Atrophied Thymus, a Tumor Reservoir for Harboring Melanoma Cells

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Abstract

Tumor metastatic relapse is the primary cause for cancer-associated mortality. Metastatic relapse is believed to arise from quantities of tumor cells that are below detectable thresholds, which are able to resist radio/chemotherapy by obtaining a dormant state and hiding in certain organs, i.e., tumor reservoirs. The thymus, a central T-cell immune organ, has been suggested to be a premetastatic tumor reservoir for B-lymphoma cells. However, it remains unknown whether the thymus is able to harbor nonlymphoid solid tumor cells, and whether chemotherapy can thoroughly eliminate cancer cells in the thymus. If chemotherapy is not able to eliminate these cells in the thymus, then what processes allow for this? Melanoma cell–inoculated and genotoxic doxorubicin-treated mouse model systems were used to determine that the thymus, particularly the atrophied thymus, was able to harbor blood stream–circulating melanoma cells. In addition, a chemotherapy-induced DNA-damage response triggered p53 activation in nonmalignant thymic cells, which in turn resulted in thymocyte death and thymic epithelial cell senescence to develop an inflammatory thymic microenvironment. This inflammatory condition induced thymic-harborred minimal tumor cells to acquire a chemoresistant state.

Implications: Here, the thymus serves as a premetastatic reservoir for nonlymphoid solid tumor cells during chemotherapy, which could be a novel target of minimal residual disease in antitumor therapy, thus preventing tumor metastatic relapse. Mol Cancer Res; 1–13. ©2018 AACR.

Introduction

Tumor metastatic relapse at distant organs several years after removal of the primary tumor and adjuvant chemotherapy poses a clinical challenge. Metastatic relapse results in a poor prognosis and is responsible for the majority of cancer-associated mortality (1, 2). There is a period between primary cure and metastatic relapse, which may be defined as a remission period, when neither symptoms nor cancer cells are detected. It remains unclear where the cancer cells hide and in what physical state they are, as well as why adjuvant chemotherapy is unable to thoroughly eradicate these undetectable tumor cells during the remission period. Emerging evidence has revealed that a few cancer cells still survive in certain organs of the body during the remission period. The period of time during which these small numbers of cancer cells in the patient survive during chemo/radiotherapy is defined as minimal residual disease (MRD), while these cancer cell–harboring organs may be defined as premetastatic niches/reservoirs. Bone marrow (BM) has been determined to be a premetastatic reservoir for disseminating malignant cells (3–7). The perivascular space of blood vessels in the lungs and liver has also been identified as these kinds of cancer niches/organs (8, 9). Recently, the thymus has also been identified as a tumor reservoir for lymphoid cancer cells (lymphomas; refs. 10, 11). We ask whether the thymus, the largest T-cell lymphoid organ in the body, can play a role as a premetastatic reservoir for nonlymphoid solid tumor cells during chemo/radiotherapy, and if so, why and how the thymus induces its harbored tumor cells to resist chemo/radiotherapy.

The thymus is a primary lymphoid organ responsible for generating functional naïve T cells and establishing self-tolerance. It undergoes a progressive and age-related involution/atrophy, attributed to the deterioration of the thymic microenvironment (12), which is composed of an integrated three-dimensional meshwork of thymic epithelial cells (TEC) and thymocytes. Previously, the thymus was paid scant attention as a cancer premetastatic reservoir. This may be due to the use of immuno-deficient athymic animal models, such as nude mice, in most cancer studies. These models with a primary immunodeficiency cannot mimic the natural processes of tumor development and immune suppression bona fide. Furthermore, the thymus is very sensitive to any physical and chemical assault, particularly chemotherapeutic toxins and radiation, which contribute to thymic atrophy and induce increased proinflammatory factors, such as IL6, thereby potentially becoming a hospitable environment for harboring tumor cells (10, 11). Solid tumor cells can enter circulation as circulating tumor cells (CTC; refs. 7, 13) and disseminate to distal organs (9), including the thymus (14).

It is well known that inflammation is a double-edged sword that is necessary for antitumor responses (15–17), but also induces tumor drug resistance (18–20). For example, the IL6-rich BM microenvironment facilitates chemoresistance...
in multiple myeloma by up-regulating various prosurvival proteins (21); and chemotherapy-induced IL6 secretion is also involved in the ability of cancer cells to acquire stem-like characteristics, i.e., generating cancer stem cells (22, 23) that possess innate resistance mechanisms to chemo/radiotherapy (24, 25). The stemness-associated features are potentially due to activation of antiapoptotic features (26) and induction of the cancer cell–intrinsically quiescent state of G0 to G1 arrest (27). The chemotherapy-resistant feature is attributed to microenvironment-induced tumor cell changes in gene expression profiles. For example, some genes are turned on, such as MAPK p38, while others are turned off, such as MAPK ERK. A high ratio of p38/ERK (activation of p38 and inhibition of ERK) induces tumor growth arrest (dormancy: a risk for cancer recurrence), while a high ERK/p38 ratio favors tumor regrowth (28–30).

