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Mutant *KRAS* Promotes NKG2D$^+$ T Cell Infiltration and CD155 Dependent Immune Evasion

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Abstract. *Background/Aim: Roles for mutant (mt) KRAS in the innate immune microenvironment in colorectal cancer (CRC) were explored. Materials and Methods: Human CRC HCT116-derived, mtKRAS-disrupted (HKe3) cells that express exogenous mtKRAS and allogenic cytokine-activated killer (CAK) cells were co-cultured in 3D floating (3DF) culture. The anti-CD155 antibody was used for function blocking and immunohistochemistry. Results: Infiltration of CAK cells, including NKG2D$^+$ T cells, into the deep layer of HKe3-mtKRAS spheroids, was observed. Surface expression of CD155 was found to be up-regulated by mtKRAS in 3DF culture and CRC tissues. Further, the number of CD3$^+$ tumor-infiltrating cells in the invasion front that show substantial CD155 expression was significantly larger than the number showing weak expression in CRC tissues with mtKRAS. CD155 blockade decreased the growth of spheroids directly and indirectly through the release of CAK cells. Conclusion: CD155 blockade may be useful for therapies targeting tumors containing mtKRAS.*

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Mutation in the *Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) is present in various human cancers and is associated with poor prognosis (1, 2). KRAS mutations are found in 30%-40% of colorectal cancers (CRCs) (2, 3). Extensive effort has been put toward the development of chemical compounds that directly target and inhibit constitutively active KRAS (4). However, such drugs have not yet reached clinical use (5).

Cancer immunotherapy has recently achieved significant progress. The application of monoclonal antibodies targeting cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4) or programmed death receptor-1 (PD-1) has promoted adaptive antitumor activity through blockade of inhibitory receptor–ligand interactions, with remarkable clinical benefits (6). Recently, the US Food and Drug Administration approved PD-1 blockade for metastatic CRC that displays mismatch repair (MMR) deficiency (7). MMR deficiency produces a large proportion of passenger mutations and subsequent transcription of neoantigens (8). T cells recognize neoantigens *via* T cell receptor–MHC class I interactions and become sensitive to immune checkpoint blockade associated with PD-1–PD-L1 interaction (9). However, CRC cells frequently escape T cell-mediated control by down-regulating the expression of MHC class I receptors (10, 11). Previous investigations demonstrate that other tumors with *KRAS* mutations, such as mutant (mt) *KRAS* pancreatic ductal adenocarcinomas, are resistant to checkpoint immunotherapies (12). Furthermore, mtKRAS down-regulates MHC class I receptors during metastasis of non-small cell lung cancer (13), suggesting that blockade of this immune checkpoint is ineffective for cancers expressing mtKRAS.

The activation of innate immune cells, including natural killer T (NKT) and (NK) cells, is currently considered a promising therapeutic approach for the treatment of various types of cancer because these cells are effective killers of cancer cells without the need for prior sensitization. This ability contrasts sharply the activation of adaptive immune cells (14, 15). Numerous studies demonstrate that functional deficiencies in NK cells are associated with an increased risk of developing various cancers (12, 16, 17). Based on this information, cytokine-activated killer (CAK) cells composed of natural killer group 2, member D (NKG2D)-positive T cells (TNK cells) have previously been shown to induce cytotoxicity against various cancers in a manner independent of MHC class I (18, 19). The signaling balance between NK-activating receptors and inhibitory receptors determines the antitumor activity of CAK cells. Activating receptors include molecules such as DNAX Accessory Molecule-1 (DNAM-1) and NKG2D. Inhibitory receptors include T cell immunoreceptor with immunoglobulin, immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), and PD-1 (20). For example, NKG2D recognizes its ligands, the UL16 binding proteins (ULBP) and MHC class I chain-related (MIC) protein, on cancer cells and is also thought to play an important role in mediating the activation of anticancer immune responses (21). In contrast, TIGIT contributes to immunotolerance by inhibiting immune responses mediated by T and NK cells through binding to its ligand on cancer cells (22). TIGIT competes with DNAM-1 for binding to their common ligands, including CD155 and CD112 (23-25). Several mechanisms of transcriptional/proteasomal regulation of immune-activating and inhibitory molecules on cancer cells, such as ULBP, MIC, CD155, and PD-L1, have been reported (26-28). However, the regulatory mechanisms of mtKRAS on the expression of immune-related molecules in tumor-immune microenvironments remain still unclear (29).

Recently, a 3D floating (3DF) culture system was established using human CRC HCT116-derived mtKRAS-disrupted cells (HKe3) that stably overexpress wild-type (wt) *KRAS* (HKe3-wtKRAS) or mtKRAS (HKe3-mtKRAS) (30, 31). HKe3-wtKRAS and HKe3-mtKRAS cells share the same genetic background except for mt*KRAS*, thereby facilitating accurate identification of molecules directly controlled by mtKRAS (30). Further, 3DF culture and co-culture are effective methods for observation of sphere formation and direct infiltration of immune cells into tumor spheroids (32). Thus, the combination of HKe3 and CAK cells in 3DF co-culture is a suitable *in vitro* model for analyzing the role of mtKRAS in the tumor-immune microenvironment.

mtKRAS was found to regulate the infiltration of CAK cells into tumors, and surface expression of CD155 was increased in HKe3-mtKRAS spheroids and in CRC tissues with mtKRAS. The latter finding suggests that mtKRAS up-regulates surface expression of CD155.

Materials and Methods

Antibodies and reagents. Anti-CD3-FITC antibody was purchased from Tonbo Biosciences (San Diego, CA, USA). Anti-MICA/B-PE, anti-CD155-PE, anti-Nectin2-FITC, anti-UL16-binding protein (ULBP)-1, anti-ULBP-2, anti-ULBP-3, and anti-CD155 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-PD-L1-PE was purchased from Immunotech Beckman Coulter (Brea, CA, USA). Anti-TIGIT-PE and anti-PD-1-PE antibodies were purchased from Biolegend (San Diego, CA, USA). Anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified anti-CD155 antibody for the functional blocking of CD155 was purchased from Biolegend. A Mini Dialysis Kit (GE Healthcare, Tokyo, Japan) was used for antibody purification in order to remove sodium azide as described previously (33). The anti-CD155 antibody for immunohistochemistry was purchased from Cell Signaling (Tokyo, Japan).

