1 **ATR kinase inhibition induces thymineless death in proliferating CD8⁺ T cells**

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SUMMARY

ATR kinase is a central regulator of the DNA damage response (DDR) and cell cycle checkpoints. However, little is known about the role of ATR in the cell cycle and the impact of DDR inhibitors in immune cells in the absence of DNA damage. We previously showed that the ATR inhibitor AZD6738 (ATRi) combines with radiation to generate delayed, CD8⁺ T cell-dependent antitumor responses in mouse models of cancer. Here, we show that ATRi induces untimely CDK1 activity during S phase in CD8⁺ T cells and this induces origin firing and simultaneous degradation of dNTP synthesis and salvage enzymes. These pleiotropic effects of ATRi in proliferating CD8⁺ T cells induce deoxyuridine contamination in genomic DNA, R loops, RNA-DNA polymerase collisions, and death. Remarkably, thymidine significantly rescues ATRi-induced CD8⁺ T cell death. Our data identifies critical considerations for the design of clinical ATRi regimens with genotoxic chemo- and radiation and immunotherapies.

KEYWORDS

ATR/CD8⁺ T cells/origin firing/deoxyuridine contamination/thymineless death

INTRODUCTION

Most chemotherapies target DNA replication forks and their efficacy is associated with both the direct killing of proliferating tumor cells and, in many cases, the stimulation of innate and adaptive anti-tumor immune responses (Bracci et al., 2014; O'Connor, 2015). One mechanism through which DNA damaging chemotherapies increase anti-tumor immune responses is the direct killing of proliferating immune cells causing transient leukocytopenia followed by a rebound proliferation of immune cell populations. A major clinical goal is to identify novel regimens that maximize the direct killing of tumor cells, while concurrently selecting for and stimulating the activity of anti-tumor immune effector cells.

The DNA Damage Response (DDR) is a signaling system that integrates nucleic acid metabolism and DNA repair with the cell cycle to safeguard genome stability (Ciccia and Elledge, 2010). DDR inhibitors (DDRi) have been developed to target tumors that have acquired inactivating mutations in a DNA repair pathway and to potentiate direct tumor cell killing by DNA damaging chemotherapies and radiotherapy. For example, Olaparib (Lynparza), the poly (ADP-ribose) polymerase (PARP) inhibitor, is approved for the treatment of tumors harboring BRCA1 or BRCA2 mutations. More recently, four ATR kinase inhibitors (we use the abbreviation ATRi's for these four inhibitors) have advanced to phase 1 and phase 2 trials: ceralasertib (AZD6738); berzosertib (M6620, VX-970); elimusertib (BAY 1895344); and RP-3500 (Foote et al., 2018; Hall et al., 2014; Roulston et al., 2021; Wengner et al., 2020). ATRi's potentiate chemotherapies that target DNA replication forks, selectively kill tumor cells with inactivating mutations in ATM, ERCC1, RNASE H2, and XRCC1, and block the upregulation of PD-L1 after radiation (Hustedt et al., 2019; Mohni et al., 2015; Roulston *et al.*, 2021; Sato et al., 2017; Vendetti et al., 2018; Vendetti et al., 2015; Wang et al., 2019). Phase I clinical trial data show that ATRi's suppress circulating monocytes and proliferating T cells, and that these populations rebound after cessation of ATRi's, as expected (Krebs et al., 2018). However, ATR inhibitor AZD6738 (we use the abbreviation ATRi for AZD6738) unexpectedly combines with radiation to generate delayed, CD8⁺ T cell-dependent responses in mouse models of cancer (Vendetti *et al.*, 2018). These preclinical and clinical findings suggest that the direct impact of ATRi in immune cells is clinically important and warrants further investigation.

ATR is an essential kinase that is activated at regions of single-stranded DNA that are generally associated with stalled and collapsed DNA replication forks and resected DNA double strand breaks (Brown and Baltimore, 2000; Cortez et al., 2001; Zou and Elledge, 2003). ATR kinase phosphorylates and activates a second essential kinase, CHK1, that phosphorylates the CDC25A phosphatase causing its degradation in the proteasome after DNA damage (Liu et al., 2000; Mailand et al., 2000; Sanchez et al., 1997). Since CDC25A removes inhibitory phosphorylations on the cyclin-dependent kinases CDK2 and CDK1 facilitating the G1/S and G2/M transitions, respectively, CDC25A degradation induces cell cycle arrest (Hoffmann et al., 1994). While the role of CDC25A degradation in cell cycle arrest after DNA damage is well-accepted, CDC25A is a dynamic protein and the ATR-CHK1-CDC25A signaling axis has also been implicated in progression through S phase in unperturbed cells (Sorensen et al., 2004). Whether ATR-CHK1-CDC25A signaling limits origin firing through S phase to prevent replication fork stalling or repairs replication forks stalled at endogenous DNA lesions or following mechanical failure in unperturbed cells is not known.

ATR_i induce origin firing across active replicons in human cancer cells and fibroblasts (Couch et al., 2013; Kwok et al., 2016; Moiseeva et al., 2017; Moiseeva et al., 2019b). However, the physiological and clinical significance of this signaling mechanism has not been established. Computational models of the spatiotemporal pattern of origin firing point to an unknown mechanism that limits additional origin firing within ~7–120 kb of an origin that fires (Lob et al., 2016). Since the ~50,000 origins that replicate the human genome are generated from an excess of ~500,000 licensed origins, we hypothesized that the first origin firing event within a replicon may be stochastic and that ATR-CHK1-CDC25A signaling may then limit additional origin firing across active replicons generating dormant or flexible origins that are passively replicated (Mahbubani et al., 1997; Sorensen et al., 2004). We further hypothesized that, through limiting origin firing across active replicons, ATR-CHK1-CDC25A signaling may accommodate the supply of dNTPs which is 50-fold less than that required to synthesize the genome in a human epithelial cell (Ferraro et al., 2010).

To test these hypotheses in a clinically important model, we interrogated the impact of ATR_i in CD8⁺ T cells *ex vivo*. We show that ATR_i induces deoxyuridine (dU) contamination in genomic

DNA, R loops, RNA-DNA polymerase collisions, and death in proliferating, but not naïve CD8⁺ T cells. Remarkably, we show that thymidine significantly rescues ATRi-induced CD8⁺ T cell death. Our results have far-reaching implications for the design of clinical trials combining ATRi with immunotherapies and/or antimetabolites and suggest that dose limiting toxicity in active clinical trials of ATRi may be associated with leukocytopenia caused by thymineless cell death.

RESULTS

ATR kinase is essential in proliferating CD8⁺ T cells *ex vivo*

To study the impact of ATRi on the activation, proliferation, and survival of CD8⁺ T cells, we used *ex vivo* activated CD8⁺ T cells derived from spleens and lymph nodes of C57BL/6 and Pmel-1 mice. We used Pmel-1 CD8⁺ T cells activated with the synthetic peptide gp100 in splenocytes when the experimental endpoint allowed the identification of CD8⁺ T cells. We used CD8⁺ T cells activated with CD3E and CD28 antibodies after purification by negative selection when contaminating cells could compromise the experimental endpoint.

To confirm the kinetics of activation and proliferation of CD8⁺ T cells *ex vivo*, we used a flow cytometry assay of cell trace violet (CTV) dye dilution. CD8⁺ T cells were fully activated (CD44^{hi}) at ~21 h and started dividing at ~24 h (Figure 1A). Once activated, CD8⁺ T cells divided 4-5 times from 24-48 h, consistent with a previous report (Yoon et al., 2010). To determine whether the activation of CD8⁺ T cells was associated with increased expression of DDR and replication proteins, we generated whole cell extracts from CD8⁺ T cells at 0 h and 30 h. Empirically we found that ATM could be used to normalize the naïve and proliferating CD8⁺ T cells proteomes (Figure 1B). ATR and deoxycytidine kinase (DCK) were expressed in naïve CD8⁺ T cells and induced significantly in proliferating CD8⁺ T cells. CHK1, the ribonucleotide reductase subunits RRM1 and RRM2, deoxyuridine 5'-triphosphate nucleotidohydrolase (DUT), and WEE1 were expressed at the limits of detection in naïve CD8⁺ T cells and highly expressed in proliferating CD8⁺ T cells.

To determine whether ATR kinase activity is required for the activation and proliferation of CD8⁺ T cells, we treated CD8⁺ T cells with ATRi (Foote *et al.*, 2018) from 0-24 h (during activation) or 24-48 h (during proliferation) and monitored survival using the viability dye eFluor 780. While ATRi had little impact on CD8⁺ T cells from 0-24 h, ATRi from 24-48 h induced death in most

proliferating CD8⁺ T cells (Figure 1C). We then examined whether ATRi affects the survival of unactivated, naïve CD8⁺ T cells maintained in interleukin 7 (IL-7). While ATRi caused a slight decrease in the survival of naïve CD8⁺ T cells from 0-24 h, ATRi from 24-48 h had no impact (Figure 1D). IL-7 can promote homeostatic proliferation and this may explain why ATRi caused a slight decrease in the survival of CD8⁺ T cells from 0-24 h (Tan et al., 2001). We also determined whether a 24h treatment with ATRi impacts the survival of exponentially dividing B16 cancer cells and primary fibroblasts (Figure 1E). ATRi did not induce significant cell death in B16 or primary fibroblasts in 24 h. Finally, we determined whether clinical inhibitors of ATM (AZD0156), PARP (Olaparib), and WEE1 (AZD1775) induced death in proliferating CD8⁺ T cells. Neither AZD0156 nor AZD1775 induced death in proliferating CD8⁺ T cells in 24 h (Figure 1F). While Olaparib induced statistically significant death in proliferating CD8⁺ T cells, this was not comparable with ATRi which induced death in most proliferating CD8⁺ T cells in 24 h.

ATR kinase is essential in previously activated, proliferating CD8⁺ T cells *in vivo*.

ATRi potentiates CD8⁺ T cell-dependent anti-tumor activity following conformal radiation in syngeneic CT26 colorectal tumors, despite transiently reducing activated CD8⁺ T cells in BALB/c host spleens (Vendetti *et al.*, 2018). To define the impact of ATRi on CD8⁺ T cell activation and proliferation *in vivo*, we treated CT26 tumor-bearing mice with 75 mg/kg of ATRi on days 1,2, and 3, and immunoprofiled CD8⁺ T cells in tumor infiltrating lymphocytes (TIL) and the periphery (spleen and draining lymph node (DLN)) on day 4. CD8⁺ T cell numbers decreased in TIL in mice treated with ATRi (Figure 2A). Relative CD8⁺ T cell numbers were not changed in the periphery by ATRi. However, spleen weight decreased in mice treated with ATRi (Figure S1A). Importantly, the percentage of proliferating CD8⁺ T cells (Ki67⁺) significantly decreased in TIL, spleen, and DLN in mice treated with ATRi (Figure 2B,C). Thus, ATRi impacts proliferating CD8⁺ T cells *in vivo* in all immunoprofiled tissues.

