

Mapping Immune Exhaustion Markers in CD8⁺ T Cells within Syngeneic Tumor Microenvironments

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Abstract

The functional state of CD8⁺ T cells within the tumor microenvironment (TME) critically determines antitumor immunity and immunotherapy outcome. However, how tumor location, tumor cell intrinsic features and progression kinetics shape CD8⁺ T cell exhaustion remains poorly defined. We performed a spatial and temporal characterization of immune infiltration and CD8⁺ T cell phenotypes across multiple syngeneic murine tumor models implanted at subcutaneous (SC), orthotopic, and metastatic sites. We examined tumor-infiltrating immune cell dynamics, immune checkpoint (IC) acquisition, and antigen-specific T cell responses at baseline and under anti-PD-L1 treatment. The immune composition of the TME varied with tumor site, developmental stage, and tumor-intrinsic features. Orthotopic and metastatic tumors displayed distinct lymphoid and myeloid infiltration profiles compared to SC tumors. Among head and neck (HN) cancer models, HPV⁻ MOC1 tumors were sensitive to PD-L1 blockade, with expansion of antigen-specific CD8⁺ T cells whereas HPV⁺ TC1 tumors remained refractory despite harboring E7-specific CD8⁺ T cells. Across models, CD8⁺ T cells acquired ICs in a temporally ordered and tumor-dependent manner. TIM-3 emerged as an early marker of exhaustion preceding PD-1 expression, and advanced exhausted populations accumulated preferentially in slower-growing tumors. Taking all models into account, CD8⁺ T cell exhaustion appeared as a dynamic and context-dependent process predominantly governed by tumor antigenic profile and growth kinetics relative to anatomical location. These findings establish a mechanistic framework for understanding T cell differentiation across tumor types and emphasize the need for spatially and temporally faithful preclinical models to guide immunotherapy development.

Introduction

Over the past decade, cancer treatment has undergone a paradigm shift from conventional approaches, such as chemotherapy and radiotherapy, toward immunotherapeutic strategies [1]. These therapies aim to harness and enhance the activity of antitumor effector cells to promote selective elimination of malignant cells. Among these, immune checkpoint blockade (ICB), that targets inhibitory receptors associated with T cell exhaustion, has demonstrated considerable clinical efficacy in several malignancies, including melanoma, non-small cell lung cancer and renal cell carcinoma [2]. However, durable responses are achieved only in a subset of patients, and many common cancer types, such as breast, prostate and colon cancers, remain largely refractory to ICB [3, 4]. Moreover, even within a single patient, heterogeneous responses are often observed across distinct tumor sites, underscoring the profound influence of the tissue-specific tumor microenvironment (TME) on therapeutic outcome.

The TME is composed of diverse cell types, including immune cells that can play both tumor-promoting and tumor-suppressing roles [5]. The density and composition of tumor infiltrating immune cells, particularly CD8⁺ T lymphocytes, play a pivotal role in antitumor immunity. Tumor antigen-specific memory and effector CD8⁺ T lymphocytes can mediate tumor rejection through rapid proliferative capacity, secretion of effector cytokines and potent cytotoxic activity [6]. The functional state of these T cells is shaped by continuous exposure to tumor-derived antigens and complex regulatory signals within

the TME [7]. However, persistent antigenic stimulation within tumors drives CD8⁺ T cells into a state of functional exhaustion (Texh), characterized by reduced effector functions and sustained expression of multiple inhibitory immune checkpoints (ICs), including programmed cell death protein-1 (PD-1), cytotoxic T lymphocyte associated antigen 4 (CTLA-4), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin-domain containing 3 (TIM-3) [8]. CD8⁺ Texh are generally characterized by impaired proliferative responses upon stimulation, and reduced cytokine production, particularly interferon (IFN)- γ . In addition, CD8⁺ Texh exhibit a distinct transcriptional program driven in part by the transcription factor and epigenetic regulator thymocyte selection-associated high mobility group box (TOX) [9], and possess a unique and stable epigenetic landscape [10]. They can be stratified into Texh progenitors (Texh-prog) that give rise to and maintain terminally exhausted (Texh-term) subsets [11, 12]. Texh-prog cells retain proliferative potential, typically express PD-1 and Slamf6, and are enriched near lymphoid structures, whereas Texh-term cells exhibit advanced exhaustion features characterized by high IC expression, diminished proliferation, and limited effector activity [12, 13]. Despite their dysfunction, exhausted CD8⁺ T cells remain crucial for antitumor immunity and serve as key targets of ICB therapy, with their presence often correlating with clinical response [14, 15]. These observations underscore the critical, albeit modulated, role of CD8⁺ Texh in tumor immunity and highlight the necessity for precise monitoring of Texh dynamics in appropriate models.

The trajectory of CD8⁺ T cell exhaustion, including the expression patterns of IC, appears to be modulated, at least in part, by strength and duration of T cell receptor (TCR) signaling [16, 17], IFN signaling [17], immunosuppressive cytokines such as interleukin (IL)-10 [18] and transforming growth factor (TGF)- β [19], further underscoring the multifaceted nature of T cell fate determination in persistent inflammatory environments. In addition, beyond tumor cell-intrinsic properties, the anatomical site of tumor growth plays a key role in shaping the TME, influencing immune cell recruitment, polarization, and function [9, 10]. In this context, T cell exhaustion may represent a dynamic and plastic process influenced by both temporal progression and spatial context. Studies have shown that primary and metastatic tumor locations can impact both tumor progression and CD8⁺ T cell targeting under ICB [16–18, 20]. Nonetheless, the extent to which anatomical localization of tumors and their intrinsic biological properties independently shape CD8⁺ T cell exhaustion phenotypes, and responsiveness to immunotherapy remains undercharacterized.

In patients, genetic heterogeneity between lesions at different sites makes it challenging to investigate tissue-specific effects on tumor development and therapy response. To address this, syngeneic mouse models, where cancer cell lines are implanted into genetically identical hosts, are valuable tools for studying immune responses and evaluating immunotherapy efficacy [21]. Orthotopic models, in which tumor cells are engrafted into their tissue of origin, more accurately reproduce the native TME compared to conventional subcutaneous grafts, thereby improving the translational relevance of preclinical studies. We have developed a panel of such orthotopic and metastatic models encompassing head and neck (HN), colorectal, and liver cancers, previously demonstrating that tissue-specific determinants shape not

only local immune landscapes [22–24] but also systemic immune responses, such as those in metastatic colorectal cancer (mCRC) [25, 26].

In the present study, we systematically investigated how tumor type and anatomical localization collectively influence immune cell infiltration, CD8⁺ T cell exhaustion dynamics, and responsiveness to ICB. Using multiple syngeneic tumor models implanted at subcutaneous, orthotopic, and metastatic sites, we characterized the temporal and spatial regulation of IC co-expression in tumor-infiltrating CD8⁺ T cells. We further dissected the proliferation and differentiation trajectories of these cells in vivo and evaluated their association with antigen specificity and response to anti-PD-L1 therapy. Our findings reveal that tumor cell intrinsic characteristic type overweighs anatomical site in dictating CD8⁺ T cell exhaustion profiles, that advanced exhaustion correlates with antigen-driven activation, and that these features critically determine sensitivity to immunotherapy. Collectively, this study establishes a comprehensive framework for understanding how tumor-intrinsic and site-specific factors shape adaptive immunity in solid tumors and underscores the value of orthotopic syngeneic models for preclinical evaluation of immunotherapies.