In this report, we first focused on determining whether the thymus is able to retain circulating solid nonlymphoid melanoma cancer cells. We then addressed how the atrophied thymic microenvironment becomes a suitable tumor reservoir during chemotherapy. Finally, we demonstrated how thymic-harbored melanoma cells acquired chemoresistance by exposure to the inflammatory factor–rich thymic microenvironment. We determined that when the thymus (lymphoid organ) is in an inflammatory condition, it is able to harbor circulating nonlymphoid solid cancer cells. This inflammatory microenvironment results from chemotherapy-induced DNA-damage responses (DDR), thereby triggering p53 activation in thymic cells. This, sequentially, results in thymocyte death and TEC senescence. Thus, an inflammatory condition is developed to facilitate thymic-harbored minimal tumor cells to acquire an antiapoptotic predominant chemoresistant feature. Together, our results have identified a novel premetastatic cancer reservoir: the thymus. We bring this novel target into the focus for antitumor therapy of nonlymphoid solid tumor cells to combat potential metastatic relapse.

**Materials and Methods**

**Mice and animal care**

All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, in accordance with guidelines of the NIH. C57BL/6 wild-type (WT) young (1–3 months old) and aged (≥18 months old, purchased from the rodent colonies of National Institute on Aging) were used. Two gene manipulated mouse colonies were also used: (i) FoxN1 conditional knockout (termed FC) mice. Once the FoxN1-flxed gene is deleted via CreERT activation by induction with three intraperitoneal (i.p.) injections of tamoxifen (TM), the thymus becomes atrophied (details in our previously published paper, ref. 31); (ii) immunodeficient NSG (NOD. Cg-Prkdc-scid, and il-2rγ<sup>−/−</sup>) mice.

**Tumor cell lines and GFP transduction, the generation of circulating tumor cell condition in mice, and subcutaneous implantation of tumor-bearing tissues**

Mouse melanoma B16F1 cells (ATCC, CRL-6323, simply termed "B16") were transduced with enhanced green fluorescence protein (eGFP) lentivirus particles containing neomycin resistance gene. On 80% confluent B16 cells, cultured in a 24-well plate with 1 mL of complete DMEM medium containing 5 μg/mL of polybrene, 12 μL of eGFP lentiviral particles (>1 × 10<sup>6</sup>, from GeneCopoeia, Cat#: LPP-EGFP-LV151-100-C) were added. Three days later, the GFP<sup>+</sup> cells were visualized by fluorescence microscopy, and GFP<sup>+</sup> cells were sorted via Influx Cell Sorter (BD Biosciences). GFP-expressing B16 cells were cultured in complete growth medium DMEM, supplemented with 500 μg/mL of G418 (for neomycin maintenance of the cells). When the confluence was about 80% to 90%, the cells were dissociated for single-cell suspension with 0.25% Trypsin-1 mmol/L EDTA solution followed by washing with 1 × PBS twice. This single-cell suspension was used for intravenous (i.v.) injection (1 × 10<sup>6</sup> cells/mouse) through retro orbital route to mimic a circulating tumor cell condition in mice. The thymus was isolated from cancer cell–inoculated mice and cut into tissue blocks. The tissue blocks were subcutaneously transplanted into immunodeficient NSG mice under the dorsal skin. Three to 4 weeks after the transplantation, the mice were sacrificed, and tumor was visualized under the skin.

**Tumor recurrence in vitro assay**

WT mice received i.v. inoculation with 1 × 10<sup>6</sup> B16-GFP melanoma cells. Three days later, the mice were treated with doxorubicin (Doxo) or PBS. The thymus, lymph nodes (LN), and lungs were adjusted to similar weight and individually cultured in a plate, and 10 to 14 days later, the GFP<sup>+</sup> cells were visualized and semiquantitatively measured with Image software.

**Flow cytometry**

To analyze the percentage of cancer cells in the thymus and LNs, single-cell suspensions were prepared using enzymatic digestion. Freshly isolated tissues were torn apart and digested at 37°C in DNase-I/Collagenase V solution, as previously described (32). The single-cell suspensions were then stained with specific antibodies on cell surface markers and/or intracellular GFP, phosphorlated-p53, or Ki67, and fixed with 2% PFA for 1 hour and permeabilized with 0.1% Triton X-100, as previously reported (32). Fluorochrome-conjugated antibodies against CD45 (30-F11), MHC-II (M5/114.15.2), EpCam (G8.8) were purchased from BioLegend. The anti-GFP (BioLegend, Cat #338002), antiphosphorylated p53 antibody (Ser15, Cell Signaling Technology, Cat #9284) with secondary antibody against CD45 (30-F11), MHC-II (M5/114.15.2), EpCam (G8.8) were purchased from BioLegend. The substrate was TMB (3,3′,5,5′-tetramethylbenzidine) and the absorbance was measured at 450 nm with the BioTek ELx800 ELISA reader.
Annexin-V–based and caspase-3–based apoptotic assays in thymocytes, TECs, and/or cultured B16 tumor cells

Thymocytes and TECs were freshly and enzymatically isolated from Doxo- or PBS-treated young WT mice. Cells were stained for surface markers, washed, and incubated in APC–Annexin-V antibody (BioLegend, Cat #640920) at a 1:20 dilution with Annexin-V buffer (10 mmol/L Hepes adjusted to pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl2) for 15 minutes at room temperature, followed by flow-cytometric analysis. In addition, we did caspase-3–based apoptotic assay with flow cytometry to confirm Annexin-V results, in which cleaved caspase-3 (Asp175) monoclonal antibody (Cell Signaling Technology, Cat #95791) and second antibody (Alexa Fluor 488–conjugated anti-rabbit IgG, Zenon, Cat #Z-25302) were used for intracellular staining. Apoptotic positive control cells were prepared by incubating cells at 55°C for 20 minutes to induce cell death before staining. Details were described in our recent publication (33).