To determine whether ATRi impedes CD8⁺ T cell activation *in vivo*, we examined expression of the early activation marker CD69 on CD8⁺ T cells in the TIL and periphery. The percentage of newly or recently activated CD8⁺ T cells (CD69⁺) was increased in TIL in mice treated with ATRi (Figure 2D). The percentage of recently activated CD8⁺ T cells (CD69⁺) in the periphery was not changed by ATRi. We further probed CD8⁺ T cell activation phenotypes using the markers CD62L

and CD44 to identify un-activated naïve (TN, CD26L^{lo} CD44^{lo}) CD8⁺ T cells and activated central memory (TCM, CD26L^{hi} CD44^{hi}) and effector/effector memory (TEM, CD26L^{lo} CD44^{hi}) CD8⁺ T cells in the TIL and periphery. Consistent with our previous findings (Vendetti et al., 2018), ATRi reduced the percentage of activated TCM and TEM CD8⁺ T cells in the spleen (Figure S1B,C). Concurrently, ATRi increased the percentage of un-activated TN CD8⁺ T cells in the spleen. No significant changes in these populations were observed in the DLN (Figure S1B,C). While the CD8⁺ TCM and TEM subpopulations in the TIL were not altered by ATRi (Figure S1B,D), the percentage of total CD62L^{lo} CD8⁺ T cells (which includes the effector/effector memory pool) decreased in TIL in mice treated with ATRi (Figure S1E,F).

To further define the population of effector CD8⁺ T cells impacted by ATRi *in vivo*, we examined KLRG1, CD127, and CD69 expression on CD62L^{lo} CD8⁺ T cells. The percentage of short lived/terminal effector CD8⁺ T cells (KLRG1⁺ CD127⁻) decreased in TIL in mice treated with ATRi (Figure 2E,F). Similarly, the percentage of KLRG1⁺ effector CD8⁺ T cells that had been previously activated and had already downregulated CD69 (KLRG1⁺ CD69⁻) decreased in TIL in mice treated with ATRi (Figure 2G,H). No significant changes in tissue resident (CD103⁺ CD69⁺) or memory precursor effector (KLRG1⁻ CD127⁺) or newly activated KLRG1⁺ effector (KLRG1⁺ CD69⁺) CD8⁺ T cells were seen in TIL in mice treated with ATRi (Figure S1G-J).

Together these data show that ATRi decreases a short-lived population of effector CD8⁺ T cells in TIL but does not impede CD8⁺ T cell activation *in vivo*. This is entirely consistent with the data presented in Figure 1 that show that CD8⁺ T cell activation is associated with significant increases in ATR and CHK1 protein expression and that ATR kinase activity is essential in activated, proliferating CD8⁺ T cells that divide 4-5 times in 24 h *ex vivo*. Thus, the impact of ATRi on CD8⁺ T cell activation and proliferation *ex vivo* is a physiologically relevant system in which to study mechanism.

Most proliferating CD8⁺ T cells are in S phase

We reasoned that the sensitivity of proliferating CD8⁺ T cells to ATRi-induced cell death relative to naïve CD8⁺ T cells and other cell types (Figure 1C-E) may be associated with their rapid cell cycle. To determine the cell cycle distribution of proliferating CD8⁺ T cells, and exponentially

dividing primary fibroblasts and B16, we labelled S phase cells with 5-ethynyl-2'-deoxyuridine (EdU) for 30 min and then identified EdU incorporation and DNA content using flow cytometry. Using these datasets, we estimated the length of G1, S, and G2/M (Figure S2A). In CD8⁺ T cells, G1 was $\sim 1.0 \pm 0.3$ h, S phase was $\sim 4.7 \pm 0.3$ h, and G2/M was $\sim 0.3 \pm 0.1$ h (Figure 3A). In fibroblasts, G1 was $\sim 13 \pm 3.3$ h, S phase was $\sim 8.2 \pm 1.8$ h, and G2/M was $\sim 7.5 \pm 2.3$ h (26%) (Figure S2B). In B16, G1 was $\sim 7.1 \pm 1.3$ h (41%), S phase was $\sim 9.3 \pm 1.3$ h, and G2/M was 0.7 ± 0.3 h (Figure S2C). The cell cycle distribution of these three different cell types is summarized in a figure wherein the circumference of the circle represents the doubling time (Figure 3B). It is striking that most proliferating CD8⁺ T cells are in S phase.

ATR kinase limits origin firing in CD8⁺ T cells

Next, we reasoned that ATRi-induced origin firing could cause DNA damage and death in rapidly proliferating CD8⁺ T cells. In mammalian cells, CDK2 and CDC7 kinase-dependent mechanisms activate the replicative helicase and initiate DNA replication at $\sim 10\%$ of licensed origins in a spatiotemporal pattern that is broadly conserved from one cell division to the next (Cayrou et al., 2011; Chagin et al., 2016). The 90% of licensed origins that are passively replicated, described as either dormant or flexible origins, are essential for genome stability (Ge et al., 2007). Origin firing between stalled forks is a simple way to recover replication that would otherwise be lost. In the vast majority of mammalian cells, the selective pressure for genome stability likely supersedes the need for rapid DNA replication and cell division. However, in effector CD8⁺ T cells the selective pressure for rapid DNA replication and cell division may supersede the need for genome stability, as at least 90-95% are destined to undergo cell death during contraction. We used ATRi to investigate whether the fundamental mechanisms that mediate origin firing in mammalian cells are conserved in proliferating CD8⁺ T cells.

To determine whether ATRi induces origin firing in proliferating CD8⁺ T cells, we first examined the hyper-phosphorylation of chromatin bound MCM4 which has been associated with excessive activation of the replicative helicase (Sheu et al., 2016). ATRi, but not ATMi or PARPi, induced a mobility shift in MCM4 in chromatin extracts prepared from proliferating CD8⁺ T cells (Figure 3C). WEE1i induced a mobility shift in MCM4 in chromatin extracts from proliferating CD8⁺ T cells, but this was at the limits of detection. ATRi and WEE1i induce similar mobility shifts in MCM4 in

chromatin extracts prepared from U2OS and BJ-TERT (Moiseeva et al., 2019a). Since WEE1 is highly expressed in proliferating CD8⁺ T cells (Figure 1B), the difference is not due to an absence of this kinase.

To determine whether ATRi induces changes in the replication timing program, we used Repli-seq. Proliferating CD8⁺ T cells were treated with ATRi for 30 min and then added 5-bromo-2-deoxyuridine (BrdU) for an additional 30 min. CD8⁺ T cells were sorted into 2N-3N and 3N-4N populations and BrdU-labelled nascent DNA was purified and sequenced. ATRi did not induce late origin firing in early S phase proliferating CD8⁺ T cells (Figure 3D; Figure S3A,B). Furthermore, the distribution of DNA synthesis between early and late-S phase was not significantly different between fibroblasts and proliferating CD8⁺ T cells (Figure S3C).

To determine whether ATRi increased DNA synthesis, we treated proliferating CD8⁺ T cells with ATRi for 30 min and then added EdU for an additional 30 min, the same incubation intervals used for Repli-seq. EdU was quantitated in 2N-3N and 3N-4N cells using flow cytometry. ATRi increased the relative fluorophore-EdU intensity in proliferating CD8⁺ T cells in both in early S phase and late S phase (Figure 3E). Thus, ATRi induces origin firing and increases DNA synthesis in proliferating CD8⁺ T cells without impacting the replication timing pattern.

ATR kinase limits CDK1-dependent origin firing across active replicons in CD8⁺ T cells

Since ATRi increased DNA synthesis in proliferating CD8⁺ T cells by >25% without impacting the replication timing pattern, we reasoned that ATR kinase activity limits origin firing across active replicons in CD8⁺ T cells. To test this, we combed DNA purified from proliferating CD8⁺ T cells, exponentially dividing B16, and primary fibroblasts. Cells were treated with ATRi for 30 min followed by 5-iodo-2-deoxyuridine (IdU) for 10 min and 5-chloro-2-deoxyuridine (CldU) for 20 min. ATRi decreased inter-origin distance in proliferating CD8⁺ T cells, B16, and fibroblasts (Figure 3F). ATRi also decreased replication track length in proliferating CD8⁺ T cells, B16, and fibroblasts. Thus, ATRi induces dormant origin firing which increases the density and reduces the velocity of replication forks in proliferating CD8⁺ T cells.

ATRi-induced dormant origin firing in U2OS and BJ-TERT is associated with a CDK1-dependent phosphorylation of RIF1 serine 2205 (serine-2153 in mouse) that disrupts an association between

RIF1 and PP1 (Moiseeva *et al.*, 2019b). RIF1 is a regulatory subunit of PP1 (Sukackaite *et al.*, 2017) and PP1 opposes CDC7 kinase activity at replication origins (Hiraga *et al.*, 2017). To determine whether these mechanisms are conserved in CD8⁺ T cells, we treated proliferating CD8⁺ T cells with titrations of CDK1i Ro-3306, CDK2i CVT-313, and CDC7i PHA-767491 for 15 min and then added ATRi for an additional 1 h. ATRi induced a mobility shift in MCM4 in chromatin extracts and this was blocked by 5 μ M Ro-3306 and 20 μ M PHA-76749, but not by CVT-313 (Figure 3G).

We generated a rabbit monoclonal antibody that identifies RIF1 only when it is phosphorylated on serine-2205 in human and serine-2153 in mouse (Figure S4). RIF1 phosphoserine-2153 was detected in proliferating CD8⁺ T cells and this was increased by ATRi and blocked by 5 μ M Ro-3306 (Figure 3H). This is the first antibody that identifies mouse RIF1. The two isoforms identified here are consistent in size with those identified in mouse embryonic stem cells genetically engineered to express RIF1-FLAG-HA (Sukackaite *et al.*, 2017).

ATR kinase-dependent CHK1 activity phosphorylates CDC25A causing its degradation in the proteasome after DNA damage (Liu *et al.*, 2000; Mailand *et al.*, 2000; Sanchez *et al.*, 1997). CDC25A degradation induces cell cycle arrest because inhibitory phosphorylations on CDK2 and CDK1 cannot be removed (Hoffmann *et al.*, 1994). ATRi induces CDK1-dependent dormant origin firing and this suggests that physiological (low level) ATR-CHK1-CDC25A signaling limits origin firing by preventing CDK1 activation (Figure 3F/G). If this premise is correct, DNA damage-induced (high level) ATR-CHK1-CDC25A signaling should degrade CDC25A and this should reverse ATRi induced origin firing. Accordingly, the sequence of treatment with a DNA damaging agent and ATRi should determine whether ATRi induces hyper-phosphorylation of chromatin bound MCM4. We treated proliferating CD8⁺ T cells and B16 with either ATRi or the ribonucleotide reductase (RNR) inhibitor hydroxyurea (HU) for 30 min and then added HU or ATRi, respectively, for an additional 30 min. When proliferating CD8⁺ T cells and B16 were treated with HU before ATRi, ATRi did not induce a mobility shift in MCM4 in chromatin extracts (Figure 3I). In contrast, ATRi induced a mobility shift in MCM4 in chromatin extracts in proliferating CD8⁺ T cells and B16. While this mobility shift was not reversed by subsequent treatment with HU, it was reduced, suggesting that free deoxyribonucleotides may be limiting for origin firing in cells treated with

ATRi and HU. Taken together, these data show that ATRi induces CDK1- and CDC7-kinase dependent origin firing across active replicons in proliferating CD8⁺ T cells (Figure 3J).

