Because we were not always able to find specific values for some of our model's parameters in the literature, and because some parameters, such as antigen concentration, are varied in B-cell activation experiments, we also performed parametric studies to evaluate the effect of those parameters on our model's results. In addition to the initial number of antigen molecules, A_0, we varied the number of Lyn and Syk molecules (L_0 and S_0), the probability of Lyn binding to and unbinding from Ig-α or Ig-β, $p_{on(Lyn)}$ and $p_{off(Lyn)}$, as well as the ratio $P_{A(Lyn)}=p_{on(Lyn)}/p_{off(Lyn)}$, the probability of Syk binding to and unbinding from Ig-α or Ig-β, $p_{on(Syk)}$ and $p_{off(Syk)}$, and the phosphorylation probability of Ig-α by Lyn ($p_{phos(Ig\alpha)}$), of Ig-β by Lyn ($p_{phos(Ig\beta)}$), and of Syk by Ig-α or Ig-β ($p_{phos(Syk)}$). In the case of Syk kinetics, because we have found a literature value for the affinity of Syk binding to Ig-α/-β ($K_A=10^6\ M^{-1}$),[39] we keep the ratio $P_{A(Syk)}$ constant ($P_{A(Syk)}=10^2$) while varying $p_{on(Syk)}$ and $p_{off(Syk)}$ in tandem.

In Supplementary Figure 1, we show the effect of setting the initial number of antigen molecules to $A_0=20$. This number corresponds to a concentration of the order of 10 molecules/μm^2, which is the lower end used in B-cell activation experiments.[7] The remaining parameter values are identical to those in Table 1. In comparison to Figure 3 ($A_0=200$), the number of bound antigen is approximately an order of magnitude less and thus scales linearly with changes in antigen number. The numbers for pBCR and aSyk are lower than those of Figure 3 but do not scale linearly with A_0. Importantly, although the numbers for bound antigen, pBCR and aSyk are different, the pattern is identical to that of Figure 4, i.e., monotonic decrease for $\mu=0$, non-monotonic for $\mu=1$ s and monotonically increasing at $\mu=10$ s, leveling off at high affinity, and pBCR=aSyk=0 for $K_A=10^5\ M^{-1}$. Similarly, when we set the number of antigen molecules at $A_0=2000$ (Supplementary Figure 2), the number of BCR–antigen complexes increases significantly (though not linearly, as the number of BCR–Ag complexes is limited by the number of BCRs $B_0=400$); however, the pattern remains broadly the same: affinity discrimination is only achieved with threshold time $\mu=10$ s. By symmetry, varying the number of BCR molecules (B_0) while keeping A_0 constant would also not qualitatively alter the results.

In Supplementary Figure 3, we have set the number of Lyn molecules $L_0=1$ and kept the remaining parameter values the same as Table 1, with $A_0=200$. Again, the number of pBCR and aSyk molecules is lower than in Figure 4 ($L_0=100$), but the pattern is identical. In this instance, the number of pBCR and aSyk does not scale linearly with changes in Lyn number. It is interesting to note that a single Lyn molecule can generate a non-negligible number of phosphorylated BCR ITAMs and activated Syk molecules.

In Supplementary Figure 4, we have set the number of Syk molecules to $S_0=100$ and kept the remaining parameter values the same as in Table 1, with $A_0=200$ and $L_0=100$. Once again, the pattern is qualitatively identical to Figure 4 ($S_0=400$). The number of aSyk scales linearly with changes in the number of Syk molecules, while upstream quantities (bound antigen and pBCR) are unaffected. We thus see that changing the concentration of antigen, Lyn and Syk molecules does not qualitatively change the pattern of affinity discrimination, although it does change the numerical values of bound antigen, pBCR and aSyk.

