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Rigidity of Circulating Lymphocytes Is Primarily Conferred by Vimentin Intermediate Filaments

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Lymphocytes need rigidity while in circulation, but must abruptly become deformable to undergo transmigration into tissue. Previously, the control of leukocyte deformability has been attributed to microfilaments or microtubules, but the present studies demonstrate the greater importance of vimentin intermediate filaments (IFs). In circulating T lymphocytes, IFs form a distinctive spherical cage that undergoes a rapid condensation into a juxtanuclear aggregate during chemokine-induced polarization. Measurements of the resistance of peripheral blood T lymphocytes to global deformation demonstrate that their rigidity is primarily dependent on intact vimentin filaments. Microtubules, in contrast, are not sufficient to maintain rigidity. Thus, vimentin IFs are a primary source of structural support in circulating human lymphocytes, and their regulated collapse is likely to be an essential element in chemokine-induced transendothelial migration. The Journal of Immunology, 2001, 166: 6640–6646.

Materials and Methods

Purification of CD4+ human T lymphocytes

PBMC were obtained from buffy coats derived from healthy donors by centrifugation through lymphoprep (ICN, Aurora, OH). Enriched populations of resting, CD4+ PBT were isolated from PBMC by immunomagnetic negative selection, essentially as described previously (14). The resulting resting CD4+ T cells (>95% purity) were suspended in medium (HBSS, without phenol red, containing 10 mM HEPES and 0.2% BSA) and either used immediately or held in suspension by rotation at 4°C for up to 24 h. Cells were placed in polypropylene microcentrifuge tubes at 10^7/ml and prewarmed to 37°C on a rocking platform for at least 1 h before use in experiments.

Mouse splenic T lymphocyte cultures

The vimentin knockout (KO) mouse (Vim1 mutation) has been previously described (15). Control 129/Sv mice were generously provided by Linda Lowe (National Cancer Institute). All animals were treated according to National Institutes of Health guidelines. Mice were sacrificed at 6–10 wk of age, and spleens were removed. Single cell suspensions were treated with ACK lysis buffer and washed. Bulk cultures of mouse spleen cells were initiated at a density of 2 × 10^6 cells/ml in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Summit Biotechnology, Ft. Collins, CO), MEM nonessential amino acids, 20 mM HEPES buffer, 1 mM pyruvate, 1 mM M 2-ME (Sigma, St. Louis, MO), 20 U/ml human rIL-2 (Hoffmann-LaRoche, Nutley, NJ), 10% rat T-STIM (Becton Dickinson Labware, Mountain View, CA), and 5 μg/ml Con A (Sigma). After 40 h of culture at 37°C, 5.9% CO2, and 99% relative humidity, the cultures were diluted 5-fold in the same medium lacking Con A. Cultures were subsequently rediluted as needed to maintain a density of 0.3–2 × 10^6 cells/ml. Splenocyte cultures were maintained for up to 14 days, and cells were used for experimentation between days 6 and 14. Flow cytometric analysis of cultures at days 6 and 8 demonstrated that the cells present were virtually all T lymphocytes.

Cytoskeletal manipulations

Suspended PBT were polarized by stimulation with 100 ng/ml recombinant human stromal cell-derived factor-1α (SDF-1α; SDF-1α; PeproTech, Rocky Hill, NJ) for 3 min or by 2-h exposure to 100 μg/ml colchicine or 10 μg/ml nocodazole (Calbiochem, La Jolla, CA) at 37°C. Microfilaments and microtubules were disrupted in spherical resting PBT by transferring cells from 37°C to 4°C for 30 min. IFs were disrupted by exposure to 50 nM calyculin A (Calbiochem) for 30 min at 37°C, followed by 30 min at 4°C. Microtubules were stabilized by 1.5-h pretreatment at 37°C with 10 μM...
taxol (Paclitaxel; Calbiochem), followed by 30 min at 4°C, with and without calyculin A treatment. Control cells remained at 37°C for an equal time after addition of equal volumes of diluent. To confirm microtubule and microfilament disruption by cold treatment, 100 μg/ml colchicine (Calbiochem) or 1 μM latrunculin B (Calbiochem) was added 1.5 h and 30 min, respectively, before transfer of cells to 4°C. Cells remained largely spherical in suspension with all treatments, and viability was >90% by trypan blue exclusion.

