## Title
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# Hedgehog signaling controls T-cell killing at the immunological synapse

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## Abstract

The centrosome is essential for cytotoxic T lymphocyte (CTL) function, contacting the plasma membrane and directing cytotoxic granules for secretion at the immunological synapse. Centrosome docking at the plasma membrane also occurs during cilia formation. The primary cilium, formed in non-haemopoietic cells, is essential for vertebrate Hedgehog (Hh) signaling. Lymphocytes do not form primary cilia, but here we found that Hh signaling played an important role in CTL killing. TCR activation, which “pre-arms” CTL with cytotoxic granules, also initiated Hh signaling. Hh pathway activation occurred intracellularly and triggered Rac1 synthesis. These events “pre-armed” CTL for action by promoting the actin remodelling required for centrosome polarisation and granule release. Thus Hh signaling plays a role in CTL, and the immunological synapse may represent a modified cilium.

Cytotoxic T lymphocytes (CTL) recognise tumor and virally infected cells via their T cell receptor (TCR). Recognition triggers a cascade of intracellular signaling that leads to the formation of the immunological synapse and polarisation of the centrosome to contact the plasma membrane (1) at the central supramolecular activation complex (cSMAC) (2) where TCR clusters within the synapse (1, 3). Cytotoxic granules move towards the docked centrosome and deliver their contents precisely at the point of TCR-mediated recognition, focussing secretion towards the target cell to be destroyed. Docking of the centrosome also occurs during cilia formation, when the mother centriole contacts the plasma membrane, forming the basal body from which the cilium extends. Although lymphocytes are one of very few cell types that do not form primary cilia (4) morphological and functional similarities can be drawn between the immunological synapse and cilia. Endocytosis and exocytosis are focussed at the point of centrosome docking in both cases (5); ciliary intraflagellar transport (IFT) proteins are found in T cells (6), and both structures form important signaling platforms (1, 2, 7, 8).

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In Hh signaling, binding of exogenous Sonic, Indian or Desert Hh (Shh, Ihh, Dhh) to the transmembrane receptor Patched (Ptch) regulates translocation of Smoothened (Smo) to primary cilia (9, 10). The ciliary localisation of Smo is required to initiate transduction of Gli-mediated transcription of target genes, including Gli1, which serves as a reporter of Hh signaling (7, 8). We asked whether proteins of the Hh pathway are expressed in T cells and whether TCR activation triggered Hh signaling. Naïve CD8 T cells and CTL derived after 4-5 days of in vitro TCR activation were isolated from OT-I TCR transgenic mice. TCR cross-linking was triggered in both naïve CD8 T cell and CTL populations using plate-bound anti-CD3 antibody (Fig.1A). In naïve CD8 T cells *Gli1* mRNA was not detected, but expression was induced upon TCR cross-linking, peaking at 12h. Controls lacking anti-CD3 showed no *Gli1* expression. CTL had low *Gli1* mRNA levels that increased 180-fold after TCR ligation (Fig. 1A). In addition the genes encoding Ptch1 and 2 receptors the signal transducer Smo, and the ligand Ihh were all expressed in both naïve CD8 and CTL (Figure S1A), and protein expression of Ptch, Gli1 and Ihh increased after TCR activation of naïve CD8 cells (Fig. 1B) and CTL (Figure S1B). Neither *Shh* nor *Dhh* were detected in CD8 T cells before or after 24h TCR activation, or EL4 and P815 target cell lines, (Fig. S1C). When TCR signaling was severely impaired by deletion of the upstream tyrosine kinase Lck (11), induction of *Gli1* was also diminished in naïve CD8 T cells (Fig. 1C,D). Thus CD8 T cells express Hh pathway components and require TCR signaling to trigger Hh signaling.

Because only T cells were present in these assays, CD8 T cells must both have synthesised and responded to Hh proteins to activate this signaling pathway. This is unusual as Hh signaling is usually paracrine, with one cell type producing Hh and another responding to this cue. We noted that Ihh was detected as a 45kDa protein, indicating that it was not fully processed into the secreted form (12, 13). This raised the possibility that Ihh might bind Ptch intracellularly. We used recombinant Ihh protein to ask whether CTL responded to exogenous Ihh. Although cross-linking of TCR triggered *Gli1* expression, stimulating CTL with extracellular Ihh alone did not. Furthermore exogenous Ihh did not enhance *Gli1* expression in response to TCR activation (Fig. S1E). Thus Ihh encounters its receptor, Ptch, intracellularly in CTL.

