Chapter II:

Review of Literature

2.1 Salt regulation and cellular homeostasis

A major part of sodium in our body is present in blood and in and around extracellular fluids of cells. Sodium contributes to the maintenance of body fluids in a normal equilibrium and plays a crucial role in osmotic balance, nerve conduction, and muscle movements [1] **(Figure 2.1)**. Most of our sodium intake comes from dietary salt that we consume and it is primarily excreted through sweat and urine. The kidney plays a vital role in blood filtration and maintaining the total amount of sodium and total water content in the body under the influence of the renin-angiotensin-aldosterone system (RAAS). In addition, the atrial natriuretic peptide (ANP) hormone regulates renal mechanisms that govern the retention or loss of sodium [2]. Any changes in these processes lead to hypertension due to sodium retention and plasma volume expansion. As per the earlier accepted notion high salt consumption increases fluid intake and subsequently fluid volume [3]. Under such conditions, the kidney eliminates the excess salt along with the excess load of water to bring normalcy. On the contrary, a low sodium concentration in the body stimulates the secretion of aldosterone from the adrenal glands, which helps retain sodium and excrete potassium. When sodium is retained, the body produces less urine, ultimately leading to an increase in blood volume. The pituitary gland releases vasopressin (also known as antidiuretic hormone), which aids the kidneys in conserving water.

Current literature helped us to understand the mechanism regulating salt balance in the body. A space station simulation study, indicated that a considerable amount of sodium is retained or excreted from the body without parallel retention of water indicating a different mode of sodium retention in the body without altering fluid balance [4-6]. Further studies on sodium retention found that sodium can be stored under the skin and tissue interstitium in an osmotically inactive form in relatively higher concentration than the plasma through its association with negatively charged GAGs [7-10]. Strong evidence for the observation was provided by a study performed using ^{23}Na-MRI and flame photometry where, a significant amount of Na^+ was found to be stored in skin non-osmotically, without subsequent fluid retention [11-13]. This observation indicated that not all body fluids strictly follow isotonicity and that skin sodium concentrations do not necessarily equilibrate with blood electrolytes

for maintaining high sodium concentration in an osmotically inactive form. Ideally, the presence of sodium gradient should generate strong edema but experimental data concluded that skin can regulate its own electrolyte balance by creating local gradients between the interstitial fluid and plasma without creating an osmotic gradient [14,15]. These findings have prompted a redefinition of the two-compartment model of sodium balance, which originally included the extracellular fluid within the intravascular and interstitial spaces. The new model now incorporates a third compartment, the intracellular space, particularly within the skin [16]. The process of deposition and clearance of sodium into the skin and interstitial tissue is not well understood. Hofmeister et.al., focused on the distribution pattern of sodium in the epidermis and dermis and proposed that skin has a functional kidney-like counter-current mechanism to maintain its sodium gradient without leading to fluid accumulation in skin interstitium [17]. Many experimental evidence has showed that Na^+ storage in interstitial tissue is associated with hypertension [18], heart disease [19], and autoimmune diseases [20] **(Figure 2.1)**. It also exhibits a pro-inflammatory response that helps in fighting infection [21]. The expenditure of such high energy to maintain such a hypertonic environment might have also evolved as a protection barrier against infection or to prevent water loss through the skin [22]. Studies also have shown that Na^+ storage between muscle and skin is gender biased. Men tend to have higher sodium content in their skin compared to their muscles, while women accumulate more sodium in their muscles, implementing its role in fighting infection [23].

In an ultra-long-term sodium balance study conducted by Rakova et.al., in men simulating a flight to Mars in a controlled environment, found that Na^+ storage and release follow rhythmic patterns—both weekly (circaseptan) and monthly (circalunar) periodicity, this was independent of increase in blood pressure or body water intake and occurred regardless of salt intake [5]. Surprisingly, studies on mice showed that high salt intake did not result in increased water consumption [21]. Instead of increasing water intake, the mice retained water, which was facilitated by the production and recycling of urea osmolytes [24]. This is known as natriuretic-ureotelic regulation which is mediated by an increased buildup of urea in the renal medulla and increased numbers of renal urea transporters [25].

