Title: Therapeutic scheduling of WEE1 inhibition preserves T cell function and promotes immune control of HPV$^+$ tumors

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Short title: Intermittent WEE1i enables immune control of HPV$^+$ tumors

Abstract

Human papillomavirus–associated oropharyngeal carcinoma (HPV$^+$ OPC) is primarily driven by viral E6 and E7 oncoproteins, which disrupt G1 checkpoint control and impose a selective dependency on WEE1-mediated G2/M regulation. While this vulnerability confers sensitivity to WEE1 inhibition, its immunologic consequences remain poorly defined. Here, we show that WEE1 inhibition elicits durable antitumor immunity in immunocompetent models of HPV$^+$ OPC. Using murine and human preclinical systems, we demonstrate that the WEE1 inhibitor ZN-c3 (azenosertib) mediates tumor control through cell-autonomous cytotoxicity and immune-dependent mechanisms requiring T cells, and conventional dendritic cells. Mechanistically, HPV$^+$ tumor cells are deficient in STING signaling and fail to mount canonical type I interferon responses *in vitro*. Instead, tumor cell–intrinsic cGAS initiates immune

activation through STING-competent host cells within the tumor microenvironment. Intermittent WEE1 inhibition preserves T cell fitness while maintaining antitumor efficacy, establishing WEE1 inhibition as an immune-enabling therapeutic strategy in HPV$^+$ OPC.

Significance

WEE1 inhibition induces durable antitumor immunity against HPV$^+$ oropharyngeal tumors through coordinated tumor cell–intrinsic DNA damage sensing and host STING–dependent immune activation. These findings establish intermittent WEE1 inhibition as an immune-permissive therapeutic strategy that enables antigen-specific T cell responses and provides a mechanistic foundation for rational combination with immunotherapy in HPV-driven malignancies.

Introduction

Human papillomavirus–associated oropharyngeal squamous cell carcinoma (HPV$^+$ OPC) incidence is rising in the United States (1, 2). Although many patients are curable, standard chemo-radiotherapy (CRT) causes substantial toxicity and fails a clinically significant subset with recurrence-prone disease (3), underscoring the need for therapies that enable safe de-escalation while improving durable tumor control.

HPV-driven carcinogenesis is mediated primarily by the viral E6 and E7 oncoproteins, which inactivate p53 and RB, respectively, disrupting G1 checkpoint control (4, 5). This creates dependence on G2–M checkpoint regulation, rendering HPV$^+$ cancers sensitive to pharmacologic WEE1 inhibition (WEE1i) (6-10). Consistent with this vulnerability, the first-generation WEE1 inhibitor AZD1775 shows activity in HPV$^+$ cancers, which we previously linked to elevated FOXM1 activity (9).

Beyond cell-autonomous cytotoxicity, WEE1i–induced DNA damage has been linked to antitumor immune responses in solid tumors, in part through activation of the cGAS–STING pathway (11-13). However, because HPV infection antagonizes tumor cell–intrinsic STING signaling (14, 15), the relevance of this pathway to WEE1i efficacy in HPV$^+$ OPC remains unclear.

Despite promising preclinical activity, clinical translation of WEE1 inhibitors has been limited by dose-limiting hematologic toxicities, particularly with AZD1775 (16, 17). Emerging evidence suggests that inhibiting WEE1 can directly affect immune cell proliferation and function (11, 12, 18), raising unresolved questions about how to elicit antitumor immunity without compromising immune fitness required for durable tumor control.

The next-generation WEE1 inhibitor azenosertib (ZN-c3) has shown favorable antitumor activity with reduced systemic toxicity in patients with advanced solid tumors (19-21), yet its immunologic effects remain undefined. Here, we used immunocompetent preclinical models of HPV$^+$ OPC to define the immune contributions to WEE1i efficacy, identify dosing strategies that preserve immune fitness, and characterize the effects of WEE1i on T cell function.

Results

Antitumor activity of the WEE1 inhibitor ZN-c3 across immunocompetent models of HPV$^+$ OPC

Given the sensitivity of HPV$^+$ tumors to WEE1i and emerging evidence that WEE1i can modulate

antitumor immunity (6, 9, 11-13), we evaluated the antitumor activity of ZN-c3 in immunocompetent models of HPV^{+} OPC. Mice were treated with ZN-c3 (60 mg/kg, 3× weekly; **Fig. 1A**) as intermittent dosing previously showed limited growth delay in xenograft models (21).

We first tested ZN-c3 in the mEERL model, a murine oropharyngeal carcinoma line transformed with HRAS and HPV16 E6/E7 and syngeneic to C57BL/6J (B6) mice (22). In the subcutaneous flank setting, ZN-c3 treatment inhibited mEERL tumor growth and prolonged survival of tumor-bearing mice compared with vehicle controls, with 1 of 10 complete responses (CRs) observed (**Fig. 1A–C**). Comparable antitumor activity was observed in MLM3 tumors, a CRT-resistant mEERL variant (23), and in mEE-F3T3 flank tumors (24), which demonstrated growth suppression including 2 of 6 CRs (**Fig. 1D, E**).

To assess efficacy in an orthotopic context, mEERL cells were implanted into the buccal mucosa of syngeneic hosts. Orthotopic tumors grew more slowly than subcutaneous flank tumors and exhibited spontaneous clearance in a subset of mice (3 of 19; **Fig. 1F**). Intermittent ZN-c3 treatment further suppressed orthotopic tumor growth and increased tumor rejection rates in both mEERL (8 of 13 CRs; **Fig. 1F**) and MLM3 tumors (3 of 6 CRs; **Sup. Fig. 1F**).

To assess target engagement, mice bearing orthotopic mEERL tumors were treated with ZN-c3 or vehicle for six hours. Immunohistochemistry showed reduced pCDK1^{Y15} levels and increased the DNA damage marker γH2AX^{S139} in ZN-c3–treated tumors compared with controls (**Sup. Fig. 1A–C**). Consistent with a class effect of WEE1i, the structurally distinct WEE1 inhibitor AZD1775 resulted in dose-dependent tumor growth suppression in mEERL flank tumors (**Fig. 1G, H**). Intermittent ZN-c3 treatment was well tolerated as assessed by body weight (**Sup. Fig. 1D, E**). Collectively, these results indicate that ZN-c3 monotherapy activity is likely mediated through on-target activity.

ZN-c3 induced durable CRs in a subset of mice (**Fig. 1F; Sup. Fig. 1F**). Mice achieving CR were maintained off treatment and remained tumor-free for more than 90 days prior to mEERL rechallenge. Upon rechallenge, cured mice rejected tumor growth, whereas tumor-naïve controls progressed (**Fig. 1I**), consistent with immunologic memory. Together, these findings indicate that antitumor responses observed across overlapping dosing paradigms and structurally distinct WEE1 inhibitors occur in models with marked WEE1i sensitivity, enabling subsequent dissection of tumor cell–autonomous versus immune-mediated mechanisms.

Antigen-specific immunity cooperates with WEE1i to promote immune control of HPV^{+} OPC

To distinguish tumor cell–autonomous effects of WEE1i from immune-mediated mechanisms, we compared the activity of low-dose AZD1775 (30 mg/kg, 3× weekly) in mEERL flank tumors grown in immunocompetent B6 mice versus immunodeficient NOD/SCID/IL2Rγ^{-}/$^{-}$ (NSG) hosts. This regimen conferred no therapeutic benefit in NSG mice but significantly delayed tumor growth and prolonged survival in B6 mice (**Fig. 2A–C**).

To further isolate tumor cell–intrinsic sensitivity to WEE1i, mEERL tumors were established in Batf3$^{-/-}$ mice, which lack conventional type 1 dendritic cells (cDC1s) required for antigen cross-presentation (25). A continuous ZN-c3 regimen (80 mg/kg, 7× weekly) induced durable CRs in 5 of 7 mice (**Fig. 2D–F**), consistent with intrinsic tumor sensitivity to WEE1i. In contrast, the intermittent ZN-c3 regimen (60 mg/kg, 3× weekly), which was highly effective in immunocompetent B6 mice, showed no activity in Batf3$^{-/-}$ hosts (**Fig. 2G–I**).

To directly assess the contribution of T cells, B6 mice bearing mEERL tumors were treated with intermittent ZN-c3 (60 mg/kg, 3× weekly) in the setting of antibody-mediated T cell depletion (**Sup. Fig. 2A–D**). T cell depletion abrogated the therapeutic benefit conferred by intermittent ZN-c3 (**Fig. 2J–L**). Depletion of either $CD8^+$ or $CD4^+$ T cells alone partially blunted therapeutic efficacy, indicating nonredundant contributions from both T cell compartments (**Sup. Fig. 2E–H**).

To assess whether antigen-specific human T cells can participate in WEE1i–mediated tumor control, we employed the $HPV16^+$ HLA-A*02:01*$^+$ *LNT-20 patient-derived xenograft (PDX) model. NSG mice bearing LNT-20 tumors received adoptive transfer of human CD8*$^+$ *T cells transduced with an HLA-A*02:01–restricted E7–specific TCR. E7 TCR T cells induced dose-dependent tumor regression (**Sup. Fig. 2I**). Intermittent ZN-c3 treatment delayed tumor growth to a similar extent in the presence or absence of E7-specific T cells (**Fig. 2M, N**), indicating that adoptively transferred $CD8^+$ T cells do not further augment WEE1i-mediated tumor control in this PDX setting. Together, these findings suggest that additional host-derived immune interactions are likely required to support the WEE1i-induced antitumor immunity.

Tumor cell-intrinsic cGAS and host STING are required for WEE1i-induced antitumor immunity

WEE1i has been reported to elicit antitumor immune responses in solid tumors through activation of cGAS–STING signaling following DNA damage (11-13). Because HPV16 antagonizes tumor cell–intrinsic STING signaling (14, 15), we sought to define the mechanisms by which ZN-c3 elicits adaptive immunity in mEERL tumors.

mEERL cells or isogenic non–HPV-transformed mouse oropharyngeal epithelial (mTE) cells were treated with ZN-c3 for 24 hours followed by drug washout. WEE1i reduced $CDK1^{Y15}$ phosphorylation in both cell lines in a dose-dependent and reversible manner (**Fig. 3A–C**). HPV16 E6/E7–transformed mEERL cells accumulated substantially higher levels of DNA damage and premature mitosis compared with mTE controls, and DNA damage in mEERL cells persisted following washout (**Fig. 3A, B, D; Sup. Fig. 3A**), consistent with our previous observations in human HPV^+ models of OPC (9). In contrast, minimal residual DNA damage was detected in mTE cells after recovery (**Fig. 3D**).

Despite robust DNA damage, inhibiting WEE1 using ZN-c3 or AZD1775 failed to induce expression of Cxcl10, Ccl5, type I interferon (IFN) transcripts, STAT1 activation, or interferon-stimulated genes (ISGs) in mEERL cells *in vitro* (**Fig. 3E; Sup. Fig. 3B, C**). Instead, ZN-c3 increased expression of CXCL1 and soluble TNFR1 (**Fig. 3E**), factors associated with immunosuppressive signaling (26). Poly(I:C), but not a double-stranded DNA analog, activated type I IFN signaling in mEERL cells, whereas the STING agonist diABZI induced *Ifnb1* and *Isg15* expression in mTE but not mEERL cells (**Fig. 3F; Sup. Fig. 3A, B**). Consistent with these findings, ZN-c3 did not induce *CXCL10* or *CD274* expression in human HPV^+ OPC models *in vitro* (**Sup. Fig. 3D, E**), indicating compromised tumor cell–intrinsic STING signaling across murine and human systems.

In contrast to these tumor cell–intrinsic *in vitro* findings, WEE1i induced a robust IFN response *in vivo*. AZD1775 treatment of mEERL tumors resulted in upregulation of *Ifna1*, *Ifnb1*, *Stat1*, and multiple ISGs, including *Cxcl9*, *Cxcl10*, and *Cd274* (**Sup. Fig. 3F**). NanoString profiling further revealed enrichment of antigen presentation, IFN signaling, and T cell activation pathways within the tumor microenvironment (TME, **Sup. Fig. 3G**).

To determine whether host STING signaling mediates this IFN response, we generated organotypic tumor slice cultures from mEERL tumors grown in WT or STING-deficient ($Sting^{gt/gt}$) hosts.

ZN-c3 induced dose-dependent IFN signaling in slices from WT but not Sting$^{gt/gt}$ hosts (Fig. 3G, H). Accordingly, ZN-c3 treatment (60 mg/kg, 3× weekly) inhibited tumor growth and prolonged survival in WT but not STING-deficient mice (**Fig. 3I, J; compare with Fig. 1A-C**).

Finally, to assess whether tumor cell–intrinsic cGAS contributes to WEE1i-induced immunity, we generated cGas-knockout mEERL cells and established slices from cGas-knockout tumors grown in WT hosts (Sup. Fig. 3H). Tumor cell–specific deletion of cGas attenuated the IFN response to ZN-c3 *ex vivo* (**Fig. 3H**) and completely abrogated therapeutic efficacy *in vivo* (**Fig. 3K, L; Sup. Fig. 3I, J**). Together, these data demonstrate that WEE1i-induced antitumor immunity requires tumor cell–intrinsic cGAS to initiate danger signaling and host STING to propagate productive immune activation within the TME.

WEE1i potentiates antitumor immunity against HPV$^+$ OPC tumors by altering the TME.

To define how WEE1i alters the TME, we performed FLEX single-cell whole-transcriptome sequencing (scRNA-seq) on dissociated mEERL tumors grown in B6 mice and treated with AZD1775 or vehicle control (**Sup. Fig. 4A**). Using established lineage-specific markers, cells were clustered into three major compartments—immune, stromal, and tumor (**Fig. 4A**). Compartment-specific gene set variation analysis revealed transcriptional programs consistent with enhanced antitumor immunity, including increased IL-2/STAT5 signaling within immune cells and concomitant upregulation of IFN signaling in tumor and stromal compartments (**Fig. 4B**). In parallel, we observed downregulation of proliferation-associated pathways, including E2F targets and KRAS signaling, within tumor and stromal cells (**Fig. 4B**). We did not detect evidence of integrated stress response (ISR) activation under the AZD1775 dosing regimen used in this experiment (**Sup. Fig. 4C**).

To further resolve TME dynamics, we subclustered cells into 13 populations encompassing endothelial cells, fibroblasts, tumor-infiltrating lymphocytes (TILs), myeloid cells, and tumor cells (**Fig. 4C; Sup. Fig. 4B**). Within the immune compartment, we observed a significant increase in the proportion of effector CD8$^+$ TILs following AZD1775 treatment (**Sup. Fig. 4D, F**). These findings were corroborated by multiplex immunohistochemistry (mIHC) staining for pan-cytokeratin, CD8, and Ly6G, which demonstrated an increased CD8:neutrophil ratio in AZD1775-treated tumors compared with vehicle controls (**Sup. Fig. 4G, H**). Analysis of the stromal compartment revealed an approximately fivefold expansion of lymphatic endothelial cells (LECs), accompanied by a reduction in immunosuppressive myofibroblastic cancer-associated fibroblasts (MyCAFs; **Fig. 4C; Sup. Fig. 4E**), consistent with a TME permissive to antitumor immune activity.

To better understand AZD1775–associated signaling within the TME, we performed cell–cell interaction analysis using the CellChat framework (27). Intermittent AZD1775 treatment profoundly reshaped ligand–receptor communication networks, increasing both the frequency and strength of inferred interactions (715 AZD1775-specific interactions versus 255 interactions in vehicle-treated tumors), with the most pronounced effects observed within the immune compartment (**Fig. 4D**). Focusing on antigen-presenting cell (APC) populations—including DCs, tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs)—as sender populations and CD4$^+$ TILs, CD8$^+$ TILs, and NK cells as receiver populations, CellChat identified several ligand–receptor programs selectively enriched in AZD1775-treated tumors. Prominent among these was enhanced CD8 receptor signaling through MHC class I expressed by APCs (**Fig. 4E**), consistent with increased antigen presentation and TIL activation. CellChat also predicted enhanced CXCR6–CXCL16 signaling

between $CD8^+$ TILs and TAMs/DCs (**Fig. 4E**), a pathway implicated in tissue localization of T cells, including tissue-resident memory and tissue-resident exhausted T cells, and associated with enhanced cytotoxic function *in vivo* (28, 29). Additionally, increased activity of the CCL3/4/6–CCR2/5 axis between neutrophils and lymphocytes in AZD1775-treated tumors is consistent with enhanced lymphocyte recruitment and persistence (**Fig. 4E-G**).

