

AWARD NUMBER: W81XWH-14-1-0397

TITLE: Targeting CD81 to Prevent Metastases in Breast Cancer

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*  
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<b>1. REPORT DATE</b> October 2015		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 30 Sep 2014 - 29 Sep 2015	
<b>4. TITLE AND SUBTITLE</b>  Targeting CD81 to Prevent Metastases in Breast Cancer				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-14-1-0397	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Shoshana Levy, PhD  E-Mail: slevy@stanford.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Stanford University 269 Campus Drive, CCSR 1105a Stanford, CA 94305-5151				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Results obtained in the first year of this project have shown that 1) the presence of the tetraspanin CD81 in a syngeneic immunocompetent <u>host</u> had an effect on metastasis and on circulating tumor cells (CTCs); 2) the presence of CD81 in the <u>tumor</u> had an effect on primary tumor growth, on metastasis, and on CTCs in a syngeneic immunocompetent wild type host.					
<b>15. SUBJECT TERMS</b> Breast cancer, CD81 knockout (CD81KO), Circulating tumor cells (CTCs), Primary tumor, Metastases Myeloid-derived suppressor cells (MDSCs), Regulatory T (Treg) cells, Wild type (WT)					
<b>16. SECURITY CLASSIFICATION OF:</b> U			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  44	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b>  Unclassified	<b>b. ABSTRACT</b>  Unclassified	<b>c. THIS PAGE</b>  Unclassified			<b>19b. TELEPHONE NUMBER</b> (include area code)

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## 1. INTRODUCTION:

We have shown that CD81 is a driver of metastases, we therefore hypothesized that inhibiting the function of CD81 by antibodies could halt metastatic spread. We will determine the mechanism(s) by which CD81 functions, and the effect of anti-CD81 mAbs on shedding of circulating tumor cells (CTCs) and on metastases using multiple breast cancer models.

**Specific Aim 1:** Determine the roles of CD81 for the metastatic phenotype in the host and in the tumor

**Specific Aim 2:** Determine the effect of anti-CD81 mAbs on CTCs and metastases

**Specific Aim 3:** Determine if the reduced metastatic phenotype of CD81KO mice is directly related to the impaired function of CD81KO myeloid-derived suppressor cells (MDSCs)

If successful, this work could not only lead to a clearer understanding of metastatic spread and growth, but more significantly, provide preclinical evidence for preventing and/or limiting metastases with a new targeted therapy. Our goal is to provide preclinical rationale for the development of a humanized anti-CD81 antibody for future use in human clinical trials. This proposal represents the first step in bringing a new-targeted therapy into the clinic aimed at metastatic breast cancer to diminish death from this disease.

## 2. KEYWORDS:

Breast cancer  
CD81 knockout (CD81KO)  
Circulating tumor cells (CTCs)  
Primary tumor  
Metastases  
Myeloid-derived suppressor cells (MDSCs)  
Regulatory T (Treg) cells  
Wild type (WT)

## 3. ACCOMPLISHMENTS:

### What were the major goals of the project?

**Specific Aim 1: Determine the roles of CD81 for the metastatic phenotype in the host and in the tumor**

**Major Task: Determine if the absence of CD81 affects CTC shedding and metastases**

End of Year 1 Accomplishments are summarized below, they focus on the role of CD81:

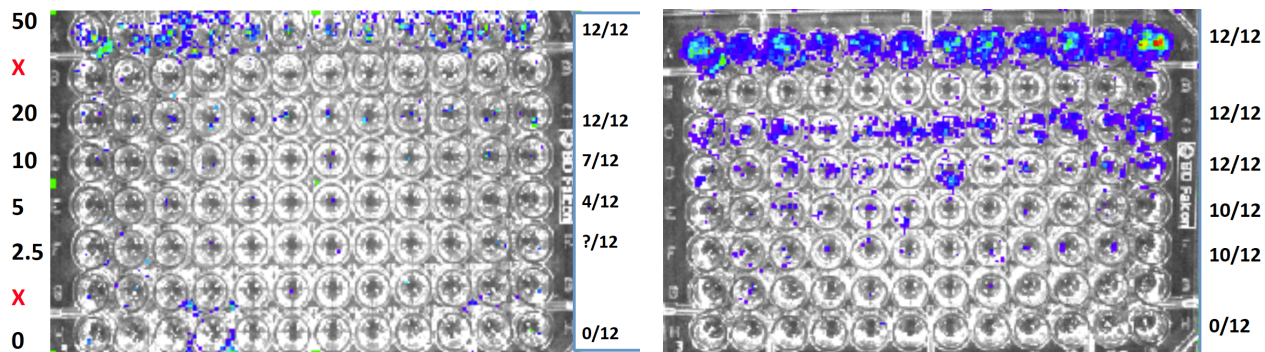
a) In the host

b) In the tumor

### What was accomplished under these goals?

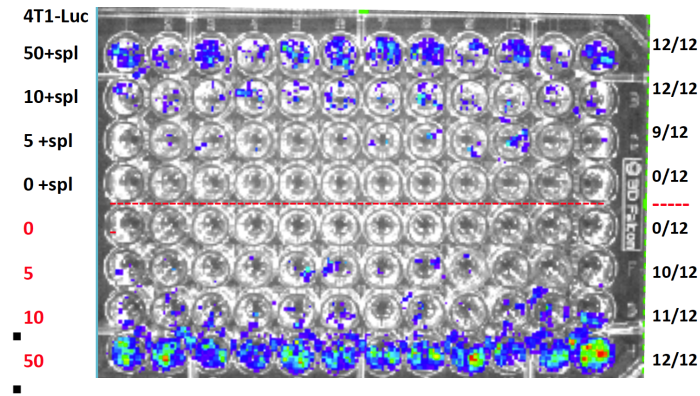
#### *Developing a sensitive assay for CTC detection*

We increased the sensitivity of CTCs detection in the 4T1 tumor model system by developing a luminescence assay that enumerates luciferase-tagged (-Luc) 4T1 cells. Briefly, the same number of 4T1-Luc cells (50, 20, 10, 5, 2.5) are deposited in 12 wells of a 96 well plates and incubated at 37C°. Luminescence was recorded on day 1 and again on day 4. The sensitivity of detection increased the increased from day 1 (left panel) to day 4 (right panel), as illustrated in Figure 1, below.



**Figure 1: Increased sensitivity of detection.** The indicated number of 4T1-Luc cells in each well of the entire row is shown on the left, the number of luminescent wells in each row is given on the right side of each panel. On day 1 (left panel) 12/12 wells give a positive signal for 50 and 20 cells/well; 7/12 for 10 cells/well, 4/12 for 5 cells/well. By day 4 (right panel) 12/12 wells are positive for 50, 20, and 10 cells/well; while 10/12 are positive for 5 and even 2.5 cells/well.

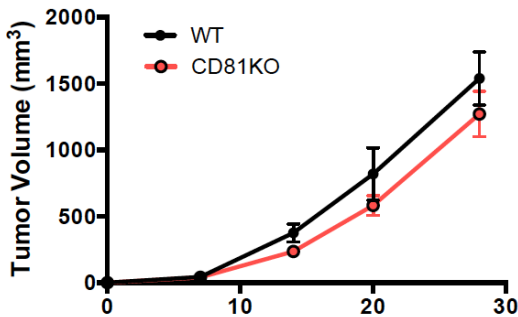
CTCs are usually a minor fraction of circulating blood cells. We therefore spiked 4T1-Luc cells into  $5 \times 10^5$  splenocytes. Figure 2 shows an image of a plate in which the upper rows contained splenocytes (+spl) whereas the lower rows did not. As can be seen, 5 cells amongst 500:000 could be detected in 9/12 wells.



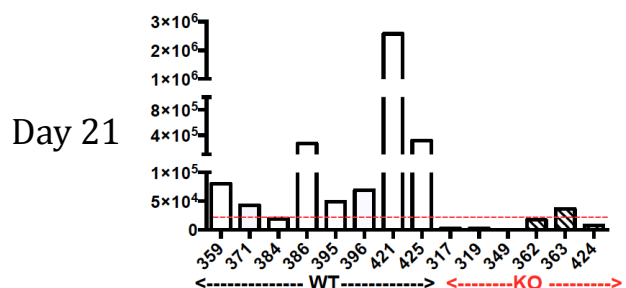
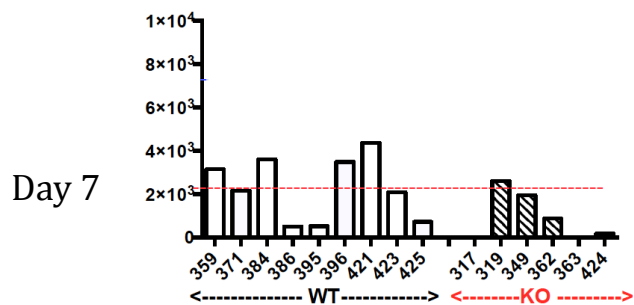
**Figure 2: Detection of luminescence is not interfered with in the presence of a large excess of non-tumor cells.** The indicated number of 4T1-Luc cells in the entire row is shown on the left, the upper half of the plate contains splenocytes in addition to the 4T1-Luc cells. The number of positive wells is shown on the right.

**a) Determining the effect of the host on CTC shedding:**

We used the above-described sensitive method to enumerate circulating 4T1-Luc cells post injection into wild type (WT) and CD81 knockout (CD81KO) hosts. As expected, tumor growth was reduced in CD81KO, compared to that of WT mice (Figure 3). Enumeration of CTCs in these tumor-bearing mice showed that WT mice harbored more 4T1-Luc circulating cells (Figure 4)



**Figure 3: Growth of primary tumors is reduced in CD81KO mice.**  $1 \times 10^4$  4T1-Luc cells were injected orthotopically into the indicated mice; tumor growth was monitored by caliper measurements.

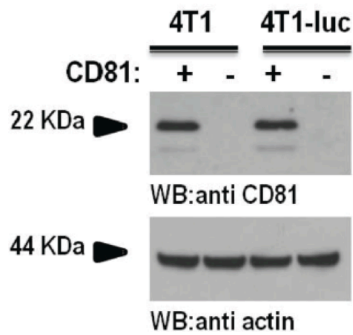


**Figure 4: Reduced number of circulating tumor cells in CD81KO mice.** CTCs were detected in 4/9 WT and in 1/6 CD81KO mice on day 7 post tumor inoculation. On day 21 CTCs were detected in 7/8\* WT and 1/6 CD81KO mice. \*one of the WT mice expired by day 21.

