THE ASSOCIATION OF TUMOR-INDUCED CHANGES IN MACROPHAGE
PHENOTYPE WITH IMMUNOSUPPRESSIVE FUNCTIONS

by

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(ABSTRACT)

During tumor growth there are a series of phenotypic and functional changes that occur in macrophages (Mφ) that ultimately lead to the immunosuppression of the tumor-bearing host (TBH). To investigate the phenotypic changes of Mφ during tumor growth, we examined the expression of the Mφ surface antigens, Mac-1, Mac-2, Mac-3, and Ia on peritoneal and splenic Mφ. In the peritoneal cavity there was no change in the percentage of Mac-1+ Mφ but a decrease in the percentage of Mac-2+, -3+, and Ia+ Mφ during tumor growth. In addition, three distinctly sized populations of peritoneal Mφ, showing differential antigen expression, also shifted during tumor growth. In the peritoneal cavity there was a decrease in the percentage of Mφ co-expressing the Mac-2, -3, and Ia antigens, leading to a shift towards Mac-1+2−3−Ia− TBH Mφ. In splenic Mφ, the percentage of Mac-1+, -2+, and -3+ Mφ increased, while the percentage of Ia+ Mφ decreased. Splenic Mφ showed an increase in the percentage of Mφ co-expressing Mac-1, -2, and -3 antigens and a decrease in the percentage of Mφ co-expressing Ia, leading to a shift towards a Mac-1+2−3+Ia− TBH Mφ. Taken together, these data suggest that tumor growth alters the phenotype of Mφ and causes a shift in Mφ subpopulations.

After measuring the phenotypic changes in Mφ during tumor growth, changes in Mφ accessory function to T cells were assessed. TBH Mφ have significantly reduced accessory activity for autoreactive T cells. This reduction is caused by decreased Ia antigen expression and increased production of the suppressor molecule, prostaglandin (PG). TBH Mφ down-regulated autoreactive T cell responsiveness to interleukin (IL)-1, IL-2, and IL-4. In addition to TBH Mφ reducing T cell responsiveness to cytokines, TBH CD4+ T cells alone were less responsive to the cytokines IL-1, IL-2, and IL-4. To examine the responsiveness of Mφ to activation signals,
lipopolysaccharide (LPS) was incubated with normal and TBH splenic Mφ and assessed for their phenotypic, functional, and cell-cycle changes. The data showed that TBH Mφ had a decreased responsiveness to LPS.

We showed that there was a shift from an Ia⁺ Mφ in the normal host to an Ia⁻ Mφ in the TBH. Concomitant with the shift in TBH Mφ Ia⁻ phenotype was a change in TBH Mφ function. Normal and TBH Ia⁻ Mφ were suppressor Mφ. TBH Ia⁻ Mφ, however, suppressed autoreactive and alloreactive CD4⁺ T cells significantly more than could their normal counterparts. Tumor growth causes quantitative and qualitative changes in Ia⁻ suppressor Mφ. Although Ia⁻ Mφ-mediated suppression seemed to be the major source of down-regulation of CD4⁺ T cells, CD8⁺ T cells were not without fault. In the TBH, there was an increase in the percentage of CD8⁺ T cells and an increase in CD8⁺ T cell-mediated suppression. In conclusion, tumor growth leads to a change in immunoregulation that causes suppression of the immune response.
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INTRODUCTION

The goal of my research was to determine how tumor growth upsets macrophage (Mφ)-mediated regulation of T cells. Specifically, I focused on how tumor growth affected Mφ phenotype and function, and how these two traits were interrelated. My research tested the hypothesis: Do tumor-induced changes in Mφ phenotype correlate with functional events, and are these changes associated with immunosuppression?

The role Mφ play in immune regulation has been extensively studied and, in the last two decades, substantial experimentation has uncovered many of the Mφ’s functional abilities in the immune system. Mφ are a heterogeneous population of cells that help make up the mononuclear phagocyte system (16,47,98,243,250,253). Mφ originate from bone marrow pluripotent stem cells, differentiate to monocytes, and as end cells take up residence in various anatomical sites as tissue Mφ (13,27,47,99,217,243). Initially, Mφ were thought to be responsible only for phagocytosis and destruction of foreign invaders (199,243,253). More recent evidence, however, shows that the Mφ’s ability to initiate and to interact with the rest of the immune system may be their most important functions (1,16,47,240-242). Mφ can serve as both positive and negative mediators of the immune system, demonstrating that Mφ may be considered immunoregulatory cells. This Mφ-mediated control of immune responsiveness can be through cell-cell interactions and/or release of cytokines. It is important to understand how Mφ regulate the rest of the immune response,
because Mϕ are the first cells to interact with foreign or aberrant antigens/cells. Following this interaction, Mϕ can process antigen and present it through their Ia molecules (14,18,168,240-242,260) to CD4⁺ helper T (T_H) cells. In addition to this cell-cell contact, Mϕ also secrete up-regulatory signals to which T cells can respond. These signals include interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), and others (1,87,126,133,139,148,183,242,245). The activation of CD4⁺ T_H cells by Mϕ begins the complex circuit of immune regulation and is pivotal to a successful immune response. Activated CD4⁺ T cells can in turn activate: (i) Mϕ through their release of lymphokines such as IL-3, IL-4, granulocyte macrophage-colony stimulating factor (GM-CSF), and interferon-γ (IFN-γ) (61,65,66,84,148,153); (ii) CD8⁺ cytotoxic T (T_C) cells through their release of IL-2 and IFN-γ (148,153,203,204); and (iii) B cells through their release of IL-4, -5, and -6 (148,153).

In addition to being a key cell in initiating an immune reaction, Mϕ also function to suppress the immune response after the antigen challenge has passed. This down-regulation is accomplished by the natural presence of suppressor Mϕ and their inhibitory monokines, such as prostaglandins and other factors (3,4,31,71,176,215,234). These factors can down-regulate: (i) T-cell responsiveness to cytokines (247,270); (ii) T-cell production of lymphokines (31,33,270); (iii) T_C-mediated cytotoxicity (22,34,77); and (iv) B cell production of antibody (6,78). This "normal" presence of suppressor Mϕ is well documented (34,119,120,135,151,176,257). Suppressor Mϕ operation insures tight regulation of the immune network. In fact, the role suppressor Mϕ play in maintaining immune homeostasis may be similar to that suppressor T cells were formerly thought to fill. Thus, Mϕ can serve as the initiators of an immune response and the terminators of an immune response. In a healthy host, this process of up- and down-regulation is finely tuned, with the effector and suppressor arms being tightly balanced.

The Mϕs' helper and suppressor role are not completely defined because unlike T cells, which are classified into distinct functional subpopulations based on their cell surface phenotype, Mϕ are not. Thus, there are nagging questions: do Mϕ represent a single population of cells that can respond to different stimuli with different functional attributes, or are they a heterogeneous group of cells representing a series of different subpopulations with defined functions? Mounting evidence

INTRODUCTION
is now available to support the notion that Mϕ heterogeneity is due to distinct subpopulations of Mϕ (16,47,243,253). Most of this earlier evidence, however, dealt with the different functional attributes based on the various mechanisms of isolation (244,250). Phenotype-to-functional correlations were not done, as were done with T cells. That is, CD4+ T cells are T_{H} cells and CD8+ T cells are T_{E} cells and/or suppressor T (T_{S}) cells.

Recent work in our laboratory (8,72-74,137) has described and correlated the phenotype of various Mϕ subpopulations with distinct functions, providing evidence that subpopulations of Mϕ exist. Work by others (130,244,249,251) also suggests that there are different subpopulations of Mϕ. The delineation of shifts in Mϕ subpopulations is especially important in many disease states, such as cancer, because Mϕ-mediated regulation of the immune response is compromised.

During tumor growth, the host's immune system becomes impaired and is unable to mount an effective response against the tumor. The cause of this immunosuppression is not completely understood, but Mϕ are one of the cell types involved in this immunosuppression. What causes these changes in Mϕ-immune regulation? Are they due to a quantitative shift in the Mϕ subpopulation responsible for up-regulating and down-regulating immune reactivity, a qualitative shift in the helper or suppressor Mϕ populations, or both? Earlier evidence (7,37,45,50,51,72-74,137) from our laboratory suggested that there is both a quantitative change and a qualitative change in Mϕ during tumor growth and that these changes lead to the loss of the Mϕ population that can up-regulate T_{H} cells and keep the suppressor Mϕ in check. Concomitant with the decreases in some Mϕ subpopulations during neoplastic growth, there is also an increase in the suppressor Mϕ population (76,137,172,177,231,236,258). These data show identifiable changes in Mϕ during tumor growth. Therefore, I undertook studies to answer how tumor growth alters Mϕ-mediated immunoregulation.

Based on the previous results (72-74,137), my study was to expand our knowledge of the changes in Mϕ-mediated regulation of the immune system during tumor growth. Specifically, I sought to answer these three questions:

1. Does tumor growth alter the phenotypic characteristics Mϕ?
2. Are there functional changes in Mφ during tumor growth that lead to an increase in their suppressive activity?

3. Can phenotypic changes be correlated with functional changes by identifying suppressor Mφ subpopulations that are responsible for the tumor-induced immunosuppression?

To address the first question, I used monoclonal antibodies (mAb) originally described by Springer et al. (95-97,208-211). These mAb are specific for the murine Mφ surface antigens Mac-1, -2, and -3. The mAb specific for La was also used. These different mAb may describe Mφ subpopulations and functional differences in Mφ. Mφ were examined for the expression of these antigens and the potential changes in their expression during tumor growth by using: (i) one-color fluorescence analyses to initially characterize Mφ; and (ii) two-color fluorescence analyses to explore the multiple expression of these various Mφ antigens. These techniques provided exciting and novel insights into Mφ subpopulations and how tumor growth affects these subpopulations. These data are presented in Chapters I and II.

To address the second question, I examined Mφ accessory function to CD4+ T cells in the syngeneic mixed lymphocyte reaction (SMLR). Mφ serve as accessory cells to autoreactive T cells by way of their cell surface antigens and release of soluble factors (14,36,62,157,158,168,260). This function of Mφ is vital to the up-regulation of T cell reactivity. The identification of abnormalities in tumor-bearing host (TBH) Mφ function would point to underlying defects in Mφ function and the potential sites at which therapeutic intervention could take place. Chapters III and IV describe many interesting facets of the nature of the tumor-induced defects in Mφ responsiveness. Chapter V examines how tumor growth changes the way TBH Mφ respond to the activating agent lipopolysaccharide (LPS).

To address the third question, I separated normal and TBH Mφ into different populations by mAb plus complement treatment and then measured for their accessory cell function. This work was done to identify the subpopulation of suppressor Mφ and how tumor growth leads to shifts in this subpopulation of Mφ. These Mφ populations were used as accessory cells to CD4+
alloreactive T cells (Chapter VI) and autoreactive T cells (Chapter VII). Chapter VI and VII provide the provocative and novel work addressing this third question.

The dissertation is divided into seven chapters dealing with the research mentioned above. The chapters are preceded by a LITERATURE REVIEW that examines the scientific background and basis for these studies. The seven chapters are arranged in chronological order and represent the sequence for uncovering the tumor-induced changes in MΦ, that is, from the early phenotypic changes to the later functional changes. After the last chapter, I discuss and summarize my results. In the SUMMARY, I emphasize how the phenotypic changes relate to the functional changes and how my observed changes in TBH MΦ subpopulations ultimately lead to the suppression of the immune response seen in TBH.
LITERATURE REVIEW

The importance of Mφ in the immune system has received much attention in recent years, because changes in Mφ function are associated with or caused by many disease states. This LITERATURE REVIEW is separated into five related topics that gives the reader a review of the pertinent literature and a background to my work. An Introduction to the Mφ is the first section and gives the reader a feel for what Mφ are and how far Mφ biology has come. Second is a review entitled Mφ Heterogeneity: Is It due to Subpopulations?. This question-title presently remains unanswered. Attempts to answer this question need to be made before the interactions of Mφ and the rest of the immune system can be completely explained. The changes in Mφ subpopulations that occur during tumor growth may be what lead to the dysfunction of Mφ-mediated regulation of the immune system. Third, in Characterization of Mφ Subpopulations, I examine the past and present methods of delineating Mφ subpopulations and what these methods tell us about Mφ heterogeneity. Fourth, in Mφ Immunoregulatory Functions, the role Mφ play in regulating the immune response is discussed. This section leads into the final section, because it examines Mφ interactions in the normal animal. The final section Mφ-Mediated Immunosuppression During Tumor Growth examines how a growing tumor alters Mφ function. The latter topic is related to the previous sections, that is, how tumor growth upsets Mφ subpopulations and the role those
subpopulations play in the immune system. The last section also serves as an introduction to the work discussed in this dissertation.

INTRODUCTION TO THE Mϕ

The phagocyte or Mϕ was the first immune cell to be discovered. In 1882 Elie Metchnikoff described a motile phagocytic cell in transparent star fish larvae. This cell would surround and attempt to engulf the thorn he stabbed into these organisms. From this initial experiment, Metchnikoff realized there may be similar cells in humans that served in the defense of the host against intruders (199). This early work led Metchnikoff to propose his theory of cell-mediated immunity and eventually led to his winning the Nobel prize in 1908. The theory of cell-mediated immunity, however, fell into disfavor in the early twentieth century because Immunologists, such as von Behring and Ehrlich, proposed the theory of humoral immunity to explain resistance to disease (199). Because of the general acceptance of the humoral theory, cell-mediated immunity as a major mechanism of immune defense did not resurface until the 1950s and 1960s.

The original function of the Mϕ was thought to be one of a nonspecific scavenger; that is, when a bacteria invaded a host and the Mϕ came into contact with the bacteria, the bacteria would be engulfed and destroyed. Since this early theory of Mϕ function, much has been learned about the role Mϕ play in the immune system (reviewed later). The immune system is a collection of cells that interact to provide protection from foreign pathogens, such as bacteria and viruses, and internal agents, such as aberrant cancerous cells. The immune system includes three major populations of leukocytes: B cells, T cells, and Mϕ. B cells are the cells responsible for humoral immunity and T cells are the cells responsible for cell-mediated immunity.

Mϕ, however, are considered the primary initiators of the immune response against antigens. Mϕ are the first cells that can come into contact with foreign or aberrant antigens and are shown
by many (reviewed later) to be the vital first cell in an immune response. Mφ are part of the mononuclear phagocyte system. Mφ originate from pluripotent stem cells in the bone marrow, differentiate to monocytes, enter the peripheral blood, and then as end cells take up residence in various anatomical sites as tissue Mφ with a variety of functions (16,47,99,243,253).

Functionally, Mφ are important in phagocytosis and destruction of foreign invaders (243,250). They can process and present antigens to Tₜ cells as well as release soluble immune modulators which can influence T-cell proliferation and activation (240-242). Mφ are also required for generating anti-tumor immunity (93,116). In addition to being a key cell in initiating an immune reaction, Mφ also play a role in the reduction of the immune response after antigen challenge has passed. This Mφ-mediated down-regulation of the immune response may be caused by suppressor Mφ and the factors Mφ release, which can suppress T cells, B cells, and Mφ (78,119,160,215). Tₜ cells are also thought to play a role in this down-regulation of the immune response. The actual role of Tₜ cells in the suppression of an immune response remains uncertain (154). This complex immune circuit is finely tuned and designed to maintain immune homeostasis. This idea of an immune network is the basis of the theory Jerne proposed in 1974, leading to his 1984 Nobel prize (101).

Mφ HETEROGENEITY: IS IT DUE TO SUBPOPULATIONS?

Mφ are a heterogeneous group of cells that have been characterized by assessing a variety of different functions (16,47,98,243,250,253). These functions are reported to be due to different subpopulations of Mφ. Mφ are important in (i) antigen presentation, (ii) antimicrobial and anti-tumor function, and (iii) phagocytosis. Yet not a single Mφ population has been reported to have
all these functions, that is, some Mφ can present antigen, some are bactericidal, and some are tumoricidal. In addition, Mφ produce over 100 different factors including proteins, lipids, nucleotide metabolites, and oxygen metabolites (223), with different populations of Mφ reported to secrete only some of these products (16,47,98,243,250,253). This apparent heterogeneity in Mφ function may be explained by the existence of distinct Mφ subpopulations.

To understand the role Mφ play in the immune system, one must know what contributes to this Mφ diversity. Are Mφ a single population of cells in different states of activation or differentiation that respond to various stimuli by exhibiting different functional attributes and, thus, appear to be a diverse population of cells (16,47,243); or does this heterogeneity reflect diverse and distinct subpopulations of Mφ that have unique and defined functions (27,47,217,243,251)?

Recent evidence (27,47,194,195,217,243,251) points to Mφ diversity being due to distinct Mφ subpopulations. Although the existence of Mφ subpopulations can explain much of the reported differences in Mφ functions, differences in Mφ activation and/or differentiation probably also contribute some of the heterogeneity. The presence of distinct Mφ subpopulations suggests that differentiation must precede the development of unique Mφ functions. Two hypotheses have been suggested to explain differentiation. One suggests that all Mφ develop from pluripotent stem cells in the bone marrow where they undergo several steps of differentiation, from monoblasts to promonocytes, before entering the peripheral blood as monocytes (16,47,243). After several days, monocytes enter various tissues and in these microenvironments Mφ receive their final differentiation signals (13,47,194,244,251). The evidence that tissue Mφ come solely from monocytes was found using chimeras and by following the in vivo kinetics of radio-labeled cells (47,243). This hypothesis is presently the most accepted.

Another hypothesis suggests that Mφ subpopulations result from differentiation along several independent lines. Recent evidence lends support to this second hypothesis. Shibata et al. (194-196), using 89Sr to destroy bone marrow cells, show at least three different pathways of Mφ differentiation. When the bone marrow was destroyed, the number of monocytes and Corynebacterium parvum-induced suppressor Mφ dropped significantly, but there was no change in the number of resident peritoneal Mφ. In addition, the number of splenic Mφ colony-forming
units (M-CFU) increased after destruction of the bone marrow, suggesting ontogenetic and environmental factors both contribute to the functional heterogeneity of Mφ. Thus, the peritoneal and splenic Mφ seem to come from self-renewing populations (bone marrow independent) while the monocytes and suppressor Mφ are bone marrow-derived. Daems and de Bakker (42) and Stewart et al. (213) show that resident peritoneal Mφ do not arise from blood monocytes but rather from stem cells in the milky spots of the peritoneal cavity. Goodman (82) has also shown the presence of hemopoietic stem cells in the peritoneal cavity. Work by Walker (251) shows that Mφ need not originate in the bone marrow. Splenic Mφ can form from M-CFU in the spleen and these Mφ are heterogeneous with respect to Mac-2 expression, Ig expression, and antigen presentation. Walker’s data show separate lines of differentiation, and that stem cells within a population can form separate subpopulations of Mφ. Van Furth and Diesselhoff-den Dulk (244) show that the murine spleen contains at least two populations of Mφ. One of these splenic Mφ populations was derived from the classical differentiation pathway in the bone marrow, while the other may be maintained by an independent self-renewal mechanism.

Mφ development is probably a result of several mechanisms, with some Mφ coming from the bone marrow through monocytes (47,243) and others (42,82,194-196,217,244,251) coming from self-renewing populations in "hemopoietic" sites distinct from the bone marrow. Mφ would terminally differentiate in the organ or tissue where they reside. The microenvironment of this final differentiation site must be responsible for some of the heterogeneity of Mφ, because different Mφ subpopulations in the same site are derived from the same progenitor pool (13,42,47,217,244,251). These sites of Mφ self-renewal probably originated from bone marrow hemopoietic stem cells during fetal ontogeny or later in life, although the origin is unknown. Taken together, the evidence suggests that there are distinct subpopulations of Mφ. Because these subpopulations may have distinct functions, it becomes important to characterize these subpopulations based on their phenotypic make up.
CHARACTERIZATION OF MΦ SUBPOPULATIONS

Because the understanding of the role MΦ play in an immune response is clouded by the heterogeneity of MΦ, it becomes necessary to characterize and identify different MΦ subpopulations. The characterization of MΦ subpopulations was one of the goals of my work. Before the widespread use of mAb, MΦ were characterized by their associated enzymes, including peroxidase and esterase (79,105,131,141,144,223). Peroxidase was a useful enzymatic marker of MΦ maturity (105,141) and esterase proved to be a constitutively produced enzyme that could be used as a MΦ pan marker (131,144). Other enzymes, such as lysozyme, proteases, peptidases, and nucleotidases, can also be detected in MΦ and some are differentially expressed in different MΦ populations (79,223,248). Other methods of characterizing MΦ have included measuring MΦ for their ability to phagocytize or opsonize foreign particles. Buoyant density centrifugation has also been used to isolate MΦ into discrete fractions (29,53,112,155,237,267). Using this last method, researchers showed differential expression of Ia and peroxidase (29,237) as well as differential production of IL-1 and prostaglandin E₂ (PGE₂) in the separated fractions of MΦ (53,112).

The development of mAb technologies allowed MΦ populations to be examined or defined by their surface antigen expression. Murine MΦ can be characterized by the expression of: Fc and complement receptors (47,250,252,253); Mac-1, -2, and -3 antigens (95-97); the F4/80 antigen (11); Ia antigen (73,237,253); and other surface antigens (225,226). Human MΦ can also be characterized by their surface antigen expression (7,15,166,175,233). These antigens proved to be useful MΦ markers, with some being MΦ pan markers (11,96), others differentiation markers (95,97), and still others activation markers (95,225,226). Some of these markers may be important in delineating MΦ subpopulations.

Because of the importance of the Mac antigens (reviewed below) and my extensive use of mAb against the Mac-1, -2, -3, and Ia surface markers, I will spend the remaining review of MΦ characterization on the Mac-1, -2, -3, and Ia MΦ surface antigens. These antigens were examined because previous work (72-74) suggested that they could be used to identify MΦ subpopulations. More
importantly, there seemed to be a shift in the subpopulations of Mφ expressing these antigens during tumor growth. The mAb against the Mφ surface antigens Mac-1, -2, and -3 were developed by Springer et al. (95-97,208-212), who also performed much of the early work exploring their expression and characteristics.

Mac-1 (CD11b) belongs to a family of cell surface adhesion molecules including lymphocyte function associated antigen-1 (LFA-1) and p150/95 (145,212,213). Mac-1, LFA-1, and p150/95 are αβ heterodimers with dissimilar 170 kD, 180 kD, 150 kD α chains, respectively, but identical 95 kD β-chains (5,212,213). Mac-1 can be found on Mφ, monocytes, granulocytes, natural killer cells (NK cells), and bone marrow cells. Mac-1 is a receptor for the C3bi component of complement (48,212). Anti-Mac-1 mAb can block Mφ and monocyte adherence and chemotaxis, suggesting Mac-1’s importance in adherence and chemotaxis (19,108,212). Adherence through the Mac-1 receptor has also been shown to induce degranulation of phagocytes and their release of H2O2 (48,190,193). Mac-1 expression increases as monocytes mature (208), and Mac-1 is continuously expressed on mature Mφ, suggesting that it is an early differentiation antigen (96).

Mac-2 is a 32 kD protein which is variably expressed on Mφ from different tissues, Mφ cell lines, and on Mφ elicited by different stimulatory agents, suggesting Mac-2 represents a late differentiation and/or activation marker (95,217,251). Mac-2 has DNA sequence homology to a rat IgE receptor and may function as such in vivo (32). Mac-2 also has lectin-like properties that may facilitate Mφ-parasite binding (32). Mac-2 does not, however, contain a classical signal peptide or transmembrane domain, suggesting that its attachment to the Mφ surface may be through another surface protein (32).

Mac-3 is a protein of variable molecular weight (92 kD to 110 kD), is found on mature Mφ, is variable expressed, and may be a late differentiation and or subpopulation marker (97,249). The function of Mac-3 is presently unknown.

The Mac-1, -2, and -3 antigens are important in defining Mφ subpopulations because they are expressed on different populations of Mφ (72,74). Garner et al. (72,74) used the expression of these Mac antigens to define different functional populations of Mφ. In the peritoneal cavity, a Mac-1+ Mφ functions as a regulator of PGE2 production and a Mac-3+ Mφ functions as a suppressor
Mφ (74). Askew et al. (8) showed that a Mac-2+ Mφ may be the primary PGE2 producer. While in the spleen, a Mac-1− Mφ may be the accessory Mφ for mitogen-stimulated T cells (72). The Mac antigens can therefore be used as markers for Mφ subpopulations. Tumor growth, however, was shown to change these populations and these changes in Mφ may be the cause of the tumor-induced immunosuppression (discussed later).

Ia antigens are the major histocompatibility complex class II markers and are needed for effective Mφ antigen presentation to T cells (240-242). Ia antigens are αβ heterodimers with a 34 kD α chain and a 28 kD β chain. A substantial volume of work has described differences in Mφ populations based on their expression of Ia. Walker et al. (255) showed that there were unique progenitor cells which gave rise to Ia+ and Ia− Mφ, demonstrating that different subpopulations of Mφ existed based on their expression of Ia. Beller and Uaane showed that Ia− Mφ not only lacked surface expression of Ia but also lacked intracellular expression of Ia (17). Thus, the expression of Ia must either be stimulation-dependent or subpopulation-dependent (1,17,18). The necessity of Ia expression on Mφ for presentation of nominal antigen to specific T H cells (240-242) and autoreactive T H cells (168) means that only Ia+ Mφ are capable of the cell-cell interactions that must occur before activation of T cells. Ia expression, however, is not correlated with phagocytic ability (237). Expression of, or lack of, Ia expression has been linked to suppressor Mφ function. Chow and Battisto (34) showed that Ia− Mφ were the final suppressor cells in the suppressor cascade for down-regulating T C cells. Others (135,152,161) have also shown that Ia− Mφ may be the important cells in the suppressor cascade, while Dorf and co-workers (120,164,165) showed that Ia+ I-J+ Mφ are the important suppressor Mφ. The cause of these differences is unknown but reported to be due to differences in culture or experimental conditions (164,165). Nevertheless, different subpopulations of Mφ can be defined by their surface markers and correlated to different specific functions.
Mφ IMMUNOREGULATORY FUNCTIONS

Even though Mφ can be defined by one of several different functions including phagocytosis, killing, or immunoregulatory, I will focus on the importance of Mφ as immunoregulatory cells. My functional studies dealt exclusively with the immunoregulatory function of Mφ. The Mφ’s prolific secretory capacity (223) makes it one of the chief immuno regulatory cells in the immune system. Mφ immunoregulation can affect the humoral (78,134,140) and cell-mediated (155,160,180-182,232) branches of an immune response. In fact, the dependence of immune reactivity on Mφ may be absolute, because when Mφ are removed from lymphocyte cultures T-cell reactivity is reduced (24,86,182,218). The importance of Mφ in initiating an immune response stems from the fact that: (i) Mφ are the first cells to encounter foreign or aberrant antigens; (ii) Mφ can process and present this antigen to Tₜ cells; and (iii) Mφ can recognize the native or whole antigen while Tₜ cells can only recognize the processed version of the antigen.

Although physical contact between Mφ and T cells through an Ia-T cell receptor interaction is necessary for T cell activation, this interaction is only the first signal (168). T cells need a second signal which is usually a soluble factor (cytokine) such as IL-1 (49,87,133,148), IL-6 (148,245), TNF-α (87,126,178,183,191), GM-CSF (69,123), or the newly discovered co-stimulatory factor (179,256). These different cytokines stimulate IL-2 or IL-4 production by Tₜ cells, thereby initiating T-cell proliferation and up-regulation of the immune response (146,148,203,204).

Mφ-induced activation and differentiation of Tₜ cells can influence Mφ themselves. Mφ respond to a variety of T cell-derived cytokines, including IL-3, IL-4, IFN-γ, TNF-α and GM-CSF. All of these cytokines can act as Mφ-activating factors (MAFs). IL-3 induces Ia expression, LFA-1 expression, and IL-1 production but not Mφ cytotoxicity (65,66). IL-4 induces Ia expression, Fc receptor expression, Mac-1, LFA-1 and p150/95 expression, and Mφ cytotoxicity (28,221,229). IFN-γ induces Ia expression, LFA-1 expression, IL-1 secretion, and Mφ cytotoxicity (2,20,28,142,170,171). TNF-α induces Mφ cytotoxicity (92,129). GM-CSF induces Ia expression, Mac-1 expression, Fc receptor expression, IL-1 secretion, and Mφ cytotoxicity

LITERATURE REVIEW
Some of these factors can induce PGE$_2$ production (91,129) that probably functions in an autoregulatory loop to control extended activation of M$\phi$ (discussed below). Many of these cytokines have synergistic or additive effects on M$\phi$. For example, Frendl and Beller (65) show that IFN-$\gamma$ and IL-3 are synergistic for induction of Ia and additive for induction of LFA-1. Heidenreich et al. (91) and Hart et al. (90) show that GM-CSF and IFN-$\gamma$ are both needed to induce TNF-$\alpha$ production. Heidenreich et al. (92) also show that TNF-$\alpha$ can act with suboptimal doses of IFN-$\gamma$ and LPS to induce M$\phi$ cytotoxicity. Some of these different MAFs may work as antagonists. te Velde et al. (228) show that IFN-$\gamma$ and IL-4 are antagonistic for Fc receptor expression on human monocytes. After M$\phi$ activation of T cells, T cells through their release of different MAFs, can activate M$\phi$.

A feature of M$\phi$ immunoregulation is that whenever M$\phi$ can enhance a particular immune response, they can also inhibit the response. M$\phi$-mediated suppression is through soluble modulators that can inhibit T-cell proliferation. These modulators can also affect M$\phi$ through an autocrine mechanism. M$\phi$ soluble suppressor molecules include low molecular weight compounds such as thymidine (214), arginase (121), complement components (186), and prostaglandins (PG) (4,30,75,81,163), as well as the larger molecular weight compounds such as the high molecular weight inhibitor found previously in our laboratory (138), the soluble immune response suppressor discovered by Aune (10), the factor(s) investigated by Chow and Battisto (34), and others (31,67,79,109,132,234).

Suppression mediated by PG has been well characterized and, because much of my research dealt with the involvement of PG in down-regulation of T-cell reactivity, I will emphasize PG-mediated suppression. PG are produced by the cyclo-oxygenase pathway of arachidonic acid metabolism. Monocytes and M$\phi$ are the primary producers of PG, particularly PGE$_2$ (75,81,124,163,215). PGE$_2$ is a small (352 molecular weight) compound with a variety of inflammatory and anti-proliferative functions, not unlike many arachidonic acid metabolites (75,80,163,215). These functions include direct suppression of T$_H$ cells or indirectly through the activation of T$_s$ cells (33,177). PGE$_2$-mediated suppression of lymphocyte proliferation is linked to changes in cAMP and is associated with inhibition of IL-2 production, IL-2 receptor expression,
and IL-1 and IL-2 responsiveness (113,247,270). PGE_2 can suppress GM-CSF production by T_n cells (174), and inhibit T_c cell (77,128) and NK cell (22) activities.

PGE_2 can act as an autocrine molecule. Snyder et al. (207) showed that PGE_2 reduced Ia antigen expression on Mφ, and Kunkel et al. (122) showed that PGE_2 down-regulated IL-1 production by Mφ. PGE_2 can also inhibit LPS-induced Mφ-mediated cytotoxicity (184) and release of oxygen metabolites (143). PGE_2 can block Mφ chemotaxis (71) and is reported to prevent Mφ-mediated phagocytosis of foreign particles (12,40). In addition, Heidenreich et al. (91) showed that GM-CSF-induced PGE_2 production inhibited the GM-CSF/IFN-γ-induced release of TNF-α. The generation of these two mediators by GM-CSF provides an autoregulatory circuit in which the later produced PGE_2 limits Mφ activation. Hancock et al. (89) showed that GM-CSF acts in an autocrine manner due to induction of PGE_2. At low doses, PGE_2 can augment TNF-induced activation of Mφ (129) and act synergistically with IL-2 on some T cell clones to increase GM-CSF production (174). PGE_2 is thought to be part of the normal process of reduction of the immune system after antigen challenge has passed. Suppression of immune reactivity by Mφ through products such as PGE_2, that inhibit activated T cells and Mφ, is thought to be by a subpopulation of Mφ called suppressor Mφ (30,75,119,120,132,165,257,263). This down-regulation may be the result of negative feedback loops in which Mφ and Mφ-T cell/B cell interactions are inhibited. This shutting off of the immune response by Mφ is a normal occurrence in Mφ-mediated immune regulation.
**Mφ-MEDIATED IMMUNOSUPPRESSION DURING TUMOR GROWTH**

In disease states such as cancer, Mφ-mediated regulation of the immune response is compromised. During tumor growth, the host's immune system becomes suppressed and Mφ are one of the cells that play a major role in this immunosuppression. Because of the Mφ's paramount role in the immune system, investigation of how tumor growth affects changes in Mφ-mediated regulation is vital.

The immune system effectively prevents tumor growth as evidenced by the general good health of most people. For unknown reasons, some cancers can develop uninhibited by the immune system, usually leading to the death of the host. How does the body allow these aberrant cancerous cells to reproduce unchecked when it has such a powerful capacity to prevent or destroy tumor growth? As mentioned above, the immune system is a complex network of interacting cells that is responsible for protecting the host against neoplastic growths. Mφ are only one of the many critical components whose cooperation is required for tumor cell rejection. Ia+ Mφ are required for the presentation of tumor antigens to T<sub>H</sub> cells (93,116). T<sub>H</sub> cells release lymphokines such as IL-2, IL-3, IL-4, IFN-γ, and GM-CSF which can activate NK cells, tumor-specific T<sub>C</sub> cells, and induce other Mφ to become tumoricidal. Upon activation, T<sub>C</sub> cells and Mφ release cytotoxic factors which destroy tumor cells (127,139,169,189,265). T<sub>C</sub> cells can release lymphotoxin (169,189) and Mφ can release TNF-α (61,123,139,183), which are cytotoxic to tumor cells. Mφ also release a variety of reactive oxygen metabolites and destructive enzymes (1,223). Thus, the immune system presents a powerful network of cells and chemicals to destroy tumor cells.

In the cases where tumor growth goes unchecked, as signals are sent between Mφ and T cells, another process induced by the cancer itself signals the body to suppress the immune response. Early work demonstrated that tumor-produced factors act on the immune system by directly suppressing immune cells (39,59,82,94,103,106) or indirectly through the induction of T<sub>S</sub> cells.
(23,35,102,106,173) and/or suppressor Mφ (6,103,110,172,230,231). PGE₂ production by tumors has also been implicated in tumor-induced immunosuppression (85,268). Because of the work showing increased production of transforming growth factor-β (TGF-β) during tumor growth and its potential role as a down-regulator of Mφ function, TGF-β has also received much attention (41,57,107). Recently, Tsuchiya et al. (236) showed that many different tumors produce a GM-CSF-like molecule which could induce suppressor Mφ. Altered or increased production of normal immunomodulators like CSF, PGE₂, and TGF-β could have profound effects on immune cells and their interactions in the immune network.

In early work from our laboratory, Elgert and Farrar (51) documented that tumor-induced immunosuppression was due to dualistic control by Tₜ cells and Mφ. The role of Mφ in this immunosuppression is pivotal and may be dominant. Mφ can be the key cell in reduction of the immune response during tumor growth (35,45,67,76,138,172) by their increased production of PGE₂ and other inhibitory molecules. Production of PGE₂ is 2-5 times higher in Mφ from TBH. This increased production, coupled with the increased numbers of Mφ, correlates with a significant increase in in vivo PG secretion. Goodwin et al. (83) showed that peripheral blood mononuclear cells from patients with Hodgkin's disease produced increased amounts of PGE₂. Gemsa et al. (76) also showed increased production of PGE₂ by Mφ during lymphoma growth. Others (45,67,137,138,172) also have shown an increased production of PG during tumor growth. Increased PGE₂ production by TBH Mφ can have many deleterious effects on the immune system, because PGE₂ can down-regulate Ia expression (207), IL-1 and IL-2 production (247,270), and IL-2 receptor expression (247). Many of these changes have been observed in TBH. Garner et al. (73) and Nagai et al. (156) demonstrated reduced Ia expression on TBH Mφ. Moldawer et al. (149) showed decreased IL-1 production by Mφ from mice bearing sarcomas and Burger et al. (26) showed decreased production of IL-2 in TBH. The data suggest that changes in immune reactivity during tumor growth could correlate with increased production of PGE₂ and induction of PGE₂-producing suppressor Mφ. A shift in this suppressor Mφ subpopulation could cause an increase in TBH Mφ-mediated suppression. Therefore, identifying and characterizing this subpopulation of Mφ has been the a goal of our laboratory and was one of my research goals.
Previously Malick et al. (136) and Garner et al. (72-74) showed a shift in Mφ subpopulations and changes in Mφ antigen expression and Mφ function during tumor growth. They showed that there were identifiable shifts in the surface antigen expression of Mac-1, -2, -3, and Ia on peritoneal (74,137) and splenic Mφ (72,73). More importantly, they correlated some of the phenotypic changes to functional changes. In the peritoneal cavity there was a shift in the phenotype of the suppressor Mφ subpopulation from Mac-3+ in the normal host to Mac-2+ in the TBH (137). The shift to a TBH Mac-2+ suppressor Mφ correlated with increased production of PGE₂ (137). In addition, the Mac-1+ subpopulation of Mφ in the normal host was an important down-regulator of Mφ-produced PGE₂ but was absent in the TBH (74). In the spleen, the accessory Mφ shifted from a Mac-1− phenotype in the normal host to a Mac-1+ Mφ in the TBH (72). These data demonstrate that there are shifts in Mφ populations during tumor growth. The recent work by Tsuchiya et al. (236) showing the induction of Mac-1+2+ suppressor Mφ by tumor-produced factors and Young et al. (269) showing a shift in alveolar Mφ from helper Mφ to suppressor Mφ during tumor growth lends further support to the theory that there is a shift in the suppressor Mφ subpopulation during tumor growth. Others also report on how changes in Mφ are associated with tumor-induced immunosuppression (76,177,246,258) and how changes in Mφ are associated with other diseases (162).

