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DIETARY ZEAXANTHIN AS A NOVEL MODULATOR OF CD8⁺ T CELL
ANTI-TUMOR IMMUNITY

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ABSTRACT

While advances in cancer immunology have profoundly transformed approaches to cancer treatment, substantial challenges continue to limit clinical responsiveness. As the key driver of cytotoxic anti-tumor immunity and existing immunotherapies, CD8⁺ T cells often exhibit signs of exhaustion and dysfunction within the immunosuppressive tumor microenvironment (TME). CD8⁺ T cell activity is governed by a combination of cell-intrinsic metabolic programs and extrinsic signals, among which nutrients comprise a major component of the extracellular factors. While much focus has been placed on nutrient availability and their classical roles in supporting biosynthesis and energy metabolism within the TME, growing evidence suggests that dietary nutrients also act as important regulators of cellular activities. Yet, the specific mechanisms underlying the regulatory significance of nutrients in modulating anti-tumor immunity remain largely unknown.

Here, we utilized a co-culture-based screening approach with a circulating blood nutrient compound library to identify novel immunomodulatory molecules. We found that zeaxanthin (ZEA), a dietary carotenoid pigment found in many fruits and vegetables, enhances the cytotoxicity of CD8⁺ T cells against tumor cells. Orally administering ZEA, but not its structural isomer lutein (LUT), promotes anti-tumor immunity in B16F10 melanoma and MC38 colorectal carcinoma models *in vivo*. To determine the molecular mechanism of how ZEA modulates CD8⁺ T cell function, we performed integrated multi-omics mechanistic studies, revealing that ZEA directly promotes T-cell receptor (TCR) stimulation at the CD8⁺ T cell membrane, leading to improved intracellular TCR signaling for effector T cell function. Strikingly, ZEA treatment augments the effectiveness of immune checkpoint inhibitor treatment *in vivo* and the cytotoxicity of human TCR gene-engineered CD8⁺ T cells *in vitro*. Our findings uncover a previously unknown

immunoregulatory function of ZEA, which has translational potential as a dietary element in bolstering immunotherapy.

Building on these findings, we sought to explore structure-activity relationships by modifying the parent nutrient compound to enhance its immunomodulatory potential. In a collaborative project, we designed and assessed the effects of structural derivatives of a previously identified immunomodulatory nutrient *trans*-vaccenic acid (TVA). We systemically modified the structure of TVA and screened for candidates more potent at tumor-killing among a library of more than 50 derivatives. We successfully identified derivative 171 as a more potent CD8⁺ T cell activator, demonstrating enhanced effector functions at lower doses compared to its parent compound. These findings highlight the promise of rational nutrient modification to improve immune outcomes. Further research is warranted to optimize the efficacy of naturally occurring immunomodulators and to advance the translational potential of incorporating diet-derived compounds into therapeutic strategies.

This dissertation describes the discovery of ZEA as a novel diet-derived modulator of CD8⁺ T cell-driven anti-tumor immunity, exploration of its molecular mechanism, and procedures to optimize the efficacy of natural compounds through structure-activity based research. This body of translational works aims to provide insights into the diet-immune axis by examining the director modulatory effects of dietary ZEA on CD8⁺ T cells and discuss the limitations and broader implications of nutrients as immunomodulators, as well as their translational potential in improving current treatment outcomes.

*For those who rooted me in love, faith, and light –
and whose sheer vitality stirred me forward.*

“Malgré la soif de disparaître, je fus prodigue dans l'attente, la foi vaillante.

Sans renoncer.”

– René Char

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CHAPTER 1

INTRODUCTION

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Dr. Fan and I collected references together. I was responsible for making the figure and writing majority of the manuscript. All authors contributed to manuscript editing.

1.1 Cancer Immunology

The dynamic interplay between cancer and the immune system has reshaped our understanding of cancer prevention, progression, and treatment [1,2]. Cancer is not merely a group of diseases with uncontrolled cell growth, but a continuous battle between malignant cells and the immune system [3]. While the immune system functions by detecting and eliminating abnormal cells, cancer has learned to evade immune surveillance and developed ways to suppress, manipulate, and escape detection by the immune system [4]. This ongoing battle has laid the foundation for the field of cancer immunology, which strives to understand how immune cells recognize, respond to, and at times fail to control tumor growth. Insights from cancer immunology research then paved the way for transformative advances in cancer immunotherapy, which harnesses and modifies the immune system to better target and eradicate cancer cells [5,6]. The development of immunotherapeutics including immune checkpoint inhibitors and adoptive cell therapy has revolutionized cancer treatment [6].

1.1.1 The innate immune system in cancer immunology

The innate immune system is the first line of defense that activates inflammatory responses against tumor cells and shapes adaptive immune responses. Innate immunity consists different cell types, each with distinct functions in recognizing and responding to cancer cells. Natural Killer (NK) cells are crucial for their ability to directly lyse tumor cells without prior sensitization, primarily through the recognition of stress-induced ligands and the absence of MHC class I molecules on cancer cells [7,8]. Dendritic cells (DCs) act as professional antigen-presenting cells, capturing tumor antigens and presenting them to T cells to initiate adaptive immunity [9,10]. Macrophages, particularly tumor-associated macrophages (TAMs), can exhibit dual roles, where

M1 macrophages promote anti-tumor immunity through secreting pro-inflammatory cytokines and phagocytosis and M2 macrophages, on the other hand, often support tumor progression by fostering immunosuppression and angiogenesis [11,12]. Neutrophils, including tumor-associated neutrophils (TANs), similarly display plasticity, where N1 neutrophils contribute to anti-tumor activity, and N2 neutrophils support metastasis and immune evasion [13,14]. Together, these innate immune cells orchestrate complex interactions within the tumor microenvironment, underscoring their potential as therapeutic targets in cancer immunotherapy.

1.1.2 The adaptive immune system in cancer immunology

The adaptive immune system is crucial in cancer immunology by providing specific and lasting responses against tumors. CD8⁺ T cells, or cytotoxic T cells, are key effectors that directly eliminate tumor cells by recognizing antigens presented on MHC class I molecules and releasing pro-inflammatory cytokines, as well as perforin and granzymes to induce apoptosis [15,16]. Neoantigens derived from tumor-specific mutations enhance CD8⁺ T cell responses, forming the basis for personalized cancer vaccines [17].

CD4⁺ helper T cells support anti-tumor immunity through differentiation into subsets like Th1 cells, which secrete IFN γ to activate macrophages and enhance CD8⁺ T cell effector functions [18,19]. However, CD4⁺ T cells subset like regulatory T (Treg) cells can suppress immune responses and promote tumor progression when overactive, presenting a significant challenge for anti-tumor immunity but also a target for treatment in immunotherapy [20–22]. More recently, innate lymphoid cells (ILCs) and $\gamma\delta$ T cells were discovered to modulate tumor microenvironments by secreting cytokines that influence immune cell recruitment and activation [23,24]. $\gamma\delta$ T cells are a unique subset of T cells that act as a bridge between innate and adaptive immunity,

recognizing stress-induced ligands on tumor cells and secreting cytokines that recruit other immune cells, again offering a promising avenue for immunotherapy [24–26].

B cells contribute through tumor-specific antibody production, antigen presentation to T cells, and cytokine secretion, although their roles can vary from suppressing to promoting tumor progression [27–29]. These adaptive and innate-like immune components highlight the complexity of cancer immunology and provide valuable targets for novel therapeutic strategies.

1.2 The Current Landscape of Cancer Immunotherapy

Cancer immunotherapy has revolutionized cancer treatment by harnessing the immune system to fight cancerous cells. At the heart of many successful strategies are CD8⁺ T cells. CD8⁺ T cells are indispensable for their ability to precisely recognize cancer-specific antigens and deliver powerful cytotoxic effects [30,31]. Breakthroughs in the development of immune checkpoint inhibitors (ICI), adoptive cell therapies (ACT), cancer vaccines, and bispecific antibodies (bsAbs) have demonstrated the immense potential of mobilizing immune cells, including CD8⁺ T cells, for anti-tumor responses [32]. However, significant challenges remain, particularly in addressing tumor immune evasion and enhancing T cell function in suppressive microenvironments [31]. As the field continues to evolve, CD8⁺ T cells remain central to the development of novel strategies aimed at improving clinical outcomes across diverse cancer types.

1.2.1 Immune checkpoint inhibitors

T cell exhaustion arises when T cells are persistently exposed to antigens, such as during chronic infections or cancer. Exhausted T cells are characterized by impaired effector functions, reduced cytokine production, and sustained expression of inhibitory receptors like PD-1, CTLA-

4, TIM-3, and LAG-3 [33–35]. While exhaustion limits immune-mediated tissue damage in chronic diseases, it poses a significant barrier to effective anti-tumor immunity in cancer [36]. In the tumor microenvironment, prolonged antigen stimulation and immunosuppressive factors lead to a progressive decline in T cell functionality, allowing tumors to evade immune surveillance [37].

To overcome this challenge, immune checkpoint inhibitor (ICI) therapies have emerged by targeting inhibitory pathways to rejuvenate exhausted T cells and restore their effector functions [38,39]. PD-1/PD-L1 and CTLA-4 are two of the more well-studied checkpoint pathways [39]. PD-1 is expressed on T cells and binds to PD-L1 on tumor cells to transmit inhibitory signals that suppress T cell activity [40]. Anti-PD-1/PD-L1 antibodies are generated to block this interaction, reinvigorating T cells and enhancing anti-tumor responses [40,41]. Similarly, CTLA-4 is expressed on activated T cells, and it competes with the co-stimulatory receptor CD28 for binding to B7 molecules, in turn dampening T cell activation. Anti-CTLA-4 antibodies disrupts this inhibition, promoting T cell priming and proliferation [42,43].

1.2.2 Adoptive cell therapy

Adoptive cell therapy (ACT) involves engineering and reinfusing patient-derived T cells to recognize and eliminated tumor cells. Currently, this approach includes chimeric antigen receptor T (CAR-T) cell therapy and T cell receptor-engineered T (TCR-T) cell therapy, both of which aim to enhance T cell recognition of tumor antigens. CAR-T cells are genetically modified to express chimeric antigen receptors, allowing them to recognize extracellular tumor antigens without MHC presentation [44]. This MHC-independent mechanism enables CAR-T cells to directly target antigens on the surface of tumor cells, making them particularly effective against

hematologic malignancies, such as B cell leukemia and lymphoma, where targets like CD19 and BCMA have been identified [45,46]. On the other hand, TCR-T cells utilize natural T cell receptors engineered to recognize intracellular tumor antigens presented on MHC molecules [47,48]. By enhancing the affinity of these receptors for peptide-MHC complexes, TCR-T cells expand the range of potential tumor targets, including neoantigens and oncogenic mutations [49].

1.2.3 Cancer vaccination

Cancer vaccines are designed to stimulate the immune system to recognize and eliminate cancer cells, offering a promising strategy for cancer prevention and treatment. These vaccines can be broadly categorized into two main types: preventative vaccines, which aim to protect against cancers caused by infectious agents, and therapeutic vaccines, which seek to treat existing malignancies by inducing robust anti-tumor immune responses [50]. Preventative cancer vaccines work by targeting infectious agents known to cause cancer, interrupting the initial steps of carcinogenesis [51]. These vaccines function by introducing viral antigens to the immune system, priming the immune system to prevent infection and subsequent tumor formation [51]. The immune response is primarily mediated by the generation of neutralizing antibodies and memory T cells [51]. The most notable examples of preventative cancer vaccines are the human papillomavirus (HPV) vaccine and the hepatitis B virus (HBV) vaccine [52]. On the other hand, therapeutic cancer vaccines aim to treat existing cancers by inducing an adaptive immune response against tumor antigens [53]. These vaccines function by presenting tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) to the immune system, thereby activating immune cells like CD8⁺ T cells and CD4⁺ T cells to attack cancer cells [53]. The mechanism of therapeutic cancer vaccines involves antigen delivery, antigen presentation, and immune activation [54].

Vaccines introduce TAAs or TSAs through various platforms, such as peptides, proteins, nucleic acids (DNA or RNA), or dendritic cells (DCs) loaded with tumor antigens [54]. Delivered antigens are processed and presented to T cells by APCs for T cell activation and the following therapeutic responses [54]. Unlike preventative vaccines, therapeutic vaccines face the challenge of overcoming the immunosuppressive TME, which often inhibits effective immune responses [55].

1.2.4 Bispecific antibodies

Bispecific antibodies (bsAbs) have garnered significant attention due to its unique mode of action by engaging two distinct antigens simultaneously [56]. Bispecific T cell engager (BiTE) is a prominent subclass of bsAbs, which brings T cells into close proximity with cancer cells and facilitates direct tumor killing [57]. With T cells playing a dominant role in current immunotherapy approaches, there is growing interest in leveraging other immune cells such as NK cells, dendritic cells, and macrophages to improve cancer immunotherapy.

1.2.5 Immunotherapies involving other cell types

Immunotherapies involving cell types beyond conventional T cells, such as natural killer (NK) cells, macrophages, dendritic cells (DCs), innate lymphoid cells (ILCs), and $\gamma\delta$ T cells, are expanding the arsenal of cancer treatment strategies. NK cells directly kill tumor cells without prior sensitization and show promise in adoptive transfer and antibody-dependent cellular cytotoxicity [58,59]. Macrophage-targeting therapies, such as anti-CD47 antibodies, enhance phagocytosis, while CSF1R inhibitors reduce tumor-promoting macrophage recruitment [60,61]. DC-based approaches, including vaccines like sipuleucel-T, enhance T cell activation via antigen presentation by antigen-loaded DCs [62,63]. ILCs and $\gamma\delta$ T cells offer novel pathways to modulate

tumor environments and target both solid and hematologic cancers [64,65]. These therapies, particularly in combination with immune checkpoint inhibitors, are paving the way for improved efficacy of and addressing resistance mechanisms in current cancer treatment strategies.

1.2.6 Challenges facing current cancer immunotherapy

Despite the remarkable progress made with current immunotherapeutic strategies, significant challenges remain. Tumor immune evasion mechanisms, such as downregulation of antigen presentation machinery and induction of an immunosuppressive tumor microenvironment (TME), often prevent robust immune responses [66]. The TME, enriched with inhibitory cytokines, regulatory immune cells, and structural barriers, suppresses T cell infiltration and activity [67]. Persistent antigen stimulation in tumors also leads to T cell exhaustion, a state where T cells lose their cytotoxic function and fail to respond effectively, even in the presence of checkpoint blockade therapies [68]. Antigen heterogeneity within tumors complicates targeted therapies, as not all cancer cells express the same antigens, enabling immune escape [69]. Additionally, treatment-related toxicities, including immune-related adverse events (irAEs) and cytokine release syndrome (CRS), present further challenges in the application of promising treatment strategies, particularly for immune checkpoint inhibitors and adoptive cell therapies [70]. High production costs and the need for personalized approaches in some immunotherapies, such as CAR-T and TCR-T cell therapies, also limit their accessibility and scalability [71].

These obstacles highlight critical knowledge gaps in our understanding of how to modulate the immune system more effectively and develop novel approaches to enhance immune cell activity without overactivation and eliciting autoimmune responses. Emerging fields like immunometabolism offer new insights into how diet-derived components influence T cell

activation and persistence, potentially revealing dietary interventions that could boost therapeutic efficacy.

1.3 The Diet-Immune Axis

Throughout evolutionary history, diet has been central to shaping human physiology and pathology, including the development and regulation of the immune system. While it is well-established that a healthy diet is essential to maintaining our physical health and preventing diseases, the definition of a “healthy diet”, the dietary components that contribute to good health, and the mechanisms behind these effects remain largely unresolved. The diet-immune axis investigates the complex interplay between diet and immunity, highlighting how dietary patterns and components influence immune responses and affect health and disease outcomes [72]. Despite growing interest in this field, much of our understanding of how diet impacts the immune system comes from epidemiological studies that reveal correlations rather than direct causation. Mechanistic studies, while rare and challenging due to the complexity of dietary mixtures and the diverse immune cells involved in the responses, are beginning to shed light on these critical interactions [73].

1.3.1 Types of diets

Different dietary patterns exert distinct effects on the immune system. The Western diet, characterized by high intake of refined sugars, saturated fats, and processed foods, has been linked to chronic inflammation and immune dysregulation, therefore contributing to metabolic diseases and cancer [74]. In contrast, the Mediterranean diet, rich in polyphenols, omega-3 fatty acids, and fiber, is associated with anti-inflammatory effects and improved immune regulation [75]. High-fat

ketogenic diets, which promote ketogenesis over glycolysis, modulate immunity by suppressing pro-inflammatory responses but can impair immune functions with prolonged use [76]. Similarly, plant-based diets rich in fiber influence gut immunity through their effects on the microbiota, promoting short-chain fatty acid (SCFA) production and Treg activity [77]. While these dietary patterns are associated with distinct immune profiles, the mechanisms underlying these effects are only beginning to be explored and understood.

1.3.2 Epidemiological studies

Epidemiological research provides much of the current knowledge linking diet to immunity. Studies like the Nurses' Health Study have shown correlations between diet and immune-mediated diseases. For example, a diet high in total fat intake, including polyunsaturated fatty acids (PUFAs), ω -6 fatty acids, and meat is linked to a higher risk of Crohn's disease and ulcerative colitis [78]. In contrast, a fiber- and fruit-rich diet is associated with lower risk for Crohn's disease and a vegetable-rich diet is associated with decreased risk of ulcerative colitis [78]. Eating a Mediterranean diet correlates with reduced risks of autoimmune diseases, such as rheumatoid arthritis, enhanced immune-associated long-term cardioprotective effect, and lower incidence of inflammatory bowel disease [79–81]. On the other hand, high consumption of processed foods and refined sugars has been associated with increased inflammation and a higher risk of chronic diseases [82]. These studies, while invaluable for establishing associations, lack the mechanistic insights necessary to fully explain how specific dietary components influence immune responses.

1.3.3 Models to study dietary effects on health and disease

Mechanistic studies of the diet-immune axis rely on experimental models that can dissect the intricate interplay between dietary components and immune responses. Germ-free and gnotobiotic mouse models are widely used to study how diets influence gut microbiota and immunity. For instance, fiber-enriched diets in germ-free mice have been shown to promote SCFA production, enhancing Treg function and suppressing inflammation [77]. Human organoid models derived from gut epithelium provide an *in vitro* platform to study the impact of dietary components on barrier integrity and mucosal immunity [83]. Human dietary intervention studies, while challenging due to inter-individual variability, offer crucial insights into short-term dietary effects on immune function. For example, omega-3 supplementation trials have demonstrated improved resolution of inflammation in patients with autoimmune conditions [84]. Despite the insights provided by these studies, the complexity of diet, comprising diverse macronutrients, micronutrients, and bioactive compounds, presents a significant challenge for identifying specific immune-modulating components.

1.3.4 Critical gap in knowledge linking diet to health and disease

Despite advances in understanding the diet-immune link, significant gaps remain. Much of the current knowledge is based on correlations from epidemiological studies, with limited mechanistic evidence linking dietary components to specific immune functions. The immune-modulatory effects of many dietary components, historically attributed to their metabolic roles, are only now being explored at the cellular level. Emerging technologies, such as high-throughput compound screens and single-cell immune profiling, are beginning to identify novel dietary immune regulators and their molecular mechanisms [85]. The convergence of diet and

immunology defines a promising modality for improving health and disease treatments. Research is needed to integrate epidemiological findings with mechanistic insights and leverage high-resolution tools to address these knowledge gaps and develop comprehensive dietary strategies to optimize immune-based therapeutics and preventative strategies.

1.4 Regulatory Effects of Dietary Components

The immunoregulatory effects of dietary components have emerged as an area of interest for recent research, particularly for their influence on CD8⁺ T cells, which play a pivotal role in cancer immunotherapy. Recent research employing unbiased screening and mechanistic approaches has identified specific lipids, amino acids, vitamins, and other metabolites as critical regulators of CD8⁺ T cell functionality, including their differentiation, effector responses, and memory formation (Figure 1.1) [73]. These findings reveal critical insights on how dietary components directly modulate signaling pathways, energy metabolism, and epigenetic programs to enhance or suppress T cell immunity.

1.4.1 Lipids

Lipids, including fatty acids, are gaining attention as key drivers of CD8⁺ T cell immunity. Identified from an unbiased library screening, *trans*-vaccenic acid (TVA) selectively regulates CD8⁺ T cells by antagonizing GPR43, traditionally known as the short-chain fatty acid receptor, and activating the cAMP-Protein Kinase A-cAMP Response Element-Binding Protein axis to promote cell proliferation, effector function, and antitumor immunity in mouse and human CD8⁺ T cells [86]. TVA not only enhances CD8⁺ T cell-mediated tumor attenuation in tumor mouse models, but also functions synergistically with ICI therapy *in vivo* and enhances killing from

bispecific T cell engager *ex vivo* [86]. Clinically, serum TVA levels correlate with patient response to Chimeric Antigen Receptor T cells therapy [86]. Moreover, linoleic acid (LA) uptake via SLC27A1 improves metabolic fitness, prevents exhaustion, and promotes a memory-like phenotype in both mouse and human CD8⁺ T cells [87]. This process involves modulating mitochondrial membrane potential and boosting mitochondria–ER contact (MERC) formation, which in turn enhances calcium signaling and mitochondrial energetics [87]. Consequently, these changes increase the antitumor efficacy of CD8⁺ T cells in tumor-bearing mice and improve Chimeric Antigen Receptor T cells cell therapy efficacy *in vitro* and *in vivo* [87].

Fatty acids also impact CD8⁺ T cell–induced immunogenicity indirectly by priming tumor cells or function synergistically with CD8⁺ T cell–dependent cytokines. Elaidic acid (EA) and Short Chain Fatty Acids enhance the cytotoxicity of CD8⁺ T cells by regulating MHC-I(MHC-I)-mediated tumor antigen presentation and creating a feedback loop with pro-inflammatory cytokines [88,89]. EA, a substrate of acyl-CoA synthetase long-chain family member 5 (ACSL5), boosts murine and human tumoral MHC-I presentation via the ACSL5–NOD-like Receptor C5 axis, where NOD-like Receptor C5 is an IFN γ -responsive *trans* activator of Major Histocompatibility Complex Class I genes [88]. EA supplementation results in suppressed tumor growth and enhanced efficacy of ICI therapy *in vivo* [88]. Clinically, higher levels of ACSL5 are correlated with better survival and prognosis in patients with lung cancer, while plasma EA concentration is higher in Non-Small Cell Lung Cancer patients who responded to ICI therapy and had better survival [88]. Short Chain Fatty Acids treatment of murine colorectal cancer (CRC) cells and organoids with microsatellite instability (MSI) induces DNA damage and activates Cyclic GMP-AMP Synthase/Stimulator of Interferon Genes signaling to upregulate genes involved in cytokine production, antigen processing, and MHC-I generation, thus enhancing

CD8⁺ T cell activation and function *in vitro* and *in vivo* [89]. Human MSI CRC samples are found to express similar gene expression patterns [89].

Arachidonic acid (AA) co-ordinates with IFN γ to induce immunogenic tumor ferroptosis [90]. CD8⁺ T cell–dependent IFN γ targets the promoter region of ACSL4 and enhances its expression, thereby increasing AA incorporation into specific phospholipid species and promoting tumor cell ROS production *in vitro* [90]. AA supplementation *in vivo* enhances tumor ferroptosis and promotes spontaneous and ICI-induced antitumor immune response [90]. Clinically, patient tumor ACSL4 levels correlate with T cell signatures and better ICI-treated patient survival [90].

In murine and Jurkat T cells, *in vitro* exposure to free fatty acids activates fatty acid metabolism, signaling through the mTOR-IRF4-PRDM1 axis to maintain elevated expression levels of SCML4, which is required for the fitness and functionality of Trm and TILs [91]. Furthermore, SCML4 epigenetically promotes the survival and effector capacity of tumor-resident CD8⁺ T cells by enhancing global H3K14 acetylation via regulating the BRPF2-ING4-HBO1 histone acetyltransferase complex [91]. Altogether, this mechanism results in enhanced T cell activation and better efficacy in combination with ICI therapy in tumor-bearing mice [91].

1.4.2 Amino acids

Systematically examining nutrient signaling processes with *in vivo* pooled CRISPR screening reveals that amino acid transporters and signaling events associated with Guanosine Diphosphate-fucose biosynthesis are novel regulators of CD8⁺ T cell terminal effector differentiation and memory T cell (T_{MEM}) formation [92]. Specifically, genetic perturbation of arginine transporter SLC7A1 and glutamine transporter SLC38A2 dampens mTORC1 signaling

and improves T_{MEM} persistence and killing capacity [92]. Additionally, protein-O-fucosyltransferase-1 regulates downstream Notch-Rbpj signaling to block terminal effector differentiation and drive context-dependent effector proliferation and T_{MEM} development for enhanced antitumor immunity [92].

Branched-chain amino acids (BCAA) accumulation upregulates Glut1 expression in $CD8^+$ T cells via mTOR and the PI3K-Akt-FoxO1 signaling axis, thus increasing glucose uptake and reprogramming glucose metabolism to enhance effector function in both mouse and human $CD8^+$ T cells [93]. Accumulation of intracellular BCAAs in $CD8^+$ T cells in mouse models results in enhanced antitumor immunity and increased efficacy of anti-PD-1 therapy [93]. Clinically, Non-Small Cell Lung Cancer patients with high BCAAs are also found to achieve better prognosis [93].

Asparagine directly binds the Proto-oncogene tyrosine-protein kinase Src family protein tyrosine kinase LCK in mouse and human $CD8^+$ T cells and induces LCK phosphorylation, enhancing LCK activity and T cell receptor (TCR) signaling, ultimately improving antitumor immunity in tumor-bearing mice [94]. In another study, extracellular asparagine uptake is required for early $CD8^+$ T cell activation *in vitro* due to low expression of asparagine synthetase, and its deprivation impairs $CD8^+$ T cell activity and effector function [95].

Higher taurine levels are observed in patients with lung cancer who responded to anti-PD-1 therapy [96]. Taurine supplementation results in better tumor attenuation and enhanced $CD8^+$ T cell function and proliferation in tumor-bearing mice [96]. Mechanistically, taurine uptake mediated by SLC6A6 enhances oxidative phosphorylation and regulates calcium and MAPK signaling via the PLC γ 1-DAG axis to promote TCR signaling and human $CD8^+$ T cell effector function [96].

1.4.3 Vitamins and metabolites

Analysis of publicly available T cell transcriptomic and proteomic data sets identifies vitamin B6 metabolism and vitamin B6–dependent transaminases as drivers of T cell activation and differentiation [97]. Inhibition of VB6 metabolism impairs T cell differentiation and activity *in vitro* and antitumor immunity *in vivo* [97]. Specifically, hypoxia-inducible transcription factor (HIF1) is associated with regulating intracellular VB6 levels via pyridoxal phosphate phosphatase, providing mechanistic insights on VB6 metabolism and T cell function [97]. Vitamin D metabolic products play regulatory roles in CD8⁺ T cells and hold potential clinical significance. Calcitriol suppresses glycolysis in autoreactive CD8⁺ T cells by downregulating the expression of GLUT1 and Lactate Dehydrogenase A and activating the AMPK signaling pathway, thereby inhibiting cell proliferation, cytotoxicity, and aberrant activation of human CD8⁺ T cells *in vitro* and in an autoimmune mouse model [98]. Clinically, circulating calcitriol levels negatively correlate with vitiligo severity [98]. In a clinical study, patients under anti-tuberculosis (TB) treatment are grouped based on serum level of vitamin D metabolite calcifediol, 25-hydroxyvitamin D (25[OH]D) [99]. This study finds that calcifediol may have beneficial effects clinically in increasing CD4⁺ and CD8⁺ T cells during anti-TB treatment [99].

