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Thymus Subset Alterations Accompanying Concomitant Tumor Immunity Mimics Phenotypic Patterns of Cytotoxic Drug Doxorubicin

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Abstract. *Background/Aim: Concomitant immunity (CIM) is a phenomenon that elicits an antitumor response not sufficient enough to destroy the primary tumor but prevents a secondary implant from growing and spreading. This study aimed to develop a method of identification of serum tumoricidal factors released into circulation during CIM and to compare the CIM-related effect to the effect elicited by the cytotoxic drug doxorubicin. Materials and Methods: SL2 tumor-bearing mice were studied at three time points – day 4, day 7, and day 11 following i.p. 5×10^5 cell implantation. Hematological effects and thymocyte immunophenotyping (CD4/CD8) data were compared to the effects induced by intravenous 10 mg/kg doxorubicin (DOX) administration to intact DBA 2 mice. The level of plasma colony stimulating factor-granulocyte macrophage (CSF-GM) was evaluated by ELISA. Results: Identical thymus histopathology and an extent of double-positive CD4+CD8+ subset depletion was found in day 11 tumor-bearing mice (TBM-11) and in DOX-administered animals. TBM-11 exhibited a leukemoid reaction with an increase in monocyte and granulocyte counts. Conversely, DOX administration was followed by severe leukocytopenia at the 72-h time point. No increase in CSF-GM was observed in mice with or without a leukemoid reaction. Conclusion: The complexity of CIM can be examined by tracking alterations in the most fragile cortical CD8+CD4+ double positive population. Thymocyte apoptosis induced by DOX and TBM-11 might be associated with different mechanisms. TBM-11 did not exhibit severe myelotoxicity as DOX did. CIM-related serum factors can be assessed and screened via thymocyte subset analysis.*

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Although radical excision of the primary tumor can save lives, it has been reported that the surgical trauma itself may activate minimal residual disease and accelerate tumor recurrence (1, 2). The mechanism behind the activation of this surgery-induced growth acceleration or tumor dissemination is poorly understood. Some of the most appealing studies have indicated the presence of concomitant immunity (CIM) (also known as concomitant resistance) accompanying primary tumor growth (3, 4). CIM is a unique phenomenon that elicits an antitumor response not sufficient enough to destroy the primary tumor but prevents a secondary implant from growing and spreading. Many experimental tumors have been demonstrated to induce the CIM effect (5-7) including SL2 lymphoma (8). Apparently, two independent components of CIM are simultaneously present in the tumor-bearing host. The one is specific cellular immunity, which is prevailing in small tumor-bearing mice and decreases with tumor progression (6). Another one is a non-specific component, possibly nonimmunological in its nature, which occurs in animals at a late stage of tumor growth and increases with tumor burden (5, 6). The non-specific component of CIM is strong but

rapidly disappears with tumor excision. Several attempts have been made to identify serum factors released into the blood capable of secondary tumor growth inhibition by a non-specific non-immune mechanism (5, 6). The attractiveness of identification of these CIM-related components is that these factors can be further studied and adopted for postoperative minimal residual disease control. However, little is known on the origin of these potential secretome constituents. In addition, the mainstream CIM studies are focusing on the clarification of an initial phase of resistance induced by experimental immunogenic tumors.

Paradoxical survival benefits obtained after primary partial tumor excision in oligometastatic human malignancies (9-11) seemingly dispute the data found in experimental CIM models. However, cases where the metastatic disease is regressing following primary tumor extirpation, are very rare. These cases might also be viewed as a manifestation of residual CIM, which still operate in spite of the primary tumor excision. Indirect evidence indicates that human malignancies are often prone to the hyperproduction of variable soluble factors capable of inducing paraneoplastic reactions. It would be of interest to get a deeper insight into a plethora of soluble factors eliciting paraneoplastic reactions, which in some cases might even contribute to better control of malignancy. For instance, human tumors might induce a paraneoplastic leukemoid reaction, which is also an attributable feature of numerous experimental tumors (12, 13). Notably, a leukemoid reaction might somehow be associated with a dramatic response of stage IV lung cancer patients undergoing single-agent immunotherapy (14). Previously, we observed that a SL2 tumor can induce a leukemoid reaction in the peripheral blood of mice and the extent of white blood cell (WBC) count increase was directly related to the size of the tumor. Unidentified factors in the serum of SL2 tumor-bearing mice (TBM) were also able to induce cytotoxicity towards SL2 cells (8).

