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IMPACT *OF CK2αWT* IN LYMPHOCYTE DEVELOPMENT WITHIN NEUROBLASTOMA

by

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DEDICATION

To my parents, Conrad and Tabitha Thomas whose unwavering love, support, and encouragement have been my greatest strength. Their belief in me, even during the most challenging times, has been a constant source of inspiration. This work is as much a reflection of their sacrifices and dedication as it is of my own. Thank you for always being there, guiding me, and believing in my potential.

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IMPACT *OF CK2αWT* IN LYMPHOCYTE DEVELOPMENT WITHIN NEUROBLASTOMA

MELODY THOMAS

ABSTRACT

Neuroblastoma (NB) is an aggressive pediatric cancer associated with aberrant oncogenic *MYCN* amplification in ~50% of high-risk cases. The amplification of *MYCN* helps promote a “cold” microenvironment, characterized by fewer CD8+ tumor-infiltrating lymphocytes, making this cancer more difficult to treat. This research utilizes zebrafish overexpressing the *alpha* subunit of casein kinase II (CK2) in lymphocytes (*CK2αWT)* and zebrafish MYCN-driven NB model to unravel the complex regulation of protein kinases on lymphocyte development. *CK2* overexpression leads to an initial decreased number of lymphocytes in the thymus yet delayed thymus involution. We also observed increased CD8+ and IgM+ cells in the kidney (bone marrow equivalent in fish) of *CK2αWT* fish in the absence of tumor development. The findings indicate altered lymphocyte development driven by CK2. Future studies are needed to further elucidate the functional relationship of CK2 and lymphocyte development and its contribution to MYCN-driven immune evasion, to develop therapeutic strategies for the treatment of NB and similar cancers.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
APC	Antigen-presenting cell
B-ALL	B-cell acute lymphoblastic leukemia
BCR	B cell receptor
Breg	Regulatory B cell
CK2	Casein kinase II
CKLF	Chemokine-like factor
DC	Dendritic cell
DPF	Days post-fertilization
GC	Germinal center
HPC	Hematopoietic precursor cell
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPF	Months post-fertilization
NB	Neuroblastoma
NK	Natural killer
PDL-1	Programmed death ligand 1
PI3K	Phosphatidylinositol-3 kinase
PTEN	Phosphatase and TENsin homolog
ROS	Reactive oxygen species

ScRNA-seq	Single-cell RNA sequencing
TAA	Tumor-associated antigen
TAP	Tumor-associated peptide
TCR	T cell receptor
TH1	T helper 1
TH2	T helper 2
TIL	Tumor-infiltrating lymphocyte
TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
Treg	Regulatory T cell
TSA	Tumor-specific antigen
WPF	Weeks post-fertilization
αα	Alpha-alpha
αβ	Alpha-beta
γδ	Gamma-delta
κƛ	Kappa-lambda
μ	Mu

INTRODUCTION

Introduction to Neuroblastoma (NB)

Neuroblastoma (NB) is a detrimental, highly heterogeneous pediatric cancer. It is the most common extracranial solid tumor in children [Chung, 2020], that accounts for 8–10% of all pediatric tumors and 15% of all pediatric deaths [Jiang et al., 2011]. NB is derived from the sympathoadrenal lineage of neural crest cells, arising from the dorsal region of the neural tube in the ectoderm [Pajanoja, et al., 2023], and most commonly occurs in the abdominal sympathetic ganglia or adrenal medulla [Bansal et al., 2022]. Among the various genetic alterations that contribute to NB, *MYCN* amplification stands out as a critical factor, driving tumor aggressiveness, increasing the likelihood of cancer reoccurrence, and is an indicator of poor patient outcomes.

The MYC proto-oncogene family consists of three paralogs: *c-MYC, MYCL, and MYCN* [Ruiz-Perez, Henley and Arsenian-Henriksson 2017]. Of those with high-risk NB, 50% of patients have aberrant expression of *MYCN* [Otte et al., 2021]. Adding to the complexity of the disease, the MYC family is essential for embryonic development. 9.5-day-old mouse models are shown to have relatively high levels of *MYCN* in the fetal brain, kidney, and neural crest [Zimmerman et al., 1986], and targeted deletion of *MYCN* in mouse neuronal cells caused ataxia, growth retardation, and behavioral abnormalities [Knoepfler et al., 2002]. Additionally, NBs are considered immunologically “cold” tumors, which hinders diagnosis and treatment, with over 60% of cases being metastatic [Cheung and Dyer 2013].

Tumor microenvironment (TME) of NB

Tumors are described as immunologically “hot” or “cold” depending on the presence, density, and arrangement of immune cells within the tumor microenvironment (TME) [Ouyang et al., 2024]. Cold tumors tend to have low amounts of tumor-infiltrating lymphocytes (TILS) [Qin et al., 2024] and remain resistant to therapies while “hot” tumors are characterized by increased immune activity, immune cell infiltration, and response to immune checkpoint inhibitors [Khosravi et al., 2024]. Additionally, the TME tends to be hypoxic in nature further exasperating immunosuppression [Heintzman et al., 2022], sometimes resulting in less than 2% oxygen levels surrounding the TME [Jing et al., 2019] compared to physiologically, healthy tissues which are composed of more than 3–19% [Zenewicz., 2017].

The TME is an extremely heterogeneous, complex ecosystem composed of immune cells, cancer cells, blood vessels, and extracellular matrices [Lei et al., 2020]. While the composition of TME differs between various cancer types, the big picture is the same: it is a battlefield. Among these components, immune cells which make up at least 50% of the cellular component, play a crucial role in influencing tumor behavior [Anderson and Simon 2020]. According to previous studies, immune cells are abundant within the TME and significantly contribute to both tumor progression and treatment response. Their interactions within the environment can either promote or inhibit tumor growth depending on the context. Given their significant role in tumor surveillance, immune cells are a key cellular component of the TME and are essential in shaping the tumor dynamics.

Immune cells present in the TME

Immune cells can be classified into two main types: innate and adaptive. Innate immunity is the body's first line of defense and provides rapid, non-specific responses [Vivier and Malissen 2004]. Innate cells include both myeloid and lymphoid lineage cells such as dendritic cells (DCs), granulocytes, monocytes, macrophages, and natural killer (NK) cells [Wang et al., 2024]. These cells are generally not "tumor supportive" or "tumor opposing" as their response to cancer is cancer context dependent. In their tumor-supportive state, macrophages produce reactive nitrogen species, reactive oxygen species (ROS) or secrete cytokines that induce proliferation of inflammation associated cancers [Maiorino et al., 2022] and can recruit immunosuppressive cells, such as regulatory T cells (Tregs), further dampening the immune response [Zhu et al., 2025]. These cells are also able to mediate cancer development and promote host defense primarily through detecting and capturing tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) and trigger the adaptive immune response [Yi et al., 2023]. Adaptive immunity is learned through experience or exposure to antigens and mainly consists of T and B cells [Wang et al., 2024]. These cells can either be pro or anti-tumor. Among the various cells, NKs and T cells are most readily involved in eliminating cancer cells [Maggi et al., 2024]. Current research tends to focus on the role of T cells due to their essential role in immune responses and their ability to directly kill tumor cells through the secretion of cytotoxic granules and lytic molecules.

