Spatial Regulation of T-Cell Signaling by Programmed Death-Ligand 1 on Wireframe DNA Origami Flat Sheets

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ABSTRACT: Programmed Death-1 (PD-1) is a coinhibitory receptor expressed on activated T cells that suppresses T-cell signaling and effector functions. It has been previously shown that binding to its ligand PD-L1 induces a spatial reorganization of PD-1 receptors into microclusters on the cell membrane. However, the roles of the spatial organization of PD-L1 on PD-1 clustering and T-cell signaling have not been elucidated. Here, we used DNA origami flat sheets to display PD-L1 ligands at defined nanoscale distances and investigated their ability to inhibit T-cell activation in vitro. We found that DNA origami flat sheets modified with CD3 and CD28 activating antibodies (FS-α-CD3-CD28) induced robust T-cell activation. Co-treatment with flat sheets presenting PD-L1 ligands separated by ∼200 nm (FS-PD-L1-200), but not 13 nm (FS-PD-L1-13) or 40 nm (FS-PD-L1-40), caused an inhibition of T-cell signaling, which increased with increasing molar ratio of FS-PD-L1-200 to FS-α-CD3-CD28. Furthermore, FS-PD-L1-200 induced the formation of smaller PD-1 nanoclusters and caused a larger reduction in IL-2 expression compared to FS-PD-L1-13. Together, these findings suggest that the spatial organization of PD-L1 determines its ability to regulate T-cell signaling and may guide the development of future nanomedicine-based immunomodulatory therapies.

KEYWORDS: DNA nanotechnology, DNA origami, PD-1 receptor, cancer immunotherapy, nanoscale spatial distribution
Mechanistic studies using supported lipid bilayers to artificially interrogate the T-cell-APC interface (also known as the immunological synapse) have revealed the formation of PD-1 microclusters on the T-cell membrane upon binding to PD-L1. The PD-1 microclusters partially colocalized with T-cell receptor (TCR) microclusters during initial cell-bilayer contact and subsequently migrated to costimulatory receptor CD28 microclusters, resulting in dephosphorylation of TCR-CD3 and CD28 complexes by recruitment of Src homology region 2 domain-containing phosphatase-2 (SHP-2). While some studies have reported that PD-1 is primarily associated with TCR signaling, others have either shown that CD28 is the preferred target or that both TCR and CD28 are equally inhibited. Furthermore, the costimulatory receptor CD2 has been shown to localize to the outer edge of the immunological synapse and boost TCR signaling, an effect which was dampened by colocalization with ligand-bound PD-1, even in the absence of CD28 engagement. Although the underlying mechanisms are not fully elucidated, these studies support the hypothesis that the spatial organization of PD-1 on the T-cell membrane is a relevant biophysical regulator for PD-1-mediated T-cell suppression. Interestingly, homodimerization of PD-L1 by nonpeptide-based small molecule inhibitors have been reported to block PD-1/PD-L1 interactions and restore TCR activity. However, a secreted PD-L1 alternative splice variant that forms homodimers was more effective in T-cell inhibition than soluble PD-L1 monomers. Therefore, the effects of spatial proximity of PD-L1 ligands on PD-1 mediated inhibition of T-cell signaling remain unclear.

DNA origami nanofabrication presents a robust and programmable approach to position individual proteins with nanoscale precision. TCR activation on bioengineered surfaces commonly utilize antibodies to CD3, a signal transduction component of the TCR complex, for a non-

Figure 1. Production of anti-CD3 IgG, anti-CD28 IgG, and PD-L1–oligo conjugates. (a) A schematic workflow of anti-CD3ε IgG, anti-CD28 IgG, and PD-L1-His conjugations to 3’ azide oligos via copper-free click chemistry and hybridization with complementary fluorescently labeled oligos for fluorescence native PAGE. The antibodies and PD-L1-His were labeled using DBCO-NHS ester and bis-sulfone-PEG₄-DBCO cross-linkers, respectively. (b) Fluorescence native PAGE of (i) anti-CD3 IgG, (ii) anti-CD28 IgG, and (iii) PD-L1–oligo conjugates before and after purification, imaged using Cy5 or SYBR Green channels and then silver stained. Unconjugated proteins and azide—oligos mixed with fluorescently labeled oligos were run as controls (purple and blue arrowheads respectively). The control for fluorescently labeled oligos alone (Fluo-oligo) shows unhybridized fluorescently labeled oligos (gray arrowhead). Black arrowheads indicate protein—oligo conjugate products, in which two PD-L1—oligo conjugate products of one and two oligos attached were formed (upper and lower bands, respectively (iii)).
antigen specific trigger.\textsuperscript{36–39} Costimulation with CD28 antibodies provides a secondary signal for T-cell activation which results in cell proliferation and the production of cytokines such as the key pleiotropic lymphocyte mitogen interleukin-2.
Here, we used the wireframe-scaffolded DNA origami strategy to produce DNA origami flat sheets that remain stable under physiological ionic strengths, unlike DNA nanostructures composed of densely packed DNA helices. These DNA origami flat sheets were functionalized to present either agonistic anti-CD3 antibodies (FS-α-CD3), antibodies against CD3 and CD28 (FS-α-CD3-CD28), or PD-L1 proteins separated by varying nanoscale distances. As a readout of T-cell activation, we analyzed the activity of a luciferase reporter driven by nuclear factor of activated T cells (NFAT) response elements in PD-1-expressing Jurkat T cells. NFAT is a family of transcription factors that are induced following TCR ligation to elicit transcription of genes involved in T-cell activation and development, including IL-2.

