Communication

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Establishment of a Novel Anti-Mouse CD73 Monoclonal Antibody C$_{73}$Mab-9 by CBIS Method

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Abstract: Cluster of differentiation 73 (CD73), also known as 5'-nucleotidase ecto, is a glycosyl-phosphatidylinositol-anchored cell surface molecule that belongs to the 5'-nucleosidase family. CD73 metabolizes extracellular AMP into adenosine and inorganic phosphate. CD73 plays a vital role in cancer-immune evasion by facilitating adenosine production. Therefore, the development of specific anti-CD73 antibodies is desired to clarify the biological and pathological functions of CD73. Moreover, developing antibodies against mouse CD73 (mCD73) is also essential for preclinical experiments. This study successfully established an anti-mCD73 monoclonal antibody (clone C$_{73}$Mab-9, rat IgG$_{2a}$, lambda) using the Cell-Based Immunization and Screening (CBIS) method. In flow cytometric analysis, C$_{73}$Mab-9 is confirmed to recognize mCD73 in mCD73-overexpressed CHO-K1 (CHO/mCD73), MUSS, and NMuMG cells. The dissociation constant values of C$_{73}$Mab-9 were 7.6 × 10^{-9} M for CHO/mCD73 and 1.2 × 10^{-9} M for MUSS cells, respectively. Furthermore, C$_{73}$Mab-9 detected mCD73 in western blot analysis. Therefore, the C$_{73}$Mab-9, established by the CBIS method, is helpful for basic research and is expected to contribute to preclinical studies.

Keywords: CD73; cell-based immunization and screening method; monoclonal antibody; flow cytometry

1. Introduction

The tumor microenvironment (TME) includes cells and secreted substances and influences the efficacy of antitumor treatments. Immunosuppressive cells, such as regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), play essential roles in the formation of TME [1-3]. The development of cancer immunotherapy, including immune checkpoint inhibitors, has been a dramatic game-changer in cancer treatment [4].

Hypoxia, critically low oxygen levels, has potently correlated with the driving tumor toward a more aggressive phenotype and a poorer prognosis for tumor patients [5,6]. Hypoxic conditions are a beneficial environment for cancer metastasis [7]. Hypoxia drives hypoxia-inducible factor (HIF)-dependent alteration of gene expression profile, which is related to proteome and metabolome [5,6,8]. Under hypoxic conditions, cancer cells release adenosine 5'-triphosphate (ATP) into the extracellular space, which is sequentially converted into adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), and adenosine (ADO) by membrane-anchored ectoenzymes CD39 and CD73 [6]. High levels of extracellular ADO in tumors play a pivotal role in evading antitumor immune responses [9]. The ADO receptor is composed of four members, A2A ADO receptor (A2AR) is predominantly expressed in most immune cells, including T cells, natural killer (NK) cells, macrophages, and neutrophils [10]. Genetic deletion of A2AR in mice rejected tumors via T cell

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function [9]. Thus, extracellular ADO produced by CD39/CD73 is one of the key regulators of the immunosuppressive TME.

CD73 is a glycosylphosphatidylinositol (GPI)-anchored 70-kDa protein encoded by the NT5E gene, which hydrolyzes extracellular AMP into ADO and phosphate. CD73 is composed of two major domains: the N-terminal domain coordinates two catalytic divalent Zn^{2+} and Co^{2+} metal ions, whereas the C-terminal domain provides a binding site for AMP to generate ADO [11,12]. In immunosuppressive TME, increasing ADO production by CD73 weakens the effects of tumor-killing of effector cells, including T cells, B cells, and NK cells [13-16]. CD73 expression is confirmed in numerous cancer types, such as glioma [17], breast [18], bladder [19], pancreas [20], ovary [21], and lung cancers [22]. CD73 expression protects cancer cells from being eliminated by the immune system. Furthermore, high CD73 expression induces resistance against chemotherapy [23,24]. On the other hand, A2AR upregulates the expression of programmed cell death-1 (PD-1) and lymphocyte activation gene 3 (LAG-3) immune checkpoint molecules in T cells [25,26]. A2AR activation also promotes the differentiation of $CD4^+$ T cells into Treg with high cytotoxic T lymphocyte antigen-4 (CTLA-4) expression [27]. CD73 and PD-1 synergistically govern $CD4^+$ T cells activation [28]. ADO does not induce migration of tumor cells without A2AR [26], demonstrating that the CD73-metabolized ADO-A2AR axis exhibits potential as a target of tumor immunotherapy. Not only expressed on the cell surface, CD73 in small extracellular vesicles also constitutes immunosuppressive environments [29,30]. A variety of clinical trials targeting CD73 are currently underway [31].

