Dimers Aren't Forever: CD80 breaks up with PD-L1

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Targeting the CTLA-4 and PD-1 "checkpoints" is an effective treatment for a number of cancers. In this issue of Immunity, *Hui et al* reveal that interaction between a CTLA-4 ligand, CD80, and its counterpart in the PD-1 pathway, PD-L1, affects both PD-1 and CTLA-4 function, raising new questions about the biological effects of using checkpoint inhibitors alone and in combination.

T cell immune checkpoints are regulatory pathways that limit the activity of T cells, often preventing autoimmune consequences. The CTLA-4 pathway was originally identified as an essential checkpoint from studies of *Ctla-4*$^{-/-}$ mice that suffer from fatal perinatal autoimmunity. Similarly, PD-1-deficient mice also have a propensity for autoimmunity albeit with somewhat milder effects compared to CTLA-4. The appreciation that such inhibitory pathways could be manipulated to enhance T cell responses to cancer ultimately led to the 2018 Nobel Prize for physiology and medicine to Allison and Honjo. Surprisingly, despite successful therapeutic manipulation, the interactions between these pathways and the molecular controls that dictate their function are still rather poorly understood. In this issue of Immunity, *Hui et* al provide new insights into this important issue.

Whilst CTLA-4 and PD-1 pathways are both important negative regulators of T cell responses, there are some clear differences between them. CTLA-4 is part of a complex system involving two ligands - CD80 and CD86 - that are shared with a second receptor, CD28. Whereas CTLA-4 is inhibitory to T cell responses, CD28 is an activating receptor, which alongside the T cell receptor provides costimulatory signals that are required for effective T cell and B cell immunity. Indeed, evidence suggests that the role of CTLA-4 is to directly oppose the activating function of CD28 and that loss of control of this pathway results in activation of self-reactive T cells. One mechanism by which CTLA-4 can effect such control is by the physical removal of their shared ligands from APCs in a process known as transendocytosis(Qureshi et al., 2011). Since CTLA-4 is highly expressed on regulatory T cells (Treg) and is required for their function(Wing et al., 2008), a plausible model is that Treg-expressed CTLA-4 regulates CD80 and CD86 levels on APCs, thereby limiting T cell costimulation in the steady state.

In contrast to CTLA-4, PD-1 appears to act as a more straightforward inhibitory receptor, acting in a cell-intrinsic manner to inhibit the cells that express it. Increased expression of PD-1 following T cell activation permits engagement by two its ligands - PD-L1 and PD-L2 - resulting in inhibitory signaling centered around ITIM and ITSM

motifs and the recruitment of the SHP-2 phosphatase. Consequently, T cells expressing PD-1 (potentially all activated T cells) can be restrained by its engagement. Unlike CTLA-4, which appears to operate early in immune responses, PD-1 is frequently associated with T cell exhaustion and may function at later stages in the immune response following chronic exposure to antigen, e.g. during viral infection or at tumor sites. Thus, blockade of CTLA-4 and PD-1 were initially thought to affect separate processes that could be manipulated independently in immunotherapy.

However, recent findings now suggest significant crosstalk between the CTLA-4 and PD-1 pathways. Over a decade ago Freeman and colleagues identified a physical interaction between CD80 and PD-L1(Butte et al., 2007). The significance of this "ligand-ligand" interaction, initially thought to take place between cells, *in trans*, was difficult to grasp as neither molecule has clear signaling capabilities. However, it now seems that CD80-PD-L1 interactions occur on the same cell membrane - *in cis* (Chaudhri et al., 2018). Moreover, the *cis* interaction between CD80 with PD-L1 prevents PD-1 engagement, thereby enhancing immune responses (Sugiura et al., 2019). That CD80-PD-L1 binding inhibits PD-1 engagement is in keeping with features evident from structural studies. PD-1-PD-L1 binding has an unusual interface whereby the IgV domains of PD-1 and PD-L1 interact at an acute angle allowing a more side-by-side interface(Lin et al., 2008). This places the PD-1 binding site on the side of the PD-L1 molecule, thereby exposing it to interference from an interacting partner expressed in the same membrane (e.g. CD80 - **see Figure 1**). Whilst CD80 normally exists as a homodimer in the membrane, the affinity of CD80 for PDL1 is greater than its affinity for itself, indicating that CD80-PD-L1 heterodimers may be preferentially formed over CD80 homodimers.

