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ABSTRACT

Tumor metastatic relapse is the major cause of 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, the central T-cell immune organ, has been suggested to be a pre-metastatic 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 bloodstream–circulating melanoma cells. In addition, 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-harbored minimal tumor cells to acquire a chemo-resistant state.

Key words: tumor reservoir, dormancy, inflammation, atrophied thymus, minimal residual disease (MRD), chemo-resistance, p53, melanoma, DNA damage response (DDR), doxorubicin.

ATROPHIED THYMUS, A TUMOR RESERVOIR FOR HARBORING MELANOMA CELLS

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DISSERTATION

Presented to the Graduate Council of the
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By

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This dissertation work is dedicated to my mom, Anna Sizova. Without her support, I wouldn't have gone this far in my scientific carrier. Her love and support have been missed.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC – Allophycocyanin;

BM – Bone marrow;

CCL – CC chemokine ligands;

CD – Cluster of differentiation;

CDK4 - Cyclin Dependent Kinase 4;

Cre^{ERT} - Cre-recombinase and estrogen-receptor fusion protein;

CTC – Circulating tumor cells;

CXCR - C-X-C chemokine receptor;

DDR – DNA damage response;

DEC2 - Differentially Expressed In Chondrocytes Protein 2;

DNA – Deoxyribonucleic acid;

Doxo - doxorubicin;

DP - double positive;

DTC – Disseminating tumor cells;

DSTCs - Disseminating solid tumor cells;

ECM – extracellular matrix;

ELISA- Enzyme-linked immunosorbent assay;

EMT – epithelial-mesenchymal transition;

Epcam - epithelial cellular adhesion molecule;

ERK - extracellular-signal-regulated kinase;

FC – FoxN1-CreER^{T} mouse;

FITC – Fluorescein isothiocyanate;

Foxn1 - Forkhead box protein N1;

GFP – Green fluorescent protein;

HSCs – Hematopoietic stem cells;

i.p. – Intraperitoneal (ly);

i.v.- Intravenous (ly);

IL - Interleukin;

KO - conditional knockout;

MAPK - mitogen-activated protein kinase;

MET - mesenchymal–epithelial transition;

MRD – Minimal residual disease;

NF-κb - nuclear factor kappa-light-chain-enhancer of activated B cells;

NSG - NOD-*scid* IL2Rgamma^{null} mouse;

PBS - Phosphate-buffered saline;

PE – Phycoerythrin;

PercP – Peridinin Chlorophyll Protein Complex;

PFA – Paraformaldehyde;

PVN - Perivascular niches;

RT PCR – Reverse transcription polymerase chain reaction;

SA-β-gal - Senescence-associated beta-galactosidase;

Scid – severe combined immunodeficiency (mice);

TAp63 - N-terminal transactivating domain, homolog of p53;

TECs – Thymic epithelial cells;

TEs – Thymic endothelial cells;

Tg - transgenic;

TGFβ - Transforming growth factor beta;

TM – Tamoxifen;

TNFα – Tumor necrosis factor alpha;

Trp53 - transformation-related protein-53, a.k.a. mouse *p53* gene;

WT – Wild-type mouse

CHAPTER I

INTRODUCTION

Tumor metastatic relapse refers to tumor recurrence at distant organs with the same type of cancer several years after removal of the primary tumor, and it is associated with adjuvant chemotherapy, which poses a clinical challenge. The patients with metastatic relapse have a poor prognosis and high mortality, and in fact metastatic relapse is responsible for the majority of cancer-associated mortality [3,4]. The reason why cancer cells cannot be found in the patients after the treatment is because there is a tumor undetectable period between the primary cure and metastatic relapse, which may be defined as a remission period, when neither symptoms nor cancer cells are detectable. It remains unclear where the cancer cells hide and in what metabolic state they are, as well as why adjuvant chemotherapy is unable to thoroughly eradicate these hiding 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 small numbers of cancer cells exist in the patient during chemo/radiotherapy is defined as minimal residual disease (MRD), while these cancer cell-harboring organs may be defined as pre-metastatic niches/reservoirs. Bone marrow (BM) has been determined as a pre-metastatic reservoir for disseminating malignant cells [5-9]. The perivascular space of blood vessels in the lungs and liver have also been identified as these kinds as cancer niches/organs [10,11]. Recently, the thymus, a lymphoid organ, has also been identified as a tumor reservoir for lymphoid cancer cells (lymphomas) [12,13]. We ask whether the thymus, the largest T cell lymphoid organ in the body, can play a role as a pre-metastatic reservoir for non-lymphoid solid tumor cells during chemo/radiotherapy. If so, we also ask why and how the thymus induces its harbored tumor cells

to resist chemo/radiotherapy. Figure 1 depicts a funnel model describing the premise for these critical questions.

The thymus is the 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 [14], which is composed of an integrated three-dimensional meshwork of thymic epithelial cells (TECs) and thymocytes. Previously, the thymus was paid scant attention as a cancer pre-metastatic reservoir. This may be due to the use of immunodeficient athymic animal models, such as nude mice, in most cancer studies. These immunodeficiency models cannot truly mimic the natural processes of tumor development and immune suppression. Furthermore, the thymus is very sensitive to physical and chemical assaults, particularly chemotherapeutic toxins and radiation, which contribute to thymic atrophy and induce pro-inflammatory factors, such as IL-6, thereby potentially becoming a hospitable environment for harboring tumor cells [12,13]. Solid tumor cells can enter circulation as circulating tumor cells (CTC) [9,15] and disseminate to distal organs [11], including the thymus, because the thymus can accept cells from the periphery [16-18].

It is well known that inflammation is a double-edged sword, which is necessary for antitumor response, [19-21] but induces tumor drug-resistance [22-24]. For example, the IL-6-rich BM microenvironment facilitates chemo-resistance in multiple myeloma by up-regulating various pro-survival proteins [25]. Additionally, chemotherapy-induced IL-6 expression is also involved in cancer cell acquisition of stem-like characteristics, i.e. generating cancer stem cells [26,27] that possess innate resistance mechanisms to chemo/radiotherapy [28,29]. The stemness-associated features are potentially due to activation of anti-apoptotic features [30] and induction of the cancer cell intrinsic quiescent state of $Gap_0 - Gap_1$ (G_0-G_1) arrest [31]. The chemotherapy resistant feature

is attributed to microenvironment-induced tumor cell intrinsic changes in gene expression profiles. For example, some genes are turned on, such as MAPK (Mitogen-activated protein kinases) p38 leading to its activation, while others are turned off, such as MAPK ERK (Extracellular signal-regulated kinases*)* for its inactivation. A high ratio of p38/ERK (activation of p38 and inhibition of ERK) induces tumor growth arrest, i.e. dormancy and a risk for cancer recurrence, while a high ERK/p38 ratio favors tumor re-growth [32-34].

In this report, we first focused on determining whether the thymus is able to retain circulating solid, non-lymphoid melanoma cancer cells. Then we addressed how the atrophied thymic microenvironment becomes a suitable tumor reservoir during chemotherapy. We revealed that in response to DNA damage the thymus acquires pro-inflammatory features. DNA-damage response triggers p53 activation in thymic cells. This, subsequently, results in thymocyte death and thymic epithelial cell (TEC) senescence. These two mechanisms can potentially result in inflammation. We determined that thymocyte cell death predominately occurs via apoptosis, which has anti-inflammatory effects [35,36,37]. However, there are other types of cell death, that promote inflammation, such as necrosis [38] and pyroptosis [39] that may also take place at early stages of responses to genotoxic agents, such as doxorubicin. Meanwhile, p53 upregulation in TECs leads to senescence, that can also contribute to induction of pro-inflammatory conditions via SASP (Senescence-associated secretory pathway) [40-42]. Finally, we demonstrated how thymic-harbored melanoma cells acquired chemo-resistance by exposure to the inflammatory factor-rich thymic microenvironment. Generally, we determined that the lymphoid organ, thymus, under an inflammatory condition is able to harbor circulating non-lymphoid solid cancer cells, and this inflammatory condition facilitates thymic-harbored tumor cells to acquire an anti-apoptotic predominant chemo-resistant feature. Together, our results have identified a novel pre-metastatic

cancer reservoir: the thymus, for harboring solid cancers. We bring this novel target into the focus for antitumor therapy to combat potential metastatic relapse.

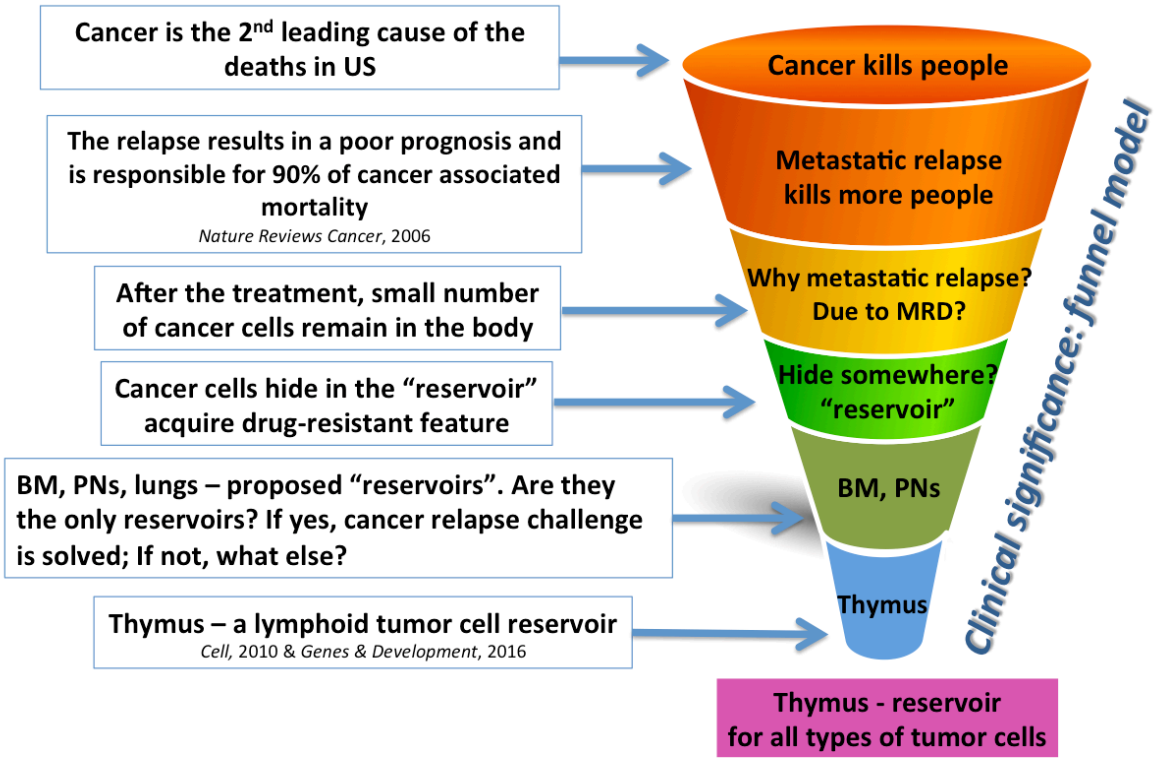

Figure 1. Funnel model demonstrating clinical significance of the project. Descending from the top to the bottom, showing how the focus of this project is narrowed down from global problems in oncology to my specific question.

BACKGROUND

Minimal residual disease

Cancer is characterized by an unlimited cell growth and has the potential to invade and spread throughout the body. It is the second leading cause of deaths globally, after cardiovascular diseases, according to the World Health Organization report for 2015 year [43].

Generally, progression of cancer development and metastatic relapse can be divided into three phases: cancer dissemination, remission, and metastatic relapse (Fig. 2). Cancer dissemination can begin during the pre-clinical stage, following with primary tumor mass resection and subsequent chemo/radio-treatments in order to eliminate the possibility of metastatic relapse. Then, the cancer patient can go into remission, when no signs of the tumor can be observed via various modern screening techniques. However, years or decades after the treatment, the same type of cancer can recur, meaning that a few cancer cells developed chemo-resistance and initiated new tumor relapse (Fig. 2, red box). It is estimated that approximately 90% of cancer patients' deaths are due to relapse and metastatic spread of tumor cells to distant sites via blood and/or lymph [3,4,44].

For most cancers, it is thought that remaining in a complete remission for five years or more implies "cancer-free" status. However, even after several years these "cancer-free" patients can experience metastatic relapse of the initial type of cancer at a distant part of the body, forming a secondary tumor. This phenomenon is termed metastatic relapse of "minimal residual disease" (MRD), which describes a disease stage during the remission period when no signs of the disease were observed, but some non-proliferative cancer cells survived in certain niches/organs of the body. It is suggested, that these "sleeping", non-proliferating cells can be

the origin of future metastases [45]. The organs that are harboring these remaining cancer cells are called "pre-metastatic reservoirs". It still remains unclear, which organs are pre-metastatic reservoirs, why anti-cancer treatment fails to eliminate all malignant cells in these pre-metastatic reservoirs, how these residual cancer cells become inactive for a substantial amount of time, and lastly, what triggers provoke cancer recurrence.

The "seeds and soil" hypothesis of metastasis

What is the relationship between a cancer pre-metastatic reservoir and harboring cancer cells? This may be partially explained through the "seeds and soil" hypothesis. It is well known that tumors have a "tendency" to give rise to metastases at specific parts of the body, such as the liver, lungs and bone [46]. The reason for this is that metastatic cancer cells ("seeds") tend to migrate into their specific "soil" organs [47]. If the soil is suitable for the seed growth, the soil is called "rich-soil". On the other hand, soil not favorable for growth, is called "unfertilized soil". In the "rich-soil", which probably resembles the primary tumor location or has the ability to assist DTCs (disseminating tumor cells) extravasation [48,49], the invading seeds grow slowly and develop a secondary tumor, whereas, in the "unfertilized soil", the seeds cannot develop into a tumor but may acquire dormant features if they are not killed. As we know, rapidly proliferating tumor cells are easily killed by chemotherapy, while slow growing tumor cells are more difficult to eliminate. This is another reason why cancer cells survive from chemotherapy when they enter "unfertilized soil" organs. It is possible that the thymus is a kind of an organ, with "unfertilized soil", because (1) it is considered a semi-immune privileged zone and (2) it lacks factors favorable for growth conditions, such as important nutrients, oxygen availability, growth factors etc.

Pre-metastatic reservoirs

Dormant DTCs may reside in specialized sites that support their survival, limit their proliferation, and quite possibly provide resistance to therapeutic agents. These sites may be referred to as "tumor reservoirs" or "tumor niches". Such proposed tumor reservoirs include bone marrow (BM) [5,7,8,50,51] and perivascular niches (PVNs) [11,52]. The communication between DTCs and their resident microenvironment, or metastatic niche, is crucial for the fate of DTCs. The microenvironment of the pre-metastatic niche is central to dormancy of the niche-harbored cancer cells. To support this matter, BM – a key site accommodating hematopoietic stem cells (HSCs), has been widely investigated as a pre-metastatic reservoir for disseminating malignant cells [5-8]. BM is one of the target organs in metastatic breast and prostate cancers. Both stromal and immune cells in the marrow can contribute to activation of dormancy in disseminated cancer cells. For example, BM stromal cells are responsible for producing quiescence-maintaining signals for BM-residing HSCs that can also provide signals to BM-harboring cancer cells [53]. Therefore, BM-harbored cancer cells can utilize the HSCs niche as a fertile ground for survival during anti-cancer treatment.

The perivascular space of blood vessels in the lung, liver and BM have been also identified as these kinds of cancer niches/organs [10,11]. In fact, microvascular endothelium would be the first of its kind encountered by disseminating tumor cells that utilize a hematogenous route to arrive at sites of metastasis. Therefore, perivascular niches (PVN), specifically endothelial cells (ECs) of certain organs may play an important role in dormancy of DTCs. This has been profoundly explored in murine models of breast cancer, where the tumor-suppressive nature of the mature microvascular endothelium is partially attributed to upregulation of specific

endothelium-derived tumor inhibitory factors, such as thrombospondin-1 (TSP-1) [11]. In contrast, sprouting endothelium producing periostin and TGFβ stimulates awakening of dormant DTCs resulting in tumor growth [11].

Recently, the thymus has been also identified as this kind of organ for lymphoid cancer cells (lymphomas) [12]. Here, we asked whether the thymus can also serve as a cancer reservoir able to harbor solid tumor cells. Evidently, all pre-metastatic niches must share some similar properties that serve to ensure cancer cell survival. Determination of novel cancer reservoirs (certain niches/organs) will make great progress in reducing or eliminating MRD-resulted cancer metastatic relapse. This explains the recent rise in interest in identifying these specific niches/organs in the body, where disseminated cancer cells may acquire chemo-resistance and remain quiescent, creating the risk of eventual metastatic relapse.

Tumor dormancy

Dormancy is a stage in cancer progression, where the cells cease dividing but survive in a quiescent state while waiting for appropriate environmental conditions to begin proliferation again. Dormant cancer cells are thought to be present in early tumor progression, in micrometastases, or left behind in minimal residual disease (MRD) after what was thought to be a successful treatment of the primary tumor. The tumor dormancy may be beneficial for a cancer patient as it may delay disease progression or in some cases never result in relapse. However, there will always be a risk because those dormant tumor cells might become re-activated in the future and lead to a new wave of migration and growth, a metastatic relapse. 20% to 45% of breast and prostate cancer patients experience distant recurrence of the disease years or decades after successful treatment of their primary cancer [54,55]. These re-occurrences of the tumors were

reported even after the tumor had been silenced for 10-20, and even 30 years [56,57]. In some cases, organ donation recipients experience new tumor development due to receiving an organ from a "cancer-free" donor who had a history of cancer [58], which is evidence that a "cancer-free" cancer patient still possesses dormant tumor cells [59]. Certainly, there is a major factor that makes this possible, as organ recipients are required to take immune suppressors.

The assumption that the thymus can harbor tumor cells and that thymic-harbored tumor cells neither develop a tumor mass (such as thymoma), nor are eradicated by chemotherapy suggests that these cells are in a chemo-resistant, dormant state. Tumor chemo-resistant dormancy occurs at two levels [7,31]: (1) *at the single-cell level*, in which the dormant cancer cells exist in a quiescent state of G0 – G1 arrest [31], which is conventional quiescent dormancy; and (2) *at the population level* [30], in which cancer cell proliferation is balanced by apoptosis, equal-cell-death, resulting in unchanged total cancer cell population numbers during drug treatment [30], which is dynamic dormancy. We postulated that thymic-harbored tumor cells may possibly acquire both quiescent dormancy and dynamic dormancy. We define these changes as a heterogeneous chemo-resistant dormancy, similar to the feature of heterogeneity of cancer itself. This heterogeneous chemo-resistant feature could be reflected in intrinsic changes in gene expression profiles. Some genes are turned on, such as MAPK (Mitogen-activated protein kinases) p38, while others are turned off, such as ERK (Extracellular signal-regulated kinases or classical MAP kinases). A high ratio of p38/ERK (activation of p38 and inhibition of ERK) induces tumor growth arrest (dormancy), while a high ERK/p38 ratio favors tumor re-growth/recurrence [32-34].

