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14. ABSTRACT

Accumulation of activated and suppressive regulatory T cells (Treg) within the tumor microenvironment (TME) is a major obstacle to the development of efficient anti-tumor immunity. Although Treg depletion can enhance anti-tumor immune responses, autoimmune sequelae can complicate this approach. To analyze the impact of transcription factor Helios on FoxP3$^+$ CD4 Treg in lymphoid tissues, we determined that Helios activates the IL2R–STAT5 pathway to enhance FoxP3 expression and maintain Treg suppressive activity. The observation that Helios-deficient Treg enhancement of anti-tumor immunity may reflect conversion of unstable Helios$^+$ Treg into T effector cells (Teff) within tumors was tested by inducing Treg lineage instability to promote anti-tumor immunity. During the first year of funding, we performed transcriptome analysis of intratumoral Treg, which revealed that Helios deficient intratumoral Treg adopt a genetic program that is typical of effector Th1 and Th2 cells. We also tested the feasibility of enhancement of anti-tumor immune responses by Treg conversion by targeting IL-23R using antibodies or genetic mouse models. Hypothesis driven analysis of the mechanism of Treg reprogramming upon blockade of IL-23 signaling is currently underway. These findings are consistent with our hypothesis that antibody-based approaches to reprogram tumor-infiltrating Treg into T effector cells represent a potential immunotherapeutic approach to the treatment of melanoma.

15. SUBJECT TERMS
tumor microenvironment, inflammation, CD4 regulatory T cells, Helios

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1. INTRODUCTION:

While immunotherapeutic approaches to melanoma have gained traction in the clinic, regulatory T cells remain an understudied area of potential clinical importance. Here we delineate the contribution of CD4 regulatory T cells (Treg) to cancer immunity and define novel and effective therapeutic approaches using multiple experimental approaches including conditional knock-out mouse models, antibody dependent Treg reprogramming and knockout generation using CRISPR/Cas9. Insights gained from this study may allow new therapeutic approaches to CD4 Treg-based cancer immunotherapy of melanoma.

2. KEYWORDS:

tumor microenvironment, inflammation, CD4 regulatory T cells, Helios

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. Definition of the contribution of the Helios TF to proliferation, survival and stable FoxP3 expression by CD4 Treg within the microenvironment of murine melanoma. Milestone(s) Achieved: *Definition of the contribution of Helios to Treg proliferation/ survival in the face of chronic inflammatory responses of tumors: establishment of a colony of FoxP3$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice. The contribution of Helios TF to IL-2 responsiveness of Treg under inflammatory conditions.* **100% complete**

Aim 2. In vivo single cell transcriptome analysis of the genes responsible for conversion of intratumoral Treg into T effector cells. Milestone(s) Achieved: *Definition of the genetic events that underlie Treg conversion and potential biomarkers of reprogramming of intratumoral Treg.*
100% complete

Aim 3. Definition of Treg pathways that inhibit Helios expression and allow Treg→Teff conversion of intratumoral but not systemic Treg. Deliverable: Candidate IL-23R Ab in development for potential humanization process. Milestone(s) Achieved: *Identification of molecular pathways that are targeted by antibodies and small molecules to reprogram tumor-infiltrating Treg into T effector cells. The contribution of IL-23R signaling to Treg stability has been validated. Ab dependent blockade and genetic deletion of IL-23R led to delayed tumor growth that is associated with Treg reprogramming*.
100% complete

What was accomplished under these goals?

Aim 1. Definition of the contribution of the Helios TF to proliferation, survival and stable FoxP3 expression by CD4 Treg within the microenvironment of a murine melanoma: We established a colony of FoxP3$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice to define the contribution of Helios to Treg proliferation/ survival in the face of chronic inflammatory responses of tumors. Our analyses revealed that Helios expression by Treg under inflammatory conditions is essential to maintain Treg stability by ensuring Treg's responsiveness to IL-2, a critical cytokine for Treg survival. We also showed that converted Treg alone may be sufficient to induce anti-tumor immunity in an adoptive transfer system. Treg from Helios conditional KO mice (Helios$^{fl/fl}$.FoxP3-Cre) in adoptive hosts produced IFNγ and delayed tumor progression. Analysis of the contribution of STAT5 expression/ activation in Helios-dependent conversion of intratumoral Treg by expression of Akt and Foxo-1 suggested that conversion of intratumoral Treg may be associated with antigen recognition and local cytokine signaling which may explain acquisition of an unstable Treg phenotype selectively within the tumor microenvironment (TME).

Aim 2. Single cell transcriptome analysis of genes associated with conversion of intratumoral Treg into T effector cells: Transcriptome analysis with small numbers of cells revealed the dominant molecular pathways correlated with Treg conversion. Analysis of the transcriptome of Treg in spleen and tumor sites with respect to Helios expression revealed a Helios-dependent Treg program within the tumor-tissue microenvironment that was associated with increased expression of genes that control the T effector cell phenotype. Our transcriptome analysis also revealed that ~50% of genes that are upregulated by Helios deficient intratumoral Treg compared to WT tumor Treg belong to STAT4 target genes. Comparison of multiple cytokine signaling suggested that IL-12 may be one of the major cytokines that can induce STAT4 activation, since chronic inflammatory conditions of tumor can deliver a signal through IL-12R on Treg and sustained activation of STAT4 can lead to induction of unstable Treg phenotype. Molecular insight obtained from gene expression profiling may now be applied to the rational design of immunotherapeutics that selectively induce Treg reprogramming in the TME.

Aim 3. **Definition of receptor-linked pathways that promote Treg →T effector conversion: targeting by antibodies**: The functional efficacy of IL-23R blocking in vitro on Treg conversion showed that engagement of IL-23R leads to Treg conversion as evidenced by downregulation of FoxP3 and CD25 and de novo expression of IFNγ. In addition, the efficacy of anti-IL23R Ab treatment to anti-tumor immunity in vivo using an MC38 colon adenocarcinoma model showed that Ab treatment significantly delayed tumor progression. Inhibition of tumor growth upon anti-IL23R Ab treatment was associated with expression of IFNγ by intratumoral Treg, which in turn de-represses activation of conventional CD4 and CD8 T cells.

Our analyses revealed that mice with selective deletion of IL23R in Treg (IL-23R$^{fl/fl}$.FoxP3-^{Cre}) almost completely suppress tumor development after inoculation with MC38 cells. Analysis of the involvement of enhanced IL12 signaling in TME-specific Treg conversion revealed that STAT4 activation is a hallmark of induction of Treg instability that is also characterized by IFNγ expression by Treg. STAT4 activation and IFNγ production by Treg correlates with TCR signaling, suggesting that the phenotypic change of Treg selectively in the TME, represents inflammatory conditions. Measurement of STAT4 activation in IL-23R deficient Treg or following anti-IL23R Ab treatment showed that IL23R-deficient Treg have increased IL12 sensitivity.

We also analyzed the molecular signature(s) that is prominent in intratumoral Treg that undergo Treg→T effector conversion using the B16 melanoma model by performing RNA-Seq. This analysis revealed that STAT4 target genes are the most upregulated in Treg that adopt an effector phenotype. Among these genes, upregulation of IL-12Rβ2 in reprogrammed Treg within the TME is highly relevant to targeting IL-23R, since blockade of IL-23R-IL-23-IL12Rb1 assembly by anti-IL-23R Ab can lead to increased formation of functional IL-12R leading to accelerated Treg conversion. We are currently testing the efficacy of targeting multiple intratumoral Treg surface molecules (e.g. GITR + IL-23R) to find the most efficient immunotherapeutics that enhance anti-tumor immune response by inducing Treg reprogramming.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report; this is the final report.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Since regulatory T cells (Treg) are one of the most abundant lymphocytes infiltrating into tumors, development of immunotherapeutics that convert their phenotype to anti-tumor effector T cells represents a novel principle. In particular, targeting cell surface molecules that are upregulated or uniquely expressed by intratumoral Treg is critically important to enhance anti-tumor immune responses without systemic inflammation. The data obtained from this study will serve as a foundation for the development of the most effective single (anti-IL-23R) or combined (Anti-GITR + anti-IL23R) therapy after engineering Fc-optimized humanized antibodies.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Hidetoshi Nakagawa, Lei Wang, Harvey Cantor, Hye-Jung Kim. New insights into the biology of CD8 regulatory T cells. *Advances in Immunology* 2018; 140_ 1-20.

Kim HJ, Cantor H, Cosmopoulos K. Overcoming Immune Checkpoint Blockade Resistance via EZH2 Inhibition. [Review] *Trends in Immunology*, 2020; 41:948-963.

Andrew Wight, Jessica M. Sido, Hidetoshi Nakagawa, Lei Wang, Hye-Jung Kim, Harvey Cantor. Requirement for IL23R to maintain Treg stability in the tumor microenvironment. *Manuscript in preparation*.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

June 5, 2019– Poster Presentation, DFCI Cancer Immunology & Virology Retreat, "*Targeting Treg-specific IL23R expression is a potent immunotherapeutic candidate*", Andrew Wight, PhD

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Harvey Cantor, M.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-3313-2478
Nearest person month worked:	1 CM
Contribution to Project:	No change
Funding Support:	N/A

Name:	Hye-Jung, Ph.D.
Project Role:	Lecturer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 CM
Contribution to Project:	No change
Funding Support:	N/A

Name:	Hidetoshi Nakagawa, M.D., Ph.D.

Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 CM
Contribution to Project:	Dr. Nakagawa was supported by a CRI-Irvington Fellowship (through 12/31/19) and subsequently support by this award for 7 months.
Funding Support:	N/A

Name:	Lei Wang, Ph.D.
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 CM
Contribution to Project:	Dr. Wang works on in vitro and in vivo studies outlined in Aims 3.2 and 3.3.
Funding Support:	Dr. Wang was supported by a Benacerraf Fellowship in Immunology (as of 1/1/2019), which allowed support of Dr. Nakagawa (above)

Name:	Andrew Wight, PhD
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 CM
Contribution to Project:	Dr. Wight performed the mechanistic studies outlined in Aim 3.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Not applicable

9. APPENDICES:

CHAPTER ONE

New Insights Into the Biology of CD8 Regulatory T Cells

Hidetoshi Nakagawa[*,†], **Lei Wang**[*,†], **Harvey Cantor**[*,†,1], **Hye-Jung Kim**[*,†]

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Contents

Abstract

Regulatory T cells are central mediators of immune regulation and play an essential role in the maintenance of immune homeostasis in the steady state and under pathophysiological conditions. Disruption of CD8 Treg-dependent recognition of Qa-1-restricted self-antigens can result in dysregulated immune responses, tissue damage, autoimmune disease and cancer. Recent progress in studies on regulatory T cells of the CD8 lineage has provided new biological insight into this specialized regulatory T cell subpopulation. Identification of the Helios transcription factor as an essential control element for the differentiation and function of CD8 regulatory T cells has led to a better understanding of the unique genetic program of these cells. Recent analyses of T-cell receptor usage and antigen recognition by Qa-1-restricted CD8 Treg have provided additional insight into the unusual biological function of this regulatory CD8 lineage. Here we summarize recent advances in our understanding of CD8 regulatory T cells with emphasis on lineage commitment, differentiation and stability.

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Abbreviations

B2M	beta 2 microglobulin
FoxP3	forkhead box P3
GC	germinal center
HLA-E	human leukocyte antigen, type E
Hsp60	heat shock protein 60
TF	transcription factor
T_{FH}	follicular helper T cell
T_{FR}	follicular regulatory T cell
Qdm	Qa-1 determinant modifier
SLE	systemic lupus erythematosus
STAT5	signal transducer and activator of transcription 5
TAP	transporter associated with antigen processing
TME	tumor microenvironment

1. INTRODUCTION

Current views of T cell subset development and function hold that the $CD4^+$ T cell lineage is comprised of distinct regulatory and effector sublineages, while the CD8 lineage is devoted to the development of effector T cells. Although this may be the case for the majority of conventional CD8 cells that recognize class Ia-restricted peptides, there is increasing evidence that a subset of CD8 cells that is restricted by class Ib Qa-1/HLA-E molecules is genetically programmed to regulate immune responses (Akane, Kojima, Mak, Shiku, & Suzuki, 2016; Kim et al., 2015; Kim & Cantor, 2011).

Early studies suggesting that immune responses were accompanied by suppressive activity initially focused on a subpopulation of $CD8^+$ T cells designated CD8 suppressors (Cantor et al., 1978; Eardley et al., 1978). Subsequent research revealed that $CD8^+$ T cells were involved in inhibition of the development of autoimmune diseases (Jiang, Zhang, & Pernis, 1992; Koh et al., 1992). Analysis of the mechanism of this $CD8^+$ T cell-dependent suppressive response suggested that inhibition depended on recognition of the MHC class Ib molecule Qa-1 by activated target T cells (Cantor et al., 1978; Jiang et al., 1995; Ware et al., 1995). Qa-1-restricted $CD8^+$ regulatory T cells (Treg) are currently one of the best characterized $CD8^+$ Treg populations, based in part on generation of Qa-1 mutant mouse models, which have allowed improved analysis of the activities of Qa-1-restricted $CD8^+$ Treg. Identification of surface markers expressed by this $CD8^+$ T cell subset has further increased our

understanding of their potential contribution to health and disease (Kim, Verbinnen, Tang, Lu, & Cantor, 2010; Kim et al., 2011).