To date, we have succeeded in developing numerous monoclonal antibodies (mAbs) targeting membrane proteins, including PD-L1 (clone L_1Mab-13) [32], TIGIT (clone TgMab-2) [33], CD20 (clone C_{20}Mab-11) [34], and EphB6 (clone Eb_6Mab-3) [35] by using the Cell-Based Immunization and Screening (CBIS) method. The CBIS method is a high-throughput screening method using flow cytometry to obtain a wide variety of antibodies that bind multiple epitopes, including extracellular modifications of membrane proteins. In this study, we have successfully established a novel anti-mouse CD73 (mCD73) mAb (clone C_{73}Mab-9) using the CBIS method that can be used for flow cytometry and western blot.

2. Materials and Methods

2.1 Cell Lines and Plasmids

LN229 (glioblastoma), Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1, mouse myeloma), and NMuMG (mouse mammary gland) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MUSS (histiocyte-like cells from spontaneous sarcoma from mice) cell line, a spontaneous malignant fibrous histiocytoma originating in mice, was provided from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). The expression plasmid of Nt5e (mouse 5' nucleotidase, ecto; mCD73) (pCMV6neoNt5e-Myc-DDK (Catalog No.: MR227439, Accession No.: NM_011851) was obtained from OriGene Technologies, Inc. (Rockville, MD, USA). The mCD73 expression vector was transfected into cell lines using the Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, LN229 and CHO-K1, which stably overexpressed mCD73 (hereafter described as LN229/mCD73 and CHO/mCD73, respectively), were stained with an anti-mCD73 mAb (clone TY/11.8; BioLegend, San Diego, CA, USA) and sorted using the SH800 cell sorter (Sony corp., Tokyo, Japan).

2.2. Antibodies

A purified anti-mouse CD73 Antibody (clone TY/11.8, rat IgG_1, kappa) was purchased from BioLegend. (San Diego, CA, USA).

2.3. Hybridoma Production

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For obtaining anti-mCD73 mAbs, a 6-week-old female Jcl: SD rat, purchased from CLEA Japan (Tokyo, Japan), was immunized with LN229/mCD73 (1×10^9 cells) via the intraperitoneal route. The LN229/mCD73 cells as immunogen were harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). As an adjuvant, Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA, USA) was mixed with the immunogen in the first immunization. Three additional injections of 1×10^9 cells of LN229/mCD73 were administered every week via the intraperitoneal route. A final booster injection was performed with LN229/mCD73 (1×10^9 cells) intraperitoneally two days before harvesting splenocytes from the immunized rat. Subsequently, splenocytes from LN229/mCD73-immunized rat and P3U1 mouse myeloma cells were fused using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN, USA).

2.4. Flow Cytometry

All cells were collected using 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells were analyzed using the SA3800 Cell Analyzer (Sony Corp.) as described previously [35].

2.5. Determination of the Binding Affinity by Flow Cytometry

CHO/mCD73 cells were suspended in 100 μL serially diluted C_{73}Mab-9 (30 μg/mL to 0.002 μg/mL) and TY/11.8 (an anti-mouse CD73 mAb, 30 μg/mL to 0.002 μg/mL), after which Alexa Fluor 488-conjugated anti-rat IgG (1:200) was added. MUSS cells were suspended in 100 μL serially diluted C_{73}Mab-9 (50 μg/mL to 0.003 μg/mL) and TY/11.8 (10 μg/mL to 0.0006 μg/mL), after which Alexa Fluor 488-conjugated anti-rat IgG (1:200) was reacted. The dissociation constant (K_D) was determined as described previously [35].

2.6. Western Blotting

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque, Inc.). Western blotting was performed using C_{73}Mab-9 (5 μg/mL), TY/11.8 (5 μg/mL), an anti-DYKDDDDK mAb (1E6, 0.5 μg/mL), and an anti-IDH1 mAb (RcMab-1, 1 μg/mL) as described previously [34].