This has important implications with respect to the boundary conditions used. The zero net flux boundary conditions we use in our model do not allow for a gradual increase in reactants within the simulation domain due to diffusional trapping of reactants at the center (due to the lower p_{diff} of receptor–ligand complexes and BCR signalosomes compared to free molecules). If we were to include such diffusional trapping, we would expect the number of BCR, antigen, Lyn and Syk molecules in the simulation domain to gradually increase over time. However, as we show that the concentration of reactants has no effect on affinity discrimination, even though we vary the number of antigen by orders of magnitude (from $A_0=200$ in the main text, to $A_0=20$ and $A_0=2000$ here), there is no change in the affinity discrimination pattern. This is also the case when we vary the number of Lyn and Syk molecules. Although we do not show it, the same applies for variation in the number of BCR molecules. Thus, even if we were to remove the zero net flux boundary conditions and include diffusional trapping, the relatively small and gradual increase in the number of reactants due to diffusional trapping would have no effect on affinity discrimination and on the conclusions of our manuscript.

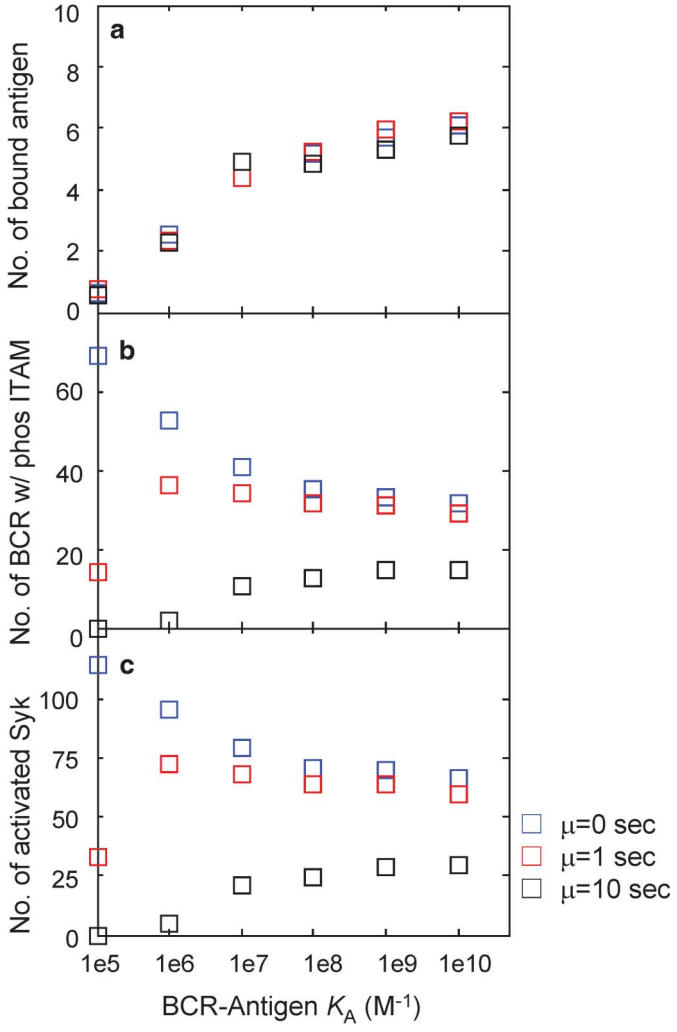
Varying $p_{on(Lyn)}$, $p_{off(Lyn)}$, as well as their ratio $P_{A(Lyn)}$, has a very weak effect on the number of pBCR and aSyk and the affinity discrimination pattern remains unaffected, as seen in Supplementary Figure 5. Even though we vary Lyn affinity two orders of magnitude above and below $K_A=10^6\ M^{-1}$, the effect on the number of pBCR and aSyk is minimal (Supplementary Figure 5a, b, d and e). Similarly, keeping Lyn affinity constant at $P_{A(Lyn)}=100$ while varying $p_{on(Lyn)}$ and $p_{off(Lyn)}$ in tandem has virtually no discernible effect on the results (Supplementary Figure 5c and f). In Supplementary Figure 6, we show that varying $p_{on(Syk)}$ and $p_{off(Syk)}$ in tandem while keeping their ratio $P_{A(Syk)}$ also does not change the affinity discrimination pattern, with only the number of aSyk being affected, and weakly at that.

