Asymmetric Cell Division of T Cells upon Antigen Presentation Uses Multiple Conserved Mechanisms


*J Immunol* published online 7 June 2010
http://www.jimmunol.org/content/early/2010/06/07/jimmunol.0903627
Asymmetric cell division is a potential means by which cell fate choices during an immune response are orchestrated. Defining the molecular mechanisms that underlie asymmetric division of T cells is paramount for determining the role of this process in the generation of effector and memory T cell subsets. In other cell types, asymmetric cell division is regulated by conserved polarity protein complexes that control the localization of cell fate determinants and spindle orientation during division. We have developed a tractable, in vitro model of naive CD8+ T cells undergoing initial division while attached to dendritic cells during Ag presentation to investigate whether similar mechanisms might regulate asymmetric division of T cells. Using this system, we show that direct interactions with APCs provide the cue for polarization of T cells. Interestingly, the immunological synapse dissemination before division even though the T cells retain contact with the APC. The cue from the APC is translated into polarization of cell fate determinants via the polarity network of the Par3 and Scribble complexes, and orientation of the mitotic spindle during division is orchestrated by the partner of inscuteable/G protein complex. These findings suggest that T cells have selectively adapted a number of evolutionarily conserved mechanisms to generate diversity through asymmetric cell division. The Journal of Immunology, 2010, 185:000–000.

Materials and Methods

Abs and constructs

Primary Abs used were rabbit anti-aPKC, rabbit anti-Scribble, rabbit anti-aPKC® (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-ASIP/PAR-3 (Invitrogen, San Diego, CA); mouse anti–PSD-95 family (Upstate Biotechnology, Lake Placid, NY); goat anti-Numb, rat anti-tubulin (Abcam, Cambridge, MA); mouse anti-Prox1 (Chemicon International, Temecula, CA); mouse anti-tubulin (Sigma-Aldrich, St. Louis, MO); rabbit anti-tubulin (Rockland, Gilbertsville, PA); rat anti–CD8-Alexa-488, rat anti-CD45, rat anti-
CD11a(LFA-1), hamster anti–CD69-FITC, rat anti–CD44-FITC, rat anti–Vsv2 TCR-PE, mouse anti–CD45.1-PE (BD Biosciences, San Jose, CA); rat anti–CD25-APC, rat anti–CD62L-APC, mouse anti–CD45.2-APC-Cy7, rat anti–CD45R-APC, and hamster anti–TCR-β-PE-Cy5.5 (eBioscience, San Diego, CA). Secondary Abs used were anti-rabbit, anti-rat, anti-mouse, anti–goat-Alexa Fluor 488, anti-rabbit, anti-mouse, anti–rat-Alexa Fluor 594/543, and anti–goat–rhodamine (Proteins, Eugene, OR). MSCV–β-ARK-C-terminal–GFP was subcloned from pRK5-Bark Ict supplied by Robert Lefkowitz (16) and aurothiomalate (ATM) was supplied by Alan Fields. Biotin-labeled hamster mAbs to the Notch ligands, Delta 1, Delta 4, Jagged 1, and Jagged 2 (17) were supplied by Hideo Yagita (Juntendo University, Tokyo, Japan).

**Mice and cells**

C57BL/6 mice, B6-Ptpcr or OT-1 mice (C57BL/6 background) (18) of 8–12 wk of age were used. Naive OT-1 CD8+ T cells were purified from spleens of mice using MACS negative selection (Miltenyi Biotec, Auburn, CA). Bone marrow cells from hind limbs of C57BL/6 mice were cultured in GM-CSF and IL-4 for 6 d to generate immature DCs (CD11c+, CD86low, CA). Bone marrow cells from hind limbs of C57BL/6 mice were cultured in IL-3, IL-6, and stem cell factor conditioned media with 20% FCS but not Delta 4 that was moderately upregulated after peptide pulsing (Supplemental Fig. 1). All experiments on mice were performed in accordance the Animal Experimentation Ethics Committee of the Peter MacCallum Cancer Centre. To generate effector and memory cells, OT-1 T cells were cultured with IL-2 or IL-15, as previously described (20) and analyzed by flow cytometry.

