Lymphocytic Infiltration and Immune Escape Mechanisms in Human Chordoma

Table of Contents

Glossary

APM	Antigen processing machinery
β2m	Beta-2-microglobulin
CAR	Chimeric antigen receptor
CD3-ζ	Cluster of differentiation 3-zeta
CSPG4	Chondroitin sulfate proteoglycan 4
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DAB+	3,3'-Diaminobenzidine (chromogen)
ER	Endoplasmic reticulum
H&E	Hematoxylin & eosin
HC-10	anti-HLA-B/C monoclonal antibody
HC-A2	anti-HLA-A monoclonal antibody
HLA	Human leukocyte antigen
HMW-MAA	High molecular weight-melanoma associated antigen
HRP	Horseradish peroxidase
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IL-2	Interleukin-2
MGH	Massachusetts General Hospital
MHC	Major histocompatibility complex
NAMB-1	anti-beta-2-microglobulin monoclonal antibody
NK	Natural killer (cell)
PAS	Periodic acid-Schiff stain
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PDGFR-β	Platelet-derived growth factor receptor-beta
TA	Tumor antigen
TAP	Transporter associated with antigen processing
TBST	Tris buffered saline with Tween 20 (solution)
TCR	T cell receptor
TNF	Tumor necrosis factor

Abstract

Recent advances in immunotherapy for cancer have led to increasing interest in the role of the immune system in the pathogenesis and treatment of various tumors. Tumor-infiltrating lymphocytes have been associated with more favorable prognoses in a number of malignancies, though the mechanism of such outcomes remains unclear. This study, to our knowledge, is the first to describe lymphocytic infiltration in chordoma. Hematoxylin and eosin stained slides from 62 randomly selected patients with chordoma treated at MGH were evaluated for lymphocyte infiltrates. Lymphocytic infiltration was found in 84% of tumors, with lymphocytes primarily distributed in the fibrous septae surrounding the physaliferous cells. Twenty-four tumors were randomly selected for immunohistochemical (IHC) analysis. All of the tumors with lymphocytic infiltration that underwent IHC staining had CD4-positive lymphocytes present, ranging from 1-300 cells per high-power field. CD8-positive lymphocytes were noted in 52% of tumors, ranging from 1-39 cells per high-power field. HLA class I antigen defects were noted in 79% of chordoma tumors. These included negative membranous and/or cytoplasmic staining patterns, weakly positive staining, and heterogeneous combinations of these defects in variable percentages of chordoma cells. Both heavy chain isoforms and beta-2-microglobulin components were affected. Our novel finding of intratumoral lymphocytes in chordoma tumors suggests that a patient's immune system mounts a response against his/her tumor. The absence of HLA class I antigen components is compatible with the possibility that a patient's immune response imposes selective pressure that facilitates the outgrowth of chordoma cell subpopulations that have developed escape mechanisms from host immune recognition. We are the first to report these original findings in chordoma, and while the clinical significance requires further evaluation,

these data have implications in optimally selecting patients for appropriate treatment regimens. We believe these data will spark further inquiry into the role of immunotherapy in the treatment of chordoma and other rare primary bone tumors.

Introduction

The Role of the Immune System in Cancer

The interaction between the immune system and malignancy has long been studied. The immune system plays a critical role in eliminating abnormal cells (1). These abnormal cells harbor molecular signatures that are recognized as non-self antigens by immune cells that are constantly surveying the cells in our body. When an immune cell, such as a cytotoxic T lymphocyte (CTL), encounters a non-self antigen, it engages in a process that leads to cell death for the abnormal or intruder cell. As will be described below, tumor antigens (TA) are processed by malignant cells and are displayed on the tumor cell surface via the human leukocyte antigen (HLA) class I antigen complex. Studies have shown that these TA are viewed as non-self antigens by circulating immune cells, leading to tumor cell destruction as a result of interactions with the immune system. These interactions, and mechanisms that allow tumors to specifically evade such interactions, which will be further described in the following sections, are what inspired this research program and led to the work performed in this thesis.

The HLA Class I Antigen Complex and Tumor Antigen Processing

The HLA system is the human analog of the major histocompatibility complex (MHC), a group of genes on chromosome 6 that encode cell surface markers and antigen processing machinery (APM) involved in the innate and adaptive immune responses, as well as in infection, autoimmunity, and malignancies (2). The HLA class I region contains genes that encode both classical and non-classical class I antigens. For the purposes of this brief introduction, only the

classical antigens will be discussed, as they are the standard molecules involved in antigen presentation and the immune interactions that will be detailed below.

The classical HLA class I antigens, HLA-A, HLA-B, and HLA-C, are normally expressed on all human cell types, with the exception of erythrocytes and trophoblasts (1). The complete HLA class I antigen is a dimer composed of a transmembrane heavy (alpha) chain and an associated light (beta) chain, beta-2-microglobulin (β2m). The β2m component is non-polymorphic and is encoded on chromosome 15, which is not part of the MHC.

One of the major roles of the HLA in human cells is to process endogenous proteins intracellularly and present short amino acid sequences on the cell surface via the HLA class I antigen complex (1). In malignant cells, these unfamiliar tumor antigens are exhibited on the cell surface, allowing for recognition by cognate T lymphocytes (3) (Fig. 1). Primed T cells recognize TA presented by the HLA class I antigen via interaction with the T cell receptor (TCR). This interaction leads to a cytotoxic effect that leads to tumor cell destruction.

Antigen processing begins with the synthesis of the heavy chain and β2m components in the endoplasmic reticulum (ER) (1). These components associate with the chaperone proteins calnexin, calreticulin, and Erp57, which stabilize the heavy and beta chains and prevent loading of self-peptides from the ER itself. The heavy and beta chains then associate with the amino acid transporter TAP, which is stabilized by the chaperone protein tapasin. In the cytosol, proteins to be degraded are ubiquitinated and thereby directed towards the proteasome. In the context of immune activation or inflammation, IFN-γ induced expression of LMP-2 and LMP-7 leads to proteasome augmentation with these new subunits. This augmented proteasome creates peptides of optimal length for HLA class I presentation, approximately 8-10 amino acids long. TAP then transports these short peptides from the cytosol into the ER, where they associate with the heavy

and beta chains, stabilizing the complex. This peptide-HLA antigen complex is then transported to the cell surface for antigen presentation. An intact HLA class I antigen complex is needed in order to present TA on the tumor cell surface for possible interaction with a TCR on specifically primed CTLs.