Tumor growth can affect changes in Mφ by altering their phenotypic and functional makeup. The cause of these changes in Mφ phenotype and function is due to a shift in Mφ subpopulations. The goal of my work was to understand this tumor-induced shift in Mφ subpopulations. The work presented in the following chapters helps further our understanding of tumor-induced alterations in Mφ subpopulations and how these alterations may lead to immunosuppression.
CHAPTER I

CHANGES IN MACROPHAGE POPULATIONS:
PHENOTYPIC DIFFERENCES BETWEEN NORMAL AND TUMOR-BEARING HOST MACROPHAGES

INTRODUCTION

Mφ are a heterogeneous group of cells exhibiting many functions during an immune response. Heterogeneity may reflect diverse and distinct subpopulations of Mφ. This hypothesis is supported by work of others (131,249,251,253) as well as ourselves (72-74). To understand Mφ functions during the immune response, these subpopulations need to be characterized and their functional relationships established. Attempts at characterizing Mφ phenotypes were done by investigating Mφ expression of surface antigens such as Ia (73,237,253), Fc and complement receptors (47,250,252,253), Mac-1, -2, and -3 expression (72,74,211,250,251), and other surface antigens
Mφ can also be fractionated by size and density (155,253). These types of Mφ subpopulation delineation are critical in studies involving TBH, since tumor growth alters the immunoregulatory properties of Mφ. We (50,51,72-74,137) and others (94,103,231,232,246) demonstrate alterations in Mφ phenotype and function contribute to tumor-induced immune dysfunction.

The goal of our current work is to characterize functional/phenotypic relationships between normal and TBH Mφ and relate this to immune dysfunction. This study is a continuation of our work (72-74,137) and involved the phenotypic characterization of Mφ subpopulations by their antigen expression. The availability of anti-Mφ mAb allows for characterization of Mφ phenotypes. Monoclonal antibodies against the Mφ surface antigens Mac-1, -2, and -3 (95-97) and the mAb against Ia antigen were used in this study. Mac-1, -2, and -3 antigens are glycoproteins found on the surface of Mφ and other cell types. Mac-1 is found on Mφ, monocytes, granulocytes, a proportion of NK cells, and bone marrow cells. The continuous expression of Mac-1 on Mφ suggests it is an early differentiation marker (96), as further evidenced by an increase in Mac-1 expression as monocytes mature (208). Mac-2 is variably expressed on Mφ populations from different tissues and on Mφ elicited by different stimulatory agents, suggesting Mac-2 may represent a differentiation and/or activation marker of Mφ in response to a specific signal (95). Mac-3 is found on Mφ and is reported to be a differentiation antigen with variable expression (97,249,251). Ia antigen expression on Mφ is needed for Mφ to be effective presenters of antigen (240-242). These studies on the Mac antigens support the notion of different Mφ populations and previous Western blotting analyses (unpublished data) suggest they are stable phenotypic markers; that is, tumor growth does not seem to alter their structure.

Expression of Mac-1, -2, -3 and Ia antigens on normal and TBH Mφ was examined by flow cytometry. A technique used by others to characterize B cells (68), NK cells (126), T cells (259), as well as Mφ (46,209,249,251). Tumor growth resulted in phenotypic changes in peritoneal and splenic Mφ. Overall percentages of TBH peritoneal Mac-2⁺, -3⁺, and Ia⁺ Mφ decreased. In contrast, the overall percentage of Mac-1⁺ Mφ did not change during tumor growth. The percentage of TBH Mac-1⁺, -2⁺, and -3⁺ splenic Mφ all increased, while TBH Ia⁺ splenic Mφ de-
creased. Three size versus antigen-expression peritoneal Mφ subpopulations were seen and changed during tumor growth. This was not the case with splenic Mφ. Taken together, these changes suggested tumor growth modulated Mφ phenotype. Combined with our previous work (72-74,137), a relationship between Mφ phenotype and tumor-induced immune dysfunction is suggested.
MATERIALS AND METHODS

**Animals**

Eight to 12 week-old male BALB/c mice (H-2^d; Dominion Labs, Dublin, VA) were used. TBH mice received intramuscular injections of $4 \times 10^5$ cells from a methylcholanthrene-induced, non-metastatic, transplantable fibrosarcoma into the left hind leg. This resulted in palpable tumors by days 10-14 and death by days 28-35 (50,51). Mice with no tumors (day 0/normal) or with tumors (7, 14, or 21 days after tumor cell injection) were used in this study. By days 10-14, there is significant suppression of concanavalin A (Con A), phytohemagglutinin (PHA), and mixed lymphocyte reaction (MLR) reactivity and suppression increases as tumor growth progresses (51,72,73,261).

To validate using untreated mice for controls, as opposed to ones with nonspecific inflammatory responses, BALB/c mice were injected with $4 \times 10^5$ allogeneic, nontumor C3H muscle cells. No significant differences in MLR and SMLR or mitogen reactivity was seen between untreated mice and mice injected with allogeneic C3H muscle cells. Furthermore, mice injected with C3H muscle cells had Mac-1, -2, -3, and Ia fluorescence patterns identical (histograms superimposable) with untreated mice; therefore, nontumor-bearing mice (Day 0/normal) were used throughout. Mice were LDH virus negative (Microbiological Associates, Bethesda, MD).

**Peritoneal Mφ Preparation**

Peritoneal exudate cells (PEC) were obtained by peritoneal lavage from 0 (day-0 represents the normal host Mφ), 7, 14, and 21 day TBH BALB/c mice 4 days after intraperitoneal injection of 2.0 ml of sterile thioglycollate. Whole PEC (no plating) labeled with antibodies were analyzed by
flow cytometry. These Mφ were selected by gating the flow cytometer on the forward angle light scatter (FALS) and 90 degree angle light scatter (90LS) pattern to exclude lymphocytes and granulocytes. PEC were also plated for 3 hr on 150 x 15 mm tissue culture plates (LUX/Miles Scientific, Naperville, IL), washed free of nonadherent cells, and recovered by scraping with a rubber policeman. Fifty nine percent and 63% of the recovered cells from the normal and TBH PEC were Mφ with each having >90% viability. Adherence and subsequent spreading did not result in significantly different light scatter patterns from that of unplated whole PEC (mean channel numbers for FALS and 90LS for whole and adherent PEC were 20 and 32 versus 20 and 33, respectively; TBH whole versus adherent PEC showed the same pattern). Adherent PEC or whole PEC treated with propidium iodide, a vital stain (192), and subsequently analyzed by flow cytometry were >93% viable. These adherent cells (>97% esterase positive) were used to set the FALS and 90LS gates, which were then used for selecting Mφ. Using FITC-labeled anti-Thy-1 and anti-IgG, -IgM, and -IgA (Cappel, Malvern, PA), we showed that the T and/or B cell contamination was <3% of the total of normal or TBH Mφ preparations.

**Splenic Mφ Preparation**

Whole spleen cells were harvested from untreated and TBH animals, and were not collected from thioglycollate-treated animals. Mφ in the WSC preparations were also selected by flow cytometry, gating on FALS and 90LS for the desired cell population. Splenic Mφ obtained by plating for 3 hr were used to select the FALS and 90LS gating parameters. Plated Mφ (>96% esterase positive) showed no significant changes from unplated splenic Mφ in their light scatter patterns (32 and 33 versus 29 and 35 for the FALS and 90LS of whole and adherent splenic cells, respectively; TBH Mφ showed similar patterns) and no loss of viability (>95%) due to the collection procedure. T and/or B cell contamination was <3% of the total of normal or TBH splenic cells measured.
Cell Lines

The hybridoma cell lines M1/70, M3/38, and M3/84 (producers of anti-Mac-1, -2, and -3 mAb, respectively) (American Type Culture Collection [ATCC], Rockville, MD) were cultured in 2 L roller bottles in RPMI-1640 medium (Hazleton Research Products, Lenexa, KS) supplemented with 10% or 20% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, VA) and 4 x 10^{-5} M 2-mercaptoethanol. Supernatants were harvested every 3 days and the cell-free supernatants were stored at -70°C. All media contained 50 mg/l Gentamicin (GIBCO, Grand Island, NY), 2 g/l NaHCO_{3}, and 25 mM HEPES (Research Organics, Cleveland, OH). The mAb MK-D6 (anti-Ia^a) and 25-9-3S (anti-Ia^b) were a gift from Dr. P.S. Nagarkatti.

Monoclonal Antibody Purification

Briefly, a pH 7.4 ammonium sulfate solution was added to hybridoma supernatants to yield a 50% saturated solution. After standing at 4°C overnight, precipitates were collected by centrifugation (10,000 x G, 30 min), resuspended as a 20X concentrate in PBS (pH 7.1), dialyzed against PBS for 48 hr, and applied to an affinity column. The affinity column was made by using goat-anti-rat antibody, heavy- and light-chain specific (Cappel), and linking it to cyanogen bromide activated-Sepharose 4B (Sigma, St. Louis, MO). The column was washed to remove any unbound protein with PBS (pH 7.1) until the absorbance at 280 nm was <0.02. The mAb fraction was then eluted with a 0.2 M glycine buffer (pH 2.3). Glycine-eluted column fractions were immediately dialysed to PBS with two changes over 48 hr. Column fractions are assayed for mAb using goat-anti-rat antibody, heavy- and light-chain specific (Cappel) in an Ouchterlony immunodiffusion test.
Positive fractions were pooled, assayed for antibody concentration, diluted to 1.0 mg/ml, sterile filtered, and stored at -70°C.

**Fluorescent Antibody Labeling**

Cell samples from 0, 7, 14, and 21 day TBH consisted of whole PEC at 4 x 10^6 cells/ml or WSC at 8 x 10^6 cells/ml. To 1.0 ml aliquots of these cells, 10 μl of mAb or normal rat immunoglobulin G (NR IgG) (Cappel) were added and incubated for 35-45 min at 4°C. This was followed by two washes with cold medium. Cells were incubated in a 1:500 dilution of FITC-labeled secondary antibody for 35-45 min at 4°C and washed twice. The secondary antibodies were: (i) affinity purified goat-anti-rat F(ab')_2 fragment FITC-labeled antibody (Cappel) for anti-Mac-1, -2, -3, and NR IgG and (ii) affinity purified goat-anti-mouse F(ab')_2 fragment FITC-labeled antibody (Cappel) for anti-Ia^d and anti-Ia^b antibodies.

**Flow Cytometry**

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics, Hialeah, FL) laser flow cytometer and cell sorter. The instrument was calibrated with fluorescent 10 micron-sized microsphere standards prior to analysis of the Mφ samples. Three parameters per cell were collected: FALS, 90LS, and green fluorescence (GFL). Laser excitation was normally 300 mW at 488 nm using a 5 W argon laser (Coherent Inc., Palo Alto, CA). All data collection was done with the multiparameter data acquisition and display system (MDADS, Coulter Electronics). FALS was collected using a linear integral, and 90LS and GFL were collected using the log integral. The two parameter histograms used for expressing the results are shown with a 64 x 64 channel resolution. Only the flow cytometry data from day-0 and day-21 (representative ex-
periments) are shown in the figures; however, the data from all time points are given in the tables. The figures represent raw data not compensated by control values. Data in the Tables have all control values subtracted. These calculations are done by the MDADS computer using the MDADS software.

**Calculation of Results**

Splenic or peritoneal Mφ were pooled from 3-6 mice and each experiment was repeated 3-4 times. All percentages were determined by the MDADS computer and represent the percentage of 10,000 cells counted per sample that were above the fluorescent intensity of the background level of nonspecific binding. Numbers in the tables represent the mean ± standard error (SE) of triplicate experiments for each time point. The number of TBH Mφ bearing a particular antigen was considered decreased or increased by comparison with normal host Mφ numbers which were considered as a maximum. Significance was checked using Student’s t test (p <0.05).
RESULTS

Mac Antigen Expression of Peritoneal Mφ

To examine the size versus fluorescent intensity (antigen expression) in normal (day-0) and TBH (7, 14, and 21 days after tumor cell injection) Mφ, mAb against the respective antigens were used (Figure 1A-F and Figure 2A-F). NR1IgG was used as the nonspecific control antibody. Size is represented on the X-axis and fluorescent intensity is represented on the Y-axis. The fluorescent intensity is related to the density of antigen expression per cell (192). By comparing the test groups (Mac-1, -2, and -3) to the control (NR1IgG) the percentage of positively labeled cells could be determined (Table 1). Percentages represent the number of cells out of 10,000 that were above the level of nonspecific binding (measured by using NR1IgG) and thus stained positive for the various markers. The numbers represent the mean \( \pm \) SE for the 3-4 experiments that were run for each point. As tumor growth progressed, Mac-1\(^+\) Mφ numbers did not change, 73% for normal host Mφ to 72% for day-21 TBH Mφ. The overall percentage of Mac-2\(^+\) and -3\(^+\) Mφ dropped significantly (p <0.05). Mac-2\(^+\) Mφ dropped from 64% to 50% (a 22% decrease) and Mac-3\(^+\) Mφ dropped from 86% to 74% (an 14% decrease) as the tumor developed. The Mφ-gated populations, as tested by FITC-labeled anti-Ig and anti-Thy-1 antibodies, had <3% B and/or T cell contamination, respectively. These cells expressed high percentages of unique Mφ markers (Mac antigens), giving further evidence the cells were Mφ. (Figure 2A and B).

The distinct subpopulations can be seen in Figure 1. The boxed-in regions represent the three size versus antigen expression groups. Only Figure 1A and B show the boxed-in regions, but they represented the sizes of all peritoneal samples examined (Figure 1C-E and all of Figure 2). These groups were based on the distinct peaks seen when depicted in three-dimensions (data not given). By using 10 micron-sized beads (channel 16 on the figures) and measuring the histograms on a linear scale, we were able to determine the size ranges of the individual populations. These size ranges were 10-16, 17-22, and 23-27 microns and were designated small-, medium- and large-sized.
Figure 1. Comparison of PERITONEAL Mac Antigen Expression on Day-0 (Normal) and Day-21 TBH Mφ: (A = Day-0 Mac-1; B = Day-21 Mac-1; C = Day-0 Mac-2; D = Day-21 Mac-2; E = Day-0 Mac-3; F = Day-21 Mac-3). The boxed-in regions shown in panel A and B of Figure 1 are representative of the three sized populations examined in all the peritoneal Mφ labeling experiments, A-F. Mφ were labeled with mAb anti-Mac-1, -2, and -3 as stated in the Materials and Methods. The arrows in panel A and B of Figure 1 represent the 10 micron-sized beads.
Figure 2. Comparison of PERITONEAL Ia Antigen Expression on Day-0 (Normal) and Day-21 TBH Mφ: (A = Day-0 Mac Antigen Control (NRIGG); B = Day-21 Mac Antigen Control (NRIGG); C = Day-0 Ia (Ia"); D = Day-21 Ia (Ia"); E = Day-0 Ia Control (Ia"); F = Day-21 Ia Control (Ia"). The boxed-in region shown in Figure 1A and B are the representative sizes for the three different populations seen in the peritoneal Mφ samples. Mφ were labeled with mAb anti-NRIGG, -Ia", and -Ia" as stated in the Materials and Methods.
Table 1. Tumor-Induced Alterations in PERITONEAL Mφ Phenotype

<table>
<thead>
<tr>
<th>Cell surface antigen</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1+</td>
<td>73 ± 4e</td>
<td>71 ± 1</td>
<td>71 ± 3</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>Mac-2+</td>
<td>64 ± 1e</td>
<td>58 ± 2</td>
<td>44 ± 1**</td>
<td>50 ± 3**</td>
</tr>
<tr>
<td>Mac-3+</td>
<td>86 ± 2e</td>
<td>86 ± 4e</td>
<td>79 ± 2</td>
<td>74 ± 1**</td>
</tr>
<tr>
<td>Ia+</td>
<td>14 ± 1†</td>
<td>28 ± 3†</td>
<td>1 ± 1**</td>
<td>6 ± 1**</td>
</tr>
</tbody>
</table>

* Percentage positive represents the number of cells out of 10,000 that were considered positively labeled (compared to the NR IgG or anti-Ia6 control); for example, 73% of the day-0 Mφ were Mac-1+, thus 7300 Mφ were positive and 2700 were negative.

* Cells were labeled as stated in Materials and Methods.

* The numbers are the mean of the different experiments for each time point ± SE. Significance was checked by using students t test (p < 0.05). Numbers marked with * are significantly different from numbers marked with ** and numbers marked with † are significantly different from numbers marked with ‡ (p < 0.05).
populations, respectively. The MDADS computer verified these sizes by giving the range of channel numbers 16-24, 30-37, and 40-46 out of a possible 64 for the three different sized populations, respectively. Since size was measured from FALS, a linear relationship between the different sizes can be made (192). The medium-sized population were approximately 50% larger than the small-sized population and the large-sized cells were approximately 50% larger than the medium-sized population, agreeing with our determined sizes.

Differences in these populations occurred as a result of tumor growth. The most obvious difference is the masking of the various peaks in the TBH samples (Figure 1). Day-21 TBH Mϕ appeared to have a single broad population with an increase in the number of unlabeled smaller-sized cells. To assess what tumor-induced changes occurred in the different sized mAb-labeled populations as compared to the different sized populations observed in normal hosts, we used the same size parameters to measure normal and TBH Mϕ populations. The percentage of positively labeled cells in each of these distinct populations is shown in Table 2. The number of large-sized Mac-1⁺ and Mac-2⁺ Mϕ significantly (p <0.05) decreased between normal (37% [19% to 12%]) and 21 days of tumor growth (38% [16% to 10%]). The large-sized Mac-3⁺ Mϕ did not show this decrease. Instead Mac-3⁺ Mϕ showed a significant 33% decrease in the number of medium-sized Mϕ from 24% to 16%. Concomitant with the decrease in large-sized Mac-1⁺ Mϕ there was a 30% increase in the number of small-sized Mϕ. In contrast, Mϕ bearing the Mac-2 and -3 antigens did not show this trend. Aggregation of cells was discounted as a cause of the differences in cell sizes by using acridine orange to assess the DNA content of the cells. Any cells showing >2N DNA content (aggregates), representing <5% of the particulate matter, were gated out.

Ia Antigen Expression of Peritoneal Mϕ

Ia antigen expression was measured by labeling cells of the H-2^e haplotype with anti-Ia^e mAb and comparing it to the nonspecific anti-Ia^e antibody control (Figure 2C-F). Normal host Mϕ Ia
Table 2. Tumor-Induced Alterations in PERITONEAL MΦ Antigen versus Size Defined Phenotypic Subpopulations

<table>
<thead>
<tr>
<th>Cell surface antigen</th>
<th>Percentage of MΦ in Each Subpopulation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Days post tumor cell inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>Mac-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30&lt;sup&gt;*&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td>Mac-2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Mac-3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>50</td>
<td>24&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ia&lt;sup&gt;+&lt;/sup&gt;</td>
<td>17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage positive represents the number of cells out of 10,000 that were considered positively labeled (compared to the NR IgG or anti-Ia<sup>+</sup> control); for example, 30<sup>%</sup>, 23<sup>%</sup>, and 19<sup>%</sup> of the day-0 Mac-1<sup>+</sup> MΦ were in the S-, M-, and L-sized categories, respectively, thus 3000, 2300, and 1900 MΦ were positive for the S, M, and L size and 2800 were negative.

<sup>b</sup> Cells were labeled as stated in Materials and Methods.

<sup>+</sup> S, M, and L represent the small- (10-16 microns), medium- (17-22 microns), and the large-sized (22-27 microns) MΦ populations, respectively.

<sup>c</sup> The numbers are the mean of the different experiments for each time point. All numbers have a SE <3%. Significance was checked by using students t test (p < 0.05). Numbers marked with * are significantly different from numbers marked with ** and numbers marked with † are significantly different than numbers marked with ‡ (p < 0.05).
antigen expression was above background control levels (Figure 2C versus E), while day-21 TBH Mφ Ia expression was nearly identical to its control (Figure 2D versus F). This confirmed what is shown in Table 1; that is, the number of peritoneal Ia⁺ TBH Mφ decreased significantly (p <0.05) from 14% to 6%. In examining Figure 2, one must keep in mind that this is raw data and no compensation was made for background nonspecific binding and autofluorescence. Thus in Figure 2, the fluorescent intensity of the TBH Mφ Ia antigen expression appeared to be greater than that of the normal host (Figure 2C versus 2D). However, this is not the case. It is simply due to the increased nonspecific binding which affected the Ia control and Ia test samples equally; Figure 2D (Ia test sample) is identical to Figure 2F (Ia control). The increase in nonspecific/background labeling by TBH peritoneal Mφ was a consistent finding and may indicate a tumor-induced change in the structure or charge of the TBH Mφ cell membrane. The binding of the anti-Ia antibodies to Mφ Fc receptors was discounted by pre-incubating Mφ with NR1IgG. By blocking with NR1IgG, we saw no differences in the levels of Ia binding when compared to the unblocked samples. Ia antigen expression dropped significantly from an initial 14% in the normal host to 6% in the day-21 TBH (58% decrease).

Unlike the Mac antigens, nearly all of the Ia⁺ Mφ were in the small-sized Mφ population (Table 2). In the normal host, 15% of the Ia⁺ Mφ were small-sized, 2% were medium-sized, and none were large-sized. By day 7 of tumor growth, the number of Mφ in the small and medium-sized populations had increased to 22% and 5%, respectively. By day 14 only 1% of the Mφ were Ia⁺ and by day 21, 4% were, with 2% in the small- and 2% in the medium-sized subpopulations. Since the total number of positive cells and number of positive cells in each subpopulation were analyzed with different programs overall percentages are slightly different between Table 1 and Table 2.
Mac Antigen Expression of Splenic Mφ

The fluorescent intensity of the Mac antigen-labels on normal and TBH splenic Mφ was similar (Figure 3), suggesting the number of antigenic markers per Mφ does not change during tumor growth. So the change was not in the density of the Mac-1, -2, or -3 antigens per Mφ but in the overall percentage of Mac-1⁺, -2⁺, or -3⁺ Mφ. This change in cell numbers is seen by comparing the differences between normal and TBH splenic Mφ (Figure 3A-F) and their respective NR IgG (nonspecific) control antibody samples (Figure 4A and B). The Figures represent the raw data and do not have the control values subtracted. TBH Mφ labeled with anti-Mac-1, -2, or -3 mAb compared to the TBH NR IgG control showed a much greater difference than the corresponding one between normal host Mφ labeled with anti-Mac-1, -2, or -3 mAb compared to their NR IgG control. Normal host Mφ had a higher level of background fluorescence than did TBH Mφ. Meaning when the percentage of positively labeled cells was calculated (test mAb minus the control Ab), the percentage of TBH Mφ labeling positive for the various Mac antigens increased (Table 3). The difference in nonspecific binding between the normal and TBH Mφ may just be another manifestation of a tumor-induced change in Mφ phenotype. The differences between the nonspecific binding capacities of splenic and peritoneal Mφ is not surprising as peritoneal and splenic Mφ are distinctly different populations of Mφ. Splenic Mφ differed from peritoneal Mφ in their expression of Mac antigens and changes that occurred as a result of tumor growth (Table 3). The percentage of Mac-1⁺ Mφ significantly increased during tumor growth from 40% to 76% (a 90% increase). The number of Mac-2⁺ Mφ (from 40% to 59%) and Mac-3⁺ Mφ (from 48% to 67%) also significantly increased (p < 0.05) during tumor growth.

Splenic Mφ showed different size characteristics than peritoneal Mφ. Instead of three distinct size versus antigen expression subpopulations, all splenic Mφ were the same size (Figure 3 and Figure 4). However, differences between the size distribution of the normal and TBH Mac-1⁺, -2⁺, and -3⁺ Mφ occurred. The average peak size of the normal host Mφ was 8-20 microns, while peak size of TBH Mφ was 8-13 microns. Even though the peaks shifted during tumor growth, no
Figure 3. Comparison of SPLENIC Mac Antigen Expression on Day-0 (Normal) and Day-21 TBH Mφ: (A = Day-0 Mac-1; B = Day-21 Mac-1; C = Day-0 Mac-2; D = Day-21 Mac-2; E = Day-0 Mac-3; F = Day-21 Mac-3). Mφ were labeled with mAb anti-Mac-1, -2, and -3 as stated in the Materials and Methods.
Figure 4. Comparison of SPLENIC Mφ la Antigen Expression on Day-0 (Normal) and Day-21 TBH Mφ: (A = Day-0 Mac Antigen Control (NR1gG); B = Day-21 Mac Antigen Control (NR1gG); C = Day-0 la (Iaα); D = Day-21 la (Iaα); E = Day-0 la Control (Iaα); F = Day-21 la Control (Iaα). Mφ were labeled with mAb anti-NR1gG, -Iαα, and -Iαβ as stated in the Materials and Methods.
Table 3. Tumor-Induced Alterations in SPLENIC Mφ Phenotype

<table>
<thead>
<tr>
<th>Cell surface antigen&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percentage of Mφ Expressing Mac and Ia Antigens&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Days post tumor cell injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Mac-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>40 ± 9&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mac-2&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>40 ± 3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mac-3&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>48 ± 3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ia&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>56 ± 1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup> Percentage positive represents the number of cells out of 10,000 that were considered positively labeled (compared to the NRlG or anti-Ia<sup>a</sup> control); for example, 40% of the day-0 Mφ were Mac-1<sup>+</sup>, thus 4000 Mφ were positively-labeled and 6000 were negative.

<sup>b</sup> Cells were labeled as stated in Materials and Methods.

<sup>c</sup> The numbers are the mean of the different experiments for each time point ± SE. Significance was checked by using student’s t test (p <0.05). Numbers marked with * are significantly different from numbers marked with ** (p <0.05).
detectable differences were seen in the numbers of cells in different sized populations (data not shown). The gated populations of splenic Mφ showed <4% B and/or T cell contamination.

**Ia Antigen Expression of Splenic Mφ**

Splenic Mφ Ia antigen expression, unlike any of the other antigens examined, decreased in both the density per cell and the number of positive cells per population (Figure 4C, D, and Table 3). The number of Ia⁺ Mφ significantly decreased (p <0.05) from 56% in the normal host to 40% in the day-21 TBH (a 29% decrease). The density of Ia antigen per cell also decreased. Two distinct TBH Ia⁺ Mφ were seen (Figure 4). Both of these populations had a lower Ia antigen density than normal host Ia⁺ Mφ. The population of TBH Ia⁺ Mφ with the higher antigen density showed a 10-20% decrease in antigen per cell, while the other population had a >50% decrease in antigen density. A significant increase in the number of TBH Mφ staining with low intensity (nearly a 4 fold increase over their normal host counterparts) occurred with a concomitant decrease (40%) in the number of Ia⁺ Mφ staining with a greater fluorescent intensity (Figure 3 and Figure 4). Fc receptor interaction played no role in anti-Ia antibody binding to Mφ, as blocking with NR1G did not significantly change the percentage of cells labeling positive with the anti-Iα mAb.
DISCUSSION

Our characterization of Mφ subpopulations using the flow cytometer supported the hypothesis that Mφ, like lymphocytes, are composed of subpopulations (72-74,137,249,251,253). These subpopulations can be separated on the basis of their size versus expression of the antigens Mac-1, -2, -3, and Ia. By analyzing Mφ based on these markers, a better understanding of Mφ diversity can be gained. When the size of mAb-defined peritoneal Mφ was measured by flow cytometry, three distinct Mφ populations were seen: 10-16, 17-22, and 23-27 micron sized populations. By following these Mφ populations as the tumor developed, we noticed a shift in TBH Mφ antigen expression which extends our earlier data (72-74,137), suggesting tumor-induced phenotypic alterations in peritoneal Mφ. Total numbers of Mac-2+ and -3+ peritoneal Mφ decreased during tumor growth, while Mac-1+ Mφ numbers remained constant. These trends confirmed our previous fluorescent microscopy results (74). We also showed no significant difference in the total number of thioglycollate-elicited normal or TBH Mφ collected, yet there was a 2.2 fold increase in the number of peroxidase-positive TBH Mφ (that is immature Mφ [47]). If Mac-2 and -3 are differentiation antigens developing late in Mφ maturation and Mac-1 is an early differentiation antigen, then the decrease in Mac-2+ and 3+ Mφ and the constant number of Mac-1+ Mφ during tumor growth could be explained. Because more immature Mφ were available in the TBH (over 2 times more), more immature Mφ travelled to the peritoneal cavity and consequently, due to their earlier stage of development/maturation, lower percentages of Mac-2+ and -3+ Mφ were found. Tumor-induced changes in the peritoneum, a site removed from tumor growth, is not surprising since tumor growth causes a generalized immunosuppressive state regardless of tumor location or etiology (104). This is especially important in light of recent reports showing tumor-produced factors released into circulation that can inhibit lymphocyte proliferation, Mφ production of factors, as well as induce suppressor cells (39,103,236,269).

In addition to examining the total number of Mac+ cells, we were also interested in how size versus antigen expression Mφ populations changed in the TBH. TBH Mφ appeared (i) to be a
more homogeneous population showing a single broad population that masks the different sized populations seen in the normal host and (ii) to shift in overall cell numbers toward a smaller cell. The number of large-sized Mφ bearing Mac-1 and -2 along with the number of medium-sized Mφ bearing Mac-3 markers decreased significantly. A structure-function relationship was seen when comparing size and Mac-1+ Mφ. Previously, we showed a disappearance in a TBH Mac-1+ Mφ functional regulatory mechanism (74,137), that in the normal host was responsible for down regulating PGE₂ production. Decreased numbers of large-sized Mac-1+ Mφ may account for the reduction in Mac-1+ Mφ-mediated regulation. Concomitant with this decrease in the large-sized Mac-1+ Mφ was an increase in the number of small-sized Mφ. This increase in smaller sized Mac-1+ and Mac-1- Mφ in the TBH is probably related to the increased influx into the peritoneal cavity of smaller immature Mφ, a more predominant cell type than in the normal host. A relationship exists between TBH Mac-1+ Mφ and peroxidase-positive Mφ (74). The increase in Mac-1+ Mφ, small-sized Mφ, and immature peroxidase-positive Mφ could be one and the same.

Ia antigen expression by peritoneal Mφ was also examined. Overall, most Ia⁺ Mφ disappeared by day 21 of tumor growth (a 58% decrease in Ia⁺ cells). The surprising increase in antigen expression seen at day 7 seemed to be a recurrent phenomena since it was reproducible as well as verifiable by SMLR experiments (CHAPTER III). This increase in Ia antigen expression may represent the hosts early hyperresponsiveness to the tumor occurring before the onset of tumor-induced immunosuppression, Ia antigen reduction, and a shift towards Ia⁻ Mφ. Ia antigen was exclusively associated with the small-sized cells in the peritoneal Mφ populations. The decrease in Ia antigen was observed by us before and may play a role in the decreased immune response in the TBH, including decreased MLR (73) and SMLR responsiveness (CHAPTER III). In the normal host, there is significantly fewer Ia⁺ Mφ in the peritoneal cavity then in the spleen and more importantly there is a significant decrease in the amount of Ia antigen per peritoneal Mφ. These changes are even more dramatic in the TBH.

Functionally, at least in the SMLR, an assay driven by the presentation of self-Ia by Mφ to autoreactive T cells (168,260), two points can be discussed; (i) peritoneal Mφ compared to splenic Mφ, especially in the normal host, are poor stimulators of autoreactive T cells because of their low
Ia antigen content (unpublished data) and (ii) TBH peritoneal and splenic Mφ compared to their normal counterparts are also poor stimulators of autoreactive T cells because of their low Ia antigen content (unpublished data). For example, normal host splenic Mφ-stimulated SMLR values were 26,909 ± 2426 counts per minute (cpm), while normal host peritoneal Mφ-stimulated SMLR values were 3215 ± 152 cpm, when TBH splenic and peritoneal Mφ were used there was a reduction to 5783 ± 330 and 2847 ± 77, respectively, in autoreactive T cell proliferation. Anatomical location of Mφ and tumor growth contributed to decreased Ia expression. How does the tumor down-regulate Ia antigen expression? This can only be speculated on. However, we have shown that TBH Mφ have increased PGE₂ production, a potent immunosuppressive agent (74,137,163). More importantly, we demonstrated that Mac-2⁺ Mφ are a principle PGE₂ producer in the TBH (74,137), and a peritoneal Mac-1⁺ PGE₂ regulating Mφ is absent in the TBH (74). Since PGE₂ can act as an autocrine agent and down-modulate Ia antigen expression, one potential method of tumor-induced control of Ia antigen becomes evident (207). The dependence of Ia antigen expression for Mφ presentation of antigen to T cells (240-242) suggests changes in Ia expression may play a vital role in decreased major histocompatibility (MHC)-restricted immune reactivity during tumor growth (73,132,156,176,177).

Splenical Mφ also demonstrated a tumor-induced shift in Mφ phenotype. Cells bearing Mac-1, -2, and -3 all increased, 90%, 48%, and 40%, respectively. The increase in the percentage of Mac-1⁺ and -2⁺ Mφ was also demonstrated using indirect fluorescent microscopy (72). The increase in Mac-1⁺ Mφ in the TBH follows a shift in the phenotype of the splenic accessory Mφ from Mac-1⁻ in the normal host to Mac-1⁺ in the TBH (72). While Mac-3⁺ Mφ were previously shown to decrease slightly, this apparent contradiction can be explained by the increased sensitivity of the flow cytometer.

Recently, Tsuchiya et al. reported that a number of different tumors produced a CSF inducing a large population of Mac-1⁺ and -2⁺ splenic suppressor Mφ (236). These Mac-1⁺ and -2⁺ Mφ suppressed the in vitro mitogen-induced proliferation of T and B cells. The induction of suppressor cells appeared to correlate with in vivo production of factors by the tumors. Their results may help explain the changes that occurred in the spleens of our TBH. In the spleens of our TBH there is
a 4-5 fold increase in the number of Mϕ (51), this, along with our flow cytometry data showing a 90% and 48% (or 1.9 fold and 1.48 fold, respectively) increase in the percentage of TBH Mac-1+ and -2+ Mϕ, translates into 6-9 times more Mac-1+ and -2+ Mϕ in the spleen. A significant increase of these suppressor Mϕ would explain why we find TBH splenic Mϕ to be suppressors of mitogen-induced, allogeneic, and autoreactive T cell responses (50,51,72,73,CHAPTER III). Others, also report that tumor growth induces changes in Mϕ function (93,103,231,232,236,246).

Splenic Mϕ, however, did not show multiple distinct size versus surface phenotype subpopulations. This is not unexpected, since Mϕ from different anatomical sites are thought to have distinct phenotypic characteristics (250,253). Instead, TBH splenic Mϕ showed a decrease in their average size. Normal host splenic Mϕ were more heterogeneous in their size distribution (8-20 microns) than were TBH Mϕ (8-13 microns). The shift to smaller sized TBH Mϕ may be due to the influx of smaller immature Mϕ into the spleen (4-5 fold increase). Splenic Ia+ Mϕ numbers decreased by 29% during tumor growth. Ia antigen density per cell also decreased. Two different antigen density populations were seen in the TBH Ia+ Mϕ, but both populations showed decreased Ia expression per cell (Fig. 4). One population showed a 10-20% decrease, while the other showed a >50% decrease in Ia antigen expression per cell. The decrease in splenic Ia+ Mϕ is supported by our earlier work (73). In translating the percentage of the number of Ia+ Mϕ to absolute in vivo numbers, we saw an increase in the total number of TBH Ia+ splenic Mϕ. This increase in Ia+ Mϕ, as with the increase in the Mac+ Mϕ, is due to the influx of peripheral Mϕ into the TBH spleen, which weighs 3-5 times more than the normal host spleen (51). Because more TBH Ia+ Mϕ are found, it could be argued the increased numbers would more than compensate for the decreased percentage. However, several lines of evidence argue against this. First, the majority of these TBH Ia+ Mϕ have <50% the amount of Ia on their surface as compared to their normal counterparts. Second, we showed in the SMLR, TBH splenic Mϕ dramatically increase their production of suppressive monokines, including prostaglandins (CHAPTER III). At the very least, increased PGE2 production, which autoregulates Ia expression (207) and suppresses T cell proliferation (163), would explain much of suppression in T cell responsiveness seen in the TBH. As

CHAPTER I 43
with peritoneal Mφ, the decrease in la antigen expression may be one of the causes/effects of tumor-induced immunosuppression.

