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SLAMF7 and TREM1 Mediate Immunogenic Cell Death in Colorectal Cancer Cells: Focus on Microsatellite Stability

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Abstract. *Background/Aim: The aim of this study was to identify the association between SLAMF7 and TREM1 and anti-PD-1 drugs, and to determine whether they are molecular targets or predictors of responses to immunotherapy through induction of immunogenic cell death. Materials and Methods: CRC cell lines over-expressing SLAMF7 and TREM1 were used to examine immunogenic and biological traits (e.g., proliferation and invasiveness) associated with factors related to anti-cancer immunity. In addition, multiplex immunofluorescence was used to examine immune cells in microsatellite instability-high (MSI-H) CRC and microsatellite stable (MSS) CRC. Results: Proliferation rate and invasiveness of TREM1-over-expressing CRC cells were significantly greater than those of control cells ($p<0.001$ and 0.031, respectively), whereas SLAMF7-over-expressing CRC cells showed the opposite traits ($p=0.005$ and 0.002, respectively). SLAMF7-over-expressing DLD-1 cells harboring MSI-H showed increased apoptosis when treated with anti-PD-1 drugs, unlike SLAMF7-over-expressing SW480 cells harboring MSS. SLAMF7-over-expressing DLD1 and SW480 cells showed a marked increase in expression of the major cytokine mediator HMGB1 when exposed to anti-PD-1 drugs. Co-administration of anti-PD-1 drugs and TREM1 inhibitors induced apoptosis only in MSI-H HCT116 cells; HMGB1 was over-expressed regardless of microsatellite status. Conclusion: Expression of TREM1 and SLAMF7 is closely associated with immunogenic cell death, and TREM1 inhibitors may be an effective adjuvant that enhances anti-PD-1-mediated immunogenic cell death in MSS CRC.*

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Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-associated mortality; unfortunately, systemic metastasis and recurrence occur in almost half of patients after curative surgery (1). Chemotherapy (combined treatment with a 5-FU-based regimen plus oxaliplatin or irinotecan) improves objective responsiveness; this can be improved further by targeted treatment using bevacizumab and cetuximab. CRC cells develop drug resistance; therefore, few cases are cured. Thus, immunotherapy based on modulating the tumor microenvironment (TME), which comprises tumor cells and immune cells is being developed (2). Immunotherapy to promote and optimize the immune status of CRC patients is likely to prolong survival. Immune responses to tumor cells have long been of great interest; for example, anti-tumor immune checkpoint blockers, particularly those targeting programmed cell death-1 (PD-1)/programmed cell death-ligand 1(PD-L1), are new lines of CRC treatment (3, 4).

Anti-PD1 drugs show outstanding responses in patients with metastatic CRC with deficient mismatch repair (dMMR). However, CRC patients with dMMR, also designated as consensus molecular subtype (CMS) I, are a very small subset of the metastatic population. These tumors are mostly characterized by dysfunction of mismatch repair genes (referred to as high-level microsatellite instability (MSI-H)); unfortunately, MSI-H becomes rarer as the tumor becomes more advanced (5-7). However, a study showed that 44.8-89% of CRC patients express PD-L1 (8), and that over-expression of PD-L1 is an independent indicator of poor prognosis. As PD-1/PD-L1 immune checkpoint blockers (ICBs) are effective only in 2.5% of patients with metastatic CRC showing MSI-H or dMMR, the majority of patients do not benefit from anti-PD1/PD-L1 regimens. This is another obstacle to general application of immunotherapy for CRC despite the responsiveness of advanced-stage MSI-H CRC to immune checkpoint blockers; thus, much research is being conducted to examine the cellular mechanisms that are activated/inhibited by combination regimens that include targeted therapy with ICBs (9-11).

Previously, we reported a risk scoring system for CRC prognosis based on the expression of 19 genes (TCA19) regulated by triggering receptor expressed on myeloid cells-1 (TREM1) or connective tissue growth factor (CTGF) (12). Of the 19 genes, down-regulation of signaling lymphocytic activation molecule F7 (SLAMF7) and up-regulation of TREM1 were identified in both primary and metastatic CRCs (13). The self-ligand receptor SLAMF7 plays roles in various immune reactions; namely, cytotoxicity, humoral immunity, autoimmunity, cell survival, cell adhesion, and lymphocyte development (14). In the presence of EAT-2, SLAMF7 has a positive effect on natural killer (NK) cell activation. NK cells are important innate immune lymphocytes that support the immune response by destroying virus-infected cells or cancer cells through targeted release of cytotoxic factors and inflammatory cytokines (15). By contrast, TREM1 is a potent amplifier of pro-inflammatory innate immune response. In addition to pro-inflammatory effects, TREM1 has other important biological functions such as promoting growth of lung tumors by regulating the malignant behavior of lung cancer cells (16, 17). CRC is a paradigm for cancers driven by chronic inflammation. Inhibition of TREM1 by LP17 has an anti-inflammatory effect that inhibits inflammation and tumor progression (18). Herein, we aimed to investigate the relationship between SLAMF7 and TREM1 and anti-PD-1 drugs, and to determine whether they may be a molecular target or predictors of response to immunotherapy.

Materials and Methods

The study protocol was approved by the Institutional Review Board of Asan Medical Center (registration No. 2015-0581).

CRC cell lines, cloning and THP-1 cells. Five CRC cell lines (DLD1, RKO, SW480, HCT116, and HT29) and human monocytic THP-1 cells were purchased from the American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Myc-DDK tagged SLAMF7 and TREM1 cDNAs were purchased from OriGene (Rockville, MD, USA). Transient transfection was performed to establish each cell mixture using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). Stable clones were selected by culturing with aminoglycoside antibiotic G418 for 10 d, and at least two different clones were generated for each cell line. M2- polarized THP-1 cells were generated by treatment with 50 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) for 24 h, followed by overnight incubation with 25 ng/ml interleukin (IL)-4 (Sigma-Aldrich) and 25 ng/ml IL-13 (Sigma-Aldrich). THP-1 cells were plated at a density of 1×10^4 cells/well and then activated by overnight incubation in the presence of lipopolysaccharide (LPS; 1 μg/ml, Sigma-Aldrich). DLD1, RKO, SW480, HCT116, HT29, and THP-1 cells were co-cultured for 5 days in chambers fitted with Transwell inserts (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell viability and invasion assay. Control and treated CRC cells were seeded onto 96-well plates to assess proliferation. Cell viability was measured daily for 5 days using a cell proliferation assay kit (CCK-8; Dojindo, Kumamoto, Japan) and a microtiter plate reader adjusted to measure absorbance at 450 nm (Tecan, Melbourne, Australia). For the invasion assay, control and treated CRC cells (2×10^5 cells) were seeded onto the upper chamber of 24-well culture plates using a Biocoat™ Matrigel invasion chamber (Becton Dickinson). Next, 3T3-fibroblast-conditioned medium was placed in the lower chamber to act as a chemoattractant. After incubation at 37˚C for 24 h, cells on the upper surface of the filter were completely removed by gently swabbing. The number of cells that passed through the filter and invaded the lower chamber were counted under a light microscope in three different fields (×100). All assays were performed in triplicate.

Western blotting and real-time reverse transcription-PCR. Protein extracts from cultured cells were resolved in 10% SDS polyacrylamide gels and transferred to a polyvinylidene difluoride (Millipore, Billerica, MA, USA) membrane for western blot (WB) analysis with antibodies specific for HMGB1 (Cell Signaling Technology, Danvers, MA, USA), PD-L1 (Cell Signaling Technology), Ewing's sarcoma-related transcript 2 (EAT-2) (Abcam, Cambridge, UK), and actin (Bethyl, Montgomery, TX, USA). The membranes were then washed with TBST and incubated for 1 h at room temperature with an appropriate HRP-conjugated secondary antibody (1:10,000; Abcam). Protein-antibody complexes were visualized using a chemiluminescence reagent (New England Nuclear, Boston, MA, USA).