Immune Escape Mechanisms in Cancer

A growing body of evidence indicates that a major obstacle to the success of immunotherapy is represented by the many escape mechanisms utilized by tumor cells to avoid recognition and destruction by the host's immune system (4). Among them are defects in the HLA class I antigen. Many malignancies have been shown to have absent or mutated expression of HLA class I antigen components, as studied and reviewed extensively by our lab (4-16). To our knowledge, this is the first study to examine HLA class I expression in human chordomas and to report similar defects in these tumors.

These immune escape mechanisms are clinically significant in malignancies due to their role in the pathogenesis of tumors (4). If a malignant cell is able to evade immune cell detection, it has the ability to grow and divide unchecked in the tumor microenvironment, leading to increased tumor burden in patients, as well as a more aggressive tumor phenotype. The significance of these HLA class I antigen defects in cancer is underscored by the interaction between immune cells and malignant cells.

One school of thought is that tumor-infiltrating lymphocytes exert an immune response against the patient's tumor. The presence of tumor-infiltrating lymphocytes has been associated with improved prognoses in numerous malignancies, including melanoma (17-20), breast cancer (21-24), lung cancer (25-29), colorectal cancers (30-51), and ovarian cancer (52-54). Data from

large clinical studies demonstrate that a robust infiltration of neoplastic lesions by specific immune cell populations, including, but not limited to, $CD8^+$ cytotoxic T lymphocytes (CTL), Th1 and Th17 $CD4^+$ T cells, natural killer (NK) cells, dendritic cells, and M1 macrophages constitutes an independent favorable prognostic indicator in several types of cancer (55). The mechanism by which this effect occurs is currently unknown, however more recently, increasing attention has been paid to the role of immunosurveillance in the pathogenesis and clinical course of cancer, with particular emphasis on the application of immunotherapy for treatment (56). The aforementioned studies suggest that the immune system plays a dynamic role in regulating tumors. The immune cell-malignant cell interaction is important in not only preventing malignant cells from colony formation, but also in keeping clonal expansion at bay. Thus, the immune system plays a role in controlling tumor progression even if it has failed in preventing tumor formation. This has led to renewed interest in understanding the mechanisms that drive these processes, as well as developing strategies to stimulate the immune system as a way to treat cancer.

Immunotherapeutic Strategies for the Treatment of Cancers

For decades now, researchers have understood the importance of utilizing immunotherapy in the treatment of cancers. However, until recently, aside from the successful use of monoclonal antibodies in certain types of cancers, we have been limited in the number of immune-mediated pathways we could utilize to treat tumors. In the past few years, significant progress has been made in bringing immunotherapeutic strategies back to the forefront of cancer management, and these have garnered considerable attention (57, 58).

Highlighting these successes are the recent studies describing the use of immune checkpoint blockade in treating various malignancies (57). The immune checkpoint molecules these therapies target are the programmed cell death protein 1 (PD-1) and the cytotoxic T lymphocyte antigen 4 (CTLA4). The binding of PD-1 on T cells with its ligands, PD-L1 and PD-L2, on tumor cells initiates an inhibitory effect that negatively regulates TCR signaling and leads to apoptosis of T cells (59). Nivolumab and pembrolizumab are examples of monoclonal antibodies directed against PD-1 (60-62). By blocking the interaction of PD-1 with its ligands, these antibodies prevent T cell exhaustion and death, and thereby boost the immune response specifically targeted against tumors. CTLA4 is expressed on helper T cells and has a similar structure to CD28. While CD28 is a costimulatory molecule that binds to the B7 family of molecules on antigen presenting cells and enhances the immune response, CTLA4 is its antagonist and initiates a cascade of inhibitory signaling that ultimately serves as an "off" switch for T cell-based immune attack (63). As another example of a negative regulator of the immune response, CTLA4 was identified as a target for blockade, and ipilimumab, a CTLA4-specific monoclonal antibody, was the first example of FDA-approved immune checkpoint blockade therapy for cancer (64).

Another immunotherapeutic strategy recently garnering significant interest is the use of chimeric antigen receptors (CARs), or artificially engineered TCRs specific to a tumor antigen. Briefly, CARs are fusions of single-chain variable fragments from monoclonal antibodies with the intracytoplasmic and transmembrane domains of the CD3-ζ molecule. In a process known as adoptive cell transfer, T cells are isolated from a patient and CTLs are modified to express the TA-specific CAR via viral vector transduction. The result is CAR-modified T cells with TCRs that are unique and specific to the tumor cells they intend to target, leading to tumor cell

destruction when reintroduced into the patients (65). Early pre-clinical and phase I clinical studies utilizing adoptive cell transfer of CAR-modified T cells in various malignancies show promise that this strategy could soon become another option in cancer therapy (66).

A final strategy that might soon offer another opportunity to take advantage of the interaction of the immune system and cancer is the advent of successful tumor antigen vaccination. Patients with tumors known to overexpress a certain antigen can be vaccinated with low-dose inactivated antigen in order to drive an antigen-specific T cell response. While these vaccines have been notoriously difficult to create and have historically yielded only modest effect in early clinical trials, newer vaccines are showing early signs of success. For example, preliminary results from an open-label phase I clinical trial vaccinating chordoma patients with heat-killed recombinant yeast modified to express the brachyury protein suggest that certain patients have an immune response to brachyury (67). While these initial results only represent a small cohort of seven chordoma patients, the study showed that patients who were vaccinated with this yeast-brachyury vaccine were able to mount both CD4 and CD8 responses with increased intracellular expression of pro-inflammatory and immune stimulating cytokines IFN-γ, TNF, and IL-2, as well as the perforin and granzyme marker CD107a.