**b) Determining the effect of the tumor on CTC shedding:**

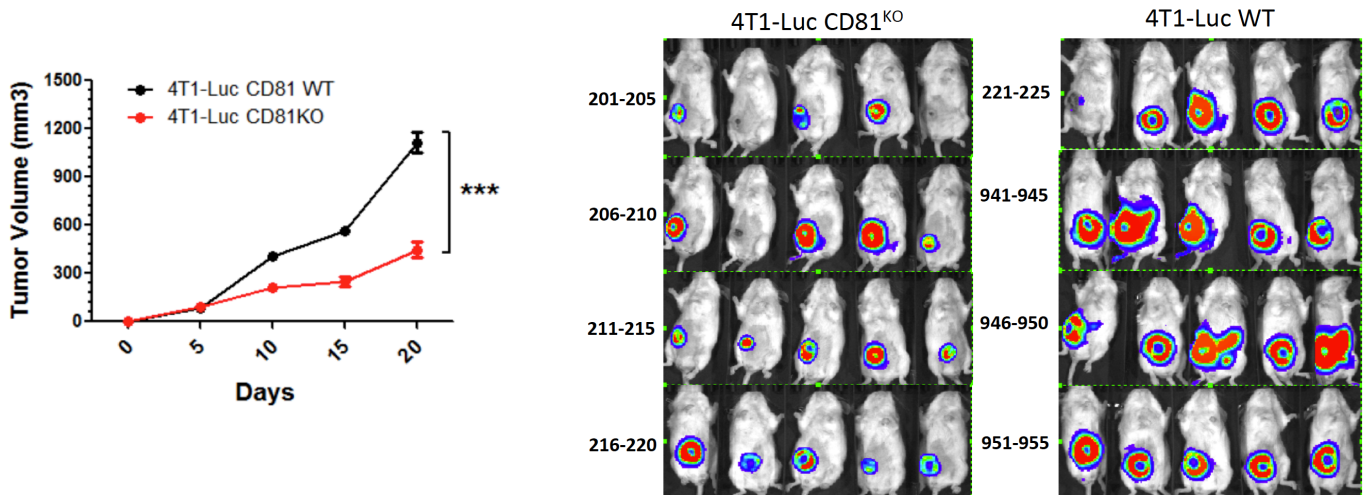
**Generation of 4T1 cells lacking CD81 (4T1CD81<sup>KO</sup>):**

We used CRISPR-Cas9 methodology to knockout CD81 in 4T1 parental and 4T1-Luc cells, Figure 5 demonstrates complete lack of CD81 expression in these 4T1 tumors.



**Figure 5: Generation of 4T1 cell lines in which CD81 was knocked out by CRISPR-Cas9 methodology.** Shown is CD81 expression in the indicated parental 4T1 cells and in cells in which CD81 was knocked out as analyzed by Western blots.

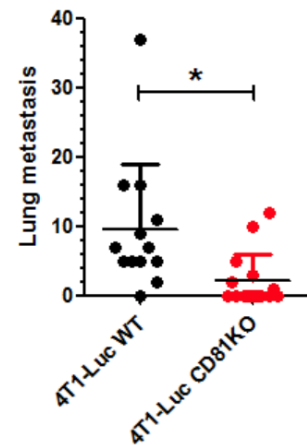
We have now tested if the presence of CD81 in the tumor is important for tumor growth and metastases in WT mice. As can be seen in Figure 6, tumor volume measured by caliper (left panel) and by luminescence (right panels) is reduced in mice injected with the 4T1CD81<sup>KO</sup>-Luc cells by comparison to parental 4T1-Luc cells.



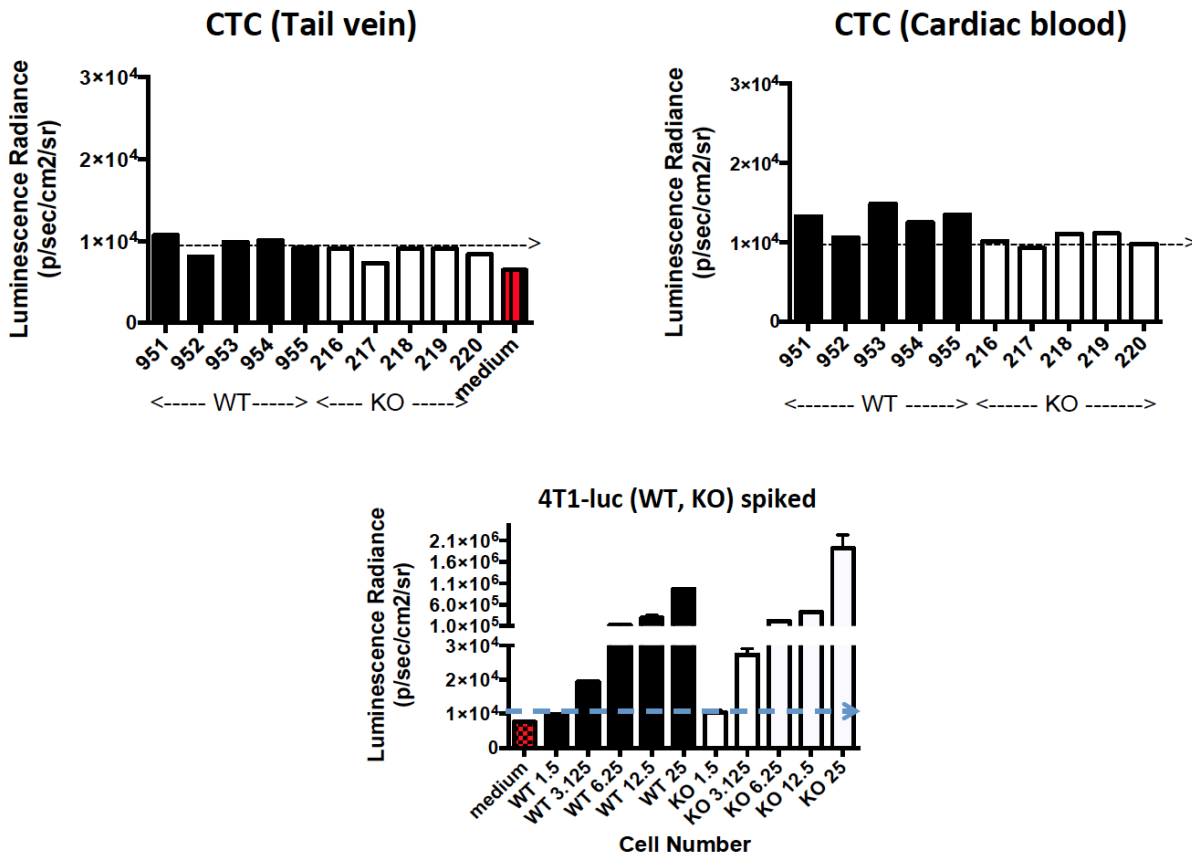
**Figure 6: Tumor growth is reduced in WT mice injected with 4T1 cells in which CD81 was knocked out.**  $1 \times 10^4$  4T1-Luc or 4T1CD81<sup>KO</sup>-Luc cells were injected orthotopically into WT mice; tumor growth was monitored by caliper measurements (left panel) and by luminescence (right panels) on day 21.

Lung metastases were also reduced in mice injected with 4T1CD81<sup>KO</sup>-Luc cells, as shown in Figure 7.

**Figure 7: Lung metastases are reduced in WT mice injected with 4T1 cells in which CD81 was knocked out.** Lung metastases were enumerated in WT mice sacrificed on day 27 post orthotopic injection of  $1 \times 10^4$  4T1-Luc or 4T1CD81<sup>KO</sup>-Luc cells.



Analysis of CTCs using the sensitive luminescence assay (shown in Figures 1 and 2) revealed fewer CTCs in WT mice that were inoculated with 4T1 tumor cells that lack CD81 (Figure 8).



**Figure 8: Fewer CTCs are detected in WT mice injected with 4T1 cells in which CD81 was knocked out.** The lower panel shows the luminescence radiated from spiked 4T1 cells (WT, black bars) and from 4T1CD81<sup>KO</sup>-Luc (KO, open bars). Detection of CTCs in individual mice inoculated with the indicated 4T1 cells is shown in the upper panels. As expected, cardiac bleeding (upper right panel) increases the sensitivity of detection by comparison to tail vein bleeding (upper left panel).

Ongoing studies focus on subtasks II and III in which lack of CD81 in both the host and in the tumor have an effect on growth and metastasis in the 4T1 tumor model.

- **What opportunities for training and professional development has the project provided?**  
The project provided an opportunity for Dr. Felipe Vences Catalan, a Postdoctoral Fellow, to study tumor biology. Specifically, he gained a deep understanding of the effect of the tumor environment on cells of the innate and adaptive immune system. Evidence for his professional development is summarized in our recent publication, which was supported in part by this award (Appendix); he has also been invited to present his results orally at both the Immunology and the Oncology Retreats at Stanford.
- **How were the results disseminated to communities of interest?**  
The results have been published and are available:  
Vences-Catalan F, Rajapaksa R, Srivastava MK, Marabelle A, Kuo CC, Levy R, Levy S. [Tetraspanin CD81 promotes tumor growth and metastasis by modulating the functions of T regulatory and myeloid-derived suppressor cells.](#) Cancer Res. 2015 Sep 1. pii: canres.1021.2015. [Epub ahead of print] PubMed PMID: 26329536.
- **What do you plan to do during the next reporting period to accomplish the goals?**  
We plan to pursue our initial goals.

