Immune consequences of protracted host - tumor interactions in a transgenic mouse model of mammary carcinoma

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Abbreviations:

LN, Lymph node; LNC, Lymph node cell; TCR, T cell receptor; MTAG, Middle T AntiGen; Tg⁺, transgene positive; Tg⁻, transgene negative; Ag, antigen; CTL, cytotoxic T lymphocyte; ConA, Concanavalin A; LPS, Lipopolysaccharide; TDLNs, Tumor-draining lymph nodes; Treg, regulatory T; MSC, myeloid suppressor cell; NKT, natural killer T; TNF- α , tumor-necrosis factor- α ; MCP-1, monocyte-chemoattractant protein-1

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ABSTRACT:

A transgenic mouse model of mammary carcinoma (MTAG) was chosen to study host – tumor interactions over an extended period where the immune system interacts continuously with a developing and progressive malignancy. MTAG mice develop multiple mammary tumors with disease progression to pulmonary metastases and better represent natural disease progression than many of the current tumor implant models. Splenocyte number was found to increase concurrently with increasing tumor burden, mainly due to an expansion in the number of CD11b⁺ cells. Lymph node cell (LNC) numbers also increased until a tumor load of approximately 8 cm³, after which the number of LNCs decreased. No increases in the percentage of NKT or regulatory T (Treg) cells were detected in the spleen or draining LNs and the percentages of Treg cells within the tumor was similar to those reported in breast cancer patients. Suppressed T cell function was seen in unfractionated splenocytes based on reduced proliferation and cytokine production, and elevated levels of MCP-1 and TNF- α were detected in the sera of tumor-bearing mice. Many of these tumor-induced effects are similar to what has been reported in human disease progression and further validates this model for preclinical studies. These changes occurred in mice with extensive tumor load, and suggest that earlier therapeutic interventions may circumvent some of these effects. The multifactorial nature of this altered immune status in tumor-bearing hosts is consistent with an “immunological syndrome” that represents the complexity of host-tumor interactions and influences the competency of host immunosurveillance.

INTRODUCTION:

A review of the current literature on breast cancer initiation and progression highlights the complex nature of the interactions between a tumor and the host immune system. Although the study of tumorigenic cell lines has given some insights on the immunobiology of breast cancer, these systems cannot reflect the intricacy of long-term *in vivo* host – tumor interactions that occur during cancer development and progression. Genetically engineered mouse models of mammary cancer have therefore become important tools in the study of breast cancer biology and immunobiology^{1,2}. The MTAG mouse was created using the MMTV-LTR promotor to specifically target expression of the polyoma virus middle T antigen oncogene to mammary tissue³. Mammary tumors develop in 100% of transgenic mice with >95% progression to pulmonary metastases, over an average 6-month lifespan. Tumor histopathology of these mice has been shown to mimic that of human breast carcinoma at both morphological (disease stage) and molecular levels⁴. Although some specific immunological aspects of various transgenic tumor models have been investigated [Mukherjee, P (2003) Glyco J 18-931; Rowse, G (1998) Cancer Res 58-315], a basic immunobiological characterization of the immunological consequences of long-term tumor progression in this, or any other similar transgenic pre-clinical cancer model, has yet to be reported.

The MTAG model allows the study of host – tumor interactions over an extended period where the immune system interacts continuously with a developing and progressive malignancy. This environment of chronic interactions better reflects a human disease setting than many of the current tumor implant models where the host is ‘assaulted’ by an acute tumor cell challenge. As a validation of the relevancy of this MTAG mouse as a model for pre-clinical studies, we investigated the consequences of tumor development and progression on the immune system,

including the development of suppressive immune cell types and mediators of inflammation that have been associated with a poor prognosis in breast cancer patients. Examples of suppressive immune cell types include tumor-associated macrophages (TAMS) ^{5, 6}, myeloid suppressor cells ^{7, 8}, natural killer (NK)T cells ^{9, 10} and regulatory T (Treg) cells ¹¹⁻¹³.

