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REVIEW

A new therapeutic target: the CD69-Myl9 system in immune responses

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Abstract

CD69 is an activation marker on leukocytes. Early studies showed that the $CD69^+$ cells were detected in the lung of patients with asthmatic and eosinophilic pneumonia, suggesting that CD69 might play crucial roles in the pathogenesis of such inflammatory diseases, rather than simply being an activation marker. Intensive studies using mouse models have since clarified that CD69 is a functional molecule regulating the immune responses. We discovered that Myosin light chain 9, 12a, 12b (Myl9/12) are ligands for CD69 and that platelet-derived Myl9 forms a net-like structure (Myl9 nets) that is strongly detected inside blood vessels in inflamed lung. CD69-expressing activated T cells attached to the Myl9 nets can thereby migrate into the inflamed tissues through a system known as the CD69-Myl9 system. In this review, we summarize the discovery of the CD69-Myl9 system and discuss how this system is important in inflammatory immune responses. In addition, we discuss our recent finding that CD69 controls the exhaustion status of tumor-infiltrating T cells and that the blockade of the CD69 function enhances anti-tumor immunity. Finally, we discuss the possibility of CD69 as a new therapeutic target for patients with intractable inflammatory disorders and tumors

Keywords CD69 · Myl9 · Inflammation · Allergy · CD69–Myl9 system · Anti-tumor immunity

Structure and function of CD69

CD69 is a type-2 glycoprotein with C-type lectin-like domains [1–3] and is expressed on the cell surface as a disulfide linked homo-dimer, with three distinct regions (an extracellular domain, a transmembrane domain, and an intracellular domain) each exerting particular functions [3]. Although CD69 has C-type lectin-like domains, it does not contain Ca^{2+}-dependent carbohydrate binding sites and thus does not function as a C-type lectin molecule [4]. CD69 has two N-glycosylation sites in humans and three in mice, and the glycosylation status of the CD69 molecule might influence its function, although this has not been clarified [3]. Interestingly, a three-dimensional structure analysis showed that homo-dimers of CD69 create a hydrophobic patch surrounded by acidic residues, which can work as a hypothetical ligand-binding site [4]. Accordingly, CD69 may bind to its ligands through such a hypothetical ligand-binding site by protein-protein interaction; however, it took a long time to discover such ligands.

Three groups recently independently reported the molecules that interact with CD69 [5–7]. The first report was published in 2014, showing that galectin-1 can bind to CD69 [6]. The authors showed that human CD69 expressed by T cells can bind to galectin-1 expressed on dendritic cells (DCs), and that the interaction negatively controls the T helper 17 (Th17) cell differentiation, at least in vitro [6]. However, galectin-1 cannot be the specific ligand for CD69 because galectin-1 is a glycan-binding protein with a carbohydrate recognition domain (CRD) that can bind to many glycosylated receptors on a variety of cells [8–11]. In 2015, another report showed that the heterodimer of S100A8 and S100A9 (S100A8/A9, calprotectin) binds to CD69, regulating the regulatory T cell differentiation [5]. S100A8/A9 is known to be secreted by granulocytes, monocytes, and DCs under inflammatory conditions and tumorigenesis. These data suggest that S100A8/A9 may be involved in inflammation and the tumor microenvironment, where CD69-expressing leukocytes are located

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[12]. In 2016, our group discovered that myosin light chain 9, 12a, 12b (Myl9/12) are functional ligands for CD69. The Myl9/12 expression is increased under conditions of an inflammatory response and creates net-like structures inside vessels, which we have termed "Myl9 nets." We think that CD69-expressing activated T cells migrate into and are maintained within the inflamed tissues through their interaction with the Myl9 nets in a system called the "CD69-Myl9 system" [7]. We will discuss the details of the CD69-Myl9 system in a later section of this review.

As we mentioned above, three groups independently reported that CD69 extracellular domains can bind to proteins. However, whether or not those interactions provide any intracellular signaling through CD69 intracellular domains has not been directly examined. CD69 may function as a kind of "adhesion molecule," but alternatively, CD69 may be a signaling molecule. The molecular mechanisms underlying the biological relevance affected by the interaction of CD69 with its interacting molecules, such as Myl9/12, S100A8/A9, and galectin-1, or unknown other molecules still need to be investigated.