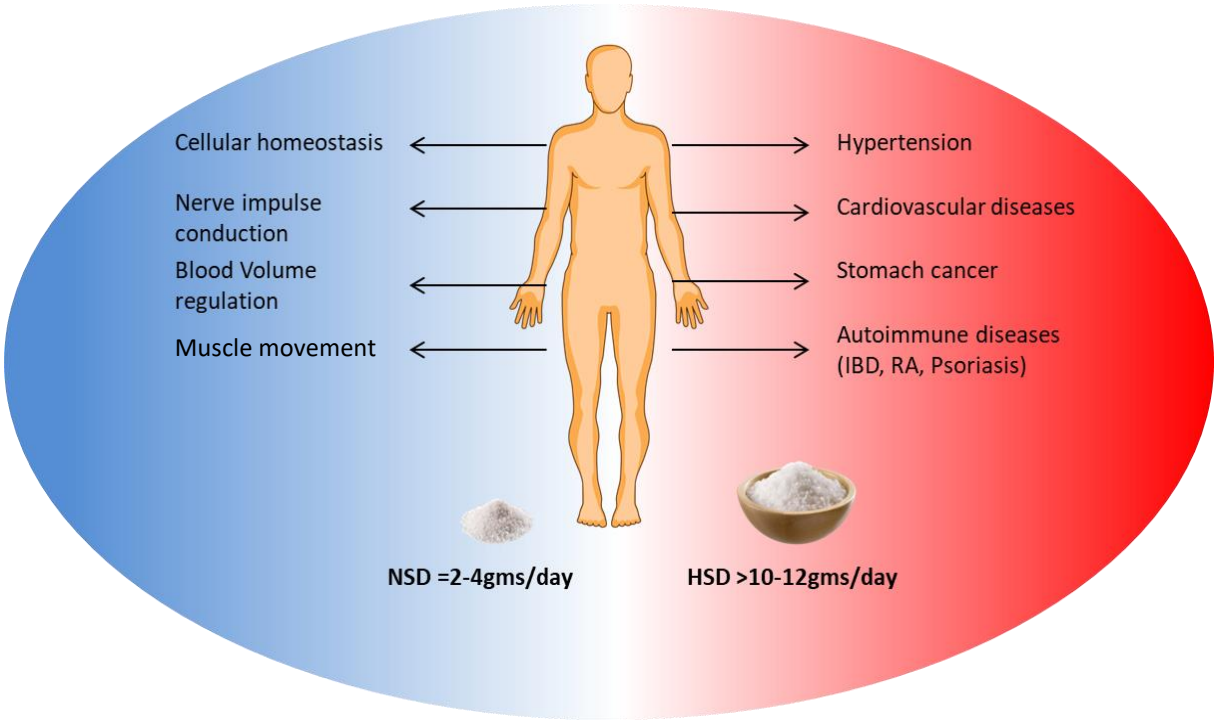

Figure 2.1: Physiological functions of salt. Effect of normal (2-4gms/day) and high (>10gms/day) concentrations of salt in regulation of various physiological responses of the body, indicating how escalating consumption of salt leads to hypertension, CVD, and Autoimmune diseases.

These studies suggested that osmotic regulation in response to high dietary salt is maintained by a complex regulatory process under the control of multiple factors including hormone fluctuation, water intake, metabolism, and efficient renal excretion. Rossitto et al. found that high salt intake leads to systemic accumulation in the body rather than specifically in the skin [26]. This phenomenon is closely tied to water balance and occurs in hypertensive patients, often associated with ageing. Interestingly due to its association with inflammation, this systemic Na^+ accumulation may contribute to various diseases, including cardiovascular diseases (CVDs), hypertension, and autoimmune diseases. However, such sodium deposition is known to induce inflammation which might serve as a defense against local and systemic infections.

2.2 Regulation of sodium homeostasis by Immune cells

The role of immune cells as extra-renal regulators of salt homeostasis and blood pressure has been reported [27-29]. Lymph vessels normally function to clear extracellular fluids,

macromolecules, and immune cells largely from the peripheral interstitium compartment. Machnik et al. were the first to report that high salt intake leads to the accumulation of sodium under the skin, resulting in increased macrophage infiltration [30]. These macrophages are known to help remove the excess sodium stored in the skin [31]. This sodium accumulation leads to an increase in the density and growth (hyperplasia) of lymphatic capillaries in the skin. High salt causes the up-regulation of the tonicity responsive enhancer binding protein (TonEBP)/NFAT5 in macrophage cells present in the interstitium leading to vascular endothelial growth factor-C (VEGF-C) secretion. VEGF-C is known to stimulate lymphatic capillary network growth which regulates the buffering of stored Na^+ and fluid volume, thus suppressing the high blood pressure associated with excess salt intake [32] [33,34]. This was further confirmed in an experiment where, depletion of MPS (mononuclear phagocyte system) which includes monocytes, macrophages, and dendritic cells (DCs), or by blocking VEGF-C signaling enhanced interstitial water retention, decreased the expression of e-NOS and heightened blood pressure in response to high salt. All these phenomena indicate how macrophage contributes locally to regulate sodium storage, blood volume, and blood pressure [30].

RNAi-based knockdown of TonEBP, which is essentially the regulator of salt removal did not affect the migration of macrophage towards the site of excess sodium deposition under the skin, conforming to the presence of an alternative mechanism regulating its salt-dependent chemotactic migration [35]. Inflamed monocytes increase reactive oxygen species (ROS) production by uncoupling NOS 3, leading to oxidative stress and hypertension [36]. Under a high-salt diet (HSD), macrophages are known to secrete inflammatory cytokines [37]. These cytokines, along with reactive oxygen species (ROS), can raise blood pressure by causing vascular endothelial dysfunction, which in turn impairs the kidneys' ability to excrete sodium [38,39]. HSD also induces cyclooxygenase-2 (COX-2) expression in macrophages. COX-2 helps prevent hypertension by activating the PGE2/EP4 receptor pathway in the kidney and skin, aiding in sodium disposal. These findings reveal that macrophages have a crucial role in managing high dietary salt levels and act as extra-renal regulators of sodium and water balance. Enhancing macrophages' ability to control salt and water balance could be significant for treating hypertension and salt-balance disorders.