Methods

Cell lines and tissue culture.

MC38 (C57BL/6 (B6) background colorectal carcinoma) (RRID: CVCL_0A67), HEPA1-6 (B6 hepatocellular carcinoma, ATCC Cat#CRL-1830), CT26 (BALB/c colorectal carcinoma; ATCC Cat#CRL-2638) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Cat#6429) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat#10270106). TC1 cells were kindly provided by Pr TC Wu (Johns Hopkins University, Baltimore, Maryland, US) and Pr Eric Tartour (INSERM UMR S970, Paris, France) and cultured at 37°C and 5% CO₂ in Roswell Park memorial Institute Medium (RPMI) 1640 GlutaMAX (Invitrogen, Cat#61870044) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat#10270106), 1% Sodium Pyruvate (Sigma, Cat#58636-100M), 1X MEM Non-Essential Amino Acid Solution (Invitrogen, Cat#11140035), 10 mmol/L HEPES (Invitrogen, Cat#15630056), 1% Penicillin-streptomycin (5000U/ml) (Invitrogen, Cat#15070063). MOC1 cells (Mouse Oral Cancer, Kerafast, Cat#EWL001-FP) were cultured at 37°C and 5% CO₂ in Iscove's Modified Dulbecco's Medium (IMDM) (MERCK, Cat#I3390-500M) supplemented with 5% FBS, 1% Penicillin-streptomycin (5000U/ml), 0.1% Insulin (SIGMA, Cat#I6634-50mg), 0.08% Hydrocortison (SIGMA, Cat#H0135-1mg), 5 µg Epidermal Growth Factor (EGF), human recombinant (MILLIPORE, Cat#01-107). [47].

Mice.

6 weeks-old female C57BL/6-Jrj and BALB/c mice were purchased from Janvier Laboratory (Le Genest-Saint-Isle, France). *TetOP-H2B-GFP (H2B-GFP^{tg})* mouse model was kindly provided by Dr Bastien Gerby (CRCT, Toulouse) [48] and in house bred in breeding area of *Centre Régional d'Exploration Fonctionnelle et de Ressources Expérimentales* (CREFRE, Toulouse, France). Mice were housed under specific pathogen

free conditions at CREFRE experimental area. All experimental protocols were approved by the regional Ethic Committee of Toulouse Biological research Federation (C2EA – 01, FRBT) and by the French Ministry for Higher Education and Research (permission number: 50124-2024070111468954). For the guidelines on animal welfare, we followed the European directive 2010/63/EU.

Tumor inoculation, monitoring and treatment.

Subcutaneous (SC) solid tumors were generated by injecting viable tumor cells ($2 \cdot 10^6$ MC38, $5 \cdot 10^4$ TC1, $5 \cdot 10^6$ MOC1, $2 \cdot 10^6$ HEPA1-6 or 10^6 CT26 cells) in the shaved-right flank of the mouse as previously described [22]. Liver metastases were established by intra-hepatic (IH) injections of viable tumor cells ($2,5 \cdot 10^5$ MC38, $5 \cdot 10^4$ TC1, $5 \cdot 10^6$ MOC1, $2 \cdot 10^6$ HEPA1-6, 10^6 CT26) as previously described [26]. Orthotopic MC38 or CT26 colorectal tumors ($5 \cdot 10^5$ MC38, 10^6 CT26 cells) were implanted intra-colon (ICol), sub-serosa into the caecum, as previously described [25, 26, 49, 50]. Orthotopic MOC1 or TC1 oral tumors ($3 \cdot 10^4$ TC1, $5 \cdot 10^6$ MOC1) were implanted intra-oral (IO), specifically within the cheek, by gently retracting the cheek with

forceps and injecting a 10 μ l volume using Hamilton syringe fitted with a 30G needle. The progression of SC and IO tumors was monitored externally by measuring the visible solid tumor masses, on the flank or the surface of the cheek, using a caliper. Mice were euthanized when tumor size reached the ethically defined size of 150 mm². Informations about cell lines are provided in Supplementary Table 1. For the proof-of-concept ICB therapy, 200g/dose of anti-PD-L1 antibody (Ab) (clone 10F.9G2, BioXcell, Cat#BE0101) or Rat IgG2b isotype control Ab (clone LTF-2, BioXcell, Cat#BE0090) were injected intraperitoneally (IP) at day (D)5, D8, D11, D14, D19 (MOC1) and D8, D11, D12, D14 and D19 (TC1).

Cell isolation and cryopreservation.

Tumors and spleen were harvested at multiple timepoints following mice sacrifice. Tumors were sliced in 1–2 mm pieces and digested for 40 minutes (min) using the Tumor Dissociation kit (Miltenyi Biotech, Cat#130-096-730) at 37°C with agitation followed by filtration through a 70 μ m cell strainer. Spleen were directly dissociated by filtration through a 70 μ m cell strainer. An Ammonium-Chloride-Potassium (ACK) buffer (BD Biosciences, Cat#555899) erythrocyte lysis step (5 min at room temperature) was performed on spleen cell suspensions. All cell suspensions were frozen in cryovials, in FBS supplemented with 10% Dimethyl sulfoxide (DMSO) (Sigma, Cat#D2650) and stored at -80°C.

Flow cytometry.

Cells were thawed and resuspended in PBS, 2.5% FBS (staining buffer), containing anti-mouse CD16/CD32 monoclonal Ab (mAb) (clone 2.4G2, 1:100; BD Biosciences, Cat#553142) and incubated 10 min on ice. For dead cell exclusion, cells were centrifuged and resuspended in PBS containing Fixable Viability Stain 700 (1:1000; BD Biosciences, Cat#564997,) or Stain 780 (1:1000; BD Biosciences, Cat#565388) for 10 min, in the dark, at room temperature. Cells were then washed twice in PBS. For

MHC I/peptide dextramer staining, cells were incubated with 20 µl of PBS with 5% FBS containing PE-labeled H-2 K^b/ KSPWFTTL dextramers (mutated peptide from P15E MOC1 antigen, Immudex, Cat# JD03702 PE 50) and PE-labeled H-2 D^b/ RAHYNIVTF dextramers (HPV16 E7₄₉₋₅₇ peptide, Immudex, Cat# JA02195 PE 15) for 30 min, in the dark, at room temperature. No wash was performed before subsequent surface staining with monoclonal antibodies (mAbs). For surface staining, cells were incubated with combinations of different anti-mouse mAbs (Supplementary Table 2), in staining buffer, for 20 min, in the dark, at 4°C. For intracellular/intranuclear staining, cells were permeabilized 30 min at 4°C using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Cat#00-5523-00) except following dextramer staining where cell were permeabilized using Fixation/Permeabilisation kit (BD Biosciences, Cat#554714) for 20 min at 4°C. Stainings with different anti-mouse mAbs (Supplementary Table 2) directed against intracellular/intranuclear markers were then performed for 30 minutes at 4°C. Flow cytometry analyses were performed using BD LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using Flowlogic software (Miltenyi).

Statistical analyses.