Cell deformation and immunofluorescence

Following cytoskeletal manipulations, cells were fixed for 10 min by transfer to 6-ml polypropylene tubes containing an equal volume of 4% paraformaldehyde (PFA) in Dulbecco’s PBS without calcium and magnesium at the same temperature. After washing twice in PBS, cells were aliquoted into 24-well tissue culture plates (10^5/well) containing 12-mm No. 1 coverslips, precoated overnight at 37°C with 100 μg/ml poly(t-Lysine) (PLL; Sigma) in borate buffered saline, and washed with distilled water before use. Each experiment was contained within a single 24-well plate. After addition of cells, plates were centrifuged in a Sorvall RT6000 centrifuge at 200 × g for 10 min. In experiments using live cells, plates and centrifugation were chilled to 4°C. Following centrifugation, plates were drained and cells fixed to the PLL substratum by addition of 4% PFA for 20 min. Chemokine- or colchicine-polarized cells were initially fixed in 3% PFA, washed, allowed to adhere to PLL at 1 × g for 30 min, and then fixed to the PLL with 2% PFA. Fixed plates were washed three times in PBS, and cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS and blocked for 1 h in PBS containing 1% BSA and 5% normal donkey serum (DS; Jackson ImmunoResearch, West Grove, PA) at room temperature. Cells were labeled for 1 h at room temperature in PBS/BSA/DS with monoclonal anti-vimentin (clone V9; Sigma), anti-CD45 (mAb 9.4; Pharmingen, San Diego, CA) and FITC-conjugated streptavidin (Jackson ImmunoResearch). Cells were permeabilized and labeled with Alexa-488-conjugated phalloidin (Molecular Probes), and relative F-actin content was quantitated by flow cytometry. Mouse splenocytes were processed for deformability analysis and flow cytometric analysis of F-actin, as described above. Immunofluorescent staining was performed as above, with the following exceptions: vimentin was detected using polyclonal goat anti-vimentin C-20 (Santa Cruz), and CD45 by biotinylated rat anti-CD45 mAb 30-F11 (PharMingen, San Diego, CA) and FITC-conjugated streptavidin (Jackson ImmunoResearch).

Confocal microscopy and deformability analysis

Samples were examined on a Zeiss LSM-410 laser-scanning confocal microscope using a 100×, 1.4 N.A. planapochromat objective and pinhole settings providing optical sections of ~0.7 μm. While viewing fields of CD45-labeled cells at an equatorial focal plane, individual cells without apparent damage or contact with other cells were randomly selected. Selected cells were then magnified using a ×8 optical zoom factor, and the smallest rectangular region of interest that could enclose the cell was drawn while focused on the adherent surface. An automated macro then acquired two perpendicular Z-scans (XZ and YZ profiles) crossing the geometric center of the XY region of interest. Digitized cell profiles were imported to Image Pro-Plus (Media Cybernetics, Silver Spring, MD), in which the width and height of each profile were measured manually by single blind analysis. Width/height division was done to control for inherent as well as potential cytoskeletal manipulation-induced variations in cell diameter. A deformability index for each cell was calculated as the average width/height ratio of the XZ and YZ profile pair. Multiple experiments were averaged, standard error calculated, and the data then normalized to the control group within each experiment set (either 37°C or 4°C controls). Projection images of cytoskeletal elements in deformed samples and chemokine- or colchicine-polarized cells were generated from serial sections (~0.7-μm section thickness) using Zeiss LSM-410 software. Samples comparing the distribution and structure of vimentin and lamin B were acquired using identical brightness and contrast settings.

Results

PBT polarize in vitro in response to a variety of stimuli, including the chemokine SDF-1α. Chemokines are an important signal regulating leukocyte polarization and extravasation in vivo, and can be used with in vitro model systems to study the rapid cytoskeletal remodeling underlying lymphocyte polarization (16). Each of the three cytoskeletal systems in resting PBT has a characteristic geometry (Fig. 1, a–c): microfilaments are pervasive in the submembranous region and enriched in peripheral processes such as microvilli (α); microtubules radiate from the microtubule organizing center (MTOC) (b); vimentin IFs form a coarsely woven cage throughout the cytoplasm (c). Cytoskeletal reorganization begins within seconds after lymphocyte stimulation with SDF-1α (Fig. 1, d–g). A tail-like projection, or uropod, develops within 1 min of stimulation. Simultaneously, F-actin-rich pseudopodia become prominent at the leading edge of the cell (d). The IF cytoskeleton is rapidly reorganized from the cytoplasmic network into a condensed juxtanuclear aggregate, which forms near the MTOC and occupies much of the uropod (e). Microtubules retract toward and become concentrated within the base of the uropod, but to a lesser degree than do the vimentin IFs (f).

