High surface expression of PD-1 has also been found to be a hallmark of T cell exhaustion, where antigen-specific CD8+ T cells lose their ability to combat tumor cells or virus-infected cells (4, 5). Antibody blockade of the PD-1 pathway is able to restore the effector functions of exhausted CD8+ T cells for tumor or viral clearance, and this approach is emerging as a promising immunotherapeutic strategy to treat a wide range of cancer and infectious diseases, e.g. nivolumab and pembrolizumab among others in the pipeline (3, 6). In addition, the more complex functions of PD-1 are evidenced by its important role in the generation and activity of induced regulatory T cells (7–9), its high expression on follicular helper CD4+ T cells (10), the improved cognitive performance in an Alzheimer’s mouse model with PD-1 blockade (11), and its expression and growth-promoting effect on certain tumor cells (12).

PD-1 is a type I transmembrane glycoprotein with a single IgV domain in the extracellular region and two tyrosine-based signaling motifs in the cytoplasmic tail: an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif. The potent inhibitory effect of PD-1 relies on the phosphorylation of the immunoreceptor tyrosine-based switch motif and subsequent recruitment of SHP-2, which attenuates TCR or B cell receptor (BCR) proximal signaling (13). The two ligands, PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC), are both type I transmembrane glycoproteins, each consisting of an IgV and an IgC domain with high similarities to other B7 family proteins (14–17). Although both ligands inhibit T cell function in vitro upon binding to PD-1, their in vivo effects are largely governed by their distinct expression patterns, with PD-L1 universally expressed, whereas PD-L2 is restricted to activated antigen-presenting cells (13). Interest-
In situ ligand interactions of murine PD-1

We also characterized the in situ interactions of mPD-1 expressed on the CHO cell surface with mPD-ligands coated on
RBCs (Fig. 2, C and D, Table 1). In contrast to hPD-1, mPD-1 binds to both ligands in situ with indistinguishable affinities: 1.69 ± 0.51 × 10⁻⁴ and 1.63 ± 0.34 × 10⁻⁴ μm⁴ for mPD-L1 and mPD-L2, respectively (Fig. 2E). However, the same Aₖₛ is composed of distinct kinetic rates: mPD-1 binds to mPD-L2 with a 2-fold faster Aₖₕ on and dissociates ~2-fold faster than mPD-L1 (Fig. 2F and G). Both in situ and solution measurements report lower ligand-binding affinities for mPD-1 than hPD-1 (Fig. 2E) (20). The murine to human Aₖₛ difference is 2.65-fold for PD-1/PD-L1 but 13-fold for PD-1/PD-L2 interactions (Fig. 2E). The reduced in situ affinities are largely due to the slower on-rate for mPD-L1 (5.46 ± 1.33 × 10⁻⁵ versus 2.24 ± 0.17 × 10⁻⁴ μm⁴ s⁻¹) and to both the slower on-rate (1.59 ± 0.40 × 10⁻⁴ versus 5.55 ± 0.23 × 10⁻⁴ μm⁴ s⁻¹) and faster off-rate (0.97 ± 0.05 s versus 0.30 ± 0.07 s) for mPD-L2, compared with the human counterparts.

In situ interactions of PD-L1 with B7-1

PD-L1 has been reported to interact with B7-1 and deliver inhibitory signals bidirectionally (22, 26). However, it remains controversial as to how strong this interaction is compared with PD-1/PD-L1 binding (20, 26). To compare their in situ kinetics, we expressed hB7-1 and mB7-1 in CHO cells and tested their binding to PD-L1-coated RBCs. B7-1 has been shown to form non-covalent dimers on the cell surface (38, 39). For both human and murine cases, however, a monomeric binding model (Equation 1) with the same kinetic parameters could be fitted simultaneously to two adhesion frequency curves generated using two sets of molecular densities of B7-1 and PD-L1 (Fig. 3, A and B), suggesting that dimeric binding did not occur under our experimental conditions (40). The in situ affinities are 1.21 ± 0.16 × 10⁻⁵ μm³ for the human interaction and 3.47 ± 1.25 × 10⁻⁴ μm³ for the murine following the same trend as the solution affinities (Fig. 3C, Table 1). The higher Aₖₛ for hB7-1/hPD-L1 is largely because of its 8.3-fold faster on-rate, although its dissociation is more rapid as well (Fig. 3, D and E). Interestingly, B7-1/PD-L1 interactions are much weaker than PD-1/PD-L1 interactions, with 37- and 49-fold Aₖₛ differences for human and murine, respectively. The differences in the in situ parameters are larger than those previously estimated by SPR experiments (20), suggesting that potential restrictions on B7-1/PD-L1 interactions are imposed by the cellular environment. Consistent with this, mPD-L1-coated RBCs generated an adhesion frequency of >50% when tested against activated CD8⁺ T cells from WT P14 mice, whereas negligible binding was observed in cells from PD-1⁻/⁻ P14 mice despite the significant levels of B7-1 expressed by both cells (data not shown).

