Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

INVITED MINI-REVIEW

TITLE: Pharmacological modulation of proton channel Hv1 in cancer therapy: Future perspectives.

Audry Fernández, Amaury Pupo, Karel Mena-Ulecia and Carlos Gonzalez

Interdisciplinary Center for Neurosciences of Valparaíso, Faculty of Sciences, University of Valparaíso, Chile.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

RUNNING TITLE: Proton channel Hv1 in cancer therapy

Corresponding Author:

Carlos Gonzalez

Interdisciplinary Center for Neurosciences of Valparaíso, Faculty of Sciences, University of Valparaíso, Chile.

Address: Pasaje Harrington 269, Playa Ancha, Valparaíso. Chile.

Phone: (56)-(32)-299 5522

Fax: (56)-(32)-250 8047

E-mail: carlos.gonzalezl@uv.cl

Text Pages: 42

Number of Tables: None

Number of Figures: 3

Number of References: 225

Number of Words:

Abstract: 232

Introduction: 644

Discussion: 11205

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

Abbreviations:

2GBI: 2-guanidinobenzimidazole

AP-1: activator protein 1

Bcl3: B-cell lymphoma 3-encoded protein

CTL: cytotoxic T lymphocytes

CTLA-4: cytotoxic T lymphocyte-associated protein 4

Erk: extracellular receptor–activated kinase

FDA: Food and Drug Administration

GM-CSF: granulocyte–macrophage colony-stimulating factor

HLA-DR: human leukocyte antigen-antigen D related

ICAM1: intercellular adhesion molecule 1

IL: interleukin

iNOS: nitric oxide synthase

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

LPS: lipopolysaccharide

MEK: mitogen-activated protein kinase kinase

MHC: major histocompatibility complex

MMP: metalloproteinase

NF-κB: nuclear factor kappa B

NOX: NADPH oxidase

PGE2: prostaglandin E2

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

ROS: reactive oxygen species

siRNA: small interfering RNA

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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 physiological values for the intracellular pH. Therefore, since 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.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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, 2006). The voltage-sensing domain of Hv1 channel comprises four trans-membrane segments (S1-S4) and is equivalent to the one present in voltage-dependent K^+ channels (Ramsey et al., 2006; Sasaki, 2006) (Fig. 1A and B). Unlike other voltage-gated channels, Hv1 lacks a pore domain (Ramsey et al., 2006; Sasaki, 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; Lee et al., 2008; Tombola et al., 2008; Fujiwara 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 invasiveness (Wang et al., 2011, 2012b, 2013a; b). However, a tumor is a very complex environment that contains tumor-promoting and anti-tumoral immune cells, but the balance amongst these populations generally favors tumor growth and metastasis (Fridman et al., 2012). In fact, it is

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

well recognized that tumors promote the expansion and recruitment of immune populations able to suppress T cell responses and support their proliferation, vascularization, and metastatic spreading (Fridlender and Albelda, 2012; Gabrilovich 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., 2010; 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 Calcinotto, 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 has previously been 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 physiological 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 pro-inflammatory 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-

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

interleukin) and TGF-β (transforming growth factor β), but also down-regulate the expression of co-stimulatory 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., 2015). The importance of the IL-12/IL-10 balance in the activation of T cells has been widely recognized since IL-12 stimulates anti-tumoral 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 L-arginine availability for T cell proliferation due to 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 towards the TME (Curiel et al., 2004), along with an increased secretion of immunosuppressive TGF-β and prostaglandin E2 (PGE2) (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; Schwitalla et al., 2013; Di Mitri et al., 2014; Lu et al., 2014; Wan et al., 2014), angiogenesis and vasculogenesis (Murdoch, 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 has recently been demonstrated for tumor-associated neutrophils (TANs). Similarly to TAMs and in a process regulated by TGF-β, the TME skews TANs polarization from anti-tumoral N1 to pro-tumoral N2 phenotype (Fridlender et al., 2009). The TGF-β-induced N2 phenotype of TANs is characterized by arginase 1 expression and low

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

production of TNF (tumor necrosis factor) and ICAM1 (intercellular adhesion molecule 1) (Fridlender et al., 2009). On the contrary, the production of several chemokines by TANs is increased in comparison to 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 anti-tumoral 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 physiological implications. The role of CTLA-4 has been associated with the blockage of co-stimulation needed for T cell activation since 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 physiological mechanisms of T cell contraction; for example, PD-L1 is

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 relevance of these regulatory pathways in the inhibition of tumor-specific T cell responses has been validated in clinical trials where patients with melanoma (Weber et al., 2008; Brahmer et al., 2012), renal cell carcinoma (Yang et al., 2007; Brahmer et al., 2012), prostate cancer (Karan and Van Veldhuizen, 2012), and non-small cell lung cancer (Brahmer et al., 2012) benefited from ipilimumab (anti-CTLA-4 antibody) or nivolumab/pembrolizumab (anti-PD-1 antibodies). Of note, an increase in the ratio of intratumoral effector T cells to Tregs that correlates with clinical benefit has been observed in patients treated with ipilimumab (Hodi et al., 2008; Liakou et al., 2008). These results, together with pre-clinical experiments (Bulliard et al., 2013; Selby et al., 2013; Simpson et al., 2013), show that anti-tumor activity of anti-CTLA-4 antibodies could be attributed, at least partially, to the depletion of Tregs. In fact, the immune checkpoint therapy demonstrates that T cell responses against tumor antigens can certainly be potentiated by releasing the key suppressive nodes.

Another important and somehow underestimated element of T cell inhibition is related to the predominant metabolic pathways in the TME. Indeed, as first described by Otto Warburg, tumor cells employ mainly aerobic glycolysis to obtain energy and sustain their high proliferative rate (Warburg et al., 1927). Tumor cells are considerably more efficient than T cells in glucose uptake (Cham and Gajewski, 2005), causing a deprivation of this important metabolite for effector T cell proliferation that depends on glycolysis (Cham et al., 2003). Additional consequences of tumor cell metabolism include a drop of TME pH due to lactate and H^+ generation in the hypoxic conditions of the tumor (Gatenby and Gillies, 2004), and the subsequent exportation of these metabolites to the tumor cell extracellular milieu through the

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

mono-carboxylate transporter and different proton extrusion systems (Bellone and Calcinotto, 2013), including the Hv1 proton channel (Wang et al., 2011). CO_2 that is released due to pyruvate decarboxylation in the mitochondria of tumor cells is converted to bicarbonate and H^+ 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*. observed that the capacity of CTLs to kill tumor cells was noticeably suppressed in acidic pH conditions (Redegeld et al., 1991). This was further corroborated in a recent report from Calcinotto *et al*. 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 (Calcinotto et al., 2012). 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 anti-tumoral efficacy of different immunotherapeutic approaches (Calcinotto et al., 2012).

This plethora of mechanisms inducing immune dysfunction can explain the relatively low success of cancer immunotherapy 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 transference, 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 towards more personalized treatment. Undoubtedly, cancer vaccines face the biggest challenge since this treatment would have to

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 adoptively 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 since ipilimumab and the two anti-PD-1 antibodies, nivolumab and pembrolizumab, were approved by the FDA (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 anti-tumoral response, such as macrophages, neutrophils, dendritic cells, and T cells. In the following sections we will discuss these elements in more detail.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 physiological values is essential for most biological 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 anti-tumoral 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*. 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 non-metastatic tissue and the poorly metastatic cell line MCF-7 (Wang et al., 2011). The inhibition of Hv1 expression with small interfering RNA (siRNA) 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

treated with siRNAs 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 since 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 $ZnCl_2$ reduced migration and induced apoptosis of SHG-44 glioma cells *in vitro*. Moreover, the administration of $ZnCl_2$ *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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 since 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

Even though 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 support tumor development or participate in anti-tumoral 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 ($O_2^{\bullet -}$) 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 towards 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

channel has been made in phagocytic leukocytes and other cell types (Murphy and DeCoursey, 2006; Morgan et al., 2009).

Effector 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 transference 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 early publication of Kaldi *et al*. described an electrogenic proton efflux in human tonsillar T cells that was activated with arachidonic acid and inhibited by Zn^{2+} and Ca^{2+} (Kaldi et al., 1996). This proton extrusion system was structurally independent of NOX and showed some properties assigned today to Hv1 in phagocytic cells (Kaldi et al., 1996). More recently it was revealed that Hv1 is expressed in T cells from human peripheral blood and the human leukemic T cell line Jurkat E6-1 (Schilling et al., 2002). Although the H^+ current amplitude was 100 times smaller in T lymphocytes than in B lymphocytes or the Jurkat E6-1 cell line, it could be significantly increased by phorbol 12-myristate 13-acetate (PMA) stimulation (Schilling et al., 2002). Correspondingly, Hv1 mRNA and protein were found in mouse T cells

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

from spleen and lymph nodes, but again, to a lesser degree than in B cells and macrophages (Sasaki et al., 2013).