We demonstrated that DNA flat sheets presenting two PD-L1 ligands separated by 200 nm (FS-PD-L1-200), but not 13 nm (FS-PD-L1-13) or 40 nm (FS-PD-L1-40), were able to reduce the levels of CD3- and CD3/CD28-triggered NFAT activity. Moreover, dose–response assays showed that NFAT activity levels decreased with increasing concentrations of FS-PD-L1-200. In addition, cell stimulation with FS-PD-L1-200 resulted in smaller PD-1 cluster sizes and decreased IL-2 mRNA expression compared to stimulation with FS-PD-L1-13. Overall, these findings demonstrate that the nanoscale spatial distribution of PD-L1 tunes T-cell signaling.

RESULTS AND DISCUSSION

Synthesis of Anti-CD3 IgG-, Anti-CD28 IgG-, and PD-L1–oligo Conjugates. In order to assemble proteins on DNA origami flat sheets, we conjugated anti-CD3 IgG, anti-CD28 IgG, and recombinant PD-L1-His-tag protein to single-stranded DNA oligos (ssDNA) designed to hybridize to CD28 IgG, and PD-L1 respectively. The protein conjugates were then visualized on either agarose gel electrophoresis to verify hybridization of protein–oligo conjugates revealed that these conjugates were stable under physiological salt conditions.

The antibodies were strangled DNA oligos (ssDNA) designed to hybridize to CD3/CD28-triggered NFAT activity. Moreover, dose–response assays showed that NFAT activity levels decreased with increasing concentrations of FS-PD-L1-200. In addition, cell stimulation with FS-PD-L1-200 resulted in smaller PD-1 cluster sizes and decreased IL-2 mRNA expression compared to stimulation with FS-PD-L1-13. Overall, these findings demonstrate that the nanoscale spatial distribution of PD-L1 tunes T-cell signaling.

Production of Antibody- and PD-L1-DNA Flat Sheets. As a platform for assembling the protein–oligo conjugates, we used a wireframe DNA origami flat sheet design which yields nanostructures that have the capacity to fold and remain stable under physiological salt conditions. The flat sheets were first folded using short ssDNA oligos (staples) which bind complementarily to regions of the p8064 phage ssDNA scaffold. Protein–oligo conjugates were then assembled onto flat sheets via hybridization with complementary staples at designated positions that protrude out of the nanostructure. Each protein binding site consists of a pair of protruding staples within a 3-tessellation triangulated tile to ensure a high yield of hybridized protein–oligo conjugates at each site.

Using this principle, we developed a panel of DNA flat sheets displaying antibody– and PD-L1–oligo conjugates at different positions (Figure 2). DNA flat sheets without any proteins (FS-empty), with one binding site in the center for anti-CD3 IgG (FS-α-CD3), and with anti-CD3 and anti-CD28 IgGs separated along the helical axis 13.6 nm (FS-α-CD3-CD28), were used as controls (Figure 2a(i)). For flat sheets containing PD-L1, we designed a single PD-L1 binding site in the center (FS-PD-L1) or two binding sites spaced 13.6, 43.5, and 202.3 nm (FS-PD-L1-13, FS-PD-L1-40, and FS-PD-L1-200) (Figure 2a(ii)). The 13.6 nm spacing was designed to display two closely spaced PD-L1 ligands. The 43.5 nm distance was created from adjoining triangle tiles to the 13.6 nm design to control the spatial distribution of PD-L1 ligands within the 35–70 nm range of TCR nanoclusters. Finally, the 202.3 nm distance was selected to space proteins at the maximum limit that can be achieved with these flat sheets. Atomic force microscopy (AFM) confirmed the self-assembly of flat sheets presenting the protein–oligo conjugates according to design, with estimated fractions of 40–65% (Figures S4–S10). As the protein–oligo conjugates are tethered to the flat sheets via a 19 bp or 21 bp oligo, we observed fluctuations in protein distances, which we quantified for FS-α-CD3-CD28 and FS-PD-L1-40 (Figures S11 and S12).