To define how WEE1i modulates antitumor immune states within the local TME in a human-relevant setting, we first analyzed immune responses in organotypic tumor slice cultures derived from treatment-naïve HPV^+ OPC specimens. To confirm if TILs can be activated in this *ex vivo* system, tumor slices were stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin, followed by dissociation and flow cytometric immunophenotyping. This stimulation induced robust $CD8^+$ TIL activation, marked by increased expression of CD25 and CD38, concomitant downregulation of memory-associated markers CD127 and TCF1, and upregulation of inhibitory receptors including CTLA-4 and LAG-3 (**Sup. Fig. 6A**).

Tumor slices were subsequently treated with ZN-c3 or vehicle control, and immunohistochemical analysis confirmed on-target drug activity (**Sup. Fig. 6B, C**). Following dissociation, unsupervised hierarchical clustering of TILs revealed heterogeneous responses to ZN-c3, including an effector-like subset characterized by increased CD25, granzyme B, and CD38 expression, a second subset enriched for memory-associated features (CD127, TCF1), and a third subset with minimal phenotypic change (**Fig. 5A–D**). To explore factors underlying this heterogeneity, we examined baseline immune features associated with divergent responses and found that increased baseline frequencies of activated ($CD38^+$) $CD4^+$ TILs, including both regulatory and conventional subsets, were negatively associated with induction of a stem-like T cell phenotype following ZN-c3 treatment (**Sup. Fig. 6D, E**).

We next examined how WEE1i modulates immune cell populations *in vivo*. Because continuous high-intensity WEE1i induces tumor regression largely independent of adaptive immunity (**Fig. 2D-F**), whereas intermittent or submaximal dosing reveals immune-dependent mechanisms (**Fig. 2; Fig 3I-L**), we performed flow cytometric immunophenotyping of mEERL tumors treated with two submaximal ZN-c3 regimens (30 or 60 mg/kg) or vehicle control (**Fig. 5E**). ZN-c3 treatment increased overall immune cell infiltration relative to vehicle-treated tumors (**Fig. 5F**). Within the $CD45^+$ leukocyte compartment, the abundance of $CD3^+$ TILs increased at both dose levels (**Fig. 5G**). While FOXP3− (non-regulatory) $CD4^+$ TILs expanded predominantly at the lower dose, $CD8^+$ TILs and NK cells increased at both doses (**Fig. 5H**). $CD8^+$ TILs exhibited an activated phenotype and lacked CD62L expression, and most expressed high levels of granzyme B, and upregulated inhibitory receptors including PD-1 and CD39 (**Sup. Fig. 5B**).

ZN-c3 treatment was associated with the expansion of two phenotypically distinct TIL populations within the TME, observed across both the $CD8^+$ and $CD4^+$ compartments. Within the PD-1^+ gate, these populations were defined by a PD-1^+TCF1^+ subset and a PD-1^+TCF1^+Ly6C^+ subset. In $CD8^+$ TILs, both populations exhibited reduced expression of terminal exhaustion markers, including Eomes, Lag3, and Tim3, relative to vehicle-treated controls (**Fig. 5I–K** and **Sup. Fig. 5C**). A parallel expansion of PD-1^+TCF1^+ and PD-1^+TCF1^+Ly6C^+ populations was also observed among $CD4^+$ TILs (**Fig. 5L, M**).

PD-1^+TCF1^+ $CD8^+$ and $CD4^+$ T cells are known to arise in tumor-draining lymph nodes, where they function as a self-renewing progenitor reservoir that sustains intratumoral effector responses and

is essential for durable tumor control during immunotherapy (30-32). To determine whether ZN-c3–induced antitumor immunity could be sustained by pre-existing TILs or instead required ongoing recruitment from secondary lymphoid organs, we pharmacologically blocked lymphocyte egress using FTY720. This intervention attenuated the therapeutic efficacy of ZN-c3, indicating that circulating T cells—and not solely resident TILs—contribute to WEE1i-mediated immune control (**Fig. 5N–P**). Consistent with these findings, ZN-c3 treatment did not increase the frequency of PD-1^{+}TCF1^{+} TILs in human HPV^{+} OPC tumor slice cultures *ex vivo* (**Sup. Fig. 6F, G**). Together, these data indicate that WEE1i promotes infiltration and maintenance of cytotoxic lymphocytes with features of functional persistence in both murine and human HPV^{+} OPC tumors.

Effect of WEE1i dosing regimen on circulating immune cells

Our data indicate that ZN-c3 can mediate tumor control through distinct mechanisms depending on dosing schedule: continuous WEE1i achieves tumor regression largely independent of adaptive immunity, whereas intermittent dosing critically requires an intact immune system. We therefore asked whether these divergent dependencies are associated with differential effects on systemic immune cell populations. To address this, we evaluated the impact of intermittent ZN-c3 dosing (30 or 60 mg/kg) versus continuous dosing (80 mg/kg) on circulating immune cells in tumor-bearing mice (**Fig. 6A**).

ZN-c3 treatment induced a dose-dependent shift in circulating immune cell composition, characterized by an increased relative frequency of T cells that coincided with a reduction in neutrophils across all dosing regimens, including continuous dosing at 80 mg/kg (**Fig. 6B**). In contrast, reductions in B cell relative frequency were largely restricted to continuous dosing at 80 mg/kg. Within the T cell compartment, the relative frequencies of CD8^{+} T cells and non-regulatory CD4^{+} T cells were largely unchanged across dosing regimens, with the exception of continuous dosing at 80 mg/kg (**Fig. 6C**). This change was driven by an increased frequency of double-negative (CD4^{-}CD8^{-}) T cells observed only under continuous dosing at 80 mg/kg (**Fig. 6C**). No significant changes were observed in the relative frequencies of circulating monocytes, macrophages, regulatory T cells, or γδ T cells across dosing regimens (**Fig. 6B, C**).

To determine how dosing schedule influences immune cell proliferation and viability, we assessed Ki-67 expression and PARP cleavage (cPARP) across leukocyte subsets. Intermittent ZN-c3 treatment had minimal impact on immune cell proliferation, whereas continuous high-dose treatment (80 mg/kg) significantly reduced proliferation, most notably among B cells **(Fig. 6D, E**). This finding was consistent with the relative decrease in circulating B cell abundance observed at this dose (**Fig. 6B**). Similarly, continuous ZN-c3 treatment resulted in the greatest increase in cleaved PARP across immune cell populations, particularly in B and T cells (**Sup. Fig. 7A-D**). Together, these data indicate that intermittent WEE1i better preserves circulating immune cell composition compared with continuous high-dose dosing.

Intermittent ZN-c3 dosing preserves T cell proliferation and function

To directly assess how ZN-c3 schedule influences T cell function, we cocultured human E7 TCR–transduced CD8^{+} T cells with UPCI-SCC90 cells (HLA-A*02:01^{+} HPV^{+} OPC) across a range of effector-to-target ratios in the presence of increasing concentrations of ZN-c3 for 6 days. Consistent with our *in vivo* observations, sustained ZN-c3 exposure resulted in dose-dependent attenuation of T cell effector activity, as measured by IFN-γ secretion (**Fig. 6F, G**). These findings indicate that

prolonged WEE1i can constrain T cell function and suggest that limiting exposure duration may mitigate these effects.

To more precisely define how dosing schedule influences T cell proliferation and signaling, human peripheral blood mononuclear cells (PBMCs) were activated with anti-CD3 and anti-CD28 antibodies and treated with ZN-c3 under continuous or intermittent regimens. ZN-c3 induced a dose-dependent reduction in inhibitory CDK1 phosphorylation, accompanied at the highest concentration (1,000 nmol/L) by accumulation of γH2AX. Both pCDK1 suppression and γH2AX accumulation were fully reversible following drug washout (**Sup. Fig. 8A–C**), indicating transient cell cycle checkpoint engagement rather than irreversible T cell injury.

Using CellTrace Violet (CTV) dilution assays, we quantified T cell proliferation as the percentage of cells undergoing division. Continuous ZN-c3 exposure at 1,000 nmol/L was associated with a modest but statistically significant reduction in the fraction of dividing $CD4^+$ and $CD8^+$ T cells, whereas no significant changes in proliferative fraction were observed under intermittent dosing at the same concentration (**Fig. 7A, C; Sup. Fig. 8D**). The reduction in dividing cells under continuous exposure was limited in magnitude (approximately 20%) and was accompanied by only a modest decrease in T cell viability (approximately 10–15%), indicating largely preserved T cell survival (**Fig. 7B; Sup. Fig. 8F**). In parallel experiments, ZN-c3 effectively suppressed proliferation of HPV^+ OPC cell lines at similar or lower concentrations, supporting a dosing window in which intermittent WEE1i preferentially preserves T cell proliferative capacity relative to tumor cells (**Sup. Fig. 9A**).

Consistent with these observations, the first-generation WEE1 inhibitor AZD1775 suppressed T cell proliferation more profoundly than ZN-c3 at comparable doses, indicating a narrower immune tolerability profile (**Fig. 7D; compare with Fig. 7C**). Analysis of dividing T cells confirmed that intermittent dosing was better tolerated than continuous exposure for both inhibitors, with ZN-c3 exhibiting a more favorable profile overall (**Fig. 7C, D; Sup. Fig. 8D, E**). Phenotypic analysis of cycling (CTV low) $CD8^+$ T cells revealed that granzyme B expression remained unchanged within the dividing population, while markers associated with T cell exhaustion (TOX, TIM-3, LAG-3) and activation (CD25) were similarly maintained (**Sup. Fig. 8G**). These data indicate that reduced proliferation under continuous WEE1 inhibition occurs without induction of canonical exhaustion programs, impaired activation, or loss of cytotoxic effector differentiation among responding T cells.

Collectively, these findings indicate that continuous WEE1i imposes a cumulative, yet reversible, constraint on T cell expansion, whereas intermittent ZN-c3 dosing preserves proliferative competence and cytotoxic effector features across both $CD4^+$ and $CD8^+$ T cell compartments, providing a mechanistic rationale for improved immune tolerability under intermittent dosing schedules.

Discussion

We found that WEE1i anti-HPV^+ OPC activity involves two distinct mechanisms: 1) tumor cell–autonomous cytotoxicity, and 2) modulation of antitumor immunity. HPV^+ tumor cells are highly sensitive to WEE1i, in part because they rely upon the G2/M checkpoint to tolerate oncogene-driven genomic instability, which is disabled by inhibiting WEE1 (6-10). Our previous work demonstrated the role of the FOXM1-CDK1 axis in driving WEE1i-sensetivity in HPV+ models of OPC (9), as well as early clinical promise of an AZD1775-based regimen in advanced HPV+ OPC (10). Here, we extend these findings by demonstrating a critical role for host immunity in the antitumor activity of both AZD1775 and the next-generation WEE1 inhibitor ZN-c3, which achieved durable responses as

single-agents in immunocompetent HPV$^+$ OPC models (**Fig. 1, 2**). Comparison of these two structurally distinct WEE1 inhibitors revealed shared antitumor and immune phenotypes consistent with on-target WEE1 inhibition, as well as differences in immune tolerance between compounds.

WEE1i elicits durable antitumor immunity that depends on CD4$^+$ and CD8$^+$ T cells and cDC1s, implicating antigen-specific immune responses in therapeutic efficacy (**Fig. 2**). HPV$^+$ OPC is an immunogenic malignancy, attributable to expression of viral antigens and other mechanisms (33). While current HPV-targeted T cell therapies largely focus on E6 and E7 (34), our findings suggest that the immunologic effects of WEE1i extend beyond simply targeting viral epitopes, and also involves complex interplay between DNA damage, innate immune sensing (cGAS/STING), and cDC1-dependent priming within a permissive TME (**Fig. 2-4**). WEE1i may thus increase antigen availability and broaden antitumor T cell specificity. Future studies incorporating additional HPV antigens or full viral genome expression will be important to define the breadth of immunity elicited by WEE1i.

In contrast to reports in other solid tumor models (11-13), WEE1i does not directly activate type I IFN signaling within HPV$^+$ tumor cells (**Fig 3E, F**), but instead provokes a type I IFN reponse within STING-proficient non-tumor cells in the TME (**Fig. 3G-J; Fig. 4H**). This mechanism may be unique to HPV$^+$ cancers, as HPV oncoproteins actively suppress STING signaling, inhibit IRF3 activation, and dampen type I IFN production within infected epithelial cells (15). Accordingly, we find that HPV$^+$ tumor cells are intrinsically STING-deficient, yet intact STING signaling within the host compartment is essential for the antitumor immunity induced by WEE1i (**Fig. 3E-J**).

While host STING signaling is required, tumor cell–intrinsic cGAS remains necessary for therapeutic efficacy (**Fig. 3K, L**). WEE1i induces DNA damage and activate cGAS–STING signaling in cDC1s within solid tumor models (12), providing a potential explanation for IFN responses we observe following ZN-c3 treatment *ex vivo* (**Fig. 3G, H**). Indeed, despite the potential engagement of innate sensing pathways in myeloid cells, we find that cGAS deletion within tumor cells ablates productive antitumor immune responses following WEE1i (**Fig. 3K, L; Sup. Fig. 3I, J**). These findings support a hierarchical model in which WEE1i-induced DNA damage is initially sensed within tumor cells and subsequently amplified by STING-competent host cells within the TME (**Fig. 4H**). Such tumor-to-host cGAS–STING communication has been described in other contexts of DNA damage–induced immunity (35-37).

Overall, WEE1i reshapes the HPV$^+$ OPC TME to favor cytotoxic lymphocyte recruitment, function, and persistence (**Fig. 4 and 5**). The antitumor efficacy of WEE1i in our preclinical models was associated with enhanced lymphocyte-mediated immune activity. A key finding of this study is that the WEE1 inhibitor ZN-c3 is both immune-permissive and immune-modulatory, defining a favorable preclinical safety profile. Unlike continuous WEE1i, which impairs T cell proliferation and function (11, 18), intermittent dosing preserves T cell fitness while maintaining antitumor efficacy (**Fig. 1 and 7**). This distinction is critical, as T cells are themselves highly proliferative and vulnerable to perturbations in cell-cycle regulation (38).

The increase in PD-1$^+$TCF1$^+$ CD8$^+$ and CD4$^+$ TILs (**Fig 5. I, K, M**) aligns with the concept that progenitor-like cells can be mobilized from secondary lymphoid organs to sustain TME immunity (31, 32), which may help explain the sensitivity of intermittent dosing to lymphocyte trafficking (**Fig. 5N-P**). In parallel, intermittent WEE1i was associated with the emergence of PD-1$^+$Ly6C$^+$ CD8$^+$ TILs that retained TCF1 expression (**Fig. 5I, J**), consistent with an activated yet progenitor-like CD8 state.

Consistent with recent reports in other solid tumors (39-41), the accumulation of Ly6C-expressing $CD8^+$ TILs has been associated with enhanced antitumor immunity and improved tumor control, potentially reflecting IFN-driven programs that support lymphoid homing, central memory differentiation, and preservation of proliferative capacity (40, 42, 43). Together, these features may contribute to durable immune surveillance in HPV^+ OPC. This is particularly relevant in HPV^+ OPC, where tumors harbor antigen-specific $CD8^+$ T cells recognizing a broad repertoire of viral antigens, including E2 and E5, and spanning diverse differentiation states with variable proliferative capacity (30). Preservation of T cell proliferative potential during therapy may therefore be a critical determinant of durable immune-mediated tumor control in this setting.

More than half of patients with recurrent or metastatic HPV^+ OPC ultimately develop incurable disease with limited therapeutic options. Neoadjuvant anti–PD-1 therapy is increasingly being incorporated into the management of advanced resectable head and neck cancers following a recent phase III trial that included patients with advanced HPV^+ OPC (44). In this setting, neoadjuvant anti–PD-1 was associated with reductions in distant metastatic failure but showed less impact on locoregional control, indicating that additional strategies are needed to further augment immunotherapeutic benefit. As an orally bioavailable agent with a favorable systemic toxicity profile and emerging immunomodulatory activity, WEE1i represents a compelling therapeutic approach for HPV^+ OPC. Our findings demonstrate that WEE1 inhibitors can function not only as direct antitumor agents but also as immune-enabling therapies, providing a rationale for combination strategies with immune checkpoint blockade or HPV-directed T cell–based therapies to improve outcomes in advanced disease.

Methods

Compound Preparation and Treatment

ZN-c3 (Zentalis Pharmaceuticals, Inc.) and AZD1775 (Selleck) were formulated in 0.5% methylcellulose and administered by oral gavage as indicated in the figure legends.

