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# Dynamics of podosome stiffness revealed by atomic force microscopy

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**Podosomes are unique cellular entities specifically found in macrophages and involved in cell–matrix interactions, matrix degradation, and 3D migration. They correspond to a core of F-actin surrounded at its base by matrix receptors. To investigate the structure/function relationships of podosomes, soft lithography, atomic force microscopy (AFM), and correlative fluorescence microscopy were used to characterize podosome physical properties in macrophages differentiated from human blood monocytes. Podosome formation was restricted to delineated areas with micropatterned fibrinogen to facilitate AFM analyses. Podosome height and stiffness were measured with great accuracy in living macrophages ( $578 \pm 209$  nm and  $43.8 \pm 9.3$  kPa) and these physical properties were independent of the nature of the underlying matrix. In addition, time-lapse AFM revealed that podosomes harbor two types of overlapping periodic stiffness variations throughout their lifespan, which depend on F-actin and myosin II activity. This report shows that podosome biophysical properties are amenable to AFM, allowing the study of podosomes in living macrophages at nanoscale resolution and the analysis of their intimate dynamics. Such an approach opens up perspectives to better understand the mechanical functionality of podosomes under physiological and pathological contexts.**

cytoskeleton | Young's modulus | microcontact printing

**M**acrophages, which derive from circulating blood monocytes and reside in most tissues, play critical roles in host defense and tissue homeostasis. In case of infections or wounds, macrophages migrate to injured sites and fulfill their functions, but in numerous pathological situations their activities can worsen inflammatory processes. Thus, regulating macrophage migration is being regarded as a promising strategy for the development of anti-inflammatory therapies. Macrophages harbor specialized structures named *podosomes*, which play a pivotal role in migration and matrix degradation (1, 2). As macrophages patrol through a variety of tissues, they encounter a variety of extracellular matrix (ECM) architectures and composition that may influence podosome formation and organization; however, the extracellular cues that regulate podosome formation and functions remain unclear.

In two dimensions, podosomes appear as unique nanoscale cellular entities anchored into the ventral plasma membrane and perpendicularly oriented from the substratum. They present a core of column-shaped F-actin surrounded at its base by adhesion proteins (i.e., integrins) and actin-binding proteins, including vinculin, talin, and paxillin (3). As shown recently by electron microscopy, podosomes are anchored into a radial F-actin network laying on the ventral membrane that interconnects podosome cores, with their top standing above the rest of this actin network (4, 5). Podosomes are very dynamic structures that present a rapid actin turnover, with a lifespan ranging from 2 to 14 min (6, 7). When macrophages are activated, their podosomes can rearrange in a particular belt-shape structure called a rosette that efficiently degrades the ECM (1, 8). Works on protease-dependent 3D migration of macrophages indicate that podosomes are key elements of this process (1, 9) and correspond to actin-rich protrusive structures invading the ECM (2, 10). Thus podosomes

are probably sites of force generation based on actin polymerization and myosin II activity (11). Although direct quantification of individual podosome forces remains difficult, evaluating podosome biophysical properties would improve our knowledge of their structure/function relationships. Over the last decade, atomic force microscopy (AFM) has been proven to be a powerful tool for the analysis of morphological aspects of cell structures at nanometer scale resolution and more importantly their rheological properties in living cells (12, 13).

Here we combined micropatterned surfaces and AFM to study podosome biophysical properties in human macrophages derived from blood monocytes. Micropatterned ECM proteins revealed that fibrinogen, compared with other matrix proteins, favors podosomes formation. This property was then used to delineate small areas for podosome formation, which was a prerequisite for resolute AFM investigations. This allowed us to accurately measure podosome height and Young's modulus in different ECM protein contexts. Finally, time-lapse AFM studies of podosome dynamics combined with the use of actin toxins or myosin II inhibitor revealed that this structure exhibits bi-periodical Young's modulus oscillations regulated by F-actin dynamics and myosin II activity.