In addition, it is possible that reservoir-harbored disseminating tumor cells can acquire a stem-like dormant phenotype, since it is known that stem cell behavior is regulated by

microenvironmental stimuli [60]. The idea of a specialized microenvironment regulating stem cell behavior postulates that a niche restricts stem cell differentiation and cell cycle entry in order to maintain a tissue's stem cell population [45,61]. Often, metastatic cancer cells are referred to as cancer initiating stem cells, according to cancer stem cell theories [62-64]. Therefore, it is suggested that the conditions of stem cell niche and pre-metastatic dormant niches have very much in common.

DDR and p53

In response to a plethora of different stress signals, p53 gets activated, mainly by phosphorylation and can stimulate apoptosis, cell cycle arrest, senescence or dormancy [65-69]. It is known that anti-cancer chemotherapy can initiate the DNA damage response (DDR) that leads to activation of p53 [70,71]. Phosphorylated p53 can induce either cell death or cell cycle arrest (i.e. cellular senescence) in order to initiate DNA repair mechanisms and to avoid genomic mutations that may result in malignancy [65,66].

In addition to the role of p53 in maintaining cell homeostasis in response to various stress stimuli, accumulating reports suggest the involvement of p53 in inflammatory reactions [72]. Generally, there are two main transcription factors, namely, p53 and NF-κB that get activated in response to stress, including DNA damage-induced stress. The phenotypic outcomes of p53 and NF-κB activation are strikingly different and generally have opposing effects. NF-κB activates transcription of positive growth regulators, various anti-apoptotic factors, and secreted attractants of the immune response (cytokines and chemokines). p53, however, induces genes that encode cell cycle checkpoint regulators, pro-apoptotic factors and secreted growth inhibitors [73]. Most of the studies describing the opposing interaction between p53 and NF-κB have been conducted on

cancer cells, although there are some reports conducted on non-malignant cells, implying that there is a crosstalk between these two transcription factors [74,75]. The crosstalk between the p53 and NF-κB pathways occurs on multiple levels, and the results of this crosstalk depend on the specific cellular context and stress stimulus. For example, it has been shown that in response to double-strand DNA breaks, p53 and NF-κB are coordinately activated and contribute together to the resultant changes in gene expression [76]. In chemotherapy-treated human macrophages, p53 is required for NF-κB – induced IL-6 production [75]. It is also proposed that NF-κB induction by p53 does not occur through classical activation of the IκB kinases and degradation of IκBα. Rather, p53 expression stimulates the serine/threonine kinase ribosomal S6 kinase 1 (RSK1), which in turn phosphorylates the p65 subunit of NF-κB, resulting in pro-inflammatory gene expression [77].

Inflammation and tumor drug-resistant dormancy

The immune system of our body provides the defense against infections as well as malignancies. Induction of inflammation during immune response to infection is an important step. However, inflammation is a double-edged sword in cancer. Inflammation may also assist in tumor immunosurveillance and inhibit development of the tumor growth [19-21]. On the other hand, many scientific reports have revealed the role of inflammation in promoting cancer development in several ways. Inflammation, and especially chronic inflammation can trigger cellular events that promote malignant transformation of normal cells into tumor cells [78]. Pro-inflammatory cytokines such as tumor necrosis factor (TNFα) and interleukin-6 (IL-6), are perhaps the best characterized pro-tumorigenic cytokines. TNFα and IL-6 can influence all stages of tumor development, including initiation, promotion, progression, and metastasis due to their ability to

activate the oncogenic transcription factors NF-κB, AP-1 (TNF) and STAT3 (upstream of IL-6) [79]. Pro-cancerous activity of inflammatory cytokines may have systemic [80,81] or localized effects. Local IL-6 produced by malignant or non-malignant cells in the tumor microenvironment has been shown to act intrinsically on tumor cells through numerous downstream mediators to support cancer cell proliferation and metastatic dissemination [82-84]. It has been shown that IL-6 -rich bone marrow microenvironment facilitates drug resistance in multiple myeloma by upregulating various pro-survival proteins [25]. Chemotherapy-induced IL-6 expression is also involved in acquiring stem-like characteristics, i.e. "stemness" (becoming cancer stem cells), which possess innate resistance mechanisms to chemo/radiotherapy [28,29]. Exogenous or tumor reservoir-expressed IL-6 is shown to induce cancer cell cycle arrest, senescence and dormancy [12,85]. Dormancy can be also induced by cancer cells themselves to maintain chemo-resistance by autocrine IL-6 signaling [86].

Overexpression of pro-inflammatory cytokines in tumor microenvironments and tumor niches can also contribute to survival and chemo-resistance of cancer cells. Inflammatory mediators can induce chemo-resistance of cancer cells by at least three ways: (I) promotion of the activation of anti-apoptotic genes; (II) induction of stemness which results in protection from chemotherapy-induced apoptosis by promoting stemness-associated features [26,27]; (III) induction of cancer cell dormancy.

In addition, current knowledge cannot fully explain the molecular mechanism of chemotherapy-induced thymic inflammation. Cancer dormancy pathways are also poorly characterized and the mechanisms of how cancer cells develop dormancy and relapse remain elusive. It is assumed that dormant cells are chemotherapy resistant because they are not dividing. However, it is still unclear what triggers cancer cell dormancy.

The thymus, a potential cancer pre-metastatic reservoir

The thymus is the largest T-lymphoid organ, which is a 3-dimensional structure with two main cellular compartments: thymocytes (accounting for ~95%) and thymic epithelial cells (TECs, accounting for <5%). Previously, the thymus was not paid sufficient attention as a cancer pre-metastatic reservoir. This may be due to the use of immunodeficient, athymic animal models in most cancer studies, and the thymus was considered useless in adults due to its involution/atrophy with aging. However, the atrophied thymus could pose a threat serving as a potential cancer pre-metastatic niche/reservoir. The thymus is endowed with features that make it a potential cancer cell reservoir, such as its status as a semi-immune privileged site without or with very low immune reactivity and inflammatory factor-rich, such as IL-6 [87], during involution, and its ability to uptake cells from the periphery (similar to the bone marrow niche).

Recently, the thymus was proposed to serve as a pre-metastatic reservoir of lymphoid cancer cells (lymphoma) [12]. It was reported that mouse B-lymphoma cells are able to enter the mouse thymus during the dissemination process. After treatment with a common chemo-agent doxorubicin, most lymphoma cells were eradicated in the lymph nodes, but not in the thymus [12,13]. This finding indicates that the thymus, especially the drug-induced involuted thymus, possess the features for induction of cancer cell chemo-resistance. The mechanism was proposed to be due to increased chronic inflammation, mainly IL-6 and Timp-1, in the thymus during the chemotherapy, and due to doxorubicin-initiated ASAP (Acute Stress-associated Phenotype) in thymic endothelial cells [13]. The thymic-endothelial cells released IL-6 and Timp-1 to promote B-lymphoma chemo-resistance in the thymus. Thus, the doxorubicin (Doxo)-treated thymus becomes a chemo-resistant reservoir that promotes the survival of lymphoid tumor cells, which is

a risk for eventual tumor relapse [12,13]. However, because thymic endothelial cells compose a very small portion (less than 1%) of thymic cells [88], we asked how such a small population of cells can induce a wide-spread thymic inflammation? There must be other unknown mechanisms to induce thymic inflammation.

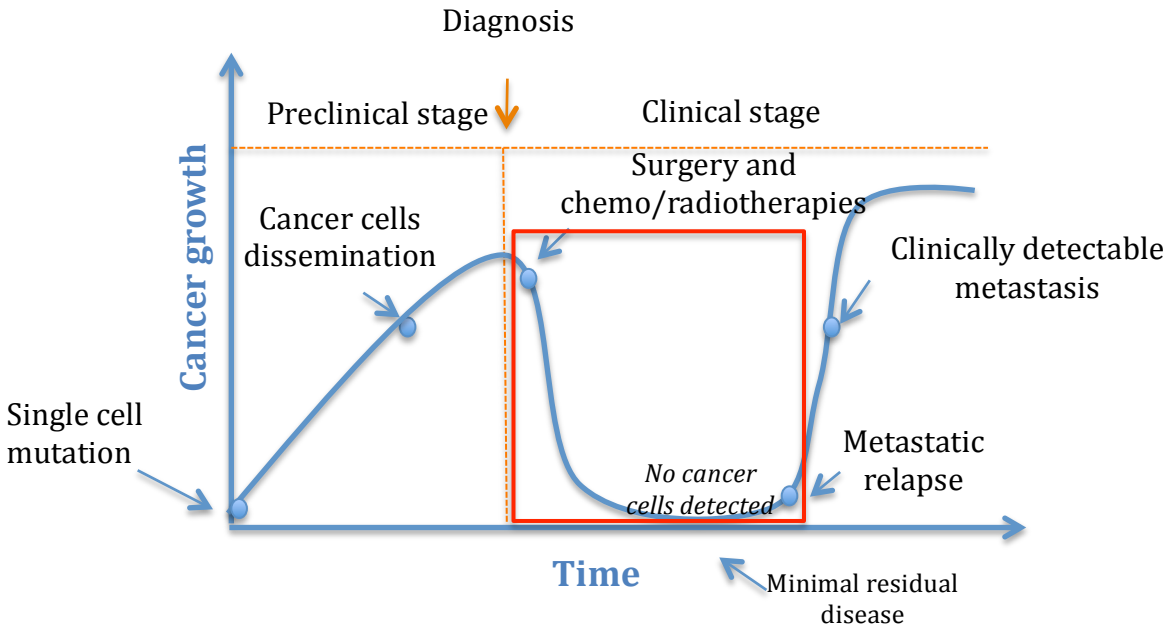

Figure 2. Cancer progression curve. The red box draws attention to the period of time followed by the removal of primary tumor and chemo/radiotherapy, where no metastatic cancer growth is observed. Possibly, few chemo-resistant cancer cells are hidden in organs, such as metastatic reservoirs. These rare cancer cells might be the source of relapse in the future.

HYPOTHESIS AND SPECIFIC AIMS

We hypothesize that chemotherapy-induced inflammatory, atrophied thymus is a suitable solid tumor cell reservoir, potentially responsible for tumor metastatic relapse.

The hypothesis has been verified through three specific aims:

Specific Aim #1. To determine the role of the thymus, which serves as a reservoir harboring solid (melanoma) tumor cells, in addition to lymphoid cancer cells.

- Sub-aim #1a: To confirm that the thymus can harbor circulating mouse melanoma cells.
- Sub-aim #1b: To compare the capacity of normal and atrophied thymic conditions (naturally-aged, genetically induced-atrophied, drug-induced-atrophied thymus) in retaining solid cancer cells.

Specific Aim #2. To determine how chemotherapy (doxorubicin) induces the inflammatory condition in thymic microenvironment, which provides favorable conditions for harboring cancer cells.

- Sub-aim #2a: To analyze how the inflammation arises from two main thymic cell populations: hematopoietic-origin thymocytes and non-hematopoietic-origin thymic epithelial cells, in response to chemotherapy treatment.
- Sub-aim #2b: To investigate whether chemotherapy-induced, DDR-associated persistent increased p53 activation is the trigger for a synergistic effect on thymocyte death and TEC

premature-senescence, which results in an thymic inflammatory condition in the entire thymus.

Specific Aim #3. To determine whether and how thymic microenvironment induces thymic-harbored melanoma cells to acquire chemo-resistant dormancy at dynamic and quiescent status.

- Sub-aim #3a: To evaluate the percentages of surviving cancer cells in the thymus and other organs, focusing on the lymph nodes (LNs) and the lung after doxorubicin treatment.
- Sub-aim #3b: To analyze dormancy-associated p38/ERK ratio, apoptotic activity and level of cell replication in thymic-harbored cancer cells after doxorubicin treatment.

PROJECT RATIONALE

It is known that tumor cells can enter circulation as circulating tumor cells (CTC) [15] and disseminate to distal organs [49]. Emerging evidence shows that the thymus can become a tumor reservoir that protects lymphoid cancer cells from chemotherapy [12,13]. We believe the thymus can also harbor melanoma cells, since solid tumors also emit circulating tumor cells (CTCs) into the blood stream [89], which can disseminate into the thymus. Subsequently, these disseminating solid tumor cells (DSTCs) become chemo-resistant in the thymus during chemotherapy, induced by the same pro-inflammatory thymic microenvironment as for lymphomas [12,13].

However, studies have paid scant attention toward determining the role of the atrophied thymus as a tumor pre-metastatic reservoir. First of all, the thymus is undergoing degeneration or atrophy, as a normal process of aging. During chemotherapy, the thymus, which is very sensitive to any assault, also undergoes acute atrophy. The atrophied thymic microenvironment exhibits

increased inflammation [2] to potentially create a hospitable tumor reservoir that promotes harboring tumor cell dormancy Secondly, athymic animal models (such as Scid, nude, and NSG mice) have been thoroughly used in cancer research. Due to inborn mutations, these mice lack the thymus, T lymphocytes and, sometimes, B lymphocytes, resulting in primary immunodeficiency. Therefore, they are highly susceptible to tumors and highly sensitive to chemotherapy from birth, and cannot mimic the natural processes of tumor dormancy and immune suppression. Instead, we used an immunocompetent mouse model (wild-type, WT) with intravenous (i.v.) inoculation of mouse melanoma cells (B16) to mimic tumor cell circulation. We also used the genotoxic drug doxorubicin, which induces DNA damage response (DDR) and p53 activation in the thymus.

Therefore, our overall rationale was that chemotherapy-induced thymic atrophy creates chronic inflammatory conditions in this low immune responsive lymphoid organ to induce tumor cells to undergo both dynamic and quiescent dormancy.

CLINICAL SIGNIFICANCE

In most cases, cancer patients in long-term remission, when no sign of the disease can be observed for several years, are classified as cancer-free. However, the doctors still avoid telling their patients that “cancer is cured”, since in rare situations cancer patients can experience recurrence of the disease years after [56,57]. Interestingly, there are a number of reports that describe transplant recipients acquiring cancer from a previously diagnosed but cured organ donor. These rare cases demonstrate the likelihood of cancer relapse after many years of tumor dormancy [58,59]. The eradication of the remaining tumor cells, which are undetectable, chemo-resistant, and

resulting in minimal residual disease (MRD), poses a significant clinical challenge. The priority for eradicating MRD is to locate the tumor reservoir, to understand the mechanisms responsible for tumor chemo-resistant dormancy, and to determine why antitumor immunity is unable to eradicate MRD. The bone viscera are a known tumor metastatic niche [8,52,90], in which harboring tumor cells are induced to acquire a dormant feature [7,11,34]. Although, the thymic environment is almost identical to that of the BM, a role for the thymus as a tumor reservoir has only started to be recognized [12,13]. It remains elusive how circulating solid tumor cells are harbored in the thymus, thereby becoming chemo-resistant during chemotherapy. Although much research has focused on the intrinsic changes in malignant tumors, there is now overwhelming evidence that the behavior of tumorigenic cells is highly influenced by their surrounding non-malignant stromal cells in the reservoir, aptly named "the seed and soil hypothesis" [91], where the microenvironment promotes cancer cell intrinsic changes leading to dormancy [33,92,93]. Although the thymus is a lymphoid organ for immature T cell development, many mature T-lymphocytes can reenter the thymus [16-18], implying that the thymus can exchange cells with those, including tumor cells, from the periphery. The thymus is also a semi-immune privileged site without, or with very low, immune reactivity. Therefore, once circulating tumor cells enter the thymus they are very difficult to eradicate. Furthermore, the thymus is highly sensitive to any assault. Chemo/radiotherapy and aging induce thymic atrophy. This results in an increase in pro-inflammatory cytokines, particularly IL-6 [12], IL-1β, etc., which potentially provide a hospitable microenvironment to promote tumor dormancy [22,82,94]. Therefore, it is clinically significant to study the role of the atrophied thymus in cancer chemoimmunotherapy to combat cancer metastatic relapse.

NOVELTY OF THE WORK

First of all, we demonstrated here that disseminating cancer cells from solid tumors (such as melanoma) can harbor in the thymus during the circulation. This has not been reported yet, although lymphoid tumor (lymphoma) has been confirmed to be harbored in the thymus [12,13]. Secondly, we generated the evidence that both thymocyte and thymic epithelial cells (TECs) under a persistent p53 activation, triggered by chemotherapy-induced DDR, are responsible for increased pro-inflammatory conditions in Doxo-treated mice. Finally, we determined the specific changes, mediated by inflammatory thymic environment induced by chemotherapy, in thymic-harbored cancer cells that lead to the induction of chemo-resistant dormancy.

CHAPTER II

RESEARCH DESIGN

Briefly, we inoculated cancer cells in the amount of $1X10^6$ intravenously (i.v.) to mimic the process of cancer cell circulation in the bloodstream. One group of mice was treated with doxorubicin intraperitoneally (i.p.) and the other was treated with PBS as a control. Doxorubicin (Doxo) is a common chemotherapeutic drug, which is used in the treatment of multiple types of cancers: lymphomas, myelomas, breast, lung, bladder etc. [95]. After several days (3-4 days) the mice were humanely sacrificed and the thymus and LNs (a pair of inguinal and a part of mesenteric) were isolated for various experiments. For flow cytometry analysis the collected tissue samples underwent enzymatic digestion with Collagenase and DNAse-I in order to examine the percentage of retained cancer cells. To identify the specific changes in chemotherapeutic drug-treated thymus, the mice received doxorubicin injections, and we proceeded with further analysis, that included flow cytometry, immunohistochemistry, ELISA, Western Blot, qRT-PCR etc. The schematic representation of the workflow is shown in Figure 3.

Figure 3. Standard experimental workflow, which is used thoroughout the project. For the experiments in Chapters III and IV mice were inoculated with $1X10^6$ B16F1 GFP^+ melanoma cells intravenously (i.v.) via retro-orbital route. Three or four days after the inoculation the mice were administered 10 mg/kg of doxorubicin intraperitoneally (i.p.) or PBS (control). On the 4^{th} day after the last doxorubicin injection, the mice were humanely sacrificed and thymuses or thymuses and/or LNs (experiments in Chapter V) were extracted for analysis. For the experiments in Chapter IV, mice were treated with either doxorubicin (experimental group) or PBS (control group) and three or four days later they were sacrificed and thymuses were taken for analysis.

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 (IACUC #2014-15/45), in accordance with guidelines of the National Institutes of Health. 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. Three genetically manipulated mouse colonies were also used: (1) *FoxN1* conditional knockout (termed FC) mice. Once the *FoxN1*-floxed gene is deleted via $CreER^T$ activation by induction with three intraperitoneal (i.p.) injections of tamoxifen (TM), the thymus becomes atrophied (details in our previously published paper [96]); (2) Immunodeficient NSG (NOD. *Cg-Prkdc-scid*, and *il-2rγ*$^{-/-}$) mice. (3) *Trp53* TM-inducible ubiquitous knockout mouse model (*Trp53* floxed/floxed crossbred with *Rosa26* Cre^{ERT}).

