

# A checkpoint cliffhanger at the dawn of placental mammals

DOI 10.1074/jbc.H120.013307

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Edited by Peter Cresswell

The PD-1 ligands PD-L1 and PD-L2 are commonly expressed on the surface of cells, where they regulate immune system activation. However, the specific role played by each ligand has been unclear. Using site-directed mutagenesis, surface plasmon resonance, and crystallography, Philips *et al.* explore the distinct features of PD-L2 and identify a specific evolutionary event linked to its appearance. This work provides a deeper understanding of how the immune system adapted to mammalian placental gestation and could be an important consideration in the development of new immune checkpoint therapies.

The immune system must be tightly regulated to avoid persistent inflammation while still being able to aggressively counter infections. The corresponding “checkpoints” are provided by negative regulatory receptor-ligand pairs (Fig. 1); these have become clinically important targets in cancer immunotherapy, where their antagonists are said to “take the brakes off” the immune system. Immune system regulation is also critical for mammalian life in another way: The emergence of the placental mammals required radical changes in the immune system to enable the half-foreign fetus to implant and live attached to its mother for weeks to months in full contact with maternal blood and thus immune cells. Despite many years of wrong theories that claimed maternal immunosuppression and fetal immaturity as reasons for fetal “indulgence,” it is now clear that the maternal immune system actively tolerates the fetus (1). Moreover, emerging knowledge suggests the fetus itself is involved in negotiating its own protection. The very first molecule secreted by the trophoblast, human chorionic gonadotrophin, drives regulatory T-cell generation (2) and modulation of maternal immune cells. Which other fetal factors are relevant for T- and B-cell modulation is not entirely known. However, it is known that signaling through the checkpoint receptor PD-1 is highly relevant for tolerance, and its ligand PD-L2 is expressed in trophoblast cells (3). In an effort to understand PD-1 interactions for cancer immunotherapy applications, Philips *et al.* (4) unexpectedly identified compensating loss- and gain-of-interaction mutations in PD-L2 that were selected at this critical point in evolution such that the interaction was at points hanging by its fingernails. Their discovery that the remodeling of the PD-L2-

PD-1 interface, which leads to a pairing structurally different from but functionally equivalent to that with PD-1's other ligand PD-L1, also opens new questions regarding the full role of PD-L2.

PD-L2 has a 3-fold higher affinity for PD-1 than PD-L1, thought to be due to a tryptophan in PD-L2 creating a larger hydrophobic surface. To test this assumption, Philips *et al.* (4) used site-directed mutagenesis of human PD-L2 and PD-L1, surface plasmon resonance measurements, and crystallographic studies of a murine PD-L2/PD-1 pair. These studies revealed that the tryptophan actually decreased interaction with PD-1 when inserted into PD-L1, and substitution of this tryptophan with alanine in PD-L2 increased affinity. Thus, the authors conclude the tryptophan actually serves as an “elbow” that creates steric hindrance. The loss of interaction due to the elbow is offset by changes in the C-D loop that enable PD-L2 to latch onto PD-1, although a glycosylation site in this latch attenuates the affinity for PD-1 somewhat. The authors confirm the small difference in affinity that results from these three adjustments leaves PD-L2 equivalent to PD-L1, at least in standard assays of PD-1 function, but does differentiate the PD-L2 interface from that of PD-L1. What was the context for this structural cliffhanger that might have as easily destroyed the ancestral PD-L2's interaction with PD-1?

To understand this aspect, the authors turned next to an evolutionary analysis. They found that the primordial PD-1 ligands had duplicated in the ancestors of modern marsupials, but marsupial PD-L2 had not evolved the elbow and latch, leaving it structurally similar to PD-L1. Instead, PD-L2 acquired these mutations in the common ancestor of all placental mammals, suggesting it seems to have contributed to the success of this transition. Indeed, many adaptations were needed to enable the transition from support of embryonic development from the yolk sac to using maternal blood as a longer-term nutrient source, allowing much longer gestational periods but also fully exposing the growing fetus to the maternal immune system. Marsupials represent an intermediate step in this process where the yolk sac is still used to supply all nutrients to the embryo, and implantation in the uterine wall stops shortly before being fully exposed to the maternal blood supply. Thus, one might speculate that it was important to differentiate PD-L1 and PD-L2 interfaces as one of many changes in the immune system arising from the continuous interaction between fetal and maternal cells, also in the periphery as a consequence of microchimerism (5), and that marsupials would be a natural intermediate in this process.

