Programmed death-1 concentration at the immunological synapse is determined by ligand affinity and availability

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Despite the importance of programmed death-1 (PD-1) for T cell inhibition, little is known about its intracellular trafficking or requirements for localization to the immunological synapse. Here, we show that in activated T cells, PD-1 is present at the plasma membrane, near the Golgi and in the trans-Golgi network. Unlike CD28 and CTLA-4, PD-1 accumulation at the synapse is extensive only when T cells interact with dendritic cells (DCs) expressing high B7-DC levels. However, B7-H1 is also critically important, especially when the DCs have little B7-DC. Despite this preference, B7-H1$^{-/-}$ DCs elicit greater cytokine secretion than B7-DC$^{-/-}$ DCs during T cell restimulation, possibly because they also express less B7-DC. PD-1 and CD28 have similar kinetics of synaptic accumulation, suggesting that the process involves T cell receptor-triggered cytoskeletal reorganization followed by ligand binding.

B7-DC | B7-H1 | costimulation | LPS | IL-4

The activation of T cells requires the engagement of T cell receptors (TCRs) by antigenic peptide–MHC complexes. The outcome of this interaction is influenced by signals from both positive (e.g., CD28) and negative (e.g., CTLA-4) costimulatory molecules. Programmed death-1 (PD-1), a fairly new member of the extended CD28/CTLA-4 family, was identified as a gene up-regulated by cell lines undergoing programmed cell death and was later reported to be induced on primary T cells, B cells, and myeloid cells after activation (1). PD-1-deficient mice develop lupus-like arthritis and glomerulonephritis on the C57/BL6 (B6) background and die of autoimmune dilated cardiomyopathy on the BALB/c background (1), suggesting an inhibitory function for PD-1 in T cell activation.

PD-1 has two ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), which are related to the ligands for CD28 and CTLA-4, B7–1 and B7–2, and have been reported to either costimulate or inhibit T cell proliferation and cytokine secretion (1). The two PD-1 ligands have different expression patterns. B7-H1 is expressed more broadly on both hematopoietic and nonhematopoietic cells (2–4), whereas B7-DC is restricted to cytokine-activated macrophages and dendritic cells (DCs) (2, 3). Analysis of B7-H1-deficient mice has shown that B7-H1 negatively regulates both CD4$^+$ and CD8$^+$ T cells (5, 6). In contrast, one line of B7-DC$^{-/-}$ mice has diminished immune responses, suggesting a positive costimulatory function for B7-DC (7). Other studies, however, including another line of B7-DC$^{-/-}$ mice (8), have shown that B7-DC can inhibit T cell responses via PD-1 (9, 10).

Administration of antibodies specific for PD-1, B7-H1, or B7-DC in mouse models of autoimmunity has had different effects on disease incidence and severity, depending on the autoimmune model (1). Some of the differences could be attributed to the differential tissue expression of the two ligands; however, another level of complexity is imposed by the suggested existence of a putative second receptor for B7-H1 and B7-DC on T cells, distinct from PD-1, that might mediate the observed costimulatory effects of these ligands on T cell responses (11–13).

Despite abundant functional and biochemical data on PD-1 and its associated signaling molecules (1, 14), relatively little is known about its intracellular localization and how it is recruited to the specialized interface between a T cell and an antigen-presenting cell (APC), known as the immunological synapse. Our work has focused on the mechanism of PD-1 concentration at the immunological synapse. Similarly to our experiments with CD28 and CTLA-4 (15), we found that ligand binding is important for the recruitment of PD-1 to the synapse of CD4$^+$ T cells forming conjugates with peptide-pulsed activated splenic DCs. Because PD-1 concentrated in a larger number of conjugates when the DCs expressed high levels of B7-DC subsequent to IL-4 treatment, relative to when they were activated with LPS, which induced high B7-H1 levels, our study suggests that B7-DC is the preferred ligand for PD-1 recruitment to the immunological synapse of T cells interacting with DCs. This finding correlates with the reported higher affinity of PD-1 for B7-DC relative to B7-H1 (16). Additional experiments with DCs lacking either B7-H1 or B7-DC confirmed that B7-DC was essential for the recruitment of PD-1 to the T cell/DC synapse, especially when the DCs were cultured with IL-4; however, when the DCs were treated with LPS, B7-H1 was also important for the accumulation of PD-1 at the synapse, suggesting that the relative significance of each ligand for PD-1 concentration may be determined by the ratio of B7-H1 to B7-DC expressed by the WT DCs.

Results

In Activated T Cells, PD-1 Localizes to an Intracellular Compartment and the Plasma Membrane. To determine the intracellular localization of endogenous PD-1 in primary T cells, day-3 activated OT-II T cells were stained with anti-PD-1 antibodies (Fig. 1 *A–F Left*). PD-1 was detectable at the plasma membrane and in an intracellular compartment that has been previously reported for T regulatory cells (17). To identify this compartment, we also stained the cells with antibodies against the Golgi matrix protein GM130, the trans-Golgi network (TGN) protein TGN38, the transferrin receptor (TfR), which recycles between endosomes and the plasma membrane), or LAMP1 (a lysosomal resident protein). The PD-1-positive vesicles were proximal to the Golgi, as determined by GM130 staining (Fig. 1*A*) and some of them colocalized with TGN38, although many of the trans-Golgi vesicles were free of PD-1 (Fig. 1*B*). In contrast, there was no extensive overlap between

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Abbreviations: APC, antigen-presenting cell; CFP, cyan fluorescent protein; DC, dendritic cell; GM-CSF, granulocyte/monocyte colony-stimulating factor; OVA, ovalbumin; PD-1, programmed death-1; TCR, T cell receptor; TfR, transferrin receptor; TGN, trans-Golgi network; Th, T helper; YFP, yellow fluorescent protein.