Cell Lines

mTE, mEERL, mEE-F3T3 and MLM3 cells were maintained in a 1:3 mixture of Ham's F-12 and DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 50 µg/mL hydrocortisone, 5 µg/mL insulin–transferrin–selenium, 0.65 ng/mL triiodothyronine, and 5 ng/mL EGF (E-media). Human cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Supplementary Table 1). All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2 and periodically tested and confirmed negative for Mycoplasma.

Animal Studies

WT C57BL/6J, $Batf3^{-/-}$, $Sting^{gt/gt}$ and NOD/SCID/IL2R$\gamma^{-/-}$ mice were purchased from the Jackson Laboratory and/or bred at the University of Pennsylvania and housed under specific pathogen-free conditions at the University of Pennsylvania and the Fred Hutchinson Cancer Center. For tumor implantation studies, mice aged 8–12 weeks were used. Experiments were randomized but not blinded. All animal experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania (IACUC #807486) and the Fred Hutchinson Cancer Center (IACUC #1413).

For subcutaneous flank tumors, mice received 1×10^6 tumor cells in 100 µL PBS/Matrigel (2:1)

subcutaneously in the right flank. For orthotopic buccal mucosa model, mice were anesthetized with isoflurane, and 0.5 × 10^6 cells in 50 μL of PBS/Matrigel (2:1) were injected submucosally into the left buccal mucosa. Tumor dimensions were measured using digital calipers, and volume was calculated as (length × $width^2$)/2. Mice were euthanized per institutional humane endpoints. For rechallenge studies, mice with established orthotopic mEERL tumors that achieved CR following ZN-c3 monotherapy and remained tumor-free for at least 90 days were rechallenged with 1 × 10^6 mEERL cells implanted in the contralateral flank. Age- and sex-matched, tumor-naïve WT B6 mice served as controls.

Generation of cGAS-Knockout Cell Lines

cGas knockout mEERL cells were generated using CRISPR–Cas9–mediated genome editing with the pSCAR system (45). Lentiviral particles encoding Cas9-GFP (pSCAR_Cas9; Addgene #162074) were produced in 293T cells using psPAX2 (#12260) and pMD2.G (#12259), both gifts from Didier Trono, and used to transduce mEERL cells. FACS-enriched GFP^+ Cas9-expressing mEERL cells were subsequently transfected with a pooled set of three synthetic single guide RNAs (sgRNAs) targeting distinct regions of murine *cGas* (Supplementary Table 2) or transduced with pSCAR_sgcGAS lentivirus, and edited cells were enriched by FACS ($mKate^+$). Cre-mediated excision was subsequently performed using integrase-deficient lentivirus expressing Cre recombinase (psPAX2-D64V; Addgene #63586) to generate GFP^-mKate^- knockout cells. Single cell clones were obtained by limiting dilution and knockout was confirmed by genomic sequencing and immunoblotting. For *in vivo* studies, 1 × 10^6 cGAS-KO cells were subcutaneously implanted into WT B6 mice.

Tumor Slice Cultures

For murine tumor slice cultures, mEERL tumors (~1,000mm^3) were embedded in 4% low-melt agarose and sectioned at 250-300 μm using a Compresstome (VF-510-0Z; speed 6, oscillations 4). Slices were cultured on cell culture inserts (Millicel, Millipore) in 24-well plates in E-media at 37°C/5% CO_2.

For human tumor slice cultures, de-identified treatment-naïve HPV^+ oropharyngeal squamous cell carcinoma specimens were obtained from patients undergoing surgical resection under an IRB-approved protocol (protocol No. 417200) with written informed consent. Age, sex and specimen descriptions are in Supplementary Table 3. Fresh specimens were processed within 2 hours and sectioned as above.

Flow Cytometry Analysis

Samples were processed in a 96-well plate format. Non-spleen samples were concentrated to 200 μL via centrifugation and vacuum aspiration, while spleen samples were directly resuspended to 200 μL. Cells were stained with Zombie NIR™ dye or LIVE/DEAD™ Fixable Blue Dead Cell Stain and CD16/CD32 blocking antibody for 10 minutes at room temperature (RT). Surface staining was performed in True-Stain™ Multi-Fluor or BD Horizon™ Brilliant Stain Buffer for 30-60 minutes at RT.

After surface staining, cells were fixed with fixation buffer (eBioscience) for 30 minutes at RT and washed with Permeabilization buffer. For intracellular staining, cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) per the manufacturer's guidelines and subsequently stained for 1 hour at RT or overnight at 4°C. Cells were then washed with Permeabilization buffer and resuspended in 200 μL FACS buffer (PBS + 2% FBS + 0.04% EDTA) for analysis. Single-color compensation controls were prepared by labeling one drop of UltraComp

447 eBeads™ Compensation Beads with 0.5–1 μL of each antibody (Supplementary Table 4-6). Data
448 were acquired on a Cytek Aurora or LSR Fortessa flow cytometer and analyzed using OmiQ or FlowJo
449 version 10.10.0.

450 **In Vitro T-Cell Functional Assays**

451 PBMCs and purified $CD8^+$ T cells were obtained from healthy volunteers with informed consent
452 obtained from all subjects in this study and apheresis was collected under University of Pennsylvania
453 IRB-approved protocol 705906 and processed by the Human Immunology Core (HIC). PBMCs were
454 labeled with CellTrace Violet (5 μmol/L) and were activated and expanded using the bead-free
455 ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies) or anti-CD3/CD28
456 microbeads (Life Technologies). Cells were treated with ZN-c3 or AZD1775 as indicated, and
457 proliferation was assessed by dye dilution using flow cytometry.

458 **Single-Cell RNA Sequencing**

459 mEERL tumors from B6 mice treated with AZD1775 (60 mg/kg, 4 doses over 8 days) or vehicle were
460 processed using the Tissue Fixation and Dissociation for Chromium Fixed RNA Profiling protocol
461 (CG000553, 10X Genomics) and the Chromium Next GEM Single Cell Fixed RNA Sample
462 Preparation Kit (1000414, 10X Genomics). Libraries were sequenced on an Illumina NextSeq2000
463 platform for 15,000 reads per cell. Raw sequencing data was processed using 10X Genomics
464 CellRanger with mm10 (GRCm38) as reference genome for specified panel of 17,348 genes and then
465 postprocessed in R using Seurat v4. Cells with fewer than 500 reads and mitochondrial gene
466 percentage of >20% were filtered out. Filtered cells were normalized, scaled and clustered in Seurat
467 v4. Cell type annotation was performed by SingleR using the mouse-cell specific markers established
468 in ImmGen Project. Gene set enrichment analysis (GSEA) was performed at pseudobulk level to
469 assess pathway-level changes across compartments (immune, stromal, tumor).

470 **Cell-Cell Interaction Analysis**

471 Ligand-receptor interactions were inferred from scRNA-seq data using CellChat v1.6.1 (27). Signaling
472 networks were compared between treatment groups, focusing on antigen-presenting cell populations
473 (dendritic cells, tumor-associated macrophages, tumor-associated neutrophils) as senders and
474 lymphocyte populations ($CD4^+$ T cells, $CD8^+$ T cells, NK cells) as receivers. Pathway-specific
475 communication probabilities were calculated and visualized.

476 **STING Pathway Functional Assays**

477 mEERL and mTE cells were treated with the STING agonist diABZI (10 μmol/L, InvivoGen) or
478 transfected with poly(I:C) (1 μg/mL, InvivoGen) or double-stranded DNA analog (ISD Naked,
479 InvivoGen) using polyethylenimine for 48 hours. Gene expression was assessed by RT-qPCR, and
480 STAT1 phosphorylation was measured by western blot as described above.

481 **T-Cell Depletion and FTY720 treatment**

482 $CD4^+$ and $CD8^+$ T cells were depleted using intraperitoneal injections of depleting antibodies: anti-
483 CD4 (clone GK1.5, Bio X Cell, 200 μg), anti-CD8α (clone 2.43, Bio X Cell, 200 μg), or combined anti-
484 CD4/CD8 for pan-T-cell depletion. Antibodies were administered 1 day before ZN-c3 initiation and
485 twice weekly thereafter. For FTY720 experiment, FTY720 (MedChemExpress) was provided in the
486 drinking water (2 μg/mL) on day 7 post inoculation. FTY720 treatment was continued throughout the

entire experimental course.

Lentiviral TCR construct:

TCR HPV E7 (Addgene #122728) lentiviral construct was generated using the third-generation lentiviral transfer vector pTRPE-eGFP-T2A-mCherry (provided by Michael C. Milone, University of Pennsylvania).

TCR HPV E7-engineered T cells

Purified human $CD8^+$ T cells were obtained from healthy volunteers through HIC as detailed above. TCR HPV E7-engineered CD8+ T cells were generated as described previously (46). Briefly, primary CD8+ T cells were activated with anti-CD3/CD28 microbeads (Life Technologies) and transduced with TCR E7 lentiviral vector particles at an MOI of 5. Cell cultures were grown in IL-7 (5 ng/ml; R&D Systems) / IL-15 (5 ng/ml; R&D Systems) supplemented T cell media. On day 5, microbeads were removed and T cells electroporation with sgRNAs targeting *TRAC* and *TRBC1/TRBC2* (Supplementary Table 2, Integrated DNA Technologies) complexed with TrueCut Cas9 Protein V2 (10 µg, ThermoFisher Scientific). Edited T cells were cultured in IL-7/IL-15 supplemented T cell media for a total of 14 days followed by Rapid Expansion Method (47). HPV16 E7-specific T cell frequencies were determined by p-HLA multimer flow cytometric assays.

In vitro cytotoxicity Assay

For cytotoxicity assays, E7 TCR-transduced CD8+ T cells were cocultured with UPCI-SCC90 target cells at effector: target ratios of 1:1 to 1:10 in the presence of ZN-c3 for 6 days. Culture supernatants were collected for IFN-γ quantification by ELISA (Human IFN-γ DuoSet, R&D Systems).

Adoptive T-Cell Therapy

LNT-20 PDXs were established and passaged in the subcutaneous flank of NSG mice as described (48). Tumor volumes were measured as above. Treatment started when tumors reached ~150-250 mm^3. Mice bearing established LNT-20 tumors received intravenous injection of 0.5-4 × 10^6 E7 TCR T cells intravenously with or without ZN-c3 treatment.

Statistical Analysis

Data are presented as mean ± SEM unless otherwise indicated. Statistical analyses and graphical representations were performed using GraphPad Prism (RRID: SCR_002798) version 9 or 10 and R (v4.2). Tumor growth significance was assessed using growth-rate–based modeling of longitudinal tumor volume measurements, fitting individual tumors to an exponential growth model and comparing estimated growth rates between treatment groups as described previously (49). Survival analyses were performed using Kaplan–Meier curves with log-rank (Mantel–Cox) tests.

Flow cytometry data, gene expression measurements, and other continuous variables were analyzed using unpaired two-tailed Student's *t* tests (for two-group comparisons) or one-way ANOVA with appropriate post-hoc multiple-comparisons tests (for three or more groups), as indicated in the figure legends. When assumptions of normality were not met, nonparametric tests were used as noted. A P value < 0.05 was considered statistically significant. Sample sizes were determined based on prior studies and institutional guidelines; no samples were excluded from analysis.

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Data Availability Statement: The data generated in this study are available upon request from the corresponding author.

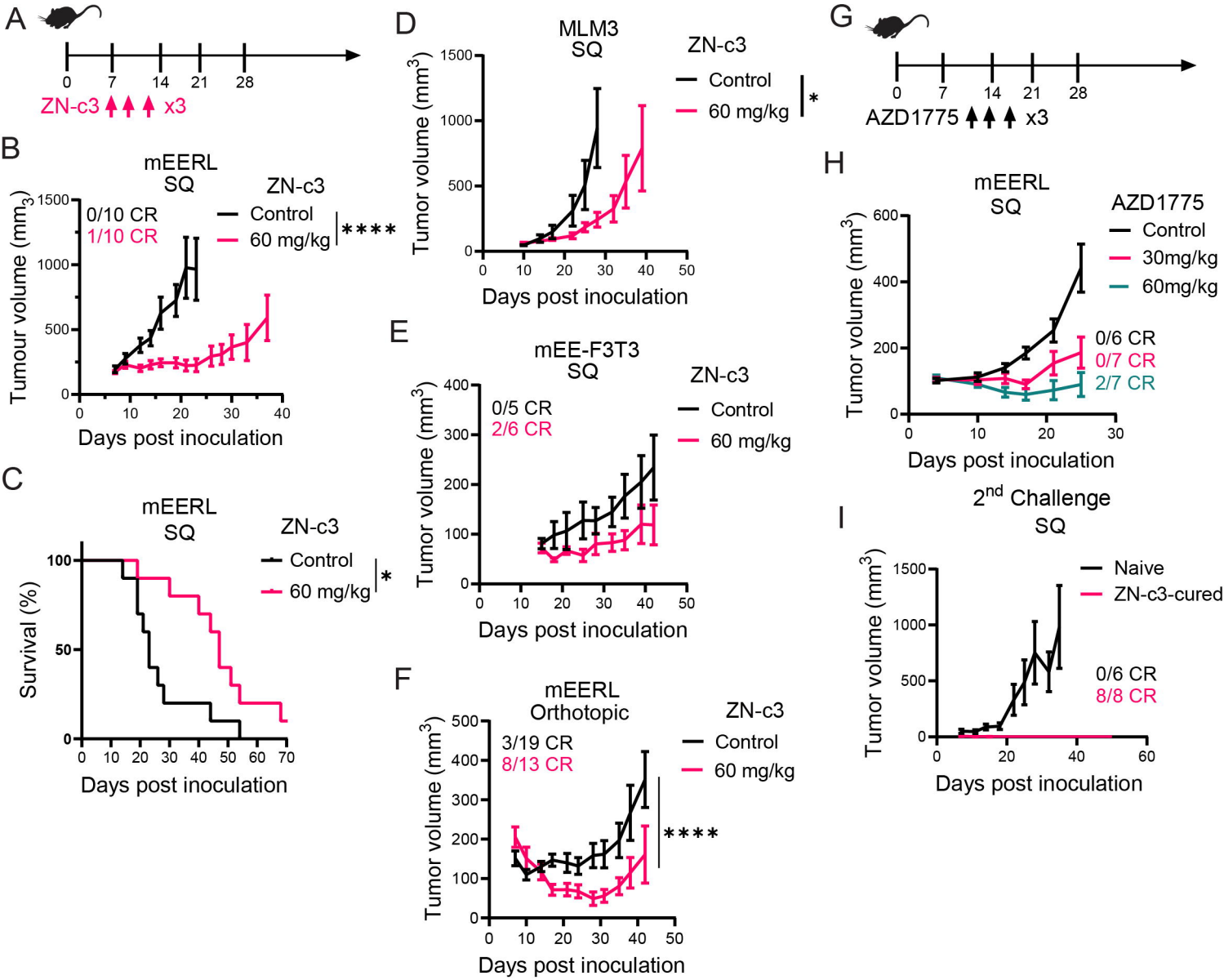
References

1. Roman BR, Aragones A. Epidemiology and incidence of HPV-related cancers of the head and neck. J Surg Oncol. 2021;124(6):920-2.
2. Sherman RL, Firth AU, Henley SJ, Siegel RL, Negoita S, Sung H, et al. Annual Report to the Nation on the Status of Cancer, featuring state-level statistics after the onset of the COVID-19 pandemic. Cancer. 2025;131(9):e35833.
3. Chow LQM. Head and Neck Cancer. New England Journal of Medicine. 2020;382(1):60-72.
4. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. Virology. 2009;384(2):324-34.
5. Roman A, Munger K. The papillomavirus E7 proteins. Virology. 2013;445(1-2):138-68.
6. Tanaka N, Patel AA, Wang J, Frederick MJ, Kalu NN, Zhao M, et al. Wee-1 Kinase Inhibition Sensitizes High-Risk HPV+ HNSCC to Apoptosis Accompanied by Downregulation of MCl-1 and XIAP Antiapoptotic Proteins. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(21):4831-44.
7. Moser R, Xu C, Kao M, Annis J, Lerma LA, Schaupp CM, et al. Functional Kinomics Identifies Candidate Therapeutic Targets in Head and Neck Cancer. Clinical Cancer Research. 2014;20(16):4274 LP-88.