For FS-PD-L1-200, the PD-L1 proteins presented at the edge of the origami tended to land on the mica surface and appear as small protrusions on the AFM images (Figures S10 and S13). In addition to AFM imaging, we immunolabeled the protein flat sheets and visualized with agarose gel electrophoresis to verify hybridization of protein–oligo conjugates to the flat sheets (Figure 2b). We observed that flat sheets functionalized with PD-L1 were recognized by Alexa Fluor 488 dye-labeled oligos, oligos modified with PD-L1 were immunolabeled with Alexa Fluor 488 dye-labeled oligos, the majority of unreacted azide–oligos were removed during the purification step (Figure 1b(i–iii) and Figure S1). Taken together, these results validate the labeling density and quality of the three protein–oligo conjugates used in this study.

Spatial Organization of PD-L1 Modulates T-Cell Signaling. To investigate the effects of PD-L1 nanoscale spatial distribution on T-cell signaling, we performed a NFAT-luciferase reporter assay in PD-1 expressing Jurkat T cells. To immobilize the flat sheets on the surface, we incorporated biotin-modified staples at four positions in the flat sheets such
that the biotins protruded from the non-protein side (Figure 3a). The biotinylated protein flat sheets were then coated on a streptavidin–biotinylated-bovine serum albumin (BSA) surface before cell stimulation. We verified the presence of biotins on the flat sheets with fluorescently labeled streptavidin (Figure S15). Given that NFAT-dependent gene expression can be activated by TCR-CD3 stimulation alone,47,48 we first stimulated PD-1-NFAT luciferase cells with flat sheets functionalized only with anti-CD3 antibody (FS-α-CD3) and measured the activation levels with increasing flat sheet concentrations (Figure S16). The NFAT-luciferase activity showed a dose-dependent response with increasing concentrations of FS-α-CD3 stimulation. In subsequent luciferase assays, we used a concentration of 0.5 nM FS-α-CD3 to obtain an activation of NFAT above background levels that could still be regulated by PD-L1 stimulation (Figure 3b). The wells were first coated with FS-α-CD3 and then PD-L1-flat sheets at 10-fold (FS-PD-L1-13, FS-PD-L1-200 at 5 nM) or 20-fold (FS-PD-L1 at 10 nM) molar excess of FS-α-CD3, to obtain equivalent molar amounts of PD-L1. To ensure that the total molar concentration of flat sheets was equivalent between conditions, FS-empty was added in FS-α-CD3, FS-PD-L1-13 and FS-PD-L1-200 conditions.
We found that FS-PD-L1-200 resulted in a considerable decrease in CD3-mediated NFAT-luciferase activity (∼60.8%) which was significantly different from FS-PD-L1 and FS-PD-L1-13 (Figure 3b). FS-PD-L1 and FS-PD-L1-13 showed no significant decrease in CD3-induced NFAT-luciferase activity, indicating that a single PD-L1 ligand or two PD-L1 ligands presented in proximity did not elicit PD-1-inhibition on TCR-CD3 signaling. To further investigate the role of PD-L1 nanoscale spatial distribution on T-cell activation in the presence of costimulation mediated by CD28, we treated cells with flat sheets functionalized with both CD3 and CD28 antibodies (FS-α-CD3-CD28) (Figure 3c). We also introduced FS-PD-L1-40 in the panel of PD-L1-flat sheet designs.

Consistent with the results obtained with FS-α-CD3, FS-PD-L1-200 induced a statistically significant inhibition of NFAT-luciferase activity. In addition, FS-PD-L1-40 showed similar NFAT-luciferase levels to those of FS-PD-L1-13, with no significant decrease compared to cells treated with FS-α-CD3-CD28 alone. Overall, the results confirm that FS-PD-L1-200, but not FS-PD-L1, FS-PD-L1-13, or FS-PD-L1-40, enabled inhibition of CD3- and CD3/CD28-mediated T-cell activation. We also verified the stability of flat sheets in cell culture media by agarose gel electrophoresis. The flat sheets remained largely intact in complete media after 3 h at 37 °C (Figure S17), indicating that the nanostructures were preserved under these cell stimulation conditions.