Exogenous nucleosides rescue ATRi-induced CD8⁺ T cell death

Exogenous nucleosides rescue genome instability associated with DNA replication in pluripotent stem cells and oncogene-induced senescence in primary fibroblasts cultured *in vitro* (Aird et al., 2013; Halliwell et al., 2020). We hypothesized that exogenous nucleosides may rescue ATRi-induced death in proliferating CD8⁺ T cells. To test this hypothesis, we used three nucleoside cocktails reported to rescue genome instability: Low rN was 250 nM adenosine (A), cytidine (C), and guanosine (G), and thymidine (T)(Aird et al., 2013); High rN was 15 μM A, C, and G, and 6 μM T; EmbryoMax, which is marketed for mouse embryonic stem cell culture applications, was 15 μM A, C, G, and uridine (U), and 6 μM T (Halliwell et al., 2020). We observed a dose-dependent rescue of ATRi-induced death in proliferating CD8⁺ T cells when exogenous nucleosides were added to the tissue culture media 2 h prior to ATRi (Figure 4A). EmbryoMax did not rescue ATRi-induced CD8⁺ T cell death as efficiently as high rN suggesting that 15 μM U had a negative impact on the survival of proliferating CD8⁺ T cells treated with ATRi.

Thymidine concentrations of 1 mM or greater are used to inhibit DNA replication and arrest cells at the onset of S phase and this could protect proliferating CD8⁺ T cells from ATRi-induced death (Bjursell and Reichard, 1973). We did not observe cell cycle arrest in proliferating CD8⁺ T cells treated with the nucleoside cocktails used here where the maximum concentration of thymidine is 12 μM (Figure 4B; Figure S5).

ATRi is a competitive ATP inhibitor and the addition of exogenous nucleosides to the media could block the activity of the inhibitor by, for example, preventing its transport into proliferating CD8⁺ T cells (Foote et al., 2018). The addition of nucleosides to proliferating CD8⁺ T cells two hours prior to ATRi did not block either the ATRi-induced mobility shift in MCM4 in the chromatin fraction or the inhibition of CHK1 phosphorylation indicating that ATRi AZD6738 still enters cells and is active in the presence of these concentrations of nucleosides (Figure 4C).

Inter-origin distance and replication fork velocity in CD8⁺ T cells treated with ATRi were not changed by the addition of nucleosides (Figure 4D). Thus, exogenous nucleosides rescue ATRi-

induced death in proliferating CD8⁺ T cells in a dose-dependent manner without impacting either the proliferation of cells, the activity of ATRi, or origin firing.

ATR kinase prevents thymineless death in proliferating CD8⁺ T cells

To determine whether a single nucleoside could rescue ATRi-induced death in proliferating CD8⁺ T cells, we treated with either 15 μM A, C, or G, or 6 μM T. Addition of 6 μM T rescued ATRi-induced death in proliferating CD8⁺ T cells with similar efficiency to high rN while addition of other individual nucleosides did not significantly affect survival (Figure 5A). We did not observe cell cycle arrest in proliferating CD8⁺ T cells treated with 6 μM T (Figure 5B; Figure S6A).

Hydroxyurea induces ATR kinase-dependent cell cycle arrest. To determine whether ATRi and HU induce death in proliferating CD8⁺ T cells through a similar mechanism, we first determined the length of treatment with ATRi and HU needed to induce death in proliferating CD8⁺ T cells. We treated proliferating CD8⁺ T cells with thymidine and 2 h later added ATRi or 5 mM HU for 6 or 9 h. Cells were then replated with or without thymidine and allowed to recover to 48 h post-activation.

ATRi for 6 h did not impact the proliferation of CD8⁺ T cells while HU induced cell cycle arrest (Figure 5C). ATRi for 6 h induced death in proliferating CD8⁺ T cells, but while thymidine increased the percentage of live cells, the change was not statistically significant (Figure S7). ATRi for 9 h induced death in proliferating CD8⁺ T cells and this was rescued by 6 μM T (Figure 5D; Figure S8). In contrast, HU for 9 h induced death in proliferating CD8⁺ T cells, and while HU-induced cell cycle arrest was rescued by 6 μM T, HU-induced CD8⁺ T cell death was not rescued by 6 μM T. We conclude that the thymidine rescue of ATRi-induced death in proliferating CD8⁺ T cells is associated with proliferation and not stalled replication forks.

Since ATRi-induced death in proliferating CD8⁺ T cells is associated with proliferation, we hypothesized that thymidine was limiting for DNA polymerases. We therefore quantitated free deoxyribonucleotide concentrations in proliferating CD8⁺ T cells treated with ATRi or HU for 1 h. Concentrations of dCTP, dCDP, dCMP, dTTP, dTDP, dTMP, and dUMP, and the concentration of dUTP decreased in proliferating CD8⁺ T cells treated with HU (Figure 6A). HU induces ATR kinase-dependent cell cycle checkpoints and increases ATR kinase-dependent DCK activity for dNTP

salvage. Since dNTPs are not used in DNA synthesis and dNTP salvage pathways are induced in proliferating CD8⁺ T cells treated with HU, concentrations of free dCTP, dTTP, dCDP, dTDP, dCMP, dTMP, and dUMP increase (Figure 6A). The concentration of dUTP decreased in proliferating CD8⁺ T cells treated with HU. This suggests that DUT activity and thymidine salvage pathways were induced in response to a decreased dTTP:dUTP ratio. The concentration of dUTP also decreased in proliferating CD8⁺ T cells treated with ATRi.

ATR kinase prevents RRM2 and DCK degradation in the proteasome

Deoxyuridine 5'-triphosphate nucleotidohydrolase (DUT) hydrolyses dUTP to dUMP and pyrophosphate, simultaneously reducing dUTP and providing dUMP for dTTP biosynthesis (Figure 6B). A high cellular dTTP:dUTP ratio is essential to avoid dU contamination in genomic DNA. The decreased concentration of free dUTP in proliferating CD8⁺ T cells treated with ATRi suggested that DUT activity had increased in response to a decreased dTTP:dUTP ratio. A key step in *de novo* dTMP biosynthesis is the reduction of UDP to dUDP by RNR. ATRi's were recently reported to induce CDK1-dependent phosphorylation and degradation of RRM2, a subunit of RNR, in Ewing sarcoma cells, acute lymphoblastic leukemia (ALL) cells, and adrenocortical carcinoma cells (Bothou et al., 2021; Koppenhafer et al., 2020; Le et al., 2017), consistent with the well-known CDK1-dependent degradation of RRM2 in G2 (D'Angiolella et al., 2012). Since ATRi and HU both decreased the concentration of dUTP, we hypothesized that ATRi may induce the CDK1-dependent degradation of RRM2 in proliferating CD8⁺ T cells.

Furthermore, the increased concentration of dCDP (dUDP is at the limits of detection) in proliferating CD8⁺ T cells treated for 1 h with HU, but not ATRi, suggested that ATR is required to mitigate the consequences of RNR inhibition. A key step in dNTP salvage is the phosphorylation of dA, dC, and dG by deoxycytidine kinase (DCK) to generate dAMP, dCMP, and dGMP. ATR phosphorylates DCK on serine-74 and this phosphorylation is associated with DCK kinase activity (Amsailale et al., 2012; Beyaert et al., 2016; Hazra et al., 2011). Since HU, but not ATRi increased the concentration of dCDP, we hypothesized that ATRi inhibited DCK in proliferating CD8⁺ T cells.

To test these hypotheses, we immunoblotted RRM1, RRM2, DCK, and DUT, in proliferating CD8⁺ T cells treated with ATRi for 1 or 4 h. HU increased RRM2 protein levels in proliferating CD8⁺ T

cells at 4 h and this was ATR kinase-dependent (Figure 6C). ATRi decreased RRM2 and DCK protein levels in proliferating CD8⁺ T cells at 1 h and 4 h. To determine whether the RRM2 and DCK were degraded in the proteasome following CDK-dependent phosphorylation, we treated proliferating CD8⁺ T cells with the 5 μ M MG132, a proteasome inhibitor, and 5 μ M Ro-3306, for 15 min prior to ATRi for 2 h. ATRi-induced a CDK1-dependent reduction in RRM2 protein and this was largely via MG132-dependent degradation in the proteasome (Figure 6D). ATRi-induced a CDK1-dependent reduction in DCK protein and this was entirely via MG132-dependent degradation in the proteasome. The ATRi-induced reduction in RRM2 and DCK were blocked by 5 μ M and 10 μ M CDK1i Ro-3306 (Figure 6E). The ATRi-induced reduction in RRM2 and DCK was not blocked by 5 μ M CDK2i CVT-313 (Figure 6F).

To determine whether ATRi induced CDK1-dependent phosphorylation(s) on RRM2, we treated proliferating CD8⁺ T cells with ATRi, with and without CDK1i, and immunoprecipitated proteins using a phospho-MAPK/CDK substrate (PXpSP or pSPXR/K) rabbit monoclonal antibody, and then immunoblotted RRM2 using a rabbit polyclonal antibody and a conformation-specific HRP-conjugated anti-rabbit antibody. Phosphospecific antibodies that identify RRM2 phosphothreonine-33 (pTPPT mouse) and phosphoserine-20 (pSPLK mouse and human), known CDK-dependent sites, are not available. RRM2 was immunoprecipitated from proliferating CD8⁺ T cells treated with ATRi (Figure 6G). Since the only sequence in RRM2 that matches the consensus of these antibodies is phosphoserine-20, we conclude that ATRi-induced the CDK1-dependent phosphorylation of pSPLK on RRM2.

To determine whether ATRi blocked the phosphorylation of DCK, we treated proliferating CD8⁺ T cells with ATRi, with and without CDK1i for 1 h, and immunoprecipitated proteins using a phospho-SQ/phospho-TQ substrate rabbit monoclonal antibody, and immunoblotted DCK using a rabbit polyclonal antibody and a conformation-specific HRP-conjugated anti-rabbit antibody. Phosphospecific antibodies that identify DCK phosphoserine-74 (pSQ) are no longer available. DCK was immunoprecipitated from proliferating CD8⁺ T cells and this was blocked by ATRi (Figure 6H). Since the only sequence in DCK that matches the consensus of these antibodies is phosphoserine-74, we conclude that DCK is phosphorylated on this site by ATR in proliferating CD8⁺ T cells.

ATR kinase is essential for genome stability in proliferating CD8⁺ T cells

Since ATRi reduced RRM2 protein and DCK phosphorylation, and presumably their activity, in proliferating CD8⁺ T cells, we hypothesized that ATRi may induce dU contamination in genomic DNA and genome instability. To quantitate DNA constituent base composition, we developed a fit-for-purpose LC-MS/MS assay that quantitated 1 rN per 20,000 bases from 1 mg DNA. We observed a significant increase in dU contamination, measured as the ratios of dU/dA and dU/T, in genomic DNA in proliferating CD8⁺ T cells treated with ATRi for 1 h (Figure 7A). dU contamination in genomic DNA is repaired by base excision repair and mismatch repair, and this can generate DNA double-strand breaks at multiple damaged sites.

To determine whether ATRi induced DNA damage signaling in proliferating CD8⁺ T cells, we quantitated γ H2AX in a flow cytometry assay. We observed a dramatic increase in the number of γ H2AX positive proliferating CD8⁺ T cells treated with ATRi for 2 and 4 h (Figure 7B). Since the number of γ H2AX positive proliferating CD8⁺ T cells after treatment with ATRi for 2 and 4 h was decreased by approximately 50% by thymidine, we reasoned that a second class of lesion might also cause cell death.