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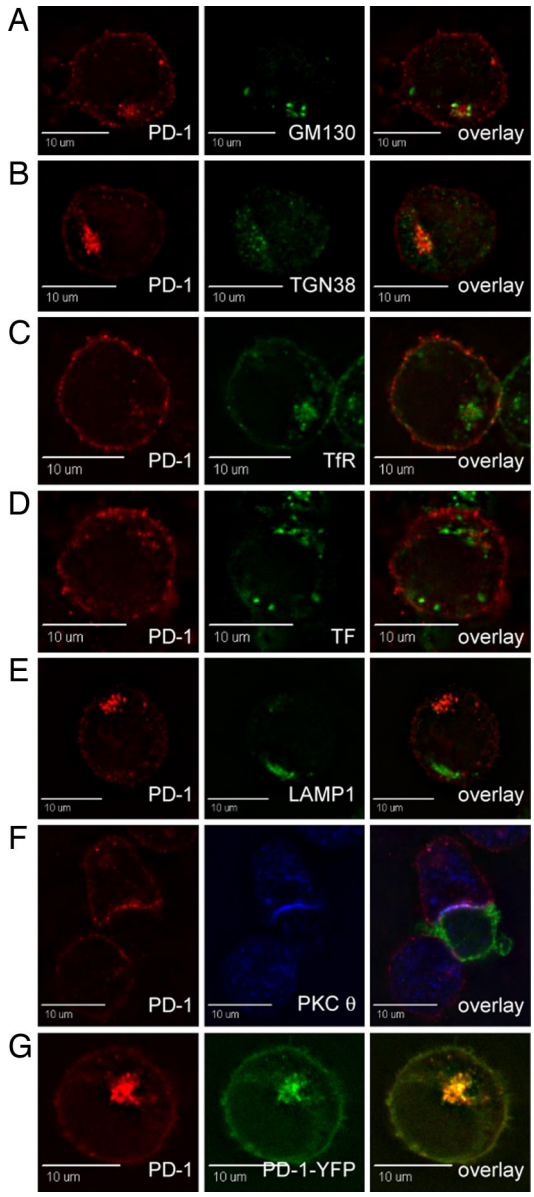

Fig. 1. Intracellular localization of endogenous and exogenous PD-1. Day-3 activated OT-II T cells (*A–F*) or OT-II T cells infected with PD-1-YFP (*G*) were stained with anti-PD-1 antibodies (red). The cells were also stained with antibodies (green) specific for GM130 (*A*), TGN38 (*B*), TfR (*C*), FITC-transferrin (TF; *D*) and LAMP-1 (*E*). In *F*, conjugates between OT-II cells and IL-4-activated B6 DCs were also stained with anti-PKC θ (blue) and anti-CD11c (green) to visualize the DCs. In *G*, PD-1-YFP is in green.

the PD-1$^+$ vesicles and recycling endosomes, visualized either with anti-TfR antibodies or fluorescent transferrin (Fig. 1 *C* and *D*), nor LAMP1$^+$ lysosomes (Fig. 1*E*). Despite localizing to this intracellular compartment, endogenous PD-1 was able to accumulate at the immunological synapse of conjugates between activated T cells and IL-4-treated DCs (Fig. 1*F*).

Because endogenous PD-1 levels decreased rapidly after the peak of expression at day 3 (not shown), we fused PD-1 to yellow fluorescent protein (YFP) and introduced it into previously activated TCR transgenic T cells via retroviral infection. When the infected T cells were stained with anti-PD-1 antibodies, there was almost perfect overlap between the antibody signal and the YFP fluorescence, suggesting that the exogenous PD-1-YFP was folding properly and localizing to the same compartments as the endogenous protein (Fig. 1*G*; colocalization data not shown).

Ligand Binding Is Important for the Concentration of PD-1 at the Immunological Synapse. To determine the requirements for PD-1 concentration at the immunological synapse, infected 5C.C7 T cells were allowed to form conjugates with B10.A splenic DCs that had been cultured in the presence of peptide, granulocyte/monocyte colony-stimulating factor (GM-CSF) and either LPS or IL-4. Both treatments resulted in high expression of B7–1, B7–2, B7-H1, and B7-DC on >90% of the DCs (Fig. 2*A*). In all cases, except for B7-DC, LPS induced slightly higher levels of the costimulatory molecules in comparison with IL-4. In the case of B7-DC, IL-4 induced high B7-DC levels on >90% of the cells, whereas the combination of LPS and GM-CSF resulted in heterogeneous up-regulation of B7-DC, which has been previously reported (2).

