards the Th1 subset and upregulating MHC expression (284,319). IL-12 induces Th1 cells to secrete IFN-γ, which facilitates the proliferation and/or activation of $CD8^+$ Tc cells, NK cells, and Mϕs, all of which can contribute to tumor regression (36,214). Many of IL-12s therapeutic effects (191) are dependent on IL-12-stimulated IFN-γ secretion by T and NK cells (48,62,63,214). IFN-γ enhances many antigen-nonspecific immune and non immune mechanisms, many of which favor tumor regression. These include direct cytotoxicity in combination with TNF-α (37), a slowing of cellular proliferation (37), induction of NO production (102,243), and inhibition of angiogenesis (179,259,292,300). IL-12 potentially activates a variety of antitumor mechanisms, some of which are antigen-specific.

IFN-γ release is also induced by IL-18. IL-18 is produced by activated Mϕs, promotes IFN-γ release (194), and inhibits the production of IL-10 by activated T cells (227,286). IL-18 augments both murine and human NK cytotoxicity (227,286) and stimulates FasL-mediated tumor cell cytotoxicity by NK cells (285). IL-18 activities suggest that it may have antitumor activity. This possibility was recently confirmed when recombinant murine IL-18 was shown to enhance the survival of Balb/c mice bearing Meth A tumors (195).

Interferon Consensus Sequence Binding Protein

IFN-γ has two important roles: (a) IFN-γ is a critical effector molecule leading to activation of Mϕs and subsequent killing (255,305) along with generating a cell-mediated immune response, and (b) IFN-γ enhances IL-12, IL-18 production (172) and inhibits IL-10 production (52) leading to the maintenance of a Th1 type response. Due to the diverse and important functions of IFN-γ, there is great interest in studying the transcription factors that are induced by IFN-γ.

The study of genes transcriptionally activated by IFN-γ has identified homologous sequences within their promoters that act as cis-acting elements to regulate the expression of genes activated by IFN-γ. The first sequence of this type was identified by Friedman and Stark (99) and was designated the IFN consensus sequence (ICS) (122). It was found that a sequence within the ICS, termed the IFN-stimulated response element (ISRE), conferred IFN inducibility on heterologous promoters (219). The ISRE is bound by members of a growing family of nuclear proteins called IFN regulatory factors (IRFs) (219) of which ICSBP is a member. These proteins share significant homology in their N-terminal domains, which allows for interaction with a consensus DNA sequence, (G/C)(A)AAAN$_{2-3}$AAA(G/C)(T/C), present in the ISRE. There is only limited homology between any of these proteins at their C-terminal ends (219). ICSBP has three functional

domains: a DNA binding domain, a transcriptional repressor domain, and a domain that enables the association with other IRFs (260).

ICSBP is an immune system-specific transcription factor (218,238) induced by IFN-γ through the GAS sequence present in its promoter (58). ICSBP is capable of stimulating transcription from IFN-γ-inducible promoters in a GAS-dependent manner (58). ICSBP binds to the ISRE and controls activities of promoters carrying this element (69). There is an ISRE element in the promoter of the IL-12 p40 gene and there are two ISRE elements in the promoter of the CD40L gene at positions -361 to -348 and -141 to -125 (283). ICSBP functions in Mϕs as a positive transcriptional activator of its own promoter (140), of IL-12 p40 promoter (253), and of the IL-18 promoter (149). Impaired production of ICSBP by tumor cell-derived factors will lead to reduced IL-12, IL-18, and CD40L expression in the TBH.

Like IL-12 p40, ICSBP is induced in Mϕs by IFN-γ and LPS in a synergistic manner (140). ICSBP induction is a consequence of STAT1 activation, which leads to stimulation of ICSBP promoter activity through the GAS element. ICSBP in turn potentiates STAT1-dependent activation of IFN-γ-responsive promoters through the GAS element (139). ICSBP is a late-acting activator of IFN-γ responsive genes involved in the elicitation of IFN-γs unique activities in immune cells (58). After STAT1 activity declines, ICSBP prolongs transcription from respective promoters thereby sustaining the

effect of IFN-γ (58). The most striking defects found in ICSBP $^{-/-}$ mice are those associated with Mϕ functions (58,109,253,309). ICSBP is capable of stimulating only a certain set of GAS elements. ICSBP selectivity is affected by other components such as chromatin structure and recruitment of other cofactors (58). The iNOS gene promoter contains a functional GAS element. It is not surprising that NO production was significantly reduced in IFN-γ-treated ICSBP $^{-/-}$ Mϕs compared with their $^{+/+}$ counterparts (102). In summary, Contursi *et al.* showed that IFN-γ sequentially activates STAT1 and ICSBP in Mϕs, thereby augmenting transcription of certain IFN-γ responsive genes whose functions are relevant to IFN-γs unique role in these cells (58).

ICSBP is expressed in human cells of the Mϕ and lymphocyte lineage (302). In IFN-γ-activated EL4 T cells, ICSBP expression is associated with activation of STAT1, which binds to the pIRE of the ICSBP promoter (218). Because levels of ICSBP expression in unstimulated APCs are low, the CD40/CD40L interaction probably stimulates induction of ICSBP in these cells in a manner similar to IFN-γ stimulation (238). Wu *et al*. showed that ICSBP $^{-/-}$ APCs are unresponsive to stimulation by CD40/CD40L interactions, suggesting that T-cell-dependent induction of IL-12 p40 also requires ICSBP (309).

To summarize, suppression of CD40L expression during tumor growth decreases IL-12 and IL-18 production, impeding T-cell IFN-γ production. IFN-γ is essential for

expression of ICSBP, which regulates CD40L, IL-12 and IL-18 expression through the ISRE. The overall effect is manifested in the form of reduced tumoricidal activity by TBH Mϕs.

Cytotoxic Effector Molecules

Mϕ cytostatic and cytotoxic activities against tumor cells contribute significantly to tumor rejection (136,150). Through secreted molecules such as NO (267) and TNF-α (90), activated Mϕs directly inhibit or kill tumor cells. Tumors may escape Mϕ cytotoxic activities by producing immunomodulatory molecules that inhibit production of cytotoxic effectors *in situ* while simultaneously promoting cytotoxic molecule production by tumor-distal Mϕs. Molecules such as NO fail to mediate antitumor activity when released in tumor distal compartments but may compromise T-cell function (27). TBH Mϕs outwardly appear to be unactivated, but they are actually primed for tumoricidal activity (46,79,184) and the production of cytokines (10,12,13), NO (16,203), and prostaglandins (10,12,13,217,230). TBH Mϕs are considered primed because they constitutively express mRNA for TNF-α (10,13,30) and other cytokines (12). After activation, these TBH Mϕs demonstrate superior capacity to kill tumors and to produce larger amounts of factors, as compared with normal host Mϕs (38,46,92).

Nitric Oxide

L-arginine-derived NO, a soluble cytotoxic molecule, is produced in activated Mϕs by iNOS (216). Several reports show that *in vitro*-activated Mϕs kill some tumor cells by a NO-dependent mechanism that can also work in the absence of cell- to-cell contact (66,123,144). NO can also act as a regulator and this will be discussed later.

NO, when synthesized in large amounts, acts as a potent cytotoxic effector molecule in immune defense (165). The damage to target cells by activated Mϕ released NO or endothelial cells is confirmed *in vitro* (19), and both necrotic and apoptotic pathways of cell death can be triggered by high doses of NO (153). At present, it is not well understood which actions of NO are responsible for the induction of apoptosis or necrosis. Primary candidates as targets for induction of cell death are nuclear and mitochondrial DNA, as well as the mitochondrial electron transfer chain and mitochondrial membrane permeability (152). NO interferes with the heme groups of the electron transfer complex IV, and NO can interact directly with DNA causing deamination. An important additional aspect is that uncoupling the electron transfer chain gives rise to enhanced production of oxygen free radicals; the free radical might react with NO, leading to the formation of the peroxynitrite anion, which is an extremely potent oxidant (152).

The role of NO in tumor biology is poorly understood. Growth of solid tumors is regulated by interactions of endothelial cells of the tumor vasculature, tumor-infiltrating immune cells such as T lymphocytes and Mϕs, and the tumor cells themselves (135). Most of these cellular components generate NO *in vitro* (241). IL-12 primes Mϕs for NO production *in vivo* (304).

TNF-α

TNF-α exerts a key role in the cytokine network. TNF-α was purified and sequenced (2) and the TNF-α-encoding gene was cloned in the mid-1980s (236,298). Since then, several biological properties of this cytokine have been demonstrated, in addition to the induction of cachexia and tumor cell lysis that originally led to its identification (34). As early as 1893, it was observed that severe infection could lead to a reduction in the size of a malignant tumor (56). This can now be attributed to the release of cytotoxic cytokines like TNF-α.

The major source of TNF-α is activated monocytes/Mϕs. Human TNF-α is synthesized as a pre-protein comprising 233 amino acids, with a molecular mass of 26 kDa. The pre-protein is cleaved by a specific metalloprotease to yield a monomeric form of 17 kDa (81).

TNF-α effects are transmitted via crosslinking of the membrane-bound receptor molecules TNF receptor I (TNFRI, p55) and TNFRII (p75) (28). The extracellular portions of both TNF receptors can be shed, and these soluble receptors retain the ability to bind TNF-α and thus can limit acute TNF-α effects (86). After binding to its membrane-bound receptors, TNF-α mediates diverse effects. Signal transduction distal to the TNF receptors involves phospholipase C and sphingomyelinases, which release ceramide from sphingomyelin and activate ceramide dependent protein kinases (81). The therapeutic application of TNF-α has been investigated in several malignant diseases, although many studies have been limited by a high rate of severe side effects (81).

Regulatory Roles of NO and TNF-α

NO generated by iNOS is more than just a cytotoxic molecule at work in immune defense; it appears to exert substantial immunoregulatory activity (152). Immune cells exhibit a specific response when exposed to NO, with substantial consequences for cytokine- and apoptosis-dependent immunoregulatory balance (152). Exposure to NO suppresses IL-2 gene expression at the level of transcription by interfering with zinc-finger transcription factor activity. NO modulates the Th1/Th2 balance by favoring Th2 responses (152). In Mϕs, NO induces transcription of the IL-12 p40 gene but not of the IL-12 p35 gene (248). Because the IL-12 $(p40)_2$ is an antagonist for IL-12 (188), this

might be a further indication of less Th1 reactivity in the presence of NO. NO inhibits MHC class II expression and increases PGE_2 production in Mϕs (118,262). NO interferes with selectin-dependent adhesion, which downregulates the accumulation of Th1 cells at sites of inflammation (155). The regulatory actions of NO released from iNOS are mainly due to modification of transcription factors, proteins controlling translation, mitochondrial functions, and apoptosis (152).

TNF-α strongly suppresses T-cell proliferation by an autocrine pathway that induces Mϕ PGE_2 and NO production (10,16). TNF-α induces peritoneal Mϕs to mediate suppression but causes splenic Mϕs to upregulate T-cell proliferation (13). TNF-α may play a suppressive role *in vivo* because peritoneal Mϕs are the strongest TNF-α producers (31,203,290), and TNF-α would always be produced in the microenvironment of these Mϕs. In our Meth-KDE tumor model, TNF-α production occurs even in tissues far removed from tumor growth suggesting the possible *in vivo* suppressor role of TNF-α (83).

Evasion Strategies of Tumors

Tumor escape from the immune response is currently the greatest impediment to the application of effective immunotherapy (231). The ideal immune response often does not

occur in cancer patients because cancer cells evolve mechanisms to evade the body's defenses. The immune surveillance theory hypothesizes that cancerous cells arise regularly, but the body eliminates them before they become harmful to surrounding tissue; only those that evade surveillance develop into tumors. Thus, there is a strong selective pressure favoring cancer cells that can avoid notice or somehow prevent themselves from being killed by the immune system.

Because cancer cells arise from normal cells, many of their evasion strategies are shared by healthy tissue. Ordinarily, the immune system does not want to kill the body's own, healthy cells. These self-cells are protected from immune responses by a variety of mechanisms including self-tolerance, sequestration of tissue from surveillance, antigen shedding, lymphocyte killing, secretion of immunosuppressive cytokines, lack of MHC II expression, lack of costimulatory molecules, and local secretion of prostaglandins neuropeptides. Many of these evasion strategies are maintained in or exploited by cancer cells. However, because cancer cells contain altered proteins, the immune system may be able to recognize tumor-specific peptides as foreign antigens. As a result, many cancer cells have additional mechanisms to escape host immunity that may include upregulation of the evasion mechanisms shared by healthy cells, downregulation of MHC I expression or peptide presentation, antigenic modulation, and failure of lymphocyte homing. These strategies arise through the selection and outgrowth of mutant cells that escape the immune system. Knowledge of the particular evasion strategies that tumors employ is

necessary to design vaccines that specifically target these strategies and may be useful to predict responsiveness to available radiation or chemotherapy (228).