The aim of this study was to investigate if progressing SL2 tumors can induce characteristic cortical thymocyte depletion *in vivo*. The immunotoxin activity can be closely monitored by the thymic histopathology and architecture rearrangements observed in rodent models (15). This method is advantageous over general toxicity tests such as animal body weight, hematological effects or other assays (16). In fact, the cortical thymocyte is potentially the most fragile body cell being rapidly triggered to undergo programmed cell death *in vivo* and *in vitro*. Characteristic rearrangements detectable in certain thymocyte subsets as a specific signature of a drug can be ascribed to immunomodulating agents such as cyclosporin (17), doxorubicin (17, 18), rapamycin (19), etoposide, lipopolysaccharide, dexamethasone (20), and some other (21).

We hypothesized that the progressing tumor eliciting non-specific CIM can be assessed by a thymocyte rearrangement signature. We compared thymocyte subsets and hematological variations observable on day 4 tumor (small, not yet eliciting CIM) *vs*. day 11 tumor (large, exhibiting CIM). The day 11 tumor was found to be able to induce thymocyte effects identical to the ones seen after doxorubicin (DOX) administration. Thymocyte phenotypic alterations observed on day 11 TBM were not associated with severe myelosuppression seen following DOX intravenous (*i.v.*) injection.

Materials and Methods

Mice and tumors. DBA/2 mice, aged 8 to 12 weeks, were obtained from a local breeding facility of State Research Institute of Innovative Medicine (SRIIM), Vilnius, Lithuania. Mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. All research protocols were approved by the Institutional Animal Care Committee and *in vivo* procedure biosafety was assured by the supervision from State Research Institute Centre for Innovative Medicine. All research protocols involving animals were approved (approval permission number G2-120) by the Institutional Animal Care Committee, Vilnius, Lithuania (Valstybinė Maisto ir Veterinarijos Tarnyba, Vilnius). The study was carried out in compliance with the ARRIVE guidelines. Animals had *ad libitum* access to pelleted feed, food supplements, and water. All animals were determined specific pathogen free. Light inhalation anesthesia with isoflurane was used for iv injections and blood sampling procedures. The SL2 lymphoma occurred as a spontaneous tumor in a DBA/2 mouse of the Chester Beatty Research Institute, UK, and acquired through prof. Den Otter, University Medical Center Utrecht, The Netherlands. SL2 cells were maintained in RPMI-1640 medium (Thermo Fisher scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific). All tumors in our study were induced by *i.p.* injection of 5×10^5 cells per mouse.

Thymus harvesting and thymocyte phenotyping. At each time point of experiment, the TBM and DOX treated mice were sacrificed by cervical dislocation after isoflurane inhalation anesthesia. Midline incision was done at the chest cavity. The sternum was carefully removed, and the thymuses were obtained and weighed. The thymuses were divided so that half of the number of the organs of different groups were fixed in 10% buffered formalin followed by paraffin-embedding, while the other half was processed for single cell suspension preparation. Paraffin-embedded parts were prepared for histological analysis: 4-μm-thick sections were cut and stained with hematoxylin and eosin (H&E). Slides were then examined by light microscopy. The determination of the size and cellularity of the cortex and medulla, lymphocyte apoptosis, cortex: medulla ratio and an increase or decrease in the epithelial component of the thymus were evaluated by three pathologists.

For single cell suspension preparation, the fragments of tissues were placed into petri dishes with a 5 ml of PBS, chopped, and placed on a 70 μm cell strainer in the centrifuge tube. Cells were centrifuged at 300×*g* at 40˚C for 5 min. Supernatant was removed and 10 ml of FACS buffer (Becton-Dickinson, San Jose, CA, USA) was added to wash. For flow cytometry (FCM) analyses, 5×10^5 cells per sample were incubated with FACS buffer containing 0.1 μg of anti-mouse FcgIII/II receptor (clone 2.4G2) for 20 min at 4˚C. Cells were then washed with FACS buffer and staining was performed using 0.5 μg of FITC anti-CD4 (clone GK1.5), and 0.5 μg of PE antiCD8a (clone 53-6.7). All antibodies were purchased from Thermo Fisher scientific (Waltham, MA, USA). Cells were stained