Because our earlier CD28/CTLA-4 studies used only activated B cells as APCs (15), we also determined the number of T cell/DC conjugates that accumulated CD28-YFP or CTLA-4-YFP at the synapse. Conjugates were selected on the basis of PKC θ concentration, a known marker for the immunological synapse (18). Representative images are shown in the top two rows of Fig. 2*B*. When the DCs were cultured in GM-CSF and either LPS or IL-4, CD28 concentrated in 98% and 95% of the conjugates with accumulated PKC θ, respectively, whereas CTLA-4 was concentrated in 81% of the cells (Fig. 2*C*). These numbers are similar to those obtained for CD28 and CTLA-4 concentration in activated T cells conjugated to LPS-activated B cells (15).

Although the concentration of PD-1 was not as extensive as that of CD28 and CTLA-4, it was able to accumulate in 47% of the conjugates when the DCs were treated with LPS and GM-CSF and in 74% of the cells when DCs were cultured in IL-4 and GM-CSF (Fig. 2*C*). As LPS up-regulated more B7-H1 on the DCs than IL-4, whereas IL-4 induced the expression of significantly more B7-DC than LPS, these results suggested that B7-DC might be the main ligand responsible for PD-1 accumulation at the synapse. This hypothesis is consistent with the reported higher affinity of PD-1 for B7-DC relative to B7-H1 (16).

To determine whether the two PD-1 ligands also accumulate at the immunological synapse, conjugates between PD-1-YFP-expressing T cells and activated DCs were stained with anti-B7-H1 and anti-B7-DC antibodies. As shown in Fig. 2*D* and supporting information (SI) Fig. 6, both ligands showed extensive concentration at the immunological synapse and overlapped with the regions of PD-1 accumulation.

The Absence of B7-DC Causes a Significant Reduction in the Number of Conjugates That Can Concentrate PD-1 at the Synapse. To determine whether B7-DC is the more important ligand for PD-1 accumulation at the synapse relative to B7-H1, we used splenic DCs from animals that lacked B7-DC as APCs in similar experiments (7). After incubation with GM-CSF and either LPS or IL-4, the B7-DC$^{-/-}$ and the WT DCs up-regulated similar levels of B7–1, B7–2, and B7-H1 at the plasma membrane, and the mutant DCs did not express B7-DC, as expected (Fig. 3*A*).

When activated OT-II TCR transgenic T cells were allowed to form conjugates with WT B6 DCs, PD-1 concentrated at the synapse of 70% of the conjugates when using LPS-treated DCs and in 87% of the cells when the DCs were cultured in IL-4 (Fig. 3 *B Top* and *C*). These numbers are slightly higher than the ones in the experiments with 5C.C7 T cells and B10.A DCs. The use of B7-DC$^{-/-}$ DCs caused a significant reduction in the fraction of the synapses that accumulated PD-1. Although PD-1 was concentrated in some cells (Fig. 3*B Middle*), in the majority of the cells, it remained uniformly distributed around the plasma membrane (Fig. 3*B Bottom*). When the B7-DC$^{-/-}$ DCs were cultured in LPS and GM-CSF, 41% of the OT-II T cells were able to accumulate PD-1, whereas only 36% of the T cells conjugated with IL-4-treated B7-DC$^{-/-}$ DCs had concentrated PD-1 at the synapse (Fig. 3*C*). The absence of B7-DC did not affect the concentration of CD28, which was used as a positive control (Fig.

