

Distinct Kinetics Features of LFA-1 and Mac-1 in Neutrophil Activation

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Abstract – LFA-1 and Mac-1, two β_2 integrin members constitutively expressed on neutrophils, mediate leukocyte recruitment cascade by binding to the same ligand of ICAM-1. The slow rolling and firm adhesion of leukocytes rely on LFA-1 while the cell crawling is dependent on Mac-1. We hypothesized that their distinct roles are likely attributed to the differences in the binding kinetics or in the diverse responses of outside-in and inside-out signaling. In this study, we compared the ICAM-1 binding features between soluble or membrane-expressed LFA-1 and Mac-1 with different affinity conformation using optical trap technique. Our data indicated that the affinity up-regulation from wide type (WT) to high affinity (HA) is off-rate dependent for LFA-1 but on-rate dependent for Mac-1. The structural bases of this new finding were found to be consistent with our previous simulations. These results furthered our understanding in their function differences under shear flow.

Keywords – LFA-1, Mac-1, ICAM-1, on-rate, off-rate.

I. INTRODUCTION

LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) and Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) are two members of β_2 integrin subfamily and share common β_2 subunit non-covalently associated with respective α subunit. They are constitutively expressed on neutrophils and mediate leukocyte recruitment at various stages via binding to the same ligand of intercellular cell adhesive molecule 1 (ICAM-1). Previous studies indicated that the slow rolling and firm adhesion of leukocytes is dependent on LFA-1 while the crawling is dependent on Mac-1 [1]. We hypothesized that their distinct roles are likely attributed to the differences in the binding kinetics to ICAM-1 or in the diverse responses of outside-in and inside-out signaling. In this study, we constructed the recombinant LFA-1 and Mac-1 proteins and compared the ICAM-1 binding features between soluble or membrane-expressed LFA-1 and Mac-1 with different affinity conformation. Also investigated were their corresponding conformational dynamics, which govern the binding kinetics. The outcomes provide an insight in understanding the

molecular mechanism for their cooperative functions and structure-function relationship.

II. MATERIALS AND METHODS

A. Reconstruction and expression of soluble β_2 integrins

To create human Mac-1 and LFA-1 Fc chimera, separate fragments encoding the ectodomains of α_L (Gln-1063), α_M (Leu-1093), and β_2 (Asn-678)-subunit fused to specially-mutated Fc domains were respectively cloned into pcDNA3.1 vectors. The Fc domains which contained T366Y or Y407T mutation were used to increase the likelihood of heterodimerization between α and β chains [2]. The high affinity mutants (LFA-1-K287C/K294C and Mac-1-Q163C/Q309C) were constructed through substitution of corresponding amino acids by cysteine in α_L and α_M -subunit as described [3]. The four recombinant Fc chimera proteins (Fig. 1A) were expressed by transiently transfected 293T cells using a calcium phosphate-mediated transfection procedure. Culture supernatant was harvested from transfected 293T cells and tested for integrin expression by ELISA and Western blotting analyses. The recombinant integrin was harvested via anti-Fc domain secondary antibodies coated on beads. Their binding affinity to ICAM-1 ligand was quantified respectively upon adhesion frequency assay using optical trap assay.

B. Reconstruction and expression of full-length β_2 integrins

Four distinct α -subunit and one common β -subunit full-length plasmids including extracellular domains, transmembrane segment, and cytoplasmic tail were reconstructed, respectively, for Mac-1-WT, Mac-1-HA, LFA-1-WT, and LFA-1-HA (Fig. 1B). Each pair of plasmid was co-transfected into 293T cells via calcium phosphate method while mock plasmid without target gene was used as control. Harvested cells were segregated into two aliquots: one was used for adhesion kinetics measurements via optical trap assay; the other was used for flow cytometry measurements where the distinct mAb of MEM-25, ICRF44 or TS1/18 was employed for staining α_L , α_M and β_2 -subunit respectively.

