Improving immunotherapy for melanoma: models, biomarkers and regulatory T cells

Rao, D.

Chapter 4

Lack of *in vivo* effect of LMK235 on regulatory T cells and melanoma growth control

Disha Rao[1], Tainá Gomes[1], Johanna A. Stunnenberg[1], Daniel S. Peeper[1,2], Christian U. Blank[1,3,4]

Abstract

The presence of regulatory T cells (Tregs) in the tumor is associated with worse prognosis in patients with melanoma. Targeting Tregs can limit tumor growth and synergize with PD-1 checkpoint blockade therapy. However, therapeutic avenues for Treg targeting remain limited. Class-specific inhibition of histone deacetylase (HDAC) can reduce both frequency and the suppressive function of Tregs. LMK235 is a class II specific HDAC inhibitor, shown to reduce Treg function *in vitro*. However, the efficacy of LMK235 in tumor models remains unexplored. We therefore evaluated the effect of LMK235 on Treg populations and its potential synergistic effects with anti-PD-1 on tumor growth control. Unexpectedly, in two syngeneic murine melanoma models (MeVa2.1.dOVA and B16.dOVA), LMK235 failed to alter Treg populations in the tumor and had limited therapeutic benefit *in vivo*.

Introduction

Immune checkpoint blockade (ICB) therapies have significantly prolonged the overall survival of patients with advanced stage cutaneous melanoma [1]. However, nearly 55% of the patients do not respond to anti-PD-1 monotherapy [2], due to intrinsic or acquired resistance mechanisms [3]. The presence of regulatory T cells (Tregs) results in a suppressive tumor microenvironment (TME), which has been associated with a lack of response to ICB therapies in melanoma [4]. Tregs are a subset of suppressive immune cells, marked by the expression of the transcription factor Forkhead box P3 (FoxP3) [5]. Under physiologic circumstances, they play an important role in maintaining peripheral tolerance to self-antigens [5]. However, their presence is detrimental in the context of anti-tumor immunity, since they suppress $CD8^+$ T cell effector functions [4]. A shift to a high Tregs to a low $CD8^+$ T cell frequency is associated with worse prognosis in melanoma [6], making Tregs an attractive therapeutic target to enhance the efficacy of immunotherapy [4]. In line with this notion, depleting Tregs in murine models results in increased effector responses and better tumor growth control [7,8]. Targeting Tregs can also increase the efficacy of PD-1 checkpoint blockade treatment in murine tumor models of melanoma [7,9,10]. However, limited therapeutic strategies are available in the clinic to specifically deplete Tregs in the tumor. Current strategies to target Tregs include Fc tail modified monoclonal antibodies against CD25 [7] and CTLA-4 [11], which induce target cell killing via antibody dependent cell cytotoxicity (ADCC). However, the expression of these markers is not exclusive to Tregs and is upregulated by effector $CD4^+$ T cells and $CD8^+$ T cells upon activation [12,13]. As a result, such strategies raise concerns regarding potential depletion of effector T cells. Therefore, testing other candidates that can specifically reduce Treg frequencies and function in the tumor is warranted.

Accumulating data have shown that histone deacetylase (HDAC) inhibitors could be beneficial in controlling tumor growth owing to their immune-modulatory properties [14,15]. Certain HDAC inhibitors can modulate the expression of FoxP3, thus altering the functionality of Tregs [16]. HDACs are grouped into class I (HDAC 1 2, 3 and 8), class IIa (HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), class III (sirtuins) and class IV (HDAC 11) [15]. While knockout (KO) of HDAC 6, HDAC 9, HDAC 10 and HDAC 11 is known to augment Treg suppressive functions, KO of HDAC 3 and HDAC 5 reduces Treg functionality [17–22]. Therefore, specific targeting of HDAC 3 or HDAC 5 might reduce Treg suppressive functions.

LMK235 is a class II (HDAC 4 and HDAC 5)-specific inhibitor that has been shown to reduce induction of Tregs [18]. However, the efficacy of targeting HDAC class II, using LMK235, in tumor models remains unexplored. We therefore evaluated the effect of LMK235 on Tregs and tested its efficacy on tumor growth control in two murine melanoma models, with the idea to create a rationale for combining LMK235 with immunotherapy in patients who do not benefit from immunotherapy alone.