Research into CD4 Treg gained critical experimental traction after definition of surface markers that allowed analysis of isolated $CD4^+$ cells with regulatory activity and identification of FoxP3 transcription factor control of this regulatory T-cell subset (Cantor, 2004; Fontenot et al., 2005; Rudensky, 2011; Sakaguchi, 2005). We have recently made similar progress in defining Qa-1-restricted CD8 Treg through definition of a triad of cell surface markers that reliably separates the 3–5% of CD8 cells that mediate Qa-1-restricted regulatory activity (Kim et al., 2011). Surface markers that reliably identified Qa-1-restricted $CD8^+$ Treg allowed tracking of their activity and stability in various inflammatory and tumor settings in which different cytokine microenvironments might influence the CD8 Treg phenotype and immune outcome. We have also used these markers to isolate CD8 Treg for genetic studies that initially identified the contribution of the Helios TF to the genetic programming of CD8 Treg (Kim et al., 2015). Increased insight into Helios-dependent Qa-1-restricted $CD8^+$ Treg has revealed new layers to the regulation of T cell immune responses, including selection of the peripheral CD8 Treg TCR repertoire, suppression of autoantibody responses and the potential contribution of CD8 Treg to anti-tumor immunity. Here we review the development and function of the major $CD8^+$ Treg cell subtype, i.e., Qa-1-restricted $CD8^+$ Treg cells.

2. TRANSCRIPTION FACTOR HELIOS SAFEGUARDS THE STABILITY OF BOTH CD4 AND CD8 TREG

During T cell maturation in the thymus, strong TCR signals can drive clonal deletion to prevent development of self-reactive conventional T cells, but also promote differentiation of regulatory CD4 and CD8 T cell lineages. In view of their intrinsic self-reactivity, the ability of Treg to maintain a stable suppressive phenotype in the face of active immune responses is critical to their functionality. Recent studies have revealed that CD4 and CD8 Treg express a common transcription factor, Helios, that is essential to the maintenance of a stable phenotype and potent suppressive activity in the context of inflammation. Helios (Ikzf2) is a member of the Ikaros family of transcription factors that includes Ikaros (Ikzf1), Aiolos (Ikzf3), Eos (Ikzf4), and Pegasus (Ikzf5). Expression of the Helios family member is restricted to T cells. During T-cell development, Helios is expressed by DN thymocytes,

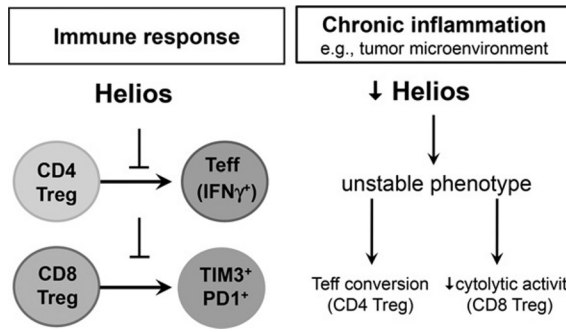

Fig. 1 Helios-dependent control of Treg stability. The Helios TF stabilizes the CD4 and CD8 Treg phenotype during immune and inflammatory responses. Genetic ablation of Helios expression in CD4 Treg results in phenotypic instability and conversion of some CD4 Treg into effector T cells. Genetic ablation of Helios in CD8 Treg results in acquisition of a dysfunctional CTL phenotype.

$CD4^{lo}CD8^{lo}$ DP cells that undergo negative selection, and by 100% of mature $FoxP3^+$ CD4 SP cells (Thornton et al., 2010). In the periphery, $FoxP3^+$ CD4 Treg and $Ly49^+$ CD8 Treg both express Helios and emerging evidence supports the critical contribution of this TF to the suppressive activity of these two distinct regulatory T cell lineages (Fig. 1).

2.1 Role of Helios in CD4 Treg Differentiation and Function

The development of functional CD4 Treg depends on the FoxP3 TF throughout life and continued expression of this TF is essential for maintenance of Treg activity and restraint of autoimmunity (Fontenot, Gavin, & Rudensky, 2003; Hori, Nomura, & Sakaguchi, 2003). Attenuation of FoxP3 expression can lead to impaired CD4 Treg activity and the development of inflammatory disorders and autoimmunity (Wan & Flavell, 2007). $FoxP3^+$ CD4 Treg maintain regulatory identity in a variety of inflammatory conditions and FoxP3 expression by thymus-derived CD4 Treg is highly stable (Miyao et al., 2012; Rubtsov et al., 2010), reflecting the action of several transcription factors, including the Runx-CBFb complex, NF-κB, GATA3, Foxo1 and Foxo3 (Rudra et al., 2012; Wohlfert et al., 2011). In contrast to mice with FoxP3 deficiency, Helios-deficient mice were initially noted to display unimpaired $FoxP3^+$ Treg development and function and did not develop autoimmunity at an early age (Cai, Dierich, Oulad-Abdelghani, Chan, & Kastner, 2009; Rudra et al., 2012; Thornton et al., 2010; Wohlfert et al., 2011). More recent studies have identified Helios

as a critical TF that ensures FoxP3$^+$ Treg stability under conditions of disturbed immune homeostasis (Kim et al., 2015). Under immunologically challenging conditions, such as aging, lymphopenia, infection, autoimmunity or cancer, Helios expression is an indispensable component of stable inhibitory activity of FoxP3$^+$ CD4 Treg (Kim et al., 2015). Helios-deficient mice develop a progressive SLE-like autoimmune disease characterized by multi-organ infiltration of immune cells, generation of a broad range of autoantibodies and autoimmune kidney pathology. LCMV infection and lymphopenic conditions in young mice also provoke an autoimmune disorder that reflects the acquisition of an unstable phenotype by FoxP3$^+$ Treg. Mechanistic studies revealed that regulation of the IL-2Ra–STAT5 pathway by Helios is essential to stabilize FoxP3 expression and maintain CD4 Treg integrity during periods of acute and chronic inflammation or stress.

A major target of regulatory T cells are self-reactive CD4 T cells that differentiate into follicular helper T cells (T_{FH}). This process requires continuous control to prevent their delivery of help to autoreactive B cells and the consequent generation of autoantibodies. In the germinal center (GC) microenvironment of different lymphoid tissues, a subpopulation of CD4 Treg, called follicular regulatory T cells (T_{FR}), expresses phosphorylated STAT5 and interacts with IL-2 producing T_{FH}. In the steady state, regulating T_{FR} responses that are directly proportioned to the concentrations of IL-2 secreted by self-reactive T_{FH} may be essential to control autoantibody responses (Liu et al., 2015). Helios-dependent activation of STAT5 modulates T_{FR} suppressive activity in the GC; defective Helios expression results in upregulation of inflammatory cytokines, including IFNγ and IL-17 and fails to control excessive and autoreactive T_{FH} responses (Kim et al., 2015; Sebastian et al., 2016).

The contribution of Helios to stabilization of the Treg phenotype in the face of inflammatory conditions includes maintenance of Treg stability within progressively growing tumors. Helios-dependent expansion of intratumoral Treg represents a major cellular element that shapes the immunosuppressive tumor microenvironment (TME). Selective Helios deficiency within CD4 Treg results in the development of an unstable Treg phenotype by intratumoral but not systemic Treg and conversion of these Tregs into Teff cells within the TME (Nakagawa et al., 2016). These changes result in enhanced anti-tumor immunity (Fig. 1). Transcriptome analysis of intratumoral Treg revealed that Helios expression maintains lineage stability by repressing alternate genetic programs associated with effector T_H differentiation and activation (Yates, Bi, Haining, Cantor, & Kim, 2018).

Helios-deficient intratumoral Treg display a high affinity for tumor-associated antigens (TAA), as judged by GITR and PD1 expression, and preferential conversion of the $GITR^{hi}PD1^{hi}$ fraction of Treg into effector T cells upon stimulation with syngeneic APC. These findings support the notion that loss of Treg stability in the absence of Helios reflects increased TCR signaling by autoreactive ex-Treg within the chronic inflammatory environment of tumors (Yates et al., 2018).

Recent analyses of genome-wide DNA methylation patterns have indicated that among the previously known Treg-specific hypomethylation sites (*FoxP3*, *Ctla4*, *Ikzf2*, and *Il2ra*), *Ctla4*, *Il2ra* and *FoxP3* are uniformly hypermethylated in both peripherally-induced Treg (pTreg) and thymus-derived Treg (tTreg) populations, while *Ikzf2* (Helios) is completely methylated in pTreg, strongly suggesting that Ikzf2 represents an epigenetic mark that distinguishes pTreg from tTreg (Delacher et al., 2017). In view of studies that identify CD4 Treg as important contributors to organismal homeostasis, shared expression of Helios by tissue Treg adds an additional layer to its contribution to maintaining Treg stability within distinct tissue micro-milieu (Burzyn et al., 2013; Cipolletta et al., 2012; Delacher et al., 2017; Sefik et al., 2015).

2.2 Role of Helios in CD8 Treg Differentiation and Function

Unlike CD4 Treg, where the contribution of FoxP3 as the key TF has been unequivocally established, the transcription factor(s) that control CD8 Treg development and function have not been defined until recently. Definition of a triad of surface markers for Qa-1-restricted CD8 Treg—$CD44^+$ $CD122^+$ $Ly49^+$—led to the isolation of CD8 Treg and identification of Helios as an essential TF for this specialized regulatory lineage (Kim et al., 2015). Helios expression has been implicated in both the survival and maintenance of suppressive function of CD8 Treg during immune responses. Definition of the genome-wide distribution of Helios binding sites in CD4 and CD8 Treg led to the interesting observation that Helios controls a substantial number of genes expressed by both of these regulatory cell lineages (Kim et al., 2015). A major pathway controlled by Helios in both CD4 and CD8 Treg is the IL-2Ra–STAT5 pathway, as attested by the observation that the reduced survival and disrupted immune homeostasis of Helios-deficient CD4 and CD8 Treg reflect reduced responsiveness to the IL-2 and IL-15 cytokines (Kim et al., 2015). The observation that CD8-specific deletion of Helios results in autoimmune disease characterized

by immune cell infiltration into multiple peripheral organs further highlights the critical contribution of CD8 Treg to the maintenance of immune homeostasis and the essential contribution of Helios to expression of the genetic program of suppressive Qa-1-restricted CD8 Treg (Fig. 1). Although Helios-dependent regulation of overlapping genetic pathways appears to stabilize the suppressive phenotype of both FoxP3$^+$ CD4 Treg and CD8$^+$ Treg, an additional component of the Helios-dependent program of CD8 Treg may include commitment to cytolytic activity and cytotoxic mechanisms of suppression, particularly after recognition of Qa-1—peptide complexes. Similar to CD4 Treg, CD8 Treg functionality extends to control of anti-tumor immune responses since, e.g., genetic disruption of CD8 Treg activity can result in enhanced antitumor immunity (Alvarez Arias et al., 2014). This notion is also supported by recent findings from single cell transcriptome analysis of CD8 T cell subpopulations found within tumors. This study identified a subset of intratumoral CD8 T cells that contains gene modules resembling Ly49$^+$ CD8 T cells including Helios expression and expression of a regulatory phenotype, consistent with the idea that CD8 Treg-mediated suppression can occur locally within tumors (Singer et al., 2017). These intratumoral Ly49$^+$ CD8 T cells express increased levels of IL-10 compared to other CD8 TILs, which may suggest that CD8 Treg can adapt to particular tissue microenvironments and exert specialized regulatory effects on tumor growth and cell homeostasis (Wang, Singer, & Anderson, 2017). In view of the upregulation of Qa-1/HLA-E after stress, excessive activation or cellular transformation, CD8 Treg are well suited to regulate tissue homeostasis and tissue integrity after inflammation or injury.

3. CD8 TREG AS AN INNATE T CELL LINEAGE

Although suppressive activity of cells within the CD8 T cell lineage has long been suggested, the identity and underlying molecular mechanism have remained enigmatic for many decades. The MHC class Ib molecule Qa-1 (HLA-E in man) was reported early on as a key molecule that mediated suppressive activity by CD8 T cells in the context of an antibody response to heterogenous foreign antigens (Eardley et al., 1978). Subsequent studies indicated that immunization with autoreactive T cells (i.e., T cell vaccination; TCV) suppressed the progression of autoimmune disease, which could be neutralized by administration of anti-Qa-1 antibodies (Panoutsakopoulou et al., 2004).

Currently, Qa-1 is thought to play a significant role in initiating CD8 T cell-mediated regulatory function.

Qa-1 in mice is the functional homolog of human leukocyte antigen-E (HLA-E) in man and represents a non-classical major histocompatibility complex (MHC) class Ib molecule encoded on chromosome 17 as the H2-T23 gene with relatively limited polymorphisms (Wolf & Cook, 1990). Similar to other class I MHC molecules, Qa-1 is expressed as a membrane-binding molecule at the cell surface with a light chain (β2-microglobulin) and a peptide in a peptide-binding pocket formed by the α1 and α2 domains of Qa-1 (Zeng et al., 2012). Qa-1 is expressed on activated T cells, B cells and dendritic cells (Kim & Cantor, 2011), and is recognized by two classes of receptors with opposing functions: (1) the T cell receptor (TCR) and (2) NKG2A/CD94 receptor. TCR engagement by Qa-1 provokes activating signals that can induce CD8 lytic activity, whereas Qa-1 binding to NKG2A/CD94 results in inhibitory signals (Vance, Kraft, Altman, Jensen, & Raulet, 1998) that dampen target cell killing (Fig. 2). In humans, the inhibitory NKG2A/CD94 complex also is a key receptor for HLA-E (Braud et al., 1998; Lee et al., 1998). In all cases, engagement between Qa-1 and its receptors is peptide-dependent. Qa-1 can present a dominant peptide termed Qa-1 determinant modifier (Qdm; AMAPRTLLL), that is, derived from the leader

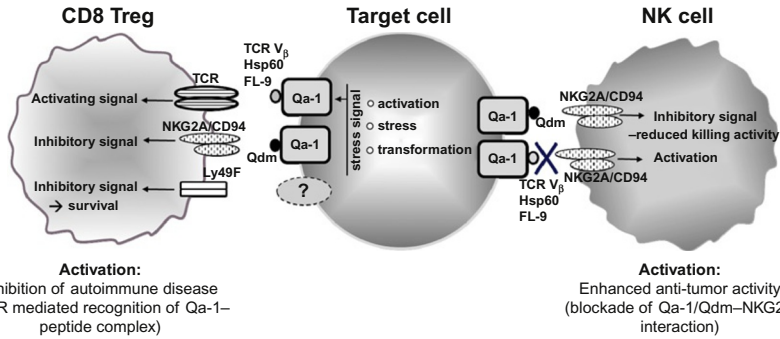

Fig. 2 Molecular interactions involved in Qa-1-dependent immune regulation. Excessive activation, cellular stress or transformation can promote presentation of characteristic peptides associated with Qa-1. Recognition of Qa-1–peptide complex by CD8 Treg via TCR can lead to elimination/suppression of these Qa-1$^+$ target cells. Expression of inhibitory Ly49 receptors by CD8 Treg may promote development and survival of intrinsically self-reactive Treg and potentiate Treg activation. Engagement of the NKG2A/CD94 receptor on both CD8 Treg as well as NK cells by Qa-1/Qdm complexes inhibits activation of CD8 Treg and NK cells. Replacement of Qdm by self-peptides derived from proteins associated with cellular activation and stress abrogates NKG2A-mediated inhibition of NK cells and promotes activation and enhancement of NK cytolytic activity.

sequence of H2-D and -L, in a process that depends on the Transporter associated with Antigen Processing (TAP) intracellular protein (Aldrich et al., 1994). The Qa-1–Qdm complex is recognized by NKG2A/CD94 receptors on NK cells (Brooks, Posch, Scorzelli, Borrego, & Coligan, 1997) and activated CD8 T cells (Fig. 2) (Bertone et al., 1999; Ponte et al., 1998; Rapaport et al., 2015). In contrast, TCR-dependent recognition of Qa-1 complexed to peptides is generally TAP-independent (Aldrich et al., 1992) and represents the major initiating step in CD8 Treg regulation of immune responses.