The growth of most tumors can be associated with an inflammatory reaction ¹⁴, which can have both pro- and anti-tumor affects. Indeed, an anti-tumor immune response requires certain inflammatory messages to develop effectively ¹⁵; however, there is mounting evidence that tumor cells and/or tumor-associated leukocytes can also produce inflammatory cytokines and chemokines that promote malignant progression. In human breast cancer increased sera levels of the inflammatory chemokine, monocyte-chemoattractant protein-1 (MCP-1), has been linked with poor prognosis ^{16, 17}. MCP-1 is a CC chemokine described as a potent macrophage recruiting molecule ^{18, 19} and has been associated with increased angiogenesis and tumor growth rate ^{16, 20}. TNF- α is another inflammatory mediator that has been detected in tumor and/or stromal cells of a number of human cancers including breast, ovarian and colorectal ^{21, 22}. There is also evidence that TNF- α has pro-tumorigenic actions ²³⁻²⁷ and an association with poor prognosis ²⁸. Therefore this study included an analysis of serum levels of a number of inflammatory mediators including MCP-1 and TNF- α .

These studies outline some of the immunological consequences of disease progression in a mouse model of spontaneously arising and progressive mammary carcinoma. Our data support the hypothesis that tumor development and progression systemically influence the content and function of various immune cell populations, the development of immunosuppressive cell types, and the production of systemic pro-inflammatory cytokines. These changes reveal the complexity of host–tumor interactions, and indicate that a multitude of immune consequences

emerge, consistent with a tumor-induced “immunological syndrome”. However, many of these changes occurred during late-stage disease when tumor burden was extensive, suggesting that therapies may be more efficacious if earlier stages of disease are targeted, when immune performance is less likely to be compromised.

MATERIALS and METHODS:

Mice

Transgenic MTAG mice on a C57BL/6 background contain the Polyoma Virus Middle T Antigen (PyMT) controlled through the MMTV Promoter (LTR) ³ and were a kind gift of Dr. S. Gendler (Mayo Clinic Scottsdale, AZ). Male MTAG mice were bred with C57BL/6 wildtype females to produce offspring that were heterozygous for the PyMT transgene. Transgene positive (Tg⁺) mice develop mammary carcinomas with 100% penetrance and progression to pulmonary metastases in > 95% of cases. Mice designated as “Aged” were >160 days of age and “Young” mice were used between the ages of 42 and 80 days. Aged MTAG mice developed tumors in multiple mammary glands and routinely had a total tumor burden of >6 cm³, whereas young mice did not possess palpable tumors. Only female mice were used in experiments and transgene negative (Tg⁻) littermates were used as age- and gender-matched controls. Mice were housed in a specific pathogen free environment and experiments were conducted according to institutional guidelines for animal care and use. Under these standards, mice could develop up to ten individual tumors and were euthanized once any single tumor mass reached 2 cm³ in size or tumors interfered with normal activity.

Culture conditions

Lymphocyte cultures were assayed in complete RPMI (cRPMI) media. Complete RPMI (GibCo, Invitrogen Corp., Carlsbad, CA) or DMEM (GibCo, Invitrogen Corp., Carlsbad, CA) contained 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 15 mM HEPES, 100 U/ml Penicillin + 100 µg/ml Streptomycin solution, 50 µM 2-mercaptoethanol and 10% heat inactivated Fetal Bovine serum (FBS) (Hyclone Laboratories Inc., Utah).

Flow Cytometric analysis

Cells were incubated with the appropriate directly conjugated monoclonal antibody (mAb) in 100- μ l FACs buffer (PBS plus 2% FBS) for 30-60 minutes at 4°C. Cells were washed three times before analysis on a FACSCalibur flow cytometer (Becton Dickinson, CA) and by CellQuest software (Becton Dickinson). The following mAbs were used for cell labeling: phycoerythrin (PE) conjugated anti-CD4, anti-CD8, anti-NK1.1, anti-Gr1 (Ly6C+Ly6G); PE-Cy5 conjugated anti-CD3; fluorescein isothiocyanate (FITC) conjugated anti-TCR, anti-CD45R, anti-CD4, anti-CD8; anti-CD4-PerCP and anti-CD25-APC were used for Treg cell staining. All the above mAbs were obtained from BD Biosciences Pharmingen (San Diego, CA). Anti-CD11b-FITC (SouthernBiotec, Birmingham, AL) was used to stain Cd11b positive cell populations. FoxP3 staining was conducted using the FoxP3 staining kit (eBiosciences, San Diego, CA) and following manufacturer protocol. All samples were run with appropriate isotype controls.