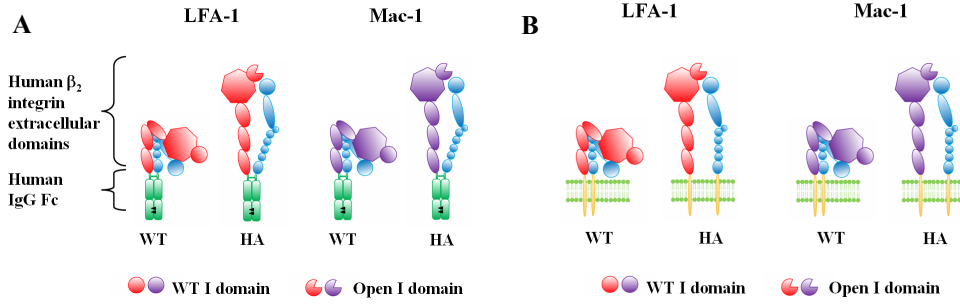


Fig. 1 Reconstruction of extracellular (A) and full-length (B) Mac-1 and LFA-1 molecules.

C. Adhesion frequency approach using Optical trap assay

An adhesion frequency assay, used to measure the kinetics and affinity of surface-bound molecule pair, has been previously described [4]. Briefly, each 10^3 β_2 integrin- and ICAM-1-coupled beads were mixed just before injecting into a customer-made glass sample cell ($\sim 14 \times 10 \times 0.5$ cm). A 6 μm -diameter bead physically adsorbing human ICAM-1 settled quickly down and adhered stably onto the surface of coverslip substrate. A floating 3 μm -diameter streptavidin coated bead, which pre-absorbed biotin-labeled goat-anti-human IgG polyclonal antibodies and then captured Fc-fused recombinant β_2 integrin, was captured by a mobile optical trap (PALM, Zeiss, Germany). ICAM-1-bearing bead was brought by a computer-controlled piezoelectric actuator into contact repeatedly with the β_2 integrin-bearing bead. The contact duration was kept constant in each 50 cycle for one bead pair and varied over a range (0.25-7s) for different pair pairs. Adhesion between two beads was staged by placing them onto controlled contact via micro-manipulation. The presence of adhesion at the end of a given contact period was detected mechanically by observing microscopically the rebounding of the 3 μm -diameter bead upon retracting it away from 6 μm -diameter bead. This contact-retraction cycle was repeated 50 times to estimate the adhesion probability, P_a , at that contact duration, t . For each recombinant β_2 integrin examined, 5 pairs of beads were used to obtain several P_a vs. t curves that correspond to different protein densities, m_r , and ICAM-1 density, m_l . Each binding curve was fitted to a small system probabilistic kinetic model,

$$P_a = 1 - \exp\{-m_r m_l A_c K_a [1 - \exp(-k_r t)]\}, \quad (1)$$

to estimate a pair of parameters: the reverse rate, k_r , and effective binding affinity, $A_c K_a$, where A_c is the contact area, which was kept constant in all experiments. Multiple pairs of (k_r , $A_c K_a$) values were obtained for each recombinant β_2 integrin to allow evaluation of the mean and standard deviation. The statistical significance of the difference between

the affinities of different surface-bound recombinant β_2 integrin was assessed by the Student t-test.

In some cases, ICAM-1-Fc was coupled onto 3 μm -diameter bead, which pre-absorbed goat-anti-human IgG Fc polyclonal antibodies. The interaction between the ICAM-1-bearing beads and 293T cells transfected by full-length of Mac-1-WT, Mac-1-HA, LFA-1-WT, and LFA-1-HA, were measured using optical trap assay, respectively.

D. Free and steered molecular dynamics simulation

The micro-structural dynamics of the complex were tested using molecular dynamics simulations (MDS) by comparing the conformational stability of LFA-1/Mac-1 α subunit I domain and the interaction between I domain-ICAM-1 D1/D3 domain.

III. RESULTS

A. Biological validation of recombinant proteins

The recombinant β_2 integrin-Fc fusion molecules were validated using ELISA and Western blotting analyses. Our data indicated that those recombinant proteins are correctly expressed. Full-length Mac-1 and LFA-1 were expressed well onto 293T cells as compared to that of control, suggesting that the transfection protocol works well (*data not shown*).