Chordoma

Chordoma is a primary malignancy of the axial skeleton originating from notochordal remnants (68). The embryologic notochord gives rise to the nucleus pulposus of intervertebral discs, which undergo malignant transformation to become chordoma. These rare tumors have an annual incidence of approximately 1 per 1 million people, and they tend to be locally aggressive and have a high rate of recurrence. Anatomically, they are primarily found in the sacrococcygeal

region and also often at the skull base involving the clivus (69). Pathologically, gross specimen are gelatinous pinkish-gray masses with solid and cystic components. Low-power microscopic examination reveals lobules of epithelioid cells encompassed in clusters by surrounding fibrous septae in a background of mucinous matrix. The pathognomonic cell type for chordoma is the physaliferous cell, which has a heavily vacuolated cytoplasm. Chordoma is immunohistochemically identified by strongly positive cytokeratin and brachyury expression, as well as weak positivity for PAS and S-100. Brachyury is a transcription factor involved in notochordal development and is overexpressed in chordoma (70).

The standard of care for chordoma is *en bloc* resection, with the goal of negative margins, and adjuvant radiation therapy (68). Unfortunately, this treatment paradigm has not drastically shifted over the past few decades, and chordoma remains a cancer ridden with significant morbidity and mortality. Effective first-line small molecule-based targeted therapy does not exist for chordoma. Rare cases of advanced PDGFR-β-positive chordomas treated with imatinib have shown a modest survival benefit and halting of disease progression, suggesting that imatinib may have some antitumor activity in chordoma (71). Imatinib has also been used as off-label salvage therapy in patients with disease refractory to conventional treatment. Chemotherapy has proven to be largely ineffective (72). Radiation therapy for chordoma has been shown to provide both therapeutic and palliative benefit in patients. While most centers provide adjuvant radiation, MGH has published a phase II clinical trial describing improved local control for chordomas receiving both neoadjuvant and adjuvant proton beam radiation therapy (73). Ultimately, the lack of curative options for chordoma led us to think about other strategies we could use to try to devise new ways to treat this disease.

Rationale and Significance

Incidental observations of dense lymphocytic infiltrates in resected human chordoma specimens by our research group, paired with the prospect of the increasing clinical relevance of these findings, led us to develop our research program described here. While immune cell infiltrates have been noted in a number of tumors, no such reports for chordoma existed prior to this study. Additionally, we sought to investigate the role of these intratumoral lymphocytes in the pathogenesis of chordoma, with specific focus on potential implications for immunotherapy. Knowledge of defects in HLA class I antigen components in chordoma provides insight into the possible mechanism(s) of immune escape in this tumor, and also aids in the development and application of appropriate immunotherapy as well as optimal selection of patients for certain treatment paradigms. The work performed in this thesis has potential future implications in chordoma therapy.

Materials and Methods

Patients with chordoma treated as the Massachusetts General Hospital (MGH) between 1989-2009 were identified and selected from the Orthopaedic Oncology database with approval from the MGH institutional review board. All slides were reviewed in tandem with a senior bone and soft tissue pathologist in order to confirm the diagnosis of chordoma and to ensure that sufficient tumor material was present on each slide.

Lymphocytic Infiltration

Hematoxylin and eosin (H&E) stained human chordoma tissue slides from 62 randomly selected patients were assessed for lymphocyte presence and histologic location. A total of 387 slides (average of 6 slides per patient, range 2-19, SD 3.1) were assessed in order to ensure adequate tumor specimens were reviewed at multiple levels within the tumor for lymphocytic infiltration and localization.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded human chordoma tissue samples from 24 tumors were randomly selected. Four-micron thick tissue sections were deparaffinized and subjected to antigen retrieval. Slides with incubated in 3% hydrogen peroxide for 20 minutes and then rinsed in TBST. Following incubation with blocking buffer, slides were incubated with anti-HLA-A (HC-A2, (74)), -HLA-B/C (HC-10, (75)), -beta-2-microglobulin (NAMB-1, (75)), -CD4 (Dako), -CD8 (Dako) monoclonal antibodies overnight at 4°C. After rinsing with TBST, slides were incubated for 45 minutes at room temperature with EnVision™+ Dual Link System-HRP goat anti-mouse immunoglobulin (Dako). Antibody binding was detected with the DAB+ Peroxidase

Substrate Kit (Dako), and the slides were counterstained with hematoxylin. Slides were then dehydrated and mounted with coverslips.

Staining results were analyzed by at least two investigators in combination with a senior bone and soft-tissue pathologist. Slides were examined throughout the tissue section at high-power (200X) magnification. For HLA class I expression in chordoma cells, both staining intensity and percentage of tumor cells staining positive were assessed. Staining intensity was scored 0 (negative), 1+ (weakly positive), or 2+ (strongly positive). Both cytoplasmic and membranous staining were considered positive for HLA-A, HLA-B/C, and beta-2-microglobulin staining. For CD4 and CD8 staining of lymphocytes, four high-power fields were randomly chosen on slides and the number of positively staining lymphocytes were counted. Membranous staining was considered positive for CD4 and CD8 staining.

Results

Lymphocytic Infiltration in Chordoma

Lymphocytic infiltration was found in 52 (84%) of the tumors removed from 62 patients. The presence or absence of lymphocytes was homogeneous in 45 (73%) tumors; 35 were positive and 10 were negative. In the remaining 17 (27%) tumors, the presence of lymphocytes was heterogeneous, with areas of lymphocyte infiltrate associated with areas with no detectable lymphocytes. The lymphocyte infiltrates were distributed in two distinct patterns; either primarily in the fibrous septae of tumor lobules or between the physaliferous cells within tumor lobules (Fig. 2).

Consistent with our findings above, 88% of the 24 tumors that underwent immunohistochemical analysis exhibited lymphocytic infiltration. Of those with lymphocytic infiltrates, 19% had lymphocytes distributed between chordoma cells within the tumor lobules. All but one of the tumors with lymphocytic infiltration had lymphocytes located within the fibrous septae of chordoma lobules. All 21 tumors with lymphocyte infiltrates had CD4-positive lymphocytes present (Fig. 3). The number of CD4-positive lymphocytes per high-power field was not uniform in the chordoma tumors, ranging from 1-300 lymphocytes per high-power field. Fifty-two percent of the tumors with lymphocytic infiltration had CD8-positive lymphocytes present. Like the CD4-positive lymphocytes, the number of CD8-positive lymphocytes was not uniform in the chordoma tumors, and ranged from 1-39 per high-power field.