Cytokine analysis

Splenocytes were cultured in an anti-CD3 (1 μ g/ml) pre-coated 24 well plate, which contained 10 μ g/ml anti-CD28 and 2×10^6 cells in 1 ml of cRPMI, at 37°C, 5% CO₂. Supernatants were harvested after 48 hours and frozen until cytokine quantification. Sera were collected from individual mice and frozen at -20°C until assayed. Samples were analyzed for cytokine concentration using the BDTM Cytometric Bead Array (CBA) Inflammation kit, which detects IL-6, IL-10, IL-12p70, MCP-1, IFN- γ and TNF- α (BD Biosciences). Assays were performed as outlined in manufacturer protocols.

Proliferation assay

Bulk splenocytes (2×10^5 cells/well) were plated in round-bottomed 96-well plates in the presence of 5×10^5 irradiated autologous splenocyte feeder cells and either ConA or LPS. After 2 days, 1

μCi of ^3H -thymidine was added to each well and the plate incubated a further 24 hours. Cells were harvested onto glass fiber filter mats using an automated plate harvester (TOMTEC, Hamden, CT). The amount of proliferation was determined by measuring ^3H -Thymidine incorporation using a Wallac liquid scintillation counter (PerkinElmer Life Sciences, IL). Data is expressed as the mean cpm ($\pm$ SD) of triplicate wells.

Statistical analysis

Tumor volumes were calculated using $W^2 \times L / 2$, where W =width and L =length of tumor mass measured in millimeters. Total tumor volume equals the sum of each individual tumor volume, which in the case of MTAG transgene positive mice could be up to 10 tumors. A two-tailed student's t test was performed on sample comparisons to determine statistically significant differences. A probability level (p) of less than 0.05 was considered statistically significant.

RESULTS & DISCUSSION:

Tumor growth in the MTAG transgenic mouse

Study of the nature of host - tumor interactions has been hampered by the paucity of preclinical model systems that accurately reflect the complex and long-term exchanges, which occur in human cancer development and progression. The MTAG transgenic mouse model of mammary carcinoma has been shown to mirror many aspects of human breast cancer development and progression. We therefore chose this mouse to immunologically validate as a preclinical model^{4, 29}. One aspect of cancer development in humans is the variability in the aggressiveness of disease progression. Tumor development and progression in MTAG mice was monitored by measuring primary tumor growth and the development of pulmonary metastases over time [Fig.1 & Table 1]. All transgene positive (Tg⁺) mice developed primary mammary tumors, which progressed to pulmonary metastases in >95% of mice more than 130 days of age [Table 1]. No histological evidence of metastases was found in young mice without palpable tumors (<85 days). Mice were found to develop primary tumors at varying ages, with the first masses becoming palpable between day 90 and 120 of life [Fig.1]. Total tumor volume was calculated as the sum of discrete tumor volumes, which could number up to 10 tumors within an individual mouse. Each primary tumor could develop at a different age and may arise in any, and up to all mammary glands. Individual tumor growth rates varied considerably and appeared independent of the tumor position (data not shown). Cancer progression in this transgenic model was therefore highly variable and should more accurately represent what occurs in human disease.

Transplantable tumor cells have already been selected for their tumorigenicity and, because of the acute, predictable kinetics and short-term nature of their disease progression,

interactions with host immune responses may not recapitulate those that occur during the course of natural tumor development and progression. Transgenic models that mirror the chronic nature of tumor-development and host – tumor interactions should therefore provide more relevant data on the consequences of tumor growth on host systems. The variability of tumor growth [Fig.1] between individual mice with differing tumor loads renders this model challenging to work with experimentally. However, it is this variability that also reinforces the relevancy of the MTAG transgenic mouse as a preclinical model of human cancer because it closely resembles the unpredictability of human disease progression. The unpredictability of tumor development and progression also suggests that transgene expression is not the only factor driving cancer progression.

