Role of Neurokinin-1 Receptor Signalling in Immunomodulation of Tumor Development

A Thesis

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by

Reshmi Suresh

Indian Institute of Science Education and Research, Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Under the guidance of

Dr. Girdhari Lal, Scientist F

National Centre for Cell Science, Pune

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Certificate

This is to certify that this dissertation entitled 'Role of Neurokinin-1 Receptor Signalling in Immunomodulation of Tumor Development' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Ms. Reshmi Suresh at National Centre for Cell Science, Pune under my supervision during the academic year 2022-2023.

Girdhari Lal

Scientist-F

Committee:

Dr. Girdhari Lal

Dr. Satyajit Rath

This thesis is dedicated to my Acha and Amma.

Declaration

I hereby declare that the matter embodied in the report entitled **'Role of Neurokinin-1 Receptor Signalling in Immunomodulation of Tumor Development'** is the results of the work I carried out at the National Centre for Cell Science, Pune, under the supervision of Dr. Girdhari Lal. The same has not been submitted elsewhere for any other degree or diploma.

Reshmi Suresh

Date: 22/05/2023

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List of Abbreviations

SP	Substance P
NKR	Neurokinin receptor
NK1R	Neurokinin-1 receptor
NK2R	Neurokinin-2 receptor
NK3R	Neurokinin-3 receptor
TACR	Tachykinin receptor
PPTA	Pre-protachykinin A
PAM	Peptidylglycine α-amidating monooxygenase
ACE	Angiotensin converting enzyme
CRC	Colorectal cancer
AP	Aprepitant/Fosaprepitant
TME	Tumor microenvironment
TIME	Tumor immune microenvironment

ABSTRACT

Neuro-immune communications play an important role in regulating various functions in homeostasis and diseases. Amongst different diseases, it plays a crucial role in modulating tumor development. Substance P (SP), a neuropeptide, is an integral part of the neuro-immune axis, and it plays a significant role in neuro-immune regulations. It promotes pro-inflammatory immune responses and affects many immune cells crucial for anti-tumor immunity. Although extensive work has been done on the effect of SP/neurokinin-1 receptor (NK1R) signaling in neurological and autoimmune disorders, very little is known about their effect on the immune cells in modulating the tumor microenvironment (TME) of different cancers. This study shows that SP-NK1R signaling decreases colorectal cancer (CRC) growth in a mouse model. Further profiling of the immune cell phenotypes using multidimensional spectral flow-cytometry analysis revealed that SP specifically affects various CD8 T cell and CD4 T cell populations within the TME. SP promotes pro-inflammatory effector and memory CD8 T cells, Th17 cells, and Th1-like Th17 cells within the TME of CRC in mice. SP not only suppresses the proportions of Tregs within the tumor, but it also reduces the expression of immune-regulatory molecules like CTLA-4, GITR, and PD-1 on different CD8 T cell and CD4 T cells. Thus, we demonstrate that SP-NK1R signaling plays a crucial role in mounting anti-tumor immune responses against colorectal cancer. In addition, our finding gives a new dimension that suggests the functional relevance of incorporating the neuroimmune axis in designing therapeutic strategies to treat colon cancer.

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Contributions

Contributor name	Contributor role
Dr. Girdhari Lal	Conceptualization Ideas
Dr. Girdhari Lal, Mr. Surojit Karmakar	Methodology
-	Software
-	Validation
-	Formal analysis
-	Investigation
Dr. Girdhari Lal	Resources
-	Data Curation
Dr. Girdhari Lal	Writing - original draft preparation
Mr. Surojit Karmakar	Writing - review, and editing
-	Visualization
Dr. Girdhari Lal	Supervision
-	Project administration
Dr. Girdhari Lal	Funding acquisition

CHAPTER 1 - INTRODUCTION

1.1. Neuro-Immune Crosstalk

A well-functioning organism is characterized by maintaining homeostasis within its body, achieved through the coordinated activity of its nervous and immune systems. Both systems can recall past challenges and can mount memory responses that swiftly and effectively respond to constantly changing circumstances. The interaction between these two systems during development, homeostasis, and illnesses has been intensively researched in preclinical and clinical studies. Several studies have established the existence of nervous system-mediated regulation of the immune system and immune system-mediated regulation of nervous functions. Neurodegenerative diseases, mental disorders, autoimmune conditions, chronic inflammation, and cancer all show signs of this bi-directional crosstalk being disrupted (Chu et al., 2020; H Veiga-Fernandes, 2016; Klein Wolterink et al., 2022). Neuro-immune communications occur either via physical contact between their respective cells or through the receptors and signaling molecules commonly expressed between these two systems (Godinho-Silva et al., 2018; Kulka et al., 2008; Pacheco, Contreras, Prado, et al., 2012) **(Figure 1)**. Neural reflexes regulate innate and adaptive immune systems by sensing pathogen associated molecules, cytokines, chemokines and other factors through sensory neurons that generates an action potential to signal to the brain stem. The central nervous system (CNS) then signals to the secondary lymphoid organs through motor neurons, which releases neurotransmitters. These neurotransmitters in turn interact with specific receptors expressed on immune cells and help regulate the immune response (Andersson & Tracey, 2012; Mishra & Lal, 2021). Cytokines released by immune cells, such as interleukins, TNF-α, and interferons, can act on neurons and glial cells through the cytokine receptors they express. This can, in turn, alter gene expression patterns, synaptic plasticity, and neuronal excitability and also stimulate the release of various neuropeptides, chemokines, and cytokines by the cells of the nervous system (O. Pascual et al., 2012; Rothwell et al., 1996). Immune cells also express receptors for different neurotransmitters, such as serotonin, acetylcholine, neuropeptides, and dopamine which augment and regulate immune responses (Halder & Lal, 2021; Karmakar & Lal, 2021; Pacheco, Contreras, & Prado, 2012). Furthermore, they are known to

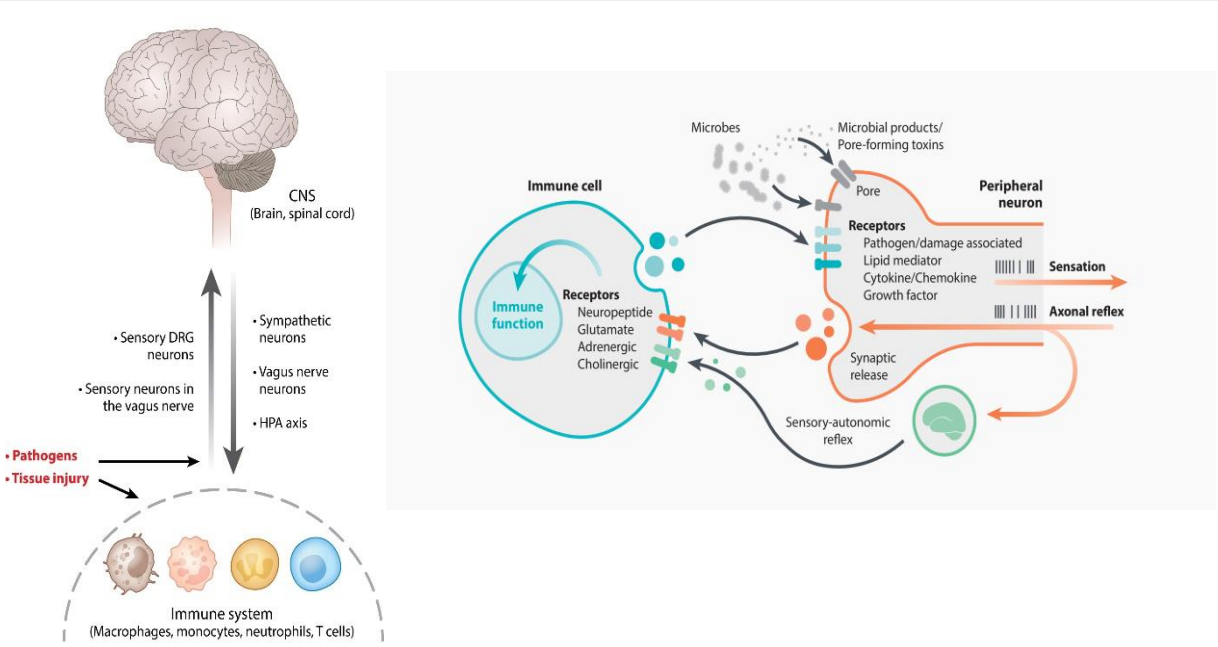


Figure 1. Neuro-immune crosstalk. The nervous system and immune system regulate each other and maintain homeostasis. This crosstalk is mediated through physical contact between neurons and immune cell and through signaling molecules like neurotransmitters, cytokines and chemokines and their receptors that are commonly expressed in both the systems. (Figures adapted from (Klein Wolterink et al., 2022; Pavlov et al., 2018))

synthesize and secrete these neurotransmitters themselves. These shared receptors and soluble factors were the primary evidence for neuro-immune crosstalk.

How the nervous system influences immune responses in health and disease pathogenesis is still being investigated. Most of the current treatments for neurological disorders, cancers, and autoimmune conditions target their respective autonomic networks. Harnessing the involvement of the neuro-immune axis in these disorders can be crucial for their treatment and prognosis.

1.2. Neurokinins: Facilitating neuro-immune crosstalk

Neurokinins or tachykinins are a class of neuropeptides ubiquitously expressed across the central nervous system (CNS), peripheral nervous system (PNS), and immune cells. They act as mediators between the nervous system and immune responses and are crucial in regulating various physiological processes (Steinhoff et al., 2014). The major neurokinin members are Neurokinin-1 (NK-1) or Substance P (SP), neurokinin-A (NKA), neurokinin-B (NKB), hemokinin-1 (HK-1), neuropeptide K (NPK), and

neuropeptide γ (NP-γ). They are recognized by their conserved sequence X-Phe-X-Gly-Leu-Met-NH2 and are encoded by alternative splicing of *Tac* genes or pre-protachykinin (*ppt)* genes (Chang et al., 1971). *Ppta* (*Tac1*) gene encodes for SP, NKA, NPK, and NP-γ. *Tac3* (*Ppt-b*) encodes NKB and *Tac4 (Ppt-c)* encodes for HK-1 and endokinins (Krause et al., 1987; Mishra & Lal, 2021). Neurokinins influence significant physiological functions like hematopoiesis, inflammation, nociception, smooth muscle contraction, cellular secretion, and proliferation (Steinhoff et al., 2014).

Neurokinins specifically interact with three G-protein coupled receptors (GPCR) called neurokinin receptors (NKR). They are encoded by three *TacR* genes, neurokinin-1 receptor (NK1R) encoded by *TACR1*, neurokinin-2 receptor (NK2R) by *TACR2* and neurokinin-3 receptor (NK3R) by *TACR3*. These receptors are expressed across the CNS, PNS, immune system, digestive system, and secretory glands in varying levels (Caberlotto et al., 2003; Grubor et al., 2004; Maggi, 1997; Steinhoff et al., 2014). Their presence across immune cell populations has been studied extensively and is known to be crucial in mediating immune responses against pathogens and malignancy. Neurokinins preferentially bind to the three NKRs with varying affinity. NK1R binds to SP the strongest, followed by NKA, then NKB. For NK2R, the order of affinity is NKA > NKB > SP, and for NK3R, it is NKB>NKA>SP (Maggi, 2000; Mishra & Lal, 2021; Regoli et al., 1994; Steinhoff et al., 2014b). NK1R is found in two isoforms: full-length NK1R (f-NK1R) and truncated NK1R (t-NK1R). f-NK1R has 407aa long with an intracellular C-terminal domain and is considered the functional variant. t-NK1R with 311aa lacks this C-terminus domain and thus lacks intracellular signaling (Lai et al., 2006; Suvas, 2017).

1.3. Substance P

SP was the first neuropeptide discovered when it was isolated from the horse intestine and brain in 1931 (v. Euler & Gaddum, 1931). Since then, it has been one of the most studied neuropeptides. It is an 11-amino acid long peptide that binds to NK1R and elicits signaling cascades leading to calcium signaling, enhanced cellular proliferation, survival, cytokine reorganization, migration, and effector functions. It is synthesized and secreted by CNS, peripheral neurons, and immune cells, including T cells, dendritic cells (DCs), macrophages, and mast cells (Janelsins et al., 2009; Mathers et

al., 2007; Weinstock et al., 2003). Hence it acts in a paracrine and autocrine manner on immune cells to regulate their functions. SP is pro-inflammatory as it stimulates immune cell activation, survival, effector functions, and migration (Steinhoff et al., 2014; Suvas, 2017).

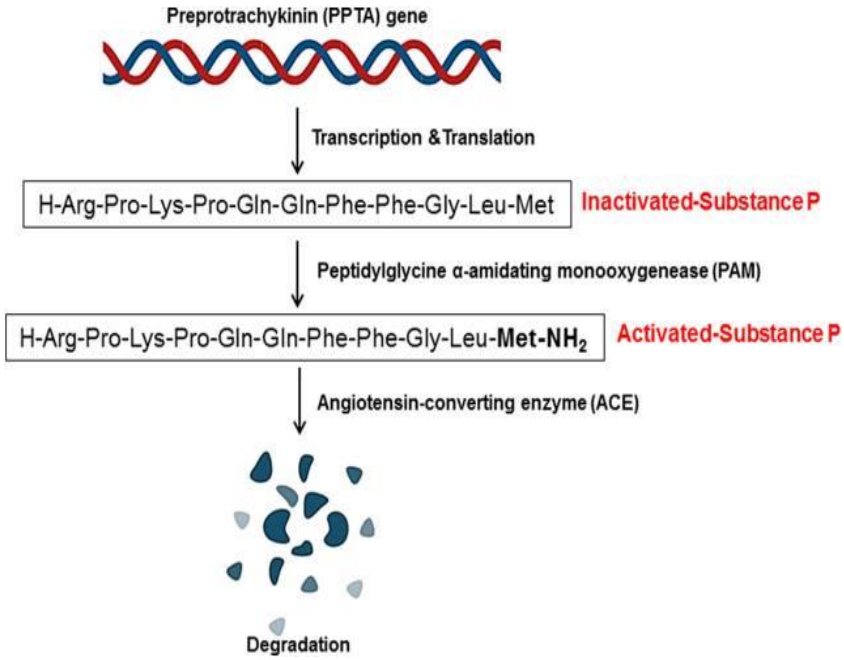


Figure 2. SP synthesis and degradation. SP is encoded by the PPTA gene which upon transcription and translation generates inactive SP. Activating enzyme PAM converts inactivated SP to its functional form by amidation. SP within cells is degraded by ACE. (Figure adapted from Mishra & Lal, 2021)

SP is encoded by the *PPTA* gene. Transcription and translation of *PPTA* generate inactive forms of SP. The inactive SP is converted to its activated form through amidation by the peptidylglycine α-amidating monooxygenase (PAM) enzyme. Following post-translational modifications, SP is packed in secretory vesicles and released out of the cell by exocytosis. SP is degraded within cells by angiotensin-converting enzyme (ACE) **(Figure 2)** (Mishra & Lal, 2021). Once released, SP binds to NK1Rs present in the same cell or nearby cells. Unbound SP is degraded by neprilysin, a metalloendopeptidase present on cell surfaces. Thus, SP has a short half-life in tissues (Skidgel et al., 1984). However, SP forms a complex with high molecular

weight factors such as fibronectin, prolonging its half-life in blood (Corbally et al., 1990).