We next asked where Ptch, Ihh and Smo localise in CTL using antibodies to detect endogenous Ihh and endogenous Smo, and Ptch1-YFP to localise the receptor (Fig. 2). In CTL, Ptch1 was found on intracellular vesicles (Fig.2A). Ihh co-localised with Ptch1 on a subset of these vesicles, which polarised towards the immunological synapse upon recognition of a target cell (Fig. 2B, C). This is consistent with the idea that Ihh-mediated signaling via Ptch1 takes place intracellularly, and we confirmed the interaction between Ihh and Ptch1 on intracellular vesicles using a proximity ligation assay (Fig. S2A). Ihh was also seen near sites of actin accumulation (Fig. S2B,C), raising the possibility that Ihh may also influence actin as shown for other Hh components (14, 15). Endogenous Smo was also associated with intracellular vesicles in CTL (Fig. 2D) that are predominantly Lamp1-positive (Fig. S2D) and distinct from the Ihh-positive compartment (Figure S2E). Live imaging of Smo-EGFP expressed in CTL showed that, upon recognition of a target cell via TCR, the intracellular pool of Smo polarised to the immunological synapse (Fig. 2E and Movie 1), analogous to the Hh-triggered translocation of Smo into the cilium (9, 10, 16, 17).

To determine whether TCR-triggered Hh signaling affects CTL-mediated killing, we made use of a genetic model in which *Smo* is conditionally deleted. Although Hh signaling is required for T cell development (18-21), T cells from mice, in which exon 1 of *Smo* is inducibly deleted in adult haemopoietic cells under Mx1-Cre control, develop normally (22, 23). CTL generated from *Smo*-deleted mice showed over 65-fold reduced levels of *Smo* mRNA relative to controls and greatly reduced Smo protein levels, (Fig. 3A, B). *Gli1* mRNA upregulation in response to TCR ligation was also reduced: CTL from control mice showed a 3-fold increase in levels of *Gli1* mRNA, whereas CTL from *Smo*-deleted mice gave only a 1.5-fold increase (Fig. 3A). TCR signaling was not altered in *Smo*-deleted CTL (Fig. 3C and S3). However when we assessed *Smo*-deleted CTL for their cytotoxic effector function we found reduced levels of target cell killing compared to control CTL (Fig. 3D) suggesting that Hh signaling contributes to CTL killing. Because centrosome polarisation to the plasma membrane is a key step in CTL mediated killing, we asked whether Hh signaling affected centrosome docking at the cSMAC during conjugate formation between CTL and target cells. *Smo*-deleted CTL showed a ~50% reduction in centrosomal docking at the cSMAC (Fig. 3E) consistent with the reduction in Smo protein levels and CTL killing.

We also confirmed that Hh signaling is important for centrosome polarisation and CTL-mediated killing using three separate inhibitors: cyclopamine and vismodegib (GDC-0449) both of which inhibit Smo (24, 25) and GANT61 which targets Gli transcription factors (26). The inhibitors reduced levels of Gli1 and Ptch protein, both targets of the Hh signaling pathway, but did not affect levels of TCR-associated kinases Lck and ERK or granzyme A and perforin, two CTL proteins required for target cell lysis (Fig. 3F, S4A). TCR signaling was also unaffected by these inhibitors (Fig. 3G, S4B). All three inhibitors diminished CTL-mediated killing in a dose-dependent manner (Fig. 3H, S4C), without impairing conjugate formation with target cells or clustering of Lck at the cSMAC in response to TCR signaling (Fig. S4E, F). Centrosome docking at the cSMAC was also reduced in conjugates formed by CTL treated with inhibitor (Fig. 3I).