The signaling capacity of CD69 molecules is still under debate, with no definitive conclusions yet drawn [3]. Interestingly, the intracellular domains of the CD69 molecule include eight serine residues in both humans and mice and two and one threonine residues in humans and mice, respectively [1, 2], suggesting the possible signaling ability of the CD69 molecule. In fact, some residues of the intracellular domain of CD69 are reported to be phosphorylated in activated lymphocytes and thymocytes [13, 14]. In addition, early in vitro studies using anti-CD69 antibody (Ab) showed that the cross-linking of the CD69 molecule in the presence of phorbol esters enhanced TCR signaling by prolonging both the Ca^{2+} influx and extracellular signal-regulated kinase (ERK)1/2 kinase activation in human T cells [14, 15]. Furthermore, the intracellular domain of CD69 was reported to be able to associate with Janus family kinase (JAK)3 and activate STAT5 and STAT1 signals, thereby regulating Th17 differentiation [16]. However, how this association occurs is unclear, as the CD69 intracellular domain has neither a JAK-binding motif (i.e., the conserved box1 motif) nor any STAT-binding tyrosine residues.

One of the best-known functions of CD69 is that CD69 negatively regulates the Sphingosine-1-phospate receptor 1 ($S1P_1$) function [17] through a system we call the "CD69–$S1P_1$ system" in this review. CD69 binds to $S1P_1$ through the juxtamembrane region of the intracellular domain of CD69 (i.e., HEGSF in humans, HEGSI in mice), and the binding of $S1P_1$ to this motif in the CD69 molecule results in the internalization of the CD69-$S1P_1$ complex [17, 18]. Since the egress of thymocytes and lymphocytes from lymphoid tissues requires the surface expression of $S1P_1$ to respond to S1P, the concentration of which is higher in blood (approximately 1 μM) and lymph (100 nM) than in lymphoid organs [19], the loss of $S1P_1$ surface expression prevents the egress of thymocytes and lymphocytes from lymphoid tissues [17, 18, 20]. Indeed, the thymus from CD69 full-length transgenic (Tg) mice showed the accumulation of mature T cells, indicating that CD69 downregulation is necessary for mature T cells to exit the thymus [21, 22]. These data suggest that CD69 expression can control the residency time of thymocytes during positive and negative selection [23]. However, CD69-deficient mice did not show any obvious defects of thymocyte development [24, 25]; therefore, the CD69 expression during positive and negative selection in the thymus may not be important for thymocyte development.

Notably, in 2018, our group showed that the CD69-$S1P_1$ system is important for the development of NKT2 cells [26]. CD69 deficiency promotes the early egress of NKT2 precursor cells from the thymus due to their early expression of $S1P_1$, thereby preventing them from becoming NKT2 cells [26]. This finding demonstrates that the residency time in the thymus that is controlled by CD69 expression is important for the proper development of a subset of T cells.

The importance of the CD69-$S1P_1$ system in the immune response upon infection has been reported [20, 27, 28]. When infection occurs, antigen-presenting cells (APCs), such as DCs, capture antigens and present them to T cells in the draining lymph nodes (LNs). Within the draining LNs, IFNα/β rapidly induces CD69 expression on T cells, which prevents T cells from leaving the LNs so that antigen-specific T cells are efficiently stimulated by APCs [20, 28]. The CD69 expression on T cells is further upregulated by antigen stimulation by APCs, which allows T cells to be retained in the LN and receive sufficient stimulation to become effector T cells until they are ready to migrate into the inflamed tissues [29]. Thus, the CD69-$S1P_1$ system is important for lymphocyte egress from lymphoid organs, such as the thymus and LNs, in both the steady state and during infection.