## Results

**Micropatterned Fibrinogen Polarizes Podosome Formation and Facilitates AFM Analyses.** To study podosomes formed on physiologically relevant substrates, we tested different ECM proteins encountered by macrophages in vivo. The percentage of macrophages spread on fibrinogen or fibronectin forming podosomes ( $\approx 60\%$ ) was significantly enhanced compared with collagen IV or gelatin ( $\approx 35\%$ ) but similar to naked glass (Fig. S1A). Because fibrinogen is mostly found at inflamed sites, we decided to focus on this protein (14). We started AFM experiments with formaldehyde-fixed macrophages, which allowed the use of correlative fluorescence microscopy. Deflection and topographical images showed the highly textured landscape of macrophages, with membrane ruffles mostly found in podosome-forming regions, as seen at the migration front of polarized macrophages (Fig. 1A). The cells appeared flat, with an average height at the cell periphery of  $\approx 400$  nm. Correlative fluorescence microscopy showed that some membrane bumps corresponded to underlying podosomes revealed by F-actin staining (arrowheads, Fig. 1A). However, these observations highlighted that discriminating podosomes in the cell landscape by the use of AFM alone was uncertain, which hindered further analyses. To address this issue, we thought that

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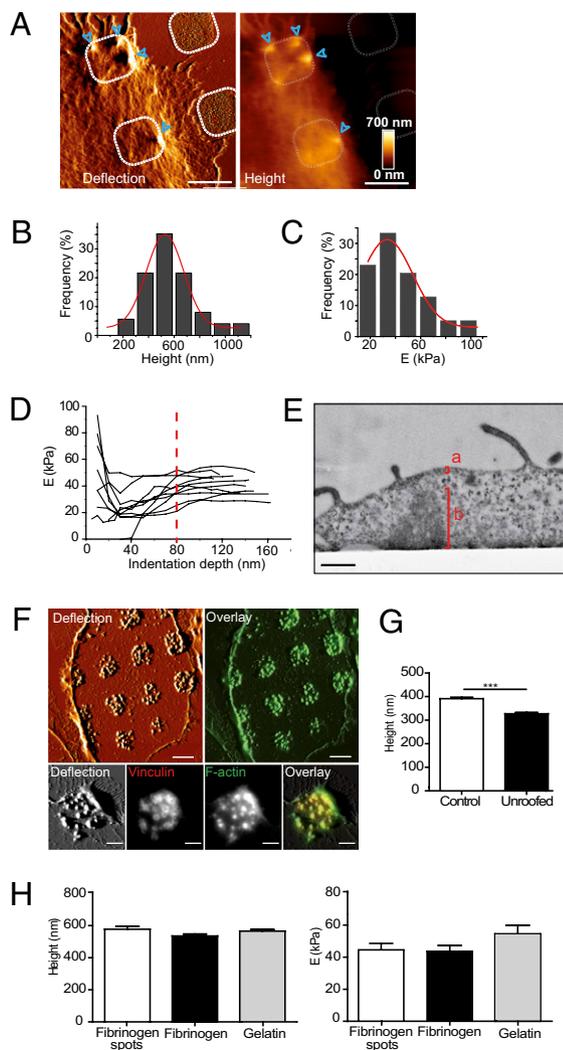
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**Fig. 2.** Biophysical properties of podosomes in living macrophages. (A) AFM deflection and topographical images of podosomes on fibrinogen spots. Dotted lines delineate fibrinogen spots underneath the cell, and blue arrowheads show podosomes. (Scale bars, 5  $\mu\text{m}$ .) (B) Podosome height values from AFM topographical images of living macrophages on micro-patterned fibrinogen exhibited a Gaussian distribution (red curve), with a mean of  $578 \pm 209$  nm ( $n = 125$  podosomes in 19 cells from seven donors). (C) The distribution of Young's modulus values was Gaussian (red curve), with a mean of  $43.8 \pm 9.3$  kPa ( $n = 39$  podosomes in 17 cells from four donors). (D) Young's modulus vs. indentation depth of podosomes. Representative curves of different podosomes are displayed ( $n = 10$  podosomes in 10 cells from five donors). Dashed line shows the average indentation depth above which the Young's modulus reaches a plateau. (E) Transmission electron micrograph of a podosome at the cell periphery: a, dorsal membrane and cortical actin; b, podosome core. (Scale bar, 500 nm.) (F) *Upper*: AFM deflection of an unroofed macrophage plated on fibrinogen spots (*Left*) and overlay of AFM deflection combined to fluorescence image (*Right*). *Lower*: Correlative microscopy of AFM deflection and fluorescence images of F-actin and vinculin of an unroofed macrophage. (Scale bars, 2  $\mu\text{m}$ .) (G) Quantification of podosome height of control and unroofed macrophages; mean values are  $392.5 \pm 110$  nm and  $328 \pm 94$  nm, respectively. Measurements were performed on at least 392 podosomes in 26 cells from at least four donors for each condition. (H) Height and Young's modulus podosomes in macrophages plated for 2 h on fibrinogen spots or nonpatterned gelatin and fibrinogen. Height and Young's modulus were measured on at least 125 podosomes in eight cells and on 17 podosomes in four cells, respectively, from at least two donors for each condition. One-way ANOVA shows that variance is not significant over the tested conditions.