Centrosome polarisation has been correlated with actin remodelling at the immunological synapse (1, 3). Carrier treated CTL reorganised actin into a distal ring at the immunological synapse, and polarised the centrosome within 5 minutes of encountering the target (Fig. S5A and Movie S2). By contrast in cyclopamine-treated CTL, actin accumulated across the immunological synapse (t=0), but failed to reorganise into the distal actin ring (Fig. S5B). Centrosome polarisation to the plasma membrane was also disrupted (Movie S3): 92% of conjugates polarised the centrosome to the synapse in control carrier-treated CTL compared with 59% of conjugates in cyclopamine-treated CTL. Actin clearance from the immunological synapse was also greatly reduced in CTL from *Smo*-deleted mice (Fig. 4A-C), and *Smo*-deleted CTL showed a 60% reduction in actin clearance from the synapse compared with control CTL. Thus Hh signaling might also be required to promote actin reorganisation at the immunological synapse.

Centrosome polarisation in T cells is driven by a process of microtubule end-on capture-shrinkage, in which microtubules emanating from the centrosome are captured at the cortex and then undergo shrinkage. Both this process and actin remodelling are mediated by Rac1 (27-30). Furthermore the Hh pathways has been implicated in Rac1-mediated actin

remodelling in neurons and fibroblasts (14, 15). Therefore we examined Rac1 expression during induction of CTL from naïve T cells. Rac1 protein levels increased after TCR stimulation of naïve T cells (Fig. 4D). By contrast both protein and mRNA levels of Rac1 were diminished in *Smo*-deleted CTL (Fig. 4E,F), suggesting that Rac1 levels were regulated by Hh signaling via a transcriptional effect.

These findings support a model for the regulation of CTL-mediated killing by Hh signaling (Fig. S5C). Naive T cells are small, round cells lacking both the cytotoxic granules and the highly developed cytoskeleton required for target cell killing. Upon TCR activation these cells develop over 4-5 days into mature CTL pre-armed with cytotoxic granules. We now show that TCR activation also triggers Hh signaling during this time which increases levels of Rac1 and thereby promotes centrosome polarization, actin remodelling, granule release and target cell killing. In this way Hh signaling pre-arms CTL with the ability to rapidly polarise the cytoskeleton and deliver the cytotoxic granules within minutes when the CTL encounters a target. Our results reveal molecular parallels between primary cilia and the immunological synapse, highlighting the possible origin of the immunological synapse as a modified cilium.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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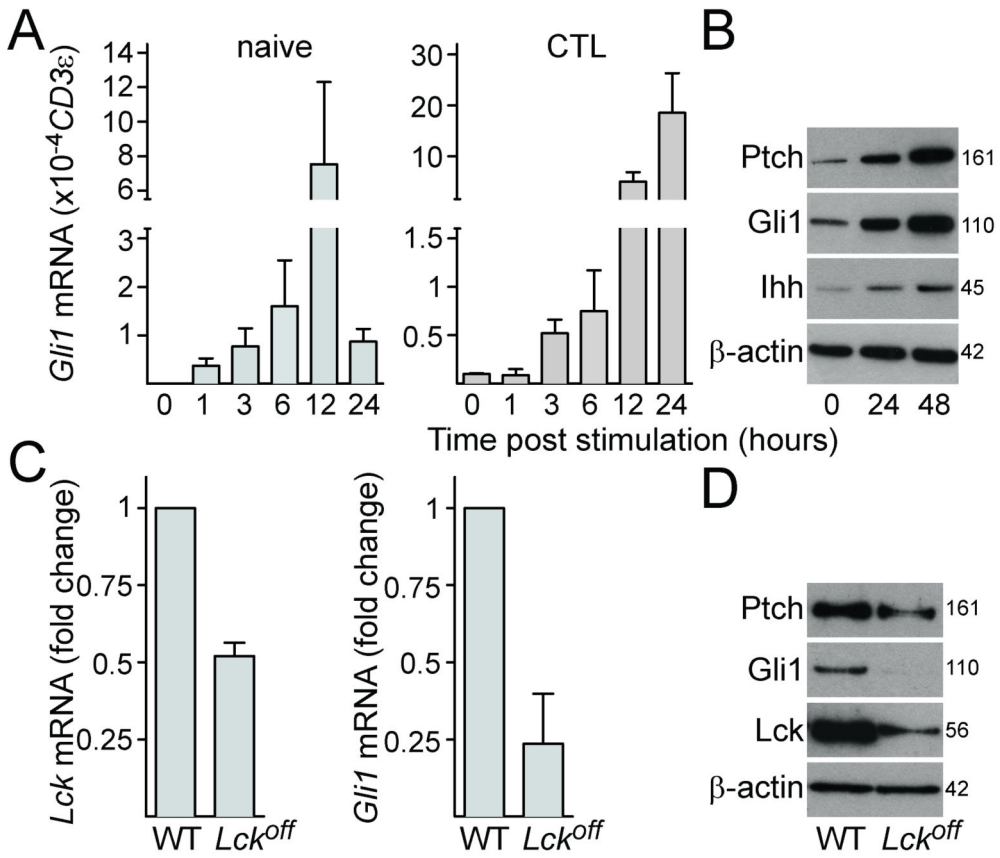