- **What opportunities for training and professional development has the project provided?**  
The project provided an opportunity for Dr. Felipe Vences Catalan, a Postdoctoral Fellow, to study tumor biology. Specifically, he gained a deep understanding of the effect of the tumor environment on cells of the innate and adaptive immune system. Evidence for his professional development is summarized in our recent publication, which was supported in part by this award (Appendix); he has also been invited to present his results orally at both the Immunology and the Oncology Retreats at Stanford.
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#### 4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**  
The results of our studies demonstrate that expression of CD81 in both the host and in the tumor affect CTC shedding.
- **What was the impact on other disciplines?**  
Results of these studies in a syngeneic mouse model should be applicable to additional tumor models.
- **What was the impact on technology transfer?**  
Development of a sensitive assay applicable to luciferase-tagged tumors.
- **What was the impact on society beyond science and technology?**  
Nothing to report

#### 5. CHANGES/PROBLEMS:

Nothing to report

#### 6. PRODUCTS:

- **Publications**
  - **Journal publications**  
Vences-Catalan F, Rajapaksa R, Srivastava MK, Marabelle A, Kuo CC, Levy R, Levy S. [Tetraspanin CD81 promotes tumor growth and metastasis by modulating the functions of T regulatory and myeloid-derived suppressor cells](#). Cancer Res. 2015 Sep 1. pii: canres.1021.2015. [Epub ahead of print] PubMed PMID: 26329536.
- **Books or other non-periodical one-time publications**  
Nothing to report
- **Other publications, conference papers and presentations**  
Nothing to report
- **Website(s) or other internet site(s)**  
Nothing to report
- **Technologies or techniques**  
Nothing to report
- **Inventions, patent applications, and/or licenses**  
Nothing to report

- **Other products**  
Nothing to report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Shoshana Levy, Ph.D.</i>
Project Role:	<i>Initiating PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1.8 (no change)</i>
Contribution to Project:	<i>Directed the project</i>
Funding Support:	

Name:	<i>Ronald Levy, M.D.</i>
Project Role:	<i>Co-Investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>.12 (no change)</i>
Contribution to Project:	<i>Consulted on the design of studies</i>
Funding Support:	

Name:	<i>Felipe Vences, Ph.D.</i>
Project Role:	<i>Post Doctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>3.6 (no change)</i>
Contribution to Project:	<i>Designed and performed the studies.</i>
Funding Support:	

Name:	<i>Ranjani Rajapaksa, Ph.D.</i>
Project Role:	<i>Research Assistant</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2.4 (no change)</i>
Contribution to Project:	<i>Performed the studies.</i>

Funding Support:	
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**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

**What other organizations were involved as partners?**

Nothing to Report

## **8. SPECIAL REPORTING REQUIREMENTS**

### **COLLABORATIVE AWARDS:**

A separate report is being submitted by the collaborating/partnering PI

### **QUAD CHARTS:**

Not applicable

## **9 APPENDICES:**

Journal article attached

## **Tetraspanin CD81 promotes tumor growth and metastasis by modulating the functions of T regulatory and myeloid-derived suppressor cells**

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<sup>1</sup>Department of Medicine, Division of Oncology, Stanford University Medical Center, Stanford, CA, USA

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**Short title:** CD81 promotes tumor growth and metastasis

**Keywords:** Tetraspanin; CD81; Metastasis; Tregs; MDSC; immune suppression

### **GRANT SUPPORT**

This work was supported by the Translational Cancer Award from Stanford Cancer Institute and the Breast Cancer Research program from the Department of Defense grant W81XWH-14-1-0397 (both grants were awarded to S. Levy). None of the authors have a conflict of interest.

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## SUMMARY

Tumor cells counteract innate and adaptive antitumor immune responses by recruiting regulatory T cells (Treg) and innate myeloid-derived suppressor cells (MDSC), which facilitate immune escape and metastatic dissemination. Here we report a role in these recruitment processes for CD81, a member of the tetraspanin family of proteins that have been implicated previously in cancer progression. We found that genetic deficiency in CD81 reduced tumor growth and metastasis in two genetic mouse backgrounds and multiple tumor models. Mechanistic investigations revealed that CD81 was not required for normal development of Treg and MDSC but was essential for immunosuppressive functions. Notably, adoptive transfer of wildtype Treg into CD81-deficient mice was sufficient to promote tumor growth and metastasis. Our findings suggested that CD81 modulates adaptive and innate immune responses, warranting further investigation of CD81 in immunomodulation in cancer and its progression.

## PRECIS

Findings demonstrate that the cell surface tetraspanin CD81 contributes to immune escape in cancer by attenuating the immunosuppressive activity of innate and adaptive cells that drive malignant progression.

## INTRODUCTION

Understanding the factors influencing tumor progression should have a great impact in preventing and treating human cancers. Tetraspanins are a family of proteins that influence a wide range of cellular functions including proliferation, adhesion, migration, differentiation, activation and cell signaling (1-2). Tetraspanins serve as membrane “docking” molecules that interact with cell surface receptors, such as integrins (3) and with intracellular signaling molecules (1-2), and have been shown to play a role in cancer progression (4-5). Tetraspanins cluster with partner proteins into so-called “tetraspanin-enriched microdomains” (TEMs). Included in these TEMs are cell surface molecules important in the immune system, such as CD19 in B lymphocytes and CD4 in T lymphocytes (6-7).

Historically, the first tetraspanin molecule was identified by a monoclonal antibody (mAb) that recognized a human “antigen associated with early stages of melanoma tumor progression”, now renamed CD63 (8-9). Expression of a specific individual tetraspanin molecule in human cancer has been correlated with either good or bad prognosis. For example, *KAI1/CD82* was originally identified as a metastasis suppressor gene in a rat prostate cancer model; subsequently the human homolog was shown to suppress metastasis in this model (10). Moreover, CD82 mRNA expression in several cancers is associated with a good prognosis (11-12). By contrast, CD151, previously identified by an anti-metastatic mAb (13) and TSPAN8, originally identified as a colon-associated antigen (14) are markers of poor prognosis. Over-expression of these tetraspanins correlates with tumor progression and metastasis (15). Corroborating the role of CD151 in tumor progression are studies in CD151-deficient mice, which develop fewer metastases than their wild type (WT) counterparts in carcinogen-induced skin cancer, melanoma (B16F10), Lewis lung carcinoma (LLC), transgenic breast cancer (MMTV-PyVmT) and adenocarcinoma prostate cancer (TRAMP) models (16-19).

## CD81 promotes tumor growth and metastasis

CD81 was originally discovered as a target of an anti proliferative antibody (TAPA-1) (20). It was subsequently identified as a cell entry receptor for hepatitis C virus (HCV) (21). It is also noteworthy that entry of sporozoites, the liver stage of the malaria parasite, requires the presence of CD81 (22). Mice lacking CD81 have additional impairments, including female infertility, and nervous system malfunctions (23-24). Although many studies have addressed the function of CD81 in infection (25) and in the immune system (26), few have studied the involvement of CD81 in tumorigenesis and metastasis. Recently, it was shown that expressing exogenous CD81 in a human melanoma cell line enhanced its migrating, invasive and metastatic abilities in a xenograft model (27). This evidence suggests that CD81 contributes to melanoma cell motility. However, the effect of host CD81 on tumor progression has not been addressed previously.

Here we used CD81-deficient hosts on both C57BL/6 and BALB/c mouse backgrounds in which we analyzed several tumor models to determine the contribution of CD81 to tumor progression and metastasis. Our results provide the first evidence that host CD81 facilitates tumor growth and metastasis. Furthermore, we demonstrate that lack of CD81 severely impairs the function of regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs). These findings provide insight to the mechanism by which CD81 modulates adaptive and innate immune responses involved in tumor growth and metastasis.

## EXPERIMENTAL PROCEDURES

### Mouse tumor cell lines

C57BL/6 tumors were purchased in 2014: Lewis lung carcinoma (LLC) from ATCC (then transfected with luciferase using pNL4.3 HIV Luc to generate LLC-luc cells); and the breast cancer E0771 from CH3 Biosystems™. The BALB/c 4T1 mammary carcinoma and 4T1-luc cells were gifted in 2012 by Dr. S. Strober and Dr. C. Contag, respectively (both at Stanford University, CA, USA). 4T1, 4T1-luc

## CD81 promotes tumor growth and metastasis

and E0771 were cultured in RPMI 1640 media (Corning® Cellgro®), LLC and LLC-luc in DMEM media (Corning® Cellgro®) both media contain 10% (vol/vol) heat-inactivated FCS (HyClone), 1% L-glutamine (Corning® cellgro®), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), and 50 µM 2ME (Gibco) at 37°C in a 5% CO<sup>2</sup> incubator. All cell lines were tested according to the manufacturer's protocol and proved to be mycoplasma free (MycoAlert Mycoplasma detection kit, Lonza).

### Mice

Mice were backcrossed to the BALB/c and C57Bl6 backgrounds more than 10 generations. Due to female infertility both colonies were maintained by breeding of *Cd81*<sup>+/-</sup> heterozygous (HT) mice. 6-12 weeks old wild type (WT), HT, and *Cd81*<sup>-/-</sup> (CD81KO) female or male littermates mice were used in these studies. All animal experiments were approved by the Stanford Administrative Panel on Laboratory Animal Care and conducted according to the Stanford University Facility and NIH guidelines. Mice were bred and housed at the pathogen-free animal facility of the Stanford University Medical Center.

### Genotyping

The following primers were used to genotype all mice: CD81FP 5'-AACCACGCTCTTGCCATCCCT-3', CD81RP 5'-CAAGGTGGCCTCTGGTCACT-3' and CD81NEO 5'-ATTCGCAGCGCATCGCCTTCT-3'. PCR conditions were as follows: DNA was denatured at 94°C, followed by 35 cycles of amplification using *Taq* DNA Polymerase (New England Biolabs); 94°C for 1 min, 55°C for 45 sec, 72°C for 1 min, and a final extension step at 72°C for 5 min. PCR products were separated in 1.5% agarose gel electrophoresis, expected size were 301 bp for WT and 565 bp for CD81KO.

## Tumor growth assays

4T1 or E0771 tumor cells were injected either orthotopically or into the tail vein (i.v.) and LLC tumor cells were injected subcutaneously (s.c) or (i.v.) as detailed in each study. Growth of primary tumors was monitored with a digital caliper measurements (Mitutoyo) and expressed as tumor volume (length x width x height) and/or by bioluminescence using the in vivo optical imaging system (IVIS 100, Xenogen). Mice were sacrificed when s.c. tumor size reached 2 cm<sup>2</sup>. Lungs metastases were visualized by injecting 15% India ink through the trachea. Following dissection, lungs were washed once with water and fixed in Fekete's solution (100ml of 70% ethyl alcohol, 10ml of formaldehyde, and 5ml of glacial acetic acid) for 24 hours. Surface white nodules metastases were counted under a dissecting microscope.

4T1 cells are resistant to 6-thioguanine, lungs from 4T1-bearing mice were collected under sterile conditions and digested with 2.5 ml of 1mg/ml collagenase type IV + containing 15 units elastase at 4<sup>0</sup>C for 75 minutes, followed by 2 washes in RPMI. Dissociated cells were resuspended in 10ml of complete media containing 60μM of 6-thioguanine (Sigma), and incubated at 37<sup>0</sup>C, 5% CO<sub>2</sub> for 10-14 days. Growing colonies were fixed by methanol, washed and stained with 0.03% methylene blue (Sigma) solution, rinsed with water and counted.