Role of CD69 on allergic respiratory inflammation

Our group has been focusing on CD69 biology with particular attention paid to the function of CD69 during immune responses, especially under inflammatory conditions. We made CD69-deficient mice and examined the influence of CD69 deficiency on airway inflammation [30]. We used an OVA-induced allergic airway inflammation model, which is a standard method of inducing allergic airway inflammation in systemically immunized mice with OVA/Alum two times on days 0 and 7 followed by challenging the mice with OVA inhalation on days 14 and 16. With this protocol, *Cd69*$^{+/+}$ wild-type mice showed massive infiltrations of eosinophils, neutrophils, lymphocytes, and macrophages into the bronchoalveolar

lavage (BAL) fluid; in contrast, the infiltration of inflammatory leucocytes was significantly ameliorated in *Cd69*$^{-/-}$ mice [30]. HE staining of lung tissues also revealed that the infiltration of leucocytes in *Cd69*$^{-/-}$ mice was significantly decreased compared to that in *Cd69*$^{+/+}$ mice. Furthermore, airway inflammation was associated with an increase in methacholine-induced airway hyperresponsiveness (AHR) in *Cd69*$^{+/+}$ wild-type mice, whereas the AHR in *Cd69*$^{-/-}$ mice was significantly reduced. These data demonstrate that CD69 deficiency ameliorated airway inflammation and reduced AHR [30]. To examine whether or not the CD69 expression on CD4T cells is important for airway inflammation, we generated ectopic CD69-expressing CD4T cells derived from *Cd69*$^{-/-}$ mice, adoptively transferred them into *Cd69*$^{-/-}$ host mice, and then induced OVA-induced allergic airway inflammation. *Cd69*$^{-/-}$ host mice with CD69-expressing CD4T cells developed airway inflammation, suggesting that CD69 expression on CD4T cells is sufficient to induce the development of airway inflammation [7, 30].

Importantly, we found that the injection of anti-CD69 Ab 1 day before the OVA inhalation strongly ameliorated airway inflammation and AHR. Another group also showed that anti-CD69 Ab treatment inhibited established airway inflammation as effectively as dexamethasone (DXM) treatment [31]. Furthermore, anti-CD69 Ab treatment together with DXM treatment completely inhibited airway inflammation [31]. The fact that anti-CD69 Ab treatment as well as CD69 deficiency can prevent airway inflammation suggested the existence of some ligands for CD69 expressed at the inflamed sites. We will discuss the details of our discovery of such ligands in a later section of this review.

Involvement of CD69 in several inflammatory disorders

Several studies using mouse models have shown evidence that CD69 plays crucial roles in the pathogenesis of not only airway inflammation but also various inflammatory disorders, such as arthritis, cigarette smoke-induced pulmonary inflammation, lung fibrosis, and colitis. We first reported that murine arthritis induced by the injection of anti-type II collagen Ab together with lipopolysaccharide (LPS) was significantly ameliorated in *Cd69*$^{-/-}$ mice due to a substantial decrease in the number of inflammatory neutrophils in the joint [24]. Another group reported that cigarette smoke-induced pulmonary inflammation [32] and bleomycin-induced lung injury fibrosis [33] were also ameliorated in *Cd69*$^{-/-}$ mice due to less infiltration of CD69-expressing macrophages, neutrophils, and lymphocytes into the lung. CD69 expression on CD4 T cells is also important for the pathogenesis of the murine colitis model. We showed that the severity of dextran sulfate sodium (DSS)-induced colitis, which is a model of human ulcerous colitis (UC), was significantly ameliorated in *Cd69*$^{-/-}$ mice [34]. Importantly, anti-CD69 Ab treatment also ameliorated the pathogenesis of DSS-induced colitis, indicating the existence of some ligands for CD69 expressed at inflamed sites.

In contrast to DSS-induced colitis which is ameliorated in *Cd69*$^{-/-}$ mice, the severity of the colitis induced by CD25^{-}CD45RB^{high} T cell injection into lymphopenic host mice, such as *Rag*$^{-/-}$ mice, was shown to be exacerbated with CD69-deficient T cells[35, 36]. It has been reported that regulatory T cells (Tregs) are crucial to prevent the onset of any pathogenesis in this colitis model, and they are induced by the injection of CD25^{-}CD45RB^{high} T cells. Since CD69 is known to be expressed on a subset of Tregs [37], CD69 may be important for the function of Tregs, which negatively regulate the immune response. As we mentioned above, the influence of CD69 deficiency differs among colitis models [34–36], possibly due to the different contributions of CD69-expressing effector T cells and Tregs in each model.

Not only murine disease models but also several early studies using samples from human patients with different diseases have revealed the possible involvement of CD69 in inflammatory disorders [3]. We have already summarized the findings of several reports demonstrating the expression of CD69 in samples from patients with various diseases [3].

