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Permalink

<https://escholarship.org/uc/item/0hf5g50t>

Journal

Cancer Research

ISSN

0008-5472

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Publication Date

2026-08-20

DOI

10.1158/0008-5472.can-25-5784

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Peer reviewed

Mathematical and Mouse Models Identify Regulatory T Cell Influx as A Key Determinant of Acquired Resistance to PD-1 Immunotherapy

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Running title:

Mathematical modeling of tumor immunosuppression

Keywords: mathematical modeling, checkpoint blockade immunotherapy, CD8⁺ T cells, Tregs, dendritic cells (DCs).

CONFLICT OF INTEREST

None reported.

ABSTRACT

The immune system can eradicate cancer, but various immunosuppressive mechanisms active within a tumor curb this beneficial response. However, unraveling the effects of multimodal interactions between tumor and immune cells and their contributions to tumor control using an experimental approach alone is time- and resource-intensive. To identify the critical immunological features associated with tumor control and escape, we built a mechanistic, structurally identifiable mathematical model of the interactions between CD8⁺ T cells, regulatory T cells (Tregs), dendritic cells, and tumor cells deeply rooted in current biological concepts. The model captured Treg accrual occurring after checkpoint blockade immunotherapy. After successfully fitting the mathematical model to experimental data from an immunogenic melanoma mouse model with acquired resistance to PD-1 immunotherapy and ensuring that the model was practically identifiable, hundreds of parameter sets were generated, each of which fit the data well and represented a unique 'virtual mouse' to capture variability across individuals. The model indicated that the initial tumor and immune conditions instruct cancer control or progression and that optimal initial ratios of immune cells exist that result in improved tumor control. The model further predicted that the Treg influx into the tumor is a key determinant of resistance to PD-1 immunotherapy. Experimental studies validated all these predictions from the model. Overall, this integrated approach of modeling and experimental validation identified crucial determinants of resistance to PD-1 immunotherapy and can be used to guide the development of more effective therapeutic strategies.

STATEMENT OF SIGNIFICANCE

The combination of mathematical models and immediate experimental validation enables time- and resource-efficient identification of Treg influx into the tumor as a crucial mechanism of secondary resistance to PD-1 immunotherapy.

INTRODUCTION

Checkpoint blockade immunotherapy (CBI) revolutionized the management of advanced cancer patients. CBI aims to remove the negative feedback mechanisms (checkpoints) that evolved to prevent autoimmunity yet also curb antitumor immunity. In metastatic melanoma, PD-1 blockade resulted in 32% of patients exhibiting progression-free survival three years after diagnosis, and its combination with α CTLA-4 raises this percentage to 39% (1). Crucially, benefits are durable: the patients who were progression-free at the three-year time point had >95% probability of being alive after 10 years (1). CBI is now approved to treat more than 20 clinical indications.

Despite these successes, 69-77% of patients progress within 10 years from the start of CBI (1). While therapy failure can be due to mutations in tumor cells that make them refractory to treatment, another leading cause of therapy resistance is the occurrence of immunosuppressive reactions within the tumor environment (2). “CBI-mediated immunosuppression” is an emerging concept emphasizing that, in addition to triggering anti-tumor immunity, CBI also unleashes immunosuppressive reactions that may decrease efficacy. Alternative checkpoint receptors including Tim3 and Lag3 have been studied as immunosuppressive adaptations to CBI (3), paving the way for their blockade to improve the therapeutic efficacy of PD-1 blockade (4,5). Macrophages are the most abundant cell type in the tumor environment, and increased interferon-gamma ($\text{IFN}\gamma$) availability after CBI instructs pro-inflammatory functions in macrophages that oppose tumor growth. Nonetheless, blockade of the PD-1/PD-L1 axis also triggers immunosuppressive responses in tumor-associated macrophages, including the expression of IL-6 and IDO (6,7). However, the most studied immune adaptation to CBI is the enhancement of $\text{CD4}^+\text{FOXP3}^+$ regulatory T cells (Tregs). Seminal papers from Drs. Sakaguchi and Nishikawa suggested that PD-1 blockade directly increases the proliferation and immunoregulatory function of tumor Tregs by enhancing T cell receptor (TCR) signaling (8,9). Moreover, our group recently showed that, in immunogenic melanoma, Treg accumulation after PD-1 blockade was due to IL-2 secretion by tumor-associated CD8^+ T cells (10). Notably, CTLA-4 blockade also results in the accrual of tumor-associated Tregs in melanoma patients (11). Mechanistically, α CTLA-4-mediated tumor Treg accrual is due to increased co-stimulation during cognate interactions between Tregs and dendritic cells (DCs) (12).

The extensive investigations of the antitumor immune response continue to provide new mechanistic details of the complexities of the tumor-immune system. Thus, we reason that unraveling the effects of multimodal

interactions between tumor and immune cells and their contributions to tumor control using an experimental approach alone would rapidly exceed the available time and resources. Mathematical modeling has been used to navigate complex parameter spaces, including immune reactions within the tumor environment (13–15). To expedite and guide experimental discovery, we built a mechanistic, structurally identifiable mathematical model of the interactions between CD8⁺ T cells, Tregs, DCs, and tumor cells that captures CBI-mediated tumor Treg accrual after both α PD-1 and α CTLA-4 monotherapy, a distinctive feature not seen in other mathematical models. We calibrated the model using experimental data on D4M-S, an immunogenic melanoma mouse model that progresses after a transient response to PD-1 CBI, and we verified that the model was practically identifiable. Our simulations indicated that tumor growth was profoundly affected by the number of tumor cells engrafted at early time points and by complex alterations in individual immune populations. We verified these predictions *in vivo* by transferring graded numbers of tumor cells and analyzing mice bearing the natural microbiota, which primes the antitumor immune response. Crucially, when we simulated PD-1 blockade, we identified Treg influx as a key parameter contributing to acquired resistance, and we validated this prediction in mice whose Tregs could not leave tumor-draining lymph-nodes. Our work illustrates how mathematical modeling can guide experimentalists in conducting focused experiments to understand therapeutic response, identify determinants of CBI resistance, and refine treatment strategies.

MATERIALS AND METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

D4M.3A and D4M.3A H2B SIINFEKL Cer (D4M-S) melanoma cells were obtained from Thorsten Mempel (Massachusetts General Hospital) and cultured in DMEM supplemented with 10% fetal calf serum (GeminiBio) under 37 °C / 5% CO₂ conditions. These cell lines are derived from male mice and have not been authenticated. We created frozen batches of each cell line that lasted for several years. Each batch was tested for mycoplasma contamination using the MycoAlert Detection Kit from Lonza, and found negative (latest test dates 5/11/2026 and 2/22/2024 for D4M and D4M-S, respectively). Cells were used within three passages from thawing, and then discarded. Details on cell lines are also reported in **Supplementary Table S1**.

Mice

Foxp3^{DTR}, CD45.1, and C57BL/6J mice were purchased from The Jackson Laboratory. C57BL/6NTac mice were purchased from Taconic Biosciences. Wildling mice were generated by rederivation of C57BL/6NTac embryos to feral dams, and thus bear the natural microbiome, mycobiome, and virome at all barrier sites (16). We obtained Wildling mice from Dr. Barbara Rehermann (NIH). Details on mouse models are also reported in **Supplementary Table S1**. Mice were enrolled in experiments at 6-10 weeks of age. D4M-S cells are originally from male mice, making them artificially immunogenic in female recipients. Because of this, we implanted D4M-S melanomas only in male mice. Mice were bred, housed, enrolled in experiments, and euthanized according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of MD Anderson Cancer Center (00002170-RN00) and the University of California Irvine (AUP25-094).

METHOD DETAILS

Model development

The mathematical model is a set of ordinary differential equations based on the interaction diagram depicted in **Fig. 1**. The full model presented in **Supplementary Fig. S1** is structurally identifiable. The model is practically identifiable when parameters K_{DN} , ρ_{EN} , K_{EN} , δ_E , and r_R are set to zero and K_N is fixed at 10^5 (red parameters in **Supplementary Fig. S1**). Model parameters are detailed in **Table 1**.

Equation for Tumor Cells

The rate of change in the number of tumor cells (N) is given by:

$$\frac{dN}{dt} = r_N N - \frac{d_N T_E N}{N + K_N}. \quad (1)$$

The first term ($r_N N$) represents tumor growth. The second term $\left(\frac{d_N T_E N}{N + K_N}\right)$ represents tumor cell death by CD8⁺ T effector cells and uses a Michaelis-Menten-like term to represent that the killing capacity of effector cells is limited (17). In the equations that follow, we let $\omega = \frac{d_N T_E N}{N + K_N}$ be multiplied by T_E and T_R associated parameters to represent the effects of the tumor antigen released upon the death of the tumor.

Equation for Inactive Dendritic Cells

The rate of change in the number of inactive dendritic cells (D_I) is given by:

$$\frac{dD_I}{dt} = \frac{\delta_I N}{1 + K_D N} + \sigma_R T_R D_A - \rho_{DE} T_E D_I - \frac{\rho_{DN} D_I N}{1 + K_{DN} N} - d_I D_I. \quad (2)$$

The first term $\left(\frac{\delta_I N}{1 + K_D N}\right)$ represents that tumor chemokines recruit D_I into the system; however, there is a saturating effect of this recruitment, modeled using a Michaelis-Menten-like term. The second term ($\sigma_R T_R D_A$) represents deactivation of DCs by CTLA-4 on Tregs. The third term ($\rho_{DE} T_E D_I$) and fourth term $\left(\frac{\rho_{DN} D_I N}{1 + K_{DN} N}\right)$ represent D_I activation by IFN γ produced by T effector cells and by tumor antigen acquisition, respectively. We model the activation of DCs by tumor antigen with a Michaelis-Menten-like term to represent that there is a limit to the number of antigens DCs can pick up. The last term ($d_I D_I$) is the natural death of D_I .

Equation for Active Dendritic Cells

The rate of change in the number of active dendritic cells (D_A) is given by:

$$\frac{dD_A}{dt} = \rho_{DE} T_E D_I + \frac{\rho_{DN} D_I N}{1 + K_{DN} N} - \sigma_R T_R D_A - d_A D_A. \quad (3)$$

The first three terms represent that D_A can be deactivated by CTLA-4 on Tregs ($\sigma_R T_R D_A$) and become activated by IFN γ produced by T_E ($\rho_{DE} T_E D_I$) or by acquiring tumor antigen $\left(\frac{\rho_{DN} D_I N}{1 + K_{DN} N}\right)$. The last term ($d_A D_A$) represents the death rate of active DCs.

Equation for CD8⁺ T Effector Cells

The rate of change in the number of CD8⁺ T effector cells is given by:

$$\frac{dT_E}{dt} = \frac{T_E D_A^2 \rho_E \omega}{(1 + K_R T_R)(1 + K_A T_E D_A)} + \frac{\rho_{EN} T_E N}{1 + K_{EN} T_E N} + \delta_E \omega + \delta_{EN} N - \frac{d_E T_E + d_{Ex} T_E N}{1 + \beta \alpha_E I}. \quad (4)$$

The first term $\left(\frac{T_E D_A^2 \rho_E \omega}{(1 + K_R T_R)(1 + K_A T_E D_A)}\right)$ describes that D_A can activate T_E via antigen presentation. This activation is inhibited by Tregs $\left(\frac{1}{1 + K_R T_R}\right)$ as well as by PD-L1 present on D_A , which is upregulated by $IFN\gamma$ that is produced by T_E $\left(\frac{1}{1 + K_A T_E D_A}\right)$. The second term $\left(\frac{\rho_{EN} T_E N}{1 + K_{EN} T_E N}\right)$ represents that the tumor is also capable of presenting antigen to T_E and triggering activation, which is also inhibited by PD-L1 present on the tumor $\left(\frac{1}{1 + K_{EN} T_E N}\right)$. The third term $(\delta_E \omega)$ and the fourth term $(\delta_{EN} N)$ represents the influx of primed T_E from the lymph node into the system, dependent on antigen shed during tumor cell death and on tumor chemokines, respectively. The last term $\left(\frac{d_E T_E + d_{Ex} T_E N}{1 + \beta \alpha_E I}\right)$ describes how T_E die $(d_E T_E)$ or become exhausted $(d_{Ex} T_E N)$ and how the amount of IL-2 that T_E can absorb prolongs their functionality and lifespan $\left(\frac{1}{1 + \beta \alpha_E I}\right)$.

