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Research Article

Immunology

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CD155 loss enhances tumor suppression via combined host and tumor-intrinsic mechanisms

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Critical immune-suppressive pathways beyond programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) require greater attention. Nectins and nectin-like molecules might be promising targets for immunotherapy, since they play critical roles in cell proliferation and migration and exert immunomodulatory functions in pathophysiological conditions. Here, we show CD155 expression in both malignant cells and tumor-infiltrating myeloid cells in humans and mice. *Cd155*^{-/-} mice displayed reduced tumor growth and metastasis via DNAM-1 upregulation and enhanced effector function of CD8⁺ T and NK cells, respectively. CD155-deleted tumor cells also displayed slower tumor growth and reduced metastases, demonstrating the importance of a tumor-intrinsic role of CD155. CD155 absence on host and tumor cells exerted an even greater inhibition of tumor growth and metastasis. Blockade of PD-1 or both PD-1 and CTLA4 was more effective in settings in which CD155 was limiting, suggesting the clinical potential of cotargeting PD-L1 and CD155 function.

Introduction

Immunotherapy is emerging as an alternative to standard anti-cancer therapies, with the successful treatment of a proportion of various advanced cancer patients with anti-programmed death 1 (anti-PD-1), anti-programmed death ligand 1 (anti-PD-L1), and/or anti-CTLA4. However, many patients still do not benefit from these immunotherapies (1–4). Among many potential innate resistance mechanisms (5), other immune checkpoint receptors become more important. One receptor-ligand complex of interest involves the Ig superfamily members CD226 (also known as DNAM-1), CD96, and TIGIT and its cognate ligands CD155 (also known as poliovirus receptor [PVR] or NECL5) and CD112 (also known as nectin 2). Analogous to the CTLA4/CD28 pathway, the immune checkpoint receptors TIGIT and CD96, together with the costimulatory receptor DNAM-1, share CD155 and/or CD112 ligands and fine-tune the antitumor immune response (6, 7). Both TIGIT and CD96 appear to be promising immune checkpoint targets, given their expression patterns and preclinical data (6, 8–12). However, just as tumors evade T cell and NK cell responses through molecules like PD-L1, expressed on tumors and infiltrating myeloid cells, CD155 expressed by tumors and activated

myeloid cells has the potential to subvert immune responses through interaction with the immune checkpoint receptors CD96 and TIGIT (6, 12, 13).

The nectin/nectin-like family of genes comprise a group of cell adhesion molecules characterized by 3 extracellular Ig-like domains, a transmembrane region, and a cytoplasmic domain (14, 15). The nectin-like protein CD155 (PVR), given its involvement in the cellular poliovirus infection in primates, is expressed at very low levels in normal tissues, including in immune, epithelial, and endothelial cells (14, 16), but is highly expressed in most tumor cells (17, 18). On host endothelial or antigen-presenting cells (APCs), CD155 is upregulated by LPS or inflammatory cytokines (19, 20) and has been shown to regulate the development and function of immune cells in noncancer settings. For example, CD155 is involved in intestinal humoral immune responses (21) and, reportedly, may also be used to positively select MHC-independent T cells in the thymus (22). Furthermore, recipient CD155-deficient mice have more severe graft versus host disease (GVHD) than do WT mice (23).

The importance of these molecules in cancer is clearly evident by the fact that high levels of CD155 expression are associated with a poor prognosis in cancer patients (24, 25). Despite *in vitro* observations that CD155 overexpression on tumor cells plays a critical role in cell motility during invasion and migration (26–28), whether CD155 plays a critical tumor cell-intrinsic role upon tumor growth and metastasis *in vivo* remains unknown. Similarly, the distribution of CD155 within nonmalignant cells of the tumor microenvironment (TME) and the relative contributions of CD155 expression on tumor versus host cells in limiting antitumor immunity or response to therapy have not been previously defined.

► Related Commentary: p. 2199

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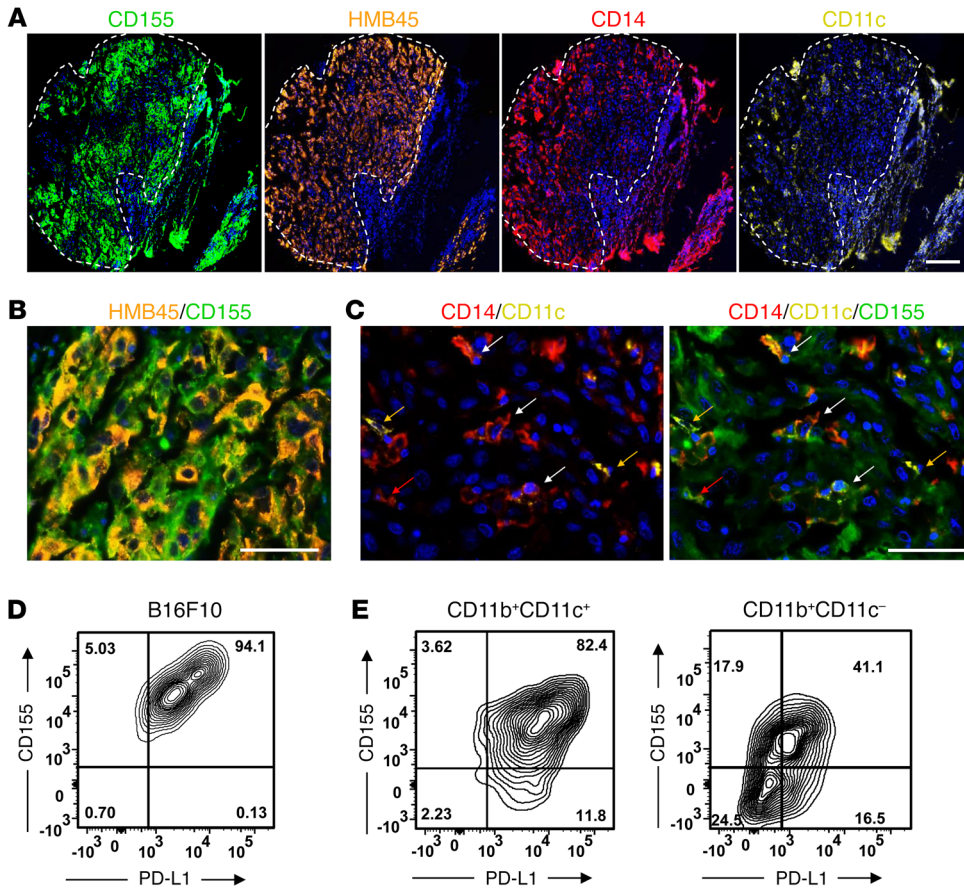


Figure 1. CD155 is expressed in malignant cells and tumor-infiltrating myeloid cells in human and mouse tumors. (A and B) Representative multiplexed IHC images of human primary cutaneous melanoma samples. CD155 (green) was distributed broadly within the carcinoma element of human melanoma, identified by HMB45 positivity (orange). Tumor-infiltrating myeloid cells were revealed by CD14 (red) or CD11c (yellow) positivity. The dotted line circumscribes HMB45⁺ tumor cells in a representative human melanoma TMA core. The merged image shows high colocalization of CD155 and HMB45. Scale bars: 200 μm (A) and 50 μm (B). (C) Colocalization of CD155 in tumor-infiltrating myeloid cells in human melanoma. CD11c (yellow) and CD14 (red) discriminated different populations of tumor-infiltrating cells, including CD11c⁺CD14⁻ DCs (yellow arrows), CD11c⁺CD14⁺ myeloid cells (white arrows), and CD11c⁻CD14⁺ monocytes/macrophages (red arrows). CD155 staining (green) was colocalized within each of these myeloid populations, as indicated in the merged panel. Scale bar: 50 μm. (A–C) Nuclei were stained with DAPI (blue) in each panel. (D and E) WT mice were injected s.c. with 1 × 10⁵ B16F10 cells (n = 5/group), and tumor samples were digested and analyzed on day 12. Tumor cells were gated by FSC^{hi}SSC^{lo}Zombie-yellow⁻CD45.2⁺ expression. (D) CD155 and PD-L1 expression on ex vivo B16F10 tumor cells is shown. (E) CD11b⁺CD11c⁺ and CD11b⁺CD11c⁻ tumor-infiltrating myeloid cell populations were gated by FSC^{hi}SSC^{lo}Zombie-yellow⁻CD45.2⁺ expression. CD155 and PD-L1 expression on these cells is shown. See also Supplemental Figures 1 and 2.

Here, we have revealed that CD155 is highly expressed on tumor and tumor-infiltrating myeloid cells in both human and mouse tumors. Moreover, we used CD155-deficient mice, bone marrow (BM) chimeras, and CD155-deleted tumors in a series of in vitro studies and mouse models of cancer to interrogate and identify both host and tumor CD155 as critical regulators of tumor development via nonredundant mechanisms.