T cells

There is a large repertoire of T cell phenotypes determined by the dimerization of their T cell receptors (TCRs). Alpha-beta heterodimerization (αβ) is the most common chain composition [Matos et al., 2017], moreover, T cells can also homodimerize and form an alpha-alpha (αα) TCR. CD8+ T cells are typically anti-tumor [Want et al., 2023] and have the ability to recognize tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) through their unique αβ TCRs [Hwang et al., 2020]. Tumor recognition is activated when the receptors bind to major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) [De Visser and Joyce 2023] they enable the recognition of tumors. CD4+ helper T cells, which also usually express αβ TCRs [Hwang et al., 2020], influence other immune cells, specifically CD8+ T cells. They can either be anti-tumorigenic in the case of T helper 1 (TH1) cells or pro-tumorigenic as mentioned above in the case of Tregs or T helper 2 cells (TH2) [Anderson and Simon 2020]. There is also a small subset of T cells that are classified as gamma-delta (γδ) that are rich in peripheral tissue [Ribot et al., 2021] and connect the innate and adaptive immune systems [Ren et al., 2022].

There are some paramount differences between αβ and γδ T cells. Ɑβ T cell activation is limited by the confines of MHC recognition. Conversely, γδ T cells are more versatile in their antigen recognition and can respond to a broader range of stimuli, since they do not need MHC presentation to become active, especially in contexts like tumor surveillance, infection, and tissue stress, but they can also become APCs [Hu et al., 2023; Shah et al., 2021]**.** Both αβ and γδ T cell infiltration has shown positive correlation in various cancer types and have oncolytic potential [Arias-Badia et al., 2024]. The ability for T cells to recognize tumor antigens is crucial for patient survival as it is a central feature of many cancer immunotherapies [Andersen., 2023]. This is typically done through their TCRs which can recognize various MHC class presentation, tumor-associated peptides (TAPs), or neoantigens [Xie et al., 2023]. However, sometimes the ability to recognize tumor cells fails.

In NB several issues arise with the T cells. Not only are there fewer TILS, such as cytotoxic CD8+ cells [Qin et al., 2024], but the T cells that are present are marginally ineffective. Several factors could potentially contribute to this phenomenon, such as chronic stimulation of T cells leading to exhaustion, the inability to recognize TAAs, high expression of immune checkpoint molecules such as programmed death ligand 1 (PD-L1) [Wienke et al., 2021] or a transcriptomic shift that causes T cells to transform from TH1 to TH2 cells. T cells are well-established players in the immune response, particularly in their role in recognizing and targeting tumor cells. While it is known how T cell-mediated immunity contributes to the TME, the influence that B cells have has not yet been elucidated.

B cells

B cells are an essential part of the adaptive immune system that make antibodies (Abs) which bind to the antigens via their B cell receptor (BCR) [Roberts et al., 2020]. There are multiple types of B cells, such as transitional B cells, which act as transitional states between immature and mature B cells as they travel between the bone marrow and secondary lymphoid tissues. Memory B cells circulate through the bloodstream post-disease and help build long-term immunities through the memory of antigens. Plasma cells or plasmacytes, release antibodies in response to antigen benign present, and B1 and B2 cells which produce a small repertoire of Abs, are more associated with early host defense and play a much larger role in bacterial infection and are involved in the adaptive immunity, respectively [Prieto and Fillipe., 2017]. B cells develop from hematopoietic precursor cells (HPC) in the kidney marrow of zebrafish, in the fetal liver, and in the fetal bone marrow of humans. There are three major developmental stages for B cells. In stage, 1 the “pro-B cells” rearrange the D and J segments of their H chain which is followed by a rearrangement of the upstream V region. After this “VDJ rearrangement”, the cells are considered “pre-

B cells”. The second stage known as the "pre-B cells" is a result of 1–2 cell divisions occurring along with the rearrangement of the gene segments encoding kappa and lambda chains (κƛ) [Pieper et al., 2013]. Once the κƛ chain is combined with the mu (μ) chain, an IgM molecule and pre-B cell receptor are formed and expressed on the cell surface. This is known as an immature B cell and marks the end of the second developmental stage [Cantor et al., 2019]. Once the immature B cells leave the bone marrow they enter the last major developmental stage. In humans, they migrate to the spleen where they will finalize early development, and further differentiate into naive, follicular, or marginal zone B cells [Pieper et al., 2013] and become a part of the peripheral B cell population. Humans also have a distinct subset population of B cells that reside in the thymus, and while these cells come from the same progenitor cells as peripheral B cells, their location for maturation is unique [Perera and Huang., 2015].

The role of B cells in NB is not well understood. B cells can have both anti-tumor and pro-tumor effects. They can act as anti-tumor by producing Abs, secreting cytokines, or generating long-lasting immune memory that results in a sustained immune response [Kinker et al., 2021]. They can also be pro-tumor through immunosuppressive B cells or regulatory B cells (Bregs) [Weißenborn et al., 2022]. Understanding the roles of these cells in the context of cancer is crucial for developing more effective treatments.

There are slight differences between B cell development in mammals and zebrafish. Zebrafish lack bone marrow and lymph nodes; thus, B cell development occurs in the head kidney marrow (which some consider the equivalent to bone marrow in mammals [Miao et al., 2021; Liu et al., 2017]), pancreas [Danilova and Steiner 2002] and a small population of B cells that migrate to the thymus for continued maturation [Perera et al., 2013]. While mammalian immunoglobulins include: IgM, IgD, IgG, IgE, and IgA [James 2022], only IgM, IgD, and IgZ are found in zebrafish [Miao et al., 2021]. It takes approximately 21 days post fertilization (dpf) for B cells to appear in the lymphoid organs in zebrafish and 7 weeks post conception to appear in the human fetal liver [Jackson et al., 2021].

Determining whether the TME is “hot” or “cold” is greatly influenced by several factors; and one of those being B cells. When B cells internalize tumor antigens and present their MHC class II complex to CD4+ T cells, T cells are able to start a cascade effect which ultimately leads to an antitumor response [Xue et al., 2024]. B cells can inhibit immune T cell and NK cell responses through the production of suppressive cytokines such as IL-10, TGF-B, or IL-35 [Leong and Bryant 2021]. This duality underscores the complexity of cell-to-cell interactions and the importance of B cells in tumor pathology. Protein kinases play a similarly critical and multifaceted role in cancer biology.