To investigate the effects of stoichiometry of FS-PD-L1-200 on NFAT-luciferase activity, we stimulated cells with increasing molar ratios of FS-α-CD3-CD28 to FS-PD-L1-200 (1:1 to 1:10). We observed a gradual decrease in NFAT-luciferase activity with increasing molar ratios and a significant reduction at 1:10 ratio of FS-α-CD3-CD28 to FS-PD-L1-200 (Figure 3d). A 1:1 ratio of FS-α-CD3-CD28 to FS-PD-L1-200 showed no changes in NFAT-luciferase activity, indicating that a high stoichiometry of FS-PD-L1-200 is required to elicit PD-1 downregulation of TCR activity in this system. On the other hand, FS-PD-L1 which contains a single PD-L1 protein, showed no significant effect on NFAT-luciferase activity even at high concentrations (Figure S18), suggesting that a high molar ratio of PD-L1 to TCR-activating antibodies is not sufficient for PD-L1 mediated inhibition of T-cell signaling.

**PD-L1-Flat Sheets Modulate PD-1 Nanocluster Size.** We next sought to investigate the nanoscale distribution of PD-1 receptors on the cell membrane upon treatment with PD-L1-flat sheets using stimulated emission depletion (STED) microscopy. As a cell model for these studies, we generated a stable Jurkat cell line expressing PD-1 fused with an intracellular self-labeling protein, SNAP-tag, which allows covalent attachment of fluorophore-coupled SNAP ligands. We confirmed PD-1-SNAP expression and PD-1 down-regulation of IL-2 secretion in these cells (Figure S19).

Consistent with the findings from the NFAT-luciferase assays, FS-PD-L1-200 stimulation of PD-1-SNAP cells resulted in a significant decrease in IL-2 gene expression (Figure S20). These results confirmed that FS-PD-L1-200 tunes TCR downstream signaling by inhibition of IL-2 expression.

For the STED imaging experiments, we used the same molar ratios of antibody- and PD-L1-flat sheets as in the NFAT-luciferase assays (0.5 nM FS-α-CD3-CD28, 5 nM FS-PD-L1-13, and 5 nM FS-PD-L1-200) to stimulate PD-1-SNAP cells.
To analyze the PD-1 receptor distribution when it is not stimulated by PD-L1, cells were stimulated with 0.5 nM FS-α-CD3-CD28 and 5 nM FS-empty. We first fluorescently labeled the flat sheets to study the distribution of antibody- and PD-L1-flat sheets on the glass surface by confocal microscopy (Figure S21). The negative correlation observed between the PD-1 receptor distribution when it is not stimulated by PD-L1, cells were stimulated with 0.5 nM FS-α-CD3-CD28 and 5 nM FS-empty. We first fluorescently labeled the flat sheets to study the distribution of antibody- and PD-L1-flat sheets on the glass surface by confocal microscopy (Figure S21). The negative correlation observed between the

Figure 5. Analysis of PD-1 nanoclusters from STED microscopy data. (a) Point cloud and corresponding cluster density maps. Representative cluster density maps from each flat sheet condition, with intensity corresponding to the local density of localized clusters (number of clusters per Gaussian kernel with bandwidth = 200 nm). Scale bar = 3 μm. (b) Example image of k-nearest neighbor analysis for a cluster of interest (middle blue cross) with the four nearest neighbors (surrounding blue crosses) and the distances $d_n$ between them (yellow lines). (c) Example image showing cluster size analysis of an individual cluster with a line profile marked and plotted (white circles), providing a full width at half-maximum (FWHM) of 68 nm from a Lorentzian fit to the data (yellow line). (d–f) Results from respective analysis: (d) peak intensity distributions from cluster density maps, (e) nearest neighbor distance distributions and (f) cluster size distributions shown as mean kernel density estimation (solid lines) of the resulting distribution from each cell ($N = 24$). Corresponding 83.4% confidence intervals of the mean are indicated by shaded areas per condition, FS-PD-L1-13 (orange), FS-PD-L1-200 (blue) and FS-α CD3-CD28 (gray). Regions of significant difference between conditions are marked by colored bars (bottom, FS-PD-L1-13/FS-α-CD3-CD28 (orange/gray), FS-PD-L1-200/FS-α-CD3-CD28 (blue/gray) and FS-PD-L1-13/FS-PD-L1-200 (orange/blue)). $N = 24$ cells for each flat sheet condition from 2 glasses from 2 days of experiment ($N_1 = 15$, $N_2 = 9$ for each condition), a total of 72 cells were analyzed (24 cells $\times$ 3 conditions).
antibody- and PD-L1-flat sheets confirmed that they did not overlap each other. We next labeled PD-1-SNAP cells with a cell membrane-permeable SNAP-Cell 647-SiR substrate and stimulated them with antibody- and PD-L1-flat sheets. PD-1-SNAP receptor distribution was imaged by depleting 647-SiR with a 775 nm depletion beam in a far-red custom-built STED system, featuring a spatial resolution within the range of 30–50 nm (Figure 4 and Figure S22). When compared with the corresponding confocal images, PD-1 receptor clusters were only localizable using STED microscopy (Figure 4a,b). To localize these PD-1 receptor clusters (Figure 4c), we followed the data preprocessing and localization pipeline as described in Figure S23. Using the localized cluster coordinates, we performed three kinds of analyses.