1.4. SP-NK1R Signaling

When SP binds to NK1R, it leads to the dissociation of Gαq/11 and Gαs subunits. Gαq/11 activates phospholipase C (PLC), and Gαs activates adenylyl cyclase (AC). This results in the generation and activation of secondary signaling molecules like inositol triphosphate (IP_3) and diacylglycerol (DAG) by PLC and the cyclic AMP (cAMP) pathway by AC. IP_3 increases intracellular calcium levels by triggering its release from ER. SP-NK1R signaling also activates the Rho-associated ROCK pathway, which can

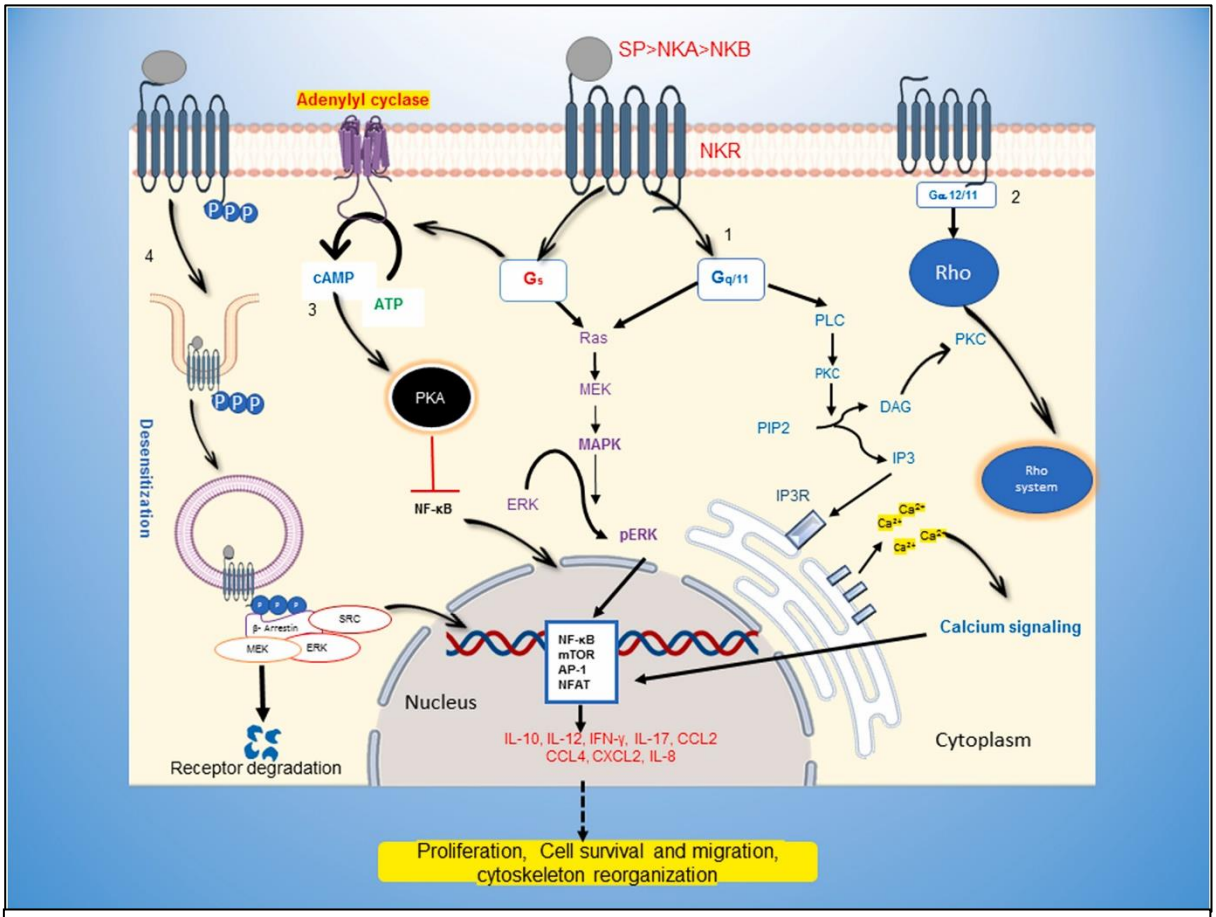


Figure 3. SP-NK1R signaling pathway. 1. SP binding to NK1R leads to the dissociation of G-protein subunits. Dissociated Gq/11 subunit activate PLC which generates secondary messengers DAG and IP3 which then activates PKC and induce intracellular Ca^{2+} release, respectively. 2. G12/13 subunit activates the Rho/Rock pathway. 3. Gs subunit activates AC that converts ATP to cAMP. This inturn binds to PKA and regulate NFκB expression. 3. β-arrestin mediated internalization of SP-NK1R complex can mediate MAPK signaling and prolong NK1R signaling before their degradation. Signaling pathways activated by neurokinin signaling activated various transcription factors that mediates the expression of several cytokines and promote cellular proliferation, survival, cytoskeletal reorganization and migration. (Figure adapted from Mishra & Lal, 2021)

cause apoptosis-independent cellular blebbing. In human astrocytoma cell lines, SP and NK1R transactivate the epidermal growth factor receptor (EGFR), activating the

mitogen-activated protein kinase (MAPK) pathway. In human fibroblast cells, SP-NK1R signaling has also been shown to induce growth-regulating arachidonic acid metabolites such as prostaglandin E2 and prostacyclin (Kähler et al., 1993). The pathways activated by SP-NK1R interaction ultimately result in the cell-specific activation of various transcription factors NFκB, mTOR, AP-1, and NFAT. These transcription factors, in turn, promote the expression of pro-inflammatory cytokines IL-2, IL-12, IFN-γ, IL-17, TNF-α, and IL-1β and regulates IL-10 and TGF-β expression. SP-NK1R-activated signaling pathways also promote several chemokines, such as CCL4, CCL2, CCL5, and CXCL8. This increases cellular activation, proliferation, cell survival, and migration. SP signaling also exhibits an anti-apoptotic effect in glioblastoma cells by activating the anti-apoptotic molecule Akt (Akazawa et al., 2009). Thus SP-NK1R signaling induces and promotes pro-inflammatory responses. Desensitization of cells to SP-signaling is mediated by β-arrestin by endosomal internalization of the SP-NK1R signaling complex. SP is degraded within the endosomes by the endothelin-converting enzyme (Pelayo et al., 2011). NK1R is then recycled back to the cell surface in an SP-concentration-dependent manner.

1.5. Role of SP-NK1R Signaling in Immune Cells

Substance P has established itself as a pro-inflammatory neuropeptide with its signaling effects on cells. The expression of neurokinin receptors in immune cells indicates its role in influencing immune responses and homeostasis. Neurokinin receptors in the bone marrow are known to regulate hematopoiesis. They also influence B cell and T cell development in the thymus (Corcoran et al., 2007).

T-cells express NK1Rs and are known to synthesize SP, thus being prone to autocrine and paracrine signaling. NK1R signaling is a crucial factor in T-cell activation, proliferation, and survival (Blum et al., 2003; Kincy-Cain & Bost, 1997; Muñoz et al., 2022a). SP-NK1R signaling is critically important for efficient Ca^{2+}-dependent activation and survival of TCR-activated CD8 and CD4 T cells. They stimulate the production of pro-inflammatory cytokines like IL-12, IFN-γ, and IL-17, which effectively

promote the differentiation of CD4 T cells into Th1 and Th17 cells (Morelli et al., 2020a). $NK1R^{-/-}$ mice show reduced T cell proliferation (Lambrecht et al., 1999). SP-NK1R promotes T-cell migration by upregulating MIP-1β and this migration is inhibited when NK1R is antagonized with CP96345 (Guo et al., 2002). It has been shown that NK1R expression in lamina propria T cells mediates intestinal inflammation in mice models. This can be reduced by antagonizing NK1R, indicating their potential in the treatment of chronic inflammatory diseases (Weinstock et al., 2003). Cytokines modulate the expression and signaling of NK1R in murine T cells. IL-12 induces SP and NK1R expression (Calvo et al., 1992). In contrast, IL-10 inhibits NK1R expression and SP production in T cells (Payan et al., 1983). Another cytokine, TGF-β can delay the SP-NK1R internalization process in intestinal T cells, prolonging its signaling and causing increased IFN-γ and IL-17 release (Beinborn et al., 2010).

Dendritic cells (DCs) express SP, NK1R, and NK2R. Bone marrow-derived DCs (BMDCs) can promote allogeneic T-cell responses through SP (Lambrecht et al., 1999). NK1R signaling also promotes the survival of BMDCs (Janelsins et al., 2009). NK1R promotes Tc1 and Th1-based immune responses through Langerhans cells in mice (Janelsins et al., 2009). The NK1R antagonist reduces dry eye disease by preventing DCs from activating Th17 (Yu et al., 2020). NK1R in B cells augments IgA secretion (Pascual et al., 1991). NK1R shows a dual effect on NK cell migration and cytotoxicity (Fu et al., 2011; Lang et al., 2003; Monaco-Shawver et al., 2010). SP increases phagocytosis in macrophages. It also promotes IL-12 production in murine macrophages and stimulates the secretion of H_2O_2, nitric oxide, and thromboxane (Arsenescu et al., 2005; Bar-Shavit et al., 1980; Hartung & Toyka, 1983). NK1R is also expressed across neutrophils, eosinophils, and mast cells (Friedman & Levi-Schaffer, 2015; Kulka et al., 2008; Serra et al., 1988).

Thus, the neurokinin-1 receptor has a predominant role in regulating immune cell function and mediating immune responses against pathogens, malignancy, and autoimmunity. Moreover, their functional role emphasizes the importance of neuro-immune crosstalk in maintaining homeostasis and in the pathogenesis of diseases.

1.6. Substance P in Cancer

Extensive studies on neurokinin signaling in cancer until now suggest a dual role of SP signaling in cancer development. The effect of SP-NK1R signaling is highly dependent on the type of tumor model being studied. SP has been shown to have an anti-tumor effect in small-cell lung carcinoma, where SP treatment reduces tumor cell growth both *in vitro* and *in vivo* (MacKinnon & Sethi, 2003; Seckl et al., 1997). Administration of aerosolized SP in mice with lung cancer inhibited tumor development and increased their survival (Harris & Witten, 2003). SP inhibited PC-3 prostate cancer cells (Friess et al., 2003). By mobilizing LAK cells, SP mounted an anti-tumor immune response against colorectal cancer (Flageole et al., 1992). In murine melanoma, SP inhibited tumor progression by increasing the cytotoxicity of T cells and NK cells and mobilizing them against tumor cells (Manske & Hanson, 2005b).

However, SP has been reported to support tumor cell proliferation and growth in several cancer models. SP stimulated glioma progression by inducing it to release cytokines and antagonizing NK1R-inhibited glioma progression (Palma et al., 1999, 2000; Carla Palma & Maggi, 2000). Enhanced expression of NK1R is associated with poor prognosis in pancreatic cancer. Moreover, SP analogs stimulated pancreatic cancer growth (Friess et al., 2003). SP signaling also promotes breast cancer cell proliferation, and NK1R expression is correlated with poor prognosis (Al-Keilani et al., 2022; Li et al., 2016).

It is clear from previous studies that SP-NK1R signaling has a dual effect on cancer that is highly dependent on the tumor model. How this signaling specifically affects the immune cell composition to promote pro-tumor or anti-tumor responses is not well known. Nevertheless, they reinforce the prominence of neuro-immune crosstalk in modulating the tumor microenvironment (TME) and influencing tumor progression.

In this study, we demonstrate the role of NK1R signaling in controlling the development of colorectal (CRC) cancer through immunomodulation of its tumor microenvironment (TME). We start by confirming the expression of neurokinin receptor genes and SP synthesizing and degradation machinery within mouse CRC cells, CD8 T, and CD4 T cells. Next, we assess the effect of agonizing NK1R on orthotropic CRC developed in C57BL/6J mice and validate the anti-tumor immune response to be mediated via SP's effect on immune cells. Additionally, we analyze the specific changes in immune cells

that occur upon SP treatment within the tumor and tumor-draining lymph nodes (tdLN). Finally, we assessed the effect of the clinically approved NK1R antagonist drug on treating chemotherapy-induced nausea and vomiting, called Fosaprepitant (AP) (Navari, 2008; Weinstein et al., 2016), on colon tumors in combination with Oxaliplatin, a platinum-based chemotherapeutic drug. Overall, this study investigates and proves the role of NK1R signaling in eliciting an anti-tumor immune response by promoting pro-inflammatory CD4 and CD8 T cells and suppressing regulatory T cells in the CRC model.

CHAPTER 2

MATERIALS AND METHODS

Mice

Six to eight weeks old C57BL/6J (RRID: IMSR_JAX:000664) and NRG (NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ), male and female mice were used in the tumor studies, were procured from the Jackson Laboratory (Marine, USA) and bred in the experimental animal facility (EAF) of NCCS. All experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC). The animal ethics numbers are NCCS/EAF/2017/B-256.

Cell lines and culture methods

Mice colon adenocarcinoma cell line MC38 was received from the NCCS cell repository. MC38 cells were cultured in complete Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM MEM non-essential amino acid, 1 mM sodium pyruvate, 10 mM HEPES, 50 µg/ml gentamycin, and 25 µg/ml penicillin/streptomycin. Cell lines were maintained at 37˚C in a 5% CO_2 incubator.

In vitro CD4 T cell culture

Purified naïve $CD4^+CD25^-CD44^-$ cells were plated at 1 × 10^5 cells per well in 0.2 ml 96-well flat-bottomed tissue culture plates coated with anti-CD3ε antibody (10 µg/ml). The culture media was supplemented with soluble anti-CD28 (2µg/ml). To differentiate naïve $CD4^+$ T cells into subsequent lineages, culture was further supplemented with cytokines and blocking antibodies accordingly for Th0: purified recombinant mouse IL-2 (10 ng/ml); Th1: purified recombinant mouse IL-12 (10 ng/ml), α-mouse IL-4 (BioXCell, clone 11B11; 10 µg/ml) and purified recombinant mouse IFN-γ (10 ng/ml); Th2: purified recombinant mouse IL-4 (10 ng/ml) and α-mouse IFN-γ (BioXCell, clone XMG1.2; 10 µg/ml); and purified recombinant human TGF-β1 (10 ng/ml); pathogenic Th17: purified recombinant mouse IL-1β (10 ng/ml), purified recombinant mouse IL-

23 (10 ng/ml) and iTreg: purified recombinant human TGF-β1. The plates were incubated at 37^{o}C in a 5% CO_2 incubator for five days. On day 4, gene expression and cytokine production was analyzed. The addition of mediators, such as signaling agonists, antagonists, or other cytokines, during the differentiation process, can also be used to study the influence of a target on T-cell differentiation.