Results

CD155 expression in malignant and nonmalignant compartments of human and mouse tumors. Profiling of CD155 mRNA expression across 19 cancers (The Cancer Genome Atlas [TCGA] data set) indicated a broad diversity of tumor types in which CD155 was upregu-

lated compared with normal, uninvolved tissue (Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI98769DS1>). *PDL1* mRNA expression was also increased in malignant tissue compared with expression in normal tissue and followed a pattern similar to that seen with CD155, however, the upregulated *PDL1* mRNA levels were significantly lower compared with those of *CD155* across each of the tumor types analyzed (Supplemental Figure 1B). Examination of CD155 protein by multiplexed IHC indicated predominant expression in HMB45⁺ melanoma cells (Figure 1, A and B), similar to previous observations in human melanoma samples (29). We also observed CD155 expression on tumor-infiltrating myeloid cells such as CD14⁺CD11c⁻ macrophages and CD14⁺CD11c⁺ myeloid cells, as well as the rarer CD14⁻CD11c⁺ DCs (Figure 1C). Further analysis revealed CD155 expression on CD163⁺ tumor-associated myeloid cells located proximal to CD3⁺ T cells (Supplemental Figure 1C), suggesting that CD155 may be associated with immunosuppressive myeloid cells. CD155 was highly expressed on all mouse tumor cell lines including B16F10 (melanoma), SM1WT1 (melanoma), and MC38 (colon cancer) lines (Supplemental Figure 2A). CD112, which shares some of the same interacting receptors (e.g., DNAM-1 and TIGIT) with CD155 (6), was expressed at very low levels on B16F10 and SM1WT1 cells and was undetectable on MC38 cells (Supplemental Figure 2A). We detected PD-L1 on B16F10 melanoma cells in vitro (data not shown), and the majority (94%) of B16F10 tumor cells coexpressed CD155 and PD-L1 in vivo (Figure 1D). Analysis of tumor-infiltrating myeloid cells in the B16F10 model also revealed substantial coexpression of CD155 and PD-L1 in both CD11b⁺CD11c⁻ and CD11b⁺CD11c⁺ myeloid cells (Figure 1E). The high levels of CD155 on tumor-infiltrating myeloid cells (Supplemental Figure 2B, right) contrasted with the low expression levels detected on DCs, NK cells, and CD4⁺ and CD8⁺ T cells in naive (Supplemental Figure 2B, left) and tumor-bearing mice (data not shown). These data not only confirmed the high prevalence of CD155 on tumor cells but also revealed similar expression levels of CD155 and PD-L1 within tumor-infiltrating myeloid cells.

Suppressed tumor growth and metastasis in Cd155^{-/-} mice is immune cell dependent. To understand the role of host CD155 in regulating tumor growth, we tested several transplantable mouse tumors in WT and *Cd155^{-/-}* mice and found that s.c. injected B16F10 (Figure 2A), SM1WT1 (Figure 2B), and MC38 (Figure 2C) tumor growth was restricted in *Cd155^{-/-}* mice compared with that seen in WT mice. Next, we examined whether loss of host CD155 regulated experimental tumor metastasis to the lungs after i.v. injection of B16F10 or LWT1 melanoma cells. We found that B16F10 (Figure 2D) and LWT1 (Figure 2E and Supplemental Figure 2I) lung metastases were significantly inhibited in *Cd155^{-/-}* mice compared with that observed in WT mice. Together, these data in transplantable tumor and lung metastasis models suggest that host CD155 plays a negative role in tumor immunity. To determine whether the inhibition of B16F10 tumor growth in *Cd155^{-/-}* mice was immune cell mediated, WT and *Cd155^{-/-}* mice were depleted of CD8⁺ T cells, CD4⁺ and CD8⁺ T cells, or NK cells. Antibody-mediated depletion of CD8⁺ T cells or CD4⁺ and CD8⁺ T cells demonstrated that the reduced B16F10 tumor growth observed in *Cd155^{-/-}* mice was mediated by CD8⁺ T cells (Figure 2F), whereas the impact on tumor growth upon NK cell depletion in *Cd155^{-/-}* mice was minimal (Figure 2F). Next, to examine whether experimental B16F10 lung metastasis in *Cd155^{-/-}* mice was immune cell mediated, WT and *Cd155^{-/-}* mice were again depleted of CD8⁺ T cells, CD4⁺ and CD8⁺ T cells, or NK cells after i.v. administration of B16F10 cells. Depletion of NK cells by anti-asialoGM1 (anti-asGM1) enhanced metastasis in *Cd155^{-/-}* and WT mice to an equivalent degree (Figure 2G), whereas no significant impact on metastasis was observed upon CD4⁺ and/or CD8⁺ T cell depletion in *Cd155^{-/-}* mice (Figure 2G). Importantly, we found that immune homeostasis was broadly normal in naive *Cd155^{-/-}* mice (Supplemental Figure 2, C-H), with equivalent compositions of all the major immune cell subsets between *Cd155^{-/-}* and WT mice. These data clearly show that although the immune cell composition of *Cd155^{-/-}* mice was similar to that of WT mice in the naive setting, loss of host CD155 reduced tumor growth and metastasis in an immune-dependent manner, with tumor growth largely controlled by CD8⁺ T cells and experimental metastasis controlled by NK cells.

To identify whether CD155 expressed by hematopoietic or nonhematopoietic cells regulates antitumor immunity, we generated BM chimeric mice (Figure 2H). C57BL/6 *Ptprc^d* (CD45.1⁺) and *Cd155^{-/-}* (CD45.2⁺) mice were irradiated twice (total radiation dose: 1,050 cGy/rad) (30, 31), and BM cells from nonirradiated *Ptprc^d* or *Cd155^{-/-}* mice were injected i.v. into each irradiated mouse to generate BM chimeric mice (Figure 2H). The efficiencies of BM reconstitution of the chimeric mice were greater than 90% (Supplemental Figure 2J). Reconstitution of *Ptprc^d* or *Cd155^{-/-}* mice with *Ptprc^d* BM did not alter B16F10 tumor growth (Figure 2I) or experimental lung metastasis (Figure 2J), while reconstitution of *Ptprc^d* or *Cd155^{-/-}* mice with *Cd155^{-/-}* BM significantly delayed B16F10 tumor growth (Figure 2I) or experimental lung metastasis (Figure 2J). Together, these data demonstrate that CD155 expression on hematopoietic cells limits antitumor immunity and promotes tumor growth and metastasis.

Reduced tumor growth and metastasis in Cd155^{-/-} mice is DNAM-1 dependent. DNAM-1 is a critical costimulatory molecule for CD8⁺

T cell- and NK cell-mediated tumor immunity *in vivo* (6) and it has been shown to be upregulated in lymphocytes in *Cd155^{-/-}* mice (32). Moreover, tumor cells expressing CD155 may impair immune function by downregulating DNAM-1 on NK cells (33). We confirmed the significantly increased expression of DNAM-1 on CD4⁺ T cells, CD8⁺ T cells, and NK cells in naive *Cd155^{-/-}* mice compared with expression in naive WT mice (Supplemental Figure 3A). Moreover, blocking CD155-receptor interactions by using an anti-CD155 mAb *in vivo* also upregulated DNAM-1 on lymphocytes in the peripheral blood and spleen (Supplemental Figure 3B and data not shown), as has been previously described (32). Importantly, these CD155-dependent alterations in DNAM-1 expression were recapitulated in the TME, since B16F10 tumor-infiltrating lymphocytes (TILs) showed that DNAM-1 was also upregulated in the lymphocytes of *Cd155^{-/-}* mice compared with that seen in WT mice (Figure 3A). We found that DNAM-1 expression was also higher on lymphocytes in chimeric mice reconstituted with *Cd155^{-/-}* BM in comparison with expression in mice reconstituted with *Ptprc^d* BM (Supplemental Figure 3C). Taken together, these data show that CD155 expression on hematopoietic cells downregulates DNAM-1 on CD4⁺ and CD8⁺ T cells and NK cells, not only during normal immune homeostasis, but also within the TME, potentially shifting the balance toward reduced antitumor immunity.

To address the functional importance of the increased DNAM-1 expression observed in TILs from *Cd155^{-/-}* mice, we used a DNAM-1-blocking mAb or depleted CD8⁺ T cells or NK cells and confirmed that B16F10 tumor inhibition in *Cd155^{-/-}* mice was DNAM-1 and CD8⁺ T cell dependent, but not reliant on NK cells (Figure 3B). A greater proportion of CD8⁺ T cells from *Cd155^{-/-}* mice produced IFN- γ after anti-CD3/anti-CD28 stimulation than did CD8⁺ T cells from WT mice (Figure 3C). NK cell function is important for host protection from lung metastasis, and the inhibition of tumor metastasis in *Cd155^{-/-}* mice was dependent on NK cells and abrogated by anti-DNAM-1 mAb (Figure 3D). We next tested the *in vitro* functions of NK cells from WT and *Cd155^{-/-}* mice. By stimulating purified NK cells from WT and *Cd155^{-/-}* mice with IL-12 and IL-18 *in vitro* (34), we found that higher proportions of NK cells from *Cd155^{-/-}* mice expressed IFN- γ (Figure 3E). We have previously shown an enhanced cytokine secretion profile and antimetastatic function of DNAM-1⁺ NK cells compared with DNAM-1⁻ NK cells (34). Consistent with this phenotype, we detected an increased concentration of IFN- γ , granulocyte macrophage-CSF (GM-CSF), and IL-6 in the supernatant of cultured NK cells from *Cd155^{-/-}* mice compared with that from WT mice (Supplemental Figure 3D). Since DNAM-1 is crucial for NK cell activity (33–35) and CD155 is not expressed on NK cells (Supplemental Figure 2B), CD155 might regulate NK cell functions by altering DNAM-1 expression *in trans*. Flow cytometric analysis showed that the higher expression of DNAM-1 in NK cells from *Cd155^{-/-}* mice was critical for the higher IFN- γ expression *in vitro* (Supplemental Figure 3E). Overall, loss of host CD155 regulated tumor growth and metastasis via upregulation of DNAM-1 on CD8⁺ T and NK cells, respectively.

