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Chemical inhibition of DNA-PKcs impairs the activation and cytotoxicity of CD4+ helper and CD8+ effector T cells

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Abstract

Modulation of T cell activity is an effective strategy for the treatment of autoimmune diseases, immune-related disorders and cancer. This highlights a critical need for identification of proteins that regulate T cell function. The kinase DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is emerging as a potent regulator of the immune system spurring interest in its use as a therapeutic target. In murine models of immune-related diseases including asthma and rheumatoid arthritis, treatment with small-molecule DNA-PKcs inhibitors decreased disease severity. Additionally, DNA-PKcs inhibitors reduced T cell-mediated graft rejection in a murine allogenic skin graft model. These *in vivo* studies suggest the use of DNA-PKcs inhibitors as immunotherapy for autoimmune and T cell-mediated disorders. In this study, we sought to further characterize the effects of DNA-PKcs inhibitors on T cells to better understand their clinical potential. We determined that inhibition of DNA-PKcs using inhibitor NU7441 and inhibitors currently in clinical trials for cancer therapy, M3184 and AZD7648, abrogated activation of murine and human $CD4^+$ and $CD8^+$ T cells as evident by reduced expression of the activation

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AUTHOR'S CONTRIBUTIONS

Ana Azevedo-Pouly and Lauren Appell – Participated in research design, performance of research, data analysis, and writing of the paper, Lyle Burdine – Participated in research design, data analysis, and writing of the paper, Lora J Rogers – performance of research and data analysis, Lauren C Morehead – performance of research and data analysis, Daniel Fil - performance of research and data analysis, Melanie Barker - performance of research, animal management, Randall Rainwater - performance of research and data analysis Zachary Waldrip – performance of research, Brian Koss - participated in research design, performance of research, data analysis and writing of the paper, Marie Schluterman Burdine – participated in research design, performance of research, data analysis, writing of the paper, and obtaining funding.

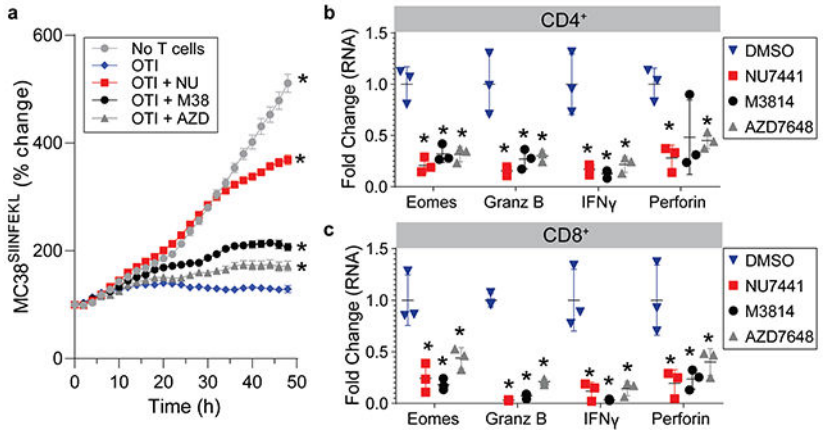
CONFLICT OF INTEREST

The authors declare no conflict of Interest with the contents of this manuscript.

markers CD69 and CD25. Furthermore, inhibition of DNA-PKcs impeded metabolic pathways and proliferation of activated T cells. This reduced the ability of OTI-CD8$^+$ T cells to kill cancer cells and the expression of IFNγ and cytotoxic genes. These results highlight a critical role for DNA-PKcs in T cells and validate future studies using DNA-PKcs inhibitors as immune modulation therapy for the treatment of immune-related diseases.

Graphical Abstract

In this study, we determined that inhibitors for the DNA damage repair kinase DNA-dependent protein kinase catalytic subunit (DNA-PKcs) significantly inhibit the activation, proliferation, and cytotoxicity of CD4+ and CD8+ T cells. These data confirm a pivot role for DNA-PKcs in T cell regulation and highlight the need for clinical precautions when using DNA-PKcs inhibitors for cancer therapy given the value of T cell immunity in controlling tumor growth.



Keywords

DNA dependent protein kinase catalytic subunit; DNA-PKcs; NU7441; CD4$^+$ helper T cells; CD8$^+$ effector T cells; T cell activation; T cell receptor; cytotoxicity; OTI; SIINFEKL-MC38; T cell metabolism

INTRODUCTION

Numerous small-molecule or cell-based immunotherapeutics are FDA-approved for the treatment of immune-related disorders and diseases. For example, inhibitors of T cell activation including tacrolimus and Janus kinase (JAK) inhibitors are successful drugs for the treatment of organ transplant rejection and rheumatologic disorders.[1,2] Further investigation of novel molecules that regulate CD4$^+$ and CD8$^+$ T cell function is imperative for the generation of additional therapeutic options for autoimmune and immune-related diseases.

