MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Pharmacological Modulation of Proton Channel Hv1 in Cancer Therapy: Future Perspectives.

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Received February 14, 2016; accepted June 2, 2016

ABSTRACT
The pharmacological modulation of the immunosuppressive tumor microenvironment has emerged as a relevant component for cancer therapy. Several approaches aiming to deplete innate and adaptive suppressive populations, to circumvent the impairment in antigen presentation, and to ultimately increase the frequency of activated tumor-specific T cells are currently being explored. In this review, we address the potentiality of targeting the voltage-gated proton channel, Hv1, as a novel strategy to modulate the tumor microenvironment. The function of Hv1 in immune cells such as macrophages, neutrophils, dendritic cells, and T cells has been associated with the maintenance of NADPH oxidase activity and the generation of reactive oxygen species, which are required for the host defense against pathogens. We discuss evidence suggesting that the Hv1 proton channel could also be important for the function of these cells within the tumor microenvironment. Furthermore, as summarized here, tumor cells express Hv1 as a primary mechanism to extrude the increased amount of protons generated metabolically, thus maintaining physiologic values for the intracellular pH. Therefore, because this channel might be relevant for both tumor cells and immune cells supporting tumor growth, the pharmacological inhibition of Hv1 could be an innovative approach for cancer therapy. With that focus, we analyzed the available compounds that inhibit Hv1, highlighted the need to develop better drugs suitable for patients, and commented on the future perspectives of targeting Hv1 in the context of cancer therapy.

Introduction
Voltage-gated proton channel (Hv1) is a membrane protein with the capability to permeate protons through membranes with absolute specificity (DeCoursey, 2008). Hv1 channel is activated upon membrane depolarization in a time-, pH (Cherny et al., 1995; Musset and Decoursey, 2012), and temperature (DeCoursey and Cherny, 1998; Kuno et al., 2009)-dependent manner. The channel is composed of three functional domains: a voltage-sensing domain and the cytoplasmic N-terminal and C-terminal domains (Ramsey et al., 2006; Sasaki et al., 2006). The voltage-sensing domain of Hv1 channel comprises four transmembrane segments (S1–S4) and is equivalent to the one present in voltage-dependent K+ channels (Ramsey et al., 2006; Sasaki et al., 2006) (Fig. 1, A and B). Unlike other voltage-gated channels, Hv1 lacks a pore domain (Ramsey et al., 2006; Sasaki et al., 2006) and proton permeation occurs through the voltage-sensing domain (Koch et al., 2008; Tombola et al., 2008; Lee et al., 2009). Hv1 channels assemble as homodimers through the interactions of the coiled-coil domains in the C-terminal region (Koch et al., 2008; Tombola et al., 2008; Lee et al., 2008; Fujiiwara et al., 2012). Monomeric channels, obtained by deletion of the C-terminal domain, are also functional (Koch et al., 2008; Tombola et al., 2008). The N-terminal domain, which contains a phosphorylation site (T29 in human Hv1) that triggers an enhanced gating behavior (Morgan et al., 2007; Musset et al., 2010; Hondares et al., 2014), plays an important role in Hv1 regulation.

Interestingly, Hv1 is expressed in tumor cells, where this channel is involved in the maintenance of intracellular pH and regulates metastasis-related properties such as migration and

ABBREVIATIONS: ClGBI, Cl-guanidinobenzimidazole; CTL, cytotoxic T lymphocytes; CTLA-4, cytotoxic T lymphocyte-associated protein 4; Erk, extracellular receptor-activated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; iNOS, nitric oxide synthase; LPS, lipopolysaccharide; MMP, metalloproteinase; NF-κB, nuclear factor kappa B; NOX, NADPH oxidase; PD-1, programmed cell death protein 1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; TCR, T cell receptor; TGF-β, transforming growth factor β; TLR, toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor; Tregs, regulatory T cells; 2GBI, 2-guanidinobenzimidazole.
Tumor-Associated Immune Dysfunctions and the Challenges of Cancer Immunotherapy

The ability of the immune system to recognize and eliminate tumor cells has been widely described in the last decades (Dunn et al., 2002; Stagg et al., 2007; Vesely et al., 2011), resulting in an enormous effort both in basic and clinical research in pursuit of feasible strategies to control cancer via immunotherapy. However, these studies have demonstrated the challenges of activating immune effectors in tumor-bearing hosts.

Dendritic cells, the most efficient among antigen-presenting cells, are defective in number and functionality in patients with prostate (Pinzon-Charry et al., 2005), breast (Pinzon-Charry et al., 2007), cervical (Lee et al., 2006), non-small cell lung (Perrot et al., 2007), hepatocellular (Ormandy et al., 2006), and pancreatic cancer (Bellone et al., 2006). The impairment in the dendritic cell compartment is mainly associated with the aberrant myelopoiesis promoted through tumor-released soluble factors; this leads to a reduced number of mature dendritic cells and to a simultaneous increase of immature myeloid cells (Gabrilovich, 2004; Gabrilovich et al., 2012). The immature myeloid cells, which expanded during this abnormal myeloid differentiation, also acquire suppressive function in response to proinflammatory signals, thus generating the population of myeloid-derived suppressor cells (Condamine and Gabrilovich, 2011; Talmadge and Gabrilovich, 2013). Myeloid-derived suppressor cells inhibit T cell responses through multiple mechanisms, including the arginase 1-mediated L-arginine depletion (Rodriguez et al., 2004, 2010), the sequestration and consumption of L-cysteine (Srivastava et al., 2010), and the generation of reactive oxygen and nitrogen species by the coordinated function of inducible nitric oxide synthase (iNOS), NOX, and arginase 1 (Gabrilovich et al., 2012; Serafini, 2013). Other main players of tumor-associated immunosuppression are regulatory T cells (Tregs), which not only secrete immunosuppressive cytokines such as IL-10 (IL-interleukin) and TGF-β (transforming growth factor β), but also downregulate the expression of costimulatory molecules on dendritic cells and therefore inhibit their capacity to activate T cells in the TME (Bauer et al., 2014; Joshi et al., 2015).

Tumor-associated macrophages (TAMs) are a population of M2-like macrophages with an impaired capacity to secrete IL-12 and an enhanced ability to produce IL-10 (Ugel et al., 2012; Serafini, 2013; Ugel et al., 2015; De Sanctis et al., 2016). Another manifestation of tumor-promoted immune dysfunctions is the impairment in antigen presentation to T cells, which is essential for both endogenous and vaccine-induced activation of tumor-specific T cells (Gabrilovich, 2004; Herber et al., 1998; Yang et al., 2010). The metabolic adaptation of tumor cells generates a tumor microenvironment (TME) with pH and redox features that additionally contribute to limit T cell function and viability (Bellone and Calcinoatto, 2013). These diverse mechanisms that we summarize in this review have limited the success of cancer immunotherapy and highlight the necessity for drugs that are able to modulate the immune dysfunctions associated with the TME.

It was previously described that the Hv1 channel supports NADPH oxidase (NOX)-mediated generation of reactive oxygen species (ROS) in leukocytes (Ramsey et al., 2009; El Chemaly et al., 2010, 2014). This process of ROS production has a critical function for the phagocytic killing of pathogens (Ramsey et al., 2009), for the regulation of antigen processing and presentation (Savina et al., 2006; Rybicka et al., 2012), and is associated with the control of T cell activation (Jackson et al., 2004; Sasaki et al., 2013). Remarkably, tumors hijack many physiologic mechanisms of host defense, including ROS generation, to avoid immune-mediated destruction. Thus, although there is no experimental evidence to date regarding the role of Hv1 proton channel in tumor-infiltrating immune cells, we hypothesize in this review that possible links exist between inflammation, NOX, and Hv1 in the context of the TME and that Hv1 potentially contributes to tumor-associated macrophage, neutrophil, dendritic cell, and T cell function. Additionally, we discuss evidence indicating the functional role of Hv1 in tumor biology, as well as possible strategies for the pharmacological inhibition of this channel. Finally, we include critical comments regarding the future perspectives of Hv1 inhibition for cancer therapy.
The importance of the IL-12/IL-10 balance in the activation of T cells has been widely recognized, because IL-12 stimulates antitumoral Th1 and cytotoxic T lymphocyte (CTL) responses, whereas IL-10 promotes tumor-supporting Th2 and Treg differentiation (Murai et al., 2009; Biswas and Mantovani, 2010; Ostrand-Rosenberg et al., 2012; Ruffell et al., 2014). Similarly to myeloid-derived suppressor cells, TAMS also reduce \( \text{L-arginine} \) availability for T cell proliferation because of their expression of arginase 1 (Rodriguez et al., 2004; Gabrilovich et al., 2012). Other features of TAMS are their impaired function as antigen-presenting cells and their ability to release CCL22, which attracts Tregs toward the TME (Curiel et al., 2004), along with an increased secretion of immunosuppressive TGF-\( \beta \) and prostaglandin E2 (Torroella-Kouri et al., 2009). Moreover, both TAMS and myeloid-derived suppressor cells facilitate tumor progression by contributing to tumor cell stemness (Cui et al., 2013; Schwaitalla et al., 2013; Di Mitri et al., 2014; Lu et al., 2014; Wan et al., 2014), angiogenesis, and vasculogenesis (Murdoch et al., 2004; Shojaei et al., 2007a,b; Schmidt and Carmeliet, 2010), as well as metastatic spreading (Hiratsuka et al., 2008; Toh et al., 2011; Bonde et al., 2012; Kitamura et al., 2015).