Varying the phosphorylation probabilities $p_{phos(Ig\alpha)}$, $p_{phos(Ig\beta)}$ and $p_{phos(Syk)}$, either together or individually, has no discernible effect on the affinity discrimination pattern, and the effect on the values of pBCR and aSyk is minimal (not shown). This is because when a Lyn or Syk molecules is attached to Ig-α/-β, a phosphorylation trial is carried out every time an Ig-α/-β with Lyn or Syk bound to it is sampled. Given that $p_{off(Lyn)}$ and $p_{off(Syk)}$ are set to 0.01 (Table 1), this implies a mean bond lifetime of 100 time steps. This implies an average of 100 phosphorylation trials for each Ig-α or Ig-β with a Lyn attached, and for each Syk attached to an Ig-α or Ig-β. Consequently, this greatly dampens the effect of $p_{phos(Ig\alpha)}$, $p_{phos(Ig\beta)}$ and $p_{phos(Syk)}$.

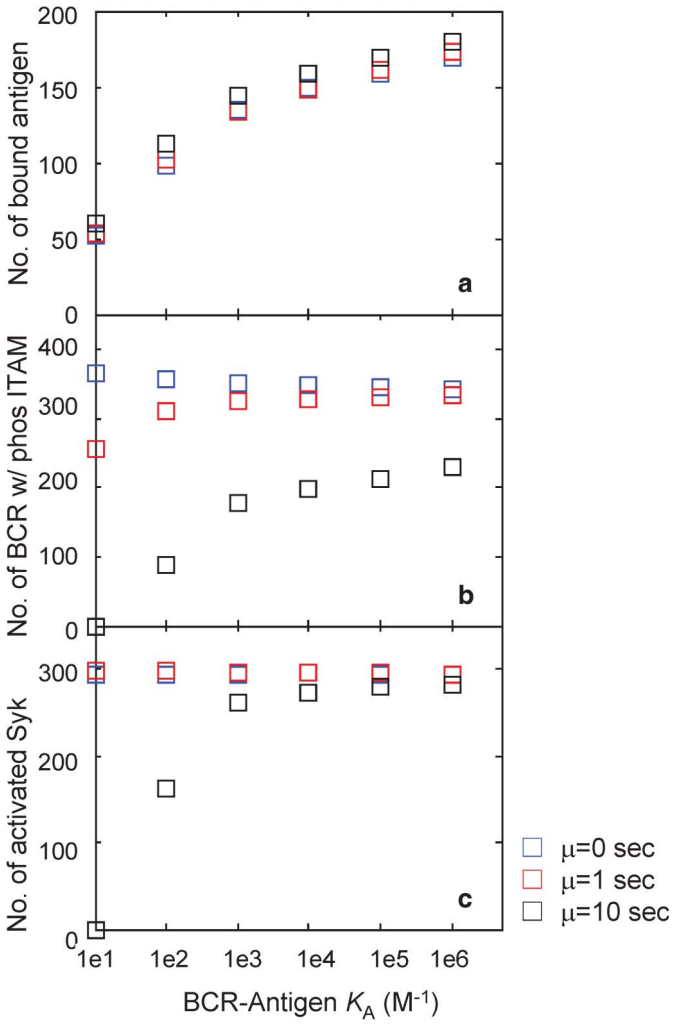
Taken together, the above results indicate that our model's behavior is relatively immune to variations in the values of those parameters that we have not been able to find literature values for. Particularly important is that the affinity discrimination pattern is qualitatively unaffected by variations in the values of the parameters mentioned above, even though the numerical values of pBCR and aSyk do change.