## Flow cytometry

Single cell suspensions from spleens, tumors and peripheral blood cells from naïve and tumor-bearing mice were filtered through a 70μm cell strainer (BD Biosciences) and resuspended in 1% BSA in PBS, then stained with fluorochrome-conjugated antibodies (Supplementary Table 1) on ice for 30 minutes. Cells were washed twice in BSA/PBS and fixed in 2% paraformaldehyde, intracellular staining of anti-FoxP3 (clone FJK-16s) was performed according to the manufacturer's protocol (eBioscience). Cells were acquired using the FACS Calibur or LSRII flow cytometers (BD

## CD81 promotes tumor growth and metastasis

Biosciences, San Jose, CA). Data analysis was performed using the FlowJo software (Treestar, Asland, OR).

### **Proliferation assays**

Purified spleen T cells from naïve mice were negatively isolated using a pan T cell isolation kit (MACS Miltenyi Biotec) and labeled with 2.5-5  $\mu$ M CFSE (Gibco, Life technologies) for 10 min (according to the manufacturers' protocols), and reactions were terminated by 10 volumes of cold 10% FCS in RPMI. Labeled naïve T cells were stimulated with anti CD3/CD28 dynabeads (Gibco, Life Technologies) in a U-bottom 96 well plate and co-cultured at the indicated ratios with regulatory T (Treg) cells from naive or tumor-bearing mice (also isolated using a MACS Miltenyi Biotec CD4+CD25+ kit according to the manufactured protocol) or with blood MDSCs from tumor-bearing mice. Co-cultures were incubated for 5 days at 37°C in a 5% CO<sup>2</sup> incubator, followed by staining with anti- CD3, CD4 and CD8 mAb. T cell proliferation was analyzed by flow cytometry using FACS Calibur. Division index and percentages of proliferating cells was calculated using FlowJo software (Treestar, Asland, OR).

### **Macrophage polarization assay**

Naïve mice were injected with sterile 3% thioglycolate 4-5 days before macrophage isolation. 4-5 days later, macrophages were collected from the peritoneum by washing several times with PBS using an 18 gauze needle. The cell pellet was centrifuged and resuspended in DMEM media containing 10% FCS and incubated for 3 hrs at 37°C in a 5% CO<sup>2</sup> incubator. Non-adherent cells were removed by washing several times with PBS. Adherent macrophages were then stimulated with 100 ng/ml of LPS and incubated with either WT or CD81KO blood MDSCs for 24 hrs at 37°C. After 24 hrs

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supernatants were collected and IL-10, IL-12p70, latent TGF- $\beta$  and IFN- $\gamma$  secretion was measured by ELISA kit (Legend Max™, Biolegend) according to the manufacturer's protocol.

### **Adoptive transfer of Tregs**

4T1 cells were injected into either WT or CD81KO donor mice and after 10 and 17 days of tumor injection spleens were collected for Treg isolation. Tregs were purified using a MACS Miltenyi Biotec CD4+CD25+ kit according to the manufactured protocol. Purified CD3+CD4+CD25+FoxP3+ from WT or CD81KO donor mice were co-injected with  $1 \times 10^4$  4T1 cells orthotopically into CD81KO recipient mice, a second dose of purified Tregs was injected one week later after tumor injection. Tumor growth was monitor by caliper and metastasis was assessed after 30 days.

### **IL-10 and latent TGF- $\beta$ determination**

Purified WT or CD81KO Tregs from tumor bearing mice were isolated as described above and culture in RPMI media supplemented with 10% FCS for 48 hrs. Supernatants were collected and IL-10 and latent TGF- $\beta$  cytokines were quantified by ELISA kit (Legend Max™, Biolegend) according to the manufacturer's protocol.

### **Statistical Analysis**

Results are presented as the mean of triplicates  $\pm$  SD of at least three independent experiments. Data were analyzed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA) by either unpaired t-test or one-way ANOVA when more than two groups were compared. Differences are indicated in the figures. A p-value of less than 0.05 was considered statistically significant.

## RESULTS

### Lack of CD81 expression in the host affects tumor growth

To investigate the role of CD81 in tumor growth we generated a Lewis Lung Carcinoma (LLC) cell line expressing luciferase (LLC-luc) to monitor tumor growth in vivo. LLC-luc cells were injected subcutaneously (s.c.) into wild type (WT), heterozygous (HT) and CD81 knockout (KO) C57BL/6 mice, followed by analysis of tumor volume by caliper measurements and bioluminescence imaging. We found that locally injected tumor growth was significantly reduced in CD81KO by comparison to WT and HT mice (**Fig 1 A, B**). We then injected the tumor intravenously (i.v.) and found that lung metastases were significantly reduced in CD81KO mice compared to WT mice (**Fig 1C, D**). These studies suggested that the lack of CD81 in the host affects tumor growth and metastases.

To ascertain the role of CD81 in the host versus the tumor, we analyzed the growth of breast cancer cells (E0771), which in contrast to LLC tumor do not express CD81 (**Supplementary Figure 1**). E0771 tumor volumes were equal in WT and HT mice, as monitored by caliper measurements (**Fig 2 A, B**). By contrast, tumor volume was considerably smaller in CD81KO mice throughout the monitoring period (**Fig 2 A, B**). Moreover, by monitoring individual tumor growth (**Fig 2A, right panel**) we observed tumor shrinkage in more than half of the CD81KO mice. Furthermore, we found that tumors regressed in 10/25 of the CD81KO mice on day 25-post injection (**Fig 2 C**). This result establishes that lack of CD81 in the host plays an important role in susceptibility to tumor growth and because a subset of the mice actually rejected the tumor, suggests that the immune system might play a role.

To establish that reduced tumor growth in CD81KO mice in these tumor models was not due to the host C57BL/6 genetic background, we moved to the 4T1 breast cancer model in BALB/c mice. This tumor expresses CD81 (**Supplementary Figure 1**). 4T1-luc cells were injected orthotopically into

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female mice and tumor growth was monitored by caliper and by bioluminescence imaging. Once again, tumor volume in CD81KO mice was reduced by comparison to their wild type littermates (**Fig 3A and B**). We also injected 4T1 breast carcinoma cells into males, tumors grew but much slower than in females, yet, tumor volume was reduced in male CD81KO BALB/c, compared to their wild type littermates (data not shown). These results indicate that CD81 deficiency in the host affects the growth of tumor cells of different histologic types and the effect is independent of the genetic background of the host.

### Diminished metastasis in CD81KO mice

Metastasis, which occurs during cancer progression, is the leading causes of death among all cancers. We therefore evaluated the role of CD81 in dissemination of tumor cells from the primary site to the lungs using a breast cancer model. 4T1 cells were injected orthotopically into the mammary fat pad of WT, HT or CD81 KO BALB/c mice and 28-30 days post injection lungs were perfused with India ink to visualize lung metastases, which appear as macroscopic white colonies on a black background (**Fig 3C, left panel**). We found significantly fewer lung metastases in both female and male CD81KO BALB/c mice in comparison to their WT and HT counterparts (**Fig 3C, right panel**). 4T1 cells are resistant to 6-thioguanine, which offers an alternative approach to the count of macroscopic colonies (28). Lungs from tumor bearing mice were digested with collagenase IV and elastase and single cell suspensions were then plated in the presence of 6-thioguanine. As expected, lungs from WT 4T1 bearing mice develop more tumor colonies in comparison to lungs from 4T1 bearing CD81 KO mice (**Fig 3D**).

To determine if the presence of CD81 in the host would affect colonization of tumor cells in the lung, 4T1 cells were injected i.v. - as with the orthotopic model we observed far fewer lung metastases in CD81KO mice by comparison to WT mice (**Fig 3E**). Thus, lack of CD81 is associated with reduced

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colonization of tumor cells in both backgrounds, in LLC-i.v.-injected C57BL/6 (**Fig 1C**) and in 4T1-i.v.-injected BALB/c mice (**Fig 3E**). Taken together, these results emphasize the importance of CD81 in the host in tumor progression and metastasis.

### **CD81 deficiency impairs regulatory T cells (Tregs) function in tumor bearing mice**

Expansion of regulatory T cells is a hallmark during cancer progression in both human and mouse (29). Treg accumulation contributes greatly to immune suppression in the tumor microenvironment promoting immune evasion, tumor growth and dissemination. Others have demonstrated that Treg depletion is effective in reducing tumor growth and metastasis of 4T1 tumor-bearing mice (30). Indeed, the percentage of Tregs in spleens of 4T1-bearing mice was increased by comparison to naïve mice (**Fig 4A, upper panel**). However an equal accumulation of Tregs was observed in 4T1-bearing WT and CD81KO spleens (**Fig 4A, lower panel**). Interestingly, CD81 is upregulated in Tregs derived from tumor bearing WT mice (**Fig 4B**).

We proceeded to analyze the function of WT and CD81KO Tregs derived from tumor-bearing mice. Equal numbers of splenic CD4+CD25+ Tregs cells were isolated from tumor-bearing mice (**Supplementary Figure 2**). Purified naïve T cells were labeled with tracking dye (CFSE) then stimulated to proliferate by beads coated with anti-CD3 and anti-CD28 mAbs (**Fig 4C upper panel**). Proliferation was also assessed in the presence of the purified CD4+CD25+ Treg cells. This analysis revealed a considerable effect of CD81 deficiency on Treg function. CD81KO Tregs were severely impaired in their ability to suppress proliferation of both CD4 and CD8 T cells in comparison to WT Tregs (**Fig 4C, D and E**). Moreover, analysis of the suppression activity of Tregs from LLC and E0771 -bearing CD81KO C57BL/6 mice confirmed impairment in this alternative genetic background (**Supplementary Figure 3**).

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Taken together these results suggest that CD81 mediates anti-tumor immune responses by affecting regulatory T cell function. Interestingly, Tregs derived from non-tumor-bearing CD81KO mice were as effective as WT Tregs in their ability to suppress T cell proliferation (**Supplementary Figure 4**). In addition, Tregs equally suppressed naïve WT and CD81KO T cells.

## CD81 deficiency impairs myeloid derived suppressor cell (MDSC) function in tumor bearing mice

MDSCs are a heterogeneous population that accumulates in response to pro-inflammatory mediators during infection or during cancer development (31). In addition to their immune suppression activity, MDSCs also promote tumor angiogenesis and metastasis (32). Moreover, MDSCs have been shown to suppress the adaptive immune response to tumors. Indeed, multiple previous studies have demonstrated sharp increases in the number of MDSCs circulating in 4T1-bearing BALB/c mice (33). Some of these studies have also revealed a suppressive effect of MDSC on T cell proliferation (34). We therefore decided to analyze the impact of CD81 on MDSC function.

