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Review

Cell Volume Regulation in Immune Cell Function, Activation and Survival

Matt Como[a] Bhavesh Reddy Koppala[b] Md Nabiul Hasan[b] Vanessa L. Han[c] Ishika Arora[c] Dandan Sun[b,d]

[a]Pennsylvania State University, State College, PA, USA, [b]Department of Neurology and Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Pittsburgh, PA, USA, [c]Shady Side Academy, Pittsburgh, PA, USA, [d]Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, PA, USA

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Abstract
The regulation of cell volume is an essential cellular process in nearly every living organism. The importance of volume regulation in immune cells cannot be understated, as it ensures proper cellular function and effective immune response. These cells utilize ion channels and transporters to maintain volume homeostasis through rapid ion transport across the cell membrane. Immune cells express mechanisms controlling regulatory volume decrease (RVD), regulatory volume increase (RVI), proliferative RVD, and apoptotic volume decrease (AVD). In this review, we summarize recent studies examining the importance of several ion channels, particularly potassium and chloride channels in regulating ion transport during osmotic stress, and in immune cell function, activation, proliferation, and death. We also review the key mechanisms functioning in immune cell proliferation and apoptosis. They serve a crucial role in maintaining adequate ionic concentrations, mediating immune cell activation, and generating proliferative pathways. These regulatory mechanisms play key roles in the function and survival of immune cells, as impaired volume regulation contributes to the pathophysiology of various disorders. A complete understanding of immune cell volume regulatory mechanisms may be a starting point for the development of therapeutic agents targeting these ion channels to treat inflammatory diseases.

Introduction

Cells must maintain a relatively consistent volume to survive and function properly. Volume alterations caused by extracellular osmolarity changes can affect the arrangement and organization of important cellular structures and cause detrimental effects on important cellular functions [1]. Volume regulation is dependent on the anionic and cationic

Dandan Sun, MD, PhD, FAHA, Professor
University of Pittsburgh School of Medicine
7016 Biomedical Science Tower-3, 3501 Fifth Ave, Pittsburgh, PA 15260 (USA)
Tel. +1-412-624-0418, Fax +1-412-648-3321, E-Mail sund@upmc.edu

permeability of the cell membrane [1]. To maintain adequate intracellular volume and proper function, cells employ various mechanisms to transport ions and water across the lipid bilayer membrane to regulate their volume [1, 2]. The lipid bilayer membrane in most cells is slightly permeable to water, but not permeable to cations or anions. Activation of the bilayer-inserted transport proteins for ions and water allows for variations in cellular volume to respond to various extracellular osmotic gradients [1]. In response to hypertonic osmotic stress, a cell shrinks when water exits the cell through aquaporins (AQP) to balance the osmotic gradient created by the increased extracellular osmolarity [1, 2]. The increased intracellular osmolarity and cell shrinkage stimulate regulatory volume increase (RVI) mechanisms to transport Na^+ and Cl^- into the cell mediated by the widely recognized ion transport protein Na^+-K^+-Cl^- cotransporter (NKCC1) with the coupled activation of the Na^+/H^+ exchanger that functions in parallel with the $Cl^-/HCO3^-$ exchanger (Fig. 1A). Conversely, extracellular hypotonicity causes rapid cell swelling that reduces intracellular osmolarity. This stimulates a specific volume regulatory process known as regulatory volume decrease (RVD), which activates K^+ and Cl^- channels that allow the ions to exit the cell along with water to reduce the intracellular volume and reestablish optimal cell volume homeostasis [3]. An increased conductive K^+ and Cl^- membrane permeability must occur to allow for K^+ and Cl^- efflux and subsequent RVD [1]. Many cells utilize coupled K^+/Cl^- cotransport or a parallel activation of K^+/H^+ and $Cl^-/HCO3^-$ exchange to control K^+ and Cl^- flux and thus volume [4]. The presence of AQP water channels enables cells to move water molecules between the inside and outside of the cell through the lipid bilayer membrane and rapidly recover the volume.

Immune cells are vital to the survival of any organism, functioning to prevent infection through various immune response mechanisms. These cells are produced in the bone marrow before they develop into mature immune cells to function in immune response mechanisms [5]. Dendritic cells, natural killer cells, lymphocytes, neutrophils, and macrophages all play vital roles in the immune system and function to remove toxic substances and environmental pathogens from the host organism and thus lower the threat of infection [5, 6]. Based on the functions, T cells are broadly divided into three categories, memory T cells, effector T cells, and regulatory T cells (Tregs) [7-9]. Naïve T-cells are matured and activated to effector T cells once they encounter a specific antigen and activated [7], while Tregs suppress other immune cells and keep the immune response in check [10]. A small fraction of effector T cells remains as memory T cells in the body for many years even when the infection is terminated and the primary response against infection is over [10] and are capable of fighting the same infection in the future [7, 10]. In this review, we generally discuss $CD4^+$ and $CD8^+$ effector T cells. Despite the important physiological functions of immune cells, the specific role of cell volume regulatory mechanisms in these cells has not been extensively studied. Because of this, the pathways mediating volume regulation in immune cells are not completely understood. In this review, we summarized the current knowledge of volume regulatory mechanisms in immune cells, particularly highlighting recent studies related to immune cell function, survival, and death.

Ion Channels in Immune Cell Volume Regulation

K^+ and Cl^- Channels in Thymocyte Volume Regulation

Thymocytes are the developing progenitor cells located in the thymus that have not yet matured into T cells, hence the majority of them are considered immature precursors [9]. When subjected to hypotonic conditions, murine thymocytes rapidly swell to their maximum volume due to the presence of AQP and subsequently followed by a relatively slow return to normal cell volume by RVD [4, 11]. Phloretin and glibenclamide, which are common volume-sensitive outwardly rectifying (VSOR) Cl^- channel blockers, were effective in suppressing RVD while maxi-anion blocker Gd^{3+} ions were less effective in inhibiting the RVD process, providing evidence that murine thymocytes possess a powerful RVD mechanism which is mediated by the VSOR Cl^- channel as the principal regulator of anion efflux during the

