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# Role of ICAM-1 polymorphisms (G241R, K469E) in mediating its single-molecule binding ability: Atomic force microscopy measurements on living cells

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ABSTRACT

Atherosclerosis (As) is characterized by chronic inflammation and is a major cause of human mortality. ICAM-1-mediated adhesion of leukocytes in vessel walls plays an important role in the pathogenesis of atherosclerosis. Two single nucleotide polymorphisms (SNPs) of human intercellular adhesion molecule-1 (ICAM-1), G241R and K469E, are associated with a number of inflammatory diseases. SNP induced changes in ICAM-1 function rely not only on the expression level but also on the single-molecule binding ability which may be affected by single molecule conformation variations such as protein splicing and folding. Previous studies have shown associations between G241R/K469E polymorphisms and ICAM-1 gene expression. Nevertheless, few studies have been done that focus on the single-molecule forces of the above SNPs and their ligands.

In the current study, we evaluated both single molecule binding ability and expression level of 4 ICAM-1 mutations – GK (G241/K469), GE (G241/E469), RK (R241/K469) and RE (R241/E469). No difference in adhesion ability was observed via cell adhesion assay or atomic force microscopy (AFM) measurement when comparing the GK, GE, RK, or RE genotypes of ICAM-1 to each other. On the other hand, flow cytometry suggested that there was significantly higher expression of GE genotype of ICAM-1 on transfected CHO cells. Thus, we concluded that genetic susceptibility to diseases related to ICAM-1 polymorphisms, G241R or K469E, might be due to the different expressions of ICAM-1 variants rather than to the single-molecule binding ability of ICAM-1.



## 1. Introduction

Atherosclerosis (As) is a complicated, vascular, inflammatory disease. The early pathological process of As is primarily mediated by adhesion molecules, which induce the transendothelial migration of leukocytes from the circulation [1]. Previous studies have demonstrated an up-regulation of ICAM-1 at lesion-prone sites. Experiments in animal models have shown reduced As in ICAM-1 gene knockout mice [2–4]. In recent years, a large number of epidemiological surveys have revealed a correlation between As and genetic factors [5–7]. ICAM-1 gene polymorphisms are of interest due to the role of ICAM in leukocyte adhesion and migration [8,9].

Intercellular adhesion molecule-1 (ICAM-1, CD54) is a member of the immunoglobulin (Ig) superfamily, which is expressed on the surfaces of various cells, including endothelial cells. It is a transmembrane glycoprotein made up of 505 amino acids with a molecular structure that is divided into three parts: an extracellular portion (453 amino acids) containing five Ig-like domains (D1–5) that function in adhesive interactions, a transmembrane region consisting of 24 residues, and a short intracellular tail made up of 28 amino acids, that is related to signal transduction [10]. The two main ligands for ICAM-1 are lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$), which are located on the surface of leukocytes. Of these, Mac-1 is considered to play a bigger role at the beginning of As formation due to its atherogenic effects [11].

During the last decade, ICAM-1 has been identified as a risk factor for several vascular inflammatory diseases [12,13]. Results

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from previous epidemiological studies into the genetics of As have shown significant linkage on chromosome 19 near the ICAM-1 structural gene [14]. Furthermore, many studies have indicated that genetic polymorphisms of ICAM-1 may be important contributors to genetic susceptibility for As [9,15]. G241R and K469E polymorphisms within the structural gene have also been shown to be related to a variety of atherosclerotic diseases [16–18].

Consistent with this, a large body of literature suggests that G241R and K469E may be associated with As [19], PAOD [20], MI [16] and other inflammatory diseases. However, the results of these studies are controversial [21,22]. During the pathological process of As, leukocyte adherence to endothelial cells depends on the amount of expression of ICAM-1 and the single-molecule force between the ICAM-1 ligands and the leukocyte surface. Both of these factors are supposed to be the underlying mechanisms. Nevertheless, few studies have focused on single-molecule force of the above SNPs and their ligands.

Atomic force microscopy (AFM) is an ultrahigh resolution (0.1 nm × 0.1 nm) type of scanning probe microscopy and a sensitive multifunctional molecular tool. It is a key instrument used in nanoscience and nanobiology. The advantages of AFM for measuring living cells have been highlighted in a large number of reports during recent decades [24]. The combination of AFM and fluorescence microscopy allows for a more detailed characterization of cellular structures and processes.