Deletion of tumor CD155 decreases tumor growth and metastasis. While CD155 is highly expressed on mouse and human tumor cells (Supplemental Figure 2A and Figure 1) (16), the comparative function of CD155 on tumor versus host cells in the TME *in vivo* is poorly understood. To elucidate the role of CD155 expression

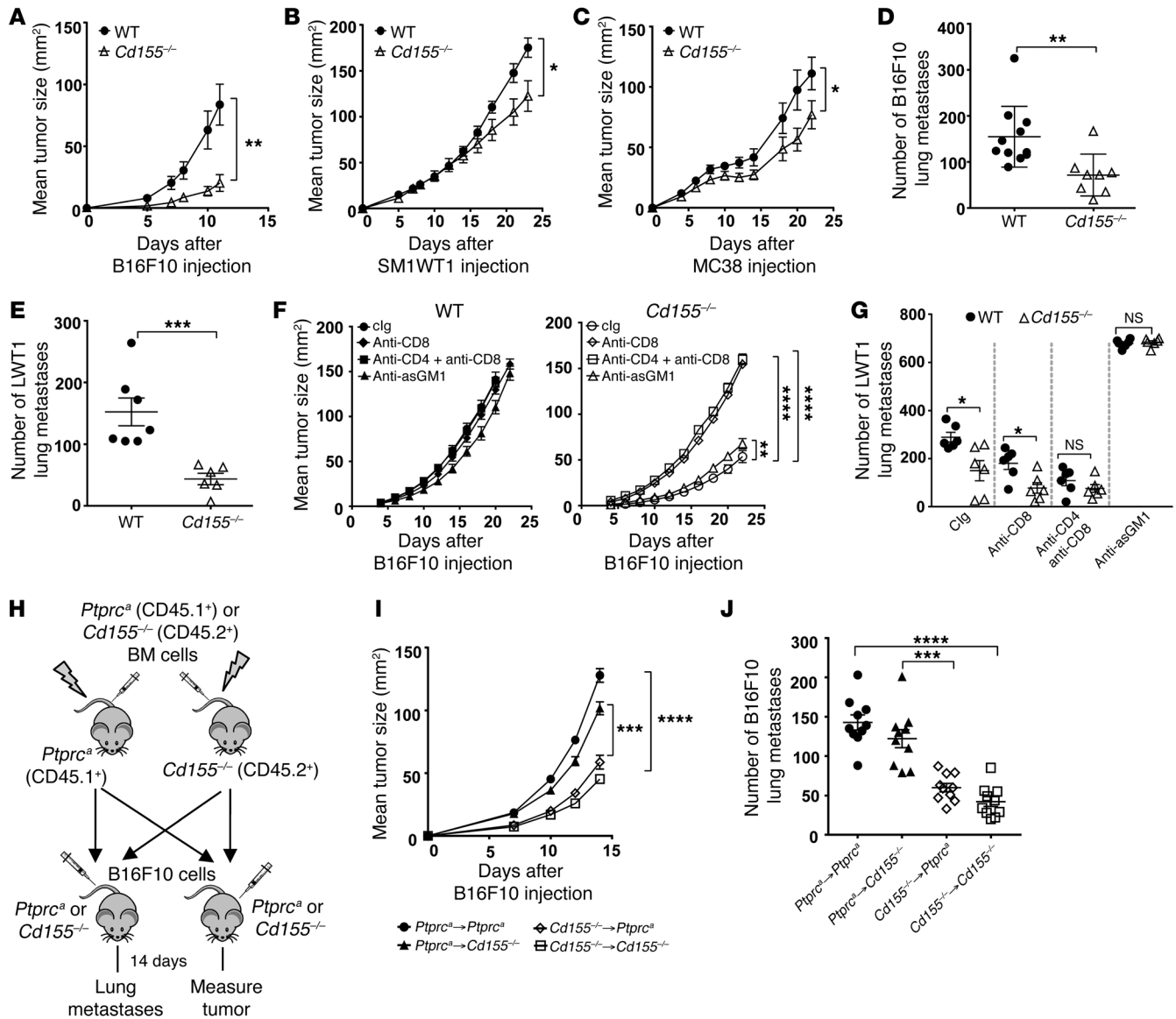


Figure 2. Tumor growth and metastasis are inhibited in *Cd155*^{-/-} mice in a BM cell-dependent manner. (A–C) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 B16F10 cells ($n = 6$ –7/group) (A) or 5×10^5 SM1WT1 cells ($n = 7$ /group) (B), or 5×10^5 MC38 cells ($n = 5$ –6/group) (C), and tumor sizes were measured at the indicated time points. (D and E) WT and *Cd155*^{-/-} mice were challenged i.v. with 2×10^5 B16F10 cells ($n = 8$ –11/group) (D) or 7.5×10^5 LWT1 cells ($n = 6$ –7/group) (E), 2 weeks after tumor inoculation, and metastatic burden was quantified in the lungs by counting colonies on the lung surface. (F and G) WT and *Cd155*^{-/-} mice were treated with 100 μ g clg, or 100 μ g anti-CD8 β (53.5.8, CD8⁺ T cell depletion), or 100 μ g anti-CD4 (GK1.5, CD4⁺ T cell depletion) plus 100 μ g anti-CD8 β (53.5.8), or 50 μ g anti-asGM1 (NK cell depletion) on days -1, 0, 7, and 14 for tumor growth, or on days -1, 0, and 7 for tumor metastasis relative to tumor inoculation. Mice were challenged s.c. with 1×10^5 B16F10 cells ($n = 5$ –6/group; the experiment was performed once) (F), and tumor sizes were measured at the indicated time points, or mice were challenged i.v. with 7.5×10^5 LWT1 cells ($n = 6$ /group) (G), and lung metastases were quantified 2 weeks after tumor inoculation. (H and I) *Ptprc*^a (CD45.1⁺) and *Cd155*^{-/-} (CD45.2⁺) mice were irradiated twice (total radiation dose: 1,050 cGy/rad), and 5×10^5 BM cells from *Ptprc*^a or *Cd155*^{-/-} mice were then i.v. injected into each irradiated mouse to construct BM chimeric mice (H). Mice were then challenged s.c. with 1×10^5 B16F10 (I) or i.v. with 2×10^5 B16F10 (J) cells, respectively ($n = 10$ /group; the experiment was performed once for both I and J). Tumor sizes were measured at the indicated time points (I), and lung metastases were quantified 2 weeks after tumor inoculation (J). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, by 1-tailed Mann-Whitney U test or 2-way ANOVA. Data indicate the mean \pm SEM and are representative of 3 experiments unless otherwise indicated. See also Supplemental Figures 2 and 3.

on tumor cells, we used the CRISPR-Cas9 system to delete CD155 (*Cd155*-KO) expression on B16F10 melanoma, LWT1 melanoma, MC38 colon adenocarcinoma, and MCA1956 fibrosarcoma cells. *Cd155*-KO tumor cell lines were generated using 2 independent, distinct single-guide RNAs (sgRNAs) (sg2 and sg6), and deletion of CD155 was verified by flow cytometry (Supplemental Figure

4, A and B). Subcutaneously injected B16F10-*Cd155*-KO (Figure 4A), LWT1-*Cd155*-KO (Figure 4B), and MC38-*Cd155*-KO (Figure 4C) tumor cells resulted in reduced tumor growth compared with matched control cells in immunocompetent WT hosts in vivo. We found that deletion of CD155 in the MCA1956 fibrosarcoma cells also significantly inhibited tumor growth compared with that seen