which was comparable to fixed cells (Fig. 2B). Moreover, the sequence of AFM images suggested that podosome formation and dynamics (lifespan  $\approx 4$  min) are amenable to AFM in living macrophages without disturbing the cells when compared with time-lapse fluorescence imaging (Fig. S3 and Movie S1).

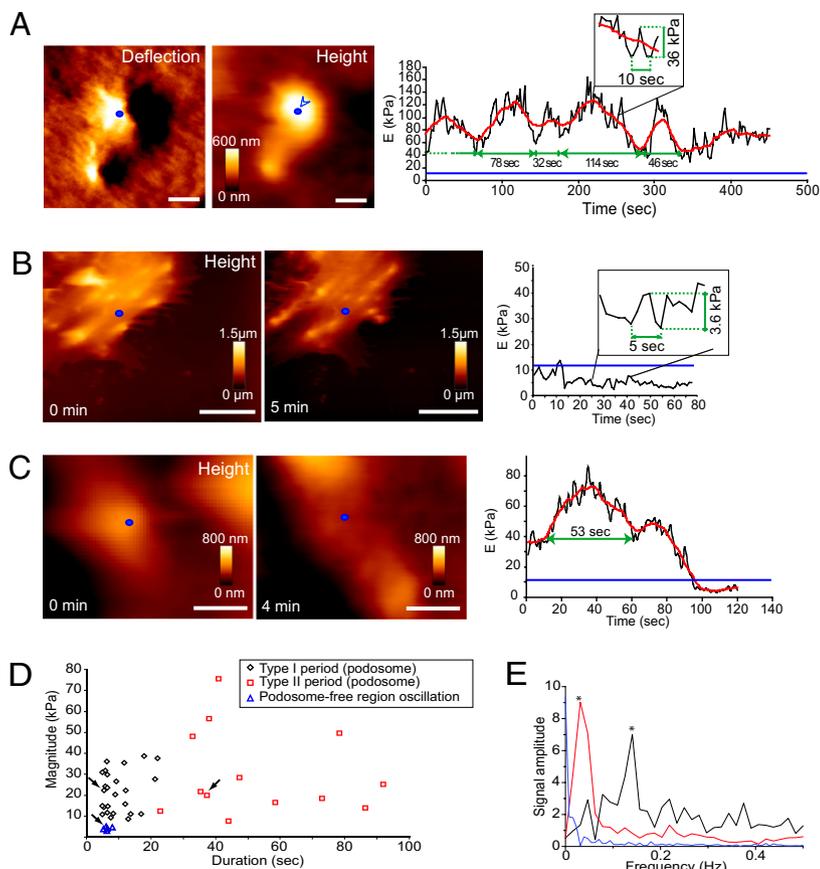
Podosome average Young's modulus in living macrophages was  $43.8 \pm 9.3$  kPa (Fig. 2C), which was fivefold higher than that of podosome-free regions ( $8 \pm 1.7$  kPa) and lower than in fixed macrophages in which formaldehyde increases biological sample stiffness (17). This value of podosome stiffness was two- to threefold higher than that of stress fibers measured by AFM in other work (12), suggesting that podosome stiffness may reflect specific properties as a matter of composition and/or architecture. Force–distance curves were also analyzed to estimate the thickness of overlying layers crossed by the AFM tip before reaching the top of the podosome core. For this purpose we studied stiffness as a function of indentation depth [as previously performed by Lu et al. on stress fibers (12)]. By postulating that under these conditions F-actin polymer harbors isotropic properties, the Young's modulus value of such polymer would be independent of indentation depth; thus, curves of stiffness as a function of the indentation would reach a plateau when the AFM tip is indenting the F-actin polymer (12). Analysis of multiple force–distance curves showed that the stiffness plateau was reached after the first 80 nm of indentation in all cases, indicating that the thickness of superficial layers crossed by the AFM tip before indenting the podosome F-actin core is  $\approx 80$  nm (Fig. 2D). To verify the accuracy of this estimation, two complementary approaches were used. First, transmission electron microscopy was performed on transversal sections of macrophages, and second, dorsal membrane was removed by unroofing to gain direct access to podosome cores. Transmission electron microscopy images of the cell periphery showed the