The presence of Hv1 in mouse T cells was further corroborated through electrophysiological measurements (Sasaki et al., 2013). Interestingly, these authors observed two-phase kinetics for ROS production in T cells following PMA stimulation. The first phase of ROS production was faster, smaller in quantity, and remained unchanged in Hv1-deficient mice, whereas the later phase was more sustained and yielded a higher amount of ROS in wild type T cells, but was deeply inhibited in those obtained from Hv1-knockout mice (Sasaki et al., 2013). ROS production in T cells begins with TCR stimulation and has been previously associated with the activation 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*. (Jackson et al., 2004) found similar phases of ROS production upon T cell stimulation to those described by Okamura *et al*. (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 towards 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 MEK (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*. have shown that sustained Erk activation triggered by the TCR inhibited IL-4 production in naive T cells, thus promoting Th1 differentiation (Jorritsma et al., 2003). Snelgrove *et al*. demonstrated that lung's T cells from mice lacking NOX2 had a Th1-biased cytokine response upon infection with *Cryptococcus neoformans* (Snelgrove et al., 2006). The Th1-prone phenotype of NOX2-deficient mice was also influenced by dendritic cells-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 to wild type dendritic cell, translating to a higher ability to induce the differentiation of ovalbumin-specific $CD4^+$ T cells towards 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 to 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 microbicidal 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 anti-tumoral T cell responses and to the promotion of tumor proliferation, angiogenesis, and metastatic spreading (Gabrilovich et al., 2012; Medina-Echeverz 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 physiological 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 (Petheő et al., 2010). Early publications detected proton

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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*. found Hv1 protein in mouse neutrophils, particularly within phagosomes, together with NOX2 components such as gp91, p22, p47 and p67 (Okochi et al., 2009). Consistently, Hv1 was detected in neutrophils from human peripheral blood, both at mRNA and protein levels (Petheő 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 co-localization with NOX2 was observed (Petheő 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*. observed enhanced acidification in the cytoplasm of human neutrophils after phagocytosis of opsonized zymosan particles when Hv1 was inhibited with Zn^{2+} (Morgan et al., 2009). In this study, the authors detected likewise a two-fold increase in the rate and the extent of cytoplasm acidification in bone marrow phagocytes from Hv1-knockout mice compared to wild type littermates (Morgan et al., 2009). Moreover, several publications have shown a substantial reduction of ROS production in neutrophils from Hv1-deficient mice when compared to 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.,

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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*. have shown 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-

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

formyl-Met-Ile-Val-Ile-Leu bacterial peptide (El Chemaly et al., 2010). Afterwards, Zhu *et al*. corroborated these findings in an *in vivo* model of peritonitis induced by intraperitoneal injection of thioglycollate (Zhu et al., 2013). In this experimental setting, the number of neutrophils infiltrating the peritoneal cavity was reduced two-fold in Hv1-deficient mice compared to wild type littermates, indicating that Hv1 channel regulates neutrophils migration towards 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*. via a western blot assay (Okochi et al., 2009).

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_2O_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*. additionally showed the glucose dependence of NOX activation and ROS production in human macrophages, a different behavior to that exhibited by human granulocytes (Musset et al., 2012a). 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 due to the elevated production of ROS sustained by this channel, which then inhibits the accumulation of vacuolar 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 anti-microbial defense against the intracellular fungus *Histoplasma capsulatum*, which causes pulmonary and disseminated histoplasmosis (Subramanian Vignesh et al., 2013). GM-CSF induced an up-regulation of metallothioneins and Zn^{2+} exporters that redirect these divalent cations away from the phagosomes and towards 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 anti-tumoral responses due to 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.* 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 (Szteyn et al., 2012). Interestingly, even though the function of Hv1 in dendritic cells is once more associated with NOX2 activity, due to 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 (MHC) 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 $CD8^+$ 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 one hour following antigen phagocytosis in dendritic cells (Sasaki, 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 alkalinization 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*. 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 (Rybicka et al., 2012). 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 Concanamycin 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 alkalinization 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.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

In another interesting study, Szteyn *et al*. evaluated the effect of bacterial LPS on Hv1 and NOX2 activity in mouse bone marrow-derived dendritic cells (Szteyn et al., 2012). 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 up-regulation of MHC, co-stimulatory molecules, cytokines, and mechanisms allowing for the migration towards 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 $p47^{phox}$ is phosphorylated by PKC, which promotes its translocation to the membrane and the subsequent assembly of other subunits such as $p40^{phox}$ and $p67^{phox}$ 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 bi-phasic 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. While it would be interesting to understand the role of Hv1 in the expression of co-stimulatory 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*. 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 secondly, 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 (Bcl3), a co-activator of NF-κB, inhibited Hv1

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

channel expression in LPS-stimulated mouse macrophages (Wang et al., 2012a), suggesting a role of the NF-κB/Bcl3 complex in Hv1 transcriptional regulation. The expression of NOX subunit $gp91^{phox}$ is also controlled by NF-κB in monocytic cell lines, demonstrating an up-regulation upon LPS stimulation that was inhibited in cells constitutively expressing IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) (Anrather, 2005). Thus, NF-κB-mediated sensing of inflammation and redox stress leads to NOX up-regulation 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 AP-1 (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).

Due to the important role of NOX in bacterial killing through the respiratory burst, it makes sense that the up-regulation 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 (Hanahan and Weinberg, 2000) as the seventh hallmark of cancer due to 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 since tumor cells produce ROS due to their metabolic activity, the hyper-activation of oxidative enzymes, the activity of oncogenes, and the increased signaling through cellular receptors (Szatrowski 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

physiological mechanism that links inflammation/redox sensing with Hv1/NOX expression and activation to their advantage. This rises the interesting hypothesis that increased and sustained inflammation, along with ROS production occurring during tumor development, could cause a NF-κB-mediated up-regulation 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 (Karin et al., 2002). NF-κB become activated by inflammatory cytokines (IL-1 and TNF-α), growth factors, oncogenes, TLR signaling, hypoxia, and acidic conditions within the TME (Karin 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 anti-apoptotic genes, along with several genes that promote proliferation, angiogenesis, invasion, and metastasis (Karin et al., 2002; Greten et al., 2004; Pikarsky et al., 2004). Tumor-infiltrating immune cells such as macrophages, neutrophils, DCs, and T cells also display NF-κB activation that facilitates IL-6, IL-1, TNF-α, and ROS secretion (Karin 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 pro-tumoral 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 firstly, Hv1 could be overexpressed on both tumor cells and tumor-infiltrating immune cells, and secondly, that Hv1 up-regulation might be driven by the inflammatory, redox, and pH conditions within the TME. NF-κB and AP-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 up-regulation 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 (H_2O_2) promotes down-regulation of CD3 ζ chain (Schmielau and Finn, 2001), whereas peroxynitrite ($ONOO^-$) causes a nitration 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 H_2O_2, contribute to the maintenance of tumor-associated myeloid cells in the immature stage and therefore inhibit their differentiation towards mature antigen-presenting cells (Kusmartsev and Gabrilovich, 2003).

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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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*. demonstrated that ROS production in macrophages is required for M2 and TAMs differentiation, but not for the polarization towards a M1 phenotype (Zhang et al., 2013). 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 up-regulation 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 since it has been demonstrated that TANs can indeed secrete ROS and, in some models, be cytotoxic for tumor cells (Dallegri et

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 due to 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.* 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 (Subramanian Vignesh et al., 2013). Neutrophils are also phagocytic cells with similar ROS-mediated pathogen destruction mechanisms, and therefore, it could be possible that an up-regulation 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*. 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 (Kasahara et al., 2016), 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 towards 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 (CXCL2, CXCL1, CCL3, 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).

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

Since 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 NOX2 activity and 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 up-regulation 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 have previously shown that NOX activity could be a relevant factor driving a tolerogenic phenotype in tumor-infiltrating dendritic cells. Kuang *et al*. demonstrated that the TME-educated dendritic cells assume a semi-mature phenotype, which causes a rapid down-regulation of CD3 ε chain and TCR itself and the subsequent apoptosis of T cells (Kuang et al., 2008). Interestingly, the negative effect of these tolerogenic dendritic cells on T cell activation

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

and viability was dependent on NOX activity and was not mediated by arginase 1, iNOS or indoleamine 2,3-dioxygenase (Kuang et al., 2008). More recently, Martner *et al*. provided evidence that the NOX2 inhibitor histamine promoted the maturation of human DCs from monocytes, and this is characterized by an increased expression of HLA-DR (human leukocyte antigen-antigen D related) and co-stimulatory molecules, along with an enhanced ability to stimulate T helper cells with Th0 phenotype (Martner et al., 2015). 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 anti-tumoral 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 anti-tumoral $CD8^+$ T cell responses through the production of immunosuppressive factors like indoleamine 2,3-dioxygenase, arginase 1, TGF-β, IL-10, and nitric oxide (Lee et al., 2003; Dumitriu et al., 2009; Norian et al., 2009; Watkins et al., 2011).

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

On the other hand, the results discussed earlier in this chapter (Jackson et al., 2004; Sasaki et al., 2013) suggest that Hv1 function could be associated with T cell contraction that is physiologically required to avoid tissue damage and autoimmunity due to exacerbated and sustained T cell responses during inflammatory conditions such as infections or aging. According to this idea, aged Hv1-deficient mice developed an autoimmune phenotype characterized by splenomegaly, increased production of anti-dsDNA antibodies, and immune complexes deposition in the kidney, which resembles human lupus erythematosus (Sasaki et al., 2013). Cancer is a disease linked to chronic inflammation (Colotta et al., 2009), and therefore, it would be very interesting to determine whether tumor-infiltrating lymphocytes overexpress Hv1 and whether this channel could be part of another mechanism contributing to the strict control of T cell activation and function within the TME. It could be hypothesized that a potential up-regulation of Hv1 expression in tumor-infiltrating T cells could support a sustained intracellular NOX-mediated ROS production that limits T cell activation and skews T helper polarization towards Th2 phenotype, thus contributing to anti-tumoral response impairment (Fig. 3A). In fact, previously discussed evidence demonstrated that NOX-mediated ROS production played an important role in constraining Th1 responses against exogenous antigens (Jackson et al., 2004; Snelgrove et al., 2006; Jendrysik et al., 2011). Thus, from the perspective of the nexus between Hv1 and NOX, an enhanced ROS production in both T cells and dendritic cells (that might happen in the TME) could limit the development of a Th1 response, which is more efficient to eliminate the tumor. Undoubtedly this is a relevant issue that should be evaluated in the future.

Although we have focused on the possible role of Hv1 mainly in the maintenance of high levels of ROS production by tumor-infiltrating immune cells, we do not disregard the idea that the Hv1 channel could also regulate other mechanisms of immunosuppression and promotion of tumor

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

development that would be interesting to study. Furthermore, if it is proven in the future that the hypotheses we have mentioned are correct, it could establish that Hv1 channel overexpression in tumor-infiltrating immune cells is a novel mechanism contributing to tumor escape that might be targeted in cancer therapy.

Pharmacological modulation of proton channel Hv1

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 Zn^{2+}, have been the gold standard for proton current inhibition (Seredenina et al., 2015). The inhibition of Zn^{2+} is inconsistent with classical voltage-dependent block, instead, Zn^{2+} binds to a superficial site on the channel and modulates gating (Cherny and DeCoursey, 1999). The effects of extracellular Zn^{2+} are strongly dependent on external pH and insensitive to internal pH, suggesting that protons and Zn^{2+} compete for external sites on Hv1 channels (Cherny and DeCoursey, 1999). Ramsey *et al*. identified the residues H140 and H193 as critical for external Zn^{2+} inhibition (Ramsey et al., 2006), and this was later confirmed when Takeshita *et al*. (Takeshita et al., 2014) structurally characterized the external Zn^{2+} 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). Zn^{2+} 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 Zn^{2+} as an Hv1 channel inhibitor is severely hampered due to its involvement in several physiological 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Kornilov et al., 2014); 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 $O_2^{\bullet-}$ 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; Groemping and Rittinger, 2005; Musset et al., 2010).

