1 Inducible T cell kinase regulates expression of cytolytic effectors and degranulation in CD8^{+}

2 cytotoxic T lymphocytes[1]

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14 ABSTRACT

15 Patients with mutations in ITK are susceptible to viral infections, particularly Epstein
16 Barr Virus, suggesting that these patients have defective function of $CD8^+$ cytolytic T
17 lymphocytes (CTLs). Here, we evaluated the direct effects of ITK-deficiency on cytolysis in
18 murine CTLs deficient in ITK, and both human and murine cells treated with an ITK inhibitor.
19 We find that ITK-deficiency leads to a global defect in the cytolysis of multiple targets. The
20 absence of ITK affected both the expansion and expression of cytolytic effectors during
21 activation, and led to a previously unappreciated intrinsic defect in degranulation. Nonetheless,
22 these defects could be overcome by prolonged exposure to IL-2 in culture, which also revealed
23 that ITK-deficient $CD8^+$ T cells were more resistant to cell death. Our results provide new
24 insight into the effect of ITK and suboptimal TCR signaling on $CD8^+$ T cell function, and how
25 these may contribute to phenotypes associated with ITK-deficiency.

26 **INTRODUCTION**

27 CD8+ cytotoxic T lymphocytes (CTLs) are critical for combatting viral infections and
28 tumors through the directed lysis of target cells. Accordingly, mutations in genes affecting CTL
29 cytolytic function have been found in a number of primary immunodeficiencies associated with
30 impaired viral clearance and tumor development.

31 Granule-dependent, contact-mediated killing of virally infected cells by CTLs is initiated
32 upon T cell receptor (TCR) engagement, which causes a series of cellular changes resulting in
33 the release of cytolytic effectors at the site of contact with target cells. These stages include the
34 initial adhesion of CTLs to target cells and the rapid accumulation of a rich cortical actin
35 network [1], which then clears to form a ring at the edge of immunological synapse, the special
36 organization of membrane and signaling proteins that forms at the interface between a T cell and
37 its target. Actin clearance is closely followed by reorientation of the centrosome [2] and the
38 polarization of cytotoxic granules toward the target cell along a reorganized microtubule network
39 [3, 4], leading to centrosome docking and granule fusion at the plasma membrane. The release of
40 lytic granule contents at the secretory domain of the synapse, including the pore-forming
41 molecule perforin, allows granzymes to enter the cytoplasm of target cells and initiate cell death
42 [5-8]. Through this ordered series of events, CTLs are able to rapidly and effectively eliminate
43 virally infected targets during an immune response.

44 In order to trigger cytolysis, TCR engagement initiates signaling cascades associated with
45 the formation of signaling complexes at the plasma membrane. Inducible T cell kinase (ITK) is a
46 non-receptor tyrosine kinase that is a component of the LAT-SLP76 signaling complex, which is
47 formed downstream of TCR activation. ITK phosphorylates PLCγ1, a key enzyme required for
48 generation of critical second messengers during TCR signaling. Accordingly, the loss of ITK

49 leads to reduced TCR-induced PLCγ1 phosphorylation and downstream impairments in Ca^{2+} flux
50 and ERK signaling, as well as altered actin cytoskeletal regulation [9-11]. Studies of $CD4^+$ T
51 cells from *Itk*$^{-/-}$ mice have shown that suboptimal TCR signaling in the absence of ITK leads to
52 dramatic effects on $CD4^+$ T cell differentiation, and altered $CD4^+$ T cell function [12-14]
53 (reviewed in [15]), including decreased IL-2 production and altered responses to IL-2 [9, 13, 16-
54 19]. Notably however, these studies have primarily focused on either total T cell or $CD4^+$ T cell
55 populations, leaving the role of ITK in $CD8^+$ T cells relatively less well explored.
56 Recently, loss of function mutations in ITK were reported in a subset of patients with
57 fulminant infectious mononucleosis triggered by Epstein Barr virus (EBV) infection [20-22]. In
58 addition, lymphomas, defective antibody responses, and a broader susceptibility to viral infection
59 were also reported in these patients (reviewed in [23]), highlighting a potential requirement for
60 ITK for proper CTL function. Intriguingly, this clinical phenotype resembles a number of other
61 primary immunodeficiencies, including X-linked lymphoproliferative syndrome (XLP-1), a
62 disease caused by mutations affecting the small adaptor molecule, signaling lymphocyte
63 activation molecule (SLAM)-associated protein (SAP). We have previously shown that CTLs
64 from SAP-deficient mice exhibit specific defects in killing B cells, despite normal cytolysis of
65 other targets [24]. Analogous observations have been made in cells from patients with XLP-1
66 [25], likely accounting for the inability of SAP-deficient CTLs to clear EBV-infected B cells.
67 The similarities in clinical phenotypes between ITK-deficiency and XLP-1 raised the question of
68 whether ITK-deficiency also similarly affects cytolytic effector function. Although *Itk*$^{-/-}$ mice can
69 mount protective immune responses against vaccinia virus, vesicular stomatitis virus, and
70 lymphocytic choriomeningitis virus [26, 27], viral clearance is delayed, likely reflecting poor
71 activation of $CD8^+$ T cells under conditions of suboptimal TCR signaling. However, whether or

72 not there were defects in granule-mediated cytolysis of specific targets, or at specific stages of
73 cytolysis, has not been well examined. A more complete examination of the role of ITK in CTL
74 effector function would be useful for better understanding the human disease.

75 Here, we used the OT-I TCR transgenic system to examine the role of ITK in $CD8^+$ T
76 cell cytolytic effector function. We found that ITK was required for killing of multiple different
77 target cells, suggesting global defects in cytolysis in the absence of ITK. Although ITK-deficient
78 $CD8^+$ T cells showed decreased expansion and expression of effector molecules after activation,
79 treatment of differentiated WT CTLs with an ITK inhibitor still led to defects in cytolysis,
80 suggesting direct effects of ITK-deficiency on the process of killing. Examination of discrete
81 stages of CTL function revealed that ITK-deficiency did not affect the early stages of killing,
82 including adhesion to targets and polarization of the centrosome and lytic granules, which were
83 intact in $Itk^{-/-}$ CTLs. Instead, ITK-deficiency in CTLs was associated with defects in
84 degranulation, a late stage of target killing, which could be recapitulated by treatment of WT
85 mouse CTLs or activated $CD8^+$ T cells from healthy human donors with an ITK-specific
86 inhibitor. Nonetheless, we also found that prolonged incubation with IL-2 could rescue these
87 defects. These results suggest that ITK plays a previously unappreciated role in lytic granule
88 secretion during CTL killing, and provide evidence for novel roles for ITK and TCR signaling in
89 regulating both early differentiation/expansion of CTLs and late stages of cytolytic activity that
90 may contribute to reduced viral clearance in patients with mutations in ITK.