RNA isolation and semi-quantitative PCR

Total RNA was isolated from purified cells using TRIZOL reagent (Invitrogen). RNA was measured, and DNA contamination was removed using DNAse I. cDNA was prepared using an Omniscript RT kit (Qiagen), and qRT-PCR was performed using a specific forward and reverse primer mix and a universal SYBR green reverse transcriptase master mix (Biorad; catalog # 1725122) in CFX96 Touch deep-well Real-Time PCR system (Biorad).

MTT assay

MC38 cells were cultured at a density of 0.3 X 10^4 cells/ well in 96 well plates (flat bottom) and were allowed to adhere for 24 hours. Then NK1R agonist, [Sar-9, Met(O2)-11]-Substance P (SP) at concentrations 5μM, 10μM, and 40μM and antagonist CP-96345, at concentrations 5μM, 10μM, and 50μM were added for 24 and 72 hours in a reduced serum medium (2% FBS containing DMEM). Then, the media was removed, washed once with PBS, and incubated with 5mg/ml MTT reagent for 3 hours at 37˚ C. Following, MTT crystals were dissolved with DMSO, and the reading was taken at 590 nm in an ELISA reader (Thermofisher).

CRC tumor model development in mice

Six to eight weeks old sex-matched different mice strains were taken and injected with MC38 mice colon cancer cells (0.5 X 10^6 cells/flank) subcutaneously in 50 μl PBS. After five or seven days of tumor inoculation, when the tumor became palpable, all the therapeutic intervention was started. When the tumor became measurable, they were measured using a digital Vernier caliper. The largest diameter of the tumor (Height) and the smallest diameter of the tumor (Width) were measured, and tumor volume was calculated using the following formula Tumor volume = Height X Width^2 /2.

Subcutaneous colon cancer model and SP treatment in C57BL/6J mice

Six to eight weeks old sex matched C57BL/6J mice were subcutaneously injected with MC38 cells (1 X 10^6 cells on each mouse flank) suspended in 50 µL PBS. One group of mice was kept as a control, and another group was treated with [Sar-9, Met(O2)-11]-Substance P (SP, 5 nm/dose) via the intraperitoneal (IP) route from the 5^{th} day of tumor cell injection. When tumors became measurable, tumor height and width were measured, and tumor volume was calculated by the formula height x $(width)^2/2$ and plotted.

Subcutaneous colon cancer model and SP treatment in NRG mice

Six to eight weeks old sex-matched NRG mice were subcutaneously injected with MC38 cells (1 X 10^6 cells on each mouse flank) suspended in 50 µL PBS. One group of mice was kept as a control, and another group was treated with [Sar-9, Met(O2)-11]-Substance P (SP, 5 nm/dose) via the intraperitoneal (IP) route from the 5^{th} day of tumor cell injection. When tumors became measurable, tumor height and width were measured, and tumor volume was calculated by the formula height x $(width)^2/2$ and plotted.

Fosaprepitant (AP) and oxaliplatin treatment in subcutaneous colon cancer model

Six to eight weeks old sex matched C57BL/6J mice were subcutaneously injected with MC38 cells (0.6 X 10^6 cells on each mouse flank) suspended in 50 µL PBS. One group of mice was kept as a control. A second group was treated with Fosaprepitant (AP) (50 mg/kg/day), a third group received oxaliplatin (OXA) (4 mg/kg/day) for six days, and a fourth group of mice received both AP and OXA treatment. All treatments were given via the intraperitoneal (IP) route from the 5^{th} day of tumor cell injection. When tumors became measurable, tumor height and width were measured, and tumor volume was calculated by the formula height x $(width)^2/2$ and plotted.

Preparation of single-cell suspension and cell staining for flow cytometry

Tumors were excised, manually disrupted into small pieces using fine forceps and scissors, and resuspended in serum-free DMEM media containing collagenase type I (0.1 mg/ml), collagenase IV (0.1 mg/ml), hyaluronidase (0.06 mg/ml), DNase I (0.02 mg/ml) and soybean trypsin inhibitor (0.1 mg/ml) and incubated for 30-60 minutes at

37˚C in a shaker incubator. The single-cell suspension was prepared by passing through a 70 µM pore-size cell strainer. Then cells were washed with PBS and stained with fluorochrome-tagged primary antibodies. Single-cell suspension of spleen and tumor-draining lymph nodes (tdLNs) were prepared by mechanical disruption of the tissues, and the cell suspension was passed through a 70 µM pore size cell strainer. RBCs were removed using ACK lysis buffer, washed with RPMI medium, and stained using specific antibodies.

Cells were incubated with fluorochrome-tagged primary antibodies for 1 hour on ice. Then, washed once with PBS, and cells were either fixed with 0.5% paraformaldehyde or proceeded for intracellular staining. For intracellular staining, cells were fixed with 1X FoxP3 fixation buffer (Biolegend) for 45 minutes on ice, followed by washing with 1X permeabilization buffer (Biolegend) and incubated with permeabilization buffer for 30 mins on ice. Cells were then incubated with fluorochrome-tagged specific antibodies in permeabilization buffer for 1 hour, washed with PBS, and fixed with 0.5% paraformaldehyde. Finally, the cells were acquired using Spectral flow cytometry (Cytek aurora, Cytek Biosciences, USA), and the data were analyzed using FlowJo, Graph Pad, and OMIQ software.

ELISA

Mouse IL-17 ELISA MAX™ Deluxe Set was purchased from BioLegend (Catalog # 43250), and ELISA was performed according to the manufacturer's protocol. Mouse IL-10 MAX™ Deluxe Set was procured from Bio Legend (Catalog# 431416), and the ELISA was performed according to the manufacturer's guidelines. Mouse IFN-γ MAX™ Deluxe Set was procured from Bio Legend (Catalog# 430806), and the ELISA was performed according to the manufacturer's guidelines. Mouse TNF-α MAX™ Deluxe Set was procured from Bio Legend (Catalog# 430906), and the ELISA was performed according to the manufacturer's guidelines. Mouse IL-1β MAX™ Deluxe Set was procured from Bio Legend (Catalog# 432604), and the ELISA was performed according to the manufacturer's guidelines.

Clustering Analysis

The data obtained following flow cytometry was appropriately gated for singlets and $CD45^+$ live cells, followed by lymphocytes expressing different markers. For the analysis, dimensionality reduction was done using the opt-SNE algorithm, followed by

clustering the cells based on the differential expression of markers using FlowSOM. Heatmaps were generated through hierarchical clustering based on the mean fluorescence intensity of the markers used. The metaclusters obtained were gated and statistically analyzed for significant changes between the SP-treated group and the control by EdgeR. A significant change was determined with reference to p-value <0.05. The significantly altered clusters were further checked for expression levels of individual markers, and phenotypes were identified.

Statistical analysis

Unpaired two-tailed Student's *t*-test was used to compare two independent groups. For comparing more than two independent groups, ANOVA with Sidak's multiple comparison tests was used. A *p-value* of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6 and GraphPad Prism 9 software (GraphPad Software, San Diego, CA).

Chapter 3 - RESULTS

3.1. MC38 colorectal cancer cells, $CD8^+$ T cells, and $CD4^+$ T cells express TACR isoforms and components of the SP synthesis and degradation system.

There is much evidence that suggests the involvement of neurokinins in modulating gut-related functions. As colorectal cancer is a major gut-associated cancer affecting the Indian population, we wanted to understand how neurokinins influence its progression. To understand its effect on CRC, we have used an orthotropic colorectal cancer model of mice CRC cell line, MC38. MC38 cell line is developed from a colorectal tumor in female C57BL/6J mice treated with N-N- dimethyl hydrazine (DMH) carcinogen treatment. MC38 CRC cells are microsatellite unstable (MSI) cells and are responsive to immune cell-mediated killing. To begin with, we first tried to understand the expression pattern of the tachykinin system in the MC38 cells and CD4 and CD8 T cells. Neurokinins signal through three major receptors TACR1, TACR2, and TACR3. The major type of neurokinin that performs many functions within the body is substance P (SP). It is synthesized within the body from a precursor pre-pro tachykinin-A (PPTA) as an inactive SP. This is activated by the enzyme PAM. After its functions, SP is degraded by the enzyme ACE. So, we have looked into the expression pattern of the TACRs and PPTA, PAM, and ACE in the MC38 cell line and naïve and in vitro activated and differentiated CD8 and CD4 T cells by qRT-PCR. We have found that the MC38 cells expressed all the TACR mRNAs with higher expression of TACR1 and TACR3 (**Figure 4A**). MC38 also has high levels of the PPTA gene and PAM but lower expression of ACE (**Figure 4B**). CD8 T cells also showed expression of TACRs and PPTA, PAM, and ACE, but their expression is decreased in an *in vitro* activated CD8 T cells with α–CD3ε and α–CD28 **(Figure 4C and 4D)**. The CD4 T cell subsets differentiated *in vitro* into Th0, Th1, Th2, Th17, and Treg subtypes showed variable expression of TACRs, PPTA, PAM, and ACE (**Figure 4E and 4F**). Among these, Th2 cells showed the highest expression of all three TACRs, followed by Th17 and Tregs. However, these three subsets also expressed increased levels of degrading enzyme ACE while Th1 cells had decreased expression of it.

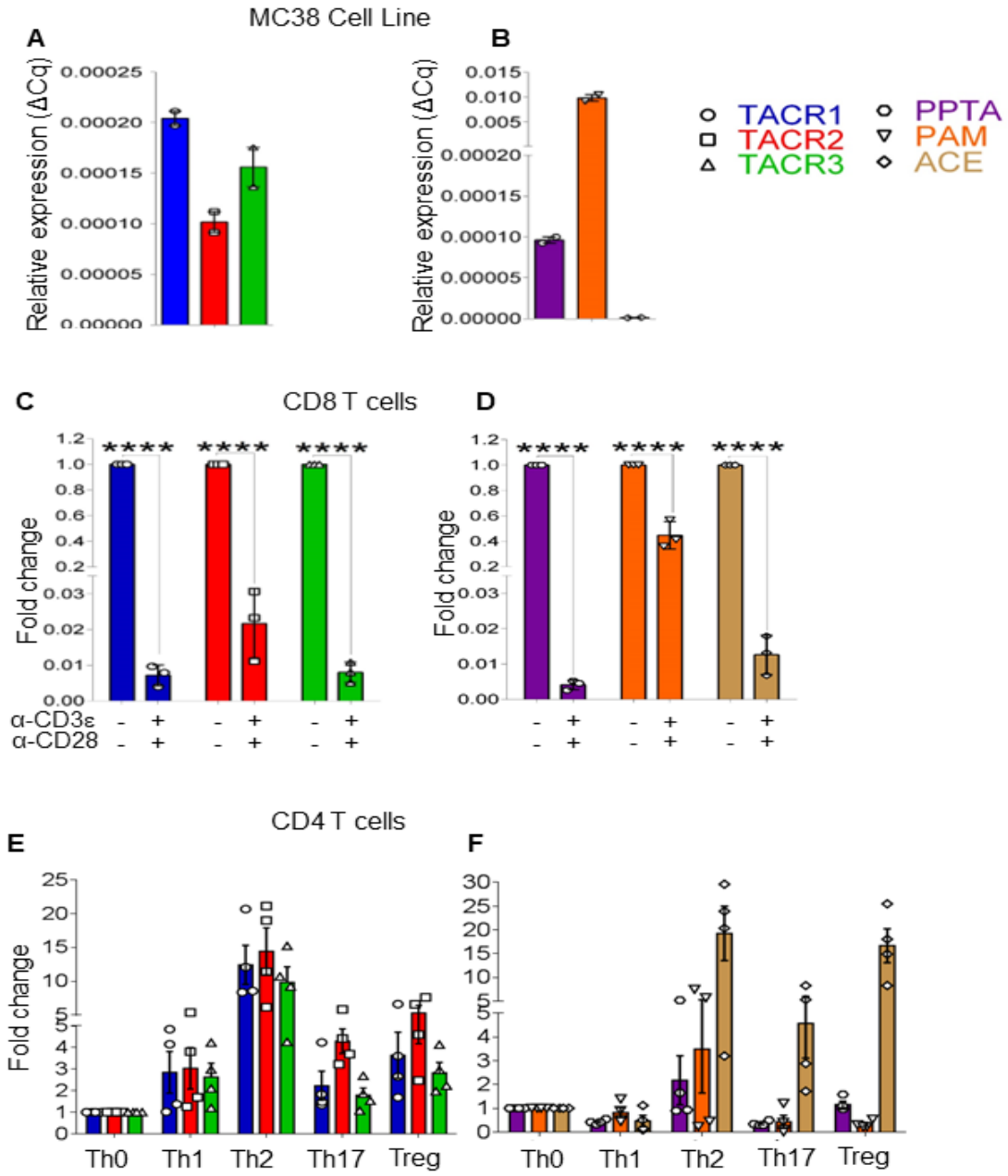


Figure 4. Characterization of TACR expression and cellular SP synthesizing system in MC38 cells, CD8 T and CD4 T cells.

(A) Relative mRNA expression of TACRs in MC38 cells measured by qRT-PCR. (B) Relative mRNA expression of PPTA, PAM and ACE in MC38 cells measured by qRT-PCR. (C) Relative mRNA expression of TACRs in naïve and in vitro activated CD8 T cells measured by qRT-PCR. (D) Relative mRNA expression of PPTA, PAM and ACE in naïve and in vitro activated CD8 T cells measured by qRT-PCR, all the fold change compared to naïve CD8 T cells. (E) Relative mRNA expression of TACRs in naïve and in vitro differentiated CD4 T cells measured by qRT-PCR. (F) Relative mRNA expression of PPTA, PAM and ACE in naïve and in vitro differentiatedCD4 T cells measured by qRT-PCR, all the fold change compared to Th0 CD4 T cells.

p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

Taken together, we have found that MC38 colon cancer cells, CD4 and CD8 T cells widely express TACRs and SP synthesis and degradation system, and this expression varies with the activation and differentiation status of CD8 and CD4 T cells.

