

TARGETING ANTIGEN PRESENTATION DEFICIENCY IN LUNG CANCER

by

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ABSTRACT

Small cell lung cancer (SCLC) is an aggressive lung cancer type of neuroendocrine origin with an exceptionally low survival rate of <7%. Despite a high neoantigen load associated with heavy chronic smoking, SCLC exhibits overall modest clinical benefit to immune checkpoint blockade (ICB). Suppression of class I major histocompatibility complex (MHC-I) represents a cell-intrinsic mechanism of resistance that enables cancer to evade antitumor immunity. The majority of SCLC exhibit low or absent MHC-I expression, which renders this tumor “invisible” to cytotoxic lymphocytes (CTLs), leading to failure to engage T cell-dependent antitumor activities. In Chapters 1-4, we discussed the current understanding of the antigen processing and presentation (APP) pathway in tumor cells, the regulatory and de-regulatory mechanisms that control MHC-I expression on the cell surface, and the landscape of therapeutic strategies that target this deficiency. In Chapter 5, we explored the molecular drivers of immunotherapy resistance and sensitivity in a clinical cohort of SCLC. In Chapter 6, we studied the immunomodulatory functions of lysine-specific demethylase 1 (LSD1) in regulating antigen presentation and immunotherapy response.

To delineate the regulatory pathways associated with long-term benefit to immunotherapy, we utilized multi-omic approaches consisting of bulk RNA-sequencing (RNA-seq), single-cell RNA-sequencing (scRNA-seq), and next generation sequencing (NGS) coupled with immunohistochemistry (IHC) analyses on a cohort of 263 patients. Neoantigen prediction was performed to annotate somatic variants and HLA alleletypes, and a ranked list of peptides was computed using NetMHC4.0-pan. To perturb LSD1 functions, we employed the irreversible inhibitor ORY-1001 (ladademstat) and RNA interference to assess changes in MHC-I expression in SCLC cell lines by flow cytometry and western blot. We then performed RNA-seq to characterize whole transcriptomic changes in SCLC cells following LSD1 inhibition. To explore effects of targeting LSD1 on T cell cytotoxicity, we engineered HLA*02:01-expressing SCLC cell lines to express endogenous NY-ESO1 or MART-1 and co-cultured with pre-

activated cognate CD8+ T cells. Finally, we treated immunocompetent mice bearing syngeneic genetically engineered mouse model (GEMM)-derived SCLC tumors with ORY-1001 and/or anti-PD-L1 to evaluate tumor growth and characterize intratumor immune activities.

We observed that the tumor microenvironment of long-term responders harbors not only an abundance of CD8 effector T cells but also activated and memory CD4 and B lymphocytes, which co-localize within tertiary lymphoid structures at the tumor edge. SCLC exhibits significant less frequency of HLA mutation and loss-of-heterozygosity compared to NSCLC yet displayed a markedly higher level of tumor mutational burden. Durable clinical benefit to immunotherapy is driven not by neoantigen count but also their immunogenic qualities. In the second project, we discovered significant anti-correlation between expressions of *KDM1A* and MHC-I/APP genes. We demonstrated that targeting LSD1 restores MHC-I cell surface expression, transcriptionally activates APP-regulatory genes, and sensitizes SCLC to an immunogenic phenotype. LSD1 inhibition functionally rescues endogenous antigen processing in SCLC and enables MHC-I-restricted T cell-mediated cytotoxicity. Finally, concurrent treatment of LSD1 inhibitor and anti-PD-L1 blockade further enhances intratumor immune infiltrates and sensitizes refractory SCLC model to ICB.

Our findings expand our current understanding of SCLC tumor-intrinsic immunogenicity and nominate LSD1 as a promising target for combinatorial immunotherapeutic approaches for resistant SCLC tumors.

BIOGRAPHICAL SKETCH

Minh Ngoc Nguyen (Evelyn) was born in Hanoi, Vietnam and moved to Virginia, United States for high school. In 2011, she completed a summer at Institute of Biotechnology to learn how to engineer low-cost H5N1 vaccines in Vietnam, under the guidance of Dr. Nguyen Tuong Van. She subsequently obtained her Bachelor of Arts at Mount Holyoke College, where she majored in Biochemistry and minored in English Literature. As a pre-medical student, she completed an internship at the Department of Medical Oncology, Vietnam National Cancer Hospital and was mentored by Dr. Dao Van Tu and Dr. Tran Van Thuan. In the subsequent years, Evelyn joined the laboratory of Dr. Michelle Farkas at the University of Massachusetts, Amherst, where she studied gene regulation of core circadian genes in polarized macrophage phenotypes. Following graduation, she moved to Boston to work at the Department of Cancer Immunology & Virology at Dana-Farber Cancer Institute, under the guidance of Dr. Wayne Marasco and Dr. Allison O'Neill. Here she worked on the discovery and engineering of humanized monoclonal antibody from phage-display scFv library and studied their therapeutic efficacy against Ewing sarcoma in humanized mouse models. In 2019, Evelyn moved from Boston to New York City to further pursue translational research and joined the Cancer Biology PhD Program at the Louis V. Gerstner Jr. Graduate School of Biomedical Sciences at MSKCC, where she is a PhD student in the laboratory of Dr. Charles Rudin. Since joining the Rudin lab, she has received co-mentorship from Dr. Andrew Chow to study mechanisms of immune evasion in lung cancer, with a focus on process of antigen processing and presentation in immunotherapy-resistant tumors.

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CHAPTER 1: OVERVIEW OF TUMOR ANTIGEN PROCESSING AND PRESENTATION

A pivotal notion in our understanding of tumor immune surveillance is the essential engagement of the antigen processing and presentation pathway to facilitate a complex interplay of both adaptive and innate immune responses. Maintenance of tissue homeostasis is licensed by the capability of the highly polymorphic major histocompatibility complex (MHC) family to present a diverse repertoire of the host proteome and its alterations to T cell subsets. The MHC surface peptidome restricts discrimination of “self” and “non-self,” thereby charting the outcome of diverse immune responses in the host tissues. In this aspect, transformed tumor cells have adapted, in many ways parallel to pathogens, undergoing strong selective immune pressure to avoid presentation of aberrant tumor-specific peptide sequences on the surface MHC complexes. To augment existing literature on this subject, this review focuses on our current understanding of tumor antigen presentation in the context of lung tissue and cancer.

1.1. The major histocompatibility complex region

MHC genes are encoded within a large cluster of >200 gene loci of 3.6Mb termed the MHC region, located on chromosome 6 (6q21.3) in humans and chromosome 17 in mice¹. This region harbors three classes of MHC (class I, class II, and class III), as well as genes involved in peptide processing and transport (i.e. *TAP1*, *TAP2*, *PSMB8*, *PSMB9*, *TAPBP*) and in innate immunity (i.e. *TNF*, *LY6G*). MHC class I and class II genes, or “human leukocyte antigen” (*HLA*) genes in humans and *H-2* genes in mice, are considered the “classical” MHC with high polymorphism, polygenism and established function in presentation of peptides to conventional T cells. In addition, the more recently discovered “non-classical” MHC-Ib genes are located within Class I region but differ from the classical genes in that they contain little to no polymorphism, with selective functions in peripheral tolerance and responses to cellular stress. The class III region is highly dense, comprised of many nonimmune genes and genes involved in complement pathway and inflammatory responses^{1,2}. Interestingly, within the MHC region

also lies ~20 non-coding RNA sequences and ~80 pseudogenes, many of which are embedded remnants of endoretroviruses. Indeed, comprehensive HLA loci mapping has revealed that about half of all HLA genomic loci contains virtually all classes of interspersed repeat elements, including SINE, LINE, LTR and DNA elements³. These elements are often co-upregulated in the presence of an inducible event activating HLA class I and class II transcripts, most often in the context of interferon signaling.

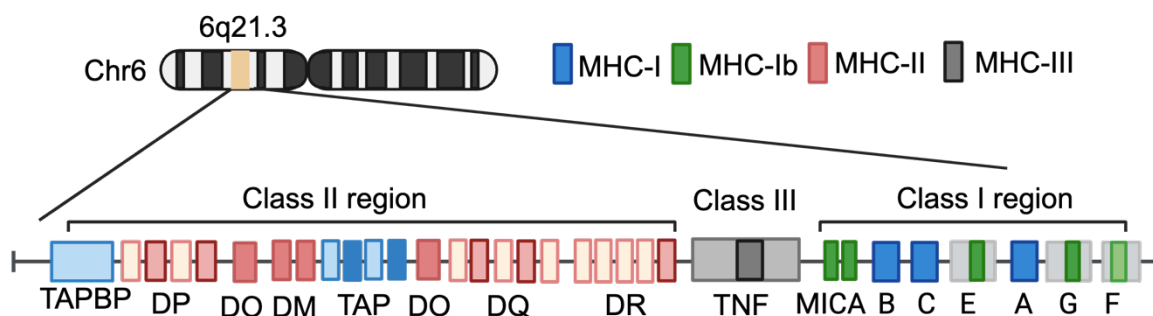


Figure 1.1 Overview of the MHC region on human chromosome 6. Three representative regions, Class I, Class II and Class III, are shown.

Human MHC class I α chain is encoded by three genes, *HLA-A*, *HLA-B* and *HLA-C*, with *H2-K*, *-D*, and *-L* representing the murine counterpart, whereas the β 2-microglobulin gene (*B2M*) is located on chromosomes 15 in humans and chromosome 2 in mice. In humans, only one allele of *B2M* is known, as opposed to seven in mice⁴. The MHC class II region encompasses genes encoding both α and β chains, termed *HLA-DR*, *-DP*, and *-DQ* in humans, and *H-2A* and *H-2E* in mice, as well as genes encoding the MHC-II peptide binding complex DM and the regulatory DO molecules. Within the class II region, most genes encode functions related to antigen processing and presentation, including the TAP transporters (*TAP1*, *TAP2*, *TAPBP*) and LMP-proteasomal subunits (*PSMB8*, *PSMB9*) (**Figure 1.1**).

The presence of multiple MHC class I and class II genes performing the same function is termed “polygenism.” In addition, each gene of the classical MHC with the exception of *DR α* , harbors multiple alleles or “polymorphism,” resulting in tens of thousands of allelic variants of

total human MHC^{5,6}. Notably, within MHC-I, HLA-B is the most polymorphic human MHC gene, comprising of 2,000 alleles⁶. This diversity enables individuals within a population to possess a distinct set of MHC allelotypes. At the allelic level, this means that most individuals possess a heterozygous set of genes for both class I and class II MHC and rarely homozygosity at the same locus^{1,7}. Polymorphism is often seen in the peptide binding pockets of MHC molecules, altering binding specificity of different peptides to the same MHC^{4,8}. As a result, the same MHC molecule can present hundreds to thousands of different peptides. Accompanied by the fact that MHC alleles are codominantly expressed, the repertoire of pathogenic antigens presented by somatic cells is directly influenced by both spectrum and fitness of the peptides and also allelic variants of an individual's HLA genotype evolved under selective pressure⁹.

1.2. Basic structure of surface peptide:MHC complex

A prerequisite for MHC complexes to be detected on the plasma membrane is the presence of a peptide bound on that MHC molecule. In the absence of peptides, both MHC class I and class II are highly unstable, improperly folded and consequently subjected to ER degradation, as discussed in the later section. Thus, a basic review of the structures of, and the inherent differences in, class I and class II peptide complexes benefit our understanding of how some tumors are deficient in surface peptide-MHC-I (pMHC) complex.

1.2.1. Structure of MHC class I complex

The mature MHC I complex consist of a 45-kDa heavy chain, a 12-kDa light chain or β 2-microglobulin (β 2-m) and a short peptide antigen. Class I MHC heavy chain is a type I glycoprotein of ~ 340 amino acids in length, with a cytoplasmic region (~30 residues), a transmembrane region, and an extracellular region with three domains (α 1, α 2, and α 3), each containing one to three N-linked glycosylation sites⁴. β 2-m is an approximately 100-amino acid non-glycosylated protein that lacks a transmembrane domain and can only locate on cell surface through attachment with all three domains of the heavy chain¹⁰. Among these domains,

$\alpha 1$ and $\alpha 2$ directly form a shallow groove to bind to peptides and interact with TCR, whereas $\alpha 3$ primarily interacts with CD8 receptor, allowing the same pMHC complex to concurrently interact with both TCR and CD8 on a single CD8⁺ T cells. It is therefore not surprising that $\alpha 1$ and $\alpha 2$ domains tend to harbor the most polymorphism. The MHC class I peptide binding groove is receptive to peptides of 8-10 residues, preferably with a hydrophobic or basic residues at the carboxy terminus. Both the amino- and carboxy- termini of the peptide interacts with and stabilizes the MHC-I molecule in a closed conformation¹¹. Each MHC molecule only needs a few conserved anchor residues to bind to a specific peptide and can associate with hundreds or thousands of different peptides. Thus, distinct MHC class I variants often differ most in the position and identity of the anchor residues of the peptides they can interact with, and the genetic and structural makeup of MHC class I directly determines the classes of antigens that can be effectively presented.

1.2.2 Structure of MHC class II complex

The MHC-II molecule is encoded by three genes *HLA-DR*, *HLA-DQ* and *HLA-DP* to form a heterodimer with an α and a β chain, forming a peptide-binding groove atop two immunoglobulin domains¹²⁻¹⁴. Polymorphism on MHC class II is exhibited mostly on the β chain while some degree of polymorphism is observed on the α chain of HLA-DQ and -DP but not -DR. Similar to MHC class I, polymorphism is displayed in and around the peptide binding groove¹⁵. Peptides bound to MHC-II are significantly longer than those on MHC-I, typically with a 9-mer core with few anchor residues and up to 12-16 amino acids in length. Unlike MHC-I-bound peptides with enclosed ends, the conserved residues at the two ends of a peptide in MHC-I are not found in MHC-II. In contrast, peptides bound to MHC-II adopt an open conformation with conformational flexibility, allowing exposed side chains to interact with TCR^{16,17}. As a result, the binding pockets of MHC class II molecules can accommodate a broader range of side chains compared to MHC class I. CD4 receptor binds to the hydrophobic

crevice at the junction of $\alpha 2$ and $\beta 2$ domains of MHC class II molecule, the interaction of which enhances sensitivity to the antigen¹⁴. Akin to CD8 and TCR on MHC class I, TCR and CD4 molecules can interact simultaneously with the same MHC class II molecule. Thus, though most likely sharing a common evolutionary origin¹⁸, MHC class I and class II have evolved to bind different types of peptide antigens and are subsequently described to be functionally distinct.

The process of antigen processing and presentation by both MHC class I and class II molecules have been extensively reviewed in the context of APCs^{19,20}. These two pathways diverge primarily in the localization of antigen source and in the trafficking of MHC molecules but largely converge in the following key steps: antigen procurement, peptide processing and selection, transport and docking antigen to MHC molecules, and export of pMHC complex for surface placement. These steps are briefly summarized below.

1.3. Endogenous peptide antigen processing and presentation via MHC class I

MHC class I molecules are ubiquitously expressed by all nucleated cells, where they present peptide fragments at the cell surface²¹. The antigen processing pathway begins in the cytosol with putative antigens derived from defective ribosomal products (DRiPs) or foreign sources undergoing degradation within the proteasomes. The proteasome exists in two conformations: (1) the 20S complex composed of seven α subunits in the outer ring and seven β subunits in the catalytic inner ring, and (2) the larger 26S proteasome harboring the 20S at its core in addition to other subunits that can degrade ubiquitin-marked proteins – 26S particle is responsible for most intracellular protein degradation^{22,23}. About 30-70% of proteins are immediately degraded after synthesis, generating a pool of peptide fragments that serve as rate limiting step for MHC class I presentation^{24,25}. Indeed, proteasomal activity dictates the C-terminus of MHC class I fragments, conforming virtually all MHC class I-bound peptides to harbor either hydrophobic or basic residues^{26,27}. Under cellular stress (i.e. IFN- γ exposure), the proteasomal complex replaces its constitutive β -subunits ($\beta 1$, $\beta 2$ and $\beta 5$) with the

inducible LMP2, LMP7, and MECL-1, forming the immunoproteasome with enhanced proteolytic efficiency^{28–30}. Putative antigens are then trafficked into the ER lumen via the transporter associated with antigen presentation (TAP) bound on the ER membrane^{24,25}. TAP transporter, comprised of TAP1 and TAP2 subunits, selectively translocates peptides of 8-13 amino acids in length into the ER. Efficiency of peptide transport drops significantly with peptides shorter than 7 residues and longer than 13-mer³¹. These nascent peptides can be further trimmed at the N-terminus, facilitated by the ER aminopeptidase associated with antigen processing (ERAAP), termed ERAP 1 and ERAP2^{32–34}. Together with the proteasomes, ERAAP facilitates the processing step of putative antigens for MHC class I presentation.

Newly synthesized MHC class I enter the ER in a partly folded state and binds to calnexin. The heterodimer is formed when the heavy chain assembles with the light chain β 2-microglobulin (β 2m), dissociating calnexin. In the absence of peptides, naïve MHC heterodimer is extremely unstable and held together by ER chaperones calreticulin, ERp57 (also known as PDIA3), and tapasin³⁵. Tapasin interacts with TAP, and together with ERp57 and calreticulin, they form the peptide-loading complex (PLC). Docking of peptides onto MHC class I is facilitated by the PLC, which also mediates the exchange of low-affinity peptides to peptides of higher affinity. Peptides not bound on MHC molecules are exported back to the cytosol by Sec61, an ATP-dependent transport complex. Binding of high-affinity peptides to MHC class I releases the PLC, and the newly assembled peptide:MHC-I complex is transported to the cell surface via exocytic pathway, completing the presentation step of the processing and presentation pathway of cytosolic peptide via MHC class I molecules.

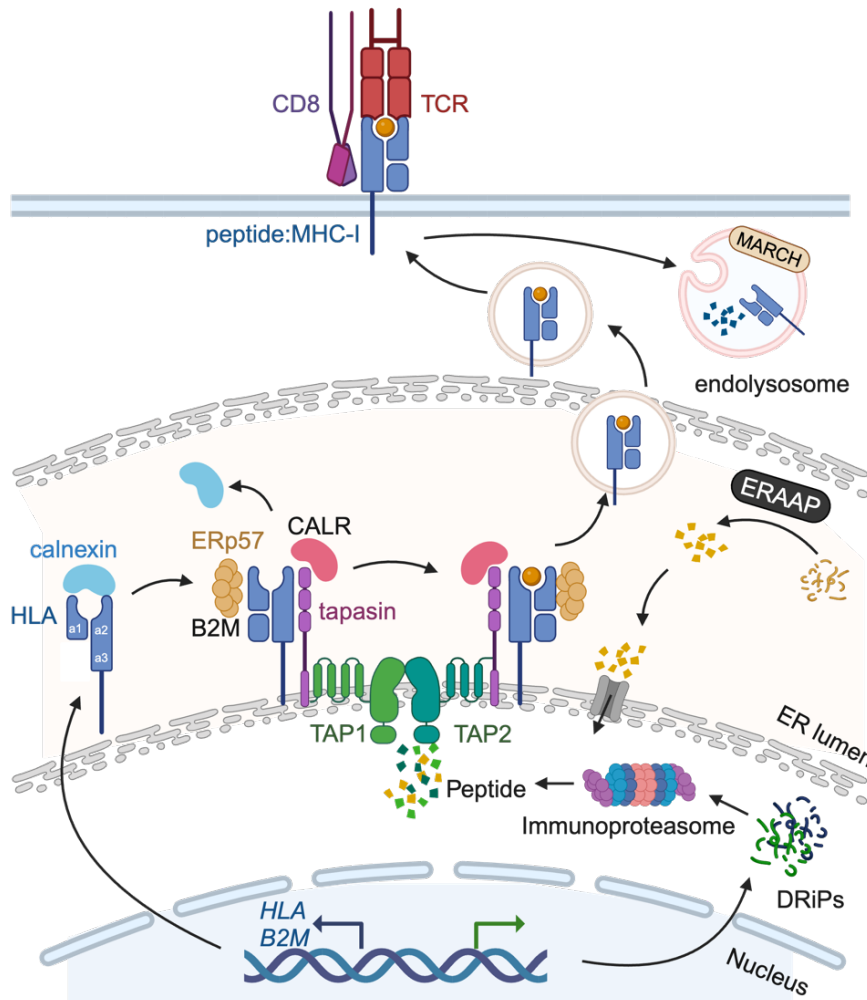


Figure 1.2. The process of endogenous antigen processing and presentation via MHC-I molecules.

Presentation of endogenous peptides on MHC-I-competent nucleated cells represents the sole pathway of MHC class I tumor antigen presentation in cancer. Endogenous peptide presentation distinguishes nucleated cells from professional APCs, i.e. conventional type 1 dendritic cells, that uniquely possess a superior capacity to present not just endogenous, but also exogenous peptides derived from engulfed pathogens and phagocytosed cells, a process called cross-presentation^{36–38}. This process has been observed primarily in the context of MHC-II, as discussed in the next section. MHC-I cross presentation is also found in a subset of APC called CD8alpha+CD103+ DCs yet remain incompletely understood³⁹. Cross-presentation via MHC class I is fundamentally defined by the merging of the extracellular cargo with the

intracellular processes, either via phagosomal rupture (cytosolic pathway) or phagosomal fusion with the ER (vacuolar pathway). Compared to the endogenous pathway, cross-presentation is highly inefficient, yielding significantly fewer pMHC complexes; however, it is still sufficient to mount CD8⁺ T cell immunity as well as peripheral tolerance³⁷. Cross-presentation of tumor antigens by cDC1s necessitates MHC class I-restricted activation of naïve CD8⁺ T cells and their subsequent differentiation into cognate cytotoxic CD8⁺ T cells in secondary lymphoid tissues, the latter of which are capable of migration and patrolling peripheral tissues where they embark on robust effector and memory responses against tumor cells³⁹. Thus, a fundamental requirement for effective antitumor T cell immunity is the capability of tumor cells to retain those antigens presented on surface MHC-I.

1.4. Exogenous peptide antigen processing and presentation via MHC class II

To date, the role of class II MHC presentation in tumors remains far less understood compared to MHC class I pathway. A primary reason was the fundamental premise that HLA class II loci are constitutively expressed only in professional APCs and thymic epithelial cells and restricted to a much lower extent in other tissues. Thus, the process of MHC class II peptide presentation has been largely attributed to APCs, including dendritic cells (cDC1/2), macrophages, and B cells, which relay two-signals activating message to naïve CD4⁺ T cells. In the context to antitumor response, help signals relayed by CD4⁺ T cells from APCs are indispensable to achieve the magnitude of CD8⁺ T cell response⁴⁰. CD8⁺ T cell response against MHC class II-negative tumor was also shown to be dependent on type 1 T helper cell signal delivered by APCs⁴¹. Thus, primed CD4⁺ T cells not only shapes the clonal expansion and differentiation into effector or memory CD8⁺ T cells but also deliver costimulatory signals and cytokine release to instruct B cell response, which together play a central role in the orchestration of tumor immunity^{42–47}.

Recent studies have indicated that MHC class II molecules are present on the surface of tumor cells with tissue-specific variability. Interestingly, airway epithelial cells, specialized to the lung, can induce expression of MHC-II during chronic inflammation of the lung, including chronic bronchitis, asthma, idiopathic pulmonary fibrosis or parainfluenza⁴⁸. In addition, the presence of clonally expanded CD4⁺T cell with cytotoxic functions that can abrogate bladder cancer cells and enhances checkpoint blockade response in an MHC class II-dependent manner^{49,50}. Thus, perhaps unsurprisingly, both NSCLC and SCLC have been shown to express MHC-II in primary tumors^{51–53}.

The fact that tumor cells stain positive for surface MHC-II supports a compelling notion that MHC-II presentation is not only exclusive to APCs but is also competent on tumor cells. Thus, it is worth revisiting our understanding of the antigen processing and presentation through MHC-II molecules, as discussed below, focusing on the distinctive differences between these two presentation pathways that rarely ever overlap in a cell. However, it should be noted that most of our understanding of MHC-II antigen presentation is derived primarily from studies on APCs, thus concerning primarily presentation of engulfed exogenous peptides^{15,54–56}. The question of whether MHC-II-positive tumor cells can present exogenous and/or endogenous tumor peptides, and what consequences of these two distinct processes might diverge in CD4⁺T cells, remain largely understudied.

The process of MHC-II antigen presentation begins with structural assembly of the α and β chains of a nascent MHC-II molecule with the invariant chain (I chain) or CD74^{57–59}. I chain is a nonpolymorphic type II transmembrane glycoprotein not encoded in the MHC region but is required for MHC-II $\alpha\beta$ stability in the absence of peptides and for targeted export of MHC-II to endosomal compartments⁶⁰. Unlike MHC class I of which peptide loading occurs in the ER, MHC class II molecules are transported from the ER membrane to intracellular vesicles called MHC-II compartments (MIIC) where peptide antigens are presented for binding^{61,62}. These

peptides derive from pathogens or aberrant proteins internalized via endocytosis, micropinocytosis or phagocytosis and degraded by proteases, mostly of cathepsin family, within the endolysosomal compartment⁶³. Thus, MHC-II peptides are often called “exogenous” peptides since they derive from outside the cells. Next, in the MIIC endocytic compartment, I chain is cleaved to yield a short peptide fragment called CLIP retained on MHC-II molecules until physically removed by HLA-DM^{64,65}. Similar to tapasin, HLA-DM acts as a proofreading protein that can select superior peptides based on stability and affinity to the respective MHC-II molecules^{66,67}. HLA-DM catalytic activity is negatively regulated by another MHC-II-associated chaperone, HLA-DO^{68,69}. HLA-DO is present in DCs, B cells and thymic epithelial cells, where they remain in the intracellular vesicles.

Importantly, besides exogenous peptide presentation, another cell-intrinsic process recently emerged that can generate endogenous peptide for MHC class II presentation^{70–72}. Autophagy, a process of nutrient conservation mechanism, shares the dependence on lysosomal degradation with the MHC-II antigen processing pathway. Evidently, about 20-30% of peptides eluted from MHC class II in B cells and DCs are of cytosolic and nuclear origin⁷³. The autophagolysosome was shown to also form from fusion of the autophagosome with the endosomal-lysosomal MIIC complex⁷⁴. Perhaps the most compelling evidence of autophagy in MHC-II presentation is the profound effects of mutation of autophagy gene 5 (ATG5) on both positive and negative selection of CD4+ T cells by thymic epithelial cells via alteration MHC class II peptidome in cortical thymic epithelial cells⁷⁵. With this evidence, it is possible that tumor cells might be able to present MHC-II peptides via autophagic mechanisms; however, to date, evidence of such capacity has not been well described in cancer.

1.5. Alternative antigen presentation mechanisms via non-classical MHC-Ib

Another class of MHC encompassing a diverse array of genes encoded both within (e.g., HLA-E, HLA-F, HLA-G, MIC-A, MIC-B) and outside (e.g., MR1, CD1a-e) the MHC region, are

collectively referred to as “nonclassical” MHC genes. While these nonpolymorphic genes exist for both class I and class II, the majority of the molecules are related to MHC class I and are thus termed “MHC class Ib” or “MHC class I-like” proteins.

While resembling classical MHC-I in structure, MHC-Ib molecules do not present peptides to conventional $\alpha:\beta$ T cells. Some members in this class lack B2M on the cell surface, display limited polymorphism, and exhibit varying degrees of tissue expression. As reviewed below, these MHC-Ib genes play an important role in “self”-monitoring and responses to cellular stress.

The most well-studied MHC-Ib gene is HLA-E, due to its function in antitumor NK cell surveillance. Located between HLA-C and HLA-A genes in the MHC region, HLA-E is an unconventional MHC gene because of its monomorphism and limited allelic diversity. HLA-E binds to very restricted subset of nonpolymorphic peptides, termed Qa-1 determinant modifiers (Qdm) in mice and VL9 in human, derived from the leader sequences of classical HLA-I molecules. Binding of peptide:HLA-E occurs in the ER but is TAP-independent. The expression level of HLA-E strongly correlates with MHC-Ia in normal tissues. HLA presentation of MHC-Ia leader sequence is recognized by CD94/NKG2A inhibitory receptors, thus inhibiting NK cytotoxic activity⁷⁶. Expression of MHC-Ia on normal tissues endows cells with the ability to evade NK cell activation and maintain peripheral homeostasis.

Another member of MHC-Ib class, HLA-G consists of 15 alleles and has seven isoforms, four membrane-bound (G1-G4) and three soluble (5-7), which bind to KIR2DL4 and CD160 on NK cells⁷⁷, as well as endothelial cells and T lymphocytes. HLA-G is expressed on fetus-derived placental cells and mediates immune tolerance during pregnancy by interacting with LILRB1, preventing NK cell-mediated cytotoxicity⁷⁸. HLA-G exhibits restricted expression in normal tissues, albeit re-expressed in some tumor types. HLA-F, expressed in various tissues, is typically not detected on the cell surface and is believed to interact with LILRB1. *HLA-E, -F, and*

-G has been shown poorly expressed in lung cancer cell lines compared to cell lines from other cancer types³.

Among MHC class I-like genes encoded outside the MHC region, CD1 family members are expressed on DCs, monocytes and some thymocytes. CD1 is structurally similar to MHC class I with a B2M attachment but is functionally resembling an MHC II in its bypass of the TAP-dependent ER processing. A distinguished feature of CD1 family member is its proclivity for hydrophobic residues, enabling CD1 molecules to present glycolipids instead of linear peptides as MHC-Ia molecules. T cells that recognize CD1 mostly lack CD4 and CD8 but still retain a diverse α :beta TCR repertoire; indeed, some CD1-restricted T cells express both TCR α and NK receptor and are termed invariant NKT (iNKT) cells.

We recognize other MHC class I-like genes mapped within and outside the MHC coding region that are not extensively reviewed here. Within the MHC coding regions lie the complement components C2, C4 and factor B or cytokine factor TNF and lymphotoxin- sometimes termed MHC class III region but are by no means related to MHC family. For example, MR1, is conservedly associated with mucosal-associated invariant T (MAIT) cells, a population of lymphocytes abundant in peripheral blood, lymphoid organs, mucosa of intestines, and about 2% of tumor immune infiltrates⁷⁹. Their functions are largely described in relation to metabolite sensing from microbes; however, a recent study described two subsets of tumor-infiltrating MAIT populations differed by expression of TRAV1-2 and SLC4A10, suggesting a potential role of these T cell subsets in the tumor microenvironment⁷⁹.

CHAPTER 2: REGULATORY MECHANISM OF TUMOR ANTIGEN PROCESSING AND PRESENTATION PATHWAY

2.1. Transcriptional regulation of classical HLA genes

2.1.1. Transcriptional regulation of MHC class I genes

Nucleated cells constitutively express MHC class I, which can be further upregulated by activation of interferon signaling, including alpha, beta and gamma. Transcription of MHC class I gene is regulated through cis-acting regulatory elements located on the proximal promoter, which harbors three structures: an enhancer A containing NF- κ B-binding sites, an IFN-stimulated response element (ISRE) serving as binding site for interferon-regulatory factors (IRF), and a conserved motif, comprised of four cis-acting S (also called W or Z), X1, X2, and Y elements termed the SXY box^{80,81}. The X1 box of SXY motif is bound by the regulatory factor complex (RFX complex) consisting of RFX5, RFXAP and p41, together forming the enhanceosome transcriptional factor complex⁸². Interestingly, RFX is not required for MHC-I presentation as RFX5-deficient mice exhibit intact MHC class I expression⁸³. However, all three regions: enhance A, ISRE and SXY motif are present at the promoter of all three class I *HLA* loci as well as *B2M* gene on chromosome 6⁸².

Comprehensive studies on MHC class I locus and its allelic variants have revealed notable differences among three HLA class I loci. Of note, the promoter region of *HLA-B* locus contains additional binding sites for USF1/2 and the transcription factor SP1 compared to *HLA-A* and *-C*⁸⁴. In addition, *HLA-A* and *HLA-B* are expressed at much higher levels compared to *HLA-C* that exhibits a restricted peptide repertoire with limited assembly process^{85,86}. *HLA-A* and *HLA-C*, but not *HLA-B*, can physically associate with the peptide loading complex⁸⁷. As a result, among three molecules, *HLA-B* undergoes quicker peptide loading and plasma membrane export, and most *HLA-B* are always loaded with peptides, compared to 30-70% for *HLA-A* and *HLA-C*⁸⁵.

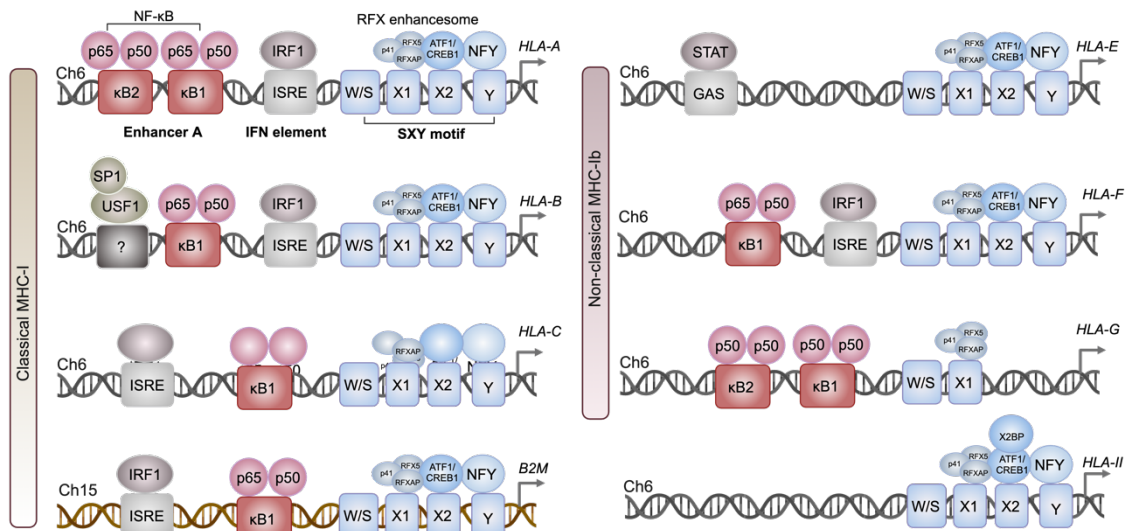


Figure 2.1. Conserved transcriptional motif among class I and class II HLA genes. All HLA genes exhibit a SXY motif with optional one or two NFkB-specific enhancer(s) and/or interferon stimulated regulatory element (ISRE).

The constitutive expression of MHC class I in most nucleated cells is primarily dependent on the function of MHC-I-specific master transcriptional regulator, NLRC5. NLRC5, or NOD-, LRR- and CARD-containing 5, harbors a caspase activation domain (CARD), a nucleotide binding domain (NBD), a nuclear localization signal (NLS) and a leucine-rich repeats terminus (LRR)⁸⁸. These domains are required for nuclear import of NLRC5 and for its transactivation of MHC class I genes. NLRC5 is expressed at the highest levels in lymphocytes, but its expression is inducible by STAT1-mediated IFN- γ activation due to the presence of a gamma-activating sequence (GAS) at the promoter of *NLRC5* gene. In addition, NLRC5 can be upregulated by type I IFNs, LPS and virus infection⁸⁴. Upon induction, NLRC5 forms into a heterodimer complex that is recruited by RFX transcription factor to the SXY module on the promoter region of MHC class I⁸⁹. NLRC5 then activates transcription of classical MHC-I genes (*HLA-A*, *-B*, and *-C*), *B2M*, *LMP2*, *TAP1*, as well as the non-classical *HLA-E*, but has no effects on MHC class II locus⁸⁸. Mice lacking NLRC5 substantially impairs MHC class I expression even in the presence of IFN- γ , deficient CD8⁺ T cell activities and subsequent failure to engage

in immunity^{90,91}. Overall, physiological regulation of MHC class I expression in nucleated cells necessitates the presence of its transactivator NLRC5.

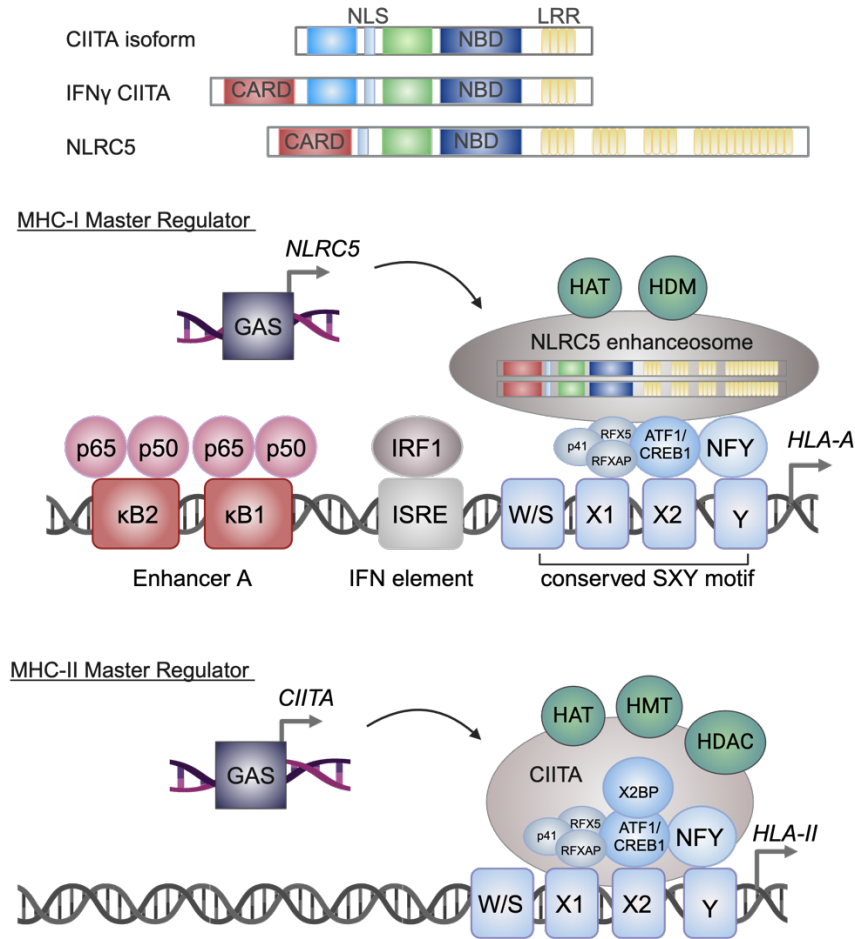


Figure 2.2. Mechanism of transcriptional activation of *HLA* genes via binding of the master regulator to the SXY conserved motifs on MHC-I class I and class II genes.

2.1.2. Transcriptional regulation of MHC class II genes

Unlike MHC class I, MHC II expression is only constitutively expressed in APCs, including DCs, B cells and macrophages⁹² and otherwise inducible in fibroblasts, epithelial cells and endothelial cells following exposure to interferon-gamma specifically, but not interferon-alpha or -beta⁹³. Similar to MHC class I, transcriptional regulation of MHC-II and MHC-II-related genes (*i.e.* *HLA-DM*, *HLA-DO*) is governed by a conserved regulatory SXY motif located within the initial 150 base pairs upstream of the transcription initiation site^{80,81}. MHC-II promoter region

lacks ISRE and enhancer A, but the SXY region of MHC-II can be recognized by diverse transcription factors, including the RFX complex. X2 box is the binding site for X2BP, CREB/ATF and Fos/Jun, and the Y box is bound by the NFY complex⁹⁴. The differences in promoter region of MHC class I and class II reflect evolutionary divergence and subsequent specified functions of these two molecules within the immune system.