First, we generated cluster density maps through kernel density estimation of the cluster localization point cloud (Figure 5a). By analyzing the local cluster densities, we observed that both FS-PD-L1-13 and FS-PD-L1-200 induced more areas with higher density of PD-1 clusters as compared to a more evenly spread PD-1 distribution in FS-α-CD3-CD28 without PD-L1 stimulation (Figure 5d and Figure S24). Despite displaying a high cell-to-cell variability in the cluster density maps (Figure S22), FS-PD-L1-13 on average resulted in more areas with higher PD-1 density (one-sided Kolmogorov–Smirnov test: FS-PD-L1-13/FS-α-CD3-CD28: P < 0.0001, FS-PD-L1-13/FS-PD-L1-200: P < 0.001) (Figure S24).

We next analyzed the nearest neighbor distances between the PD-1 cluster localizations (Figure 5b and 5e). FS-PD-L1-13 and FS-PD-L1-200 both resulted in distributions of PD-1 clusters that were shifted toward shorter distances as compared to FS-α-CD3-CD28, with ranges of significant difference (nonoverlapping confidence intervals) at 134–211 and 131–233 nm, respectively (Figure 5e). The largest differences in mean probability density appeared at 160 nm (FS-PD-L1-13) and 155 nm (FS-PD-L1-200). Because of the increased probability at shorter distances and the fixed k in the analysis, we additionally saw a significant decrease in the mean probability density distributions of FS-PD-L1-13 and FS-PD-L1-200 as compared to FS-α-CD3-CD28 at 412–532 and 431–511 nm, respectively. Moreover, the maximum cumulative difference in the number of nearest neighbor distances between FS-PD-L1-200 and FS-α-CD3-CD28 was found at the crossover point of the two probability density functions at 264 nm, indicating more neighbors were within 264 nm for FS-PD-L1-200. For FS-PD-L1-13 compared to FS-α-CD3-CD28, the maximum cumulative difference was found at 237 nm. Overall, these results indicate that there is a significant effect on nearest neighbor distances of PD-1 clusters induced by FS-PD-L1-200 and FS-PD-L1-13.

Finally, FS-PD-L1-13 resulted in significant differences in PD-1 cluster sizes as compared to FS-α-CD3-CD28, with a clear distribution shift toward larger cluster sizes (>100 nm) (Figure 5c,f). In contrast, FS-PD-L1-200 induced a cluster size distribution more like FS-α-CD3-CD28, and the two conditions were more likely to form smaller PD-1 cluster sizes (<100 nm) than FS-PD-L1-13. A small subpopulation of larger PD-1 nanoclusters was also observed in FS-PD-L1-200, which significantly differed with FS-α-CD3-CD28 at 111–123 nm (Figure 5f). The maximum cumulative differences (crossover points) indicate that FS-PD-L1-200 was more likely to result in PD-1 cluster sizes above 76 nm than FS-α-CD3-CD28, while FS-PD-L1-13 showed a maximum difference at 94 and 103 nm as compared to FS-α-CD3-CD28 and FS-PD-L1-200, respectively.

Overall, FS-PD-L1-13 and FS-PD-L1-200 resulted in areas of dense PD-1 clusters of similar cluster–cluster distances. FS-PD-L1-200 mostly induced formation of smaller PD-1 nanoclusters (50–100 nm), while FS-PD-L1-13 mostly generated larger PD-1 nanoclusters (>100 nm). The latter could be triggered by the 13 nm PD-L1 interligand distance on the flat sheets bringing two PD-1 receptors in proximity, which is inseparable by STED imaging. Interestingly, FS-PD-L1-200 also triggered a small subset of large PD-1 nanoclusters (>120 nm) which could be caused by the ends of flat sheets meeting each other, thus bringing multiple PD-L1 proteins in proximity.

