MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 41, Number 5, 2022

DOI: 10.1089/mab.2022.0016

KLMab-1: An Anti-human KLRG1 Monoclonal Antibody for Immunocytochemistry

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Immune checkpoint molecules have received attention as targets of cancer immunotherapy. Killer cell lectin-like receptor subfamily G member 1 (KLRG1) is one of the immune checkpoint molecules expressed in $CD4^+$ T, $CD8^+$ T, and natural killer (NK) cells. KLRG1 exhibits antiviral and antitumor immunity, and its expression in T and NK cells is upregulated by viral infectious diseases and some tumors. Thus, monoclonal antibodies (mAbs) for KLRG1 would be useful tools for the diagnosis and immunotherapy against viral infectious diseases and cancers. We have developed anti-human KLRG1 (hKLRG1) mAb (clone KLMab-1, mouse IgG_1, kappa) by the Cell-Based Immunization and Screening method. We have also demonstrated that KLMab-1 recognizes both exogenous and endogenous hKLRG1 in flow cytometry. In this study, we first showed that KLMab-1 and its recombinant mAb (recKLMab-1) bound to exogenous hKLRG1 overexpressed in Chinese hamster ovary (CHO)-K1 cells, but not in parental CHO-K1 cells, in immunocytochemistry. We next showed that both mAbs detected endogenous hKLRG1 expressed in human NK cells. These results demonstrate that KLMab-1 and recKLMab-1 are available for immunocytochemistry.

Keywords: KLRG1, KLMab-1, monoclonal antibody, immunocytochemistry

Introduction

T cells and natural killer (NK) cells play crucial roles in antiviral and antitumor immunity.[1–4] During the response to viral infection or cancer development, T cells are activated, expanded, and differentiated into effector and memory T cells. Activated NK cells migrate to the infected or tumor site, and the NK cells eliminate the target cells through the production of cytokines and exhibition of cytolytic activity. In contrast, the activities of T and NK cells are suppressed by immune checkpoint molecules, including programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).[5]

Accordingly, immune checkpoint molecules have drawn attention as targets for cancer immunotherapy. In fact, specific monoclonal antibodies (mAbs) against PD-1 and CTLA-4 have provided great advances in the medical treatment of cancers.[6] However, developing novel mAbs against other immune checkpoint molecules has been required because the number of patients who respond to anti-PD-1 or anti-CTLA-4 mAbs is limited.[7]

Killer cell lectin-like receptor subfamily G member 1 (KLRG1), a lectin-like type II transmembrane protein, is an immune checkpoint molecule expressed in $CD4^+$ T, $CD8^+$ T, and NK cells.[8–10] It harbors an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region. Upon binding to KLRG1 ligands including E-cadherin, KLRG1 evokes inhibitory signaling through recruitment of Src homology 2 domain-containing inositol polyphosphate 5-phosphatase 1 (SHIP1) and Src homology region 2 domain-containing phosphatase 2 (SHP2) to ITIM. Then, KLRG1 attenuates interferon γ production in T and NK cells, and suppresses NK cell-mediated cytotoxicity.[8,10–16]

The mechanism contributes to the progression of viral infection and tumor by KLRG1. Moreover, the expression of KLRG1 is increased in NK cells of virus-infected mice and

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T cells of cervical and colorectal cancer patients.[8,17,18] These reports have suggested that KLRG1 can be a target molecule for the diagnosis and immunotherapy of viral infectious diseases and some cancers.

We have established mAbs against cell surface-expressing membrane proteins by the Cell-Based Immunization and Screening, including CCR3,[19–21] CCR8,[22–25] CCR9,[26,27] CD10,[28,29] CD19,[30] CD20,[31,32] CD44,[33] CD133,[34] EpCAM,[35,36] HER3,[37] PD-L1,[38] podoplanin,[39–53] TIGIT,[54] and TROP2.[55,56] We have also established an antihuman KLRG1 (hKLRG1) mAb (clone KLMab-1; mouse IgG_1, kappa), which reacts to endogenous and exogenous hKLRG1 in flow cytometry.[57] In this study, we showed that KLMab-1 and its recombinant mAb (recKLMab-1) are available for immunocytochemistry against endogenous and exogenous hKLRG1.

Materials and Methods

Cell lines

Chinese hamster ovary (CHO)-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). CHO-K1 cells overexpressed with human KLRG1 (CHO/hKLRG1) were established previously.[57] CHO-K1 and CHO/hKLRG1 were cultured in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc.). The cells were maintained in a humidified atmosphere at 37°C and 5% carbon dioxide. Human NK cells (donor lot. 4022602, purity >70%) were purchased from Takara Bio (Shiga, Japan).

