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

Jane Oliaro,*,† **Vanessa Van Ham,*** **Faruk Sacirbegovic,***,† **Anupama Pasam,***,†
Ze'ev Bomzon,*,‡ **Kim Pham,** *,‡ **Mandy J. Ludford-Menting,*** **Nigel J. Waterhouse,***,†
Michael Bots,* **Edwin D. Hawkins,*** **Sally V. Watt,*** **Leonie A. Cluse,*** **Chris J. P. Clarke,***
David J. Izon,§ **John T. Chang,**¶ **Natalie Thompson,**‖ **Min Gu,**‡ **Ricky W. Johnstone,***
Mark J. Smyth,*,† **Patrick O. Humbert,**# **Steven L. Reiner,**¶ **and Sarah M. Russell***,†,‡

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 disseminates 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: 367–375.

U pon activation, a naive T cell proliferates to generate the different T cell subsets required for both an immediate response and an immune memory (1). How the activation of a single-parent T cell can control multiple pathways of differentiation in the T cell progeny remains controversial. A parental CD8+ T cell, for example, may have the potential to develop into both effector and memory cells, with the outcome determined by extrinsic factors such as environmental signals or stimulus strength (2). Alternatively, T cells may divide asymmetrically after Ag presentation, leading to molecularly distinct daughter cells with different effector and memory fate potential (3–5).

In vivo imaging has revealed much about the dynamics of T cell–dendritic cell (DC) interactions (6–8) and would be the ideal tool to analyze the molecular events after T cell conjugation with

APCs and subsequent activation and proliferation. Although current technology using two-photon microscopy can accurately assess the duration of contacts and the functional consequences of these interactions (9–12), it does not have the resolution to assess the distribution of individual proteins in single cells. Fixed imaging analysis of dividing cells ex vivo in response to *Listeria* infection has revealed that asymmetric cell division (ACD) of T cells may dictate T cell memory and effector fates (4). However, in this approach, the history of the dividing cell is lost, making it difficult to extrapolate information about the mechanism of ACD, in particular, the cue for polarity.

To overcome these limitations, we have developed an experimental in vitro system that enables the molecular analysis of single progenitor T cells undergoing their first division during interaction with an APC. This model provides an excellent system with which to image individual T cells undergoing division in response to contact with APCs, and evaluate the three requirements for ACD: 1) a 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 (13–15). Using this system, we elucidate each of the three conditions required for ACD in T cells and show that T cells have adapted a number of evolutionarily conserved mechanisms to regulate polarity and mitotic spindle orientation during ACD.

Materials and Methods

Abs and constructs

Primary Abs used were rabbit anti-aPKC, rabbit anti-Scribble, rabbit anti-PKCθ (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–Vα2 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–TCRβ-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 (Molecular Probes, Eugene, OR). MSCV-β-ARK-C-terminal-GFP was subcloned from pRK5-Bark 1CT 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-Ptpcra 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$^+$, CD86^{low}, and MHC-II^{low}) for use as APCs (19). A proportion of these bone marrow-derived APC expressed the Notch ligands Delta 1, Jagged 1, and Jagged 2, but not Delta 4 that was moderately upregulated after peptide pulsing (Supplemental Fig. 1). All experiments on mice were performed in accordance with 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 terminus 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 transfected 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-Ptpcra (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 8×10^4 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% CO$_2$ 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 overlayed for 40 h and nonadherent cells washed off. Cells were then fixed with 3.7% (w/v) paraformaldehyde in 100 mM Pipes, 5 mM MgSO$_4$, 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. Maximum intensity projections of Z 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 using 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. 1*A*). 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