1.4.4 Other dietary components

Playing a bioenergetic role, exogenous lactate displaces glucose as the preferred carbon source to fuel the Tricarboxylic Acid cycle, enhancing metabolic capacity and effector marker expression of activated mouse or human CD8⁺ T cells *in vitro* [100].

Extracellular magnesium (Mg²⁺) sensing via costimulatory molecule LFA-1 augments CD8⁺ T cell activation and effector function [101]. Mg²⁺-dependent LFA-1 headpiece opening and

extension trigger focal adhesion kinase phosphorylation and enhance downstream ERK1/2 and cJUN activities in mouse and human CD8⁺ T cells [101]. A range of magnesium depletion, Lymphocyte Function-associated Antigen knockout, and immunotherapy mouse models were employed to demonstrate magnesium's impact on CD8⁺ T cell function *in vivo* [101]. Clinically, low serum magnesium levels are associated with faster disease progression and shorter overall survival in patients treated with Chimeric Antigen Receptor T cells cell and ICI therapies [101].

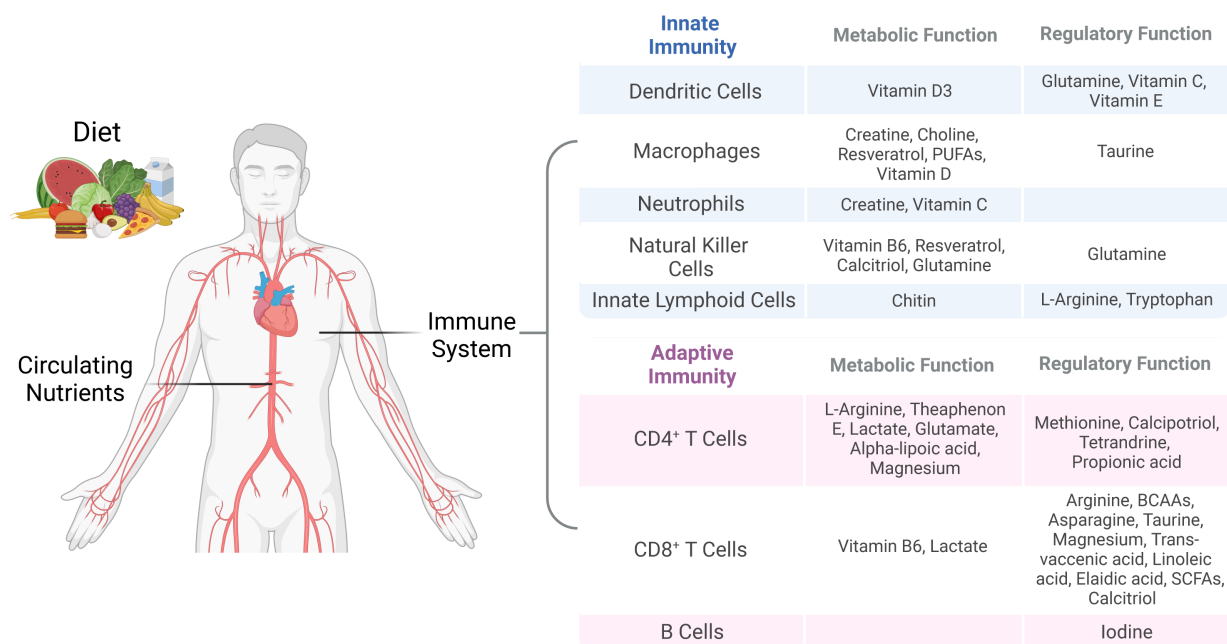


Figure 1.1 ZEA augments CD8⁺ T cell surface TCR stimulation.

A summary of a group of recent discoveries of metabolic or regulatory functions of dietary components on various innate and adaptive immune cell populations.

1.5 Significance and Overarching Question

The immune system's ability to recognize and eliminate cancer cells is critical for maintaining physical health, and immunotherapy has revolutionized cancer treatment by enhancing these natural defenses. Concurrently, growing evidence highlights the significant role of diet in modulating immune responses, particularly through its effects on CD8⁺ T cells, which play a central role in anti-tumor immunity. Despite this progress, our understanding of how specific dietary components regulate immune cell function at the mechanistic level remains limited. Current knowledge is largely derived from epidemiological studies that establish correlations between dietary patterns and immune-related outcomes, but these studies often do not explain the causative pathways or detailed molecular mechanisms. This gap in knowledge underscores the need for mechanistic studies to unravel the complex interplay between dietary components and immune cell regulation.

To address this gap, this thesis research focuses on circulating nutrients, which offer several advantages for mechanistic studies. By studying nutrients already present at detectable physiological levels in the bloodstream, this approach bypasses the complexities associated with identifying dietary origins or variability in nutrient intake. Circulating nutrients are more likely to exert direct biological effects on immune cells and present a lower risk of toxicity compared to pharmacological interventions. Furthermore, focusing on circulating nutrients allows for a controlled and targeted examination of their impact on immune cells, particularly CD8⁺ T cells, under physiologically relevant conditions.

To systematically identify and characterize immunoregulatory nutrients, our research employs a compound library-based screening approach that has proven successful in uncovering novel immune modulators. For example, our lab identified *trans*-vaccenic acid (TVA), an animal-

derived fatty acid as a regulator of CD8⁺ T cell function using our circulating blood nutrient library [86]. TVA enhances CD8⁺ T cell activation and effector function via the cAMP-PKA-CREB axis and in turn significantly improves anti-tumor immunity [86]. Building on this success, this thesis research identifies zeaxanthin (ZEA), a plant-derived carotenoid, as novel modulator of CD8⁺ T cell activity in the context of anti-tumor immunity. Unlike TVA, ZEA operates through distinct mechanisms that are dispensable to TVA's pathway. Importantly, we found that the combination of these nutrients results in a synergistic enhancement of CD8⁺ T cell function. These findings imply the importance of an inclusive and balanced diet to harness the full spectrum of immunoregulatory benefits of diverse nutrients. While the human diet is inherently a dynamic and complex mixture, the idea that the availability of specific nutrients can significantly influence immunity introduces a paradigm shift in how we view nutrition's role in health and disease treatment.

The overarching question driving this thesis research is: How do dietary components regulate CD8⁺ T cell function through distinct and potentially synergistic pathways? Current literature lacks answers to how diverse dietary components work together or through independent mechanisms to regulate immune cells in the context of anti-tumor immunity. This question addresses a critical gap by moving beyond correlation-based studies to investigate causative mechanisms and combinatorial effects of distinct nutrients. By systematically identifying and characterizing these interactions, this research aims to establish a mechanistic foundation for nutrient-based strategies, ultimately informing how dietary interventions can enhance immune resilience and improve disease outcomes.

1.6 Dissertation Overview

This dissertation explores the intersection of cancer immunology and the diet-immune axis, proposing novel strategies to enhance the efficacy of current immunotherapies through dietary modulation of CD8⁺ T cells. The following chapters outline the conceptual framework, experimental discoveries, and translational potential of this research.

Chapter 1 provides essential background on cancer immunology with a focus on the role of CD8⁺ T cells in immunotherapy and the limitations of existing treatments. Chapter 1 also introduces the emerging role of the diet-immune axis as a promising avenue to enhance CD8⁺ T cell activity and effector function. Evidence from recent studies suggests that dietary components not only serve metabolic functions, but also function as regulatory molecules impacting diverse immune cell populations (Figure 1.1). This chapter establishes the rationale for investigating dietary components as immunoregulators to complement current immunotherapy strategies with the goal to achieve better treatment outcomes.

Chapter 2 describes the surprising discovery of an unexplored immunomodulatory role of dietary zeaxanthin on T cell receptor signaling of CD8⁺ T cells. Through an unbiased library-based screening approach, we identified zeaxanthin as a modulator of CD8⁺ T cell activation, proliferation, and effector function in the context of anti-tumor immunity. Integrated multi-omics mechanistic studies reveal that zeaxanthin directly promotes T-cell receptor (TCR) stimulation on CD8⁺ T cell surface, leading to improved intracellular TCR signaling for effector T cell function. Hence, zeaxanthin treatment augments effectiveness of immune checkpoint inhibitor *in vivo* and human TCR-engineered CD8⁺ T cells to induce cell death against co-cultured tumor cells. This chapter highlights the potential clinical implications of dietary zeaxanthin as a supplement to improve existing cancer immunotherapy.

Chapter 3 explores the potential to chemically modify and optimize natural dietary components to enhance their efficacy in regulating CD8⁺ T cell function. By conducting structure-activity research (SAR), we aim to identify key chemical features required for immunomodulatory activity. This work was done in collaboration with Dr. Junhong Xiang from the lab of Dr. Chuan He (University of Chicago). Dr. Xiang synthesized a library of more than 50 derivatives of trans-vaccenic acid (TVA). By modifying the structure of TVA and screening for its effects on CD8⁺ T cell activity, we identified derivative 171 that significantly enhanced the efficacy comparing with the parent compound TVA both *in vitro* and *in vivo*.

Chapter 4 discusses the overall conclusions, limitations, future directions, as well as broader implications of the body of works presented in this dissertation. In addition, we also explored how combinations of immunomodulatory dietary components may synergistically regulate T cell activity. These combinatorial studies demonstrate additive or synergistic effects of nutrients of diverse origin on CD8⁺ T cell activation and effector function, pushing us to consider the mechanistic basis for a balanced diet.

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CHAPTER 2

ZEAXANTHIN AUGMENTS CD8⁺ EFFECTOR T CELL FUNCTION BY ENHANCING TCR STIMULATION

Contribution of Authors:

This chapter consists of the following manuscript (under revision):

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I was responsible for conducting the experiments, analysis, and making figures except for the following: Dr. Jiacheng Li (co-first author) tested ZEA derivatives and analyzed results from KAS-seq, RNA-seq, and microbiome studies. Dr. Hao Fan performed Fig. 4O and Fig. S3C. Dr. Rukang Zhang contributed to *in vivo* experiments and performed Fig. S1E. Dr. Zhicheng Xie performed Fig. 3C-D and Fig. S3A-B. Dr. Jiayi Tu performed Fig. S4N. Hardik Shah analyzed Fig. S1B. All authors contributed to manuscript editing. I was responsible for conducting the rest of the experiments, analysis, and making figures.

2.1 Introduction

The evolution of diet has played an essential role in shaping human physiology and pathology, including the development of the immune system and its responses to environmental stimuli [1-3]. However, despite extensive epidemiological studies, the mechanistic understanding of how different diets impact the human immune system is very limited due to the intricate nature of the immune system, whole organism metabolism, and the vast diversity of dietary components available. Emerging studies on the diet-immune axis have uncovered diet-derived nutrients that, besides performing their metabolic functions to provide energy and biosynthesis building blocks, serve immunoregulatory functions to influence diverse immune cell populations [4]. We thus developed an approach to focus on the bioactivity of individual nutrients, which allows us to conduct mechanistic studies to decipher the links between diet and immunity, despite the vast diversity of foods and dietary origins. We assembled a library of “blood nutrients” that are commercially available, including dietary supplements, inorganics, organic metabolites, peptides, and lipids, and screened for circulating blood nutrients that affect CD8⁺ T cell function. We uncovered that dietary nutrient *trans*-vaccenic acid (TVA) selectively promotes effector CD8⁺ T cell function and anti-tumor immunity *in vivo*, through TVA-mediated antagonism of an immunomodulatory G-protein coupled receptor (GPCR), GPR43 on cell surface of CD8⁺ T cells [5].

Here, using this blood nutrient compound library, we applied a co-culture-based screening approach to identify circulating nutrients that function as immunomodulators to affect the cytotoxicity of mouse Pmel-1 CD8⁺ T cells against mouse B16F10 melanoma tumor cells. We uncovered zeaxanthin (ZEA), a dietary nutrient found in many fruits and vegetables, as an

unexpected immunomodulator, which enhances CD8⁺ effector T cell function and consequently improving anti-tumor immunity *in vivo*.

ZEA is a non-provitamin A carotenoid synthesized only by plants and microorganisms, which can be found in grains such as corn and corn products, as well as many green leafy vegetables and is commonly used as a feed additive and colorant for birds, swine, and fish [6-8]. While more than 700 carotenoids are found in nature, only 40-50 have nutritional values and about 20 of them reach detectable levels in human circulation and tissues. ZEA and its structural isomer lutein (LUT) are among the most prevalent circulating carotenoids in the human body, but they can only be incorporated into the human circulation through consumption from diet and dietary supplements [9,10]. The chemical structures of ZEA and LUT differ only in the location of a double bond in one cyclic ring, resulting that ZEA has two β -ionone rings while LUT has a β -ionone ring and an ϵ -ionone ring [11]. Both ZEA and LUT are found to concentrate at the macular region of the human retina, with ZEA being the dominant component in the central macula and LUT distributed more broadly throughout the retina [12]. Because of their blue-light filtering and antioxidant roles, ZEA and LUT are commonly consumed as dietary supplements to protect the retina and eye tissues for vision health. Previous studies have revealed ZEA's role in photoprotection, energy transfer, and antioxidation [12,13]. However, the distinct immunomodulatory functions of ZEA and LUT in the context of anti-tumor immunity were previously unknown.

Here, we show that ZEA interacts with the TCR complex and promotes stimulation on the cell surface of CD8⁺ T cells, leading to enhanced TCR complex formation, activation of intracellular TCR signaling to augment CD8⁺ effector T cell function, and consequent anti-tumor immunity.

2.2 Results

Carotenoid nutrient ZEA enhances cytotoxicity of CD8⁺ T cells and anti-tumor immunity

Using our blood nutrient library [5] including dietary supplements, lipids, peptides, organic metabolites, and inorganics (Figure 2.1A), we performed a co-culture screen to identify circulating nutrients that influence the cytotoxicity of mouse Pmel-1 CD8⁺ T cells stimulated by anti-CD3/CD28 antibodies against co-cultured B16F10-luc2 mouse melanoma cells (Figure 2.2A). The screen results (Figure 2.2B, *lower*) show that ZEA, but not its structural isomer LUT (Figure 2.2B, *upper*), effectively enhanced Pmel-1 mediated cell death of B16F10-luc2 cells. In addition, ZEA treatment but not LUT augmented the ability of OT-I CD8⁺ T cell-mediated cytotoxicity to induce cell death against co-cultured B16-OVA cells (Figure 2.2C).

We next found that *in vivo* tumor growth of poorly immunogenic B16F10 cells (Figure 2.2D) was significantly attenuated in syngeneic mice receiving oral gavage of ZEA, but not in the control mice receiving LUT, compared to B16F10 syngeneic mice receiving oral gavage of control vehicle (Figure 2.2E). We detected increased levels of ZEA and LUT in the tumor interstitial fluid (TIF) of tumors in the syngeneic mice (Figure 2.1B), whereas oral administration of neither ZEA nor LUT affected body weights of syngeneic mice (Figure 2.1C). Similar results were obtained using mouse colon cancer MC38 cells in syngeneic mice receiving oral gavage of ZEA or vehicle control (Figure 2.2F).

Moreover, we found that depletion of CD8⁺ T cells by anti-CD8 antibodies in B16F10 syngeneic tumor-bearing mice (Figure 2.2G and 2.1D) resulted in abolishment of ZEA-mediated reduction of tumor growth (Figure 2.2H). In contrast, depletion of CD4⁺ T cells by anti-CD4 antibodies had minimal effects on ZEA-mediated tumor growth in B16F10 syngeneic mice (Figure

2.1E-2.1F). These data together suggest that ZEA reprograms CD8⁺ T cells and consequently enhances anti-tumor immunity.

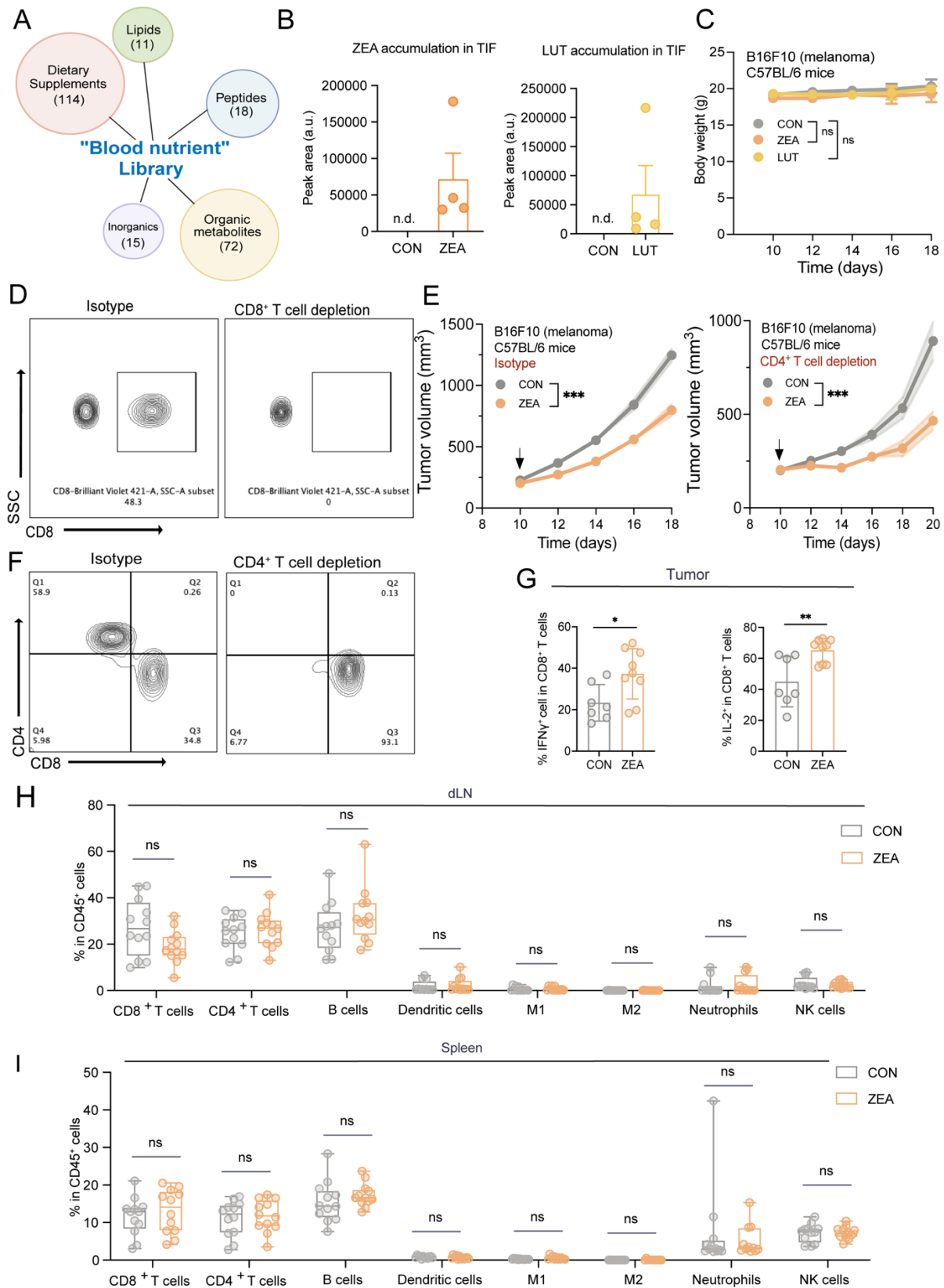


Figure 2.1 ZEA functions through CD8⁺ T cell immunity *in vivo*.

Figure 2.1 ZEA functions through CD8⁺ T cell immunity *in vivo*.

- (A) Diagram showing the composition of the blood nutrient library for cell-based screening.
- (B) ZEA (left) or LUT (right) levels in B16F10 tumor-bearing mice TIF were measured by mass spectrometry ($n = 4$).
- (C) Effect of orally administered ZEA or LUT (500mg/kg b.w.) on the body weight of B16F10 tumor-bearing C57BL/6 mice ($n = 6$ mice).
- (D) Representative flow cytometry plots showing CD8⁺ T cell depletion efficiency.
- (E) Effect of orally administered ZEA (500mg/kg b.w.) on B16F10 tumor growth in C57BL/6 mice treated with isotype control (*left*) or CD4⁺ T cell depleting antibodies (*right*) ($n \geq 9$ mice).
- (F) Representative flow cytometry plots showing CD4⁺ T cell depletion efficiency.
- (G) Effects of orally administered ZEA (500mg/kg b.w.) on IFN γ and IL-2 levels in tumor-infiltrating CD8⁺ T cells ($n \geq 7$ mice).
- (H) Effect of orally administered ZEA (500mg/kg b.w.) on the composition of leukocytes in the dLN ($n \geq 7$ mice).
- (I) Effect of orally administered ZEA (500mg/kg b.w.) on the composition of leukocytes in the spleen ($n \geq 7$ mice).
- Data are mean $\pm$ s.e.m (B, C, E) or mean $\pm$ s.d. (G-I). P values are calculated using Two-way ANOVA (C, E) or Student's two-sided unpaired t-test (B, G-I) (ns, not significant; * $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$).

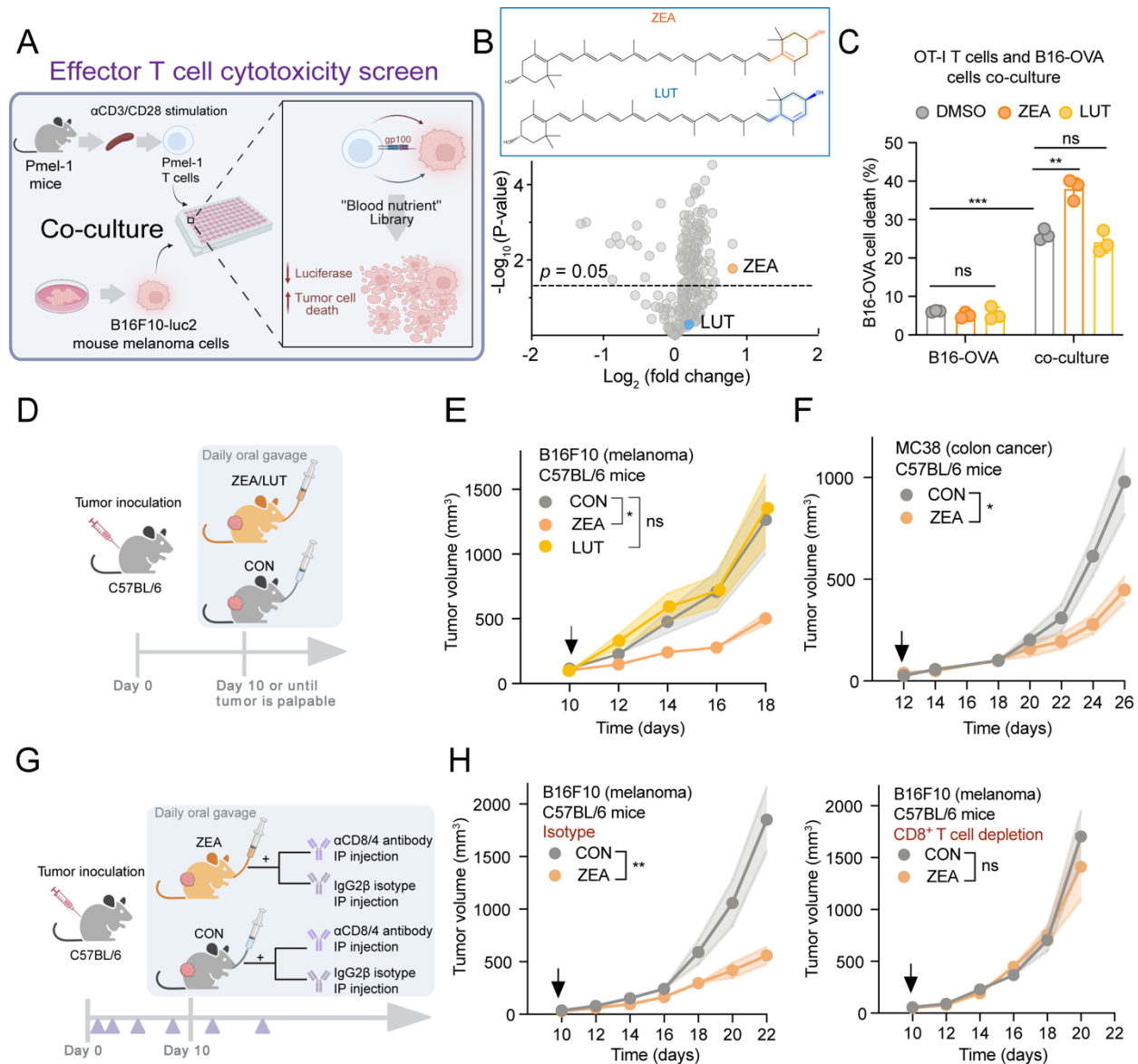


Figure 2.2 Oral ZEA enhances CD8 $^+$ T cell-mediated anti-tumor immunity.

(A) Schematic depicting experimental design for effector T cell cytotoxicity screen. This schematic was generated using BioRender.com.

(B) Chemical structures of ZEA and LUT (*upper*), and volcano plot showing results from the library screen with ZEA and LUT highlighted (*lower*).

(C) Effects of 5 μ M ZEA or LUT on B16-OVA cell death when treating B16-OVA cells alone and treating OT-I primary murine T cell and B16-OVA cell co-culture ($n = 3$).

(D) Schematic depicting experimental design for *in vivo* tumor-bearing mouse model. This schematic was generated using BioRender.com.

(E) Effect of orally administered ZEA or LUT (500mg/kg b.w.) on B16F10 tumor growth in C57BL/6 mice ($n = 6$ mice).

Figure 2.2 Oral ZEA enhances CD8⁺ T cell-mediated anti-tumor immunity (Cont'd).

(F) Effect of orally administered ZEA (500mg/kg b.w.) on MC38 colon cancer growth in C57BL/6 mice ($n = 6$ mice).

(G) Schematic depicting experimental design for CD8⁺ (or CD4⁺) T cell depletion experiment. This schematic was generated using BioRender.com.

(H) Effect of orally administered ZEA (500mg/kg b.w.) on B16F10 tumor growth in C57BL/6 mice treated with isotype control (*left*) or CD8⁺ T cell depleting antibody (*right*) ($n = 8$ mice). Data are mean $\pm$ s.e.m (E,F,H) or mean $\pm$ s.d. (C). P values are calculated using Two-way ANOVA (E,F,H) or one-way ANOVA with Dunn's multiple comparisons test (C) (ns, not significant; * $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$).