Total RNA was extracted from CRC cell lines using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Next, cDNA was synthesized from total RNA by amplification using random primers and SuperScript II RT (Invitrogen). Quantitative real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on a LightCycler 96 using the SYBR Green I Master Mix (Roche, Mannheim, Germany), in a total volume of 20 μl. The PCR conditions were as follows: initial denaturation at 95˚C for 10 min, followed by 45 cycles of amplification (95˚C for 10 s, 60˚C for 10 s, and 72˚C for 20 s). Gene expression determined by RT-qPCR was normalized to expression of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used to detect genes related to for cancer immunity are listed in Table I.

Multiplexed immunofluorescence with image acquisition and quantitative analysis. MSI-H and MSS tissues from 10 CRC patients each were used for immunofluorescence multiplex staining, which was performed using the Perkin-Elmer Opal kit (Perkin-Elmer, Waltham, MA, USA). MSI status was based on the results of pathological studies, and tissue samples were acquired as 4 μm-thick serial sections cut from formalin-fixed paraffin-embedded (FFPE) blocks. After deparaffinization, the slides underwent five sequential rounds of multiplex immunohistochemistry using the following primary antibodies: anti-CD68 (ab955, Abcam), anti-CD73 (ab175396, Abcam), anti-SLAMF7 (ab192526, Abcam), anti-TREM1 (LS-B10694, LSBio, Seattle, WA, USA), and anti-PD-L1 (#13684, Cell Signaling Technology). Nuclei were visualized by staining with DAPI. Spectral information from a multiplexed panel of targets was captured by the Vectra 3.0 Automated Quantitative Pathology Imaging System (Perkin-Elmer). Each individually stained section (CD68-Opal 780, CD73-Opal 690, SLAMF7-Opal

Table I. *Primers for real time RT-PCR for cancer-immunity cycles genes.*

Genes	Primer and target sequence 5'-3'	RefSeq	Product (bp)	Tm ˚C
GAPDH	Forward: TTCTTTTGCGTCGCCAGCCGA Reverse: GTGACCAGGCGCCCAATACGA	NM_002046.7	96	60
BTLA	Forward: TGGGTCATACCGCTGTTCTGCA Reverse: CTGCTTGCCATTTCGTCCTTGG	NM_001085357	123	60
CD8A	Forward: ACTTGTGGGGTCCTTCTCCTGT Reverse: TGTCTCCCGATTTGACCACAGG	NM_001768	106	60
CXCL10	Forward: GGTGAGAAGAGATGTCTGAATCC Reverse: GTCCATCCTTGGAAGCACTGCA	NM_001565	134	60
HMGB1	Forward: GCGAAGAAACTGGGAGAGATGTG Reverse: GCATCAGGCTTTCCTTTAGCTCG	NM_002128	134	60
IL6	Forward: AGACAGCCACTCACCTCTTCAG Reverse: TTCTGCCAGTGCCTCTTTGCTG	NM_000600	132	60
IL13	Forward: GAGGATGCTGAGCGGATTCTG Reverse: CACCTCGATTTTGGTGTCTCG	NM_002188	85	60
LAG3	Forward: GCAGTGTACTTCACAGAGCTGTC Reverse: AAGCCAAAGGCTCCAGTCACCA	NM_002286	143	60
PD-L1	Forward: TGCCGACTACAAGCGAATTACTG Reverse: CTGCTTGTCCAGATGACTTCGG	NM_001267706	150	60

620, TREM1-Opal 570, PD-L1-Opal 520, and DAPI) was used to establish the spectral library of the fluorophores required for multispectral analysis. The phenotyping feature was trained to choose cells with high confidence (≥60%). In addition to phenotyping, expression of immune cells was analyzed using the double positivity scoring method. This method categorized the cells as showing positive or negative intensity according to the threshold score, which was evaluated using the normalized count value for each antibody [provided by the Inform® software (Perkin-Elmer)]. For double positivity scoring analysis, the number of cells expressing two immune markers simultaneously was counted by exporting the score for each marker using the Spotfire™ program (Perkin-Elmer). Multispectral imaging and immune cell quality were analyzed by phenotyping and double positivity scoring analysis. The density of the immune-infiltrates was calculated from the mean number of immune infiltrates in all evaluated blocks.

Apoptosis and necrosis. TREM1 antagonist peptides (LP17: LQVEDSGLYQCVIYQPP; and LR12: LQEEDAGEYGCM), and the corresponding sequence-scrambled negative control peptides (LP17-s: EDSQCVIGLYQPPLQVY; and LR12-s: YQMGELCAGEED), were chemically synthesized as -COOH terminally-amidated peptides (Peptron, Daejeon, Republic of Korea). Apoptosis induced by nivolumab, pembrolizumab, and TREM1 inhibitors (LP17 and LR12) was measured by analysis of annexin V-FITC and PI signals (BD annexin V-FITC Apoptosis detection kit 1, Becton Dickinson). Briefly, DLD1 and SW480 cells in 6-well plates were treated with or without nivolumab or pembrolizumab for 48 h or 72 h. HCT116 and HT29 cells in 6-well plates were treated with LP17 or LR12 with or without nivolumab or pembrolizumab for 48 h or 72 h. Cells were analyzed using the BD FACSDiva 8.0 program and a FACScan flow cytometer (Becton Dickinson).

Immunogenic cell death. For the HMGB-1 ELISA, cells in 24-well plates were treated as indicated. CRC cell supernatants were collected after 48 h. The assay was performed using the HMGB1 ELISA kit (ST51011, IBL International, Hamburg, Germany), according to manufacturer's instructions, with the following modifications. Briefly, plates containing the standards, a positive control, and treated samples were incubated for 24 h at 37˚C. After the medium was discarded, wells were washed five times with 400 µl of diluted wash buffer. An anti-HMGB1-horseradish peroxidase-conjugated antibody was added to each well for 2 h at 25˚C. After washing again, a color solution (100 µl) containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide was added to each well for 30 min at 25˚C. Stop (0.35M sulfuric acid) solution (100 µl) was then added to each well and the contents were mixed by shaking the plates gently. Absorbance was measured at 450 nm (reference wavelength of 600–650 nm) within 60 min.

Statistical analysis. Cell proliferation, invasion, and mRNA expression by over-expressing cells and control cells were compared using an unpaired Student's *t*-test. Cell viability, apoptosis, and immunogenic cell death (ICD) of CRC cells treated with or without immunotherapeutic agents were compared using a paired t-test. Comparison of immune cells between MSI-H CRC and MSS CRC cells was also carried out using the unpaired Student's *t*-test or the Mann-Whitney *U*-test. All calculations were conducted using SPSS software (version 21; SPSS Inc., Chicago, IL, USA). A *p*-value of <0.05 was considered significant.