Overall, the data suggested Mφ subpopulations exist in both the same anatomical site in the host and different sites (spleen versus peritoneal cavity) and tumor growth altered these populations. This extends previous work by ourselves (50,51,72-74,137) and others (93,103,231,232,236,246) showing TBH Mφ to be important contributors to immunosuppression. Although we cannot say conclusively whether these subpopulations represent stable phenotypic subpopulations or transient populations, we can say tumor growth changes the phenotypic makeup of TBH Mφ subpopulations. Two-color analyses support the one-color data showing that phenotypic changes in Mφ occur during tumor growth (CHAPTER II). This will allow us to make substantive comments about the relationship of the various Mac+ populations, the expression of Mac-antigens on la+ versus la− populations and la expression on various Mac− cells. We continue to relate Mφ phenotype to function and have shown that changes in Mφ subpopulations are caused by tumor-altered immune responses. To definitively prove this structural-functional relationship, sorting and testing of these subpopulations is needed. The characterization, isolation, and use of these Mφ subpopulations will allow for further delineation of the phenotype to function relationship, especially in the TBH, of the heterogeneous group of cells known as Mφ.
CHAPTER II

TWO-COLOR FLOW CYTOMETRIC ANALYSIS OF
THE EXPRESSION OF MAC AND MHC CLASS II
ANTIGENS ON MACROPHAGES DURING TUMOR
GROWTH

INTRODUCTION

Mφ have diverse functions that may reflect the existence of distinct subpopulations of Mφ (62-74,137,249-251,253). To understand Mφ functions during the immune response, these subpopulations need to be characterized and their functional relationships established. Subpopulation delineation is critical in studies involving TBH, because tumor growth changes the immunoregulatory properties of Mφ (50,51,72,74,103,137,138,149,231,236,246,269). During tumor growth, changes occur in Mφ phenotype and function. Phenotypic changes in TBH Mφ include
changes in surface antigen (Mac-1, Mac-2, Mac-3, and Ia) expression (72-74,137,156,236), size distribution (CHAPTER I), and peroxidase staining (74). Concomitant functional changes in TBH Mφ include decreased accessory cell function in the MLR (50,73,74,136,231), the SMLR (56), and mitogen-induced T cell proliferation (51,72,73,236,269). These functional changes result from an altered suppressor Mφ population (72,74,137,236) and a decreased production of enhancing factors (149,246). If the phenotype of the suppressor Mφ could be identified (reported here) and then correlated with its specific function, a greater understanding of the mechanism of TBH Mφ-mediated control of immune reactivity would emerge.

This study continues our earlier work on phenotypic characterization of Mφ subpopulations (72-74). Monoclonal antibodies (mAb) to the Mφ surface antigens Mac-1, -2, -3 and Ia were used. Mac-1 (CD11b) is found on Mφ, monocytes, granulocytes, some NK cells, and bone marrow cells and is a receptor for the complement component, C3b (48,96,208). The continuous expression of Mac-1 suggests that it is an early differentiation antigen; Mac-1 expression increases as monocytes mature (208). Mac-2 is variably expressed on Mφ from different tissues and on Mφ elicited by different stimulatory agents, suggesting that Mac-2 may represent a differentiation and/or activation marker (95). Mac-2 is now thought to be an IgE receptor with lectin-like properties that may facilitate parasite-Mφ binding (32). Mac-3 is found on Mφ and is a differentiation antigen with variable expression (97,249). Ia antigen is the class II major histocompatibility complex marker and is needed for effective Mφ antigen presentation to T cells (240-242). In this study, we investigated the dual expression of Mφ surface markers by flow cytometry. Changes in TBH Mφ phenotype were not caused by a generalized inflammatory response but by tumor growth. Tumor growth led to phenotypic changes in peritoneal and splenic Mφ populations resulting in a shift in Mφ subpopulations. Combined with our previous work, a relationship between TBH Mφ phenotype and immune dysfunction is suggested.
MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (H-2^d, Dominion Labs) were used. TBH mice received intramuscular injections of $4 \times 10^5$ cells from a methylcholanthrene-induced, non-metastatic, transplantable fibrosarcoma into the left hind leg. These injections led to palpable tumors by days 10-14 and death by days 28-35 (50.51). By days 10-14, there is significant suppression of Con A, PHA, MLR, and SMLR reactivity and suppression increases as tumor growth progresses (51,72,73,261). Mice with 21-day old tumors were used throughout. Functional or phenotypic changes in the TBH were not caused by a general inflammatory response to the tumor, because mice (BALB/c normal mice) injected with their own tissue or C3H nontumor tissue showed no significant differences from normal unexposed mice in their Mϕ surface antigen phenotypes. Previous results also showed that suppression was not caused by an inflammatory response (74). Therefore, normal unmanipulated mice served as the source of normal host Mϕ.

Peritoneal Mϕ Preparation

PEC) were obtained by lavage from normal and TBH mice 4 days after intraperitoneal injection of 2.0 ml of sterile thioglycollate. No significant differences are seen between the number of peritoneal Mϕ collected from untreated normal, inflammatory control, or TBH mice (74; data not shown). Mϕ were selected by gating the flow cytometer on the FALS and 90LS pattern to exclude lymphocytes and granulocytes. PEC were also plated for 3 hr on 150 x 15 mm tissue culture plates (LUX/Miles Scientific), washed, and recovered. Adherence and subsequent spreading did not lead to significantly different light scatter patterns from unplated whole PEC (data not
shown). Adherent PEC or whole PEC treated with propidium iodide, a vital stain, and subsequently analyzed by flow cytometry were >93\% viable. These adherent cells (>97\% esterase positive) were used to set the FALS and 90LS gates, which were then used for selecting Mϕ. Using FITC-labeled anti-Thy-1 and anti-IgG, -IgM, and -IgA (Cappel), we showed that the T and/or B cell contamination was <3\% of the total of normal or TBH Mϕ preparations.

**Splenic Mϕ Preparation**

WSC were harvested from untreated normal and TBH animals (no thioglycollate). No significant differences were seen between the number of splenic Mϕ collected from untreated normal or inflammatory control mice (data not shown). There was, however, a 3-5 fold increase in the number of TBH splenic Mϕ (51). Splenic Mϕ were selected by flow cytometry, gating on FALS and 90LS for the desired cell population. Splenic Mϕ obtained by plating for 3 hr were used to select the FALS and 90LS gating parameters. Plated Mϕ (>96\% esterase positive) showed no significant changes from unplated splenic Mϕ in their light scatter patterns (data not shown). T and/or B cell contamination was <3\% of the total of normal or TBH splenic cells measured.

**Cell Lines**

The hybridoma cell lines (ATCC) were cultured in roller bottles in RPMI-1640 medium (Hazelton Research Products) supplemented with 10\% heat-inactivated FCS (Flow Laboratories) and 4 x 10\(^{-5}\) M 2-mercaptoethanol. Supernatants were harvested every 3 days and the cell-free supernatants were stored at -70°C. All media contained 50 mg/l Gentamicin (GIBCO), 2 g/l NaHCO\(_3\), and 25 mM HEPES (Research Organics).
Monoclonal Antibody Purification

Briefly, a pH 7.4 ammonium sulfate solution was added to hybridoma supernatants to yield a saturated solution. After standing overnight, precipitates were collected by centrifugation, resuspended as a 20X concentrate in PBS, dialyzed against PBS, and applied to an affinity column. The mAb fraction was then eluted with a glycine buffer. Glycine-eluted column fractions were immediately dialysed to PBS. Column fractions are assayed for mAb using goat-anti-rat antibody, heavy- and light-chain specific (Cappel) in an Ouchterlony immunodiffusion test. Positive fractions were pooled, assayed for antibody concentration, diluted to 1.0 mg/ml, sterile filtered, and stored at -70°C.

Biotin Labeling of mAb

Anti-Mac-1, -Mac-2, and -Mac-3 antibodies were labeled with biotin (Research Organics). Affinity purified mAb, when used for biotin labeling, were dialyzed into a 0.1 M NaHCO₃ buffer (pH 8.2-8.6). Twenty-five to 50 mg of biotin was dissolved in 1.0 ml of a DMSO solution (Sigma). Dialyzed mAb were incubated at room temperature for 2 hr in a 1/10 w/v biotin/mAb, dialyzed to 3 changes of PBS, and stored at -70°C. mAb were assayed for biotin binding by comparing the fluorescent efficiency between cells labeled with biotin-mAb and followed by R-PE (R-phycoerythrin)-avidin D (Vector Laboratories, Burlingame, CA) and cells labeled with mAb and followed by goat anti-rat-FITC.
Fluorescent Antibody Labeling

Cell samples from normal and TBH mice consisted of whole PEC at 4 x 10^6 cells/ml or WSC at 8 x 10^6 cells/ml. To 1.0 ml aliquots of these cells, the first antibody was added (anti-Mac-1, -2, -3, -Ia or NR1IgG or normal mouse immunoglobulin G [NMIgG]) (Cappel) and incubated for 35-45 min. This treatment was followed by three washes with cold medium. Cells were incubated in FITC-labeled secondary antibody for 35-45 min and washed 3 times in PBS. The secondary antibodies were affinity purified goat-anti-rat F(ab')2 fragment FITC-labeled antibody (Cappel) for anti-Mac-1, -2, -3, and NR1IgG and affinity purified goat-anti-mouse F(ab')2 fragment FITC-labeled antibody (Cappel) for anti-Iaα and NMIgG antibodies. NR1IgG was the control antibody for the anti-Mac mAb and NMIgG was the control antibody for the anti-Ia mAb. After labeling Mφ with the first antibody (anti-Mac-1, -2, -3, Ia, NR1IgG or NMIgG) plus the corresponding FITC-labeled secondary antibody, Mφ were incubated with the second surface target antibody (biotin-labeled anti-Mac-1, -2, -3, NR1IgG or NMIgG) for 35-45 min, washed 3 times in PBS, incubated with R-PE for 35-45 min, and washed 3 times. This treatment was carried out at 4°C. These conditions allow effective antibody binding and maintain cell viability while minimizing membrane turnover and antibody internalization. Because this procedure may cause artifactual double labeling, we ran several additional controls. Including, blocking studies, in which we added an excess of NR1IgG or NMIgG to block the free-binding sites on the secondary step FITC-labeled antibody. This procedure demonstrated that <5% of the red fluorescent intensity was caused by the biotin-labeled mAb binding to the second antigen binding site of the divalent FITC-labeled secondary antibody. Furthermore, we added biotin-labeled rat-anti-mouse (Accurate Chemical & Scientific Corp., Westbury, NY) (plus R-PE) to Mφ labeled with a primary Mφ mAb followed by the corresponding FITC-conjugated antibody. This procedure allowed us to examine the amount of red fluorescence that was due to nonspecific binding of the biotin-labeled antibody to the FITC-conjugated second step antibody. This control showed that <3% of all Mφ were labeled with the biotin-tagged antibody.
Flow Cytometry

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics) laser flow cytometer and cell sorter. The instrument was calibrated with fluorescent 10 micron-sized microsphere standards before analysis of the Mφ samples. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc.). All data collection was done with the MDADS (Coulter Electronics). FITC-labeled mAb was measured as the log of the green fluorescence and R-PE-labeled mAb was measured as the log of the red fluorescence. The two parameter histograms (green fluorescence versus red fluorescence) used for expressing the results are shown as a 64 x 64 channel resolution histogram. For the two-color analysis, the histograms were divided into four quadrants based on the control samples. The figures represent raw data. Data in the tables show the percentage of Mφ in each quadrant. These calculations were done by the MDADS computer using the MDADS "quadstat" software.

Calculation of Results

Splenic or peritoneal Mφ were pooled from 3-6 mice and each experiment was repeated 3-4 times. All percentages were determined by the MDADS computer and represent the percentage of 10,000 cells present in each quadrant. Numbers in the tables represent the mean of triplicate experiments. The number of TBH Mφ bearing a particular antigen was considered decreased or increased by comparison with normal host Mφ numbers which were used as a maximum. Significance was checked using Student’s paired t test (p <0.05).
RESULTS

*Surface Marker Expression of Nontumor Tissue Unexposed or Exposed Normal Host Mφ*

To show whether changes in TBH Mφ phenotype were due to tumor growth or a generalized or specific inflammatory response to a stimulus different from the tumor, we first assessed whether changes in Mφ phenotype can be caused by an inflammatory response to nontumor tissue. BALB/c mice were injected into the left hind leg with $4 \times 10^5$ cells (this mimic's the treatment TBH mice received) of their own nontumor tissue or C3H nontumor tissue and used 21 days later. These mice were used to test whether a general inflammatory response causes the changes in Mφ phenotype? The data showed that there were no significant differences between the normal unexposed mice and the 21-day inflammatory mice (Table 4). These data are consistent with our previous results (74) demonstrating the lack of suppression or changes in Mφ phenotype due to an inflammatory response and points to the cause of changes in TBH mice as being tumor-induced.

*Dual Antigen Expression on Peritoneal Mφ*

To examine the antigen expression in normal and TBH peritoneal Mφ, mAb against the respective antigens were used (Figure 5 and Figure 6). By comparing the test groups (dual-labeled Mac-1, -2, -3, and Ia) to the control (NRIgG and/or NMIgG), the percentage of labeled cells per 10,000 counted could be determined for each quadrant (Table 5). During tumor growth, the percentage of double-labeled Mac-1$^+2^+$, Mac-1$^+3^+$, and Mac-2$^+3^+$ Mφ significantly decreased. Mac-1$^+2^+$ Mφ decreased 12%, Mac-1$^+3^+$ Mφ decreased 15%, and Mac-2$^+3^+$ Mφ decreased 24%. The double-labeling procedure detected a unique population of normal and TBH
Table 4. Analysis of Changes in Mφ Phenotype Caused by Nontumor Tissue

### Percentage of Peritoneal Mφ Expressing Antigens

<table>
<thead>
<tr>
<th></th>
<th>Unexposed</th>
<th>Inflammatory$^{b}$ (Self)</th>
<th>Inflammatory$^{c}$ (Foreign)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1</td>
<td>77</td>
<td>82</td>
<td>78</td>
</tr>
<tr>
<td>Mac-2</td>
<td>71</td>
<td>68</td>
<td>69</td>
</tr>
<tr>
<td>Mac-3</td>
<td>90</td>
<td>92</td>
<td>85</td>
</tr>
<tr>
<td>Ia</td>
<td>12</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>

### Percentage of Splenic Mφ Expressing Antigens

<table>
<thead>
<tr>
<th></th>
<th>Unexposed</th>
<th>Inflammatory$^{b}$ (Self)</th>
<th>Inflammatory$^{c}$ (Foreign)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1</td>
<td>65</td>
<td>59</td>
<td>65</td>
</tr>
<tr>
<td>Mac-2</td>
<td>70</td>
<td>70</td>
<td>68</td>
</tr>
<tr>
<td>Mac-3</td>
<td>72</td>
<td>75</td>
<td>73</td>
</tr>
<tr>
<td>Ia</td>
<td>57</td>
<td>60</td>
<td>54</td>
</tr>
</tbody>
</table>

$^{a}$ Percentage is the number of cells per 10,000 counted that were considered positively labeled (compared to the control). The percentages are means of triplicate experiments. Significance was checked by using Student’s paired $t$ test ($p<0.05$).

$^{b}$ Inflammatory (Self) represents normal BALB/c mice injected with $4 \times 10^5$ BALB/c nontumor cells and tested 21 days later.

$^{c}$ Inflammatory (Foreign) represents normal BALB/c mice injected with $4 \times 10^5$ C3H nontumor cells and tested at 21 days.
Figure 5. Dual-Label Analysis of Normal and TBH PERITONEAL Mφ Antigen Expression: Column 1 is the two-color fluorescence pattern of normal host peritoneal Mφ and column 2 is the two-color fluorescence pattern of TBH peritoneal Mφ. The symbols to the left and underneath each pair of histograms represent the two surface antigens measured in each histogram. The bars/quadranats were calculated from the control sample and then set so they remained the same throughout all the test samples. Mφ were labeled with anti-Mac-1, -2, -3, or 1a mAb. See Table 5 for percentages of cells in each quadrant.
Figure 6. Dual-Label Analysis of Normal and TBH PERITONEAL Mφ Antigen Expression: Column 1 is the two-color fluorescence pattern of normal host peritoneal Mφ and column 2 is the two-color fluorescence pattern of TBH peritoneal Mφ. The symbols to the left and underneath each pair of histograms represent the two surface antigens measured in each histogram. The bars/quadrants were calculated from the control sample and then set so they remained the same throughout all the test samples. Mφ were labeled with anti-Mac-1, -2, -3, or Ia mAb. See Table 5 for percentages of cells in each quadrant.
Table 5. Two-Color Analysis of PERITONEAL Mφ

Percentage of Normal Mφ Expressing Mac/1a Antigens

<table>
<thead>
<tr>
<th>Cell Surface (Ag/Ag)</th>
<th>Quadrants</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 (Ag⁺/Ag⁻)</td>
<td>2 (Ag⁺/Ag⁺)</td>
<td>3 (Ag⁻/Ag⁻)</td>
<td>4 (Ag⁻/Ag⁺)</td>
</tr>
<tr>
<td>Mac-2/Mac-1</td>
<td>0</td>
<td>74*</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Mac-1/Mac-3</td>
<td>8</td>
<td>71*</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Mac-1/1a</td>
<td>61</td>
<td>16*</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Mac-2/Mac-3</td>
<td>0</td>
<td>79*</td>
<td>9</td>
<td>12</td>
</tr>
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<td>Mac-2/1a</td>
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</tr>
<tr>
<td>Mac-3/1a</td>
<td>77*</td>
<td>13*</td>
<td>10</td>
<td>0</td>
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</table>

Percentage of TBH Mφ Expressing Mac/1a Antigens

<table>
<thead>
<tr>
<th>Cell Surface (Ag/Ag)</th>
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* Percentage is the number of cells per 10,000 counted that were considered positively labeled (compared to the control) in each quadrant of the two-color analysis. The percentages are means of triplicate experiments. Significance was checked by using Student's paired t test (p < 0.05). Normal host percentages marked with * are significantly different from their TBH counterparts marked with **.
Mac-1$^{-}$2$^{-}$3$^{-}$ and Mac-1$^{-}$2$^{-}$3$^{+}$ Mφ. The percentage of these Mφ populations did not change significantly during tumor growth. There were no detectable single-labeled Mac-2$^{+}$ or Ia$^{+}$ peritoneal Mφ. In fact, TBH Mac-2$^{-}$Ia$^{-}$ Mφ significantly increased from 27% to 42%. The number of Mac-1$^{+}$Ia$^{+}$, Mac-2$^{+}$Ia$^{+}$, and Mac-3$^{+}$Ia$^{+}$ Mφ significantly decreased (40%, 38%, and 38%, respectively) during tumor growth. The number of Mac-1$^{+}$Ia$^{-}$ Mφ did not change during tumor growth. Mac-2$^{+}$Ia$^{-}$ Mφ decreased 17% during tumor growth and TBH Mac-3$^{+}$Ia$^{-}$ decreased 14%. Although there seems to be a contradiction in some panels of Figure 5 between the number of cells that appear in Figure 5 and the number shown in Table 5, there is not. In the Mac/Ia tests (panel 2 and 4), a significant number of cells are Mac$^{-}$Ia$^{-}$ as shown in Table 5; however, they are hard to see in Figure 5 because they form a tight peak in quadrant 3. Additionally, some steric interactions between the different anti-Mac mAb may occur. But the reverse reactions were also done; that is, cells treated with anti-Mac-1 mAb first then anti-Mac-2 mAb, or anti-Mac-2 mAb then anti-Mac-1 mAb, showed no significant differences in percentages. These results suggested that although there may be interactions between the mAb, the percentages of cells labeled was not artifactual. The Mφ-gated populations, as tested by FITC-labeled anti-Ig and anti-Thy-1 antibodies, had <3% B and/or T cell contamination. These cells expressed high percentages of Mac antigens, substantiating that the cells were Mφ.

**Dual Antigen Expression on Splenic Mφ**

To examine the dual antigen expression of normal and TBH splenic Mφ, they were labeled with anti-Mac and -Ia$^{a}$ mAb (Figure 7 and Figure 8). In comparing the test groups (dual-labeled) to the controls (NR IgG and/or NM IgG), the percentage of cells per 10,000 counted was determined for each quadrant (Table 6). The number of Mac-1$^{+}$2$^{+}$3$^{+}$ Mφ significantly increased during tumor growth. Mac-1$^{+}$2$^{+}$ TBH Mφ increased 26%, Mac-1$^{+}$3$^{+}$ TBH Mφ increased 38%, and Mac-2$^{+}$3$^{+}$ TBH Mφ increased 30%. The percentage of Mac$^{+}$Ia$^{-}$ TBH Mφ also significantly in-
Figure 7. Dual-Label Analysis of Normal and TBH SPLENIC Mφ Antigen Expression: Column 1 is the two-color fluorescence pattern of normal host splenic Mφ and column 2 is the two-color fluorescence pattern of TBH splenic Mφ. The symbols to the left and underneath each pair of histograms represent the two surface antigens measured in each histogram. The bars/quadrants were calculated from the control sample and then set so they remained the same throughout all the test samples. Mφ were labeled with anti-Mac-1, -2, -3, or Ia mAb. Table 6 for percentages of cells in each quadrant.
Figure 8. Dual-Label Analysis of Normal and TBH SPLENIC Mφ Antigen Expression: Column 1 is the two-color fluorescence pattern of normal host splenic Mφ and column 2 is the two-color fluorescence pattern of TBH splenic Mφ. The symbols to the left and underneath each pair of histograms represent the two surface antigens measured in each histogram. The bars/quadrants were calculated from the control sample and then set so they remained the same throughout all the test samples. Mφ were labeled with anti-Mac-1, -2, -3, or Ia mAb. Table 6 for percentages of cells in each quadrant.
Table 6. Two-Color Analysis of SPLENiC Mφ

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Percentage of TBH Mφ Expressing Mac/Ia Antigens

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* Percentage is the number of cells per 10,000 counted that were considered positively labeled (compared to the control) in each quadrant of the two-color analysis. The percentages are means of triplicate experiments. Significance was checked by using Student’s paired t test (p < 0.05). Normal host percentages marked with * are significantly different from their TBH counterparts marked with **.
creased: Mac-1⁺Ia⁻, Mac-2⁺Ia⁻, and Mac-3⁺Ia⁻ Mφ increased 147%, 171%, and 135%, respectively. In contrast, the percentage of Ia⁺ Mφ decreased significantly in the TBH, and there was also a decrease in Ia density per Mφ. Mac-1⁺Ia⁺ TBH Mφ decreased 60%, Mac-2⁺Ia⁺ TBH Mφ decreased 65%, and Mac-3⁺Ia⁺ decreased 61%. TBH splenic Mφ had a more homogenous fluorescence pattern than did normal host splenic Mφ. The gated populations of splenic Mφ showed <4% B and/or T cell contamination.
DISCUSSION

Mφ, like lymphocytes, are thought to have distinct subpopulations. Previously, we used single-label analysis to characterize Mφ phenotype (CHAPTER I). Because single-label analysis does not show multiple antigen expression per cell, we turned to double-labeling methods. We demonstrated that tumor growth affected the phenotype (double-labeled antigen expression) of Mφ in the peritoneal cavity and the spleen.

During tumor growth, the percentage of Mac-1⁺2⁺, Mac-1⁺3⁺, and Mac-2⁺3⁺ peritoneal Mφ decreased. The percentage of Mac-2⁺Ia⁻ and Mac-3⁺Ia⁻ TBH Mφ also decreased. There was no change in the percentage of Mac-1⁺Ia⁻ Mφ, suggesting that the overall percentage of TBH Mφ expressing Mac-1 did not change. What changed was the percentage of TBH Mφ expressing different combinations of Mac-1, Mac-2, and Mac-3 antigens. The results suggested that there was a shift towards TBH Mac-1⁺2⁻3⁻ Mφ. These changes were not due to an inflammatory response to the tumor as experiments with mice injected with nontumor tissue showed no significant changes in Mφ antigen expression. Thus, changes in TBH Mφ were due to the effects of tumor growth. These data are supported by our single-label analysis (CHAPTER I). These Mac-1⁺2⁻3⁻ TBH Mφ would be expected to be immature Mφ because Mac-2 and -3 are late differentiation antigens, while Mac-1 is an early differentiation antigen (95-97,208,249). An increase in immature Mac-1⁺2⁻3⁻ TBH Mφ is consistent with previous work showing a significant increase in immature peroxidase positive Mφ (74). An increased percentage of phenotypically (and functionally) immature peroxidase-positive Mac-2⁻3⁻ TBH Mφ may explain why TBH Mφ have a decreased accessory cell ability and an increased suppressive nature (50,51,72,74,103,137,138,149,231,236,246,269).

Previously, we showed normal host Mac-1⁺ Mφ were responsible for down regulating PGE₂ production and that this population was absent in the TBH (74). A shift in the suppressor Mφ from a Mac-3⁺ Mφ in the normal host to a Mac-2⁺ Mφ occurred in the TBH (74,137). A decrease in Mφ expressing Mac-1 or Mac-3 (decreased Mac-1⁺2⁺, Mac-2⁺3⁺, and Mac-1⁺3⁺ Mφ) could account for the loss of the normal host Mac-1⁺ Mφ-mediated PGE₂ control and Mac-3⁺
Mφ-mediated suppression. Although there is no change in Mac-1+ TBH Mφ phenotype, there is a functional aberration in this Mφ subpopulation (74). Mac-2+ Mφ may be the primary PGE2 producers in the TBH (74,137). The loss of the Mφ population that controls PGE2 production could more than overcome the decrease in Mac-2+ Mφ. Because there is a 2 fold increase in PGE2 production in peritoneal TBH Mφ (45,138), a decrease in the percentage of Mac-2+ Mφ did not seem to affect PGE2 production or TBH suppressor Mφ activity. Elsewhere, we have addressed the functional attributes of the various Mφ populations by using the flow cytometer to sort the various Mac+ populations (8).

The double-labeling procedure detected a unique population of normal and TBH Mac-1+2-3- and Mac-1-2+3+ Mφ. The percentage of Mφ in these populations did not change during tumor growth and there are no functions currently associated with them. Our functional studies using sorted Mφ populations is answering some of the functional questions of the different populations of Mφ (8). The number of Mac-1+ Ia+, Mac-2+ Ia+, and Mac-1+ Ia+ Mφ significantly decreased during tumor growth. These data are supported by previous data from us (73,CHAPTER I) and others (156). Decreased Ia expression on TBH Mφ limits their ability to act as antigen presenting cells (240-242) and accessory cells in Ia-dependent reactions, such as the SMLR (157,168).

In the spleen, the number and percentage of Mac-1+2+, Mac-1+3+, and Mac-2+3+ Mφ significantly increased during tumor growth. The increase in Mac-1+ Mφ in the TBH follows a shift in the phenotype of the splenic accessory Mφ from Mac-1+ in the normal host to Mac-1+ in the TBH (72). A recent report (236) suggests that different tumors produced a colony stimulating factor that induced Mac-1+ and -2+ splenic Mφ, which would suppress the in vitro mitogen-induced proliferation of T and B cells. Suppressor cell induction correlated with in vivo production of factors by these tumors. In our model, there is a 4-5 fold increase in the number of TBH splenic Mφ (51). Flow cytometry data showed a 30% increase in the percentage of TBH Mac-1+2+ Mφ. This percentage increase translates into 2-3 times more Mac-1+2+ Mφ in the spleen. A significant increase of these suppressor Mφ would explain why TBH splenic Mφ suppress mitogen-induced, allogeneic, and autoreactive T cell responses. Recently, it was also shown that

CHAPTER II 63
*Lactobacillus casei* can induce Mac-1+ and -2+ Mφ populations in the spleen (162). However, these Mφ were Mac-3- and showed cytotoxic activity. Perhaps that is the difference between the TBH and bacterial systems, Mac-1+2+3+ Mφ are suppressor Mφ and Mac-1+2+3- Mφ are cytotoxic Mφ.

The percentage of splenic Ia+ Mφ decreased significantly in the TBH, and there was also a decrease in Ia antigen density per Mφ. Mφ presentation to antigen-specific and autoreactive T cells depends on Ia expression (157,168,240-242), so changes in Ia expression would play a vital role in decreased MHC-restricted immune reactivity during tumor growth. Because Mφ with the Ia- phenotype are associated with suppressor activity (34), a 2 fold increase in TBH Ia- Mφ plus the four- to five-fold increase in TBH splenic Mφ (51), could explain the increased suppressor activity seen in TBH Mφ. The data that the suggested splenic Mφ shift from a Mac-1+2+3+ Ia+ normal host helper/accessory Mφ to a Mac-1+2+3+ Ia- TBH suppressor Mφ. This shift partially explains why the TBH is in a state of tumor-induced suppression and why immune reactivity is significantly suppressed by TBH Mφ.

In contrast to TBH peritoneal Mφ, TBH splenic Mφ showed a more homogenous fluorescence pattern than did normal host splenic Mφ. TBH splenic Mφ were more homogenous in size and because cell size correlates with antigen density, they would be expected to have a more uniform surface marker expression. The shift to smaller-sized TBH Mφ may be because of the influx of smaller immature Mφ into the spleen, there are 4-5 fold more Mφ in the TBH spleen than in the normal host spleen (51).

In conclusion, the data suggested that tumor growth changed the surface antigen expression of Mφ and that subpopulations of Mφ may exist in the spleen and the peritoneal cavity. Furthermore, the data showed that Mφ from different sites were phenotypically distinct, correlating with the distinct functional characteristics of each. This work extends previous work by ourselves (50,72-74,137,138) and others (56,103,149,231,236,246,169) showing that tumor growth changes Mφ characteristics leading to tumor-induced immunosuppression. Although these Mφ populations may represent either stable phenotypic subpopulations or transient populations, we can say that tumor growth changes the surface marker expression of TBH Mφ. How these changes correlate
with function will be the next major issue addressed. As we characterize Mφ phenotypically and functionally, we hope to further delineate the role of Mφ in the immune response and how tumor growth upsets this balance.
CHAPTER III

TUMOR-INDUCED ALTERATION IN
MACROPHAGE ACCESSORY CELL ACTIVITY ON
AUTOREACTIVE T CELLS

INTRODUCTION

Mφ are important accessory cells in the functioning of immune responses. In the SMLR, an assay driven by the presentation of self-Ia antigens by non-T cells to autoreactive T cells, Mφ serve as accessory cells by virtue of their ability to express and present self-Ia antigens to autoreactive T cells (14). This Mφ (Ia)-T cell interaction is the first signal in autoreactive T cell regulation (168).

The responder cell in the mouse SMLR is an autoreactive T cell, a L3T4+, Thy-1+, Ia−, and Lyt-2− cell (14,224), capable of responding to the presentation of self-Ia molecules alone from autologous accessory cells (36,157,158) but not Ia plus antigen (159). This is not unusual since the
immune system is self-centered and regulates itself by looking inward (101). Autoreactive T cells perform several regulatory functions in vitro such as help/amplification (36,117,157,158,264), suppression (36,100,201), cytotoxicity (235), and anti-tumor responses (238). Recently, autoreactive T cell clones have been shown to stimulate naive L3T4+ T cells and participate in a T-T network in vitro and in vivo (111,157,159,222). Autoreactive T cells have also been extensively studied in diseased hosts. Patients with lymphoproliferative diseases such as chronic lymphocyte leukemia (88) or Hodgkin's disease (56) have defective SMLR responses. Autoreactive T cells have important immunoregulatory functions and are essential for immune homeostasis.

Tumor growth alters Mφ phenotypic and functional characteristics. This includes decreased control of PGE₂ production (74,137), a decrease in Mφ regulatory capabilities, as well as a shift in the Mφ surface antigens, Mac-1, -2, -3, and Ia (72-74,137). Tumor growth suppresses mitogen-induced proliferation and MLR reactivity by altering Mφ accessory functions (51,73,74). Since tumor growth changes the Mφ ability to interact in the immune response and autoreactivity is altered by diseased states, we were interested in knowing what effect tumor growth had on the Mφ ability to act as an accessory cell to autoreactive T cells.

In the present study we observed that Mφ from BALB/c mice bearing a transplantable methylcholanthrene-induced tumor, inhibited autoreactivity. The suppression was found to be associated with decreased Ia antigen expression on TBH Mφ and increased suppressor activity by these Mφ which was partly caused by increased PG production.
MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Dominion Labs) were used throughout the experiments. Mice, designated TBH, received intramuscular injections of a single-cell suspension of $4 \times 10^5$ cells from a methylcholangthrene-induced nonmetastatic transplantable fibrosarcoma into the left hind leg. This resulted in palpable tumors by days 10-14 and death by days 28-35 (51). After the tumor becomes palpable, significant suppression of Con A, PHA, and MLR reactivity follows and this suppression increases as tumor growth progresses (51,72,73,261). In these kinetic studies, the increasing immunosuppression was due to the time of tumor-bearing as well as the size of the tumor (51,261). Mice were LDH virus negative (Microbiological Associates). Mice with no tumors (day 0/normal) or with tumors (7, 14, or 21 days after tumor cell injection) were used in this study. To confirm the use of untreated mice for controls as opposed to ones with large nonspecific inflammatory responses, BALB/c mice injected with $4 \times 10^5$ allogeneic, nontumor C3H muscle cells were also run as controls to determine whether immunosuppression was due to an inflammatory response or the tumor.

Medium

All cells were grown in RPMI-1640 (Hazelton) medium with 10% heat-inactivated FCS (Gibco) and $4 \times 10^{-5} \text{M}$ 2-mercaptoethanol (Sigma). All media contained 50 mg/l gentamicin (Gibco), 2 g/l NaHCO$_3$ and 25 mM HEPES (Research Organics).
Spleens were removed and placed in a wire sieve. The collected WSC suspensions were re-suspended in medium with 10% FCS and then plated or separated on a nylon wool column and further purified by antibody plus complement (C) treatment. The plated preparation provided the source of Mϕ, which were the stimulator cells in the SMLR. The nylon wool column and antibody plus C-treated preparation provided the source of L3T4+ T cells, which were the responder cells in the SMLR. The source of T cells were normal mice. T cells were collected by incubating WSC on a nylon wool column for 30 min at 37°C. Nonadherent cells were eluted from the column and centrifuged. The resulting nonadherent cells were further depleted of Ia+ B cells, Mϕ, and other contaminating cells by resuspending in supernatants containing mAb anti-Lyt-2 (3.155), anti-IAd (MK-D6), anti-immature T cell and B cell (J11d), and anti-IEd (14-4-4) for 30 min at 4°C, and followed by two washes. The cells were then incubated at 37°C for 30 min in a 1:12 dilution of low-tox-M rabbit C (Cedarlane Laboratories Ltd., Hornby, Canada). After three washes, the L3T4+ T cells were resuspended to 4 x 10⁶ cells/ml in medium containing 10% FCS and 4 x 10⁵ cells were added per well of a “U” bottom 96-well plate (Flow Laboratories). Splenic Mϕ (>96% esterase positive) were collected by plating the WSC on plastic 150 x 15 mm plastic plates (Lux/Miles Scientific) for 3 hr, washing vigorously to remove the nonadherent cells, scraping with a rubber policeman to remove the adherent cells (Mϕ), and centrifuged at 500 x g. After centrifugation, Mϕ were counted and resuspended to 4 x 10⁶ cells/ml. Mϕ were X-irradiated for 4 min at 50 kV (2000 rads) (TFI Minishot II X-iradiator, New Haven, CT). Four x 10⁵ cells were added per well (greater or lesser cell numbers resulted in decreased response, see results), except when titrations were done. Then, 4 x 10⁵ day-0 Mϕ were added to each well along with varying doses (from 4 x 10⁵ to 5 x 10³ cells/well) of day-0 (control), -7, -14, and -21 TBH Mϕ. For indomethacin treatment, indomethacin at 10⁻⁷ M was added to the SMLR. This concentration was previously shown to be effective and nontoxic (137). The plates were incubated for 4 days at 37°C. Six hr before termination and harvest of the cells, each reaction mixture was pulsed with 1 μCi of tritiated thymidine (³H-TdR; specific activity 6.7 Ci/mM, Dupont NEN Research Products).
Cells were harvested onto glass fiber filters (Whatman 934-AH, Thomas, PA) and counted in a 6895 betatrac liquid scintillation counter (Tm Analytic, Elk Grove Village, IL).

**Fluorescent Antibody Labeling**

Mouse spleens were excised and put through a wire sieve. The single suspension was centrifuged at 500 x g and suspended to 8 x 10⁶ cells/ml. Cell samples of day-0, -7, -14, and -21 TBH WSC at 8 x 10⁶ cells/ml, were dispensed in 1.0 ml aliquots. To these 1.0 ml reaction mixtures, 10 µl of mAb, anti-IA⁺ (MK-D6) or anti-IA⁺ (25-9-3S) were added and allowed to incubate for 35-45 min at 4°C. This was followed by two washes with cold media. The cells were incubated in a 1:500 dilution of FITC-labeled IgG F(ab')₂ fragment goat-anti-mouse antibody (Cappel Laboratories) for 35-45 min at 0°C, and finally washed twice. Splenic Mφ were selected by flow cytometry by gating on the unique Mφ FALS and 90LS pattern.