**Transfections and transductions**

For generation of naive CD8+ OT-1 T cells expressing GFP or the C terminal of β-adrenergic kinase fused to GFP (16), hematopoietic stem cells were harvested from livers of OT-1 (Ly5.2) 13.5–14.5 embryos and cultured in IL-3, IL-6, and stem cell factor conditioned media with 20% FCS for 3 d. Phoenix-E cells were transduced by calcium phosphate and the supernatant containing recombinant retrovirus used to transduce the stem cells. Transduced cells were sorted by flow cytometry based on GFP expression and injected into the tail vein of lethally irradiated B6-Ptpcr (Ly5.1) mice to reconstitute their hematopoietic system. After reconstitution, CD8+ T cells were isolated from the spleen using MACS negative selection and the GFP+ cells sorted by flow cytometry for use in experiments. In some instances, 40 μM ATM was added to the cultures 20 h after addition of the T cells to the DCs.

**Live imaging**

For live cell imaging of dividing T cell–DC conjugates, 4 × 10^5 DCs were seeded into a glass bottom 35 mm culture dish (MatTek, Ashland, MA) and left to adhere overnight. DCs were then incubated with 1 μM SIINFEKL (1 h, 37°C), washed, and overlaid with 8 × 10^5 naive OT-1 T cells. The cocultures were left for 40 h prior to imaging. Differential interference contrast (DIC) and GFP images were captured on a TCS SP5 confocal microscope (Leica Microsystems, Deerfield, IL) fitted with a temperature controlled chamber maintained at 37°C and 5% CO2 using a 40× air objective (NA 0.85). Images were captured using Leica LAS AF Lite software every 2 min. All image analysis and manipulation was performed using Leica LAS AF Lite software or MetaMorph Imaging Series 7 software (Universal Imaging, Downingtown, PA).

**Immunofluorescent image analysis**

For immunofluorescent staining of DC–T cell conjugates, DCs were adhered overnight onto 8-well chamber slides (Nalge Nunc, Rochester, NY) and incubated with 1 μM SIINFEKL for 1 h at 37°C. Naive T cells were overlaid for 40 h and nonadherent cells washed off. Cells were then fixed with 3.7% (w/v) parafomaldehyde in 100 mM Pipes, 5 mM MgSO4, 10 mM EGTA and 2 mM DTT (10 min, room temperature [RT]), then washed twice and permeabilized in 0.1% Triton X-100 in 50 mM Tris–HCl (pH 7.6) (5 min, RT). Cells were then labeled with primary Abs, followed by detection with Alexa Fluor-conjugated secondary Abs (Molecular Probes) and mounted in Prolong antifade (Molecular Probes). For examples of control staining for immunofluorescence, see supplementary data (Supplemental Fig. 2). The slides were examined at RT using a Fluoview FV1000 confocal microscope (Olympus, Melville, NY) mounted with a 60× oil immersion objective (NA 1.42). Three-dimensional images of the cells were acquired with an optical distance of 0.5 μm between slices. Maximal intensity projections of the sections spanning the entire cell were used for all analyses and in each representative image throughout the text. Dividing T cells selected for analysis had a single contact site with the DC (DC shown at bottom of all images), enabling designation of proximal (P, adjacent to the DC) versus distal regions (D, away from the DC). Mitotic cells were identified by the pattern of α-tubulin fluorescence, which was also used to draw regions to define the two poles of the mitotic spindle in early mitotic cells and the proximal or distal daughter cells using MetaMorph software. The regions were then overlaid onto the protein image. To remove background fluorescence from the images, a top hat filter was applied using a circular structure element with a radius equal to the average radius of the two marked cells. MetaMorph software was utilized to calculate the integrated fluorescence in the proximal or distal daughter, the ratio (P – D/P + D) was applied and the results plotted using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

**Statistics**

The p values for polarization of individual proteins were generated using a one sample t test comparing (P – D/P + D) values to zero. To compare polarization between pairs of conditions, a two-tailed t test was used. The p values smaller than 0.05 were considered significant.

