



Coordinated regulation of scaffold opening and enzymatic activity during CARD11 signaling

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The activation of key signaling pathways downstream of antigen receptor engagement is critically required for normal lymphocyte activation during the adaptive immune response. CARD11 is a multidomain signaling scaffold protein required for antigen receptor signaling to NF- κ B, c-Jun N-terminal kinase, and mTOR. Germline mutations in the *CARD11* gene result in at least four types of primary immunodeficiency, and somatic *CARD11* gain-of-function mutations drive constitutive NF- κ B activity in diffuse large B cell lymphoma and other lymphoid cancers. In response to antigen receptor triggering, CARD11 transitions from a closed, inactive state to an open, active scaffold that recruits multiple signaling partners into a complex to relay downstream signaling. However, how this signal-induced CARD11 conversion occurs remains poorly understood. Here we investigate the role of Inducible Element 1 (IE1), a short regulatory element in the CARD11 Inhibitory Domain, in the CARD11 signaling cycle. We find that IE1 controls the signal-dependent Opening Step that makes CARD11 accessible to the binding of cofactors, including Bcl10, MALT1, and the HOIP catalytic subunit of the linear ubiquitin chain assembly complex. Surprisingly, we find that IE1 is also required at an independent step for the maximal activation of HOIP and MALT1 enzymatic activity after cofactor recruitment to CARD11. This role of IE1 reveals that there is an Enzymatic Activation Step in the CARD11 signaling cycle that is distinct from the Cofactor Association Step. Our results indicate that CARD11 has evolved to actively coordinate scaffold opening and the induction of enzymatic activity among recruited cofactors during antigen receptor signaling.

The signaling pathways that control cellular behavior frequently rely on scaffold proteins to translate receptor triggering at the cell surface into the assembly of multiprotein complexes

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that execute changes in gene expression and cellular effector function. Signaling scaffolds provide important nodes of regulation, and their ability to influence other proteins in a signal-responsive manner can be modulated by a variety of mechanisms, including induced conformational change, regulated cellular localization, phosphorylation, and other post-translational modifications. Understanding how scaffold proteins signal and how they are regulated is key to appreciating how signaling pathways attain biological specificity and how the dysregulation of signaling pathways contributes to disease.

CARD11 is a critical scaffold protein that operates in the adaptive immune system to relay antigen receptor engagement on B and T lymphocytes into the activation of NF- κ B, JNK,⁴ and mTOR pathways (1–4). CARD11 function is essential for the efficient elimination of multiple human pathogens. At least four types of primary immunodeficiency in humans have recently been described that result from germline mutations in the *CARD11* gene (5–14). While CARD11 signaling is essential for normal B and T cell responses, its signaling is highly regulated by several mechanisms to prevent unwarranted lymphocyte activation (1, 15–17). Furthermore, gain-of-function CARD11 mutations are found in ~10% of cases of the activated B cell-like subtype of diffuse large B cell lymphoma and account for the constitutive activation of NF- κ B that is required for the aberrant proliferation of this lymphoma (18, 19). Gain-of-function CARD11 mutations have also been observed in other diffuse large B cell lymphoma subtypes and in other B and T cell leukemias and lymphomas (1, 20).

CARD11 is composed of a series of protein–protein interaction domains that nucleate the assembly of a multiprotein complex in response to T cell receptor (TCR) and B cell receptor (BCR) triggering. Prior to signal initiation, CARD11 is kept in a closed, inactive state by an Inhibitory Domain (ID) that prevents the binding of cofactors in part through intramolecular interactions involving the CARD, LATCH, Coiled-coil, L3, and GUK domains (21–24). ID function is mediated by four Repressive Elements (REs) that work cooperatively and with redundancy to prevent spontaneous signaling. Upon receptor

⁴ The abbreviations used are: JNK, c-Jun N-terminal kinase; TCR, T cell receptor; BCR, B cell receptor; ID, inhibitory domain; RE, repressive element; KD, knockdown; NT, nontarget; IE, inducible element; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; reQM, repressive element quadruple mutant; β -gal, β -galactosidase; IPLB, immunoprecipitation lysis buffer; DIPLB, denaturing immunoprecipitation lysis buffer; MS, mass spectrometry.

engagement, the autoinhibitory action of the REs is neutralized in a poorly understood process that converts CARD11 into an open, active state that is receptive to the binding of cofactors including Bcl10, MALT1, and HOIP, the catalytic subunit of the linear ubiquitin chain assembly complex, as well as several other proteins (1). This receptor-induced RE neutralization is thought to involve the signal-inducible phosphorylation of three serine residues within the ID, including Ser-564, Ser-567, and Ser-577 (21, 25, 26). However, it has remained unclear how exactly the modification of these residues contributes to CARD11 signaling activity and whether these residues are required subsequent to RE neutralization and cofactor association.

The signal-induced recruitment of HOIP, Bcl10, and MALT1 to CARD11 leads to the action of HOIP on Bcl10 to generate LinUb_n-Bcl10, which then associates with the IKK γ subunit of the IKK complex in a step required for the induction of IKK kinase activity (27). It has remained unclear whether recruitment of HOIP (enzyme) and Bcl10 (substrate) to CARD11 is sufficient to elicit Bcl10 conjugation or whether other steps are also required. Similarly, MALT1 recruitment to CARD11 precedes MALT1-mediated cleavage events that modulate pathway output, including those involving HOIL1, CYLD, A20, RelB, and others (28). However, it is not clear whether MALT1 recruitment to CARD11 is sufficient to activate its proteolytic activity.

In this report we investigate the function of Inducible Element 1 (IE1) within the CARD11 ID and surprisingly find that IE1 is required both for CARD11 opening and, independently, for the maximal activation of HOIP and MALT1 enzymatic activity subsequent to their recruitment to CARD11. IE1 thus provides a small element in CARD11 that ensures coordination of at least two steps in the CARD11 signaling cycle.

Results

The IE1 element within the ID is required for inducible CARD11 signaling

In a prior study (23), our analysis of a series of CARD11 deletion mutants suggested that a region within the ID between residues 530 and 616 was required for inducible TCR/CD28 signaling to NF- κ B, whereas other portions of the ID were dispensable. To further define how this region contributed to signal-induced CARD11 activity, we performed a deletion analysis in the context of Δ 530–616, a CARD11 variant that is missing a portion of the ID but retains REs 1 and 4 (Fig. 1, A and B). CARD11 constructs were assayed in an RNAi-rescue transient transfection assay in which the activation of the I κ B κ -IFN-LUC NF- κ B luciferase reporter downstream is monitored following anti-CD3/anti-CD28 cross-linking of Jurkat T cells with stable lentiviral-mediated knockdown of CARD11 (KD-CARD11). This reporter assay has previously been shown to quantitatively reflect TCR-induced NF- κ B nuclear translocation and DNA-binding activity (29). As previously described (29), KD-CARD11 cells failed to appreciably activate NF- κ B upon TCR/CD28 triggering, as compared with the control Jurkat T cell line that expresses a nontarget (NT) shRNA, but signaling could be rescued upon expression of WT murine

CARD11, which is resistant to the shRNA targeting human CARD11 (Fig. 1, C and D). We cloned six overlapping regions of CARD11 into the Δ 530–616 construct and assayed them for anti-CD3/anti-CD28-induced NF- κ B activation. Inducible signaling was observed with the 536–610 region, the 561–610 region, and the 536–586 region, but not with the 568–610 region or the 536–578 region (Fig. 1, C and D). This suggested that the interval between Ser-561 and Ala-586 would be necessary and sufficient to support inducible CARD11 signaling to NF- κ B in the Δ 530–616 context. This prediction was borne out by the Δ 530–616 + 561–586 construct (Fig. 1, C and D). We refer to this region between Ser-561 and Ala-586 as Inducible Element 1 (IE1).