**Flow Cytometry**

Flow cytometric analysis was performed on an EPICS V, Model 752 (Coulter Electronics) laser flow cytometer and cell sorter. The instrument was calibrated with fluorescent microsphere standards prior to analysis of the Mφ samples. Three parameters per cell were studied: FALS, 90LS, and GFL. Laser excitation was normally 300 mW at 488 nm using a 5 W argon laser (Coherent Inc.). All data collection was done with the MDADS from Coulter Electronics Inc. FALS was collected using a linear integral and 90LS and GFL were collected using the log integral. Histograms showing cell number as a function of fluorescence were collected at a resolution of 256 channels and gated on FALS and 90LS dual parameter histogram 64 x 64 channels resolution defining the cell population of interest. The total number of cells counted per sample were 10,000.
Statistics

Splenic M\(
\) for flow cytometry were collected from 3-6 mice and each experiment was repeated 3-4 times. All percentages were collected from the MDADS computer. Three to six mice were used for each time point in the SMLR. Four duplicate wells were run for each test and each experiment was repeated 3 times. All numbers on graphs were statistically tested for significance by the students $t$ test (p <0.05).
RESULTS

Kinetic Analysis of Normal and TBH Mφ Stimulatory Capacity

To examine if the differences between normal and TBH Mφ stimulatory capabilities were due to differences in the time of incubation or cell numbers, a concomitant kinetic study (3, 4, and 5 days) and a titration of cells (2 x 10⁵ to 8 x 10⁵ cells/well) was done. A 4-day incubation was optimal for normal and TBH Mφ-induced T-cell proliferation (Figure 9). For normal host Mφ (day-0), the optimal number of stimulator cells was found to be 4 x 10⁵ cells/well on all 3 days of the assay. Titration beyond 8 x 10⁵ or less than 2 x 10⁵ cells/well resulted in further decreases in T cell responses (data not shown). Similar results were obtained for day-21 TBH Mφ, except these cells, in the range of 2 x 10⁵ to 8 x 10⁵ cells/well, caused significant inhibition. The decrease in the TBH Mφ-induced T-cell reactivity could not be attributed to a general inflammatory response. Mice injected with allogeneic C3H muscle cells and assessed at 21 days after injection showed no significant differences in mitogen, MLR, and SMLR reactivity as well as no demonstrable change in Mac-1, -2, -3, and IA surface antigen expression as assessed by flow cytometry (data not shown). For instance in the SMLR, un.injected normal mice had cpm of 4849 ± 525, normal mice injected with C3H muscle cells 21 days previously had cpm of 5045 ± 715 (no significant differences between the two normals), while mice (TBH) injected with tumor cells 21 days before had cpm of 1145 ± 80 (p <0.05, TBH cpm were significantly lower than normal un.injected or injected mice cpm). And previous work by us showed no difference in peroxidase staining of Mφ from normal untreated mice and normal mice injected with allogeneic, nontumor tissue 21 days before (74).
Figure 9. Kinetic Study of SMLR Reactivity: The X-axis represents the various dilutions of MΦ used in the titration study. The Y-axis represents the cpm that result from the $^3$H-TdR incorporation by proliferating T cells. Symbols represent the source of MΦ and the length of time the SMLR was incubated: (□) = Day-0 MΦ, 3-day incubation; (◇) = Day-0 MΦ, 4 day incubation; (X) = Day-0 MΦ, 5-day incubation; (△) = Day-21 TBH MΦ, 3-day incubation; (○) = Day-21 TBH MΦ, 4-day incubation; (+) = Day-21 TBH MΦ, 5-day incubation. Background counts for all irradiated MΦ were <600 cpm and for all purified L3T4$^+$ T cells were <3000 cpm.
Kinetic Study of Autoreactivity Using Normal and TBH Mφ as Accessory Cells

Day-0 (normal), -7, -14, and -21 TBH splenic Mφ were next used as stimulator cells and normal host L3T4+ T cells were used as responder cells to examine the effect of tumor growth on autoreactivity. No change in reactivity occurred when day-0 Mφ and day-7 TBH Mφ (26,909 and 27,515 cpm, respectively) were used as stimulator cells (Figure 10). By day 14, the counts had decreased to 18,390 cpm (a 32% decrease). Using day-21 TBH Mφ, reactivity decreased to 5783 cpm (a 78% decrease). The decrease in autoreactivity observed at 14 and 21 days of tumor growth was significantly (p <0.05) different from their normal counterparts. The decrease in autoreactivity caused by day-14 and -21 TBH Mφ can not be attributed to increased Lyt-2+ suppressor T-cell activity, because anti-Lyt-2 + C treatment did not restore autoreactivity (data not shown).

Ia Antigen Expression of Splenic Mφ

Splenic Mφ Ia antigen expression was assessed during tumor growth by flow cytometry. Splenic Mφ Ia antigen expression decreased in both the number of positive cells per population and density per cell (Figure 11 and Table 7). The number of Ia+ Mφ decreased from 59% in the normal host to 38% in the day-21 TBH (a 36% decrease). The density of Ia antigen per cell also decreased in day-21 TBH Mφ. Two populations of Ia+ Mφ were seen in day-14 and -21 TBH, one population had a 10-20% decrease in Ia antigen expression per cell, and the other had a >50% decrease in Ia antigen expression per Mφ when compared to day-0 Mφ. In addition, the number of cells observed with the lower fluorescent intensity (lower antigen density) increased nearly 4 times, while the number of cells staining with the higher fluorescent intensity decreased by nearly 40% as tumor growth progressed from day 0 to day 21.
Figure 10. Mφ Accessory Ability in the SMLR: The X-axis represents Mφ from hosts with various stages of tumor growth (0, 7, 14, and 21 days of tumor growth). The Y-axis represents the cpm that result from $^3$H-TdR incorporation into proliferating T cells. Background counts for irradiated 0-, 7-, 14-, and 21-day Mφ were 275, 123, 305, and 160 cpm, respectively. Background counts for the purified L3T4+ T cells were 1063 cpm.
Figure 11. Flow Cytometry Data of Day-0, -7, -14, and -21 TBH Mϕ: Mϕ were labeled with anti-IA^d (left column) and compared to the nonspecific antibody control, anti-IA^a (right column). The X-axis represents increasing size and the Y-axis is increasing fluorescent intensity (a measure of antigen density).
Table 7. Tumor-Induced Decrease of Ia\(^+\) Splenic M\(\phi\)

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<thead>
<tr>
<th>Days post tumor cell inoculation</th>
<th>Percentage of Ia(^+) M(\phi)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>14</td>
<td>54</td>
</tr>
<tr>
<td>21</td>
<td>38</td>
</tr>
</tbody>
</table>

* Percentage Ia\(^+\) cells represents the number of cells labeled with the specific mAb corrected for the number of cells labeled with the nonspecific control mAb.
**Mφ Modulation of Autoreactivity**

To determine if the SMLR hyporesponsiveness was due solely to a reduction in Mφ Ia expression or if TBH Mφ were actively involved in mediating decreased reactivity, we admixed different doses of day-0 (control) -7, -14, and -21 TBH Mφ with normal host Mφ (Figure 12). Four x 10^5 normal host (day-0) Mφ were mixed with varying numbers of normal or TBH Mφ. No significant differences were seen when different doses of day-0, day-7 or day-14 TBH Mφ were mixed with 4 x 10^5 normal host Mφ. However, different doses of day-21 TBH Mφ when mixed with normal host Mφ, suppressed the autoreactive T cell response. When 4 x 10^5 day-21 TBH Mφ were added to normal host Mφ, reactivity decreased by 92% (from 36,629 with day-0 Mφ to 2962 cpm with day-21 TBH Mφ). No significant differences were seen when day-7 and -14 TBH Mφ were added in at 4 x 10^5 cells. Suppression was also seen using 4 x 10^4 day-21 TBH Mφ. However, when 4 x 10^3 day-21 TBH Mφ were admixed with normal host Mφ no suppression was observed (no significant differences). These data therefore demonstrated a titratable suppression by TBH Mφ, not simply a reduction in autoreactivity caused by decreased TBH Mφ Ia expression.

**PG-Mediated Suppression by Normal and TBH Mφ**

The contribution of PG to the suppression of the autoreactive T-cell responsiveness by normal and day-21 TBH Mφ was assessed by adding indomethacin at 10^{-7} M to the SMLR. Previously, we showed that TBH Mφ secrete 2 times more PGE₂ than their normal counterparts. In peritoneal Mφ, PGE₂ production increases from 4.4 nM in the normal host to 7.4 nM in the TBH (45). In splenic Mφ, PGE₂ production increases from 0.6 nM in the normal host to 1.0 nM in the TBH (137). These amounts of PGE₂ are above the minimum level that we found to suppress T cell reactivity (45). Using L3T4⁺ autoreactive T cells, we showed PGE₂ significantly suppresses autoreactivity in a dose dependent manner in the range of 0.5 nM to 100 nM (45). The physiological
Figure 12. Effect of Admixing TBH Mφ with Normal Mφ on the SMLR: Different doses of TBH Mφ are shown on the X-axis (1 = $4 \times 10^5$, 2 = $4 \times 10^6$, 3 = $4 \times 10^7$, 4 = $4 \times 10^8$, and 5 = $4 \times 10^9$ day-0 Mφ only), were added to $4 \times 10^5$ day-0 Mφ. The Y-axis represents cpm and is a measure of $^3$H-TdR incorporation into proliferating T cells. Symbols represent the source of Mφ added to the SMLR: (□) = day-0 Mφ, (△) = day-7 Mφ, (◇) = day-14 Mφ, (○) = day-21 Mφ, and (X) = no addition, only $4 \times 10^5$ day-0 Mφ. Background counts for irradiated Mφ were 551, 851, 1215, and 1283 cpm, respectively for 0-, 7-, 14-, and 21- day Mφ. Background counts for the L3T4+ T cells were 1904 cpm.
levels of PGE$_2$ secreted by Mφ can suppress T-cell proliferation. PG contributed to the down regulation of normal and TBH Mφ-stimulated autoreactivity (Figure 13). The addition of indomethacin to the normal host Mφ-stimulated SMLR significantly ($p < 0.05$) increased the response (24,375 to 38,681 cpm; a 59% increase). The addition of indomethacin to the SMLR stimulated by TBH Mφ resulted in a 99% increase in reactivity (7963 to 15,883 cpm). TBH Mφ-stimulated autoreactivity, however, only returned to 65% of the level of the normal Mφ-stimulated autoreactivity, indicating the presence of other suppressor circuits. This suppression was not due to contaminating Lyt-2$^+$ suppressor T cells which can down-regulate autoreactive T cells (107,156,231), and can be preferentially activated by TBH Mφ (unpublished data), because pretreatment of normal and TBH Mφ with anti-Lyt-2 plus C did not restore T-cell reactivity.
Figure 13. Effect of Indomethacin on the SMLR: The X-axis represents the Mφ from the normal host (day-0) and the TBH (day-21). The Y-axis represents the cpm that result from $^3$H-TdR incorporation into proliferating T cells. Symbols represent the treatment of the SMLR with/without indomethacin: $\square$ = without indomethacin (no treatment), and $\square$ = with $10^{-7} M$ indomethacin added. Background counts for irradiated 0-, and 21-day Mφ were 1079, and 2314 cpm, respectively. Background counts for the purified L3T4$^+$ T cells were 2907 cpm.
DISCUSSION

We are studying the importance of Mφ in tumor-induced immunosuppression (51,72-74,137,261). In this study, we investigated tumor growth effects on the ability of splenic Mφ to serve as accessory cells to autoreactive T cells. Mφ are accessory cells because they express Ia antigen and release soluble modulators. Mφ provide the first two signals to the T cell (168). Autoreactive T cells maintain immune homeostasis (help/amplification/suppression) (36,100,117,157,158,201,264). Recently, it was shown that human CD4+ autoreactive T cells (L3T4+ in the mouse) can activate tumor cytotoxic CD8+ T cells (238). Altered SMLR responses are reported in many diseased states (56,88). Because Mφ serve as accessory cells to autoreactive T cells and we (51,72-74,137,261) and others (77,103,206,216,231,269) have reported that Mφ (particularly TBH Mφ) are responsible for immune dysfunction, we investigated the importance of TBH Mφ in autoreactive T-cell activity.

As the tumor grew, autoreactivity decreased when Mφ were used as accessory cells. Day-7 TBH Mφ did not cause a significant decrease in autoreactivity. When day-14 and -21 TBH Mφ were used as accessory cells, there was a 32% and 78% decrease, respectively, in reactivity. The distinct responses of autoreactive T cells to normal and TBH Mφ were not caused by differences in incubation times or cells numbers. The decrease in Ia antigen expression among TBH Mφ paralleled the loss in autoreactivity. Day-0 and day-7 TBH had the same number of Ia+ Mφ and Ia antigen density per Mφ, while day-14 and -21 TBH showed a 9% and 36% decrease, respectively, in total Ia+ Mφ and a decrease in the density of Ia antigen per cell. Tumor-induced reduction in Ia antigen expression was reported by us (73) and others (156,176). Decreased Ia antigen expression reflects a decrease in allogeneic MLR reactivity (73). Decreased Ia is important in the down regulation of the SMLR, since blocking Ia with specific antibody suppresses SMLR reactivity in a dose dependent fashion (157,158), and furthermore, resting autoreactive T cells need the first signal (Mφ Ia antigens) to become responsive to IL 1 and IL 2 (168). Because Ia molecule presentation drives the SMLR; we investigated whether the reduction in reactivity was due solely to a
decrease in Mϕ Ia antigen expression. When the number of TBH Mϕ increased 2-4 fold (that is, increasing the number of Ia+ stimulators), autoreactive T-cell activity did not increase, as might be expected if the only defect in TBH Mϕ accessory capabilities was a lack of Ia+ stimulators. Increasing the number of TBH Mϕ by 2-4 fold further depressed the already low TBH Mϕ-induced SMLR reactivity. The reduction in TBH Ia+ Mϕ only partly explained the decrease in autoreactivity. In addition to the importance of the phenotypic shift towards an Ia- TBH Mϕ in decreased Mϕ-T cell contact, it was recently shown that Ia- Mϕ are functionally suppressor Mϕ (34).

Tumor-bearing host Mϕ can suppress T-cell responsiveness (51,72-74,137,261). Is this the case with autoreactivity as measured by the SMLR? We addressed this question by admixing various concentrations of TBH Mϕ into a constant number of normal host Mϕ. If the decrease in Ia antigen on TBH Mϕ caused autoreactive T-cell hyporesponsiveness, then following the addition of day-21 TBH Mϕ to normal host (day-0) Mϕ, autoreactivity would be similar to control levels, since normal host Mϕ would present Ia antigen to the autoreactive T cells. Because of the presence of other suppressor circuits, TBH Mϕ suppressed the ability of even normal Mϕ to present Ia antigens. When day-7 and -14 TBH Mϕ were added to normal host stimulators, there was no decrease in reactivity. This suggests that changes in the functional capabilities occurred after day 14, even though day-14 TBH Mϕ already have tumor-induced phenotypic changes (73,74). Functional changes follow phenotypic changes in the TBH. The decrease in reactivity with day-14 TBH Mϕ occurred only when day-14 TBH Mϕ were used alone as stimulators, but not when used with day-0 Mϕ. The decrease in reactivity with day-14 TBH Mϕ seemed to be only caused by reduced cell surface Ia antigens on accessory Mϕ. However, day-21 TBH Mϕ caused decreased autoreactive T-cell activity when used as stimulator cells alone or when added to normal host Mϕ. This suppression was titratable and returned to normal levels when the number of day-21 TBH Mϕ was reduced. After 14 days of tumor growth, in addition to reduced Ia expression, functional changes in the Mϕ also caused reduced autoreactivity.

The nature of this suppression is not known, but speculation is possible. Mϕ produce many immunoregulatory compounds (223) including PGE₂, a powerful immunosuppressive agent
Tumor growth results in increased PGE₂ production by Mϕ (83,85,137) and a disappearance of a Mϕ subpopulation that controls or regulates PGE₂ production (74,137). PGE₂ is a strong negative modulator of Ia antigen expression, IL 2 receptor expression, and IL 2 secretion (207,247,270). Increased PGE₂ production by TBH Mϕ has the potential to change T-cell reactivity. To examine this PG effect, indomethacin was used. Indomethacin addition to the SMLR stimulated by normal or TBH Mϕ lead to a significant increase in autoreactivity. Autoreactive T cell responsiveness increased 59% with normal host Mϕ and 99% with TBH Mϕ. Mϕ not only stimulate autoreactive T cells by their la presentation but also down regulate autoreactive T cells by their monokine secretion. Secretion of PG and other suppressive monokines in the normal host would suggest that the natural presence of suppressor Mϕ maintain and control "normal" immune homeostasis (206,216,257,263). The TBH Mϕ-stimulated SMLR returned to only 65% of the normal host Mϕ-stimulated SMLR level (without indomethacin), suggesting other suppressor pathways, like the high molecular weight inhibitor we found in Mϕ supernatants (138). Tumor-induced functional changes in suppressor Mϕ might include quantitative and qualitative changes in suppressive agents (72,74,137,156,269).

Day-21 TBH splenic and peritoneal Mϕ have altered phenotypes and functions (74,CHAPTER I,CHAPTER II) that cannot be attributed to an inflammatory response against the tumor. There are shifts in Mϕ cell surface antigen expression and phenotypically distinct subpopulations during tumor growth (72-74,137), and decreases and differences in Mϕ functional capabilities in the allogeneic MLR (72-74). The tumor might induce a unique population of suppressor Mϕ or alter "normal" Mϕ so they become suppressor Mϕ. Others also report differences in Mϕ as a result of tumor growth (103,231,269).

In summary, these findings suggest that TBH Mϕ suppressed autoreactivity in at least two ways that are not necessarily mutually exclusive: (i) decreased Ia antigen expression and (ii) active suppression by soluble factors like PG. The exact mode of the tumor-induced Mϕ-mediated suppression is not known and further investigation is needed. We are examining the effect of Ia⁺ sorted normal and TBH Mϕ on autoreactive T cells to see if TBH Ia⁺ Mϕ are as effective as normal host Ia⁺ Mϕ and if Ia⁻ Mϕ are important. To understand the scope of tumor-mediated control of
autoreactivity, TBH L3T4+ autoreactive T cells need to be investigated. Recent evidence shows that TBH autoreactive T cells have a decreased responsiveness not caused by decreased in vivo cell numbers, even when normal host Mφ are used as accessory cells (CHAPTER IV). They also have a decreased responsiveness to IL-1, IL-2, and IL-4 (CHAPTER IV). By investigating a structure to function relationship, we hope to identify the phenotype of the controlling Mφ and the cellular mechanisms in the normal and TBH immune responses. This information will aid the development of treatment modalities for hypoactive (cancer and AIDS) and hyperactive (autoimmunity) immune responses.
CHAPTER IV

TUMOR MODULATION OF AUTOREACTIVITY:
DECREASED MACROPHAGE AND
AUTOREACTIVE T CELL INTERACTIONS

INTRODUCTION

$\text{M}^\phi$ are important regulatory cells in immune responses. In the SMLR, an in vitro assay of autoreactivity, $\text{M}^\phi$ are accessory cells that express and present self-Ia antigens to autoreactive T cells (14,36,157,158). Autoreactive T cells respond to $\text{M}^\phi$-Ia antigens not foreign antigen such as the fetal calf serum in the culture medium (158). This $\text{M}^\phi$ (Ia)-T cell interaction is the necessary first signal in autoreactive T cell regulation (168), as anti-Ia antibodies block this signal (157,158). $\text{M}^\phi$ also release soluble modulators that either up- or down-regulate autoimmunity. The responder cell in the SMLR is a $\text{L3T4}^+$, Thy-1$, \text{Ia}^-$, Lyt-2$^-$ autoreactive T cell (14,224). Autoreactive T
cells perform regulatory functions in vitro like help/amplification (36,117,157,158), suppression (36,100,117,201), cytotoxicity (235), and anti-tumor responses (238). Autoreactive T cell clones stimulate naive L3T4+ T cells and participate in a T-T cell network in vitro and in vivo (111,159,222). Autoreactivity has also been extensively studied in diseased hosts and shown to be defective (56,88). These facts suggest autoreactive T cells have important immunoregulatory functions and are essential for immune homeostasis.

Previously, we showed that Mφ from TBH suppressed autoreactive T cells by at least two different mechanisms; decreased Ia expression and increased suppressor molecule production (CHAPTER III). This change in TBH Mφ phenotype and function is one of many, we have observed during our examination of tumor-induced immunosuppression. Phenotypically, tumor growth caused changes in the Mφ surface antigen expression of Mac-1, -2, -3 and Ia (72-74,CHAPTER I,CHAPTER II). Functionally, TBH Mφ suppress MLR reactivity (72-74), SMLR reactivity (CHAPTER III), mitogen reactivity (51,73,261), and have an increased production of and alteration in suppressive factors (74,137,138). Furthermore, we have related phenotypic changes in TBH Mφ to certain functional abnormalities (72-74,137,138). In addition to changes in Mφ during tumor growth, TBH T cells are also affected; they are less responsive in the MLR (38,45,261).

To continue our study of how tumor growth suppresses autoreactivity, we investigated the contribution of TBH Mφ and TBH T cells to decreased autoreactivity. TBH Mφ significantly suppressed both normal and TBH T-cell reactivity by releasing PG and other suppressive factors. TBH T cells were significantly less responsive to Mφ accessory cells compared to their normal counterparts. TBH T cells were less responsive to IL-1, IL-2, or IL-4 than were normal T cells. The data suggest that tumor growth changes immune reactivity by affecting the immune response at the level of the Mφ and the T cell and may affect T_{H1} and/or T_{H2} subsets of T\_H cells.
MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Dominion Labs) were used. Mice were LDH virus negative (Microbiological Associates). A single-cell suspension of $4 \times 10^5$ cells from a methylcholanthrene-induced nonmetastatic transplantable fibrosarcoma was injected i.m. into the left hind leg of the TBH 3 weeks before use. Palpable tumors form by days 10-14 and death occurs by days 28-35 (51). Tumor growth significantly suppresses mitogen, contact sensitivity, MLR, and SMLR reactivity (51,72,73,CHAPTER III), but immunosuppression is not caused by a generalized inflammatory response (74).

Medium

All cells were grown in RPMI-1640 (Hazleton) medium with 10% heat-inactivated FCS (Gibco) and $4 \times 10^{-5}$ M 2-ME (Sigma). All media contained 50 mg/l gentamicin (Gibco), 2 g/l NaHCO$_3$ and 25 mM HEPES (Research Organics).

SMLR

Briefly, the collected WSC suspensions were plated for 2 hr (150 x 15 mm plastic plates: Lux/Miles Scientific). T cells were collected by incubating the plastic nonadherent WSC on a nylon wool column. Nylon wool nonadherent cells were eluted from the column and further depleted of Ia$^+$ B cells, Mφ, and other contaminating cells by resuspending in supernatants containing
monoclonal antibody anti-Lyt-2 (3.155), anti-IA<sup>+</sup> (MK-D6), anti-immature T cell and B cell (J11d), and anti-IE<sub>b</sub> (14-4-4). The cells were then incubated in a 1:12 dilution of C (Low-Tox-M rabbit C; Cedarlane Laboratories Ltd.) and washed. The L3T4<sup>+</sup> T cells (≤1% contaminating M<sub>ϕ</sub> and B cells) were resuspended to 4 x 10<sup>6</sup> cells/ml in medium with 10% FCS, and 4 x 10<sup>5</sup> cells were added per well of a "U" bottom 96-well plate (Flow Laboratories). Splenic M<sub>ϕ</sub> (>96% esterase positive) were collected by washing the plates vigorously to remove the nonadherent cells and scraping with a rubber policeman to remove the adherent cells (M<sub>ϕ</sub>). M<sub>ϕ</sub> were counted and resuspended to 4 x 10<sup>6</sup> cells/ml. M<sub>ϕ</sub> were X-irradiated for 4 min at 50 kV (2000 rads) (TFI Minishot II X-iradiator). Four x 10<sup>5</sup> cells, in medium containing 10% FCS, were added per well and incubated for 4 days at 37°C. Six hr before termination and harvest of the cells, each reaction mixture was pulsed with 1 μCi of ³H-TdR (specific activity 6.7 Ci/mM, Dupont NEN Research Products). Cells were harvested onto glass fiber filters (Whatman 934-AH) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic).

**Indomethacin Treatment**

Indomethacin at a final concentration of 10<sup>-7</sup> M was added to the SMLR cultures. This concentration is an effective inhibitor of the arachidonic acid pathway for PG synthesis and is nontoxic to immune cells (137). The same indomethacin dose was used when a combination of either indomethacin and interleukins or indomethacin and PGE<sub>2</sub> was added to the SMLR.

**IL-1, IL-2, IL-4 Treatment**

The IL dose for optimum responsiveness was determined by titration (data not shown). This concentration was then added to the SMLR and used throughout. The optimal dose of IL-1
(cell-line derived IL-1 [specific activity $10^8$ units/ml]: Genzyme, Boston, MA) was found to be 1 unit/well; for recombinant IL-2 ([specific activity $3 \times 10^6$ NIH units/ml]: Genzyme), the optimal dose was found to be 2 BRMP units/well; and for recombinant IL-4 ([specific activity $10^8$ units/ml]: Genzyme), the optimal dose was found to be 20 units/well. When combinations of the interleukins were added to the SMLR, the same doses were used.

**PGE$_2$ Treatment**

PGE$_2$ (Sigma) was added into the SMLR at final concentrations ranging from 1 nM to 100 nM. This range includes the physiological level of PGE$_2$ released by normal and TBH Mφ (45,138). To negate the effect of endogenous PGE$_2$, indomethacin was added with the PGE$_2$.

**Statistics**

Pooled cells from three to six mice were used for each experiment. Four replicate wells were run for each test and each experiment was repeated 3 times. All numbers in tables and data points on graphs were tested for significance by Student’s $t$ test ($p < 0.05$).
RESULTS

Reaction and Cell Number Kinetics between Normal and TBH Mφ and Autoreactive T Cells

Earlier examination of TBH Mφ Ia expression showed that decreased Ia expression was one way TBH Mφ controlled autoreactivity (CHAPTER III). Therefore to assess other ways tumor growth decreases autoreactivity, various combinations of T-cell responders and Mφ accessory cells were used (Table 8). The data suggest TBH Mφ significantly suppress normal and TBH autoreactive T-cell responsiveness and that TBH autoreactive T cells are significantly less responsive than normal autoreactive T cells. To examine whether the differences between normal and TBH Mφ and L3T4+ autoreactive T cells were due to variations in incubation times or cell numbers, a concomitant kinetic study (3, 4, and 5 days) and a cell titration (1 x 10^5 to 12 x 10^5 cells/well) were done. A 4-day incubation gave optimal autoreactivity for normal and TBH L3T4+ autoreactive T cells when using normal host Mφ as accessory cells (Figure 14), although TBH T-cell autoreactivity was only 62% of normal host T-cell proliferation. At 3 and 5 days, T-cell reactivity decreased, but TBH autoreactive T-cell responsiveness was always significantly lower than the normal counterpart. No significant differences were seen between normal and TBH T-cell reactivity when using TBH Mφ. TBH Mφ suppressed autoreactivity irrespective of the T-cell source at all time points. When TBH Mφ were used as accessory cells, they suppressed normal T-cell autoreactivity by 68% and TBH T-cell autoreactivity by 49%. Assays cultured for less than 3 days or longer than 5 days had significantly depressed responses in all cell populations (data not shown).

To investigate whether the differences between normal and TBH Mφ accessory cell capabilities were caused by quantitative differences in TBH Mφ, normal and TBH Mφ were titrated into a constant number of normal and TBH autoreactive T cells (Figure 15). When normal or TBH Mφ were added to either normal or TBH T cells, the optimal number of stimulator cells was found to be 4 x 10^5 cells/well. While Figure 15 shows only the 4-day titration, this pattern was similar
Table 8. Tumor-Induced Decrease in SMLR Reactivity

<table>
<thead>
<tr>
<th>T cell Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mφ Source&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NORMAL</td>
</tr>
<tr>
<td>NORMAL</td>
<td>72,938 ± 565&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBH</td>
<td>59,715 ± 3480</td>
</tr>
</tbody>
</table>

<sup>a</sup> Normal or TBH L<sub>3</sub>T<sub>4</sub><sup>+</sup> autoreactive T-cell responders were collected as stated in the Materials and Methods.

<sup>b</sup> Normal or TBH Mφ accessory cells were collected as stated in the Materials and Methods.

<sup>c</sup> <sup>3</sup>H-TdR incorporation by T cells is expressed as cpm ± SE.
Figure 14. Time Course Kinetics of Autoreactive T Cell Proliferation: Different cell combinations were incubated for 3, 4, or 5 days. The X-axis represents incubation time and the Y-axis represents $^3$H-TdR incorporation by proliferating T cells. Symbols designate these combinations: (●) = normal host L3T4$^+$ T cells plus normal host Mφ; (+) = normal host L3T4$^+$ T cells plus TBH Mφ; (○) = TBH L3T4$^+$ T cells plus normal host Mφ; and (▲) = TBH L3T4$^+$ T cells plus TBH Mφ.
Figure 15. Admixture of Normal and TBH Mϕ with Normal or TBH Autoreactive T Cells: The X-axis represents the number of Mϕ added and the Y-axis represents $^3$H-TdR incorporation by proliferating T cells. Symbols designate these combinations: (*) = normal host L3T4$^+$ T cells plus normal host Mϕ; (+) = normal host L3T4$^+$ T cells plus TBH Mϕ; (◇) = TBH L3T4$^+$ T cells plus normal host Mϕ; and (▲) = TBH L3T4$^+$ T cells plus TBH Mϕ.
for days 3 and 5 of the assay (data not shown). These results support previous work showing that TBH Mφ-mediated suppression is due to qualitative differences in TBH Mφ, not quantitative or cell number differences (CHAPTER III).

In Figure 14, we see that TBH T cells are only 62% as reactive as normal T cells. To assess whether the 38% decrease in TBH T-cell autoreactivity was caused by a qualitative difference in TBH T cells, various concentrations of normal and TBH autoreactive T cells were added into a constant number of Mφ (Figure 16). Maximum T-cell proliferation for either normal or TBH autoreactive T cells stimulated with normal host Mφ occurred with $4 \times 10^5$ T cells/well. When TBH Mφ were used, maximum T-cell responsiveness occurred with $4 \times 10^5$ to $12 \times 10^5$ T cells (no significant differences, $p < 0.05$). Because $4 \times 10^5$ T cells/well gave maximum T-cell reactivity when normal host Mφ were used, this number of T cells was used throughout. The decrease in TBH Mφ-induced SMLR reactivity could not be attributed to a general inflammatory response (74). Furthermore, this suppression was not due to contaminating Lyt-2$^+$ T$\text{\textsubscript{S}}$ cells which can down-regulate autoreactive T cells (159), because pretreatment with anti-Lyt-2 plus complement did not restore T-cell reactivity.

**PG-Mediated Suppression of Autoreactivity**

Because TBH Mφ produce more PGE$_2$ than their normal counterparts (45,137), the role of PG in the suppression of autoreactivity was assessed by adding $10^{-7}$ M indomethacin to autoreactive T cells. PG contributed to the down regulation of both normal and TBH Mφ-stimulated T-cell proliferation (Table 9). The addition of indomethacin to the normal host Mφ-stimulated cultures significantly increased normal host T-cell responses 48% and TBH T-cell responses 50%. The addition of indomethacin to TBH Mφ-stimulated cultures increased normal T-cell reactivity 104% and TBH T-cell reactivity 89%. These percentages are deceiving because with TBH Mφ, normal and TBH T-cell reactivity returned to only 70% and 63%, respectively,
Figure 16. Admixture of Normal and TBH Autoreactive T Cells with Normal or TBH Mφ: The X-axis represents the number of T cells added and the Y-axis represents $^3$H-TdR incorporation by proliferating T cells. Symbols designate these combinations: (*) = normal host L3T4+ T cells plus normal host Mφ; (+) = normal host L3T4+ T cells plus TBH Mφ; (○) = TBH L3T4+ T cells plus normal host Mφ; and (▲) = TBH L3T4+ T cells plus TBH Mφ.
Table 9. PG Modulation of SMLR Reactivity

<table>
<thead>
<tr>
<th>Cell Source*</th>
<th>Indomethacin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>NOR-T:NOR-Mφ</td>
<td>65,156 ± 2109</td>
</tr>
<tr>
<td>NOR-T/TBH-Mφ</td>
<td>30,985 ± 1352</td>
</tr>
<tr>
<td>TBH-T/NOR-Mφ</td>
<td>46,286 ± 3590</td>
</tr>
<tr>
<td>TBH-T/TBH-Mφ</td>
<td>27,964 ± 1557</td>
</tr>
</tbody>
</table>

* Cell source is the cell combination used in the assay. They were collected as stated in the Materials and Methods. Abbreviations represent the following: NOR-T = normal host L3T4+ autoreactive T cells; TBH-T = TBH L3T4+ autoreactive T cells; NOR-Mφ = normal host Mφ; and TBH-Mφ = TBH Mφ.

* 10^{-7} M indomethacin was added.

* ^3H-TdR incorporation by T cells is expressed as cpm ± SE.
of normal unstimulated autoreactivity levels, showing that other suppressor circuits exist in the TBH (138).

Direct Role of PGE₂ on Autoreactivity

Our earlier studies showed that the titration from 1-100 nM includes the physiological level (1-20 nM) of PGE₂ released by Mφ (45,138) Pure PGE₂ was added to assess its effect on autoreactivity (Figure 17). PGE₂ was added to the cultures at 1, 10, 20, and 100 nM. To ensure that endogenous PG would not affect the results, indomethacin was added to the assay concomitantly with the PGE₂. In a dose dependent manner, PGE₂ suppressed both normal and TBH T-cell autoreactivity with either normal or TBH Mφ as accessory cells. However, using either normal or TBH Mφ, normal T cells were more susceptible to the suppressive effects of PGE₂ than TBH T cells. A 100 nM dose of PGE₂ caused a 79% decrease in normal host T-cell responsiveness but only a 58% decrease in TBH T-cell responsiveness when normal host Mφ accessory cells were used. Similar results were seen with TBH Mφ at 100 nM PGE₂. Normal host T-cell autoreactivity decreased by 64% and TBH autoreactivity by 37%. The decreased susceptibility of TBH autoreactive T cells to the suppressive effects of PGE₂ mirrors previous results showing that TBH T cells in the allogeneic MLR are less susceptible to the effects of PG (45). No significant differences were seen between experiments run with or without indomethacin except that 1 nM PGE₂ suppressed TBH Mφ-stimulated T-cell proliferation (data not shown).

IL-1, IL-2, and IL-4 Modulation of Autoreactive T-Cell Proliferation

IL-1, IL-2, IL-4 or combinations of these were mixed with TBH Mφ or L3T4⁺ autoreactive T cells to determine if differences were due to a defect in the production of, or reactivity to, the
Figure 17. Effect of PG on Autoreactivity: PGE₂ was added at final concentrations ranging from 1-100 nM. In addition, to prevent endogenous PG production, indomethacin was added at a final concentration of 10⁻⁷ M. The X-axis shows the concentration of PGE₂ added and the Y-axis represents ³²P-TdR incorporation by proliferating T cells. Symbols designate the following: (*) = normal host L3T4⁺ T cells plus normal host Mφ; (◊) = normal host L3T4⁺ T cells plus TBH Mφ; (+) = TBH L3T4⁺ T cells plus normal host Mφ; and (▲) = TBH L3T4⁺ T cells plus TBH Mφ.
Figure 18. Effect of IL-1, IL-2, and IL-4 on Autoreactivity: IL-1 was added at 1 unit per well, IL-2 was added at 2 units per well, and IL-4 was added at 20 units per well. The X-axis represents the interleukins added (NONE/no addition, IL-1, IL-2, IL-4, or their combinations) and the Y-axis represents $^3$H-TdR incorporation by proliferating T cells. Symbols designate these combinations: (2) = normal host L3T4 $^+$ T cells plus normal host Mφ; (3) = normal host L3T4 $^+$ T cells plus TBH Mφ; (4) = TBH L3T4 $^+$ T cells plus normal host Mφ; and (5) = TBH L3T4 $^+$ T cells plus TBH Mφ.
cytokines (Figure 18). The addition of IL-1 led to a significant increase in both normal and TBH autoreactive T-cell reactivity (19% and 32%, respectively) when normal host Mφ were used. TBH T-cell proliferation stimulated with IL-1 was still only 72% as reactive as normal host T cells stimulated with IL-1. Proliferation of normal T cells driven by TBH Mφ increased 20% when IL-1 was added. No significant differences were seen between control (no IL-1 addition) and IL-1-stimulated TBH T-cell proliferation when TBH Mφ accessory cells were added. Adding IL-2 significantly increased the proliferation of all cell combinations. Normal and TBH T-cell autoreactivity increased 62% and 83%, respectively, with normal host Mφ and 46% and 23%, respectively, with TBH Mφ. When IL-4 was added, normal and TBH T-cell reactivity increased 71% and 58%, respectively, with normal host Mφ accessory cells and 57% and 53%, respectively, when TBH Mφ were the accessory cells. Simultaneous addition of IL-1 and IL-2, IL-1 and IL-4, and IL-2 and IL-4 caused synergistic or at least additive responses, since autoreactivity was increased more than with any cytokine alone. The addition of IL-1 and IL-2 increased normal and TBH T-cell autoreactivity 111% and 136%, respectively, with normal host Mφ accessory cells. When TBH Mφ were used as accessory cells, normal and TBH T-cell proliferation increased, but only 88% and 50%, respectively. When the IL-1 and IL-4 combination was added, normal and TBH T-cell autoreactivity increased 95% and 107%, respectively, with normal host Mφ and 86% and 80%, respectively, with TBH Mφ. The addition of IL-2 and IL-4 enhanced T-cell proliferation to a greater extent than did any of the other combinations. For example, normal and TBH T-cell proliferation increased 168% and 141%, respectively, when IL-2 and IL-4 were given in the presence of normal host Mφ. If IL-2 and IL-4 were given in the presence of TBH Mφ, normal and TBH T-cell proliferation increased 99% and 87%, respectively. It is interesting that the combination of TBH autoreactive T cells plus TBH Mφ (the cell combination occurring in vivo in the TBH) reacted significantly less to the cytokines than did the cell combination of normal host T cells and TBH Mφ even though their controls (no addition of cytokines) were similar. A cytokine dose response was done (data not shown) to show that decreased TBH T-cell reactivity was not caused by a simple quantitative defect in cytokine production. As the interleukin dose was increased, TBH T-cell responsiveness increased but never equaled normal host T-cell responsiveness.
**IL-1, IL-2, and IL-4 Stimulation of Accessory Cell-Depleted L3T4⁺ T Cells**

Cytokines were added to L3T4⁺ T cells alone (no Mφ added; Figure 19) to isolate T-cell responsiveness to the cytokines from a combination of Mφ-T cell interactions and cytokine responsiveness. Both normal and TBH L3T4⁺ T-cell responses were significantly enhanced by the addition of IL-1, IL-2, and IL-4. Normal host T-cell proliferation increased 128% with IL-1, 353% with IL-2, and 352% with IL-4, while TBH T-cell proliferation increased 60% with IL-1, 161% with IL-2, and 215% with IL-4. The combination of IL-1 and IL-2 enhanced normal T-cell reactivity 635% and TBH T-cell reactivity 304%. IL-1 and IL-4 increased normal T-cell proliferation 513% and TBH T-cell proliferation 245%, less than the combinations of either IL-1 and IL-2 or IL-2 and IL-4. IL-2 and IL-4 increased normal T-cell proliferation 928% and TBH T-cell proliferation 537%. In each case, however, TBH T-cell proliferation never equaled (significantly lower, p < 0.05) normal host T-cell proliferation. IL-1-, IL-2-, and IL-4-stimulated TBH T-cell autoreactivity was still only 74%, 61%, and 70%, respectively, of normal host T cell levels. When the combinations of IL-1 and IL-2, IL-1 and IL-4, and IL-2 and IL-4 were added, TBH T-cell responsiveness was only 58%, 60%, and 66%, respectively, of stimulated normal host T cells.

