

INSIGHTS

# Costimulatory connections: CARMIL2 and CD28

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In this issue of *JEM*, Zhang et al. (<https://doi.org/10.1084/jem.20250339>) report that a gain-of-function mutation in CARMIL2, previously identified in patients, is sufficient to replace the contribution of CD28 to NF- $\kappa$ B activation and downstream effects in T cells, including in the setting of an anti-tumor response.

Work by Malissen and colleagues reported in this issue (Zhang et al., 2025) extends previous work from this group and others on an adaptor protein known as CARMIL2 (also known as RLTPR) and its critical function for mediating costimulatory signals downstream of the receptor CD28. Thus, the authors demonstrate, using a novel mouse model, that a gain-of-function mutation first identified in human CARMIL2 can substitute for most physiological functions of CD28, including in the context of anti-tumor immunity.

Early activation and proliferation of T cells requires three distinct sets of signals: one arising from interaction of the TCR with peptide/MHC complexes on APCs or target cells ("Signal 1"), another from interaction of a costimulatory receptor (e.g., CD28) with corresponding ligands on APCs or target cells ("Signal 2"), and a third from autocrine or paracrine signaling provided by a cytokine, most often IL-2 ("Signal 3"). Whether CD28 provides quantitative or qualitative signals to enhance T cell activation has been the subject of much debate, although before the discovery of CARMIL2, the preponderance of the evidence pointed to a quantitative effect, at least at the level of signaling from the receptor to the nucleus (Acuto and Michel, 2003).

One of the signaling pathways augmented by CD28 costimulation is the classical NF- $\kappa$ B pathway. In conjunction with TCR/CD3 signals, CD28 engagement enhances the protein kinase C (PKC)-mediated phosphorylation of CARMA1 (also known as Card11), which promotes its nucleation of a

protein complex that also contains Bcl10 and MALT1, commonly referred to as the "CBM" complex (Thome and Weil, 2007). Formation of this complex is required for activation of the I $\kappa$ B kinase (IKK) complex by TCR and CD28. Active IKK then phosphorylates I $\kappa$ B, leading to its turnover and the nuclear entry of NF- $\kappa$ B dimers (mostly commonly RelA/p50), which drive expression of numerous immune response genes, including those encoding the critical cytokine IL-2 (Schulze-Luehrmann and Ghosh, 2006).

Receptor-proximal signaling proteins known to participate in CD28 costimulation include PI3K, Lck, Grb2, and PKC  $\theta$ . However, none of these is unique to CD28, versus TCR and/or IL-2r. The discovery of CARMIL2 as a molecule uniquely recruited to a CD28 signaling complex represented a major advance in understanding CD28-dependent signaling. Malissen and colleagues initially identified CARMIL2 through a functional screen, using N-ethyl-N-nitrosourea (ENU) mutagenesis of mice carrying a gain-of-function allele of LAT<sup>Y136F</sup>, to discover secondary mutations that would reverse the LAT mutant phenotype (Liang et al., 2013). Thus, the mutation, a point mutation (termed *basilic*) in the leucine-rich region (LRR), not only reverted the LAT<sup>Y136F</sup> phenotype but on its own led to a defect in CD28-dependent T cell activation, as well as an impairment in the development of thymic-derived regulatory T cells (Treg). The function of CARMIL2 was further elucidated in a 2016 publication by the Malissen group, wherein they demonstrated that the scaffolding function of



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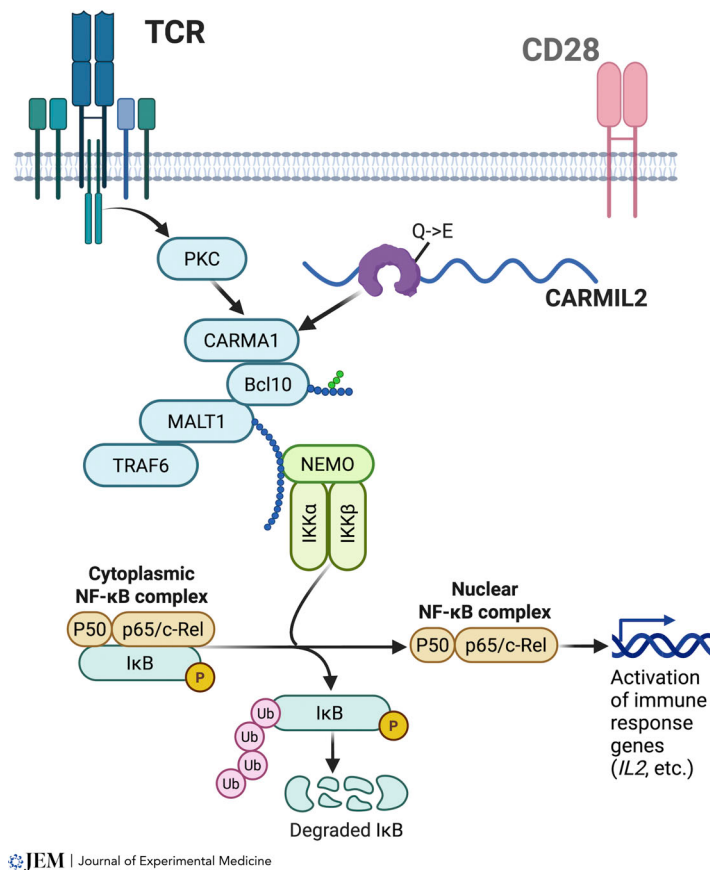
CARMIL2 is most critical for its role in CD28 costimulation (Roncagalli et al., 2016). Specifically, mutation of the N-terminal pleckstrin homology domain, LRR (as with the *basilic* mutation), or C-terminal proline-rich region was sufficient to impair rescue of CD28-dependent IL-2 production in CARMIL2 KO T cells, while mutation of the actin-capping protein interaction domain was not.