The sample size for animal studies were guided by previous murine studies in our laboratory [25]. Tumor growth and flow cytometry and were analyzed using GraphPad Prism software. For group comparison, Student's *t* test, Mann-Whitney test and analyses of variance (ANOVAs) (Tukey's were used following evaluation of normality using Shapiro-Wilk normality test. For tumor sizes monitoring, two-way ANOVA (Sidak) was used. $P < 0.05$ was considered statistically significant for all assays, and individual *P* values are denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Results

Immune cell infiltration in tumors varies with stage and anatomical location

To assess how tumor location influences the immune composition of the TME, we implanted various syngeneic tumor cell lines into SC, orthotopic (primary tumor: intra-oral (IO), intra-colon (ICol) or intra-hepatic (IH)) and metastatic (liver) sites. The models encompassed HN cancers, represented by two C57BL/6 (B6) background cell lines differing in human papilloma virus 16 (HPV16) E7 expression (non-expressing^{(negative(neg))} MOC1 and expressing^{(positive(pos))} TC1 cells). It also included colorectal cancer models, with cell lines from both B6 (MC38) and BALB/c (CT26) backgrounds, and hepatocarcinoma modeled using the B6 background HEPA1-6 cell line (**Fig. 1a**). Tumors were harvested at both early and late stages, and the composition of lymphoid and myeloid infiltrates was characterized by flow cytometry (**Fig. S1a to S1c**). We compared the immune TME, at early and late stages of tumor development, either within each tumor type across different anatomical sites, or, within the same location, between the tumor models with distinct antigenic profiles. All tumor types showed infiltration by both lymphoid and myeloid cells (**Fig. 1b to 1g**). Consistent with our previous findings [22], we observed substantial heterogeneity in the TME immune cell composition for the same tumor cell line

when implanted at different anatomical sites (**Fig. 1b to 1g**). Regarding myeloid cell populations, in addition to tumor location, their proportions also varied according to tumor cell origin, mouse genetic background and tumor development stage. Tumors from HN HPV^{pos} TC1 tumor model contained the highest proportions of myeloid subsets (**Fig. 1b to 1d**). HPV^{pos} TC1 tumors contained the highest proportion of macrophages at both early and late stages (**Fig. 1h**). In comparison, HPV^{neg} MOC1 tumors exhibited the greatest abundance of MDSC (**Fig. 1i**). In CRC models, immune infiltration patterns were different according to mouse genetic backgrounds. For instance, CT26 BALB/c tumors, regardless of anatomical location, showed higher macrophage infiltration compared with MC38 B6 tumors (**Fig. 1j**). Consistent with our previous observations in the MC38 tumor model [25], the overall proportion of myeloid cells increased over time (**Fig. 1k**). The proportion of tumor-infiltrating T cells also varied by tumor location across all models (**Fig. 1e to 1g**), emphasizing the role of the surrounding tissue environment in shaping adaptive immune cell recruitment and retention in the TME [22]. Notably, at early stages, SC HEPA1-6 tumors displayed the highest proportion of total T cells compared to the other tumors (**Fig. 1l**). Furthermore, MC38 tumors exhibited higher infiltration by conventional CD4⁺ (Tconv, FoxP3⁻) (**Fig. 1e to 1g**) and CD8⁺ (**Fig. 1m**) T cells compared to CT26 tumors, particularly at orthotopic and metastatic locations, reflecting features of an immune inflamed tumor phenotype in this model. In HN tumor models, a higher proportion of CD8⁺ T cell infiltration was observed in HPV^{neg} MOC1 compared with HPV^{pos} TC1 tumors (**Fig. 1m, Fig. S1e**), suggesting that MOC1 tumors may have a greater potential to generate an anti-tumor immune response and respond to CD8⁺ T cell-targeted immunotherapy.

Figure 1. Spatial and temporal heterogeneity of immune cell infiltration across syngeneic tumor models. (a) B6 mice were implanted with MOC1, TC1, MC38, CT26 and HEPA1-6 cells at different anatomical sites (Mets = metastasis; Ortho = orthotopic (intra-oral (IO) or intra-colon (ICol) or intra-hepatic (IH)); SC = subcutaneous) as depicted. (b to g) Proportion of (b to d) myeloid-derived suppressive (MDSC), dendritic cells (DC), macrophages (Macro) and (e to g) CD8⁺ T cells (CD8), CD4⁺ conventional T cells (Tconv) and CD4⁺ regulatory T cells (Treg) at early (top panels) and late (bottom panels) stages of tumor progression in (b and e) SC ($n = 5-10$), (c and f) orthotopic ($n = 5-10$) and (d and g) metastatic site ($n = 5-10$). Proportion of (h) Macro in early orthotopic TC1 tumors versus the other tumors, (i) MDSC in early orthotopic MOC1 tumors versus the other tumors, (j) Macro in early orthotopic CT26 versus MC38 tumors, (k) myeloid cells in early versus late MC38 Mets, (l) T cells in HEPA1-6 tumors versus the other tumors. (m) Percentage of CD8⁺ T cells at early stage in all sites ($n = 5-10$). Green * indicate statistics between tumor types and black * indicate statistics between location within each tumor type. Each dot represents an individual mouse. Student's *t* test, Mann-Whitney test and One-way ANOVA with Tukey's test were used. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Sequential acquisition of ICs reflects progressive differentiation and proliferation of tumor-specific CD8 T cells

To further characterize the temporal dynamics of IC acquisition, we utilized doxycycline-inducible *H2B-GFP^{tg}* mice, an in vivo model that enables the tracking of cell division via GFP dilution [31, 32]. Following a five-week doxycycline pulse to GFP-label cells, doxycycline was withdrawn (chase period), and mice were implanted IO or SC with MOC1 tumors on day 0 (**Fig. 4a**). During the doxycycline chase period, tumors were collected at various timepoints (**Fig. 4a**). We then assessed the in vivo cell division kinetics of IC-expressing CD8⁺ T cell subpopulations by monitoring the progressive loss of GFP expression (**Fig. 4b**). At the end of the pulse period, on the day of tumor implantation, virtually all leukocytes expressed H2B-GFP, as confirmed in spleens from control mice (**Fig. 4b**). Over time, GFP intensity gradually decreased in total leukocytes, without any significant change in their overall abundance (**Fig. 4b and 4c**). By day 30, GFP expression was largely absent in leukocytes (**Fig. 4b**), limiting reliable analyses of tumor immune infiltrate to the early timepoints, up to day 9. CD8⁺ T cells infiltrating MOC1 tumors, regardless of implantation site, exhibited decrease in GFP expression over time (**Fig. 4d**) indicating cell proliferation. This proliferation was paralleled by an increased frequency of tumor-infiltrating CD8⁺ T cells over time (**Fig. 4e**), indicating that the loss of GFP is associated with enhanced tumor infiltration and proliferation of tumor-specific CD8⁺ T cell. Tumor-infiltrating CD8⁺ T cells, including P15E-specific cells, showed a loss of GFP content as early as day 5, which further decreased by day 9. This dynamic paralleled the increase in both total CD8⁺ T-cell infiltration and the frequency of P15E dextramer⁺ cells (**Fig. 4d to 4g**). These findings suggest sustained activation and proliferation of tumor-specific T cells. The progressive loss of GFP expression was associated with the stepwise acquisition of ICs in both IO and SC MOC1 tumors, with QP CD8⁺ T cell subpopulations displaying the lowest GFP signal (**Fig. 4h**). Together, these results indicate that ICs are acquired in a temporally ordered manner as CD8⁺ T cells undergo progressive activation and proliferation in response to tumor.