Tumors Circumvent Mϕ-mediated Cytotoxicity

Although *in vitro* investigations established that Mϕs can kill tumor cells while leaving normal cells unharmed (1,92,150), Mϕ-derived cytotoxic molecules may not be effective during *in vivo* tumor growth (92,150,164,280). This finding suggests that tumors somehow interfere with the activity or production of cytotoxic molecules, leading to the reduction of Mϕ cytotoxic activity (38,54). Many tumor-derived molecules such as IL-4, IL-6, IL-10, TGF-β, and PGE_2, deactivate or suppress activated-Mϕ-mediated cytotoxic activity (5,208,272). Although TBH Mϕs normally are primed for enhanced cytotoxicity, tumor supernatants suppress activated-Mϕ tumoricidal activity (10,13,15,16,46,47,89,107,132,134,320), and tumor-derived cytokines and chemotactic molecules fail to stimulate Mϕ TNF-α and NO production (290). In our tumor model, LPS activation of isolated TAMs failed to induce NO production *in vitro* (208). Furthermore many tumors (especially spontaneously arising neoplasms) have mechanisms to resist toxicity from one or more Mϕ-derived cytotoxic molecules (92,150,297). Tumors can stimulate Mϕs to produce cytotoxic molecules through tumor soluble or membrane-bound tumor-associated antigens (TAAs) (134,320), extracellular

matrix proteins (ECM) (121), or receptor-mediated binding of Fc portions of Ab (71,92) attached to tumor cells (297). Although Mϕ production of TNF-α and NO imparts cytotoxic and suppressor activities, tumor growth also increases Mϕ production of the noncytotoxic suppressor molecules PGE_2, TGF-$\beta_{1,}$ and IL-10 (208). The simultaneous action of several Mϕ-derived molecules is required for lysis of many spontaneous tumors, suggesting that the inhibition of one type of cytotoxic molecule may be sufficient for the tumor to escape lysis (297).

Tumor Cell-Derived Cytokines Inhibit Th1-Type Immune Responses

Cell-mediated responses tend to be optimal in the eradication of tumors (33,92). Tumor cell-derived IL-4, IL-10, TGF-β (16,53,189,272) and PGE_2 (16,32) suppress the necessary cell-mediated responses supported by Th1-type cells during cancer, while supporting the largely ineffective humoral responses maintained by Th2-type cells. Tumors may create environments that inhibit Mϕ cytotoxicity by either directly suppressing it (8,122) or by inhibiting Th1 type cells that induce Mϕ tumoricidal actions (240). Tumor cell-derived IL-10 blocks tumor-induced Mϕ-mediated suppression of lymphocyte proliferation (15), unlike TGF-β_1 and other tumor-derived cytokines. Therefore tumor-derived IL-10 blocks Mϕ cytotoxicity at the tumor site (16,199,239). At the same time, IL-10 can directly suppress proliferation of alloantigen- or mitogen-

stimulated Th cells (15,199). Studies show that IL-10 preferentially downregulates Th1-type cell activity by inhibiting Mϕ co-stimulatory activity, which is necessary for Th1-type cell activation (199). Th1-type cells promote Mϕ activation by producing IFN-γ, whereas Th2-type cells suppress Mϕ accessory and cytotoxic activities by producing IL-4 and IL-10 (239). IL-10 hinders Mϕ NO and TNF-α production (15) and may reduce IFN-α and IL-12 production (204,208), which is unfavorable for the generation of Th1-type cells (246).

TGF-β-Mediated Immunosuppression

TGF-β may be the most potent immunosuppressive factor to be characterized (162). It affects proliferation, activation, and differentiation of the cells that participate in both innate and acquired immunity. TGF-β inhibits immunoregulatory cytokine production (87), including monocyte production of the important regulatory cytokine IL-12 (42,129). Several studies suggest that TGF-β-induced functional inhibition may be associated with modulation of the expression of surface receptors important for cell activation and growth (200). As a principal natural suppressor, TGF-β is a potent inhibitor of Tc cell differentiation (250), suggesting that this factor may affect the development of tumor-reactive Tc cells *in vivo*. Studies *in vivo* showed that TGF-β administration led to inhibition of T cell responses to viruses and allogeneic tumors (97). TGF-β may block

the signaling pathway initiated by cytokines (233). TGF-β inhibits IL-12/IL-18-induced IFN-γ production in NK cells and T cells (129,162). TGF-β may inhibit IL-12 mediated T-cell proliferation through an effect on the IL-12 transduction pathway (53). TGF-β reduces Mϕ NO production by inhibiting iNOS activity (291) and NO production from Mϕs (74). It is clear from these observations that TGF-β exerts immunosuppressive activities that could favor tumor growth *in vivo* (53).

IL-10-Mediated Immunosuppression

IL-10 is spontaneously secreted by a variety of human tumors (103). It was initially identified as a factor produced by Th2 cells that inhibits cytokine synthesis by Th1 cells (70). This effect was attributed to inhibition of the accessory function of Mϕs, including downregulation of MHC class II expression, leading to impaired antigen presentation to reactive T cells (96). TGF-β may help shift the Th1-Th2 balance towards Th2 by favoring the development of Th2 type cells via IL-10 overproduction and the inhibition of Th1-type responses (178). Given the spontaneous secretion of IL-10 by tumor cells, it is tempting to speculate that Th1 function is downregulated during tumor growth. In our experiments, IL-10 secreted by the tumor suppresses ICSBP production which leads to downregulation of Th1 function (Martins and Elgert, unpublished observations). Peng *et al*. claim that exogenous rIL-10 inhibited CD40L expression (235). Th1 type cytokines

promote cell-mediated immunity, and thus IL-10 mediated inhibition is likely to be beneficial to tumor survival. Local secretion of IL-10 can render tumor cells totally insensitive to Tc lysis (29,186), suggesting that this cytokine is an important contributor to downregulation of the anti-tumor immune response (53).

To summarize, suppression of CD40L expression during tumor growth correlates with decreased IL-12 and IL-18 production, and reduced T-cell IFN-γ production. IFN-γ is essential for expression of ICSBP, which regulates IL-12 and IL-18 expression through the ISRE. Tumor cell-derived factors (TGF-β_1 IL-10, and PGE_2) could also directly impair ICSBP expression. The overall effect is manifested in the form of reduced tumoricidal activity by TBH Mϕs.

SECTION II

TUMOR CELL-DERIVED TGF-β AND IL-10 DYSREGULATE MACROPHAGE ACTIVITIES THROUGH INHIBITION OF INTERFERON CONSENSUS SEQUENCE BINDING PROTEIN

ABSTRACT

Tumors evade immune responses, in part, through the release of suppressor signals that dysregulate host effector cell function. In this study, we demonstrate that tumor-derived suppressor molecules impede host antitumor immune function through dysregulation of Mϕ cytotoxic and effector functions. Tumor cell-derived supernatant suppressed primary Mϕ production of the cytotoxic effectors, tumor necrosis factor (TNF)-α and nitric oxide (NO), and a correlative decrease in direct Mϕ-mediated tumor cytotoxicity was observed using primary Mϕs incubated with Meth-KDE supernatants. Tumor-derived factors also dysregulated primary Mϕ production of proinflammatory cytokines, interleukin (IL)-12 and IL-18, and parallel studies confirmed similar suppression of these cytokines in tumor-bearing host (TBH) Mϕs. These data compliment T cell studies that revealed a deficit in IFN-γ production and CD40L expression in the TBH. Because these data collectively suggest a defect in IFN-γ-associated activities, we evaluated expression of interferon consensus sequence binding protein (ICSBP), a critical transducer of activation signal pathways in macrophages (Mϕ). ICSBP expression was dysregulated in TBH Mϕs, and tumor supernatants similarly inhibit expression. Depletion studies revealed that IL-10 and transforming growth factor β_1 (TGF-β_1), but not prostaglandin E_2 (PGE_2), impaired ICSBP, suggesting that abrogation of these factors *in situ* might restore Mϕ function and enhance antitumor efficacy.

INTRODUCTION

A functional immune system is a potential barrier to tumor development and progression. To evade these mechanisms, tumors may produce immunosuppressive factors that obstruct immunologic antitumor responses. As tumors progress, growth kinetics hasten and the tumor surpasses the control capacity of the immune system, leading to progressive decreases in cell-mediated antitumor responses and accelerated disease (195). We and others have shown that tumor cell-derived factors such as interleukin (IL)-10, transforming growth factor (TGF)-β, and prostaglandin E_2 (PGE_2) induce systemic immunosuppression and selectively alter macrophage (Mϕ) expression of pro-inflammatory cytokines and other potential mediators of immunosuppression (16,82,169). These factors subvert Mϕ function to favor tumor growth (83). Because restoration of Mϕ antitumor function is a desirable goal for clinical therapy of tumors, we used our well-established tumor model to determine the effect of tumor-derived suppressor molecules on Mϕ and T cell effector function and cytokine production. We show that tumor cell-derived factors dysregulate Mϕ function and activity, both directly (decreased NO, TNF-α, IL-12, IL-18 production, and ICSBP expression) and indirectly (reduced T cell IFN-γ production and CD40L expression).

A mechanism by which tumors may modulate Mϕ activity involves the control of interferon (IFN)-γ responsivenes. IFN-γ plays a central role in Mϕ-T cell interactions. T cells produce IFN-γ in response to activation by antigen-presenting cell (APC)-derived cytokines IL-12 and IL-18 (4,194). IFN-γ primes Mϕs for enhanced production of cytotoxic effector molecule and proinflammatory cytokines, including IL-12 and IL-18. Thus, IFN-γ is a central effector molecule in expanding and maintaining effective immune responses. Tumors may subvert adaptive immunity by disrupting this system by modulating cytokine production, inhibiting receptor expression, or dysregulating intracellular signal pathways. We (204,206,207) and others (51,119) described tumor-induced suppression of Mϕ IL-12 and $CD4^+$ T cell IFN-γ production demonstrating that tumors inhibit cytokine release. Because tumor growth modulates IFN-γ-induced Mϕ IL-12, IL-18, and subsequent T-cell IFN-γ production, we hypothesized that tumor-derived factors may interfere with Mϕ responsiveness to IFN-γ. We evaluated Mϕ expression of the central signal transduction molecule interferon consensus sequence binding protein (ICSBP) in normal and TBH Mϕs. The transcription factors that control IFN-γ activity form an integral part of a host's immune response. Interferon consensus sequence binding protein (ICSBP) is unique among transcription factors controlled by IFN-γ activity in that it is expressed exclusively in cells of the immune system (238). IFN-γ directly induces ICSBP expression in peritoneal Mϕs (238). ICSBP regulates IL-12 (109,253) and IL-18 (148) expression through the ISRE in the promoter of these genes.

In this study, we describe tumor-induced dysregulation of ICSBP expression in both primary *ex vivo* TBH Mϕs and normal Mϕs cultured with tumor-derived factors.

MATERIALS AND METHODS

Murine Tumor Model

Eight to twelve-week old Balb/c (H-2^d) male mice (Harlan Sprague-Dawley, Madison, WI) were used. A Balb/c nonmetastatic methylcholanthrene-induced transplantable fibrosarcoma, designated Meth-KDE was used as described (85). The Meth-KDE fibrosarcoma induces significant immunosuppression through the production of the soluble suppressor cytokines IL-10, TGF-β_1, and PGE_2, as described (16). Tumors were induced by intramuscular (i.m.) injection of 4 x 10^5 transplanted Meth-KDE cells and palpable tumors developed within 10 days. TBH Balb/c mice were used 21 days post tumor induction because tumor-induced Mϕ suppressor activity is maximal at this time, without cachexia or necrosis.

Tumor Cell Culture and Supernatant Preparation

The Balb/c-derived fibrosarcoma designated Meth-KDE was used to generate tumor-derived supernatants. Meth-KDE cells were purified as described (208) and maintained by diluting 1:10 in fresh medium every fifth day. Tumor cell supernatant was obtained by culturing the purified Meth-KDE tumor (4 x 10^6 cells/ml at 37°C, 5% CO_2) in 24-well

culture plates (Corning, Corning, NY) in a total volume of 1.0 ml complete RPMI medium. Cell-free Meth-KDE supernatants were obtained by culturing the purified Meth-KDE tumor (4 x 10^6 cells/ml at 37°C, 5% CO_2) in 24-well culture plates (Corning, Corning NY) in a total volume of 1.0 ml complete RPMI medium. Cell-free Meth-KDE supernatants were collected by centrifugation (400 x g, 5 min) at 72 h (optimal time) and passed through a 0.4-micron filter.

Fresh Meth-KDE supernatants were depleted of IL-10 or TGF-β_1 as described (16). Briefly, tumor supernatant (1.0 ml) was incubated (2 h at 37°C) in 24-well plates that were coated with cytokine-specific Abs. Prior to addition of the tumor supernatants, plates were coated with anti-IL-10 or anti-TGF-β_1 by adding concentrated Ab preparations to wells, incubating overnight at 4°C, and washing three times with phosphate buffered saline (PBS). Wells were blocked with sterile PBS containing 5% fetal bovine serum (FBS) for 2 h at room temperature and washed three times with PBS. Meth-KDE supernatants (500 μl/well) were incubated in coated plates or control plates (non-Ab coated, serum-treated wells) for 2 h at 37°C and then immediately transferred into assays. PGE_2-depleted tumor supernatants were prepared by incubating Meth-KDE cells, as described, with 10^{-7} M indomethacin (Sigma Chemical Co., St. Louis, MO), an arachidonic acid pathway inhibitor. Resulting supernatants did not contain detectable PGE_2, as measured by specific enzyme-linked immunosorbent assay (ELISA; Advanced Magnetics, Boston, MA).