We hypothesized that ATRi-induced dormant origin firing in genes may result in both R loops at sites of transcription and primer synthesis. To test this hypothesis, we generated genomic DNA from B16 and proliferating CD8⁺ T cells treated with the topoisomerase inhibitor camptothecin (TOP1i) or ATRi for 1 h using conditions that preserve R loops and RNA primers (Chedin et al., 2021; Ginno et al., 2012). We immunopurified RNA-DNA hybrids from restriction endonuclease digested DNA using S9.6 and after extensive washing liberated the DNA from beads using proteinase K. Denatured DNA was dot blotted using an anti-single-stranded DNA antibody. RNA-DNA hybrids were induced by both TOP1i and ATRi (Figure 7C).

Origins preferentially fire in gene promoters rather than gene coding regions (Chen et al., 2019; Petryk et al., 2016). Transcription starts in early G1 phase, peaks at the G1/S boundary, and then fades during the early stages of S phase (Bertoli et al., 2013). These mechanisms that coordinate transcription and DNA replication may be critical in proliferating CD8⁺ T cells that have a short G1 phase (Figure 3A-B). We used the proximity ligation assay (PLA) to quantitate collisions between

DNA replication (identified by PCNA) and RNA transcription (identified by RNA polymerase II (Pol II) phosphoserine-5) complexes. We observed an increase in PCNA-RNA polymerase PLA signals in proliferating CD8⁺ T cells treated with ATRi for 1 h (Figure 7D). This increase in PCNA-RNA polymerase-PCNA PLA signals was observed in both S phase cells (EdU⁺) and, to a lesser extent, EdU⁻ cells. However, CD8⁺ T cells divide extremely fast, and during a 1 h treatment with ATRi, ~20 % of cells are anticipated to exit S phase (~4.7±0.3 h/1 h).

DISCUSSION

ATRi's have advanced to phase 1 and phase 2 trials and NCI's Experimental Therapeutics Network has formed Project Teams to develop and prioritize ATRi's in clinical trials. In this study, we show that ATRi induces untimely CDK1 activity in S phase in proliferating CD8⁺ T cells, and that this induces origin firing across active replicons and simultaneous degradation of dNTP synthesis and salvage enzymes. These effects of ATRi in proliferating CD8⁺ T cells lead to dU contamination in genomic DNA, R loops, transcription-DNA replication machinery collisions, and death in proliferating CD8⁺ T cells, but not in naïve CD8⁺ T cells, B16 melanoma, or primary fibroblasts after a 24 h treatment. ATRi-induced death in proliferating CD8⁺ T cells was significantly rescued by thymidine treatment. This may provide an explanation for how ATRi's suppress proliferating T cells causing dose-limiting toxicity in patients.

ATRi's are not inert agents that only have activity when combined with a DNA damaging agent. ATRi's fundamentally change DNA replication in cells and the effect is greater in proliferating CD8⁺ T cells than naïve CD8⁺ T and cancer cells. Thus, ATRi's may cause transient leukocytopenia without impacting naïve populations, allowing rebound proliferation of immune cell populations in both the tumor microenvironment (TME) and periphery. In the TME, many sub-populations of T cells in various degrees of dysfunction (exhausted T cells, regulatory T cells) are being continuously stimulated to proliferate. This *in situ* proliferative signal may represent an unappreciated target of ATRi's, such that certain populations are selectively targeted due to their underlying proliferation and replicative stress. Furthermore, recent preclinical studies demonstrate that ATRi's potentiates type I interferon (IFN1) signaling, production of pro-inflammatory cytokines/chemokines, myeloid immune cell infiltration, and antigen presentation after radiation (Dillon et al., 2019; Feng et al., 2020). Taken with our previous findings that ATRi

and radiation promote CD8⁺ T cell-dependent anti-tumor responses (Vendetti *et al.*, 2018), these studies collectively link DNA damage following ATRi and radiation, pro-inflammatory cytokine signaling, recruitment and activation of the innate immune system, and activation of the adaptive immune system to mediate anti-tumor responses.

Our observation that ATR limits origin firing across active replicons in proliferating mouse CD8⁺ T cells *ex vivo*, as well as in mouse and human cancer cell lines and fibroblasts, argues that the underlying mechanism is fundamental to DNA replication in these species. Consistent with this premise, we show that ATRi induces a CDK1 kinase-dependent phosphorylation on RIF1 serine-2153 (serine-2205 human) in proliferating CD8⁺ T cells. We previously showed that RIF1 serine-2205 phosphorylation disrupts an association between PP1 and RIF1 in human U2OS and BJ-TERT (Moiseeva *et al.*, 2019b). Others showed that PP1 opposes CDC7 kinase-dependent origin firing in human cells (Hiraga *et al.*, 2017). Taken together, these data are consistent with a model in which ATR-CHK1-CDC25A signaling prevents the CDK1 kinase-dependent phosphorylation on RIF1 that would disrupt the localization of PP1 that opposes CDC7-dependent origin firing across active replicons. Accordingly, ATRi induces the CDK1 kinase-dependent phosphorylation on RIF1 and this disrupts the localization of PP1, causing CDC7-dependent origin firing across active replicons (Figure 7F, right). In this model, ATR signaling limits origin firing in the absence of either DNA lesions or mechanical failures.

Our data showing that HU and ATRi have different effects in proliferating CD8⁺ T cells are entirely consistent with the model suggested above. HU inhibits RNR activating a DDR that is anticipated to induce ATR-CHK1-CDC25A-dependent cell cycle checkpoints (Figure 5C) and increase ATR kinase-dependent DCK activity for dNTP salvage. Since dNTPs are not used in DNA synthesis and dNTP salvage pathways are induced in proliferating CD8⁺ T cells treated with HU, concentrations of free dCTP, dTTP, dCDP, dTDP, dCMP, dTMP, and dUMP increase (Figure 6A). The decreased concentration of dUTP in proliferating CD8⁺ T cells treated with HU (Figure 6A) suggests that DUT activity was increased in response to a decreased dTTP:dUTP ratio. While mechanisms that control DUT activity are not known, DUT hydrolyses dUTP to dUMP and pyrophosphate, simultaneously reducing dUTP to prevent dU contamination in genomic DNA and providing dUMP for the *de novo* synthesis of dTTP.

ATRi inhibits DCK (Figure 6H) and induces untimely CDK1 activity during S phase in proliferating CD8⁺ T cells, and this increases DNA synthesis (Figure 3E) and causes RRM2 and DCK degradation in the proteasome (Figure 6C). Remarkably, these effects do not cause cell cycle arrest (Figure 5C), or change dCTP, dTTP, dCDP, dTDP, dCMP, dTMP, and dUMP concentrations in proliferating CD8⁺ T cells at 1 h (Figure 6A). The decreased concentration of dUTP in proliferating CD8⁺ T cells treated with ATRi (Figure 6A) suggests that DUT activity had increased in response to a decreased dTTP:dUTP ratio. Our observation that the decrease in the concentration of dUTP in CD8⁺ T cells treated with ATRi was less than those treated with HU, suggesting that ATR may be required to increase DUT activity in response to a decreased dTTP:dUTP ratio. The increased DNA synthesis, and modest decrease in the concentration of free dUTP, could explain why dU contamination in genomic DNA is observed in proliferating CD8⁺ T cells treated with ATRi, but not HU (Figure 7A). dU contamination in genomic DNA is repaired by base excision repair (BER) mechanisms initiated by uracil DNA glycosylases, primarily UNG, and mismatch repair (MMR). The removal of uracil by UNG generates an abasic site which is cleaved by APE1 to generate a single-strand break (SSBs). ATRi's are anticipated to concentrate dU contamination in genomic DNA at replication forks, and the concentration of dU contamination may be further increased by futile cycles of BER repair synthesis when the dTTP:dUTP ratio is low. This could result in APE1-induced SSBs on opposite strands of the helix that generate DSBs at multiple damaged sites, and subsequently, cell death. This is entirely consistent with our observation of that ATRi induced γ H2AX in proliferating CD8⁺ T cells at 2 and 4 h (Figure 7B). Since the number of γ H2AX positive proliferating CD8⁺ T cells treated with ATRi decreased by approximately 50% by thymidine, we reasoned that a second class of lesion might also cause cell death.

In addition to our observation that ATRi induce dU contamination in genomic DNA, we also found that ATRi induce R loops and transcription-DNA replication machinery collisions in proliferating CD8⁺ T cells. We anticipate that the extent to which ATRi will induce dU contamination in genomic DNA in cells is a function of the amount of ongoing DNA synthesis, determined by both the length of S phase and the rate of dTTP biosynthesis. We anticipate that the extent to which ATRi will induce R loops and DNA polymerase-RNA polymerase machinery collisions in cells is a function of the separation of RNA and DNA synthesis, determined by the relative length of S and G1 phase.

As G1 is abridged to approximately 1 h in proliferating CD8⁺ T cells, most of the transcription must be concomitant with DNA replication. This is a fundamental difference between proliferating CD8⁺ T cells, and cancer cells and fibroblasts. Pol II can synthesize 70 bp/sec and e.g. it would take Pol II approximately 44 min to transcribe just ATM (184,490 bp) without pausing (Darzacq et al., 2007). Accordingly, the number of active RNA polymerases and DNA polymerases in genes is likely to be greater in proliferating CD8⁺ T cells than cancer cells or fibroblasts, and the number of ATRi-induced R loops and DNA polymerase-RNA polymerase machinery collisions is likely to be higher. Further work is needed to quantitate ATRi-induced DNA damage in different cell types.

In summary, our work serves to highlight the direct impact of ATRi in CD8⁺ T cells and points to a role for ATRi's as immune modulators. Our work also provides novel insights for the combinatorial application of ATRi with genotoxic therapies, including anti-metabolites, and immunotherapies in a clinical setting. Considering the wide interest in ATRi, CHK1i and WEE1i, which have a similar impact on origin firing in cancer cells, our insights may be used to design novel therapeutic approaches aimed at generating anti-tumor immunity.

MATERIALS AND METHODS

Antibodies

Antibodies used in this study are summarized in [Table SM1](#).

Inhibitors

AZD6738, AZD0156, Olaparib, and AZD1775 were provided by AstraZeneca. Other inhibitors used in this study are Ro-3306 (CDK1i, Selleckchem S7747), CVT-313 (CDK2i, Santa Cruz 199986-75-9), PHA-767491 (CDC7i, Selleckchem S2742), (S)-(+)-camptothecin (TOP1i, MilliporeSigma C9911), and MG132 (proteasome inhibitor, Selleckchem S2619).

Mice

C57BL/6, Pmel-1 TCR transgenic (B6.Cg-*Thy1^o*/Cy Tg(TcraTcrb)8Rest/J), and female BALB/c mice were purchased from Jackson Laboratories.

Cell lines

B16-F10 (ATCC CRL-6475) and CT26 (ATCC CRL-2638) were cultured in DMEM and RPMI, respectively, containing 10 % FBS, 100 U/mL penicillin and 100 mg/mL streptomycin (all Lonza). Cells were routinely tested for mycoplasma.