3.2. SP treatment suppresses tumor growth by eliciting an anti-tumor immune response.

To test the effect of SP on tumor growth, orthotropic CRC is developed in C57BL/6J mice and five-day post-tumor inoculation, one group of mice received SP treatment (5nm/mice/day) intraperitoneally, and another group kept as control (**Figure 5A**). The SP treatment significantly reduced tumor growth compared to the control (**Figures 5B and 5C**). To understand its mechanism, we have looked into the effect of SP treatment on the cancer cells and the immune cells that impacts tumor growth. SP elicit NKR signaling in both tumor and immune cells resulting in their activation, proliferation, migration, and effector functions. Since SP has been reported to be a mitogen in several studies of various cancers, we first assessed the effect of SP on *in vitro* cultures of MC38 cells using an MTT assay. We have found that SP treatment increased the viability of MC38 cells, whereas an increased dose of NK1R antagonist CP93456 significantly reduced it. **(Figure 5F).** This reflected the results of many previous studies on different cancer cells (Garnier et al., 2015; Golestaneh et al., 2022; Rosso et al., 2012). Further, to evaluate whether the suppressed tumor growth was mediated by immune cells modulated by SP, we developed an MC38 tumor model in the NRG mice **(Figure 5D)**. NRG mice are devoid of T cells, B cells, and NK cells, and we found that in the absence of functional adaptive immune cells, SP treatment did not decrease tumor growth, rather it increased tumor growth in NRG mice, but the changes were non-significant **(Figure 5E)**.

Altogether, we have found that NK1R signaling increases the viability of cancer cells *in vitro*. This signaling also increased tumor growth in immune-deficient mice, but in immunocompetent mice, NK1R agonism reduced tumor growth. Further, we have tried to understand the role of NK1 signaling on the immune modulation in the tumor.

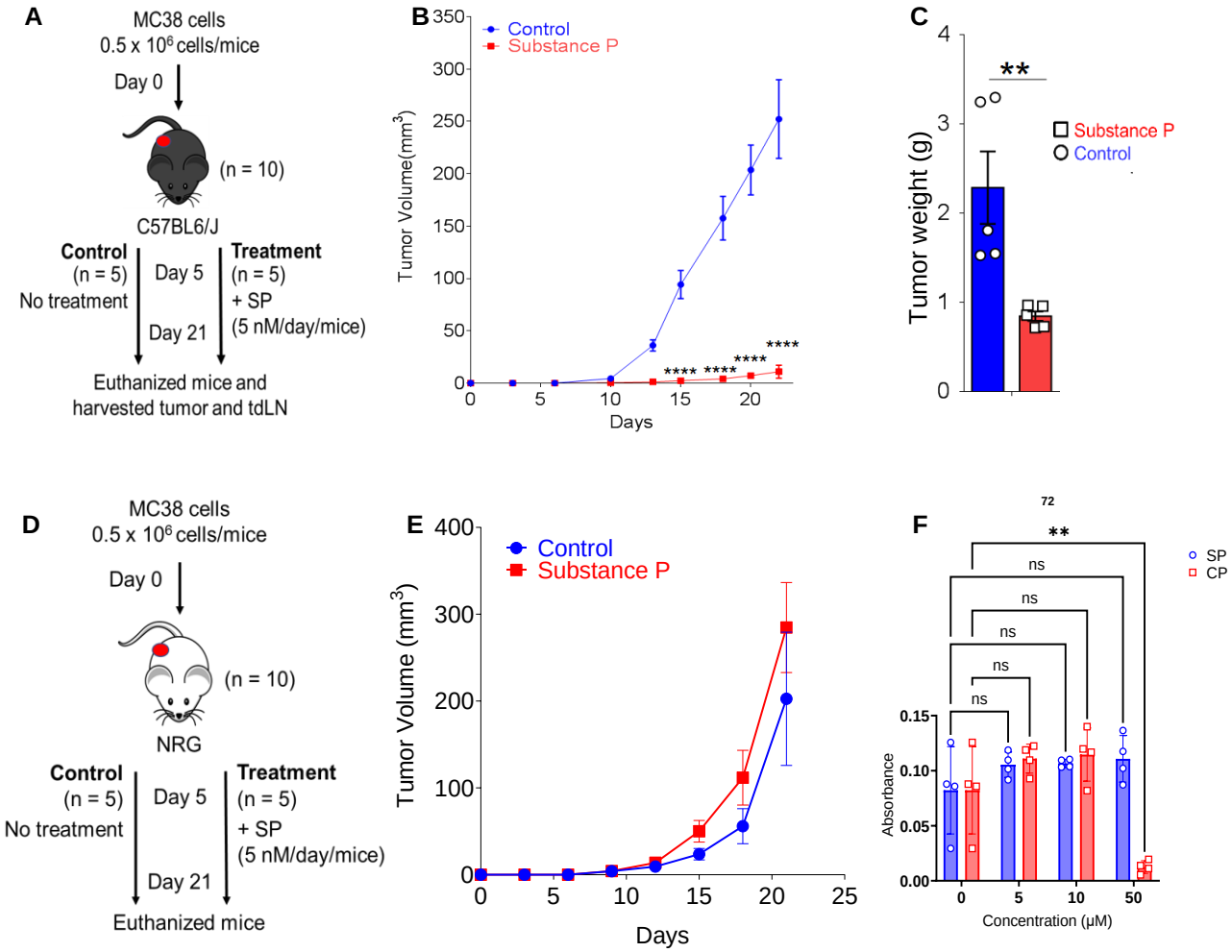


Figure 5. SP treatment reduces tumor growth *in vivo* through immune cells.
(A) Experimental design of SP treatment given to MC38 tumor induced C57BL/6J mice.
(B) Comparison of tumor volume data of MC38 tumor treated with SP injection (5 nM/day) with control tumor that received no treatment.
(C) Comparison of tumor weight data of MC38 tumor treated with SP injection (5 nM/day) with control tumor that received no treatment (n = 5 mice/group; two independent experiment).
(D) Experimental design of SP treatment given to NRG mice with MC38 tumor induced.
(E) Comparison of tumor volume data of MC38 tumor treated with SP injection (5 nM/day) with control tumor that received no treatment in NRG mice (n = 5 mice/group).
(F) MTT analysis of SP-treated and CP-treated MC38 cells.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

3.3 NK1R signaling enhances T-bet$^+$ and RORγt$^+$ effector, central memory, and tissue resident memory CD8 T cells within the tumor and tdLNs.

To distinguish the immune cell sub-populations that were affected by SP treatment in tumor responses, cells isolated from tumor and tdLNs following SP treatment were characterized based on multiple cell-surface and intracellular markers by multicolor spectral flow cytometry. The acquired high-dimensional data was analyzed by performing clustering analysis and dimensionality reduction algorithms t-SNE and FlowSOM to assess immune cell perturbations upon SP treatment (see Materials and Methods). We have identified several unique immune cell clusters within the tumor and tdLNs that showed significant changes between the SP-treated and control groups. Within tumors, we could identify fourteen clusters that showed significant changes following SP treatment, out of which five were CD8 T cell clusters (**Figure 6A**). Further, when we looked into the median fluorescence intensity (MFI) of different markers on the intra-tumoral CD8 T cells, we found that SP treatment significantly enhanced the expression of the activation and pro-inflammatory markers on the CD8 T cells such as CD69, ICOS, and CD44, T-bet and RORγt **(Figure 6B)**. There was also an increase in the central memory markers CCR7 and CD62L **(Figure 6B)**. We have also found increased expression of the CXCR5 on the intratumoral CD8 T cells. CXCR5 is the receptor of the chemokine CXCL13, which is very important for the formation of the tertiary lymphoid structures (TLS) within the tumor. We also found an overall decrease in the expression of anti-inflammatory markers amongst CD8 T cells **(Figure 6B)**. These included the regulatory markers FOXP3, GITR, CTLA-4, and PD-1.

Then we performed the meta-clustering analysis of lymphocytes gated on live CD45$^+$ cells from the tumor and found five significantly altered CD8 T cell clusters between the SP-treated and control groups. We then analyzed the phenotypes of these specific CD8 T cell clusters based on the mean expression of various markers within individual clusters (**Figures 7A and 7B**). The clusters and their phenotype are depicted in Figure 6B and given in **Table 1**. These clusters (Clusters 13,15,18, 19, and 23) increased significantly in the SP-treated tumors and were pro-inflammatory in nature. Cluster 13 (CD25^{mid}CXCR5$^+$CD62L$^-$ cells) consisted of activated effector memory CD8 T cells, and Cluster 15 (CD25$^+$CD44$^+$CCR7$^+$CD62L$^-$CXCR5$^+$PD1$^+$ cells) were of effector memory subtype. Cluster 18 cells which are CD25$^+$CD62L$^+$CXCR5$^+$RORγt$^+$

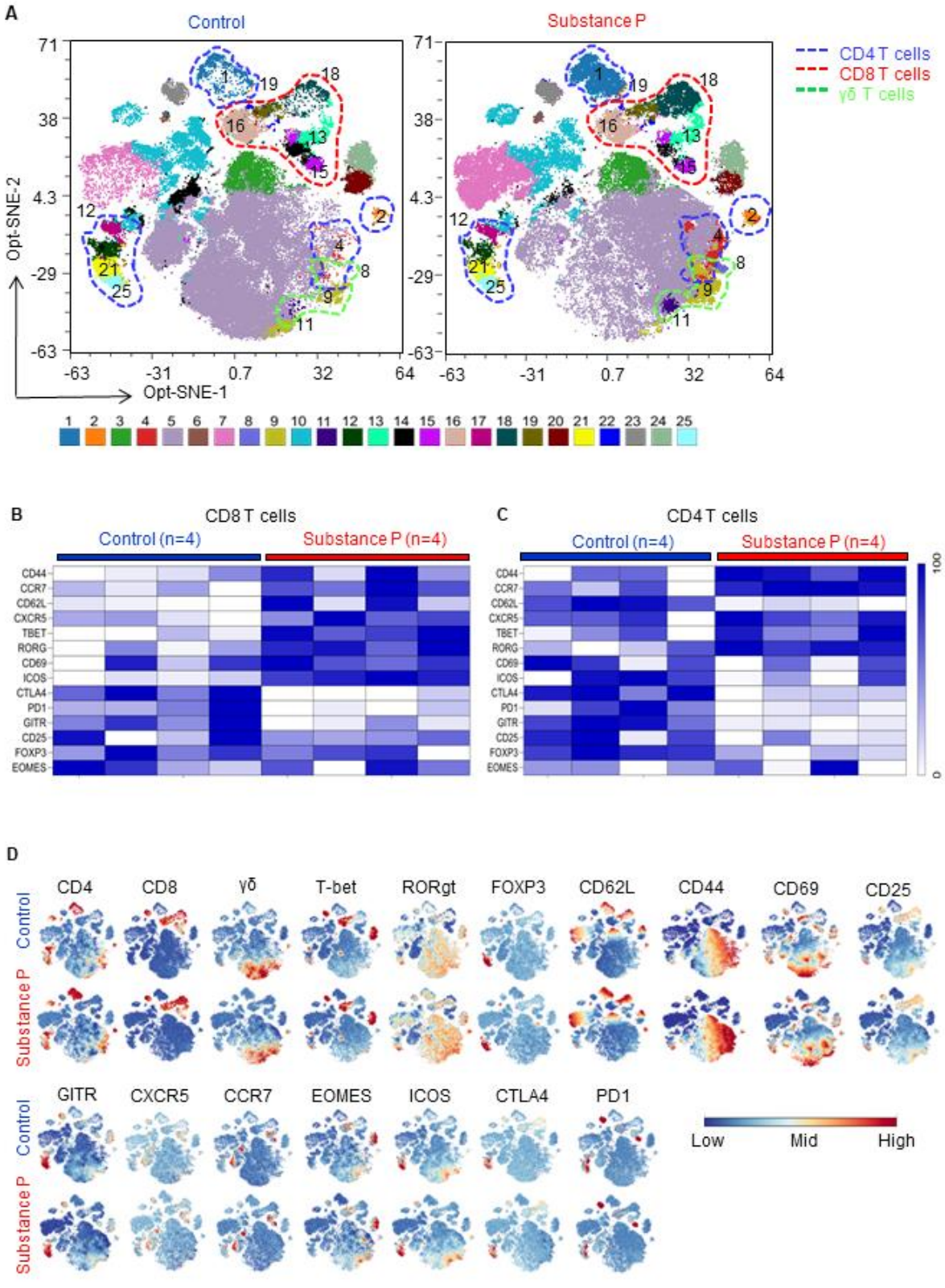

A
Control
Substance P
CD4 T cells
CD8 T cells
γδ T cells
Opt-SNE-2
Opt-SNE-1
71
38
4.3
-29
-63
-31
0.7
32
64
B
CD8 T cells
Control (n=4)
Substance P (n=4)
C
CD4 T cells
Control (n=4)
Substance P (n=4)
CD44
CCR7
CD62L
CXCR5
TBET
RORG
CD69
ICOS
CTLA4
PD1
GITR
CD25
FOXP3
EOMES
100
0
D
CD4
CD8
γδ
T-bet
RORgt
FOXP3
CD62L
CD44
CD69
CD25
Control
Substance P
GITR
CXCR5
CCR7
EOMES
ICOS
CTLA4
PD1
Low
Mid
High