MHC class II deficiency was revealed to arise not from mutations within the MHC genes but rather from mutations in the regulatory enhanceosome required for transcriptional activation of class II genes, with the master regulator MHC class II coactivator (CIITA) being of utmost importance. CIITA is both necessary and sufficient to activate MHC-II in IFN- γ -inducible cells⁹³. In the absence of CIITA, MHC-II cannot be transcriptionally induced by IFN- γ , and CIITA itself is induced by IFN- γ , which precedes MHC-II transcript induction by several hours⁹⁵. Phylogenetics studies revealed that CIITA is the closest relative of NLRC5 in humans⁹⁶. Patients manifesting MHC class II impairment exhibit four complementing gene defects: CIITA, RFXANK, RFX5 and RFXAP, with the latter three encoding a multimeric complex RFX that binds to X-box motif at the promoter region of all MHC class II genes^{97,98}. RFX enhanceosome recruits CIITA to the promoter region where CIITA exerts its control over both constitutive and inducible transcription of MHC-II genes^{99–101}. RFX defects results in deficiency in expression of MHC class II while virtually having no effect on MHC class I expression⁸².

While RFX and the enhanceosome components are ubiquitously expressed, CIITA expression is tightly regulated and cell-type specific⁹³. CIITA is constitutively expressed in dendritic cells via Promoter I, in B cells via Promoter III, and in other cell types, induced by IFN- γ via Promoter IV. Promoter IV sequence consists of IFN- γ activated sequence (GAS), an E-box and an IFN response element (IRE) site. Similar to MHC-I, transcriptional activation of CIITA is controlled by the JAK/STAT pathway via STAT-1, IRF-1 and USF-1 binding to GAS, IRE and E-box of CIITA promoter, respectively¹⁰². In addition, genome wide RNAi screen has revealed that

CIITA might act in concert with a large network of other transcription factors to regulate MHC-II expression in specific tissues¹⁰³. It has recently been shown that transcriptional regulation of MHC class I and class II are intricately intertwined. The CIITA-RFX complex can transactivate both MHC class II, MHC class I and B2M^{104,105}. The MHC class I transactivator NLRC5 also cooperates with the same transcription factor recognized by CIITA to induce MHC class I gene expression, suggesting a potential mechanism for cross regulation of MHC genes⁸⁹.

2.2. Epigenetic regulation of classical HLA genes

Genes encoding members of the MHC antigen presentation pathway can be silenced or activated by epigenetic mechanisms. Studies performed over a decade ago have demonstrated the role of NLRC5, the master regulator of MHC class I, in engaging histone-modifying enzymes. In particular, NLRC5 can dampen the silencing H3K27me3 modification on MHC-I promoters to activate its transcription in response to IFN- γ treatment¹⁰⁶. Furthermore, NLRC5 interacts with the CREB/ATF1 transcription factors to engage in histone acetyltransferase transcriptional activation⁸⁹. These studies underscore dynamic of epigenetic control over MHC-I gene expression.

In MHC class II, IFN- γ stimulation has been shown to enhance acetylation of histones H3 and H4 on the inducible Promoter IV of MHC-II, driven by the dynamic of CBP/p300 and SWI/SNF complex^{107–109}. At the same time, trimethylation of H3K4 are deposited to the same region by displacement of the histone methyltransferase EZH2 from CIITA Promoter IV, potentially as early epigenetic events¹¹⁰. Furthermore, CIITA was shown to interact with histone acetyltransferases and can facilitate chromatin remodeling, enhance promoter clearance, and recruit the transcriptional apparatus¹¹¹. Together, these studies demonstrate that even under physiological conditions, both MHC class I and class II antigen presentation pathways are epigenetically regulated. Relying on this dependence of epigenetic modifiers to achieve homeostasis, transformed tumor cells have devised novel mechanisms to enhance silencing

and dampen activation of genes encoding the antigen processing and presentation pathway, details of which will be explored in the later section.

2.3. Other regulatory mechanism of MHC-I antigen presentation

2.3.1. Surface MHC complex durability

In the context of MHC class I, intact MHC molecules are necessary but not sufficient to elicit successful peptide surface presentation²¹. The mature MHC class I complexes undergo rigorous quality control during folding and assembly in the endoplasmic reticulum (ER). Pulse-chase studies have revealed that a significant proportion of MHC class I molecules fails to engage suitable peptides^{85,112}. Misfolded and unbound MHC class I molecules are subject to degradation through the ER-associated degradation (ERAD) pathway, involving ubiquitylation and subsequent dislocation from the ER to the cytosol for proteasomal degradation^{113,114}. In addition, studies on immunosuppressive mechanisms of viruses have identified several viral proteins that exhibit a RING domain resembling the human MARCH protein. MARCH family contains an E3 ligase membrane-associated RING finger that ubiquitylates target proteins to induce their internalization and lysosomal degradation. In humans, MARCH 4 and MARCH9 have been shown to downregulate surface MHC-I molecules¹¹⁵.

MHC class II is constitutively expressed on APCs, enabling efficient and timely presentation of antigens to CD4⁺ helper T cells. The processes of antigen uptake, processing and durability of antigens are cell-type specific, dictating the specific functions of each cell type during tissue inflammation. Interestingly, the cytoplasmic tail of the MHC-II β chain harbors a conserved lysine residue, which serves as a target for ubiquitination by MARCH-1, another E3 ligase. MARCH-1 is expressed exclusively on APCs where it localizes to the endosomal membrane to regulate recycling of both MHC-I and MHC-II molecules at steady state^{113,116}. During inflammation, MARCH-1 ubiquitination is functionally repressed, allowing mature APCs to accumulate and constitutively sustain p:MHC complexes on the cell surface during tissue

migration^{116–118}. Thus, ubiquitination controls the life cycle of MHC molecules in their trafficking between endosomal compartments and the plasma membrane, and their capacity to remain on the cell surface for antigen presentation.

2.3.2. Accessory components of the presentation machinery

Prior studies have shown that selected individuals with MHC class I deficiency syndrome can still harbor normal levels of MHC-I transcript and protein synthesis; however, few MHC class I molecules ever reach the cell surface^{119–121}. These individuals were found to exhibit either deletions or mutations in TAP1/2 subunits of the TAP complex, with associated defects in class I antigen presentation, reduction of CD8+ T cells and relative expansion of NK and $\gamma\delta$ T cells^{119,122}. Further studies in TAP-deficient cells confirmed that the TAP transporter is indeed required for MHC-I presentation of endogenous peptides in nucleated cells^{119,122,123}. These studies underscore that the functional capabilities of tumor pMHC-I requires not just the presence of intact MHC-I molecules but also absence of defects in members of the antigen presentation machinery.

The premise that MHC class II is uniquely associated with professional APC arises not from exclusive expression of MHC-II molecules, but from the functions and expressions of costimulatory signals and members of the processing and presentation pathway. The invariant chain (CD74) and cathepsin S are well expressed in virtually all APCs (DCs, macrophages, monocytes and B cells, with the exception of pro-B cells) but are absent on ILCs, T cells and stem cells¹⁵. Covalent inhibitors of cathepsins alters MHC-II antigen presentation and are utilized in the context of autoimmune disease controls¹²⁴. Similarly, the gamma interferon-inducible lysosomal thiol reductase (GILT), constitutively present in late endocytic compartments of APCs, is required for MHC-II-restricted presentation to CD4+ T cells¹²⁵. In addition, in the absence of HLA-DM, cells exhibit defects in peptide loading and exchange, resulting in increased self-reactivity and defective negative selection^{126–128}. It is unknown if cells

of different tumor types express functional level of these factors, but like MHC class I, the presence of intact MHC molecules is necessary but not sufficient to elicit the highly regulated process of antigen presentation.

2.3.3. Inducible antigen presentation via interferon signaling

Cytokines, particularly interferons, play a pivotal role in regulating the expression of both MHC class I and MHC class II molecules during immune responses. Interferon- α (IFN- α) and IFN- β specifically increase MHC class I expression across various cell types¹²⁹. In contrast, IFN- γ enhances the expression of both MHC class I and MHC class II molecules^{130,131}. Additionally, IFN- γ has the capacity to induce MHC class II expression on certain cell types that typically do not constitutively exhibit MHC-II. Activation of interferon signaling leads to activation of STAT dimer and subsequent of IRF-1, which translocates to the nucleus and binds to ISRE region located on the promoters of MHC-I genes or of CIITA transactivator of MHC-II, in turn transcriptionally activating MHC gene expression.

Furthermore, interferons contribute to augmenting the antigen-presenting function of MHC class I molecules by triggering the expression of critical components within the intracellular machinery. As mentioned above, treatment with interferon type I or type III can induce expression of TAP1/2, LMP2/7, ERp57, as well as MHC-I molecules. Expression of NLRC5 was also shown to be inducible by IFN- γ due to a Gamma-activating sequence motif on its promoter. NLRC5 has been shown to negatively regulate TLR4 and RIG-I-MDA5 signaling, whose function is to recognize cytosolic RNA derived from pathogenic infection or from transcription of retroviral elements and mediate type I interferon response¹³². It is possible that NLRC5 regulates MHC-I transcription as a mediator of cellular stress response and homeostasis.

In the context of tumor-associated inflammation involving activation of interferon signaling, it is challenging to tease out which steps in the antigen presentation machinery are

independent of such interferon response. However, in tumors with mutations in the interferon signaling pathway, antigen presentation has been shown to significantly suppressed¹³³.

Functional interferon signaling can instruct the environment of tumor cells with inflammatory cytokines that might play diverse roles in initiation, sustaining and expansion of antitumor response. One consequence of such inflammatory environment is the induction of chronic antigen stimulation, a series of events that might be deleterious to tissues without proper resolution. Given this context, some immune cold tumors, such as several subtypes of lung cancers associated with chronic smoke-induced inflammation, might have not always been “cold” but rather already resolved this problem of constant antigen exposure in the early events of tumorigenesis: immune pressure might have promoted selection and expansion of clones that have successfully evaded both adaptive and innate immune surveillance. In the next chapter, we will explore mechanisms that allow cancers to evade antigen processing and presentation and subsequent resistance to immunotherapy.

2.3.4. Autophagy pathway in MHC class I tumor antigen presentation

Autophagy is an evolutionarily conserved nutrient sensing mechanism that replenish energy and cellular components recycled from lysosomal degradation of cytoplasmic proteins and damaged organelles, subsequently maintaining metabolic homeostasis and survival. Three mammalian autophagic mechanisms have been described: macroautophagy, microautophagy and chaperone-mediated autophagy, with the Atg-dependent microautophagy pathway being predominantly conserved in mammals.

Under cellular metabolic stresses, the dissociation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) from the Atg1/ULK complex activates the creation of the pre-autophagosome, consisted of the ubiquitin-like Atg12 and Atg8 (also known as microtubule-associated protein 1A/1B-light chain 3 [LC3]), and their associated conjugation systems (Atg7 and Atg10). Cleavage of LC3 by Atg4 to form LC3-II enables the newly formed autophagosome to fuse with lysosomal membrane, after which autophagosome contents are degraded to

generate cellular components. The autophagic machinery is coupled with other lysosomal pathways including endocytosis and phagocytosis, thus associated with the process of pathogen-derived protein degradation in both innate and adaptive immunity, which has been most notably observed in APCs.

As mentioned in section 2.3.1, surface MHC-I bound to endogenous peptides can be internalized back into the endosomal compartment, where they can be degraded in the lysosomes or transported back to the plasma membrane. In dendritic cells deficient in autophagy, MHC class I expression is sustained on the cell surface as a consequence of decreased pMHC-I endocytosed degradation^{134,135}.

In the context of MHC-I presentation of exogenous peptides (discussed in Section 1.3), autophagic tumor cells can act as a source of tumor peptides presented by APCs, however, the role of autophagy in MHC-I cross presentation has been debated. Loss of Atg5 or Atg7 was shown to have no effects on DCs MHC-I cross presentation in some cases¹³⁶ but not others¹³⁷. To date, the single cell type thought to be capable of cross-presenting antigens in LN and spleens are CD8+ CD103+ DCs, especially in the lung, where CD103+ subset is the only one capable of migrating from the lung^{138–140}. Overall, the role of cross-presentation of MHC-I peptides remains to be further studied in tumor cells.

CHAPTER 3: MECHANISMS OF TUMOR ANTIGEN PRESENTATION EVASION

Presentation of tumor peptide antigen via surface MHC molecules requires repletion of ample immunogenic neoantigen repertoire that can prime, elicit and sustain a durable functional T cell response. However, immune surveillance can also exert potent forces on tumor genome during tumor evolution. Disruption of the antigen presenting machinery is thought to be a mechanism for which tumor cells respond to such immune-editing pressure. Tumor clones that exhibit robust antigen presentation might have been selectively eliminated or acquired additional escape mechanisms to evade immune surveillance.

Multi-omic analysis of 18 TCGA tumor types demonstrated acquired mutations in antigen presentation pathway components, including B2M, and HLA alleles, highlighting functional loss of antigen presentation as an effective pan-cancer strategy of resistance to cytolytic pressure¹⁴¹. Cellular processes leading to loss of APM were previously discovered in the context of pathogen infection or chronic inflammation. Infectious agents have evolved various strategies to suppress presentation of itself on surface MHC, thus evading both CD8+ cytotoxic T cell and NK cell surveillance. Various tumors have mirrored these pathogenic mechanisms to interfere with HLA transcription regulation or with regulators of its pathway. Here we discuss the various mechanisms that tumor cells have evolved to evade antigen presentation via MHC-I.

3.1. Genetic alterations in MHC-I genes

Over the past few years, numerous studies have illustrated the presence of elevated somatic mutation rate in HLA genes in many solid tumors^{142–144}. Most notably, a pan-cancer analysis of ~8,000 tumors and matched health tissue have comprehensively characterized the mutational landscape of class I HLA genes. In this study, somatic HLA mutations were found at the rate of 3.3% (266 out of 7,930 individuals), the highest being in head and neck squamous cell carcinoma (HNSCC) with 7.9%¹⁴². Lung squamous cells exhibit enriched HLA mutations in *HLA-A* gene, whereas some tumors, i.e. liver cancer, were found to entirely lack HLA mutations. In subsequent studies, mutations in HLA genes were found in only 3 out of 90 NSCLC patients

in the TRACERX cohort¹⁴⁵. Furthermore, various studies on metastatic melanoma reported that point mutations, deletion or allelic loss of *B2M* were significantly enriched in non-responders of pembrolizumab¹⁴⁶, an observation also observed in ICB-resistant lung cancer¹⁴⁷. However, subsets of *B2M*-mutant tumors can still achieve clinical benefit to immunotherapy if these samples are microsatellite instability (MSI)-high. Nonetheless, loss of *HLA* or *B2M* represents an effective yet uncommon pan-cancer mechanism of resistance to T cell-based immunotherapy¹⁴⁸.

The frequency of genetic loss of HLA genes is strongly tumor-type specific. In some cancers, i.e. DLBCL, somatic inactivation of *B2M* or HLA genes is reported in 80% of patients, resulting in negative surface MHC-I expression¹⁴⁹. Mutations of *HLA* or *B2M* genes tend to be recurrent, with loss-of-function mutations strongly biased to localize to exon 4, where the TCR-recognition domain is encoded¹⁴². Other frequent mutations localize to the peptide binding domains of HLA molecules, potentially affecting antigen presentation. Reduction in class I activity is also co-enriched with mutations in interferon signaling and lymphocyte attractant chemokines, suggesting that mutations of HLA are strongly selected by immune pressure¹⁴².

Unlike the rare events of *HLA* somatic mutations, loss of heterozygosity (LOH) of HLA represents a much more frequently observed mechanism of HLA genetic loss. Montesion et al. investigated the landscape of LOHHLA in 83,644 patients across 59 disease groups and identified HLA-I LOH at a 17% rate and significantly associated with worse overall survival in NSCLC¹⁵⁰. This frequency is nearly identical that found in another pan-cancer study from 610 patients of 15 tumor types, where HLA LOH was identified in 18% of patients, varying from 4% in liver cancer to the highest again in HNSCC (40%)¹⁵¹. In the TRACERx study, HLA loss of heterozygosity is frequently observed in over half of lung adenocarcinoma and 78% of squamous cell carcinomas¹⁴⁵. Interestingly, NK cell infiltration is increased in tumor heterozygous for HLA-C alleles but not in HLA-C LOH tumors, suggesting compromised KIR-dependent tumor elimination by NK cells by LOHHLA¹⁵². Together, these studies reveal that

genomic alterations of HLA genes are clinically relevant but generally infrequently observed across solid tumors, with the frequency being strongly influenced by tumor types.

3.2. Genetic alterations in regulators of antigen presentation machinery

Members of the peptide-loading complex were initially discovered by their necessary functions in the MHC-I antigen processing and presentation pathway. Genetic loss of the TAP transporter genes abrogates capacity of somatic cells to present endogenous antigen, evidently shown in TAP-mutant cell lines^{153–158}, validating its role as a positive regulator of MHC-I surface expression. Among other members of APM, LMP-2 and LMP-7 have been shown to be important but not required for MHC-I expression. Mutations in these inducible catalytic of immunoproteasome did not abrogate MHC-I antigen presentation in NSCLC but significantly deplete peptide repertoire, reflecting the presence of other accessory mechanisms that can generate suitable peptides¹⁵⁹. Similar outcome occurred when calreticulin is impaired, resulting in reduced surface antigen display^{160,161}. Expression of the master transcriptional regulator *NLCR5* is also highly correlated with *HLA*, *B2M*, and *TAP* genes, and deficiency of which selectively hinders MHC-I-restricted T cell recognition^{162,163}. Like *HLA* genes, *NLRC5* also harbors promoter hypermethylation, copy number loss and mutations that enable immune evasion in both cancer cells and quiescent stem cells¹⁶⁴. Last, interferon signaling plays a crucial role in the inducible expression of components of the antigen presentation pathway. An in vivo screen identified tumor evasion is attributed to defects of *STAT1*, *JAK1/2*, *IFNGR1/2*, and *APLNR* of the IFN- γ signaling pathway¹⁶⁵, an observation similarly recapitulated in primary tumors resistant to ICB¹⁴⁶. These cumulative findings suggest that antigen presentation relies not only on the integrity of HLA genes but also on the proper functioning of components within and preceding the antigen presentation machinery.

3.3. Epigenetic suppression of MHC-I antigen presentation

A more comprehensive landscape of epigenetic regulation of antigen presentation is discussed in Chapter 4, in parallel with current findings on therapeutic strategies to rescue

MHC-I surface expression in preclinical studies. In brief, the promoter regions of HLA genes are subjected both histone and DNA modification as a mechanism of gene silencing^{166,167}. Genome-wide study of 347 patients with diffuse large B-cell lymphoma uncovered the association of acquired deficiency in both MHC-I and MHC-II expression with activating *EZH2* mutations¹⁶⁸. This deficiency could be due to EZH2 trimethylating the K27 tail of histone H3 on the promoters of HLA genes, leading to transcriptional silencing. MHC-I and MHC-II suppression is often accompanied by loss of upstream regulators of the pathway, including NLRC5 and CIITA, both of which associated with poor clinical outcome¹⁶⁸. As discussed in chapter 4, epigenetic silencing carries a profound potential to diminish HLA expression, contributing to immune evasion during the course of tumorigenesis.

3.4. Depletion of neoantigen pool

As discussed in Section 1.3, peptide antigen serves as a rate limiting factor for loading and export of MHC-I to the plasma membrane. Based on structural conformation and binding affinity, different *HLA* haplotypes displays specific preferences for a distinct set of neoantigens. In return, the types of neoantigens being presented on MHC-I and MHC-II molecules also determine the specificity of T cell responses¹⁶⁹. Depletion in quantity and quality of tumor-specific neoantigens can diminish MHC-I surface expression as well as the strength, clonality and durability of a T cell response. Loss of neoantigens can occur through copy-number loss, suppression of neoantigen transcripts, epigenetic silencing, or post-translational modification. In addition, the primary sources of MHC-I peptide antigen, DRiPs, can also be degraded via autophagy pathway¹⁷⁰.

A recent report of 1,017 recurrent oncogenic mutations across 9,176 patients demonstrate that oncogenic mutation frequency is inversely correlated with the likelihood of being presented on autologous MHC-I, implying that recurrent neoantigens that are shared among tumor samples are likely poorly presented¹⁷¹. Similarly, MHC II exhibits stronger selection pressure on oncogenic mutations, which are either presented either in most patients or

in none¹⁷². Studies on other cancer types have confirmed the association of neoantigen immunoediting with the patient germline HLA heterozygosity¹⁵², in which tumors with intact HLA alleles exhibit significantly lower expression of neoantigens. In addition, evidence of promoter hypermethylation and neoantigen epigenetic silencing are also found in tumor subclones, suggesting an influence of selective immune pressures in lung cancer¹⁵². Immune pressure may result in tumors expressing few immunogenic HLA-I-restricted neoantigens, a feature attributed to prolonged immunoediting. In contrast, in tumor with CD8+ T cell exclusion phenotypes and in nonsmokers, neoantigens are less abundant but have an increased likelihood of being presented to T cells¹⁷³. Together, these findings indicate that immunological pressures can shape both the quality and quantity of neoantigen repertoire during tumor evolution¹⁷⁴.

3.4. Blockade of surface MHC-I cycling

As noted in section 2.3.1, surface MHC-I molecules, when bound to endogenous peptides, have the capacity to undergo internalization into the endosomal compartment. Within this compartment, there is the possibility of either degradation in the lysosomes or transportation back to the plasma membrane. Taking advantage of this trafficking course, breast cancer overexpresses MAL2 to promote rapid endocytosis of tumor antigens via interaction with endosomal proteins¹⁷⁵. Furthermore, oncogenic proteins *BRAF*^{V600E} also cooperate to upregulate internalization of HLA-A2 through modulation of the cytoplasmic tails, resulting in only transient expression of surface MHC-I¹⁷⁶. *BRAF* mutations are present in 25% of melanoma, reflecting their potentially broader effect in other tumors. In addition, promotion of MHC-I surface degradation can also be accomplished via autophagy receptor NBR1 in PDAC¹⁷⁷, a mechanism mimicking that of virus-mediated evasion strategies. However, further studies are needed to understand this post-translational regulation of MHC class I, especially the process of MHC biogenesis, trafficking and degradation in cancer immune evasion.

3.5. Non-classical MHC-Ib and NK cell evasion

The loss of major histocompatibility complex (MHC) class I on the surface of tumor cells raises questions regarding how these tumors evade natural killer (NK) cell surveillance. Viruses have long achieved suppression of MHC-I via selective inhibition of MHC class I cell surface export, functionally shutting down the peptide loading complex and blocking endoplasmic reticulum export, all of which have been described above and reviewed elsewhere¹⁷⁸. However, the consequences of these actions result in NK cell recognition, activation, and subsequent killing^{176,179,180}. In both humans and mice, NK cells express a heterodimer comprising two C-type lectin-like receptors, CD94 and NKG2. Within the NKG2 receptor family, NKG2A functions as an inhibitory receptor with a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), the inhibition of which has been shown to potentiate antitumor immunity in various tumors¹⁸¹. NKG2A is not only expressed on nearly half of peripheral blood NK cells but also found on human peripheral blood CD8 T cells both at steady state and during chronic antigen stimulation¹⁸². In both cell types, NKG2A/CD94 binding to HLA-E blocks NK cells and effector T cell function. Thus, the binding of HLA-E to MHC-I-derived peptide fragments enables NK cells to sample MHC-I variants in target cells, a tolerance phenomenon often referred as “missing self” recognition.

As observed in cancers, upregulation of HLA-E in the absence of classical HLA-I expression may facilitate immune escape by suppressing NK responses. In tumors with downregulated MHC-I, HLA-E and HLA-G expression by tumors has been reported. HLA-E expression is associated with CD8+ responses elicited by MHC-E epitopes¹⁸³, including cryptic epitopes that are undetected by conventional mapping technique but can represent <10% of the class I immunopeptidome¹⁸⁴. Tumor-specific cryptic peptides can likely escape self-tolerance and serve as immunotherapy targets. Indeed, HLA-E was found to not only present HLA-I-derived signals but also pathogen-derived peptides to CD8+ T cells^{185–187}. In HLA-I-deficient

tumors, NKG2A+ CD8+ T cells develop a TCR-independent cytotoxic mechanism with innate-like features. This inhibitory function of NKG2A+ CD8+ T cells can be restored upon NKG2A:HLA-E blockade¹⁸⁸. Cancer cells may have evolved additional evasion mechanisms to preserve HLA-E surface expression, similar to those observed in the context of pathogens^{185,189}. This evasion strategy theoretically enables tumors to evade both antigen-agnostic NK cells through HLA-E upregulation and CD8 T cell recognition through MHC class I downregulation.

Another class of MHC-Ib relevant to NK cell activation includes seven MIC genes, with MICA and MICB being the only ones with protein synthesis. These genes are predominantly expressed in fibroblasts and epithelial cells, playing pivotal roles in the initiation of innate immunity in scenarios devoid of interferon production. MICA and MICB are recognized by NKG2D receptor expressed on NK cells, $\gamma\delta$ T cells and select CD8 T cells. NKG2D stands apart from other inhibitory receptors of the NKG2 family of NK cell receptors; in the absence of ITIM, NKG2D contains an activating domain associated with the adaptor protein DAP10¹⁹⁰. Similar to human MICA/MICB, murine ULBP or RAET1 e are MHC-I-like molecules that can bind to NKG2D during pathogenic infections or tumor transformation, leading to subsequent elimination by NK, $\gamma\delta$ T cells and some cytotoxic $\alpha\beta$ T cells¹⁹⁰.

Compared to NSCLC, SCLC cells exhibit significantly lower levels of MICA/B both in transcript and protein levels^{191,192}. MICA expression is significantly associated with neuroendocrine phenotype, with YAP1-high cell lines exhibiting remarkably higher MICA than ASCL1 and NEUROD1 tumors. Furthermore, NKG2DL expression is negatively correlated with c-MYC, which has been associated with the NEUROD1 subtype in SCLC¹⁹². Within this context, expression of MICA/B was shown to be epigenetically regulated by H3K27Ac-dependent promoter inaccessibility. MYC further collaborates with histone deacetylase 3 (HDAC3) to deacetylate the promoter region of MICA/B, at histone 3 lysine 9 (H3K9Ac). This collaborative

action effectively eliminates the transcriptional activation signal, contributing to the epigenetic silencing of NKG2DL on tumor cells. This phenomenon can in part explain why NK cells were found to be conspicuously absent from NEUROD1-expressing SCLC tumors¹⁹³. Employing a pan-HDAC inhibitor resulted in the derepression of MICA expression in SCLC cells with elevated levels of ASCL1. In addition, treatment with a pan-HDAC inhibitor trichostatin A (TSA) proved sufficient to enhance both NK-cell recruitment and activation in allograft experiments¹⁹¹. Further investigations have demonstrated that while cytotoxic NK cells did not exert a discernible impact on primary tumor formation, their activation emerged as a pivotal determinant in orchestrating the metastatic dissemination of SCLC. Notably, NKG2DL also serves as a target for recognition by CD8⁺ effector T cells, exemplifying yet another instance of epigenetic mechanisms employed by SCLC cells to evade detection by various components of the immune system. In summary, these cumulative findings highlight the interplay among various classes of MHC-I in coordinating antitumor immunity. They underscore the comprehensive strategies employed by cancer cells to avoid the presentation of tumor antigens to both innate and adaptive immune responses.

CHAPTER 4. THERAPEUTIC STRATEGIES FOR ANTIGEN PRESENTATION DEFICIENCY

Building on the preceding section, this chapter focuses on the array of therapeutic strategies aimed at reactivating antigen presentation, with a central focus on epigenetic mechanisms. A crucial requirement for the efficacy of these strategies is the genetic integrity of HLA genes and those encoding the antigen presentation pathway. Therapeutic targeting of MHC-I presentation becomes unfeasible in the event where there is genetic loss of function in one of the pathways explored below. Since tumor cell presentation of MHC-I antigen presentation constitutes the last step in the sequential order of recognition and resolution by adaptive immunity, it is crucial to acknowledge that even if the strategies discussed here were all to take effect, tumor cells might still evade immune surveillance by other mechanisms. It is also important to highlight that while many of these epigenetic approaches may impact the phenotype and activities of immune cells, immune cells are not the primary focus of our discussion in this context. We solely focus our discussion on impacts of diverse therapeutic approaches on MHC-I presentation of tumor cells.

4.1. Targeting epigenetic pathways for antigen presentation deficiency

Heritable patterns of altered histone modifications and chromatin structure are a central process in cell development, differentiation, and lineage decision. These diverse chromatin remodeling effects can result in a progressive shift from open chromatin configuration to a more condensed state. Coupled with these changes in chromatin structure are covalent modifications of DNA and histone, together resulting in establishment and maintenance of cellular identity or a commitment for a phenotypic transition. Epigenetic mechanisms have been implicated in nearly every tumor-intrinsic activity, including their ability to process and present antigen¹⁹⁴.

4.1.1 Overview of classes of epigenetic modifiers

The fundamental epigenetic changes encompass DNA methylation, histone modifiers and readers, chromatin remodelers, microRNAs and alternative chromatin structures¹⁹⁵. Within

this section, we explore the initial three classes of epigenetic modifiers, delving into their functions in the regulation of gene expression and examining their impact on cancer cells. Of note, many epigenetic targets discussed below have advanced to clinical stage for treatment of both NSCLC and SCLC, the details of these clinical applications have been reviewed elsewhere^{196,197}.

a. DNA methylation

Over the last 40 years, one of the most well-documented epigenetic changes during tumorigenesis is the observation of DNA hypermethylation on cancer-associated gene promoters¹⁹⁸. Methylations of the 5-carbon on cytosine residues (5mC) can occur within the CpG islands found in the promoter regions of nearly half of the mammalian genomes; this modification is most typically associated with transcriptional gene silencing, and subsequently loss of that gene function^{194,199}. During normal development or in adult stem cells, 60% of gene promoters harbor CpG islands that remain unmethylated^{195,200,201}. In cancer cells, 5-10% of CpG island promoters are hypermethylated, many of which turned out to be tumor suppressor genes whose loss of functions represents a hallmark of cancer biology^{195,200,201}.

The landscape of DNA methylation is established by a family of DNA methyltransferase (DNMT) enzymes consisting of DNMT1, DNMT3A and DNMT3B²⁰². *De novo* methylation patterns are deposited by DNMT3A/B, whereas DNMT1 mostly maintains these marks over the course of DNA replication. Interestingly, when both DNMT1 and DNMT3B are simultaneously deleted in human colon cancer cells, tumor cells lose 95% of their genome-wide methylation^{203,204}, highlighting the potency of DNA methyltransferase activity across the whole genome.

In addition, another interesting observation in the global methylation patterns in cancer cell is the global DNA hypomethylation that are distinct from CpG-rich islands of the promoter region. DNA can be demethylated by ten-eleven translocation (TET) family of proteins, which catalyzes the oxidation of 5mC to 5hmC to remove methyl groups and might contribute to

transcriptional repression²⁰⁵. DNA methylation marks are interpreted, or “read,” by methyl-binding proteins (MBD family)^{206,207}.

Evidence of promoter hypermethylation of tumor suppressor genes, i.e *MGMT*, *CDKN2A* (p16^{INK4}, p16^{INK4A}, *CDK4I*, *MTS1*, and *p16*), and *DAPK*, was first observed in NSCLC to associate with poor prognosis. Loss of function of *CDKN2A* is also frequently observed in lung squamous cell carcinoma (LUSC), a subtype of NSCLC²⁰⁸. Consequentially, overexpression of DNMTs have been observed in about half of patients with NSCLC^{209–212}, and strongly associated with chronic smoking^{210,213}. The initial development of DNA demethylating inhibitors, 5-azacytidine (5-aza, AZA) and its deoxy derivative, 5-aza-2'-deoxycytidine (DAC), did not result in clinical benefit as a monotherapy in NSCLC, in part due to the transient effects and high toxicity^{214,215}. Treatment with AZA or DAC did not prevent methylation marks from depositing back into CpG islands once the inhibitor is removed²¹⁵. This observation has since inspired the clinical development of irreversible pharmacological modulators of chromatin remodeling.

Similar to NSCLC, tumor-specific promoter hypermethylation has been detected in >70% of SCLC tumors, with loci including *RB1*, *NEUROD1*, *REST*, *BCL2*, *ZNF423*, *TCF21*, and *TREX1*, with promoter hypermethylation strongly associated with overexpression of *EZH2*^{216–218}. The degree of hypermethylated promoter in SCLC was also found to be greater than other tumor types in TCGA; however, genome-scale studies have not established a correlation of global hypermethylation to patient outcome in SCLC^{217,218}. Interestingly, TREX1 activity has been associated with chromothripsis and its absence results in activation of cGAS-STING mediated type I interferon response and systemic inflammation, suggesting a role of hypermethylation in tumor immune phenotype^{219,220}. On the other hand, promoter hypermethylation of caspase-8, FAS, and TRAIL-R1 contributes to resistance to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in SCLC cell lines, which can be restored via either DAC (decitabine) alone or combination of DAC and HDAC inhibitors^{221,222}. In addition, POU2F3 has been described as a master transcriptional regulator of the tuft cell-like lineage

(POU2F3 subtype); promoter hypermethylation of POU2F3 has been previously observed in other cancer type, suggesting a potential role of DNA methylation in defining SCLC subtype lineages. SCLC tumors can be stratified into epitypes based on their “CpG-island methylator phenotype” status, with hypermethylated status associated with worse outcome²²³.

b. Histone modifications

Across multiple cancer types, hypermethylation of promoter region of tumor suppressor genes has been attributed to not just DNA methylation alone, but also high prevalence of histone modifications, including primarily histone acetylation and methylation, which together determine whether a gene can be transcriptionally activated or repressed. Of note, besides the major classes of histone modifications discussed below, 14 other histone modifications have been detected, including phosphorylation, ubiquitination, sumoylation, deamination, and others²⁰⁶, which are substantially less studied in the context of tumorigenesis and tumor immune evasion^{224,225}.

Histone methylation

Histone modifications orchestrate chromatin structure and gene expression patterns through covalent marks on the four histone proteins comprising the primary structure of the nucleosome (H2A, H2B, H3, and H4). The landscape of histone methylation is regulated by three classes of histone modifiers: the JmjC demethylase (KDM) class, the LSD1 demethylase (KDM) class and the KMT methyltransferases. Substrate specificity and mechanism of action of these KMT and KDM have been previously reviewed²²⁶. In brief, the KMT class encompasses 33 members: KMT1A-F, KMT2A-H, and KMT3A-G, KMT5A-C, KMT8A-F, and KMT-4, -6, and -7. Of note, most KMTs have alternative aliases, notably KMT1E referred as SETDB1, KMT2A-E as MLL1-5, KMT6 as DOT1, and KMT6 as EZH2. The LSD class only has two members: LSD1 (KDM1A) and LSD2 (KDM1B), while the JmjC KDM includes 19 members from KDM2A-B, 3A-C, 4A-D, 5A-D, 6A-B, 7A-C to KDM8²²⁶. Both methyltransferases (KMTs) and demethylases (KDMs) exhibit a high level of specificity for distinct lysine residues; thus, to cover the full

spectrum of methylation would exceed the scope of this review. Below we highlight the notable members of the KMT and KDM family known to be relevant in the context of antigen presentation.

It is now understood from studies performed over two decades ago that distinct methylation marks on histone H3 serve as signals to distinguish transcriptionally active chromatin from transcriptionally inactive regions²²⁷. Specifically, methylation of lysine 9 (Lys9) in the histone H3 tail is closely linked to transcriptionally repressive chromatin, while methylation of lysine 4 (Lys4) on H3 identifies the transcriptionally active chromatin bordering heterochromatin^{227–230}. High-resolution genome-wide mapping have linked H3K27me1, H3K9me1, and H3K36me3 to gene activation, whereas trimethylation of H3K27me3, H3K9me3 and H3K79me3 are associated with gene silencing²³¹.

Among the methyltransferases, H3K9 methylation is catalyzed by KMT1A/B/C and E, with KMT1E (SETDB1) catalyzing H3K9me2/me3 marks, KMT1A/B specific for H3K9me1 to H3K9me3 methylation, and KMT1C/D catalyzing H3K9me1 and H3K9me2²²⁶. On the other hand, H3K4 methylation is accomplished by the Set1-containing complex (SET1A/B and MLL1-4), later named COMPASS, with H3K4me3 associated with active transcription. On the other hand, H3K27me3 repressive marks are catalyzed by EZH2 (KMT6) subunit of the Polycomb-repressive complexes 2 (PRC2), which establishes repressive chromatin and transcriptional silencing. In contrast, the demethylases KDM1A/B carry methyl-group removal of H3K4me1 and me2 to repress active transcription. KDM2A/B catalyzes methylation of H3K36, KDM3A-C on H3K9, and 3K27me1/me2/me3 are removed by KDM6A/B and KDM7A/N²²⁶. Together, the tight balance between KMTs and KDMs maintain cell fate and genomic stability.

In the context of lung cancer, DNA methylation and histone methylation in promoter regions coordinately control gene expression. These marks together silence global gene expression and significantly contribute to tumorigenesis and tumor evolution, including differentiation to a neuroendocrine subtype in SCLC. In genes that are central to initiation and

maintenance of neuroendocrine lineage (*NEUROD1*, *NEUROG2*, *OLIG2*, *ASCL1*, *NKX* genes) and subsequent survival of SCLC cells, promoter hyper-accessibility has been observed. Interestingly, methylation modifiers, including MLL (*KMT2A*), MLL2 (*KMT2D*), KDM5A, and KDM6A are often mutated in SCLC, with MLL2 mutated in ~8% of SCLC, and KDM6A at 3-4%^{232,233}. In SCLC, KMT2C (*MLL3*) is preferably mutated in metastatic SCLC and has been shown to directly bind to regions of DNMT3A to activate its expression²³⁴.

Our group and others have shown that the histone demethylase LSD1, or KDM1A, is over-expressed in SCLC compared to NSCLC and normal lung tissues²³⁵. Mutations in KDM1A gene is rarely observed in SCLC²³⁵. However, LSD1 activity is dependent on INSM1 and GF1B interaction, and inhibition of LSD1 leads to downregulation of *ASCL1* and subsequent activation of NOTCH1 signaling in SCLC²³⁵⁻²³⁷. NOTCH signaling is deemed as the central player in neuroendocrine differentiation^{236,238-241} thus linking histone modification with neuroendocrine lineage differentiation. In this aspect, the efficacy of LSD1 inhibitors have been shown to correlate with an epigenetic signature of DNA hypomethylation²⁴².

EZH2, the enzymatic subunit of the polycomb repressive complex (PRC), is highly overexpressed in SCLC, depositing H3K27 trimethylation to promoters that are often coupled with DNA hypermethylation²¹⁸. EZH2 expression is regulated by E2F, the elongation factor and member of the pRB-E2F pathway essential for SCLC formation²⁴³. Loss of function or copy number loss of RB1, the repressor of E2F, is nearly universally observed in SCLC tumors, which might contribute to hyperactivation of E2F and subsequently upregulation of EZH2 in SCLC. Hyperactivity of EZH2 can further suppress TGF-beta-SMAD pathway that contributes to *ASCL1* overexpression and subsequently SCLC tumorigenesis²⁴⁴. Substantial efforts in generating preclinical models for SCLC have demonstrated a mechanism of acquired chemoresistance, a factor contributing to its exceptionally high lethality, is attributed to EZH2 activity. Chemoresistance is associated with suppression of *SLFN11*, a DNA-damage response gene, via promoter H3K27me3 deposition by EZH2. Together, these studies underscore the

central role of histone methylation in orchestrating the initiation and progression of SCLC tumors.