Cell culture. HKe3, HKe3-wtKRAS, HKe3-mtKRAS, and HKe3-CD155 cells were maintained as described previously (30, 31). Human peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy volunteers who provided written informed consent. PBMCs were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 5% human serum, 2000 units/ml penicillin (Meijiseika, Tokyo, Japan), and 10 μg/ml streptomycin (Meijiseika) as previously described (34).

3DF cell culture. Cells were seeded in round-bottomed 96-well plates with ultralow attachment surfaces (Corning Inc., Corning, NY, USA). Cells were cultured in a CO_2 incubator as described previously (30, 35, 36).

Flow cytometry. Cancer cells in 2D culture were detached from culture plates using Accutase Solution (Promo Cell, Heidelberg, Germany). In 3DF culture, cancer spheroids were harvested by centrifuging 96-well plates for 5 min at 200 × *g* and then separated into individual cells using Accutase Solution. PBMCs, CAK, and cancer cells were washed twice in cold phosphate-buffered saline (PBS), suspended in FACS buffer (5% bovine serum albumin and 0.05% sodium azide in PBS), and incubated with fluorophore-conjugated antibodies in FACS buffer for 60 min at 4˚C. Fluorescence was detected using an FC500 flow cytometer (Beckman Coulter) and expressed as relative median fluorescence intensity (MFI) or percentage above baseline as determined with FlowJo software (Tree Star, Inc., San Carlos, CA, USA).

Western blotting. Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail (Roche, Basel, Switzerland)) and were subjected to immunoblotting as described previously (37). Quantitative analysis of immunoblots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Patients and tumor samples. Formalin-fixed, paraffin-embedded tissues were obtained from eight patients with CRC who underwent surgery at Fukuoka University Hospital. The study protocol was approved by the Institutional Review Board of Fukuoka University (No. U19-08-006). *KRAS* mutations in unstained paraffin sections containing cancer cells were detected at the laboratory of SRL Inc.

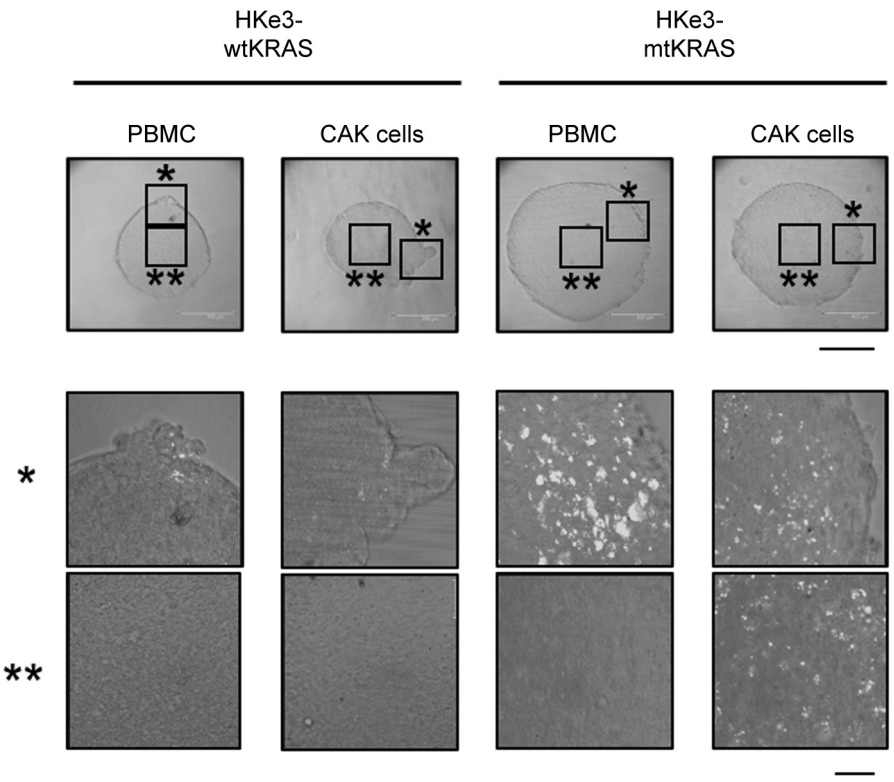

Figure 1. *mtKRAS promotes the infiltration of cytokine-activated killer (CAK) cells into spheroids. Upper panels represent images of spheroids of HKe3 wild-type (wt) KRAS (left) and Hke3 mutant (mt) KRAS (right) co-cultured with peripheral blood mononuclear cells (PBMCs) or CAK cells. Scale bar, 500 μm. Enlarged images of areas enclosed by black lines in the upper panels are shown for the middle (*: the superficial part of spheroids) and lower panels (**: the central part of spheroids). The middle and lower panels represent merged images for both calcein AM and normal light signals. Scale bar, 50 μm.*

(Fukuoka, Japan) using polymerase chain reaction reverse sequence-specific oligonucleotide (PCR-rSSO). A summary of patients' characteristics is provided in Table I. All participants provided informed written consent. The research was conducted per the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001.

