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Science

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Asymmetric T Lymphocyte Division in the Initiation of Adaptive Immune Responses

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A hallmark of mammalian immunity is the heterogeneity of cell fate that exists among pathogen-experienced lymphocytes. We show that a dividing T lymphocyte initially responding to a microbe exhibits unequal partitioning of proteins that mediate signaling, cell fate specification, and asymmetric cell division. Asymmetric segregation of determinants appears to be coordinated by prolonged interaction between the T cell and its antigen-presenting cell before division. Additionally, the first two daughter T cells displayed phenotypic and functional indicators of being differentially fated toward effector and memory lineages. These results suggest a mechanism by which a single lymphocyte can apportion diverse cell fates necessary for adaptive immunity.

The clonal selection theory of adaptive immunity suggests that proliferation of a single lymphocyte should provide sufficient function for acute defense (effector cells), as well as the regenerative capacity to maintain the selected lineage (memory cells). Throughout metazoan development and homeostasis, a conserved mechanism is used to confer disparate fates among daughter cells. This mechanism, known as asymmetric cell division, involves polarized alignment of determinants of cell fate perpendicular to the mitotic spindle, thus ensuring the unequal inheritance of critical molecules and divergence of daughter cell fates (1). Asymmetric cell division could thus represent a potential mechanism to ensure that appropriate diversity of cell fate arises from the clonal descendants of a single lymphocyte during an immune response.

T cell interaction with antigen-presenting cells is characterized by orientation of the actin and tubulin cytoskeleton and asymmetric segregation of signaling and adhesive proteins toward the site of intercellular contact (2). With sustained signaling to the T cell, intercellular conjugation can last for hours in vitro (3). Although the immunological synapse has not been fully resolved in vivo (4, 5), recent time-lapse imaging of lymphoid tissue suggests that an activated T cell undergoes sustained interaction with antigen-bearing dendritic cells (DCs) during the interval preceding its first cell division (5–11).

If synapse-like polarity occurs during the prolonged contact with DCs that precedes the first T cell division, it is possible that asymmetry might ensue. Stable orientation of the original microtubule organizing center (MTOC) in a synapse-proximal position through the time of mitosis would result in the duplicate centrosome moving to the distal pole during prophase. If segregation of signaling components was also maintained during the interval of sustained contact, then their orientation at a pole of the mitotic spindle would fulfill the hallmarks of asymmetric division. Consequently, we explored the possibility that asymmetric cell division can initiate differentiation associated with the adaptive immune response of T cells.

Segregation of immune receptors and ancestral polarity proteins in mitotic T cells. Whether initial cytokinesis of naïve T cells occurs during contact with DCs, or after dissociation from DCs, has not been resolved (5, 8, 12, 13). We therefore tested the potential for asymmetric division by isolating T cells that appeared to be preparing for their first division in vivo and examining their subcellular characteristics ex vivo (14). Naïve mouse CD8+ T cells transgenic for the P14 T cell receptor were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and adoptively transferred into wild-type recipients. These recipients had been infected 24 hours previously with intravenous recombinant Listeria monocytogenes bacteria expressing a specific antigenic gp33-41 peptide epitope (gp33–L. monocytogenes) (14). At 32 hours after transfer, undivided donor T cells (represented by the brightest CFSE peak)

were sorted by flow cytometry and examined by confocal microscopy. This approach ensured that we were examining T cells preparing for their first division.

Undivided (parental) T cells were stained with antibodies against β-tubulin and various T cell signaling proteins and readily identified as activated blasts by their increased cell size (fig. S1). Most of the blasts contained a single MTOC, indicating that they were activated, but premitotic, cells (Fig. 1A, far left). In more than 90% of such cells, the centrosome colocalized with a polarized patch containing leukocyte function-associated antigen–1 (LFA-1), CD8, and CD3 (Fig. 1A, far left). This polarity was not a direct result of the adoptive-transfer process, because transferred naïve T cells that had not been exposed to antigen exhibited diffuse staining of the same receptors (fig. S2). In contrast to the polarity exhibited by components known to participate in immunological synapse formation, the localization of CD90, a nonsynaptic transmembrane receptor, was found to be diffuse in T cells from infected recipients (Fig. 1A).