DNA-PKcs is a 460 kDa phosphatidylinositol 3-kinase-related, serine/threonine kinase. In its canonical role, DNA-PKcs functions as a DNA damage repair kinase recruited to double-strand break foci where it recruits repair proteins to initiate non-homologous end joining (NHEJ).[3] DNA-PKcs inhibitors, highly-specific, synthetic small-molecule

ATP-competitors including NU744, M3814 and AZD7648, sensitize tumor cells to radiation and chemotherapy by reducing DNA damage repair resulting in decreased tumor growth.[4–8] Therefore, the DNA-PKcs inhibitors M3814 and AZD7648 have advanced to clinical trials as antineoplastic adjuvants in combination with radiation, chemotherapy and immunotherapy in Phase I/II clinical trials.[9,10] Interestingly, DNA-PKcs is emerging as a regulator of the immune system. While it is known that DNA-PKcs is required during mammalian development for lymphocyte maturation due to its role in DNA repair, studies have identified functions for DNA-PKcs in mature lymphocytes.[11–13] For instance, in a murine asthma model, Mirsha *et al.* reported dust mite antigens activated DNA-PKcs which was required for an efficient Th2-mediated inflammatory response.[14] Ghonim *et al.* determined in a similar asthma model that DNA-PKcs regulated differentiation of $CD4^+$ T cells into Th1 and Th2 subtypes by altering expression of the transcription factors Gata3 and Tbet.[15] In both studies, treatment with DNA-PKcs inhibitors reduced the severity of asthma-related symptoms. We determined that DNA-PKcs is required for the activity of the transcription factors NFAT, NFκB and EGR1 and production of the cytokines IL2, IL6, IFNγ and TNFα.[16–18] Moreover, we showed that treatment with the DNA-PKcs inhibitor NU7441 extended the survival of transplanted murine allogenic skin grafts by reducing T cell-graft infiltration and inflammatory cytokine production.[17] These studies highlight the immunosuppressive effects of DNA-PKcs inhibitors *in vivo*. However, the effects of DNA-PKcs inhibitors, specifically inhibitors in clinical trials, on T cells have not been thoroughly investigated but is critical for understanding potential clinical benefits. Here, we sought to characterize the effects of DNA-PKcs inhibition on T cells *in vitro*. We determined that treatment with NU7441 and the inhibitors currently in clinical trials, M3814 and AZD7648, significantly disrupted the activation, metabolism and cytotoxic function of murine and human $CD4^+$ and $CD8^+$ T cells. These data suggest a novel role for DNA-PKcs in T cell activation and support evaluating the use of inhibitors as therapy for immune-related disorders. Importantly, they highlight the need for clinical precautions when using M3814 and AZD7648 for cancer therapy given the value of T cell immunity in controlling tumor growth.

RESULTS

Inhibition of DNA-PKcs impairs activation of $CD4^+$ and $CD8^+$ T cells

As observed previously, stimulation of human Jurkat T cells results in an increase in DNA-PKcs activation via phosphorylation of the activation site s2056.[16,19] Treatment with inhibitors NU7441, M3814,AZD7648 reduced s2056 phosphorylation in T cells (Figure 1a). To gain better insight into how DNA-PKcs inhibition affects T cells, we assessed the effect of inhibitors on T cell activation.[16,17] Splenic $CD4^+$ T cells isolated from wildtype mice were stimulated with αCD3/CD28 and monitored for activation via expression of two prominent cell surface activation markers, CD69 and CD25. Activation elevated expression of CD69 and CD25 in $CD4^+$ T cells. However, inhibition of DNA-PKcs via NU7441 or M3814 prevented the increase in double positive CD69 and CD25 cells by approximately 57%, 65% at 24 h and by 83%, 59% at 48 h respectively compared to untreated activated cells (Figure 1b, c). Although activation was reduced with AZD7648 treatment in both $CD4^+$ and $CD8^+$ T cells the difference did not reach significance. Similar results were also seen

in primary mouse $CD8^+$ T cells where all inhibitors reduced the presence of $CD69^+/CD25^+$ cells at 24 and 48 h. (Figure 1d). These results suggest DNA-PKcs inhibition abrogates T cell receptor (TCR)-induced activation of $CD4^+$ and $CD8^+$ T cells.

Metabolism and proliferation in activated T cells are altered by DNA-PKcs inhibition

Immediately following TCR-induced activation, T cells undergo metabolic changes to accommodate for increased cellular proliferation and function.[20] This includes an increase in glucose uptake and aerobic glycolysis. Therefore, we monitored metabolic changes in $CD4^+$ and $CD8^+$ T cells by measuring the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis. We first employed the OVA-specific TCR transgenic OTI mouse model where $CD8^+$ T cells express a TCR designed to recognize the OVA class I antigen SIINFKEL.[21] OTI-$CD8^+$ T cells isolated from mouse spleens were activated with the SIINFKEL peptide at varying concentrations with or without NU7441 and ECAR measured. As expected, T cell activation profoundly induced ECAR and glycolytic metabolism. However, inhibition of DNA-PKcs with NU7441 altered aerobic glycolysis engagement and reduced maximal ECAR levels at all peptide concentrations (Figure 2a, b). Treatment with M3814 or AZD7648 showed similar effects on OTI-$CD8^+$ T cells with significantly reduced ECAR levels (Figure 2c, d). We confirmed this effect by showing that inhibition of DNA-PKcs with NU7441 prevented aerobic glycolysis engagement and the increase in ECAR in $CD4^+$ and $CD8^+$ T cells activated with αCD3/CD28 (Figure 2e, f). These data are consistent with an abrogation in T cell activation.

DNA-PKcs activates the serine/threonine kinase AKT via phosphorylation of serine 473.[18,22] AKT phosphorylation following T cell stimulation is required for the shift to aerobic glycolysis. Loss of AKT activation is known to result in a disruption of T cell metabolism following stimulation.[20,23] We show that treatment with all three DNA-PKcs inhibitors significantly reduced AKT s473 phosphorylation in primary mouse splenocytes activated with αCD3/CD28 highlighting a potential mechanism by which inhibitors disrupt T cell metabolism (Figure 2g).