Immunohistochemical analysis. Colorectal cancer tissues were fixed in 10% formalin, processed into paraffin blocks, sectioned (4-μm thickness), deparaffinized, and hydrated in descending alcohol dilutions. Sections were heated in 10 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0) in a microwave (800 W) for 20 min to retrieve epitopes before staining and were then immersed in 3% hydrogen peroxide in water for 10 min at room temperature (RT) to block endogenous peroxidase activity. Sections were subsequently washed in Tris-buffered saline (TBS) and incubated with anti-human CD155 antibody (1:200) overnight at 4˚C. Sections were washed again in TBS and incubated for 1 h at RT with EnVision reagent conjugated horseradish peroxidase (Dako, Carpinteria, CA, USA). Immunoreactive proteins were visualized with 3,3'-diamino-benzidine (Dako), followed by counterstaining with Mayer's hematoxylin for 1 min. Specimens were viewed using a BZ-X710 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan).

Generation of cytokine-activated killer cells. PBMCs were activated in KBM551 medium (Kohjin Bio, Saitama, Japan) supplemented with 5% human serum, 2000 U/ml penicillin, 10 μg/ml streptomycin, 2000 U/ml recombinant human interleukin-2 (IL-2), and 0.5% albumin (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) in T-75 flasks coated with 2 μg/ml anti-CD3 monoclonal antibody (OKT3, Janssen Pharmaceutical K.K., Tokyo, Japan). After 5 days of activation, these cells were collected as CAK cells.

Infiltration of spheroids by CAK cells. HKe3-mtKRAS cells were seeded at 1000 cells/well into round-bottomed 96-well plates with an ultralow attachment surface for 3DF culture. Cells were cultured for 4 days in a CO_2 incubator as described previously (36). CAK cells were stained with calcein AM (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. About 2,000 cells/well calcein AM-stained CAK cells were then added into the culture medium of spheroids. After 48 h, HKe3-mtKRAS spheroids were collected, and any CAK cells that had infiltrated into the spheroids were imaged using a TCS-SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany).

Immunofluorescence labeling. Immunofluorescence labeling was performed overnight at 4˚C using cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA) as previously described (35). DAPI

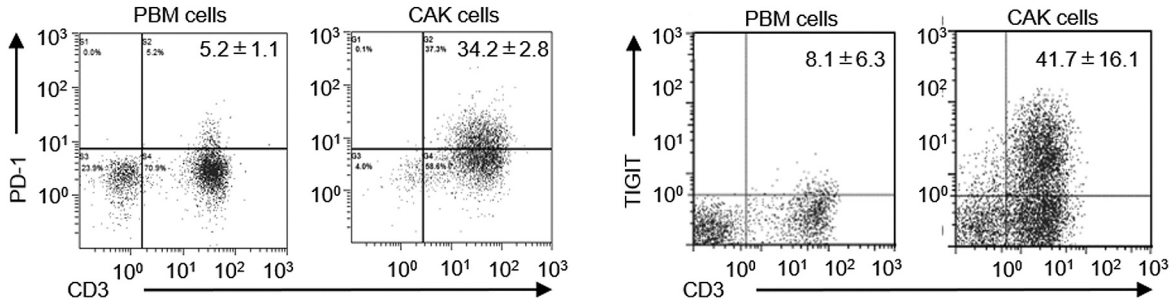

Figure 2. *Increased expression of inhibitory natural killer (NK) receptors on the surface of cytokine-activated killer (CAK) cells. Activation of inhibitory NK receptors on the surface of peripheral blood mononuclear cells (PBMCs) or CAK cells. Flow cytometric profiles of CD3 (x-axis) and PD-1 (right panel) or TIGIT (y-axis) (left panel) on PBMCs or CAK cells. The numbers indicate the percentage of cells in each gate±SD from three biological replicates.*

and CytoPainter Phalloidin-iFluor 555 Reagent (Abcam, Cambridge, UK) were used for the counterstaining of spheroid surfaces.

Co-culture of cancer and CAK cells. Cancer cells (HKe3-wtKRAS, 3000 per well; HKe3-mtKRAS, 1000 per well) were seeded in round-bottomed 96-well plates with ultralow attachment surfaces for 3DF culture. Cancer cells were cultured for 4 days in a CO_2 incubator as described previously (30, 36). Co-culture of these cells with CAK cells (cancer/CAK cell ratio of 1:40) was then initiated, and this time point was considered day 0. Images of cells were taken using a BIOREVO BZ9000 microscope (Keyence), and spheroid area was measured using a BZ Analyzer (Keyence) as described previously (35, 38). Growth rates of tumor spheroids were calculated based on the changes in spheroid area on days 0, 3, and 7.

Detection of apoptotic cells in spheroids. Apoptotic cells were stained on day 7 using a Magic Red Caspase 3&7 Assay Kit (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA) according to the manufacturer's instructions. Cells were imaged using a TCS-SP5 laser scanning confocal microscope (Leica).

Statistical analyses in cell culture experiments. Statistical analyses were performed using unpaired two-tailed Student's *t*-tests. All *p*-values less than 0.05 were considered statistically significant.

Results

mtKRAS promotes the infiltration of CAK cells into spheroids. To confirm that PBMCs and CAK cells target CRC spheroids with or without mtKRAS, PBMCs and CAK cells were stained by green fluorescence and subsequently co-cultured with HKe3-wtKRAS spheroids or HKe3-mtKRAS spheroids. PBMCs and CAK cells weakly were found to infiltrate HKe3-wtKRAS spheroids (Figure 1). In contrast, PBMCs and CAK cells readily infiltrated into the shallow layer of HKe3-mtKRAS spheroids (Figure 1). Notably, CAK cells also infiltrated into the deep layer of HKe3-mtKRAS spheroids, suggesting a role of mtKRAS in immune cell infiltration.