Although much less frequent than the premitotic blasts, mitotic cells (those in prophase through anaphase) could be identified by the presence of two oppositely facing MTOCs (Fig. 1A). Like their premitotic counterparts, these mitotic T cells exhibited pronounced polarized distribution of LFA-1, CD8, and CD3 (Fig. 1A). As with premitotic blasts, the synaptic components colocalized with one MTOC, which consequently represented one of the two poles of the mitotic spindle (Fig. 1A). The initial cell divisions we observed in this experimental setting of CD8+ T cell responses were, therefore, characterized by partitioning of signaling proteins perpendicular to the mitotic spindle, a morphology reminiscent of asymmetric cell division.

We next examined if asymmetric T cell division could also be detected in CD4+ helper T cell responses. Leishmania-specific T cell receptor (TCR) transgenic CD4+ T cells were labeled with CFSE and adoptively transferred into wild-type recipients that had been infected 24 hours earlier with L. major (14). At 32 hours after the transfer, undivided blasts were examined for the localization of LFA-1, CD4, and the receptor for interferon-γ (IFNγR), which polarizes to the immunological synapse during activation of CD4+ T cells in vitro (15). All three receptors colocalized with the MTOC of premitotic blasts and remained asymmetrically partitioned in mitotic CD4+ T cells in vivo (Fig. 1C). Asymmetric segregation of signaling molecules may, thus, be a feature of both major T cell subsets undergoing the first division during an immune response.

On the basis of their suggested role during T cell migration and activation in vitro (16), we next examined the localization of ancestral regulators of asymmetric cell division, protein kinase C–ζ (PKCζ) and Scribble, in CD8+ T cells preparing for division. PKCζ is a mamma-

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lian homolog of atypical PKC, a component of an essential cell polarity complex containing Par-3 and Par-6 (1, 17). Scribble is a signature component of another key polarity complex that is often positioned opposite the atypical PKC-containing complex (17). In premitotic T cell blasts, PKCζ was observed to localize opposite the MTOC and persisted into mitosis at this asymmetric position at the putative distal pole (Figs. 1A and 2). In contrast, Scribble colocalized with the MTOC at the putative synaptic pole of premitotic blasts (fig. S3), and this, too, persisted into mitosis (Fig. 2). Thus, an ancestral polarity network appeared to be established before mitosis and persisted into M phase with characteristic orientation at opposite poles of the mitotic spindle.

Sustained synapsis required for asymmetry during mitosis. To determine whether the asymmetry we observed in the initial mitotic T cell

was related to its sustained contact with pathogen-associated DC before division, we examined two alternative stimuli eliciting cell division in vivo. In the first, transgenic T cells were transferred into lymphopenic RAG1-deficient recipients to initiate cell division in the absence of pathogen. This form of division is called acute homeostatic proliferation and does not depend on specific recognition of foreign antigen by T cells, but instead is initiated in response to self-antigen in an environment devoid of other lymphocytes (18). In the second model, a foreign microbial stimulus was used, but in recipients lacking intercellular adhesion molecule 1 (ICAM1) and so unable to support LFA-1–dependent interactions between T cells and DCs (19). This mutation was chosen because such integrin-cadherin interactions have been shown to be critical for organizing asymmetric cell division in mammalian skin (20) and because DCs lacking ICAM1

do not support the sustained intervals of T cell contact that characterize productive immune responses (21).

In both the uninfected lymphopenic mice and the infected ICAM1-deficient recipients, vigorous cell division of the transferred T cell population was supported (Fig. 3C, fig. S4). There was, however, a substantial loss of asymmetry in mitotic T cells retrieved from the mutant mice, with diffuse distribution of synaptic and polarity proteins and no apparent orientation of these proteins to the mitotic spindle (Fig. 1, A and B). These findings suggest that events during the prolonged contact between T cell and DC preceding the first T cell division, rather than general mitogenic signaling, are required to establish asymmetry.