The regulatory role of protein kinases

Protein kinases are enzymes that regulate key signaling pathways involved in cell growth, survival, and metastasis, making them pivotal in the development and progression of tumors. These enzymes are vital in regulating multiple cellular processes, enabling phosphorylation cascade events through phosphorylation transcription factors or receptor proteins [Cipak., 2022; Manning et al., 2002]. Protein kinase research has traditionally focused on phosphatidylinositol-3 kinase (PI3K)/Akt/mTOR and Ras/mitogen-activated protein kinase (MAPK) pathways [Smiles et al., 2023], however the role of casein kinase II (CK2) is known to be dysregulated across multiple cancers [Firnau and Brieger., 2022; Zhou et al,. 2021]. CK2 mutation is implicated in altered Hedgehog signaling, (PI3K)/Akt, inactivation of Phosphatase and TENsin homolog (PTEN), a tumor suppressor, and activation of proto-oncogenes in various cancers [Chua et al., 2017]. Thus, the role of CK2 in NB survival and progression is poorly understood.

The purpose of this study

In this study we utilized *Danio rerio*, zebrafish, to investigate how CK2 modulation in both T and B cells impacts tumor immunity and development, and the underlying mechanisms of CK2 in B cell development.

The benefits of a zebrafish model

Zebrafish make an excellent model to study NB for several reasons. First, tumor progression occurs more rapidly in zebrafish than in mice. NB tumor detection takes 9–13 weeks in mice [Teitz et al., 2011], while it can be detected in as little as 2 dpf in zebrafish [Corallo et al 2016]. Second, zebrafish have huge egg clutch sizes and reproduce very quickly. At peak sexual maturity, they can produce 200–300 eggs weekly [Liew and Orban., 2013]. Third, lymphocyte development is conserved between humans and zebrafish [Bajoghli et al., 2019; Liu et al., 2017].

Similar to humans, zebrafish demonstrate innate and adaptive immunity and have two primary lymphoid organs, the kidney marrow and thymus, which also shrink with age as in humans [Miao et al., 2021]. Additionally, NB development in zebrafish is analogous to human NBs histologically, immunologically, and microstructurally [Corallo et al., 2016]. Finally, approximately 80–82% of disease-related human genes have an orthologous counterpart in zebrafish [Miao et al., 2021; Sakai et al., 2018].

METHODS

Zebrafish husbandry

Zebrafish husbandry and care was performed by the Chobanian & Avedisian School of Medicine aquatic facility with approved protocols from the Institutional Animal Care and Use Committee.

Subcloning

To generate F0 founder fish (*tg(lck:CK2αWT)*), *lck:CK2* construct**,** human cDNA of wild-type *CK2α (CK2αWT)* was amplified using PCR from pZW6 plasmid vector (AddGene, Watertown, MA, USA). PCR products were subsequently cloned into the *I-SceI-lck/pKS* or *I-SceI-rag2/pKS* vector, containing a zebrafish *lck* or *rag2* promoter sequence and flanked by the I-SceI endonuclease recognition sites. The forward primer contained an Age-I enzyme site and *CK2α* translation start sequence. The reverse primer contained the *CK2α* translation termination codon and a ClaI enzyme site. PCR-purified fragments were digested with AgeI-HF and ClaI restriction enzymes and then cloned into the respective *I-SceI/pKS* vector through AgeI-HF and ClaI sites. PCR primer pairs used for amplification of *CK2α* cDNA are listed in table 1 [Zhu et al., 2021].

Zebrafish line generation and genotyping

To generate F0 founder fish, the *I-SceI-lck:CK2αwt-I-SceI* and *I-SceI-rag2:CK2αwt-I-SceI* constructs were microinjected with meganuclease (New England Biolabs, Ipswich, MA, USA) into *nacre (mitfa-/-)* single-cell zebrafish embryos. The *I-SceI-lck:CK2αwt-I-SceI* construct was co-injected with the *I-SceI-rag2:mCherry-I-SceI* construct. The founder fish were obtained from the A. Thomas Look Laboratory at Dana-Farber Cancer Institute and outcrossed with AB wild-type background zebrafish, and their progeny were screened for the presence of human *CK2α* gene by gene-specific PCR using the primers listed in table 2 to create the *tg(lck:CK2; rag2:mCherry; lck:EGFP)* fish line as previously described [Rembold et al., 2006]. To generate the *tg(dβh:MYCN;dβh:EGFP*;*rag2:mCherry; lck:EGFP*) and *tg(dβh:MYCN: lck:CK2; rag2:mCherry; lck:EGFP)* fish lines, *tg(dβh:MYCN) fi*sh were crossed with either *tg(rag2:mCherry; lck:EGFP*) or *tg(lck:CK2; rag2:mCherry; lck:EGFP)* respectively.

Resulting embryos were raised and screened at 21 dpf under a fluorescent dissecting microscope (Olympus) to identify EGFP-expressing cell masses in the neural crest region to confirm the presence of the MYCN transgene as well as EGFP and RFP-expressing cell masses in the thymic region to be able to visualize T cells and B cells respectively. At 6 weeks post-fertilization (wpf), the sorted *tg(MYCN;EGFP;lck:EGFP;rag2:mCherry)* or *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* fish had 1–3 mm of their fins-clipped to extract genomic DNA for PCR amplifications using gene-specific primers and Taq DNA polymerase (New England Biolabs) to identify fish with the mutant *CK2αWT* gene. *Tg(lck:CK2αWT;lck:EGFP;rag2:mCherry)* or *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* fish were monitored and imaged weekly for thymus development and quantified using ImageJ software (National Institute of Health, NIH).

Fluorescence microscopy

Embryos and fish were imaged by fluorescence microscopy at 20x magnification (Echo Revolve) and quantified using ImageJ software (National Institute of Health, NIH) [Qin et al., 2024].

Confocal imaging

Fish were euthanized as described in Institutional Animal Care and Use Committee protocols, subsequently fixed in 4% paraformaldehyde (Thermo Fisher Scientific) at 4°C overnight with gentle agitation, washed with phosphate-buffered saline containing 0.1% Tween 20 (Thermo Fisher Scientific), equilibrated in 30% sucrose at 4°C overnight, and frozen at −80°C. The frozen fish were embedded in 1% low-melting agarose (Life Technologies) and in optimal cutting temperature compound (Sakura Finetek) and were imaged using LSM 710-Live Duo Confocal microscope (Zeiss).