Identification of Myosin light chain 9, 12a and 12b as ligands of CD69

The experimental findings that anti-CD69 Ab treatment prevents inflammatory diseases, such as allergic inflammation and DSS-induced colitis, suggested that a CD69 ligand should exist somewhere in inflamed tissues and that anti-CD69 Ab treatment may prevent the interaction between CD69 and the CD69 ligand, thus inhibiting the CD69 function that is crucial for the pathogenesis of inflammatory responses. To identify such ligands for CD69, we prepared recombinant murine CD69 extracellular protein (GST-His-CD69EC) and performed a pull-down assay. Because our previous study suggested that the CD69 ligand should exist in the bone marrow (BM) [38], we used BM lysates to purify the molecules that interact with GST-His-CD69EC protein. We detected the specific interacting proteins, which were then purified and identified as Myl9 by liquid chromatography with tandem mass spectrometry [7].

Myl9 is a regulatory component of myosin protein and small protein whose molecular weight is about 19 kDa. Myl9 is known to interact with non-muscle myosin heavy chain (Myh9, Myh10, and Myh14), especially Myh9. Myl9 belongs to the myosin light chain family of molecules, which has 10 family members, and Myl9 has high homology to Myl12a (94%) and Myl12b (94%).

To determine whether or not CD69 and Myl9 can truly physically interact with each other, we generated recombinant murine Myl9 and confirmed its interaction with CD69 using an immuno-precipitation assay as well as an enzyme-linked immunosorbent assay. We further confirmed that CD69 also physically interacts with Myl12a and Myl12b, at least in vitro. Myl9 consists of two EF-hand domains with a small N-terminal region that are highly positively charged with several lysine residues [7]. We made several Myl9 mutants with the positively charged lysine residues removed from the N-terminal region of the Myl9 protein and found that the positively charged N-terminal region is indeed required for the interaction with CD69 [7]. Subsequently, we used the positively charged N-terminal region of Myl9 as an immunogen and made two monoclonal and several polyclonal Abs. We confirmed that all antibodies recognized Myl9 and that two monoclonal Abs also recognized Myl12a and Myl12b; therefore, we named these Abs mouse anti-Myl9/12 monoclonal Ab (114-2G9; KAN Research Institute Inc.) and rabbit anti-Myl9/12 monoclonal Ab (F-6; Abwiz Bio Inc.). We also confirmed that the two monoclonal Abs and several polyclonal Abs were functional Abs capable of inhibiting the interaction between CD69 and Myl9, Myl12a, or Myl12b [7].

We next examined the effects of anti-Myl9/12 Ab injection on the pathogenesis in airway inflammatory responses using an OVA-induced airway inflammation model and house dust mite (HDM)-induced airway inflammation model. Airway inflammation was significantly ameliorated in the mice treated with anti-Myl9/12 Abs, in a manner similar to that of anti-CD69 Ab treatment [7]. These results indicate that CD69-expressing cells interact with Myl9 somewhere in the inflamed tissues, and these interactions result in the pathogenesis of airway inflammation. Both anti-CD69 and anti-Myl9/12 Ab treatments inhibited the interaction between CD69 and Myl9, thereby preventing airway inflammation (Fig. 1).

Myl9 expression in inflamed tissues

Regarding where the CD69 ligands—Myl9, Myl12a, and Myl12b—are expressed in inflamed tissues, since Myl9 is considered to be a cytosolic protein, it is unusual that it can work as a ligand for CD69 that is expressed on the cell surface of activated leukocytes. To reconcile this issue, we first performed immunohistochemistry experiments to identify where the Myl9/12 proteins were expressed within the inflamed tissues using confocal microscopy [7]. Interestingly, we detected the Myl9/12 protein within the blood vessels but not inside the inflamed lungs. Furthermore, the Myl9/12 expression was barely detected in the lung from healthy mice, suggesting that this expression was induced within the blood vessel upon exposure to inflammatory stimuli. To analyze the expression profiles of Myl9 inside blood vessels in greater detail, we obtained a slightly thicker section of inflamed lungs than usual and analyzed the expression of Myl9. Interestingly, we found that Myl9 formed a net-like structure inside the vessels in inflamed lungs. Accordingly, we decided to call this net-like structure of Myl9 as “Myl9 nets” [7] (Fig. 1). We also found that platelets and leucocytes were tangled in Myl9 nets and that Myl9 nets existed together with the von Willebrand factor (vWF) inside blood vessels [7].