The general significance of Qa-1-restricted CD8 T cells in regulation of overall immune responses was revealed from studies of Qa-1-deficient mice, which developed exaggerated CD4 responses following Herpes Simplex viral infection or immunization with self-peptides, including proteolipid protein, PLP (Hu et al., 2004). Although these studies indicated that Qa-1-restricted regulation depended on lysis of activated Qa-1^+ CD4 T cells, Qa-1-deficient mice did not develop spontaneous autoimmune disease. The absence of an autoimmune phenotype in these mice reflected the action of two opposing Qa-1-dependent signaling pathways coupled to the TCR or to NKG2A/CD94. Definition of the separate TCR-dependent contribution of Qa-1-restricted CD8 T cells to self-tolerance awaited generation and analysis of Qa-1 mutant mice. The B6.Qa-1-D227K strain expresses an amino acid exchange mutation (Arg → Lys, pos 227) that disrupts Qa-1–CD8 coreceptor binding, while preserving the Qa-1–NKG2A/CD94 interaction (Lu, Kim, Werneck, Cantor, 2008). The B6.Qa-1-D227K strain develops a spontaneous autoimmune disease relatively late in life, at around 6 months of age, characterized by a SLE-like pathology, including glomerulonephritis and elevated antinuclear antibody (ANA) production (Kim et al., 2010). The most prominent histological feature of this disease in B6.Qa-1-D227K mice is enlarged and numerous germinal centers that progressively increase with age and reflects loss of Qa-1-restricted regulation of T_{FH} cells and consequent increased autoantibody production.

An intriguing characteristic of CD8 Treg was the expression of a memory phenotype that depended on IL15 and distinguishes this subset from conventional CD8 cytotoxic effector lymphocytes. Discovery of IL15 dependency opened the door to identification of a characteristic set of CD8 Treg surface markers. Analysis of CD8 T cells that expressed the CD122 component of the IL15 receptor expressed a triad of surface markers: CD44, CD122, and Ly49. These triad$^+$ CD8 T cells but not CD44$^+$ CD122$^+$Ly49$^-$ CD8 T cells suppressed anti-NP antibody production in Rag2$^{-/-}$ hosts

adoptively transferred with CD8 T cells, B cells and $CD25^{-}$ CD4 T cells followed by immunization with NP-KLH/CFA (Kim et al., 2011).

The Ly49 marker is a member of the Ly49 receptor family that generally recognizes class I MHC and is expressed mainly by natural killer (NK) cells and NK T cells. Unlike NK cells, CD8 Treg express only inhibitory members of the Ly49 family (Ly49A, Ly49C/I, Ly49G and Ly49F) and 90% of CD8 Treg express the Ly49F receptor, which is not significantly expressed by NK cells. Although the ligands of Ly49A, Ly49C/I and Ly49G are H2-K^{b} and/or H2-D^{b}, the ligand for Ly49F has not been identified in the B6 mouse strain (Schenkel, Kingry, & Slayden, 2013) and the contribution of Ly49 to CD8 Treg biology is unclear. Although Ly49 inhibitory signals reduce CD8 Treg activation, they may also protect CD8 Treg from activation-induced cell death (Fig. 2). Since CD8 Treg recognize self-peptides presented by Qa-1, Ly49-dependent inhibitory signals may be required for Treg survival, expansion, and possibly function.

Most of the key biological properties of CD8 Treg differ from CD4 Treg, with the exception of a common requirement for the Helios TF. However, there are some remarkable similarities between CD8 Treg and NK T cells (Table 1; Fig. 3). Both subsets are restricted by non-classical MHC class Ib, Qa-1 and CD1d, respectively. Both CD8 Treg and NKT cells express a homologous set of stimulatory and inhibitory receptors: TCR, NKG2A and Ly49. Both CD8 Treg and NKT cells express a memory phenotype and IL15 is necessary for their development and function. CD8 Treg express regulatory activity via target cell killing that depends on perforin (Kim et al., 2010) as does the effector function of NK T cells (Fig. 3). There are also critical differences between CD8 Treg and NKT cells, including the canonical transcription factors of CD8 Treg and NK T cells which are Helios and PLZF, respectively (Kim et al., 2015; Savage et al., 2008). Moreover, during thymic development, Qa-1-restricted CD8 Treg are selected at the DP stage by hematopoietic cells and differentiate into the CD8 SP lineage, while NKT cells selected at the DP stage by hematopoietic cells become CD4 SP then DN immature NKT cells (Table 1) (Das, Sant'Angelo, & Nichols, 2010; Urdahl, Sun, & Bevan, 2002).

4. THE CONTRIBUTION OF CD8 TREG IN DISEASE SETTINGS

As discussed above, the contribution of CD8 Treg to regulation of autoimmune disease was initially studied using B6.Qa-1-D227K mutant mice. In the steady state, CD8 Treg inhibit excessive autoantibody

Table 1 A Comparison of $CD8^+$ Treg With $CD4^+$ Treg and NKT Cells

	$CD4^+$ Treg	$CD8^+$ Treg	NKT Cells
Selection in the thymus	Thymic epithelial cells	Thymic hematopoietic cells	Thymic hematopoietic cells
MHC restriction	MHC class II	MHC class Ib (Qa-1)	MHC class Ib (CD1)
Major transcription factor	FoxP3/Helios	Helios	Promyelocytic leukemia zinc finger (PLZF)
Antigens	Tissue-specific self-antigens	Stress-associated antigens, i.e., HSP60, FL-9 peptide	Lipid antigens
TCR repertoire	Polyclonal	Limited repertoire?	Limited repertoire (mouse: $V_\alpha 14\ V_\beta 7$)
Surface markers	CD25, GITR, Nrp-1, etc.	$CD44^+ CD122^+ Ly49^+$ triad	$CD44^+ CD122^+ NK1.1^+$
Cytokine dependence	IL-2	IL-15	IL-15
Major function	Inhibition of excessive immune response, tissue homeostasis	Inhibition of excessive immune response	Cytolysis, immune regulation

Comparison of CD8 Treg with canonical CD4 Treg shows that, with the exception of the contribution of Helios to phenotypic stability of both Treg, thymic selection, MHC restriction, antigen recognition and TCR repertoire are quite distinct. In contrast, there are striking similarities between key features of CD8 Treg and NKT cells (Engel et al., 2016; Kim et al., 2015).

production by controlling T_{FH} cell activity and preventing the development of an SLE-like autoimmune disorder (Kim et al., 2010). In response to immunization with exogenous antigen (NP-KLH), although acute responses seen in the first week (day 7) do not show differences between WT and B6.Qa-1-D227K mutant mice, at later stages (days 21–30) CD8 Treg were essential for return to a baseline state, whereas B6.Qa-1-D227K mutant mice continued to develop strong inflammatory responses (Kim et al., 2010). In an acute viral infection model using Lymphocytic choriomeningitis virus (LCMV), CD8 Treg controlled the number of CD8 effector cells and production of pro-inflammatory cytokines, including IFNγ, resulting in protection of the host from sustained inflammation and consequent tissue damage (Holderried, Lang, Kim, & Cantor, 2013).

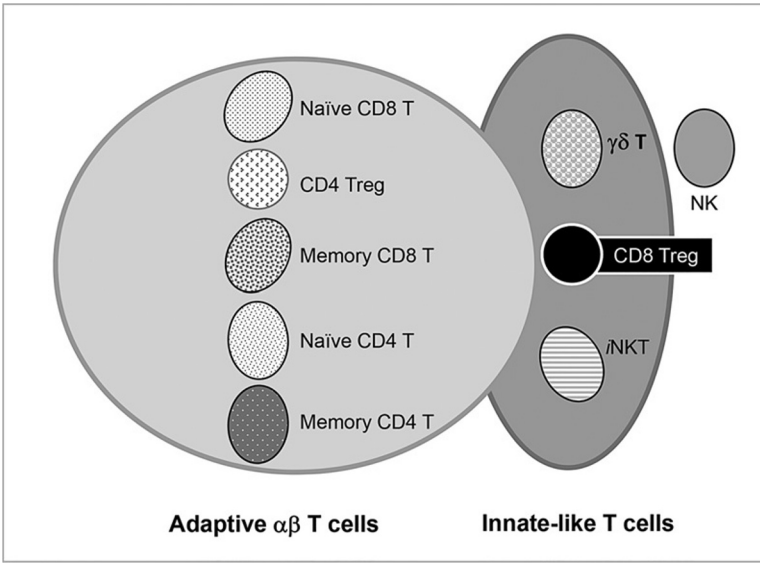

Fig. 3 $CD8^+$ Treg can be grouped with innate immune cells. Expression of NK-associated markers (Ly49, NKG2A, etc.), intrinsic activation status ($CD44^+CD122^+$) and perforin-dependent immediate cytolytic activity suggests that CD8 Treg may belong to innate T cells similar to NKT cells. CD8 Treg and γδ T, NK, and *i*NKT cells share common genes, including those for NK cell receptors, suggesting transcriptional commonality and genetic characteristics that resemble innate immune cells (Engel et al., 2016; Kim et al., 2015; Vivier & Anfossi, 2004; Vivier et al., 2011).

More recently, studies of the potential contribution of CD8 Treg have been extended to tumor immunity. In the B16-OVA melanoma tumor model after GVAX immunization, CD8 Treg contributed to inhibition of anti-tumor effects as judged by increased T_{FH} cells in both tumors and spleens, increased effector CD8 T cells in tumors and diminished intratumoral $Ly49^+$ CD8 Treg in B6.Qa-1-D227K mutant hosts (Alvarez Arias et al., 2014). Studies of this model also revealed inhibition of TAA-specific antibody production by CD8 Treg, as judged by anti-OVA antibody production against the B16-OVA tumor (Alvarez Arias et al., 2014).

In addition to the impact of CD8 Treg on effector T-cell responses, one must also consider the potential influence of CD8 Treg on NK responses to Qa-1-expressing tumor cells. Recent studies suggest that Qa-1 expression by tumor cells may allow evasion of the immune response in the context of immunotherapy (Manguso et al., 2017). However, the potential interactions

among tumor-expressed Qa-1 and its receptors, NKG2A and TCR, as well as the relevant Qa-1-reactive cell subsets have not been defined.

5. ANTIGEN RECOGNITION BY CD8 TREG

Recognition of Qa-1-associated self-peptides by CD8 Treg requires replacement of the dominant Qdm peptide by other peptides. Several mechanisms that may regulate this peptide presentation shift have been reported, including a malfunction in antigen–peptide processing machinery of normal or neoplastic cells. Defects of TAP, tapasin or endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) can increase the peptide bound to Qa-1 (Li et al., 2004; Nagarajan et al., 2016; Oliveira et al., 2010) and increase potential immunogenicity. This mechanism may underlie potential Qa-1-restricted CD8 T cell surveillance of transformed cells and compensate for reduced peptide presentation by classical MHC class I molecules. A second mechanism may depend on changes in immune cell status from a quiescent state to an activated or pathogenic state. For example, Hsp60 is expressed by activated immune cells and targeted by CD8 Treg (Soloski & Metcalf, 2001). Hsp60-derived peptide-loaded dendritic cell immunization can protect mice from EAE disease progression (Chen et al., 2007) and decrease the severity of collagen-induced arthritis (Leavenworth, Tang, Kim, Wang, & Cantor, 2013). Peptides derived from TCR $V_\beta 8$ expressed by MOG-specific pathogenic CD4 T cells (Jiang et al., 1995, 1998) may also contribute to the regulatory function of Qa-1-restricted CD8 T cells. TCR $V_\beta 8$-specific Qa-1-restricted CD8 T cells induced by staphylococcal enterotoxin B (SEB)-specific $V_\beta 8$ CD4 T cell vaccination may also recognize $V_\beta 8^+$ CD4 T cells specific for myelin basic protein (Jiang et al., 1995). Together, these studies strongly suggest that $V_\beta 8$-specific Qa-1-restricted CD8 T cells may control EAE (Jiang et al., 1998).