In the current study, AFM was used to investigate the single molecule binding force of GK, GE, RK, and RE to Mac-1 in living cells to determine whether binding force changes after mutation and to further define the role of G241R and K469E SNPs in atherosclerotic diseases.

## 2. Materials and methods

### 2.1. Cells and reagents

Human umbilical vein endothelial cells (HUVECs) and Chinese hamster ovary (CHO) cells were kindly provided by Dr. Wei Chen (Beijing Institute of Microbiology and Epidemiology, China). HUVEC and CHO cells were cultured in medium (M200+LSGS, Cascade Biologics, USA) and MEM (GIBCO USA) containing 10% fetal bovine serum (Hyclone, USA), respectively. The origin and isolation of donated HUVECs has been described previously [25] and were used with permission from the local Ethics Committee. The pMD18-T and pEGFP-N1/pcDNA3.1+ vectors were purchased from TaKaRa (Japan) and Invitrogen (USA), respectively. The 3-Mercaptopropyl trimethoxysilane (MPTMS) was purchased from ACRO. N-hydroxysuccinimide-poly-ethylene glycol-maleimide (NHS-PEG-MAL, MW 3400) was purchased from Laysan Bio (China).

### 2.2. cDNA constructions and mutations

Total RNA was extracted from the cultured HUVECs per the standard technique described in the Qiagen RNA Kit (Germany). Six primers were designed that contained BamH1 and HindIII restriction sites at the 3′and 5′ ends (Table 1) to produce single gene mutations.

**Table 1**
Primer sequences used for PCR amplification.

| Primers | Sequences for primers |
|---|---|
| U1 | 5′-CCA AGC TTC CTC GCT ATG GCT CCC AGC AG-3′ |
| L1 | 5′-CTG GAT CCA AGG GAG GCG TGG CTT GTG TGT T-3′ |
| U2 | 5′-CAC GGT CAC CTC GCG GGT GAC CTC-3′ |
| L2 | 5′-GAG GTC ACC CGC GAG GTG ACC GTG-3′ |
| U3 | 5′-TCC CTG GAC AGG CTG TTC CCA-3′ |
| L3 | 5′-TGG GAA CAG CCT GTC CAG GGA-3′ |

First, cDNA was made using total RNA as a template and oligodT as the primer. The coding region of ICAM-1 was amplified by PCR using U1/L1 as primers. The PCR product was determined to be the GK genotype of ICAM-1 by DNA sequencing. Three additional ICAM-1 gene mutations (GE, RK, RE) were then generated by overlapping PCR. Briefly, GE was generated using GK as a template and U1/L2 and U2/L1 as primers, which encoded the E substitution at codon 469 to produce DNA fragments 1 and 2. After verification using agarose gel analysis, a third round of PCR was carried out to produce the GE genotype using fragments 1 and 2 as the template and U1/L1 as the primers. Similarly, RK was generated using GK as a template and U1/L1/U3/L3 as primers, which encoded the R substitution at codon 241. RE was generated using RK as a template and U1/L1/U2/L2 as primers.

All PCR products were ligated into pMD18-T vectors for sequencing and subcloned into the HindIII and BamH1 restriction sites of pEGFP-N1/pcDNA3.1+. Recombinant plasmids were analyzed by digestion with restriction enzymes.

### 2.3. Cell transfection and the establishment of stable cell lines

Before transfection, CHO cells were grown to 80–90% confluence. Transfection was accomplished with Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions.

For transiently transfection, CHO cells were transfected with pcDNA3.1+ plasmids encoding GK, GE, RK, RE respectively together with CMVβ(a plasmid encoding β-galactosidase, Invitrogen) to control transfection efficiency. After 48 h incubation, CHO cells were stained with ICAM-1 antibody FITC-MEM111 (Biolegend).

For stable transfection, the recombinant plasmids GK-GFP, GE-GFP, RK-GFP, RE-GFP and control GFP were transfected into CHO cells. After incubation for 48 h, the cell monolayers were washed, trypsinized and resuspended. Each genotype was then plated in a separate 24-well plate and selection was done with 1000 μg/ml G418. To ensure consistent ICAM-1 expression, positive clones with similar fluorescence intensities were selected and isolated after 7 days. The selected clones were then expanded in preparation for the adhesion assay and AFM measurements. Confocal laser scanning microscope was used to verify the correct location of the 4 ICAM-1 protein mutations on the cell surface (Fig. 1C).