Cryosections for immunofluorescence (IF) or SA-β-Gal (senescence) staining

Cryosections (5–6 μm thick) were stained as described previously (32). The tissue was fixed with acetone, and then stained with various antibodies. The primary antibodies used were anti-K8 (Troma-1 supernatant), anti-GFP (B2; Santa Cruz Biotechnology, Cat #sc-8996), anti-p21 (C-19; Santa Cruz Biotechnology, Cat #sc-397), anti-p16 (F-12; Santa Cruz, Cat #sc-1661), or anti-Tap63 (N-16; Santa Cruz Biotechnology, Cat #sc-8609). Based on primary antibody species, the secondary antibodies used were Cy3-conjugated or Alexa Fluor 488–conjugated donkey anti-rabbit or anti-rat IgG (Jackson ImmunoResearch Lab) or (Invitrogen, Cat #Z-25302). For senescence-associated β-galactosidase (SA-β-Gal) staining, cryosections of young and aged, as well as FC mouse thymus tissues (8 μm thick), were analyzed for SA-β-gal activity using a senescence β-Galactosidase staining kit according to the manufacturer’s protocol (Cell Signaling Technology, Cat #9860) and counterstained with nuclear fast red (RICCA Chemical #R5463200) solution.

Western blot analysis of p53 expression

The whole thymus was subjected to homogenization and protein extraction in RIPA lysis buffer (Sigma, Cat #R0278), containing 1× protease inhibitor cocktail (Sigma, Cat #P8340) and 1× phosphatase inhibitor cocktail (Sigma, Cat #P0044). Protein, ~30 μg/lane, was loaded under reducing conditions for a direct Western blot assay with anti-phosphorylated p53 antibody (Ser15; Cell Signaling Technology, Cat #9284) and anti-total p53 antibody (Santa Cruz Biotechnology, Cat #sc-6243), respectively. Housekeeper GAPDH or β-actin was used as an internal loading control. Positive protein bands were visualized through SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Cat #34095) and scanned by a C-Digit Scanner (LI-COR).

Transwell cell culture and in vitro cell death and proliferation analysis

B16 cancer cells with 10% to 30% confluence were treated with 3 μmol/L Doxo for 8 hours. Thymuses of young C57/BL6 mice injected with PBS or Doxo (2 times for 10 mg/kg mouse weight) were placed on the membrane of the transwell and cocultured for 3 to 5 days. Then, cancer cells were detached from the bottom of the plate using a nonenzymatic dissociation solution (Sigma, Cat #c5789) and used for further flow-cytometric analysis of Annexin-V–based apoptosis and/or Ki67 (BioLegend, Cat #652404) proliferation assay, and/or evaluation of dormancy phenotype by flow-cytometric analysis using p-p38/p-ERK ratio (Cell Signaling Technology, Cat #4551S and #4375S).

Statistical analysis

For evaluation of group differences, an unpaired two-tailed Student t test was used assuming equal variance. Differences were considered statistically significant at values of *, P < 0.05 and **, P < 0.01.

Results

The thymus, particularly the atrophied thymus, can harbor melanoma (nonlymphoid solid tumor) cells with a regrowth capacity

In order to determine whether the thymus, a lymphoid organ, was able to harbor circulating nonlymphoid solid tumor cells, we i.v. injected mouse melanoma cells (B16 cells transduced with enhanced green fluorescent protein, eGFP) into immunocompetent WT mice, which arbitrarily mimics the situation in which circulating solid tumor cells (13) disseminate to distal organs (9). Seven to 10 days after the inoculation, we were able to detect GFP+ melanoma cell clusters in the normal thymus (Fig. 1A, top). Although thymic tissues usually show autofluorescence signals, we can easily distinguish between this and the PBS-inoculated control mice (PBS-Ctr; Fig. 1A, bottom) and the B16-GFP+ cell-inoculated mice (Fig. 1A, top). To confirm whether these GFP+ cell clusters were the inoculated GFP+ melanoma cells, we isolated the thymuses from both B16-GFP and PBS-Ctr group mice and transplanted these thymuses (either cancer cell–bearing or control) into immunocompromised NSG (NOD. Cg-Pkdcl-scid, and il-2reg−/−) mice subcutaneously (procedure shown in Fig. 1B, left). Around 4 weeks after the transplantation, we found that tumors developed under the skin of the NSG mice transplanted with the thymus from melanoma-inoculated mice (Fig. 1B, right), but we did not find any developed tumor until 8 weeks after the graft in the NSG mice transplanted with the thymus of PBS-Ctr mice (image not shown). This evidence suggests that the body’s largest T lymphoid organ, the thymus, is able to harbor nonlymphoid solid tumor cells, in addition to lymphoid fluid cancer cells (10, 11), with a regrowth capacity.