Equation for Regulatory T Cells

The rate of change in the number of Tregs (T_R) is given by:

$$\frac{dT_R}{dt} = T_R D_A^2 (r_R + \rho_R \omega) + \delta_R \omega + \delta_{RN} N - \frac{d_R T_R}{1 + \beta \alpha_R I}. \quad (5)$$

The first term $(T_R D_A^2 (r_R + \rho_R \omega))$ describes how Tregs are activated by D_A through self-antigens $(r_R T_R D_A^2)$ or via tumor antigens $(\rho_R \omega T_R D_A^2)$. The second term $(\delta_R \omega)$ and the third term $(\delta_{RN} N)$ assume there is an influx of T_R from the lymph node into the system dependent on antigen shed during tumor cell death and tumor chemokines, respectively. The last term $\left(\frac{d_R T_R}{1 + \beta \alpha_R I}\right)$ represents T_R death $(d_R T_R)$ and how IL-2 can prolong T_R lifespan $\left(\frac{1}{1 + \beta \alpha_R I}\right)$.

Equation for IL-2

The rate of change in the concentration of IL-2 (I) is given by:

$$\frac{dI}{dt} = \gamma_P T_E - \gamma_C I (\alpha_E T_E + \alpha_R T_R). \quad (6)$$

The first term $(\gamma_P T_E)$ describes that IL-2 is produced by T_E , and the second term $(\gamma_C I (\alpha_E T_E + \alpha_R T_R))$ represents that IL-2 is consumed by T_E and T_R . We assume IL-2 to be in local equilibrium as there is limited diffusion of IL-2 around its producers (18), which gives

$$I = \frac{T_E}{\gamma' (\alpha_E T_E + \alpha_R T_R)}, \quad (7)$$

where $\gamma' = \frac{\gamma_C}{\gamma_P}$.

Structural identifiability

To assess the structural identifiability of our mathematical model, we used the Julia software package `StructuralIdentifiability.jl` (<https://docs.sciml.ai/StructuralIdentifiability/stable/>).

Data used for model parametrization

Tumor Growth Data

We measured D4M-S tumor growth at six time points in five mice. The first tumor measurement was taken on day 6 using calipers. All tumors were able to be measured using calipers, so no data was censored. For a list of previously published tumor growth data, please refer to **Supplementary Table S1**.

Immune Cell Dynamics Data

We quantified the number of immune cells present in the tumor over time. In this experiment, mice were implanted with D4M-S tumors, and 4-5 mice were sacrificed at each timepoint (days 4, 7, 10, 13, 17, and 32). The number of immune cells per mg of tumor was quantified using flow cytometry.

Unit Conversions

Since our tumor data was measured in volume (mm^3) or mass (mg) and our mathematical model tracked the number of cells over time, we converted the data from tumor volume or mass to the number of tumor cells by assuming that 10^5 tumor cells is equivalent to 1 mg of tumor or 1 mm^3 of tumor, according to reference (19). For immune cell data points without a corresponding tumor measurement on the same day the immune cells were quantified, we extrapolated or interpolated the tumor size and used that value for the conversion.

Initial Conditions

We estimated the initial conditions using the experimental tumor growth and immune cell dynamics data. All mice were injected with 2 million tumor cells on day 0. However, we cannot be certain that all 2 million were successfully implanted, so we used day 4 as our initial simulated time point. We used the average number of immune cells quantified on day 4 as the initial condition for each immune cell type. The first tumor measurement was taken on day 6, so we estimated the tumor size on day 4 (tumor initial condition) using linear interpolation between day 0 (2 million cells injected) and the first measured tumor size on day 6. Although the tumor size may

increase exponentially at the early stages of growth, both linear and exponential interpolation yield approximately the same result over the short time scale used (day 0 to day 6). The two methods differ by only about 11.85% in their predicted number of tumor cells at day 4.

Model parametrization

We first worked to parameterize the model by searching the literature for available data that allowed us to infer parameter values (**Table 1**). For parameters we were unable to determine from the literature, we used the constrained optimization function '*fmincon*' in MATLAB to minimize the weighted least squares residual chi-square cost function

$$\chi^2(\mathbf{p}) = \sum_{i=1}^n \left(\frac{y_i^{obs} - y_i^{sim}(\mathbf{p})}{\sigma_i} \right)^2$$

where $\mathbf{p}$ is the set of parameter values, y_i^{obs} is the average of the log-transformed tumor and immune cell experimental data at the i^{th} time point, y_i^{sim} is the simulated tumor-immune model evaluated at the i^{th} time point, σ_i is the standard deviation of the i^{th} time point, and n is the total number of time points measured. Given that we did not have biological ranges for many of the estimated parameters, we let the bounds for '*fmincon*' be $[1e-19, \text{Inf}]$. In total, we estimated 21 parameters in the structurally identifiable model and 15 parameters in the practically identifiable model.

Practical identifiability

To assess the practical identifiability of our mathematical model, we performed profile likelihood analysis (20). In short, a model is practically identifiable if the confidence interval (CI) for each parameter is finite and bounded on both sides (21). We first calibrated the structurally identifiable model to data and used the parameter set to obtain the minimized cost function ($\chi^2 = 15.6$). We defined the threshold for a confidence interval corresponding to a 95% confidence level as $\chi_{min}^2 + 3.84$, where 3.84 is the 95th percentile of the chi-squared distribution with one degree of freedom. We evaluated the profile likelihood of one parameter at a time by fixing that parameter across a range of values - first using a large range and then narrowing in on the region of interest. For each fixed value, we re-optimized the model fit over all remaining parameters to determine the lowest achievable cost function. If the resulting profile exceeded the threshold on both sides of the minimum χ^2 value or was bound at

zero on the left side while also crossing the threshold on the right side of the minimum χ^2 value, then we concluded that the parameter was practically identifiable. One parameter was deemed practically unidentifiable in the structurally identifiable model. Further, five of the parameters that were practically identifiable when bounded by zero achieved the best model fit when their values were near zero. We either fixed these parameters at zero, or in the case of K_N , fixed the value to be 10^5 to model the fact that very small tumors may not be efficiently recognized by the immune system (22). These parameters are highlighted in red in **Supplementary Fig. S1** and **Table 1**. We re-optimized the fit of the updated model and repeated the profile likelihood analysis. We found that all parameters in the updated model were now practically identifiable.

Latin hypercube sampling

To generate additional parameter sets ('virtual mice') to use for analysis, we utilized the confidence intervals identified for each estimated parameter in the practically identifiable model to perform Latin hypercube sampling (LHS) using the '*lhsdesign*' function in MATLAB. We generated 1 million parameter sets and identified sets that fell within one standard deviation of the last time point for each cell type. Additionally, we required that the parameter sets fell within one standard deviation of the second to last time point of the T effector cell data, as this is where the number of T effector cells peaked in experimental data. This resulted in 342 parameter sets that met the inclusion criteria. We note that the low acceptance rate of parameter sets is a natural consequence of how the CIs are constructed. Profile likelihood analysis fixes one parameter and allows all the others to adjust to compensate, thereby removing the coupled, multidimensional relationships that exist among the parameters. As a result, each CI reflects the plausible range for a single parameter in isolation rather than the joint behavior of the full parameter set. Therefore, the 15-dimensional parameter space resulting from the LHS spans every combination of individual parameter values within their respective CIs. The region that actually reproduces the data, however, is a much smaller subset of this space—one shaped by the multidimensional relationships that the univariate CIs do not fully capture. The mismatch between the broadly defined sampled space and the narrow, truly data-consistent region is a main driver of the low acceptance rate.

The effect can be illustrated by varying the width of the CIs. Using 99% CIs, which enlarge the range of each parameter and therefore expand the 15-dimensional space, we could accept only 22 of the one million parameter sets generated by LHS (a 0.002% acceptance rate). Note that these parameter sets are newly generated, as the

CI has changed. In contrast, using 90% CIs shrinks the range of each parameter and the overall sampling space, allowing us to accept 996 out of one million parameter sets (a 0.1% acceptance rate). This confirms that the larger the sampled space, the smaller the fraction of sets that fall within the subregion consistent with the data, and vice versa.

Further, an analysis of the pairwise linear correlations between parameters indicates that only three pairs of parameters (δ_I and K_D ; K_R and ρ_E ; d_{Ex} and δ_{EN}) showed statistically significant moderate correlations (Pearson's correlation coefficients: 0.6, 0.64, and 0.66, respectively). In addition, using principal component analysis, we find that each principal component explained less than 14% of the variance in the parameter sets. Altogether, these results suggest that the multiparameter relationships are nonlinear and could exhibit higher-order correlations. A full investigation of the complex relationships between the parameters is beyond the scope of this paper.

Simulating immunotherapy treatments

Immunogenicity Reduction

To simulate the change in immunogenicity between D4M-S and D4M cell lines, we decreased the parameters associated with immunogenicity ($\rho_E, \rho_R, \delta_{EN}, \delta_R$) by 90%.

CD8 Depletion

Mice were treated with FTY720 (a drug that inhibits the efflux of lymphocytes from the lymph node) and α CD8 to deplete effector T cells beginning on day 8. The mice were sacrificed on day 14 and tumor weight analyzed. We simulated this experiment in our mathematical model by decreasing all T cell influx terms by 65% ($\delta_{EN}, \delta_R, \delta_{RN}$) and removing all T_E cells in the simulation on day 8. Simulations ended on day 14 and tumor weight was calculated.

Treg Numbers after α PD-1 Treatment

To identify the number of Tregs per mg of tumor in our model after α PD-1, we simulated α PD-1 treatment from day 12 to day 18 (as per reference (10)) by reducing the PD-L1 parameter (K_A) by 18%. The numbers of Tregs and tumor cells on day 18 were then calculated to determine the number of Tregs per mg of tumor.

Treg Depletion

To simulate Treg depletion, we decreased the Treg initial condition by 90%.

Treg Numbers after α CTLA-4 Treatment

To identify the number of Tregs per mg of tumor in our model after α CTLA-4, we simulated α CTLA-4 treatment from day 12 to day 18 by reducing the CTLA-4 parameter (σ_R) by 50%, which resulted in a tumor Treg increase similar to what we observed experimentally (12). The numbers of Tregs and tumor cells on day 18 were then calculated to determine the number of Tregs per mg of tumor.

Simulation of PD-1 CBI

Mice were treated with α PD-1 from day 12 or 13 to day 40 or 42. To simulate this treatment, we reduced the PD-L1 parameter (K_A) by 8%, 18%, or 28% from day 12 to 42. Simulations were categorized as “Complete Responders” if the tumor size reached a threshold of 0.1 cells or less on day 90 and “Non-Responders” otherwise.

Reduction of Individual Parameters after α PD-1 Treatment

We simulated α PD-1 treatment by reducing the PD-L1 parameter (K_A) by 18% from day 12 to 42. Individual parameters were decreased by 2% throughout the entire simulated time. Simulations were categorized as “Tumor Death without Treatment” if the 2% parameter reduction alone resulted in a tumor size of 0.1 cells or less on day 90, “Complete Responders” if the tumor size reached a threshold of 0.1 cells or less on day 90 with the combined simulated therapy, and “Non-Responders” otherwise.

Tumor growth in vivo and antibody treatments

Mice received a subcutaneous injection of 1×10^6 D4M cells or 2×10^6 D4M-S cells. In the indicated experiments, 2×10^5 D4M-S cells were injected. To perform flow cytometry analysis, we injected two tumors per mouse 1 cm off the midline on the abdomen. PD-1 blockade was achieved by the administration of 200 μ g α PD-1 (29F.1A12) i.p. every other day. We injected 200 μ g rat IgG i.p. as an isotype control. Tumor growth after CD8 depletion was conducted as per reference (10). Briefly, 8-day-old D4M-S tumors were treated with FTY720 (1mg/kg i.p. every other day) and α CD8 or rat IgG antibodies (300 μ g i.p. every third day). Tumor weight was determined six days after treatment started.

