Mechanical strength determines \( \text{Ca}^{2+} \) transients triggered by the engagement of \( \beta_2 \) integrins to their ligands

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**A R T I C L E  I N F O**

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**A B S T R A C T**

Lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1) are key adhesion receptors to mediate neutrophil (PMN) recruitment and intracellular calcium (\( \text{Ca}^{2+} \)) signaling. Binding of LFA-1 and Mac-1 to their ligands is essential in triggering \( \text{Ca}^{2+} \) transients and activating \( \text{Ca}^{2+} \)-dependent kinases involved in cytoskeletal remodeling and migratory function. While mechanical forces are critical in regulating integrin-mediated \( \text{Ca}^{2+} \) transients, it is still unclear how the bond strength of \( \beta_2 \)-integrin-ligand pair affects \( \text{Ca}^{2+} \) responses. Here three typical ligands with known mechanical features with LFA-1 and Mac-1 in our previous work were adopted to quantify their capabilities in inducing \( \text{Ca}^{2+} \) transients in adherent PMNs under shear flow. Data indicated that LFA-1 dominates \( \text{Ca}^{2+} \) transients in PMNs on intercellular adhesive molecule 1 (ICAM-1) and junctional adhesion molecule-A (JAM-A), while Mac-1 mediates \( \text{Ca}^{2+} \) transients induced by receptor for advanced glycation end products (RAGE), consistent with their corresponding bond strengths. These results link \( \beta_2 \) integrin-ligand bond strength with \( \text{Ca}^{2+} \) transients in PMNs, suggesting high bond strength gives rise to strong \( \text{Ca}^{2+} \) response especially under physiological-like shear flow. The outcomes provide a new insight in understanding the mechanical regulatory mechanisms of PMN recruitment.

**1. Introduction**

Polymorphonuclear leukocytes (PMNs), also known as neutrophils, play an essential role in the innate immune system by initiating killing or repair mechanisms at the site of infection or tissue injury [1]. PMNs circulating in the blood are recruited to the inflammatory sites through a well-defined multistep cascade mediated by specific adhesion molecules and their ligands [2,3]. Two \( \beta_2 \) integrin members, lymphocyte function-associated antigen-1 (LFA-1, \( \alpha_\text{L} \beta_2 \), CD11a/CD18) and macrophage-1 antigen (Mac-1, \( \alpha_\text{M} \beta_2 \), CD11b/CD18) are key adhesion receptors in this process to initiate both adhesive bond formation and intracellular signals [4]. Multiple \( \beta_2 \) integrin ligands are presented on the surface of an endothelial cell (EC), including intercellular cell adhesion molecule 1 (ICAM-1), junctional adhesion molecule A (JAM-A) and receptor for advanced glycation end products (RAGE), promoting PMN adhesion, crawling and transmigration in different tissues [3,5]. While the respective roles of binding these ligands to \( \beta_2 \) integrins are extensively investigated, it is not completely clear how these ligands initiate diverse intracellular signaling under blood flow.

Calcium (\( \text{Ca}^{2+} \)) response is one of the major signaling triggered by \( \beta_2 \) integrin binding. The cytosolic free \( \text{Ca}^{2+} \) level contributes to key cellular responses in PMNs including activation, chemotaxis, degranulation and phagocytosis [6]. \( \text{Ca}^{2+} \) mediates PMN activation during recruitment through its central role in downstream signaling of chemokine ligation and adhesion function [7], and modulates vesicular integrin recycling, cytoskeletal rearrangements and uropod retraction during PMN migration [1]. Moreover, \( \text{Ca}^{2+} \) regulates the production of reactive oxygen species through activation of the NADPH oxidase via \( \text{Ca}^{2+} \)-dependent protein kinase C and promotes tumor necrosis factor-induced degranulation [1]. Changes in the spatiotemporal distribution of intracellular \( \text{Ca}^{2+} \) concentration are highly related with PMN behaviors and can be mediated by several ligand-receptor interactions such as the engagement of G-protein coupled receptors (GPCRs), Fc\( \gamma \)-receptors (Fc\( \gamma \)Rs), selectins and integrins [8,9]. \( \text{Ca}^{2+} \) dynamics of individual PMNs is closely associated with their respective functional states, along with phenotypic changes from one cell state to another [1]. \( \text{Ca}^{2+} \) transients following PMN adhesion are the cause of cytoskeletal arrangement and cell spreading [10]. In migrating PMNs, \( \text{Ca}^{2+} \)
enriches at the leading edge of pseudopod projection and directs localized cytoskeletal activation and cell migration [8,11]. Meanwhile, binding of LFA-1 and Mac-1 to their ligands is essential in triggering Ca2+ transients and activation of Ca2+-dependent kinases involved in cytoskeletal remodeling and migratory function [12–15]. Therefore, the correlation between mechanical loading of integrins and intracellular Ca2+ transients is of great interest to understand the mechanotransduction during PMN recruitment [7].