Primary fibroblasts isolation and culture

Primary fibroblasts were isolated from the ears of 6-8 week Pmel-1 mice as described previously (Khan and Gasser, 2016). Fibroblasts were cultured in DMEM containing 15 % FBS, 100 U/mL penicillin, 100 mg/mL streptomycin (all Lonza), 1x MEM NEAA, 1 mM sodium pyruvate, 25 mM HEPES pH 8.0, 120 μ M β -mercaptoethanol (all Gibco), and 250 ng/mL amphotericin-B (Sigma-Aldrich).

CD8⁺ T cell isolation, activation, and culture

CD8⁺ T cells were extracted from spleen and lymph nodes of 6-8 week C57BL/6 and Pmel-1 mice. To obtain single cell suspension, spleens and lymph nodes were mechanically processed between frosted glass slides and filtered through 70 μ m cell strainers (Corning). Erythrocytes were lysed in 150 mM NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA pH 8.0. Pmel-1 CD8⁺ T cells were activated by incubating with R10 media (RPMI containing 10 % FBS, 100 U/mL penicillin and 100 mg/mL streptomycin (all Lonza), 1x MEM NEAA, 1 mM sodium pyruvate, 5 mM HEPES pH 8.0, 50 μ M β -mercaptoethanol (all Gibco)) supplemented with 1 μ M human gp100 (25-33) (Eurogentec) and 50 U/mL IL-2 (PeproTech) for 24 h. Naïve CD8⁺ T cells were cultured in R10 media containing 5 ng/mL IL-7 (R&D systems). C57BL/6 CD8⁺ T cells were purified using the EasySep Mouse CD8 T Cell Isolation Kit (Stemcell technologies) according to the manufacturer's instructions. C57BL/6 CD8⁺ T cells were activated by resuspending into R10 media containing 50 U/mL IL-2 (PeproTech) and 2 μ g/mL anti-CD28 antibody (BD) and then plated in a culture plate pre-coated with 10 μ g/mL anti-CD3 ϵ antibody (Biolegend). Culture medium was exchanged to R10 containing IL-2 and the cell density was adjusted to be 0.5 – 1x10⁶ cells/ml every 24 h.

Proliferation and survival assays

For the proliferation assay, 5x10⁶ cells/mL were stained in 8 mg/mL Cell Trace Violet (CTV, Fisher) in PBS for 10 min. Staining was quenched the addition of with 5x the volume of R10 media and then activated as above. Cells were collected on ice and surface antigens were identified using

the antibodies indicated ([Table SM1](#)) for 15 min before staining for 10 min with eFluor780 viability dye (1:4000, ThermoFisher). Cells were fixed in Fixation/Permeabilization reagent (eBioscience) and uncompensated data was collected using a LSRFortessa cytometer and FACSDiva Software (BD Biosciences). Compensation and analyses were performed using FlowJo v10 software. Gating is shown in [Figure SM1](#).

Nuclease insoluble chromatin fractionation and immunoblotting

Purification of nuclease insoluble chromatin (NIC) and immunoblotting of hyperphosphorylationMCM4 was performed as described previously (Moiseeva *et al.*, 2017). Soluble fractions were blotted for the antibodies listed in [Table SM1](#).

In vivo treatments and immunophenotyping

CT26 cells (approximately 5×10^5 in RPMI) were subcutaneously injected into the right hind flank of 8-10 week old mice. Treatment started 7-10 days later when tumors reached ~ 60 -120 mm³. Mice were treated daily for 3 days with 75 mg/kg AZD6738 or vehicle in a volume of 10 μ L/g of bodyweight, as described previously (Vendetti *et al.*, 2018). AZD6738 was dosed (in 50 % H₂O, 40 % Propylene Glycol, 10 % DMSO) by oral gavage. Spleens, tumor-draining lymph nodes (DLN, right inguinal) and CT26 tumors were excised from mice at day 4. Tissues were processed to generate single cell suspensions, as described previously (Vendetti *et al.*, 2018). Briefly, spleens and DLN were mechanically dissociated between frosted glass slides and filtered through 70 μ m cell strainers (Corning). Tumors were injected in multiple locations with a total of 1.5 mL RPMI containing 50 μ g/mL Liberase DL research grade (Roche) and 10 U/mL DNase I (Sigma), incubated 3 min at room temperature, cut into small pieces, incubated in a total volume of 5 mL Liberase DL/DNase solution for 15 min at 37°C, mechanically dissociated between frosted glass slides, filtered through 70 μ m cell strainers (Corning), vortexed at low speed for 90 sec, and filtered again through new 70 μ m cell strainers (Corning). Erythrocytes were lysed in 150 mM NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA pH 8.0. for 30 sec (spleens) or 10 sec (tumors). Cells suspensions were counted using a Scepter 2.0 or 3.0 handheld counter (Millipore) and seeded at 1.5×10^6 cells in 96-well round bottom plates for staining. Cells were blocked in FSC buffer (2% FBS/1x PBS) containing 0.5 μ g anti-CD16/32 antibody (TruStain FcX Plus, BioLegend) for 10 min at 4°C to block

non-specific binding of antibodies via Fc receptors, stained in FSC buffer containing antibodies to surface antigens, Brilliant Stain Buffer Plus (1:5, BD Biosciences), and True-Stain Monocyte Blocker (1:20, BioLegend) for 15 min at 4°C, stained with eFlour780 viability dye (1:4000, ThermoFisher) for 10 minutes at 4°C to label dead/dying cells, fixed and permeabilized in eBioscience Fixation/Permeabilization reagent (ThermoFisher) for 15 min at room temperature, and when performing nuclear staining of Ki67, stained for 45 min at room temperature in eBioscience 1x Permeabilization Buffer (ThermoFisher) containing anti-mouse Ki67 antibody. Uncompensated data were collected with a BD LSRFortessa 4-laser cytometer and BD FACSDiva software. Compensation and data analyses were performed in FlowJo V10 software. Single color compensation controls included single stained OneComp eBeads (ThermoFisher) and single stained spleen or DLN samples and matching unstained cells. Fluorescence minus one (FMO) controls were included where appropriate to empirically determine gating. Antibody panel for surface antigens are shown in **Table SM1** and gating strategies are shown in **Figures SM2/3**.

Cell cycle analysis

For cell cycle analysis, asynchronous populations of the cells were labeled with 10 µM EdU for 30 minutes at 37 °C. Cells were harvested, washed with PBS, and fixed in cold 70 % ethanol on ice for 30 minutes. Fixed cells were permeabilized using 1x Saponin permeating solution (Alfa Aesar) diluted in 1% BSA in PBS for 15 min. EdU was identified using EdU Click-It kit (Thermofisher, C10632) according to the manufacturer's instructions. EdU-labeled cells were washed with 1x Saponin permeating solution and resuspended in 200 – 300 µl PBS containing 200 nM FxCycle™ Far Red Stain (Thermofisher, F10348) and 0.1 mg/mL RNase A (Thermo Scientific, EN0531. Data were collected with a Accuri C6 cytometer and software (BD Biosciences). Data analyses were performed using Flowjo v10 software.

Repli-seq

Library preparation was performed as described previously (Moiseeva et al., 2019a). Sequencing was performed on an Illumina HiSeq (GENEWIZ). Raw Repli-seq reads were trimmed and filtered for quality using Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Reads were aligned using

bowtie2 (Langmead and Salzberg, 2012) against GRCm38 (mm10). Genome-wide RT profiles were constructed, scaled, and pooled for analysis, as described previously (Marchal et al., 2018). Briefly, Log2 ratios of early versus late read counts were calculated for 50kb non-overlapping windows, Loess smoothed at 300kb windows. Data were visualized using IGV (Robinson et al., 2011). Repli-seq data have been deposited in the Gene Expression Omnibus database (accession no. GSE183412).

DNA combing

30 minutes post treatment, 25 μ M IdU was added for 10 minutes followed by 200 μ M CldU for 20 minutes (both MP Biomedical). Cells were harvested and washed twice with PBS. Cells were then resuspended in PBS, mixed with an equal volume of 1 % low melting point agarose (Bio-Rad, 161-3111), and allowed to solidify in plug molds (Bio-Rad, 170-3713). Agarose plugs were incubated in 2 mg/mL proteinase K, 50 mM EDTA, 1 % Sarkosyl, and 10 mM Tris pH 7.5 overnight at 50 °C. The buffer was replaced and the incubation was continued for an additional 6 h. Plugs were washed 5 times in TE50 (10 mM Tris-HCl pH 7.0, 50 mM EDTA) and stored at 4 °C or washed 3 more times in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Each plug was melted in 200 μ l TE at 68 °C for 30 minutes and then digested using β -agarase I (BioLabs, M0392L) at 42 °C overnight. MES buffer, pH 5.5 was gently added to increase the volume to 2 mL and the mixture was incubated at 68 °C 30 min. DNA was combed onto CombiCoverslips™ using the molecular combing system (Genomic Vision). Cover slips were baked for 2 h at 60 °C. DNA strands were denatured in 1 M NaOH and 1.5 M NaCl for 8 min, neutralized in PBS, and dehydrated with subsequent 5 minutes incubations with 70, 90, and 100 % ethanol. Halogenated nucleotides were identified with anti-IdU (1:20, BD Biosciences, 347580) and anti-CldU (1:50, Abcam, ab6326) antibodies for 1 h at 37 °C. Slides were then stained with anti-mouse Alexa 594 (1:50, Invitrogen, A11005) and anti-rat Alexa 488 (1:50, Invitrogen, A21470), followed by anti-ssDNA (1:50, Millipore, MAB3034), and then anti-mouse Alexa 647 (1:50, Invitrogen, A21235), each incubated for 30 min. Slides were dehydrated in ethanol as described above and mounted using ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, P36971). DNA fiber images were acquired with a Nikon Ti inverted fluorescence microscope using NIS Elements v5.3 at 60x magnification and analyzed using Adobe Photoshop Elements 15 and ImageJ software.

Quantification of free nucleotides by LC-HRMS

Nucleotides were analyzed from cells by LC-HRMS as previously described (Kuskovsky et al., 2019). Cell pellets were extracted by addition of a 50 μ L (20 ng/ μ L) mix of all stable isotope labeled internal standards (1000 ng/sample) in 80:20 (v/v) methanol:water followed by 1 mL of -80°C 80:20 (v/v) methanol:water before homogenization by probe tip sonication, incubation at -80°C for 30 min, centrifugation at 17,000 rcf for 10 minutes at 4°C , evaporation of the supernatant to dryness, then resuspended in 50 μ L 95:5 water: methanol. An Ultimate 3000 UHPLC equipped with an autosampler kept at 6°C and a column heater kept at 55°C using a HSS C18 column (2.1 $\times$ 100 mm i.d., 3.5 μ m; Waters) was used for separations. Solvent A was 5 mM DIPEA and 200 mM HFIP and solvent B was methanol with 5 mM DIPEA 200 mM HFIP. The gradient was as follows: 100% A for 3 min at 0.18 mL/min, 100% A at 6 min with 0.2 mL/min, 98% A at 8 min with 0.2 mL/min, 86% A at 12 min with 0.2 mL/min, 40% A at 16 min and 1% A at 17.9 min-18.5 min with 0.3 mL/min then increased to 0.4 mL/min until 20 min. Flow was ramped down to 0.18 mL/min back to 100% A over a 5 min re-equilibration. For MS analysis, the UHPLC was coupled to a Q Exactive HF mass spectrometer (Thermo Scientific) equipped with a HESI II source operating in negative mode. The operating conditions were as follows: spray voltage 4000 V; vaporizer temperature 200°C ; capillary temperature 350°C ; S-lens 60; in-source CID 1.0 eV, resolution 60,000. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were 45 and 10 (arbitrary units), respectively. Single ion monitoring (SIM) windows were acquired around the [M-H] $^{-}$ of each analyte with a 20 m/z isolation window, 4 m/z isolation window offset, 1e6 ACG target and 80 ms IT, alternating in a Full MS scan from 70-950 m/z with 1e6 ACG, and 100 ms IT. Data was analyzed in XCalibur v4.0 and/or Tracefinder v5.1 (Thermo Scientific) using a 5 ppm window for integration of the peak area of all analytes. Standards used as calibrants and isotope labeled internal standards are indicated in the [Table SM2](#), all were used without further purification, and no adequate commercially available diphosphate standard was found therefore the monophosphate was used as a surrogate standard.