Given the established role of ICs in attenuating the effector functions of tumor-specific CD8⁺ T cells to effectively eliminate tumor cells and considering the increased proportions of advanced exhausted CD8⁺ T cells in MOC1 tumors (**Fig. 2i**), we next investigated the efficacy of immunotherapy in these HN cancer models. Mice bearing SC MOC1 or TC1 tumors were treated with anti-PD-L1, as a proof-of-concept ICB. Relative to TC1, MOC1 tumors exhibited greater sensitivity to immunotherapy, with complete responses observed in 3 out of 11 treated mice and partial responses in 5 out of 11 (**Fig. 5a and 5b**). TC1 SC tumors were completely unresponsive to ICB, exhibiting no observable therapeutic response (**Fig. 5c and 5d**). Anti-PD-L1 treatment significantly enhanced the infiltration of MOC1 tumors by P15E-specific CD8⁺ T cells (**Fig. 5e**), whereas the proportions of tumor-infiltrating HPV16 E7-specific CD8⁺ T cells remained unchanged in treated mice harboring TC1 tumors (**Fig. 5f**). Notably, the robust infiltration by P15E-specific CD8⁺ T cells in MOC1 tumors was systematically associated with tumor regression, whereas the TC1 model, lacking such infiltration, showed no tumor regression (**Fig. 5e, 5f**). These results suggest that the response to ICB is associated with ongoing tumor recognition by antigen-specific T cells that progress along the exhaustion trajectory.

Figure 4. Progressive loss of GFP expression parallels stepwise acquisition of IC in Head and Neck cancer model. (a) *H2B-GFP^{tg}* mice received doxycycline (DOX) pulse during 5 weeks, DOX chase occurred at day (D)0 when mice were implanted with MOC1 cells at subcutaneous and intra-oral locations as depicted. Tumors were harvested (harv) at day 5, 9 and 30. Percentage of (b) Green Fluorescent Protein (GFP) in leukocytes (leuko) and (c) leukocytes in total cells from naive spleens at different time points ($n = 3$ for D0; $n = 6$ for D5 and 9; $n = 5$ for D30). (d to h) Percentage of (d, f, h) GFP⁺ cells, (e) CD8⁺ T cells and (g) P15E-specific CD8⁺ T cells, among (d) CD8⁺ T cells, (e and g) leukocytes, (f) P15E-specific CD8⁺ T cells, (h) the most frequent IC-expressing CD8⁺ T cell subpopulations (subpop) at day 9 in MOC1 SC (left panels) and IO (right panels). Each dot represents an individual mouse. One-way ANOVA with Tukey's test was used. $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Discussion

CD8⁺ T lymphocytes are central mediators of anti-tumor immunity, and their phenotypic and functional states within the TME critically determine therapeutic outcome. Here, using multiple syngeneic murine tumor models across distinct anatomical sites, we provide an integrated spatial and temporal analysis of CD8⁺ T cell exhaustion. By comparing tumors of varying antigenicity, growth kinetics, and tissue location, we show that tumor-intrinsic factors primarily dictate the dynamics of IC acquisition and the establishment of T cell exhaustion.

Notwithstanding the importance of tumor cell intrinsic factors, our study extends prior work from us [22, 26] and others [33, 34], demonstrating that, in addition, the anatomical site of tumor growth modulates immune infiltration and therapy response. Consistent with these findings, we found substantial heterogeneity in immune cell composition between SC, orthotopic and metastatic tumors, despite their derivation from identical tumor cell lines. These site-specific immune landscapes likely reflect distinct tissue-resident stromal and immune cues influencing cell recruitment, migration and polarization. Furthermore, we show that temporal progression adds another layer of complexity, as the TME evolves markedly between early and late tumor stages. Together, these findings highlight the importance of considering both spatial and temporal variables when modeling antitumor immunity and predicting immunotherapy outcomes.

Beyond differences between the TME immune composition, our data reveal profound variations in CD8⁺ T cell functional states across models. In HN cancer models, the HPV⁻MOC1 tumors displayed higher CD8⁺ T cell infiltration and greater sensitivity to anti-PD-L1 therapy than the HPV⁺ TC1 tumors. These results contrast with human HN cancers, where HPV positivity is associated with improved ICB responsiveness [35, 36]. This discrepancy may arise from intrinsic features of the murine models: TC1 cells, transformed with HPV16 E6/E7 and Ras oncogenes [37], may not faithfully mimic the immunobiology of HPV⁺ oral tumors, while MOC1 cells, derived from carcinogen-induced oral squamous carcinoma [38], more accurately recapitulate the mutational and immunologic landscape of HPV⁻ HN cancers [39]. In addition, the higher macrophage infiltration and more immunosuppressive milieu in TC1

tumors may further restrain T cell effector functions and explain their ICB resistance [40]. This suggested that the slower progression of MOC1 tumors could permit more extensive CD8⁺ T cell activation, reflected by acquisition of IC.

A key insight from our analysis is that the trajectory of CD8⁺ T cell exhaustion, defined by the stepwise co-expression of multiple ICs, is shaped primarily by tumor-intrinsic properties, likely antigenicity, rather than anatomical context. In the MOC1 model, CD8⁺ T cells progressively acquired advanced exhausted phenotypes (TP and QP subpopulations), associated with high TOX expression and loss of Slamf6, indicative of a transition toward terminal exhaustion. Conversely, in the more aggressive TC1 and HEPA 1–6 models, CD8⁺ T cells failed to reach fully exhausted states, suggesting that rapid tumor progression may limit the adaptive antitumor response. These findings support the use of models in which the extent and sequence of IC expression reflect both the duration and intensity of antigenic stimulation to study exhaustion-related mechanisms.

Using the *H2B-GFP* model, we provide in vivo evidence that ICs acquisition occurs in a temporally ordered manner as CD8⁺ T cells are activated and proliferate in the tumor microenvironment. In MOC1 tumors, TIM-3⁺PD-1⁻ cells displayed lower proliferation, as indicated by higher GFP content, identifying them as an early, less-advanced subset that precedes the emergence of fully exhausted populations. Notably, TIM-3 expression appeared before substantial PD-1 upregulation in MOC1 tumors, unlike in TC1 or CT26 tumors, suggesting that the timing of ICs expression is influenced by the tumor context. This observation challenges the view of PD-1 as an universal early marker of exhaustion [41, 42] and indicates that high TIM-3 expression, although generally associated with more advanced states of T-cell exhaustion [43], may also be detected at earlier stages in a tumor-dependent manner. These findings support the emerging view of T cell exhaustion as a dynamic differentiation continuum shaped by persistent antigen exposure and tumor-specific microenvironmental cues [44]. Thus, while ICs expression is associated with progressive T-cell dysfunction, the kinetics of individual receptors may not strictly reflect a defined stage of exhaustion and may instead be modulated by factors such as antigen load and inflammatory signaling.

The accumulation of QP exhausted CD8⁺ T cells in MOC1 tumors coincided with improved responsiveness to anti-PD-L1 therapy, reinforcing the concept that exhausted but antigen-specific T cells remain critical mediators of therapeutic benefit. In this model, the presence of P15E-specific CD8⁺ T cells, expressing high levels of ICs and TOX indicates ongoing antigen-driven activation and supports the notion that advanced exhaustion may represent a regulated differentiation endpoint rather than irreversible dysfunction. In addition, in the MOC1 tumor model, we observed an early accumulation, from day five, of tumor antigen-specific CD8⁺ T cells. At this early timepoint, many ICs are already detectable by flow cytometry analyses, in CD8⁺ T cells. This supports prior findings [29] and aligns with the concept that exhaustion begins early during T cell activation in tumors. The timing of assessment of exhaustion in preclinical studies is therefore critical. Indeed, in clinical settings, the detection of exhausted CD8⁺ T cells in pre-treatment and early on-treatment tumor samples correlates with improved response to ICB

[45], implying that a pre-existing antitumor T cell response has been activated. Conversely, exhaustion signatures in late on-treatment and post-treatment samples are associated with poor outcome [46], indicating failed tumor clearance despite therapeutic intervention.