Results

LMK235 modulates Treg induction *in vitro* without altering conventional T cells
Since inhibition of HDAC5 lowers expression of FoxP3 [18], we first set-out to confirm the effect of LMK235 on TGFβ-mediated induction of murine FoxP3$^+$ Tregs. We observed a dose-dependent reduction in the frequency of Tregs in the presence of LMK235 **(fig 1A)**. We then evaluated whether LMK235 treatment impacts on activation and proliferation of murine CD4$^+$ and CD8$^+$ T cells. Whereas the higher dose of LMK235 significantly reduced CD4$^+$ T cell activation **(fig 1B)**, there was no effect on the activation of CD8$^+$ T cells **(fig 1C)**, as measured by the expression of the activation marker CD69. In addition, LMK235 reduced proliferation of CD4$^+$ T cells, upon stimulation in the presence of anti-CD3 and anti-CD28, whereas the proliferation of CD8$^+$ T cells remained unaltered **(fig 1D-F)**.

We then examined whether the observed effect can be reproduced using human T cells. Owing to the slight effect of the higher dose of LMK235 on reduced CD4$^+$ T cell activation in the murine setting **(fig 1B)**, we lowered the dosing for human T cell analysis. In line with the murine setting, we observed a significant reduction of human Treg induction upon treatment with LMK235 **(fig 1G)**. Moreover, at the tested doses of LMK235, we did not observe any loss of viability **(fig 1H)** or proliferation **(fig 1I-J)** of CD3$^+$ T cells upon activation with anti-CD3 and anti-CD28, in the presence of LMK235. Since knockout of HDAC5 has been shown to reduce interferon-gamma (IFN-γ) production by CD8$^+$ T cells [18], we assessed if HDAC5 inhibition via LMK235 also hinders cytokine production. IFN-γ production by both CD4$^+$ and CD8$^+$ T cell subsets remained intact in the presence of LMK235 **(fig 1K)**. In summary, our *in vitro* data indicated that LMK235 might be a promising compound to lower intra-tumoral Treg frequency, without impairing proliferation and effector functions of CD4$^+$ or CD8$^+$ T cells.

Treatment with LMK235 does not alter Treg subsets or tumor growth control *in vivo*
PD-1 blockade on Tregs has been shown to augment their suppressive function, potentially limiting the efficacy of PD-1 blockade-mediated tumor growth control [9]. In line with this notion, Treg targeting synergizes with PD-1 blockade [7,9,10]. We therefore hypothesized that LMK235 could be a likely candidate to target Tregs *in vivo*, alone or in combination with PD-1-blockade, thereby improving tumor growth control. We tested this using the MeVa2.1.dOVA melanoma model, which has been previously shown to be responsive to Treg depletion [23]. Moreover, PD-1 blockade alone has no effect in this model allowing us to evaluate a potential synergistic effect with LMK235 [23].

In contrast to our *in vitro* findings, treatment with LMK235 did not alter the frequency of Tregs in the spleen or in the tumor of MeVa2.1.dOVA tumor bearing mice, indicating a lack of effect systemically and in the tumor **(fig. 2A, B)**. In line with our *in vitro* data, there