8. Xu C, Nikolova O, Basom R, Mitchell RM, Shaw R, Moser R, et al. Functional precision medicine identifies novel druggable targets and therapeutic options in head and neck cancer. Clinical Cancer Research. 2018.
9. Diab A, Gem H, Swanger J, Kim HY, Smith K, Zou G, et al. FOXM1 drives HPV+ HNSCC sensitivity to WEE1 inhibition. Proceedings of the National Academy of Sciences. 2020;117(45):28287-96.
10. Mendez E, Rodriguez CP, Kao MC, Raju S, Diab A, Harbison RA, et al. A phase I clinical trial of AZD1775 in combination with neoadjuvant weekly docetaxel and cisplatin before definitive therapy in head and neck squamous cell carcinoma. Clinical Cancer Research. 2018;24(12):2740-8.
11. Jiao X, Liu J, Wu Y, Zhong Q, Zhu L, Wang L, et al. Sequential treatment with PARPi and WEE1i enhances antitumor immune responses in preclinical models of ovarian cancer. Sci Transl Med. 2025;17(811):eadu6989.
12. Ng, II, Zhang Z, Xiao K, Ye M, Tian T, Zhu Y, et al. Targeting WEE1 in tumor-associated dendritic cells potentiates antitumor immunity via the cGAS/STING pathway. Cell Rep. 2025;44(6):115733.
13. Taniguchi H, Caeser R, Chavan SS, Zhan YA, Chow A, Manoj P, et al. WEE1 inhibition enhances the antitumor immune response to PD-L1 blockade by the concomitant activation of STING and STAT1 pathways in SCLC. Cell Rep. 2022;39(7):110814.
14. Lau A, Gray EE, Brunette RL, Stetson DB. DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. Science. 2015;350(6260):568-71.
15. Shamseddine AA, Burman B, Lee NY, Zamarin D, Riaz N. Tumor Immunity and Immunotherapy for HPV-Related Cancers. Cancer discovery. 2021;11(8):1896-912.
16. Do K, Wilsker D, Ji J, Zlott J, Freshwater T, Kinders RJ, et al. Phase I Study of Single-Agent AZD1775 (MK-1775), a Wee1 Kinase Inhibitor, in Patients With Refractory Solid Tumors. J Clin Oncol. 2015;33(30):3409-15.
17. Fu S, Yao S, Yuan Y, Previs RA, Elias AD, Carvajal RD, et al. Multicenter Phase II Trial of the WEE1 Inhibitor Adavosertib in Refractory Solid Tumors Harboring CCNE1 Amplification. J Clin Oncol. 2023;41(9):1725-34.
18. Tjeerdsma RB, Ng TF, Roorda M, Bianchi D, Yang S, Bonnet C, et al. WEE1 inhibitors trigger GCN2-mediated activation of the integrated stress response. Nat Commun. 2025;16(1):11598.
19. Kim D, Chung H, Liu W, Jeong K, Ozmen TY, Ozmen F, et al. Cyclin E1/CDK2 activation defines a key vulnerability to WEE1 kinase inhibition in gynecological cancers. NPJ Precis Oncol. 2025;9(1):3.
20. Jameson NM, Kim D, Lee C, Skrable B, Shea A, Guo X, et al. The Selective WEE1 Inhibitor Azenosertib Shows Synergistic Antitumor Activity with KRASG12C Inhibitors in Preclinical Models. Cancer Res Commun. 2025;5(2):240-52.
21. Ma J, Liu W, Li J, Kim D, Kim S, Levy A, et al. Azenosertib Is a Potent and Selective WEE1 Kinase Inhibitor with Broad Antitumor Activity Across a Range of Solid Tumors. Mol Cancer Ther. 2025;24(8):1171-85.
22. Hoover AC, Spanos WC, Harris GF, Anderson ME, Klingelhutz AJ, Lee JH. The role of human papillomavirus 16 E6 in anchorage-independent and invasive growth of mouse tonsil epithelium. Archives of Otolaryngology - Head and Neck Surgery. 2007;133(5):495-502.
23. Vermeer DW, Coppock JD, Zeng E, Lee KM, Spanos WC, Onken MD, et al. Metastatic model of HPV+ oropharyngeal squamous cell carcinoma demonstrates heterogeneity in tumor metastasis. Oncotarget; Vol 7, No 17. 2016.
24. Khan N, Akagi K, Jiang S, Dunn JD, Jiang B, Xiao W, et al. Human Papillomavirus Integration

Induces Oncogenic Host Gene Fusions in Oropharyngeal Cancers. Cancer Discov. 2025;15(9):1927-48.
25. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science. 2008;322(5904):1097-100.
26. Felix J, Savvides SN. Mechanisms of immunomodulation by mammalian and viral decoy receptors: insights from structures. Nat Rev Immunol. 2017;17(2):112-29.
27. Jin S, Plikus MV, Nie Q. CellChat for systematic analysis of cell-cell communication from single-cell transcriptomics. Nat Protoc. 2025;20(1):180-219.
28. Di Pilato M, Kfuri-Rubens R, Pruessmann JN, Ozga AJ, Messemaker M, Cadilha BL, et al. CXCR6 positions cytotoxic T cells to receive critical survival signals in the tumor microenvironment. Cell. 2021;184(17):4512-30 e22.
29. Park SL, Painter MM, Manne S, Alcalde V, McLaughlin M, Sullivan MA, et al. Tissue-resident exhausted and memory CD8(+) T cells have distinct ontogeny, function and role in disease. Nat Immunol. 2025.
30. Eberhardt CS, Kissick HT, Patel MR, Cardenas MA, Prokhnevska N, Obeng RC, et al. Functional HPV-specific PD-1(+) stem-like CD8 T cells in head and neck cancer. Nature. 2021;597(7875):279-84.
31. Shen Y, Connolly E, Aiello M, Zhou C, Chappa P, Song H, et al. Combination radiation and alphaPD-L1 enhance tumor control by stimulating CD8+ PD-1+ TCF-1+ T cells in the tumor-draining lymph node. Nat Commun. 2025;16(1):3522.
32. Cardenas MA, Prokhnevska N, Sobierajska E, Gregorova P, Medina CB, Valanparambil RM, et al. Differentiation fate of a stem-like CD4 T cell controls immunity to cancer. Nature. 2024;636(8041):224-32.
33. Conarty JP, Wieland A. The Tumor-Specific Immune Landscape in HPV+ Head and Neck Cancer. Viruses. 2023;15(6).
34. Nagarsheth NB, Norberg SM, Sinkoe AL, Adhikary S, Meyer TJ, Lack JB, et al. TCR-engineered T cells targeting E7 for patients with metastatic HPV-associated epithelial cancers. Nat Med. 2021;27(3):419-25.
35. Ablasser A, Schmid-Burgk JL, Hemmerling I, Horvath GL, Schmidt T, Latz E, et al. Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. Nature. 2013;503(7477):530-4.
36. Gentili M, Kowal J, Tkach M, Satoh T, Lahaye X, Conrad C, et al. Transmission of innate immune signaling by packaging of cGAMP in viral particles. Science. 2015;349(6253):1232-6.
37. Samson N, Ablasser A. The cGAS-STING pathway and cancer. Nat Cancer. 2022;3(12):1452-63.
38. Vendetti FP, Pandya P, Clump DA, Schamus-Haynes S, Tavakoli M, diMayorca M, et al. The schedule of ATR inhibitor AZD6738 can potentiate or abolish antitumor immune responses to radiotherapy. JCI Insight. 2023;8(4).
39. Boulton S, Singh S, Organ B, Thomas J, Rezaei R, Gill R, et al. Oncolytic vaccinia virus encoding constitutively active EPAC remodels the tumor microenvironment to enhance therapeutic efficacy with chemotherapy and surgery. J Immunother Cancer. 2026;14(1).
40. Tu HF, Tao J, Hu MH, Fan D, Tsai YC, Wu TC, et al. Type I Interferon Modulates the Function of Ly6C High-Expressing Naive CD8(+) T Cells to Promote an Antitumor Response. Vaccines (Basel). 2025;13(3).