The importance of CARMIL2 for human physiology was revealed in two 2016 papers, wherein autosomal recessive mutations in CARMIL2 were found to be associated with a novel primary immunodeficiency (Sorte et al., 2016; Wang et al., 2016). The study of Casanova and colleagues reported detailed cellular and functional analyses that thoroughly delineated the impact of CARMIL2 loss-of-function on immune homeostasis, with particularly significant effects on the activation of CD4<sup>+</sup> T cells and B cells (Wang et al., 2016). Other reports identified a distinct naturally occurring mutation in

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Schematic of signaling from the gain-of-function CARMIL2 Q→E mutant to NF-κB. As described in the manuscript of Zhang et al. (2025), the Q→E mutation in the leucine-rich repeat region of CARMIL2 bypasses the contribution of CD28 to TCR-dependent NF-κB signaling. P, phosphorylation; Ub, ubiquitination. Created in BioRender. Kane (2025) <https://BioRender.com/xelg4vu>.

CARMIL2 that led to a gain-of-function phenotype, found in people with a subset of T cell malignancies known as cutaneous T cell lymphomas (CTCL) (Park et al., 2017; Uchida et al., 2021). More specifically, this is a glutamine to glutamic acid (Q→E) replacement in the LRR domain. In the present paper (Zhang et al., 2025), the authors have engineered mice to express the analogous mutation in mouse CARMIL2. These studies reveal that the Q→E mutation is sufficient to replace most known CD28 functions that require CARMIL2 in vivo, including T cell activation, production of IL-2, Treg differentiation, and anti-tumor immunity. Notably, however, the Q→E gain-of-function mutation rescued neither development of invariant natural killer T (iNKT) cells nor the full suppressive activity of Treg in CD28-deficient mice. These functions may therefore require other CD28-dependent signals, such as activation of the PI3K signaling pathway.

Interestingly, Malissen and colleagues show that Q→E CARMIL2 augments the function of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with the latter including an adoptive transfer model of tumor immunotherapy (Zhang et al., 2025). CD28 is thought to be more critical for promoting CD4<sup>+</sup> T cell responses, while 4-1BB is more important for CD8<sup>+</sup> T cells, at least under some circumstance (Watts, 2005). This is consistent with previous findings that CARMIL2 loss-of-function impacted CD4<sup>+</sup> T cells more profoundly than CD8<sup>+</sup> T cells in mice (Liang et al., 2013) and mainly CD4<sup>+</sup> T cells and B cells in humans (Wang et al., 2016). Thus, the gain-of-function allele of RLTPR analyzed in the current paper, while replacing most CD28 functions, may also be revealing additional T cell (and B cell) biology not dependent on CD28. This possibility is also consistent with a recent report that loss-of-function in CARMIL2 causes more profound immune defects in humans compared with loss of CD28 function (Lévy et al., 2023).

As reported by Malissen and colleagues, the CARMIL2 Q→E mice did not seem to develop signs of autoinflammation or malignant transformation, at least up to ~1 year after birth. This is somewhat surprising, given the link of the analogous mutation in patients to CTCL (Park et al., 2017; Uchida et al., 2021). However, in mice, the mutation is present throughout T cell development, whereas in patients with CTCL, the Q→E mutation appears to arise somatically. Thus, it may be that T cell development is also altered in these mice, which could help explain the absence of immune dysregulation. This is also interesting in the context of a recent study demonstrating that a naturally occurring fusion involving CARMA1, a protein in the same pathway as CARMIL2, confers superior activity to chimeric antigen receptor-T (CAR-T) cells, again without apparent transformation (Garcia et al., 2024). This is despite the fact that the fusion in question was first identified in patients with Sezary syndrome, a subtype of CTCL (Wang et al., 2015).

Finally, one of the more intriguing aspects of this paper is the apparent bypassing of CTLA-4 and PD-1 checkpoint control of CD8<sup>+</sup> T cells afforded by the CARMIL2 Q→E mutation (Zhang et al., 2025). Although CTLA-4 and PD-1 function by different mechanisms, their inhibitory effects appear to converge on CD28 costimulation as a target (Walker, 2017). Indeed, the effect of the CARMIL2 Q→E mutation is shown to be comparable to blockade of PD-1 in a syngeneic mouse tumor model (Zhang et al., 2025). It will be interesting to see if future studies further develop manipulation of CARMIL2 for tumor immunotherapy, including CAR-T approaches.

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