Surprisingly, T lymphocytes and dendritic cells (DCs) were found to induce hypertension under the influence of high dietary salt exposure [40]. DCs under the influence of high salt cause the production of immunogenic isoketals [41] and ROS generation [42], these ROS cause renal T-cell activation, oxidative damage and fluid retention [43]. Guzik et al. successfully showed the role of T-cells in angiotensin II-mediated genesis of high blood pressure and involvement of inflammation as under cause of hypertension. T-cells are activated by angiotensin II, a hormone that increases blood pressure. This activation leads to the production of more inflammatory cytokines and further infiltration of T-cells into critical tissues, exacerbating hypertension [44]. T cells interact with other immune cells, such as dendritic cells (DCs), which produce reactive oxygen species (ROS) and immunogenic isoketals under high salt conditions. The isoketals generated in DCs upon exposure to high salt leads to the activation of CD8+ T cells, proliferation and production of IFN-γ and IL-17A by inducing inflammation and oxidative damage [45] thus paving the way for high salt diet-mediated hypertension. The development of hypertension often involves abnormal immune cell function, which under the influence can either help regulate or further aggravate hypertension. Thus, understanding how immune cells function under high salt stress conditions is of utmost importance.

2.3 Sodium accumulation in tumor microenvironment

Sodium ions are found throughout the human body and play a crucial role in all cellular activities in the body. Sodium ion is distributed across the cell membrane. At the cellular level, maintaining a difference in ion concentration across the cell membrane is crucial. The concentration of sodium ions inside and outside the cell is managed very preciously in a dynamic fashion with intracellular sodium concentration (ISC) of the cell in the range of ~10–15 mM and extracellular sodium concentration (ESC) in the range of ~140–150 mM [46]. This concentration is maintained in a narrow range in order to maintain the integrity of the plasma membrane to avoid cell membrane damage because of osmolality. This balance of sodium ions is maintained with the help of different sodium channels like Sodium-Calcium Exchanger (NCX), Voltage Gated Sodium Channels (VGSCs), Sodium Hydrogen exchanger 1 (NHE1), Epithelial Sodium Channels (ENaC) and Sodium-Potassium Adenosine Triphosphatase (Na^+/K^+-ATPase) [47-50]. However, in the case of cancer, the expression and function of these

ion channels are known to be altered leading to a change in the Total Sodium Concentration (TSC) from a mean of 50 mM to 145 mM, which is 2-3-fold higher than the normal tissue [46,51]. Recently, studies have reported that the sodium concentration in malignant tissue is significantly higher than the surrounding normal tissue [52-54]. ^{23}Na-MRI is a precise imaging technique that can provide access to study the temporal and spatial dynamics of sodium ions (Na^+) in tumors in vivo. This non-invasive imaging technique directly probes TSC including intracellular-sodium concentration (ISC) and extracellular-sodium concentration (ESC) sodium ions in tumor tissue. In general, the sensitivity of ^{23}Na-MRI is significantly lower than that of ^{1}H-MRI due to the lower tissue concentration of sodium compared to hydrogen and the inherently lower gyromagnetic ratio. However, technological advancements using higher magnetic power (7 or 9.4 Tesla ultra-high field MRI), along with diffusion-weighted imaging (DWI) helped in improving the imaging capability of ^{23}Na-MRI to predict early treatment outcomes of neoadjuvant chemotherapy in breast cancer [55,56]. ^{23}Na-MRI provides a better, non-invasive method for the timely tracking of pathophysiological, morphologic and biochemical characteristics of tumor for patients with advanced and inoperable breast cancer.

Although the exact reason for the increase in sodium in tumor is not known, recent evidence suggests pathologic changes such as tissue injury, tumor revascularization, edema, apoptosis/ necrosis and inflammation could lead to dysfunction off Na^+-K^+-ATPase leading to an increase in sodium content in tumor microenvironment. This raises an obvious question: What is the direct impact of high salt on deposition on cancer and immune cells present in TME? The effect of high salt on various cells in TME is discussed below.

2.4 Role of High Salt in Influencing Immune cell function

In the 19th century, Rudolf Virchow found the presence of immune cells inside tumors, which gave the primary idea of a potential connection between inflammation and cancer. Since then, hundreds of literatures have shown that an inflammatory microenvironment is an indispensable part of all tumors. Tumor microenvironment contains immune cells of diverse origins like macrophages, neutrophils, mast cells, MDSCs, DCs, NK cells and lymphocytes like T and B cells [57]. These immune cells interact with each other and tumor microenvironment by

means of different pro-inflammatory and anti-inflammatory cytokine and chemokine production. Immune cells in tumor microenvironment can exist either in an anti-tumorigenic or pro-tumorigenic immune state based on their activation and signals from surrounding immune and cancer cells. The state of immune activation determines whether the balance will shift towards inflammation that promotes tumor growth or towards anti-tumor immunity.

