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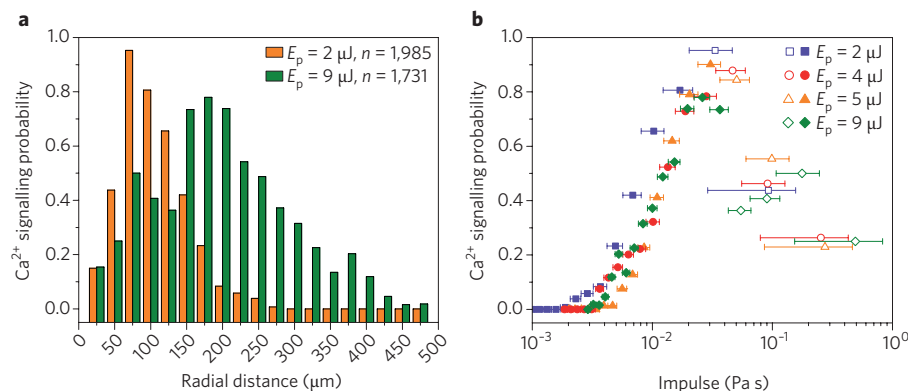
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## Reply to 'Mechanism for microtsunami-induced intercellular mechanosignalling'

**Luo *et al.* reply** — We thank He *et al.*<sup>1</sup> for their Correspondence regarding our recent paper<sup>2</sup>. They offer an alternate hypothesis for our observed microtsunami ( $\mu$ tsunami)-initiated  $\text{Ca}^{2+}$  signalling ( $\mu$ T-ICS) that differs from our claim of mechanical activation of stretch-sensitive G protein-coupled receptors (SS-GPCRs) by shear flow. Instead, they assert that  $\mu$ tsunami exposure initiates the release of  $\text{Ca}^{2+}$  signalling molecules, including ATP, from the cell immediately below the site of laser radiation. These molecules then diffuse to neighbouring cells and activate purinergic receptors, including those coupled to GPCRs, resulting in a propagating intercellular  $\text{Ca}^{2+}$  wave. They cite previous studies<sup>3–5</sup> that use femtosecond-laser microbeam irradiation to initiate  $\text{Ca}^{2+}$  signalling in primary rat hippocampal cells and astrocytes as well as immortalized human kidney (HEK293T) and cervical cancer (HeLa) cells. These  $\text{Ca}^{2+}$  waves have higher velocities ( $10\text{--}40\ \mu\text{m s}^{-1}$ ) and smaller spatial extent ( $\sim 200\ \mu\text{m}$  radius) as compared with  $\mu$ T-ICS waves ( $4.5\ \mu\text{m s}^{-1}$  and  $\sim 400\ \mu\text{m}$  radius, respectively) reported in our paper. Importantly, our studies employed human vascular endothelial cells (HUVECs) whose physiological function requires mechanosensitivity to hemodynamic shear stress.

The papers cited by He *et al.*<sup>3–5</sup> examine the roles of purinergic P2X (ligand-gated ion channels) and P2Y (GPCR) receptors in femtosecond laser microbeam induced  $\text{Ca}^{2+}$  signalling. Cells were treated with pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and Reactive Blue 2 (RB2) to provide broad inhibition of P2X and P2Y receptors, respectively. Because  $\text{Ca}^{2+}$  signalling was unaffected by PPADS but reduced by more than 70% with RB2, the authors concluded that P2Y receptors play a central role in femtosecond laser induced signalling of astrocytes<sup>5</sup>. However, purinergic receptor subtypes vary significantly between cell lines, as do their susceptibility to antagonists such as PPADS and RB2 (ref. 6). In fact, HUVECs express purinergic receptors P2X<sub>1</sub>, P2X<sub>6</sub>, P2X<sub>7</sub>, and P2Y<sub>1</sub> (refs 7,8) that are not susceptible to the action of these compounds<sup>6,8</sup>.

He *et al.*<sup>1</sup> suggest that 2-APB inhibition of  $\mu$ T-ICS does not elucidate the underlying signalling mechanisms because 2-APB acts at the IP<sub>3</sub> receptor well downstream of the activation of SS-GPCRs. Our demonstration of 2-APB suppression of  $\text{Ca}^{2+}$  signalling in response to both exogenous ATP administration<sup>9</sup> and  $\mu$ tsunami exposure<sup>2</sup>,



**Figure 1** | Mechanical dose-response of  $\mu$ T-ICS. **a**, Probability of  $\mu$ T-ICS versus radial location for laser pulse energies of 2  $\mu\text{J}$  and 9  $\mu\text{J}$  showing tunability of the spatial extent of  $\text{Ca}^{2+}$  signalling. **b**, Signalling probability versus impulse for laser pulse energies ranging from 2–9  $\mu\text{J}$ . Closed symbols refer to cell locations outside the  $\mu\text{CB}$  radius and open symbols refer to regions within the maximum radius of the  $\mu\text{CB}$ .

left open the possibility that purinergic receptors play a principal role in  $\mu$ T-ICS. However, in Supplementary Fig. 1 of our paper<sup>2</sup>, we presented data showing minimal  $\text{Ca}^{2+}$  signalling in HUVEC cultures when exposed to cell lysate, which presumably contains the signalling molecules released by HUVECs resulting from  $\mu$ tsunami exposure.