proximity between the dorsal plasma membrane and the electron-dense F-actin core of podosomes anchored into the ventral membrane (Fig. 2E). The dorsal plasma membrane is supported by a thin cortex of F-actin, with a total thickness (membrane and F-actin cortex) ranging from 60 nm to 80 nm. Thus, the fluid phase of the cytoplasm probably flows out of the region when the AFM tip applies pressure on the plasma membrane. Direct measurement of F-actin core by AFM after cell unroofing showed a difference of  $64 \pm 7$  nm when compared with intact cells. This value corresponded to the thickness of the dorsal membrane and its associated actin cortex that were removed during the unroofing process (Fig. 2F and G). In these experiments, intact and unroofed cells were fixed with glutaraldehyde instead of formaldehyde, and this could explain why podosome height values were lower than those measured before. Altogether the three approaches indicated that the AFM tip reaches the top of the podosome F-actin after the first 80 nm of indentation. In our experimental setup, the total indentation depth on podosomes in living macrophages ranged from 120 nm to 170 nm, which ensured that the AFM tip indented the F-actin core of podosomes. Because podosome formation was modulated differently depending on the nature of the ECM protein (Fig. S1), we wondered whether their physical properties could be influenced by the underlying ECM protein. Podosome height and stiffness on nonpatterned gelatin were compared with fibrinogen patterned or not (Fig. 2H), and no significant differences were observed. These results suggest that podosomes have constant structural properties independent of the nature and the organization of the ECM.

**Dynamics of Podosome Young's Modulus Revealed by Time-Lapse AFM.** F-actin turnover in podosomes has been shown to be highly dynamic (5–7), hence we examined how podosome Young's modulus behaves over their lifespan. The AFM force–distance curves approach was used to track single podosome stiffness dynamics as fast as one measure per second over several minutes.