**Fig. 1. TCR activation triggers Hh signaling and expression of Hh components in CD8 T cells**

**(A)** Graphs showing mRNA levels of *Gli1* in naïve CD8 T cells (left) and CTL (right) at times shown after TCR cross-linking with plate-bound anti-CD3 antibody relative to *CD3ε* as a reference gene. Similar results were obtained using *TBP* as a reference gene; n=3 (naïve) or 2 (CTL); data are mean +/− SD. Cells plated without anti-CD3 showed no *Gli1* induction over 12h. **(B)** Immunoblot analysis of protein expression of Ptch, Gli1, Ihh and actin at 0, 24 and 48h after TCR stimulation in naïve CD8 T cells; n=3. Molecular weights are shown in kDa. Similar results were also obtained from CD8 T cells derived from C57BL/6 and BALB/c mice (not shown). **(C** and **D)** Naïve CD8 T cells were purified from spleens of WT or Lck$^{off}$ mice and stimulated for 12h with plate-bound anti-CD3 antibody. **(C)** Graphs showing mRNA levels of *Lck* (left) and *Gli1* (right) in Lck$^{off}$ CD8 T cells relative to WT control; n=2, data are mean +/− SD. **(D)** Immunoblot analysis of Ptch, Gli1, Lck and actin in Lck$^{off}$ and WT control CD8 T cells after 12h of TCR stimulation; n=3. Molecular weights are shown in kDa.

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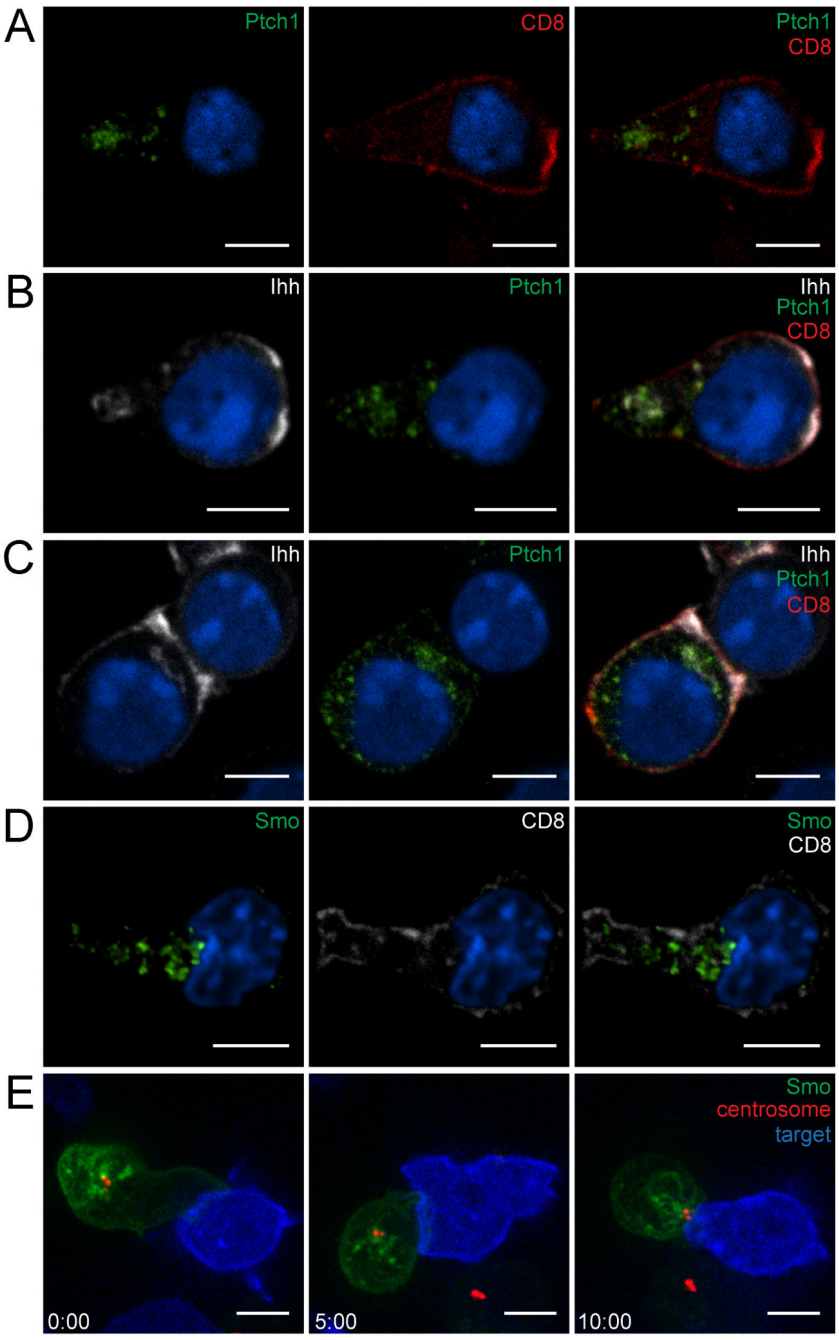


