

## RESEARCH HIGHLIGHT



## Inducing Z-DNA overcomes immune checkpoint blockade resistance

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Cell Research (2022) 32:871–872; https://doi.org/10.1038/s41422-022-00705-y

**Inducing immunogenic cell death in tumors holds promise in stimulating anti-tumor immunity. A recent study published in *Nature* reported that a small molecule previously shown to induce the formation of Z-DNA activates ZBP1-dependent cell death and renders tumors sensitive to immune checkpoint blockade-based immunotherapy.**

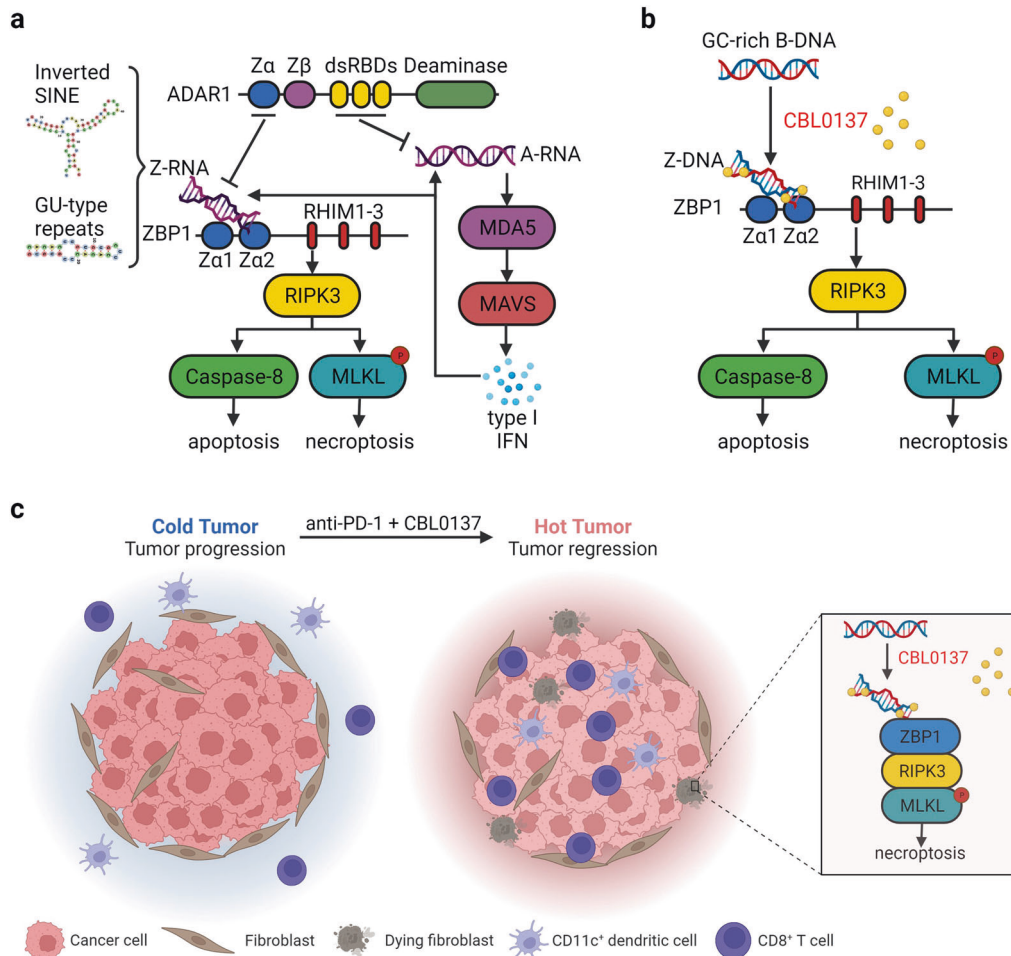
Cancer immunotherapy by immune checkpoint blockade (ICB) has achieved remarkable success in individual cases; however, these are still restricted to a small number of patients. Therefore, novel approaches to enhance anti-tumor immunity are urgently needed. Induction of immunogenic cell death, such as necroptosis, in tumor cells was shown to synergize with ICB to induce anti-tumor immune responses in mouse models.<sup>1</sup> The RNA-editing enzyme adenosine deaminase acting on RNA 1 (ADAR1) has emerged as a novel checkpoint conferring resistance to ICB therapy.<sup>2</sup> ADAR1 catalyzes adenosine-to-inosine (A-to-I) editing in RNA, which prevents the formation of long double-stranded RNA (dsRNA) that activates the cytosolic RNA sensor melanoma differentiation-associated protein 5 (MDA5) inducing type I interferon (IFN) responses. The IFN-inducible ADAR1p150 isoform possesses a Za domain that recognizes nucleic acids with a left-handed double helix structure, termed Z-nucleic acids (Z-NAs), including Z-RNA and Z-DNA. The only other protein in mammals known to harbor Za domains is the IFN-inducible protein Z-DNA-binding protein 1 (ZBP1), which has emerged as a Z-NA sensor inducing cell death, anti-viral immunity and inflammation.<sup>3</sup> This raises the possibility that ADAR1p150 might inhibit ZBP1-mediated cell death by repressing the accumulation of Z-NAs, a mechanism potentially contributing to ICB therapy resistance.