Increased expression of inhibitory NK receptors on the surface of CAK cells. CAK cells have been previously shown to express NK-activating receptors including DNAM-1 and NKG2D (19). Flow cytometry was used to assess the expression levels of inhibitory NK receptors on CAK cells. The percentages of $CD3^+PD\text{-}1^+$ and $CD3^+TIGIT^+$ cells in the CAK cell population were 6.6-fold and 5.1-fold higher than that in PBMCs, respectively (Figure 2), suggesting that PD-1 and TIGIT play key roles during the transition from PBMCs to CAK cells.

mtKRAS up-regulates the surface expression of CD155 in CRC spheroids. Flow cytometry using cells dispersed from 3DF culture was used to examine the expression levels of ligands of NK receptors, including DNAM-1, NKG2D, PD-L1, and TIGIT, in HKe3-wtKRAS or HKe3-mtKRAS (Figure 1a, b). No significant differences between mean fluorescence intensities (MFI) of ligands between wt and mt spheroids were observed (Figure 3a). However, surface expression levels of CD155 (DNAM-1 and TIGIT ligands) in both HKe3-wtKRAS and HKe3-mtKRAS cells in 3DF culture were higher than the levels in 2D culture. Further, MFI of CD155 in HKe3-mtKRAS spheroids was 1.57-fold higher than MFI in HKe3-wtKRAS spheroids (Figure 3b; $*p<0.05$), suggesting that mtKRAS is involved in the up-regulation of CD155. No significant differences in CD155 protein levels were observed in lysates between HKe3-wtKRAS and HKe3-mtKRAS cells from 3DF and 2D culture (Figure 3c). These results suggest that surface expression of CD155 in CRC spheroids is up-regulated by mtKRAS, although the total levels of CD155 protein remain unchanged.

Surface expression of CD155 in CRC tissues with mtKRAS. Immunohistochemistry was used to examine the differences in expression levels of CD155 between CRC tissues with or without mtKRAS *in vivo*. Two-thirds of samples with