**Results**

*T cells divide while attached to an APC and show asymmetry of the polarity network*

*T* cells maintain prolonged interactions with APCs in vivo, and stable interactions are critical for T cell activation (9, 21). ACD after *Listeria* infection in vivo requires ICAM, suggesting that prolonged interactions between *T* cells and APCs also orchestrate ACD (4), but the polarizing cue could not be identified in this experimental system. To determine whether APCs can provide a cue for polarity during mitosis, we established an in vitro system to study long-term interactions between *T* cells and DCs. Naive CD8+ *T* cells from OT-1 transgenic mice expressing a TCR specific for an *OVA* peptide were cultured with preadhered, peptide-pulsed DCs. We seeded the cells at low densities to enable imaging of individual cells, and performed flow cytometric analysis of CFSE-labeled T cells to determine the time of first division under these conditions (40 h, Supplemental Fig. 3). Time-lapse microscopy of established T cell–DC conjugates over this period showed that nearly all the T cells remained attached to the DCs during mitosis (172/182 cells from five experiments; see Fig. 1A). These data demonstrate that long-term interactions between T cells and DCs in vitro can lead to division on the DCs, suggesting that the DCs can provide a cue for the establishment of the polarity required for a T cell to undergo ACD.

Polarity in many cells is regulated by complexes of evolutionarily conserved polarity proteins known as the Scribble and Par complexes, which antagonize each other to define molecularly distinct regions of the cell (22). We, and others, have determined that both the Scribble complex (including Scribble, lethal giant larvae, and discs large [Dlg]) and the Par complex (including Par3, Par6, and atypical protein kinase C [aPKC]) are expressed and polarized in T cells and are important in a number of T cell functions (19, 23–27). In support of a possible role in T cell ACD, we previously found that Scribble and aPKC were asymmetrically distributed in mitotic T cells responding to *Listeria* infection (4). To determine whether these, and other polarity proteins, were asymmetrically distributed in the dividing OT-1 *T* cells, and to definitively ascribe localization to proximal or distal cells relative to the DCs, we fixed T cell–DC conjugates for immunofluorescent staining of α-tubulin and polarity proteins, and used confocal microscopy to capture fluorescent and DIC images of dividing cells. For a description of the imaging protocol and quantification of fluorescence see supplemental data (Supplemental Fig. 4). We used the DIC image and the pattern of tubulin staining to identify the phase of mitosis, to draw regions delineating the distal and proximal halves of the dividing T cells, and to quantitate fluorescence of the costained polarity proteins in each half of the dividing cells. The mitotic cells were classified into early (prophase, metaphase, and anaphase) and late (early, mid, and late
telophase) mitotic phases to assess the polarization of each polarity protein over the course of division (Fig. 1B; images show representative early and late mitotic cells stained for the indicated proteins; scatter plots represent quantitation of dividing cells). Both aPKC and Par3 polarized significantly to the distal side of the cell in early mitotic T cells and maintained this asymmetry during late mitosis. The localization of Dlg showed greater spread between proximal and distal cells compared with Scribble, possibly due to the Dlg family (DlgF) Ab detecting all four Dlg isoforms (Dlg1–4) in T cells (19), which might localize differently. However, Scribble and DlgF were both significantly polarized to the proximal cell in early and late mitosis. These data show that T cells can divide asymmetrically while in contact with an APC.

**ACD segregates Numb to the distal daughter and affects effector and memory fate**

We next assessed whether ACD of OT-1 T cells correlated with any apparent differences in the daughters that could lead to distinct fates, such as differently sized daughter cells, or differences in distribution of cell fate determinants. We first measured the area of the proximal and distal daughters of 191 cells captured in late telophase and found the sizes to be very similar (Supplemental Fig. 5, 13907 versus 13514 ± 440 arbitrary units) indicating that ACD of T cells does not establish gross differences in size that might cause differences in cell fate. Unequal inheritance of Numb and Prospero can dictate cell fate in Drosophila sensory organ precursors and

![FIGURE 1. Polarity proteins are asymmetric in mitotic T cells interacting with DC.](image)
neuroblasts (28), and Numb is asymmetrically distributed in mitotic CD8\(^+\) T cells following Listeria infection (4). Staining for Prox-1 (the mammalian homolog of Prospero) (29) showed it was not polarized in dividing T cells (Supplemental Fig. 6); whereas, Numb was polarized to the distal pole of mitotic cells, and this asymmetry was maintained through to cytokinesis (Fig. 2A). These data demonstrate the differential segregation of the cell fate determinant, Numb, into the distal daughter cell of a dividing T cell attached to a DC.