HLA Class I Antigen Defects in Chordoma

Of the 24 tumors tested, 29% were completely negative for HLA-A heavy chain staining (Fig. 4, Fig. 5). Thirty-three percent of tumors had less than 60% of chordoma cells stain

positively for the HLA-A heavy chain. Of these, 25% were weakly positive and another 25% were strongly positive but only stained rare chordoma cells (less than 10% of the tumor).

For the HLA-B/C heavy chain isoform, 17% of tumors tested were completely negative, and an additional 46% of tumors had less than 50% of chordoma cells stain positively for this HLA class I antigen component. Of these 11 tumors with decreased cytoplasmic and membranous staining of chordoma cells, 36% stained only rare chordoma cells (less than 10% of the tumor) and 36% were weakly positive.

While no tumors were completely negative for the beta-2-microglobulin component, 17% exhibited positive staining of less than 60% of chordoma cells and 46% of tumors were only weakly positive.

In total, 19/24 (79%) of the tumors tested exhibited a defect in at least one of the components of the HLA class I antigen. No tumors were pan-negative. All component-specific monoclonal antibodies resulted in staining patterns ranging the full spectrum from completely negative to strongly positive staining of various percentages of chordoma cells.

Discussion

Tumor-infiltrating lymphocytes have been shown to portend an improved prognosis in patients with a number of malignancies (18, 23, 25, 28, 33, 35, 38, 40-42, 44, 45, 48, 49, 52, 53, 55). Our results are the first to describe and characterize lymphocytic infiltration in human chordoma tumors. Our data suggests that these lymphocytes represent an immune response by the host patient mounted against his/her tumor. The result of this interaction is likely manifested in the absent or decreased expression of HLA class I antigen components in tumor cells. This is consistent with the possibility that these intratumoral lymphocytes impose selective pressure on the physaliferous cells, which leads to the outgrowth of specific malignant cell subpopulations that have developed mutations that allow for escape from immunosurveillance. Our study, which is the first to describe lymphocytic infiltration and HLA class I antigen defects in chordoma tumors, contributes to an early mechanistic understanding of the interaction between the host immune system and the pathogenesis of chordoma in patients. This knowledge has implications in better understanding the role the immune system plays in regulating chordomas, classifying and prognosticating patient outcomes on the basis of their tumor and lymphocytic phenotype, and optimally selecting patients for appropriate therapy, whether that be through immunomodulation or other strategies.

The results of recent clinical trials and the FDA approval of monoclonal antibodies specific to immune checkpoint molecules such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1) in various types of malignancies have rekindled interest in the role of immune surveillance in oncology and in the application of immunotherapy for the treatment of malignant diseases (60, 61, 76-89). An increasing number of recent studies

suggest that the CTLA4 and PD-1 pathways are utilized by tumor cells to evade immune cell-mediated destruction (57). These pathways represent mechanisms for negative regulation of the T cell priming and effector phases, respectively, serving as a stopgap for unchecked immune responses and thus allowing tumors to escape T cell interactions (77). Immunotherapy focused on checkpoint blockade interferes with these negative regulators of T cells in order to drive an antitumor immune response. Tumeh *et al*. found that patients with higher numbers of $CD8^+$, PD-1^+, and PD-$L1^+$ cells in metastatic melanoma tumors responded more favorably to treatment with pembrolizumab, a humanized monoclonal antibody directed against PD-1 (62). Thus, our findings of high rates of lymphocytic infiltration in chordoma tumors, as well as subtype analysis showing significant $CD4^+$ and $CD8^+$ infiltrates, is consistent with the possibility that lymphocytic infiltration could serve as a predictor of prognosis for patients.

Based on our proposed connection between intratumoral lymphocytes in chordoma and HLA class I antigen defects in the physaliferous cells, we postulate that the clinical behavior and phenotype of patients' tumors can be identified and used as a biomarker for the activity of immunotherapeutic agents against the tumor. In patients with an intact HLA class I antigen complex, many immunotherapeutic strategies are potential options. For example, checkpoint blockade therapies, such as the monoclonal antibodies directed against PD-1 and CTLA4 mentioned above, CAR-based T cell therapy, and tumor antigen vaccines could be utilized to treat patients. However, in patients with defects in HLA class I antigen components, many immunotherapy options are not possible. In this subset of patients, an argument can be made for more aggressive treatment paradigms, such as *en* bloc surgical resection with neoadjuvant and adjuvant proton radiation therapy. This therapeutic modality has been shown in a phase II clinical trial to provide effective local control which is durable for at least five years (73). With

the advent of newer CAR-based immunotherapies, tumors with defects in HLA class I antigen processing and presentation may still be able to be targeted by T cells if tumor-specific cell surface molecules are identified. The HLA class I antigen complex is utilized to present normally intracellular tumor antigen peptides on the cell surface for immune surveillance by T cells. However, molecules that are routinely transported to the cell surface by the ER-Golgi apparatus do not require intact HLA machinery. With adoptive cell transfer utilizing CARs, a patient's T cells can be engineered artificially to recognize specific tumor cell surface markers, and can then be reintroduced into the patient for cytotoxic effect. A novel example of this strategy that our lab is currently working on in chordoma using CAR-modified T cells directed against the chondroitin sulfate proteoglycan 4 (CSPG4) is detailed below in the "Suggestions for Future Work" section.

We hope these data will stimulate further studies investigating the efficacy of immunotherapeutics in malignancies, specifically chordoma, and their mechanism of action.