Guanidine 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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). Since 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 (ClGBI) (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 ClGBI'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 (Takeshita 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, as it is possible that the sequence incorporated from Ci-VSP, in the region of S2–S3, locally perturbs the structure, disrupting some native interactions and changing its register (Li et al., 2015). Unfortunately, as 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 histological 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 pro-tumoral and anti-tumoral 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 up-regulation of Hv1 in tumor-infiltrating lymphocytes might enhance the exhaustion of tumor-specific T cells

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

(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 anti-tumoral 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 up-regulation of Hv1 channel in dendritic cells could also impair the cross-priming of tumor antigens due to 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 since 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 anti-tumor CTLs. It cannot be completely disregarded, however, that Hv1 inhibition might affect cross-priming by inducing excessive antigen degradation. If this is the case, the negative effect of a therapy based

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 anti-tumoral T cells and might impair the polarization of TAMs towards M2 phenotype since ROS is required for macrophage differentiation into M2 functional state (Zhang et al., 2013) (Fig. 3B). Additionally, the migration of neutrophils towards 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. Besides 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.; Uhlen et al., 2015). The functional role of Hv1 is still uncharacterized in most of these tissues, with some exceptions; in airway epithelium,

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 (temporally 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 anti-tumoral 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 due to 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 due to 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 beneficiated with strategies that specifically deliver the drug in the TME, such as intratumoral inoculation or coupling with monoclonal antibodies specific for tumor antigens.

Since 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

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

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 be also 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.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

AUTHORSHIP CONTRIBUTIONS:

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

REFERENCES:

Abbas AK, Lichtman AH, and Pillai S (2014) *Cellular and Molecular Immunology*, 8th ed. Elsevier Saunders, Canada.

Aggarwal BB, Vijayalekshmi RV, and Sung B (2009) Targeting Inflammatory Pathways for Prevention and Therapy of Cancer: Short-Term Friend, Long-Term Foe. *Clin Cancer Res* **15**:425–430.

Alabi AA, Bahamonde MI, Jung HJ, Kim JI, and Swartz KJ (2007) Portability of paddle motif function and pharmacology in voltage sensors. *Nature* **450**:370–375.

Anrather J (2005) NF- B Regulates Phagocytic NADPH Oxidase by Inducing the Expression of gp91phox. *J Biol Chem* **281**:5657–5667.

Antony S, Wu Y, Hewitt SM, Anver MR, Butcher D, Jiang G, Meitzler JL, Liu H, Juhasz A, Lu J, Roy KK, and Doroshow JH (2013) Characterization of NADPH oxidase 5 expression in human tumors and tumor cell lines with a novel mouse monoclonal antibody. *Free Radic Biol Med* **65**:497–508.

Babcock DF and Pfeiffer DR (1987) Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem* **262**:15041–15047.

Babcock DF, Rufo GA, and Lardy HA (1983) Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc Natl Acad Sci U S A* **80**:1327–1331.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* **392**:245–252.

Barese CN, Goebel WS, and Dinauer MC (2004) Gene therapy for chronic granulomatous

disease. *Expert Opin Biol Ther* **4**:1423–1434.

Bauer CA, Kim EY, Marangoni F, Carrizosa E, Claudio NM, and Mempel TR (2014) Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. *J Clin Invest* **124**:2425–2440.

Becker S, Wasser S, Hauses M, Hossle JP, Ott MG, Dinauer MC, Ganser A, Hoelzer D, Seger R, and Grez M (1998) Correction of Respiratory Burst Activity in X-Linked Chronic Granulomatous Cells to Therapeutically Relevant Levels after Gene Transfer into Bone Marrow CD34 + Cells. *Hum Gene Ther* **9**:1561–1570.

Bellone G, Carbone A, Smirne C, Scirelli T, Buffolino A, Novarino A, Stacchini A, Bertetto O, Palestro G, Sorio C, Scarpa A, Emanuelli G, and Rodeck U (2006) Cooperative induction of a tolerogenic dendritic cell phenotype by cytokines secreted by pancreatic carcinoma cells. *J Immunol* **177**:3448–3460.

Bellone M and Calcinotto A (2013) Ways to enhance lymphocyte trafficking into tumors and fitness of tumor infiltrating lymphocytes. *Front Oncol* **3**:231.

Biswas SK and Mantovani A (2010) Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* **11**:889–896.

Bittinger F, González-García JL, Klein CL, Brochhausen C, Offner F, and Kirkpatrick CJ (1998) Production of superoxide by human malignant melanoma cells. *Melanoma Res* **8**:381–387.

Bonde A-K, Tischler V, Kumar S, Soltermann A, and Schwendener RA (2012) Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. *BMC Cancer* **12**:35.

Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM,

Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, and Wigginton JM (2012) Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *N Engl J Med* **366**:2455–2465.

Bronte V, Chappell DB, Apolloni E, Cabrelle A, Wang M, Hwu P, and Restifo NP (1999) Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. *J Immunol* **162**:5728–5737.

Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, Wilson NS, Dranoff G, and Brogdon JL (2013) Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *J Exp Med* **210**:1685–1693.

Calcinotto A, Filipazzi P, Grioni M, Iero M, De Milito A, Ricupito A, Cova A, Canese R, Jachetti E, Rossetti M, Huber V, Parmiani G, Generoso L, Santinami M, Borghi M, Fais S, Bellone M, and Rivoltini L (2012) Modulation of Microenvironment Acidity Reverses Anergy in Human and Murine Tumor-Infiltrating T Lymphocytes. *Cancer Res* **72**:2746–2756.

Carey PD, Wakefield CH, and Guillou PJ (1997) Neutrophil activation, vascular leak toxicity, and cytolysis during interleukin-2 infusion in human cancer. *Surgery* **122**:918–926.

Cevik MO, Katsuyama M, Kanda S, Kaneko T, Iwata K, Ibi M, Matsuno K, Kakehi T, Cui W, Sasaki M, and Yabe-Nishimura C (2008) The AP-1 site is essential for the promoter activity of NOX1/NADPH oxidase, a vascular superoxide-producing enzyme: Possible involvement of the ERK1/2-JunB pathway. *Biochem Biophys Res Commun* **374**:351–355.

Cham CM, and Gajewski TF (2005) Metabolic Mechanisms of Tumor Resistance to T Cell Effector Function. *Immunol Res* **31**:107–118.

Cham CM, Xu H, O'Keefe JP, Rivas F V, Zagouras P, and Gajewski TF (2003) Gene Array and Protein Expression Profiles Suggest Post-transcriptional Regulation during CD8+ T Cell Differentiation. *J Biol Chem* **278**:17044–17052.

Chambard JC and Pouyssegur J (1986) Intracellular pH controls growth factor-induced ribosomal protein S6 phosphorylation and protein synthesis in the G0-G1 transition of fibroblasts. *Exp Cell Res* **164**:282–294.

Cherny VV and DeCoursey TE (1999) pH-dependent inhibition of voltage-gated H(+) currents in rat alveolar epithelial cells by Zn(2+) and other divalent cations. *J Gen Physiol* **114**:819–838.

Cherny VV, Markin VS, and DeCoursey TE (1995) The voltage-activated hydrogen ion conductance in rat alveolar epithelial cells is determined by the pH gradient. *J Gen Physiol* **105**:861–896.

Chiche J, Ilc K, Laferriere J, Trottier E, Dayan F, Mazure NM, Brahimi-Horn MC, and Pouyssegur J (2009) Hypoxia-Inducible Carbonic Anhydrase IX and XII Promote Tumor Cell Growth by Counteracting Acidosis through the Regulation of the Intracellular pH. *Cancer Res* **69**:358–368.

Cho DY, Hajighasemi M, Hwang PH, Illek B, and Fischer H (2009) Proton secretion in freshly excised sinonasal mucosa from asthma and sinusitis patients. *Am J Rhinol Allergy* **23**:10–13.

Colotta F, Allavena P, Sica A, Garlanda C, and Mantovani A (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30**:1073–1081.

Condamine T and Gabrilovich DI (2011) Molecular mechanisms regulating myeloid-derived

suppressor cell differentiation and function. *Trends Immunol* **32**:19–25.

Cui TX, Kryczek I, Zhao L, Zhao E, Kuick R, Roh MH, Vatan L, Szeliga W, Mao Y, Thomas DG, Kotarski J, Tarkowski R, Wicha M, Cho K, Giordano T, Liu R, and Zou W (2013) Myeloid-Derived Suppressor Cells Enhance Stemness of Cancer Cells by Inducing MicroRNA101 and Suppressing the Corepressor CtBP2. *Immunity* **39**:611–621.

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, and Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10**:942–949.

Dallegri F, Ottonello L, Ballestrero A, Dapino P, Ferrando F, Patrone F, and Sacchetti C (1991) Tumor cell lysis by activated human neutrophils: analysis of neutrophil-delivered oxidative attack and role of leukocyte function-associated antigen 1. *Inflammation* **15**:15–30.

De Sanctis F, Solito S, Ugel S, Molon B, Bronte V, and Marigo I (2016) MDSCs in cancer: Conceiving new prognostic and therapeutic targets. *Biochim Biophys Acta* **1865**:35–48.

DeCoursey TE (2015) The Voltage-Gated Proton Channel: A Riddle, Wrapped in a Mystery, inside an Enigma. *Biochemistry* **54**:3250–3268.

DeCoursey TE (2008) Voltage-gated proton channels. *Cell Mol Life Sci* **65**:2554–73.

DeCoursey TE and Cherny VV (2007) Pharmacology of voltage-gated proton channels. *Curr Pharm Des* **13**:2400–2420.

DeCoursey TE and Cherny VV (1993) Potential, pH, and arachidonate gate hydrogen ion currents in human neutrophils. *Biophys J* **65**:1590–1598.

DeCoursey TE and Cherny VV (1998) Temperature dependence of voltage-gated H+ currents in

human neutrophils, rat alveolar epithelial cells, and mammalian phagocytes. *J Gen Physiol* **112**:503–522.

DeCoursey TE and Cherny VV (1996) Voltage-activated proton currents in human THP-1 monocytes. *J Membr Biol* **152**:131–140.