OTI-$CD8^+$ T cells have impaired cytotoxicity following DNA-PKcs inhibition

We next sought to determine whether the defects in T cell activation mediated by DNA-PKcs inhibition translated into functional deficits. To do so, we analyzed the response of cytotoxic OTI-$CD8^+$ T cells to SIINFEKL-expressing tumor cells. OTI-$CD8^+$ T cells were incubated with $MC38^{SIINFKEL}$ colon cancer cells at a ratio of 4:1 for 48 h and viability of tumor cells analyzed. While untreated OTI-$CD8^+$ T cells effectively killed tumor cells, NU7441, M3814 and AZD7648 treated T cells had a significant decrease in cytotoxic function (Figure 3a). Ensuing activation, T cells generate proteases and cytolytic proteins that mediate target cell killing. Such molecules include the transcription factor eomes, granzyme B and perforin and the cytokine IFNγ. All three DNA-PKcs inhibitors significantly reduced expression of these proteins by αCD3/CD28-activated mouse splenic $CD4^+$ and $CD8^+$ T cells. These results are consistent with a disruption in T cell cytotoxicity (Figure 3b).

Inhibition of DNA-PKcs reduces activation of primary human T cells

Lastly, to provide relevance to human disease, we evaluated the effects of DNA-PKcs inhibition on human $CD4^+$ and $CD8^+$ T cells. T cells isolated from donated human PBMCs were activated with αCD3/CD28 and monitored for changes in activation, proliferation and metabolism with or without inhibitor treatment. As seen in the above experiments using murine T cells, DNA-PKcs inhibitors NU7441 and M3184 significantly decreased expression of the activation markers CD69 and CD25 in both human $CD4^+$ and $CD8^+$ T cells 48 and 96 h after αCD3/CD28 stimulation (Figure 4a, b, c). AZD7648 also reduced expression of activation markers but to a lesser extent. Furthermore, the inhibition of DNA-PKcs with NU7441, M3814 or AZD7648 significantly reduced proliferation of human $CD3^+$ T cells (Figure 4d). DNA-PKcs inhibition also disrupted metabolic pathways in activated human T cells. We observed a reduction in maximal ECAR levels and aerobic glycolysis engagement in NU7441 treated-$CD3^+$ T cells compared to control (Figure 4e). Taken together, these data confirm that DNA-PKcs inhibitors have a significant negative impact on T cell function and support previously published *in vivo* findings that DNA-PKcs inhibitors have immunosuppressant properties.

DISCUSSION

Re-evaluation of currently available drugs for alternative uses is a promising and cost-effective method for rapidly developing new treatments options. Small-molecule DNA-PKcs inhibitors are in clinical trials for cancer treatment, however; recent studies indicate that DNA-PKcs inhibitors have immunosuppressive effects *in vivo* suggesting their potential use as therapy for immune-related diseases and disorders.[24] Our study confirms this potential by demonstrating that inhibition of DNA-PKcs has a significant effect on T cell activation highlighting for the first time a role for DNA-PKcs in transduction of early TCR signaling of both $CD4^+$ and $CD8^+$ T cells. Importantly, we show that inhibitors diminished the ability of $CD8^+$ T cells to lyse antigen-specific tumor cells in co-culture assays. These data indicate that DNA-PKcs inhibition has a significant effect on T cell function and align with previous *in vivo* studies.[14,15,17] Our data are supported in a study by Wang *et al.* which showed DNA-PKcs is activated in αCD3/CD28-stimulated aged-$CD4^+$ T cells and inhibition with NU7441 reduced $CD4^+$ T cell activation and proliferation.[19] Our study further extends these effects to the clinical inhibitors M3814 and AZD7648 and show DNA-PKcs has a similar role in $CD8^+$ T cells as inhibitors have the same effects on these cells. In slight contrast to our studies, Tsai *et al.* reported that DNA-PKcs inhibitors increased expression of CD25 and the activation marker CD44 (CD69 was not analyzed) in $CD4^+$ and $CD8^+$ T cells.[25] Interestingly, however, treatment with DNA-PKcs inhibitors alone reduced T cell proliferation and the infiltration of T cells into tumors resulting in an increase in tumor growth. This supports the idea that DNA-PKcs inhibition suppresses T cell function. This side effect should be considered when using DNA-PKcs inhibitors for cancer therapy where T cell activity is important for tumor control and may account for disappointing outcomes in current clinical trials. Importantly, it highlights the use of DNA-PKcs inhibitors for immunosuppression therapy.

We report one potential mechanism by which DNA-PKcs inhibitors curtail T cell function is through reduction of AKT activation, a known phosphorylation target of DNA-PKcs required for T cell activities.[27] We show that all three DNA-PKcs inhibitors reduced phosphorylation of AKT at s473. Inhibition of AKT has widely been published to hinder T cell activation and alter T cell metabolism. In fact, Wang *et al.* also observed a decrease in AKT activation with NU7441 treatment of DNA-stimulated aged $CD4^+$ T cells which resulted in a decrease in proliferation and activation.[19] While this paper focuses on the effects of small-molecule DNA-PKcs inhibition on T cells to evaluate therapeutic use, we are actively investigating molecular mechanisms by which DNA-PKcs regulates T cell activation to uncover novel targets for future therapeutic strategies.