3. Results

3.1. Development of Anti-mCD73 mAbs Using the CBIS Method

To establish anti-mCD73 mAbs, we employed the CBIS method using mCD73-overexpressed cells. Anti-mCD73 mAb-producing hybridomas were screened by using flow cytometry (Figure 1). A female Jcl:SD rat was intraperitoneally immunized with LN229/mCD73 (1×10^9 cells/time) every week, 5 times in total. Subsequently, rat splenocytes and P3U1 myelomas were fused by PEG1500. Hybridomas were seeded into 96-well plates with HAT-containing medium, after which the screening using flow cytometry was conducted to extract CHO/mCD73-reactive and parental CHO-K1-nonreactive supernatants of hybridomas. We successfully obtained some highly CHO/mCD73-reactive supernatants of hybridomas. After limiting dilution and additional investigations, we finally established the highly sensitive clone C_{73}Mab-9 (rat IgG_{2a}, lambda).

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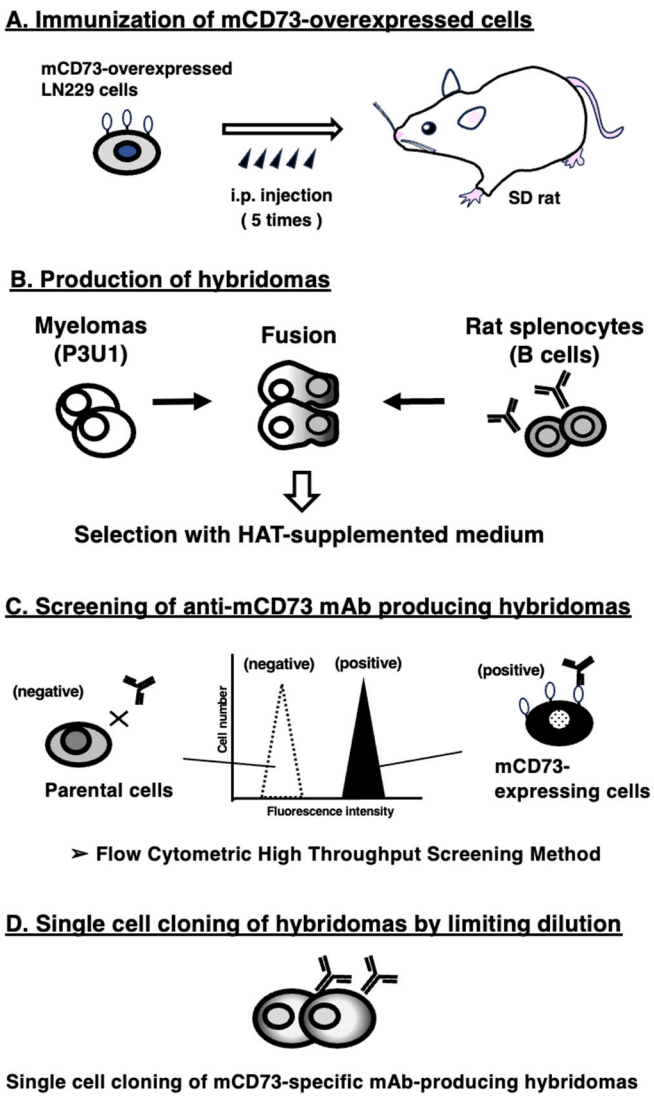

Figure 1. A schematic depiction of the CBIS method for developing anti-mCD73 mAbs. The simplified procedure flow of mAb development using the CBIS method. (A) LN229/mCD73 cells were intraperitoneally immunized into a female Jcl: SD rat. (B) The spleen cells from an antigen-immunized rat were fused with P3U1 myeloma cells. (C) The culture supernatants of hybridoma were screened by flow cytometry using CHO-K1 and CHO/mCD73 to select mCD73-specific mAb-producing hybridomas. (D) Single hybridoma clones were obtained by limiting dilution, followed by additional screening. Finally, C_{73}Mab-9 (rat IgG_{2a}, lambda) was successfully established.

3.2. Evaluation of The Antibody Reactivity Using Flow Cytometry

Flow cytometric analysis was conducted using C_{73}Mab-9 and a commercially available anti-mouse CD73 mAb (clone TY/11.8) against CHO-K1 and CHO/mCD73 cells. Results indicated that C_{73}Mab-9 and TY/11.8 recognized CHO/mCD73 dose-dependently (Figure 2A). Reactivity is almost identical between C_{73}Mab-9 and TY/11.8 to CHO/mCD73 (Figure 2A). Both C_{73}Mab-9 and TY/11.8 never reacted with parental CHO-K1 cells even at a concentration of 10 μg/mL (Figure 2B). Thus, C_{73}Mab-9 can detect mCD73 in flow cytometry.