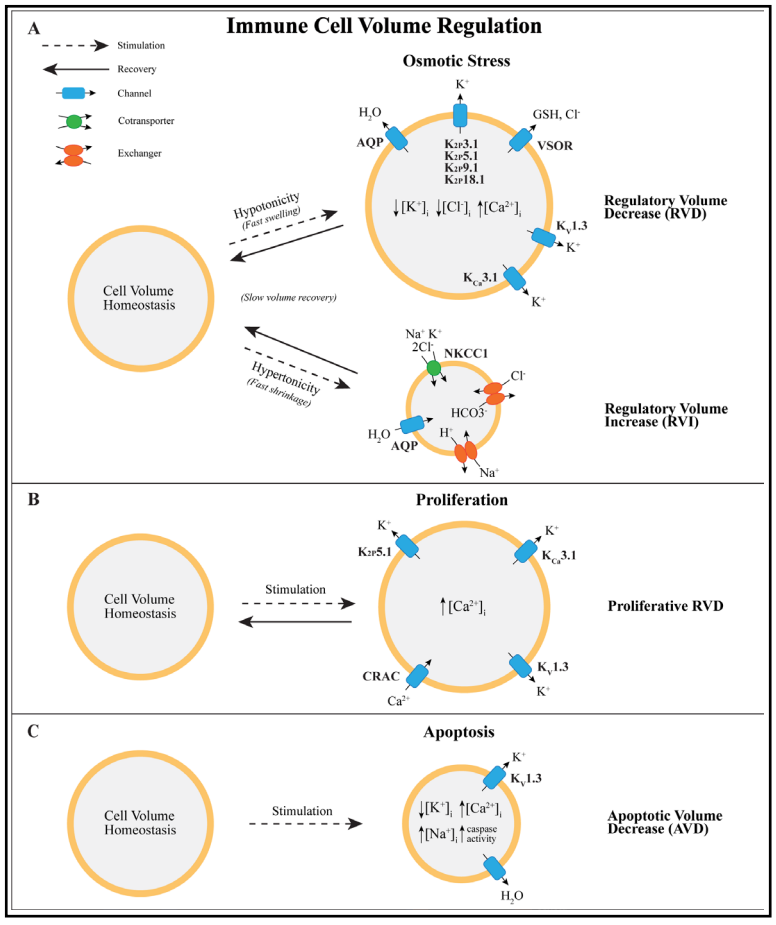

Fig. 1. A summary of the ion channels utilized during immune cell volume regulation, apoptosis, and proliferation. (A) Pathways of osmoregulation through RVD and RVI are shown. Hypotonic stress causes immune cell swelling. To accomplish RVD, AQP water channels mediate water efflux. $K_{2P}3.1$, $K_{2P}5.1$, $K_{2P}9.1$, and $K_{2P}18.1$ regulate K^+ efflux. VSOR Cl^- channels control the efflux of anions and organic compounds to reduce cellular volume. $[Ca^{2+}]_i$ increases and activated $K_{Ca}3.1$. Cl^- conductance depolarizes the cell membrane due to Cl^- efflux and stimulates $K_V1.3$ along with K^+, Cl^-, and water efflux to restore normal cellular volume. (B) Mechanisms of immune cell proliferative volume regulation. $K_{2P}5.1$, $K_{Ca}3.1$, and $K_V1.3$ control K^+ efflux through RVD during immune cell proliferation. K^+ efflux via $K_V1.3$ hyperpolarizes the cell membrane, leading to an increase in $[Ca^{2+}]_i$. Binding of Ca^{2+} to CaM stimulates $K_{Ca}3.1$ during cell proliferation. (C) A summary of ion channels utilized during immune cell AVD. $K_V1.3$ mediates K^+ efflux. Increased $[Ca^{2+}]_i$, $[Na^+]_i$, and caspase activity is seen during immune cell apoptosis, while an overall reduction in $[K^+]_i$ occurs.

process [4, 12]. VSOR anion channels, as one of the fundamental pathways of RVD in thymocytes, not only remove Cl^- but also various organic molecules (Fig. 1A) [13]. A recent study found that glutathione (GSH) cellular efflux via the VSOR channel and organic anion transporters also play a role in volume regulation in thymocytes [13]. GSH is a tripeptide antioxidant found in most cells that helps maintain cellular homeostasis by reacting with oxidizing species before their interference with and damage of cellular components [14]. Under typical isotonic conditions, thymocyte GSH release is relatively low, but when thymocytes increased their volume in response to hypotonic conditions, concentrations of extracellular GSH increased significantly [13]. It is also important to note that swelling-prompted GSH efflux in thymocytes was significantly reduced after treatment with ion transport inhibitor 4-acetamido 4'isothiocyanostilbene-2,2'-disulphonic acid (SITS) and channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) [11]. SITS treatment, which inhibits volume regulation in thymocytes, effectively blocks various anion channels as well as the $Cl^-/HCO3^-$ transporter [12]. It should be noted that the majority of the immune cell investigations of channel physiology and volume regulatory biophysics discussed in this review utilized anisosmotic media, which application in many important discoveries of cell volume regulation in red blood cells has been recently discussed by Peter K. Lauf and Norma C. Adragna [15].

Potassium ion channels allow for rapid K^+ transport down its electrochemical gradient and are key players in the function of both excitable and non-excitable cells of nearly every organism. Potassium channel blockade in murine T lymphocytes has been investigated to better understand the role that these channels play during the volume regulatory mechanisms of RVD and RVI. Various concentrations of K^+ channel blocker $BaCl_2$ considerably reduced murine thymocyte shrinkage when exposed to a hypotonic solution. A concentration of tetraethylammonium (TEA, 5 mM) ions, an organic cation that blocks various K^+ channels, effectively eliminated an RVD response, providing additional support that the activation of K^+ channels is essential for effective volume regulation in thymocytes [12, 16]. Taken together, thymocyte volume regulation under hypotonic conditions includes the proper function of the VSOR Cl^- channel as well as K^+ channels to remove ions and organic molecules. However, future studies are required to identify specific types of K^+ channels and VSOR Cl^- channels in these cells.

K^+ Channels in Lymphocyte Volume Regulation

T lymphocytes are considered mediators of immunity, and it is known that both $CD4^+$ and $CD8^+$ T lymphocytes promptly swell when subjected to a hypotonic solution. However, they ultimately fail to demonstrate RVI under hypertonic conditions, displaying only a minimal change in cell volume following shrinkage [17]. Cells utilize three major types of K^+ channels, characterized by the number of transmembrane (TM) and pore-forming (P) domains they contain, including voltage-gated K_v channels (six TM domains plus one P domain), inwardly rectifying K channels (two TM domains and one P domain), and two-pore domain K^+ channels (four TM domains and two P domains) [18]. In humans, $K_V1.3$ functions to regulate the cellular membrane potential and promote the influx of Ca^{2+} through CRACs and thus plays a role in the regulation of T cell activation, proliferation, and cytokine and chemokine secretion [19, 20]. There are distinct differences in K^+ channel composition and function in murine T cells versus human T cells. Human T cells express only the $K_V1.3$ channel, while in addition to $K_V1.3$, mouse $CD4^+$ T cells express $K_V1.1$, $K_V1.3$, and $K_V1.6$. Mouse $CD8^+$ T cells, on the other hand, express the $K_V3.1$ channel instead of $K_V1.3$ [19, 21].