Asymmetric inheritance of fate determinants in the first daughter cells. To determine if asymmetric segregation of synaptic proteins
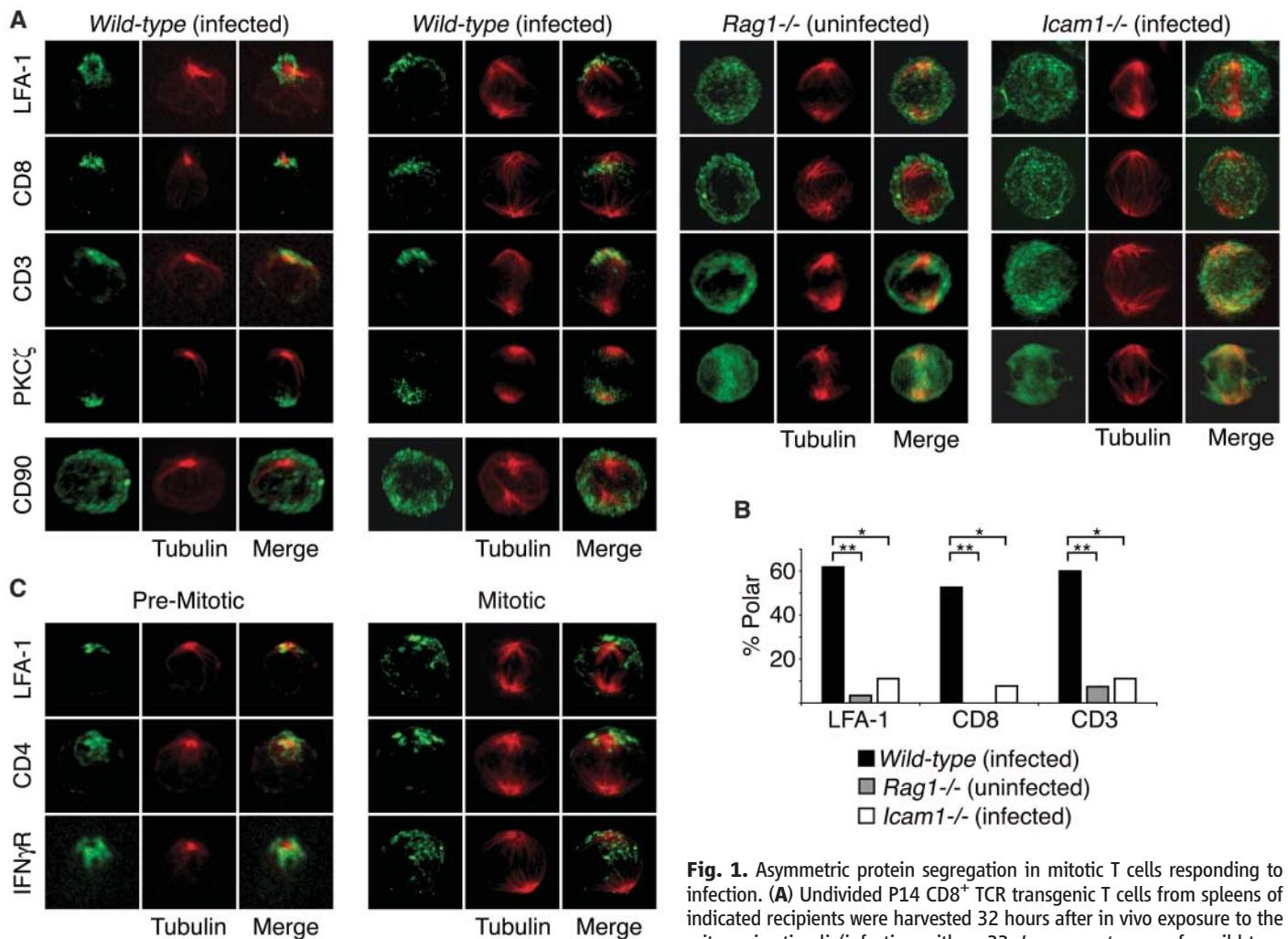
Fig. 1. Asymmetric protein segregation in mitotic T cells responding to infection. (**A**) Undivided P14 CD8⁺ TCR transgenic T cells from spleens of indicated recipients were harvested 32 hours after in vivo exposure to the mitogenic stimuli (infection with gp33–L. monocytogenes for wild-type and Icam1⁻/⁻ mice; lymphopenia for Rag1⁻/⁻ mice) and examined by confocal microscopy after staining for β-tubulin (red) and LFA-1, CD3, CD8, PKCζ, or CD90 (green). Panels are representative of nine or more mitotic cells per group. Far left panels are premitotic blasts from infected wild-type recipients (large cell, but single MTOC); all remaining panels are mitotic cells (dual, oppositely facing MTOCs). (**B**) Quantification of polarized receptors on mitotic CD8⁺ T cells represented in (A). The number of mitotic cells examined for each receptor (per group of wild-type, Rag1⁻/⁻, and Icam1⁻/⁻ mice, respectively) was as follows: LFA-1 (21, 30, 9); CD8 (19, 27, 13); CD3 (15, 27, 9). *P ≤ 0.025; **P ≤ 0.001. (**C**) Undivided donor Leishmania-specific CD4⁺ TCR transgenic T cells from infected wild-type recipients were harvested from draining lymph nodes after 32 hours and stained with antibodies against β-tubulin (red) and LFA-1, CD4, or IFNγR (green). Polarity of LFA-1, CD4, and IFNγR was observed in 60% (n = 15), 78% (n = 9), and 71% (n = 14) of mitotic cells, respectively.