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

B16F1 mouse melanoma 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 1ml of complete DMEM medium containing 5μg/ml of polybrene, 12ul of eGFP lentiviral particles (>1 x 10^8, from GeneCopoeia, Cat#: LPP-EGFP-LV151-100-C) were added. Three days later, the GFP^+ cells were visualized by fluorescence

microscopy, and GFP^{hi} 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 80-90%, the cells were dissociated for single cell suspension with 0.25% Trypsin-1 mM EDTA solution followed by washing with 1X PBS twice. This single cell suspension was used for intravenous (i.v.) injection (1×10^6 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 thymic tissue blocks were subcutaneously transplanted into immunodeficient NSG mice under the dorsal skin. Three – four weeks after the transplantation, the mice were sacrificed and the tumor was visualized under the skin.

Tumor recurrence in-vitro assay

Wild-type (WT) mice received i.v. inoculation with 1×10^6 B16-GFP melanoma cells. Three days later, the mice were treated with doxorubicin or PBS. The thymus, LNs, and lungs were adjusted to similar weight and individually cultured in a plate, and 10-14 days later, the GFP^+ cells were visualized and semi-quantitatively measured with Image J software.

Flow cytometry

To analyze the percentage of cancer cells in the thymus and LNs, single-cell suspensions were prepared using enzymatic digestion as follows. Freshly isolated thymic

tissues were torn apart and digested at 37°C in DNase-I/Collagenase V solution for 30 min, as previously described [2]. The single-cell suspensions were then stained with specific antibodies for thymic cell surface markers, including CD45, (30-F11), MHC-II (M5/114.15.2), EpCAM (G8.8) (BioLegend), and then fixed with 2% PFA for 1 hour and permeabilized with 0.1% Triton X-100, followed by intracellular staining of anti-GFP, -phosphoralated-p53, or -Ki67, as previously reported [2].

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, Cat #9284) with secondary Alexa Fluor 488–conjugated donkey anti-Rabbit IgG antibody (Invitrogen, Cat #Z-25302) were used. Flow cytometry was performed using an LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo software.

ELISA assay for inflammatory cytokines in thymic tissues

Thymic tissues were freshly isolated and homogenized in RIPA buffer (Sigma, Cat#R0278). Total protein concentrations were measured using BCA Protein Assay Kit from ThermoFisher (Cat#23227). IL-6, IL-1β, and TNFα were quantified by ELISA (from BioLegend, Cat#431305, Cat#432605, and Cat#430905), following the manufacturer's instructions. Standard curves for IL-6, IL-1β, and TNFα were generated with a range of 0 – 500pg/ml standard protein provided with the ELISA kits. Samples were run in duplicate and the data represent the mean of multiple animals (indicated in the figures). The substrate was TMB (3,3',5,5'-tetramethylbenzidine) and the absorbance

was measured at 450nm 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 doxorubicin (Doxo)- or PBS-treated young WT mice. Cells were stained for surface markers, washed, and incubated in Allophycocyanin (APC) - Annexin-V antibody (BioLegend, Cat# 640920) at a 1:20 dilution with Annexin-V buffer (10mM Hepes adjusted to pH7.4, 140mM NaCl and 2.5mM $CaCl_2$) for 15 minutes at room temperature, followed by flow cytometric analysis. In addition, a caspase-3-based apoptotic assay with flow cytometry was performed to confirm Annexin-V results, in which cleaved caspase-3 (Asp175) monoclonal antibody (Cell Signaling, Cat#9579T) 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 WT normal thymic cells at 55ºC for 20 minutes to induce cell death before the staining. Details described in our recent publication [2].

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

Cryosections (5 - 6μm thick) were stained as described previously [96]. The freshly isolated thymic tissue was cut into cryosections (5 - 6μm thick) with a cryostat. The slides were fixed with acetone and blocked with 10% Donkey-derived serum, then stained with various antibodies. The primary antibodies used were anti-Keratin-8 (Troma-1 supernatant); anti-GFP (B2) (Santa Cruz, Cat#sc-9996); anti-p21 (C-19) (Santa

Cruz, Cat#sc-397); anti-p16 (F-12) (Santa Cruz, Cat#sc-1661), or anti-TAp63 (N-16) (Santa Cruz, 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 1x protease inhibitor cocktail (Sigma, Cat #P8340) and 1x 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, Cat#9284) and anti-total p53 antibody (Santa Cruz Biotech, 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

Cultured B16 cancer cells with 10 - 30% confluence were treated with 2.5μM doxorubicin for 8 hours. Thymuses isolated from young C57/BL6 mice injected twice with PBS or Doxo (10mg/kg mouse weight) were placed on the upper-well membrane of the transwell and co-cultured with B16 cells on the lower-well bottom for 3 – 5 days. Then cancer cells were detached from the bottom of the plate using a non-enzymatic dissociation solution (Sigma, Cat#c5789) to be analyzed with flow cytometric analysis for Annexin-V-based apoptosis and/or Ki67 (BioLegend, Cat#652404) proliferation, and/or evaluation of dormancy phenotype by flow cytometric analysis using phosphorylated (p)-p38/p-ERK ratio (Cell Signaling, Cat# 4551S and Cat#4375S). Details are described in the Fig. 17.

Real-time RT-PCR for gene expression analysis

Total RNAs were isolated with TRIzol reagent and reverse transcribed (RT) to cDNA with the SuperScriptIII cDNA kit (Invitrogen/ThermoFisher Scientific). Real-time RT-PCR was performed with TaqMan reagents and primers (*NF-κ*B subunit-1, *Ccl25*, *Cxcl12* and housekeeping GAPDH and 18s RNA; the primers and probes were purchased from Invitrogen). The relative expression levels of mRNAs from the thymuses were internally normalized to both GAPDH and 18s RNA levels, then compared to a ΔΔCT value from pooled PBS-treated samples, which was always arbitrarily set as 1.0 in each real-time PCR reaction.

Statistics

For evaluation of group differences, an unpaired two-tailed Student's *t*-test was used assuming equal variance. Differences were considered statistically significant at values of * $p < 0.05$ and ** $p < 0.01$.

CHAPTER III

THE THYMUS, PARTICULARLY THE ATROPHIED THYMUS, CAN HARBOR MELANOMA (NONLYMPHOID SOLID TUMOR) CELLS WITH A CAPACITY FOR REGROWTH

Identifying body sites/organs, in which tumor cells can persist in an indolent state as single disseminated tumor cells (DTCs) or as small micrometastatic clusters for a prolonged period of time is a vital problem in oncology. At this moment, it is unfeasible to detect such sites / niches with modern technology. Hematologic cancers or cancers of the immune system can originate and disseminate into lymphatic organs, such as bone marrow (BM), lymph nodes, and spleen [50,97]. Recently, the thymus has been proposed to be one of the dissemination sites [12,13]. Hematologic cancers can disseminate into non-lymphatic organs, such as the liver, lungs, and brain [98]. Also, there is evidence that solid cancers can also disseminate into BM and LNs [5,7,99]. However, there was no report that solid cancer cells can disseminate into the body's largest T lymphatic organ – thymus. Therefore, the next logical step is to test whether solid cancer cells can also disseminate into the thymus. This chapter addresses Specific Aim 1, presenting the evidence that the thymus can be a novel site of disseminated cancer cells originated from solid tumors.

Specific Aim #1

To determine the role of the thymus, which serves as a reservoir harboring solid (melanoma) tumor cells, in addition to lymphoid cancer cells.

- Sub-aim #1a: To confirm that the thymus can harbor circulating mouse melanoma cells.
- Sub-aim #1b: To compare the capacity of normal and atrophied thymic conditions (naturally-aged, genetically induced-atrophied, drug-induced-atrophied thymus) in retaining solid cancer cells.

Working hypothesis: The thymus can serve as a tumor reservoir for circulating melanoma cells.

Rationale

It is reported that bone marrow (BM) can be a cancer reservoir for such non-hematologic cancers as prostate and breast cancer [5,7,54]. The thymus has many similar characteristics to the BM. Both BM and the thymus are the source of lymphopoietic cytokines, both rely on stromal cells to foster the differentiation of hematopoietic precursors, and both develop hypoxic conditions [100-102]. Therefore, the thymus is a potential site for harboring non-hematologic cancer cells. Recently it was demonstrated that the thymus can serve as a pre-metastatic reservoir for circulating B-lymphoma cancer cells in a mouse model [12,13]. We asked whether the thymus can serve as a pre-metastatic reservoir for solid tumor cells, utilizing a mouse model of melanoma.

Results

The thymus can harbor melanoma (nonlymphoid solid tumor) cells with a capacity for a regrowth

- Sub-aim #1a: To confirm that the thymus can harbor circulating mouse melanoma cells. We found that upon intravenous (i.v.) inoculation, GFP^+ mouse melanoma cancer cells circulate and harbor in the thymic tissue. We were able to detect them via flow cytometric analysis and immunostaining assay of frozen tissues.

In order to test our hypothesis for this aim, we i.v. inoculated GFP^+ melanoma cells into young WT mice. First, we verified that the injection of tumor cells was a success, by observing multiple tumor lesions that developed in the lungs 7-10 days post-injection (Fig. 4). 10-12 days after the inoculation, the mice still appeared to be relatively healthy and we humanely sacrificed them to analyze the thymic tissue. We found GFP^+ mouse melanoma cells in frozen thymic tissue sections from melanoma-inoculated mice (Fig. 5A). With flow cytometry approach, we also found cancer cells, which are the CD45-negative and GFP-positive population, in the mouse thymus (Fig. 6A, left panel). The results suggest that melanoma cancer cells can be harbored in the thymic tissue.

Notably, the number of melanoma cells in the thymus is very low (Fig. 5). To ask whether these low tumor cell numbers are still sufficient to develop a tumor mass, we subcutaneously transplanted the thymus tissue blocks from melanoma-inoculated young WT mice into NSG mice (the experimental schema is shown in Fig. 5B). Approximately 30 days after the transplantation, the grafted tissue blocks developed a neo-tumor under the NSG mouse skin (Fig. 5B). This procedure was repeated in three mice with identical

results. The tumor mass developed from the thymic tissues containing replicating cancer cells in the young WT thymus was visually observed on ~27th day post-surgery. As a control, we subcutaneously transplanted the thymus tissue blocks from PBS injected mice into NSG mice, and we did not find any tumor mass growth at day ~30. The transplanted thymic tissue blocks were absolved under the skin of NSG mice (photo not shown). The results indicate that circulating solid tumors are able to disseminate and harbor in the thymus, which creates a potential for MRD and these thymic-harbored cancer cells are able to enter metastatic process eventually.

Atrophied thymus retains the ability to harbor the circulating melanoma cells

- *Sub-aim #1b:* To compare the capacity of the atrophied thymus (naturally-aged, genetically induced-atrophied, drug-induced-atrophied thymus) vs. "normal" thymus to retain cancer cells.

As shown in Fig. 5 the "normal" thymus of young wild-type (WT) mice can harbor circulating GFP^+ melanoma cells. However, the thymus is an organ with high sensitivity to any insults, such as chemo/radio-therapy, resulting in atrophy or involution. We wanted to know the role of the atrophied thymus in harboring these circulating cancer cells. Aging induces thymic atrophy. Decline of *FoxN1* expression can also trigger thymic atrophy. In our conditional FoxN1 knockout (FC, $Foxn1^{fx/fx}$-Cre^{ERT}) mouse model, acute thymic atrophy can be induced by intraperitoneal (i.p.) injection of tamoxifen (TM) [96]. We inoculated B16 GFP^+ melanoma cells into aged (with natural thymic involution) and FC (with induced acute thymic atrophy) mice, and we found that these atrophied thymuses contained an increased percentage of melanoma cells compared to that in

young wild-type mice with normal thymic conditions (Figs. 6C-D). The results imply that the atrophied, altered thymic microenvironment favors circulated melanoma cells harboring.

Discussion

To combat tumor metastatic relapse, it is important to identify pre-metastatic reservoirs, because they contribute to MRD, potentially resulting in eventual tumor relapse. Several cancer pre-metastatic reservoirs, such as BM, which preserve disseminating malignant cells [5,7,9], and the perivascular space of blood vessels in the lung and liver, which serve as cancer niches [9,11], have been determined. However, in all likelihood, these are not the only tissues that can serve as tumor reservoirs contributing to MRD, because the challenge of tumor metastatic relapse is still unsolved. The thymus has been determined to be a B-lymphoma pre-metastatic reservoir in recent studies [12,13]. We confirmed the role of the thymus in serving as a pre-metastatic reservoir not only for lymphoid cancer, but also for nonlymphoid melanoma cells.

The model we use in Figs. 4-6 to mimic Circulating Tumor Cells (CTCs) was generated through an i.v. inoculation of B16 tumor 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 circulation rather than natural dissemination, this model has its advantages [103], including controllable numbers of cells delivered to each mouse, comparability between each experiment, short waiting time for evidence, and easy observation by flow cytometery and fluorescent microscopy. This model may not be ideal for studying the mechanism of natural 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. Also a previous study has already demonstrated that B16 melanoma growing under the skin of mice can metastasize into different organs, including the thymus of 6 out of 20 mice [104]. Nevertheless, the goal of our study is to investigate whether the thymus can serve as a reservoir for chemo-resistant cancer cells.

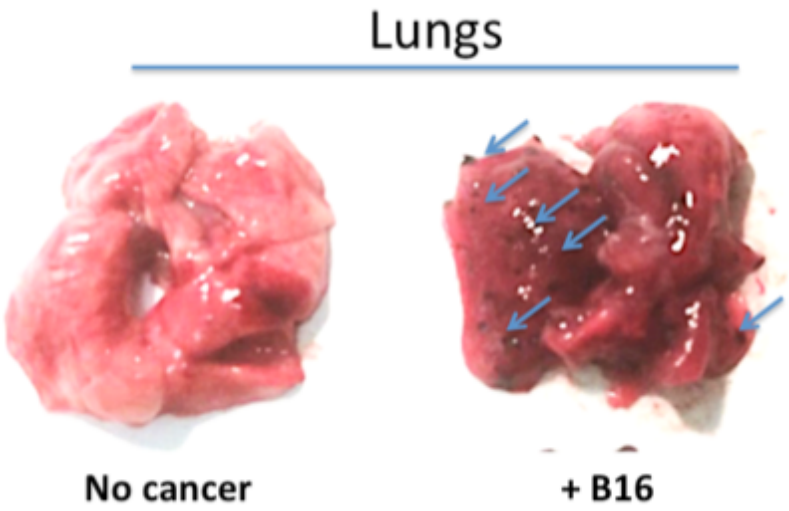

Figure 4. Representative photos of the lungs from the mice 10 days after i.v. inoculation with melanoma cells. Starting from ~10 days after inoculation with B16 melanoma cells, the lungs develop multiple cancer lesions *(right, indicated with arrows)*. The photo of the lungs from the mice without any cancer cell inoculation is provided for control *(left)*.

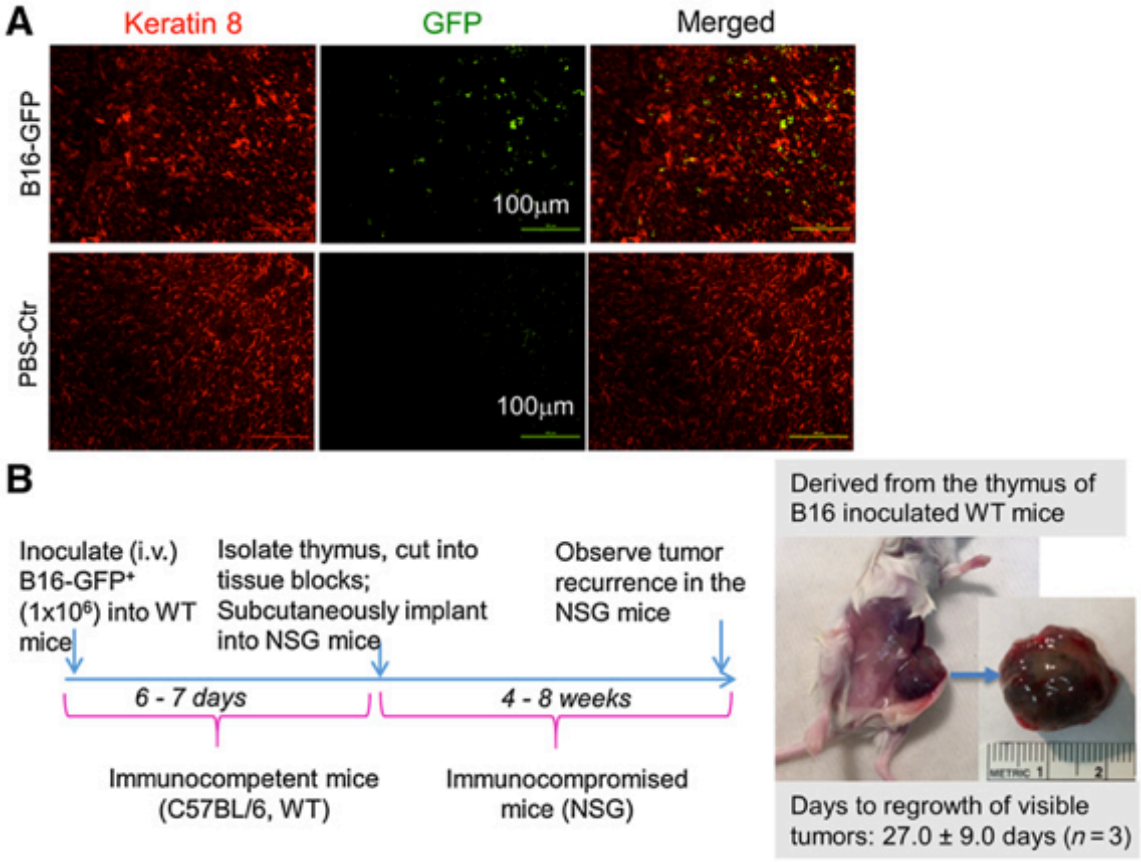

Figure 5. The thymus is able to harbor melanoma (nonlymphoid tumor) cells with a capacity for regrowth. Mice were i.v. inoculated with GFP (Green Fluorescent Protein)-transduced mouse B16F1 (termed B16-GFP) melanoma cells ($1x10^6$ per mouse) or PBS for control (PBS), 1 week after the inoculation the 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. 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).

