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## The adaptor protein Crk in immune response

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### Abstract

The adaptor proteins Crk, including CrkI, CrkII, and CrkL, are important signal molecules that regulate a variety of cellular processes. Considerable progress has been made in understanding the roles of the Crk family proteins in signal transduction, with a focus on cellular transformation and differentiation. However, since Crk was identified in 1988, very few studies have addressed how Crk regulates the immune response. Recent work demonstrates that Crk proteins function as critical signal molecules in regulating immune cell functions. Emerging data on the roles of Crk in activation and inhibitory immunoreceptors signaling suggest that Crk proteins are potential immunotherapeutic targets in cancer and infectious diseases. The aim of this review is to summarize recent key findings regarding the role of Crk in immune responses mediated by T, B, and natural killer (NK) cells. In particular, the roles of Crk in NK cells function are discussed.

### Keywords

Immune response; Adaptor protein; Natural killer cell; CT10 regulator of kinase; Inhibitory receptor; Cytotoxicity

## INTRODUCTION

The v-crk oncogene was isolated by two separate groups: Mayer *et al.*<sup>1</sup> from chicken tumor virus no.10 (CT10) retrovirus, and Tsuchie *et al.*<sup>2</sup> from avian sarcoma virus (ASV1). Based on its ability to increase cellular tyrosine phosphorylation and transform primary chicken embryo fibroblasts, Mayer *et al.*<sup>1</sup> named this new oncogene *crk*, for CT10 regulator of kinase, and the oncogene product p47gag-crk.<sup>3</sup> Three years later, Reichman *et al.*<sup>4</sup> cloned and sequenced a complementary DNA encoding the cellular homologue of the transforming oncogene v-crk of avian sarcoma virus CT10. Following the sequencing of the cellular Crk (c-Crk) in chickens,<sup>4</sup> two isoforms of Crk (CrkI and CrkII, hereinafter referred to as 'Crk') and Crk-like (CrkL), which are encoded by separate genes, were identified in humans by two different groups.<sup>5, 6</sup> Since then, extensive studies have been focused on the roles played by Crk in cell growth, motility, apoptosis, transcription, and transformation in the field of cell biology.<sup>7–9</sup> Finding the proteins that interact with Crk in normal or transformed

fibroblasts has represented a main effort during the past three decades. Several reviews have discussed the physiological and pathological function of Crk,<sup>8, 10, 11</sup> signaling complex formation,<sup>7, 12</sup> roles of Crk in human cancer,<sup>13</sup> and roles of Crk in cytoskeletal actin dynamics.<sup>14</sup> Few studies on Crk have discussed the function of Crk in lymphocytes.<sup>15, 16</sup> The field of immunology now needs to direct attention to the role of Crk in the regulation of lymphocytes.

The interactions between Crk/CrkL and other signal proteins have been discussed already in other reviews.<sup>7, 8, 16</sup> This review focuses on the roles of Crk and CrkL in immune responses mediated by T, B, and NK cells, with a focus on human NK cells. This review divides into seven main parts: (1) the structure of the Crk family proteins, (2) the regulation of Crk activities, (3) the role of Crk in regulating T cell functions, (4) the role of Crk in regulating B cell functions, (5) the role of Crk in NK cell functions, (6) the role of Crk in other immune cell functions, and (7) perspectives. The main points that this review addresses are that:

1. Crk not only plays numerous roles in cell biology but also Crk has several specific roles in regulating lymphocyte functions.
2. Our understanding of Crk has been advanced by the recent published studies on the roles of Crk in human primary NK cells, of which Crk may function as a 2-way molecular switch to control NK cell-mediated cytotoxicity.