In contrast to microfilaments and microtubules, lymphocyte IFs remain largely unstudied. There is no established function for the vimentin IF network of circulating lymphocytes, nor for its rapid redistribution and compaction during lymphocyte polarization. Because circulating lymphocytes exist in a mechanically stressful environment, we hypothesized that the IF network provides mechanical support to the cell. Therefore, we examined the contributions of vimentin IFs to the rigidity of resting, spherical lymphocytes. We adapted an assay (17) that assesses global cell deformability by...
FIGURE 2. Analysis of PBT deformability after cytoskeletal manipulations (a). Representative confocal XZ and YZ profiles (Z-scans) of anti-CD45-labeled PBT following high g-force centrifugation onto adhesive substrate, control (37°C) compared with sample treated with phosphatase inhibitor calyculin A (CA) at 4°C. Dashed boxes in lower panels illustrate measurement of relative width (adherent surface) vs height of cells following deformation. b, Flow cytometric quantitation of F-actin depolymerization by cold exposure compared with 37°C controls and cold plus 1 μM latrunculin B. Data represent mean FITC-phalloidin intensity from 40,000 cells from four donors/experiments, ±SEM, normalized to 37°C control. c, Projection images of deformed cells demonstrating microtubule disruption (top row, MT) after cold exposure compared with microtubules stabilized by taxol pretreatment. Disruption of vimentin IFs by exposure to calyculin A (bottom row, IF). Bars = 5 μm.

Analyzing the extent of deformation during centrifugation. Suspended cells, either live or briefly fixed, were centrifuged at high g-force onto PLL-coated coverslips. Cells are compressed against the coverslip during centrifugation, and the highly adhesive PLL surface preserves the contact imprint following centrifugation. After fixation to the PLL and fluorescent labeling of the plasma membrane, the extent of cell deformation was analyzed by confocal microscopy. A representative example is shown in Fig. 2a, in which the deformability of two PBT preparations is compared: resting PBT at 37°C (all cytoskeleton intact) vs cells at 4°C pretreated with the phosphatase inhibitor calyculin A (all cytoskeleton disrupted, see below). Analysis of the width vs height of cells in the control preparation reveals that the normal cell is virtually spherical, indicating significant resistance to deformation. In contrast, the specimen with all three filaments disrupted shows diminished height, and broadened width at the base, indicating deformation during centrifugation. For quantitative comparisons, we calculated a deformability index as the average width/height from paired XZ-YZ profiles of each cell; in the example shown, the deformability index for the control cell is 0.93 compared with 1.66 for the 4°C plus calyculin A sample.

To evaluate the contributions of vimentin IFs and other cytoskeletal elements to lymphocyte rigidity using this assay, it was necessary to establish experimental conditions that selectively disassembled subsets of cytoskeletal filaments while preserving spherical shape and cell viability. Placing PBT at 4°C for 30 min was highly effective for disrupting both F-actin and microtubules. Cold exposure causes an ~80% loss of F-actin (Fig. 2b) as well as a substantial disruption of the microtubule network (Fig. 2c), while leaving the cells spherical and IFs intact. Vimentin IFs were disrupted by exposure to the protein phosphatase inhibitor calyculin A. Because vimentin disassembly is regulated by phosphorylation (18), treatment of cells with the calyculin A completely abolished IF structure (Fig. 2c). Alternative methods for disrupting IFs, including acrylamide exposure (19) and microinjection of vimentin-disruptive mimetic peptides (20), were ineffective, toxic, or impractical for use with spherical PBT. Pretreatment with the microtubule-stabilizing agent taxol prevented microtubule depolymerization during cold plus calyculin A exposure. However, attempts to preserve F-actin during cold exposure using the stabilizing agent jasplakinolide were not successful, as cell morphology and the distribution of F-actin became highly irregular.