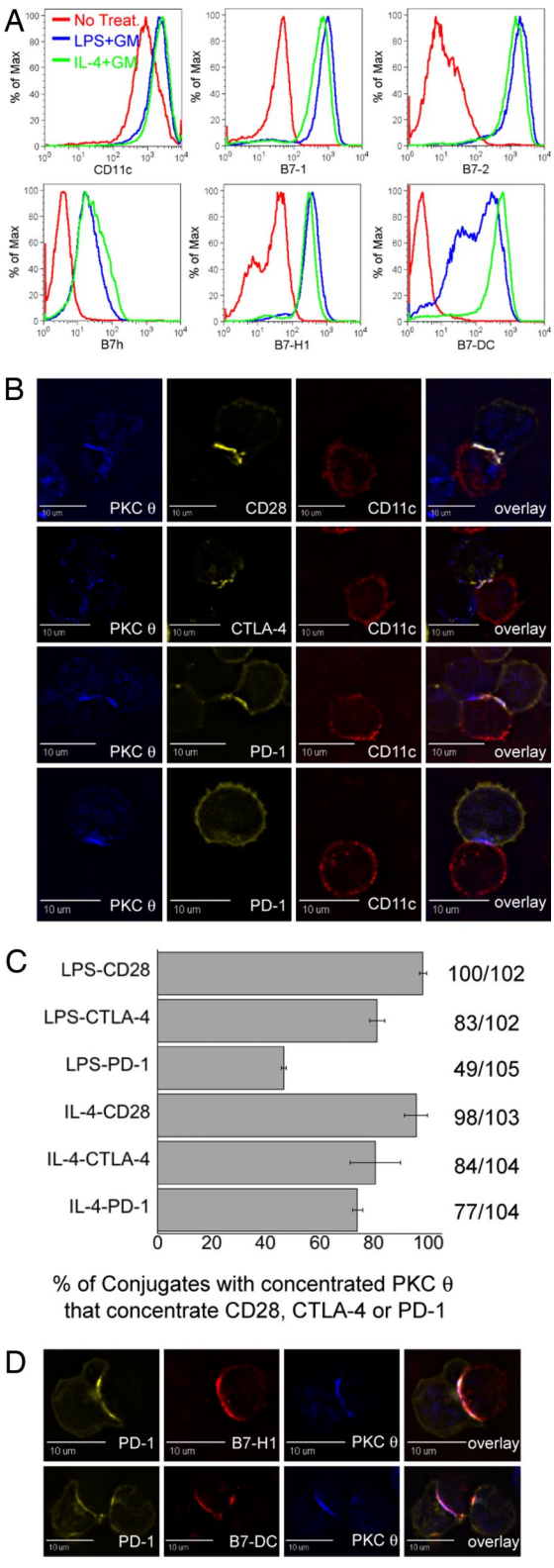

Fig. 2. PD-1 can concentrate at the synapse of conjugates between infected 5C.C7 T cells and activated splenic DCs. (*A*) Splenic DCs from B10.A mice were purified by CD11c-positive selection and cultured in GM-CSF and either LPS or IL-4 for 16 h. The untreated DCs are in red, LPS-activated DCs are in blue, and the IL-4-cultured DCs are in green. (*B*) 5C.C7 T cells were infected with CD28-YFP (top row), CTLA-4-YFP (second row), or PD-1-YFP (bottom two rows). Conjugates with concentrated PKC θ (blue) at the synapse were scored for the localization of the costimulatory molecules (yellow) to the synapse. A representative cell with concentrated PD-1 is in the third row, and a cell with uniform distribution of PD-1 is in the bottom row. The DCs are visualized with anti-CD11c antibodies (red). (*C*) The statistics from three experiments with at least 30 conjugates are shown as mean and SD (error bars). The numbers after the bars represent the number of conjugates that had concentrated the molecule of interest of the total number of conjugates counted. (*D*) B7-H1 and B7-DC (red) accumulate at the immunological synapse of conjugates between 5C.C7 T cells infected with PD-1-YFP (yellow) and moth cytochrome c-pulsed DCs. PKC θ is in blue.

3*C*). These data clearly suggest that B7-DC is important for PD-1 accumulation at the synapse. Similar results were obtained in experiments using infected DO11.10 T cells and BALB/c WT or B7-DC$^{-/-}$ DCs (SI Fig. 7), suggesting that despite the difference in phenotype of PD-1$^{-/-}$ mice on the BALB/c and the B6 backgrounds (19, 20), B7-DC is equally important for PD-1 recruitment.

In the Presence of Low Levels of B7-DC, B7-H1 is Essential for the Concentration of PD-1 at the Synapse. To evaluate the importance of B7-H1 for the recruitment of PD-1 to the immunological synapse, we used splenic DCs from B7-H1$^{-/-}$ B6 mice (5) as APCs for OT-II TCR transgenic T cells. WT and B7-H1$^{-/-}$ DCs up-regulated similar levels of B7–1 and B7–2 after incubation in GM-CSF and either LPS or IL-4 (Fig. 4*A*). As expected, the knockout DCs did not express B7-H1; however, they also had about half as much B7-DC as WT cells (Fig. 4*A*).

In conjugates between infected OT-II T cells and B7-H1$^{-/-}$ DCs, PD-1 was observed to concentrate at the synapse of some cells (Fig. 4*B Upper*), whereas in others, it remained uniformly distributed around the plasma membrane (Fig. 4*B Lower*). As expected, the lack of B7-H1 did not affect CD28 accumulation at the synapse but it was important for PD-1 concentration, because fewer OT-II T cells recruited PD-1 to the synapse in its absence (Fig. 4*C*). This trend was especially true when LPS-treated DCs were used as APCs, where the number of T cells that could concentrate PD-1 decreased by 60% (Fig. 4 *C* and *D*). The defect was smaller when the B7-H1$^{-/-}$ DCs were cultured in IL-4, where the number of T cells with concentrated PD-1 at the synapse decreased by 23% (Fig. 4 *C* and *D*). These data suggest that when the DCs express high levels of B7-DC, B7-H1 is not important for PD-1 recruitment to the synapse; however, when the cells have little B7-DC, B7-H1 is important for the synaptic concentration of PD-1.

The Complete Absence of B7-H1 in the Presence of Low B7-DC Levels Leads to Greater Cytokine Secretion Compared with the Complete Absence of B7-DC Alone. To determine whether the apparent PD-1 ligand preferences translate into differential inhibition of activated OT-II T cells, we used DCs from WT, B7-H1$^{-/-}$, or B7-DC$^{-/-}$ mice as APCs for *in vitro* restimulation experiments. Surprisingly, even though B7-DC was better at recruiting PD-1 to the immunological synapse, the previously activated OT-II T cells secreted more IFN-γ, IL-2, IL-6, and TNF upon restimulation with B7-H1$^{-/-}$ DCs than when they were stimulated with B7-DC$^{-/-}$ DCs (Fig. 5). When the OT-II T cells were restimulated with B7-DC$^{-/-}$ DCs, they produced the same or slightly higher amounts of the cytokines relative to restimulations with WT DCs (Fig. 5). There was no difference in the proliferation of OT-II T cells, as measured by carboxyfluorescein diacetate succinimidyl ester dilution, upon re-stimulation with the three kinds of DCs (data not shown). These results suggest that although B7-DC is the preferred ligand for PD-1 recruitment to the synapse, the decreased expression levels of B7-DC coupled with the complete deficiency of B7-H1 allow greater T cell activation and cytokine secretion than the absence of B7-DC alone.