91 **MATERIALS AND METHODS**

92 **Mice**

93 Wild type (WT) OT-I [28], *Itk*$^{-/-}$ [29] OT-I TCR transgenic, and C57Bl/6 (Jackson
94 Laboratories) mice were maintained in a Specific Pathogen Free facility and used between 6-10
95 weeks of age. For *in vitro* experiments, cells from either male or female mice were used. Animal
96 husbandry and experiments were performed in accordance with approved protocols by the
97 National Human Genome Research Institute Animal Use and Care Committee at the National
98 Institutes of Health.
99

100 **Cell culture**

101 To generate *in vitro* activated mouse CTLs, splenocytes from WT or *Itk*$^{-/-}$ OT-I mice were
102 harvested and stimulated at 0.5x10^6 cells/mL with 10nM OVA$_{257-264}$ peptide (AnaSpec) for 3
103 days in 10% complete media (RPMI 1640 plus 10% FBS, 2mM L-glutamine, 50U/mL
104 penicillin/streptomycin, and 50μM β-mercaptoethanol). Cells were then washed twice and
105 resuspended in complete media plus 10 IU/mL recombinant human IL-2 (rHIL-2). Cells were
106 washed once and seeded in fresh media at 0.5x10^6 cells/mL every 48 hours. All experiments
107 were performed with CTLs between 6 and 7 days after primary *in vitro* stimulation, unless
108 otherwise indicated. Resting B cells were purified by negative selection with anti-CD43
109 microbeads (Miltenyi) and activated with 1μg/mL LPS from *E. coli* (Enzo Life Sciences) in 10%
110 complete media for 2-3 days before use as targets in assays. EL4 and MC57 cell lines were
111 maintained in complete Dulbecco's modified Eagle's medium (DMEM) plus 5% FBS. Blood
112 from healthy donors was obtained at the NIH Clinical Center under NIH Clinical Center IRB-
113 approved protocol 99-CC-0168 "Collection and Distribution of Blood Components from Healthy

114 Donors for In Vitro Research Use." Peripheral blood mononuclear cells (PBMCs) were isolated
115 from whole blood by density-gradient centrifugation using Lymphocyte Separation Medium (MP
116 Biomedical), washed twice in phosphate buffered saline (PBS), and resuspended at $1x10^6$
117 cells/mL. One mL of cells was then added to each well of a 24-well plate and placed at 37° C.
118 Mixed buffy coats for anti-allogeneic stimulation were irradiated and resuspended at $1x10^6$
119 cells/mL, and phytohemagglutinin (PHA) was added to the buffy coats at 2μg/mL. To stimulate
120 lymphocytes, 1mL of activated buffy coat was added to each well for a final ratio of 1:1
121 stimulators:responders in 1μg/mL PHA. PHA blasts were split as needed, and $CD8^+$ T cells
122 isolated using a $CD8^+$ T cell isolation kit (MACS Miltenyi). Bulk $CD8^+$ T cells were cultured for
123 use in experiments. For inhibition experiments, previously activated WT OT-I CTLs or human
124 $CD8^+$ T cells were pre-treated for 10 minutes at 37°C with the ITK inhibitor, 10n (gift of Craig
125 Thomas, NCATS, Bethesda, MD), at indicated concentrations and used directly in assays
126 without washing.
127

128 **Staining and flow cytometry**

129 For staining surface markers, cells were washed and blocked in FACS buffer (PBS plus
130 1% FCS) in the presence of Fc block. Samples were stained with anti-CD8α (clone 53-6.7,
131 BioLegend), anti-CD25 (clone 7D4, eBioscience), anti-CD62L (clone MEL-14, eBioscience),
132 anti-CD69 (clone H1.2F3, eBioscience), anti-Vα2 (clone B20.1, BD Biosciences), anti-CD244
133 (clone C9.1, BD Biosciences), anti-Ly108 (clone 13G3, BD Biosciences), or anti-CD27 (clone
134 LG.3A10, BioLegend), at 4 C for 30 minutes in FACS buffer, protected from light, followed by
135 fixation with 4% paraformaldehyde (PFA, Electron Microscopy Sciences). For intracellular
136 staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) at 4 C

137 for one hour. Samples were then stained with anti-granzyme B (clone GB11, BD Biosciences)
138 for one hour at 4 C, protected from light. For phospho-antibody staining, cells were fixed with
139 4% paraformaldehyde, methanol-permeabilized at -20 C, and stained for 60 minutes at 4 C with
140 anti-phosphoS6 (clone D57.2.2E, Cell Signaling) in PBS plus 1% Triton X-100 and 0.5% bovine
141 serum albumin (BSA). Data were acquired on either a Calibur1 or LSRII flow cytometer (BD)
142 and analyzed using FlowJo software (Tree Star).
143

144 **Proliferation assay**

145 To evaluate the proliferative capacity of cells, splenocytes were stained with 1μM Cell
146 Trace Violet (CTV, Life Technologies) in PBS at 37 C for 10 minutes. Stained cells were
147 washed 3 times with complete media and then stimulated in the presence of $OVA_{257\text{-}264}$. Cells
148 were collected at indicated time points, stained with anti-CD8α (clone 53-6.7, BioLegend) and
149 evaluated via flow cytometry.
150

151 **Cytotoxicity assays**

152 *In vitro* cytolytic activity was determined using either a lactate dehydrogenase (LDH)
153 release or flow-based assay. For LDH release, CytoTox Non-radioactive Cytotoxicity Assays
154 (Promega) were used according to the manufacturer's instructions. Briefly, targets were pulsed
155 with 1μM $OVA_{257\text{-}264}$ peptide for 1 hour at 37°C, washed twice, and resuspended in phenol red-
156 free RPMI with 2% FBS (assay buffer). Activated CTLs were washed and resuspended in assay
157 buffer, added to 96 well plates and titrated in assay buffer. Targets or assay buffer were added to
158 wells to achieve appropriate effector:target ratios and control groups, and plates were incubated
159 for indicated times at 37°C. Supernatants were then transferred to unused plates containing assay

160 substrate and the OD read at 490nm on a Thermomax plate reader to measure lactate
161 dehydrogenase (LDH) release. Percent cytotoxicity was calculated per manufacturer's
162 suggestions. For the flow-based method, targets were stained with 1μM Cell Trace Violet (CTV,
163 Life Technologies) as described above. Targets were either left unpulsed or pulsed with 1μM
164 $OVA_{257-264}$ peptide for 1 hour at 37°C, washed twice, and resuspended in assay buffer. Activated
165 CTLs were washed and resuspended, added to 96 well plates and titrated in assay buffer. Non-
166 pulsed, or pulsed targets were added to wells with CTLs to achieve appropriate effector:target
167 ratios; additional wells set up with CTLs or target cells alone to control for spontaneous cell
168 death. After indicated times at 37°C, plates were centrifuged, and supernatants discarded. Cells
169 were then stained with anti-CD8α (clone 53-6.7, BioLegend) antibodies and LiveDead green
170 (Life Technologies), washed with FACS buffer, and fixed with 4% PFA. Plates were read on a
171 LSRII instrument using a high throughput sampler. For analysis, the CTV+ LiveDead+
172 population represents the target cells that have been killed, while the CTV+ LiveDead-
173 population represents the remaining viable target cells in each well. Percent cytotoxicity was
174 calculated as: 100 – [(viable CTV+ cells in sample)/(viable CTV+ cells in control)] x 100, where
175 CTV+ cells in sample are cells in experimental wells, and viable CTV+ cells in control are cells
176 in wells without T cells.