In situ interactions of human B7-1 with CD28 and CTLA-4

To better orient the in situ kinetics of the PD-1-ligand interactions, we analyzed the in situ interactions of hB7-1 with...
**Table 1**

| Interaction       | CHO      | Purified | $A_{\text{off}} K_{\text{on}}$  | $k_{\text{off}}$ | $A_{\text{off}} K_{\text{on}}$ |
|-------------------|----------|----------|---------------------------------|-----------------|---------------------------------|
| hPD-1/PD-L1       | hPD-1    | hPD-L1   | $4.74 \pm 0.30 \times 10^{-4}$ | $0.50 \pm 0.03$ | $2.24 \pm 0.17 \times 10^{-4}$ |
| hPD-1/PD-L2       | hPD-1    | hPD-L2   | $2.12 \pm 0.56 \times 10^{-3}$ | $0.30 \pm 0.07$ | $5.55 \pm 0.23 \times 10^{-4}$ |
| hB7–1/PD-L1       | hB7-1    | hPD-L1   | $1.21 \pm 0.16 \times 10^{-4}$ | $1.31 \pm 0.35$ | $1.52 \pm 0.22 \times 10^{-5}$ |
| hB7–1/CD28        | hB7-1    | hCD28    | $2.68 \pm 0.05 \times 10^{-4}$ | $1.41 \pm 0.20$ | $3.78 \pm 0.53 \times 10^{-4}$ |
| hB7–1/CTLA-4      | hB7-1    | hCTLA-4  | $1.63 \pm 0.21 \times 10^{-4}$ | $0.70 \pm 0.02$ | $1.13 \pm 0.001 \times 10^{-4}$ |
| mPD-1/PD-L1       | mPD-1    | mPD-L1   | $1.69 \pm 0.51 \times 10^{-4}$ | $0.33 \pm 0.02$ | $5.46 \pm 1.33 \times 10^{-5}$ |
| mPD-1/PD-L2       | mPD-1    | mPD-L2   | $1.63 \pm 0.34 \times 10^{-4}$ | $0.97 \pm 0.05$ | $1.59 \pm 0.40 \times 10^{-4}$ |
| mB7–1/PD-L1       | mB7-1    | mPD-L1   | $3.47 \pm 1.25 \times 10^{-4}$ | $0.51 \pm 0.03$ | $1.84 \pm 0.74 \times 10^{-4}$ |

**Figure 2.** *In situ* and solution kinetics of human and murine PD-1-ligand interactions. A–D, adhesion frequency curves of CHO cells expressing hPD-1 (A and B) or mPD-1 (C and D) tested against RBCs coated with PD-L1 (A and C) or PD-L2 (B and D) of the respective species. Different curves represent independent measurements with different receptor and ligand densities. E–G, *in situ* effective affinity ($A_{\text{off}} K_{\text{on}}$, E) *in situ* off-rate ($k_{\text{off}}$, F), and *in situ* on-rate ($A_{\text{off}} K_{\text{on}}$, G) were derived from data shown in A–D using Equations 1 and 2. The corresponding solution kinetics were replotted from published SPR measurements using the same protein constructs (20). Data represent the mean ± S.D. of measurements in A and B.
CD28 and CTLA-4, the best-studied interactions in the B7-CD28 family (Fig. 4). CTLA-4 binds to the same ligands as CD28 and antagonizes its costimulatory signaling via multiple mechanisms (41). The kinetic basis manifests as a 10-fold higher solution affinity for B7-1/CTLA-4 (42), which is further enhanced by bivalent binding on the cell membrane (43, 44). The in situ affinities follow the same trend but display a 60-fold difference (2.68 × 10^−4 versus 1.63 × 2.0 × 10^−7 μm^2; Fig. 4C). Given that low densities of B7-1 were used to reduce dimerization on the membrane, the much larger difference in in situ affinity versus solution affinity suggests that the in situ interactions of these proteins are highly differentially modulated by the cellular environment, i.e., native B7-1 is much less favored for binding to CD28 than CTLA-4, potentially due to the membrane constraints (e.g., orientation on the membrane) missed in solution measurement. Although the difference in solution affinity was largely attributed to the smaller k<sub>off</sub> of B7-1/CTLA-4, the in situ off-rates are different by only 2-fold (Fig 4D). Instead, the in situ on-rate of B7-1/CTLA-4 binding is 50-fold higher than that of B7-1/CD28 binding (1.13 ± 0.001 × 10^−2 versus 3.78 ± 0.53 × 10^−4 μm^4s<sup>−1</sup>; Fig. 4E), accounting for the 60-fold higher in situ affinity of CTLA-4.