Changes in lymphocyte populations during progressive tumor growth

Prior to the characterization of T cell function, an investigation into the consequences of tumor growth on lymphocyte numbers within the spleen and draining lymph nodes (LNs) of female MTAG mice, with varying levels of tumor burden, was conducted [Fig. 2A]. The total numbers of splenocytes increased progressively with increasing tumor burden so that the spleens of mice with extensive tumor load contained 3-4 fold more cells than controls. The expansion in splenocyte numbers correlated with overall tumor burden. The total number of lymph node cells (LNCs) from the draining LNs of tumor-bearing MTAG mice increased progressively until a total tumor burden of between 8 and 12 cm³ was reached, after which a decrease in total LNC numbers was observed. Similar to our observations in the MTAG mouse, enlargement of the tumor-draining LNs (TDLNs) has been reported during human cancer^{30, 31}.

Further analysis of lymphocyte subsets in the spleens of four pairs of individual mice (tumor-bearing denoted as A, B, C, D and their age/gender matched controls denoted as W, X, Y, Z) was then conducted. Mice with the highest tumor burden had the biggest spleens, whereas mice with lower tumor loads, (although still extensive compared to transplantable tumor models), had splenocyte counts closer to controls [Fig. 2B]. This may indicate homeostatic control mechanisms in the transgenic mouse that have been overcome in implantable tumor models, because mice with implanted tumors routinely show splenomegaly with less tumor loads^{32, 33}. Flow cytometric analysis of the composition of the MTAG spleens showed a marked increase in the percentage of macrophage-like populations (i.e., CD11b/Mac-1⁺ cells) that directly correlated with tumor load [Table 2]. Therefore, as total tumor burden and splenocyte number increased, there was a concomitant amplification in both the percentage and absolute number of this population, as determined by CD11b-staining [Table 2 & Fig.3]. This resulted in a corresponding decrease in the percentage of T cells, although the absolute number of T lymphocytes remained constant. This is in contrast to several tumor implant models and reports on human disease progression^{34, 35}, where a decrease in the absolute number of peripheral T cells was observed.

No bias in the reduction of a particular T cell subpopulation was observed because the percentages of both CD4⁺ and CD8⁺ T cell subsets were similarly decreased, and the CD4/CD8 cell ratio was maintained [Table 2]. The changes in splenocyte populations were more strikingly represented when CD3/CD11b ratios were determined. However, the increase in CD11b⁺ cell number became evident only in mice with a relatively high tumor burden (i.e., >2 cm³) [Fig. 3], which contrasts with some tumor implant models where this increase is seen in mice with much lower tumor loads^{32, 33}. The majority of these CD11b⁺ cells were found to be Gr1⁺ with their

percentage and absolute number increasing progressively with increasing total tumor burden.

The splenocyte profiles of young Tg^- and Tg^+ (with no palpable tumor growth) mice were similar to that of aged Tg^- mice (data not shown), suggesting that the altered splenocyte profile seen in the aged tumor-bearing Tg^+ mice resulted from tumor load rather than transgene expression.

A longitudinal study where complete blood counts (CBC) were determined in real-time following blood draws at different points during the lives of both Tg^+ and Tg^- mice (data not shown) demonstrated that the expansion in myeloid cells, measured by differences in CBC, was not seen in the blood until very late in disease progression ($> 8 \text{ cm}^3$). This did not reflect the gradual accumulation of these cells in the spleen as tumor load increased. Therefore, CBC profiles were an unsuitable readout for monitoring disease progression in this model.

Cells within the TDLNs are some of the first immune components to be exposed to tumor cells and/or their products. The composition of these LN populations may therefore determine the nature of the response of host – tumor interactions. Analysis of specific lymphocyte populations within the TDLNs of mice with extensive tumor loads ($>6 \text{ cm}^3$) revealed a significant increase in the percentages of B cells ($CD45R/B220^+$), $CD11b^+$ and $CD11b^+Gr1^+$ cells [Fig. 4]. However, there was variability in the results of individual mice, particularly in the percentage of $CD45R^+$ cells, which showed a very high increase in 3 out of the 10 mice tested. A marked increase in B lymphocyte numbers in the TDLNs of cancer patients has also been reported in adenocarcinomas (colon and breast) while in other tumors (melanoma and squamous cell carcinoma) the increase was less pronounced³¹. There was a significant decrease in the percentage of $CD8^+$ cells but not $CD3^+$ or $CD4^+$ T cells populations in these LNC preparations. The data indicates that tumor progression resulted in an altered composition of immune cell

populations present in the host spleen and LNs and suggests a dysregulation of homeostatic controls and possible immune response skewing.