T lymphocyte swelling has been seen to stimulate ATP-dependent Cl_{swell} currents and perform RVD to reestablish cell size even under continuous hypotonic conditions [22]. Swelling-activated Cl^- efflux (Cl_{swell}) depolarizes the cell membrane, causing a conformational change that opens Kv channels [23]. These channels are important in controlling K^+ flux during cell volume regulation [23]. The $K_V1.3$ channel is an extensively studied subset of potassium channels found in immune, muscular, and neuronal cells [17, 19, 20, 23]. $K_V1.3$ activation also requires increased Ca^{2+} influx, resulting in increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and stimulating the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$, and in turn promoting cell membrane depolarization [17, 22, 24]. Collectively, as illustrated in Fig. 1A, increased cellular volume during cell swelling triggers Cl^- conductance, depolarizing the lipid bilayer membrane due to net Cl^- efflux and stimulating $K_V1.3$ [25]. The exit of K^+ and Cl^- from the cell along with water results in an overall decrease in cellular volume [25]. Since both $K_V1.3$ and $K_{Ca}3.1$ regulate T cell membrane potential and thus promote the entry of Ca^{2+} through CRACs, the subsequent increase in $[Ca^{2+}]_i$ leads to $K_{Ca}3.1$ activation that results in hyperpolarization of the lipid bilayer membrane and more Ca^{2+} influx [25, 26].

Two-pore domain (K_{2P}) potassium channels are also known to play key roles in volume regulatory mechanisms of T lymphocytes. Recently, K_{2P} channels $K_{2P}3.1$ (TASK1), $K_{2P}5.1$ (TASK2), $K_{2P}9.1$ (TASK3), and $K_{2P}18.1$ (TRESK) have been focal points of research in investigating lymphocyte volume regulation [17]. In particular, $K_V1.3$ and $K_{2P}5.1$ have been determined to be the primary contributors of RVD in murine T lymphocytes, as K^+ channel blockers targeting these channels caused the most significant RVD inhibition in wild-type $CD4^+$ cells experiencing hypotonic stress [17]. Also, the expression patterns of various potassium channels employed during volume regulation were altered following $K_{2P}5.1$ knockout [17]. Complete knockout of $K_{2P}18.1$ also demonstrated a weakened RVD response, but to a lesser degree [17].

Significant progress has also been seen in research studying volume regulatory mechanisms of human $CD4^+$ T lymphocytes. Andronic et al. found that when subjected to anisotonic solution, human T cells either shrank or swelled as determined by the osmotic pressure gradient [27]. Both naive and stimulated human $CD4^+$ T lymphocytes demonstrated RVD but did not fully restore cell volume within 20 minutes [28]. As expected, exposure to hypertonic conditions leads to cell shrinkage. However, neither naive nor stimulated cells were capable of performing RVI to restore homeostatic cell volume [28]. The use of various ion channel blockers allows researchers to study the impact of ion transporters in cell volume regulatory mechanisms. It was found that Stichodactyla helianthus toxin (ShK) used to block $K_V1.3$ currents effectively inhibited the RVD process in human T lymphocytes [28]. However, ShK was more effective at blocking $K_V1.3$ in unstimulated naive cells, suggesting that $K_V1.3$ provides a greater contribution to the RVD response in unstimulated human T lymphocytes, compared to stimulated T lymphocytes [28]. Particularly, treatment with the K^+ channel blockers anandamide and A293 to inhibit $K_{2P}3.1$ and $K_{2P}9.1$ function significantly suppressed the RVD process in both naive and stimulated human T lymphocytes, confirming their significance in regulating cellular volume [28]. $K_{2P}5.1$ is also essential for immune cell function and volume regulation, as it is understood to influence cytokine production and cell proliferation [29]. In naïve T lymphocytes, blockage of $K_{2P}5.1$ by quinidine displayed a substantial inhibition of RVD [28]. Similarly, quinidine blocked RVD in stimulated cells at reduced concentrations, suggesting that $K_{2P}5.1$ is functionally important for RVD in human T lymphocytes regardless of activation status [28]. Although quinidine effectively blocked $K_{2P}5.1$ and RVD in human T lymphocytes it also inhibits various calcium-activated K^+ channels as well as KCl cotransport. Higher concentrations of 100 μM quinidine also blocks $K_{2P}18.1$ [28]. Another important two-pore domain potassium channel in human T lymphocyte osmoregulation is $K_{2P}18.1$ (TRESK). It was found that treatment with the channel blocker bupivacaine significantly reduced RVD efficiency and function in both naive and stimulated human T lymphocytes [28].

Considering the aforementioned data reporting significant inhibition of RVD in human T lymphocytes following treatment with various pharmacological channel blockers, it can be assumed that K_{2P} and K_V channels perform critical functions in RVD mechanisms in human T lymphocytes and thus are essential for effective volume regulation in immune cells. Although T lymphocyte volume regulation may vary depending on activation status, $K_V1.3$, $K_{2P}5.1$, and $K_{2P}18.1$ are hypothesized to be responsible for human T lymphocyte RVD and play key roles in volume regulatory pathways.

Water Channels in Platelet Volume Regulation

Platelets similarly require a relatively consistent volume to function correctly. Platelets are small, anucleate cells that play key roles in various immune response mechanisms and are known to secrete several functionally important molecules [30, 31]. Because of these essential functions, impaired platelet function caused by improper volume regulation can lead to blood clotting and thus have damaging effects such as myocardial infarction, stroke, or pulmonary embolism [31]. To ensure proper function, platelets employ a variety of volume regulatory mechanisms. AQP water channel mediates the passive membrane permeability of water in platelets. The chief controller of volume regulatory mechanisms relies on the changes in concentration of various organic osmolytes and the osmotic imbalance created by the transport of solutes [32]. One study examined the role of these AQPs in platelet volume regulation and found that exposure to the AQP6- stimulant $HgCl_2$ quickly leads to swelling, suggesting that AQP6 is present in platelets and functions in volume regulation [30]. Specifically, Hazama et al. (2002) determined that treatment with 10 μM $HgCl_2$ increased the open probability of AQP6 in oocytes within 10 seconds [33]. It must be noted that treatment with $HgCl_2$ normally inhibits AQP-mediated water transport, but AQP6 is stimulated by $HgCl_2$. Hazama's group reported that following the addition of 100 μM $HgCl_2$, a 2.6-fold increase of Na^+ influx in AQP6 oocytes was seen. They reported that the mechanism of AQP6 activation for both water permeability and ion conductance is due to the binding of Hg^{2+} to the Cys-155

or Cys-190 residues in each monomer [33]. Variations in water permeability resulted in proportionate changes in ion conductance, suggesting that each monomer forms a pore region for water and ions rather than a single permeation in the center of the homotetramer. Thus, AQP6 functions also as an anion channel activated by low pH or $HgCl_2$ as stated above [33]. These findings help explain why the activation of AQP6 can generate cellular volume changes and thus play a key role in volume regulation. As discussed earlier, water transport is crucial for proper cell volume regulation, and AQP channels function to control water flux across cell membranes. Thus, reduced AQP function inhibits the process of volume regulation [34]. To further examine possible mediators of AQP-related platelet volume regulation, G proteins have been investigated. G (guanine nucleotide-binding) proteins are coupling proteins that play key roles in the regulation of various ion channels by transmitting extracellular signals to the inside of the cell [35]. Mastoparan, a tetradecapeptide found in wasp venom, enhances GTPase activity of particular heterotrimeric G proteins [30, 36]. Treatment of platelets with mastoparan displayed similar results to that of $HgCl_2$, causing platelets to quickly swell. It was determined that just seconds after the addition of $HgCl_2$ and mastoparan, platelet swelling was described by a linear function of time followed by a period where cell swelling began to slow [30]. This suggests that the stimulation of selective AQPs and heterotrimeric G proteins leads to unimpeded water and ion entry, further indicating that they are significant in regulating water transport during platelet volume regulation [30].