177 To examine cytolytic activity *in vivo*, indicated numbers of previously activated WT or
178 ITK-deficient CTLs, or a PBS control were adoptively transferred via retro-orbital injection into
179 naïve WT C57BL/6 hosts. LPS-activated B cell targets from WT GFP mice were labeled with
180 either 0.2μM or 2μM Cell Proliferation Dye eFlour450 (eBioscience) and left unpulsed or pulsed
181 with 1μM $OVA_{257-264}$ peptide for 1 hour at 37°C, respectively. B cells were then mixed at a 1:1
182 ratio and transferred via retro-orbital injection into mice 24 hours after delivery of CTLs. Spleens

183 were harvested at indicated time points and populations analyzed via flow cytometry.

184 Transferred B cells were distinguished from recipient B cells by gating on the GFP positive

185 population, and peptide pulsed versus non-pulsed targets based on the intensity of eFluor450

186 fluorescence. Percent cytotoxicity was calculated as follows: % cytotoxicity = 100 –

187 $([(T_{pulsed}/T_{non\text{-}pulsed})/(C_{pulsed}/C_{non\text{-}pulsed})] \times 100)$, where T_{pulsed} is the percentage of peptide-pulsed

188 targets harvested from spleens of recipients, $T_{non\text{-}pulsed}$ is the percentage of non-pulsed targets

189 harvested from spleens of recipients, C_{pulsed} is the percentage of peptide-pulsed targets harvested

190 from spleens of PBS recipients, and $C_{non\text{-}pulsed}$ is the percentage of non-pulsed targets harvested

191 from spleens of PBS recipients.

192

193 **Conjugate assays**

194 For FACS-based conjugate assays, LPS-activated primary B cells, EL4, or MC57 targets

195 were stained with 0.1μM carboxyfluorescein diacetate succinimidyl ester (CFSE), and pulsed

196 with peptide at indicated concentrations for 1 hour at 37°C or left non-pulsed as a control. After

197 washing, targets were mixed with previously activated CTLs at a 2:1 T:target ratio in 96-well

198 round bottom plates, spun down, and incubated for 20 minutes at 37°C. Cells were washed and

199 stained with anti-CD8α-PerCPCy5.5 or –APC (clone 53-6.7, BioLegend) and conjugates

200 enumerated via flow cytometry, where the $CD8^{+}$CFSE double positive population represented T

201 cells forming conjugates with targets.

202

203 **Degranulation assays**

204 For degranulation assays [30], activated CTLs were stimulated in plates coated with anti-

205 CD3ε (BioXCell) or mixed at a 1:1 ratio with peptide-pulsed or non-pulsed targets at 37°C in the

206 presence of anti-CD107a-PE or –APC (clone 1D4B, Biolegend) labeled antibody. At indicated

207 time points, plates were placed on ice and cells transferred into cold PBS, stained with anti-

208 CD8α and anti-CD107a-FITC (clone 1D4B, eBioscience) antibodies, and analyzed via flow

209 cytometry.

210

211 **RNA isolation and analysis**

212 Total RNA was isolated from WT and *Itk*$^{-/-}$ CD8^{+} T cells at indicated time points using

213 the RNeasy Mini Kit (Qiagen) and was reverse transcribed with random hexamer primers and the

214 M-MLV Reverse Transcriptase (Applied Biosystems). Quantitative RT-PCR was performed on

215 Step One Plus Real-time PCR System (Applied Biosystems) using TaqMan assays (Applied

216 Biosystems) for indicated genes. Samples were normalized to 18S RNA and data are expressed

217 as relative to WT levels using the $2^{-\Delta\Delta CT}$method.

218

219 **Immunofluorescence confocal microscopy**

220 To prepare conjugates for immunofluorescence microscopy, targets were pulsed with

221 1μM $OVA_{257-264}$ at 37°C for 1 hour, washed twice, and resuspended in pre-warmed phenol red-

222 free RPMI (imaging media). Activated CTLs were washed and resuspended in imaging medium

223 and mixed with peptide-pulsed targets at a 1:1 ratio. Cells were incubated at 37°C for 15 minutes

224 to allow conjugate formation and then plated on glass multi-well slides previously coated with

225 0.01% poly-L-lysine for 5 minutes at 37°C. Cells were fixed and permeabilized with cold

226 methanol on ice or fixed at room temperature with 2% paraformaldehyde for 5 minutes, followed

227 by several washes in PBS. Methanol-fixed cells were blocked for 30 minutes at room

228 temperature in 1% BSA in PBS plus Fc block (blocking buffer). PFA-fixed cells were quenched

229 for 10 minutes with 5mM glycine, and permeabilized and blocked with 0.2% saponin in blocking
230 buffer for 30 minutes at room temperature. Cells were incubated with primary antibodies
231 (polyclonal actin and γ-tubulin, Sigma, and polyclonal granzyme B, Abcam) in blocking buffer
232 for 1 hour at room temperature, washed in either blocking buffer or blocking buffer containing
233 0.2% saponin, followed by a 45-minute incubation with secondary antibodies at room
234 temperature, and washed several times. Samples were preserved using ProLong Gold with DAPI
235 (Life Technologies) and no. 1.5 cover glass (VWR), and imaged using an Axio Observer Z1
236 microscope (Carl Zeiss Inc.). For quantification of centrosome and granule polarization,
237 structures in the uropod (or distal 1/3) of T cells were scored as "distal." Structures in the main
238 body (or middle third) of the T cell polarized toward the target cell were scored as "partial," and
239 structures in contact with the plasma membrane at the immunological synapse were scored as
240 "polarized." Granules were scored as "dispersed" when seen localized throughout more than one
241 section of the body of the cell.
242

243 **Transmission electron microscopy (TEM)**

244 WT and $Itk^{-/-}$ OT-I splenocytes that were frozen after three days activation in the presence
245 of $OVA_{257-264}$ as described above, were thawed into medium plus IL-2 and incubated for 48
246 hours to allow for the recovery and expansion of CTLs. CTLs were then either used unlabeled or
247 incubated overnight with horseradish peroxidase (HRP, Boehringer Ingelheim) to label granules,
248 washed and used to generate CTL:target cell conjugates by incubating for 20-60 min with EL-4
249 targets previously pulsed with 1μM, 10nM, or 1nM $OVA_{257-264}$. Conjugated samples were fixed
250 in 1.5% glutaraldehyde/2% paraformaldehyde and processed for TEM analysis, as previously
251 described [31, 32]. Samples were analyzed using a FEI Tecnai G2 Spirit BioTWIN transmission

252 EM (Eindhoven, The Netherlands) and images captured with an Eagle 4K CCD camera using
253 FEI TIA software.
254

255 **Statistical analysis**

256 All statistical analyses (Student's t tests, paired sample tests, and two-way ANOVA)
257 were performed using Microsoft Excel or GraphPad Prism software. P values less than 0.05 were
258 considered statistically significant.

259 **RESULTS**

260 **ITK-deficient CTLs have impaired cytolytic effector function against targets**

261 Previous studies on the effects of ITK-deficiency on murine $CD8^+$ T cell function
262 revealed both decreased and delayed viral clearance, accompanied by decreased CTL expansion
263 *in vivo* and *in vitro* [26, 27]. However, whether there are defects against distinct targets, such as
264 B cells, and whether there are specific defects in the distinct stages of cytolysis on an individual
265 cell basis, have not been examined. To evaluate the effects of ITK on $CD8^+$ T cell cytotoxicity,
266 we used the OT-I TCR transgenic mouse model. T cells from OT-I mice express a clonal TCR
267 that recognizes a peptide, $OVA_{257\text{-}264}$, in the context of $H2K^b$ [28]. This system allowed us to
268 evaluate killing of different targets presenting the same antigen in a controlled environment,
269 using defined numbers of effectors and targets. Furthermore, although ITK-deficient mice show
270 altered thymic development of $CD8^+$ T cells, expression of the OT-I transgene largely rescues
271 these phenotypes [33].