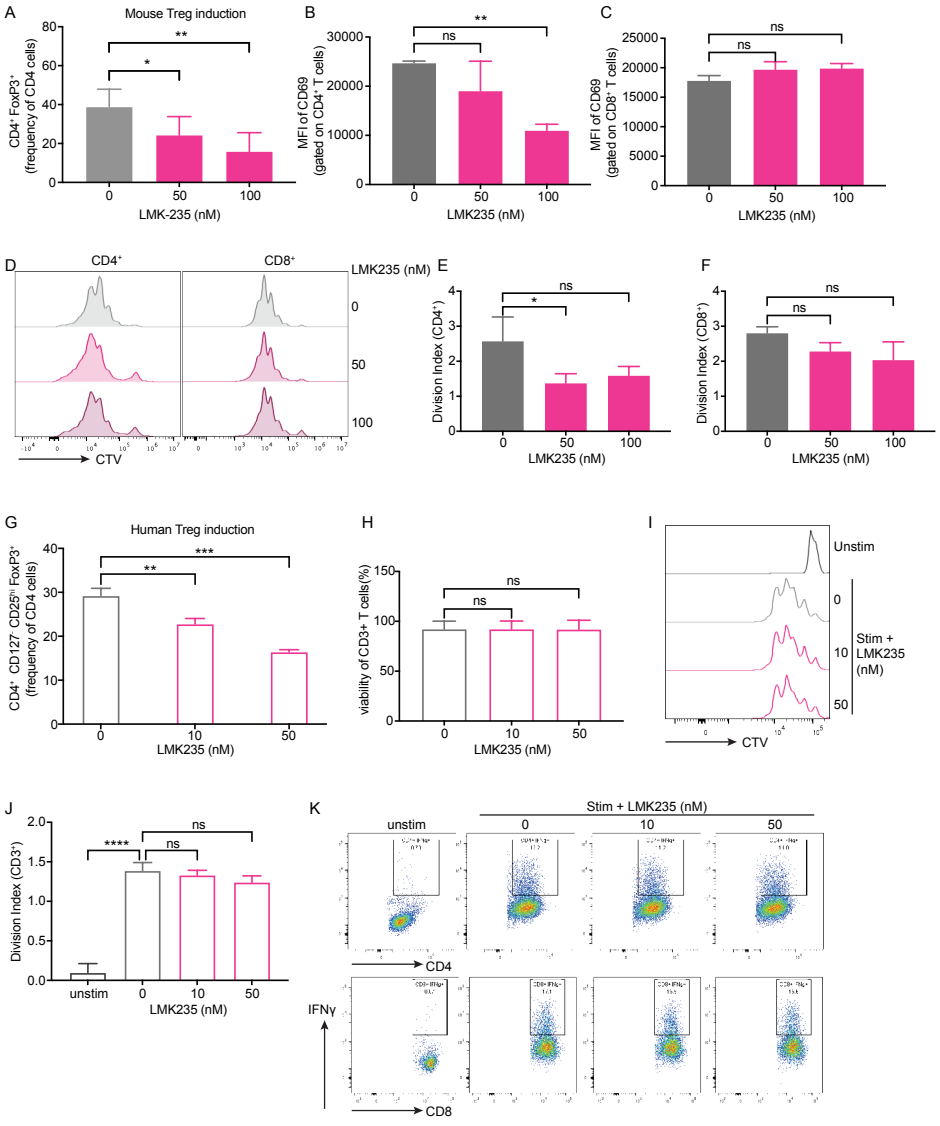

Fig 1: LMK235 modulates Treg induction *in vitro* without altering conventional T cells
(A) Percentage of induced Tregs upon stimulating purified CD4$^+$ T cells obtained from spleen of C57BL/6 mice in the presence of TGFβ and LMK235 (n=at least 3 independent experiments). (B-F) Kit purified CD8$^+$ or CD4$^+$ T cells from spleen of C57BL/6 mice were stimulated for 72h in the presence of LMK235. MFI of CD69 on (B) CD4$^+$ and (C) CD8 T cells at the end of 72h stimulation. (D) Representative CellTrace^{TM} Violet dilution and calculated division index indicating proliferation of (E) CD4$^+$ and (F) CD8$^+$ T cells (n=3 technical replicates). (G) Percentage of induced Tregs after 72h stimulation of purified CD4$^+$ T cells obtained from healthy donor PBMCs in the presence of TGFβ along with indicated doses of LMK235 (n=3 independent experiments). (H-K) PBMCs from healthy donors were stimulated with anti-human CD3 and anti-human CD28 for 72h with different doses of LMK235. (H) Viability of CD3$^+$ T cells, (I) Representative CellTrace^{TM} Violet dilution and (J) calculated division index indicating the proliferation of CD3$^+$ T cells in the PBMC pool (n=3 technical replicates). (K) Representative flow cytometry plots indicating IFN-γ production at 72h (n=2 technical replicates). Statistical significance was estimated by comparing each group to untreated control using one-way ANOVA followed by Dunnett's multiple comparisons test. Error bars represent S.D. ns not significant, * *p*-value<0.05, ** *p*-value<0.01, *** *p*-value<0.001, **** *p*-value<0.0001.

was no effect on the total CD8⁺ T cells or specifically, on the OVA (SIIINFEKL-peptide)-reactive CD8⁺ T cell population in the tumor **(fig. 2C, D)**. Treatment with LMK235 alone was not sufficient to control the growth of MeVa2.1.dOVA tumors **(fig. 2E, F)**. While there was a trend towards an improved tumor growth control **(fig. 2F)** and prolonged survival **(fig. 2G)** upon combining LMK235 with anti-PD-1 in this model, these did not reach statistical significance.