Our findings provide a mechanistic framework to interpret our previous observations in patients with epithelial malignancies [27, 28]. In human tumors, we showed that the co-expression of PD-1, TIGIT, CTLA-4 and TIM-3 in CD8⁺ T cells was associated with distinct degrees of exhaustion and could be linked to clinical response to ICB [27]. However, because human tumors are typically analyzed at discrete time points, the temporal sequence of ICs acquisition during tumor progression cannot be directly reconstructed. The use of syngeneic mouse models therefore provides a complementary approach, allowing us to longitudinally track the emergence and co-expression of these receptors within the same tumor context. Our data show that the progressive accumulation of ICs observed in human tumors is recapitulated in mice, while further revealing that the order of IC acquisition can vary according to tumor-intrinsic features. Thus, the mouse models extend our observations in patients by providing temporal resolution to a process that can only be inferred from the phenotypic states captured in human tumor samples. Importantly, this supports the concept that the co-expression of multiple ICs reflects a dynamic differentiation trajectory rather than a fixed or universally ordered sequence, with the precise kinetics shaped by the tumor context.

Collectively, our findings demonstrate that CD8⁺ T cell exhaustion is not a uniform process, but a dynamic, tumor-dependent continuum shaped by intrinsic tumor properties, antigenicity, and growth kinetics. Anatomical site and tissue context modulate the composition of the TME but do not fundamentally alter the trajectory of exhaustion once initiated. This has critical implications for preclinical modeling and translational interpretation: models must be carefully selected to capture both the antigenic and temporal dimensions of immune regulation. Furthermore, the identification of antigen-specific, multi-ICs-expressing CD8⁺ T cells as correlates of responsiveness highlights their potential as predictive biomarkers for immunotherapy efficacy. These findings underscore the need for physiologically relevant orthotopic and metastatic models in preclinical immuno-oncology and pave the way for strategies aimed at modulating exhaustion trajectories to optimize therapeutic benefit.

Abbreviations

ACK

Ammonium-Chloride-Potassium

B6

C57BL/6

CTLA-4

cytotoxic T lymphocyte associated antigen 4

D

day

DC

dendritic cells

DMSO

Dimethyl sulfoxide

DP

double-positive

FBS

fetal bovine serum

GFP

green fluorescent protein

HN

head and neck

HPV16

human papilloma virus 16

IC

immune checkpoint

ICol

intra-colon

ICB

immune checkpoint blockade

IFN

interferon

IH

intra-hepatic

IL

interleukin

IMDM

Iscoe's Modified Dulbecco's Medium

IP

intraperitoneally

Macro

macrophages

mCRC

metastatic colorectal cancer

MDSC

myeloid-derived suppressive

Mets

metastatic

MFI

mean fluorescence intensity

min

minutes

IO

intra-oral

Ortho

orthotopic

PD-1

programmed cell death protein-1

PD-L1

PD-1 ligand

QP

quadruple positive

RPMI

Roswell Park memorial Institute Medium

SC

subcutaneous

SP

single-positive

Subpop

subpopulations

Tconv

CD4⁺ conventional T cells

TCR

T cell receptor

Texh

Exhausted T cells

Texh-prog

Texh progenitors

Texh-term

T terminally exhausted

TGF

transforming growth factor

TIGIT

T cell immunoreceptor with Ig and ITIM domains

TME

tumor microenvironment

TOX

thymocyte selection-associated high mobility group box

Treg

CD4⁺ regulatory T cells

TP

Declarations

Competing interests

The authors declare that they have no competing interests.

Author Contribution

Designing research studies: M.A. and C.D. Development of methodology: V.F., M.M. and C.D. Conducting experiments: V.F., M.M., Y.B., L.S., A.S., CM.S. Acquiring and analysing data: V.F., M.M., Y.B., L.S. Providing reagents/mice: B.G. Writing the manuscript: VF. J.P.D., M.A., C.D.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