METHODS

Reagents and antibodies

Flow cytometric antibodies: anti-CD4 eFlour 450, anti-CD8α eFlour 450, goat anti-rabbitt IgG (Invitrogen, Carlsbad, CA #69-0032-80, 48-004-82, 48-0081-82, A32733); anti-CD25 FITC and anti-CD69 APC/Cy7 antibodies (BioLegend, San Diego, CA, #102006, 104526); anti-DNA-PKcs (Cell Signaling, Danvers, MA, #38168). Western blot antibodies - GAPDH antibody (ThermoFisher, Waltham, MA, cat #MA5-15738); pAKT 473 (Cell Signaling, #4060), AKT (Cell Signaling, #2938), pDNA-PKcs 2056 (ThermoFisher cat #PA5-78130). Inhibitors: NU7441, M3814 (Nedisertib), AZD7648 (Selleckchem, Houston, TX, cat #S2638, S8586, S8843). T cell activation antibodies– purified anti-CD3ε and purified anti-CD28 (BioLegend, cat #100340, 102116). For all experiments, supplemented RPMI (RPMI-1640 supplemented with 10% fetal bovine serum, penicillin-streptomycin, Sodium Pyruvate, MEM NEAA, 30 U mL^{-1} recombinant IL2 (ThermoFisher) and 55 μM β-mercaptoethanol) was used.

Mice

C57Bl/6 and OT-I mice were housed and bred in specific pathogen-free conditions at Arkansas Children's Research Institute and the University of Arkansas for Medical Sciences. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences/Arkansas Children's Research Institute.

$CD8α^+$ and $CD4^+$ T cell isolation

Single-cell suspensions enriched for naive $CD8α^+$ or $CD4^+$ T cells from mouse spleens were isolated. Briefly, spleens were minced and passed through a 70-μm-nylon cell strainer into PBS. After washing in PBS, T cells were isolated from mouse splenocytes using Naïve $CD4^+$ T or $CD8α^+$ MACS T cell isolation kits (Miltenyi Biotech, San Jose, CA, USA, #130–104-453, #130–104-075) according to the manufacturer's protocol. For *in vitro* co-culture experiments, OTI^+ T cells were purified using $CD8α^+$ enrichment as detailed above.

Primary cell culture

Murine T cell culture—$CD4^+$ and $CD8\alpha^+$ T cells were cultured as follows: plates were coated with 5 μg mL^{-1} anti-CD3ε (or with PBS alone) for 12–18 h at 4 °C. Cells were then plated at 1–2 x 10^6 cells mL^{-1} in supplemented RPMI and either left unstimulated, stimulated with the plate-bound anti-CD3ε and 5 μg mL^{-1} of soluble anti-CD28, or stimulated and treated with NU74411 (2–5 μM) M3814 (5–10 μM), or AZD7648 (5–10 μM) for 24–48 h.

Human T cell culture

$CD3\varepsilon^+$, $CD4^+$ and $CD8\alpha^+$ T cells were isolated using the negative selection Human T cell Isolation Kit (StemCell Technology, Vancouver, Canada, cat #17951). Cells were plated at 1 x 10^6 cells mL^{-1} in T cell growth media (StemCell Technology, cat #10981) and activated using ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (StemCell Technology, cat #10970) with or without NU74411 (2 μM), M3814 (5 μM), or AZD7648 (5 μM) for 48 h. Jurkat T cells were cultured in supplemented RPMI and either left unstimulated, stimulated with the plate-bound anti-CD3ε and 5 μg mL^{-1} of soluble anti-CD28, or stimulated and treated with NU74411 (5 μM) M3814 (10 μM), or AZD7648 (10 μM) for 24 h.

Flow cytometry

Isolated $CD4^+$ and $CD8\alpha^+$ T cells were stained after activation and flow cytometry was performed. Cells were stained for cell surface markers and resuspended in eBioscience flow cytometry staining buffer (Invitrogen, cat #00-4222-26). Viability was assessed by staining with 7-AAD Viability Staining Solution (BioLegend, 420404). anti-DNA-PKcs (Cell Signaling, cat #38168) and goat anti-rabbit IgG (Invitrogen, cat #A32733).

Metabolic analysis

The extracellular acidification rate (ECAR) was measured using the Seahorse XFe bioanalyzer. 2×10^5 T cells per well (≥ 8 wells per sample) were spun onto Cell-Tak (Corning)–coated seahorse 96-well plates and preincubated at 37°C for approximately 20 min in the absence of CO_2. ECAR was measured in XF media (nonbuffered RPMI 1640 containing 10 mmol L^{-1} glucose, 2 mmol L^{-1} l-glutamine and 1 mmol L^{-1} sodium pyruvate) under basal conditions and in response to activation (SIINFEKL or anti-CD3/28), 2 μmol L^{-1} oligomycin and 10 mmol L^{-1} 2-Deoxy-D-glucose.

***In vitro* killing assays—**Co-culture experiments were performed as previously reported.[26] Briefly, T cell killing assays were carried out using preactivated $CD8^+$ T cells isolated from spleens of OTI (effector) mice. Target cells ($MC38^{SIINFEKL}$) expressing a nuclear RFP (IncuCyte® NucLight™ Red) were plated for 16 h prior to culturing with T cells. The cocultures were conducted at a target: effector (T:E) ratio of 4:1 for 48 h in 96 well live cell imaging plates. Plates are automatically imaged every two h with an ImageExpress confocal HT.AI microscope (Molecular Devices, San Jose, CA) in the RFP channel. At each timepoint, 9 unique sites were imaged, and the total number of RFP^+ (target) cells were quantified. Plates were imaged for up to 48 h, and values were normalized to target cell counts at t = 0.