persists through mitosis, we examined cells undergoing cytokinesis. Sorted, undivided T cells that had already responded to various in vivo stimuli were cultured in media containing cytochalasin B, to inhibit actin polymerization, before imaging (*14*). This resulted in the arrest of cytokinesis in parent T cells attempting to divide, yielding conjoined twin daughter sets. Costaining of CD8 and PKCζ revealed unequal protein inheritance in daughter T cells subsequent to the in vivo immune response (Fig. 3A). CD8 and PKCζ consistently segregated to opposite daughters, in accord with the synapse-distal localization of PKCζ in premitotic and mitotic blasts. In contrast, cells undergoing acute homeostatic proliferation, representing non–antigen-driven cell division, displayed uniform inheritance of CD8 and PKCζ to both daughters (Fig. 3A). Similar results were obtained from cytokinetic cells examined directly ex vivo that were not cultured in cytochalasin B (fig. S5).

The asymmetric segregation of CD8 between conjoined twin daughter cells suggested that it might be possible to measure flow cytometric evidence for this receptor disparity in nascent daughter populations. In infected wild-type recipients, bimodal distribution of CD8 and LFA-1 staining was evident in bulk populations of daughter T cells (Fig. 3B). In contrast, daughter T cells that had emerged from homeostatic proliferation exhibited a unimodal distribution of co-receptor staining (Fig. 3C). The bimodal staining evident in the first T cell division of the immune response may, thus, have represented proximal and distal daughter populations, with a greater and lesser abundance of synaptic receptors, respectively. However, why the putative distal daughter was not observed to proliferate substantially beyond the first division (Fig. 3, B and C) is not evident and remains to be explored.

To ascertain whether signaling pathways involved in cell fate might be asymmetrically partitioned in CD8$^+$ T cells, we examined IFNγR in cells responding to gp33–*L. monocytogenes*. Consistent with its proximal location in blasts (fig. S6), preferential inheritance of IFNγR was observed in the putative proximal daughter of conjoined twin sets, based on its segregation away from the putative distal daughter, which inherits the greater portion of PKCζ (Fig. 4). Early in the immune response, the ligand for IFNγR might be secreted by CD8$^+$ T cells themselves (fig. S6), neighboring natural killer cells, or DCs (*22*). In activated CD8$^+$ T cells, IFN-γ signaling induces the key transcription factor, T-bet (fig. S7), which is preferentially expressed in effector cells compared to the memory lineage (*23, 24*). It is thus conceivable that differences in IFN-γ signaling could directly or indirectly influence fate-associated gene expression in the first daughter T cells during an immune response.

Unequal inheritance of Numb, an inhibitor of Notch signaling, is another ancestral hallmark of asymmetric cell division (*1*). In conjoined twin cells, we observed preferential inheritance of Numb in the proximal daughter, based on its cosegregation to daughters that inherited greater CD8 (Fig. 4). Inheritance of Numb by proximal daughters was consistent with its reported localization at the immunological synapse in vitro (*25*). Disparity in Notch signaling could contribute to initial fate divergence, because Notch ligands have been found to be induced on pathogen-stimulated DCs (*26*). Like the asymmetric inheritance seen for IFNγR, that of Numb appeared specific to daughter cells responding to infection, because only symmetric segregation of these proteins was detected in the conjoined twin sets arising from acute homeostatic division (Fig. 4). Segregation of critical compo-

nents of two fate-determining signaling pathways thus provides a possible mechanism for asymmetric T cell division to generate intraclonal diversity during the immune response.

Asymmetric division yielding effector- and memory-fated progeny. Consistent with the predicted role for asymmetric cell division in specifying different daughter fates, we observed a pronounced disparity in phenotypic markers of effector and memory cells between the putative proximal and distal daughters, respectively. The putative proximal daughters (possessing more abundant CD8) were larger in size, had increased granularity, and expressed low levels of CD62L but higher levels of CD69, CD43, CD25, and CD44 (Fig. 5A, fig. S8), a profile consistent with the effector lineage (*27*). In contrast, distal daughters (those with less abundant CD8) had a profile more consistent with the central memory lineage (*27*); these cells were smaller, less granular, and expressed high levels of CD62L but lower levels of CD69, CD43, CD25, and CD44 (Fig. 5A, fig. S8). We also observed coordinate functional disparity in proximal and distal daughters. Proximal daughters exhibited greater expression of the effector gene products, IFN-γ and Granzyme B (Fig. 5B), whereas distal daughters exhibited greater expression of interleukin-7 receptor α (IL-7Rα) mRNA (Fig. 5C), a marker of early memory precursors (*28*).