Immune Cell Collection and Culture

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

T cells were collected by plating pooled normal host or TBH-derived whole spleen cells for 2 h in glass plates and collecting the nonadherent cell fraction. Red blood cells were lysed with 0.83% ammonium chloride (Sigma).

To obtain purified $CD4^+$ T cells, the nonadherent cell fraction was treated with anti-CD8 (ATCC clone; 3.155), anti-IA^d (ATCC clone; MK-D6), and anti-B cell and immature T-cell (ATCC clone; J11d) monoclonal antibodies and complement (Accurate). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Media and Reagents

Mϕs were cultured in serum-free RPMI-1640 medium with 2 mM L-glutamine (Sigma). All media contained 50mg/L gentamicin sulfate (Tri-Bio Laboratories, State College, PA), 25 mM sodium bicarbonate ($NaHCO_3$), and 25 mM HEPES buffer (Sigma). RPMI-1640 medium was endotoxin-free (<10 pg/ml) as assessed by the *Limulus* amebocyte lysate assay (Sigma). T cell cultures were supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype 026:B6) was purchased from Sigma. Recombinant murine IFN-γ (specific activity 2.98 x 10^6 U/ml; endotoxin content <10 pg/ml) was purchased from Genzyme, Inc. (Cambridge, MA).

Mϕ Nitrite Production

Either 4 x 10^6 normal host or TBH peritoneal Mϕs were cultured in 96-well flat-bottom tissue culture plates (Corning Cell Wells) without or with 72 h Meth-KDE supernatants (1:2 dilution). 50 U/ml IFN-γ, or a combination of 50 U/ml IFN-γ and 1 μg/ml LPS was added at the start of incubation. Supernatants were collected after 48 h (optimal incubation time) for nitrite assessment following centrifugation (400 x g, 5 min). Mϕ viability remained >95% throughout the culture period as determined by the MTT assay

(201). Nitrite levels in culture supernatants were measured using the Griess reagent (113). Briefly, 100 μl Mϕ supernatants were added to 100 μl Griess reagent (0.1% naphthylenediamine dihydrochloride, 1.0% sulfanilamide, 2.5% H_3PO_4, Sigma), incubated at room temperature for 10 min, and absorbance read at 570 nm (MR 600 microplate absorbance reader; Dynatech Laboratories, Alexandria, VA). A sodium nitrite (Sigma) standard curve was used to calculate nitrite content in supernatants. Nitrite was not detected in RPMI-1640 medium alone.

Mϕ-Mediated Cytotoxicity Assays

To assess direct cytotoxic activity of Mϕs against Meth-KDE tumor cells, Mϕs (2 x 10^5 cells) were added to 1 x 10^5 tumor cells. Cytotoxicity was measured using a modification of the Alamar blue™ (Biosource International, Camarillo, CA) colorimetric viability assay as previously described (209). Briefly, Meth-KDE cells were seeded into 96-well flat-bottom tissue culture plates in 100 μl RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals) and incubated for 24 h. IFN-γ- and LPS-activated Mϕs cultured without or with 72 h Meth-KDE supernatants (1:2 dilution) were added and culture continued for 24 h in the presence of 10 nM actinomycin-D (Sigma), an antiproliferative agent used to halt tumor cell proliferation during the assay period. Following 20 h of culture, 20 μl (10% of the well volume) of Alamar blue™ indicator

dye was added to each well. In the presence of viable cells, Alamar blue™ dye is reduced to a colored product detectable at 580 nm. Four hours after addition of the indicator dye, tumor cell viability was assessed by measuring absorbance at 570 nm on a MR 600 microplate absorbance reader.

Quantfication of Cytokine Production

IL-12, IL-18, TNF-α and IFN-γ production was induced by culturing Mϕs as described for NO production. Cell free supernatants were collected following centrifugation and assayed by specific ELISA (R&D Systems) per the manufacturers recommended protocol.

Western Blot Analysis of IL-18 and ICSBP

For western blot analysis of Mϕ ICSBP and IL-18, normal host and TBH peritoneal Mϕs were cultured as described. Cells were lysed with 50 mM Tris-Cl, pH 7.6, containing 10 μg/ml leupeptin and aprotinin (Sigma) and 300 mM NaCl. Membranes were pelleted by centrifugation (12,000 x g), and protein concentration was determined using the Sigma Lowry protein assay kit (Sigma). Protein (15 μg) was denatured by boiling in β-

mercaptoethanol (2-ME, 5%) and separated by SDS-PAGE gel electrophoresis using a 10% ProtoGel (National Diagnostics, Atlanta, GA) vertical gel, and transferred to nitrocellulose. The membrane was blocked using 5% non-fat milk (ICSBP) or PBS supplemented with 0.5% Tween-20 (IL-18). ICSBP was specifically detected using polyclonal rabbit anti-mouse ICSBP Ab (Zymed Laboratories, Inc., South San Francisco, CA) at 2 μg/ml and HRP-conjugated goat-anti-rabbit IgG (Transduction Laboratories) at 1:2,000 dilution. IL-18 was detected using monoclonal goat anti-mouse IL-18 (R&D Systems) at 0.2 mg/ml and HRP-conjugated rabbit-anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used at 1:20,000 dilution. Bound ICSBP and IL-18 were visualized using luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and documented on Kodak Biomax film after 30 sec. exposure.

Flow Cytometry

Mϕs and T cells from the spleens of normal host and TBH animals were analyzed for CD40 and CD40L expression by flow cytometry using a Becton-Dickinson FACSCaliber and CellQuest software (BD, Franklin Lakes, NJ). CD40 expression was analyzed using FITC-conjugated hamster anti-mouse CD40 monoclonal antibody and CD40L expression was analyzed using PE-conjugated hamster anti-mouse CD40L monoclonal antibody. T cells were gated using APC-conjugated anti-mouse TCR and Mϕs were gated using APC-

conjugated anti-mouse CD14. All antibodies used for flow cytometry analyses were purchased from BD-Pharmingen, La Jolla, CA.

Statistics and Calculations

Cells from six to 10 normal host or TBH mice were pooled for each experiment. Triplicate cultures were tested for all samples in the Griess reagent tests, cytotoxicity assays and ELISAs. All experiments were repeated at least three times and representative experiments are shown. Data are averages +/- SEM of triplicate independent determinations. All data points on graphs were tested for significance by Student's *t* test.

RESULTS

Tumor Cell-Derived Factors Downregulate Primary Mϕ Cytotoxic Effector Function

LPS activates cytotoxic activity and effector function by IFN-γ primed normal host Mϕs (167,177,182). Tumors induce functional and phenotypic changes that alter tumor associated (74,169) or tumor-distal (16) Mϕ responsiveness to activating agents, and tumor cell-derived supernatants inhibit activation of RAW 264.7 Mϕs (208). To assess the effect of tumor cell supernatants on primary Mϕs, normal host Mϕs were cultured without or with 72 h Meth-KDE-derived cell-free supernatants (1:2 dilution). Priming and activation with IFN-γ (50 U/ml) and LPS (1 μg/ml, optimal dose), respectively, enhanced normal host Mϕ NO production, but the addition of tumor-derived supernatants suppressed NO production regardless of priming and activation signals (Figure 1).

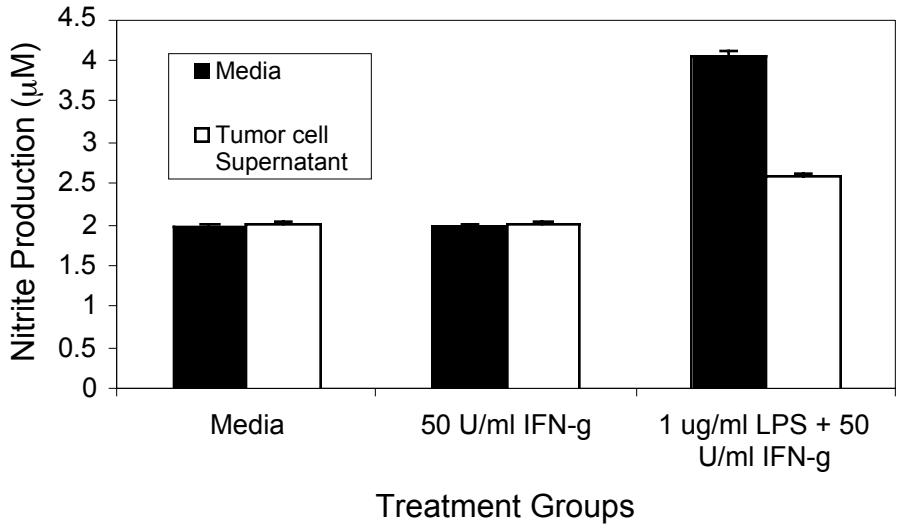


Figure 1. Tumor cell-derived supernatants inhibit Mϕ NO production. Four x 10^6 thioglycollate-elicited Balb/c normal host peritoneal Mϕs were cultured in 200 μl serum-free medium without or with 72 h Meth-KDE supernatants (1:2 dilution). 50 U/ml IFN-γ, or a combination of 50 U/ml IFN-γ and 1 μg/ml LPS was added at the start of incubation and supernatants were collected after 48 h (optimal incubation time) for nitrite assessment using the Griess reagent test. Data are averages +/- SEM of triplicate independent determinations from one of three experiments that have similar results.

Because tumor-derived factors functionally alter LPS-induced peritoneal Mϕ NO production, and the regulation of TNF-α is fundamentally coupled to NO (203), we also examined the effect of tumor cell-derived supernatants on TNF-α. Activation with LPS significantly increased Mϕ TNF-α production, and tumor supernatant suppressed TNF-α production. Priming and activation with optimal doses of IFN-γ (50 U/ml) and LPS (10 μg/ml), further enhanced normal host Mϕ TNF-α production, but even optimal priming and activation could not prevent tumor supernatant-induced suppression of TNF-α production (Figure 2).

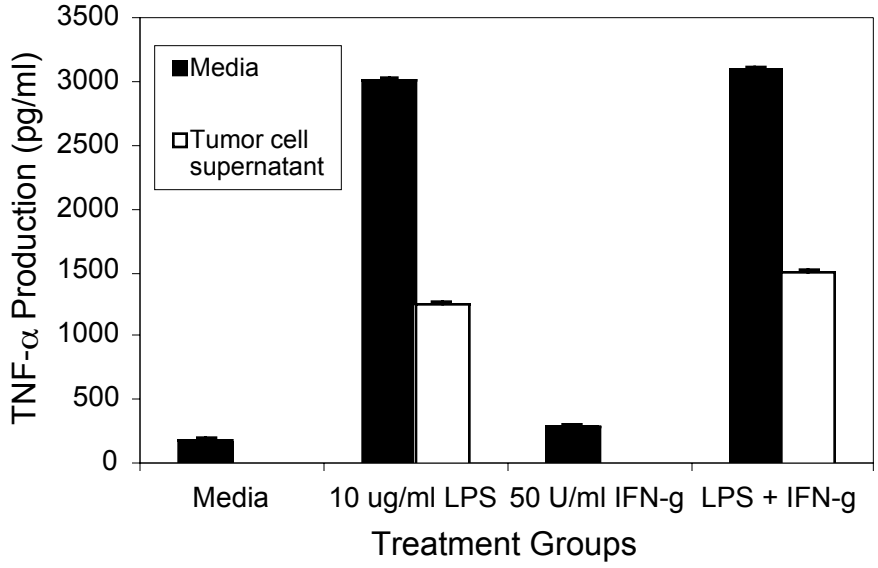


Figure 2. Mϕ TNF-α production is inhibited by tumor cell supernatants. Four x 10^6 thioglycollate-elicited Balb/c normal host peritoneal Mϕs were cultured in 200 μl serum-free medium without or with 72 h Meth-KDE supernatants (1:2 dilution). 10 μg/ml LPS, 50 U/ml IFN-γ or a combination of the two were added at the start of incubation and supernatants were collected and TNF-α protein levels were determined. Data are averages +/- SEM of triplicate independent determinations from one of three experiments that have similar results.

To evaluate whether tumor-derived factors dysregulate Mϕ antitumor cytotoxicity, IFN-γ and LPS-activated Mϕs were added to growing Meth-KDE tumor cells and cytotoxicity was measured using a modification of the Alamar blue™ colorimetric viability assay (3) (Figure 3). IFN-γ and LPS treatment activated Mϕs for enhanced cytotoxicity against the purified adherent Meth-KDE tumor cell line. IFN-γ and LPS-activated normal host Mϕs demonstrated significantly greater cytotoxic activity against Meth-KDE cells when compared to similarly treated Mϕs cultured with 72 h Meth-KDE-derived cell-free supernatants (1:2 dilution). These data suggest that activated normal host Mϕs possess cytotoxic activity, but, tumor-derived supernatants alter the capacity of these Mϕs to be cytotoxic to the tumor.