Tumor measurement

For tumor growth studies, we implanted only one tumor and measured three times a week with an electronic caliper. Tumor volume was estimated using the formula $0.5 \times a \times b^2$, where a is the maximum and b is the perpendicular tumor diameter. Immunotherapy administration ended on day 40 after tumor injection. When tumors reached a maximum diameter of > 15 mm or both diameters > 10 mm, mice were sacrificed.

Bone marrow chimeras

We created bone marrow chimeras by irradiating mice at 750 rads (X-ray source). Irradiation doses were established to ensure engraftment of $8-10 \times 10^6$ donor bone marrow cells with minimal lethality. To investigate the impact of early Treg influx on tumor growth, we reconstituted CD45.1 hosts with a 1:1 mixture of Foxp3^{DTR} and either S1pr1^{ff} or S1pr1^{ff} x UBC^{creERT2} bone marrow (C57BL/6 background), generously provided by Dr. Susan Schwab (New York University). Bone marrow chimeras were enrolled in experiments two months after transplantation to ensure hematopoietic reconstitution. Chimeras were given drinking water with 40mg/ml Sulfamethoxazole and 8mg/ml Trimethoprim for 1 month after irradiation to prevent opportunistic infections. We confirmed engraftment by flow cytometry analysis of blood. Tamoxifen treatment consisted of oral gavage (15 mg in 75 μ l EtOH + 425 μ l corn oil) on day -5 followed by four i.p. daily injections of 2 mg (in 10 μ l EtOH + 40 μ l corn oil). Chimeras were injected i.p. with a 25 μ g/kg attack dose of diphtheria toxin (Calbiochem) on day 0 followed by a 5 μ g/kg dose on days 1-3 (23). We verified depletion of Foxp3^{DTR} Tregs by diphtheria toxin through flow cytometry analysis of blood.

Flow cytometry

Tumors were finely minced and digested for 30 min at 37 °C using DMEM 10% FCS with 1.5 mg/ml Collagenase IV (Worthington) and 50 U/ml DNase I (Roche). All samples were filtered through a 40 μ m strainer.

We stained 8×10^6 cells and identified dead cells with Live-or-dye 750/777 (1:1000), diluted in PBS, for 30 min at 4 °C. On an aliquot of cells, we measured the absolute cell numbers using AccuCheck flow cytometry counting beads (Invitrogen). The remainder of the cells were further processed. To decrease nonspecific Ab binding, cells were incubated with 5 μ g/ml FcBlock for 10 min at 4 °C. Extracellular antibody staining was carried out at 4 °C for 20 minutes in FACS buffer (PBS, 0.5% BSA, 2 mM EDTA). Cells were then permeabilized using the Foxp3 fixation-permeabilization buffer (Invitrogen), while intracellular staining was performed at 4 °C for 30 min in Foxp3

wash buffer. We acquired the samples on a NovoCyte Quanteon flow cytometer and analyzed the data using FCS Express and FlowJo.

T cell and APC panel: To enumerate and identify tumor specific T cells we used α CD45, α CD8, α CD4, α Foxp3, and a SIINFEKL tetramer. To evaluate exhaustion, we stained with α CD366 (Tim3), and α TCF1. To count APCs, we used a lineage cocktail of antibodies against TCR β , B220, Gr-1, and NK1.1, as well as α H-2K^b (MHC-I), α IA/IE (MHC-II), α CD135, and α CD11c. After gating for live CD45⁺ Lin⁻ IA/IE⁺ cells, DCs were gated as CD11c⁺ CD135⁺.

Cytokine panel: To evaluate the function of exhausted tumor T cells, we stimulated them with plate-bound α CD3 ϵ (10 μ g/ml) and α CD28 (10 μ g/ml) in the presence of brefeldin A (5 μ g/ml) for eight hours at 37 °C. Cells were stained with α CD45, α CD8, α CD4, α Foxp3, α CD366 (Tim3), α TCF1, α IFN γ , and α TNF antibodies.

Chimera reconstitution panel: To verify reconstitution in the S1PR1 chimeras, we stained ACK-lysed blood with α CD4, α CD8, α CD25, α CD45.1, α CD45.2, and identified Foxp3^{DTR} Tregs with GFP.

An exhaustive list of the antibodies used in this study is shown in **Supplementary Table S1**.

Statistical analysis

The extension of survival in mice that did not reject tumors followed a normal distribution (Shapiro-Wilk test); therefore, we calculated p-values using ANOVA. Tumor growth data were also distributed normally (QQ plot estimation on a square root transformation of the data in the TumGrowth package), allowing us to calculate p-values using a linear mixed effect model. In the other cases, we employed Mann-Whitney U, Wilcoxon signed-rank, and Kruskal-Wallis tests. To compare survival curves, the log-rank test was used. P values less than 0.05 were considered statistically significant.

Data Availability

All data generated in this study are available on GitHub (<https://github.com/ImmunoimagingLab/Sousa-CancerResearch-2026>) and from the corresponding author upon request.

Code Availability

All code used for this analysis is available on GitHub (<https://github.com/ImmunoimagingLab/Sousa-CancerResearch-2026>).

RESULTS

A mechanistic mathematical model of key tumor immunity features.

We developed a mathematical model of anti-tumor and immunosuppressive mechanisms occurring within the tumor immune microenvironment to study the immune response to PD-1 inhibition. PD-1 binding to PD-L1 and PD-L2 recruits phosphatases in proximity to TCR and co-stimulatory receptors, decreasing signaling efficiency and T cell activation (24,25). The main target of PD-1 inhibition is CD8⁺ T cells (26), yet other components of the tumor immune environment, including Tregs (8,10) and DCs (27), are directly or indirectly affected. For these reasons, we developed a mathematical model encompassing CD8⁺ T cells, Tregs, and DCs at different stages of activation. While the tumor is heterogeneous, there exist niches whereby CD8⁺ T cells, Tregs, and DCs are well-mixed with tumor cells (10), allowing us to specifically model mechanisms occurring within these niches using ordinary differential equations (ODEs).

To build our system of ODEs, we made assumptions rooted in current knowledge of the tumor immune environment (**Fig. 1**). First, we assumed that without immune cells, tumor cell (N) accumulation was exponential (28). The tumor environment produces chemokines attracting inactive DCs (DI), tumor antigen-specific CD8⁺ effector T cells (TE), and Tregs (TR) to the tumor site. Tumor cells also present antigens, which activate CD8⁺ effector T cells, and in turn kill tumor cells. As per Breart et al., 2008, we assume that the killing capacity of CD8⁺ T cells is limited (17). Upon death, tumor cells release tumor-associated antigens, which contribute to DC activation (29). DCs can also be activated by IFN γ produced by CD8⁺ T cells (27). Active DCs (DA) present antigens to both CD8⁺ T cells (30) and Tregs (12), activating them. In line with available literature, we also assume that a single DC can engage multiple T cells (31). IFN γ produced by CD8⁺ T cells also upregulates PD-L1 (32) on both activated DCs and tumor cells, and the subsequent binding to PD-1 on CD8⁺ T cells results in their deactivation (26). In agreement with recent data, we also posited that Treg activation by inhibition of PD-1 on their surface was negligible (10). We factored in CD8⁺ T cell exhaustion upon repeated activation by cognate antigens presented in the tumor environment (33), and its reversal by IL-2 (34). The main source of IL-2 in the tumor environment is CD8⁺ T cells (10), and IL-2 is modeled to be in a local equilibrium, in agreement with evidence of limited diffusion of IL-2 around its producers (18). IL-2 is taken up by both CD8⁺ T cells (35) and Tregs (10) to increase their lifespan. However, Tregs express the high-affinity IL-2 receptor at greater levels than effector T cells, outcompeting them for IL-2 usage (36). Treg activation inhibits CD8⁺ T cell function via adenosine

production (37) and cytokines including TGF β , IL-10, and IL-35 (38,39). Tregs also inhibit the expression of CD80 and CD86 on DCs via CTLA-4 (12). These biological assumptions are translated into a system of ODEs in **Supplementary Fig. S1** and **Methods**.

Mathematical model captures *in vivo* tumor growth and immune cell dynamics.

Before calibrating our mathematical model (**Supplementary Fig. S1**) to experimental data, we checked the structural identifiability of the model using the Julia package '*StructuralIdentifiability.jl*'. Structural identifiability of a mathematical model ensures that a unique parameterization can exist based solely on the structure of the equations of the model (21). We first performed a literature search and identified biological values for nine parameters (**Table 1**). By fixing these parameters as constants, the model was rendered structurally identifiable.

We chose to apply our model to cutaneous melanoma because it is responsive to checkpoint immunotherapies, including α PD-(L)1 monotherapy, yet many patients develop acquired resistance (2). To enable the model to be predictive and quantitatively accurate, we calibrated it using newly generated experimental data. We first assessed the dynamics of tumor growth in the immunogenic melanoma cell line D4M-S, a derivative of D4M melanoma expressing the immunodominant peptide OVA₂₅₇₋₂₆₄ (SIINFEKL). All mice developed tumors and displayed a transient stagnation of tumor growth before progression (**Fig. 2A**). We then quantified the numbers of active and inactive DCs, CD8⁺ T cells, and Tregs at multiple time points during tumor development using flow cytometry (**Fig. 2B-C**). Tumor-associated DCs were recognized based on expression of MHC-II, CD11c, and CD135, and further divided into inactive or active DCs, indicated by the low or high expression of MHC molecules (**Supplementary Fig. S2A**). We defined Tregs by the co-expression of CD4 and Foxp3 (**Supplementary Fig. S2B**). Tumor antigen-specific CD8⁺ T cells were identified using SIINFEKL-specific tetramers. To distinguish functional from exhausted CD8⁺ T cells, we stained them with antibodies recognizing Tcf1 and Tim3, retaining cells with any expression of Tcf1 and low expression of Tim3 as functional (**Supplementary Fig. S2C**). We chose such a gating strategy after drawing small regions along the Tcf1 – Tim3 continuum, and quantifying concomitant production of IFN γ and TNF, a hallmark of T cell functionality (40), in each region (**Supplementary Fig. S2D**). We set the baseline expression of IFN γ and TNF to be that of Tcf1^{hi} cells. Cytokine production peaked in Tcf1^{lo} Tim3⁻ T cells and then decreased below the baseline in Tim3^{int-hi} T cells, which we considered exhausted (**Supplementary Fig. S2E**). Using these strategies, we were able to quantify how many immune cells populated

the tumor environment during progression (**Fig. 2C**). Together with parameters reported in the literature (**Table 1**), we calibrated the model to this experimental data (**Supplementary Fig. S2F; Methods**). Using this parameter set, we assessed the practical identifiability of the model. Practical identifiability ensures that each parameter value has a finite confidence interval and that each unique parameter set maps to a unique model output, which allows model predictions to be reliable (21). Using profile likelihood analysis (20) (**Methods**), we found one parameter (K_{EN}) that was not practically identifiable (**Supplementary Fig. S2G**). Further, five of the parameters that were practically identifiable when bounded by zero achieved the best model fit when their values were near zero (**Supplementary Fig. S2H**). Because of this, we fixed the values of r_R , ρ_{EN} , δ_E , and K_{DN} at zero, effectively removing these parameters from the model. This also removes K_{EN} since its contribution to the model vanishes when ρ_{EN} is zero. For the final parameter in this analysis, K_N , we observed that the likelihood profile was essentially unchanged in the interval from zero to 10^5 . Thus, we chose to fix K_N at 10^5 to model the fact that very small tumors may not be efficiently recognized by the immune system (22). These changes (highlighted in red in **Supplementary Fig. S1** and **Table 1**) did not impact how well the model fit the data after recalibration (**Fig. 2D**), allowing for practical identifiability (**Supplementary Fig. S2I**) and a simplification of the model. We do not exclude that the biological mechanisms removed could be relevant in the tumor environment, yet our current data do not allow evaluation of their importance. Notably, the practically identifiable model also fit previously published experimental data (**Supplementary Fig. S2J-K**). The practically identifiable model will be used in the remainder of our analysis.