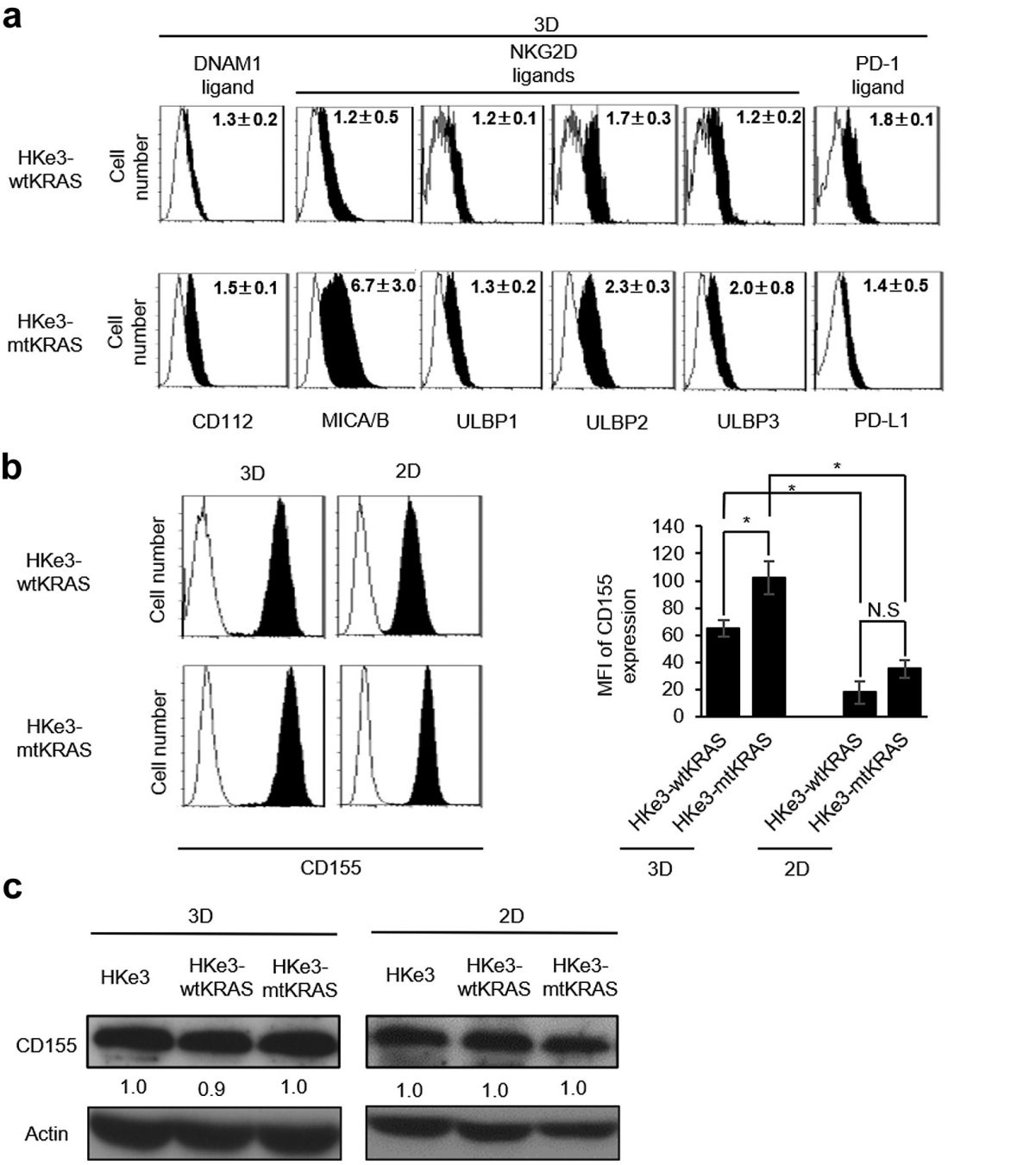

Figure 3. *Mutant (mt) KRAS up-regulates the surface expression of CD155 on spheroids. (a) The surface expression of CD112, MICA/B, ULBP1, ULBP2, ULBP3, and PD-L1 on HKe3, HKe3 wild-type (wt) and mutant (mt) spheroids. FACS profiles of ligands of natural killer receptors on HKe3-wtKRAS spheroids and HKe3-mtKRAS spheroids. The x-axis is fluorescence intensity, and the y-axis is the number of cells. The white histograms show unstained control cells, and the black histograms show cells stained with specific antibodies. Histograms are representative of three biological replicates, and the insert number on each histogram indicates MFI±SD. (b) Surface expression of CD155 on HKe3-wtKRAS cells and HKe3-mtKRAS cells in 2D or 3DF culture on day 4. FACS profiles of CD155 on HKe3-wtKRAS spheroids and HKe3-mtKRAS spheroids (left panel). The x-axis is the fluorescence intensity, and the y-axis is the number of cells. The white histograms show the unstained control cells and the black histograms show cells stained with anti-CD155 antibody. Histograms are representative of three biological replicates (left panel), and the MFIs±SD of CD155 in HKe3-wtKRAS cells and HKe3-mtKRAS cells in 2D and 3DF cultures are shown in the right panel. *p<0.05, n.s.: not significant. (c) The expression levels of CD155 protein in HKe3 cells, HKe3-wtKRAS cells, and HKe3-mtKRAS cells in 2D or 3DF culture. β-actin was used as a loading control. Levels of proteins were quantified by densitometry and normalized to actin levels. Values are relative ratios to levels in HKe3 spheroids. Data are representative of three biological replicates.*

Table I. *Clinicopathological details of patients.*

Patient Number	Age	Tumor location	Invasion depth	KRAS	CD155 staining
#1	68	Descending colon	SS	Wild type	Weak
#2	68	Descending colon	SS	Wild type	Weak
#3	77	Transverse colon	SS	Wild type	Strong
#4	49	Ascending colon	SS	Mutant (G12D)	Strong
#5	69	Transverse colon	SE	Mutant (G12V)	Weak
#6	80	Sigmoid colon	SS	Mutant (G12D)	Weak
#7	76	Transverse colon	SS	Mutant (G12D)	Strong
#8	79	Ascending colon	SS	Mutant (G12D)	Strong

SS; subserosa, SE; Serosa exposed.

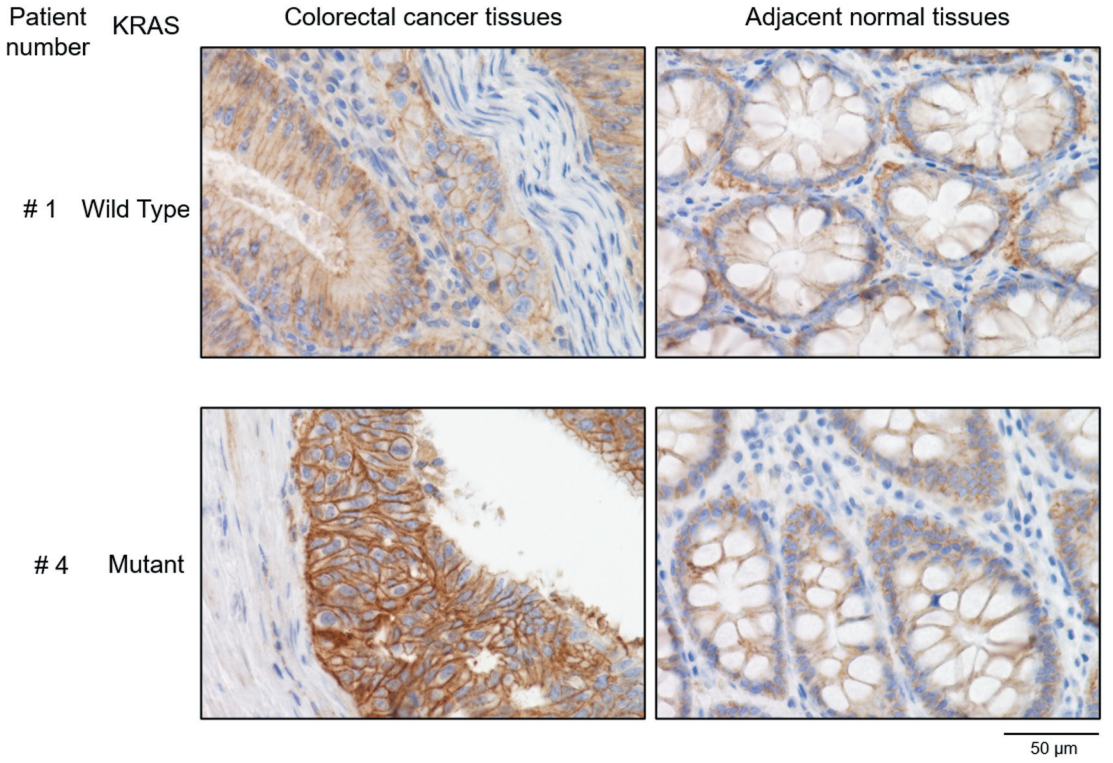

Figure 4. *The surface expression of CD155 is up-regulated in colorectal cancer (CRC) tissues with mutant (mt) KRAS. The expression levels of CD155 protein in CRC tissues with wild-type KRAS (upper left panel), mtKRAS (lower left panel), and paired adjacent normal tissues (right panel) were determined by immunohistochemistry. Scale bar, 50 μm.*

wtKRAS show weak membrane staining for CD155 in CRC tissues, similar to staining observed in adjacent normal colorectal tissues (Table I and Figure 4, upper panel). Sixty percent of samples with mtKRAS showed more prominent membrane staining for CD155 in CRC tissue than in adjacent normal colorectal tissues (Table I and Figure 4, lower panel). Together with the results shown in Figure 3, these findings suggest that mtKRAS up-regulates the surface expression of CD155 in CRC tissues.