**PG-Mediated Suppression of IL-1-, IL-2-, and IL-4-Mediated Autoreactive T-cell Responsiveness**

By adding indomethacin and IL-1, IL-2, or IL-4, we examined the role Mφ-released PG play in the down regulation of T-cell cytokine responsiveness. The addition of indomethacin and IL-1, IL-2, or IL-4 significantly enhanced autoreactivity (Figure 20). The addition of both agents in-
Figure 19. Role of Cytokines on Accessory Cell-Depleted Autoreactive T-Cell Proliferation: IL-1 was added at 1 unit per well, IL-2 was added at 2 units per well and IL-4 was added at 20 units per well. The X-axis represents the interleukins added (NONE/no addition, IL-1, IL-2, IL-4, or their combinations) and the Y-axis represents $^3$H-TdR incorporation by proliferating T cells. Symbols designate these combinations: ($\Xi$) = normal host L3T4+ autoreactive T cells; (SS) = TBH L3T4+ autoreactive T cells.
Figure 20. Role of PG on IL-1-, IL-2-, and IL-4-Mediated Autoreactive T-Cell Proliferation: Indomethacin (10^{-7} M) was added to IL-1- (1 unit per well), IL-2- (2 units well), or IL-4- (20 units per well) stimulated cultures to measure the role of PG in the down regulation of IL-1-, IL-2-, and IL-4-mediated T-cell proliferation. The X-axis shows what was added (NONE/no addition, IL-1, IL-2, IL-4, Indomethacin, or their combinations) and the Y-axis represents {sup}3H-{TdR} incorporation by proliferating T cells. Symbols designate the following: (□) = normal host L3T4+ T cells plus normal host Mφ; (□) = normal host L3T4+ T cells plus TBH Mφ; (□) = TBH L3T4+ T cells plus normal host Mφ; and (□) = TBH L3T4+ T cells plus TBH Mφ.
creased T-cell responsiveness to a greater extent than did either alone, suggesting that Mφ PG production inhibits the T cell's ability to use the cytokines. IL-1 and indomethacin increased normal host T-cell autoreactivity by 112% and TBH T-cell autoreactivity by 148% when normal host Mφ were the accessory cells. With TBH Mφ as the accessory cells, normal autoreactive T-cell reactivity increased by 191% and TBH autoreactive T-cell reactivity increased by 119%. The response to IL-2 and indomethacin was similar to that for IL-1 and indomethacin, except that IL-2 enhances T-cell reactivity better than IL-1. Normal and TBH T-cell reactivity with normal host Mφ stimulators increased 162% and 198%, respectively. With TBH Mφ, normal T-cell reactivity increased 196% and TBH T-cell reactivity increased 131%. IL-4 and indomethacin enhanced normal and TBH T-cell proliferation with normal host Mφ 166% and 176%, respectively. With TBH Mφ, normal and TBH T-cell reactivity increased 230% and 183%, respectively. The addition of indomethacin and IL-1, IL-2, or IL-4 to TBH autoreactive T cells plus TBH Mφ was 100% of the normal SMLR control.
DISCUSSION

Tumor growth compromises the immune system leading to immunosuppression of the TBH. Previously, we showed that suppressor Mφ and suppressor T cells were responsible for suppression of mitogen and allogeneic MLR reactivity (51). In the present study, we investigated the effect of tumor growth on Mφ accessory cell abilities and T-cell autoreactivity. Mφ are accessory cells for autoreactive T-cell responders because they express and present Ia antigen and release soluble immune modulators (14,36,157,158,168). Mφ provide the necessary first signals that lead to autoreactive T-cell proliferation and maturation (157,158,168). The Mφ (Ia)-autoreactive T-cell interaction drives autoreactivity. Reactivity is not driven by foreign antigen such as FCS (158). Autoreactive T cells are vital to immune homeostasis because they serve as regulatory cells (help/amplification/suppression) (36,100,117,157,158,201) and as effector cells (cytotoxicity) (235). Furthermore, autoreactive T cells are important in anti-tumor immunity (238). Because altered autoreactive responses occur in many diseased states (56,88), we were interested in how tumor growth interrupts autoreactivity.

As a tumor grows, there is a systematic decrease in the Mφ ability to act as an accessory cell to autoreactive T cells (CHAPTER III). We show that by day 14 of tumor growth, TBH Mφ are not effective accessory cells to autoreactive T cells. This decreased accessory activity corresponded to the decreased Ia antigen expression found on Mφ from day-14 TBH. In contrast, Mφ from day-21 TBH have a decreased accessory capability caused by the quantitative decrease in Ia expression and increased secretion of suppressor molecules (CHAPTER III). In these ways, tumor growth changes Mφ functional abilities. TBH Mφ significantly suppress normal and TBH autoreactive T-cell responsiveness. This correlates with previous data showing that TBH Mφ suppress mitogen-induced proliferation, allogeneic MLR, and SMLR reactivity (51,72-74,CHAPTER III). Others also show that TBH Mφ are responsible for suppression of T-cell reactivity (103,231,269).
PG contributed to the down-regulation of normal and TBH Mφ-stimulated T-cell proliferation, suggesting that Mφ can stimulate autoreactive T cells and down-regulate autoreactive T cells by their monokine secretion. Adding indomethacin to normal host Mφ-stimulated normal or TBH T cells increased reactivity by almost 50%. When indomethacin was added to TBH Mφ-stimulated cultures, normal host autoreactive T-cell reactivity increased over 100% and TBH autoreactive T-cell reactivity increased by almost 90%. PG are responsible for part of the increase in TBH Mφ-mediated suppression and, in the normal host, for maintaining immune homeostasis. Increased PGE₂ production by TBH Mφ is noted by others (76,163,172). PGE₂ can down-regulate IL-2 receptor expression, IL-2 production, and responsiveness to IL-1 and IL-2 (247,270). PGE₂ also regulates IL-1 production (122) and Ia expression (207) in Mφ. Because there is a 2 fold increase in PGE₂ production in TBH Mφ (45,138) and a 4-5 fold increase in TBH splenic Mφ (51), there is a potential of an 8-10 fold increase in in vivo PGE₂ production in the TBH. An increase of this magnitude could account for part of the immunosuppression seen in the TBH.

Indomethacin partially restored TBH Mφ-stimulated autoreactivity (less than 75% as reactive as the normal control), indicating that PG account for only part of the suppressive capabilities of TBH Mφ. We show that TBH Mφ release a high molecular weight inhibitor which suppresses MLR T-cell reactivity and IL-2 production (138). Other suppressor factors could also contribute to the TBH Mφ-mediated suppression (10,34). Because various tumor cells actively produce TGF-β which can down-regulate Ia expression on accessory cells (41), the decreased stimulatory ability of TBH Mφ may be partly caused by TGF-β exposure. We are currently testing for this in our system. How might tumor growth control Mφ function? There is a shift in Mφ phenotype and function during tumor growth (72-74,137). Because normal host Mφ secrete PG and other suppressive monokines, there may be suppressor Mφ that normally maintain and control immune homeostasis (10,36,206,216,257,263). A tumor-induced dysfunction of the suppressor Mφ population may be why TBH Mφ are so suppressive. This scenario is supported by data showing a shift from Mac-3⁺ suppressor Mφ in the normal host to Mac-2⁺ PGE₂-secreting suppressor Mφ in the TBH (74,137). A PGE₂-regulatory Mφ is absent in the TBH (74). Furthermore, our recent evidence (CHAPTER II) showing an increase in splenic TBH Mac-1⁺2⁺ Mφ is supported by
Tsuchiya et al. (236). They show that several different tumors secrete a colony stimulating factor that induces a population of splenic Mac-1+2+ suppressor Mφ (236). Besides the change in TBH Mac antigen expression, we also see a decrease in Ia+ TBH Mφ (73,CHAPTER I,CHAPTER III). This shift to an Ia- Mφ is interesting because Ia- Mφ are the final suppressor cells in Tc cell regulation (34). Others show a Ts cell product needs Mφ processing before it can inhibit antibody secretion (10). In both these cases, normal Mφ are suppressive. Therefore, in a situation where the host is not stressed, suppressor Mφ maintain immune homeostasis. If, as our data suggests, there is a shift or increase in the suppressor Mφ population, a dysfunction in the “normal” suppressor Mφ, a lack of a controlling Mφ, or a combination of these in the TBH, a possible scenario for tumor-induced changes in Mφ-mediated accessory abilities exist.

In addition to their suppressor activity, TBH Mφ also have fewer up-regulatory signals. As shown previously, there is a decrease in the percentage of TBH Ia+ Mφ and a decrease in Ia expression per Mφ (CHAPTER III). Because Ia expression is necessary for Mφ presentation to antigen-specific T cells and autoreactive T cells (157,158,168,242), tumor-induced decreases in Ia expression would be detrimental to the host. When PGE₂ down regulates Ia expression (207), increased PGE₂ production by TBH Mφ might be autoregulatory. The inability of TBH Mφ to act as accessory cells may be caused not only by decreased Ia expression, but also by decreased IL-1 production. Further tests are necessary to verify what we can only infer from the data. Others show a decrease in IL-1 production by TBH Mφ (149). In fact, PGE₂ down regulates IL-1 production (122), so increased TBH PGE₂ secretion could be a method to control IL-1 production.

Tumor growth also affects TBH T cells. TBH autoreactive T cells are significantly less responsive to Mφ signals (60-80% as reactive) and exogenously added interleukins (60-75% as reactive) than their normal counterparts. These data suggest that tumor growth affects Mφ accessory capabilities and also L3T4+ autoreactive T cells and Tα cells. Decreased TBH T-cell responsiveness was not due to contaminating Lyt-2+ Tα cells because anti-Lyt-2 plus C had no effect on the decreased T-cell responsiveness. Furthermore, in this system, TBH L3T4+ T cells did not serve as Tα cells or Tα cell inducers, as add back experiments with L3T4+ TBH T cells did not suppress normal host T-cell responsiveness (data not shown). The addition of IL-1, IL-2, and IL-4 signifi-
icantly enhanced normal and TBH T-cell autoreactivity. TBH T-cell autoreactivity, however, was significantly less than its normal counterpart, suggesting TBH T cells were less responsive to IL-1, IL-2, and IL-4. A recent finding demonstrating the role of PGE₂ in T-cell suppression supports our results (247). PGE₂ suppresses or inactivates an early T-cell activation step. Furthermore, PGE₂ can inhibit IL-1 and IL-2 responsiveness, block IL-2 receptor expression, and block IL-2 secretion. This PGE₂-mediated suppression is irreversible (at least during in vitro culture). TBH T-cell hyporesponsiveness may be explained by the in vivo interaction of TBH T cells with the potential 8-10 fold increase in PGE₂.

TBH Mφ significantly suppress normal and TBH T-cell autoreactivity even with added IL-1, IL-2, and IL-4. Endogenous PG contributed to the down regulation of T-cell responsiveness to IL-1, IL-2, and IL-4, because indomethacin addition significantly enhanced autoreactive T-cell cytokine responsiveness. Normal and TBH T-cell autoreactivity was significantly enhanced above the control when normal host Mφ were used as accessory cells. This enhanced reactivity was seen when TBH Mφ were used as accessory cells and normal host T cells were used as responder cells, but the T-cell response was still significantly lower than when normal host Mφ were the accessory cells. When TBH Mφ were added to TBH T cells, responsiveness returned to unstimulated levels. TBH autoreactivity was never above the "baseline" normal level, supporting our previous suggestion of other suppressor circuits in the TBH (138). When examining the effect of PGE₂ on autoreactivity, we noticed TBH autoreactive T cells were less susceptible to the inhibitory effects of PGE₂ than normal autoreactive T cells (42% versus 66% suppression). This correlates with previous data demonstrating the anomaly seen in the TBH of increased PGE₂ production by Mφ but decreased inhibition by PGE₂ of TBH T cells (45). The reason for this decreased inhibition of TBH T cells may be answered by the recent work of Vercammen et al. (247). They showed that PGE₂ blocks an early T-cell activation step which prevents resting T cells from entering the cell cycle and consequently preventing proliferation. Activated or actively dividing T cells, however, that have passed this early step are less affected by PGE₂. There are increased numbers of proliferating T cells in the TBH. Thus, TBH T cells, as a population, would be less susceptible to the inhibitory effects of PGE₂.
Recently, $T_H$ cells were classified into two distinct subsets, the $T_{H1}$ and $T_{H2}$ subsets (153). Subset differentiation is based on the pattern of lymphokine secretion, responsiveness, and reported function. $T_{H1}$ secrete IL-2, respond to it in an autocrine manner, and are responsible for Mφ activation (153,219). $T_{H2}$ secrete IL-4 and respond to it in an autocrine manner but also need IL-1 to proliferate. IL-1 and IL-4 are synergistic for $T_{H2}$ growth (134,153). Functionally, $T_{H2}$ provide help for antibody secretion (153), although there are some arguments about the strict functional classification of the two subsets (44). If tumor growth alters one or both subsets of $T_H$ cells, a fully functional immune response is impossible.

We show that TBH T cells are less responsive to IL-1, IL-2, IL-4, and their combinations, suggesting that both $T_{H1}$ and $T_{H2}$ subsets may be affected by tumor growth. The suppressed response may be caused by decreased IL-1 receptor, IL-2 receptor, and IL-4 receptor expression. Although needing further proof, PGE$_2$ does down-regulate IL-2 receptor expression which would lead to decreased IL-2 responsiveness (247), so something similar may occur with IL-1 receptors and IL-4 receptors because IL-1 and IL-4 responsiveness is decreased. Our data and others suggest that there is a decrease in responsiveness to cytokines and a decrease in cytokine production (26,122,149,247). Tumor growth may regulate T-cell activity in two ways: by decreased T-cell responsiveness to cytokines, perhaps because of fewer receptors; and by decreased production of lymphokines. These changes would have profound effects on the immune system. Because $T_{H1}$ activate Mφ through interferon-γ (153,219), a decrease in $T_{H1}$ would lead to fewer "activated" Mφ for tumor cell killing and decreased Mφ Ia antigen expression for Mφ/T cell interactions. Because $T_{H2}$ help B cells by secreting IL-4, IL-5, and IL-6 (153), defects in $T_{H2}$ would cause decreased antibody secretion and antibody-dependent cellular cytotoxicity. Furthermore, defects in $T_{H2}$ cells would lead to decreased Ia expression on Mφ because $T_{H2}$ secrete IL-4 and IL-4 induces Ia expression on Mφ (221).

In summary, these findings suggest that tumor growth changes TBH Mφ and TBH T cells. These changes include increased production of suppressor factors, such as PGE$_2$, and decreased expression of Ia antigen by TBH Mφ. In TBH autoreactive T cells, changes include decreased responsiveness to cytokines. With the discovery of two subsets of $T_H$ cells, the data suggest tumor
growth may affect $T_\mu^1$ and $T_\mu^2$ cells. The tumor-induced effect on $T_\mu^1$ and $T_\mu^2$ could cause a cyclic suppression of immune reactivity. That is, $T_\mu$ cells are needed to activate $M\phi$ and $M\phi$ are needed to activate $T$ cells, creating a "catch 22" with neither cell activating the other and each needing the other to be activated. The tumor seems to have found an optimal method of preventing its own destruction. Continued research into overcoming tumor-induced suppression is vital to understanding tumor growth and to treating cancer patients.
CHAPTER V

NORMAL AND TUMOR-BEARING HOST SPLENIC
MACROPHAGE RESPONSES TO
LIPOPOLYSACCHARIDE

INTRODUCTION

Mφ have a diverse range of functions in the immune response. Mφ can be accessory cells, effector cells, or secretory cells. Their functional ability can be up- or down-regulated by a variety of factors (1,20,227), including LPS. Activation by agents such as LPS increases Mφ "competence" and causes many changes in Mφ function. These changes include increased bactericidal and tumoricidal ability (1,127,169,227), and increased regulatory ability (2,77). Mφ physiological changes include increases or decreases in monokine secretion, responses to cytokines, and antigen expression (1,9,49,188). In addition to the functional and phenotypic changes in activated Mφ,
there are changes in Mφ molecular characteristics. mRNA for CSF-1, c-fos, IL-1, and TNF are induced in LPS-treated Mφ (2). Furthermore, it is reported that activated Mφ lose their ability to proliferate (2). Activation may lock the Mφ into a functionally specific state so that it has a limited potential to carry out multiple functions yet has an increased ability to carry out a specific function.

During tumor growth, there are changes in TBH Mφ. These include changes in the expression of the Mac-1, -2, -3, and Ia surface antigens (72-74,137,156,CHAPTER I,CHAPTER II), Mφ accessory function in the MLR (50,73,74,137,231), the SMLR (56,CHAPTER III), and mitogen reactivity (51,72,73,246,269). TBH Mφ significantly suppress immune reactivity. This suppressive nature of TBH Mφ is partly due to an increase in the secretion of suppressive factors such as PGE₂ (76,137) and a decrease in enhancing molecules such as IL-1 (149). We show that there is a shift in Mφ subpopulations during tumor growth from helper Mφ to suppressor Mφ (73,137,CHAPTER I,CHAPTER II). This shift is supported by the recent work of others (236,269).

Previously, we showed that LPS can activate TBH peritoneal Mφ for tumor cell killing at concentrations that do not activate normal host Mφ (25) and normal and TBH peritoneal Mφ have different responses to LPS (9). We speculate that TBH peritoneal Mφ are primed by the tumor and LPS provides the second signal necessary to induce tumoricidal activity, while normal host Mφ are not primed and so can not be activated by low doses of LPS. Primed TBH Mφ may not be fully activated in vivo because of the release of suppressive factors, such as PGE₂; this may be one way the tumor controls Mφ-mediated suppression. Because TBH splenic Mφ act as suppressor cells to alloreactive and autoreactive T cells (50,51,56,73,74,137,231,236,269,CHAPTER III), the effects of LPS on normal and TBH splenic Mφ responses were of interest.

In this paper, we examined the effects of LPS treatment on several characteristics of normal and TBH splenic Mφ, including the expression of the Mφ surface markers Mac-1, -2, -3, and Ia, the accessory function in the MLR and the SMLR, cell-cycle kinetics, and RNA expression. We show that there are different phenotypic, functional, and cell-cycle kinetics of normal and TBH Mφ to the LPS activation signals.
MATERIALS AND METHODS

Animals

Eight to twelve week-old male BALB/c and C3H mice (H-2^d and H-2^k, respectively; Dominion Labs) were used. TBH mice received intramuscular injections of 4 \times 10^5 cells from a methylcholanthrene-induced, non-metastatic, transplantable fibrosarcoma in the left hind leg. This led to palpable tumors by days 10-14 and death by days 28-35 (50,51). By days 10-14, there was significant suppression of Con A, MLR, and SMLR reactivity and suppression increased as tumor growth progresses (51,73,231). Suppression or phenotypic changes in the TBH are not caused by a general inflammatory response to the tumor (74,CHAPTER I,CHAPTER III).

Medium

Cells were cultured in complete RPMI-1640 medium (Hazelton) with 10% heat-inactivated FCS (Gibco), 4 \times 10^{-5} M 2-mercaptoethanol (Sigma), 50 mg/l gentamicin (Gibco), 2 g/l NaHCO_3 and 25 mM HEPES (Research Organics).

Monoclonal Antibody Purification

Hybridoma cell lines (ATCC) were cultured in roller bottles in complete RPMI medium. Supernatants were harvested every 3 days and saturated with a pH 7.4 ammonium sulfate solution. After standing overnight, precipitates were collected by centrifugation, resuspended as a 20X concentrate in PBS, dialyzed against PBS, and applied to an affinity column. The column was washed
with PBS to remove any unbound protein. The mAb fraction was then eluted with a glycine buffer and immediately dialysed against PBS. Column fractions were assayed for mAb using goat-anti-rat antibody, heavy- and light-chain specific (Cappel) in an Ouchterlony immunodiffusion test. Positive fractions were pooled, assayed for antibody concentration, diluted to 1.0 mg/ml, sterile filtered, and stored at -70°C.

**Collection and Activation of Mφ**

WSC were harvested from normal and TBH animals and resuspended in complete RPMI medium. The WSC were plated on plastic tissue culture plates (LUX/Miles Scientific) for 3 hr. Mφ were collected by scraping with a rubber policeman, centrifuging, and resuspending in complete medium (> 96% Mφ by esterase staining). At intervals, LPS W S. typhosa (Difco, Detroit, MI) was added to the culture plates at a final concentration of 10 µg/ml (9). Mφ were incubated with LPS (10 µg/ml) or left untreated as controls for 3-hr or 24-hr. After the LPS treatment, Mφ were collected, washed free of LPS, and resuspended at 4 x 10⁶ cells/ml. These Mφ were used in: (i) phenotypic studies [Mφ labelled with mAb Mac-1, -2, -3, or Ia for flow cytometric analysis]; (ii) functional studies [Mφ used as accessory cells in the MLR and SMLR]; or (iii) cell-cycle studies [stained with acridine orange (AO) for DNA and RNA characterization by flow cytometric analysis].

**Fluorescent Antibody Labeling**

Cell samples from normal and TBH mice consisted of 4 x 10⁶ WSC/ml. To 1.0 ml aliquots of these cells, mAb or NRlG or NMlG (Cappel) was added and incubated for 35-45 min. This treatment was followed by three washes with cold medium. Cells were incubated in FITC-labeled
secondary antibody for 35-45 min and washed 3 times. The secondary antibodies were affinity purified goat-anti-rat F(ab')\_2 fragment FITC-labeled antibody (Cappel) for anti-Mac-1, -2, -3, and \textbf{NR1G} and affinity purified goat-anti-mouse F(ab')\_2 fragment FITC-labeled antibody (Cappel) for anti-Ia\^d and \textbf{NM1G} antibodies. The entire treatment was carried out at 4\textdegree C to allow effective antibody binding and maintain cell viability while minimizing membrane turnover and antibody internalization. Using FITC-labeled anti-Thy-1 and anti-IgG, -IgM, and -IgA (Cappel), we showed that the T and/or B cell contamination was <3% of the total of normal or TBH M\phi preparations.

\textbf{MLR}

The MLR consisted of L3T4\(^+\) BALB/c (\textit{H-2}\(^{b}\)) responder T cell populations and C3H (\textit{H-2}\(^{a}\)) stimulator cells. L3T4\(^+\) T cells were prepared by separating WSC suspensions on a nylon wool column and purified by antibody plus C treatment. C3H spleen cells were plated on tissue culture grade plates (Lux/Miles) for 3-hr and irradiated at 2000 rads (TFI Minishot II X-iradiator). BALB/c responder cells in complete RPMI medium were added at 2 \(\times\) 10\(^5\) cells/well, and the C3H stimulator cells were added at 4 \(\times\) 10\(^5\) cells/well in a 96-well "U" bottom microculture dish (Flow Laboratories). Irradiated M\phi were added to the MLR at 0\%, 5\%, 10\%, 20\%, and 50\% of the number of C3H stimulator cells. Corresponding crowding controls were done. The plates were cultured (96-hr, 37\textdegree C), pulsed with \textsuperscript{3}H-TdR (ICN, Irvine, CA), harvested, and counted (TM Analytical).

\textbf{SMLR}

The SMLR is a reaction between L3T4\(^+\) responder T cells and autologous accessory splenic M\phi. L3T4\(^+\) T cells were prepared as in the MLR. The L3T4\(^+\) T cells were resuspended to 4 x
$10^6$ cells/ml in complete medium, and $4 \times 10^5$ cells were added per well of a "U" bottom 96-well plate (Flow Laboratories). Splenic $M\phi$ (>96% esterase positive) were collected and purified by plating (adherence to plastic, LUX/Miles) and resuspended to $4 \times 10^6$ cells/ml. $M\phi$ were X-irradiated at 2000 rads. Four $\times 10^5$ cells, in complete medium, were added per well of the plates. The plates were cultured (96-hr, 37°C), pulsed with tritiated thymidine, harvested, and counted.

**AO Staining**

After 3 hr or 24 hr, $M\phi$ were removed from culture plates, centrifuged, resuspended in 0.1 ml RPMI medium with 20% FCS, and treated with 0.2 ml of solution A (0.1% Triton-X-100, 0.08 N HCl, and 0.15 N NaCl). After gentle mixing (4°C, 15 sec), 0.5 ml of solution B (0.16 µg/ml AO, 1 mM EDTA-Na, 0.15 M NaCl, in phosphate-citric acid buffer, pH 6.0) was added to stain the cells (9,43). These cells were then immediately analyzed on the flow cytometer.

**Flow Cytometry**

Flow cytometric analyses were performed on an EPICS V, Model 752 laser flow cytometer and cell sorter (Coulter Electronics). Four parameters per cell were measured: FALS, 90LS, red fluorescence, and green fluorescence. Laser excitation was 300 mW at 488nm using a 5 W argon laser (Coherent Inc.). All data was collected with the MDADS (Coulter Electronics). 10,000 cells were counted per sample. All calculations were performed using the MDADS software.
Calculation of Results

Splenic Mφ were pooled from 3-6 mice and each experiment was repeated 3-4 times. Four duplicate wells were run for each test sample in the MLR. All percentages for the flow cytometry data were computed using the MDADS software. Numbers were tested for statistical significance by Student's t test (p < 0.05).
RESULTS

Surface Antigen Expression

The effect of LPS on the percentage of normal and TBH Mφ expressing Mac-1, -2, -3 and Ia was assessed by labeling Mφ with anti-Mac-1, -2, -3, or Ia mAb after a 3-hr or 24-hr LPS treatment. Corresponding 3-hr and 24-hr plating-only controls were done also. The percentage of normal or TBH Mφ expressing the Mac-1, -2, -3, or Ia antigens did not change within each group after a 3-hr incubation with LPS (Table 10). There were significant differences, however, between the percentages of Mac-1\(^+\), -2\(^+\), -3\(^+\), and Ia\(^+\) normal or TBH Mφ, which was consistent with a previous report (CHAPTER I). In contrast, there was differential antigen expression after the 24-hr LPS incubation (Table 11). In the normal host, a 24-hr LPS treatment caused a 53%, 22%, 14%, and 9% decrease in the percentages of Mac-1\(^+\), -2\(^+\), -3\(^+\), and Ia\(^+\) Mφ, respectively. In contrast, a 24-hr LPS treatment of TBH Mφ caused a 49% and 11% increase in the percentages of Mac-1\(^+\) and -2\(^+\) Mφ, respectively, and a 12% and 11% decrease in the percentages of Mac-3\(^+\) and Ia\(^+\) Mφ, respectively. 24-hr plating alone also caused changes in antigen expression. From 3-hr to 24-hr of plating, the percentage of Mac-1\(^+\) Mφ increased 28% in the normal host and decreased 31% in the TBH. There was a 11% increase in normal host Mac-2\(^+\) Mφ and no change in the percentage of TBH Mac-2\(^+\) Mφ. Mac-3\(^+\) Mφ percentages remained constant. There were 12% and 8% decreases in normal and TBH Ia\(^+\) Mφ between 3 hr and 24 hr of plating.

Modulation of MLR Reactivity by LPS-Treated Mφ

To examine the differential effects of LPS on Mφ accessory function, normal and TBH Mφ were either untreated or pretreated with LPS for 3 hr or 24 hr and added to the MLR (BALB/c:
Table 10. Percentage of Mφ Expressing Mac or Ia Antigens After 3-hr LPS Treatment

<table>
<thead>
<tr>
<th>Antigen</th>
<th>NORMAL</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO LPS</td>
<td>LPS</td>
<td>NO LPS</td>
<td>LPS</td>
</tr>
<tr>
<td>Mac-1</td>
<td>46 ± 5*</td>
<td>49 ± 6*</td>
<td>70 ± 7**</td>
<td>73 ± 4**</td>
</tr>
<tr>
<td>Mac-2</td>
<td>42 ± 4*</td>
<td>39 ± 1*</td>
<td>64 ± 5**</td>
<td>62 ± 5**</td>
</tr>
<tr>
<td>Mac-3</td>
<td>46 ± 5*</td>
<td>48 ± 4*</td>
<td>70 ± 6**</td>
<td>66 ± 3**</td>
</tr>
<tr>
<td>Ia</td>
<td>53 ± 3*</td>
<td>55 ± 6*</td>
<td>36 ± 2**</td>
<td>39 ± 2**</td>
</tr>
</tbody>
</table>

* Cells were labeled as stated in Materials and Methods.

b Percentage represents the number of cells per 10,000 counted that were positively labeled compared to the NR1gG or NM1gG control. The numbers are the mean of different experiments ± SE. Significance was determined by Student’s t test (p<0.05). Normal host numbers marked with a * are significantly different from their TBH counterparts marked with a **.
Table 11. Percentage of Mφ Expressing Mac or Ia Antigens After 24-hr LPS Treatment

<table>
<thead>
<tr>
<th>Antigen</th>
<th>NORMAL</th>
<th></th>
<th>TBH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO LPS</td>
<td>LPS</td>
<td>NO LPS</td>
</tr>
<tr>
<td>Mac-1</td>
<td>74 ± 6**†</td>
<td>21 ± 5**‡</td>
<td>39 ± 7**†</td>
</tr>
<tr>
<td>Mac-2</td>
<td>53 ± 4**†</td>
<td>31 ± 3**‡</td>
<td>59 ± 2**†</td>
</tr>
<tr>
<td>Mac-3</td>
<td>50 ± 3**†</td>
<td>36 ± 1**‡</td>
<td>68 ± 3**†</td>
</tr>
<tr>
<td>Ia</td>
<td>41 ± 1**†</td>
<td>32 ± 2**‡</td>
<td>28 ± 3**†</td>
</tr>
</tbody>
</table>

* Cells were labeled as stated in Materials and Methods.

† Percentage represents the number of cells per 10,000 counted that were positively labeled compared to the NR1gG or NM1gG control. The numbers are the mean of different experiments ± SE. Significance was determined using Student's t test (p < 0.05). Normal host numbers marked with a * are significantly different from their TBH counterparts marked with a **. The no LPS treatment numbers marked with a † are significantly different from the LPS treatment numbers marked with a ‡.
H-2^ responder cells and C3H: H-2^ stimulator cells) at different percentages of stimulator cells (0% [control], 5%, 10%, 20%, 50%, and 100%). Mφ were treated identically to those used for the examination of cell surface antigen expression. No significant differences were noted in the accessory function among the combinations of 3-hr control or 3-hr LPS-treated normal Mφ over the range of Mφ percentages added (Figure 21). However, when untreated and LPS-treated TBH Mφ were added to the MLR at a 100% dose, they significantly suppressed alloreactivity by 53% and 60%, respectively.

Mφ that were preincubated with LPS for 24 hr showed differences in their accessory function (Figure 22). A 24-hr LPS incubation did not affect normal host Mφ accessory function at any Mφ dose used. In contrast, TBH Mφ suppressed T-cell reactivity after a 24-hr LPS incubation. A dose of 10% TBH Mφ, treated with LPS for 24 hr, suppressed alloreactivity by 57%. An increasing dose of Mφ suppressed alloreactivity significantly, with a 100% dose of Mφ suppressing T-cell reactivity by 87%. The 24-hr plating-alone also caused TBH Mφ to suppress T-cell reactivity. A 100% dose of TBH plated-only Mφ suppressed T-cell reactivity by 68%. TBH Mφ treated with LPS for 24 hr were significantly more suppressive than their untreated counterparts. Suppression was not caused by crowding, because the addition of extra C3H stimulatory cells enhanced MLR reactivity (data not shown).

Modulation of SMLR Reactivity by LPS-Treated Mφ

The alteration of accessory function of normal and TBH Mφ to autoreactive T cells was assessed after preincubating Mφ with or without LPS for 3 hr or 24 hr (Figure 23). Autoreactive T cells respond to Mφ surface antigens and their released soluble factors (168). Normal host Mφ treated with LPS for 3 hr had accessory functions similar to their untreated controls. With TBH Mφ, however, a 3-hr LPS treatment led to a 32% increase in Mφ accessory ability as measured by increased autoreactive T-cell responsiveness. Even though LPS-treated TBH Mφ helped
Figure 21. Modulation of MLR Reactivity by 3-hr LPS-Treated MΦ: The X-axis represents the percentage of MΦ added to the MLR (0% [control], 5%, 10%, 20%, 50%, 100%). The Y-axis represents ³H-TdR incorporation by proliferating T cells. The MΦ were added to the MLR after the following treatments: normal host MΦ 3-hr plating no LPS (●), normal host MΦ 3-hr plating plus LPS (+), TBH MΦ 3-hr plating no LPS (♦), and TBH MΦ 3-hr plating plus LPS (▲).
Figure 22. Modulation of MLR Reactivity by 24-hr LPS-Treated Mϕs: The X-axis represents the percentage of Mϕ added to the MLR (0% [control], 5%, 10%, 20%, 50%, 100%). The Y-axis represents $^3$H-TdR incorporation by proliferating T cells. The Mϕ were added to the MLR after the following treatments: normal host Mϕ 24-hr plating no LPS (♦), normal host Mϕ 24-hr plating plus LPS (+), TBH Mϕ 24-hr plating no LPS (●), and TBH Mϕ 24-hr plating plus LPS (▲).
Figure 23. Modulation of AMLR Reactivity by LPS-Treated Normal and TBH Mφ: The X-axis represents the treatment of the Mφ prior to AMLR addition. The Y-axis represents \(^{3}\)H-Tdr incorporation by proliferating autoreactive T cells. The following symbols represent: N = normal host Mφ; T = TBH Mφ; NO = no LPS treatment; L = LPS treatment; □ = 3-hr incubation with or without LPS; and □ = 24-hr incubation with or without LPS.
autoreactivity, they were still less than 50% as effective as normal host Mφ even after LPS treatment. The 24-hr plating with or without LPS caused a significant decrease in normal or TBH Mφ accessory activity, with the LPS treatment leading to the greatest decrease. A 24-hr LPS treatment caused 26% and 63% decreases, respectively, in the normal and TBH Mφ ability to act as accessory cells to autoreactive T cells. The 24-hr plating alone led to a 28% decrease in normal host Mφ accessory ability but caused no significant difference in the TBH Mφ accessory ability.

**Cell-Cycle Kinetics and RNA Levels of Mφ**

To measure the effect of LPS on Mφ at the molecular level, AO was used to measure both DNA, for cell-cycle kinetics, and RNA levels. Table 12 shows that the percentages of normal host Mφ in G\textsubscript{0}/G\textsubscript{1} did not change significantly after a 3-hr LPS treatment (82.3% before and 84.3% after). Mφ plated for 24 hr with or without LPS had a decreased percentage of cells in G\textsubscript{0}/G\textsubscript{1}. Treatment with LPS for 24 hr led to a significant increase in the percentage of cells in G\textsubscript{2}/M. There was little change in total RNA expression after a 3-hr LPS treatment, but RNA levels increased after a 24-hr incubation (data not shown). After the 24-hr LPS treatment, there was an increased percentage of Mφ entering late G\textsubscript{1}. Because this increase was not seen in the plating-alone group, the increase can be attributed to the LPS treatment. Table 12 also shows that the percentages of TBH Mφ in G\textsubscript{2}/G\textsubscript{1} increased (77.8% to 86.8%) after the 3-hr LPS treatment. Mφ that were plated for 24 hr with or without LPS had a decreased percentage of cells in G\textsubscript{0}/G\textsubscript{1}, but the percentages for the two groups were similar (54.5% with and 59.1% without LPS). Plating TBH Mφ increased the percentage of Mφ in S phase. The effect of LPS on the cell-cycle kinetics of TBH Mφ was diminished by 24 hr. There was an increase in RNA after a 24-hr LPS treatment (data not shown) and an increase in the percentage of Mφ in late G\textsubscript{1}. Normal and TBH Mφ had a decrease in the percentage of cells in G\textsubscript{0}/G\textsubscript{1} after 24 hr. This decrease was more dramatic in the TBH even though
Table 12. Percentage of Normal and TBH Mφ in Each Phase of the Cell-Cycle

<table>
<thead>
<tr>
<th>TREATMENT*</th>
<th>NORMAL</th>
<th></th>
<th></th>
<th>TBH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G&lt;sub&gt;0&lt;/sub&gt;/G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>S</td>
<td>G&lt;sub&gt;2&lt;/sub&gt;/M</td>
<td>Late G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Early G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>G&lt;sub&gt;0&lt;/sub&gt;/G&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>NO3</td>
<td>82.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.1</td>
<td>0.6</td>
<td>21</td>
<td>77.8</td>
<td>19.9</td>
</tr>
<tr>
<td>L3</td>
<td>84.3</td>
<td>15.2</td>
<td>0.5</td>
<td>17</td>
<td>86.8</td>
<td>10.7</td>
</tr>
<tr>
<td>NO24</td>
<td>77.2</td>
<td>20.5</td>
<td>2.3</td>
<td>21</td>
<td>59.2</td>
<td>39.9</td>
</tr>
<tr>
<td>L24</td>
<td>68.4</td>
<td>19.5</td>
<td>12.1</td>
<td>31</td>
<td>54.5</td>
<td>41.5</td>
</tr>
</tbody>
</table>

* Cells were treated as stated in Materials and Methods. Symbols represent the following: NO3, 3-hr plating without LPS; L3, 3-hr LPS; NO24, 24-hr plating without LPS; and L24, 24-hr LPS.