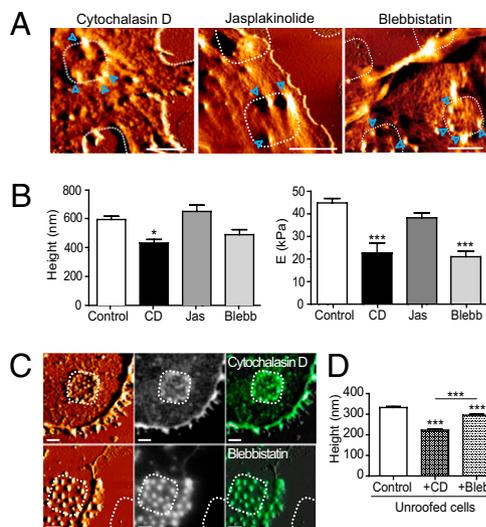
Fig. 3A displays representative deflection and topographical images of a podosome, with its corresponding stiffness over time curve extracted from the successive force curves. Two types of stiffness variation were reproducibly found: first, small and rapid oscillations (average duration  $9.8 \pm 5.1$  s, average magnitude  $21.7 \pm 9.9$  kPa) that we called type I (black curve). Second, larger variations of the average stiffness were found (red curve) and were called type II (average duration  $52.9 \pm 22.5$  s, average magnitude  $30.3 \pm 20.5$  kPa). During these experiments, variations of podosome relative height were estimated from the force–distance curves by considering the offset piezo position and the contact point position calculated from the Hertzian model (*SI Materials and Methods*). Podosome height variations averaged at a magnitude of  $55 \pm 27$  nm, which was considered to be negligible because they represent less than 10% of podosome height (Fig. S44). To verify that podosome stiffness variations were not disturbed by movements of the overlying dorsal plasma membrane, stiffness dynamics of podosome-free regions were examined (Fig. 3B). Stiffness variations in such areas greatly differed from podosomes in terms of magnitude ( $3 \pm 0.6$  kPa) and duration ( $6 \pm 0.9$  s). These results helped us to define a podosome stiffness threshold that was set at 12 kPa (corresponding to the  $E_{\text{mean}} + 2$  SD of podosome-free areas; blue line in Fig. 3B). As shown in Fig. 3C, when podosomes disappeared, a sudden decrease of average stiffness was observed, dropping down to podosome-free area stiffness values, and this was confirmed by the height reduction observed on the topographical image (Fig. 3C). The different types of variations encountered over several podosomes are represented as a dot plot (Fig. 3D), which highlighted that type I and type II variations segregated differently, even when considering podosome-to-podosome heterogeneity (Table S1). To assess the peri-

odicity of type I and type II variations, Fourier transforms of podosome stiffness over time curves were analyzed. The representative Fourier transforms of a podosome presented in Fig. 3E show frequency maxima at 0.14 Hz for type I and 0.031 Hz for type II. These frequencies correspond to periods of 7.1 s for type I and 32.2 s for type II, which are reasonably close to the measured periods (6 s for type I and 32 s for type II). As a control, Fourier transforms of podosome-free regions did not show any periodicity (Fig. 3E). Altogether our results reveal that podosomes harbor particular biperiodic stiffness oscillations throughout their lifespan.

**Biophysical Properties of Podosomes Reflect F-actin Core Properties and Depend on Myosin II Activity.** To determine whether podosome biophysical properties reflect actin-related processes, two actin-targeting toxins, cytochalasin D (CD) and jasplakinolide (Jas), were used. CD is a cell-permeant toxin that inhibits actin polymerization by capping the barbed ends of F-actin filaments and blocking the addition of new G-actin monomers, thus favoring F-actin filament depolymerization and severing (18). Treatment of macrophages with  $2 \mu\text{M}$  CD induced the rapid disruption of F-actin radial network and cortex, but podosome F-actin cores remained (Fig. 4A, Figs. S5 and S64, and Movie S2). In accordance with CD affecting F-actin polymerization, podosome height was reduced by 30% (Fig. 4B). This effect of CD was confirmed by measuring F-actin core height directly in unroofed macrophages, which was reduced by 33% (Fig. 4C and D). In parallel with the height reduction induced by CD, a 50% decrease of stiffness was observed in living macrophages (Fig. 4B), showing that the disturbance of F-actin stability profoundly impaired podosome biophysical properties. Jas is a cell-permeant toxin that stabilizes F-actin (19). Cells treated with 250 nM of Jas did not show any