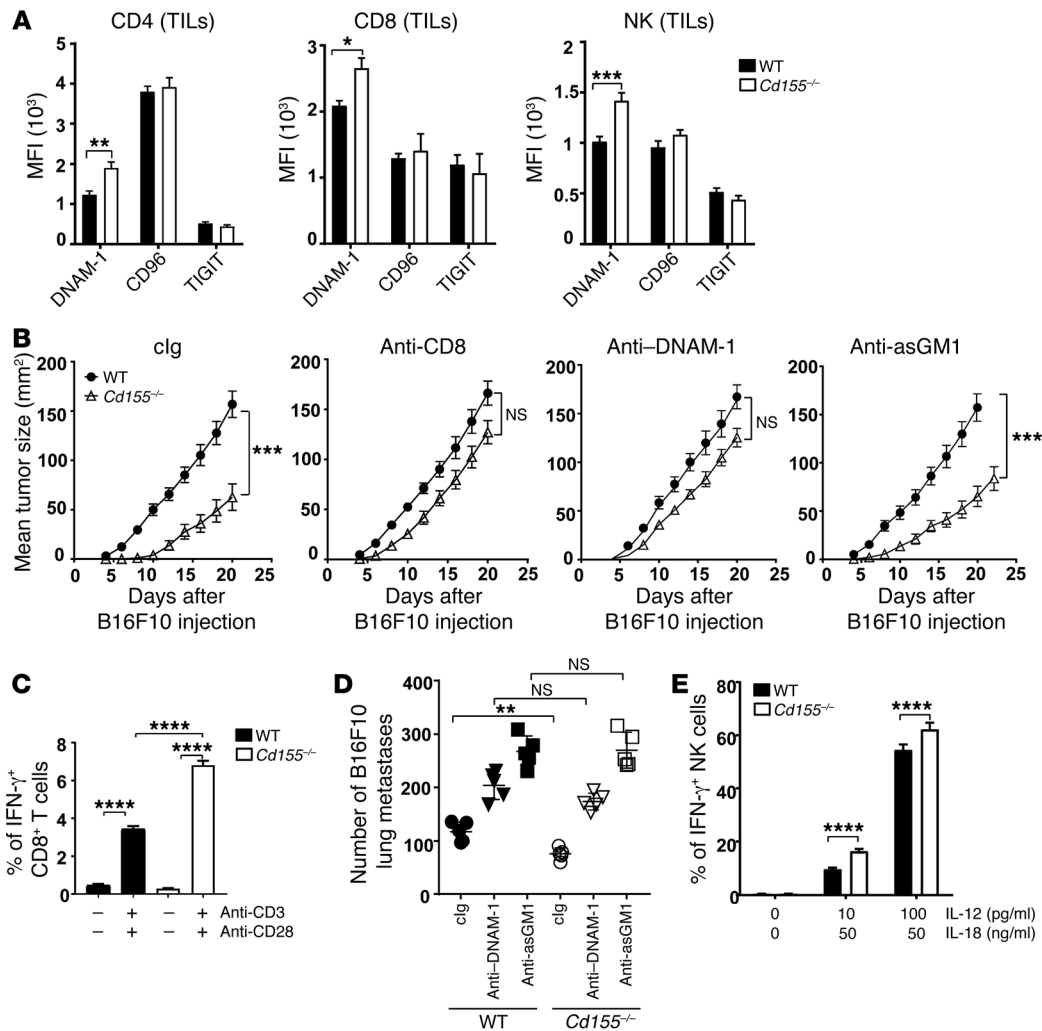


Figure 3. Inhibition of tumor growth and metastasis in *Cd155*^{-/-} mice is DNAM-1 dependent. (A) DNAM-1, CD96, and TIGIT expression was analyzed on day 12 on tumor-infiltrating CD4⁺ T cells, CD8⁺ T cells, and NK cells after inoculation of 1 × 10⁵ B16F10 cells in WT and *Cd155*^{-/-} mice (n = 5/group). The mean fluorescence intensity (MFI) is shown. (B) Tumor sizes were measured at the indicated time points for WT and *Cd155*^{-/-} mice (n = 5–6/group) treated with 100 μg clg, 100 μg anti-CD8β (clone 53.5.8), 250 μg anti-DNAM-1 (clone 480.1), or 50 μg anti-asGM1 on days -1, 0, 7, and 14, relative to inoculation of 1 × 10⁵ B16F10 cells (the experiment was performed twice). (C) Splenocytes from naive C57BL/6 WT and *Cd155*^{-/-} mice (n = 3) were stimulated with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 for 24 hours, and the expression of IFN-γ on CD8⁺ T cells was analyzed by flow cytometry (data are representative of 2 experiments). (D) WT and *Cd155*^{-/-} mice (n = 5/group) were injected i.v. with 1 × 10⁵ B16F10 melanoma cells and treated on days -1, 0, and 7, relative to tumor inoculation with 250 μg clg (clone 1-1), 250 μg anti-DNAM-1 (clone 480.1), or 50 μg anti-asGM1. Metastatic burden was quantified in the lungs by counting colonies on the lung surface 14 days after tumor cell inoculation (n = 5/group; the experiment was performed once). (E) NK cells from the spleens of naive C57BL/6 WT and *Cd155*^{-/-} mice (n = 4) were purified and stimulated with the indicated concentrations of IL-12 and IL-18 for 24 hours, and the expression of IFN-γ on NK cells was analyzed by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, by 1-tailed Mann-Whitney U test or 1-way ANOVA (A–E). Data indicate the mean ± SEM and are representative of 3 experiments unless otherwise indicated. See also Supplemental Figure 3.

in MCA1956 control cells in WT mice. Interestingly, mice injected with MCA1956-*Cd155*-KO cells exhibited spontaneous regression of their tumors (Figure 4D).

Next, we determined the relative contribution of tumor cell-intrinsic CD155 on metastasis using experimental and spontaneous metastasis models. Experimental metastasis of i.v. injected *Cd155*-KO tumor cells (B16F10, LWT1, MC38) was significantly reduced compared with the matched control tumor cells in WT mice (Figure 4, E–G). We also corroborated our findings in a model of spontaneous metastasis. Using the CRISPR-Cas9 system, we deleted CD155 in the highly metastatic 4T1.2 mammary carcinoma cell line (36) (Supplemental Figure 4C).

4T1.2 control and 4T1.2-*Cd155*-KO (sg6) cells were injected orthotopically into the mammary fat pads of BALB/c WT mice, tumors were resected on day 14, and spontaneous tumor metastases in the lungs were assessed on day 30. We analyzed survival in another independent cohort treated identically. While the primary orthotopic growth was not significantly altered by CD155 loss on day 14 (data not shown), our analyses showed that the spontaneous metastatic potential of 4T1.2-*Cd155*-KO cells was significantly reduced (Figure 4H) and that survival was significantly increased (Figure 4I). Taken together, these data demonstrate that CD155 expression on tumor cells is critical for tumor cell growth and metastasis in vivo.

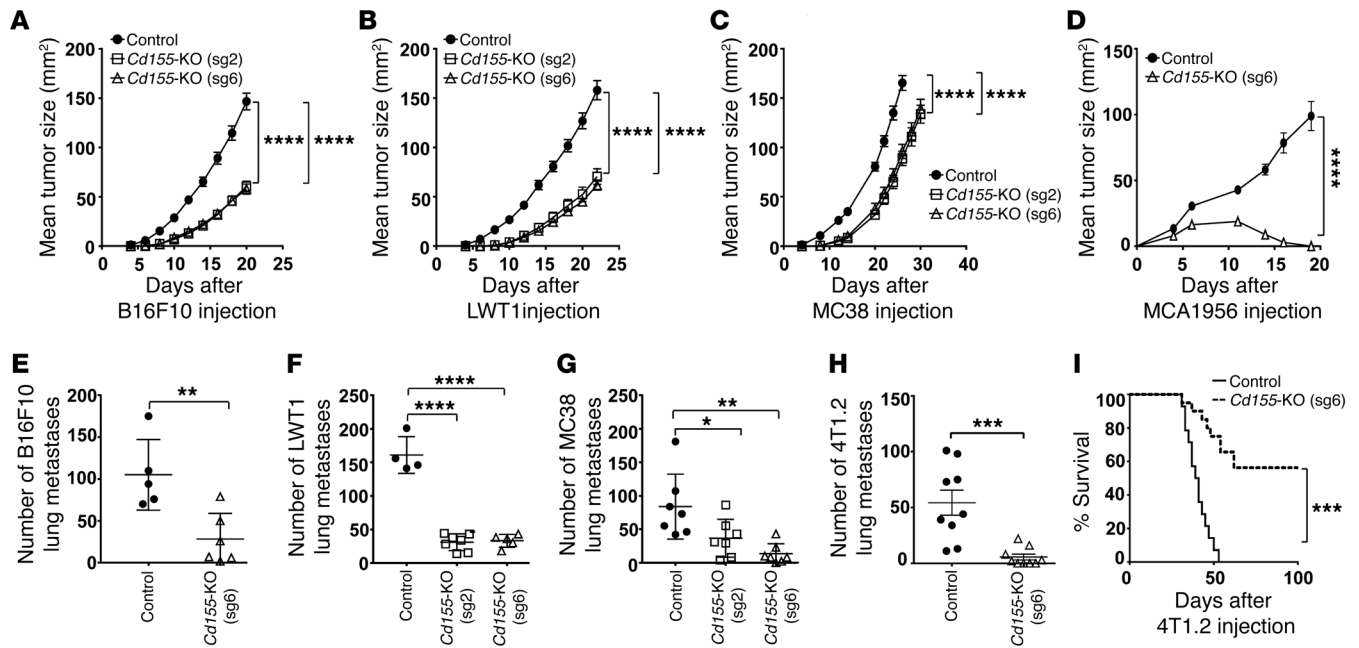


Figure 4. Deletion of tumor CD155 decreases tumor growth and metastasis. (A–D) WT mice were injected s.c. with 1×10^5 B16F10 control or B16F10-*Cd155*-KO (sg2 and sg6) cells ($n = 6$ /group) (A); 5×10^5 LWT1 control or LWT1-*Cd155*-KO (sg2 and sg6) cells ($n = 5$ –6/group) (B); 5×10^5 MC38 control or MC38-*Cd155*-KO (sg2 and sg6) cells ($n = 5$ /group) (C); or 1×10^6 MCA1956 control or MCA1956-*Cd155*-KO (sg6) cells ($n = 5$ /group) (D), and tumor sizes were measured at the indicated time points. (E–G) WT mice were injected i.v. with 2×10^5 B16F10 control or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ –6/group) (E); 7.5×10^5 LWT1 control or LWT1-*Cd155*-KO (sg2 and sg6) cells ($n = 4$ –7/group) (F); or 2×10^5 MC38 control or MC38-*Cd155*-KO (sg2 and sg6) cells ($n = 7$ /group) (G). Metastatic burden in the lungs was quantified by counting colonies on the lung surface 14 days after tumor inoculation. (H and I) Female BALB/c WT mice were injected into the mammary fat pad with 5×10^4 4T1.2 control or 4T1.2-*Cd155*-KO cells, and tumors were resected on day 14. In 1 group, the metastatic burden was quantified in the lungs by counting colonies on the lung surface on day 30 ($n = 9$ /group) (H), and in another group, the survival was monitored (I) ($n = 14$ –20/group; experiment was performed twice). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, by 1-tailed Mann–Whitney *U* test or 2-way ANOVA. Data indicate the mean \pm SEM and are representative of 3 experiments unless otherwise indicated. See also Supplemental Figure 4.

Tumor cell-intrinsic CD155 is critical for tumor cell migration and survival. Although loss of tumor CD155 reduced tumor growth and metastasis in vivo, it remained unknown whether this effect was lymphocyte dependent. Surprisingly, we found that B16F10-*Cd155*-KO and LWT1-*Cd155*-KO tumor cells grew significantly more slowly in both WT mice and lymphocyte-deficient *Rag2*^{-/-}*γc*^{-/-} mice than did control tumor cells (Figure 5, A and B). Moreover, our data indicated that lymphocyte-deficient *Rag2*^{-/-}*γc*^{-/-} mice i.v. injected with B16F10-*Cd155*-KO or LWT1-*Cd155*-KO cells had significantly fewer lung metastases than did mice injected with control tumor cells (Figure 5, C and D). It is thought that overexpression of CD155 promotes cell migration and proliferation (14, 27). Limiting dilution assays revealed that B16F10-*Cd155*-KO (Figure 5E) and 4T1.2-*Cd155*-KO (Supplemental Figure 5A) cells had a significantly hindered ability to form colonies compared with control cells. Using the scratch assay, we demonstrated that B16F10-*Cd155*-KO cells (Figure 5, F and G) and 4T1.2-*Cd155*-KO cells (Supplemental Figure 5B) had slower cell migration rates than did the matched control tumor cells. In addition, we observed a larger number of apoptotic cells in the B16F10-*Cd155*-KO cells when cultured under hypoxic (1% O₂) or low serum (1% serum) conditions compared with that seen in control cells (Figure 5, H and I).

Reexpression of full-length mouse CD155 in B16F10-*Cd155*-KO (sg6) cells to generate B16F10-*Cd155*-KO (sg6)-*Cd155ov* cells

demonstrated restored expression levels (Supplemental Figure 6A) and function in vitro (Supplemental Figure 6, B and C) and in vivo (Supplemental Figure 6D). Collectively, these data provide evidence that loss of CD155 on tumor cells alters the cell-intrinsic function, resulting in reduced tumor growth and metastasis in a lymphocyte-independent manner, possibly due in whole or in part to reductions in tumor cell survival under limiting conditions.