FIGURE 1. Polarity proteins are asymmetric in mitotic T cells interacting with DC. *A*, Naive OT-1 CD8^{+} T cells expressing GFP were cultured with peptide-pulsed DCs. The cocultures were left for 40 h prior to imaging and then imaged by time-lapse microscopy every 2 min. Still images show an example of a T cell dividing while attached to a DC, time stamp shows time of progression through mitosis. Scale bar, 25 μm. *B*, Naive OT-1 CD8^{+} T cells dividing in response to Ag presentation by DCs were fixed and stained to determine the ratio of proximal to distal polarization of aPKC, PAR-3, Scribble, and DlgF (three experiments; 73, 61, 66, and 80 cells were analyzed, respectively, representative images on the left). Tubulin (red, Alexa-546) and aPKC, Par3, Scrib, and DlgF (green, Alexa-488). Images were collected with a 60× oil immersion objective as indicated in *Materials and Methods*. Positive and negative values indicate proximal and distal polarization, respectively. Each point on the graph represents an individual cell and the green bar represents the mean. Scale bars, 10 μm.
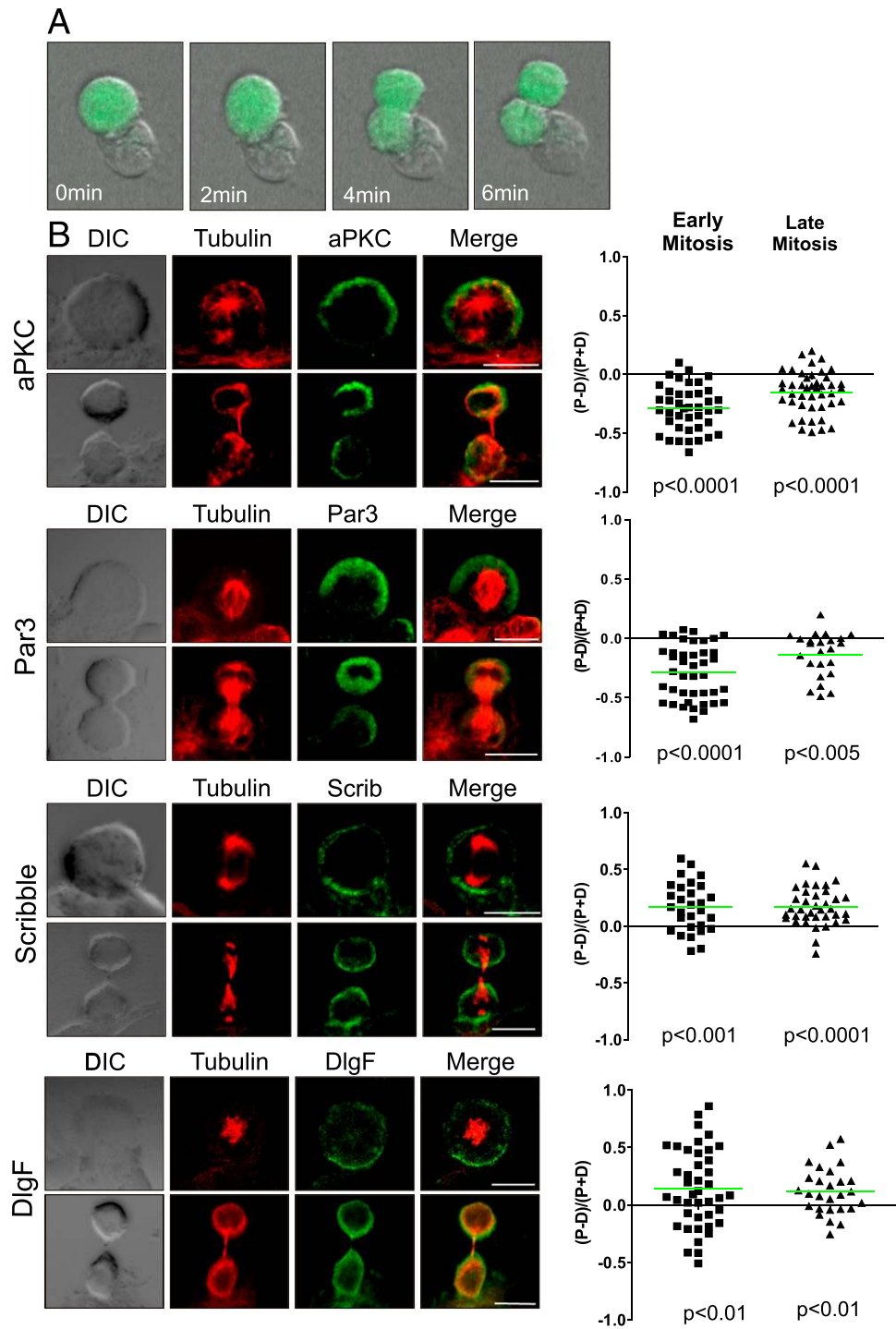
telophase) mitotic phases to assess the polarization of each polarity protein over the course of division (Fig. 1*B*; images show representative early and late mitotic cells stained for the indicated proteins; scatter plots represent quantitation of dividing cells). Both aPKC and Par3 polarized significantly 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