Flow cytometry

Tumors were dissected under a fluorescent microscope (Olympus) and dissociated in a mixture of RPMI 1640 (Corning), 0.025 µg/ml Liberase (Roche), 0.6µg/ml DNase I (ThermoFisher), and 1x Pen/strep (Corning), washed in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Sigma) and filtered with a 40-µm filter (Falcon). Fish kidneys were dissected, dissociated, and filtered in RPMI 1640 supplemented with 10% FBS. Single-cell suspensions were stained with the primary antibodies: rat anti-fish CD4 (1:100, Clone 6D1, Bio Cosmo, CAC-NIH-NA-01), CD8 (1:100, Clone 2C3, Bio Cosmo, CAC-NIH-NA-02)(*45-47*), or rabbit/mouse anti-fish IgM (1:100, ThermoFisher) in PBS containing 10 U/mL Heparin (Sigma), 10% FBS, and 1x Pen/strep, for 30 min at 4°C. Cells were then stained with the secondary antibody, goat anti-rat APC (1:500; Invitrogen, A10540), goat anti-human (1:200; Jackson Immuno, 109-136-170), for 30 min at 4°C, and counterstained with DAPI (1:5,000, ThermoFisher, 62248) or FITC anti-rabbit IgG (1:400; Thermofisher) for 1 hour at 4°C. Analysis was performed on an LSRFortessa flow cytometer (BD Biosciences), and Fluorescent-Activated Cell

Sorting (FACS) was performed on a FACSAria II (BD Biosciences) [Qin et al., 2024].

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 using a log-rank test for Kaplan-Meier and unpaired two-tailed t-tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

CK2 overexpression in lymphocytes prolongs immune cell presence in the thymus

Previous research has demonstrated CK2, is ubiquitously expressed in eukaryotic cells and is needed for immune cell development, proliferation, and activation [Zho et al.] However, its role in NB has yet to be elucidated. Interestingly, previous research has demonstrated that knockdown of the CK2*α* subunit in mice increases some subsets of lymphocytes (e.g. CD4+ and B cells) but not all (e.g. CD8+) [Wei et al., 2021]. Given this complex relationship, it was important to further investigate how CK2 impacts immune cell development.

To characterize the role of CK2 in T and B lymphocytes, first, we overexpressed human CK2 (*CK2αWT*) under the zebrafish *lck* promoter, which is expressed in both T and B zebrafish lymphocytes. Specifically, co-injection of *tg(lck:CK2αWT)* and *tg(rag2:mCherry)* at the single-cell stage allows researcher to monitor the development of both T and B cells, since *rag2* recombination is essential for VDJ arrangement in both T and B cells [Moshe., 2001]. Since the thymus is one of the main locations for lymphocyte development and maturation, it was essential to investigate how the thymus is impacted. Normally the zebrafish thymus shrinks during puberty [Miao et al., 2021], which is around 4 months post-fertilization (mpf) [Lam et al., 2002]. To assess the impact of CK2 overexpression, utilizing fluorescence microscopy, we imaged 6 wpf and 8 mpf zebrafish. As expected, imaging revealed green and red fluorescence in *tg(lck:EGFP)* and *tg(rag2:mCherry*) controls as well as in tg(lck:*CK2αWT;rag2:mCherry)* 6 wpf zebrafish. However, we found that *CK2αWT* caused lasting red fluorescence in the thymus of 8 mpf zebrafish as well, which is not observed in *tg(lck:EGFP)* or *tg(rag2:mCherry)* controls (figure 1) and suggests *CK2αWT* promotes survival of lymphocytes in the thymus.

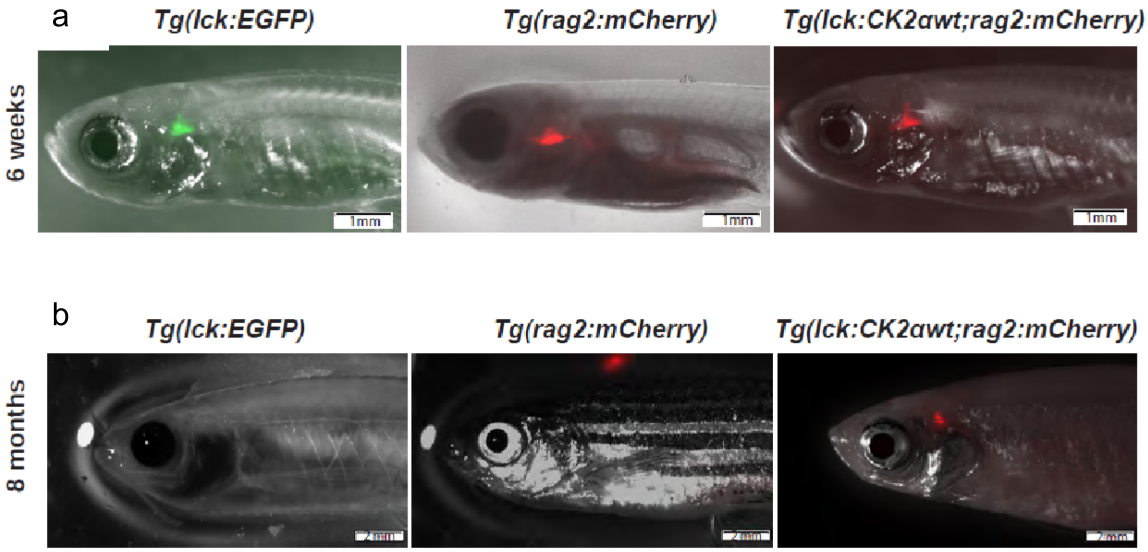

Figure 1*:* CK2 overexpression in lymphocytes prolongs immune cell presence in the thymus

Representative images of control enhanced green fluorescent protein in transgenic *tg(lck:EGFP)* (left panels), red fluorescent protein in transgenic *tg(rag2:mCherry*) (middle panels) zebrafish and overlay of transgenic *tg:(lck:CK2αwt;rag2:mCherry*) (right panel) of zebrafish at 6 wpf (a) or 8 mpf (b). n=6

Scale bars in (a) = 1 mm and (b) = 2 mm.

Data collected by Kelly Miao

CK2 overexpression increases the percentage of Cd8+ and IgM+ lymphocytes in the kidney

To further unveil how lymphocyte development is impacted by CK2 overexpression, flow cytometry was performed on 1.5-year-old fish. We detected a statistically significant increase in the percentage of Cd8+ cells in CK2 overexpression zebrafish compared to AB, wild-type siblings (figure 2a). There was also a prominent increase in the percentage of IgM+ cells present in CK2 overexpression compared to AB, wild-type siblings (figure 2c). Overexpression of *CK2αWT* did not impact the percentage of Cd4+ cells in the kidney of 1.5-year-old zebrafish (figure 2b).