Antibodies

The development of KLMab-1 was described in our previous report.[57] To generate a recombinant KLMab-1 (recKLMab-1), V_H and C_H cDNAs of KLMab-1 were subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and V_L and C_L cDNAs of KLMab-1 were subcloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). An anti-hKLRG1 mAb (clone SA231A2) was purchased from BioLegend (San Diego, CA). Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Immunocytochemistry

For immunocytochemistry of CHO-K1 and CHO/hKLRG1 cells, the cells were attached to an acid-wash coverslip and were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (4% PFA/PBS) for 10 minutes. Subsequently, the cells were incubated with the blocking buffer (PBS supplemented with 0.2 mM Ca^{2+}, 2 mM Mg^{2+}, and 0.5% bovine serum albumin) for 30 minutes, primary antibodies (10 μg/mL in the blocking buffer) for 1 hour, and Alexa Fluor 488-conjugated anti-mouse IgG (1:400 dilution in the blocking buffer) for 45 minutes.

For immunocytochemistry of NK cells, the suspension of NK cells was centrifuged at 270 × *g* for 5 minutes at room temperature, and the cell pellet was suspended in and fixed with 4% PFA/PBS for 10 minutes. The cells were further suspended in the blocking buffer for 30 minutes, primary antibodies (10 μg/mL in the blocking buffer) for 2 hours, and Alexa Fluor 488-conjugated anti-mouse IgG (1:400 dilution in the blocking buffer) for 45 minutes. 4',6-Diamidino-2-phenylindole (Thermo Fisher Scientific, Inc.) was used for nuclear staining of CHO-K1, CHO/hKLRG1, and NK cells. Fluorescence images were acquired using a 40× objective on a digital microscope (BZ-X800; Keyence, Osaka, Japan).

Results

Our flow cytometric analysis revealed that CHO/hKLRG1 cells highly express hKLRG1 on the cell surface.[57] In this study, we applied KLMab-1 and recKLMab-1 in immunocytochemistry using CHO/hKLRG1 cells and found that KLMab-1 and recKLMab-1, but not buffer control, bound to CHO/hKLRG1 cells (Fig. 1A). In particular, hKLRG1 was strongly detected at the plasma membrane. Both mAbs did not react to CHO-K1 cells (Fig. 1B). A commercially available anti-hKLRG1 mAb (clone SA231A2) also bound to CHO/hKLRG1 cells, but not CHO-K1 cells (Fig. 1A, B). This result shows that KLMab-1 and recKLMab-1 recognize exogenous hKLRG1 in immunocytochemistry.

We previously showed that KLMab-1 detects endogenously expressing hKLRG1 in human NK cells in flow cytometry.[57] In this study, we incubated NK cells with KLMab-1 and recKLMab-1 and found that both mAbs, as well as SA231A2, bound to NK cells (Fig. 1C). This result demonstrates that KLMab-1 and recKLMab-1 recognize endogenous hKLRG1 in immunocytochemistry.

Discussion

In this study, we demonstrated that KLMab-1 and recKLMab-1 were applicable for immunocytochemistry against endogenous and exogenous hKLRG1. The mAbs would become useful tools for the detection of hKLRG1-positive T and NK cells in viral infectious diseases and cancers.

KLMab-1 and recKLMab-1 provided images with high signal-to-noise ratios against not only exogenous hKLRG1 overexpressed in CHO-K1 cells but also endogenous hKLRG1 expressed in NK cells. Our previous study showed that KLMab-1 weakly detected endogenous hKLMab-1 in flow cytometry.[57] We suppose that KLMab-1 and recKLMab-1 are suitable for immunocytochemistry. Moreover, KLMab-1 and recKLMab-1 strongly detected hKLRG1 at the plasma membrane, which was clearly represented in CHO/hKLRG1 cells. The result indicates that the mAbs would be able to identify the intracellular distribution of hKLRG1, especially by colabeling the cells with any organelle markers. The information about the intracellular distribution of hKLRG1 would provide advantages in uncovering the unknown functions of KLRG1.

To uncover the KLRG1's roles and to detect KLRG1-positive cells, other applications of KLMab-1 and recKLMab-1 are also required. In the future, we would like to test other applications, including immunohistochemistry, immunoprecipitation, and Western blotting.

Furthermore, confirming the availability of KLMab-1 and recKLMab-1 in viral infectious diseases and cancers is one of our goals. Some studies have demonstrated that