Expression of CD155 at the invasion front of CRC tissues with mtKRAS is associated with the infiltration of CD3-positive T lymphocytes. Prominent expression of CD155 was hypothesized to be associated with the infiltration of immune cells. Immunohistochemistry of CD3 using CRC tissues (Table I) was performed to test this hypothesis. Serial sections of paraffin-embedded tissues from CRC patients with wtKRAS or mtKRAS were stained with HE (upper panel) and for CD155 (middle panel) and CD3 (lower panel)

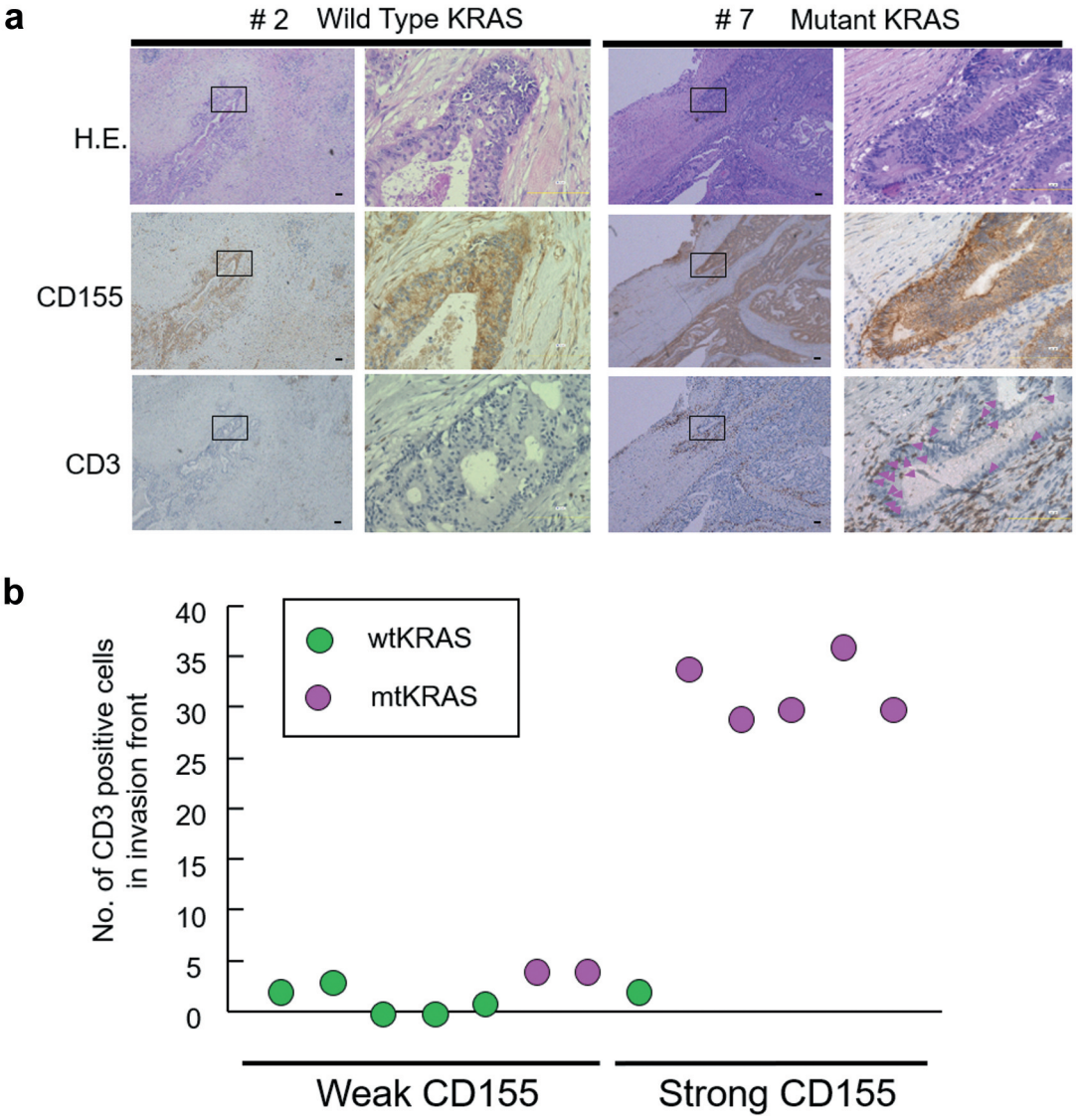

Figure 5. *Prominent expression of CD155 at the invasion fronts of colorectal cancer (CRC) tissues with mutant (mt) KRAS is associated with the infiltration of CD3-positive T lymphocytes. (a) Upper panels are representative images of HE staining. The expression levels of CD155 protein (middle panel) and CD3 protein (lower panel) in CRC tissues with wild-type (wt) KRAS (left panel) and mtKRAS (right panel) were determined by immunohistochemistry. Scale bar, 100μm. (b) The circle represents the individual invasion front with wtKRAS (green) or mtKRAS (pink). Y-axis is the number of CD3-positive cells at the invasion front.*

by immunohistochemistry. The numbers of tumor-infiltrating lymphocytes were counted in 13 randomly selected invasion fronts. Seven lesions showed weak CD155 expression (Figure 5a, left panel and Figure 5b), and six lesions showed prominent CD155 expression (Figure 5a, right panel and Figure 5b). A majority of invasion fronts (83.3%) with prominent CD155 expression showed increased numbers of infiltrating CD3-positive T lymphocytes (Figure 5a, right panel and Figure 5b). Notably, among lesions with prominent CD155 staining, all lesions with high infiltration numbers were identified as CRC with mtKRAS (Figure 5a, right panel and Figure 5b). Results suggest that both prominent CD155 expression and mtKRAS signals are important for infiltration by lymphocytes.