A series of studies of pig insulin β chain-derived peptide-specific Qa-1-restricted CD8 T cells using 6C5 transgenic mice has addressed key developmental issues (Sullivan, Kraj, Weber, Ignatowicz, & Jensen, 2002). T cells expressing the 6C5 TCR differentiated into the CD8 SP lineage in thymus and appear as $CD8^+$ T cells in the periphery. Their development is severely impaired in thymectomized recipient B6 mice reconstituted with bone marrow cells from 6C5 mice. Moreover, CD8 SP differentiation is markedly reduced in β2M-deficient or Qa-1b-negative (Tla^a) strains, but not in $K^{b-/-}D^{b-/-}$ mouse models. These findings suggest that 6C5 T cells are

positively selected by Qa-1 (or molecules encoded by Tla^b), along with β2 microglobulin. Although peptide presentation by Qa-1 to mature 6C5 T-cells was not TAP-dependent, the TAP pathway was required for 6C5 T cell development in the thymus. Additional analyses suggest that 6C5 T cells may be selected by either thymic epithelial cells or hematopoietic cells, since successful development was observed in B6 hosts reconstituted with 6C5.B2M$^{-/-}$ bone marrow cells as well as B6.B2M$^{-/-}$ hosts reconstituted with B6.6C5 bone marrow cells (Sullivan et al., 2002).

An intriguing finding concerning TCR chain usage by Qa-1 peptides and CD8 cells was shown in studies of FL9-specific Qa-1-restricted CD8 T cells. FL9-specific CD8 T cells preferentially use TRAV9d-3 (84%)/TRAJ21 (36%) and TRBV5 (40%)/TRBJ2-7 (50%), suggesting a similarity with NKT cells which use an invariant Vα chain (Vα14-Jα18) (Park et al., 2001). Qa-1-restricted CD8 T cells generated in response to TAP-deficient cells also displayed restricted but different TCR α chain usage (3/3 TRAV13D-4). One of these clones (Ln12) specific for Mediator complex subunit (Med) 15-derived peptide (RLIIHFRDI) was selected for transgenic mouse generation (Ln12 tg) and analysis of its thymic development. Surprisingly, Ln12 thymocytes differentiated into CD8 SP cells in both B6 as well as Qa-1-deficient mice (Doorduijn et al., 2018). The molecule responsible for selection of Ln12 T cells into CD8 SP in Qa-1-deficient mice was not identified. Possibly, this finding may reflect cross-reactivity with another MHC molecule. A paralog molecule of Qa-1 that is encoded in the complex H2-T loci of the B6 strain (H2-T11) has been duplicated in evolution (Ohtsuka, Inoko, Kulski, & Yoshimura, 2008). Since many of the peptides presented by Qa-1 may also be presented by this paralog (Chen et al., 2014), its potential contribution to development of Qa-1-restricted Treg and their interaction with self-peptides expressed by normal and neoplastic cells is of great interest. Additional questions remain and include the identity of molecules that select Qa-1-restricted T cells in the thymus and the role of TAP in Qa-1-restricted T cell selection and peripheral functionality.

Although Hsp60-specific Qa-1-restricted CD8 T cells and Vβ8-specific Qa-1-restricted CD8 T cells can regulate activated CD4 T cells, it is unclear whether FL9 or Ln12 T cells can be classified as regulatory cells, in view of their description as effector cells that target mutated tumor cells. Both FL9 and Ln12 T cells express a memory phenotype ($CD44^+CD122^+$), suggesting IL15 dependency and potential antigen exposure in the steady state. Possibly, small amounts of these antigenic peptides are degraded by TAP or ERAAP and presented by Qa-1 on activated or stressed cells

(Table 1). It has been reported that T cell activation induces ER stress (Kamimura & Bevan, 2008; Pino et al., 2008), resulting in an unfolded protein response (UPR) primed by IRE1α and resulting in MHC class I presentation that is altered by mechanisms that include TAP1 downregulation via XBP1 (Bartoszewski et al., 2011). The possibility that Qa-1-restricted recognition of tumor-associated peptides may also underlie Qa-1-restricted regulation of stressed immune cells requires additional analyses.

In sum, there is increasing evidence that both major T-cell lineages—CD4 and CD8—are divisible into effector pathways and a Helios-dependent regulatory path. We have reviewed experimental data indicating that Qa-1-restricted CD8 Treg are a unique Helios-dependent regulatory lineage that expresses a characteristic set of TCRs specific for HLA-E/Qa-1-associated self-ligands. Increased insight into the molecular and cellular events that control their development, differentiation and function should provide a window into a novel component of self-tolerance and immune homeostasis. These findings also have broad implications for new avenues of therapy, based on manipulation of antigen-specific TCR^+ CD8 Treg in the settings of autoimmunity and cancer.

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Trends in Immunology

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REVIEWS

Review

Overcoming Immune Checkpoint Blockade Resistance via EZH2 Inhibition

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Recent progress in cancer immunotherapy highlights the power of the immune system to control tumors, although a small patient subset responds to current immunotherapies. Additional approaches to mobilize antitumor immunity are required to overcome primary and acquired resistance to immunotherapy such as immune checkpoint blockade (ICB). Emerging evidence shows that targeting epigenetic elements that promote tumor progression and inhibit immune cell activity can enhance antitumor immunity by reshaping the tumor microenvironment (TME). Here, we review the pleiotropic functions in tumor and immune cells of enhancer of zeste homolog 2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2), with a focus on EZH2 inhibition as a potentially promising approach to enhance current immunotherapies and improve patient outcomes for certain cancers.

Highlights

Immune checkpoint blockade (ICB) has revolutionized the current cancer treatment paradigm, but low response rates require combination strategies to overcome primary and acquired ICB resistance.

Enhancer of zeste homolog 2 (EZH2) influences several key aspects of the tumor microenvironment that can contribute to ICB resistance, making it an attractive target to overcome ICB resistance in the clinic.

EZH2 inhibition can lead to increased T regulatory cell trafficking, impaired T regulatory cell capacity, increased antigen presentation, and increased antitumor immunity.

EZH2 inhibition has potentiated ICB in several preclinical models and can overcome acquired resistance to ICB in preclinical models of prostate cancer and head and neck cancer.

Additional studies are needed to understand the full potential of EZH2 inhibition as a possible strategy to improve antitumor immunity.

EZH2 inhibition in combination with ICB is currently being evaluated in the clinic.

Epigenetics and Immunotherapy

Advances in our understanding of the complex relationship between tumor cells and the immune response have resulted in a paradigm shift in cancer immunology as well as new and more effective approaches to cancer immunotherapy. **Immune checkpoint blockade (ICB)** (see Glossary) enables the adaptive immune response to recognize and kill tumor cells and has revolutionized the standard of care for several cancers, including melanoma, non-small cell lung cancer (NSCLC), and Hodgkin's lymphoma [1,2]. Despite these efforts, the success of ICB is currently limited to a small subset of patients that harbor highly immunogenic tumor types and evidence of a pre-existing immune response [3]. Tumors with low rates of mutations, high amounts of immunosuppressive factors, or a paucity of **tumor-infiltrating lymphocytes (TILs)** are less likely to respond to current checkpoint inhibitors [3,4]. Thus, strategies are needed to overcome both primary resistance to ICB as well as acquired resistance that can develop in initial ICB responders. Approaches that counteract immune escape mechanisms of the tumor and the **tumor microenvironment (TME)** may have the most potential to overcome ICB resistance mechanisms. These considerations have stimulated intense efforts to identify new classes of immune-modifying therapies to complement current approaches and extend the reach of tumor immunotherapy to more patients. A major focus of this effort has been to better understand the contribution of epigenetics in shaping the TME, and to harness the therapeutic potential of epigenetic inhibitors to boost antitumor immunity and improve current immunotherapy protocols.

Epigenetic regulation of gene expression can allow immune cells to modify their phenotype according to environmental cues, including those provided by the TME [5–8]. Recent discoveries have highlighted the contribution of epigenetic mechanisms, such as DNA methylation and **histone modification**, to regulate both antitumor immunity and self-tolerance [6]. Tumors also utilize epigenetic modifications to dampen immunity by disrupting key interactions that promote effective antitumor responses [8,9]. Epigenetic-based therapies that counter these immune

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evasion tactics may prime the immune response and complement current checkpoint or other immunotherapy approaches [10].

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase and the catalytic subunit of polycomb repressive complex 2 (PRC2) (Box 1). EZH2 catalyzes the mono-, di-, and **trimethylation of lysine 27 of histone H3 (H3K27me3)** [11]; a histone mark associated with compacted chromatin and repressed transcription. EZH2 plays a role in the normal biology of several cell types, including immune cells. Dysregulated EZH2 function has been implicated in the development of several cancer types in mice and humans (Box 1) [12] and can contribute to immune evasion, in part by suppressing intratumoral antigen presentation, immune cell migration, and enhancing **CD4$^+$ T regulatory (Treg) cell** suppressive activity [5,8]. These functions make EZH2 an attractive therapeutic target that may complement current immunotherapy approaches.

The oncogenic role of EZH2 in several cancer types has resulted in the development of EZH2 inhibitors (EZH2i) that are now being utilized in the clinic, including tazemetostat, which has been approved by the FDA for treatment of epithelioid sarcoma and follicular lymphoma [13]. Successful strategies that combine EZH2i with ICB depend on an understanding of the impact of EZH2 on the differentiation and function of immune cell types in the TME, as well as insight into how the pleiotropic effects of EZH2 inhibition can overcome mechanisms of ICB resistance. Here, we review the impact of EZH2 on the functional differentiation of **CD4$^+$ T helper (Th)** and regulatory cell lineages, the CD8$^+$ cytotoxic T lymphocytes (CTL), as well as **natural killer (NK) cells** and myeloid cells (Figure 1). We then summarize our current understanding of TME-modifying effects of EZH2 inhibition, and potential of strategies that combine EZH2i with ICB.

EZH2 in CD4$^+$ T Cell Differentiation and Function

CD4$^+$ Th cells normally coordinate activation of the immune response through differentiation into distinct lineages that include the Th1, Th2, Th17, and **T follicular helper (Tfh) cell** subsets; each

Box 1. Dual Roles of EZH2 in Tumor Biology

The PRC includes PRC1 and PRC2, which play a major role in transcriptional regulation and are required for long-term epigenetic silencing of chromatin, as well as stem cell differentiation and early embryonic development. PRC1 may limit access of TFs to chromatin and inhibit gene expression. PRC2 displays histone methyltransferase activity and primarily methylates histone H3 on lysine 27 (H3K27) to mark transcriptional silencing of chromatin. PRC2 is required for initial targeting of a genomic region to be silenced, while PRC1 stabilizes silencing and underlies cellular memory of the silenced region after cellular differentiation. EZH2 is a histone-lysine N-methyltransferase enzyme encoded by the *EZH2* gene that participates in histone methylation and transcriptional repression. Although mutation or overexpression of EZH2 has been linked to many types of cancer, there is emerging evidence for a central role of EZH2 in regulating the functional differentiation of immunological cells.

EZH2 plays a complex role in tumor biology, with both oncogenic and tumor-suppressive roles depending on the context. The contribution of dysregulated EZH2 function to tumorigenesis has been extensively studied. For example, heterozygous activating point mutations in the *EZH2* enzymatic domain have led to aberrant accumulation of H3K27me3, promoting epigenetic reprogramming of B cells, and contributing to the development of follicular lymphoma and DLBCL [72–75]. Additionally, mutations in components of the SWI/SNF complex, which normally serves to antagonize PRC2 function, can lead to loss of SWI/SNF function and aberrant EZH2 activity in certain cancers [76,77]. Also, overexpression of EZH2 has been associated with rapid tumor progression in multiple cancer types, including breast, bladder, endometrial, prostate, and melanoma [78–81]. By contrast, EZH2 nonsense mutations and inactivating deletions have been identified and characterized in myelodysplastic syndromes, myeloproliferative neoplasms, and human T cell lymphoblastic leukemias, suggesting that EZH2 can exert tumor suppressive activities in these cellular contexts [82–85]. Thus, EZH2 appears to play a dual role in tumor cell biology as either an oncogene, or a tumor suppressor, depending on the cellular, genetic, and tumor contexts in which its function is altered. Nevertheless. the known oncogenic roles of EZH2 have led to the development of several EZH2i [13]. Published reports have demonstrated efficacy of EZH2i in multiple preclinical models in immunodeficient mice and have provided a rationale for several clinical trials currently underway testing EZH2i (reviewed elsewhere in [86]).