To further clarify the possible involvement of Myl9 nets in the pathogenesis of human inflammatory disorders, we next analyzed the polyps from eosinophilic chronic rhinosinusitis (ECRS) patients [7]. ECRS is an intractable upper airway inflammatory disease, and half of patients also suffer from intractable asthma. The patients often develop nasal polyps that are infiltrated with eosinophils, lymphocytes, and plasma cells [39] and are treated with surgical resection; however, the recurrence rate is high, so a new, more effective therapy is desired. We detected significant amounts of Myl9 nets in the polyps from ECRS patients, not only inside of blood vessels but also in the perivascular regions of vessels. Furthermore, we detected CD69-expressing cells entangled in Myl9 nets, suggesting that CD69-expressing cells interact with Myl9 nets and become trapped in the polyps, resulting in exacerbated inflammation [7, 40]. These data suggest that Myl9/12 plays crucial roles in the pathogenesis of human chronic inflammatory disorders.

The CD69-Myl9 system regulates inflammatory responses

How is the expression of Myl9/12 induced inside of blood vessels under inflammatory conditions? Since Myl9, Myl12a, and Myl12b are cytosolic proteins, some mechanisms are required to transport them outside of cells. We first considered the possibility that inflammatory signal damages endothelial cells, leading to their cell death so that Myl9 can be transferred outside of the cells. To prove this possibility, we attempted to detect the Myl9/12 expression on the cell surface of blood vessels by flow cytometry; however, we failed to detect the significant expression of Myl9 on the surface of endothelial cells. Furthermore, we were not able to detect the merged staining of Myl9/12 and PECAM-1, which is a marker of endothelial cells, in confocal microscopy analyses. Instead, we detected Myl9/12 further inside the areas of PECAM-1 staining, suggesting that Myl9/12 is merely attached to the surface of endothelial cells and not expressed by endothelial cells [7].

Myl9 is a microparticle that is reportedly released from activated platelets [41]. Myh9 is known to form a complex with Myl9 that is highly expressed in platelets [42, 43]. These data suggested that Myl9 should be contained in platelets and that Myl9 may be released from the activated platelets upon

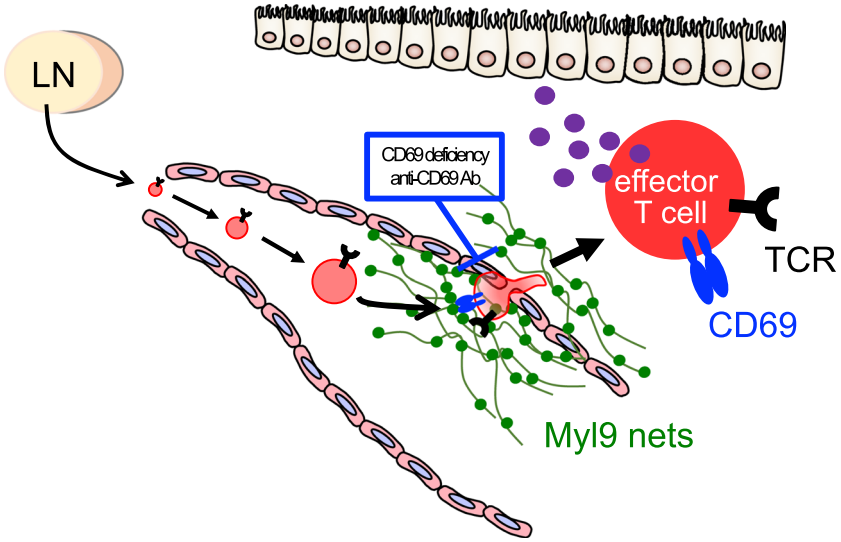

Fig. 1 The CD69–Myl9 system in inflamed tissues. Upon inflammation, antigen-specific T cells are primed in the draining LNs and migrate into the inflamed region through the bloodstream. At the inflamed site, the platelet-derived Myl9 molecule is detected inside the blood vessel, forming a net-like structure called an "Myl9 net." Myl9 nets serve as a kind of "platform," allowing CD69-expressing antigen-specific T cells to migrate into the inflamed tissues where they produce effector cytokines and chemokines to promote inflammatory responses. CD69 deficiency or Ab treatments against CD69 and Myl9 prevent the recruitment of effector T cells into the inflamed tissues (and their maintenance in the inflamed tissues under conditions of chronic inflammation), thereby preventing inflammation