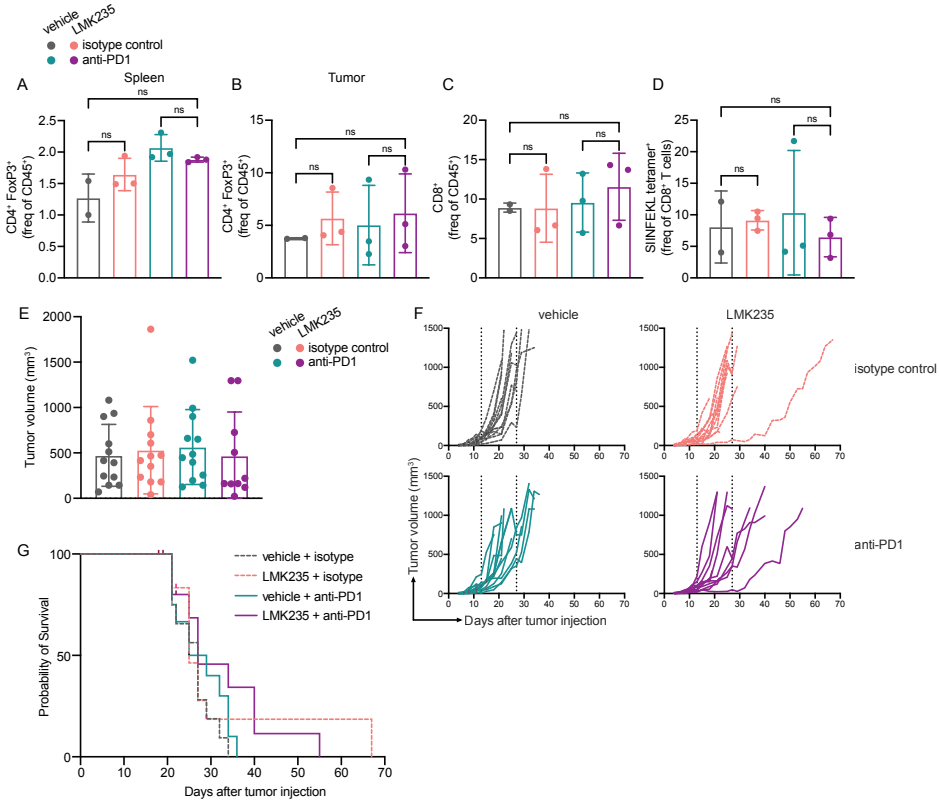

Fig 2: LMK235 does not alter Treg populations *in vivo* in MeVa2.1.dOVA model
(A-G) C57BL/6 mice were injected s.c in the right flank with MeVa2.1.dOVA tumors. Treatment with combinations of LMK235 (i.p, 5mg/kg, daily), isotype control/anti-PD-1 antibodies (i.p, 100 μg, twice per week) began at average tumor size of 100 mm³. (A-D) Flow cytometry analysis of tumor and spleen obtained from satellite mice after 7 days of treatment depicting frequency of CD4⁺ FoxP3⁺ Tregs in the (A) spleen and (B) tumor; frequency of (C) CD8⁺ T cells and (D) SIINFEKL Tetramer⁺ CD8⁺ T cells in the tumor. (E) MeVa2.1.dOVA tumor volume across groups at end-point (day 21 after tumor injection). Each dot represents one mouse. Statistical significance across groups was estimated using one-way ANOVA followed by Sidak's multiple comparisons test. (F) Tumor growth of individual mice across groups (dotted lines indicate treatment duration). (G) Survival of MeVa2.1.dOVA tumor bearing mice until end point tumor volume of 1500 mm³. Truncated events represent early termination due to tumor ulceration. Survival analysis was performed using Log-rank test. Error bars represent SD. ns not significant.

To rule out the possibility that the lack of efficacy of LMK235 was tumor model-specific, we evaluated its efficacy in another melanoma model; B16.dOVA. We first confirmed the responsiveness of this model to Treg depletion **(fig. S1)**. As earlier described [7], a single injection of Treg-depleting anti-CD25 antibody resulted in a significant reduction in the frequency of Tregs in the spleen and in the tumor **(fig. S1A, B)**. This subsequently resulted in a significant increase in the ratio of $CD8^+$ T cells and Tregs in the tumor **(fig. S1C)**. Treg depletion in this model significantly lowered tumor volume and prolonged the survival of mice **(fig. S1D-F)**. Since we observed a higher magnitude of response of B16.dOVA to Treg depletion **(fig. S1E)**, compared with that of MeVa2.1.dOVA model [23], we tested the efficacy of combining LMK235 with anti-PD-1 in B16.dOVA model as well. However, in line with our previous findings, we did not observe any LMK235-mediated effect on Treg population systemically or in the tumor *in vivo* **(fig. 3A, B)**. There was also no effect on the frequency of total $CD8^+$ T cells or on OVA-reactive $CD8^+$ T cells in the tumor **(fig.**