Proliferation assays

Proliferation of human $CD3^+$ T cells isolated from PBMCs was determined using hemocytometer to determine total cells 48 and 96 h post activation.

Real-time PCR

Following treatment as described, cells were lysed with Trizol (Qiagen, Hilden, Germany, cat #15596026) and total RNA was isolated using Zymo Direct-zol RNA MiniPrep (Zymo, Irvine, CA, USA, cat #R2052) following the manufacturer's protocol. cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA, #1708841) according to the manufacturer's instructions. PowerUP SYBR Green master mix (Applied Biosystems, Waltham, MA, USA, #A25742) was used for real-time PCR detection with primers listed below. Reactions were performed in technical triplicate and ΔΔCt method was used to calculate relative expression of the target transcripts normalized to TBP transcript levels. Primers are listed in Supplementary table 1.

Western Blot

Samples were lysed in 0.5% SDS RIPA buffer with protease inhibitors (Thermo Scientific, cat #78425) and phosphatase inhibitors (Roche, San Jose, CA, # 04906837001) followed by sonication. Samples were heated in LDS (lithium dodecyl sulfate) loading buffer then loaded into 4–12% bis-tris gels (Thermo Scientific cat #NW04122BOX) followed by transfer to a PVDF membrane. Membrane was probed for AKT, pAKT, pDNA-PKcs, DNA-PKcs and Gapdh. Imaging was performed with a GE ImageQuant LAS4000. Image J software was used for normalization.

Statistical analysis

Analysis of significance was determined with a standard *t*-test and expressed as the mean ± standard deviation. Data were analyzed using GraphPad Prism 7. $P \leq 0.05$ was considered significant. Assays were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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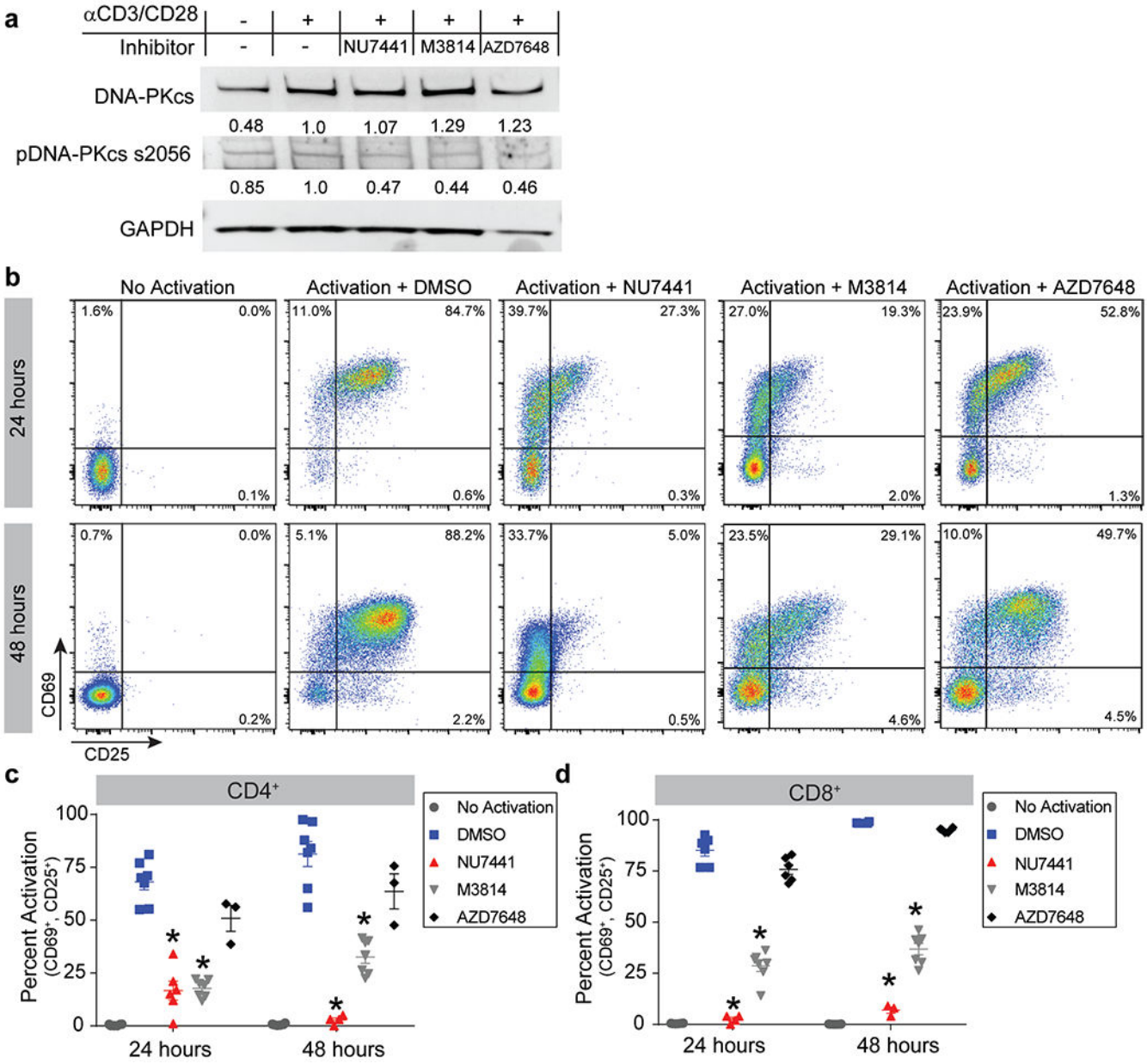

Figure 1. Inhibition of DNA-PKcs impairs CD4$^+$ and CD8$^+$ T cell activation.