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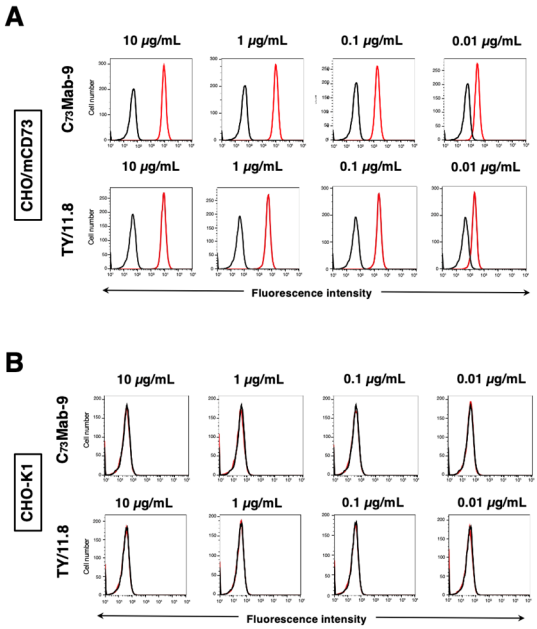

Figure 2. Flow cytometric analysis of anti-mCD73 mAbs. CHO/mCD73 (A) and CHO-K1 (B) cells were treated with 0.01–10 μg/mL of C73Mab-9 or TY/11.8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line showed a no-primary antibody-treated control.

3.3. Evaluation of the Antibody Reactivity Against Endogenously mCD73-Expressing Cells Using Flow Cytometry

Next, further flow cytometric analysis was conducted using C73Mab-9 and TY/11.8 against MUSS and NMuMG, which endogenously express mCD73. Results indicated that C73Mab-9 and TY/11.8 recognized MUSS dose-dependently (Figure 3A). Against another mCD73-expressing NMuMG cell line, C73Mab-9 and TY/11.8 also recognized dose-dependently (Figure 3B) with almost identical reactivity. Thus, C73Mab-9 can detect endogenous mCD73 in flow cytometry.

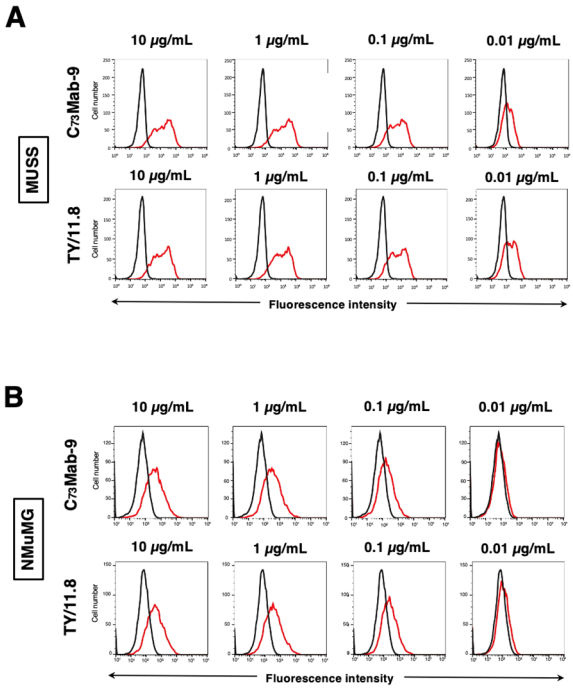

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Figure 3. Flow cytometry of anti-mCD73 mAbs against endogenously mCD73-expressing cells. MUSS (A) and NMuMG (B) cells were treated with 0.01–10 μg/mL of C_{73}Mab-9 or TY/11.8 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. The black line showed a no-primary antibody-treated control.

3.4. Calculation of The Apparent Binding Affinity Of Anti-mCD73 mAbs Using Flow Cytometry

The binding affinity of C_{73}Mab-9 and TY/11.8 was assessed with CHO/mCD73 using flow cytometry. The results indicated that the K_D values of C_{73}Mab-9 and TY/11.8 for CHO/mCD73 were 7.6×10^{-9} M and 5.7×10^{-9} M, respectively (Figure 4A). Furthermore, the K_D values of C_{73}Mab-9 and TY/11.8 for MUSS were 1.2×10^{-9} M and 5.6×10^{-10} M, respectively (Figure 4B). These results demonstrate that C_{73}Mab-9 can recognize mCD73 with high affinity to the cell surface mCD73.