41. Piranlioglu R, Lee E, Ouzounova M, Bollag RJ, Vinyard AH, Arbab AS, et al. Primary tumor-induced immunity eradicates disseminated tumor cells in syngeneic mouse model. Nat Commun. 2019;10(1):1430.
42. Jaakkola I, Merinen M, Jalkanen S, Hanninen A. Ly6C induces clustering of LFA-1 (CD11a/CD18) and is involved in subtype-specific adhesion of CD8 T cells. J Immunol. 2003;170(3):1283-90.
43. Hanninen A, Maksimow M, Alam C, Morgan DJ, Jalkanen S. Ly6C supports preferential homing of central memory CD8+ T cells into lymph nodes. Eur J Immunol. 2011;41(3):634-44.
44. Uppaluri R, Haddad RI, Tao Y, Le Tourneau C, Lee NY, Westra W, et al. Neoadjuvant and Adjuvant Pembrolizumab in Locally Advanced Head and Neck Cancer. N Engl J Med. 2025;393(1):37-50.
45. Dubrot J, Lane-Reticker SK, Kessler EA, Ayer A, Mishra G, Wolfe CH, et al. In vivo screens using a selective CRISPR antigen removal lentiviral vector system reveal immune dependencies in renal cell carcinoma. Immunity. 2021;54(3):571-85 e6.
46. Blanchard T, Faridi P, Xu C, Bear AS, Rasool RU, Huang G, et al. LOXHD1 is an oncofusion-regulated antigen of ewing sarcoma. Sci Rep. 2025;15(1):13007.
47. Jin J, Gkitsas N, Fellowes VS, Ren J, Feldman SA, Hinrichs CS, et al. Enhanced clinical-scale manufacturing of TCR transduced T-cells using closed culture system modules. J Transl Med. 2018;16(1):13.
48. Sannigrahi MK, Rajagopalan P, Lai L, Liu X, Sahu V, Nakagawa H, et al. HPV E6 regulates therapy responses in oropharyngeal cancer by repressing the PGC-1alpha/ERRalpha axis. JCI Insight. 2022;7(18).
49. Hather G, Liu R, Bandi S, Mettetal J, Manfredi M, Shyu WC, et al. Growth rate analysis and efficient experimental design for tumor xenograft studies. Cancer Inform. 2014;13(Suppl 4):65-72.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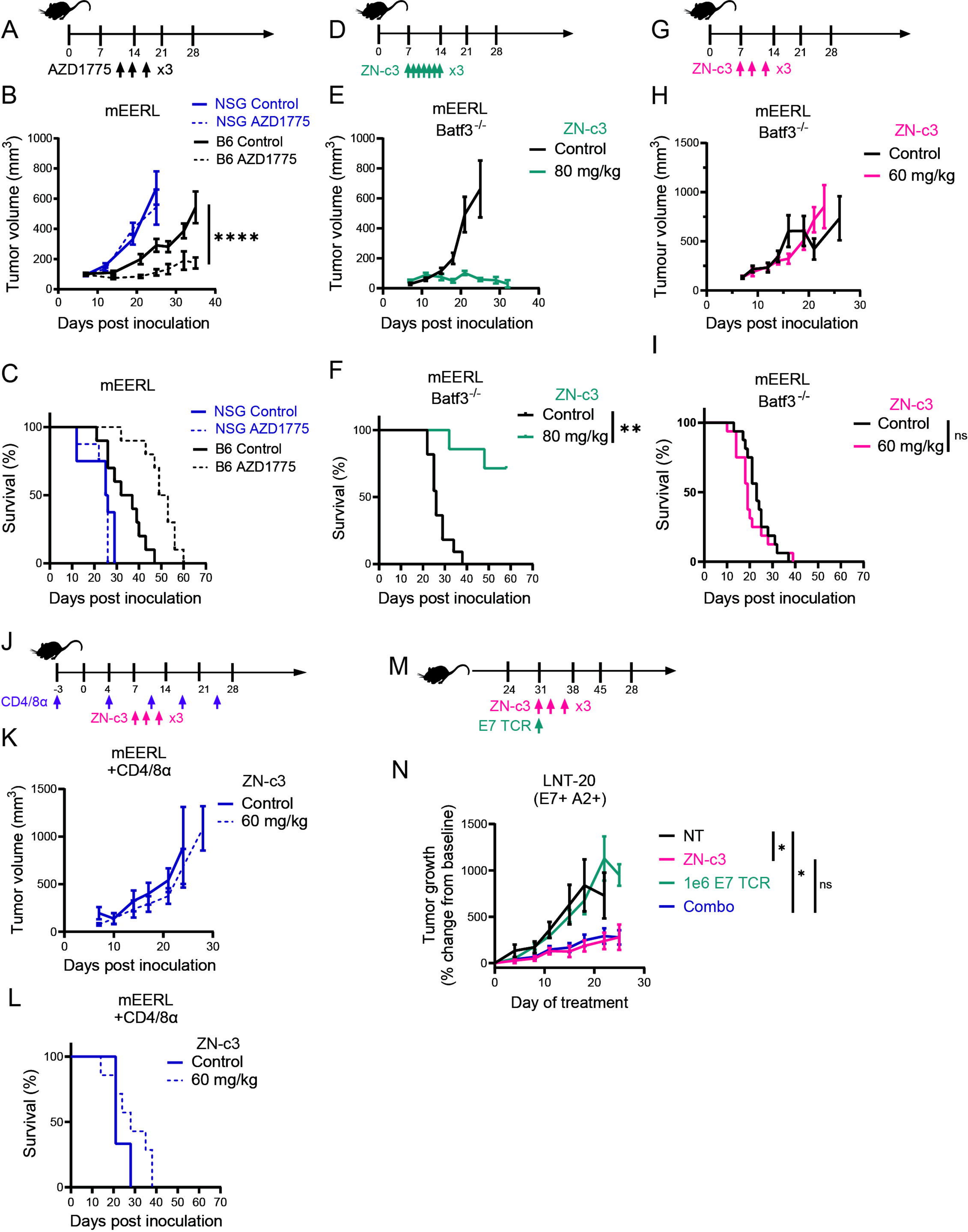
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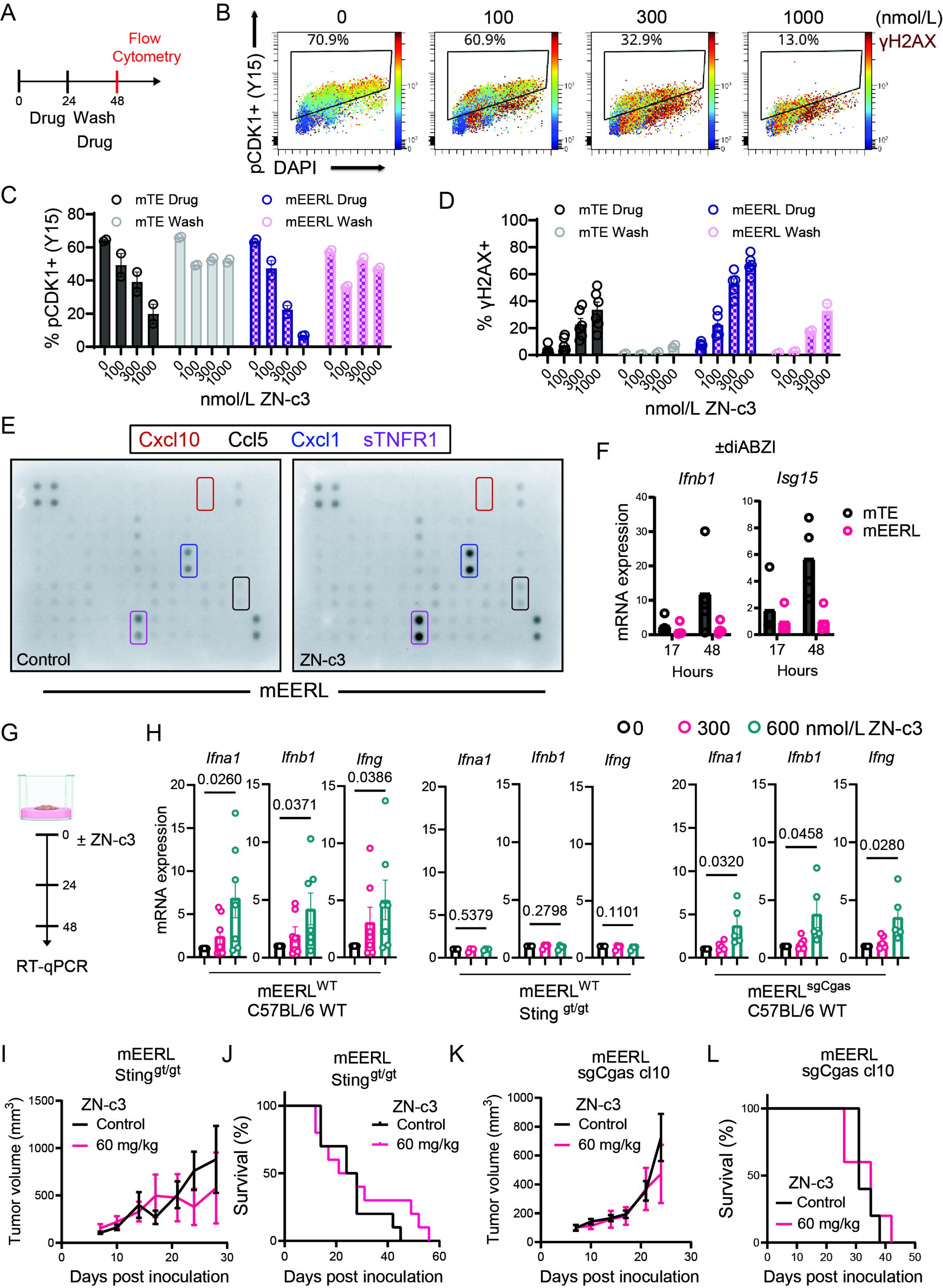
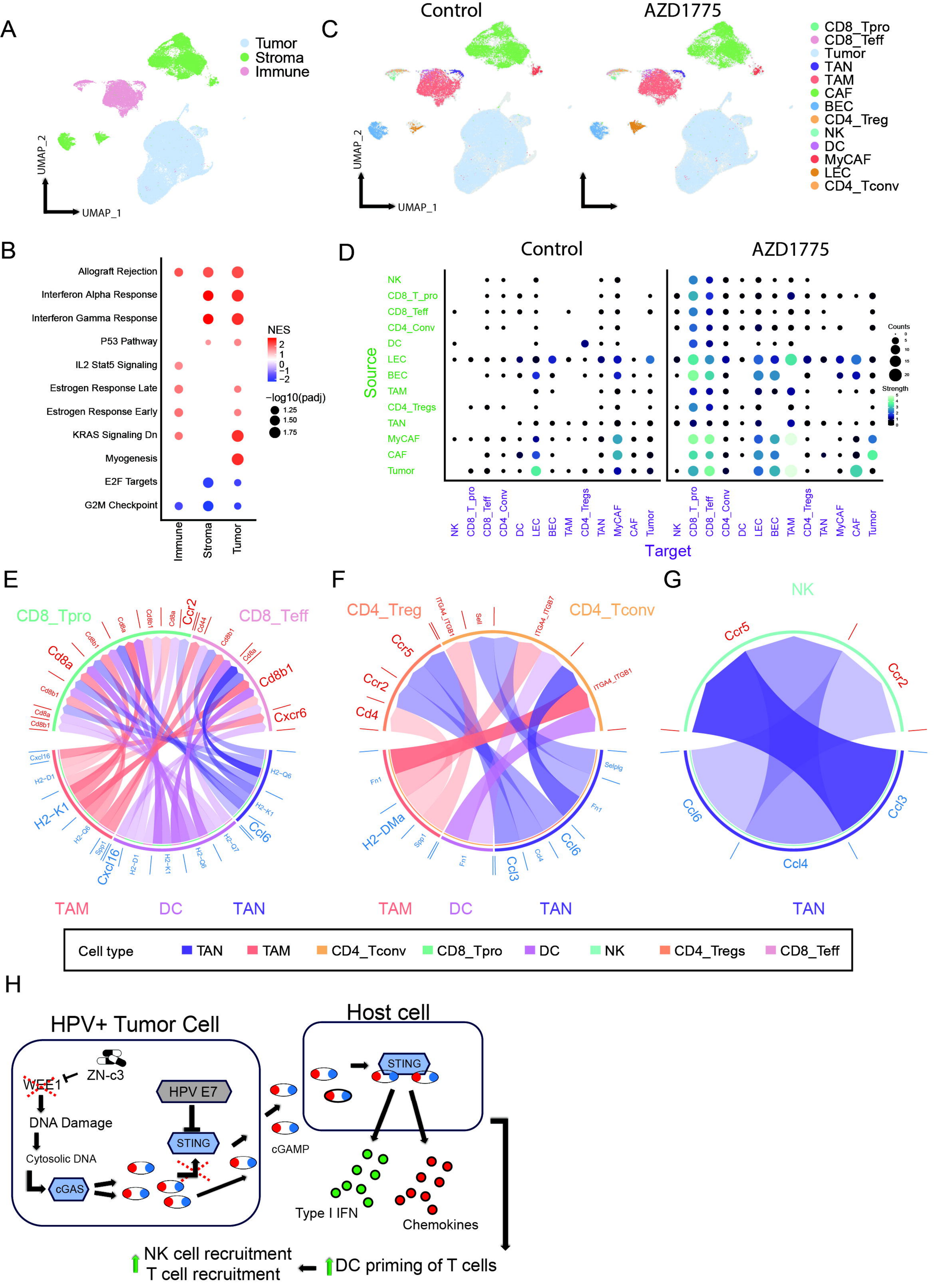
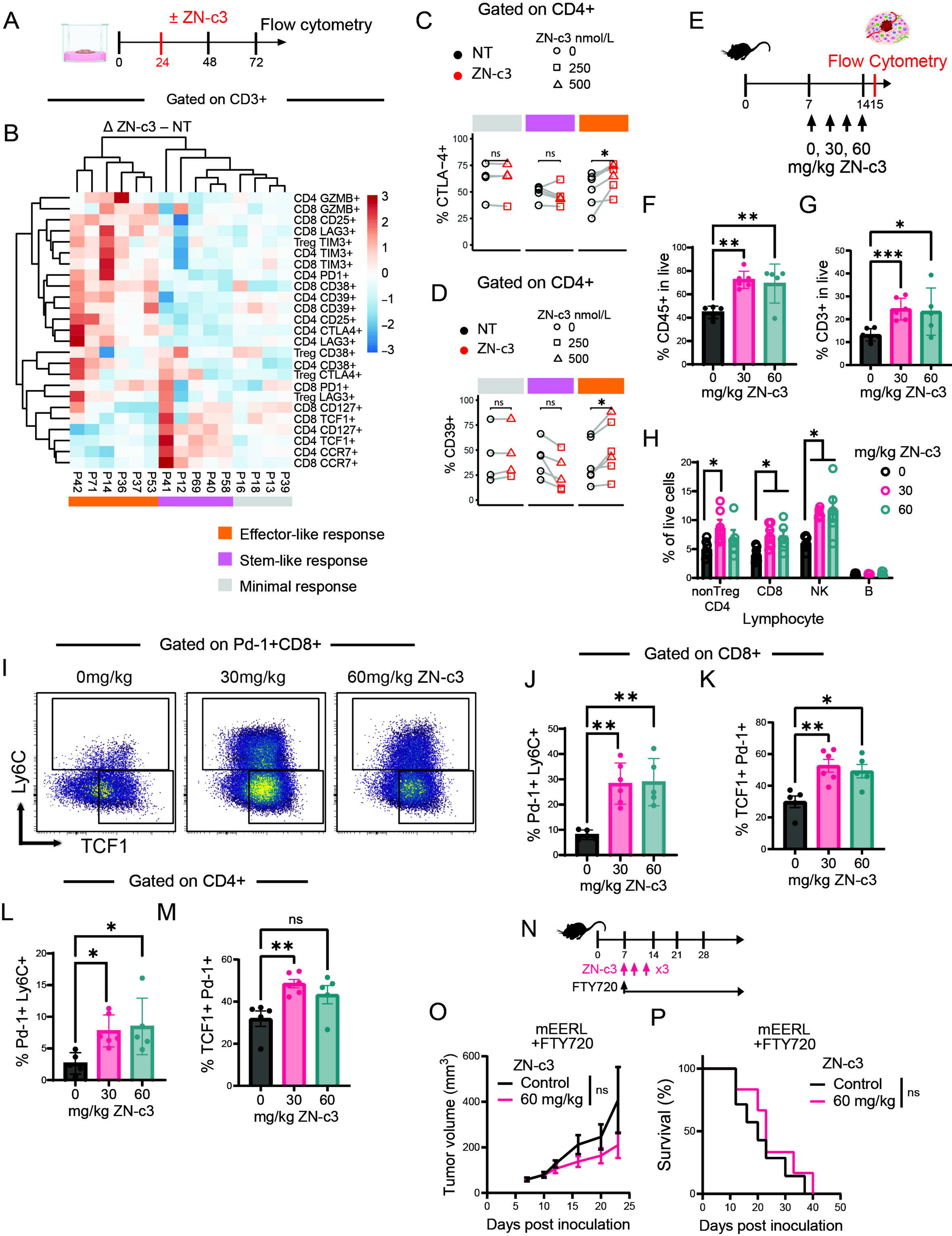
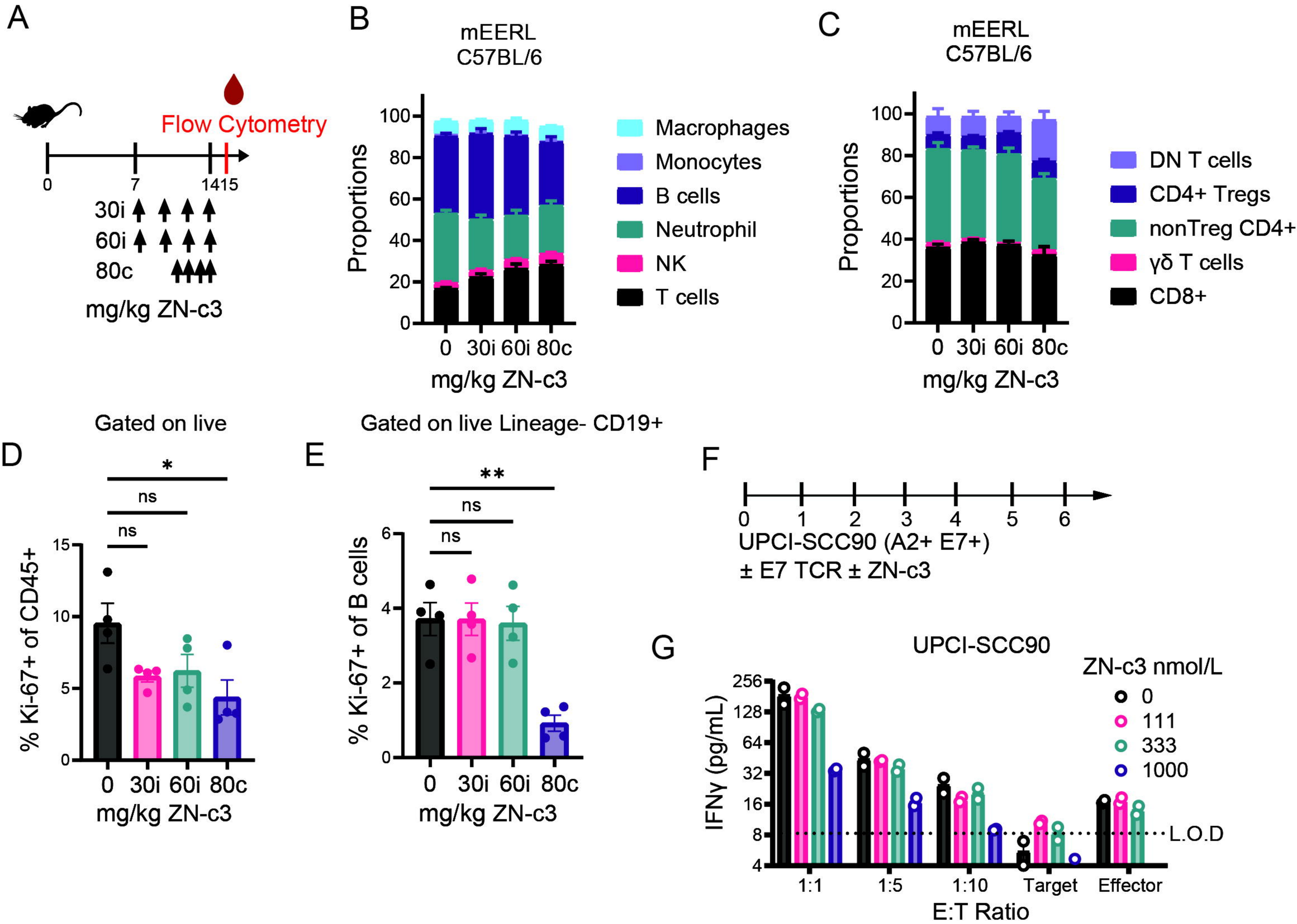
Survival (%)
Days post inoculation
M
24 31 38 45 28
ZN-c3 x3
E7 TCR
N
LNT-20
(E7+ A2+)
NT
ZN-c3
1e6 E7 TCR
Combo
*
*
ns
Tumor growth
(% change from baseline)
Day of treatment


A
Flow Cytometry
0
24
48
Drug Wash Drug
B
0
100
300
1000
(nmol/L)
70.9%
60.9%
32.9%
13.0%
γH2AX
pCDK1+ (Y15)
DAPI
C
mTE Drug
mTE Wash
mEERL Drug
mEERL Wash
% pCDK1+ (Y15)
nmol/L ZN-c3
D
% γH2AX+
nmol/L ZN-c3
E
Cxcl10
Ccl5
Cxcl1
sTNFR1
Control
ZN-c3
mEERL
F
±diABZI
Ifnb1
Isg15
mRNA expression
Hours
mTE
mEERL
G
± ZN-c3
RT-qPCR
H
0
300
600 nmol/L ZN-c3
Ifna1
Ifnb1
Ifng
mRNA expression
0.0260
0.0371
0.0386
0.5379
0.2798
0.1101
0.0320
0.0458
0.0280
mEERL WT
C57BL/6 WT
mEERL WT
Sting gt/gt
mEERL sgCgas
C57BL/6 WT
I
mEERL Sting gt/gt
ZN-c3
Control
60 mg/kg
Tumor volume (mm3)
Days post inoculation
J
mEERL Sting gt/gt
Survival (%)
K
mEERL sgCgas cl10
L
mEERL sgCgas cl10