**Fig. 2. Ihh, Ptch1 and Smo are localised on intracellular vesicles that polarise towards the immunological synapse**

OT-I CTL transduced with Ptch1-YFP and labelled with antibodies to YFP (green), CD8 (red) and Ihh (white). **(A, B)** show OT-1 CTL alone and (C) conjugated with EL4 target cells. **(D)** Endogenous Smo (green) and CD8 (white) in OT-I CTL. Single *x-y* confocal sections are shown. Nuclei are stained with Hoechst (blue). (A:n>75;B,C:n>35;D:n>85. 2-4 independent experiments each). **(E)** Individual frames of a movie (Suppl. Movie 1) showing OT-I CTL nucleofected with Smo-EGFP and PACT-RFP (centrosome marker, red), forming

a synapse with EL4 target cells (blue). Time after initial contact is shown in minutes (n=33). Scale bars: 5μm.



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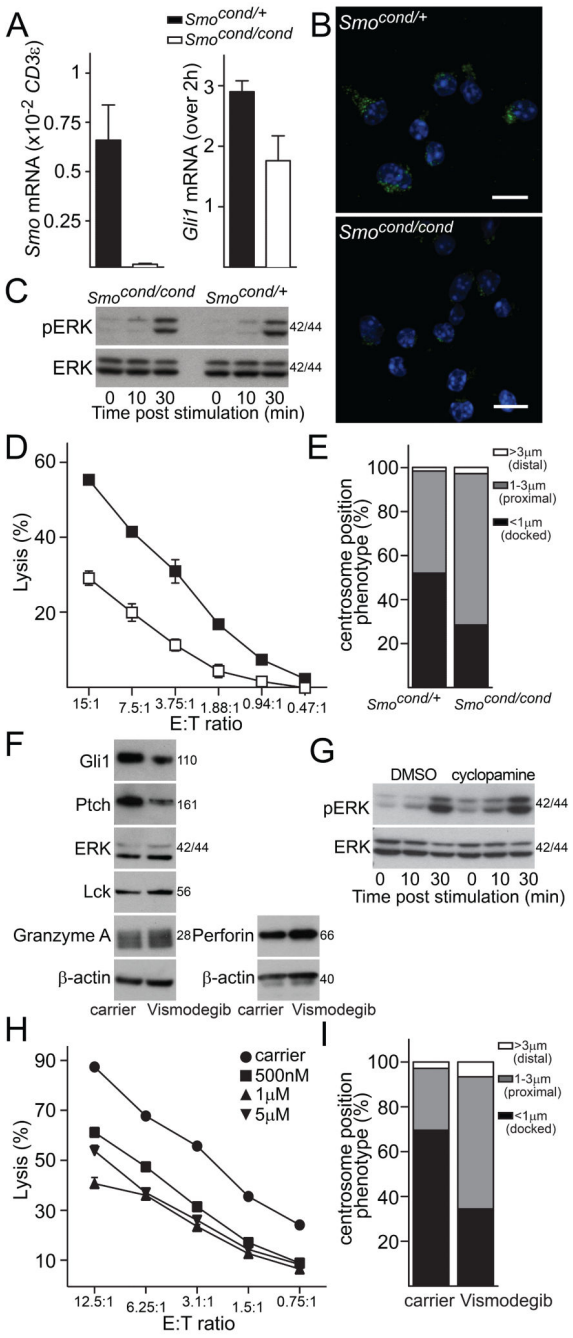