Deletion of CD155 from host and tumor cells enhances the suppression of tumor progression. We next injected control or *Cd155*-KO tumor cells i.v. or s.c. into WT or *Cd155*^{-/-} mice (Figure 6A) to monitor the role of CD155 in metastasis and tumor growth. Surprisingly, deletion of CD155 on both host and B16F10 tumor cells resulted in the strongest reduction of lung metastases compared with *Cd155*-KO cells in WT mice or control cells in *Cd155*^{-/-} mice (Figure 6B). The inhibition of metastasis upon deletion of CD155 from either host or LWT1 tumor cells was so significant, that combined deletion of CD155 from both host and tumor cells was without any further effect (Figure 6C). Deletion of CD155 from both host and B16F10 tumor cells also further decreased s.c. tumor growth (Figure 6D). While we also observed a similar effect with LWT1 tumor cells (Figure 6E and Supplemental Figure 7), deletion of CD155 from both host and MC38 tumor cells was not additive in this model (Figure 6F). These data suggest that blocking CD155 on tumor and host cells could be a rational strategy to inhibit tumor growth and metastasis.

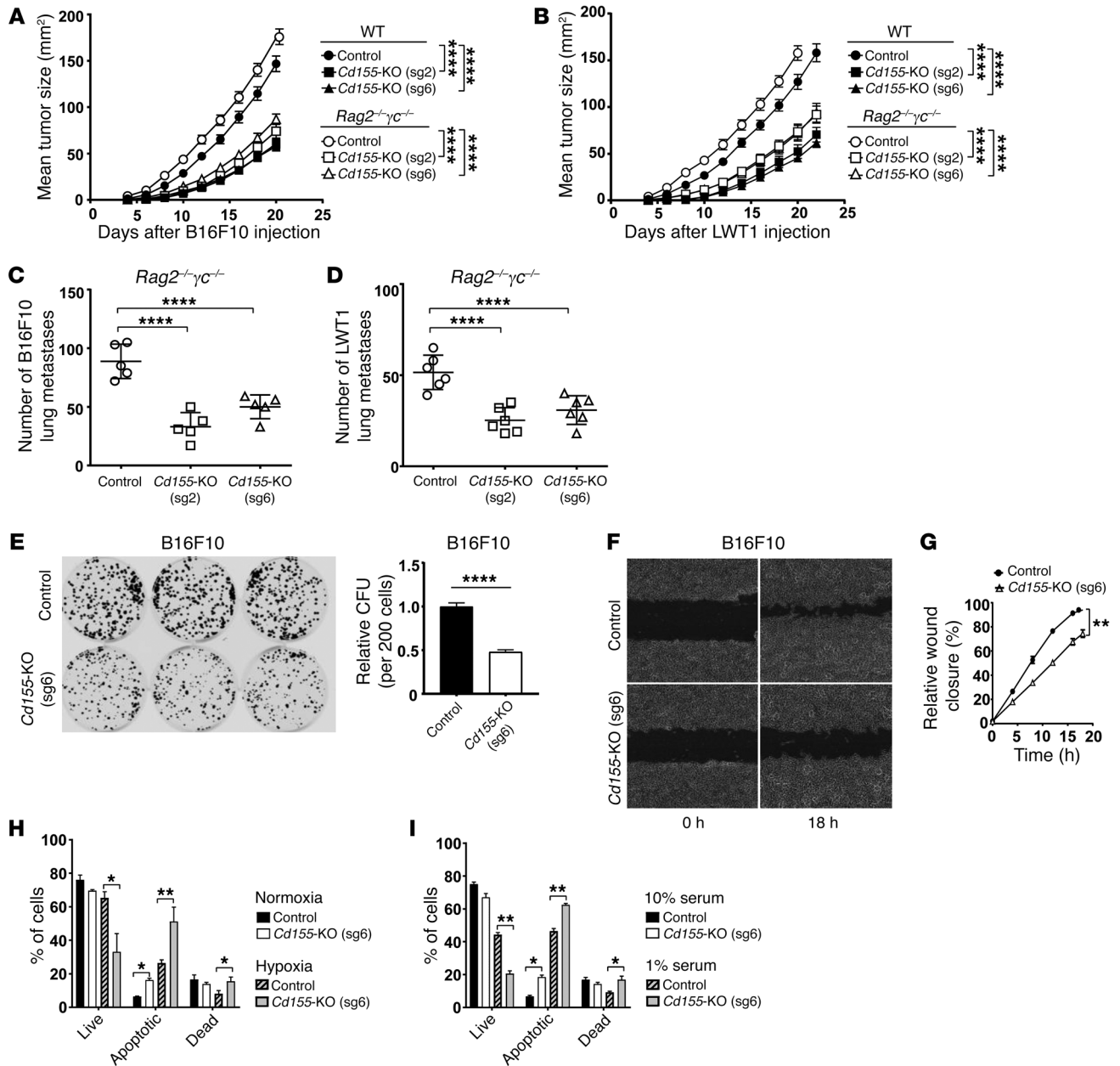


Figure 5. Tumor-intrinsic CD155 is critical for tumor cell migration and survival. (A and B) WT and *Rag2*^{-/-}*γc*^{-/-} mice were injected s.c. with 2 × 10⁵ B16F10 control or B16F10-*Cd155*-KO (sg2 and sg6) cells (*n* = 6/group) (A), or 5 × 10⁵ LWT1 control or LWT1-*Cd155*-KO (sg2 and sg6) cells (*n* = 5–6/group) (B), and tumor sizes were measured at the indicated time points (the experiment was performed once). (C and D) *Rag2*^{-/-}*γc*^{-/-} mice were injected i.v. with 5 × 10⁴ B16F10 control or B16F10-*Cd155*-KO (sg6) cells (*n* = 5/group) (C), or 1 × 10⁵ LWT1 control or LWT1-*Cd155*-KO (sg6) cells (*n* = 6/group) (D), and the metastatic burden was quantified in the lungs by counting colonies on the lung surface 14 days after tumor cell inoculation. (E) B16F10 control and B16F10-*Cd155*-KO (sg6) cells (200 cells/well; *n* = 3/group) were cultured in 6-well plates for 6 days and stained with crystal violet to assess relative CFU. Images of the colonies of B16F10 control and B16F10-*Cd155*-KO (sg6) cells are shown, and the relative CFU was assessed (representative of 3 experiments). (F and G) B16F10 control or B16F10-*Cd155*-KO (sg6) cells (1 × 10⁵ cells/well) were cultured in 96-well plates. Wounds were made, and wound-healing images were monitored and analyzed, and the percentage of relative wound closure was determined. (H and I) B16F10 control and B16F10-*Cd155*-KO (sg6) cells (1 × 10⁵ cells/well) were cultured in 24-well plates under normoxic or hypoxic conditions (*n* = 4/group) (H) or in media supplemented with 10% or 1% serum (*n* = 4/group) (I), and apoptosis was evaluated 48 hours later with annexin V and 7-AAD. (H and I) Graphs show the representative rate of cell death. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001, by 1-tailed Mann-Whitney *U* test or 2-way ANOVA. Data indicate the mean ± SEM and are representative of 3 experiments unless otherwise indicated. See also Supplemental Figure 4D and Supplemental Figure 5.

Effects of combined host and tumor CD155 loss are DNAM-1 dependent. We observed the greatest reductions in s.c. tumor growth or tumor metastasis upon the combined loss of both host and tumor CD155 (Figure 6, B, D, and E). We noticed that TILs showed the

highest DNAM-1 expression in *Cd155*^{-/-} mice bearing CD155-KO tumors (Supplemental Figure 8A), whereas CD96 was unchanged (Supplemental Figure 8B). To test whether the increase in DNAM-1 resulted in an increased antitumor response, we s.c. injected B16F10