Results

SLAMF7 and TREM1 affect CRC cell proliferation and invasion. Cell proliferation was measured using two clones of CRC cells over-expressing SLAMF7 and TREM1. Compared with that of control cells, proliferation of TREM1-over-expressing HT29 cells increased by 1.8-fold on Day 5, whereas that of SLAMF7 DLD1 cells decreased by a

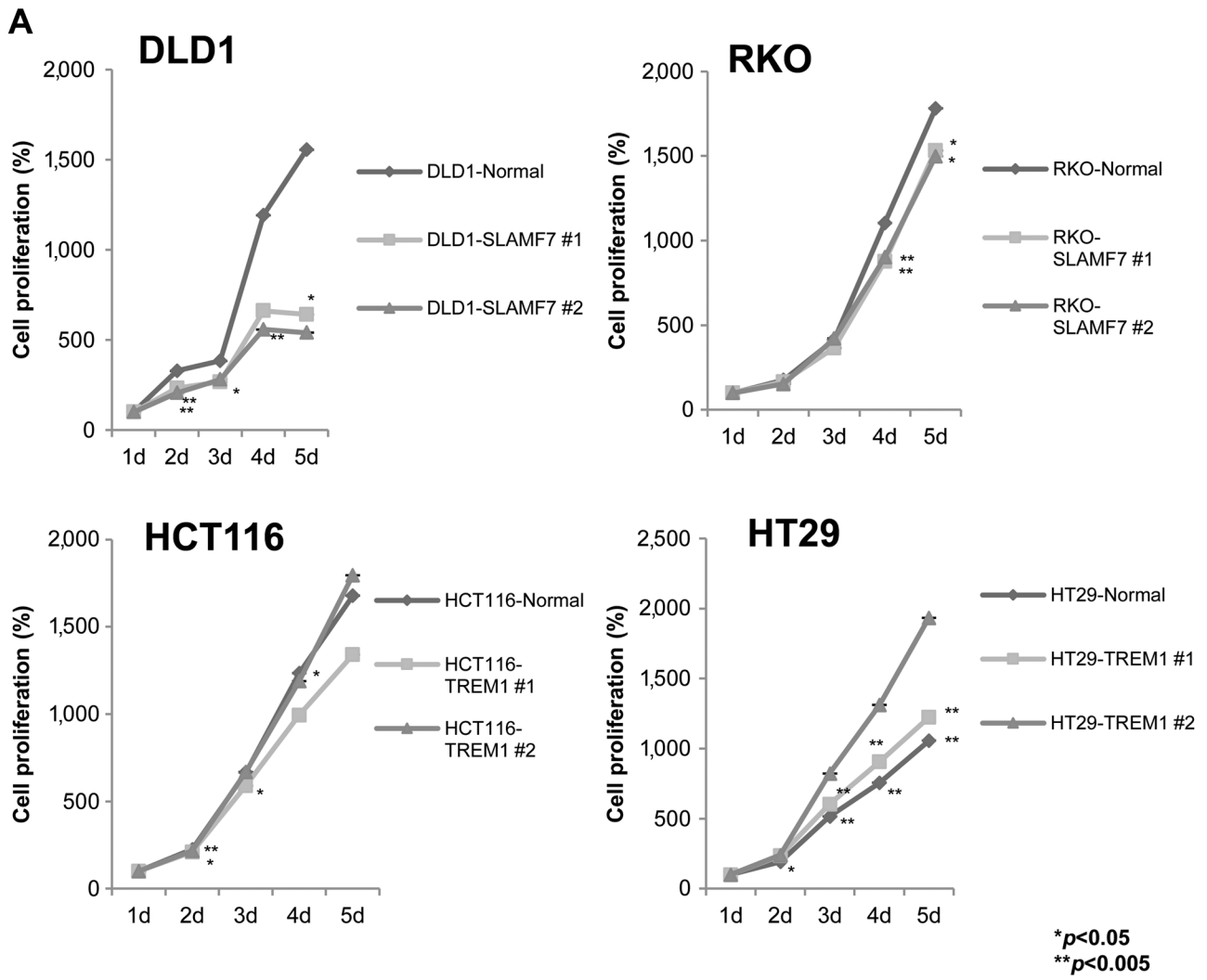


Figure 1. *Continued*

third (Figure 1A). TREM1-over-expressing HCT116 and HT29 cells were twice as invasive as control cells ($p<0.001$ and 0.031, respectively), whereas SLAMF7-over-expressing DLD1 cells and RKO cells showed reduced invasiveness (DLD1 cells, $p=0.002$) (Figure 1B).

SLAMF7 acts as a stimulatory factor in cancer immunity. To investigate the role of SLAMF7 during the cancer immunity cycle, we co-cultured SLAMF7-over-expressing CRC cells with THP-1 cells for 5 days and examined expression of immune activators. qPCR analysis revealed that *HMGB1* expression increased in SLAMF7-over-expressing DLD1 and RKO cells co-cultured with THP-1 monocytes (Figure 2A). When SLAMF7-over-expressing DLD1 cells were co-cultured with THP-1 monocytes, THP-1 M2 macrophages, or LPS-activated THP-1 monocytes, expression of both C-X-C motif chemokine ligand 10 (*CXCL10*) and *IL-6* increased (Figure 2A). Factors showing increased expression by qPCR were verified by WB. SLAMF7-over-expressing DLD1 and RKO cells showed increased expression of HMGB1 when co-cultured with THP-1 monocytes (Figure 2C). EAT-2 expression increased only in DLD1 cells; expression increased markedly upon co-culture with THP-1 macrophages (Figure 2C). PD-L1 expression decreased significantly in SLAMF7-over-expressing cells, and also decreased when co-cultured with the THP-1 cell line (Figure 2C).

TREM1 acts as an inhibitory factor during cancer immune response. TREM1-over-expressing HCT116 and HT29 CRC cells were co-cultured for 5 days with THP-1 cells and expression of immune inhibitors was analyzed by qPCR and WB. qPCR analysis revealed expression of *lymphocyte-*