Oral ZEA improves recruitment and activation of tumor-infiltrating CD8⁺ T cells

Flow cytometry analyses revealed that oral ZEA supplementation to B16F10 syngeneic mice (Figure 2.3A) resulted in an increased CD8⁺ T cell population in the CD45⁺ tumor-infiltrating leukocyte (TIL) population. In contrast, tumor-infiltrating populations of CD4⁺ T cells, B cells, dendritic cells, M1 and M2 macrophages, neutrophils, and natural killer (NK) cells were not altered in B16F10 syngeneic mice by oral ZEA supplementation, compared to mice receiving vehicle control (Figure 2.3B). Further analysis of tumor-infiltrating CD8⁺ T cells with additional representative markers revealed that oral ZEA supplementation promoted CD8⁺ T cell function with increased expression levels of representative activation marker CD69, co-stimulatory receptor ICOS, and cytokines including TNF α , IFN γ , and IL-2 (Figure 2.3C and 2.1G). Oral ZEA also resulted in increased CD4⁺ T helper 1 (Th1) but reduced CD4⁺Foxp3⁺ T_{reg} cell populations in B16F10 tumors, while it has minimal effects on other tumor-infiltrating CD4⁺ T cell populations including Th2, Th17, Th9, and Th22 (Figure 2.3D). In contrast, oral ZEA supplementation did not affect cell populations of diverse immune cells in dLNs or spleens in B16F10 syngeneic mice (Figure 2.1H-2.1I). Lastly, oral ZEA did not significantly alter the diversity and composition of the gut microbiota (Figure 2.4). Given that Th1 cells are critical in

cellular immune responses against intracellular viruses and cancer cells, including activation of cytotoxic T cells, and that Treg cells suppress CD8⁺ T cells, these results together suggest that oral ZEA enhances recruitment and activation of CD8⁺ effector T cells to tumors for improved anti-tumor immunity [14, 15].

Consistent with these findings, we next found that ZEA but not LUT treatment had direct effects on and effectively promoted mouse primary CD8⁺ T cell function (Figure 2.3E) with increased expression levels of proliferation marker Ki-67, activation marker CD69, and cytokines including TNF α , IFN γ , and IL-2 (Figure 2.3F). Given that ZEA and LUT are structurally similar but have distinct effects on CD8⁺ T cells, we next explored the structure-activity relationship (SAR) by testing several natural compounds that share structural similarity with ZEA, all of which are available in dietary sources (Figure 2.3G). Notably, only ZEA and D5 (fucoxanthin) significantly promoted CD8⁺ T cell activation, despite that all compounds have long chains of conjugated double bonds (Figure 2.3H). This result suggests that the antioxidant properties of conjugated double bonds in these compounds are not sufficient to mediate enhanced activation of CD8⁺ T cells. Instead, the unique structure of symmetrical conjugated polyene capped with six-membered rings is crucial for ZEA to enhance CD8⁺ T cell activation. In addition, the different six-membered ring structures at both ends of the double bonds resulted in the differential bioactivity of ZEA compared to D1, D2, D4, and D5 for enhanced CD8⁺ T cell activation, while D3 with an open-chain structure and D6 with a short-chain monomer lacked bioactivity (Figure 2.3H). These data together suggest that the unique structure and conformation of ZEA are vital for its bioactivity to enhance CD8⁺ T cell activation and function.

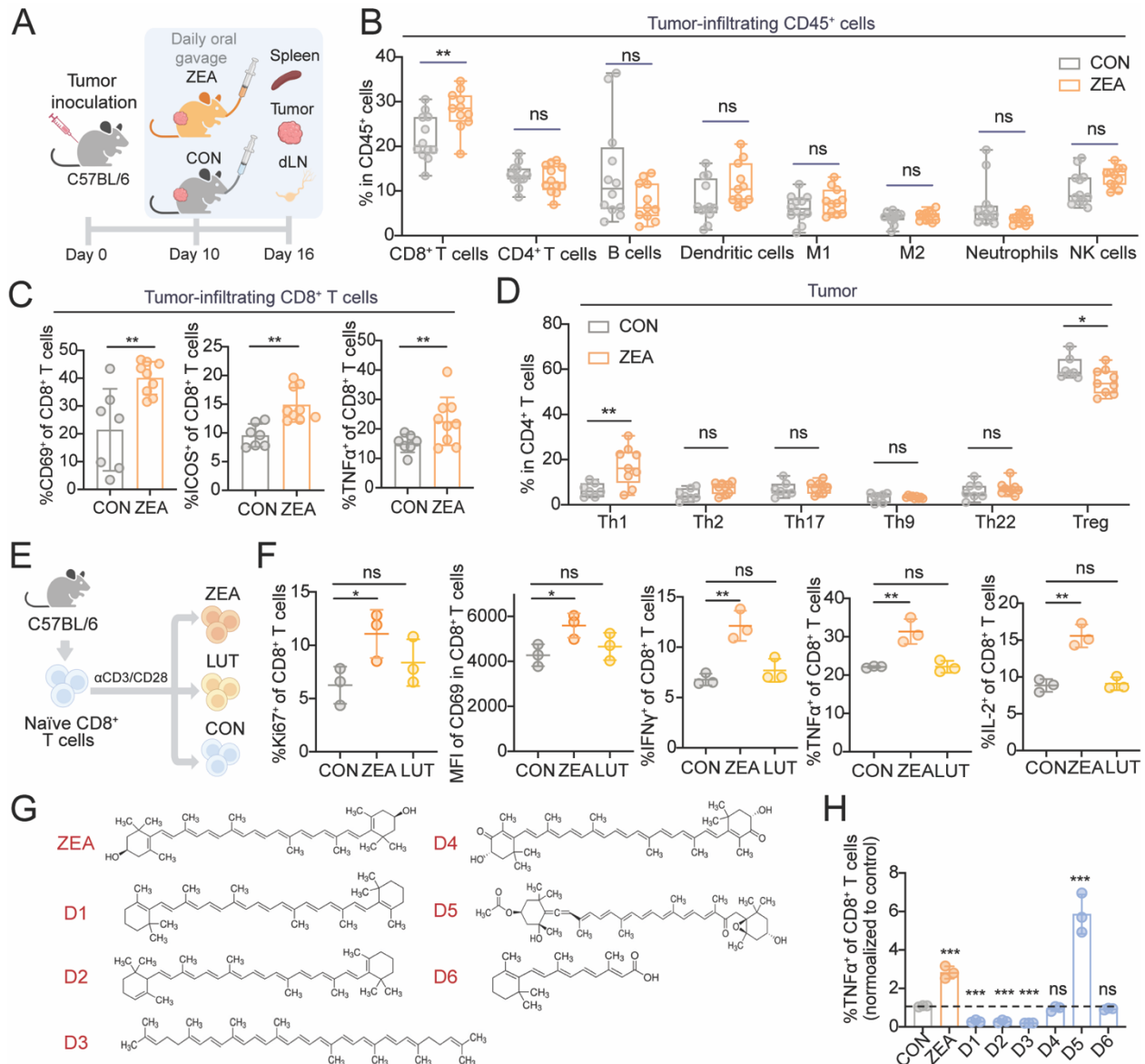


Figure 2.3 ZEA reprograms CD8⁺ T cells *in vivo* and *in vitro*.

(A) Schematic depicting experimental setup for harvesting the spleen, tumor, and draining lymph node (dLN) to examine zeaxanthin's effect on leukocytes. This schematic was generated using BioRender.com.

(B) Effect of orally administered ZEA (500mg/kg b.w.) on the composition of tumor-infiltrating leukocytes ($n \geq 7$ mice).

(C) Effects of orally administered ZEA (500mg/kg b.w.) on tumor-infiltrating CD8⁺ T cell activation, co-stimulation, and pro-inflammatory cytokine TNF α production ($n \geq 7$ mice).

(D) Effects of orally administered ZEA (500mg/kg b.w.) on tumor-infiltrating CD4⁺ T cell composition ($n \geq 7$ mice).

(E) Schematic depicting experimental setup for *in vitro* primary murine CD8⁺ T cells treatment. This schematic was generated using BioRender.com.

Figure 2.3 ZEA reprograms CD8⁺ T cells *in vivo* and *in vitro* (Cont'd).

(F) Effects of 5 μ M ZEA or LUT treatment on α CD3/CD28 stimulated murine CD8⁺ T cell proliferation, activation, and pro-inflammatory cytokine levels ($n = 3$).

(G) Chemical structures of ZEA and 6 natural structural analogs of ZEA.

(H) Effect of treatment with 5 μ M ZEA or ZEA derivatives on primary murine CD8⁺ T cell TNF α production ($n = 3$).

Data are mean $\pm$ s.d. P values are calculated using Student's two-sided unpaired t-test (B,C,D) or one-way ANOVA with Dunn's multiple comparisons test (F,H), (ns, not significant; *0.01 $<p<0.05$; **0.001 $<p<0.01$).

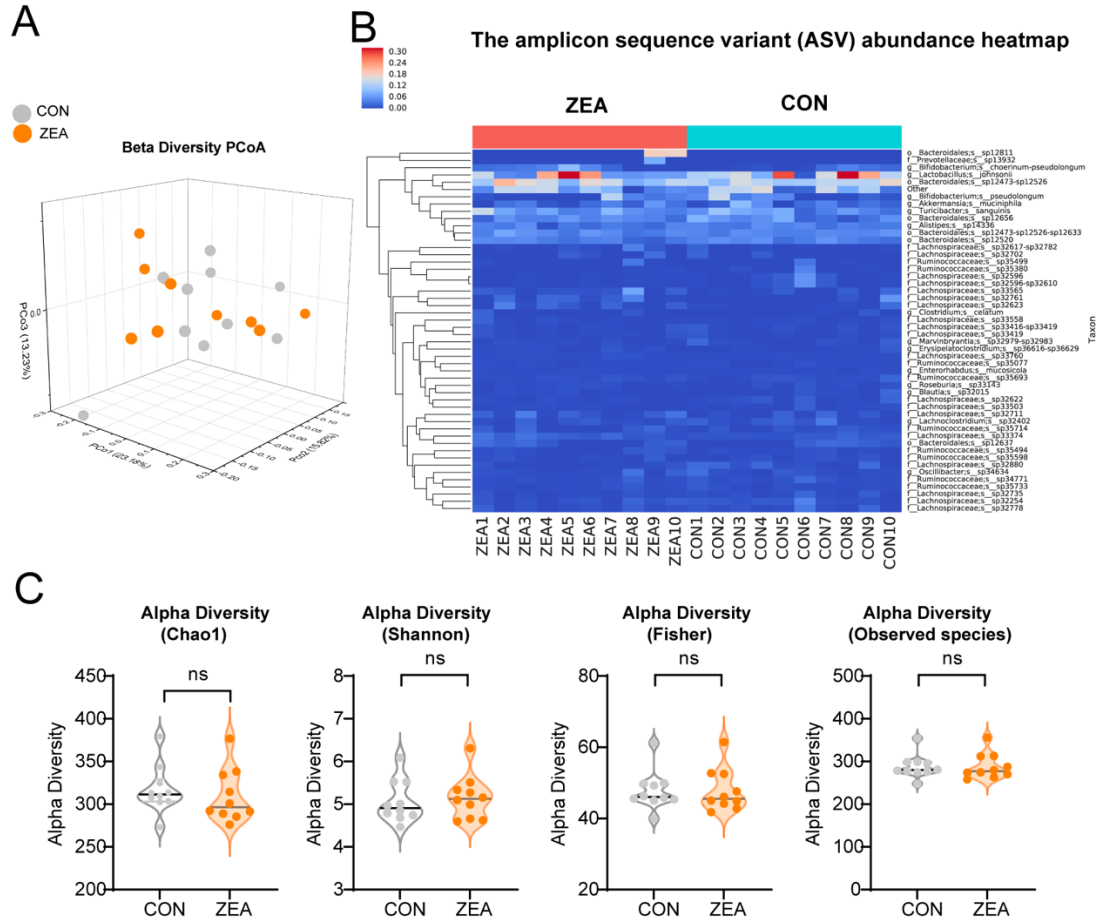


Figure 2.4 Oral ZEA has minimal effects on gut microbiota.

(A) Principal Coordinate Analysis (PCoA) plot showing the beta diversity of fecal microbiota from mice of ZEA and CON group. Each point represents the microbial community of an individual mouse, with different colors or shapes indicating different groups ($n = 10$ mice).

(B) Heatmap showing the microbial composition of the samples at the species level with the top fifty most abundant species identified. Each row represents the abundance for each taxon, with the taxonomy ID shown on the right. Each column represents the abundance for each sample, with the sample ID shown at the bottom. Group information is indicated by the colored bar located on the top of each column. Hierarchical clustering was performed on samples based on Bray-Curtis dissimilarity. Hierarchical clustering was also performed on the taxa so that taxa with similar distributions are grouped together ($n = 10$ mice).

(C) Violin plot comparing the alpha diversity of fecal microbiota across CON group and ZEA group. Alpha diversity was assessed using Chao1 Index, Shannon Index, Fisher Index, or Observed species. Each violin shape represents the distribution of diversity values within a group, with wider sections indicating a higher density of samples at specific diversity levels. Individual sample points are overlaid on the plot, and the horizontal line within each violin denotes the median. Statistical significance between groups was determined using Kruskal-Wallis test with p -values displayed on the plot ($n = 10$ mice) (ns, not significant).

ZEA augments CD8⁺ T cell surface TCR stimulation

To determine the mechanisms underlying ZEA's bioactivity in enhancing CD8⁺ T cell function, we performed a kethoxal-assisted single-stranded DNA sequencing (KAS-seq) (40 min-2 hours) to investigate the initial influences of ZEA treatment on mouse primary CD8⁺ T cells by capturing global transcription dynamics and enhancer activity [16]. Functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the top-ranking altered genes from the genome-scale KAS-seq results (Figure 2.5A) revealed T cell receptor (TCR) signaling pathway among the top-enriched ontologies in ZEA treated CD8⁺ T cells.

TCR signaling in CD8⁺ T cells is triggered when the cell surface TCR binds to a Major Histocompatibility Complex Class I (MHC I)-bound peptide antigen. This interaction triggers the assembly of the TCR signaling complex and activates downstream intracellular signaling cascades to turn on CD8⁺ effector T cell function including cytokine production and cytotoxicity [17]. Given that polar carotenoids including ZEA and LUT can insert into plasma membranes and alter the membrane fluidity, we examined the effects of ZEA or LUT treatment on TCR synapse complex formation on the cell surface of mouse primary CD8⁺ T cells upon stimulation with anti-CD3/CD28 antibodies, or on the cells with overnight pre-stimulation (Figure 2.5B) [18, 19]. Flow cytometry analysis revealed that ZEA treatment for 5, 15, and 30 min effectively enhanced TCR synapse complex formation on mouse primary CD8⁺ T cells assessed by increased levels of TCRb, CD3e, and CD28 on CD8⁺ T cell surface upon stimulation (Figure 2.5C; *left two panels*). In contrast, LUT treatment had minimal effects on TCR synapse complex formation on mouse primary CD8⁺ T cell surface upon stimulation (Figure 2.5C; *right two panels*). Moreover, we performed confocal microscopy to visualize cell surface TCR synapse complex formation on mouse primary CD8⁺ T cells upon stimulation with anti-CD3/CD28 antibodies. ZEA treatment for

5, 15, or 30 min effectively promoted TCR synapse complex formation with increased detection of cell surface TCRb, CD3ε, and CD28 on mouse primary CD8⁺ T cells (Figure 2.5D). Similar results were obtained from flow cytometry (Figure 2.6A) and confocal microscopy (Figure 2.6B) analyses using mouse primary CD8⁺ T cells with overnight pre-stimulation by anti-CD3/CD28 antibodies for overnight prior to treatment with ZEA or LUT. Note that the formation of TCR synapse complex started decreasing at 30 minutes after stimulation and ZEA treatment (Figure 2.5C and 2.6A, *left two panels*), suggesting start of internalization of TCR synapse complex. TCR internalization represents a crucial mechanism for downregulating TCR to prevent excessive T cell activation, which starts within minutes to several hours upon stimulation depending on cell types and stimulation conditions [20]. We found that ZEA had minimal effects on phorbol 12-myristate 13-acetate (PMA)-induced TCR internalization (Figure 2.6C) [21]. These results together suggest that ZEA treatment enhances TCR stimulation by promoting TCR synapse complex formation on the cell surface of CD8⁺ T cells, but it does not affect TCR internalization.

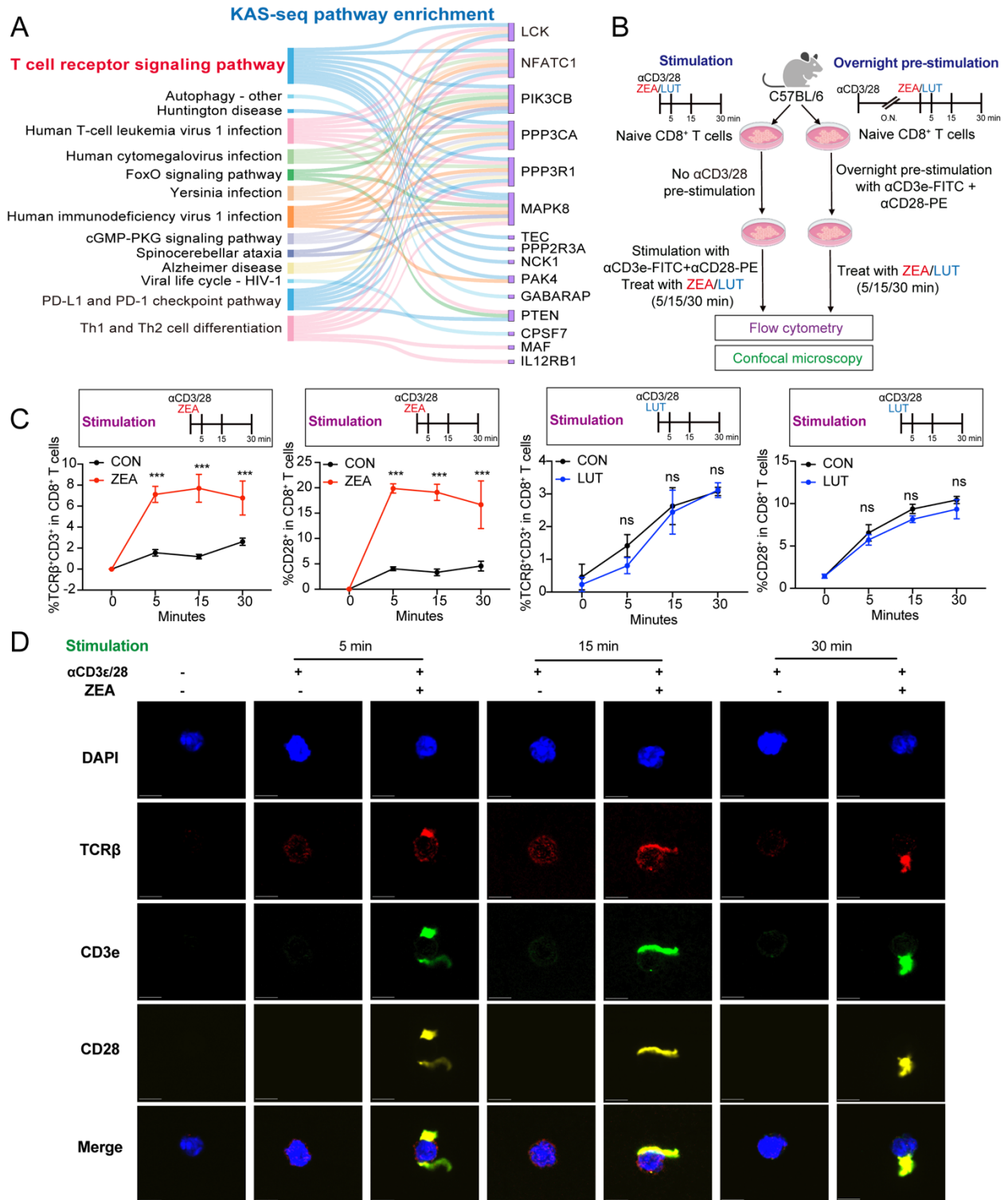


Figure 2.5 ZEA augments CD8⁺ T cell surface TCR stimulation.

(A) KEGG pathway enrichment for upregulated genes generated from KAS-seq analysis of α CD3/CD28 stimulated murine CD8⁺ T cells treated with 5 μ M ZEA compared to cells treated with DMSO control ($n = 3$).

Figure 2.5 ZEA augments CD8⁺ T cell surface TCR stimulation (Cont'd).

(B) Schematic depicting experimental setup for flow cytometry and confocal microscopy detection of α TCR β , α CD3 ϵ , and α CD28 antibody binding in murine CD8⁺ T cells with or without 5 μ M ZEA or LUT treatment with different stimulation strategies. This schematic was generated using BioRender.com.

(C) Flow cytometry analysis showing the effects of 5 μ M ZEA or LUT on α TCR β , α CD3 ϵ , and α CD28 antibody binding in murine CD8⁺ T cells at 5 min, 15 min, and 30 min after treatment and stimulation with α CD3 ϵ and α CD28 antibodies ($n = 3$).

(D) Representative confocal microscopy images showing the effects of 5 μ M ZEA on α TCR β , α CD3 ϵ , and α CD28 antibody binding in murine CD8⁺ T cells at 5 min, 15 min, and 30 min after treatment and stimulation with α CD3 ϵ and α CD28 antibodies ($n = 3$).

Data are mean $\pm$ s.d. P values are calculated using Two-way ANOVA (ns, not significant; *** $p < 0.001$).

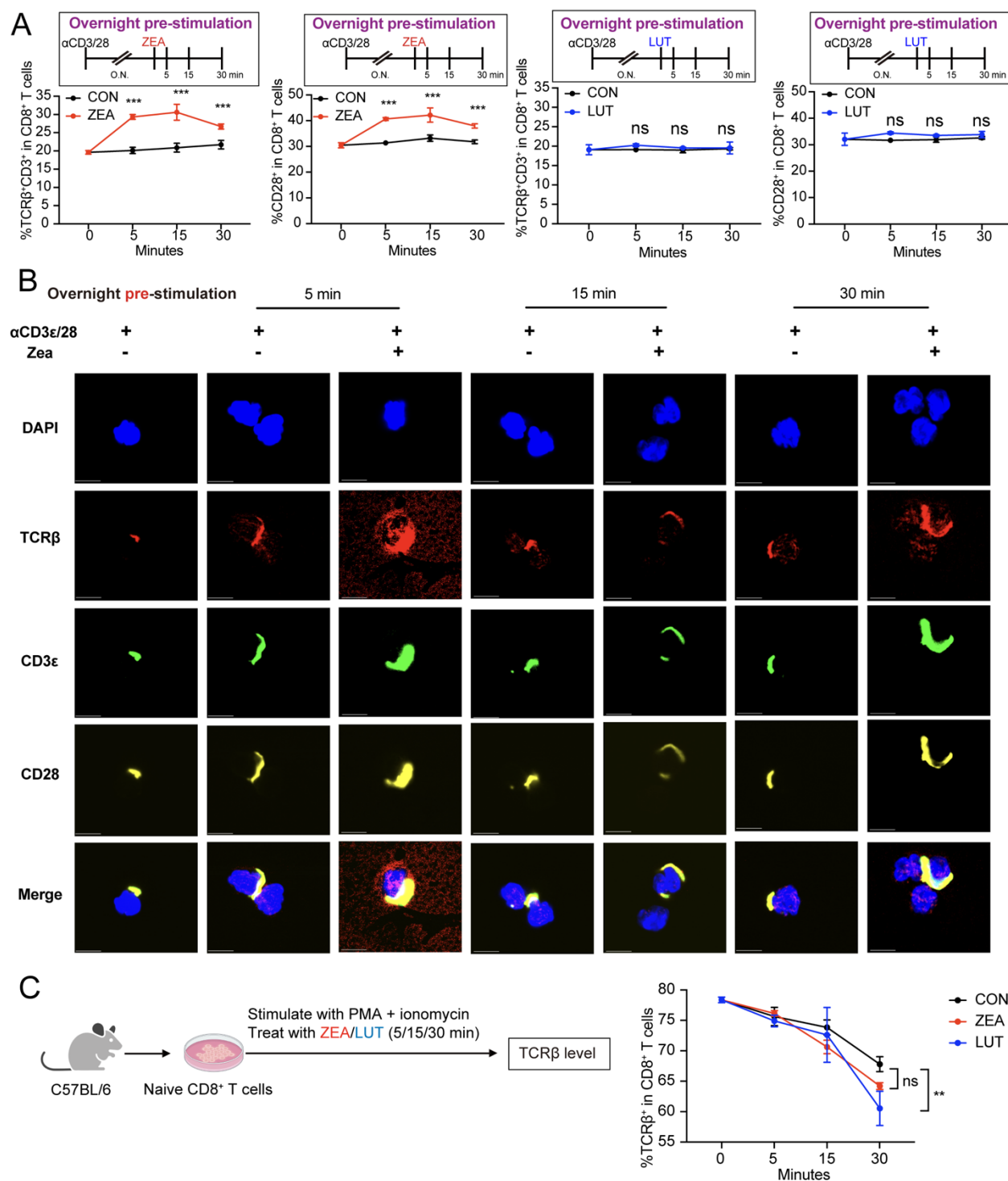


Figure 2.6 ZEA enhances TCR formation on CD8⁺ T cells upon stimulation.

(A) Flow cytometry analysis showing the effects of overnight pre-stimulation with α CD3 ϵ and α CD28 antibodies and 5 min, 15 min, and 30 min treatment of 5 μ M ZEA or LUT on α TCR β , α CD3 ϵ , and α CD28 antibody binding ($n = 3$).

Figure 2.6 ZEA enhances TCR formation on CD8⁺ T cells upon stimulation (Cont'd).

(B) Representative confocal microscopy images showing the effects of overnight pre-stimulation with α CD3 ϵ and α CD28 antibodies and 5 min, 15 min, and 30 min treatment of 5 μ M ZEA on α TCR β , α CD3 ϵ , and α CD28 antibody binding ($n = 3$).

(C) Schematic depicting experimental setup for flow cytometry detection of TCR β level in mouse CD8⁺ T cells with or without 5 μ M ZEA or LUT treatment with PMA/ionomycin. This schematic was generated using BioRender.com (*left*). Flow data summary shows the effects of stimulation with PMA/ionomycin and 5 min, 15 min, and 30 min treatment of 5 μ M ZEA or LUT on TCR β level ($n = 3$).

Data are mean $\pm$ s.d. P values are calculated using Two-way ANOVA (ns, not significant; *** $p < 0.001$).