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
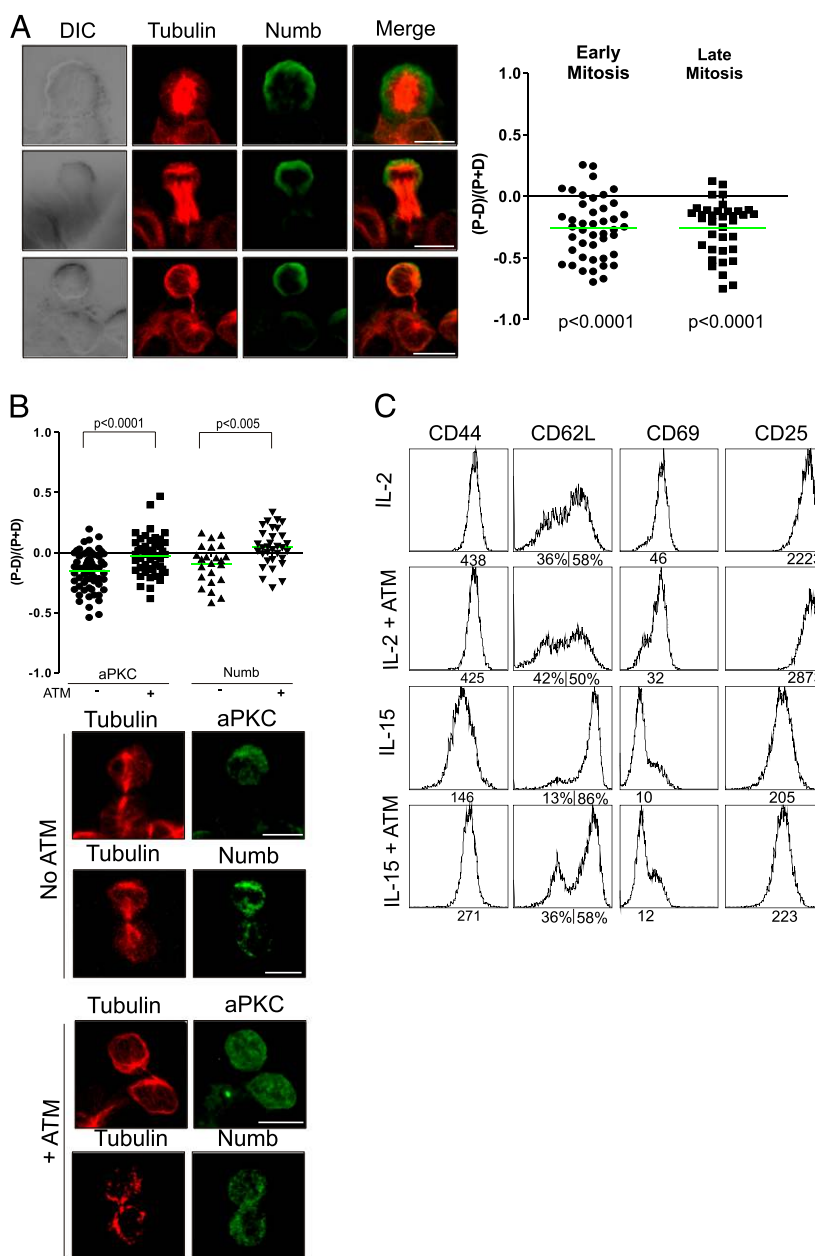