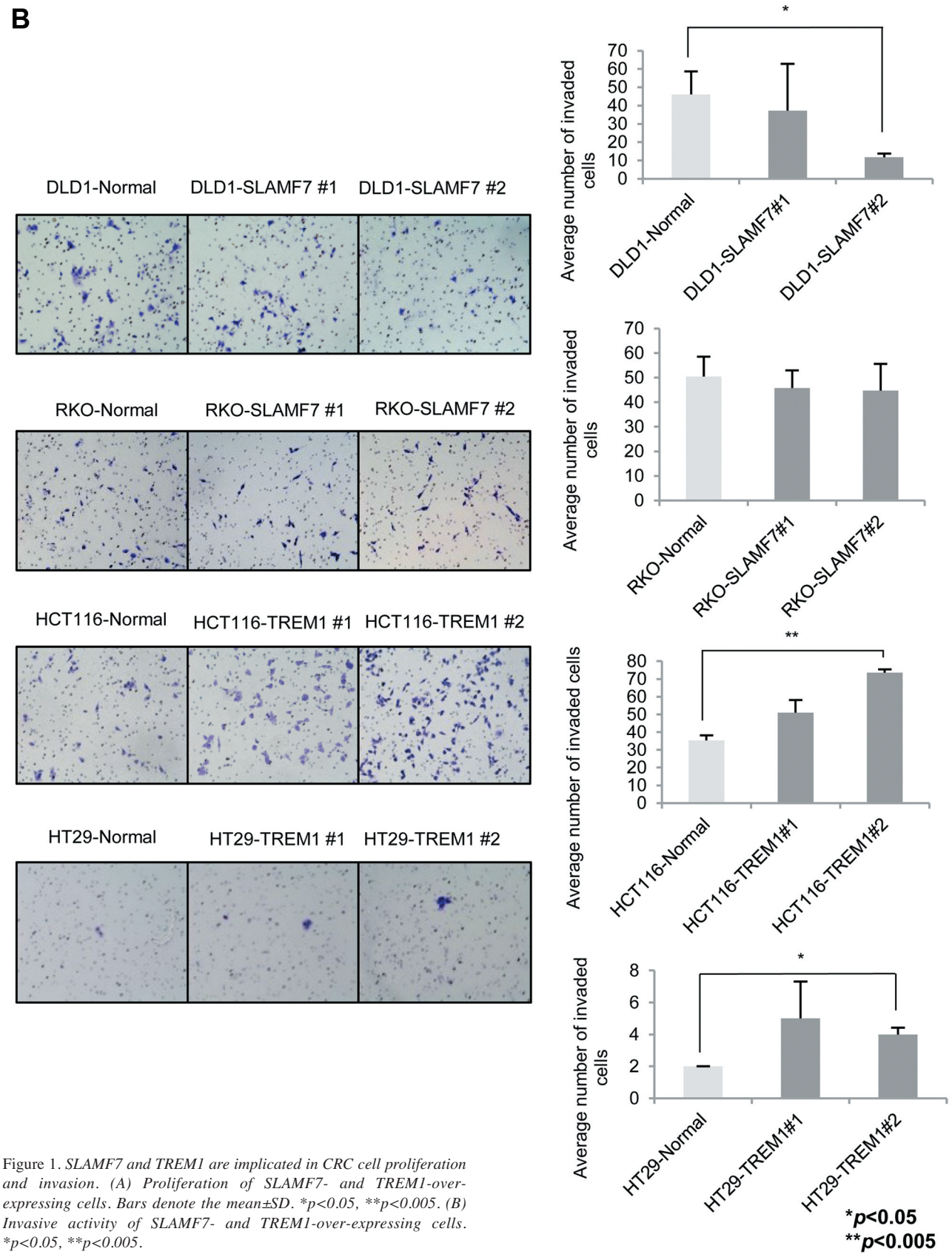
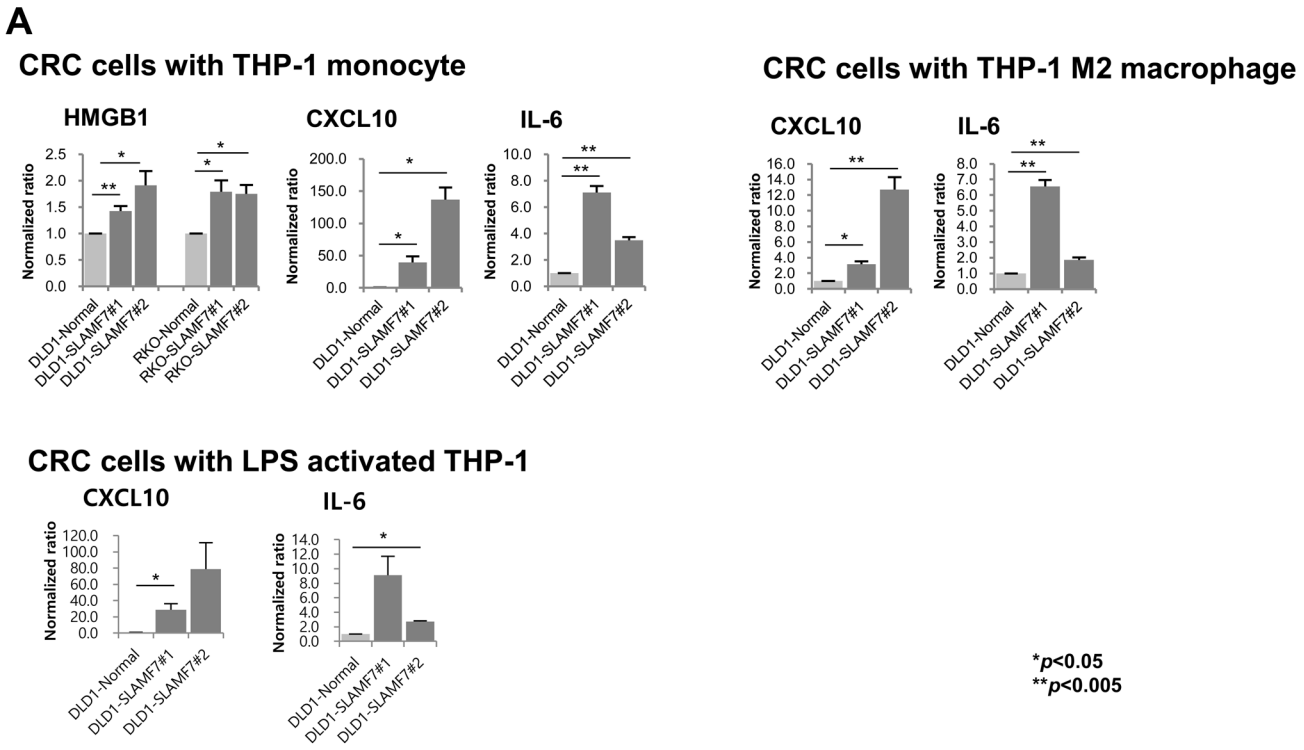


Figure 1. *SLAMF7 and TREM1 are implicated in CRC cell proliferation and invasion. (A) Proliferation of SLAMF7- and TREM1-over-expressing cells. Bars denote the mean±SD. *p<0.05, **p<0.005. (B) Invasive activity of SLAMF7- and TREM1-over-expressing cells. *p<0.05, **p<0.005.*



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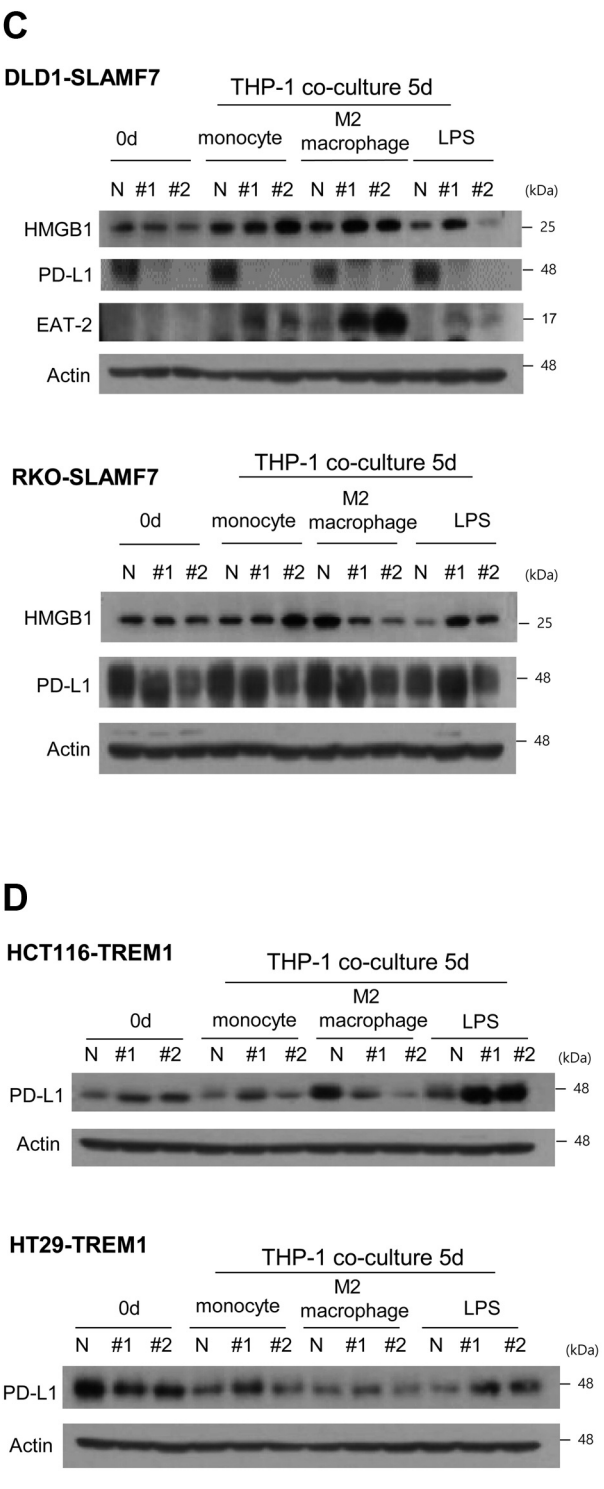