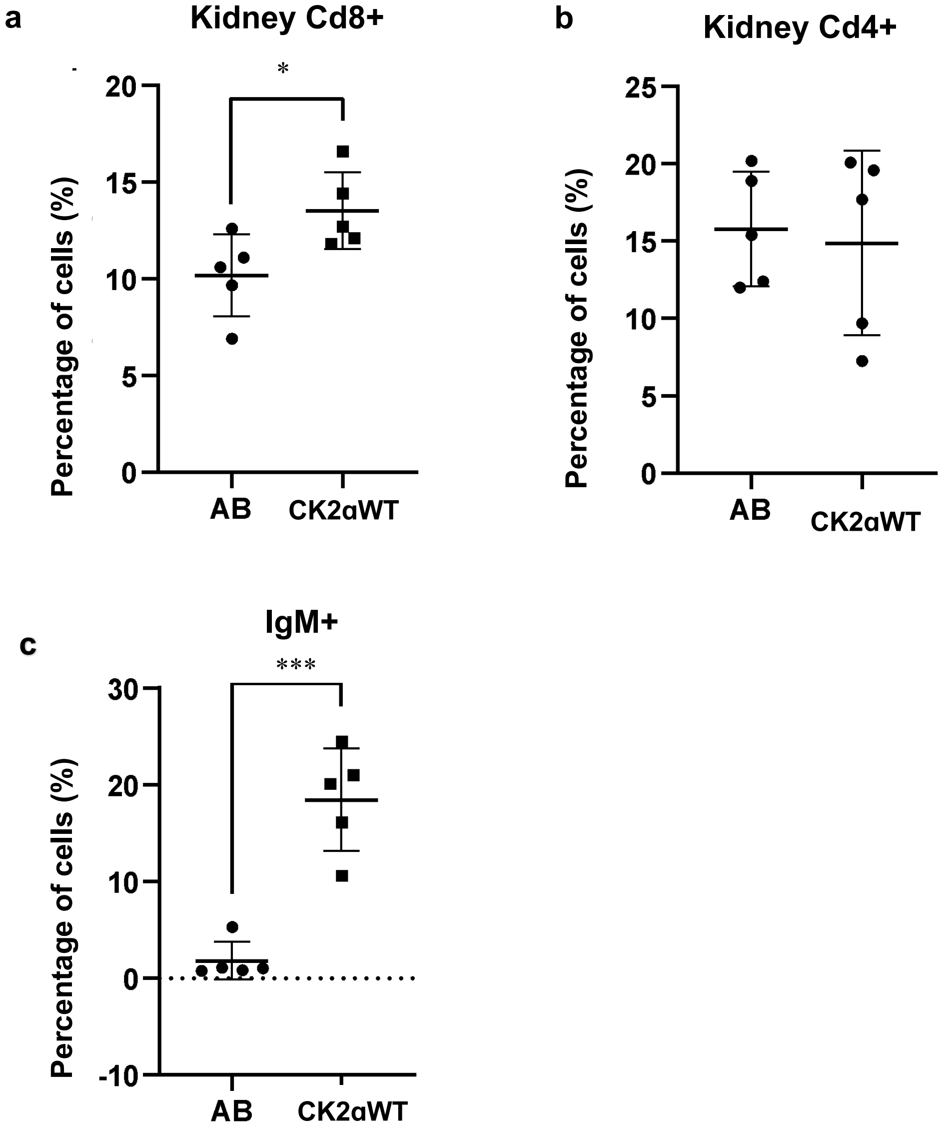

Figure 2: CK2 overexpression increases the percentage of Cd8+ and IgM+ lymphocytes in the kidney

Flow cytometric analysis of lymphocytes in the kidney of 1.5-year-old *tg(lck:CK2αWT*) or AB zebrafish. (a) Cd8+, (b) Cd4+, (c) IgM+. (n=5)

Data collected by Kelly Miao

CK2αWT decreases the presence of thymic immune cells in MYCN overexpression zebrafish model

Given CK2 overexpression led to a shift in the percentage of lymphocytes in the kidney, altered fluorescence intensity in the thymus of 1.5-year-old zebrafish, and considering that CK2 is frequently dysregulated in multiple cancers [Firnau and Brieger., 2022; Zhou et al., 2021], these changes also highlight the important of studying CK2's effect within a cancer context. In MYCN-driven NBs overexpressing various kinases have more aggressive tumors, are characterized by a decrease in the number of TILs, and have poor response to immune checkpoint inhibitors [Ouyang et al., 2024]. Interestingly, prior studies have shown that CK2 itself is not an oncogene [Chua et al., 2017]. To better understand the role of CK2 in this setting, we utilized a MYCN-driven NB model which is both genetically and molecularly similar to human NB to investigate how CK2 overexpression in lymphocytes influences lymphocytes in the thymus in a cancer context.

Fluorescence microscopy performed on 21 dpf fish revealed immune cells present in the thymus in *tg(MYCN;EGFP;lck:EGFP;rag2:mCherry)* control zebrafish and *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* zebrafish, although both *tg(lck:EGFP)* and *tg(rag2:mCherry)* fluorescence appeared dimmer in the *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* group compare to the control (figure 3).

Taken together with figure 2, immune cells could be simply drawn away from the TME and thymus. Additionally, because NB is described as an immunologically “cold” tumor [Cheung and Dyer 2013], there could be secretion of chemokines, prompting a tumor proliferative environment through inhibition of TILs. Previous studies have unmasked that MYCN-driven NBs secrete chemokine-like factor (*CKLF)*, attracting immunosuppressive CD4+ Tregs [Qin et al., 2024], which could downstream inhibit the additional proliferation of T cells [Sojka et al., 2008]

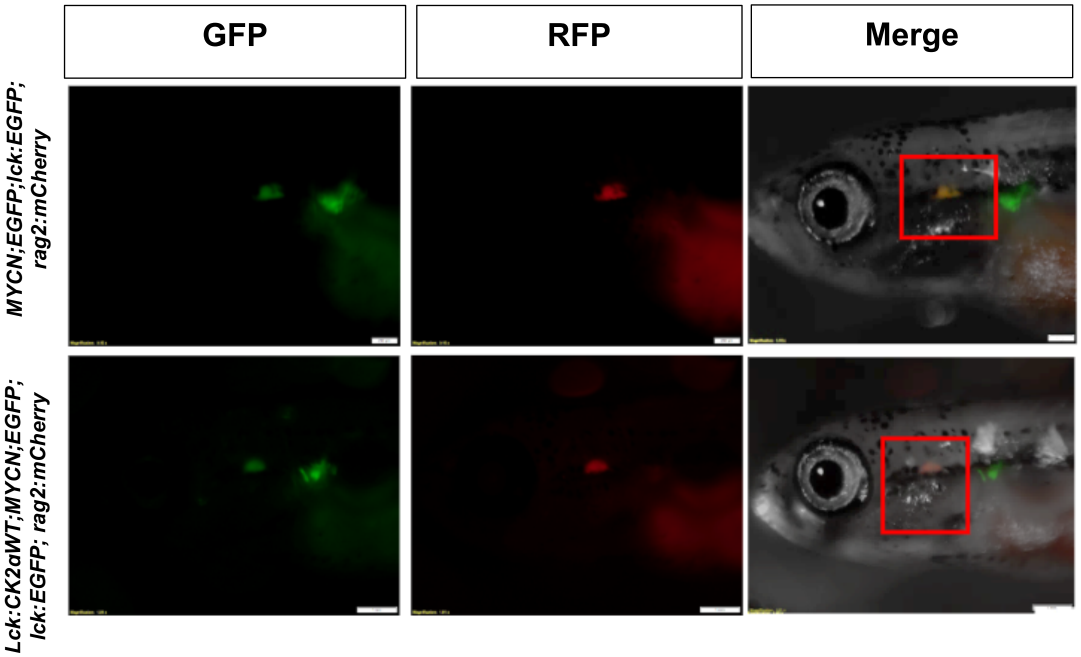

Figure 3: *CK2αWT* decreases the presence of thymic immune cells in MYCN overexpression zebrafish model