FIGURE 2. Polarity network orchestrates asymmetric distribution of the cell fate determinant, Numb. *A*, The ratio of proximal/distal polarization was assessed as in Fig 2 for Numb in different phases of mitosis (three experiments; 80 cells). Representative images on left, scale bars, 10 μm. *B*, The ratio of proximal/distal polarization was assessed as in Fig. 2 for aPKC (61 and 50 cells) and Numb (57 and 66 cells) with or without treatment with ATM (two experiments). Representative images below, scale bars, 10 μm. Tubulin (red, Alexa-546) and aPKC and Numb (green, Alexa-488). Images were collected with a 60× 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 (CD62L^{hi}, CD44^{med}, CD69$^-$, CD25$^-$, Supplemental Fig. 8) developed into subsets characteristic of effector (CD62L^{lo}, CD44^{hi}, CD69^{med}, and CD25^{hi}) and memory cells (CD62L^{hi}, CD44^{med}, CD69^{lo}, and CD25^{med}), respectively (Fig. 2C). Treatment of the T cell–DC conjugates with ATM 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 ATM, 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. LFA-1 was enriched at the contact site in early mitosis but this did not result in significant polarization to the proximal cell. However, the synapse marker, PKCθ and distal pole marker, CD45, were significantly polarized in early mitosis (Fig. 4). The relatively even distribution of all these proteins at late mitosis suggests that, although the immunological synapse might play an important role in dictating the axis of polarity (perhaps related to the recruitment of the MTOC to the interface), differential inheritance of T cell receptor-associated signaling molecules is unlikely to be important for fate determination in this system.

ACD of T cells uses conserved mechanisms to coordinate polarity with the orientation of the spindle

ACD requires not only polarization of proteins, but also alignment of the mitotic spindle with the axis of polarity (14, 15). In
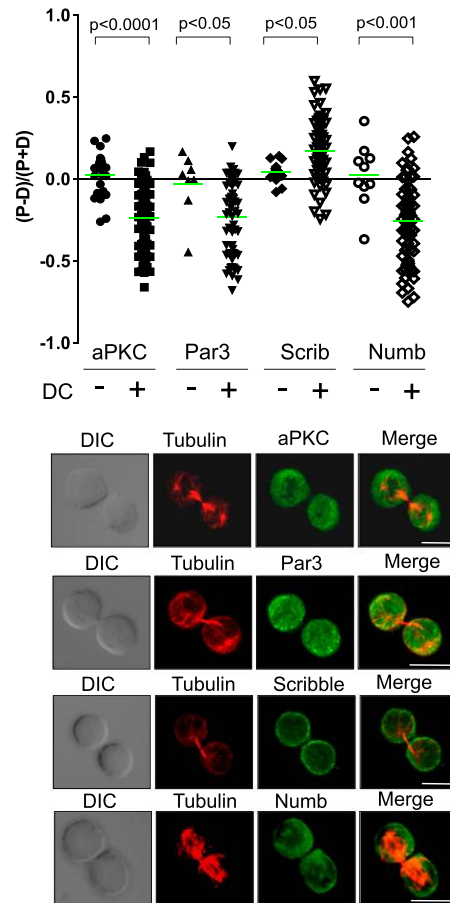

FIGURE 3. ACD of T cells requires contact with the APC. The ratio of proximal/distal polarization was assessed as in Fig. 2 for aPKC (24 cells), Scribble (14 cells), PAR-3 (8 cells), and Numb (10 cells) in mitotic cells unattached to a DC (representative images below) compared with mitotic cells attached to a DC. Tubulin (red, Alexa-546) and aPKC, Par3, Scribble, and Numb (green, Alexa-488). Images were collected with a 60× oil immersion objective as indicated in *Materials and Methods*. Scale bars, 10 μm.

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 links Dlg 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 Gα$_I$ (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 Gβγ proteins with overexpression of the β-adrenergic receptor kinase C-terminal domain (β-ARK) (16).