Lastly, we investigate the effect of reversible receptor modifications. In all the preceding results, both here and in the main manuscript, we assumed that once a BCR binds antigen long enough to satisfy the threshold time requirement to become capable of binding Lyn, the modification was irreversible. Even if the antigen subsequently detached, the BCR would remain signaling capable for the duration of the simulation. In Supplementary Figure 7, we investigate what happens if the BCR loses the ability to bind Lyn if the antigen detaches, i.e., receptor modification is reversible. Lyn can thus only bind to BCR that both satisfy the threshold time requirement and have antigen bound to them. As can be seen in Supplementary Figure 7, changing this model assumption has a minimal effect on the results of our model. For threshold time $\mu=0$ s, the number of pBCR no longer decreases monotonically with affinity, but it is still impossible to discriminate between affinity values. For $\mu=1$ s and $\mu=10$ s, the only difference between the results of Supplementary Figure 7 and those of Figure 4 in the main text is that the number of pBCR molecules is lower than in Figure 4 for all affinity values (which follows from the fact that

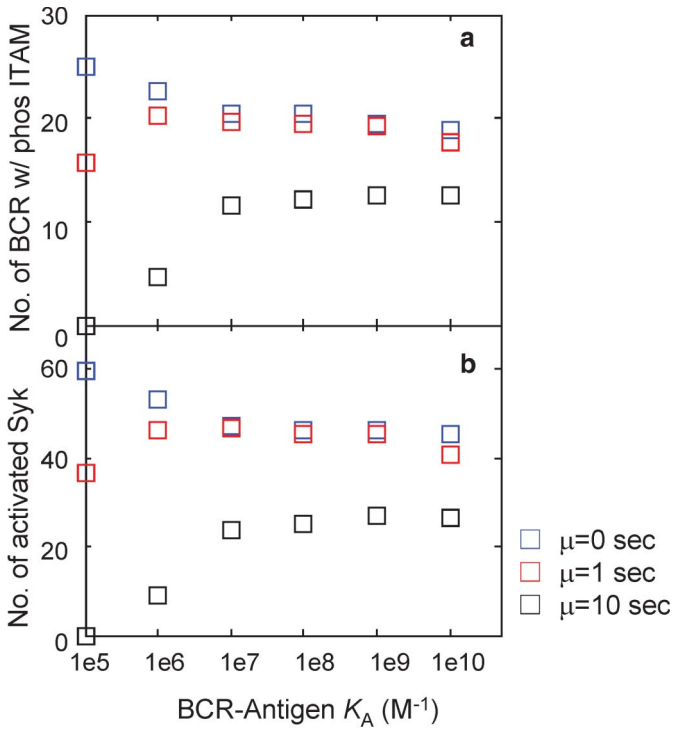
from the set of all BCRs that satisfy the kinetic proofreading requirement, only the subset that has bound an antigen can bind Lyn), but the qualitative behavior is identical. It is only possible to distinguish between affinity values with threshold time $\mu=10$ s. The results for aSyk in Figure 7 are identical to those of Figure 4, which follows from the fact that the requirement for bound antigen only applies to Lyn binding with BCR, not Syk. As long as the Ig-α or Ig-β are phosphorylated, Syk can bind to them, regardless of whether the BCR has bound an antigen. Thus, there is no difference between the two figures for the rules governing Syk binding, and the histograms are identical.