**Fig. 3.** Podosome stiffness dynamics measured by time-lapse AFM. (A) Young's modulus variations over time of a podosome in a living human macrophage on fibrinogen patterns, with images of its AFM deflection and topography. (Scale bars,  $0.5 \mu\text{m}$ .) Blue dot indicates AFM tip position, and height at this point is  $545$  nm. *Right*: Curve of stiffness over time obtained by recording a single force–distance curve per second. Black curve corresponds to raw values, with small oscillations of stiffness (type I, *Inset*). Larger variations, type II, were identified on the smoothed curve (red curve). Blue line indicates the threshold of podosome rigidity ( $12$  kPa) calculated from podosome-free region values. (B) Young's modulus variations over time of a podosome-free region in a living human macrophage. Images are AFM topography of a podosome-free region before (*Left*) and after (*Center*) the recording of single force curves every second (*Right*). *Inset*: Magnified part with typical stiffness variations. (Scale bars,  $5 \mu\text{m}$ .) (C) Young's modulus variations over time during podosome disruption. Images are AFM topography of a podosome before (*Left*) and after (*Center*) the recording of single force curves every second (*Right*). Heights values at the blue point before and after recording are equal to  $720$  nm and  $520$  nm, respectively. (Scale bars,  $1 \mu\text{m}$ .) (D) Scatter plot of magnitude as a function of duration for type I and type II stiffness variations from podosomes and podosome-free areas. Each dot represents the mean of magnitude and duration in the stiffness dynamics acquired for at least 13 podosomes in 10 cells from three donors for each condition. (E) Representative Fourier transforms (FT) of type I (black curve), type II (red curve), and podosome-free regions oscillations (blue curve) of the E (kPa) time series dynamic. \*Type I  $\text{pic}_{\text{max}} = 0.140$  Hz; type II  $\text{pic}_{\text{max}} = 0.031$  Hz. Arrows in D display the values extracted semi-automatically from the same stiffness dynamics. FT analysis was performed on 10 podosomes and five podosome-free regions stiffness dynamics, as described in *SI Materials and Methods*.

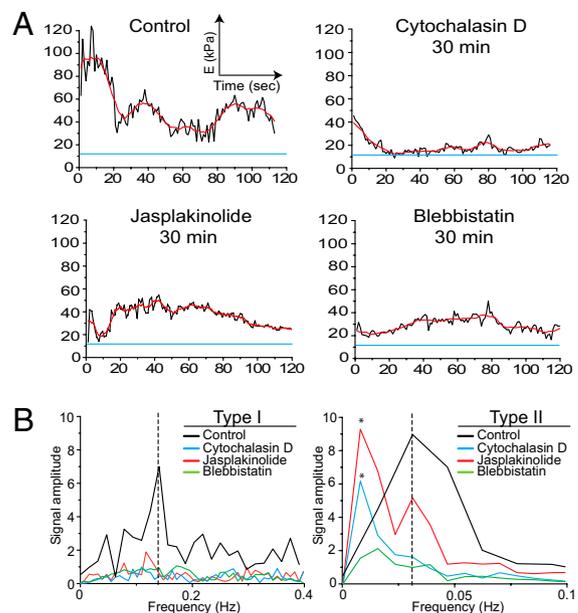


**Fig. 4.** Effects of cytoskeleton-disturbing drugs on podosome biophysical parameters. (A) AFM deflection images of a macrophage treated for 20 min with CD, Jas, or Blebb; blue arrowheads show podosomes. (Scale bars, 5  $\mu\text{m}$ .) (B) Height and Young's modulus of podosomes in macrophages; DMSO-treated cells (control) or treated with CD, Jas, or Blebb, up to 60 min. Experiments were performed on at least 14 podosomes in 10 cells from three donors. (C) AFM deflection image (Left), fluorescence microscopy of F-actin (Center), and merge images (Right) of an unroofed macrophage treated 20 min with CD or Blebb. (Scale bar, 2  $\mu\text{m}$ .) (D) Quantification of podosome height of unroofed macrophages and unroofed macrophages treated with CD or Blebb; mean values are  $328 \pm 94$  nm,  $219 \pm 89$  nm, and  $291 \pm 87.5$  nm, respectively. Values were obtained from at least 260 podosomes in 10 cells from three donors for each condition.

apparent modifications of their F-actin network (Fig. 4A, Figs. S5 and S6B, and Movie S3); moreover, there was no modification of podosome height or stiffness (Fig. 4B). These results show that our experimental setup provides a direct estimation of the effects of actin toxins on podosome F-actin core.