An emerging role in tumor progression was recently demonstrated for tumor-associated neutrophils (TANs). Similarly to TAMS and in a process regulated by TGF-\( \beta \)-\( \beta \), the TME skews TANs polarization from antitumoral N1 to protumoral N2 phenotype (Fridlender et al., 2009). The TGF-\( \beta \)-induced N2 phenotype of TANs is characterized by arginase 1 expression and low production of TNF (tumor necrosis factor) and intercellular adhesion molecule 1 (Fridlender et al., 2009). On the contrary, the production of several chemokines by TANs is increased in comparison with naive neutrophils, suggesting a function of TANs in the recruitment of other immune cells to the TME (Fridlender and Albelda, 2012). Interestingly, N2 depletion in tumor-bearing mice caused an increase of intratumoral activated CD8\(^+\) T cells (CD137\(^+\)CD25\(^-\)) and consequently a reduction in tumor growth, indicating the immunosuppressive function of TANs (Fridlender et al., 2009). Furthermore, TANs promote tumor initiation and growth (Houghton et al., 2010), angiogenesis (Nozawa et al., 2006), and metastasis formation (Kowanetz et al., 2010).

The elucidation of the role of T cell inhibitory molecules PD-1 (programmed cell death protein 1) and CTLA-4 (cytotoxic T lymphocyte-associated protein 4) in restraining antitumoral responses gave birth to one of the most successful immunotherapeutic interventions for cancer to date: the immune checkpoint therapy. CTLA-4 and PD-1 are both expressed upon T cell activation (Sharma and Allison, 2015), but they seem to have different physiologic implications. The role of CTLA-4 has been associated with the blockade of costimulation needed for T cell activation because it recognizes the B7 molecules on the surface of antigen-presenting cells with higher affinity than CD28 (Walunas et al., 1994; Krummel and Allison, 1995). CTLA-4 is also constitutively expressed in Tregs, where it is relevant for suppressive function and therefore for the inhibition of tumor-specific T cell responses (Wing et al., 2008; Ise et al., 2010). PD-1 inhibits downstream T cell receptor (TCR) signaling pathways and recognizes ligands (PD-L1 and PD-L2) broadly expressed in many cell types (Sharma and Allison, 2015), suggesting that the function of PD-1 is to control T cell-mediated target cell destruction. Tumors take advantage of these physiologic mechanisms of T cell contraction; for example, PD-L1 is expressed in different types of tumors whereas host dendritic cells, myeloid-derived suppressor cells, and macrophages can express both PD-L1 and PD-L2 (Munn and Bronte, 2016). The regulatory role of these immunotherapeutic approaches (Calcinotto et al., 2012) in which the authors showed an impairment of CTL proliferation, cytokine production, and lytic activity just by maintaining in vitro the CTLs at the pH levels observed within the TME. More interestingly, the CTLs recovered their functionality when the milieu was adjusted to normal tissue pH by membrane-bounded carbonic anhydrase IX, reinforcing the low pH values of the TME (Supuran, 2008; Chiche et al., 2009). The acidic nature of the TME has a severe negative effect in the functionality and viability of effector T cells; lymphocytes die at the low pH values wherein tumor cells are able to live and proliferate (Lugini et al., 2006). IL-2-induced T cell proliferation is also inhibited in the pH range of the TME (Ratner, 1990). In an early publication, Redegeld et al. (1991) observed that the capacity of CTLs to kill tumor cells was noticeably suppressed in acidic pH conditions. This was further corroborated in a recent report from Calcinozzo et al. (2012) in which the authors showed an impairment of CTL proliferation, cytokine production, and lytic activity just by maintaining in vitro the CTLs at the pH levels observed within the TME. More interestingly, the CTLs recovered their functionality when the milieu was adjusted to normal tissue extracellular pH, and treatment of tumor-bearing mice with a drug able to counteract the pH drop in the TME enhanced the antitumoral efficacy of different immunotherapeutic approaches (Calcinozzo et al., 2012).

This plethora of mechanisms inducing immune dysfunction can explain the relatively low success of cancer immunotherapies to date and point out the need to find more multifactorial...
and creative approaches to tackle this subject. The idea that cancer therapy (vaccines, monoclonal antibodies, adoptive T cell transfer, or low molecular weight inhibitors) could be based on the mutational neoantigens expressed by each patient’s tumor at different time points (Schumacher and Schreiber, 2015) is pushing the field toward more personalized treatment. Undoubtedly, cancer vaccines face the biggest challenge, because this treatment would have to overcome the previously mentioned impairment on antigen presentation and T cell activation. In this sense, it is important to find novel adjuvants and immunomodulators with the ability to reduce tumor-induced immunosuppression in addition to having the features of those routinely used for preventive vaccination (Fernandez et al., 2014). Another strategy to circumvent the problems of antigen presentation by adaptively transferring already activated tumor-specific T cells has led to two main approaches: the transference of tumor-infiltrating lymphocytes and the generation of T cells with chimeric antigen receptors (Rosenberg and Restifo, 2015). Immune checkpoint therapy has proven effective in unleashing endogenous tumor-specific T cell responses otherwise suppressed by the tumor, and this therapy is a reality today for patients with certain tumors, because ipilimumab and the two anti-PD-1 antibodies, nivolumab and pembrolizumab, were approved by the Food and Drug Administration in 2011 and 2014, respectively (Sharma and Allison, 2015). However, these immunotherapeutic approaches cannot ignore the immunosuppressive TME, highlighting the necessity for a combination of those strategies with a strategy that targets the most relevant mechanisms of tumor escape from effector immune cells. There is work in progress aimed to normalize tumor vasculature; to inhibit myeloid-derived suppressor cells, TAMs and Tregs; and to recover dendritic cell-mediated antigen presentation and T cell function (Whiteside, 2010; Gabrilovich et al., 2012; Joyce and Fearon, 2015; De Sanctis et al., 2016). Nonetheless, there is still an unexplored opportunity for finding novel drugs that target ion channels playing a role in the acidification of TME, a strategy that may reduce the negative impact of low pH in effector lymphocytes. Additionally, the modulation of these ion channels could regulate the function of immune cells essential for the antitumor response, such as macrophages, neutrophils, dendritic cells, and T cells. In the following sections we will discuss these elements in more detail.

Proton Channel Hv1 in Tumor Biology

A significant role of the Hv1 proton channel in tumor biology is beginning to emerge. The maintenance of intracellular pH within physiologic values is essential for most biologic mechanisms occurring in any cell, and tumor cells are not an exception. In fact, crucial processes such as proliferation, motility, metastasis, and apoptosis are regulated by intracellular pH in both normal and tumor cells (Chambard and Pouyssegur, 1986; Perona and Serrano, 1988; Schlappack et al., 1991; Gottlieb et al., 1995). Thus, counteracting the drop in intracellular pH, caused by the tumor’s high glycolytic rate that converts glucose to acidic metabolites in the hypoxic conditions of the TME, is a matter of survival for tumor cells (Gatenby and Gillies, 2004). The associated acidification of the extracellular milieu contributes to the suppression of antitumor T cell responses (Lugini et al., 2006; Calcinotto et al., 2012), an additional advantage of this process that facilitates tumor evasion from the immune system.