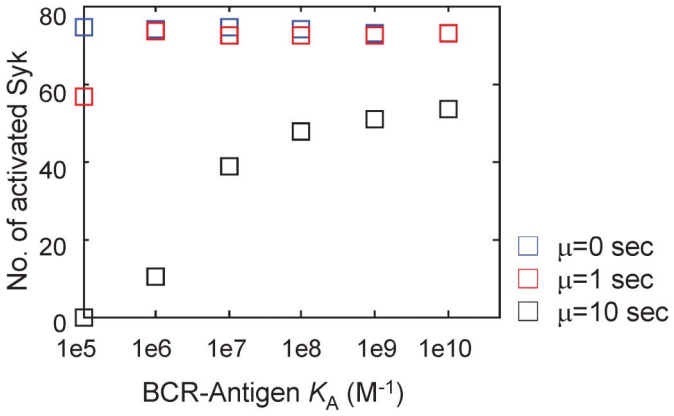
Supplemental Figure 1 Effect of A_0=20 molecules on the mean number of bound antigen (**a**), pBCR (**b**) and aSyk (**c**) molecules. In this set of *in silico* experiments, the initial number of antigen molecules is set to A_0=20 molecules (compared to A_0=200 in the main text), which approximately corresponds to a concentration of 10 molecules/μm^2. The remaining parameter values are identical to those in Table 1. The affinity discrimination pattern is identical to that of Figure 4 of the main text, even though the number of bound antigen, pBCR and aSyk molecules are different from Figure 4. Affinity discrimination is only achieved with threshold time μ=10 s. BCR, B-cell receptor; ITAM, immunoreceptor tyrosine-based activation motif.



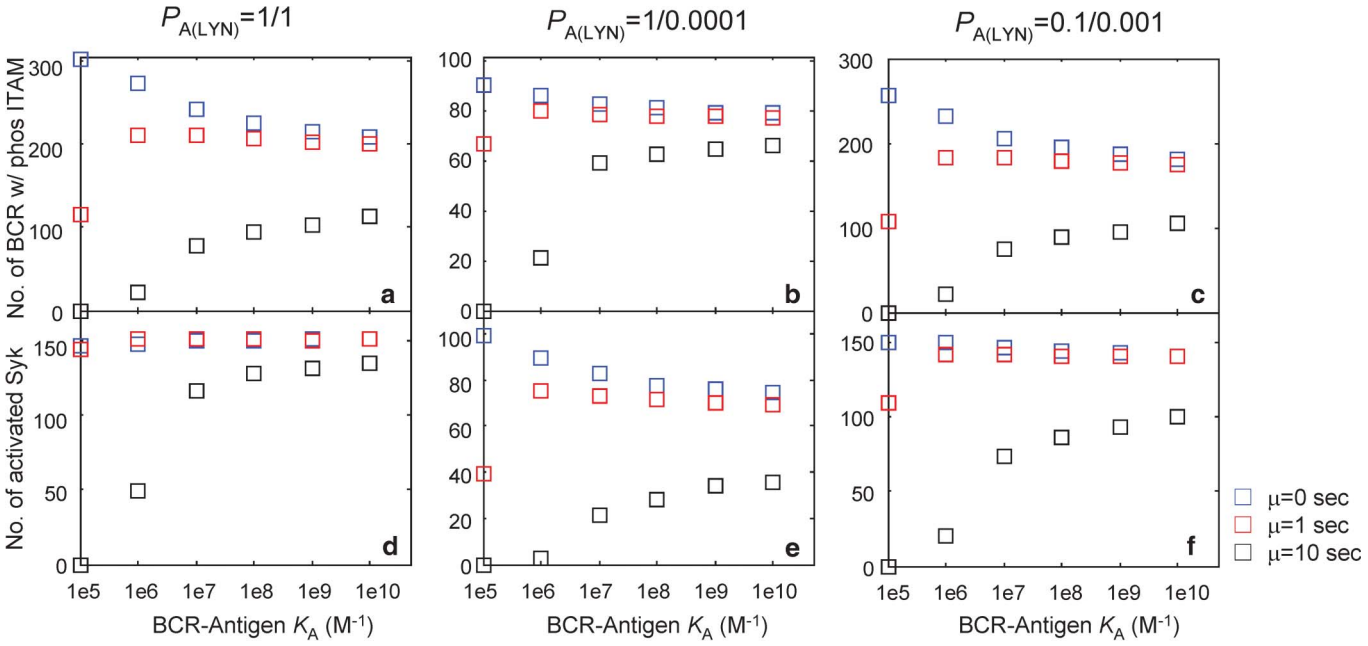
Supplemental Figure 2 Effect of A_0=2000 molecules the concentration of antigen on the mean number of bound antigen (**a**), pBCR (**b**) and aSyk (**c**) molecules. In this set of *in silico* experiments, the initial number of antigen molecules is set to A_0=2000 molecules (compared to A_0=200 in the main text), which approximately corresponds to a concentration of 1000 molecules/μm^2. The remaining parameter values are identical to those in Table 1. The affinity discrimination pattern is broadly similar to that of Figure 4 of the main text, even though the number of bound antigen, pBCR and aSyk molecules are different from Figure 4. Affinity discrimination is only achieved with threshold time μ=10 s. BCR, B-cell receptor; ITAM, immunoreceptor tyrosine-based activation motif.