(a) Western blot of total and phosphorylated s2056 DNA-PKcs expression in human Jurkat T cells with no activation, activation (αCD3/CD28), or activation plus inhibitor. Inhibitors NU7441 (5 μM), M3814 (10 μM) and AZD7648 (10 μM) reduced phosphorylation at site s2056. The Western blot is a representative of 3 independent experiments with expression normalized to Gapdh and activation alone. N = 3. Mouse splenic naïve CD8$^+$ or CD4$^+$ T cells were activated (αCD3/CD28) in the presence of vehicle (DMSO), 5 μM NU7441, 10 μM M3814 or 10 μM AZD7648 for 24 and 48 h. Flow cytometric analysis of CD25 and CD69 activation markers (viability 7-AAD) expression was then performed. **(b)** Representative staining for each CD4$^+$ T cell experimental group. Percentage of CD69$^+$/

$CD25^+$ **(c)** $CD4^+$ or **(d)** $CD8^+$ T cells was reduced following inhibitor treatment. Error bars S.D. of n ≥ 3. *$P \leq 0.05$.

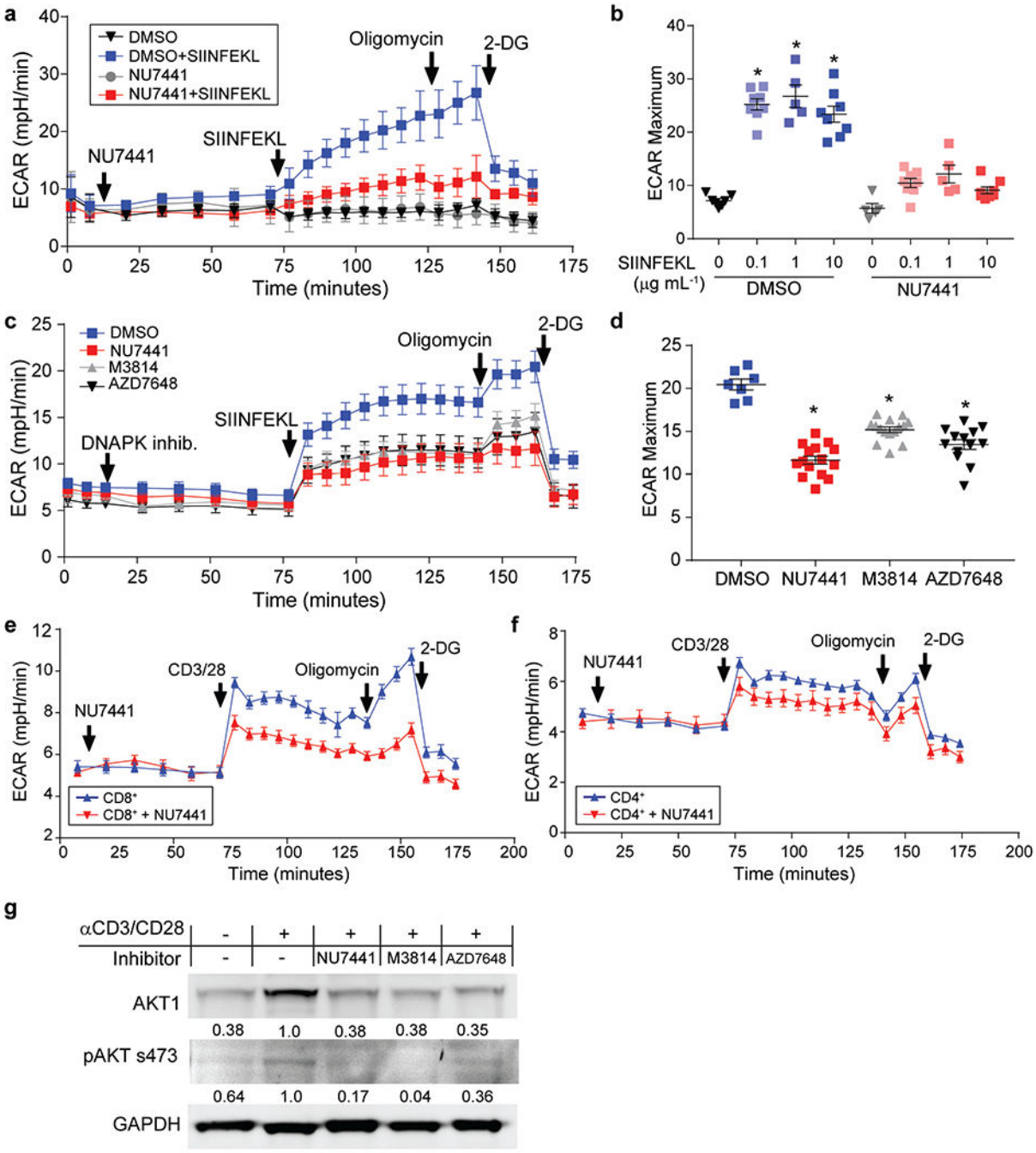

Figure 2. Inhibition of DNA-PKcs prevents engagement of aerobic glycolysis following activation regardless of antigen concentration and augments AKT phosphorylation.