The polarization of Numb with Par3 to the same daughter cell is similar to the colocalization of Numb with Par3 in migrating epithelial cells (30), but differs from the opposition of the Par3 complex and Numb in dividing neuroblasts (31). However, in both cases, the localization of Numb is dependent on the Par3 complex, and specifically on phosphorylation by aPKC (32). To test whether Numb might be similarly regulated by aPKC in asymmetrically dividing T cells, we treated the T cell–DC conjugates with ATM. ATM (“Gold”) has been used to treat rheumatoid arthritis for decades, but its mechanism of action has been unclear (33, 34). However, ATM was recently shown to inhibit the interaction between aPKC and Par6, and is currently under investigation as a treatment for cancer (35). Treatment of the cells with ATM caused only negligible delay in T cell proliferation in response to Ag presentation (Supplemental Fig. 7), but led to a significant reduction in the asymmetric polarization of aPKC to the distal pole of dividing T cells (Fig. 2B), indicating that ATM disrupted polarity during ACD. Compatible with a possible role for aPKC in regulating polarity of Numb during ACD, treatment with ATM also significantly reduced segregation of Numb to the distal cell (Fig. 2B). Thus, although treatment with ATM enabled protracted interactions between T cells and DCs and activation of the T cells, the asymmetric distribution of aPKC and Numb to the distal daughter was abrogated.

To assess whether the disruption of polarity by ATM treatment might correlate with altered T cell fate, we cultured OT-1 CD8\(^+\) T cells under conditions that induce effector or memory differentiation, using IL-2 and IL-15 as previously described (20). Memory T cells are characterized by their long-term homeostatic turnover, multipotency, and rapid recall after secondary infection. However, they can also be more generally identified by the expression of specific surface

**FIGURE 2.** Polarity network orchestrates asymmetric distribution of the cell fate determinant, Numb. A, The ratio of proximal/distal polarization was assessed as in Fig 2 for Numb in different phases of mitosis (three experiments; 80 cells). Representative images on left, scale bars, 10 \(\mu\)m. Representative images below, scale bars, 10 \(\mu\)m. Tubulin (red, Alexa-546) and aPKC and Numb (green, Alexa-488). Images were collected with a 60\(\times\) oil immersion objective as indicated in Materials and Methods. C, Expression of the surface markers CD44, CD62L, CD69, and CD25 on OT-1 CD8\(^+\) T cells cultured under effector (IL-2) and memory (IL-15) conditions with or without treatment with ATM. The geometric mean of the individual peaks, or % cells gated, is shown under the plots. Data are representative of three experiments.
markers (2). After treatment with IL-2 and IL-15, naive CD8+ T cells (CD62Lhi, CD44med, CD69+, CD25+, Supplemental Fig. 8) developed into subsets characteristic of effector (CD62Llo, CD44hi, CD69lo, and CD25lo) and memory cells (CD62Llo, CD44med, CD69lo, and CD25med), respectively (Fig. 2C). Treatment of the T cell–DC conjugates with ATG 20 h prior to first cell division had no effect on differentiation into effector T cells, as assessed by each of the four markers (Fig. 2C, compare first and second row). In contrast, after treatment with 40 μM ATG, the cells cultured in conditions designed to induce memory differentiation showed a shift toward a more effector-like phenotype with upregulation of CD44 and downregulation of CD62L (Fig. 2C, compare third and fourth row). No differences were observed for CD69 and CD25 expression between untreated and treated T cell–DC conjugates. These data suggest that ACD, regulated by the polarity network, might impact on T cell fate decisions.