Evidence indicates that tumors use the Hv1 proton channel, one of the most efficient mechanisms existing in the body for proton extrusion and pH regulation, to support their survival and development (Fig. 2A). Most of the studies performed so far have been done in breast cancer models. Wang et al. (2011) first demonstrated that the Hv1 proton channel is highly expressed in both metastatic human breast tumor tissue and metastatic breast cancer cell lines such as MDA-MB-231, but not in nonmetastatic tissue and the poorly metastatic cell line MCF-7. The inhibition of Hv1 expression with small interfering RNA induced a significant intracellular pH decrease in MDA-MB-231 cells and deterred extracellular milieu acidification (Wang et al., 2011, 2012b), indicating a dominant role of Hv1 channel in proton extrusion in these tumor cells. The knockdown of Hv1 channel also diminished the proliferation of MDA-MB-231 cells in vitro (Wang et al., 2012b). In the supernatant of MDA-MB-231 cells treated with small interfering RNAs targeting Hv1, reduced secretion and activation of extracellular matrix-degenerating proteases, such as metalloproteinases (MMP2 and MMP9), was detected (Wang et al., 2011, 2012b). The former effect was associated with intracellular pH reduction, because MMP2 and MMP9 activity is pH regulated (Wang et al., 2011). Therefore, the inhibition of proton channel Hv1 produced an impairment in the migration and invasion capabilities of the MDA-MB-231 metastatic cell line in vitro (Wang et al., 2011), and this, together with the sustained proliferation, is an important property for tumor progression and metastasis (Hanahan and Weinberg, 2011). In fact, the implantation of Hv1 knockdown MDA-MB-231 cells in nude mice produced significantly smaller tumors than the inoculation of MDA-MB-231 cells expressing Hv1 (Wang et al., 2012b), corroborating the relevance of Hv1 proton channel for the biology of this type of tumors in vivo.

Similar results were obtained in glioma and colorectal cancer. Hv1 was expressed in the highly metastatic SHG-44 glioma cell line, but was nearly undetectable in poorly metastatic U-251 cells (Wang et al., 2013b). The inhibition of Hv1 activity with ZnCl2 reduced migration and induced apoptosis of SHG-44 glioma cells in vitro. Moreover, the administration of ZnCl2 in vivo after implantation of SHG-44 cells significantly delayed tumor growth in nude mice (Wang et al., 2013b). Similarly, Hv1 expression was observed in the SW620 human cell line, a highly metastatic colorectal cancer cell line wherein this channel regulated intracellular pH and played an important role in the cell’s migration and invasion capabilities (Wang et al., 2013a).

In patients with breast and colorectal cancer, the expression of Hv1 was correlated with tumor size, tumor classification, clinical stage, Her-2 status (breast cancer), and p53 status (colorectal cancer) (Wang et al., 2012b, 2013a). Furthermore, higher expression of Hv1 was associated with poor prognosis in both types of human cancers (Wang et al., 2012b, 2013a). These findings suggest the use of Hv1 as a novel biomarker for diagnosis and prognosis of breast and colon cancer, but this will require an important effort of validation before acceptance in clinical practice. Another key question to address in the future is whether Hv1 could be a biomarker for other types of tumors and its relevance in the biology of these tumors. What seems to be rather clear is the potentiality of Hv1 as a target
for cancer therapy, because it has an important function in controlling proliferation, apoptosis, invasiveness, and migration of cancer cells, along with the TME acidification that contributes to tumor-induced immunosuppression (Fig. 2A).

**Role of Proton Channel Hv1 in Immune Cells Associated with Tumor Development**

Although the role of Hv1 proton channel in tumor immunology has not been addressed thus far, there is evidence of the expression and function of Hv1 in immune cells that either supports tumor development or participates in antitumoral responses. Among these cells we will analyze T cells, neutrophils, macrophages, and dendritic cells.

Most of the studies done with immune cells have focused on the role of Hv1 as a functional partner and regulator of NOX enzymes. During the NOX enzyme-catalyzed reaction, two electrons from cytoplasmic NADPH translocate through the plasma membrane or phagosome membrane to generate superoxide anion ($\text{O}_2^{-}$) from molecular oxygen and two protons are released in the cytoplasm (Henderson et al., 1987). This could potentially lead to a fatal drop of intracellular pH in phagocytic cells with a high activity of NOX2 (Demaurex et al., 1993). Additionally, the activity of NOX enzymes is inhibited by membrane depolarization toward positive voltages and cytoplasmic acidification arising from its own functioning (DeCoursey et al., 2003; Morgan et al., 2009). Therefore, these cells require mechanisms of proton extrusion and pH regulation to avoid the potentially negative effects of both cytoplasmic acidification and membrane depolarization. In this fashion, an important link between NOX and Hv1 proton channel has been made in phagocytic leukocytes and other cell types (Murphy and DeCoursey, 2006; Morgan et al., 2009).

**Effectors T Cells.** Nowadays, there is no question of the key role of T cells in the control of cancer development. The strength of this concept relies on the undeniable role of T cells in tumor immune surveillance (Vesely et al., 2011) and the diverse mechanisms of immune dysfunction that are focused in dampening T cell activation and function (Gabrilovich et al., 2012; Bellone and Calcinotto, 2013; Joyce and Fearon, 2015). The recent success in cancer patients of immunotherapeutic approaches directed to the pathways involved in T cell contraction (Sharma and Allison, 2015) or the adoptive transfer of activated tumor-specific T cells (Rosenberg and Restifo, 2015) has deeply contributed to demonstrate the relevance of T cell responses for cancer therapy.

Unfortunately, there are few studies addressing the expression and function of Hv1 proton channel in T lymphocytes. An
significant increase of activated CD44-high T cells (CD4 with the control of T cell function. Supporting this idea, a second event relying on NOX and Hv1 might be associated generation seems to be required for T cell activation, the higher wave of ROS in T cells. Whereas the first event of ROS taining NOX activity long enough to generate the second and second phase of sustained ROS production required NOX and coincidently in their experimental conditions only the T cell stimulation to those described by Sasaki et al. (2013), et al. (2004) found similar phases of ROS production upon activation in human and mouse T cells is triggered by TCR of a phagocyte-type NOX (Jackson et al., 2004). Indeed, NOX activation in human and mouse T cells is triggered by TCR stimulation with anti-CD3 antibody and also requires a second signal coming from Fas-Fasl interaction. Jackson et al. (2004) found similar phases of ROS production upon T cell stimulation to those described by Sasaki et al. (2013), and coincidentally in their experimental conditions only the second phase of sustained ROS production required NOX activation (Jackson et al., 2004). Although there is a contradiction between both groups regarding the NOX dependence of the first wave of ROS, probably caused by the different stimuli used to activate T cells, it is clear that a sustained ROS production in T cells requires Hv1 (Sasaki et al., 2013) and NOX (Jackson et al., 2004; Sasaki et al., 2013). This evidence points toward a relevant function of Hv1 channel for maintaining NOX activity long enough to generate the second and higher wave of ROS in T cells. Whereas the first event of ROS generation seems to be required for T cell activation, the second event relying on NOX and Hv1 might be associated with the control of T cell function. Supporting this idea, a significant increase of activated CD44-high T cells (CD4 and CD8) was detected in aged Hv1-deficient mice and also in young Hv1-knockout mice infected with lymphocytic choriomeningitis virus (Sasaki et al., 2013). Likewise, the inhibition of ROS production in T cells from NOX-deficient mice was connected with an enhanced and prolonged TCR-mediated activation of mitogen-activated protein kinase kinase-Erk (extracellular receptor-activated kinase) signaling pathway (Jackson et al., 2004).

Additionally, T cells from NOX-knockout mice were skewed to the production of Th1 cytokines (Jackson et al., 2004), a process that, according to a previous publication, might be associated with enhanced Erk activation. In more detail, Jorritsma et al. (2003) have shown that sustained Erk activation triggered by the TCR inhibited IL-4 production in naive T cells, thus promoting Th1 differentiation. Snelgrove et al. (2006) demonstrated that lung’s T cells from mice lacking NOX2 had a Th1-biased cytokine response upon infection with Cryptococcus neoformans. The Th1-prone phenotype of NOX2-deficient mice was also influenced by dendritic cell-produced cytokines. In this regard, dendritic cells lacking NOX2 that were matured in vitro with a mixture of IFN-γ and bacterial lipopolysaccharide (LPS) showed an increased secretion of the Th1-polarizing cytokine IL-12p70 in comparison with wild-type dendritic cell, translating to a higher ability to induce the differentiation of ovalbumin-specific CD4+ T cells toward a Th1 phenotype (Jendrysik et al., 2011). Thus, Hv1 function could aid in sustaining the activity of NOX to generate enough ROS to constrain a potentially harmful exacerbated Th1 response. To our knowledge there is only one report on the influence of Hv1 proton channel in cytokine production; however, this paper showed no differences in the ability of Hv1-deficient T cells to differentiate into Th1 or Th17 phenotypes compared with wild-type T cells (Sasaki et al., 2013). These results were obtained in vitro using wild-type dendritic cells and cytokine cocktails, and therefore before a conclusion can be drawn, other experiments should be done using T cells differentiated in vivo in Hv1-deficient mice during infection or in tumor-bearing mice. Nonetheless, this could also be a reflex of the difference between Hv1 and NOX deficiency, and the remaining generation of ROS in the absence of Hv1 might be enough to keep a normal balance of cytokine production and Th polarization. Considering the relevant role of cytokines and chemokines in shaping the immune response, further research is needed to understand the role of Hv1 in the production of these proteins in T cells and in antigen-presenting cells.