Figure 2. *SLAMF7 and TREM1 play roles in the cancer immunity cycle. (A and C) SLAMF7-over-expressing CRC cells and THP-1 cells were co-cultured for 5 days and expressions of activators of the cancer immunity cycle were analyzed by qPCR and western blotting. (B and D) TREM1-over-expressing CRC cells and THP-1 cells were co-cultured for 5 days and expressions of activators of the cancer immunity cycle were analyzed by qPCR and western blotting. *p<0.05, **p<0.005.*

activation gene 3 (*LAG-3)* (p=0.012, HCT116 clone #1; p=0.036, HT29 clone #2) and HMGB1 (p<0.001, clone #1 and 0.021, clone #2, HCT116; p=0.018, clone #1 and <0.001, clone #2, HT29) upon co-culture with THP-1 monocytes, whereas co-culture with LPS-activated THP-1 monocytes resulted in expression of *PD-L1* (p=0.052, clone #1 and p<0.001, clone #2, HCT116) and HMGB1 (p<0.001, HCT116 clone #1). In addition, expression of *PD-L1* and the pro-inflammatory cytokine *IL-13* was detected upon co-culture with THP-1 M2 macrophages (p<0.001, HCT116 clone #1) (Figure 2B). WB analysis revealed that TREM1-over-expressing HCT116 cells showed increased expression of PD-L1 upon co-culture with THP-1 monocytes; this increased further upon co-culture with LPS-activated THP-1 cells (Figure 2D).

Quantification of immune cells among MSI-H CRC and MSS CRC. Multispectral imaging and immune cell quality were demonstrated by phenotyping analysis with MSI-H CRC and MSS CRC patients (Figure 3A). The number of TREM1 (+) cells among total immune infiltrating cells was significantly greater in MSI-H patients than in MSS patients (p=0.016) (Figure 3B). The density of TREM1+ CD73+ double-positive cells was significantly greater in MSI-H patients than in MSS patients (Figure 3C).

Cell viability and apoptotic cell death after treatment with immunotherapeutic agents. MSI-high DLD1 cells were more susceptible to pembrolizumab and nivolumab than the MSS SW480 cell line. In particular, the survival rate fell by 27.5% in the presence of pembrolizumab, showing a statistically significant difference (p=0.046). The viability of SLAMF7-over-expressing DLD1 cells was similar (clone #1) or slightly higher (clone #2) than that of control cells when treated with pembrolizumab. The effect of nivolumab was more pronounced; the viability of clone #1 was 42.1% lower, and that of clone #2 was 56.6% lower, than of control cells (p=0.046 for clone #2).

Pembrolizumab treatment of SLAMF7-over-expressing SW480 cells induced cell death to a greater extent than in control cells; the viability of clone #1 fell by 27.8% and that of clone #2 fell by 50.6%. Even when cells were exposed to nivolumab, there was a reduction in viability (19.09% for clone #1 and 26.8% for clone #2, Figure 4A). In addition, the viability of TREM1-over-expressing HCT116 and HT29 cells was markedly lower in the presence of LP17 and LR12 peptides than in the presence of anti-PD-1 drugs alone (Figure 4A).

Apoptosis of SLAMF7-over-expressing DLD1 cells increased in the presence of pembrolizumab and nivolumab; the increase in apoptosis induced by pembrolizumab was statistically significant (p=0.014 for clone #2) (Figure 4B). Co-administration of pembrolizumab plus LR12 peptides

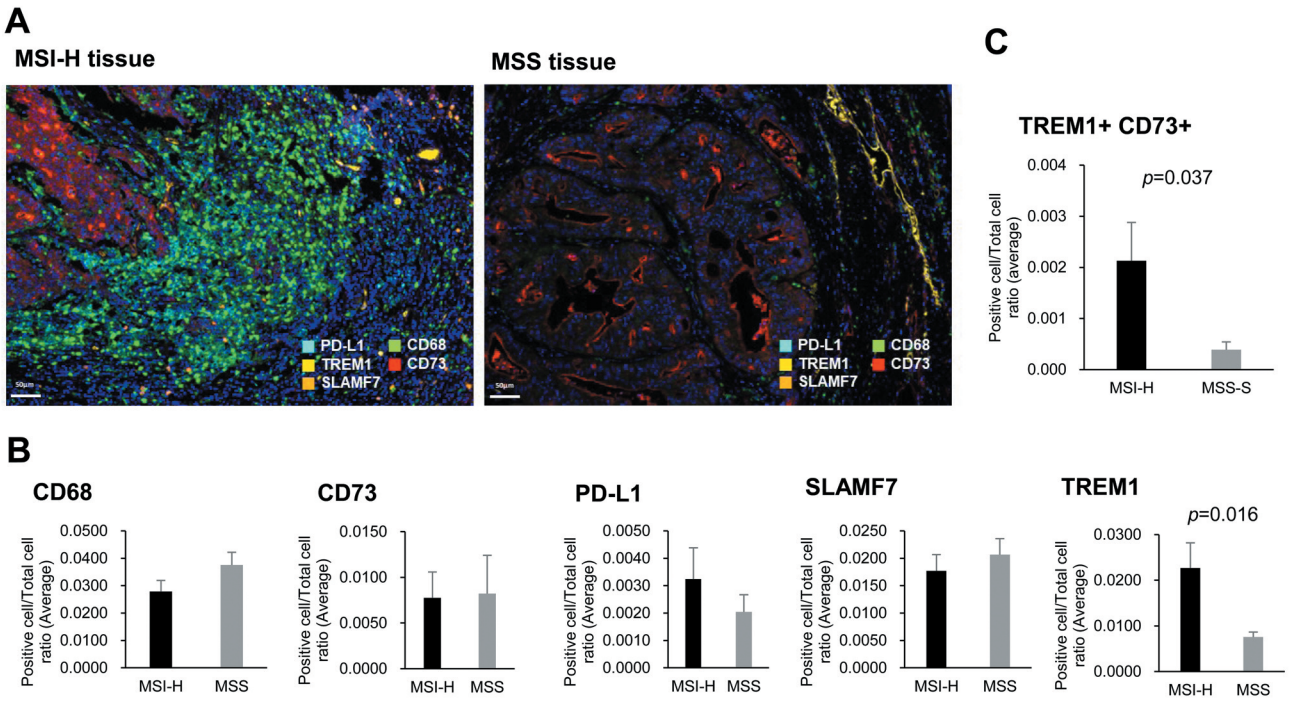

Figure 3. *Quantification of immune cells between MSI-H CRC and MSS CRC. (A) Representative images of multispectral immune-histochemical staining of MSI-H CRC and MSS CRC patients (mag. 20×, scale bars, 50 μm). (B) Comparison of immune marker expression by MSI-H CRC and MSS CRC patients. (C) Co-expression of TREM1 and CD73, as assessed using the double positivity scoring method.*

increased apoptotic cell death in TREM1-over-expressing cell lines when compared with that in control cells. When HCT116 cells were treated (or not) with pembrolizumab plus LR12 peptides, there was a statistically significant increase in apoptosis in TREM1-over-expressing cells compared with control cells. (p=0.038) (Figure 4C).