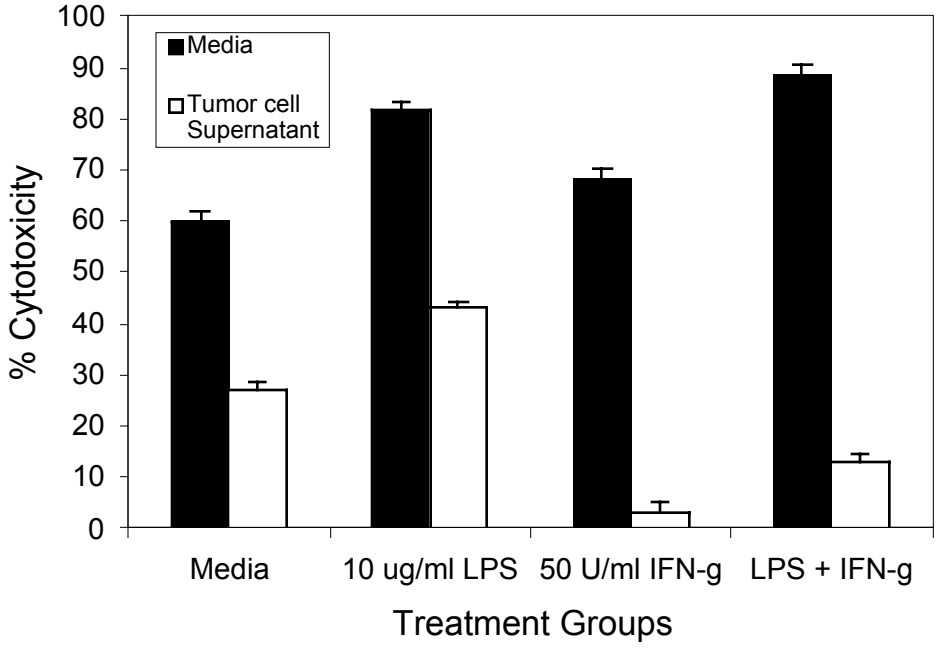
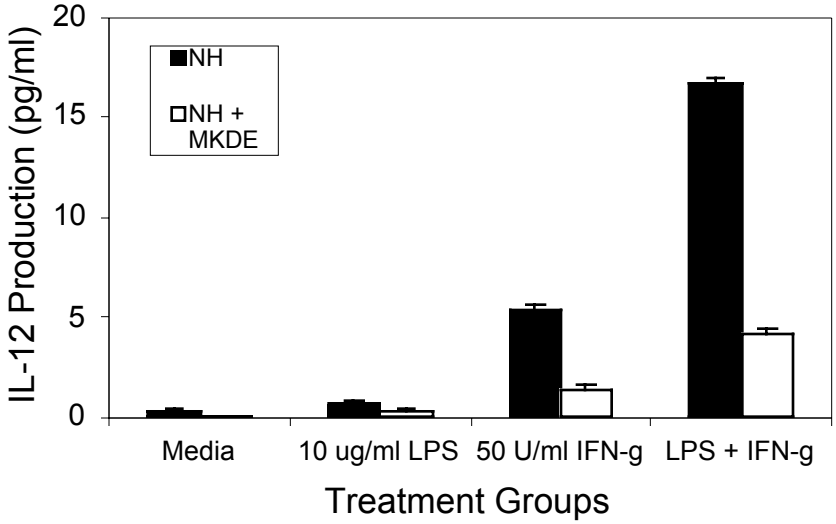


Figure 3. Tumor cell-derived factors impair Mϕ cytotoxic activity. Mϕs (2 x 10^5 cells) from untreated and LPS or IFN-γ-treated normal and tumor-bearing Balb/c mice were added to 1 x 10^5 Meth-KDE tumor cells and cultured for 24 h in the presence of 10 nM actinomycin-D. Cytotoxicity was measured using a modification of the Alamar blue ™ colorimetric viability assay. Percent cytotoxicity was calculated using the formula [1- [(control - test) / test]] x 100. Data are averages +/- SEM of triplicate independent determinations from one of three similar experiments.

Tumor Cell Supernatants Impair Mϕ Production of IL-12p70 Heterodimer

In the presence of TBH Mϕs, T-cell proliferative responses are suppressed, suggesting that neoplastic tissues subvert Mϕ function to favor tumor growth (83). Therefore, we determined whether tumor-derived factors compromise primary Mϕ production of proinflammatory effector cytokines. In our model system, tumor-derived factors such as PGE_2, TGF-β_1 and IL-10 induce systemic dysregulation of Mϕ functions (18,85). Because tumor growth dysregulates NO and TNF-α production by primary Mϕs, we cultured normal host Mϕs, as described, without or with 72 h Meth-KDE supernatants and measured IL-12 production using a p70-specific ELISA (Figure 4A). IL-12 production was strongly inhibited, regardless of activation, by tumor-derived factors. These data are in agreement with previous observations of tumor-dysregulated IL-12 in Mϕ cell lines (204) and TAMs (119). We further evaluated primary TBH peritoneal Mϕs (Figure 4B). Tumor growth significantly inhibited IFN-γ (50 U/ml)-induced IL-12 production. LPS enhanced IFN-γ-primed TBH Mϕ IL-12 production as compared to untriggered Mϕs, but the level of IL-12 production was significantly less than similarly treated normal host Mϕs.

A



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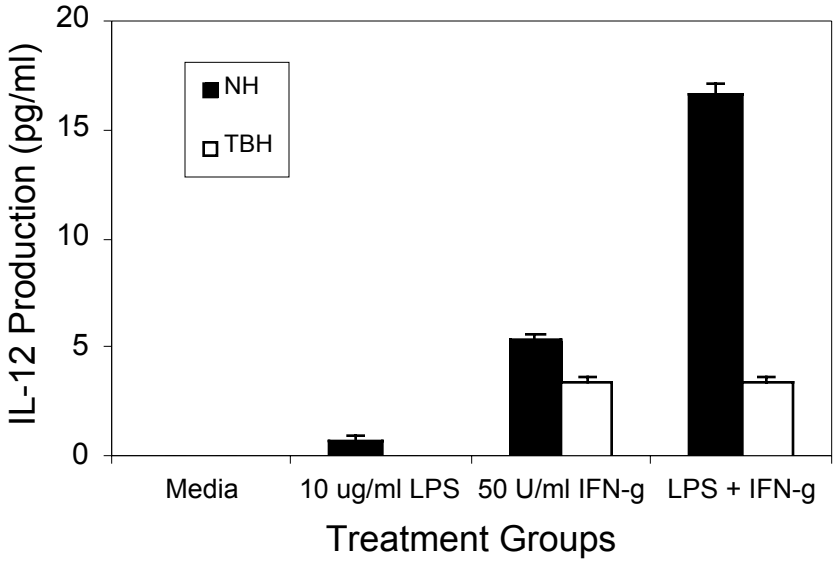


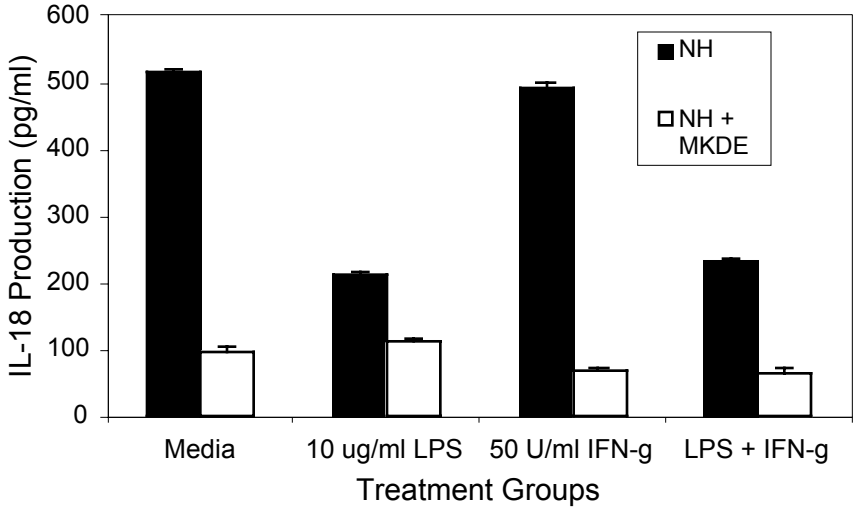
Figure 4.A. Mϕ IL-12p70 production is impaired by tumor cell-derived factors. Either 4 x 10^6 normal host or TBH peritoneal Mϕs were cultured in 200 μl serum-free medium without or with 72 h Meth-KDE (MKDE) supernatants (1:2 dilution). 10 μg/ml LPS, 50 U/ml IFN-γ or a combination of the two were added at the start of incubation and supernatants were collected after 12 h and assayed for IL-12 heterodimer by p70 specific ELISA. Tumor supernatant modulated IL-12 production regardless of activation. Data are averages +/- SEM of triplicate independent determinations from one of three experiments that have similar results. **B. Mϕ IL-12p70 heterodimer production is dysregulated by tumor growth.** Four x 10^6 normal host or TBH peritoneal Mϕs were cultured in 200 μl serum-free medium with 10 μg/ml LPS, 50 U/ml IFN-γ or a combination of the two. After 12 h, supernatants were collected and assayed for IL-12 heterodimer by p70 specific ELISA. Data are averages +/- SEM of triplicate independent determinations from one of three similar experiments.

Mϕ IL-18 Production Is Downregulated by Tumor Cell-derived Factors

IL-18 is a recently described cytokine with IL-12-like activity (75-77). IL-18 is up to 1000 times more potent than IL-12 as a stimulator of IFN-γ (226). We examined the effect of tumor-derived factors (Figure 5A) and tumor growth (Figure 5B) on IL-18 production. Because tumor growth dysregulates IL-12 production by primary Mϕs, we cultured normal host Mϕs, as described, without or with 72 h Meth-KDE supernatants and measured IL-18 production using an IL-18-specific ELISA (Figure 5A). IL-18 production was strongly inhibited, regardless of activation, by tumor-derived factors.

To determine if tumor growth impairs primary Mϕ IL-18 production, normal host and TBH peritoneal Mϕs were treated as described for IL-12 production. Total cellular proteins were harvested, separated on 10% polyacrylamide gels, electroblotted to nitrocellulose, and probed with goat anti-mouse IL-18 (Figure 5B). The supernatants were tested for IL-18 production using an IL-18 specific ELISA. Priming and activation with IFN-γ and LPS, respectively, enhanced IL-18 expression, but the level of IL-18 production was significantly less in primary TBH Mϕs compared to similarly treated normal host Mϕs.

A



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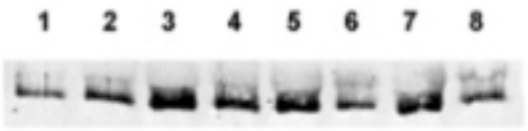

Figure 5A. Mϕ IL-18 production is downregulated by tumor cell-derived factors. Either 4 x 10^6 normal host or TBH peritoneal Mϕs were cultured in 200 μl serum-free medium without or with 72 h Meth-KDE (MKDE) supernatants (1:2 dilution). 10 μg/ml LPS, 50 U/ml IFN-γ or a combination of the two were added at the start of incubation and supernatants were collected at 12 h and IL-18 protein levels were determined. Tumor supernatant modulated IL-18 production regardless of activation. Data are averages +/- SEM of triplicate independent determinations from one of three experiments that have similar results. **B. TBH Mϕs have lower levels of IL-18 production**. Either 4 x 10^6 normal host or TBH peritoneal Mϕs were cultured in fresh medium (lanes 1 & 2), LPS (10 μg/ml, lanes 3 & 4), IFN-γ (50 U/ml, lanes 5 & 6) or LPS (10 μg/ml) + IFN-γ (50 U/ml, lanes 7 & 8). Cell lysates were obtained after 12 h and IL-18 was detected.

Tumor Growth Suppresses T Cell IFN-γ Production and Costimulatory Molecule Expression.

IL-12 and IL-18 both promote cell-mediated immune responses and synergize to induce IFN-γ production (75,76,223,281). Normal host or TBH T cells were stimulated with 8 μg/ml (optimal concentration) Con A or a combination of phorbol myristate acetate (PMA) + calcium ionophore (CaI), and IFN-γ production was measured using an IFN-γ-specific ELISA (Figure 6). Normal host T cells, on Con A stimulation and PMA + CaI addition, produced significantly more IFN-γ than similarly treated TBH T cells.

Further, we determined whether tumor growth dysregulated T cell CD40L expression. Flow cytometry revealed that Mϕ CD40 expression is unaffected by tumor growth (Figure 7A). In contrast, primary TBH T cell CD40L expression is suppressed, as compared with primary normal host T cells (Figure 7B). There was no detectable CD40 and CD40L expression on T cells and Mϕs, respectively (data not shown).