272 To generate effector CTLs, splenocytes from WT and ITK-deficient OT-I mice were
273 stimulated *in vitro* with $OVA_{257\text{-}264}$ peptide for three days, followed by culture in IL-2 to allow
274 expression of cytolytic effectors and acquisition of cytolytic capabilities (Figure 1A). Consistent
275 with their TCR signaling defects, $Itk^{-/-}$ OT-I $CD8^+$ T cells initially exhibited delayed proliferation
276 (Figure 1B), measured by the dilution of the amine reactive dye, Cell Trace Violet (CTV), as
277 well as differences in the initial induction and down-regulation of TCR surface activation
278 markers (Figure 1C). However, by day six, when CTLs are functional to kill, the mean
279 fluorescence intensity of surface markers including Vα2, CD69, CD25, as well as the percentage
280 of $CD62L^+$ cells, were grossly similar between WT and $Itk^{-/-}$ OT-I $CD8^+$ T cells (Figure 1C).
281 Similarly, expression of SLAM family members and other costimulatory molecules such as

282 CD27, which is mutated in another immunodeficiency associated with increased susceptibility to
283 EBV, was similar between WT and ITK-deficient CTLs (data not shown). Therefore, we used
284 day 6-7 CTLs to compare cytolysis between WT and *Itk*$^{-/-}$ cells.

285 CTLs from SAP-deficient patients, who have clinical phenotypes similar to ITK-deficient
286 patients, show defects in cytolysis of EBV-infected B cells but normal killing of other targets
287 [25, 34-36]. To evaluate whether *Itk*$^{-/-}$ OT-I CTLs also showed specific defects in killing B cells,
288 we used three target cell types that were pulsed with 1μM of $OVA_{257-264}$ peptide: LPS-activated
289 B cells from WT C57Bl/6 mice, the EL4 thymoma lymphocyte cell line, and the MC57 non-
290 lymphocyte fibrosarcoma cell line. While *in vitro* activated WT OT-I CTLs could kill WT B
291 cells effectively, CTLs from *Itk*$^{-/-}$ OT-I mice exhibited impaired cytotoxicity against B cell
292 targets (Figure 2A). Defects were confirmed using a flow-based assay where death of targets and
293 CTLs could be individually monitored and were observed at all time points tested, up to 8h of
294 cytolysis (data not shown). Defects were also seen in an *in vivo* transfer model, where activated
295 CTLs from *Itk*$^{-/-}$ OT-I mice had impaired cytotoxicity against co-transferred WT activated B cell
296 targets pulsed with $OVA_{257-264}$ peptide (Supplemental figure 1). However, unlike CTLs from
297 SAP-deficient mice that show defective killing primarily of B cell targets [24], ITK-deficient
298 CTLs also failed to efficiently kill both the peptide-pulsed EL4 T lymphocyte (Figure 2B) and
299 MC57 fibrosarcoma cell lines (Figure 2C). Together these data suggest that unlike SAP-
300 deficiency, ITK–deficiency leads to a global defect in cytolysis by CTLs.

301

302 **ITK-deficient cells show decreased expression of effector molecules**

303 Following activation, $CD8^+$ T cells differentiate into CTLs, which express cytolytic
304 effector molecules critical for the cytolysis of target cells. Notably, expression of several of these

305 effector molecules is dependent on mTOR and AKT-mediated pathways [37, 38]. We have
306 recently found that ITK-deficient $CD4^+$ T cells show impaired activation of mTOR, as evidenced
307 by decreased phosphorylation of ribosomal protein S6, which is phosphorylated by S6 Kinase, a
308 direct target of the mTORC1 complex [13]. Similarly, we observed decreased phosphorylation of
309 S6 during early activation of ITK-deficient $CD8^+$ T cells (Figure 3A). Consistent with these
310 observations, both granzyme B (Figure 3B) and perforin (Figure 3C) were reduced in *in vitro*
311 activated *Itk*$^{-/-}$ OT-I CTLs when compared with WT cells. Thus, ITK-deficiency prevents full
312 expression of cytolytic effectors.
313

314 **ITK-deficiency also results in an intrinsic impairment in degranulation**

315 To determine whether reduced expression of effector molecules was the sole cause of
316 reduced cytotoxicity in the absence of ITK, we treated previously activated WT OT-I CTLs with
317 10n, an inhibitor of ITK, immediately prior to their use. This treatment allowed for short-term
318 inhibition of ITK during cytolysis, while minimizing effects on differentiation. Notably,
319 incubation of WT CTLs with 10n during the cytolysis assay reproduced the defects in killing
320 seen in activated ITK-deficient OT-I CTLs (Figure 3D). Similarly, treatment with 10n of allo-
321 activated human $CD8^+$ T cell blasts generated from healthy peripheral blood mononuclear cell
322 (PBMC) donors also led to impaired killing of P815 targets, a mouse mastocytoma cell line
323 expressing high levels of Fc receptors that can present anti-CD3, making it a target for human
324 $CD8^+$ T cells (Figure 3E). These data suggest that while suboptimal TCR signaling in the
325 absence of ITK affects the kinetics of activation and the expression of cytolytic effectors,
326 impaired killing is not fully attributed to altered activation and differentiation of *Itk*$^{-/-}$ $CD8^+$ T
327 cells.

328

329 **$Itk^{-/-}$ CTLs show normal adhesion and actin ring formation**

330 To better understand the roles of ITK and TCR signaling in regulating cytolytic activity,
331 we examined how ITK-deficiency affects discrete stages of CTL function. Killing by CTLs is
332 initiated when TCR engagement triggers adherence of CTLs to targets. Similar to defects
333 observed in $Itk^{-/-}$ $CD4^+$ T cells [39], freshly isolated $Itk^{-/-}$ OT-I $CD8^+$ T cells showed decreased
334 adhesion to B cell targets in a flow-based adhesion assay (Supplemental figure 2A). However,
335 once activated, $Itk^{-/-}$ OT-I CTLs were able to effectively form conjugates with either peptide-
336 pulsed LPS-activated WT B cell targets (Figure 4A) or EL4 targets (Supplemental figure 2B),
337 when compared with WT CTLs. These results suggest that once CTLs are generated, impaired
338 adhesion is unlikely to contribute to defects in killing by $Itk^{-/-}$ CTLs.

339 Adhesion is accompanied by the accumulation and subsequent centralized clearance of
340 actin at the immunological synapse [3, 4, 31]. Previous work had shown that ITK-deficient $CD4^+$
341 T cells have defects in actin polarization, likely due to a kinase-independent scaffolding role for
342 ITK in stabilizing VAV1-SLP76 interactions during signaling [11]. To examine actin
343 organization in $Itk^{-/-}$ OT-I CTLs, we evaluated actin localization in CTLs by
344 immunofluorescence confocal microscopy (Figure 4B). Actin accumulation at the CTL:target
345 interface appeared normal in $Itk^{-/-}$ OT-I CTLs. Furthermore, the ring-like organization of the
346 actin cytoskeleton, as evaluated in 3-dimensional reconstruction of z-stacks turned *en face*, did
347 not differ between WT and $Itk^{-/-}$ cells in conjugates with peptide-pulsed LPS-activated B cells
348 (Figure 4B and C) or EL4 targets (Supplemental figure 2C). This, in combination with their
349 normal adhesion, suggested that once activated, CTLs do not require ITK for the early stages of
350 their interactions with target cells.