**Simulation of complex formation at the T cell/DC interface**

Due to the complex nature of the PD-1 and B7-1 network of interactions, the net outcome will depend on the types of complexes formed by each receptor against a background of competition for ligands. To gain quantitative insights into the behavior of this system, we simulated molecular complex formation involving PD-1 and B7-1 on T cells with PD-L1 and PD-L2 on DC coupled with molecular diffusion of proteins between contacting and non-contacting membrane compartments. The equations and parameter are the same as previously reported (20) except that the kinetic parameters only were used in the present study. Examining the fraction of individual molecular complexes formed reveals the fast establishment (within 30 s) of a steady state over time wherein PD-1/PD-L1 becomes the dominant interaction species (79%) despite its ~3-fold lower in situ affinity than PD-1 for PD-L2 (Fig. 5A). The dominance of PD-L1 is largely explained by its 15-fold higher (80,372 versus 5,243 molecules/cell) expression level versus PD-L2 on mature DC (20). Steady-state analysis wherein the number of PD-L1 or PD-L2 on the DC is varied further reveals a range between 2,000 and 200,000 molecules/cell where the fractions of PD-1/PD-L1 and PD-1/PD-L2 complexes changes dramatically (Fig. 5, B and C). This range is physiologically feasible particularly for PD-L1 (20, 45), suggesting that the contributions of PD-L1 versus PD-L2 to complex formation is highly regulated by their expression dynamics. In contrast, increasing B7-1 expression on the T cell did not significantly affect the fraction of PD-1/PD-L1 bonds until reaching a level of >20,000 molecules/cell (Fig. 5D), which is much higher than the estimated level of <4,000 molecules/cell (46).

**Discussion**

The critical role of PD-1 in maintaining peripheral T-cell tolerance and its key suppressive effect on exhausted T cells have made it a promising therapeutic target for restoring T cell functions in a wide range of cancers and infectious diseases. To better understand the biophysicochemical basis of PD-1’s function, a spatiotemporal map of ligand binding of PD-1 and...
related molecules with specified kinetic properties at the intercellular junction is needed, which have not been reported previously. Here we performed a detailed **in situ** characterization of these kinetics, which combined with the simulation of molecular complex formation at the interface of an activated T cell and a mature DC, provide several insights as discussed below.

Molecular recognition on the T cell membrane is largely governed by the intrinsic properties of the binding sites within a receptor-ligand pair, the net effects on binding of which are usually characterized by SPR analysis or thermodynamic approaches using soluble recombinant polypeptides truncated before the membrane anchor. These measurements in conjunction with structural studies have been invaluable for understanding the binding step of molecular recognition. However, applying the solution-phase results directly to the cellular environment is not necessarily straightforward as at the cell membrane, the orientation, organization, diffusion, and even structures of the proteins are likely to be subject to modulation by the cellular environment. To mimic such physiological situations, we probed the binding to native receptors expressed on the CHO cell membrane with recombinant ligands anchored on the RBC membrane, a configuration better reflecting the representation of the native receptors and a close approximation of the native ligands. We and others have shown in multiple molecular systems that binding kinetics measured **in situ** often differ dramatically from the equivalent solution measurements. For example, the **in situ** kinetics of TCR/pMHC extend over a much wider dynamic range than solution kinetics and correlate better with the functional potency of pMHC ligands (29–34). They are also sensitive to perturbations of the cellular environment such as inhibition of actin polymerization with latrunculin A or disruption of membrane microdomains with cholesterol oxidase (28, 29). Therefore, instead of the intrinsic physicochemical properties of the binding site only, the **in situ** kinetics measured by our assays represent a potentially tunable property of the molecular recognition in the cellular context. The context dependence of such interactions at the very least is illustrated in the molecular interactions measured in this study by the expanded dynamic range of **in situ** affinities compared with their solution counterparts. The solution affinities from the weakest interaction (mB7-1/PD-L1) to the strongest (hB7-1/CTLA-4) spans 200-fold, whereas the **in situ** affinities have a range of almost 4 orders of magnitude (Fig. 6A). Interestingly,
Figure 5. Mathematical simulations of interactions of PD-1, PD-ligands, and B7-1 at the T cell/DC interface. A, fraction of PD-1-PD-L1 and PD-1-PD-L2 complexes over time. B and C, fraction of PD-1-PD-L1 and PD-1-PD-L2 complexes at steady state with varying numbers of PD-L1 (B) or PD-L2 (C) on the mature DC. D, fraction of PD-1-PD-L1, PD-1-PD-L2, and PD-L1/B7-1 complexes at steady state with varying numbers of B7-1 on the activated T cell.