The polarity cue for ACD requires contact with the DCs, but not sustained polarization of classic immunological synapse markers during mitosis

We next investigated how the polarity cue provided by the DCs is transmitted to the dividing T cell. Cells such as the fertilized Caenorhabditis elegans zygote retain memory of a previous polarity cue, and in these cells polarity is maintained by proteins such as Par3 (14, 36). The asymmetry previously observed in mitotic cells separated from contact with APCs suggests a similar possibility for T cells (4). Indeed, the recent identification of a molecule, CRTAM, which can interact with Scribble to sustain CD3/CD28-Ab–mediated polarity after the cells have disengaged, supports this notion (37). We therefore investigated the dependence of the asymmetric localization of aPKC, Par3, Scribble, and Numb on the interaction with the APC at the time of mitosis. The distribution of fluorescence in dividing T cells attached to a DC was compared with the distribution of fluorescence in the rare cells captured dividing while unattached to a DC. In the absence of DC, aPKC, Par3, Scribble, and Numb were not polarized (Fig. 3). This suggests that, where ACD is controlled by Ag presentation, memory of the contact is not sufficient for polarity at the time of division, and that contact with the DC is necessary not only to establish polarization at the initiation of Ag presentation, but also to maintain this asymmetry through to the onset of mitosis.

Ag presentation initially involves the formation of an immunological synapse, with the recruitment of T cell receptor-associated signaling molecules and the microtubule organizing center (MTOC) to the interface with the DCs (38). We therefore determined whether proteins that are normally associated with the immunological synapse might transmit the polarity cue from the DCs, by assessing whether they are also polarized to the interface in the dividing T cells. CD8 was not polarized to the proximal cell at either early or late mitosis, but showed localization to some distal cells in early mitosis (Fig. 3). This suggests that, where ACD is controlled by Ag presentation, memory of the contact is not sufficient for polarity at the time of division, and that contact with the DC is necessary not only to establish polarization at the initiation of Ag presentation, but also to maintain this asymmetry through to the onset of mitosis.

To assess whether the Pins/G protein pathway regulated spindle orientation in T cells, we attempted to disrupt G protein signaling by sequestering Giβγ proteins with overexpression of the β-adrenergic receptor kinase C-terminal domain (β-ARK) (16). In some instances, such as division of Drosophila male germ cells, the orientation of the mitotic spindle is defined by the polarization of the MTOC at interphase (39). After duplication, one centrosome remains anchored in this position by microtubules, and the other relocates to the opposite side of the nucleus (39, 40). The stable recruitment of the MTOC to the interface with the DCs raised the possibility that it might also orientate the mitotic spindle during mitosis. However, staining of fixed, dividing T cell/DC conjugates for α-tubulin was not compatible with this, as the tubulin condensed in the center of the cells before the centrosomes separated to opposite poles of the cell (Fig. 5A, 55 of 60 cells at prophase were in the central third of the cell, with five slightly distal).

An alternative means of dictating spindle orientation linksDlg to trimeric G protein signaling to coordinate the orientation of the spindle body with the axis of polarity (41). In Drosophila neuroblasts,Dlg can recruit Pins (partner of inscuteable, also known as LGN in mammals) (31, 42) which in turn reinforces polarity and orients the spindle of neuroblasts and mammalian neuronal precursors by binding to Goαi (41, 43, 44). We found that Pins (45) was expressed in T cells (Supplemental Fig 9), and polarized to the distal side of the asymmetrically dividing T cell (Fig. 5B). To assess whether the Pins/G protein pathway regulated spindle orientation in T cells, we attempted to disrupt G protein signaling by sequestering Giβγ proteins with overexpression of the β-adrenergic receptor kinase C-terminal domain (β-ARK) (16).
β-ARK expression in asymmetrically dividing neural progenitors in the developing mouse neocortex disrupted the orientation of the mitotic spindle (46), and we assessed the effect on spindle orientation in T cells. We reconstituted mice with OT-1 hematopoietic stem cells transduced with a control GFP construct and the β-ARK-GFP construct. Analysis of the peripheral blood of reconstituted mice demonstrated that GFP+ T cells developed in these animals (Supplemental Fig. 10). We then assessed spindle orientation, based on tubulin staining, of both GFP and β-ARK-GFP transduced T cells dividing in contact with peptide-pulsed DCs (Fig. 5C). Of the control cells transfected with GFP, 75% showed an angle >30 degrees from the DC interface, compatible with ACD. However, inhibition of G protein signaling significantly reduced the number of cells with an axis compatible with ACD (75 versus 30%), and the majority of the cells had a spindle almost parallel to the interface with the DCs. These data combined indicate that CD8+ T cells not only use the evolutionarily conserved polarity network to polarize cell fate determinants, but also use the Pins/G protein module to align the mitotic spindle with the axis of polarity.