B. Binding of soluble β_2 integrins to ICAM-1

To compare the binding kinetics of recombinant LFA-1 and Mac-1 to their common ligand, adhesion frequency was quantified using an optical trap assay. At the given contact duration of 0.25-7.0 s, the adhesion frequency exhibited a transition phase when $t < 2$ s and then reached a plateau (*points*), which fitted well with the model predicted using Eq. 1 (*lines*) (Figs. 2A-B). Estimated kinetic parameters

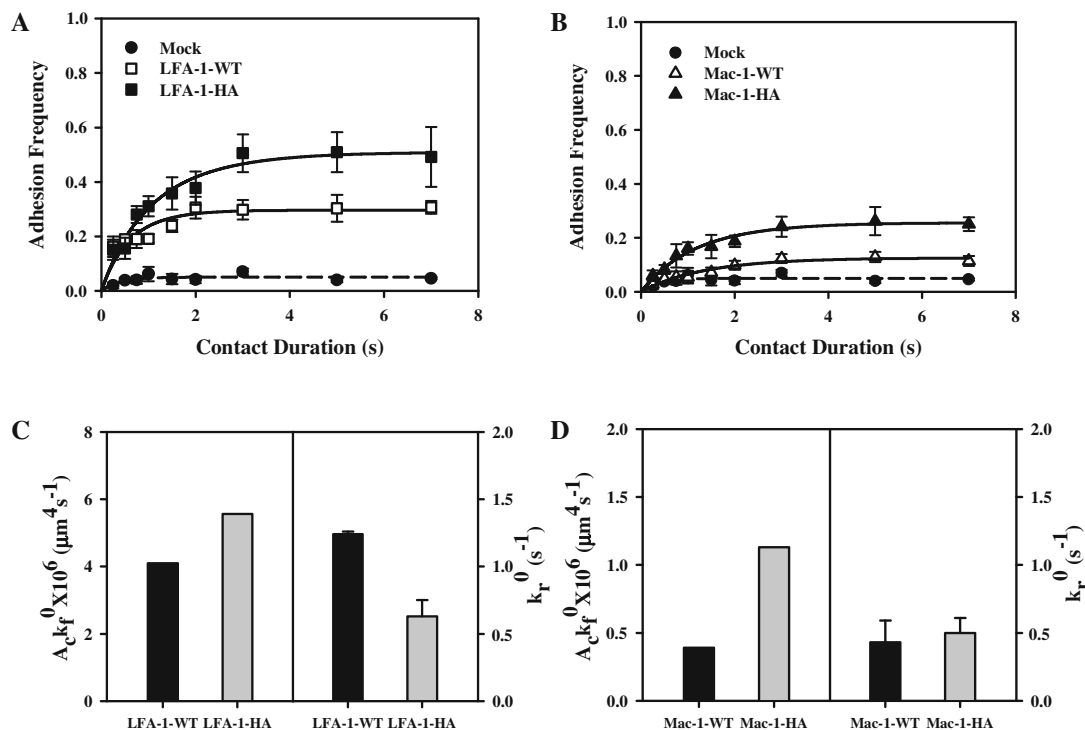


Fig. 2 Binding curves (A, B) and kinetic parameters (C, D) of extracellular recombinant LFA-1 (A, C) and Mac-1 (B, D) to ICAM-1. $A_c k_f^0$ and k_r^0 are the lumped on-rate and off-rate, respectively

indicated that the affinity difference between HA and WT is off-rate dependent for LFA-1 (Fig. 2C) but on-rate dependent for Mac-1 (Fig. 2D), which is presumably related to their distinct roles in initiating the leukocyte recruitment.

We also compared the binding affinity difference between LFA-1 and Mac-1 in the same state. For example, 3.5-fold lower affinity for WT Mac-1 is attributed to the inverse correlation of 2.9-fold lower off-rate but 10.5-fold higher on-rate, as compared to WT LFA-1. On the other hand, the off-rate is similar but the affinity is 4.1-fold lower for HA Mac-1 than that for HA LFA-1, mainly due to the 4.9-fold difference in the on-rate (Figs. 2C-D). These results were not only consistent with the previous observations in the binding differences between the two receptors, but they also proposed the distinct mechanisms of kinetic regulation in state transition between WT and HA states.