ZEA reprograms CD8⁺ T cells by enhancing intracellular Ca²⁺ signaling and NF- κ B pathway

We next sought to explore the downstream intracellular TCR signaling cascades that are crucial for ZEA-enhanced CD8⁺ T cell activation and function using mouse primary CD8⁺ T cells (Figure 2.7A). We designed integrated, temporal mechanistic studies, including (1) TCR-specific phospho-antibody array (5 min) for changes of proximal TCR signaling cascades following ZEA treatment; (2) conventional phospho-antibody array (40 min-6 h) for downstream cellular signaling changes; and (3) RNA-seq (24 h) for whole-transcriptome analysis. TCR complex assembly upon antigen recognition activates tyrosine kinase Lck, leading to consequent phosphorylation and activation of signaling molecules including ZAP-70 and phospholipase C γ (PLC γ). This initiates the activation of transcription factors such as NF- κ B and NFAT, as well as a signaling cascade involving Ca²⁺ release from endoplasmic reticulum for CD8⁺ T cell functions including cytokine production and cytotoxicity [22-25]. Indeed, TCR phospho-antibody array analysis on mouse primary CD8⁺ T cells treated with ZEA revealed increased phosphorylation levels of ZAP-70, Lck, PLC, LAT, and NF- κ B, and reduced phosphorylation levels of NFAT as activation and nuclear

localization of NFAT requires dephosphorylation by Ca^{2+} -dependent phosphatase calcineurin (Figure 2.7B) [26]. In contrast, LUT treatment had minimal effects on phosphorylation levels of these key proximal and downstream signaling molecules of the TCR synapse complex in CD8^+ T cells (Figure 2.8A). The results of the conventional cell phospho-antibody array (Figure 2.8B-2.8C) also revealed increased phosphorylation and activation of proteins in the JAK-STAT and ERK pathways, which are known to be activated by tumor necrosis factor (TNF) in CD8^+ T cells, in mouse primary CD8^+ T cells treated with ZEA, but not in cells treated with LUT [27, 28].

Consistent with these findings, principal component analysis (PCA) of RNA-seq results revealed that control mouse primary CD8^+ T cells and cells treated with LUT can be grouped together and are separated from mouse primary CD8^+ T cells treated with ZEA (Figure 2.8D), suggesting that ZEA selectively alters CD8^+ T cells while LUT has minimal effects on these cells. Moreover, functional KEGG pathway analysis and global Gene Set Enrichment Analysis (GSEA) of RNA-seq results revealed that ZEA treatment enhanced the expression of the genes enriched in the T cell receptor signaling pathway (Figure 2.7C) [29, 30]. Notably, ZEA treatment upregulated the expression of genes enriched in interferon-stimulated effector CD8^+ T cells more effectively than LUT did (Figure 2.7D), correlating with enhanced CD8^+ T cell function following ZEA treatment.

We next sought to determine which downstream pathway(s) of the TCR synapse complex is required for ZEA function. ZEA but not LUT treatment significantly increased phosphorylation levels of TCR downstream signaling molecules including $\text{CD3}\zeta$, Lck, ZAP-70, LAT, and S6 kinase that is a downstream effector of PI3K-AKT pathway (Figure 2.8E-2.8I). Consistent with these findings, Lck or PLC γ inhibitors abolished ZEA-dependent enhancement CD8^+ T cell function (Figure 2.7E-2.7F). 9, PLC γ is a downstream signaling effector of TCR for rapid release

of Ca^{2+} from the ER lumen to cytosol [31]. Indeed, we found that ZEA but not LUT treatment effectively increased intracellular Ca^{2+} level in CD8^{+} T cells, assessed by increased Ca^{2+} -bound indicator Fluo-4 (Figure 2.7G). Consistent with this finding, treatment with specific inhibitors targeting Ca^{2+} -dependent phosphatase calcineurin and its downstream substrate transcription factor NFAT obliterated ZEA-enhanced CD8^{+} T cell function, assessed by $\text{TNF}\alpha$ production (Figure 2.7H-2.7I). Moreover, we found that treatment with NF- κ B inhibitor (Figure 2.7J), but not inhibitors targeting ERK or JAK that are activated by TNF (Figure 2.8J-2.8K), also abolished ZEA-enhanced CD8^{+} T cell function assessed by $\text{TNF}\alpha$ production. Taken together, our results reveal that TCR signaling cascades involving the Lck-PLC- Ca^{2+} -NFAT and NF- κ B pathways are crucial for ZEA-enhanced CD8^{+} T cell function.

ZEA augments T cell-based immunotherapies

Our studies show that animal-derived TVA and plant-derived ZEA enhance CD8^{+} effector T cells and anti-tumor immunity through different molecular and signaling mechanisms [5]. Consistent with these findings, combined treatments with different dosages of TVA and ZEA resulted in synergistic effects to enhance primary mouse CD8^{+} T cell function *in vitro* (Figure 2.7L, 2.8L, and 2.8M). Moreover, we found that oral ZEA supplementation combined with anti-PD-1 antibody, a representative form of immune checkpoint inhibitor (ICI) therapy, showed synergistic attenuation of B16F10 tumor growth *in vivo* (Figure 2.7K) [32]. In addition, ZEA treatment was significantly more potent than LUT treatment in enhancing human CD8^{+} T cell activation and function assessed by the production of cytokines, including $\text{IFN}\gamma$, IL-2, and $\text{TNF}\alpha$, (Figure 2.7M). Furthermore, ZEA treatment improved *in vitro* cytotoxicity of human tumor antigen-specific TCR gene-engineered T (TCR-T) cells derived from peripheral blood CD8^{+} T

cells from healthy donors (Figure 2.7N). For example, ZEA treatment was significantly more potent than LUT in enhancing cytotoxicity of HLA-A*02-restricted NY-ESO-1-specific (19305DP) TCR-T cells derived from a healthy donor against co-cultured HLA-A*02⁺NY-ESO-1⁺ A375 melanoma cells (Figure 2.7O; *upper*), while both ZEA and LUT have minimal direct cytotoxic effects on A375 cells (Figure 2.7O; *lower*). Similar enhancement by ZEA, but not LUT, was observed using T cells from another donor and HLA-A*02-restricted NY-ESO-1-specific (AL) TCR gene (Figure 2.7P) [33]. Moreover, consistent with our findings using mouse primary CD8⁺ T cells, treatment with PLC γ inhibitor abolished ZEA-enhanced cytotoxicity of TCR-T cells on A375 cells (Figure 2.8N). These findings together align with the concept that ZEA potentially improves clinical efficacy of T cell-based immunotherapies.

Hereby our findings reveal a previously unknown immunomodulatory function of ZEA, a carotenoid antioxidant known for its role in eye health. Dietary ZEA can be found in many fruits and green leafy vegetables such as kale, spinach, broccoli, peas, and lettuce, durum wheat and corn, as well as egg yolks because common feeds for chickens contain grains such as corn and vegetables enriched with ZEA [34]. It is widely recognized that plant-based or vegan diets can potentially benefit the human immune system and response [35]. However, the underlying mechanistic link between plant-derived nutrients and human immunity remains largely unknown. Our strategy focusing on individual diet-derived nutrients despite the vast diversity of food and diet origins makes mechanistic studies feasible to explore nutritional influences on human health and the immune system. Using this approach, we successfully identified dietary TVA derived from animal-based diets⁵ and hereby ZEA as a plant-produced nutrient, both of which have direct yet mechanistically distinct influence on CD8⁺ effector T cells and anti-tumor immunity. Therefore,

our approach has broad implications to uncover unprecedented roles of nutrients in human health and pathologic responses.

Our findings support the high translational potential of ZEA, as a natural food component, to serve as a dietary supplement that enhances clinical outcomes of immunotherapies such as ICIs and TCR-T cell therapy. By investigating the physiological influences of diverse diet-derived nutrients on the immune system, our studies contribute to a comprehensive understanding of how dietary nutrients shape human biology, which is crucially informative for dietary choices that may be beneficial for maintaining human health, reducing disease risk, and improving response to therapies. Our studies on TVA from animal-based diets and ZEA from plant-based foods demonstrate that the scientific and mechanistic understanding of how nutrients from different diets influence human health is still limited [5]. The findings that TVA and ZEA have mechanistically distinct effects on CD8⁺ effector T cells and anti-tumor immunity, as well as that combined treatment with TVA and ZEA achieves synergy to enhance CD8⁺ T cell function, together suggest that a balanced diet may provide complementary benefits to human health.

TCR signaling plays a vital role in activation and differentiation of CD8⁺ T cells, while stronger signaling strength of TCR generally promotes cytotoxic activity [36]. Our findings suggest that ZEA enhances cytotoxicity of CD8⁺ T cells through multiple intracellular TCR signaling cascades, which may act in concert to achieve fulfillment of effective cytotoxicity. Our results demonstrate a crucial coordination between the Lck-PLC-Ca²⁺-NFAT axis and the NF-κB pathway to mediate the pro-cytotoxic effects of ZEA on CD8⁺ T cells. Targeting either of them results in abolishment of ZEA-enhanced CD8⁺ T cell function, suggesting that the Lck-PLC-Ca²⁺-NFAT axis and the NF-κB pathway are indispensable to each other. In contrast, the ERK and JAK-STAT pathways are likely downstream effectors of TNF that is produced by CD8⁺ T cells when

TCR signaling is activated upon antigen recognition, thus treatment with specific inhibitors of ERK or JAK did not abolish ZEA-enhanced CD8⁺ T cell function assessed by TNF α production. These findings also support a crucial role of TCR signaling cascades involving Ca²⁺ signaling and the NF- κ B pathway in promoting the cytotoxic activity of CD8⁺ T cells.

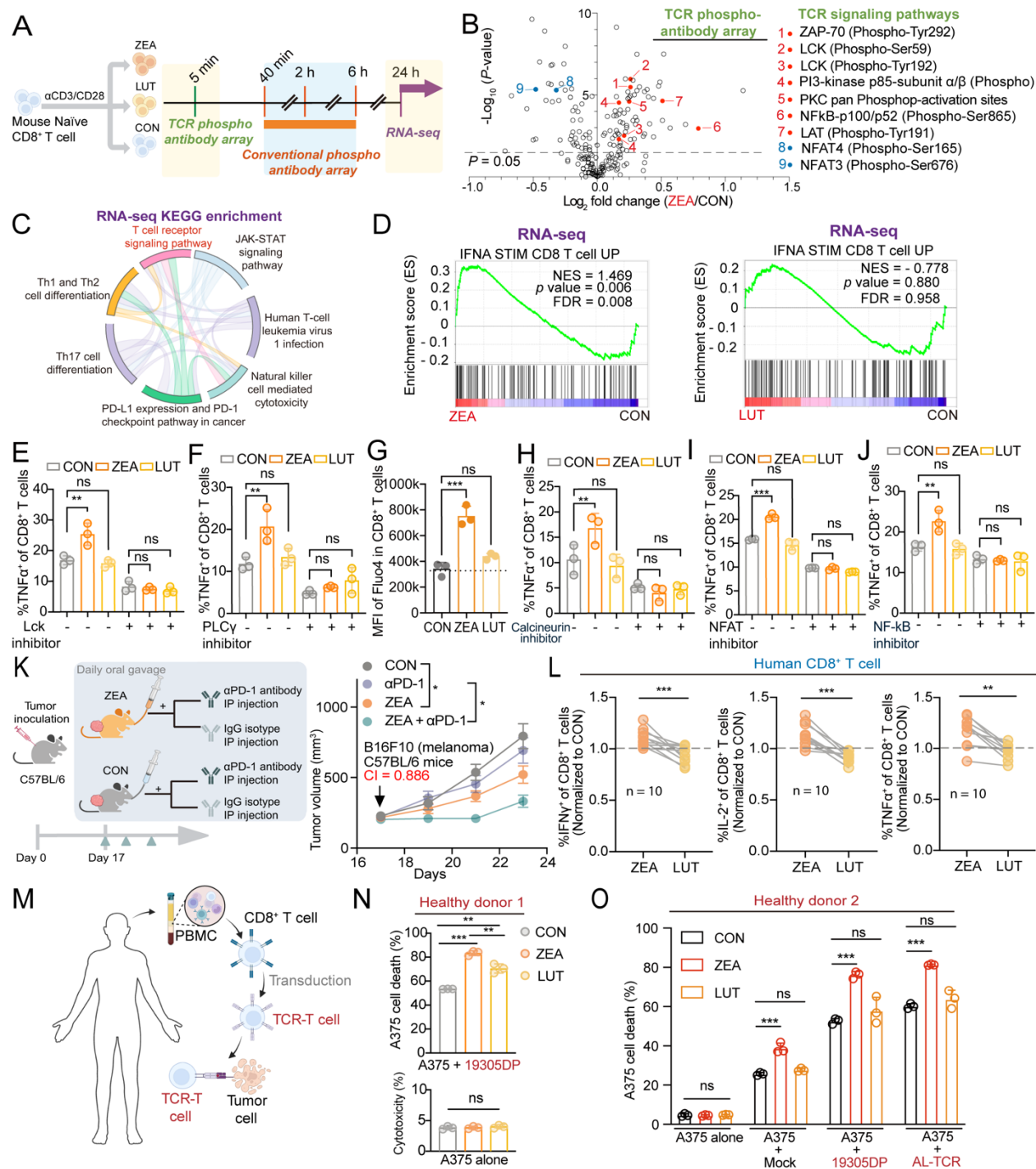


Figure 2.7 ZEA enhances TCR signaling cascades and effectiveness of T cell-based immunotherapies. (A) Schematic depicting temporal, mechanistic studies performed with murine CD8⁺ T cells treated with and without 5 μ M ZEA or LUT. (B) Volcano plot showing the effects of 5 min ZEA treatment on TCR-related protein phosphorylation (*left*). A list of changed protein phosphorylation in TCR signaling pathways (*right*).

Figure 2.7 ZEA enhances TCR signaling cascades and effectiveness of T cell-based immunotherapies (Cont'd).

(C) Chord diagram showing KEGG pathway enrichment of differentially expressed genes between ZEA treatment and DMSO in RNA-seq ($n = 3$).

(D) GSEA analysis of the gene set of IFN α stimulated CD8 $^{+}$ T cell upregulated genes after 24 h ZEA or LUT treatment ($n = 3$).

(E) Effect of LCK inhibitor PP2 (200 nM) treatment on ZEA-dependent murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(F) Effect of PLC γ inhibitor 3-Nitrocoumarin (500 nM) treatment on ZEA-dependent murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(G) Effect of 5 μ M ZEA or LUT treatment on calcium indicator fluo4 levels in murine CD8 $^{+}$ T cells ($n = 3$).

(H) Effect of calcineurin inhibitor cyclosporin A (7nM) treatment on ZEA-dependent murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(I) Effect of NFAT inhibitor (1 μ M) treatment on ZEA-dependent murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(J) Effect of NF- κ B inhibitor INK4 (2 μ M) treatment on ZEA-dependent murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(K) Effect of TVA (10 μ M) and ZEA (5 μ M) combined treatment on murine CD8 $^{+}$ T cell effector function assessed by TNF α levels ($n = 3$).

(L) Effect of α PD-1 antibody on B16F10 tumor growth in C57BL/6 mice orally supplemented with (CI = 0.886) or without ZEA ($n \geq 9$ mice).

(M) Effect of 5 μ M ZEA or LUT on human CD8 $^{+}$ T cell effector function assessed by IFN γ , IL-2, and TNF α levels in human CD8 $^{+}$ T cells isolated from PBMCs of healthy donors ($n = 10$).

(N) Schematic depicting experimental design for human TCR-T transduction and *ex vivo* co-culturing with tumor cells to assess TCR-T cell-mediated cytotoxicity. This schematic was generated using BioRender.com.

(O) Effect of 5 μ M ZEA or LUT on human NY-ESO-1-specific TCR-T cell-mediated cytotoxicity against A375 melanoma cells. Human CD8 $^{+}$ cells were derived from a healthy donor.

(P) Effect of 5 μ M ZEA or LUT on A375 melanoma cells alone, human untransduced (mock) CD8 $^{+}$ cells against A375 melanoma cells, T cell human 19305DP TCR-T cell-mediated cytotoxicity against A375 melanoma cells, and human AL-TCR-T cell-mediated cytotoxicity against A375 cells. Human CD8 $^{+}$ cells were derived from a second healthy donor.

Data are mean $\pm$ s.e.m (L) or mean $\pm$ s.d. (E-K, M-P). P values are calculated using Two-way ANOVA (E-F,H-L,P), one-way ANOVA with Dunn's multiple comparisons test (G,M,O), or Student's two-sided paired t-test (L), (ns, not significant; *0.01< p <0.05; **0.001< p <0.01; *** p <0.001).

Also see Figures S4 and Table S3-S6.

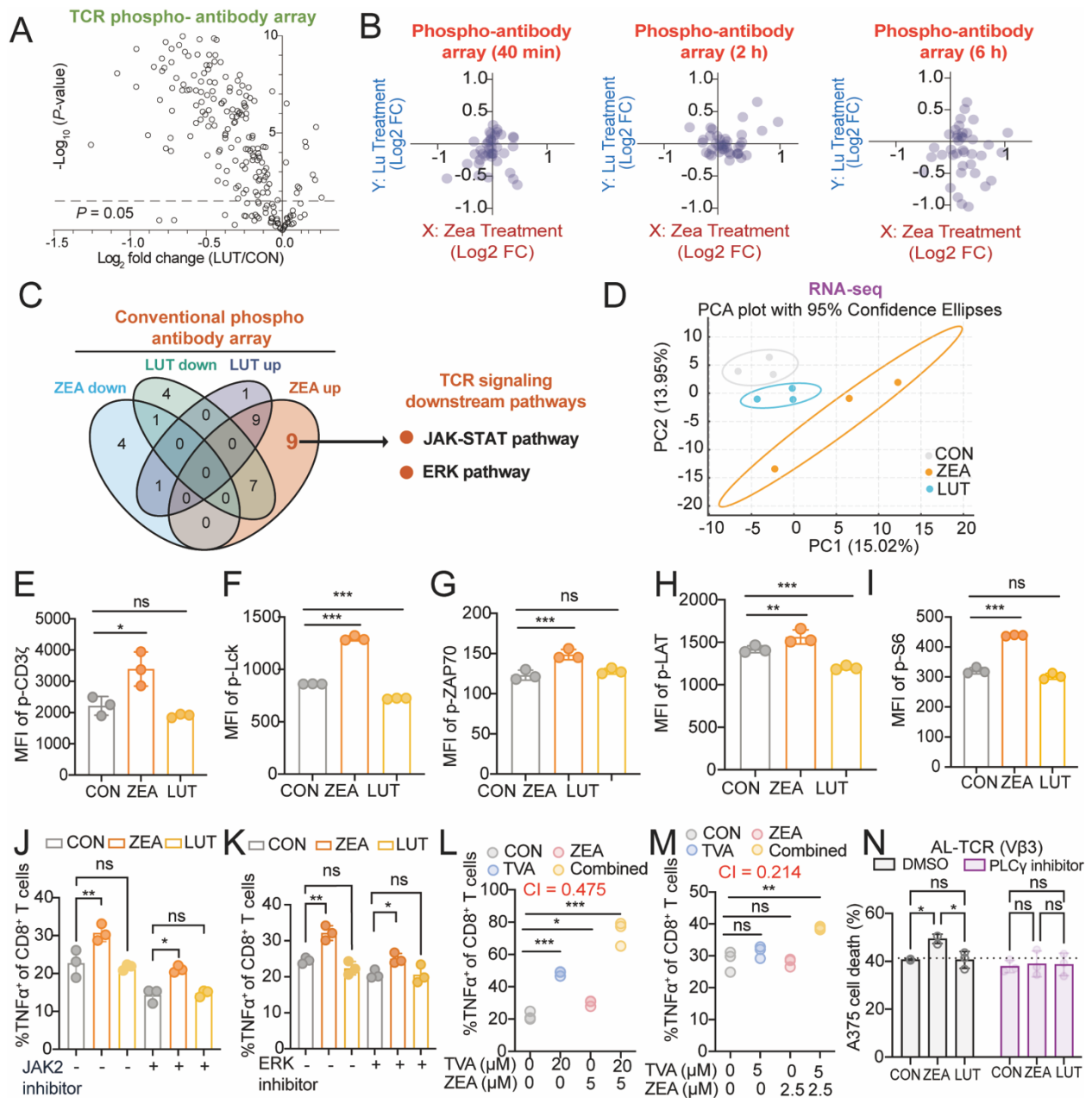


Figure 2.8 ZEA reprograms mouse primary CD8 $^{+}$ T cells by enhancing TCR signaling.

(A) Volcano plot showing the effects of 5 min LUT treatment on TCR-related protein phosphorylation.

(B) Volcano plot showing fold changes of all protein phosphorylation from conventional phosphor-antibody array experiment analyzed by relative pixel density after 40 min, 2 h, and 6 h of ZEA or LUT treatment versus DMSO control treatment.

(C) Venn diagram showing changes ($\geq 20\%$) in protein phosphorylation analyzed by relative pixel density after 6 h of ZEA or LUT treatment versus DMSO control treatment. TCR downstream signaling pathways upregulated by ZEA treatment were listed.

Figure 2.8 ZEA reprograms mouse primary CD8⁺ T cells by enhancing TCR signaling (Cont'd).

(D) PCA plot showing the clustering of RNA-seq samples based on gene expression profiles. The x-axis (PC1) and y-axis (PC2) represent the first two principal components, respectively. Each point represents an individual sample, with different colors. Clustering patterns highlight similarities and differences in transcriptomic profiles among groups.

(E) Flow cytometry analysis showing the effect of 5 min ZEA or LUT (5 μ M) treatment on murine CD8⁺ T cell CD3 ζ phosphorylation ($n = 3$).

(F) Flow cytometry analysis showing the effect of 5 min ZEA or LUT (5 μ M) treatment on murine CD8⁺ T cell Lck phosphorylation ($n = 3$).

(G) Flow cytometry analysis showing the effect of 5 min ZEA or LUT (5 μ M) treatment on murine CD8⁺ T cell ZAP70 phosphorylation ($n = 3$).

(H) Flow cytometry analysis showing the effect of 5 min ZEA or LUT (5 μ M) treatment on murine CD8⁺ T cell LAT phosphorylation ($n = 3$).

(I) Flow cytometry analysis showing the effect of 4 h ZEA or LUT (5 μ M) treatment on murine CD8⁺ T cell S6 ribosomal protein phosphorylation ($n = 3$).

(J) Effect of JAK2 inhibitor AZD1480 (500 nM) treatment on ZEA-dependent murine CD8⁺ T cell effector function assessed by TNF α levels ($n = 3$).

(K) Effect of ERK inhibitor U0126 (100 μ M) treatment on ZEA-dependent murine CD8⁺ T cell effector function assessed by TNF α levels ($n = 3$).

(L) Effect of TVA (20 μ M) and ZEA (5 μ M) combined treatment on murine CD8⁺ T cell effector function assessed by TNF α levels ($n = 3$).

(M) Effect of TVA (5 μ M) and ZEA (2.5 μ M) combined treatment on murine CD8⁺ T cell effector function assessed by TNF α levels ($n = 3$).

(N) Effect of PLC γ inhibitor 3-Nitrocoumarin (500 nM) treatment on ZEA-dependent human AL-TCR-T cell-mediated cytotoxicity against A375 cells ($n = 3$).

Data are mean $\pm$ s.d. P values are calculated using Two-way ANOVA (J-N), or One-way ANOVA (E-I), (ns, not significant; *0.01 < p < 0.05; **0.001 < p < 0.01; *** p < 0.001).

2.3 Discussion

Our study reveals carotenoid nutrient ZEA as a novel immunomodulator that enhances CD8⁺ T cell effector function and boosts antitumor immunity both *in vitro* and *in vivo*. By amplifying TCR stimulation and signaling, ZEA strengthens CD8⁺ T cell activation and cytotoxicity, enhancing immune responses against cancer cells. ZEA further reprograms CD8⁺ T cells by potentiating through TCR signaling to improve intracellular calcium signaling and activate the NFAT pathway, highlighting an unexpected immunomodulatory role of ZEA that can directly influence immune cell physiology. Together with earlier discoveries of immunomodulatory nutrients like TVA, our studies offer a new perspective on how nutrients impact distinct immune pathways.

While it is well-established that diet has a huge impact on human health, including immune responses to cancer, our understanding of the “how” is very limited due to the complexity of the diet and the immune system. At a molecular level, nutrients were traditionally seen as metabolic fuels that supports biosynthesis and energy production. But that view is changing. There’s growing evidence of nutrients acting as signaling molecules that directly influence cellular behavior. This new paradigm suggests that diet can shape immunity not just passively through metabolism but also actively as regulators. However, the vast majority of dietary nutrients haven’t been examined for potential immunoregulatory roles. While only a handful of dietary nutrients have defined immunoregulatory roles, our current understanding of the diet-immune interaction is still fragmented.

Due to the complexity of the human diet, our approach is building a circulating blood nutrient library with nutrients that are known to circulate in the bloodstream at detectable levels. Therefore, circulating nutrients are physiologically relevant because they are naturally bioavailable.

These nutrients are stable in circulation and likely to reach immune cells in distal organs. And the fact that they persist in the blood suggests that they may have evolved bioactive or signaling functions. By focusing on circulating nutrients, we increase the chances that any candidates we identify will be both mechanistically interesting and also translationally relevant. Our approach enables an unbiased and systemic way of identifying novel bioactive nutrients.

For our cell-based screening, we used 2x the physiological concentration reported in the human metabolome database (www.hmdb.ca). This concentration was chosen to capture modest effects that might be missed at baseline levels and to increase the likelihood of detecting biological activity without exceeding a range that is considered biologically relevant or safe. Following this, we performed a series of dose-response experiments to determine the optimal concentration for further *in vitro* studies.

Current studies on ZEA have primarily focused on its antioxidant properties and roles in ocular health or direct killing of tumor cells, however, its physiological relevance in immune modulation remains underexplored. Our mechanistic study for the first time linked ZEA with TCR signaling and effector functions against tumor cells, helping to fill critical knowledge gaps on the biological and regulatory activities of ZEA.

ZEA and LUT as polar carotenoids can insert into plasma membranes and alter the membrane fluidity [18,19]. Future studies are warranted to elucidate the potential effects of ZEA on plasma membrane properties that may contribute to alteration of TCR clustering on the cell surface of CD8⁺ T cells, leading to enhanced formation of TCR complex and immune synapse formation. It will also be crucial to determine the structural basis that distinguishes the distinct bioactivity of ZEA and LUT to promote cytotoxicity of CD8⁺ T cells, despite their structural similarity. Our studies on several natural homologs of ZEA revealed that the antioxidant properties

of conjugated double bonds in their long chains are dispensable in terms of enhancing cytotoxicity of CD8⁺ T cells. The unique structure of ZEA with symmetrical conjugated polyene and two specially arranged six-membered rings at both ends is vital for its bioactivity to promote CD8 T cell activation. Note that D5 (fucoxanthin) has more potent effects than ZEA to enhance TNF- α production by CD8⁺ T cells. Fucoxanthin is also a plant-produced carotenoid nutrient found in marine products such as seaweed, which has anti-cancer effects [37, 38]. Future studies are warranted to further evaluate fucoxanthin as a natural, dietary nutrient for its translational potential to enhance CD8⁺ T cell function and related immunity *in vivo*. Future detailed structure-activity relationship (SAR) studies on ZEA and fucoxanthin will provide insights into the design and development of new derivatives with improved potency for CD8⁺ T cell activation and for optimizing efficacy of T cell-based therapies as a dietary supplement.