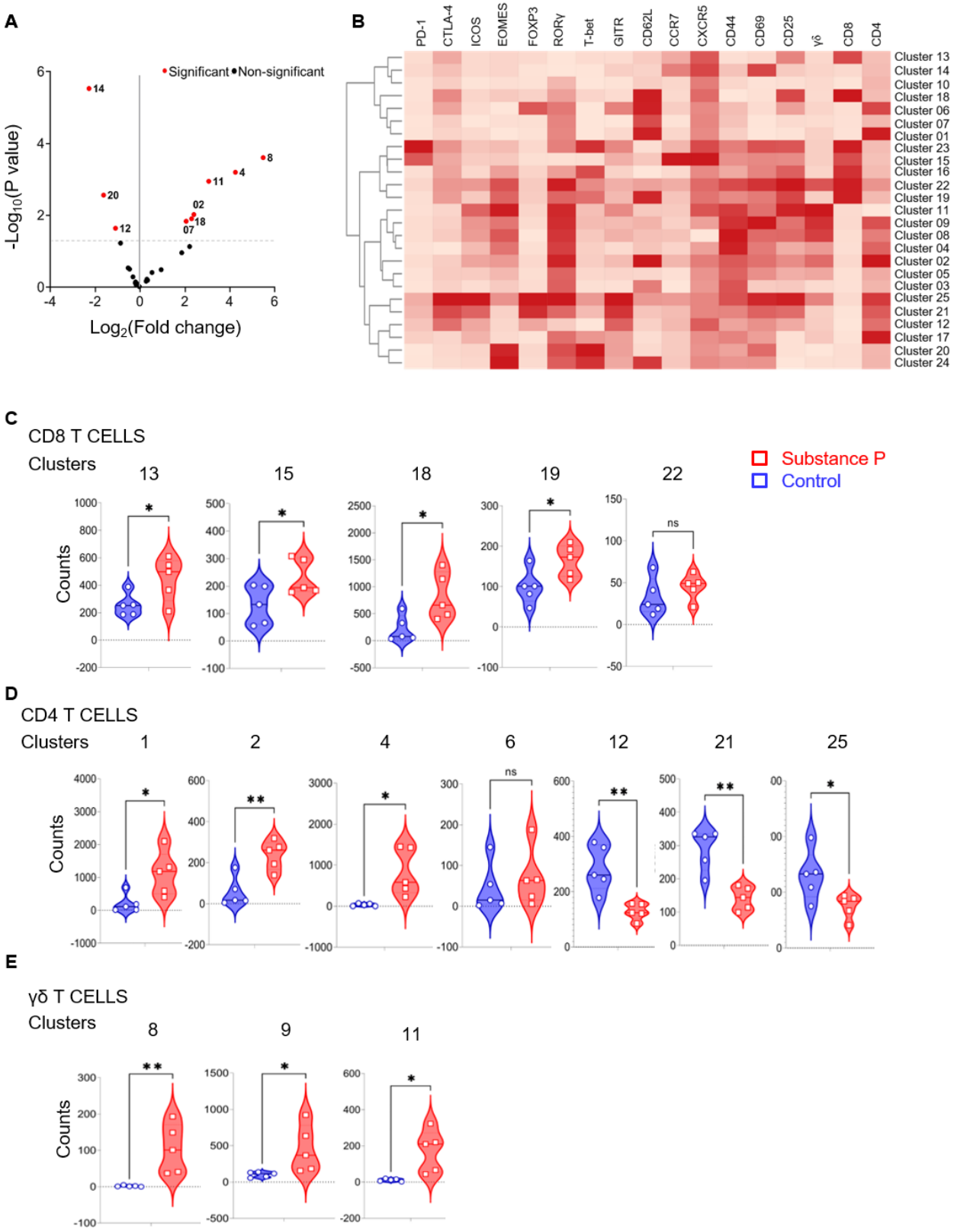
Figure 6. SP promotes pro-inflammatory markers and suppress immunoregulatory markers in CD8 T and CD4 T cell clusters.
(A) t-SNE plots of metaclusters of intratumoral immune cells gated on CD45+ live lymphocytes from control and SP treated tumor. Significantly altered metaclusters as per volcano plot and hierarchical clustering of mean expression level of markers is marked on the figure.
(B) Heat map of expression level of different markers in CD8 T cells of control and SP-treated tumor.
(C) Heat map of expression level of different markers in CD4 T cells of control and SP-treated tumor.
(D) t-SNE plots of different markers (mentioned in the figure) across cell meta-clusters in control and SP-treated tumors.

corresponds to Tc17 CD8 T cells with a strong capacity to home to follicles of lymphoid structures in the TME (Chen et al., 2019; Krieg et al., 2018) and have enhanced effector functions (Wieland et al., 2018). Cluster 23 is of effector memory phenotype of CD8 T cells that are $CD69^{+}CD44^{+}CD62L^{-}$T-$bet^{+}$ $PD1^{+}$ expression. Cluster 19 is of activated central memory phenotype with $CD69^{+}CD44^{+}CD62L^{+}CXCR5^{low}$ expression and has a coexpression of T-bet and RORγt, which is associated with enhanced effector function and increased cytotoxicity against tumors **(Figure 7C).**

Table 1. Immunophenotype of intra-tumor CD8 T cell clusters that changed significantly between SP-treated group and control group.

$CD8^{+}$ Clusters	Change	Expression
13	Increasing*	$CD8^{+}CD25^{mid}CXCR5^{+}CD62L^{-}$ cells
15	Increasing*	$CD8^{+}CD25^{+}CD44^{+}CCR7^{+}CD62L^{-}CXCR5^{+}PD1^{+}$ cells
18	Increasing*	$CD8^{+}CD25^{+}CD62L^{+}CXCR5^{+}ROR\gamma t^{+}$ cells
19	Increasing*	$CD8^{+}CD25^{+}CD69^{+}CD44^{+}CD62L^{+}CXCR5^{low}$T-$bet^{+}$ $ROR\gamma t^{+}EOMES^{+}$ cells
22	Non-significant	$CD8^{+}\gamma\delta^{mid}CD25^{+}CD69^{+}CD44^{+}CXCR5^{+}ROR\gamma t^{+}EOMES^{+}$ cells

p value significant levels *<0.05, **<0.01, ns = non-significant


A
Significant
Non-significant
-Log10(P value)
Log2(Fold change)
14
20
12
8
4
11
02
18
07
B
PD-1
CTLA-4
ICOS
EOMES
FOXP3
RORγ
T-bet
GITR
CD62L
CCR7
CXCR5
CD44
CD69
CD25
γδ
CD8
CD4
Cluster 13
Cluster 14
Cluster 10
Cluster 18
Cluster 06
Cluster 07
Cluster 01
Cluster 23
Cluster 15
Cluster 16
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Cluster 11
Cluster 09
Cluster 08
Cluster 04
Cluster 02
Cluster 05
Cluster 03
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1
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12
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γδ T CELLS
Clusters
8
9
11
Counts

Figure 7. SP alters CD8 T, CD4 T and γδ T cell clusters in the tumor to support anti-tumor immune responses.
(A)Volcano plot of significantly changed clusters in tumor following SP treatment generated using EdgeR algorithm. Significantly changed clusters are denoted as green circles above threshold value (0.05).
(B) Heat map of mean expression level of different markers in n = 25 cell clusters obtained through hierarchical clustering within the SP-treated and control tumors.
(C) Statistical comparisons of significantly altered CD4 T cell clusters following SP treatment.
(D) Statistical comparisons of significantly altered CD8 T cell clusters following SP treatment.
(E) Statistical comparisons of significantly altered γδ T cell clusters following SP treatment.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

Additionally, FACS analysis of CD8 T cells showed significantly increasing T-bet$^+$RORγt$^+$ cells, RORγt$^+$, and T-bet$^+$ CD8$^+$ T cells in terms of total percentage and absolute count of cells (**Figure 8**). Central memory (CCR7$^+$CD62L$^+$) and effector cell population (EOMES$^-$T-bet$^+$) of CD8 T cells also showed a significant increase within

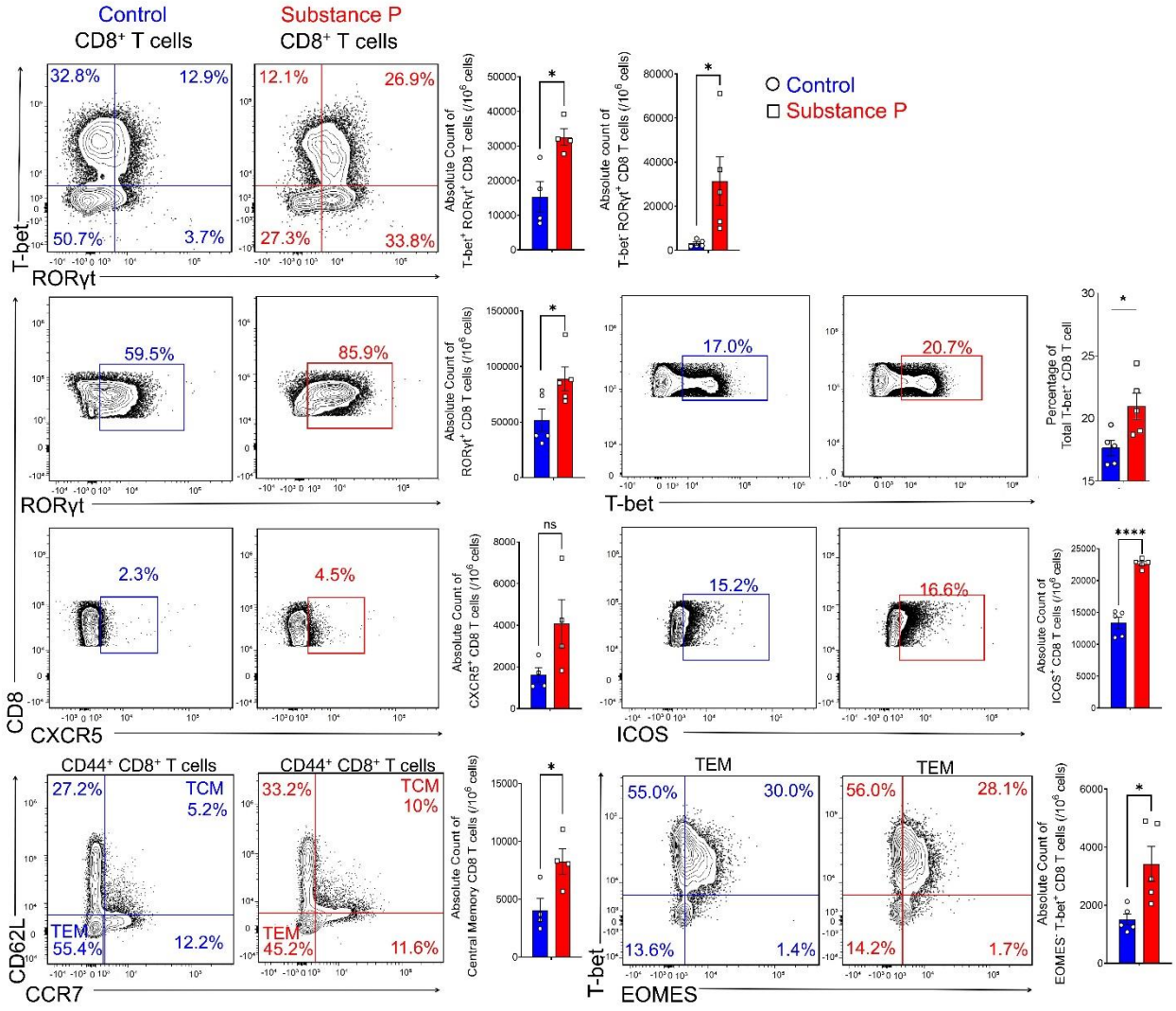

Figure 8. Flow cytometric comparative plot of different cell surface markers and transcription factors in CD8 T cells from control and SP treated tumors and statistical comparison of absolute count of cellular population of various subsets of CD8 T cells from control and SP treated mice.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001, ns=non-significant.

the tumor. This aligned with our previous results from the clustering analysis of cells based on their mean and median marker expression.

Overall, these data indicate that SP treatment significantly enhances the pro-inflammatory anti-tumor CD8 T cell populations within the TME. These CD8 T cells are of Tc1 and Tc17 phenotypes. They are not only efficient in the formation of memory CD8 T cells, but they also have the capacity to home to tertiary lymphoid structures within the tumor and to secondary lymphoid organs, which in turn dramatically enhance their anti-tumor capacity. The functions of the CD8 T cells in a tumor are regulated by CD4 T-helper (Th) cells both positively and negatively. So, next, we have tried to understand the effect of the NK1R signaling on the CD4 T cells in the tumor.

3.4. NK1R signaling enhances pro-inflammatory Th17 and Th1-like Th17 CD4 T cells and suppresses regulatory Treg cells in the TME and tdLNs of colon tumors.

Flow cytometric analysis of tumor and tdLN cells revealed significant changes in CD4 T cell sub-populations between SP-treated and control groups (**Figure 6A**). Analyzing the mean expression pattern of selected markers in CD4 T cells between these two groups showed heightened levels of pro-inflammatory markers and diminished regulatory markers in both the tumor and tdLNs of SP-treated animals (**Figure 6C**). SP treatment significantly enhanced the expression of the RORγt, CD44, CXCR5, and CCR7 and reduced the expression of CD25, CD69, CD62L, FOXP3, CTLA-4, PD1, and GITR on the intra-tumoral CD4 T cells (**Figure 6C**).

Dimensionality reduction, followed by clustering analysis by FlowSOM and t-SNE, provided unique CD4 T cell clusters that were altered within the TME of the SP-treated group when compared to the control (**Figure 7A**). Three of these clusters were significantly increased, and three decreased in SP-treated tumors (**See Table 3**). Similar to the overall expression pattern in the heat map (**Figure 7B**), all the clusters of CD4 T cells significantly increased and were characterized to be RORγt^{+}. Cluster 1 and cluster 2 are central memory CD4 T cells due to their expression of CD62L. Cluster 4 was CD62L^{-}CCR7^{-}CXCR5^{+} cells, which classifies them as follicular helper T

cells (Tfh) **(Figure 7D)**. Moreover, SP treatment significantly decreased the cell clusters 12, 21, and 25 within the tumor (**Figure 7D**). These clusters were $CD25^+FOXP3^+CTLA\text{-}4^+GITR^+$ cells, which are predominant immune-regulatory markers. Clusters 21 and 25 were Tregs as they are $CD25^+FOXP3^+$, and these cells

Table 2. Immunophenotype of intra-tumor CD4 T cell clusters that changed significantly between SP-treated group and control group.

$CD4^+$ Clusters	Change	Expression profile
1	Increasing*	$CD4^+CD62L^+ROR\gamma t^{mid}$ cells
2	Increasing**	$CD4^+\gamma\delta^{mid}CD69^{mid}CD44^{mid}CD62L^+ROR\gamma t^+$ cells
4	Increasing*	$CD4^+CD69^{mid}CD44^+CD62L^-CCR7^-CXCR5^+$ $ROR\gamma t^+EOMES^+$ cells
6	Non-significant	$CD4^+CD25^+CD62L^+ROR\gamma t^+FOXP3^+GITR^+$ cells
12	Decreasing**	$CD4^+CD69^+CD62L^-CXCR5^+GITR^+CTLA\text{-}4^+$ cells
21	Decreasing**	$CD4^+CD69^+CD25^+CD62L^{low}CXCR5^{low}CCR7^{low}$ $CD44^+FOXP3^+GITR^+ICOS^+CTLA\text{-}4^+$ cells
25	Decreasing*	$CD4^+CD69^+CD25^+CD44^+CD62L^+CCR7^+$ $CXCR5^+FOXP3^+GITR^+ICOS^+CTLA\text{-}4^+$ cells

p value significant levels *<0.05, **<0.01, ns = non-significant

are more immunosuppressive in nature due to the expression of immune checkpoint receptors such as CTLA4 and GITR **(Table 2)**.