Analyzing PBT rigidity using the conditions described above, we find distinct changes in deformability following disruption of different cytoskeletal elements. The combined disruption of both the actin and microtubule cytoskeletons by cold treatment leads to a small (~14%) but statistically significant increase in cell deformability (Fig. 3a). By comparison, subsequent disruption of vimentin IFs by calyculin A results in a pronounced (additional ~38%) increase in lymphocyte deformability. Similar results were obtained using live cells not fixed before centrifugation. The use of fixed cells was necessary to permit comparisons between 37°C (all cytoskeleton intact) and 4°C samples. As shown above, PBT F-actin is very temperature sensitive. Fixation before centrifugation allowed both 37°C and 4°C samples to be processed within a single multiwell plate without the risk of temperature-dependent changes occurring during the procedure. Although fixed cells are more rigid than live, presumably due to protein cross-linking, the increase in deformability following IF disruption is nearly identical in the two preparations (Fig. 3b). These data show that IFs, in the absence of microfilaments or microtubules, are sufficient to provide significant resistance to deformation by external force. The decreased rigidity observed following the codisruption of microfilaments and microtubules indicates that they also contribute to the rigidity of the spherical cell, either independently or via interactions with each other or other cellular components, but to a much lesser extent than do the IFs. To ensure that cold treatment was sufficient to fully disrupt both microtubules and F-actin, cells were pretreated with colchicine or the actin-depolymerizing agent latrunculin B; no increase in cell deformability compared with cold treatment alone was observed (data not shown).

The rigidity of the nucleus may be an important determinant of cellular deformability, particularly in cells such as lymphocytes that have a high nuclear to cytoplasmic volume ratio (17). The inner surface of the nuclear membrane is lined with a meshwork of protein called the nuclear lamina, a function of which may be mechanical support (21, 22). A major component of lymphocyte nuclear lamina is the type V IF protein lamin B (23). Although the lamin B nucleoskeleton can be disassembled by serine phosphorylation (24), this does not occur in lymphocytes during the calyculin A treatment that we used to disassemble vimentin (Fig. 4). Furthermore, Western blot analysis shows that lamin B remains insoluble following calyculin A treatment, while the cytoplasmic...
vimentin IFs are solubilized (not shown). During chemokine-induced polarization, the lymphocyte nucleus becomes irregularly shaped without apparent change in lamin B nuclear envelope association (Fig. 4). This indicates that the nucleus can be deformed without disruption of the nuclear lamina. These findings support the conclusion that the measured increase of lymphocyte deformability induced by calyculin A is due to the disruption of the cytoplasmic vimentin cytoskeleton and is not a reflection of changes in the properties of the nucleus.

It is possible that calyculin A treatment increases the phosphorylation of other cytoskeletal/structural components, and thus affects PBT deformability by means other than IF disruption. For example, calyculin A is a potent inhibitor of myosin light chain phosphatase, and therefore may alter actin-myosin contractility. Our assay measures passive resistance to deformation, particularly in experiments using fixed cells. As such, only intact major support structures are likely to contribute to cell rigidity; the effects of calyculin A on nonstructural proteins, or on previously dismantled cytoskeletal elements such as the F-actin of cold cells, are unlikely to be major factors. Nonetheless, the potential for non-IF-related effects of calyculin A necessitated the use of alternative means of achieving IF-free cells. As described above, several other methods of IF disruption were not feasible in human PBT. Therefore, we examined the deformability of cultured splenocytes derived from wild-type (WT) and vimentin KO mice (15).

Freshly isolated splenocytes from normal mice expressed low levels of vimentin protein by Western blot, and IF networks were not clearly visible by immunofluorescence. After several days in culture, vimentin expression is substantially up-regulated and splenocytes become highly polarized, with morphology and cytoskeletal organization equivalent to chemokine-stimulated human PBT (Fig. 5). It is noteworthy that vimentin-deficient splenocytes exhibit normal polarization, forming a uropod near the MTOC, thus demonstrating that vimentin IFs are not required for polarization and uropod formation. To obtain a homogenous spherical population suitable for analysis of global deformability, cultured WT and KO splenocytes were incubated at 4°C for 2 h. Following cold exposure, the IF distribution of WT splenocytes reverted from a uropod-localized aggregate to an extended cytoplasmic network, comparable to that of resting human PBT. WT and KO cells had