Mechanical forces are critical regulators in integrin-mediated Ca2+ transients. Our recent study indicates that mechanical features of ECs affect PMN Ca2+ response, and such stiffness-dependent Ca2+ response is associated with β2 integrin activation [16]. Ca2+ influx is shear-dependent in rolling PMNs, and contributes to β2 integrin adhesion and subsequent cell polarization [7]. shear forces of blood flow transmitted through high-affinity LFA-1 induce their colocalization with Ca2+ channel Orai1 and activate a local bursting of Ca2+ concentration [11,17]. Tensile forces exerted on cells are balanced by the adhesive forces generated by integrin-ligand interactions during PMN adhesion and migration [18]. However, it is still unclear how mechanical forces of integrin-ligand bonds affect Ca2+ signaling in PMNs.

In a previous work, we have quantified the mechanical features of various LFA-1- or Mac-1-ligand bonds [19]. We found that the mechanical strength for LFA-1–ICAM-1/JAM-A bonds are much higher than those for Mac-1–ICAM-1/JAM-A bonds and Mac-1–RAGE bonds are stronger than LFA-1–RAGE bonds. Here we hypothesized that distinct bond strengths of β2 integrin-ligand pair govern their ligand diversity in regulating Ca2+ transients. We compared the capabilities of LFA-1 and Mac-1 to induce Ca2+ transients in adherent PMNs on various ligands under static or shear flow conditions and discussed the correlation between bond strength and outside-in signaling.

2. Materials and methods

2.1. Antibodies and reagents

Soluble mouse ICAM-1, RAGE and JAM-A with human IgG Fc chimeras were from R&D Systems (Minneapolis, MN). LEAF™ purified rat anti-mouse CD11a (M17/4) and CD11b (M1/70) blocking monoclonal antibodies (mAbs) and isotype-matched irrelevant control (RTK2758, RTK4530) mAbs were obtained from Biolegend (San Diego, CA). Fluorescent Ca2+ indicator Fluo-4 AM (F14201) and solubilizer Pluronic™ F-127 (P3000MP) were from Invitrogen (Carlsbad, CA). Goat anti-human IgG Fc polyclonal antibodies (pAbs), bovine serum albumin (BSA), and chemotactic peptides N-Formyl-Met-Leu-Phe (fMLF) were all from Sigma-Aldrich (St. Louis, MO).

2.2. Murine PMN isolation

All the animal tests were granted permission by the Institutional Animal and Medicine Ethical Committee (IAMEC) at the Institute of Mechanics, Chinese Academy of Sciences. Bone marrow (BM) PMNs were freshly isolated from total 16 male C57BL/6 mice supplied by Vital River Laboratories (Beijing, China) as previously described [20–24]. The experiments were conducted separately with young, sexually mature, 8–12-week mice to provide healthy and functionally competent PMNs. The BM cells were collected from flushing femurs and tibias of both hind legs with Dulbecco’s phosphate-buffered saline (DPBS) supplemented with 0.5% BSA and 2 mM EDTA. The cell suspension was filtrated by a 70-μm pore size cell strainer (BD Biosciences, Franklin Lakes, NJ), followed by centrifugation at 300 × g for 10 min. Sedimental cells were resuspended in DPBS and isolated by an equilibrium centrifugation of Ficoll-Hypaque density gradient (Histopaque-
Fluorescent images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) to measure the intracellular Ca$^{2+}$ levels in PMNs. For each adherent cell within the FOV, the mean fluorescence intensity ($F$) was obtained by subtracting acquired Ca$^{2+}$ signals by background signals frame-by-frame and the lowest $F$, from time-lapsed images was denoted as baseline fluorescence intensity $F_0$. The normalized ratio of $F_t$/F0 was defined as relative fluorescence intensity (RFI) to reflect the intracellular Ca$^{2+}$ changes (Fig. 1C). A RFI threshold value of 1.5 was set in this work to define those remarkable Ca$^{2+}$ spikes in PMNs [29]. A spike with a peak value more than 1.5 was denoted as a specific event. The number of spiking Ca$^{2+}$ peaks and the maximum value (peak value) of RFI was recorded in each case to represent the changes in Ca$^{2+}$ signal.