**Fig. 3. Hh signaling is required for CTL killing and centrosome polarisation to the immunological synapse**

**(A)** qPCR analysis of *Smo* mRNA expression in *Mx1-Cre* $Smo^{cond/cond}$ and control *Mx1-Cre* $Smo^{cond/+}$ CTL relative to *CD3ε* as a reference gene (left); n=4, data are mean +/− SD. qPCR analysis showing increase in *Gli1* mRNA levels 2h after TCR activation with plate-bound anti-CD3 antibody for *Mx1-Cre* $Smo^{cond/+}$ and *Mx1-Cre* $Smo^{cond/cond}$ CTL relative to unstimulated (right); n=3, data are mean +/− SD. **(B)** Representative staining of endogenous Smo (green) expression in *Mx1-Cre* $Smo^{cond/+}$ and *Mx1-Cre* $Smo^{cond/cond}$ CTL. Nuclei

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stained with Hoechst (blue); n>100. Scale bars: 5μm. **(C)** *Mx1-Cre Smo*$^{cond/+}$ and *Mx1-Cre Smo*$^{cond/cond}$ CTL were stimulated with plate-bound anti-CD3 antibody for times indicated and blotted for protein expression of pERK and ERK; n=2. Molecular weights are shown in kDa. **(D)** Percentage lysis of P815 target cells by *Mx1-Cre Smo*$^{cond/+}$ and *Mx1-Cre Smo*$^{cond/cond}$ CTL at effector:target (E:T) ratios shown (n=6). **(E)** Centrosome position relative to clustered Lck was classified as <1μm (docked), 1-3μm (proximal) or >3μm (distal) as percentage of conjugates of *Mx1-Cre Smo*$^{cond/+}$ (n=129) and *Mx1-Cre Smo*$^{cond/cond}$ (n=121) with P815 targets as depicted in Figure S4 (n=3), data are mean +/− SD. **(F)** Immunoblots of cell lysates from OT-I CTL treated with 5μM vismodegib for 36h, probed with antibodies against Gli1, Ptch, ERK, Lck, granzyme A, perforin and actin; n=2. **(G)** OT-I CTL treated with 10μM cyclopamine for 24h, stimulated with plate-bound anti-CD3 antibody for times indicated and blotted for protein expression of pERK and ERK; n=2. Molecular weights are shown in kDa. **(H)** Percentage lysis of EL4 target cells by OT-I CTL treated with vismodegib at concentrations stated; n=5. x-axis shows varying CTL effector to target (E:T) ratios. **(I)** OT-I CTL treated with vismodegib (5μM) were labelled with antibodies against Lck, γ-tubulin, and CD8. Quantitation of centrosome polarisation after treatment is shown (n>60).