Quantification of nucleosides incorporated into the genome

Cells were resuspended in TE buffer containing 62.5 μ g/mL proteinase K (Invitrogen, AM2546), 62.5 μ g/mL RNase A (Thermo Scientific, EN0531), and 0.5 % SDS and incubated overnight at 37°C .

Genomic DNA was purified by phenol/chloroform extraction and resuspended in RNase/DNase free water. 5 µg DNA was digested with 5 µl RNase H (NEB, M0297), 3 µl Hind III (Fisher, FD0504), 3 µl EcoRI (Fisher, FD0274), and 3 µl Bam HI (Fisher, FD0054) in RNase H buffer (NEB, M0297) overnight at 37 °C. Digested DNA was purified using the GeneJET PCR Purification Kit (Thermo Scientific, K0702). DNA was further digested into single nucleosides using DNA Degradase Plus (Zymo Research, E2021).

To quantitate DNA constituent base composition, a fit-for-purpose LC-MS/MS assay was implemented on a 1290 Infinity II Autosampler and Binary Pump and a SCIEX 6500+ triple quadrupole mass spectrometer. Chromatographic separation was conducted on an Inertsil ODS-3 (3 µm x 100 mm 2.1 mm) reverse phase column at ambient temperature with a gradient mobile phase of methanol and water with 0.1% formic acid. MRM transitions of all analytes and isotopic internal standards were monitored to construct calibration curves. We were able to quantitate 1 rN per 20,000 bases from 1 mg DNA.

R-loop purification and dot blot

Genomic DNA was prepared as above. 5 µg DNA was digested with 3 µl Hind III (Fisher, FD0504), 3 µl EcoRI (Fisher, FD0274), and 3 µl Bam HI (Fisher, FD0054) +/- 5 µl RNase H (NEB, M0297) in RNase H buffer (NEB, M0297) overnight at 37 °C. Digested DNA was incubated with 50 µl of protein A/G agarose beads (Santa Cruz, sc-2003) and 10 µl mouse serum (MP Biomedicals, 152282) in 1.5 mL PBS/0.5% Triton X100 for 2 h at 4 °C. Digested DNA was then incubated with 50 µl of protein A/G agarose beads and 5 µl anti-S9.6 (Millipore, MABE1095) overnight at 4 °C. Agarose beads were washed 3x for 3 min in PBS/0.5% Triton X100 binding buffer followed by PBS wash for 3 min. The beads were then incubated with 3 µl proteinase K for 2 h at 37 °C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation. DNA was denatured in 50 µl TE and mixed with 50 µl of 0.8 M NaOH/20 mM EDTA solution for 10 min at 95°C then placed on ice. The solution was neutralized using sodium acetate pH 7.0 and DNA sample was transferred to Whatman Hybond N+ Blotting Membrane (Millipore Sigma, Z761079). Nucleic acids were cross-linked by to the membrane by baking at 60 °C for 30 min followed by UV exposure. The membrane was blocked for 1 h with 5 % milk in TBST and blotted for anti-ssDNA (1:1000, Millipore, MAB3034).

γH2AX analyses

Cells were harvested, washed with PBS, and fixed in cold 70 % ethanol on ice for 30 minutes for immediate staining or stored at -20 °C until use. Fixed cells were washed with PBS and permeabilized using 1x Saponin permeating solution (Alfa Aesar) diluted in 1% BSA in PBS for 15 min. Cells were stained with FITC anti-mouse γH2AX (1:250 in FCS buffer) for 30 min. γH2AX-labeled cells were washed with 1x Saponin permeating solution and resuspended in 200 – 300 μl PBS containing 200 nM FxCycle™ Far Red Stain (Thermofisher, F10348) and 0.1 mg/mL RNase A (Thermo Scientific, EN0531). Data were collected with a Accuri C6 cytometer and software (BD Biosciences). Data analyses were performed using Flowjo v10 software.

Proximity ligation assay (PLA)

After treatment, cells were collected on glass slides using a cytospin (Thermo Scientific) at 2000 rpm for 10 min. Cells were fixed with 4 % paraformaldehyde in PBS for 15 min and permeabilized in 0.1 % Triton X-100 for 10 min. Cells were washed with PBS, blocked with the blocking solution (Duolink) for 1 h and incubated overnight with anti-PCNA (1:5,000, ab 92552 (AbCam)) and anti-phospho-RNA polymerase II (S5) (1:100,000, ab5408 (AbCam)) in the Ab diluent solution (Duolink) at 4 °C. PLA reactions were performed using the following kits from Duolink: anti-rabbit PLUS (DUO92002), anti-mouse MUNUS (DUO92004), PLA detection reagent RED (DUO92008), and wash buffers, fluorescence (DUO8249). Slides were washed with PBS and EdU-labeled using EdU Click-It kit (Thermofisher, C10632). Cells were mounted using ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, P36971). Images were acquired with a Nikon Ti inverted fluorescence microscope using NIS Elements v5.3 at 60x magnification and analyzed using Adobe Photoshop Elements 15 and ImageJ software.

Study approval

Experimental procedures were approved by the University of Pittsburgh Animal Care and Use Committees and performed in accordance with the relevant guidelines and regulations.

ACKNOWLEDGEMENTS

We thank Mark O'Connor for providing AZD6738, AZD0156, AZD1775, and Olaparib.

This work was supported by R01CA236367 and R01CA204173 (CJB), R21CA259457 and DP2GM146320 (YG), R00 CA207871 (HUO), R37CA240625 (NWS and KMA), 1DP2OD024156 (GMD) and R50CA211241 from the NIH, and PRG1477 from the Estonian Research Council (TNM). This project used the Animal Facility, Cancer Pharmacokinetics and Pharmacodynamics Facility, and the Cytometry Facility that are supported in part by award P30CA047904 from the NIH.

AUTHOR CONTRIBUTIONS

NS, FPV, AJC, JJD, SSH, DP, AB, YNG, and CJB designed, performed, and analyzed experiments. HUO, NWS, and JHB designed and analyzed experiments. TNM, YW, YNG, KMA, and GMD made significant academic contributions. NS, FPV, AJC, and CJB wrote the paper.

CONFLICT OF INTEREST

The authors declare no competing interests.

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Figures

Figure 1: ATRi selectively induces death in proliferating CD8⁺ T cells *ex vivo*.

A. Proliferation of Pmel-1 CD8⁺ T cells activated with gp100 *ex vivo*. CTV histograms of live, CD44^{hi} CD8⁺ T cells at indicated time post-activation are shown. The number of cell divisions is shown. Red histograms are unactivated CD8⁺ T cells. **B.** Immunoblots of CD8⁺ T whole cell extracts prepared at 0 and 30 h post-activation. **C.** CD8⁺ T cells were activated and treated with 5 μ M AZD6738 (ATRi) from 0 - 24 h (blue) or 24 - 48 h (red). At 48 h, the percentage of live CD8⁺ T cells (eFluor 780⁻CD8⁺TCR β ⁺) was quantitated. **D.** Naïve CD8⁺ T cells were maintained in IL-7 *ex vivo* (unactivated) and treated with 5 μ M ATRi from 0 - 24 h or 24 - 48 h. At 48 h, the percentage of live CD8⁺ T cells was quantitated. **E.** Exponentially dividing B16 cells or primary fibroblasts were treated with ATRi for 24 h the percentage of live cells was quantitated. **F.** CD8⁺ T cells were activated and treated with 5 μ M ATRi, 500 nM ATMi (AZD0156), 5 μ M PARPi (Olaparib), or 500 nM WEE1i (AZD1775) from 24 - 48 h. At 48 h, the percentage of live CD8⁺ T cells was quantitated. **C-F.** Mean and SD bars shown. *:P < 0.05; **: P < 0.01; ***: P < 0.001, ****: P < 0.0001 by ANOVA with Tukey's multiple comparisons.

Figure 2: ATRi selectively induces death in proliferating CD8⁺ T cells *in vivo*.

A-H. Immunoprofiling of tissues harvested on day 4 from CT26 tumor-bearing mice treated with 75 mg/kg AZD6738 (ATRi) or vehicle (Veh) on days 1-3. **A.** Quantitation of tumor-infiltrating (TIL) CD8⁺ T cells (per mg of tumor) and quantitation of spleen and tumor-draining lymph node (DLN) CD8⁺ T cells (as a percentage of total CD45⁺ cells). **B.** Representative contour plots of Ki67⁺ expression in CD8⁺ T cells in the TIL, spleen, and DLN. **C.** Quantitation of proliferating (Ki67⁺) CD8⁺ T cells in the TIL, spleen, and DLN. **D.** Quantitation of CD69⁺ CD8⁺ T cells in the TIL, spleen, and DLN. **E.** Representative contour plots of KLRG1 and CD127 expression on CD62L^{lo} CD8⁺ T cells in the TIL. **F.** Quantitation of KLRG1⁺CD127⁻ CD62L^{lo} CD8⁺ T cells (as a percentage of total CD8⁺ T cells) in the TIL. **G.** Representative contour plots of KLRG1 and CD69 expression on CD62L^{lo} CD8⁺ T cells in the TIL. **H.** Quantitation of KLRG1⁺CD69⁻ CD62L^{lo} CD8⁺ T cells (as a percentage of total CD8⁺ T cells) in the TIL. **A-D.** n = 7 mice total (6 DLN for ATRi) from 2 independent experiments, each with 3-4 mice per arm. **E-H.** n = 4 (Veh) or 5 (ATRi) mice from one experiment. **A, C-D, F-H.**

Mean and SD bars shown. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$ by two-tailed, unpaired t-test.

Figure 3: ATR kinase limits origin firing across active replicons in proliferating CD8⁺ T cells.