**FIGURE 3.** Quantitative analysis of PBT deformation following selective cytoskeletal disruption. *a*, Increased deformability of calyculin A + 4°C-treated lymphocytes (no intact cytoskeleton) compared with 4°C-treated cells (IF intact, microfilament (MF) and microtubule (MT) disrupted) and 37°C controls (all cytoskeleton intact). Data derived from 90 cells from nine experiments/blood donors, normalized to 37°C controls, ± SEM. *b*, Fixed cells are less deformable than live cells, but show the same increased deformability following IF disruption. Data from 30 cells from three experiments/donors, normalized to fixed cell 4°C controls, ± SEM.

**FIGURE 4.** Calyculin A exposure disrupts cytoplasmic vimentin IFs, but not nuclear lamin B IFs. Lamin B distribution (red, *left column*) vs vimentin (green, *center column*) in controls, calyculin A + 4°C-treated, and SDF-stimulated PBT. During PBT polarization, the nucleus becomes irregularly shaped. IFs are retracted to the uropod pole of the nucleus, while Lamin B remains evenly distributed. Images are projections generated from confocal serial sections of fluorescently labeled cells. Bar = 5 μm.

**FIGURE 5.** Polarization and cytoskeletal organization of cultured normal (WT, *left column*) and vimentin KO (right) mouse splenocytes. Confocal micrographs showing the distribution of microtubules (red, MT), vimentin IF (green, IF), and actin microfilaments (blue, MF). Bar = 5 μm.
and KO mice, experiments, using splenocyte cultures derived from three pairs of normal normalized to WT samples. Data are from 95 cells in five independent tentative analysis of relative deformability of spherical cultured splenocytes Splenocytes from vimentin KO mouse are significantly FIGURE 6. Spleenocytes from vimentin KO mouse are significantly more deformable than spleenocytes from normal mice \( p < 0.01 \). Quantitative analysis of relative deformability of spherical cultured spleenocytes normalized to WT samples. Data are from 95 cells in five independent experiments, using spleenocyte cultures derived from three pairs of normal and KO mice, \( \pm \) SEM.

lymph node, and thymus have been reported to have collapsed IF networks (29). These observations suggest that the redistribution of the IFs is a fundamental feature of lymphocyte polarization, and thus to the process of lymphocyte extravasation. However, little had been known about the function(s) of the IF network in circulating lymphocytes, nor of the role of its collapse during lymphocyte polarization and transendothelial migration.

To determine how IF collapse during lymphocyte polarization might relate to changes of cellular deformability, we examined the contribution of vimentin filaments to the rigidity of spherical cells. Our findings demonstrate that the IFs of spherical human PBT, in the absence of microtubules and microfilaments, are sufficient to maintain the cells’ resistance to extensive deformation. Consequently, to achieve the extreme level of deformation necessary for transendothelial migration, the IF cytoskeleton of the circulating lymphocyte would require modification. Because disassembly of vimentin filaments into vimentin monomers would require significant energy expenditure, the compaction of the IF network

FIGURE 8. Microtubules do not provide resistance to deformation in the absence of other cytoskeletal elements in human PBT. Quantitative deformability analysis of cells with taxol-stabilized microtubules. PBT with taxol-stabilized microtubules are as deformable as cells with no intact cytoskeleton. Data from four experiments, four donors, 40 cells, \( \pm \) SEM normalized to 4°C control.

FIGURE 7. Microtubule disruption-induced PBT polarization and IF collapse. Projection images showing PBT polarization and IF collapse following microtubule disruption by 2-h colchicine treatment (100 \( \mu g/ml \)) at 37°C. F-actin (a, blue), microtubules (b, green), vimentin IF (c, red), and overlay (d). Bar = 5 \( \mu m \).