In summary, recent studies demonstrate that immune cells such as thymocytes, T lymphocytes, and platelets express cell volume regulatory membrane proteins such as K^+ channels, Cl^- channels, and AQP water channels. The conserved RVI and RVD mechanisms are not particularly consistent between cell subtypes, however, as different immune cells employ a diverse array of cellular mechanisms to control RVD, and some immune cells fail to employ mechanisms of RVI during hypertonic stress. However, AQP water channels, $K_V1.3$, VSOR Cl^- channels, and two-pore domain potassium channels $K_{2P}5.1$ and $K_{2P}18.1$ are all dominant mechanisms in RVD to restore volume homeostasis.

Role of Volume Regulatory Mechanisms in Inflammasome Immune Response

Dendritic cells (DCs) are widely understood to coordinate T cell-mediated adaptive immune responses, often via DC migration [37]. The migration of these cells caused by inflammatory stimuli and the subsequent immune response requires various volume regulatory mechanisms [38]. Gröbner et al. (2014) found that treating wild-type DCs with lipopolysaccharide (LPS) quickly increases the size of the cell [38]. Toll-like receptor 4 (TLR4) functions in signaling DC migration, and it was found that LPS-treated DCs lacking TLR4 displayed impaired cell swelling and chemokine (C-C motif) ligand 21 (CCL21) directed migration, which are important processes for the adaptive immune response [38]. This provides evidence that DCs rely on LPS/TLR4 signaling to regulate cell volume and subsequent migration. Calcium-activated K^+ channels ($K_{Ca}3.1$) also play an important role in DC swelling and volume regulation [38]. Particularly, it has been found that cell swelling is impaired in DCs lacking $K_{Ca}3.1$ upon treatment with LPS in comparison to wild-type DCs [38]. Moreover, $[Ca^{2+}]_i$ steadily increased in wild-type DCs following treatment with LPS while $K_{Ca}3.1$-deficient DCs displayed a less significant increase in $[Ca^{2+}]_i$, suggesting that $K_{Ca}3.1$ is likely required for $[Ca^{2+}]_i$ alterations provoked by LPS [38]. It is understood that ion channels play a role in the conversion of DCs to a migratory phenotype and that DCs subsequently respond to treatment with LPS by displaying a rapid increase in cytosolic Ca^{2+} from both intracellular and extracellular Ca^{2+} stores [39]. The subsequent activation of $K_V1.3$ in conjunction with an overall increase in $[Ca^{2+}]_i$ is understood to be functionally important in LPS-mediated DC migration [39]. Thus overall, the opening of both $K_V1.3$ and $K_{Ca}3.1$ channels are effective by maintaining the negative membrane potential and regulate the electrochemical driving force for the influx of Ca^{2+} through CRAC channels that are ultimately required for various immune response mechanisms [39]. Changes of $[Ca^{2+}]_i$ are significant

in immune response mechanisms, as cell swelling and the activation of mechanosensitive Ca^{2+} channels near the leading edge of the migrating immune cell are hypothesized to cause increased $[Ca^{2+}]_i$ during immune response [40]. Thus, it is important to recognize that $K_{Ca}3.1$ plays a key role in $[Ca^{2+}]_i$ homeostasis, DC swelling, and LPS-treated DC migration, suggesting K^+ channels mediating cell swelling and volume regulation are crucial for proper DC function [38].

Macrophages play key roles in the immune response process of many organisms and are vital cellular components of the immune system that engulf foreign material, microbes, and dead cells [48]. Macrophages also consume, digest, and recycle upwards of 200 billion red blood cells every day, a metabolic process that is required for the survival of the host organism [41]. A key element in macrophage immune response function is a multiprotein complex called the NLRP3 (nucleotide-binding domain and leucine-rich repeat pyrin 3 domain) inflammasome. It is important in the regulation of immune cell homeostasis and plays a role in the immune response by detecting pathogenic signals [42]. The NLRP3 inflammasome can sense key volume regulatory mechanisms such as cell swelling and RVD (Fig. 2), and this swelling can serve as a signal for NLRP3 inflammasome activation [42]. The NLRP3 inflammasome is upregulated by decreased levels of intracellular K^+, suggesting it likely functions in cell volume regulation [43, 44]. The NLRP3 inflammasome also mediates the release of interleukin-1β (IL-1β), a cytokine protein known to influence the pathogenesis of various neurological diseases [43, 45, 46]. IL-1β acts as a pro-inflammatory cytokine and is important in immune response mechanisms following infection or injury [47]. Exposing macrophages to variations in extracellular osmolarity has been seen to influence response mechanisms of volume regulation. In particular, Compan et al. (2012) found that when human THP-1 macrophages are exposed to hypotonic conditions, the cells swell, perform RVD, and release the caspase-1-dependent cytokines IL-18 and IL-1β (Fig. 2). Like in T cells, K^+ efflux is necessary for proper RVD function and immune response, as blocking K^+ exit in macrophages allowed for cell swelling but eliminated both RVD and IL-1β release [42]. Treating macrophages with NPPB inhibited RVD and mature IL-1β release but did not affect K^+ efflux caused by cell swelling, indicating that K^+ efflux is required but not sufficient for IL-1β release caused by cell swelling [42]. These findings suggest that an effective immune response is dependent on proper volume regulation in macrophages.

