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2 **mechanical force sensitivity and threshold**

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19 **Running title:** Mechanical force analyses for the activation of IgM-BCRs or isotype-switched BCRs

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Abstract

B lymphocytes use B cell receptors (BCRs) to sense the physical features of the antigens. However, the sensitivity and threshold for the activation of BCRs resulting from the stimulation by mechanical forces are unknown. Here we addressed this question using a double-stranded DNA based tension gauge tether system serving as a predefined mechanical force gauge ranging from 12 to 56 pN. We observed that IgM-BCR activation is dependent on mechanical forces and exhibits a multi-threshold effect. In contrast, the activation of isotype-switched IgG- or IgE-BCR only requires a low threshold of less than 12 pN, providing an explanation for their rapid activation in response to antigen stimulation. Mechanistically, we found that the cytoplasmic tail of the IgG-BCR heavy chain is both required and sufficient to account for the low mechanical force threshold. These results defined the mechanical force sensitivity and threshold that are required to activate different isotypic BCRs.

Introduction

B lymphocytes are responsible for the protective antibody responses arising from the recognition of the pathological antigens by the surface expressed B cell receptor (BCR) (Kurosaki et al., 2010). The BCR is composed of a membrane-bound immunoglobulin (mIg) and a non-covalently associated heterodimer of Ig α and Ig β in a 1 mIg: 1 Ig α -Ig β heterodimer stoichiometry (Schamel and Reth, 2000, Tolar et al., 2005). BCRs are distinguished from other types of receptors by their ability to recognize a wide range of antigen molecules. In addition to the BCR's ability to recognize antigen diversity, the activation of BCR signaling is also efficiently regulated by the presentation of variable forms of antigens. These forms include antigen density (Liu et al., 2010a, Fleire et al., 2006), antigen affinity (Liu et al., 2010a, Fleire et al., 2006), antigen valency (Bachmann et al., 1993, Liu and Chen, 2005, Liu et al., 2004), the Brownian mobility feature of the antigen (Wan and Liu, 2012) and the stiffness feature of the substrates presenting the antigen (Wan et al., 2013, Zeng et al., 2015). All these results suggest that the BCR is an extraordinary receptor which can efficiently discriminate the chemical and physical features of an antigen ligand.

Numerous early studies have investigated how the chemical cues from the antigen determine the strength of the signaling cascade mediated by the BCR (Pierce and Liu, 2010, Harwood and Batista, 2010). However, chemical cues are not the only type of external information that is delivered to the BCRs by antigens. In fact, physical cues are another layer of important information derived from the antigens for the purposes of regulating B cell activation and subsequent responses (Liu et al., 2015). For example, a recent study by Tolar and his colleagues demonstrated that B cells used the

mechanical forces to rupture the bonds between BCRs and membrane-bound antigens. The authors found that only the high affinity BCR and antigen microclusters would be internalized for antigen processing, giving a new mechanism to explain B cell affinity discrimination (Natkanski et al., 2013). Physiologically, the physical cues can also come from the stiffness of the substrates presenting the antigens. Stiffness usually describes the extent that an object can resist deformation in response to an applied force. Stiffness is quantified by Young's modulus with a measurable unit of Pascal (Pa or N/m^2 or $\text{m}^{-1} \cdot \text{kg} \cdot \text{s}^{-2}$) (Discher et al., 2005). B cells could encounter the antigens presented on substrates with various levels of stiffness *in vivo* (Bachmann and Jennings, 2010). For example, viral capsid substrates presenting antigen exhibited a high degree of stiffness (45,000-1,000,000 kPa) (Mateu, 2012), while substrates presenting antigen on the membrane of the host cell infected by virus showed a medium level of stiffness (0.01-1000 kPa) (Nemir and West, 2010). Additionally, B cells can also efficiently acquire antigens that are associated with extra cellular matrix (ECM) in the tissue (Ciechomska et al., 2014), which is well documented to exhibit an extraordinary range of stiffness from 0.012 to 20 kPa (Bao and Suresh, 2003, Paszek et al., 2005, Engler et al., 2004, Nemir and West, 2010). Soluble antigen in plasma has also been detected, and displayed a remarkably low level of stiffness (several Pa) (Araujo Gde et al., 2012). More recently, Kam and his colleagues quantified the traction forces between T cell receptors (TCRs) and the pillar substrates presenting antigens (Bashour et al., 2014). Indeed, significant shape changes of the substrates were observed due to the bending of the pillars by TCRs after TCR and antigen recognition (Bashour et al., 2014). Since stiffer substrates are known to be more

84 resistant to shape changes (Trappmann et al., 2012), it is reasonable to expect that
85 antigens presented on stiffer or softer substrates will inevitably produce and deliver
86 different mechanical forces to the BCRs. Indeed, our recent study showed that the
87 activation of a B cell is very sensitive to the stiffness of the substrates presenting the
88 antigens (Wan et al., 2013, Zeng et al., 2015).

89 Thus, all these studies suggest that physical cues, such as mechanical force, comprise
90 important external information delivered to the BCRs by antigens for the purposes of
91 regulating B cell activation and subsequent responses. However, the mechanism
92 regulating the strength of the BCR activation in response to mechanical forces that are
93 delivered to the BCR by the antigens on the grounding substrates remains unknown
94 (Pierce and Liu, 2010). There are several important questions which need to be
95 addressed. First of all, does the BCR itself have mechanosensing capability, or do B
96 cells perform mechanosensing through the conventional mechanosensors such as
97 lymphocyte function-associated antigen 1 (LFA-1) (Chen et al., 2010, Chen et al.,
98 2012). Second, is there a threshold for BCR activation from the mechanical forces that
99 are delivered to the BCR by the antigen? Third, how sensitive will the BCR be toward
100 different mechanical forces above that threshold in the initiation of BCR activation?
101 Fourth, B cells use different isotypes of BCRs to recognize antigens and initiate
102 transmembrane activation signaling (Pierce and Liu, 2010, McHeyzer-Williams and
103 McHeyzer-Williams, 2005). Mature naïve B cells use IgM-BCRs, while memory B
104 cells mainly use IgG-BCRs along with a small fraction that use IgE-BCRs. We do not
105 know how IgM-BCRs expressed by naïve B cells and IgG-BCRs (or IgE-BCRs)
106 expressed by memory B cells show differences in terms of the sensitivity and

threshold toward mechanical forces in their activation.

A major obstacle to answer all of these questions is that it is technically challenging to set up an experimental system with predefined mechanical forces between BCR and antigen, which shall then be linked to an assay to accurately measure BCR activation. This is especially challenging since it is known that BCR activation may begin seconds after the recognition of the BCR and the antigen (Pierce and Liu, 2010). To overcome these technical difficulties, we took advantage of a platform utilizing a dsDNA based tension gauge tether (TGT) that was recently developed by Ha and his colleagues (Wang and Ha, 2013). DNA is an excellent molecule showing force application geometry that has been accurately calculated and validated by *in vitro* single molecule force measurements (Albrecht et al., 2003, Lang et al., 2004). Ha and his colleagues used these TGT sensors to probe the tension on a single integrin-ECM ligand (cyclic RGDfK) bond required for cell adhesion. Here, we modified this TGT system by conjugating the B1-8-BCR specific antigen, 4-Hydroxy-3-nitrophenylacetyl (NP), to the ligand chain of the TGT system (NP-TGT). By doing so, we acquired a series of 8 TGT sensors with predefined mechanical force of 12, 16, 23, 33, 43, 50, 54 and 56 pN respectively. We analyzed the activation of BCRs in response to these TGT molecules using high resolution high speed live cell imaging techniques via total internal reflection fluorescence microscopy (TIRFM). This allowed us to determine the sensitivity and threshold for the mechanical force signal in the activation of IgM-BCR or isotype-switched IgG-BCR and IgE-BCR.

Results

The construction of B1-8-BCR specific NP-TGT mechanical force sensors

130 To further our understanding of how mechanical forces influence BCR activation, we
131 constructed the NP-TGT sensors by modifying a dsDNA based TGT system (Wang
132 and Ha, 2013). Each NP-TGT molecule is composed of two single-stranded DNA
133 (ssDNA) molecules with different modifications (Figure 1A, B). The first ssDNA
134 molecule is biotin-conjugated at different positions to provide a defined range of
135 rupture force anchoring positions as illustrated in Figure 1B. In the original version of
136 the TGT system utilized by Ha and his colleagues (Wang and Ha, 2013), the second
137 ssDNA molecule was conjugated with a well characterized integrin ligand, cyclic
138 RGDfk peptide, to provide an integrin binding site for quantifying the mechanical
139 force spectrum (12, 16, 23, 33, 43, 50, 54 and 56 pN) in the activation of integrin
140 molecules. As stated by the authors (Wang and Ha, 2013), TGT molecules with
141 modifications can provide an experimental system for the study of many other types of
142 receptors. Here, we conjugated the B1-8-BCR specific antigen, NP, to the second
143 ssDNA molecule (Figure. 1A, B, Figure 1—figure supplement 1A). Since we just
144 exchanged the cyclic RGDfk peptide with the NP hapten antigen and did not change
145 the sequence and design of these dsDNA based TGT sensors, our NP-TGT system
146 shall have the same tension gauge scales as the one used by Ha and his colleagues
147 (Wang and Ha, 2013). In our experimental system, each NP-TGT molecule can be
148 recognized by the B1-8-IgM-BCRs (Figure 1A). Thus the NP-TGT sensor that is
149 immobilized on the surface of coverslip would be ruptured if the mechanical force
150 applied by the B1-8-IgM-BCR is larger than the predefined tension force of a certain
151 NP-TGT sensor. A key feature of dsDNA based mechanical force sensor is that the
152 specific force value of each sensor is not an absolute value but presents a distribution

with a full width at half maximum (FWHM) of 5 pN for unzipping rupture mode and 15 pN for shearing rupture mode (Lang et al., 2004). So, precisely, the actual range of any given NP-TGT sensor represents a distribution with the most possible value of rupture force at 12, 16, 23, 33, 43, 50, 54 and 56 pN respectively.

We first determined whether the NP-TGT sensor can trigger the activation of the B cells expressing B1-8-IgM-BCR. We used a similar protocol as reported (Wang and Ha, 2013) to tether the highest force NP-TGT molecule (mean rupture force 56pN in the condition they used) with NP conjugated ssDNA on coverslip pre-coated with neutravidin. We also used the same TGT molecule without NP conjugation as a negative control (NC) (Figure 1B). DyLight 649 AffiniPure Fab Fragment Goat Anti-Mouse IgM, μ Chain Specific antibodies was used to pre-label the B1-8-IgM-BCRs on J558L cells (J558L cells expressing B1-8-IgM-BCR) before TIRFM imaging experiment as reported in our previous studies (Liu et al., 2010a). We found that J558L cells expressing B1-8-IgM-BCR initiated the activation responses as quantified by the dramatic accumulation of BCRs into the contact interface of B cells with coverslip presenting 56 pN NP-TGT sensor and formed a typical B cell immunological synapse (IS) as illustrated by the time lapse TIRFM images (Figure 1C-D, Video 1). These results were not observed with the negative control (NC) 56 pN TGT without NP conjugation (Figure 1C-D, Video 1). Additional experiments showed that neutravidin would not dissociate from the coverslip during the 10 min time course of our experiments (Figure 1—figure supplement 1B, C) and NP-TGT can only be attached to coverslip in a neutravidin dependent manner (Figure 1—figure supplement 1D). Further experiments also indicated that the non-specific attachment of NP-TGT

176 molecules if any on the coverslip without pre-coated neutravidin cannot induce the
177 synaptic accumulation of BCRs (Figure 1—figure supplement 1E).

178 Since TGT is a dsDNA based molecule, we were concerned that dsDNA may directly
179 stimulate B cells in a BCR-independent manner. To check it, we examine the NP-TGT
180 triggered responses of primary mature naïve B cells from wild type C57BL/6 mice,
181 which does not express NP specific B1-8-IgM-BCRs (Figure 1E). These primary B
182 cells did not accumulate BCRs into the B cell IS in response to coverslip presenting
183 NP-TGT sensor compared to the case of coverslip alone, although a dramatic
184 accumulation was readily observed in the case of encountering coverslip that was
185 coated with anti-mouse IgM surrogate antigens as a positive control (Figure 1E). The
186 B cell activation is known to be very sensitive to the density of antigen on the surface
187 of the antigen presenting substrates (Liu et al., 2010a, Fleire et al., 2006). To exclude
188 such a variant, we then used NP-specific antibodies to quantify the density of each
189 NP-TGT sensor (Figure 1F). Thus, we established the B1-8-BCR specific NP-TGT
190 sensor system which showed a predefined mean mechanical force gauge ranging from
191 12 to 56 pN.

192 **The synaptic accumulation of the IgM-BCRs is dependent on mechanical forces**
193 **and exhibits a multi-threshold effect**

194 We compared the response of naïve B1-8-IgM-BCR expressing B cells after immune
195 recognition of these series of NP-TGT molecules. Numerous early studies showed that
196 after BCR-antigen recognition, B cells immediately begin to spread over the
197 antigen-containing surfaces to form the B cell IS, and attempt to acquire the antigens
198 by actively accumulating BCRs and antigens into the B cell IS (Liu et al., 2010b, Liu

199 et al., 2010c, Seeley-Fallen et al., 2014, Fleire et al., 2006, Liu et al., 2010a). Thus, we
200 first examined the B cell spreading response and the concomitant accumulation of
201 BCRs into the B cell IS by TIRFM imaging using J558L cells expressing
202 B1-8-IgM-BCR in NP-TGT based experimental system (Figure 2A). The results
203 suggested that NP-TGT sensors with higher mechanical force generally triggered more
204 aggressive spreading response and enhanced BCR synaptic accumulation than
205 NP-TGT molecules with lower mechanical force (Figure 2A-C). More strikingly, a
206 careful examination of the response of B cells toward these series of NP-TGT sensors
207 with mechanical forces ranging from 12 to 56 pN suggested that there are three levels
208 of thresholds. The low-force (12-16 pN) NP-TGT sensors triggered a weak activation,
209 whereas the middle-force (23-43 pN) NP-TGT sensors initiated a medium level
210 activation, and the high-force (50-56 pN) NP-TGT sensors accounted for a strong
211 activation (Figure 2A). This unique pattern of the dependence on mechanical forces
212 suggested that IgM-BCR activation exhibits a multi-threshold effect. Specifically, an
213 increase of the mechanical force from one threshold to another threshold dramatically
214 enhanced the activation of the naïve IgM-BCRs. However, within these three
215 threshold barriers, increasing the mechanical force, such as from 23 pN to 33 pN or to
216 43 pN (the medium level threshold), will not result in dramatically enhanced
217 activation responses of IgM-BCRs.