CD81 is expressed on the surface of MDSCs (**Figure 5A**), however its absence does not affect the maturation of MDSCs as naïve CD81KO, HT and WT C57Bl6 and BALB/c mice have similar percentage (10-20%) of blood MDSCs (**Figure 5B**). Even 4T1-tumor-bearing mice, which accumulate circulating MDSCs rapidly post tumor inoculation, show an equal increase over time in both WT and CD81KO mice (**Figure 5C**). Similarly, MDSCs equally accumulate in the spleen, the primary tumor site and in the lungs (data not shown), independent of CD81 presence (**Figure 5D**).

Next we tested if CD81KO MDSCs suppress T cell proliferation. As expected, MDSCs from WT 4T1-bearing mice suppressed the proliferation of naïve CD4<sup>+</sup> T cells, whereas CD81KO MDSCs were severely impaired in their ability to suppress this proliferation (**Figure 5F, G**). Similarly, CD8<sup>+</sup> cell

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proliferation was suppressed by WT MDSCs, but to a considerably lesser extent by CD81KO MDSCs derived from tumor-bearing mice (**Figure 5H**).

Several mechanisms by which MDSCs mediate immune suppression include arginine depletion through ARG-1 dependent consumption and L-cysteine deprivation via its consumption and sequestration (35-36). However, Arg1 expression and other related genes did not differ between WT and CD81KO MDSC (**Supplementary Figure 5A,B**)

Generation of oxidative stress, which is caused by the production of ROS and reactive nitrogen species, is another pathway utilized by MDSCs to mediate suppression (37), but we did not detect any difference in ROS production between WT and CD81KO MDSCs (**Supplementary Figure 6**).

MDSCs have also been shown to modulate innate immune cells by polarizing M1 to M2 macrophages (38). As expected, WT MDSCs strongly polarize M1 macrophages into M2, as evident by secretion of high amounts of IL-10 and by the expected inhibition in IFN- $\gamma$  and IL-12 secretion; however, CD81KO MDSCs similarly polarize M1 into M2 macrophages (**Figure 5E**).

## CD81 in Tregs promotes tumor growth and metastasis

To determine if reduced tumor growth and metastasis was due to the impaired immune suppression in the absence of CD81, we adoptively transferred either WT or CD81KO Tregs from tumor bearing animals together with 4T1 breast cancer cells into CD81KO recipient mice (**Figure 6A**). Mice that received WT Tregs had increased tumor volumes in contrast to mice that received CD81KO Tregs (**Figure 6B**). Furthermore, lung metastases were increased upon adoptive transfer of WT but not CD81KO Tregs (**Figure 6C**). However, when MDSCs were adoptively transferred no differences were observed in tumor growth and metastasis (data not shown). Tregs mediate immune suppression by different mechanisms such as expressing inhibitory receptors that blocks activation of

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effectors cells. However, while CD81 expression was increased in WT Tregs, both WT and CD81KO Treg expressed similar levels of CTLA-4, PD-1, OX-40, ICOS, CD137 and GITR (**Supplementary Figure 7**). Finally we also tested the ability of Tregs to secrete IL-10 and TGF- $\beta$ ; cytokines known to mediate immune suppression. Although both WT and CD81KO Tregs secreted similar amounts of TGF- $\beta$  (**Figure 6D**), IL-10 secretion was diminished in CD81KO Tregs (Figure 6E).

Taken together, the absence of CD81 in Tregs (**Figures 4, 6**) and in MDSCs (**Figure 5**) impairs their T cell suppressive function.

## DISCUSSION

Over the past years several impairments have been described in CD81KO mice (23-24, 39-40). However, none of the studies have evaluated the contribution of CD81 in the host during cancer progression. Here we report for the first time that CD81 deficiency in the host has a profound effect on tumor growth and metastasis in two genetic backgrounds of CD81KO mice.

Tetraspanins are widely expressed in the body. Hence, tumor cells which arise from normal tissues also express these proteins. Indeed some tetraspanin members have been shown to play a role in cancer progression. A definite role for CD151 was demonstrated in two independently derived CD151KO mice that were challenged with several tumor models (16, 19). In the same studies, diminished metastasis in CD151KO hosts was suggested to be due to impaired adhesion and trans-endothelial migration of CD151 expressing tumor cells (19).

Numerous studies have demonstrated that 4T1 tumors induce a strong suppressive microenvironment with an accumulation of MDSCs and Treg cells. Tregs, a subset of CD4+CD25+ T cells, infiltrate tumors and suppress antitumor activity of effector T cells. Previous studies have demonstrated that depletion of Tregs by anti CD25+ antibodies completely abrogates metastasis of

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4T1 tumors (41). The fact that Tregs in 4T1-bearing mice upregulated CD81 expression suggested that CD81 could potentially mediate Treg function. As expected, Tregs accumulated in 4T1-bearing mice; however an equal increase was seen in both WT and CD81KO mice. Remarkably, the suppression ability of these tumor-induced Tregs was severely impaired in CD81KO mice. Moreover, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferated in the presence of CD81KO Tregs, but not in the presence of WT Tregs. CD81KO Tregs were not only impaired in the 4T1 BALB/c tumor model, CD81KO Tregs in E0771 and LLC tumor bearing C57BL/6 mice were also impaired in their ability to suppress T cell proliferation. Importantly, we demonstrated that CD81KO Tregs failed to suppress the anti-tumor immune response when adoptively transferred with tumor cells into CD81KO recipients, whereas WT Tregs promoted tumor growth and metastasis, establishing a link between reduced tumor growth and metastasis with impaired immune suppression in the absence of CD81.

Modulating the function of CD81 on Tregs might have an important clinical application, as current therapies aimed at blocking Treg: T cell interactions have been shown to reverse tumor induced-immune suppression (42). Furthermore, effective immunotherapeutic anti-tumor maneuvers, such as the combination of local irradiation combined with anti-PD-L1 therapy, highly reduces MDSC in the treated mice (43).

4T1 tumors secrete GM-CSF, IL-1 $\beta$ , IL-6 and TGF- $\beta$  that induce a rapid accumulation of Gr1<sup>+</sup> MDSCs (44-45), which impair anti-tumor immune responses and promote metastasis either directly, or indirectly via Treg activation (46). Indeed, reagents that deplete MDSCs, such as anti-Gr1 antibodies (47) or peptibodies targeting S100 family proteins (48), have demonstrated anti-tumor responses. Here we showed that CD81 is also expressed on MDSCs, which rapidly accumulate in blood, and to a lesser extent in spleen and at the primary tumor site, although their percentages were similar in CD81KO and WT tumor bearing mice. However, CD81 deficiency on this innate immune

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cell subset resulted in impaired suppression of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation. Intriguingly we did not see a difference in tumor growth when MDSCs were adoptively transferred, which could be explain by the short lifespan of MDSC in contrast to the lifespan of Tregs.

The fact that CD81KO mice have normal numbers of Tregs and MDSCs under steady state and in tumor bearing animals suggests that CD81 is not needed for development of these two subsets. By contrast, their function was severely impaired in tumor-bearing mice, both Tregs and MDSCs failed to suppress T cell proliferation. We propose that CD81 modulates the function of both of these immune suppressive cell populations. Additional molecules that modulate the function of immune suppressive cells include B7-H4, a member of the B7 family. MDSCs derived from B7-H4KO mice suppressed T cell proliferation more potently than their WT counterparts (49). Conversely, lack of macrophage migration inhibitory factor (MIF) or CD40 on MDSCs impaired T cell suppression (34, 50). On the other hand molecules, such as Epstein-Barr-virus induced gene 3 (Ebi3, which encodes IL-27b) and interleukin-12 alpha (which encodes IL-12a/p35), were shown to modulate Treg function (51). Ebi3KO and IL-12alphaKO Tregs had significantly reduced regulatory activity in vitro and also failed to cure inflammatory bowel disease in vivo, by comparison to WT Tregs.

Tregs and MDSCs modulate immune cells by plethora of mechanisms. Broadly, these mechanisms can be subdivided into cell-cell contact-dependent (FasL-FAS, PD-1/PD-L1, CTLA-4/CD80-CD86) and those mediated mainly by secretion of immune-modulators molecules, such as IL-10, TGF- $\beta$ , IL-37, IL-35, etc. review in (32), or by sequestering factors required by effectors cells, such as decreasing the cysteine (36) and glutathione pools (52). Additional mechanisms, observed in experimental mice models have shown that MDSCs, which are found at premetastatic distant organ sites, enable recruitment of colonizing tumor cells thereby promoting metastasis, review in (53). Although we explored some of these inhibitory mechanism that MDSCs or Tregs use, we only found

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that CD81KO Tregs derived from tumor bearing mice have reduced IL-10 secretion. Although the exact mechanism(s) by which CD81 modulates Treg and MDSCs function still need(s) further investigation, it is clear that the presence of CD81 in the host has a major effect on tumor growth and that this effect is mediated partially by the immune system.

In regard to host-tumor interactions, it is well established that tumor cells secrete exosomes that modulate the microenvironment (54). CD81 and other tetraspanins are well-known markers of exosomes (55). Furthermore, a recent paper demonstrated that exosomes secreted by fibroblasts increased metastasis of MDA-MB-231 human breast cancer cells to the lungs of immunocompromised mice, importantly, the deletion of CD81 from these exosomes highly reduced metastasis of these tumor cells (56). On the other hand, uptake of exosomes was shown to require the presence of the integrin molecule CD29 and CD81, knocking down both molecules inhibited exosome uptake by mesenchymal stem cells (57). In view of these studies it is intriguing that while naïve CD81KO and WT Tregs suppressed T cell proliferation equally, when tumor was on board, the function of CD81KO Tregs was impaired. One possible scenario is that CD81KO Tregs have an intrinsic defect in the uptake of exosome from tumor cells, which is then followed by an inability to activate and suppress effector T cells.

In summary, we report that CD81 deficiency greatly contributes to tumor development as evident by reduced tumor growth and metastasis in three different tumor models in two different genetic backgrounds. We also demonstrate that the suppressive function of Tregs and MDSCs is impaired in CD81KO tumor bearing mice. Ongoing studies are aimed at determining the contribution of CD81 on the host vs the tumor cells in growth and metastasis.

## AUTHOR CONTRIBUTIONS

FVC, RR, MS, AM, RL and SL designed, performed and analyzed the experiments and edited the manuscript. CCK generated LLC-Luc cell line. FVC and SL wrote the manuscript.