A recent study by Zhang et al.<sup>4</sup> in *Nature* reports that ADAR1 prevents the accumulation of Z-RNAs activating ZBP1 and that ZBP1 activation with a small molecule inducing Z-DNA formation could overcome resistance to ICB. Using a monoclonal antibody against Z-DNA (clone Z22, validated by the same group to also detect Z-RNA<sup>5</sup>), the authors observed gradually increasing nuclear Z-NA staining that was sensitive to RNase, but not to DNase treatment, in ADAR1-deficient cells, suggesting that Z-RNA accumulates in the absence of ADAR1. Interestingly, the accumulation of Z-RNA was dependent on and enhanced by type I IFN signaling (Fig. 1a). By sequencing Z22-immunoprecipitated RNA from IFN $\beta$ -treated ADAR1-knockout cells, the authors identified two classes of endogenous Z-RNA sources, both of which localized in 3'-untranslated regions (UTRs) of interferon-stimulated genes (ISGs). One class are dsRNAs derived from inverted short interspersed nuclear elements (SINES),

which are preferred editing substrates of ADAR1 (Fig. 1a). The other class are GU-type simple repeats capable of folding into the shape of a dumbbell, which showed little evidence of editing (Fig. 1a). Based on reconstitution experiments with catalytically inactive or Za mutant ADAR1p150, the authors concluded that ADAR1 represses Z-RNA accumulation via editing-dependent and -independent mechanisms. Using the Z22 antibody, the authors then showed that endogenous Z-RNAs co-localized with ZBP1 and were enriched in ZBP1 pull-downs in ADAR1-knockout cells, suggesting that Z-RNA is a ligand for ZBP1. Moreover, IFN $\beta$  treatment triggered rapid ZBP1-mediated apoptosis and necroptosis in ADAR1-deficient cells, suggesting that loss of ADAR1 causes Z-RNA accumulation activating ZBP1-mediated cell death (Fig. 1a). These results suggest that ZBP1-mediated cell death might contribute to the previously reported observation that ADAR1 loss in tumor cells overcomes resistance to ICB. Indeed, a recent study showed that myeloid cell-specific ADAR1 knockout could suppress tumorigenesis in a ZBP1-dependent manner, indicating that ZBP1-mediated death of ADAR1-deficient myeloid cells within the tumor microenvironment exerts anti-tumor effects possibly by stimulating anti-tumor immunity.<sup>6</sup>