Table I. *Thymic and hematological parameters determined in mice bearing SL2 tumor at different time points vs. mice injected with doxorubicin.*

Parameter		Experimental groups				
		Control	TBM-4	TBM-7	TBM-11	DOOX
Mice weight, g		19±1.6	21±0.7	21±1.4	30±3.5*	19±1.7
Thymus weight, mg		47±4.7	39±4.2	25±9.1*	9.9±2.3*	10.0±1.6*
Cortical cellularity		N	N	↓	↓↓	↓↓
Thymus immunophenotype	CD4–CD8– (%)	8.0±0.81	8.7±0.35	8.0±2.12	17.2±9.19*	17.6±5.37*
	CD4+CD8+ (%)	79.4±0.88	79.6±0.78	75.2±2.62*	41.3±23.76*	42.1±8.06*
	CD4+CD8– (%)	8.2±0.13	7.7±0.64	11.7±0.64*	23.2±8.41*	23.8±2.27*
	CD4–CD8+ (%)	4.5±0.21	4.2±0.49	5.3±021	18.3±6.15*	16.6±1.36*
RBCs, M/μl		10.0±0.33	9.8±0.57	9.9±0.21	10.0±0.42	9.8±0.46
WBCs, k/μl		7.6±0.60	7.5±0.28	7.5±0.42	10.9±0.92*	3.0±0.77*
Lymphocytes, k/μl		6.3±0.62	5.9±0.28	6.0±0.64	5.4±0.92	2.5±0.76*
Monocytes, k/μl		1.1±0.14	1.2±0.07	1.3±0.07	3.3±0.07*	0.3±0.45*
Granulocytes, k/μl		0.3±0.08	0.4±0.07	0.4±0.07	2.3±0.07*	0.2±0.05*
Platelets, k/μl		789±49.9	746±134.4	728±22.6	767±79.9	692±82.2

TBM-4: Tumor bearing mice on day 4; TBM-7: TBM on day 7; TBM-11: TBM on day 11; DOX: mice injected with doxorubicin 72 h before blood and thymus sampling; RBC: red blood cells; WBC: white blood cells; N: normal cortical to medullar ratio ~2:1. *$p<0.05$ *vs.* control mice.

at 4˚C for 30 min and then washed with FACS buffer. At least 10,000 cells were acquired on a FACS-LSRII flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analysis was performed using FlowJo 8.6.3 software (Tree Star, Ashland, OR, USA).

Blood sampling, doxorubicin injection and hematology analysis. Blood samples (125 μl) were taken from the retroorbital plexus from control, TBM and DOX treated mice in tubes containing EDTA as an anticoagulant. Each blood sample was gently inverted several times to ensure complete mixing with the anticoagulant. After centrifugation at 5,000×*g* for 10 min, plasma samples were separated and stored at –20˚C until analysis. Complete blood counts (CBC) were analyzed using ABX Micros ESV 60 within an hour of sampling. Doxorubicin hydrochloride (2 mg/ml) (Ebewe, Unterach, Austria) was obtained from a hospital pharmacy. Mice were injected at a dose of 10 mg/kg *i.v. via* plexus orbitalis.

CSF-GM quantification in ascites fluid and plasma. Ascites fluid (AF) was collected at the same time points as the plasma. Ascites was harvested, centrifuged at 1,000×*g* for 15 min at 4˚C, and the supernatant was stored at –20˚C. Samples of cell-free ascites and plasma were tested for colony stimulating factor-granulocyte macrophage (CSF-GM) presence by GMCSF Pre-coated ELISA Kit (BioLegend, San Diego, CA, USA) according to the manufacturers' instruction.

Statistical analysis. The statistical tests were performed using STATISTICA 12.0 (TIBCO Software Inc Palo Alto, CA, USA). All the results are presented as means and standard error (mean±S.E.). Significance was considered at values of $p<0.05$.