Glossary

Cancer-associated fibroblasts (CAFs): comprise a heterogeneous population of cells involved in tissue remodeling to support cancer invasion and metastasis; support immune tolerance within the TME by promoting suppressive cells and eradicating effector cells.
CAR (chimeric antigen receptor)-T-cell therapy: chimeric antigen receptor T cells genetically modified to contain a TCR that specifically targets antigens within a patient's tumor.
CD4$^+$ T helper (Th) cells: further differentiate into multiple Th subsets (Th1, Th2, and Th17) in response to developmental and environmental cues.
CD4$^+$ T regulatory (Treg) cells: characterized by their ability to suppress immune responses by other cells; express CD25, reflecting their sensitivity to survival factor IL-2; also express Foxp3.
CD8$^+$ T memory (Tmem) cells: epigenetic modifications are involved in the transition from naïve T cells to CD8$^+$ Tmem cells; some of these modifications persist after antigen clearance, establishing epigenetic memory that allows faster activation upon re-encounter with antigen. Certain effector genes, for example, *IFNG*, are not expressed but are transcriptionally poised for fast expression upon activation.
***CDKN2A*:** human gene (chromosome 9, band p21.3) is ubiquitously expressed in many tissues and cell types; codes for two proteins, INK4 family members p16 and p14ARF, which act as tumor suppressors by regulating the cell cycle. Somatic mutations of *CDKN2A* are common in most human cancers; germline mutations of *CDKN2A* are associated with familial melanoma, glioblastoma, and pancreatic cancer.
Degron: portion of a protein important in regulating protein degradation rates. Known degrons include short amino acid sequences, structural motifs, and exposed amino acids (often lysine or arginine) located anywhere in the protein.
Dendritic cells (DCs): antigen-presenting cells of the mammalian immune system whose primary function is to process and present antigen to the surface of T cells, acting as messengers between the innate and adaptive immune systems. DCs are usually not abundant at tumor sites, but increased

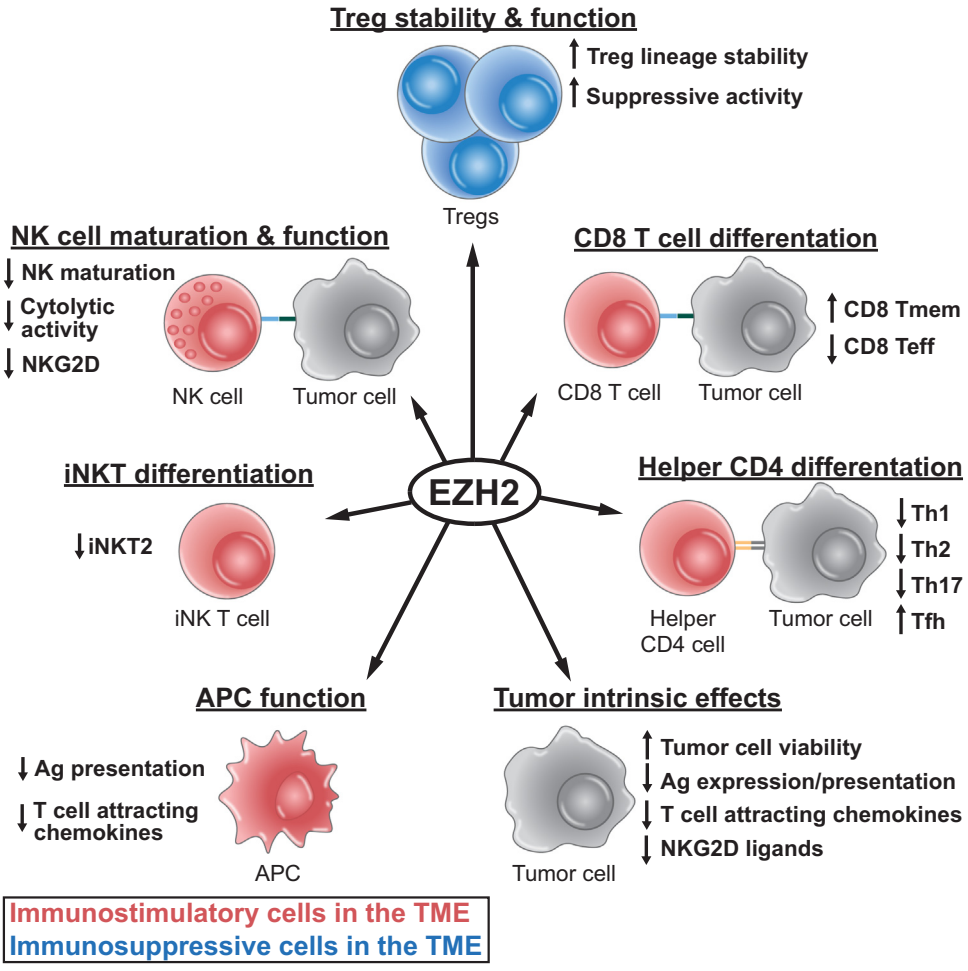


Figure 1. Role of EZH2 in the Biology of Immune Cells in the Tumor Microenvironment (TME). Enhancer of zeste homolog 2 (EZH2) is expressed in many immune cells (discussed here in mice and humans) and controls their distinct function. EZH2 expression in T cells generally dampens their antitumor responses. EZH2-dependent gene repression restricts the differentiation of T helper (Th) subsets Th1, Th2, and Th17, promotes T follicular helper (Tfh) cell differentiation and function, and increases lineage stability and suppressive activity of regulatory $CD4^+$ T regulatory (Treg) cells. Emerging evidence suggests that EZH2 promotes $CD8^+$ T memory (Tmem) precursor identity while silencing $CD8^+$ T effector (Teff) cell differentiation and function. Studies suggest that EZH2 inhibits natural killer (NK) cell activation, survival, and cytotoxicity. EZH2 also controls invariant natural killer T (iNKT) cell differentiation and function by inhibiting canonical iNKT transcription factor (TF) PLZF. In addition, EZH2 can control the antitumor immune response by suppressing antigen presentation by professional antigen presenting cells (APCs) and tumor cells.

plays a distinct role in antitumor immunity. Several reports underscore the role of EZH2 in limiting the differentiation and plasticity of naïve T cells (Table 1; Figure 2). T cell-specific deletion of *Ezh2* in mice *(CD4-Cre; Ezh2$^{fl/fl}$)* leads to increased T cell cytokine production by several Th lineages, indicating that Ezh2 normally silences expression of lineage-specific cytokines [14,15]. Ezh2 repression of Th1 and Th2 differentiation is also associated with H3K27me3 repressive epigenetic marks at the *Ifng* and *Tbx21* loci and the *Il4* and *Gata3* loci in mice, respectively [15,16]. This suggests that EZH2 inhibition can lead to increased expression of effector cytokines by $CD4^+$ T cells.

densities of DC populations have been associated with improved clinical outcomes for certain cancers.

DNA methyltransferase (DNMT): family of enzymes catalyzing the transfer of a methyl group to DNA, serving a wide variety of biological functions. Due to their epigenetic effects, some DNMT inhibitors are under investigation for certain cancer treatments.

Genetically engineered mouse models (GEMMs): developed by deleting, overexpressing, or mutating genes known to be strongly associated with a specific condition; here, tumor formation. Useful for immunotherapy assessment because of their fully competent immunity and their similarity to human tumor growth.

Histone modification: form of post-translational modification of histone proteins, including methylation, phosphorylation, acetylation etc., regulating gene expression by altering chromatin structure.

Immune checkpoint blockade (ICB): immune checkpoints include a number of inhibitory pathways hardwired into the immune system for maintenance of self-tolerance and modulation of the immune response to microbes and tumors. Many depend on ligand–receptor engagement, such as PD-L1–PD1 or B7.1(2)–CTLA-4. Antibody blockade of these interactions can enhance immune responses. Anti-CTLA-4 and anti-PD-1 are antibody prototypes of this class of cancer ICB immunotherapeutics.

Invariant natural killer T (iNKT) cells: T cells that recognize self and foreign lipid antigens in the context of CD1d (nonpolymorphic MHC-I-like molecule) and express effector cytokines, including IFNγ and IL-4 within minutes after antigen recognition. The vast majority of iNKT cells express T cell receptor (TCR)α (Vα14/Jα18 in mice; Vα24/Jα18 in humans) paired with a restricted set of TCRβ, and thus are considered invariant.

***In vitro*-induced Treg cells (iTregs):** $CD4^+$ Tregs generated from conventional $CD4^+$ T cells *in vitro* with conditions that induce FoxP3 expression, including stimulation with (i) anti-CD3, anti-CD28 antibodies, IL-2, and transforming growth factor-β (Tgf-β) and (ii) anti-CD3 antibodies, Tgf-β, and DCs.

MHC class I and II: cell surface molecules that present peptides derived from protein antigens to $CD8^+$ or $CD4^+$ T cells respectively. MHC class I is

Table 1. Summary of the Effects of EZH2 Activity and Inhibition on Key Immune Cells and the TME

Cell type	EZH2 activity on immune cell identity and function	Effects of EZH2 inhibition on the TME	Species (Refs)
$CD4^+$ Th	Repression of Th1, Th2, and Th17 polarization by targeting T-bet, Gata3, and RORα	Induction of Th1 chemokine expression	Mice [14,16,17,20,87]
$CD8^+$ Teff	Complex and stage-specific role in $CD8^+$ Teff and Tmem differentiation	Promotion of T cell trafficking to the TME by induction of attractant chemokines CXCL9 and CXCL10	Mice [32–37] Humans [36]
Treg	EZH2 is highly induced in Tregs following engagement of the CD28 co-stimulatory receptor; its expression helps to stabilize an activated functional phenotype	Represses Treg phenotype; promotes $CD4^+$ Teff differentiation	Mice [5,28–30] Humans [27,31]
Tfh	EZH2 occupancy and H3K27 modifications of Tfh genes, including the *Bcl6* master TF, increases EZH2 expression	Impact of EZH2 inhibition on ectopic germinal center formation in the TME needs further evaluation	Mice [18,21] Humans [18]
NK	Suppression of NK cell development and cytolytic activity by regulating NKG2D and *GzmB* expression	Increases gene expression required for NK cell activation, survival, and cytotoxicity	Mice [38] Humans [38]
iNKT	Negatively regulates proinflammatory differentiation of iNKT cells by repressing PLZF and Gata3 expression	Upregulates CD1d on tumor cells upon combination treatment with RARα ligand and enhancement of iNKT cell-mediated tumor killing activity [88]	Mice [42,43]
Macrophage	Implicated in the maintenance of proinflammatory macrophage polarization and survival	Potentially increases proinflammatory TAM and decreases anti-inflammatory TAM. Additional studies needed.	Mice [15,55] Humans [54,55]
MDSC	Potentially limits MDSC formation	Potentially leads to increases in MDSCs. Additional studies needed	Mice [89]

EZH2-dependent regulation of gene expression may also contribute to dampening Th17 differentiation, as evidenced from increased IL-17 production from *Ezh2*-deficient $CD4^+$ T cells during Th17 cell-driven transfer colitis in mice [14]. Recent studies have also shown that expression of the central Th17 transcription factor (TF) – RORα – is potentiated in mouse embryonic fibroblasts after either Ezh2 knockdown or overexpression of an Ezh2 mutant lacking enzymatic activity in mouse embryonic fibroblasts; this suggests that Ezh2-mediated methylation of RORα can promote its degradation and inhibit Th17 development [17]. Collectively, these findings also suggest that targeting Ezh2 may modulate Th lineage-dependent immune responses in mice.

Tfh cells are a specialized $CD4^+$ T cell subset that plays a central role in the induction of protective antibody responses to pathogens and have recently emerged as a key component of the TME [18,19]. Recent analyses of immune landscapes in human colon cancers using systems biology revealed that CXCL13-dependent infiltration of Tfh cells into the TME and formation of lymphoid-like follicles inversely correlated with tumor progression and recurrence [18]. Tfh differentiation in mice and humans depends on the expression of the *BCL6* master TF, while Tfh migration into B cell follicles depends on expression of the *CXCR5* gene [20]. Global mapping of *Ezh2* promoter occupancy and H3K27 modifications of Tfh lineage-associated genes, such as *Bcl6* during murine viral infection by the Armstrong (Arm) strain of lymphocytic choriomeningitis virus (LCMV-Arm), has revealed that *Ezh2* gene occupancy is associated with both transcriptional activation and repression of the mouse Tfh genes, *Bcl6* and *Arf*, respectively [21]. The noncanonical role of Ezh2 as a transcriptional coactivator of *Bcl6* may reflect a Ser21 phosphorylation

expressed ubiquitously while MHC class II is expressed in general by professional antigen-presenting cells, including B cells and DCs.

Myeloid-derived suppressor cells (MDSCs): heterogeneous population of cells consisting of immature myeloid cells; expand during cancer, inflammation, and infection, and display a remarkable ability to suppress T cell responses.

NK (natural killer) cells: type of cytotoxic lymphocytes of the innate immune system with antiviral, anticancer, and anti-graft-versus-host disease properties.

Tertiary lymphoid structures: developed in non-lymphoid tissues at sites of chronic inflammation (including tumors) and affected tissues in patients with autoimmune disease.

T follicular helper (Tfh) cell: specialized $CD4^+$ T cell subset; provides help to B cells triggering them to produce antibodies within germinal centers in secondary lymphoid structures.

Therapeutic cancer vaccine: designed to treat existing tumors through stimulation of the immune system with cancer antigens or autologous tumor cells from a patient.

Trimethylation of lysine 27 of histone H3 (H3K27me3): epigenetic modification to the chromatin structure protein histone H3; associated with repression of gene transcription via the formation of heterochromatic regions.

Tumor-associated antigens (TAAs): present on some tumor cells and also some normal cells; can trigger an immune response in the host; are useful markers for identifying tumor cells with diagnostic tests and potential target candidates in cancer therapy.

Tumor-associated macrophages (TAMs): contribute to tumor initiation, progression, and metastasis by inhibiting T-cell mediated immunity and stimulating tumor angiogenesis; generally characterized by expression of anti-inflammatory cytokines, scavenging receptors, angiogenic factors, and proteases; together, contribute to an immunosuppressive TME.

Tumor-infiltrating lymphocytes (TILs): subpopulation migrating to tumors, recognizing and attacking cancer cells upon infiltration. High numbers of TILs in tumors may be

modification of Ezh2 because pS21–Ezh2 is detected predominantly in Tfh cells during LCMV-Arm infection in mice [21]. Moreover, Ezh2 repression of cyclin-dependent kinase inhibitor 2A (Cdkn2a) has also been reported to diminish cellular apoptosis and promote robust Tfh differentiation, since ablation of p19Arf, a product from the ***Cdkn2a*** locus, alleviates enhanced apoptosis of Ezh2-deficient (*Ezh2*$^{-/-}$) Tfh cells [21]. While Ezh2 regulation of the Th1/Th2 lineage-specific genetic program may depend mainly on repressive histone methylation, control of Tfh gene expression by Ezh2 may combine repressive and activating elements [21]. Further studies are warranted to understand the impact of EZH2 inhibition on Tfh differentiation within the infection context, as well as in the TME in different cancer types, in view of increasing evidence that the formation of ectopic **tertiary lymphoid structures** containing Tfh cells may represent a positive prognostic sign during immunotherapy. This is also based on correlative findings between B/Tfh cell signatures and ICB responsiveness in patients with melanoma and renal cell carcinoma [19].

indicative of an improved clinical outcome.