IE1 residues required for inducible CARD11 signaling

We next individually mutated IE1 residues in the context of full-length CARD11 and used the same RNAi-rescue assay to compare each mutant's signaling to that of WT at comparable expression levels (Fig. 2, A–E). The most deleterious effects on TCR signaling to NF- κ B were observed as a result of mutation of residues Ser-564, Ser-567, Pro-573, Ser-577, Ile-578, and Arg-581, all of which reduced signaling output to 20% or less than that of WT CARD11. The mutation of residues Ile-568, Ala-570, Glu-571, Pro-572, Gly-574, Val-579, and Arg-580 also reproducibly impaired CARD11 signaling in this context to levels \sim 30–50% that of WT CARD11. These data indicated that multiple residues spanning the region between Ser-564 and Arg-581 are important for IE1 activity (Fig. 2F).

IE1 is required for CARD11 opening

The CARD11 signaling cycle downstream of antigen receptor triggering proceeds along a series of steps. In the first step, CARD11 is converted from a closed, inactive state to an open scaffold capable of binding cofactors. This step, which we refer to as the Opening Step, involves the neutralization of the autoinhibitory action of the four redundant REs in the ID, which prevent CARD11 from binding cofactors prior to receptor engagement. In the next step, signaling cofactors bind to opened CARD11 in what we refer to as the Cofactor Association Step. Following cofactor association, the kinase activity of the IKK complex is induced, leading to the phosphorylation of I κ B proteins and their subsequent ubiquitinylation and degradation, which allows for stable NF- κ B nuclear translocation. Ultimately, CARD11 returns to an inactive state in a step we refer to as the Complex Disassembly Step.

In principle, IE1 residues could be important for the Opening Step, for the Cofactor Association Step via participation in protein–protein interfaces that form as cofactors bind CARD11, or for some other CARD11-dependent step in signaling. We initially suspected that the residues would be required for the Opening Step because previous studies have implicated the phosphorylation of Ser-564, Ser-567, and Ser-577 as required for the antigen receptor-induced neutralization of the autoinhibitory function of the ID and the subsequent binding of cofactors to CARD11, including Bcl10 and MALT1 (21, 25, 26). Ser-564 has been described as a substrate of PKC θ or PKC β , Ser-567 has been described as a substrate of IKK β , and Ser-577 has been described as a substrate of an unidentified kinase (21,

The IE1 element controls two steps in CARD11 signaling

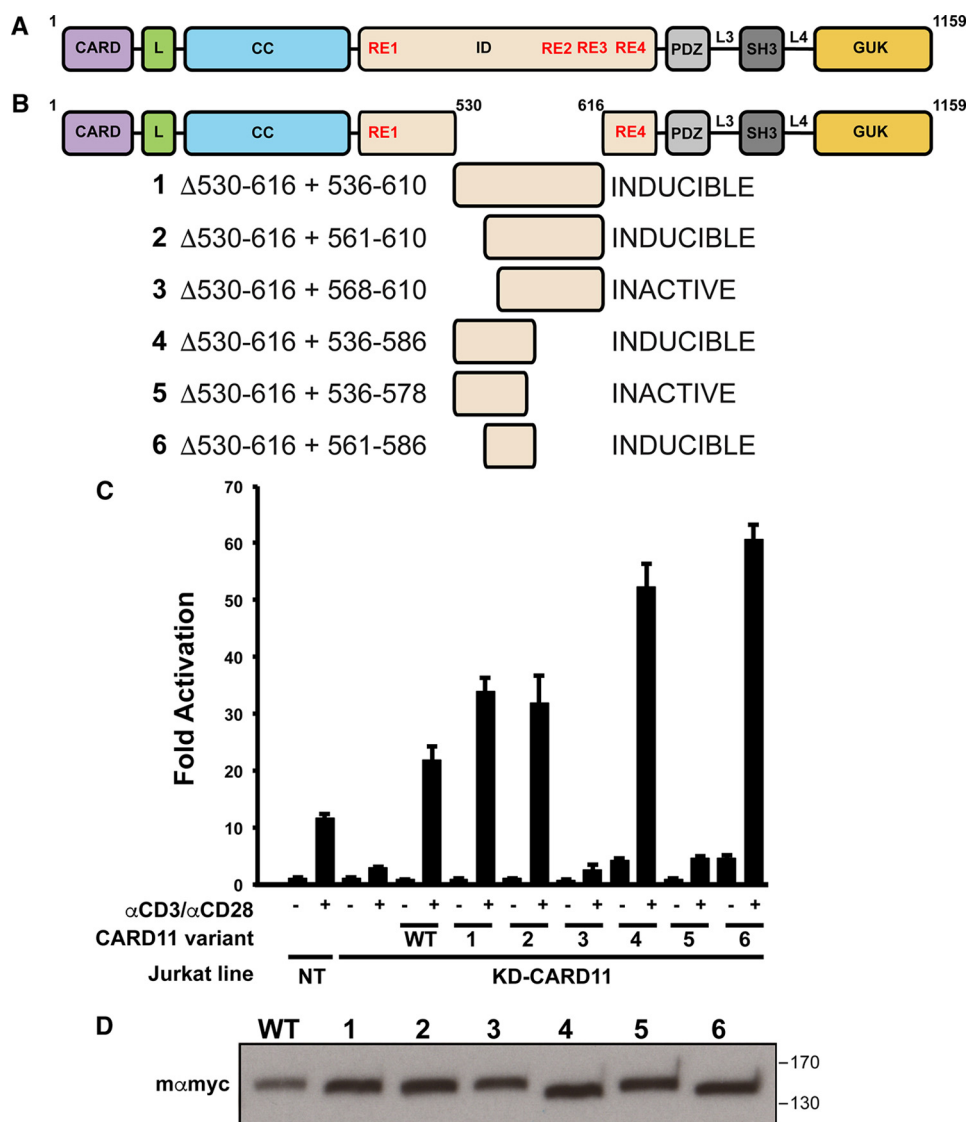


Figure 1. IE1 within the CARD11 ID is required for inducible signaling to NF- κ B. *A*, CARD11 domain structure. *B*, schematic of the constructs assayed. *C*, Jurkat T cells in which CARD11 was stably knocked down (KD-CARD11) or control cells expressing a control shRNA (NT) were transfected with CSK-LacZ and Ig κ_2 -IFN-LUC in the presence of expression vectors for the indicated Myc-tagged CARD11 variants and stimulated with anti-CD3/anti-CD28 cross-linking for 5 h as indicated. A two-tailed unpaired Student's *t* test with unequal variance resulted in the following *p* values for the values obtained under stimulated conditions as compared with that observed with WT CARD11: *construct 1*, *p* = 0.0042; *construct 2*, *p* = 0.048; *construct 3*, *p* = 0.0025; *construct 4*, *p* = 0.0012; *construct 5*, *p* = 0.0057; *construct 6*, *p* = 0.000049. *D*, HEK293T cells were transfected with the same amounts of each expression vector used in *C*, and lysates were probed by Western blotting using anti-Myc primary antibody to indicate the relative expression level of each variant. β -gal activity, driven by CSK-LacZ, was used to calculate equivalent amounts of lysate for Western blotting analysis.

25, 26). In addition, previous work has mapped the binding determinants for Bcl10, HOIP, and other positive cofactors to CARD11 domains that lie outside of the ID (22, 27), arguing against a role for IE1 in a CARD11:cofactor protein–protein interface.