351

352 ***Itk*$^{-/-}$ CTLs exhibit polarized centrosomes and lytic granules during target cell cytolysis**

353 Following immunological synapse formation, the centrosome reorients toward the

354 interface between T and target cells, thus directing lytic granules toward their target for effective

355 killing [3, 4]. Studies suggest that centrosome polarization in T cells requires PLCγ1 activation,

356 but is a DAG signaling-dependent, calcium-independent process [40]. Because ITK directly

357 phosphorylates PLCγ1, which is responsible for DAG production during TCR signaling, we

358 hypothesized that impaired centrosome reorientation could contribute to defects in cytolysis in

359 *Itk*$^{-/-}$ CTLs. To evaluate this question, we co-stained for actin and γ-tubulin as a marker for the

360 centrosome, and examined their localization using confocal immunofluorescence (IF)

361 microscopy in WT and ITK-deficient CTLs (Figure 5A). We found that ITK-deficient CTLs

362 polarized their centrosomes as efficiently as WT in response to peptide-pulsed EL4 targets

363 (Figure 5B). Nonetheless, we found that Ca^{2+} mobilization in response to anti-CD3 stimulation

364 was still markedly reduced in activated CTLs in the absence of ITK (Supplemental figure 2D),

365 confirming that PLCγ1 activation was still impaired in ITK-deficient CTLs [10, 17, 41].

366 The polarization of lytic granules has also been linked to the strength of TCR signaling

367 in the OT-I system, where weak signals generated by low avidity ligands induced centrosome

368 polarization without triggering concomitant lytic granule polarization [32, 42]. These data

369 suggest that reorientation of the centrosome and polarization of lytic granules can be decoupled

370 under suboptimal TCR-triggering conditions. Although the absence of ITK during TCR

371 engagement also results in impaired TCR signaling, we found polarization of lytic granules in

372 *Itk*$^{-/-}$ OT-I CTLs was equivalent to WT CTLs in response to peptide-pulsed EL4 targets, as

373 evaluated by granzyme B staining (Figure 5C and 5D). Thus, once activated CTLs are generated,

374 ITK is not required for centrosome or lytic granule polarization toward targets, despite defective
375 activation of PLCγ1.
376

377 **TCR-triggered degranulation is reduced in the absence of ITK**

378 The final stage of granule-dependent killing of target cells by CTLs is degranulation of
379 the cytotoxic granules, which leads to the killing of target cells. To evaluate degranulation, we
380 used a flow-based secretion assay that measures cycling of lysosomal associated membrane
381 protein 1 (LAMP1) to the cell surface in response to TCR stimulation [30]. Although there was
382 some degree of variability, we found that *Itk*$^{-/-}$ OT-I CTLs exhibited reduced degranulation, as
383 measured by LAMP1 cycling, in response to either plate-bound anti-CD3 (Figure 6A), or
384 activated targets, including both peptide-pulsed LPS-activated WT B cell (Figure 6B) and EL4
385 cell targets (Figure 6C). Importantly, intracellular staining confirmed that total LAMP1 content
386 was equivalent between WT and ITK-deficient CTLs (Supplemental figure 3A), suggesting that
387 reduced degranulation in the absence of ITK was not due to differences in total LAMP1 content
388 between WT and ITK-deficient CTLs.

389 To confirm that impaired degranulation by *Itk*$^{-/-}$ CTLs was due to the loss of ITK activity,
390 we treated previously activated WT OT-I CTLs with increasing concentrations of the ITK-
391 inhibitor, 10n, during degranulation assays. Inhibitor treatment of WT CTLs led to a reduction in
392 degranulation similar to that seen in *Itk*$^{-/-}$ OT-I CTLs (Figure 6D). In contrast, upstream
393 processes such as adhesion were not affected by the ITK inhibitor (Supplemental figure 3B).
394 Furthermore, treatment of allo-activated human CD8^{+} T cells with 10n also led to impaired
395 degranulation (Figure 6E), again supporting a defect in degranulation that was independent of
396 impaired differentiation and expression of lytic effectors. Together, these results suggest that

397 ITK activity plays a previously unappreciated role in degranulation, the final stage of CTL
398 killing, without affecting upstream events.
399

400 **Transmission electron microscopy revealed subtle differences in the immunological**
401 **synapses formed by WT and ITK-deficient CTLs and their targets**

402 To help understand the cellular basis for the reduction in LAMP1 cycling observed in
403 FACS-based secretion assays, we examined the immunological synapse in WT and ITK-
404 deficient CTL:target conjugates using transmission electron microscopy (TEM), which provides
405 more detailed structural information at a higher resolution than can be obtained using
406 immunofluorescence techniques. TEM images revealed that ITK-deficient CTLs could establish
407 normal contact sites with peptide-pulsed EL4 targets, and form secretory clefts indicative of
408 mature immunological synapses (Figure 7A). Consistent with our observations by
409 immunofluorescence microscopy, TEM images showed polarized centrosomes and accumulation
410 of granules at the synapse in both WT and ITK-deficient CTLs, Furthermore, the greater
411 resolution allowed by TEM revealed that, as in WT cells, the polarized centrosomes of ITK-
412 deficient cells moved right up to the cell surface and were associated with organized
413 microtubules under the contact site membrane (Figure 7A), indicating centrosome docking and
414 microtubule network reorganization were also unaffected in these cells.

415 In WT CTLs, the reorganized microtubule network directs granules to secretory sites
416 where they dock and release their contents, resulting in the rapid cytolysis of target cells. TEM
417 images of WT CTLs conjugated to targets showed accumulation of released material in the gaps
418 between the two cells (Figure 7Aa) and swollen ER in target cells (Figure 7Ba), indicative of
419 apoptosis induced by lytic protein release after granule exocytosis. While some dying targets

420 with swollen ER were also present in preparations using ITK-deficient CTLs (Figure 7Bb), most
421 conjugates showed little released material between the cells and were associated with healthy-
422 looking targets, even when ITK-deficient CTLs were incubated with targets for longer time
423 periods (40 minutes or more) (Figure 7Ac and C). *Itk*$^{-/-}$ CTLs associated with non-apoptotic
424 targets still showed docking of centrosomes and accumulation of granules along microtubules
425 around the polarized centrosome (Figure 7Ac and C). However, unlike MUNC13-4-deficient
426 cells, a secretory mutant blocked at exocytosis [43], there was no accumulation of granules
427 trapped along the synapse at docking sites at the membrane. This suggests killing either was
428 delayed or blocked at a post-polarization, pre-granule docking step in these cells.
429

430 **Degranulation and cytotoxicity are restored in ITK-deficient CTLs after prolonged culture**
431 **in IL-2**

432 IL-2 has long been known to enhance lymphocyte cytotoxicity in culture, particularly for
433 natural killer (NK) cells [44]. However, very little is understood about the contribution of IL-2 to
434 CTL degranulation, in part due to the requirement for IL-2 for generation of CTLs. Since IL-2
435 culture restores cytotoxic capabilities in NK cells from patients with primary
436 immunodeficiencies that exhibit defects in lymphocyte degranulation [45], we asked how
437 prolonged exposure to IL-2 affected degranulation and cytolysis in ITK-deficient CTLs.