### 2.4. Flow cytometric analysis

Transiently transfected CHO cells were analyzed using flow cytometry. β-Galactosidace activity was measured with β-galactosidase Assay Kit (Beyotime, China). Data were normalized using a ratio of mean fluorescence intensity (MFI)/β-galactosidase activity.

GK-GFP, GE-GFP, RK-GFP, and RE-GFP-expressing CHO cells and control cells were analyzed and quantified using flow cytometry. The transfection efficiency was measured using the MFI and Gate%.

### 2.5. Leukocyte isolation

Human PBMCs (peripheral blood mononuclear cells) were isolated from whole blood taken from healthy volunteers. All studies involving humans were done with permission from the local Ethics Committee. PBMCs were separated with EZ-sep (Dakewe Biotech, China) and washed twice in PBS supplemented with 2% FBS. PBMCs were labeled with WGA (wheat germ agglutinin, Invitrogen) for 10 min and washed 2–4 times to remove residual WGA. After centrifugation, the cell pellet was resuspended at $2 \times 10^6$ cells/ml in RPMI 1640 (GIBCO) and used immediately.

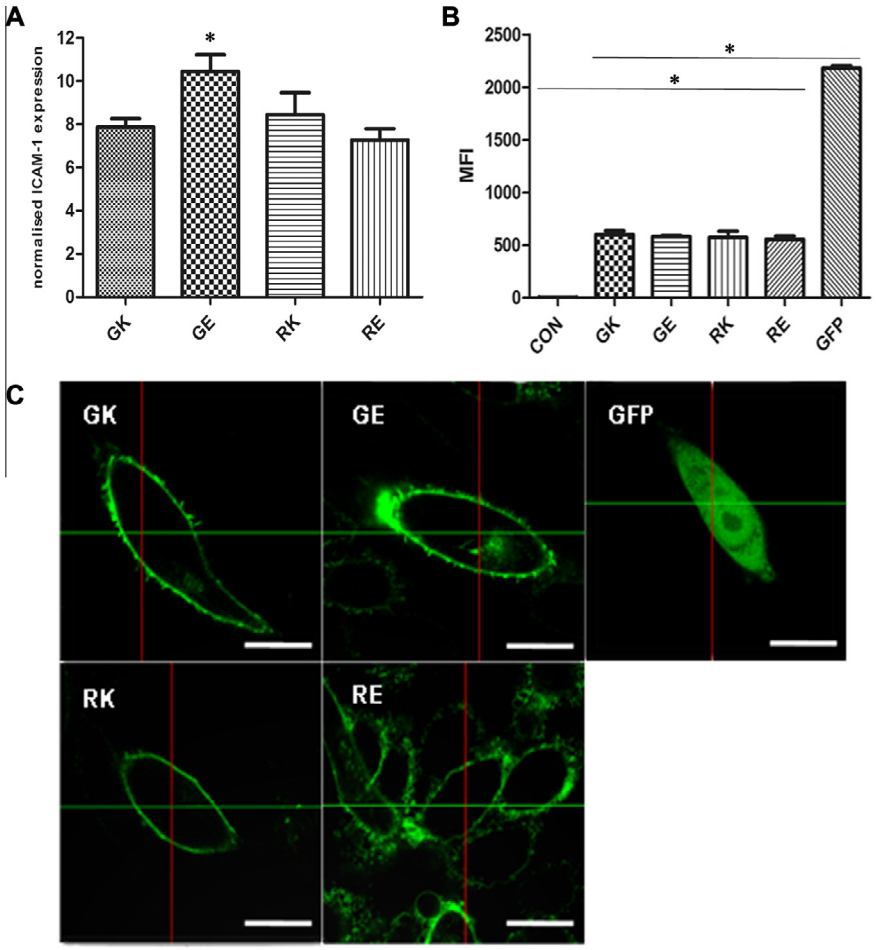


**Fig. 1.** Expression levels among different genotypes of ICAM-1. (A) ICAM-1 expression on the surface of transiently transfected CHO cells. Expression of ICAM-1 was calculated by MFI/β-galactosidase activity ±SEM. $n = 3$. *GE *vs.* the other three mutations. (B) Expression of ICAM-1 was determined by flow cytometric analysis. There was no significant difference among GK, GE, RK, or RE expression in CHO cells. $n = 3$. *$P < 0.05$ *vs.* ICAM-1 mutations. Bars indicate mean ± SEM. (C) CHO cells expressing mutant ICAM-1-GFPs and control GFP. The expression was observed by confocal microscopy. Bar = 15 μm.