The thymus undergoes atrophy generating a proinflammatory microenvironment once it receives any physical and chemical assaults, including chemotherapy. We wanted to determine whether the atrophied thymus was more hospitable for harboring nonlymphoid solid tumor cells. We compared two types of atrophied thymuses. One is the FoxN1 conditional gene knockout (FC) thymus in adult young mice, which has an inducible defect in TEC homeostasis (31). The other is naturally aged thymus from over 18-month-old mice. With the same strategy as in Fig. 1A, we found that both types of atrophied thymuses were able to harbor higher proportion of melanoma cells than the normal young thymus did with analyses of both flow cytometry (Fig. 1C) and immunofluorescence staining (Fig. 1D). This phenotype can also be observed in the atrophied thymus of FC mice with i.v. inoculation of lymphoid lymphoma cells (Supplementary Fig. S1). These results indicate that the microenvironment in the atrophied thymus is a particularly suitable environment for harboring both lymphoid and nonlymphoid solid cancer (melanoma) cells.
Chemotherapeutic drug induces thymic atrophy to generate an inflammatory microenvironment, attributed to a combination of thymocyte death and TEC senescence.

The capacity of the atrophied thymus to harbor malignant tumor cells with the ability for regrowth is related to the thymic inflammatory microenvironment (10, 11), which results from chemoradiotherapy. Chemotherapeutic drugs can induce cell stress-associated DDR, resulting in cell death or and senescence (34, 35). In the thymus, chemotherapeutic drugs impact on not only malignant tumor cells, but also potentially nonmalignant thymic cells, which consist of hematopoietic thymocytes and nonhematopoietic TECs (36). To verify whether chemotherapy could induce thymic atrophy, and how inflammatory thymic microenvironment was generated, we treated mice with a commonly used antitumor genotoxic drug Doxo to observe changes in the young healthy murine thymus. As shown in Fig. 2A, mice were inoculated with mouse GFP-transduced B16F1 (termed B16-GFP) melanoma cells (1 x 10^6 per mouse) or PBS for control (PBS-Ctr), 1 week after the inoculation the thymuses were examined.

**Figure 1.** The thymus, particularly the atrophied thymus, is able to harbor melanoma (nonlymphoid tumor) cells with a capacity for regrowth. Mice were i.v. inoculated with mouse GFP-transduced B16F1 (termed B16-GFP) melanoma cells (1 x 10^6 per mouse) or PBS for control (PBS-Ctr), 1 week after the inoculation the thymuses were examined. A, Thymic cryosection (from young WT mice) fluorescence staining shows one representative result of GFP^+ cells in the B16-GFP-inoculated thymus (top) but not in the PBS-Ctr thymus. The data are representative of 5 biological replicates in each group with essentially identical results. B, Left, Experimental schema of thymic tissues from WT mice in A to NSG mice. Right, Tumor regrowth from the thymic tissue of B16-GFP-inoculated WT mice under the NSG mouse skin. However, no tumor growth was observed from the thymic tissues of PBS-Ctr mice (image is not shown). The image is a representative result from at least three independent experiments (n = animal numbers). C, Left, Flow-cytometric plots show gates of thymic-harbored B16-GFP melanoma cells in the thymuses of three types of mice (from left to right): Young WT, FoxN1 conditional gene knockout (FC), and WT naturally aged mice. Right, A summarized result of % GFP^+ cancer cells in the thymuses shown in a bar graph. A Student t test was used to determine statistical significance between groups, and data are expressed as mean ± SEM. D, Freshly isolated thymic cryosections from 3 groups of mice (same as C) were stained with fluorescence antibodies and visualized. One of the representative results shows GFP^+ cells in the B16-GFP-inoculated thymuses (top), but not in the PBS-Ctr thymuses (bottom). Data are representative of 3 biological replicates in each group with essentially identical results.
The last injection, the thymuses were collected for analysis. The thymuses in Doxo-treated mice were dramatically reduced in size, weight, and total thymocyte numbers (Fig. 2A). We also found that an increase in proinflammatory cytokines in the thymuses of the two groups. A summarized result of three types of proinflammatory cytokines in the thymuses of the two groups. Left, Concentration of cytokine product in pg/mg of thymic protein; right, relative production in fold changes (baseline set as the average of cytokine concentrations in PBS-treated group as 1). A Student t test was used to determine statistical significance between two groups. All data are expressed as mean ± SEM. The P values are shown in each panel, and each symbol represents an individual animal sample.