## THE STRUCTURE OF THE CRK FAMILY PROTEINS

In 1992, Matsuda *et al.*<sup>5</sup> isolated two species of human Crk cDNAs, named CrkI and CrkII, from human embryonic lung cells. *CrkII* is located on chromosome 17p13.3 in humans.<sup>4, 5</sup> In 1993, ten Hoeve *et al.*<sup>6</sup> identified the Crk-like gene, *CrkL*. *CrkL* is located on chromosome 22q11.21 in humans.<sup>6</sup> The *CrkL* gene product is predicted to have a molecular mass of 36 kDa.<sup>6</sup> CrkI (28 kDa), an alternately spliced form of CrkII (40 kDa), contains only one Src homology 2 (SH2) and one Src homology 3 (SH3) domain illustrated by multiple sequence alignment<sup>17</sup>, as shown in Figure 1. CrkI lacks the regulatory phosphorylation site and the c-terminal SH3 domain. CrkII and CrkL proteins contain one SH2 domain and two SH3 domains, named SH3N (N-terminal SH3 domain) and SH3C (C-terminal SH3 domain), respectively, as shown in Figure 2. A comparison of the structures of CrkII and CrkL reveals that the SH2 domain of CrkII has an extra stretch of 17 amino acids (N'-PPVPPSPAQPPPGVSPS-C') that is rich in proline residues (proline-rich region, PRR), as shown in Figure 3. According to the nuclear magnetic resonance (NMR) structural studies by Kobashigawa *et al.*<sup>18</sup>, the PRR is located on the CrkII's outer surface. The PRR is a specific SH3 domain-binding motif, such as Abelson murine leukemia kinase (Abl) and p85.<sup>19</sup> However, this finding cannot explain the observation that CrkL (which possesses an SH2 domain, but without PRR in SH2), but not CrkII, is a preferred substrate of the breakpoint cluster region (Bcr)-Abl protein tyrosine kinases (PTKs) in Bcr-Abl-transformed cells.<sup>6, 20, 21</sup> Recent studies by Jankowski *et al.*<sup>22</sup> reveal that CrkL forms a constitutive complex with Abl because of different domain organizations between CrkL and CrkII, which may explain the strong preference of Bcr-Abl for CrkL. Table 1 summarizes the comparison of CrkII and CrkL in different aspects. Based on comparisons of the structures of CrkII and CrkL and their regulatory mechanisms, the consensus is that CrkII and CrkL

each plays its own essential role in the regulation of cell function. CrkII and CrkL may not have redundant functions and cannot be compensated by each other. Recently, Prosser *et al.*,<sup>23</sup> using the cloning of receptor targets (CORT) technique, identified a novel member of the Crk family proteins, CrkIII that contains a truncated SH3C. In summary, Crk family proteins are relatively small proteins that contain both SH2 and SH3 domains; however, the functions of the different domains are controversial and complex, as detailed below.

## THE REGULATION OF CRK ACTIVITIES

Generally, the intracellular signaling by Crk proteins is mediated by the interaction of the SH2 domain with phosphotyrosine residues, as well as the interaction of the SH3 domain with PRRs. Because Crk proteins lack the intrinsic catalytic activity that kinases possess, Crk proteins usually are classified as the adaptor protein family. Functionally, CrkI exhibits more transforming activity than does CrkII, as evidenced by the important observation that the cell lines expressing the CrkI protein grow as massive tumors in nude mice, whereas CrkII-expressing cells do not grow in nude mice.<sup>5</sup> Several studies show that the SH3C domain and the linker region in CrkII are responsible for this difference, in which the SH3C domain can downregulate CrkII activity.<sup>18, 24–26</sup> As for the regulation of CrkII and CrkL, the control of CrkII and CrkL activity is multilayered, as summarized as follows:

The first layer of regulation is mediated by phosphorylation at Tyrosine 207 (Tyr207) in CrkL and Tyrosine 211 (Tyr211) in CrkII.<sup>7</sup> In 1994, Feller *et al.*<sup>27</sup> proposed that the phosphorylated Tyr221 of CrkII could bind to its own SH2 domain, which prevents the SH3N of CrkII from binding other proteins or signal molecules. Tyr207 in CrkL is phosphorylated by Abl kinase.<sup>28, 29</sup> Upon phosphorylation of CrkL at Tyr207, the SH2 domain interacts with the pTyr207-X-X-P region in the same molecule,<sup>22</sup> similar to CrkII.<sup>18, 30</sup> Consistent with this concept, using a fluorescence resonance energy transfer-based probe by sandwiching a truncated form of CrkL with a variant of yellow fluorescent protein (YFP), also called Venus, and an enhanced cyan fluorescent protein (ECFP), Mizutani *et al.*<sup>31</sup> demonstrated that intramolecular binding of the SH2 domain to phosphorylated Tyr 207 in CrkL increases FRET efficiency. A similar result is observed in CrkII by fusing YFP and CFP with the N- and C-termini of a truncated CrkII molecule.<sup>32</sup> Hofmann *et al.*<sup>33</sup> and Cotton *et al.*,<sup>34</sup> using the site-specific chemical modification of protein to label the N- and C-termini of a truncated CrkII molecule (lacking the regulatory C-terminal SH3 domain), observed a decrease in the fluorescence resonance energy transfer efficiency (indicating an increase in distance between SH2 and SH3N domains) upon phosphorylation of the truncated CrkII by Abl. Binding pTyr207 to its own SH2 domain results in preventing the SH2 domain of CrkL from interacting with other phosphorylated signaling molecules, such as paxillin,<sup>22</sup> which indicates that phosphorylated CrkL may function as a negative regulator of signaling pathway. This concept is in line with studies from human NK cells, in which phosphorylated Crk dissociates from signal molecules a 125–135 kDa scaffold protein (p130) Crk-associated substrate (p130Cas) and Casitas B-lineage lymphoma (Cbl) protein during NK cells inhibition induced by engagement of two structurally distinct inhibitory receptors, CD94-NKG2A and killer-cell immunoglobulin-like receptor (KIR).<sup>35</sup> Therefore, pTyr207/221 and the SH2 domain collaboratively function as the first layer of regulation. However, when and where phosphorylated Crk dissociates with

signal molecules at the immunological synapses during inhibition in immune cells remains unclear.