### 2.6. Cell adhesion assay

GK-GFP, GE-GFP, RK-GFP, and RE-GFP-expressing CHO cells and control CHO cells were grown to confluence in a 48-well plate (5 wells per genotype). The, cells were washed with PBS, the labeled human PBMCs ($1.6 \times 10^5$ cells/well) were added and then allowed to adhere for 30 min at 37 °C. The unbound PBMCs were then carefully removed with PBS washing. Ten randomly selected optical fields (1.1 $mm^2$/field) per well were visualized and PBMCs were counted using a fluorescence microscope. The adhesion assay was repeated three times under the same conditions.

### 2.7. Single-molecule force spectroscopy

An AFM silicon nitride ($Si_3N_4$) tip (NP-10, Veeco, Santa Barbara, CA, USA) was functionalized according to previously reported procedures [25]. The tip was cleaned, hydroxylated in a plasma reactor and dried with nitrogen. It was then transferred into MPTMS solution to silanized. The tip was then immersed in N-hydroxy-succinimide-polyethyleneglycol-maleimide (NHS-PEG-MAL, Laysan Bio, USA) solution for 2 h and then immersed in Mac-1 recombinant protein (R&D) solution for 1 h at room temperature. Using this method, the link-protein (Mac-1) would link to the silanized tip and would remain flexible. The Mac-1-modified tip was then rinsed and stored in PBS at 4 °C until use.

A PicoSPM with PicoScan 3000 controller (molecular imaging) and a fluorescence microscope (Olympus) were used to achieve the single-molecule force measurement of ICAM-1/Mac-1. First, a 35 mm culture dish, containing one of the genotypes of the ICAM-1-expressing CHO cells, was placed on the AFM stage and fluorescent proteins were observed using a fluorescence microscope (U-MWU2 filter set: Ex: BP 460–490 nm, Em: LP 510 nm, Olympus) to determine the target gene location. The Mac-1-coated AFM tip was then used to repeatedly contact a randomly selected target location to get force-distance curves for the ICAM-1/Mac-1 interaction at the similar loading rate of $(1.2 \pm 0.2) \times 10^3$ pN $s^{-1}$. The calibrated spring constant of the cantilever was 0.076 $Nm^{-1}$, the contact time was 10 ms and the retraction rate was 5 μm $s^{-1}$. Three to five cells per dish were measured for each genotype and 1000 force-distance curves were recorded for each cell. At least four dishes were measured in every experiment.

### 2.8. Statistical analysis

All the data were analyzed by SPSS 16.0 statistic software. One-way ANOVA was used to compare means of different parameters among all the groups in the Cell adhesion assay, Flow cytometric analysis, ICAM-1/Mac-1 Binding probability and force measurements. Paired-samples *t*-test was used to compare means of binding probability of ICAM-1/Mac-1 before and after ICAM-1 blocked. *P* values <0.05 were considered as statistically significant.