FIGURE 4. ACD of T cells requires contact with the DCs, but not a sustained immunological synapse. The ratio of proximal/distal polarization was assessed as in Fig. 2 for synapse and distal pole proteins CD8 (40 cells), LFA-1 (66 cells), PKCθ (79 cells), and CD45 (58 cells) (three experiments). Representative images on left. Tubulin (red, Alexa-546) and CD8, LFA-1, CD45, PKC-θ (green, Alexa-488). Images were collected with a 60× oil immersion objective as indicated in *Materials and Methods*. Scale bars, 10 μm.
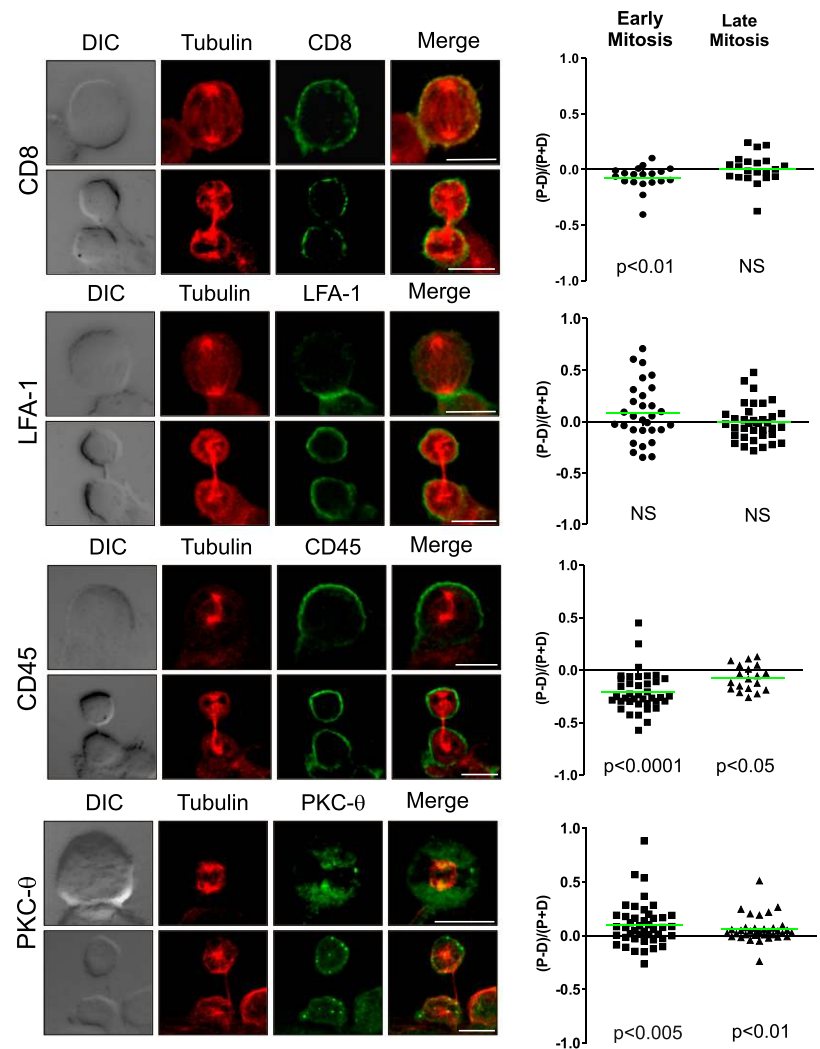
β-ARK expression in asymmetrically dividing neural progenitors in the developing mouse neocortex disrupted the orientation of the mitotic spindle (46), and we assessed the effect on spindle orientation in T cells. We reconstituted mice with OT-1 hematopoietic stem cells transduced with a control GFP 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
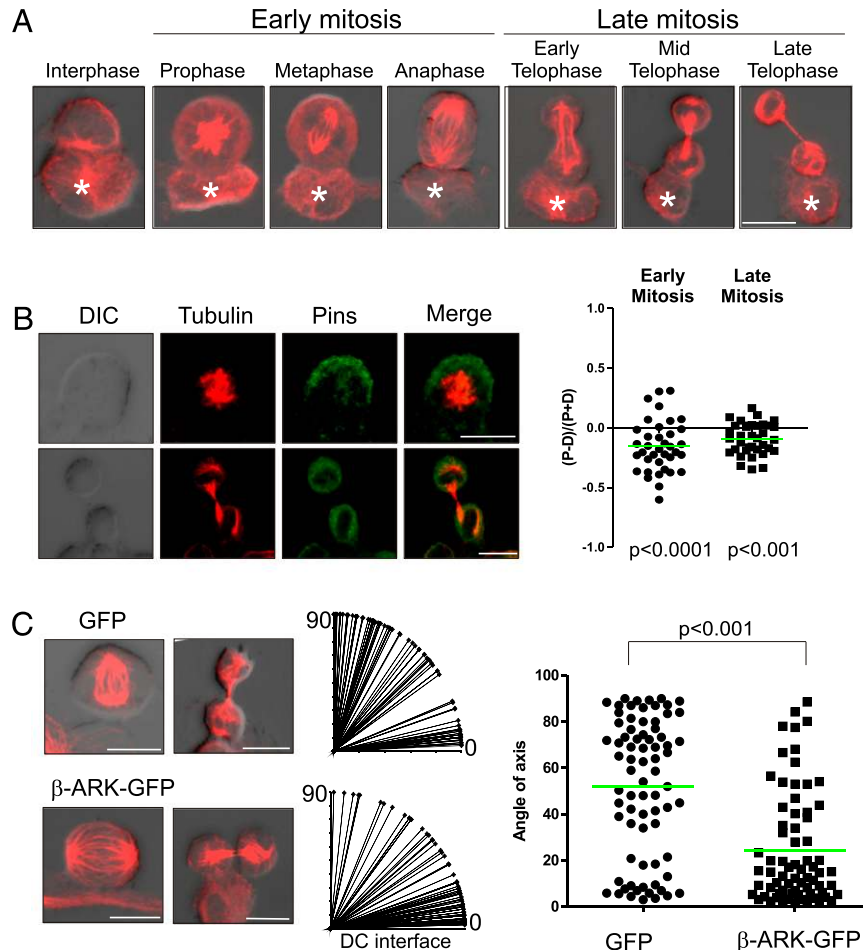
FIGURE 5. ACD of T cells uses the Pins/G protein module to orient the spindle. OT-1 CD8+ T cells dividing while attached to a DCs were (A) stained for tubulin and scored for orientation of the MTOC or (B) stained for tubulin and Pins to assess the ratio of proximal or distal polarization (three experiments; 73 cells), Tublin (red, Alexa-546) and Pins (green, Alexa-488). Images were collected with a 60× oil immersion objective as per *Materials and Methods*, or (C) naive OT-T cells expressing GFP (n = 75) or β-ARK-GFP (n = 70) were incubated with DCs and stained for tubulin to mark T cells in division. Image J software was used to draw a line through the axis of division based on the position of the centrosomes, using the DC interface as the horizontal axis and used to calculate the angle of spindle axis relative to the DC interface and plotted on the right (two experiments, representative images on left). Scale bars, 10 μm.