Changes in lymphocyte function with cancer progression

The above set of four pairs of mice [Fig.2B & Table 2] were further analyzed for changes in splenocyte function and whether these changes could be correlated with the levels of tumor burden or their splenocyte profiles. When unfractionated splenocytes from individual Tg⁺ mice (A, B, C & D), with varying levels of tumor burden, were tested in mitogen-induced proliferation assays and compared with Tg⁻ controls (W, X, Y & Z), reduced levels of proliferation were observed, which inversely related to overall tumor burden [Fig. 5A]. There was a reduction in both the proliferation of T cells, stimulated by ConA (2 µg/ml), and B cells, stimulated with LPS (10 µg/ml). Splenocytes from mice with the highest tumor loads displayed the greatest reduction in proliferative responses compared with their Tg⁻ littermate controls. Mitogens are routinely used to test the immune response in clinical settings, and are therefore appropriate reagents for testing the immune function of lymphocytes in animal models.

A decrease was also seen in the ability of unfractionated T cells to produce IFN-γ; with T cells from CD3/CD28 stimulated splenocyte preparations of tumor-bearing mice producing less IFN-γ than normal controls [Fig. 5B]. The reduction in the proliferative responses of these splenocyte preparations could be explained, at least in part, by a quantitative decrease in the number of T cells within the well. However, it is important to note that decreased T cell function was still seen in mice with lower tumor burdens that possessed similar numbers of T cells (e.g., Mouse B) compared with Tg⁻ control mice. This indicated that the reduced proliferative capacity of these cells had either a qualitative aspect or was due to the presence of a suppressive cell type.

Since reduced proliferation was seen in the splenocyte preparations with similar T cell numbers, and because purified T cells from such mice displayed comparable proliferative capacity when compared to control preparations (submitted manuscript), these data lends further evidence for the presence of a suppressive cell type/s, within the splenocyte preparations, that was responsible for the reduced proliferative capacity of splenocytes from the spleens of tumor-bearing mice.

There was no obvious impairment of immune function in Tg⁺ mice due to transgene expression since cells from aged Tg⁻ and young Tg⁺ or Tg⁻ mice exhibited similar proliferative capacity [Fig.5C]. Because the suppressed response was observed with bulk splenocytes, these mixed populations would also be present in the spleen *in vivo*; we therefore postulate that a similar *in vivo* suppressive effect on T cell function can occur. Immune suppression is also supported by the observation that lymphadenopathy occurs during early disease but then lymph node size and LNC number is reduced in mice with late stage or extensive tumor burden [Fig.2A]. These suppressed immune responses may explain, at least in part, the lack of consistently successful cancer immunotherapies, which by their very nature require the ability of the host to mount a competent immune response.

Changes in suppressive cell types as tumors progressed

There are a number of suppressive cell types that have been reported to have immune inhibitory activity in tumor-bearing animal models and cancer patients. These include Treg cells (CD4⁺CD25⁺FoxP3⁺)^{11, 12}, NKT cells^{9, 10} and CD11b⁺Gr1⁺ myeloid suppressor cells (MSCs)^{7, 8}. An analysis of these populations within the MTAG model showed that the percentage of NKT cells in both the spleen and TDLNs was not significantly different between tumor-bearing and control mice [Table 2 & Fig.4], although a change in their function could not be ruled out.