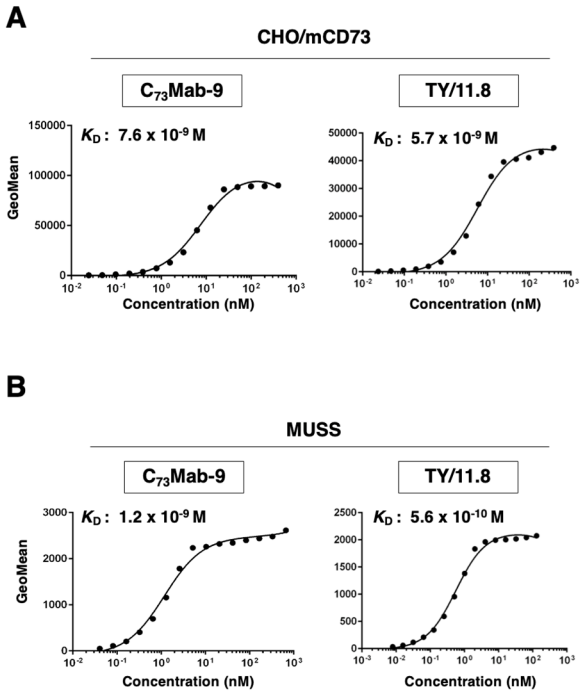

Figure 4. Determination of the binding affinity of anti-mCD73 mAbs. (A) CHO/mCD73 cells were suspended in 100 μL of serially diluted C_{73}Mab-9 (30 μg/mL to 0.002 μg/mL) and TY/11.8 (30 μg/mL to 0.002 μg/mL). (B) MUSS cells were suspended in 100 μL of serially diluted C_{73}Mab-9 (50 μg/mL to 0.003 μg/mL) and TY/11.8 (10 μg/mL to 0.0006 μg/mL). Following primary mAbs treatment, cells were incubated with Alexa Fluor 488-conjugated anti-rat IgG. Subsequently, the geometric mean values from fluorescence data were collected using the SA3800 Cell Analyzer, following the calculation of the K_D by GraphPad PRISM 6 software.

3.5. Western Blot Analyses Using Anti-mCD73 mAbs

We investigated whether C_{73}Mab-9 can be applied to western blot analysis by analyzing CHO-K1 and CHO/mCD73 cell lysates. The estimated molecular weight of the mCD73 protein is approximately 70,000. As shown in Figure 5, C_{73}Mab-9 could detect mCD73 as the major band between 63 to 75 kDa in CHO/mCD73 cell lysates, while no band was detected in parental CHO-K1 cells. Another anti-mCD73 mAb, TY11.8, could not detect any band in CHO/mCD73 cell lysates. An anti-DYKDDDDK mAb (clone 1E6) was applied for positive control because mCD73 was FLAG-tagged in CHO/mCD73. An anti-IDH1 mAb (clone RcMab-1) was used for internal control. These results indicate that C_{73}Mab-9 can recognize denatured-mCD73 in mCD73-overexpressed cells in western blot analyses.

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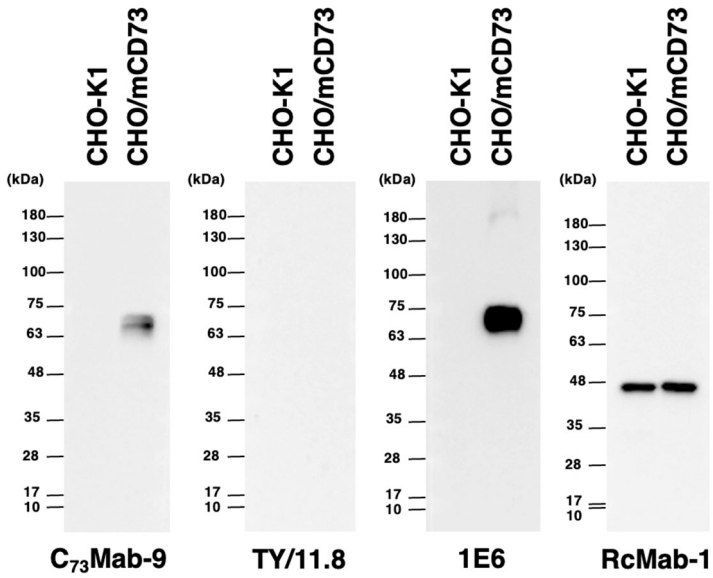