Conclusions

We are the first to describe and characterize lymphocytic infiltration in chordoma. Lymphocytic infiltration was noted in 84% of tumors, with distributions primarily in the fibrous septae of tumor lobules, but also amongst physaliferous cells. In tumors that underwent IHC analysis, the rate of lymphocytic infiltration was comparable. All tumors with lymphocytic infiltration had CD4 T cells present, and 52% of tumors with intratumoral lymphocytes had CD8 T cells present. In total, 79% of the tumors examined displayed defective expression of at least one component of the HLA class I antigen. These defects of HLA class I heavy chain (HLA-A or HLA-B/C) or light chain (β2m) expression ranged from completely absent expression to varying combinations of weakly positive staining or a low percentage of tumor cells staining positive. No tumors were negative for all HLA class I antigen components and each monoclonal antibody used exhibited the full range of staining patterns, suggesting our data is robust and not a result of tissue artifact or sample preparation. We conclude that the high rate of lymphocytic infiltration in chordoma tumors is likely a result of the patient's immune system mounting a response against their tumor. These data suggest that host immune cells apply selective pressure on physaliferous cells, leading to the outgrowth of malignant cell populations that have developed defects in the HLA class I antigen. These defects represent a mechanism of immune escape by chordoma tumors, and is important in understanding the pathogenesis of the disease and is paramount in understanding how to develop and apply immunotherapeutic strategies to the right patients based on individual tumor phenotypes.

Suggestions for Future Work

The work presented in this thesis represents the early stages of understanding the role of the immune system in chordoma progression and treatment. Before applying immunotherapeutic strategies to patients, a mechanistic understanding of how the host immune system interacts with the tumor and how tumors attempt to escape immune attack is necessary. Our novel findings of lymphocytic infiltration in chordoma and HLA class I antigen defects in physaliferous cells are the building blocks for further investigation into the immune-mediated pathogenesis of chordoma and potential for immunotherapy. We plan to further explore the interaction between chordoma and a patient's immune system with the work outlined below, and we hope that this research program and our early, novel findings will stimulate future work in this field to help promote the development of new immunotherapeutic strategies for chordoma patients.

Expression of HLA Class I Antigen Processing Machinery Components in Chordoma

Tumor antigen presentation requires intracellular tumor peptide processing, as described in the Introduction. While defects in the HLA class I antigen heavy and beta chains has implications at the tumor cell surface, improper or incomplete TA processing in the cytosol and ER can also lead to immune escape. Issues with TA processing will affect TA peptide loading, and will therefore prevent T cell recognition of tumor cells.

Our lab has created a unique panel of monoclonal antibodies directed towards APM components, including tapasin, TAP1, TAP2, calreticulin, calnexin, LMP-2, LMP-7, and Erp57. Some of these APM component-specific antibodies are not available commercially. IHC experiments examining human chordoma tissue sections for defects in HLA class I APM

components would be informative in assessing the full spectrum of this mechanism of immune escape utilized by chordoma. We have begun preliminary validation experiments using these monoclonal antibodies, and plan on reporting these results in a subsequent manuscript.

Expression of Immune Checkpoint Molecules in Chordoma

Clinical trials utilizing monoclonal antibodies directed against the immune checkpoint molecules PD-1 and CTLA4 have been successful in a variety of tumors, as previously described. Given that we have identified lymphocytic infiltration in chordoma with both CD4 and CD8 T cells, it is possible that PD-1/PD-L1/PD-L2 and CTLA4/B7 interactions play a role in chordoma as well. IHC analysis of human chordoma tissue sections for expression of these molecules could provide insight into the possible use of these immune checkpoint blockade therapies in chordoma patients. There are both commercially available and proprietary monoclonal antibodies that could be used to characterize expression of these molecules. PD-L1 expression has recently been noted in osteosarcoma, another primary malignant bone tumor (90). However, to date, no one has reported expression of PD-L1/PD-L2 in human chordoma.

Use of Chordoma-Specific CARs to Modify T cells Against Chordoma Cell Lines

Chimeric antigen receptors, as previously described, provide a novel opportunity to genetically engineer T cells with TCRs specific to TAs. Chondroitin sulfate proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA), is a cell surface molecule involved in multiple signaling pathways important for tumor cell growth, survival, migration, and metastasis (91). A study from our group has shown that chordomas overexpress CSPG4 (92). Our collaborators, utilizing anti-CSPG4 monoclonal antibodies

generated in our laboratory, have very recently designed and validated second-generation CARs with specificity for CSPG4 (93). Our lab has also been able to successfully culture and expand human chordoma cell lines, namely UCH-1, in order to perform *in vitro* studies using human cells.

We received the anti-CSPG4 CAR from our collaborator and are planning to use retroviral vector transduction of the CAR into peripheral blood T lymphocytes in order to generate anti-CSPG4 CAR-modified T cells. Analysis of cytolysis after co-culturing these CAR-modified T cells with chordoma cell lines would provide insight into the efficacy of anti-CSPG4 immunotherapy, specifically CAR-based strategies, in the treatment of human chordoma. If successful, these *in vitro* results could lead to the use of anti-CSPG4 CAR-modified T cells *in vivo* animal studies, and ultimately adoptive cell transfer phase I clinical trials in human patients with chordoma.

Effect of Radiation on HLA Class I Antigen and APM Component Expression in Chordoma

Experiments performed *in vitro* and in animal models in collaboration with researchers at the National Cancer Institute have shown that low-dose irradiation can upregulate HLA class I APM component expression and can enhance the cell surface expression of calreticulin (94). These phenotypic changes increase the immunogenicity of tumor cells and their recognition by cognate T cells.

In light of these new findings, it is possible that radiation therapy could serve multiple roles in the treatment of chordoma. While radiation therapy is part of the standard of care clinically, it may also provide immunogenic modulation of tumors that enhance antigen

processing and presentation, allowing for improved T cell effect. At the Stephan L. Harris Center for Chordoma Care at MGH, *en bloc* surgical resection with neoadjuvant and adjuvant proton beam radiation therapy has been shown in a phase II clinical trial to provide durable local control (73). As a result of this treatment modality, we are in a unique position to study the effects of radiation on HLA class I antigen and APM component expression. Patients treated at MGH often have pre-radiation tissue biopsies performed in order to confirm the diagnosis of chordoma prior to initiating treatment. Thus, we have access to pre- and post-radiation chordoma samples from individual patients. IHC experiments using the monoclonal antibodies utilized in this thesis, as well as the ones directed against APM components we are validating (as noted above), can elucidate if radiation increases the immunogenicity of chordoma tumors. This would further justify more widespread and aggressive use of radiation in the treatment of chordoma patients.