438 To compare degranulation between cells cultured in IL-2 for different amounts of time,
439 we activated $CD8^+$ OT-I T cells in culture each day for a series of five days, resuspending the
440 cells in fresh media plus IL-2 every 48 hours (Figure 8A). This provided us with CTLs at
441 different time points after primary activation that could be assayed for degranulation on the same
442 day. Under these conditions, cells that had been in culture longer were exposed to an additional

443 round of IL-2 stimulation. We noted that viability in WT OT-I CTLs began to decrease after
444 eight days of culture, from an average of 83.3% on day 7 to 25.8% on day 9, as evaluated by a
445 membrane permeable dye. However, viability of *Itk*$^{-/-}$ OT-I CTLs was less affected over the same
446 time in culture, changing only from 76% on day 7 to 62.8% on day 9 (Supplementary figure 4).
447 Thus, ITK-deficient cells were more resistant to cell death. Prolonged exposure to IL-2 also
448 restored degranulation in viable *Itk*$^{-/-}$ OT-I CTLs, to levels equivalent to those seen in viable WT
449 OT-I CTLs that were stimulated for the same period of time (Figure 8B). Notably, this was not
450 secondary to reduced degranulation in the viable WT cells, but rather to an increase in ITK-
451 deficient cells.

452 To determine whether augmented degranulation in ITK-deficient CTLs during prolonged
453 culture in IL-2 translated into increased cytolysis, we evaluated killing of peptide-pulsed EL4
454 target cells by CTLs. As was observed for degranulation, prolonged incubation with IL-2 rescued
455 the ability of ITK-deficient CTLs to kill targets, as evidenced by both LDH release and flow-
456 based assays which can evaluate cell death specifically in targets (Figure 8C, and data not
457 shown). Together these data suggest that prolonged IL-2 treatment can restore both
458 degranulation and cytolysis in ITK-deficient OT-I CTLs.

459 **DISCUSSION**

460 ITK is an important modulator of TCR signaling, required for maximum PLCγ1
461 activation and calcium signaling in T cells. Previous work has suggested that although *Itk*$^{-/-}$ mice
462 can mount a protective immune response against viral infection, the kinetics of viral clearance
463 were delayed in absence of ITK. This information, coupled with reports that patients with
464 mutations in ITK are particularly susceptible to EBV and other viral infections, led us to ask how
465 ITK-deficiency directly affects the process of killing by CTLs. We found that, unlike cells
466 deficient in SAP that exhibit specific defects in cytolysis of B cells, ITK-deficient cells exhibit
467 global defects in cytolysis. We further found that ITK-deficiency affects both CTL expansion
468 and expression of cytolytic effectors during activation, and leads to an intrinsic defect in
469 degranulation. Nonetheless, these defects could be overcome by prolonged exposure to IL-2 in
470 culture. Our results provide new insight into the effects of ITK and suboptimal TCR signaling on
471 $CD8^+$ T cell function, and how these may contribute to phenotypes associated with ITK-
472 deficiency in humans.

473 Given altered actin accumulation in ITK-deficient $CD4^+$ T cells [11], we were surprised
474 to find that the early stages of CTL killing were normal in the absence of ITK. Indeed, *ex vivo*
475 $CD8^+$ T cells from *Itk*$^{-/-}$ OTI mice exhibited reduced adhesion to target cells, similar to ITK-
476 deficient $CD4^+$ T cells. However, once CTLs were fully activated, ITK-deficiency did not affect
477 adherence or actin recruitment to target cells. This suggests that the cells that expanded during *in*
478 *vitro* activation were now more functional. We speculate that this rescue may be at least partially
479 attributable to the presence of IL-2, which can rescue adhesion and other defects in NK cells
480 from patients with other primary immunodeficiencies (see below) [45-47].

481 Downstream of TCR engagement, the activation of PLCγ1 leads to the hydrolysis of PIP_2
482 to generate two major second messengers: DAG and IP_3. Localized DAG gradients generated by
483 TCR activation serve as a polarizing signal, regulating centrosome reorientation toward target
484 cells by recruiting PKC isozymes [41, 48]. Although ITK is important for the full activation of
485 PLCγ1 in T cells, we found that centrosome docking at the synapse, microtubule reorganization,
486 and lytic granule polarization toward target cells appeared normal in *Itk*$^{-/-}$ CTLs in conjugates
487 with either dying or live targets. Thus, the DAG gradient generated in ITK-deficient CTLs
488 appears to be sufficient to drive cell polarization. Whether ITK-deficiency alters expression or
489 activity of other regulators of DAG, such as DAG kinases, remains an intriguing question.
490 The other major product of PLCγ1, IP_3, triggers store-operated calcium entry into the cell
491 through the action of ER calcium sensors, STIM1 and 2, and the calcium release-activated
492 channel (CRAC), ORAI1, at the plasma membrane. Consistent with impaired PLCγ1 activation,
493 ITK-deficient CTLs show defective Ca^{2+} mobilization. It is therefore of interest that there is an
494 absolute dependence on calcium for lytic granule secretion. Although the precise signals that
495 couple surface receptor signaling to degranulation machinery in CTLs are still not fully
496 understood, CTLs do not degranulate in the presence of EGTA, and cytotoxic lymphocytes from
497 patients with mutations in STIM1 or ORAI1 have defects in secretion, while polarization in these
498 cells remains intact [49-51] (and data not shown). Similarly, *Itk*$^{-/-}$ $CD8^+$ T cells exhibit defects in
499 degranulation, while not affecting earlier stages of CTL polarization. Nonetheless, we note that
500 in our hands, treatment with ionomycin, a calcium ionophore that bypasses TCR signaling to
501 induce calcium flux, could not rescue degranulation in *Itk*$^{-/-}$ CTLs in the presence of TCR
502 stimulation, despite inducing strong Ca^{2+} influx (data not shown). Indeed, although both human
503 CTLs and T cell clones can degranulate in response to ionomycin alone [49, 52], this is less clear

504 for primary murine CTLs. Thus calcium flux may be necessary, but not sufficient, for
505 degranulation in primary murine CTLs, at least under the conditions we have examined.