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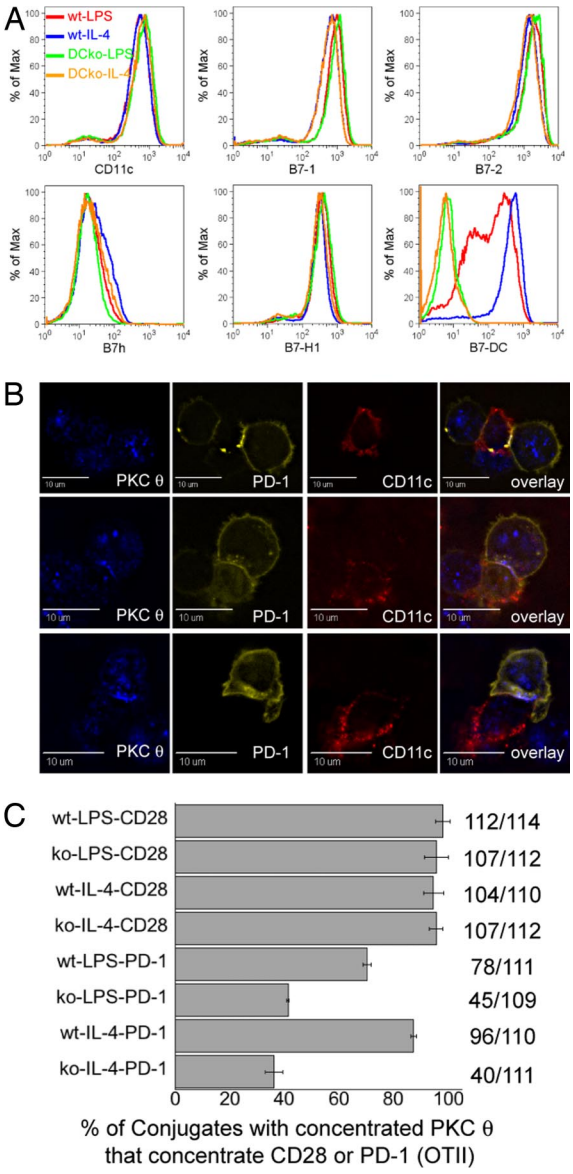

Fig. 3. B7-DC may be the main ligand responsible for the concentration of PD-1 at the immunological synapse. (*A*) Splenic DCs from WT or B7-DC$^{-/-}$ B6 mice were purified and cultured as in Fig. 2. The WT DCs are in red (LPS) and blue (IL-4), and the B7-DC$^{-/-}$ cells are in green (LPS) and orange (IL-4). (*B*) OT-II T cells were infected with CD28-YFP or PD-1-YFP. Conjugates with concentrated PKC θ (blue) at the synapse were scored for the localization of either costimulatory molecule (yellow) to the synapse. CD11c is in red. (*Top*) PD-1 at the synapse between OT-II T cells and WT DCs. (*Middle* and *Bottom*) Conjugates between OT-II T cells and B7-DC$^{-/-}$ DCs. A representative cell with concentrated PD-1 is in *Middle*, and a cell with uniform distribution of PD-1 is in *Bottom*. (*C*) The statistics from three experiments with at least 30 conjugates are shown as mean and SD (error bars). The numbers after the bars are the same as in Fig. 2.

CD28 and PD-1 Concentrate at the Immunological Synapse with Similar Kinetics. To further clarify the mechanism of PD-1 accumulation to the synapse, we compared the kinetics of CD28-CFP (cyan fluorescent protein) and PD-1-YFP recruitment with the synapse. During live imaging experiments using coinfected OT-II T cells and peptide-pulsed WT B6 DCs cultured in IL-4, CD28 and PD-1 accumulated simultaneously at the synapse of 48 of 60 cells (80%) and remained concentrated there for the length of imaging (SI Fig. 8 and SI Movie 1). This colocalization was sometimes in patches at the site of T cell/DC contact (SI Fig. 8, 16:20 time point),