To assess at which step IE1 functions, we used retroviral transduction to stably express WT CARD11 and the six IE1 mutants with the strongest phenotypes in the CARD11-KO Jurkat T cell line (27), which is CARD11-deficient as a result of lentiviral-mediated CRISPR/Cas9 genome editing. Each of these CARD11 variants contained two FLAG tags at its C terminus. We first assayed the recruitment of Bcl10, HOIP, and MALT1 to CARD11 following treatment of cells with PMA/ionomycin, which mimics TCR signaling, using an anti-FLAG immunoprecipitation assay (Fig. 3, *A* and *B*). Each of the six IE1

mutations assayed prevented the inducible recruitment of Bcl10, HOIP, and MALT1 to CARD11. The binding of Bcl10, HOIP, and MALT1 cofactors to CARD11 is regulated by the four REs in the ID, which prevent cofactor association with CARD11 in part through intramolecular interactions that involve the CARD, LATCH, Coiled-coil, L3, and GUK, and which are thought to sterically block cofactor binding (23, 24, 27). Bcl10 and HOIP robustly and constitutively bind to CARD11 variants that lack the REs or the entire ID (22, 27). Although Bcl10 binding requires the CARD and Coiled-coil of CARD11 in the Δ ID context (22), HOIP binding requires only the Coiled-coil (27) and is thus recruited independently of Bcl10. The fact that these IE1 mutants affect the binding of cofactors that are recruited independently and that are recruited to intramolecular targets of the REs suggested that

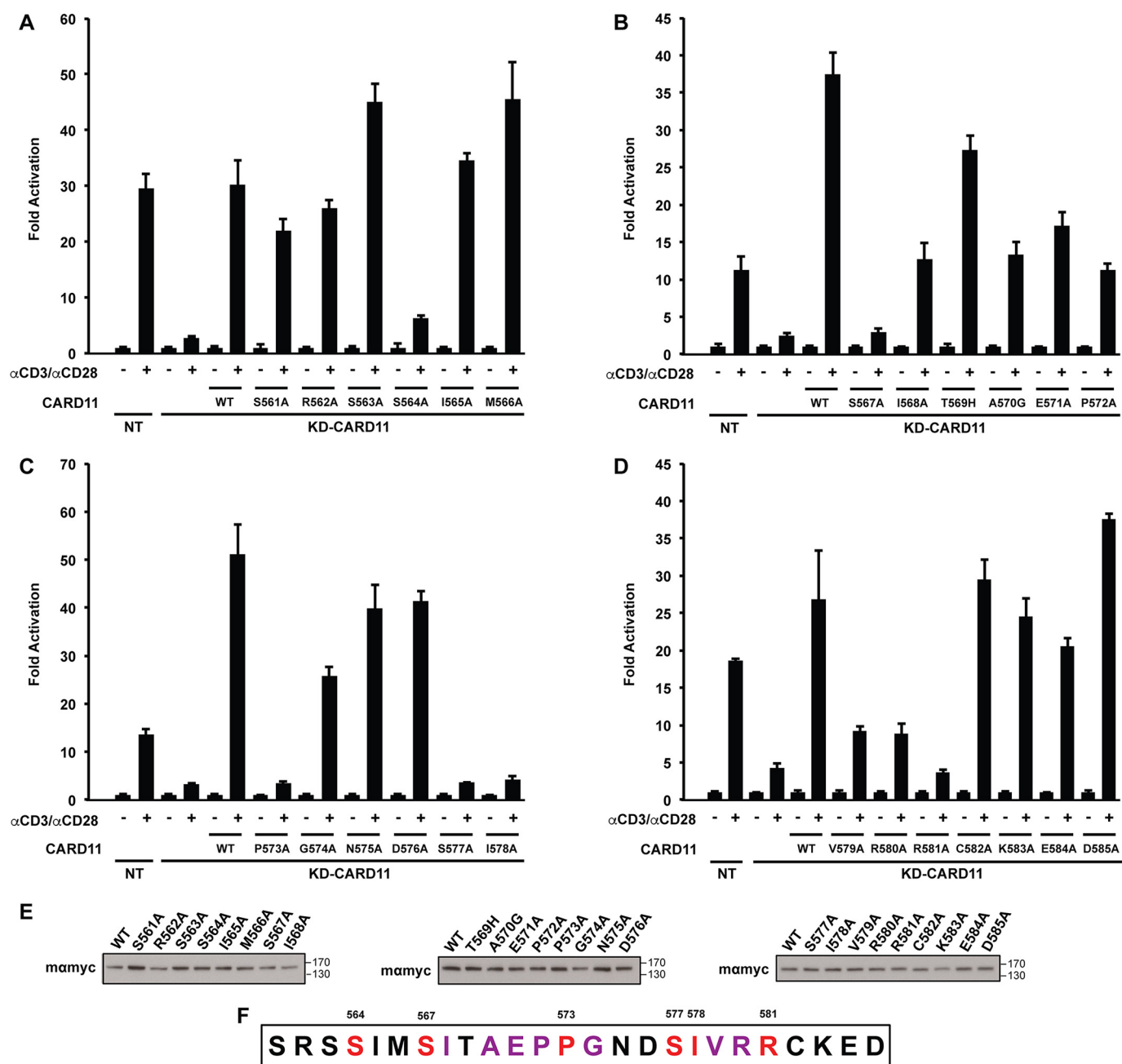


Figure 2. IE1 residues important for inducible CARD11 signaling to NF- κ B. A–D, KD-CARD11 or NT Jurkat T cells were transfected with CSK-LacZ and Ig κ ₂-IFN-LUC in the presence of expression vectors for the indicated Myc-tagged CARD11 variants and stimulated with anti-CD3/anti-CD28 cross-linking for 5 h as indicated. A two-tailed unpaired Student's *t* test with unequal variance resulted in the following *p* values for the values obtained under stimulated conditions as compared with that observed with WT CARD11: S561A, *p* = 0.033; R562A, *p* = 0.56; S563A, *p* = 0.18; S564A, *p* = 0.011; I565A, *p* = 0.14; M566A, *p* = 0.12; S567A, *p* = 0.0019; I568A, *p* = 0.00038; T569H, *p* = 0.029; A570G, *p* = 0.00080; E571A, *p* = 0.0010; P572A, *p* = 0.0021; P573A, *p* = 0.0049; G574A, *p* = 0.012; N575A, *p* = 0.14; D576A, *p* = 0.072; S577A, *p* = 0.0049; I578A, *p* = 0.0047; V579A, *p* = 0.037; R580A, *p* = 0.029; R581A, *p* = 0.025; C582A, *p* = 0.15; K583A, *p* = 0.071; E584A, *p* = 0.064; and D585A, *p* = 0.11. E, HEK293T cells were transfected with the same amounts of each expression vector used in A–D, and lysates were probed by Western blotting using anti-Myc primary antibody to indicate the relative expression level of each variant. β -gal activity, driven by CSK-LacZ, was used to calculate equivalent amounts of lysate for Western blotting analysis. F, the amino acid sequence of the IE1 region is depicted. Residues whose mutation resulted in 20% or less activity than that of WT CARD11 in the analysis in A–E are depicted in red. Those whose mutation resulted in ~30–50% the activity of WT CARD11 in the analysis in A–E are depicted in purple.

these six IE1 residues are indeed required for the Opening Step in the CARD11 signaling cycle.

The recruitment of Bcl10 and HOIP to CARD11 during signaling results in the HOIP-mediated conjugation of Bcl10 with linear ubiquitin chains to form LinUb_n-Bcl10, a signaling intermediate that associates with the IKK γ subunit of the IKK complex and whose levels determine the extent of signaling output

to NF- κ B (27). Each of the six IE1 mutants analyzed abrogated the production of LinUb_n-Bcl10 in response to PMA/ionomycin treatment (Fig. 3C), consistent with their prevention of the Opening Step, which allows Bcl10 and HOIP to bind CARD11.

We also assayed the CARD11-mediated induction of MALT1 protease activity by assaying the anti-CD3/anti-CD28-induced production of a previously described cleavage