Phagocytes. Neutrophils and macrophages are the most proficient phagocytic cells from the innate immunity. After phagocytosis of invading pathogens, these cells produce high amounts of ROS in a microbial mechanism named respiratory burst (Abbas et al., 2014). Thus, these phagocytes represent a first line of host defense during infection with pathogens. In cancer, macrophages and neutrophils have been linked to the inhibition of antitumoral T cell responses and to the promotion of tumor proliferation, angiogenesis, and metastatic spreading (Gabrilovich et al., 2012; Medina-Echeverzer et al., 2014). Although the expression of Hv1 channel has not yet been described in TAMs or TANs, there is evidence demonstrating the important role of this protein for the physiologic functions of macrophages and neutrophils in the host defense against invading pathogens.

Neutrophils. The best characterization of Hv1 role in supporting the function of NOX and the subsequent production of ROS has been obtained in granulocytes, a cell population that displays high expression of the Hv1 proton channel (Petheö et al., 2010). Early publications detected proton currents in human neutrophils with the selectivity, pH, and voltage dependence characteristics nowadays assigned to Hv1 proton channel (DeCoursey and Cherny, 1993; Demaurex et al., 1993). Afterward, Okochi et al. (2009) found Hv1 protein in mouse neutrophils, particularly within phagosomes, together with NOX2 components such as gp91, p22, p47, and p67. Consistently, Hv1 was detected in neutrophils from human peripheral blood, both at mRNA and protein levels (Petheö et al., 2010). These authors showed that the Hv1 channel was localized not only in the plasma membrane, but.
also in the membrane of intracellular granules where a partial colocalization with NOX2 was observed (Pethö et al., 2010).

Evidence has established that after pathogen phagocytosis in neutrophils, Hv1 channel aids in the maintenance of NOX2 long-term activity that allows for ROS-mediated pathogen killing. As mentioned previously, NOX activity produces proton accumulation in the cytosol and membrane depolarization, two factors that Hv1 channel-mediated proton extrusion should counteract to avoid NOX inhibition. Supporting this potential role of Hv1 channel, neutrophils isolated from Hv1-deficient mice had reduced cytoplasmic pH and higher membrane depolarization than wild-type neutrophils upon activation of NOX2 with PMA (El Chemaly et al., 2010). Similarly, Morgan et al. (2009) observed enhanced acidification in the cytoplasm of human neutrophils after phagocytosis of opsonized zymosan particles when Hv1 was inhibited with Zn\(^{2+}\). In this study, the authors detected likewise a twofold increase in the rate and the extent of cytoplasm acidification in bone marrow phagocytes from Hv1-knockout mice compared with wild-type littermates (Morgan et al., 2009). Moreover, several publications have shown a substantial reduction of ROS production in neutrophils from Hv1-deficient mice compared with wild-type neutrophils (Okochi et al., 2009; Ramsey et al., 2009; El Chemaly et al., 2010). Of note, in Hv1-deficient neutrophils the NOX2 complex expression levels are unmodified (Okochi et al., 2009) and the electron current generated due to NOX2 activity is maintained (Morgan et al., 2009; El Chemaly et al., 2010), indicating that the inhibition of ROS production observed in Hv1-deficient neutrophils is associated with the eventual inhibition of NOX2 in the absence of the compensatory effect of Hv1.

As expected from their reduced NOX2-mediated ROS production, bone marrow neutrophils from Hv1-knockout mice exhibited an impaired ability to kill serum-opsonized Staphylococcus aureus in vitro (Ramsey et al., 2009). Interestingly, the phagocytosis of heat-inactivated, serum-opsonized Staphylococcus aureus was not affected in the absence of Hv1 in these cells, suggesting that the observed inefficient bacterial killing is associated with inhibition of ROS production and not with a defect in bacterial uptake (Ramsey et al., 2009). However, the authors failed to see a deficiency in bacterial clearance after in vivo inoculation of Staphylococcus aureus, Pseudomonas aeruginosa, and Burkholderia cepacia in Hv1-deficient mice. The unchanged ability to eliminate bacteria in vivo suggests that ROS production is not completely inhibited in Hv1-deficient mice and the remaining small activity of NOX2 is sufficient to complete this task (Seredenina et al., 2015). In line with this idea, it has been demonstrated that normal granulocytes potentially produce much higher amount of NOX2-mediated ROS than what is actually required for bacterial clearance (Becker et al., 1998; Dinauer et al., 1999; Barese et al., 2004).

El Chemaly et al. (2010) showed that, in the absence of Hv1 proton channel, other important functional features of neutrophils are affected. The increased membrane depolarization observed in Hv1-deficient neutrophils when NOX2 is activated reduced Ca\(^{2+}\) influx and caused impaired actin depolymerization (El Chemaly et al., 2010). Consequently, neutrophils lacking Hv1 channel displayed a diminished ability to migrate in vitro in response to the chemoattractant N-formyl-Met-Ile-Val-Ile-Leu bacterial peptide (El Chemaly et al., 2010). Afterward, Zhu et al. (2013) corroborated these findings in an in vivo model of peritonitis induced by intraperitoneal injection of thioglycollate. In this experimental setting, the number of neutrophils infiltrating the peritoneal cavity was reduced twofold in Hv1-deficient mice compared with wild-type littermates, indicating that Hv1 channel regulates neutrophils migration toward the sites of inflammation in vivo (Zhu et al., 2013).

**Monocytes/Macrophages.** Proton currents with features corresponding to Hv1 channel have been detected in the human THP-1 monocytic cell line (DeCoursey and Cherny, 1996), in mouse macrophages from the peritoneal cavity (Kapus et al., 1993; Okochi et al., 2009), and in human peripheral blood monocytes (Musset et al., 2012a). The Hv1 protein was also found in mouse peritoneal macrophages by Okochi et al. (2009) via a Western blot assay.

The function of Hv1 in macrophages is associated with ROS production during respiratory burst, although the evidence for this in macrophages is scarcer than in neutrophils. Perhaps the first direct demonstration of Hv1 involvement in NOX-mediated ROS production was obtained in human peripheral blood monocytes where H\(_2\)O\(_2\) production was significantly inhibited with Zn\(^{2+}\) (Musset et al., 2012a), a divalent cation commonly employed to block Hv1 function (DeCoursey and Cherny, 2007). In this publication, Musset et al. (2012a) additionally showed the glucose dependence of NOX activation and ROS production in human macrophages, a different behavior to that exhibited by human granulocytes. In a more recent paper, Hv1 protein was detected via Western blot and immunostaining in the phagosomes of mouse bone marrow-derived macrophages, an organelle that also contains the components of NOX2 complex (El Chemaly et al., 2014). The authors shed more light on the functional role of Hv1 during the respiratory burst by demonstrating that Hv1 channel sustained phagosomal ROS production through the delivery of protons within the lumen of the phagosome, although the reduction in phagosomal ROS generation was slightly smaller in macrophages than in neutrophils from Hv1-deficient mice (El Chemaly et al., 2014). This incomplete inhibition of ROS generation could be related to the fact that intraphagosomal acidification is maintained in macrophages by proton channel Hv1 and vacuolar ATPase. Conversely, in neutrophils, Hv1 aids in the maintenance of a phagosome neutral pH because of the elevated production of ROS sustained by this channel, which then inhibits the accumulation of vacuum ATPase (El Chemaly et al., 2014).

Another study linked Zn\(^{2+}\) deprivation and Hv1 channel activation in macrophage phagosomes with granulocyte-macrophage colony-stimulating factor (GM-CSF) activity during antimicrobial defense against the intracellular fungus Histoplasma capsulatum, which causes pulmonary and disseminated histoplasmosis (Subramanian Vignesh et al., 2013). GM-CSF induced an upregulation of metallothioneins and Zn\(^{2+}\) exporters that redirect these divalent cations away from the phagosomes and toward the Golgi apparatus. In line with these results, GM-CSF also promoted an increased expression of Hv1 proton channel in macrophages through a STAT3- and STAT5-dependent fashion. In addition, ROS production was enhanced in wild-type infected macrophages treated with GM-CSF but was significantly attenuated in macrophages obtained from Hv1-knockout mice and NOX-deficient mice, indicating the involvement of NOX-Hv1 in this process. Thus, this strategy of Zn\(^{2+}\) deprivation triggered by GM-CSF enhanced the expression and activity of Hv1 proton channel and the NOX-mediated generation of ROS.
in phagosomes from infected macrophages (Subramanian Vignesh et al., 2013).

Dendritic Cells. Dendritic cells, able to prime naive T lymphocytes and regulate their differentiation patterns, are the most efficient antigen-presenting cells in the body (Abbas et al., 2014), and it is clear that dendritic cells are essential players in antitumor responses because of their ability to capture, process, and present tumor antigens to activate tumor-specific T cells.