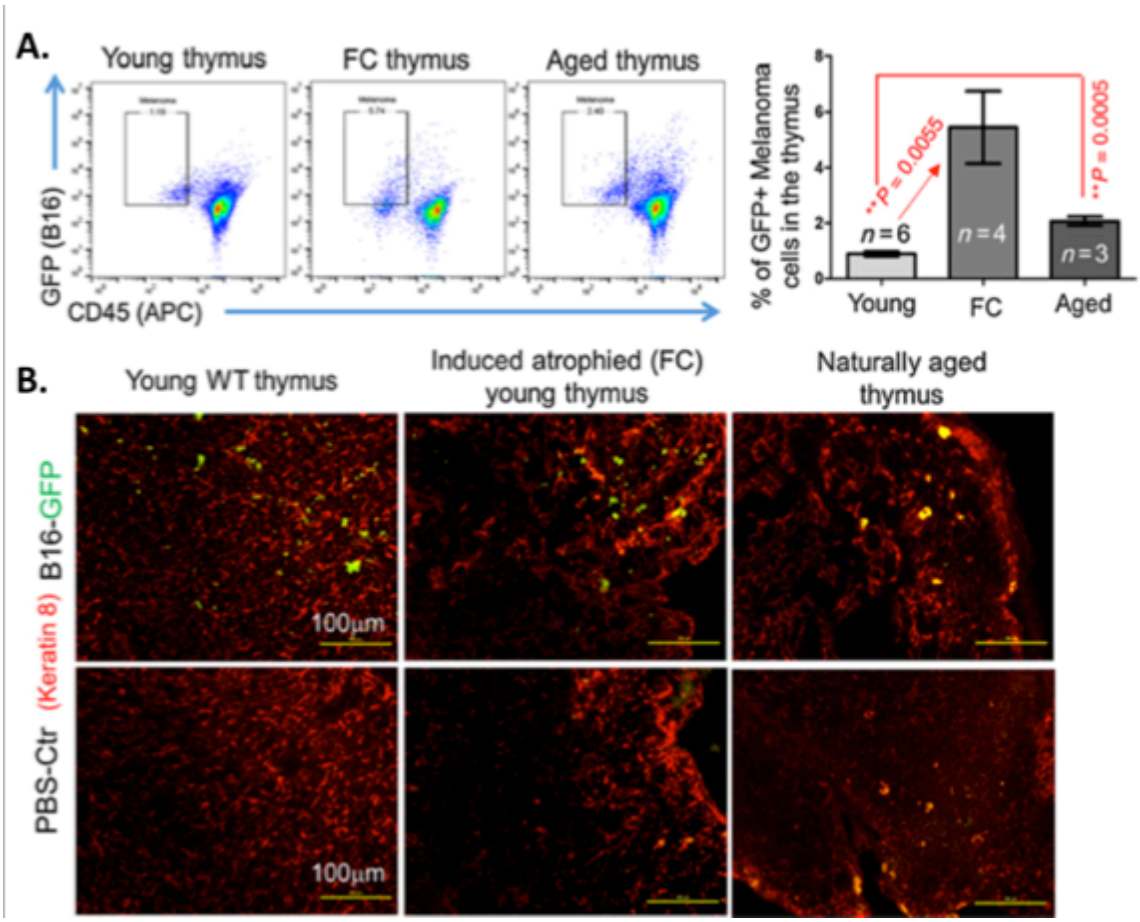

Figure 6. The atrophied thymus retains the capacity to harbor melanoma. A. *Left,* Flow-cytometric plots show gates of thymic-harbored B16-GFP melanoma cells in the thymuses of three types of mice (from left to right): Young WT, *FoxN1* conditional gene knockout (FC), and WT naturally aged mice. *Right,* 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 (standard error of the mean). The *P* values are shown in each panel, and "n" represents animal numbers. **B**, Freshly isolated thymic cryosections from three groups of mice 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 three biological replicates in each group with essentially identical results.

CHAPTER IV

CHANGES IN THYMIC MICROENVIRONMENT UNDER GENOTOXIC CHEMOTHERAPY

In the previous chapter, we showed that cancer cells can be harbored in the atrophied thymus, such as aged-initiated naturally atrophied and conditionally gene knockout-induced FC atrophied thymuses. In the present study we demonstrated that chemotherapy with doxorubicin can also induce acute thymic atrophy. Thymic atrophy results in dramatic changes in thymic microenvironment. We speculate that these changes may influence the capacity of the thymus to harbor cancer cells. In order to answer the question why the thymus, especially the atrophied thymus, can serve as a tumor reservoir for melanoma cells it is necessary to study about the specific changes in the thymic tissue in response to genotoxic drug administration. In this chapter we will describe specific DNA-damage-induced alterations in thymic microenvironment.

Specific #2

To determine how chemotherapy induces the inflammatory condition in thymic microenvironment, which provides favorable conditions for harboring cancer cells.

- *Sub-aim #2a*: To analyze how inflammation arises from two main thymic cell populations: thymocytes of hematopoietic origin and thymic epithelial cells of non-hematopoietic origin, in response to chemotherapy.

- *Sub-aim #2b*: To investigate whether drug-induced, DDR-associated persistent increase of p53 activation is the trigger leading to a synergistic effect of thymocyte death and TEC premature-senescence, which resulted in an overall pro-inflammatory thymic condition.

Working hypothesis: We hypothesized that genotoxic chemotherapeutic drugs, such as doxorubicin, induce persistent DDR-associated p53 activation in two main thymic cell subsets: thymocytes and TECs, thereby resulting in massive apoptosis of thymocytes and senescence in TECs. This may result in a synergistic release of pro-inflammatory cytokines into the thymic microenvironment.

Rationale

Chemotherapy induces dramatically increased inflammation in the thymus, which has been previously reported [12]. It has been demonstrated that the inflammation arises from chemotherapy-resulted DDR-induced cell death [12] and ASAP (Acute Stress-associated Phenotype) in TEs [13] (Thymic Endothelial cells, which composed less than 1% of the cells in the thymus) [88]. ASAP is characterized by drug-induced TE senescence and release of IL-6 and other inflammatory factors [13]. It is unlikely that these few cells induce inflammation of the entire thymus. Therefore, other thymic cells are potentially involved in the thymic inflammatory process.

In our previous studies, we found that p53 is persistently activated during age-related thymic atrophy, accompanying increased thymic cell apoptosis [96] and thymic epithelial cell senescence [2], as well as persistent chronic inflammation. This led us to the

idea that chemotherapy-induced thymic atrophy accompanied with increased inflammation could have a similar molecular mechanism to age-associated thymic atrophy. In addition, we hypothesized that p53 gene would be associated with these processes based on its well-known functions in promoting both cellular apoptosis and senescence [68,69], as well as the fact that activation of p53 is commonly triggered by the cell stress–associated DDR [70,71,105].

Results

Chemotherapeutic drug induces thymic atrophy to generate an inflammatory microenvironment, attributed to a combination of thymocyte death and TEC senescence

- *Sub-aim #2a*: To analyze how inflammation arises from two main thymic cell populations: thymocytes of hematopoietic origin and thymic epithelial cells of non-hematopoietic origin, in response to chemotherapy.

Young WT mice were injected (i.p.) with doxorubicin and evaluated the thymic involution and subsequent changes in thymic microenvironment were evaluated. In the thymus, chemotherapeutic drugs impact not only malignant tumor cells, but also potentially nonmalignant thymic cells, which consist of hematopoietic thymocytes and nonhematopoietic TECs [106]. We found that genotoxic Doxo-treatment initiates activation of phospho-p53 and expression of total p53 in the thymus. In the thymocytes the activated p53 induces thymocyte apoptosis, while in the TECs we observed the phenotype of cellular senescence.

As shown in Fig. 7A, mice were intraperitoneally (i.p.) injected with doxorubicin

(Doxo) at 8 – 10 mg/kg body weight or PBS for three consecutive days (once a day), and three days after the last injection, their thymuses were observed. Our results show that the ratios of thymic weight/body weight were decreased in doxorubicin-treated mice, compared to PBS-treated littermate controls (Fig. 7A, left and middle panels), which can be explained by DDR-resulted insult to the thymus from the general toxic effect of chemotherapy on the thymus. We also observed significant decrease in the number of thymocytes in drug-treated mice (Fig. 7A, right panel). In addition, we found an increased pro-inflammatory condition (after 4–5 Doxo injections at 10 mg/kg) induced by the chemotherapeutic drug in the atrophied thymuses, exhibited by increased IL-6, IL-1β, and TNFα compared to those in the normal thymuses (Fig. 7B). The results confirm that chemotherapy indeed induces thymic atrophy and establishes a pro-inflammatory thymic microenvironment.

Changes in thymic microenvironment are potentially correlated with DDR-triggered activation of p53 gene

- *Sub-aim #2b*: To investigate whether drug-induced, DDR-associated persistent increase of p53 activation is the trigger leading to a synergistic effect of thymocyte death and TEC premature-senescence, which resulted in an overall pro-inflammatory thymic condition.

To determine the underlying mechanism of doxorubicin-induced thymic atrophy along with an inflammatory thymic microenvironment, our assumption was that the effect comes from cell death of thymocytes in combination with senescence in thymic epithelial cells. We tested these parameters in the thymuses of Doxo-treated and PBS-

treated (PBS-Ctr) young WT mice (Fig. 8). Using Annexin-V–based apoptotic assay, we found that apoptosis of thymocytes ($CD45^+$ $EpCam^-$) from Doxo-treated mice was significantly increased, while apoptosis of TECs ($CD45^-$ $EpCam^+$) was only moderately increased, compared to their PBS-Ctr counterparts (Fig. 8A, left). In order to confirm this result, we tested activation of 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 [106]. The results were consistent with the Annexin-V–based apoptotic assay (Fig. 8A, right). The results indicate that during Doxo-treatment, thymocytes are the cells contributing predominantly to apoptotic cell death. To test the possibility of necrotic cell death involvement, we specifically looked at dead cells population by gating SSC low and FSC low population in a flow cytometry dot plot (Fig. 8B). The dead cell population was increased in Doxo-treated thymus (Fig. 8B, *right),* compared to PBS-treated control (Fig. 8B, *left).* Among the entire dead cell population, we observed a significant increase of solely-apoptotic cells in Doxo-treated thymus (Fig. 8C, grey area of the stacked bar graph). The results suggest that apoptosis is the main mechanism of cell death in the thymic cells during genotoxic chemotherapy treatment. However, it does not exclude the possibility that other pro-inflammatory types of cell death, such as necrosis, take place in earlier time points during doxorubicin administration.

Since apoptosis in TECs was not a main phenotype, we asked whether the activation of senescence in TECs was changed, which is related to senescence-associated secretory phenotype (SASP). Immunostaining of frozen thymic tissue from Doxo-treated mice showed upregulation of senescence-associated markers p21 and $p16^{INK4A}$, which

suggests cell cycle arrest leading to the activation of drug-induced cell senescence (Fig. 9A). 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. 9B). To verify that the senescent phenotype was in the TEC population, we stained the thymic cryosections with the TAp63 marker – an isoform of a transcription factor *Trp63*, which is related to thymic stromal cells' senescence and is expressed only in TECs [2] (Fig. 9C). To further prove that senescence occurs in TECs, we did co-staining of p21 and Tap63. The Tap63 (expressed in thymic epithelial cells can be observed co-localizing (yellow) with p21 (cell cycle arrest-associated molecule). This co-localization, which was reported in our previous publication [2], provides further evidence that senescence occurs in TECs during chemotherapy (Fig. 10).

Because both cellular apoptosis and senescence are associated with p53, to confirm our proposed molecular mechanism, it is essential to demonstrate phosphoralated-p53 (p-p53) levels after the Doxo-treatment. To verify p53 correlation to chemotherapy-induced thymic cellular apoptosis and senescence, we first measured phosphorylated (activated) p53 (P-p53) and total p53 in the thymus from Doxo-treated mice with Western blot assay and found that both were indeed increased compared to those from PBS-Ctr mice (Fig. 11).

To find out which thymic cell populations were responsible for the increase of P-p53, we further performed intracellular staining with anti-P-p53 antibody for flow cytometry analysis. We found that Doxo-treatment increased the percentage of P-p53 cells in both hematopoietic thymocytes and nonhematopoietic TECs (Fig. 12A and B). However, it exhibited a uniform increase in TECs (small standard deviation), but a

heterogeneous increase in thymocytes (a large variability) of the Doxo-treated mice (indicated by a red-line circle in Fig. 12B).

The 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.

To further confirm that persistent increase of P-p53 is responsible for the thymic inflammatory condition via increased massive apoptotic cell death in thymocytes and senescence in TECs after genotoxic doxorubicin treatment, it is important to generate a proof-of-concept model, whereby inhibition of *p53* activation should lead to a reduction of the chemotherapy-induced thymic inflammation. However, we did not obtain a proper mouse model. This model will be considered in the LIMITATIONS chapter.

Discussion

Chemotherapeutic drugs can induce cell stress–associated DDR, resulting in cell death and/or senescence [107,108]. In the thymus, chemotherapeutic drugs impact not only malignant tumor cells, but also potentially nonmalignant thymic cells, which consist of hematopoietic thymocytes and nonhematopoietic TECs [106]. Our data identified that after chemotherapy, thymic inflammation arises mainly from a synergistic effect of cell death (mainly of thymocytes) and senescence (mainly of TECs), although we do not exclude cell death in TECs as well. In other words, the thymic inflammation arises from chemotherapy-effected nonmaliganant thymic cells. The cell death and senescence phenotypes in thymic cells were mechanistically due to drug-associated DDR-triggered activation of the p53 gene, which induces both apoptotic cell death [68] and cellular

senescence [69], which is illustrated in Fig.13. Cellular senescence can be both beneficial and deleterious, when it happens in malignant cells because it may arrest tumor cell growth; but it is deleterious when it happens in nonmalignant stromal cells, because the cells then develop SASP-associated inflammation [41]. TECs, mainly undergoing senescence during chemotherapy, are probably the cells involved in SASP-associated inflammation in the thymus. In addition, senescent stromal cells could promote a tumor epithelial-to-mesenchymal transition [40], which establishes a condition for cancer stem cell generation that can either suppress tumor growth (such as immune-mediated inflammation) or induce tumor cell progression or chemo-resistance [109,110].

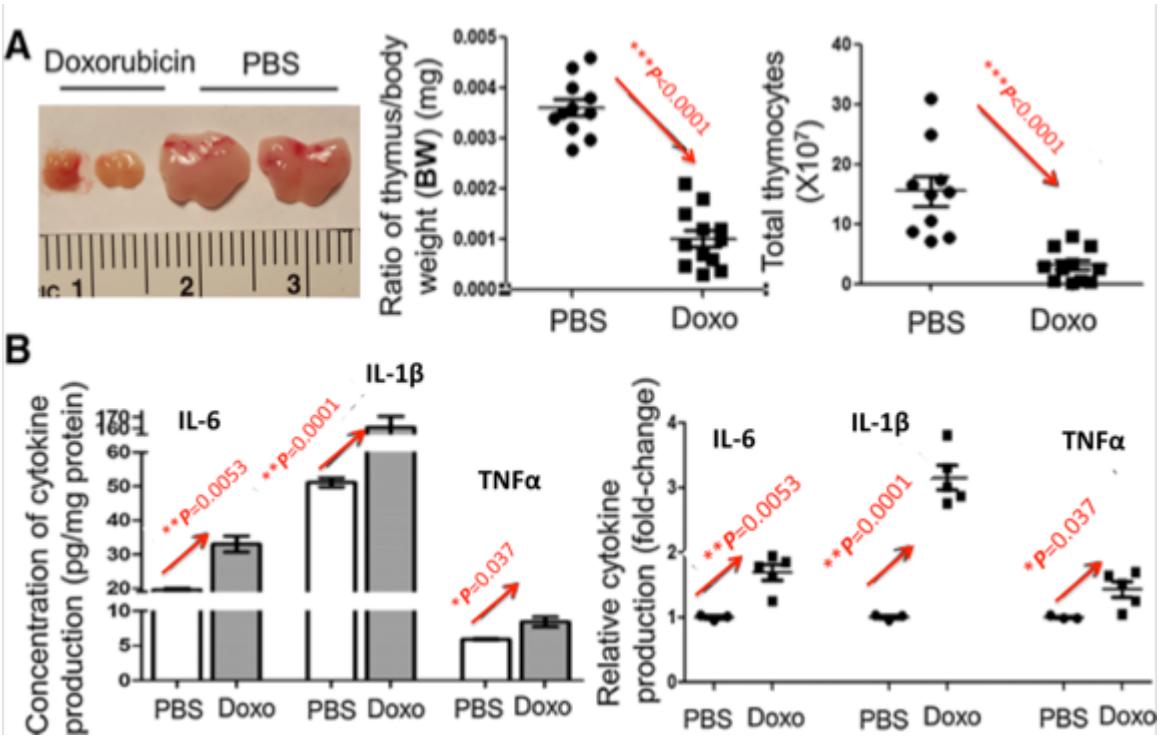

Figure 7. Chemotherapy induces thymic atrophy, generating an inflammatory thymic microenvironment. Young WT mice were intraperitoneally (i.p.) injected with either genotoxic drug Doxo at 10 mg/kg body weight or PBS once a day for 4–5 times, with an interval resting day in between the days. Three to five days after the last injection, the thymuses were collected for analysis. **A**, *Left*, A representative image of the thymuses shows the thymic atrophy in the Doxo-treated group, but not in the PBS-Ctr group; *middle*, a summary of ratios of thymus/body weight (BW) in mg; *right,* a summary of total thymocyte numbers in the two groups. **B**, 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.