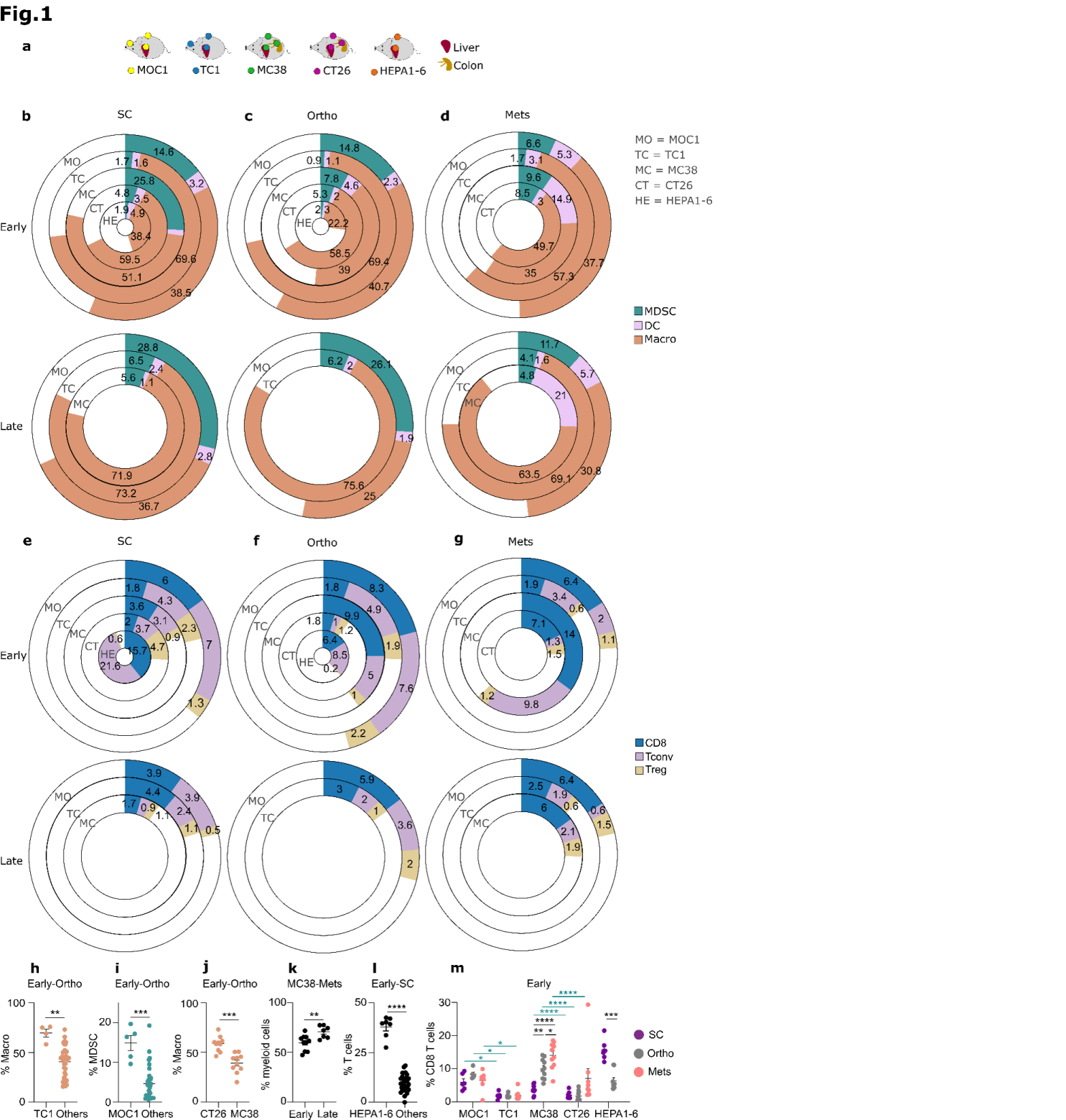


Figure 1

Spatial and temporal heterogeneity of immune cell infiltration across syngeneic tumor models. (a) B6 mice were implanted with MOC1, TC1, MC38, CT26 and HEPA1-6 cells at different anatomical sites (Mets = metastasis; Ortho = orthotopic (intra-oral (IO) or intra-colon (ICol) or intra-hepatic (IH)); SC = subcutaneous) as depicted. (b to g) Proportion of (b to d) myeloid-derived suppressive (MDSC), dendritic cells (DC), macrophages (Macro) and (e to g) CD8⁺ T cells (CD8), CD4⁺ conventional T cells (Tconv) and

CD4⁺ regulatory T cells (Treg) at early (top panels) and late (bottom panels) stages of tumor progression in (b and e) SC ($n = 5-10$), (c and f) orthotopic ($n = 5-10$) and (d and g) metastatic site ($n = 5-10$). Proportion of **(h)** Macro in early orthotopic TC1 tumors versus the other tumors, **(i)** MDSC in early orthotopic MOC1 tumors versus the other tumors, **(j)** Macro in early orthotopic CT26 versus MC38 tumors, **(k)** myeloid cells in early versus late MC38 Mets, **(l)** T cells in HEPA1-6 tumors versus the other tumors. **(m)** Percentage of CD8⁺ T cells at early stage in all sites ($n = 5-10$). Green * indicate statistics between tumor types and black * indicate statistics between location within each tumor type. Each dot represents an individual mouse. Student's *t* test, Mann-Whitney test and One-way ANOVA with Tukey's test were used. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Fig.2

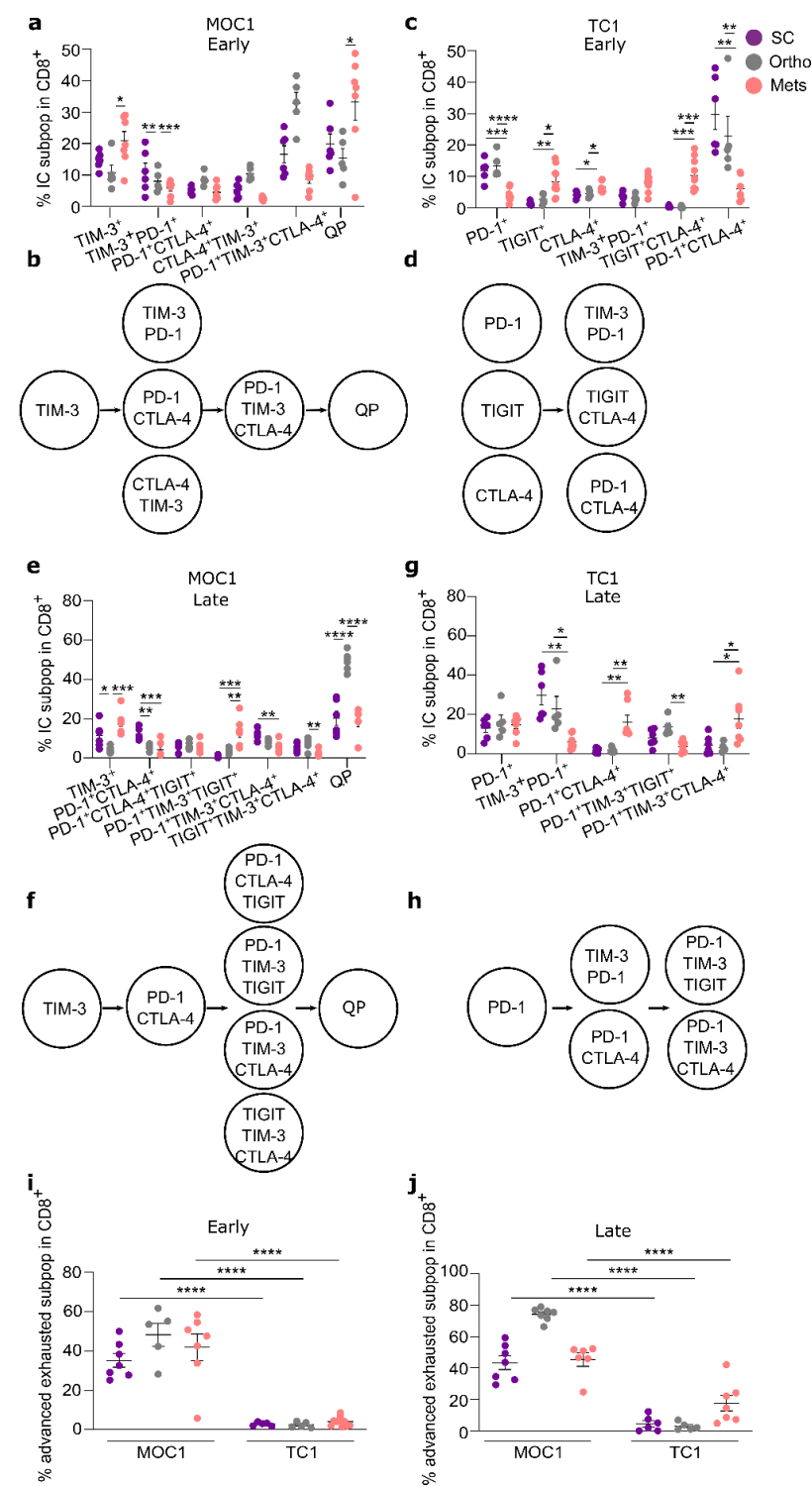


Figure 2

Expression of immune checkpoints in CD8⁺ T cells in HPV^{neg} and HPV^{pos} head and neck cancer models. Mice were implanted with tumors as described in Fig 1a. **(a, c, e, g)** Percentages and **(b, d, f, h)** representation of immune checkpoints (IC) expressing CD8⁺ T cells subpopulations (subpop) in **(a, b, e, f)** HPV^{pos} MOC1 (*n* = 5-7) and **(c, d, g, h)** HPV^{neg} TC1 (*n* = 4-7) tumors at **(a to d)** early and **(e to h)** late stages at subcutaneous (SC), orthotopic (Ortho) and metastatic (Mets) sites. Mean proportions, among

CD8⁺ T cells, for the subpop was >5%, for at least one of the three site of implantation [27]. (i and j) Percentage of advanced exhausted CD8⁺ T cell subpopulations (triple and quadruple IC positive subpopulations) in (i) early and (j) late MOC1 and TC1 tumors at all sites. Each dot represents an individual mouse. One-way ANOVA with Tukey's test was used. **P* ≤ 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig.3