Figure 6. Summary of in situ kinetics of PD-1 and B7-1 interactions. A, correlation of solution affinity with in situ affinity. The solution and in situ affinities for LFA-1/ICAM-1 interaction were from Refs. 36 and 51). B, calculated confinement lengths according to the Bell model ($r = in situ K_d$/solution $K_d$). C and D, summary of in situ on-rate (C) and in situ off-rate (D).
the enhanced dynamic range does not undermine the general agreement between in situ and solution measurements, as the ranking of in situ affinities correlates almost perfectly with that of the solution measurements (Fig. 6A). Neither does the enhancement come from a simple transformation uniformly applied to all interactions. For example, the conversion from solution to in situ affinities according to the method of Bell (47, 48) gives variable confinement lengths, a characteristic length reflecting the volume of the search space for molecular binding on the cell surface (Fig. 6B). Such variation suggests differential perturbation of in situ affinities by the cellular environment. The mechanisms, which are likely complex, may work upon differences in effective molecular length and orientation (48–50). Moreover, it is likely that the in situ on-rate, not off-rate, will be subject to the largest effects. The on-rate governs bond formation and hence depends on processes affecting the encounter rate of the interacting molecules at the intercellular junction, which accounts for the major differences in in situ versus in-solution binding. The off-rate, on the other hand, is determined by the durability of a bond after it forms and, hence, depends to a greater extent on the physicochemical property at the interface of the molecular complex. This line of reasoning is strongly supported by the remarkable correlation between the in situ on-rate and the in situ affinity (Fig. 6C). The in situ on-rate also spans a similar dynamic range as the in situ affinity, whereas the in situ off-rate changes within a 4-fold range (Fig. 6, C and D). Overall, our data emphasize the significance and advantages of in situ kinetic studies of membrane receptor-ligand interactions, which in contrast to the off-rate dominated solution affinities (20, 42) are more highly dependent on on-rate effects that, as suggested by our data, are a potentially tunable property of the in situ interactions.