2.5. Statistical analysis

Data were presented as the mean ± SEM. Significant differences among multiple groups were identified by two-way analysis of variance (ANOVA), followed by a Holm-Sidak test. $P$ values less than 0.05 were considered statistically significant.

3. Results

3.1. Ca$^{2+}$ transients in adherent PMNs on various ligands

To evaluate whether diverse ligand binding affects the β3 integrin-mediated Ca$^{2+}$ transients, we imaged in real time the time courses of Ca$^{2+}$ oscillation in PMNs loaded with Fluo-4 during LFA-1– or Mac-1–ligand binding (Fig. 1A) and compared the capabilities of three typical ligands, ICAM-1, RAGE and JAM-A, to trigger the intracellular Ca$^{2+}$ signaling (Fig. 2). Since low-affinity LFA-1 could not induce Ca$^{2+}$ flux [11], we stimulated PMNs with fMLF to mimic in vivo inflammation microenvironment, activate PMN adhesion and enhance the fraction of high-affinity integrins and the baseline level of Ca$^{2+}$. No control experiments in the absence of fMLF were performed since the un-stimulated PMNs could not adhere to the ligand-coated chamber substrate (data not shown). Fig. 1B and C displayed typical time-lapsed Ca$^{2+}$ transients in a single PMN, exhibiting several Ca$^{2+}$ spikes within a 10-min duration. The spike number and peak value of Ca$^{2+}$ transients derived from the RFI–time curve were plotted against each ligand type to analyze their capabilities in inducing Ca$^{2+}$ response (Fig. 2). Data indicated that the Ca$^{2+}$ transients triggered by ICAM-1 binding under static condition are comparable to that of RAGE binding and the capability of JAM-A to mediate Ca$^{2+}$ transients under static condition is lowest among the three ligands.

Physiological-like shear stress in the range between 1 and 10 dyne/cm$^2$ is reported to regulate integrin activation and subsequent Ca$^{2+}$ flux [11,30]. The results showed that low shear stress (1 dyne/cm$^2$) augments significantly both the spike number (Fig. 2A) and peak value
(Fig. 2B) of Ca^{2+} transients induced by ICAM-1 or JAM-A, probably by inducing more high-affinity β2 integrins and opening up mechanosensitive Ca^{2+} channels [17]. However, the peak value of Ca^{2+} transients return to the static level under high shear stress (10 dyne/cm^2), likely stemming from dissociation of β2 integrin-ligand bonds or shear-induced cleavage of β2 integrins from cell surface [31]. RAGE-mediated Ca^{2+} transients exhibit a shear-independent feature, implying a less effective impact of integrin-RAGE bonds on mechanotransduction. Collectively, distinct features of Ca^{2+} transients were uncovered for these ligands, suggesting their diverse roles in regulating PMN recruitment.