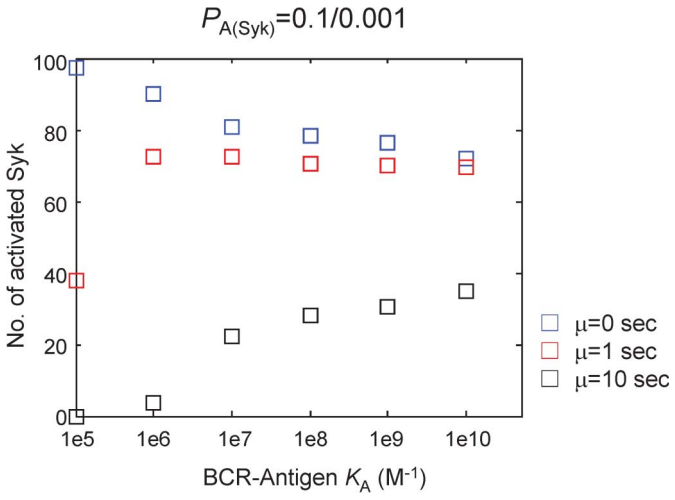
Supplemental Figure 3 Effect of varying the concentration of Lyn on the mean number of pBCR (**a**) and aSyk (**b**) molecules. Antigen binding occurs upstream of Lyn binding; hence, the number of bound antigen molecules is unaffected and not shown. In this set of *in silico* experiments, the initial number of Lyn molecules is set to $L_0=1$ molecule (compared to $L_0=100$ in the main text), with the remaining parameter values the same as Tab le 1 and $A_0=200$. We note that the affinity discrimination pattern is similar to that of Figure 4 of the main text, and that even a single Lyn molecule can generate non-negligible numbers of signaling-active molecules. BCR, B-cell receptor; ITAM, immunoreceptor tyrosine-based activation motif.