Cell cytoskeleton architecture and dynamics are mostly regulated by actomyosin complexes as observed during phagocytosis (20). Myosin IIA localizes into F-actin radial network at the vicinity of podosomes (Fig. S5) and has been shown to regulate podosome dynamics (3, 21); therefore, we assessed the role of myosin II activity in the regulation of podosome biophysical properties. For this purpose, macrophages were treated with 50  $\mu\text{M}$  of blebbistatin (Blebb) and analyzed by fluorescent microscopy and AFM. In accordance with previous works (22), myosin IIA localization was profoundly affected by Blebb treatment, whereas most podosomes remained in our conditions (Fig. 4A and Fig. S5). AFM experiments showed that Blebb weakly impaired podosome height (confirmed on unroofed cells; Fig. 4C) while reducing podosome stiffness by 53% (Fig. 4B). Therefore, myosin II does not seem to regulate podosomes architecture per se but tunes their viscoelastic properties, probably by regulating actin filaments tension.

Next the effects of CD, Jas, and Blebb were examined on the dynamical aspects of podosome Young's modulus. At different time points after drug addition, time-lapse AFM data were acquired (Fig. 5A). The three drugs markedly affected the magnitude of Young's modulus variations, even though Jas did not show any effect on static analyses. Then, in order to estimate how type I and type II periods were specifically affected in these conditions, Fourier transforms were analyzed (Fig. 5B). The periodicity of the type I periods was abolished by the three drugs, whereas type II periods were abolished by CD and Blebb but remained with a reduced magnitude in the presence of Jas. Overall, our results show that the periodicity of podosomes Young's modulus dynamics depends on F-actin integrity, treadmilling, and myosin II activity.



**Fig. 5.** Effects of cytoskeleton-disturbing drugs on podosome stiffness dynamics. (A) Representative Young's modulus variations over time of podosomes in a living human macrophage on fibrinogen patterns before (control) and during treatment with CD, Jas, or Blebb. At least 14 podosomes in 10 cells from three donors were studied for each condition. (B) Representative Fourier transform of type I and type II oscillations of the Young's modulus time series dynamic before (control) and during treatment with CD, Jas, or Blebb. Dashed lines indicate type I peak<sub>max</sub> = 0.140 Hz, type II peak<sub>max</sub> = 0.031 Hz. \*Artificial peaks whose value corresponds to the entire recording time.

## Discussion

In this work, we assessed podosome physical properties by combining the use of AFM, micropatterned surfaces, and correlative fluorescence microscopy. Such a strategy allowed us to perform nanoscale analysis of podosomes in living macrophages, further improving our knowledge of podosome physical aspects and dynamics.

When testing the patterning of different ECM proteins on podosome formation, we observed various effects dependent on protein type and/or patterning. The fact that macrophages responded to micropatterned gelatin by avoiding protein spots to form their podosomes was unexpected because gelatin is commonly used to assess podosome degradative activity. Another interesting result was that fibronectin promoted podosomes formation when nonpatterned, whereas podosomes formed mostly out of the fibronectin spots on micropatterned substrates (compare Fig. S1A with Fig. S1E). Thus, podosome formation seems to be regulated by the nature and distribution of the ECM protein. However, the comparison of podosome physical properties in different contexts, either in rosette or individualized, or on different ECM proteins showed that podosomes have steady height and stiffness, indicating that they are mostly regulated at the initiation step rather than at their architectural level. Because the ECM proteins used in this study are known to recruit different integrin families— $\beta 2$  and  $\beta 3$  for fibrinogen,  $\beta 1$  and  $\beta 3$  for fibronectin, and  $\beta 1$  for collagen IV and gelatin (14, 23)—we propose that  $\beta 2$  integrin ligands, here fibrinogen, deliver a strong podosome triggering signal, whereas  $\beta 1$  integrin ligands may have the opposite effect (24). Because podosomes belong to the migration machinery, our results suggest that ECM composition and organization could influence macrophages by defining distinct “functional” zones guiding their migration paths.