Having verified that our model is structurally and practically identifiable, we used the confidence intervals obtained in the profile likelihood analysis to perform Latin hypercube sampling (41) and generate 342 ‘virtual mice’, each having a unique set of immunological parameters yet capturing the dynamics of the experimental data (**Fig. 3A**; details in **Methods**). We then conducted a series of *in silico* experiments using these virtual mice to verify that our model can capture known biological behaviors. First, we simulated low immunogenicity by decreasing parameters associated with tumor immunogenicity ($\rho_E, \rho_R, \delta_{EN}, \delta_R$; details in **Methods**) and found that tumors grew exponentially, which matched the behavior seen in non-immunogenic D4M tumors (**Fig. 3B**). The unrestricted growth of D4M-S tumors upon depletion of CD8⁺ T cells *in vivo* was also successfully simulated using our model (**Fig. 3C**). The model further captures the expected tumor rejection upon depletion of Tregs (**Supplementary Fig. S3A**). A distinguishing feature of our model is that it can capture immunosuppressive

reactions triggered by checkpoint immunotherapies, such as the tumor Treg increase that follows treatment with α PD-1 (**Fig. 3D**) or α CTLA-4 (**Supplementary Fig. S3B**) that is seen in both mice and patients (10–12). Therefore, these virtual mice represent the variability seen across individuals and were used for further simulations and analyses.

Differential impact of tumor and immune initial conditions on tumor growth dynamics.

In D4M-S tumor growth experiments, we typically observe transient stagnation of growth before progression. This occurrence is also observed in other mouse models (10,23,42) and is reminiscent of tumor dormancy in patients (43). However, in a minority of our experiments (2/7), the tumors grew monotonically (**Fig. 4A**, triangles). To determine the cause of varying D4M-S tumor growth behaviors, we investigated how early tumor immune interactions impact tumor development by perturbing the initial number of tumor and immune cells at the start of each simulation (initial conditions). When we fixed the parameter values of the virtual mice and increased the initial conditions of all cell types, we found that the simulated tumors exhibited monotonic growth (**Fig. 4A**, simulations in orange), suggesting that the variability of D4M-S growth behavior in the experiments may be due to the initial conditions of the tumor and immune system. This result held true even when we simulated only an increase in the tumor initial condition (**Supplementary Fig. S4**). Thus, an increased initial number of tumor cells is sufficient for the tumor to escape immune regulation. Next, we simulated the growth dynamics of D4M-S tumors upon decreasing the initial conditions of all cell types, finding decreased tumor growth (**Fig. 4B**). We validated these predictions experimentally (**Fig. 4C**), further indicating that the initial size of the tumor and the quantity of immune cells play a role in D4M-S growth dynamics.

We next sought to understand how perturbations to the immune cell initial conditions alone could impact tumor outcomes. The accumulation of immune cells into tumors is a key determinant of their outcome. For instance, predominant influx of Tregs instructs tumor growth, while infiltration of CD8⁺ T cells leads to cancer rejection (44). Thus, we varied the initial conditions of each immune cell type, both individually and in combination, and quantified the mean tumor size of all simulations on day 35 (**Fig. 5A and Supplementary Fig. S5**). Tumor growth was sensitive to individual changes in the initial conditions of T effector cells and Tregs (**Fig. 5A**), though the Treg initial conditions had a larger impact. When looking at combinations of initial immune cell alterations, there

exists a threshold in which increasing the T effector cell initial condition while also increasing the Treg initial condition results in a worse outcome, even when the T effector cell initial conditions are increased more than those of the Tregs. It is not until the T effector cell initial condition is increased past this threshold that the model predicts improved tumor outcomes. The specific value of this rejection threshold depends on the initial number of both active and inactive DCs. The model further suggests that the DCs modulate much of the immune response, as any increase in initial conditions of inactive or active DCs results in decreased tumor size (**Supplementary Fig. S5**).

To validate these model predictions, we compared the initial immune states and tumor growth of specific pathogen free (SPF) and Wildling mice (**Fig. 5B**). Wildling mice are C57BL/6 mice rederived into feral mothers, thus bearing the natural microbiome, mycobiome, virome, and parasitome (16), and have a more mature immune system compared to SPF mice (45). We found that while the tumor size on day 4 was similar in SPF and Wildlings, the Wildlings had higher levels of every immune cell type (**Fig. 5C**). Since our model was calibrated to data from SPF mice, we considered our simulations with no fold change (FC) in the immune cell initial conditions (FC = 1.0) to be that of the SPF mice (**Fig. 5A**). Comparatively, this would mean that the Wildling mice would be represented in **Fig. 5A** by the square corresponding approximately to a FC of 2.5 for inactive DCs, 1.5 for active DCs, 2.5 for T effector cells, and 1.5 for Tregs. Under these immune cell initial conditions, our model predicts that SPF mice would have a tumor comprising approximately 100 million cells by day 35, whereas Wildling mice would have rejected their tumor. This prediction is validated by the results in **Fig. 5D**, in which three of five Wildling mice rejected D4M-S melanomas, whereas none of the SPF mice did.

Mathematical model accurately simulates the experimental response to α PD-1.

We next investigated whether our mathematical model could recapitulate the experimental response to PD-1 immunotherapy. We reanalyzed the growth curves of α PD-1-treated D4M-S melanomas (published in (10)), observing no therapeutic effect in 15% of mice (3/20), a transient tumor control followed by progression in 55% of mice (11/20), which resembles acquired resistance to CBI, and complete rejection in 30% of mice (6/20). For subsequent analyses, we classified mice as complete responders if they rejected the tumor (30%) and non-responders otherwise (70%, **Supplementary Fig. S6A**). Then we mathematically simulated α PD-1 immunotherapy by reducing the value of the parameter K_A , representing the deactivation of CD8⁺ T cells by PD-

L1 expressed on active DCs (**Supplementary Fig. S1, Methods**). We found that an 8% PD-1 blockade allowed tumor rejection in a minority of the virtual mice, while 28% blockade led to tumor rejection in almost all cases (**Supplementary Fig. S6B-C**). Notably, a PD-1 blocking efficiency of 18% enabled the model to recapitulate the 30% responder / 70% non-responder ratio observed in the experimental data (**Fig. 6A-C and Supplementary Fig. S6D**). Thus, our mathematical model was calibrated to recapitulate the outcome of α PD-1 immunotherapy in mice. Moreover, our simulations indicated that an increasingly efficient PD-1 blockade would correlate with enhanced antitumor efficacy.

Treg influx hinders response to α PD-1.

To identify key determinants of acquired resistance to PD-1 immunotherapy, we used our α PD-1-treated simulations and first characterized the differences between responders and non-responders by identifying which parameters exhibited significantly different values (**Fig. 7A and S7A**). Eight parameters showed statistically significant differences between the two groups: the Treg influx rate (δ_R), the antigen presentation from active DC to Tregs and CD8⁺ T cells (ρ_R , ρ_E), the tumor chemokine mediated influx of CD8⁺ T cell (δ_{EN}), the tumor growth and killing rates (r_N , d_N), the activation of DCs by IFN γ (ρ_{DE}), and the saturating constant of inactive DC influx (K_D) (**Fig. 7A**). Next, to identify additional interventions that would enhance the therapeutic efficacy of non-responders, we decreased each parameter individually by 2% and quantified the total change in response to α PD-1 immunotherapy as compared to the original simulations (**Fig. 7B, Methods**). We found that the response rate was enhanced by individually decreasing the PD-L1 amount on active DCs (K_A), the direct suppression of CD8⁺ T cell function by Tregs (K_R), the tumor chemokine-mediated Treg influx rate (δ_{RN}), and the tumor growth rate (r_N). Since K_A and K_R were not significantly different between responders and non-responders (**Supplementary Fig. S7A**), we excluded them from further analysis. We also excluded r_N , the tumor proliferation rate, from further analysis as decreasing its value and seeing improved tumor control is trivial. Therefore, Treg influx, reflected in both parameters δ_R and δ_{RN} , was statistically different between complete and non-responders and greatly increased the response of non-responders to α PD-1 immunotherapy when decreased. To experimentally validate that reduced Treg influx to the tumor could enhance the response to α PD-1 immunotherapy, we generated mice in which approximately half of the Tregs are deficient in S1PR1, a receptor necessary for leaving tumor-draining lymph nodes (10). To do so, we irradiated CD45.1 mice and reconstituted

them with a 1:1 mixture of Foxp3^{DTR} and either *S1pr1^{fl/fl}* (CTRL) or *S1pr1^{fl/fl}* x UBC^{creERT2} (*S1PR1^{KO}*) bone marrow. Treatment with tamoxifen would delete S1PR1 from half of the lymphocytes, preventing their egress from the tumor-draining lymph node and subsequent invasion of the tumor. At the same time, diphtheria toxin (DT) administration would deplete normal Tregs, but not other cells, making S1PR1 deficiency specifically targeted at Tregs. Upon hematopoietic reconstitution, we challenged mice with D4M-S tumors, administered α PD-1 or an isotype control starting on day 12, and monitored tumor growth dynamics (**Fig. 7C**). We analyzed the change in tumor size after the start of immunotherapy of each experimental group. In general, isotype control antibodies did not prevent tumor progression in both CTRL and *S1PR1^{KO}* mice, even if one mouse experienced delayed progression and one showed tumor rejection in the *S1PR1^{KO}* group (**Fig. 7D**). Administration of PD-1 immunotherapy to control animals transiently reduced tumor size, yet progression ensued in most of the mice; in fact, PD-1 treatment did not result in a statistically significant tumor reduction compared to no treatment. Conversely, PD-1 inhibition in the *S1PR1^{KO}* group significantly dampened tumor progression (**Fig. 7D and Supplementary Fig. S7B**). While CTRL and *S1PR1^{KO}* mice had a median survival of 33 days and α PD-1 administration extended it to 45 days, the association of PD-1 blockade and inhibition of Treg influx extended the median survival to 79 days (**Fig. 7E**). Such lifespan extension was due to slower progression of tumors in the α PD-1 + *S1PR1^{KO}* groups compared to all other groups including α PD-1 monotherapy, as demonstrated by the analysis of time-to-death in the mice that did not reject their tumors (**Fig. 7F**). Therefore, our mathematical model identified dampening Treg influx as the most promising aspect of tumor immunity (out of 24 parameters) that synergized with PD-1 blockade to delay or avoid the development of secondary resistance to α PD-1 immunotherapy.

DISCUSSION

In this study, we developed a mechanistic, structurally identifiable mathematical model grounded in current biological knowledge to identify the most critical immunological features that influence tumor growth. Once we successfully fit the model to experimental data, we ensured that it was also practically identifiable and could recapitulate known biological behaviors such as increased tumor growth in non-immunogenic cancers and upon depletion of T effector cells, increased tumor control upon depletion of Tregs, and the immunosuppressive reactions triggered by checkpoint immunotherapies. By conducting simulations on hundreds of ‘virtual mice’ to capture biological variability, we identified the engraftment of tumor cells and specific alterations to the immune system early after tumor establishment as critical factors influencing tumor control or escape. Crucially, we used the model to simulate α PD-1 immunotherapy and identified Treg influx as a key determinant of acquired resistance to it. Our *in silico* predictions were validated using focused experiments.

Several mathematical models of the tumor immune system have been previously developed and have contributed to the investigation of mechanisms of cancer control (13–15). Some of these models include immunomodulatory mechanisms (46–48), yet only one considers how checkpoint immunotherapy supports immunosuppression (49). However, they assume that IL-2 increases Treg proliferation in the tumor, whereas our model aligns with recent studies that suggest IL-2 helps to enhance the lifespan of Tregs (10). Moreover, distinctive features of our mathematical model are that it considers how antigen-presenting cells (APCs) (12) and effector T cells (10) support tumor Tregs, the effect of IL-2 on T cell exhaustion (34), and how these aspects are influenced by checkpoint blockade. These factors allowed us to capture the Treg increase observed after α PD-1 and α CTLA-4 checkpoint immunotherapy (10,12), which is key to identifying the determinants of acquired resistance to CBI.