<sup>a</sup> Percentage represents the number of cells in each phase of the cell cycle as determined by the MDADS cell cycle software. 10,000 cells were counted. Cells were labeled with AO as stated in the Materials and Methods.
there were more normal host Mφ entering G2/M. Plating for 24 hr affected TBH Mφ cell-cycle kinetics more than it affected normal host Mφ.
DISCUSSION

Normal and TBH Mϕ had different phenotypic, functional, and cell-cycle responses to LPS. A 3-hr LPS treatment had little affect on normal or TBH Mϕ phenotypic characteristics when compared with untreated controls. The phenotypic differences between untreated normal and TBH Mϕ were reported previously (CHAPTER I,CHAPTER II). In contrast, a 24-hr LPS treatment led to differences between normal and TBH Mac⁺ and Ia⁺ Mϕ. A recent report supports our data that different stimuli can alter the Mac antigen expression of Mϕ (162). A complete correlation of phenotypic changes to functional changes cannot be done because only the Mac-1 and Ia antigens have known functions. Mac-1 (CD11b) is found on Mϕ, monocytes, granulocytes, NK cells, and bone marrow cells (96) and may be an early differentiation antigen and the complement fragment 3b receptor (48,96). Mac-2 is variably expressed on Mϕ from different tissues and on Mϕ elicited by different stimulatory agents, suggesting it may represent a differentiation and/or activation marker (95). Mac-3 is found on Mϕ and is a differentiation antigen with variable expression (97). Ia antigen is needed for Mϕ to effectively present to antigen specific and autoreactive T cells (168,241).

After a 3-hr plating with or without LPS, TBH Mϕ suppressed alloreactive T cells but only when a high dose (100%) of Mϕ was added. This percentage of TBH Mϕ suppressed both allogeneic and autoreactive T-cell responsiveness. The 24-hr LPS-treated and untreated TBH Mϕ also suppressed alloreactive T cells. Regardless of LPS treatment, TBH Mϕ suppressed autoreactive T cell-responsiveness. The TBH Mϕ populations that suppressed alloreactive and autoreactive T-cell reactivity had a large percentage of Mac-1⁺, -2⁺, and Ia⁻ Mϕ, the reported phenotype of suppressor Mϕ (34,236). The 24-hr plating-alone tests acted differently than expected. The 24-hr plating caused an increase in the percentage of Mac-1⁺ normal host Mϕ and a decrease in the percentage of Mac-1⁺ TBH Mϕ, yet normal host Mϕ did not suppress alloreactive T-cell reactivity and TBH Mϕ did. The reason for this response is unknown but may result from
the act of plating itself which induces RNA production and monokine secretion. These monokines could change the Mφ function predicted by its phenotype.

Mφ are accessory cells for autoreactive T-cell responders because they express and present Ia antigen to autoreactive T cells (168). Mφ provide the necessary first signals that lead to autoreactive T-cell proliferation and maturation (168). Because presentation of Ia by Mφ is the driving force for Mφ induced autoreactive T-cell proliferation, the decrease in TBH Ia+ Mφ would account for the decreased autoreactive T-cell responsiveness. These data demonstrate that the phenotypic changes in Mφ induced by LPS could have a direct effect on Mφ accessory function.

In addition to the LPS-induced phenotypic changes and their effects on Mφ function, LPS alters the monokine secretion of Mφ. LPS induces IL-1, TNF, and PGE₂ secretion (49,113,188). Differential production of these monokines could affect the regulatory functions of Mφ. Because TBH Mφ significantly suppressed T-cell reactivity, PGE₂ production may have increased. TBH Mφ produce significantly more PGE₂ than normal host Mφ and this increased PGE₂ production inhibits T-cell proliferation (76,137). In contrast to the suppression of T cells by the other TBH Mφ populations, 3-hr LPS-treated TBH Mφ had a significantly increased accessory function for autoreactive T cells. This increase could be due to increased IL-1 and TNF secretion, both of which can enhance T-cell reactivity. Nevertheless, autoreactive T-cell responsiveness with the 3-hr LPS-treated TBH Mφ was still less than 50% of the normal host control. In the future, we will test the differential secretion of these monokines directly. Normal host Mφ did not seem to respond to the LPS with a change in accessory function, with the exception that 24-hr LPS treated Mφ had a lower accessory activity to autoreactive T cells. This decrease was probably caused by the decrease in Ia expression on these Mφ. Because LPS did not affect splenic Mφ as dramatically as we saw with peritoneal Mφ (9,25), we are currently examining the effect of IFN-γ plus LPS, the classical two-step activation scenario (1,2,20), and its affect on normal and TBH splenic Mφ characteristics.

Resident Mφ can synthesize DNA and proliferate (254), while activated Mφ lose their ability to proliferate (2). In the cell-cycle, cells that are in G₁ require higher levels of RNA before they can enter S phase and begin proliferating. Cells in G₁ that have high RNA levels are in late G₁,
while those in G₁ with low RNA levels are in early G₁. Because LPS may activate TBH Mφ and not normal host Mφ (25), there should be differences in levels of DNA and RNA synthesis. Cells that are resting or not synthesizing DNA are in the G₀/G₁ phase, cells that are synthesizing DNA are in the S phase, and cells that are waiting to divide are in the G₂/M phase. For convenience, cells in G₀/G₁ are called resting cells while cells in S and G₂/M are called proliferating cells.

The cell-cycle data suggested that LPS can drive both normal and TBH Mφ to synthesize DNA and/or proliferate as evidenced by the increase of Mφ in S or G₂/M. However, only normal host Mφ showed a dramatic increase of Mφ in the G₂/M phase, suggesting they are the only Mφ moving completely through the cell-cycle. This increase in normal host Mφ in the G₂/M phase implies that they are not being activated by LPS because activated Mφ do not proliferate. This idea was supported by the functional data which showed no change in normal host Mφ accessory ability after a 24-hr LPS treatment. In contrast, TBH Mφ have a large increase of Mφ in S phase after 24 hr with or without LPS but no correspondingly large increase in G₂/M. This suggests that TBH Mφ can synthesize DNA (S phase) but are being prevented from completing the cell cycle. Being held in a particular phase of the cell cycle could be responsible for the TBH Mφ suppressor activity.

Mφ in different phases of the cell-cycle express different surface markers and have different functions (254). This hypothesis would be applicable to our system because both 24-hr TBH Mφ populations (with or without LPS) suppressed T-cell reactivity and had a large percentage of Mφ in S phase. The cause of the TBH Mφ cell-cycle block is unknown but may be due to incomplete signal transduction in TBH Mφ. LPS may only partially activate TBH splenic Mφ rendering them incapable of acting as efficient accessory cells or effector cells.

The data suggest that normal and TBH Mφ respond to the LPS signal differently. There are different changes in the surface antigen, accessory function, and molecular characteristics of normal and TBH LPS-treated Mφ. TBH Mφ were suppressive to both allogeneic and autoreactive T cells. In most cases, LPS seemed to augment TBH Mφ suppressor activity while not affecting normal host Mφ, suggesting that TBH splenic Mφ may respond differently to activation signals in vivo. This inability to respond to immune signalling may be one way tumor growth controls Mφ function and its own survival. If TBH Mφ are unable to be activated for functions such as tumoricidal
ability and instead act as suppressor cells, the tumor should have a greatly increased chance of survival.
CHAPTER VI

SUPPRESSION OF CD4+ T CELLS TO FOREIGN MHC ANTIGENS BY TUMOR-INDUCED Ia-
MACROPHAGES AND CD8+ T CELLS

INTRODUCTION

Mφ are important regulatory cells in immune responses; they are responsible for up- and down-regulating other immune cells (75,242). This regulation is accomplished by cell-cell contact through Mφ cell surface receptors such as Ia and by the release of soluble factors such as IL-1, IL-6, TNF-α, and PGE₂. The nature of these regulatory signals and the mechanisms that control their function is vital to understanding the process of an immune response. This understanding is particularly important for diseases such as cancer in which the host is immunocompromised.
Tumor growth is known to suppress alloreactivity (55,74,231), autoreactivity (56,CHAPTER III,CHAPTER IV), and antigen (52) and mitogen (51,236) reactivity; however, the mechanisms remain uncertain. The cause of this suppression may be changes in monokine secretion, either increased suppressor molecule production (76,137,138,269) or decreased enhancer molecule production (149). In addition to functional changes, tumor growth also causes phenotypic changes in Mϕ surface antigen expression (74,137,CHAPTER I,CHAPTER II). Even though our recent work has centered primarily on the role TBH Mϕ play in immunosuppression, early work suggested that TBH T_{3} cells may also be responsible for the immunosuppression (35,51).

The goal of this study was to extend our understanding of how tumor growth suppresses CD4^{+} T-cell responsiveness to foreign MHC class II antigens (alloreactivity). Because earlier evidence (51) hinted that the immunosuppression seen in the TBH may be due to changes in suppressor Mϕ and T cells, we investigated the role each played in the suppression of CD4^{+} T-cell activity. In this study, we examined (i) whether tumor growth causes a shift in the Ia^{+}/Ia^{-} Mϕ ratio and in the CD8^{+}/CD4^{+} T cell ratio and (ii) whether tumor growth causes a qualitative shift in TBH Ia^{-} Mϕ and TBH CD8^{+} T cell suppressor function. Like Chow and Battisto (34), we showed that Ia^{-} Mϕ were suppressor Mϕ. More important, however, was our demonstration of a significant increase in the number and suppressor function of TBH Ia^{-} Mϕ and CD8^{+} T cells. Besides differences in normal and TBH Mϕ and CD8^{+} T-cell functions, there were also differences in cellular interplay between those cells, which were partly due to PG. The data suggested that tumor growth may change immune reactivity by two different mechanisms: (i) by affecting an increase in Ia^{-} Mϕ numbers and suppressive function and (ii) by altering the CD8^{+}/CD4^{+} T cell ratio and the suppressor function of CD8^{+} T cells. These mechanisms can act separately, but in the TBH they act together to have an additive negative effect on CD4^{+} T-cell responsiveness.
MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Dominion Labs) were used. Mice were LDH virus negative (Microbiological Associates). A single-cell suspension of $4 \times 10^5$ cells from a methylcholanthrene-induced nonmetastatic transplantable fibrosarcoma was injected i.m. into the left hind leg of the TBH 3 weeks before use. Palpable tumors form by days 10-14, and death occurs by days 28-35 (51). Three weeks of tumor growth significantly suppresses T-cell reactivity, but immunosuppression is not caused by a generalized inflammatory response (74,CHAPTER I,CHAPTER II). To further confirm that immunosuppression was not caused by a general inflammatory response, C3H muscle tissue was injected into normal untreated BALB/c mice. When spleen cells were collected from 21-day C3H-tissue-exposed BALB/c mice, there were no significant differences in alloreactivity between the C3H-tissue injected and untreated BALB/c mice (data not shown).

Medium

All cells were grown in RPMI-1640 (Hazelton) medium with 10% heat-inactivated FCS (Gibco) and $4 \times 10^{-5}$ M 2-ME (Sigma). All media contained 50 mg/l gentamicin (Gibco), 2 g/l NaHCO$_3$ and 25 mM HEPES (Research Organics).
Fluorescent Antibody Labeling

Briefly, cell samples from normal and TBH mice consisted of WSC at 8 x 10⁶ cells/ml. Before labeling, WSC were depleted of RBC by treatment with 0.83% NH₄Cl and of B cells by anti-Ig (Cappel, Malvern, PA) plus C (Low-Tox-M rabbit C; Cedarlane Laboratories Ltd.). To 1.0 ml aliquots of these cells, mAb (MK-D6, anti-IA⁺; 3.155, anti-Lyt-2) or NRlG or NMlG (Cappel) was added and the antibody-treated cells were incubated for 35-45 min at 4°C. This treatment was followed by three washes. These cells were then incubated in FITC-labeled secondary antibody for 35-45 min at 4°C, followed by three washes. The secondary antibodies were affinity-purified goat-anti-rat FITC-labeled antibody (Cappel) for anti-Lyt-2 and NRlG and affinity-purified goat-anti-mouse FITC-labeled antibody (Cappel) for anti-IA⁺ and NMlG antibodies. NRlG was the control antibody for the anti-Lyt-2, and the anti-L3T4 mAb and NMlG were the control antibodies for the anti-IA⁺ mAb. These conditions allowed effective antibody binding and maintained cell viability while minimizing membrane turnover and antibody internalization.

Flow Cytometry

Flow cytometric analyses were performed on an EPICS V, Model 752 (Coulter Electronics) laser flow cytometer and cell sorter. This instrument was calibrated with fluorescent 10-micron-sized microsphere standards before analysis of the MΦ samples. Laser excitation was 300 mW at 488 nm using a 5 W argon laser (Coherent Inc.). All data collection was done with the MDADS (Coulter Electronics). FITC-labeled mAb was measured as the log of the green fluorescence.
**Mφ and CD8⁺ T Cell Preparation**

BALB/c WSC were plated for 2 hr (150 x 15 mm plastic plates; Lux/Miles Scientific), and the splenic Mφ (>96% esterase positive) were collected by washing the plates to remove the nonadherent cells and scraping with a rubber policeman to remove the adherent cells (Mφ). For Ia⁻ Mφ preparation, collected Mφ were treated with anti-Ia⁺ (MK-D6) for 30 min, washed, incubated in a 1:12 dilution of C (Cedarlane Laboratories Ltd.) for 30 min more, and washed again. This procedure enriched the preparation for Ia⁻ Mφ (>97%) as determined by FACS analysis. Mφ (unseparated or Ia⁻) were counted and resuspended to 4 x 10⁶ cells/ml. Mφ were X-irradiated with 2000 rads (TFI Minishot II X-iradiator). CD8⁺ T cells were collected by incubating plastic and nylon wool column nonadherent spleen cells in anti-L3T4 (Gk1.5) for 30 min, washed, incubated in a 1:12 dilution of C (Cedarlane) for 30 min more, and washed again. After anti-L3T4 mAb plus C treatment, the remaining cells were resuspended to 4 x 10⁶ cells/ml. CD8⁺ T cells were X-irradiated with 2000 rads (TFI Minishot II). Mφ or CD8⁺ T cells added to MLR cultures in three concentrations: 4 x 10⁴ (10%), 1 x 10⁵ (25%), and 2 x 10⁵ (50%) cells/well in medium containing 10% FCS. The percentage of Mφ or CD8⁺ T cells added to MLR cultures represents the number of those cells compared to the number of C3H allogeneic stimulator cells added.

**MLR**

BALB/c WSC suspensions were plated for 2 hr (Lux Scientific). T cells were collected by incubating the plastic nonadherent WSC on a nylon wool column. Nylon wool nonadherent cells were eluted from the column and further depleted of Ia⁺ B cells, Mφ, and other contaminating cells by resuspending them in supernatants containing mAb anti-Lyt-2 (3.155), anti-Ia⁺ (MK-D6), anti-immature T cell and B cell (J11d), and anti-IE⁺ (14-4-4). The cells were then incubated in a 1:12 dilution of C (Cedarlane) and washed. The L3T4⁺ (CD4⁺) T cells (<1% contaminating
Mφ and B cells) were resuspended to 4 x 10^6 cells/ml in medium with 10% FCS, and 2 x 10^5 cells were added per well of a "U" bottom 96-well plate (Flow Laboratories). C3H allogeneic stimulator cells were collected by plating spleen cells for 2-3 hr, washed of nonadherent cells (depleted of Mφ), X-irradiated (2000 rads, TFI Minishot II), resuspended to 8 x 10^6 cells/ml in media containing 10% FCS, and added at 4 x 10^5 cells/well. Plates were incubated for 4 days at 37°C. Six hr before termination and harvest of the cells, each reaction mixture was pulsed with 1 μCi of ³H-TdR (specific activity 6.7 Ci/mM, Dupont NEN Research Products). Cells were harvested onto glass fiber filters (Whatman 934-AH) and counted in a 6895 Betatrac liquid scintillation counter (Tm Analytic).

**Indomethacin Treatment**

Indomethacin at a final concentration of 10⁻⁷ M was added to the MLR cultures. This concentration is an effective inhibitor of the arachidonic acid pathway for PG synthesis and is nontoxic to immune cells (137).

**Statistics/Calculation of Results**

Pooled cells from 3 to 6 mice were used for each experiment. For the flow cytometry data, all percentages were determined by the MDADS computer and represent the percentage of 10,000 cells that were considered positively labeled after the negative control was subtracted. For the MLR functional studies, 4 replicate wells were run for each test, and each experiment was repeated 3 times. All numbers in tables and data points on graphs were tested for significance by Student's t test (p <0.05).
RESULTS

Shifts in Mφ and T Cell Subpopulations

Because previous evidence suggested that shifts in Mφ and T cell subpopulations were important in tumor-induced immunosuppression (51), we measured the percentages of normal and TBH Ia+ and Ia− Mφ and CD8+ and CD4+ T cells by labeling splenic Mφ and T cells with a primary mAb (anti-IAα [Mφ] or anti-Lyt-2 and -L3T4 [T cells]) plus a secondary FITC-labeled antibody. There was a decrease in TBH Ia+ Mφ (from 54% to 36%) and a corresponding increase in TBH Ia− Mφ (Table 13). In addition, Ia expression per Mφ decreased (shown by the decreases in mean and peak fluorescence). There was an increase in the percentage of CD8+ T cells (34% to 48%) and a decrease in the percentage of CD4+ T cells (66% to 52%) during tumor growth. There was, however, no change in CD8 or CD4 expression per T cell (no change in mean or peak fluorescence). Tumor growth thus changes the Ia+/Ia− Mφ and the CD8+/CD4+ T cell ratios.

Normal and TBH Unseparated and Ia− Mφ Accessory Function: Dose-Dependent Suppression of CD4+ T-Cell Responsiveness to Foreign Allo-Antigens

The implication of the preceding Mφ changes was assessed through examination of differences between the accessory function of normal or TBH unseparated or Ia− Mφ. Changes in accessory function were determined by addition of different doses (10% and 50%) of syngeneic Mφ to MLR cultures. These doses were used because earlier work showed that Mφ affect T cells in a concentration-dependent manner (37). To determine if Ia− Mφ were suppressor Mφ, Ia+ Mφ were depleted by anti-IAα plus C. The Ia− Mφ population was contaminated by <4% Ia+ Mφ. At a low dose of Mφ (10%), normal host unseparated Mφ significantly increased by 36% the
Table 13. Flow Cytometric Analyses of Changes in Splenic Mφ and T Cells

<table>
<thead>
<tr>
<th>Immune Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Days of Tumor Growth&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cell Ratio (%)</th>
<th>Mean Fluorescence&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>21</td>
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<td>CD8&lt;sup&gt;+&lt;/sup&gt;/CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
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<td>21</td>
<td>48/52</td>
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<sup>a</sup> Mφ and T cells were collected as stated in the Materials and Methods.

<sup>b</sup> Days of tumor growth represent the days since tumor cell inoculation.

<sup>c</sup> Cells were labeled with anti-IA<sup>e</sup>, anti-Lyt-2, or anti-L3T4 mAb, as stated in the Materials and Methods. Figures in the columns headed Ia<sup>+</sup> or Ia<sup>-</sup> splenic Mφ, and CD8<sup>+</sup> or CD4<sup>+</sup> splenic T cells are the percentage of cells labeled after correction for the control. SE on all percentages was <10%.

<sup>d</sup> Mean and peak fluorescence were calculated by the MDADS computer. Mean fluorescence represents the average channel number (out of a maximum of 64 channels) for the fluorescence of the labeled cells. Peak fluorescence represents the channel number at which the maximum number of cells appeared.
CD4+ T-cell response to foreign class II antigens (Figure 24), whereas a low dose of TBH unseparated Mφ suppressed alloreactivity by 14%. High doses (50%) of normal and TBH unseparated Mφ significantly suppressed T-cell responsiveness by 20% and 77%, respectively. Normal and TBH Ia− Mφ, however, were significantly more suppressive than their unseparated Mφ populations, and TBH Ia− Mφ were significantly more suppressive than normal host Ia− Mφ. A low dose of normal host Ia− Mφ enhanced T-cell reactivity by 27%; in the TBH, a low dose of Ia− Mφ suppressed T-cell responsiveness by 29%. High doses of normal and TBH Ia− Mφ suppressed reactivity 53% and 86%, respectively. When even higher doses of these cells were added, the trends remained the same (data not shown). The data suggested that (i) Ia− Mφ were suppressor Mφ and (ii) tumor growth significantly increased their activity. Suppression by unseparated Mφ was probably due to Ia− Mφ.

Lyt-2+(CD8+) T Cell-Mediated Suppression of CD4+ T-Cell Alloreactivity

Because previous results suggested that T s cells may also be responsible for tumor-induced immunosuppression (51), we explored the role of CD8+ T cells in tumor-induced suppression of CD4+ T cells to foreign MHC class II antigens. We added different doses (10% and 25%) of BALB/c CD8+ T cells into MLR cultures. TBH CD8+ T cells were significantly more suppressive than normal host CD8+ T cells (Figure 25). A low dose (10%) of normal host CD8+ T cells suppressed alloreactivity by only 15%; a low dose of TBH CD8+ T cells suppressed reactivity 31%. With a high-dose (25%), normal and TBH CD8+ T cells suppressed reactivity 43% and 55%, respectively. Results of even higher doses of normal and TBH CD8+ T cells showed the same trends, with TBH CD8+ T cells (at all doses used) being significantly more suppressive than were normal host CD8+ T cells (data not shown). TBH CD8+ T cells are qualitatively more suppressive than their normal host counterparts.
Figure 24. Changes in Mφ Accessory Activity Affects Alloreactivity: Different combinations of normal and TBH Mφ were added to the MLR, and alloreactivity was measured. The X-axis represents $^3$H-TdR incorporation by proliferating L3T4+ T cells, and the Y-axis represents the different treatment groups, sources, and concentrations of Mφ added.
Figure 25. Changes in CD8⁺ (Lyt-2⁺) T-Cell Suppressor Activity Affects Alloreactivity: Different concentrations of normal and TBH CD8⁺ T cells were added to the MLR, and alloreactive T-cell responsiveness was measured. The X-axis shows ³H-TdR incorporation by proliferating L3T4⁺ T cells, and the Y-axis represents the dose and source of added CD8⁺ T cells.
Mφ-CD8+ T Cell Interplay Affects CD4+ T-Cell Alloreactivity: Normal and TBH Mφ Plus Normal Host CD8+ T Cells

To assess the interplay between Ia− Mφ and CD8+ T cells and the role this interplay has in mediating suppression of CD4+ T cells, both cell types were added at different concentrations into MLR cultures. When a low dose (10%) of normal or TBH Mφ (unseparated or Ia−) were added along with a low dose (10%) of normal host CD8+ T cells, there was no additional suppression (Figure 26). In fact, a low dose of unseparated normal host or Ia− Mφ enhanced reactivity (removed suppression) by 41% and 26%, respectively. T-cell proliferation, however, was still significantly lower than when a low number of Mφ alone were added to MLR cultures. When a low dose of TBH unseparated or Ia− Mφ plus a low dose of normal host CD8+ T cells was added, T-cell responsiveness was suppressed to the same extent as were cultures with CD8+ T cells alone. With a high dose (50%) of unseparated normal host Mφ, there was no increased suppression. However, unseparated TBH Mφ or normal or TBH Ia− Mφ plus a low dose of CD8+ T cells suppressed T-cell proliferation 65%, 28%, and 81%, respectively. When a high dose of normal host CD8+ T cells and any Mφ population were added to the MLR (Figure 27) results were similar to those for any Mφ population plus a low dose of normal host CD8+ T cells (compare Figure 26 and Figure 27). A low dose of unseparated normal host or Ia− Mφ plus a high dose of normal host CD8+ T cells increased alloreactivity 50% and 21%, respectively; a low dose of unseparated TBH or Ia− Mφ combined with a high dose of CD8+ T cells also led to increased reactivity (21% and 17%, respectively). With a high dose of unseparated normal host Mφ plus CD8+ T cells, T-cell responsiveness increased 20%; however, if normal host Ia− Mφ were mixed with CD8+ T cells there was no change. This result was the same as the T-cell response when CD8+ T cells were added alone to the MLR culture. In contrast, when unseparated TBH or Ia− Mφ were used, CD8+ T cell-mediated suppression increased (49% and 74%, respectively). However, suppression of CD4+ T-cell responsiveness observed when these Mφ were combined with CD8+ T cells was still less than when Mφ were used alone. The results suggest that the effects from
Figure 26. Changes in Mφ-CD8* T-Cell Interactions (Variable Mφ Doses and Low-Dose Normal Host CD8* T cells) Affect Alloreactivity: Various concentrations of normal and TBH unseparated and Ia* Mφ were added with a low dose of normal host CD8* T cells to the MLR. The X-axis represents 3H-TdR incorporation by proliferating L3T4* T cells, and the Y-axis shows the treatment groups.
Figure 27. Changes in Mφ-CD8+ T-Cell Interactions (Variable Mφ Doses and High-Dose Normal Host CD8+ T Cells) Affects Alloreactivity: Various concentrations of normal and TBH unseparated and Ia- Mφ were added with a high dose of normal CD8+ T cells to the MLR. The X-axis represents 3H-TdR incorporation by proliferating L3T4+ T cells, and the Y-axis shows the treatment groups.
normal and TBH Mφ combined with normal host CD8+ T cells are dose and cell ratio dependent, but Mφ-mediated suppression dominated normal host CD8+ T cell-mediated suppression.

*Mφ-CD8+ T Cell Interplay Affects CD4+ T-Cell Responsiveness: Normal and TBH Mφ Plus TBH CD8+ T Cells*

To explore the effects of normal and TBH Mφ-TBH CD8+ T cell interplay, Mφ and CD8+ T cells were added to MLR cultures (Figure 28). The addition of a low dose of normal or TBH unseparated Mφ, or normal host Ia− Mφ, to MLR cultures increased CD4+ T-cell proliferation to foreign class II antigens by 84%, 51%, and 51%, respectively. The addition of a low dose of TBH Ia− Mφ plus a low dose of TBH CD8+ T cells to MLR cultures caused no change in T-cell responsiveness. A high dose of normal host Mφ plus a low dose of TBH CD8+ T cells increased alloreactivity (38%). Unseparated TBH Mφ or normal or TBH Ia− Mφ suppressed reactivity by 58%, 12%, and 85%, respectively. Again, suppression with any Mφ group plus CD8+ T cells was less than or equal to Mφ-mediated suppression alone. When a high dose of TBH CD8+ T cells plus Mφ was added to MLR cultures (Figure 29), only normal host unseparated Mφ removed suppression. Unseparated TBH Mφ and normal host Ia− Mφ plus CD8+ T cells did not further suppress T-cell proliferation. TBH Ia− Mφ plus a low dose of TBH CD8+ T cells suppressed alloreactivity by 44%. The suppression resulting from a low dose of TBH Ia− Mφ combined with a high dose of TBH CD8+ T cells was greater than that from either alone. With a high dose of Mφ plus a high dose of TBH CD8+ T cells, the alloreactive response was suppressed to a greater extent than by Mφ or CD8+ T cells alone. Normal and TBH unseparated Mφ and normal and TBH Ia− Mφ suppressed reactivity by 16%, 66%, 41%, and 96%, respectively. Thus, like Mφ-normal host CD8+ T cell interactions, Mφ-TBH CD8+ T cell interactions are dose-dependent but, more important, with Mφ plus TBH CD8+ T cells, there is an additive suppression of the CD4+ T cell response to foreign MHC antigens.
Figure 28. Changes in Mϕ-CD8⁺ T-Cell Interactions (Variable Mϕ Doses and Low-Dose TBH CD8⁺ T Cells) Affect Alloreactivity: Various concentrations of normal and TBH unseparated and Ia⁻ Mϕ were added with a low dose of TBH CD8⁺ T cells to the MLR. The X-axis represents ³H-TdR incorporation by proliferating L3T4⁺ T cells, and the Y-axis shows the treatment groups.
Figure 29. Changes in Mϕ-CD8⁺ T-Cell Interactions (Variable Mϕ Doses and High-Dose TBH CD8⁺ T Cells) Affect Alloreactivity: Various concentrations of normal and TBH unseparated and Ia⁻ Mϕ were added with a high dose of TBH CD8⁺ T cells to the MLR. The X-axis represents ³H-TdR incorporation by proliferating L3T4⁺ T cells, and the Y-axis shows the treatment groups.
PG-Mediated Suppression of CD4⁺ T-Cell Reactivity

Mφ release PG that can down regulate T-cell responsiveness (75). But is Ia⁻ Mφ-mediated suppression PG mediated? Indomethacin (1 x 10⁻⁷ M) was used to measure the role of PG in Mφ-mediated suppression. When indomethacin was added to MLR cultures containing normal or TBH unseparated or Ia⁻ Mφ, alloreactivity increased 10-50% (Figure 30). No significant differences were seen between normal or TBH unseparated and Ia⁻ Mφ populations, suggesting that the suppressor ability of Ia⁻ Mφ was not solely due to PG production.

In addition to down-regulating T-cell proliferation, TBH Mφ produce more PG (74,76,137,138); therefore, we wanted to explore the possibility that changes in Mφ PG production were related to changes in Mφ-CD8⁺ T cell interactions. Indomethacin was added to MLR cultures when both Mφ and CD8⁺ T cells were present (Figure 31A-B). The cell combinations represented in Figure 31A and B are the expected normal and TBH in vivo combinations, respectively. The addition of indomethacin increased alloreactivity significantly (81% to 151%) in the normal host (Figure 31A). When indomethacin was added to MLR cultures containing normal host Mφ and CD8⁺ T cells, the different cultures increased to the same value; that is, there was no significant difference between reactivity before or after indomethacin treatment. And when indomethacin was added to MLR cultures containing TBH Mφ and CD8⁺ T cells (Figure 31B), alloreactivity was also significantly increased (96% to 118%). However, only with a low dose of TBH Mφ (plus a low or high dose of TBH CD8⁺ T cells) was alloreactivity equal to or greater than the control (the MLR cultures with no Mφ or CD8⁺ T cells added). The addition of indomethacin to the combination of high numbers of TBH Mφ and low numbers of TBH CD8⁺ T cells increased alloreactivity; however, T-cell proliferation was still only 84% of the MLR culture without added Mφ or CD8⁺ T cells (53,684 cpm compared to 63,741 cpm). With a high dose of Mφ and CD8⁺ T cells, indomethacin treatment increased T-cell reactivity to only 57% of the control (36,315 cpm).
Figure 30. Mφ-Mediated Suppression of Alloreactivity is PG-Mediated: Indomethacin (10⁻⁷ M) was added to cultures containing varying doses of normal and TBH unseparated and Ia⁻ Mφ. The X-axis represents ³H-TdR incorporation by proliferating L3T4⁺ T cells, and the Y-axis represents the treatment groups.
Figure 31. PG Affects MΦ-CD8⁺ T-Cell Interactions: Indomethacin (10⁻⁷ M) was added to cultures containing various doses of normal host MΦ and CD8⁺ T cells (A) and various doses of TBH MΦ and CD8⁺ T cells (B). The X-axis represents ³H-TdR incorporation by proliferating LYT4⁺ T cells, and the Y-axis represents the treatment groups.
compared to 63,741 cpm). The unseparated and Ia− Mϕ populations both seemed to produce equal amounts of PG because no significant differences were seen (data not shown). Indomethacin was also added to cultures with CD8+ T cells alone, and there were no significant differences in T cell reactivity before or after indomethacin addition (data not shown). The data suggest that changes in secretion of monokines such as PG during tumor growth may affect the Mϕ-CD8+ T cell interplay and lead to reduced CD4+ T cell responsiveness.
DISCUSSION

In hosts with diseases such as cancer the role of suppressor cells, or at least the changes in their role, is not clear. In this work our goal was to continue our investigation of tumor-induced immunosuppression by exploring the tumor-induced phenotypic and function shifts in Ia\(^-\) M\(\phi\)- and CD8\(^+\) T cells. We first investigated the effect of tumor growth on M\(\phi\)-mediated suppression -- results suggest that Ia\(^-\) M\(\phi\) are suppressor M\(\phi\). Ia\(^-\) M\(\phi\) were more suppressive than unseparated M\(\phi\) populations and, more important, TBH Ia\(^-\) M\(\phi\) were significantly more suppressive than normal host Ia\(^-\) M\(\phi\). The work of Chow and Battisto (34) showing that normal host Ia\(^-\) M\(\phi\) can act as the final suppressor cell in suppression of T\(_c\) cell reactivity supports our work. Others have reported that Ia\(^-\) M\(\phi\) may be responsible for inducing T\(_s\) cells (135); while others reported that Ia\(^+\) M\(\phi\) were responsible for inducing T\(_s\) cells (164). The reasons for these differences were reported to be due to differences in experimental conditions (164). Nevertheless, our results clearly showed that Ia\(^-\) M\(\phi\) suppressed CD4\(^+\) T cells. We showed an increase in TBH Ia\(^-\) M\(\phi\) and a decrease in Ia expression per M\(\phi\) (CHAPTER I,CHAPTER II). The increased percentage of TBH Ia\(^-\) M\(\phi\) coupled with 3-5 times more M\(\phi\) in the spleen of the TBH (51) would mean a 2-3 fold increase in in vivo TBH Ia\(^-\) M\(\phi\). These data strongly suggest that Ia\(^-\) M\(\phi\) are suppressor M\(\phi\) and that tumor growth leads to a shift favoring these suppressor M\(\phi\). Ia\(^+\) M\(\phi\) may be "helper" M\(\phi\) but, because the unseparated M\(\phi\) populations were contaminated with a significant number of Ia\(^-\) M\(\phi\), Ia\(^+\) "helper" M\(\phi\) function may be masked by Ia\(^-\) suppressor M\(\phi\), except at low-doses. Uncovering the function of Ia\(^+\) M\(\phi\) is important and is currently under study. Why would Ia\(^-\) M\(\phi\) be more suppressive? Because Ia expression is needed for antigen presentation (241,242), a decrease or absence of Ia expression on M\(\phi\) would make them ineffective APC for antigen-specific T cells, leading to lower immune responsiveness. But in our experimental system, M\(\phi\) were not acting as APC; they served as accessory cells by their ability to secrete soluble factors. Thus Ia\(^-\) M\(\phi\) must suppress T-cell reactivity through some soluble mediator. We are currently examining this hypothesis.
Tumor- or Mφ-produced CSF, PGE$_2$, and/or TGF-β, which can down regulate or inhibit Ia expression or induction, could be responsible for the shift toward TBH Ia$^-$ Mφ (41,207,262). Additionally, PGE$_2$ and TGF-β are potent inhibitors of T-cell proliferation (75,107,163). Therefore, tumor survival would be increased because the tumor could suppress T-cell proliferation directly and could induce Ia$^-$ suppressor Mφ through CSF, PGE$_2$, and TGF-β production. In addition, TBH suppressor Mφ in an autocrine manner (through their own production of CSF or PGE$_2$) could maintain their Ia$^-$ state, recruit additional Ia$^-$ suppressor Mφ, and suppress T-cell reactivity.

In addition to TBH Mφ, there is also a second tumor-induced “arm” of suppression. TBH T cells can also act as suppressor cells (35,51). T$_S$ cells are generally considered to be CD8$^+$ T cells. T$_C$ cells are also CD8$^+$ T cells and have been shown to produce IFN-γ, which suppresses B-cell responsiveness, antibody production, T$_H$2 cell-induced B cell help, T$_H$2 cell proliferation, and cytokine responsiveness (69,70). Whether T$_C$ and T$_S$ are different cells is a nagging question that is beyond the scope of this paper. Nevertheless, CD8$^+$ T$_C$ and T$_S$ cells can mediate suppression of alloreactivity (69,70,114). We showed that there was an increase in the percentage of CD8$^+$ T cells per spleen. Because of the 2-4 fold increase in TBH splenic T cells, there was a concomitant increase in TBH CD8$^+$ T cell numbers, leading to an increase in the CD8$^+$/CD4$^+$ T cell ratio. A change in the CD8$^+$/CD4$^+$ T cell ratio is also seen in patients with AIDS. This change in the CD8$^+$/CD4$^+$ T cell ratio could contribute to the immunosuppression seen in TBH, as it is reported to be in AIDS. However, because TBH CD4$^+$ T cells are less responsive to IL-1, IL-2, and IL-4 than are normal host CD4$^+$ T cells (CHAPTER IV), and TBH CD8$^+$ T cells are more suppressive than normal host CD8$^+$ T cells, something more than a simple quantitative shift in T cell numbers must be responsible for the immunosuppression. The data show a significant increase in TBH CD8$^+$ T cell suppressor ability. What caused this additional suppressive ability is unclear. Perhaps, like Mφ, TBH CD8$^+$ T cells change their production of lymphokines such as IFN-γ or GM-CSF. IFN-γ can suppress B-cell and T-cell responses (69,70) and GM-CSF can help T cells proliferate, induce Ia expression, and activate Mφ (58,123,262); thus, changes in the production of these lymphokines by TBH CD8$^+$ T cells would lead to decreased alloreactivity.
Taken together, the data suggested to us that tumor growth had two independent mechanisms (Ia - Mφ- and CD8 + T cell-mediated) for suppressing CD4 + T-cell proliferation. In vivo these cells do not work independently of one another, so the question that remained: Do these two cells interact and, if so, could a change in their interaction be responsible for the increased suppression of CD4 + T cells? Earlier evidence suggested that Mφ induced Lyt-2 + T cells (136) or T s cells (135,164). Therefore, we added various doses of Mφ and CD8 + T cells to MLR cultures. With low doses of any type of Mφ, CD8 + T cell-mediated suppression was lessened or removed; at high doses of Mφ only normal-host, unseparated Mφ removed CD8 + T cell suppression. These differences in Mφ-CD8 + T-cell interactions may be due to the ratio or concentration dependence of the cellular interactions. At low doses of Mφ, the secreted monokines may preferentially activate CD8 + T cells to act as T c cells and thus respond to the foreign allogeneic cells by producing IL-2, IFN-γ, or GM-CSF. At a high dose, on the other hand, Mφ may produce "too much of a good thing", or a suppressive factor such as PGE 2, which can suppress T-cell proliferation. Low-dose Mφ enhancement and high-dose Mφ suppression of T-cell reactivity were seen earlier (37). Only with a high dose of TBH CD8 + T cells plus Mφ was suppression of CD4 + alloreactive T cells greater than with Mφ or CD8 + T cells alone. In all other cases, Mφ-mediated suppression dominated, suggesting that Mφ-mediated nonspecific T-cell control may be a more important regulatory pathway than CD8 + T cell-mediated nonspecific control. Because high numbers of TBH Mφ and CD8 + T cells together in vitro have an additive effect on suppression, the observed in vivo increase in splenic TBH Mφ and CD8 + T cells could point to the cause of the tumor-induced immunosuppression.