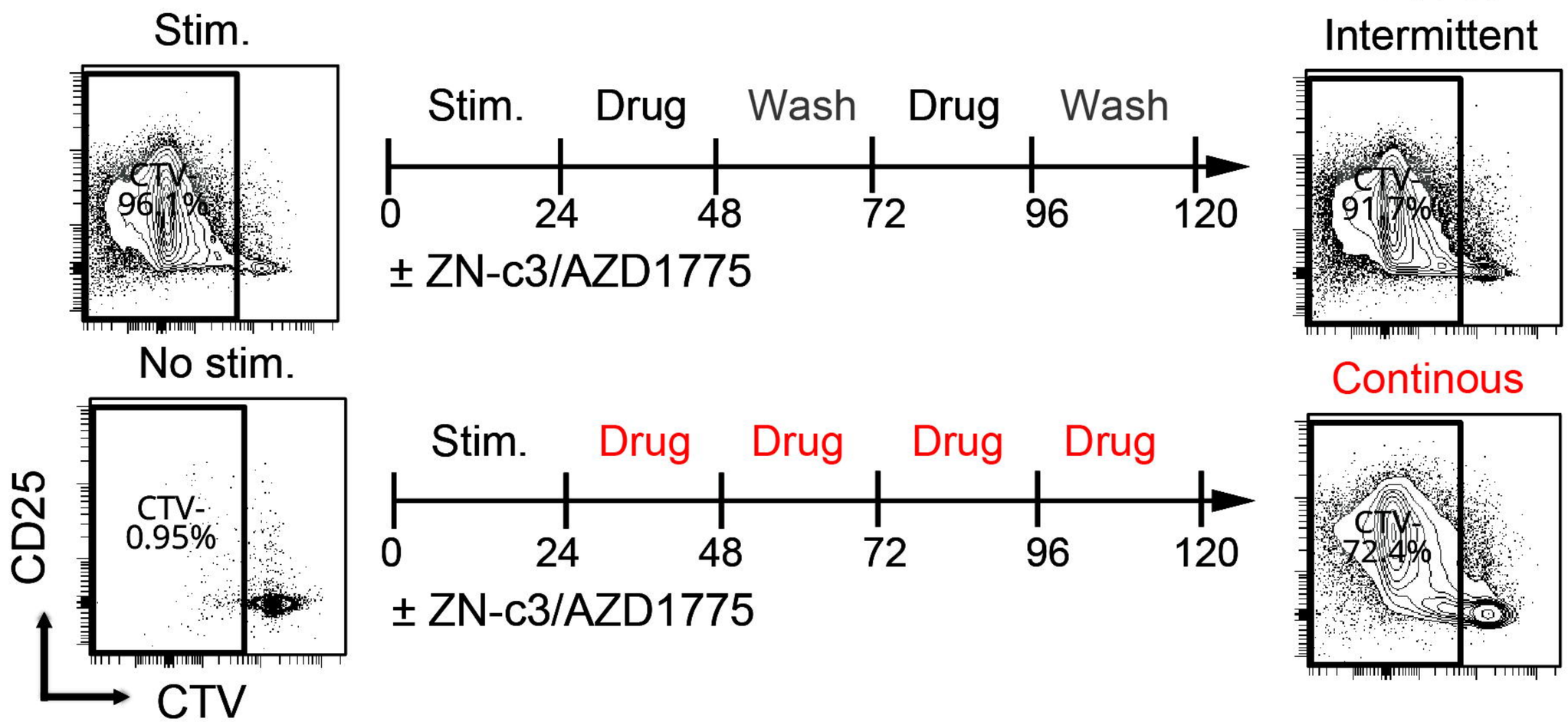

A
Tumor
Stroma
Immune
UMAP_2
UMAP_1
C
Control
AZD1775
CD8_Tpro
CD8_Teff
Tumor
TAN
TAM
CAF
BEC
CD4_Treg
NK
DC
MyCAF
LEC
CD4_Tconv
B
Allograft Rejection
Interferon Alpha Response
Interferon Gamma Response
P53 Pathway
IL2 Stat5 Signaling
Estrogen Response Late
Estrogen Response Early
KRAS Signaling Dn
Myogenesis
E2F Targets
G2M Checkpoint
Immune
Stroma
Tumor
NES
−log10(padj)
D
Control
AZD1775
Source
Target
Counts
Strength
E
CD8_Tpro
CD8_Teff
TAM
DC
TAN
F
CD4_Treg
CD4_Tconv
TAM
DC
TAN
G
NK
TAN
Cell type
TAN
TAM
CD4_Tconv
CD8_Tpro
DC
NK
CD4_Tregs
CD8_Teff
H
HPV+ Tumor Cell
Host cell
ZN-c3
WEE1
DNA Damage
Cytosolic DNA
cGAS
HPV E7
STING
STING
cGAMP
Type I IFN
Chemokines
NK cell recruitment
T cell recruitment
DC priming of T cells

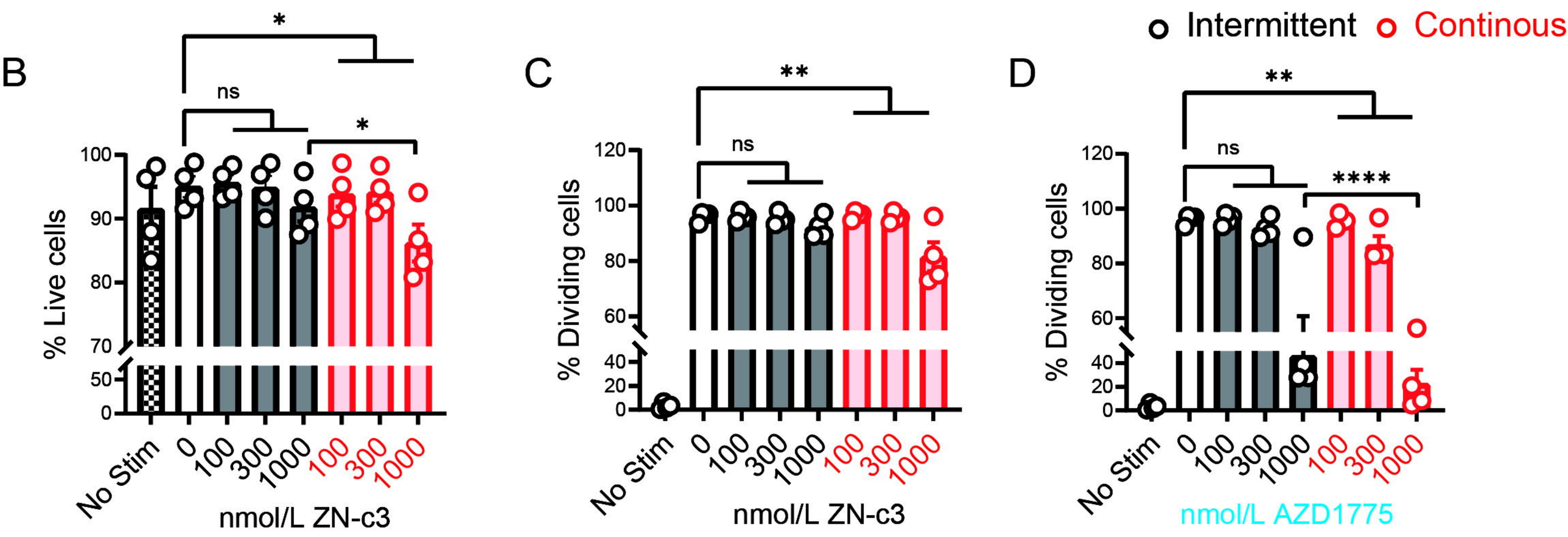

A
± ZN-c3
Flow cytometry
0
24
48
72
Gated on CD3+
B
Δ ZN-c3 – NT
CD4 GZMB+
CD8 GZMB+
CD8 CD25+
CD8 LAG3+
Treg TIM3+
CD4 TIM3+
CD8 TIM3+
CD4 PD1+
CD8 CD38+
CD4 CD39+
CD8 CD39+
CD4 CD25+
CD4 CTLA4+
CD4 LAG3+
Treg CD38+
CD4 CD38+
Treg CTLA4+
CD8 PD1+
Treg LAG3+
CD8 CD127+
CD8 TCF1+
CD4 CD127+
CD4 TCF1+
CD4 CCR7+
CD8 CCR7+
P42 P71 P14 P36 P37 P53 P41 P12 P69 P40 P58 P16 P18 P13 P39
Effector-like response
Stem-like response
Minimal response
C
Gated on CD4+
NT
ZN-c3
ZN-c3 nmol/L
0
250
500
% CTLA−4+
ns
ns
*
D
Gated on CD4+
% CD39+
E
Flow Cytometry
0
7
1415
0, 30, 60
mg/kg ZN-c3
F
% CD45+ in live
**
**
mg/kg ZN-c3
G
% CD3+ in live
*
***
H
% of live cells
mg/kg ZN-c3
nonTreg CD4
CD8
NK
B
Lymphocyte
Gated on Pd-1+CD8+
I
0mg/kg
30mg/kg
60mg/kg ZN-c3
Ly6C
TCF1
Gated on CD8+
J
% Pd-1+ Ly6C+
K
% TCF1+ Pd-1+
Gated on CD4+
L
% Pd-1+ Ly6C+
M
% TCF1+ Pd-1+
ns
N
0
7
14
21
28
ZN-c3 x3
FTY720
O
mEERL
+FTY720
ZN-c3
Control
60 mg/kg
ns
Tumor volume (mm3)
Days post inoculation
P
mEERL
+FTY720
ZN-c3
Control
60 mg/kg
ns
Survival (%)
Days post inoculation

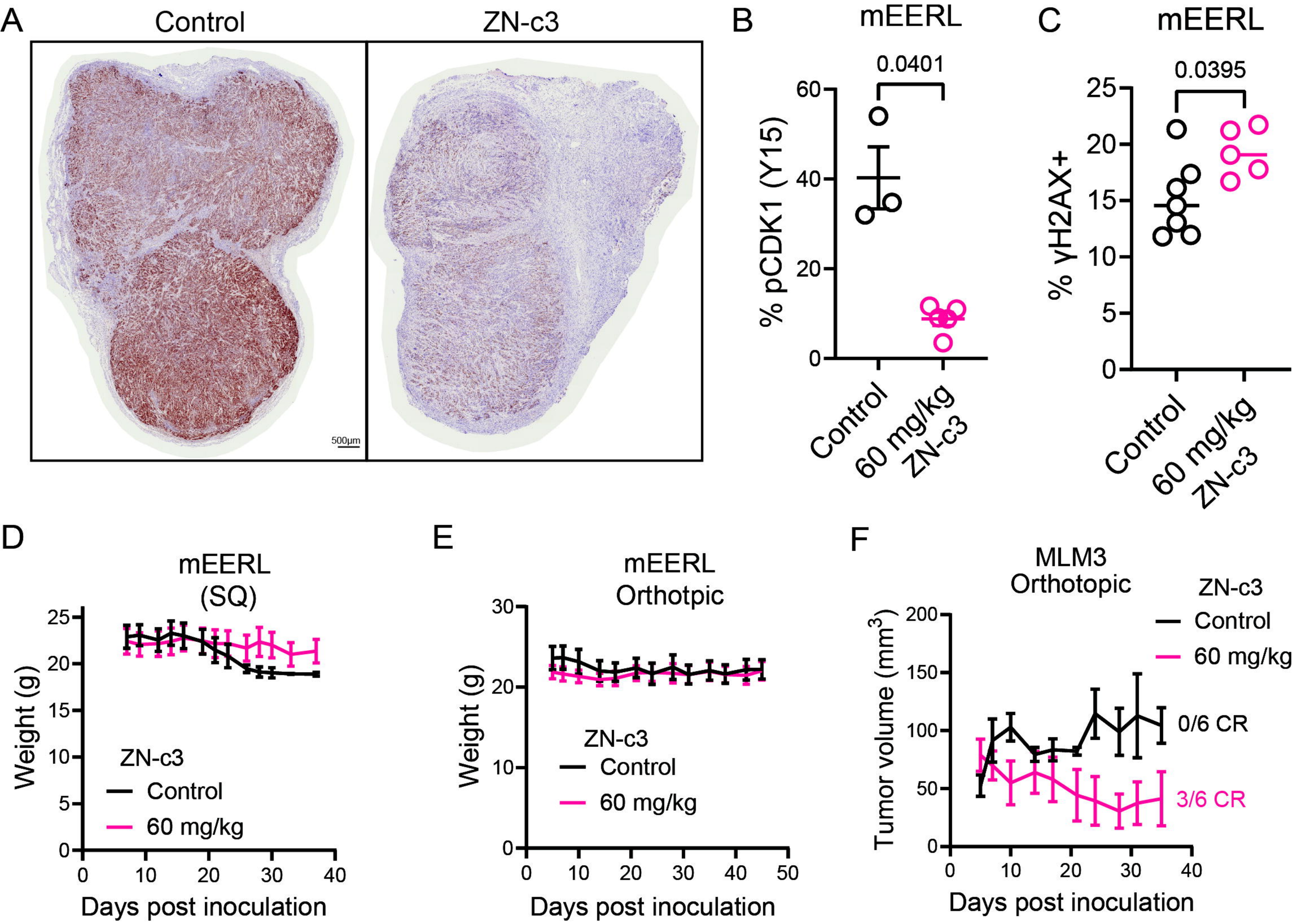

A
Flow Cytometry
0
7
14
15
30i
60i
80c
mg/kg ZN-c3
B
mEERL
C57BL/6
Proportions
Macrophages
Monocytes
B cells
Neutrophil
NK
T cells
mg/kg ZN-c3
C
mEERL
C57BL/6
Proportions
DN T cells
CD4+ Tregs
nonTreg CD4+
γδ T cells
CD8+
mg/kg ZN-c3
Gated on live
D
% Ki-67+ of CD45+
ns
ns
*
mg/kg ZN-c3
Gated on live Lineage- CD19+
E
% Ki-67+ of B cells
ns
ns
**
mg/kg ZN-c3
F
UPCI-SCC90 (A2+ E7+)
± E7 TCR ± ZN-c3
G
UPCI-SCC90
ZN-c3 nmol/L
0
111
333
1000
IFNγ (pg/mL)
L.O.D
1:1
1:5
1:10
Target
Effector
E:T Ratio