Summary

Immunotherapy for cancer has recently garnered significant interest due to the successes of clinical trials utilizing immune checkpoint blockade therapy in a various tumors. Chordoma, a rare primary malignancy of the axial skeleton, is a locally aggressive and highly recurrent tumor associated with significant morbidity and mortality. Surgery and radiation have remained the mainstays of treatment for the past few decades largely due to ineffective cytotoxic chemotherapy and the lack of first-line small molecule-based targeted therapeutics. With the resurgence of interest in immunotherapeutics for cancer, the work in this thesis makes early breakthroughs in our understanding of the role of the immune system in chordoma pathogenesis at a pivotal time. To our knowledge, we are the first to report lymphocytic infiltration in a high percentage of resected human chordoma tumors. Intratumoral lymphocytes have been shown to be associated with improved prognoses in a number of other malignancies. We also are the first to describe HLA class I antigen defects in a significant number of chordoma tumors. Nearly 80% of tumors displayed defective expression of at least one component of the HLA class I antigen. Taken together, these findings support the possibility that patients mount an immune response against their tumor, and that these intratumoral lymphocytes impose selective pressure on malignant cells, leading to the clonal expansion of cells with the ability to evade immunosurveillance through defective tumor antigen presentation. These immune mechanisms involved in chordoma pathogenesis are critical for prognostication, tumor phenotyping, directing appropriate immunotherapy to the right patient cohorts, optimally selecting patients for various treatment regimens, and stimulating further research into the interplay between the host immune system and chordoma.

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Tables and Figures

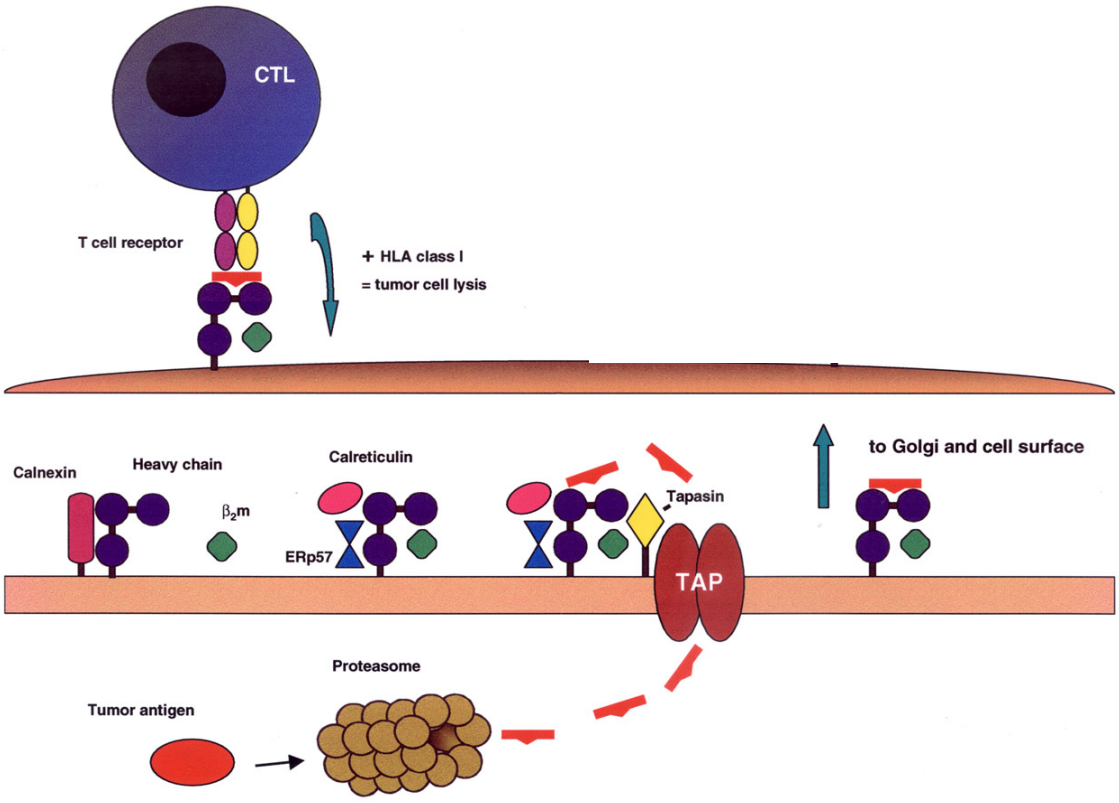

Figure 1. HLA class I APM preparing TA peptides for presentation to T cells at the cell surface. The proteasome generates peptides of appropriate length (8-10 amino acids) that are transported to the ER by the transporter TAP. In the ER, peptides are loaded on the HLA class I heavy chain-β_2m complexes. The tri-molecular complex is then transported through the Golgi secretory pathway to the cell surface for presentation of TA-derived peptides to cognate T cells. APM, antigen processing machinery; β_2m, beta-2-microglobulin light chain; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; HLA, human leukocyte antigen; TA, tumor antigen.

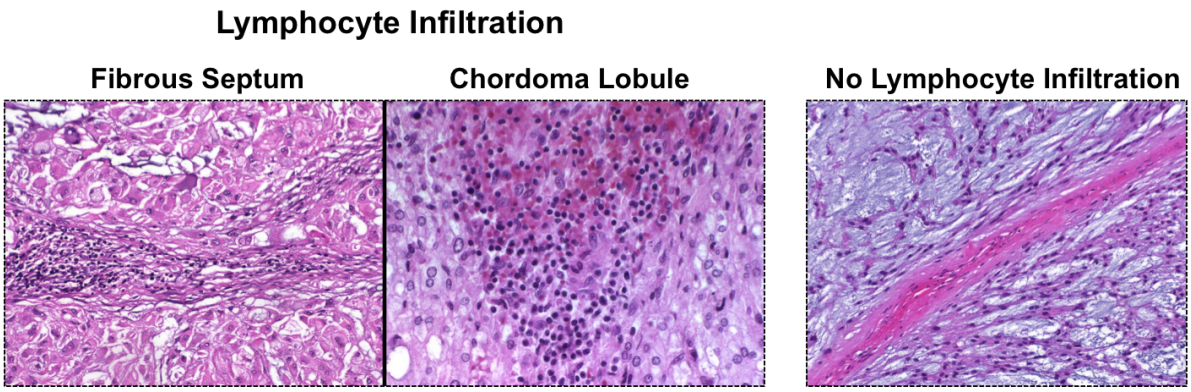

Figure 2. Lymphocytic infiltration in chordoma. H&E staining of human chordoma tissue detailing the pathologic distribution of lymphocytes within the tumor microenvironment (left) in comparison to human chordoma tissue without lymphocytic infiltration (right). The more common pattern for lymphocytic infiltration was within the fibrous septae. H&E, hematoxylin & eosin.