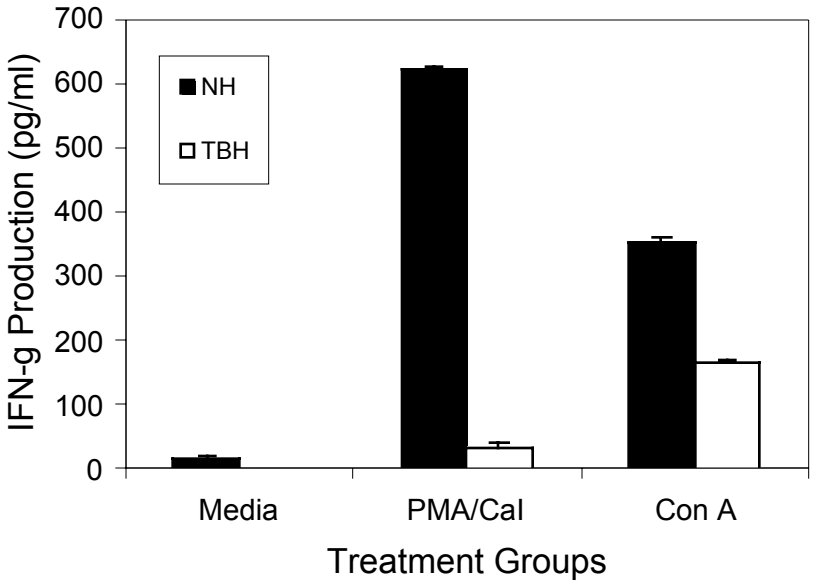

Figure 6. Tumor growth compromises T cell IFN-γ production. 4 x 10^6 purified $CD4^+$ T cells from either normal host or TBH Balb/c mice were cultured with 8 μg/ml Con A or a combination of PMA + CaI. After 12 h supernatants were collected and assayed for IFN-γ production by an IFN-γ specific ELISA. Data are averages +/- SEM of triplicate independent determinations from one of three similar experiments.

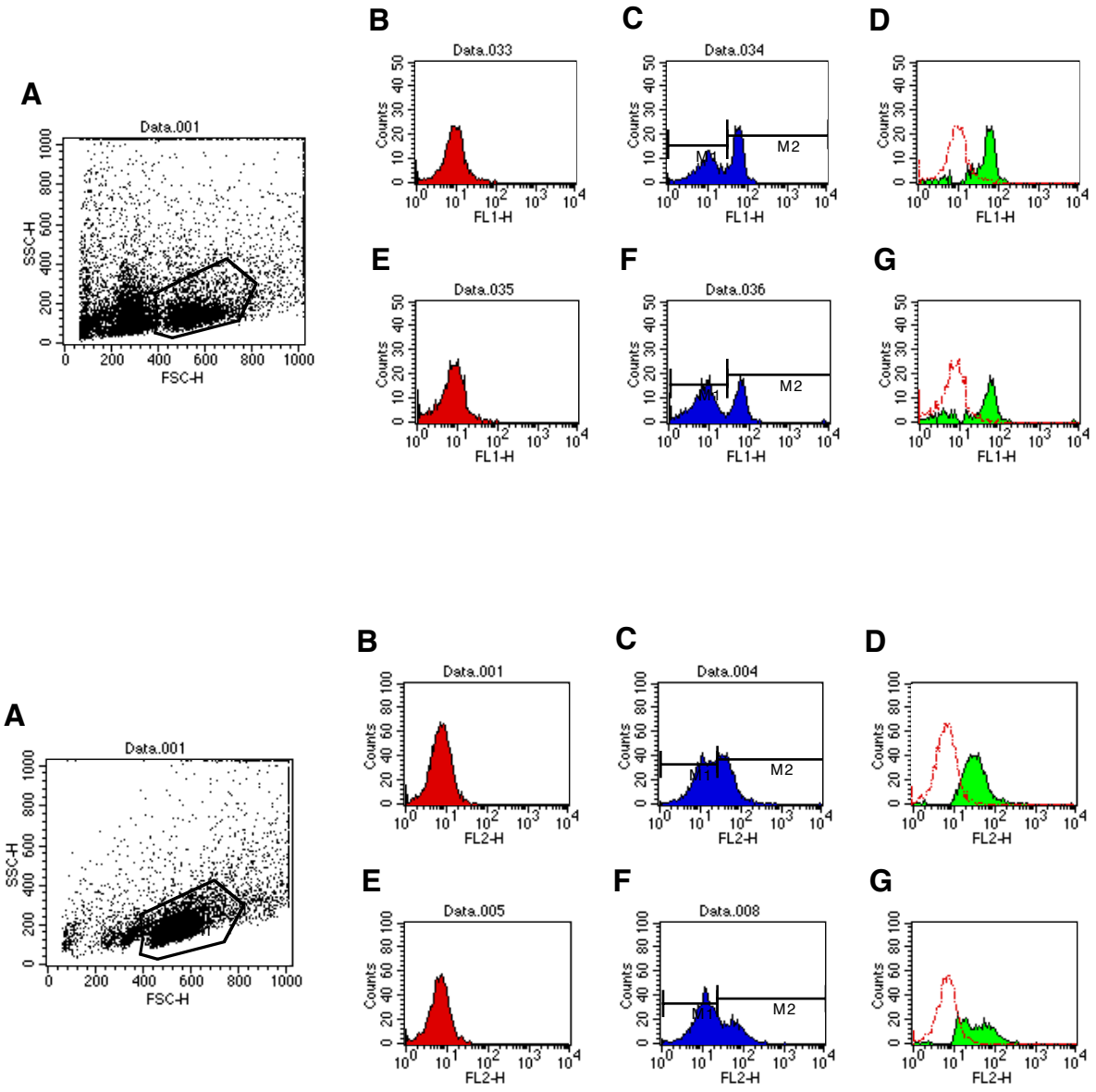

Figure 7A. TBH Mϕ CD40 expression is unaltered. 1 x 10^6 normal host (panel B - panel D) or TBH (panel E - panel G) peritoneal Mϕs were stained with FITC-conjugated hamster anti-mouse CD40 monoclonal antibody and CD40 expression was analyzed by flow cytometry. There was no discernable difference in CD40 expression on normal host and TBH peritoneal Mϕs. **B. TBH T-cell CD40L expression is decreased**. Either 1 x 10^6 normal host (panel B - panel D) and TBH (panel E - panel G) T cells from the spleen were stained with PE-conjugated hamster anti-mouse CD40L monoclonal antibody and CD40 expression was analyzed by flow cytometry. TBH T cells (panel G) express approximately 50% less CD40L than similarly treated normal host T cells (panel D).

ICSBP Expression Is Impaired in TBH Mϕs

IFN-γ induces ICSBP expression in cells of lymphocyte or macrophage lineage. ICSBP in turn regulates IL-12 (109,253) and IL-18 (148) gene expression. Because tumor growth can inhibit Mϕ production of IFN-γ-inducible factors, such as IL-12 and IL-18, we assessed the effect of tumor growth on the important signal transduction molecule ICSBP (Figure 8). Primary normal host or TBH peritoneal Mϕs ($4x10^6$ cells) were treated with IFN-γ (50 U/ml) and LPS (10 μg/ml) or a combination of the two. Total cellular proteins were harvested, separated on 10% polyacrylamide gels, electroblotted to nitrocellulose, and probed with rabbit anti-mouse ICSBP. IFN-γ regulated ICSBP expression was impaired by tumor growth. This is especially evident when the cells were activated with LPS after IFN-γ priming.

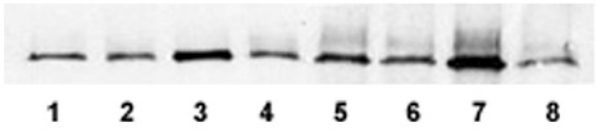

Figure 8. TBH Mϕ ICSBP expression is impaired. Either 4 x 10^6 normal host or TBH peritoneal Mϕs were cultured in fresh medium (lanes 1 & 2), LPS (10 μg/ml, lanes 3 & 4), IFN-γ (50 U/ml, lanes 5 & 6) or LPS (10 μg/ml) + IFN-γ (50 U/ml, lanes 7 & 8). After 12 h incubation, lysates were obtained and ICSBP was detected.

Tumor-Derived TGF-β_1 and IL-10 Dysregulate ICSBP Production.

The Meth-KDE fibrosarcoma produces several immunomodulatory factors, including TGF-β_1, IL-10 and PGE_2 (16,83), all of which could potentially modulate ICSBP-mediated activation of Mϕs. To determine the role of TGF-β_1 and IL-10 in the tumor-induced modulation of ICSBP-mediated Mϕ activation, Meth-KDE supernatants were depleted of these cytokines by Ab absorption using anti-TGF-β_1 and anti-IL-10 [as described in (16)]. The role of PGE_2 was investigated using the arachidonic acid inhibitor indomethacin (10^{-7} M) to treat Meth-KDE cells, thus producing PGE_2-free supernatants. Next, normal host Mϕs (4.0×10^6) were cultured with LPS (10 μg/ml) + IFN-γ (50 U/ml) and supplemented with fresh (see Figure 9a - lane B) or factor-depleted (see Figure 9b, lanes 2, 4, & 6) Meth-KDE supernatant (1:2 dilution). Similarly, activated normal host Mϕ cultures were treated with exogenous rTGF-β_1, rIL-10, or $rPGE_2$ at levels approximately equal to those measured in 72-h Meth-KDE cell supernatants (16). After 12 h, lysates were prepared and assayed for ICSBP expression.

Meth-KDE tumor cell-derived supernatant downregulated LPS + IFN-γ induced normal host Mϕ ICSBP expression (Figure 9a). Depletion of TGF-β_1 (Figure 9b - lane 2) from tumor supernatant partially reversed suppression in activated cultures. Addition of physiologic levels (16) of recombinant TGF-β_1 (10.0 ng/ml, Figure 9b - lane 1) suppressed ICSBP expression following activation with LPS + IFN-γ.

In a manner similar to TGF-β_1, IL-10 modulated normal host Mϕ ICSBP expression. Depletion of IL-10 from the tumor cell-derived supernatant (Figure 9b - lane 4) reversed suppression in LPS + IFN-γ-activated cultures. A physiologically relevant dose of recombinant IL-10 (3.0 U/ml) (16) suppressed LPS + IFN-γ-activated ICSBP expression (Figure 9b - lane 3).

In contrast to TGF-β_1 and IL-10, tumor-derived PGE_2 may not play a significant role in tumor-induced suppression of Mϕ activation. Supernatant from indomethacin (10^{-7} M) - treated Meth-KDE cells inhibited ICSBP expression (Figure 9b - lane 6), and exogenous PGE_2 (25.0 ng/ml) did not suppress LPS + IFN-γ-activated ICSBP expression compared with TGF-β_1 or IL-10. Collectively these data suggest that Meth-KDE derived TGF-β_1 and IL-10 are the primary mediators of tumor-induced suppression of Mϕ activation.

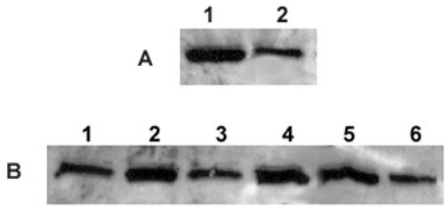

Figure 9: Tumor-derived TGF-β_1 and IL-10 impair Mϕ ICSBP production. Either 4 x 10^6 primary normal host (NH) peritoneal Mϕs were cultured in 200 μl serum-free medium without (A, lane 1) or with (A, lane 2) 72 h Meth-KDE supernatants (1:2 dilution). 10 μg/ml LPS + 50 U/ml IFN γ was added at the start of incubation. Some cultures were supplemented with TGF-β_1 (10.0 ng/ml, B-lane 1), IL-10 (3.0 U/ml, B-lane 3), and PGE_2 (25.0 ng/ml, B-lane 5) and parallel cultures were treated with TGF-β_1 (B-lane 2), IL-10 (B-lane 4), and PGE_2 (B-lane 6) - depleted Meth-KDE supernatants (1:2 dilution). After 12 h lysates were prepared and assayed for ICSBP production. Representative data from one of three similar experiments are shown.

DISCUSSION

This study showed that the *in vivo* nonmetastatic Meth-KDE fibrosarcoma, through the production and release of immunomodulatory factors such as TGF-β_1, IL-10 and PGE_2, adversely altered Mϕ and T cell effector function and cytokine production. Tumor-derived factors generated Mϕs that were tumoricidally dysfunctional. We showed this by demonstrating suppressed production of the cytotoxic effector molecules, TNF-α and NO after culture of primary normal host Mϕs with Meth-KDE tumor cell-derived supernatants. These studies correlate with our results showing a corresponding decrease in direct Mϕ-mediated tumor cytotoxicity when primary normal host Mϕs are cultured with tumor-derived factors. The suppressive role of the tumor-derived factors was further demonstrated by downregulation of IL-12 and IL-18 production in primary normal host Mϕs incubated with Meth-KDE supernatants and in *ex vivo* TBH Mϕs. These results correlate with experiments that revealed a deficit in TBH T-cell IFN-γ production and CD40L expression. These results led us to examine the role of ICSBP, an IFN-γ inducible, immune system-specific transcription factor (218,238). ICSBP expression was impaired in primary TBH and normal host Mϕs incubated with Meth-KDE supernatants. Blocking studies suggested that IL-10 and TGF-β_1 but not PGE_2 impaired ICSBP expression.