Discussion
The question of whether memory cells arise in a linear developmental progression from effector cells, or whether certain progeny of an activated T cell have a predetermined propensity for differentiation into memory cells has generated much discussion (5, 47, 48). The latter model is supported by the discovery that the first daughters arising from activation of a naive T cell could be segregated on the basis of cell surface markers to discriminate cells that have potential for memory differentiation (4). The observation of asymmetry during the first division also provided compelling evidence that ACD plays a role in this predetermination (4). Conversely, a recent study concluded that memory cells can arise from effector cells (identified by Granzyme B expression), providing support for the linear progression model (49). However, the system used did not exclude the possibility that transcription of Granzyme B mRNA might occur before the first division, followed by asymmetric polarization of cell fate determinants into the daughter cells. Resolution of this issue will depend on continuing development of more sophisticated tools with which to dissect when, where, and how the master regulators of cell fate are switched on. To test whether ACD can contribute to T cell fate decisions, we describe in this study a tractable system with which the early events in activation of naive T cells can be monitored. We demonstrate that T cells undergo ACD, show evidence that ACD can dictate cell fate and identify conserved mechanisms by which ACD is controlled. Our study defines the key elements required for ACD: 1) the cue to dictate the axis of polarity, 2) asymmetry of proteins along this axis, and 3) alignment of the mitotic spindle with the axis of polarity.

The polarity cue
Like Drosophila male germ cells and larval neuroblasts (15), T cells can use direct contact with an adjacent cell to orchestrate

![Figure 4](http://www.jimmunol.org/)
polarity throughout cell division. In the model we have studied, the cue comes from contact with the DC, but the immunological synapse does not seem to be involved per se by the time of division (as indicated by even distribution of TCR signaling components). It is possible that other cues, such as Notch ligands, Wnt signaling components, or integrins might play a role in maintaining the axis of polarity until the point of division. Our data show that peptide-pulsed DCs are capable of providing the cue, but the plethora of different cues that dictate polarity in T cells (50) suggests that other forms of Ag presentation, or other polarity cues such as chemokines, might dictate different forms of ACD in T cells, resulting in differing effects on cell fate determination. This concept is supported by observations that context can alter both the molecular distributions during ACD and the fate decisions of Drosophila neuroblasts and sensory organ precursors (51, 52). Further support comes from the differences in polarity observed between this system and the ex vivo system studied by Chang et al. (4), which has been proposed that de novo establishment of the spindle might allow for flexibility in determining the proportion of cells undergoing ACD (14). By analogy, it is possible that differences in Ag presentation might allow fine-tuning of the immune response by regulating orientation of the spindle to dictate the proportion of cells undergoing ACD.

Alignment of the mitotic spindle

T cells differ from Drosophila male germ cells (53), in that the spindle orientation of T cells is not dictated by retention of the MTOC and centriole to the cell–cell interface, but is oriented de novo. This organization is more similar to the first division of embryonic Drosophila neuroblasts (53), where it has been proposed that de novo establishment of the spindle might allow for flexibility in determining the proportion of cells undergoing ACD (14). By analogy, it is possible that differences in Ag presentation might allow fine-tuning of the immune response by regulating orientation of the spindle to dictate the proportion of cells undergoing ACD.

Our data also show that Pins localizes to the same daughter cell as the Par3 complex in the dividing T cells, suggesting that the interaction between the polarity proteins and spindle organization in T cells is similar to ACD of Drosophila sensory organ precursors (55). Interestingly, these modules can cooperate or antagonize in
different contexts (22), and it has been proposed that the proximity of Pins and Par3 dictates the different size of sensory organ precursor daughters (55). However, this does not seem to be the case in T cells, as the proximal and distal daughters were identical in size. It is clear that different cell types, such as Drosophila neuronal and sensory organ precursors, use similar conserved polarity molecules, organized into different functional modules, to meet cell-specific requirements for ACD (15). The unique molecular processes regulating ACD of T cells described in this study suggests that T cells have adopted a number of these evolutionarily conserved mechanisms. It is likely that, as in other cell systems, the modules coordinating ACD of T cells described in this study suggests that T cells organized into different functional modules, to meet cell-specific sensory organ precursors, use similar conserved polarity molecules, technical assistance, reagents, and helpful comments.