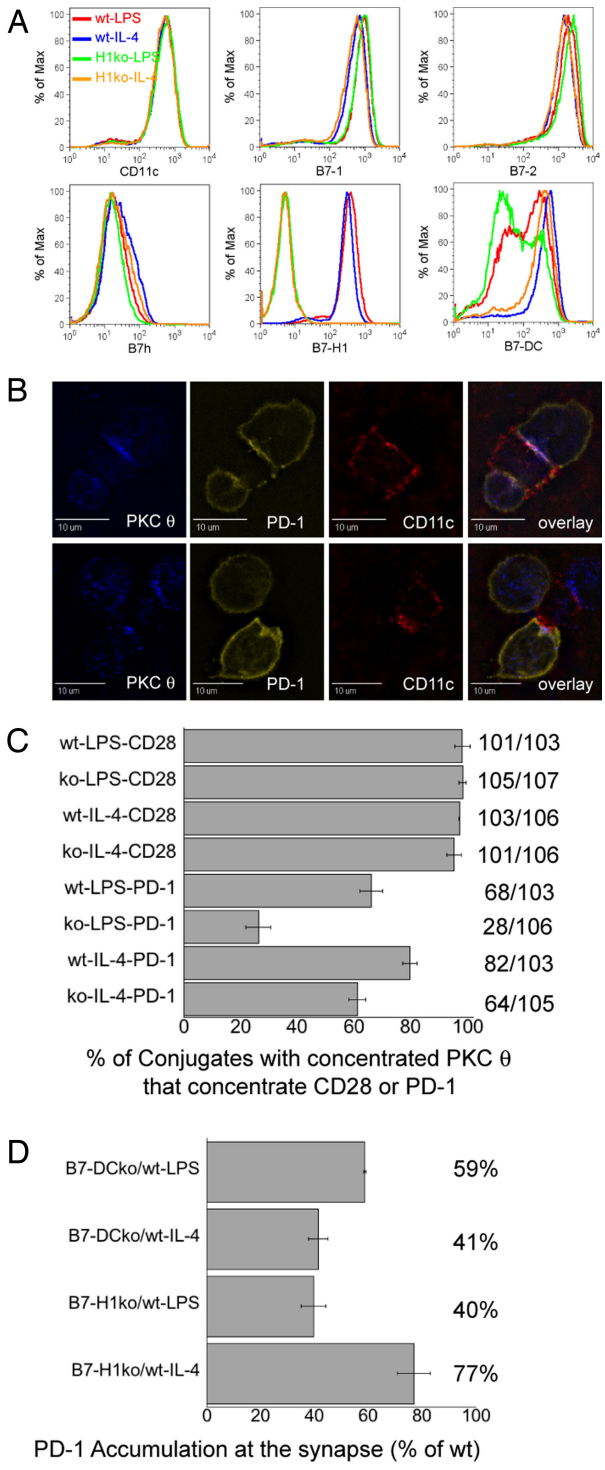

Fig. 4. In the absence of high B7-DC levels, B7-H1 is also important for the concentration of PD-1 at the immunological synapse. (*A*) Splenic DCs from WT or B7-H1$^{-/-}$ B6 mice were purified and cultured as in Fig. 2. The WT DCs are in red (LPS) and blue (IL-4), and the B7-H1$^{-/-}$ cells are in green (LPS) and orange (IL-4). (*B*) OT-II T cells were infected with CD28-YFP or PD-1-YFP. PKC θ is in blue, PD-1 is in yellow, and CD11c is in red. The images show conjugates using B7-H1$^{-/-}$ DCs. (*Upper*) A representative cell with concentrated PD-1. (*Lower*) A cell with uniform distribution of PD-1. (*C*) The statistics from three experiments with at least 30 conjugates are shown as mean and SD (error bars). The numbers after the bars are the same as in Fig. 2. (*D*) The ratios of the fractions of concentrated cells (knockout DCs relative to WT DCs) from three experiments using OT-II T cells and WT, B7-H1$^{-/-}$, or B7-DC$^{-/-}$ DCs that were treated with GM-CSF and either LPS or IL-4 are shown as mean and SD (error bars).

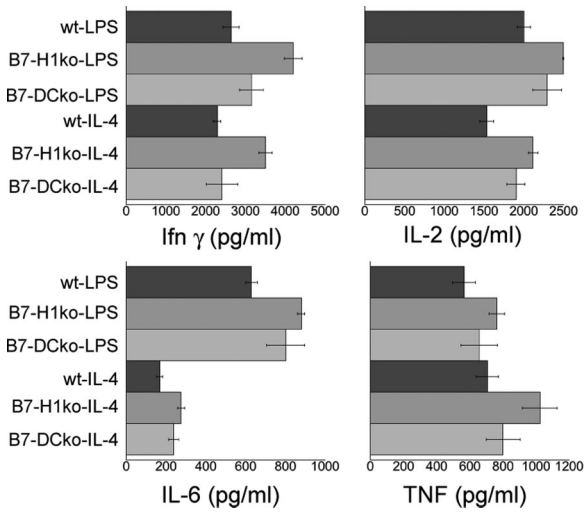

Fig. 5. Activated OT-II T cells secrete larger amounts of cytokines upon restimulation with peptide-pulsed B7-H1$^{-/-}$ DCs than when stimulated with B7-DC$^{-/-}$ or WT DCs. Day-8 activated OT-II T were restimulated at a 1:1 ratio with irradiated LPS- or IL-4-activated DCs from B6 WT, B7-H1$^{-/-}$, or B7-DC$^{-/-}$ mice. Supernatants were harvested 24 h after restimulation and assayed for cytokines by using cytometric bead array kits. A representative cytokine secretion profile from the restimulations using 4 nM OVA peptide is shown. The graphs show mean and SD (error bars) from triplicate wells in one of five independent experiments. IFN-γ, IL-2, IL-6, and TNF were the only detectable cytokines.

whereas in other cells the two molecules appeared to form a bona fide immunological synapse (SI Fig. 8, from 21:00 on). In 5 of the 60 cells (8%), CD28-CFP and PD-1-YFP initially concentrated simultaneously in the same regions, but at later time points PD-1 became uniformly distributed around the plasma membrane, whereas CD28 remained concentrated at the synapse (SI Fig. 9 and SI Movie 2). Finally, in 7 of the 60 cells (12%), CD28 became concentrated at the synapse but PD-1 remained uniform for the length of imaging (SI Fig. 10 and SI Movie 3).