Since ODE simulations typically model a variable over time, it is useful to have temporal data to fit the model to and enable quantitative analysis and predictions. However, time-course experiments can quickly become expensive and time-consuming. We conducted a pseudo-temporal experiment to quantify immune cells across tumor development by sacrificing five mice at each time point of interest (**Fig. 2B and C**). This experiment provided a correlation between time and immune cell counts but could not examine cell counts longitudinally

within the same animal. Such repeated-measure experiments are typically limited to monitoring tumor growth or collecting samples from accessible tissues (e.g., blood) over time. Recently, intravital dynamic microscopy emerged as a technology that provides time- and space-resolution of cell motility and activation (10,12,50), and allows longitudinal imaging of the same area days apart (12). We foresee that intravital microscopy will become a prime source for spatio-temporal data for modeling purposes in the future.

Our model shows that the number of tumor and immune cells at early time points ('initial conditions') predicts cancer control or escape (**Figs. 4 and 5**). This approach models the impact of tumor size when the tumor is first recognized by the immune system. Such a moment does not occur immediately after tumor initiation (22), but likely varies with tumor type, mutational burden, and host-intrinsic factors. Further, the importance of immune cell initial conditions can be extended to changes in the tumor immune environment before or immediately after a major immunological intervention (e.g., CBI). In support of this statement, a new report demonstrated that recent COVID-19 vaccination improved the outcome of CBI (51). While type I IFN production had a major role, it would be interesting to test whether mRNA vaccines alter 'initial conditions' to sensitize cancers to checkpoint immunotherapy. Moreover, early-on-therapy changes in tumor size (52) or in the composition of the tumor immune infiltrate (53) predict response to immunotherapy. Thus, how could 'initial conditions' be manipulated to maximize the effectiveness of CBI? In a perspective article, Dr. Lieping Chen argued that increasing immune effector function in the presence of immunosuppressive mechanisms may not result in tumor control, akin to increasing pressure in a clogged water pipe (54). Accordingly, our model and the work of another group (55) predict that the inhibition of immunosuppressive cells leads to better tumor control than potentiating effector cells. This notion finds experimental correlates in the increased effectiveness of PD-1 blockade after immune interventions to limit Treg response, such as inhibition of ICOSL (10), administration of depleting α CTLA-4 mAbs (56), or treatment with cyclophosphamide (57).

We predict that an increasingly efficient PD-1 blockade would correlate with enhanced antitumor efficacy (**Supplementary Fig. S6**). One of the most commonly used α PD-1 mAbs in humans, nivolumab, occupies PD-1 at 60-80% efficiency at the clinically relevant doses of 0.3 to 3 mg/kg (58), while computer simulations predict pembrolizumab to be slightly more efficient (59). Thus, there is an unmet need and a compelling rationale to

generate α PD-1 mAbs with enhanced affinity for clinical use. Notably, the recently developed antibody sintilimab has greater affinity for PD-1 than nivolumab and pembrolizumab (60). The therapeutic efficacy of these mAbs is currently being compared.

The comparison of parameter values between virtual mice responding or not to PD-1 blockade (**Fig. 7A**) and our parameter perturbation analysis (**Fig. 7B**) converged on Treg influx into the tumor as a key determinant of resistance to PD-1 CBI. We validated this prediction experimentally (**Fig. 7C-F**), providing a compelling rationale for developing drugs that block Treg recruitment to tumors. Unfortunately, this task is difficult due to the lack of specificity and the redundancy of the chemokine system. Antibodies targeting CCR4, necessary for Treg homing to a variety of cancers (61), did not synergize with PD-1 CBI because they inhibited both Tregs and antitumor CD8⁺ T cells (62). Moreover, CCR8, expressed specifically in tumor Tregs, may not be necessary for Treg homing to tumors (63). These limitations might be overcome by the development of bispecific antibodies capable of targeting activated Tregs (e.g., α CCR8, α OX-40, α CTLA-4) while blocking chemokine receptors guiding Tregs into cancers, such as CCR4, CCR5, or CXCR3 (61). Our simulations also provided other valuable insights into the mechanisms of PD-1 immunotherapy against immunogenic melanoma. Since increased tumor cell death (d_N) and tumor antigen presentation by DCs (ρ_E) correlate with better melanoma control, we speculate that PD-1 blockade will synergize with immunogenic chemotherapy, as shown in lung cancer models (64). Additionally, our model confirms that PD-L1 expressed by DCs (K_A) has a key immunosuppressive role, as observed in a mouse model of colon cancer (65). Finally, our analysis sheds some light on the relative importance of Treg-mediated inhibition of APCs (σ_R) or effector T cells (K_R) during PD-1 CBI: the latter may play a more prominent immunosuppressive role. This hypothesis awaits experimental validation.

Recent studies have shown 10-year progression-free survival in 31% of metastatic melanoma patients treated with the combination of α PD-1 and α CTLA-4 as compared to 23% with α PD-1 or 6% with α CTLA-4 alone (1). While this is a major improvement from previous metastatic melanoma treatments, the majority of patients eventually cease to respond to state-of-the-art checkpoint blockade immunotherapy. This consideration illustrates the need for further studies to uncover optimal therapeutic strategies. Seminal work showed that mathematical modeling can predict the optimal temporal combinations of preventive antitumor vaccination in

mouse models (66). While our current work focuses on α PD-1 monotherapy, the model we present here can simulate CTLA-4 inhibition, blockade of effector T cell help to Tregs (similar to the effect of α ICOSL (10)), or restriction of T cell exhaustion (resembling a possible mechanism of action of α LAG-3(33)), and can be used to identify combinations for optimal therapeutic efficacy in a time-efficient manner.

Limitations of the study

As is the case with all mathematical models, we did not include every possible biological characteristic that contributes to cancer progression and control. This leaves ample room for model expansion to consider additional immunosuppressive cells, such as macrophage polarization away from pro-inflammatory functions or the accumulation of T regulatory type 1 (Tr1) cells, and the impact of various cytokines, including IL-12 (27) and type-I and II IFNs (67). The model could also be improved by including the pharmacodynamics and pharmacokinetics of each immunotherapy to more accurately simulate drug administration and dynamics. In addition, we did not model neovascularization and extracellular matrix remodeling, which crucially modulate immune cell recruitment and motility as well as tumor growth and metastasis. These aspects can be implemented following (68,69). Finally, our work is parameterized using mouse data, but it will be crucial to extend the model to human data to help guide the development of clinical studies.

ACKNOWLEDGMENTS

This work is funded by pilot grants from the Chao Family Comprehensive Cancer Center (CFCCC) and the Cancer Systems Biology Center of the University of California Irvine, the DoD Team grant ME220176P1, and R01CA287116 (to F. Marangoni). R. S. Sousa and S. N. Geels are supported by NSF-GRFP fellowships 1000330047 and 2235784, and A. Moshensky by the NIH-NIAMS F31 AR083279. S. A. Villalta is supported by R01 NS120060, which also provided a diversity supplement to R. S. Sousa. M. Di Pilato is a Cancer Prevention and Research Institute of Texas (CPRIT) Scholar in Cancer Research. J. S. Lowengrub wishes to acknowledge support from P30 CA062203, P01CA288662, and DMS-1763272 and the Simons Foundation (594598QN) for an NSF-Simons Center for Multiscale Cell Fate Research. The authors thank Dr. Susan Schwab (New York University) for the donation of S1pr1^{ff} x UBC^{creERT2} bone marrow. The graphical abstract was created in BioRender: Marangoni, F. (2026) [https:// BioRender.com/5dh0dja](https://BioRender.com/5dh0dja).

CONTRIBUTIONS

R.S.S., J.S.L., and F.M. devised the mathematical model and wrote the paper. R.S.S. analyzed the mathematical model, performed the numerical simulations, and fit the model to data. S.N.G., A.M., and C.M. performed wet-lab experiments. M.D.P. performed experiments on D4M cells. S.A.V. provided scientific guidance. J.S.L. and F.M. supervised the study.

REFERENCES

1. Wolchok JD, Chiarion-Sileni V, Rutkowski P, Cowey CL, Schadendorf D, Wagstaff J, et al. Final, 10-Year Outcomes with Nivolumab plus Ipilimumab in Advanced Melanoma. *N Engl J Med* 2025;392:11–22.
2. Schoenfeld AJ, Hellmann MD. Acquired Resistance to Immune Checkpoint Inhibitors. *Cancer Cell* 2020;37:443–55.
3. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* 2016;7:10501.
4. Yan Z, Wang C, Wu J, Wang J, Ma T. TIM-3 teams up with PD-1 in cancer immunotherapy: mechanisms and perspectives. *Mol Biomed* 2025;6:27.
5. Tawbi HA, Schadendorf D, Lipson EJ, Ascierto PA, Matamala L, Castillo Gutiérrez E, et al. Relatlimab and Nivolumab versus Nivolumab in Untreated Advanced Melanoma. *N Engl J Med* 2022;386:24–34.
6. Tsukamoto H, Fujieda K, Miyashita A, Fukushima S, Ikeda T, Kubo Y, et al. Combined blockade of IL6 and PD-1/PD-L1 signaling abrogates mutual regulation of their immunosuppressive effects in the tumor microenvironment. *Cancer Res* 2018;78:5011–22.
7. Su X, Yu Y, Zhong Y, Giannopoulou EG, Hu X, Liu H, et al. Interferon- γ regulates cellular metabolism and mRNA translation to potentiate macrophage activation. *Nat Immunol* 2015;16:838–49.
8. Kamada T, Togashi Y, Tay C, Ha D, Sasaki A, Nakamura Y, et al. PD-1+ regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc Natl Acad Sci U S A*. 2019;116:9999–10008.
9. Kumagai S, Togashi Y, Kamada T, Sugiyama E, Nishinakamura H, Takeuchi Y, et al. The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nat Immunol* 2020;21:1346–58.
10. Geels S, Moshensky A, Sousa R, Murat C, Bustos M, Walker B, et al. Interruption of the Intratumor CD8+ T cell:Treg Crosstalk Improves the Efficacy of PD-1 Immunotherapy. *Cancer Cell* 2024;42:1051–66.
11. Sharma A, Subudhi SK, Blando J, Scutti J, Vence L, Wargo J, et al. Anti-CTLA-4 immunotherapy does not deplete Foxp3 β regulatory T cells (Tregs) in human cancers. *Clin Cancer Res* 2019;25:1233–8.
12. Marangoni F, Zhakyp A, Corsini M, Geels SN, Carrizosa E, Thelen M, et al. Expansion of tumor-associated Treg cells upon disruption of a CTLA-4-dependent feedback loop. *Cell* 2021;184:3998-4015.e19.
13. Mahasa KJ, Ouifki R, Eladdadi A, Pillis L de. Mathematical model of tumor–immune surveillance. *J Theor Biol* 2016;404:312–30.
14. Kirschner D, Panetta JC. Modeling immunotherapy of the tumor - Immune interaction. *J Math Biol* 1998;37:235–52.
15. De Pillis LG, Gu W, Radunskaya AE. Mixed immunotherapy and chemotherapy of tumors: Modeling, applications and biological interpretations. *J Theor Biol* 2006;238:841–62.
16. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, et al. Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science* 2019;365:aaw4361.
17. Breart B, Lemaître F, Celli S, Bousso P. Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. *J Clin Invest* 2008;118:1390–7.
18. Oyler-Yaniv A, Oyler-Yaniv J, Whitlock BM, Liu Z, Germain RN, Huse M, et al. A Tunable Diffusion-Consumption Mechanism of Cytokine Propagation Enables Plasticity in Cell-to-Cell Communication in the Immune System. *Immunity*. 2017;46:609–20.