A few studies have addressed the expression and involvement of Hv1 proton channel in dendritic cells, although again none of the evidence is linked to cancer pathology. Szteyn et al. (2012) detected mRNA encoding for Hv1 in mouse bone marrow-derived dendritic cells and demonstrated that this channel is functionally active through whole cell patch-clamp experiments. Interestingly, although the function of Hv1 in dendritic cells is once more associated with NOX2 activity, because of the specialization of these cells, the NOX2/Hv1 pair has been shown to participate in the regulation of antigen presentation rather than respiratory burst.

To prevent the loss of peptide fragments that could be displayed in major histocompatibility complex molecules to be recognized by T cells, the extent of antigen degradation after phagocytosis is tightly controlled in dendritic cells, whereas neutrophils and macrophages extensively destroy the phagocytosed particles (Savina and Amigorena, 2007). Amigorena et al. established that the NOX2 enzyme is a critical regulator of phagosomal degradation of extracellular antigens in dendritic cells (Savina et al., 2006). The authors demonstrated that bone marrow-derived dendritic cells lacking NOX2 showed an increased phagosomal degradation of ovalbumin and consequently performed an impaired cross-presentation and activation of OT-I transgenic C57 T cells specific for SIINFEKL ovalbumin-peptide (Savina et al., 2006). Of note, NOX2 is associated with the phagosomes for a longer period in dendritic cells than in neutrophils and macrophages, and this enzyme remains active beyond 1 hour after antigen phagocytosis in dendritic cells (Savina et al., 2006; Mantegazza et al., 2008; Rybicka et al., 2012). Two different mechanisms have been proposed to explain this function of NOX2. The first mechanism proposes that NOX2 is recruited and activated in early phagosomes of dendritic cells and causes the alkalization of the phagosomal lumen through the production of low, but sustained, levels of ROS with the associated proton consumption (Savina et al., 2006). This alkaline pH inhibits the function of proteolytic enzymes in the phagosomes, thus protecting certain antigen integrity for subsequent entry in the antigen presentation machinery. In contrast, Rybicka et al. (2012) observed that the phagosomes of bone marrow-derived dendritic cells do acidify and demonstrated that, at least in their experimental conditions, NOX2 regulates phagosomal proteolysis through a ROS-mediated diminishing of the reductive capacity of phagosomes required for optimal cysteine cathepsin function. Likewise, these authors observed that NOX2 activity is sustained through the charge compensation provided by the translocation of protons into the phagosomal lumen. Thus, bone marrow dendritic cells from Hv1-deficient mice showed reduced ROS production in their phagosomes, but the production of ROS was only completely blunted when both Hv1 and vacuolar ATPase were simultaneously inhibited (Rybicka et al., 2012), suggesting that Hv1 proton channel and vacuolar ATPase cooperate in dendritic cells phagosomes to provide charge compensation and maintain NOX2 activity. Furthermore, the inhibition of vacuolar ATPase with Concanaamycin B induced a marked reduction of ovalbumin degradation within the phagosomes of dendritic cells (Savina et al., 2006). This effect was explained by an increased alkalization of the phagosome’s lumen when NOX2 was active in the absence of the compensatory function of vacuolar ATPase, causing lysosomal proteases inhibition (Savina et al., 2006). It would be very interesting to study the role of Hv1 in antigen degradation and subsequent presentation to T cells, and whether Hv1 deficiency has a similar consequence in this process than vacuolar ATPase inhibition.

In another interesting study, Szteyn et al. (2012) evaluated the effect of bacterial LPS on Hv1 and NOX2 activity in mouse bone marrow-derived dendritic cells. LPS is the prototypic agonist for TLR4 (TLR-toll-like receptor) and induces dendritic cell maturation and secretion of Th1-polarizing cytokines (Iwasaki and Medzhitov, 2010; Kawai and Akira, 2010). The maturation process in dendritic cells is characterized by a reduction in antigen uptake and processing, along with an upregulation of major histocompatibility complex, costimulatory molecules, cytokines, and mechanisms allowing for the migration toward secondary lymphoid organs (Banchereau and Steinman, 1998). Upon acute stimulation with LPS, dendritic cells initially showed an increase in both Hv1 and NOX2 activity (Szteyn et al., 2012). This promotion of Hv1 and NOX2 activity was mediated by protein kinase C (PKC), known to be involved in the signaling of TLRs (Loegering and Lennartz, 2011). In fact, PKC phosphorylates Hv1 in the T29 triggering an enhanced gating mode (Morgan et al., 2007; Musset et al., 2010; Hondares et al., 2014). During this enhanced gating mode, the channel displays increased maximum proton conductance, suffers a 40 mV hyperpolarizing shift of the entire proton conductance-voltage relationship, and opens faster and closes more slowly, increasing the likelihood of channel opening (Morgan et al., 2007; Musset et al., 2010; Hondares et al., 2014). Furthermore, the NOX2 subunit p47phox is phosphorylated by PKC, which promotes its translocation to the membrane and the subsequent assembly of other subunits such as p40phox and p67phox to form the active protein complex (Groemping and Rittinger, 2005). However, after 24 hours from LPS stimulation, a reduction in Hv1 mRNA and proton extruding activity was observed in these dendritic cells, along with a diminished production of ROS (Szteyn et al., 2012). This biphasic effect of LPS is in accordance with the previously discussed role of Hv1/NOX2 in antigen presentation; initially the increase in Hv1 could sustain NOX2 activity and ROS production needed to avoid extensive degradation of the antigens because it temporarily corresponds to the antigen processing and presentation phase. Over time, the inhibition of Hv1 might impair the NOX2-mediated accumulation of ROS, potentially leading to the destruction of T cell epitopes and reflecting changes associated with dendritic cell maturation. Although it would be interesting to understand the role of Hv1 in the expression of costimulatory molecules and in the secretion of cytokines, both elements required in conjunction with antigen processing to activate T cells, these aspects were not investigated.

Potential Regulation and Function of Hv1 Proton Channel in the TME: Lessons from the Mechanisms of Host Defense against Pathogens. NOX and Hv1 expression and activation are coordinately regulated during
respiratory burst, and Musset et al. (2009) summarized different evidence and suggested that at least two mechanisms are involved in coordinating NOX and Hv1 channel activity (Musset et al., 2009). First, these proteins share the same agonists and, second, in phagocytes the activation of even a reduced number of NOX molecules leads to a fast and deep membrane depolarization that conduces to the coordinated opening of Hv1 proton channel (Musset et al., 2009). Additionally, PKC phosphorylates both Hv1 and NOX, regulating Hv1 channel gating (Musset et al., 2010) and NOX complex assembly (Vignais, 2002; Groemping and Rittinger, 2005) in leukocytes. Interestingly, PKC participates in the signaling pathways triggered by TLRs (Loegering and Lennartz, 2011), thus providing a link between the sensing of pathogens or self-damage and the activation of Hv1/NOX necessary for pathogen destruction and antigen presentation.

Of note, some transcription factors have been described that regulate the expression of both NOX and Hv1 channel, and the nuclear factor kappa B (NF-κB) is worth mentioning. The silencing of B-cell lymphoma 3-encoded protein, a coactivator of NF-κB, inhibited Hv1 channel expression in LPS-stimulated mouse macrophages (Wang et al., 2012a), suggesting a role of the NF-κB/B-cell lymphoma 3-encoded protein complex in Hv1 transcriptional regulation. The expression of NOX subunit gp91phox is also controlled by NF-κB in monocyteic cell lines, demonstrating an upregulation upon LPS stimulation that was inhibited in cells constitutively expressing nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Anrather et al., 2006). Thus, NF-κB-mediated sensing of inflammation and redox stress leads to NOX upregulation and activation that further increases ROS production in innate immune cells, a process that probably also requires the coordinated expression and activation of Hv1 proton channel to sustain long-term functioning of NOX. In line with this idea, it has been suggested that the activator protein 1 transcription factor responding to redox stress is involved in the regulation of both Hv1 (Seredenina et al., 2015) and NOX expression (Cevik et al., 2008).