218 We also validated these observations by analogously examining the activation
219 response of primary mature naïve B cells expressing B1-8-IgM-BCR from IgH
220 B1-8/B1-8 Igk^{-/-} transgenic mice (referred to as primary naïve B cells expressing
221 B1-8-IgM-BCR) (Hauser et al., 2007) upon the recognition of these NP-TGT sensors

(Figure 2B, Figure 2—figure supplement 1A). Furthermore, we were concerned that the density of the NP-TGT sensors on coverslip might affect the pattern of the activation response of the naïve IgM-BCRs. Thus, we quantified the density (number of molecules/ μm^2) of NP-TGT sensors on the coverslip when was coated with different incubation concentration (2, 5, 10, 50 nM) of NP-TGT sensor (Figure 2—figure supplement 1B-E) following a published protocol (Liu et al., 2010a, Wang and Ha, 2013). At the density of 4.0 NP-TGT molecule/ μm^2 (5 nM incubation concentration), we can capture the same pattern of the activation response of IgM-BCRs (Figure 2—figure supplement 1F-G). At the very low density of 0.3 NP-TGT molecule/ μm^2 (2 nM incubation concentration), we found the only 56 pN NP-TGT sensor can very mildly trigger the BCR accumulation, while both 12 pN and 43 pN NP-TGT failed to trigger B cell activation (data not shown), suggesting that we cannot use the incubation concentration of 2 nM for these experiments. Thus, it is clear that the activation of the IgM-BCR is extremely sensitive to the changes in mechanical forces exceeding the thresholds such as the increase in the forces from 12 pN to 23 pN instead of 16 pN, or from 23 pN to 56 pN instead of 43 pN. In the following experiments, we chose the 12 pN, 43 pN and 56 pN NP-TGT molecules in each of the three threshold barriers (low, medium and high) for further analyses.

To investigate the temporal dependence of BCR accumulation into the B cell IS on mechanical forces, we took advantage of high speed time lapse TIRFM imaging to test the dynamics of BCR accumulation into the B cell IS and the growing size of contact area starting with the initial immune recognition of BCRs with 12 pN, 43 pN and 56 pN NP-TGT sensors (Figure 2C-E, Video 2). As expected, we found that the synaptic

accumulation of the BCRs in the entire 10 min time lapse was dependent on mechanical forces with high force NP-TGT sensor (56 pN) exhibiting the highest accumulation, medium force NP-TGT sensor (43 pN) showed medium-level accumulation, and low force NP-TGT sensor (12 pN) had the lowest level of accumulation, consistent with the results from the abovementioned endpoint experiments. Using these NP-TGT molecules, we also showed that there were no obvious internalization during the 10 min time course of our experiments (Figure 2—figure supplement 2A-C), consistent with the published studies (Fleire et al., 2006, Natkanski et al., 2013). These results suggest that the internalization of BCR and antigen molecules did not contribute to the different levels of the accumulation of the IgM-BCRs into the B cell IS in response to different NP-TGT sensors with different mean rupture forces. Indeed, B cells that were pretreated with monodansylcadaverine (MDC), an inhibitor to block B cell internalization (Liu et al., 2010a, Tolar et al., 2005), also exhibited the dependent on mechanical forces in the synaptic accumulation of the IgM-BCRs (Figure 2—figure supplement 2D-F).

The volume of the IgM-BCR microcluster produced by different NP-TGT sensors is dependent on mechanical forces and exhibits a similar multi-threshold effect

BCR microclusters have been demonstrated to serve as the most basic platform for the investigation of the initiation of BCR signaling by our studies and those of others (Pierce and Liu, 2010, Harwood and Batista, 2010). We thus assessed the volume of the BCR microclusters produced by the series of NP-TGT sensors by quantifying the fluorescence intensity (FI) of each individual BCR microcluster. We mathematically fitted each BCR microcluster using a 2D Gaussian function as reported earlier (**Source**

code 1) (Liu et al., 2010a). We similarly observed that the volume of the BCR microclusters is dependent on the strength of the mechanical force with 56 pN NP-TGT sensors producing much bigger and brighter BCR microclusters than 43 pN and 12 pN NP-TGT molecules (Figure 3A-B). The multi-threshold effect was also observed when we analyzed the volume of the generated BCR microclusters. NP-TGT molecules with low mean rupture force (12-16 pN) triggered the formation of small BCR microclusters, whereas NP-TGT sensors with medium mean rupture force (23-43 pN) initiated medium-level volume BCR microclusters, and NP-TGT molecules with high mean rupture force (50-56 pN) accounted for bigger and brighter BCR microclusters (Figure 3A-B). Thus, the volume of the IgM-BCR microcluster produced by different NP-TGT sensors is dependent on mechanical forces and exhibits a similar multi-threshold effect.

The strength of IgM-BCR signaling is dependent on mechanical forces

Next, we examined the initiation of BCR signaling in B cells when encountering the 12 pN, 43 pN and 56 pN NP-TGT molecules by quantifying the accumulation of pSyk, pPLC γ 2, and pTyr molecules into the B cell IS using the TIRFM image analysis method as reported in our earlier studies (Liu et al., 2010b) and those of others (Liu et al., 2012a, Fleire et al., 2006). All of these signaling molecules play essential roles in the transmembrane signaling transduction of BCR. For example, Syk binds to the phosphorylated Immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic tail of Ig α and Ig β , and subsequently Syk will undergo auto-phosphorylation at multiple tyrosine sites within its linker regions to be converted to a signaling active form (Saouaf et al., 1994). The phosphorylated Syk

also provides docking sites for PLC γ 2 (Weber et al., 2008). We found that the membrane proximal recruitment of each of these signaling molecules is dependent on mechanical forces with the 56 pN NP-TGT sensors showing the highest recruitment, while the 43 pN NP-TGT sensors had a medium level of recruitment and the 12 pN NP-TGT sensors had the lowest level of recruitment (Figure 4A-C), consistent with the results from the abovementioned experiments quantifying the synaptic accumulation of the BCR molecules (Figure 2A-B). We also analyzed the volume of these signaling molecule microclusters by quantifying the FI of the microclusters, and confirmed that the FI of pSyk, pPLC γ 2 and pTyr microclusters produced by different NP-TGT molecules is dependent on mechanical force with a similar multi-threshold effect (Figure 4D-F). Thus, it was clear that the strength of the initiated IgM-BCR signaling is dependent on mechanical forces.

The outside-in activation of integrin enhances the strength of IgM-BCR activation but does not change its patterned dependence on mechanical forces

We explored whether the conventional mechanosensor integrin LFA-1 expressed on the surface of B cells influences the patterned dependence on the mechanical forces in B cell activation. To address this question, we co-tethered both NP-TGT sensors and the adhesion molecule, intercellular adhesion molecule 1 (ICAM-1, which is a ligand for integrin LFA-1) on the surface of coverslip and similarly performed TIRFM imaging experiments. The addition of ICAM-1 dramatically enhanced the synaptic accumulation of the BCRs (Figure 5A), suggesting the increased sensitivity to antigen stimulation in the initiation of B cell activation, consistent with the published studies in both the BCR (Carrasco et al., 2004) and T cell receptor system (Bachmann et al.,

1997, Dustin, 2009). However, it was clear that the outside-in activation of integrin did not change the fact that B cell activation is dependent on mechanical forces with a similar multi-threshold effect (Figure 5B). To further confirm this conclusion, we inactivated the function of focal adhesion kinase (FAK), a member of the non-receptor protein-tyrosine kinase family, that is known to play a key role in the activation of integrin signaling pathways (Yu et al., 2012, Bashour et al., 2014, Slack-Davis et al., 2007). We pretreated B cells with FAK specific inhibitor PF573-228 (Slack-Davis et al., 2007). We found that the pretreated B cells still maintained the general patterned dependence on mechanical forces with a multi-threshold effect (Figure 5C-D, Figure 5—figure supplement 1A). However it was clear that the high-end (56 pN) and medium-level (43 pN) but not low-end (12 pN) mechanical force threshold was more influenced by the inactivation of integrin, suggesting that the breakthroughs of the medium-level and high-end threshold of mechanical forces are partially supported by the inside-out activation of integrin.

The high-end but not low-end mechanical force threshold is dependent on myosin IIA in IgM-BCR activation

Motor proteins including myosin IIA and dynein are known to play important roles in B cell activation. There are recent studies showing that B cells utilize the contraction forces generated by myosin IIA to rupture the interaction between the BCR and antigen molecules (Natkanski et al., 2013), while dynein is required for the retrograde motile feature of the BCR microclusters into the center of B cell IS (Schnyder et al., 2011). Thus, we assessed the contribution of these two motor proteins to the patterned dependence on mechanical forces during IgM-BCR activation. Unexpectedly, the

inactivation of myosin IIA did not dramatically change the dependence on mechanical forces, however we observed a significant drop in the strength of the synaptic accumulation of the BCRs for the high threshold NP-TGT sensor at 56 pN only, and no drop was observed for the medium-level threshold NP-TGT sensor at 43 pN, nor the low threshold NP-TGT sensor at 12 pN (Figure 5E-F, Figure 5—figure supplement 1B). On the other hand, when inactivating dynein, we did not observe significant changes in the BCR accumulations compared to DMSO controls (Figure 5G-H, Figure 5—figure supplement 1C). All of these results suggest that the breakthroughs of the high-end but not the medium-level and low-end thresholds of mechanical forces are supported by myosin IIA.

The activation of isotype-switched IgG-BCRs or IgE-BCRs on memory B cells requires either no tension or a mechanical force below 12 pN

B cells use different isotypes of BCRs to recognize antigens and to initiate transmembrane activation signaling. Mature naïve B cells use IgM-BCRs (termed naïve IgM-BCR thereafter), while memory B cells mainly use IgG-BCRs (termed memory IgG-BCR thereafter) with a fraction of these use IgE-BCRs (termed memory IgE-BCR thereafter). Memory B cells are responsible for the rapid antigen recall humoral responses upon vaccine immunization (Pierce and Liu, 2010, McHeyzer-Williams and McHeyzer-Williams, 2005). Here, we explore the sensitivities and thresholds toward mechanical forces in the activation of memory B cells expressing isotype-switched IgG-BCRs or IgE-BCRs. We addressed this question using J558L cells expressing memory B1-8-IgG-BCR in the same experimental system that has been used for the naïve B1-8-IgM-BCR-expressing J558L cells as

described in detail above. Surprisingly, we observed a totally different pattern in terms of the dependence on mechanical forces for the activation of memory IgG-BCRs. Each of the three NP-TGT sensors producing mechanical forces at 12, 43 or 56 pN similarly drove the cells to undergo spreading responses and the synaptic accumulation of BCRs or pSyk signaling molecules into the IS (Figure 6A-D). This suggested that the activation of memory IgG-BCRs requires either no tension or a mechanical force below the mean rupture force of the lowest-force NP-TGT we used (12 pN). Similar patterned activation of memory IgG-BCR was observed at different surface density of NP-TGT sensors on coverslip (Figure 6—figure supplement 1A-C). Furthermore, a similar phenomenon was observed when examining the activation of B1-8 primary B cells expressing memory IgG-BCRs that were derived from an *in vitro* class-switch response following our published protocol (Liu et al., 2010b) (Figure 6E-F). We also used high speed TIRFM imaging to test the dynamics of memory IgG-BCR accumulation into the B cell IS starting with the initial immune recognition of memory IgG-BCRs with 12 pN, 56 pN and NP non-conjugated control TGT molecules within a 13 min time course (Figure 6G-H, Video 3). It was clear that 12 pN and 56 pN NP-TGT molecules similarly drove the accumulation of BCRs into the B cell IS. All of these results show that the activation of the memory IgG-BCR is independent on mechanical forces ranging from 12 to 56 pN as tested in our NP-TGT experimental system.

Next, we examined the dependence on mechanical forces in terms of the activation of memory IgE-BCRs in Ramos human B cells expressing memory B1-8-IgE-BCRs. Here, we similarly observed that the activation of memory IgE-BCRs require either no

tension or a mechanical force below 12 pN (Figure 6I, J). As a system control, it was clear that the activation of the naïve B1-8-IgM-BCRs expressed in Ramos B cells was also dependent on mechanical force with a multi-threshold effect (Figure 6K, L). Conclusively, it was clear that the activation of the memory IgG-BCRs or IgE-BCRs requires either no mechanical force or a force lower than that provided by the 12 pN NP-TGT, highlighting the significant low threshold for the activation of memory IgG-BCR or IgE-BCR that are typically expressed on memory B cells compared to the case of the naïve IgM-BCR on mature naïve B cells. All these results provide new explanations for the rapid and high-titered IgG antibody responses upon re-encounter with antigen by memory B cells.

The lower mechanical force threshold of memory IgG-BCR activation is dependent on its cytoplasmic tail

The unexpected extremely low mechanical force threshold (might be less than 12 pN or even lower) for the memory IgG-BCR but not the naïve IgM-BCR activation drove us to ask the next question: why does the memory IgG-BCR behave differently from the naïve IgM-BCR? This question is especially intriguing as both memory IgG-BCR and naïve IgM-BCR use the exact same BCR component, the Ig α and Ig β heterodimer, to initiate BCR signaling. In contrast, the mIgG and the mIgM are the BCR components to recognize the antigens (Schamel and Reth, 2000, Tolar et al., 2005). In addition to the constant region of the Ig, mIgG and mIgM also differ greatly in their cytoplasmic domain. In fact, mIgM has only three-amino acids in its cytoplasmic tail (KVK), while all mIgG subtypes have 28-amino acid cytoplasmic tails, which are extremely conserved across species (Liu et al., 2012b, Liu et al., 2010a, Reth, 1989,

Tarlinton, 1997). Early mice model studies utilizing biochemical assays and live cell imaging demonstrated that the cytoplasmic tail of mIgG is both necessary and sufficient to confer an enhanced activation of IgG-BCR expressing memory B cells compared to the case of IgM-BCR expressing naïve B cells (Wakabayashi et al., 2002, Waisman et al., 2007, Horikawa et al., 2007, Kaisho et al., 1997, Martin and Goodnow, 2002, Liu et al., 2010b, Liu et al., 2012b, Engels et al., 2014, Engels et al., 2009). To examine the contribution of the cytoplasmic tail of mIgG in the low mechanical force threshold for the activation of the memory IgG-BCR, we took advantage of the four types of J558L cells expressing naïve B1-8-IgM-BCR and memory B1-8-IgG-BCR with cytoplasmic tail swapped forms as reported in our previous study (Liu et al., 2010b): (1) memory B1-8-IgG-BCR; (2) mIgG swapped with a mIgM cytoplasmic tail (memory B1-8-IgG-BCR equipped with a mIgM cytoplasmic tail, termed GGM thereafter); (3) naïve B1-8-IgM-BCR; (4) mIgM swapped with a mIgG cytoplasmic tail (naïve B1-8-IgM-BCR equipped with a mIgG cytoplasmic tail, termed MMG thereafter) (Figure 7A). We found that the J558L cells expressing GGM showed the force dependent activation by accumulating more BCRs and pSyk into the IS with the higher mean rupture force NP-TGT molecules, similar to the case of J558L cells expressing naïve B1-8-IgM-BCR (Figure 7B-D). In contrast, it was apparent that the J558L cells expressing MMG behaved similarly to the case of J558L cells expressing memory B1-8-IgG-BCR in a force independent manner (Figure 7E-G). All of these results suggested that the lower mechanical force threshold of memory IgG-BCR activation depends on its cytoplasmic tail.