19. Del Monte U. Does the cell number 10(9) still really fit one gram of tumor tissue? *Cell Cycle* 2009;8:505–6.
20. Raue A, Kreutz C, Maiwald T, Bachmann J, Schilling M, Klingmüller U, et al. Structural and practical identifiability analysis of partially observed dynamical models by exploiting the profile likelihood. *Bioinformatics* 2009;25:1923–9.
21. Wieland F-G, Hauber AL, Rosenblatt M, Tönsing C, Timmer J. On structural and practical identifiability. *Curr Opin Syst Biol* 2021;25:60–9.
22. Gil Del Alcazar CR, Trinh A, Alečković M, Jimenez ER, Harper NW, Oliphant MUJ, et al. Insights into Immune Escape During Tumor Evolution and Response to Immunotherapy Using a Rat Model of Breast Cancer. *Cancer Immunol Res* 2022;10:680–97.
23. Marangoni F, Zhang R, Mani V, Thelen M, Ali Akbar NJ, Warner RD, et al. Tumor Tolerance–Promoting Function of Regulatory T Cells Is Optimized by CD28, but Strictly Dependent on Calcineurin. *J Immunol* 2018;200:3647–61.
24. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* 2012;209:1201–17.
25. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* 2017;355:1428–33.
26. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJM, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014;515:568–71.
27. Garris CS, Arlauckas SP, Kohler RH, Trefny MP, Garren S, Piot C, et al. Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN- γ and IL-12. *Immunity* 2018;49:1148–1161.e7.
28. Zhou H, Mao B, Guo S. Mathematical Modeling of Tumor Growth in Preclinical Mouse Models with Applications in Biomarker Discovery and Drug Mechanism Studies. *Cancer Res Commun* 2024;4:2267–81.
29. Cabeza-Cabrero M, Cardoso A, Minutti CM, Pereira da Costa M, Reis e Sousa C. Dendritic Cells Revisited. *Annu Rev Immunol* 2021;39:131–66.
30. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the Tumor Myeloid Compartment Reveals Rare Activating Antigen-Presenting Cells Critical for T Cell Immunity. *Cancer Cell* 2014;26:638–52.
31. Bousso P, Robey E. Dynamics of CD8⁺ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol* 2003;4:579–85.
32. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 2012;4:127ra37.
33. Andrews LP, Butler SC, Cui J, Cillo AR, Cardello C, Liu C, et al. LAG-3 and PD-1 synergize on CD8⁺ T cells to drive T cell exhaustion and hinder autocrine IFN- γ -dependent anti-tumor immunity. *Cell* 2024;187:4355–4372.e22.
34. Beltra JC, Abdel-Hakeem MS, Manne S, Zhang Z, Huang H, Kurachi M, et al. Stat5 opposes the transcription factor Tox and rewires exhausted CD8⁺ T cells toward durable effector-like states during chronic antigen exposure. *Immunity* 2023;56:2699–2718.e11.

35. Kahan SM, Bakshi RK, Ingram JT, Hendrickson RC, Lefkowitz EJ, Crossman DK, et al. Intrinsic IL-2 production by effector CD8 T cells affects IL-2 signaling and promotes fate decisions, stemness, and protection. *Sci Immunol*. 2022;7:1–15.
36. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in T reg cell function. *Nat Immunol*. 2016;17:1322–33.
37. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007;204:1257–65.
38. Chen M-L, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–24.
39. Sawant D V., Yano H, Chikina M, Zhang Q, Liao M, Liu C, et al. Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion. *Nat Immunol* 2019;20:724–35.
40. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment. *J Virol* 2003;77:4911–27.
41. Helton JC, Davis FJ. Latin hypercube sampling and the propagation of uncertainty in analyses of complex systems. *Reliab Eng Syst Saf* 2003;81:23–69.
42. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 2007;450:903–7.
43. MacKie RM, Reid R, Junor B. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. *N Engl J Med* 2003;348:567–8
44. Darrasse-Jèze G, Bergot AS, Durgeau A, Billiard F, Salomon BL, Cohen JL, et al. Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice. *J Clin Invest* 2009;119:2648–62.
45. Rehmann B, Graham AL, Masopust D, Hamilton SE. Integrating natural commensals and pathogens into preclinical mouse models. *Nat Rev Immunol* 2025;25:385–97.
46. Leon K, Garcia K, Carneiro J, Lage A. How regulatory CD25+CD4+ T cells impinge on tumor immunobiology: the differential response of tumors to therapies. *J Immunol* 2007;179:5659–68.
47. Robertson-Tessi M, El-Kareh A, Goriely A. A mathematical model of tumor-immune interactions. *J Theor Biol* 2012;294:56–73.
48. Feng P, Navaratna M. Role of regulatory T cells on a simple tumor-immune interaction system. *Commun Nonlinear Sci Numer Simul* 2021;98:105765.
49. Siewe N, Friedman A. TGF-β inhibition can overcome cancer primary resistance to PD-1 blockade: A mathematical model. *PLoS One* 2021;16:e0252620.
50. Marangoni F, Murooka TT, Manzo T, Kim EY, Carrizosa E, Elpek NM, et al. The Transcription Factor NFAT Exhibits Signal Memory during Serial T Cell Interactions with Antigen-Presenting Cells. *Immunity* 2013;38:237–49
51. Grippin AJ, Marconi C, Copling S, Li N, Braun C, Woody C, et al. SARS-CoV-2 mRNA vaccines sensitize tumours to immune checkpoint blockade. *Nature* 2025;8089:488-97.

52. Hopkins AM, Kichenadasse G, Karapetis CS, Rowland A, Sorich MJ. Early tumor shrinkage identifies long-term disease control and survival in patients with lung cancer treated with atezolizumab. *J Immunother Cancer* 2020;8:e000500.
53. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell* 2017;171:934-949.e15.
54. Sanmamed MF, Chen L. A Paradigm Shift in Cancer Immunotherapy: From Enhancement to Normalization. *Cell* 2018;175:313–26.
55. Kreger J, Roussos Torres ET, MacLean AL. Myeloid-Derived Suppressor–Cell Dynamics Control Outcomes in the Metastatic Niche. *Cancer Immunol Res* 2023;11:614–28.
56. Ha D, Tanaka A, Kibayashi T, Tanemura A, Sugiyama D, Wing JB, et al. Differential control of human Treg and effector T cells in tumor immunity by Fc-engineered anti–CTLA-4 antibody. *Proc Natl Acad Sci USA* 2019;116:609–18.
57. Mkrtichyan M, Najjar YG, Raulfs EC, Abdalla MY, Samara R, Rotem-Yehudar R, et al. Anti-PD-1 synergizes with cyclophosphamide to induce potent anti-tumor vaccine effects through novel mechanisms. *Eur J Immunol* 2011;41:2977–86.
58. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: Safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 2010;28:3167–75.
59. Elassaiss-Schaap J, Rossenu S, Lindauer A, Kang S, De Greef R, Sachs J, et al. Using Model-Based “learn and Confirm” to Reveal the Pharmacokinetics-Pharmacodynamics Relationship of Pembrolizumab in the KEYNOTE-001 Trial. *CPT Pharmacometrics Syst Pharmacol* 2017;6:21–8.
60. Wang J, Fei K, Jing H, Wu Z, Wu W, Zhou S, et al. Durable blockade of PD-1 signaling links preclinical efficacy of sintilimab to its clinical benefit. *MAbs* 2019;11:1443–51.
61. Mempel TR, Marangoni F. Guidance factors orchestrating regulatory T cell positioning in tissues during development, homeostasis, and response. *Immunol Rev* 2019;289:129–41.
62. Maeda Y, Wada H, Sugiyama D, Saito T, Irie T, Itahashi K, et al. Depletion of central memory CD8+ T cells might impede the antitumor therapeutic effect of Mogamulizumab. *Nat Commun* 2021;12:7280.
63. Whiteside SK, Grant FM, Gyori DS, Conti AG, Imianowski CJ, Kuo P, et al. CCR8 marks highly suppressive Treg cells within tumours but is dispensable for their accumulation and suppressive function. *Immunology* 2021;58:7250-7.
64. Pfirschke C, Engblom C, Rickelt S, Cortez-Retamozo V, Garriss C, Pucci F, et al. Immunogenic Chemotherapy Sensitizes Tumors to Checkpoint Blockade Therapy. *Immunity* 2016;44:343–54.
65. Oh SA, Wu D-C, Cheung J, Navarro A, Xiong H, Cubas R, et al. PD-L1 expression by dendritic cells is a key regulator of T-cell immunity in cancer. *Nat Cancer* 2020;1:681–91.
66. Palladini A, Nicoletti G, Pappalardo F, Murgo A, Grosso V, Stivani V, et al. In silico modeling and in vivo efficacy of cancer-preventive vaccinations. *Cancer Res* 2010;70:7755–63.
67. Benci JL, Xu B, Qiu Y, Wu TJ, Dada H, Twyman-Saint Victor C, et al. Tumor Interferon Signaling Regulates a Multigenic Resistance Program to Immune Checkpoint Blockade. *Cell* 2016;167:1540-1554.e12.
68. Macklin P, McDougall S, Anderson ARA, Chaplain MAJ, Cristini V, Lowengrub J. Multiscale modelling and nonlinear simulation of vascular tumour growth. *J Math Biol* 2009;58:765–98.
69. Franssen LC, Lorenzi T, Burgess AEF, Chaplain MAJ. A Mathematical Framework for Modelling the Metastatic Spread of Cancer. *Bull Math Biol* 2019;81:1965–2010.

70. Hou TZ, Qureshi OS, Wang CJ, Baker J, Young SP, Walker LSK, et al. A Transendocytosis Model of CTLA-4 Function Predicts Its Suppressive Behavior on Regulatory T Cells. *J Immunol* 2015;194:2148–59.
71. Waters E, Williams C, Kennedy A, Sansom DM. In Vitro Analysis of CTLA-4-Mediated Transendocytosis by Regulatory T Cells. *Methods Mol Biol* 2023;2559:171–87.
72. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103⁺ DCs. *J Exp Med* 2009;206:3115–30.
73. Burton OT, Bricard O, Tareen S, Gergelits V, Andrews S, Biggins L, et al. The tissue-resident regulatory T cell pool is shaped by transient multi-tissue migration and a conserved residency program. *Immunity* 2024;57:1586-1602.e10.
74. Voisinne G, Nixon BG, Melbinger A, Gasteiger G, Vergassola M, Altan-Bonnet G. T Cells Integrate Local and Global Cues to Discriminate between Structurally Similar Antigens. *Cell Rep* 2015;11:1208–19.
75. Höfer T, Krichevsky O, Altan-Bonnet G. Competition for IL-2 between regulatory and effector T cells to chisel immune responses. *Front Immunol* 2012;3:1–9.

TABLES WITH LEGENDS

Paramete	Description	Value or Range	Units	Source
r_N	Tumor proliferation rate	[0.146, 0.187]	(day) ⁻¹	Estimated
d_N	Tumor killing rate by T_E	[18.69, 28.48]	(N / T_E) / day	Estimated, (18)
K_N	Saturation of killing	100000	N	Estimated
δ_I	D_I influx rate	[0.00199, 0.00305]	(D_I / N) / day	Estimated
K_D	Saturation of D_I influx	[3.31x10 ⁻⁸ , 1.2x10 ⁻⁷]	N^{-1}	Estimated
σ_R	D_A deactivation rate by CTLA-4 on T_R	0.5688	$T_R^{-1}(\text{day})^{-1}$	(70,71)
ρ_{DE}	D_I activation rate via IFN γ	[0.043, 1.46]	$T_E^{-1}(\text{day})^{-1}$	Estimated
ρ_{DN}	D_I activation rate via tumor antigen	[0.0047, 0.0134]	$N^{-1}(\text{day})^{-1}$	Estimated
K_{DN}	Saturation of D_I activation	4.10x10 ⁻¹¹	N^{-1}	Estimated
d_I	D_I death rate	0.099	(day) ⁻¹	(72)
d_A	D_A death rate	0.099	(day) ⁻¹	(72)
ρ_E	T_E activation rate by D_A antigen presentation	[2.11x10 ⁻⁶ , 8.9x10 ⁻⁶]	$N^{-1}D_A^{-2}$	Estimated
K_R	T_E suppression rate by T_R	[6.98x10 ⁻⁵ , 2.53x10 ⁻⁴]	T_R^{-1}	Estimated
K_A	D_A PD-L1 negative feedback rate on T_E	[0.568, 1.22]	$T_E^{-1}D_A^{-1}$	Estimated
ρ_{EN}	T_E activation rate by tumor presenting antigen	101	$N^{-1}(\text{day})^{-1}$	Estimated
K_{EN}	Tumor PD-L1 negative feedback rate on T_E	1.081	$T_E^{-1}N^{-1}$	Estimated
δ_E	T_E influx rate due to priming of tumor-specific T_E	6.07x10 ⁻¹¹	T_E / N	Estimated
δ_{EN}	T_E influx rate due to chemokines produced by tumor environment	[7.81x10 ⁻⁵ , 4.03x10 ⁻⁴]	(T_E / N) / day	Estimated
d_E	T_E death rate	0.1695	(day) ⁻¹	(48,73)
d_{Ex}	T_E exhaustion rate	[6.13x10 ⁻⁹ , 2.71x10 ⁻⁸]	$N^{-1}(\text{day})^{-1}$	Estimated
β	Rate IL-2 prolongs T cell lifespan	[6.68x10 ⁻⁵ , 2.74x10 ⁻³]	(T cells/IL-2)/CD25	Estimated
α_E	Number of IL-2 receptors on T_E	4640	CD25 / T_E	(74,75)
r_R	T_R activation due to non-tumor, self antigens	5.7x10 ⁻¹⁵	$D_A^{-2}(\text{day})^{-1}$	Estimated
ρ_R	T_R activation rate due to tumor-released antigens	[5.67x10 ⁻²¹ , 1.95x10 ⁻¹⁷]	$N^{-1}D_A^{-2}$	Estimated
δ_R	T_R influx rate due to priming of tumor-specific T_R	[0.0022, 0.012]	T_R / N	Estimated
δ_{RN}	T_R influx rate due to chemokines produced by tumor environment	[0.0018, 0.0031]	(T_R / N) / day	Estimated
d_R	T_R death rate	0.0826	(day) ⁻¹	(73)
α_R	Number of IL-2 receptors on T_R	40000	CD25 / T_R	(74,75)
γ_p	IL-2 production rate by T_E	864000	(IL-2 / T_E) / day	(75)
γ_c	IL-2 consumption rate	95.04	(CD25) ⁻¹ (day) ⁻¹	(75)