DeCoursey TE and Ligeti E (2005) Regulation and termination of NADPH oxidase activity. *Cell Mol Life Sci* **62**:2173–2193.

DeCoursey TE, Morgan D, and Cherny VV (2003) The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature* **422**:531–534.

Demaurex N, Grinstein S, Jaconi M, Schlegel W, Lew DP, and Krause KH (1993) Proton currents in human granulocytes: regulation by membrane potential and intracellular pH. *J Physiol* **466**:329–344.

Di Mitri D, Toso A, Chen JJ, Sarti M, Pinton S, Jost TR, D'Antuono R, Montani E, Garcia-Escudero R, Guccini I, Da Silva-Alvarez S, Collado M, Eisenberger M, Zhang Z, Catapano C, Grassi F, Alimonti A, D'Antuono R, Montani E, Garcia-Escudero R, Guccini I, Da Silva-Alvarez S, Collado M, Eisenberger M, Zheng Z, Catapano C, Grassi F, and Alimonti A (2014) Tumour-infiltrating Gr-1+ myeloid cells antagonize senescence in cancer. *Nature* **515**:134–137.

Dinauer MC, Li LL, Björgvinsdóttir H, Ding C, and Pech N (1999) Long-term correction of phagocyte NADPH oxidase activity by retroviral-mediated gene transfer in murine X-linked chronic granulomatous disease. *Blood* **94**:914–922.

Dumitriu IE, Dunbar DR, Howie SE, Sethi T, and Gregory CD (2009) Human Dendritic Cells Produce TGF-beta 1 under the Influence of Lung Carcinoma Cells and Prime the Differentiation of CD4+CD25+Foxp3+ Regulatory T Cells. *J Immunol* **182**:2795–2807.

Dunn GP, Bruce AT, Ikeda H, Old LJ, and Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**:991–998.

Eder C and DeCoursey TE (2001) Voltage-gated proton channels in microglia. *Prog Neurobiol* **64**:277–305.

El Chemaly A, Nunes P, Jimaja W, Castelbou C, and Demaurex N (2014) Hv1 proton channels differentially regulate the pH of neutrophil and macrophage phagosomes by sustaining the production of phagosomal ROS that inhibit the delivery of vacuolar ATPases. *J Leukoc Biol* **95**:1–13.

El Chemaly A, Okochi Y, Sasaki M, Arnaudeau S, Okamura Y, and Demaurex N (2010) VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification. *J Exp Med* **207**:129–139.

Fernandez A, Oliver L, Alvarez R, Fernandez LE, Lee KP, and Mesa C (2014) Adjuvants and myeloid-derived suppressor cells: Enemies or allies in therapeutic cancer vaccination. *Hum Vaccines Immunother* **10**:3251–3260.

Fischer H (2012) Function of Proton Channels in Lung Epithelia. *Wiley Interdiscip Rev Membr Transp Signal* **1**:247–258.

Fischer H, Widdicombe JH, and Illek B (2002) Acid secretion and proton conductance in human airway epithelium. *Am J Physiol - Cell Physiol* **282**:C736–C743.

Fridlender ZG and Albelda SM (2012) Tumor-associated neutrophils: friend or foe? *Carcinogenesis* **33**:949–955.

Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, and Albelda SM (2009) Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: “N1” versus “N2” TAN. *Cancer Cell* **16**:183–194.

Fridman WH, Pagès F, Sautès-Fridman C, and Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**:298–306.

Fujiwara Y, Kurokawa T, Takeshita K, Kobayashi M, Okochi Y, Nakagawa A, and Okamura Y (2012) The cytoplasmic coiled-coil mediates cooperative gating temperature sensitivity in the voltage-gated H+ channel Hv1. *Nat Commun* **3**: 816.

Gabrilovich D (2004) Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol* **4**:941–952.

Gabrilovich DI, Ostrand-Rosenberg S, and Bronte V (2012) Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* **12**:253–268.

Gatenby RA and Gillies RJ (2004) Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**:891–899.

Gottlieb RA, Giesing HA, Zhu JY, Engler RL, and Babior BM (1995) Cell acidification in apoptosis: granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuolar H(+)-ATPase. *Proc Natl Acad Sci U S A* **92**:5965–5968.

Greten FR, Eckmann L, Greten TF, Park JM, Li Z-W, Egan LJ, Kagnoff MF, and Karin M (2004) IKKβ Links Inflammation and Tumorigenesis in a Mouse Model of Colitis-Associated Cancer. *Cell* **118**:285–296.

Groemping Y and Rittinger K (2005) Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem J* **386**:401–416.

Guzik TJ, Korbut R, and Adamek-Guzik T (2003) Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol* **54**:469–487.

Haglund S, Almer S, Peterson C, and Söderman J (2013) Gene Expression and Thiopurine

Metabolite Profiling in Inflammatory Bowel Disease – Novel Clues to Drug Targets and Disease Mechanisms? *PLoS One* **8**:e56989.

Hanahan D and Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. *Cell* **144**:646–674.

Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**:57–70.

Henderson LM, Chappell JB, and Jones OT (1987) The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H+ channel. *Biochem J* **246**:325–329.

Herber DL, Cao W, Nefedova Y, Novitskiy S V, Nagaraj S, Tyurin VA, Corzo A, Cho H-I, Celis E, Lennox B, Knight SC, Padhya T, McCaffrey T V, McCaffrey JC, Antonia S, Fishman M, Ferris RL, Kagan VE, and Gabrilovich DI (2010) Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med* **16**:880–886.

Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, Shibuya M, Akira S, Aburatani H, and Maru Y (2008) The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol* **10**:1349–1355.

Hodi FS, Butler M, Oble DA, Seiden M V, Haluska FG, Kruse A, MacRae S, Nelson M, Canning C, Lowy I, Korman A, Lautz D, Russell S, Jaklitsch MT, Ramaiya N, Chen TC, Neuberg D, Allison JP, Mihm MC, and Dranoff G (2008) Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proc Natl Acad Sci* **105**:3005–3010.

Hole PS, Zabkiewicz J, Munje C, Newton Z, Pearn L, White P, Marquez N, Hills RK, Burnett AK, Tonks A, and Darley RL (2013) Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. *Blood*

122:3322–3330.

Höll M, Koziel R, Schäfer G, Pircher H, Pauck A, Hermann M, Klocker H, Jansen-Dürr P, and Sampson N (2016) ROS signaling by NADPH oxidase 5 modulates the proliferation and survival of prostate carcinoma cells. *Mol Carcinog* **55**:27–39.

Hondares E, Brown MA, Musset B, Morgan D, Cherny VV, Taubert C, Bhamrah MK, Coe D, Marelli-Berg F, Gribben JG, Dyer MJS, DeCoursey TE, and Capasso M (2014) Enhanced activation of an amino-terminally truncated isoform of the voltage-gated proton channel HVCN1 enriched in malignant B cells. *Proc Natl Acad Sci* **111**:18078–18083.

Hong L, Kim IH, and Tombola F (2014) Molecular determinants of Hv1 proton channel inhibition by guanidine derivatives. *Proc Natl Acad Sci* **111**:9971–9976.

Hong L, Pathak MM, Kim IH, Ta D, and Tombola F (2013) Voltage-Sensing Domain of Voltage-Gated Proton Channel Hv1 Shares Mechanism of Block with Pore Domains. *Neuron* **77**:274–287.

Houghton AM, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, Stolz DB, Land SR, Marconcini LA, Kliment CR, Jenkins KM, Beaulieu KA, Mouded M, Frank SJ, Wong KK, and Shapiro SD (2010) Neutrophil elastase–mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med* **16**:219–223.

Inoue J, Gohda J, Akiyama T, and Semba K (2007) NF-κB activation in development and progression of cancer. *Cancer Sci* **98**:268–274.

Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, Murphy TL, and Murphy KM (2010) CTLA-4 suppresses the pathogenicity of self antigen–specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol* **11**:129–135.

Iwasaki A and Medzhitov R (2010) Regulation of adaptive immunity by the innate immune

system. *Science* **327**:291–295.

Jackson SH, Devadas S, Kwon J, Pinto LA, and Williams MS (2004) T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol* **5**:818–827.

Jendrysik MA, Vasilevsky S, Yi L, Wood A, Zhu N, Zhao Y, Koontz SM, and Jackson SH (2011) NADPH Oxidase-2 Derived ROS Dictates Murine DC Cytokine-Mediated Cell Fate Decisions during CD4 T Helper-Cell Commitment. *PLoS One* **6**:e28198.

Jin C, Sun J, Stilphen CA, Smith SME, Ocasio H, Bermingham B, Darji S, Guha A, Patel R, Geurts AM, Jacob HJ, Lambert NA, and O'Connor PM (2014) HV1 Acts as a Sodium Sensor and Promotes Superoxide Production in Medullary Thick Ascending Limb of Dahl Salt-Sensitive Rats. *Hypertension* **64**:541–550.

Jorritsma PJ, Brogdon JL, and Bottomly K (2003) Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive CD4+ T cells. *J Immunol* **170**:2427–2434.

Joshi NS, Akama-Garren EH, Lu Y, Lee DY, Chang GP, Li A, DuPage M, Tammela T, Kerper NR, Farago AF, Robbins R, Crowley DM, Bronson RT, and Jacks T (2015) Regulatory T Cells in Tumor-Associated Tertiary Lymphoid Structures Suppress Anti-tumor T Cell Responses. *Immunity* **43**:579–590.

Joyce JA and Fearon DT (2015) T cell exclusion, immune privilege, and the tumor microenvironment. *Science* **348**:74–80.

Juhasz A, Ge Y, Markel S, Chiu A, Matsumoto L, van Balgooy J, Roy K, and Doroshow JH (2009) Expression of NADPH oxidase homologues and accessory genes in human cancer cell lines, tumours and adjacent normal tissues. *Free Radic Res* **43**:523–532.

Kaldi K, Szaszi K, Koncz G, Susztak K, and Ligeti E (1996) Arachidonic acid activatable electrogenic H+ transport in the absence of cytochrome b558 in human T lymphocytes. *FEBS Lett* **381**:156–160.

Kapus A, Romanek R, Qu AY, Rotstein OD, and Grinstein S (1993) A pH-sensitive and voltage-dependent proton conductance in the plasma membrane of macrophages. *J Gen Physiol* **102**:729–760.