Histone acetylation

Modifications in histone acetylation include histone acetyltransferases (HATs) and histone deacetylases (HDACs), adding or removing acetyl groups to the lysine side chain of histone proteins, respectively. Histone acetylated marks deposited by HATs are associated with “open” chromatin, while the activity of HDAC restores lysine chain positive charges to maintain nucleosomes in a compacted and transcriptionally “silent” state. HDACs target both histones and non-histone proteins, where acetylation is interpreted by a class of “readers” called the bromodomain and extra-terminal domain (BET) family, which have been shown to be involved in transcription regulation and DNA damage response.

HDACs are commonly overexpressed in NSCLC and have been implicated in malignant transformation. These enzymatic “erasers” transcriptionally repress cell-cycle checkpoints, the inhibition of which induces apoptosis and autophagy in cancer cells. In the past, nonselective HDAC inhibitors as monotherapy were poorly tolerated in patients with NSCLC, and the combination approach with erlotinib did not show any improvement in progression-free survival²⁴⁵. However, combination of DNMT and HDAC dual inhibition significantly improved objective and durable responses in refractory NSCLC²¹⁵. This synergy has led to other clinical studies testing dual epigenetic therapy in combination with ICB in SCLC^{246,247}.

CREBBP, a HAT, is one of the most commonly recurrent gene mutations in human SCLC^{233,248,249}. In a Rb1/Trp53/Crebbp-deficient mouse model, *Crebbp* loss modifies the global acetylation of H3K27, activates epithelial-to-mesenchymal (EMT) transition and accelerates SCLC tumor progression. Even though clinical study of HDAC inhibitor in recurrent SCLC did not yield success a decade ago, recent study has revealed *CREBBP* inactivation significantly associated with enhanced sensitivity to HDAC inhibitor, opening up a new therapeutic avenue

for *CREBBP*-mutant SCLC²⁵⁰. Furthermore, KAT6B, another HAT that can acetylate H3K23, is also commonly inactivated in SCLC and implicated as a tumor suppressor²⁵¹.

c. Chromatin remodelers

The fundamental constituent unit of chromatin is the nucleosome, which exists either in condensed state impeding gene activation, or an open state poised for active transcription.

The most prominent member of the family of chromatin remodelers is the switching defective/sucrose nonfermenting (SWI/SNF) family, also called the BRG1-associated factor. SWI/SNF exists as a heterogenous multimeric configuration including two mutually exclusive catalytic subunits SMARCA2 (Brahma or BRM) or SMARCA4 (BRM/SWI2-related gene 1, or BRG1) and multiple conserved core proteins (SMARCB1, SMARCC1/2).

Mutations in components of the SWI/SNF chromatin remodeling complex are frequently observed in both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)^{196,252}. *SMARCA4* mutations are among the most common recurrent alterations in NSCLC^{253,254} and are generally considered a tumor suppressor whose loss of function associates with poor prognosis^{255,256}. Loss of BRG1 induces alterations in nucleosome positioning and chromatin reorganization, resulting in inactivation of tumor suppressor genes. Initial studies demonstrate that *SMARCA4* mutations contribute to enhance sensitivity to immunotherapy²⁵⁷. In SCLC, pathogenic mutations in *SMARCA4* are enriched in SCLC tumors lacking ASCL1, NEUROD1, and POU2F3. Recent studies have demonstrated that chromatin remodelers often cooperate with other histone and DNA methylation enzymes to modulate chromatin conformation. Truncating mutations in the bromodomain-containing subunit of SWI/SNF (PBRM1/BAF180) has also been reported in SCLC²³². PBRM1 loss increases expression of interferon-stimulated genes (ISGs) and subjects tumor cells to enhanced T cell-mediated cytotoxicity, inferring a role of PBRM1 in MHC-I antigen presentation²⁵⁸. Recent studies have confirmed that chromatin remodelers often exert their functions as a multimeric complex, an example of which is the NFIB transcription factor^{259–261}. NFIB is upregulated in 5-15% of SCLC^{260,261} and interacts with many

histone-modifying partners, including HDAC3 and EZH2, thereby regulating histone acetylation patterns and H3K27 trimethylation²⁶².

Collectively, these studies have provided insights into the intricately complex dynamics of epigenetic remodeling, influencing not only tumorigenicity but also the sustained orchestration of cellular identity and phenotypic plasticity. This complexity of epigenetic regulation is not imparted solely through the extensive array of epigenetic marks but also is intricately shaped by the direct interactions among various complexes.

These observations gain significance especially in the context of tumor cell intrinsic immunogenicity. As illustrated later (in Chapter 6), we have uncovered a mechanism wherein the transformation of a tumor cell to a phenotype conducive to immune surveillance and immunotherapeutic sensitivity is associated with a subversion of its tumorigenic and lineage-defining identity, and with widespread alterations in global gene expression.

4.1.2 Direct transcriptional activation of HLA genes through promoter bivalency

Promoter bivalency was first described in embryonic stem cells as a highly conserved regulatory property of pluripotency during development²⁶³. The primary characteristic of these chromatin regions is the co-localization of transcriptionally activating H3K4me3 and repressive H3K27me3 marks, establishing a structural “poised” state allowing for precise tissue-specific gene expression during differentiation^{264,265}. In hematopoietic stem cells, ~3000 bivalent promoters have been found to mark transcriptionally “ready” genes²⁶⁴, many of which were found bivalent not only in stem cells but also in differentiated cells of adult tissues and human tumors^{266–269}.

Methylation of H3K27 enriched in silenced genes is catalyzed by the PRC2 complex and recognized as a reversible mechanism of transcriptional repression^{231,270,271}. This evolutionary conserved PRC2 complex consists of primarily four subunits EZH1/EZH2, SUZ12, EED and RbAp46/48, with only EZH1/2 exhibiting the enzymatic activity for H3K27 di- and tri-methylation. Interestingly, in the absence of PRC2, H3K27 are predisposed to acetylation, and H3K27ac has

been found to co-localize with H3K4me3 (review below) marking active promoters. On the other hand, histone methylation of H3K4 in mammalian cells is exerted by the methylase activity of the SET1A/B and MLL1-4 (KMT2A, -B, -C, -D) of the COMPASS-like complexes. While SET1A/B exerts global histone H3K4me3 activity, MLL2 (KMT2B) is mainly responsible for trimethylated H3K4 presented at bivalent regions, and MLL1 (KMT2A) seems to be redundant despite being expressed in ESCs. Other members of the MLL family, including MLL3 (KMT2C) and MLL4 (KMT2D), mostly regulate H3K4-mono-methylated marks at enhancer regions.

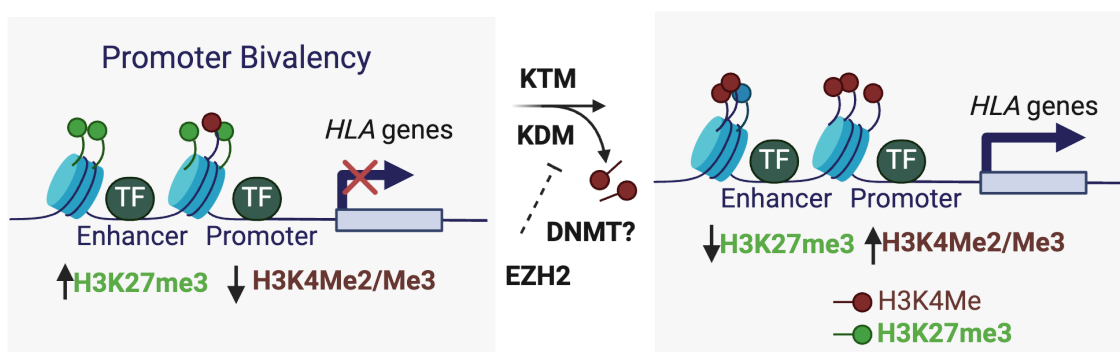


Figure 4.1. Transcriptional activation of HLA genes via epigenetic regulation at the promoter region of *HLA* genes. H3K27me3 marks are regulated by EZH2 complex and H3K4me2 marks can be regulated by KDM1A and other histone methyltransferases and demethylases. HLA genes can be transcriptionally activated via downregulating H3K27me3 and upregulating H3K4me/me2 histone marks.

Histone H3K4me3 is typically associated with actively transcribed genes, and histone H3K27me3 marks gene repression. The balance of H3K4me3 and H3K27me3 marks enable for rapid activation or silencing of specific genes associated with identity maintenance, stem-like plasticity and lineage differentiation. Thus, the discovery that the promoters of *HLA* genes are bivalent in cancer cells is quite interesting, inferring a tumor-intrinsic function of *HLA* in tumor cell identity. In a genome wide CRISPR/Cas9 screen, Burr et al. identified the top negative regulators of MHC-I encoding for subunits of the PRC2 complex, including *EED* and *SUZ12*, with *EZH2* being an intermediate hit²⁷². Neuroblastoma and SCLC are two tumor types with the lowest expression of MHC-I APP genes, and in both cases, *EZH2* was found to be

overexpressed. Subsequent genetic perturbation and pharmacological inhibition of the PRC2 complex restored MHC-I expression, enhanced type III interferon signaling and rescued T cell recognition²⁷². Most importantly, in this study, MHC-I gene promoters were shown to be occupied by both H3K4me3 and H3K27me3 marks in phenotypically similar neuroblastoma, SCLC and embryonic neural crest cells, suggesting a conservation of MHC-I repression across cell types of neuroendocrine origin. To date, bivalent promoters have not been observed on HLA genes in non-neuroendocrine tumor types, inferring a potential lineage-specific role of bivalency in maintaining neuroendocrine lineage via repressing MHC-I expression, the mechanism of which is partially mediated by H3K27me3-dependent transcriptional silencing.

In some human cancers, bivalent loci are not occupied by just histone modifications but also display *de novo* DNA methylation associated with transcriptional silencing^{273,274}. Unlike the reversible state of bivalency, DNA methylation is considered a stable silencing pathway. Thus, bivalency was thought to serve as an epigenetic “fingerprint,” foreshadowing regions of stable gene silencing by DNA hypermethylation resulting in lineage commitment²⁷⁵. It remains unknown whether DNA hypermethylated marks physically co-locate with histone H3K27me3 on bivalent promoters of HLA genes and whether DNMT cooperates with EZH2 to mediate HLA transcriptional silencing. The mechanism of action and timing of DNMTs and the PRC2 complex in the context of promoter bivalency remains to be understood. However, it has been long recognized that inhibition of DNMTs can rescue MHC-I antigen processing and presentation in cancer cells, albeit through completely different mechanisms, as discussed below.

4.1.3 Transcriptional activation of putative immunogenic neoantigens

A persistent concept in understanding the MHC-I antigen presentation pathway involves the important rate-limiting step of peptide processing. As a result, epigenetic modification of tumor-associated neoantigen has recently emerged as another mechanism for which surface MHC-I can be restricted.

Cancer testis antigens (CTAs) are often expressed only in immune-privileged organs and otherwise kept repressed in normal tissues and human malignancies²⁷⁶. When aberrantly expressed, they subject somatic cells harboring these CTAs to both humoral and cellular immunity²⁷⁷. A subset of these CTAs, including MAGEA4, MAGEA9 and NYESO1 can be transcriptionally reactivated by DNMT inhibition, suggesting the central role of DNA promoter hypermethylation in their silencing^{278–281}. Even though DNA methylation is considered a primary activator of CTAs, other histone modifiers have emerged as cooperative agents with DNMT. For example, HDAC inhibitor can sufficiently induce CTA transcripts but the effect is enhanced in combination with DNMT inhibitor²⁸². Similarly, concurrent loss of KMT6, KDM1 and KDM5B enhanced DNMT-mediated activation of CTAs²⁸³. Pharmacologically induced global demethylation, with or without histone modulation, reactivates subsets of immunogenic cancer antigens, which can readily bind to MHC-I to be expressed on the cell surface.

Another emerging class of immunogenic neoantigens is derived from genome embedded viral transcripts from past pathogenic encounters. The observation that silent transposable elements can be transcriptional reactivated by DNMT inhibition was first discovered in 1985 with the discovery of 5-aza²⁸⁴. Transposable elements constitute over 40% of the human genome and consist of LINEs, SINEs and endogenous retroviruses (ERVs). This finding was corroborated by studies investigating the identities of 300 Aza-induced genes transcripts in breast, colorectal, ovarian and NSCLC cancers, which revealed induction of not only HLA-transcripts in over 40% of cell lines²⁸⁵ but also genes encoding cancer testis antigen (CTA) and human endogenous retrovirus (ERVs)^{286,287}. Furthermore, Aza-induced ERV transcripts have also been found in melanoma, endometrial cancers, and choriocarcinoma, representing a pan-cancer mechanism that is not tumor-type specific^{286,288}.

In the case of ERV transcripts, DNA methylation is not the primary mechanism of active repression. SETDB1, a histone methyltransferase (KMT) that deposits H3K9 di- and tri-methylation on both histone tails and methylated DNA to transcriptionally silence genes, was

first found to mediate ERV suppression in AML cells²⁸⁹. Subsequent deletion of SETDB1 stimulated expression of ERV-derived antigens²⁹⁰. SETDB1 has been reported to act on broad domains in both melanoma and Lewis cell carcinoma but specifically enriched in transposable elements that encode MHC-I immunogenic antigens, which can be directly recognized by CD8+ T cells²⁹¹. Furthermore, the PRC2 complex was also recently shown to repress transposable elements in many solid tumors^{292–294}. A common outcome for de-repression of ERVs is the formation of cytosolic dsRNA; however, whether these EZH2-induced transcripts would encode for peptides bound on MHC-I remains understudied.

In a parallel study, SETDB1 was shown to cooperate with KDM5B to repress ERV transcription²⁹⁵. Unlike SETDB1's KMT role, KDM5B belongs to the class of Jumonji C domain (JmjC)-containing histone lysine demethylase (KDM) that can remove di- or trimethylation marks, together suggesting the cross functional roles of different histone modifying enzymes²⁹⁶. The other KDM class, LSD1, can also demethylate H3K4 and H3K9 mono and di-methylated marks to transcriptionally silence or activate specific genes. Loss of LSD1 was shown to not only directly transcribe ERVs but also demethylate non-histone proteins, enabling the accumulation of cytosolic dsRNA²⁹⁷; similarly, besides SETDB1, it is unknown whether specific ERVs re-expressed by histone demethylase inhibitors would bound to MHC-I on the cell surface.

To date, SETDB1-induced ERV represents the strongest evidence that targets of epigenetic modifiers encode for immunogenic neoantigens able to elicit robust T cell responses. More importantly, in many of these studies, the intensity of ERV transcript induction was many folds higher following epigenetic inhibition than in non-treated cells²⁸⁶. ERV transcripts can be hundreds to thousands of times more stable than ssRNA²⁸⁷. Thus, if some of these ERVs encode peptides bound on MHC-I, then one consequence of such reactivation is the surplus of neoantigen pools to potentiate a higher frequency of TAP transport and a much faster turnover of MHC-I surface export, leading to MHC-I resurfacing.

4.1.4 Systemic activation of cell-intrinsic innate immune signaling

Although ERVs can encode for neoantigen, the activation of these transcripts presents an immunological dilemma in the cell. Transcripts of these endogenous retroviruses elicit the formation of cytosolic dsRNA, ssRNA and ssDNA of multiple length, the presence of which in the cytosol triggers a cascade of viral sensing machinery resembling that against virus-derived nucleic acids^{286,287}. Nucleic acid sensors and adaptors have co-evolved with other innate immune signaling pathway as a conserved mechanism of pattern recognition. Depending on the length and nature of these ERVs, they can be recognized by endosomal Toll-like receptor 3/7 (TLR3/7) (dsRNA), the adaptor for which is MyD88. RIG-I recognizes short cytosolic dsRNA, while MDA-5 recognizes long cytosolic dsDNA, both of which activate MAVS upon nucleic acid recognition. Meanwhile, ssRNA can also be reverse transcribed to dsDNA that activates the cGAS/STING/TBK1 pathway²²⁰. One shared outcome of these diverse choices of pattern recognition is the activation of IRF3/7/NK-kB that elicits an intracellular type I and type III interferon via STAT1/IRF7-dependent positive feedback. In turn, interferon signaling activation upregulates expressions of most members of the MHC class I machinery, transcriptionally activate MHC-I and PD-L1 leading to their expression on the tumor cell surface. In this pathway, MHC-I resurfacing is a byproduct of interferon signaling, which was activated in the aftermath of cytosolic nucleic sensing.

It was first described in the late 1990s that low-dose of the DNMT1 inhibitor (DNMTi) DAC can upregulate IFN-stimulated genes (ISG)^{298,299}. In the following years, interferon signaling linked to DNMT inhibition was described in many pre-clinical cancer models as a mechanism for HLA-I re-expression^{285–287,300–302}. Akin to DNMT, EZH2 silences ERVs primed for interferon activation in small cell lung cancer²⁹⁴ and prostate cancer²⁹³. Besides DNMT and EZH2, epigenetic targets that utilize the same mechanism include HDAC^{303–307}, SETDB1^{289,291,295}, KMT1A/SUV39H1^{308,309}, KDM5B²⁹⁵ and KDM1A/LSD1^{297,310,311}. To date, this

“viral mimicry” mechanism represents the most well-documented mechanism of epigenetic interference in the MHC-I antigen presentation pathway^{312–314}.

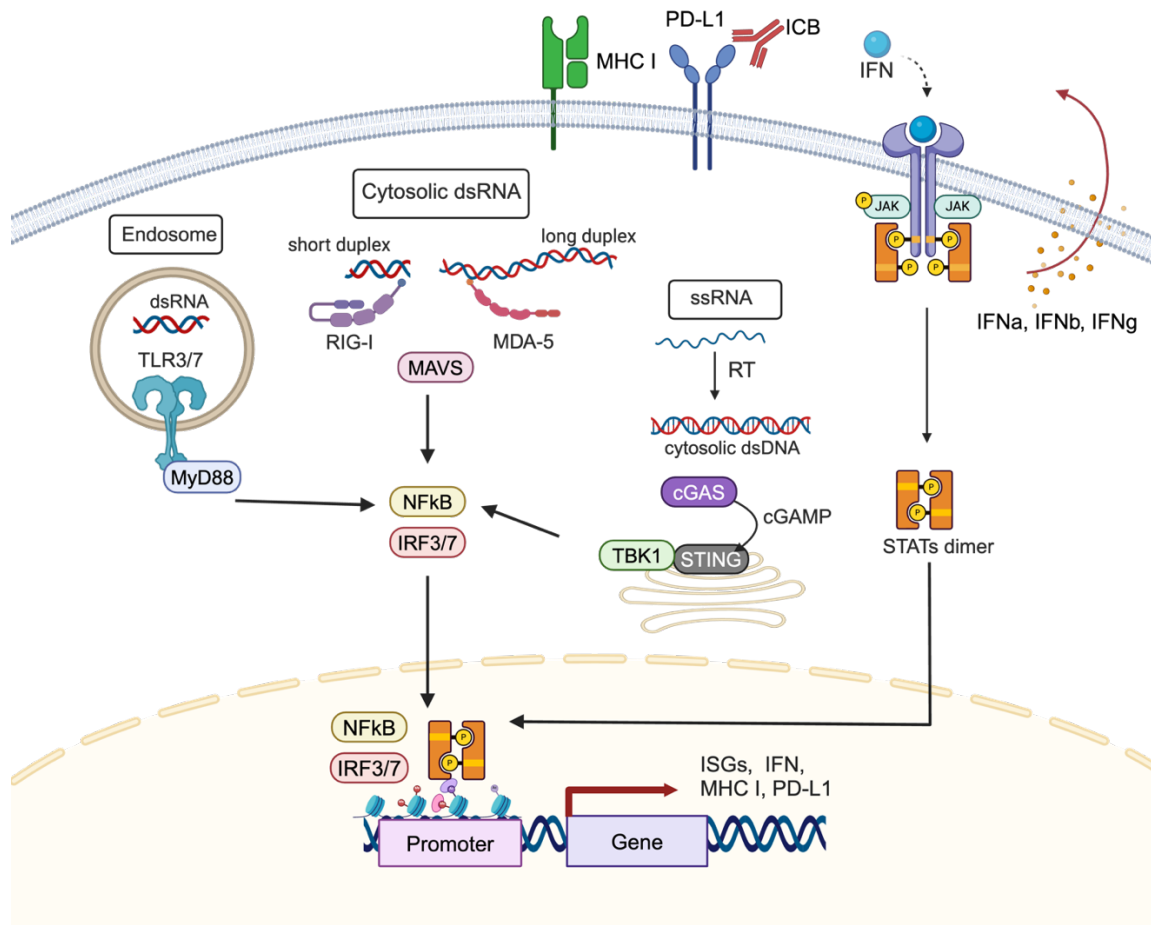


Figure 4.2. Induction of intrinsic innate signaling via cytosolic and endosomal nucleic sensors. Cytosolic nucleic acids can consist of dsRNA or ssRNA. Cytosolic dsRNA can be recognized by RIG-I or MDA-5, and ssRNA can be reverse-transcribed to form dsDNA which can activate the CGAS-STING pathway. Endosomal dsRNA is recognized by Toll-like receptors 3/7, which together with MAVS activation, signals NFκB activation and downstream signaling, leading to expression of interferon-stimulated genes.

Evidently, phenotypic synergy occurs when combining multiple classes of epigenetic targets. The Baylin group was the first to discover treatment by trichostatin A (TSA), a specific inhibitor of HDAC, that can synergistically cooperate with DAC, leading to transcription reactivation of tumor suppressors in NSCLC^{315,316}. Subsequently, these studies are corroborated by other findings that DNMT and HDAC co-inhibitions further upregulates

interferon type I transcriptional program, accompanied by upregulation of dsRNA and antigen presentation machinery^{246,305,306,317}. Similarly, DNMTi immune-sensitizing effect can be enhanced by combination with KDM5 inhibitor³¹⁸. One common denominator of these studies is a single shared requirement for STAT1-mediated interferon signaling. These findings seemingly set apart the mechanism reported Burr et. al., described in section 4.1.1 of this chapter, where inhibition of the PRC2 complex can activate MHC-I transcripts independently of STAT1 activation. Interestingly, in the same study, MHC-I re-expression was still mediated by the master transactivator NLRC5, which is IFN- γ -inducible, suggesting that the diverse mechanisms of MHC-I resurfacing are not mutually exclusive but evidently cooperative.

In conclusion, current studies attempting to dissect the mechanism of epigenetic rescue of MHC-I antigen presentation have failed to establish a step-wise process involving specific epigenetic changes. These pathways do not appear to be sequential of each other nor mutually exclusive. Transcripts of HLA genes, its neoantigens and ERV transcripts, genes encoding nucleic acid sensors, as well as systemic interferon signaling/activation have been found to be co-upregulated in the same cells following inhibition of specific epigenetic targets. A potential explanation of this observation could be distinct techniques: the phenotypic characterization of cells harboring such positive phenotype requires cell collection for bulk RNA-sequencing performed many days or weeks after the first treatment. Our groups and others have shown that short-term studies could not recapture the phenotype, and as a result, latency might lead to a missing window where the consequential steps could have been observed.

Another more likely rationale for this systemic orchestration of immunogenicity is the wide spectrum of effects elicited by epigenetic interference that are often not targeted to specific genes but instead occur on a global genome scale. To support this hypothesis, the MHC-I region on chromosome 6, as discussed in Section 1.1., is a highly gene-dense region occupied by many non-HLA genes, including genes encoding interferon-stimulated targets. Indeed, nearly half of the genes in the MHC-I region are embedded by transposable elements, which might

explain why many ERVs transcripts are co-expressed alongside with HLA transcriptional activation. The close physical proximity could serve as an evolutionary conserved mechanism for swift presentation of viral products on the cell surface to present to members of adaptive immunity.

4.2. Non-epigenetic mechanisms for tumor MHC-I induction

In recent years, alternative mechanisms of antigen presentation regulation have emerged in pre-clinical studies. Compared to studies on epigenetic perturbation, the findings described here are quite limited. However, one hypothesis that has gained the most traction is the established role of autophagy, a pathway that is now recognized as therapeutically targetable. The mechanism of autophagy regulation of MHC-I presentation has been discussed in Section 2.3.4, thus hereby we only focus on pharmacological progresses. One of the main sources of autophagy derives from defective ribosomal products (DRiPs), which also gives rise to many peptides presented by MHC-I. Thus, these two processes compete for the same substrates, limiting the rate of peptides loaded onto TAP transporter. Studies from over a decade ago have demonstrated that inhibition of autophagy by 3-MA, a PI3K inhibitor, led to enhancement of proteasomal degradation and induction of MHC-I restricted presentation¹⁷⁰. Of note, this mechanism echoes the problem with epigenetic repression of neoantigen in which the substrates of MHC-I molecules have to be restored. However, autophagy restriction controls post-transcriptional generation of peptides, whereas epigenetic targets modulate their transcription. Indeed, a recent study has implicated an epigenetic function in regulating autophagy via m6A methylation of ULK1, ATG5 and ATG7 mRNA, leading to its degradation^{319,320}. In another study on *LKB1*-mutant lung cancer, ULK1 inhibition was found to activate immunoproteasome activity, subsequently restoring antigen presentation³²¹. This tumor type exhibits features of high tumor mutational burden yet poor capacity for antigen presentation, with MHC-I transcription appearing intact, suggesting that antigen processing, not antigen presentation, is the bottleneck to unlock MHC-I peptide loading.

Lastly, surface peptide:MHC-I molecules are also the target for lysosomal degradation as a step in the life cycle of MHC-I expression (discussed in section 2.3.1). In pancreatic tumor cells with reduced surface MHC-I, peptide:MHC-I complexes were found predominantly in the autophagosome and lysosomal compartment¹⁷⁷. Inhibition of autophagy by chloroquine restores surface levels of MHC-I and leads to improved antigen presentation and sensitivity to immune checkpoint blockade¹⁷⁷. Interestingly, this pathway has been previously observed as a mechanism for constitutive expression of MHC-I on APCs¹³⁴. Similarly, turnover of antigen-loaded MHC-I complex is mediated by subunits of the early endosome (i.e. MAL2), which rapidly recycles surface MHC-I, leading to immune evasion¹⁷⁵. As a validation, genes encoding endosomal trafficking have been discovered in a genome wide screen identifying lineage-specific genes modulating MHC-I immunosurveillance³²². Together, these observations underscore the need for a pan-cancer discovery effort for therapeutic targets that can efficiently restrain autophagy and promote MHC-I mediated antitumor immunity.

CHAPTER 5. NEW INSIGHTS INTO SMALL CELL LUNG CANCER IMMUNITY

5.1. Small cell lung cancer overview

Small cell lung cancer is a high-grade carcinoma of neuroendocrine origin, representing about ~15% of all lung cancer cases³²³. Two-thirds of SCLC cases harbor distant metastases at diagnosis, reflecting the highest concentration of circulating tumor cells (CTCs) among all solid tumors³²⁴. In the absence of oncogenic driver mutations, efforts to develop targeted therapies have not yielded success; as a result, there is currently no FDA-approved targeted therapy for SCLC. Until 2018, platinum- etoposide (EP) doublet chemotherapy was the standard of care for patients with metastatic SCLC; since then, the standard of care has shifted to doublet chemotherapy with the addition of PD-(L)1 directed immunotherapy. SCLC patients respond exceptionally well to chemotherapy or chemoimmunotherapy initially, with response rate over 60% even in patients with advanced metastatic diseases³²³. However, most patients experience relapse within a year, resulting in a median survival of only slightly over one year for patients with metastatic disease at the time of diagnosis. The addition of immunotherapy to the standard of care has changed the disease trajectory for a minority of patients – there is a tripling of 3-year survival from 5.6% to 17% with some patients achieving durable response – but most patients continue to die of disease. Due to the prevalence of early widespread metastasis, limited therapeutic options, and an exceptionally high mortality rate, SCLC remains an NCI-designated “recalcitrant” cancer³²⁵.

SCLC is strongly associated with tobacco exposure: 95-98% of SCLC patients are current or former smokers³²⁶. A strong smoking signature is present in its genetic landscape, with concurrent loss of function mutations or deletions in *TP53* and *RB1* tumor suppressor genes in nearly all cases²³³. Development of tumors phenocopying clinicohistopathology features of SCLC in genetically engineered mouse models (GEMM) requires inactivation of both tumor suppressor genes^{327–332}. Every mouse model of SCLC to date harbors this concomitant loss. Both p53 and RB inactivation appear to play a role in regulating the distinct

neuroendocrine differentiation and lineage plasticity of SCLC, as similarly observed in *TP53*- and *RB-1*-deficient neuroendocrine prostate tumors^{333,334}. Other tumor suppressor genes have also been found to be commonly mutated in SCLC via genomic profiling and subsequent validation in mouse models, including *CREBBP*²⁵⁰, *KAT6B*²⁵¹, *PTEN*^{335,336}, *CDKN2A*³³⁷, *KDM6A*³³⁸ and *NOTCH*^{233,240,339}. Interestingly, virtually all every gene in this list either encodes an epigenetic modifier or are products of direct epigenetic silencing, most commonly promoter hypermethylation and DNA methylation (discussed in Section 4.1).

The stratification of NE expression in SCLC is intertwined with relative expression of four specific transcription factors: achaete-scute homolog 1 (*ASCL1*), neurogenic differentiating factor 1 (*NEUROD1*), and POU class 2 homeobox 3 (*POU2F3*), and yes-associated protein 1 (*YAP1*) defining four consensus-established molecular subtypes SCLC-A, SCLC-N, SCLC-P and SCLC-Y, respectively²⁴⁸. SCLC-A subtype represents the majority of SCLC cases, with high expression of NE markers (i.e. chromogranin A, synaptophysin). SCLC-N was previously called “variant” neuroendocrine subtype and is associated with high *MYC*. SCLC-P and SCLC-Y are considered non-neuroendocrine, with *YAP1* being a rare subtype and in many cases grouped together as *ASCL1/NEUROD1/POU2F3*-negative rather than a distinct subtype^{340,341}. *ASCL1* has been implicated as a required transcription factor for tumor formation in murine models^{332,342}. Of note, these subtypes are not considered mutually exclusive and do not encompass every subtype identified in patient samples, as other transcription factor (i.e. *ATOH1*) have also been proposed to define distinct cell states in SCLC³⁴³. Histopathological and single-cell transcriptomic studies in clinical samples, patient-derived xenografts, and GEMM have revealed the co-existence of multiple subtypes within a tumor and evidence of subtype conversion³⁴⁴, reflecting high degree of intratumoral heterogeneity and plasticity of this tumor type^{338,343,345,346}. Preliminary understanding of subtype plasticity has pointed to the members of the *MYC* oncogene family, frequently amplified in SCLC, as well as the H3K27 histone

demethylase KDM6A, as key determinants of SCLC subtype transitions, including differentiating ASCL1-high and NEUROD-1 high SCLC^{332,338}.

Initial transcriptomic analyses and subsequent single-cell profiling of clinical samples have demonstrated distinct degrees of NE differentiation in these four subtypes, with SCLC-A and SCLC-N classified as “NE-high” tumors, and SCLC-P and SCLC-Y considered “NE-low” subtypes. The binary classification as “NE-high” and “NE-low” tumors, defined based on expression of pre-established NE gene set signatures, might also provide a more robust distinction^{248,347,348}. Capacity for lineage differentiation, inherent in its neuroendocrine (NE) histology, has been implicated as a mechanism for acquired chemotherapy resistance^{348–351} and has also been associated with its immunosuppressive phenotype^{348,351}. The presence of an “inflamed” subtype has been defined – the “inflamed” profile overlaps with low NE signatures, and is characterized by relatively high expression of antigen presentation machinery, high expression of immune-related genes, including costimulatory and checkpoint ligands, interferon signaling and lineage receptors of T cells lymphocytes^{341,348,352}. This low-NE, “inflamed” signature has been associated with clinical benefit from immune checkpoint blockade, as further discussed in the next section. Interestingly, Notch pathway has been found to inhibit neuroendocrine differentiation in lung development^{238,353} and in SCLC tumors,^{236,241} and its activation is associated with ICB response³⁵¹; evidently, restoration of Notch signaling can sufficiently drive non-NE features in tumors³³⁹. Interestingly, the differentiation from SCLC-A to SCLC-N to non-NE phenotype is driven by MYC-regulated Notch signaling³⁴⁴. In chapter 6, we demonstrate the plasticity potential of NE-driven immunogenicity is governed by an epigenetic mechanism, where the conversion from NE-high to NE-low phenotype is coupled with induction of tumor-intrinsic immunogenicity that governs treatment sensitivity to anti-PD-L1 blockade.

In the following sections, we discuss newly identified correlates of SCLC response to immunotherapy from recently published retrospective studies and review our current understanding of SCLC immunogenic capacity, including its intrinsic ability to engage with

cytotoxic lymphocytes. We also revisit results from past histopathological, single cell transcriptomic, and spatial profiling studies to phenotype the tumor immune microenvironment of SCLC. Lastly, we report findings from our own comprehensive multi-omic characterization of nearly 300 SCLC patients treated at MSKCC during the period of 2013-2021, that provides new insights into SCLC immunity.

5.2. Current understanding of determinants of SCLC sensitivity to ICB

The emergence of immune checkpoint blockade has significantly improved prognosis for many solid tumors, including NSCLC. Despite a high tumor mutational burden^{354,355} that is associated with high neoantigen frequency (further discussed in Section 5.9), results from clinical studies on ICB in SCLC have been disappointing for most patients. Overall, these data demonstrate durable clinical responses to ICB in a minority of patients, 10 to 15%, and a lack of evident benefit in the majority. As monotherapy options, nivolumab was initially granted accelerated approval (contingent on subsequent success in phase 3 confirmatory trials) for third-line treatment of patients with SCLC in 2018 based on results from the phase 1/2 CheckMate 032 study but was subsequently withdrawn in 2020 following disappointing data from the phase 3 CheckMate 331 (second line)³⁵⁶ and CheckMate 451 (maintenance following 1L)³⁵⁷ studies. Similarly, another PD-1 inhibitor pembrolizumab received accelerated approval in 2019 for relapsed 2L+ ES-SCLC, after KEYNOTE-028 and KEYNOTE-158 demonstrated apparent efficacy³⁵⁸; however, as a survival advantage was not seen in the overall population in the phase 3 KEYNOTE-604 study³⁵⁹ pembrolizumab approval was similarly withdrawn. For chemoimmunotherapy, ipilimumab in combination with EP failed to prolong overall survival for extensive-stage SCLC patients^{360,361}. An exception to these disappointing results was the introduction of anti-PD-L1 atezolizumab to 1L EP regime for patients with ES-SCLC in the Impower133 study that significantly improved overall survival – although the median was improved only 2 months, a significant separation in the tail of the curve improved long-term survival in a minority of patients³⁶². Another anti-PD-L1 blocking antibody, durvalumab, was

investigated in the CASPIAN study in combination with EP and demonstrated a similar 2-month improvement in median survival, but a marked improvement in the fraction of patients with long term survival (5.6 vs. 17% at 3 years)³⁶³. In the large majority of patients with disease progression, there is currently no 2L+ immunotherapy options and no established clinical biomarker that can predict response^{355,364–366}. Together, these data demonstrate a critical need in the field to (1) understand the basic biology of SCLC immune microenvironment, (2) investigate determinants of SCLC sensitivity to immunotherapy, and (3) derive novel therapeutic options to overcome primary and acquired treatment resistance in this tumor.

Large retrospective meta-analyses investigating correlates of durable clinical response to immunotherapy have yielded interesting findings. First, expression of MHC-I molecules turned out to be the most significant and recurrent correlative stratifying durable response to ICB in SCLC. This finding was uncovered in CheckMate032³⁶⁷, the largest study of ICB alone in patients with SCLC, that investigates correlates of clinical benefit in 286 pretreated SCLC tumors. Such association of MHC-I IHC positivity and survival benefit have been confirmed in other smaller cohorts³⁵². SCLC is widely found to suppress MHC-I expression^{352,368,369}, despite displaying a high tumor mutational burden. MHC-I and CD8-I values are highly correlative, with tumor infiltrating CD8+ T cells also associated with improved prognosis^{366,367,370}. Unlike in many other solid tumors, PD-L1 expression is almost universally low and is not predictive of benefit in SCLC^{355,371}, whereas the role of tumor mutational burden (TMB) as a biomarker for immunotherapy in relapsed SCLC has not been confirmed in larger prospective datasets^{355,372}.

Prior investigations of the tumor immune microenvironment on SCLC have relied on traditional antibody-based detection techniques including immunohistochemistry (IHC) or immunofluorescence (IF). In recent years, advances in spatial profiling, with or without coupled single-cell transcriptomic technologies, have expanded the depth of our understanding of diverse cell type localization, cell-cell interactions, clonal heterogeneity, and the role of stromal components in tumor tissue. However, a comprehensive characterization of the SCLC immune

compartment has not been achieved. Single cell technologies, while excelling at characterizing intra-tumoral heterogeneity and subtype divergence^{345,350}, have yet to delineate the tumor immune microenvironment of SCLC due to limited tumor-infiltrating immune cells, small sample size, and methods of tissue extraction^{345,350,373,374}. Subsets of CD8+TILs demonstrated cytotoxic and exhaustion features, suggestive of antigenic encounter in the tumor. Despite some insights, scRNA-seq data sacrifices architectural information of the tumor tissues. Orchestration of antitumor immunity in other solid tumors has been attributed to not just cytolytic activities but also the stromal compartments, which have not yet been characterized in SCLC. Furthermore, there is currently no scRNA-seq study performed specifically on cohorts treated with immunotherapy. Together, these findings necessitate the significance of multi-modal approaches to understand the mechanisms of immune surveillance and evasion in SCLC.

5.3. Cohort overview

In the present study, we systemically profiled 263 patients with small cell lung cancer (SCLC) who were treated at Memorial Sloan Kettering Cancer Center between 2008-2019 (**Figure 5.1A; TableS1**). Only patients with “pure” or *de novo* SCLC, defined by WHO histopathological classification^{323,375}, were included. The majority of patients (168/263; 64%) were diagnosed at extensive stage (ES), and a small subset of limited-stage (LS) patients (20/95; 21%) eventually progressed to ES during follow-up (**Figure 5.1B; Supplementary Table 5.1**). Of these patients, 121 tumor samples derived from primary lesions, 105 metastatic regions were sampled at diagnosis and 37 others were retrieved from metastases at relapse or progression (**Figure 5.1C**).

As part of clinical care, 248 (94.2%) patients underwent Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT)³⁷⁶. Reflective of its biology, >90% and 80% of samples harbor mutations in *TP53* and *RB1*, respectively (**Supplementary Figure 5.1A**). Smoking signatures are prevalent in most samples, and *KMT2D*, *TERT*, *PTEN* and *NOTCH1* are frequently mutated in this cohort (**Supplementary**

Figure 1A-B)²³³. Subtype classification by IHC identified 57/103 (55%) samples of SCLC-A subtype, 25% of SCLC-A/N mixed subtype, 8% SCLC-A/N mixed subtype, and less than 3% of SCLC-P and SCLC-Y subtype (**Figure 5.1D; Supplementary Table 5.1**). 7 samples stained negative for all subtype markers. Subtype identification by immunohistochemistry highly correlates with bulk-RNA-seq data (**Supplementary Figure 1C-F**). Overall, our cohort captures a balanced spectrum of clinical features and subtype heterogeneity frequency typically observed in SCLC patient samples.