An important question that has not been fully addressed previously is how strong PD-1-ligand binding as well as PD-L1/ B7-1 interactions are compared with the interactions of antigen receptors and other co-stimulatory and co-inhibitory receptors. With enhanced resolution the in situ kinetics now defines a more precise ranking of such interactions of interest (Fig. 6A). Using the interaction between LFA-1 and ICAM-1 as a reference, the solution $K_d$ is 185 nM when the I-domain of LFA-1 is open, corresponding to the high affinity interaction (51). The solution affinity for the human B7-1/CTLA-4 interaction is moderately lower ($K_d$ = 420 nM). The SPR measurements of bivalent PD-1/PD-ligand interactions (i.e., Fc fusion proteins) yielded $K_d$ values ranging from 0.01 μM to 0.8 μM (22, 24, 26, 27), whereas the monomeric interactions are much weaker ($K_d$ = 2–8 μM) (20). In contrast, the in situ affinity of B7-1/CTLA-4 is 3-fold higher than that of LFA-1/ICAM-1, and PD-1/PD-ligand in situ affinities span the range from intermediate to strong, with the strongest (hPD-1/hPD-L2) comparable to the high affinity LFA-1/ICAM-1 interaction. Similar kinetic advantages for in situ binding of these inhibitory receptors is also evident for comparisons based on another co-stimulatory receptor, CD28, interacting with B7-1; i.e., interactions that are weaker in solution are similar or even stronger on the cell membrane, whereas the differences of stronger interactions become further amplified, giving rise to 2–5-fold lower confinement lengths than CD28 (Fig. 6B). Therefore, the relatively high in situ affinities of PD-1/PD-ligand interactions in comparison to the low solution affinities may provide a better understanding of PD-1’s potent inhibitory function to counter activating signals by the TCR and co-stimulatory receptors such as CD28. On the other hand, the in situ affinities of PD-1-ligand binding are considerably lower than that of CTLA-4, suggesting that distinct mechanisms for their inhibitory effects are at play. This notion is also supported by the fact that CTLA-4 shares the same ligands (B7-1 and B7-2) with the co-stimulatory receptor CD28 and is also evidenced by the different signaling pathways that are thought to act downstream of CTLA-4 and PD-1 (52). Notably, our in situ measurements indicate that both human and mouse B7-1/PD-L1 interactions are much weaker than interactions with their canonical receptors; the $A_{K_d}$ of B7-1/PD-L1 binding is 37–49-fold, 20-fold, or 3-log lower than that of PD-1/PD-L1 binding, B7-1/CD28 binding, or PD-1/CTLA-4 binding, respectively. The differences are much larger than the previous solution measurements, manifesting >10-fold higher confinement lengths (Fig. 6B). Again, the differences seem to have their source from the in situ on-rate instead of off-rate (Fig. 6, C and D), suggesting that these interactions occur less favorably in the cellular context. Accordingly, in silico simulations using these kinetic rates imply that B7-1 will only ever have a negligible effect on the fraction of PD-1/PD-L1 bonds formed at physiological levels of B7-1 expression. These results suggest that these interactions would only be functional when very abundant B7-1 and PD-L1 are present, which then would restrict the significance of this interaction to some particular physiological or pathological conditions in contrast to the broader and more potent effect of PD-1 in maintaining peripheral tolerance. Overall, understanding how these molecules interact in situ provides a basis for delineating this network of interactions under physiological conditions, where the interplay is likely to be affected by expression and affinity-based competition and signaling-based cellular regulation. The in situ kinetic parameters obtained here would also be a useful source for mathematical simulations of membrane receptor-ligand interactions in pharmacodynamics and thus facilitate drug development targeting this network of molecules.

We found distinct binding kinetics for human versus murine PD-1 interactions as well as for PD-L1 versus PD-L2 of both species, as also revealed by solution kinetic studies (20, 24, 27). These differential binding properties may be related to the structural differences reported earlier. Compared with mPD-1, hPD-1 has different positioning of the FG loop and also replaces the C’ loop stand with a flexible loop. Both these regions contribute to ligand binding as shown by an NMR structure (20). The recent structure of a hPD-1/hPD-L1 complex further shows significant plasticity associated with the ligand binding of hPD-1; the CC’ loop that adopts an open conformation in apo-hPD-1 closes upon binding to hPD-L1, whereas the CC’ loop of apo-mPD-1 already displays a closed conformation (21). Also, the two PD-1-ligands may bind to PD-1 via structurally distinct mechanisms. Thermodynamic analysis reveals an entropically driven process for hPD-1/hPD-L1 binding, whereas a large enthalpic term is found for the hPD-1/hPD-L2 interaction (20). All these kinetic and structural differences raise the possibility of differential signaling capacities by PD-L1 versus PD-L2. On
the other hand, their functions could be highly regulated by their distinct expression patterns; PD-L1 is universally expressed, whereas PD-L2 expression is restricted to professional antigen-presenting cells. Combining the kinetic and expression profiles, our model simulations demonstrate that the contribution of individual ligands on DCs in forming molecular complexes with PD-1 on T cell is likely to be wholly dominated by their expression dynamics. This result seems to argue against the non-redundant role of PD-1/PD-L2 interaction unless it triggers distinct signaling outcomes by virtue of its enhanced on-rate and capacity for rebinding and its somewhat slower off-rate. Together these structural and kinetic differences between human and murine PD-1 and their interactions with their two ligands imply that PD-1 is subject to continuous selection pressure during evolution, the optimization of which might be critical for balancing immune tolerance with the challenge of dealing with fast-evolving pathogens.