The IE1 element controls two steps in CARD11 signaling

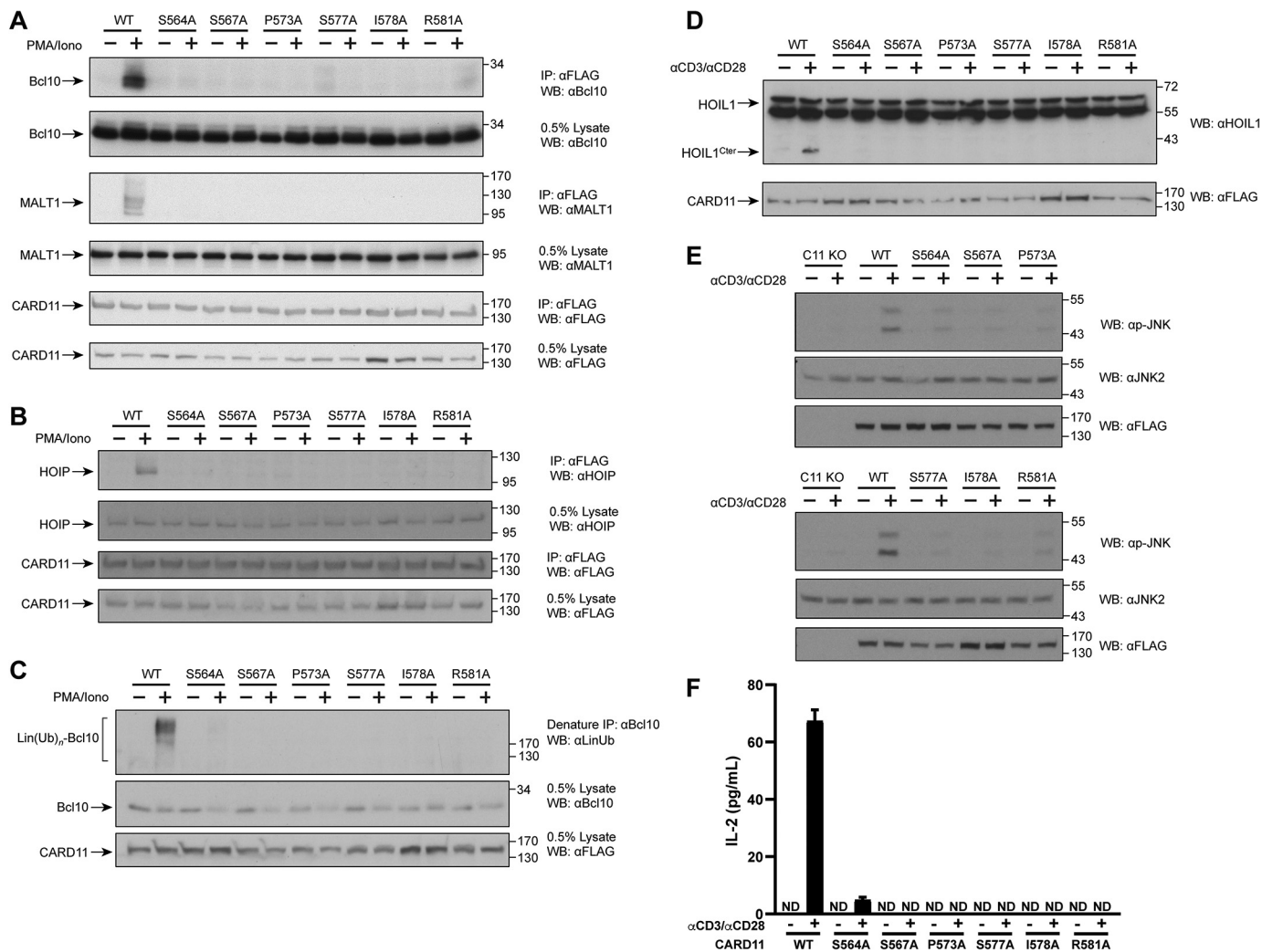


Figure 3. IE1 is required for inducible binding of Bcl10, MALT1, and HOIP to CARD11. A and B, CARD11-KO Jurkat T cells were retrovirally transduced to express the indicated FLAG-tagged CARD11 variants and stimulated with PMA/ionomycin (*iono*) for 30 min as indicated. Immunoprecipitations (IP) were performed with anti-FLAG antibodies as described under “Experimental procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies. C, CARD11-KO Jurkat T cells stably expressing the indicated FLAG-tagged CARD11 variants were stimulated with PMA/ionomycin for 30 min as indicated. Immunoprecipitations were performed with anti-Bcl10 antibodies under denaturing conditions as described under “Experimental procedures” and analyzed by Western blotting with the indicated primary antibodies. D and E, CARD11-KO Jurkat T cells stably expressing the indicated FLAG-tagged CARD11 variants were stimulated with anti-CD3/anti-CD28 for 30 min as indicated. The lysates were analyzed by Western blotting with the indicated primary antibodies. F, CARD11-KO Jurkat T cells stably expressing the indicated FLAG-tagged CARD11 variants were stimulated with anti-CD3/anti-CD28 for 24 h as indicated. The levels of IL-2 present in the media were determined by ELISA. ND, not detectable; KO, knockout.

product of MALT1 action on HOIL1, HOIL1^{Cter} (30–32). As shown in Fig. 3D, each of the six IE1 mutants analyzed prevented the stimulation of MALT1 protease activity, consistent with their prevention of the Opening Step that permits MALT1 recruitment to CARD11 (Fig. 3A), which is known to occur through Bcl10 (29, 33). We obtained similar results when assaying the appearance of cleavage products of CYLD, another MALT1 substrate (data not shown).

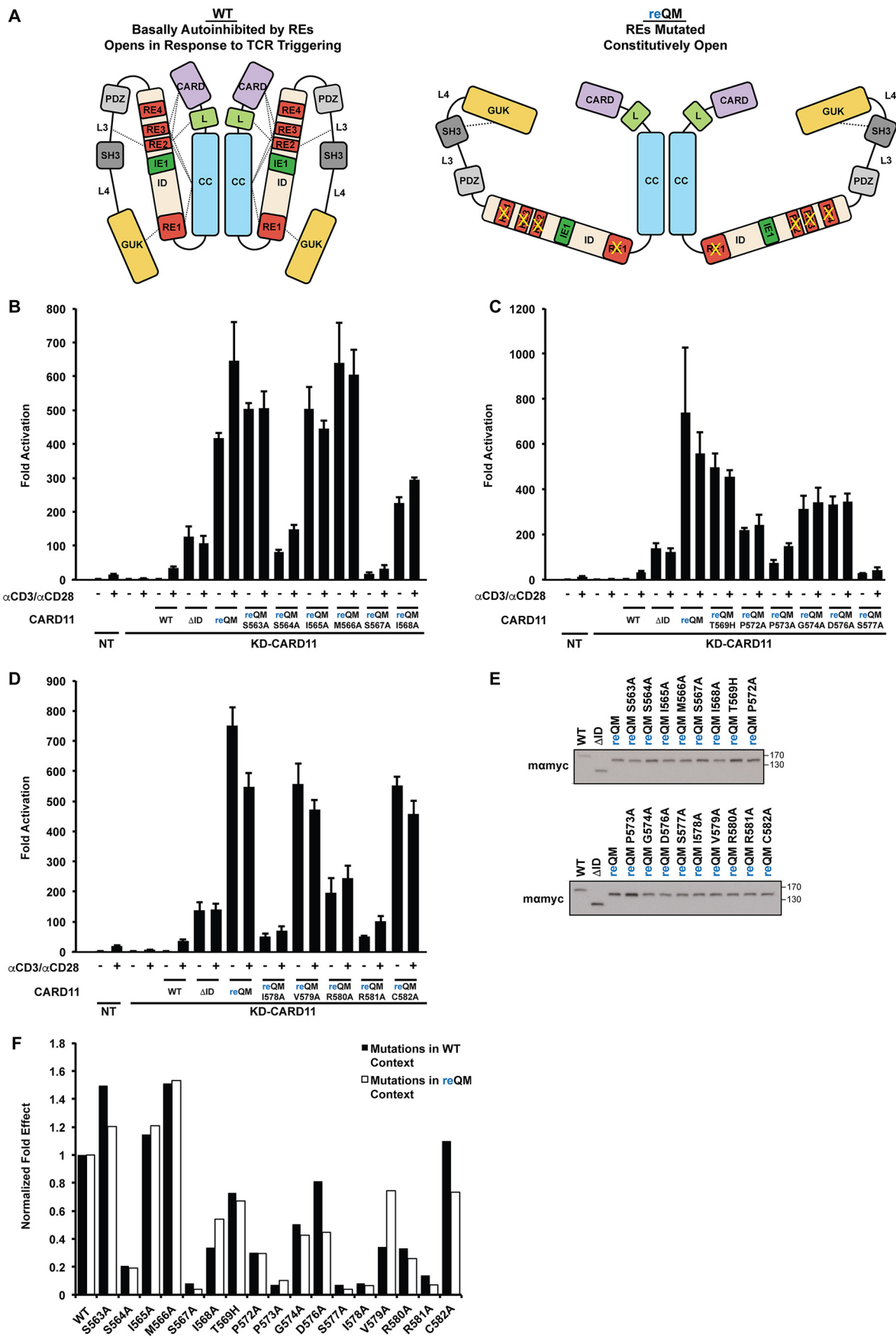
We next monitored the activation of JNK2 signaling downstream of anti-CD3/anti-CD28 cross-linking, which depends on CARD11 and on Bcl10 recruitment (34–36). While WT CARD11 restored signaling to JNK2 in CARD11-KO cells, each of the six IE1 mutations deleteriously affected JNK2 activation, as revealed by Western blotting with anti-phospho-JNK antibodies (Fig. 3E).