506 Once granules reach the plasma membrane, microscopy has revealed them to be highly
507 dynamic structures that must dock before fusion and exocytosis can occur [4]. Previous work has
508 suggested that efficient granule docking, defined as the tethering of vesicles to the plasma
509 membrane (reviewed in [53]), is dependent on strength of TCR signaling [32, 42, 54]. Other
510 studies have described distinct morphological phenotypes in CTLs that are unable to kill targets
511 but lack different components of the secretory pathway. For example, cells lacking MUNC13-4
512 accumulate polarized granules trapped at docking sites along the synapse membrane, indicating
513 these cells were blocked at the exocytic event [43, 55]. In contrast, cells lacking RAB27a
514 accumulate granules around the centrosome and along microtubules, suggesting a failure to
515 dissociate from the microtubule network and/or dock at the plasma membrane [56]. The TEM
516 studies presented here indicate that, like *Rab27a*$^{-/-}$ cells, ITK-deficient CTLs bound to non-
517 apoptotic targets accumulate granules on microtubules around the polarized centrosome but do
518 not show evidence of docked granules along the synaptic membrane. While expression of
519 *Rab27a,* as well as *Unc13d, Stx11, Syt7, Snap23*, which all encode regulators of docking during
520 degranulation, was equivalent in WT and ITK-deficient CTLs (data not shown), we cannot rule
521 out impaired levels or localization of these proteins, nor defective secondary modifications, due
522 to the limited availability of good antibodies. Moreover, it should be noted that since exocytosis
523 and docking occur rapidly in WT CTLs, cells blocked at the pre-docking step can appear
524 morphologically similar to normal cells and may only be identified by lack of target death. Thus,
525 it is not possible to distinguish definitively whether the apparent lack of target death in individual
526 conjugates with ITK-deficient CTLs is because killing is indeed blocked or just taking place

527 more slowly. Nonetheless, together with the decrease in target killing observed, these high-
528 resolution images provide clues as to how the absence of ITK may affect the process of
529 degranulation, and raise the possibility that there is a delay or block in the transfer of granules
530 from the microtubule network to the membrane and/or in granule docking itself.

531 Although our inhibitor data suggests that ITK plays a role in TCR-triggered
532 degranulation, intrinsic defects in degranulation are not the only problem in *Itk*$^{-/-}$ CTLs. We find
533 that ITK-deficient cells do not proliferate as well as WT CD8^{+} cells, consistent with previous
534 studies [27], and show reduced expression of the downstream effectors, granzyme B and
535 perforin. Previous work has shown that through the HIF1α pathway, mTORC1 signaling controls
536 a diverse transcriptional program including the expression of cytolytic effectors in T cells [37].
537 Consistent with these findings, we show here that ITK-deficient CD8^{+} T cells have altered
538 mTORC1 signaling at early time points during activation. Together, these results suggest that
539 ITK-deficiency in CD8^{+} T cells has two consequences. First, when naïve CD8^{+} T cells in the
540 periphery encounter antigen, a suboptimal TCR signal in the absence of ITK generates a reduced
541 population of CTLs that express less granzyme B, perforin and perhaps other effectors. Upon
542 second antigen encounter, this population of sub-optimally activated CTLs is less capable of
543 degranulating efficiently in the absence of ITK. In this scenario, the lytic granules that do
544 undergo fusion may contain less granzyme B and perforin, thus making ITK-deficient CTLs poor
545 cytotoxic lymphocytes that are less effective at clearing virally infected targets at early time
546 points during infection.

547 However, we further show that prolonged culture of ITK-deficient CTLs in IL-2 rescued
548 degranulation and cytolysis of target cells. The loss of ITK in CTLs is reminiscent of many
549 phenotypes associated with altered peptide or reduced TCR signaling. It is therefore possible that

550 in our system, IL-2 provides a synergizing signal that enables complete activation of CTLs in the
551 absence of optimal TCR signaling. How IL-2 exerts these effects remains an important question.
552 Expression of surface markers in ITK-deficient CTLs improved after addition of IL-2 on day 3
553 of culture, suggesting direct effects on transcription. Likewise, the expression of granzyme B
554 also improves after prolonged culture (>8 days) of ITK-deficient CTLs with IL-2 (data not
555 shown). Alternatively, there may also be other non-transcriptional effects of IL-2. For example,
556 defective NK cytotoxicity in the absence of Wiskott-Aldrich Syndrome protein (WASp)
557 improves with IL-2. Intriguingly, IL-2 stimulation of NK cells *in vitro* led to increased
558 phosphorylation of the WASp homolog WAVE2, [45], suggesting that IL-2 directly affects
559 activation of signaling intermediates. It is interesting to speculate that such IL-2-mediated effects
560 may contribute to the eventual clearance of viral infections in ITK-deficient mice. Furthermore,
561 although not the focus of this work, prolonged culture of ITK-deficient CTLs in IL-2 also
562 revealed that they are less susceptible to death. This is consistent with previous reports that ITK-
563 deficient $CD4^+$ cells are resistant to apoptosis under certain conditions [57]. Whether impaired
564 cell death contributes to the lymphoproliferative syndrome associated with ITK-deficiency in
565 patients is unknown, but it is interesting to note that cells from XLP-1 patients show a defect in
566 restimulation-induced cell death that is thought to contribute to the lymphoproliferation in this
567 disorder [58]. Whether there are other defects in human $CD8^+$ T cells deficient in ITK that make
568 patients particularly susceptible to EBV remains an important question.
569 Overall, this work demonstrates two significant effects of ITK-deficiency on CTL
570 function: first, decreased expansion and impaired expression of effectors and second, a
571 potentially novel role for ITK in regulating degranulation in CTLs without affecting upstream
572 processes such as adhesion or cell polarization. Importantly, we also offer additional evidence

573 for the role of IL-2 in integrating TCR and costimulatory signaling pathways for the generation

574 of fully functional CTL responses. Together, this work provides insight into the defects that may

575 account in part for the particular susceptibility to viral infections observed in patients with

576 mutations in ITK and other TCR signaling components.

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779

780 **FIGURE LEGENDS**

781 Figure 1: ITK-deficient CTLs express similar surface marker levels after activation. (A)
782 Schematic of activation of OT-I splenocytes. (B) Proliferation of $CD8^+$ T cells in total *ex vivo*
783 splenocytes cultures from WT or ITK-deficient OT-I mice stimulated in the presence of 10nM
784 $OVA_{257-264}$ peptide, evaluated with Cell Trace Violet (CTV). Histogram overlay depicts WT
785 (black) or *Itk*$^{-/-}$ (grey) $CD8^+$ T cells examined directly *ex vivo* (dotted lines) or after 48 hours of
786 culture (solid lines). (C) Representative histograms of surface marker staining on WT (black) or
787 *Itk*$^{-/-}$ (grey) OT-I $CD8^+$ T cells at indicated time points during *in vitro* activation. IL-2 was added
788 after 72 hours of culture. Data are representative of one of greater than three independent
789 experiments.

790

791 Figure 2: ITK-deficient CTLs have impaired cytolytic function against targets. *In vitro* cytolysis
792 of (A) LPS-activated WT B cells, (B) EL4, or (C) MC57 targets pulsed with 1μM $OVA_{257-264}$
793 peptide by WT OT-I (black) or *Itk*$^{-/-}$ OT-I (grey) CTLs at decreasing CTL:target ratios, as
794 measured by LDH-release assays. Data for LPS-activated B cells are representative of one of
795 greater than three independent experiments, EL4 targets are representative of greater than ten
796 experiments, and MC57 targets are representative of one of two experiments. Graphs show mean
797 of triplicate wells ± SD. Similar results were obtained with a flow-based cytolysis assay.