Results

Tumor and Doxorubicin impact on thymus size. The ascitic SL 2 tumor following *i.p.* injection of 5×10^5 cells exhibited rapid proliferation with a median time of animal kill of 15 days (range=13-21 days). The *i.v.* injection of DOX on day 3 at a dose of 10 mg/kg results in a median survival time of 25 days (range=19-33 days) indicating that SL2 lymphoma is sensitive to DOX treatment. TBM showed an increase in body weight on day 11, which is a characteristic feature of ascites accumulation in the peritoneal cavity (Table I). Mice treated with DOX as well as mice bearing SL2 tumor on day 11 exhibited a dramatic decrease in thymus size, whereas on day 4 TBM revealed no change in thymus mass.

Histopathology and flow cytometry. Histological examination of the thymus revealed a decreased number of cortical cells in TBM starting from day 7 and advancing on day 11. These morphological changes were associated with depletion of double-positive CD4+CD8+ cells alongside with an increase in the double-negative (CD4-CD8-) and single positive compartment (both CD4-CD8+ and CD4+CD8-). Identical changes were seen in mice treated with DOX. The thymus of the control mice showed normal- cytoarchitecture with an abundance of lymphocytes and well expressed cortical/medullar gradient (Figure 1A). There were no differences in the thymus histopathology of DOX-treated mice or mice bearing Day 11 tumor (Figure 1B and C). The cortical regions of TBM-11 and DOX treated mice exhibit apoptosis of the lymphocytes, lower cell density, and lower cellularity. Regions of tissue tearing, potentially due to lack of cell-to-cell adhesion among the apoptotic cells were also seen in TBM- and DOX-treated animals. In the thymus of control mice there were rare apoptotic bodies. The medullary region in the thymus of TBM- and DOX-treated mice increased due to cortical depletion and lower cellularity. Tingible-body macrophages were more visible in DOX and TBM-11 thymuses and showed a "starry night" pattern.

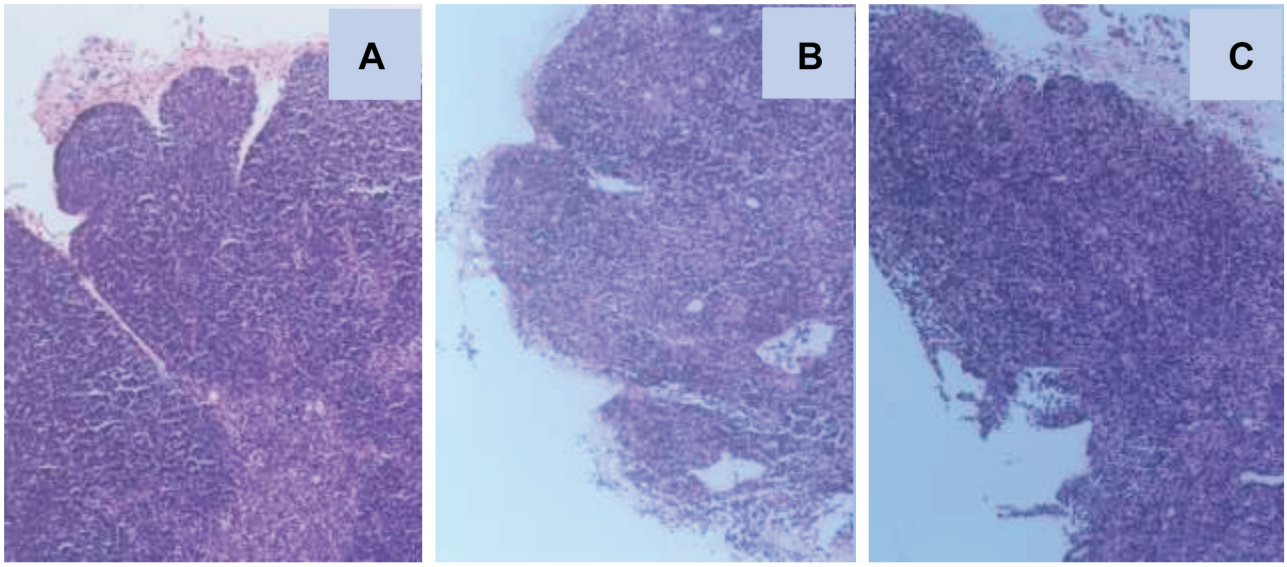

Figure 1. *Cross sections of control (A), Day 11 tumor bearing (B), and doxorubicin-treated (C) mice. (H&E, 100×).*

Hematological effects. The typical suppression of WBC counts was seen 72 h following DOX injection. Leukocytes, lymphocytes, monocytes, and granulocytes declined 1.5-3.7-fold well in line with the myelotoxicity patterns known for DOX. TBM exhibited a gradual increase in counts of monocytes and granulocytes beginning from day 7.