Most studies on the immune cells in tumor microenvironment vicinity have shown that the balance is profoundly tilted towards pro-tumor which further leads to the activation of oncogenes. A Plethora of literature has postulated the direct role of chronic inflammation in tumorigenesis, an interesting point was raised in a review by Grivennikov et.al that “not all chronic inflammatory diseases increase cancer risk”, and in the case of certain chronic inflammatory diseases like psoriasis and rheumatoid arthritis (RA), tumor is often found to be reduced whereas other diseases like inflammatory bowel disease (IBD) or chronic hepatitis were often related in tumor promotion [57]. The molecular mechanism that causes a specific subset of immune cells to be anti-tumorigenic in one type of cancer and pro-tumorigenic in another, is still not fully understood. Although it is interesting to assume that under the path of tumor progression, both anti-tumor and pro-tumor immunity must coexist at different points. These conclusions give rise to the next question: What is the mechanism behind the conversion of body's defense mechanism i.e., Immune system (anti-tumorigenic) to aid tumor progression (pro-tumorigenic)? The majority of leukocytes present in tumor microenvironment are of myeloid cells origin and most abundant in number apart from DCs, Tie-2-expressing monocytes, and neutrophils [58], which can either be in pro or anti-tumorigenic state and secrete complex inflammatory cytokines and chemokines which can either orchestrate or suppression tumor growth.

Understanding the effect of high dietary salt on immune cells becomes of utmost importance when dealing with diseases involving immune cells such as hypertension, CVDs, autoimmune diseases and cancer. Inflammation is body's defense mechanism against various types of non-self-antigen. Inflammation followed by resolution is crucial for the effective removal of the infection without damage to self-antigens. During an infection, damaged epithelial and endothelial cells release factors that initiate the inflammatory cascade. This process primarily attracts neutrophils, followed by monocytes, natural killer cells, and mast cells. Monocytes

then differentiate into macrophages and dendritic cells, which secrete various cytokines and chemokines. These secretions help activate the effector arm of the immune system, including B cells and T cells, which are involved in developing immunity and removing antigens **(Figure 2.2)**. Following clearance of infection if inflammation persists, then it can lead to chronic inflammatory conditions which increases the risk of developing cancer, autoimmune diseases (for example, inflammatory bowel disease is associated with colon cancer) and inflammatory conditions including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), inflammatory bowel disease (IBD) [59]. Underlying infections and chronic inflammation account for 15–20% of all cancer-related deaths globally. [60]. Diet and various environmental factors have been known to activate the body's immune system via various mechanisms. Several studies have shown that dietary salt shifts the balance of the immune system towards the pro-inflammatory side.

2.5 Role of salt in influencing macrophage cell function

High salt has also been known to affect the tissue infiltration and transformation of macrophages to classical M1 form, which are known to aggravate CNS autoimmunity [61]. The activation state of classical, M1 proinflammatory macrophages (activated with LPS), was also shown to be exaggerated in the presence of high salt [22] . In macrophages, salt inhibited the activation of primary Bone marrow-derived macrophages (BMDM), stimulated with IL-4 and IL-13 by blocking the mTOR and AKT signaling pathways, which are necessary for differentiation into the functional M2 phenotype [62]. Zhang et.al. studied the direct impact of high salt exposure on the activation state of macrophages and found that high salt levels enhanced LPS-induced macrophage activation and suppressed interleukin 4-induced macrophage activation. Additionally, they found that genes associated with anti-inflammatory functions were also downregulated. Interestingly, the activation state induced by sodium, M(Na), appears to be distinct from those induced by LPS M(LPS) or IFN-γ M(IFN-γ) [63]. Despite a significant overlap of pro-inflammatory genes among all three states, LPS uniquely induces a set of anti-inflammatory genes to help limit prolonged inflammation. However, this was not the case with M(Na), and in fact, it displayed suppression of anti-inflammatory genes. Also, the level of these anti-inflammatory genes in, M(Na) showed decreased or unchanged expression for more than

24h as compared to M(LPS). This indicates salt induces a different subtype of macrophage polarization M(Na), than the conventional M1 or M2 [63].

