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Co-inhibitory receptor programmed cell death protein 1 targets co-stimulatory CD28

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T-cell inhibitory receptors balance the activation signals provided by peptide/major histocompatibility complex and costimulatory receptors (1). The interplay of activation and inhibition signals directs immunoregulatory functions that control T-cell memory, differentiation, and exhaustion. Several inhibitory receptors are expressed by T lymphocytes and have non-redundant roles in regulating these functions. Functional manipulations of these receptors, primarily with use of blocking antibodies (2) and dominant negative receptors (3), are being explored in the context of autoimmunity and cancer treatment, and have yielded promising results.

Programmed cell death protein 1 (PD-1) is an inhibitory receptor that upregulates following T-cell activation and was originally described as being overexpressed in apoptotic cells (4). *In vitro* experiments have shown that this receptor exhibits inhibitory activity in B and T cells—cytokine production, proliferation, and cytotoxic activity (5,6). Furthermore, PD-1 knockout mice exhibited an increased frequency of autoimmune disease; C57BL/6 mice developed glomerulonephritis and arthritis (7,8), and BALB/c mice developed cardiomyopathy (9), both of which reinforced the inhibitory role of this receptor. The seminal work led by Dr. Rafi Ahmed has demonstrated that, in a model of chronic lymphocytic choriomeningitis virus infection, the exhausted CD8+ T cells, which are functionally impaired due to frequent restimulation, showed increased levels of PD-1. The effector function of these T cells was recovered by blocking the interaction of this receptor with its ligand (PD-L1) and using an antibody; this showed that PD-1 has a major role in maintaining the exhausted phenotype (10). This work has laid the foundation for the development of PD-1/PD-L1 blocking antibodies and their use in the clinic where they have exhibited efficacy in the treatment of diverse types of cancer; it has been approved to treat melanoma, B-cell lymphoma, bladder cancer, lung cancer, and head and neck cancer (11).

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Despite this important role in T-cell biology and the development and clinical use of blocking antibodies, little is known about the signaling pathways activated by PD-1 and which proteins are recruited to its cytoplasmatic domains. This limits the potential improvements that can be applied to anti-PD1 therapies. Hui *et al.*, from Drs. Mellmann's and Vale's group, recently described a new mechanism involved in PD-1 inhibitory receptor signaling that shows that the CD28 receptor is the primary target for dephosphorylation by PD-1-recruited SHP-2 phosphatase (12). This study provides a new perspective in understanding the inhibitory signals induced by PD-1 in T cells and has implications for cancer immunology and immunotherapy.

Several studies have attempted to biochemically elucidate the PD-1 pathway; some of these studies have achieved distinct results (12,13). PD-1 has two tyrosine-based conserved motifs in the cytoplasmatic domain called immunoreceptor tyrosine-based inhibition motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). Upon interaction with one of its ligands (PD-L1 or PD-L2), these domains are phosphorylated by Lck kinase and can recruit proteins that inhibit proximal T-cell receptor (TCR) signaling (14). Early studies with human T cells showed that phosphatases—SHP-1 and SHP-2, but not SHIP—are recruited by these motifs and that ITSM was crucial for this interaction (13). A second study also showed evidence of SHP-1 and SHP-2 recruitment, and demonstrated that proximal signaling components, such as ZAP70, CD3ζ, and PKCθ, were dephosphorylated after PD-1 engagement (15). However, some studies have demonstrated that only SHP-2 is recruited by PD-1 microclusters and that they colocalize with TCRs to inhibit T-cell activation (16,17). PD-1 was also shown to inhibit several downstream signaling pathways associated with cell cycle progression, proliferation, and survival such as AKT, Ras/MAPK (18), and Bcl-xL (13). While these multiple observations have contributed to our understanding of the role of PD-1 inhibition, the precise target of PD-1 has been elusive.

Using an artificial membrane system called the large unilamellar vesicles (LUVs), Hui *et al.* investigated the PD-1 pathway by reconstituting key components of the TCR signaling pathway and evaluating which proteins are recruited and will interact with the PD-1 cytoplasmatic domain. This cell-free system allows for the addition of both soluble and membrane proteins at their physiologic levels in an effort to mimic the signaling events that occur within the T-cell membrane. The interaction was analyzed using a technique called fluorescence resonance energy transfer (FRET) where a pair of proteins of interest are loaded with different fluorophores; the excitation of the donor fluorophore emits energy in the form of a photon that can excite the acceptor fluorophore if these proteins are close enough (i.e., in the case of an interaction). The interaction can be detected by measuring a decrease in the fluorescence of the donor fluorophore or an increase in the fluorescence emitted by the acceptor fluorophore. Despite lacking the complexity of cellular systems, this artificial system allows for a cleaner assessment of protein-protein interactions in a controlled environment. It also allows for the testing of different protein concentrations and their impact on signaling output.