Discussion
Regulated rigidity is of fundamental importance to lymphocyte trafficking and function. Lymphocytes must be sufficiently rigid to survive the considerable physical stresses of circulation, but must rapidly become highly flexible to exit the vasculature and enter tissue. A prominent feature of the cytoskeletal reorganization during lymphocyte polarization is the retraction and condensation of vimentin IFs into a dense perinuclear aggregate at the uropod (27). Lymphocyte polarization in vitro, whether spontaneous or in response to chemokines, invariably includes the collapse of the IF network. In vivo, lymphocytes within specific regions of spleen,
without disassembly during polarization would be an efficient and rapid means of increasing cell deformability. We thus propose that the collapse of the IF network during lymphocyte polarization satisfies the requirement for increased cell deformability during extravasation.

Using the same techniques, we have demonstrated that cultured splenocytes from vimentin KO mice are significantly more deformable than their WT counterparts. These data support our findings using calyculin A to disrupt the IF network of human PBT. Vimentin KO mice are viable and superficially normal (15); as in other KOs, compensatory mechanisms may exist to replace some of the normal functions of vimentin. As yet there are no reported abnormalities in the development or function of the immune system of the vimentin KO mouse. The low expression of vimentin in mouse splenocytes before culture, as well as the relative cold resistance of their F-actin, indicates that fundamental differences exist between mouse splenocytes and human PBT. The present studies do not address the physiologic functions of mouse leukocyte vimentin; cultured splenocytes provided us the means by which to compare the deformability of vimentin-rich and vimentin-deficient lymphocytes without pharmacologic manipulations. We are currently investigating whether mouse PBT are more similar than splenocytes to human PBT; and whether the loss of vimentin has any physiologic consequence to mouse leukocytes, such as changes in survival and/or tissue distribution.

The contribution of IFs demonstrated in these studies does not dismiss the potential importance of microfilaments, actin-myosin contractility, or microtubules to the overall mechanical properties of human lymphocytes. Indeed, the cytoskeleton of lymphocytes and other cells most likely behaves as a composite material (30), in which the combined characteristics and interactions of microfilaments, microtubules, and IFs determine the mechanical properties of the cell as a whole (5, 30). The limited flexibility of the IF cytoskeleton in circulating lymphocytes would be expected to allow them to endure extreme hydrodynamic and mechanical stress while still permitting sufficient cellular pliability to traverse restricted passages, such as pulmonary capillaries, without its reorganization. Within the vasculature, reorganization of the more labile actin cytoskeleton might rapidly change cortical stiffness, while the vimentin IFs continue to maintain a level of core resistance to deformation. In addition to global resistance to deformation, local membrane stiffness is likely to be important for maintaining membrane integrity and distributing locally applied force to the coarsely woven vimentin cage. Based on the location and properties of the actin cortical cytoskeleton, it is likely to play a major role in this function. The shallow angle (i.e., high radius of curvature) of the cell membrane, where the cell meets the substratum in Fig. 2a, suggests that local stiffness is minimal when the three cytoskeletal systems are disabled.

Our findings expand an emerging body of evidence that IFs contribute to cellular mechanical stability. Fibroblasts derived from vimentin KO mice are more fragile than normal cells (31). Similarly, human and mouse epidermal cells with mutations in the IF protein keratin tend to rupture when mechanically stressed (32–34). Vimentin IFs are highly resistant to extraction and disassembly under a variety of conditions, suggesting that they are more stable than microtubules or microfilaments within the ionic conditions of the cytoplasm. However, they are not static elements, as their structure can be modulated by phosphorylation (18) and they continuously undergo subunit exchange (35). The mechanical stability provided by IFs may be attributed in part to their unique physicochemical properties. Isolated vimentin networks are flexible at low strain, but rigidify in response to increasing external force, and resist breakage at stress levels beyond those that disrupt microfilaments and microtubules (30). The interactions of IFs with other cellular components and their distribution in the cytoplasm may also contribute to their role as structural supports (20, 36). IFs interact with the plasma membrane, the nucleus, and other cytoskeletal elements (37), and these interactions may regulate the position of the nucleus and mediate the transmission of external forces throughout the cell (5, 20, 36).

Our studies demonstrate that vimentin IFs form a cage in circulating spherical lymphocytes that provides their primary protection against deformation; although durable in circulation, the lymphocyte vimentin cage is rapidly collapsed during polarization/transmigration. The collapse of the vimentin network during chemokine-induced lymphocyte polarization is one of the most rapid known examples of global IF reorganization. Understanding the biochemical events underlying IF remodeling and the functions of vimentin in other aspects of lymphocyte biology will be important areas for future study.

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