exposure to inflammatory stimuli. To prove this idea, we first prepared platelet-rich plasma from mouse sera and examined whether or not platelets actually contains Myl9, Myl12a, and Myl12b. We found that platelets contained significant amounts of Myl9 mRNA, whereas the Myl12a and Myl12b mRNA expression was very low [7]. Importantly, in vitro thrombin stimulation prompted platelets to produce Myl9 outside of cells [7], suggesting that Myl9 is indeed produced by platelets upon inflammation and creates Myl9 nets inside of blood vessels. To examine whether or not the Myl9 detected on the luminal surface of blood vessels in inflamed lung was actually derived from the activated platelets, we injected the platelet depletion Ab anti-GP1bα Ab into mice 1 day before and during OVA inhalation. The Myl9 expression was significantly decreased in the mice treated with anti-GP1bα Ab, demonstrating that the Myl9 expression in inflamed lung was indeed derived from activated platelets.

Upon inflammation, blood vessels are damaged, and platelets are immediately activated to repair the injured vessels by clot formation. Importantly, the function of platelets is not only hemostasis but also the activation of the coagulation system to produce microparticle, including various coagulation factors, which activates the immune system. We think that, upon inflammation, activated platelets produce Myl9 together with various coagulation factors, which results in the formation of clots including Myl9, the creation of Myl9 nets, and the promotion of the efficient activation of the immune system. Activated CD69-expressing antigen-specific T cells bind to the Myl9 nets inside of the vessels, which promote the efficient recruitment of these cells into the inflamed tissues. We named this system the CD69-Myl9 system (Fig. 1). As mentioned in an earlier section, we have also detected Myl9 nets within tissues, such as ECRS polyps and in cases of chronic inflammatory disorder; we therefore think that the CD69-Myl9 system causes antigen-specific T cells to be retained in the inflamed tissues, which results in the exacerbation of inflammation.

One issue raised regarding the CD69-Myl9 system is when and where CD69 expression is induced on antigen-specific T cells. Antigen-specific T cells are known to be first activated in the draining LN and then move to the inflamed site through the blood stream [3, 17, 20, 28]. Interestingly, CD69 is first upregulated upon primary activation within LNs and then downregulated, which is necessary for the egress of antigen-specific T cells from the LNs. Accordingly, antigen-specific T cells in the blood stream should not express CD69, and indeed, our data showed that no antigen-specific T cells express CD69 in the peripheral blood. However, we detected CD69-expressing antigen-specific T cells in the lung vasculature of mice with airway inflammation [7]. These data suggest that an inflamed environment promotes the CD69 expression in antigen-specific T cells in the vasculature. One possible reason for this is that the cytokine-rich environment in inflamed sites induces CD69 expression on T cells that had been primed by specific antigens in the draining LNs. Indeed, some inflammatory cytokines are known to be able to activate lymphocytes and are likely present in inflamed tissues [3, 44–49]. Another possibility is that antigen-presenting cells may exist within the Myl9 nets in the inflamed vasculature.

In summary, the CD69-Myl9 system regulates inflammatory responses. Upon inflammation, platelets are activated and produce Myl9 to form Myl9 nets in the vasculature. CD69-expressing antigen-primed T cells attach to the Myl9 nets, through which they are efficiently recruited into the inflamed tissues (Fig. 1). In chronically inflamed tissues, such as ECRS polyps, Myl9 nets are often detected even in the perivascular regions, which may cause inflammatory lymphocytes to be retained within the tissues and exacerbate inflammation [3].

Role of CD69 in cancer immunology

Since CD69 plays an important role in cell retention in tissues, its expression on lymphocytes may have a unique function in the tumor microenvironment. Three groups independently reported that the blockade of the CD69 function by either genetic knockout or the administration of anti-CD69 Abs resulted in the augmentation of anti-tumor responses [50–53].