Because of the important role of NOX in bacterial killing through the respiratory burst, it makes sense that the upregulation of this enzyme is needed during pathogen-induced inflammation. Interestingly, chronic cancer-related inflammation was added by Mantovani et al. to the original definition of Hanahan and Weinberg (2000) as the seventh hallmark of cancer because of its role in promoting tumor cell proliferation and survival, angiogenesis, metastasis, and evasion from the adaptive immune response (Colotta et al., 2009). Redox stress is another well-established feature of the TME, because tumor cells produce ROS due to their metabolic activity, the hyperactivation of oxidative enzymes, the activity of oncogenes, and the increased signaling through cellular receptors (Sztawrowski and Nathan, 1991; Bittinger et al., 1998; Liou and Storz, 2010). Furthermore, immune cells within the TME also release ROS (Gabrilovich et al., 2012) or modulate the redox environment through cysteine depletion (Yan et al., 2009; Srivastava et al., 2010). Hence, a relevant question is whether tumors also use the physiologic mechanism that links inflammation/redox sensing with Hv1/NOX expression and activation to their advantage. This raises the interesting hypothesis that increased and sustained inflammation, along with ROS production occurring during tumor development, could cause a NF-κB-mediated upregulation of Hv1 proton channel in partnership with NOX, not only in tumor cells, but also in tumor-infiltrating immune cells. Indeed, it has already been established that NF-κB activation could be a link between inflammation and tumor progression (Karim et al., 2002). NF-κB becomes activated by inflammatory cytokines (IL-1 and TNF-α), growth factors, oncogenes, TLR signaling, hypoxia, and acidic conditions within the TME (Karim and Greten, 2005; Aggarwal et al., 2009; Vendramini-Costa and Carvalho, 2012). Of note, the Hv1 channel is involved in the maintenance of the acidic nature of the TME (Wang et al., 2011, 2012b), a condition that activates NF-κB and could further reinforce Hv1 expression to create a positive feedback loop. In premalignant cells, NF-κB activation induces the expression of antiapoptotic genes, along with several genes that promote proliferation, angiogenesis, invasion, and metastasis (Karim et al., 2002; Greten et al., 2004; Pikarsky et al., 2004). Tumor-infiltrating immune cells such as macrophages, neutrophils, dendritic cells, and T cells also display NF-κB activation that facilitates IL-6, IL-1, TNF-α, and ROS secretion (Karim and Greten, 2005; Inoue et al., 2007). Correspondingly, NOX isoforms have been detected in several solid tumors (Antony et al., 2013; Meitzler et al., 2014; Höll et al., 2016) and hematopoietic malignancies (Juhasz et al., 2009; Hole et al., 2013), where they modulate protumoral characteristics that resemble the effects of NF-κB (Maraldi et al., 2009; Meitzler et al., 2014; Sanchez-Sanchez et al., 2014; Liu et al., 2015); in fact, it has been shown that NOX isoform induction is at least partially mediated by NF-κB (Wu et al., 2013; Roy et al., 2015). Moreover, NOX activity and ROS production in macrophages is required for Treg induction (Kraaij et al., 2010), a key population in controlling T cell responses during inflammatory conditions such as cancer.

Altogether, the multiple evidence discussed previously suggests that first, Hv1 could be overexpressed on both tumor cells and tumor-infiltrating immune cells, and second, that Hv1 upregulation might be driven by the inflammatory, redox, and pH conditions within the TME. NF-κB and activator protein 1 transcription factors, among others, could be mediating the regulation of Hv1 within the TME with or without NOX. The expression and functional role of Hv1 has been studied in some cancer cells (Wang et al., 2011, 2012b, 2013a,b), but has not been described yet in the immune cells contributing to the TME. Hv1 upregulation in tumor-infiltrating myeloid cells could be advantageous for cancer progression because it can sustain NOX-mediated generation and release of high amounts of ROS, factors that contribute to inflammation (Guzik et al., 2003; Reuter et al., 2010), and to the impairment of tumor immune surveillance (Schmielau and Finn, 2001; Nagaraj et al., 2007) (Fig. 3A). For example, hydrogen peroxide (H2O2) promotes downregulation of CD3 ζ chain (Schmielau and Finn, 2001), whereas peroxynitrite (ONOO−) causes a nitrification of TCR components that abrogates the capacity of T cells to recognize their cognate peptide (Nagaraj et al., 2007), both elements disabling T cell activation and function. Peroxynitrite also induces CCL2 chemokine nitration that prevents effector T cell infiltration into the tumor (Molon et al., 2011). Additionally, high intracellular levels of ROS, mainly H2O2, contribute to the maintenance of tumor-associated myeloid cells in the immature stage and therefore inhibit their differentiation toward mature antigen-presenting cells (Kusmartsev and Gabrilovich, 2003).
Fig. 3. Hypothetical function of proton channel Hv1 in immune cells associated with the tumor microenvironment. (A) The chronic inflammation and redox stress might induce an upregulation of Hv1 and NOX in TAMs, TANs, dendritic cells, and T cells within the TME. In TAMs and TANs, Hv1 channel could sustain NOX activity to generate high levels of ROS, which dampens T cell function by inducing the loss of TCR \( \zeta \) chain, contributes to enhance TANs infiltration, reinforces inflammation, and polarizes TAMs toward M2 phenotype. Hv1 could be involved in the impairment of tumor antigens cross-presentation through an exacerbated control of antigen degradation, a process also mediated by NOX. The elevated intracellular levels of ROS, produced by NOX/Hv1 in T cells upon TCR recognition, might trigger signaling pathways leading to T cell contraction and may shift T cell polarization into Th2. These elements suggest a tumor-promoting function of Hv1 channel in the TME. (B) The pharmacological inhibition of Hv1 channel could significantly reduce ROS production within the TME, thus recovering the impairment of T cell signaling, skewing T cell polarization to Th1, and
In the particular case of macrophages, ROS production has been classically associated with M1 activation and function, whereas TAMs are generally linked to a M2 phenotype (Mantovani et al., 2009). Nonetheless, recently the topic of macrophage differentiation has been subjected to a revision; it is now rather clear that M1-M2 dichotomy is an oversimplification of a broader number of intermediate differentiation stages regulated by the activation stimulus (cytokines, TLR agonists, growth factors, immune complexes) (Murray et al., 2014). This holds true also for TAMs classification, especially considering the complexity of the TME in regard to the factors driving macrophage differentiation. In fact, TAMs can simultaneously express genes corresponding to both the M1 and M2 phenotype, with the prototypic example of arginase 1 and iNOS, indicating that ROS production is also a relevant mechanism of T cell suppression exerted by TAMs (Kusmartsev and Gabrilovich, 2005; Van Ginderachter et al., 2006; Ugel et al., 2015). Furthermore, Zhang et al. (2013) demonstrated that ROS production in macrophages is required for M2 and TAMs differentiation, but not for the polarization toward a M1 phenotype. Thus, it is likely that an increase in Hv1 expression could aid in the maintenance of ROS production in TAMs (Fig. 3A). Supporting this idea, it was shown that GM-CSF induced an upregulation of Hv1 expression and increased its proton permeation activity in macrophages during infection, sustaining an enhanced production of ROS by NOX enzyme (Subramanian Vignesh et al., 2013). Curiously, GM-CSF is produced in high amounts in the TME, which alters myelopoiesis in such a way that favors the accumulation of suppressor cells instead of mature dendritic cells (Tsuchiya et al., 1988; Bronte et al., 1999; Serafini et al., 2004). Therefore, it could be feasible that the high levels of GM-CSF within the TME trigger Hv1-mediated enhanced production of ROS in TAMs. A similar analysis regarding ROS production could be done for TANs (Fig. 3A). Like TAMs, the classification of TANs into N2 phenotype is an oversimplification, because it was demonstrated that TANs can indeed secrete ROS and, in some models, be cytotoxic for tumor cells (Dallegri et al., 1991; Carey et al., 1997; Fridlender et al., 2009), two features classically assigned to N1 neutrophils in resemblance to macrophage differentiation. However, tumor cells are more resistant than normal cells to ROS-mediated apoptosis because of their higher expression of antioxidant mechanisms (glutathione, superoxide dismutase, catalase, and others) (Reuter et al., 2010). Thus, our hypothesis is that an increased ROS production potentiated by enhanced Hv1 activation and function in TANs could be more deleterious for immune cells than for cancer cells (Fig. 3A). Subramanian Vignesh et al. (2013) demonstrated that an increase in the expression and activity of Hv1 channel can lead to high amounts of NOX-produced ROS in macrophages during respiratory burst. Neutrophils are also phagocytic cells with similar ROS-mediated pathogen destruction mechanisms, and therefore, it could be possible that an upregulation of Hv1 might cause a rise of ROS production in TANs, but this is a theory that needs to be tested experimentally. Supporting this idea though, Kasahara et al. (2016) showed that neutrophils lacking GM-CSF receptor signaling had reduced NOX activity, and consequently, their ability to kill Aspergillus fumigatus during respiratory burst was impaired, suggesting that a GM-CSF-mediated mechanism of Hv1/NOX regulation could apply likewise in neutrophils. Hv1 also has an important role in the migration of neutrophils toward the sites of inflammation (El Chemaly et al., 2010; Zhu et al., 2013). This function of Hv1 controlling neutrophil migration might very well apply in the context of the TME, because it is a site of inflammation where many cytokines (TNF-α, IFN-γ) and chemokines (CXC1, CXCL1, CXCL3, CXCL6) are released to attract neutrophils (Fridlender and Albelda, 2012) (Fig. 3A). Furthermore, the recruitment of neutrophils is a key factor triggering the mobilization of other tumor-supporting immune cells such as macrophages (by secreting CCL2 and CCL7) and Tregs (through CCL17) into the TME (Curiel et al., 2004; Fridlender and Albelda, 2012).