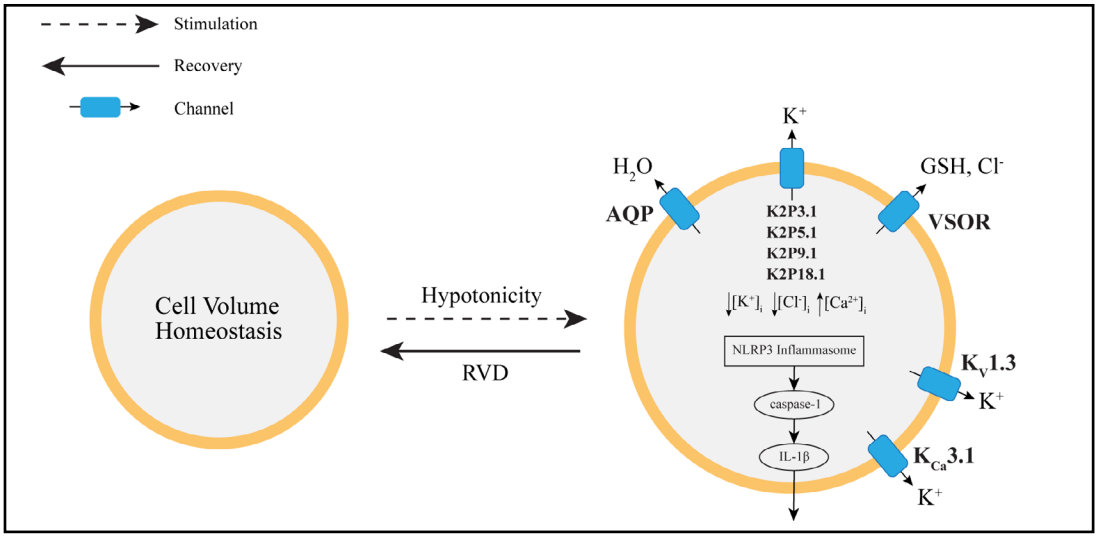

Fig. 2. NLRP3 inflammasome protein complex of macrophages is activated by efflux of K^+ during hypotonicity. Cell swelling and subsequent release of K^+ leads to conformational change of the NLPR3 inflammasome resulting in the activation of the NLPR3 inflammasome. NLPR3 activation leads to the activation of caspase-1 enzyme and allows for the release of IL-1β, necessary for host defense-responses against pathogens and injury.

Transforming growth factor β-activated kinase 1 (TAK1) is known to function in the activation of the NLRP3 inflammasome [48]. Transient receptor potential (TRP) channels are ion channels permeable to Ca^{2+} that are known to regulate $[Ca^{2+}]_i$ and TAK1 phosphorylation, which is a required step in its activation [42]. Hypotonic extracellular conditions were seen to induce TAK1 phosphorylation, while inhibition of RVD blocked TAK1 activation, suggesting that macrophage swelling acts as a signal for NLRP3 inflammasome activation [42]. Further, inhibition of TRP channels prevented TAK1 phosphorylation, indicating that TAK1 is crucial in the downstream signaling of TRP channels for the activation of the NLRP3 inflammasome in cells under hypotonic stress [42]. It is known that caspase-1 activation functions in the proinflammatory effects of various neurological diseases linked to neuronal swelling like epilepsy or stroke [46, 49, 50]. Inhibition of TRP channels was seen to significantly inhibit the activation of caspase-1 and the release of IL-1β during hypotonic stress [42]. Mice-derived macrophages deficient in caspase-1 or NLRP3 also displayed a lack of IL-1β release following exposure to a hypotonic solution but this did not affect the RVD process [42]. Activation of caspase-1 induced by a hypotonic solution is known to be dependent on the efflux of intracellular K^+ [42]. This is important to note, as it is a required prerequisite process for NLRP3 inflammasome activation [50]. Utilizing extracellular concentrations of Mg^{2+} or TRP channel blockers to prevent TRPM7 function delayed the process of RVD in macrophages and decreased the release of IL-1β [42]. Reduced levels of extracellular Mg^{2+} are known to have the opposite effect, stimulating TRPM7 and accelerating RVD and IL-1β release in hypotonic conditions [42]. In contrast, macrophages subjected to hypertonic extracellular conditions lead to cell shrinkage and resulted in an RVI response to reestablish homeostatic volume, but did not display cytokine release [42]. Although the complete pathways required for NLRP3 activation are not fully understood, it is likely that pharmacological agents targeting TRP channels and signaling pathways of cell volume regulatory mechanisms can be key aspects of future studies and treatment of inflammatory diseases [42]. Research investigating macrophage volume regulatory mechanisms may examine the specific signaling pathways of ion transport and inflammasome activation to better understand the exact molecular conditions of human inflammatory diseases.

Ion Channels and Transporters in Immune Cell Proliferation

Ion channels such as K^+ channels and volume regulated anion channels (VRACs) not only play a role in acute cell volume regulation in response to osmotic stress but also are involved in RVD during cell proliferation [22]. As previously discussed, VSORs enable the outward current of Cl^- to restore cellular volume [51]. VRACs are activated to expel Cl^- ions and other osmolytes during changes in tonicity, particularly in hypotonic stress (Fig. 2). Cl^- and osmolyte efflux are the cell's RVD mechanism to restore normal cell volume [52]. Thus, it is important to note the similarity between VRACs and VSORs where both channels produce an outward rectifying current of Cl^-, which allows the cells to mediate RVD [51, 53]. $K_v1.3$ plays an integral role in promoting immune cell proliferation and abrogating apoptotic events via osmoprotective proteins and pathways [54, 55]. In addition, $K_{Ca}3.1$/IK channels are also essential for immune cell activation and proliferation [56]. $K_{Ca}3.1$/IK channels have six transmembrane proteins and a cytoplasmic domain with an attachment site for calmodulin (CaM) [57]. Particularly, four CaM molecules bind to the $K_{Ca}3.1$ channel where each lobe of CaM has different roles in Ca^{2+} detection [58]. The C-lobe acts independently through constitutive binding to the channel whereas the N-lobe is a Ca^{2+}-dependent sensor [58]. The N-lobe undergoes a conformational change when Ca^{2+} binds to CaM, which induces a conformational change in the channel resulting in the opening of the channel pore and allowing more Ca^{2+} to enter the cell [58]. Increased $[Ca^{2+}]_i$ coupled with His358 phosphorylation induces different proliferative effects on cells [56, 57].

Both $K_v1.3$ and $K_{Ca}3.1$ channels are involved in $CD4^+$ T lymphocyte proliferation [21]. $K_v1.3$ and $K_{Ca}3.1$ are recruited to the immunological synapse, formed between T cells and

their target cell or antigen presenting cell [59]. The expression of $K_V1.3$ and $K_{Ca}3.1$ in human effector memory $CD8^+$ T cells depends on the expression of the interleukin (IL)-7 receptor alpha (IL-7Rα) chain [60]. $CD8^+$ T cells expressing low levels of IL-7Rα displayed reduced $K_V1.3$ activity as well as weak $K_{Ca}3.1$ channel conductance [60]. Chronically activated T cells demonstrate high $K_v1.3$ channel expression [21]. Tregs also express $K_V1.3$ channels [59]. Compared to naïve cells, Tregs expressed a significantly larger membrane surface area and decreased $K_V1.3$ channel density [59]. K^+ channels play a crucial role during T cell activation, stabilizing the membrane potential during Ca^{2+} influx [59]. Specifically, these K^+ channels regulate the membrane potential via membrane hyperpolarization caused by K^+ efflux [61]. An increase in cell size without concomitant K^+ channel upregulation leads to poor control of the membrane potential, ultimately resulting in insufficient Ca^{2+} signaling and a reduced propensity for cell proliferation [59]. Thus, during T cell activation, reduced $K_V1.3$ channel expression is commonly seen in Tregs from patients with multiple sclerosis, compared to naïve cells [59].