130 patients were treated with immunotherapy as part of first- or second-line therapy for ES-SCLC (**Figure 5.1A**). At the time of receiving immunotherapy, all patients were extensive stage. 105 patients (81%) received immune checkpoint blockade (ICB) as monotherapy and 25 patients received ICB in combination with chemotherapy (**Supplementary Table 5.2**). Following treatment, 67% of patients experienced either stable disease (SD) or progressive disease (PD), and complete response and partial response were recorded in 27% patients (2 and 32, respectively) (**Supplementary Table 5.2**). Durable clinical benefit (DCB) to immunotherapy was observed in 27 patients (20.7%), defined as progression-free survival (PFS) of 6 months or longer from the start of immunotherapy treatment till date of progression or last follow-up. Overall survival (OS) was calculated from start of treatment to death/last-follow-up, whichever occurs first. The presence of DCB was employed to delineate clinical covariates associated with immunotherapy sensitivity in this cohort.

Samples in this cohort underwent a combination of genomic, transcriptomic and protein expression analyses (**Figure 5.1E-F**). Paired whole exome sequencing was performed on 125 samples (**Figure 5.1E-F**). Transcriptomic analyses were conducted on 47 samples for bulk RNA-seq, and 8 samples from our previously published cohort³⁴⁵ were re-analyzed for single-cell RNA-seq (scRNA-seq) (**Figure 5.1E-F**). Whole-tissue immunohistochemistry (IHC) was performed on 103 samples (39%) for multiple biological markers including molecular subtypes (ASCL1, NEUROD1, POU2F3, YAP1), neuroendocrine features (INSM1), and immune activities

(CD3, CD20, MHC-I, and PD-L1) (**Figure 5.1A, E-F**). Together, we leveraged these pan-omic modals to interrogate the heterogeneous immunophenotypes of SCLC that drive their capacity for immune evasion and therapeutic sensitivity.

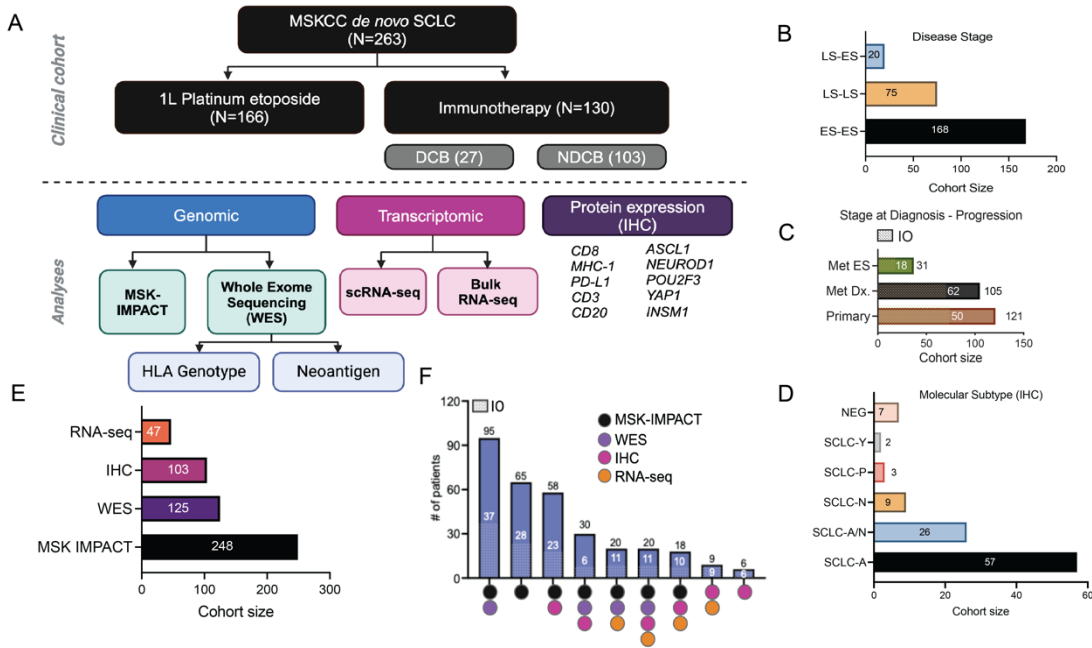


Figure 5.1. Cohort overview (A) Cohort overview with the respective analyses performed (B) Distribution of patient disease stage (C) Presence of primary vs. metastatic samples (D) Bar graph cohort molecular subtypes identified by IHC (E) Bar graph showing number of samples with respective analyses (F) Bar graph showing number of samples with shared analyses. Shaded area reflecting patients receiving immunotherapy.

5.4. Immune checkpoint blockade benefit is partially driven by CD8 effector presence

We first sought to characterize features of the tumor immune microenvironment associated with durable clinical response in our SCLC cohort. Candidates for biomarkers of immunotherapy sensitivity have emerged from several retrospective studies on relatively small cohorts (~20 patients)^{351,352} but were most affirmatively shown from results of CheckMate-032, the largest clinical study of ICB monotherapy in SCLC³⁶⁷. In this latter report, markers of high abundance of CD8 effector cells demonstrate significant favorable clinical outcome to PD-1 blockade monotherapy. Recent pre-clinical studies performed in immunotherapy-resistant SCLC tumors also demonstrated robust sensitization to anti-PD-L1 inhibitor following re-engagement of CD8 T cells²³⁵. To validate the significance of CD8 positivity, we performed whole-tissue profiling of 66 samples via IHC protein expression analysis and reported absence or low

presence of CD8 in nearly two thirds of samples (Density <100; n=43) (**Figure 5.2A**). In a subset with quantifiable positivity defined as normalized density ≤ 100 , univariate analysis demonstrated concurrent disease-free and overall survival benefit (HR = 0.81; 95% CI: 0.41-1.59, and HR = 0.65; 95% CI: 0.33-1.32, respectively) though p-values were not statistically significant (**Figure 5.2B-C**). As our sample size for CD8 analysis was comparable to that of the CheckMate-032, we postulated that the lack of statistical power might arise from the heterogeneity of this effector subset. We then sought to identify the relative localization of CD8 T cells relative to the tumor parenchyma. As expected, the majority of our SCLC samples are devoid of any presence of CD8+ T cells, reflecting an “immune-desert” phenotype³⁷⁷ (**Figure 5.2D; left panel**). Even in tumors with high CD8 positivity, effector T cells are mostly restricted to the invasive margin of the stroma without infiltration into the tumor parenchyma, suggestive of the classical “immune-excluded” phenotype (**Figure 5.2D, middle panel**). Previous studies have described tumors with an immune-excluded phenotype are more resistant to immunotherapy^{378,379}, hence some of our CD8-high samples do not confer benefit to ICB. In only one sample, we found dense clusters of T cells infiltrating into the tumor bed, reflecting the true “inflamed” phenotype (**Figure 5.2D, right panel**), yet the patient is not an IO responder. Our data demonstrate that the classical model of CD8 abundance is important but not a major driver of therapeutic vulnerability and that other features of immune microenvironment might influence divergent clinical outcomes.

5.5. Activated and memory CD4 T and B lymphocytes shape the tumor microenvironment of long-term responders

To delineate the comprehensive molecular features of regressed SCLC tumors, we deployed bulk RNA-seq analysis on 30 patient tumors, including 5 responders (DCB) and 25 non-responders (NDCB). Profiling for gene expression patterns revealed a distinct gene set signature comprised of 47 genes that significantly differentiates between DCB and NDCB

(Figure 5.2E). Interestingly, the top differentially expressed genes encode markers of homing, activation, differentiation, and maturation associated with B lymphocytes **(Figure 5.2G-H)**. B cell-associated pathways were also the most significant and highly enriched pathways in SCLC tumors of ICB responders **(Figure 5.2F)**. Expression of B cell homing *CXCL13*, mature B cells, *CD79A*, *CD79B*, germinal center *MZB1* and B cell differentiation *IRF4*, *IGLL5* are significantly enriched long-term responders **(Figure 5.2G-H)**. In addition, we identified CXCR3 receptor binding, the receptor of CXCL9, a key T cell-specific chemokine, to be significantly enriched in DCB. CXCL9 was found among the top 10 most differentially expressed genes in regressed tumor and has been demonstrated to necessitate effector functions associated with favorable response in other tumor types³⁸⁰ **(Figure 5.2F-G)**. CXCR3 is found in activated CD8+ T cells, enabling their differentiation into terminal effector phenotype^{381,382}. We indeed found markers of T cell activation and cytolytic activities, *CD38*, *GZMA* and *IFNG* as well as lymphocyte differentiation, to be elevated in sensitive tumors **(Figure 5.2I-J)**. These data imply that the tumor microenvironment of long-term responders is driven by not only CD8+ effector abundance but also by presence of other lymphocyte activities.

Memory T cells during antigen rechallenge secretes IFN- γ that upregulates CXCL9 on stromal cells to further recruit CXCR3+ memory CD8+ cells^{383,384}. To interrogate whether immune infiltrates of sensitive tumors exhibit activated and memory lymphocytic features, we employed two deconvolution methods to estimate fractions of specific immune compartments within the patient whole transcriptomes³⁸⁵. QuantIseq analysis revealed markedly higher level of total immune infiltrates in responding compared to resistant tumors **(Figure 5.2K)**. Significantly higher fractions of CD8+ T cells, CD4+ T cells and B cells differentiate DCB and NDCB tumors **(Figure 5.2L-N)**. To gain insights on these CD4 and B cell subsets, we employed a second deconvolution method, CIBERSORT, to identify 22 functionally distinct immune fractions³⁸⁶. Our data demonstrated markedly larger presence of plasma and memory B cells exhibiting differentiated and memory-like features to favor outcome **(Figure 5.2O)**. Similarly,

significantly higher fraction of CD4⁺ memory activated T cells was observed in responding tumors (**Figure 5.2P**). CD4 T regulatory trends to be remarkably higher in resistant tumors, while CD4 naïve and T follicular helper cells partially drive the higher presence of total CD4 T cells in DCB. None of the other immune cell types differ between DCB and NDCB (data not shown). These findings suggest that the major immune activities in SCLC responders are shaped by not just effector T cells but also via activated, differentiated and memory-like B and CD4 lymphocytes.

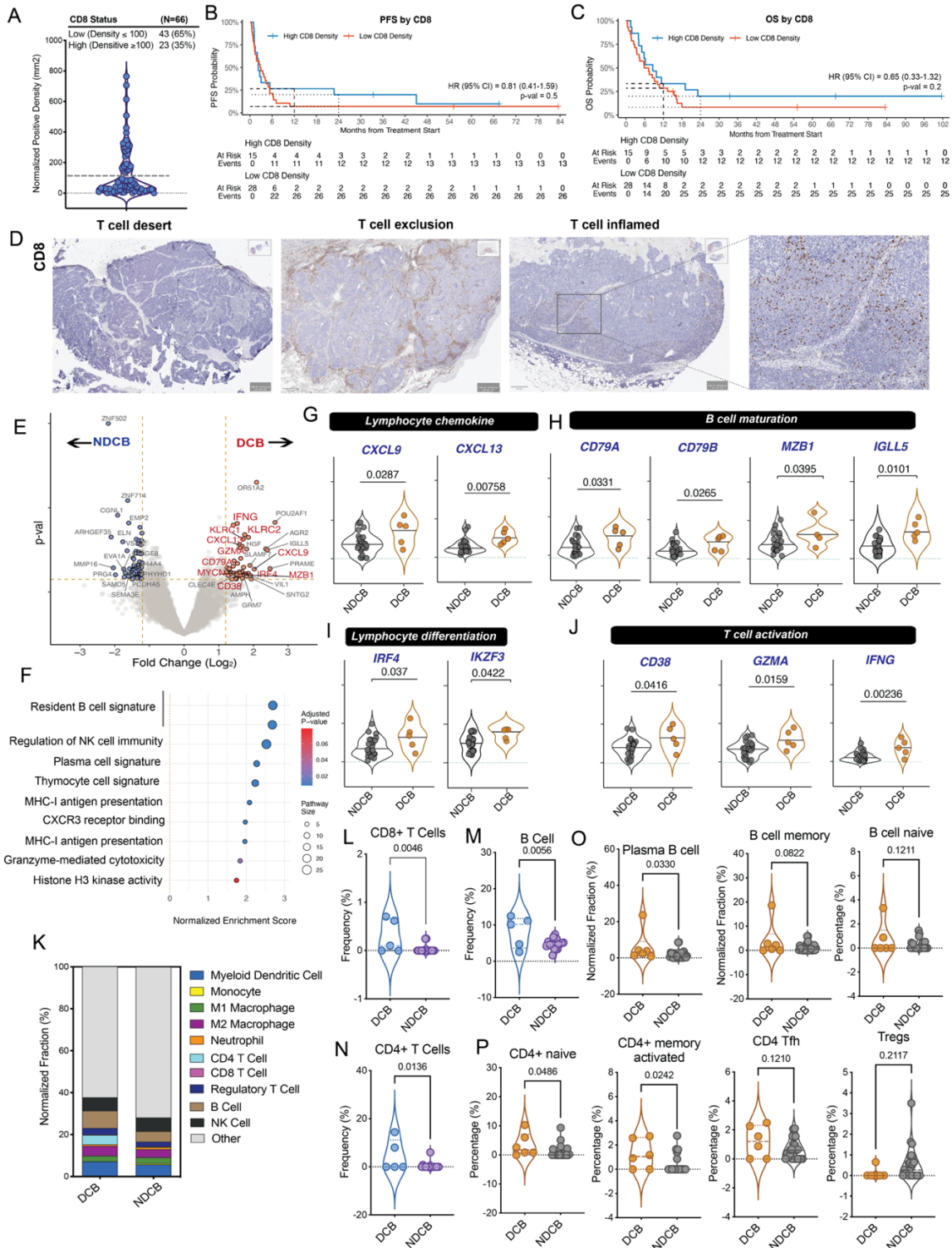


Figure 5.2. Molecular determinants of ICB durable clinical benefit in SCLC. (A) Distribution of CD8 status defined as normalized positive density by IHC (B) Kaplan Meier curve for PFS stratified by CD8-high or CD8-low (C) Kaplan Meier curve for OS stratified by CD8-high or CD8-low (D) IHC images of three patients with diverse T cell phenotype (E) GSEA for top pathways enriched in DCB (F) Top DEGs between DCB and NDCB (G) DEGs of lymphocytic chemokine (H) DEGs of B cell markers (I) DEGs encoding for lymphocytic differentiation (J) DEGs encoding for T cell

activation markers (K) Quantiseq analysis of normalized immune cell fraction. (L-N) Immune cell fractions between DCB and NDCB. Quantification of B cell subsets (O) and T cell subsets (P) by CIBERSORT.

5.6. scRNA-seq identified specific functional states of T subsets associated with immunotherapy response

Currently available single-cell transcriptomes of SCLC lack clinical correspondence with response to immunotherapy, and public scRNA-seq dataset of pan-cancer studies do not typically include SCLC as a lung cancer type. To capture the specific phenotypes of T cell subsets that inform favorable prognosis in SCLC patients, we utilized our previously reported comprehensive single-cell atlas of 21 SCLC freshly isolated tumors³⁴⁵. Eight 3' scRNA-seq transcriptomes totaling 6,389 CD45+ single cells were utilized to project 14 immune cell clusters on Uniform Manifold Approximation and Projection (UMAP) (**Figure 5.3A**). T cell population comprises the majority of tumor immune infiltrates (74%) (**Figure 5.3A**). This population is highly heterogeneous, consisting of at least 6 T cell clusters, with CD8+ T memory and CD4+ T cells seemingly exhibit the highest cell count, likely accounted by sample-related variance (**Figure 5.3B**). Using batch correction normalization, we observed minimal batch effects across samples (**Figure 5.3C**). Among T cell subsets, conventional CD4 T cells exhibit high level of *CD69*, *IL7R*, *CD44* as well as naïve markers *SELL*, *CCR7* and *TCF7*, whereas CD4 regulatory T cells is marked by expression of the canonical transcription factor *FOXP3* (**Figure 5.3D**). Among CD8 subsets, effector CD8 displays enriched *GZMB*, *CCL5*, *CST7*, *NKG7*, *GZMH*. Exhausted CD8 T cells are marked by expression of *GZMK*, *CST7*, *CD38* as well as co-stimulatory markers *ICOS*, *CTLA4*, *TIGIT*, *PDCD1*, *TOX*, *LAG3* and *HAVCR2* (**Figure 5.3D**). Memory T cell subset displays slightly lower cytotoxicity markers, including *GZMB*, *GZMK*, *CCL5*, *CST7*, *CD44*, *NKG7*, *GZMH* compared to other CD8 subsets (**Figure 5.3D**). Overall, expression profiles of our single-cell T cell clusters align with previous reports in other pan-cancer studies³⁸⁷.

To phenotype specific transcriptional states of these T cell clusters, we employed the pan-cancer T cell atlas of ~300,000 single-cell T cells isolated from 171 ICB patients spanning 16 cancer types, which capture the comprehensive transcriptional states of 24 CD8+ and 12+ tumor-reactive T cell signatures, including signatures of naïve, activation/effector, exhaustion, and cytotoxicity³⁸⁷. Once projected these diverse transcriptional states on CD4 and CD8 subsets, we reported that the majority of tumor CD8 infiltrates display strong activation phenotype, followed by cytotoxicity and exhaustion features (**Figure 5.3E**). In contrast, the majority of CD4 infiltrates are naïve and activated-like with minimal to no cytotoxic features (data not shown). Focusing on T cell subsets in responders, we next evaluated 3,219 single cells representing five patients who received ICB in our cohort. Normalized count fraction reflects heterogeneous T cell infiltrates across samples (**Figure 5.3F**). In DCB tumors, we reported significantly higher frequency of CD8+ effector cells, total CD4 T cells and markedly lower regulatory CD4 T cells, validating our previous observation with 30 bulk-RNA-seq patients (**Figure 5.3G**). Interestingly, higher density of CD8+ exhausted T cells were observed in long-term responders, perhaps reflecting the higher level of tumor reactivity associated with prolonged antigen challenge (**Figure 5.3G**). Sensitive tumors also display 7-fold higher T effector to regulatory T cell ratio compared to non-responders (**Figure 5.3H**). Of note, among other members of innate and adaptive immunity, B cells, NK cells and DCs were only present in scant number in the tumor infiltrate (DC = 30 cells, B cells = 33 cells, NK = 376 cells in IO cohort), limiting our ability to comprehensively phenotype their transcriptomic diversity.

We next evaluated 19 specific transcriptional states of CD8-expressing T cells as previously described. Effector population of responders exhibit significantly elevated degree of activation, cytotoxicity, cytokine activity, IFN gamma response, glycolysis and TCR signaling (data not shown), compared to resistant tumors (**Figure 5.3I**). Aligned with our normalized count data, exhaustion features are also elevated among CD8 residing within DCB tumors (**Figure**

5.3I). Segmentation for specific CD8 and CD4 T subsets confirmed that the exhausted CD8 T cells in sensitive tumors are more functionally intact than those derived from non-responders, displaying enhanced features of activation, cytotoxicity, TCR signaling, and cytokine and chemokine activities (**Figure 5.3K**). Within CD4 T cell subsets, we computed for enrichment of 14 distinct CD4 functional states. In agreement with our annotated cell count, functional features of CD4 Tregs, i.e. TCR signaling and cytokine activities, are markedly repressed compared to Tregs from non-responders (**Figure 5.3J-K**). By phenotyping specific T cell states associated with immunotherapy sensitivity, our data indicate that a favorable tumor microenvironment of SCLC comprises of highly heterogeneous T cell subsets with not only higher cell density but also with differentially activated and functional transcriptional states.

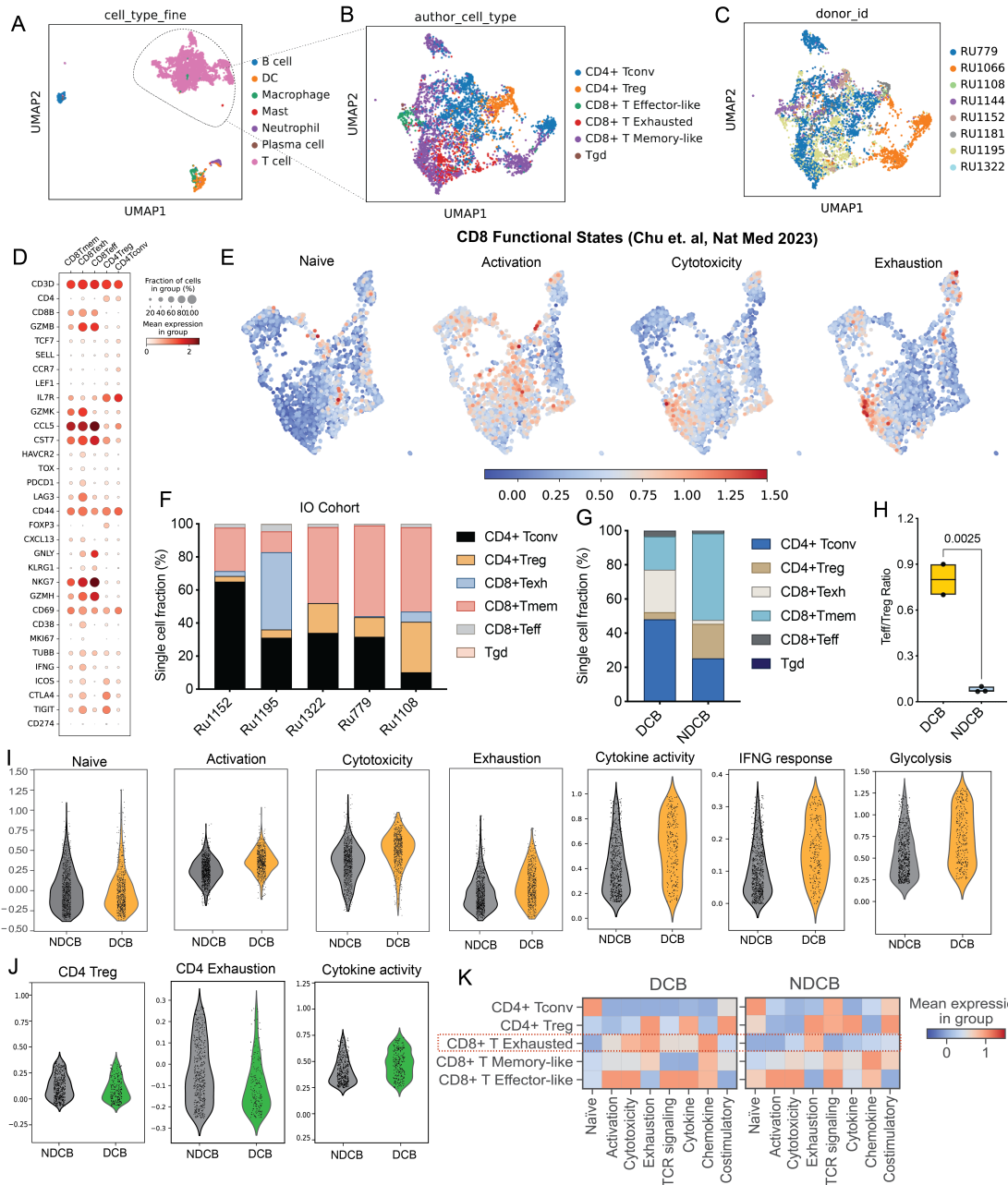


Figure 5.3. scRNA-seq analysis of infiltrating T cell subsets in SCLC responders. (A) UMAP of immune subsets in 8 SCLC patients (Ru1195; Ru1066; Ru1322; Ru1108; Ru1152; Ru779; Ru1144; Ru1181) (B) UMAP of 6 T cell subsets (C) Sample distribution within UMAP of T cells (D) Bubble plot describing representative genes of each T cell subset (E) UMAP projection of four CD8 T cell signatures on total CD8 T cells (F) Distribution of normalized T cell fraction in 5 patients in immunotherapy cohort (G) Relative comparison between fraction count of specific T cell subsets between DCB and NDCB (H) Effector CD8 to regulatory CD4T cell ratio (I) Violin plots of 7 CD8 T cell transcriptional signatures (J) Violin plots of 3 CD4 T cell states (K) Matrix plot of 5 subclusters of T cells and 8 T cell signatures.

5.7. Memory and activated CD4 and B cell populations constitute tertiary lymphoid structures in SCLC tumors

Given the significant presence of CD4+T and B cell in tumors of IO responders (**Figure 5.2E-P**), we next aimed to elucidate the cooperative potential of these lymphocyte subsets within the tumor microenvironment. We undertook whole-issue staining for CD3 and CD20 markers, respectively for pan-T and B cells, across 56 tumors. Our analyses depicted a striking pattern that B cells are found almost exclusively as co-clusters with T cells, forming three-dimensional large lymphoid aggregates resembling tertiary lymphoid structures (TLS) (**Figure 5.4A-B**). The presence of TLS in nonlymphoid reflects byproducts of lymphoid neogenesis tissues and has been associated with chronic inflammation and progressive immunity^{388,389}. Here we report that TLS are highly prevalent in a third of SCLC tumors (21/56; 38%) (**Figure 5.4C**), where they reside not as one discrete structure but as many clusters of comparable size in the stroma near the tumor periphery (**Figure 5.4A**). The spatial anatomy of many of these TLS exhibit highly organized, or “mature,” internal structures, characterized by a discrete center formed exclusively by B cells surrounded by a distinct ring of T cell-rich region³⁸⁸ (**Figure 5.4B**). The striking resemblance of these structures to secondary lymphoid organs (SLO) indicates they are indeed true TLS and not lymphoid cell clusters. This finding is also supported by evidence of TLS maturity, where these B cell areas are observed to be much larger compared to T cell zone, which have been associated with regions of active germinal center reactions and B cell class switching³⁹⁰ (**Figure 5.4B**).

We next quantified specific features of TLS that associate with favorable ICB benefit including size, composition, and degree of internal organization (“maturity”). We found no significant difference in overall TLS size (**Figure 5.4D**), or the percentage of CD3 pan-T or CD20 pan-B cells in each TLS region (**Figure 5.4G-H**). Though responders exhibit slightly higher average number of mature TLS per sample, this feature can be influenced by whole tissue size (**Figure 5.4F**). We thus normalized fraction of mature TLS over the total number of

TLS present in the tissue and showed that DCB tumors comprise of significantly more mature TLS fraction compared to resistant tumors (**Figure 5.4E**). Dichotomy of TLS presence in the tumor tissue illustrates that the presence of TLS improves PFS (HR = 0.5, 95% CI: 0.21-1; p-val = 0.055) and trends towards better prognosis by OS (HR = 0.53; 95% CI: 0.25-1.09) (**Figure 5.4I-J**). The lack of significance in PFS might be attributed to other features of TLS, including size and cellular composition, that were not a component of clinical analysis. These cumulative observations highlight that maturity degree of TLS might serve as an emerging biomarker for immunotherapy in SCLC.

TLS are widely recognized to retain fundamental features of a lymphoid organ, with follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs) forming the underlying stromal framework to organize specific lymphocyte regions. These structures not only express adhesion molecules and chemokines allowing for homing, trafficking, and egress of immune cells to and away from lymphoid organs, but they also provide the necessary costimulatory signals to activate and differentiate immune cells in response to a specific antigenic challenge^{391,392}. In NSCLC, the correlative value of TLS features driving immunotherapy response depends on the presence of not just follicular B cells but also mature DCs and activated CD4 T cells^{393–396}. To assess specific immune subsets in TLS-containing tumors, we utilized CIBERSORT deconvolution algorithm profiled on transcriptomic data of primary SCLC tumors. Agnostic of TLS size, maturity and location, tumors with TLS exhibit significantly higher memory and plasma B cells, driving the robust difference in total B cells (**Figure 5.4K**). In addition, levels of activated mature DCs, but not resting DCs, are highly expressed in tumors with TLS (**Figure 5.4L**). Similarly, subsets of memory activated and naïve CD4 T cells, not CD8+ T clusters, strongly drive the higher presence of total T cells in TLS-positive tumors (**Figure 5.4M**). No other immune cell subsets significantly differ between TLS-positive and TLS-negative tumors (data not shown).

Our data hereby implicate that the elevated signatures of activated and memory lymphocytes previously observed in the bulk transcriptomes of responders might arise from within the tertiary lymphoid structures. Indeed, *CXCL13*, previously named B-lymphocyte chemoattractant, is selectively expressed in B cell follicles of secondary lymphoid organs (SLO), where they facilitate a CXCL13 gradient responsible for homing of CXCR5-expressing B cells to B cell follicle region^{397,398}. *CXCL13* was found to be the top DEG highly upregulated in DCB (**Figure 5.2E**). CXCR5-CXCL13 has been extensively shown to necessitate lymphoid neogenesis and facilitate SLO development. Evidence of transitory B cell stages is marked by elevation of *MZB1*, encoding marginal zone B cells (MZB), serving as a prerequisite for plasma and memory B cell differentiation³⁹⁹. Similarly, *CD79A* and *CD79B* encode for B-cell receptor, and, upon BCR recognition, subsequent B cell class-switched activity, GC formation and generation of plasma cells requires activation of B cell transcription factor *IRF4*. All of these established markers of germinal center formation and B cell differentiation were found to be significantly enriched in tumor transcriptome of long-term immunotherapy responders (**Figure 5.2E-J**). We next procured a gene signature defining TLS associated with response and examined gene pathways stratifying clinical response for TLS-containing tumors (**Figure 5.4O**). We discovered that despite all exhibiting TLS, sensitive tumors present much higher T cell chemotaxis and *in situ* CD8 T cell differentiation, whereas resistant tumors are enriched in signatures associated with neuronal development and lung differentiation (**Figure 5.4O**). Together, we report for the first time the presence of TLS in the tumor microenvironment of SCLC patient tumors and comprehensively characterized the molecular and phenotypic features of TLS associated with durable benefit to standard-of-care immunotherapy.

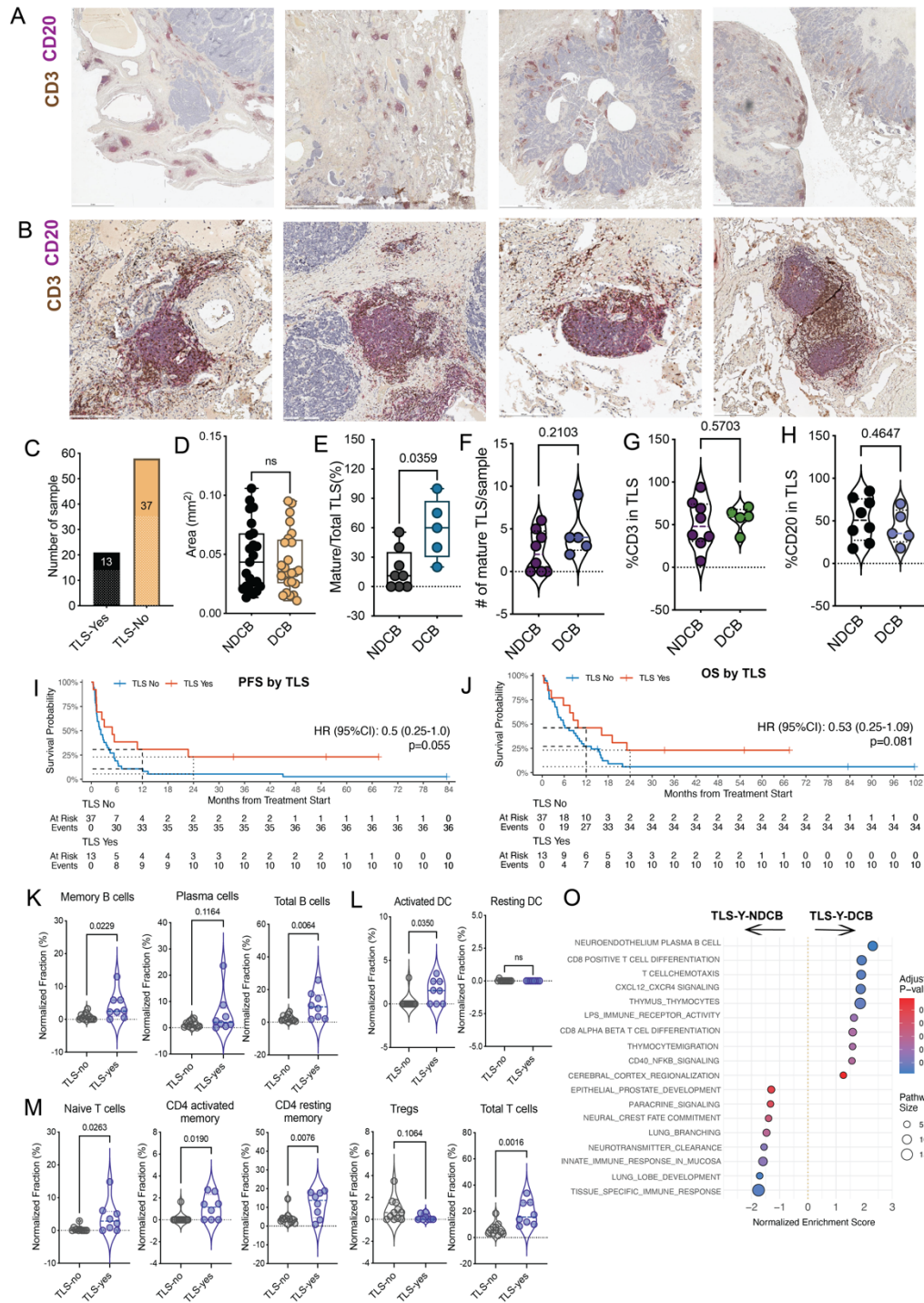


Figure 5.4. Characteristics of tertiary lymphoid structures in SCLC. (A) Location of TLS in the tumor stroma. Data representing 4 different primary samples. (B) B-cell and T cell region in the TLS. Data representing 4 different primary samples. (C) Frequency of SCLC in cohort (D)-(H) Features of TLS between responders (DCB) and non-responders (NDCB) (I) PFS Kaplan Meier curve (J) OS Kaplan Meier Curve. CIBERSORT characterization of B cell subsets (K), DC subsets (L) and T cell subsets (M). (O) GSEA pathways differentially enriched between DCB tumors with or without the presence of TLS.

5.8. Loss of function mutations of *HLA* genes are rarely observed in SCLC tumors

Next, we explored potential tumor-intrinsic regulatory mechanisms that govern the capacity to subvert immune surveillance in SCLC. Positive expression of MHC-I determined by IHC has been the predominant and most frequently observed indicator of sustained response to ICB in SCLC^{352,367}. To validate these findings, we quantitated MHC-I expression in 66 patient tumors, 43 of which were treated with immunotherapy (**Figure 5.5A**). Cox regression model carrying a positivity cut-off of H-score ≥ 50 demonstrated significantly strong clinical benefit in both disease-free survival (HR = 0.40; 95%: 0.21-0.77; p-val = 0.005) and OS (HR = 0.32, 95% CI: 0.16-0.64; p-val <0.001) in patients with high tumor MHC-I (**Figure 5.5B-C**). Probability of survival beyond 2 years was ~25% for MHC-I-high cohort, versus 0% for MHC-I low group (**Figure 5.5B-C**). Enriched tumor MHC-I is the most significant biomarker of immunotherapy sensitivity described in this cohort.

Despite serving as a strong predictive biomarker for SCLC, most SCLC tumors downregulate MHC-I on the tumor surface^{235,352,367}. We then sought to interrogate genetic mechanisms of loss of surface tumor MHC-I through genomic profiling. First, we genotyped every patient's germline and matched tumor HLA alleles and confirmed that *HLA-B* exhibits the most allelic diversity, harboring 55 unique alleletypes predominantly driven HLA-B*08:01 (13%), HLA-B*35:01 (11%) and HLA-B*44 (10%) (**Figure 5.5D**). At half that spectrum, HLA-A encompasses 27 unique alleles and HLA-C 26 alleles. This diversity is faithful to our understanding of HLA-B, representing the most polymorphic human MHC gene with over 2,000 alleles⁶. In addition, HLA-A and HLA-C, but not HLA-B, must physically associate with the peptide loading complex⁸⁷. As a result, HLA-B can undergo quicker peptide loading and plasma membrane export, and most HLA-B are always loaded with peptides, compared to 30-70% for HLA-A and HLA-C⁸⁵. In our cohort, HLA-C is the least diverse of all three alleles, driven primarily by HLA-C*07 in one third of the cohort population (33%) (**Figure 5.5D**). The markedly

more diverse HLA-A and HLA-B repertoire can be accounted by their expression, which is at much higher levels compared to HLA-C that exhibits a restricted peptide repertoire with limited assembly process^{85,86}.

Evidence that these HLA genotypes can exert selective influence on tumor immunopeptidome and subsequent response to ICB have emerged in recent years^{400–404}. The size of peptidome in different HLA variants can differ by orders of magnitude⁴⁰⁵. A recent study has identified a value termed *Promiscuity* (*Pr*) to characterize the breadth and specificity of different HLA-I genotypes, which reported that NSCLC patients harboring MHC-I genotypes with poor peptide binding specificity (high *Pr*) were 62% less likely to respond to ICB⁴⁰⁴. On the other hand, MHC-I alleles are classified into 12 “supertypes,” each with distinct shared peptide binding predilection – these 12 subtypes together cover the majority of common human HLA-A and HLA-B alleles⁴⁰⁶. In melanoma, patients carrying B44 superfamily of *HLA-B*, attributed to B*18:01, B*44:02, B*44:03, B*44:05 and B*50:01 alleletypes, were reported to clinically benefit from ICB⁴⁰⁰. Importantly, members of B44 supertype exhibit low *Pr* values and their association to ICB response have been observed in different cohorts of NSCLC^{404,407}. However, in our data, we did not observe any significant association of ICB to a particular supertype, including the B44 superfamily (**Supplementary Figure 5.2A-E**). The only supertype that has a marginal relationship with durable benefit is the presence of A03 supertype (HR = 0.63; 95% CI: 0.39-1.00; p-val =0.051), indicating that previously described association of HLA supertypes to ICB response is likely tumor-specific.

Utilizing patient HLA alleletype repertoire, we next systematically characterize the spectrum of loss-of-function mutations in HLA genes in 263 patients in our cohort. Annotation of somatic variants using PolySolver^{142,145} identified cohort frequency of HLA-mutations at ~1%. The most recurrent mutated regions are exon 3 (n=2; HLA-A*02:07) and exon 4 (n=2; HLA-

A*26:01), encoding for the α_2 peptide-binding domain and α_3 CD8-coreceptor-binding domains of HLA molecule, respectively (**Figure 5.5E**). These mutations when occurred lead to functional loss of antigen-presentation capacity of MHC-I molecules. To validate our finding in a larger dataset, we profiled somatic mutations of *HLA-A*, *HLA-B*, *HLA-C* and *B2M* in 1,034 tumor samples from the cBioportal clinical sequencing cohorts. Similarly, SCLC tumors representing 950 patients from 8 combined studies rarely harbor *HLA* alterations, exhibiting ~1% mutational frequency on *HLA-A* and *HLA-C*, and <2% on *HLA-B* and *B2M* (**Supplementary Figure 5.3A**). On *HLA-B*, frameshift insertion is also observed most frequently on exon 3 and exon 4, whereas *B2M* alterations are mostly deep deletion of exon 1 that likely leads to functional abrogation¹⁴² (**Supplementary Figure 5.3B**). The frequency reported in SCLC aligns with results from previous pan-cancer studies, in which HLA genes are mutated in about 3% of >7,000 tumors in the TCGA cohort. Rate of HLA mutation in SCLC is comparable to LUAD (3%) but much less than squamous cell carcinoma samples (8%)^{142,145}. We did not observe any significant relationship between class I *HLA* mutations and prognosis, likely due to the small number of alterations (**Supplementary Figure 5.3C-D**). These data strongly imply that genetic loss of function does not frequently occur and cannot account for the prevalent loss of surface MHC-I in SCLC tumors.