We hypothesized that the Doxo-induced inflammatory thymic microenvironment should be attributed to thymic cell death and/or cellular senescence. Then, we checked these parameters in the thymuses of Doxo-treated and PBS-treated (PBS-Ctr) young WT mice (Fig. 3). Using Annexin-V-based apoptotic assay, we found that apoptosis in thymocytes from Doxo-treated mice was increased, while apoptosis in TECs was not, compared with their PBS-Ctr counterparts (Fig. 3A, left). In order to confirm this result, we checked cleaved caspase-3 in these cells. Activation of caspase-3 plays a central role in the execution-phase of cell apoptosis induced by either intrinsic (via p53) or extrinsic (via TNF receptor) apoptotic pathways (37). The results were consistent with our Annexin-V-based apoptosis assay (Fig. 3A, right). We further asked what happened in TECs after Doxo-treatment. We examined a senescent phenotype in these TECs, with a positive control group of 8-month-old FC thymus that has increased TEC senescence (32). We noted that two senescence-associated molecules, p21 (CDKN1A) and p16INK4A, were increased in the thymuses of Doxo-treated mice (Fig. 3B). To confirm this, we performed SA-β-gal staining (a senescence marker) of thymic cryosections and observed that a senescent phenotype had developed in the thymus of Doxo-treated mice (Fig. 3C). To verify the senescent phenotype was in the TEC population, we stained the thymic cryosections with TAp63 marker, which is related to senescence and is expressed only in TECs (32). Expression of TAp63 was indeed increased in the thymuses of Doxo-treated mice (Fig. 3D). In addition, we costained the thymic cryosections with TAp63 and p21 and confirmed an almost complete colocalization of TAp63 (increased expression in senescent TECs) versus p21 (senescence-associated molecule; Supplementary Fig. S3), which we reported in our previous publication (32). This further proves that senescence occurs in TECs during chemotherapy. Therefore, the finding that Doxo-treatment resulted DDR-induced changes in the thymus have a feature of apoptosis mainly in thymocytes and senescence mainly in TECs has been confirmed.

We know that nonmalignant thymic cells mainly include two populations: hematopoietic thymocytes and nonhematopoietic TECs. Our results reveal that the chemotherapeutic drug affects both thymic cell populations, and the proinflammatory condition is synergistically composed of both increased cell death in thymocytes accompanied by increased cellular senescence in TECs. Increased cellular senescence may be further involved in the senescence-associated secretory phenotype (SASP; refs. 38, 39) to participate and/or enhance the development of a thymic proinflammatory condition, and senescence-associated IL6 and IL8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities (40).
Figure 3.
Chemotherapy (Doxo-treatment) induces increased apoptosis in thymocytes and senescence response in TECs. Mice were treated with 3 consecutive i.p. injection Doxo (10 mg/kg) or PBS once a day. Three days after the last injection, the thymuses were collected for analysis. A, A summary of apoptotic analysis of thymocytes (gated on the CD45^+EpCam^-/CD45^-EpCam^+ population) and TECs, gated on the CD45^-/EpCam^+ population) with Annexin-V assay (left) and cleaved caspase-3 assay (right), respectively. Relative fold changes were based on setting an average of % Annexin-V^+ cells or % cleaved caspase-3^+ cells in PBS-Ctr thymocytes and TECs as 1, respectively. All data are expressed as mean ± SEM. The P values are shown between two compared groups, each symbol represents an individual animal sample.

B, Representative thymic cryosections with immunofluorescence staining show the images of keratin-8 (counterstaining) vs. p21 in the top row and p16^INK4A in bottom row from the thymuses of three types of mice. Eight-month-old FC thymus served as aged control because it is similar to the 18-month-old naturally aged thymus (33).

C, Representative images of thymic cryosections with SA-β-gal staining (blue clusters) vs. nuclear fast red counterstaining from three types of thymuses as B. D, Representative fluorescence images of thymic cryosections with TAp63 (a senescent TEC marker; ref. 32) staining (red clusters) vs. keratin-8 counterstaining from three types of thymuses as B. Arrows in B-D show typical positive cell clusters. Image data are representative of 3 to 4 animals in each group with essentially identical results. The rightmost column is semiquantitative data obtained via ImageJ software, and each symbol represents the ratio of % positive area per scope (9–14 tissue scopes were recorded for each animal) of the tissues from 3–4 animals in each group.
Changes in thymic microenvironment are potentially correlated with DDR-triggered activation of p53 gene

To determine the potential mechanism of drug-induced thymocyte apoptosis and TEC senescence, we hypothesized that p53 gene would be involved in these processes based on its function in promoting both cellular apoptosis and senescence (41, 42), as well as the fact that activation of p53 is commonly triggered by the cell stress--associated DDR (34, 43). To verify its correlation to chemotherapy-induced thymic cellular apoptosis and senescence here, we first measured phosphorylated (activated) p53 (P-p53) and total p53 in the thymus from Doxo-treated mice with Western blot assay and found both were increased compared with those from PBS-Ctr mice (Fig. 4A). Then, we determined which thymic cell populations had increased P-p53 with flow-cytometric analysis. We found that Doxo-treatment increased the percentage of P-p53+ cells in both hematopoietic thymocytes and nonhematopoietic TECs (Fig. 4B and C). However, it exhibited uniform increase in TECs (small standard deviation), but a heterogeneous increase in thymocytes (a large variability) of the Doxo-treated mice (indicated by a broken-line circle in Fig. 4C). Our results demonstrate that both increased cell death and development of senescence in the thymus during chemotherapy are associated with activation of p53 in nonmalignant thymic cells, which is triggered by antitumor drug-induced DDR stress.