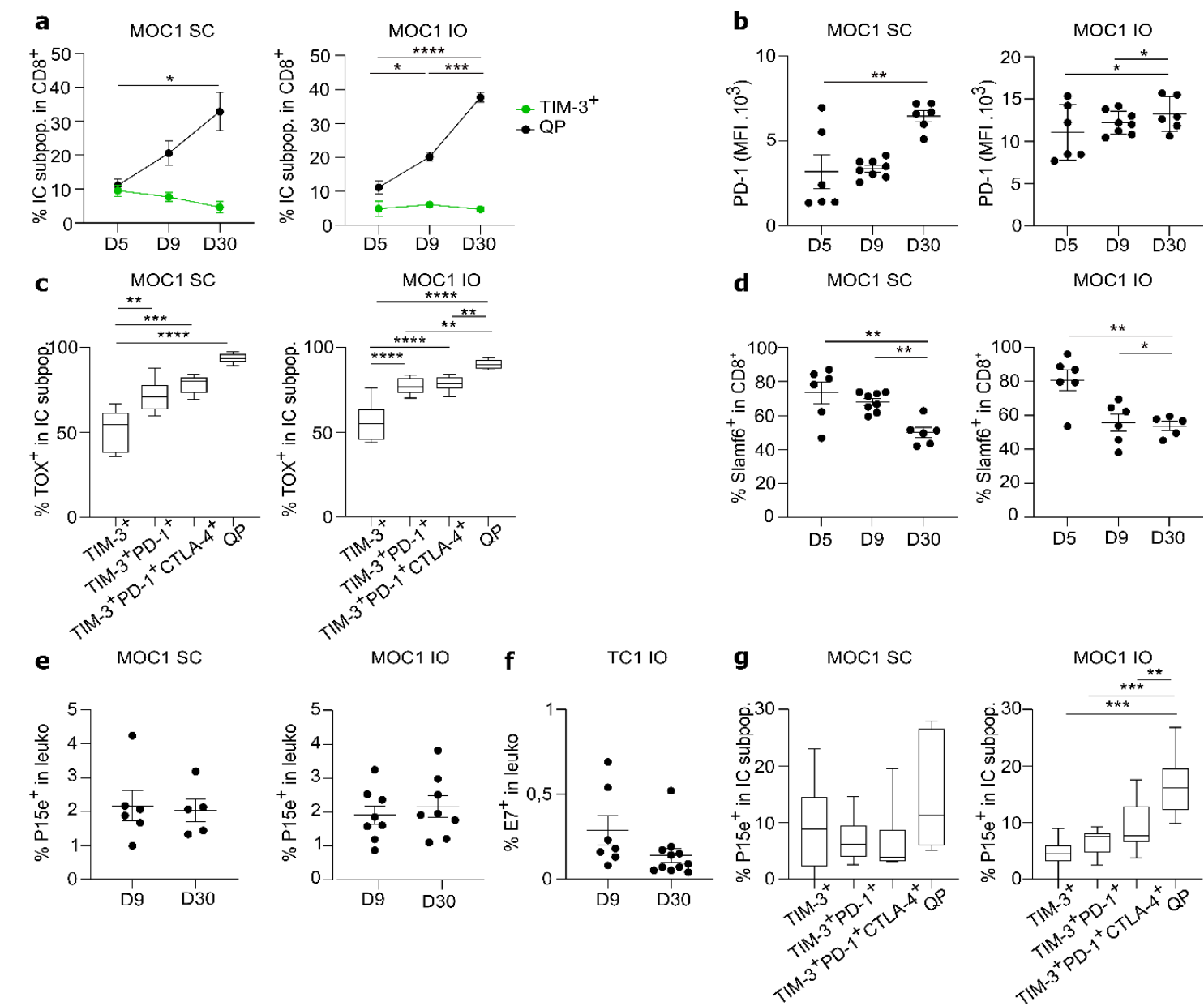


Figure 3

Exhaustion in CD8 T cells profiles in Head and Neck cancer. Mice were implanted on day 0 with MOC1 or TC1 cells subcutaneously (SC) and orthotopically (intra-orally, IO). (a) Percentage of immune checkpoint (IC)-expressing CD8⁺ T-cell subpopulations (subpop), slightly exhausted (single positive TIM3⁺) or advanced exhausted (quadruple positive (QP)), (b) mean of fluorescence intensity (MFI) of PD-

1 on CD8⁺ T cells, **(c)** percentage of TOX⁺ cells among the most frequent IC-expressing CD8⁺ T-cell subpopulations infiltrating MOC1 SC and IO tumors, at day 9, **(d)** percentage of Slamf6⁺ cells among CD8⁺ T cells and **(e, g)** percentage of P15e-specific CD8⁺ T cells detected by dextramer staining within (e) leukocytes (leuko) and (g) the most frequent IC-expressing CD8⁺ T cell subpopulations in MOC1 SC (left panels) and IO (right panels) tumors at day 9. **(f)** Percentage of dextramer E7-specific CD8⁺ T cells among leukocytes in TC1 SC tumors. Each dot represents an individual mouse. One-way ANOVA with Tukey and Student's *t* test were used. **P* ≤ 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Fig.4