There have been numerous reports of increased levels of Treg cells in the peripheral blood of cancer patients³⁶. Some researchers described increases in this cell type in breast cancer patients¹² while other studies demonstrated no difference between the numbers of Treg cells in the blood of women with breast cancer and normal donors¹³. The percentage and number of Treg cells in the spleen, at the site of tumor [Fig.6A] and in the TDLNs [Fig. 6B], was determined by staining with CD4 and CD25 then confirmed by FoxP3 staining. The percentage of CD4⁺CD25⁺FoxP3⁺ cells in the tumor ranged from 26.9% to 84.8% with a mean of 61.0% [Fig.6A]. There was no statistical difference in the percentage of Treg cells in the TDLNs of Tg⁺ mice with extensive tumor load and their Tg⁻ controls [Fig.6B]. An increase in the percentage of Treg cells in the spleens of MTAG mice was not seen until very late in disease progression (data not shown), when mice had extensive tumor load (> 10 cm³) and were approaching death. This level of tumor load is probably irrelevant to a clinical setting; therefore, any increase in the percentage of these cells may not be meaningful. However, due to the occurrence of lymphadenopathy and splenomegaly, the actual number of Treg cells within the TDLNs and spleen increased, although not preferentially compared to other T cell populations. Also, it is unknown whether there was a change in the function of these cells within tumor-bearing hosts. Therefore, in this MTAG model the data is similar to a recent report that demonstrated no increase in the Treg suppressive cell population in the peripheral blood of patients¹³.

It is becoming apparent that Treg cells in the tumor infiltrate may be more detrimental to immunotherapy attempts than systemic Treg cells. It has recently been shown that, although there is an accumulation of CD4⁺CD25⁺ T cells in the tumor infiltrate of breast cancer patients, only about 50% of these cells are FoxP3⁺³⁷. This is similar to our observation that many of the tumor infiltrating CD4⁺CD25⁺ T cells in the MTAG model are FoxP3⁻ [Fig.6A], and offers

additional support that the MTAG model is an appropriate preclinical animal model for human breast carcinoma.

As described previously in this manuscript, an increase in the percentage and absolute number of CD11b⁺ cells occurred in the spleens of tumor-bearing animals. A high number of these CD11b⁺ cells were also found to express Gr1 (Ly6C & Ly6G) [Fig.3], which is the reported phenotype of the myeloid suppressor cell population^{38,39}. A significant increase in these cells was also seen in the TDLNs [Fig.4]. Myeloid suppressor cells (MSCs) have been demonstrated in a number of transplantable tumor models and in some cancer patients. Their accumulation has been shown to cause generalized immune dysfunctions through a number of suppressive mechanisms³⁹. The presence of the aforementioned and other immune suppressive cell types may therefore inhibit therapeutic efforts and have lately become targets for consideration in combined therapies.

Tumor-bearing animals demonstrated increased serum levels pro-inflammatory cytokines

There is mounting evidence for a link between inflammation and an increased incidence of cancer development and progression. An inflammatory environment may have pro-tumorigenic influences and can enhance the movement of tumor and stromal cells to increase the metastatic potential of the primary tumor^{40,41}. Increases in serum levels of certain inflammatory mediators has been described in cancer patients and have been used as a measure of disease progression and therapy efficacy^{42,43}. An analysis of some of these indicators in this model was therefore undertaken to reinforce the relevancy of the MTAG mouse as a preclinical model. The levels of certain systemic inflammatory cytokines were determined by analysis of serum samples from Tg⁺ and Tg⁻ mice with varying levels of tumor burden. No significant levels of, or

differences in, IFN- γ , IL-2, IL-4, IL-5, IL-6, IL-10 or IL-12p70 cytokines were found in the sera. Two inflammatory mediators, MCP-1 and TNF- α , were found to be elevated in the sera of tumor-bearing mice, compared to age-matched Tg⁻ mice and younger Tg⁺ mice. Similar levels of MCP-1 and TNF- α have also been detected in the sera of breast cancer patients^{17, 44} and is further evidence of the relevancy of this model. The increased MCP-1 and TNF- α levels in the serum suggest a systemic inflammatory effect within these tumor-bearing hosts.