The authors have shown that Lck—which is a tyrosine kinase involved in the first steps TCR signaling—phosphorylated the PD-1 cytoplasmatic domain. Phosphorylated tyrosines in ITIM and ITSM form docking sites for proteins containing SH2 domains. This prompted the

authors to test different proteins (SHIP-1, Grb2, Csk, ZAP70, SHP-1, and SHP-2) that have this domain. Among these, SHP-2 showed the highest interaction with the phosphorylated PD-1 cytoplasmatic domain and both ITSM and ITIM were necessary for the interaction; this is in contrast with some results that have been reported in the literature (13). Using increased concentrations of PD-1, they showed that, while SHP-1 was also recruited, the preference for SHP-2 was 29-fold higher.

In an experiment designed to evaluate the targets of SHP-2 activity, several key components of the proximal TCR signaling pathway (ICOS, CD28, LAT, CD3ζ, p85a, ZAP70, Gads, and SLP76) were added to the LUV membrane in their physiologic concentrations. This experiment showed that CD28 is the preferential target for SHP-2 dephosphorylation, demonstrating a much more pronounced dephosphorylation compared with previously described targets of the PD-1 pathway such as CD3ζ and ZAP70. Even though ICOS has a similar intracellular motif (YxxM) it was not dephosphorylated, which showed significant specificity to SHP-2 activity. CD28 dephosphorylation occurred even at very low concentrations of PD-1 and showed a dose-dependent behavior that increased with higher PD-1 levels. Surprisingly, CD3ζ was barely dephosphorylated and ZAP70 dephosphorylation was only observed at higher PD-1 concentrations. These results were further corroborated in OT-I CD8+ cells where the authors showed that PD-1 colocalizes with CD28 after TCR/CD28 triggering. Moreover, Jurkat T cells that express PD-1 after coincubation with Raji PD-L1+ cells showed pronounced and fast (~2 min) dephosphorylation of CD28 with minimal alterations in CD3ζ, ZAP-70, SLP76, and LAT. Using the same system, the authors also showed that there was a decrease in the recruitment of the PI3K SH2 domain to CD28.

The results presented in this publication provide a new understanding about the pathways inhibited by PD-1. The preferential inhibition of a costimulatory pathway over CD3ζ and ZAP70—which are two proteins that have fundamental roles in T-cell activation—suggests that CD28 has an active role in the maintenance of T-cell effector function. This hypothesis is in line with recent results published in the same issue of *Science* that showed that CD28 signaling is crucial to the recovery of T-cell effector function after treatment with PD-1/PD-L1 blocking antibodies (19). Moreover, lung cancer patients have exhibited increased CD8+ PD-1+ T cells in their blood after treatment with PD-1 blockade with most of these cells expressing CD28 (20). The early increase in frequency of this population was associated with a clinical benefit in patients.

Since tumor cells generally do not express CD80 and CD86 (the ligands for CD28), these ligands may be provided by cells present in the tumor microenvironment such as infiltrating antigen presenting cells (APCs). There is a recent report showing that response to PD-L1 blocking therapy is related to the infiltration of dendritic cells (DCs) that were shown to express CD86 (21). Another possibility is that the antitumor T cells receive CD80/CD86 signals from APCs residing in the lymph nodes. There is evidence in the literature that removal of tumor-draining lymph nodes prevents the antitumor response induced by PD-L1 blockade in the MC38 tumor model (22). Supporting this notion is a recent paper that describes a CD8+ PD-1+ T-cell subpopulation that resides in lymph nodes, has a gene signature that resembles memory precursors/hematopoietic stem cells, and proliferates in

response to PD-1 blockade. This population also expresses CD28 and generates most of the terminally differentiated exhausted T cells responsible for the antitumor activity while maintaining its stemness (23).

The results of Hui *et al.* suggest that PD-1+ T cells are being inhibited mainly by dephosphorylation of the CD28 costimulatory receptor. While CD28 is not expressed in terminally differentiated T cells, its expression can be detected in less differentiated T-cell subsets. Based on the results discussed above, one possibility is that PD-1-blocking antibodies may be acting upon a specific CD28+ T-cell subset that has not yet exhausted, which is important for the generation of terminally differentiated effector T cells and amplification of the response. This idea counteracts the classical view that PD-1-blocking antibodies induce a reversal of the exhaustion phenotype, which has yet to be confirmed in different models. Furthermore, understanding the role of PD-1 in relation to costimulatory domains provides a rationale to develop next-generation adoptive T-cell therapies for cancer (24). Given the importance of CD28 signaling for CD4 and CD8 T cells, it remains to be seen if these findings about the PD-1 pathway can be applied to both subpopulations (25). Based on these results, future combinational approaches, involving manipulations of PD-1 and CD28 pathways, have the potential to increase the efficacy of immunotherapy.

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