798

799 Figure 3: ITK-deficient CTLs show decreased both expression of lytic effector molecules, and
800 additional defects in cytolysis. (A) Whole splenocytes from WT (black) or *Itk*$^{-/-}$ (grey) OT-I
801 $CD8^+$ T cells were activated for six hours in the presence of 10nM $OVA_{257-264}$ peptide, stained
802 and analyzed for pS6 (S235/236) using flow cytometry. Histogram is representative of three

803 independent experiments. (B) Granzyme B expression levels measured by flow cytometry in day
804 7 *in vitro* activated WT (black) or *Itk*$^{-/-}$ (grey) CTLs. Histogram is representative of greater than
805 three independent experiments. (C) *Prf* mRNA transcript levels in day 7 *in vitro* activated WT
806 (black) or *Itk*$^{-/-}$ (grey), determined by qRT-PCR. Data are representative of two independent
807 experiments. (D) *In vitro* cytolysis of EL4 targets pulsed with 1μM $OVA_{257-264}$ peptide by
808 previously activated ITK-deficient (grey) or WT OTI CTLs treated with indicated concentrations
809 of the ITK inhibitor, 10n (red, green, and blue) immediately before cytolysis assay, or left
810 untreated as a control (black). Graph shows mean of triplicates ± SD, and is representative of
811 three independent experiments. (E) *In vitro* cytolysis of P815 target cells pulsed with 2μg/mL
812 OKT3 by allo-reactive human $CD8^+$ T cells generated from two independent healthy donors and
813 treated with 10n immediately before cytolysis assay. Graph shows mean of triplicates ± SD at 4-
814 hour time points using 20:1 CD8:target cell ratio. Data representative of two experiments using
815 cells from two healthy donors each.
816
817 Figure 4: *Itk*$^{-/-}$ CTLs have normal adhesion and actin ring formation during immunological
818 synapse formation. (A) Adhesion after 20 minutes conjugation of previously activated WT
819 (black) or *Itk*$^{-/-}$ (grey) CTLs to LPS-activated WT B cells pulsed with 1μM $OVA_{257-264}$ or
820 unpulsed B cells as a control. Graph represents mean ± SD of percent $CD8^+$ $target^+$ cells in total
821 $CD8^+$ events. Data are representative of one of greater than three experiments. (B)
822 Representative images of maximum projections of WT or *Itk*$^{-/-}$ CTLs in conjugate pairs with
823 LPS-activated WT B cell targets pulsed with 1μM $OVA_{257-264}$ (first and third columns,
824 respectively). 1μm slice of reconstructed z stacks rotated in the yz plane (second and fourth
825 columns). Nuclei (blue), CD8 (red), actin (green). Scale bars = 5μm. (C) Quantification of actin

826 ring formation at the immunological synapse between WT (black) and *Itk*$^{-/-}$ (grey) CTLs and
827 LPS-activated WT B cell targets. Bars represent mean ± SEM from more than three independent
828 experiments (WT total n=105, *Itk*$^{-/-}$ total n=82).
829
830 Figure 5: *Itk*$^{-/-}$ CTLs show normal centrosome and lytic granule polarization during cytolysis of
831 targets. (A) Centrosome reorientation shown as maximum projections. Nuclei (blue), EL4 targets
832 (red), actin (green), γ-tubulin (white). Centrosome location highlighted by yellow arrowheads.
833 Scale bars = 5μm. (B) Quantification of centrosome reorientation in WT (black, total n=129) and
834 *Itk*$^{-/-}$ (grey, total n=128) CTLs. (C) Lytic granule polarization shown as maximum projections.
835 Nuclei (blue), EL4 targets (red), actin (green), granzyme B (white). Lytic granule location
836 highlighted by yellow arrowheads. Scale bars = 5μm. (D) Quantification of lytic granule
837 polarization in WT (total n=127) and *Itk*$^{-/-}$ (total n=151) CTLs in conjugates with EL4 targets
838 pulsed with 1μM $OVA_{257-264}$ peptide. Bars in panels B and D represent mean ± SEM from
839 greater than three independent experiments.
840
841 Figure 6: ITK-deficient CTLs exhibit reduced TCR-triggered degranulation. Degranulation
842 measured in a flow-based LAMP1 cycling assay in WT (black) or *Itk*$^{-/-}$ (grey) CTLs in response
843 to (A) plate-bound anti-CD3, 1μM $OVA_{257-264}$-pulsed (B) LPS-activated B cell or (C) EL4
844 targets. Each line represents paired mice from an independent LAMP1 cycling experiment.
845 *P<0.05, **P<0.01 calculated by paired sample t-tests. (D) Degranulation in response to 0 or
846 5μg/mL plate-bound anti-CD3 in WT OT-I CTLs (black), WT OT-I CTLs pre-treated with the
847 ITK inhibitor, 10n, at 0.03 μM (blue), 0.1 μM (green), 0.2 μM (yellow) or 0.3 μM (red), or *Itk*$^{-/-}$
848 OT-I CTLs (grey). Graph representative of two independent experiments. (E) Degranulation in

allo-reactive human CD8+ T cells from two healthy donors treated with increasing concentrations of 10n in response to 5μg/mL of plate-bound OKT3. Graph represents data using the same donors as in Figure 3E and is representative of one of two independent experiments.

Figure 7. The centrosome and granules polarize to the synapse in *Itk*$^{-/-}$ CTL conjugated to targets. TEM images of the immune synapse region of WT and *Itk*$^{-/-}$ CTL without (Aa-b) or with (Ac; B-C) pre-incubation of HRP overnight to load granules, and conjugated to targets for 20 (Aa-b) 40 (Ac and Cb) or 60 (B and Ca) minutes in the presence of $OVA_{257-264}$ peptide. Granules are identified by white asterisks (Aa-b) or as electron dense structures (Ac and B-C). (A) Top: thin section images showing tight polarization of centrioles and microtubule networks are organized from the membrane at the contact site in both WT and *Itk*$^{-/-}$ cells. The mother centriole barrel is just outside the plane of the membrane in these images to show the ends of the microtubule-organizing sub-distal appendages, which form the focus of microtubule polarization at the membrane (black asterisk). Granules (white asterisks, a-b; electron-dense structures, c) lie on microtubules aligned from the membrane (e.g. white arrowheads, c). Bottom: higher power images of the centrosome-docking region shown in the top images. Debris is seen in the synaptic cleft between WT CTL and its target. (B) Semi-thick (200-300nm) sections showing an example of WT and *Itk*$^{-/-}$ CTLs with polarized electron-dense HRP-loaded granules conjugated to apoptotic target cells, indicated by vacuolation of the target cell ER. The WT image (a) transects the contact site in the plane of the polarized centrosome barrel (black asterisk). (C) Thin (50-70nm) section images showing profiles of electron dense HRP-loaded granules polarized to the contact site of *Itk*$^{-/-}$ CTLs in conjugates where the target cells appear non-apoptotic and healthy.

871 The centrosome (black asterisk) is slightly retracted back from the membrane with granules

872 aligned along the retracted microtubules in (a). G=Golgi. N=nucleus. Scale bars=500nm.

873

874 Figure 8: Degranulation and cytotoxicity are restored in ITK-deficient CTLs after prolonged

875 culture in IL-2. (A) Schematic of $CD8^+$ T cell activation and IL-2 addition. (B) Percent of WT

876 (black) or $Itk^{-/-}$ (grey) CTLs cycling LAMP1 in response to 5μg/mL of plate-bound anti-CD3

877 evaluated at indicated days after the start of primary activation. (C) *In vitro* cytolysis of EL4

878 targets pulsed with 1μM $OVA_{257-264}$ peptide by WT OT-I (black) or $Itk^{-/-}$ OT-I (grey) CTLs at a

879 20:1 effector:target ratio, evaluated at indicated days after primary activation. Graphs show

880 percent cytotoxicity ± SD at 4-hour time points and are representative of three independent

881 experiments. *P<0.05, **P<0.01, ***P<0.001 calculated by Student's T-test.