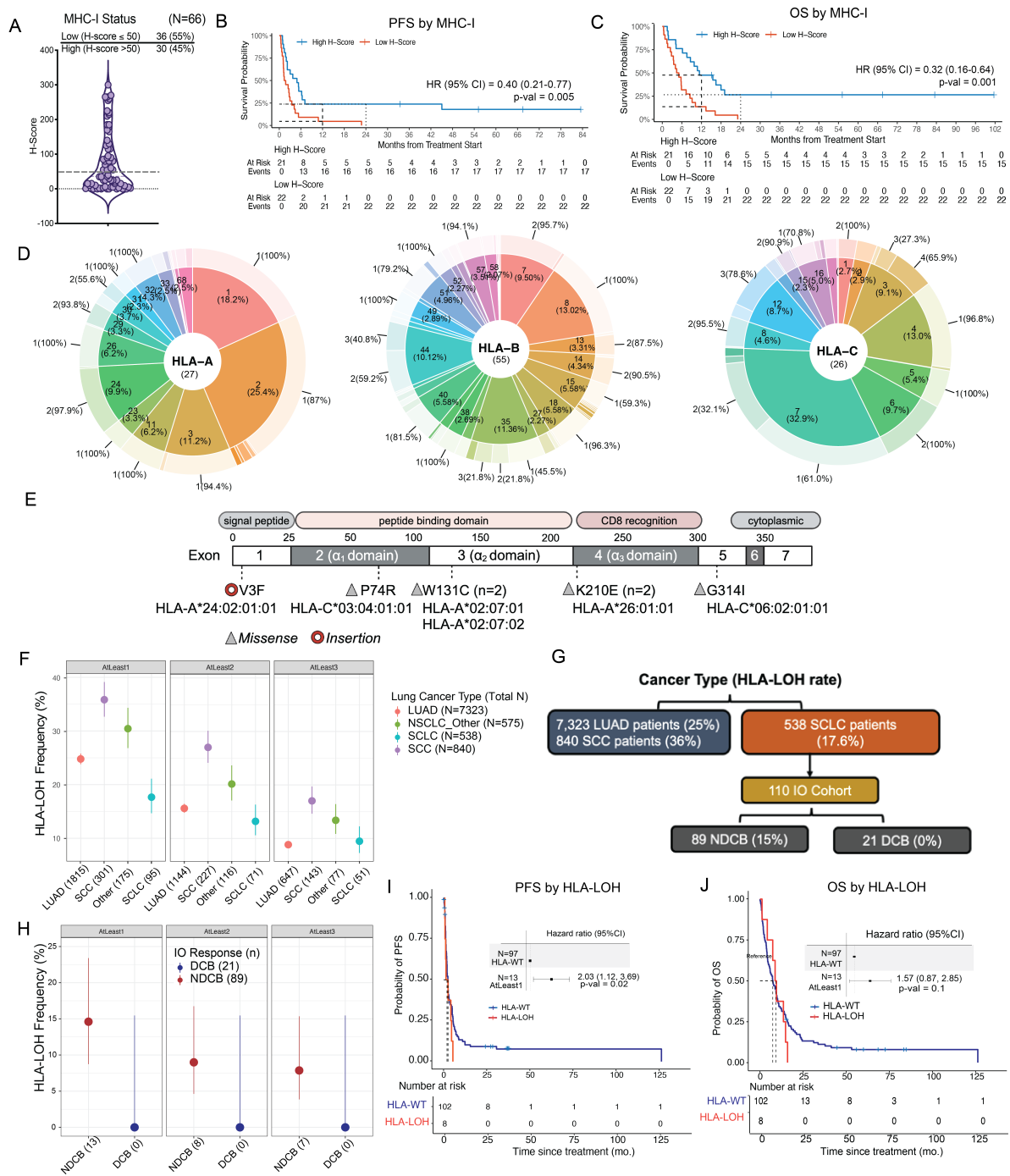


Figure 5.5. HLA alleletype repertoire in cohort (A) Distribution of HLA-A, -B, and -C in this cohort. The number of unique alleletypes for each loci is presented in bracket. (B) Loss of heterozygosity of HLA genes between lung adenocarcinoma (LUAD), squamous cell carcinoma (SCC), NSCLC of unknown subtype (NSCLC_Other), and small cell lung cancer (SCLC). All p-values are significant. (C) Comparison of frequency of LOHHLA between responders (DCB) and non-responders (NDCB). (D) Kaplan Meier curve for LOHHLA and PFS (E) Kaplan Meier curve for LOHHLA and OS.

5.9. Loss of heterozygosity dominates the spectrum of HLA loss in SCLC patients

Loss of heterozygosity of HLA locus (LOHHLA), defined as the loss of either maternal or paternal HLA allele, represents another genomic alteration that leads to widespread loss of peptide:MHC-I complexes on the cell surface. In a pan-cancer study of 59 different solid tumor encompassing >83,000 patient samples, SCLC is ranked among 10 tumors with lowest prevalence of HLA loss of heterozygosity¹⁵⁰. This frequency is markedly lower than NSCLC but at a similar rate as other tumors of neuroendocrine origin including prostate neuroendocrine cancer¹⁵⁰. To investigate the frequency and impact of LOHHLA in this cohort, we utilized LOHHLA, an algorithm estimating haplotype-specific copy number from every tumor and germline HLA locus in a patient. This computational method has overcome several limitations reported in conventional copy-number techniques, attributed to the polymorphic nature of the MHC region and resultant poor alignment to reference genome. LOHHLA can establish copy number of HLA predictions at similar or higher specificity than other HLA machine learning algorithms^{151,408}. Furthermore, LOHHLA has previously been applied to estimate HLA LOH in 90 NSCLC tumors in the TRACERx cohort¹⁴⁵.

We computed LOHHLA on MSKCC clinical sequencing cohort comprised of 538 SCLC samples, 217 of which are comprehensively profiled in this study. Frequency of HLA-LOH of SCLC was compared against 7,323 samples of lung adenocarcinoma (LUAD), 840 samples of squamous lung cancer, and other NSCLC cancer type (N=575) using binomial statistical tests (**Figure 5.5F**). We observed the frequency of HLA-LOH in at least one HLA allele in 95 patients, representing 17.6% cohort. In contrast, HLA-LOH occurs at significantly higher in 25% of LUAD ($p=1.09e-52$) and 36% of SCC tumor samples ($p=5.09e-36$), akin to what has been reported in a smaller cohort that SCC exhibiting one of the highest prevalence of HLA-LOH^{145,150} (**Figure 5.5G**). Using the same calling algorithm, the TRACERx studies previously reported HLA-LOH in 40% of NSCLC¹⁴⁵, followed by a smaller study suggesting frequency of 47%⁴⁰⁹. This

discrepancy could be attributed to these studies' small sample size (n=19-90 samples), compared to our cohort representing >7,000 primary NSCLC tumors.

Evidence of association of HLA-LOH to immunotherapy response has been established in other cancer types. In metastatic colorectal cancer patient treated with adoptive T cell transfer, evidence of treatment resistance was associated with LOH of HLA-C*08:02 haplotype, required for presentation of KRAS G12D mutation for T cell recognition⁴¹⁰. Thus, we next evaluate the degree of influence of HLA-I LOH on clinical outcome. We found that the presence of HLA-LOH is restricted to non-responders at the rate of 15% (**Figure 5.5G-H**). None of 21 IO responders in our cohort harbors any LOHHLA, either of one, two or three HLA-I genes (**Figure 5.5G-H**). Importantly, the presence of intact HLA significantly lengthens disease-free survival (HR = 2.03; p=0.02), whereas overall survival is marginally improved (**Figure 5.4I-J**).

Of note, additional studies implying other genetic features of HLA genotype as determinants of ICB have been performed^{400,402,403,411}. These features include HLA zygosity⁴⁰¹, germline HLA evolutionary divergence (HED)⁴⁰⁰, and germline variance^{402,411}. However, HLA zygosity and HED analyses have failed to demonstrate association to clinical benefit in NSCLC^{404,407,412}. In addition, MHC-I genotype might be biased to strongly bind to viral and pathogenic peptides over peptides derived from recurrent oncogenic mutations, and immunogenic tumor MHC-I peptides likely arose from passenger mutations rather than oncogenic drivers⁴⁰². Overall, the spectrum of HLA genotype influence on ICB response is likely tumor- and treatment- specific and needs to be further explored on multiple cancer types.

Our cumulative data demonstrates the largest study to date that examines the landscape of HLA genetic loss in SCLC. We show that SCLC largely appears to retain the intrinsic genomic capacity to process antigens for MHC-I presentation but might have lost capacity for presentation through other alternative mechanisms of silencing. The collective evidence here

indicates that alternative mechanisms of MHC-I loss, including epigenetics, may be at play in orchestrating the observed decline in MHC-I expression on the tumor surface.

5.10. HLA-LOH tumors harbor more neoantigen counts than wild-type HLA

The presence of MHC-I molecules on the plasma membrane requires preceding steps of binding to a peptide antigen to provide selective structural stabilization of the peptide:MHC-I complex. However, a substantial proportion of neoantigens originates from passenger mutations and are dispensable for tumor sustenance. We first sought to explore the landscape of MHC-I neoantigens present in SCLC patient tumors.

Tumor mutational load has long been implied as a surrogate of cognate neoantigen genesis. We first assessed the degree of tumor mutation burden (TMB) across different lung cancer types and reported that SCLC tumors harbor a significantly higher TMB compared to LUAD ($p=4.9e-21$) (**Supplementary Figure 5.4A**). We confirm that TMB strongly correlate with predicted neoantigen count ($R^2=0.79$, $p\text{-val} = 6.64e-28$) (**Supplementary Figure 5.4B-C**). To examine the potentials of these mutations to become a putative neoantigen, we then performed an established computational neoantigen prediction pipeline that has been validated to generate tumor-regressing cancer vaccine⁴¹³ (**Figure 5.6A**). Briefly, annotated variants derived from matched tumor and normal tissue sequencing underwent binding prediction algorithm NetMHC4.0-pan to evaluate the likelihood of a particular variant to bind autologous HLA, which subsequently returned a ranked list of putative neoepitopes (**Figure 5.6A**). 94% of putative neoantigens derived from a missense mutation (**Supplementary Figure 5.4D**), and chromosome 1 harbors the highest number of somatic variants compared to other regions (**Supplementary Figure 5.4E**).

We then inquired whether genomic loss of HLA exerts a selective pressure on the tumor antigen repertoire. The number of somatic variants and putative neoantigens expressed on

HLA-LOH tumors were markedly higher compared to intact HLA-WT SCLC (**Figure 5.6B-C**). In the event of LOH HLA, the capability of tumor presentation is reduced to 50%. Another genetic or epigenetic event on the other allele can likely abolish the capacity of that particular MHC-I to display peptide antigen. In this aspect, tumors with intact HLA strongly reduce the amount of neoantigens, implying a selection force to sustain immune evasion. To understand the preferential bias of a particular neoantigen to the HLA-WT, for each neoantigen we determined their binding predilection and aggregated the total frequency of all neoantigens in a patient to bind to the lost vs. kept HLA. We discovered that the majority of patients exhibit more neoantigens that preferentially bind to the lost allele compared to the intact HLA (**Figure 5.6D-E**). Thus, HLA-LOH represents a powerful immunoediting mechanism that enables large quantity of neoantigen to never become presented. Together, our cumulative data here demonstrates that despite a reputedly high TMB associated with diverse neoantigen repertoire, SCLC tumors restrict the number and degree of immunogenic neoAgs that can likely be presented on MHC-I through selective mechanism of HLA-LOH.

5.11. Responders exhibit more immunogenic neoAgs compared to non-responders

Following neoantigen count, epitopes were qualitatively assessed for various parameters of immunogenicity to determine their relative likelihood to elicit a T cell response. Binding affinity is considered one of these parameters as no antigen with a BA>5000nM has been reported to trigger T cell activity. Qualitative parameters encompass two primary features: (1) agretopicity and (2) immunogenicity (**Figure 5.6A**). Agretopicity is determined by the ratio of BA of the variant to BA of the wild-type copy⁴¹⁴. Immunogenicity, developed by Immune Epitope Database (IEDB), calculates the probability of TCR recognition event based on peptide sequence structure independent of binding affinity⁴¹⁵. Agretopicity and immunogenicity both independently assess for enrichment of T-cell specific epitopes and a variation of these parameters have been

applied to assess neoantigens of long-term survivors in pancreatic cancer^{416–418}. Calling only unique variants leads to an impression that SCLC tumors harbor a huge neoantigen load (**Figure 5.6F**). However, integration of qualitative restrictions significantly dropped the number of high-quality neoAgs, indicating that the majority of MHC-I neoAg in SCLC tumors are non-immunogenic (Ag >0.1 or Ig<0.1) (**Figure 5.6F**). Restricting qualitative benchmarks reported the average number of immunogenic neoantigens per patient range from 5-25 peptides (**Figure 5.6F**). Assessing for the specific HLA alleletype, we found that neoantigens bound on members of HLA-A*02 family are collectively more immunogenic compared to those recognized by HLA-B*15 family (**Supplementary Figure 5.4F**). In addition, in-frame insertions generate neoAgs with the highest average Ig score compared to other classes of mutation (**Supplementary Figure 5.4G**).

We next evaluated the qualitative measures of neoantigen repertoire that are preferentially displayed on MHC-I molecules from responding tumors. Overall total neoAg count, expression level, binding affinity, and agretopicity do not significantly differ between sensitive and resistant tumors (**Figure 5.5G-I; Supplementary Figure 5.4H**). However, when we restrict immunogenicity to 0.1 and 0.3 thresholds, we observed a markedly higher immunogenic neoAgs in DCB versus NDCB (**Figure 5.6J-K**). The fact that these neoantigens are more immunogenic in regressed tumors might explain for our report of elevated TCR signaling, cytotoxicity and exhaustion features associated with DCB single T cell subsets. Concatenating all qualitative filters do not result in any difference between the two cohorts (**Supplementary Figure 5.4I**). Even though DCB harbor more immunogenic neoAgs than NDCB, neoAgs from two cohorts do not significantly differ in the degree of immunogenicity calculated as distance of the variant Ig score from that of the wild-type copy (**Supplementary Figure 5.4J**).

Finally, we assessed for epitope sharedness across patients. As observed in most cancer types, 99.8% of epitopes in SCLC tumors are unique to the individual patients

(Supplementary Figure 5.4K). 29 sequences with BA<500nM were shared between 2 patients, and only 2 were found overlapped among 3 patients **(Figure 5.6L)**. Among the gene of origin of shared epitopes, we discovered an epitope derived from frameshift deletion within *ANTXR2* (A357Pfs*52) to display markedly lower BA and higher Ig scores compared to other gene derivatives of shared epitopes **(Figure 5.6M-N)**. Interestingly, this neoepitope was found to be expressed in a long-term responder of immune checkpoint blockade (OS ~15 months). Together, we demonstrated that tumors of immunotherapy survivors do not generally carry a significantly larger pool of neoantigens, but their epitopes are preferentially more immunogenic than those found in resistant tumors. Most neoantigens in SCLC are rare; however, shared epitopes are present across patients. can allow SCLC to be amenable to the development of TCR therapies as a supplementary immunotherapeutic approach to immune checkpoint inhibitor therapies.

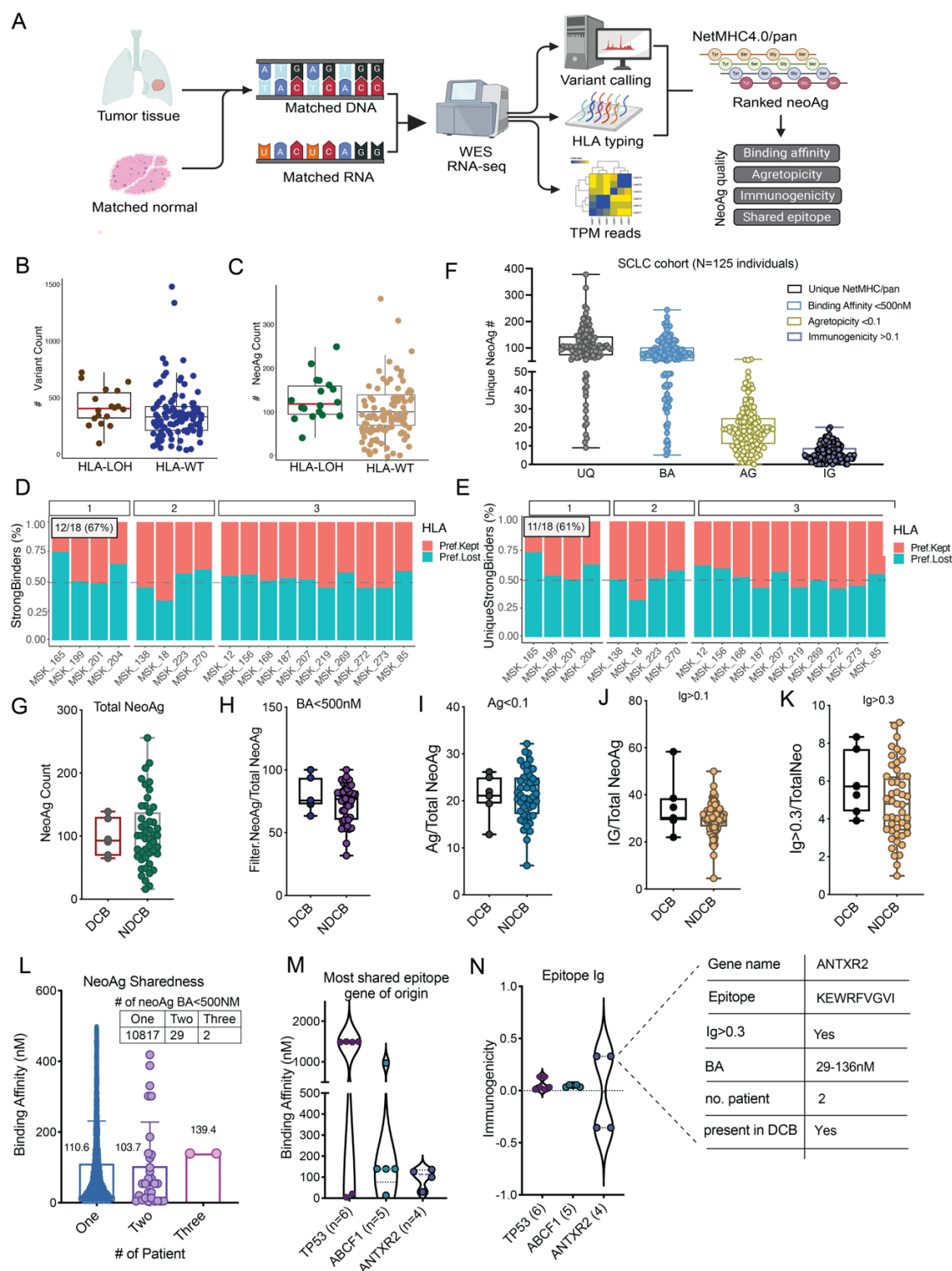


Figure 5.6. Qualitative profiling of neoantigen repertoire in SCLC. (A) Overview of neoantigen prediction algorithm. Distribution of variant count (B) and putative neoantigen count (C) between tumors of intact HLA and LOHHLA. (D) Percentage of strong-binding neoantigens that preferentially bind to the kept HLA allele vs lost HLA allele (E) Percentage of unique strong-binding neoantigens that preferentially bind to the kept HLA allele vs lost HLA allele. (F) Count of putative neoantigens that pass all qualitative benchmarks. Total neoAg count (G), fraction of neoAg with

binding affinity (BA) <500nM (H), fraction of neoAg with agretopicity <0.1 (I), fraction of neoantigen with immunogenicity >0.1 (J), fraction of neoantigen with immunogenicity >0.3 between responders (DCB) and non-responders (NDCB). (L) Binding affinity of all neoantigens that are unique (one) or shared between two patients (two) or three patients (three). (M) Average binding affinity for all neoantigens that encode for *TP53*, *ABCF1* and *ANTXR2* genes. (N) Immunogenicity of all neoantigens that encode for either *TP53*, *ABCF1*, or *ANTXR2* genes. Table displaying characteristics of epitope sequence derived from *ANTXR2* gene.

5.12. Summary

Currently available retrospective studies on SCLC patients predominantly relied on bulk tumor transcriptomes to dissect the molecular determinants of long-term responders to immune checkpoint therapies. Here we employed multi-omic approaches combining bulk RNA-seq, scRNA-seq, next generation sequencing and protein analysis to delineate comprehensive immunophenotypic features of SCLC tumors that shape long-term benefit to immune checkpoint therapies.

In our immunotherapy-treated cohort, the presence of CD8 positivity as defined by IHC is not sufficient to confer durable clinical benefit. We found that most CD8-high samples exhibit excluded phenotype, characterized by presence of high effector cell density at the tumor stroma. Subsequent scRNA-seq characterized that CD8 infiltrates in regressed tumors are more transcriptionally functional compared to those in resistant tumors, displaying higher TCR signaling, interferon response, glycolysis, and cytotoxicity. Our study was the first to utilize scRNA-seq to infer immune cell phenotypes associated with immunotherapy sensitivity.

In profiling for molecular signatures of immunotherapy sensitivity, we identified enrichment of B and T cell-specific markers respective to activated and memory phenotypes. B cells were found to exclusively reside within TLS in the stroma at the periphery of the tumor edge, where they self-organize into a B-cell rich region surrounded by T-cell-rich zone. The presence of TLS may imply a focal immunoreactivity to SCLC, serving as an intratumoral location for cognate lymphocytic activation and differentiation. In tumors from long-term ICB responders, CD4⁺ T exhibit high activation markers that may aid functions of cytotoxic CD8⁺ T

cells and other innate immune subsets, including enriched presence of activated DCs in TLS-containing tumors that can assist in differentiation of naïve to effector CD8⁺ T cells *in situ*.

Here we described for the first time the presence of tumor-infiltrating TLS. The presence of TLS enables APCs and lymphocytes to bypass traffic to and from the peritumoral lymph nodes. Subsequent studies remain to illustrate whether SCLC TLS derive from chronic smoke-induced inflammation or as a direct result of tumorigenesis. Our cumulative data illustrate tumors of long-term survivors exhibit a robustly more heterogeneous and transcriptional robust states attributed not only to CD8⁺ effector functions but other lymphocytes.

We sought to address the question of how tumor cells become deficient in their antigen presentation capacity. We presented the largest genomic study to date that characterizes genomic features of *HLA* genes in >500 SCLC and >7000 LUAD. SCLC tumors indeed typically possess the genomic capacity for antigen processing and presentation, with LOF mutations occurred only in <2% and LOHHLA at <20% of SCLC, which are significantly lower compared to frequencies observed in LUAD or SCC. This finding posits the involvement of alternative epigenetic mechanisms that predominantly drive MHC-I loss.

In addition, despite intact HLA loci and a diverse neoantigen repertoire, our cumulative data demonstrate a persisting restriction in the quality of these neoantigens which predominantly display poor features of epitope immunogenicity. However, responders exhibit more immunogenic neoantigens, reflecting higher degree of TCR signaling observed in regressed tumors. LOHHLA may contribute to driving loss of antigen presentation, as tumors harboring LOHHLA exhibits markedly higher number of neoantigens that preferentially bind to the lost allele.

Our collective findings substantially add to the current understanding of the spectrum of diverse tumor-extrinsic and -intrinsic mechanisms that enable SCLC to invade immune surveillance. This study underscores the imperative integration of multi-platform approaches to

deepen our understanding of the tumor immune microenvironment for the purpose of advancing immunotherapeutic strategies in SCLC and other MHC-deficient tumors.

CHAPTER 6. LSD1 AS AN EPIGENETIC TARGET FOR ANTIGEN PRESENTATION DEFICIENCY IN SMALL CELL LUNG CANCER

This chapter was adapted from *DOI: 10.1016/j.jtho.2022.05.014* (PMID: 35691495)¹.

6.1. Overview of Lysine-specific demethylase 1 (LSD1)

LSD1/KDM1A was the first histone lysine demethylase discovered by Yang Shi and colleagues⁴¹⁹. Equipped with a flavin adenine dinucleotide (FAD)-dependent amine oxidase domain, KDM1A demethylates H3K4me2 and H3K4me1, thereby influencing gene expression primarily as a transcriptional repressor^{419,420}. In SCLC, LSD1 has been recently recognized as a potential therapeutic target due to a tumor cell intrinsic function in growth and viability⁴²¹. In a subset of SCLC, LSD1 inhibition was shown to activate NOTCH signaling and downregulate ASCL1, both of which control aspects of neuroendocrine development^{421–423}. Few studies have also kindled interests in LSD1 as an immunomodulatory target in solid tumors^{424–427}. However, its ability to alter the immunosuppressive microenvironment of SCLC has not been previously characterized. In this study, we identified LSD1 as a potent regulator of MHC-I-mediated antigen presentation and intrinsic immunogenicity in SCLC.

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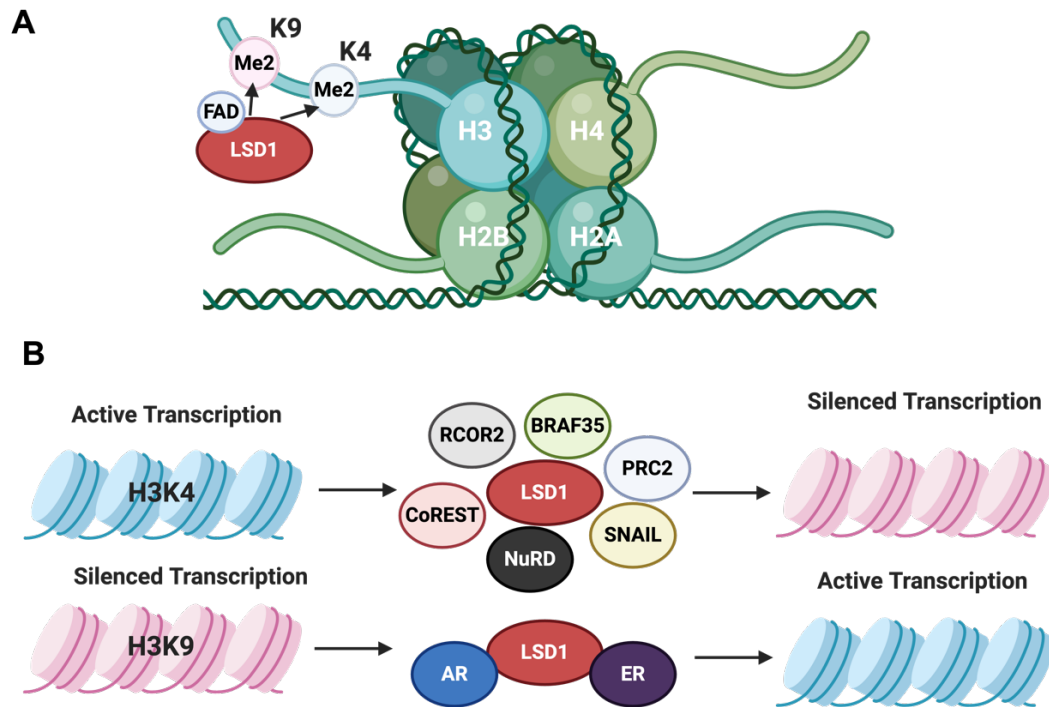


Figure 6.0. Overview of the histone lysine demethylase LSD1. (A) LSD1 was first discovered as a FAD-containing domain capable of removing mono- and di-methyl group from lysine chains on the H3 histone tails. (B) Subsequent studies uncovered that LSD1 activity requires CoREST for structural stabilization, along with other epigenetic modifiers including the EZH2-containing PRC2, SNAIL, NuRD and RCOR2. Together these cofactors form a multimeric complex to drive gene transcription silencing. In prostate cell lines, LSD1 was further shown to bind to AR and ER to demethylate the repressive H3K9me marks, in turn activating expression of prostate cancer-related genes.

6.2. Negative correlation of LSD1 with MHC-I antigen presentation in SCLC

To investigate LSD1 expression in SCLC relative to other cancer types, we queried expression of the *KDM1A* gene in 1,153 cell lines of the Broad Cancer Cell Line Encyclopedia (CCLE) Portal^{428,429}. *KDM1A* expression is more highly expressed in SCLC than in any of the other 28 cancer types (**Figure 6.1A**). High expression of *KDM1A* in SCLC cell lines was similarly reflected in primary tumors, and mutational analyses of both datasets supported overexpression as the major pathological feature as mutations of *KDM1A* are rare (<2%) (**Figure 6.1B-C**).

Next, to analyze correlative gene expressions of *KDM1A* and genes encoding endogenous antigen presentation via MHC-I, we queried three SCLC cell line datasets: CCLE (n=50)^{430,431}, University of Texas Southwestern (UTSW) 2017 dataset (SCLC Consortium cBioportal) (n=70)⁴³² and the Sanger/Massachusetts General Hospital (Genomics of Drug Sensitivity in Cancer [GDSC]) (n=74)⁴³³ (**Supplementary Figure 6.1A-C**). In all three databases, we discovered significant inverse correlation between transcriptomic levels of *KDM1A* mRNA and *HLA-A*, *HLA-B*, *HLA-C*, and *B2M*, as well as the peptide transporters (*TAP1*, *TAP2*, *TAPBP*) and subunits of the immunoproteasomes (*PSMB8* or LMP-2, *PSMB9* or LMP-7). To collectively interpret correlation from these datasets, we utilized the publicly available CellMiner-SCLC toolkit (<https://discover.nci.nih.gov/ScicCellMinerCDB/>)⁴³⁴, which integrates and normalizes microarray and RNA-seq from five independent sources into a dataset labelled “SCLC-Global” (**Figure 6.1D**). Integrating different methodologies, these datasets uniformly demonstrated a statistically significant negative correlation between *KDM1A* and multiple key genes required for antigen presentation, leading us to posit that LSD1 might regulate MHC-I-mediated antigen presentation in SCLC.

6.3. LSD1 inhibition rescues MHC-I expression in SCLC cell lines

To perturb LSD1 demethylase function, we employed the irreversible FAD-dependent LSD1 inhibitor, ORY-1001. Prior studies have indicated effective inhibitory concentrations of ORY-1001 at nanomolar range^{421,435}. Importantly, we did not observe substantial *in vitro* growth inhibition of 20 human SCLC cell lines after treatment with increasing concentrations of ORY-1001 up to 10 μ M (**Figure 6.2A**). Next, we evaluated baseline expression of MHC-I molecules in 10 different SCLC models and validated that with the transcriptomic data from CCLE (**Supplementary Figure 6.2A-B**). Two models with the lowest *B2M* mRNA transcripts, H211 and H82, also displayed the lowest MHC-I cell surface expression (**Supplementary Figure**

6.2A-B). In contrast, H196 had strongest cell surface level of MHC-I, in agreement with its transcriptional assessment (**Supplementary Figure 6.2A-B**). Interestingly, H211 and H82 ranked among the top 5 cell lines with highest *KDM1A* expression yet retained 90-100% cell viability following treatment with 1 μ M ORY-1001 (**Figure 6.2A; Supplementary 6.2B**). Another SCLC cell line, H69, similarly exhibited strong anti-correlation of *KDM1A* and *B2M* expression (**Supplementary Figure 6.2B**). We employed these three models to further assess the immunomodulatory effects of LSD1 inhibition.

To determine whether LSD1 inhibition elicits concentration- or time-dependent effects, we initially evaluated changes in total cell surface MHC-I expression of H211 cells following treatment with varying doses of ORY-1001 over time (**Supplementary Figure 6.2C**). After 4 days, minimal changes in MHC-I levels were observed at any concentration. Interestingly, at day 7, we began to detect an increase in MHC-I expression, which further intensified at day 10 with marked upregulation in a subset of cells and relatively minimal dose-dependency (**Supplementary Figure 6.2C**). These data suggest that latency, rather than concentration, might primarily contribute to LSD1-specific regulation of downstream targets in SCLC.

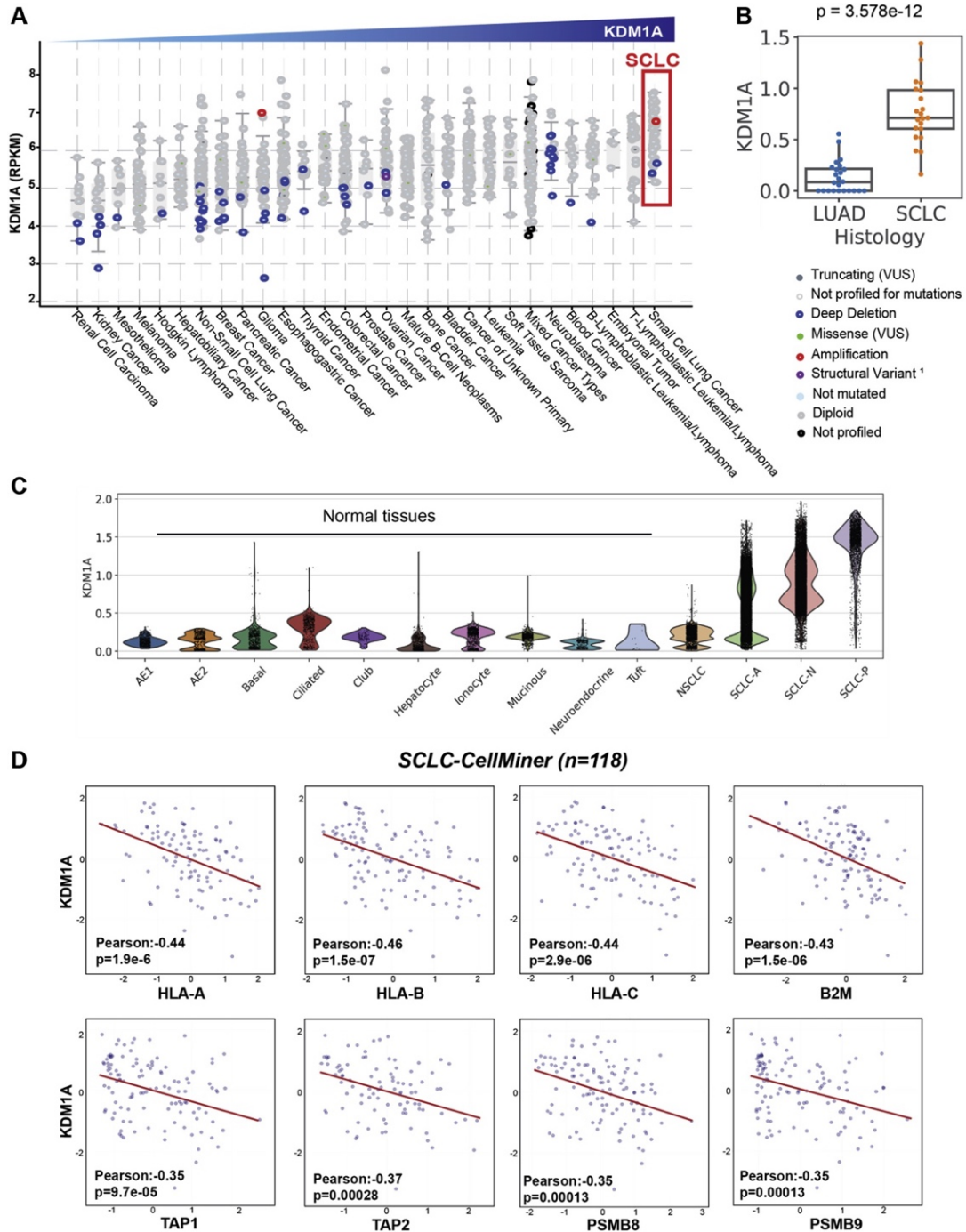


Figure 6.1. LSD1 expression and its correlation with antigen presentation pathway in small cell lung cancer (SCLC) cell lines. (A) LSD1 (KDM1A) expressed at higher levels in small cell lung cancer than any other tumor type. KDM1A mRNA expression was profiled in 29 unique cancer types; data from Cancer Cell Line Encyclopedia (CCLE) (Broad, 2019) (Downloaded from cBioportal). (B-C) KDM1A mutational profiles in SCLC cell lines (CCLE) (B) and in SCLC patient samples (George et al. 2015) (C) indicating rare mutations. (D) Pearson's correlations indicate significant anticorrelation between LSD1 (KDM1A) expression and HLA-A, HLA-B, HLA-C, B2M, TAP1, TAP2, LMP-2 (PSMB8), LMP-7 (PSMB9) from SCLC-Global (downloaded from CellMiner). Values were calculated as RNAseq RPKM ($\log_2(\text{value}+1)$).

To validate that the observed MHC-I upregulation is an LSD1-specific but not H211-specific event, we evaluated expression of mono-methylated H3K4 (H3K4me) in three biological replicates of H211, H69, and H82 after treatment with 1 μ M ORY-1001. In all three cell lines, we found a substantial increase in H3K4me at days 7 and 10, but not at day 4 relative to vehicle treatment. This time-dependent effect was concordant with what we previously observed in flow cytometry (**Figure 6.2B; Supplementary Figure 6.2C**). We then assessed whether targeting LSD1 with ORY-1001 could similarly rescue B2M protein expression. Indeed, we only noticed marked increases in B2M at later time points of days 7 and 10, but not at day 4 relative to vehicle treatment (**Figure 6.2B**). ORY-1001 did not affect LSD1 protein level but specifically inhibited its enzymatic activity, resulting in increased H3K4me. To further substantiate that MHC-I upregulation is indeed an LSD1-dependent phenomenon, we performed a doxycycline-inducible knockdown of LSD1 by shRNA. Effective knockdown of LSD1 expression was achieved with all four shRNA constructs, but not with a luciferase-non-targeting control (**Supplementary Figure 6.2D**). Similar to results observed from ORY-1001 treatment, we detected noticeably increased B2M protein level following doxycycline dose-dependent knockdown of LSD1 (**Figure 6.2E**).

Next, we evaluated the functional effect of LSD1 inhibition in inducing MHC-I expression. Exposure to low-dose IFN- γ has been shown to markedly upregulate MHC-I level^{436–438}, and an impairment in IFN- γ signaling is associated with ICB resistance^{439,440}. We compared the intensity of MHC-I re-activation following incubation with 1 μ M ORY-1001 for 10 days, IFN- γ (10ng/ml) for 24 hours, or a combination of both. LSD1 inhibition by ORY-1001 induced MHC-I expression in all three cell lines (**Figure 6.2C-D**). Interestingly, in H211, with undetectable MHC-I at baseline, concurrent treatment with ORY-1001 and IFN- γ induced higher MHC-I expression than either agent alone (**Figure 6.2C-D**). Together, these data demonstrate that perturbation of

LSD1 with pharmacological inhibition or RNA interference is sufficient to recover MHC-I expression in SCLC cell lines.