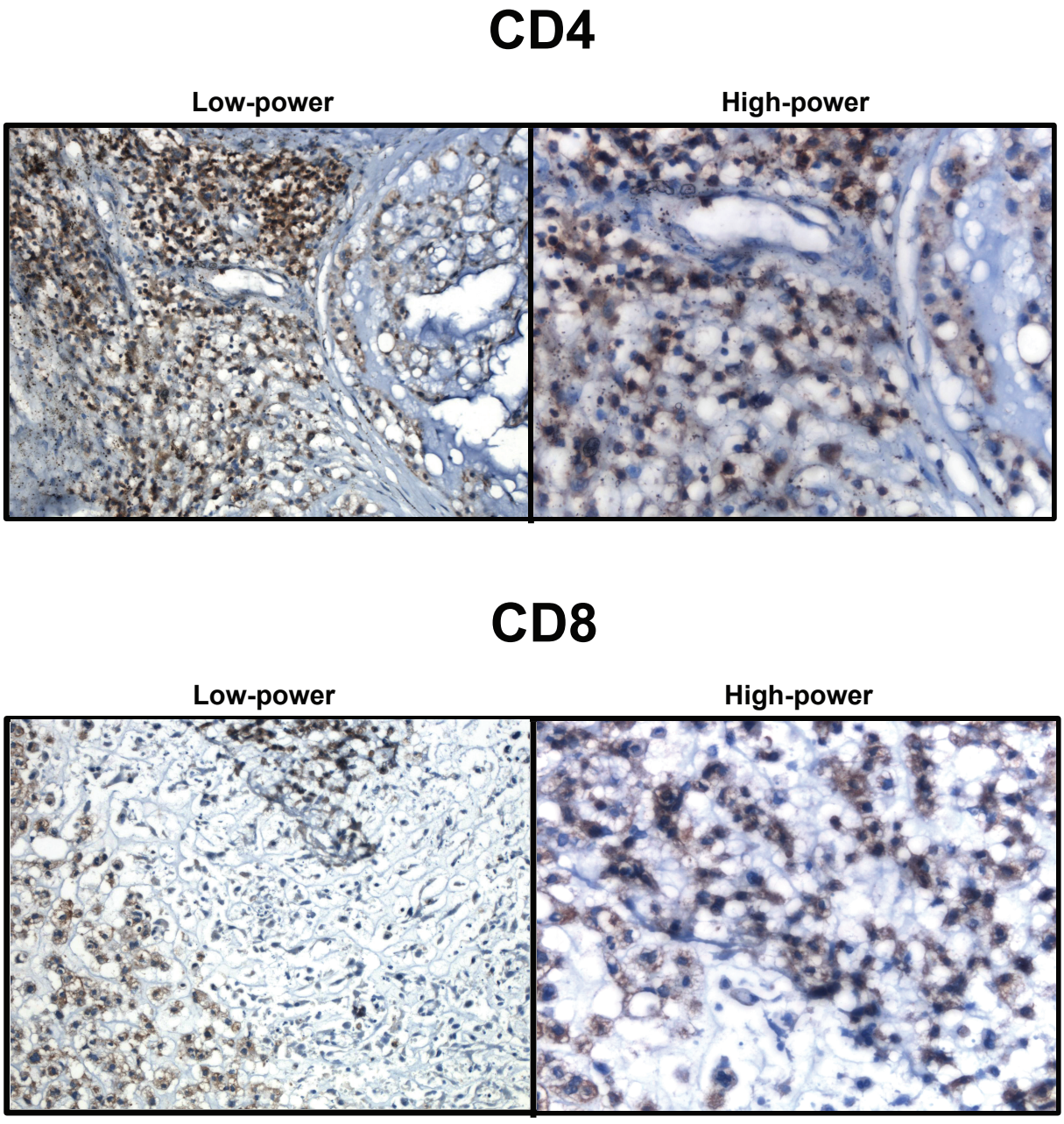

Figure 3. IHC analysis of tumor-infiltrating lymphocytes in human chordoma tumors. Both CD4 and CD8 T lymphocytes were noted chordoma specimens. Low-power images taken at 200x total magnification. High-power images taken at 400x total magnification. Interpretation of IHC staining was confirmed by a senior bone and soft tissue pathologist.