Tumor microenvironment (TME): interactive cellular milieu surrounding a tumor; includes blood vessels, immune cells, cytokines and chemokines, fibroblasts, and the extracellular matrix.

EZH2 Control of Tregs

Tregs maintain immunological self-tolerance and homeostasis by suppressing inflammatory responses, and can play a major role in suppressing antitumor immunity [22–25]. Expression of the Foxp3 TF is essential to the differentiation and function of Tregs [26,27]. Early analysis of Foxp3-dependent mechanisms of chromatin remodeling and regulation of gene expression in mouse T cells has suggested that Foxp3-containing complexes, which harbor Ezh2, promote H3K27me3 modifications of Foxp3-bound loci that are associated with silencing of genes normally expressed by conventional CD4$^+$ T effector (Teff) cells [28]. However, the nature and function of these multimolecular complexes have not been well defined, and their activity may depend on the location and activation state of Tregs (Box 2) [29].

Recent studies have indicated that Ezh2 is the most highly induced chromatin modifier of mouse Treg cells following engagement of the CD28 co-stimulatory receptor, and its expression helps to stabilize the functional phenotype of activated Tregs [30]. Since upregulated genes in activated *Ezh2*-deficient Treg (*Foxp3-GFP-hcre;Ezh2*$^{fl/fl}$) are mainly Foxp3 target genes that are also expressed by conventional CD4$^+$ Teff cells, it is likely that Ezh2 normally represses a CD4$^+$ Teff cell phenotype after activation [30]. Indeed, studies of mouse MC38 tumor models as well as human colorectal cancer, NSCLC, and breast cancer demonstrate EZH2 expression and associated H3K27me3 marking of tumor-infiltrating Tregs. This in turn suggests that targeting EZH2 expression in intratumoral Tregs might be a potentially effective approach to enhance antitumor immunity [5,27,31].

EZH2 in CD8$^+$ T Cell Differentiation

The development of potent and durable antitumor immunity depends in part on a highly controlled process of CD8$^+$ T cell activation, proliferation, terminal differentiation, and memory. The epigenetic landscapes of naïve, CD8$^+$ Teff and **CD8$^+$ T memory (Tmem) cells** include

Box 2. Context-Dependent Effects of EZH2 on Treg Phenotype

Recent analyses of Foxp3-dependent regulation of gene sets that are up- or downregulated in Tregs compared with conventional CD4$^+$ T cells using a Foxp3 alanine scan library revealed that the impact of Foxp3 binding to enhancer regions in mice depended on the formation of functionally distinct multimolecular complexes that either activated or repressed gene expression [29]. For example, association of Foxp3 with repressive cofactors including EZH2 and Ikzf3 promoted Foxp3-dependent repression through recruitment of NurD and Polycomb assembly, while Foxp3-dependent clustering of activating cofactors (e.g., RelA, Kat5, and Ikzf2) favored enhanced activation of mouse Treg-associated genes [29]. Thus, Foxp3-dependent shaping of the Treg transcriptional signature and consequent Treg activity reflects the combination of these effects on classes of multimolecular Foxp3-containing complexes, which are themselves determined by extrinsic factors, including cellular activation state and external environmental stimuli or cues.

characteristic shifts in patterns of permissive H3K4me3 and repressive H3K27me3 marks during T cell differentiation [32]. Analysis of mouse $CD8^+$ T cells that express **tumor-associated antigen (TAA)**-specific T cell receptors (TCRs), such as Pmel-1 in B16 melanoma, indicates that Ezh2 is essential for the development and maintenance of memory precursors by activating Id3-dependent Tmem development, while silencing *Id2, Prdm1* (*Blimp-1*), and *Eomes* drives effector differentiation and function [33].

By contrast, analysis of $CD8^+$ T cell differentiation by single-cell RNA-sequencing (RNA-Seq) during mouse LCMV infection suggests that Ezh2 activity during the early stage of $CD8^+$ Teff proliferation might contribute to $CD8^+$ Teff differentiation [34,35]; in part through H3K27me3-mediated repression of proapoptotic genes [36]. Moreover, genome-wide profiling of the chromatin-associated H3K27ac and H3K27me3 marks in Tmem precursor and terminally -differentiated $CD8^+$ Teff cells during acute LCMV-Arm infection in mice has suggested that Ezh2/PRC2 inhibits an early polyfunctional stage and promotes a high number of differentiated Teff progeny [37].

The studies described above suggest a complex role for EZH2 in $CD8^+$ Teff and Tmem differentiation that may also reflect the different experimental systems used (e.g., Pmel-1 in melanoma tumor models vs LCMV infection). The impact of EZH2 on $CD8^+$ T cells activated by TAAs requires more intensive analysis of clinically relevant tumor models, as well as more precise characterization of $CD8^+$ T cells at distinct stages of intratumoral differentiation.

EZH2 in NK and NKT Cell Differentiation and Function

NK cells are innate lymphocytes endowed with potent cytolytic activity that protects against microbial infections and tumor growth. EZH2-dependent epigenetic control of gene expression plays a crucial role in NK cell lineage differentiation and function [38]. Genetic deletion (Vav1-Cre, $Ezh2^{fl/fl}$ mice) or pharmacological inhibition (UNC1999 and EPZ005687) of Ezh2 activity in hematopoietic stem progenitor cells (HSPCs) increases NK cell lineage development by promoting survival and differentiation of NK precursors in mice [38]. Enhanced NK cell generation in the absence of Ezh2 activity in mice is accompanied by increased expression of genes involved in NK activation, survival, and cytotoxicity, including *Klrk1* (encoding the activating receptor, NKG2D), *IL2ra*, *IL7r*, and *Gzmb* [38]. Increased killing of lymphoma cells by $NKG2D^+$ NK cells following treatment of HSPCs with EZH2i opens up the possibility that EZH2 inhibition might be able to promote NK-mediated antitumor responses [38].

EZH2 might also negatively regulate the proinflammatory differentiation of **invariant natural killer T (iNKT) cells**; an innate T cell population that recognizes lipid antigens and has emerged as a player in antitumor immunity [39,40]. Similar to Th lineage differentiation, iNKT cells are controlled by the innate T cell-specific TF PLZF that regulates early development, and further differentiation into distinct iNKT cell subsets is dependent on a distinct set of TFs that promote lineage restriction in mice, (e.g., T-bet^+ iNKT1, $Gata3^+$ iNKT2, and $ROR\gamma t^+$ iNKT17) [39–41].

Analysis of mice with a CD4-dependent *Ezh2* deletion (CD4-Cre;$EZH2^{fl/fl}$) has revealed that loss of *Ezh2* results in a dramatic increase of iNKT2 cells that produce large amounts of Th2 cytokines, including interleukin (IL)-4 and IL-13 relative to wild-type (WT) mice; however, the impact on iNKT1 and iNKT17 cells is modest [42]. Moreover, ChIP-Seq analysis in this study indicated that Ezh2 directly targeted the *Zbtb16* gene encoding PLZF. Therefore, increased development of iNKT2 cells in Ezh2-deficient mice may reflect derepression of PLZF and Gata3 (key TF for Th2 differentiation) secondary to diminished H3K27me3 marks at these gene loci relative to WT

mice [42]. This suggests that EZH2 inhibition can induce skewing of NKT cells towards a Th2-like subset.

A chromatin-independent function of EZH2 in NKT cells has also been suggested based on the observation that EZH2 directly methylates PLZF protein at lysine residue K430, leading to increased ubiquitination and degradation through the formation of a methyl **degron** in humans and mice [17,43]. Moreover, sustained PLZF expression in *Ezh2*-deficient mice (CD4-Cre;Ezh2$^{fl/fl}$) promoted expansion of PLZF^{hi} NKT cells in the absence of Ezh2-PRC2-mediated gene silencing, suggesting that maintenance of NKT cell homeostasis occurred in an Ezh2-mediated manner [43]. An increased understanding of EZH2-dependent control of genetic programs in NK cells and iNKT cells might allow rational approaches to boost innate tumor immunosurveillance via EZH2 inhibition.

EZH2 Inhibition and Reshaping of the TME

The contribution of EZH2 to the differentiation and function of lymphocyte subsets summarized above suggests that EZH2 inhibition has the potential to enhance antitumor immunity in certain cancers. However, a more informed prediction of the potential impact of EZH2 inhibition on antitumor immunity requires consideration of the pleiotropic effects of EZH2 and EZH2 inhibition on both tumor cells and immune cells shaping the TME (Table 1; Figure 2). Here, we summarize evidence that EZH2 contributes to several of the key mechanisms of innate and acquired resistance to ICB and highlight how the TME-modifying effects of EZH2 inhibition may counteract these mechanisms to overcome ICB resistance (Figure 3, Key Figure).

EZH2 Inhibition Promotes CD8$^+$ Teff Cell Function and Trafficking to the TME

Trafficking of CD8$^+$ T cells into the TME and cytotoxic killing of tumor cells are key steps in anticancer immunity, and exclusion of T cells from the TME is a key tactic of immune evasion. T cell recruitment is facilitated by secretion of chemokines including CXCL9 and CXCL10 by tumor-resident **dendritic cells (DCs)** [44]. Suppression of these chemokines by EZH2 impairs T cell trafficking into the TME, and EZH2 inhibition can reverse this effect. For example, EZH2i treatment of five mouse ovarian cancer cell lines increased CXCL9 and CXCL10 expression *in vitro* [45]. EZH2 inhibition, in combination with **DNA methyltransferase (DNMT)** inhibition, in the ID8 mouse ovarian tumor model elevated CXCL9 and CXCL10 expression, increased effector cell trafficking, and potentiated ID8 tumor killing *in vivo* following anti-programmed death-ligand 1 (PD-L1) antibody treatment, compared with anti-PD-L1 antibody treatment alone. Furthermore, immunohistochemical analysis of 186 high-grade serous ovarian tumor patient samples showed that increased EZH2 expression in tumor cells correlates with decreased CD8$^+$ T cell infiltration and poorer prognosis [45]. In another study, *in vitro* tumor necrosis factor (TNF)-α treatment of mouse melanoma cell lines B16-F10 and RIM-3 led to Ezh2-dependent CXCL9 and CXCL10 downregulation that was reversed by EZH2i treatment. Furthermore, knockdown of *Ezh2* expression in B16-F10 tumor cells increased *in vivo* production of CXCL9 and CXCL10, while EZH2i combined with IL-2 complexes or anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) promoted CXCL9 and CXCL10 expression as well as CD8$^+$ T cell infiltration and tumor regression, compared with untreated tumors [46]. EZH2-mediated suppression of T cell-attracting chemokines was further supported by a recent study confirming increased CXCL9 and CXCL10 production following EZH2i treatment of B16-F10 and MB49 mouse melanoma cell lines *in vitro*, and MB49 tumor-bearing mice *in vivo*, compared with controls [47].

In some cancers, increased CD8$^+$ T cell infiltration correlates with poorer prognosis, suggesting an immunosuppressive TME [48]. Of note, studies have shown that infiltrating T cells can lead

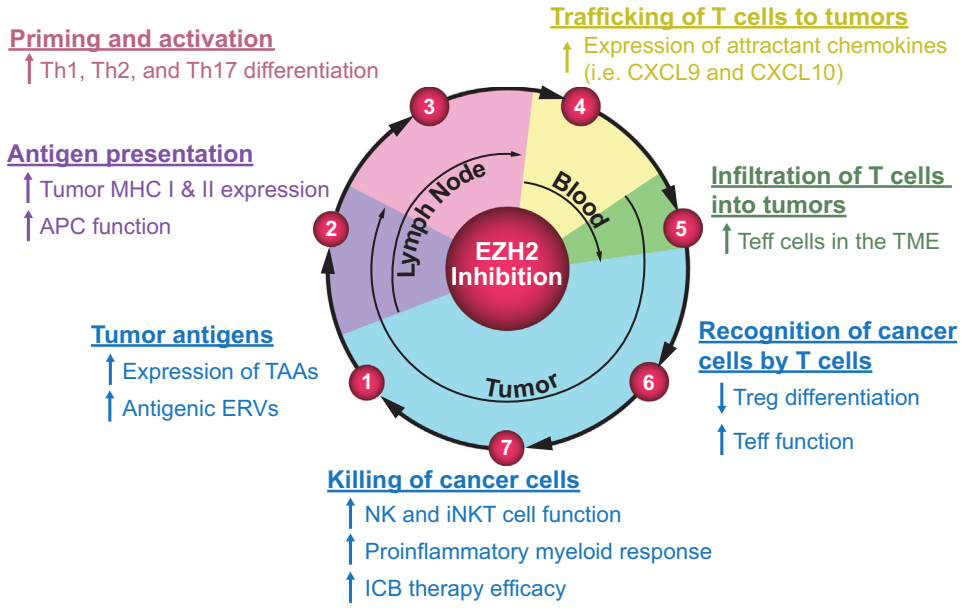
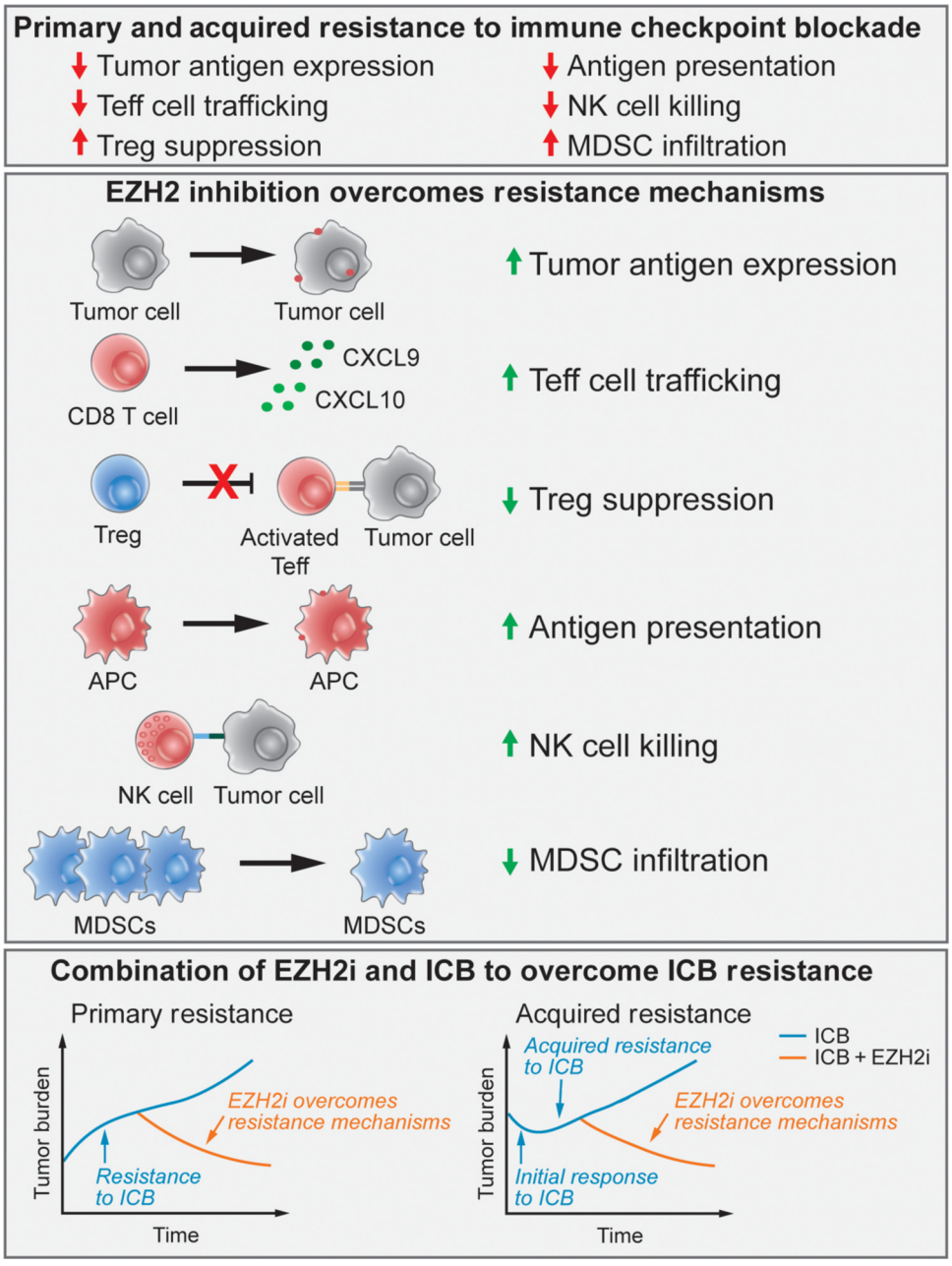