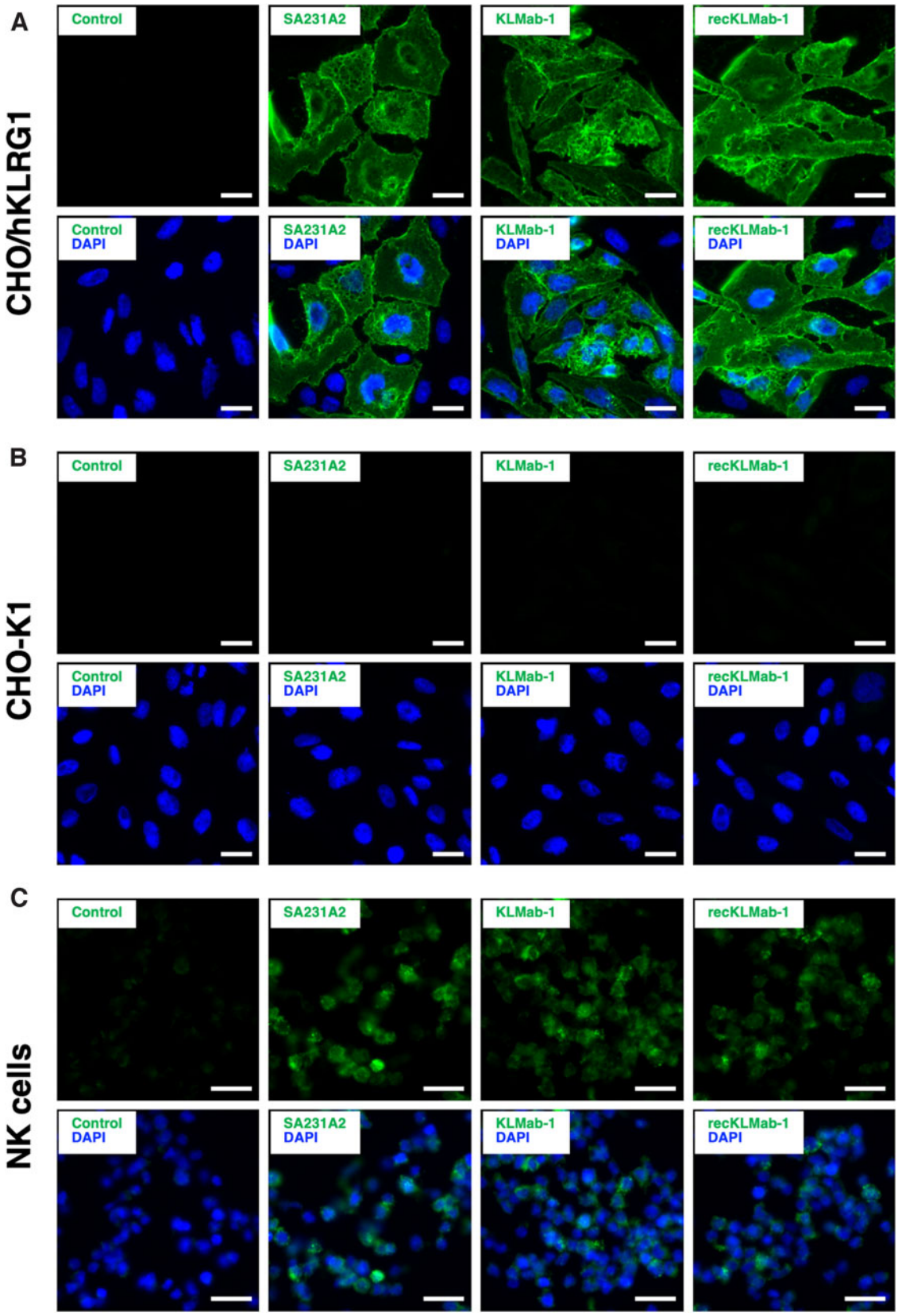

FIG. 1. Immunocytochemistry of hKLRG1 using KLMab-1 and recKLMab-1. **(A, B)** CHO/hKLRG1 cells **(A)** or CHO-K1 **(B)** cells were incubated with buffer control, SA231A2 (10 μg/mL), KLMab-1 (10 μg/mL), or recKLMab-1 (10 μg/mL) for 1 hour. Subsequently, the cells were incubated with Alexa 488-conjugated anti-mouse IgG and DAPI for 45 minutes. **(C)** Immunocytochemistry of endogenously expressing hKLRG1. NK cells were incubated with SA231A2 (10 μg/mL), KLMab-1 (10 μg/mL), or recKLMab-1 (10 μg/mL) for 2 hours. Subsequently, NK cells were incubated with Alexa 488-conjugated anti-mouse IgG and DAPI for 45 minutes. Scale bars, 20 μm. CHO, Chinese hamster ovary; DAPI, 4',6-diamidino-2-phenylindole; hKLRG1, human KLRG1; KLRG1, Killer cell lectin-like receptor subfamily G member 1; NK, natural killer.

anti-KLRG1 mAb reduces cytokine productions in KLRG1-overexpressed NK cells[8] and KLRG1$^+$ CD4$^+$ T cells.[58] An anti-KLRG1 mAb reduces the progression of breast cancer cells.[59] In addition, blocking of KLRG1 signaling in CD8$^+$ T cells by anti-E-cadherin antibody reduces the proliferation of the cells.[60] These studies would promote us to investigate the antitumor and/or antiviral effects of KLMab-1 and recKLMab-1.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP22ama121008 (to Y.K.), JP22am0401013 (to Y.K.), JP22bm1004001 (to Y.K.), JP22ck0106730 (to Y.K.), and JP21am0101078 (to Y.K.).

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Received: April 6, 2022
Accepted: September 26, 2022