In an in-vivo experiment aimed to study the effect of an HS diet on monocytes and macrophages, 11 healthy humans were subjected to a 2-week low and high salt diet. Blood analysis revealed that high salt consumption increased monocyte expression of CCR2, a chemokine receptor that mediates monocyte infiltration towards the site of inflammation. Additionally, an increase in plasma MCP-1 levels, trans-endothelial migration of monocytes, and skin macrophage density was observed. This study explains how macrophages infiltrate the skin interstitium under high salt diet conditions, as CCR2 plays a key role in monocyte chemotaxis towards high salt. The team also noted that skin macrophage HLA-DR (a pro-inflammatory marker) gene expression increased, while CD206 (an anti-inflammatory marker) gene expression decreased, confirming a pro-inflammatory state of macrophages in the skin. The high salt diet also led to an increase in neutrophils and basophils in the blood [64]. Macrophages activated by interleukin 4 (IL-4) and IL-13 through STAT6 activation exhibit strong inhibitory activity against T cells [65], This aligns with the observation that a high salt diet (HSD) induces the expression of pro-inflammatory genes and suppresses anti-inflammatory gene expression in macrophages. Macrophages being the most abundant immune cells in tumor microenvironment. The pro-inflammatory effect of high salt on macrophages provides a hint for the beneficial role of salt in inducing macrophage-based tumor suppression. Further studies on the effect of high salt on macrophage function are hence necessary

2.6 Role of high salt in influencing T and Dendritic cell's function

T-lymphocytes are center to fighting against cancer cells because of its key function of antigen-directed cytotoxicity [66]. T cells can polarize either in TH1, TH2 or TH17 depending upon the inflammatory microenvironment in which they get polarized and contribute to host defense or promote autoimmunity [67]. Cytotoxic CD8+ T cells are the most potent agents in the adaptive immune system's fight against cancer, forming the core of cancer immunotherapy [68]. whereas, CD4+ T cells play a vital role in boosting the functions of antigen-presenting cells and enhancing the activity of CD8+ T cells [69]. $FOXP3^{+}$ Treg cells are known to suppress T cells by secretion of immunosuppressive cytokines like TGF-β and IL-10. Conversely, high salt inhibits

Treg function by inducing IFN-γ production in these cells, suggesting that high salt impairs Treg function while maintaining T cell-mediated cytotoxic activity [70]. Kleinewietfeld, et.al., showed that high salt boosted induction TH17 cells and pathogenic IL-23-dependent TH17 cells produced from CD4+ helper cells, which are known for augmenting the development of autoimmune diseases [71]. Many studies have shown that although TH17 cells are the primary source of IL-17A in salt-enhanced colitis [72], many other immune cells can also secrete IL-17A and IL-17F. These cells include CD8+ T cells, CD4-/CD8- α/β T lymphocytes, γ/δ T cells, NK cells, neutrophils, and innate lymphoid cells (ILC). [73,74]. The role of Th-17 cells in tumor immunity is complex and context-dependent, exhibiting both pro- and anti-tumor effects however excessive infiltration of Th-17 cells in mammary gland TME was found to be associated with poor disease outcome, suggesting that high salt could potentially improve disease outcomes [75]. Further studies on how high salt alters the inflammatory microenvironment will help advance the development of novel treatment approaches.

Dendritic cells (DCs) are crucial antigen-presenting cells (APCs) of the immune system. They monitor their microenvironment and play a key role in inducing immune responses by activating effector T cells. However, the impact of high salt on DC cells is less studied and controversial e.g. high salt level in the kidney medulla, impairs DCs maturation and suppresses the release of IL-12p70 required for activation of the TH1 cells [76]. It is apparent to consider that the condition in the renal medulla is different from a typical tumor microenvironment and requires further studies to confirm the effect of high salt on DC cells in tumor microenvironment. In contrast to those studies, other research has shown that dendritic cells (DCs) exhibit anti-tumor effects in a sodium-rich milieu, enhancing their ability to present antigens and thereby promoting T-cell activation via MHC-II [41]. In salt-sensitive mice, high salt stimulates DCs to induce potent IFN-γ and IL-17A secretion from CD4+ and CD8+ T cells, which is known to counteract the immunosuppressive effects of Treg cells in the tumor microenvironment [40]. These dendritic cells (DCs) not only present cancer cell surface peptides and MHC complexes to activate naive T cells but also produce soluble factors influenced by environmental cues at the inflammatory site. Therefore, high salt intake can affect the immune response by enhancing the pro-inflammatory functions of macrophages and

T cells. This shift can help transform the immunosuppressed tumor microenvironment into a pro-inflammatory state, aiding in the clearance of malignant cells.