Discussion

429 B cells produce protective antibodies against pathogens. Antibody response is
430 originated from the activation of B cells upon BCR and antigen recognition. As
431 mentioned earlier, the antigens recognized by B cells *in vivo* exhibit great diversities
432 including antigen density (Liu et al., 2010a, Fleire et al., 2006), antigen affinity (Liu et
433 al., 2010a, Fleire et al., 2006), antigen valency (Bachmann et al., 1993, Liu and Chen,
434 2005, Liu et al., 2004), Brownian mobility feature of the antigen (Wan and Liu, 2012)
435 and the stiffness of the substrates presenting the antigen (Wan et al., 2013, Zeng et al.,
436 2015). All these studies demonstrated that BCR is an extraordinary receptor which can
437 efficiently discriminate the chemical and physical features of an antigen ligand. That
438 raises a long-standing question in immunological studies that how possible the
439 strength of BCR activation can be so complicated and efficient to combine sensitivity,
440 threshold, speed, specificity, affinity and substrate stiffness discriminatory power?

441 In this report, we modified the dsDNA based TGT developed by Ha and his colleagues
442 (Wang and Ha, 2013) to acquire a series of 8 NP-TGT sensors with predefined mean
443 rupture force from low to high respectively (12, 16, 23, 33, 43, 50, 54 and 56 pN at the
444 condition in the original study). The use of these NP-TGT molecules in combination
445 with the high resolution high speed live cell imaging technique through TIRFM allows
446 us to investigate several intriguing questions: (1) Does the BCR itself possess any
447 mechanosensing properties? (2) Is there a threshold for BCR activation from the
448 mechanical forces that are delivered to BCR by antigen? (3) How sensitive the BCR
449 will be toward different mechanical forces above that threshold in BCR activation?

450 Supported by NP-TGT system, for the first time we demonstrated that IgM-BCR
451 activation is in a mechanical force dependent manner by providing evidence that high

mean rupture force NP-TGT sensors which can stand for high force load triggered more aggressive spreading response and the enhanced BCR signaling than low mean rupture force NP-TGT sensors. Since such a patterned dependence on mechanical force in the initiation of IgM-BCR does not strictly rely on integrin or adhesion molecule, we propose that IgM-BCR alone could function as a mechanosensor. The performance of IgM-BCRs in TGT based mechanical force experimental system is mostly consistent with the reported findings on integrin molecules (Wang and Ha, 2013). However IgM-BCR and integrin also exhibit some obvious differences in their respective responding pattern to the same mechanical force spectrum (12 pN ~ 56 pN) produced by TGT sensors.

First of all, 12 pN NP-TGT sensor, the one enduring the smallest mechanical force, can induce the mild but consistent activation of B1-8-naïve-IgM-BCR when was compared to the control TGT without the conjugated NP antigen. Though, it is a fact that the strength of such activation produced by 12 pN NP-TGT sensor is dramatically weaker than those produced by other NP-TGT molecules bearing higher force (such as 56 pN NP-TGT). In contrast, integrin does not seem to get activated under 12 pN or even 16 pN TGTs (Wang and Ha, 2013). These results suggested that the initiation of BCR signaling could be extremely sensitive to mechanical forces. BCR might rely on such fine sensitivity to initiate its signaling cascade during the surveillance for non-self-antigens that could be of very low density. Subsequently, those initially weak signals could be further boosted by other well characterized mechanisms through activating co-receptors (such as CD19) (Depoil et al., 2008) or co-stimulatory factors (such as LFA-1) (Tseng et al., 2008, Dustin et al., 2006, Carrasco et al., 2004, Arana et

475 al., 2008a, McLeod et al., 2004, Spaargaren et al., 2003, Arana et al., 2008b).

476 Secondly, IgM-BCR exhibits an obvious multi-threshold effect in response to the

477 NP-TGT sensors enduring different mechanical forces from 12 pN to 56 pN. The

478 low-force 12-16 pN NP-TGT sensors triggered a weak activation, whereas the

479 middle-force 23-43 pN NP-TGT sensors initiated a medium-level activation, and the

480 high-force 50-56 pN NP-TGT sensors accounted for a strong activation. Within these

481 three thresholds, there is a quite obvious barrier effect as an increase in mechanical

482 force, such as from 23 pN to 33 pN or to 43 pN within the medium-level mechanical

483 force barrier, will not result in any dramatically enhanced activation responses of

484 IgM-BCRs. Instead, the activation of IgM-BCR is extremely sensitive to the changes

485 of mechanical forces exceeding these barriers, such as the increase of the mechanical

486 force from 12 pN to 43 pN or from 43 pN to 56 pN. We speculate that these

487 observations might reflect some key features of dsDNA-derived mechanical force

488 sensor that each sensor represents a distribution of force value with an FWHM of 5 pN

489 for unzipping rupture mode and 15 pN for shearing rupture mode (Lang et al., 2004).

490 Thus, the actual rupture force of any given TGT sensor shall not be viewed as a fixed

491 value, but rather a distribution with “shoulder overlap” to the nearby TGT sensors. We

492 also speculate that these findings would potentially deliver key meanings with

493 biological significance. Different from many other receptors, IgM-BCR composes a

494 mIgM and a heterodimer of Ig α and Ig β in a 1 mIg: 1 Ig α -Ig β stoichiometry with

495 mIgM, the biosensor for antigens, and Ig α -Ig β heterodimer, the BCR signaling

496 initiator (Schamel and Reth, 2000, Tolar et al., 2005). The antigenic signal needs to be

497 delivered from the antigen binding region on the extracellular domain of mIgM to the

498 signaling active Immunoreceptor tyrosine-based activation motif (ITAM) on the
499 cytoplasmic domain of both Ig α and Ig β . It is still a big open question in terms of the
500 molecular mechanism of how exactly BCR transduces physical and chemical signals.
501 Here we tried to interpret our data in a way that such multi-threshold effect of the
502 activation of IgM-BCRs reflects the multi-threshold progress of delivering these
503 mechanical forces induced potential conformational changes from mIgM to Ig α and
504 Ig β heterodimer complex. It is also our speculation that such multi-threshold likely
505 provides a chance for the proof-testing of the mechanical forces applied to IgM-BCR
506 during its activation. All these speculations are under our further extensive
507 investigation.

508 These observed differences that were induced by different NP-TGT sensors cannot
509 exclude the contribution of life time of the bonds between BCR and NP-TGT sensors.
510 Although, the contribution from mechanical forces and the life time of the BCR with
511 the NP-TGT molecules cannot be absolutely separated. This especially intrigues a
512 question when considering the recent study by Zhu and his colleagues (Liu et al.,
513 2014), showing that the mechanical force prolongs life time of catch bond between
514 TCR and agonist pMHC, but decreases the life time of slip bond between TCR and
515 antagonist pMHC antigen. The authors showed that both the value of the force and the
516 life time of the force are important factors in TCR activation. The situation with BCR
517 shall be more complicated as different from TCR, BCR is a bivalent binding receptor,
518 stating that one BCR can bind to two NP-TGT molecules. Additionally, the recent
519 advance of the super-resolution imaging studies demonstrated that BCRs form obvious
520 nanoclusters with 20-50 IgM-BCRs per nanocluster on quiescent B cells (Mattila et al.,

2013). All these features of BCR demonstrated the high possibility of multivalent bonds based mechanical forces in regulating the initiation of B cell activation. Additionally, Tolar and his colleagues recently used AFM to examine the rupture forces between B1-8-IgM-BCR and NP antigen (Natkanski et al., 2013). Their study provides useful reference information to better understand the relationship between rupture forces and lifetime of a single bond B1-8-IgM-BCR and NP antigen.

It shall be noted that the values of the rupture forces of TGT sensors were all calculated based on the data measured by magnetic tweezers system with a loading rate of 0 pN/s (constant force with a time under force mode of 2 sec during the measurements). Rupture force measuring systems using AFM-based versus magnetic tweezers-based approaches show many differences including but not limited to different spring constants, different loading rate and different time under load (Hatch et al., 2008, Albrecht et al., 2003, Lang et al., 2004). Thus, all these differences prevented the feasibility of a direct comparison of the mechanical force value that is acquired from different kind of measurements (constant force *versus* constant loading).

From this point of view, it would be of great importance to use the constant force measurements to examine the interaction of B1-8-BCR with NP antigen, and to investigate how the magnitude and duration of mechanical forces will influence B cell activation. During the measurement, the time of the force under load shall be carefully picked up because it is known that such a parameter greatly influence the measured value of the rupture forces (Natkanski et al., 2013, Lang et al., 2004, Wang and Ha, 2013). As mentioned above a constant force mode with a time under force of 2 sec was used to measure the rupture force of these TGT sensors by magnetic tweezers

(Wang and Ha, 2013). The relevant time scale of rupturing NP-TGT sensors in our experimental system shall be at the seconds to dozens of seconds level with consideration from the recent studies by Tolar and his colleagues (Natkanski et al., 2013).

In this report, we examined the molecule requirements of the patterned dependence on mechanical forces in IgM-BCR activation. The source accounting for the applied force would be quite diverse in a live cell. In the case of BCR, motor proteins including myosin IIA and dynein molecules are known to be the mechanical force provider. Myosin IIA executes the vertical forces to BCR microclusters to rupture the bonds between BCRs and membrane-bound antigens, and such event is used by B cells to discriminate antigen affinity (Natkanski et al., 2013). Dynein is shown to be required for the retrograde motile feature of BCR microclusters into the center of the B cell immunological synapse, suggesting dynein would more likely execute a lateral force (Schnyder et al., 2011). We assessed the contribution of myosin IIA and dynein in the patterned dependence on mechanical forces of IgM-BCR activation. Unexpectedly, the inactivation of either myosin IIA or dynein did not dramatically changed the patterned dependence on mechanical forces in IgM-BCR activation, suggesting the formation of the basic barrier for the patterned multi-threshold effect does not strictly rely on myosin IIA or dynein. However, in primary naïve B cells expressing B1-8-IgM-BCR expressing B cells with inactivated myosin IIA, we observed a significantly decreased accumulation of BCR in response to high-end 56 pN NP-TGT sensor only, and such drop was not observed in the case of medium-level threshold 43 pN NP-TGT sensor or low-end 12 pN NP-TGT sensor. No such drop was observed in each of these

567 multi-threshold NP-TGT molecules after inactivating dynein. All these data drive us to
568 speculate that the break-through of the high-end but not the medium-level or low-end
569 mechanical force threshold might be contributed by myosin IIA. In contrast, dynein
570 does not seem to play an obvious role in the establishment of the barriers of
571 mechanical forces.

572 The unique multi-threshold pattern in IgM-BCR activation is not strictly mediated by
573 the outside-in or inside-out activation of the integrin molecules as addition of neither
574 ICAM-1 nor FAK inhibitor changed that patterned dependence on mechanical forces
575 in IgM-BCR activation. However, in FAK inactivated B cells, we observed a
576 significantly decreased accumulation of BCR in response to high-end 56 pN NP-TGT
577 sensor and medium-level threshold 43 pN NP-TGT sensor, while no such drop was
578 observed in the case of low-end 12 pN NP-TGT sensor. We speculate from these data
579 that the break-through of the high-end and medium-level threshold but not low-end
580 mechanical force barriers might be contributed by the inside-out activation of integrin.
581 This is in consistent with the role of myosin IIA as discussed above. Myosin IIA
582 bound actin filaments are physically connected with integrin through the key focal
583 adhesion molecules talin and vinculin and was shown to be important for the function
584 of TCR microclusters (Ilani et al., 2009). Inactivation of either myosin IIA or FAK
585 affects the high-end mechanical force barrier for IgM-BCR activation seems to
586 suggest that these two molecules may regulate B-cell activation through similar
587 mechanism at least partially.

588 The most striking observation of this report is that the activation of isotype-switched
589 IgG-BCR and IgE-BCR on memory B cells only requires either no tension or a

590 mechanical force below 12 pN since 12 pN, 43 pN or 56 pN NP-TGT sensors
591 similarly drove the activation of IgG-BCR (or IgE-BCR). All these results highlighted
592 the significant low force requirement to initiate the activation of IgG-BCR or
593 IgE-BCR that are expressed on memory B cells compared to the case of IgM-BCR on
594 mature naïve B cells, implicating a new possible mechanism to explain the rapid and
595 high-titered IgG antibody responses upon re-encounter with antigen of memory B
596 cells. It is worth noting that the recent study by Ha and his colleagues showed that the
597 activation of Notch receptor also requires either no tension or a single molecule force
598 smaller than 12 pN (Wang and Ha, 2013). It is of great interest to address the
599 underlying fundamental mechanism of the different mechanical force requirements for
600 different receptors. In this report, we investigated which domain of mIgG resulted in
601 the low or none requirements on mechanical forces for IgG-BCRs compared to the
602 case of IgM-BCRs since both of these two forms of BCRs use the same signaling
603 initiation component, Ig α and Ig β heterodimer. By a systemic swap experiment we
604 demonstrated that the lower threshold on mechanical forces of memory IgG-BCR
605 activation is dependent on its cytoplasmic tail. At this moment, we are addressing the
606 question of why an evolutionarily conserved cytoplasmic tail confers such low
607 threshold on mechanical forces for the activation of memory IgG-BCR. We
608 hypothesize that the cytoplasmic tail of mIgG (or mIgE) might have some capabilities
609 to facilitate the conformational changes of the “umbrella-opening like” cytoplasmic
610 domain of Ig α and Ig β complex during the transmembrane signaling transduction of
611 BCR activation (Tolar et al., 2005).

612 In conclusion, all these results define the sensitivity and threshold for mechanical

force that is required to activate IgM-BCRs and isotype-switched IgG-BCR and IgE-BCR, highlighting the significant contribution of mechanical force signals in the enhanced activation of isotype-switched IgG-BCR and IgE-BCR expressing memory B cells in rapid and high-titered antigen recall responses.