Using a FRET approach Hui *et al.,* demonstrate this cis-interaction between CD80 and PD-L1 in the membrane and, in agreement with Sugiura et.al., show that this prevents PD-1 binding to PD-L1. What then is the impact of PD-L1 on the binding of CD80 to its own receptors - CD28 and CTLA-4? Here the work by Hui *et.al.,* reveals interesting new concepts. At first glance one might expect that PD-L1 would have little impact on CD80 binding to its receptors, since structural studies place the CD80-CTLA-4 interaction (Stamper et al., 2001) on the opposite face of the molecule to the PD-L1 site. Hui *et al* tested these interactions using a number of different approaches. TIRF microscopy revealed that the introduction of PD-L1 into supported bilayers containing CD80 does not disrupt CD28 clustering, indicating no effect on CD28. The surprise came when they looked at CD80-CTLA-4 interactions. Here, the introduction of PD-L1 alongside CD80 reduced CTLA-4-Ig binding to Raji cells. This could be reversed by the introduction of the clinically approved anti-PD-L1 antibody (Atezolizimab), which blocks the CD80-PD-L1 interaction **(Figure 1)**. The authors go on to show that mutation of the CD80 dimer interface and the introduction of PD-L1 both have similar effects, reducing CD80 FRET and leading them to conclude that PD-L1–CD80 interactions specifically disrupt the CD80 homodimer. Furthermore, the authors also used a CTLA-4 transendocytosis assay to show that CTLA-4 can specifically reduce CD80 expression on Raji cells and that this downregulation is inhibited by PD-L1 co-expression and restored by Atezolizimab. Together these data suggest that PD-L1 influences CTLA-4 function by affecting its interaction with CD80. These concepts are supported by *in vivo* experiments where tumor infiltrating APCs are stained for CD80 before or after anti-PD-L1 treatment. Here again the inclusion of anti-PD-L1 antibody reduced CD80 expression, consistent with improved targeting of CD80 by CTLA-4. Conversely, anti-CTLA-4 Ab increased CD80 surface expression in the presence of PD-L1, although it should be noted that anti-CTLA-4 can achieve this independently (Ovcinnikovs et al., 2019).

That the CTLA-4-CD80 interaction is disrupted by PD-L1 whereas CD28 binding to CD80 remains intact is intriguing. This initially seems paradoxical given the superior affinity of CTLA-4 for CD80 compared to CD28 (Collins et al., 2002). In effect, a strong interaction is being disrupted whilst a weak one remains intact, despite CD28 and CTLA-4 sharing overlapping ligand binding sites. However, it is important to realise that whilst CTLA-4-CD80 interactions are impacted by PD-L1, they are not in fact blocked (in contrast to PD-L1-PD-1 interactions). Indeed, the authors show that monomeric CTLA-4 is not prevented from binding to CD80-PD-L1 complexes in biacore assays. The answer therefore appears to relate to the fact that CD80-CTLA-4 binding is normally a highly avid dimer-dimer lattice. The disruption of the CD80 homodimer by PD-L1 appears to affect the overall avidity, such that the interaction with CTLA-4 is now weakened. Since CD28 only binds monovalently to CD80 it is unaffected by whether CD80 is a homo- or hetero- dimer. A further surprise is that transendocytosis of CD80 is so markedly disrupted, given that CTLA-4 is clearly capable of transendocytosis of CD86, which is a low affinity monomeric target(Collins et al., 2002; Qureshi et al., 2011).