Together, our results indicate that the nanoscale spatial organization of PD-L1 regulates PD-1-mediated inhibition of T-cell activation. Interestingly, the nonoverlapping surface distributions of antibody- and PD-L1-flat sheets (Figure S21) imply a separation between PD-L1 proteins in FS-PD-L1-200 and CD3/CD28 activating antibodies in FS-α-CD3-CD28 of at least 50 nm. This suggests that proximity between ligand bound PD-1 and activating antibody bound-TCR and CD28 is not a requirement for PD-L1-mediated inhibition of T-cell activation in this system. Further, we demonstrated that FS-PD-L1, FS-PD-L1-13, and FS-PD-L1-40 presented PD-L1 proteins to T cells at fixed positions that were not conducive to inhibition of T-cell activation. This suggests that the nanoscale organization of PD-L1 proteins can be exploited to prevent inhibition of T-cell activation, in a manner that is orthogonal to PD-1 blockade using anti-PD-1 antibodies.

CONCLUSIONS

In this study, we used wireframe DNA origami as a tool to investigate the effects of the nanoscale spatial organization of PD-L1 on T-cell activation. We demonstrated that DNA origami flat sheets with a single PD-L1 protein or two PD-L1 proteins separated by 13 or 40 nm did not cause inhibition of CD3/CD28-mediated T-cell signaling. In contrast, DNA origami flat sheets with two PD-L1 proteins separated by 200 nm, and presented at the edges of the nanostructures, suppressed T-cell signaling. Overall, our results provide insights into the spatial relationships between PD-1/PD-L1 immune checkpoints and T-cell signaling. These findings can provide a foundation for rationally designed nanotherapeutic-based platforms in cancer immunotherapy.49,50

METHODS

Antibody Conjugation. Mouse anti-human CD3e IgG2a (OKT3, eBioscience) and mouse anti-human CD28 IgG1 (CD28.2, eBioscience) at a concentration of 1 mg/mL were buffer-exchanged to 1× PBS, pH 7.4 using Zeba Spin Desalting columns, 7K MWCO (ThermoFisher Scientific). DBCO-NHS reagent (Click Chemistry Tools) was resuspended in N,N-dimethylformamide at 10 mM concentration. This was added to the antibody mixes at a molar ratio of 10:1 and incubated on ice for 2 h. The reaction was quenched with 75 mM Tris–HCl, pH 8 on ice for 15 min. Nonreactive DBCO-NHS esters were removed by buffer-exchange to 1× PBS, pH 7.4 using Zeba Spin Desalting columns, 7K MWCO. Azide-modified oligos, Tag 1 and Tag 2 (Table S1) were added to anti-CD3e IgG2a and CD28 IgG1, respectively, at a molar ratio of 2:1 based on the molality of DBCO incorporated. The reactions were then incubated overnight at 4 °C. The conjugates were added into Amicon Ultra-0.5 centrifugal filter units with Ultracel-50 membrane (Merck Millipore) coated with 5% Pluronic-F127 (w/v) (ThermoFisher Scientific) and washed 8

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times with 1 × PBS, pH 7.4 (centrifugation 14,000 × g, 2 min) to remove unconjugated oligos. After washing, antibody–oligo conjugates were concentrated to about 100 μL volume. Antibody–oligo conjugate concentrations were determined using the Micro BCA Protein Assay kit (ThermoFisher Scientific) according to manufacturer’s instructions.

**PD-L1 Conjugation.** Recombinant human PD-L1 His-tag (9049-B7-100, R&D Systems) was reconstituted with 1 × PBS, pH 6.3 according to manufacturer’s guidelines. The protein was conjugated to an azide-modified oligo, Tag 3 (Table S1) using bis-sulfone-PEG3-DBCO compound (Click Chemistry Tools) as previously described, with a molar ratio of 1:2 (molarity of DBCO incorporated: molarity of oligo). Protein–oligo conjugates were purified using an Amicon Ultra-0.5 centrifugal filter unit with Ultracel-30 membrane (Merck Millipore) coated with 5% Pluronic-F127 (w/v) (ThermoFisher Scientific).