Figure 2. Enhancer of Zeste Homolog 2 (EZH2) Inhibition Exerts Multiple Effects on the Cancer Immunity Cycle. The cancer immunity cycle describes the intricate balance that the immune system must strike to combat tumor cells without causing autoimmunity [90]. This stepwise process begins with (1, 2) expression and presentation of tumor-associated antigens (TAAs), followed by (3) priming and activation of effector cells that (4–7) traffic to the tumor site to kill tumor cells. Several studies have characterized the role of EZH2 in limiting the plasticity and trafficking of both regulatory and effector cells to the tumor microenvironment (TME), as well as in decreasing cytokine signaling that is essential throughout the cancer immunity cycle [44,45,47]. EZH2 inhibition can alleviate this repression to stimulate the release and presentation of tumor antigens and increase the trafficking and function of effector immune cells in the TME [8]. As a result, EZH2 inhibition has the potential to boost anticancer immunity by promoting multiple aspects of the cancer immunity cycle. Abbreviations: ERV, endogenous retrovirus; ICB, immune checkpoint blockade; iNKT, invariant natural killer T cell; NK , natural killer; Teff, $CD4^+$ T effector cells; Th, T helper; Treg, $CD4^+$ T regulatory (Treg) cells.

to increased EZH2 expression and epigenetic-mediated dampening of tumor immunity. For example, a survey of melanoma patient data in The Cancer Genome Atlas (TCGA) revealed a strong correlation between $CD8^+$ T cells and expression of PRC2 complex components, including EZH2 [46]. This observation was validated in three experimental melanoma models in immunocompetent mice: B16-F10 and two **genetically engineered mouse models (GEMMs)** of melanoma. Immunotherapy with either IL-2 complexes or anti-CTLA-4 antibody resulted in an increase in Ezh2 mRNA and protein expression localized to areas of increased lymphocytic infiltrates, relative to controls [46]. However, these findings could not be recapitulated when the same immunotherapy was directed against these melanoma lines *in vitro* or in *Rag1*$^{-/-}$ mice (which lack B and T cells). This suggests a putative link between T cell infiltration and increased Ezh2 expression in melanoma tumors [46]. Recently, immunohistochemistry in 1603 human kidney tumor samples assessed on tissue microarrays revealed a correlation between $CD8^+$ T cell infiltration, increased EZH2 expression, and poorer prognosis in renal cell carcinoma [48]. Additionally, first-line therapy of ipilimumab led to increased EZH2 expression in peripheral blood $CD4^+$ T cells compared with baseline in metastatic melanoma patients. Of note, in experimental models, T cell activation through CD28 signaling – often following anti-CTLA-4 antibody treatment – also increases EZH2 expression in $CD8^+$ T cells and may dampen their effector capacity [5]. Indeed, a reversal of this process has been reported after EZH2i administration in mouse and human cells [30,47]. These studies suggest that EZH2 counterbalances

Key Figure

Tumor-Modifying Effects of Enhancer of Zeste Homolog (EZH2) Inhibition Can Overcome Resistance Mechanisms and Sensitize Resistant Tumors to Immune Checkpoint Blockade (ICB)



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T cell activation and dampens $CD8^+$ Teff-mediated antitumor immunity in the TME (presumably, therapeutically targeting EZH2 via EZH2i might reverse this form of immune evasion).

As the lack of effector TILs is a key hurdle for current immunotherapies, the TME-modifying effects of EZH2i may have the potential to boost ICB efficacy in patients that otherwise would not respond to immunotherapy. As described above, EZH2 expression in tumor and immune cells serves to limit $CD8^+$ Teff trafficking to tumors, while increased EZH2 expression after T cell activation might also contribute to innate and acquired resistance to ICB. Taken together, clinical observations and experimental studies to date highlight the potential of EZH2i to overcome this hurdle and extend the reach of ICB in the clinic.

Support for this hypothesis comes from an analysis of biopsy samples from a patient with poorly differentiated chordoma who was enrolled in an open-label, dose-escalation, and dose-expansion, single-group Phase I study of the EZH2i tazemetostat in patients with relapsed or refractory INI1-negative tumors (ongoing) (NCT02601937[i]). In Phase II of this study, immunohistochemistry of post-tazemetostat treatment on this patient revealed increased tumor infiltration of $Ki67^+$ $CD8^+$ T cells with concurrent increase in PD-1 expression, as well as upregulated PD-1 and LAG3 expression by stromal $CD8^+$ T cells, relative to pre-tazemostat treatment. These data suggest that EZH2i might contribute to modulating an antitumor $CD8^+$ T cell response in this patient [49]. In this case study, concurrent treatment with ICB and/or radiation revealed evidence of increased proliferating CTLs, as well as Tregs in metastatic lung lesions, where an abscopal effect was observed [49]. Thus, combination therapy with ICB might be effective in reinvigorating effective antitumor Teff cell functions, although further studies are warranted to confirm these effects.

EZH2 Inhibition Can Diminish the Impact of Tregs in the TME

Increased Treg activity relative to Teff cell activity in the TME can hinder the effectiveness of ICB, and EZH2i has been suggested to potentially help in overcoming this hurdle. Targeted deletion of *Ezh2* in Tregs (*Foxp3*^{cre}*Ezh2*$^{fl/fl}$ mice) or with EZH2i can potentiate antitumor immune responses through loss of Treg stability in the TME [30,47]. Compared with WT, MC38, TRAMPC2, and B16F10 tumors in *Foxp3-GFP-hcre;Ezh2*$^{fl/fl}$ mice with Treg-specific *Ezh2* deficiency showed delayed growth, and were associated with increased production of IL-2, interferon (IFN)γ, and TNF by *Ezh2*-deficient tumor-infiltrating Tregs [5]. These studies also showed that EZH2i could selectively target intratumoral Tregs without a systemic change in Treg function, suggesting that its clinical application might result in minimal adverse autoimmune effects, although this remains to be tested [30]. In a coculture system, EZH2i in both murine and human $CD4^+$ T cells resulted in a dose-dependent reduction in ***in vitro*-induced Tregs (iTregs)** and reduced suppressive capacity of human iTregs, compared with controls [47]. Additionally, RNA-Seq analysis of EZH2 inhibition in murine iTregs resulted in upregulated expression of inflammatory pathway molecules compared with untreated controls – as demonstrated by increased concentrations of proinflammatory cytokines in the supernatant of cultured murine iTregs [47].

Figure 3. Primary and acquired resistance to ICB involves multiple immune evasion mechanisms. These mechanisms include reduced tumor antigen expression and presentation, reduced $CD8^+$ T effector (Teff) cell trafficking, increased T regulatory (Treg) cell suppression, decreased presentation of tumor antigens by antigen-presenting cells (APCs) and tumors, reduced natural killer (NK) cell killing, and potentially increased myeloid-derived suppressor cell (MDSC) infiltration (top panel). EZH2 plays a role in suppressing antitumor immunity by promoting many of these immune evasion tactics and EZH2 inhibition can reverse these resistance mechanisms (middle panel). EZH2i has the therapeutic potential to sensitize tumors that otherwise would be resistant to ICB (bottom panel, left) and to resensitize tumors that have acquired resistance after initial response to ICB (bottom panel, right).

Similarly to $CD8^+$ T cells, EZH2 is induced in activated murine and human Tregs *in vitro* [30,47]. Accordingly, anti-CTLA-4 antibody treatment with ipilimumab in patients with metastatic melanoma or metastatic prostate cancer led to increased EZH2 expression in peripheral blood Tregs, compared with baseline expression prior to treatment [47]. Furthermore, EZH2i improved the response resulting from anti-CTLA-4 antibody treatment in MB49 bladder and B16-F10 murine models through diminished Treg suppressive activity and increased $CD4^+$ and $CD8^+$ Teff activity [47,50]. These data provided the rationale for a current active Phase I/II multicenter, single-arm study of the objective response to EZH2i CPI-1205 plus ipilimumab in patients with histologically or cytologically confirmed advanced solid tumors (NCT03525795[ii]) (see Clinician's Corner).

Taken together, the data described above support a model in which EZH2 can be induced in activated T cells, and under certain contexts, contribute to the maintenance of Tregs and decrease proinflammatory cues to $CD8^+$ T cells. EZH2 inhibition is an attractive candidate therapeutic strategy that might be combined with ICB to treat some tumor types (Figure 2).

Effects of EZH2 Inhibition on Innate Immunity in the TME

NK cell-mediated tumor cell killing represents a first line of immune activation that induces a proinflammatory cascade in the TME, and evidence suggests that EZH2 inhibition boosts this through changes to both tumor cells and NK cells [51]. In addition to the role of EZH2 in limiting the maturation and activation of NK cells as described earlier, EZH2 inhibition may regulate the expression of NK cell-activating ligands. Specifically, EZH2i treatment of human hepatocellular carcinoma (HCC) cell lines SH-HEP-1 and PLC/PRF/5 led to the expression of the NK cell-activating ligand ULBP1, as well as NK cell-mediated killing of the HCC lines in direct *in vitro* cytotoxicity assays [51]. EZH2 inhibition in HT1376, a human bladder cancer cell line with mutations in the switch/sucrose nonfermentable (SWI/SNF) complex, resulted in tumor cell death in xenograft mouse models and this was mediated, in part, through activation of NK cells [52]. RNA-Seq and quantitative PCR studies performed on HT1376 tumors treated with EZH2i showed an increase in NK cell-activation markers, including IFNγ, relative to control mice. Furthermore, immunohistochemistry confirmed that EZH2 inhibition led to increased expression of NK cell markers CD56 and NCR1, relative to controls [52].

NK cells act as a first line of defense against tumor initiation, and since progression can activate the adaptive antitumor response, EZH2 inhibition might augment the efficacy of ICB by enhancing NK cell-mediated tumor cell killing. Additionally, the Th1 chemokine expression profile induced by EZH2 inhibition might also boost NK cell activity in the TME, since NK cells can be recruited by CXCL9 and CXCL10 [53]. It will thus be of value to determine if EZH2 inhibition can boost the efficacy of current NK cell-based therapies, such as antibody- and cytokine-based therapies, as well as adoptive NK cell transfer aimed therapies.

Tumor-associated macrophages (TAMs) can enhance tumor cell survival and proliferation and promote an immunosuppressive microenvironment that supports tumor progression. ChIP-seq analysis of primary human macrophages after treatment with IFNγ showed increased H3K27me3 on proinflammatory genes, such as *TNFRSF11A, PPARG, and RANK* [54]. While EZH2 is suggested to maintain suppression of these human macrophage genes after IFNγ treatment, short hairpin (sh)RNA knockdown of EZH2 showed that it was not essential for suppression [54]. Conversely, EZH2 has been implicated in the maintenance of proinflammatory macrophage survival and polarization of murine macrophages through repression of miRNA let7-c and subsequent expression of PAK1, a key determinant of the proinflammatory macrophage phenotype [15]. Recently, EZH2i treatment overcame ICB

resistance in the HiMYC PCa transgenic tissue transplant mouse model of prostate cancer, in part by increasing proinflammatory and decreasing anti-inflammatory TAMs [55]. Although these results require thorough validation, it is possible that the cytokine milieu generated by EZH2 inhibition and T cell activation in this model might shift the TME towards an inflammatory setting and shift macrophage polarization towards a proinflammatory phenotype. These contradictory reports on the effect of EZH2 on TAM polarization and function highlight the need for more extensive studies of the myeloid component of the TME for different tumor settings to more accurately predict the clinical TAM response to EZH2i (see Outstanding Questions).