Finally, we assayed the ability of IE1 mutants to support the TCR-induced production of IL-2, a functionally important

gene target of CARD11 signaling (15). As shown in Fig. 3F, each of the six IE1 mutations deleteriously affected IL-2 secretion in response to anti-CD3/anti-CD28 treatment, underscoring the critical role for IE1 in T cell function.

IE1 residues required subsequent to CARD11 opening

We fully expected that IE1 functioned only at the Opening Step and would not be required for subsequent steps in the CARD11 signaling cycle subsequent to RE neutralization. To test this prediction, we assessed the effect of 17 individual IE1 mutations in the context of repressive element quadruple mutant (reQM), a construct previously described as re1 re2 re3 re4 (23), in which all four REs in the CARD11 ID have been simultaneously mutated and which is 640-fold more active than WT CARD11 because of its constitutively open, active state (depicted in Fig. 4A). We reasoned that any residue required solely for the Opening Step would inhibit signaling when



The IE1 element controls two steps in CARD11 signaling

mutated in the WT context but would not affect signaling when mutated in the reQM context, which represents CARD11 subsequent to RE neutralization. The activity of each variant was compared with that of WT CARD11, the ID-deleted Δ ID construct, and the parental reQM at comparable levels of expression in the RNAi-rescue assay in KD-CARD11 Jurkat T cells (Fig. 4, B–E). Contrary to our prediction, the mutation of Ser-564, Ser-567, Pro-573, Ser-577, Ile-578, and Arg-581 in the reQM context resulted in dramatic reductions in signaling activity. In addition, the mutation of Ile-568, Pro-572, Gly-574, Asp-76, and Arg-580 also had reproducible deleterious effects on reQM signaling potential (Fig. 4, B–E). These results indicated that remarkably, all of these residues are critical for CARD11 function subsequent to RE neutralization and must be required for a step in signaling in addition to and distinct from the Opening Step.

Interestingly, we compared the relative effect of each mutation in the WT and reQM contexts in their respective assays by representing their signaling activity as a fraction of their parental construct containing IE1 unmutated (Fig. 4F). The profile of effects of these 17 mutations is remarkably similar in these two contexts.

IE1 is required subsequent to cofactor binding for maximal CARD11-induced HOIP and MALT1 enzymatic activity

Subsequent to the Opening Step, IE1 could be required for the Cofactor Association Step or for some other step in CARD11 signaling. To determine which steps subsequent to RE neutralization require IE1, we reconstituted CARD11-KO cells with the constitutively active FLAG-tagged reQM and with reQM variants containing S567A, S577A, I578A, and R581A mutations. We compared these mutants' ability to associate with endogenous Bcl10, HOIP, and MALT1 to that of WT full-length CARD11 in the absence of upstream signaling. Consistent with previous work (23, 24), anti-Bcl10 immunoprecipitations from cell lysates revealed that the reQM variant constitutively associated with Bcl10, whereas WT CARD11 did not (Fig. 5A). Furthermore, the S567A, S577A, I578A, and R581A mutations had no significant effect on the ability of reQM to bind Bcl10 (Fig. 5A), indicating that these residues do not participate in a CARD11:Bcl10 protein–protein interface. Thus, although Ser-567, Ser-577, Ile-578, and Arg-581 in IE1 are required for Bcl10 binding to WT CARD11 during induced signaling (Fig. 3A), these residues do not regulate Bcl10 binding once the REs in the ID have been neutralized, consistent with a critical role for these residues during the Opening Step.

Similarly, anti-HOIP immunoprecipitations from the same cell lysates indicated that HOIP constitutively bound reQM, but not WT CARD11 in unstimulated cells (Fig. 5B), and that the S567A, S577A, I578A, and R581A mutations had no significant effect on this binding in the context of reQM (Fig. 5B), even though the same mutations abrogated HOIP binding in WT CARD11 undergoing stimulated signaling (Fig. 3B). The probing of anti-FLAG immunoprecipitates for MALT1 similarly suggested that MALT1 associates to a greater extent with reQM than with WT CARD11 and that Ser-567, Ser-577, Ile-578, and Arg-581 are not required for this association in the reQM context (Fig. 5C). These binding studies with Bcl10, HOIP, and MALT1 suggested that the negative impact of IE1 mutations in the reQM context (Fig. 4) could not be accounted for by a negative impact on the binding of these cofactors. Rather, the requirement for these residues for maximal reQM context signaling must be due to a role for IE1 in one or more steps subsequent to the Opening and Cofactor Association Steps.

We next assayed lysates from these cells for the presence of LinUb_n-Bcl10. Expression of reQM led to the spontaneous production of LinUb_n-Bcl10, while WT CARD11 did not, in unstimulated cells (Fig. 5D). Surprisingly, while S567A, S577A, I578A, and R581A reQM mutants appeared to be fully competent to spontaneously recruit Bcl10 and HOIP (Fig. 5, A and B), no LinUb_n-Bcl10 was detectable in cells expressing these mutants (Fig. 5D). The data suggest that these IE1 residues are required for the maximal activation of HOIP E3 ligase activity subsequent to the recruitment of HOIP and Bcl10 to CARD11.

Finally, we assayed the spontaneous production of HOIL1^{Cter}, the cleavage product of MALT1 proteolytic action on HOIL1. Expression of reQM resulted in spontaneous MALT1-mediated HOIL1 cleavage, while WT CARD11 did not induce this in the absence of upstream signaling (Fig. 5E). The S567A, S577A, I578A, and R581A reQM mutants resulted in significantly reduced accumulation of HOIL1^{Cter}, despite their apparently equivalent ability to bind MALT1 (Fig. 5C). The data suggest that these IE1 residues are required for the maximal activation of MALT1 protease activity in a step that is distinct from the recruitment of MALT1 to CARD11 through Bcl10. The requirement for IE1 in reQM-induced HOIP and MALT1 enzymatic activities can account for the deleterious effect of IE1 mutations on reQM signaling to NF- κ B (Fig. 4). Furthermore, this role of IE1 indicates that there is a previously unrecognized Enzyme Activation Step in the CARD11 signaling cycle that is

Figure 4. IE1 residues important for CARD11 signaling subsequent to RE neutralization. A, schematic of the reQM variant used to analyze the contribution of IE1 to signaling following RE neutralization, compared with WT CARD11. B–D, KD-CARD11 or NT Jurkat T cells were transfected with CSK-LacZ and Ig κ_2 -IFN-LUC in the presence of expression vectors for the indicated Myc-tagged CARD11 variants and stimulated with anti-CD3/anti-CD28 cross-linking for 5 h as indicated. A two-tailed unpaired Student's *t* test with unequal variance resulted in the following *p* values for the values obtained under unstimulated conditions as compared with that observed with reQM: reQM S563A, *p* = 0.0041; reQM S564A, *p* = 0.00015; reQM I565A, *p* = 0.14; reQM M566A, *p* = 0.080; reQM S567A, *p* = 0.00028; reQM I568A, *p* = 0.00018; reQM T569H, *p* = 0.21; reQM P572A, *p* = 0.061; reQM P573A, *p* = 0.039; reQM G574A, *p* = 0.081; reQM D576A, *p* = 0.092; reQM S577A, *p* = 0.034; reQM I578A, *p* = 0.0017; reQM V579A, *p* = 0.020; reQM R580A, *p* = 0.00024; reQM R581A, *p* = 0.0022; and reQM C582A, *p* = 0.012. E, HEK293T cells were transfected with the same amounts of each expression vector used in B–D, and lysates were probed by Western blotting using anti-Myc primary antibody to indicate the relative expression level of each variant. β -gal activity, driven by CSK-LacZ, was used to calculate equivalent amounts of lysate for Western blotting analysis. F, profile of effects of IE1 mutations in WT and reQM contexts. The mean fold activation achieved with each mutant in the full-length CARD11 context following anti-CD3/anti-CD28 treatment, normalized to that observed with WT CARD11 in the same experiment, is plotted with *black bars* using the data presented in Fig. 2. The mean fold activation achieved with each mutant in the reQM context in the absence of anti-CD3/anti-CD28 treatment, normalized to that observed with parental reQM in the same experiment, is plotted with *white bars* using the data presented in B–D.