### 3.2. Role of LFA-1– or Mac-1–ICAM-1 engagement in triggering Ca^{2+} transients

ICAM-1 is the most effective and widely tested ligand for mediating PMN adhesion and transmigration [3,32–35]. LFA-1 and Mac-1 play distinct functions that LFA-1 initiates slow rolling and firm adhesion but Mac-1 mediates cell crawling in PMN recruitment when binding to ICAM-1. These distinct functions are governed by their different binding capacities and mechanical strengths [36–38]. Here the capabilities of LFA-1–ICAM-1 and Mac-1–ICAM-1 interactions to induce Ca^{2+} transients were compared by pre-incubating PMNs with anti-LFA-1 or anti-Mac-1 blocking mAbs respectively (Fig. 3). Data indicated that LFA-1 dominates the Ca^{2+} spike number triggered by ICAM-1 binding under static condition (Fig. 3A) and the peak value of Ca^{2+} transients tends to decrease after LFA-1 or Mac-1 blocking (Fig. 3B), even not significantly. At low shear stress, LFA-1 and Mac-1 compensate each other in the resulted Ca^{2+} spike number (Fig. 3A) and play equal roles in the peak value of Ca^{2+} transients (Fig. 3B). At high shear stress, only LFA-1 blocking reduces ICAM-1-mediated Ca^{2+} response significantly (Fig. 3), implying a crucial role of LFA-1-ICAM-1 bonds in triggering Ca^{2+} signal under high shear stress.

### 3.3. Role of LFA-1– or Mac-1–RAGE engagement in triggering Ca^{2+} transients

RAGE engages in thioglycollate- or trauma-induced leukocyte recruitment mainly through Mac-1 binding [39,40]. Here the results indicated that LFA-1 and Mac-1 work cooperatively in mediating Ca^{2+} spiking induced by RAGE binding under static condition (Fig. 4A), where only the role of Mac-1–RAGE binding is significant for the peak value of Ca^{2+} transients (Fig. 4B). LFA-1 blocking can increase the spike number under shear flow presumably by compensating more Mac-1–RAGE bonds instead (Fig. 4A). Accordingly, Mac-1 blocking reduces significantly the peak value of Ca^{2+} transients triggered by RAGE under shear flow (Fig. 4B) and also decreases the spike number at high shear stress (Fig. 4A), suggesting Mac-1–RAGE bonds play a more effective role in mediating Ca^{2+} response.

### 3.4. Role of LFA-1– or Mac-1–JAM-A engagement in triggering Ca^{2+} transients

JAM-A mainly locates at endothelial junctions and promotes PMN transmigration by interacting with LFA-1 [41,42]. Here the data indicated that the spike number and peak value of Ca^{2+} transients triggered by JAM-A binding under static condition is lowest among all the non-blocking groups (Fig. 2). LFA-1 blocking cannot further reduce the static Ca^{2+} response in PMNs on JAM-A, while Mac-1 blocking increases the spike number significantly and the peak value slightly (even not significantly) probably due to the formation of more LFA-1–JAM-A bonds (Fig. 5). When Ca^{2+} transients triggered by JAM-A binding are enhanced by low shear stress, LFA-1 and Mac-1 compensate each other in the spike number, presenting the null effect of β2 integrin blocking (Fig. 5A). Meanwhile, LFA-1 dominates the peak value of Ca^{2+} oscillation (Fig. 5B). At high shear stress, LFA-1 blocking decreases Ca^{2+} response in PMNs on JAM-A moderately, while Mac-1 blocking increases the number of Ca^{2+} spikes by compensating more LFA-1–JAM-A bonds.

### 4. Discussion

Intracellular Ca^{2+} signaling is highly mechanosensitive and modulated by various mechanical stimuli. Shear stress enhances and quickens the cytoplasmic Ca^{2+} bursting of PMNs on immobilized E– or P-selectins [43,44], initiates Ca^{2+} influx mediated by transient receptor potential vallinooid type 4 (TRPV4) channel [45], primary cilium [46] or mitochondrial ATP [47] of ECs, and triggers a longitudinally-propagating, global Ca^{2+} wave via activation of purinergic signaling in atrial myocytes [48]. Stretch induces Ca^{2+} influx in vascular smooth muscle cells through plasma membrane stretch-activated channels (SACs) [49], increases Ca^{2+} spark in cardiomyocytes upon a mechanism that requires cytoskeletal integrity [50], and evokes Piezo1-dependent Ca^{2+} signaling and ATP release in urothelial cell [51]. Extracellular matrix stiffness modulates Ca^{2+} signaling of ECs in response to vascular endothelial growth factor (VEGF) [52], while mechanical vibration causes global Ca^{2+} response in ECs [53]. Evidently, significant alterations in Ca^{2+} signaling induced by mechanotransduction may be involved in many of physiological and pathological responses.