The authors declare that they have no conflicts of interest with the contents of this article.

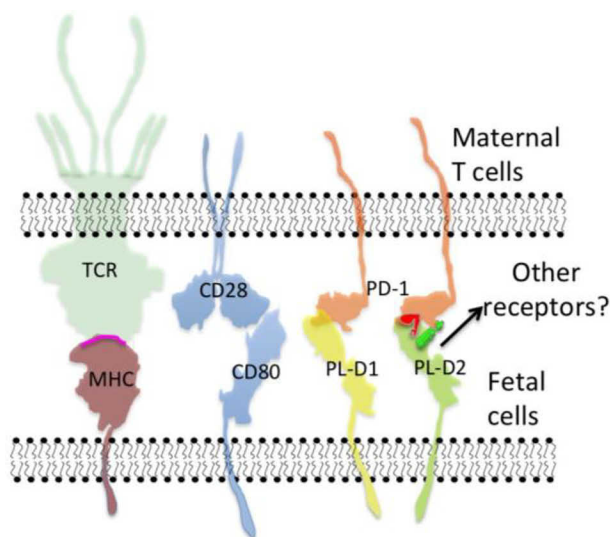
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*J. Biol. Chem.* (2020) 295(14) 4381–4382 **4381**



**Figure 1. Key antigen recognition, costimulatory, and checkpoint molecules in T cell activation and tolerance.** TCR signals are amplified by CD28-mediated co-stimulation. The PD-1 checkpoint antagonizes CD28-mediated costimulation to help maintain tolerance. The offsetting “elbow” and “latch” mutations in PD-L2 to maintain interactions with PD-1 are shown as *red* and *green* cartoons. It is possible this also gave PD-L2 the ability to interact with new receptors that have a role in T cell activation (see text).

However, other data complicate this speculative interpretation. For example, whereas PD-L2 is expressed in the mouse and human placenta (6), only antibodies to PD-L1, not PD-L2, induce pregnancy failure in mice (7). Similarly, experiments with PD-L1 knockout mice verified that PD-L1 is required in the mother for successful pregnancy (7). Thus, there is no clear answer for whether or why mutations in PD-L2 that changed the interface with PD-1 were under selective pressure.

One alternative explanation relates to a controversial area in the checkpoint field, regarding a possible positive costimulatory function of PD-L2 (its ability to enhance, rather than inhibit, T-cell activation) (8). Recently, a glycosylphosphatidylinositol-anchored ligand called Rgmb has been identified in mice that seems to account for the positive costimulatory contribution of PD-L2 (9). Interestingly, mutational analysis reveals that Rgmb uses the key latch residue in PD-L2 identified by Philips *et al.* One could speculate that the interactions with additional binding partners, particularly molecules with positive activities, might have driven the divergence of PD-L1 and PD-L2, leaving PD-L1 with a more purely negative regulatory role. It would be interesting to know whether Rgmb or other alternative PD-L1/2 ligands still exist in marsupials, such as Tasmanian devils (10). Tasmanian devils might be surprisingly useful model organisms, as transmissible tumors threaten these endangered marsupials. The effort to save this species has motivated study of immune surveillance with generation of Tasmanian devil-specific anti-PD-1 and anti-PD-L1 antibodies. These studies could be extended to determine whether Tas-

manian devils have Rgmb and whether it binds to PD-L2, which might not be expected. Perhaps PD-L2 of placental mammals needed to take on a positive role in immune defense to offset stronger immune suppression through PD-1 and PD-L1, which was needed to tolerate the placenta. Lack of placentation and facial tumors in Tasmanian devils may be limitations of the seemingly safer evolutionary strategy of avoiding the cliff edge and not radically changing PD-L2.

Philips *et al.* (4) have carried out an impressive analysis that changes the model for the PD-1/PD-L2 interaction and provides us with new clues and puzzles to understand the dramatic evolutionary events that unfolded at the dawn of placental mammals. Understanding these basic mechanisms of tolerance will help us not only to better understand immunological aspects of human pregnancy, but also to apply this knowledge in therapeutic strategies for fostering transplant tolerance and stopping immune escape mechanisms of tumors.

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