Because of the extensive research on $K_V1.3$ and $K_{Ca}3.1$ channels, their mechanisms are largely known providing us a better understanding of how immune cells rely on $K_V1.3$ and $K_{Ca}3.1$ activation to survive and replicate. $K_{Ca}3.1$ channels can affect T cell proliferation by modulating $[Ca^{2+}]_i$ and T cell viability by regulating RVD processes [56]. Li et al. (2018) investigated T lymphocytic $K_{Ca}3.1$ channel in hypertensive (high blood pressure) patients to observe their effects on T lymphocytic proliferation [62]. Normally, hypertension is linked to inflammatory pathways in the innate immune cells which are responsible for eliminating pathogens [63]. Immune responses against pathogens include reactive oxygen species (ROS) and cytokine production [64] resulting in increased immunoinflammatory responses of T cells which contributes to hypertension [64, 65]. Li et al. reported increased $K_{Ca}3.1$ channel expression in $CD4^+$ T cells in hypertensive patients than the healthy control participants, suggesting $K_{Ca}3.1$ channel is vital to regulating hypertension [62]. Furthermore, when hypertension curbing drug, candesartan was administered, $K_{Ca}3.1$ channel expression was reduced in the T lymphocytes of the hypertensive patients by an unknown mechanism [62]. Li et al. also showed that they are crucial for T cell proliferation and consequent immune cell inflammation [62]. These studies also provide insights on targeting ion channels to reduce inflammation (which is linked to immune cell proliferation) and in turn, hypertension [62, 63].

Now, the ionic basis of immune cell proliferation will be addressed. Proliferative pathways stem from $[Ca^{2+}]_i$ fluctuations due to $K_{Ca}3.1$ or $K_V1.3$ channel activation, and intracellular Ca^{2+} store and Ca^{2+} release [17, 66, 67]. In lymphocytes, Ca^{2+} is released from Ca^{2+} stores in the endoplasmic reticulum (ER) through the second messenger inositol–1,4,5–trisphosphate (InsP3) to increase $[Ca^{2+}]_i$ [66]. This increase in intracellular Ca^{2+} can also be dependent on the activation of $K_V1.3$ channel which creates K^+ efflux causing the membrane to hyperpolarize due to the loss of positively charged K^+ that consequently favor Ca^{2+} influx [68]. Although the exact mechanism of $K_V1.3$ activation is unknown, researchers have suggested that ionic concentrations play a crucial role in cellular proliferation [67]. The rise in intracellular Ca^{2+} drives the cell to utilize Ca^{2+}-dependent transcription which activates certain proliferative pathways [66]. In particular, Ca^{2+} release from ER activated CRAC channels and plays a predominant role in sustained Ca^{2+} influx into lymphocytes (Fig. 3). T cell receptor (TCR) stimulation causes the production of InsP3 which releases the Ca^{2+} from ER through InsP3R, resulting in reduced Ca^{2+} in the ER [20]. The fall in Ca^{2+} in ER leads to the dissociation of Ca^{2+} from the EF hand of STIM1 (a calcium sensor) and facilitates the migration of STIM1 to the cell membrane where it binds to ORAI1 to activate CRAC channel and Ca^{2+} entry into cells (Fig. 3) [20, 69]. Sustained Ca^{2+} influx from CRACs activates CaM-dependent protein phosphatase calcineurin and the nuclear factor protein NFAT [20, 70]. Then, NFAT translocates to the nucleus and initiates transcription of IL-2 which is responsible for T cell proliferation and activation even in the absence of antigens (Fig. 3) [22, 69].

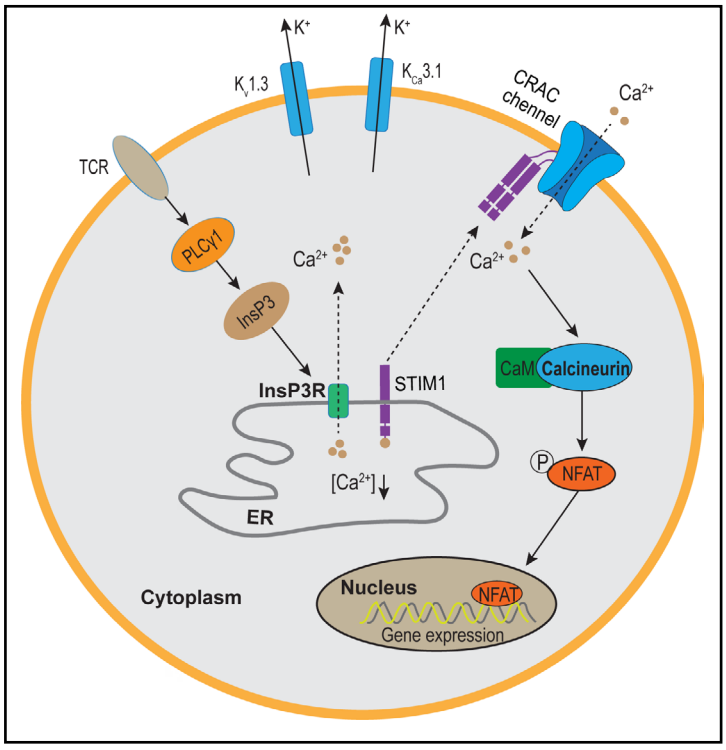

Fig. 3. Schematic illustration of ORAI1 and STIM1 in forming CRAC channel and NFAT activation in response to increased intracellular Ca^{2+}. STIM1, is a Ca^{2+} sensor that is bound to the endoplasmic reticulum (ER). Release of ER Ca^{2+} via InsP3R leads to dissociation of Ca^{2+} from the binding site of STIM1, which enables it to dissociate from the ER and migrates to the cell membrane where it binds to ORAI1 to activate CRAC channel and Ca^{2+} entry into cells. Sustained Ca^{2+} influx into cytosol via CRAC leads to the activation of Ca^{2+}-dependent enzyme calcineurin via its binding to calmodulin (CaM) and NFAT dephosphorylation leading to its translocation into nucleus, initiating transcription and cytokine expression.

NFAT5 activates T cells allowing T cells to adapt to anisotonic conditions through intracellular signaling and osmoprotective proteins [71]. NFAT5 activation in splenocytes stimulates the expression of osmoprotective proteins such as TauT (taurine transporter) and SMIT (Na^+/myo-inositol cotransporter) that enable them to regulate cellular activity in response to changes in osmolarity [56]. However, Kino et al.'s (2009) study implicated Brx (protein kinase A- anchoring protein 13) induced NFAT5 activation, which upregulated osmoprotective protein expression [72]. Regardless of the NFAT5 activation mechanism, NFAT5 regulates cellular osmolarity suggesting it is essential for lymphocyte proliferation in the spleen and thymus [72]. Not only does NFAT5 upregulate osmoprotective protein expression, but it also attenuates p53 expression which normally suppresses immune cell growth [73]. In T cells and macrophages, NFAT5 activation is triggered in response to microbial pathogens and inflammatory stimuli, indicating its essential role in protecting cells from foreign threats and ionic heterostasis [74].