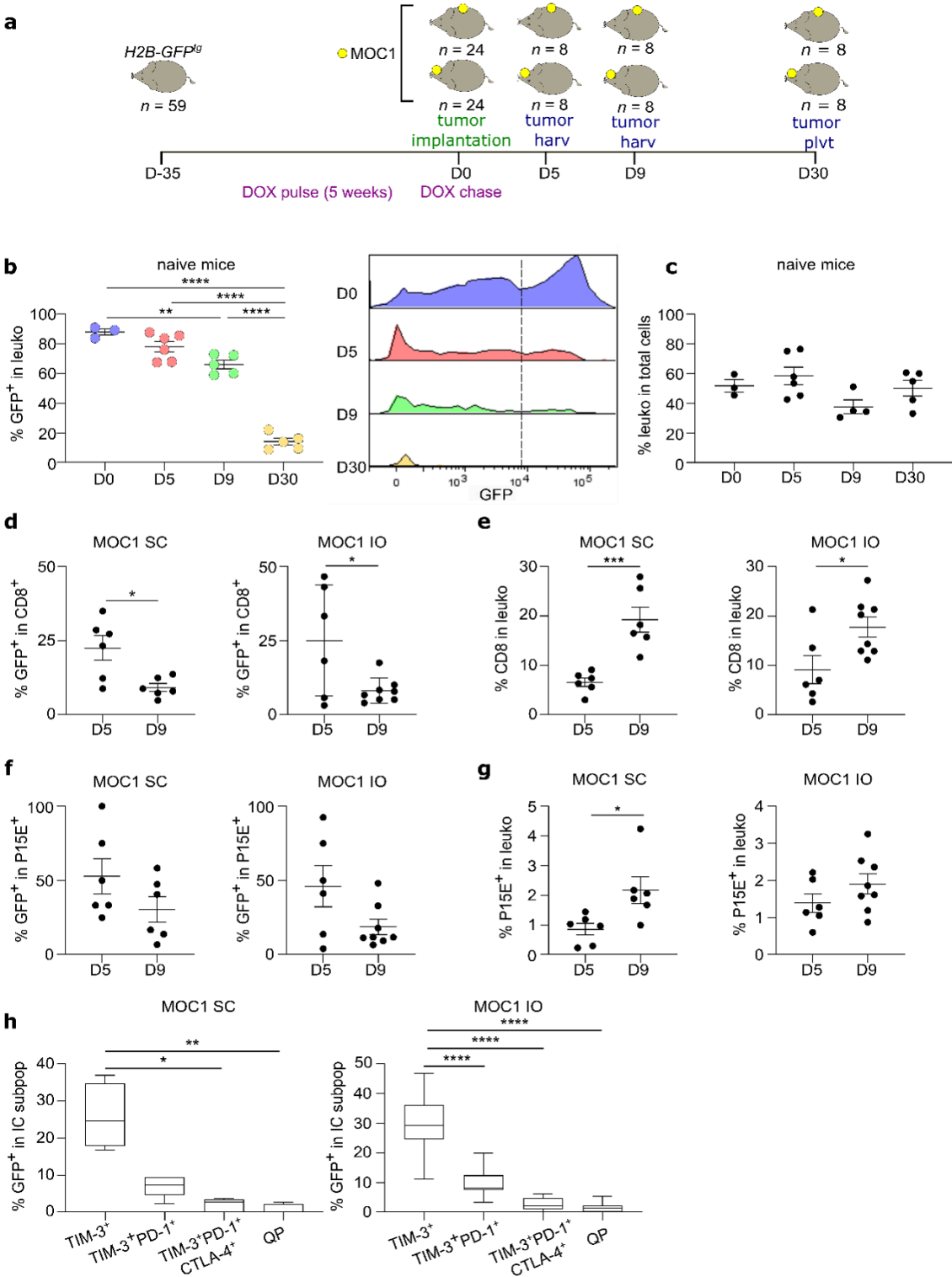


Figure 4

Progressive loss of GFP expression parallels stepwise acquisition of IC in Head and Neck cancer model. (a) *H2B-GFP^{tg}* mice received doxycycline (DOX) pulse during 5 weeks, DOX chase occurred at day (D)0 when mice were implanted with MOC1 cells at subcutaneous and intra-oral locations as depicted. Tumors were harvested (harv) at day 5, 9 and 30. Percentage of (b) Green Fluorescent Protein (GFP) in leukocytes (leuko) and (c) leukocytes in total cells from naive spleens at different time points ($n = 3$ for

D0; $n = 6$ for D5 and 9; $n = 5$ for D30). (d to h) Percentage of (d, f, h) GFP⁺ cells, (e) CD8⁺ T cells and (g) P15E-specific CD8⁺ T cells, among (d) CD8⁺ T cells, (e and g) leukocytes, (f) P15E-specific CD8⁺ T cells, (h) the most frequent IC-expressing CD8⁺ T cell subpopulations (subpop) at day 9 in MOC1 SC (left panels) and IO (right panels). Each dot represents an individual mouse. One-way ANOVA with Tukey's test was used. * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Fig.5

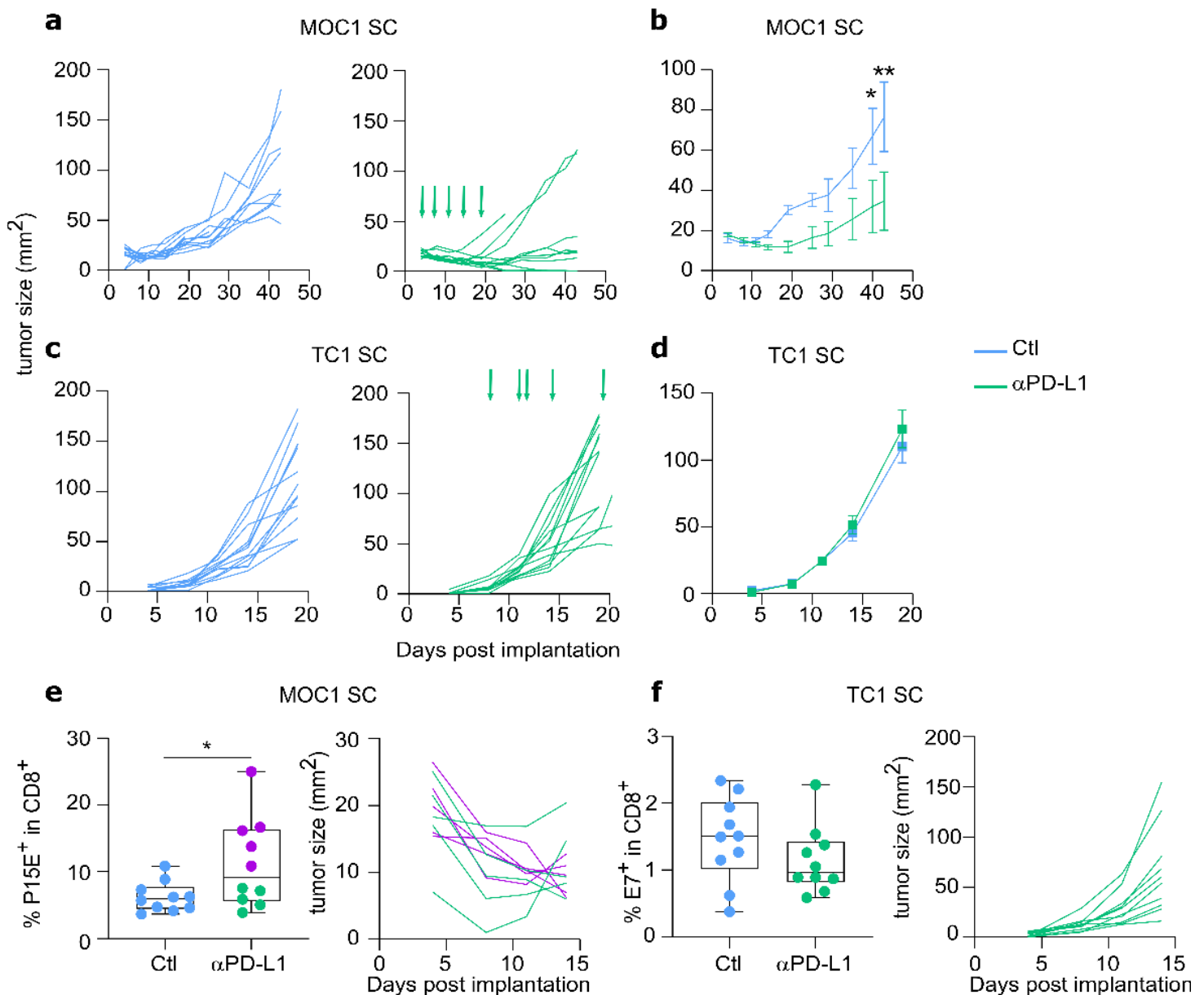


Figure 5

Immunotherapy expands tumor-specific CD8 T cells. Mice were implanted subcutaneously (SC) with MOC1 and TC1 cells and treated with anti-PD-L1 (aPD-L1) or isotype control (Ctl) antibodies on day (D)5, D8, D11, D14, D19 (MOC1 model) and D8, D11, D12, D14 and D19 (TC1 model) (aPD-L1 treatment

indicated by arrows). **(a, c)** Individual and **(b, d)** average SC tumor sizes in aPD-L1-treated ($n = 24$) and control ($n = 24$) groups in (a, b) MOC1 and (c, d) TC1 tumor-bearing mice. **(e and f)** Percentage of (e) P15E- and (f) E7-specific CD8⁺ T cells in (e) MOC1 and (f) TC1 SC tumors (Ctl ($n = 10$), aPD-L1 ($n = 10$)) (left panels) and (e) MOC1 and (f) TC1 SC tumor sizes in aPD-L1-treated mice (right panels) on day 19. Purple points and lines on (e) indicate tumors with P15E-specific CD8⁺ T cell infiltration above the median ($n = 5$). Each dot represents an individual mouse. Sidak, one-way ANOVA with Tukey and Student's t test were used. $*P \leq 0.05$, $**P < 0.01$.

Supplementary Files

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