A prediction of these observations is that the effector-fated daughters will terminally differentiate, without substantial regenerative capacity. In contrast, memory-fated daughters would retain long-term developmental plasticity to both self-renew and terminally differentiate, consistent with the recognized features of memory T cells (*29*). Consequently, both effector and memory precursors should be capable of protection against an acute challenge, whereas delayed challenge should be better controlled by the memory-like precursors (*28*). To test this prediction, we sorted putative proximal and distal daughters from primary recipients and transferred them to a new set of naïve secondary recipients that were challenged immediately or at 30 days after transfer with gp33–*L. monocytogenes* to assess protection (*14*). At 30 days after transfer, distal daughters were indeed found to provide measurably better protection than was found with proximal daughter T cells (Fig. 5D). However, proximal daughters provided equal or better protection than their distal counterparts when challenged immediately after transfer (Fig. 5D). The functional properties of the first two daughters were thus consistent with our assignment as effector and memory precursors on the basis of phenotypic and functional markers.

Discussion. The function of the immunological synapse, as it has been studied in vitro, has remained enigmatic (*30*). The emerging recognition that the parent T cell and DC undergo prolonged contact in vivo relatively late after initial activation (*5–11*) prompted us to consider the possibility of a previously unknown function

**Fig. 2.** Bipolar segregation of conserved regulators of asymmetric cell division during mitosis. Cells from infected wild-type recipients were harvested as in Fig. 1A and stained for CD3 (green), β-tubulin (blue), and LFA-1, PKCζ, or Scribble (red). Colocalization of CD3 with LFA-1 and Scribble was observed in 94% (*n* = 16) and 100% (*n* = 10) of cells, respectively. PKCζ was polarized opposite CD3 in 90% (*n* = 10) of cells.
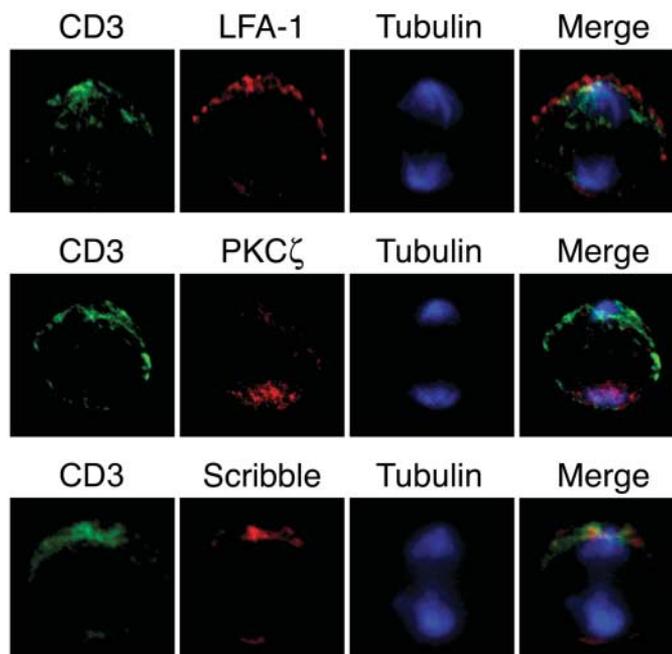
Fig. 3. Disparity in synaptic and polarity protein inheritance of daughter CD8$^+$ T cells. (**A**) Undivided P14 transgenic CD8$^+$ T cells from infected wild-type or uninfected $Rag1^{-/-}$ recipients were cultured in media containing cytochalasin B for 4 hours at 37°C to arrest cytokinesis. Cells were then stained for CD8 (green), PKCζ (red), β-tubulin (blue), and DNA (grayscale). Cells undergoing cytokinesis were identified by pronounced cytoplasmic cleft by brightfield and dual nuclei and were scored for asymmetric segregation of staining between conjoined daughter cells. Merges of CD8 with PKCζ were superimposed onto both the tubulin and brightfield images (fourth and seventh columns, respectively). Asymmetric segregation of CD8 in wild-type versus $Rag1^{-/-}$ mice occurred in 69% ($n = 29$) versus 14% ($n = 35$) of twin sets, respectively. The percent asymmetric segregation of PKCζ was 62% ($n = 34$) in wild-type and 14% ($n = 29$) in $Rag1^{-/-}$ mice. For both molecules, the incidence of asymmetry was significantly greater in cells from wild-type compared to $Rag1^{-/-}$ mice ($P \leq 0.001$). In costaining experiments where both molecules asymmetrically partitioned, CD8 and PKCζ segregated to opposite daughters in 100% ($n = 9$) of wild-type twin sets. (**B**) Differential abundance of synaptic proteins in the first daughter T cells responding to infection. CFSE-labeled transgenic CD8$^+$ T cells from infected wild-type recipients were analyzed by flow cytometry 48 hours after infection. Division, represented by CFSE dilution (x axis), and receptor abundance (y axis) of all transferred cells (left upper panel) and CD8$^+$ transferred cells (right upper panel) are shown. Receptor abundance (x axis) of electronically gated daughter cells (second brightest CFSE peak only) is displayed below. (**C**) Comparison of CD8 abundance in daughter cells arising after infection (left) versus acute homeostatic division (right).