Despite evidence that EZH2 might contribute to DC function in autoimmune models, there are limited data concerning the potential effects of EZH2 inhibition on DC function in the TME, and its impact on antitumor immunity [56,57]. Given the importance of DCs in antitumor immunity, increased insight into the effects of EZH2 inhibition on the recruitment and function of tumor-resident DC and myeloid populations is required.

EZH2 Inhibition Can Increase Antigen Expression, Processing, and Presentation in the TME

Decreased tumor antigen presentation by **MHC class I and II** molecules impairs recognition and targeting by activated Teff cells, representing a key immune evasion mechanism [58]. Several studies have shown that EZH2 mediates suppression of MHC I and II, and suggest that EZH2 inhibition can restore immunogenicity of some tumors and boost responses to ICB. A genome-wide CRISPR-Cas9 screen of the human K562 MHC-I-deficient cancer cell line identified a PRC2-dependent mechanism that coordinates silencing of key MHC I components, including MHC I heavy chain genes [8]. Specifically, genetic ablation of PRC2 components, including EZH2 (*EZH2* knockout), in cells from a small cell lung carcinoma GEMM led to increased MHC I expression *in vitro*, while EZH2 inhibition restored MHC I expression and promoted $CD8^+$ T cell-mediated tumor cell killing in coculture assays [8]. In another preliminary study, EZH2 inhibition led to upregulated MHC I, *Tap1* and *B2m* genes in organoids derived from a prostate cancer GEMM. Although requiring further validation, these data were supported by analysis of human prostate cancer RNA-Seq data showing that low EZH2 expression correlated with increased IFNγ signaling and antigen presentation genes *B2m* and *Hla-a*, compared with patient samples with higher expression of EZH2 [55]. Similarly, TCGA data in head and neck squamous cell carcinoma (HNSCC) revealed a negative correlation between EZH2 expression and MHC I components, including B2M and several HLA molecules [50]. EZH2i treatment of a panel of human and murine HNSCC cell lines increased MHC I mRNA expression, and led to ovalbumin antigen presentation in two mouse HNSCC cell lines. Notably, EZH2 inhibition was able to overcome ICB resistance mechanisms in MOC1-esc1, an anti-PD-1-resistant mouse model of HNSCC, since combined treatment with EZH2i and anti-PD-1 antibody led to significant inhibition of tumor growth compared with either intervention alone [50].

Of note, EZH2 binds to the promoter that drives CIITA – the IFNγ-inducible regulator of MHC II expression by non-antigen presenting cells – and RNAi of *EZH2* leads to an increase in CIITA expression in human uveal melanoma cell lines [59]. In another study, EZH2 knockdown was also shown to increase CIITA and MHC II protein expression in the human breast cancer cell line MDA MB 435 and its highly metastatic variants [60]. Recently, studies on a panel of 247 diffuse large B cell lymphoma (DLBCL) patients noted a link between *EZH2* activating mutations and diminished MHC I and II expression; this was confirmed in mice transplanted with mutant Ezh2 compared with WT control tumors [61]. Accordingly, EZH2 inhibition increased antigen presentation by DLBCL cell lines bearing EZH2 mutations compared with EZH2 WT cell lines.

Clinician's Corner

Increased EZH2 activity plays a role in the oncogenesis and proliferation of some tumor types, notably follicular lymphoma and SMARCb1/INI1-negative tumors such as epithelioid sarcoma and poorly differentiated chordoma. EZH2i are well tolerated in the clinic, rendering them attractive combination partners for ICB [47,91]. To identify the patients who might benefit from EZH2 inhibition in combination with ICB, a more comprehensive understanding is needed regarding the pleiotropic effects of EZH2i on the immune landscapes of different tumor types. In addition to its TME-modifying effects, EZH2 inhibition has the advantage of inducing tumor-autonomous cell death in certain tumor types, and this combined effect might be leveraged in the clinic. Identification of biomarkers of clinical response would greatly impact the success of combination strategies. Assessment of TME modifications induced by EZH2i in tumors from patients currently enrolled in clinical trials could provide valuable information on the potential impact of these inhibitors in different clinical settings when combined with immunotherapy.

Therapeutic strategies inhibiting the epigenetic drivers of oncogenesis while increasing effective T cell tumor infiltration and antigen presentation might provide an ideal combination approach with ICB to yield clinically active antitumor immune responses, potentially in tumor types that have previously been less responsive to ICB alone. It will be essential to design the translational endpoints of current EZH2i/ICB combination trials to define the effects on the TME, and determine the indications and patient populations that might reveal the greatest therapeutic benefit. For example, initial treatment with EZH2i might prime the TME for more efficient ICB, while sustained EZH2 inhibition might lead to a negative feedback loop with some cell types in the TME. Since there are conflicting reports regarding the effect of EZH2i on activated T cells, future translational data are needed to increase our understanding of the effects of EZH2 inhibition on other cellular subsets within the TME. A thorough assessment of TIL populations in current EZH2i trials could provide valuable information to inform optimal combination settings.

Box 3. Induction of Tumor Antigens and Endogenous Retroviruses

Inducing expression of human endogenous retroviruses (ERVs) and TAAs has recently gained attention as a potential therapeutic strategy to boost tumor cell susceptibility to immunotherapies. This is highlighted by studies showing that DNMT inhibitors can induce ERV re-expression in human tumor cell lines, stimulating innate immune activation [64,65]. EZH2i function has been recently shown to induce stimulated 3 prime antisense retroviral coding sequences (SPARCS) in human SCLC cell lines – a novel set of ERVs that can trigger innate immune signaling [64,66]. EZH2 has been shown to drive the suppression of tumor antigens encoded by oncoviruses such as Epstein–Barr virus [67]. It will be interesting to see if EZH2i can induce a T cell response in the TME via the expression and presentation of these antigens. Further studies are warranted to determine the impact of EZH2 inhibition on ERV and tumor antigen expression in the clinic.

Together, these studies have shown that EZH2 inhibition or genetic ablation of Ezh2/EZH2 can increase antigen presentation, tumor immunogenicity, and enhanced tumor killing in combination with ICB [8,55].

Moreover, these reports indicate that tumors can co-opt EZH2 function to suppress antigen presentation – a phenomenon that might be therapeutically exploited with EZH2i. A known mechanism of acquired resistance to ICB is the emergence of mutations in B2M that prevent antigen expression, and thus, NK cell therapy has been suggested to potentially provide a means to eliminate those B2M-mutated cells that would otherwise be spared due to MHC expression [62,63]. While EZH2 inhibition clearly cannot override B2M mutations and enhance MHC I and antigen presentation, it is worth considering whether a combination of EZH2i and NK cell therapy might in theory enhance immunity against these tumors.

Tumors also evade recognition by $CD8^+$ Teff cells through suppression of TAAs. Double-stranded RNA and endogenous retroviruses that enhance an adaptive immune response combined with EZH2 inhibition may induce expression of tumor antigens and endogenous retroviruses that are normally repressed by EZH2 (Box 3) [64–67]. Collectively, these data provide strong evidence that EZH2 inhibition may boost tumor immunogenicity by increasing the expression of both tumor antigens and antigen presenting activity to elicit more efficient adaptive immune responses (Figure 2). Further investigation is warranted to determine if EZH2 inhibition results in the reversal of viral latency that can upregulate expression of antigens recognized by virus-specific Teff cells, such as Epstein–Barr virus antigens in the case of B cell lymphomas.

Concluding Remarks

The complexity and diversity of the TME-modifying potential of EZH2 inhibition underscores the need for rational approaches to combination immunotherapy treatments. It is essential to determine how various TME-modifying effects of EZH2 inhibition may enhance immunotherapy in the context of different tumor types. Several clinical trials are underway with small-molecule EZH2i for cancer types that have an oncogenic dependency on this activity. As EZH2 inhibition affects both tumor cells as well as the TME, insight into the pleiotropic effects of EZH2i in these patients might help predict clinical responses. Additionally, understanding the indication-specific and patient-specific TME-modifying effects of EZH2i may better inform rational immunotherapy combinations. As with current immunotherapy approaches, the effect of EZH2i on the TME will likely vary not only for different cancers, but also for individuals within a cancer type, and it will be essential to consider this when designing combination trials (see Outstanding Questions). EZH2i currently under investigation in clinical trials are generally well tolerated [13], rendering them attractive combination partners. Combination trials with EZH2i and ICB are emerging and can inform the design of future combinations. Tumor types that are poorly immunogenic with low numbers of mutations, including prostate, ovarian, and breast cancer, may be relevant for combining EZH2 inhibition with ICB [68]. In addition, the potential for EZH2 inhibition combined with other immunotherapy

Outstanding Questions

How does the role of EZH2 in B cell biology relate to the TME and antitumor immunity? EZH2 is required for B cells to form germinal centers through repression of cyclin-dependent kinase inhibitors [92] and plasma cell differentiation through plasma cell transcriptional program repression [93]. Since the formation of tertiary lymphoid structure can be a positive prognostic indicator for certain tumors [19,94,95], further studies need to determine whether EZH2 inhibition in the context of tumor development might modulate B cell responses to tumors in lymphoid organs and ectopic follicles within the TME.

How does EZH2 inhibition affect nonimmune compartments that shape the TME, such as CAFs? CAFs comprise a heterogeneous population of cells that are involved in tissue remodeling to support cancer invasion and metastasis [96]. CAFs support immune tolerance within the TME by promoting suppressive cells such as **myeloid-derived suppressor cells (MDSCs)**, TAMs, and Tregs and by eradicating Teff cells and inhibiting NK cells. Thus, CAFs are major players in shaping the TME and may favor cancer progression. There are limited and conflicting data regarding the role of EZH2 and CAFs in the TME. In order to maximize the TME modulating potential of EZH2 inhibition, it will be important to understand the role of EZH2 in CAFs in various indications.

How does EZH2 inhibition affect the myeloid compartment within different TME contexts? Approaches that unleash the antitumor myeloid compartment within the TME may widen the breadth of immunotherapy. Given conflicting reports regarding the role of EZH2, in these cells and the clear context-specific nature of EZH2 in this immune compartment, it will be crucial to elucidate the influence of EZH2 inhibition on the myeloid compartment within the TME; this may enable an understanding potential combination treatments of EZH2i with ICB and/or with myeloid-targeting immunotherapy approaches.

What is the optimal combination schedule for EZH2i plus ICB? Additional preclinical experiments to determine the most effective scheduling for EZH2i treatments relative to ICB are warranted.

approaches, such as **CAR (chimeric antigen receptor)-T cell therapy** and **therapeutic cancer vaccines**, remains unexplored (see Outstanding Questions). An understanding of how EZH2 inhibition can affect less-well-studied TME cell types, including B cells and **cancer-associated fibroblasts (CAFS)**, is also needed to elucidate the full potential of this therapeutic strategy to extend the reach of ICB. EZH2 inhibition clearly has the potential to overcome key ICB resistance mechanisms, such as low immune cell infiltration of the TME, immunosuppression, and decreased antigen expression (Figure 3). Additionally, the interplay between histone methylation and DNA methylation is well established and targeting both types of modifications in certain cancer types might provide the best chances for successful combinations with ICB [69–71]. In summary, the diverse TME-modifying effects of EZH2 inhibition might serve to unleash the potential of current immunotherapies, and further studies are needed to determine the most effective strategy for combining EZH2 inhibition with current immunotherapy approaches.

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Resources

[i]https://clinicaltrials.gov/ct2/show/NCT02601937

[ii]https://clinicaltrials.gov/ct2/show/NCT03525795

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For example, initial treatment with EZH2i may prime the TME for more efficient ICB, while sustained EZH2i treatment may lead to a negative feedback loop with some cell types in the TME. It will be important to reconcile the conflicting reports regarding the effect of EZH2 inhibitors on activated T cells, and to increase our understanding of the effects of EZH2i on other cellular subsets within the TME. These preclinical data as well as a thorough assessment of TIL populations in current EZH2i trials could provide valuable information to inform optimal combination scheduling.

What is the potential of EZH2 inhibition beyond its combination with ICB? Given the role of EZH2 in multiple immune cell types, it will be important to determine which current immuno-oncology therapies will benefit from the TME-modifying effect of EZH2i. For example, could the increased tumor antigen presentation and T cell trafficking and activation induced by EZH2 inhibition augment the effects of CAR-T cell therapy or therapeutic cancer vaccines? A more in-depth understanding of how other immune constituents of the TME are affected by EZH2 inhibition could aid in the rational design of clinical trials with these additional immunotherapy approaches.

What upstream stimuli induce upregulation of PRC2/EZH2 expression or activity and influence the TME? Cell cycle regulators such as Myc and E2F may control EZH2 transcription in cancer types, including breast and prostate cancer; also, there is evidence that HIF1a may directly upregulate EZH2 expression under hypoxic conditions [97–100]. Additionally, KRAS activating mutation-induced MEK/ERK and PI3K/AKT signaling, as well as NF-κB and MUC1-C signaling have all been implicated in induction of EZH2 expression [101–103]. Notably, T cell activation, including via anti-CTLA-4 antibodies, can induce the expression of EZH2, which might lead to suppression of T cell function [30,47]. This recent discovery may lead to a deeper understanding of how EZH2 inhibition could exploit normal physiological roles of EZH2 that have been co-opted by tumors to enhance immune evasion. It will be important to determine if activating/inhibitory or polarizing signals

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in other immune cell types also influence EZH2 expression to uncover additional vulnerabilities that might be targeted with EZH2i.

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