The first report showing that CD69 is involved in anti-tumor responses was published in 2003 [50], and the same group later followed up on their study [51]. The authors showed that the growth of MHC-I-deficient tumors (RMA-S and RM-1) was significantly reduced in *Cd69*$^{-/-}$ mice and that the survival of tumor-bearing *Cd69*$^{-/-}$ mice was better than that of *Cd69*$^{+/+}$ mice [50]. Furthermore, the administration of anti-CD69 Abs also prevented tumor growth [50, 51]. Mechanistically, the authors showed that the number of NK and T cells was increased in tumor-bearing *Cd69*$^{-/-}$ mice and that TGFβ production by NK and T cells was reduced [50, 51]. Since TGFβ suppresses anti-tumor responses [54–56] by inhibiting T cell infiltration into tumors [55, 56] or by affecting the exhaustion status of T cells [57], it is reasonable that a reduction in TGFβ production in tumor-bearing *Cd69*$^{-/-}$ mice results in the augmentation of the anti-tumor immune responses. In 2017, another group reported that anti-CD69 Ab treatment for BALB/c mice prevented the growth of Renca renal cell carcinoma cells. The authors showed that anti-CD69 Ab treatment augmented the anti-tumor responses by enhancing the T cell proliferation, IL-2 production, and cytotoxic activity. Furthermore, this group showed that the combination of a dendritic cell-based vaccine and anti-CD69 Ab synergistically inhibited tumor growth [52], suggesting the potential utility of combination therapy using anti-CD69 Ab.

In 2018, our group showed that the growth of 4T1 (breast cancer), CT26 (colon cancer), and A20 (diffuse large B cell lymphoma) cells was prevented in *Cd69*$^{-/-}$ mice and that *Cd69*$^{-/-}$ mice had less lung metastasis than *Cd69*$^{+/+}$ mice [53]. We also found that the number of tumor-infiltrating T cells and the production of effector cytokines were significantly increased in tumor-bearing *Cd69*$^{-/-}$ mice [53]. Importantly, CD69-deficient tumor-infiltrating CD8 T cells showed a less-exhausted phenotype than CD69-sufficient tumor-infiltrating CD8 T cells, indicating important roles of CD69 in inducing the exhaustion of tumor-infiltrating T cells [53]. Furthermore, we showed that anti-CD69 Ab injection into tumor-bearing mice prevented lung metastasis, with tumor-infiltrating CD8 T cells shown to be less exhausted than the mice injected with control Ab [53].

However, how the CD69 expression promotes exhaustion of tumor-infiltrating CD8 T cells is unclear. Considering the function of CD69, which plays an important role in tissue retention [3], one possible mechanism is that CD69 expression promotes the retention of tumor-infiltrating CD8 T cells within the tumor; therefore, the cells may receive chronic tumor antigen stimulation, which results in T cell exhaustion (Fig. 2). Since there seem to be several mechanisms by which CD69 expression promotes the retention of cells in tissue, depending on the situation (i.e., normal or under inflammatory conditions, lymphoid organs or peripheral tissues, etc.) [3], further experiments will be required to clarify whether or not CD69 is involved in the retention of its expressing cells within the tumor. Another possibility is that CD69-mediated signaling itself may be involved in the induction of exhaustion in tumor-infiltrating T cells. In fact, previous reports have shown that CD69 engagement promotes TGFβ production [50, 51] and that TGFβ induces exhaustion of memory CD8 T cells [57]. Further experiments are required to elucidate the detailed mechanisms underlying the CD69-mediated induction of T cell exhaustion and inhibition of anti-tumor responses.