Given ZEA's dietary origin, it is important to consider the role of the gut microbiota in mediating ZEA's *in vivo* effects. Our data showed no significant changes in the microbial composition following oral gavage of ZEA. This suggests that ZEA does not reshape the microbiota at the compositional level to affect anti-tumor immunity. However, this does not preclude the possibility that microbial metabolism may still influence ZEA's bioactivity through more subtle functional or metabolomic changes.

As a lipid-soluble carotenoid, ZEA's relatively low bioavailability in the bloodstream presents a challenge for its therapeutic application. To enhance its therapeutic potential, future studies could benefit from developing derivatives or delivery systems to improve ZEA's bioavailability and absorption. Thus, leveraging SAR studies and formulation strategies (e.g., lipid nanoparticles) may improve ZEA's biological efficacy.

Our findings suggest that ZEA preferentially modulate CD8⁺ T cell with minimal functional enhancement in CD4⁺ T cells. This selectivity raises important mechanistic questions. One potential explanation lies in the differences in coreceptor and TCR affinity. CD8⁺ T cells typically engage peptide-MHC I complexes with higher affinity, possibly leading to more responsive and better enhancement of downstream pathways. On the other hand, CD4⁺ T cells rely on MHC II and may have different thresholds or feedback loops that render them less susceptible to ZEA modulation. To further understand this specificity, future studies would examine how ZEA impacts downstream signaling cascades in CD4⁺ T cell population and its subsets. It's possible that ZEA enhances signaling strength or duration in CD8⁺ T cells but not in CD4⁺ counterparts due to differences in coreceptor usage and thus signaling architecture.

To examine ZEA's effect under distinct immunological landscapes *in vivo*, we employed two tumor models with different immune profiles: B16F10 melanoma and MC38 colorectal carcinoma. B16F10 tumors are poorly immunogenic, characterized by low CD8⁺ T cell tumor infiltration and limited immune responsiveness. MC38 tumors are highly immunogenic and has better CD8⁺T cell infiltration and a more inflamed TME. Remarkably, ZEA oral gavage effectively attenuate tumor growth in both contexts, suggesting that its efficacy is not constrained by tumor type or immune context. These findings highlight ZEA's potential as a broadly effective immunomodulatory compound.

The synergy observed between ZEA and anti-PD-1 therapy further underscores ZEA's translational potential to overcome immune resistance and enhance immunotherapy outcomes. A compelling future direction would be to examine whether ZEA enhances anti-tumor immunity by reinvigorating exhausted CD8⁺ T cells or expanding the pool of tumor-reactive T cells within the TME.

ZEA's ability to improve cancer cell killing in human TCR-T co-culture experiments suggests its potential for optimizing adoptive T cell therapies. These *in vitro* findings warrant future studies using preclinical mouse models as well as in other cellular therapies such as CAR-T cell therapies. However, given that CAR-T cells do not rely on the endogenous TCR complex, the extent to which ZEA can modulate their function remains uncertain. Future directions should include testing ZEA in additional human tumor cell lines with diverse antigen profiles and evaluating its impact on CAR-T cell therapy.

Furthermore, the discovery of ZEA fills a critical gap in our understanding of nutrient-immune interactions and highlights the potential of targeting specific nutrient pathways to enhance anti-tumor immunity. While ZEA and TVA function through distinct immune mechanisms, their integration may offer synergistic therapeutic potential and provide a foundation for rational nutrient-based combination strategies to amplify immunotherapy outcomes.

Lastly, the significance of this discovery goes beyond cancer immunotherapy. By regulating critical signaling pathways of CD8⁺ T cells, ZEA could have broader applications in managing autoimmune diseases, infectious diseases, and other conditions that require immune modulation. This work emphasizes the regulatory significance of a plant-based circulating nutrient and the specific nutrient-immune interactions that can fine-tune immune responses.

2.4 Material and Methods

Mice

Animal experiments were conducted and designed according to protocols approved by the Institutional Animal Care and Use Committee of The University of Chicago. C57BL/6 mice (6-8 weeks), Pmel-1 mice (6-8 weeks), and OT-I mice (6-8 weeks) were obtained from The Jackson Laboratory. Mice were housed in a temperature (23-25 °C) and humidity-controlled colony room, maintained on a 12-h light/dark cycle (08:00 to 20:00 light on) with standard food and water provided ad libitum and environmental enrichments in a pathogen-free facility.

Primary cells

Mouse naïve CD8⁺ T cells were isolated from the spleen of C57BL/6 mice (6-8 weeks), Pmel-1 mice (6-8 weeks), or OT-I mice (6-8 weeks) by magnetic bead purification using EasySep™ Mouse naïve CD8⁺ T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. Naïve CD8⁺ T cells were activated in vitro for 18 hours with plate-bound anti-mouse CD3 (1µg/mL; Biolegend) and soluble anti-mouse CD28 (0.5 µg/mL; Biolegend) antibodies in complete Click's media (Fujifilm Irvine Scientific) at 37°C and 5% CO₂ incubator for further experiments.

Human naïve CD8⁺ T cells were isolated by magnetic bead purification using EasySep™ Human naïve CD8⁺ T Cell Isolation Kit (Stemcell Technologies) from peripheral blood mononuclear cells (PBMCs) obtained via Ficoll gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare) from whole blood. Human naïve CD8⁺ T cells were activated in vitro for 18 hours with plate-bound anti-human CD3 (1µg/mL; Biolegend) and soluble anti-human CD28 (0.5

µg/mL; Biolegend) antibodies in Click's media (Fujifilm Irvine Scientific) at 37°C and 5% CO₂ incubator for further experiments.

Human CD8⁺ untransduced mock T cells, 19305DP TCR-T cells, and AL-TCR-T cells were provided by Dr. Kunle Odunsi. Mock and transduced TCR-T cells were cultured in RPMI 1640 medium at 37°C and 5% CO₂ incubator for further experiments.

Cell lines

All cell lines were authenticated by genomic short tandem repeat (STR) profiling at the University of Chicago Integrated Genomics Core (EIGC) upon purchase and at least annually as appropriate.

Mouse melanoma cell line B16F10-luc2 and mouse melanoma cell line B16F10 were purchased from ATCC. Mouse melanoma cell line B16-OVA was provided by Dr. Hongbo Chi. Mouse colorectal adenocarcinoma cell line MC38 was purchased from Kerafast. B16F10 and MC38 cells were cultured in Dulbecco Modified Eagle Medium (DMEM). B16F10-luc2 and B16-OVA were cultured in the Click's media (Fujifilm Irvine Scientific) prior to co-culture experiments to assess murine T cell-mediated cytotoxicity. Human melanoma cell line A375 was provided by Dr. Kunle Odunsi. Human melanoma A375 cells were cultured in RPMI 1640 medium prior to co-culture experiments to assess TCR-T cell-mediated cytotoxicity. All the cells were cultured at 37°C and 5% CO₂. Cell line experiments were conducted and designed according to protocols approved by the Institutional Biosafety Committee (IBC) of the University of Chicago.

Effector T cell cytotoxicity screen

T cells were isolated from Pmel-1 mouse by magnetic bead purification using EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies) and seeded at a density of 5×10^6 cells/well in a 6-well plate pre-coated with anti-mouse CD3 (Biolegend) and anti-mouse CD28 (Biolegend) for overnight stimulation. 5×10^5 stimulated Pmel-1 cells were then co-cultured with 1×10^5 B16F10-luc2 cells (E:T = 5:1) and treated with each of the compounds in the blood nutrient library for 24 hours. Bright-Glo Luciferase Assay System (Promega) was used to measure luminescence level as a readout for B16F10-luc2 cell death. Luminescence was measured on the BioTek Synergy H4 Hybrid Reader.

Co-culture cytotoxicity experiments

To determine mouse OT-I T cell-mediated tumor cytotoxicity, OT-I T cells were isolated using EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies) and seeded at a density of 5×10^6 cells/well in 6-well plate (pre-coated with anti-CD3 and anti-CD28) for overnight stimulation. B16-OVA cells were stained with CellTracker Deep Red dye (Invitrogen) according to manufacturer's instruction prior to co-culturing with OT-I T cells. 5×10^5 stimulated OT-I cells were then co-cultured with pre-stained 1×10^5 B16F10-OVA cells (E:T = 5:1) and treated with DMSO control, 5 μ M ZEA (Sigma-Aldrich) or 5 μ M LUT (Sigma-Aldrich) for 24 hours. B16-OVA cells alone were treated under the same conditions. All cells were collected, stained with PI, and analyzed by flow cytometry.

To determine human TCR-T cell-mediated tumor cytotoxicity, human TCR-T cells were co-cultured at a density of 3×10^5 cells/well (E:T = 3:1) with 1×10^5 human melanoma A375 cells and treated with or without 5 μ M ZEA (Sigma-Aldrich) or LUT (Sigma-Aldrich) for 24 hours. A375 cells

alone were treated at the same conditions. All cells were collected, stained with Annexin V-APC and propidium iodide (PI), and analyzed by flow cytometry.

Mouse models

For C57BL/6 mice tumor models, mice were anesthetized with isoflurane, shaved at the injection site, and then injected subcutaneously in the abdominal flank with 2×10^5 B16F10 or MC38 cells for tumor development. Daily oral gavage of ZEA, LUT, or vehicle control (PBS+1% Tween-80) was administered once tumors reached a size of 100 mm³. Mice's body weight and tumor size were measured every other day. Tumor sizes were measured using a caliper, and tumor volumes were calculated using the following formula: tumor volume = (Length x Width²)/2. Mice were euthanized at humane endpoints or on days 15-16 for tissue collection.

For antibody-mediated T cell depletion models, C57BL/6 mice (6-8 weeks) were injected subcutaneously in the abdominal flank with 2×10^5 B16F10 cells and then injected intraperitoneally with six doses of depleting antibodies (anti-CD8a, BioXCell) or isotype control (rat IgG2b isotype control, BioXCell) on day 1 (200 µg), 2 (200 µg), 4 (200 µg), 8 (200 µg), 12 (200 µg) and 16 (200 µg) relative to tumor injection (day 0). Depletion efficiency was assessed by staining for CD8 (Biolegend) or CD4 (Biolegend) and flow cytometry analysis. Tumor sizes were measured using a caliper and tumor volumes were calculated using the following formula: tumor volume = (Length x Width²)/2. Mice were euthanized at humane endpoints.

For the anti-PD-1 treatment mouse model, C57BL/6 mice (6-8 weeks) were injected subcutaneously in the abdominal flank with 2×10^5 B16F10 cells for tumor development. Daily oral gavage of ZEA or vehicle control (PBS+1% Tween 80) was given to tumor-bearing mice after tumors became palpable. On days 3, 6, 9, 12, and 15, 250 µg anti-PD-1 (BioXCell) or IgG control

(BioXCell) was injected intraperitoneally per mouse. Tumor sizes were measured using a caliper and tumor volumes were calculated using the following formula: tumor volume = (Length x Width²)/2. Mice were euthanized at humane endpoints.

CD45⁺ tumor-infiltrating leukocyte isolation

Tumor tissues were dissected from euthanized tumor-bearing mice, minced into small pieces (≤ 2 mm) using a scalpel in a dish, and then transferred to a 14 mL round-bottom tube containing 5 mL tumor digestion medium (500 μ L Collagenase/Hyaluronidase Solution, 750 μ L 1 mg/mL DNase I Solution, and 3.75 mL RPMI 1640 Medium). After incubation at 37°C for 25 min on a shaking platform, the digested tumor tissues were transferred into a 70 μ m mesh nylon strainer on a 50 mL conical tube, pushed through the strainer using the rubber end of a syringe plunger, and rinsed with the recommended medium. After centrifugation at 300 $\times$ g for 10 min at room temperature with the brake on low, the resulting cell pellets were added 10 mL of ammonium chloride solution for incubation at room temperature for 5 minutes, followed by centrifugation at 300 $\times$ g for 10 minutes at room temperature with the brake on low. The resulting cell pellets were re-suspended at $1-10 \times 10^6$ cells/mL in PBS and then subjected to CD45⁺ tumor-infiltrating leukocyte isolation.

Mouse Spleen lymphocyte isolation

Mouse spleens were disrupted with a syringe plunger in a 70 μ m strainer, filtered to a 50 mL tube, washed with PBS, and centrifuged at 300 $\times$ g for 10 minutes. The resulting cell pellets were re-suspended with 2 mL red cell lysis buffer (Invitrogen), incubated at room temperature for 10 minutes, and centrifuged at 300 $\times$ g for 5 minutes after adding 15 mL PBS. The resulting

cell pellets were designated splenocytes and used for the following experiments. For cytokine detection, T cells were stimulated with a cell activation cocktail (Biolegend) for 4hr, and then intracellular staining for cytoplasmic protein was performed, followed by flow cytometry analysis.

Mouse draining lymph node lymphocyte isolation

Draining lymph nodes were disrupted with a syringe plunger in a 70 μ m strainer filtered to a 50 mL tube, washed with PBS, and centrifuged at 300 $\times$ g for 10 minutes. The resulting cell pellets were designated dLN lymphocytes and used for the following experiments.

Flow cytometry analysis

Murine primary cells isolated from tumor, spleen, draining lymph node were stained with fluorescent antibodies and analyzed by flow cytometry.

For experiments with live/dead criteria, cells were first stained with Fixable Viability Dyes (FVD) (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequent surface marker staining was performed in Cell Staining Buffer (Thermo Fisher Scientific).

Intracellular staining for flow panels containing nuclear proteins was performed using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. For intracellular staining of cytoplasmic proteins, eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) was used according to the manufacturer's instructions.

For phospho-antibody staining, cells were incubated with FVD (cell viability dye) for 15 min at room temperature in a tube, re-suspended with 200 μ l pre-warmed 1 $\times$ BD Phosflow

Lyse/Fix buffer directly into the tube, and incubated at 37 °C for 10–15 min, followed by centrifugation at 300g for 5 min. The resulting cell pellets were washed once with FACS buffer, permeabilized with 200 µl of BD Phosflow Perm Buffer III for 45 min on ice, and centrifuged at 300g for 5 min. The cell pellets were washed again with FACS buffer, centrifuged at 300g for 5 min, and incubated with antibodies in FACS buffer for 45 min-1 hour at room temperature.

Mouse anti-CD11b antibody was used for the myeloid cell (CD11b⁺) marker. After gated with CD11b⁺ cells, anti-F4/80 and Gr1 antibodies were used for macrophage (Gr1⁺F4/80⁺) markers, the anti-CD11c antibody was used for dendritic cells (CD11c⁺) marker, anti-CD16, and CD63 antibodies were used for neutrophils (CD16⁺CD63⁺) markers, the anti-CD14 antibody was used for monocytes (CD14⁺) marker. For CD4 subtypes analysis, CD3⁺ cells were gated within the CD45⁺ population, and CD4⁺ and CD8⁺ cells were subsequently identified. For CD45⁺CD4⁺ cells, further analysis was performed to identify subtypes: Treg cells were identified as Foxp3⁺ CD25⁺ within the CD45⁺CD4⁺ population. In the Foxp3⁻ (Treg⁻) population, Th1 cells were gated as IFNγ⁺, Th2 cells as IL4⁺, Th17 cells as RORγt⁺, Th9 cells as IL9⁺, and Th22 cells as AhR⁺.

Data were collected with LSR-Fortessa 4-15, Attune NxT 4-14, Aurora Red/Blue, or Agilent NovoCyte Quanteon flow cytometer and analyzed using FlowJo v10.8.1.

Cell culture treatment

For all *in vitro* ZEA (Sigma-Aldrich) or LUT(Sigma-Aldrich) treatments, ZEA or LUT were added to the cell culture in the dark, and the cell culture plates were covered with aluminum foil to avoid light throughout the duration of incubation.

Murine naïve CD8⁺ T cells were isolated from C57BL/6 mice, stimulated, and subjected to the following treatments. For *in vitro* inhibitor treatment, stimulated murine CD8⁺ T cells were

pre-treated with LCK inhibitor PP2 (200 nM), PLC γ inhibitor 3-Nitrocoumarin (500 nM), calcineurin inhibitor cyclosporin A (7 nM), NFAT inhibitor VIVIT (1 μ M), NF- κ B inhibitor INH14 (2 μ M), JAK2 inhibitor AZD1480 (500 nM), or ERK inhibitor U0126 (100 μ M) for 1-2 hours, followed by treatment with 5 μ M ZEA (Sigma-Aldrich), 5 μ M LUT (Sigm-Aldrich), or DMSO control for 24 hours. Cells were then stained with FVD and treated with Cell Activation Cocktail (with Brefeldin A) (Biolegend) for 4-6 hours before intracellular staining to detect pro-inflammatory cytokine levels.

For *in vitro* derivative treatment, stimulated murine CD8⁺ T cells were treated with 5 μ M ZEA (Sigm-Aldrich), or 5 μ M of each of the derivatives D1-6 for 24 hours before intracellular staining to detect pro-inflammatory cytokine levels.

Intracellular calcium detection with Fluo4 calcium indicator

Murine naïve CD8⁺ T cells were isolated from C57BL/6 mice and loaded with fluo-4 using Fluo-4 Direct Calcium Assay Kit (ThermoFisher Scientific) according to manufacturer's instructions. Loaded cells were then stimulated with soluble anti-CD3 ϵ (1 μ g/ml; Clone:145-2c11) and anti-CD28 (0.5 μ g/ml; Clone: 37.51) antibodies, and simultaneously treated with 5 μ M ZEA (Sigma-Aldrich), 5 μ M LUT (Sigm-Aldrich), or DMSO control for 30 min, followed by flow cytometry analysis.

TCR complex analysis with flow cytometry and confocal microscopy

Murine naïve CD8⁺ T cells were isolated from C57BL/6 mice. For short-term stimulation conditions, murine naïve CD8⁺ T cells were stimulated with anti-CD3 ϵ -FITC (1 μ g/ml; Clone:145-2c11) and anti-CD28 -PE (0.5 μ g/ml; Clone: 37.51) antibodies, and simultaneously treated with

5 μ M ZEA (Sigma-Aldrich), 5 μ M LUT (Sigma-Aldrich), or DMSO control for 5 min, 15 min, or 30 min. For overnight pre-stimulation conditions, murine naïve CD8⁺ T cells were stimulated with anti-CD3 ϵ -FITC (1 μ g/ml; Clone:145-2c11) and anti-CD28 -PE (0.5 μ g/ml; Clone: 37.51) antibodies and incubated overnight in Click's medium at 37°C and 5% CO₂ before centrifugation and treating anti-CD3 ϵ -FITC (1 μ g/ml; Clone:145-2c11) and anti-CD28 -PE (0.5 μ g/ml; Clone: 37.51) antibodies again simultaneously with 5 μ M ZEA (Sigma-Aldrich), 5 μ M LUT (Sigma-Aldrich), or DMSO control for 5 min, 15 min, or 30 min.

For confocal microscopy analysis, cells were collected and stained with anti-TCR β -APC antibody, fixed and stained with DAPI (1 μ g/ml), and subject to analysis using Leica SP8 Confocal Microscope. For flow cytometry analysis, cells were collected and stained with FVD anti-CD8-BV421 and anti-TCR β -APC antibodies, followed by flow cytometry analysis.

Kethoxal-assisted single-stranded DNA sequencing (KAS-seq) and data analysis

Primary murine CD8⁺ T cells were isolated from the spleens of 8-week-old female C57BL/6 mice using the EasySep™ Mouse Naïve CD8⁺ T Cell Isolation Kit (STEMCELL Technologies, 19858). A total of 10,000,000 primary Naïve CD8⁺ T cells were activated overnight with α CD3/CD28 antibodies, followed by treatment with 5 μ M ZEA for 40 minutes or 2 hours. DMSO-treated cells served as controls. Each condition included three biological replicates. Samples for KAS-seq were processed following the protocol described previously with minor modifications [45]. Cells were lysed using a lysis buffer containing 0.1% NP40, 0.1% Tween-20, 0.01% Digitonin, 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂, and incubated on ice for 3 minutes. The lysed cell pellets were resuspended in 100 μ L of 10 mM N3-Kethoxal in PBS and incubated at 37°C with shaking at 500 rpm for 10 minutes. Cells were then collected for

genomic DNA (gDNA) purification using the PureLink Genomic DNA Mini Kit (Invitrogen). Approximately 1 μ g of purified gDNA in a 100 μ L reaction volume was incubated with 1 mM DBCO-PEG4-biotin (Sigma) in PBS at 37°C for 1.5 hours with gentle shaking to achieve biotinylation. Biotinylated gDNA was further purified using the DNA Clean & Concentrator-5 kit (Zymo Research) and fragmented to approximately 250 bp by sonication. Biotinylated DNA fragments were captured using 10 μ L of Dynabeads MyOne Streptavidin C1 (Thermo Fisher) following the manufacturer's instructions. DNA was eluted from the beads by heating in 15 μ L of H₂O at 95°C for 10 minutes. Library preparation was performed using the xGen Methyl-Seq DNA Library Prep Kit (IDT), and the final libraries were sequenced on the Illumina NovaSeq 6000 platform.

For bioinformatics analysis, we used the Trim Galore package to trim low-quality sequences, adapter sequences, and primer sequences from paired-end KAS-seq raw FASTQ files. Reads shorter than 30 bp were discarded. The remaining reads were aligned to the reference genome (mm10) using Bowtie2 (v2.3.3.1) with the alignment carried out under default parameters. The SAM files were then converted and sorted into BAM files using SAMtools sort (v1.9) [46, 47]. Duplicate reads were removed with the assistance of Picard MarkDuplicates (v1.141), and the uniquely mapped reads were extended to 150 bp to match the average length of DNA fragments, using the awk command.. The extended data, in BED file format, were converted to BedGraph files using bedtools genomecov, and subsequently transformed into BigWig files through bedGraphToBigWig. Shell scripts for KAS-seq data mapping and quality control can be accessed at GitHub (<https://github.com/Ruitulyu/KAS-Analyzer>). For peak calling, MACS2 was utilized to identify broad KAS-seq peaks, and 2kb bins overlapping with KAS-seq peaks were used to detect differentially generated time-course KAS-seq signals. Promoters were defined as regions spanning

1kb upstream and 1kb downstream of the transcription start site (TSS), while gene bodies were defined from 1kb downstream of the TSS to the transcription end site (TES). Differential analysis of time-course KAS-seq data at promoter and gene body regions was performed using DESeq2 software [48]. KEGG pathway enrichment analysis was performed using the DAVID platform.

T-Cell Receptor (TCR) phospho-antibody array

Murine naïve CD8⁺ T cells were isolated, stimulated with soluble anti-CD3e and anti-CD28, then immediately treated with DMSO control, 5 µM ZEA or 5µM LUT for 5 min followed by TCR-related phosphoprotein profiling using T-Cell Receptor Phospho Antibody Array kit (Full Moon BioSystem) according to the manufacturer's instructions.

Conventional phospho-antibody array

Murine naïve CD8⁺ T cells were isolated, stimulated overnight, and then treated with DMSO control, 5 µM ZEA or 5µM LUT for 40 min, 2 hours, or 6 hours, followed by phosphoprotein profiling using Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, ARY003C) according to the manufacturer's instructions. Analysis was performed using Fiji for relative pixel density comparison.

16S rRNA sequencing

Feces were collected from C57BL/6 mice (6-8 weeks) orally administered with ZEA (500 mg/kg b.w.) or vehicle control (PBS + 1% Tween 80) daily for 3 weeks (10 samples per group), and then subjected to microbiome 16S sequencing by Zymo Research (Irvine, CA). In brief, The ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research) was used to extract DNA using an

automated platform. Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research). The bacterial 16S primers amplified the V3-V4 region of the 16S rRNA gene. The sequencing library was prepared using an innovative library preparation process in which PCR reactions were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research), then quantified with TapeStation® (Agilent Technologies) and Qubit® (Thermo Fisher Scientific). The ZymoBIOMICS® Microbial Community Standard (Zymo Research) was used as a positive control for each DNA extraction, if performed. The final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in. For Bioinformatics Analysis, unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline [49]. Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 [50].

RNA-Seq and data analysis

Spleens were harvested from 8-week-old female C57BL/6 mice, and Naïve CD8⁺ T cells were isolated using the EasySep™ Mouse Naïve CD8⁺ T Cell Isolation Kit (STEMCELL Technologies, 19858). For each sample, a total of 6,000,000 primary Naïve CD8⁺ T cells were

activated overnight with α CD3/CD28 antibodies, followed by treatment with 5 μ M ZEA, 5 μ M LUT, or DMSO for 24 hours ($n=3$). After treatment, cells were collected and washed once with PBS, followed by RNA extraction using RNeasy Mini Kit according to the manufacturer's instructions. Library preparation and sequencing were performed by Novogene Co, Ltd (Sacramento, California). RNA samples with RIN>7.5 were used for Illumina Next Generation Sequencing. Alignment was performed by STAR software⁴⁰ with the reference genome being mm10. Post-alignment quantification was performed using featureCounts software [51]. Differential gene analysis was conducted using DESeq2, with multiple testing corrections applied using FDR [48]. Enrichment analysis was carried out using clusterProfiler 4.0, and gene set enrichment analysis was performed using GSEA 4.3.2. [52, 53].

TIF Zeaxanthin and Lutein detection by Liquid Chromatography-Mass Spectrometry (LC-MS)

To 5 μ L of Tumor interstitial fluid (TIF) and plasma sample, 225 μ L of ice-cold methanol containing 0.1mg/mL butylated hydroxytoluene and 750 μ L of methyl-tert-butyl ether were added followed by mixing for 15 min at 500 rpm and 15°C. The phase separation was induced by adding 225 μ L of ice-cold water and a vortex for 15 min at 15°C and 500 rpm then samples were spun down at 18,000g at 15°C for 15 min. The upper 650 μ L of organic layer was dried using the Genevac EZ 2.4 elite evaporator. The dried samples were re-suspended in 50 μ L of isopropanol/acetonitrile, vortexed for 15 min at 15°C and 2000 rpm, and centrifuge at 18,000g at 15°C for 15 min before collecting the supernatant in the amber color LC-MS vials. The exposure to lights were minimized during the extraction process. Zeaxanthin and Lutein were separated on the BEH C18 column (Waters Corporation, 2.1x150 mm, 2.5 μ m) connected to a Vanquish

Horizon UHPLC system and IQ-X tribrid mass spectrometer. The column temperature, injection volume, and flow rate were 45°C, 5 µL, and 0.4 mL/min, respectively. The mobile phase A (MPA) was 60/40 acetonitrile/water, 10 mM ammonium formate+0.1% formic acid and MPB was 89.1/9.9/0.99 isopropanol/acetonitrile/water, 10 mM ammonium formate +0.1% formic acid. The chromatographic gradient was 0 min: 10%B, 2.00 minutes (min): 10% B, 9 min: 100%B, 10.50 min: 100%B, 11.00 min: 10%B, 14.00 min: 10%B. The MS parameters were as follows: spray voltage: 3600 V for positive ionization, sheath gas: 40, auxiliary gas: 10, sweep gas: 1, ion transfer tube temperature: 300°C, vaporizer temperature: 350°C, RF lens(%): 60. The isobaric compounds, zeaxanthin, and lutein were isolated by quadrupole and targeted MS/MS fragmentation was performed with the normalized HCD energy(%):20, maximum injection time of 54 milliseconds and normalized AGC target (%): 50 followed by detection in orbitrap analyzer at 30K resolution. The data acquisition was performed using the Thermo Scientific Xcalibur and data analyses using the Tracefinder 5.1. Zeaxanthin and lutein are chromatographically separated and have distinct fragmentation patterns: Zeaxanthin 568.4264 m/z → 476.3642m/z (Quantifier) and lutein 568.4266 m/z → 338.2601m/z. The retention time and fragmentation patterns from the TIF/serum samples were matched with the respective reference standards.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism 8 and 9 or using the software indicated above. Details of statistical analysis are described in the figure legends or in the STAR Methods section above.