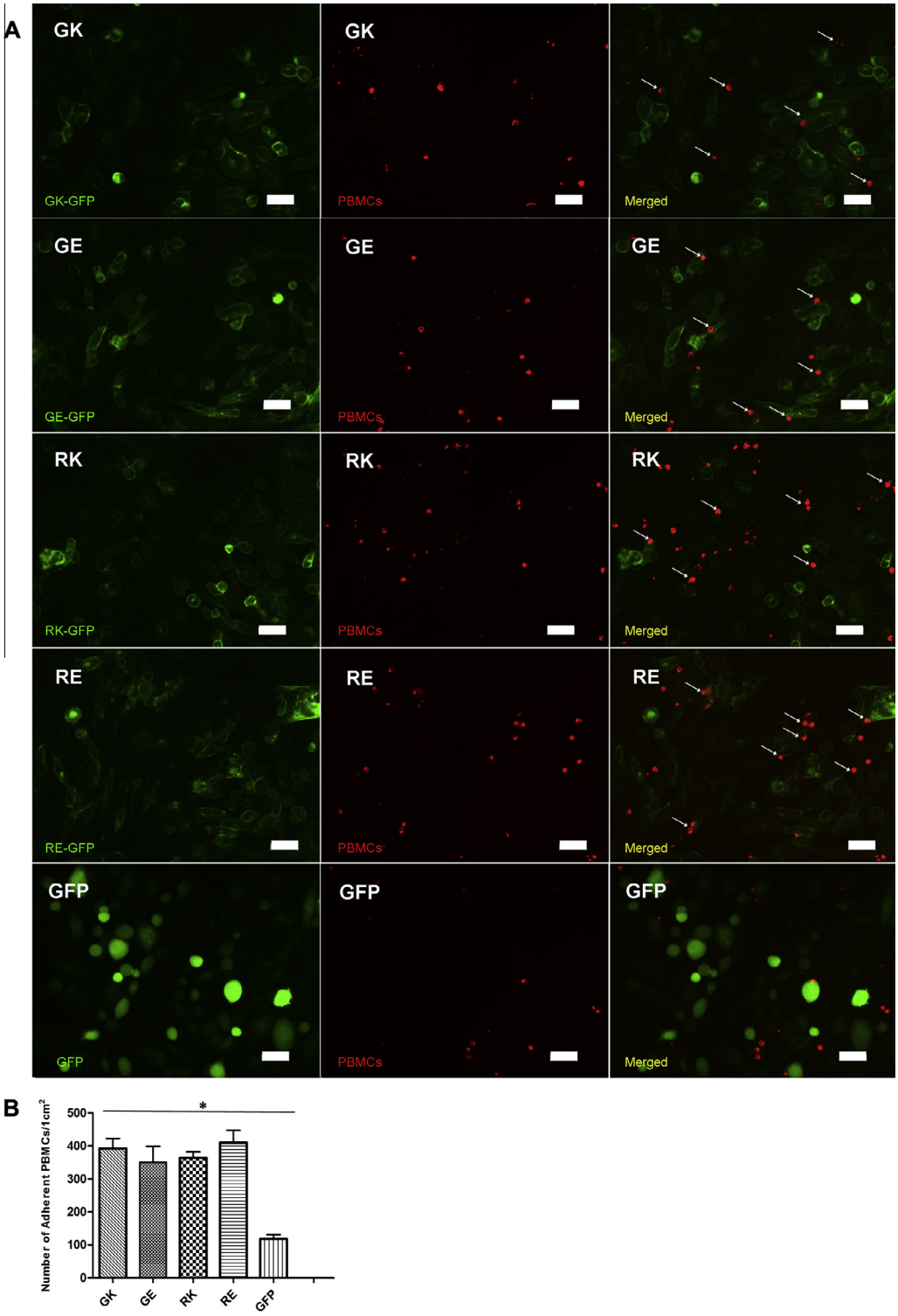


**Fig. 2.** Adhesion of PBMCs to CHO cells transfected with different genotypes of ICAM-1. (A) PBMCs adhere to the surface of CHO cells transfected with GK, GE, RK, or RE genotype of ICAM-1(arrows). Bar = 40 μm. (B) Adhesion number of PBMCs to GK, GE, RK, or RE was not significantly different $^{*}P < 0.05$ *vs*. ICAM-1 mutations. $n = 3$. Bars indicated mean ± SEM.

## 3. Results

### *3.1. Expression of polymorphic mutations of the ICAM-1 gene in CHO cells*

The four mutations of ICAM-1 were transiently transfected into CHO cells together with CMVβ to control for transfection efficiency. Results suggested that expression of GE genotype was significantly increased compared with the other three genotypes (Fig. 1A).

On the other hand, CHO cell lines that can consistently express the GK, GE, RK, and RE genotypes of ICAM-1 were successfully established. Since the current study was focused on the single molecule force of ICAM-1, CHO cells with similar expression levels were selected to rule out the impact of gene expression. As illustrated in Fig. 1B, flow cytometry analysis showed that there was