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## FIGURE LEGENDS

**Fig 1. Reduced lung (LLC-1) tumor growth and metastasis in CD81KO C57BL6 mice. (A)**  $0.5 \times 10^6$  Lewis lung carcinoma (LLC-luc) cells were injected subcutaneously into either WT, HT or CD81KO C57BL6 male and female mice (WT 10/10, HT:10/10, KO: 10/10). Bioluminescence of LLC-luc-bearing mice was imaged at day 7 post injection and tumor growth of individual mice was monitored by caliper measurements. **(B)** pooled caliper measurements (pooled data include 20 WT, 20 HT and 20 KO mice **(C,D)**).  $1 \times 10^6$  LLC-luc cells were injected intravenously into WT or CD81KO mice and lungs were collected on day 16 and perfused with India ink to visualize tumor metastasis (each dot represents one mouse). Mean and SEM are shown.

**Fig 2. Reduced breast E0771 tumor growth in CD81KO C57BL/6 mice. (A)**  $0.5 \times 10^6$  E0771 breast tumor cells were injected subcutaneously into either WT, HT or CD81KO C57BL6 female mice in three independent experiments (10 mice in each group WT, HT, KO) and tumor growth was monitored by caliper measurements. **(left panel)** macroscopic tumor sizes **(right panel)** tumor growth in individual mice. **(B)** tumor growth of pooled caliper measurements (data include 25 mice in each group WT, HT and KO). Mean and SEM are shown. **(C)** Percentage of E0771 tumor bearing mice at day 25 post tumor injection (WT 0/25, HT: 1/25, KO: 10/25).

**Fig 3. Reduced breast cancer (4T1) tumor growth and metastasis in CD81KO BALB/c mice. (A and B)**  $1 \times 10^4$  4T1-Luc tumor cells were injected subcutaneously into either wild type (WT) or CD81KO BALB/c female mice (5 WT, 5 KO). Tumor growth was monitored every 5 days by caliper measurements and weekly by bioluminescence imaging (shown is day 21 post tumor injection) **(A)** Tumor growth of individual mice **(B)** pooled caliper measurements data **(C)**. Lung metastases developed after subcutaneous injection of 4T1 tumor cells were visualized by India ink infusion on day 28 post tumor inoculation, as shown on the left panel, and quantified (each dot represents one

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mouse WT:30, HT:18, KO:38). **(D)** Lungs from 4T1-bearing WT or CD81KO mice injected s.c. with  $1 \times 10^4$  4T1 cells were collected on day 28, digested with collagenase and elastase for 75 min at  $4^\circ\text{C}$  and dissociated cells were plated and incubated at  $37^\circ\text{C}$  for 10-14 days in media containing 60mM of 6-thioguanine. Tumor metastasis were visualized by staining with methylene blue and quantified **(E)**  $2.5 \times 10^4$  4T1 tumor cells were injected intravenously into WT or CD81KO BALB/c mice. On day 21 lungs were collected and perfused with India ink to visualize tumor metastasis (each dot represents one mouse). Mean and SEM are shown.

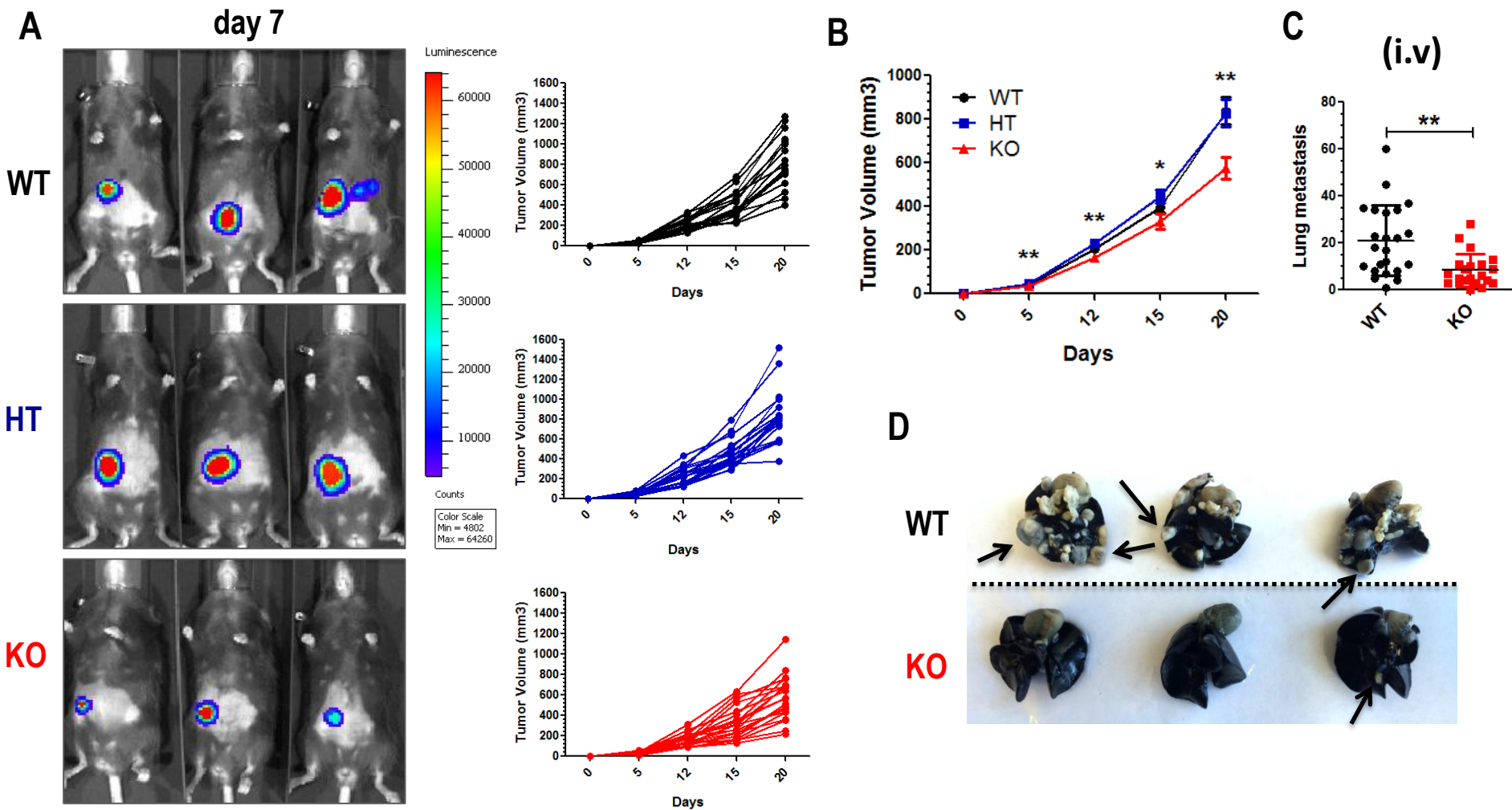
**Fig 4. Tregs are less suppressive in 4T1-bearing CD81KO mice. (A, upper panel)** Percentage of CD3+CD4+CD25+FoxP3+ cells in naive vs. 4T1-bearing WT mice. **(A, lower panel)** Percentage of splenic Tregs (CD4+CD25+FoxP3+) in 4T1 tumor bearing WT, HT and CD81KO BALB/c mice. **(B)** CD81 expression on splenic FoxP3+ cells in naive and 4T1 bearing WT mice. **(C, D, E)** Purified WT and CD81KO splenic Tregs from tumor bearing mice were co-cultured at the indicated Treg:CD4+ ratios with CFSE labeled naive CD3/CD28 stimulated T cells. **(C)** CD4+ T cell proliferation was analyzed after five days and showed as histograms. **(D)** Division index quantification of CD4+ and **(E)** CD8+ T cells of 5 independent experiments are shown.

**Fig 5. Myeloid derived suppressor cells (MDSCs) increase in 4T1-bearing CD81KO mice, but their suppressive function is reduced. (A)** CD81 expression on MDSC's CD11b+Gr1+, shaded histogram CD81KO MDSCs, open histogram WT MDSCs. **(B)** Percentage of MDSCs in the blood of naive WT, HT or CD81 KO BALBc and C57BL/6 mice **(C)** Percentage of blood MDSCs (CD11b+Gr1+) at the indicated times in 4T1-bearing WT and CD81KO BALB/c mice **(D)** Percentage of MDSCs in blood, spleen and tumor sites on day 28 post s.c tumor injection of  $5 \times 10^4$  4T1 cells. **(E)** Peritoneal macrophages from WT BALB/c mice were purified and stimulated with LPS (100 ng/ml) and co-culture with or without WT or CD81KO MDSCs derived from 4T1-bearing mice at 1:2 ratio for