Karan D and Van Veldhuizen P (2012) Combination immunotherapy with prostate GVAX and ipilimumab: safety and toxicity. *Immunotherapy* **4**:577–580.

Karin M, Cao Y, Greten FR, and Li Z-W (2002) NF-κB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**:301–310.

Karin M and Greten FR (2005) NF-κB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* **5**:749–759.

Kasahara S, Jhingran A, Dhingra S, Salem A, Cramer RA, and Hohl TM (2016) Role of Granulocyte-Macrophage Colony-Stimulating Factor Signaling in Regulating Neutrophil Antifungal Activity and the Oxidative Burst During Respiratory Fungal Challenge. *J Infect Dis* **213**:1289–1298.

Kawai T and Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**:373–384.

Kitamura T, Qian B-Z, and Pollard JW (2015) Immune cell promotion of metastasis. *Nat Rev Immunol* **15**:73–86.

Koch HP, Kurokawa T, Okochi Y, Sasaki M, Okamura Y, and Larsson HP (2008) Multimeric nature of voltage-gated proton channels. *Proc Natl Acad Sci* **105**:9111–9116.

Kornilov P, Peretz A, Lee Y, Son K, Lee JH, Refaeli B, Roz N, Rehavi M, Choi S, and Attali B

(2014) Promiscuous gating modifiers target the voltage sensor of Kv7.2, TRPV1, and Hv1 cation channels. *FASEB J* **28**:2591–2602.

Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, Yu L, Ross J, Korsisaari N, Cao T, Bou-Reslan H, Kallop D, Weimer R, Ludlam MJC, Kaminker JS, Modrusan Z, van Bruggen N, Peale F V, Carano R, Meng YG, and Ferrara N (2010) Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A* **107**:21248–21255.

Kraaij MD, Savage NDL, van der Kooij SW, Koekkoek K, Wang J, van den Berg JM, Ottenhoff THM, Kuijpers TW, Holmdahl R, van Kooten C, and Gelderman KA (2010) Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species. *Proc Natl Acad Sci* **107**:17686–17691.

Krummel MF and Allison JP (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**:459–465.

Kuang D, Zhao Q, Xu J, Yun J, Wu C, Zheng L, and Alerts E (2008) Tumor-Educated Tolerogenic Dendritic Cells Induce CD3 ε Down-Regulation and Apoptosis of T Cells through Oxygen-Dependent Pathways. *J Immunol* **181**:3089–3098.

Kulleperuma K, Smith SME, Morgan D, Musset B, Holyoake J, Chakrabarti N, Cherny VV, DeCoursey TE, and Pomès R (2013) Construction and validation of a homology model of the human voltage-gated proton channel hHV1. *J Gen Physiol* **141**:445–465.

Kuno M, Ando H, Morihata H, Sakai H, Mori H, Sawada M, and Oiki S (2009) Temperature dependence of proton permeation through a voltage-gated proton channel. *J Gen Physiol* **134**:191–205.

Kusmartsev S and Gabrilovich DI (2003) Inhibition of myeloid cell differentiation in cancer: the

role of reactive oxygen species. *J Leukoc Biol* **74**:186–196.

Kusmartsev S and Gabrilovich DI (2005) STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. *J Immunol* **174**:4880–4891.

Lee BN, Follen M, Rodriquez G, Shen D-Y, Malpica A, Shearer WT, and Reuben JM (2006) Deficiencies in myeloid antigen-presenting cells in women with cervical squamous intraepithelial lesions. *Cancer* **107**:999–1007.

Lee JR, Dalton RR, Messina JL, Sharma MD, Smith DM, Burgess RE, Mazzella F, Antonia SJ, Mellor AL, and Munn DH (2003) Pattern of recruitment of immunoregulatory antigen-presenting cells in malignant melanoma. *Lab Invest* **83**:1457–1466.

Lee SY, Letts JA, and Mackinnon R (2008) Dimeric subunit stoichiometry of the human voltage-dependent proton channel Hv1. *Proc Natl Acad Sci U S A* **105**:7692–7695.

Lee SY, Letts JA, and MacKinnon R (2009) Functional Reconstitution of Purified Human Hv1 H+ Channels. *J Mol Biol* **387**:1055–1060.

Li Q, Shen R, Treger JS, Wanderling SS, Milewski W, Siwowska K, Bezanilla F, and Perozo E (2015) Resting state of the human proton channel dimer in a lipid bilayer. *Proc Natl Acad Sci* **112**:E5926–E5935.

Liakou CI, Kamat A, Tang DN, Chen H, Sun J, Troncoso P, Logothetis C, and Sharma P (2008) CTLA-4 blockade increases IFN -producing CD4+ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci* **105**:14987–14992.

Liou GY and Storz P (2010) Reactive oxygen species in cancer. *Free Radic Res* **44**:479–496.

Lishko P V, Botchkina IL, Fedorenko A, and Kirichok Y (2010) Acid Extrusion from Human Spermatozoa Is Mediated by Flagellar Voltage-Gated Proton Channel. *Cell* **140**:327–337.

Liu X, Pei C, Yan S, Liu G, Liu G, Chen W, Cui Y, and Liu Y (2015) NADPH oxidase 1-

dependent ROS is crucial for TLR4 signaling to promote tumor metastasis of non-small cell lung cancer. *Tumor Biol* **36**:1493–1502.

Loegering DJ and Lennartz MR (2011) Protein Kinase C and Toll-Like Receptor Signaling. *Enzyme Res* **2011**:1–7.

Lu H, Clauser KR, Tam WL, Fröse J, Ye X, Eaton EN, Reinhardt F, Donnenberg VS, Bhargava R, Carr SA, and Weinberg RA (2014) A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. *Nat Cell Biol* **16**:1105–1117.

Lugini L, Matarrese P, Tinari A, Lozupone F, Federici C, Iessi E, Gentile M, Luciani F, Parmiani G, Rivoltini L, Malorni W, and Fais S (2006) Cannibalism of Live Lymphocytes by Human Metastatic but Not Primary Melanoma Cells. *Cancer Res* **66**:3629–3638.

Mahaut-Smith MP (1989) The effect of zinc on calcium and hydrogen ion currents in intact snail neurones. *J Exp Biol* **145**:455–464.

Mantegazza AR, Savina A, Vermeulen M, Perez L, Geffner J, Hermine O, Rosenzweig SD, Faure F, and Amigorena S (2008) NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood* **112**:4712–4722.

Mantovani A, Sica A, Allavena P, Garlanda C, and Locati M (2009) Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum Immunol* **70**:325–330.

Maraldi T, Prata C, Vieceli Dalla Sega F, Caliceti C, Zambonin L, Fiorentini D, and Hakim G (2009) NAD(P)H oxidase isoform Nox2 plays a prosurvival role in human leukaemia cells. *Free Radic Res* **43**:1111–1121.

Martner A, Wiktorin HG, Lenox B, Ewald Sander F, Aydin E, Aurelius J, Thoren FB, Stahlberg A, Hermodsson S, and Hellstrand K (2015) Histamine Promotes the Development of

Monocyte-Derived Dendritic Cells and Reduces Tumor Growth by Targeting the Myeloid NADPH Oxidase. *J Immunol* **194**:5014–5021.

Medina-Echeverz J, Aranda F, and Berraondo P (2014) Myeloid-derived cells are key targets of tumor immunotherapy. *Oncoimmunology* **3**:e28398.

Meitzler JL, Antony S, Wu Y, Juhasz A, Liu H, Jiang G, Lu J, Roy K, and Doroshow JH (2014) NADPH Oxidases: A Perspective on Reactive Oxygen Species Production in Tumor Biology. *Antioxid Redox Signal* **20**:2873–2889.

Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, and Viola A (2011) Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J Exp Med* **208**:1949–1962.

Morgan D, Capasso M, Musset B, Cherny VV, Ríos E, Dyer MJS, and DeCoursey TE (2009) Voltage-gated proton channels maintain pH in human neutrophils during phagocytosis. *Proc Natl Acad Sci U S A* **106**:18022–18027.

Morgan D, Cherny VV, Finnegan A, Bollinger J, Gelb MH, and DeCoursey TE (2007) Sustained activation of proton channels and NADPH oxidase in human eosinophils and murine granulocytes requires PKC but not cPLA 2 α activity. *J Physiol* **579**:327–344.

Munn DH and Bronte V (2016) Immune suppressive mechanisms in the tumor microenvironment. *Curr Opin Immunol* **39**:1–6.

Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, and Kronenberg M (2009) Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* **10**:1178–1184.

Murdoch C (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of

tumors and other ischemic tissues. *Blood* **104**:2224–2234.

Murphy R and DeCoursey TE (2006) Charge compensation during the phagocyte respiratory burst. *Biochim Biophys Acta - Bioenerg* **1757**:996–1011.

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege J-L, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, and Wynn TA (2014) Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* **41**:14–20.

Musset B, Capasso M, Cherny VV, Morgan D, Bhamrah M, Dyer MJS, and DeCoursey TE (2010) Identification of Thr29 as a critical phosphorylation site that activates the human proton channel Hvcn1 in leukocytes. *J Biol Chem* **285**:5117–5121.

Musset B, Cherny VV, and DeCoursey TE (2012a) Strong glucose dependence of electron current in human monocytes. *AJP Cell Physiol* **302**:C286–C295.

Musset B, Cherny VV, Morgan D, and DeCoursey TE (2009) The intimate and mysterious relationship between proton channels and NADPH oxidase. *FEBS Lett* **583**:7–12.

Musset B, Clark RA, DeCoursey TE, Petheo GL, Geiszt M, Chen Y, Cornell JE, Eddy CA, Brzyski RG, and El Jamali A (2012b) NOX5 in Human Spermatozoa: expression, function, and regulation. *J Biol Chem* **287**:9376–9388.

Musset B and Decoursey T (2012) Biophysical properties of the voltage gated proton channel H(V)1. *Wiley Interdiscip Rev Membr Transp Signal* **1**:605–620.

Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, and Gabrilovich DI (2007) Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* **13**:828–835.

Norian LA, Rodriguez PC, O'Mara LA, Zabaleta J, Ochoa AC, Cella M, and Allen PM (2009) Tumor-Infiltrating Regulatory Dendritic Cells Inhibit CD8+ T Cell Function via L-Arginine Metabolism. *Cancer Res* **69**:3086–3094.

