CD155, also called NECL5 or TAGE4, is a member of the immunoglobulin superfamily that was initially identified as a poliovirus receptor, and its expression is significantly upregulated in tumor cells and correlated with poor clinical outcomes in various types of cancer, such as lung adenocarcinoma. While CD155 has a role in promoting cell movement and proliferation, it’s also known as a molecule that regulates antitumor immunity. It is a shared ligand for DNAM-1 and TIGIT. The binding of CD155 to DNAM-1 enhances the cytotoxicity of NK cells and CD8+ T cells, resulting in tumor elimination. In contrast, the binding of CD155 to TIGIT on these cells induces an inhibitory signal and suppresses antitumor immunity. A recent study showed that the single nucleotide polymorphism rs1058402G>A causing a mutation to Thr from Ala at residue 67 of CD155 is associated with worse overall survival of patients with small cell lung cancer and suggested that this is caused by the decreased affinity of mutant CD155 for DNAM-1 as a result of the 3D structural analysis. Unexpectedly, however, we found that the mutation increased the binding affinity for TIGIT rather than decreased the binding affinity for DNAM-1 and induced a stronger signal than WT CD155. Our results suggest that the mutation suppresses tumor immune responses by generating a stronger inhibitory signal in immune cells in the tumor microenvironment.

**KEYWORDS**
A67T, CD155, DNAM-1, PVR, TIGIT

**INTRODUCTION**
CD155, also called NECL5 or TAGE4, is a member of the immunoglobulin superfamily that was initially identified as a poliovirus receptor, and its expression is significantly upregulated in tumor cells and correlated with poor clinical outcomes in various types of cancer, such as lung adenocarcinoma. While CD155 has a role in promoting cell movement and proliferation, it’s also known as a molecule that regulates antitumor immunity. It is a shared ligand for DNAM-1 and TIGIT. The binding of CD155 to DNAM-1 enhances the cytotoxicity of NK cells and CD8+ T cells, resulting in tumor elimination. In contrast, the binding of CD155 to TIGIT on these cells induces an inhibitory signal and suppresses antitumor immunity. A recent study showed that the single nucleotide polymorphism rs1058402G>A causing a mutation to Thr from Ala at residue 67 of CD155 is associated with worse overall survival of patients with small cell lung cancer and suggested that this is caused by the decreased affinity of mutant CD155 for DNAM-1 as a result of the 3D structural analysis. Unexpectedly, however, we found that the mutation increased the binding affinity for TIGIT rather than decreased the binding affinity for DNAM-1 and induced a stronger signal than WT CD155. Our results suggest that the mutation suppresses tumor immune responses by generating a stronger inhibitory signal in immune cells in the tumor microenvironment.

**Key Words**
A67T, CD155, DNAM-1, PVR, TIGIT

**Abstract**
CD155 is a shared ligand for activating and inhibitory immunoreceptors DNAX accessory molecule 1 (DNAM-1), also called CD226, and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), which are expressed on natural killer (NK) cells and T cells, and positively and negatively regulates tumor immune responses, respectively. A recent study showed that the single nucleotide polymorphism rs1058402G>A causing a mutation to Thr from Ala at residue 67 of CD155 is associated with worse overall survival of patients with small cell lung cancer and suggested that this is caused by the decreased affinity of mutant CD155 for DNAM-1 as a result of the 3D structural analysis. Unexpectedly, however, we found that the mutation increased the binding affinity for TIGIT rather than decreased the binding affinity for DNAM-1 and induced a stronger signal than WT CD155. Our results suggest that the mutation suppresses tumor immune responses by generating a stronger inhibitory signal in immune cells in the tumor microenvironment.

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However, we unexpectedly found that the mutation increased the binding affinity of mutant CD155 for TIGIT rather than decreased the binding affinity for DNAM-1 and induced a stronger TIGIT signal in reporter cells and NK cells than WT CD155.

2 MATERIALS AND METHODS

2.1 Reporter cells and transfectant

The NFAT-GFP reporter 2B4 or BaF/3 cell line provided by H. Arase (Osaka University, Osaka, Japan) was transduced with plasmid DNA encoding the extracellular and transmembrane regions of human TIGIT or DNAM-1 fused with the intracellular region of CD3ζ chain or Fcγ chain. The human TIGIT reporter cell line was further transduced with plasmid DNA encoding the full length of human DNAM-1. To establish A2058 cell transfectant expressing WT or mutant CD155, CD155 knock-down A2058 cell line was retrovirally transduced with plasmid DNA encoding the full length of WT or mutant CD155.

2.2 Generation of recombinant human CD155-Fc fusion protein (CD155-Fc)

CD155-Fc was generated using the Expi293™ Expression System (Thermo Fisher Scientific). Expi293F™ cells were transfected with plasmid DNA encoding the extracellular region of WT or mutant CD155 fused with Fc of human IgG1 according to the user guide.

2.3 Binding affinity assay

Ni-nitrilotriacetic acid (Ni-NTA) biosensor (ForteBio) was coated with His-tagged recombinant human DNAM-1 (200µg/ml; R&D Systems) or His-tagged recombinant human TIGIT (100µg/ml; R&D Systems). WT and mutant CD155-Fc (156nM, 312nM, and 625nM) were used as analytes for association with coated His-tagged recombinant human DNAM-1 or TIGIT. All doses of each analyte were selected because the R² value was over 0.94. Quantifications were done using the global mode of biolayer interferometry technology system (ForteBio). The times for a series of analysis steps were determined, as previously reported.9

2.4 Reporter assay

Each well of 96 well plates (Nunc MaxiSorp™ flat-bottom, Thermo Fisher Scientific) was coated with 1mg/ml of DOTAP (CAYMAN CHEMICAL), followed by 3.1, 6.3, 12.5, or 25.0µg/ml of WT or mutant CD155-Fc. The reporter cells (2×10⁴/well) were then seeded onto the wells and incubated at 37°C for 16.5 hours. The GFP expression of reporter cells was analyzed by flow cytometry.