C. Binding of membrane-expressed β_2 integrins to surface-bound ICAM-1

To further confirm that the novel kinetic regulation is more physiologically relevant, full-length β_2 integrins were transfected into 293T cells and the binding to ICAM-1 coated on

beads was measured using optical trap assay at contact duration of 0.5-7.0 s. As exemplified in Fig. 3A, the data (points) also fitted well with the predictions (lines). More importantly, the estimated kinetic parameters supported the opposite regulation mechanisms of Mac-1 and LFA-1 when they switched from low to high affinities. It was found that the affinity transition for Mac-1 is on-rate dependent since the off-rate is similar but the on-rate is 1.8-fold lower from WT and HA state (gray bars) whereas it is off-rate dependent for LFA-1 because the on-rate is similar but the off-rate yields 1.7-fold difference (black bars) (Fig. 3B).

D. Structural differences of state transition between LFA-1 and Mac-1

These results were also consistent with MDS simulations in the two aspects from on-rate and off-rate viewpoints: 1) the binding “pocket” keeps accessible to the ligand for LFA-1 but switches from close to open for Mac-1 when the receptor transits from low affinity (LA) to HA state, suggesting that Mac-1 binding could be on-rate dependent; 2) forced lifetime of LFA-1-ICAM-1 bond is significantly enhanced when LFA-1 transits from LA to HA state,

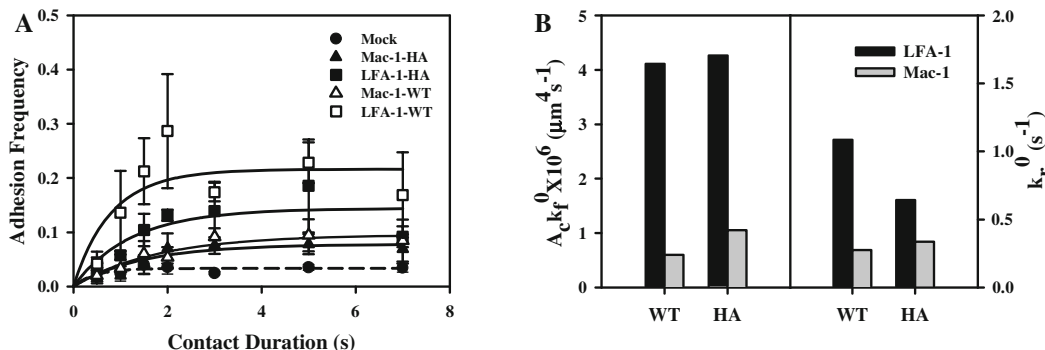


Fig. 3 Binding curves (A) and kinetic parameters (B) of full-length recombinant LFA-1 and Mac-1 to ICAM-1.

$A_c k_f^0$ and k_r^0 are the lumped on-rate and off-rate, respectively.

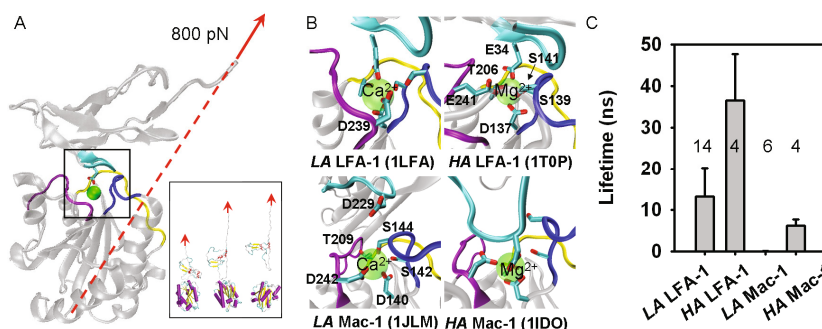


Fig. 4 Key interactions between LFA-1/Mac-1 I domain and ICAM-1 D1/D3 domain (B) and bond lifetime (C) estimated via SMD simulation with 800-pN constant force pulling C-terminal of ICAM-1 D1 or D3 domain (A).

implying that the interaction might be off-rate related [5] (Fig. 4). These simulations further the understandings in their function differences and structure-function relationship.

IV. CONCLUSION

Kinetic comparison between LFA-1 and Mac-1 to ICAM-1 indicated that the affinity up-regulation from WT to HA is off-rate dependent for LFA-1 but on-rate dependent for Mac-1, which is consistent with conformational difference between LFA-1 and Mac-1. These results shed a light on their biological functions of β_2 integrin from the viewpoint of distinct binding kinetics and structural features.

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