Representative images of *tg(MYCN:EGFP*) (left), *tg(rag2:mCherry*) (middle) and overlay of EGFP and RFP images of 21 dpf zebrafish *tg(MYCN;EGFP;lck:EGFP;rag2:mCherry)* (top) and *tg(lck*:*CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* (bottom) scale bar=200µm (inserts) n=6

Data collected by Kelly Miao

CK2αWT causes a decrease in *tg(rag2:mCherry)* thymic fluorescence intensity, both in and out of tumor context

We quantified red fluorescence intensity in the thymus of *tg(rag2:mCherry)* cells of *CK2αWT* zebrafish with or without *MYCN* overexpression. There is a statistically significant decrease in the *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* compared to their *tg(MYCN;EGFP;lck:EGFP;rag2:mCherry)* control ($p < 0.05$, n= 5 to 12) and interestingly, there was an even more significant reduction in the *tg(lck:CK2αWT;lck:EGFP;rag2:mCherry)* group compared to their *tg(lck:EGFP;rag2:mCherry)* control ($p < 0.0001$, n= 5 to 12) (figure 4). These results suggest that *CK2αWT* shuttles developing *tg(rag2:mcherry)* cells towards the kidney and away from the thymus. Additionally, these results suggest CK2 overexpression may not directly promote cancer, due to its ability to also decrease red fluorescence intensity both in and out of tumor context.

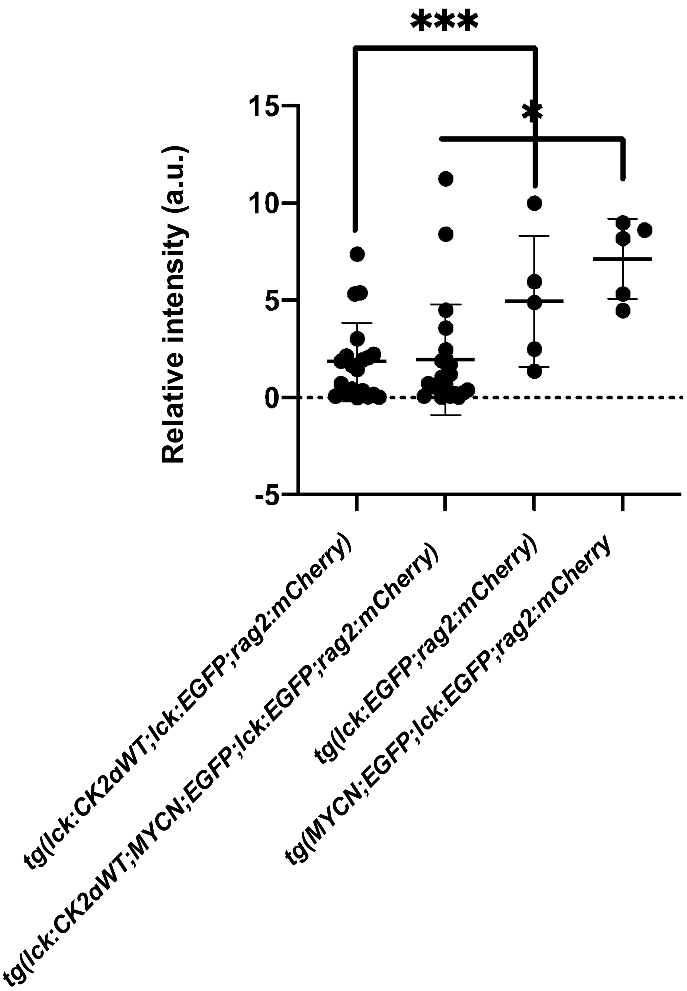

Figure 4: *CK2αWT* causes a decrease in *tg(rag2:mCherry)* thymic florescence intensity, both in and out of tumor context

Quantification of total thymus intensity based on red fluorescence intensity of *tg(lck:CK2αWT;lck:EGFP;rag2:mCherry), tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry), tg(lck:EGFP;rag2:mCherry) and tg(MYCN;EGFP;lck:EGFP;rag2:mCherry)* cells in 21 dpf fish (n = 5 or 12).
**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Data collected by Kelly Miao

CK2αWT overexpression causes lower numbers of *tg(rag2:mCherry*) lymphocytes within the thymus of MYCN overexpression zebrafish

Next, we further perturbed the effects of overexpression of CK2 in lymphocyte recruitment to the thymus of NB model zebrafish. Confocal microscopy was performed on zebrafish 21 dpf to further characterize the phenomenon that immune cells are negatively affected by CK2 overexpression. Visualization of *tg(lck:CK2αWT;MYCN;EGFP;lck:EGFP;rag2:mCherry)* shows there are fewer immune cells present (figure 5b) when compared to the tg(*MYCN;EGFP;lck:EGFP;rag2:mCherry)* control group (figure 5a). The quantification of this data shows that the reduction is significant (figure 5c).

The lack of lymphocytes visualized at 21 dpf could be acting to support the tumor due to the lack of crosstalk between CD8+T cells and anti-tumor lymphocytes. This could be brought on by crosstalk between CD4+ Tregs and immune cells, such as B cells, in the thymus. Evidence has shown that Tregs can kill antigen-presenting B cells in a perforin-dependent manner [Vignali et al., 2008], and other NB models have shown an increase in Tregs in the TME [Qin et al., 2024].