Materials and methods

Cells, antigens, antibodies and plasmid constructs

Mouse J558L cells stably expressing Ig α -YFP and high affinity version of NP-specific B1-8-mIgM-CFP or mIgG-CFP were constructed and maintained as previously described (Liu et al., 2010a). J558L B cells and Human Ramos B cells were cultured in RPMI 1640 medium containing 10% FBS, penicillin and streptomycin antibiotics (Invitrogen) as described (Liu et al., 2010c, Sohn et al., 2011). Both of these B cell lines were gifts from Dr. Susan K. Pierce (NIAID, NIH, USA). Primary B1-8 specific mature naïve B cells were negatively isolated from spleens of the IgH B1-8/B1-8 Igk $^{-/-}$ transgenic mice as reported previously (Liu et al., 2010a). Following our published protocol (Liu et al., 2010b), the spleen B cells from IgH B1-8/B1-8 Igk $^{-/-}$ transgenic mice were incubated with 40 μ g/ml LPS and 20 ng/ml recombinant mouse IL-4 (rm-IL-4) for 3 days to induce the isotype-switch to IgG1-BCR.

DyLight 649 AffiniPure Fab Fragment Goat Anti-Mouse IgM, μ Chain Specific (Jackson ImmunoResearch) and Alexa Fluor 647 AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) were used for IgM and IgG staining accordingly. In short, the cells were stained with 100 nM antibody on ice for 10 min, after washing three times with PBS, the cells are ready for further experiments. FITC-conjugated mouse IgG2a anti-NP antibody named Mouse IgG2a isotype control

antibody was purchased from Miltenyi Biotec.

Preparation of B1-8 NP specific tension gauge tethers (NP-TGTs)

NP-TGTs were prepared following the published protocols (Wang and Ha, 2013) with modifications. To specifically activate B cells, we conjugate NP (4-Hydroxy-3-nitrophenylacety) hapten to one DNA strand (single strand DNA, ssDNA) of the double strand DNA (dsDNA) of TGTs. Briefly, NP-e-Aminocaproyl-OSu (Biosearch Technologies) were conjugated to ssDNA with NH₂ group modification. The sequence is as below:

5'-CAC AGC ACG GAG GCA CGA CAC-NH₂-3'

The other strand of the TGT has a biotin tag at a pre-designed position for performing different rupture force points and binding to the coverslip through biotin-neutravidin bond. The sequence is as below:

5'-GTG TCG TGC CTC CGT GCT GTG-3' with biotin label at position 1, 2, 4, 7, 11, 15, 18, and 21 base, which form 12, 16, 23, 33, 43, 50, 54 and 56 pN respectively.

NP-ssDNA and biotin-ssDNA were further annealed in the annealing buffer following the protocol from Invitrogen.

Coverslip (VWR International) were pretreated with stripping buffer (H₂SO₄:H₂O₂=7:3), washed and dried before were glued to the disposable 8-well chamber frame (Nunc Lab-Tek chamber). And then 200 µg/ml neutravidin were added to the coverslip, after incubation for 30 min, extensive washing was performed. NP-TGTs were then loaded to the coverslip at the concentration of 50 nM for 30 min at room temperature for the purpose of tethering NP-TGTs to the coverslip. After carefully washing with PBS, the coverslip was blocked with 1% casein

(Weight/Volume) in PBS for 30 min. And then, the NP-TGT conjugated coverslip was ready for use. The cells were then loaded on the surface for reaction at 37 degree for 10 min, if no specific indications.

Preparation of planar lipid bilayers (PLBs)

PLBs were prepared following our published protocol (Liu et al., 2010a, Wan and Liu, 2012), which biotinylated NP8-BSA were attached to biotin lipid through streptavidin.

In brief, biotin liposomes were prepared by sonication of 1,2-Dioleoyl-sn-Glycero-3-phosphocholine and 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine-cap-biotin (Avanti Polar Lipids) in a 25:1 molar ratio in PBS at a lipid concentration of 2.5 mM. The PLBs were prepared in 8-well chambers (Nunc Lab-Tek) with the coverslip cleaned by nanostrip buffer. The coverslip was incubated with 0.1 mM biotin liposomes in PBS for 20 min at room temperature. After washing with 10 ml PBS, the PLB was incubated with 40 nM streptavidin for 15 min and excessive streptavidin was washed away with 10 ml PBS. And then the streptavidin-containing PLBs were incubated with 30 nM biotinylated, NP8-BSA (pre-mixed Alexa488 conjugated NP8-BSA with non-fluorescent conjugated NP8-BSA at 1:10 molar ratio) for 15min. After washing, PLBs were blocked with 1 % (Weight/Volume) Casein in PBS for 30 min at 37°C and washed thoroughly for further use.

Molecule imaging by Total Internal Reflection Fluorescence Microscope (TIRFM) and Confocal Fluorescence Microscope

TIRFM images were acquired by an Olympus IX-81 microscope equipped with a TIRF port, Andor iXon+ DU-897D electron-multiplying CCD camera, Olympus 100 ×

1.49 N.A. objective lens. The acquisition was controlled by Metamorph software (Molecular Devices). The excitation area is about $11600\ \mu\text{m}^2$. Laser power was measured at the head of the objective lens by placing the laser beam at a perpendicular orientation to the imaging plane. The laser power of 647 nm laser in BCR imaging is about $7.61\ \mu\text{W}$ (2% output power only in the configuration of Metamorph supported operating panel; 647 nm laser is 10 mW if using 100% output power). The laser power of 488 nm laser for the imaging of NP-TGT sensors by FITC-conjugated NP-specific antibodies is about $22.9\ \mu\text{W}$ (5% output power only in the configuration of Metamorph supported operating panel), and the laser power is about $377.1\ \mu\text{W}$ in single molecule imaging based experiment for the quantification of surface density of NP-TGT sensors (20% output power only in the configuration of Metamorph supported operating panel; 488 nm laser is 3 mW if using 100% output power). For the imaging options, the exposure time was 100 ms for 512×512 pixels image, unless specially indicated. All the images are confirmed not over exposed by the software. Confocal images were acquired by the Olympus FLUOVIEW FV1000 confocal laser scanning microscope with a $100 \times$ oil objective lens. The reconstructed side view images were processed by Bitplane Imaris. All the images were analyzed and processed with Image J (NIH, U.S.) software as indicated. The mean fluorescence intensity (MFI) and the total fluorescence intensity (Total FI), as arbitrary unit, of BCRs and signaling molecules accumulated to the immunological synapse were calculated based on the intensity and area analysis as described (Liu et al., 2012b, Lakadamyali et al., 2004, Liu et al., 2010b, Liu et al., 2010c).

Intracellular immunofluorescence staining and molecules imaging

The recruitment of signaling molecules into the immunological synapse of B cells stimulated by NP-TGTs was imaged by TIRFM, and the detection of internalized BCR is imaged by Confocal following our previously published protocol (Liu et al., 2012b, Liu et al., 2010a, Liu et al., 2010b, Liu et al., 2010c). In brief, BCRs were pre-stained and then B cells were loaded to the chambers for reaction with NP-TGTs for 10 min followed by 4% paraformaldehyde fixation. After washed with 10 ml PBS, the B cells were permeabilized with 0.1% Triton X-100 and then blocked with 100 µg/ml goat non-specific IgG (Jackson ImmunoResearch Laboratory). Subsequently, cells were stained with different primary antibodies including phospho-Zap-70(Tyr319)/Syk (Tyr352) antibody (Cell Signaling Technology), phospho-PLCγ2 (Tyr759) antibody (BD), phospho-PI3K (Tyr458) antibody (Cell Signaling Technology), anti-phosphotyrosine (Millipore Upstate) at 37 °C for 1h. After washed with 10 ml PBS, B cells were stained with secondary antibody Alexa Fluor 568-conjugated F(ab')₂ goat antibody specific for rabbit or mouse IgG (Invitrogen) as previously described (Liu et al., 2012b). Images were analyzed by software Image J (NIH, U.S.) following our published protocols (Liu et al., 2012b, Liu et al., 2010b, Liu et al., 2010c, Liu et al., 2010a).

Treatment of B cells with pharmaceutical inhibitor

For inhibitor studies, primary mature naïve B cells from IgH B1-8/B1-8 Igk^{-/-} transgenic mice were pretreated with myosin IIA inhibitor Blebbistatin (Hung et al., 2013), FAK inhibitor PF573-228 (Sigma-Aldrich) (Slack-Davis et al., 2007), dynein inhibitor HPI-4 (Sigma-Aldrich) (Firestone et al., 2012) , or internalization inhibitor Monodansylcadaverine (MDC) (Sigma-Aldrich) (Liu et al., 2010a) before the imaging

experiment. Briefly, B cells were pretreated with 50 μ M Blebbistatin at 37 degree for 30 min, 1 μ M PF573-228 at 37 degree for 30 min, 30 μ M HPI-4 at 37 degree overnight, or 100 μ M MDC for 30 min at room temperature to block the function of myosin IIA, FAK, or dynein respectively. As a control, B1-8 primary B cells were pretreated with DMSO for 30 min at 37 degree, or overnight as the control for HPI-4 inhibitor, or 30 min at room temperature as the control for MDC inhibitor.

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Conflict of Interest Disclosure:

The authors declare no financial or commercial conflict of interest.

References:

- ALBRECHT, C., BLANK, K., LALIC-MULTHALER, M., HIRLER, S., MAI, T., GILBERT, I., SCHIFFMANN, S., BAYER, T., CLAUSEN-SCHAUMANN, H. & GAUB, H. E. 2003. DNA: a programmable force sensor. *Science*, 301, 367-70.
- ARANA, E., HARWOOD, N. E. & BATISTA, F. D. 2008a. Regulation of integrin activation through the B-cell receptor. *J Cell Sci*, 121, 2279-86.
- ARANA, E., VEHLow, A., HARWOOD, N. E., VIGORITO, E., HENDERSON, R., TURNER, M., TYBULEWICZ, V. L. & BATISTA, F.

754 D. 2008b. Activation of the small GTPase Rac2 via the B cell receptor regulates B cell adhesion and
755 immunological-synapse formation. *Immunity*, 28, 88-99.

756 ARAUJO GDE, S., FONSECA, F. L., PONTES, B., TORRES, A., CORDERO, R. J., ZANCOPE-OLIVEIRA, R. M., CASADEVALL, A.,
757 VIANA, N. B., NIMRICHTER, L., RODRIGUES, M. L., GARCIA, E. S., SOUZA, W. & FRASES, S. 2012. Capsules from
758 pathogenic and non-pathogenic *Cryptococcus* spp. manifest significant differences in structure and ability to
759 protect against phagocytic cells. *PLoS One*, 7, e29561.

760 BACHMANN, M. F. & JENNINGS, G. T. 2010. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.
761 *Nat Rev Immunol*, 10, 787-96.

762 BACHMANN, M. F., MCKALL-FAIENZA, K., SCHMITS, R., BOUCHARD, D., BEACH, J., SPEISER, D. E., MAK, T. W. & OHASHI, P.
763 S. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation.
764 *Immunity*, 7, 549-57.

765 BACHMANN, M. F., ROHRER, U. H., KUNDIG, T. M., BURKI, K., HENGARTNER, H. & ZINKERNAGEL, R. M. 1993. The
766 influence of antigen organization on B cell responsiveness. *Science*, 262, 1448-51.

767 BAO, G. & SURESH, S. 2003. Cell and molecular mechanics of biological materials. *Nat Mater*, 2, 715-25.

768 BASHOUR, K. T., GONDARENKO, A., CHEN, H., SHEN, K., LIU, X., HUSE, M., HONE, J. C. & KAM, L. C. 2014. CD28 and CD3
769 have complementary roles in T-cell traction forces. *Proc Natl Acad Sci U S A*, 111, 2241-6.

770 CARRASCO, Y. R., FLEIRE, S. J., CAMERON, T., DUSTIN, M. L. & BATISTA, F. D. 2004. LFA-1/ICAM-1 interaction lowers the
771 threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity*, 20, 589-99.

772 CHEN, W., LOU, J., EVANS, E. A. & ZHU, C. 2012. Observing force-regulated conformational changes and ligand
773 dissociation from a single integrin on cells. *J Cell Biol*, 199, 497-512.

774 CHEN, W., LOU, J. & ZHU, C. 2010. Forcing switch from short- to intermediate- and long-lived states of the alphaA
775 domain generates LFA-1/ICAM-1 catch bonds. *J Biol Chem*, 285, 35967-78.

776 CIECHOMSKA, M., WILSON, C. L., FLOUDAS, A., HUI, W., ROWAN, A. D., VAN EDEN, W., ROBINSON, J. H. & KNIGHT, A. M.
777 2014. Antigen-specific B lymphocytes acquire proteoglycan aggrecan from cartilage extracellular matrix
778 resulting in antigen presentation and CD4+ T-cell activation. *Immunology*, 141, 70-8.

779 DEPOIL, D., FLEIRE, S., TREANOR, B. L., WEBER, M., HARWOOD, N. E., MARCHBANK, K. L., TYBULEWICZ, V. L. & BATISTA, F.
780 D. 2008. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in
781 response to membrane-bound ligand. *Nat Immunol*, 9, 63-72.

782 DISCHER, D. E., JANMEY, P. & WANG, Y. L. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science*,
783 310, 1139-43.

784 DUSTIN, M. L. 2009. Modular design of immunological synapses and kinapses. *Cold Spring Harb Perspect Biol*, 1,
785 a002873.

786 DUSTIN, M. L., TSENG, S. Y., VARMA, R. & CAMPI, G. 2006. T cell-dendritic cell immunological synapses. *Curr Opin*
787 *Immunol*, 18, 512-6.

788 ENGELS, N., KONIG, L. M., HEEMANN, C., LUTZ, J., TSUBATA, T., GRIEP, S., SCHRADER, V. & WIENANDS, J. 2009.
789 Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic
790 costimulation to class-switched B cells. *Nat Immunol*, 10, 1018-25.

791 ENGELS, N., KONIG, L. M., SCHULZE, W., RADTKE, D., VANSHYLLA, K., LUTZ, J., WINKLER, T. H., NITSCHKE, L. & WIENANDS,
792 J. 2014. The immunoglobulin tail tyrosine motif upgrades memory-type BCRs by incorporating a Grb2-Btk
793 signalling module. *Nat Commun*, 5, 5456.

794 ENGLER, A. J., GRIFFIN, M. A., SEN, S., BONNEMANN, C. G., SWEENEY, H. L. & DISCHER, D. E. 2004. Myotubes
795 differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff
796 microenvironments. *J Cell Biol*, 166, 877-87.

797 FIRESTONE, A. J., WEINGER, J. S., MALDONADO, M., BARLAN, K., LANGSTON, L. D., O'DONNELL, M., GELFAND, V. I.,
798 KAPOOR, T. M. & CHEN, J. K. 2012. Small-molecule inhibitors of the AAA+ ATPase motor cytoplasmic dynein.
799 *Nature*, 484, 125-9.