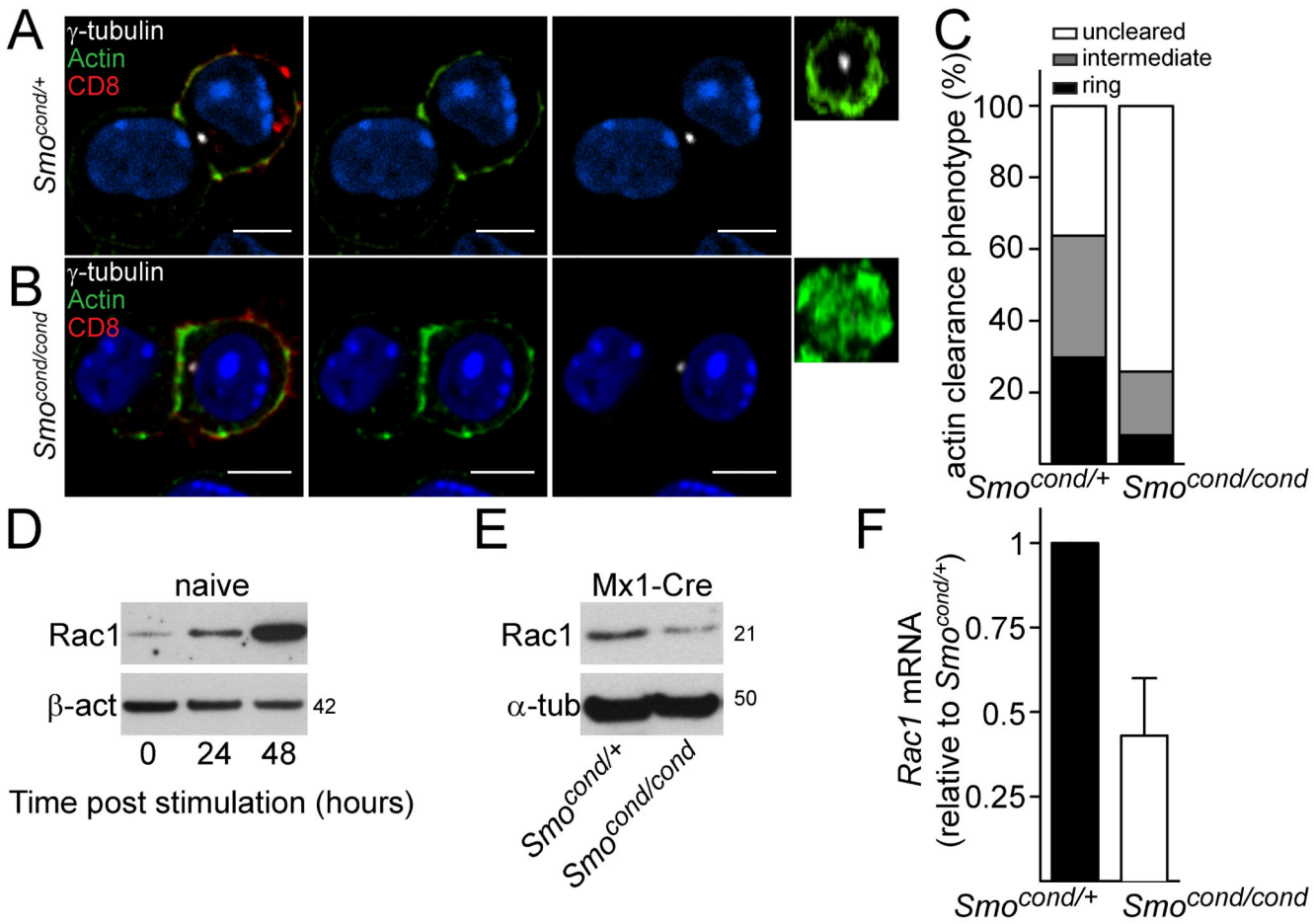


**Fig. 4. Hh signaling in CTL controls Rac1 expression and actin reorganisation at the immunological synapse**

**(A-C)** *Mx1-Cre Smo*$^{cond/+}$(A) and *Mx1-Cre Smo*$^{cond/cond}$ CTL (B) were conjugated to P815 target cells for 15min, fixed and stained using antibodies against CD8, γ-tubulin, and actin. Single *x-y* confocal sections and *en face (y-z)* constructions through the synapse are shown, demonstrating that the actin ring does not form properly in *Mx1-Cre Smo*$^{cond/cond}$ CTL. Nuclei stained with Hoechst (blue). Scale bars: 5μm. **(C)** Quantitation of actin clearance at the immunological synapse, depicting the percentage of CTL in which actin remains distributed throughout the synapse (not cleared), show an intermediate phenotype or is cleared to form an actin ring (*Smo*$^{cond/+}$n=47; *Mx1-Cre Smo*$^{cond/cond}$*n*=62). Immunoblot analyses of protein expression of Rac1, actin and calnexin **(D)** at 0, 24 and 48h after TCR stimulation in naïve CD8 T cells (n=3) and **(E)** *Mx1-Cre Smo*$^{cond/+}$ and *Mx1-Cre Smo*$^{cond/cond}$ CTL (n=2). Molecular weights are shown in kDa. **(F)** Graph showing mRNA levels of *Rac1* in *Mx1-Cre Smo*$^{cond/cond}$ CTL relative to *Mx1-Cre Smo*$^{cond/+}$ control CTL n=3, data are mean +/− SD.




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