Finally, flow cytometric comparative analysis of intratumoral CD4 T cells showed increased memory CD4 T cells in SP-treated tumors. We observed significantly increased $T\text{-}bet^+ROR\gamma t^+CD4^+$ T cells and $ROR\gamma t^+$ CD4 T cells. Whilst the Th1-like Th17 and Th1 cells increased, we saw significantly decreased $FOXP3^+CD4^+$ T cells in SP-treated tumors. CD4 T cells bearing CTLA-4, PD-1, and GITR also decreased

significantly in SP-treated tumors. CXCR5^{-}CD4^{+} T cells (T-helper cells) increased within the tumor of SP-treated mice. Moreover, amongst follicular CD4 T cells (CXCR5^{+}CD4^{+}), we saw that SP treatment significantly enhanced Tfh cells and decreased the follicular regulatory CD4 T (Tfr) cells (**Figure 9**).

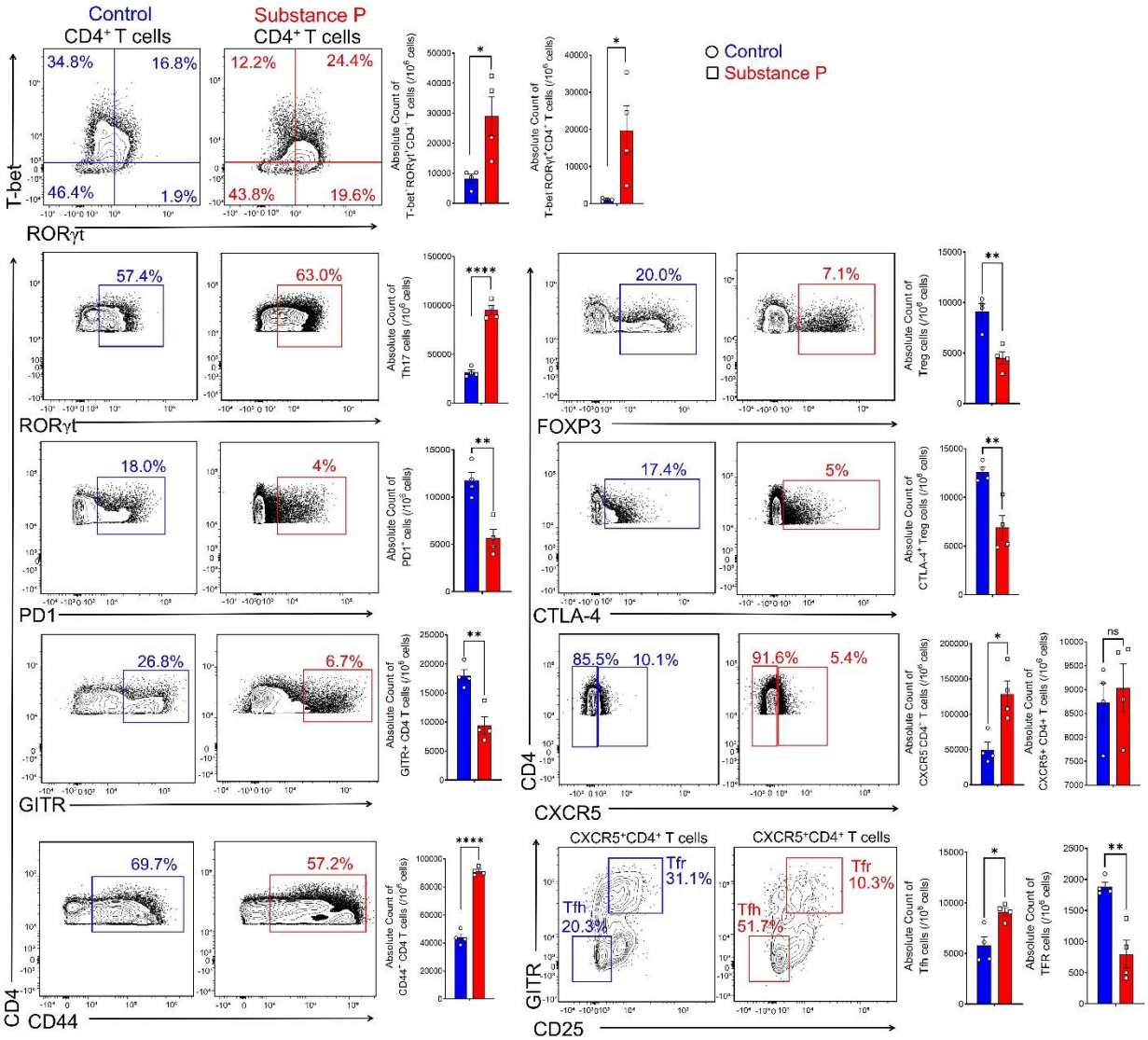

Figure 9. Flow cytometric comparative plot of different cell surface markers and transcription factors in CD4 T cells from control and SP treated tumors and statistical comparison of absolute count of cellular population of various subsets of CD4 T cells from control and SP treated mice.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001, ns; non-significant.

Altogether, external SP administration enhanced the pro-inflammatory Th17 CD4 T cells and Th1-like Th17 cells within the tumor and draining lymph nodes along with decreasing Treg populations. By doing this, SP may enhance the effector functions of the CD8 T cells within the tumor and subsequently reduce tumor growth.

3.5. NK1R signaling promotes anti-tumor γδ T cell clusters within the TME.

Apart from CD4 and CD8 T cells, other immune cells also affect the tumor progression directly or indirectly affect the cytotoxic functions of the CD8 T cells. Multidimensional flow cytometric analysis revealed SP treatment significantly altered three γδ T cell clusters within the tumors. These three clusters (11, 8, and 9) increased significantly upon SP treatment (**Figure 7E**), and all three clusters were $CD69^{+}CD25^{+}CD44^{+}ROR\gamma t^{+}EOMES^{+}$ (**See Table 3. And Figure 7B**). Though we haven't performed a detailed analysis of the γδ T cell subtypes, our data indicates SP treatment enhances overall inflammatory properties in the intra-tumoral γδ T cell.

Table 3. Immunophenotype of tumor γδ T cell clusters that changed significantly between SP-treated group and control group.

$\gamma\delta^{+}$ Clusters	**Change**	**Expression Profile**
11	Increasing*	$\gamma\delta^{+}CD25^{+}CD69^{+}CD44^{+}CD62^{low}CXCR5^{+}ROR\gamma t^{+}EOMES^{+}ICOS^{+}$ cells
8	Increasing**	$\gamma\delta^{+}CD4^{low}CD69^{+}CD25^{+}CD44^{+}CD62^{low}ROR\gamma t^{+}EOMES^{+}$ cells
9	Increasing*	$\gamma\delta^{+}CD4^{low}CD69^{+}CD25^{+}CD44^{+}CXCR5^{+}CD62^{low}$T-$bet^{mid}ROR\gamma t^{+}EOMES^{+}ICOS^{+}$ cells

p value significant levels *<0.05, **<0.01, ns = non-significant

3.6. SP-NK1R signaling enhances Tc1, Tc17, Th1-like Th17 and Th17 cells in the tumor draining lymph nodes.

Not only within the tumor, but we have also found changes in the CD8 T cell populations within the tdLNs (Inguinal, axillary, and brachial) similar to the tumor. We have found SP treatment changed the frequency of seven CD8 T cell clusters (**Figures 10A and 10B**). Four clusters out of this showed a significantly increased in SP treatment than control tumor-bearing mice. Within the tdLNs, there was a significant increase in RORγ^{+} CD8^{+} T (Cluster 14, 16, and 21) cells like that of tumors (**See Table 4**.). Phenotype of these clusters was derived from the clustered heatmap of the markers (**Figure 10C**). Clusters 7, 14, and 16 were central memory T cells with their CD62L^{+}CCR7^{+} phenotype (**Figure 10D**). Their pro-inflammatory and anti-tumor function is marked by the high expression of RORγt and low expression of regulatory markers. The presence of increased activation and memory markers within these clusters and low expression of regulatory markers is thus reflective of the pro-inflammatory, anti-tumor CD8 T cell population within the tdLN.

Table 4. Immunophenotype of tdLN CD8 T cell clusters that changed significantly between SP-treated group and control group.

CD8^{+} Clusters	Change	Expression
7	Increasing*	CD8^{+}CCR7^{+}CD62L^{+} cells
14	Increasing**	CD8^{+}CD44^{+}CCR7^{+}CD62L^{+}RORγt^{+} cells
16	Increasing*	CD8^{+}CD25^{+}CD62L^{+}RORγt^{+}GITR^{+}CTLA-4^{-} cells
21	Increasing*	CD8^{+}CD44^{+}CD62L^{low}CCR7^{mid}RORγt^{+} cells

Flow cytometric comparative analysis of CD8 T cells expressing these markers also showed significantly increased T-bet^{+}, RORγt^{+}, and RORγt^{+}T-bet^{+}CD8^{+} T cells in SP-treated. There was a significant increase in overall memory CD8 T cells (CD44^{+}CD8^{+}) and effector memory CD8 T cells (CD62L^{-}CCR7^{+}CD44^{+}CD8^{+}), and PD1^{+}CD8^{+} T cells significantly decreased (**Figure 11**).

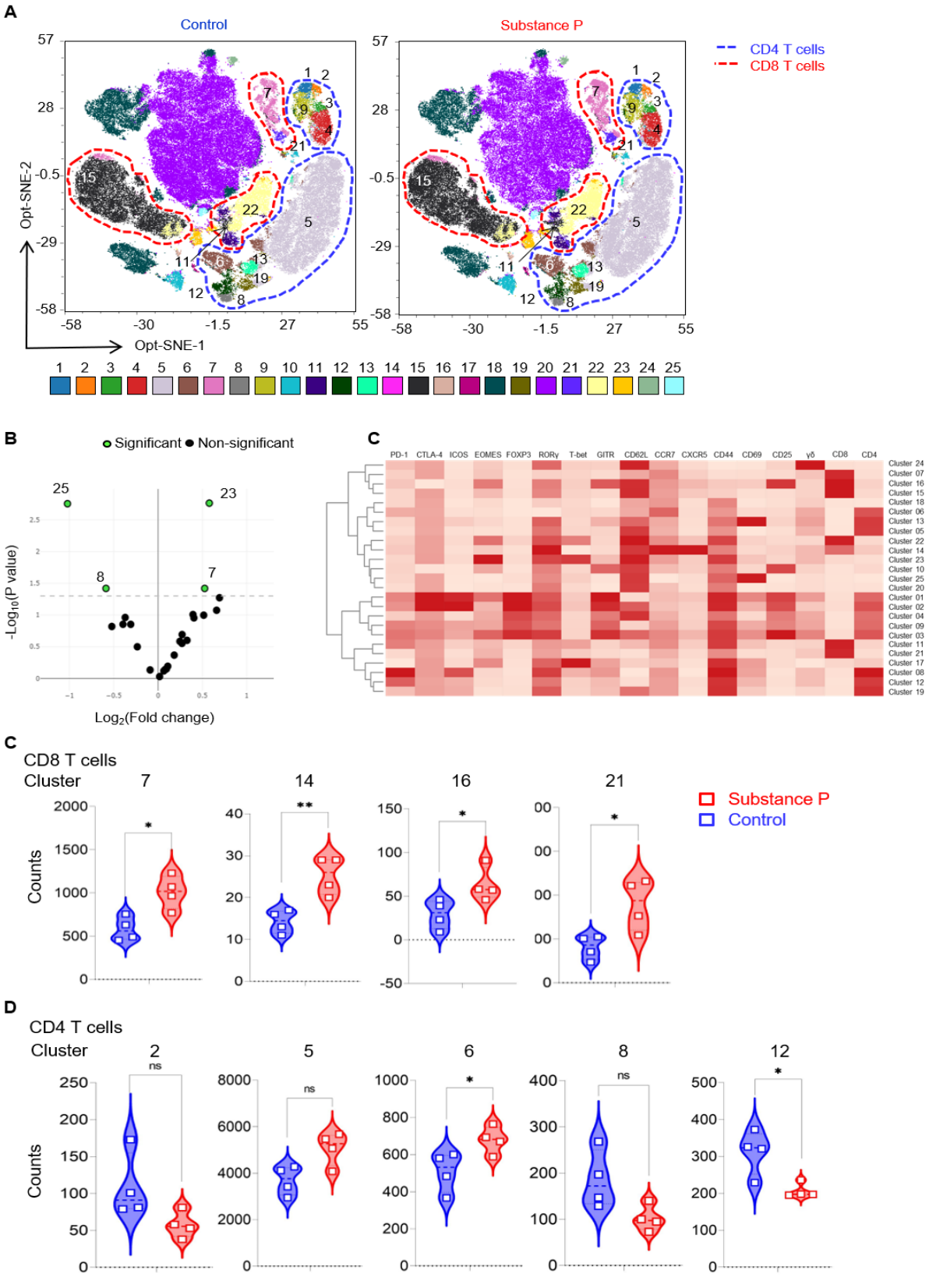
A
Control
Substance P
CD4 T cells
CD8 T cells
Opt-SNE-2
Opt-SNE-1
B
Significant
Non-significant
-Log10(P value)
Log2(Fold change)
C
PD-1 CTLA-4 ICOS EOMES FOXP3 RORγ T-bet GITR CD62L CCR7 CXCR5 CD44 CD69 CD25 γδ CD8 CD4
C
CD8 T cells
Cluster
Counts
Substance P
Control
D
CD4 T cells
Cluster
Counts

Figure 10. SP treatment alters immune cell clusters in tdLN to promote pro-inflammatory anti-tumor immune response.
(A) Opt-SNE plots of metaclusters of tdLN cells gated on CD45+ live lymphocytes from control and SP treated tumor. Significantly altered metaclusters as per volcano plot and hierarchical clustering of mean expression level of markers is marked on the figure.
(B) Heat map of mean expression level of different markers in n = 25 cell clusters obtained through hierarchical clustering within the SP-treated and control tdLNs.
(C) Volcano plot of significantly changed clusters in tumor following SP treatment generated using EdgeR algorithm. Significantly changed clusters are denoted as green circles above threshold value (0.05).
(D) Statistical comparisons of significantly altered CD8 T cell clusters following SP treatment.
(E) Statistical comparisons of significantly altered CD4 T cell clusters following SP treatment.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