To further test the roles of SS-GPCRs in  $\mu$ T-ICS, we designed experiments to seek a differential response between exogenous ATP administration and  $\mu$ tsunami stimulation. We first examined exogenous ATP-initiated  $\text{Ca}^{2+}$  signalling (ATP-ICS) in HUVECs treated with PPADS and RB2. When administered alone, or in combination (at doses as high as 100  $\mu\text{M}$ ) they proved ineffective in suppressing ATP-ICS. This result casts serious doubt as to the effectiveness of PPADS and RB2 to investigate the role of purinergic receptors in HUVECs.

Instead, we administered HUVECs with U-73122, a drug that blocks the activation of phospholipase C (PLC) just downstream of GPCR activation. Treatment with 20  $\mu\text{M}$  U-73122 reduced  $\mu$ T-ICS by nearly 70% but did not significantly reduce ATP-ICS (Supplementary Fig. 1). These results, along with the inhibition of both  $\mu$ T-ICS and ATP-ICS by 2-APB, demonstrates that  $\mu$ T-ICS occurs along the GPCR–PLC–IP<sub>3</sub> pathway. Moreover, we treated HUVECs with 200 ng ml<sup>-1</sup> pertussis toxin and 100  $\mu\text{M}$  suramin, both of which disrupt G protein coupling with differing efficacy and specificity. In these cases,  $\mu$ T-ICS was reduced by over 87% and 95%, respectively, (Supplementary Figs 2,3) whereas

exogenous ATP-ICS was reduced only by 9% and 5%, respectively. These results demonstrate that  $\mu$ T-ICS can be inhibited without significantly affecting ATP-ICS, further supporting our hypothesis that  $\mu$ T-ICS is initiated through the activation of SS-GPCRs by the  $\mu$ tsunami-associated shear stresses.

There are also fundamental differences in the operative physical processes when using an 80 MHz train of highly focused 100 fs pulses of 800 nm radiation<sup>10,11</sup> versus single 500 ps pulses of 532 nm radiation<sup>11–13</sup> used in our study. These differences are comprehensively elucidated in the Supplementary Information, rebutting the assertion made by He *et al.* that a train of femtosecond laser pulses can mimic the mechanical stimulation provided by the  $\mu$ tsunami initiated by a single 500 ps laser pulse. In further support of our contention that  $\mu$ T-ICS is mechanical in nature, we examined the potential 'dose response' relationship between  $\mu$ T-ICS probability and shear stress impulse (SSI) in a manner similar to our previous work, which established such a relationship between SSI and cell fate<sup>13,14</sup>. To test for a similar relationship between  $\mu$ T-ICS probability and SSI we examined the  $\mu$ T-ICS using pulse energies of 2–9  $\mu\text{J}$ , corresponding to maximum cavitation bubble diameters of 140–270  $\mu\text{m}$ . Figure 1a shows the  $\mu$ T-ICS probability and demonstrates that the spatial extent of  $\mu$ T-ICS (from  $\sim 200\ \mu\text{m}$  to  $\sim 500\ \mu\text{m}$ ) is dependent on the laser pulse energy, which determines the spatial distribution of SSI. Importantly, when consolidating the data of  $\mu$ T-ICS probability

versus shear stress impulse obtained from four different pulse energies, we discover a single 'universal curve' indicating a one-to-one relationship between these two parameters (Fig. 1b). This provides additional evidence that  $\mu\text{T-ICS}$  is produced through cellular exposure to SSI. Lastly, we disagree with He *et al.* that slow dynamics of the  $\mu\text{T-ICS}$  wave ( $\sim 5 \mu\text{m s}^{-1}$ ) relative to the impulsive dynamics of the  $\mu\text{tsunami}$  makes mechanical stimulation of signalling improbable. The difference in timescale is irrelevant as the kinetics of  $\mu\text{T-ICS}$  is dominated by the kinetics of  $\text{IP}_3$  generation, molecular transport and signalling within the cell, well downstream from the impulsive shear stress.

Collectively, our new data and analysis supports the hypothesis that  $\mu\text{tsunami}$  SSI stimulates the activation of SS-GPCRs that acts along the GPCR-PLC- $\text{IP}_3$  pathway to

govern the probability of  $\text{Ca}^{2+}$  signalling for any given cell in a HUVEC culture. □

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