800 FLEIRE, S. J., GOLDMAN, J. P., CARRASCO, Y. R., WEBER, M., BRAY, D. & BATISTA, F. D. 2006. B cell ligand discrimination

through a spreading and contraction response. *Science*, 312, 738-41.

HARWOOD, N. E. & BATISTA, F. D. 2010. Early events in B cell activation. *Annu Rev Immunol*, 28, 185-210.

HATCH, K., DANILOWICZ, C., COLJEE, V. & PRENTISS, M. 2008. Demonstration that the shear force required to separate short double-stranded DNA does not increase significantly with sequence length for sequences longer than 25 base pairs. *Phys Rev E Stat Nonlin Soft Matter Phys*, 78, 011920.

HAUSER, A. E., JUNT, T., MEMPEL, T. R., SNEDDON, M. W., KLEINSTEIN, S. H., HENRICKSON, S. E., VON ANDRIAN, U. H., SHLOMCHIK, M. J. & HABERMAN, A. M. 2007. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity*, 26, 655-67.

HORIKAWA, K., MARTIN, S. W., POGUE, S. L., SILVER, K., PENG, K., TAKATSU, K. & GOODNOW, C. C. 2007. Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med*, 204, 759-69.

HUNG, W. C., CHEN, S. H., PAUL, C. D., STROKA, K. M., LO, Y. C., YANG, J. T. & KONSTANTOPOULOS, K. 2013. Distinct signaling mechanisms regulate migration in unconfined versus confined spaces. *J Cell Biol*, 202, 807-24.

ILANI, T., VASILIVER-SHAMIS, G., VARDHANA, S., BRETSCHER, A. & DUSTIN, M. L. 2009. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. *Nat Immunol*, 10, 531-9.

KAISHO, T., SCHWENK, F. & RAJEWSKY, K. 1997. The roles of gamma 1 heavy chain membrane expression and cytoplasmic tail in IgG1 responses. *Science*, 276, 412-5.

KUROSAKI, T., SHINOHARA, H. & BABA, Y. 2010. B cell signaling and fate decision. *Annu Rev Immunol*, 28, 21-55.

LAKADAMYALI, M., RUST, M. J. & ZHUANG, X. 2004. Endocytosis of influenza viruses. *Microbes Infect*, 6, 929-36.

LANG, M. J., FORDYCE, P. M., ENGH, A. M., NEUMAN, K. C. & BLOCK, S. M. 2004. Simultaneous, coincident optical trapping and single-molecule fluorescence. *Nat Methods*, 1, 133-9.

LIU, B., CHEN, W., EVAVOLD, B. D. & ZHU, C. 2014. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell*, 157, 357-68.

LIU, B., CHEN, W. & ZHU, C. 2015. Molecular Force Spectroscopy on Cells. *Annu Rev Phys Chem*.

LIU, C., MILLER, H., ORLOWSKI, G., HANG, H., UPADHYAYA, A. & SONG, W. 2012a. Actin reorganization is required for the formation of polarized B cell receptor signalosomes in response to both soluble and membrane-associated antigens. *J Immunol*, 188, 3237-46.

LIU, W., CHEN, E., ZHAO, X. W., WAN, Z. P., GAO, Y. R., DAVEY, A., HUANG, E., ZHANG, L., CROCETTI, J., SANDOVAL, G., JOYCE, M. G., MICELI, C., LUKSZO, J., ARAVIND, L., SWAT, W., BRZOSTOWSKI, J. & PIERCE, S. K. 2012b. The scaffolding protein synapse-associated protein 97 is required for enhanced signaling through isotype-switched IgG memory B cell receptors. *Sci Signal*, 5, ra54.

LIU, W. & CHEN, Y. H. 2005. High epitope density in a single protein molecule significantly enhances antigenicity as well as immunogenicity: a novel strategy for modern vaccine development and a preliminary investigation about B cell discrimination of monomeric proteins. *Eur J Immunol*, 35, 505-14.

LIU, W., MECKEL, T., TOLAR, P., SOHN, H. W. & PIERCE, S. K. 2010a. Antigen affinity discrimination is an intrinsic function of the B cell receptor. *J Exp Med*, 207, 1095-111.

LIU, W., MECKEL, T., TOLAR, P., SOHN, H. W. & PIERCE, S. K. 2010b. Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling. *Immunity*, 32, 778-89.

LIU, W., PENG, Z., LIU, Z., LU, Y., DING, J. & CHEN, Y. H. 2004. High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity. *Vaccine*, 23, 366-71.

LIU, W., WON SOHN, H., TOLAR, P., MECKEL, T. & PIERCE, S. K. 2010c. Antigen-induced oligomerization of the B cell receptor is an early target of Fc gamma RIIB inhibition. *J Immunol*, 184, 1977-89.

MARTIN, S. W. & GOODNOW, C. C. 2002. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol*, 3, 182-8.

MATEU, M. G. 2012. Mechanical properties of viruses analyzed by atomic force microscopy: a virological perspective. *Virus Res*, 168, 1-22.

848 MATTILA, P. K., FEEST, C., DEPOIL, D., TREANOR, B., MONTANER, B., OTIPOBY, K. L., CARTER, R., JUSTEMENT, L. B.,
849 BRUCKBAUER, A. & BATISTA, F. D. 2013. The actin and tetraspanin networks organize receptor nanoclusters to
850 regulate B cell receptor-mediated signaling. *Immunity*, 38, 461-74.

851 MCHEYZER-WILLIAMS, L. J. & MCHEYZER-WILLIAMS, M. G. 2005. Antigen-specific memory B cell development. *Annu*
852 *Rev Immunol*, 23, 487-513.

853 MCLEOD, S. J., SHUM, A. J., LEE, R. L., TAKEI, F. & GOLD, M. R. 2004. The Rap GTPases regulate integrin-mediated
854 adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J Biol*
855 *Chem*, 279, 12009-19.

856 MLINARIC-RASCAN, I. & YAMAMOTO, T. 2001. B cell receptor signaling involves physical and functional association of
857 FAK with Lyn and IgM. *FEBS Lett*, 498, 26-31.

858 NATKANSKI, E., LEE, W. Y., MISTRY, B., CASAL, A., MOLLOY, J. E. & TOLAR, P. 2013. B Cells Use Mechanical Energy to
859 Discriminate Antigen Affinities. *Science*, 340, 1587-1590.

860 NEMIR, S. & WEST, J. L. 2010. Synthetic materials in the study of cell response to substrate rigidity. *Ann Biomed Eng*, 38,
861 2-20.

862 PASZEK, M. J., ZAHIR, N., JOHNSON, K. R., LAKINS, J. N., ROZENBERG, G. I., GEFEN, A., REINHART-KING, C. A., MARGULIES,
863 S. S., DEMBO, M., BOETTIGER, D., HAMMER, D. A. & WEAVER, V. M. 2005. Tensional homeostasis and the
864 malignant phenotype. *Cancer Cell*, 8, 241-54.

865 PIERCE, S. K. & LIU, W. 2010. The tipping points in the initiation of B cell signalling: how small changes make big
866 differences. *Nat Rev Immunol*, 10, 767-77.

867 RETH, M. 1989. Antigen receptor tail clue. *Nature*, 338, 383.

868 SAOUAF, S. J., MAHAJAN, S., ROWLEY, R. B., KUT, S. A., FARGNOLI, J., BURKHARDT, A. L., TSUKADA, S., WITTE, O. N. &
869 BOLEN, J. B. 1994. Temporal differences in the activation of three classes of non-transmembrane protein
870 tyrosine kinases following B-cell antigen receptor surface engagement. *Proc Natl Acad Sci U S A*, 91, 9524-8.

871 SCHAMEL, W. W. & RETH, M. 2000. Monomeric and oligomeric complexes of the B cell antigen receptor. *Immunity*, 13,
872 5-14.

873 SCHNYDER, T., CASTELLO, A., FEEST, C., HARWOOD, N. E., OELLERICH, T., URLAUB, H., ENGELKE, M., WIENANDS, J.,
874 BRUCKBAUER, A. & BATISTA, F. D. 2011. B cell receptor-mediated antigen gathering requires ubiquitin ligase Cbl
875 and adaptors Grb2 and Dok-3 to recruit dynein to the signaling microcluster. *Immunity*, 34, 905-18.

876 SEELEY-FALLEN, M. K., LIU, L. J., SHAPIRO, M. R., ONABAJO, O. O., PALANIYANDI, S., ZHU, X., TAN, T. H., UPADHYAYA, A. &
877 SONG, W. 2014. Actin-binding protein 1 links B-cell antigen receptors to negative signaling pathways. *Proc Natl*
878 *Acad Sci U S A*, 111, 9881-6.

879 SLACK-DAVIS, J. K., MARTIN, K. H., TILGHMAN, R. W., IWANICKI, M., UNG, E. J., AUTRY, C., LUZZIO, M. J., COOPER, B.,
880 KATH, J. C., ROBERTS, W. G. & PARSONS, J. T. 2007. Cellular characterization of a novel focal adhesion kinase
881 inhibitor. *J Biol Chem*, 282, 14845-52.

882 SOHN, H. W., KRUEGER, P. D., DAVIS, R. S. & PIERCE, S. K. 2011. FcRL4 acts as an adaptive to innate molecular switch
883 dampening BCR signaling and enhancing TLR signaling. *Blood*, 118, 6332-41.

884 SPAARGAREN, M., BEULING, E. A., RURUP, M. L., MEIJER, H. P., KLOK, M. D., MIDDENDORP, S., HENDRIKS, R. W. & PALS, S.
885 T. 2003. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J Exp Med*, 198,
886 1539-50.

887 TARLINTON, D. 1997. Antigen presentation by memory B cells: the sting is in the tail. *Science*, 276, 374-5.

888 TOLAR, P., SOHN, H. W. & PIERCE, S. K. 2005. The initiation of antigen-induced B cell antigen receptor signaling viewed in
889 living cells by fluorescence resonance energy transfer. *Nat. Immunol.*, 6, 1168-76.

890 TRAPPMANN, B., GAUTROT, J. E., CONNELLY, J. T., STRANGE, D. G., LI, Y., OYEN, M. L., COHEN STUART, M. A., BOEHM, H.,
891 LI, B., VOGEL, V., SPATZ, J. P., WATT, F. M. & HUCK, W. T. 2012. Extracellular-matrix tethering regulates stem-cell
892 fate. *Nat Mater*, 11, 642-9.

893 TSENG, S. Y., WAITE, J. C., LIU, M., VARDHANA, S. & DUSTIN, M. L. 2008. T cell-dendritic cell immunological synapses
894 contain TCR-dependent CD28-CD80 clusters that recruit protein kinase C theta. *J Immunol*, 181, 4852-63.

VASCOTTO, F., LANKAR, D., FAURE-ANDRE, G., VARGAS, P., DIAZ, J., LE ROUX, D., YUSEFF, M. I., SIBARITA, J. B., BOES, M., RAPOSO, G., MOUGNEAU, E., GLAICHENHAUS, N., BONNEROT, C., MANOURY, B. & LENNON-DUMENIL, A. M. 2007. The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *J Cell Biol*, 176, 1007-19.

WAISMAN, A., KRAUS, M., SEAGAL, J., GHOSH, S., MELAMED, D., SONG, J., SASAKI, Y., CLASSEN, S., LUTZ, C., BROMBACHER, F., NITSCHKE, L. & RAJEWSKY, K. 2007. IgG1 B cell receptor signaling is inhibited by CD22 and promotes the development of B cells whose survival is less dependent on Ig alpha/beta. *J Exp Med*, 204, 747-58.

WAKABAYASHI, C., ADACHI, T., WIENANDS, J. & TSUBATA, T. 2002. A distinct signaling pathway used by the IgG-containing B cell antigen receptor. *Science*, 298, 2392-5.

WAN, Z. & LIU, W. 2012. The growth of B cell receptor microcluster is a universal response of B cells encountering antigens with different motion features. *Protein Cell*, 3, 545-58.

WAN, Z., ZHANG, S., FAN, Y., LIU, K., DU, F., DAVEY, A. M., ZHANG, H., HAN, W., XIONG, C. & LIU, W. 2013. B cell activation is regulated by the stiffness properties of the substrate presenting the antigens. *J Immunol*, 190, 4661-75.

WANG, X. & HA, T. 2013. Defining single molecular forces required to activate integrin and notch signaling. *Science*, 340, 991-4.

WEBER, M., TREANOR, B., DEPOIL, D., SHINOHARA, H., HARWOOD, N. E., HIKIDA, M., KUROSAKI, T. & BATISTA, F. D. 2008. Phospholipase C-gamma2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *J Exp Med*, 205, 853-68.

YU, Y., FAY, N. C., SMOLIGOVETS, A. A., WU, H. J. & GROVES, J. T. 2012. Myosin IIA modulates T cell receptor transport and CasL phosphorylation during early immunological synapse formation. *PLoS One*, 7, e30704.

ZENG, Y., YI, J., WAN, Z., LIU, K., SONG, P., CHAU, A., WANG, F., CHANG, Z., HAN, W., ZHENG, W., CHEN, Y. H., XIONG, C. & LIU, W. 2015. Substrate stiffness regulates B-cell activation, proliferation, class switch, and T-cell-independent antibody responses in vivo. *Eur J Immunol*, 45, 1621-34.

Figure Legends

Figure 1:

The construction of B1-8-BCR specific NP-TGT mechanical force sensor system

(A) Schematic representation of the NP-TGT and NP-specific B1-8-BCR expressing B cells. NP-TGT molecule is immobilized on the surface of coverslip, which will get ruptured if the mechanical force applied by the B1-8-BCR is larger than the pre-defined tension force of a certain NP-TGT (for example 56 pN is depicted in the figure). FITC-conjugated anti-NP antibody is used to quantify the molecule density of each different type of NP-TGT sensors tethered on coverslip.

(B) The dsDNA geometries and pre-defined tension force of all eight NP-conjugated TGT sensors and one control TGT without NP conjugation.

(C) Representative TIRFM images showing the dynamics of the synaptic accumulation of BCRs from J558L cells expressing B1-8-IgM-BCR in contact with coverslip presenting 56 pN NP-TGT sensor or control TGT (NC) at the indicated time points. Scale bar is 1.5 μm .

(D) Comparisons of averaged traces showing the dynamic accumulation of BCRs as demonstrated in (C) in a 13 min TIRFM imaging time course. Bars represent mean $\pm$ SEM. Data were from at least 20 cells over three independent experiments.