We have observed that *ex vivo* primary tumor cells and tumor cell lines (derived from the primary mammary tumors of these transgenic mice) secreted high levels of MCP-1 into the culture media. A broad spectrum of other tumor cell lines (eg. brain, lung, renal and other breast cancer lines) have also been shown to produce MCP-1 protein⁴⁵. Since the number of CD11b⁺ cells is greatly increased in mice with large tumor loads [Table 2 & Fig.3] and monocytes are known to produce both MCP-1 and TNF- α , the source of the higher levels of MCP-1 in the sera could be either the increasing tumor burden and/or the increasing number of CD11b⁺ cells. It has been postulated that the interaction of mammary tumor cells, which produce MCP-1, and monocytic cells, which secrete TNF- α , contribute to tumor progression through a cytokine cross-talk mechanism⁴¹. MCP-1 can also inhibit the generation of tumor-reactive T cells⁴⁶ and polarize immunity in a type II direction⁴⁷. Therefore, increased levels of MCP-1 in the blood may skew the immune system towards a type II inflammatory response that may undermine effective type I and anti-tumor defense mechanisms⁴⁸. Indeed, MCP-1 expression has been related to tumor aggressiveness in a number of tumor types including carcinoma of the breast, bladder and ovaries⁴⁹. MCP-1 and other inflammatory mediators, or the pathological consequences of their presence, may therefore be targets for anti-tumor therapy and have already been used as a measure of therapeutic efficacy.

CONCLUSIONS:

In this MTAG transgenic mouse model of tumor growth, changes in immune cell populations, their reduced function, the presence of immune suppressive cell types and increased levels of serum inflammatory mediators, occurred as a consequence of long-term tumor progression. These changes mirror what has been reported in human breast cancer patients and therefore support the relevancy of this transgenic model for preclinical studies. However, these alterations in the populations and function of cells of the host immune system occurred at late stage disease when tumor load is extensive. The multifactorial consequences of tumor progression suggest that not a single, but rather a combination of factors contribute to altered immune status in a tumor-bearing host, resulting in a tumor-induced “immunological syndrome”. Moreover, the protracted nature of host – tumor interactions in this model will permit the study of earlier stages of disease progression and the analysis of therapeutic interventions that otherwise would not be possible in the abbreviated time-frame that is characteristic of current tumor implant models.

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FIGURE LEGENDS:

Figure 1: Growth of autochthonous tumor. Tumor progression was monitored by measuring each discrete tumor within the individual mice ($n=15$). Total tumor volume was calculated by the addition of all discrete mammary tumor volumes (up to 10 per mouse), present in each individual mouse. Measurements were taken every week until a tumor size reached ethical limits or adversely affected normal activity.

Figure 2: Changes in splenocyte and LNC numbers as tumor load increases. The number of splenocytes and LNCs from a pool of LNs from individual Tg⁺ mice with varying amounts of tumor load were determined. Total tumor volume (cm³) was calculated from the addition of all discrete mammary tumor volumes, present in each individual mouse (up to 10 per mouse). (A) Total numbers of splenocytes and LNCs were graphed against the total tumor volume range of the individual mice. Error bars represent the SD of mice at that range of tumor volume. The number of mice per range of total tumor volume was between 5 and 30. (B) Total splenocyte counts of 4 individual Tg⁺ mice with varying tumor loads (denoted A, B, C & D), and their corresponding Tg⁻ gender - matched littermate controls (denoted W, X, Y & Z respectively).

Figure 3: The percentage of CD11b+ cells in the spleens of tumor-bearing MTAG mice increases with increasing tumor burden. Splenocytes from MTAG mice with various tumor loads (1.9 to 12.5 cm³), and their age/gender-matched controls were stained for CD11b and Gr-1 surface markers using monoclonal antibodies and analyzed by flow cytometry. Total tumor volume was calculated from the addition of individual mammary tumor volumes, present in each Tg+ mouse.

Figure 3

Figure 3: The percentage of CD11b+ cells in the spleens of tumor - bearing MTAG mice increases with increasing tumor burden. Splenocytes from MTAG mice with various tumor loads (1.9 to 12.5 cm³), and their age/gender - matched controls were stained for CD11b and Gr - 1 surface markers using monoclonal antibodies and analyzed by flow cytometry. Total tumor volume was calculated from the addition of individual mammary tumor volumes, present in each Tg+ mouse.