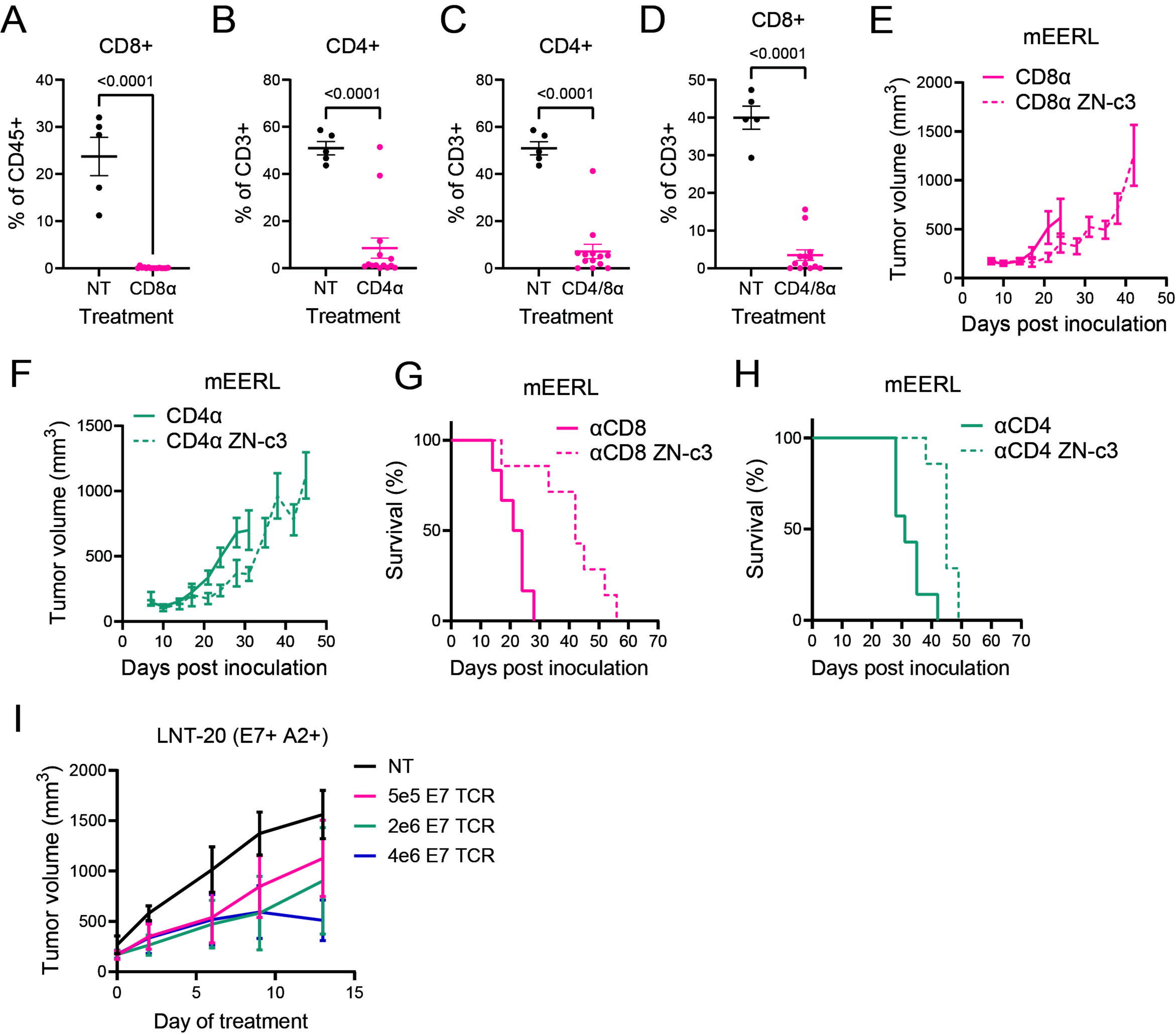
Gated on CD8+

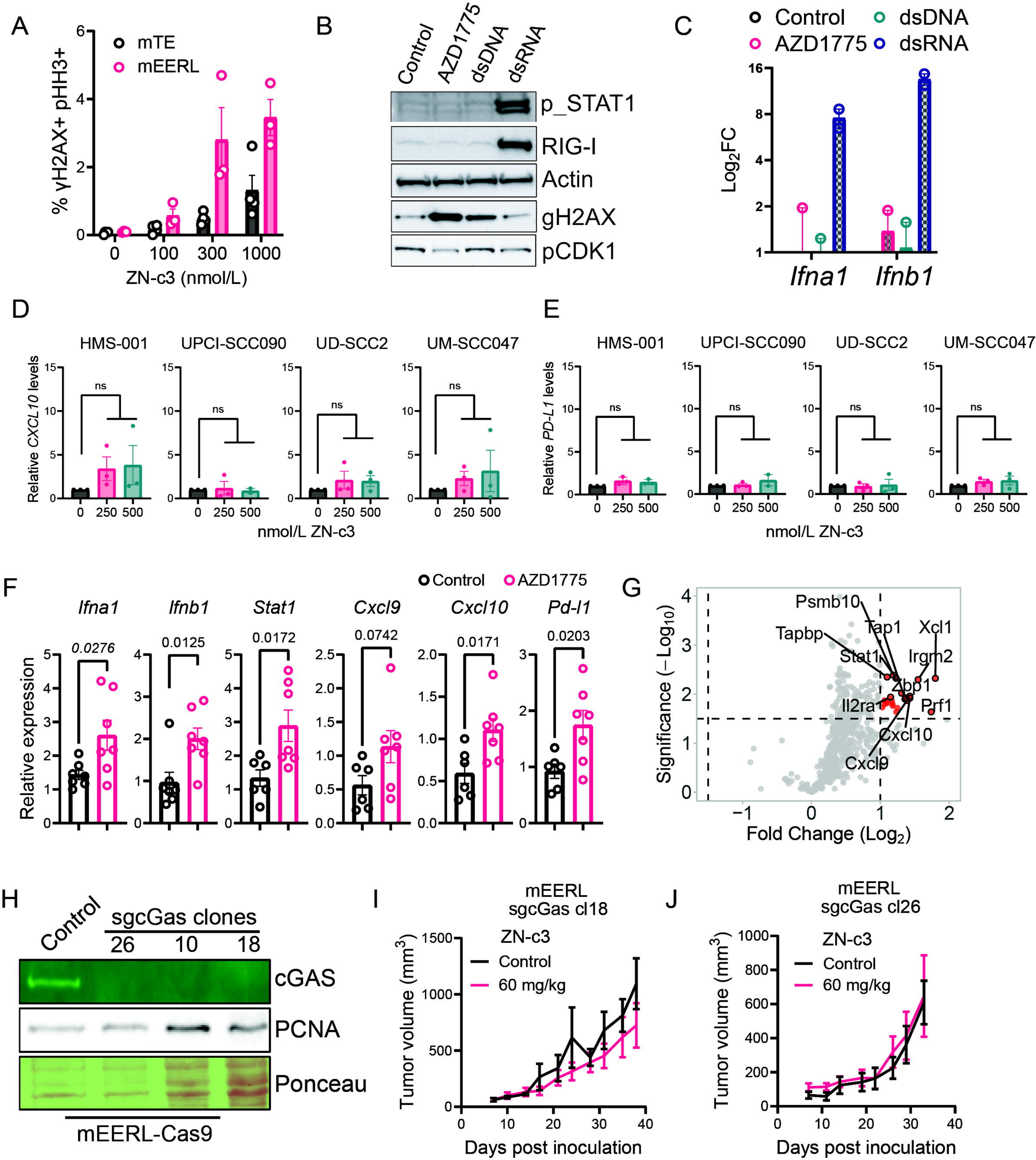
A

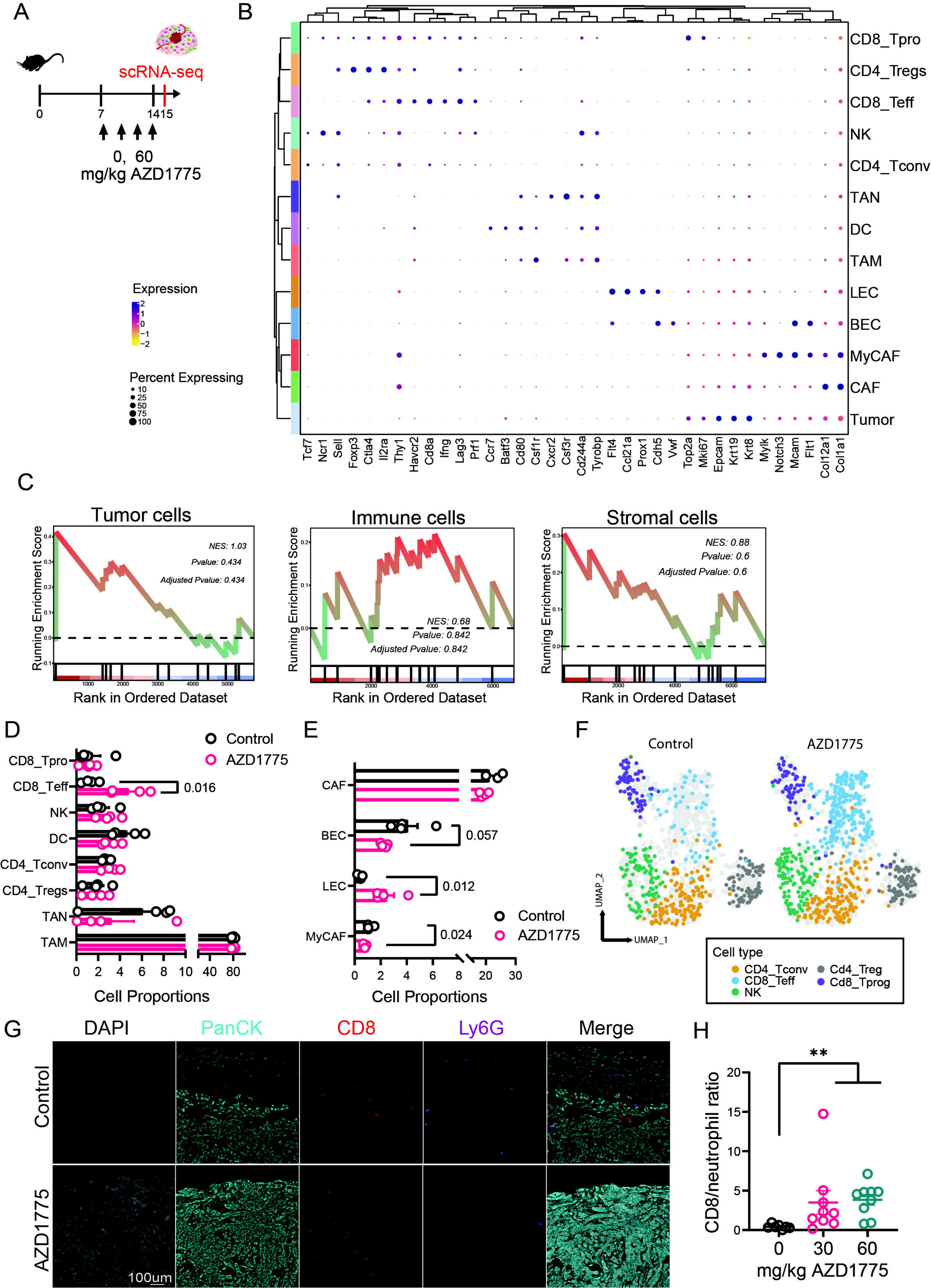


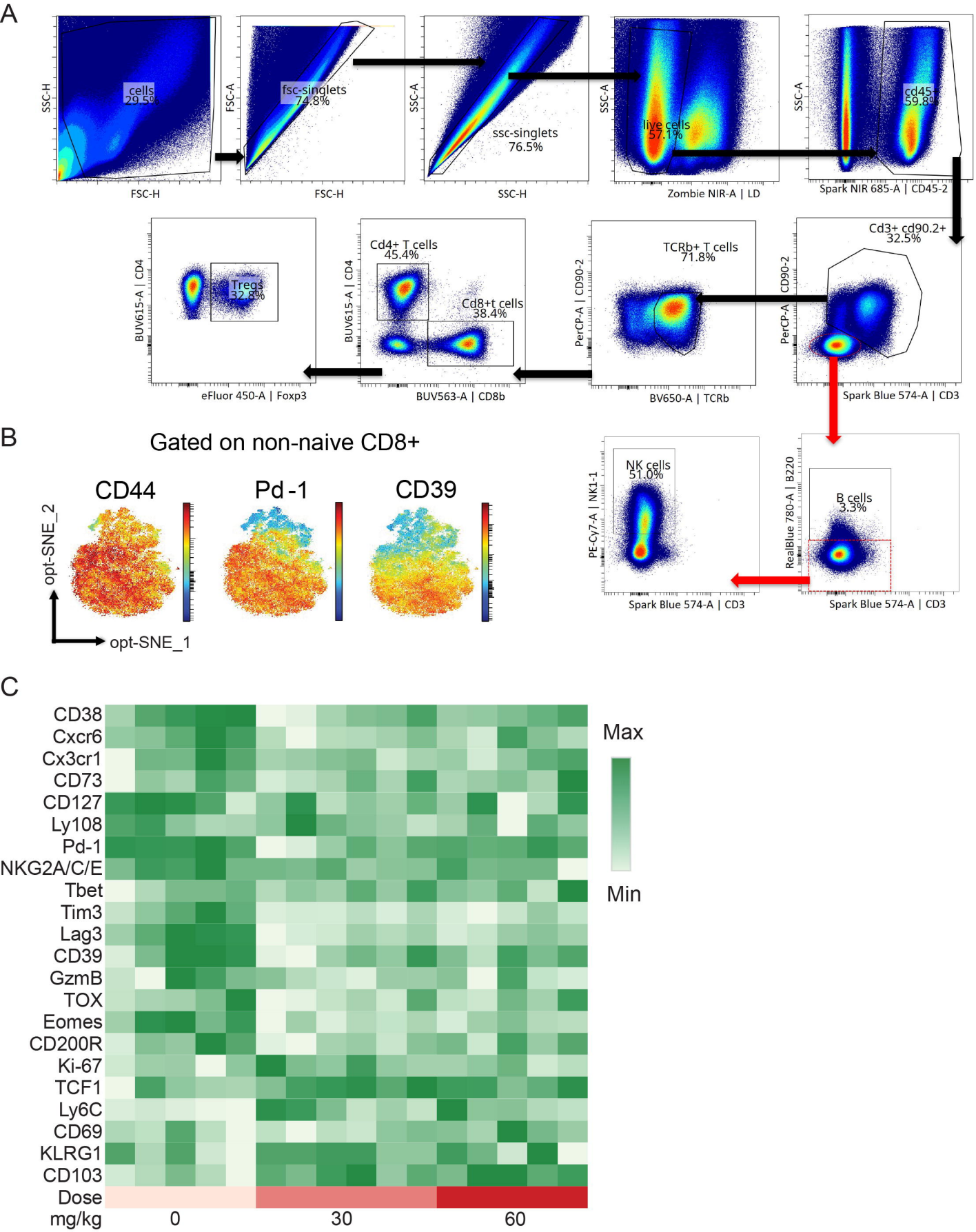


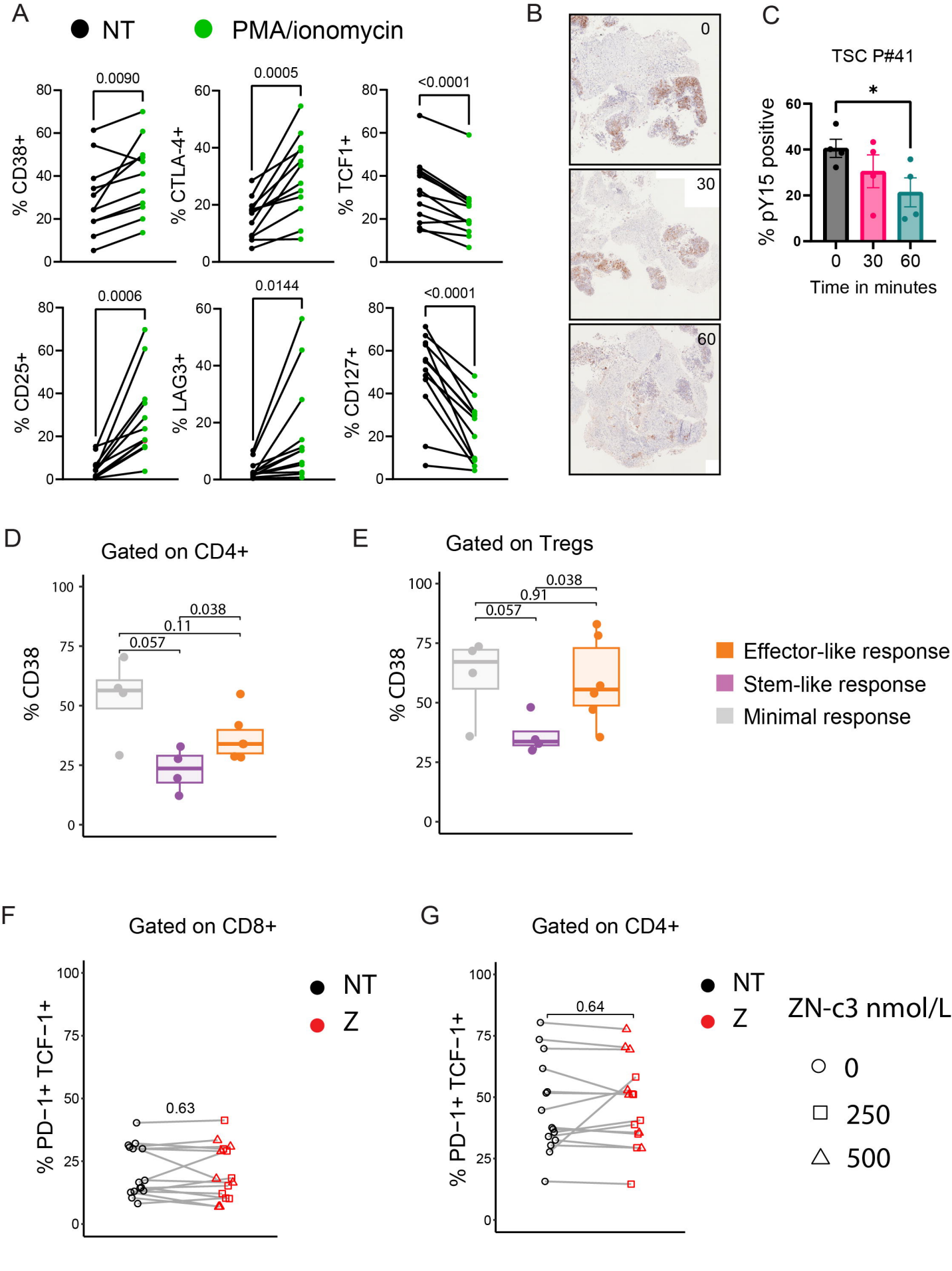

A
Control
ZN-c3
500µm
B
mEERL
0.0401
% pCDK1 (Y15)
60
40
20
0
Control
60 mg/kg ZN-c3
C
mEERL
0.0395
% γH2AX+
25
20
15
10
5
0
Control
60 mg/kg ZN-c3
D
mEERL (SQ)
Weight (g)
25
20
15
10
5
0
ZN-c3
Control
60 mg/kg
0
10
20
30
40
Days post inoculation
E
mEERL Orthotpic
Weight (g)
30
20
10
0
ZN-c3
Control
60 mg/kg
0
10
20
30
40
50
Days post inoculation
F
MLM3 Orthotopic
ZN-c3
Control
60 mg/kg
Tumor volume (mm3)
200
150
100
50
0
0/6 CR
3/6 CR
0
10
20
30
40
Days post inoculation