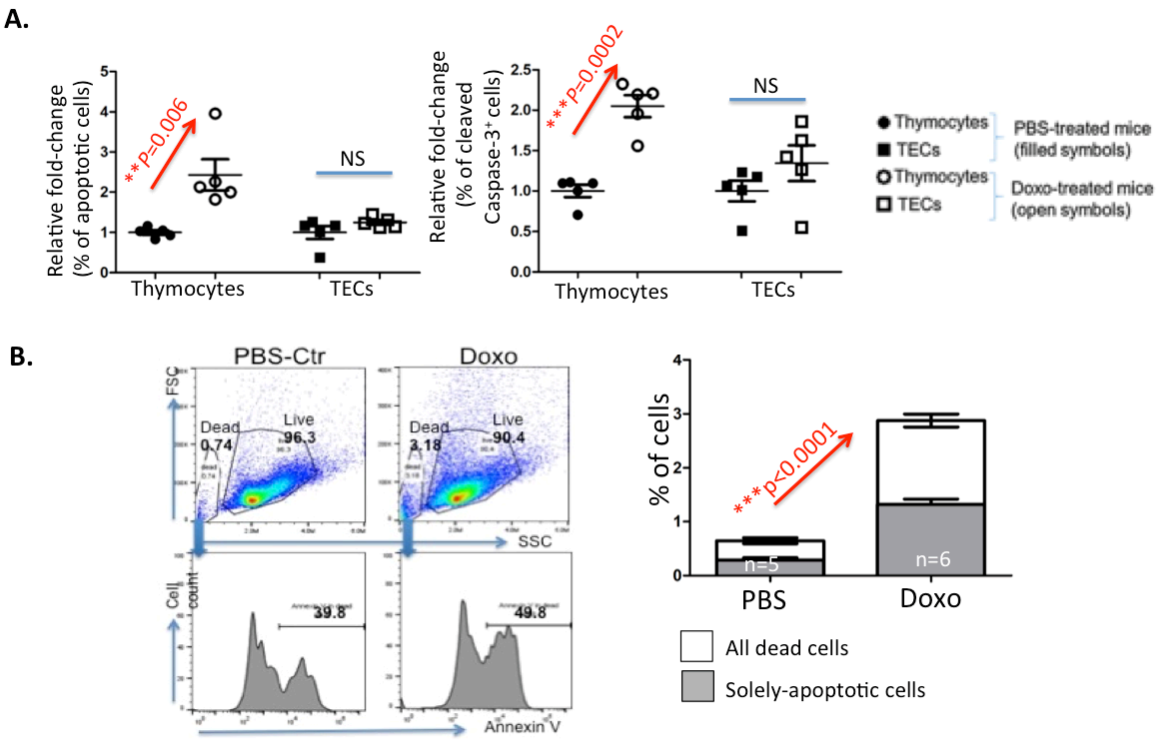

Figure 8. Chemotherapy (Doxo-treatment) induces increased apoptosis in thymocytes. Mice were treated with three consecutive i.p. injections of Doxo (10 mg/kg) or PBS once a day. Three days after the last injection, the thymuses were collected for analysis. **A**, A summary of apoptotic analysis of thymocytes (gated on the $CD45^{+}EpCam^{-neg}$ population) and TECs, gated on the $CD45^{neg}EpCam^{+}$ population) with Annexin-V assay (l*eft*) and cleaved caspase-3 assay (*right*), respectively. Relative fold changes were based on 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.** *Upper panel:* representative flow cytometry plots gating on dead vs.live population in PBS- and Doxo-treated thymuses. *Lower panel:* representative histogram gating on $Annexin^{+}$ cells inside the "dead" cell population. **C.** Stacked bar graphs demonstrating % of solely-apoptotic cells (grey-colored area) among % of dead cells (white-colored area). 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.

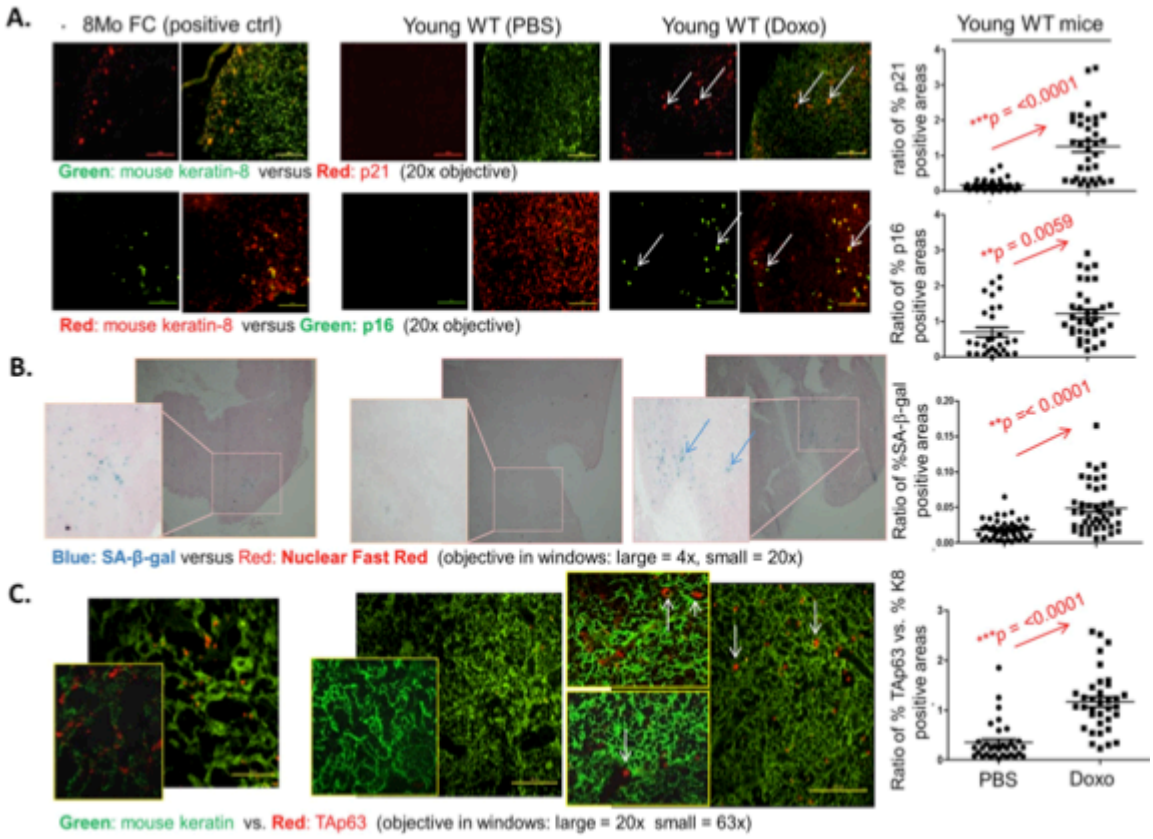

Figure 9. Chemotherapy (Doxo-treatment) induces senescence response in TECs. **A**, Representative thymic cryosections with immunofluorescence staining show the images of keratin-8 (counterstaining) vs. p21 in the top row and $p16^{INK4A}$ in bottom row from the thymuses of three types of mice. Eight-month-old FC thymus served as aged control because it is similar to the 18-month-old naturally aged thymus [1]. **B,** Representative images of thymic cryosections with SA-β-gal staining (blue clusters) vs. nuclear fast red counterstaining from three types of thymuses as **A. C**, Representative fluorescence images of thymic cryosections with TAp63 (a senescent TEC marker [2] staining (red clusters)) vs. keratin-8 counterstaining from three types of thymuses as **B**. Arrows in A–C 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 Image J software, and each symbol represents the ratio of % positive area per image (9–14 tissue images were recorded for each animal) of the tissues from 3–4 animals in each group.

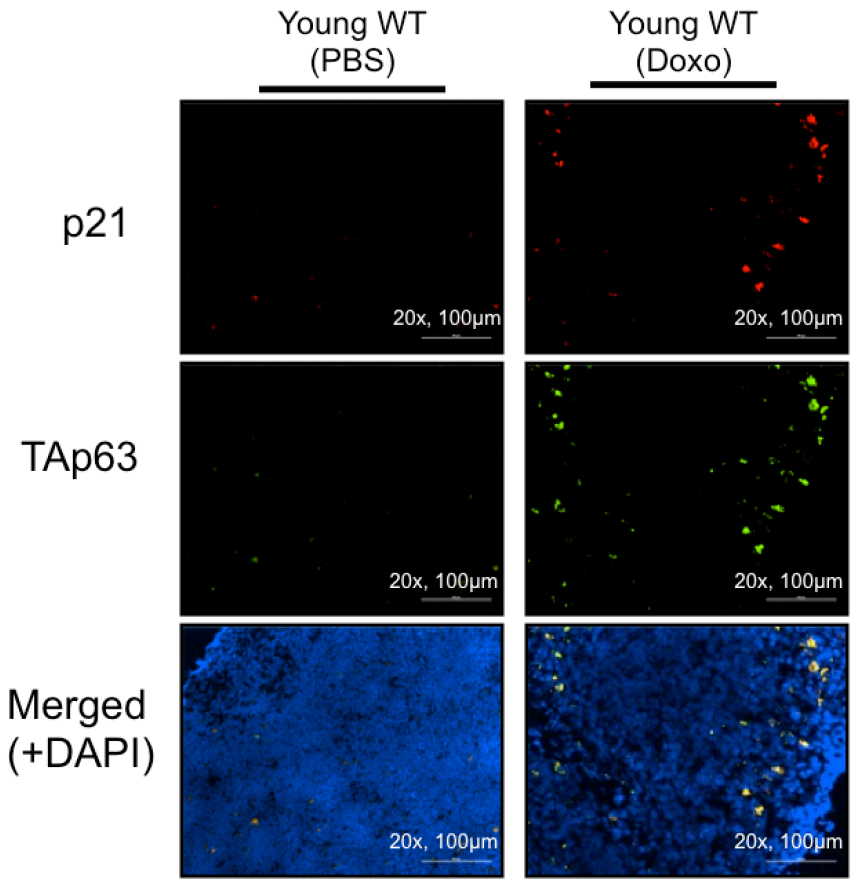

Figure 10. Co-localization of TA63 and p21 in the thymic cryosections from mice treated with doxorubicin and PBS, respectively. Thymic cryosections of young WT mice, which were intraperitoneally injected with either PBS (*left panels*) or doxorubicin (Doxo, *right panels*), were stained with p21 (red), Tap63 (green), and DAPI (blue).

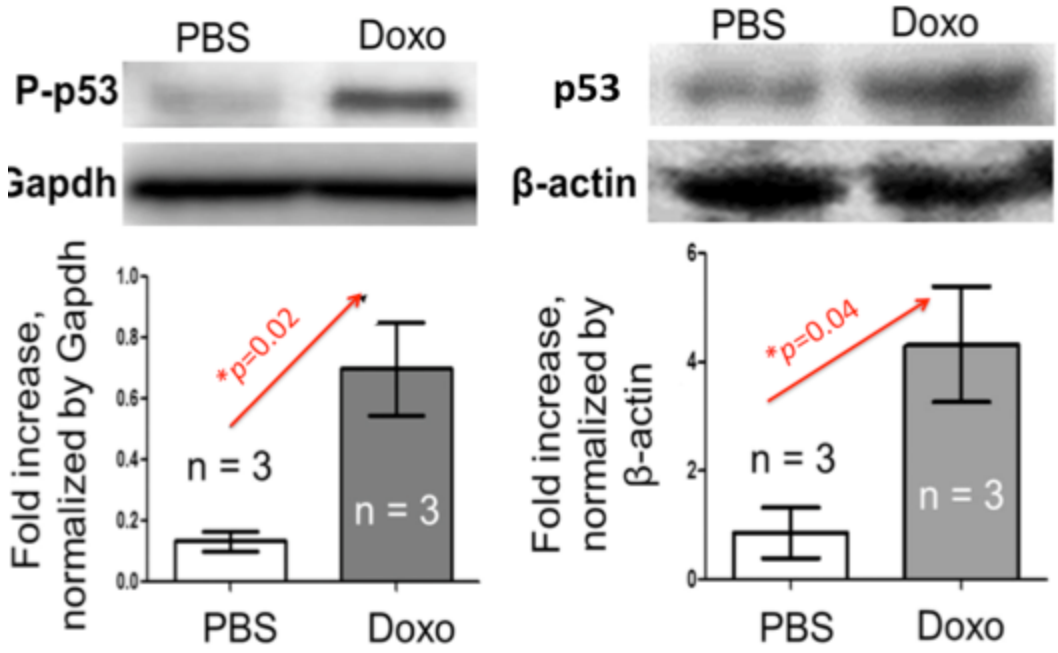

Figure 11. Chemotherapy (Doxo-treatment) induces activation of p53 in the thymus. With the same treatment as those in Fig. 3. Western blot analysis of phosphorylated p53 (P-p53; *left*) and total p53 (*right*) in the thymuses of the two groups. A Student t test was used to determine statistical significance 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. "n" represents animal numbers.

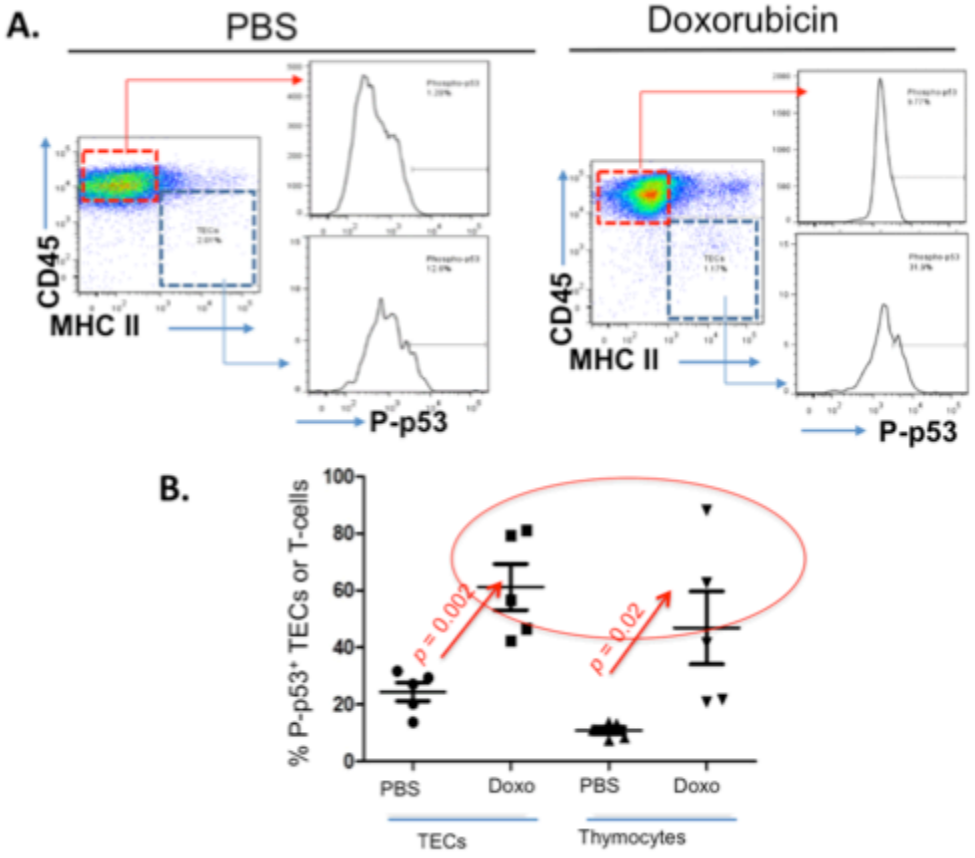

Figure 12. Chemotherapy (Doxo-treatment) induces activation of p53 in the thymus (Cont.). A. Flow-cytometric gate strategy of thymocyte ($CD45^{+}$ $MHC\text{-}II^{neg}$) and TECs ($CD45^{neg}$ $MHC\text{-}II^{+}$), as well as P-$p53^{+}$ cell populations. **B,** A summary of percentages of P-$p53^{+}$ 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.

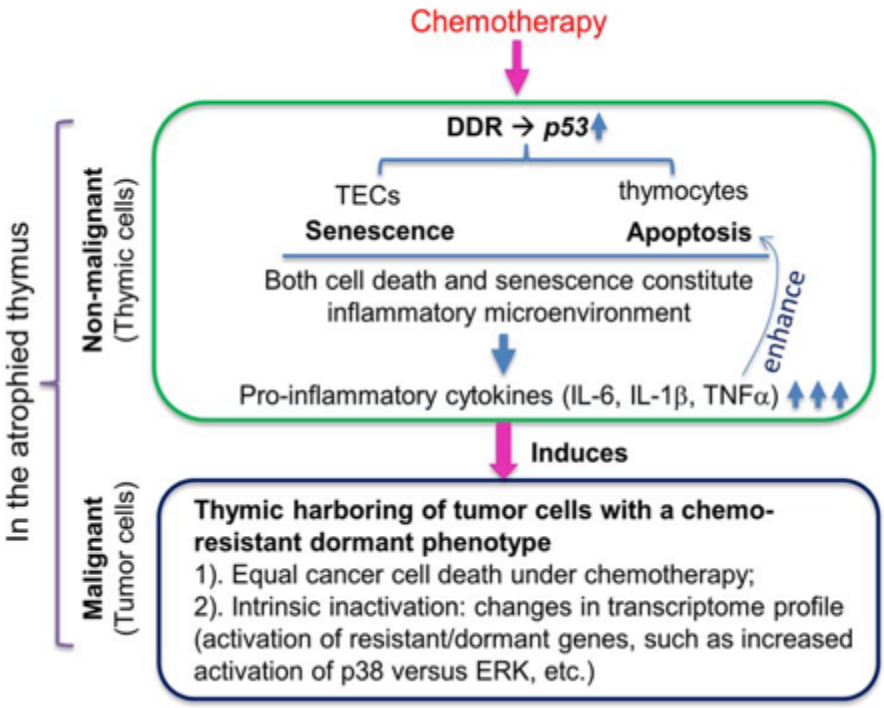

Figure 13. Schematic representations of potential mechanisms by which drug-induced changes in the atrophied thymic microenvironment subsequently induced thymic-harbored cancer cells to display chemo-induced dormant phenotype.

CHAPTER V

CHEMOTHERAPY INDUCES A CHEMORESISTANT PHENOTYPE IN THYMIC-HARBORED MELANOMA CELLS

Chemotherapy is one of the adjuvant types of anti-cancer treatment, following surgery. For late stage cancers, chemotherapy and radiotherapy become the only available treatment options. However, chemotherapeutic drugs are known to stimulate tumor cell chemoresistance [111,112]. Resistance to therapy can be either acquired or *de novo* [113]. Acquired resistance occurs when new genetic mutations accumulate in tumor cells over time and result in therapy-resistant cells. *De novo* drug resistance can either be soluble-factor mediated drug resistance or cell-adhesion mediated drug resistance [113]. While most research has focused on tumor cell autonomous mechanisms of chemoresistance, it seems the tumor niche microenvironment can also play a role in the development of chemoresistance and in malignant progression, thereby influencing the development of novel therapies in clinical oncology. The interaction of cancer cells and stromal components, such as fibroblasts, and the ECM (extracellular matrix) via surface receptors, such as integrins, induce cell-adhesion mediated drug resistance [114]. Additionally, chemokines, growth factors and cytokines that are secreted from the stromal cells are known to induce the soluble factor-mediated drug resistance of cancer cells via induction of dormancy [115,116]. In our model of thymic-harbored melanoma cells, we also suggest that secreted soluble factors in Doxo-altered thymic microenvironment benefit cancer cell chemoresistance by induction of cancer cell dormancy. This chapter

provides the evidence of thymus-harbored melanoma cells acquiring chemo-resistant phenotype following the changes in the thymic microenvironment.

Specific Aim #3

To determine that the interaction between the thymic microenvironment and the thymic-harbored solid tumor cells induces chemo-resistant dormancy at dynamic and quiescent states.

- *Sub-aim #3a:* To evaluate the percentages of surviving cancer cells in the thymus and other organs, focusing on the lymph nodes (LNs) and the lung after doxorubicin treatment.
- *Sub-aim #3b:* To analyze dormancy-associated p38/ERK ratio, apoptotic activity, and level of cell replication of thymic-harbored cancer cells after Doxo-treatment.

Working hypothesis: We hypothesize that the doxorubicin-altered thymic microenvironment induces intrinsic changes in the harboring cancer cells to protect them from chemotherapy, which is involved in cancer cell dormancy (dynamically and quiescently).