A. EdU versus DNA histograms of proliferating CD8⁺ T cells and exponentially dividing primary fibroblasts. The percentage of cells in each cell cycle phase and the doubling time was used to estimate the length of G1, S, and G2/M. **B.** Cartoon of the lengths of cell cycle and each phases of proliferating CD8⁺ T cells, B16 cancer cells, and primary fibroblasts drawn to scale. **C.** Immunoblot of MCM4 in nuclease insoluble fraction in proliferating CD8⁺ T cells treated with 5 μ M ATRi, 500 nM ATMi (AZD0156), 5 μ M PARPi (AZD2281/Olaparib), or 500 nM Wee1i (AZD1775) for 1 h. **D.** Repli-seq analyses of the replication timing program of proliferating CD8⁺ T cells treated with DMSO (blue) or 5 μ M AZD6738 (red) for 1 h. Upper – whole genome. Lower – chromosome 1. Sequencing data are deposited at GEO (code: GSE183412). **E.** EdU incorporation in early (2-3N) and late (3-4N) S phase in proliferating CD8⁺ T cells treated with DMSO or 5 μ M ATRi for 1h. 10 μ M EdU was added from 30-60 min. **F.** DNA fiber analyses of proliferating CD8⁺ T cells, B16, and primary fibroblasts treated with DMSO or 5 μ M ATRi for 1 h. Cells were treated with IdU from 30-40 min and CldU from 40-60 min of the treatment. **G.** Immunoblots of MCM4 in nuclease insoluble fraction in proliferating CD8⁺ T cells treated with increasing concentrations of Ro-3306 (CDK1i), CVT-313 (CDK2i), or PHA-767491 (CDC7i) for 75 min. Cells were treated with ATRi from 15-75 min. **H.** Immunoblot of RIF1 phosphoserine-2153 (S2205 in human) in whole cell extracts of proliferating CD8⁺ T cells treated with increasing concentrations of Ro-3306 for 75 min. Cells were treated with ATRi from 15-75 min. **I.** Immunoblot of MCM4 in the nuclease insoluble fraction of proliferating CD8⁺ T cells or B16 treated with 5 μ M ATRi and/or 5 mM HU in the sequence indicated. **J.** Model showing that ATR limits origin firing across active replicons. Green circles indicate origins that fired and red circles indicate origins that have not fired. **E-F.** Mean and SD bars shown. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$ by two-tailed, unpaired t-test.

Figure 4: Nucleosides rescue proliferating CD8⁺ T cells from ATRi-induced cell death.

A. Proliferating CD8⁺ T cells were treated with nucleoside cocktails at 22 h and ATRi at 24 h. For x2, proliferating CD8⁺ T cells were treated with nucleoside cocktails at 22 h and 30 h. At 48 h, the percentage of live CD8⁺ T cells (eFluor 780-CD8⁺TCRβ⁺) was quantitated. Low rN is 250 nM A, C, G, and T. High rN is 15 μM A, C, G, and 6 μM T. EmbryoMax is 15 μM A, C, G, U, and 6 μM T. **B.** CTV histograms of live (eFluor 780-CD44^{hi}CD8⁺) cells at 24 h (grey) or 48 h (blue) treated with nucleosides and ATRi as described in panel A. Number of cell divisions is indicated. **C.** Immunoblot of MCM4 in nuclease insoluble fraction in proliferating CD8⁺ T cells treated low rN or high rN for 2 h prior to 5 μM ATRi for 1 h. Immunoblots of CHK1, CHK1 phosphoserine-345, and GAPDH are from the soluble fraction. **D.** DNA combing analyses of proliferating CD8⁺ T cells treated with high rN for 2 h prior to 5 μM ATRi for 1 h. Cells were treated with IdU for from 30-40 min and CldU from 40-60 min during the treatment with ATRi. **A and D (right panel).** Mean and SD bars shown. *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-way ANOVA with Tukey's multiple comparisons. **D (left panel).** *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by two-tailed, unpaired t-test.

Figure 5: Thymidine rescues proliferating CD8⁺ T cells from ATRi-induced cell death.

A. Proliferating CD8⁺ T cells were treated with either 15 μM A, C, G, or 6 μM T, or high rN at 22 h and ATRi at 24 h. At 48 h, the percentage of live CD8⁺ T cells (eFluor 780-CD8⁺TCRβ⁺) was quantitated. **B.** CTV histograms of live (eFluor 780-CD44^{hi}CD8⁺) cells at 24 h (grey) or 48 h (color) treated with nucleoside(s) and ATRi as described in panel A. Number of cell divisions is indicated. **C.** Proliferating CD8⁺ T cells were treated with 6 μM T at 22 h and either ATRi or HU at 24 h. At 30 h, cells were replated with and without 6 μM T. CTV histograms of live (eFluor 780-CD44^{hi}CD8⁺) cells at 24 h (grey), and 30 h and 48 h (with recovery) for ATRi- and HU-treated (6 h) samples (DMSO treated histograms in black; thymidine treated histograms in pink). Number of cell divisions is indicated. **D.** Proliferating CD8⁺ T cells were activated and treated with 6 μM T at 22 h and either ATRi or HU at 24 h. At 33 h, cells were replated with and without 6 μM T. At 48 h, the percentage of live CD8⁺ T cells (eFluor 780-CD8⁺TCRβ⁺) was quantitated. **A and D.** Mean and SD bars shown. *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-way ANOVA with Tukey's multiple comparisons.

Figure 6: ATRi induces degradation of RRM2 and DCK in proliferating CD8⁺ T cells.

A. Quantitation of cellular deoxyribonucleosides in proliferating CD8⁺ T cells treated with DMSO, 5 μ M ATRi, or 5 mM HU. **B.** Cartoon of nucleoside metabolism. **C-F.** Immunoblots of proliferating CD8⁺ T whole cell extracts. **C.** Proliferating CD8⁺ T cells were treated with 5 μ M ATRi and 5 mM HU for 1 or 4 h. **D.** Proliferating CD8⁺ T cells were treated with 5 μ M MG132 (protease inhibitor) and 5 μ M Ro-3306 (CDK1i) for 15 min prior to ATRi for 2 h. **E.** Proliferating CD8⁺ T cells were treated with increasing concentrations of Ro-3306 (CDK1i) for 15 min followed by 5 μ M ATRi for 1 h. **F.** Proliferating CD8⁺ T cells were treated with increasing concentrations of CVT-313 (CDK2i) for 15 min followed by 5 μ M ATRi for 1 h. **G-H.** Whole cell extracts were generated from proliferating CD8⁺ T cells treated with 5 μ M ATRi and 5 μ M CDK1i for 1 h. **G.** Proteins immunopurified with anti-pSPXK antibodies (phospho-MAPK/CDK substrates) were immunoblotted for RRM2. **H.** Proteins immunopurified with an anti-pSQ antibody (phospho-ATM/ATR substrates) were immunoblotted for DCK. **A.** Mean and SD bars shown. *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-way ANOVA with Dunnett multiple comparisons.

Figure 7: ATRi-induces genome instability in proliferating CD8⁺ T cells.

A. Quantitation of dU contamination in genomic DNA prepared from proliferating CD8⁺ T cells treated with 5 μ M ATRi or 5 mM HU for 1 h. **B.** Quantitation of γ H2AX in proliferating CD8⁺ T cells treated with 6 μ M thymidine for 2 h followed by 5 μ M ATRi for 2 h or 4 h (upper). γ H2AX versus DNA plots of proliferating CD8⁺ T cells treated with 6 μ M thymidine for 2 h followed by 5 μ M ATRi for 2 h (lower). **C.** B16 and proliferating CD8⁺ T cells were treated with 10 μ M camptothecin (TOP1i) or 5 μ M ATRi for 1 h. Genomic DNA was prepared and digested with restriction endonucleases, +/-RNASE H. DNA fragments were immunopurified with anti-RNA-DNA hybrid antibody (S9.6). Immunopurified DNA was denatured and dot blotted using an anti-single-stranded DNA antibody. **D.** Representative images from the PLA of Pol II phosphoserine 5 and PCNA in proliferating CD8⁺ T cells treated with 5 μ M ATRi for 30 min. 10 μ M EdU was added during the last 15 min of treatment. **E.** Quantitation of PLA described in panel D. **F.** Cartoon summarizing the mechanisms through which ATRi induces genome instability in CD8⁺ T cells. **A-B,**

1046 **E.** Mean and SD bars shown. **A.** *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-
 1047 way ANOVA with Dunnett multiple comparisons. **B and E.** *: P < 0.05, **: P < 0.01, ***: P < 0.001,
 1048 ****: P < 0.0001 by one-way ANOVA with Tukey's multiple comparisons.

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Figure S1: A. Weight of whole spleens harvested on day 4 from CT26 tumor bearing mice treated with ATRi (75 mg/kg AZD6738) or vehicle (Veh) on days 1-3. **B-J.** Immunoprofiling of tissues harvested on day 4 from CT26 tumor bearing mice treated with ATRi (75 mg/kg AZD6738) or vehicle (Veh) on days 1-3. **B.** Representative contour plots of CD8⁺ T cells with naïve (TN, CD62L^{hi}CD44^{lo}), central memory (TCM, CD62L^{hi}CD44^{hi}), and effector/effector memory (TEM, CD62L^{lo}CD44^{hi}) phenotypes in the tumor-draining lymph node (DLN), spleen, and tumor infiltrate (TIL). **C.** Quantitation of TN, TCM, and TEM CD8⁺ T cells in the DLN and spleen. **D.** Quantitation of TCM and TEM CD8⁺ T cells in the TIL. **E.** Representative contour plots of CD62L expression on CD8⁺ T cells in the tumor-draining lymph node (DLN) and tumor infiltrate (TIL). **F.** Quantitation of CD62L^{hi} and CD62L^{lo} CD8⁺ T cells in the TIL. **G.** Representative contour plots of CD69 and CD103 expression on CD8⁺ T cells in the DLN and TIL. **H.** Quantitation of CD103⁺CD69⁺ (tissue resident) CD8⁺ T cells in the TIL. **I.** Quantitation of KLRG1⁺CD127⁺ CD62L^{lo} (memory precursor effector) CD8⁺ T cells (as a percentage of total CD8⁺ T cells) in the TIL. **J.** Quantitation of KLRG1⁺CD69⁺ CD62L^{lo} CD8⁺ T cells (as a percentage of total CD8⁺ T cells) in the TIL. **A-D.** n = 7 mice total per group (6 DLN for ATRi) from 2 independent experiments, each with 3-4 mice per group. **E-J.** n = 4 (Veh) or 5 (ATRi) mice. **A, C-D, F, H-J.** Mean and SD bars shown. *: P < 0.05, **: P < 0.01 by two-tailed, unpaired t-test. Brackets not shown for comparisons that were not statistically significant.

Figure S2: A. Summary of doubling times and estimated lengths of G1, S, and G2 phases in CD8⁺ T cells, B16, and primary fibroblasts. The percentage of cells in each cell cycle phase and the doubling time was used to estimate the length of G1, S, and G2/M. **B.** Doubling time of B16 was previously reported (Danciu et al., 2013; Fidler, 1975) while that of primary fibroblasts was calculated with the equation $(t_2 - t_1)/(3.32 \cdot \log(n_2/n_1))$ using linear part of the growth curve. n_1 is the cell number at the time point t_1 and n_2 is the cell number at the time point t_2 . **C.** EdU versus DNA histograms of B16. Mean and SD bars shown.

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Fidler, I.J. (1975). Biological behavior of malignant melanoma cells correlated to their survival in vivo. *Cancer research* 35, 218-224.