CSF-GM concentration in plasma. Plasma and ascitic fluid samples tested for CSF-GM did not reveal significantly increased levels at any time point of tumor progression. All CSF concentrations evaluated were below 16.5 ng/ml (the manufacturer declared the lowest accuracy level).

Discussion

The mice bearing large SL2 tumors exhibited a significant reduction in the cortical CD4+CD8+ thymocyte population – a result identical to the one seen after the administration of the cytotoxic drug DOX. The effects elicited by TBM-11 and DOX on peripheral blood leukocytes were the opposite: TBM-11 provoked leukocytosis, whereas DOX caused severe myelotoxicity. We hypothesize that SL2 TBM released factors into circulation that might exhibit diverse profiles of cytotoxic activity as compared to the plain cytoablative effect of DOX. There is also a likelihood that TBM induce the release of several factors, some of which might stimulate leukopoiesis, whereas others induce cytotoxicity detectable on cortical thymocytes (Figure 2). Serum cytotoxicity was noticeable already on days 2 and 4 in SL2 TBM in our earlier studies (8). The simultaneous cytotoxicity towards tumors alongside hematopoietic stimulation might be an appealing feature in the search for refined postoperative control of minimal residual disease. Ruggiero *et al*. (5) demonstrated that potential candidates for these factors generating the CIM phenomenon might be related to low molecular weight agents such as tyrosine isomers. We hypothesized that factors similar to CSF-GM might be associated to the CIM effect in our SL2 model. However, there was no CSF-GM increase in the blood or ascitic fluid in late stage TBM in our study. The paraneoplastic leukemoid reaction in humans is considered to be associated with the overproduction of hemopoietic factors by tumors or following host-tumor interaction (22-24). The inability to detect CSF-GM protein by ELISA did not mean that there is no colony-stimulating activity in murine serum, which can be observed using a GMC-FC bioassay (25). Some studies have demonstrated that murine tumors are able to elicit leukemoid reactions alongside concomitant immunosuppression (22, 26). No thymus subset analysis was reported in these studies. Notably, cytokines similar to CSF are able to elicit contrasting effects: a) pro-tumorigenic or b) therapeutic (22). Some pro-tumorigenic effects of CSF-GM and CSF-G were attributed to the activity that promote tumor vascular expansion (27). Human recombinant CSF-GM as an adjunct to intensive chemotherapy in patients are currently used to facilitate recovery of severe chemotherapy-induced myelosuppression. In our study, the hematological effects of TBM-11 might be associated with multiple factors secreted by the tumor itself or as a result of tumor-host interaction. The factors eliciting the antitumor effect and promoting myelopoiesis might not be the same.