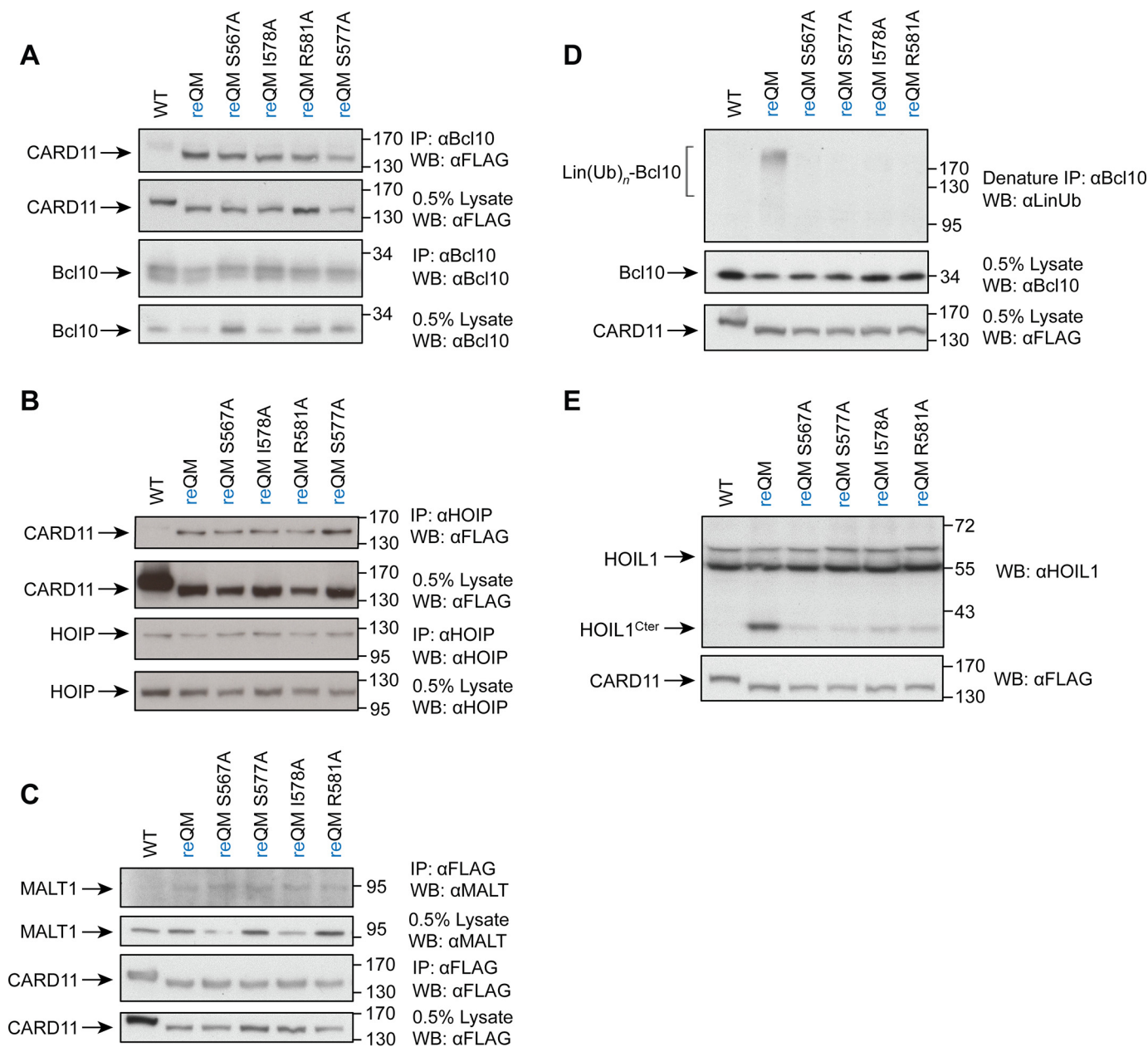


Figure 5. IE1 is required for HOIP and MALT1 enzymatic activity following cofactor binding. CARD11-KO Jurkat T cells were retrovirally transduced to express the indicated FLAG-tagged CARD11 variants as indicated. Immunoprecipitations (IP) were performed with anti-Bcl10 (A), anti-HOIP (B), or anti-FLAG (C) antibodies as described under “Experimental procedures” and analyzed by Western blotting (WB) with the indicated primary antibodies. D, immunoprecipitations were performed with anti-Bcl10 antibodies under denaturing conditions as described under “Experimental procedures” and analyzed by Western blotting with the indicated primary antibodies. E, lysates were analyzed by Western blotting with the indicated primary antibodies.

distinguishable from the Opening and Cofactor Association Steps.

Discussion

Our analysis of IE1 reveals several insights about the CARD11 signaling cycle, which proceeds from a closed, inactive state to an open, active scaffold and then back to the closed state (Fig. 6). First, IE1 functions in the CARD11 Opening Step that occurs in response to antigen receptor engagement. IE1 mutations in CARD11 prevent the inducible binding of Bcl10, MALT1, and HOIP to CARD11 and subsequent signaling events that depend on cofactor recruitment, including LinUb_n-Bcl10 generation and the cleavage of substrates by MALT1.

Bcl10 and HOIP bind independently to CARD11 with different domain requirements and can robustly bind the constitutively open CARD11 variants reQM and ΔID in an IE1-independent manner, indicating that IE1 residues do not form part of the protein–protein interfaces that recruit Bcl10 or HOIP. Consistent with this conclusion, IE1 mutations that prevent Bcl10, MALT1, and HOIP from associating with CARD11 during inducible signaling do not affect the binding of these factors to the constitutively open reQM variant. IE1 must therefore control the Opening Step in which CARD11 is converted from an autoinhibited state into an open, active scaffold capable of assembling a signaling complex. The IE1 residues involved in opening include Ser-564, Ser-567, and Ser-577, three serines