(E) Primary mature naïve B cells from wild type C57BL/6 mice expressing non-NP-specific IgM-BCR did not initiate the activation when encountering 56 pN NP-TGT sensor compared to the response of the same B cells encountering 56 pN TGT sensor without NP conjugation. Biotin-conjugated goat anti-mouse IgM

surrogate antigens were used as a positive control (PC) to efficiently drive the synaptic accumulation of IgM-BCRs in B cell activation. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were from at least 30 cells over three independent experiments.

(F) Quantification of the mean fluorescence intensity (MFI) of FITC-conjugated NP-specific antibodies on the surface of coverslip tethering the same amount of NP-TGT sensors. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. The surface density is 29.0 molecule/ μm^2 , seeing more in **Figure 2—figure supplement 1**.

Figure 2:

The synaptic accumulation of the IgM-BCRs is dependent on mechanical forces and exhibits a multi-threshold effect

(A-B) Statistical quantification of the synaptic recruitment of IgM-BCR in J558L cells expressing naïve B1-8-IgM-BCR **(A)** and primary naïve B cells expressing B1-8-IgM-BCR **(B)**. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data are from at least 40 cells over three independent experiments.

(C) Representative TIRFM images showing the dynamics of the synaptic accumulation of IgM-BCRs from J558L cells expressing naïve B1-8-IgM-BCR in contact with coverslip presenting 12 pN, 43 pN or 56 pN NP-TGT sensors at the indicated time points. Scale bar is 1.5 μm .

(D-E) Comparisons of averaged traces showing the dynamic accumulation of naïve

IgM-BCRs into the immunological synapse **(D)** and the growing features of the size of contact area **(E)** for J558L cells expressing naïve B1-8-IgM-BCR as demonstrated in **(C)** in a 10 min TIRFM imaging time course. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data are from at least 20 cells over two independent experiments.

Figure 3: The volume of the IgM-BCR microcluster produced by different NP-TGT sensors is dependent on mechanical forces and exhibits a similar multi-threshold effect

(A) Representative original (top panel), pseudo-colored 2D (middle panel), and 2.5D Gaussian images (bottom panel) of typical BCR microclusters induced by 12, 16, 23, 33, 43, 50, 54, and 56 pN NP-TGT sensors. Scale bar is 1.5 μ m.

(B) Statistical comparison of FI of hundreds of BCR microclusters in the immunological synapse in J558L cells expressing naïve B1-8-IgM-BCR encountering NP-TGT sensors with indicated tension force. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data are from at least 30 cells over three independent experiments.

Figure 4:

The strength of IgM-BCR signaling is dependent on mechanical forces

(A-C) Statistical quantification of the synaptic recruitment of pSyk **(A)**, pPLC γ 2 **(B)**, and pTyr **(C)** in primary naïve B cells expressing B1-8-IgM-BCR that were placed on coverslip presenting 12 pN, 43 pN or 56 pN NP-TGT sensors.

(D-F) Statistical comparison of the volume of pSyk (D), pPLC γ 2 (E) or pTyr (F) microclusters in J558L cells expressing naïve B1-8-IgM-BCR that were produced by 12 pN, 43 pN or 56 pN NP-TGT molecules.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were at least from 30 cells of three independent experiments.

Figure 5:

The patterned dependence on the mechanical forces of IgM-BCR activation does not rely on LFA-1 mediated adhesion and dynein, and is only partially dependent on myosin IIA

(A, B) The synaptic accumulation of IgM-BCRs in primary naïve B cells expressing B1-8-IgM-BCR in contact with the indicated types of NP-TGT sensors with or without ICAM-1 co-tethering. Cross comparison strategy were used in these figures.

(C-H) The synaptic accumulation of IgM-BCRs in primary naïve B cells expressing B1-8-IgM-BCR in contact with the indicated types of NP-TGT probes. In this experiment, primary naïve B cells expressing B1-8-IgM-BCR were pre-treated with DMSO as a control in combination with FAK inhibitor (C-D), myosin IIA inhibitor (E-F) or dynein inhibitor (G-H) following a protocol that was detailed in materials and methods section. Cross comparison strategy were used in these figures.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were at least from 30 cells in each group of three independent experiments.

Figure 6: The activation of isotype-switched IgG-BCRs or IgE-BCRs on memory B cells requires either no tension or a mechanical force below 12 pN

(A, B) Statistical quantification of the synaptic accumulation of IgG-BCR and the volume of the contact area of J558L cells expressing memory B1-8-IgG-BCR encountering 12 pN, 43 pN or 56 pN NP-TGT sensors.

(C, D) Statistical analyses of synaptic accumulation of pSyk accumulation **(C)** and the volume of pSyk microcluster **(D)** in response to 12 pN, 43 pN or 56 pN NP-TGT sensors.

(E, F) Quantification of the synaptic accumulation of IgG-BCRs **(E)** or pSyk **(F)** in memory B cells expressing isotype-switched B1-8-IgG-BCR from B1-8 Tg mice that were placed on coverslip presenting 12 pN, 43 pN, or 56 pN NP-TGT probes.

(G) Representative TIRFM images showing the dynamics of the synaptic accumulation of IgG-BCRs from J558L cells expressing memory B1-8-IgG-BCR in contact with coverslip presenting 12 pN, 56 pN NP-TGT sensor, or control TGT (NC) molecule at the indicated time points. Scale bar is 1.5 μm .

(H) Comparisons of averaged traces showing the dynamic accumulation of memory IgG-BCRs as demonstrated in **(G)** in a 13 min TIRFM imaging time course. Bars represent mean $\pm$ SEM. Data were from at least 20 cells over three independent experiments.

(I-L) Statistical analyses of the synaptic accumulation of two types of chimeric BCRs and pSyk, Human mIgE heavy chain **(I, J)** or Human mIgM heavy chain **(K, L)** with mouse B1-8 variable region in human Ramos B cells encountering 12 pN, 43 pN, or 56 pN NP-TGT sensors.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were from at least 30 cells in each group of three independent experiments.

Figure 7:

The lower mechanical force threshold of IgG-BCR activation is dependent on its cytoplasmic tail

(A) Schematic illustration of the strategy of swapping the cytoplasmic tail of B1-8-IgG- or B1-8-IgM-BCRs.

(B-G) Quantification of the synaptic accumulation of BCRs in J558L cells expressing memory B1-8-IgG-BCR **(B)**, J558L cells expressing memory B1-8-IgG-BCR equipped with a mIgM cytoplasmic tail, termed GGM **(C)**, J558L cells expressing naïve B1-8-IgM-BCR **(E)** and J558L cells expressing naïve B1-8-IgM-BCR equipped with a mIgG cytoplasmic tail, termed MMG **(F)**. Also given are the synaptic accumulations of pSyk in GGM **(D)** and MMG cells **(G)**.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were from at least 30 cells in each group of three independent experiments.

Supplement Figure Legends

Figure 1—figure supplement 1: The quality control of NP-TGT sensor based experimental system

(A) The quality control of the purified NP-ssDNA by mass spectrum.

(B, C) No obvious dissociation of the neutravidin was detected in a 10 min incubation time course. In B, two-color TIRF images showing the formation of a typical immunological synapse (IS) of a single B cell (BCR, red color) and the corresponded Alexa488-conjugated neutravidin within the B cell IS. Also given are control neutravidin TIRF images from an area without B cells. Scale bar is 5 μ m. In C, the statistical analyses of the MFI of Alexa488-conjugated neutravidin from the area on coverslip without B cells (no cell region) versus the case within the area of B cell IS (beneath the cell) that were induced by 12 pN, 43 pN or 56 pN NP-TGT sensors and a negative control 56 pN TGT without NP-conjugation (NC).

(D) Statistical analyses for the MFI of FITC-conjugated NP-specific antibodies to show that NP-TGT can only be coated to coverslip in a neutravidin dependent manner.

(E) Quantification of the synaptic accumulation of IgM-BCRs in J558L cells expressing naïve B1-8-IgM-BCR to show that non-specific NP-TGT that were tethered on coverslip in a neutravidin independent manner cannot activate B cells. J558L cells expressing naïve B1-8-IgM-BCR were either placed on neutravidin-coated coverslip that were incubated with 56 pN NP-TGT sensor or placed on neutravidin-non-coated coverslip that were incubated with indicated types of NP-TGT sensors. NC represents 56 pN TGT sensor without NP-conjugation.

In figure C, D, and E, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed

for the statistical comparisons. Data were from at least 30 cells or 20 measurements in each group of two independent experiments.

Figure 2—figure supplement 1: The contact area after IgM-BCR activation is dependent on mechanical forces with multi-threshold effects and such a pattern is still evident at low density of NP-TGT sensor

(A) Statistical analyses of the size of the contact area of primary naïve B cells expressing B1-8-IgM-BCR from B1-8 Tg mice when encountering of the indicated types of NP-TGT sensors.

(B) The representative image of NP-TGT molecule indicated by FITC-conjugated NP-specific antibodies within a counting area ($473.1 \mu\text{m}^2$). Scale bar is $1.5 \mu\text{m}$.

(C) The conversion and strong linear correlation between the MFI and the density of FITC-conjugated NP-specific antibody on coverslip. NP-specific antibody was used to indicate the density of NP-TGT sensor on coverslip.

The surface density is quantified at the appropriate incubation concentration of NP-TGT sensor to achieve well-separated and approximately round spots in TIRF imaging **(B)**, which were subsequently analyzed by a Matlab supported 2D Gaussian fitting code (**Source code 1**) to perform the counting as reported in our previous studies (Liu et al., 2010a). The equation of the fitted linear regression is: Surface density (per counting area, about $473.1 \mu\text{m}^2$) = $2.42 \times \text{MFI}$, R square value for the linear fitting is 0.99.

(D) Quantification of the MFI of NP-specific antibody on the coverslip at different incubation concentration of NP-TGT sensor.

(E) Surface density of NP-TGT sensors at different incubation concentration when were coated on coverslip as calculated by combining the data in C and D.

(F, G) Quantification of the synaptic accumulation of IgM-BCRs in J558L cells expressing naïve B1-8-IgM-BCR (F) or primary naïve B cells expressing B1-8-IgM-BCR (G) that were placed on coverslip coated with surface density of 4.0 molecule/ μm^2 of 12 pN, 43 pN, and 56 pN NP-TGT sensors. Bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were from at least 30 cells over three independent experiments.

Figure 2—figure supplement 2: The patterned dependence on the mechanical forces of IgM-BCR activation does not rely on BCR internalization

(A) Representative confocal images showing the efficient internalization of BCR and antigen molecules in primary naïve B cells expressing B1-8-IgM-BCR from B1-8 Tg mice that were interacted with soluble NP8-BSA for 10 min. Scale bar is 1.5 μm .

(B) Side view confocal images showing the lack of internalization of the pre-stained BCR molecules in primary naïve B cells expressing B1-8-IgM-BCR from B1-8 Tg mice that were placed on coverslip presenting 12 pN, 43 pN or 56 pN NP-TGT sensors for 10 min. B cells were pre-stained with DyLight 649 AffiniPure Fab Fragment Goat Anti-Mouse IgM, μ Chain Specific antibodies before the imaging experiment. Scale bar is 2 μm .

(C) Statistical quantification of the percentage of B cells with internalization of BCRs as represented in A and B.

(D) Statistical quantification to show that B cell internalization can be blocked by

MDC inhibitor in the condition that the B cells were pre-treated by MDC before were activated for 10 min by soluble NP8-BSA as represented in **A**.

(E, F) The synaptic accumulation of IgM-BCRs in primary naïve B cells expressing B1-8-IgM-BCR from B1-8 Tg mice in contact with the indicated types of NP-TGT sensors. In this experiment, B cells were pre-treated with either DMSO or MDC following a protocol as detailed in Methods section. Cross comparison strategy were used in these figures.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed *t* tests were performed for the statistical comparisons. Data were at least from 30 cells or 15 measurements in each group of three independent experiments.

Figure 5—figure supplement 1: Functional test of pharmaceutical inhibitors

(A-C) Positive control experiments were performed to show that each inhibitor used in **Figure 5** is working. These experiments showed that FAK inhibitor significantly reduced the size of the contact area of B cells that were placed on coverslip presenting antigens, consistent with the reported function of FAK inhibitor (Mlinaric-Rascan and Yamamoto, 2001) **(A)**; Myosin IIA inhibitor dramatically reduced the proportion of B cells with internalized Alexa488-conjugated NP8-BSA antigen in the soluble format after reaction for 30 min, consistent with the reported function of Myosin IIA inhibitor (Vascotto et al., 2007) **(B)**; Dynein inhibitor dramatically reduced the formation of cSMAC structure in B cell that were placed on planar lipid bilayers presenting antigens for 20 min, consistent with the reported function of Dynein inhibitor (Schnyder et al., 2011) **(C)**.

In all of these plots, bars represent mean $\pm$ SEM. Two-tailed t tests were performed for the statistical comparisons. Data were at least from 30 cells or at least 15 measurements in each group of three independent experiments.

Figure 6—figure supplement 1: Quantification of the accumulation of IgG-BCR in recognition of NP-TGT sensors at different surface density

(A-C) Quantification of the synaptic accumulation of IgG-BCRs in J558L cells expressing memory B1-8-IgG-BCR that were placed on coverslip coated with NP-TGT sensors at a density of 29.0 molecule/ μm^2 **(A)**, 19.0 molecule/ μm^2 **(B)**, and 0.3 molecule/ μm^2 **(C)**. 12 pN NP-TGT, 56 pN NP-TGT and 56 pN TGT molecule without NP conjugation (NC) were used in the experiment. Bars represent mean $\pm$ SEM. Two-tailed t tests were performed for the statistical comparisons. Data were from at least 30 cells over three independent experiments.

Video Legends

Video 1:

Time lapse images showing the dynamics of the synaptic accumulation of BCRs from J558L cells expressing naïve B1-8-IgM-BCR in contact with coverslip presenting 56 pN NP-TGT or control TGT (NC) sensor. Scale bar is 1.5 μm . The movie was recorded with a 4 s time interval and is shown at 30 frames per second. Related to **Figure 1C**.

Video 2:

1170 Representative time lapse TIRFM images showing the dynamics of the synaptic
1171 accumulation of IgM-BCRs from J558L cells expressing naïve B1-8-IgM-BCR in
1172 contact with coverslip presenting 12 pN, 43 pN or 56 pN NP-TGT sensor. Scale bar is
1173 1.5 μ m. The movie was recorded with a 4 s time interval and is shown at 30 frames
1174 per second. Related to **Figure 2C**.

1175

1176 **Video 3:**

1177 Time lapse images showing the dynamics of the synaptic accumulation of IgG-BCRs
1178 from J558L cells expressing memory B1-8-IgG-BCR in contact with coverslip
1179 presenting 12 pN, 56 pN NP-TGT or control TGT (NC) sensor at the indicated time
1180 points. Scale bar is 1.5 μ m. The movie was recorded with a 4 s time interval and is
1181 shown at 30 frames per second. Related to **Figure 6G**.