Discussion

The goal of our study was to examine the intracellular localization of PD-1 in activated CD4$^+$ T cells and to determine whether B7-H1 and B7-DC differ in their ability to concentrate PD-1 at the synapse. In day-3 activated T cells, PD-1 was present at the plasma membrane, near the Golgi, and in the TGN (Fig. 1 *A* and *B*), suggesting that after its synthesis, PD-1 traffics through these compartments en route to the plasma membrane. PD-1 did not appear to extensively localize to recycling endosomes (Fig. 1 *C* and *D*), nor to LAMP1$^+$ lysosomes (Fig. 1*E*), suggesting that either PD-1 is internalized via a different endocytic pathway, or that once it enters those compartments, it loses this particular antibody epitope. This intracellular localization is distinct from that of CD28 and CTLA-4 (21) and could account for the observed rapid up-regulation of PD-1 induced by TCR stimulation of T regulatory cells (17).

Because of limited molecular data on the ligand-binding domain of PD-1 (22), we used the preferential up-regulation of B7-H1 and B7-DC on splenic DCs that had been activated with either LPS or IL-4 as a way to determine whether one ligand is better at recruiting PD-1 to the synapse. Our observation that the synaptic concentration of PD-1 is always higher when the activated T cells interact with IL-4-cultured DCs expressing high levels of B7-DC relative to LPS-treated DCs that have more B7-H1 suggests that B7-DC is the preferred ligand for PD-1 accumulation at the synapse. This trend was observed with 5C.C7, OT-II, and DO11.10 TCR transgenic T cells (Figs. 2–4 and SI Fig. 7) and is consistent with the higher affinity of PD-1 for B7-DC relative to B7-H1 (16).

To test this hypothesis directly, we used DCs from B7-DC$^{-/-}$ mice on two different genetic backgrounds, both of which were significantly impaired in their ability to concentrate PD-1 at the synapse, especially when the DCs were cultured in IL-4 (Fig. 3 and SI Fig. 7). The fact that the higher-affinity ligand is better at recruiting PD-1 to the synapse is reminiscent of our earlier observation that B7–1, which has higher affinity and avidity for CTLA-4 than B7–2, is the main ligand for CTLA-4 concentration at the synapse (15).

An unexpected difference in the case of PD-1 was the finding that B7-H1 is also very important for the synaptic concentration of PD-1, especially when the DCs have low B7-DC levels after LPS activation (Fig. 4). It should be reiterated, however, that the B7-H1$^{-/-}$ DCs also expressed less B7-DC than WT cells, complicating the analysis of the individual contribution of each ligand to PD-1 recruitment to the synapse. Furthermore, the relatively minor defect in PD-1 concentration at the synapse when the B7-H1$^{-/-}$ DCs were treated with IL-4 to up-regulate B7-DC (Fig. 4) suggests that a certain minimal amount of B7-DC is both necessary and sufficient to trigger the extensive synaptic concentration of PD-1. However, in the absence of high B7-DC levels, B7-H1 is critical for PD-1 accumulation at the synapse.

Surprisingly, even though B7-DC was better at recruiting PD-1 to the immunological synapse and should have caused stronger T cell inhibition, activated OT-II T cells secreted more IFN-γ, IL-2, IL-6, and TNF upon restimulation with B7-H1$^{-/-}$ DCs compared with B7-DC$^{-/-}$ DCs (Fig. 5). This difference could be caused by the reduced B7-DC expression on B7-H1$^{-/-}$ DCs. Alternatively, the imperfect correlation between the differential synaptic recruitment of PD-1 and the relative inhibition of cytokine production mediated by B7-H1 or B7-DC could reflect a superimposed effect caused by the engagement of a second, as yet unidentified, costimulatory receptor (7). Finally, the recent report that B7-H1, but not B7-DC, can specifically bind B7–1 and inhibit T cells (23) provides an additional explanation for the observed discrepancy. The absence of B7-H1 from DCs might eliminate two inhibitory pathways for activated T cells, one via PD-1 and the other via B7–1. In contrast, the absence of B7-DC would only affect the former pathway, while leaving the second one intact. This could result in greater inhibition of T cell activation upon stimulation with B7-DC$^{-/-}$ DCs relative to B7-H1$^{-/-}$ DCs, even though less PD-1 is recruited to the synapse in the absence of B7-DC, as observed in our study.

Our live cell imaging experiments revealed that the kinetics of PD-1 recruitment to the synapse are very similar to those of CD28. In the majority of the cells, both CD28 and PD-1 initially concentrated in the same regions of the T cells that were in contact with DCs, whereas at later time points PD-1 was sometimes observed to become more uniformly distributed (SI Figs. 8 and 9 and SI Movies 1 and 2). These observations are consistent with a model of PD-1 recruitment similar to the one already proposed for CD28 and CTLA-4 (15, 24). TCR engagement by antigenic peptide–MHC complexes triggers the reorganization of the actin cytoskeleton that leads to the initial concentration of CD28 and PD-1 at the immunological synapse. B7-H1 and B7-DC can then stabilize PD-1 at the synapse, either because they prevent its diffusion away from the interface or because they generate a signal that leads to further PD-1 accumulation, possibly by its release from the observed intracellular compartment. The higher affinity of B7-DC for PD-1 correlates with its superior ability to stabilize PD-1 at the synapse relative to B7-H1.