Figure S3: A. Repli-seq analyses of proliferating CD8⁺ T cells (blues) and primary fibroblasts (reds). Upper – whole genome. Lower - chromosome 1. Sequencing data are deposited at GEO (code: GSE183412). **B.** Repli-seq analyses of proliferating CD8⁺ T cells treated with DMSO (blues) or 5 μ M AZD6738 (black) for 1 h. **C.** Genome-wide distribution of the replication timing of 50 kb genomic windows determined by repli-seq. Comparison of proliferating CD8⁺ T cells treated with DMSO (blues) or 5 μ M AZD6738 (black) (left panel) or proliferating CD8⁺ T cells treated with DMSO (blues) and primary fibroblasts treated with DMSO (reds) (right panel).

Concentration (ng/mL)	1,000.00	500.00	250.00	125.00	62.50	31.25	15.62	7.81	3.90	1.95	Blank		
Dilution	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000	1:64,000	1:128,000	1:256,000	1:512,000	Blank	Titer	Coating
107C2-2	2.792	2.739	2.778	2.686	2.567	2.387	2.202	1.867	1.495	1.067	0.056	>1:512,000	A
107C2-2	0.054	0.057	0.060	0.055	0.052	0.057	0.066	0.052	0.055	0.058	0.057	<1:1,000	B

Figure S4: A. A rabbit monoclonal antibody was generated against a synthetic peptide CKVRRV(pSer)FADPI. ELISA of the hybridoma supernatant shows selectivity against phosphopeptide A: CKVRRV(pSer)FADPI vs. nonphosphopeptide B: CKVRRVSFADPI. **B.** GFP-RIF1 WT (wild-type) or GFP-RIF1 S2205A mutated were expressed in 293T cells. Cells were treated with ATRi for 1 h and whole cell extracts were generated and immunoblotted using the purified rabbit monoclonal antibody from A. Upper panel is a long exposure that shows that GFP-RIF1 WT, but not GFP-RIF1 S2205A mutated, is recognized by the rabbit monoclonal antibody in cells treated with ATRi. Middle panel is a typical exposure that shows that endogenous RIF1 is recognized by the rabbit monoclonal antibody in whole cell extracts of 293T cells treated with ATRi. **C.** Whole cell extracts of 293T cells treated with vehicle or ATRi were treated with calf intestinal phosphatase (CIP). RIF1 is recognized by the rabbit monoclonal antibody in whole cell extracts of 293T cells treated with ATRi and this is reversed by treatment with CIP.

Figure S5:

Representative overlays of CTV (proliferation) histograms for proliferating CD8⁺ T cells treated with DMSO and different nucleosides analyzed at 24 h (gray) or 48 h (blue), corresponding to Figure 4A. Red histogram represent unactivated samples. CTV histograms are within live, CD44^{hi}CD8⁺ gates.

Figure S6: Representative overlays of CTV (proliferation) histograms for proliferating CD8⁺ T cells treated with DMSO and different nucleosides analyzed at 24 h (gray) or 48 h (blue), corresponding to Figure 5A. Red histogram represent unactivated samples. CTV histograms are within live, CD44^{hi}CD8⁺ gates.

Figure S7: Percentage of live CD8⁺ T cells (eFluor 780⁻CD8⁺TCRβ⁺) was quantitated for the experiment described in Figure 5C. Mean and SD bars shown. Statistics represent one-way ANOVA with Tukey's multiple comparisons where *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001. Brackets not shown for comparisons that were not statistically significant and between DMSO and ATRi treated samples for clarity.

Figure S8: Representative overlays of CTV (proliferation) histograms at 24 h (gray), 33 h or 48 h post stimulation for proliferating CD8⁺ T cells treated with ATRi or HU and DMSO (black) or thymidine (pink) , corresponding to the experiment described in Figure 5D. CTV histograms are within live, CD44^{hi}CD8⁺ gates.

SUPPLEMENTAL METHODS

Table SM1: Antibodies used in this work.

Antibody	Dilution	Manufacturer	Catalog #
CD8⁺ T cell activation			
Anti-mouse CD28		BD Biosciences	553294
Anti-mouse CD3E		BioLegend	100331
Proliferation/ survival assay			
AF647 anti-mouse CD44 (IM7)	1:500	BioLegend	103018
PE anti-mouse CD8 α (53-6.7)	1:250	BioLegend	100708
AF488 anti-mouse TCR- β (H57-597)	1:250	BioLegend	10925
<i>In vivo</i> immunophenotyping			
BV650 anti-mouse CD4 (clone GK1.5)	1:500	BD Biosciences	563232
BV786 anti-mouse CD62L (clone MEL-14)	1:1000	BD Biosciences	564109
AF488 anti-mouse TCR- β (H57-597)	1:250	BioLegend	109215
AF647 anti-mouse Ki67 (clone 16A8)	1:200	BioLegend	652408
BV421 anti-mouse CD69 (clone H1.2F3)	1:250	BioLegend	104545
BV510 anti-mouse CD4 (clone GK1.5)	1:500	BioLegend	100449
BV510 anti-mouse CD45 (clone 30-F11)	1:250	BioLegend	103138
BV650 anti-mouse CD127 (IL-7R α) (clone A7R34)	1:100	BioLegend	135043
PE anti-mouse/human CD44 (clone IM7)	1:500	BioLegend	103007
PE anti mouse/human KLRG1 (MAFA) (clone 2F1/KLRG1)	1:250	BioLegend	138407
PE-Cy7 anti-mouse CD8 α (clone 53-6.7)	1:250	BioLegend	100721
PerCP-Cy5.5 anti-mouse CD103 (clone 2E7)	1:50	BioLegend	121416
TruStain FcX PLUS anti-mouse CD16/32 (clone S17011E)	1:100	BioLegend	156604
AF647 anti-mouse CD8 (clone KT15)	1:500	MBL Intl	D271-A64
Western blot			
ATM (D2E2)	1:1000	Cell Signaling	2873
ATR	1:1000	Cell Signaling	2790
CHK1 (2G1D5)	1:1000	Cell Signaling	2360
Phospho-CHK1 (S345) (133D3)	1:1000	Cell Signaling	2348
RRM1	1:1000	Cell Signaling	3388
RRM2 (N1C1)	1:1000	GeneTex	GTX103193
DCK	1:1000	Abcam	ab96599
DUT	1:1000	LSBio	LS-C80791
WEE1 (D10D2)	1:1000	Cell Signaling	13084
MCM4 (D3H6N)	1:1000	Cell Signaling	12973
α -Tubulin	1:1000	Cell Signaling	2144
RIF1 pS2205 (CUSTOM)	1:5000	Genescript	107C2-2
ssDNA	1:1000	Millipore	MAB3034

DNA Combing			
IdU (BrdU)	1:20	BD Biosciences	347580
CldU (BrdU)	1:50	abcam	ab6326
ssDNA	1:50	Millipore	MAB3034
anti-mouse AF594	1:50	Invitrogen	A11005
anti-rat AF488	1:50	Invitrogen	A21470
anti-mouse AF647	1:50	Invitrogen	A21235
Immunoprecipitation			
Phospho-MAPK/CDK Substrates (PXS*P or S*PXR/K) (34B2)	1:50	Cell Signaling	2325
Phospho-ATM/ATR Substrate (S*Q) (D23H2/D69H5) MultiMab™	1:50	Cell Signaling	9607
S9.6		Millipore	MABE1095
PLA			
PCNA	1:5000	Abcam	ab92552
phospho-RNA polymerase II (S5)	1:100000	Abcam	ab5408
γH2AX flow cytometry			
FITC anti-mouse γH2AX	1:250	BioLegend	613404

Table SM2: Reagents used for the quantitation of free nucleotides by LC-HRMS.

Reagent	Manufacturer	Catalog #
Optima LC-MS grade water	Thermo Fisher Scientific	W6-4
Optima LC-MS grade acetonitrile	Thermo Fisher Scientific	A955-4
Optima LC-MS grade methanol	Thermo Fisher Scientific	A456-4
1,1,1,3,3,3-hexafluoro 2-propanol (HFIP)	Sigma-Aldrich	105228
Diisopropylethylamine (DIPEA)	Sigma-Aldrich	D125806
AMP standard	Sigma-Aldrich	1930
dAMP standard	Sigma-Aldrich	D6375
ATP standard	Sigma-Aldrich	A26209
dATP standard	Sigma-Aldrich	D6500
dTMP standard	Sigma-Aldrich	T7004
dTTP standard	Sigma-Aldrich	T0251
CMP standard	Sigma-Aldrich	C1006
dCMP standard	Sigma-Aldrich	D7625
CTP standard	Sigma-Aldrich	C1506
dCTP standard	Sigma-Aldrich	D4635
UMP standard	Sigma-Aldrich	U6375

Figure SM1: Flow cytometry gating strategy for CD8⁺ T cells *ex vivo*.

Any unstable portions of the run were gated out prior to analysis. *Ex vivo* cultured CD8⁺ T cells were gated first with scatter gating (FSC-A vs. SSC-A) to exclude debris followed by single cell gating (FSC-A vs. FSC-H) to remove doublets/clumps. For proliferation assay, CD8⁺ T cells were identified as CD8⁺TCRβ⁺ cells within live population. CTV proliferation dye dilution was further characterized in CD44^{hi} population within these cells.

Supplemental Figure SM2.

Flow cytometry gating strategy for CD8⁺ T cell profiling in spleen, TIL, and DLN. Any unstable portions of the run were gated out prior to analysis. After single cell gating (FSC-A vs. FSC-H) to remove doublets/clumps and scatter gating (FSC-A vs. SSC-A) to exclude debris, CD8⁺ T cells were identified as CD8⁺CD4⁻TCRβ⁺ cells within the live, CD45⁺ immune cell population. CD8⁺ T cells were further profiled to identify proliferating (Ki67⁺), newly activated (CD69⁺), naïve (TN, CD62L^{lo}CD44^{lo}), central memory (TCM, CD62L^{hi}CD44^{hi}), and effector/effector memory (CD62L^{lo}CD44^{hi}) CD8⁺ T cells. Example plots are shown for spleen and TIL for all gating following single cell and scatter gating.

Supplemental Figure SM3.

Flow cytometry gating strategy for effector CD8⁺ T cell profiling in TIL and DLN. Any unstable portions of the run were gated out prior to analysis. After single cell gating (FSC-A vs. FSC-H) to remove doublets/clumps and scatter gating (FSC-A vs. SSC-A) to exclude debris, CD8⁺ T cells were identified as TCRβ⁺CD8⁺CD4⁻ cells within the live cell population. Tissue resident CD8⁺ T cells in the TIL were identified as CD103⁺CD69⁺. Expression of CD62L was examined on CD8⁺ T cells in the TIL and DLN, and the CD62L^{lo} CD8⁺ T cell population (which includes effector/effector memory CD8⁺ T cells) in the TIL was further profiled for expression of KLRG1, CD127, and CD69 to identify short-lived/terminal effector (KLRG1⁺CD127⁻), memory precursor effector (KLRG1⁻CD127⁺), newly activated KLRG1⁺ effector (KLRG1⁺CD69⁺), and previously activated KLRG1⁺ effector (KLRG1⁺CD69⁻) CD8⁺ T cells. Example plots are shown for DLN and TIL for all gating following single cell and scatter gating, and for TIL following gating of CD62L^{lo} CD8⁺ T cells. Both TIL and DLN fluorescence minus one (FMO) controls were used to empirically determine gating for KLRG1, CD127, and CD69, and are shown as overlaid plots (TIL in red, DLN in blue).