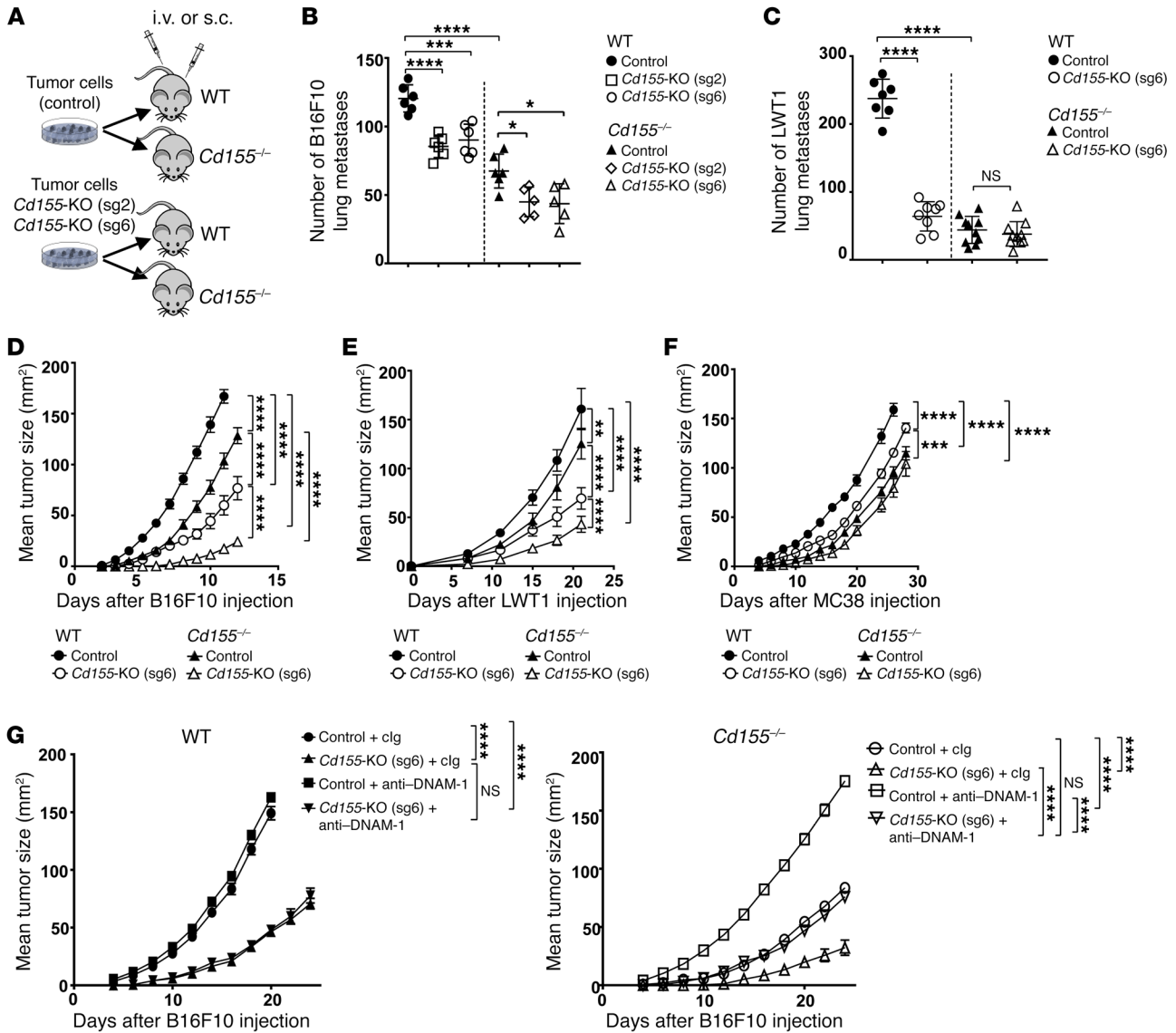


Figure 6. Deletion of CD155 from host and tumor enhances the suppression of tumor progression. (A) Experimental protocol for the induction of primary s.c. or lung metastases with control or *Cd155*-KO (sg2 and/or sg6) tumor cells in WT or *Cd155*^{-/-} mice. (B) WT and *Cd155*^{-/-} mice were injected i.v. with 1×10^5 B16F10 control, B16F10-*Cd155*-KO (sg2), or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ -6/group). Metastatic burden was quantified in the lungs by counting colonies on the lung surface 14 days after tumor cell inoculation (the experiment was performed once). (C) WT and *Cd155*^{-/-} mice were injected i.v. with 5×10^5 LWT1 control or LWT1-*Cd155*-KO (sg6) cells ($n = 7$ -10/group). Fourteen days after tumor inoculation, the metastatic burden was quantified in the lungs by counting colonies on the lung surface (the experiment was performed once). (D-F) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 B16F10 control or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ /group) (D); 5×10^5 LWT1 control or LWT1-*Cd155*-KO (sg6) cells ($n = 8$ -9/group) (E); or 5×10^5 MC38 control or MC38-*Cd155*-KO (sg6) cells ($n = 5$ /group) (F), and tumor sizes were measured at the indicated time points. (G) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 B16F10 control or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ /group). A dose of 250 μ g clg (clone 1-1) or anti-DNAM-1 (clone 480.1) mAb was injected on days -1, 0, and 7, relative to tumor inoculation (experiment was performed once). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, by 2-way ANOVA. Data indicate the mean \pm SEM and are representative of 3 experiments unless otherwise indicated. See also Supplemental Figure 6.

control or B16F10-*Cd155*-KO cells into WT or *Cd155*^{-/-} mice and treated them with control Ig (clg) or anti-DNAM-1-blocking mAb. In accordance with earlier results (Figure 3, B and D), anti-DNAM-1 mAb treatment reversed the B16F10 tumor growth inhibition in *Cd155*^{-/-} mice (Figure 6G). Interestingly, anti-DNAM-1 mAb treatment did not affect the growth of B16F10-*Cd155*-KO tumors in WT mice, but increased B16F10-*Cd155*-KO tumor growth in *Cd155*^{-/-} mice (Figure 6G). These results show that the immune-mediated antitumor effect in *Cd155*^{-/-} mice was DNAM-1 dependent.

Reduced CD155 increases the activity of PD-1 and/or CTLA4 blockade. Given that the combined loss of CD155 in tumor and host cells increased sensitivity to DNAM-1-dependent antitumor immunity, we wanted to test whether immune checkpoint blockade therapies were more effective in a CD155-deficient host and/or TME. When using the anti-PD-1-sensitive MCA1956 tumor model, low-dose anti-PD-1 alone was far more effective in *Cd155*^{-/-} mice than it was in WT mice (Figure 7A). We achieved similar results with a higher dose regimen of anti-PD-1 alone against s.c.

MC38 tumors (Figure 7B). To test the immune checkpoint blockade further, we established B16F10 control or B16F10-*Cd155*-KO s.c. tumors in WT or *Cd155*^{-/-} mice and s.c. blocked PD-1 and CTLA4 with mAbs (Figure 7C). As expected, combined PD-1 and CTLA4 blockade significantly reduced B16F10 control tumor growth in WT mice and also in *Cd155*^{-/-} mice (Figure 7C). However, the anti-tumor response to combined PD-1 and CTLA4 blockade was significantly greater in mice with *Cd155*-deleted B16F10 tumors than in those with B16F10 control tumors, and this enhanced response to combined anti-PD-1 and anti-CTLA4 therapy was consistent in both in WT and *Cd155*^{-/-} hosts (Figure 7C). This enhanced activity of PD-1 and CTLA4 blockade was also superior in the absence of host and/or tumor CD155, even when controlling the effect for tumor size when immune checkpoint blockade was first applied (Figure 7D). This showed that the antitumor effect of blocking PD-1 or PD-1 and CTLA4 was more effective in *Cd155*^{-/-} mice compared with the CD155-rich environment in WT mice and that tumor suppression was greatest in the complete absence of host and tumor CD155. We also used coblockade of TIGIT and CD96 to demonstrate the enhanced activity of immune checkpoint blockade in the complete absence of tumor CD155 (Figure 7E). Notably, all therapies, including single PD-1 blockade, dual TIGIT and CD96 blockade, and triple PD-1, TIGIT, and CD96 blockade, were more effective against B16F10-*Cd155*-KO s.c. tumors (Figure 7E, right) than against B16F10 control tumors (Figure 7E, left). Thus, these data suggest that blockade of CD155 function may enhance antitumor responses to contemporary immune checkpoint blockade and perhaps other immunotherapies.

Discussion

CD155 has pleiotropic functions relevant to cancer, including an ability to affect immune responses, via binding to the immunomodulatory receptors DNAM-1 (CD226), CD96, and TIGIT, as well as tumor cell-intrinsic activities that regulate proliferation, adhesion, and migration. Despite this knowledge, we still lack a detailed understanding of the relative contributions of host and tumor CD155 expression to tumor development, metastasis, and antitumor immunity. Here, we show that CD155 is expressed broadly in the TME in mouse models of cancer and human melanoma. We found that CD155 was not only expressed at high levels on tumor cells, but also on tumor-infiltrating myeloid cells. Using genetic deletion in preclinical models, we show that CD155 expression in tumor and hematopoietic cells contributed to tumor progression via nonredundant mechanisms. The cumulative magnitude of CD155-dependent signals from both sources not only influenced net tumor growth but also responsiveness to immune checkpoint therapies. Thus, blocking CD155 could be a rational strategy to improve current immunotherapeutic approaches.

While high expression of CD155 on tumor cells has been described (24, 25, 29), the present study reveals a role for host CD155 in fine-tuning the immune response against tumors. We found that germline deletion of CD155 had no major overt effect on baseline immune homeostasis; however, loss of CD155 in the host cells significantly decreased tumor growth and metastasis in a T cell- or NK cell-dependent manner, respectively. This immune-dependent reduction in tumor growth or metastasis was reversed by blocking DNAM-1, an activating receptor for CD155 that is present

on infiltrating T cells and NK cells, and correlated with enhanced levels of DNAM-1 in TILs. Using BM chimeras, we demonstrate that the CD155-dependent downregulation of DNAM-1 on TILs is dependent on hematopoietic cells, consistent with the increase in CD155 expression observed in tumor-infiltrating myeloid cells.

The immunomodulatory role of DNAM-1 in tumor immune surveillance is well established (6), and activating signals through DNAM-1 are effectively counterbalanced by the inhibitory receptors for CD155, CD96, and TIGIT (7). Our functional preclinical results are consistent with the observed dynamic downregulation of DNAM-1 and subsequent hyporesponsiveness of primary human NK cells upon CD155 exposure (33) and the decreased levels of DNAM-1 on tumor-associated NK (37, 38) and CD8⁺ TILs from patients with melanoma (39). Altogether, these results highlight the functional consequence of locally regulated DNAM-1 expression on TILs and indicate that host-derived CD155 signals are capable of disrupting the normal balance between activating and inhibitory lymphocyte signaling, leading to reduced antitumor immune responses.

In contrast to the immune cell-dependent role for host CD155, tumor-expressed CD155 promotes tumor growth and metastasis via distinct, tumor cell-intrinsic mechanisms.

Deletion of CD155 substantially reduced tumor cell migration rates and cell viability during low serum or hypoxic conditions, leading to a lower overall tumor and metastatic burden in vivo. The greater effect on metastatic burden may reflect the impact of CD155 loss on the migration of tumor cells, since successful tumor metastasis requires effective tumor cell movement. The molecular mechanism of the tumor-intrinsic activity of CD155 remains unresolved. It is currently unclear why the reduced tumor growth and metastasis observed upon loss of tumor-expressed CD155 was limited to tumor-intrinsic changes and did not apparently affect tumor immunity directly but, nonetheless, these observations highlight the nonredundant activities of host- versus tumor-expressed CD155. In fact, we observed that the cumulative loss of both host- and tumor-derived CD155 led to the greatest reductions in tumor growth or metastasis. These results are consistent with the general correlation of CD155 overexpression and unfavorable prognosis in non-small-cell lung cancer (NSCLC) (40), pancreatic cancer (25), cholangiocarcinoma (24), sarcoma (41), and melanoma (29), but suggest that the totality of CD155 expression, including assessment of expression on both tumor and infiltrating myeloid cells, may be more predictive of poor outcomes in cancer patients. The prevalence of CD155 overexpression in human tumors is well documented (25, 29, 40), and high CD155 expression is known to be a consequence of oncogenic signals (e.g., oncogenic Ras), growth factor signaling, genotoxic stress (42), or induction by chemotherapies (43). Importantly, the nonredundant functional activities of CD155 originating from distinct compartments described herein indicate that host-derived CD155 negatively affects T cell and NK cell responses, irrespective of CD155 expression on tumor cells.