2.5 NK cell degranulation assay

NK cells were purified from the human peripheral blood, as described.9 NK cells were cultured in 24-well plates at 37°C for 24 hours in RPMI 1640 complete medium containing 10% FBS and 50μM 2-mercaptoethanol in the presence of 100ng/ml IL-2 (BD Biosciences). IL-2-activated NK cells were incubated on ice with anti-human DNAM-1 (TX94) antibody, anti-human TIGIT antibody (MBSA43; Invitrogen) or isotype mouse IgG1 (MOPC-21; BioLegend), and then cocultured at 37°C in a 5% CO₂ incubator for 5 hours with WT or mutant CD155-expressing A2058 cells (E:T = 2:1) in the presence of GolgiStop™ protein transport inhibitor (1/2000; BD Bioscience) and APC-conjugated anti-human CD107a (1/200, H4A3; BioLegend) in 96-well round-bottomed plates.

2.6 Epidemiological analysis

The frequency of the mutant CD155 was analyzed using a data-set of exome sequences from the Genome Aggregation Database v2.1.1 (gnomAD; https://gnomad.broadinstitute.org/). Variant ID is 19-45150614-G-A.

2.7 Statistical analysis

Paired and unpaired student t tests were used for statistical analysis. P < 0.05 was considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 The mutation (Ala67Thr) increased the binding affinity of CD155 for TIGIT and enhanced the TIGIT signal

A previous study suggested that the mutation increases the distance of binding sites between CD155 and an activating receptor DNAM-1 by using the 3D structural analysis, suggesting a weakening binding affinity of mutant CD155 for DNAM-1.8 This study led us to analyze the binding affinity of WT and mutant CD155 for DNAM-1. Unexpectedly, however, the binding affinity of mutant CD155 for DNAM-1 was rather greater than that of WT CD155 (Figure 1A). Consistently, mutant CD155 generated a stronger DNAM-1 signal than did WT CD155 in a reporter cell assay (Figure S2A). Thus, our results were inconsistent with the previous report.8 A possible explanation for the differences from the previous study is a difference in organisms of DNAM-1. In the present study, we used human CD155 and human DNAM-1, while the previous study used human CD155 and mouse DNAM-1 as a template for computational 3D structural analysis. As the homology of the extracellular domain of DNAM-1 between mice and human is 54%, this difference may have affected the results.
To clarify how the mutation is associated with worse overall survival of patients with small cell lung cancer, we next analyzed the binding affinity for an inhibitory receptor TIGIT. The affinity of mutant CD155 for TIGIT was also greater than that of WT CD155 (Figure 1B). Moreover, mutant CD155 generated a stronger signal than WT CD155 in the human TIGIT reporter assay (Figure S2B). We further analyzed the TIGIT signal using the reporter cells expressing both DNAM-1 and TIGIT (Figure S2C). As a result, mutant CD155 generated a stronger TIGIT signal than WT CD155 even in the presence of the expression of a shared ligand DNAM-1 with TIGIT (Figures 1C and S2D).

### 3.2 Mutant CD155 decreased NK cell activation in a TIGIT-dependent manner

To examine whether the mutation indeed affects antitumor immune responses, we cocultured human NK cells derived from healthy volunteers with A2058 cell transfectant expressing WT or mutant CD155. We found that the transfectant expressing mutant CD155 induced lower CD107a expression than did the transfectant expressing WT CD155. However, the blocking of the interaction between CD155 and TIGIT with an anti-TIGIT neutralizing antibody canceled the lower expression of CD107a on NK cells cocultured with the transfectant expressing mutant CD155. In contrast, the anti-DNAM-1 neutralizing antibody had no effect on the CD107 expression on NK cells (Figures 2 and S3A,B), suggesting that decreased NK cell activation was dependent on increased TIGIT signal.

### 4 CONCLUSION

Finally, we analyzed the mutation frequency using the Genome Aggregation Database (gnomAD). We found that 7.7% of people...
worldwide had the mutation, of which 7.3% were heterozygotes and 0.4% were homozygotes. In particular, more than 10% of Asians and Americans were found to have the mutation (Table 1). Our results suggest that cancer patients with the mutation have accelerated tumor progression through enhanced TIGIT signaling. Therefore, TIGIT blockade therapy may be more beneficial for patients with the mutation, and analyzing the presence of the mutation may help select patients for TIGIT blockade therapy. However, as our study used artificially generated proteins and transfectants, further studies are needed to analyze how mutant CD155 on tumor cells affects immune cells in the future.

## AUTHOR CONTRIBUTIONS
All authors have contributed significantly and are in agreement with the content of the manuscript.

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## DISCLOSURE
The authors have no conflict of interest.

## ETHICS STATEMENT
Approval of the research protocol by an Institutional Reviewer Board: N/A.
Informed Consent: N/A.
Registry and the Registration No. of the study/trial: N/A.
Animal Studies: N/A.

## ORCID
Tomohi Matsuo https://orcid.org/0000-0001-5630-5100
Akiko Iguchi-Manaka https://orcid.org/0000-0002-0338-130X
Kazuko Shibuya https://orcid.org/0000-0002-8203-2342

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## SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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