Abundant literature shows that NFAT5 activation is imperative in the maintenance of normal intracellular water and ion concentration in immune cells. However, NFAT5 first requires an auxiliary export domain (AED) into the cytoplasm during tonicity changes enabling NFAT5 to maintain cellular homeostasis in hypotonic environments and allow for cell proliferation in hypertonic environments [75-77]. Because optimal water and ionic concentrations (specifically high $[K^+]_i$) are necessary for immune cell proliferation and viability, NFAT5 is essential for cell survival [55, 78]. To exemplify the importance of AED in hypotonic environments, Tong et al. (2006) deleted AED, and observed less hypotonicity induced-NFAT5 export [75]. However, it is also important to consider the effects of hypertonicity on NFAT5. Drews-Elger et al. (2009) further analyzed cell cycle progression and production of osmoprotective genes through hypertonicity induced- NFAT5 pathways in wild-type T lymphocytes and $NFAT^{-/-}$ (NFAT5 deficient) T lymphocytes [77]. Drews-Elger et al. (2009) reported that exposure of wild-type T lymphocytes to hypertonic environments leads to halted cell cycle, upregulation of p53 and p21 (proteins that are activated during DNA damage), followed by an NFAT5-dependent adaptive phase [77] that entails NFAT5 expression to combat osmotic stress [79]. NFAT5 expression triggers osmoprotective

gene expression including expression of the SMIT and TauT [77]. TauT increases taurine concentrations, an osmolyte responsible for protein stabilization, cell volume regulation, and combating oxidative stress [80]. Additionally, SMIT is responsible for rectifying cell shrinkage or cell volume increase resulted from cells exposure in anisotonic environments [81]. Collectively, SMIT and TauT are essential to resist changes in tonicity [80, 81]. The NFAT5$^{-/-}$ T lymphocytes showed minimal osmoprotective protein production and drastically reduced cell viability [77]. Kaesler et al. (2012) showed that apoptosis was induced in activated TauT$^{-/-}$ T cells, pointing to the phenomenon that T cell proliferation is dependent on cell volume regulation [80]. Without TauT, decreased cell viability in TauT$^{-/-}$ T cells exemplifying TauT's importance in conferring resistance during cell volume changes [80]. Moreover, the NFAT5$^{+/+}$ cells exhibited increased cellular viability in hypertonic condition due to osmoprotective protein production of STIM1/2 and TauT, while the NFAT5$^{-/-}$ T lymphocytes displayed reduced cellular viability and little osmoprotective gene expression, indicating that the activation of NFAT5 and specific protein upregulation are essential to cell survival and proliferation [77]. Thus, without these essential osmoprotective genes and NFAT5 activation, cell proliferation cannot progress, and cellular apoptosis may occur [77]. As mentioned before, ORAI1 and STIM2 are two essential proteins for T cell proliferation (Fig. 3) [68, 82] and downregulation of ORAI1 and STIM2 decreases T cell proliferation [83]. STIM1/2 and ORAI1 production in T cells influences cellular homeostasis to prevent ionic, especially, Ca^{2+} imbalances and apoptosis [17]. ORAI1 and STIM1/2 function is dependent on both Ca^{2+} entry, via CRAC channels, $K_{Ca}3.1$ channels, and K^+ efflux through $K_V1.3$ channels (Fig. 3) [84]. K^+ efflux coupled with Ca^{2+} influx induces alterations in gene expression and increase T cell proliferation, while K^+ efflux coupled with Cl^- efflux regulates cell volume by adjusting ionic imbalances [56, 84]. Taken together, cell volume regulation of T lymphocytes and activation of ion channels in varying osmotic conditions will induce mechanistic T cell activation and proliferation while suppressing apoptosis. Due to our extensive knowledge of $K_V1.3$ and $K_{Ca}3.1$ channels, therapeutic potential lies in using $K_V1.3$ and $K_{Ca}3.1$ inhibitors to suppress multiple sclerosis, inflammatory bowel disease, atherosclerosis, and hypertension, and other forms of inflammation [62, 63, 85].

Ion Channels and Transporters in Immune Cell Apoptotic Volume Decrease and Apoptosis

Ion channels play a key role in immune cell apoptosis by regulating ionic homeostasis and volume. Reduced $[K^+]_i$ and apoptotic volume decrease (AVD) are characteristic features of early stage apoptosis (Fig. 1C) [86]. In lymphocytic apoptosis, increased K^+ efflux resulting in depletion of $[K^+]_i$, leads to cell shrinkage [87]. The large K^+ efflux triggers AVD, caspase activity, and DNA fragmentation [70]. In addition to the K^+ efflux, there is also efflux of anions and intracellular water [87]. Overall, scientific literature has attributed the channel induced K^+ efflux for water loss, and AVD [86-88]. Further studies reveal the underlying mechanisms for the direct effect of $K_V1.3$ channel inhibition on osmotic balance and eventual caspase activity during apoptosis. Before we discuss the direct effect of $K_V1.3$ channel inhibition on immune cells, it is important to discuss protein kinase B (AKT) which activates an anti-apoptotic pathway and induces RVI responses in immune cells [89-91]. To confirm the anti-apoptotic role of PI3K/AKT activation, Bergermann et al. (2019) administered a PI-3K/AKT inhibitor, resulting in 75% cell death demonstrating AKT's integral role as an anti-apoptotic protein [91]. The findings from Bergermann et al.'s (2019) study points to the importance of $K_v1.3$ channels on the mitochondria in PI-3K/AKT activation [91], but how plasma membrane $K_v1.3$ channels affect the PI3K/AKT activation remains to be determined. $K_V1.3$'s importance in apoptosis is further backed by findings that prevention of a decrease in $[K^+]_i$ due to K^+ efflux reduced AKT-mTOR signaling in T cells [92]. T cells with constitutively active AKT expression showed resistance against the inhibitory effect of $[K^+]_e$ which augments AKT's role in immune cells [92]. This leads us to discuss AKT's role in RVI responses to replenish

intracellular NaCl and water for maintaining proper cellular osmolarity (Fig. 1C) [93]. Without proper intracellular water and ion concentrations, cell death occurs. When the AKT pathway was inhibited in S49 (osmotic sensitive lymphoma) cells, they exhibited decreased RVI responses, leaving the cells in a shrunken state [90]. However, it is worth noting RVI's effect on apoptosis-primed cells. RVI serves as a compensatory mechanism for the cell before apoptosis takes place (Ruiz-Martínez et al. 2011). Furthermore, Ruiz Martínez et al. (2011) also found inhibition of AKT resulted in increased apoptosis [94]. Thus, cells can prevent apoptosis by activating an RVI response to restore $[H_2O]_i$ and ion concentrations [93-95]. However, in lymphocytes, apoptosis is a highly regulated process so once apoptosis is induced, it is nearly impossible to initiate another mechanism to prevent it and restore normal cellular functions [94, 96]. Bortner et al. (2012) reported that RVI inhibition in S49 cells (osmotic lymphoma 4-15) resulting from non-functional AKT left the cells in a shrunken state [90].