Inflammatory thymic microenvironment of chemotherapeutic drug-treated mice confers tumor cells toward a chemoresistant phenotype

Based on evidence that the inflamed atrophied thymus is able to harbor nonlymphoid solid cancer cells during chemotherapy, we wanted to know why the chemotherapy is not able to completely eradicate the thymic-harbored cancer cells and whether these thymic-harbored tumor cells are conferred a chemoresistant phenotype by this inflammatory condition. We inoculated B16-GFP melanoma cells (1 × 10^6 per injection) to young WT mice, and 3 days after the inoculation, we treated these tumor-bearing mice with Doxo for 3 days (8–10 mg/kg, once a day for 3 consecutive days). Three days after the last drug treatment, we compared the tumor cells in the thymus and LNs and found that the ratio of percentage of melanoma cells in thymus versus lymph nodes was increased (Fig. 5A), implying that chemotherapy killed cancer cells more efficiently in the LNs than it did in the thymus. In other words, cancer cells harbored in the thymus were more resistant to chemotherapy. Because Doxo treatment usually results in autofluorescent interference during flow-cytometric assay in Peridinin Chlorophyll Protein Complex (PerCP) and Alexa Fluor 488 (AF488) channels (Supplementary Fig. S2), we made an effort to avoid using PerCP-conjugated antibodies, and strictly set up a cutoff for the positive population for the GFP channel. In addition, we directly visualized the inoculated GFP+ melanoma cell clusters with immunofluorescent staining of thymic cryosections from mice with the two treatments (Doxo or PBS) and confirmed that a proportion of these clusters was increased in the thymus from Doxo-treated mice (Fig. 5B). In order to further confirm that chemotherapy has little effect on the thymic-harbored cancer cells, we compared the absolute melanoma cell numbers using a flow-cytometric approach (Fig. 5A, left) after inoculation and treatment with/without Doxo. We observed that the absolute cell numbers of the GFP+ melanoma cells in the thymuses from Doxo-treated mice were not reduced (Fig. 5C). The results indicate that the chemotherapeutic drug treatment induces thymic atrophy and dramatically reduced thymic mass, but this did not significantly affect numbers of thymic-harbored cancer cells.

We next attempted to investigate whether the chemotherapeutic drug was able to kill melanoma cells equally in the thymus, LNs, and lungs. The lungs are the most suitable metastatic site for melanoma (44). We inoculated B16 melanoma cells into young WT mice and then treated the mice with Doxo or PBS twice (10 mg/kg, once a day for 2 consecutive days). Three days after the last drug treatment, we isolated the thymus, LNs, and lungs, and cultured these three tissues using PerCP-conjugated antibodies, and strictly set up a cutoff for the positive population for the GFP channel. In addition, we directly visualized the inoculated GFP+ melanoma cell clusters with immunofluorescent staining of thymic cryosections from mice with the two treatments (Doxo or PBS) and confirmed that a proportion of these clusters was increased in the thymus from Doxo-treated mice (Fig. 5B). In order to further confirm that chemotherapy has little effect on the thymic-harbored cancer cells, we compared the absolute melanoma cell numbers using a flow-cytometric approach (Fig. 5A, left) after inoculation and treatment with/without Doxo. We observed that the absolute cell numbers of the GFP+ melanoma cells in the thymuses from Doxo-treated mice were not reduced (Fig. 5C). The results indicate that the chemotherapeutic drug treatment induces thymic atrophy and dramatically reduced thymic mass, but this did not significantly affect numbers of thymic-harbored cancer cells.

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Figure 4.

Chemotherapy (Doxo-treatment) induces activation of p53 in the thymus. With the same treatment as those in Fig. 3. A, Western blot analysis of phosphorylated p53 (P-p53; left) and total p53 (right) in the thymuses of the two groups. B, Flow-cytometric gate strategy of thymocytes (CD45+MHC-II-***+) and TECs (CD45+MHC-II**), as well as P-p53+ cell populations. C, A summary of percentages of P-p53+ cells in TECs and thymocytes of PBS-Ctr and Doxo-treated groups, respectively. A Student t test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. The P values are shown in each panel, and each symbol or "n" represents an animal or animal numbers.
adjusted to similar weight in separate plate wells for about 2 weeks. On the final day of the culture, we visualized the cancer cell regrowth via GFP clusters under the microscope (Fig. 6A) and semiquantitatively measured these green cellular clusters with ImageJ software (Fig. 6B). We found that the inoculated cancer cells in both the thymic and lung tissues from mice with chemotherapy could regrow, but regrowth of cells from the thymic tissues was greater than that from the lung tissues. We set up the baseline for our calculations as follows: the percentage of GFP+ cell cluster area in PBS-treated mice as 100% (because the cancer cells in cancer cell–bearing tissues without drug treatment should fully regrow). The percentage of GFP+ cancer cell cluster area in Doxo-treated mice was evaluated in comparison with the 100% regrowth baseline in the corresponding tissue with PBS-treatment using ImageJ software (Fig. 6B). In addition, we seldom found any cancer cell regrowth from the LN tissues of Doxo-treated mice.