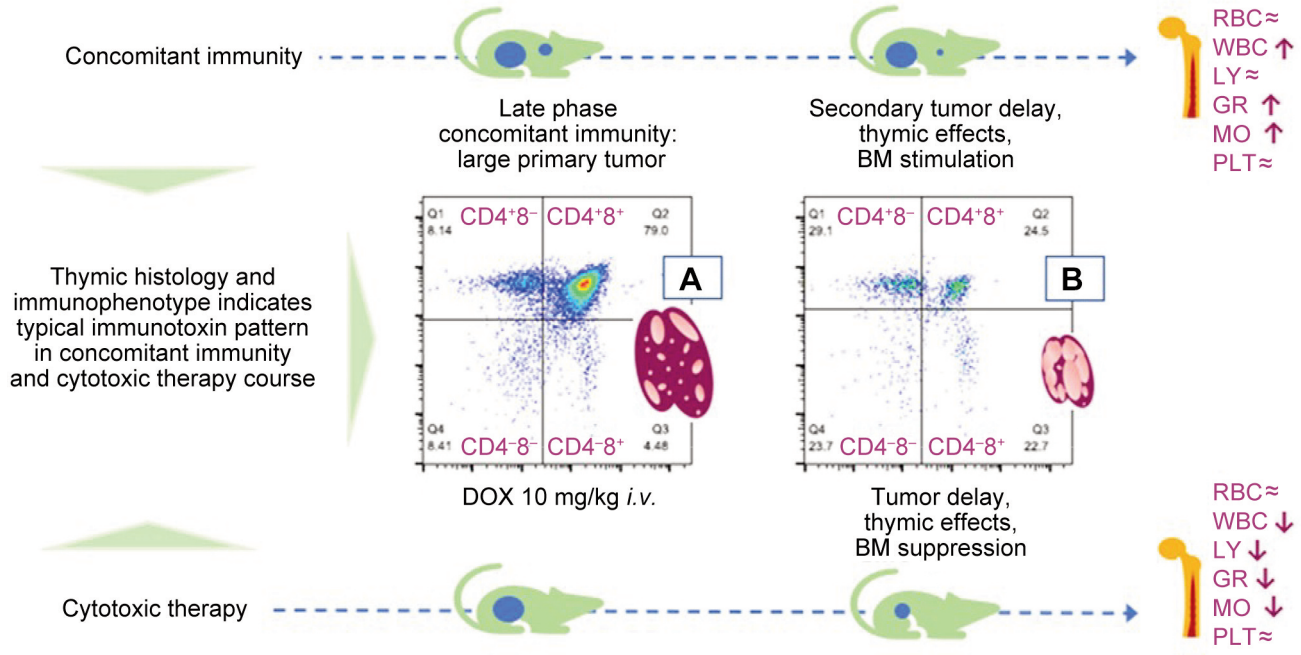

Figure 2. *Large tumor [(day 11 tumor-bearing mice (TBM)] and cytotoxic drug doxorubicin (DOX) induce identical changes in the thymus and opposite alterations in peripheral blood. Before entering late-stage tumor and at the moment of DOX administration, (A) thymus exhibit normal histopathology and characteristic CD4/CD8 staining with no hematological effects. Cortical depletion with double positive decrease is detectable 72 h after DOX administration or in TBM-11 animals (B). However, myelosuppression induced by DOX is not seen at any stage of TBM. At later stages, TBM exhibit characteristic leukemoid reaction.*

The non-specific component of CIM is known to be unrelated to the T-cell-dependent cytotoxic mechanisms, involved in a classical immunological rejection (5). The serum from mice bearing non-immunogenic tumors exhibited a growth-inhibitory activity proportional to the intensity of CIM (8). The low molecular weight agents, which were initially identified in the serum of CIM, might be used as anti-metastatic agents. For instance, an antimetastatic capacity with minimal myelotoxicity was demonstrated in a racemetyrosine phase II clinical trial (28). This agent is somewhat similar to the group of low molecular weight drug candidates detectable in CIM models (5).

The concept of metastatic dissemination occurring at the late stage of tumor development is currently being challenged (1). Disseminated metastatic cells may evade immune surveillance and survive in a state of dormancy that is influenced by the organ microenvironment. During this time, the growth of cells is paused and micrometastatic deposits survive to enter the acceleration phase in response to external stimuli, sometimes decades later (29). The exact mechanisms of this phenomenon are not entirely elucidated but several factors are known to be involved. For instance, surgical tumor manipulation can result in a manyfold rise in circulating tumor cells (2, 3). Surgery may prompt immune evasion by triggering down-regulation of cellular responses (3). The increase in tumor growth after surgery was also associated with macrophage dysfunction, which was proportional to the extent of surgery (29). Surgery-induced increases in the levels of growth or angiogenetic factors might provoke an accelerated progression of undetectable dormant metastases (26). Transfusions, general anesthesia, postoperative infectious complications, inflammatory or coagulative dysfunction can additionally activate surgery-related relapse or metastatic dissemination (30). All these data are rather contradictory and further investigation of multiple components responsible for the CIM effect are needed. CIM is possibly "not working" in some cases and the existence of the "concomitant enhancement" phenomenon in some models has also been advocated. For instance, post-operative regression, although rare, has been reported in certain cases of metastatic disease (10, 11). A similar effect was reproduced in murine models (6). The most frequently reported clinical cases of regression were linked to pulmonary metastases following nephrectomy. Paradoxically, even these cases might not contradict the concept of metastasis control by the CIM effect. The CIM-related soluble factors might be working in a pattern of sinecomitant immunity, which has been demonstrated to exist following excision of a primary tumor (6). Sinecomitant immunity is defined as a host resistance lasting