Experimental procedures

Cells and proteins

CHO cells (ATCC) were cultured in RPMI 1640 (Cellgro) supplemented with 10% FBS (Cellgro), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM HEPES and transfected with pcDNA3.1 encoding hPD-1, mPD-1, or hB7-1 using nucleofection (Lonza). To generate stable cell lines, transfected cells were subjected to G418 selection (0.4 mg/ml) and multiple rounds of FACS sorting for uniform surface receptor expression. CHO mB7-1 cells were a generous gift of Dr. Periasamy Selvaraj (Emory University, Atlanta, GA).

His6-tagged and biotinylatable proteins hPD-L1, hPD-L2, mPD-L1, and mPD-L2 were produced in CHO cells using approaches described previously (42, 53, 54). Biotinylation was performed in vitro using the BirA biotin-protein ligase kit (Avidity).

Protein coating on RBC surface

According to a protocol approved by the Institutional Review Board of the Georgia Institute of Technology, human RBCs were isolated from healthy donors, biotinylated with various concentration of Biotin-X-NHS, functionalized with saturating amount of streptavidin (SA), and washed (29). SA-coated RBCs were then incubated with biotinylated recombinant proteins and washed before adhesion frequency assay or flow cytometric analysis.

Site density measurement

CHO cell transfectants and protein-coated RBCs were stained for 30 min at 4°C in 100 μl of FACS buffer (PBS without Ca2+ or Mg2+, 5 mM EDTA, 2% FBS) with 10 μg/ml PE-conjugated antibodies: anti-hPD-1 (clone MIH4), anti-hPD-L1 (clone MIH1), anti-hPD-L2 (clone MIH18), anti-mPD-1 (clone J43), and anti-mB7-1 (clone 1G10/B7) were from BD Pharmaningen. Anti-mPD-L1 (clone MIH5), anti-mPD-L2 (clone TY25), and anti-hB7-1 (clone 2D10.4) were from eBioscience. Fluorescently labeled cells together with BD QuantiBRITE PE beads were analyzed using the BD LSR II flow cytometer (BD Biosciences). Molecular site densities were calculated following the instructions of QuantiBRITE PE beads with the cell area derived from diameter measured using cell Multisizer (Beckman Counter).

Adhesion frequency assay

The theoretical framework and detailed procedures have been reported previously (29, 35, 55). As shown in Fig. 1, a CHO-expressing PD-1 and a RBC coated with PD-Ligand were repetitively brought in contact for a well defined duration (t_{c}) with a constant contact area (A_{c}). Adhesion frequency (P_{a}) was calculated over scoring 50 contact cycles, with each giving 1 for adhesion or 0 for no adhesion based on the deflection of the RBC membrane upon separation (Fig. 1A). The adhesion frequency curve P_{a} versus t_{c} monotonically increased with contact duration then plateaus, and the P_{a} versus t_{c} curve changed shape with the PD-1-ligand and level with the receptor (m_{r}) and ligand (m_{l}) densities (Fig. 1B). This curve reflects the kinetic nature of the molecular bond formation and dissociation at the cellular interface and can be well fitted to the following equation assuming a single step second-order forward, first-order reverse reaction between monomeric receptor and ligand (35).

\[
P_{a} = 1 - \exp\left(-m_{r}m_{l}K_{a}[1 - \exp(-k_{off}t_{c})]\right) \quad (Eq. 1)
\]

The effective in situ affinity (A_{k_{on}}) and off-rate (k_{off}) were then determined from least-mean-square fitting in conjunction with measurement of receptor and ligand densities using flow cytometry. In situ on-rate was further calculated as

\[
A_{k_{on}} = A_{k_{a}} \times k_{off} \quad (Eq. 2)
\]

Simulations of complex formation in the PD-1/PD-ligands/B7-1 system

The mathematical model for simulating the interactions of PD-1, PD-1-ligands, and B7-1 has been published previously (20). The same set of equations and parameters was used except for the in situ binding kinetics being the numbers measured in this study. Briefly, the model describes the case where an activated T cell expressing PD-1 and B7-1 makes contact with a mature DC with PD-L1 and PD-L2. The molecular interactions at the cell-cell interface and the diffusion across different membrane compartments were integrated into coupled ordinary differential equations. The fraction for each complex in the interface was simulated over time to obtain steady-state values. Analysis of complex fractions at steady-state were examined against varying numbers of PD-L1, PD-L2, or B7-1.

Author contributions—K. L., S. J. D., and C. Z. designed the experiments. K. L. performed most of the experiments. K. L. and C. Z. analyzed the data. X. C. made the recombinant proteins. A. T. performed the in silico simulation.

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