Rationale

Since it is reported that an inflammatory thymic condition drives drug-resistance in lymphoma during chemotherapy [12,13], we wanted to know if solid tumor cells under the same conditions can be induced towards drug-resistant dormancy as we discussed in the background section. The drug-resistant dormancy usually includes dynamic and/or

quiescent states So-called “dynamic dormancy” refers basically to a dormancy at “tumor mass” or “cell population” level, where part of the cancer cell population is undergoing replication and the other part of the cell population is in a dormant state or undergoing apoptosis; while “quiescent dormancy” refers to a single cell level, where each individual cancer cell is at non-replicating dormant state. The former refers to a balance of cell death and replication, while the latter is involved in changes in transcriptome profiles of chemo-resistant cancer cells, exhibiting enhanced expression of anti-apoptotic genes, a decrease of cell cycle genes, as well as an increased ratio of p38/ERK, which is an indication of dormancy. A high ratio of P-p38/P-ERK (activation of p38 and inhibition of ERK) induces tumor growth arrest, i.e., dormancy, while a low P-p38/P-ERK ratio favors tumor regrowth/recurrence [32,33,117].

Results

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

- *Sub-aim #3a:* To evaluate the percentages of surviving cancer cells in the thymus and other organs, focusing on the lymph nodes (LNs) and the lung after doxorubicin treatment.

Based on evidence shown in Chapter IV that the inflamed atrophied thymus favors harboring of 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 exhibit a chemo-resistant phenotype induced by this inflammatory condition. We inoculated B16-GFP melanoma cells ($1x10^6$

per injection) to young WT mice. Three days after the inoculation, we injected (i.p.) these tumor-bearing mice with Doxo for three days (8–10 mg/kg, once a day for three consecutive days). Three days after the last drug injection, 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 in Doxo-treated group compared to PBS-treated group (Fig. 14A), 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.

In addition, we directly visualized the inoculated GFP melanoma cell clusters with immunofluorescent staining of thymic cryosections from mice from both treatment groups (Doxo or PBS) and confirmed that GFP^+ melanoma cell clusters were increased in the thymus from Doxo-treated mice (Fig. 14B). 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. 15) 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. 15). 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 growth [118]. We inoculated B16 GFP^+ melanoma cells into young WT mice and then treated the mice with Doxo or PBS twice (10 mg/kg,

once a day for two consecutive days). Three days after the last drug treatment, we isolated the thymus, LNs, and lungs, adjusted these tissues to similar weight in separate culture plate wells, and cultured these three tissues for about two weeks. On the final day of the culture, we visualized the cancer cell regrowth via GFP^+ clusters under the microscope (Fig. 16A) and semi-quantitatively measured these green cellular clusters with Image-J software (Fig. 16B). 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 the 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 Image J software (Fig. 16B). 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 doxorubicin is not able to equally kill cancer cells in the thymus, LNs, and lung. Particularly, the chemotherapeutic drug had the least effect on thymic-harbored cancer cells from Doxo-treated mice. This is due to an inflammatory environment, which may confer the harbored cancer cells toward a chemo-resistant feature. Although these cancer cells cannot be completely eradicated in the thymus, they are unlikely to develop into a tumor in the thymus, because we never observed thymoma development in these mice. Therefore, the atrophied thymus is only asserted to play a role as a potential tumor reservoir and not a metastatic relapse site. The cancer cells in the lungs were also not

completely eradicated, in accordance with previous findings identifying the lungs as a known tumor reservoir [11].

Thymus from chemotherapeutic drug-treated mice potentially modulates the thymic-harbored cancer cells to acquire an antiapoptotic feature

- *Sub-aim #3b:* To analyze dormancy-associated p38/ERK ratio, apoptotic activity and the level of cell replication in thymic-harbored cancer cells after Doxo-treatment.

If cancer cells acquire either dynamic or quiescent dormant features, their metabolic profile should be altered. This is an intrinsic change, which is potentially induced by the inflammatory thymic microenvironment. Specifically, expression of genes involved in cell cycle and proliferation may be decreased and the balance of p38 Mapk/ERK expression may be increased, in which a high ratio indicates cell dormancy [119,120]. In this Aim, we used a novel experimental system that we developed. It is an *in vivo* drug-treatment in mice to induce thymic involution, followed by an *in vitro* culture to confer the inflammatory condition from this involuted thymus through a transwell to cultured tumor cells, which are pre-treated with antitumor drug (Doxo) (work schema is shown in Fig. 17). Because thymic-harbored cancer cells were able to resist chemotherapy, we found that the drug-induced inflammatory soluble factor–rich microenvironment [12] in the thymus was involved in this modulation of the proliferative and apoptotic features in the thymic-harbored cancer cells.

The results showed that when the atrophied thymus from Doxo-treated mice was co-cultured with Doxo-pre-treated B16 cancer cells separated by a membrane in a

transwell (Fig. 18A, group #4), these cancer cells were modulated to become more resistant to Doxo-induced apoptosis, showing a reduced percentage of Annexin-V^+ cells (Fig. 18A: 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). However, in the control group, the thymus was from PBS-treated mouse (Fig. 18A, group #3) and showed to be less able to confer resistance to Doxo-induced apoptosis (Fig. 18A: the unfilled peak). With the same transwell co-culture system, we did not find a significant difference in proliferation between the experimental group (Doxo-pre-treated B16 cancer cells co-cultured with Doxo-treated thymus) and control group ((Doxo-pre-treated B16 cancer cells co-cultured with PBS-treated thymus) (Fig. 18B: an arrow in the left labeled with "Doxo-treated thymus," and the rightmost striped bar on the right). The results indicate that the atrophied thymus from Doxo-treated mice is able to modulate drug pre-treated cancer cells to enhance their anti-apoptosis, but does not affect their proliferation, suggesting that the atrophied thymus from Doxo-treated mice indeed confer an anti-apoptotic feature to the thymic-harbored cancer cells. The mediators are soluble factors, which can pass through the membrane, and could be pro-inflammatory factors secreted from the drug-induced thymus.

Furthermore, we wanted to know why proliferation in these cancer cells was unchanged. It could be related to a quiescent dormant feature. Changes in the ratio of p38/ERK are associated with quiescent dormancy. Therefore, 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., quiescent dormancy, while a high P-ERK/P-p38 ratio favors tumor regrowth/recurrence

[32,33,117]. We found that the ratio of P-p38/P-ERK was significantly increased in the cancer cells under the transwell co-cultured with the atrophied thymus from Doxo-treated mice (Fig. 19, the rightmost filled striped bar in right). These results can explain why proliferation in these cancer cells was unchanged and indicate that the atrophied thymus from Doxo-treated mice confers Doxo-pretreated cancer cells with a relative quiescent dormant feature.

Discussion

To combat tumor metastatic relapse, it is important to determine how the microenvironment in pre-metastatic reservoirs modulates harboring tumor cells, how they become drug-resistant, and how they retain MRD.

Here, we determined how chemotherapy induces the thymus to act as a tumor reservoir and how this reservoir can modulate the thymic-harbored tumor cells to acquire a chemoresistant feature, the dark side of chemotherapy. When patients with cancer receive chemotherapy, there is the risk of damaging healthy tissues, including creating an atrophied and inflamed 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. There is the risk that thymic-harbored dormant cancer cells could eventually develop a new tumor once conditions are suitable for them to disperse to distant organs.

Taken together, we demonstrated that thymic-harbored cancer cells in the inflammatory thymus possess the capacity to resist chemotherapy through anti-apoptosis

accompanied by unchanged proliferation. The fact that no significant changes in cell proliferation were observed and an increased capacity to resist apoptosis was also observed, suggests activation of dormancy at a “dynamic” or “tumor mass” level. Meanwhile, thymus-modulated intrinsic changes in molecular activation, such as increased P-p38 and/or decreased P-ERK indicate most of the cancer cells are at quiescent state. Upregulation of intrinsic markers of cell dormancy demonstrates the activation of dormancy on a “single cell level”. When both types of dormancy are taken place, the concept is termed a “heterogeneous dormancy”. This provides us with the evidence that a heterogeneous dormancy occurs with the thymic-harbored cancer cells during doxorubicin treatment.

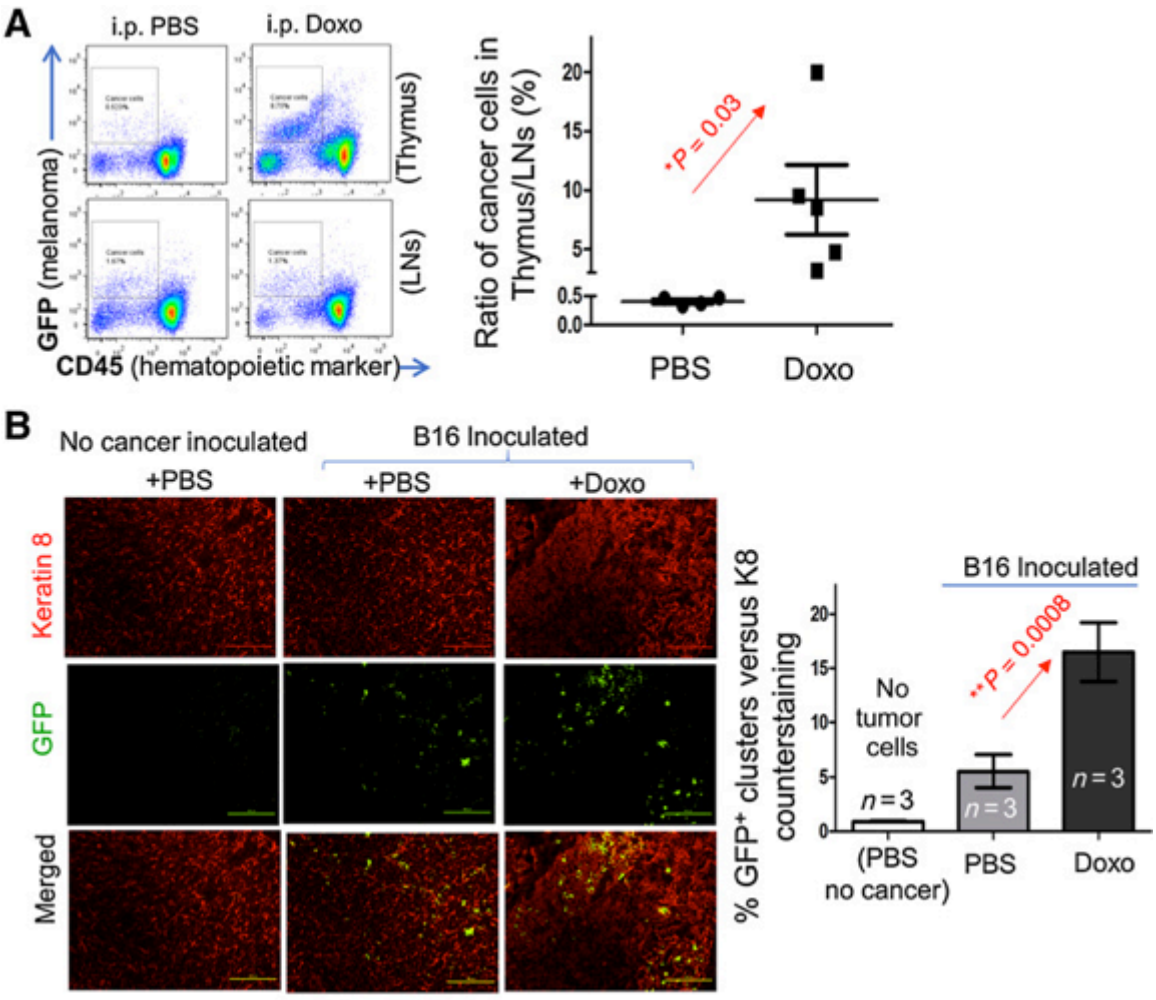

Figure 14. Chemotherapy induces a chemoresistant phenotype in thymic-harbored melanoma cells. With the same melanoma (B16-GFP) inoculation and then the same Doxo-treatment as those in Fig. 3. Three to five days after the last drug injection, the thymuses and inguinal and mesenteric LNs were isolated for analysis. **A,** *Left,* A flow-cytometric gate strategy shows melanoma cells (GFP^+CD45^{-neg} population) in the thymuses (*top*) and LNs (*bottom*); right, summarized ratios of % melanoma cells in thymus/LN of PBS-Ctr and Doxo-treated groups. **B,** *Left,* Immunofluorescence staining images of GFP^+ melanoma cell clusters (Green) in Keratin-8 TEC background (Red) of three differently treated mouse thymuses. The bar in the image is 100 nm. *Right*, A summary of GFP^+ melanoma cell clusters in the thymuses of three differently treated mice. The GFP^+ cell cluster images were semiquantitatively analyzed using Image J software. All data are expressed as mean ±SEM. The *P* values are shown in each panel, and "n" represents animal numbers.

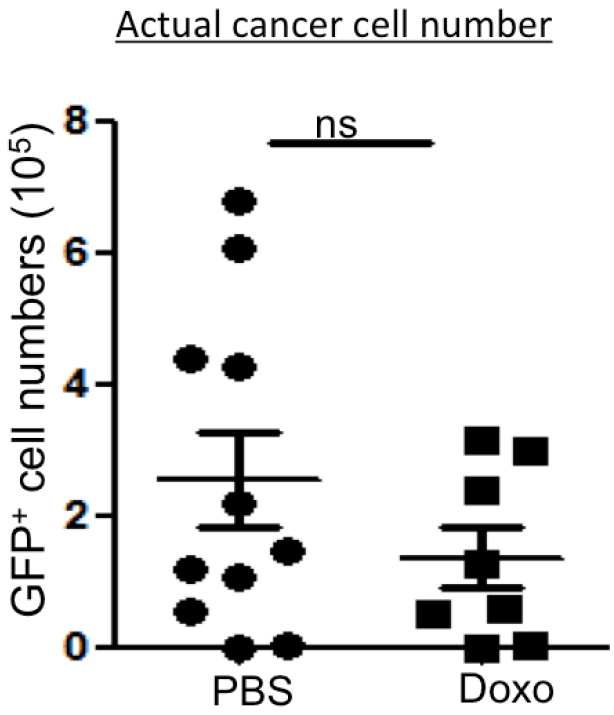

Figure 15. Chemotherapy induces a chemoresistant phenotype in thymic-harbored melanoma cells. With the same melanoma (B16-GFP) inoculation and then the same Doxo-treatment as those in Fig. 3. A summarized flow-cytometric data for absolute cancer cell numbers (gated on $GFP^{+}CD45^{-neg}$) in the thymuses from B16 cell–inoculated and PBS-Ctr or Doxo-treated mice. 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, each symbol represents an individual animal.

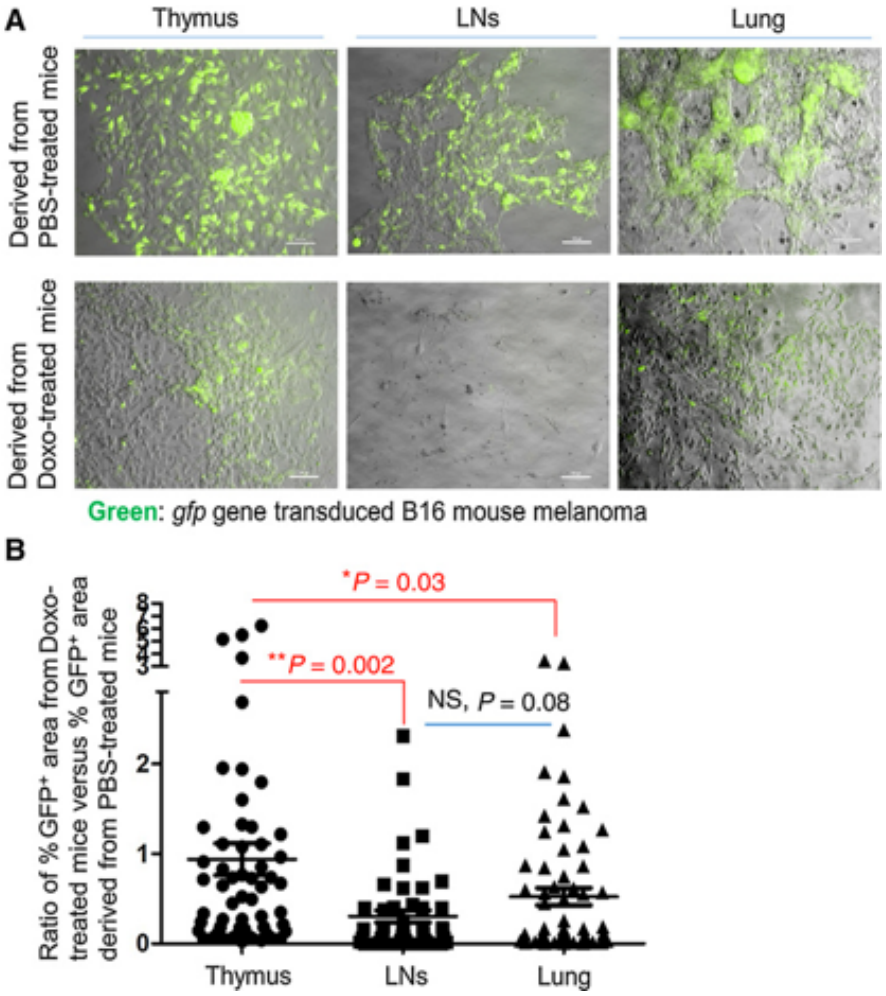

Figure 16. Capacity of cancer cell regrowth from the thymus is greater compared with other organs from the mice with chemotherapy. WT young mice were inoculated with B16-GFP cancer cells, and then treated with Doxo or PBS, same setting as in Fig. 3. Three days after the last drug injection, the thymus, LNs, and lungs were freshly isolated. These three tissues with similar weight were cut into small pieces and homogenized, then each organ was subsequently cultured in separate plate wells for about two weeks. On the final day of the culture, GFP^+ cell clusters were visualized and semiquantitatively measured with ImageJ software. **A**, Representative images of GFP^+ melanoma cells in culture from three types of tissues (the thymus, LNs, and lung) of PBS-Ctr (*top*) and Doxo-treated (*bottom*) groups, respectively. **B,** Summarized ratios of % of GFP^+ areas derived from Doxo-treated mice to the areas derived from PBS-treated mice. Each symbol represents ratio of % positive area per image of the tissues from a total of 4 animals in each group. This experiment was repeated at least four times. All data are expressed as mean ±SEM. The *P* values are shown in each panel.