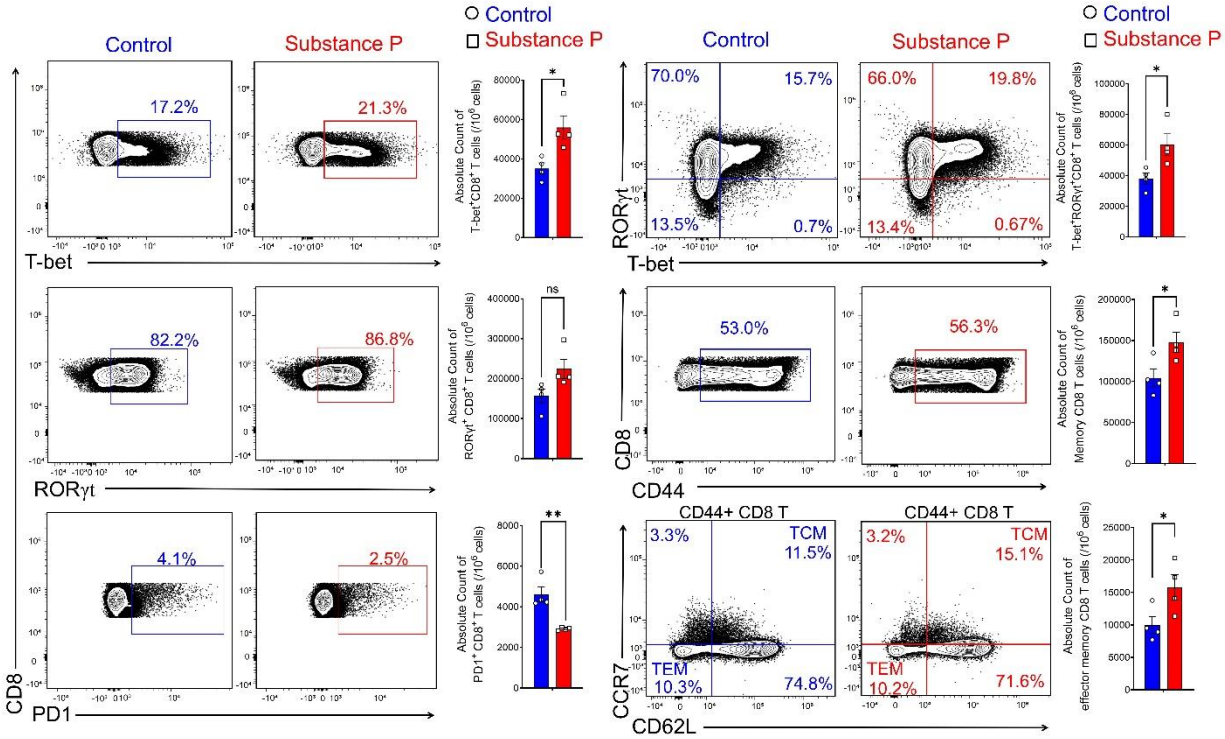

Figure 11. Flow cytometric comparative plot of different cell surface markers and transcription factors in CD8 T cells from tdLN of control and SP treated tumor-bearing mice and statistical comparison of absolute count of cellular population of various subsets of CD8 T cells from control and SP treated mice. p value significant levels *<0.05, **<0.01, ***<0.001, ns; non-significant.

Similar analysis on tdLN cells also identified different CD4 T cell clusters corresponding to different CD4 T cells that are increased in the SP-treated group (**Figures 10A and 10B**). Among them, cluster 6 was significantly increased, which is RORγt$^+$, corresponding to the Th17 CD4 T cell phenotype. In addition, SP treatment also significantly reduced cluster 12, which were CD69$^+$CD62L$^-$CXCR5$^+$GITR$^+$CTLA4$^+$

cells, corresponding to the regulatory cells (**Figure 10E and Table 4**). The flow cytometric analysis showed a significantly increased population of overall memory CD4 ($CD44^+CD4^+$) T cells and T-helper cells ($CXCR5^-CD4^+$) along with significantly decreased $CTLA\text{-}4^+CD4^+$ and $PD1^+CD4^+$ T cells in SP-treated group (**Figure 12**).

Table 5. Immunophenotype of tdLN CD4 T cell clusters that changed significantly between SP-treated group and control group.

$CD4^+$ Clusters	Change	Expression profile
2	Non-significant	$CD4^+CD62L^+ROR\gamma t^{mid}$ cells
5	Non-significant	$CD4^+\gamma\delta^{mid}CD69^{mid}CD44^{mid}CD62L^+ROR\gamma t^+$ cells
6	Increasing**	$CD4^+CD69^{mid}CD44^+CD62L^-CCR7^-CXCR5^+ ROR\gamma t^+EOMES^+$ cells
8	Non-significant	$CD4^+CD25^+CD62L^+ROR\gamma t^+FOXP3^+GITR^+$ cells
12	Decreasing**	$CD4^+CD69^+CD62L^-CXCR5^+GITR^+CTLA4^+$ cells

p value significant levels *<0.05, **<0.01, ***<0.001, ns; non-significant.

Overall, the immunomodulation within the tdLNs of SP-treated tumor-bearing mice partially reflected the significant changes in their corresponding tumor immune cell populations. There was an increase in overall memory CD4 and CD8 T cell population, Tc1 and Tc17 cells, and Th17 and Th1-like Th17 cells within the tdLNs of SP-treated mice. Also, SP treatment decreased CD4 and CD8 T cells expressing immune-regulatory markers in the tdLNs. Altogether these changes may contribute to the tumor-suppressing effect of SP treatment.

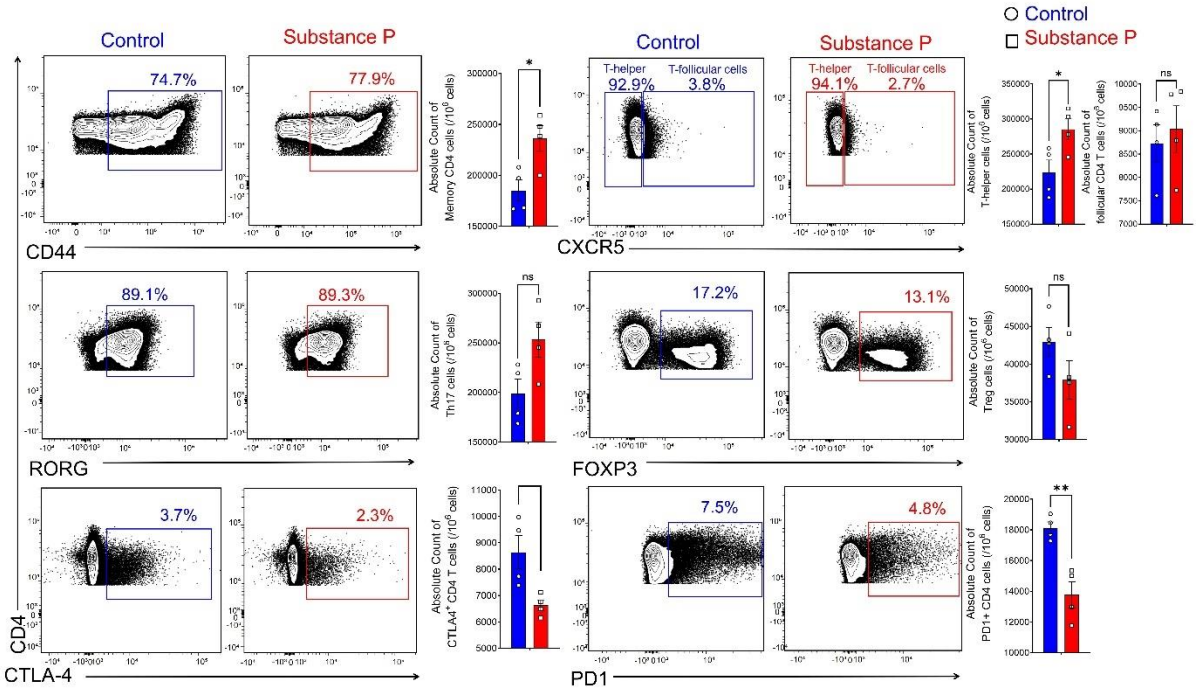

Figure 12. Flow cytometric comparative plot of different cell surface markers and transcription factors in CD4 T cells from tdLN of control and SP treated tumor-bearing mice and statistical comparison of absolute count of cellular population of various subsets of CD4 T cells from control and SP treated mice.
p value significant levels *<0.05, **<0.01, ***<0.001, ns; non-significant.

3.7. SP treatment increases pro-inflammatory IL-17 cytokine and reduces anti-inflammatory IL-10 in the serum while maintaining the IFN-γ levels.

Further, to observe whether the changes in the immune cell subtypes have any functional relevance, we have looked into whether NK1R signaling affected the serum levels of the different cytokines in the tumor conditions by ELISA. We have found that SP significantly enhances the serum levels of IL-17 and reduces the serum levels of IL-10 and TNF-α (**Figures 13A, 13B, and 13D**). This is a direct reflection of enhanced ROR-γt+ T cells and reduced FOXP3+ T cells in tumor and tdLN. But the TNF-α plays a dual role in tumor progression. However, we haven't found any significant changes in the serum levels of IFN-γ and IL-1β (**Figure 13C-13D**).

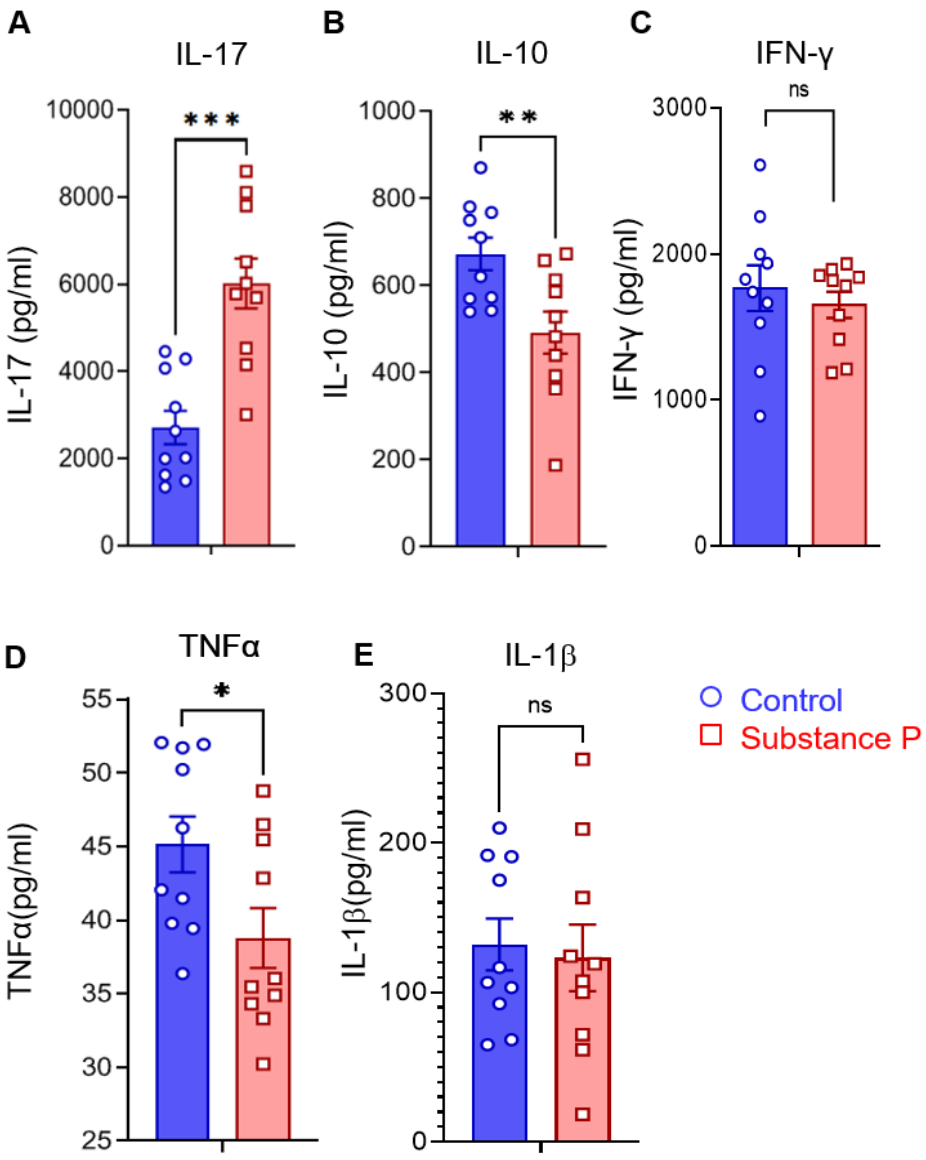

Figure 13. SP affects serum cytokine levels of tumor bearing mice.
(A) Serum IL-17 ELISA of the serum samples from the control and SP treated MC38 tumor bearing mice.
(B) Serum IL-10 ELISA of the serum samples from the control and SP treated MC38 tumor bearing mice.
(C) Serum IFN-γ ELISA of the serum samples from the control and SP treated MC38 tumor bearing mice.
(D) Serum TNFα ELISA of the serum samples from the control and SP treated MC38 tumor bearing mice.
(E) Serum IL-1β ELISA of the serum samples from the control and SP treated MC38 tumor bearing mice.
p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

So, our data indicate that SP treatment significantly enhances the functional pro-inflammatory CD4 and CD8 T cells and reduces anti-inflammatory cellular phenotypes, which may contribute to reduced tumor growth.