Acknowledgments
We thank Cameron Nowell, Sarah Ellis, Anne Sossinka, Robert Lefkowitz, Fengwei Yu, Bill C. Patrick Metz, Vikrim Palanivel, Helena Richardson, Natasha Harvey, Alan Fields, Hideo Yagita, and Sally Dunwoodie for technical assistance, reagents, and helpful comments.

Disclosures
The authors have no financial conflicts of interest.

References


Figure S1: Bone-marrow derived dendritic cells were stained with biotin-conjugated hamster monoclonal antibodies to mouse Delta 1, Delta 4, Jagged 1 and Jagged 2 (kindly supplied by Hideo Yagita) and detected with a secondary streptavidin-PE antibody (BD Biosciences) and analysed by flow cytometry. **A.** Untreated BM-derived dendritic cells. **B.** BM-derived dendritic cells were first incubated with 1 μM SIINFEKL peptide at 37°C for 1 hour, then stained for the above antibodies.
A Tubulin Rabbit IgG Dapi Merge

B Tubulin Goat IgG Dapi Merge

C Tubulin CD8 Dapi Merge

Figure S2: Control antibody staining. For immunofluorescent staining of DC-T cell conjugates, DCs were adhered overnight onto 8-well chamber slides (Nalgene Nunc, IL, USA) and incubated with 1 μM SIINFEKL for 1 hour at 37°C. Naïve OT-I T cells were overlayed for 40 hours and non adherent cells washed off. Cells were then fixed and permeabilised (see materials and methods) and labelled with (A) goat polyclonal IgG antibodies (Chemicon) or (B) rabbit polyclonal antibodies to CD46 (Crimeen-Irwin et al. (2003) JBC 278:46927-46937) or (C) anti-CD8 antibody (BD Biosciences) followed by detection with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and mounted in Prolong antifade with Dapi (Molecular Probes). The slides were examined at room temperature using a Fluoview FV1000 confocal microscope (Olympus) mounted with a 60x oil immersion objective (NA 1.42). Each image was taken with identical settings.
Figure S3: Time of first division of OT-I T cells in response to peptide-pulsed dendritic cells. Naive CD8\(^+\) T cells were purified from the spleens of OT-I mice by MACS negative selection and labelled with carboxyfluorescein succinimidyl ester (CFSE: Sigma), such that each cell division results in a detectable halving of the fluorescence. T cells were co-cultured with bone-marrow derived DC and analyzed by flow cytometry to determine the time of first division. Histograms show naive OT-I cells cultured with non-pulsed DCs (no proliferation) and SIINFEKL-pulsed DC showing approximately 10% of cells in first division and approx 1% of cells in second division.
Daughter | Area | Fluorescent Intensity | (P-D)/(P+D)
--- | --- | --- | ---
Proximal | 15145 | 137246 | -0.5
Distal | 16776 | 405908 |
**Figure S4**: Confocal analysis of dividing OT-1 T cells attached to dendritic cells (DC). A. Images represent two fields of T cells and DCs following co-culture in vitro. Each image shows one dividing cell (a,b) and undivided T cells (c,d,e) stained for Numb (green) and Tubulin (red). B. Magnified images of T cells highlighted in left panels demonstrating polarization of Numb to the distal daughter/side in a and b respectively, distal polarization of Numb in undivided T cells attached to a DC (c,d) and a T cell that would not be analyzed as it sits on top of the DC (e) and polarization relative to the DC cannot be defined. C. Shows the detailed analysis of the image shown in Figure 1 (top panel). The DCs are identified and distinguished from the OT-1 T cells based on morphology and membrane characteristics. The DC is orientated at bottom of all figures in the manuscript (DC is cropped for better emphasis on T cells) and dividing T cells were only selected for analysis if they had a single contact site with the DC enabling designation of proximal versus distal regions. All images in the figures were taken using a Fluoview FV1000 confocal microscope (Olympus) mounted with a 60x oil immersion objective (NA 1.42). 