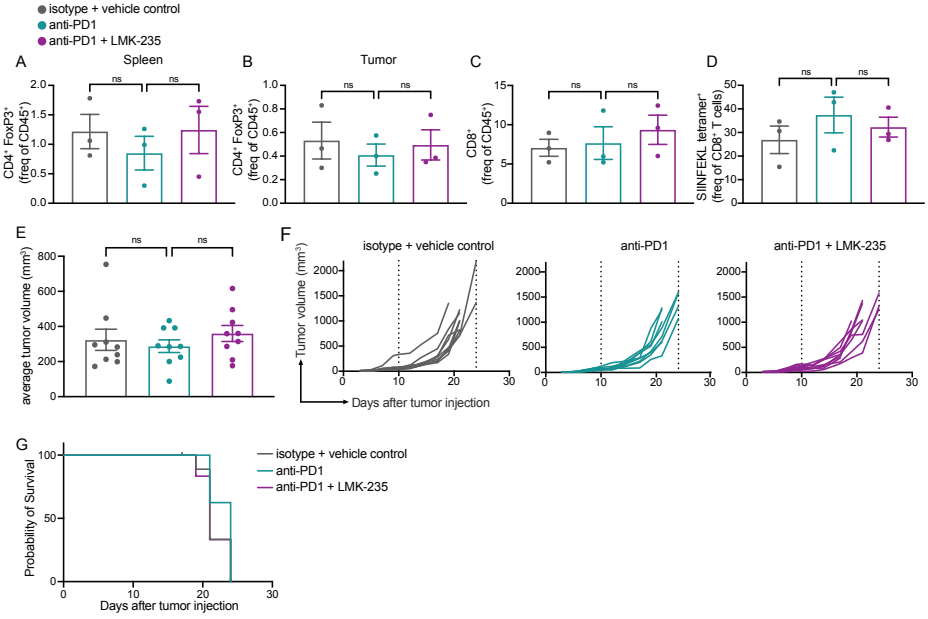

Fig 3: LMK235 does not alter Treg populations *in vivo* in B16.dOVA model
(A-G) C57BL/6 mice were s.c injected in the right and left flanks with B16.dOVA tumors. Treatment with combinations of LMK235 (i.p, 5 mg/kg, daily), isotype control/ anti-PD-1 antibodies (i.p, 100 µg, twice per week) began at average tumor size of 100 mm^3. (A-D) Flow cytometry analysis of tumor and spleen obtained from satellite mice after 5 days of treatment depicting frequency of $CD4^+FoxP3^+$ Tregs in the (A) spleen (each dot represents one mouse) and (B) tumor; frequency of (C) $CD8^+$ T cells and (D) SIINFEKL $Tetramer^+$ $CD8^+$ T cells in the tumor. (E) Average tumor volume of dual flank across groups at end-point (day 17 after tumor injection). Each dot represents average of dual flank for one mouse, unless otherwise stated. Statistical significance across groups was estimated using one-way ANOVA followed by Sidak's multiple comparisons test. (F) Tumor growth of individual mice (average of dual flank) across groups with dotted lines indicating treatment duration. (G) Survival of B16.dOVA tumor bearing mice across groups. Truncated events represent early termination due to tumor ulceration. Survival analysis was performed using Log-rank test. Error bars represent SEM. ns not significant.

3C, D). LMK235, in combination with anti-PD-1 failed to control tumor growth in B16.dOVA model and there was no effect on survival of tumor-bearing mice **(fig. 3E-G)**.

In summary, testing in two different melanoma models, which both are responsive to Treg depletion, LMK235 failed to modulate the Treg population *in vivo,* and subsequently did not improve anti-PD-1- mediated tumor growth control. We therefore conclude that LMK235, alone or in combination with anti-PD-1, has limited therapeutic benefit in melanoma and does not warrant further testing in clinical trials.

Discussion

The presence of Tregs is known to hinder anti-tumor immune responses across a wide range of tumor types, including melanoma [6]. Despite recent advances, clinical targeting of Tregs in melanoma patient tumors remains a hurdle [4,24]. Based on previous data, we hypothesized that specific targeting of HDAC5 could reduce Treg frequencies, improving anti-tumor immune responses. HDAC5 inhibition might also have additional benefits by directly impacting tumor cell proliferation and can modulate anti-tumor immune responses [25], thereby limiting tumor growth.