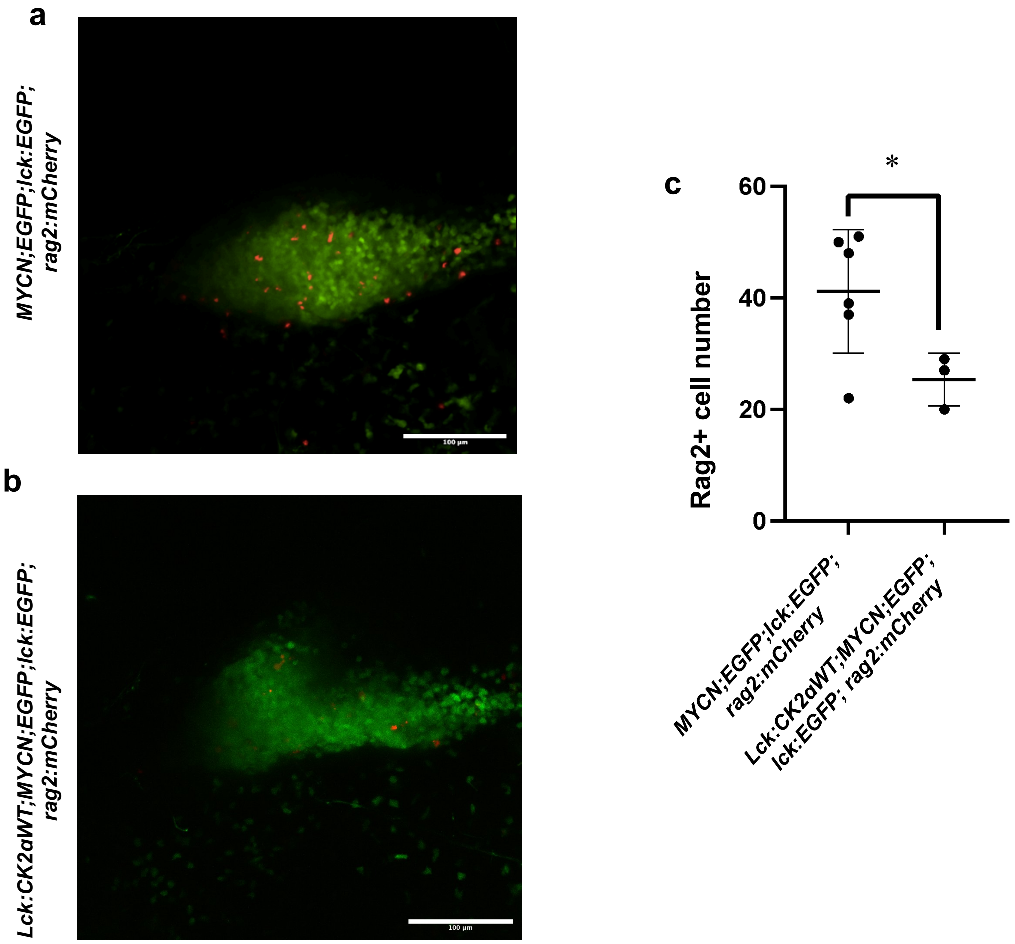

Figure 5: *CK2αWT* overexpression causes lower numbers of *tg(rag2:mCherry)* lymphocytes within the thymus of MYCN overexpression zebrafish

(a)Overlay of EGFP and red fluorescent protein images of the thymus of *tg(MYCN;EGFP;lck:EGFP)* and *tg(rag2:mCherry*) of 21 dpf zebrafish (n=6) scale bar= 100µm
(b) Overlay of EGFP and red fluorescent protein images of the thymus of *tg(*l*ck:CK2αWT;MYCN;EGFP;lck:EGFP)* and tg(*rag2:mCherry*) (n=3) scale bar= 100µm
(c) quantification of *tg(rag2:mCherry*) thymic cells in tg(l*ck:CK2αWT;MYCN;EGFP;lck:EGFP:rag2:mCherry)* compared to *tg(MYCN;EGFP;lck:EGFP;rag2;mCherry)* control (n= 3 or 6).
**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Data collected by Kelly Miao

DISCUSSION

Neuroblastoma (NB) is the most common extracranial solid tumor in children. Originating from neural crest cells and primarily affecting the sympathetic nervous system, NB is difficult to treat due to its aggressive nature, most commonly caused by amplification of MYCN, its tendency to evade the immune system, and its propensity to metastasize. Previous studies have shown that while there is initial recruitment to the TME, evident by increases in CD8+ T cells, over time, there is a subsequent reduction [Mina et al., 2015; Qin et al., 2024], possibly due to T cell exhaustion.

Protein kinases play a significant role in regulating cellular processes such as growth, survival, and differentiation, and their dysregulation is known to exacerbate tumor aggressiveness. CK2 has been implicated in the progression of various cancers [Firnau and Brieger., 2022] but its specific role in NB remains poorly understood. Further research is needed to fully elucidate its contribution to the malignancy's aggressiveness and potential as a therapeutic target.

Using a CK2 driven MYCN overexpressed zebrafish model to mimic NB, the findings in this study reveal the intricate relationship between protein kinases, tumor development and immune response. CK2 significantly impacts immune cells by causing lasting thymus fluorescence, delaying thymic involution (possibly through delaying apoptosis), and shuttling the naive lymphocytes away from the thymus but towards the kidney. Specifically, our CK2 overexpression model showed a significant increase in the percentage of CD8+ and IgM+ cells in the kidney.

There are several reasons why we could be seeing a preferential upshift in the percentage of CD8+ T cells and B cells but not CD4+ cells. These results could be due to CD4+ T cells shifting towards TH1 response, causing an increase in the proliferation and differentiation of CD8+ cells through the production of varying cytokines such as IL-2, IL-12, IL-15 and, IL-21 [Kudryavtsev et al., 2022]. Also, since the kidney marrow is the major site for B cell development and CK2ɑWT is known to preferentially mature B cells over T cells [Wei et al., 2021], proliferating B cells in the kidney could preferentially recruiting CD8+ cells through presentation of MHC-I molecules. Additionally, different cytokines or signal pathways (e.g., IFN-γ) could preferentially activate CD8+ T cells or B cells without influencing CD4+ T cells [Bhat et al., 2017]. Specific disease states in which these cytokines are produced in higher amounts or under specific conditions could cause CD8+ T cells and B cells to proliferate, while CD4+ T cells remain unaffected due to different receptor or signaling dependencies.

Much research has focused on the development of immune checkpoint inhibitors. However, these treatments fail in high-risk NB patients. Providers currently use HDACs [Shendy et al., 2022] or retinoic acid, a vitamin A derivative as second-line treatments to induce NB differentiation as the most common treatment forms [Zeineldin et al., 2022] or other combinations of chemotherapy and radiotherapy [Krystal and Foster., 2023]. Despite these interventions, half of NB patients often experience relapse, and the 3-year survival rate is not marginally better with these forms of treatments [Kohler et al., 2002]. Newer treatments for high-risk NB are attempting to target the immune system. This is exemplified by Anti-GD2 immunotherapy (dinutuximab). While this treatment produces an initial tumor size decrease in the majority of patients [Furman et al., 2022] and has shown great short-term survival rates, five- and 10-year survival rates are still unknown. Additionally, this treatment does not work for all patients due to the heterogeneity of NBs.

Further research is necessary to understand the link between CK2 and tumor proliferation. In recent years, it has become clear that CK2 plays a role in not only the intrinsic processes of tumor cells but also the regulations of the immune response to tumors specifically through modulation of T cells, macrophages, and DCs. Research has demonstrated that reduction of CK2 increases MHC-II class expression in lymphocytes [Larson et al., 2020]. It could be advantageous to look at the HES1/Notch1 signaling pathway in conjunction with CK2 overexpression too, as previous studies have shown that CK2 knockdown promotes maturation and differentiation by increasing HES1/Notch1 signaling in B cells, [Hong and Benveniste., 2021].