(a) Representative trace of OTI-CD8$^+$ T cells isolate from mouse spleens stimulated with the SIINFEKL peptide (1 μg mL^{-1}) while being monitored by a Seahorse XF96 metabolic flux analyzer. T cells treated with NU7441 (2 μM) 1 h prior to stimulation had decreased glycolytic rate (ECAR). Oligomycin (ATP synthase inhibitor) and 2-DG (glycolysis inhibitor) were used as controls. **(b)** Maximal ECAR of OTI-CD8$^+$ T cells activated for 48 h with different concentrations of SIINFEKL peptide was decreased with

NU7441 treatment. Error bars = S.D., $*P \leq 0.05$. **(c)** Representative trace of OTI-$CD8^+$ T cells stimulated with the SIINFEKL peptide (1 μg mL^{-1}) with or without NU7441 (2 μM), M3814 (5 μM) or AZD7648 (5 μM) while being monitored by a Seahorse XF96 metabolic flux analyzer. **(d)** Maximal ECAR of OTI-$CD8^+$ T cells activated for 48 h with SIINFEKL peptide was decreased following NU7441 (2 μM), M3814 (5 μM) or AZD7648 (5 μM) compared to no treatment. Error bars = S.D., $*P \leq 0.05$. **(e, f)** Representative trace of isolated mouse splenic naïve **(e)** $CD8^+$ or **(f)** $CD4^+$ T cells activated (αCD3/CD28) in the presence of vehicle (DMSO) or 2 μM NU7441. NU7441 treatment reduced the glycolytic rate in both $CD4^+$ and $CD8^+$ T cells. n = 3 independent experiments. **(g)** The Western blot of mouse primary splenocytes activated (αCD3/CD28) shows reduced phosphorylation of s473 of AKT following treatment with 5 μM NU7441, 10 μM M3814 or 10 μM AZD7648 for 24 h. Representative blot of normalized expression of AKT1 and pAKT to GAPDH for the three treatment groups is shown. n = 3 independent experiments.

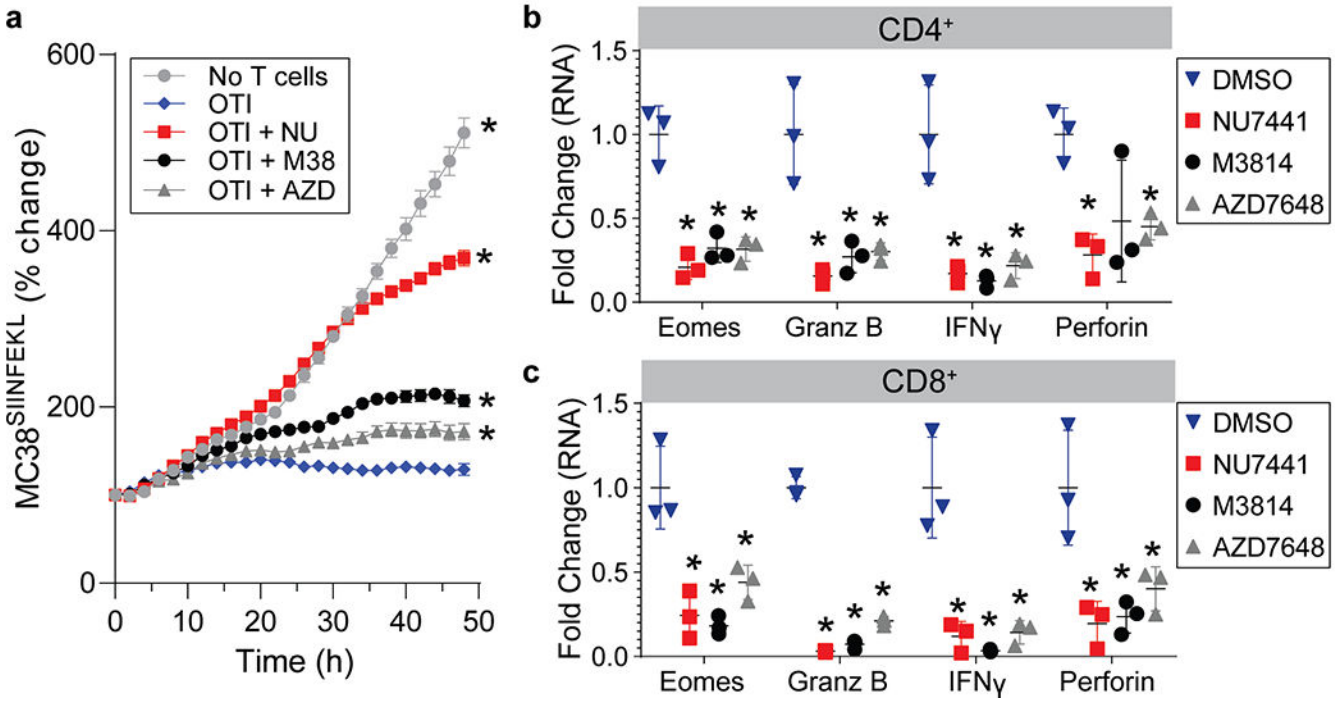

Figure 3. Loss of DNA-PKcs activity inhibits cytotoxicity of CD8$^+$ T cells.

OT-I CD8$^+$ T cells isolated from mouse spleens were activated (αCD3/CD28) in the presence of vehicle (DMSO) or 2 μM NU744, 5 μM M3814, or 5 μM AZD7648 for 72 h. **(a)** Activated OT-I CD8$^+$ T cells were then co-cultured with the MC38^{SIINFEKL} cell line at an Effector: Target ratio of 4:1 for 48 h. Plates are automatically imaged every 2 h and at each timepoint the total numbers of RFP$^+$ (Target) cells was quantified. Cell count values are normalized to target cell counts at t = 0. Error bars = S.D. of 9 unique sites. *$P \leq 0.05$. **(b, c)** Cytotoxic gene expression at 48 h in **(b)** CD4$^+$ and **(c)** CD8$^+$ T cells was determined using qPCR. Error bars represent the S.D. of n = 3 independent experiments. *$P \leq 0.05$.