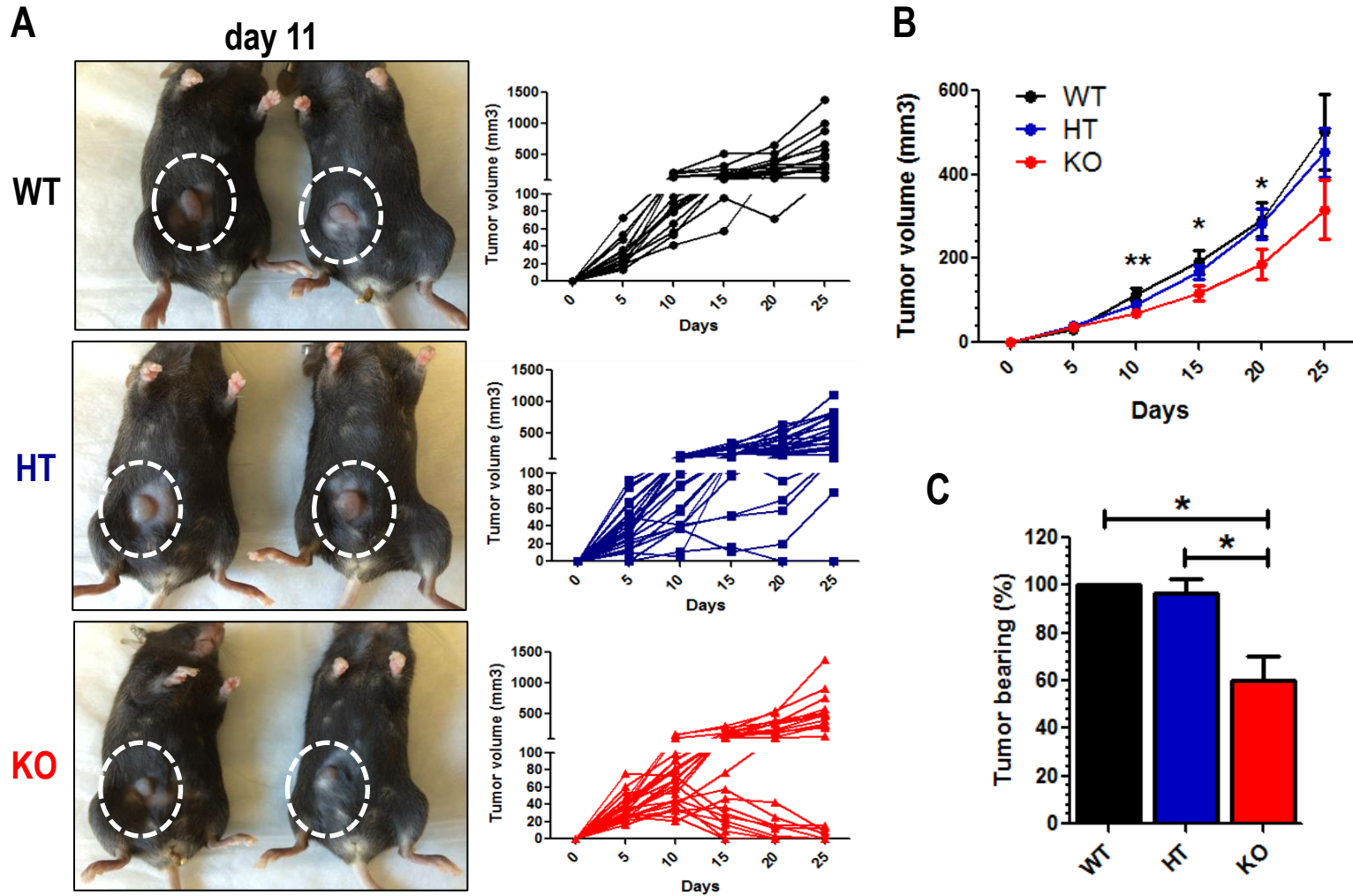
## CD81 promotes tumor growth and metastasis

24 hrs. Supernatants were collected and IL-10, IL-12p70 and IFN- $\gamma$  were measured by ELISA. **(F,G,H)** Blood MDSCs from 4T1 -bearing WT and CD81KO mice were co-cultured at the indicated ratios with CFSE labeled naive CD3/CD28 stimulated T cells. **(G)** CD4+ or CD8+ **(H)** T cell proliferation was analyzed after five days and shown as histograms and percentages of proliferating T cells were quantified.

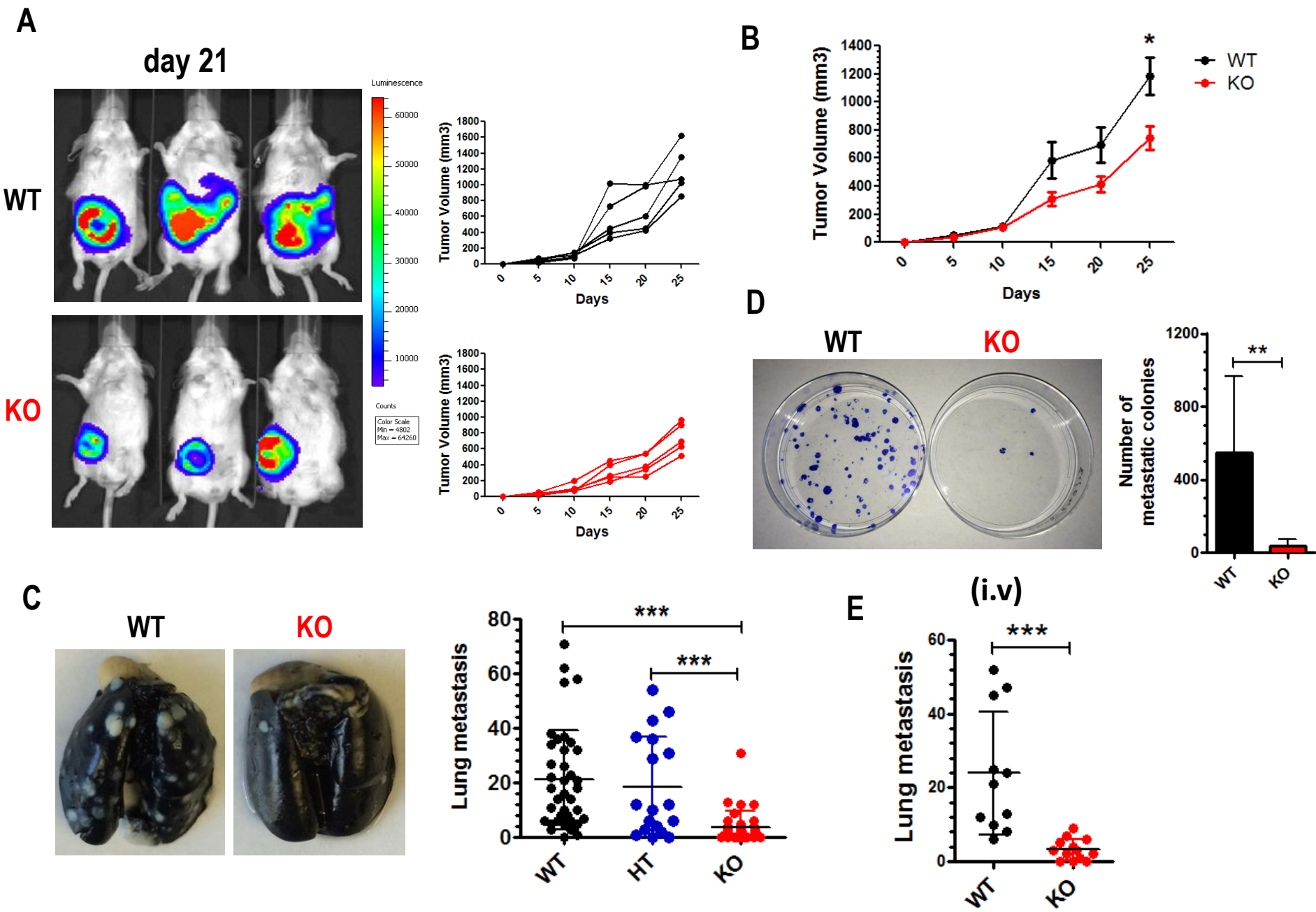
**Fig 6. CD81 in Tregs promotes tumor growth and metastasis.** **(A)** Adoptive transfer of WT or CD81KO Tregs scheme: 4T1 were injected into WT or CD81KO donor mice and Tregs were purified on day 10 and 17 post tumor injection. Purified WT or CD81KO Tregs (CD3+CD4+CD25+FoxP3+) were then co-injected with 4T1 tumor cells into CD81KO recipient mice. A second transfer of Tregs was given after 7 days post tumor injection. **(B)** Tumor growth was monitored by caliper and **(C)** lung metastasis was assessed by India ink staining. **(D)** Latent TGF- $\beta$  and **(E)** IL-10 secretion was measure by ELISA of purified WT or CD81KO Tregs from 4T1 bearing mice after 2 days in culture.