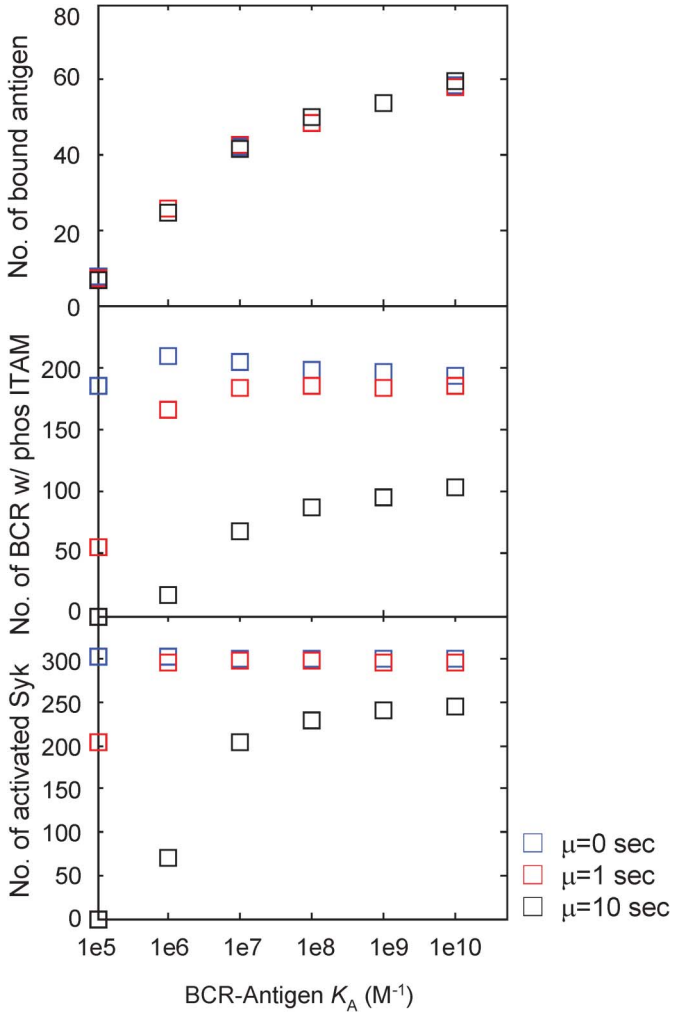
Supplemental Figure 4 Effect of varying the concentration of Syk on the mean number of aSyk molecules. In this set of *in silico* experiments, the initial number of Syk molecules is set to $S_0=100$ molecules, with the remaining parameter values the same as in Table 1, and $A_0=200$, and $L_0=100$. The affinity discrimination pattern is the same as in Figure 4 of the main text, even though the number of aSyk is different. BCR, B-cell receptor.



Supplemental Figure 5 Effect of varying Lyn kinetics on the number of pBCR (**a**–**c**) and aSyk (**d**–**f**) molecules. In **a** and **d**, the affinity of Lyn for Ig-α/β is set to $K_A=10^4\ M^{-1}$, two orders below the value of $K_A=10^6\ M^{-1}$ used in Table 1. In **b** and **e**, the affinity of Lyn for Ig-α/β is set to $K_A=10^8\ M^{-1}$, two orders above the value of $K_A=10^6\ M^{-1}$ used in Table 1. In **c** and **f**, the affinity of Lyn is set to $K_A=10^6\ M^{-1}$ as in Table 1, but the values of $p_{on(Lyn)}$ and $p_{off(Lyn)}$ are set to $P_{A(Lyn)}$=0.1/0.001, in contrast to $P_{A(Lyn)}$=1/0.01 used previously. In all cases, the affinity discrimination pattern is identical to that seen in Figure 4 of the main text, indicating that B-cell affinity discrimination is largely independent of Lyn kinetics. BCR, B-cell receptor; ITAM, immunoreceptor tyrosine-based activation motif.



Supplemental Figure 6 Effect of varying Syk kinetics on the number of aSyk molecules. The affinity of Syk is set to the literature value of $K_A = 10^6\ M^{-1}$ used in Table 1, but the values of $p_{on(Syk)}$ and $p_{off(Syk)}$ are set to $P_{A(Syk)}$=0.1/0.001, in contrast to $P_{A(Syk)}$=1/0.01 used in the main manuscript. The affinity discrimination pattern observed is identical to that of Figure 4 of the main text, indicating that B-cell affinity discrimination is not dependent on Syk kinetics. BCR, B-cell receptor.



Supplemental Figure 7 Effect of reversible receptor modification. In this figure, the parameter values are identical to those of Figure 4 in the main text; however, Lyn can only bind BCRs that satisfy both the kinetic proofreading requirement and the requirement that they have bound at least one antigen. The number of pBCR is generally lower than that in Figure 4, though the qualitative behavior is roughly the same. It is only possible to discriminate between affinity values for threshold time μ=10 s. The values for aSyk are identical to those of Figure 4, as the bound antigen requirement only applies to Lyn binding, not Syk. BCR, B-cell receptor; ITAM, immunoreceptor tyrosine-based activation motif.