Table 2.1 List of antibodies

REAGENT	SOURCE	IDENTIFIER
Rat anti-IgG2b isotype	BioXCell	Cat#BE0090; Clone#LTF-2; RRID:AB_1107780.
Mouse anti-CD8 α	BioXCell	Cat#BE0061; Clone#2.43; RRID:AB_1125541.
Mouse anti-PD-1(CD279)	BioXCell	Cat#BE0146; Clone# RMP1-14; RRID:AB_10949053.
Mouse anti-CD4	BioXCell	Cat#BE0288; Clone# GK1.5; RRID:AB_2687811
Mouse FITC anti-CD3 ϵ Antibody	Biolegend	Cat#100305; Clone#145-2C11; RRID:AB_312670.
Mouse PE anti-CD28 Antibody	Biolegend	Cat#102105; Clone#37.51; RRID:AB_312870.
Mouse APC anti-TCR β Antibody	Biolegend	Cat#109211; Clone#H57-597; RRID:AB_313434.
Human FITC anti-TNF- α Antibody	Biolegend	Cat#502906; Clone#MAb11; RRID:AB_315258.
Human APC anti-IL-2 Antibody	Biolegend	Cat#310605; Clone#W19046A; RRID:AB_3083299.
Human PE anti- IFN- γ Antibody	Biolegend	Cat#383405; Clone#W19227C; RRID:AB_2924584.
Purified anti-human CD3 Antibody	Biolegend	Cat#317302; Clone# OKT3; RRID:AB_571927.
Purified anti-human CD28 Antibody	Biolegend	Cat#302902; Clone#CD28.2; RRID:AB_314304.
Purified anti-mouse CD3 Antibody	Biolegend	Cat#100202; Clone#17A2; RRID:AB_312659.
Purified anti-mouse CD28 Antibody	Biolegend	Cat#102102; Clone#37.51; RRID:AB_312867.
Human/mouse/rat FITC anti-CD278 (ICOS) Antibody	Biolegend	Cat#313505; Clone#C398.4A; RRID:AB_416329.
Mouse PE/Cyanine5 anti-CD69 Antibody	Biolegend	Cat#104509; Clone#H1.2F3; RRID:AB_313112.
Mouse PE/Cyanine5 anti-CD4 Antibody	Biolegend	Cat#100409; Clone#GK1.5; RRID:AB_312694.
Mouse Brilliant Violet 421™ anti-IL-2 Antibody	Biolegend	Cat#503825; Clone#JES6-5H4; RRID:AB_10895901.
Mouse PE anti-IL-2 Antibody	Biolegend	Cat#503807; Clone#JES6-5H4; RRID:AB_315301.
Mouse APC anti-IL-2 Antibody	Biolegend	Cat#503810; Clone#JES6-5H4; RRID:AB_315304.
Mouse APC anti-CD45.2 Antibody	Biolegend	Cat#109813; Clone#104; RRID:AB_389210.
Mouse APC anti-IFN- γ Antibody	Biolegend	Cat#505810; Clone#XMG1.2; RRID:AB_315404.
Mouse PE anti- IFN- γ Antibody	Biolegend	Cat#505808; Clone#XMG1.2; RRID:AB_315402.
Mouse PerCP/Cyanine5.5 anti-TNF- α Antibody	Biolegend	Cat#506321; Clone#MP6-XT22; RRID:AB_961435.
Mouse FITC anti-TNF- α Antibody	Biolegend	Cat#506304; Clone#MP6-XT22 RRID:AB_315425.
Mouse Brilliant Violet 711™ anti-CD8a Antibody	Biolegend	Cat#100747; Clone#53-6.7; RRID:AB_11219594.

Table 2.1 List of antibodies (Con'd)

Mouse Brilliant Violet 421™ anti-FOXP3 Antibody	Biolegend	Cat#126419; Clone#MF-14; RRID:AB_2565933.
Mouse APC anti-CD3 Antibody	Biolegend	Cat#100235; Clone#17A2; RRID:AB_2561455.
PE anti-RPS6 Phospho (Ser235/Ser236)	Biolegend	Cat#608603; Clone#A17020B; RRID:AB_2750251.
Mouse FITC anti-F4/80 Recombinant Antibody	Biolegend	Cat#157309; Clone#QA17A29; RRID:AB_2876535.
Mouse APC anti-Ly-6G (Gr1) Antibody	Biolegend	Cat#127613; Clone#1A8; RRID:AB_1877163.
Mouse/human APC anti-CD11b Antibody	Biolegend	Cat#101211; Clone#M1/70; RRID:AB_312794.
Mouse PerCP anti-CD11c Antibody	Biolegend	Cat#117325; Clone#N418; RRID:AB_893236.
Alexa Fluor® 647 anti-mouse CD16 Antibody	Biolegend	Cat#158021; Clone#S17014E; RRID:AB_2904300.
PE/Cyanine5 anti-mouse CD28 Antibody	Biolegend	Cat#102108; Clone#37.51; RRID:AB_312873.
Mouse PE/Cyanine7 anti-CD14 Antibody	Biolegend	Cat#123315; Clone#Sa14-2; RRID:AB_10641133.
Mouse/human PE anti-Ki-67 Antibody	Biolegend	Cat#151210; Clone#11F6; RRID:AB_2716008.
Mouse/human PE anti-Phospho-CD247 (CD3zeta) (Tyr142)	Invitrogen	Cat#12-2478-42; Clone#3ZBR4S; RRID:AB_2744700.
PE anti-Lck Phospho (Tyr394)	Biolegend	Cat#933103; Clone#A18002D; RRID:AB_2820203.
PE anti-ZAP70 Phospho (Tyr292) Antibody	Biolegend	Cat#693803; Clone#A16038B; RRID:AB_2686946.
Alexa Fluor® 647 anti-LAT Phospho (Tyr171) Antibody	Biolegend	Cat#946603; Clone#A20005D; RRID:AB_2936765.
PE Mouse anti-S6 (pS235/pS236)	BD Biosciences	Cat#560433; Clone#N7-548; RRID:AB_2827879.
FITC anti-mouse CD3e Antibody	BD Biosciences	Cat#553062; Clone#145-2C11; RRID:AB_394595.
Mouse Alexa Fluor® 647 anti-IL-9 Antibody	Biolegend	Cat#514107; Clone#RM9A4; RRID:AB_2126637.
Mouse BV510 anti-IFN-γ Antibody	Biolegend	Cat#505841; Clone#XMG1.2; RRID:AB_2562187.
Mouse BV605 anti-IL-4 Antibody	Biolegend	Cat#504125; Clone#11B11; RRID:AB_2562101.
Mouse PE anti-Ahr Antibody	Biolegend	Cat#162703; Clone#W16012C; RRID:AB_2892335.
Mouse BV421 anti-RORγt Antibody	BD Biosciences	Cat#562894; Clone#Q31-378; RRID:AB_2687545.
Mouse APC anti-FOXP3 Antibody	Proteintech	Cat#APC-65089; Clone#3G3; RRID:AB_2882984.
Mouse BUV563 anti-CD69 Antibody	BD Biosciences	Cat#741234; Clone#H1.2F3; RRID:AB_2870786.
Mouse BV650 anti-CXCR5 Antibody	Biolegend	Cat#145517; Clone#L138D7; RRID:AB_2562453.

Table 2.2 List of chemicals, peptides, and recombinant proteins

REAGENT	SOURCE	IDENTIFIER
Human IL-2 IS	Miltenyi Biotec	Cat#130-097-743
Mouse IL-2 IS	Miltenyi Biotec	Cat#130-120-662
Mouse IL-7	Thermo Fisher Scientific	Cat#50813299
Mouse IL-15	Thermo Fisher Scientific	Cat#50813273
Fixable viability dye 780	R&D	Cat#65-0865-14
DNase I	Millipore	Cat#EN0521
N-methylpyrrolidone	Sigma-Aldrich	Cat#PHR1352 CAS:872-50-4
TWEEN-80	Sigma-Aldrich	Cat#P1754
Lck inhibitor PP2	Sigma-Aldrich	Cat#529576
PLCy inhibitor 3-Nitrocoumarin	MedChemExpress	Cat#HY-111919
Calcineurin inhibitor Cyclosporine	Sigma-Aldrich	Cat#1158504
NFAT Inhibitor	Cayman Chemical	Cat#13855-1
NF- κ B inhibitor INK4	MedChemExpress	Cat#HY-13453
JAK2 inhibitor AZD1480	MedChemExpress	Cat#HY-10193
ERK inhibitor U0126	MedChemExpress	Cat#HY-12031A
Collagenase/Hyaluronidase	Stemcell Technologies Inc	Cat#07912
Ammonium Chloride Solution	Stemcell Technologies Inc	Cat#07800
Zeaxanthin (CAS:144-68-3)	Sigma-Aldrich	Cat#14681
Zeaxanthin (CAS:144-68-3)	aablocks	Cat# AA00BWRX
Zeaxanthin (CAS:144-68-3)	Wuxi AppTech	Cat#144-68-3
Lutein (CAS:127-40-2)	Wuxi AppTech	Cat# LN01318215
Lutein (CAS:127-40-2)	Sigma-Aldrich	Cat#204-840-0
Lutein (CAS:127-40-2)	Angene Chemicals	Cat#AG000WIY(AGN-PC-0PIJ3S)
β -Carotene (CAS:7235-40-7)	Sigma-Aldrich	Cat#C4582
($\pm$)- α -Carotene (CAS:432-70-2)	Sigma-Aldrich	Cat#74283
Lycopene (CAS:502-65-8)	Sigma-Aldrich	Cat#75051
Astaxanthin (CAS:7542-45-2)	Sigma-Aldrich	Cat#SML0982
Fucoxanthin (CAS:3351-86-8)	Sigma-Aldrich	Cat#16337
Retinoic acid (CAS:302-79-4)	Sigma-Aldrich	Cat#R2625
Ficoll® Paque Plus	Sigma-Aldrich	Cat#GE17-1440-03

Table 2.3 List of commercial assay reagents

REAGENT	SOURCE	IDENTIFIER
Annexin V apoptosis detection kit I	BD Biosciences	Cat#556547
EasySep™ Mouse T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19851
EasySep™ Mouse Naïve CD8+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19858
EasySep™ Mouse Naïve CD4+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19765
EasySep™ Mouse CD8+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19853
EasySep™ Mouse CD4+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19852
EasySep™ Human T Cell Isolation Kit	Stemcell Technologies Inc	Cat#17951
EasySep™ Human CD8+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#17953
Bright-Glo Luciferase Assay System	Promega	Cat# E2620
CaspaseGlo 3/7	Promega	Cat#G8090
Cell Activation Cocktail (with Brefeldin A)	Biolegend	Cat#423304
eBioscience 7-AAD Viability Staining Solution	Thermo Fisher Scientific	Cat#00-6993-50
eBioscience IC Fixation Buffer	Thermo Fisher Scientific	Cat#00-8222-49
eBioscience Intracellular Fixation & Permeabilization Buffer Set	Thermo Fisher Scientific	Cat#G762684
FoxP3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat#00-5523-00
Phosflow Perm Buffer III	Biolegend	Cat# 558050
Phosflow Lyse/Fix buffer	BD Biosciences	Cat#558049
Fluo-4 Direct™ Calcium Assay Kit	Fisher Scientific Company	Cat#F10471
Purelink RNA Mini Kit	Fisher Scientific Company	Cat#12183018A
Pierce™ Rapid Gold BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#A53225
QIAprep Spin Miniprep Kit	Qiagen	Cat#27106
RBC lysis buffer	Invitrogen	Cat#00-4300-54
T-Cell Receptor Phospho Antibody Array	Full Moon Biosystems	Cat#PTC188
Antibody Array Assay Kit	Full Moon Biosystems	Cat#KAS02
Proteome Profiler Phospho-Kinase Array Kit	R&D Systems	Cat#ARY003C

Table 2.4 List of deposited data

	SOURCE	IDENTIFIER
RNA-seq data: effect of Zeaxanthin treatment on gene expression of mouse CD8 ⁺ T cells	This paper	GEO: GSE282703
KAS-seq data	This paper	GEO: GSE282686
Microbiome 16S rRNA sequencing data	This paper	NCBI SRA: PRJNA1189274

Table 2.5 List of software

SOFTWARE	SOURCE	LINK
ImageJ	Schneider et al. [39]	https://imagej.net/ij/download.html
GraphPad Prism 9	GraphPad Software	RRID:SCR_002798 https://www.graphpad.com/
FlowJo v10.8.1	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/flowjo-v10-software
FragPipe	Nesvilab	https://fragpipe.nesvilab.org/
STAR	Dobin et al. [40]	https://github.com/alexdobin/STAR/releases
featureCounts	Liao et al. [41]	https://subread.sourceforge.net/
DESeq2	MI Love et al. [42]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
clusterProfiler	Wu et al. [43]	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
Origin (Pro), Version 2021	OriginLab Corporation	https://www.originlab.com/demodownload.aspx
GSEA 4.3.3	Subramanian et al. [30]	https://www.gsea-msigdb.org/gsea/downloads.jsp
KAS-Analyzer	Lyu et al. [44]	https://github.com/Ruitulyu/KAS-Analyzer
Leica Application Suite X	Leica Microsystems	RRID:SCR_013673 https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/

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CHAPTER 3

STRUCTURE-ACTIVITY RELATIONSHIP (SAR) RESEARCH IDENTIFIES NOVEL IMMUNOMODULATORY MOLECULE

This chapter consists of unpublished research:

I am responsible for *in vitro* experiments, data analysis, making the figures, and manuscript writing unless specified otherwise. Dr. Junhong Xiang designed the derivatives for the library screen. Dr. Junhong Xiang and I are both responsible for animal studies.

3.1 Introduction

Dietary interventions play a vital role in shaping immune responses. A growing body of evidence suggests that specific nutrients and bioactive compounds display immunomodulatory properties, capable of enhancing T cell functions and modulating inflammatory responses [1]. In previous chapters, we explored the intricate relationship between diet and the immune system in the context of cancer immunology. Specifically, we demonstrated that zeaxanthin, a plant-based natural dietary nutrient, can improve CD8⁺ T cell effector functions by modulating TCR signaling, thereby bolstering anti-tumor immunity. While our research on the novel immunomodulatory effects of natural compounds has provided insights into CD8⁺ T cell biology and function, we sought to expand these findings by exploring strategies to further optimize CD8⁺ T cell-based anti-tumor immunity.

In our previous research, we identified a long chain *trans*-fatty acid TVA as an animal-derived nutrient that regulates CD8⁺ T cell function by unexpectedly antagonizing short-chain fatty acid receptor G protein-coupled receptor GPR43 and activating the downstream cAMP-PKA-CREB axis [2]. TVA is a natural compound that the human body cannot synthesize. Instead, it is obtained through dietary sources, primarily from derived foods including beef, lamb, and dairy products, including milk and butter.

Fatty acids are a major branch of the lipid family that is ubiquitous in our diet. They exhibit remarkable diversity and serve as essential components in biological processes [3–6]. Increasing evidence suggests that fatty acids play a crucial role in regulating tumor immunity [7–10]. Given the crucial role of fatty acids in anti-tumor immunity, we utilized a medicinal chemistry and chemical biology approach to optimize the regulatory effects of fatty acids in the context of tumor immunotherapy aiming to uncover new therapeutic targets and mechanisms. Additionally, these

studies may allow for the discovery of candidate compounds with improved efficacy in clinical application.

The discovery of TVA as a natural immunomodulator that regulates CD8⁺ T cell activation independently of the conventional lipid metabolism revealed novel therapeutic targets and innovative utilization of naturally occurring dietary nutrients. Based upon these findings, this study focuses on the structure-activity relationship (SAR) of TVA to expand our knowledge on lipid-based immunomodulation. By systemically modifying its structure, we aim to develop derivatives with enhanced efficacy in modulating CD8⁺ T cell function and to advance our understanding of lipids as immunoregulators by bridging the chemical structure with mechanisms of action.

TVA and its derivatives represent a novel class of lipid-based immunoregulators that act through direct target-mediated signaling. Moreover, TVA's natural presence in the diet suggests that optimized derivatives could have fewer toxicity concerns compared to synthetic small molecule drugs. This research expands on the growing interest of lipids as immunoregulators and provides insights into how structural modifications of lipids can be leveraged for therapeutic applications in cancer immunotherapy.

3.2 Results

Construction of a fatty acid derivative library

Through structural analysis of unsaturated fatty acids and the molecular framework of TVA, we identified four key structural components that define its bioactivity: (A) the carboxyl group, (B) the central fatty acid chain, (C) the unsaturated double bond, and (D) the terminal fatty acid chain.

Using TVA as a template, we designed and synthesized derivatives through four major modification strategies (Figure 3.1A).

1. Modification of the carboxyl group (A). To assess the impact of the exposed carboxyl group on bioactivity, we performed esterification and amidation reactions. These modifications allow us to determine how changes in the carboxyl moiety influence the compound's ability to enhance CD8⁺ T cell function.
2. Modification of the central fatty acid chain (B). The length of the B-chain was systematically adjusted to examine how variations in chain length affect bioactivity. Given that fatty acid chain length can influence membrane permeability and receptor interactions, this modification aims to optimize TVA's stability and effectiveness.
3. Modification of the unsaturated double bond (C). We performed hydrogenation reactions to reduce the double bond and obtain a fully saturated carbon chain, allowing us to investigate the role of unsaturation in T cell activation. Geometric and positional isomerism of the double bond was explored by altering cis/trans configurations, shifting the double bond position, and modifying double bond numbers. The Julia olefination reaction was used to construct trans double bonds, while the Wittig reaction was applied to generate cis double bonds. Additionally, we replaced the double bond with alternative unsaturated

functional groups such as aromatic rings (benzene, heteroaromatic rings) to evaluate their effects on activity.

4. Modification of the terminal fatty acid chain (D). We altered the aldehyde structure in Julia olefination and Wittig reactions to introduce modifications in the terminal chain (D). The length of the terminal fatty acid chain was varied, and additional functional groups such as double bonds or hydroxyl groups were introduced at the terminal position (R_2), allowing us to assess their influence on T cell activation.

By systematically modifying and evaluating each of the four structural regions (A, B, C, and D), we first examine the effects of deleting individual regions on bioactivity. Then, through diverse structural modifications and bioactivity assessments, we identify the essential components required for CD8⁺ T cell activation. Applying the above modifications, we compiled a fatty acid library consisting of 50 lipid-based derivatives using TVA's structure as the foundational template (Figure 3.1B).

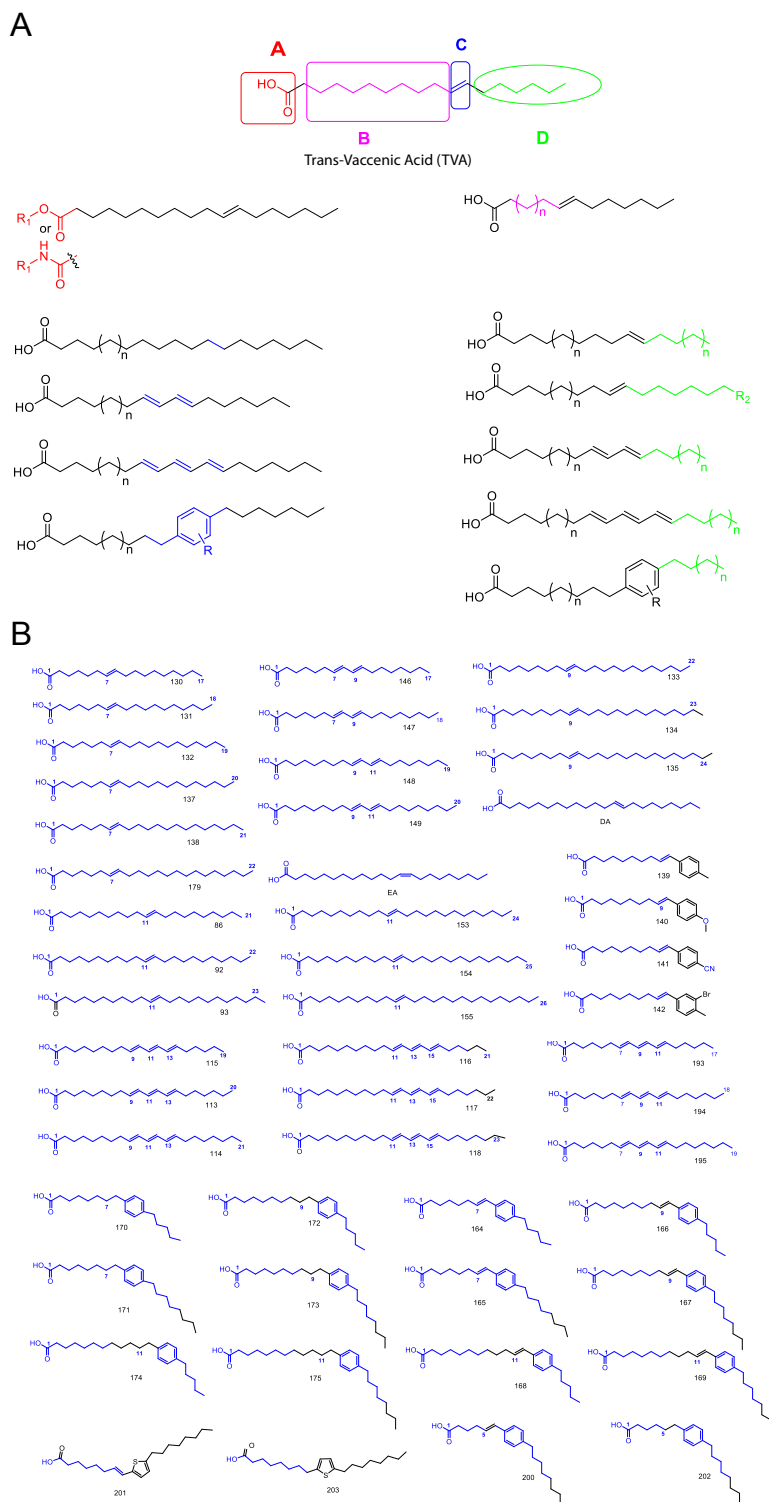


Figure 3.1 *Trans*-vaccenic acid (TVA) derivative library

(A) Structural analysis and modification strategies of TVA to generate the derivative library.
 (B) Structures included in the TVA derivative library.

Derivative 171 enhances CD8⁺ T cell activation and tumor-killing efficacy

Using TVA as a positive control, we performed two screens to assess the efficacy of each derivative from the newly synthesized fatty acid derivative library. Screen 1 evaluates CD8⁺ T cell activation after treatment of each derivative in comparison with the DMSO control and TVA (Figure 3.2A). Obtaining top bioactive candidates from screen 1, we used screen 2 to examine CD8⁺ T cell-dependent tumor killing by co-culturing OT-I T cells and B16-OVA cells and treated with top derivative candidates (Figure 3.2A). Additionally, we also assessed the direct toxicity on B16-OVA cells and OT-I T cells in screen 2 (Figure 3.2A and C). Combining the results from both screens, we identified derivative 171 as the top candidate in enhancing both CD8⁺ T cell activation and CD8⁺ T cell-mediated tumor cytotoxicity without presenting direct toxicity to OT-I T cells (Figure 3.2B-C). Structurally, derivative 171 replaces the double bond in TVA with a phenyl ring, which serves as an unsaturated center (Figure 3.2D). This modification enhances hydrophobic interactions, including potential π - π stacking with aromatic side-chain amino acids in target proteins. Furthermore, the introduction of the phenyl ring alters the molecular conformation, increasing rigidity and potentially influencing binding affinity. Additionally, the extended carbon chain, approximately two carbons longer, may affect flexibility, lipophilicity and membrane permeability, further modulating its bioactivity. Moreover, 171 does not show direct effects on the proliferation of CD8⁺ T cell or B16-OVA cells (Figure 3.2E-F).

In comparison to TVA, derivative 171 replaces the double bond with a phenyl ring, which serves as an unsaturated center (Figure 3.2D). This modification enhances hydrophobic interactions, including potential π - π stacking with aromatic side-chain amino acids in target proteins. Furthermore, the introduction of the phenyl ring alters the molecular conformation, increasing rigidity and potentially influencing binding affinity. Additionally, the extended carbon

chain, approximately two carbons longer, may affect flexibility, lipophilicity and membrane permeability, further modulating its bioactivity.

To evaluate 171's effect *in vivo*, we administered 171 to syngeneic mice bearing immunogenic B16F10 cells through oral gavage (Figure 3.3A). Derivative 171 reduced *in vivo* B16F10 tumor growth more effectively in comparison to TVA at 150 mg/kg b.w. (Figure 3.3B). We closely monitored the body weight during 171 administration and did not find significant impacts on mice body weight, suggesting that it is unlikely to have overt toxicity (Figure 3.3C). Furthermore, CD8⁺ T cell depletion via anti-CD8 antibody treatment (Figure 3.3D) abolished the effect of 171 on tumor growth attenuation, demonstrating that CD8⁺ T cells are essential for 171's anti-tumor effects (Figure 3.3E). Altogether, 171 is a novel and effective natural compound derivative that promotes CD8⁺ T cell activation and tumor killing *in vitro* and suppresses tumor growth *in vivo*.