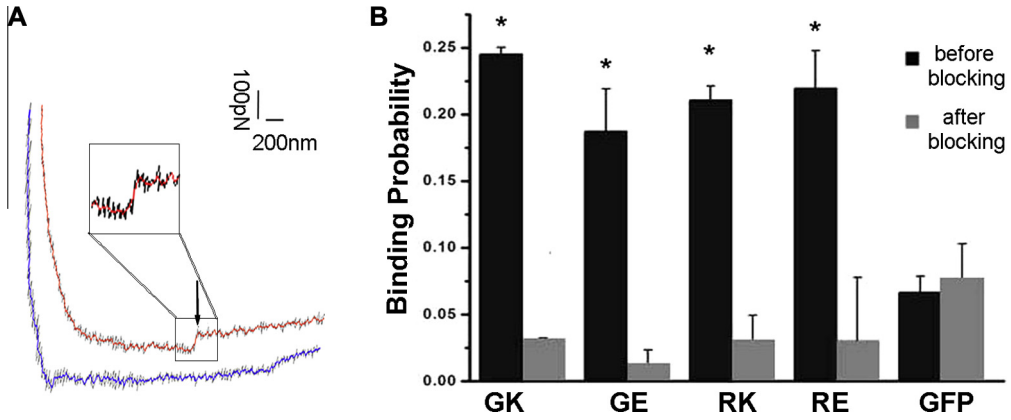


**Fig. 3.** Binding probability of ICAM-1 mutations and Mac-1. (A) Force–displacement traces of ICAM-1/Mac-1. The smooth blue curve means no force was determined between the tip and the cell. The abrupt shift (arrow) in the red curve represents adhesion between the tip and the cell. (B) Binding probability of GK, GE, RK, and RE with Mac-1. $^{*}P < 0.05$ *vs*. after blocking. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

no significant difference in the expression of different ICAM-1 mutations in CHO cells.

### 3.2. Adhesion of PBMCs to mutations of ICAM-1

As showed in Fig. 2, the adhesion of PBMCs to ICAM-1-expressing CHO cells was significantly higher compared to control CHO cells ($P < 0.05$, GK, GE, RK, RE *vs*. control). However, there was no significant difference in the number of adherent PBMCs among the GK, GE, RK, or RE genotypes of ICAM-1. The results suggest that there is no difference in adhesion among the GK, GE, RK, or RE genotypes of ICAM-1 on a cellular level.

### 3.3. Measurement of single-molecule force of mutations of ICAM-1/Mac-1

To further clarify whether the above two SNPs affect the adhesion avidity of ICAM-1, AFM was used to examine the single molecule binding force of GK, GE, RK, and RE to Mac-1 in living cells. The Mac-1 binding site is located in the third extracellular domain of ICAM-1 protein, which is also the location of G241R. Therefore, Mac-1 was used to coat the AFM tips that made contact with the ICAM-1 mutations. During the measurement process, the modified tip was directed to the ICAM-1-expressing CHO cells with the help of optical and fluorescence imaging of cells using the combined AFM/fluorescence microscopy system. Typical force curves are displayed in Fig. 3A for the ICAM-1/Mac-1 interaction. The red curve designates the specific ICAM-1/Mac-1 interaction force when the tip was retracted from a CHO cell. Thus, the red curve demonstrates the force obtained with the Mac-1-coated tip in a single ICAM-1/Mac-1 pair, whereas the blue curve indicates that there was no interaction force between the Mac-1-coated tip and ICAM-1-blocking cells.

To assure the binding of single pairs of molecules, the concentration of Mac-1 was adjusted to 10ug/ml, while the concentration of CHO cells was kept at a low density. The force were obtained at the similar loading rate of $(1.2 \pm 0.2) \times 10^3$ pN $s^{-1}$, the retraction rate was set to 5 μm $s^{-1}$, and the contact time was set to 10 ms, so that the binding probability could be varied from 10% to 30%. As shown in Fig. 3B the binding probabilities of GK, GE, RK, and RE were all <30%, which means that most of the observed binding events originated from a single ligand-receptor pair interaction. The antibody, MEM111, was used to block ICAM-1 on the tested cells. We then measured binding probabilities with Mac-1-coated tips before/after blocking cells under the same conditions. The results showed that the binding probability was decreased by ~10-fold after blocking ICAM-1, confirming that the forces were ICAM-1/Mac-1 interaction forces (Fig. 3B).

The force (mean ± S.D.) was determined as 40.29 ± 3.30 pN, 40.58 ± 2.05 pN, 39.42 ± 4.09 pN and 41.20 ± 2.71 pN for GK/Mac-1, GE/Mac-1, RK/Mac-1, and RE/Mac-1, respectively (Fig. 4A). Fig. 4B shows the characteristic binding force distribution of ICAM-1/Mac-1 obtained by Gaussian fitting. The results are based on thousands of force–distance curves for each genotype by employing SPIP software and all results were obtained from three independent sets of experiments. The parametric statistical analysis (One-way ANOVA) indicated that there was no significant difference in the adhesion force measured for any of the four genotypes.