Whether or not all tumor-infiltrating CD8 T cells are critical for initiating a response to cancer immunotherapy is unclear. Several reports have shown that tumor-resident CD8 T cells were present in the tumor microenvironment and associated

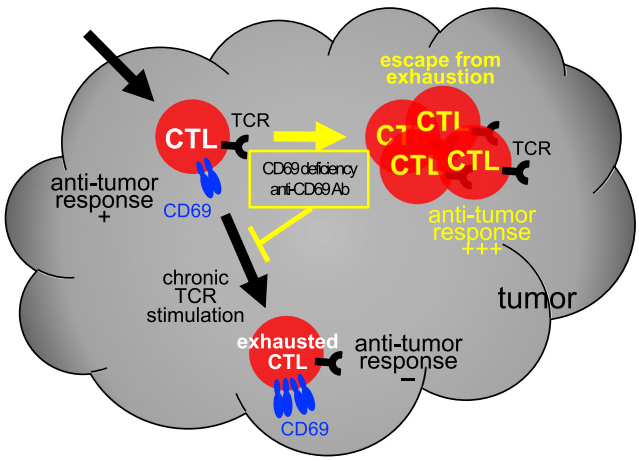

Fig. 2 CD69 regulates T cell exhaustion in the tumor microenvironment. In the tumor microenvironment, antigen-specific cytotoxic CD8 T cells (CTLs) exert anti-tumor responses. However, CTLs that dwell for a long time within the tumor receive chronic stimulation by tumor antigens, resulting in their exhaustion and subsequent dampening of their anti-tumor responses. CD69 deficiency or anti-CD69 Ab treatment prevents CTLs from becoming exhausted, keeping the anti-tumor responses high in order to suppress tumor progression

with a favorable prognosis in patients with several malignant tumors [58–62]. Tumor-resident CD8 T cells are characterized by the surface expression of CD69 and CD103. Importantly, these cells expanded significantly after anti-PD-1 Ab treatment in metastatic melanoma patients [63]. Recently, tumor-reactive CD8 T cells have been reported to show the $CD103^{+}CD39^{+}$ phenotype [64]. Consistent with this notion, cancer-unrelated tumor-infiltrating CD8 T cells lacked CD39 expression [65]. Interestingly, $CD103^{+}CD39^{+}$ tumor-reactive CD8 T cells co-expressed CD69 [64, 65]. These findings suggest that $CD69^{+}$ tumor-infiltrating CD8 T cells play a critical role in anti-tumor immunity in human cancer, and CD69 may be an ideal therapeutic target for cancer immunotherapy.

While increasing evidence that CD69 works as a suppressor of anti-tumor responses [50–53], there is a report showing that the CD69 expression is positively important for anti-tumor responses against melanoma in the skin [66]. In that study, the authors showed that the CD69 expression on tumor antigen-specific tissue-resident memory CD8 T cells is required for melanoma-immune equilibrium in the skin [66]. The immune system not only eliminates cancer cells but also prevents the outgrowth and spread of cancer cells that still exist by escaping eradication in a system called "cancer-immune equilibrium" [66, 67]. These data suggest that CD69-expressing memory T cells can play positive roles in the cancer-immune equilibrium, although CD69 expression on tumor antigen-specific CD8 T cells is important for the induction of the exhaustion, playing negative roles in eliminating cancer cells. Regarding the future use of anti-CD69 Ab as a new therapy, further carefully conducted experiments will definitely be required in order to clarify which kinds of cancers can be effectively treated by anti-CD69 Ab administration.

Conclusions

In this review, we summarized the current knowledge regarding CD69 biology and the importance of CD69 in regulating inflammatory responses and tumor immunity. The CD69 expression on effector T cells is important for exacerbating inflammation, whereas that on tumor antigen-specific T cells induces T cell exhaustion, thereby dampening anti-tumor immunity. Since the effects of anti-CD69 Ab treatment vary depending on the situation (inflammatory disorder vs. cancer), it will be crucial to elucidate the detailed mechanisms underlying how CD69 is involved in different immune responses. Importantly, in both cases, CD69 facilitates the tissue retention of its expressing cells.

We need to carefully assess the usage of anti-CD69 Ab in each situation. By collaborating with pharmaceutical companies, we have established humanized anti-CD69 and anti-Myl9/12 monoclonal antibodies. Furthermore, we are currently examining the efficacy of anti-CD69 Ab treatment for combination therapy with immune-checkpoint inhibitors, CAR-T cell therapy, and NKT cell therapy. We think that both anti-CD69 and anti-Myl9/12 may be promising new therapeutic tools for treating inflammatory disorders and/or cancer patients.

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Compliance with ethical standards

Conflicts of interest T. Nakayama has received research fund from Genefrontier (Chiba, Japan). M.Y. Kimura, R. Koyama-Nasu, and R. Yagi declare that they have no conflict of interest.

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