Mϕs influence immune responses in diverse and fundamental ways. Capabilities such as antigen presentation, cytokine production and tumoricidal activities contribute to the role of Mϕs in host defense. Mϕs have been shown to play a crucial role in the defense against tumors (8,122,273,314). Tumor cell-derived factors dysregulate Mϕs in several ways. We demonstrated that tumor-derived factors suppress TNF-α and NO production from primary normal host Mϕs with a corresponding decrease in direct Mϕ-mediated cytotoxicity in the TBH. This can be explained because TNF-α may induce or enhance the production of other cytokines that mediate antitumor immune responses, such as the T cell stimulatory cytokine IL-12, both directly (171) and indirectly through increased T cell production of IFN-γ (168). Furthermore, TNF-α can promote the generation of $CD8^+$ T cell-mediated antitumor cytotoxicity through reversal of TGF-β_1-induced inhibition (111), and TNF-α-dependent production of reactive nitrogen intermediates can mediate Mϕ tumoricidal activity through IFN-γ and IL-2 (64). Furthermore, TNF-α may enhance tumor cell sensitivity to NO, rendering tumors susceptible to even moderate levels of NO suggesting that tumors may escape tumor-proximal Mϕ-mediated cytotoxicity by overproducing Mϕ-deactivating cytokines that inhibit local NO and TNF-α production.

We show that tumor cell-derived factors downregulate IL-12 and IL-18 production by primary normal host Mϕs, which corresponds to a decrease in IL-12 and IL-18 production in TBH Mϕs. Downregulation of IL-12 and IL-18 production

correlated with lowered IFN-γ production in TBH T cells. Tumor-induced IL-12 and IL-18 downregulation may be detrimental to TBH survival because IL-12 induces high levels of IFN-γ, which can mediate a variety of antitumor effects. IL-18 induces IFN-γ production by activated murine and human T cells, in synergy with IL-12 (4,210). The upregulation of IL-18Rα gene expression by IL-12 may explain its capacity to increase IL-18 induced IFN-γ production by T cells (317). IL-18 is one of the most promising cytokines with antitumor activity (76). IL-18 when administered to mice as an adjuvant for increased tumor immunogenecity caused enhanced NK-cell activation, production of IL-2, and sequentially decreased IL-10 levels. IL-18 stimulated primarily NK-mediated antitumor effects against interperitoneal (i.p.) growing Meth A sarcoma, induced cytotoxic $CD4^+$ T cells, and evoked immunological memory (195). IL-18 initially stimulates a non-specific arm of the immune response and then develops a specific cytotoxic T-cell-mediated immune response (195).

Since IL-18 stimulates IFN-γ production, the suppressive effect of the tumor cell supernatants is amplified. IFN-γ production promotes immune recognition of tumor cells by enhancing MHC molecule expression and stimulates cytotoxicity mediated by NK cells, T lymphocytes, and Mϕs (37). IFN-γ promotes tumor regression through the inhibition of cell proliferation, direct toxic effect on cells in combination with TNF-α, and induction of iNOS (37). Along with a deficit in IFN-γ production in TBH Mϕs, CD40L expression was lower on TBH T cells. CD40/CD40L interactions are important

for T cell-dependent activation of Mϕs (114). Inefficient CD40/CD40L interactions resulted in decreased Mϕ functions (234). CD40/CD40L interactions play a critical role in the induction of antitumor immunity, especially in the activation of cytotoxic T cells (279). CD40L expression is upregulated by IFN-γ production (137). The paucity of IFN-γ production could explain the observed reduction in CD40L expression in our tumor model.

ICSBP is an immune system specific transcription factor (218,238), which is induced by IFN-γ through the GAS sequence present in its promoter (58). ICSBP plays a critical role in Mϕs after activation by IFN-γ. Politis *et al*. (238) and Wu *et al*. (308) suggest that the CD40/CD40L interaction stimulates ICSBP production in APCs, in a way that is similar to stimulation by IFN-γ. ICSBP regulates IL-12p40 gene activation in Mϕs and, as a consequence, IFN-γ-dependent host resistance (253). ICSBP is a critical factor for IL-18 promoter activity, performing a dominant regulatory role in the inducible expression of IL-18 (148). Because tumor-derived factors and tumor growth modulated IFN-γ-induced Mϕ production of IL-12 and IL-18 and ICSBP has been implicated in the control of IL-12p40 and IL-18 gene expression we assessed whether tumor growth dysregulated the IFN-γ signaling pathway through differential regulation of ICSBP. We measured ICSBP expression in TBH Mϕs and primary Mϕs incubated with Meth-KDE supernatants. ICSBP expression was dysregulated in TBH Mϕs, and tumor supernatants inhibited ICSBP expression in primary normal host Mϕs. Because the Meth-KDE

fibrosarcoma produces several immunomodulatory factors, including IL-10, TGF-β_1 and PGE_2, we next assessed the effects of individual factors on ICSBP expression. TGF-β_1, and IL-10 produced by the tumor, modulated ICSBP expression in primary Mϕs.

These results suggested that tumor cell-derived IL-10 and TGF-β_1 significantly downregulate Mϕ activation *in situ* and that abrogation of these tumor-derived factors might restore Mϕ function and enhance antitumor efficacy. The tumor cell-derived factors downregulated primary Mϕ production of the immunostimulatory cytokines, IL-12 and IL-18, which are potent inducers of IFN-γ production. The downregulation of IFN-γ production dysregulated Mϕ signalling pathways due to the impairment of ICSBP production. Mϕ function was also altered indirectly via lower CD40L expression on TBH T cells. Finally, impaired ICSBP production was manifested in the inability of primary Mϕs to be tumoricidal. The release of these inhibitory molecules (TGF-β_1, IL-10 and PGE_2) by the tumor upsets the normal balance of the immune system, leading to altered Mϕ function and immunosuppression. These data emphasize the importance of ICSBP in antitumor activity. They suggest that restoration of ICSBP production *in vivo* could enhance the tumoricidal activity of primary Mϕs.

CONCLUSIONS

This study advanced our knowledge of mechanisms of tumor escape from the immune response. My work focussed on the ability of the tumor to outsmart the immune response by breaking a critical link between innate and adaptive immunity. This link (the CD40/CD40L interactions between Mϕs and T cells, respectively) plays a pivotal role in the generation of protective cell-mediated immunity. We have demonstrated, using flow cytometry, lowered CD40L expression on TBH T cells (see Figure 9b).

Studies show that CD40/CD40L interactions are critical for the priming and expansion of $CD4^+$ Th cells and $CD8^+$ Tc cells in response to antigen (174). Mackey *et al*. (174) showed that both $CD4^+$ and $CD8^+$ effector T cell generation appears to be significantly impaired in the absence of CD40/CD40L interactions. CD40-deficient mice exhibit impaired antitumor responses and are incapable of generating protective cell-mediated tumor immunity (176). Several lines of evidence suggest that CD40 signals are crucial for T cell-dependent Mϕ activation (279). CD40 ligation is the most potent stimulus to upregulate the expression of important co-stimulatory molecules like ICAM-1, CD80, and CD86 (279). CD40/CD40L interaction is critical for the induction of antitumor immunity, especially its essential role in the induction of cytotoxic T cells (279). We believe that decreased APC function or decreased Mϕ functions may be

explained by inefficient CD40/CD40L interactions via lower IL-12, IL-18 and as a consequence IFN-γ production.

IFN-γ plays particularly important roles in Mϕs, which include elicitation of antipathogenic and antitumor activity, stimulation of chemokine/cytokine production, and enhanced antigen presentation (305,314). IFN-γ aids in the induction of CD40 and CD40L expression, leading to effective antitumor immunity (137). The production of T cell-derived IFN-γ is induced by IL-12 and IL-18. IL-18 is generated after the interaction between activated T cells and APCs (like Mϕs) via the CD40/CD40L interaction (261). IFN-γ induces ICSBP expression in activated T cells and Mϕs. Expression and activity of DNA-binding proteins like ICSBP are contingent on the CD40/CD40L interaction (unpublished observation, Martins and Elgert). ICSBP plays an essential role in the activation of the IL-12p40, IL-18, and CD40L genes, and, as such, this transcription factor can directly regulate IFN-γ-dependent host defense (58,109,149,253,308,309). These findings encouraged us to explore the existence of an immunosuppressive loop in the TBH. This loop could be generated by dysregulated CD40/CD40L interactions as a consequence of impaired CD40 and/or CD40L expression.

Having shown CD40L expression on T cells of the TBH to be impaired, we started characterizing the loop. Using western blotting and ELISAs, we found that IL-12 (see Figure 4a & b) and IL-18 (see Figure 5a & b) expression was impaired in TBH Mϕs.

Impaired IL-12 expression is consistent with the finding that CD40/CD40L interactions are required for T cell-dependent production of IL-12 by murine Mϕs and that a defect in the T cell-dependent induction of IL-12 may contribute to the immune status of an individual lacking CD40L (137). IL-18 induces IFN-γ production by activated T cells in synergy with IL-12 due to the upregulation of IL-18Rα gene and corresponding surface expression by IL-12 (159). IL-12 upregulates the expression of CD40L on T cells at both the protein and RNA level (235), possibly through the phosphorylation of STAT4 by IL-12 and binding of STAT-4 to the CD40L promoter (235). This effect of IL-12 on CD40L expression could lead to increased cytokine production by Mϕs and potentiation of the antitumor response.

Tumor growth significantly impaired T-cell IFN-γ production (see Figure 6). IFN-γ regulates a variety of important immunologic programs. It is the predominant cytokine during Th1-dominated immune reactions and participates during antigen presentation (37,318). It is the prototypical Mϕ-activating cytokine, regulating transcription through proteins belonging to the interferon regulatory factor family that include ICSBP (139). IFN-γ promotes the immune recognition of tumor cells and stimulates T cell and Mϕ-mediated cytotoxicity (37). Boehm *et al.* (37) show that IFN-γ has other activities that may promote tumor regression, such as inhibition of angiogenesis, inhibition of cell proliferation, direct toxic effect on cells in combination with TNF-α, and induction of iNOS.

Politis *et al*. (308) showed that IFN-γ induces ICSBP expression in activated T cells and Mϕs. The paucity of IFN-γ production in our tumor model system suggested a lesion in ICSBP production. ICSBP expression was impaired in TBH Mϕs and in NH Mϕs after incubation with Meth-KDE supernatants (Figure 7). This suggested that tumor-derived factors can modulate ICSBP expression. TGF-β_1, and to a lesser extent IL-10, modulated ICSBP expression in primary Mϕs (see Figure 8). We showed that the Meth-KDE fibrosarcoma produces these immunosuppressive cytokines (TGF-β_1 and IL-10) along with PGE_2 (16,208).

Wu *et al*. (238) suggested that the CD40/CD40L interaction stimulates ICSBP production in APCs, similar to that by IFN-γ stimulation. ICSBP is a positive transcriptional activator of IL-12p40, IL-18, and CD40L induction (58,109,149,253,283,308,309). ICSBP acts as a central switch in the immunosuppressive loop. Impaired ICSBP expression would imply lowered IL-12, IL-18, IFN-γ, and CD40L expression and impaired cytotoxicity of TBH Mϕs against the tumor. This impaired cytotoxicity is the culmination of the immunosuppressive loop. It is manifested in the form of lowered NO (see Figure 1) and TNF-α (see Figure 2) production. In addition to lower levels of cytotoxic effector molecules (NO and TNF-α) the Alamar blue assay demonstrated a significant difference in the ability of normal host Mϕs to induce cytotoxicity of Meth-KDE cells in the presence of tumor cell supernatants (see Figure 3).

To summarize, decreased CD40L expression may disrupt Mϕ activation pathways, leading to impaired production of immunostimulatory cytokines, IL-12 and IL-18 by TBH Mϕs. Disruption of CD40L expression, via dysregulation of IL-12 and IL-18 production, impeded T-cell IFN-γ production, which in turn exacerbated Mϕ dysfunction. In addition, we showed that IFN-γ-induced ICSBP expression is impaired in TBH Mϕs due to tumor cell-derived TGF-β and to a lesser extent, IL-10. ICSBP in turn can induce CD40L, IL-12 and IL-18 expression. In effect, disruption of the CD40/CD40L interaction via lowered T cell CD40L expression generates an immunosuppressive loop that may lead to tumor survival and growth. This was demonstrated by impaired cytotoxicity via impaired TNF-α and NO production by normal host Mϕs against Meth-KDE tumor cells in the presence of tumor cell supernatants. Collectively, these studies show that multiple antitumor mechanisms may be enhanced by the restoration of CD40L expression.

MODEL OF TUMOR-INDUCED Mϕ DYSFUNCTION VIA DYSREGULATION OF CD40/CD40L INTERACTIONS

As essential components of the immune system, Mϕs influence immune responses in diverse and fundamental ways, including phagocytosis, antigen presentation, cytokine production, and tumoricidal activities (110). Mϕs are key components of immunity. Mϕs present targets for tumors to evade, thereby enhancing tumor survival and growth. Tumors may inhibit Mϕ production of immunostimulatory cytokines or suppress the expression of co-stimulatory molecules leading to inefficient stimulation of tumor-reactive T cells. Tumors may prevent the synthesis of transcription factors essential for the production of integral cytokines. Our research suggests that tumors evade host immune responses by dysregulating CD40/CD40L interactions between Mϕs and T cells. Tumor-induced lowered CD40L expression on T cells leads to dysregulation of CD40/CD40L interactions. Decreased CD40L expression disrupts Mϕ activation pathways, leading to impaired production of the immunostimulatory mediators IL-12 and IL-18 by TBH Mϕs. IL-12 and IL-18 production by normal host Mϕs is lowered upon incubation with tumor-derived supernatants, demonstrating the role of the tumor cell-derived factors IL-10, TGF-β_1, and PGE_2. Disruption of CD40L expression, via dysregulation of IL-12 and IL-18 production, impedes T-cell IFN-γ production, which in turn exacerbates Mϕ dysfunction. IFN-γ production is essential for ICSBP expression. TBH Mϕs have lowered ICSBP expression, which participates in the regulation of

CD40L, IL-12, and IL-18 expression through the ISRE. TGF-β_1 and IL-10 are the tumor-derived factors that impair ICSBP expression.