## 4. Discussion

In the current study, both the expression level and the single-molecule ability of ICAM-1 polymorphisms GK, GE, RK, RE were determined. The results indicated that expression of GE genotype is significantly higher than the other 3 genotypes. However, there is no significant difference in the single-molecule binding force among these four variants of ICAM-1 and the Mac-1 ligand.

In recent years, an increasing number of clinical studies have suggested that two SNPs of ICAM-1, K469E and G241R, can influence the functions of ICAM-1 protein. However, there is no consensus regarding how the two SNPs influence ICAM-1 functions, namely which allele may be the impact factor. As suggested in a meta-analysis [26], the K allele of the ICAM-1 K469E gene might predispose one to cardiovascular disease (CAD). Holder et al. [23] have demonstrated that the GE genotype may increase cell surface expression of ICAM-1. In addition, a study done by Bielinski [27] showed that the K469E polymorphism is related to sICAM-1, but is not associated with ischemic heart disease. In other words, allele E appeared to increase ICAM-1 expression but did not contribute to morbidity.

These conflicting results may be explained by the different gene expressions and single-molecule binding abilities of GK, GE, RK, and RE. Although both of these two factors can affect the functions of ICAM-1 protein, the majority of previous studies only focused on its expression level [28–30] rather than on the single-molecule binding force. Meanwhile according to the studies of Ponthieux A [8] and Oh H-M [19], we noticed that the location of G241R and K469E in the ICAM-1 protein may be a potential factor to change the molecular binding ability. G241R is located in the third immunoglobulin domain of ICAM-1 protein. The G/R241 mutation results in a change from glycine to arginine, which has been shown