for a certain period of time and develops against a second tumor challenge after excision of the primary tumor. Sinecomitant inhibition was postulated to be antigen-specific, probably representing an extension of a specific component of concomitant immunity (6). Therefore, lung metastasis regression following nephrectomy can also be attributed to CIM factors still circulating in the blood after tumor excision (31). Serum factors released into circulation before tumor extirpation might persist in the blood for several months after surgical intervention. Notably some cytotoxic tumor drugs used in cancer chemotherapy can remain in tissues for prolonged periods after discontinuation of therapy. Interestingly, platinum compounds administered some 20 years ago can be tracked in the circulation of testicular cancer survivals (32). The duration of retention of many cytotoxic drugs for several years after treatment in tissues is well reported (33, 34). Extirpation of primary tumor does not necessarily abruptly remove the residual activity of potential CIM-related serum factors. The significantly higher content of non-dividing cancer cells persisted in a study of mice that had primary tumor compared to mice that did not (6). This study revealed that a CIM induced by the primary tumor can maintain cancer cell dormancy or mitigate metastatic spread rather than be directly tumoricidal. The extirpation of a primary tumor might turn into a signal triggering the proliferation of micrometastases that were dormant before surgical intervention. However, a significant impact of residual circulating factors following a surgical procedure might correct the postoperative outcomes. Interestingly, more than 70% of early-stage prostate cancer patients are known to have preoperative bone marrow dissemination, regardless of stage, Gleason score, PSA, or any evidence of systemic disease (35). The primary tumor in these cases might play an important role in delaying or retarding the growth of secondary deposits. However, identification of tumor-associated CIM soluble factors especially in the human system is rather difficult. Here, we propose to use the fragile cortical thymocyte as a test system to screen and identify CIM-related factors.

Histopathological thymocyte analysis can be a strait forward indicator of cytotoxic factors released into the circulation. Somewhat similar findings in different tumor models have also been reported. Our thymocyte data with SL2 tumor in DBA/2 mice appeared to be identical to changes induced by DOX in C57/6BL mice (17). However, we did not see a significant increase in numbers of Hassall corpuscles (HC) neither in DOX injected mice including C57/6BL nor in SL2 bearing DBA2 mice as it was reported for DOX injected B6C3 mice (18). HC association with DOX-induced senescence might be a finding which we did not duplicate in our study. We assume that the structures classified as HC can be quantitated and applied in CIM-related factor screening if rodent strain and age are considered. The occurrence of HC is rare in rodents and is strain-dependent in mice. Immunohistochemical analysis of intracellular cytokines or other molecules present in medullar cells – dendritic, neutrophils, HC, and others – might be an additional tool in testing CIM factors with antitumor activity. We observed similarities between alterations caused by TBM-11 and DOX only in classical thymocyte subsets: double-positive CD4+CD8+, double negative CD4-8-, and single positives CD4+8- or CD4-8+. The signature of drug or tumor-induced rearrangements in the thymus might be significantly extended if multiple other immunophenotype characteristics are considered. For instance, the CD4+CD25+FOX3+ thymic subset in a rapamycin study (19) or CD25, CD44, CD24, CD5, and CD25 markers (20) were also explored as indicators of thymocyte toxicity. Some mechanisms of cortical thymocyte depletion might be specific to certain chemotherapeutic drug. However, the final outcome is always simple cortical thymocyte depletion. We propose that thymocyte apoptotic fragility can be explored as a reliable test system for CIM factor screening and identification.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

GZ, DCh, AD and VP participated in the study design, carried out all experiments and drafted the manuscript. JJ and LZ performed flow cytometry analysis. KK, MJ and MVZ assisted in cell handling, histopathology, and hematological analysis. All Authors read and approved the final manuscript.

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