Fig. 4. Asymmetric inheritance of fate determinants in the first daughter T lymphocytes. Cells obtained and analyzed as in Fig. 3A were costained for IFNγR and PKCζ (upper rows) or CD8 and Numb (lower rows), plus β-tubulin and DNA. Asymmetric segregation of IFNγR in wild-type versus $Rag1^{-/-}$ mice occurred in 77% ($n = 13$) versus 7% ($n = 14$) of twin sets, respectively. The percent asymmetric segregation of Numb was 67% ($n = 18$) in wild-type and 13% ($n = 15$) in $Rag1^{-/-}$ mice. For both IFNγR and Numb, the incidence of asymmetry was significantly greater in cells from wild-type compared to $Rag1^{-/-}$ mice ($P \leq 0.001$). In costaining experiments where both molecules asymmetrically partitioned, IFNγR and PKCζ segregated to opposite daughters in 100% ($n = 5$) of wild-type twin sets, while CD8 and Numb segregated to the same daughter in 100% ($n = 8$) of wild-type twin sets.

for the synapse; namely, that it helps coordinate asymmetric cell division. At its simplest level, a mechanism for asymmetric cell division might provide a solution to an essential problem facing adaptive immunity. If an immune response were to begin from the activation and proliferation of a single antigen-specific naïve T cell, simultaneous assignment of effector and memory properties to two different daughters would ensure acute elimination of the microbe, while preserving
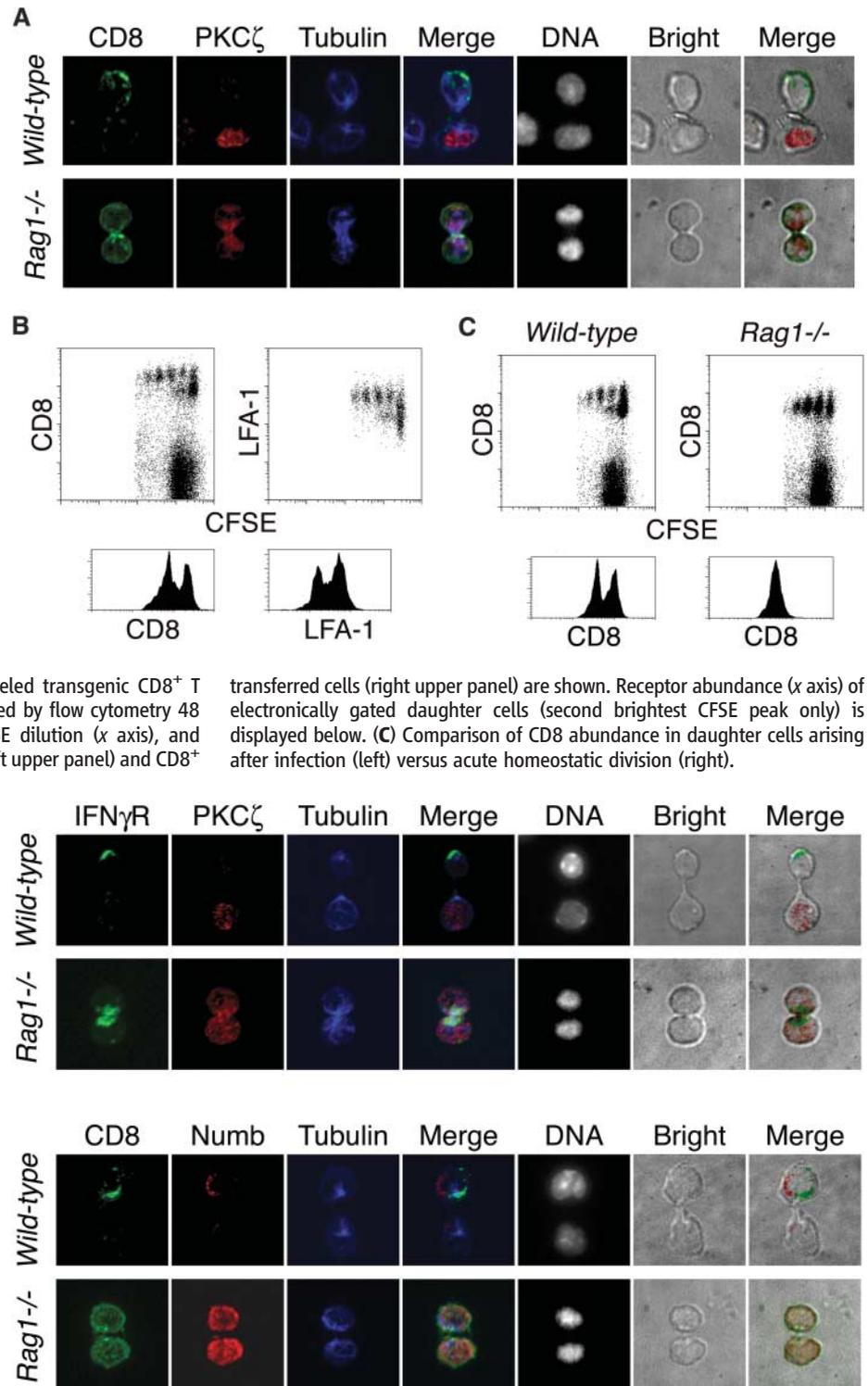
the useful clone through a memory lineage in the event that all effector cells die. After resolution of infection, the slow, cytokine-driven proliferation of memory T cells might then involve symmetric divisions to maintain stable numbers of similarly fated daughter cells. This might be akin to the symmetric homeostatic division we observed for naïve T cells transferred to antigen-free, lymphopenic mice. Upon secondary exposure to the pathogen, antigen-presenting DCs might