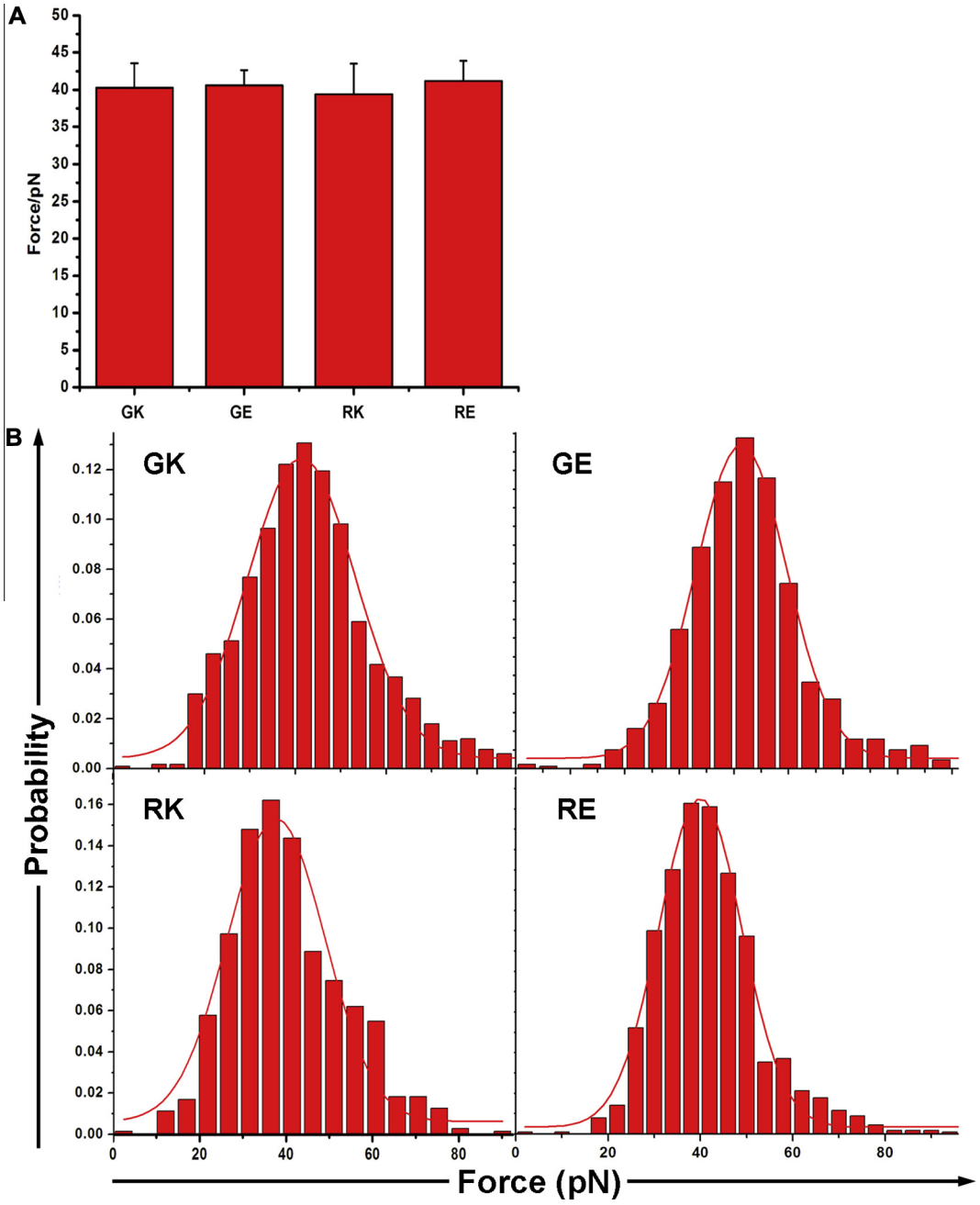


**Fig. 4.** Single-molecule binding force of ICAM-1 mutations and Mac-1. (A) Single-molecule binding force of ICAM-1 mutations (GK, GE, RK, and RE) with Mac-1 were determined by AFM with Mac-1 modified tips. There was no significant difference in the adhesion force among the four genotypes. (B) Histogram of binding force of GK, GE, RK, and RE with Mac-1.

to be of importance in binding to the leukocyte integrin Mac-1 [8]. K469E is located in the membrane proximal region after the fifth immunoglobulin domain of the ICAM-1 protein and is considered to be relevant to the protein dimerization of ICAM-1 according to Oh HM [19]. This dimerization appears to enhance the binding ability of ICAM-1. Therefore, it is necessary to determine whether the SNPs can influence the single-molecule binding force of ICAM-1.

In the current study, we firstly demonstrated that more ICAM-1 were expressed on the CHO cell transiently transfected with plasmid encoding GE genotype, which confirmed the conclusion of Oh HM et.al. Then the single-molecule binding force of GK, GE, RK, and RE was observed and compared in two ways. First, the adhesion ability of CHO cells expressing GK, GE, RK, and RE were observed. Second, AFM was used to directly determine directly the binding force and to confirm the results obtained through observation.

Stable CHO cell lines enabled us to select cells with approximate expression to better focus on changes in the single-molecule force of ICAM-1. On the other hand, AFM measurements confirmed the adhesion assay findings at a molecular level (Fig. 4). The parametric statistical analysis indicated that there was no significant difference in the adhesion force measured for GK, GE, RK, or RE. This demonstrates that 241(G → R) and 469(K → E) polymorphisms of ICAM-1 may not change the single molecule binding force with the Mac-1 ligand.

Based on the binding probability measurement, the results proved the vital role of ICAM-1 in adhesion process and ICAM-1/Mac-1 specific binding. Binding probability of GK, GE, RK, and RE

with Mac-1 is about 25% while the binding probability of control group with Mac-1 is 7%. The significantly lower binding probability of the control group validated the role of ICAM-1 in adhesion from the perspective of molecular mechanics. Furthermore, after blocking Mac-1, the binding probability of GK, GE, RK, and RE with Mac-1 is sharply decreased, confirming the receptor-ligand specific single-molecule binding of ICAM-1/Mac-1 (Fig. 3B).

In conclusion, genetic susceptibility to diseases related to ICAM-1 polymorphisms, G241R or K469E, may be due to the different expressions of ICAM-1 variants rather than to the single-molecule binding force of ICAM-1. This potentially helps to clarify the pathophysiological mechanisms of the above-mentioned diseases and provides evidence for possible treatments, especially at a molecular level.

## 5. Ethical approval

All patients gave written informed consent for their participation. This study was approved by the Ethics Committee of General Hospital of Chinese People's Armed Police Forces with the following reference number: WJ20120915 and conforms to the principles outlined in the Declaration of Helsinki.

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