The promising effect of LMK235 (known to inhibit HDAC4 and HDAC5) *in vitro* prompted us to investigate the effect of this compound on tumor growth *in vivo*. In line with the previous findings [18], we again observed reduced induction of Tregs *in vitro* upon treatment with LMK235, while the proliferation and functionality of $CD8^+$ T cells remained unhindered. However, we could not translate our *in vitro* findings into the *in vivo* setting in two different syngeneic murine melanoma models. While the toxicity associated with pan-HDAC inhibition on immune cell populations [26] was not observed with LMK235, there was limited therapeutic benefit in melanoma mouse models.

We observed a slight, but insignificant, reduction in tumor growth upon combining LMK235 with PD-1 blockade in MeVa2.1.dOVA model. It is therefore tempting to speculate that increasing the dosage or frequency of LMK235 administration might further improve tumor growth control and prolong survival of MeVa2.1.dOVA tumor bearing mice. Although we have not evaluated the target inhibition by LMK235 in our study, the tested dose of LMK235 has been previously shown to increase histone acetylation *in vivo* [27]. Furthermore, we observed reduced proliferation of effector T cells *in vitro* using high doses of LMK235 (data not shown). Such effect of high dose LMK235 on effector T cells would be detrimental for tumor growth control. Hence, we did not increase the dose of LMK235 in our *in vivo* setting.

To our knowledge, the efficacy of HDAC5 targeting using LMK235 *in vivo* has not been previously evaluated. We have shown that the effect of LMK235 on reducing FoxP3

expression *in vitro* could not be recapitulated in tumor-bearing mice. In addition, we found no synergistic effects of LMK235 combined with anti-PD-1 in melanoma models. Future studies aiming to evaluate HDAC5 inhibition in melanoma tumor models should therefore consider alternative compounds.

Materials and methods

Mice strains

C57BL/6JRj (C57BL/6) mice were purchased from Janvier and maintained at the Netherlands Cancer Institute under standard housing conditions. Female mice at the age of 8 weeks were used for experiments involving tumor growth. For *in vitro* experiments, spleen from either male or female mice between the age of 8-15 weeks were used. All animal experiments were performed in accordance with institutional and national guidelines and were approved by the experimental animal committee of the Netherlands Cancer Institute.

Cell lines and culture conditions

MeVa2.1.dOVA tumor cell line [23] was cultured in DMEM/F-12 advanced media (Life Technologies) supplemented with 10% Heat Inactivated Fetal Bovine Serum (HI-FBS), penicillin/streptomycin (pen-strep) (Sigma-Aldrich), and L-glutamine (Gibco™). B16.dOVA cell line was cultured in DMEM media (Life Technologies) supplemented with 10% HI-FBS, and pen-strep (Sigma-Aldrich). Cells were maintained under hygromycin (0.15 mg/mL, Gibco™) selection pressure for ovalbumin (OVA) expression.

PBMC isolation and culture

Peripheral blood monocytes (PBMCs) were obtained from healthy donor buffy coat by separation on a Ficoll (cat: 11743219, ThermoFisher Scientific) density gradient. Where relevant, either Dynabeads™ Untouched™ Human CD4 T Cells Kit (cat: 11346D) or human CD8 T cells kit (cat: 11348D, ThermoFisher Scientific) was used for T cell isolation as per manufacturer's instructions. Cells were cultured in RPMI media substituted with 10% HI-FBS, pen-strep, 100 IU/mL human Interleukin-2 (IL-2) and 50 μM 2-mercaptoethanol.

Where mentioned, CellTrace™ Violet (CTV) (cat: C34557, ThermoFisher Scientific) labelling was performed by incubating cells with CTV (1.25 μM diluted in 1X phosphate buffered saline) at 37 °C for 10 minutes, followed by washing with excess culture media. Cells were stimulated using plate-bound anti-human CD3 (1 μg/ml) (clone: OKT3, cat: 15171506, ThermoFisher Scientific) and soluble anti-human CD28 (1 μg/ml) (clone: CD28.2, cat: 16028985, ThermoFisher Scientific) antibodies for 72h. During stimulation, cells were treated with LMK235 (SelleckChem) at the indicated doses.

Human Treg induction

For Treg induction, kit purified CD4 T cells were obtained as described in the earlier section. Cells were stimulated, using anti-human CD3 and anti-human CD28, as described in the earlier section in the presence of recombinant human TGFβ1 (5 ng/ml) (cat: 100-21, Peprotech) for 72h. In addition, cells were treated with LMK235 at the indicated doses.