Mechanotransduction-induced Ca^{2+} signaling is modulated by the mechanical features of receptor-ligand interactions. Catch bonds formed between T cell receptor (TCR) and agonist peptide-major histocompatibility complex (pMHC) trigger Ca^{2+} signaling in T cells, whereas slip bonds formed between TCR and antagonist pMHC fail to activate [54]. High-affinity LFA-1 is necessary for the amplification of Ca^{2+} flux in the presence of shear flow, while low-affinity LFA-1 could not induce Ca^{2+} responses [11]. Therefore, bond strength might be critical to mechanical stimuli-induced Ca^{2+} signaling. In this study, we attempted to correlate the mechanical strength of LFA-1 or Mac-1 to respective ligands with their capabilities in inducing Ca^{2+} signaling in PMNs. LFA-1 dominates Ca^{2+} transients in adherent PMNs on ICAM-1...
Our data correlate the mechanical strength of LFA-1 reduced by RAGE binding (Fig. 4), all of which are consistent with their in vivo especially under physiological-like shear recruitment cascade and maximizing Ca\(^{2+}\) signaling in PMNs [7]. Here Binding of LFA-1 and Mac-1 to ICAM-1 is essential for initiating classic ligands are tissue- and stimulus-specific in an dose-dependent manner [55]. Few works have reported the recruitment during in vivo mechanical strength in triggering Ca\(^{2+}\) signaling, which provides an insight into the mechanotransduction and outside-in signaling of PMN [56], therefore JAM-A expressed on junctions and the surfaces of PMNs [56], therefore JAM-A expressed on endothelial tight junctions and the surfaces of PMNs [56], therefore JAM-A expressed on PMNs might engage in homophilic binding with those JAM-As coated on the chamber substrate in this study (Fig. 5). The homophilic interactions could also initiate the Ca\(^{2+}\) transients in PMNs triggered by the engagement of \(\beta_2\) integrin to RAGE or JAM-A. Specifically, JAM-A locates onto both the endothelial tight junctions and the surfaces of PMNs [56], therefore JAM-A expressed on PMNs might engage in homophilic binding with those JAM-As coated on the chamber substrate in this study (Fig. 5). The homophilic interactions could also initiate the Ca\(^{2+}\) transients in PMNs triggered by JAM-A binding, which might lead to the abnormal increase of Ca\(^{2+}\) spike number after Mac-1 blocking (Fig. 5A).

Collectively, we focus in this study on the contributions of bond mechanical strength in triggering Ca\(^{2+}\) signaling, which provides an insight into the mechanotransduction and outside-in signaling of PMN recruitment. It should also be mentioned that the downstream signaling of LFA-1 or Mac-1-mediated Ca\(^{2+}\) transients may not be the same for distinct ligands. One possibility is that LFA-1 and Mac-1 utilize different calcium-dependent kinases to trigger adherent activation and migration of PMNs.

This in vitro study applied a flow chamber assay to mimic PMN recruitment during inflammation and gave a brief description of the Ca\(^{2+}\) transients in PMNs triggered by distinct ligands under physiological-like shear flow. However, the expression and distribution of multiple ligands are tissue- and stimulus-specific. The matrix stiffness, vessel geometry and blood hemodynamics in different tissues complicate the microenvironment faced by PMNs in vivo. Therefore, the Ca\(^{2+}\) responses of PMNs in specialized in vivo microenviornment might be different and need further investigation.

Declarations of interest

The authors declare no conflicts of interest, financial or otherwise.

CRediT authorship contribution statement

Xinyu Shu: Conceptualization, Investigation, Visualization. Ning Li: Conceptualization, Validation, Writing - original draft, Data cura\(\text{tion}\), Writing - review & editing. Dandan Huang: Methodology. Yan Zhang: Writing - review & editing. Shouqin Lü: Writing - review & editing. Mian Long: Conceptualization, Writing - review & editing, Supervision.

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