The IE1 element controls two steps in CARD11 signaling

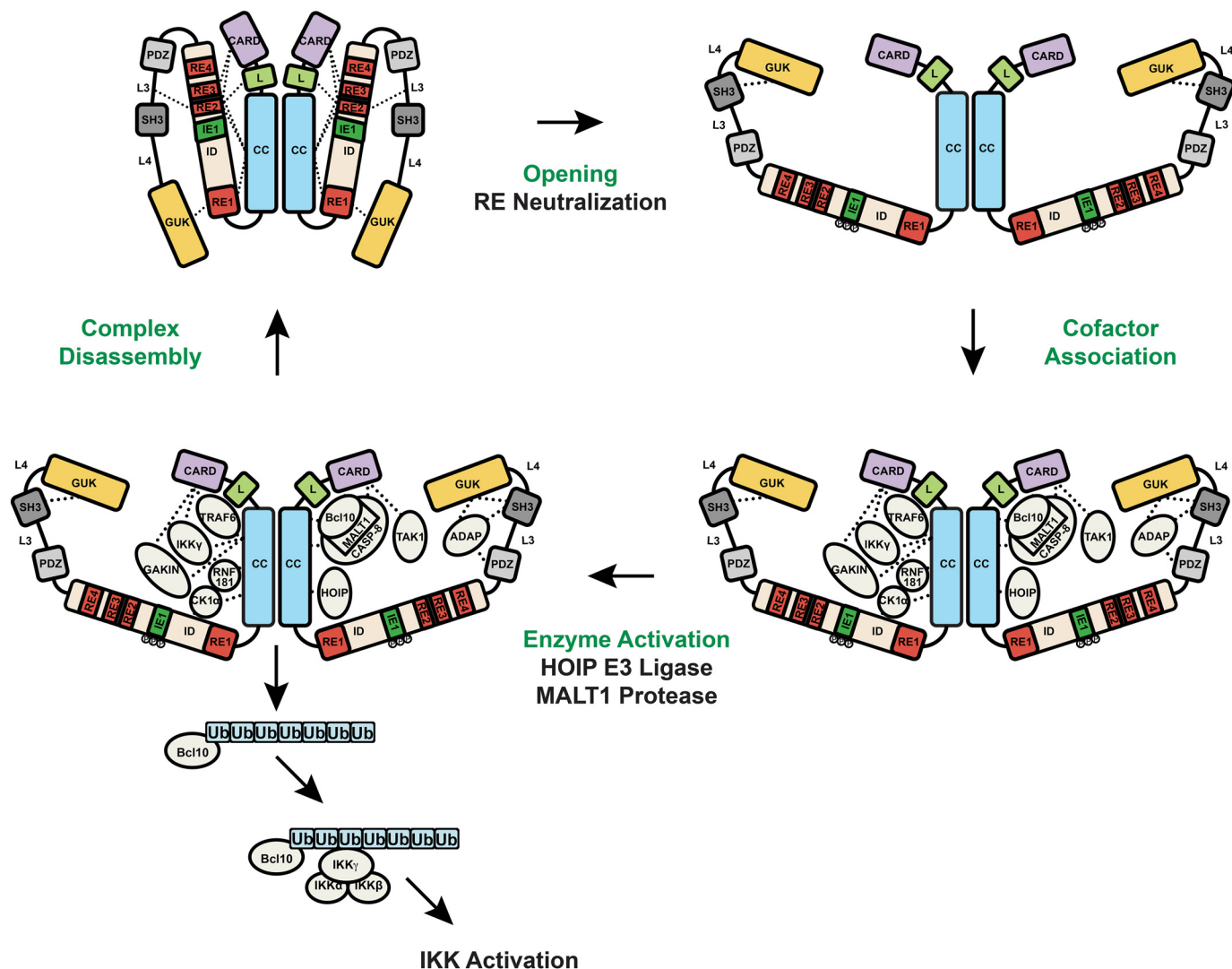


Figure 6. Four steps in the CARD11 signaling cycle. In the Opening Step, antigen receptor signaling causes the neutralization of the Repressive Elements in the Inhibitory Domain and the conversion of CARD11 from an inactive state to an open state that is capable of binding multiple cofactors. In the Cofactor Association Step, signaling cofactors associate with CARD11. Several known cofactors are depicted, adapted from Bedsaul *et al.* (1). In the Enzyme Activation Step, the E3 ligase activity of HOIP is activated, leading to the conjugation of Bcl10 with linear ubiquitin chains and the production of LinUb_n-Bcl10, which associates with the IKK complex through IKK γ to promote kinase activation. The proteolytic activity of MALT1 is also activated during the Enzyme Activation Step, leading to the cleavage of HOIL1 and CYLD and possibly other substrates. In the Complex Disassembly Step, cofactors dissociate from CARD11 and the scaffold returns to the closed, inactive state.

previously implicated as phosphorylation sites required for CARD11 signaling activity, although Pro-573, Ile-578, and Arg-581 are just as critical. It is likely that other regions of CARD11 are also required for the Opening Step and work with IE1 to make CARD11 receptive to the binding of other proteins in the Cofactor Association Step.

Second, our study of IE1 mutations in the constitutively open and active reQM context reveals that there is an Enzyme Activation Step in CARD11 signaling that is distinct from the Cofactor Association Step (Fig. 6). The reQM variant, in which all four REs in the ID have been mutated, constitutively binds Bcl10, HOIP, and MALT1. Although IE1 mutations in the reQM context do not impair the binding of Bcl10, HOIP, or MALT1, the mutations do impair HOIP enzymatic activity on Bcl10 and MALT1 enzymatic activity on its proteolytic substrates. Therefore, the corecruitment of Bcl10 and HOIP is not sufficient to induce HOIP action on Bcl10 to produce LinUb_n-

Bcl10, and the recruitment of MALT1 through Bcl10 to CARD11 is not sufficient to induce MALT1 action on HOIL1. Examination of CYLD cleavage products revealed that MALT1 action on CYLD similarly occurs in the presence of reQM but is diminished by IE1 mutations (data not shown). IE1 appears to be required in the CARD11 signaling cycle for the Enzyme Activation Step in addition to being required for the Opening Step.

Third, it is extremely likely that the same pathway component that recognizes IE1 in the Opening Step recognizes IE1 in the Enzyme Activation Step. The effects of IE1 single amino acid substitutions on inducible CARD11 signaling are remarkably similar to the effects in the context of reQM (Fig. 4F). The parallel effects of these side-chain substitutions in IE1 suggest that the same interface involving IE1 governs both opening, which allows cofactor binding, and the activation of HOIP and MALT1 enzymatic activity following their recruitment to CARD11. How does IE1 function at these two signaling steps? It

is possible that IE1 is recognized by and recruits an unknown factor in *trans* that is required for the Opening Step and that is also subsequently required for the Enzyme Activation Step. Alternatively, IE1 may participate in intramolecular interactions with another region of CARD11 that support an induced conformation of CARD11 in which REs can no longer repress, that makes CARD11 accessible to cofactor binding, and that promotes the enzymatic action of HOIP and MALT1 on their targets, perhaps by providing the appropriate spatial juxtaposition of enzymes and substrates. It is also possible that IE1 may contribute to a direct, CARD11-mediated allosteric activation of HOIP and MALT1. Our attempts at binding studies with CARD11 protein fragments have not yet revealed evidence of intramolecular binding, but these negative results are not conclusive. It is important to note that Ser-564, Ser-567, and Ser-577 may be recognized at the Opening and Enzymatic Activation Steps when phosphorylated or when unphosphorylated. Supporting the latter possibility, these residues are clearly important for signaling in the context of reQM, which can activate NF- κ B in the absence of TCR triggering or PMA/ionomycin, which activates upstream kinases including PKC θ . The phosphorylation of Ser-564 and Ser-577 has been observed by MS in stimulated T cells (25). Ser-564 was also identified in a search for potential PKC substrates (21, 25), and Ser-567 was found to be a substrate of IKK β (26). Further work is required to firmly establish whether the phosphorylation of these residues is obligately required for IE1 function during the CARD11 signaling cycle or whether their phosphorylation plays inhibitory or regulatory roles. The ID and IE1 within it are predicted to have little or no secondary structure (23), but it is possible that IE1 adopts a rigid structure upon phosphorylation or upon binding to an intramolecular target in CARD11 or to another protein in *trans*.

Finally, because IE1 is required for Opening and Enzymatic Activation Steps, likely via the interaction with the same target, our data suggest that in the WT CARD11 signaling cycle, these two steps are coordinately regulated. The signaling pathway appears to have evolved to employ the same components in the opening of CARD11 and in the activation of cofactor enzymatic activities to relay signaling from the antigen receptor to NF- κ B. It is currently thought that the TCR and BCR signaling pathways employ identical signaling steps between the CARD11 Opening Step and NF- κ B activation (1). However, all of our studies were done in the context of TCR signaling in T cells, and further work will be required to confirm a strict role for IE1 in BCR signaling in B cells.

CARD10 and CARD14 are homologous to CARD11, but their mechanisms of signaling are not understood as well. CARD10 mediates NF- κ B activation downstream of the epidermal growth factor receptor, angiotensin II receptor, and lysophosphatidic acid receptor (37), whereas CARD14 transmits IL-17 receptor and Dectin-1 signaling to NF- κ B in keratinocytes (38). Each of these proteins contains a short region C-terminal to their Coiled-coil domain that displays homology to IE1, including at the six positions revealed by our analysis to be most critical (Fig. 7). It would be fruitful to explore the roles of these regions in CARD10 and CARD14 in the context of their relevant signaling pathways.

mCARD11	SIMSITAEPGND SIVRR
hCARD11	SIMSITAEPGND SIVRR
mCARD10	NRLSILPFPPSAGS ILRR
hCARD10	NRLSILPFPPSAGS ILRR
mCARD14	SFRSSSPMP PSQQSLYKR
hCARD14	SFRSSSPMP PSQQSLYKR

Figure 7. Homology between CARD11, CARD10, and CARD14 in the IE1 region. The following regions are aligned for comparison: murine CARD11 residues 564–581, human CARD11 residues 559–576, murine CARD10 residues 557–574, human CARD10 residues 517–534, murine CARD14 residues 470–487, and human CARD14 residues 470–487.