Figure 5. The detection of mCD73 by the western blotting. The boiled cell lysates of CHO-K1 and CHO/mCD73 (10 μg/lane) were electrophoresed and transferred onto PVDF membranes. The membranes were incubated with 5 μg/mL of C_{73}Mab-9, 5 μg/mL of TY/11.8, 0.5 μg/mL of 1E6, and 1 μg/mL of RcMab-1 and subsequently with horseradish peroxidase-conjugated anti-rat or anti-mouse immunoglobulins.

4. Discussion

Upregulation of CD73 and CD39 has been observed in tumors and immune cells not only under hypoxia but also in response to cytokines such as transforming growth factor-β and interleukins [36,37]. These secreted factors also concomitantly upregulate the A2AR expression. The high concentrations of ATP can be observed in the extracellular milieu of solid tumors [38]. Moreover, it is well known that a wide variety of cytokines are released to TME by tumor cells and immune-related cells [39,40]. Thus, ADO production within tumors provides a complex mechanism for evading immune approaches. Interestingly, CD73 might act as a tumor promoter independent of ADO production. CD73 induces adhesiveness and invasiveness of tumors, and transactivation of receptor tyrosine kinases by binding extracellular matrix proteins and clustering on the cell surface, respectively [41-43]. Simultaneous inhibition of CD73 and epidermal growth factor receptor significantly induces tumor cell death and suppresses migration of cancer cells [44]. It is essential to know the epitope of mAb to predict its inhibitory effect on the target protein activity. Anti-CD73 mAb cocktail targeting two different epitopes potently inhibits tumor growth than the single treatment [45]. We plan to identify the epitope of C_{73}Mab-9 to clarify whether it binds near the catalytic domain of mCD73, and whether it has an inhibitory effect on the catalytic activity of mCD73 in preclinical models.

CD73 correlates with the initiation of epithelial-mesenchymal transition (EMT) and cancer stemness in ovarian cancer [46]. Also in hepatocellular carcinoma, CD73 upregulation and A2AR activation contribute to the induction of EMT, metastatic features, and cancer stem cell traits through PI3K/AKT signaling and SOX9 stabilization [47,48]. CD73 influences cancer progression not only by immune evasion but also by promoting malignant phenotypes. Moreover, cancer-associated fibroblasts (CAFs), a tumor stromal cell, highly express CD39 and CD73, which enable the production of extracellular ADO in cancers [49,50]. A high abundance of CAFs was associated with a worse prognosis in colorectal cancer patients [50]. A2AR activation triggers the proliferation of CAFs, followed by tumor cell growth in non-small cell lung cancer [51]. CD73-ADO-A2AR axis plays multiple roles in cancer malignancy in tumor tissues, and more detailed analysis may expand its potential as a therapeutic target for numerous cancers. C_{73}Mab-9 could be a helpful tool for evaluating mCD73-related biological responses in mouse models. Previously, we have successfully developed

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anti-mouse CD39 mAbs, clones C_{39}Mab-1 and C_{39}Mab-2, by the CBIS method [52,53]. These established mouse CD39 and CD73-targeting mAbs could significantly contribute to the analysis of the adenosine pathway.

CD73-targeted clinical studies have been conducted against solid tumors. Anti-CD73 mAbs, such as oleclumab (human mAb), CPI-006 (humanized mAb), BMS-986179 (hybrid IgG_1/IgG_2 antibody), and NZV930 (human mAb), have been used in clinical studies. Furthermore, CD73-targeting small molecules, including AB680 and LY3475070, have also been evaluated for solid tumors [31]. In addition to the role in cancer progression, CD73 is a promising therapeutic target that antibodies and small-molecule compounds can target to block its function. Furthermore, more significant antimetastatic effects have been observed in CD73/A2AR dual blockade by anti-mCD73 mAbs and A2AR inhibitors than either single treatment in the mouse melanoma lung metastasis model [54]. In future studies, we will investigate the antitumor efficacy of C_{73}Mab-9 in mouse models.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) for studies involving animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest involving this article.

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