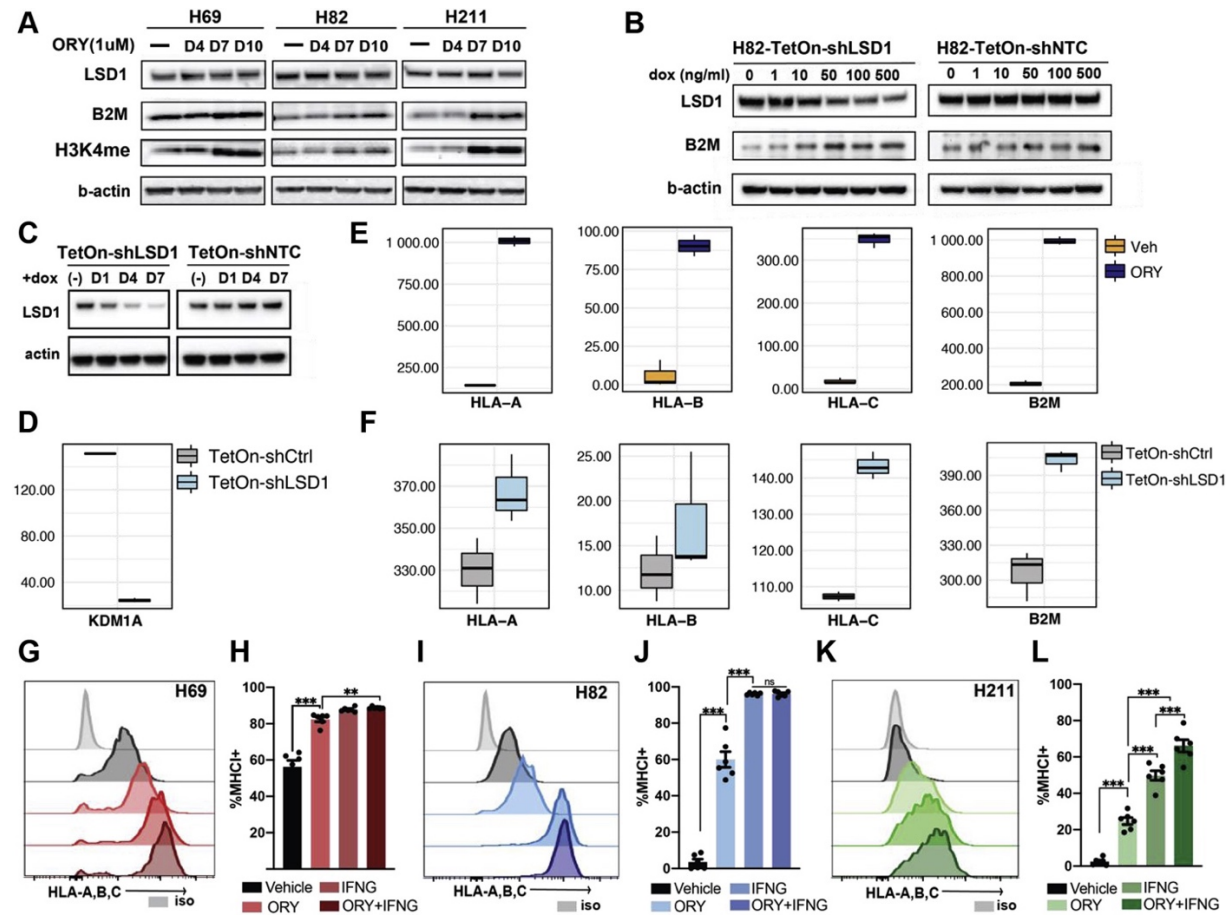


Figure 6.2. Induction of MHC-I expressions following LSD1 ablation in SCLC. (A) 20 human SCLC cell lines were subjected to treatment with LSD1 inhibitor ORY-1001 at increasing concentrations up to 10μM for 7 days. Cell titer glo viability assay was performed to quantitate percentage of viability after drug treatment. (B) Immunoblots of H69, H211 and H82 treated with 1μM ORY-1001 for 4, 7 and 10 days before cell lysates were collected and blotted for LSD1, B2M, di-methylated H3K4 and beta-actin protein expression. (C) Flow cytometry analysis was performed on H69, H211 and H82 treated with either 1μM ORY-1001 for 10 days, 10ng/ml IFN-γ for 24 hours, or its combination to assess cell-surface expression of MHC-I by HLA-A,B,C antibodies. (D) Levels of increased MHC-I expression were quantitated against baseline expression from two independent experiments. Statistical significance was performed with two-way ANOVA using Geisser-Greenhouse correction; ns p>0.12; * p< 0.033; ** p<0.002; and *** p<0.001. (E) Doxycycline-inducible shRNA knockdown of LSD1 (sh#1227) or non-targeting control (NTC) (shLuc-Ctrl) was performed on H82 to assess LSD1, B2M and beta-actin expression after LSD1 suppression.

6.4. LSD1 inhibition transcriptionally activates antigen presentation and induces intrinsic immunogenicity

We next conducted transcriptomic analysis to determine the extent to which the antigen presentation machinery was re-activated upon LSD1 targeting. Gene set enrichment analysis (GSEA) revealed significant enrichment of gene sets defining the antigen presentation machinery in both H82 and H211 following LSD1 inhibition by ORY-1001 (**Figure 6.3A-B**). Analyses of differentially expressed genes (DEGs) highlighted those of the endogenous peptide antigen presentation pathway, including *HLA-A*, *HLA-B*, *HLA-C*, *B2M*, *TAP1/2*, *PSMB8/9*, to be among the most significantly upregulated genes following LSD1 inhibition (**Figure 6.3C-D**). We further validated the role of LSD1 as a transcriptional regulator of key regulators of antigen presentation in H211 and H82 by qPCR (**Figure 6.3E-F**).

We next assessed additional biological processes associated with antigen presentation by MHC-I molecules. Preceding peptide:MHC-I assembly, multi-subunit proteasomes must degrade large cytosolic polypeptides into antigenic peptides suitable for transport to the endoplasmic reticulum by TAP transporters and for binding to MHC-I molecules. We profiled gene expressions of MHC-I molecules, comprised of the alpha chain and B2M, as well as the MHC-I peptide loading complex, including tapasin (*TAPBP*), which is required for the MHC-I heterodimer to associate with TAP transporters (*TAP1*, *TAP2*). We found that most genes encoding these components were upregulated in ORY-1001-treated SCLC cells (**Figure 6.3G**). Interestingly, we observed significant induction of the NOD-like receptor CARD domain containing 5 (NLRC5) gene, a member of the NLR family of regulators of inflammatory responses, following LSD1 inhibition (**Figure 6.3G**). The NLRC5 promoter has been previously shown to be inducible by IFN- γ , and NLRC5 is considered an MHC-I trans-activator due to its essential function in MHC-I transcription regulation^{84,88}. Upregulation of NLRC5 suggests that transcriptional induction of MHC-I by LSD1 inhibition might involve activation of interferon signaling.

Consistent with this hypothesis, gene set enrichment and DEGs of H82 and H211 following LSD1 inhibition revealed consistent induction of innate immune-related processes, including response to type I and type III interferons, cellular defense against viruses, and interferon-stimulated genes reflecting pan-inflammatory processes (**Figure 6.3G-I**). Expression of viral defense factors, including the endosomal sensor TLR-7, the adaptor MyD88, the transcription factor NF κ B and several interferon-regulating factors (IRFs) were significantly upregulated (**Figure 6.3G**). TLR-7 can signal through MyD88 to activate NF κ B and induce transcriptions of pro-inflammatory cytokines such as type I interferons. Importantly, in H211 cells, we noticed increased induction of interferon- α receptor IFNAR, which is a common receptor recognizing both IFN- α and IFN- β interferons (data not shown). IFNAR signals through STAT1-STAT2 to interact with IRF9, which together binds to the promoters of interferon stimulated genes (ISGs). Indeed, we noticed induced expression of STAT1, IRF9 and ISGs following LSD1 inhibition (**Figure 6.3G**). Significant enrichment of interferon response signaling in LSD1-inactivated cells was further confirmed by GSEA and gene ontology analysis (**Figure 6.3H-I**). Induction of interferon signaling in SCLC following LSD1 inhibition was further validated by RT-qPCR, demonstrating consistent induction of *IFNA* and *IFNB*, and cell line-selective induction in *IFNG* in H82 (**Figure 6.3J-K**). Collectively, these data demonstrate that LSD1 perturbation transcriptionally upregulates MHC-I antigen presentation machinery and primes SCLC into an immunogenic phenotype.

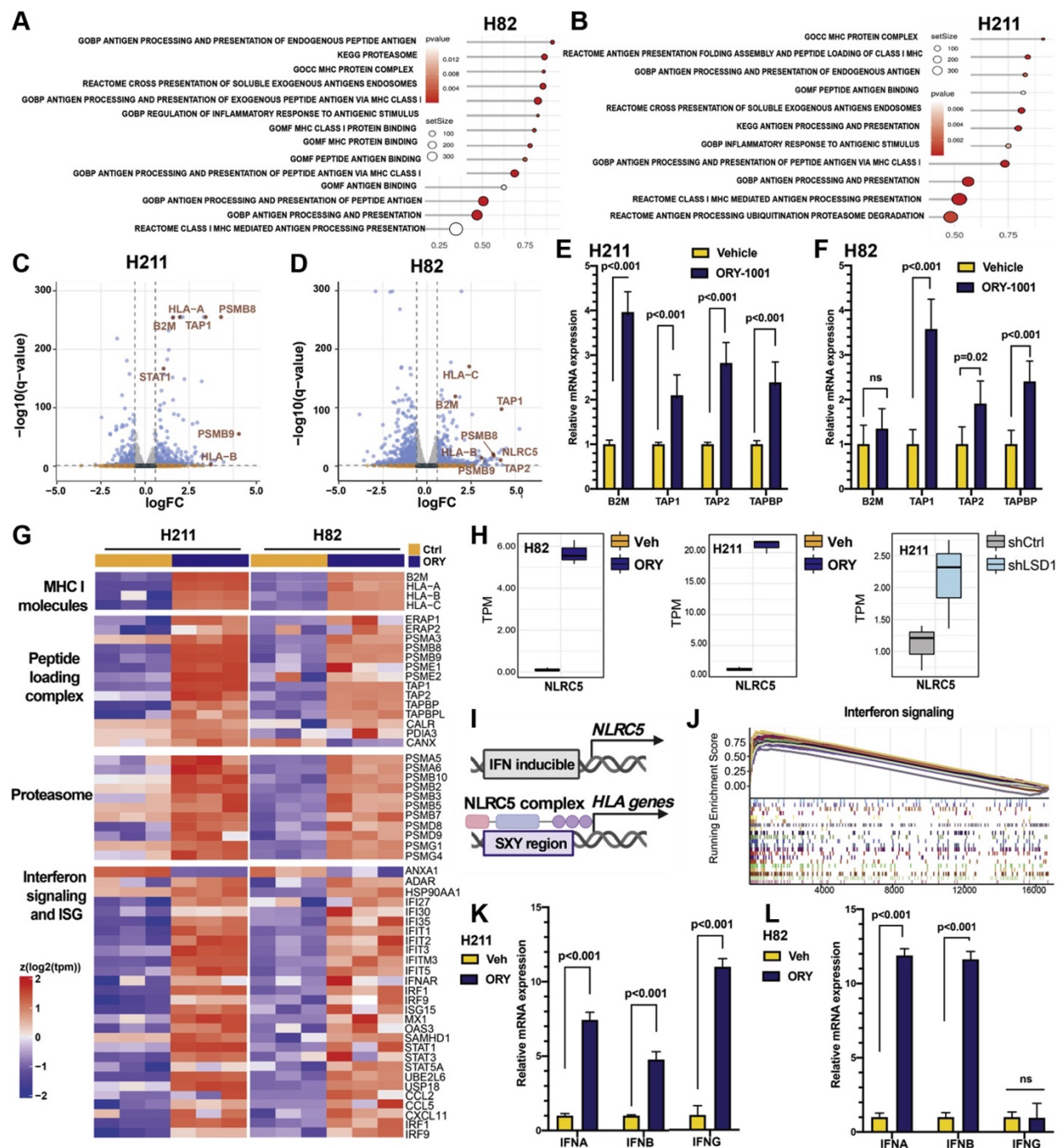


Figure 6.3. Transcriptomic characterization of SCLC cells following LSD1 inhibition. Cells treated with 1 μ M ORY-1001 or vehicle for 10 days were isolated and subjected to RNA-seq. Pathway enrichment analyses for antigen presentation pathways (APP) are shown as dot plots for (A) H211 and (B) H212. Each dot represents a gene set significantly enriched after LSD1 inhibition. Red indicates significant p-values. Size reflects number of genes in each gene set. (C-D) Volcano plots showing differential gene expression as beta value (Sleuth-based estimation of log2 fold change) in (C) H211 and (D) H212. APP-related genes are highlighted in red. (E-F) Increased relative mRNA expression of APP-related genes were confirmed by RT-qPCR for (E) H211 and (F) H212. (G) Heatmap reporting mRNA expression of MHC-I molecules, MHC-I peptide complex, proteasomal components and parts of the viral response and pan-inflammatory signatures including interferon-stimulated genes in H211 and H212 cell lines treated with 1 μ M ORY-1001 or vehicle (Ctrl). (H-I) Gene set enrichment analyses for interferon signaling represented as

enrichment scores sorted by rank list (H) or as dot plots for each individual pathway (I). (J-K) Increased relative mRNA expression of interferon genes were confirmed by RT-qPCR for (J) H211 and (K) H82.

6.5. LSD1 inhibition leads to loss of neuroendocrine signatures in neuroendocrine SCLC

SCLC subtypes have been defined by differential expression of transcription factors ASCL1, NEUROD1, POU2F3, and YAP1⁴⁴¹. Intra-tumoral heterogeneity with co-expression of more than one of these factors and tumor evolution from one predominant subtype to another have been observed in clinical and experimental models. ASCL1 and NEUROD1-positive tumors demonstrate strong neuroendocrine features and comprise >80% of all SCLC. Previous studies have demonstrated that LSD1 inhibition by ORY-1001 in a subset of SCLC cells could activate NOTCH signaling⁴²¹ and downregulate ASCL1⁴²², both of which have been associated with differentiation to a less strongly neuroendocrine phenotype. We examined NOTCH targets in H211 (POU2F3+) and H82 (NEUROD1+) and found that ORY-1001 activated NOTCH targets in both cell lines regardless of neuroendocrine phenotype (**Supplementary Figure 6.3A**). Next, we investigated whether LSD1 inhibition could specifically alter gene expression associated with a neuroendocrine phenotype. We applied the neuroendocrine signature gene sets from Cai et al. 2021⁴⁴² to our dataset. In the non-neuroendocrine/POU2F3 cell line H211, we did not observe any change in neuroendocrine signatures despite an evident shift in immunogenicity (**Supplementary 6.3B; Figure 6.3**). In contrast, the neuroendocrine/NEUROD1 line H82 exhibited coordinate suppression of multiple neuroendocrine genes, consistent with differentiation into a more non-neuroendocrine phenotype (**Supplementary Figure 6.3B**). We then applied a recently defined NEUROD1 subtype-specific signatures⁴⁴³ to further characterize the H82 phenotype after LSD1 inhibition and confirmed a loss of NEUROD1-specific signatures in this cell line (**Supplementary Figure 6.4**). Overall, our data suggest that targeting LSD1 may substantially alter patterns of gene expression influencing both immunologic responsiveness and subtype specificity in SCLC.

6.6. LSD1 inhibition rescues antigen-specific T cell-mediated recognition and cytotoxicity

To explore the functional consequences of MHC-I induction, we next assessed the effects of LSD1 inhibition on antigen-specific, cytotoxic T cell response. H82, H211 and H69 all express the HLA-A*02:01 allele (**Supplementary Figure 6.5A**). We generated luciferase-expressing derivatives of these lines and subjected them to LSD1 inhibition by 1 μ M ORY-1001 or vehicle. These cells were subsequently pulsed with the HLA-A*02:01-restricted tumor antigen NY-ESO1 peptide (SLLMWITQC), which preferentially binds to cell surface HLA-A2 with high affinity. Following antigen pulse, we co-cultured these SCLC cells with cognate CD8 T lymphocytes (CTLs) cells recognizing the HLA-A2-NY-ESO1 peptide complex (**Figure 6.4A**). These CTLs were isolated from a healthy CMV seropositive patient and pre-activated with autologous monocytes pulsed with NYESO1 peptides. As a negative control, we employed another SCLC line, H526, in which baseline MHC-I expression could be augmented by LSD1 inhibition almost up to IFN- γ -treated level, but which lacks HLA-A*02:01 expression (**Supplementary Figure 6.5B**).

Following incubation, we measured luciferase activity to characterize antigen-specific T cell killing. At higher T cell (effector) to tumor (target) ratios (E:T), we noticed low cytotoxicity activity in the vehicle treated group, which is consistent with the low-level presence of MHC-I (**Figure 6.4B; Supplementary Figure 6.2A**). At baseline, H69 was more effectively lysed than H211 and H82, consistent with their relative expression of MHC-I (**Supplementary Figure 6.2A-B; Supplementary Figure 6.5C**). In contrast to the vehicle-treated cells, markedly enhanced cytotoxicity was observed in H82, H211, and H69 pretreated with ORY-1001 (**Figure 6.4B**). We did not observe any T cell-dependent lysis of H526 even after LSD1 inhibition, substantiating the notion that NY-ESO1 presentation was restricted to HLA-A2 (**Figure 6.4B**). To confirm that the observed T cell killing activity was antigen-specific, we pulsed SCLC cells

with an unspecific HLA-A02:01-restricted antigen, MART-1/MLANA peptide (ELAGIGILTV) prior to co-incubation with NYESO-1 CTLs. MART-1 pulsed cells failed to be cleared by NY-ESO1 T cells in either treatment group, confirming that T cell mediated killing was antigen-specific (**Figure 6.4B**). Because CD8 T cells release IFN- γ after recognition of the MHC-I-peptide complex and during lysis of target cells, we performed ELISA on the co-culture media to quantify IFN- γ levels after target clearance. IFN- γ was significantly released in the media of H211 and H69 cells pretreated with ORY-1001 (**Figure 6.4C**). A similarly elevated level of IFN- γ was detected in H82 co-culture after LSD1 inhibition though the difference was not statistically significant. As expected, ORY-1001 pretreatment did not increase IFN- γ levels in the co-culture media of H526 cells (**Figure 6.4C**). Together, these results further support that the MHC-I induction by LSD1 inhibition was sufficient to enable MHC-I restricted, antigen-specific T cell lysis.

Transcriptional activation of TAP-dependent antigen presentation machinery (**Figure 6.3A-F**) led us to hypothesize that LSD1 inhibition could restore endogenous peptide processing in SCLC. These SCLC cell lines were previously shown to be antigen presentation deficient but TAP-competent⁴³⁶. To test our hypothesis, we engineered the SCLC cells to stably express either NYESO1-luciferase or MART1-luciferase peptides endogenously. We also employed the T2 cell line, which lacks a functional TAP complex and expresses an empty HLA-A*0201 on the cell surface, as an additional control⁴⁴⁴. To test the inducibility of endogenous peptide processing by LSD1 inhibition, we subjected these transduced cell lines to LSD1 inhibition or vehicle treatment before co-incubation with NYESO1-specific T cells (**Figure 6.4D**). After the co-culture period, we were readily able to detect lysis of target cells in LSD1-treated cells but not in vehicle-treated controls (**Figure 6.4E**). Absence of T cell killing was observed in both TAP-deficient T2 cells and MART1-expressing cells, indicating that T cell recognition was

dependent on functional endogenous peptide processing (**Figure 6.4E**). Consistent with these results, IFN- γ induction was observed in the co-culture media of ORY-1001-treated NY-ESO-expressing SCLC relative to vehicle-treated NY-ESO1-specific populations (**Figure 6.4F**), or to ORY-1001-treated MART-1-expressing cells (**Figure 6.4G**). Together, these data demonstrate that inhibition of LSD1 is necessary and sufficient to activate the endogenous antigen processing and presentation machinery of SCLC, which allows for antigen-specific recognition and clearance by cognate CD8 T cells.

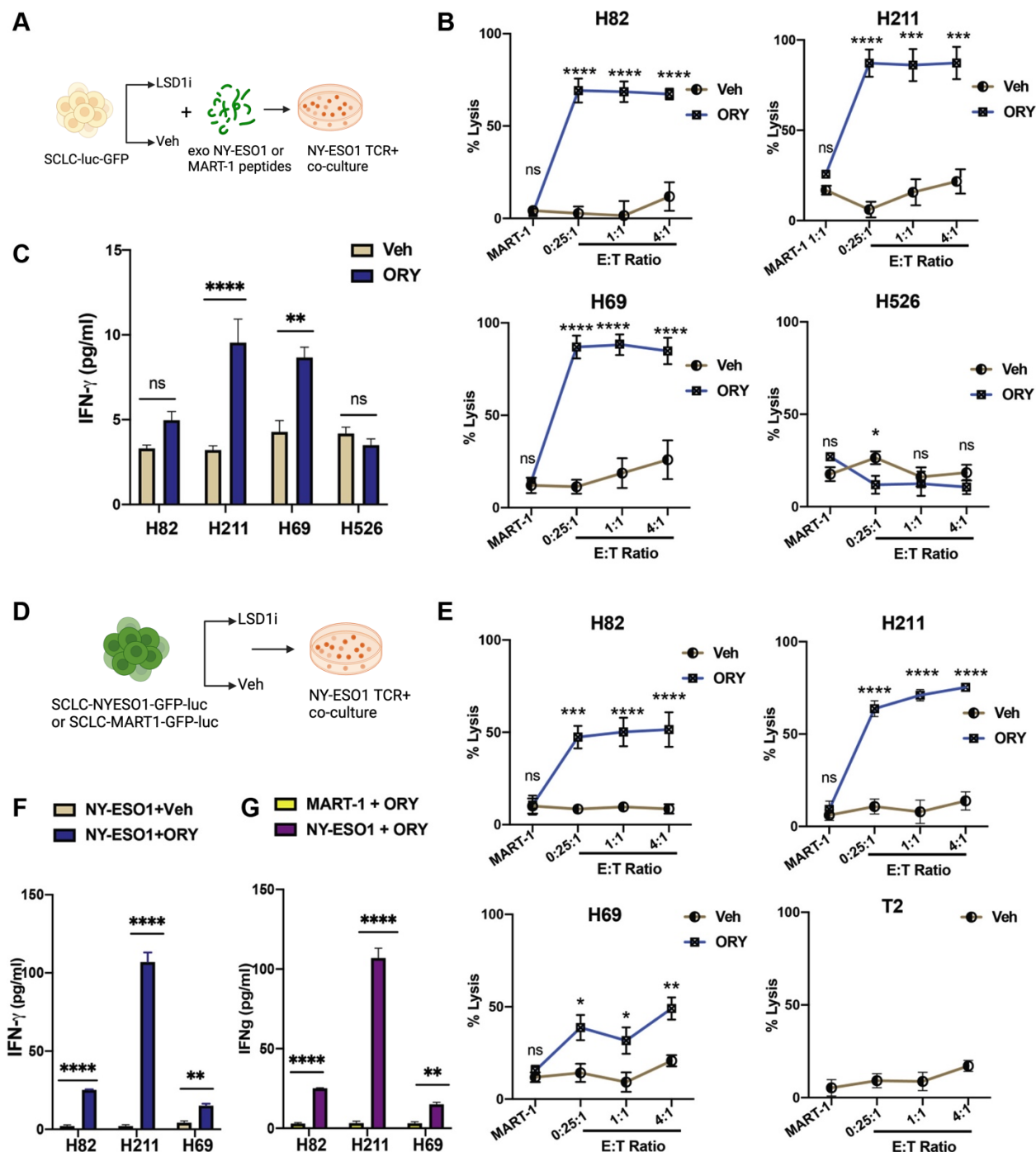


Figure 6.4. LSD1 inhibition sensitizes SCLC cells to T cell-mediated cytotoxicity. (A) Workflow of T cell co-culture experiment with pulsed peptides. H82, H211, H69 and H526 expressing luciferase were treated with either 1 μ M ORY-1001 or vehicle for 10 days before short-term exposure to NY-ESO1 or MART-1 peptides (5 μ g/ml). NY-ESO1-reactive CD8⁺T cells were introduced at specific effector-to-target (E:T) ratio and co-incubated for 18 hours. (B) Luciferase assay was performed to determine the percentage of live cells remaining in each co-culture condition compared to control samples without effector cells. For cells pulsed with NY-ESO-1 antigens values were calculated at 0.25:1, 1:1 and 4:1 ratios. Cells pulsed with MART-1 antigen were co-cultured at a 1:1 ratio with NY-ESO-1+ CD8⁺T cells. (C) Levels of human IFN- γ present in the culture media after co-incubation period were quantified using ELISA. (D) Workflow of T cell co-culture experiment with endogenous peptides. H82, H211, H69 and T2 cells

expressing either NY-ESO1-luciferase or MART-1-luciferase were treated with either 1 μ M ORY-1001 or vehicle for 10 days before NY-ESO1+ CD8+T cells were introduced at specific effector-to-target (E:T) ratio and co-incubated for 18 hours. (E) Percentage of live cells remaining in each co-culture condition compared to control samples without effector cells were assessed using a luciferase assay. For cells pulsed with NY-ESO-1 antigens values were calculated at 0.25:1, 1:1 and 4:1 ratios. Cells pulsed with MART-1 antigen were co-cultured in 1:1 ratio with NY-ESO-1+ CD8+ T cells. T2 cells, which lack TAP transporters and express an empty HLA-A*0201 allele of the MHC-I on the cell surface, served as a negative control. (F-G) Levels of human IFN- γ present in the culture media after co-incubation period were quantified using ELISA. Comparison between treatment conditions was assessed in NY-ESO-1-expressing cells lines in (F) or in MART-1- and NY-ESO-1 expressing cells treated with 1 μ M ORY-1001 (G).

6.7. LSD1 inhibition augments antitumor immune response of PD-L1 checkpoint

blockade in SCLC

The restoration of antigen presentation and activation of intrinsic immunogenicity by ORY-1001 highlights the possibility that targeting LSD1 might enhance sensitivity of SCLC tumors to immunotherapies. To explore this idea, we utilized tumors derived from a genetically engineered syngeneic model of SCLC driven by biallelic loss of *Trp53*, *Rb1* and cooperative deletion of the Rb family member *p130*⁴⁴⁵. Tumors were subcutaneously implanted into syngeneic hybrid B6129F1 mice (H2Kb haplotype). To evaluate effects of LSD1 inhibition on tumor growth, we began our treatment groups seven days after tumor injection, including cohorts treated with vehicle and IgG isotype, anti-PD-L1 (300 μ g/week), ORY-1001 (50 μ g/week), or combination of LSD1 and PD-L1 co-inhibition. PD-L1 blockade did not cause any change in tumor growth as compared to vehicle control (**Supplementary Figure 6.6A**). ORY-1001 monotherapy demonstrated limited antitumor efficacy. However, significant growth reduction was observed in the tumors treated with ORY-1001 and anti-PD-L1 (**Supplementary Figure 6.6A-C**). Mice treated with combination therapy experienced a significant survival benefit compared to PD-L1 blockade treatment alone or to control group (**Supplementary Figure 6.6B**). No significant weight loss was observed in any cohort (**Supplementary Figure 6.6D**).

To characterize the effects of LSD1 inhibition on the tumor immune microenvironment of SCLC, we initiated treatment with ORY-1001 and/or anti-PD-L1 when average tumors volumes reached 80-120 mm³ (**Figure 6.5A**). Cohorts of tumor-bearing mice were randomized and

treated with control vehicle, anti-PD-L1 (300 μ g/week), ORY-1001 (50 μ g/week), or combination and followed for two treatment cycles before euthanasia on day 18. As before, PD-L1 blockade therapy had no effect on tumor progression in this model of SCLC (**Figure 6.5B-C**). However, significant tumor reduction was observed in the ORY-1001 monotherapy, which was further enhanced when combined with PD-L1 blockade (**Figure 6.5B-C**), suggesting that LSD1 inhibition could enhance response to PD-L1 blockade in this immuno-resistant model. Next, we harvested tumors and analyzed the presence of tumor immune infiltrates. Tumors derived from combination treatment demonstrated significantly higher MHC-I expression than other groups, with tumors from the ORY-1001 alone cohort also exhibiting higher MHC-I compared to either control or PD-L1-treated tumors (**Figure 6.5D**). In addition, we found significantly more CD45+ immune cell infiltrates in the combination-treated tumors, suggesting cooperative recruitment of infiltrating immune cells (**Figure 6.5E**). While we did not observe any overall changes in CD3+ or CD4+ T cell fractions (**Supplementary Figure 6.6E-F**), we observed significantly more infiltrating CD8+ cytotoxic T cells (**Figure 6.5F**), and in particular higher levels of activated CD8+ T cells in groups given ORY-1001 (**Figure 6.5G**). These results indicate that targeting LSD1 effectively sensitizes SCLC to PD-L1 blockade therapies.

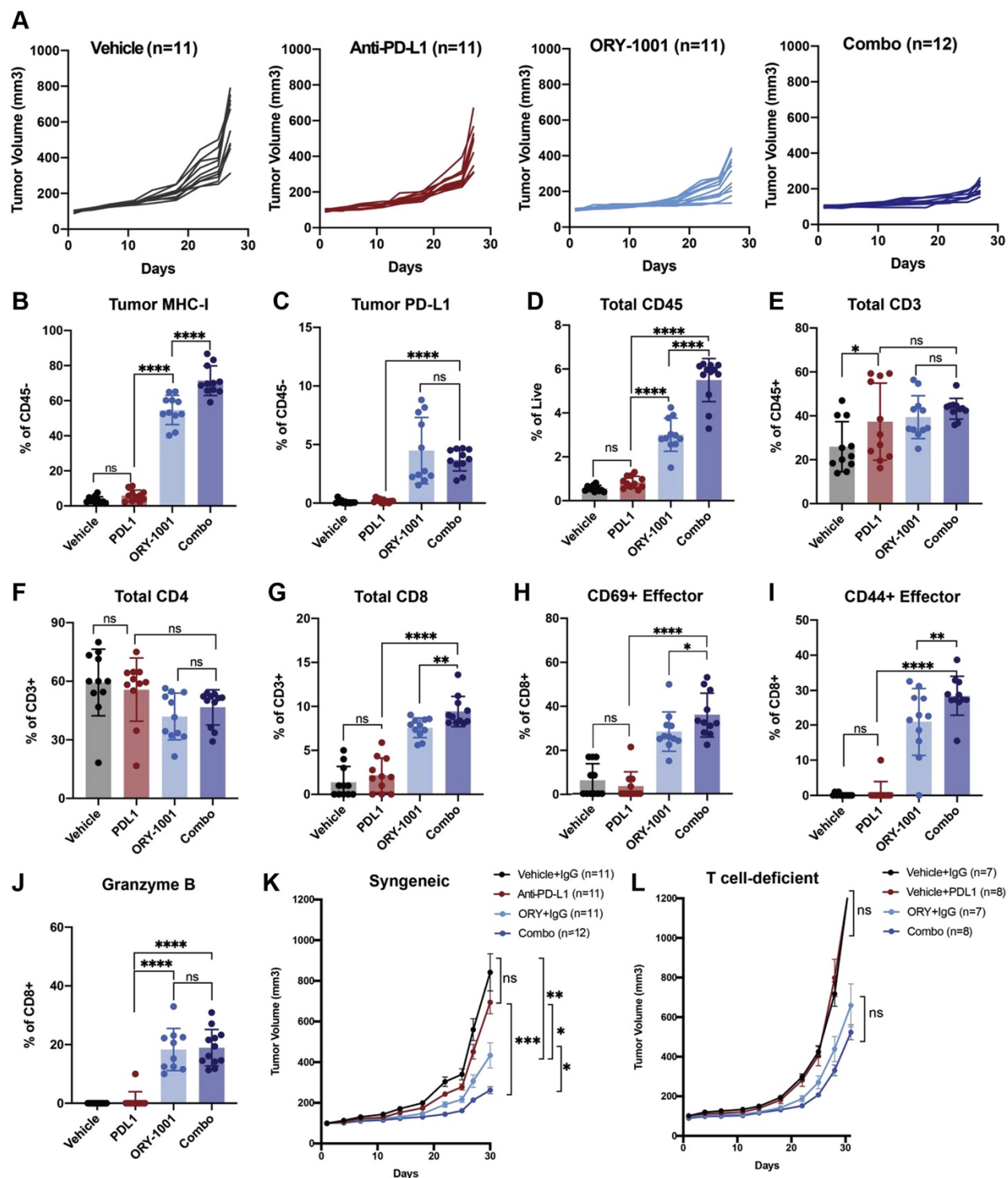


Figure 6.5. LSD1 inhibition rescues immune checkpoint blockade resistance and promotes anti-tumor immunity in a syngeneic SCLC model. (A) Schematic of animal experiment. *Rb1*^{-/-}, *p53*^{-/-}, *p130*^{-/-} RPP cells were subcutaneously grafted on B6129J female 6–8-week-old mice until tumors reached 80–120mm³ before treatment with control, ORY-1001 (50μg/animal/week), anti-PD-L1 antibody (300 μg/animal/week) or the combination on a two-week-on, two-week-off basis. Tumors were collected and analyzed once control tumors reached 1200mm³. (B) Tumor growth curves (mean ± SEM) from four treatment groups: vehicle and isotype IgG (Control) (n=5), vehicle and anti-PD-L1 antibody (PD-L1) (n=5), ORY-1001 and isotype IgG (ORY-1001) (n=4), and ORY-1001 and anti-PD-L1 antibody (ORY-

1001+PD-L1) (n=5). P values were calculated by Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001. (C) Individual tumor curves representing replicate variance within each treatment group. (D-G) RPP tumors were dissociated into single cells. 4 million live cells were subjected for immune profiling by flow cytometry. Representative plots and cumulative data for the tumors are shown. Flow cytometry analysis for CD45- tumor cells (D), CD45+ infiltrating immune cells (E), CD45+CD8+ cytotoxic T cells (F) and CD45+CD8+CD44+CD25+ activated T cells (G). Percentages in (D) and (E) were normalized to live cells defined as Ghost Violet 510 negative staining. Percentages in (F) and (G) were normalized to CD45+CD3+ T cells. P values were calculated by one-way ANOVA analysis corrected for multiple comparisons with Turkey's test; *P<0.032; ** P<0.0021 **, *** P< 0.0002; **** P<0.0001.

6.8. Summary

Immune checkpoint blockade therapies have revolutionized cancer treatment and resulted in major shifts in standard of care treatments for many solid tumors. An essential mechanism on which these T cell-based immunotherapies depend to achieve efficacy and durability is antigen presentation by MHC-I. Despite an abundance of mutation-derived neoantigens, SCLC tumors exhibit low or absent of MHC-I expression which may contribute to an immunosuppressive phenotype and resultant failure to achieve benefit from ICB therapy in the majority of patients⁴³⁸. Therapeutic reactivation of antigen presentation represents a salient strategy to enhance sensitivity to ICB in SCLC. Previous work has reported that inhibition of EZH2, an epigenetic modifier, can induce MHC-I expression in neuroendocrine tumors including SCLC^{437,438}.

In this study, we have identified LSD1 as another potent epigenetic regulator of MHC-I-mediated antigen presentation. We demonstrate a strong inverse correlation of *KDM1A* and genes involved in MHC-I presentation and further show that selective inhibition of LSD1 can significantly restore MHC-I cell surface expression, transcriptionally activate the antigen presentation pathway, trigger intrinsic interferon signaling and amplify systemic immunogenicity. Furthermore, we provided evidence that pharmacological inhibition of LSD1 is sufficient to rescue MHC-I-restricted cytotoxic T cell recognition and lysis in several SCLC models. Combination treatment with an LSD1 inhibitor and PD-L1 blockade augmented tumor growth inhibition in an immunocompetent immune-refractory model of SCLC. Importantly, we also

demonstrated markedly higher MHC-I expression, enhanced recruitment of immune infiltrates and significantly more cytotoxic and activated CD8+ T cells in tumors treated with LSD1 inhibitor. Together, our findings indicated that targeting LSD1 is a viable approach to therapeutically rescue MHC-I expression, ameliorate immune suppression and enhance SCLC sensitivity to ICB. While the degree of anti-tumor response observed in the immunocompetent allografts from a genetically engineered mouse model of SCLC is modest, greater response might be anticipated in the high tumor mutation burden tumors of patients with SCLC, given the exceptional neoantigen burden associated with heavy tobacco exposure.

Recent studies have characterized a non-neuroendocrine subtype representing <15% of all SCLC, that exhibits immune-inflamed signatures, higher MHC-I expression, and more promising response to immunotherapy^{438,442}. However, the majority of SCLC exhibits strong neuroendocrine features and low MHC-I expression, suggesting a need to modulate the immune-intrinsic profile in neuroendocrine SCLC tumors. Here, we demonstrated that induction of immunogenicity and restoration of MHC-I expression can be achieved in neuroendocrine SCLC tumors through LSD1 inhibition. We validated the previously observed LSD1-mediated activation of NOTCH signaling⁴²¹. Notably, a recent study reported that overexpression of NOTCH *in vitro* could increase MHC-I expression in SCLC⁴⁴⁶, but inactivating mutations of NOTCH in 25% of SCLC represents a hurdle to immuno-sensitize this tumor exclusively through this pathway⁴⁴⁷. We further found evidence of neuroendocrine plasticity induced by LSD1 inhibition, in particular reduction of the neuroendocrine phenotype in a NEUROD1-expressing SCLC cell line. In addition, we detected loss of NEUROD1-specific signatures in the NEUROD1-positive line, which might have translational implication as NEUROD1 tumors are associated with a particularly immune-cold SCLC subtype^{193,448}. The co-occurrence of non-neuroendocrine phenotype and enhanced immunogenicity have been reported in SCLC, but we here

demonstrated enhanced immunogenicity without evident changes in neuroendocrine profile in the POU2F3-positive model. The relationship between neuroendocrine plasticity and immunogenicity in SCLC remains incompletely characterized and warrants further investigation.

Our findings contribute to the emerging data nominating epigenetic targets as immune modulators. LSD1 inhibition has been reported to activate transcription of endogenous double-stranded RNA (dsRNA) and interferon signaling in breast cancer cell lines and to enhance response to ICB in melanoma mouse models⁴⁴⁹. This induction of tumor-intrinsic immunity by LSD1 inhibition resembles that reported in other epigenetic regulators, including DNA-methyltransferase 1 (DNMT1) and EZH2, in other cancer types^{450–453}. We supplement this theme by demonstrating that LSD1 inhibition in SCLC can enhance MHC-I expression and elicit cooperative benefits when combined with ICB. This study provides further support for the hypothesis that deficiency in antigen presentation can be epigenetically reversed through pharmacological means. Of direct translational relevance, several LSD1 small molecule inhibitors are being actively investigated in patients with SCLC. Our study provides pre-clinical support for clinical trials combining LSD1 inhibition with immune checkpoint blockade as a promising approach to SCLC, with potential implications for other MHC-I-deficient tumors.

CHAPTER 7: FUTURE DIRECTION – ANTIGEN PRESENTATION BEYOND MHC CLASS I

Cancer immunotherapies have revolutionized treatment outcome in many solid tumors; however, most patients fail to sustain long-term response to immunotherapy. A systemic understanding of the biological mechanisms underlying efficacy and evasion of immunotherapeutic holds profound promise for development of novel immunotherapeutic strategies. Many immunotherapeutic approaches hinge upon the recognition and elimination of tumor cells by CD8 T cell subsets, which relies on the dual processes of endogenous T cell priming facilitated by APCs and the presentation of tumor antigens by MHC-I molecules. Our discussion thus far indicates a pervasive modulation of HLA-I surface expression mediated by a diverse array of mechanisms, including genetic, epigenetic, post-translational modifications, transcriptional regulation and protein trafficking. Preliminary findings suggest that tumors employ multiple strategies simultaneously to attenuate HLA-I presentation, underscoring the complexity of immune evasion mechanisms in the tumor microenvironment. A substantial portion of the preceding discussion underscores this pivotal role of restoring or inducing MHC class I molecules on the cell surface. However, the field is expanding beyond the confines of class I molecules to grasp the intricate processing and presentation mechanisms of other classes of MHC molecules and their corresponding peptide and non-peptide substrates. This trajectory is imperative to achieve a comprehensive understanding of the spectrum of antigen presentation mechanism and its implications in immune surveillance and escape.