The fact that in the absence of high levels of B7-DC, B7-H1 is critically important for the concentration of PD-1 at the synapse is consistent with a number of studies showing that B7-H1 provides an important inhibitory signal to T cells. This is especially true for CD8$^+$ T cells, where high PD-1 levels on virus-specific T cells have been shown to correlate with functional exhaustion and antibody-mediated B7-H1 blockade during chronic viral infection restores T cell proliferation, cytokine

secretion, and cytolysis (25). Similarly, B7-H1 expressed by nonhematopoietic cells in the pancreas could delay disease in a diabetes model, whereas B7-H1 and B7-DC on nonlymphoid hematopoietic cells could not (26). Finally, multiple studies have reported that B7-H1 expression by B7-DC-negative tumors provides an important mechanism for tumor escape from immune destruction (1). All of these reports emphasize the important inhibitory signal for T cell function mediated by B7-H1 in the absence of B7-DC. However, in disease models involving T helper (Th) 2-dependent cytokine production and the upregulation of B7-DC (e.g., asthma and experimental allergic conjunctivitis), anti-B7-DC, but not anti-B7-H1, antibodies exacerbate disease, suggesting that B7-DC is the main ligand mediating inhibition (27, 28).

The combined data from multiple studies, along with our own observations presented here, are consistent with partially redundant functions of the PD-1 ligands, where both B7-H1 and B7-DC are capable of inhibiting T cell responses via PD-1. Because B7-DC is better able to recruit PD-1 to the synapse because of its higher affinity relative to B7-H1 (16), it can mediate stronger T cell inhibition in disease models that involve high surface levels of B7-DC on professional APCs (asthma, conjunctivitis). In contrast, in cases of low B7-DC expression (diabetes, tumor models, chronic viral infection?), B7-H1 is the more important ligand mediating T cell inhibition. Such a mechanism for PD-1 recruitment to the immunological synapse is somewhat different from the one observed in the case of CD28 and CTLA-4, where the two costimulatory molecules have distinct ligand preferences, rooted in the defined hierarchy of receptor/ligand affinities and avidities. The observations presented here provide a mechanism and a rationale for the disparate results obtained in the numerous studies of the effects of anti-B7-H1 and anti-B7-DC antibodies on disease incidence and severity in mouse models of autoimmunity. Together with our earlier report that Th1 and Th2 cells can modulate the expression of B7-H1 and B7-DC on mouse macrophages (3), the present study underscores the complex interplay between molecules expressed by cells from both the innate and the adaptive arms of the immune system.

Materials and Methods

Conjugate Formation and Fluorescence Microscopy. DCs from B10.A, B6 WT, B7-H1$^{-/-}$, B7-DC$^{-/-}$, BALB/c WT, or B7-DC$^{-/-}$ mice were activated with LPS or IL-4 in the presence of GM-CSF and 5 μM moth cytochrome *c* or ovalbumin (OVA) peptide for 16 h. Conjugates between the DCs and infected 5C.C7, OT-II, or DO11.10 T cells were formed for 20 min at 37°C, subsequent to which the cells were adhered to slides, fixed, and processed for fluorescence microscopy as described (15).

Cells were imaged on a DMIRB/E inverted microscope (Leica, Wetzlar, Germany), using a 1.32 NA ×63 oil-immersion objective as described (15). Image stacks consisting of 15–20 planes spaced 0.5 μm were collected with a Photometrics CoolSNAP HQ camera (Roper Scientific, Tucson, AZ), operated by the Slidebook software (Intelligent Imaging Innovations, Denver, CO). The image stacks were deconvolved by using the nearest-neighbor algorithm of Slidebook.

Live cell experiments were performed with a heated stage system (Bioptechs, Butler, PA) set to 37°C. WT B6 DCs were cultured in 5 μM OVA, GM-CSF, and IL-4 for 16 h before addition to a heated culture dish in RPMI medium 1640 plus 3% FCS and 25 mM Hepes. OT-II T cells expressing CD28-CFP and PD-1-YFP were then added to the dish. Bright-field, CFP, and YFP images were acquired approximately every 20 s by using the ×63 objective.

***In Vitro* Restimulation Experiments.** OT-II T cells were activated and rested overnight in RPMI medium 1640 without IL-2 on day 7 after activation. DCs from B6 WT, B7-H1$^{-/-}$, or B7-DC$^{-/-}$ mice were purified by CD11c positive selection, activated with GM-CSF and either LPS or IL-4 for 16 h, and then irradiated (2,000 rads). Activation cultures contained equal numbers of T cells and DCs. The OVA peptide was titrated from 0.5 μM to 0.8 nM. Supernatants were harvested 24 h after restimulation and assayed for cytokines secreted by the OT-II T cells by using cytometric bead array kits (BD Biosciences, San Jose, CA) for mouse Th1/Th2 cytokines (simultaneously measuring IL-2, IL-4, IL-5, IFN-γ, and TNF) and the mouse inflammation kit (simultaneously measuring IL-6, IL-10, IL-12p70, IFN-γ, TNF, and monocyte chemoattractant protein 1). The assays were performed according to the manufacturer's instructions. For additional details, see *SI Text*.

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