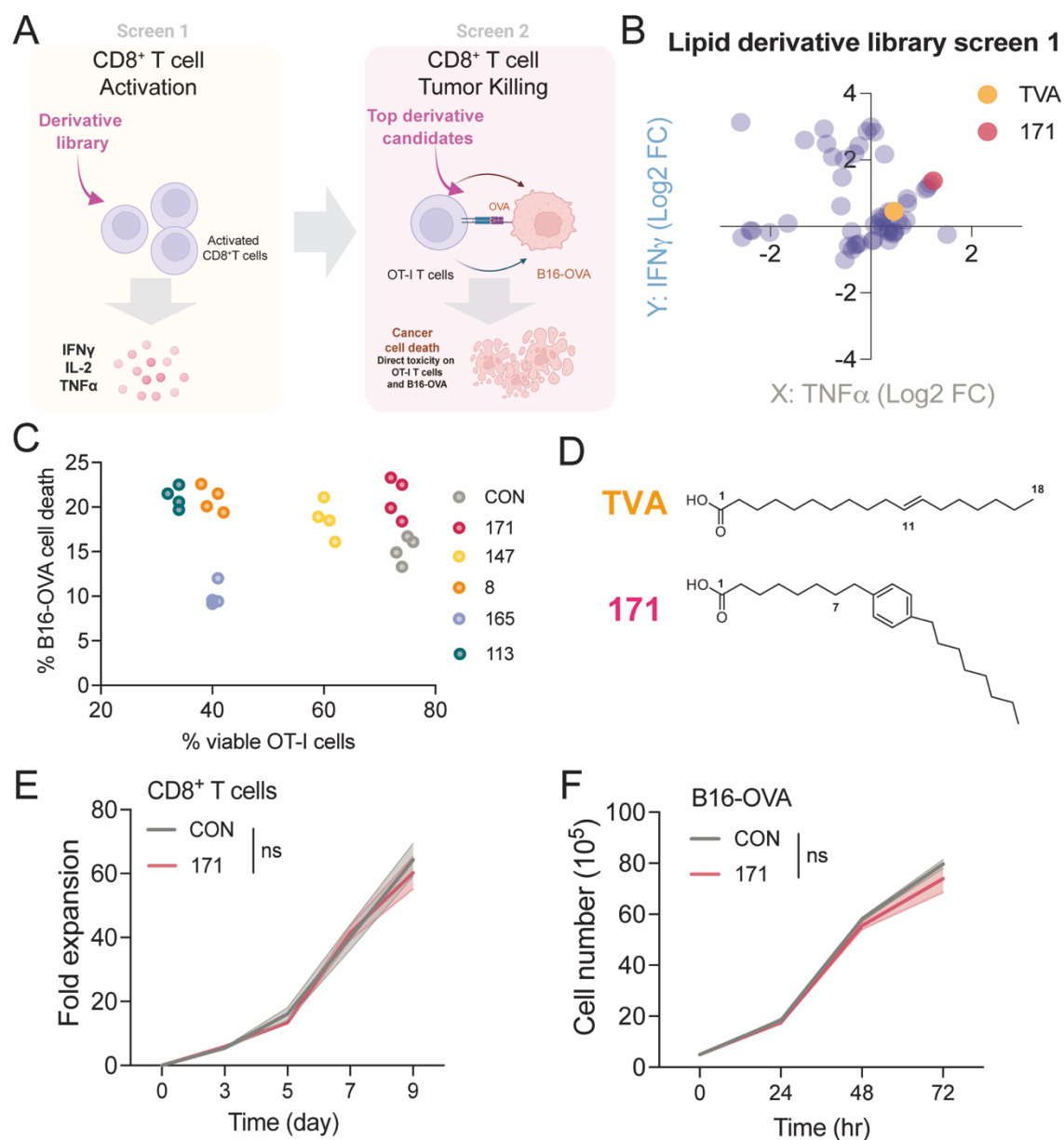


Figure 3.2 Derivative 171 enhances CD8⁺ T cell activation and cytotoxic efficacy

(A) Schematic depicting experimental design for CD8⁺ T cell activation (screen 1) and CD8⁺ T cell-dependent tumor killing (screen 2). This schematic was generated using BioRender.com.

(B) Results from CD8⁺ T cell activation screen.

(C) Results from CD8⁺ T cell-dependent tumor killing screen. (D) Chemical structure of TVA and 171.

(E) Effects of 171 (10 μ M) on CD8⁺ T cell proliferation.

(F) Effects of 171 (10 μ M) on B16-OVA cell proliferation.

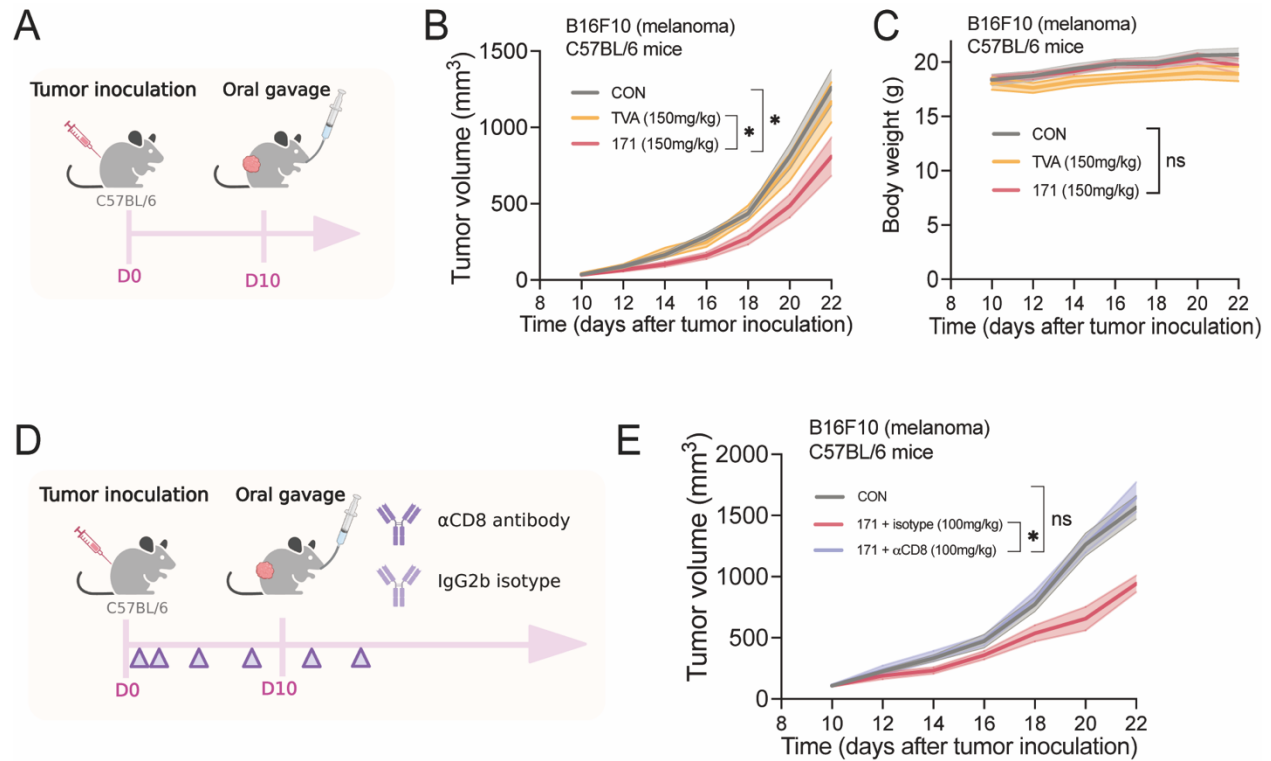


Figure 3.3 Oral 171 enhances CD8⁺ T cell-mediated anti-tumor immunity

(A) Schematic depicting experimental design for *in vivo* tumor-bearing mouse model. This schematic was generated using BioRender.com.

(B) Effect of orally administered TVA or 171 (150 mg/kg b.w.) on B16F10 tumor growth in C57BL/6 mice ($n \geq 8$ mice).

(C) Effect of orally administered TVA or 171 (150 mg/kg b.w.) on the body weight of B16F10 tumor-bearing C57BL/6 mice ($n \geq 8$ mice).

(D) Schematic depicting experimental design for CD8⁺ T cell depletion experiment. This schematic was generated using BioRender.com.

(E) Effect of orally administered 171 (100 mg/kg b.w.) on B16F10 tumor growth in C57BL/6 mice treated with isotype control or CD8⁺ T cell depleting antibody ($n \geq 6$ mice).

Oral 171 enhances CD8⁺ T cell tumor infiltration and effector function

To investigate the effect of 171 on tumor-infiltrating T cells, we analyzed the composition and effector markers of tumor-infiltrating lymphocytes (TIL) in B16F10 tumor-bearing mice after oral 171 administration (Figure 3.4A). Flow cytometric analysis indicated that derivative 171 selectively increased CD8⁺ T cell tumor infiltration, while the percentage of CD4⁺ T cells remained unaffected within the total tumor-infiltrating CD45⁺ leukocyte population (Figure 3.4B-C).

Next, we evaluated the functional state of TILs following oral 171 treatment by key effector molecule levels. Tumor-infiltrating CD8⁺ T cells showed significant increase in production of pro-inflammatory cytokines IFN γ and TNF α , as well as cytotoxic molecule granzyme B (GZMB), indicating enhanced effector functions and cytotoxic potential (Figure 3.4D-F). On the other hand, tumor-infiltrating CD4⁺ T cells showed no significant changes in IFN γ production or FOXP3 expression, suggesting that oral 171 is unlikely to affect helper T cell activity or regulatory T cell populations in tumors (Figure 3.4G-H). Our results suggest a targeted role of derivative 171 in boosting CD8⁺ T cells-mediated anti-tumor immunity.

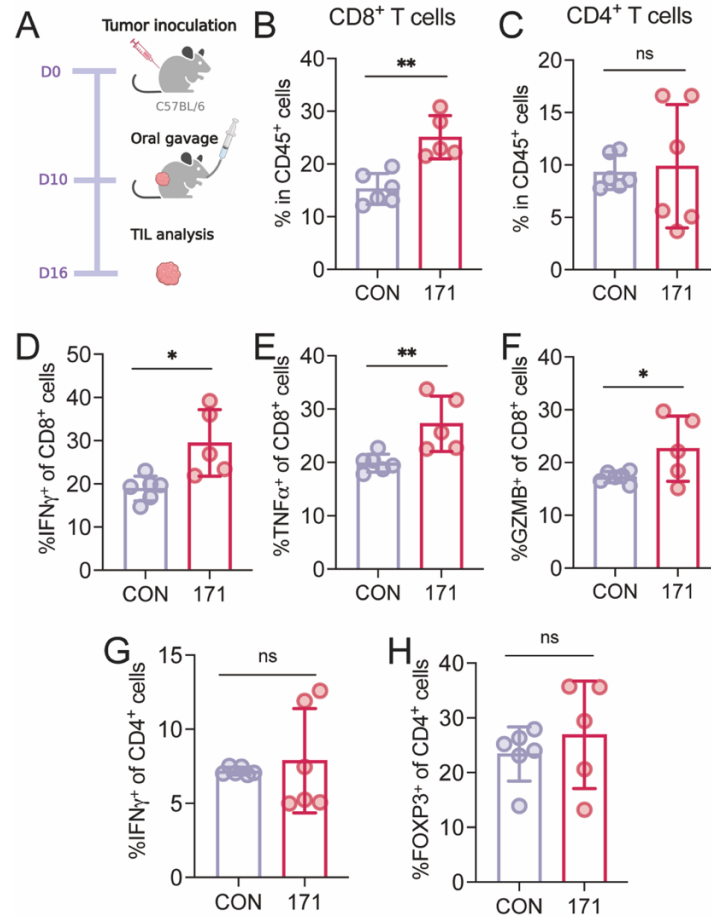


Figure 3.4 Oral 171 selectively enhance CD8⁺ T cell tumor infiltration and effector function

(A) Schematic depicting experimental setup for harvesting tumors to examine the effect of 171 on tumor-infiltrating T cells *in vivo*. This schematic was generated using BioRender.com.

(B) Effect of orally administered 171 (100 mg/kg b.w.) on percentage of CD8⁺ T cells within tumor-infiltrating lymphocytes ($n \geq 5$ mice).

(C) Effect of orally administered 171 (100 mg/kg b.w.) on percentage of CD4⁺ T cells within tumor-infiltrating lymphocytes ($n = 6$ mice).

(D) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD8⁺ T cell IFN γ production ($n \geq 5$ mice). (E) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD8⁺ T cell TNF α production ($n \geq 5$ mice).

(E) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD8⁺ T cell TNF α production ($n \geq 5$ mice).

(F) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD8⁺ T cell GZMB production ($n \geq 5$ mice).

(G) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD4⁺ T cell IFN γ production ($n = 6$ mice).

(H) Effects of orally administered 171 (100 mg/kg b.w.) on tumor-infiltrating CD4⁺ T cell FOXP3 expression ($n \geq 5$ mice).

3.3 Discussion

We explored structure-activity relationship (SAR) optimization of *trans*-vaccenic acid (TVA) as a strategy to optimize CD8⁺ T cell-based anti-tumor immunity following the identification of naturally occurring immunomodulatory molecules [11]. Through SAR-based chemical modifications, we identified derivative 171 as a more potent T cell activator than its parent compound TVA, demonstrating enhanced cytokine production and anti-tumor immune function both *in vitro* and *in vivo*.

Identifying natural nutrients with immunomodulatory effects represents a promising avenue for developing the next-generation immunotherapies [12]. However, the efficacy of naturally occurring compounds is often limited by the bioavailability, potency, and incomplete mechanistic understanding [13,14]. Our SAR-drive approach aims to address some of these limitations by systematically modifying TVA's structure to enhance its immunomodulatory properties.

While some findings in this study remain preliminary, our research provides compelling evidence that SAR-driven optimization can further amplify CD8⁺ T cell effector function. However, to fully understand the therapeutic relevance of these approaches, further investigations are warranted. Future studies on 171 will include combination treatment with current cancer immunotherapies including immune-checkpoint inhibitor (ICI) therapy and adoptive cell transfer (ACT) therapies. Moreover, although we have not seen any effects on body weight or change in behavior in our tumor mouse models treated with 171, future research should further characterize the compound by evaluating its pharmacokinetics, distribution, bioavailability, as well as its long-term effects on health and behavior. These properties would be important to determine the feasibility of translating chemically modified nutrient-derivatives into therapeutic applications.

Overall, our findings lay the groundwork for future preclinical and translational research, with the ultimate goal of leveraging nutrient-based immunomodulator and strategically integrate nutritional interventions with current immunotherapeutic to enhance immune function and improve cancer treatment outcomes.

3.4 Material and Methods

Mice

Animal experiments were conducted and designed according to protocols approved by the Institutional Animal Care and Use Committee of The University of Chicago. C57BL/6 mice (6-8 weeks), Pmel-1 mice (6-8 weeks), and OT-I mice (6-8 weeks) were obtained from The Jackson Laboratory. Mice were housed in a temperature (23-25 °C) and humidity-controlled colony room, maintained on a 12-h light/dark cycle (08:00 to 20:00 light on) with standard food and water provided *ad libitum* and environmental enrichments in a pathogen-free facility.

Primary cells

Mouse naïve CD8⁺ T cells were isolated from the spleen of C57BL/6 mice (6-8 weeks) or OT-I mice (6-8 weeks) by magnetic bead purification using EasySep™ Mouse Naïve CD8⁺ T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. Naïve CD8⁺ T cells were activated *in vitro* for 18 hours with plate-bound anti-mouse CD3 (1µg/mL; Biolegend) and soluble anti-mouse CD28 (0.5µg/mL; Biolegend) antibodies in complete Click's media (Fujifilm Irvine Scientific) at 37°C and 5% CO₂ incubator for further experiments.

Cell lines

All cell lines were authenticated by genomic short tandem repeat (STR) profiling at the University of Chicago Integrated Genomics Core (EIGC) upon purchase and at least annually as appropriate.

Mouse melanoma cell line B16F10 were purchased from ATCC. Mouse melanoma cell line B16-OVA was provided by Dr. Hongbo Chi. B16F10 were cultured in Dulbecco Modified Eagle Medium (DMEM). B16-OVA was cultured in the Click's media (Fujifilm Irvine Scientific) prior to co-culture experiments to assess murine T cell-mediated cytotoxicity. All the cells were cultured at 37°C and 5% CO₂. Cell line experiments were conducted and designed according to protocols approved by the Institutional Biosafety Committee (IBC) of the University of Chicago.

CD8⁺ T cell activation screen

T cells were isolated from C57BL/6 mice (6-8 weeks) mouse by magnetic bead purification using EasySep™ Mouse Naïve CD8 Mouse T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. Naïve CD8⁺ T cells were activated *in vitro* for 18 hours with plate-bound anti-mouse CD3 (1µg/mL; Biolegend) and soluble anti-mouse CD28 (0.5µg/mL; Biolegend) antibodies in complete Click's media (Fujifilm Irvine Scientific) at 37°C and 5% CO₂ incubator. Activated CD8⁺ T cells were treated with each derivative from the lipid derivative library for 24 hours. Then cells were then stained with FVD and treated with Cell Activation Cocktail (with Brefeldin A) (Biolegend) for 4-6 hours before intracellular staining to detect pro-inflammatory cytokine levels.

CD8⁺ T cell tumor killing screen

To determine mouse OT-I T cell-mediated tumor cytotoxicity, OT-I T cells were isolated using EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies) and seeded at a density of 5×10^6 cells/well in 6-well plate (pre-coated with anti-CD3 and anti-CD28) for overnight stimulation. B16-OVA cells were stained with CellTracker Deep Red dye (Invitrogen) according to manufacturer's instruction prior to co-culturing with OT-I T cells. 5×10^5 stimulated OT-I cells were then co-cultured with pre-stained 1×10^5 B16F10-OVA cells (E:T = 5:1) and treated with DMSO control or top derivative candidates for 24 hours. B16-OVA cells or OT-I cells were also treated alone under the same conditions. All cells were collected, stained with PI, and analyzed by flow cytometry.

Cell proliferation assay

Tumor cell proliferation assays were conducted by seeding 5×10^5 B16-OVA cells in a 6-well plate and record cell number daily for 3 days using TC20 Automated Cell Counter (Bio-Rad). CD8⁺ T Cell proliferation assays were conducted by isolating mouse naïve CD8⁺ T cells from the spleen of C57BL/6 mice (6-8 weeks) by magnetic bead purification using EasySep™ Mouse Naïve CD8⁺ T Cell Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions. Naïve CD8⁺ T cells were seeded at a density of 5×10^6 cells/well in a 6-well plate (pre-coated with anti-CD3 and anti-CD28) for overnight stimulation before seeding at 1×10^5 cells/well in a 48-well plate and adding 10ng/ml recombinant human IL-2 (Miltenyi Biotec). Cells numbers were recorded daily and expanded twice every 2 to 3 days and moved to 24-well plates when necessary for 9 days using TC20 Automated Cell Counter (Bio-Rad).

Mouse models

For C57BL/6 mice tumor models, mice were anesthetized with isoflurane, shaved at the injection site, and then injected subcutaneously in the abdominal flank with 2×10^5 B16F10 cells for tumor development. Daily oral gavage of derivative 171 or vehicle control was administered once tumors reached a size of 100 mm^3 . Mice's body weight and tumor size were measured every other day. Tumor sizes were measured using a caliper, and tumor volumes were calculated using the following formula: tumor volume = (Length x Width²)/2. Mice were euthanized at humane endpoints or on days 15-16 for tissue collection.

For antibody-mediated T cell depletion models, C57BL/6 mice (6-8 weeks) were injected subcutaneously in the abdominal flank with 2×10^5 B16F10 cells and then injected intraperitoneally with six doses of depleting antibodies (anti-CD8a, BioXCell) or isotype control (rat IgG2b isotype control, BioXCell) on day 1 (200 μg), 2 (200 μg), 4 (200 μg), 8 (200 μg), 12 (200 μg) and 16 (200 μg) relative to tumor injection (day 0). Depletion efficiency was assessed by staining for CD8 (Biolegend) and flow cytometry analysis. Tumor sizes were measured using a caliper and tumor volumes were calculated using the following formula: tumor volume = (Length x Width²)/2. Mice were euthanized at humane endpoints.

CD45⁺ tumor-infiltrating leukocyte isolation

Tumor tissues were dissected from euthanized tumor-bearing mice, minced into small pieces ($\leq 2 \text{ mm}$) using a scalpel in a dish, and then transferred to a 14 mL round-bottom tube containing 5mL tumor digestion medium (500 μL Collagenase/Hyaluronidase Solution, 750 μL 1 mg/mL DNase I Solution, and 3.75 mL RPMI 1640 Medium). After incubation at 37°C for 25 min on a shaking platform, the digested tumor tissues were transferred into a 70 μm mesh nylon strainer

on a 50 mL conical tube, pushed through the strainer using the rubber end of a syringe plunger, and rinsed with the recommended medium. After centrifugation at 300×g for 10 min at room temperature with the brake on low, the resulting cell pellets were added 10 mL of ammonium chloride solution for incubation at room temperature for 5 minutes, followed by centrifugation at 300×g for 10 minutes at room temperature with the brake on low. The resulting cell pellets were re-suspended at $1-10 \times 10^6$ cells/mL in PBS and then subjected to CD45⁺ tumor-infiltrating leukocyte isolation.

Mouse Spleen lymphocyte isolation

Mouse spleens were disrupted with a syringe plunger in a 70 µm strainer, filtered to a 50 mL tube, washed with PBS, and centrifuged at 300×g for 10 minutes. The resulting cell pellets were re-suspended with 2 mL red cell lysis buffer (Invitrogen), incubated at room temperature for 10 minutes, and centrifuged at 300×g for 5 minutes after adding 15 mL PBS. The resulting cell pellets were designated splenocytes and used for the following experiments. For cytokine detection, T cells were stimulated with a cell activation cocktail (Biolegend) for 4hr, and then intracellular staining for cytoplasmic protein was performed, followed by flow cytometry analysis.

Flow cytometry analysis

Murine primary cells isolated from tumor, spleen, draining lymph node were stained with fluorescent antibodies and analyzed by flow cytometry. For experiments with live/dead criteria, cells were first stained with Fixable Viability Dyes (FVD) (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequent surface marker staining was performed in Cell Staining Buffer (Thermo Fisher Scientific).

Intracellular staining for flow panels containing nuclear proteins was performed using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. For intracellular staining of cytoplasmic proteins, eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) was used according to the manufacturer's instructions.

For phospho-antibody staining, cells were incubated with FVD (cell viability dye) for 15 min at room temperature in a tube, re-suspended with 200 μ l pre-warmed $1\times$ BD Phosflow Lyse/Fix buffer directly into the tube, and incubated at 37 °C for 10–15 min, followed by centrifugation at 300g for 5 min. The resulting cell pellets were washed once with FACS buffer, permeabilized with 200 μ l of BD Phosflow Perm Buffer III for 45 min on ice, and centrifuged at 300g for 5 min. The cell pellets were washed again with FACS buffer, centrifuged at 300g for 5 min, and incubated with antibodies in FACS buffer for 45 min–1 hour at room temperature.

For CD4 subtypes analysis, CD3⁺ cells were gated within the CD45⁺ population, and CD4⁺ and CD8⁺ cells were subsequently identified. For CD45⁺CD4⁺ cells, further analysis was performed to identify subtypes: Treg cells were identified as Foxp3⁺ within the CD45⁺CD4⁺ population. Data were collected with LSR-Fortessa 4-15 or Attune NxT 4-14 flow cytometer and analyzed using FlowJo v10.8.1.

Quantification and statistical analysis

Statistical analysis was conducted using GraphPad Prism 8 or using the software indicated above. Details of statistical analysis are described in the figure legends or in the STAR Methods section above.

Table 3.1 List of antibodies

REAGENT	SOURCE	IDENTIFIER
Rat anti-IgG2b isotype	BioXCell	Cat#BE0090; Clone#LTF-2; RRID:AB_1107780.
Mouse anti-CD8 α	BioXCell	Cat#BE0061; Clone#2.43; RRID:AB_1125541.
Mouse FITC anti-CD3 ϵ Antibody	Biolegend	Cat#100305; Clone#145-2C11; RRID:AB_312670.
Mouse PE anti-CD28 Antibody	Biolegend	Cat#102105; Clone#37.51; RRID: AB_312870.
Mouse PE/Cyanine5 anti-CD69 Antibody	Biolegend	Cat#104509; Clone#H1.2F3; RRID:AB_313112.
Mouse PE/Cyanine5 anti-CD4 Antibody	Biolegend	Cat#100409; Clone#GK1.5; RRID:AB_312694.
Mouse Brilliant Violet 421™ anti-IL-2 Antibody	Biolegend	Cat#503825; Clone#JES6-5H4; RRID:AB_10895901.
Mouse PE anti-IL-2 Antibody	Biolegend	Cat#503807; Clone#JES6-5H4; RRID:AB_315301.
Mouse APC anti-IL-2 Antibody	Biolegend	Cat#503810; Clone#JES6-5H4; RRID:AB_315304.
Mouse APC anti-CD45.2 Antibody	Biolegend	Cat#109813; Clone#104; RRID:AB_389210.
Mouse APC anti-IFN- γ Antibody	Biolegend	Cat#505810; Clone#XMG1.2; RRID:AB_315404.
Mouse PE anti- IFN- γ Antibody	Biolegend	Cat#505808; Clone#XMG1.2; RRID:AB_315402.
Mouse PerCP/Cyanine5.5 anti-TNF- α Antibody	Biolegend	Cat#506321; Clone#MP6-XT22; RRID:AB_961435.
Mouse FITC anti-TNF- α Antibody	Biolegend	Cat#506304; Clone#MP6-XT22 RRID:AB_315425.
Mouse Brilliant Violet 711™ anti-CD8a Antibody	Biolegend	Cat#100747; Clone#53-6.7; RRID:AB_11219594.
Mouse Brilliant Violet 421™ anti-FOXP3 Antibody	Biolegend	Cat#126419; Clone#MF-14; RRID:AB_2565933.
Mouse APC anti-CD3 Antibody	Biolegend	Cat#100235; Clone#17A2; RRID:AB_2561455.
PE/Cyanine5 anti-mouse CD28 Antibody	Biolegend	Cat#102108; Clone#37.51; RRID: AB_312873.
Mouse/human PE anti-Ki-67 Antibody	Biolegend	Cat#151210; Clone#11F6; RRID:AB_2716008.
Mouse BV510 anti-IFN- γ Antibody	Biolegend	Cat#505841; Clone#XMG1.2; RRID:AB_2562187.
Mouse APC anti-FOXP3 Antibody	Proteintech	Cat#APC-65089; Clone#3G3; RRID:AB_2882984.
Mouse BUV563 anti-CD69 Antibody	BD Biosciences	Cat#741234; Clone#H1.2F3; RRID:AB_2870786.