Na/H exchanger 1 (NHE1) is another transporter that often mediates the direct influx of extracellular Na^+ along with osmotic water movement, resulting in RVI to counteract AVD [97]. NHE1 is considered as the primary mechanism to regulate RVI in many cell types [97]. AKT phosphorylates NHE1 in HeLa cells and stimulates NHE1-mediated cation influx and water into the cell, initiating the RVI to prevent apoptosis [93, 95]. Maeno et al. (2006) reported that Hela cells failed to undergo RVI in response to TNF-α and staurosporine-induced AVD with NHE1 inhibition [98]. Moreover, many of the immune cell functions, such as phagocytosis and cytokine production, are regulated by cytosolic pH (pH_i). NHE1 is one of the main pHi regulators [99] and it is possible that NHE1 can play a role in maintaining the RVI of different immune cells such as macrophages, lymphocytes, etc. However, to date, there is no direct evidence that NHE1 regulates immune cell RVI.

Caspase activation is an important indicator of apoptosis [54]. GSH depletion has been a defining indicator of lymphoid apoptosis via cytochrome C (CytC) release, ROS production, and increased caspase activity. Depletion of $[K^+]_i$ is one of the many cues that facilitates apoptotic mechanisms [100]. Along with GSH efflux, $[K^+]_i$ loss potentiates apoptosome formation, cell fragmentation, and CytC release to cytosol which activates caspase cascade and initiates apoptosis [100]. However, an abundance of AKT in the mitochondrial membrane can phosphorylate proteins like ATP synthase to increase ATP production and HK-II (hexokinase II) to suppress Ca^{2+} induced CytC release [101, 102].

The Na^+/K^+-ATPase (Na^+ pump) is another crucial K^+ regulatory mechanism that maintains a low $[Na^+]_i$ and high $[K^+]_i$, and plays an important role in apoptosis regulation [103]. It has been shown that inhibition of the Na^+ pump by ouabain (Na^+ pump blocker) leads to ionic imbalances like increased $[Na^+]_i$ and loss of $[K^+]_i$, and FasL-induced apoptosis [103]. Shortly after Jurkat cells were exposed to FasL, H2O2 (a ROS) was generated leading to enhanced caspase activity and decreased cell count [83]. Na^+ pump inhibition via ouabain and FasL leads to AVD, which was reflected by decreased light scattering properties of Jurkat cells, indicating a reduction in cell size [103]. Additionally, Yin et al. (2007) reported that administering FasL/ouabain in Jurkat cells reduced cell counts and increased apoptosis through Na^+/K^+-ATPase pump impairment, which is supported by the study of Panayiotidis et al. (2010) [103, 104]. FasL changes protein levels of the Na^+/K^+-ATPase pump to induce immune cell apoptosis [103, 104]. However, the Na^+/K^+-ATPase pump protein was constantly expressed in caspase 8 deficient cells in the presence of FasL, suggesting that caspase 8 activation is necessary for FasL to induce Jurkat cell apoptosis [104]. Furthermore, it is worth noting that GSH loss may take place before K^+ efflux, emphasizing that GSH loss is independent of K^+ loss [104]. However, GSH loss regulates apoptotic body formation, cell fragmentation, and executes the caspase activity during the execution phase, all of which regulate and facilitates K^+ loss and lead to apoptosis [104].

In addition to intracellular K^+ homeostasis, the Na^+/K^+-ATPase pump also affects $[Ca^{2+}]_i$, which plays a role in immune cell apoptosis. When the Na^+/K^+-ATPase is inhibited, Ca^{2+} is released from intracellular stores, resulting in increased $[Ca^{2+}]_i$ [103]. There is also a

correlation between FasL-induced apoptosis and an increase in $[Ca^{2+}]_i$ where $[Ca^{2+}]_i$ rise is necessary during the late stages of apoptosis [103]. Furthermore, it is important to note that Oubain-mediated inhibition of the Na^+-K^+-ATPase function can increase in $[Ca^{2+}]_i$ via stimulating reversal operation of Na/Ca exchanger (NCX) since ouabain causes increased $[Na^+]_i$, and there is a larger driving force for Ca^{2+} to be transported inwardly as Na^+ transported outwardly [103]. Ajiro et. al's (2008) reported a five-fold increase in $[Ca^{2+}]_i$ and a decrease in $[K^+]_i$ during thymocyte apoptosis, which induced DNA degradation. However, maintaining normal $[K^+]_i$ during late stage apoptosis inhibited Ca^{2+}-dependent DNA degradation pathways [105]. Collectively, changes in $[Ca^{2+}]_i$ play an important role in the regulation of other ion channel activity, enzymatic activity, and gene expression [104, 105]. Dynamic and delicate regulation of these signaling processes controls immune cell survival and death. Increased $[Na^+]_i$, $[Ca^{2+}]_i$, and decreased $[K^+]_i$ occur when the Na^+ pump is impaired and leads to immune cell apoptosis via activation of caspase and ROS-mediated cell damage. These findings further demonstrate the importance of intracellular ionic and cell volume homeostasis in maintaining cell viability.

Conclusions and Future Research Perspectives

Volume regulation of immune cells is fundamentally important for proper immune response and cell survival. Immune cell osmoregulation relies on several ion channels, particularly potassium and chloride channels. These ion channels are key in controlling RVD and RVI to maintain cell volume homeostasis in the event of osmotic stress in thymocytes, lymphocytes, platelets, dendritic cells, and macrophages. In particular, thymocyte function relies on the VSOR Cl^- channel to regulate anion efflux and reestablish normal cell volume. Potassium channels $K_{2P}5.1$, and $K_{2P}18.1$ play crucial roles in the regulation of T lymphocyte cell volume, while platelets primarily utilize AQP water channels to control cellular volume. Moreover, volume regulation of macrophages is important for the formation and function of the NLRP3 inflammasome which is functionally important in various immune response mechanisms. $K_V1.3$ and $K_{Ca}3.1$ have also been identified as integral mechanisms to maintain immune cell volume homeostasis and proper cellular functions in diverse environmental conditions. Ionic imbalances and cell volume dysregulation induce responsive pathways to compensate for the fluctuations in ionic and volume dysregulation which are harmful to immune cells.

Future research might focus on the environmental and molecular causes of immune cell shrinkage and mechanisms controlling RVI. Current knowledge of the role of ion channels in immune cell RVI is limited (particularly for thymocytes, lymphocytes, and macrophages). This includes the investigation of the signaling pathways and molecular requirements for the stimulation of ion channels necessary during RVI, including changes of ionic homeostasis for immune cell proliferation and apoptosis. A further understanding of immune cell shrinkage and mechanisms of volume recovery may be important for future clinical research analyzing therapeutic agents for various inflammatory diseases.

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Disclosure Statement

The authors declare no potential conflicts of interest exist.

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