Because they are able to capture and cross-present tumor antigens to tumor-specific CTLs, a central role for dendritic cells in antitumor immune surveillance has been recognized (Vesely et al., 2011). For the same reason, several therapeutic cancer vaccines require the involvement of dendritic cells to generate efficient CD8+ T cell immunity (Palucka and Banchereau, 2013). Therefore, the modulation of the antigen processing capacity of dendritic cells is essential to coordinate T cell responses against the tumor in either scenario. Interestingly, Hv1 collaborate with vacuolar ATPase to sustain ROS production in dendritic cell phagosomes (Rybicka et al., 2012). These low levels of ROS that are maintained for a relatively long period contribute to limit the extent of antigen degradation in the phagosomes of dendritic cells, which is critical for antigen cross-presentation (Savina et al., 2006). When these last results linking Hv1/NOX with the control of antigen processing are extrapolated to the particularities of the TME, our hypothesis is that a putative upregulation of Hv1/NOX in the tumor-infiltrating dendritic cell phagosomes could lead to a higher production of ROS in the lumen of the phagosomes that might disturb the delicate control of the optimal level of antigen degradation (Fig. 3A). In this situation, a pH or redox balance change within phagosome could seriously compromise the degradation of the antigen; if too much antigen degradation affects cross-presentation (when NOX activity is inhibited), too little degradation (probably when NOX activity is increased) could have the same result. Other authors previously showed that NOX activity could be a relevant factor driving a tolerogenic phenotype in tumor-infiltrating dendritic cells. Kuang et al. (2008) demonstrated that the TME-educated dendritic cells assume a semimature phenotype, which causes a rapid downregulation of CD3e chain and TCR itself and the subsequent apoptosis of T cells. Interestingly, the negative effect of these tolerogenic dendritic cells on T cell activation and viability was dependent on NOX activity and was not mediated by arginase 1, iNOS, or promoting tumor-antigen cross-presentation by dendritic cells. Hv1 blockage might also reduce the infiltration of immunosuppressive TANs, TAMs, and Tregs, and hinder TAMs differentiation into tumor-promoting M2 phenotype. Therefore, the inhibition of Hv1 channel could tip the balance between antitumoral and tumor-supporting immune populations toward tumor rejection.
indoleamine 2,3-dioxygenase (Kuang et al., 2008). More recently, Martner et al. (2015) provided evidence that the NOX2 inhibitor histamine promoted the maturation of human dendritic cells from monocytes, and this is characterized by an increased expression of human leukocyte antigen-antigen D related and costimulatory molecules, along with an enhanced ability to stimulate T helper cells with Th0 phenotype. Remarkably, EL-4 tumor-bearing mice treated in vivo with histamine showed a higher accumulation of intratumoral dendritic cells that associated with a significant reduction of tumor growth. These consequences of histamine treatment were not observed in NOX-deficient mice, indicating that the effects of histamine were mediated by the NOX2 regulation (Martner et al., 2015).

Even in the case that Hv1/NOX expression and activity remain unchanged in tumor-infiltrating dendritic cells, allowing an efficient cross-presentation of tumor antigens, this does not mean that the process would end in the activation of tumor-specific CTLs. In fact, several additional mechanisms have been described that promote a profound impairment of dendritic cell function in tumor-bearing hosts. For example, the amount of adenosine and hypoxia within the TME induced dendritic cells to promote the differentiation of T helper cells into Th2 instead of the more potent antitumoral Th1 phenotype (Yang et al., 2010). Other authors have shown that dendritic cells in tumor bearing hosts have an intracellular accumulation of lipids that limits the processing of soluble proteins and the subsequent activation of tumor-specific T cell responses (Herber et al., 2010). Additionally, dendritic cells in the TME can actively suppress antitumoral CD8+ T cell responses (Herber et al., 2010). Currently, there is no a therapeutic drug that targets the Hv1 channel (Seredenina et al., 2015). Since the 1980s (Mahaut-Smith, 1989), divalent cations, principally Zn2+, have been the gold standard for proton current inhibition (Seredenina et al., 2015). The inhibition of Zn2+ is inconsistent when Takeshita et al. (2014) structurally characterized the external Zn2+ binding site, which is located in each Hv1 monomer and composed by residues equivalent to H140, H193, E119, and D123 in human Hv1 (Takeshita et al., 2014). Zn2+ binds to a closed conformation of Hv1 and prevents the S4 segment movement in response to membrane depolarization and subsequent proton conduction (Cherny and DeCoursey, 1999; Takeshita et al., 2014). Nevertheless, the therapeutic use of Zn2+ as an Hv1 channel inhibitor is severely hampered because of its involvement in several physiologic processes.

Promiscuous gating modifiers targeting voltage-sensing domains inhibit Hv1 proton currents. One such inhibitor is Hanatoxin, a tarantula venom, which binds to a conserved motif among different voltage-sensing domains (Alabi et al., 2007). Two structurally related non toxin gating modifiers, NH17 and NH29, stabilize Kv7.2 potassium channel in the closed and open states, respectively (and the opposite with TRPV1 channel), and also affect Hv1 currents (Kornilov et al., 2014). The effects of NH29 and NH17 on Hv1 proton channels are similar to those exerted on Kv7.2 and opposite to those observed with TRPV1 channel; external exposure to 50 μM of NH17 significantly inhibited murine Hv1 currents by ∼34%, and external application of 50 μM NH29 increased murine Hv1 proton currents (Kornilov et al., 2014). There are several proton current inhibitors with potentially indirect effects. Compounds such as 4-aminopyridine, amantadine, amiloride, D600, nicardipine, imipramine, DM, chlorpromazine, clozapine, haloperidol, and rimantadine inhibit proton currents (Song and Yeh, 2012; Shin and Song, 2014;
Shin et al., 2015), probably by a local increase of intracellular pH when the neutral drug crosses the membrane (DeCoursey and Cherny, 2007). DEPC (diethylpyrocarbonate) inhibits NOX-dependent proton current in human eosinophils and O$_2^-$ production on human neutrophils probably through modification of NOX2 hemes (Seredenina et al., 2015). Epigallocatechin-3-gallate might interfere with Hv1 channel activity by modifying the lipid bilayer structure (Seredenina et al., 2015). Proton currents are pharmacologically enhanced by unsaturated long-chain fatty acids such as arachidonic acid and others (DeCoursey and Cherny, 1993; Seredenina et al., 2015). Arachidonic acid is known to trigger multiple signaling pathways, including PKC (Morgan et al., 2007). The observed activation of proton currents upon arachidonic acid treatment could be explained by Hv1 channel PKC-mediated phosphorylation along with an increased activity of NOX (Vignais, 2002; Groenming and Rittinger, 2005; Musset et al., 2010).

Guandine derivatives are the only potential specific inhibitors of Hv1 known to date (Hong et al., 2013, 2014). 2-Guanidinobenzimidazole (2GBI) (Fig. 1C) binds the Hv1 channel from the intracellular side of the membrane and acts as potential channel blocker by accessing the core of the voltage-sensing domain when the channel is in the open conformation (Hong et al., 2013). Four Hv1 residues are involved in the binding of 2GBI: D112, F150, S181, and R211 (Hong et al., 2014). Because 2GBI is too polar to permeate the cytoplasmic membrane, its usefulness as a potential drug to inhibit Hv1 is hampered (Pupo and Gonzalez León, 2014), and can be only used as a pharmacological tool (Hong et al., 2013). Fortunately, a simple modification of 2GBI leads to Cl-guanidinobenzimidazole (CIGBI) (Fig. 1C), which can permeate the cellular membrane and access the intracellular side of the channel to block Hv1 with an increased apparent binding affinity (Hong et al., 2014). Despite this, two main concerns remain regarding CIGBI’s potential as a putative lead compound for the development of inhibitory drugs against Hv1: its relatively low potency and the uncertainty of its fine specificity against Hv1 (Pupo and Gonzalez León, 2014).

Large high-throughput screens and rational designs are required for the development of specific and potent therapeutic Hv1 proton channel inhibitors (and activators) with good drug-like properties. The structure of a closed conformation of mHv1cc, a chimeric construction of Hv1 (Takehita et al., 2014), provided the first support for structure-based approaches for the development of Hv1 drugs (Fig. 1A). Nevertheless, this structure should be used with caution, because it is possible that the sequence incorporated from Cig-VSP in the region of S2–S3, locally perturbs the structure, disrupting some native interactions and changing its register (Li et al., 2015). Unfortunately, because there is no structure of the open conformation of Hv1, drug design projects should rely on the diverse set of Hv1 molecular models (Pupo et al., 2014) (Fig. 1B).