Table 1: Parameter descriptions and values used in the mathematical model.

The parameters fixed or set to zero in the practically identifiable model are highlighted in red.

FIGURE LEGENDS

Figure 1. Tumor-Immune Model Schematic

Depiction of Tumor-Immune Model interactions between tumor cells (N), inactive (DI) and active (DA) dendritic cells, tumor-specific CD8⁺ T effector cells (TE), regulatory T cells (TR), and interleukin-2 (IL-2). Black arrows, promotion; red arrows, inhibition; gray arrow, state transition; dashed arrows, only present in structurally identifiable model.

See also **Figure S1**.

Figure 2. Model captures *in vivo* tumor growth and immune cell dynamics.

(A) Mean growth curve of D4M-S tumors in mice, $n = 5$. Error bars depict one standard deviation.

(B) Experimental scheme to quantify immune cell dynamics in mice bearing D4M-S melanomas.

(C) Number of the indicated cell type per mg of tumor over time. $n = 4 - 5$ mice per group. Bar represents the mean number of cells.

(D) Practically identifiable model fit to tumor (left) and immune cell (right) experimental data. Tumor volume from **Fig. 2A** was converted to number of tumor cells and immune data in **Fig. 2C** was converted to total number of immune cells based on the tumor growth data (see **Methods** for more details). Black dots are the mean tumor size from (A) (left) and mean number of immune cells (right). Error bars depict one standard deviation.

See also **Figure S2**.

Figure 3. Model recapitulates known biological behaviors.

(A) Depiction of the 342 unique parameter sets generated via Latin hypercube sampling, shown with experimental data from **Fig. 2A-C**.

(B) Unaltered model simulations (solid line, mean of parameter sets from **Fig. 3A**) fit to immunogenic D4M-S tumor growth data (purple dots) from **Fig. 2A** compared to model simulations with decreased immunogenicity (dashed line), which is now comparable to non-immunogenic D4M tumor growth data (black dots). D4M tumor growth data $n = 4$ mice. Clouds around experimental data depict one standard deviation.

(C) Tumor weight in mice with D4M-S melanomas treated or not with FTY720 and CD8-depleting mAbs (left). $n = 9 - 10$ mice per group. Model simulations ($n = 342$ per group) are depicted on the right.

(D) Simulated number of Tregs per mg of tumor, in the presence or absence of α PD-1 mAbs. $n = 342$ /group. Compare to Figure S7N of Geels et al, Cancer Cell 2024.

In C and D bars depict mean, p values determined by Wilcoxon signed-rank test. See also **Figure S3**.

Figure 4. Initial tumor and immune conditions contribute to tumor outcome.

(A) Unaltered model simulations (purple line, mean of fits from **Fig. 3A**) fit to D4M-S “dipping” tumor growth data (black circles) from **Fig. 2A**. Model simulations with increased tumor and immune cell initial conditions (orange line), are comparable to D4M-S “monotonic” tumor growth data (white triangles). The D4M-S “monotonic” tumor growth experiment ($n = 5$ mice) was published in Geels et al, *Cancer Cell* 2024. Clouds around mean simulations and error bars on data depict one standard deviation.

(B) Unaltered model simulations (purple line, mean of fits from **Fig. 3A**) and model simulations with decreased tumor and immune cell initial conditions (orange line). Clouds around mean simulations depict one standard deviation.

(C) Growth curves of D4M-S tumors in mice injected with 2×10^6 (left, red) and 2×10^5 (right, black) tumor cells. Thin lines represent individual mice; thick lines represent mean of group; $n = 5$ mice/group.

See also **Figure S4**.

Figure 5. Specific alterations in immune cell initial conditions result in increased tumor control.

(A) Mean tumor size at final simulation time (day 35) for the indicated fold change in TE, TR, DA, and/or DI initial condition. $n = 342$ simulations/square.

(B) Experimental scheme to assess initial immune conditions and tumor growth dynamics in SPF and Wildling mice bearing D4M-S melanomas.

(C) Tumor weight and number of immune cells per mg of tumor on day four after tumor implantation. Bar represents mean tumor weight or number of cells; $n = 5$ mice/group.

(D) Growth curves of D4M-S tumors in SPF (left) and Wildling (right) mice. Each line represents an individual mouse; $n = 5$ mice/group. Numbers in the lower right corner represent the quantity of complete tumor rejections.

See also **Figure S5**.

Figure 6. Model captures the experimental response to α PD-1.

(A) Simulated response to α PD-1 treatment classified as “Complete Responders” (green, tumors that are fully rejected) and “Non-Responders” (red, all other tumors). Dashed lines indicate treatment start and stop time. α PD-1 blocking efficiency is set to 18%; $n = 342$ simulations. See **Methods** for simulated treatment details.

(B) Simulated outcome to α PD-1 treatment by response type.

(C) Experimental outcome to α PD-1 treatment by response type, as displayed in **Fig. S6A**.

See also **Figure S6**.

Figure 7. Treg influx hinders response to α PD-1.

(A) Difference between parameter values in simulations classified as “Non-Responders” (red) and “Complete Responders” (green) with simulated α PD-1 treatment. Black bars depict mean; $n = 342$ simulations; p values determined by Mann-Whitney U test.

(B) Simulated response to α PD-1 treatment with blocking efficiency set to 18% after decreasing an individual parameter by 2% of its original value. “Non-Responders”, red; “Complete Responders”, green; simulations that resulted in tumor death without CBI by only decreasing parameter value, gray. $n = 342$ simulations/bar. See Methods for simulated treatment details.

(C) Experimental scheme for Treg influx depletion.

(D) D4M-S tumor growth in mice with or without decreased Treg influx rates in the presence or absence of α PD-1. Individual mice shown in each group (left) and median tumor growth upon setting the tumor size of sacrificed mice to the last measurement (right). $n = 5 - 7$ per group. p values by a linear mixed effect model with Holm’s post-test calculated on the tumor growth of individual mice.

(E) Survival curves of the mice in D. $n = 5 - 7$ per group. p values by Logrank test.

(F) Analysis of lifespan duration after removal of the mice that completely rejected the tumor. $n = 4 - 6$ per group. p values by one-way ANOVA with Benjamini-Krieger-Yekutieli post-test.

See also **Figure S7**.