3D images of the cells were acquired with an optical distance of 0.5 μm between slices. D. Example of individual Z sections for the protein of interest, aPKC, shown in C. E. Tubulin staining overlaid on DIC was used to draw regions to define the two poles of the mitotic spindle in early mitotic cells and the proximal or distal daughter cells in late mitosis using MetaMorph® software. The regions were then overlaid onto the image of the protein of interest (eg aPKC). In order to remove background fluorescence from the images, a top hat filter was applied using a circular structure element with a radius equal to the average radius of the two marked cells. For quantitation, MetaMorph® software was using to calculate the integrated fluorescence in the proximal (P) or distal (D) region, the ratio (P-D/P+D) was applied.
**Figure S5**: Area of proximal and distal daughter cells of dividing OT-I T cells at late telophase. CD8⁺ T cells from OT-I mice were cultured with peptide-pulsed DC and at 40 hrs, the cells were fixed and stained for α-tubulin and protein markers. MetaMorph software was used to draw regions around the proximal (closest to the DC) and distal (away from the DC) daughter cells and the area calculated in arbitrary units. The column graph shows no significant difference in area measurement between proximal and distal daughter cells at late telophase. SEM of 191 cells from at least 7 experiments is shown.
Figure S6: Polarization of Numb and Prox-1 in naive T cells dividing on a DC. Naive OT-I T cells dividing in response to antigen presentation by DC were fixed and stained to determine the ration of proximal and distal polarization of Numb (80 cells, 3 experiments) and Prox-1 (52 cells, 2 experiments). Positive and negative values indicated proximal and distal polarization respectively. Each point on the graph represents an individual cell and the green bar represents the mean. Data shows distal polarization of Numb and symmetric polarization of Prox-1.
**Figure S7:** Effect of ATM treatment on OT-1 T cells. **A.** Proliferation of OT-1 T cells in the presence or absence of aurothiomalate (ATM). CD8⁺ T cells from OT-1 mice were CFSE-labelled and cultured with peptide-pulsed DC for 3 days. In some instances, 25 μM or 40 μM ATM was added to the co-cultures 20 hrs following addition of the T cells. At each time point, the cells were harvested and analyzed by flow cytometry. OT-1 T cells proliferated under each condition, with a slight delay in proliferation of T cells treated with ATM. **B.** Phenotype of T cells treated with 40 μM ATM 48 hrs post activation with peptide-pulsed DC. T cells were harvested and stained with fluorophore-conjugated antibodies to the surface proteins as labelled.
Figure S8: Expression of the surface markers, CD44, CD62L, CD69 and CD25 on naive OT-I T cells. CD8+ T cells were purified from the spleens of OT-I mice by MACS negative selection and stained for CD44, CD62L, CD69 and CD25 and analyzed by flow cytometry. Ninety percent of cells were positive for CD62L and negative for CD69 and CD25. Geometric means, or % cells gated, are shown below the peaks.
Figure S9: Detection of Pins protein in naive T cells and T cell lines. Cell lysates were prepared from purified splenic naive CD8+ T cells, Hut78 and Hut102 T cell lines. Pins expression was detected by Western blot using a rabbit anti-Pins antibody. Western blot analysis using this antibody shows detection of a major 75 kDa band as previously reported for mouse liver and brain (Yu et al., (2002) Journal of Cell Science 116: 887-896).
Figure S10: Analysis of the T cells in the peripheral blood of mice reconstituted with GFP constructs. OT-I hematopoietic stem cells were retrovirally transduced with either GFP alone or β-ARK-C-terminal GFP constructs and used to reconstitute lethally irradiated syngeneic mice. At 6 weeks post-injection of transduced cells, the peripheral blood of the mice was analyzed for reconstitution efficiency (donor vs host cells) and the donor cells analyzed for ratios of B and T cells based on CD45R and TCRβ expression respectively. TCRβ positive cells were then assessed for GFP expression.