This immunosuppressive loop (CD40/CD40L expression → IL-12 production → IL-18 production → IFN-γ production → ICSBP production → IL-12/IL-18/CD40L) leads to the inability of: Mϕs to cross-prime $CD8^+$ cytotoxic T cells, differentiation of T cells to the Th1 type, production of the cytotoxic molecules NO and TNF-α, and impaired cytotoxicity of TBH Mϕs against the Meth-KDE tumor and corresponding ineffectiveness in host anti-tumor immunity. Restoration of T cell CD40L expression and stimulation of Mϕs through CD40 permits Mϕs and T cells to support the functions of other immune cells. This is especially important because Mϕs and T cells represent a potent antitumor modality through their capacity to mediate a multifaceted attack on the tumor.

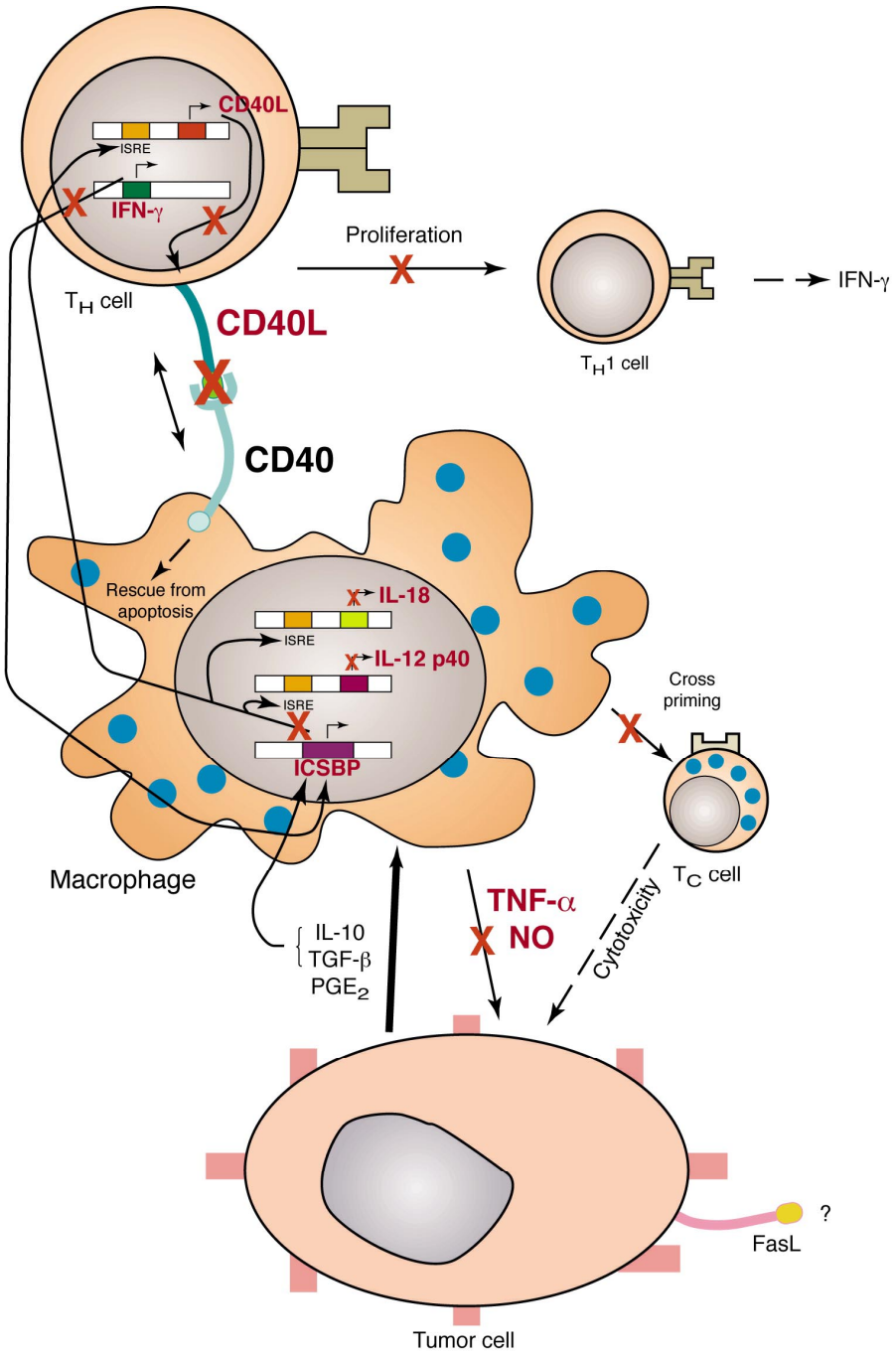

Figure 10. Proposed model of tumor-induced immunosuppression of Mϕ tumoricidal activities: Impaired Mϕ cytotoxic activity is due, in part, to decreased TNF-α and NO production, caused by reduced levels of IL-12 and IL-18. The consequence of lowered IL-12 and IL-18 levels is decreased IFN-γ production from T cells. Because IFN-γ is required for ICSBP induction, there will be a lesion in ICSBP transcription. TGF-β and IL-10 also alter ICSBP expression. ICSBP binds to the ISRE in the promoter of the *IL-12p40, IL-18,* and *CD40L genes* and regulates their expression. Thus, CD40-CD40L interactions are essential for an antitumor immune response; the dysregulation of CD40L expression contributes to tumor-induced Mϕ-mediated immune dysfunction and corresponding ineffectiveness in tumor immunity.

SUGGESTED FUTURE INVESTIGATIONS

My studies demonstrate how the TBH immune response may be subverted by disruption of CD40/CD40L interactions. There are still a number of questions, the answers to which can only be obtained by carrying out more experiments. Specifically, these questions need to be answered:

- ***What are the IL-12, IL-18, IFN-γ, ICSBP, CD40, and CD40L mRNA levels in normal host and TBH Mϕs?*** The paucity of the immune response in the TBH was explained on the basis of observed protein levels. Experiments showing the same effect with mRNA levels would help determine whether this is a transcriptional phenomenon or a translational phenomenon. This is important due to the variation in mRNA synthesis and degradation rates.

- ***How are IL-18 levels suppressed during tumor growth?*** IL-18 is a potent activator of T cell function that is up to 1000 times more potent as a stimulator of IFN-γ than IL-12. Without significant IFN-γ production, the link between innate and acquired immune responses is broken. I have shown that IL-18 levels are decreased during tumor growth. A useful experiment would study the ICSBP-mediated regulation of IL-18 gene expression. The IL-18 coding sequence (with a reporter gene like chloramphenicol acetyl-transferase, CAT) along with the promoter region could be

transfected into normal host and TBH Mϕs. The IL-18 promoter has an ICSBP binding site. By measuring reporter gene expression it can be determined if the lowered levels of IL-18 are a consequence of insufficient/inefficient binding of ICSBP to the IL-18 promoter sequence (147,148).

- ***How can the TBH immune response be restored?*** I have shown that the disruption of CD40/CD40L interactions (due to lowered levels of CD40L expression) plays a role in the suppression of the immune responses. The best way to show this would be by transfecting TBH peritoneal Mϕs with adenovirus carrying the CD40L gene (146). This would help in two ways:

  1. It would obviate the requirement for T cells as CD40L molecules would now be expressed on the surface of Mϕs.
  2. It would allow Mϕs to autoactivate themselves (through CD40/CD40L interactions on the Mϕ cell surface).

  Once transfectants are obtained, these Mϕs can be returned to the animals by innoculating the tumors with Mϕs infected with adenovirus carrying the CD40L gene. The size of each tumor can be assessed three times weekly and recorded as the average tumor area ± standard error by measuring the largest perpendicular diameters. A decrease in tumor size would confirm the importance of the CD40/CD40L interaction.

- ***Is lowered IL-18 expression a direct result of tumor-mediated reduction in caspase-1 activity?*** Caspase-1 cleaves pro-IL-18 to form mature IL-18. ICE itself is expressed in the form of pro-ICE, hence a normal sandwich ELISA for ICE will not detect the active form. Thus a "functional" ICE ELISA can be carried out to detect "functional" ICE (303). The ELISA takes advantage of the fact that pro-ICE does not bind to the ICE inhibitor but functional ICE does. Briefly, 96-well plates should be coated with avidin-D (Vector Laboratories, Burlingame, CA), washed with PBS, and blocked with BSA. Samples incubated with the biotinylated irreversible ICE inhibitor (Ac-Tyr-Val-Lys-(biotin)-Asp-(acyloxy)-methyl-ketone) can be added to these plates. After a 2 h incubation at room temperature and washing with PBS, the samples should be incubated with R105 antibody (Merck Research Laboratories). R105 is a rabbit polyclonal antiserum generated against the p20 and p10 ICE forms (functional), but it also recognizes the p45 (non-functional) form. The bound antibody can be detected with HRP-conjugated goat anti-rabbit antibody and developed with o-phenylenediamine. Color development should be stopped with H_2SO_4 and absorbance read at 490nm using a MR600 ELISA plate reader (Dynatech) (303).

Alternatively, ICE western blots can be carried out by obtaining the lysates of cultured normal host and TBH Mϕs and detecting ICE by incubating with R105 antibody, which will be detected with peroxidase conjugated goat anti-rabbit antisera,

and analyzed by chemiluminescence on X-OMAT film (Eastman Kodak, Rochester, NY) (303).

- ***Is Gamma Activated Transcription Activator (GATA)-3 expression increased in the TBH?*** Recently, there have been indications that link a variety of molecular factors to Th subset development. The transcription factor GATA-3 is selectively expressed in Th2 cells and induces the expression of Th2 cytokines in developing Th1 cells (160). Ectopic expression of GATA-3 in developing Th1 cells significantly inhibits IFN-γ production, as well as enhancing IL-4 and IL-5 production (91). GATA-3 inhibits production of IFN-γ by developing Th1 cells in the complete absence of IL-4 (91). Thus, antagonism of Th1 development by GATA-3 may facilitate rapid divergence of Th subsets toward a Th2 phenotype in concert with other factors. A tumor, via the production of IL-10/TGF-β/PGE_2, may drive GATA-3 expression. This could explain the paucity of IFN-γ production and the lack of antitumor responsiveness in the TBH. Thus in the presence of the tumor, increased GATA-3 expression in T cells would imply lower IFN-γ levels and hence impaired expression of ICSBP in Mϕs, eventually leading to impaired Mϕ antitumor response to the tumor.

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APPENDIX A

ABBREVIATIONS

- ATCC, American Type Culture Collection
- Ab, antibody
- APC, antigen presenting cell
- ^{51}Cr, chromium-51
- Con-A, Concanavalin A
- cNOS, constitutive nitric oxide synthase
- Tc, cytotoxic T lymphocyte
- DMSO, dimethyl sulfoxide
- ELISA, enzyme-linked immunosorbent assay
- ECM, extracellular matrix
- FasL, Fas ligand
- FBS, fetal bovine serum
- GATA-3, Gamma-activated transcription activator
- GM-CSF, granulocyte-macrophage colony-stimulating factor
- ICSBP, interferon consensus sequence binding protein
- iNOS, inducible nitric oxide synthase
- IFN-α, interferon-alpha
- IFN-β, interferon-beta

- IFN-γ, interferon-gamma
- IL-1, interleukin 1
- IL-2, interleukin 2
- IL-4, interleukin 4
- IL-5, interleukin 5
- IL-10, interleukin 10
- IL-12, interleukin 12
- IL-18, interleukin 18
- i.m., intramuscular
- i.p., intraperitoneal
- ISRE, interferon-stimulated response element
- LPS, lipopolysaccharide
- Mϕ, macrophage
- 2-ME, β-mercaptoethanol
- Meth-KDE, methylcholanthrene-induced nonmetastatic murine fibrosarcoma
- MHC, major histocompatibility complex
- NK cell, natural killer cell
- NO, nitric oxide
- NH, normal host
- ND, not defined
- NFκB, nuclear factor kappa B

- PBS, phosphate buffered saline
- PEM, peritoneal exudate macrophages
- PGE_2, prostaglandin E_2
- RT-PCR, reverse transcription-polymerase chain reaction
- s.c., subcutaneous
- SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- SEM, standard error of the mean
- TAA, tumor associated antigen
- TCR, T-cell receptor
- TGF-β_1, transforming growth factor -$beta_1$
- Th, T helper cell
- TMB, tetramethyl benzidine
- [^{3}H]-TdR, tritiated thymidine
- TAM, tumor-associated macrophage
- TBH, tumor-bearing host
- TNF-α, tumor necrosis factor-alpha

APPENDIX B

Colorimetric mRNA Quantitation - IFN-γ (Quantikine mRNA, R&D Systems)

The IFN- γ Quantikine mRNA kit can be used to determine IFN- γ mRNA levels colorimetrically rather than using a radisotope as in northern blots. It is less time consuming and more accurate than northern blotting. This kit can be used to determine IFN- γ mRNA levels in T cells of normal host and TBH mice.