Validation of antibody–oligo conjugates and PD-L1–oligo conjugates was performed by hybridizing complementary Alexa Fluor-labeled oligos (Supporting Information Table S2) for 1 h at 37 °C as previously described. Antibody–oligo conjugates (1.3 μM) were mixed with complementary Alexa Fluor 467–oligos (4 μM) and PD-L1–oligo conjugates (5 μM) were mixed with Alexa Fluor 488–oligos (2 μM) and the mixes were run on a native 4–20% Mini-Protean TGX Precast gel (Bio-Rad) in 1× Tris-glycine buffer at 200 V for 34 min. The gels were imaged with ImageQuant LAS 4010 system (GE Healthcare) with fluorescence transillumination and then stained using Pierce Silver Stain kit (ThermoFisher Scientific).

**Protein Flat Sheet Production.** Staple strand sequences for folding the 3-tessellation flat sheet were described previously. The 216 core staples were obtained from Integrated DNA Technologies at 216 core staples were obtained from Integrated DNA Technologies at 200 μM in water in three 96-well plates. Staple mixes for folding each flat sheet design were prepared to a final concentration of 463 nM based on the pipetting schemes outlined in Supporting Information Figure S25 with some core staples replaced by protruding/modified staples (Tables S3 and S4). The flat sheets were produced as previously described with the following minor modifications. In the folding reactions, the p8064 scaffold (Tålibit) was mixed at concentration of 20 μM in water in three 96-well plates. Staple strands sequences for folding the 3-tessellation flat sheet were described previously. The 216 core staples were obtained from Integrated DNA Technologies at 216 core staples were obtained from Integrated DNA Technologies at 200 μM in water in three 96-well plates. Staple mixes for folding each flat sheet design were prepared to a final concentration of 463 nM based on the pipetting schemes outlined in Supporting Information Figure S25 with some core staples replaced by protruding/modified staples (Tables S3 and S4). The flat sheets were produced as previously described with the following minor modifications.

**AFM Imaging.** After Sepharose purification, protein–DNA flat sheets were imaged on a disc of mica fastened with epoxy adhesive to the center of a microscope slide and enclosed by a plastic ring attached to the slide using repro rubber. The flat sheets were diluted to 1 nM in TE-Mg buffer (5 mM Tris base, 1 mM EDTA, 10 mM MgCl₂, pH 8.0), and 10 μL was pipetted onto freshly cleaved mica. After 30 s, 4 μL of 5 mM NiSO₄ was added and incubated for a further 4.5 min. The surface was then rinsed with 1 mL of 0.1 μM-filtered TE-Mg buffer after which 1.5 mL of filtered TE-Mg buffer was added to the mica disk for imaging. Imaging was performed in liquid using a JPK Instruments NanoWizard 3 Ultra AFM with a Bruker AC40 cantilever in AC mode.

**Immunolabeling and Agarose Gel Electrophoresis for Characterization of Protein Flat Sheets.** Flat sheets hybridized with anti-CD3 IgG and anti-CD28 IgG (4 μL volume) were mixed with 40 nM Alexa Fluor 647-conjugated AflFinnPure rabbit anti-mouse IgG, Fcy fragment specific (no. 315-605-008, Jackson ImmunoResearch). Similarly, flat sheets hybridized with PD-L1 were mixed with Alexa Fluor 488-conjugated anti-PD-L1 antibody (R&D Systems).

The mixes were incubated for 15 min at room temperature before being loaded onto 2% agarose gels (0.5x TBE, 11 mM MgCl₂, and 0.5 mM ethidium bromide) and ran at 90 V for 3 h in an ice bath. The gel was imaged in the ImageQuant LAS 4010 system (GE Healthcare), using UV, Cy5 and SYBR Green transillumination. The final concentration of the protein–DNA flat sheets was determined by the ratio of gel band intensity before and after Sepharose purification and calculated using the concentration of flat sheets prior purification as a reference.

**Cell Culture.** Jurkat E6-1 (ATCC), PD-1-NFAT luciferase reporter (BPS Bioscience) and PD-1-SNAP-Jurkat cells were maintained in complete RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ThermoFisher Scientific). The cell lines were routinely screened for mycoplasma contamination (Eurofins Genomics).