Experimental procedures

Cell lines

HEK293T and Jurkat T-cell lines were cultured as described previously (15). Stable CARD11 knockdown Jurkat T cells (KD-CARD11) and control cells expressing the shNT hairpin were previously generated (15). CARD11-KO Jurkat T cells generated by CRISPR/Cas9-mediated genome editing were described previously (27).

Expression constructs

Sequence-verified N-terminal Myc-tagged murine CARD11 mutant and deletion constructs were cloned by standard molecular biology techniques and QuikChange site-directed mutagenesis (Stratagene) using WT CARD11 and the reQM CARD11 construct, a variant in which all four repressive elements are mutated that was previously referred to as re1 re2 re3 re4 (23).

Antibodies

Antibodies used were as follows. Mouse mAb against c-Myc (9E10, sc-40), mouse mAb against Bcl10 (331.3, sc-5273), rabbit polyclonal antibody against Bcl10 (H-197, sc-5611), mouse mAb against MALT1 (B-12; sc-46677), mouse mAb against MALT1 (D-1; sc-515389), and mouse mAb against RBCK1/HOIL1 (H-1; sc-393754) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against Bcl10 (A303–579A) and rabbit polyclonal antibody against HOIP/RNF31 (A303–560) were obtained from Bethyl Laboratories. Mouse mAb against HOIP/RNF31 (MAB8039) was purchased from R&D Systems. Mouse mAb against linear ubiquitin (clone LUB9; MABS451) was from EMD Millipore. Rabbit (F7425) and mouse (M2; F1804) antibodies against the FLAG epitope were from Sigma. Rabbit polyclonal antibody against phospho-SAPK/JNK Thr-183/Tyr-185 (81E11; 4668) and rabbit polyclonal antibody against JNK2 (4672) were purchased from Cell Signaling Technology.

HEK293T protein expression assay

5×10^5 cells in 2 ml of Dulbecco's modified Eagle's medium were plated into each well of a 6-well plate 22–26 h prior to transfection. Using the calcium phosphate method, the cells were transfected with 200 ng of pCSK-LacZ, 1500 ng of Ig κ_2 -IFN-LUC, and 100–400 ng of each Myc-tagged CARD11 variant. Total amount of DNA per transfected sample was kept constant at 3 μ g via supplementation with empty pcDNA3 vector. The medium was changed 22–24 h post-transfection, and cells were harvested 40–44 h post-transfection. The cells were

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incubated with 500 μ l of Reporter Lysis Buffer (Promega) on ice for 10 min, and cell debris was cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. To measure β -gal activity, 3 μ l of lysate was loaded into 96-well plates for the chemiluminescent β -gal reporter gene assay (Roche). Chemiluminescence was measured by a luminometer (Berthold Technologies TriStar LB 941) integrating for 10 s after a 2-s delay. β -gal activity was used to normalize lysates for transfection efficiency and extract recovery. Normalized lysates were resolved on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Millipore), and analyzed by Western blotting to determine relative expression and standardized nanogram amounts of each expression vector as described previously (23).

Transient transfections and NF- κ B luciferase reporter assay of CARD11 knockdown Jurkat T cells

5×10^5 Jurkat T cells in 2 ml were plated into each well of a 6-well plate. NT and KD-CARD11 Jurkat cells were transiently transfected with 200 ng of pCSK-LacZ, 1500 ng of Ig κ_2 -IFN-LUC, and 100–400 ng of each Myc-tagged CARD11 variant using LT-1 (Mirus) with 3 μ g of total DNA according to the manufacturer's instructions. In all experiments, each sample was supplemented with empty pcDNA3 vector to keep the total expression vector constant. The cells were stimulated 40–44 h later in 1 ml of RPMI medium with or without 1 μ g/ml each of mouse anti-human CD3 (BD Pharmingen 555329), mouse anti-human CD28 (BD Pharmingen 555725), and anti-mouse IgG1 (BD Pharmingen 02231D). 4–5 h later, the cells were lysed in 150 μ l of Reporter Lysis Buffer for 10 min at room temperature, debris was removed as described above, luciferase and β -gal activities were measured using 50 and 25 μ l of lysate respectively, and fold activation was calculated as described previously (23). All results show the averages (\pm S.D.) of triplicate samples from one experiment, representative of two or three performed for each group of CARD11 variants.

Reconstitution of CARD11-knockout Jurkat T cells with CARD11 variants

Each CARD11 variant with an N-terminal Myc tag and two C-terminal FLAG tags were stably expressed in the context of the pCLB3B retroviral vector, a Moloney murine leukemia virus-based vector carrying blasticidin resistance (27). Retroviruses were packaged, CARD11-KO Jurkat T cells were infected, and Jurkat T-cell lines were maintained in puromycin and blasticidin as described previously (27).

Endogenous coimmunoprecipitations in Jurkat T cells

For each experiment, samples consisting of 5×10^7 or 1×10^8 CARD11-KO Jurkat T cells stably expressing CARD11 variants were resuspended in 10 ml of RPMI medium with or without 50 ng/ml PMA (Sigma) and 1 μ M ionomycin (Sigma) for 30 min at 37 °C. The cells were incubated 10 min on ice, spun at 1400 rpm for 5 min, and lysed in 1.5 ml of immunoprecipitation lysis buffer (IPLB) with protease inhibitor mixture (Sigma, P8340) as described previously (24). Lysates were incubated with 2–4 μ g of indicated antibodies for 4 h at 4 °C with rotation, further incubated with a 20- μ l bed volume of protein A- or protein G- conjugated Sepharose (GE Healthcare) overnight at

4 °C with rotation, and washed with IPLB four times for 10 min at 4 °C with rotation. All immunoprecipitates and cell lysate input samples were boiled in SDS loading buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes for Western blotting with the indicated antibodies. The results shown are representative of at least two repeats of each experiment.

Denatured coimmunoprecipitation

5×10^7 Jurkat T cells per sample were resuspended in 10 ml of RPMI medium with or without 50 ng/ml PMA (Sigma) and 1 μ M ionomycin (Sigma) for 30 min at 37 °C. The cells were incubated 10 min on ice, spun at 1400 rpm for 5 min, and lysed in 1.5 ml of Den-IPLB (DIPLB) as described previously (27). To assess Bcl10 polyubiquitination, SDS was added to cell lysates to a concentration of 1%, and lysates were heated at 95 °C for 5 min, diluted with DIPLB to a final concentration of 0.1% SDS, incubated with 4 μ g of anti-Bcl10 antibody for 4 h at 4 °C with rotation, further incubated with a 20 μ l bed volume of protein A- conjugated Sepharose overnight at 4 °C with rotation, and washed with DIPLB four times for 10 min at 4 °C with rotation. Immunoprecipitates and cell lysate input samples were prepared for Western blotting as described above. The results shown are representative of at least two repeats of each experiment.

HOIL-1L cleavage and JNK activation

Jurkat T cells were stimulated with or without 1 μ g/ml each of mouse anti-human CD3, mouse anti-human CD28, and anti-mouse IgG1 in 1 ml of RPMI medium for 30 min. The cells were incubated on ice for 10 min, spun at 1400 rpm for 5 min, and lysed in IPLB. 1×10^6 cells per sample were boiled in SDS loading buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes for Western blotting using the indicated antibodies. The results shown are representative of at least two repeats of each experiment.

IL-2 ELISA

To measure IL-2 production, 5×10^5 CARD11-KO Jurkat T cells stably expressing full-length CARD11 variants were stimulated in 200 μ l of RPMI medium alone or RPMI medium containing 5 μ g/ml each of mouse anti-human CD3 (BD Pharmingen, 555329), mouse anti-human CD28 (BD Pharmingen, 555725), and rat anti-mouse IgG1 (BD Pharmingen, 553440) in a 96-well plate. After 24 h, culture supernatants were harvested via centrifugation at 1400 rpm for 5 min. The amount of IL-2 present in the supernatants was quantified using the human IL-2 uncoated ELISA kit (Invitrogen, 88-7025-88) according to the manufacturer's instructions. Optical density at 450 and 570 nm (reference) was measured using a Beckman Coulter AD340 plate reader. The results shown are representative of at least two repeats of each experiment.

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