Nozawa H, Chiu C, and Hanahan D (2006) Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci* **103**:12493–12498.

Okochi Y, Sasaki M, Iwasaki H, and Okamura Y (2009) Voltage-gated proton channel is expressed on phagosomes. *Biochem Biophys Res Commun* **382**:274–279.

Ormandy L-A, Farber A, Cantz T, Petrykowska S, Wedemeyer H, Horning M, Lehner F, Manns M-P, Korangy F, and Greten T-F (2006) Direct ex vivo analysis of dendritic cells in patients with hepatocellular carcinoma. *World J Gastroenterol* **12**:3275–3282.

Ostrand-Rosenberg S, Sinha P, Beury DW, and Clements VK (2012) Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol* **22**:275–281.

Palucka K and Banchereau J (2013) Dendritic-Cell-Based Therapeutic Cancer Vaccines. *Immunity* **39**:38–48.

Perona R and Serrano R (1988) Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. *Nature* **334**:438–440.

Perrot I, Blanchard D, Freymond N, Isaac S, Guibert B, Pacheco Y, and Lebecque S (2007) Dendritic Cells Infiltrating Human Non-Small Cell Lung Cancer Are Blocked at Immature Stage. *J Immunol* **178**:2763–2769.

Petheő GL, Orient A, Baráth M, Kovács I, Réthi B, Lányi Á, Rajki A, Rajnavölgyi É, and Geiszt M (2010) Molecular and Functional Characterization of Hv1 Proton Channel in Human

Granulocytes. *PLoS One* **5**:e14081.

Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, and Ben-Neriah Y (2004) NF-κB functions as a tumour promoter in inflammation-associated cancer. *Nature* **431**:461–466.

Pinzon-Charry A, Ho CSK, Maxwell T, McGuckin MA, Schmidt C, Furnival C, Pyke CM, and López JA (2007) Numerical and functional defects of blood dendritic cells in early- and late-stage breast cancer. *Br J Cancer* **97**:1251–1259.

Pinzon-Charry A, Maxwell T, and Lopez JA (2005) Dendritic cell dysfunction in cancer: A mechanism for immunosuppression. *Immunol Cell Biol* **83**:451–461.

Pupo A, Baez-Nieto D, Martínez A, Latorre R, and González C (2014) Proton channel models. *Channels* **8**:180–192.

Pupo A and Gonzalez León C (2014) In pursuit of an inhibitory drug for the proton channel. *Proc Natl Acad Sci* **111**:9673–9674.

Ramsey IS, Moran MM, Chong JA, and Clapham DE (2006) A voltage-gated proton-selective channel lacking the pore domain. *Nature* **440**:1213–1216.

Ramsey IS, Ruchti E, Kaczmarek JS, and Clapham DE (2009) Hv1 proton channels are required for high-level NADPH oxidase-dependent superoxide production during the phagocyte respiratory burst. *Proc Natl Acad Sci* **106**:7642–7647.

Ratner S (1990) Lymphocytes stimulated with recombinant human interleukin-2: relationship between motility into protein matrix and in vivo localization in normal and neoplastic tissues of mice. *J Natl Cancer Inst* **82**:612–616.

Redegeld F, Filippini A, and Sitkovsky M (1991) Comparative studies of the cytotoxic T lymphocyte-mediated cytotoxicity and of extracellular ATP-induced cell lysis. Different

requirements in extracellular Mg2+ and pH. *J Immunol* **147**:3638–3645.

Reuter S, Gupta SC, Chaturvedi MM, and Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic Biol Med* **49**:1603–1616.

Rodriguez PC, Hernandez CP, Morrow K, Sierra R, Zabaleta J, Wyczechowska DD, and Ochoa AC (2010) L-arginine deprivation regulates cyclin D3 mRNA stability in human T cells by controlling HuR expression. *J Immunol* **185**:5198–5204.

Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, and Ochoa AC (2004) Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res* **64**:5839–5849.

Rosenberg SA and Restifo NP (2015) Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**:62–68.

Roy K, Wu Y, Meitzler JL, Juhasz A, Liu H, Jiang G, Lu J, Antony S, and Doroshow JH (2015) NADPH oxidases and cancer. *Clin Sci* **128**:863–875.

Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CMT, Pryer N, Daniel D, Hwang ES, Rugo HS, and Coussens LM (2014) Macrophage IL-10 Blocks CD8+ T Cell-Dependent Responses to Chemotherapy by Suppressing IL-12 Expression in Intratumoral Dendritic Cells. *Cancer Cell* **26**:623–637.

Rybicka JM, Balce DR, Chaudhuri S, Allan ERO, and Yates RM (2012) Phagosomal proteolysis in dendritic cells is modulated by NADPH oxidase in a pH-independent manner. *EMBO J* **31**:932–944.

Sanchez-Sanchez B, Gutierrez-Herrero S, Lopez-Ruano G, Prieto-Bermejo R, Romo-Gonzalez M, Llanillo M, Pandiella A, Guerrero C, Miguel JFS, Sanchez-Guijo F, del Canizo C, and

Hernandez-Hernandez A (2014) NADPH Oxidases as Therapeutic Targets in Chronic Myelogenous Leukemia. *Clin Cancer Res* **20**:4014–4025.

Sasaki M (2006) A Voltage Sensor-Domain Protein Is a Voltage-Gated Proton Channel. *Science* **312**:589–592.

Sasaki M, Tojo A, Okochi Y, Miyawaki N, Kamimura D, Yamaguchi A, Murakami M, and Okamura Y (2013) Autoimmune disorder phenotypes in Hvcn1-deficient mice. *Biochem J* **450**:295–301.

Savina A and Amigorena S (2007) Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev* **219**:143–156.

Savina A, Jancic C, Hugues S, Guermonprez P, Vargas P, Moura IC, Lennon-Duménil AM, Seabra MC, Raposo G, and Amigorena S (2006) NOX2 Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. *Cell* **126**:205–218.

Schilling T, Gratopp A, DeCoursey TE, and Eder C (2002) Voltage-activated proton currents in human lymphocytes. *J Physiol* **545**:93–105.

Schlappack OK, Zimmermann A, and Hill RP (1991) Glucose starvation and acidosis: effect on experimental metastatic potential, DNA content and MTX resistance of murine tumour cells. *Br J Cancer* **64**:663–670.

Schmidt T and Carmeliet P (2010) Blood-vessel formation: Bridges that guide and unite. *Nature* **465**:697–699.

Schmielau J and Finn OJ (2001) Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res* **61**:4756–4760.

Schumacher TN and Schreiber RD (2015) Neoantigens in cancer immunotherapy. *Science* **348**:69–74.

Schwitalla S, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, Canli O, Heijmans J, Huels DJ, Moreaux G, Rupec R a., Gerhard M, Schmid R, Barker N, Clevers H, Lang R, Neumann J, Kirchner T, Taketo MM, Van Den Brink GR, Sansom OJ, Arkan MC, and Greten FR (2013) Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **152**:25–38.

Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, and Korman AJ (2013) Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res* **1**:32–42.

Serafini P (2013) Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly. *Immunol Res* **57**:172–184.

Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, and Borrello I (2004) High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* **64**:6337–6343.

Seredenina T, Demaurex N, and Krause K (2015) Voltage-Gated Proton Channels as Novel Drug Targets: From NADPH Oxidase Regulation to Sperm Biology. *Antioxid Redox Signal* **23**:490–513.

Sharma P and Allison JP (2015) The future of immune checkpoint therapy. *Science* **348**:56–61.

Shin H, Kim J, and Song J-H (2015) Clozapine and olanzapine inhibit proton currents in BV2 microglial cells. *Eur J Pharmacol* **755**:74–79.

Shin H and Song JH (2014) Antipsychotics, chlorpromazine and haloperidol inhibit voltage-gated proton currents in BV2 microglial cells. *Eur J Pharmacol* **738**:256–262.

Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, Fuh G, Gerber HP, and Ferrara N (2007a) Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol* **25**:911–920.

Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale F V, van Bruggen N, Ho C, Ross J, Tan M, Carano RA, Meng YG, and Ferrara N (2007b) Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* **450**:825–831.

Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, Peggs KS, Ravetch J V, Allison JP, and Quezada SA (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti–CTLA-4 therapy against melanoma. *J Exp Med* **210**:1695–1710.

Snelgrove RJ, Edwards L, Williams AE, Rae AJ, and Hussell T (2006) In the absence of reactive oxygen species, T cells default to a Th1 phenotype and mediate protection against pulmonary Cryptococcus neoformans infection. *J Immunol* **177**:5509–5516.

Song JH and Yeh JZ (2012) Dextromethorphan inhibition of voltage-gated proton currents in BV2 microglial cells. *Neurosci Lett* **516**:94–98.

Srivastava MK, Sinha P, Clements VK, Rodriguez P, and Ostrand-Rosenberg S (2010) Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res* **70**:68–77.

Stagg J, Johnstone RW, and Smyth MJ (2007) From cancer immunosurveillance to cancer immunotherapy. *Immunol Rev* **220**:82–101.

Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, and Deepe GS (2013) Granulocyte Macrophage-Colony Stimulating Factor Induced Zn Sequestration Enhances Macrophage Superoxide and Limits Intracellular Pathogen Survival. *Immunity* **39**:697–710.

Supuran CT (2008) Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* **7**:168–181.

Szatrowski TP and Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* **51**:794–798.

Szteyn K, Yang W, Schmid E, Lang F, and Shumilina E (2012) Lipopolysaccharide-sensitive H+ current in dendritic cells. *AJP Cell Physiol* **303**:C204–C212.

Takeshita K, Sakata S, Yamashita E, Fujiwara Y, Kawanabe A, Kurokawa T, Okochi Y, Matsuda M, Narita H, Okamura Y, and Nakagawa A (2014) X-ray crystal structure of voltage-gated proton channel. *Nat Struct Mol Biol* **21**:352–357.

Talmadge JE and Gabrilovich DI (2013) History of myeloid-derived suppressor cells. *Nat Rev Cancer* **13**:739–752.

Tissue expression of HVCN1. The Human Protein Atlas. http://www.proteinatlas.org/ENSG00000122986-HVCN1/tissue.

Toh B, Wang X, Keeble J, Sim WJ, Khoo K, Wong W-C, Kato M, Prevost-Blondel A, Thiery J-P, and Abastado J-P (2011) Mesenchymal Transition and Dissemination of Cancer Cells Is Driven by Myeloid-Derived Suppressor Cells Infiltrating the Primary Tumor. *PLoS Biol* **9**:e1001162.