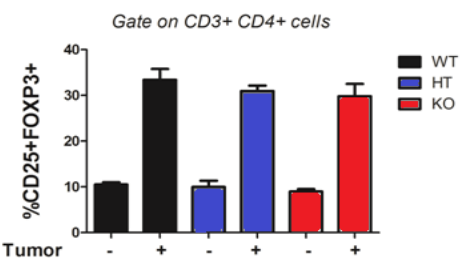
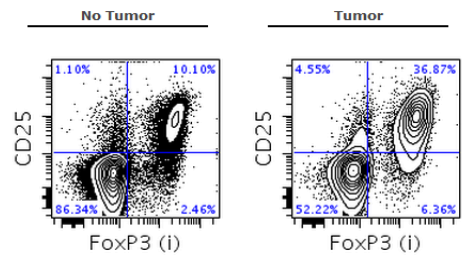
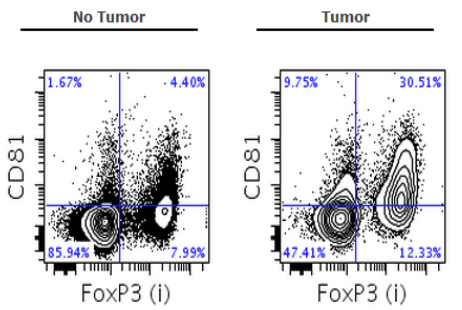
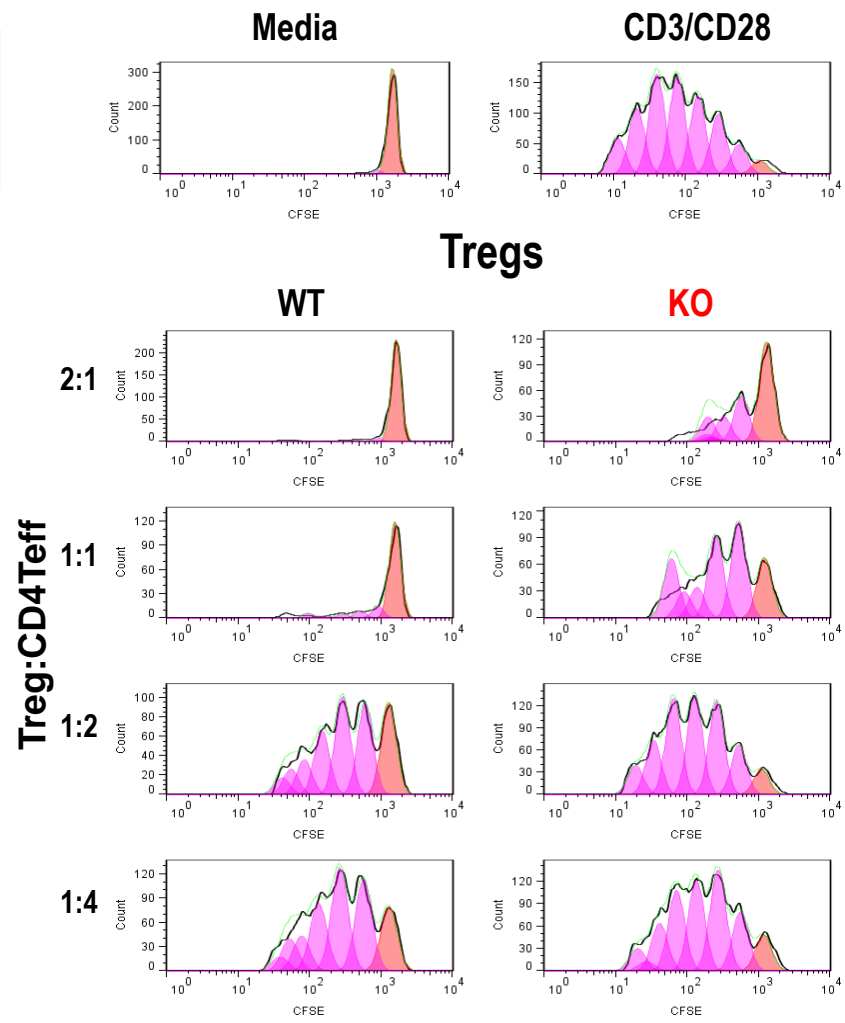
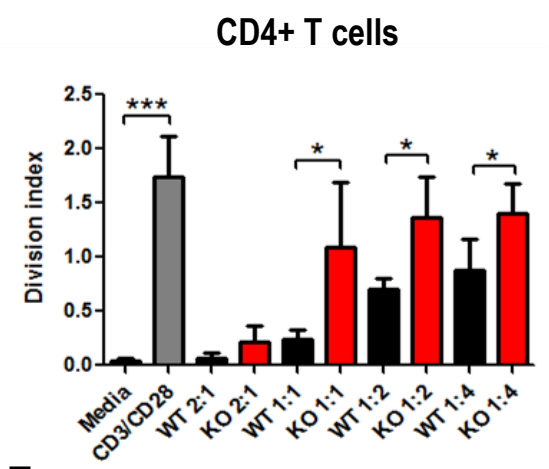
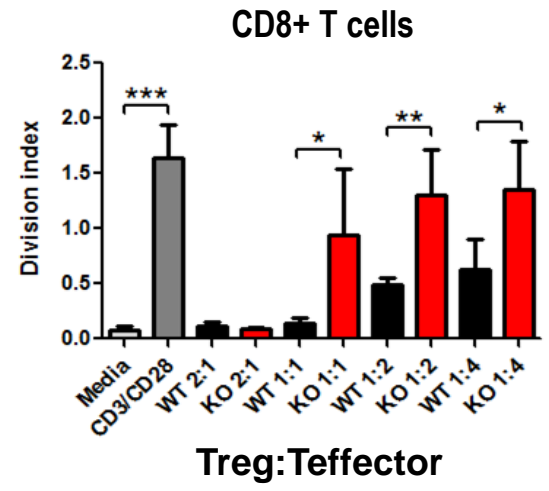
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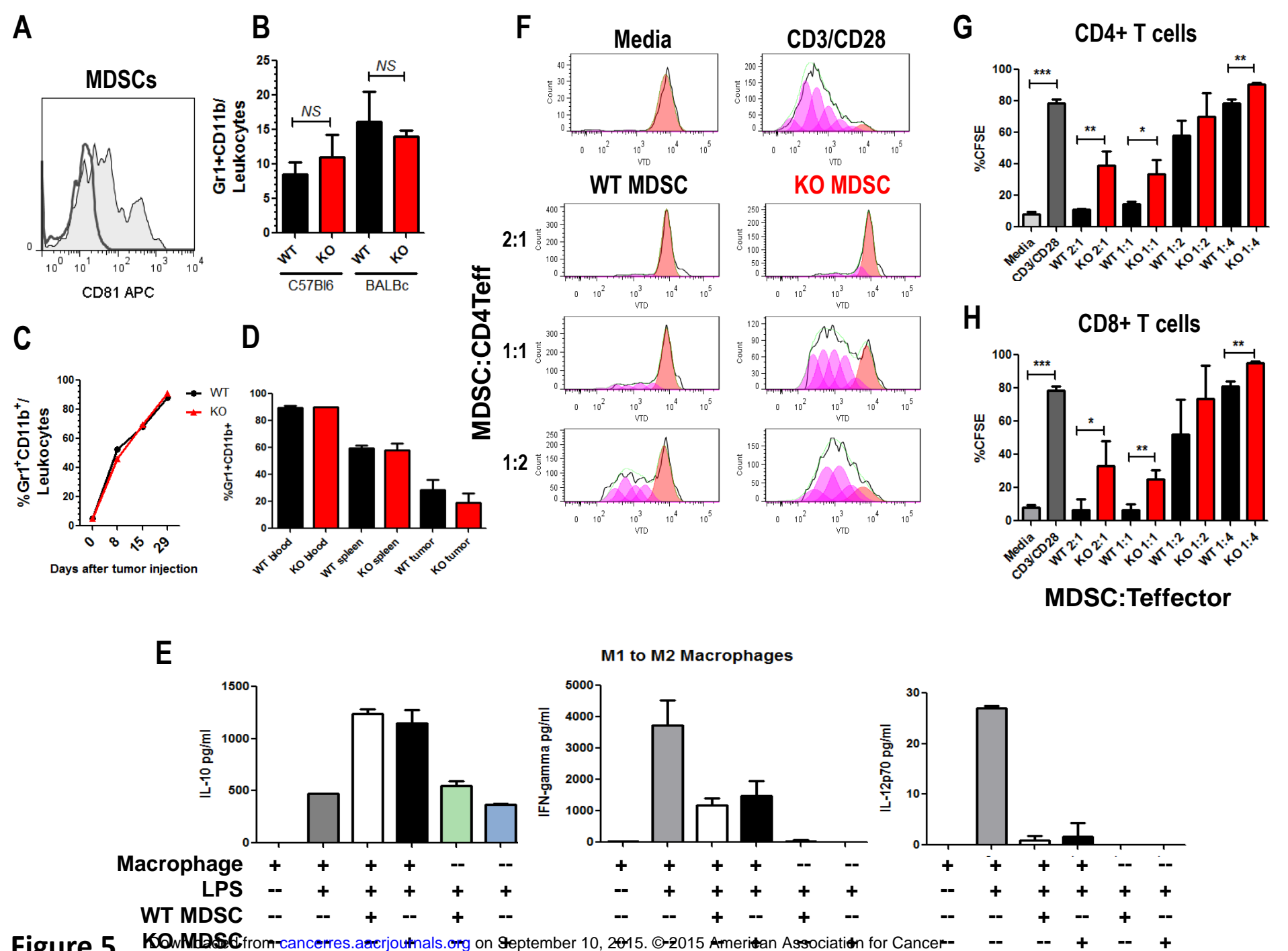


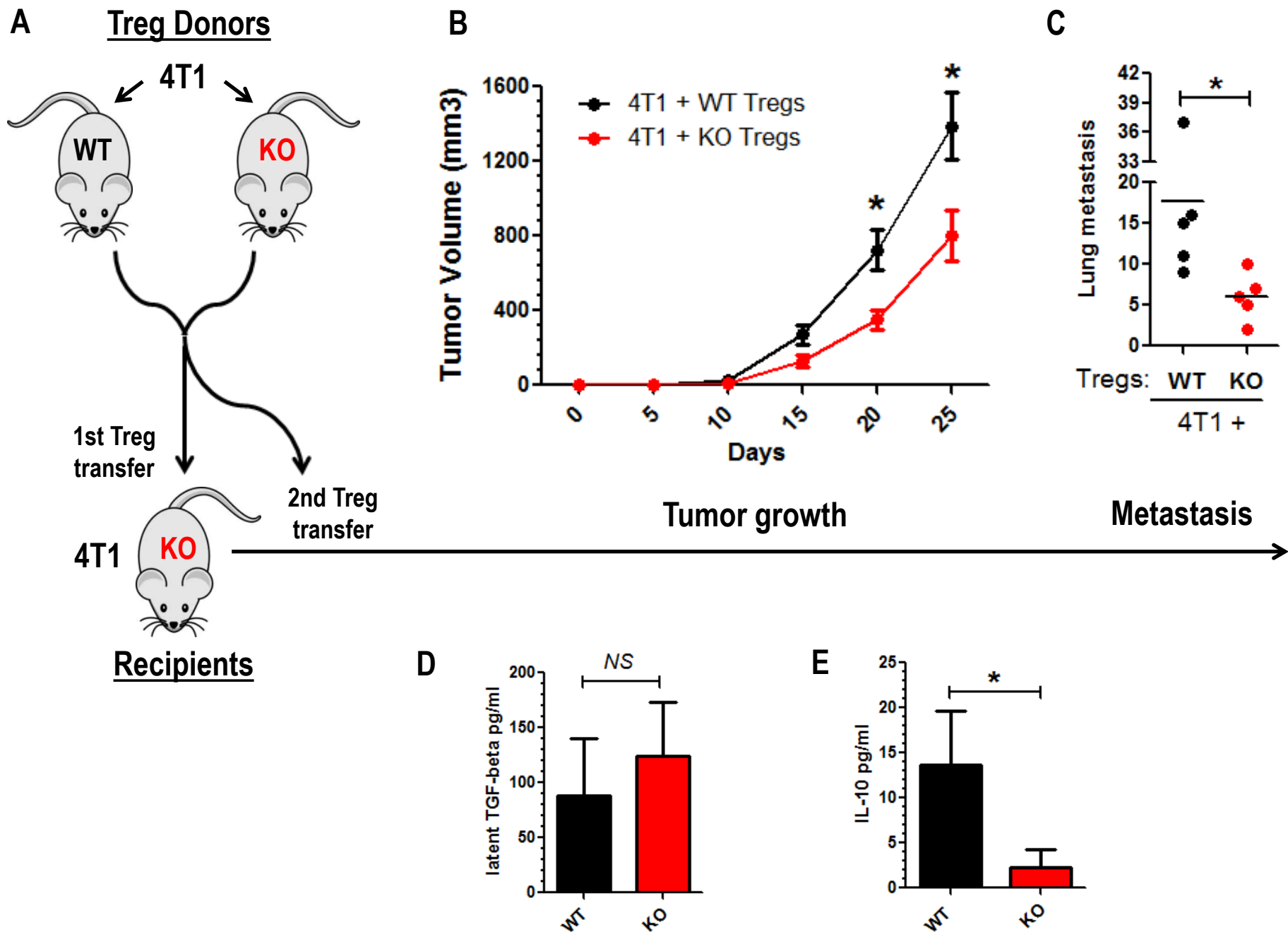
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**A****B****C****D****E****Figure 4**





**Figure 6**

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## Tetraspanin CD81 promotes tumor growth and metastasis by modulating the functions of T regulatory and myeloid-derived suppressor cells

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*Cancer Res* Published OnlineFirst September 1, 2015.

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