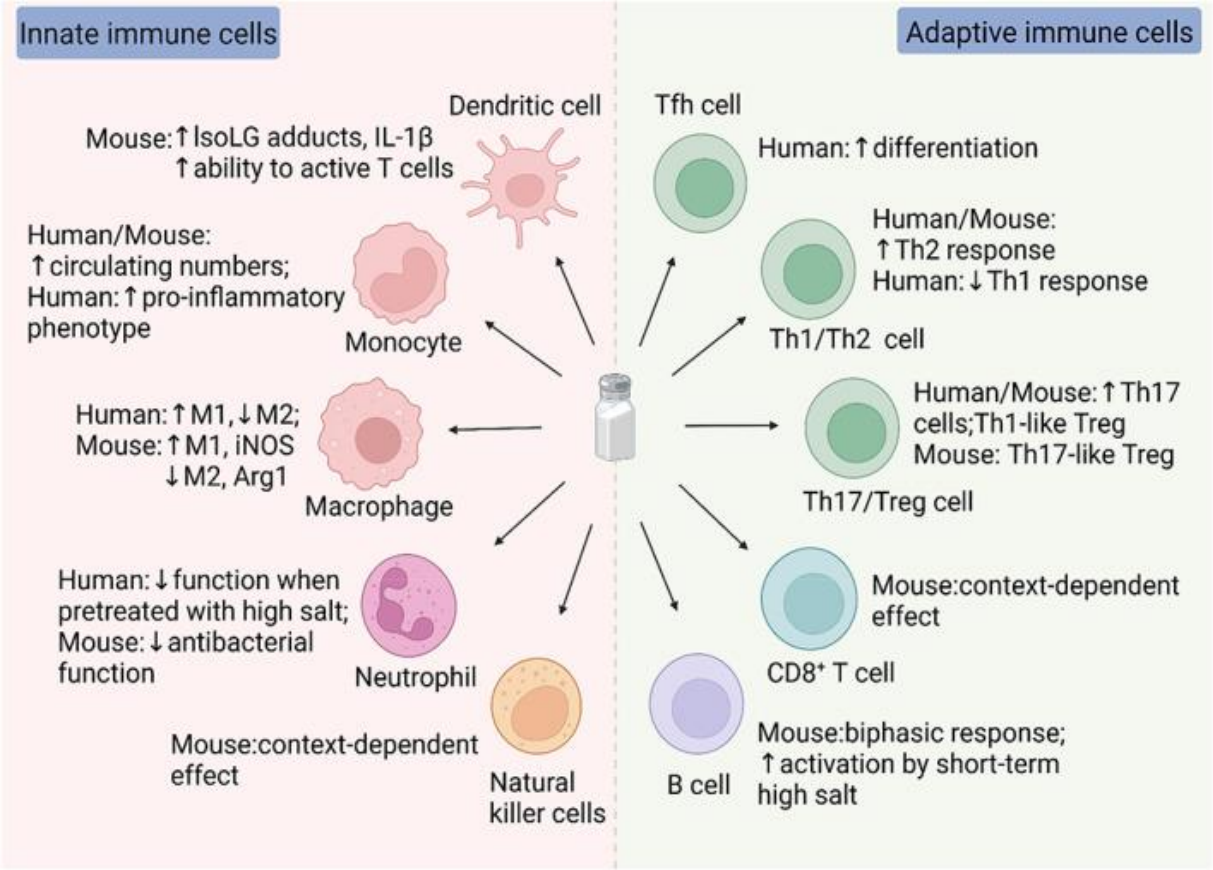

Figure 2.2: Effect of high salt on modulating immune cell functions. High salt induces increases infiltration of immune cells in tumor microenvironment and modulates the immune cells by shifting their immune state into a pro-inflammatory state. **Image adapted** from Xian Li et.al. Journal: Cell proliferation (2022). Refer to [77]

2.7 Mediators of Immunosuppressive state in TME

Tumor microenvironment which consists of numerous immune cells (T cells, NK cells, Myeloid cells) especially tumor-associated macrophages (TAM), which are the most abundant myeloid cells in tumor vicinity are key orchestrators of cancer-related inflammation. Normally, immune cells play crucial roles in defending the body against infections and diseases. However, cancer cells hijack these functions, presenting themselves as normal cells in need of support. This manipulation tricks immune cells into aiding the cancer's growth and survival rather than attacking it. Nevertheless, the phenotypic state of TAMs is strongly under the control of the

surrounding microenvironment. Studies have shown metabolites produced by cancer cells can significantly influence the activation and differentiation of immune cells within the TME e.g. High glucose consumption by cancer cells (Warburg effect) can lead to competition for nutrients in the TME, depriving immune cells of essential glucose needed for their functions [78]. The end product of high glucose uptake leads to high lactate production. Lactate can acidify the TME, which can impair the function of cytotoxic T-cells and NK cells while promoting the activity of immunosuppressive cells like Tregs and MDSCs [79]. Also because of high proliferation, cancer cells rapidly deplete amino acids like tryptophan and arginine from the TME. The depletion of these amino acids can inhibit the function of effector T cells and enhance the suppressive function of Tregs and MDSCs [80]. Apart from these cancer cells also secrete growth factors like VEGF, FGF, EGF and matrix metalloproteinases to enhance their invasion and metastatic properties [81,82]. Macrophages in tumor microenvironment are generally in the M2 phase and hence the likely culprits for mediating pro-tumor functions. INF-γ, GM-CSF secreted by immune cells in TME can induce M1 macrophage activation which express high levels of proinflammatory cytokines that are responsible for antitumor immunity via TNF-a, IL-1, IL-6, IL-12 or IL-23, MHC molecules, and ROS [57]. TH2 cell-derived IL-4 and IL-13 are essential for orchestrating the activation of M2 macrophages. These M2 macrophages, in turn, secrete cytokines such as IL-10, IL-1, prostaglandin E2 (PGE2), and matrix metalloproteinase-7 (MMP-7), all of which play a significant role in promoting tumor growth. Although the inflammatory microenvironment is densely populated with immune cells, some interesting studies have shown an inverse relationship between T cells and macrophages in the tumor bed. Typically, T cells are found in peripheral zones, while macrophages are embedded within the tumor cells [83,84]. This could possibly bean added advantage to tumor growth given the tumor architecture and close proximity of pro-tumor M2 TAMs.