FIGURE 1

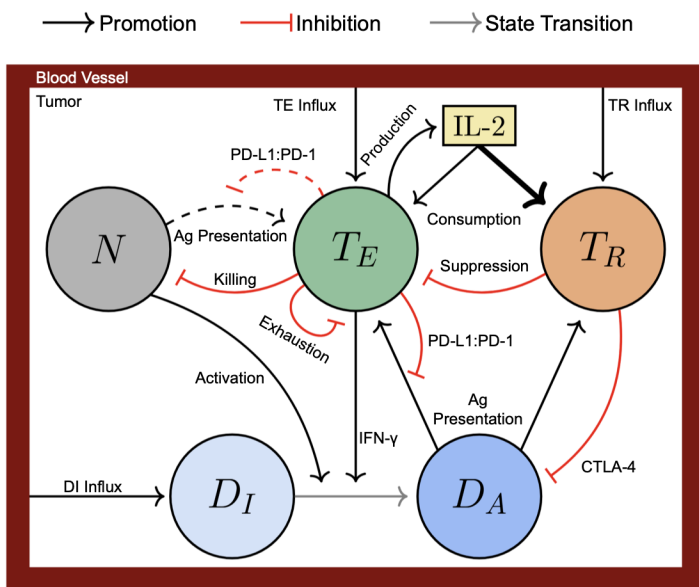


FIGURE 2

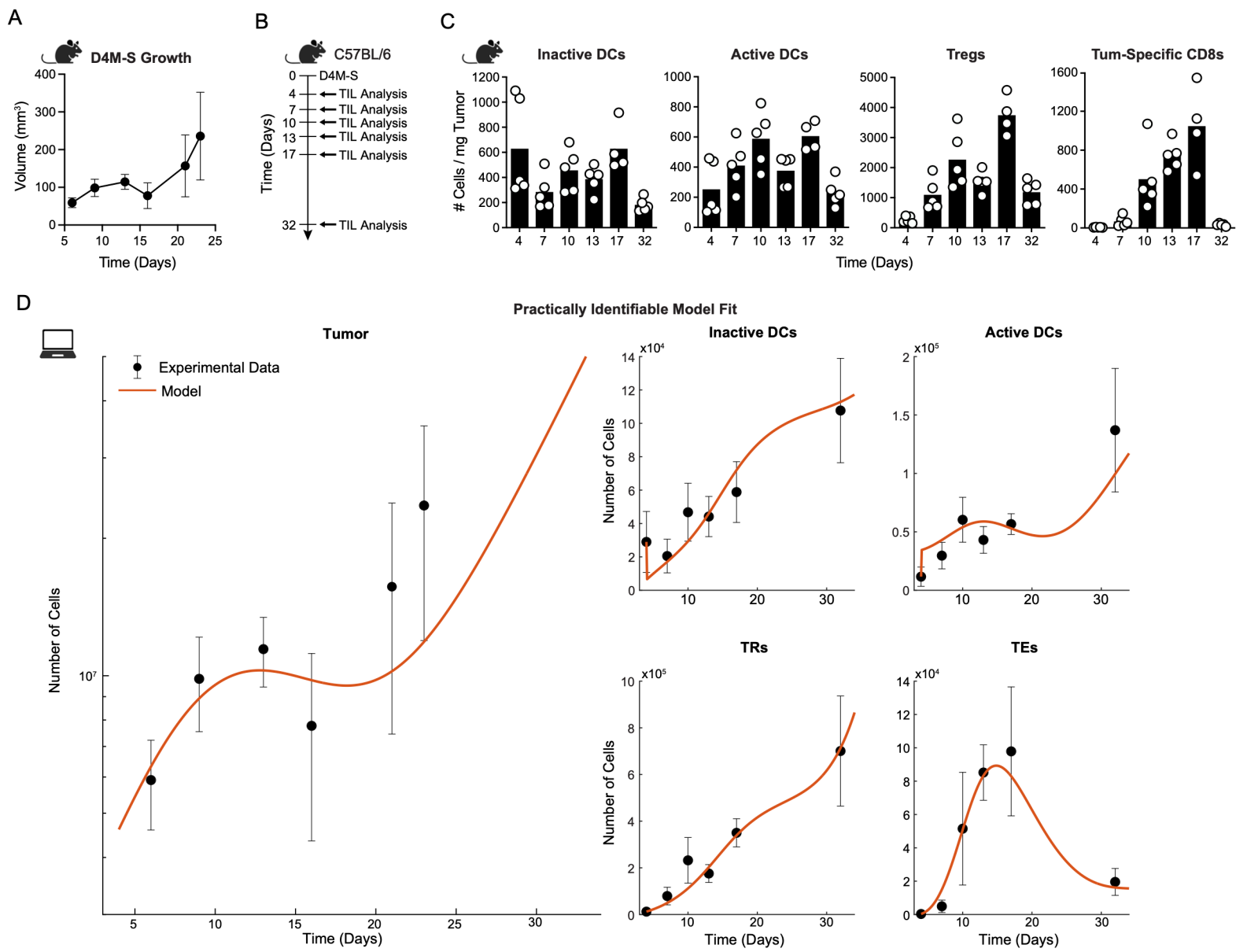


FIGURE 3

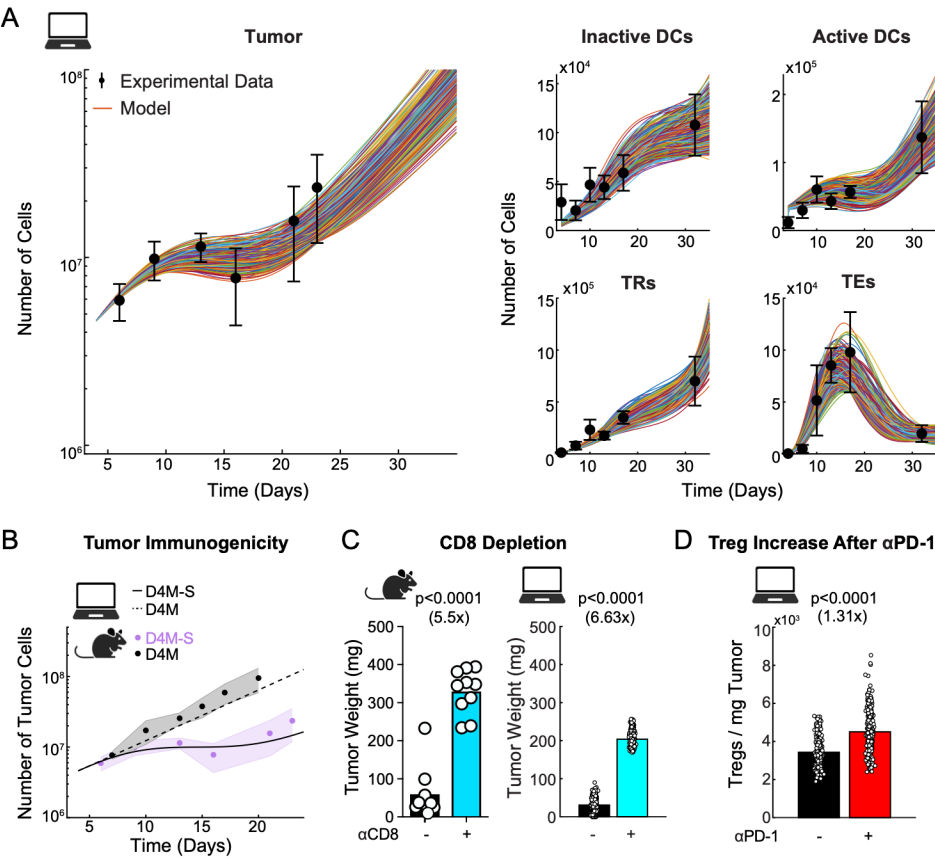


FIGURE 4

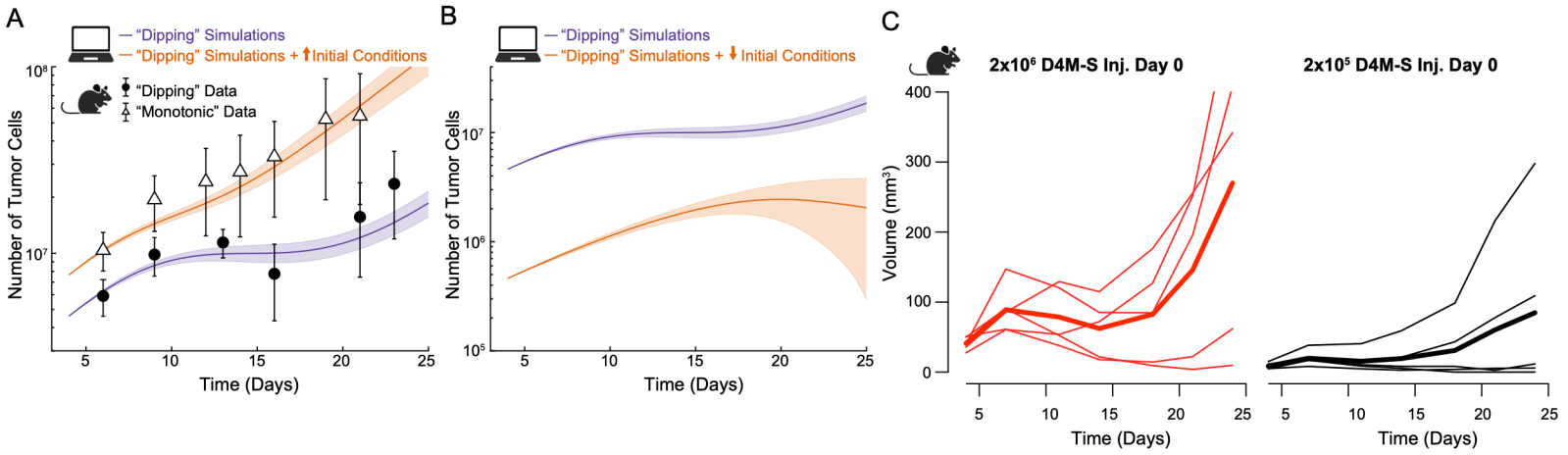


FIGURE 5

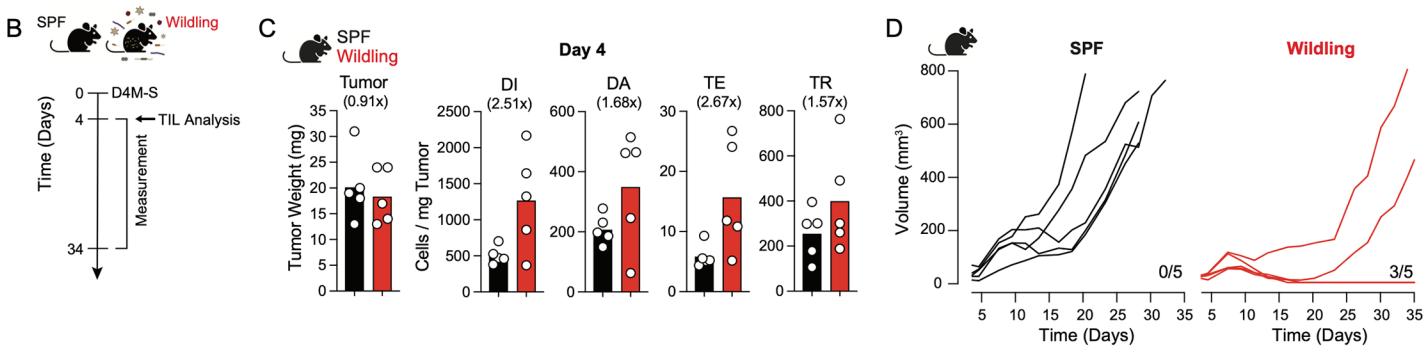
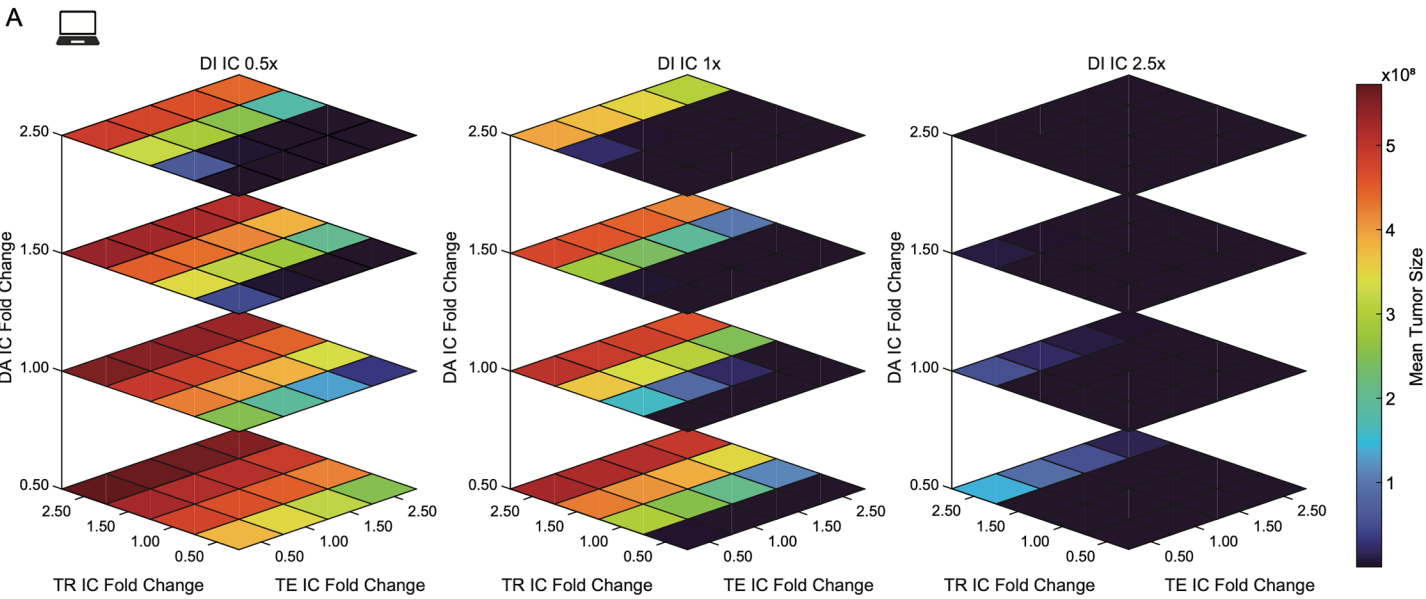


FIGURE 6

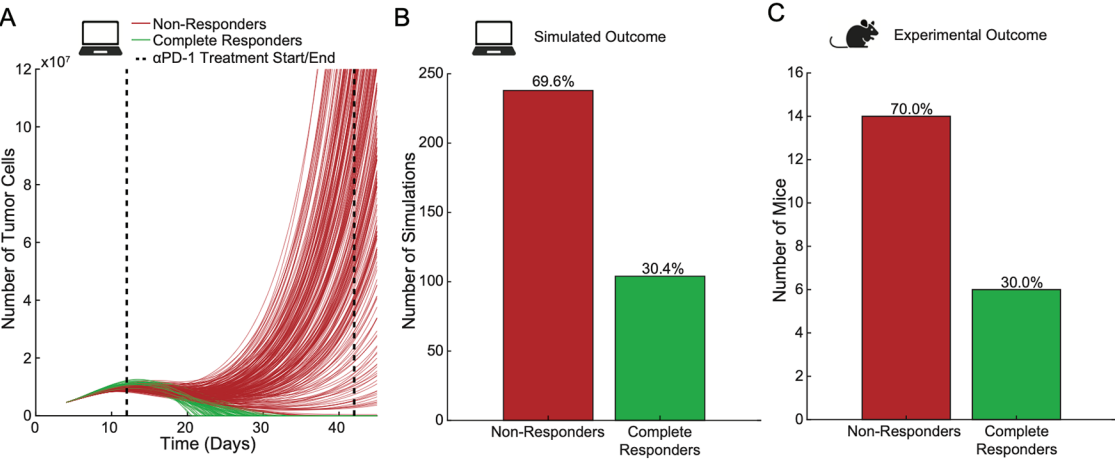


FIGURE 7