Like the CD155 results in the present study, recent preclinical evaluations have highlighted different temporal kinetics and functional roles for PD-L1 on host and tumor cells (44, 45) and demonstrated the immunosuppressive capacity of PD-L1 emanating from immune cells independently of tumor-derived PD-L1. These results may provide a mechanistic explanation for

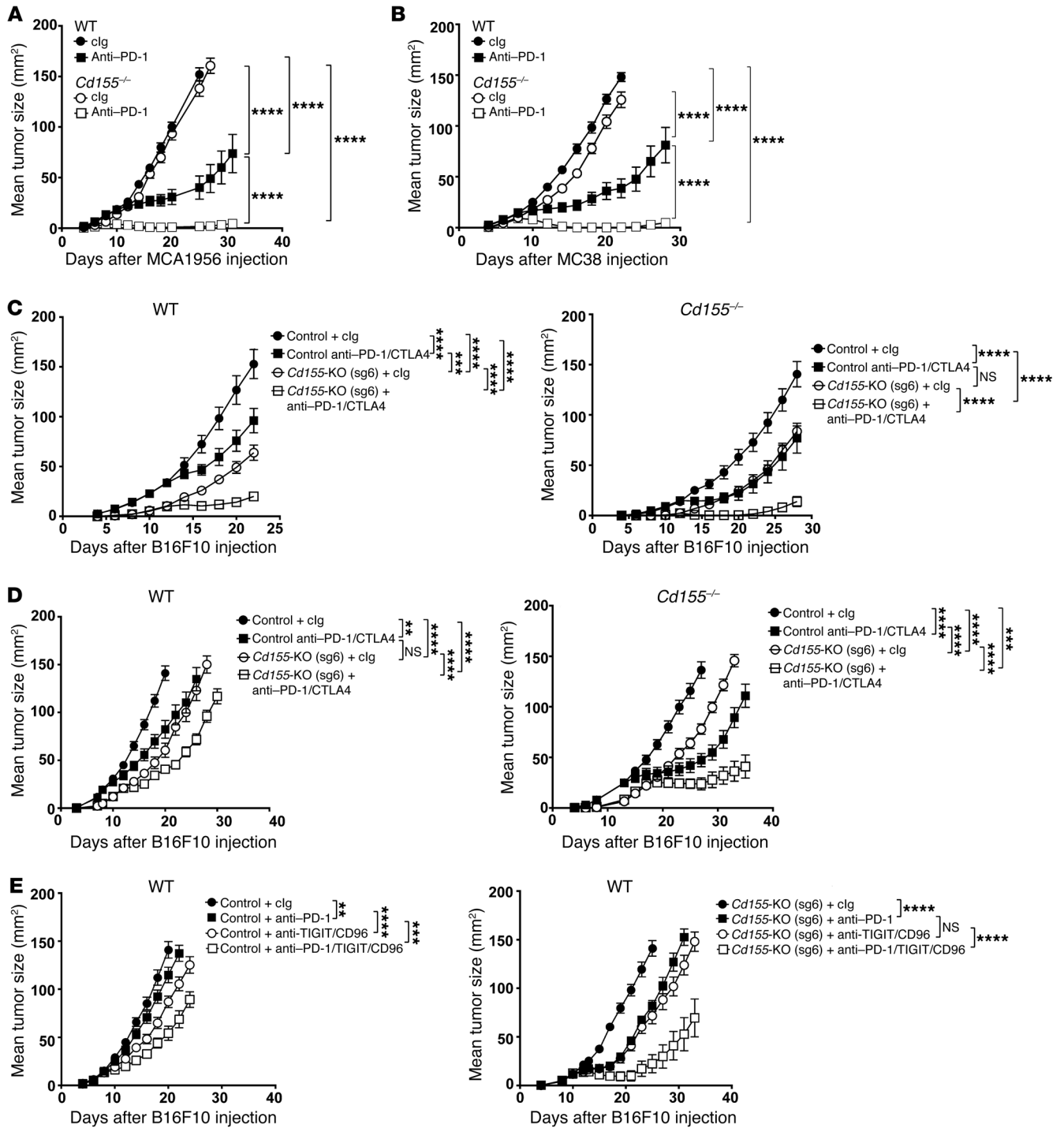


Figure 7. Blockade of PD-1 pathway combined with CD155 deletion enhances tumor growth suppression. (A) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^6 MCA1956 cells ($n = 10$ /group). clg or anti-PD-1 mAb (50 μ g) was injected on days 8, 12, 16, and 20, relative to tumor inoculation. (B) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 MC38 cells ($n = 5$ –6/group). clg or anti-PD1 mAb (250 μ g) was injected on days 8, 12, 16, and 20, relative to tumor inoculation. (C) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 B16F10 control or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ /group). Mice were injected with 250 μ g clg or anti-PD-1/CTLA4 mAb on days 12, 15, 18, and 21, relative to tumor inoculation. (D) WT and *Cd155*^{-/-} mice were injected s.c. with 1×10^5 B16F10 control or B16F10-*Cd155*-KO (sg6) cells ($n = 5$ /group). clg or anti-PD-1 and anti-CTLA4 mAbs (250 μ g) were injected into WT mice with B16F10 control cells on days 8, 10, 12, and 14; WT mice with B16F10-*Cd155*-KO (sg6) cells on days 12, 14, 16, and 18; *Cd155*^{-/-} mice with B16F10 control cells into on days 13, 15, 17, and 19; *Cd155*^{-/-} mice with B16F10-*Cd155*-KO (sg6) cells on days 17, 19, 21, and 23, relative to tumor inoculation. (E) WT mice were injected s.c. with 1×10^5 B16F10 control (left) or B16F10-*Cd155*-KO (sg6) (right) cells ($n = 5$ /group). A dose of 250 μ g clg, anti-PD-1 or anti-TIGIT and anti-CD96, or a combination of these mAbs was injected i.p. into WT mice with B16F10 control tumors on days 8, 10, 12, and 14 and into mice with B16F10-*Cd155*-KO (sg6) tumors on days 10, 12, 14, and 16, relative to tumor inoculation. (A–E) Tumor sizes were measured at the indicated time points. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, by 2-way ANOVA. Data indicate the mean \pm SEM. All experiments were performed once, except the experiment reflected in A, which was performed twice.

the greater predictive value of immune cell PD-L1 expression in responses to PD-1- or PD-L1-blocking antibodies in patients with bladder or urothelial cancer (46). We found that CD155 was not only highly expressed within the malignant component of human and mouse tumors, but was also expressed on tumor-infiltrating myeloid cells. Interestingly, the expression pattern of CD155 closely matched that of PD-L1, with substantial CD155 and PD-L1 coexpression within tumor cells and infiltrating myeloid cells from mouse tumors. CD155 loss in the host had a more profound impact on immune-mediated tumor control, despite the relatively lower total CD155 expression levels in tumor-infiltrating myeloid cells versus levels in tumor cells. Collectively, our observations that host-derived CD155 functionally impacts antitumor immunity and that CD155 was associated with infiltrating T cells suggest that CD155 upregulation is an underappreciated adaptive response to immune cells that contributes to immune evasion. Conflicting observations from *in vitro* studies regarding CD155 regulation by IFN- γ have been reported (39, 47), and it is currently unclear whether CD155 and PD-L1 are regulated by common or distinct mechanisms. Defining the mechanisms and kinetics for CD155 regulation in the TME remains a critical question.

Not only was coexpression of CD155 and PD-L1 detected in the present study, but the greater efficacy of anti-PD-1 therapy in a CD155-deficient TME we observed revealed the functional cross-talk between these 2 immunosuppressive factors. These observations are consistent with the additive suppressive effect of CD155 and PD-L1 on melanoma-specific T cells (39, 47) or the activation of melanoma tumor antigen-specific T cells after combined CD155 and TIGIT and PD-L1 and PD-1 blockade (39, 47) and suggest that combining therapies that selectively block the immunosuppressive activities of CD155 along with checkpoint inhibitor blockade would provide greater antitumor benefit to cancer patients. Our experiments with B16F10 melanomas using triple PD-1, TIGIT, and CD96 blockade support this hypothesis. The future design of anti-CD155 antibodies or small-molecule inhibitors of CD155 that do not affect DNAM-1-CD155 interactions may well lead to new combination therapies.

In conclusion, we have demonstrated what to our knowledge is a previously unrecognized immunosuppressive role for host-derived CD155 that is independent of tumor-derived CD155. Separately, tumor-expressed CD155 promotes tumor growth and metastasis via tumor-intrinsic mechanisms related to cell migration and survival. Loss of both host- and tumor-derived CD155 led to the greatest reduction in tumor growth and improved responses to anti-PD-1 or combined anti-PD-1 and anti-CTLA4 blockade, substantiating the nonredundant properties of CD155 from disparate sources. The preclinical functional results were correlated with the substantial expression of CD155 observed in both tumor cells and infiltrating myeloid cells from human and mouse tumors, revealing potential strategies for optimal immunotherapy combinations and highlighting the importance of assessing CD155 expression in both compartments as a preferred approach to predicting prognostic outcomes or response to immunotherapies.

Methods

Mice. C57BL/6, C57BL/6 *Ptprca*^d (CD45.1⁺), and BALB/c WT mice were purchased from the Walter and Eliza Hall Institute for Medi-

cal Research or the ARC Animal Resource Centre or were bred at the QIMR Berghofer Medical Research Institute. *Cd226*^{-/-} and *Rag2*^{-/-} γ *c*^{-/-} mice have been previously described (34). C57BL/6 *Cd155*^{-/-} mice (originally generated by Yoshimi Takai, Kobe University, Kobe, Japan) were provided by Stephen Gasser (Department of Microbiology, National University of Singapore). All mice were bred and maintained at the QIMR Berghofer Medical Research Institute and were used at 6 to 14 weeks of age. All mice were sex- and age-matched, and groups of 5 to 10 mice were used per experimental assay. No mice were excluded on the basis of preestablished criteria in any of the studies, and no active randomization was applied to any experimental group. The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome.