Tombola F, Ulbrich MH, and Isacoff EY (2008) The Voltage-Gated Proton Channel Hv1 Has Two Pores, Each Controlled by One Voltage Sensor. *Neuron* **58**:546–556.

Torroella-Kouri M, Silvera R, Rodriguez D, Caso R, Shatry A, Opiela S, Ilkovitch D, Schwendener RA, Iragavarapu-Charyulu V, Cardentey Y, Strbo N, and Lopez DM (2009) Identification of a Subpopulation of Macrophages in Mammary Tumor-Bearing Mice That Are Neither M1 nor M2 and Are Less Differentiated. *Cancer Res* **69**:4800–4809.

Tsuchiya Y, Igarashi M, Suzuki R, and Kumagai K (1988) Production of colony-stimulating factor by tumor cells and the factor-mediated induction of suppressor cells. *J Immunol* **141**:699–708.

Ugel S, De Sanctis F, Mandruzzato S, and Bronte V (2015) Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages. *J Clin Invest* **125**:3365–3376.

Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA-K, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist P-H, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, and Ponten F (2015) Tissue-based map of the human proteome. *Science* **347**:1260419–1260419.

Van Ginderachter JA., Movahedi K, Hassanzadeh Ghassabeh G, Meerschaut S, Beschin A, Raes G, and De Baetselier P (2006) Classical and alternative activation of mononuclear phagocytes: Picking the best of both worlds for tumor promotion. *Immunobiology* **211**:487–501.

Vendramini-Costa DB and Carvalho JE (2012) Molecular link mechanisms between inflammation and cancer. *Curr Pharm Des* **18**:3831–3852.

Vesely MD, Kershaw MH, Schreiber RD, and Smyth MJ (2011) Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* **29**:235–271.

Vignais PV (2002) The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. *Cell Mol Life Sci* **59**:1428–1459.

Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, and

Bluestone JA (1994) CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1**:405–413.

Wan S, Zhao E, Kryczek I, Vatan L, Sadovskaya A, Ludema G, Simeone DM, Zou W, and Welling TH (2014) Tumor-Associated Macrophages Produce Interleukin 6 and Signal via STAT3 to Promote Expansion of Human Hepatocellular Carcinoma Stem Cells. *Gastroenterology* **147**:1393–1404.

Wang VY, Huang W, Asagiri M, Spann N, Hoffmann A, Glass C, and Ghosh G (2012a) The Transcriptional Specificity of NF-κB Dimers Is Coded within the κB DNA Response Elements. *Cell Rep* **2**:824–839.

Wang Y, Li SJ, Pan J, Che Y, Yin J, and Zhao Q (2011) Specific expression of the human voltage-gated proton channel Hv1 in highly metastatic breast cancer cells, promotes tumor progression and metastasis. *Biochem Biophys Res Commun* **412**:353–359.

Wang Y, Li SJ, Wu X, Che Y, and Li Q (2012b) Clinicopathological and Biological Significance of Human Voltage-gated Proton Channel Hv1 Protein Overexpression in Breast Cancer. *J Biol Chem* **287**:13877–13888.

Wang Y, Wu X, Li Q, Zhang S, and Li SJ (2013a) Human voltage-gated proton channel hv1: a new potential biomarker for diagnosis and prognosis of colorectal cancer. *PLoS One* **8**:e70550.

Wang Y, Zhang S, and Li SJ (2013b) Zn2+ induces apoptosis in human highly metastatic SHG-44 glioma cells, through inhibiting activity of the voltage-gated proton channel Hv1. *Biochem Biophys Res Commun* **438**:312–317.

Warburg O, Wind F, and Negelein E (1927) The metabolism of tumors in the body. *J Gen Physiol* **8**:519–530.

Watkins SK, Zhu Z, Riboldi E, Shafer-Weaver KA, Stagliano KER, Sklavos MM, Ambs S, Yagita H, and Hurwitz AA (2011) FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer. *J Clin Invest* **121**:1361–1372.

Weber JS, O'Day S, Urba W, Powderly J, Nichol G, Yellin M, Snively J, and Hersh E (2008) Phase I/II Study of Ipilimumab for Patients With Metastatic Melanoma. *J Clin Oncol* **26**:5950–5956.

Whiteside TL (2010) Inhibiting the inhibitors: evaluating agents targeting cancer immunosuppression. *Expert Opin Biol Ther* **10**:1019–1035.

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, and Sakaguchi S (2008) CTLA-4 Control over Foxp3+ Regulatory T Cell Function. *Science* **322**:271–275.

Wu LJ, Wu G, Sharif MRA, Baker A, Jia Y, Fahey FH, Luo HR, Feener EP, and Clapham DE (2012) The voltage-gated proton channel Hv1 enhances brain damage from ischemic stroke. *Nat Neurosci* **15**:565–573.

Wu Y, Lu J, Antony S, Juhasz A, Liu H, Jiang G, Meitzler JL, Hollingshead M, Haines DC, Butcher D, Roy K, and Doroshow JH (2013) Activation of TLR4 Is Required for the Synergistic Induction of Dual Oxidase 2 and Dual Oxidase A2 by IFN- and Lipopolysaccharide in Human Pancreatic Cancer Cell Lines. *J Immunol* **190**:1859–1872.

Yan Z, Garg SK, Kipnis J, and Banerjee R (2009) Extracellular redox modulation by regulatory T cells. *Nat Chem Biol* **5**:721–723.

Yang JC, Hughes M, Kammula U, Royal R, Sherry RM, Topalian SL, Suri KB, Levy C, Allen T, Mavroukakis S, Lowy I, White DE, and Rosenberg SA (2007) Ipilimumab (Anti-CTLA4 Antibody) Causes Regression of Metastatic Renal Cell Cancer Associated With Enteritis

and Hypophysitis. *J Immunother* **30**:825–830.

Yang M, Ma C, Liu S, Shao Q, Gao W, Song B, Sun J, Xie Q, Zhang Y, Feng A, Liu Y, Hu W, and Qu X (2010) HIF-dependent induction of adenosine receptor A2b skews human dendritic cells to a Th2-stimulating phenotype under hypoxia. *Immunol Cell Biol* **88**:165–171.

Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, and Liu Z-G (2013) ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Res* **23**:898–914.

Zhu X, Mose E, and Zimmermann N (2013) Proton channel HVCN1 is required for effector functions of mouse eosinophils. *BMC Immunol* **14**:24.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES:

This work was supported by the National Fund for Scientific and Technological Development of Chile [FONDECYT Grant 1160261]; a postdoctoral fellowship from the Interdisciplinary Center for Neurosciences of Valparaíso; and a doctoral fellowship from the National Commission for Scientific and Technological Research of Chile. The Interdisciplinary Center for Neurosciences of Valparaíso is a Millennium Institute supported by the Millennium Initiative of the Ministry of Economy, Development and Tourism of Chile.

The authors declare no conflict of interest.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS:

Figure 1. Structure of voltage-gated proton channel, Hv1, and some of its inhibitors. (A) Structure of the mHv1cc chimeric channel (PDB file 3WKV (Takeshita et al., 2014)) representing the membrane domain in a closed conformation, plus N and C-terminal domains fragments. The backbone is shown as a cartoon colored in a blue-yellow-red gradient from the N to the C-terminus. **(B)** Model of the membrane domain of human Hv1 channel in the open conformation (R2D model from (Kulleperuma et al., 2013)), represented as in **A**. **(C)** Examples of the only group of specific Hv1 inhibitors known to date, the guanidine derivatives. Left: 2-guanidinobenzimidazole (2GBI), right: Cl-guanidinobenzimidazole (ClGBI) (Hong et al., 2013, 2014). The compounds are represented as sticks colored by atom type (C: cyan, N: blue, H: white and Cl: green).

Figure 2. Role of proton channel Hv1 in tumor biology. (A) Hv1 proton channel extrudes the excess of protons generated during tumor cell metabolism, maintaining intracellular pH (pHi) within physiological values and contributing to the acidification of the extracellular pH (pHo). This function of Hv1 supports tumor cell proliferation, migration and invasiveness, whereas extracellular pH acidification impairs T cell proliferation, cytotoxicity, and induces apoptosis of T cells in the same conditions where tumor cells survive, thus promoting tumor growth. **(B)** The pharmacological inhibition of Hv1 channel could lead to a fatal decrease of the intracellular pH in tumor cells, and this might hinder their proliferation, migration and invasiveness, in addition to promoting their apoptosis. The associated increase in the extracellular pH could also potentiate T cell function through enhanced proliferation and cytotoxicity, together with decreased apoptosis. In consequence, the inhibition of Hv1 could contribute to tumor rejection.

Molecular Pharmacology Fast Forward. Published on June 3, 2016 as DOI: 10.1124/mol.116.103804
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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 up-regulation 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 ζ chain, contributes to enhance TANs infiltration, reinforces inflammation, and polarizes TAMs towards 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 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 anti-tumoral and tumor-supporting immune populations towards tumor rejection.