then coordinate another episode of asymmetry in the memory T cell, leading to a terminally differentiated effector lineage, as well as a self-renewing memory lineage. Such a model is consistent with the stem cell–like features that have been proposed for memory T cells (*29*, *31*) and is one that we are currently investigating by examining the morphology and phenotype of memory T cells and their daughters in the response to a secondary challenge. Whether the

Fig. 5. Daughter CD8$^+$ T cells acquiring disparate fates during immunity. (**A**) CFSE-labeled CD8$^+$ T cells were analyzed as in Fig. 3, B and C, with antibodies against CD8 and one of the following molecules: CD62L, CD69, CD43, CD25, or CD44. Phenotypic markers of effector and memory cells (*x* axis) and CD8 abundance (*y* axis) of electronically gated daughter cells (second brightest CFSE peak only) are shown. Cell size represents forward light scatter; granularity represents side light scatter. Fluorescence intensity of surface receptor staining is listed in fig. S8. (**B**) Cells were analyzed as in (A), with antibodies to detect intracellular IFN-γ or Granzyme B. (**C**) IL-7Rα mRNA was measured by means of real-time reverse transcription–polymerase chain reaction from sorted proximal and distal daughters. (**D**) Sorted proximal and distal daughters were transferred into new naïve recipients. Untransferred control and recipient mice were challenged with *L. monocytogenes* either immediately (Acute) or 30 days (Delayed) after transfer.