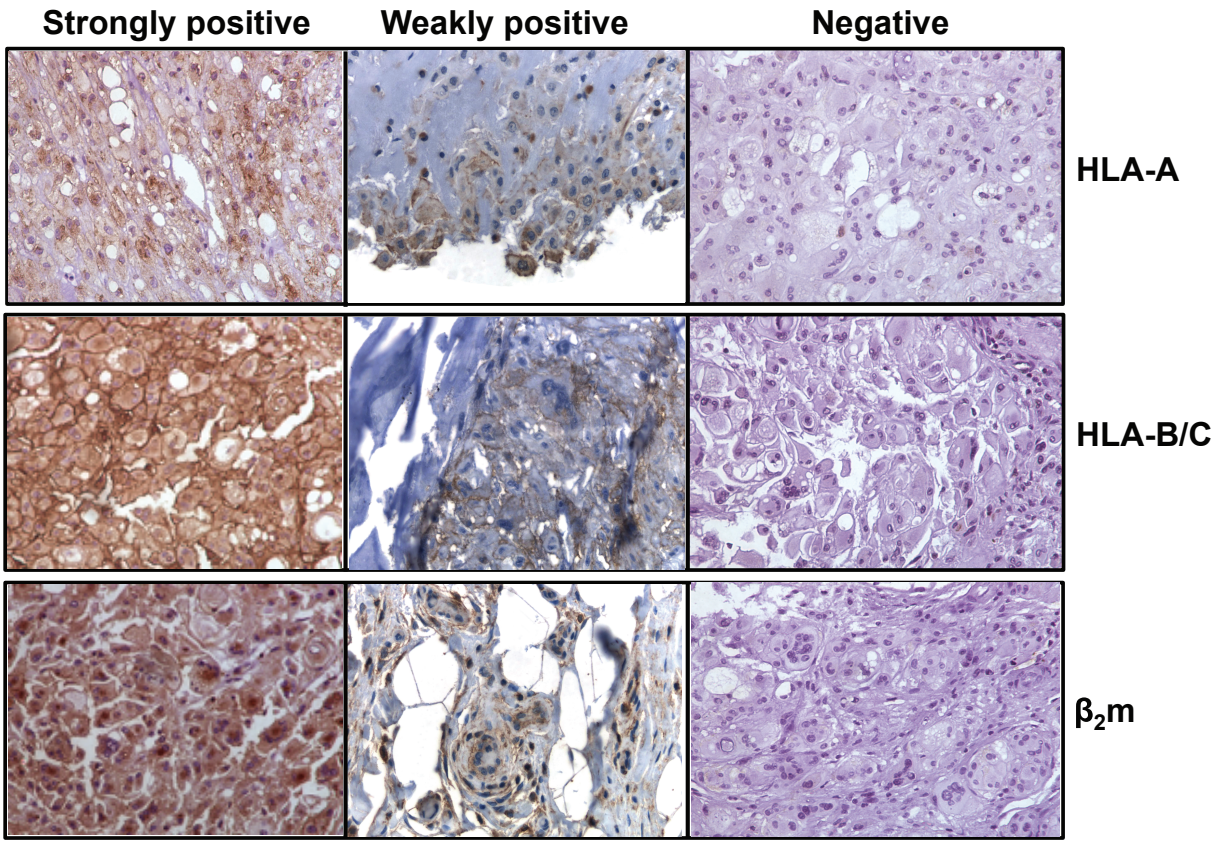

Figure 4. IHC analysis of HLA class I antigen component defects in human chordoma tumors. Human chordoma tissue sections were stained with monoclonal antibodies directed against the HLA class I heavy chain isoforms (HLA-A, HLA-B/C) and the light chain (β_2m). Staining was scored by both intensity (strongly positive, weakly positive, negative) and percentage of tumor cells staining positive. Scoring was performed independently by at least 2 investigators and was confirmed independently by a senior bone and soft tissue pathologist. β_2m, beta-2-microglobulin light chain; HLA, human leukocyte antigen.

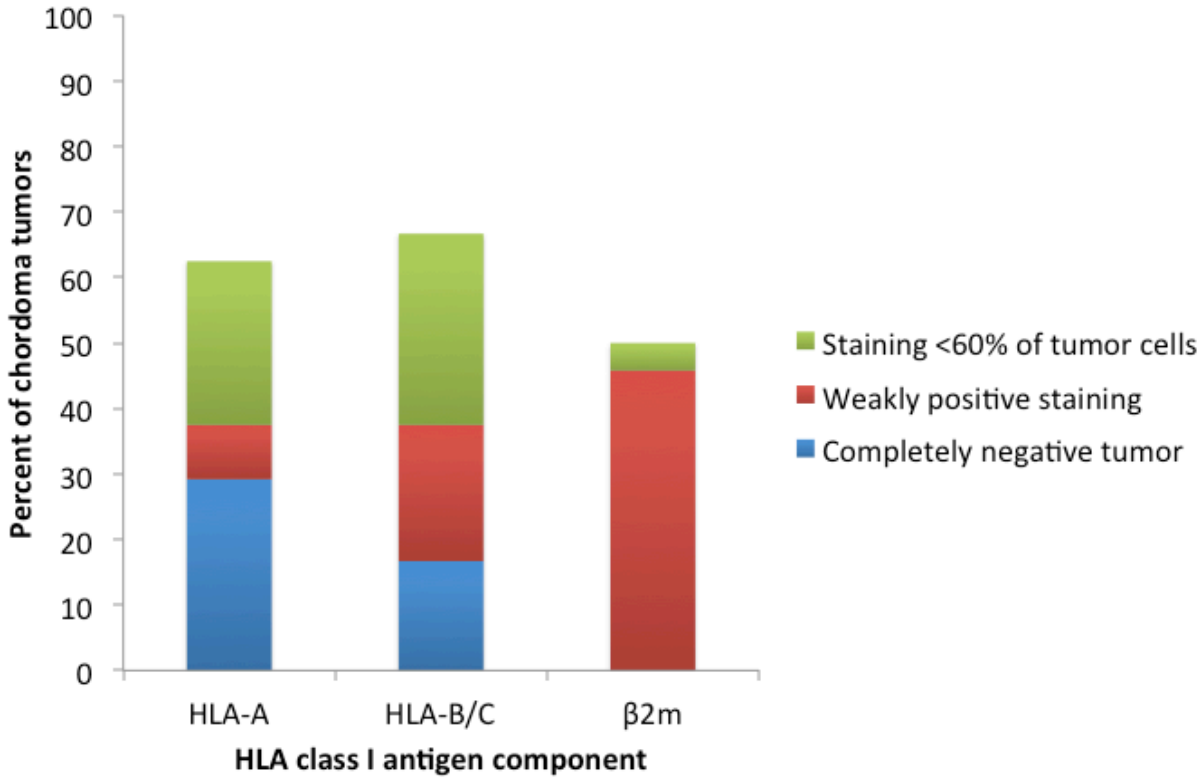

Figure 5. Frequency of HLA class I antigen defects in 24 human chordoma tumors. HLA class I antigen component expression was determined via IHC analysis. β_2m, beta-2-microglobulin light chain; HLA, human leukocyte antigen.