Taken together, our results suggest that the chemotherapeutic drug is not able to equally kill cancer cells in the thymus, LNs, and lung. Particularly, the thymus from Doxo-treated mice harbored more cancer cells. This is due to an inflammatory environment, which may direct the harbored cancer cells toward a chemoresistant feature. Although these cancer cells cannot be completely eradicated in the thymus, they are unlikely to develop a tumor in the thymus, because we never observed thymoma development in these mice. Therefore, the atrophied thymus is only a potential tumor reservoir. The cancer cells in the lungs were also not completely eradicated, in accordance with previous findings identifying the lungs as a known tumor reservoir (9).
Thymus from chemotherapeutic drug-treated mice potentially modulates the thymic-harbored cancer cells to acquire an antiapoptotic feature

Because thymic-harbored cancer cells were able to resist chemotherapy, we assumed that the drug-induced inflammatory soluble factor–rich microenvironment (10) in the thymus modulates the cellular features (proliferation and apoptosis) in the thymic-harbored cancer cells. We designed a novel analysis system, through an in vivo (by drug-induced changes in the thymus) plus in vitro (by a transwell coculture system, to confer these changes to a drug-pretreated cancer cells) system (work schema is shown in Fig. 7A). The results showed that when the atrophied thymus from Doxo-treated mice was cocultured with Doxo-pretreated B16 cancer cells in a transwell (Fig. 7A, group #4), these cancer cells were more resistant to Doxo-induced apoptosis, with a reduced percentage of Annexin-V+ cells (Fig. 7B: the filled peak, indicated by an arrow in the left histograms panel labeled with “Doxo-treated thymus,” and the rightmost filled striped bar in the right). With the same transwell coculture system, we did not find a significant difference in proliferation (Fig. 7C: an arrow in the left labeled with “Doxo-treated thymus,” and the rightmost striped bar on the right). The cellular features of decreased apoptosis with unchanged proliferation in the cancer cells cocultured with the atrophied thymus from Doxo-treated mice suggests that drug-induced thymic soluble factors, mostly proinflammatory factors, indeed confer antiapoptotic feature to the thymic-harbored cancer cells.

Furthermore, we wanted to answer why proliferation in these cancer cells was not changed. We believe that the thymic-harbored cancer cells could obtain intrinsic changes via modulation by the inflammatory (Doxo-treatment) thymus. We performed intracellular staining of P-p38 and P-ERK with flow-cytometric analysis, because a high ratio of P-p38/P-ERK (activation of p38 and inhibition of ERK) induces tumor growth arrest, i.e., dormancy, while a high P-ERK/P-p38 ratio favors tumor regrowth/recurrence (28–30). We found that the ratio of P-p38/P-ERK was significantly increased in the cancer cells under the transwell coculture with the atrophied thymus from Doxo-treated mice (Fig. 7D, the rightmost filled striped bar in right). The results indicate that the atrophied thymus from Doxo-treated mice confers Doxo-pretreated cancer cells with a relative dormant feature.

Taken together, thymic-harbored cancer cells in the inflammatory thymus possess the capacity to resist chemotherapy through...
Figure 7.
The atrophied thymus from Doxo-treated mice modulates Doxo-pretreated melanoma cells toward exhibiting increased antiapoptosis but unchanged proliferation, and increased ratio of P-p38-to-P-ERK. 

A, Experimental schema of thymic modulation on B16 melanoma cells. In this system, we i.p. injected young WT mice with Doxo as we did in Fig. 3. We isolated the thymuses from the Doxo-treated and PBS-Ctr mice, respectively, and cut the thymuses into tissue pieces. We loaded the same weight of thymic tissue blocks on top of transwells to coculture with Doxo-pretreated B16 melanoma cells in monolayer culture on the bottom wells. Three days after the coculture, in which the thymus and monolayer B16 cells were separated by the transwell membrane, the B16 cancer cells were analyzed with flow-cytometric assays.

B, Results of Annexin-V-based apoptosis assay. Left, Representative histograms show Annexin-V⁺ B16 melanoma cells with or without modulation by the thymuses from PBS- and Doxo-treated mice, respectively. Right, Summarized results show % of Annexin-V⁺ PBS/Doxo-pretreated B16 cancer cells in 4 different groups (the bars from left to right match the order of the group #1 to #4 in A).

C, Results of Ki67-based proliferation assay. Left, Representative histograms show Ki67⁺ proliferative B16 melanoma cells with or without modulation by the thymuses of PBS- and Doxo-treated mice, respectively. Right, Summarized results show % of Ki67⁺ PBS/Doxo-pretreated B16 cancer cells in 4 different groups (the bars from left to right match the order of the groups #1 to #4 in A).

D, Results of intracellular staining of P-p38 and P-ERK, respectively. Left, Representative histograms show P-p38⁺ and P-ERK⁺ B16 melanoma cells with or without modulation by the thymuses of PBS- and Doxo-treated mice, respectively. Right, Summarized results show ratios of P-p38⁺ to P-ERK⁺ PBS/Doxo-pretreated B16 melanoma cells in 4 different groups (the bars from left to right match the order of the groups #1 to #4 in A). All data are expressed as mean ± SEM. The P values are shown in each panel, and “n” represents animal numbers.
antiapoptosis accompanied by unchanged proliferation, attributed to thymus-modulated intrinsic changes in molecular activation, such as increased P-p38 and decreased P-ERK, which induces a feature of drug-resistant dormancy.