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 suggests that the ACD of Chang et al. is orchestrated by a cue other than the peptide-pulsed DCs used in this study. The copolarization of Numb and CD8 in mitotic cells activated by *Listeria* (compatible with Numb recruitment to the putative proximal cell) (4) suggests that Numb can orientate differently under different stimuli (e.g., peptide dose versus in vivo infection) and that different polarity cues, by altering ACD, might regulate different fate outcomes

Maintaining polarity through division

The stable recruitment of the MTOC to the interface with the DC suggests that the polarity cue might be translated to the polarity network via microtubules (53). In *Drosophila* larval neuroblasts at

metaphase, Dlg is transiently polarized by astral microtubules via the kinesin H chain, Khc-73 (42). In support of a similar mechanism in T cells, the mammalian homolog of Khc-73, GAKIN, is also localized by interactions with microtubules, and can interact with Dlg in T cells (54). These data, and analogies with other cell systems, indicate that the initial polarity triggered by TCR signaling is translated into stable polarity by antagonistic interactions between the Scribble and Par3 polarity complexes, which are maintained over the tens of hours until cell division. An involvement of the Par3 complex in this process is supported by the disruption in aPKC and Numb polarization on treatment with ATM, which can functionally inhibit the Par3 complex by preventing interactions between Par6 and aPKC (35).

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 will be arranged differently according to the context in which T cells divide, facilitating the highly regulated diversity that characterizes the immune system. The observations presented in this study suggest new approaches and tools that will enable the elucidation of the role of ACD in the generation of memory and effector precursor cells, and perhaps in other immune cell fate decisions.

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Disclosures

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