Pembrolizumab and nivolumab increase HMGB1 expression in SLAMF7- and TREM1-over-expressing CRC cell lines. In both DLD1 and SW480 cell lines, HMGB1 expression increased after treatment with pembrolizumab and nivolumab (p=0.012 and 0.01, respectively in DLD1 cells, and p=0.006 and 0.036, respectively in SW480 cells) (Figure 5A). Treatment with pembrolizumab led to over-expression of HMGB1 in SLAMF7-over-expressing SW480 cells (clone #1) (p=0.004).

Over-expression of HMGB1 was observed in TREM1-over-expressing HCT116 cells (clone #2) treated with pembrolizumab plus LR12 (p=0.006) (Figure 5B). In this case, addition of LR12 increased expression of HGMB1 to a greater extent than pembrolizumab alone (p=0.012) (Figure 5B). LR12 showed similar effects when administered with nivolumab. Over-expression of HMGB1 was observed in TREM1-over-expressing HCT116 cells (clone #2) treated with nivolumab plus LR12 (p=0.017) (Figure 5B). Finally, expression of HMGB1 in TREM1-over-expressing MSS HT29 cells increased further in the presence of LP17 plus pembrolizumab (p=0.004 for clone #1; p=0.045 for clone #2) (Figure 5C).

Discussion

SLAMF7 acts as an immune activator. Here, we show that proliferation and invasiveness of a SLAMF7-over-expressing CRC cell line were inhibited. The association between SLAMF7 and other immune activators linked to cancer immunity was examined by qPCR and WB. qPCR revealed increased expression of *HMGB1*, *CXCL10*, and *IL-6* when SLAMF7-over-expressing CRC cells were co-cultured with THP-1 cells. In particular, WB revealed increased expression of HMGB1.

CXCL10 drives T helper type 1 (Th1) polarization; Th1 cells produce interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-2, and increase anti-tumor immunity by stimulating cytotoxic T lymphocytes, NK cells, natural killer T (NKT) cells, and macrophages (19). HMGB1 is released by activated macrophages, monocytes, dendritic cells, and NK cells in response to inflammation and infection. In addition, HMGB1 released by dying tumor cells may suppress M2 polarization of macrophages and prevent tumor regrowth and resistance to treatment (20).

Expression of PD-L1, which has an immunosuppressive function, was reduced to a greater extent in SLAMF7-

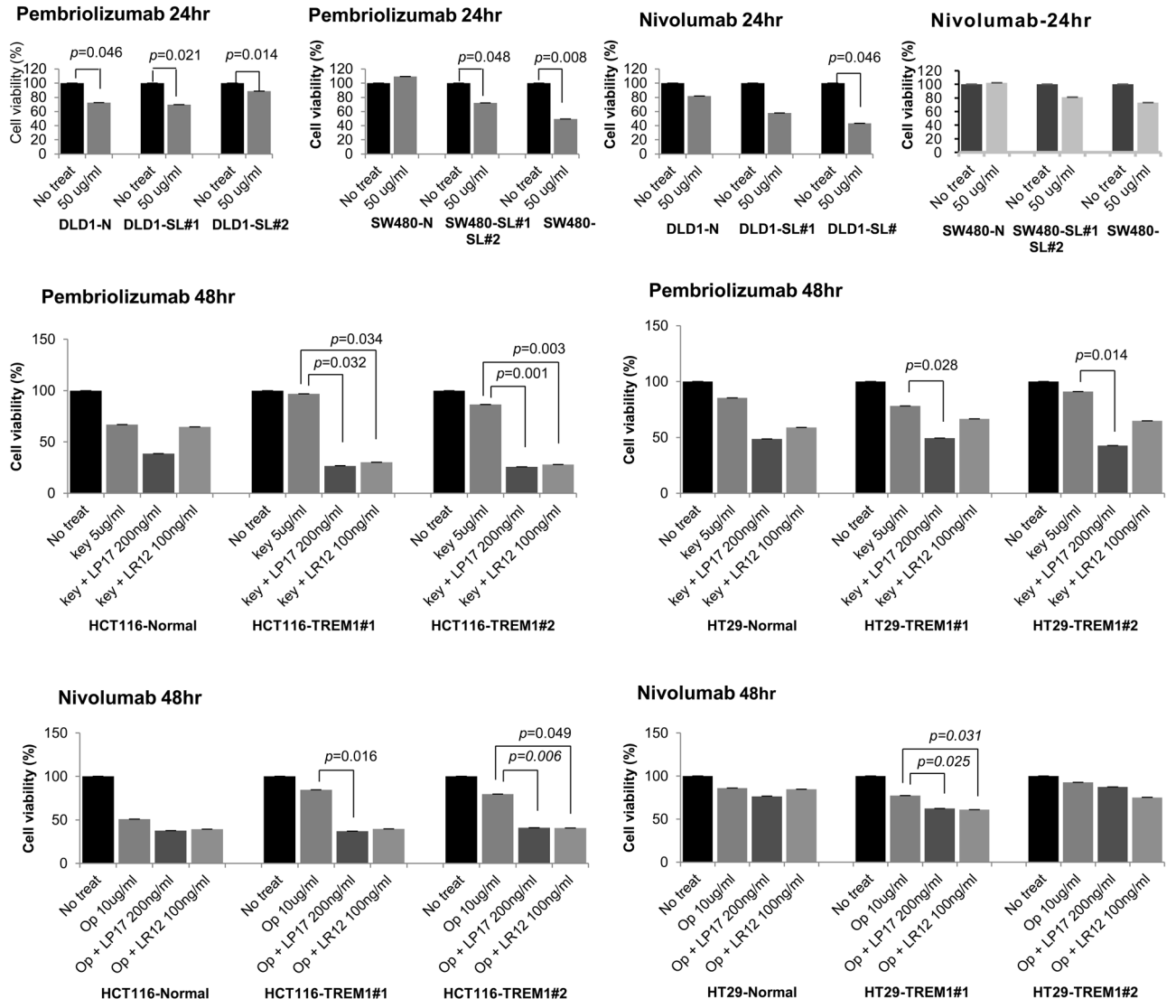

Figure 4. *Continued*

over-expressing CRC cells than in control cells, and decreased significantly when SLAMF7-over-expressing CRC cells were co-cultured with THP-1 cells. We found that SLAMF7-over-expressing CRC cells exhibited reduced viability and increased apoptotic cell death when treated with pembrolizumab and nivolumab. Multiple myeloma cells show high expression of SLAMF7; thus, many studies have examined the therapeutic effects of the anti-SLAMF7 antibody elotuzumab (21). However, because expression of SLAMF7 is low in CRC, inducing increased expression in CRC cells could be a therapeutic strategy (22).

TREM1 is over-expressed by CRC cells; indeed, and we found that TREM1-over-expressing cells showed augmented proliferation and invasion. Expression of immune inhibitors PD-L1 and IL-13 increased when TREM1-over-expressing CRC cells were co-cultured with THP-1 cells. TREM1 plays a role in colon inflammation and tumorigenesis, and has emerged as a prognostic indicator (23). Therefore, inhibiting TREM1 with synthetic peptides attenuates cell-mediated inflammation, migration, and proliferation. Indeed, we showed that the small peptide LP17, spanning the complementary-determining region 3 (CDR3) of TREM1, binds competitively to TREM1 to reduce its activity, thereby accelerating