CD155 KO with CRISPR-Cas9 in tumor cells. CD155 small oligonucleotides (sgRNAs) were designed following the rules described by the Broad Institute (<http://www.genome-engineering.org/>). CD155 sgRNAs were subcloned into the PX330 vector (Addgene, 42230) according to the 1-step protocol. CD155 sgRNAs containing the PX330 plasmid or the empty vector were transfected into B16F10, MC38, LWT1, 4T1.2, or MCA1956 cell lines together with GFP-expressing plasmids (pRp-GFP). GFP⁺ cells were sorted using a FACSAria II Cell Sorter (BD Biosciences) 2 days later and were cultured *in vitro*. After 7 to 10 days, CD155⁻ cells were sorted to obtain CD155-KO cell lines. The primers for CD155 sgRNAs are detailed in the supplemental materials.

CD155 overexpression in B16F10-*Cd155*-KO (sg6) cells. B16F10-*Cd155*-KO (sg6) cells were transfected with a CD155 plasmid (pCMV3-mPVR, Sino Biological, catalog MG50259-UT), and CD155⁺ cells were sorted 2 days after transfection. The sorted CD155⁺ cells were then cultured in 200 μ g/ml hygromycin B for 3 weeks to produce cells with stable expression of murine CD155. The cells that expressed stable CD155 at levels similar to those in B16F10 control cells were sorted and named B16F10-*Cd155*-KO (sg6)-*Cd155ov* cells.

Cell culture. B16F10 melanoma (American Type Culture Collection [ATCC]), B16F10 control, B16F10-*Cd155*-KO (sg2/sg6), MC38 colon carcinoma, MC38 control, and MC38-*Cd155*-KO (sg2/g6) cells were cultured in complete DMEM media containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamax, HEPES, and sodium pyruvate; LWT-1 melanoma, LWT1 control, LWT1-*Cd155*-KO (sg2/sg6), 4T1.2 mammary carcinoma, 4T1.2 control, 4T1.2-*Cd155*-KO (sg2/sg6), MCA1956 fibrosarcoma, MCA1956 control, and MCA1956-*Cd155*-KO (sg2/sg6) cells were cultured in complete RPMI media containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamax, HEPES, and sodium pyruvate and maintained at 5% and 10% CO₂. Cell injection and monitoring procedures were described in previous studies (11, 12, 36, 48).

***In vivo* tumor growth.** For primary tumor growth experiments, B16F10 (1×10^5), SMIWT1 (5×10^5), MC38 (1×10^5 to 5×10^5), or MCA1956 (1×10^6) cells were s.c. injected into mice in a final volume of 100 to 200 μ l (day 0). Subcutaneous primary tumor growth was measured using digital calipers, and tumor sizes were recorded. At the indicated time points, the tumors were excised and subjected to immunological analysis by flow cytometry. For primary metastases, 2×10^5 B16F10 cells, 7.5×10^5 SMIWT1 cells, or 2×10^5 MC38 cells were injected i.v. into the tail vein, lungs were harvested on day 14, and metastatic colonies on the surface of the lungs were counted using a dissecting microscope.

Spontaneous tumor metastasis. For spontaneous metastasis and post-surgery survival experiments, 5×10^4 4T1.2 tumor cells or 4T1.2-

Cd155ko (sg2/sg6) tumor cells were inoculated into the fourth mammary fat pad of BALB/c mice. On the day after injection, mice were anesthetized, the primary tumor surgically removed, and the wound closed with surgical clips. Survival of the mice was monitored. Lungs were harvested and fixed, and metastatic colonies were identified under a dissecting microscope.

In vivo treatments. Some groups of mice received either anti-CD4 (clone GK1.5) and anti-CD8 β (clone 53.5.8) to deplete T cell subsets or anti-asGM1 to deplete NK cells. Some mice were treated with cIg (clone 1-1), anti-CD155 (clone 4.24), or anti-DNAM-1 (clone 480.1) mAb with schedules and doses as indicated in the figure legends. For immunotherapy, some groups of mice received anti-PD-1 (clone RMP1-14) and/or anti-CTLA4 (clone UC10-4F10) mAb (both from Bio X Cell) or anti-TIGIT D265A (clone 4B1, mouse IgG1 Fc dead, provided by Bristol-Myers Squibb) and anti-CD96 (clone 3.3 rat IgG1) as indicated in the figure legends.

Flow cytometry. Tumors, peripheral lymphoid tissues, and blood were harvested from WT and *Cd155*^{-/-} mice and treated mice. Single-cell suspensions of spleens or BM were depleted from erythrocytes as previously shown and stained with mAbs in phosphate PBS containing 1% (v/v) FBS and 2.5 mM EDTA. Dead cells stained with 7-AAD (BioLegend) or Zombie yellow were excluded from analysis. The antibodies used are detailed in the Supplemental information. Serum cytokine levels (GM-CSF, IFN- γ , IL-6, IL-10, MIP-1 β) were determined with a CBA Flex Set Multiplex Cytokine Bead Array (BD Biosciences). Cell apoptosis was visualized by staining with annexin V and 7-AAD (BD Biosciences).

CD8⁺ T cell and NK cell assays. Freshly purified splenocytes were cultured in 96-well, U-bottomed plates in complete RPMI medium supplemented with plate-bound anti-CD3 (1 μ g/ml) and soluble anti-CD28 (2 μ g/ml) in the presence of brefeldin A (3 μ g/ml; eBioscience). Intracellular IFN- γ was analyzed by flow cytometry in CD8⁺ T cells after 24 hours of culture. NK cells from the spleens of C57BL/6 WT and *Cd155*^{-/-} mice were enriched by MACS technology (NK Cell Isolation Kit II; Miltenyi Biotec) using an AutoMACS Pro Separator (Miltenyi Biotec). Freshly purified NK cells were plated in 96-well, U-bottomed plates in complete RPMI medium supplemented with mouse IL-12 (10–100 pg/ml; eBioscience) and mouse IL-18 (50 ng/ml; R&D Systems). Cytokine production was analyzed in the supernatant and in NK cells after 24 hours of culture.

BM chimera construction. CD45.1⁺ *Ptprc*^a WT mice and CD45.2⁺ *Cd155*^{-/-} mice (used as recipient mice; 9–10 mice per group) were irradiated twice with a total dose of 1,050 cGy, as before. BM cells (5 \times 10⁶ to approximately 10 \times 10⁶) from *Ptprc*^a mice or *Cd155*^{-/-} mice were then i.v. injected into the irradiated mice to construct BM chimera mice. Neomycin water was given to these mice for 3 weeks. After confirming the BM reconstruction by flow cytometry of peripheral blood, B16F10 cells were i.v. injected (2 \times 10⁵) or s.c injected (1 \times 10⁵) into the BM chimeric mice, respectively. Lung metastases were counted 14 days later, and tumor size was measured at the time points indicated in the figure legends.

IncuCyte scratch assay. B16F10 LWT1 4T1.2 control and B16F10 LWT1 4T1.2-*Cd155*-KO (sg6) cells were cultured in 96-well plates and grown to 80% to 100% confluency. Cells were then scratched with a Scratch WoundMaker (Essen BioScience). Photomicrographs of the scratched fields were captured every 3 hours and monitored with the IncuCyte Live Cell Imaging System (Essen BioScience). The degree of migration was analyzed until the time points indicated in the figure

legends using IncuCyte Scratch Wound Cell Migration Software and is represented as a percentage of wound closure.

Multispectral IHC, image processing, and analysis of human melanoma. Multiplex IHC analysis was carried out on FFPE samples (4- μ m thick sections) indirectly stained using PerkinElmer Tyramide Plus (Opal) reagents. Human melanoma tumor tissue (ME1004a) (1.3-mm-diameter cores) was obtained from US Biomax. Paraffin sections were dewaxed and rehydrated using a Leica XL Autostainer and treated with 0.5% H₂O₂ in TBS for 5 minutes to quench endogenous peroxidase. Antigen retrieval and antibody stripping were achieved using a home microwave (LG model MS2540SR/00). All incubations were carried out at room temperature. Multispectral images were of TMA slides acquired using the Vectra 3.0 Automated Quantitative Pathology Imaging System (PerkinElmer). Cores were only excluded if no analyzable tissue was present or the nuclear DAPI staining was not clear. Spectral unmixing and tissue and cell segmentation were performed using inForm software (PerkinElmer), allowing for the simultaneous evaluation of all markers in each tissue sample. Two TMA tumor tissue samples from each core collected from different FFPE blocks were analyzed through two different panels: CD3/CD163/CD155/HMB45/DAPI and CD14/CD11c/CD155/DAPI. All pixel intensity and shape-size measurements from segmented images were exported to a file format compatible with the flow and image cytometric data analysis software FCS Express 6 Plus (De Novo Software). Image brightness and contrast were adjusted in order to match the background values established for data quantification.

Statistics. Statistical analysis was determined with GraphPad Prism 7 (GraphPad Software). A 1-tailed Mann-Whitney *U* test was used for comparisons of 2 groups. Significance of differences was also calculated by log-rank *t* test for Kaplan-Meier survival analysis or 2-way ANOVA as necessary. Tukey's multiple comparisons tests were utilized unless otherwise indicated. Differences between 2 groups are shown as the mean \pm SD or the mean \pm SEM. *P* values of less than 0.05 were considered statistically significant.

Study approval. All mouse experiments were approved by the QIMR Berghofer Medical Research Institute animal ethics committee (Herston, Queensland, Australia).

Author contributions

XYL, WCD, and MJS conceived and designed the study. XYL, ID, AL, VA, KS, DB, JL, ARG, MB, KN, WCD, and MJS handled data acquisition and interpretation. KT, TB, MWLT, SJ, and SWL provided key materials. XYL, ID, WCD, and MJS wrote and revised the manuscript. MJS and WCD supervised the study.

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