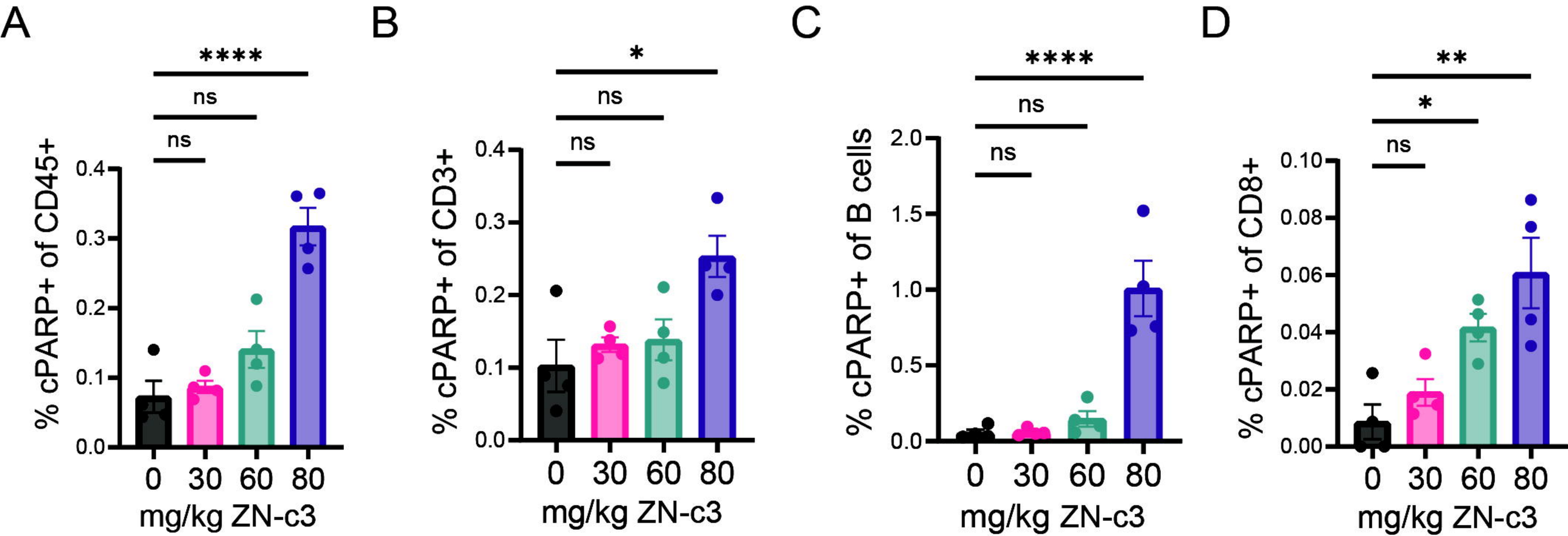

A
CD8+
<0.0001
% of CD45+
NT
CD8α
Treatment
B
CD4+
<0.0001
% of CD3+
NT
CD4α
Treatment
C
CD4+
<0.0001
% of CD3+
NT
CD4/8α
Treatment
D
CD8+
<0.0001
% of CD3+
NT
CD4/8α
Treatment
E
mEERL
CD8α
CD8α ZN-c3
Tumor volume (mm3)
Days post inoculation
F
mEERL
CD4α
CD4α ZN-c3
Tumor volume (mm3)
Days post inoculation
G
mEERL
αCD8
αCD8 ZN-c3
Survival (%)
Days post inoculation
H
mEERL
αCD4
αCD4 ZN-c3
Survival (%)
Days post inoculation
I
LNT-20 (E7+ A2+)
NT
5e5 E7 TCR
2e6 E7 TCR
4e6 E7 TCR
Tumor volume (mm3)
Day of treatment

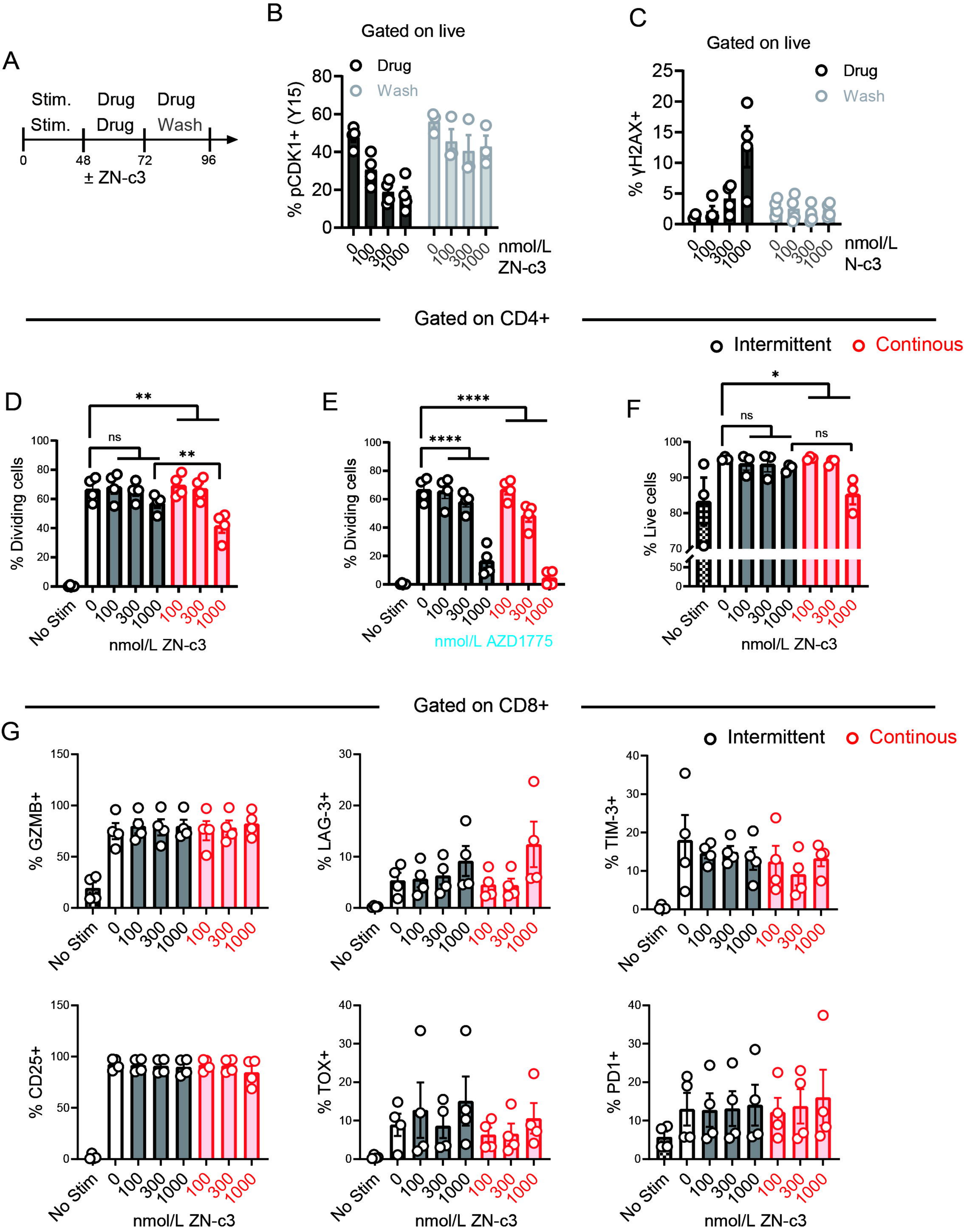

A
mTE
mEERL
% γH2AX+ pHH3+
ZN-c3 (nmol/L)
B
Control
AZD1775
dsDNA
dsRNA
p_STAT1
RIG-I
Actin
gH2AX
pCDK1
C
Control
AZD1775
dsDNA
dsRNA
Log2FC
Ifna1
Ifnb1
D
HMS-001
UPCI-SCC090
UD-SCC2
UM-SCC047
Relative CXCL10 levels
ns
nmol/L ZN-c3
E
Relative PD-L1 levels
F
Control
AZD1775
Ifna1
Ifnb1
Stat1
Cxcl9
Cxcl10
Pd-l1
Relative expression
0.0276
0.0125
0.0172
0.0742
0.0171
0.0203
G
Psmb10
Tapbp
Tap1
Xcl1
Stat1
Irgm2
Zbp1
Il2ra
Prf1
Cxcl10
Cxcl9
Significance (−Log10)
Fold Change (Log2)
H
Control
sgcGas clones
26
10
18
cGAS
PCNA
Ponceau
mEERL-Cas9
I
mEERL
sgcGas cl18
ZN-c3
Control
60 mg/kg
Tumor volume (mm3)
Days post inoculation
J
mEERL
sgcGas cl26
ZN-c3
Control
60 mg/kg
Tumor volume (mm3)
Days post inoculation

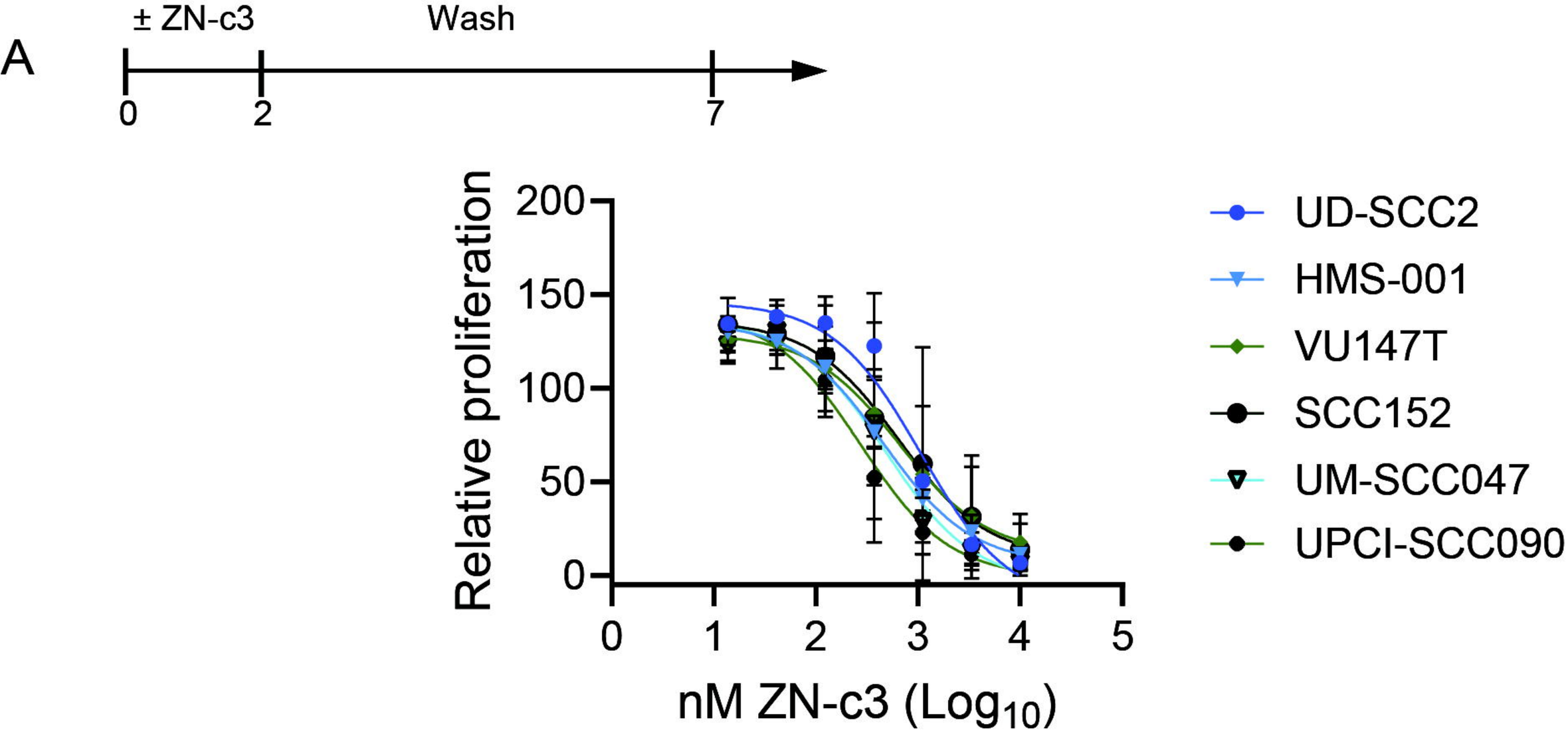

A
scRNA-seq
0
7
1415
0, 60
mg/kg AZD1775
B
Expression
Percent Expressing
CD8_Tpro
CD4_Tregs
CD8_Teff
NK
CD4_Tconv
TAN
DC
TAM
LEC
BEC
MyCAF
CAF
Tumor
Tcf7 Ncr1 Sell Foxp3 Ctla4 Il2ra Thy1 Havcr2 Cd8a Ifng Lag3 Prf1 Ccr7 Batf3 Cd80 Csf1r Cxcr2 Csf3r Cd244a Tyrobp Flt4 Ccl21a Prox1 Cdh5 Vwf Top2a Mki67 Epcam Krt19 Krt8 Mylk Notch3 Mcam Flt1 Col12a1 Col1a1
C
Tumor cells
NES: 1.03
Pvalue: 0.434
Adjusted Pvalue: 0.434
Immune cells
NES: 0.68
Pvalue: 0.842
Adjusted Pvalue: 0.842
Stromal cells
NES: 0.88
Pvalue: 0.6
Adjusted Pvalue: 0.6
Running Enrichment Score
Rank in Ordered Dataset
D
Control
AZD1775
0.016
Cell Proportions
E
0.057
0.012
0.024
F
Control
AZD1775
UMAP_1
UMAP_2
Cell type
CD4_Tconv
CD8_Teff
NK
Cd4_Treg
Cd8_Tprog
G
DAPI
PanCK
CD8
Ly6G
Merge
100um
H
**
CD8/neutrophil ratio
mg/kg AZD1775


A
cells
29.5%
SSC-H
FSC-H
fsc-singlets
74.8%
FSC-A
FSC-H
ssc-singlets
76.5%
SSC-A
SSC-H
live cells
57.1%
SSC-A
Zombie NIR-A | LD
cd45+
59.8%
SSC-A
Spark NIR 685-A | CD45-2
Cd3+ cd90.2+
32.5%
CD90-2
PerCP-A
Spark Blue 574-A | CD3
TCRb+ T cells
71.8%
PerCP-A | CD90-2
BV650-A | TCRb
Cd4+ T cells
45.4%
Cd8+t cells
38.4%
BUV615-A | CD4
BUV563-A | CD8b
Tregs
32.8%
BUV615-A | CD4
eFluor 450-A | Foxp3
B
Gated on non-naive CD8+
CD44
Pd-1
CD39
opt-SNE_2
opt-SNE_1
NK cells
51.0%
PE-Cy7-A | NK1-1
Spark Blue 574-A | CD3
B cells
3.3%
RealBlue 780-A | B220
Spark Blue 574-A | CD3
C
CD38
Cxcr6
Cx3cr1
CD73
CD127
Ly108
Pd-1
NKG2A/C/E
Tbet
Tim3
Lag3
CD39
GzmB
TOX
Eomes
CD200R
Ki-67
TCF1
Ly6C
CD69
KLRG1
CD103
Dose
mg/kg
0
30
60
Max
Min


A
NT
PMA/ionomycin
% CD38+
0.0090
% CTLA-4+
0.0005
% TCF1+
<0.0001
% CD25+
0.0006
% LAG3+
0.0144
% CD127+
<0.0001
B
0
30
60
C
TSC P#41
% pY15 positive
*
0
30
60
Time in minutes
D
Gated on CD4+
% CD38
0.038
0.11
0.057
E
Gated on Tregs
% CD38
0.038
0.91
0.057
Effector-like response
Stem-like response
Minimal response
F
Gated on CD8+
% PD−1+ TCF−1+
0.63
NT
Z
G
Gated on CD4+
% PD−1+ TCF−1+
0.64
NT
Z
ZN-c3 nmol/L
0
250
500


A
****
ns
ns
% cPARP+ of CD45+
0.4
0.3
0.2
0.1
0.0
0
30
60
80
mg/kg ZN-c3
B
*
ns
ns
% cPARP+ of CD3+
0.4
0.3
0.2
0.1
0.0
0
30
60
80
mg/kg ZN-c3
C
****
ns
ns
% cPARP+ of B cells
2.0
1.5
1.0
0.5
0.0
0
30
60
80
mg/kg ZN-c3
D
**
*
ns
% cPARP+ of CD8+
0.10
0.08
0.06
0.04
0.02
0.00
0
30
60
80
mg/kg ZN-c3


A
Stim.
Stim.
Drug
Drug
Drug
Wash
0
48
72
96
± ZN-c3
B
Gated on live
Drug
Wash
% pCDK1+ (Y15)
nmol/L
ZN-c3
C
Gated on live
Drug
Wash
% γH2AX+
nmol/L
N-c3
Gated on CD4+
Intermittent
Continous
D
% Dividing cells
No Stim
nmol/L ZN-c3
E
% Dividing cells
nmol/L AZD1775
F
% Live cells
nmol/L ZN-c3
Gated on CD8+
Intermittent
Continous
G
% GZMB+
% LAG-3+
% TIM-3+
% CD25+
% TOX+
% PD1+
nmol/L ZN-c3



	UD-SCC2	HMS-001	VU147T	SCC152	UM-SCC047	UPCI-SCC090
IC50	1067	454.2	651.5	645.1	483.5	274.3