First, tumors that effectively downregulate antigen presentation may have adopted various tactics to evade 'missing-self' surveillance by natural killer (NK) cells (**Figure 7.1**). This mechanism relies on expression of non-classic MHC-I molecules, i.e. HLA-E and HLA-G, that remain to be explored in the context of tumor cells. Understanding their characteristics, including transcriptional regulation, substrate repertoire and regulatory dynamics, holds the key to advancing therapeutic interventions by harnessing the untapped potential of NK cells.

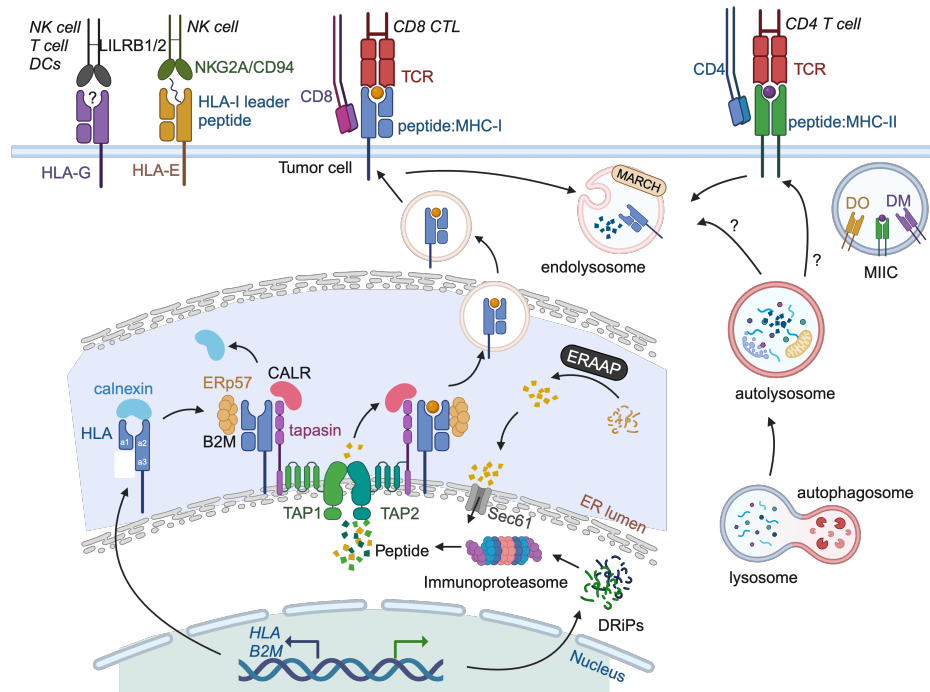


Figure 7.1. Tumor antigen presentation landscapes beyond MHC-I.

Secondly, restoration of MHC class II is crucial for enabling CD4 T cells with cytotoxic properties. Clinical studies are crucial for unraveling the pathways involved in the generation of diverse helper T-cell subsets and the presentation of immunogenic MHC Class II epitopes, distinct from tolerogenic counterparts. MHC-II presentation employs the endosomal pathway that can converge with autophagy, a cellular mechanism pivotal for maintaining cellular homeostasis. Autophagy has emerged as a pathway through which endogenous antigens can be delivered to MHC-II molecules, facilitating their presentation to CD4⁺ T cells. Furthermore, recent findings suggest that autophagy plays a role in MHC class I cross-presentation of tumor antigens, thereby contributing to the activation of CD8⁺ T cells. Additional investigations pave the way to elucidate the interplay between different autophagy pathways and the presentation of both MHC class I and class II antigens during adaptive responses against tumors and self-antigens. Consequently, these insights may elucidate novel therapeutic avenues to address deficiency in antigen presentation and offer significant therapeutic benefits in cancer patients.

SUPPLEMENTARY TABLE

Supplementary Table 5.1. Cohort Summary

Patient Characteristics		All Patients (N=263) No. (%)	1L Platinum Cohort (N=189) No. (%)	IO Cohort (N=130) No. (%)
Median age at diagnosis (range)		66 (20, 91)	66 (20, 91)	66 (20, 91)
Gender (%)	Female	128 (48.7)	89 (47.1)	57 (43.8)
	Male	135 (51.3)	100 (52.9)	73 (56.2)
Cigarette smoking status (%)	Former/current, median 40 pack years	241 (91.6)	173 (91.5)	120 (92.3)
	Never	22 (8.4)	16 (8.5)	10 (7.7)
Stage at initial diagnosis (%)	Limited-stage	95 (36.1)	43 (22.8)	38 (29.2)
	Extensive-stage	168 (63.9)	146 (77.2)	92 (63.0)
Metastasis (%)	Brain (%)	60 (22.8)	50 (26.5)	36 (27.7)
	Liver (%)	100 (38.0)	85 (45.0)	59 (45.4)
Analysis	MSK-IMPACT	248 (94.3)		115 (88.5)
	MSK-IMPACT + Whole Exome Sequencing (WES)	125 (47.5)		54 (41.5)
	Immunohistochemistry (%)	103 (39.1)		65 (50.0)
	RNA-seq (%)	47 (17.9)		30 (23.1)
	RNA-seq + IHC	9 (3.4)		9 (6.92)
	WES + IHC	58 (22.1)		23 (17.7)
	WES + IHC+ RNA-seq	20 (7.6)		11 (8.5)
	WES + RNA-seq	20 (7.6)		11 (8.5)
Subtype (n=113) (H-score >= 100)	ASCL1	79 (30.0)		
	NEUROD1	26 (9.9)		
	ASCL1/NEUROD1	18 (6.8)		
	POU2F3	3 (1.1)		
	YAP1	2 (0.8)		
	Negative	12 (4.6)		
PD-L1 status (IHC) (%)	Positive	4 (1.5)		
	Negative	99 (37.6)		
INSM1 status (IHC) (H-score >=50) (%)	High/med	70 (26.6)		
	Low/neg	33 (12.5)		
Tumor Mutation Burden (TMB) Mean (range)		9.3 (1-29)	9.1 (1.8-29)	8.8 (1.8-21.9)

Supplementary Table 5.2. Summary of Clinical Responses

Immunotherapy Cohort (N=130) No. (%)			
Immunotherapy (n=130)	Monotherapy received (%)		105 (81)
	Combined with chemotherapy (%)		25 (19)
Best overall response No. patients (%)	Complete response (CR)		2 (1.5)
	Partial response (PR)		32 (25)
	Stable disease (SD)		15 (11.5)
	Progressive disease (PD)		71 (55)
	Not Applicable		10 (8)
Median progression free survival in months (range)	Extensive Stage		1.9 (0, 126)
Median overall survival in months (range)	Extensive-stage*		7.3 (0, 126)
Immunotherapy Received (%)	Cisplatin+etoposide+atezolizumab	1L	23 (18)
	Nivolumab	2L+	19 (15)
	Ipilimumab + Nivolumab	2L+	82 (63)
	Other	2L	5 (4)
	Other	1L	1 (0)
Lines of Therapy Prior to Immunotherapy (%)	0		24 (18)
	1		78 (60)
	2		20 (15)
	>=3		9 (7)
First-Line Therapy (%)	Chemo		106 (47)
	ChemoIO		24 (18)

Supplementary Table 6.1. Antibodies used for western blot

Antibody	Source	Catalog #	Dilution
beta-microglobulin	Cell Signaling	#59035	1:1000
Di-Methyl-Histone H3 (Lys4)	Cell Signaling	#9725	1:1000
beta actin	Cell Signaling	#4970	1:1000
LSD1	Cell Signaling	#2184	1:1000

Supplementary Table 6.2. Probes used for RT-qPCR

Gene	Source	Assay ID
IFN- α	Thermo Fisher	Hs03044218_g1
IFN- β	Thermo Fisher	Hs01077958_s1
IFN- γ	Thermo Fisher	Hs00989291_m1
B2M	Thermo Fisher	Hs00187842_m1
TAP1	Thermo Fisher	Hs00388675_m1
TAP2	Thermo Fisher	Hs00241060_m1
TAPBP	Thermo Fisher	Hs00917451_g1
GAPDH	Thermo Fisher	Hs99999905_m1

Supplementary Table 6.3. Antibodies used for flow cytometry

	Fluorophores	Marker	Source	Clone	Catalog#	Dilution
Mouse	Ghost violet 510	Live/Dead	Tonbo		13-0870-T500	1/100
	Pacific Blue	CD45	BioLegend	30-F11	103126	1/100
	PE/Dazzle594	CD3	BioLegend	17A2	100246	1/100
	APC/Cy7	CD4	BioLegend	RM4-5	100526	1/400
	PE Cy7	CD8	BioLegend	53-6.7	100722	1/800
	PE	CD69	BioLegend	H1.2F3	104532	1/50
	BUV395	CD25	BD	PC61	564022	1/100
	BV605	PD1	BioLegend	29F.1A12	135220	1/100
	BV711	CD44	BioLegend	IM7	103057	1/100
	BUV737	NKp46	BD	29A1.4	565085	1/100
	FITC	H2Kb	BioLegend	AF6-88.5	116506	1/100
	BV421	TIM3	BioLegend	RMT3-23	119723	1/200
	BV785	PDL1	BioLegend	10F.9G2	124302	1/100
	APC	Granzyme B	BioLegend	505810	396408	1/50
	PerCP Cy5.5	FoxP3	eBioscience	FJK-16s	45-5773-82	1/100

Human	HLA-A,B,C	APC	BioLegend	W6/32	311410	1/64
	HLA-A2	FITC	BioLegend	BB7.2	343304	1/200
	Mouse IgG2a, kappa	Legend	BioLegend	MOPC-173	400220	1/400

Supplementary Table 6.4. Neuroendocrine signature gene list

Adapted from Cai et al 2021

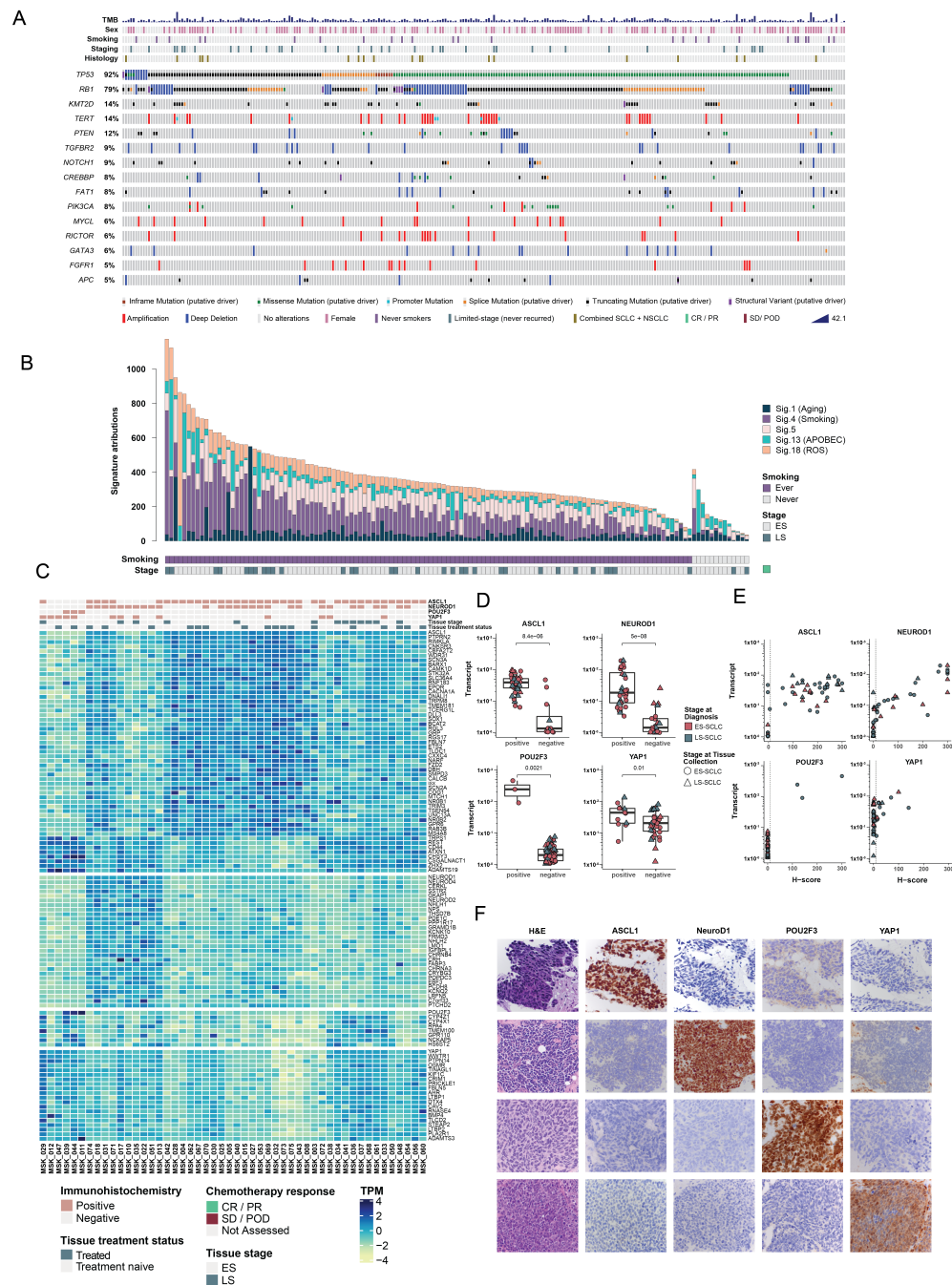
SNAP25	ITGB4
AP3B2	HFE
SYP	NABP1
BEX1	LGALS3
NCAM1	IFITM3
RUNDC3A	TGFBR2
SCG3	NT5E
KIF1A	WWTR1
TUBB2B	EMP1
INSM1	ANXA3
TMEM198	IL18
CRMP1	EPHA2
CHGA	S100A16
RAB39A	CYR61
UNC13A	YAP1
MAPK8IP1	RHBDF1
KIF5C	AHNAK
PGBD5	ADGRG6
GNG4	GPRC5A
SCAMP5	ANXA1
GNAO1	CAV1
CHGB	MYOF
DPYSL5	CAV2
SOGA3	OSMR
CHRNA2	
GPX8	

Supplementary Table 6.5. NEUROD1-specific signatures

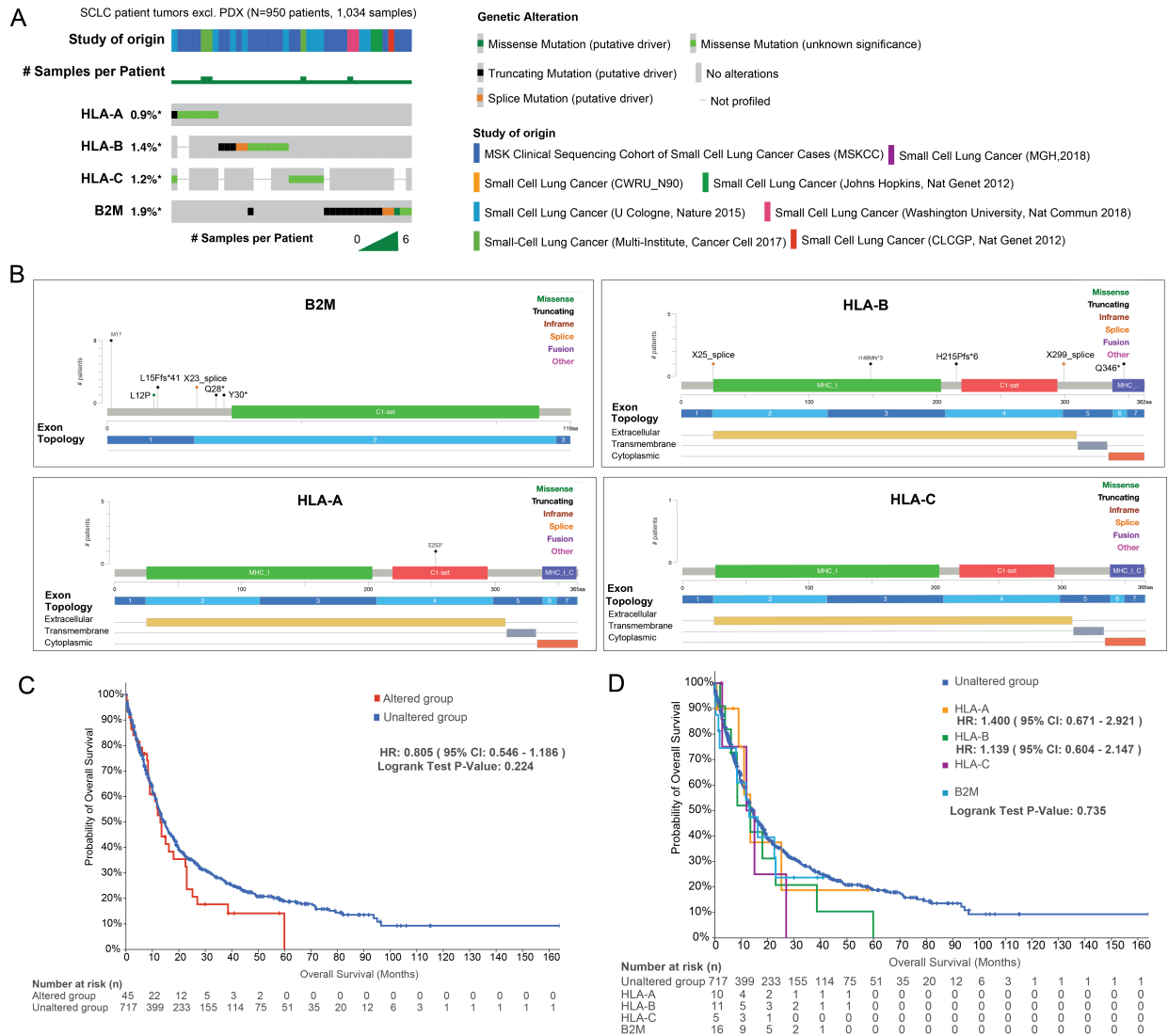
Adapted from Chan et al 2021

ZFP36L1	MFAP4	TPT1	TUBB2B	ASCL1	LSM4
ADCYAP1	RCN1	TMEM233	PPP1R14B	BASP1	MAD2L2
HLA-B	MTRNR2L12	DLK1	TUBA1B	C12ORF75	MARCKSL1
HLA-E	PRAME	DUSP6	UCHL1	CD24	MEST
SIX1	MYO6	ELAVL2	SOX4	CDK2AP1	MGST1
HSPA1A	POMC	ETFB	PTMS	CFL1	MIF
HSPA2	NDUFA4L2	CD99	RBP1	COTL1	MLLT11
ID1	PGF	APOE	TUBA1A	COX17	MTRNR2L8
ID3	NEUROD1	GKAP1	RTN1	CTXN1	NAA38
IER3	NEUROD4	FBLN1	TFF3	DHPS	KRT7
IGF2	OLFM1	SSTR2	STMN1	FAM3B	
IGFBP2	HLA-A	GDF15	SEC11C	FTH1	
SAT1	HLA-C	SST	SOX2	FXD6	
ITM2C	PEG10	VIM	PERP	GRP	
S100A11	FAM213A	GAS2	TPD52	HMGN2	
RRBP1	CD63	HSPA1B	AC090498.1	HOXB5	
HES6	CHGA	HIST1H4C	NDUFB4	ICA1	
RPRML	CHGB	TUBB	NDUFA3	IGFBP5	
RGS10	COL1A2	UQCRC1	AES	KLF6	

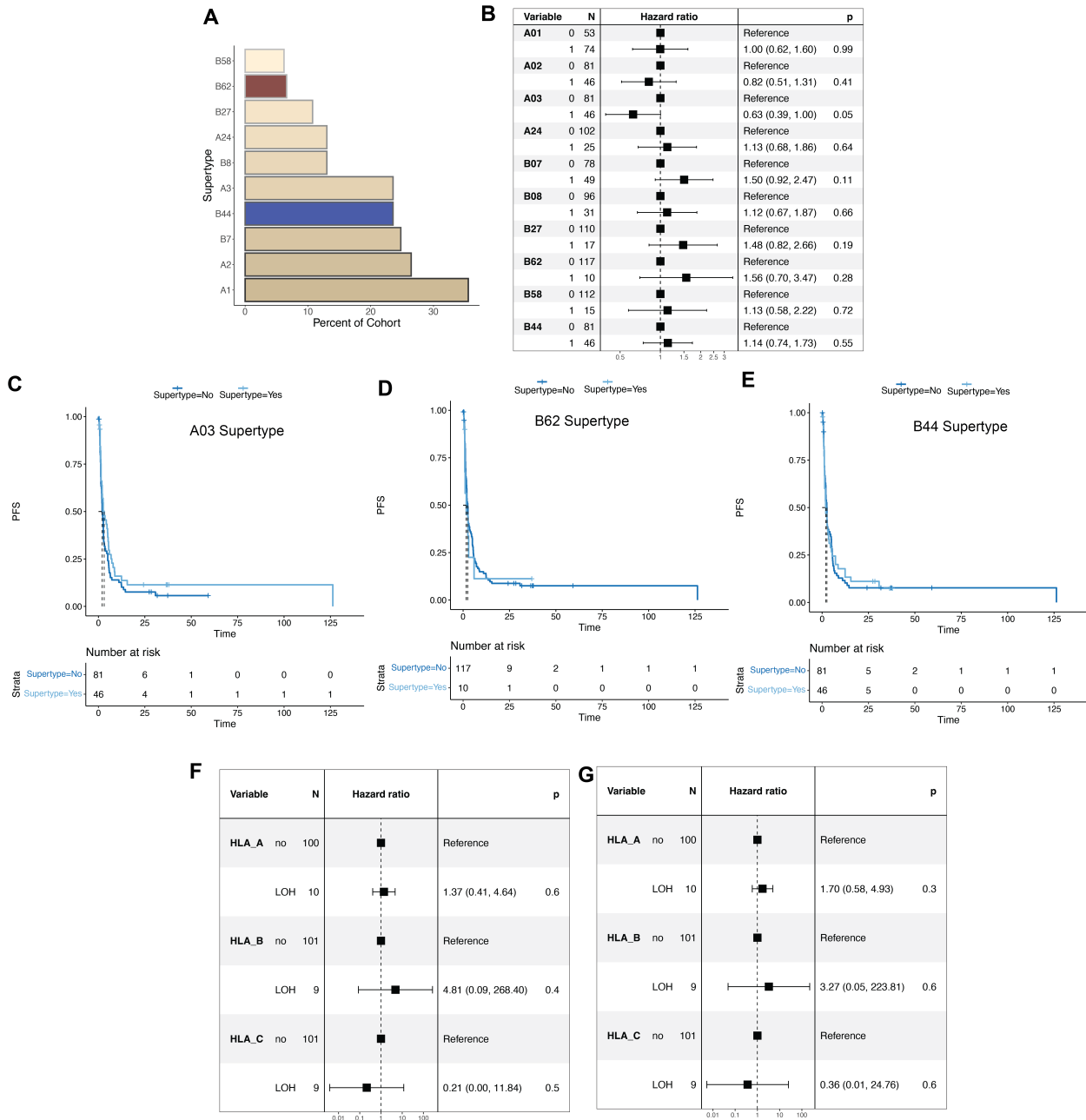
SUPPLEMENTAL FIGURE



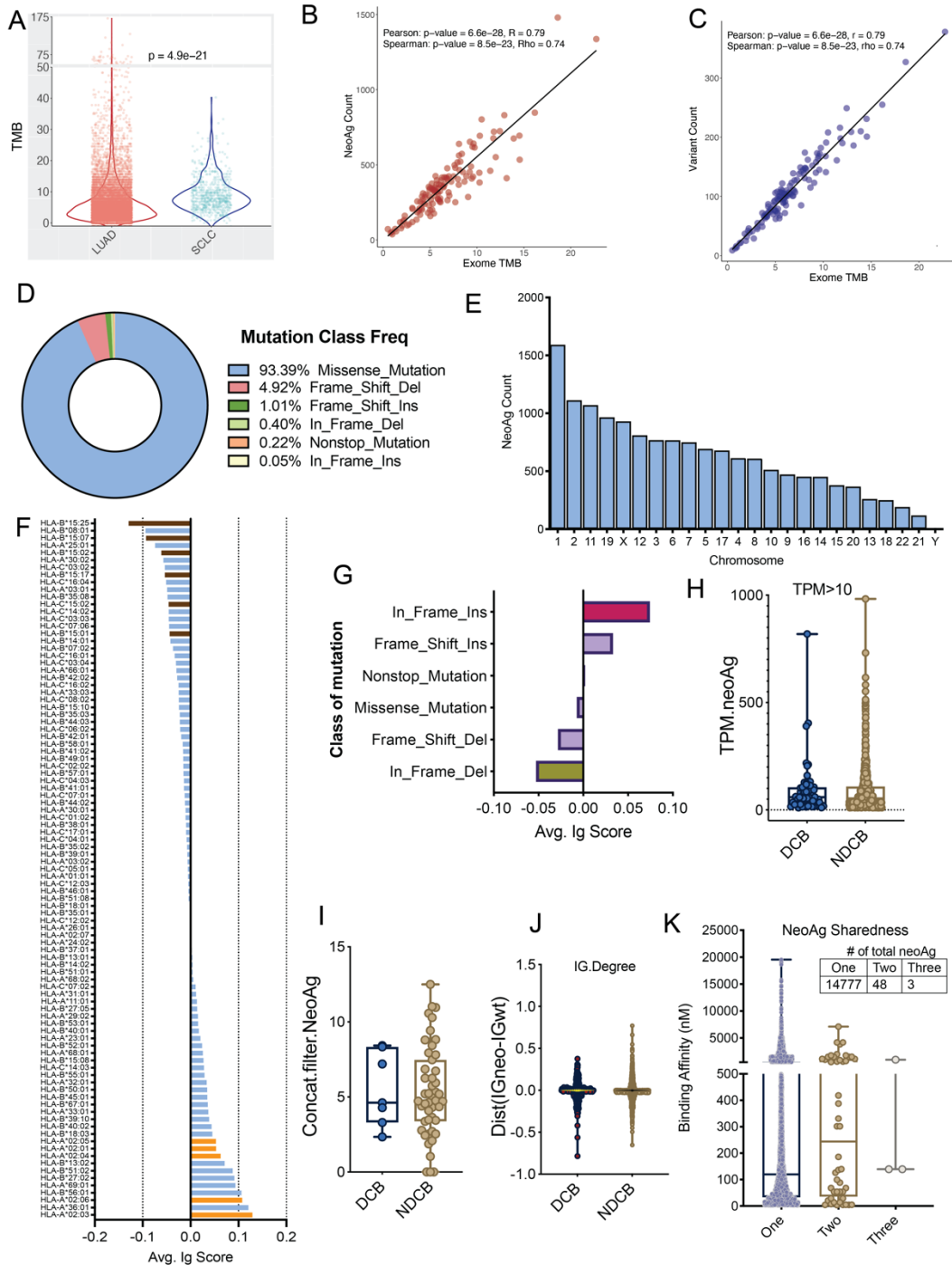
Supplementary Figure S5.1. Cohort overview (A) Oncoprint of the most frequent mutations in cohort (B) Profiling for common mutational signatures (C) Heatmap indicating transcriptional profiles associated with each molecular subtype (D) Comparison of expression transcripts and positivity definition (E) Correlation of subtype vs H-score (F) H&E staining and molecular subtype staining for SCLC tumors.



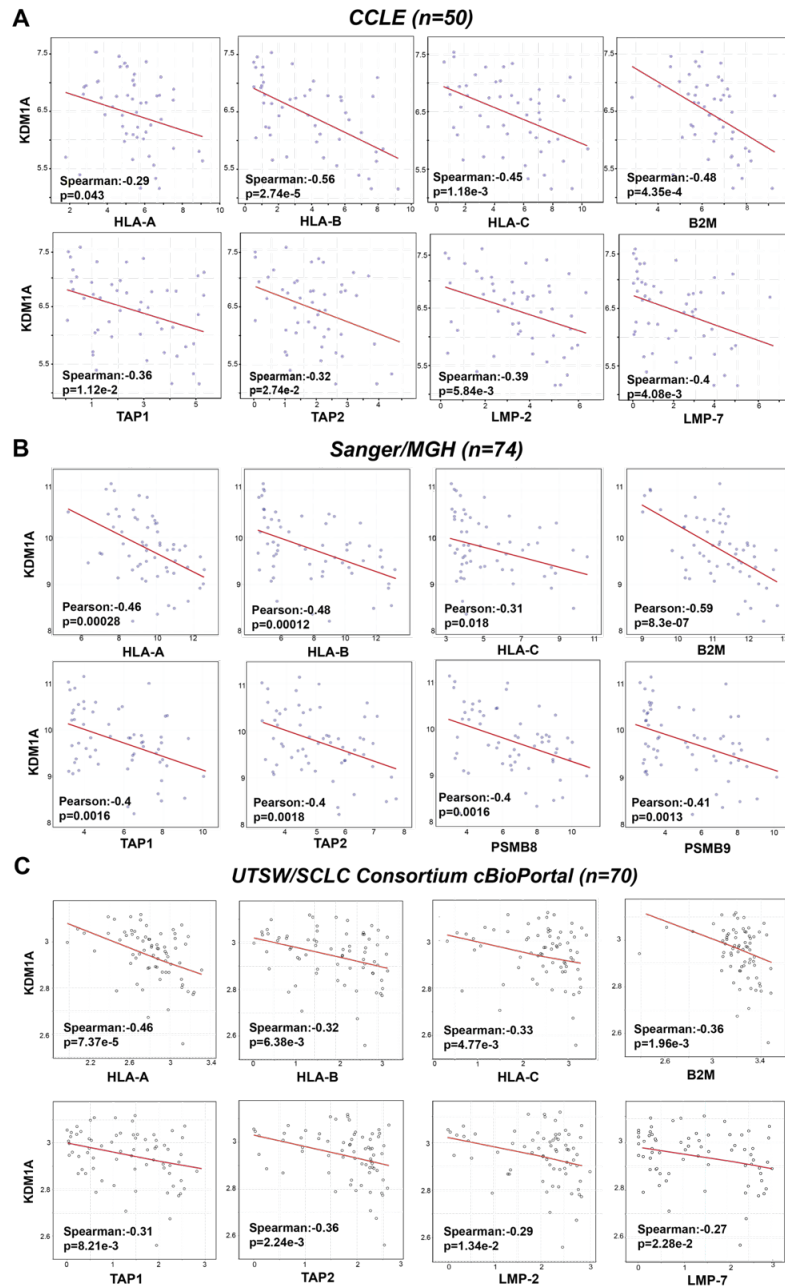
Supplementary Figure S5.2. HLA mutation in SCLC cohort (A) Oncoprint of HLA and B2M mutational spectrums in 950 patients and 1,034 samples from the cBioportal public database (B) Mutation localization for B2M, HLA-A,-B, and -C, excluding variants of unknown significance (C) and (D) Probability of overall survival for all altered HLA (C) or segmented by specific HLA genes (D)



Supplementary Figure S5.3. HLA supertype characterization (A) Cohort distribution of nine HLA supertypes. (B) Forest tree displaying hazard ratio for each supertype. Probability of survival of A03 supertype (C), B62 supertype (D), and B44 supertype (E). Hazard Ratio for PFS of LOH of HLA (F) and OS (G).

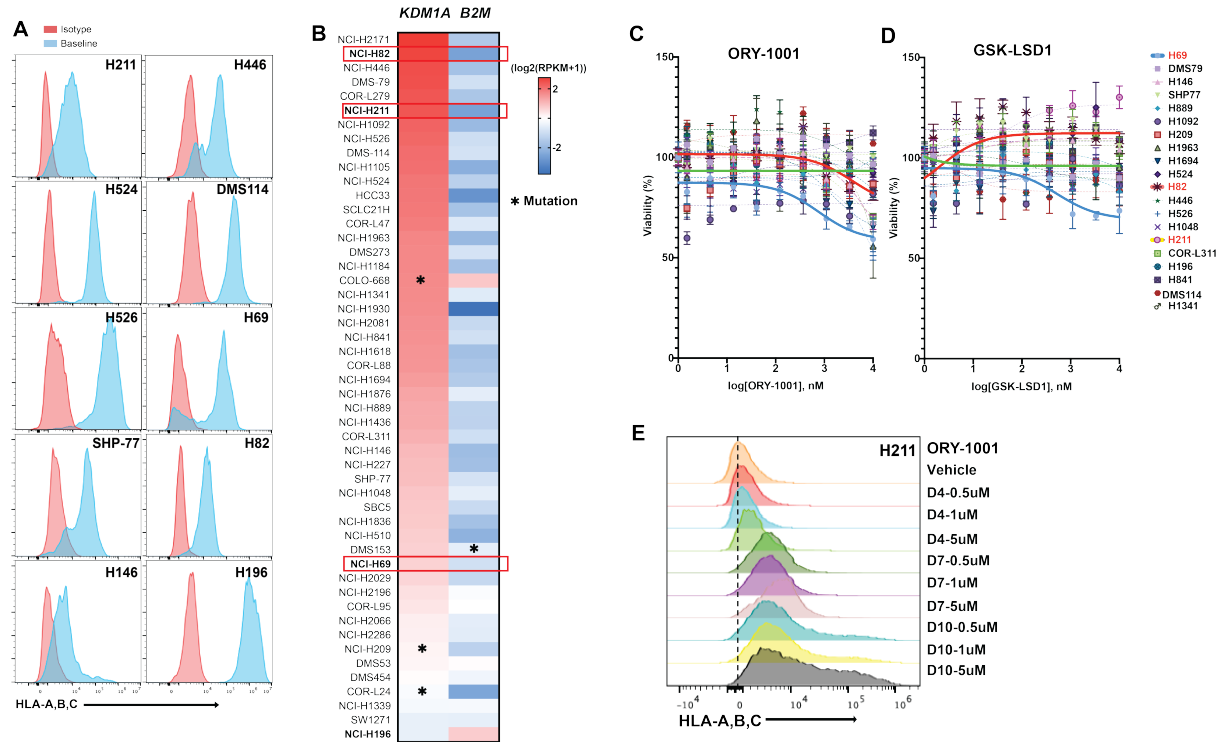


Supplementary Figure S5.4. TMB and neoantigen characterization. (A) TMB of LUAD and SCLC (B) Scatterplot for TMB and neoantigen (C) Scatterplot for TMB and variant count (D) Frequency of mutation classes (E) Distribution of neoantigen across chromosomes (F) Immunogenicity score of all neoantigens predicted to specific HLA allelotype (G) Immunogenicity score of neoantigens associated with different classes of mutation (H) Transcript per million RNA expression of neoantigens from DCB and NDCB (I) Fraction of neoantigens passing all qualitative benchmarks between DCB and NDCB (J) Distance of immunogenicity score of neoantigen vs that of wildtype allele between DCB and NDCB (K) Neoantigen binding affinity among unique neoantigens (one), or those shared between two (two) or three (three) patients.

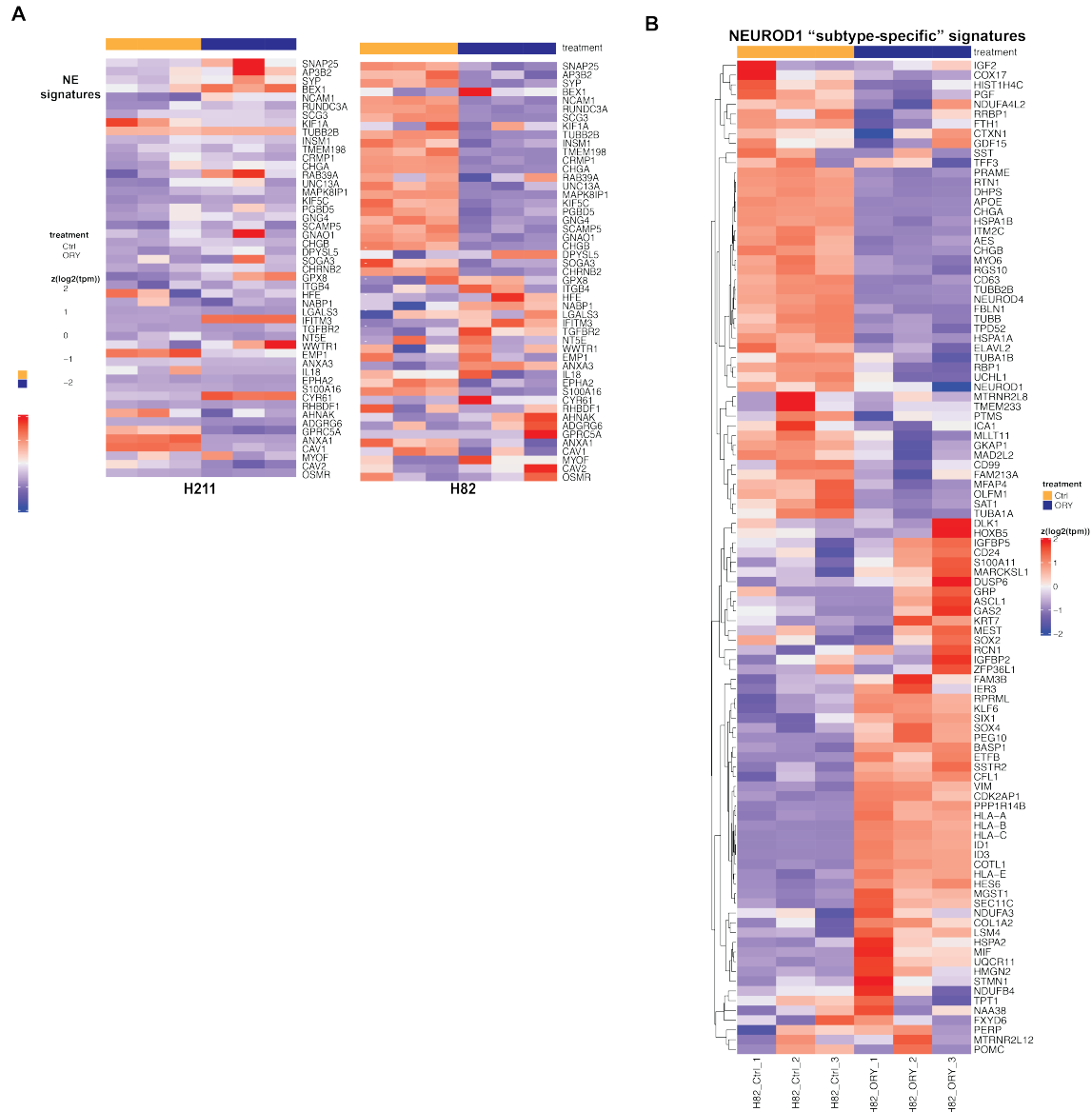


Supplementary Figure S6.1. LSD1 expressed at higher levels in small cell lung cancer than any other tumor type. Spearman's and Pearson's correlations indicate significant anticorrelation between LSD1 (*KDM1A*) expression and HLA-A, HLA-B, HLA-C, B2M, TAP1, TAP2, LMP-2 (*PSMB8*), LMP-7 (*PSMB9*) from (A) Cancer Cell Line Encyclopedia (CCLE) database (Broad, 2019) (downloaded from cBioportal-MSKCC) and (B) Sanger/Massachusetts General Hospital (MGH) dataset (downloaded from CellMiner) (C) University of Texas Southwestern (UTSW) (downloaded from SCLC Consortium, cBioportal database). Values were calculated as RNAseq RPKM ($\log_2(\text{value}+1)$).