2.8 The contradictory Role of High Salt in cancer progression

A high salt diet has been directly associated with the deregulation of immune cells by tilting them towards a pro-inflammatory state that helps in restoring the characteristic function of immune cells in killing the tumor cells. Multiple studies have tried to understand the possible link between salt eating habits and cancer progression. However, the role of high salt and cancer progression is a topic of ongoing debate, with only a few studies suggesting a potential

pro-tumorigenic effect of high salt. In one of the Epidemiological studies, the association between high dietary salt consumption and gastric cancer was proposed [85-87]. There exists a strong relationship between Infection with *Helicobacter pylori* risk factor of gastric cancer, but is not a sufficient cause for cancer development[88,89]. A high salt diet strips the inner mucus membrane of the stomach and can thus synergize *Helicobacter pylori* infection to develop into gastric cancer [90]. Amara et.al showed that high sodium chloride levels along with IL-17 a pro-inflammatory cytokine, played a synergistic role in breast cancer cell proliferation, induction of VEGF-A, an angiogenic stress factor and ROS formation that is important in tumor progression [91,92]. Induction of VEGF-A, involves tonicity-responsive enhancer binding protein (TonEBP), a transcription factor involved in the regulation of cellular osmolarity [93]. Interestingly, high salt is also known to induce a Th17 differentiation of naïve CD4+T-cells, which plays a key role in T-cell mediated cancer progression as discussed above [94]. alt-inducible kinases (SIKs) were first studied for their role in regulating adrenocorticotropic hormone-mediated gene expression to help maintain sodium and potassium balance. Further research revealed that SIK3 mediates the combined effect of IL-17 and high salt levels on cancer cell proliferation and metastasis [95]. Chen et al. demonstrated that a high-salt diet accelerated breast cancer progression and lung metastasis in MMTV-PyVT mice. Additionally, it increased the levels of Th17 cells in the bloodstream, tumor tissue, and draining lymph nodes [96]. Several studies have demonstrated that NaV channels are present in non-excitable cells, including microglial cells and macrophages. In these cells, NaV channels were found to affect endosomal acidification in phagocytic cells and podosome formation in migratory immune cells, thereby influencing their capacity to target and kill cancer cells. [97]. Voltage-gated sodium channels are also significantly upregulated in many cancer cells compared to normal cells, where they are thought to facilitate ECM degradation and rapid migration through tumor tissue [98]. Studies on sodium ion channels like NaV1.5 and NaV1.6 isoforms which are notably overexpressed in breast and cervical cancer respectively, are linked to cancer recurrence, metastasis development, and reduced patient survival [99,100]. Additionally, a different class of sodium channel, hypertonicity-induced cation channels (HICCs), specifically α-ENaC, has been linked to the proliferation of human hepatoma (HepG2) cells [101]. However, it is important to know the overexpression of sodium channels and

voltage-gated sodium channels are affected by many factors like hormonal levels of aldosterone and insulin, pro-inflammatory cytokines like TNF-α and IL-1β, environmental factors like oxygen and diet, genetic factors like mutation and epigenetic modifications and ageing [102-105]. Most likely, the overexpression of sodium channels responsible for increased proliferation and metastasis of cancer might not be solely due to increased sodium accumulation.

Table 2.1: List of literature studies on the effect of high salt performed in animal model.

Sr. No.	Animal Model	Cancer	Effect	Molecular Mechanism	Reference
1.	C57BL/6, BALB/c, and RAG1−/− mice	B16F10 and LLC melanoma cell	A high-salt diet (HSD) enhances tumor control by altering immune responses, affecting effector and regulatory T cells. It also shifts gut microbiota composition, stimulating natural killer (NK) cells and promoting antitumor immunity.	HSD induces natural killer (NK) cell-mediated tumor immunity by inhibiting PD-1 expression while enhancing IFN-γ and serum hippurate.	[106]
2.	C57Bl/6 mice	Py230 syngeneic breast cancer	The high salt-expanded CD4+T cells exhibited tumour-specific cytotoxicity against Py230 breast cancer cells and reduced in vivo syngeneic tumor growth	High salt induces an effector phenotype in tumor-primed CD4+ T cells through NFAT5-mediated signaling pathways, enhanced metabolic activity, and specific cytokine stimulation.	[107]
3.	C57BL/6 and BABL/c mice	4T1 induced mammary and B16F10 cells induced	High salt regulates the differentiation of MDSCs in the tumor niche, leading to the removal of local immune suppression and inhibition of tumor progression.	HSD elevates local sodium chloride concentration in tumors, inducing osmotic stress, reducing cytokine production, and regulating MDSC function. It promotes	[108]