CAK cells under CD155 and PD-1 blockade suppress growth of HKe3-mtKRAS spheroids in a 3DF co-culture system via induction of luminal apoptosis. Time-dependent changes in spheroid area in 3DF co-cultures were used to assess cytotoxic capacity of CAK cells under a CD155 and PD-1 blockade for HKe3-mtKRAS *in vitro*. A CD155 function blocking antibody was used as described previously (33). Spheroid areas treated

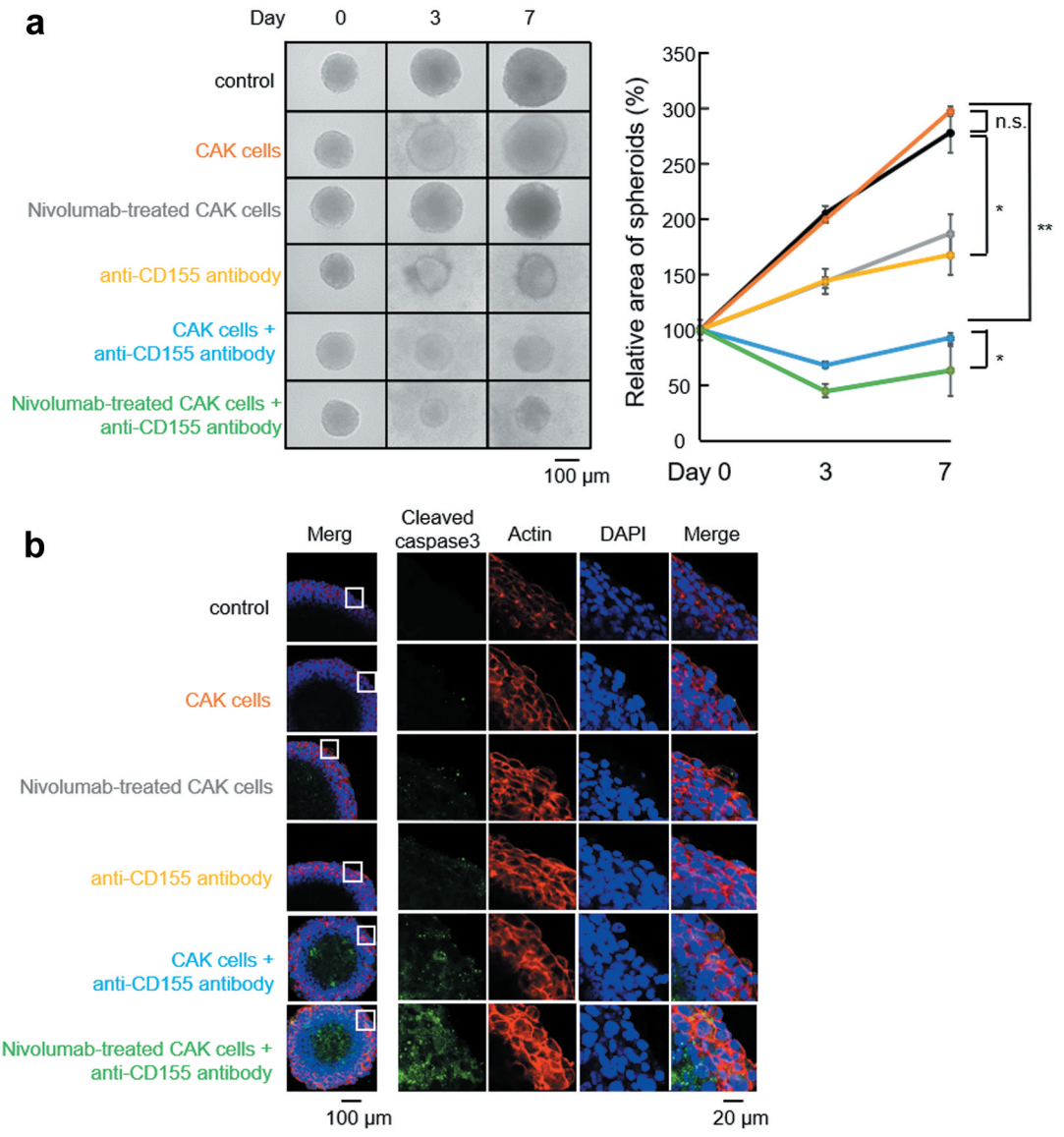

Figure 6. *Cytokine-activated killer (CAK) cells under CD155 and PD-1 blockade suppress the growth of HKe3 mutant (mt) KRAS spheroids in a 3DF co-culture system via the induction of luminal apoptosis. (a) Representative images of HKe3-mtKRAS spheroids from three biological replicates (left panel). Scale bar, 100 μm. The right panel is the relative areas of HKe3-mtKRAS spheroids incubated with CAK cells, nivolumab-treated CAK cells, anti-CD155 antibody, CAK cells with anti-CD155 antibody, nivolumab-treated CAK cells, or nivolumab-treated CAK cells with anti-CD155 antibody in 3DF culture. Mean±SD from three technical replicates are shown. *$p<0.05$. **$p<0.001$. n.s.: not significant. (b) Representative signals for cleaved caspase-3 in HKe3-mtKRAS spheroids from three biological replicates. Enlarged images of the areas enclosed by white lines in the left panels are shown. Cleaved caspase-3, green; actin, red; DAPI, blue. Scale bar, 100 μm (low-magnification images) or 20 μm (high-magnification images).*

with anti-CD155 antibody were 1.7-fold smaller than controls (Figure 6a; *$p<0.05$), suggesting growth suppression by blocking CD155-associated signals. Direct cytotoxicity of CAK cells was assessed by co-culturing with HKe3-mtKRAS spheroids. No significant differences in spheroid area were observed between HKe3-mtKRAS spheroids treated with CAK cells alone and the control (Figure 6a). CAK cell cytotoxicity may be suppressed by an immune checkpoint. However, spheroid areas in co-culture with CAK cells with anti-CD155 antibody were 3.2-fold smaller compared to those co-cultured with CAK cells alone (Figure 6a; **$p<0.01$) and 1.8-fold smaller than spheroids treated with CD155 antibody alone. CD155 blockade may release the cytotoxic capabilities of CAK cells in addition to suppressing spheroid growth.