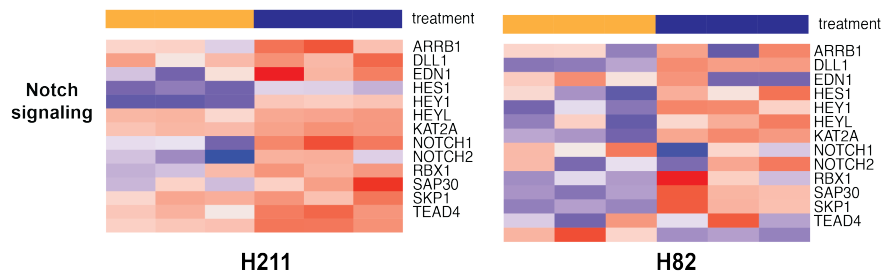
Supplementary Figure 2



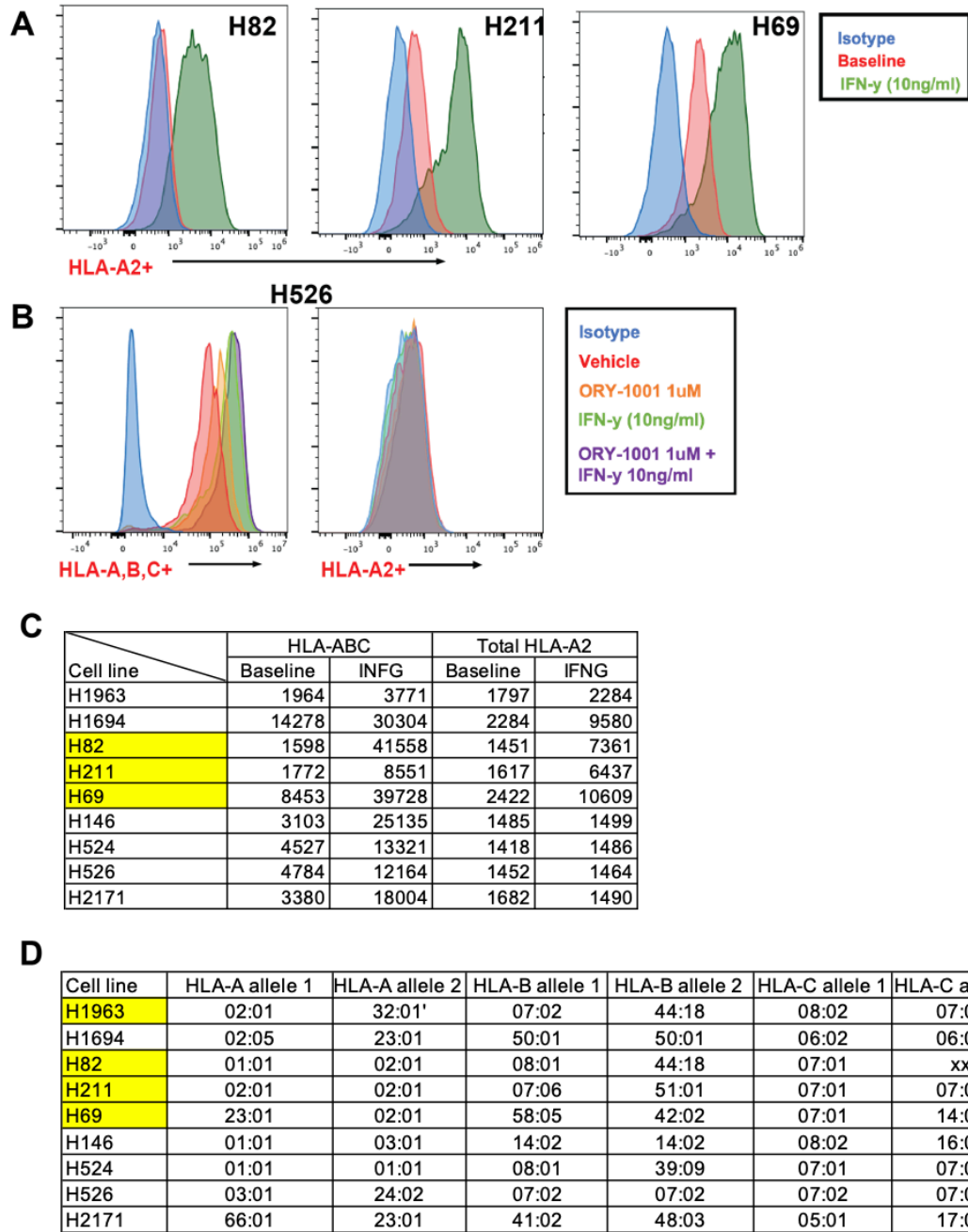
Supplementary Figure S6.2. Baseline cell surface MHC-I and gene expression in SCLC cell lines. (A) Baseline expression of total MHC-I cell surface expression assessed in 10 different SCLC cell lines by flow cytometry. (B) Heatmap detailing the anti-correlation of LSD1 and B2M and mutational features in SCLC cell lines from the CCLE dataset. Three cell lines H82, H211 and H69 were selected for subsequent analyses. (C) H211 cells were treated with varying concentrations of ORY-1001 after several time points to assess concentration- and time-dependent effects of LSD1 inhibition on MHC-I expression by flow cytometry. (D) Doxycycline-inducible shRNA knockdown of LSD1 or non-targeting control was performed for five different shRNA constructs on H82 following treatment with doxycycline (100ng/ μ l) at 1, 4 and 7 days.



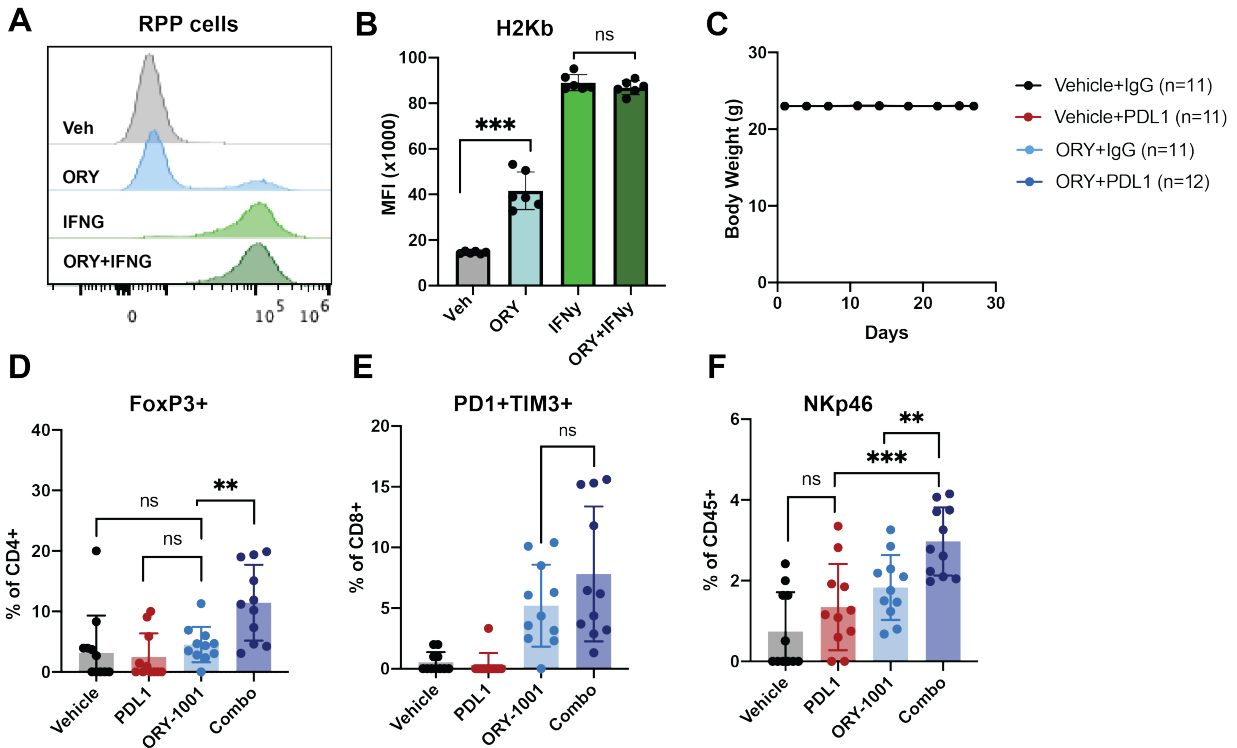
Supplemental Figure S6.3. LSD1 inhibition activates Notch signaling and reduces neuroendocrine gene expression in SCLC. Heatmap indicating Notch signaling upregulation (A) and downregulation of neuroendocrine signature genes (B) in H82 and H211 following treatment with 1 μ M ORY-1001 or vehicle.



Supplemental Figure S6.4. NEUROD1 subtype loses NEUROD1-specific signatures following LSD1 inhibition. NEUROD1-specific signatures were compiled from Chan et al 2021 and applied for H82 transcriptomic analyses following treatment with 1 μ M ORY-1001 or vehicle.



Supplemental Figure S6.5. HLA-A2 expression in SCLC cell lines. (A) H82, H211 and H69 express HLA-A2 as assessed by flow cytometry following incubation with 10ng/ml IFN- γ for 24 hours. (B) Total MHC-I expression, but not HLA-A2 allele, was upregulated in H526 following LSD1 inhibition and incubation with 10ng/ml IFN- γ for 24 hours. (C) Summary of evaluation of total MHC-I expression and cell-surface HLA-A2 allele-type by flow cytometry.



Supplemental Figure S6.6. LSD1 inhibition by ORY-1001 confers significant tumor growth reduction and survival advantage. *Rb1*^{-/-}, *p53*^{-/-}, *p130*^{-/-} RPP cells were subcutaneously grafted into B6129J female 6–8-week-old mice. Seven days later, treatment commenced on a three-week-on, two-week-off basis with either vehicle and isotype IgG (Control), vehicle and anti-PD-L1 antibody (PD-L1), ORY-1001 and isotype IgG (ORY-1001), or ORY-1001 and anti-PD-L1 antibody (ORY-1001+PD-L1). (A) Tumor growth curves (mean ± SEM) from four treatment groups: Control (n=7), PD-L1 (n=7), ORY-1001 (n=7), and ORY-1001+PD-L1 (n=6). (B) Survival curve showing significant survival advantage in ORY-1001 and anti-PD-L1 antibody combination group compared with anti-PD-L1 antibody alone. (C) Individual tumor curves representing replicate variance within each treatment group. (D) Body weight changes in this experiment. (E-F) Flow cytometry analysis for CD3+ and CD3+CD4+ T cells. Percentages in (E) and (F) were normalized to CD45+ and CD3+ T cells, respectively. P values were calculated by one-way ANOVA analysis corrected for multiple comparisons with Turkey's test.

MATERIAL AND METHODS²

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patient cohort

The present study consisted of 263 *de novo* SCLC patients treated at Memorial Sloan Kettering Cancer Center between 2008 and 2019. We limited this analysis to samples that were classified by pathologists as “pure” or *de novo* SCLC, defined by WHO histopathological classification^{323,375}, as opposed to “combined” SCLC, characterized by discrete subregional features of squamous cell carcinoma (SCC) or lung adenocarcinoma (LUAD). This study was approved by MSK Institutional Review Board (IRB) for biospecimen collection and analysis. All patients provided written informed consent for tumor sequencing via approved IRB protocol (MSKCC IRB #12-245). A retrospective review of electronic medical records was performed to obtain detailed clinical, demographic, pathology and treatment information.

Clinical response definition

In total, this cohort includes 130 patients who received any form of immunotherapy (49.4%). Durable clinical benefit (DCB) from immunotherapy was observed in 27 patients (20.7%), defined as progression-free survival (PFS) of 6 months or longer from the onset of immunotherapy treatment.

METHOD DETAILS

Survival Analysis

130 SCLC patients treated with immune checkpoint blockade therapies were analyzed for survival benefit adjusting for clinical covariates of interest, as specified in the figure legends. Follow-up time amongst patients who are alive is 59.14 months (IQR: (38.72, 76.89)). Analyses of median survival, progression-free survival (PFS) and overall survival (OS) were computed

² Related to Chapter 5

using R version 4.3. 4.3.2 with the tidyverse (v2.0.0), gtsummary (v1.7.2), survival (v3.5.7) and ggsurvfit (v1.0.0) packages. PFS was calculated from treatment start date until date of progression, death, or last follow-up date, whichever occurs first. OS was calculated from treatment start date until date of death or last follow-up date. Progression events were defined as patients that experienced progression or death, whichever first occurs. 11 patients experienced a PFS event before or on the IMPACT Report date. All PFS calculations were restricted to patients who were alive and progression-free at the time of IMPACT report. Patients who did not experience progression or death events were censored at the last follow-up date. Kaplan-Meier curves are plotted with 95% log confidence intervals. Comparisons between MHC-I status, CD8 status and TLS status were performed for both PFS and OS using the log-rank test. Univariable Cox proportional hazard models were performed to investigate associations between PFS or OS and clinical covariates of interest. Follow-up time amongst patients who are alive was reported using the median and interquartile range (IQR.) All tests were two-sided and a p-value < 0.05 was considered statistically significant. For sensitivity analyses, PFS and OS were further analyzed accounting for left truncation using IMPACT report date via the Kaplan-Meier method with left truncation.

Targeted Capture RNA Sequencing and Quality Control

47 libraries corresponding to an equal number of samples were sequenced. Approximately 200-500 ng of RNA extracted from FFPE slides with a DV200 range between 3-99 per sample were used for RNA library construction using the KAPA RNA Hyper library prep kit (Roche, Switzerland). The number of pre-capture PCR cycles was adjusted based on the quality and quantity of RNA extracted from the samples. Custom adapters with 3bp unique molecular indexes (UMI) (Integrated DNA Technologies, USA) and sample-specific dual-index primers (Integrated DNA Technologies, USA) were added to each library. The quantity of cDNA libraries

was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA) and the quality was assessed by TapeStation Genomic DNA Assay (Agilent Technologies, US). Approximately 500 ng of each cDNA library was pooled for hybridization capture with the IDT Whole Exome Panel V1 (Integrated DNA Technologies, US) using a customized capture protocol modified from NimbleGen SeqCap Target Enrichment system (Roche, Switzerland). The captured cDNA libraries were then sequenced on an Illumina HiSeq 4000 in paired end (2X100bp) to a target of 50M read pairs per sample according to manufacturer's instructions. Image analysis and base calling were conducted by the HiSeq Control Software. Raw sequence data generated from Illumina HiSeq were converted into fastq files, and demultiplexed FASTQs were aligned to the human genome reference hg19/GRCh37 using STAR (version 2.7.3a)⁴⁵⁴ and deduplicated from the combination of UMI sequence and alignment coordinate using UMI-tools (version 1.0.1)⁴⁵⁵. Transcript counts and abundance were quantified from RNA-seq reads using Kallisto (version 0.46.2) from the unique read pairs and summarized at the gene level using the R library Sleuth (version 0.30.0)⁴⁵⁶. Quality control metrics for the sequenced libraries included the (1) number of unique pass filter (PF) read pairs, (2) deduplication ratio i.e. total/unique PF read pairs, (3) number of genes with 0 count mapped unique PF read pairs, and 3) RNA categories from Picard CollectRnaSeqMetrics. One sample was excluded from further downstream analyses due to low deduplication ratio and failing to reach a minimum of 10M unique PF read pairs and was composed of 10% ribosomal RNA. The remaining 46 libraries had a deduplication ratio ranging from 60 to 92% and between 14M and 82M unique PF read pairs.

Differential Gene Expression and Pathway Analyses in RNA-seq

Expression measures were estimated and normalized at the gene level from the aligned and deduplicated alignments using Kallisto. Differential expression at the gene level was computed using a moderated *t*-statistics with a Bayes adjusted denominator using the R/Bioconductor

library limma (version 3.44.1)^{457,458}. Genes were marked as statistically significant if Sleuth-based estimation of log2 fold change is 1.2-fold difference or higher and a p-value of less than 0.05.

Gene Set Enrichment Analysis was performed^{459,460} on the unique transcript abundances as implemented in the R/Bioconductor library GSVA (version 1.36.0)⁴⁶¹. Genes were ranked on p value scores computed as $-\log_{10}(\text{p value}) * (\text{sign of beta})$. The significance level of enrichment was evaluated using permutation test, and the *p* value was adjusted by BH procedure. Any enriched gene sets with adjusted *p* value less than or equal to 0.05 were regarded as statistically significant. These analyses were executed with the R package ClusterProfiler version 3.18.1.48. The log of the normalized transcript per million values for statistically significant genes within the interested pathways was rescaled using a z-score transformation.

Pathways and gene sets of interest were procured from GSEA-msigdb database

(<https://www.gsea-msigdb.org/gsea/index.jsp>) and Gene Ontology resources

(<http://geneontology.org/>) representing biological processes in (H) Hallmark, (C5)

Reactome⁴⁶² (C2) KEGG⁴⁶³ (C5) Gene Ontology, (C6) Oncogenic Signature (C7) Immunologic Signature and (C8) Cell Type Signature Gene Sets of the MSigDB database⁴⁶⁴. Tumor infiltrating immune cell deconvolution and quantification was performed using quanTIseq⁴⁶⁵ which uses least squares regression of the unique transcript abundances to a signature matrix of marker genes of known immune cell types and implemented in the R/Bioconductor library quantiseqr (version 1.2.0) and immunedeconv⁴⁶⁶.

Data and codes availability

Processed data and R scripts for the enclosed analyses will be provided on GitHub at

<https://github.com/ndbrown6/MSK-RNA-SCLC>.

Immunohistochemistry

Immunohistochemistry (IHC) for neuroendocrine (synaptophysin, chromogranin, CD56, and INSM1) and subtype markers (ASCL1, NEUROD1, POU2F3 and YAP1) was performed on whole sections of patient samples with available tissue as previously described^{467,468}. Cases were also stained for MHC1 (clone A4, Ebioscience) and CD8 (clone C8/144B, Dako), according to the manufacturer protocols, using Leica Bond RX automated stainer. For neuroendocrine, subtype, and MHC1 markers, a histoscore (H-score) was used to quantitate the level of expression of each marker, which was calculated by the product of labeling intensity (1+ weak, 2+ moderate, 3+ strong) and the percentage of positive tumor cells (0-100%). Slides stained for CD8 were annotated to delineate the tumor area, and the positivity of CD8 T lymphocytes was quantified using HALO image analysis software (Indica Labs) within the designated area. The tumors were classified into cold/deserted, inflamed, or excluded based on the density and predominant localization of the CD8 T cells. Tumors with no or rare intratumoral or peritumoral CD8 T cells were designated as cold/deserted, while those with at least a mild CD8 T cell infiltrate were considered either inflamed or excluded, depending on the localization of the infiltrate. While the inflamed tumors were designated as those containing intratumoral (intimately admixed with tumor cells) and peritumoral (adjacent to tumor cells/nests within stroma) CD8 T cells, the excluded tumors were those with the CD8 infiltrate seen exclusively at the immediate tumor edge.

Tertiary lymphoid structures detection and quantification

TLS histopathology staining was performed at Histowiz, Inc Brooklyn, using the Leica Bond RX automated system (Leica Microsystems) with a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded on paraffin, and sectioned at 4µm. FFPE slides were dewaxed using xylene and alcohol based dewaxing solutions. For CD3 staining, epitope retrieval was performed by heat-induced epitope retrieval (HIER) of the formalin-fixed, paraffin-embedded tissue using Citrate based pH 6 solution (Leica Microsystems,

AR9961) for 20 mins at 95 C. The tissues were first incubated with peroxide block buffer (Leica Microsystems), followed by incubation with the rabbit anti-CD3 (Ab16669) at 1:100 dilution for 30 mins, followed by DAB rabbit secondary reagents: polymer, DAB refine and hematoxylin (Bond Polymer Refine Detection Kit, Leica Microsystems) according to the manufacturer's protocol. For CD20 staining, epitope retrieval was performed by heat-induced epitope retrieval (HIER) of the formalin-fixed, paraffin-embedded tissue using Citrate based pH 6 solution (Leica Microsystems, AR9961) for 10 mins at 95 C. The tissues were first incubated with peroxide block buffer (Leica Microsystems), followed by incubation with the rabbit anti-CD20 (PA5-17601) at 1:2000 dilution for 30 mins, followed by Red rabbit secondary reagents: polymer, Red refine and hematoxylin (Bond Polymer Refine Detection Kit, Leica Microsystems) according to the manufacturer's protocol. The slides were dried, coverslipped (TissueTek-Prisma Coverslipper) and visualized using a Leica Aperio AT2 slide scanner (Leica Microsystems) at 40X.

TLS were identified by training a random forest classifier algorithm to separate out TLS from tissue, any artifacts and background/white space. Once the TLS areas were identified a size threshold was set at 10,000um² and any structure below the size threshold was excluded from any further analysis. The analysis then identified positive and negative cells based on either CD3 and CD20 staining within each defined TLS area on each slide. Positive and negative cells were identified using the Halo Multiplex IHC algorithm v3.4.1 by first defining the settings for the hematoxylin counterstain, followed by setting thresholds to detect both the CD3 DAB stain (Halo threshold settings 0.2, 2.5, 2.5) and CD20 Fast Red stain (Halo threshold settings 0.18, 2.5, 2.5). Results were reported as numbers of CD3 and CD20 positive cells and the area and perimeter of each TLS.

MSK IMPACT Data Processing

Targeted next-generation sequencing via MSK-IMPACT was performed on DNA extracted from tumor and matched normal DNA samples, with somatic mutations (substitutions and small

insertions and deletions), gene-level focal copy number alterations, and structural rearrangement^{376,469}. TMB from IMPACT is defined as the number of non-synonymous mutations divided by the total genomic target region, as previously described⁴⁶⁹.

Exome Data Processing

Existing libraries that corresponded to clinical MSK-IMPACT sequencing were recaptured with IDT exome capture. 74–500 ng of barcoded library was captured by hybridization using either the SureSelectXT Human All Exon V4 (Agilent 5190-4632) or xGen Exome Research Panel v1.0 (IDT) according to the manufacturer's protocol. PCR amplification of the post-capture libraries was carried out for 8 cycles. Samples were run on either HiSeq 4000 or HiSeq 2500 in rapid mode in a 100-bp or 125-bp paired-end run using the HiSeq 3000/4000 SBS Kit or HiSeq Rapid SBS Kit v2 (Illumina) or on NovaSeq 6000 in a 100-bp paired-end run using the NovaSeq 6000 SBS v1 Kit and an S2 flow cell (Illumina). Demultiplexed FASTQ files were aligned and processed using TEMPO (<https://github.com/mskcc/tempo>). Reads were aligned to the GRCh37 assembly of human reference genome with Burroughs-Wheeler Aligner (BWA)-MEM⁴⁷⁰. Read groups were annotated and base quality recalibration was performed using Toolkit (GATK)⁴⁷¹.

HLA Typing and Loss of Heterozygosity

Total allele-specific copy number alterations and tumor purity and ploidy were determined using FACETS (Fraction and Allele-Specific Copy Number Estimates from Tumor Sequencing) (v0.5.14, <http://github.com/mskcc/facets>)⁴⁷². Each tumor and matched normal specimen was processed in a two-pass manner: an initial run for purity and ploidy estimation followed by a second run for focal event detection. FGA and whole-genome doubling were calculated from the resulting FACETS using facets-suite V2⁴⁷³.

HLA typing was performed using PolySolver¹⁴² (POLYmorphic loci reSOLVER) (<http://www.broadinstitute.org/cancer/cga/polysolver>) to infer *HLA-A*, *HLA-B*, and *HLA-C* alleles from whole-exome data or MSK-IMPACT data. Pie-chart distribution of unique HLA alleles

across samples was created using “webr” package. Classification of nine HLA Supertypes was defined as previously described⁴⁷⁴. To assess for loss of heterozygosity (LOH) of the HLA region, we utilized LOHHLA¹⁴⁵ method using the following thresholds: copy number of an HLA allele < 0.5 and p-value comparing pairwise differences in logR of the two alleles < 0.001, adjusted so that each read was counted once.

Somatic Variant Calling

Whole exome sequencing data from the clinical cohort was used for variant calling for non-synonymous mutations. Somatic variants including single nucleotide point mutations, small insertions and deletions were identified in tumor-normal pairs using the union of Mutect2⁴⁷⁵ and Strelka2⁴⁷⁶. Exome TMB was calculated by TEMPO as the number of non-synonymous mutations in coding regions covered by the baits. Variants were filtered and annotated using the following exclusion measures: variant allele frequency (VAF) <0.05, tumor alternate read count of fewer than 3 variant reads, tumor read depth < 20, and matched normal coverage < 10, along with variants corresponding to RepeatMasker-annotated low-complexity⁴⁷⁷ and common variants that appear at allele frequencies >0.01 in GNOMAD⁴⁷⁸

Neoantigen Prediction and Profiling

Computational pipeline for predicting neoantigens have been previously described. In brief, putative neoantigen binding affinity was computed using NetMHC-pan4.0⁴⁷⁹, which predicts binding of a particular variant sequence >8 amino acids to any MHC molecule of known sequence using artificial neural networks (ANNs). The method was trained on combination of > 180,000 quantitative binding data totaling 172 human MHC molecules as well as MHC-eluted ligands derived from mass spectrometry (MS) covering 55 HLA and mouse alleles. A list of ranked peptides was generated which includes the peptide sequences of respective potential ligands, HLA alleles, and binding affinity in nanomolar units of all putative peptides. An affinity threshold of IC50 <500nM, a cutoff previously demonstrated to filter an epitope's capacity to

elicit a T cell response, was selected⁴⁸⁰. Strong binders were identified if found among the top 0.5% percentile rank of all predicted peptides, and weak binders are defined as those above % rank of the threshold of the strong binders but below the 2% Rank threshold. Neoantigens were considered expressed if ≥ 1 RNA-seq read was identified to support the mutated base and the gene had a TPM of 10.

Agretopicity (Ag), also called “wild-type/mutant binding affinity fold change” estimates the degree of difference of a neoantigen’s binding affinity to that of the corresponding wild-type sequence. Agretopicity was calculated for neoantigens derived from non-synonymous SNVs using agReto (<https://github.com/mskcc/agReto>)^{481–483}. Briefly, wildtype version of each resulting peptide was identified and calculated against the difference in binding affinity of the mutant compared. Low Ag score was defined as values < 0.1 .

Immunogenicity score^{484–486} was developed by IEDB (<http://tools.iedb.org>) that examines the properties of the amino acid sequence, composition of the side chains, and their respective non-anchor position within the peptide to predict MHC-I epitope on any predicted peptide independent of MHC restriction. By default, primary candidates responsible for MHC binding are masked at the first, second and C-terminus residues. Residues from position 3-8 are evaluated to compute a propensity score reflecting the degree of likelihood that the residues are involved in a T cell recognition event^{485,486}. The higher the positive score, the higher probability of T cell recognition, whereas a negative score suggests a low likelihood of recognition.

Data analysis

Analysis of lung cancer subtypes was performed only on IMPACT samples where other analyses of the cohort, such as calling HLA alleles and LOH of the HLA region, combined IMPACT and exome samples, preferentially using When the exome results. comparing TMB to survival and LOH status, only IMPACT TMB was used due to the non-linear relationship between IMPACT and exome TMB. Correlations were calculated in R using “cor.test”. Cox

proportional hazard model was used to compare genomic variables to PFS and OS in patients treated with IO using “survival” package in R. Plots for the hazard ratios were created using the “forestmodel” package.

MATERIALS AND METHODS³

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and cell lines

NCI-H69, DMS79, NCI-H146, SHP-77, NCI-H889, NCI-H1092, NCI-H209, NCI-H1963, NCI-H524, NCI-H82, NCI-H446, NCI-H526, NCI-H211, COR-L311, NCI-H196, and DMS114 were maintained in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. NCI-H841, NCI-H1048, and NCI-1694 were maintained in RPMI supplemented with 10% FBS, 1% penicillin/ streptomycin, 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30nM Sodium selenite (Gemini Bio-Products Cat.400-145), 10nM hydrocortisone (Sigma-Aldrich Cat. H6909), and 10nM beta estradiol (Sigma-Aldrich Cat. E4389). NCI-H1341 were maintained in ACL-4 media without serum supplemented with 1% penicillin/streptomycin. Primary NY-ESO1 T cells were cultured in RPMI supplemented with 10% human serum (Gemini Bio Cat.100-512), 1% penicillin/streptomycin, 0.1% amphotericin, 1mM sodium pyruvate (Thermo Fisher Cat. 11350070), non-essential amino acids (Millipore Sigma Cat. 32160406), and Glutamax (Thermo Fisher Cat. 35050061). All cell lines were cultured in a 5%CO₂ incubator at 37°C and passaged every 2-3 days. Identities of cell lines were confirmed by STR profiling, and all lines were frequently tested for mycoplasma.

Mouse models

All animal experiments were approved by and used in accordance with animal care guidelines from the Memorial Sloan Kettering Cancer Center (MSKCC) Animal Care and Use Committee. Female B6129J/s mice (6-8 weeks old) were purchased from Taconic Biosciences and housed in accredited facilities under pathogen-free conditions. Prior to all experiments, purchased mice

³ Related to Chapter 6

were allowed one week to acclimate to housing conditions at Memorial Sloan Kettering Cancer Center Animal Facility.

METHOD DETAILS

Cell viability assay

All cell lines were cultured in a 5%CO₂ incubator at 37°C and passaged every 2-3 days. 1000 cells were seeded as triplicates in 96-well black opaque plates with lid (Corning Cat. #3916) in 50uL normal growth media the day before drug treatment. Drug titrations were performed in normal growth media at 2X concentration. 50uL of drug-containing media was then added so that column 11 wells contained the highest drug concentration, column 3 containing the lowest, and column 2 containing vehicle instead of drug. Outside wells were filled with culture media (RPMI complete) to prevent precipitation. Drug concentrations are indicated in dose/response curves for ORY-1001 (Selleckchem; Cat. #S7795) or DMSO as vehicle. Cells were cultured in a 5%CO₂ at 37°C for 7 days during which cells were passaged every 3 days and replenished with fresh media with indicated concentration. After 7 days, Cell Titer Glo Luminescent Cell Viability Assay (Promega; Cat.G7570) was performed according to manufacturer's instructions. Cell viability was calculated as percentage of average count of drug treated cells and average count of vehicle treated cells.

Flow Cytometry

Human SCLC were blocked with TruFCX and stained (30min, 4°C C) with appropriate dilutions of anti-human HLA-A,B,C antibodies. Murine tumors were dissociated into single cells using GentleMACS according to standard protocol. Single cell suspensions were Fc-blocked with antiCD16/32 antibody and stained with appropriate anti-mouse antibodies. Antibody clone and dilutions are listed in Supplementary Table 2. Murine populations from Figure 6.5 were gated as follow: CD45-MHC-I+ (tumor cells), CD45+, CD45+CD3+ (total T cells), CD45+CD3+CD4+, CD45+CD3+CD8+, and CD45+CD3+CD8+CD44+CD25+ (activated T

cells). Stained cells were acquired on a Cytex Aurora Flow Cytometer, and the data were analyzed using FlowJo software (version 10.6, BD Biosciences). Debris, doublets and dead cells were excluded based on forward and side scatter, side scatter height vs side scatter width, forward scatter area vs forward scatter height, and Fixable Viability Dye Zombie NIR or Ghost Violet 510, unless otherwise indicated.

Immunoblots

Protein extraction and western blot were performed as previously described⁴⁸⁷. 30-50ug of protein were extracted and normalized using BCA Protein Assay Kit (Thermo Fisher Cat #23225) and diluted with NuPAGE LDS sample buffer 4X (Invitrogen Cat #NP0007), loaded on Bis-Tris Gel 4-12% (Invitrogen Cat. #NP0322) and electrophoresed before transferring with Trans-Blot Turbo RTA Mini LF PVDF Transfer Kit (Bio-Rad). Blots were incubated with blocking buffer at room temperature for 1 hour before overnight incubation with primary antibody. Blots were washed 4-6X with 1%PBST before secondary incubation with anti-rabbit, HRP-conjugated antibodies/ Chemiluminescent detection was performed on iBright Imaging Systems (Thermo Fisher). All antibodies used for this procedure are listed in Supplementary Table 3.

RT-qPCR

RNA extraction, reverse transcription and quantitative PCR were performed as followed. RNA extractions were performed using the RNeasy Plus Mini Kit (Qiagen, Cat # 74034) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from RNA by reverse transcription PCR using Superscript IV VILO Master Mix (Thermo Fisher Cat #11756050). Real time PCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Cat #4444556) following manufacturer's protocol. Triplicate PCR reactions were run on StepOnePlus Real-Time PCR System (Applied Biosystems) and $2^{-\Delta\Delta}$ method were used to calculate comparative Ct. and normalized to *GAPDH*. Fluorescent probes used in this study are included in Supplementary Figure 6.1.

RNA sequencing

RNA extraction and sequencing was done in collaboration with Genewiz. Total RNA was extracted from fresh frozen cell pellet samples using QIAGEN RNeasy Plus Universal mini kit following manufacturer's instructions (QIAGEN, Hilden, Germany). Extracted RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA).

RNA sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA).

The sequencing libraries were clustered on 2 lanes of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq instrument (4000 or equivalent) according to manufacturer's instructions. The samples were sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

RNA-seq Analysis

Transcript counts and abundances were quantified from RNA-seq reads using Salmon v1.1.0⁴⁸⁸. RNA-seq raw reads were mapped to 25 mer indexed hg38 genome by Salmon. With default settings, mapping validation (`--validateMappings`), bootstrapping with 30 re-samplings (`--numBootstraps`), sequence specific biases correction (`--seqBias`), coverage biases correction (`--posBias`) and GC biases correction (`--gcBias`) were also enabled. Transcript to gene mapping based on Ensembl 92⁴⁸⁹, normalized by size factor at gene level, and differential gene expression analyses were processed on Salmon output files using Sleuth v0.30.0 in gene mode⁴⁹⁰. Differentially expressed genes were identified using the Wald test. Genes were marked as significant if the False Discovery Rates, q , calculated using the Benjamini-Hochberg method, was less than 0.05, and beta (Sleuth-based estimation of log2 fold change) > 0.58, which approximately correlated to a log2 fold change of 1.5 in our data.

Gene set enrichment analysis (GSEA)⁴⁹¹ was performed on full sets of differential gene expression results on the previously mentioned comparisons. Genes were ranked on p value scores computed as $-\log_{10}(p \text{ value}) * (\text{sign of beta})$. Gene set annotations include all gene sets deposited in the Molecular Signatures Database (MSigDB v7.0.1)^{492,493}. The significance level of enrichment was evaluated using permutation test and the p value was adjusted by Benjamini-Hochberg procedure. Any enriched gene sets with adjusted p value ≤ 0.05 were regarded as significant. This analyses were executed with the R package ClusterProfiler v3.18.1⁴⁹⁴.

Pathways and genesets of interest were procured from GSEA-msigdb database (<https://www.gsea-msigdb.org/gsea/index.jsp>) and Gene Ontology resources (<http://geneontology.org/>). Interested pathways were highlighted in H82 and H211 GSEA dot plots. The log of the normalized TPM values for significant genes within the interested pathways, were rescaled using a z-score transformation, and plotted in a heatmap using the ComplexHeatmap v2.7.10⁴⁹⁵ Library in R 4.0.4⁴⁹⁶.

Lentiviral construction and synthesis

shRNA sense and antisense guide sequences were generated using splashRNA from coding sequence of *KDM1A* transcript, variant 1 (NM_001009999) following previously described protocols^{497,498}. Four antisense constructs were generated from splashRNA: #1897 (TAAATAAGGTTTGACTCGTGG), #392 (TTCTGAATAATACTCATCTTCT), #1227 (TAAGGTAAGATGTAGCTTCTAG) and #806 (TAAATAACTGTGAACCTCGGTGG) and subsequently synthesized to include XhoI and EcoRI restriction sites. Cloning into LT3GEPIR vector backbone was performed using the following primers: **miRE-Xho-short-fw** (5'-AGAAGGCTCGAGAAGGTATATTGC-3') and **miRE-EcoPlasmid-rev** (5'-GCTCGAATTCTAGCCCCTTGAAGTCCGAGG-3'). LT3GEPIR vector and primer sequences were provided as a gift from Levine Lab.

NY-ESO1- and MART-1-GFP lentiviral expression plasmids were purchased from SinoBiological (Cat# HG15611-ACGLN and Cat# HG12253-ACGLN, respectively). Luciferase-GFP vectors were obtained from System Biosciences (Cat #LL310PA-1; pLL-CMV-rFLuc-T2A-GFP-mPGK-Puro). Lentiviral production and transduction were performed as previously described⁴⁹⁹. For transduced cell lines from Figure 6.4, H82, H211, H69 and T2 cells were first transduced with luciferase-neomycin vector and underwent neomycin selection for 7 days prior to transduction with NY-ESO1-GFP or MART-1-GFP lentiviruses.

Cytotoxicity Assay

Luciferase-GFP SCLC cell lines were plated into 6 well plates and treated with 5uM ORY-1001 for 10 days in complete RPMI media. Media was replenished every 3 days. Human NY-ESO1-TCR CD8+ T cells were isolated from a healthy CMV seropositive patient, pre-activated with autologous monocytes pulsed with NY-ESO1 peptides and subsequently cultured in RPMI media supplemented with human serum, +IL-12 (100IU/ml). After treatment, SCLC cells were plated to 96-well white opaque plates with lid (Cat. #). For exogenous peptide assay

(Figure 6.4A), cells were pulsed with 5ug/ml of NY-ESO-1 peptide (Sequence) or MART-1/MLANA peptide (sequence) for 2 hours before excess peptide is removed. For endogenous peptide assay, NY-ESO-1-luc-GFP or MART-1-luc-GFP were pre-treated with ORY-1001 or vehicle before co-incubation. In both assays, co-incubation with NY-ESO-1-TCR CD8+ T cells were performed at appropriate Effector:Target ratio as indicated in the Figure Legend. After 18 hours, culture media was collected for ELISA. Promega Luciferin was added to each well of the plate, incubated for 15minutes and luminescence signal was recorded on the Synergy HT1.

ELISA

90µl of co-culture media was collected from each well, spun down to remove debris and subjected to quantitative human IFN- γ ELISA performed using the Quantikine ELISA Immunoassay (R&D Systems, Cat #DIF50C) according to manufacturer's instruction. ELISA plates were read using a microplate reader set at 450nm with wavelength correction set to 540nm.

Animal model and treatment

4×10^6 RPP single cells were injected subcutaneously in a 1:1 mixture of PBS and Matrigel (Thermo Fisher #CB40234) into the flanks of 6-week-old female B6129F1 mice (Taconic). Treatment commenced either at seven days after injection or once the mean tumor volume reached approximately 100 mm³, unless otherwise indicated in the figure legend. Mice were randomized and treated with either vehicle (0.5% Methylcellulose; oral gavage) and rat IgG2b isotype (BioXCell #BE0090), vehicle and anti-PD-L1 antibody (300µg/animal/week, IP) (BioXCell #BE0101), ORY-1001 (50ug/animal/week, oral gavage) and isotype, or a combination of anti-PD-L1 and ORY-1001. Tumors were measured twice a week using calipers and their volumes were calculated as width² x length x 0.5. Statistical significance was determined by Student's t-test.

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