Mouse T cell culture and Treg induction

Spleens were harvested from C57BL/6 mice and mashed on a 70 μm cell strainer to obtain single cell suspension. After red blood cell lysis using RBC lysis buffer (cat: 420301, BioLegend), $CD4^+$ T cells were isolated using Dynabeads™ Untouched™ mouse CD4 cell kit (cat: 11415D, ThermoFisher Scientific) as per manufacturer's instructions. Cells were cultured in RPMI media substituted with 10% HI-FBS, pen-strep, 100 IU/mL recombinant mouse IL-2 and 50 μM 2-mercaptoethanol. Cells were stimulated for 72h in the presence of plate-bound anti-mouse CD3 (0.5 μg/ml) (clone: 145-2C11, cat: 16003186, eBioscience™) and soluble anti-mouse CD28 (1 μg/ml) (clone: 37.51, cat: 16003185, eBioscience™) antibodies. During stimulation, recombinant mouse TGFβ1 (3 ng/ml) (cat: 763102, BioLegend) was added to induce Tregs with/without indicated doses of LMK235.

***In vivo* experiments**

For MeVa2.1.dOVA cell line, $0.3x10^6$ cells suspended in 50 μL of PBS and an equal volume of matrigel (cat: 354234, Corning®) were injected s.c into the shaven right flank of C57BL/6 mice. For B16.dOVA tumor cell line, $0.5x10^6$ cells suspended in 50 μL of PBS and an equal volume of matrigel were injected s.c into the shaven right flank or both right and left flanks of C57BL/6 mice as indicated. Tumor outgrowth was followed by two-dimensional caliper measurements of the greatest longitudinal diameter (length) and the greatest transverse diameter (width). Tumor volume was calculated by the modified ellipsoidal formula: Tumor volume = length × width x (width/2). Tumor volumes were compared across groups at the time point at which the first mouse was killed, thereby referred to as the end-point. Mice were terminated at the time at which the tumor volume was $\geq 1500 mm^3$ for single flank tumors or a cumulative tumor volume $\geq 2000\ mm^3$ for dual flank tumors. In addition, if tumors developed ulceration, mice were terminated and therefore censored for survival analysis. Furthermore, satellite mice were terminated at the indicated time-point after start of treatment for flow cytometry analysis.

Treatment began when the average tumor volume of all mice reached $100\ mm^3$. LMK235 (5 mg/kg dissolved in 10% DMSO in phosphate buffered saline, SelleckChem) was administered once daily intraperitoneally (i.p). Anti-PD-1 (clone: RMP1-14, BioXCell) or its relevant isotype control (clone: 2A3, BioXCell) was injected two times per week

i.p (200 µg per dose per mouse). Treatment lasted for 14 days. For depletion of regulatory T cells (Tregs), mice were injected once i.p with anti-mouse CD25 m2a (200 µg per mouse; Evitria) as described [7] on day 3 after tumor injection.

For flow cytometry analysis, single cell suspension was obtained as follows: tumor tissues were extracted and single cell suspensions were obtained by mechanical disruption of the tissue by slicing, followed by 1h enzymatic digestion at 37 °C in medium containing 2 mg/mL collagenase A (Roche) and 1 mg/mL DNAse (Sigma-Aldrich). The suspension was filtered through a 70 µm cell strainer to remove debris. Single cell suspension of splenocytes was obtained by mashing followed by RBC lysis as described in the earlier section.

Flow cytometry analysis

Single cell suspensions were obtained from tissues harvested from *in vivo* study as described in the earlier section. For *in vitro* studies, cells were harvested at the experimental end-point and Fc blocking was performed using 20% HI-FBS in 1X PBS (for human samples) or anti-mouse CD16/CD32 (cat: 14-0161-85, eBioscience™; for mouse samples). This was followed by surface staining for extracellular antibodies. Dead cells were excluded using LIVE/DEAD™ fixable dead cell stain kit (cat: L34968 or L34976, Invitrogen™). For intracellular staining, cells were permeabilized using Intracellular Fixation & Permeabilization Buffer Set (cat: 00552100, eBioscience™) according to the manufacturer's protocol. This was followed by intracellular staining. Antibody details are listed in **table 1**. Cells were finally suspended in FACS buffer (2% HI-FBS in 1X phosphate