**Perspectives of the Pharmacological Inhibition of Proton Channel Hv1 for Cancer Therapy**

The pharmacological inhibition of Hv1 proton channel represents an attractive and novel approach for targeting the immunosuppressive TME. From the perspective of the tumor cells, the blockage of proton extrusion through Hv1 leads to a substantial drop in intracellular pH, causing an impairment in the secretion of MMP2 and MMP9, diminished invasiveness and migratory properties, and in vivo tumor growth reduction (Wang et al., 2011, 2012b, 2013a,b) (Fig. 2B). This evidence has been obtained in human breast, glioma, and colorectal cancer cell lines, suggesting that it could be a general mechanism for tumors of different localizations and histologic type. Although further research is needed to corroborate these findings in several human tumors in vivo, in patients with breast and colorectal cancer, the expression of Hv1 proton channel in the tumor tissue was correlated with tumor size, tumor classification, clinical stage, and a worst prognosis (Wang et al., 2012b, 2013a). The other advantage of Hv1 inhibition is the associated reduction in the acidification of the extracellular milieu, another element of T cell dysfunction in the TME (Redegeld et al., 1991; Lugini et al., 2006; Calcinotto et al., 2012) (Fig. 2B).

Interestingly, it is possible to speculate, based on the previously discussed evidence, that Hv1 proton channel could also be overexpressed in innate and adaptive immune cells recruited to the TME. In this case, the analysis is far more complex and should consider the potential outcome of Hv1 inhibition either in protumoral and antitumoral populations.

Probably the most effective mechanism for tumor cell elimination is mediated by T lymphocytes, where the Hv1 channel regulates higher ROS production that is observed in a delay moment after TCR signaling (Sasaki et al., 2013). Our hypothesis is that Hv1-mediated ROS production is involved in the contraction of T cell responses, and from that perspective an upregulation of Hv1 in tumor-infiltrating lymphocytes might enhance the exhaustion of tumor-specific T cells (Fig. 3A). Therefore, the blockage of Hv1 would probably increase the amount of activated tumor-specific T cells within the TME (Fig. 3B). Supporting this idea, higher frequency of activated T cells was found in Hv1-deficient mice in conditions such as aging and viral infection, situations that are similar to cancer in that they are linked to inflammation (Sasaki et al., 2013).

To properly activate antitumoral T cells, it is necessary for tumor antigens to be cross-presented by dendritic cells, a process that requires certain preservation of the antigen after phagocytosis. Hv1 acts together with vacuolar ATPase to sustain NOX-mediated ROS production in the phagosomes of dendritic cells, which inhibits the activity of proteolytic enzymes, limits the extent of antigen degradation, and enables the cross-priming of CD8$^+$ T cells (Savina et al., 2006; Rybicka et al., 2012). A possible upregulation of Hv1 channel in dendritic cells could also impair the cross-priming of tumor antigens because of insufficient degradation of the antigens (Fig. 3A). In this sense, a particular comment should be made about the potential advantage of inhibiting Hv1 proton channel instead of NOX2 enzyme in dendritic cells due to the regulation of antigen presentation. NOX2 deficiency leads to a profound impairment in ROS production in the phagosomes of dendritic cells, and this abrogates cross-presentation due to exacerbated degradation of the antigen (Savina et al., 2006). Conversely, Hv1-deficient dendritic cells have diminished, but measurable amounts of ROS, because vacuolar ATPase is functional and can fulfill some charge compensation for the activity of NOX2 in phagosomes (Rybicka et al., 2012). Therefore, the pharmacological inhibition of Hv1 could help to restore in certain degree the
normal levels of phagosomal processing of tumor antigens (Fig. 3B), whereas the targeting of NOX2 would more likely cause an impairment in the cross-priming of antitumor CTLs. It cannot be completely disregarded, however, that Hv1 inhibition might affect cross-priming by inducing excessive antigen degradation. If this were the case, the negative effect of a therapy based on blocking Hv1 function could be solved by a combination with peptide vaccines, adoptive transference of dendritic cells already loaded with tumor antigens, or another feasible strategy that circumvents antigen processing.

TAMs and TANs are potent suppressors of T cell function within the TME. In these populations Hv1 channel might aid to sustain the production of high levels of ROS, which impairs T cell function through several mechanisms (Schmielau and Finn, 2001; Nagaraj et al., 2007; Molon et al., 2011) (Fig. 3A). Thus, the inhibition of Hv1 could lead to a diminished ability of TAMs and TANs to suppress antitumoral T cells and might impair the polarization of TAMs toward M2 phenotype, because ROS is required for macrophage differentiation into M2 functional state (Zhang et al., 2013) (Fig. 3B). Additionally, the migration of neutrophils toward the sites of inflammation is regulated by Hv1 channel (El Chemaly et al., 2010; Zhu et al., 2013), suggesting that in tumor-bearing hosts, the inhibition of Hv1 could lead to a reduced intratumoral infiltration of TANs (Fig. 3B). A diminished infiltration of TANs could be accompanied by a decreased recruitment of tumor-supporting Tregs and TAMs (Curiel et al., 2004; Fridlender and Albelda, 2012) (Fig. 3B).

A relevant issue that must be addressed in any therapy is potential toxicity. In addition to immune system-related cells and tissues, there is evidence of Hv1 expression (RNA and/or protein level) at different tissues, such as the brain (cerebral cortex, hippocampus, and lateral ventricle), endocrine tissues (thyroid and adrenal glands), muscles (heart, skeletal, and smooth), liver and gallbladder, gastrointestinal track, kidney and urinary bladder, testis and prostate, female tissues (endometrium, fallopian tube, ovary, and placenta), skin and adipose and soft tissue (tissue expression of HVCN1, The Human Protein Atlas. http://www.proteinatlas.org/ENSG00000122986-HVCN1/tissue; Uhlen et al., 2015). The functional role of Hv1 is still uncharacterized in most of these tissues, with some exceptions: in airway epithelium, Hv1 regulates the extracellular pH in airway surface liquid (Fischer et al., 2002; Cho et al., 2009; Fischer, 2012) and Hv1 plays multiple roles in human sperm (Babcock et al., 1983; Babcock and Pfeiffer, 1987; Lishko et al., 2010; Musset et al., 2012b). Then, Hv1 inhibition can potentially affect airway epithelium pH regulation and sperm capacitation (temporarily hampering male fertility). The effects of Hv1 inhibition in other human tissues remain to be studied. Therefore, it is very important to determine the balance between the antitumoral effect and the associated toxicity when studying any drug targeting Hv1. Of note, Hv1-deficient mice develop some degree of autoimmunity associated with aging, but a life-threatening toxicity has not been described (Sasaki et al., 2013). Furthermore, these mice are able to clear bacterial infections in vivo (Ramsey et al., 2009). Similarly, Hv1-deficient rats lack a fatal/severe phenotype (Jin et al., 2014). The phenotypes of Hv1-deficient animal models cannot be directly extrapolated to the human scenario because of some differences regarding Hv1 expression among species (Lishko et al., 2010), and no human deficiency of Hv1 is known (DeCoursey, 2015). Nonetheless, these results suggest that the pharmacological inhibition of Hv1 channel could be feasible because of tolerable toxicity and potential relevant effect in both tumor cell biology and tumor-infiltrating immune cells. Moreover, a therapy directed to the Hv1 proton channel in cancer can be benefitted with strategies that specifically deliver the drug in the TME, such as intratumoral inoculation or coupling with monoclonal antibodies specific for tumor antigens.

Because cancer needs a multifactorial strategy, it is desirable that Hv1-based therapy could be used together with the standard of care for this disease and with the novel developing therapies. From our point of view, the inhibition of Hv1 could be a strategy to target the TME that could be combined with cancer vaccines, monoclonal antibodies, immune checkpoint therapy, adoptive T cell transference, and low molecular weight inhibitors, etc. Although more direct experimental evidence needs to be obtained regarding the role of Hv1 in the TME, there is no doubt that it is a novel approach for cancer therapy that is worth exploring more seriously. With that in mind, a larger effort should be made in the design and development of efficient drugs targeting Hv1 channel that could be used in cancer patients.

Hv1 inhibition can also be beneficial for the treatment of other pathologies: Alzheimer’s disease (Eder and DeCoursey, 2001), ischemic liver disease, atherosclerosis, Parkinson’s disease (DeCoursey and Ligeti, 2005), ischemic stroke (Wu et al., 2012), and Crohn’s disease (Haglund et al., 2013), but a complete analysis of this is beyond the scope of the current review.

Acknowledgments

ASPET thanks Dr. Katie Strong for copyediting of this article.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Fernández, Pupo, Mena-Ulecia, and Gonzalez.

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