There are many complicated regulatory mechanisms involved in CK2's role in the TME and it would be useful to understand the influence that CK2 overexpression has in dysregulating B cell infiltration in the lymphoid organs.

Future studies could unveil the role of CK2 by performing flow cytometry and qRT-PCR for B cells to study the transcriptomic and gene shifts to further unveil the role of how CK2 overexpression affects B cells.

There should also be an increased focus on the Th17/Treg axis in NB since they can play a role in promoting tumor growth and can be immune to immunotherapies [Lei et al., 2020] Additionally, previous studies have suggested that Tregs can inhibit tumor suppressing B cells by inhibiting T cell-dependent B cell immunoglobulin responses, further complicating immune system interactions within the TME [Gonzalez-Figueroa et al., 2021]. Although previous studies have suggested that there are minimal Bregs present in NBs [Weißenborn et al., 2022], this aspect warrants further investigation given few studies thoroughly explored this potential connection. Additional research is needed to confirm or challenge this conclusion and to explore the role of Bregs in NB, as this information could offer valuable insights into tumor progression and therapeutic strategies.

In addition to further unveiling the mechanisms of CK2 in various signaling pathways, future experimental strategies should be extended to show later stages of tumorigenesis to better understand the progression of NB caused by MYCN amplification. Additionally, incorporating metastatic models could provide insight into how CK2 affects surrounding tissues, highlighting its broader impact on TME dynamics. As cancer progresses to later stages, it often becomes more resistant to chemotherapy, thus investigating how CK2 contributes to immune evasion in these advanced stages could provide critical information to help researchers develop more effective strategies for combatting chemotherapy resistance.

In our MYCN+ zebrafish model, expression of *tg(lck:EGFP;rag2:mCherry)* has proven to be valuable in studying immune cell behavior in real time. However, the core factors causing the fluorescence intensity change of *tg(lck:EGFP;rag2:mCherry)* have yet to be elucidated. Fluctuations in *tg(lck:EGFP;rag2:mCherry)* could indicate T cell activation, exhaustion, or alterations in T cell populations. Continued research efforts focusing on the causes of these changes in fluorescence could reveal critical insight into the pathways that lead from an initial T cell response to T cell tumor tolerance, illustrating how tumors drive T cells from anti-tumor to pro-tumor phenotypes which is a critical event in cancer progression.

Although significant progress has been made in understanding the role of T cells in tumor immunity, B cells are less well studied, particularly in zebrafish models. There is a critical need to develop B cell markers in zebrafish to track developmental or transitional staging. Since B cells are responsible not only for Ab production but also for T cell recruitment, having an increased repertoire of B cell-specific markers would allow for more detailed studies of B cell lineage and function within the TME and could lead to new therapeutic targets for modulating B cell response in cancer.

While the research presented reveals that CK2 overexpression does cause a shift in the subtypes of lymphocytes present, more work should be done to describe the transcriptomic shift. A research study has shown that in high-risk B-cell acute lymphoblastic leukemia (B-ALL), another aggressive pediatric cancer, CK2's phosphorylation inhibited the IKAROS tumor suppressor by downregulating the *eIKZF1* gene, ultimately causing resistance to the chemotherapy doxorubicin [Song et al., 2020]. Single-cell RNA sequencing (ScRNA-seq) is a powerful tool that can be used to analyze the gene expression profiles of individual cells. ScRNA-seq can provide a more detailed understanding of the heterogeneity exhibited in the immune cell populations in tumors since this avenue has yet to be explored in NB. Both T and B cells can exhibit a range of phenotypes, from proinflammatory to immunosuppressive. ScRNA-seq can further reveal how different subtypes of lymphocytes in the TME adopt a pro or anti-tumor phenotype as they interact with tumor cells. By examining the transcriptomic profiles of individual B cells in tumors, researchers can identify new therapeutic targets and better understand the immune landscape within tumors.

The location of immune cells within tumors is critical for determining how they will respond to tumor cells. Tertiary lymphoid structures (TLS) are organized areas within tumors where immune cells organize like lymphoid organs [Zhao et al., 2024]. TLSs contain a T cell zone and a B cell zone. Primary follicles in TLSs lack germinal center (GC) reactions, and secondary follicles in the B cell zone contain GCs, supporting the idea that TLSs are a place for the maturation of B cells as seen through B cell differentiation, and class switching [Laumont and Nelson., 2023].

Interestingly, it is thought that TLS are only present in “disease states” such as chronic inflammation, autoimmune disease, and cancer [Xie et al., 2024]. While the exact mechanism driving TLS formation remains unclear, the formation of mature TLS in a tumor context often correlates with a positive prognosis for cancer patients [Bao et al., 2024]. Evaluating the location of lymphocytes within TLSs provides pertinent information about how immune cells respond to the tumor. In gastric cancer, there is a positive correlation between patient survival and the production of TLSs and the density of immune cells correlates with the eradication of tumor cells (such as CD8+PD-1+ T cells), and the number of T and B cell interactions were highly enriched at the tumor core when compared to non-TLS forming microenvironments [Xie et al., 2025]. By studying the molecular and cellular dynamics of TLS, particularly the involvement of B cells, researchers could potentially uncover strategies to enhance the immune system's ability to target tumors by inducing the formation of TLSs.

Sex bias in cancer research is a notable aspect to also consider. Cancer affects men more than women generally [Jackson et al., 2022], and this is also true for NB [Kong et al., 2022]. While some researchers have found that males also have an overall worse survival rate [Yan et al., 2020], some forms of NB have shown to have a worse overall survival rate in high-risk females [Raleigh et al., 2022]. This is particularly relevant as protein kinase expression differs by sex in brain tissue [Chen et al., 2022]. Additionally, estrogen receptors can influence CK2 activity, which could impact tumor development in a sex-dependent manner [Williams et al., 2016; Williams et al., 2009]. Therefore incorporating sex as a variable in our models is clinically relevant. Sexing zebrafish at 21 dpf has been difficult however, technological advancements tests such as TaqMan PCR, a qRT-PCR technique that utilizes sex-determining markers, should be implemented to understand the nuanced roles of CK2 in tumor proliferation, in future studies [King et al., 2020].

TABLES

Table 1: PCR primer pairs used for amplification of CK2α cDNA for subcloning

Primer sequences (5′-3′)
Forward: 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCACCGGTATGTCGGGACCCGTGCCAAGCAG-’3 Reverse: 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATCGATTTACTGCTGAGCGCCAGCGGCAG-’3

Table 2: PCR primers used for genotyping CK2αWT

Primer sequences (5′-3′)
Forward: 5’-ATGTCGGGACCCGTGCCAAGCAG-’3 Reverse: 5’-TTACTGGCTTGAGAATTTTAAC-’3

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