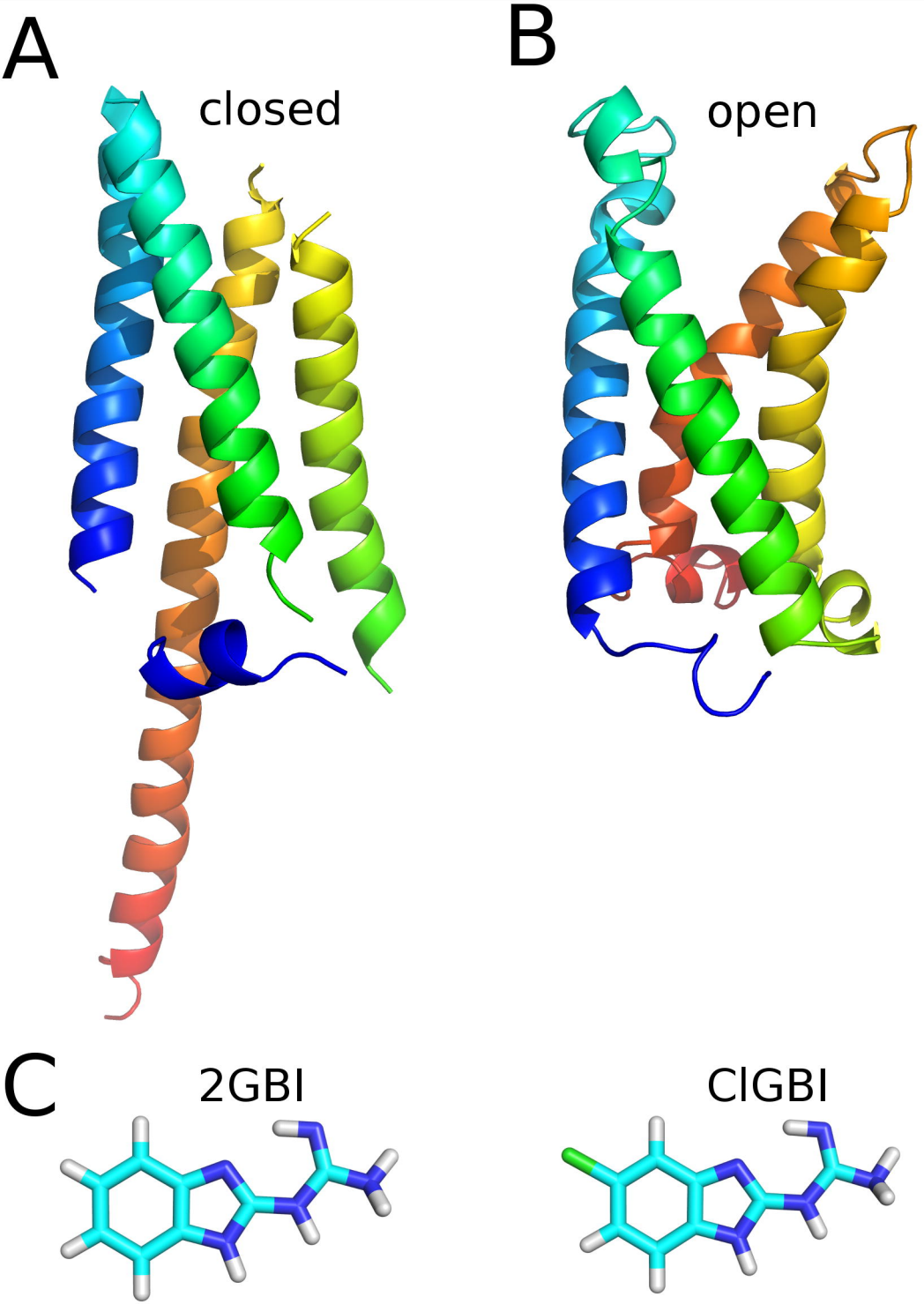

Figure 1

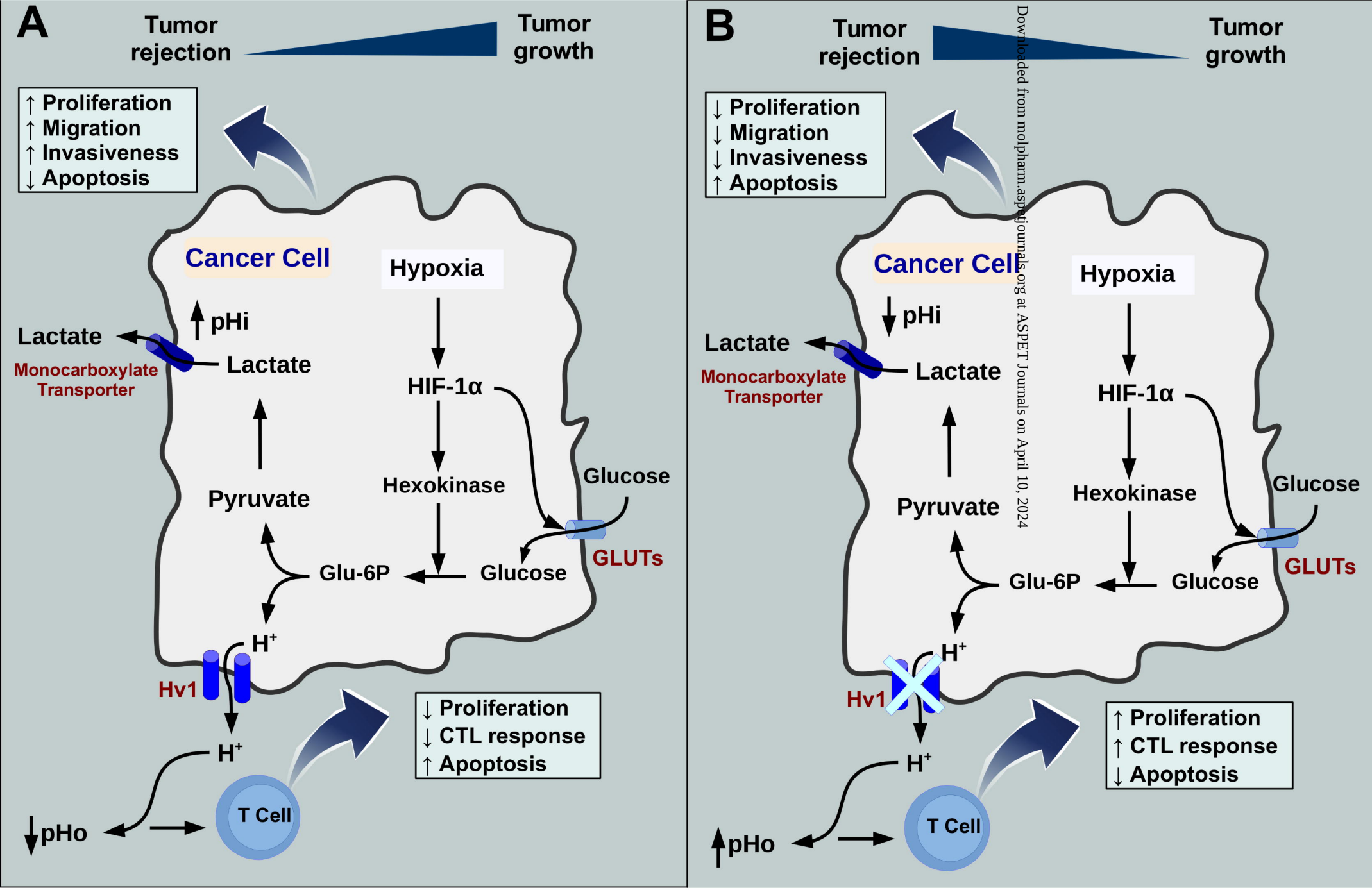

Figure 2

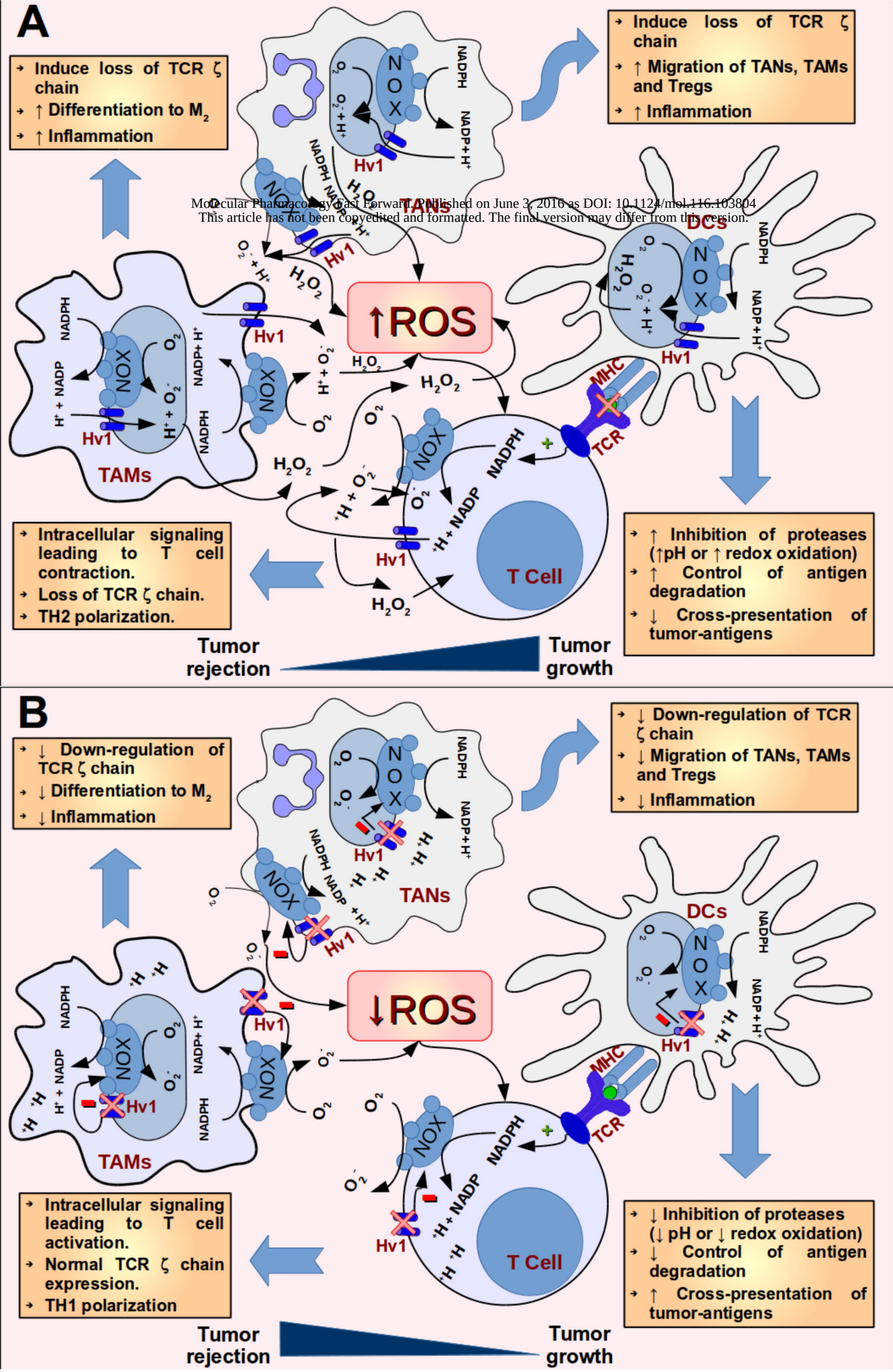

Figure 3