1182

1183 **Source code legend:**

1184 **Sourec code 1- Matlab supported 2D Gaussian fitting code.**

Figure.1

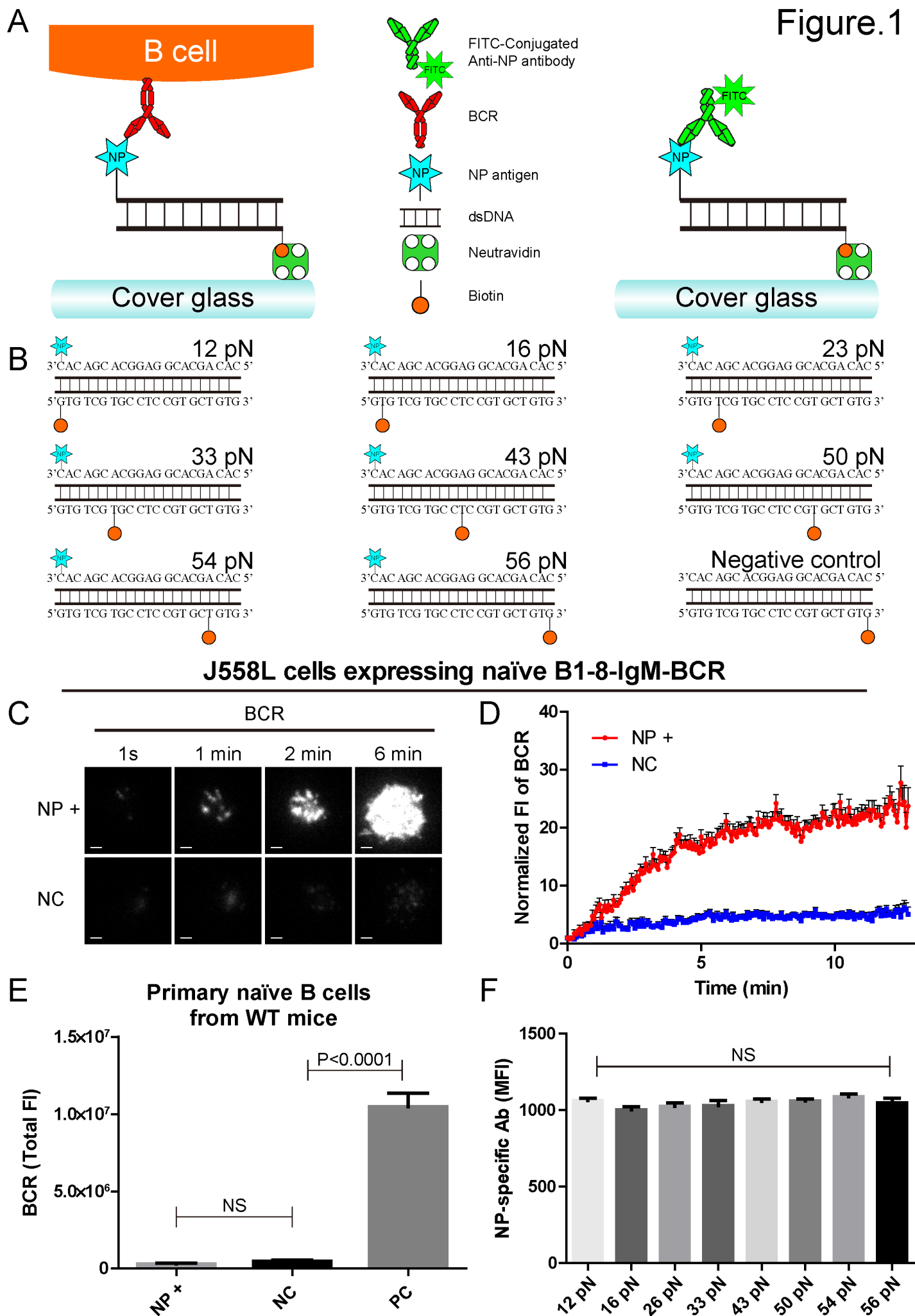
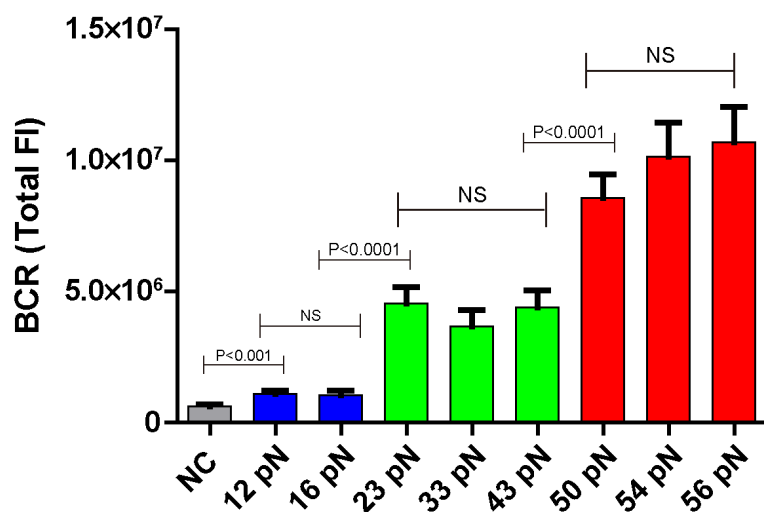
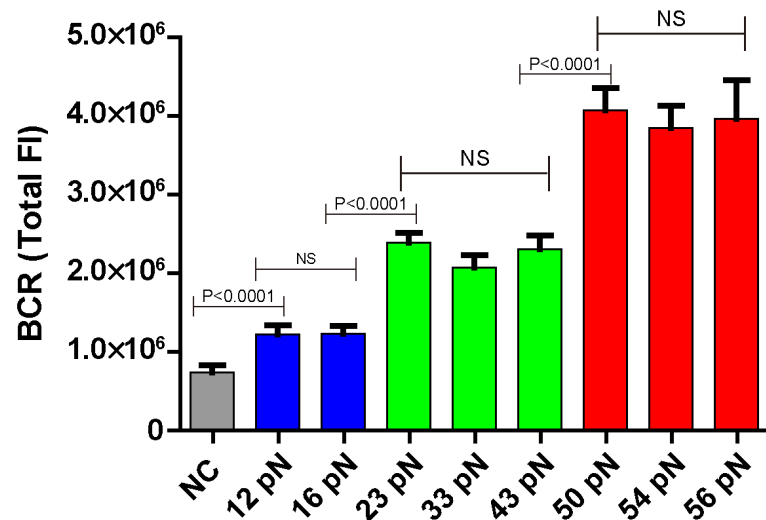


Figure. 2

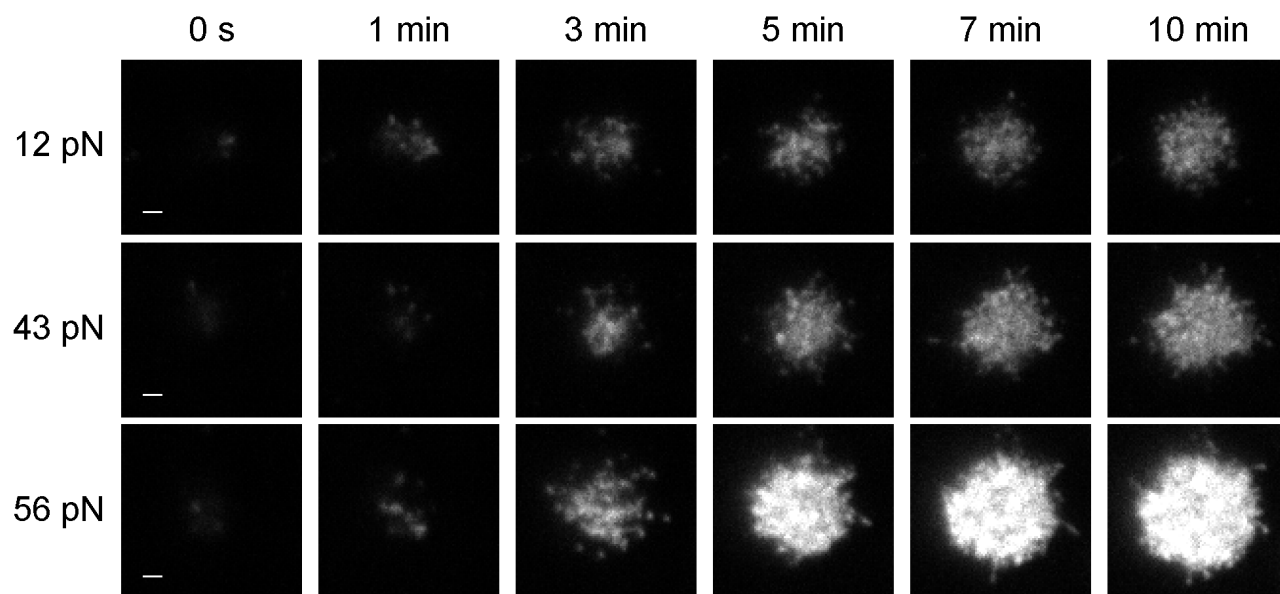
A

J558L cells expressing naïve B1-8-IgM-BCR

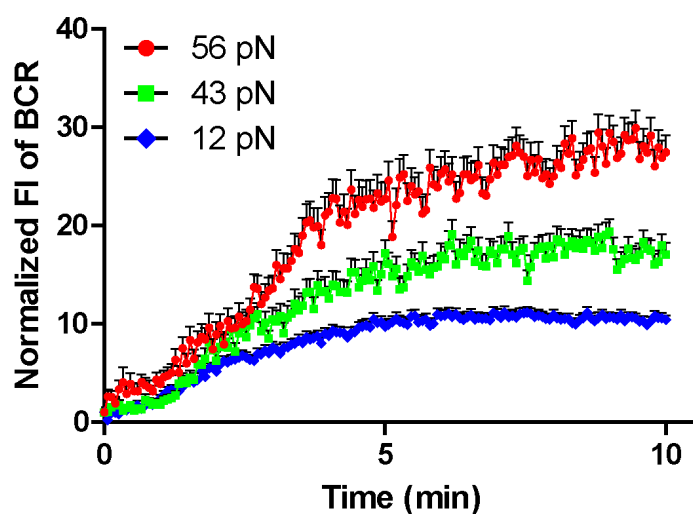
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Primary naïve B cells expressing B1-8-IgM-BCR**J558L cells expressing naïve B1-8-IgM-BCR**

C



D



E

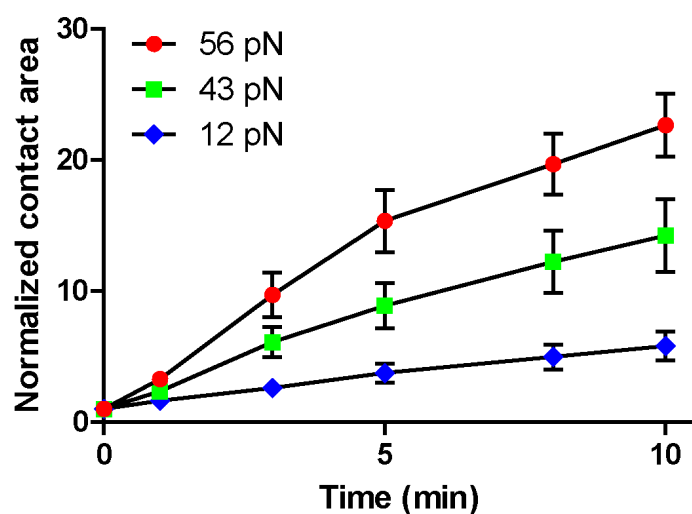
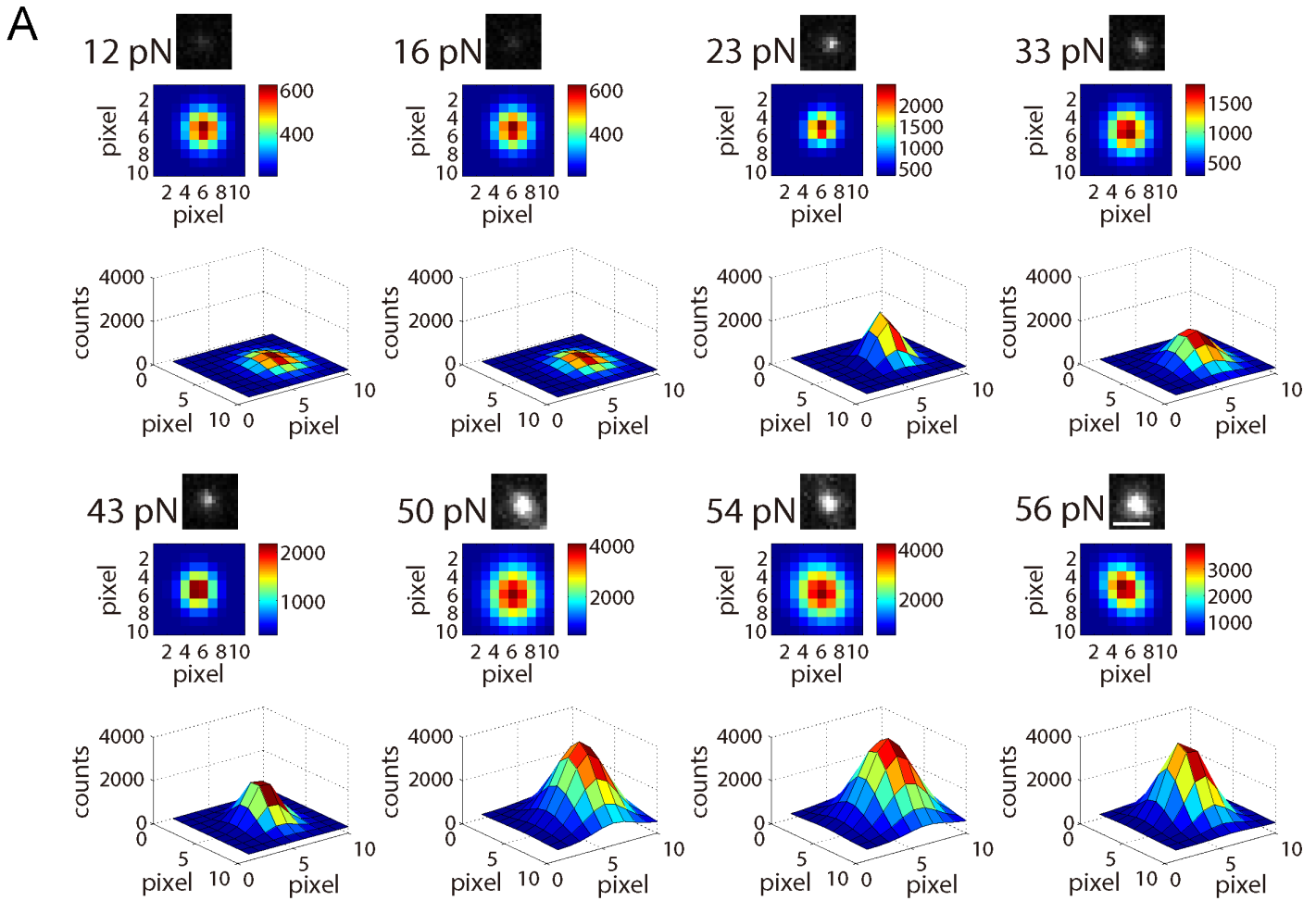
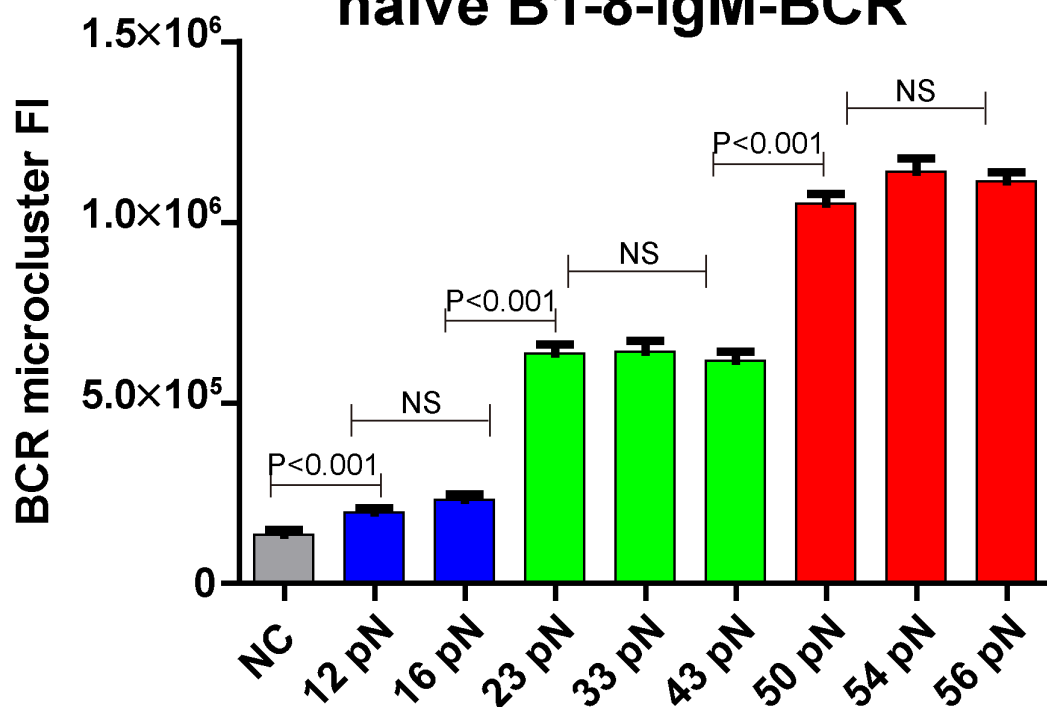