Figure 5: T cell and B cell function is reduced as tumor load increases. Total splenocyte numbers of 4 tumor - bearing Tg⁺ mice and their Tg⁻ age - matched controls were plotted against total tumor burden. (A) Fractions of splenocytes from these individual mice were then placed in a proliferation assay with either ConA (final concentration of 2 μ g/ml) or LPS (final concentration of 10 μ g/ml) mitogen for stimulation. Values represent the cpm of tritiated thymidine incorporation from triplicate wells and error bars are the standard deviations. (B) Splenocytes were also placed in culture with anti - CD3 (1 μ g/ml) and anti - CD28 (10 μ g/ml) stimulation for 48 hrs before harvesting culture supernatants for cytokine analysis. The 4 pairs of mice illustrated are representative of more than 10 similar experiments that have analyzed greater than 24 age/gender matched pairs of mice. (C) Splenocyte preparations from aged Tg⁺ and Tg⁻ and young Tg⁺ and Tg⁻ mice were placed in a standard proliferation assay against a titration of ConA mitogen. Values represent the cpm of tritiated thymidine incorporation from triplicate wells and error bars illustrate the standard deviation.

Figure 6: Analysis of Treg cell populations within the tumor and TDLNs. Cellular preparations were stained for the CD4 and CD25 surface markers. Treg phenotype was confirmed by FoxP3 staining. Graphs show the percentage of CD4+ cells that are also CD25+ and FoxP3+. (A) A single cell preparation of tumor cells was derived from a pool of tumors from individual mice. Samples were gated on the lymphocyte population of cells for acquisition and analysis. (B) The TDLNs from individual Tg+ mice with extensive tumor burden (ie. > 6 cm³), or their age/gender - matched Tg - controls, were pooled and dissociated into a single cell suspension before cell staining. Bars on the graph represent the mean of the 10 samples and the p value was obtained using a two - tailed students t test.

Figure 7: Tumor - bearing mice have elevated serum levels of MCP - 1 and TNF - α . The sera from 25 Tg+ mice with extensive tumor load and their age/gender - matched Tg - controls was analyzed for inflammatory cytokines / chemokines by cytometric bead array (CBA). Graphs illustrate the results for MCP - 1 and TNF - α in pg/ml. Bars on the graph represents the mean values and the p value was obtained by using a unpaired student ' s ' t test.

Table 1: The number of pulmonary metastases in individual MTAG mice of different ages and varying tumor loads.

Age^a (days)	Total Tumor Volume (mm³)	# Mets^b
171	11967	27
171	8728	28
171	7944	8
177	7333	23
150	2395	2
150	1523	3
150	3067	4
122	825	3
122	1796	2
85	54	0
85	23	0
85	0	0

^a Of individual mice

^b Number of pulmonary metastases

Table 2: The percentages and absolute numbers of splenocyte populations from the spleens of tumor-bearing Tg⁺ or control Tg⁻ mice.

ID _a	Total	Cell surface markers										Cell Subset Ratios					
	spleno	CD3		CD4		CD8		CD11b		CD45R		NK1.1		CD3/CD11b		CD4/CD8	
	No. ^b	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Transgene negative mice																	
W	105.0	34.0	32.4	23.3	22.2	13.4	12.8	3.8	3.6	59.3	56.5	3.6	3.5	8.9	8.9	1.7	1.7
X	94.5	27.5	29.1	17.9	18.9	12.0	12.7	5.4	5.7	64.4	68.2	2.4	2.6	5.1	5.1	1.5	1.5
Y	73.5	25.3	34.4	13.5	18.4	8.1	11.1	3.1	4.2	49.7	67.6	1.3	1.7	8.1	8.1	1.7	1.7
Z	117.0	36.8	31.5	26.3	22.4	13.9	11.9	3.5	3.0	62.4	53.4	1.4	1.2	10.5	10.6	1.9	1.9
Transgene positive mice																	
A	404.0	51.0	12.6	28.8	7.1	18.1	4.5	89.5	22.2	209.0	51.8	1.4	0.3	0.6	0.6	1.6	1.6
B	91.5	20.4	27.3	15.9	17.4	9.4	10.3	5.5	6.0	56.6	61.8	1.0	1.1	3.7	3.7	1.7	1.7
C	130.0	28.4	21.8	17.6	13.5	13.3	10.2	21.4	16.5	64.4	49.6	2.4	1.8	1.3	1.3	1.6	1.3
D	317.0	31.0	9.8	20.1	6.3	12.6	4.0	93.1	29.3	81.3	25.7	0.0	0.0	0.3	0.3	1.6	1.6

^a Mouse identifier. Mice A, B, C and D are tumor-bearing animals matched with their corresponding littermate controls W, X, Y and Z^b Number of splenocytes (x 10⁶)