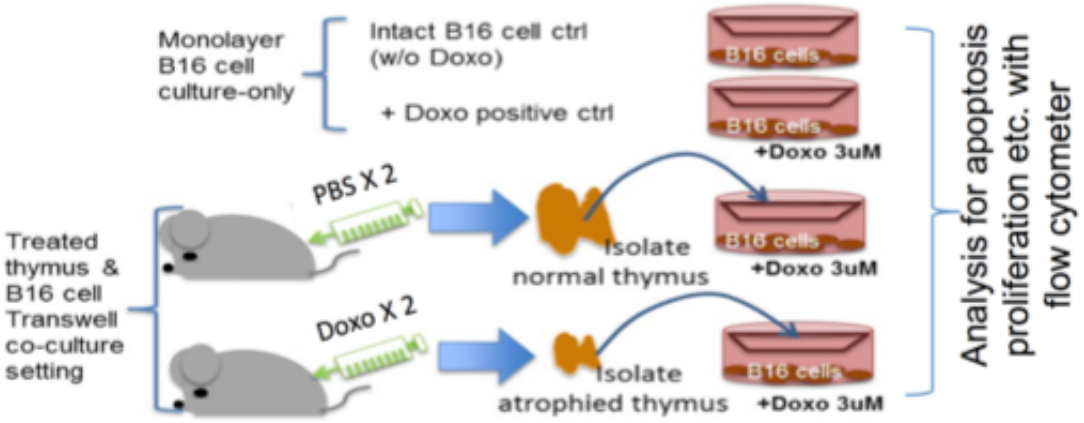

Figure 17. The atrophied thymus from Doxo-treated mice modulates Doxo-pretreated melanoma cells toward exhibiting increased anti-apoptosis but unchanged proliferation, and increased ratio of P-p38-to-P-ERK. Experimental schema of thymic modulation of B16 melanoma cells. In this system, young WT mice were i.p. injected with Doxo as shown in Fig. 3. The thymuses from the Doxo-treated and PBS-Ctr mice, respectively, were isolated and cut the thymuses into tissue pieces. The same weight of thymic tissue blocks were loaded on top of transwells to co-culture with Doxo-pretreated B16 melanoma cells in monolayer culture on the bottom wells. Three days after the co-culture, in which the thymus and monolayer B16 cells were separated by the transwell membrane, the B16 cancer cells were analyzed with flow cytometric assays.

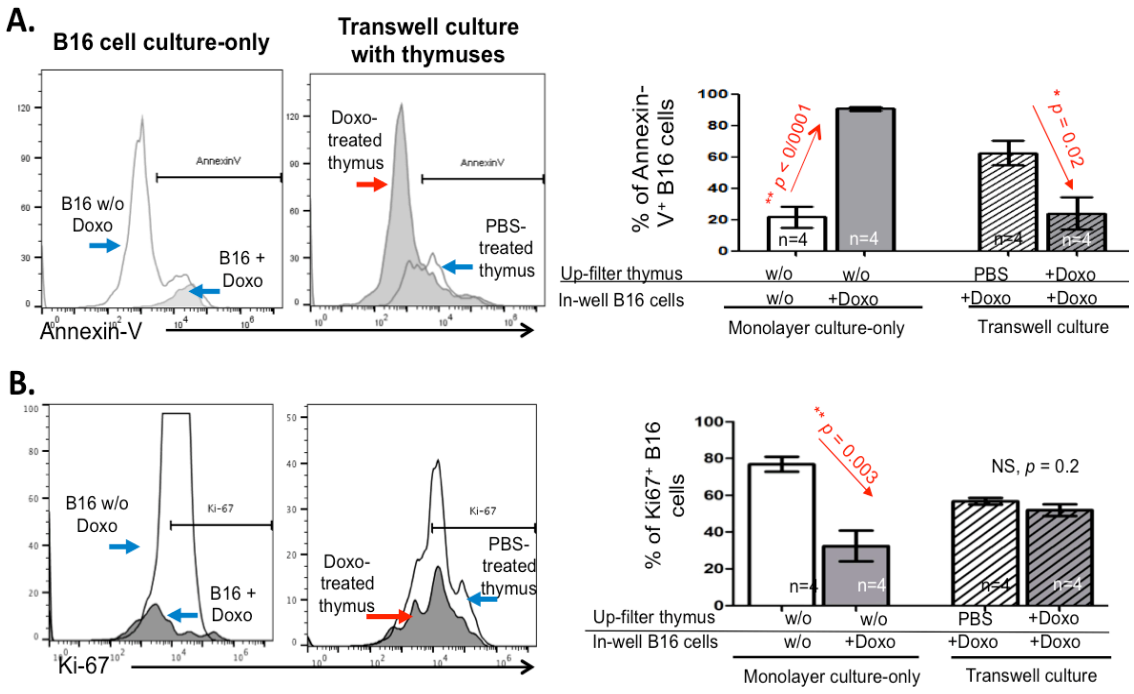

Figure 18. The atrophied thymus from Doxo-treated mice modulates Doxo-pretreated melanoma cells toward exhibiting increased anti-apoptosis but unchanged proliferation. A, Results of Annexin-V–based apoptosis assay. *Left*, Representative histograms show Annexin-V$^+$ B16 melanoma cells with or without 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). **B,** 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). All data are expressed as mean ±SEM. The *P* values are shown in each panel, and "n" represents animal numbers.

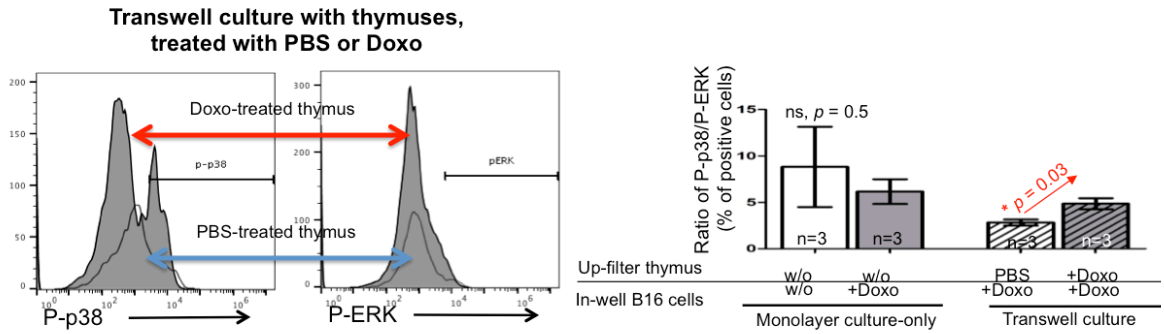

Figure 19. The atrophied thymus from Doxo-treated mice modulates Doxo-pretreated melanoma cells toward exhibiting increased ratio of P-p38-to-P-ERK. Results of intracellular staining of P-p38 and P-ERK, respectively. *Left,* Representative histograms show P-p38$^+$ and P-ERK$^+$ B16 melanoma cells with 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.

CHAPTER VI

CONCLUSIONS AND DISCUSSION

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 chronic inflammation. It is 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 [12]. However, these lymphoma cells were unable to circulate in great numbers into the thymus, and they do not expand in the thymus, as well, since we did not find a thymoma developed when we tested the lymphoma model (data not shown). Therefore, it is unlikely that the majority of thymic inflammation comes from death of the small number of tumor cell in the thymus. The inflammation was also proposed to come from thymic endothelial cells, associated with acute stress–associated phenotype (ASAP)-related secretion [13,85]. This is also unlikely because vasculature-related thymic endothelial cells represent a very small portion, contained within 8% of the UEA-1^{-neg} *and Ly51*$^{-neg}$ subsets in 5% of the *CD45*$^{-neg}$ population, i.e., less than 0.4% of total thymic cells based on a flow-cytometric assay [88]. Therefore, it is difficult to explain how so few cells could act as the predominant source of thymic inflammation.

The thymus is a unique organ and very sensitive to any assault, especially, chemo/radiotherapy, which induces acute thymic atrophy associated with inflammation. Our data identified that under chemotherapy, thymic inflammation arises from a synergistic effect of cell death (mainly in thymocytes) and senescence (mainly in TECs),

although we do not exclude cell death in TECs as well. In other words, the thymic inflammation arises from chemotherapy-induced non-malignant thymic cell death and senescence, rather than the harbored malignant tumor cells *per se*. The cell death and senescence phenotype in thymic cells could be explained mechanistically due to drug-associated DDR-triggered activation of the p53 gene, which induces both cell apoptosis [68] and cellular senescence [69], which is illustrated in Fig.13. On the other hand, cellular senescence can be beneficial when it happens in malignant cells because it may arrest tumor cell growth, but it is deleterious when it happens in nonmalignant stromal cells, because the cells develop SASP-associated inflammation [41]. TECs, mainly undergoing senescence during chemotherapy, are probably the cells involved in SASP-associated inflammation in the thymus. In addition, senescent stromal cells, that establish a condition for cancer stem cell generation and cancer metastasis, could promote an epithelial-to-mesenchymal transition [40]. As to inflammation, it is a double-edged sword that can either suppress tumor growth (such as immune reaction-mediated inflammation) or induce tumor cell progression or chemoresistance [23,78,109,121].

The problem of tumor cell chemo-resistance still remains one of the greatest challenges in oncology. Several processes may be involved in cancer cell chemo-resistance, such as activation of anti-apoptotic mechanisms, inhibition of cell replication and induction of cell dormancy. P38 MAPK can play a dual role as a regulator of cell death, thus it can either mediate cell survival or cell death through different mechanisms, including apoptosis, since the specific function of p38 MAPKs in apoptosis appears to depend on the cell type, the stimuli, and/or the isoform [122,123]. However, a high P-p38/P-ERK is reported to be an indicator for cellular dormancy [32,33,117]. In the BM (bone

marrow) TGFβ2 (tumor growth factor beta 2) induced a low ERK/p38 signaling ratio, which induced the metastasis suppressor DEC2. In turn, DEC2 (Differentially Expressed In Chondrocytes Protein 2) repressed CDK4 (Cyclin Dependent Kinase 4) and induced p27 to induce bone marrow DTC quiescence [119].

The anti-apoptotic feature with inactive state in thymic-harbored tumor cells may reflect tumor cell dormancy at single-cell levels with intrinsic changes. These cells may display stem cell-like properties (termed "stemness"), which possess innate resistance mechanisms to chemo/radiotherapy [27].

In addition, we cannot exclude the possibility that some, if not all, thymic-harbored cancer cells still retain sensitivity to chemotherapy, and they may undergo a low level of apoptosis and proliferation to maintain limited total tumor cell numbers in the thymus. This is termed "equal cell death." If this is the case, the thymic-harbored tumor cells exhibit a heterogeneous dormant state, which makes these cancer cells neither likely to manifest as a tumor mass in the atrophied thymus, such as thymoma, nor to be thoroughly eradicated in the atrophied thymus under the chemotherapy. Taken together, this study demonstrates that the body's largest T lymphoid organ, the thymus, and especially the chemotherapy-induced atrophied thymus, indeed provides a chemoprotective microenvironment for thymic-harbored cancer cells. Therefore, the thymus plays a role of a cancer pre-metastatic reservoir during chemotherapy. We brought this new target, which could be responsible for cancer relapse, into focus for efficient cancer therapy. Considering this target during chemotherapy will potentially lead to efficient therapeutic interventions to combat tumor recurrence.

CHAPTER VII

LIMITATIONS

P53 is an essential, but not the only contributor to genotoxic damage-induced changes in the thymus

To further confirm that persistent increase of P-p53 is responsible for the thymic inflammatory condition via increased massive apoptosis in thymocytes and senescence in TECs after genotoxic doxorubicin treatment, it is important to use a *p53* gene conditional knockout mouse model to eliminate p53 expression in thymocytes and TECs. Therefore, in order to investigate this idea, we generated *Trp53* floxed homozygous mice crossbred with *Rosa26* Cre^{ERT} transgenic mice. In such a mouse model the ubiquitous deletion of *p53* gene can be achieved through a Tamoxifen-induced (20ug/g i.p. x3) activation of Cre-recombinase (Fig. 20). These mice are termed *Trp53* cKO mice (*Trp53*fx/fx;Rosa26 Cre^{ERT}). These mice received similar treatment as described in previous experiments in Chapter IV: treatment with doxorubicin with subsequent evaluation of apoptosis, TEC senescence, and thymic inflammatory condition. The preliminary data show that Doxo-treated p53 cKO mice still experienced thymic involution with massive apoptosis of thymocytes and TEC senescence (Fig. 21). Additionally, we observed secretion of IL-6 in thymic microenvironment of these mice post-doxorubicin injection, and found that IL-6 was still increased (Fig. 22). Inoculation with B16 GFP^+ melanoma cells also resulted in a phenotype similar to what we observed previously with WT mice: melanoma cells still harbored in the thymus after Doxo-treatment (Fig. 22). These results demonstrated that in

this model, ubiquitous *p53* gene deletion does not lead to rescue of the thymus phenotype. The results imply that ubiquitous knockout of p53 is a limitation that cannot uncover the mechanisms underlying p53-correlative thymic cell death and senescence. However, since we have shown that anti-tumor chemotherapy treatment induces p53 up-regulation in non-malignant thymic cells (thymocytes and TECs), it may be beneficial to utilize another mouse model with p53 gene deletion specifically in these thymic cells. In order to confirm the role of p53 in thymic cells, we must down-regulate p53 only in thymic cells, *rather than all cells in the body (ubiquitously*). Particularly, we must avoid down-regulation of p53 in macrophages and neutrophils, since it has been shown that these highly-reactive immune cells exhibit systemic pro-inflammatory phenotype with the loss of p53 [124]. In order to knock-out or knock-down p53 specifically in thymocytes we should utilize a transgenic mouse strain expressing Cre^{ERT} recombinase under the control of the *Lck* (lymphocyte protein tyrosine kinase) promoter. This system enables excision of loxP-flanked sequences of Trp53 specifically in thymocytes [125]. Meanwhile, utilizing $K5Cre^{ERT}$ mice will allow us to knock-out p53 specifically in epithelial cells, including thymic epithelial cells [126].

It is also possible that p53 is the key, but not the sole, player in reshaping the thymic condition upon genotoxic chemotherapy treatment. We propose that a p53-independent signaling pathway may exist. In addition to p53, DDR is known to activate NF-κB [127, 128]**.** Doxorubicin-induced DDR has been shown to inactivate IKB-α, an endogenous inhibitor of NF-κB translocation to the nucleus and subsequently NF-κB activity [129]. Additionally, it has been shown that NF-κB may be activated independently from p53 in primary cells under genotoxic stress conditions [130]. Indeed, we observed that

Nf-κB1 (subunit of NF-κB complex) is upregulated in thymus tissue upon Doxo-treatment (Fig. 23).

However, p53- and NF-κB- initiated downstream pathways have opposite effects. For example, while NF-κB typically activates pro-proliferative and anti-apoptotic genes, p53 often activates anti-proliferative and pro-apoptotic genes [131]. Nevertheless, a number of studies have shown that NF-κB is simultaneously upregulated along with p53 [75,132]. DNA-damaging agents also activate NF-κB in a canonical IKK complex-dependent fashion. However, in contrast to classical or alternative NF-κB signaling pathways, this genotoxic signaling cascade is initiated in the nucleus instead of via membrane-bound receptors [127]. As a result of pro-survival mechanisms, activation upon NF-κB engaging during DDR cell survival may be encouraged. An example of such cell survival may be activation of NF-κB – induced senescence. NF-κB is proposed to be a core molecule in SASP [133,134]. Together with data of p53 activation (Fig. 11), which is a trigger of SASP [41,135], increased pro-inflammatory cytokines (Fig. 7) along with increased NF-κB (Fig. 23) the results suggest that doxorubicin-induced DDR activates SASP. Additionally, depending on the severity of genotoxic stress and cell type NF-κB may be employed to promote programmed cell death [136,137]. It has been shown that T-lymphocytes undergo apoptosis via NF-κB activation during treatment with DNA damaging agents [138]. We propose that in the absence of p53 signaling in *Trp53* cKO mice, NF-κB may contribute to apoptosis of thymocytes and senescence of TECs during Doxo-treatment as well.

The p53-dependent pathway for DNA damage-induced apoptosis has been very well studied, however, not much is known about the p53-independent pathway, even though DNA damage-induced apoptosis was shown in cell lines that lack p53 expression

[139]. Doxorubicin can mediate cell death via two main pathways: inhibition of Topoisomerase-II enzyme to induce DNA damage and the formation of ROS [95]. It has been shown that ROS activation can also induce cell death via apoptosis and necrosis [140,141]. Additionally, ROS have been shown to induce senescence in normal keratinocytes via epigenetic regulation of $p16^{INK4A}$ [142]. We propose that ROS involvement in re-shaping of the thymic microenvironment during doxorubicin administration should also be considered in future studies.

Metastatic model

We believe that our findings will establish evidence that the thymus, especially the involuted thymus, acts as a pre-metastatic reservoir for circulating lymphoid or non-lymphoid cancer cells that can become chemo-resistant during chemotherapy. However, our metastatic model of direct injection of cancer cells into bloodstream is imperfect, since it does not truly represent the process of cancer cells entering into blood circulation under physiological condition. Naturally, each metastatic cancer cell must undergo a complex cascade of metastatic events, that is driven by specific molecular pathways. The upregulation of specific genes is essential for multiple steps necessary for metastasis, such as: resistance to anoikis, EMT (epithelial-mesenchymal transition), resilience to the shear force of the blood stream, MET (mesenchymal-epithelial transition) etc.[143-145]. However, for our experiments we inoculated cultured trypsinized cancer cells directly into the bloodstream, as an artificial means of establishing cancer cell circulation. However, this model has its advantages [103], including controllable numbers of cells delivered to each mouse, comparability between each experiment, short waiting time for

evidence, and easy observation by flow cytometry and fluorescent microscopy. This model may not be ideal for studying the mechanisms 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.

Doxo autofluorescence

Another important limitation is that the drug doxorubicin has its own fluorescence property that may influence the results, especially in PE (Phycoerythrin) and PerCP (Peridinin Chlorophyll Protein Complex) channels (excitation of ~470nm and emission of ~570nm) (Fig. 24). To avoid this problem, it is important to have a doxorubicin-only control (without inoculation with fluorescence-tagged cancer cells) with every experiment and try to avoid using PE and PerCP conjugated antibodies.