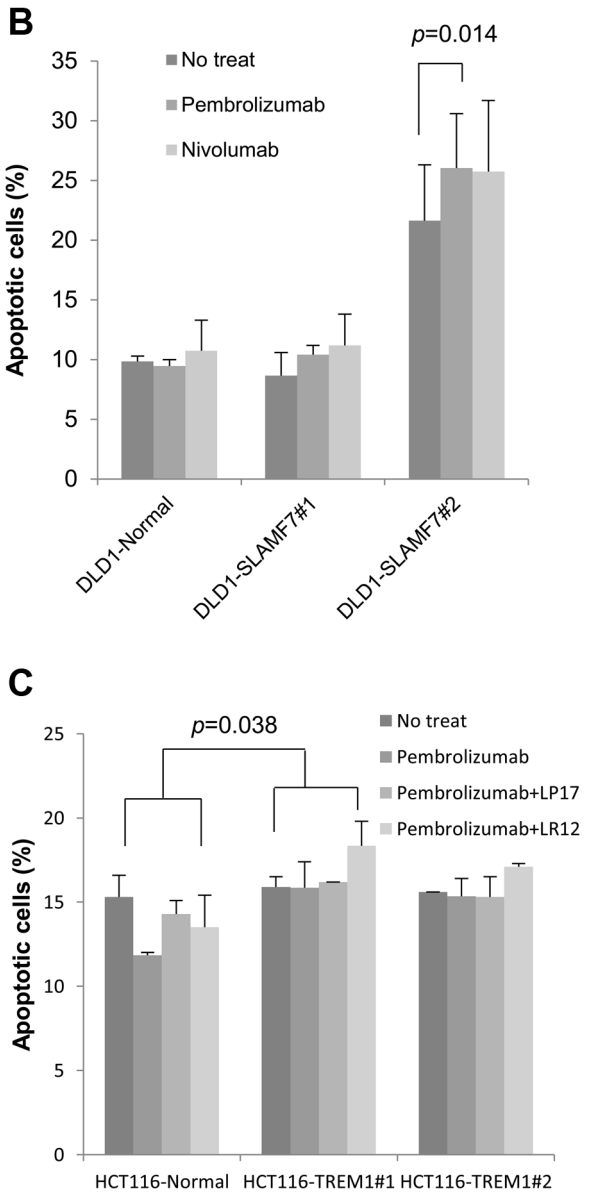

Figure 4. *Cell viability and apoptosis of SLAMF7- and TREM1-over-expressing CRC cells treated with PD-1 drugs. (A) Viability of DLD1, SW480, HCT116, and HT29 cells (CCK8 assay). (B) Effect of pembrolizumab or nivolumab on apoptotic cell death of SLAMF7-over-expressing DLD1 cells. (C) Effects of pembrolizumab or nivolumab plus LP17 or LR12 on apoptotic cell death of TREM1-over-expressing HCT116 cells.*

inflammation and CRC progression (24). In addition, a TLT-1 derived peptide (LR12) is a pharmacological adjuvant that inhibits TREM1 (25). LR12 does not bind directly to TREM1; rather, it prevents ligation of the TREM1 ligand, which then inhibits activity of TREM1. The apoptotic effects of the anti-PD-1 regimen alone on tumor cells without an immune microenvironment is negligible or weak. However, several studies demonstrated apoptotic effects of anti-PD-1 on cancer when administered concurrently with chemotherapeutic drugs such as cisplatin or an immune-sensitizer (26, 27). We found that TREM1-over-expressing CRC cells showed greater apoptotic cell death when treated with LP17 or LR12 plus anti-PD-1 than they did when treated with anti-PD1 alone. This indicates that these peptides are potential adjuvant molecules that may augment anti-PD1 treatment; in vivo trials using patient-derived xenograft or organoids should be conducted to confirm this.

In this study, we found that expression of HMGB1 by TREM1-over-expressing HCT116 cells and HT29 cells increased upon co-culture with THP-1 monocytes/macrophages. A previous study shows that necrotic cell lysate from THP-1 cells activated by murine hepatocytes containing HMGB1 were able to induce an inflammatory response that was blocked by recombinant soluble TREM1. Thus, the TREM1/HMGB1 interaction likely plays an important role in promoting inflammatory responses and liver damage (28). We also found that co-administration of pembrolizumab or nivolumab plus TREM1 inhibitors increased expression of HMGB1 by TREM1-over-expressing CRC cells. HMGB1 is a marker of immunogenic cell death. Depending on the initiating stimulus, cancer cell death can be immunogenic or non-immunogenic. Immunogenic cell death involves cell surface structural changes and leads to release of pro-inflammatory cytokines, chemokines, and immunogenic factors (29). Immunogenic cell death biomarkers include exposure of pro-apoptotic calreticulin on the outer plasma membranes, extracellular release of non-histone chromatin HMGB1, and ATP secretion during apoptosis (30). In CRC cells, HMGB1 induces a non-apoptotic type of cell death that lacks caspase activation and characteristic morphological features (31). Here, we found that MSI-H DLD1 CRC cells over-expressing SLAMF7 showed increased apoptosis when treated with anti-PD-1 drugs; there was no difference in the SW480 cell line, which is MSS. By contrast, expression of HMGB1 increased when DLD1 and SW480 cells over-expressing SLAMF7 were treated with anti-PD-1 drugs.

Nivolumab and pembrolizumab are the first two anti-PD-1 mAbs approved by the US Food and Drug Administration. They target epitopes on PD-1 with high affinity and specificity. There is a clear developmental role for PD-1 inhibition in MSI-H CRC; however, an alternative would be required for MSS CRC. New drugs combinations are needed urgently (32, 33). In the case of TREM1, co-administration of anti-PD-1 drugs and TREM1 inhibitors increased apoptosis only in the HCT116 cell line (which is MSI-H), but HMGB1 expression was detected in both HCT116 cells and HT29 cells (which is MSS). Combined administration of agents such as anti-PD-1 drugs and TREM1 antagonists can reactivate immunogenic cell death in CRC tumors regardless of MSI status.