What are the implications of these new insights on our understanding of both pathways? As ever, with checkpoint biology there seem to be few straightforward answers. The simplest concept is that in the context of high CD80 expression, PD-L1 is no longer able to bind PD-1. Here, one would view CD80 as a regulator of the PD-1 pathway. However, the data from Hui *et al.,* provide a different perspective and suggest that PD-L1 is also a regulator of the CTLA-4 pathway. Accordingly, as PD-L1 levels increase, CD80-PD-L1 heterodimers dominate and CTLA-4 becomes less functionally active against CD80. Ultimately this could lead to activation of the PD-1 pathway via free PD-L1 but simultaneously impair CTLA-4 (but not CD28) interactions via CD80-PD-L1 heterodimers. What confounds interpretation of these ideas at present is that the functional importance of CTLA-4 interactions with CD80, compared to CD86, are still unknown. Furthermore, one needs to consider the impact of CD86 which is unaffected by PD-L1 yet can still influence both CD28 and CTLA-4 function. Whilst the authors suggest important considerations, there is as yet no simple model for predicting functional outcome of co-blockade with anti-CTLA-4 and anti-PD-L1. What is clear from these data is that blockade of PD-1 compared to PD-L1 is unlikely to be functionally equivalent. Nonetheless, the ability of PD-L1 to regulate CD80-CTLA-4 interactions is presumably not the only reason for synergy between CTLA-4 and PD-1 pathways, since this only applies to PD-L1 and not PD-1 antibodies. Ultimately it seems that there is still much more to understand about how these critical regulatory pathways intersect.

Figure legend

PD-L1 breaks up the CD80 homodimer and disrupts the CTLA-4-CD80 lattice

A). The CTLA-4-CD80 interaction is an unusually avid lattice involving covalent CTLA-4 dimers interacting with non-covalent CD80 dimers that associate in the plasma membrane. The critical site for CD80 homo-dimerisation is marked in red. **B).**CD80 can also form heterodimers with PD-L1 which affects the formation of the CTLA-4 lattice and occupies the PD-L1-PD-1 binding site shown in green. Thus CD80-PD-L1 interactions affect both the CTLA-4 and PD-1 pathways. **C).** Note the angle of binding between PD-1 and PD-L1 is unusual and allows this interaction site to be disrupted *in cis* by CD80. **D).** Atezolizimab (anti-PD-L1) binds to PD-L1 at a site which prevents CD80 binding and disrupts PD-1-PDL1 interactions. In this setting CD80 is now free to form homodimers and lattice interactions with CTLA-4 can be restored.

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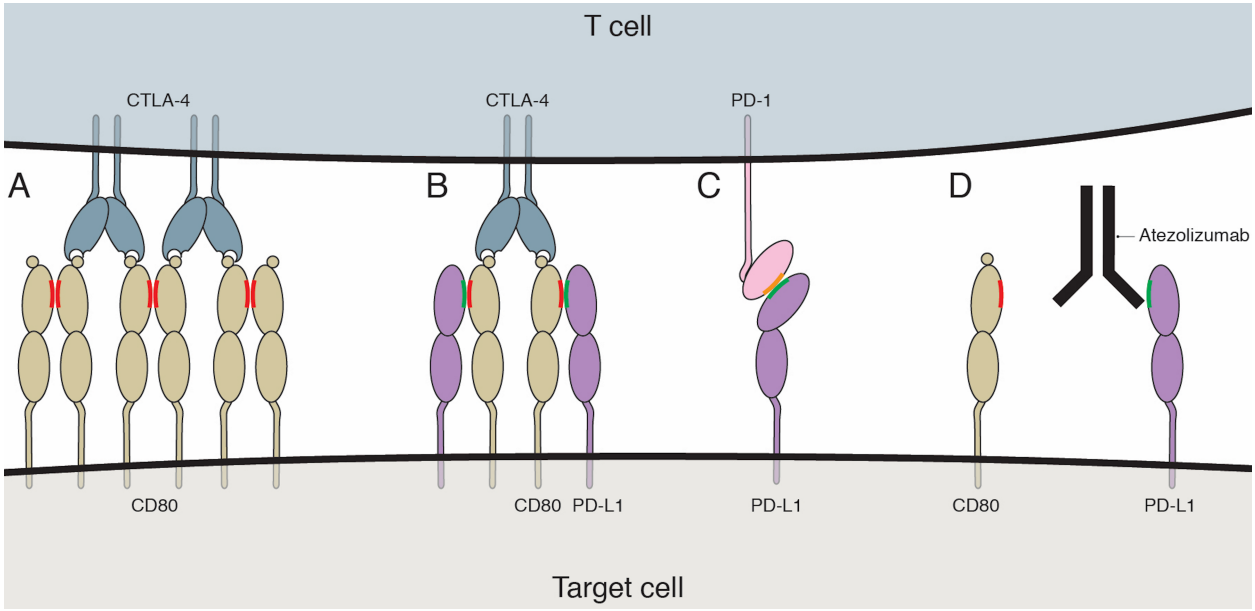

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