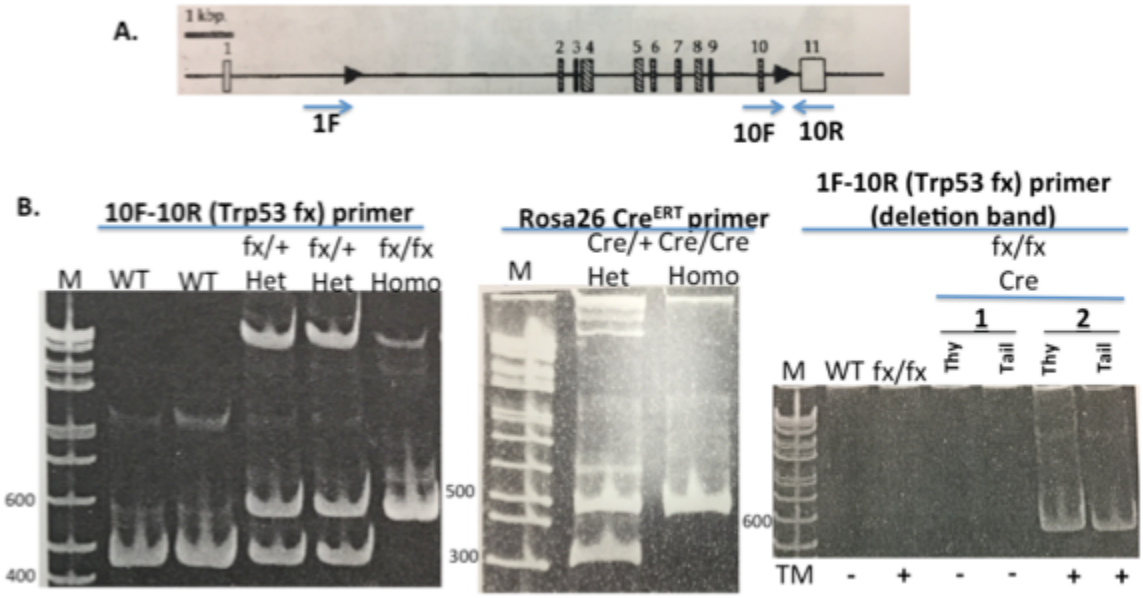

Figure 20. Trp53 deletion after TM-induced Cre recombinase activation is confirmed in Trp53 cKO mice. A. Schematic representation of *Trp53* conditional gene knockout system: *Trp53* functional domain is flanked by two loxP sites (termed "fx"). After introduction of CreERT transgene (Tg) into these mice by crossbreeding and induction with tamoxifen (TM, only for CreERT Tg), the loxP-flanked exon sites are excised and the *Trp53* gene loses its function (knockout). B. Representative acrylamide gel image of polymerase chain reaction (PCR) products to identify the offspring carrying *Trp53* floxed sites (*left*), Rosa26 CreERT Tg *(middle*), TM-induced deletion of *Trp53* gene *(right)*. TM injection was used to activate CreERT recombinase and initiate the deletion of *Trp53* floxed sites ubiquitously.

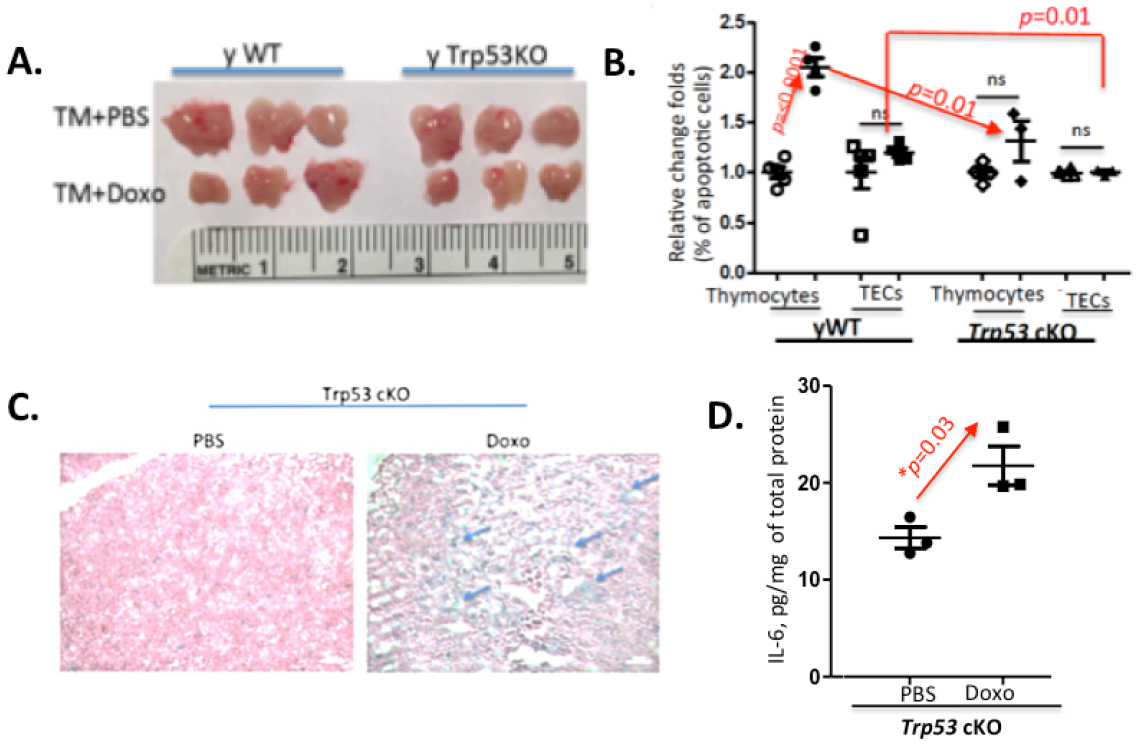

Figure 21. p53 deletion does not protect the thymus from DDR. With the same melanoma (B16-GFP) inoculation and the same Doxo-treatment as those in Fig. 3. Three to five days after the last drug injection, the thymuses were isolated for analysis. **A,** *left,* representative image of the thymuses shows the thymic atrophy in the Doxo-treated groups of WT and Trp53cKO mice. **B,** A summary of apoptotic analysis of thymocytes (gated on the $CD45^{+}$ $EpCam^{-neg}$ population) and TECs, gated on the $CD45^{neg}EpCam^{+}$ population) with Annexin-V assay. **C,** a representative picture of SA-β-gal increase in Doxo-treated group of Trp53cKO mice. **D,** a pro-inflammatory IL-6 is increased in Doxo-treated Trp53 cKO mice. Concentration of cytokine product in pg/mg of thymic protein; 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.

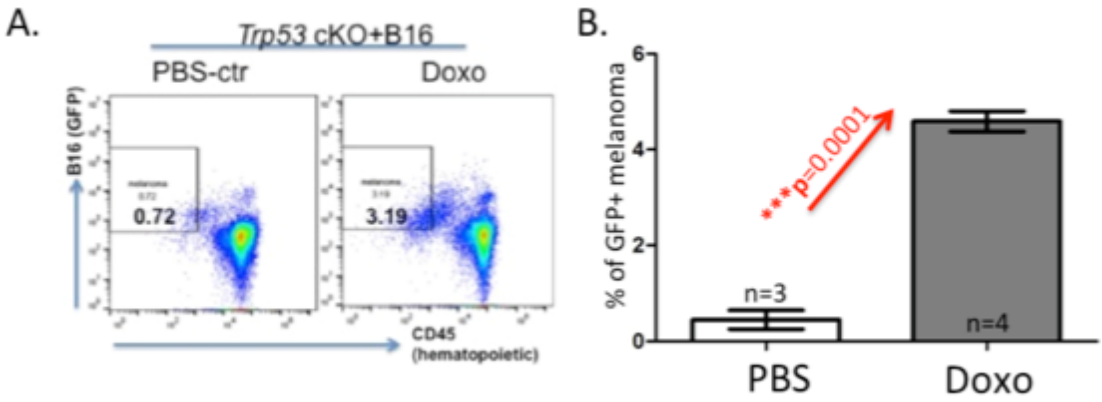

Figure 22. Trp53-deficient thymus retains the capacity to harbor B16F1 cancer cells. With the same melanoma (B16-GFP) inoculation and then the same Doxo-treatment as those in Fig. 3. Three days after the last drug injection, the thymuses were isolated for analysis. **A,** A flow-cytometric gate strategy shows melanoma cells ($GFP^{+}CD45^{-neg}$ population) in the thymuses of PBS-treated (l*eft*) and Doxo-treated (*right*) mice. **B,** summarized ratios of % melanoma cells in the thymus of PBS-Ctr and Doxo-treated groups. 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 "n" represents number of the animals in the experiment.

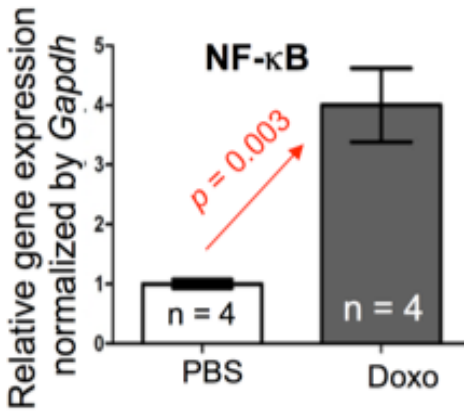

Figure 23. Nf-ϰB is overexpressed in the Doxo-treated thymus. With the same Doxo-treatment as those in Fig. 3. Three days after the last drug injection, the thymuses were isolated for gene expression analysis. Summary of real-time RT-PCR results showing relative increased expression of NF-ϰB subunit-1 in Doxo-treated thymus. 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 "n" represents number of the animals in the experiment.

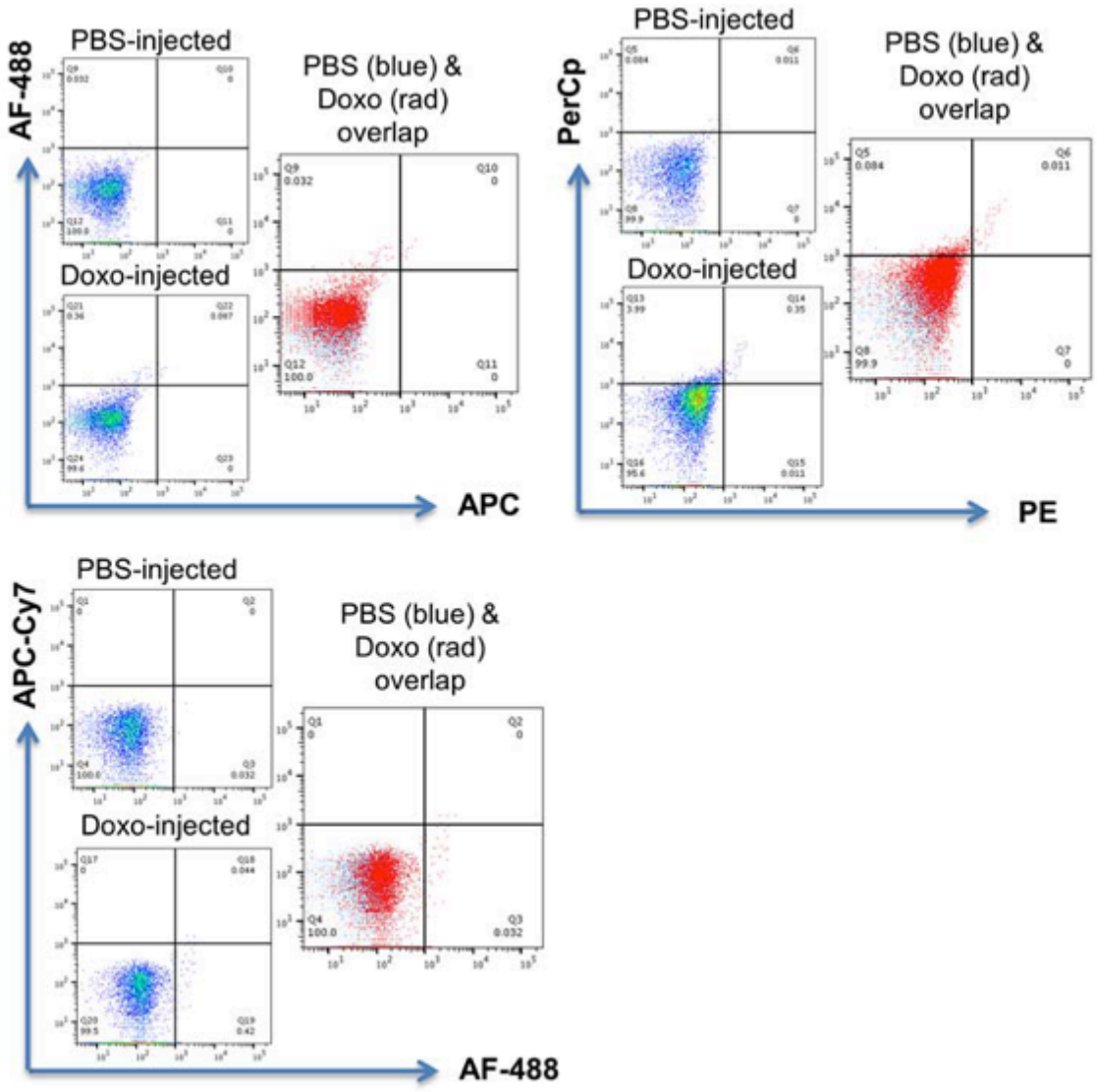

Figure 24. Doxorubicin fluorescence. Representative flow cytometric dot plots show doxorubicin (Doxo) auto-fluorescence in different flow cytometric channels. The result suggests not to use PerCP, and strictly set up a cutoff of a positive threshold for GFP (AF-488) channel.

CHAPTER VIII

FUTURE DIRECTIONS

This project has primarily determined some answers related to cancer metastatic reservoir, and has also raised other profound questions that can be answered in the future studies.

Cancer cells into the thymus: possible routes of entry

One question is *how* cancer cells circulate into the thymus: only passively through blood stream, or in combination with active attraction by certain molecules? Firstly, it is probably a correct assumption that metastatic cancer cells can enter the thymus passively via blood circulation, since the thymus lacks afferent lymphatic vessels [146]. Secondly, it is possible that there is active attraction of cancer cells with thymus-producing chemokines. It is now thought that chemokines play a significant role in organ-selective cancer metastasis, because cancer cell migration and metastasis share many similarities with leukocyte trafficking [147]. Naturally, in order to attract early T-cell progenitors from the bone marrow, the thymus releases CXCL12, CCL25, CCL21 etc. chemokines [148]. CXCL12 is a ligand for CXC chemokine receptor 4 (CXCR4) and it is known to be the most common chemokine receptor overexpressed in human cancers. More than 23 different human malignancies, including breast cancer, ovarian cancer, melanoma, and prostate cancers, express CXCR4 [149,150]. The murine model of metastatic melanoma B16 is known to constitutively express levels of CXCR4 [151] and its overexpression is shown to increase pulmonary and hepatic metastasis [152]. In addition, overexpression of CCR7, a

receptor to another thymus-derived chemokine CCL25, by B16 melanoma cells leads to enhanced metastasis to draining lymph nodes [153].

We propose that thymic chemokines, such as CXCL12, CCL25 and CCL21, may be involved in active recruitment of disseminating cancer cells into the thymus. We generated evidence that there is a significant increase of *Cxcl12* and *Ccl21* gene expression after the treatment with doxorubicin (Fig. 25). This evidence encourages us to do future experiments using corresponding chemokine receptor-knockdown mouse models to shed more light on this question.

Long-term cancer recurrence from the thymus niche

This study proposes that retention of dormant cancer cells in the thymus may increase the risk for cancer patients' metastatic relapse, even to those in confirmed long-term remission. So far, there is no direct evidence as to whether or how these thymic-harbored cancer cells reserve the capacity for recurrence. On one hand, if these tumor cells are going to remain in a permanent dormant state indefinitely and stay in the thymus without emitting from the thymus, the patients are not under any risk for relapse. Conversely, if there is even a slight possibility that these cells can "awaken" and metastasize when conditions are favorable, it is analogous to having a "time bomb" in the patient's body. Long-term *in vivo* studies are important to prove whether or not thymus-harbored cancer cells are capable of causing recurrent metastasis and initiating secondary tumors at distant organs. In this case, thymectomy for eligible patients may become a reasonable option.

Thymus soil is "unfertilized"

The fact that thymic-harbored cancer cells remain in the thymic tissue, but do not initiate tumor growth (thymoma) *in situ,* remains intriguing. According to our data obtained from multiple experiments, at least 12 days post-inoculation, even in young WT PBS-treated mice, cancer cells do not form a tumor mass in the thymus. However, when these cancer cells are provided with suitable conditions, instantaneous recurrence is observed (Fig. 5). This suggests that an intact thymic condition, even without chemotherapy-induced alterations, restricts solid cancer cell growth *in situ*. It is essential to investigate why this unaltered young thymic microenvironment serves as a niche for disseminated melanoma cells, but inhibits growth of these cells.

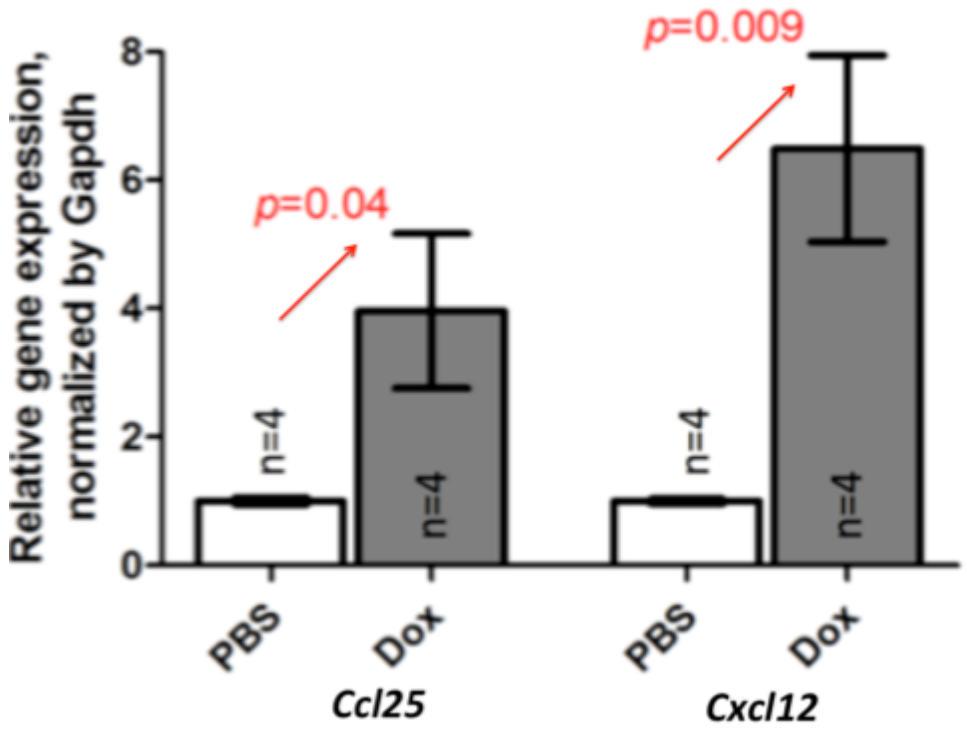

Figure 25. Thymic chemokines were increased after Doxo-treatment. RT-PCR results of thymic chemokines *Ccl25* and *Cxcl12*. Relative *Ccl25* and *Cxcl12* gene expression in PBS- vs. Doxo-treated thymuses. With the same Doxo-treatment as those in Fig. 3. Three days after the last drug injection, the thymuses were isolated for gene expression analysis. Summary of real-time RT-PCR results showing relative increased expression of NF-ϰB subunit-1 in Doxo-treated thymus. 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 "n" represents the number of the animals in the experiment.

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