Table 1: List of antibodies used for flow cytometry analysis

Name	Clone	Conjugate (Catalogue number)	Manufacturer
Anti-mouse CD4	GK1.5	Brilliant Violet 785™ (100453)	BioLegend
Anti-mouse FoxP3	FJK-16s	APC (17-5773-82) PE (12-5773-82)	eBioscience™
Anti-mouse CD8	53-6.7	FITC (11-0081-85) PerCP-eFluor710 (46-0081-82)	eBioscience™
Anti-mouse CD45.2	104	FITC (109806) PE-Cyanine7 (560696)	BioLegend BD Biosciences
Anti-mouse SIINFEKL H2-K^b tetramer	-	PE	Kind gift from group of Ton Schumacher
Anti-human CD4	SK3	FITC (345768)	BD Biosciences
Anti-human CD25	M-A251	PE (560989)	BD Biosciences
Anti-human CD127	A019D5	Brilliant Violet 650™ (351325)	BioLegend
Anti-human FoxP3	236A/E7	APC (174777-41)	eBioscience™
Anti-human CD3	UCHT1	AF647 (557706)	BD Biosciences
Anti-human CD8	SK1	PE (345773) APC (555369)	BD Biosciences
Anti-human IFNγ	B27	APC (554702)	BD Biosciences

buffered saline) and acquired using LSR Fortessa™ (BD Biosciences) or Intellicyt® iQue Screener PLUS (Sartoruis). Flow cytometry data analysis was performed using FlowJo (version 10.7.1, BD Life Sciences).

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Graphpad Software Inc., v9). Two-tailed Student's t test was used to compare means across two groups. Mean values across more than two groups were compared using one-way ANOVA followed by correcting for multiple comparisons using Sidak's test or Dunnett's test, as described in the respective figure legend. Survival analysis was done using Log-rank test. p-value<0.05 was considered statistically significant.

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Supplementary figure

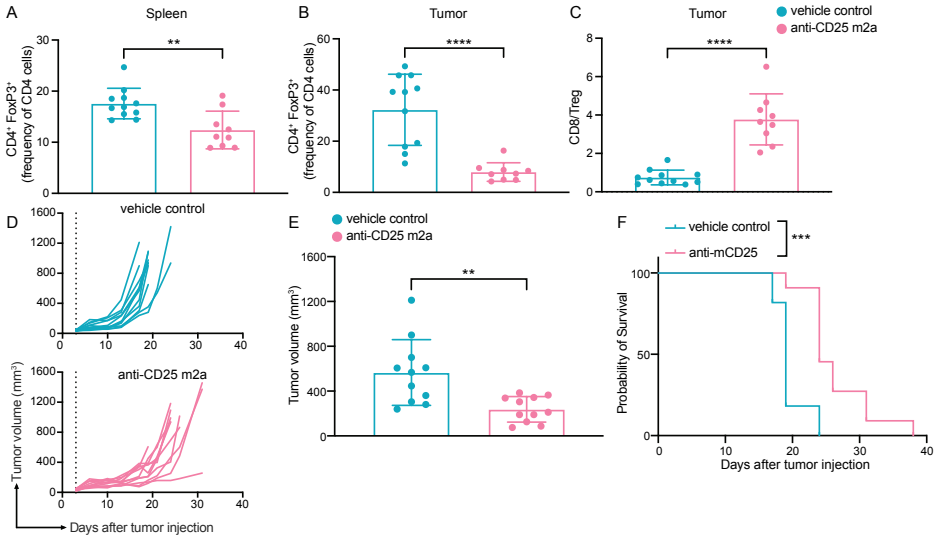

Fig S1. (Supplement to fig 3): B16.dOVA as a suitable model to evaluate efficacy of Treg targeting agents
(A-F) C57BL/6 mice were injected s.c with B16.dOVA tumor cells on the right flank. On day 3 after tumor injection, mice received vehicle control or Treg depleting anti-mouse CD25 m2a antibody (i.p, 100 μg). (A-C) Flow cytometry analysis indicating frequency of Tregs in (A) spleen and (B) tumor and (C) ratio of $CD8^+$ T cells to Tregs in tumors at end-point. (D) Tumor growth across days in individual mice. Dotted line indicates treatment day. (E) Average tumor volume across groups on day 17 after tumor injection (last time point at which all mice across groups were alive). Each dot represents one mouse. Groups were compared using two-tailed unpaired Student's t-test. Error bars represent SD. (F) Survival of B16.dOVA tumor bearing mice treated with control or anti-mouse CD25 m2a antibody. Statistical significance was estimated using Log-rank test. ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001.

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Affiliations:
[1] Division of Molecular Oncology and Immunology, The Netherlands Cancer Institute
[2] Department of Medical Oncology, The Netherlands Cancer Institute
[3] Department of Internal Medicine III, Hematology and Medical Oncology, University Medical Center of Regensburg
[4] Oncode Institute

Correspondence:
Prof. dr. Christian U. Blank
c.blank@nki.nl