**Luciferase Reporter Assay.** For surface immobilization of protein flat sheets, 10 μg/mL of biotinylated-BSA (29130, ThermoFisher Scientific) was codified in a 96-well plate for 30 min at 37 °C. The wells were washed five times with 1 × PBS, pH 7.4 and then coated with 10 μg/mL streptavidin (21122, ThermoFisher Scientific) for 30 min at 37 °C. The wells were first coated with either FS-empty (10.5 nM), FS-α-CD3 (0.5 nM), or FS-α-α-CD3-CD28 (0.5 nM) in 1× PBS, pH 7.4 for 30 min at 37 °C. After washing five times with PBS, FS-α-CD3 or FS-α-α-CD3-CD28-coated wells were either coated with FS-empty (10 nM), FS-PD-L1 (10 nM), FS-PD-L1-13 (5 nM), FS-PD-L1-40 (5 nM), or FS-PD-L1-200 (5 nM) for 30 min at 37 °C. After incubation, the wells were subjected to five washes of PBS. Wells precoated with 5 nM of FS-PD-L1-13, FS-PD-L1-40, and FS-PD-L1-200 were treated with another coating of 5 nM FS-empty, to ensure all wells had a similar level of flat sheet coating. After washing with PBS, PD-1-NFAT cells were seeded at 2 × 10⁴ cells per well and incubated for 3 h at 37 °C, 5% CO₂. Cells were lysed with 1 × Glo Lysis buffer (Promega), and luciferase reporter activity was measured using Bright-Glo Luciferase Assay System (Promega) in a Varioskan LUX multimode microplate reader.

**Statistical Analysis.** Statistical significances for Figure 3, Supporting Information Figure S18, S19c and S20 were evaluated by one-way ANOVA and Tukey multiple pairwise comparison with R package ggplot2. The number of biological repeats was N = 3, unless otherwise stated. Shapiro-Wilk and Levene’s tests were employed to check for violations of normality and homogeneity of variances. Statistical significance for the mean cell distributions in Figure 5d-f was evaluated comparing the confidence intervals, extracted as explained under “STED image pre-processing and cluster localization”. Nonoverlapping confidence intervals correspond to a significant difference in a 95% Student’s t test (p < 0.05), while overlapping confidence intervals similarly correspond to no significant difference. Statistical significance for the pooled distributions in Figure S24, based on data presented in Figure 5d in the Results section, was evaluated with one-sided two-sample Kolmogorov-Smirnov tests, testing the hypothesis that distribution A is bigger than distribution B. Sample Preparation for STED Microscopy. Unless otherwise stated, incubations were performed at room temperature. Round cover glasses (18 mm, 1.5H) were cleaned with acetone and isopropanol and dried with N₂ gas. Each cover glass was placed on a parafilm containing a drop (120 μL) of 1 mg/mL biotinylated-BSA (ThermoFisher Scientific) for 5 min to allow adsorption to the glass surface. Cover glasses were then washed twice in 1 × PBS and coated with 0.5 mg/mL streptavidin (ThermoFisher Scientific) in the same manner. After washing twice in 1 × PBS, each cover glass was incubated with a drop of FS-α-α-CD3-CD28 (0.5 nM) for 10 min. The coated cover glasses were washed twice and then incubated with 5 nM of either FS-empty, FS-PD-L1-13 or FS-PD-L1-200. PD-1-SNAP cells were labeled with 5 μM SNAP-Cell-647-SiR (New England Biolabs) according to manufacturer’s instructions at 37 °C for 30 min with gentle mixing at 300 rpm. The labeled cells were stimulated in the flat sheet-coated coverslips in a 12-well plate for 10 min and then fixed with 4% paraformaldehyde in 1 × PBS for 20 min. The cover glasses were carefully washed three times in PBS before mounting in Mowiol mounting media.

**STED Microscopy Setup and Imaging.** The STED microscopy imaging was performed with a custom-built STED setup operating in the far-red wavelength regime as previously described. The 647-SiR
The results from each of the three analyses were presented as the mean kernel density estimation of the individual kernel density estimations of the distributions from each cell. The 83.4% confidence interval for the mean was calculated using the implementation in the scikit-image package. Graphs were prepared using the matplotlib package.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.0c10632.

Additional methods, DNA sequences, quantifications of native-PAGE images, reducing SDS-PAGE, vHelix design of DNA flat sheet nanostructures, AFM images and their quantification, SPR analysis, agarose gel images, NFAT-luciferase experiments, flow cytometry, Western blots, IL-2 Elisa, IL-2 qPCR, confocal microscopy images, STED images, and pipetting schemes (PDF).

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Author Contributions

T.F. planned, performed, and analyzed experiments related to protein conjugation, DNA origami, and cell assays. J.A. and I.T. designed the STED experiments. J.A. performed and analyzed the STED and confocal microscopy experiments, J.S. performed and analyzed the AFM imaging, and E.A. performed and analyzed the SPR experiments. A.I.T. conceived and supervised the study. T.F. wrote the manuscript with input from all authors; all authors contributed to the manuscript revision and gave approval to the final version.

Notes

The authors declare no competing financial interest.

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