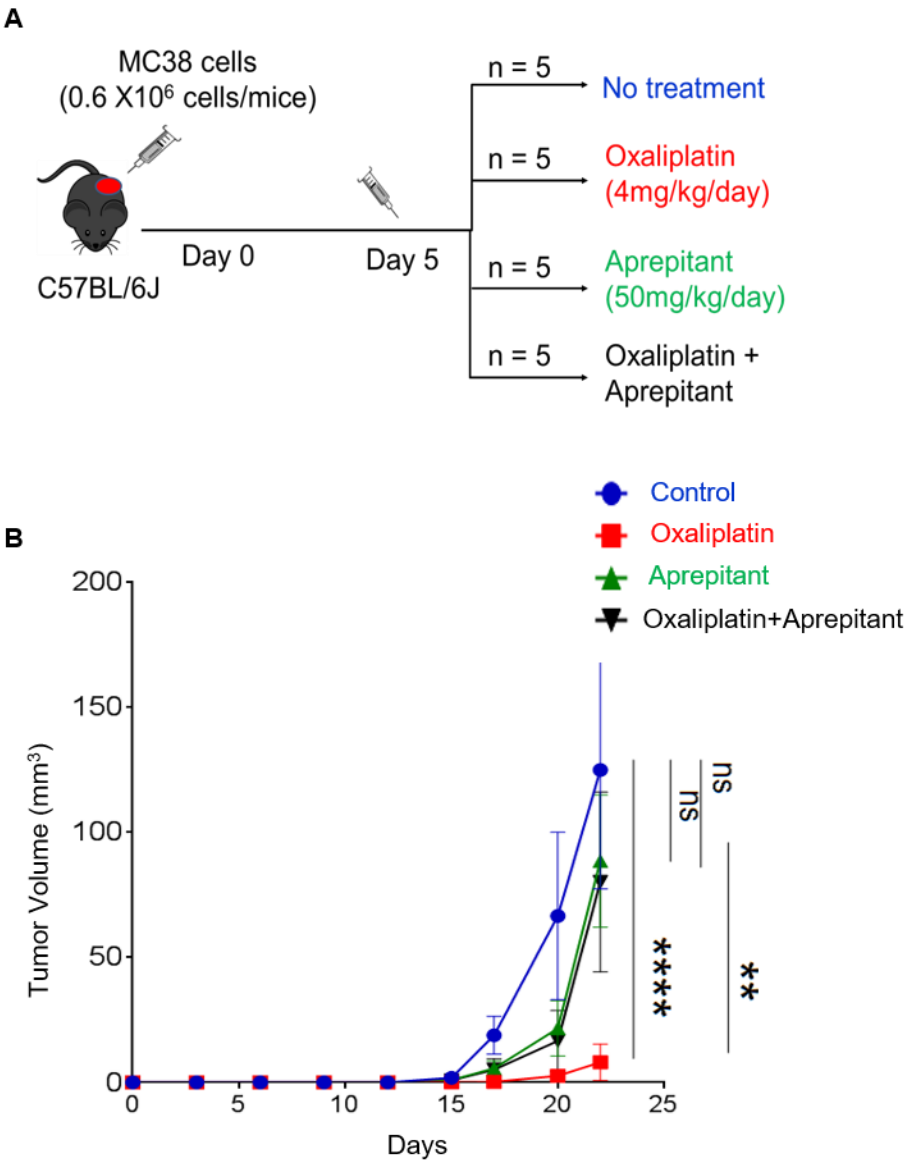

Figure 14. NK1R antagonist reduces the efficacy of chemotherapy drug.
(A) Experimental design of AP and oxaliplatin treatment in MC38 tumor bearing C57BL6/J mice.
(B) Comparison of tumor volume data of MC38 tumor treated with p value significant levels *<0.05, **<0.01, ***<0.001, ****<0.0001.

3.8. NK1R Antagonist, when combined with oxaliplatin, decreases its efficacy in treating colon cancer.

Fosaprepitant is a clinically approved NK1R antagonist drug used to treat chemotherapy-induced nausea (Weinstein et al., 2016). It is not known how they will affect tumor growth, given the role of NK1R in eliciting immune responses. We developed an orthotropic tumor by subcutaneously injecting MC38 cells in C57BL/6J mice (**Figure 14A**). After the tumor became palpable, we separated the mice into four groups. One group received NK1R antagonist fosaprepitant (AP), one group received oxaliplatin (OXA), one group received both, and the fourth one was kept as control. We have observed combination of AP and OXA significantly increased tumor growth than OXA-only treated mice which controlled tumor growth efficiently (**Figure 14B**). AP itself did not bring about any significant change in the tumor growth when compared to untreated controls. Therefore, the use of NK1R antagonists along with chemotherapeutic drugs like oxaliplatin can decrease the efficacy of the chemotherapeutic drugs in controlling colon cancer growth.

CHAPTER 5
DISCUSSION

Substance-P is an established pro-inflammatory neuropeptide. NK1R signaling in immune cells promotes lymphocyte activation, proliferation, and migration and stimulates effector functions (Douglas & Leeman, 2011). SP-NK1R signaling was found to be crucial in T cell activation and survival and stimulate the release of pro-inflammatory cytokines like IFN-γ, IL-17, and IL-12. They influence T helper (Th) cell differentiation towards Th1 and Th17 in the psoriasis model (Morelli et al., 2020b). Previous works have also shown their role in suppressing regulatory immune cells and promoting the pathogenesis of acute inflammatory disorders, autoimmunity, and neurogenic inflammation (Ahmed et al., 1995; Mishra & Lal, 2021; Quartara et al., 2009; Weinstock et al., 2003). Given their ability to promote pro-inflammatory immune cells and suppress immune regulatory pathways, SP can have an important part to play in tumor growth and development. Previous studies on SP-NK1R signaling in cancer have shown both tumor-promoting (Asadi et al., 2012; Friess et al., 2003; Li et al., 2016; Muñoz et al., 2022b; C Palma et al., 1999) and anti-tumor effect based on the tumor model (Flageole et al., 1992; Friess et al., 2003; Harris & Witten, 2003; Manske & Hanson, 2005a). However, most of these works were performed in either *in vitro* settings or in tumor models in immune-deficient mice. But, since SP has a significant role in immune regulation, we have assessed the role of neurokinin signaling in an immunogenic tumor model. Therefore, this study aimed to determine what effect NK1R signaling would have on the murine colorectal cancer (CRC) model.

MC38 mice colon cancer cell lines express all the neurokinin receptors (TACRs; TACR1, TACR2, and TACR3), and they also have the capacity to synthesize and secrete active substance P due to the presence of the PPTA gene and PAM enzyme. However, these cells have a much lower capacity to degrade substance P because of the reduced expression of ACE enzyme. Apart from the cancer cell lines, different immune cell subsets also have variable expression of the components of the substance P system. Both CD4 and CD8 T cells are important components of adaptive immune system that regulates tumor progression. Both naïve and in vitro activated

CD8 T cells also expressed TACR and cellular SP components. However, activation reduced the expression of the TACRs and components of SP synthesis and degradation in CD8 T cells. *In vitro* differentiated CD4 T cell subsets also show variable expression of the TACRs and components of the substance P system.

We then assessed the effect of SP-NK1R signaling on CRC growth *in vivo.* As SP is known to modulate a variety of functions in the gut, CRC can be a great model to understand its effect on the tumor. External administration of SP significantly reduced tumor growth. But, SP enhances the viability of MC38 cancer cells *in vitro,* whereas NK1R antagonist CP-93456 significantly reduced it. Since NK1R is expressed by both tumor cells and immune cells, SP can have a bidirectional role in regulating cancer and immune cells. To dissect this, we have used immune-compromised mice to observe the effect of SP on the MC38 tumor growth in the absence of immune cells. In that setting, SP administration showed a tumor-promoting effect on the MC38 colon cancer growth. Therefore, the anti-tumor effects of the SP in wild-type cells are predominantly mediated through the regulation of the immune cells affecting cancer growth.

To identify these immune cell populations responsible for the anti-tumor effect, we have used multicolor spectral flow cytometry and clustering analysis on the tumor-infiltrating cells and tdLN cells. Overall, SP increased the effector and memory T cell and reduced the regulatory T cell population within the tumor. Specifically, SP increased Follicular Tc17 and circulatory Tc17 CD8 T cells. It also enhanced T-bet^{+}RORγt^{+} CD8 T cells. SP treatment significantly decreased the expression of different regulatory markers like FOXP3, CTLA-4, GITR, and PD-1 on CD8 T cells. Similar changes can also be found in the tumor-draining lymph nodes. SP also enhanced the Th17 and Th1-like Th17 cell populations and significantly decreased CD4 Treg cells within the tumor. Apart from that, SP treatment also reduced the expression of immune checkpoint molecules such as GITR, CTLA-4, and PD1 on the Tregs, suggesting the impaired suppressive capacity of those cells. Apart from CD4 and CD8 T cells, SP also enhanced the infiltration of pro-inflammatory $\gamma\delta$ T cells within the tumor and tdLN, suggesting their involvement in the anti-tumor response in SP treatment. However, more detailed experiments are needed to dissect various subtypes of γδ T cells and their anti-tumor effect in this condition.

Though we have seen that SP mobilizes the anti-tumorigenic CD4 and CD8 T cells and reduces the regulatory T cells in the tumor, the major effects of these cells are mediated through either direct cell-mediated killing or by releasing various cytokines in the microenvironment. SP also significantly enhanced the serum levels of pro-inflammatory cytokine IL-17A and reduced the serum levels of regulatory cytokine IL-10. These changes in the cytokine levels in the serum directly reflect the cellular changes that we have observed within tumors and tdLNs. However, we haven't found much change in the serum levels of IFN-γ. There are many recent evidence suggesting a pro-tumorigenic role of IFN-γ where increased levels of IFN-γ were found to be associated with a tumor-promoting microenvironment *(*Gocher *et al.*, 2022; Zagorulya *et al*., 2023). Therefore, we suggest our results to be in favor of the tumor regression observed within colon cancer-bearing mice.

NK1R antagonists like fosaprepitant (AP) are clinically approved and widely used treatments for chemotherapy-induced nausea based on NK1R's neurological role in eliciting spontaneous gut movements (Weinstein *et al*., 2016). However, their impact on the efficacy of the chemotherapeutic agent and tumor progression is not well understood, considering NK1R's crucial role in eliciting efficient immune responses. We have observed that agonism of NK1R receptors has anti-tumor potential. So, hypothetically the NK1R antagonism must have a detrimental effect on the tumor progression. To test this hypothesis, we investigated the effect of AP on tumor growth and the efficacy of anti-tumor effects of oxaliplatin *in vivo*. AP significantly decreased the efficacy of oxaliplatin in reducing tumors in mice. This suggests the crucial effect NK1R signaling on the TIME is not only responsible for the regulation of tumor growth but also has an impact on the efficacies of common chemotherapeutic agents used to target tumor progression.

In summary, our work showed that SP-NK1R signaling controls CRC growth in mice by enhancing the effector and memory CD8 T cells, Th17, and Th1-like Th17 cells and suppressing the regulatory T cell responses within the tumor microenvironment (**Figure 15**).

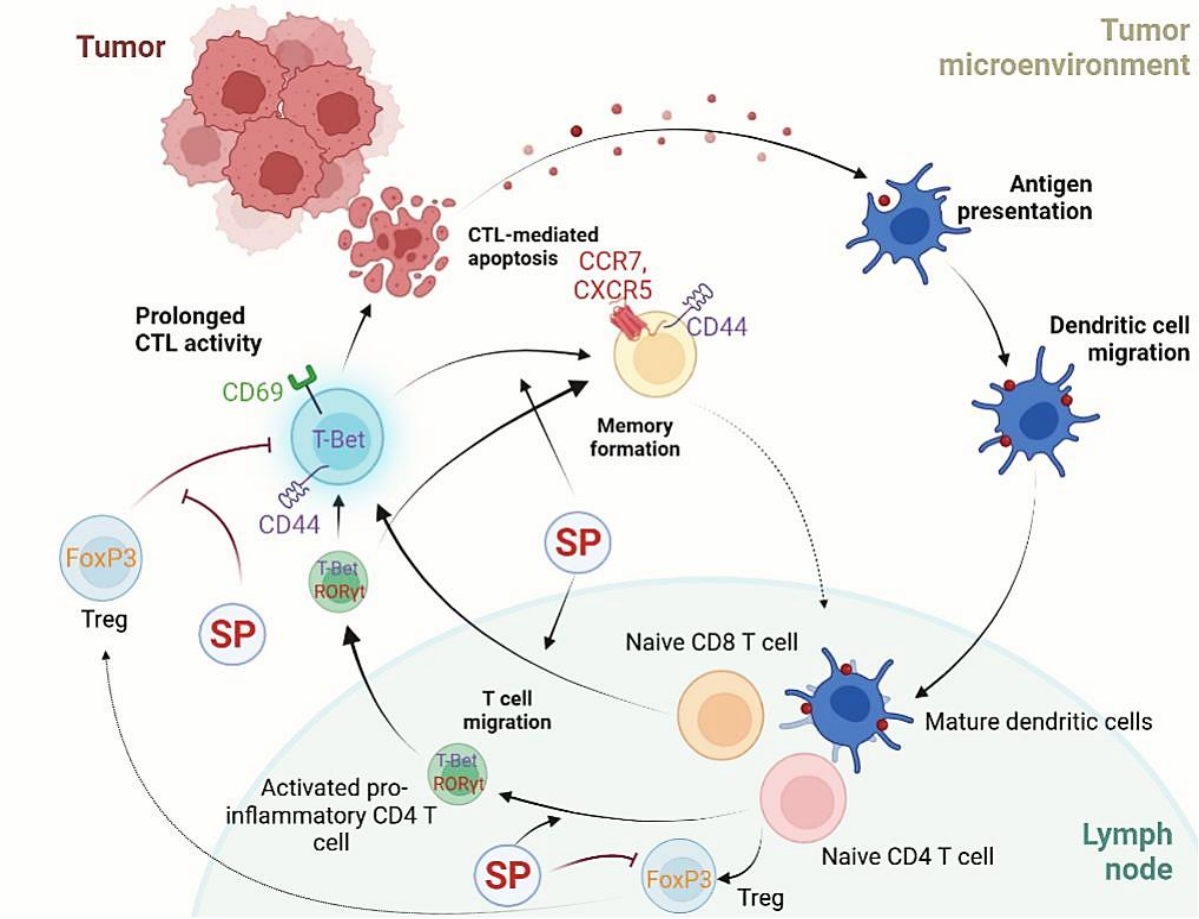

Figure 15. Graphical depiction of substance P's immunomodulatory action in the tumor microenvironment and draining lymph node of CRC. SP promotes effector and memory CD8 T cells and pro-inflammatory Th17 and Th1-like Th17 cells within the TME. SP suppress Treg cells and henceforth support anti-tumor immune response.

Therefore, by promoting anti-tumor immune cells and suppressing the immunoregulatory cells, SP was able to reduce colon cancer growth in mice. We could also emphasize the importance of neuro-immune crosstalk in constituting tumor development and how their influence needs to be considered for efficient anti-tumor therapies.

However, further work is required to establish the causality of this immunomodulation by NK1R signaling in detail. Cytokine profiling of SP-treated tumor and tdLNs can validate the anti-tumor effect of immunocellular modulation seen above. The antigen specificity of SP-augmented immune responses can be assessed with OT-I and OT-II expressing CRC cells that is specifically recognized by OVA specific CD8 T and CD4 T cells respectively *in vivo*. How exactly NK1R signaling initiates a pro-inflammatory immune response within the TME is still unknown. SP treatment on tumor bearing

$NK1R^{-/-}$ mice can directly show the role of SP-NK1R in the overall immune response against tumor. Similar experimental protocol on mice model with cell-specific knockout of NK1R receptors, mainly $ROR\gamma t^{+}$ or $FOXP3^{+}$ cell-specific knockout of NK1R. Studying the effect of NK1R signaling on Th17 or Treg differentiation by blocking Akt-mTOR pathway with rapamycin in SP-treated *in vitro* cultures of naïve CD4 T cells can provide insights into the effect of NK1R signaling on CD4 T-cell differentiation, since the Akt/mTOR pathway is the major signaling pathway driving RORγt or FOXP3 expression. In conclusion, our study has demonstrated a clear effect of neuro-immune crosstalk on tumor development through neurokinin signaling. This establishes the importance of SP-NK1R signaling and several other neuro-immune regulatory pathways as potential therapeutic targets for efficient treatment of different cancers, that could be studied extensively.

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