Similar results were observed for PD-1 blockade. PBMCs were treated with nivolumab (a PD-1 inhibitor) during CAK cell

activation; nivolumab-treated CAK cells were subsequently co-cultured with HKe3-mtKRAS spheroids (Figure 6a). The spheroid areas in cultures with nivolumab-treated CAK cells were 1.5-fold smaller compared to spheroids cultured with untreated CAK cells (Figure 6a). PD-1 blockade may also release the cytotoxic capability of CAK cells.

Finally, spheroid areas in co-culture with nivolumab-treated CAK cells with anti-CD155 antibody were 1.5-fold smaller than that of spheroids co-cultured with CAK cells with anti-CD155 antibody (Figure 6a; $*p<0.05$). CAK cells subjected to a combination blockade of CD155 and PD-1 had greater efficacy against CRC spheroids containing mtKRAS than CAK cells blockaded against either target alone. Overall, mtKRAS appears to protect cells from the attack by CAK cells.

Apoptotic activity was assessed to address whether the influence of CD155 blockade extends to apoptosis. HKe3-mtKRAS in 3DF co-culture for 7 days was evaluated for cleaved caspase-3/caspase-7 using confocal microscopy. Luminal space was distinguished by counterstaining surfaces of spheroids using DAPI and phalloidin. Luminal apoptosis was detected in spheroids cultured with CAK cells and with anti-CD155 antibody (Figure 6b). Apoptosis was further augmented in spheroids cultured with nivolumab-treated CAK cells with anti-CD155 antibody.

Discussion

Previous studies show that CD155 expression is up-regulated in many human malignancies, including CRC, and is correlated with tumor progression and unfavorable prognosis (39-42). Furthermore, oncogenic Ras up-regulates CD155 expression *via* MEK/ERK signaling in mouse NIH3T3 cells (43), suggesting that mtKRAS transcriptionally controls CD155 expression in CRC expressing mtKRAS. In this study, mtKRAS did not increase the total amount of CD155 protein but increased the expression of CD155 protein on the surface of CRC spheroids in 3DF culture (Figure 3b, c). In clinical samples, mtKRAS was associated with increased levels of CD155 on cell membranes in CRC tissues expressing mtKRAS (Figure 4). Further, the surface expression levels of CD155 protein in both HKe3-wtKRAS and HKe3-mtKRAS spheroids in 3DF culture were much higher than in 2D culture (Figure 3b). These results suggest that the mtKRAS-CD155 axis plays a key role in the 3D microenvironment. Previously, mtKRAS was shown to inhibit cellular polarity and luminal apoptosis in 3D culture (35), suggesting that the surface expression of various proteins on spheroids is altered under these conditions. Another recent study shows that mtKRAS affects the cellular sorting system of CRC cells (44), raising the possibility that mtKRAS also controls sorting of CD155.

Many studies have shown that extensive NK infiltration is associated with advanced disease and may even facilitate cancer development (45). The exact role of the tumor-infiltrating NK cells and the correlation between their presence and poor prognosis remains still unclear. An increased number of $CD3^+PD1^+TIGIT^+$ CAK cells that infiltrate into spheroids from CRC cells with mtKRAS was observed (Figure 1). Similarly, $CD3^+$ T cells were found to infiltrate into invasion fronts of CRC tissues with mtKRAS (Figure 5). mtKRAS may promote the infiltration of lymphocytes in CRC, and the identification of tumor-infiltrating lymphocytes at the invasion fronts of CRC samples may help determine the severity of prognosis.

Single CD155 blockade suppresses the area of spheroids and increases signals of cleaved caspase-3 (Figure 6), and the CD155 antibody may directly induce apoptosis. Furthermore, both anti-CD155 antibody and nivolumab similarly unleash the cytotoxic capabilities of CAK cells and coordinately promote antitumor immunity in the co-culture system (Figure 6). CD155–TIGIT or PD-L1–PD-1 interactions may act as an immune checkpoint inside spheroids. In cytokine-induced killer (CIK) cells, activated by CAK-cell medium (including IL-2 and CD3 antibodies) with interferon γ, treatments that block PD-1 or TIGIT are reportedly effective at increasing the cytotoxic action of CIK cells to targeted tumor cells (46, 47). Both blockades may act similarly as immune checkpoints for CIK/CAK cells. TIGIT has a stronger affinity for CD155 than DNAM1, and the CD155–TIGIT interaction is recognized as a strong immune inhibitory system in NKT and NK cells (48).

Consistent with the present study, where dual blockade of CD155 and PD-1 suppresses growth of HKe3-mtKRAS spheroids (Figure 6), dual blockade of TIGIT and PD-1 suppresses tumor growth in a murine glioblastoma model (49). Inhibiting both CD155–TIGIT and PD-L1–PD-1 pathways is thus more effective than inhibiting CD155–TIGIT alone. When comparing CD155 and TIGIT blockades, it appears that TIGIT blockade targets only the extrinsic signal due to predominant expression of TIGIT on immune cells (50), whereas CD155 blockade, targets both intrinsic and extrinsic signals and thus could be expected to be more efficient than a TIGIT blockade for CAK/CIK-treated patients with CRC containing mtKRAS.

In conclusion, the co-culture system mimicking microenvironments allowed a demonstration that mtKRAS modulates the surface expression of CD155 and that the infiltration of CAK cells may directly induce cancer cell proliferation. Patient samples show similar expression patterns and infiltration of CD3-positive cells. mtKRAS suppresses antitumor immunity by enhancing TIGIT–CD155 interactions. Thus, CD155 blockade may be useful, in the future, for treating cancers that express mtKRAS.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

KN, TM, TS, SS and TT designed the study. KN, SI, MU, TI, AS and TT carried out *in vitro* experiments and drafted the manuscript. TK, MA, SN, YY, SH and KN carried out immunohistochemical analysis. KN, MA, SN, KN, TS, SS and TT, participated in the data interpretation. KN, AS, SS, and TT prepared the final manuscript. As the principal investigator, TT supervised the study. All Authors read and approved the final manuscript.

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