Mφ-produced PG is one chemical messenger that down-regulates T-cell reactivity. PG can down-regulate IL-1 and IL-2 production (122,247,270), Ia (207) and IL-2 receptor expression (247), and IL-1, IL-2, and IL-4 responsiveness (247,CHAPTER IV). The addition of indomethacin to cultures with Mφ significantly increased T-cell reactivity. No differences were seen in PG production between unseparated and Ia - Mφ, probably because there was a significant number of Ia - Mφ in the whole population (especially in the TBH). TBH Mφ show increased PGE 2 production (74,76,137,138); however, the addition of indomethacin to cultures with TBH Mφ did not increase
CD4+ T-cell responsiveness to normal host levels, suggesting that other suppressor pathways may play a role (10,34,138). Indomethacin treatment was also used to assess if changes in Mφ and CD8+ T-cell interactions were PG-regulated. Indomethacin-restored MLR cultures with normal host Mφ and CD8+ T cells were enhanced. All these restored populations increased to the same cpm (no significant differences), suggesting that PG may be one of the messengers in the normal host Mφ-CD8+ T-cell interaction. In the TBH, however, the results suggested that other pathways besides PG were present and were responsible for the suppression of reactivity to foreign antigens.

In conclusion, we showed that TBH Mφ and CD8+ T cells are significantly more suppressive than their normal host counterparts. This fact, coupled with increased numbers in vivo, suggests a potential cause of suppression of CD4+ T cells to foreign MHC antigens. In addition to the increase in suppression of CD4+ T cells by Ia− Mφ and CD8+ T cells, we also showed that CD4+ T cells are less responsive to IL-1, IL-2, and IL-4 (CHAPTER IV). Taken together, these results suggest that one of the sites of tumor-induced dysfunction of CD4+ T cells is at the level of Ia− Mφ and CD8+ T cells. For an effective immune response to be generated against foreign allo-antigens -- or, more important, in the case of cancer to tumor allo-antigens -- immune cells must first recognize their self-antigens. Thus, the Mφ-T cell interactions that occur between self cells are the basis for all immune responses (101). Therefore, we undertook, in a companion study (CHAPTER VII), the correlation between the tumor-induced shift from Ia+ to Ia− Mφ and the increase in Ia− Mφ and CD8+ T cell suppressor function in suppression of CD4+ autoreactive T<sub>H</sub> cells. In a broader context, the tumor controls its own survival by altering the intracellular communication between Mφ and T cells, which leads to a defective immune response.
CHAPTER VII

TUMOR-INDUCED SUPPRESSION OF CD4+ AUTOREACTIVE HELPER T CELLS BY Ia- MACROPHAGES AND CD8+ T CELLS

INTRODUCTION

Mφ are accessory cells that display Ia markers and release soluble modulators that either up- or down-regulate T cells. In the SMLR, an in vitro measure of autoreactivity, Mφ are the chief stimulatory/regulatory cells because of their Ia expression and release of soluble modulators (14,36,157,168). Autoreactive T cells do not respond to foreign antigen such as FCS in the culture medium (157), but rather to self Ia molecules (14,36,157,168). This reaction to self is important because before the host can respond to nonself it must recognize self (101). Mφ Ia presentation to autoreactive T cells is the first signal in autoreactive T cell regulation (168), since blocking anti-
bodies prevent T-cell proliferation (157). Autoreactive T cells are L3T4+, Thy-1+, Ia-, and Lyt-2- (14,224). Autoreactive T cells perform many important regulatory functions (14,36,117,118,157,168,235,238), stimulate naive L3T4+ T cells, and participate in a T-T cell network (111,159). These functions suggest that autoreactive T cells are vital to immune regulation and "normal" immune responsiveness.

Autoreactivity has been extensively studied in diseased hosts and shown to be defective in these hosts (56,88,115). Our recent work supports this conclusion (CHAPTER III,CHAPTER IV). We showed that TBH Mφ suppress autoreactive T cell responsiveness and that this suppression is partly due to decreased Ia expression and increased PG-secretion by TBH Mφ (CHAPTER III). In addition, we demonstrated that TBH autoreactive T cells were less responsive to Mφ stimulation, and to IL-1, IL-2, and IL-4 responsiveness (CHAPTER IV). We conclude that tumor growth affects both Mφ accessory cell function and autoreactive T\(_\alpha\) cell reactivity.

Recently, we discovered that a tumor-induced shift in TBH Ia- suppressor Mφ and CD8+ T cells was responsible for the suppression of CD4+ T cell reactivity to foreign class II MHC antigens (CHAPTER VI). These data caused us to start a study on the mechanisms of tumor-mediated suppression of autoreactivity by investigating tumor-induced changes in Ia- Mφ and CD8+ T cells. Our goals here were to examine: (i) the changes in Ia- Mφ-mediated function and their relationship to decreased autoreactivity; (ii) the changes in CD8+ T cell-mediated function and their relationship to decreased autoreactivity; and (iii) the mechanisms of Mφ- or CD8+ T cell-mediated activity. In this report, we demonstrate that tumor growth leads to changes in Ia- Mφ and CD8+ T cell function and interactions which affect autoreactive T\(_\mu\) cells.
MATERIALS AND METHODS

Animals

Eight to 12 week-old male BALB/c mice (Dominion Labs) were used. Mice were LDH virus negative (Microbiological Associates). A single-cell suspension of $4 \times 10^5$ cells from a methylcholanthrene-induced nonmetastatic transplantable fibrosarcoma was injected i.m. into the left hind leg of the TBH 3 weeks before use. Palpable tumors form by days 10-14 and death occurs by days 28-35 (51). Immunosuppression was not caused by a general inflammatory response, since normal BALB/c mice injected with C3H muscle tissue 3 weeks before showed no significant differences in their autoreactive response compared with normal untreated mice (data not shown; CHAPTER I,CHAPTER III).

Medium

All cells were grown in RPMI-1640 (Hazelton) medium with 10% heat-inactivated FCS (Gibco) and $4 \times 10^{-5} M$ 2-ME (Sigma). All media contained 50 mg/l gentamicin (Gibco), 2 g/l NaHCO$_3$, and 25 mM HEPES (Research Organics).

$M\phi$ and CD8$^+$ T Cell Preparation

Briefly, BALB/c splenic $M\phi$ (>96% esterase positive) were collected by washing the plated WSC to remove nonadherent cells and scraping with a rubber policeman to remove the adherent
cells. For Ia" Mφ preparation, collected Mφ were treated with anti-IAα (MK-D6) plus C (Cedarlane Laboratories Ltd.). This procedure enriched for Ia" Mφ (>97%) as determined by FACS analysis. Mφ (unseparated or Ia"") were counted and resuspended to 4 x 10⁶ cells/ml in medium containing 10% FCS. Mφ were X-irradiated with 2000 rads (TFI Minishot II X-irradiator). CD8" T cells were collected by anti-L3T4 (Gk1.5) plus C. After anti-L3T4 mAb plus C treatment, the cells were resuspended to 4 x 10⁶ cells/ml in medium containing 10% FCS. CD8" T cells were X-irradiated with 2000 rads (TFI Minishot II).

**SMLR**

BALB/c WSC suspensions were plated for 2 hr (Lux/Miles). T cells were collected by incubating the plastic nonadherent WSC on a nylon wool column. Nylon wool nonadherent cells were eluted from the column and further depleted of Ia" B cells, Mφ, and other contaminating cells by being resuspended in supernatants containing mAb anti-Lyt-2 (3.155), anti-IAα (MK-D6), anti-immature T cell and B cell (J11d), and anti-IEα (14-4-4). The cells were incubated in a 1:12 dilution of C and washed. The L3T4" T cells (<1% contaminating Mφ and B cells) were resuspended to 4 x 10⁶ cells/ml in medium with 10% FCS, and 4 x 10⁵ cells were added per well of a "U" bottom 96-well plate (Flow Laboratories). Four x 10⁵ unseparated Mφ stimulator cells were also added per well. Four x 10⁵ cells/well of Mφ and L3T4" T cells make up the "base" SMLR. Four x 10⁴ (10%), 2 x 10⁵ (50%), and 4 x 10⁵ (100%) Mφ or CD8" T cells/well were added to the SMLR. The percentage of additional Mφ or CD8" T cells added to some SMLR represents the number of those cells compared to the number of normal or TBH Mφ stimulator cells added. Indomethacin was added to the SMLR cultures at a final concentration of 1 x 10⁻⁷ M. This concentration is an effective inhibitor of the arachidonic acid pathway for PG synthesis and is nontoxic to immune cells (137). Plates were incubated for 4 days at 37°C. Six hr before termination and harvest of the cells, each reaction mixture was pulsed with ³H-TdR (Dupont NEN
Research Products). Cells were harvested onto filters (Whatman 934-AH) and counted in a 6895 Betatrac liquid scintillation counter (Tri Analytic).

**Statistics/Calculation of Results**

Pooled cells from 3 to 6 mice were used for each experiment. For the SMLR functional studies, four replicate wells were run for each test, and each experiment was repeated 3 times. All numbers in tables and data points on graphs were tested for significance by Student's t test (p <0.05).
RESULTS

Unseparated and Ia\(^-\) M\(\phi\) Accessory Function

The response of autoreactive T cells to unseparated and Ia\(^-\) M\(\phi\) was measured by adding normal and TBH unseparated and Ia\(^-\) M\(\phi\) to L3T4\(^+\) autoreactive T cells (Figure 32). Ia\(^-\) M\(\phi\) were collected by depleting the Ia\(^+\) M\(\phi\) with anti-IA\(d\) plus C. Unseparated TBH M\(\phi\)-stimulated autoreactivity was significantly less (only 43% as reactive) than unseparated normal host M\(\phi\)-stimulated autoreactivity (Figure 32). When normal and TBH Ia\(^-\) M\(\phi\) were added, autoreactivity was 3% and 4%, respectively, of unseparated M\(\phi\)-stimulated autoreactivity. The data demonstrate that the Ia\(^-\) M\(\phi\) population was depleted of Ia\(^+\) M\(\phi\) because without Ia expression, a necessity for autoreactive T-cell reactivity (168), autoreactive T cells can not proliferate. The purity of the Ia\(^-\) M\(\phi\) population was confirmed by FACS analysis showing >96% Ia\(^-\) M\(\phi\) (data not shown).

Normal and TBH Ia\(^-\) M\(\phi\) Suppress CD4\(^+\) Autoreactive T Cells

To investigate the role of Ia\(^-\) M\(\phi\) in autoreactivity, additional doses of normal and TBH Ia\(^-\) M\(\phi\) were added to normal host M\(\phi\)-stimulated SMLR cultures. These additional M\(\phi\) were added to a “base” number of CD4\(^+\) autoreactive T cells and M\(\phi\). This “base” number of M\(\phi\) needs to be added to the CD4\(^+\) T cells because without Ia\(^+\) M\(\phi\) there will be no autoreactive T cell responsiveness (14,36,157,168). Normal or TBH unseparated M\(\phi\) addition to SMLR cultures served as the appropriate crowding controls. Various doses of M\(\phi\) were used because previous results showed that M\(\phi\) activity was dose-dependent (37). Percentage of M\(\phi\) added was equal to the number of additional normal or TBH unseparated or Ia\(^-\) M\(\phi\) above the base of 4 x 10\(^5\).
Figure 32. Tumor Growth Affects Mφ Accessory Function, Leading to Suppression of Autoresitivity: Normal and TBH unseparated and la− Mφ were added to SMLR cultures. The X-axis shows the different Mφ added, and the Y-axis shows the 3H-TdR incorporation by proliferating autoreactive T cells.
Mφ/well. Ia⁻ Mφ were collected by depleting the Ia⁺ Mφ with anti-Ia⁺ plus C. The addition of normal and TBH Ia⁻ Mφ to normal host Mφ-stimulated SMLR cultures significantly suppressed T-cell responsiveness in a dose-dependent manner (Figure 33A). TBH Ia⁻ Mφ were, however, significantly more suppressive than their normal host counterparts. With a low dose (10%) of Mφ, normal and TBH Ia⁻ Mφ decreased autoreactivity 4% and 52%, respectively, compared to 0% and 25%, respectively, when unseparated normal and TBH Mφ (control) were added. Similar patterns of suppression occurred when medium (50%) and high (100%) dose of normal or TBH Ia⁻ or unseparated Mφ were used. By a high dose of Mφ, normal and TBH Ia⁻ Mφ suppressed autoreactivity 69% and 97%, respectively, and normal and TBH unseparated Mφ suppressed autoreactivity 20% and 72%, respectively.

Do normal and TBH Ia⁻ Mφ suppress "base" TBH Mφ-stimulated SMLR reactivity? When a low dose of normal or TBH Ia⁻ Mφ was added to TBH Mφ-stimulated SMLR cultures, autoreactive T-cell responsiveness decreased 0% and 29%, respectively, while the addition of unseparated normal or TBH Mφ did not suppress T-cell reactivity (Figure 33B). A medium dose of normal or TBH Ia⁻ Mφ suppressed autoreactivity 27% and 42%, respectively; and a medium dose of unseparated normal or TBH Mφ suppressed CD4⁺ T⁺ cell responsiveness 0% and 15%. Similar trends of suppression continued when high doses of Mφ were used. The data were consistent with other studies, suggesting that Ia⁻ Mφ are suppressor Mφ (34,CHAPTER VI) and that tumor growth significantly increases their activity (CHAPTER VI). Suppression of autoreactive T cells by the unseparated Mφ populations was dose-dependent, was consistent with previous data (37), and was probably due to Ia⁻ Mφ.

**CD8⁺ (Lyt-2⁺) T Cell-Mediated Suppression of Autoreactive T Cells**

Since Mφ are not the only source of suppression during tumor growth (35,51,CHAPTER VI), we explored the role of CD8⁺ T cells in tumor-induced suppression of autoreactivity. We added
Figure 33. Ia⁻ Mφ Suppress Autoreactive T-Cell Responsiveness: Different concentrations of normal and TBH unseparated and Ia⁻ Mφ were added to the SMLR. Figure 33A is the normal host Mφ-stimulated SMLR, and Figure 33B is the TBH Mφ-stimulated SMLR. The X-axis shows the percentage of additional Mφ added, and the Y-axis shows the ³H-TdR incorporation by proliferating CD4⁺ autoreactive T cells.
different doses of CD8\(^+\) T cells into normal host M\(\phi\)-stimulated SMLR cultures. CD8\(^+\) T cells were significantly more suppressive than normal host CD8\(^+\) T cells at all doses. A low dose (10\%) of normal host CD8\(^+\) T cells enhanced autoreactive T-cell responsiveness 8\%; a low dose of TBH CD8\(^+\) T cells suppressed reactivity 41\%. When a medium dose (50\%) of normal or TBH CD8\(^+\) T cells was added to SMLR cultures, autoreactive T-cell responsiveness decreased 30\% and 56\%, respectively. With a high dose (100\%) of normal or TBH CD8\(^+\) T cells, responsiveness was suppressed 52\% and 74\%, respectively. Figure 34A shows that TBH

When normal or TBH CD8\(^+\) T cells were added to TBH-stimulated SMLR cultures (Figure 34B), the results were similar to the results for the addition of normal and CD8\(^+\) T cells to normal host M\(\phi\)-stimulated autoreactivity. TBH CD8\(^+\) T cells are significantly more suppressive than normal host CD8\(^+\) T cells at all doses; that is, moving from a low, medium, and high dose of normal or TBH CD8\(^+\) T cells, autoreactivity progressively decreased. The data showed a shift in the suppressor function of CD8\(^+\) T cells during tumor growth. The expected in vivo combination of TBH M\(\phi\) and TBH CD8\(^+\) T cells was significantly more suppressive than the expected in vivo normal host combination.

**PG-Mediated Suppression of Autoreactive T Cells**

Because M\(\phi\) release PG that suppresses T-cell proliferation and tumor growth increases PG secretion (45,76,138,163), we examined whether normal or TBH Ia\(^-\) M\(\phi\)-mediated suppression was PG-mediated. Indomethacin at a final concentration of 1 \(\times 10^{-7}\) \(M\) was used. Indomethacin treatment of normal or TBH M\(\phi\)-stimulated SMLR cultures containing additional normal or TBH unseparated or Ia\(^-\) M\(\phi\), increased normal host M\(\phi\)-stimulated autoreactivity (Figure 35A) and TBH M\(\phi\)-stimulated autoreactivity (Figure 35B) by 20-60\%. Indomethacin-restored SMLR cultures co-cultured with normal or TBH Ia\(^-\) M\(\phi\) were significantly less reactive than SMLR cultures containing additional normal or TBH unseparated M\(\phi\) populations. Furthermore, autoreactive
Figure 34. CD8+ T Cells Suppress CD4+ Autoreactive T-Cell Proliferation: Various concentrations of normal and TBH CD4+ or CD8+ T cells were added to SMLR cultures. Figure 34A is the normal host Mφ-stimulated SMLR, and Figure 34B is the TBH Mφ-stimulated SMLR. The X-axis represents the percentage of CD8+ T cells added, and the Y-axis represents 3H-TdR incorporation by proliferating CD4+ T cells.
T-cell proliferation after indomethacin treatment of SMLR cultures containing TBH Ia- Mφ was significantly less than such proliferation after indomethacin treatment of SMLR cultures containing normal host Ia- Mφ.

In addition to suppressing T-cell proliferation directly, PG could also affect CD8+ T-cell function. Therefore, we wanted to explore the possibility that changes in Mφ PG production were related to changes in Mφ-CD8+ T cell interactions. Indomethacin was added to SMLR cultures when both Mφ and CD8+ T cells were present. The addition of indomethacin to normal host Mφ-stimulated SMLR cultures containing varying doses of normal or TBH CD8+ T cells (Figure 36A), significantly increased reactivity. The addition of varying doses of CD4+ T cells served as the crowding control. The decrease in autoreactivity, as CD4+ T cell numbers were increased, is consistent with previous data showing that optimal autoreactive T-cell proliferation is dose-dependent (CHAPTER III). Irrespective of cell dose, cultures containing TBH CD8+ T cells were less reactive than cultures with normal host CD8+ T cells. The addition of TBH CD8+ T cells and indomethacin to SMLR cultures showed that TBH CD8+ T cells were 2-3 times more suppressive than their normal host counterparts. Because indomethacin-restored cultures did not return to control levels other suppressor factors besides Mφ produced-PG must be regulating CD8+ T cell function. Indomethacin-restored TBH Mφ-stimulated cultures containing normal or TBH CD8+ T cells also showed increased CD4+ T-cell reactivity (Figure 36B). However, the presence of CD8+ T cells in TBH Mφ-stimulated SMLR cultures caused significantly less reactivity than when normal host CD8+ T cells were present. The expected TBH in vivo combination of TBH CD8+ T cells and TBH Mφ was the most suppressive to autoreactive T₈ cell proliferation and was the least responsive to indomethacin treatment. These results suggest that TBH Mφ and TBH CD8+ T cells have an altered function because there are changes in their cytokine production and responsiveness.
Figure 35. Addition of Indomethacin Restores Autoreactive T-Cell Proliferation: Various concentrations of normal and TBH unseparated and Ia- Mφ were added along with indomethacin (1 x 10^{-7} mM) to SMLR cultures. Figure 35A is the normal host Mφ-stimulated SMLR, and Figure 35B is the TBH Mφ-stimulated SMLR. The X-axis represents the percentage of additional Mφ added, and the Y-axis represents 3H-TdR incorporation by proliferating CD^+ autoreactive T cells.
Figure 36. Indomethacin Restores Autoreactive T-Cell Responsiveness in Cultures with Normal or TBH Mφ and CD8+ T Cells: Various concentrations of normal and TBH CD4+ or CD8+ T cells were added along with indomethacin to SMLR cultures. Figure 36A is the normal host Mφ-stimulated SMLR, and Figure 36B is the TBH Mφ-stimulated SMLR. The X-axis shows the percentage of CD8+ T cells added to cultures, and the Y-axis shows 3H-TdR incorporation by responding CD4+ autoreactive T cells.
DISCUSSION

The SMLR is an \textit{in vitro} measure of autoreactivity, a vital reaction between self cells that must occur before the host can recognize foreign antigens (101). Autoreactive T cells respond to the presentation of Ia and the release of soluble modulators by Mφ. Ia\(^{+}\) Mφ provide the first signals that drive autoreactive T-cell proliferation and maturation (168). Autoreactive T cells do not respond to foreign antigen such as the FCS in medium (157). Autoreactive T cells are necessary to immune regulation because they serve a variety of roles in the immune system such as help, amplification, suppression, and cytotoxicity (111,117,118,159,224,235). In addition, autoreactive T cells are important in anti-tumor immunity (238). Because autoreactive T cells are necessary for an immune response, and defective autoreactivity occurs in many diseased states (56,88,115), we investigated how tumor growth affects autoreactivity.

In this work, our goal was to examine the role TBH Ia\(^{-}\) Mφ and CD8\(^{+}\) T cells play in suppressing autoreactive T\(_{H}\) cells. Chow and Battisto (34) have shown that, in the normal host, Ia\(^{-}\) Mφ can act as the final suppressor cell in suppression of T\(_{C}\) cell reactivity. Our previously reported studies support their findings. We described a mechanism for tumor-induced suppression of CD4\(^{+}\) T cells to foreign class II MHC antigens (CHAPTER VI). We have also shown a quantitative and qualitative shift in TBH Ia\(^{-}\) Mφ (CHAPTER VI); that is, there is a 2-3 fold increase in \textit{in vivo} TBH Ia\(^{-}\) Mφ numbers and an increase in TBH Ia\(^{-}\) Mφ-mediated suppression of alloreactivity. In this current study, we demonstrated that Ia\(^{-}\) Mφ significantly suppressed autoreactive T-cell responsiveness and that tumor growth led to a significant shift in suppressor Mφ function. Coupled with our previous results (CHAPTER VI), these new findings strongly suggest that Ia\(^{-}\) Mφ may be the first "prong" of tumor-induced immunosuppression.

Why would Ia\(^{-}\) Mφ suppress autoreactive T cells? Ia expression is vital for induction of autoreactive T cells (14,36,157,168); therefore, decreases in or absence of Ia antigen on TBH Mφ would make them unable to act as accessory cells for autoreactive T cells, leading to suppression of autoreactivity. The quantitative loss of Ia markers may be due to PGE\(_{2}\), TGF-β, and/or CSF.
These cytokines can down-regulate Ia expression and are produced in excess in the TBH (41,207,262). In our experimental system, however, Ia- Mφ were not acting alone as accessory cells, because they were being titrated into cultures containing Ia+ Mφ. If a defect in Ia expression was the only difference between Ia+ and Ia- Mφ, then autoreactivity would not be suppressed by Ia- Mφ because the Ia+ Mφ would be able to express and present Ia to autoreactive T cells. The addition of even the lowest dose of Ia- Mφ was capable of suppressing autoreactivity, suggesting that suppression must be mediated by some soluble suppressor factors. But why is a decrease in Ia expression connected to a change in monokine secretion or suppressor function? Perhaps, as with most cellular responses, there are multiple effects and, in this case, the changes in a yet unknown mechanism include down-regulation of Ia expression and increase in suppressor function.

What is the role of Ia+ Mφ in this model? They are helper Mφ but, because the unseparated Mφ population is contaminated with a significant number of Ia- Mφ, Ia+ helper Mφ function can get masked by the overriding suppression mediated by Ia- suppressor Mφ. The question, however, is not whether Ia+ Mφ are helper Mφ, which indeed they are for autoreactive T cells (168), but rather, Does tumor growth also change TBH Ia+ Mφ function? These questions are currently under study using FACS sorted Ia+ Mφ.

The second "prong" of the tumor-induced suppression of autoreactivity is mediated by CD8+ T cells. In this study, we showed that TBH CD8+ T cells were significantly more suppressive than normal host CD8+ T cells. Our finding here that CD8+ T cells mediate suppression of autoreactive T_{H} cells is consistent with early work showing that T_{S} cells may be responsible for decreased mitogen responsiveness, alloreactivity, and autoreactivity (51,159) during tumor growth. What causes this increased T cell-mediated suppression? It is not due to increased CD8+ T cell numbers, as we showed previously (CHAPTER VI). Although a change in the CD8+/CD4+ T cell ratio probably does play a role in in vivo immunosuppression much as it does in AIDS patients, tumor-induced qualitative changes in T cells also are present. These qualitative changes are evident when equal numbers of normal or TBH CD8+ T cells are added to SMLR cultures; TBH CD8+ T cells were significantly more suppressive than normal host CD8+ T cells. But what mechanisms are responsible for this increased suppression by TBH CD8+ T cells? Perhaps, like Mφ, TBH CD8+
T cells have an altered production of lymphokines such as IFN-γ or GM-CSF. IFN-γ can suppress B-cell and T-cell responses (69,70), and GM-CSF helps in T-cell proliferation and Mφ Ia induction and activation (58,123,262). Changes in the production of these lymphokines by TBH CD8+ T cells would lead to decreased autoreactivity.

The production of PG by Mφ is one way Mφ can control T-cell reactivity. PG can down-regulate IL-1, IL-2, and IL-4 responsiveness (247,270,CHAPTER IV), IL-2 receptor (247) and Ia expression (207), and IL-1 production (122). Indomethacin treatment significantly enhances all groups of cell combinations; however, indomethacin addition to cultures with TBH Mφ did not increase autoreactivity to normal host levels, probably because TBH Mφ secrete other suppressor factors in addition to PG (10,34,138). Previous evidence hinted that Mφ may regulate CD8+ T cells (136). Is this regulation PG-mediated? The addition of indomethacin to SMLR containing CD8+ T cells was used to measure any potential Mφ-CD8+ T cell interactions that were PG regulated. The data showed that PG play a role in both normal or TBH Mφ and normal or TBH CD8+ T cell interplay. However, because cultures with normal or TBH CD8+ T cells added do not increase to the same level as cultures containing additional CD4+ T cells (control), Mφ-produced PG may not play as great a role in Mφ-CD8+ T cell interactions as they do in Mφ CD4+ T cell interactions. The differences could also result from CD8+ T cell-produced IFN-γ, which would not be sensitive to indomethacin. The combination of TBH CD8+ T cells and TBH Mφ (expected TBH in vivo combination) was the most suppressive to autoreactive T, cell proliferation and the least responsive to indomethacin treatment, suggesting that tumor growth can change TBH Mφ and T cell secretory patterns.

Tumor growth thus leads to changes in the function of TBH Mφ and TBH T cells. TBH Ia- Mφ and TBH CD8+ T cells were significantly more suppressive than their normal host counterparts and this fact, coupled with increased numbers in vivo, suggests a potential cause of suppression of autoreactivity. In this report, however, we did not investigate changes in the autoreactive T cells themselves. Previous data show that autoreactive T cells are less responsive to IL (CHAPTER IV) and may also be responsible for suppression of immune responsiveness against tumors (118). In summary, our present findings suggest that tumor growth leads to at least two distinct but not
mutually exclusive mechanisms of "attack" of autoreactive T-cells. TBH Ia- Mφ and CD8+ T cells can suppress autoreactivity independently of each other; however, when they are both present, such as in vivo, suppression would be additive. The cause of the tumor-induced shift towards suppressor Mφ and T cells needs to be understood before we can begin to control tumor growth. Questions remain. What are the molecular mechanisms controlling the changes in these immune cells? Can the changes be reversed or prevented? We are currently doing experiments at the molecular level to try to answer these questions.
SUMMARY

Results presented in the preceding chapters suggest that a tumor-induced shift in Mφ sub-populations is responsible for the immunosuppression seen in TBH. The data showed that there is a change in Mφ phenotype during tumor growth, a change in Mφ function during tumor growth, and a correlation between the phenotypic changes and the functional changes. We also identified a suppressor Mφ population in normal hosts that was altered during tumor growth. This work, in conjunction with previous studies (72-74,137), suggests a mechanism for tumor-induced immunosuppression.

The three questions we set out to answer were:

1. Does tumor growth alter Mφ phenotypic characteristics?
2. Are there functional changes in Mφ during tumor growth that lead to their increase in suppressive function?
3. Can phenotypic changes be correlated with functional changes by identifying suppressor Mφ sub-populations that are responsible for tumor-induced immunosuppression?

To answer the first question, we charted the changes in TBH Mφ surface antigen expression. mAb to the surface antigens Mac-1, -2, -3, and la were used to delineate the shifts in Mφ sub-populations during cancer growth. Previous work showed that there was a shift in TBH Mφ sub-
populations and that these changes could be measured by examining the changes in the Mac-1, -2, -3, and Ia surface antigens (72-74,137). This early work, however, used the now dated technique of UV fluorescence microscopy which is only able to give general quantitative information about the labeled cells. Our goal was to use the flow cytometer to more directly quantify the changes in these surface antigens, and more importantly, examine Mφ antigen expression on a per cell basis. The flow cytometer allows the user to examine individual cells and thus study changes in antigen expression directly on each cell rather than on a population basis as is done with the fluorescent microscope.

By using the flow cytometer to chart the kinetics of the Mφ antigen shift during tumor growth, several exciting discoveries were made. In the normal host, the data suggested that Mφ, like lymphocytes, may be composed of distinct subpopulations (72,74,130,244,249-253), and in the TBH, the data supported the notion that there is a tumor-induced shift in Mφ subpopulations (72,74,76,177,236,258,269). The initial studies examined the one-color fluorescence analyses of the various Mφ antigens and the size versus antigen expression comparison of these antigens on Mφ. Overall, the percentage of peritoneal Mac-1+ Mφ remained constant, while the percentage of Mac-2+ and -3+ peritoneal Mφ decreased during tumor growth. The percentage of Ia+ peritoneal Mφ also decreased. These results confirmed the previous fluorescent microscopy results of Garner et al. (74), who showed a decrease in the percentage of Mac-2+ and Ia+ Mφ and no change in the percentage of Mac-1+ Mφ. They did, however, show an increase in the percentage of Mac-3+ peritoneal Mφ. The differences are probably due to the increased sensitivity of the flow cytometer compared to UV microscopy.

Three distinct-sized Mφ populations were seen in the peritoneal cavity. When examining the size versus antigen expression on these different sized-Mφ in the normal and TBH, we noticed that TBH Mφ were a more homogeneous population and that there was a shift towards a smaller cell. The percentage of large-sized Mac-1+ and -2+ Mφ and the percentage of medium-sized Mac-3+ Mφ decreased significantly. Concomitant with this decrease in the large-sized Mac-1+ Mφ was an increase in the percentage of small-sized Mφ. The decrease in large-sized Mac-1+ Mφ during tumor growth may account for the disappearance of the Mac-1+ subpopulation of Mφ responsible
for PGE₂ regulation (74,137). A loss of this PGE₂-regulatory population during tumor growth would cause an increase in PGE₂ production. A 2-3 fold increase in PGE₂ production was detected in TBH Mφ (45,138). The increase in small-sized Mφ during tumor growth was probably caused by the increase in immature Mφ. The presence of peroxidase indicates immature Mφ (141) and our previous report showed a relationship between TBH Mφ and peroxidase-positive immature Mφ (74). Ia⁺ Mφ were small-sized and this Ia⁺ population nearly disappeared during tumor growth. Others also report decreased Ia antigen expression on TBH Mφ (156), and this loss of TBH Ia⁺ Mφ may cause decreased Mφ-T cell interactions (168,240-242).

There were also tumor-induced changes in splenic Mφ Mac-1⁻, -2⁻, -3⁻, and Ia antigen expression. The percentage of TBH Mac-1⁺, -2⁺, and -3⁺ splenic Mφ increased. The increase in Mac-1⁺ Mφ in the TBH follows a shift in the phenotype of the splenic accessory Mφ from Mac-1⁻ in the normal host to Mac-1⁺ in the TBH (72). The work of Tsuchiya et al. (236) supports our phenotypic findings. The percentage of splenic Ia⁺ Mφ decreased during tumor growth and there was a decrease in the density of Ia antigen expression. As with peritoneal Mφ, decreased Ia expression on TBH Mφ plays a role in tumor-induced immunosuppression (168,240-242). This phenotypic data is supported by the previous phenotypic studies of Garner et al. (72). The association between Mφ size and marker expression was not seen with splenic Mφ. We did see a decrease in the average size of TBH splenic Mφ, which correlates with the increase in immature Mφ found in TBH spleens (51).

After examining normal and TBH peritoneal and splenic Mφ antigen expression using one-color analyses, we took advantage of the powerful properties of the flow cytometer and examined Mφ for the multiple expression of the Mac-1, -2, -3, and Ia antigens by two-color fluorescence analyses. This technique could show (i) if there are distinct subpopulations of Mφ expressing various combinations of antigens and (ii) if there is a shift in these populations during tumor growth.

Two-color analyses of normal and TBH peritoneal Mφ showed a decrease in the percentage of TBH Mac-1⁺2⁺, Mac-1⁺3⁺, and Mac-2⁺3⁺ peritoneal Mφ. The percentage of TBH Mac-2⁺Ia⁻ and Mac-3⁺Ia⁻ Mφ also decreased, yet no change was seen in the percentage of TBH
Mac-1 \textsuperscript{+} Ia\textsuperscript{-} M\phi. These results suggest that there is an overall shift towards a TBH Mac-1 \textsuperscript{+} 2\textsuperscript{-} 3\textsuperscript{-} Ia\textsuperscript{-} peritoneal M\phi. Mac-2 and -3 are late differentiation antigens (95,97,217), while Mac-1 is an early differentiation antigen (96,208,217); thus, Mac-1 \textsuperscript{+} 2\textsuperscript{-} 3\textsuperscript{-} TBH M\phi would be expected to be immature M\phi. Because immature M\phi probably exhibit decreased accessory function, an increase in immature TBH Mac-1 \textsuperscript{+} 2\textsuperscript{-} 3\textsuperscript{-} Ia\textsuperscript{-} M\phi could lead to decreased M\phi-mediated immune functions.

The data collected from the two-color analyses were consistent with the one-color data presented above and, in combination, the two studies helped to explain the previously reported functional studies (72-74,137). It was reported that a Mac-1 \textsuperscript{+} M\phi, responsible for down-regulating PGE\textsubscript{2} production, was absent in the TBH (74,137) and that there was a shift from a Mac-3\textsuperscript{+} suppressor M\phi to a Mac-2\textsuperscript{+} suppressor M\phi during tumor growth (74). The decreased percentage of Mac-1 \textsuperscript{+} 2\textsuperscript{+}, Mac-2\textsuperscript{-} 3\textsuperscript{+}, and Mac-1 \textsuperscript{+} 3\textsuperscript{+} M\phi could account for the disappearance of the Mac-1 \textsuperscript{+} M\phi-mediated PGE\textsubscript{2} control and Mac-3\textsuperscript{+} M\phi-mediated suppression in the normal host. Malick et al. (137) showed that TBH Mac-2\textsuperscript{+} peritoneal M\phi are the primary PGE\textsubscript{2} producers and that there is over a 2 fold increase in PGE\textsubscript{2} production. Others (73,83,85,103,104,172) also report a significant increase in PGE\textsubscript{2} production by TBH M\phi. The recent work of Askew et al. (8) using sorted populations of peritoneal M\phi confirmed these studies. This work, however, shows that there is a decrease in the percentage of Mac-2\textsuperscript{+} M\phi. This apparent contradiction can be explained by the work of Askew et al. (8). They showed that the changes in the individual TBH Mac\textsuperscript{+} sub-populations were not as great as the change in the whole population. That is, the data suggested that tumor growth altered the regulatory network of TBH suppressor M\phi. A dysfunction in TBH M\phi-mediated regulation of immune responsiveness is reported by others (23,76,103,173,176,246,258,269).