Figure.3



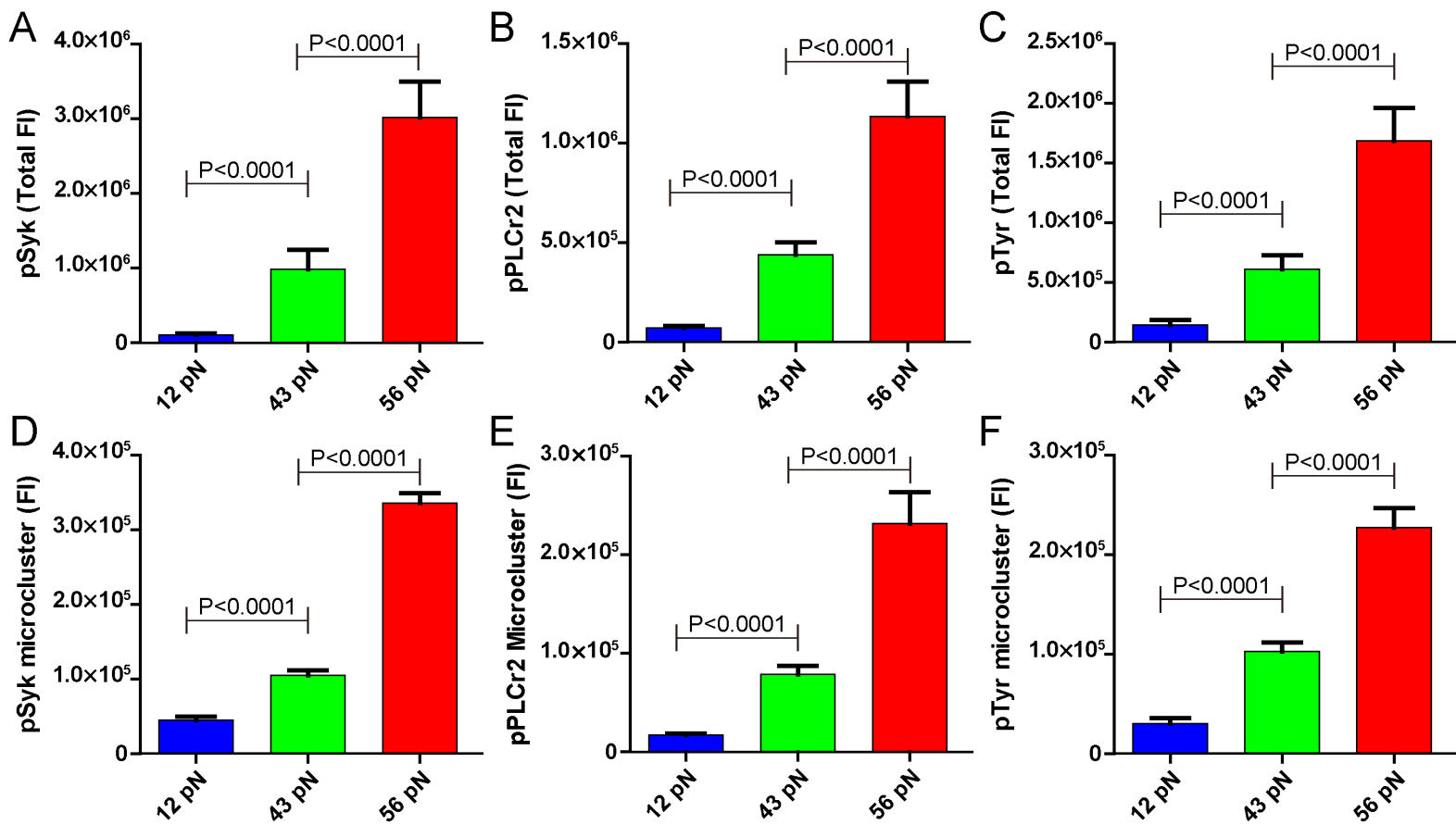
B

**J558L cells expressing
naïve B1-8-IgM-BCR**



Primary naïve B cells expressing B1-8-IgM-BCR

Figure. 4



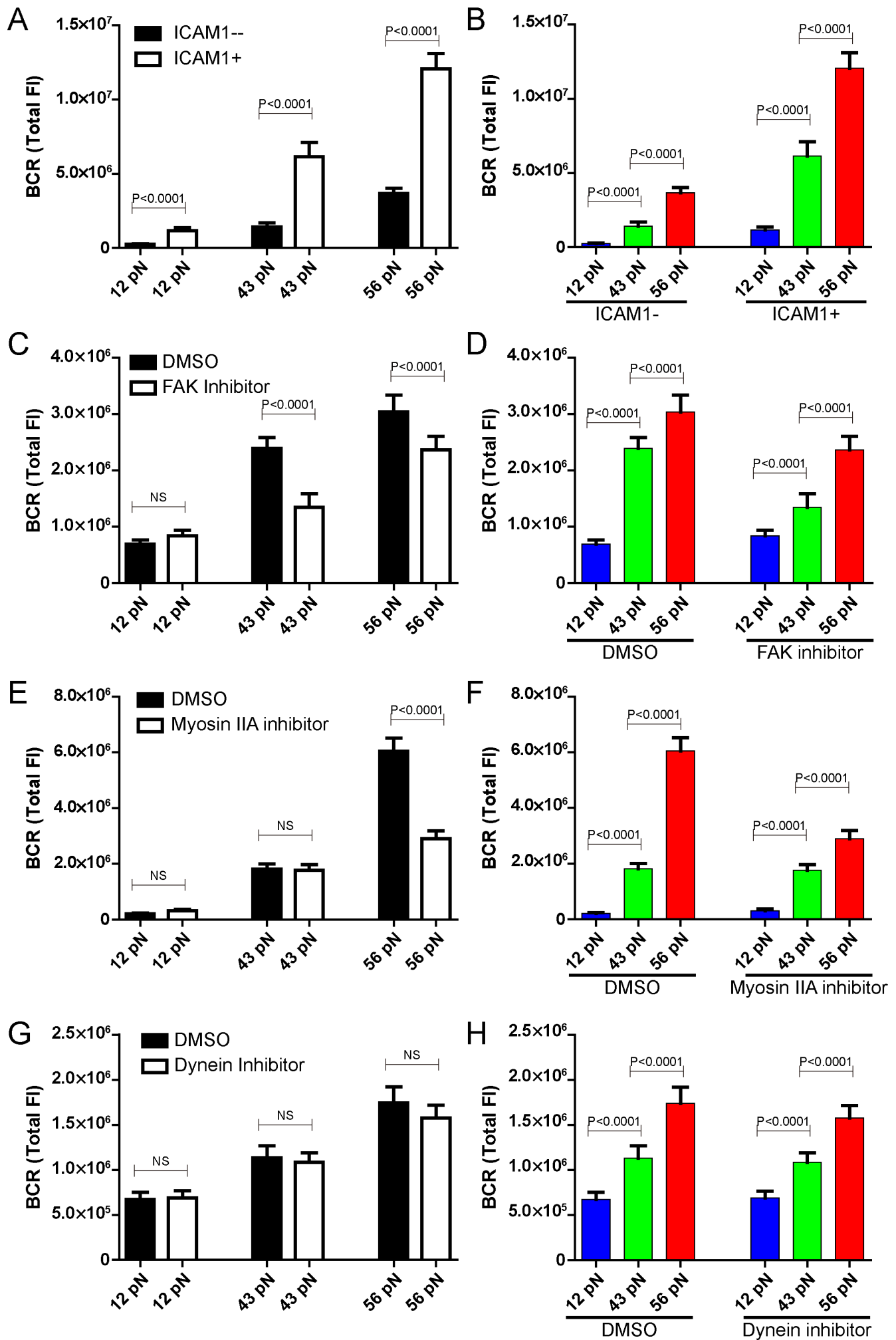


Figure.6

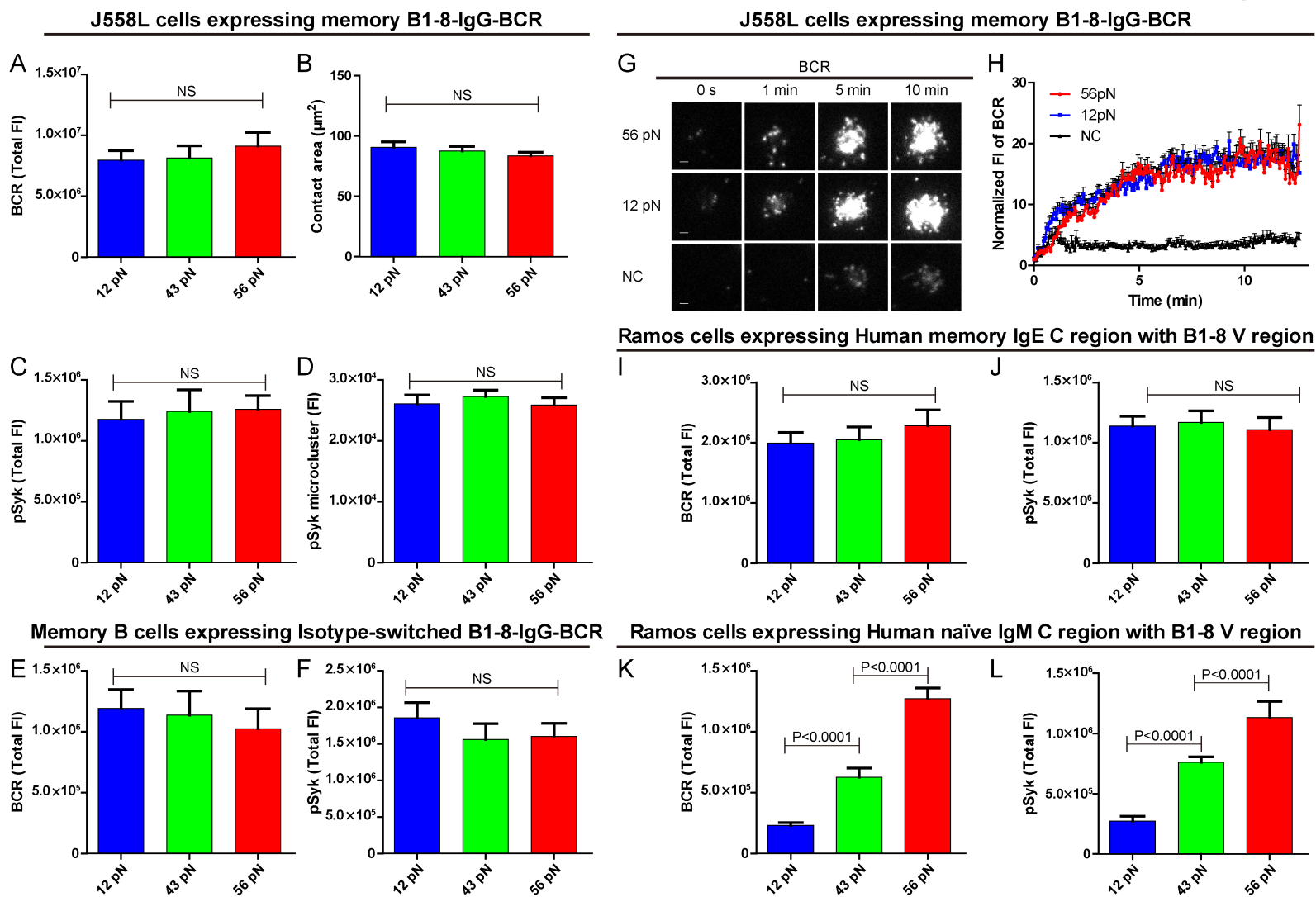


Figure.7