Discussion

To combat tumor metastatic relapse, it is important to identify premetastatic reservoirs, because they retain MRD, resulting in eventual tumor relapse. Several cancer premetastatic reservoirs, such as BM, which preserve disseminating malignant cells (3–7), and the perivascular space of blood vessels in the lung and liver, which serve as cancer niches (8, 9), have been determined. However, in all likelihood, these are not the only tissues that can serve as tumor reservoirs for harboring MRD, because the challenge of tumor metastatic relapse is still unsolved. The thymus has been determined to be a B-lymphoma premetastatic reservoir in recent studies (10, 11). We confirmed the role of the thymus serving as a premetastatic reservoir not only for lymphoid cancer cells (Supplementary Fig. S1), but also for nonlymphoid melanoma cells. We also determined how chemotherapy induces the thymus to form a tumor reservoir and how this reservoir can modulate the thymus-harbored tumor cells to acquire a chemoresistant feature, the dark side of chemotherapy. When patients with cancer receive chemotherapy, there is risk to damage healthy tissues, including creating an atrophied and inflammatory thymus that potentially harbors circulating cancer cells. The inflammatory thymic microenvironment, in turn, protects and modulates its harbored cancer cells to enter a chemoresistant state with intrinsic signaling alteration. This is a risk because these thymus-harbored dormant cancer cells could eventually develop a new tumor once conditions are suitable for them to disperse to distant organs.

The model we use in Figs 1, 5, and 6 to mimic CTCs was generated through an i.v. injection of B16 melanoma cells. We recognized that this is a relatively artificial means of mimicking spontaneously tumor cell spread. However, by studying the distribution of CTC via the bloodstream rather than metastasis itself, this model has its advantages (45), including controllable numbers of cells delivered to each mouse, and comparability between each experiment, short waiting time for evidence, and easy observation by flow cytometer and fluorescent microscopy. This model may not be ideal for studying the mechanism of metastasis, but it is suitable for determining tumor reservoirs, and particularly for preliminary assessment of how the reservoir microenvironment interacts with its harbored tumor cells during chemotherapy. The question is whether melanoma cells can spontaneously spread into the thymus. A previous study has already demonstrated that B16 melanoma growing under the skin of mice can metastasize to the thymus in 6 of 20 mice (14).

Chemo/radiotherapy is a necessary adjunct treatment in cancer therapy. However, this treatment not only kills cancer cells, but also induces cancer stromal cell DDR to increase inflammation. It was unclear which cell types are the main source of the thymic inflammation during chemotherapy. Based on the B-lymphoma model in a previous report, it seems that the thymic inflammation arose from lymphoma cell death induced DDR (10). However, these lymphoma cells were unable to circulate in great numbers into the thymus, and they do not expand in the thymus. (We did not find thymoma developed when we tested the lymphoma model; Supplementary Fig. S1 and data not shown.) Therefore, it is unlikely that the majority of thymic inflammation comes from the small number of thymic-harbored tumor cell death. The inflammation was also proposed to come from thymic endothelial cells, associated with acute stress-associated phenotype (ASAP)-related secretion (11, 40). This is also unlikely because vasculature-related thymic endothelial cells represent very small portion, contained within 8% of the UEA-1 

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www.aacrjournals.org Mol Cancer Res; 2018

Published OnlineFirst July 13, 2018; DOI: 10.1158/1541-7786.MCR-18-0308

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chemotherapy-induced atrophied thymus, indeed provides a chemoprotective microenvironment, playing the role of a cancer premetastatic reservoir during chemotherapy. We brought a new target responsible for cancer relapse into focus. Considering this target during chemotherapy will potentially lead to efficient therapeutic interventions to combat tumor recurrence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: O. Sizova, D.-M. Su
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Acknowledgments
The authors thank Dr. Alakananda Basu (Director of the Cancer Biology Program at UNTHSC) and Rance Berg (Director and Graduate Advisor at UNTHSC) for critical reading of the manuscript and Dr. Xiangle Sun (Core Facility at UNTHSC) for flow cytometer technical support. We also thank Dr. Michael T. Hemann (Department of Biology at MIT) for kindly providing the Eμ-Myc;P19ARF−/−-B cell lymphoma cell line.

This work was partially supported by The American Association of Immunologists (AAI) Careers in Immunology Fellowship Program, awarded to O. Sizova.

Received March 28, 2018; revised June 1, 2018; accepted June 22, 2018; published first July 13, 2018.

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Molecular Cancer Research

Atrophied Thymus, a Tumor Reservoir for Harboring Melanoma Cells

Olga Sizova, Denis Kuriatnikov, Ying Liu, et al.

Mol Cancer Res Published OnlineFirst July 13, 2018.

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Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-18-0308

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http://mcr.aacrjournals.org/content/suppl/2018/07/13/1541-7786.MCR-18-0308.DC1

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