		melanoma cancer		MDSC differentiation into M1-type macrophages through P38-mediated NFAT5 activation	
4.	MMTV-PyVT /Nju mice	-	High salt accelerates the growth of breast cancer and metastasis. Increases the level of Th17 cell, which secretes IL-17, which influences cancer growth	High salt causes increased frequency of TH17 cells, which further aggravates cancer via MAPK/ERK pathway	[96]
5.	C57BL/6 mice and RAG2−/−	B16F10 melanoma cells and Lewis Lung carcinoma (LLC)	High salt inhibited tumor growth and increased anti-tumor immunity	Enhanced anti-tumor immunity by a functional inactivation of MDSCs	[109]
6.	4T1 mouse	Mammary	Improved Fitness and Survival rate Reduced Tumor growth and Metastasis	Calorie restriction, Decreased Ki67 and Increased p53	[88]
7.	C57BL/6 mice	Azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colon cancer model	Bamboo salt decreased colon length shortening, weight-to-length ratios, and tumor counts	Modulating apoptosis (Bax, BCL-2, p21 and p53) and colon inflammation-related gene expression (TNF-a, IL-6, and IL-1b)	[110]
8.	C57 mice	4T1 induced Lung cancer	Systemic hypertonicity reduced metastatic burden as demonstrated by reduced lung nodules	-	[111]

Contrary to the above, new studies on the effect of high salt have more evidently shown anti-tumor effect of high salt in mice models. The details of the studies are listed in Table 2.1. 4T1 mouse mammary tumor model fed with high salt showed significantly less metastatic foci in lungs. HS-fed mice showed high expression of the tumor suppressor gene p53 in tumors and reduced food intake, which was interpreted as a reason for its antitumor effect through mimicking calorie restriction [88]. Another interesting study showed the impact of high salt diet on tumor growth in two different mice models (B16F10 melanoma cell and Lewis Lung carcinoma)[109]. It was found that HSD significantly reduces tumor growth by increasing the level of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) which are known for its tumor suppressive activity, thereby resulting in improved defense against tumors. High salt also showed increased infiltration of CD4+ T cells and anti-tumorigenic gene expression of TNF-α, IFN-γ and NOS-2 [109]. A recent report published by Wei et.al., supported the tumor inhibiting effect of HSD, which tend to occur by regulation of MDSCs differentiation [108]. The study found that HSD increases the local concentration of sodium chloride in tumor tissue. Thymus, livers, spleens and tumors were hyperosmolar as compared to control mice and level of salt in tumor tissues was remarkably higher. This high osmotic stress reduced both the production of cytokines like IL-6, and IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) required for MDSCs expansion and accumulation in tumor microenvironment. M-MDSCs under the influence of proper signaling molecules differentiate either into macrophages or DCs cells. The team found that while the amount of DCs in the tumor tissue did not alter considerably, HSD dramatically raised the number of macrophages in the tissue. [108].

Another study showed high salt diet increased *Bifidobacterium* abundance and heightened gut permeability, leading to intratumor localization of *Bifidobacterium [106]*. This enhances NK cell functions by inhibiting PD-1 expression and facilitating tumor regression by inducing simultaneous expressions of Serum Hippurate and IFN-γ [106]. Programmed death-ligand 1 (PD-L1) plays a crucial role in cancer primarily through its interaction with the programmed cell death protein 1 (PD-1) receptor on immune cells. Cancer cells typically have higher levels of PD-L1 compared to normal cells, which binds to PD-1 on T cells [112]. This interaction inhibits the activation and function of T cells, preventing them from attacking the cancer cells. Many

cancers exploit the PD-L1/PD-1 pathway to evade immune checkpoint blockade therapies. It is commonly believed that high salt can tune immunosuppressed cells in TME towards a pro-inflammatory state. Thus, anti-tumor effect of high salt has largely accounted for the induction of inflammation mediated by MDSCs, Th17 and NK cells suggesting potential implications of salts in cancer immunotherapy. However, there are no literature that discusses the direct effect of high salt on breast cancer cells and their impact on the crosstalk with TAMs. This emphasizes the need for further research to elucidate its influence on cancer cells and the tumor microenvironment.

Considering this gap in the field, the thesis work aimed to study the direct effect of high NaCl treatment on cancer cells and on the cross-talk of cancer cells and TAMs. To investigate the following objectives were set:

2.9 Objectives of the study

1) To study the effect of high dietary salt on tumorigenic properties of breast cancer cell lines.

2) To study the effect of high dietary salt on the crosstalk between cancer cells and macrophages / TAMs *in vitro*.

2.10 References

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