Four days after infection, quantitative bacterial burdens in spleens were determined. Mean bacterial burden of recipient mice (left to right, *n* = 3, 4, 4, 4) is expressed as a percentage relative to the burden of untransferred control mice. All groups were significantly protected relative to untransferred controls (*P* ≤ 0.006). After delayed challenge, distal daughters afforded significantly better protection than proximal daughters (**P* = 0.03).
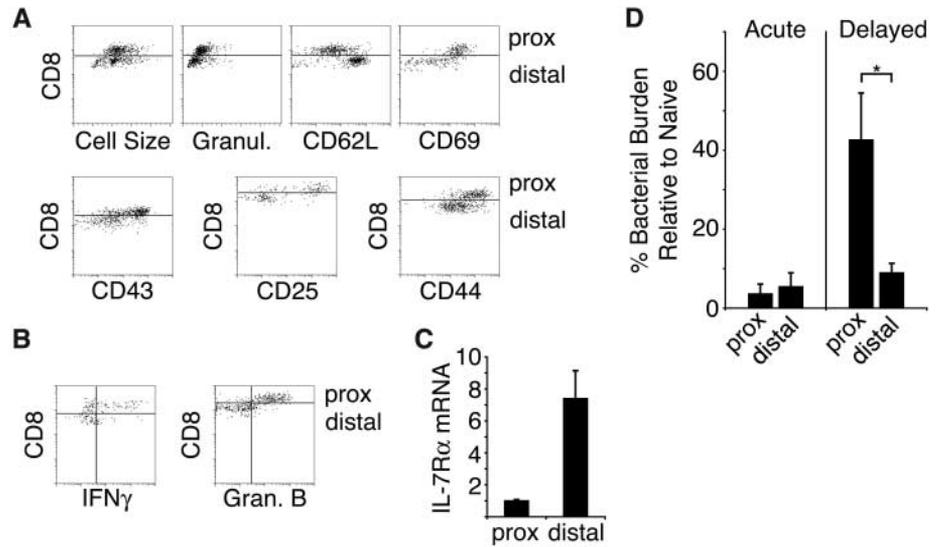
asymmetric daughters of naïve T cells are actually fated to become specialized subsets other than effector and memory cells, moreover, cannot be excluded at this time.

Although the progeny of a newly activated CD8$^+$ T cell may be capable of dividing and differentiating without further exposure to antigen (*32*, *33*), it has been suggested that efficient CD4$^+$ T cell responses may require continued exposure to antigen by the initial daughter cells (*12*, *34*), raising the possibility that more than one round of asymmetric division might occur. Reiterative rounds of asymmetry during the initial clonal expansion of a CD4$^+$ T cell might, thus, facilitate the diversification of effector choices [T helper cell 1 (T$_H$1), T$_H$2, and T$_H$17] in addition to, or instead of, simply generating effector and memory progeny. In this way, the spectrum of CD4$^+$ T cell effector fates, which arise only after cell division (*35*), might all be represented in the initial clonal burst. Thereafter, only the most useful of the diverse progeny might undergo further selection, on the basis of the type of pathogen encountered. Indeed, the determinants that are segregated in the initial CD8$^+$ T cell division are also presumptive regulators of CD4$^+$ T$_H$1-T$_H$2 lineage choice (*15*, *26*, *36–39*). With ever-improving methodology for in vivo imaging and lineage tracing, it may soon be possible to construct a detailed fate map of a single T cell clonal burst during a variety of immune challenges. For now, however, the role of asymmetric cell division in diversifying CD4$^+$ T cell fate remains to be formally evaluated.

Although information from the environment is essential in choice of cell fate, the facultative milieu of a migrating cell has often made it difficult to identify the most critical signals. It is tempting to speculate that the prolonged interlude with the antigen-presenting cell, which seemingly coordinates the asymmetry of T cell division,

might be mirrored in the behavior of other dividing lymphocytes and in the interactions of metastatic cancer cells with the cellular or extracellular components of their provisional niche. There remain numerous avenues to formally test the prevalence and importance of asymmetric cell division in adaptive immunity and in mammalian development and homeostasis. Extensions of the framework presented here might, thus, expand the principle of asymmetric cell division to various stages of B and T lymphocyte immunity, or provide insight into the elusive circumstances preceding asymmetric division of hematopoietic and neoplastic stem cells (*40*, *41*).

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42. We thank M. Birnbaum and E. Williamson for the generous use of and assistance with the confocal microscope; and N. Takemoto, J. Northrup, A. Pasam, K. Pham, L. Ng, S. Daxini, and J. Laverty for assistance and discussion. Supported by NIH grants AI042370, AI053827, and AI061699 and the Abramson Family (S.L.R.); NIH grants CA114114 and AI069380 (W.W.); NIH training grants DK007066 (J.T.C.), AI055428 and GM007170 (V.R.P.), AI007532 (A.M.I.), and CA87812 (A.B.); and the Peter MacCallum Cancer Centre, National Health and Medical Research Council, and Australian Research Council (S.M.R.).