Table 3.2 List of chemicals, peptides, and recombinant proteins

REAGENT	SOURCE	IDENTIFIER
Human IL-2 IS	Miltenyi Biotec	Cat#130-097-743
Fixable viability dye 780	R&D	Cat#65-0865-14
DNase I	Millipore	Cat#EN0521
N-methylpyrrolidone	Sigma-Aldrich	Cat#PHR1352 CAS:872-50-4
Collagenase/Hyaluronidase	Stemcell Technologies Inc	Cat#07912
Ammonium Chloride Solution	Stemcell Technologies Inc	Cat#07800

Table 3.3 List of commercial assay reagents

REAGENT	SOURCE	IDENTIFIER
Annexin V apoptosis detection kit I	BD Biosciences	Cat#556547
EasySep™ Mouse T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19851
EasySep™ Mouse Naïve CD8+ T Cell Isolation Kit	Stemcell Technologies Inc	Cat#19858
Cell Activation Cocktail (with Brefeldin A)	Biolegend	Cat#423304
eBioscience IC Fixation Buffer	Thermo Fisher Scientific	Cat#00-8222-49
eBioscience Intracellular Fixation & Permeabilization Buffer Set	Thermo Fisher Scientific	Cat#G762684
FoxP3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat#00-5523-00
Purelink RNA Mini Kit	Fisher Scientific Company	Cat#12183018A
QIAprep Spin Miniprep Kit	Qiagen	Cat#27106
RBC lysis buffer	Invitrogen	Cat#00-4300-54

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CHAPTER 4

DISCUSSION

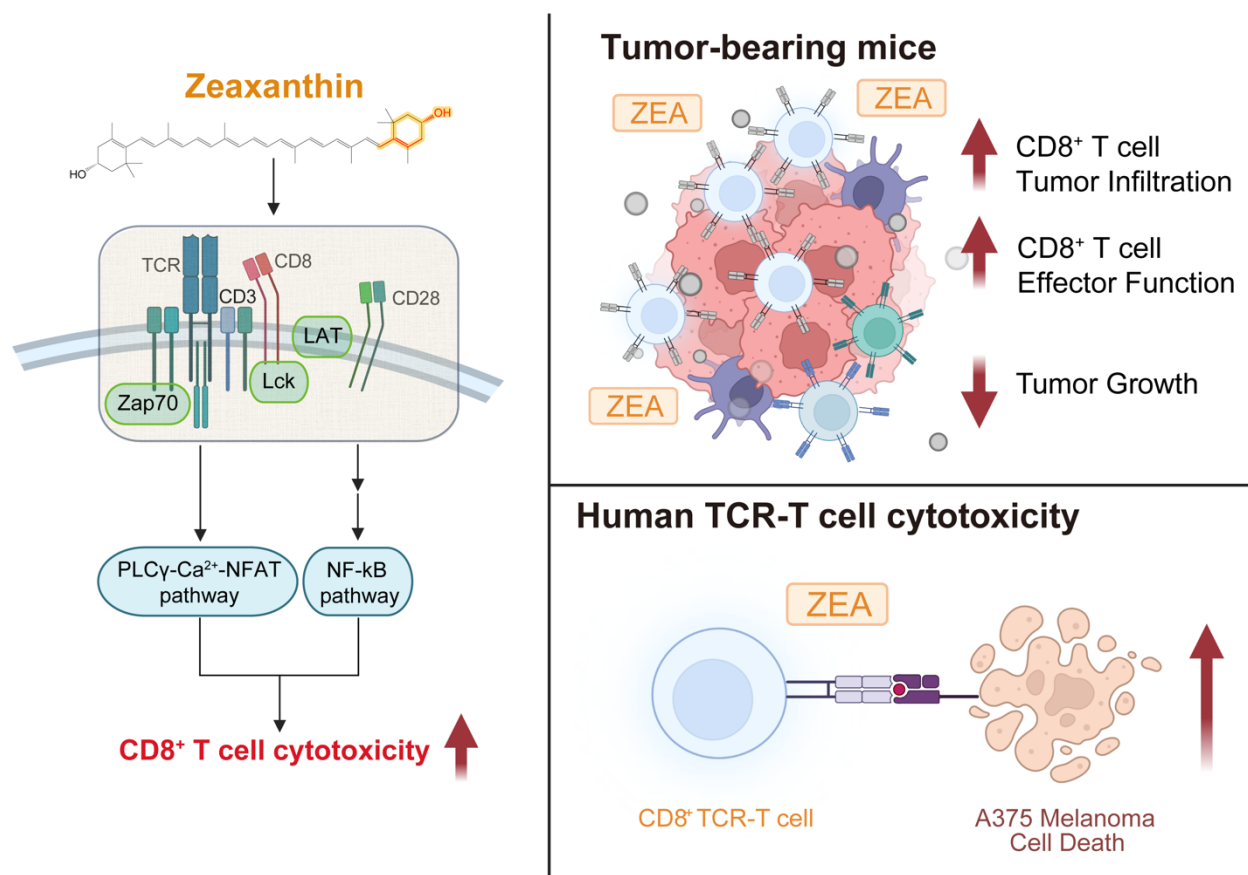


Figure 4.1 Dietary zeaxanthin as a novel modulator of CD8⁺ t cell anti-tumor immunity

4.1 Summary

The immune system's ability to recognize and eliminate cancerous cells relies heavily on the function of CD8⁺ T cells [1]. However, within the tumor microenvironment (TME), T cells often undergo suppression and become dysfunctional, limiting their effectiveness in anti-tumor immunity [2]. In recent years, the regulatory roles of nutrients have been explored and many nutrients have emerged as immunoregulatory molecules that play a crucial role in modulating immune cell metabolism, differentiation, activity, and survival [3]. Various classes of nutrients, including fatty acids, amino acids, vitamins, and polyphenols, have been shown to shape immune function through metabolic and signaling pathways [4,5]. This dissertation explores the regulatory roles of diet-derived nutrients in CD8⁺ T cell-mediated anti-tumor immunity and strategies to optimize the efficacy of natural nutrients through structure-activity relationship (SAR) research.

In Chapter 2, we performed a comprehensive screen to examine how circulating nutrients could modulate T cell-mediated tumor killing. Our discovery of a xanthophyll carotenoid zeaxanthin (ZEA) that functions through enhancing TCR signaling pathways uncovered a previously unknown mechanism of a novel immunomodulatory natural compound (Figure 4.1). Notably, ZEA improved CD8⁺ T cell function not only in mouse models but also in human TCR-T cells when co-culturing against tumor cells, highlighting its relevance across species and therapeutic platforms. In Chapter 3, we extended this work systematically altered the structure of *Trans*-vaccenic acid (TVA), a naturally occurring CD8⁺ T cell modulator previously identified by our lab, to build a novel library of derivatives. From this screen, we found derivative 171, which showed improved CD8⁺ T cell activation and tumor-killing capacity at lower doses than its parent compound. Together, these findings illuminate a new class of diet-derived immunomodulators and demonstrate the feasibility of structure-based optimization to enhance their efficacy. The effects

in human TCR-T cell systems underscore the translational potential of these compounds, supporting further investigation into their integration with existing immunotherapies.

Our current understanding of anti-tumor immunity is largely based on immunological mechanisms that focus on the functional effects of T cells, while the precise molecular mechanisms underlying these effects remain less characterized. Our approach not only identifies previously unknown natural immunomodulators, but also combines modulation of immune functions with the specific intracellular signaling pathways that the natural compound engages with. Our lab has developed an unbiased library-based workflow that integrates high-throughput compound screening, *in vitro* and *in vivo* phenotype validation, temporal mechanistic studies, and target verification to tell a more holistic story of the effects of previously unknown immunomodulatory compounds. Using this approach, we successfully identified novel regulatory functions of ZEA and TVA [6]. Working with collaborators with chemistry expertise, we then strategically optimize the bioactivity of the parent compound through systemic alterations of its structure, as exemplified by our discovery of derivative 171 as a more potent T cell modulator than TVA. By leveraging this comprehensive workflow, we can not only discover new immunoregulatory compounds but also gain deeper mechanistic insights on the diet-immune axis.

The collective insights from this dissertation reinforce the importance of comprehensive screening and mechanistic studies to identify novel immunomodulatory compounds and optimizing their activity using SAR. By bridging nutrient-based immune modulation with detailed mechanistic studies, this framework provides a strategic and powerful platform for improving current immunotherapeutics and developing next-generation interventions [7]. Ultimately, these findings contribute to the broader goal of elucidating the mechanistic link between dietary nutrients

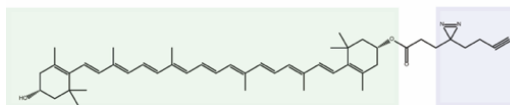
and immunity and leveraging unique nutritional interventions to overcome immune suppression, enhance anti-tumor immunity, and improve therapeutic outcomes in cancer and beyond.

4.2 Limitations

4.2.1 Identifying a specific target

Although we would like to improve the mechanistic understanding of ZEA's function of TCR signaling, obtaining direct evidence supporting the interaction between ZEA and the TCR complex is highly technically challenging. Despite the weak intrinsic fluorescence of ZEA and available protocol to image ZEA with confocal resonance Raman microscopy at the tissue level, imaging techniques are currently unable to achieve high-resolution visualization of ZEA at the cellular level [8–10]. In hope to gain more insights of ZEA's mechanism, we have developed two ZEA-derived probes to detect interaction between ZEA and proteins in the TCR complex on CD8⁺ T cells (Figure 4.2). However, two potential challenges of this approach include: (1) although the chemical synthesis is feasible, due to the unique structure of ZEA, the synthesis process, particularly the chiral resolution and purification, is complex and requires supercritical fluid chromatography (SFC). Thus, the synthesis takes more than 30 days and the quality of the final product is not 100% guaranteed. (2) even if we obtained results suggesting interaction use these probes, they would not support a direct binding between ZEA and key proteins of TCR complex as we cannot exclude the possibility that other protein factors might be part of the “super-complex” of ZEA-TCR, which mediate the association between ZEA and TCR complex.

1. Zea-derived electrophilic covalent probe
for pull-down and proteomics study



2. Zea-derived fluorescent probe
for confocal imaging study

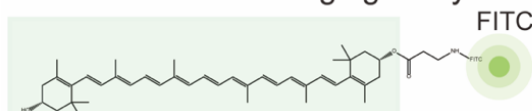


Figure 4.2 ZEA-derived probes designed to identify specific targets

4.2.2 Quantifying nutrients without stable isotope-labeled standards

To determine the exact concentration of ZEA and LUT in the plasma and in tumor-infiltrating fluids (TIF) of tumor-bearing mice orally administered with ZEA and LUT, stable isotope-labeled standards are typically used to correct for matrix effect, extraction variability and instrument fluctuations [11–13]. These standards co-elute with the target compounds to ensure accurate measurement [13]. However, since isotope-labeled carotenoid standards are not available for our analysis, we determined the relative abundance of ZEA and LUT in the TIF in comparison to mice orally administered with vehicle control to demonstrate that the carotenoids can accumulate in the tumor microenvironment and exert their functions. One of the future directions is to measure the absolute concentration of ZEA and LUT in the plasma and in TIF so that we can use the effective concentrations in preclinical models and the physiological values found in human circulation to gain a better understanding of a safe and effective range for ZEA to function. To quantify ZEA and LUT, the alternative strategies include standard addition approach, where we spike known amounts of the compounds into plasma to create a calibration curve to account for the matrix effects, and external calibration, where we generate the calibration curve in water or

methanol and spike-in known amounts of the compounds to compare expected vs. measured concentrations [14]. Multiple experiments would be needed to validate these approaches before applying them to our samples.

4.2.3 Dietary nutrient studies and their therapeutic applications

While dietary nutrients have demonstrated promising results as immunomodulatory agents, several key limitations must be considered when translating these findings into clinical applications. Factors such as bioavailability, whole-organism metabolism, long-term effects, and solubility would all influence the efficacy and practicality of using nutrients as therapeutic interventions. Understanding these limitations is critical for designing optimized nutrient-based interventions.

Bioavailability refers to how efficiently a nutrient is absorbed, distributed, metabolized, and utilized by the body [15]. Many bioactive compounds, including fatty acids and polyphenols, exhibit low oral bioavailability due to poor absorption in the gastrointestinal (GI) tract, first-pass metabolism in the liver, or degradation before reaching systemic circulation [16–18]. One of the advantages of our circulating nutrient library approach is that all the compounds included are present at quantifiable levels in circulation, meaning that they are stable enough to last and not completely degraded before reaching the bloodstream. However, differences in solubility and metabolism among these nutrients still present significant challenges for their effective delivery and biological activity.

While some compounds in the screen are water-soluble and readily bioavailable, ZEA is a fat-soluble carotenoid, and its absorption, stability, and bioavailability could be greatly influenced by its method of delivery [19,20]. Due to its lipophilic nature, ZEA is absorbed in the intestine via

micellar incorporation, a process that requires dietary fat and biliary salts [21]. However, to avoid confounding effects from exogenous lipid interactions, we did not include additional lipids for ZEA delivery in our research beyond the lipoproteins naturally present in fetal bovine serum (FBS) added to the culture media. This was done to ensure that any observed immune modulation was due to ZEA itself, rather than secondary interactions with dietary fats or emulsifiers. Due to this reason, the impact of lipid-based delivery on ZEA's immunomodulatory function remains unclear. It is unknown whether co-administration with common dietary lipids could enhance or later ZEA's mechanism of action, potentially influencing T cell activation and effector function. Future studies could explore whether co-administration *in vivo* with lipids and lipid-based delivery systems could enhance ZEA's bioavailability and bioactivity.

Another key challenge when working with carotenoids like ZEA and LUT is that they are often sensitive to light [22]. Therefore, we avoided light exposure during handling and storage of both ZEA and LUT. This presents an important consideration not only in research settings but also for supplement formulation.

4.3 Future Directions

4.3.1 Carotenoid-Membrane Interaction

Both ZEA and LUT are known for their roles as dietary supplements for eye health. ZEA and LUT are inserted into the membrane of retinal cells in a perpendicular or close to perpendicular manner due to their polar hydroxyl groups [23–25]. Given this property, we hypothesize that ZEA may similarly insert into the plasma membrane of CD8⁺ T cells, leading to biophysical changes that further influence their immune functions [26]. Since the TCR complex is formed by multiple membrane proteins, and its activation and clustering are strongly influenced by the membrane

properties, ZEA's insertion into the plasma membrane could alter membrane rigidity and lipid raft composition, resulting in an indirect modulation of the TCR complex for stronger downstream signaling [26]. As mentioned previously, the current technology is unable to image ZEA and LUT for their localization at the cellular level. Some alternative approaches to test this hypothesis would be testing the changes in membrane properties after *in vitro* ZEA and LUT treatment, using synthetic lipid bilayers to measure and compare ZEA and LUT incorporation, as well as checking for changes in TCR clustering after ZEA and LUT treatment [27,28]. If technically feasible, we could use cryoEM to look at changes in membrane proteins, especially the TCR complex, post-treatment or use other imaging techniques to obtain evidence that more directly demonstrates the carotenoid-membrane interaction [29].

4.4 Broader Implications

4.4.1 Combination effects of immunomodulatory nutrients

Leveraging synergistic interactions between immunomodulatory nutrients and bioactive compounds to enhance immune function is an additional strategy to enhance immune function beyond what individual nutrients can achieve alone [30]. In chapter 2, we explored the roles of a plant-derived nutrient ZEA and an animal-derived fatty acid TVA in promoting CD8⁺ T cell-mediated anti-tumor immunity through distinct pathways (Figure 2.4). Combining ZEA and TVA treatment resulted in a greater enhancement of CD8⁺ T cell cytokine production compared to their individual effects (Figure 2.4). This result highlights the potential combining nutrients for additive or synergistic effects to optimize immune responses.

To further examine this concept, we investigate the different combination effects of additional immunomodulatory nutrients identified by our lab as independent CD8⁺ T cell

modulators with less known mechanisms. We combined TVA or newly identified very long-chain fatty acid (VLCFA) nervonic acid (NVA) with long-chain fatty acid eicosadienoic acid (EDA). NVA is primarily found in nuts and seeds, fish oils, and plant oils, while EDA is mostly found in animal fats [31,32]. We hypothesize that these fatty acids modulate CD8⁺ T cell function through distinct pathways and that combining these bioactive compounds could lead to additive or synergistic effects on CD8⁺ T cell activation and cytokine secretion. Therefore, we built upon prior findings in the lab and explores whether specific combinations of nutrient compounds can fine-tune CD8⁺ T cell responses for improved anti-tumor activity.

To investigate the potential synergistic effects of immunomodulatory fatty acids TVA, NVA, and EDA, we evaluated various combination schemes and their impact on CD8⁺ T cell effector function assessed by TNF α production (Figure A1). When TVA (10, 20, and 40 μ M) was combined with EDA (15 μ M), we observed a significant increase in TNF α production compared to each nutrient alone at that concentration (Figure A2). We performed combination index (CI) analysis using Bliss-Independence Model and all three TVA combinations with EDA resulted in a synergistic effect, as indicated by a CI value below 1 (Figure A2). Combining NVA (5, 10, 20 μ M) treatment with EDA (15 μ M) also resulted in a significant enhancement of TNF α production compared to single nutrients at the same concentration (Figure A3). Combination index (CI) analysis using Bliss-Independence Model also showed a synergistic effect in all three NVA combinations with EDA, as indicated by a CI value below 1 (Figure A3). These findings highlight the potential of nutrient combinations to enhance immune function through synergistic metabolic or signaling interactions.

Here, we explored the combination effects of the natural dietary long-chain fatty acid immunomodulator EDA, with either TVA or NVA. All three fatty acids (TVA, NVA, and EDA)

have been identified as independent CD8⁺ T cell activators by our lab. While TVA's mechanism has been characterized, the mechanisms of EDA and NVA remain less understood. However, the enhanced immune responses observed with their combination suggests that they likely function through distinct pathways, complementing each other to further optimize CD8⁺ T cell activation and effector function.

Beyond single nutrient optimization through SAR studies, our findings show that combining distinct immunomodulatory nutrients provides an alternative strategy to enhance T cell function. The synergistic effects on cytokine production suggest that different nutrients may act through complementary metabolic or signaling pathways. Our study demonstrates opportunities for strategizing nutritional interventions to optimize immune responses and supports the concept of a balanced diet is critical for the most benefits. In fact, both synergistic and antagonistic results from nutrient combinations can give us valuable insights on the interactions between natural compounds and their mechanisms of actions. While our *in vitro* combination of nutrients studies suggests promising improvements in CD8⁺ T cell effector function, *in vivo* mouse models are essential to assess whether the synergistic effects we have observed translate into more efficient tumor attenuation without affecting the overall health of the mice.

4.4.2 Nutrients vs. whole foods vs. diets

This dissertation focuses on deciphering the mechanistic links between single nutrients and immune function, providing insights into how specific bioactive molecules influence CD8⁺ T cell function. While studying individual nutrients or synergism among a few nutrients allows for more precise mechanistic understanding, it is important to consider the broader dietary context, including the role of whole foods, dietary patterns, the method of cooking in shaping immune

responses [33,34]. The challenge lies in determining how isolated nutrients function within complex biological systems and whether their effects are influenced when consumed as part of a whole food or dietary regimen.

Research on single nutrients involves studying their effects in isolation, allowing us to identify specific targets and signaling pathways as well as specific functional outcomes. However, in real-world settings, nutrients do not act alone. For example, fat-soluble nutrients like ZEA and vitamin E require lipid co-factors for optimal absorption, whereas nutrients could interact with the gut microbiota to produce bioactive metabolites that influence the immune function or directly impacting the microbiota composition for an effect [35–37]. These interactions highlight the need to consider our diet as dynamic systems, rather than just collections of isolated nutrients.

The overall macronutrient composition has been associated with different immune responses. For example, diets rich in healthy fats, fiber, and phytonutrients, such as the Mediterranean diet, have been linked to enhanced immune function and reduced inflammation [38]. On the other hand, Western diets high in processed sugars and saturated fats can promote chronic inflammation and immune dysfunction [39]. These correlations suggest that the composition of the diet, rather than just individual nutrient intake affects the overall immune health.

Dietary patterns may also play a role in modulating immune responses. Studies on intermittent fasting, time-restricted feeding, and caloric restriction suggest that when nutrients are consumed can be just as important as what is consumed [40]. For example, nutrients involved in T cell metabolism may exert different effects on whether they are ingested in a fed or fasting state. Similarly, caloric restriction has been shown to enhance immune surveillance and reduce age-related immune decline [41].

While this dissertation primarily focuses on understanding the impacts and molecular mechanisms of individual nutrients, these findings should ultimately be contextualized within the broader dietary composition and patterns. Future research could explore how feeding patterns, food matrices, and long-term dietary habits influence immune function to bridge the gap between mechanistic discovery and real-world application.

4.4.3 Balancing immune amplification and autoimmune risks

The potential to enhance immune function through nutrient-based strategies presents both therapeutic opportunities and challenges, particularly in striking a balance between boosting anti-tumor immunity and avoiding autoimmunity or excessive inflammation [42]. While optimizing CD8⁺ T cell effector functions is beneficial for cancer immunotherapy, it is crucial to ensure that these approaches do not inadvertently drive autoreactive T cell activation, which could lead to autoimmune complications [43,44]. A potential strategy is to explore targeted delivery mechanisms to restrict interventions to the tumor microenvironment and confine immune activation to cancerous tissues. Understanding the determining factors that distinguishes effector T cells that attack infected or cancerous cells from autoreactive T cells is also crucial for developing strategies that are specific to the abnormal cells and do not provoke off-target immune activation that enhances the risks for systemic autoimmunity.

To determine whether ZEA induces unintended systemic immune activation, we assessed cytokine levels in healthy mice following oral supplementation. Here, 20 mice were randomly assigned to receive either ZEA (500 mg/kg b.w.) or vehicle control, and we measured relative plasma levels of key pro- and anti-inflammatory cytokines. As shown in Figure B in the appendix, there are no significant differences in cytokine levels between ZEA-supplemented and control

mice, indicating that ZEA does not elicit a detectable systemic cytokine response in the absence of cancer or immune stimulation.

4.4.4 Applications beyond cancer

While our research primarily focuses on enhancing CD8⁺ T cell function for cancer immunotherapy, the principles of nutrients as immunomodulators could extend beyond oncology. The ability to precisely tune immune responses has implications for autoimmune diseases, chronic inflammatory conditions, infectious immunity, and age-related immune decline or immunosenescence [45–47]. These broader applications emphasize the need for enhanced understanding of the mechanistic links between immune cells and immunomodulatory agents as well as novel strategies to fine-tuning immune responses under diverse conditions.

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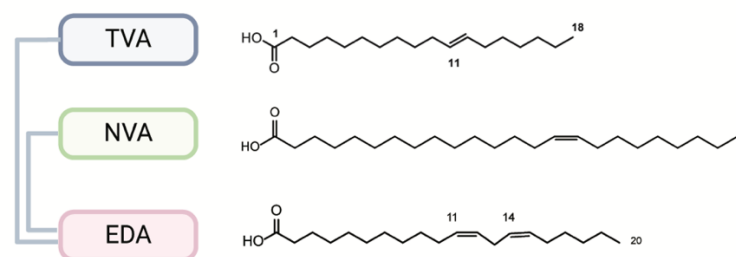
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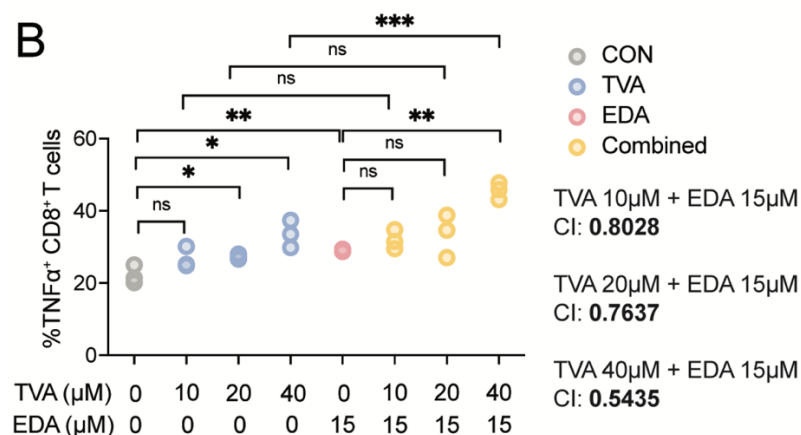
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APPENDIX A: Combination of immunomodulatory nutrients results in synergistic effects

A



B



C

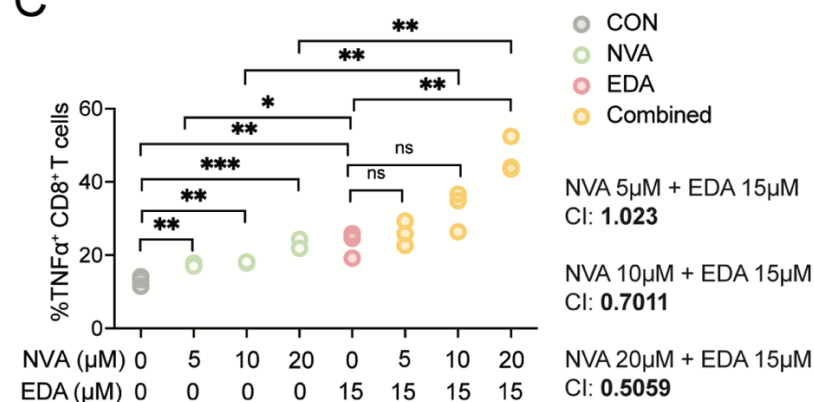


Figure A Combinations of TVA, NVA, and EDA promotes CD8⁺ T cell effector function

(1) Schematics of combination strategy and fatty acid structures. (2) Effects of TVA (10, 20, 40 μ M) and EDA (15 μ M) on murine CD8⁺ T cell effector function assessed by TNF α levels individually and in combination ($n = 3$). Combination index calculation is based on the Bliss-Independence Model. CI < 1 indicates synergy. (3) Effects of NVA (5, 10, 20 μ M) and EDA (15 μ M) on murine CD8⁺ T cell effector function assessed by TNF α levels individually and in combination ($n = 3$). Combination index calculation is based on the Bliss-Independence Model. CI < 1 indicates synergy.

APPENDIX B: Effects of ZEA supplementation on plasma cytokine levels in healthy mice

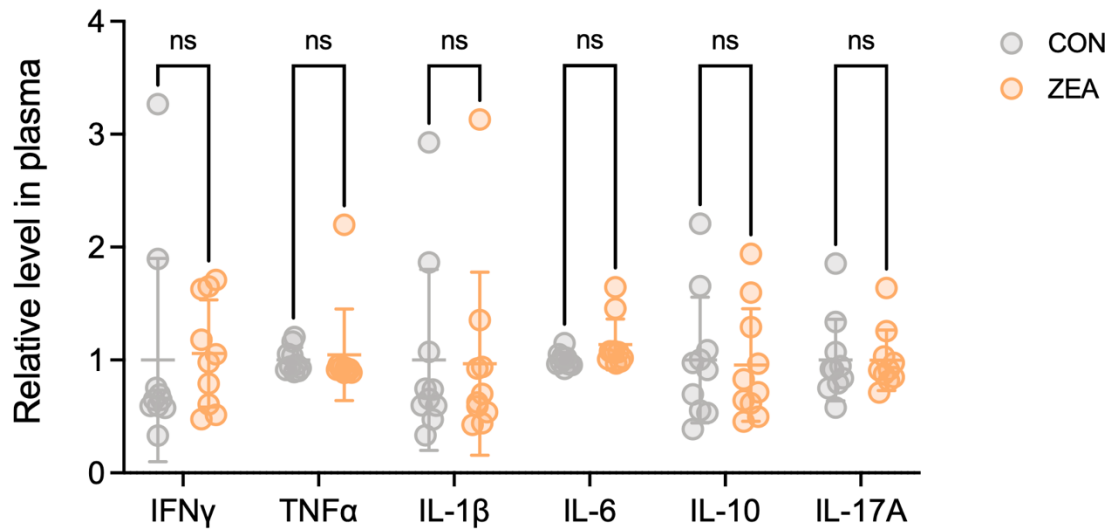


Figure B Oral ZEA supplementation does not impact systemic cytokine levels in healthy mice

Twenty healthy mice were randomly assigned to ZEA or control (CON) groups ($n = 10$). Mice were under oral ZEA (500 mg/kg b.w.) or vehicle control supplementation for a week, then evaluated for relative cytokine levels using LEGENDplex mouse inflammation panel.