Kits required:

Quantikine mRNA Base Kit (Cat# RN000)

- Hybridization Plate
- Streptavidin Plate
- Calibrator Diluent
- Sample Diluent
- Anti-digoxigenin Conjugate
- Wash Buffer Concentrate
- Substrate
- Substrate Diluent
- Amplifier
- Amplifier Diluent
- Stop Solution

- Plate Sealers
- Float Collar

Quantikine mRNA Probes and Calibrator Kit (Cat# RN485)

- Probes are supplied as a 6X concentrated stock solution (1.1 ml).
- RNA Calibrator is supplied as an 1800 attomole/ml (amol/ml) stock solution (1.1 ml).

Quantitation of gene specific mRNA is an important tool used routinely in the study of gene expression and function. Common methods include Northern Blot, slot blot, RT-PCR and Ribonuclease Protection Assay (RPA). Quantikine mRNA is a novel method that can be used to quantitate cytokine-speific mRNA at low levels. It is sensitive, specific and accurate without the known disadvantages of the traditional methods. Northern blot analysis is labor intensive, takes several days to perform and is unsuitable for multiple mRNA analyses. With quantitative RT-PCR precise and accurate results are difficult to obtain because of the exponential nature of PCR amplification. Ribonuclease protection assays are labor intensive and typically require the use of a radioisotope.

Quantikine mRNA is a colorimetric microplate assay that can be completed in less than one day, while providing the sensitivity of a Northern blot. Total RNA or poly $(A)^+$ RNA may be used. Multiple mRNA targets can be analyzed on the same plate.

PRINCIPLE OF THE ASSAY

Gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes are hybridized with RNA samples in a microplate. The RNA/probe hybrid is captured by transferring the hybridization solution to a streptavidin-coated microplate. Following a wash to remove unbound material, an anti-digoxigenin alkaline phosphatase conjugate is added. A substrate solution is added after washing away unbound conjugate. Color develops in proportion to the amount of gene-specific mRNA in the original sample upon addition of an amplifier solution. Color development is stopped and the intensity of the color is measured.

SAMPLE PREPARATION

Purification of high quality RNA is important for the accurate quantitation of cytokine-specific mRNA. Both poly A^+ RNA and total RNA are suitable as samples for the assay. Total RNA should be purified using a modified guanidine isothiocyanate-acid phenol/chloroform extraction (single step method). Poly A^+ RNA should be purified using R&D Systems Poly A^+ RNA isolation kit (Cat# MBK-001-25). The concentration of RNA samples should be determined at A_{260} using absorbance values in the linear range of the spectrophotometer, typically 0.1-1.0. An optical density of 1.0 at A_{260} is equal toan

RNA concentration of 40 μg/ml. The A_{260}/A_{280} ratio for RNA samples should be 1.8-2.2. RNA samples should be stored at ≤ -70^{0}C.

PROCEDURE

1. Wash the hybridization plate 2X with wash buffer. Remove excess wash buffer by decanting or aspirating. Invert the plate and blot against clean paper towels.
2. Add 50 μl of cytokine-specific probes to the designated wells.
3. Add 150 μl of Calibrator or diluted sample to the designated wells. Cover with a plate sealer.
4. Apply the float collar to the hybridization plate and incubate the plate for 60 min in a 65^{0}C water bath.
5. Remove unused microplate strips from the streptavidin plate frame. Return them to the foil pouch containing the dessicant pack, reseal.
6. Wash the streptavidin plate 2X with wash buffer and remove excess wash buffer as described in step 1.
7. Transfer 150 μl from each well of the hybridization plate to the washed streptavidin plate and apply a new plate sealer.
8. Incubate for 60 min at room temperature on a shaker set at 500 ± 50 rpm.
9. Wash the streptavidin plate 4X with wash buffer and remove excess wash buffer.

10. Add 200 μl of anti-digoxigenin conjugate to each well and cover with a new plate sealer.
11. Incubate for 60 min on a shaker at room temperature.
12. Wash the streptavidin plate 6X with wash buffer and remove excess wash buffer.
13. Add 50 μl of substrate solution to each well and cover with a new plate sealer.
14. Incubate for 60 min on a shaker at room temperature. **Do not wash**.
15. Add 50 μl of amplifier solution to each well and cover with a new plate sealer.
16. Incubate for 60 min on a shaker at room temperature. **Do not wash**.
17. Add 50 μl of stop solution to each well.
18. Determine the optical density of each well within 30 min, using a microplate reader at 490 nm with a correction wavelength of 650/690 nm. This will correct for optical imperfections in the plate.

APPENDIX C

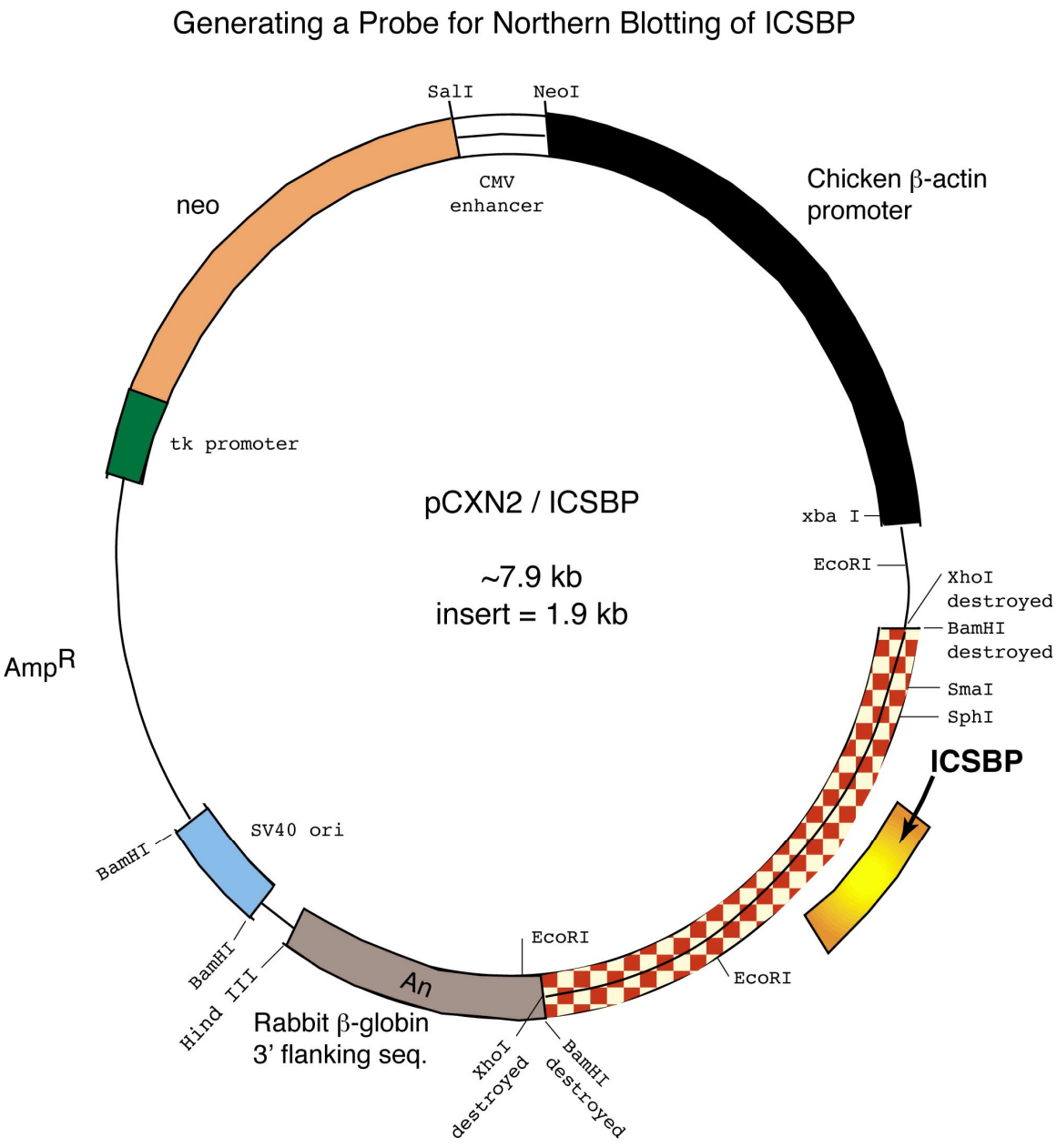

Figure 11: The pCXN2/ICSBP plasmid (~7.9 kb) containing murine ICSBP cDNA (insert = 1.9 kb)

The pCXN2/ICSBP plasmid (~7.9 kb) (196) containing murine ICSBP cDNA (insert = 1.9 kb) was provided by Dr. Tom Tamura (M.D., Ph.D., tamurat@mail.nih.gov) in the lab of Dr. Keiko Ozato (ozatok@mail.nih.gov). The plasmid is supplied at a concentration of 1μg/ml (5 μl). The vector is described by Minucci *et al*. (196). The ICSBP cDNA can be used to generate a probe for detection of ICSBP mRNA. Probe can be generated by *EcoR1* digestion to generate an ~ 1.4 kb fragment. (See Figure 11). The probe will be generated to determine ICSBP mRNA levels in normal host and TBH Mϕs. The radioactive probe serves to specifically label ICSBP mRNA from total cellular RNA in northern blotting.

Making Random Primed ^{32}P-Labeled Probes (using Boehringer Mannheim Kit)

1. Mix DNA template and water to a final concentration of 100 ng DNA in 4 μl.
2. Heat at 100^{0}C for 2 minutes and chill immediately on ice.
3. Mix together:

    4 μl DNA template and water

    *3 μl dATP, dGTP, dTTP (premixed 1:1:1)

    *2 μl reaction mix (containing random hexamer primers)

    10 μl ^{32}P-dCTP (Amersham Redivue AA0005 2000-3000 Ci/mmol)

    1 μl Klenow

    * = provided with Boehringer Mannheim kit

1. Incubate for 30 minutes at 37^{0}C.

2. Add 2 μl 0.2 M EDTA and 28 μl TE.

3. Purify over spin columns.

Alpha ^{32}P calculation of specific activity

1. Label the scintillation tubes (**B,1,2**)
2. Plug in 'seal-a-meal' thermal sealer.
3. Fill three plastic scintillation bags with scintillation fluid.
4. Seal the blank bag and place it into the B tube.
5. Add 1 μl of probe to each bag except for control B.
6. Seal bags and place into the labeled scintillation tubes.
7. Calculate specific activity using scintillation counter.

Ryan Stephen Martins

Ryan Stephen Martins was born in Bombay, India on October 15, 1976. He earned a Bachelor of Science degree in Life Sciences (Biotechnology option) in 1997 from St. Xavier's College, Bombay, India. In 1998, he completed his Master of Science - Part 1 in Biotechnology from the University of Bombay. During his undergraduate education, Ryan worked as a Research Associate in the Microbiology and Cell and Molecular Biology Departments of Hoechst Marion Roussel (now Aventis Pharmaceuticals) in Bombay, India.

Ryan began graduate studies in 1998 and completed his thesis entitled "Tumor-bearing host macrophage dysfunction: Role of CD40/CD40L interactions" under the guidance of Dr. Klaus D. Elgert. Ryan's research at Virginia Tech led to one first authored and two-second authored manuscripts either published or submitted. His work was presented at four local, state, regional, and national meetings of professional societies, including the American Association of Immunologists, Society for Leukocyte Biology, Mid-Atlantic Immunology Conference, Virginia Academy of Science and Virginia Tech Graduate Research Symposium. Ryan received 2nd place for his presentation at the Virginia Academy of Science. Ryan also received 1st place in the Natural and Biological Sciences category in the 17th Annual Research Symposium at Virginia Tech. Ryan's research was funded, in part, by grants from the Virginia Academy

of Science, Sigma Xi, Graduate Research Development Program of Virginia Tech, and Department of Biology. He was awarded travel grants by the Society for Leukocyte Biology and Graduate Student Assembly of Virginia Tech.

While Ryan was completing his Master's degree at Virginia Tech, he put in a lot of effort into teaching. He taught laboratory classes in General Biology (7 sections), General Microbiology (3 sections) and Immunology (4 sections). In addition to his teaching responsibilities, he directed students in the Biological Sciences Initiative Teaching Internship Program, Fralin Fellowship Program, and Minority Academic Opportunities Internship Program. Ryan maintained a good student evaluation ratings throughout.

Ryan was a member of some university committees. He served as the graduate student assembly representative on the Intellectual Properties Committee and the Athletics Committee. He was a member of the Biology Graduate Student Association. Ryan is an active member of several professional societies, including The American Association of Immunologists, Society for Leukocyte Biology, and Virginia Academy of Sciences.

Ryan will be pursuing a career in patent law at Chiron Corporation in Emeryville, California after his graduation.