Two-color analyses of splenic M\phi showed that the percentage of Mac-1 \textsuperscript{+} 2\textsuperscript{+}, Mac-1 \textsuperscript{+} 3\textsuperscript{+}, and Mac-2\textsuperscript{-} 3\textsuperscript{+} M\phi significantly increased during tumor growth. These dual-antigen changes in TBH splenic M\phi confirm the single-antigen studies presented above. Nanno et al. (162) reported that bacteria such as \textit{Lactobacillus casei} can induce Mac-1 and -2 antigen expression and cytotoxicity in splenic M\phi. These cytotoxic M\phi, however, were Mac-3\textsuperscript{-}. The data suggest that the Mac-1 \textsuperscript{+} 2\textsuperscript{+} 3\textsuperscript{+}
Mφ reported here are suppressor Mφ and the Mac-1+2+3- Mφ reported by Nanno et al. (162) are cytotoxic Mφ. It will be necessary to investigate what causes the differential induction of cytotoxic versus suppressor Mφ. Does tumor growth selectively induce Mac-3+ suppressor Mφ and bacteria such as *Lactobacillus casei* selectively induce Mac-3- cytotoxic Mφ? If so, the data would point to unique Mφ differentiation pathways and could lead to possible sites of therapeutic intervention. Further investigation into this difference in Mac-3 expression and Mφ cytotoxic function versus suppressor function is necessary and may point to a Mφ subpopulation delineation marker. In contrast to the increase in the percentage of Mφ expressing the Mac antigens, there was a decrease in the percentage of Mφ expression the Mac+Ia+ phenotype. The shift from Ia+ Mφ in the normal host to Ia- Mφ in the TBH is important not only because Mφ-T cell interactions depend on Ia expression (168,240-242), but also because an Ia- Mφ phenotype is associated with suppressor activity (34,135,151,152). Taken together, the data point to TBH splenic Mφ which have the Mac-1+2+3+Ia- phenotype.

The phenotypic data (both one- and two-color analyses) suggest that tumor growth leads to changes in the subpopulations of Mφ in the peritoneal cavity and the spleen. The phenotypic changes in peritoneal and splenic Mφ subpopulations during tumor growth are summarized in Table 14 (one-color analyses) and Table 15 (two-color analyses). The Tables show that in the peritoneal cavity there are shifts towards TBH Mac-1+2-3-Ia- Mφ because of the decreased numbers of Mφ expressing Mac-2, -3, and Ia. In the spleen, the Tables show that there are shifts towards TBH Mac-1+2+3+Ia- Mφ because of the increased numbers of Mφ expressing Mac-1, -2, and -3 and the decreased numbers of Mφ expressing Ia. The phenotypic studies presented in this dissertation extend previous work, showing that alterations in TBH Mφ may lead to tumor-induced immunosuppression (72-74,137). The phenotypic studies were augmented by studying the functional changes that occur in Mφ during tumor growth.

The second question asked was, "Are there functional changes in Mφ during tumor growth that lead to their immunosuppressive function?" To answer this question, we investigated how tumor growth alters splenic Mφ accessory function to autoreactive CD4+ T<sub>H</sub> cells. Functional changes were measured using the accessory function of splenic Mφ. Splenic Mφ were used exclu-
Table 14. Summary of the Changes in Mφ Single-Antigen Expression During Tumor Growth

<table>
<thead>
<tr>
<th>Cell surface antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peritoneal</th>
<th>Splenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>--&lt;sup&gt;c&lt;/sup&gt;</td>
<td>↑</td>
</tr>
<tr>
<td>Mac-2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Mac-3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Ia&lt;sup&gt;+&lt;/sup&gt;</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mφ were harvested from the peritoneal cavity or spleen of normal and 21-day TBH mice.

<sup>b</sup> Cells were labeled by indirect immunofluorescence and analyzed on the flow cytometer.

<sup>c</sup> The following symbols represent: "--", no change in the percentage of Mφ expressing this antigen was seen during tumor growth; "↑", an increase in the percentage of Mφ expressing this antigen during tumor growth; and "↓", a decrease in the percentage of Mφ expressing this antigen during tumor growth.
Table 15. Summary of the Changes in Mφ Dual-Antigen Expression During Tumor Growth

<table>
<thead>
<tr>
<th>Cell surface antigen(^b)</th>
<th>Peritoneal</th>
<th>Splenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1(^+) 2(^+)</td>
<td>↓(^c)</td>
<td>↑</td>
</tr>
<tr>
<td>Mac-1(^+) 3(^+)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Mac-2(^+) 3(^+)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Mac-1(^+) 1a(^+)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Mac-2(^+) 1a(^+)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Mac-3(^+) 1a(^+)</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

\(^a\) Mφ were harvested from the peritoneal cavity or spleen of normal and 21-day TBH mice.

\(^b\) Cells were dual-labeled by indirect immunofluorescence using a FITC-labeled Ab as the label for the first antigen and R-PE as the label for the second antigen and then analyzed on the flow cytometer.

\(^c\) The symbols represent the following: "↑", an increase in the percentage of Mφ expressing this antigen during tumor growth; and "↓", a decrease in the percentage of Mφ expressing this antigen during tumor growth.
sively because peritoneal Mφ are poor accessory cells to autoreactive T cells even in the normal host. In the peritoneal cavity, there is a low percentage of Ia+ Mφ (<14% in the normal host) each with a low density of Ia antigen per cell (mean channel number 22 compared to 40 for splenic Mφ).

Mφ can act as accessory cells to autoreactive CD4+ T cells through their expression of Ia antigen and release of soluble immune modulators. Mφ provide the first two signals to autoreactive T cells -- Ia antigen and then IL-1 (14,157-159,168). The investigation of Mφ accessory function to autoreactive T cells is important because of the regulatory role autoreactive T cells play in the immune system. Autoreactive T cells maintain immune homeostasis through their help/amplification (36,54,63,111,117,157,158,187,202) and suppression (36,55,100,114,117,201) of immune reactivity. Autoreactive T cells are also important mediators of anti-tumor immunity (118,197,198,238). Because of the regulatory role autoreactive T cells play, they are a vital and necessary component of the normal immune response. Autoreactivity is changed during autoimmune diseases (115,147,185,200,266) and tumor growth (56,88). Therefore, we were interested in how tumor-induced changes in Mφ function affects autoreactivity. Mφ accessory function to autoreactive T cells provides an effective model for exploring normal and TBH Mφ-mediated function. The SMLR serves as the in vitro model for measuring autoreactivity (14,260).

Decreased Mφ accessory function to autoreactive T cells was observed during tumor growth. This decrease is supported by previous reports showing that TBH Mφ have a reduced accessory ability for mitogen-induced T-cell proliferation (236,261), alloreactivity and autoreactivity (38,45,47,56,88,137), and contact sensitivity (52,60). In examining the kinetics of tumor growth on Mφ function, we showed that the Mφ's ability to stimulate autoreactivity decreased inversely with increasing days of tumor growth. Concomitant with the decrease in accessory function, there was a decrease in TBH Mφ Ia antigen expression. Others (156) have reported a tumor-induced reduction in Ia antigen expression that correlated with decreased Mφ accessory function.

Was the decreased TBH Mφ accessory function due to decreased Ia expression and/or an active suppressive mechanism? TBH Mφ can actively suppress T-cell responsiveness by producing suppressive factors (33,76,83,85,103,104,137,231,268). To examine whether reduced TBH
M\(_\phi\)-controlled autoreactivity was due to active suppression or to reduced Ia expression, various concentrations of TBH M\(_\phi\) were admixed with normal host M\(_\phi\). The admixture of TBH M\(_\phi\) significantly suppressed normal host M\(_\phi\)-stimulated autoreactivity. This suppression by TBH M\(_\phi\) may be due to increased production of suppressor monokines (33,76,83,85,103,104,137,231,269). Only the admixture of day-21 TBH M\(_\phi\), however, suppressed normal host M\(_\phi\) accessory function. Day-7 and day-14 TBH M\(_\phi\) did not, even though there was a significant decrease in Ia expression on day-14 TBH M\(_\phi\). The admixing data suggest that changes in the functional capabilities occurred after 14 days of tumor growth, while the phenotypic changes occurred after 7 days of tumor growth. Our phenotypic studies support the preceding suggestion, because no significant differences were seen in splenic M\(_\phi\) Mac-1, -2, -3, and Ia antigen expression until 14 days of tumor growth. This sequence of events, the phenotypic changes then the functional changes, suggests a growing tumor may selectively induce an early set of changes that are then followed by a later set of changes.

What could cause the suppression of autoreactivity by TBH M\(_\phi\)? M\(_\phi\) produce many suppressive monokines (30,31,67,79,109,121,186,214,215,234). One of the most widely studied suppressor factors is PGE\(_2\) (4,30,75,81,163). Tumor growth leads to a significant increase (over 2 fold) in PGE\(_2\) secretion by M\(_\phi\) (74,76,83,137) and a disappearance of the M\(_\phi\) subpopulation responsible for regulating PGE\(_2\) production (74). The increase in PGE\(_2\) production by TBH M\(_\phi\) and the increase in M\(_\phi\) numbers would cause a significant rise in in vivo PGE\(_2\) production. We confirmed these earlier studies (45,72,74,137,138) showing that TBH M\(_\phi\) PG production is an important negative modulator of T-cell reactivity, by using indomethacin. The addition of indomethacin significantly enhanced normal and TBH M\(_\phi\) accessory function. Because indomethacin-restored TBH M\(_\phi\) did not return autoreactivity to normal host levels, other suppressor monokines distinct from PG, must be produced by TBH M\(_\phi\). The secretion of suppressive monokines by normal host M\(_\phi\) shows that M\(_\phi\) in the normal host are responsible for down-regulating immune reactivity. These M\(_\phi\) may be a suppressor subpopulation of M\(_\phi\), which others (4,34,76,176,257) have suggested are present in normal hosts. In the TBH, increased PGE\(_2\)
would down-regulate immune reactivity and may point to a shift in the suppressor Mφ subpopulation (discussed later).

PGE₂ is a strong negative modulator of Ia antigen expression (207), IL-1 production (122), TNF-α production (90), Mφ-mediated cytotoxicity (184), and other Mφ functions (12,40,71,160). In addition, PGE₂ can directly suppress T cells through inhibition of IL-2 production, IL-2 receptor expression, and IL-1 and IL-2 responsiveness (247,270). PGE₂ can also down-regulate IFN-γ production (75,163). Thus, an increased production of PGE₂ during tumor growth would inhibit Mφ accessory and killing function and T-cell responsiveness.

In the process of examining functional differences in Mφ during tumor growth, autoreactive T₉ cells were also examined for changes during tumor growth. The data showed that tumor growth also affected autoreactive CD4⁺ T₉ cells. TBH autoreactive T cells were significantly less reactive to normal or TBH Mφ. But why were they less reactive to Mφ accessory activity, even when normal accessory Mφ were used? Were TBH autoreactive T cells less responsive to immune enhancing cytokines such as IL-1, IL-2, or IL-4 than their normal host counterparts? The answer was yes. The addition of IL-1, IL-2, and IL-4 significantly enhanced normal and TBH T-cell autoreactivity. TBH autoreactive T-cell responsiveness, however, was significantly less than that of the normal host, suggesting that tumor growth upsets the ability of T cells to respond to positive stimuli. A recent finding (247) demonstrating that the inhibitory action of PGE₂ is on an early T-cell activation step, suggests an answer to the reduced reactivity of TBH autoreactive T cells to enhancing cytokines. If Mφ-secreted PGE₂ blocks an early stage of T-cell activation, then potentially even up-regulatory signals would not be able to overcome this inhibition. That is, if TBH Mφ-produced PGE₂ blocks an early stage of the T cell cell cycle before the T cell becomes responsive to IL-1, IL-2, or IL-4, TBH T cells would be unresponsive to enhancing factors. Additional evidence (247) supporting this suggestion showed that PGE₂-mediated inhibition was irreversible. The addition of indomethacin to SMLR cultures, in which IL-1, IL-2, or IL-4 were added, significantly enhanced autoreactive T-cell cytokine responsiveness, demonstrating that PG do play a role in the down-regulation of T-cell cytokine responsiveness. Because the addition of
indomethacin to cultures containing TBH Mφ did not return reactivity to normal host levels, other TBH Mφ suppressor factors were also affecting interleukin responsiveness.

What causes TBH Mφ accessory function to change? Do TBH Mφ have a differential response to MAFs when compared to normal host Mφ? To address this point, the responses of normal and TBH Mφ to LPS were examined. LPS is a MAFs that has been used by others (1,2) to examine Mφ activation in the normal host. Activated Mφ have an increased ability to act in an immune response. We therefore wanted to explore if TBH Mφ have a different or decreased response to LPS. The data showed that normal and TBH Mφ respond to the LPS signal differently. Different changes were seen in surface antigen, accessory function, and molecular characteristics of normal and TBH LPS-treated Mφ. A 24-hr LPS treatment of TBH Mφ led to a significant increase in the percentage of Mac-1⁺, -2⁺, and Ia⁻ Mφ, the reported phenotype of suppressor Mφ (34,236). This increase in the TBH suppressor Mφ subpopulation correlated with the increase in suppression of allogeneic and autoreactive T cells by TBH Mφ. LPS seemed to increased TBH Mφ suppressor activity while not affecting normal host Mφ suppressor activity, suggesting that TBH splenic Mφ respond differently to activation signals. The differences in normal and TBH Mφ function after LPS treatment may reflect the differences in monokines induced by LPS, although this is unknown. LPS can induce IL-1, TNF-α, and PGE₂ secretion (1,21,167,184,239). Differential production of these monokines would affect T-cell reactivity. Because TBH Mφ became significantly more suppressive after LPS treatment, the increased suppression may be due to PGE₂ production. Increased PGE₂ secretion, however, was not examined. Recent work (unpublished data) examining the differential response of normal and TBH Mφ to MAFs such as IFN-γ and GM-CSF supports this work. TBH Mφ were significantly less responsive to IFN-γ and GM-CSF than their normal host counterparts.

The third question, "Is there a correlation between phenotypic changes and functional changes in TBH Mφ?", was asked because of the increasing evidence that changes in Mφ during cancer growth are due to shifts in Mφ subpopulations (72-74,76,137,231,258). To examine if there were changes in Mφ subpopulations during tumor growth, we concentrated on Ia⁻ Mφ, a phenotype of Mφ that we showed was significantly increased during tumor growth. Ia⁻ Mφ were also re-

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ported to suppress Tc cell activity (34). In this work, the goal was to investigate Ia- Mφ as the source of the tumor-induced immunosuppression. To address the functional changes in TBH Ia- Mφ, we examined Mφ accessory function to alloreactive and autoreactive CD4+ T cells. The different Mac+ populations of Mφ were not examined for suppressor function due to our initial difficulty in sorting splenic Mφ, particularly from the normal host. The investigation of the different Mac+ populations is necessary and certainly should be one of our future goals (mentioned later).

We first investigated the change in the Ia+/Ia- Mφ ratio. We showed an increase in the percentage of TBH Ia- Mφ, that corresponded to previous work (73,156), showing decreased Ia expression on TBH Mφ. The increased percentage of TBH Ia- Mφ, coupled with the increased numbers of Mφ in vivo, would mean a potential 2-3 fold increase in TBH Ia- Mφ. In examining the effect of tumor growth on Ia- Mφ-mediated suppression, we showed that Ia- Mφ were suppressor Mφ for both alloreactive and autoreactive CD4+ T cells. Ia- Mφ were more suppressive than unseparated Mφ populations and TBH Ia- Mφ were significantly more suppressive than normal host Ia- Mφ. Whole or unseparated Mφ were the corresponding controls for comparison to Ia- Mφ. We used unseparated Mφ instead of Ia+ Mφ because an insufficient number of cells would be collected if splenic Mφ were sorted. The unseparated population was an appropriate control because our interest was in the role Ia- Mφ play in suppression of T-cell reactivity, not in the role Ia+ Mφ play. Investigation of Ia+ Mφ is, however, necessary in light of their suspected role as a helper Mφ (168,240-242), and certainly should be a future goal.

The results suggest that Ia- Mφ are suppressor Mφ and that tumor growth leads to a quantitative and qualitative shift in this Mφ subpopulation. Others (135,152,161) also report that Ia- Mφ can function as suppressor Mφ in the normal host (119,120,164,165). Dorf et al. reported that Ia+ Mφ may act in the suppressor cascade with Tc cells. The reasons for the differences are unknown, but as Moser et al. (151,152) suggest, the differences may be due to two different Mφ-mediated pathways of suppression; one controlled by Ia+ Mφ and one controlled by Ia- Mφ. Nevertheless, my results show a subpopulation of Mφ which can act as suppressor cells and that are changed during tumor growth. The relationship of these TBH Ia- suppressor Mφ to the TBH Mac-1+2+ suppressor Mφ reported by Tsuchiya et al. (236) remains unknown.

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What could cause the tumor-induced shift from an Ia$^{+}$ Mϕ to an Ia$^{-}$ Mϕ? CSF, PGE$_2$, and TGF-β can negatively regulate Ia expression (41,207,262). Tumor cells and TBH Mϕ can secrete these factors. Therefore, it is possible that aberrant or excessive production of CSF, PGE$_2$, and/or TGF-β during tumor growth could be responsible for the increase in Ia$^{-}$ TBH Mϕ. The correlation between the loss of Ia expression on TBH Mϕ and increased suppressor function is unclear. The loss of Ia expression, however, could be an additional consequence of the tumor-induced shift from a helper Mϕ to a suppressor Mϕ.

Because TBH Mϕ are not the only culprits involved in tumor-induced immunosuppression (35,51,173), we also investigated if tumor growth affected CD8$^{+}$ T cell-mediated function. Are there two tumor-induced “arms” of suppression -- Mϕ and T cells? The investigation of tumor-induced changes in CD8$^{+}$ T cells showed CD8$^{+}$ T$_{C}$ or T$_{S}$ cells suppressed alloreactivity and autoreactivity and that tumor growth significantly enhanced their suppressor function. We also saw a shift in the CD8$^{+}$/CD4$^{+}$ T cell ratio due to increased percentages of CD8$^{+}$ T cells and decreased percentages of CD4$^{+}$ T cells. A change in the T cell ratio would lead to a decreased immune response. A change in the CD8$^{+}$/CD4$^{+}$ T cell ratio is seen in patients with AIDS and plays a major role in the immunosuppression seen in these patients. The shift in the T cell ratio could certainly lead to changes in immune reactivity, however, the qualitative changes in the T cells themselves probably play a far greater role. As mentioned previously, TBH CD4$^{+}$ T cell are significantly less responsive to IL-1, IL-2, IL-4, and Mϕ than their normal host counterparts, while TBH CD8$^{+}$ T cell are significantly more suppressive than their normal host counterparts. The increased suppressive ability of TBH CD8$^{+}$ T cells is unclear. Perhaps, like Mϕ, TBH CD8$^{+}$ T cells have an altered production of lymphokines. CD8$^{+}$ T cells can secrete IFN-γ and GM-CSF (69,70). IFN-γ can suppress B-cell and T-cell responses. IFN-γ may be one of the primary T cell secreted negative mediators of the immune response (69,70). Additionally, IFN-γ can stimulate PGE$_2$ production in Mϕ (1,21), which seems like an anomaly because PGE$_2$ can down-regulate IFN-γ production (75,163). This apparent contradiction could be caused by differences in the responsiveness of CD4$^{+}$ and CD8$^{+}$ T cells to these factors. Perhaps Mϕ-produced PGE$_2$ inhibits CD4$^{+}$ T$_{H}$ cell production of IFN-γ but induces IFN-γ production by CD8$^{+}$ T$_{CIS}$ cells. This differential
responsiveness to PGE$_2$ could be the reason we saw increased IFN-$\gamma$ production by TBH CD8$^+$ T cells. GM-CSF, on the other hand, can help T cells proliferate, induce Ia expression, and activate Mφ (58,64,69,84,123,205). The positive signal GM-CSF provides can be inhibited by IFN-$\gamma$ (70). An increase in IFN-$\gamma$ and a decrease in GM-CSF production by TBH CD8$^+$ T cells would lead to decreased alloreactivity and autoreactivity. Increased production of IFN-$\gamma$ by TBH CD8$^+$ T cells was recently shown to be the cause of the TBH CD8$^+$ T cell-mediated suppression (unpublished data), as blocking studies with anti-IFN-$\gamma$ mAb removed T cell inhibition.

The data from the preceding paragraphs suggest that tumor growth has two independent mechanisms (Ia$^-$ Mφ- and CD8$^+$ T cell-mediated) for suppressing CD4$^+$ T-cell proliferation. But the question remains, “What is the interaction between these two suppressor cells?” Earlier evidence suggested that Mφ could induce T$_{\text{R}}$ cells (33,136). Therefore, we explored the interaction of Ia$^-$ Mφ and CD8$^+$ T cells. Different responses were seen depending on the concentration of Mφ or T cells used as well as the source of the cells, normal or TBH. Mφ-mediated suppression dominated CD8$^+$ T cell-mediated suppression except when a high-dose of TBH CD8$^+$ T cells and Mφ were present, as would be expected in the TBH. In this case, suppression of CD4$^+$ T$_{\text{R}}$ cells was additive, with the suppression-mediated by Mφ and CD8$^+$ T cells together being greater than either alone.

Increased PGE$_2$ production by TBH Mφ has a wide range of effects on the immune system (33,76,163,215), including a suspected role as an inducer of T$_{\text{S}}$ cells (173). To address the role PGE$_2$ play in Mφ-CD8$^+$ T cell interactions in our system, we added indomethacin to MLR and SMLR cultures. In the MLR, indomethacin completely removed the suppression of alloreactivity mediated by the combination of normal host Mφ and CD8$^+$ T cells. In contrast, indomethacin only partly restored alloreactivity when TBH Mφ and CD8$^+$ T cell were present. These results suggest that PGE$_2$ may be one of the primary signals in normal host Mφ-CD8$^+$ T-cell control of alloreactive T cells; while in the TBH, other signals besides PGE$_2$ must be important. In the SMLR, the addition of indomethacin only partly restored the suppression of autoreactivity when normal or TBH Mφ and CD8$^+$ T cells were present. The SMLR data suggest that PGE$_2$ plays a minor role in the Mφ-CD8$^+$ T cell-mediated control of autoreactive T cells in the normal and
TBH. The differences in the role PGE₂ plays in these two systems (alloreactivity versus autoreactivity) probably stem from the need for tighter control of autoreactivity. Autoreactivity, because of its very nature (self-reactivity) needs to be more tightly regulated and this regulation probably involves several different suppressor circuits.

The preceding findings suggest that tumor growth changes Mφ and both CD4⁺ and CD8⁺ T cells. The results showed an increase in the suppression mediated by TBH Mφ and CD8⁺ T cells and a decrease in TBH CD4⁺ T-cell reactivity. Mφ changes during tumor growth were dominant in the tumor-induced immunosuppression compared with the changes in TBH T cells.

My model of how tumor-induced changes in Mφ and T cells affect the immune system is shown in Figure 37. The top panel shows the interaction of Mφ and T cells in the normal host and the bottom panel shows the interactions of Mφ and T cells in the TBH. During tumor growth the number of Mac-1⁺2⁺3⁻Ia⁻ Mφ (suppressor Mφ) increases and the number of Mac-1⁺2⁺3⁺Ia⁺ (helper Mφ) and Mac-1⁺2⁺3⁻Ia⁻ (cytotoxic Mφ) Mφ decreases. In addition to an increase in suppressor Mφ numbers, there is also an increase in their suppressor function due to increased PGE₂ production. This increased PGE₂ production would down-regulate Ia expression and IL-1 secretion by helper Mφ and presumably inhibit cytotoxic Mφ function. PGE₂ would inhibit CD4⁺ T-cell proliferation through the down-regulation of IL-2 receptors and IL-1, IL-2, and IL-4 responsiveness. The suppression of TH cells would lead to decreased humoral and cell-mediated immunity. Mφ-produced PGE₂ could also activate T₅ cells which through their increased production of IFN-γ would provide additional negative signals to TH cells. Decreased TH cell activity would lead to decreased Mφ activation because of a reduced secretion of MAFs.

Overall, the data show that there are phenotypic and functional changes in Mφ during tumor growth. Phenotypically, there are shifts in the surface antigen-defined Mφ subpopulations that correlate with the examined shifts in TBH Mφ suppressor function. The demonstration that the phenotypic changes correlated with the functional changes culminated in the identification of the Ia⁻ suppressor subpopulation of Mφ. Furthermore, there were quantitative and qualitative shifts in this subpopulation of Mφ, thus supporting my original hypothesis that tumor growth leads to a change in suppressor Mφ. My work in conjunction with the previous studies in our laboratory
Figure 37. Model of the Effects of Tumor Growth on Mφ-Mediated Accessory Activity: Panel A shows the proposed interaction of Mφ in the normal host and panel B shows how tumor growth upsets this Mφ-mediated immune reactivity. The arrows/lines represent the following: the thick arrow is the major pathway and the dotted arrow is the minor pathway.
and the work of others (51,72-74,76,93,137,230,231,236,269) provides a better understanding of the
tumor-induced changes in the immune system and the role Mφ play in this immunosuppression.
My work contributes to the understanding of the underlying cellular defects in the immune system
caused by a growing tumor. Several questions left unanswered by my work need, to be addressed.

1. Which of the phenotypically-defined Mac+ Mφ is functionally the suppressor population and
what is the correlation between the Mac+ Mφ and Ia- Mφ? To more definitively describe the
suppressor Mφ subpopulation and how tumor growth affects this subpopulation, a more
complete phenotypic and functional characterization of these Mφ populations is required. To
answer this question, the different population of normal and TBH Mφ would have to be sorted
on the flow cytometer and then analyzed for function in the MLR and SMLR. In addition
to answering the question of which Mφ is the suppressor subpopulation, these results would
also tell us if there are helper Mφ (Ia+ Mφ?) and cytotoxic Mφ (Mac-3- Mφ?). These results
would give a better understanding of not only the changes in Mφ subpopulations during tumor
growth, but also provide valuable evidence into the delineation of Mφ subpopulations.

2. What are the differences in cytokine production of normal and TBH Mφ subpopulations and
CD4+ and CD8+ T cells? My results only hinted at the potential differences in cytokine
production. To answer this question, Mφ and T cells would need to be examined using
methods that: (i) can measure the amount of factor actually secreted; and (ii) can measure the
amount of mRNA made. By using these methods, we could examine the mechanisms of
tumor-induced alterations in cytokine production. The understanding of how tumor growth
changes Mφ and T cell factor production may allow us to explain how tumor growth regulates
Mφ and T cells. Is the site of control at the transcriptional or translational level or is the site
of control at the level of secretion? By knowing the site of regulation, additional studies could
be done to examine the mechanisms behind this dysregulation. Are there differences in the
DNA binding proteins? Are there enhancer or suppressor elements which are turned on or
off? That is, where is the sight of the changes in intracellular signalling? Because tumor
growth, at least in our model, is not due to an underlying genetic defect, the cause of the
tumor-induced changes in Mφ and T cells must be aberrant intercellular and intracellular signalling.

The answers to these kinds of studies in conjunction with the work presented in this dissertation will give valuable insights into what changes occur in Mφ during tumor growth. More importantly, these answers will tell us mechanistically how tumor growth changes Mφ. The understanding of the mechanisms involved in the tumor-induced changes in Mφ provides a much more substantial base with which to develop a potential cure. Without the underlying knowledge of how tumor growth alters Mφ function, we only have a phenomenon. Thus, as we learn more about tumor growth, we will be able to determine the molecular origins of tumor-induced changes in the immune system.
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Appendix A. CHARACTERIZATION OF MACROPHAGE PHENOTYPE

One-Color Fluorescent Analysis

1. Use at least 3 mice for peritoneal Mφ; however for splenic Mφ, 3-5 mice should be used.

2. Prepare a single-cell suspension of either peritoneal or splenic Mφ and place the cells into ice cold medium.

3. After collecting the Mφ, dispense the Mφ (use from $1 \times 10^5$ to $4 \times 10^5$ cells/ml, bring cells up to 1.0 ml total) into 2.0 ml Sarstedt centrifuge tubes. These tubes make convenient sample holders.

4. To label the cells with mAb, add the mAb or the appropriate control antibody (must determine optimal dose ahead of time; usually between 10 µl and 100 µl) to the 1.0 ml aliquots of the cells. This treatment is done in the 2.0 ml Sarstedt tubes.
5. The cells are incubated for 35-45 min at 4°C. This incubation is followed by three washes with cold medium.

6. Next, the appropriate secondary FITC-tagged antibody is added. Cells are incubated in 1.0 ml of a 1:500 dilution of FITC-labeled secondary antibody for an additional 35-45 min at 4°C, and washed 3 times.

7. Cells are then analyzed on the EPICS V, laser flow cytometer at the Virginia Regional College of Veterinary Medicine.

Two-Color Fluorescent Analysis

1. Before doing two-color analysis of cells, the second mAb must be biotin-labeled. This labeling is done by the following technique.

   a. Affinity purified mAb (works better than straight supernatant) at 2-5 mg protein/ml (a must) is dialyzed over night (3-4 changes of the buffer) into a 0.1 M NaHCO₃ buffer (pH 8.2-8.6). Make up about 1 liter of the NaHCO₃ buffer.

   b. Twenty-five to 50 mg of biotin is dissolved in 1.0 ml of a straight DMSO solution.

   c. Dialyzed mAb are incubated at room temperature for 2-4 hr in a 1/10 w/v biotin/mAb solution (that is, 1 mg biotin to 10 ml of mAb [at 2-5 mg protein/ml]) on a rocker plate, and then dialyzed over night with PBS (3-4 changes).
d. Antibody must be sterile filtered and stored at -70°C.

e. The efficiency of biotin-labeling can be measured by comparing cells labeled with the biotin-coupled mAb followed by phycoerythrin-avidin D, to cells labeled with the biotin-coupled mAb followed by a FITC-tagged secondary antibody.

2. If peritoneal Mφ are collected, 3 mice can be used. If splenic Mφ are collected, 3-5 should be used.

3. Prepare a single-cell suspension of Mφ in PBS. Mφ need to be treated in PBS for all steps of the double-labeling procedure, because RPMI medium has biotin in it which interferes with the binding of R-phycoerythrin.

4. After collecting the Mφ, dispense the Mφ (1.0 ml aliquots of Mφ at 1 x 10^6 to 4 x 10^6 cell/ml) into 2.0 ml Sarstedt centrifuge tubes. These tubes make convenient sample holders.

5. To fluorescently label the cells, Mφ are incubated with the first mAb (in the Sarstedt tubes) for 35-45 min in cold PBS. This treatment is followed by three washes with cold PBS.

6. Cells are then incubated in 1.0 ml of 1:500 dilution of appropriate FITC-labeled secondary antibody for 35-45 min and washed 3 times in cold PBS.

7. Next, Mφ are incubated with the second mAb (this mAb has been biotin-labeled) for 35-45 min and then washed 3 times in cold PBS.

8. The Mφ are incubated with R-phycoerythrin for 35-45 min and washed 3 times in cold PBS. (I found that 5 µl-15 µl of R-phycoerythrin into 0.5 ml of PBS was suffi-
cient, however, it is probably important to determine the appropriate dilution of R-
phycoerythrin for each experimental system.)

9. Mϕ are then analyzed on the Virginia Regional College of Veterinary Medicine’s
EPICS V flow cytometer.
Appendix B. CHARACTERIZATION OF MACROPHAGE FUNCTION

*Allogeneic Mixed Lymphocyte Reaction (MLR)*

1. Use 3-4 BALB/c mice and 3-4 C3H mice for a three-plus plate assay. If you need to collect Mφ also, use 4-5 BALB/c mice.

2. BALB/c splenic single-cell suspensions are depleted of RBC by 0.83% NH₄Cl treatment (4 ml of 0.83% NH₄Cl are added to the cell pellet and incubated for 2 min, then add 46 ml of medium and spin) and then the remaining cells are washed twice.

3. Mφ are plated (medium containing 5% heat-inactivated FCS works best) for 2-3 hr.

4. T cells are collected by incubating the plastic nonadherent WSC in 5% FCS on a nylon wool column for 30 min in the 37°C incubator. The nylon wool column is prepared by rinsing the column 4-5 times with straight medium and 2-3 times with medium containing 5% FCS. The cells are added to the nylon wool column as fol-
lows. After centrifugation, the cells are resuspended to 3.0 ml and this 3.0 ml sample added to the column and carefully let them soak into the column. A top layer of 5.0 ml of medium containing 5% FCS is added and the column covered with parafilm before placing in the incubator. (Note, nylon wool columns may not be necessary because antibody plus C does an effective job.)

5. Nylon wool nonadherent cells are eluted from the column, centrifuged, and further depleted of Ia+ B cells, Mφ, and other contaminating cells by resuspending them in 100 μl of anti-Lyt-2 Ab (ATCC clone 3.155), 100 μl of anti-Iaα Ab (ATCC clone MK-D6), and 100 μl of anti-immature T cell and B cell Ab (ATCC clone J11d) for 30-40 min at 4°C. The key to doing the antibody procedure is to keep the volume to a minimum so that the antibodies don’t become too dilute to be effective. Therefore, only add about 0.5 ml of cold medium before adding the antibodies. This treatment should keep the volume under 1.0 ml.

6. The cells are washed once to remove excess antibody.

7. The cells are then incubated in a 1:10 dilution of C (300 μl of C plus 2.7 ml of medium) for 30-40 min at 37°C, remaining cells are pelleted, and washed 3 times.

8. The L3T4+ (CD4+) T cells (<1% contaminating Mφ and B cells) are resuspended to 4 x 10^6 cells/ml in complete medium (10% FCS and 10^-5M 2-ME), and 2 x 10^5 cells are added per well to a "U" bottom 96-well plate.

9. C3H allogeneic stimulator cells are collected by plating C3H WSC for 2-3 hr in medium containing 5% FCS, washed of nonadherent cells (depleted of Mφ), depleted of RBC by a 2 min treatment in 0.83% NH₄Cl, washed twice, X-irradiated (4 min at 50 kV), resuspended to 8 x 10^6 cells/ml in complete medium, and added at 4 x 10^5 cells/well.

Appendix B. CHARACTERIZATION OF MACROPHAGE FUNCTION

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10. Plates are incubated for 4 days at 37°C.

11. Six hr before termination and harvest of the cells, each reaction mixture is pulsed with 0.5 μCi of $^3$H-TdR (100 μl $^3$H-TdR/10 ml RPMI medium).

12. Cells are harvested onto glass fiber filters using the MASH unit and dried overnight.

13. Filter disks are removed, placed in the sealable counting vials with 2 ml of scintilene, capped, and then placed in the reusable counting vials.

14. Samples are counted in the Betatrac liquid scintillation counter.

**Syngeneic Mixed Lymphocyte Reaction (SMLR)**

1. To do a large SMLR experiment you will need 5-6 BALB/c mice. On average, 3 mice usually give you enough Mφ for 1-1.5 plates.

2. Spleens are removed and a single-cell suspension is made.

3. RBC are removed by a treatment in 0.83% NH₄Cl. Four ml of 0.83% NH₄Cl are added to the cell pellet, incubated for 2 min, mixed with 46 ml of medium, spun down, and then washed 3 times.

4. WSC suspensions are plated in medium with 5% FCS for 2-3 hr.

5. The nonadherent cells (provides the source of L3T4⁺ T cells, which are the responder cells in the SMLR) are removed, centrifuged, and placed on a nylon wool column in
5% heat inactivated FCS for 30 min in the 37°C incubator. The nylon wool column is prepared as stated above.

6. Nonadherent cells are eluted from the column and centrifuged. The resulting non-adherent cells are further depleted of la⁺ B cells, Mϕ, and other contaminating cells as stated above.

7. The cells are then incubated at 37°C for 30-40 min in a 1:10 dilution of C (300 µl of C plus 2.7 ml of medium), remaining cells are pelleted, and then washed 3 times.

8. The remaining L3T4⁺ T cells are resuspended to 4 x 10⁶ cells/ml in complete medium (contains 10% FCS and 10⁻⁵ M 2-ME) and 4 x 10⁵ cells are added per well of a "U" bottom 96-well plate.

9. The plated adherent cells provide the source of Mϕ, which are the stimulator/accessory cells in the SMLR.

10. Splenic Mϕ are collected by plating the WSC on plastic 150 x 15 mm plastic plates for 2-3 hr, washing vigorously to remove the nonadherent cells, scraping with a rubber policeman to remove the adherent cells (Mϕ), and centrifuged.

11. Mϕ are resuspended to 4 x 10⁶ cells/ml and X-irradiated for 4 min at 50 kV.

12. Four x 10⁵ cells are added per well.

13. Plates are incubated for 4 days at 37°C.

14. Six hr before termination and harvest of the cells, each reaction mixture is pulsed with 0.5 µCi of ³H-TdR.

15. Cells are harvested onto glass fiber filters and let sit over night to dry.
16. Samples are counted in the liquid scintillation counter.
VITA

Andrew David Yurochko was born in Munich, West Germany in 1963. He graduated from Virginia Polytechnic Institute and State University in 1985 after completing a B.S. in Biochemistry, with a minor in biology and chemistry. After graduation, he returned to Virginia Tech in 1985 to pursue a Ph.D. degree in Immunology under the tutelage of Dr. Klaus D. Elgert. While at Virginia Tech, he worked in the area of macrophage biology and tumor immunology. This research lead to the presentation of several awards, including two tuition waivers, a tuition award, a Society of Leukocyte Biology Student Travel Award, and the Virginia Tech Dissertation Year Cunningham Fellowship. In addition, Mr. Yurochko received several grants in conjunction with Dr. Elgert to fund his research, including two Sigma Xi Grants, two Biomedical Research Support Grants, and a F.E. Rippel Foundation Grant. This research was presented, in part, at the 1987 and 1988 Virginia Tech Graduate Research Symposium meetings, the 1988 and 1989 Society of Leukocyte Biology Meetings, the 1989 and 1990 Federation of American Societies for Experimental Biology meetings, and the 1990 Virginia Division of the American Cancer Society meeting. Portions of this work are published in eight articles in Cancer Immunology Immunotherapy, Cellular Immunology, Cytometry, Immunological Investigations, Immunology Letters, and Immunobiology. Several more papers have been submitted for publication or are in preparation.