Zhang et al. then searched for small molecules capable of inducing Z-NA formation to activate ZBP1-mediated cell death. In a screen, they identified CBL0137, a compound that was shown previously to induce robust Z-NA formation in the nucleus.<sup>7</sup> In contrast to ADAR1 loss, CBL0137 induced nuclear Z22 antibody staining that was sensitive to DNase but not RNase treatment, arguing that this corresponds to Z-DNA (Fig. 1b). Similar to ADAR1 loss, accumulation of Z-DNA in CBL0137-treated cells caused ZBP1-mediated apoptosis and necroptosis (Fig. 1b). Notably, in immunoprecipitation experiments, ZBP1-enriched DNA sequences largely overlapped with Z22-enriched DNA sequences in CBL0137-treated cells and were mainly mapped to the 5'-UTRs of L1Md A and L1Md T long interspersed nuclear elements (LINEs), arguing that these sequences are prone to forming Z-DNA to activate ZBP1. Using CBL0137 as a tool to trigger ZBP1-dependent cell death, the authors examined its potential anti-tumor effects using syngeneic mouse melanoma models. Intratumor injection of CBL0137 induced Z-DNA formation in cells of the tumor microenvironment including infiltrating fibroblasts. Moreover, CBL0137 treatment triggered MLKL phosphorylation in tumor stromal fibroblasts in wild-type (WT) but not *Zbp1*<sup>-/-</sup> mice. The authors further showed that combined treatment with CBL0137 and anti-PD-1 antibody, but not either agent alone, induced tumor regression in WT but not *Zbp1*<sup>-/-</sup> mice in mouse B16-F10 and YUMMER1.7 melanoma models. Mechanistically,

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**Fig. 1 Sensing of Z-NAs triggers ZBP1-dependent cell death and potentiates ICB responses.** **a** ADAR1 prevents type I IFN-mediated accumulation of dsRNA, including A-RNA and Z-RNA. Unedited inverted SINEs and GU-type simple repeats localized in ISG 3'-UTRs represent two main classes of Z-RNA, which activate ZBP1, leading to both apoptosis and necroptosis. **b** CBL0137 promotes B-to-Z transition of DNA in the nucleus, which in turn activates ZBP1-mediated apoptosis and necroptosis. **c** CBL0137 triggers ZBP1-dependent cell death, likely necroptosis, in tumor stromal fibroblasts, which synergizes with anti-PD-1 treatment to induce tumor regression. Panel **c** was adapted from "Cold vs Hot Tumors" by Biorender.com (2022), retrieved from <https://app.biorender.com/biorender-templates>. The figure was created with Biorender.com (License No.: DG2426M4R2).

the authors could show that CBL0137 in combination with anti-PD-1 induced the recruitment of activated CD8<sup>+</sup> T cells and CD11c<sup>+</sup> dendritic cells into the tumors to potentiate ICB responses *in vivo*, which depended on ZBP1 expression in host cells (Fig. 1c). Interestingly, intratumor CBL0137 injection combined with anti-PD-1 triggered an abscopal effect in the B16-OVA tumor model, suggesting that activating ZBP1 in cells of the tumor microenvironment can induce potent adaptive immune responses against the tumor cells. Based on these findings, the authors concluded that ZBP1-dependent necroptosis induced by CBL0137 in tumor stromal fibroblasts reverses resistance to ICB in mouse models of melanoma (Fig. 1c). However, a functional role of necroptosis in CBL0137-induced anti-tumor immunity was not formally demonstrated in the paper. Although the cell culture assays as well as the detection of phosphorylated MLKL in tumor fibroblasts support that ZBP1 might trigger anti-tumor immunity by inducing necroptosis, functional studies in MLKL-deficient mice, the gold standard for demonstrating a role of necroptosis, were not performed. Therefore, the involvement of necroptosis in this anti-tumor response remains to be experimentally demonstrated.

In conclusion, Zhang and colleagues identified CBL0137 as a small molecule inducing ZBP1-mediated cell death and showed

that when injected into tumors, it synergizes with ICB to induce tumor regression. These promising results in mouse models warrant further studies to assess whether CBL0137 could overcome resistance to ICB in human cancer.

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## ADDITIONAL INFORMATION

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