In this study AFM is revealed as a versatile tool for the study of multiple parameters at a scale suiting individual podosomes. Delineating podosome formation to small areas by using micro-

patterned fibrinogen was an asset for the use of AFM. We were able to precisely measure height and Young's modulus of hundreds of podosomes and estimate the effects of different drugs that affect F-actin treadmilling and stability, as well as myosin II activity. The three drugs affected podosome properties at different levels: CD affected podosome height and stiffness, Blebb affected podosome stiffness without impairing much podosome height, and Jas had an effect on the stiffness dynamics only. These results highlight that AFM allows a refined analysis of multiple aspects of podosome structure and behavior.

Podosome Young's modulus dynamics show type I and type II overlapping periods, suggesting that at least two different processes coexist. A clear understanding of these phenomena will require future in-depth studies; but meanwhile, some hypothesis may be drawn. The duration of type I variations ( $\approx 10$ – $20$  s) is in the same range as that of actin turnover in podosomes measured by fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP) ( $\tau_{1/2} \approx 20$  s) (5, 6). The impairment of type I periods by CD and Jas reinforced this hypothesis because both drugs affect actin turnover. Blebb also abolished type I periods, suggesting that microfilament tension regulated by myosin II impacts actin treadmilling, as has been found for other cell structures (25, 26). Type II periods are more profound and durable than type I variations and are also actomyosin-related processes because they were abolished by CD and Blebb and were to a lesser extent affected by Jas. Different observations suggest that these oscillations may result from tangential forces transmitted through the radial F-actin between podosomes, because actomyosin complexes are mostly found in these areas (11). Moreover, CD rapidly disrupted the F-actin radial network, thus disturbing myosin IIA localization (Fig. S5). Finally, although Jas did not disturb much F-actin organization or myosin IIA localization (Fig. S5), it was shown recently that Jas can affect some myosin II functions (27). Altogether this work leads us to propose that F-actin treadmilling and actomyosin complex activities generate periodic forces at podosomes. Because these processes have been proposed to participate in the protrusion of podosomes into the ECM (10, 11), the involvement of their periodicity during macrophage migration remains to be elucidated. Podosome biophysical properties are

likely to be coupled with their mechanosensing activity. However, investigation of podosome properties in macrophages plated on soft matrix is challenging as these cells tend to round up and form less podosomes (28). Although the exploration of podosome biomechanics is just beginning, here we show that AFM offers the opportunity to directly quantify biophysical processes in podosomes.

## Materials and Methods

Detailed procedures are provided in *SI Materials and Methods*.

**Cell Culture.** Human monocytes were isolated from blood of healthy donors and differentiated for 7 d into macrophages in the presence of human macrophage colony-stimulating factor.

**Atomic Force Microscopy.** A NanoWizard II (JPK Instruments) AFM mounted on an inverted optical microscope was used for all experiments. Silicon nitride cantilevers (MLCT-AUHW; Veeco) with a nominal spring constant of 10 pN/nm [corrected using the thermal noise method (29)] were used. All AFM images and measurements were obtained in contact mode in liquid.

**AFM height measurements.** Fixed cells were analyzed in PBS liquid buffer at room temperature. For AFM analysis of living cells, a temperature-controlled sample holder filled with RPMI Hepes (pH 7.4) buffer as culture media was used.

**Elasticity measurements.** The determination of the elastic modulus from the force–distance curves was performed using a modified Hertz model (30, 31). During elasticity measurements, force–distance curves were recorded continuously at 2 Hz (1 s per approach–retract force curve).

**Statistical analysis.** A *t* test or one-way ANOVA analysis with Tukey's multiple comparisons posttest was performed using GraphPad Prism. On each graph, SEs are displayed. Statistical significance is indicated as follows: \*\*\**P* < 0.001; \*\**P* < 0.01; \**P* < 0.05.

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