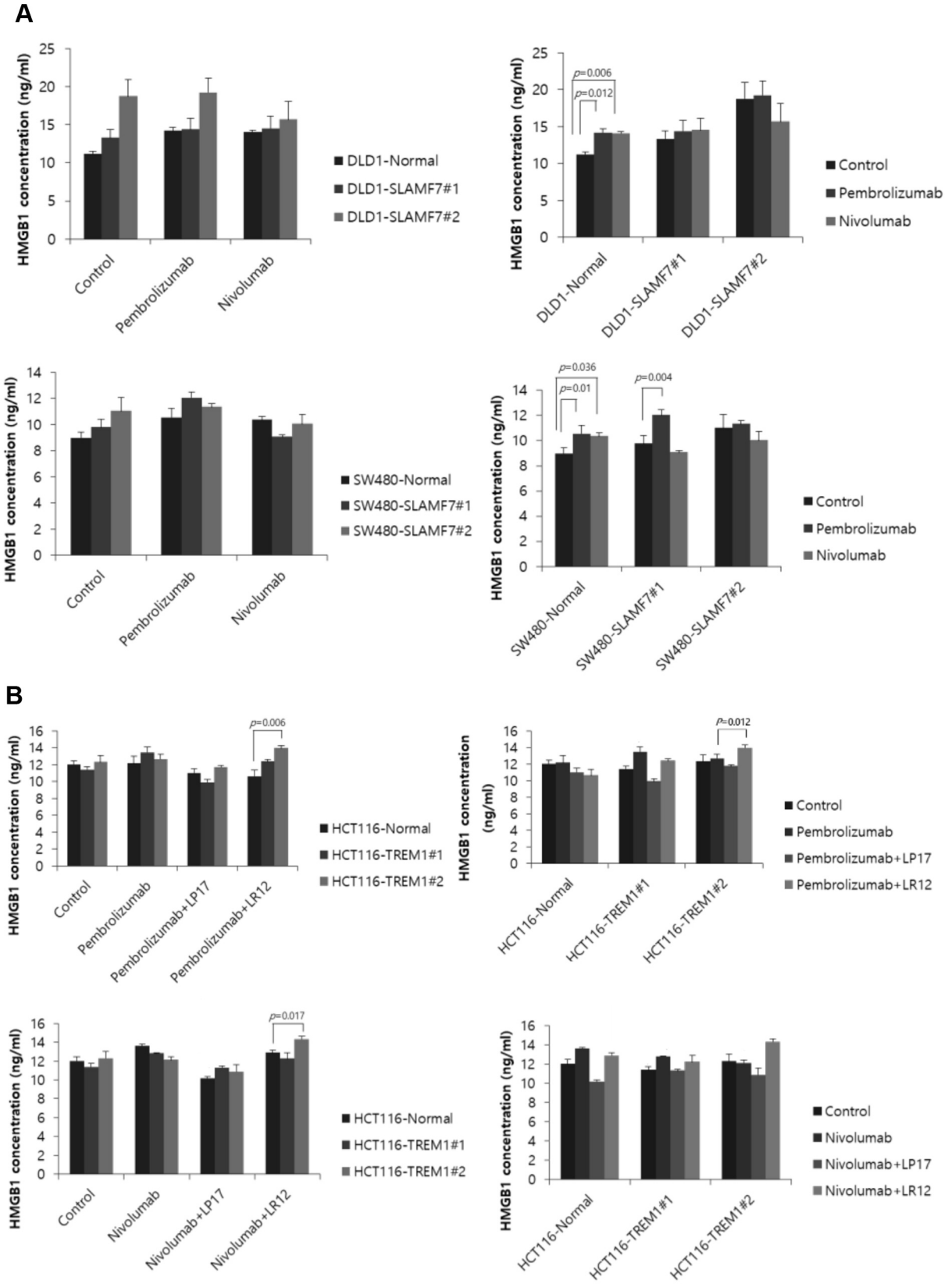

Figure 5. *Continued*

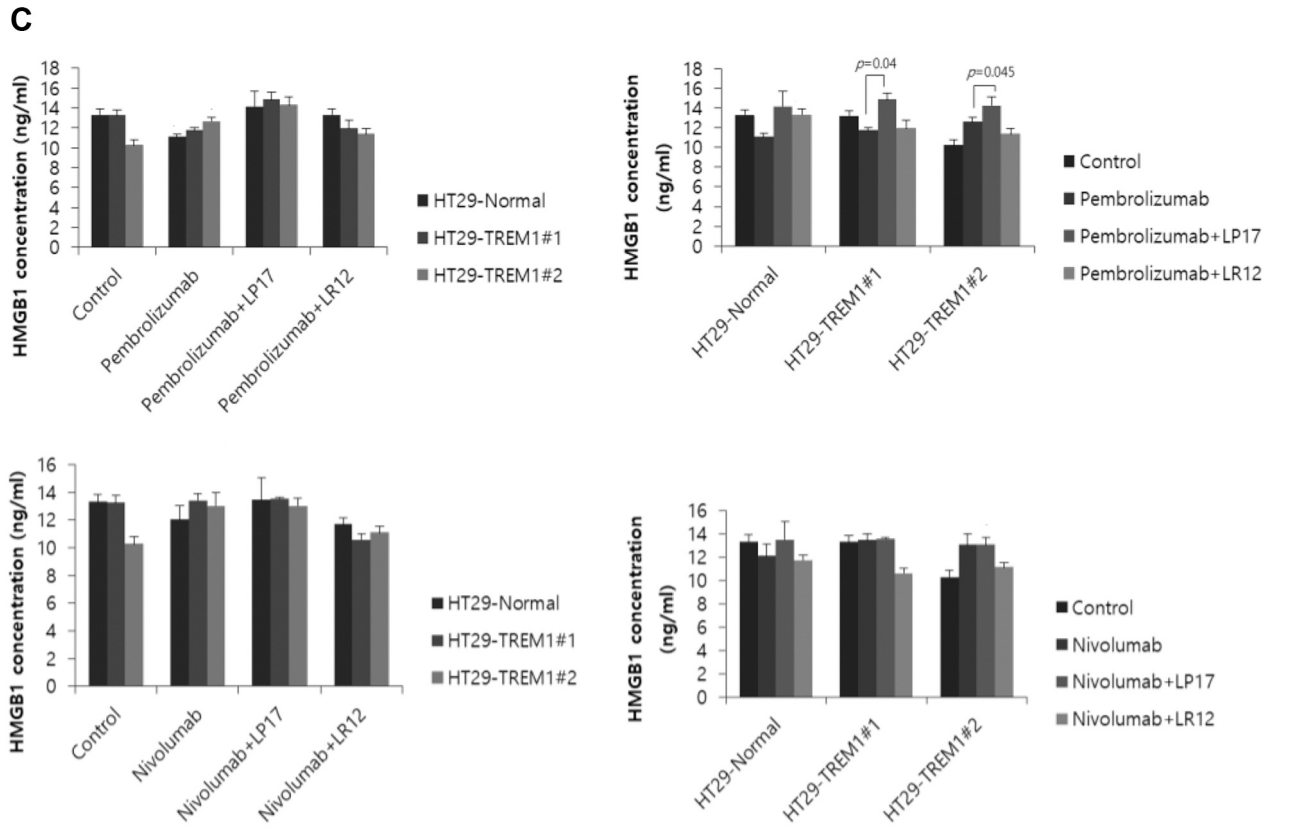

Figure 5. *Drug treatment increases expression of HMGB1 by SLAMF7- and TREM1- over-expressing CRC cells. (A) Effect of pembrolizumab or nivolumab on HMGB1 expression by SLAMF7- over-expressing CRC cells. (B) Effect of pembrolizumab or nivolumab plus LP17 or LR12 on HMGB1 expression by TREM1-over-expressing HCT116 cells. (C) Effect of pembrolizumab or nivolumab plus LP17 or LR12 on HMGB1 expression by TREM1-over-expressing HT29 cells.*

This study has several limitations. First, we likely omitted a number of immunogenic reactions associated with SLAMF7 and TREM1 because we did not consider them to be our primary focus with respect to interaction with ICBs. Second, given the limited availability of dMMR CRC cells, we may have missed the effects of these molecules on immunoediting. Third, the observed discrepancy between our findings and those of other reports requires further investigation to clarify their roles in CRC carcinogenesis and progression. In addition, we need to perform an upfront trial using patient-derived xenograft (PDX) or similar CRC xenograft to identify the link between TREM1 interactions and T cell activity.

In conclusion, our results strongly suggest that TREM1 and SLAMF7 are associated with immunogenic cell death. It has long been an issue whether the response of MSS tumors to immunotherapy can be modified; from this perspective TREM1 inhibitors may be an effective adjuvant for immunotherapy in MSS patients, particularly when combined with anti-PD-1 drugs.

Conflicts of Interest

The Authors declare no potential conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: Seon Ae Roh, Jin Cheon Kim; Methodology: Seon Ae Roh, Seon-Kyu Kim, Jin Cheon Kim; Resources: Seon Ae Roh, Yi Hong Kwon, Jong Lyul Lee, Seon-Kyu Kim; Data curation: Seon Ae Roh, Yi Hong Kwon, Jong Lyul Lee, Seon-Kyu Kim, Jin Cheon Kim; Investigation: Seon Ae Roh, Yi Hong Kwon, Jong Lyul Lee, Seon-Kyu Kim, Jin Cheon Kim; Formal analysis: Seon Ae Roh, Jin Cheon Kim; Funding acquisition: Seon Ae Roh, Jin Cheon Kim; Project administration: Seon Ae Roh, Jin Cheon Kim; Supervision: Jin Cheon Kim; Manuscript writing: Seon Ae Roh, Jin Cheon Kim. All Authors read and approved the manuscript.

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