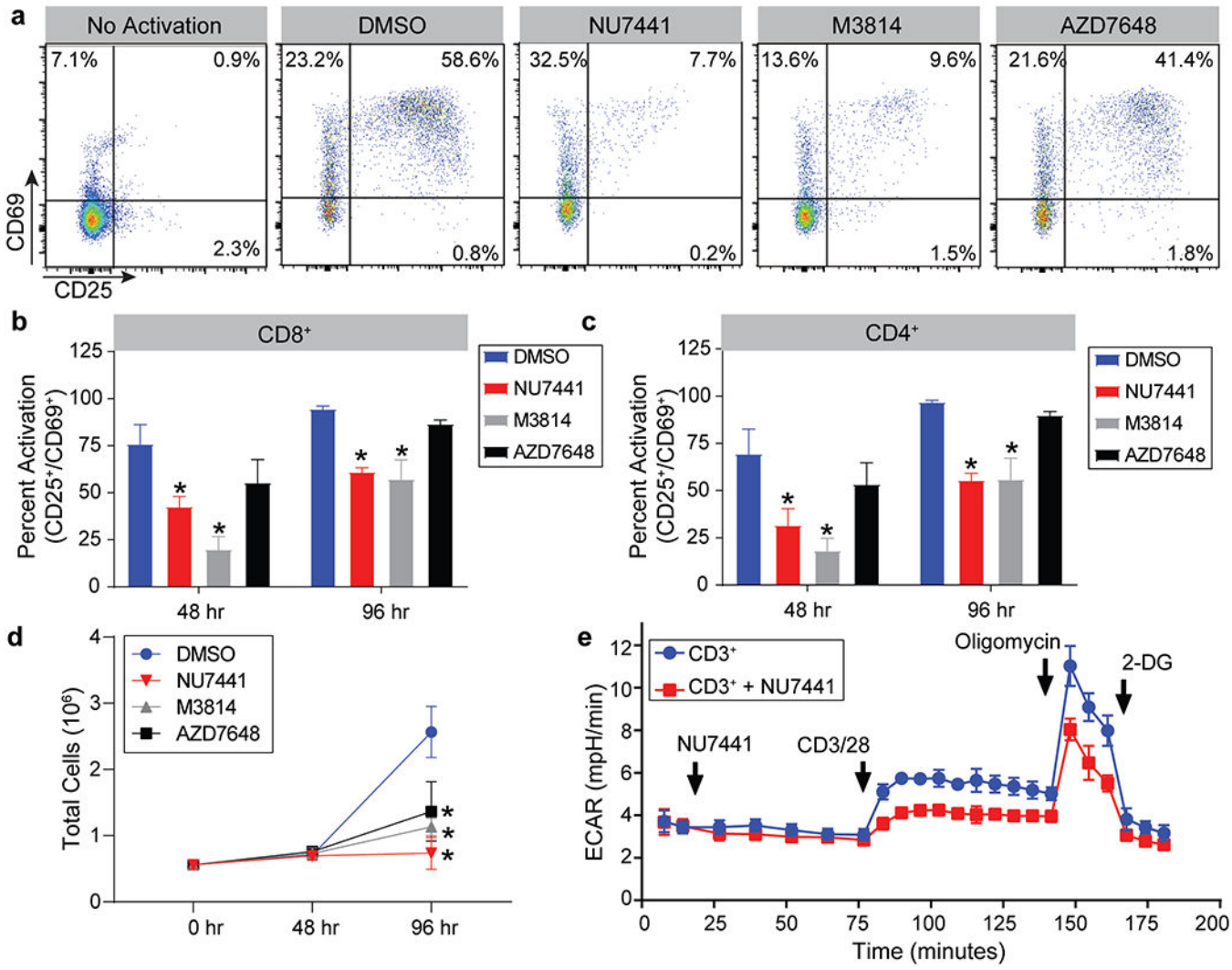

Figure 4. Inhibition of DNA-PKcs reduces activation and proliferation of human T cells.

(a) Representative staining of activation markers CD25 and CD69 by flow cytometry following activation of $CD4^+$ T cells isolated from human PBMCs in the presence of NU7441 (2 μM), M3814 (5 μM) or AZD7648 (5 μM). Percentage of $CD69^+/CD25^+$ **(b)** $CD8^+$ or **(c)** $CD4^+$ T cells decreased following inhibitor treatment. Error bars S.D. of n ≥ 3. *$P \leq 0.05$ **(d)** Proliferation of human $CD3^+$ T cells isolated from PBMCs was determined using hemocytometer to determine total cells 48 and 96 h post activation. Error bars S.D. of n ≥ 3. *$P \leq 0.05$ **(e)** Representative trace (three independent experiments were performed) of human $CD3^+$ T cells stimulated with αCD3/CD28 while being monitored by a Seahorse XF96 metabolic flux analyzer. T cells treated with NU7441 (2 μM) 1 h prior to stimulation had decreased glycolytic rate (ECAR). Oligomycin (ATP synthase inhibitor) and 2-DG (glycolysis inhibitor) were used as controls.