The second layer of regulation is mediated by the regulatory SH3C domain. In general, SH3C functions as a negative regulatory unit because the SH3C contains elements that constrain binding to the SH2 and SH3N domains.<sup>18, 36</sup> Indeed, CrkI, which lacks the SH3C domain, shows more transforming activity than does CrkII, which may involve phosphorylation of Y221 in CrkII and the SH3C domain.<sup>8, 25</sup> However, a recent study demonstrated that the SH3C domain has positive effects via phosphorylation of Tyr251 residue in the RT-loop structure (a loop in SH3 structure named after arginine, R, and threonine, T residues), as shown in Figure 3. Phosphorylation of Tyr251 in a conserved PNAY sequence by Abl *in vitro* enhances activation of Abl by providing binding sites for the SH2/PTB (phosphotyrosine-binding domain) containing proteins.<sup>37, 38</sup> In contrast to this observation, Jankowski *et al.*<sup>22</sup> used molecular mass determination to demonstrate that CrkL exists as a monomer in solution and that only one Tyrosine site (Tyr207) is phosphorylated by Abl kinase *in vitro*. The more detailed discussion of the regulatory role of SH3C has been described.<sup>8</sup> In summary, the regulatory SH3C domain provides the second layer of regulation, and the exact role of SH3C in regulating CrkII and CrkL proteins remains controversial.

The third layer of regulation is mediated by prolyl cis-trans isomerization.<sup>39</sup> A recent finding by Sarkar *et al.*<sup>36, 39</sup> demonstrates that isomerization of chicken CrkII requires the SH3N-SH3C linker region, which CrkI lacks. The authors found that a proline (Pro238 in chicken, similar to conserved residue Pro237 in humans) on the linker tethering the two SH3 domains of the Crk adaptor protein interconverts between the *cis* and the *trans* conformations (Figure 3). In the *cis* conformation, the two SH3 domains interact intramolecularly, forming an auto-inhibitory conformation. In this auto-inhibition, SH2 may interact with SH3 domains, and other signal molecules cannot bind to Crk. In the *trans* conformation, Crk exists in an extended conformation (also referred to as uninhibited conformation) that constitutes approximately 10% of total Crk molecules in the absence of any intermolecular binding partners, but it serves to activate the protein upon ligand binding. Interconversion between the *cis* (auto-inhibited) and *trans* (activated) conformations is accelerated by the action of peptidyl-prolyl isomerase A, also known as cyclophilin A (CypA).<sup>36</sup> Functionally, phosphorylated Crk is similar to the *cis* form *in vitro*, in which Crk cannot bind to other signal molecules. Whether Crk molecules in the *cis* form have the same physiological outcomes as that of increased phosphorylation in the cell system or *in vivo* is not clear. The concept needs to be verified to determine if human CrkL or CrkII has similar proline isomerization in live cells and what receptor controls this process. Given the conserved Pro238 feature among all Crk proteins, the possibility exists that proline isomerization is present in other Crk proteins, such as CrkL or CrkII in humans. It will be of interest to examine the correlation of *cis* and *trans* conformations with the phosphorylation and un-phosphorylation of Crk *in vivo* and to determine how a cytosolic protein, CypA, affects the biological outcome in immune cells.

In summary, the activities of CrkII and CrkL are regulated not only by the tyrosine within the CrkII/CrkL and SH3C, but also by the key proline mediated isomerization (Pro238 in chicken), which further complicates the regulation of the Crk family proteins.

## THE ROLE OF CRK IN REGULATING T-CELL FUNCTIONS

The function of Crk in T lymphocytes has been reported to form multi-protein complexes through T cell receptor (TCR) stimulation with a specific focus on the CD4<sup>+</sup> T cell line - the Jurkat cell line. For example, Cbl becomes tyrosine-phosphorylated and associated with Crk upon TCR stimulation.<sup>40-43</sup> The formation of complex is summarized in Table 2. The detailed interaction of Crk family proteins in lymphocytes has been reviewed by Isakov<sup>15</sup> and Gelkop *et al.*<sup>16</sup> In the following sections, several critical signal molecules involved with Crk family proteins will be classified on the basis of TCR signaling, costimulatory receptor signaling, cytokine signaling, and T cell development.

### Crk in TCR signaling

Cbl, an E3-ligase that catalyzes protein ubiquitination,<sup>44, 45</sup> is the first protein found to regulate TCR signaling via Crk interaction upon anti-CD3 signaling.<sup>41-43, 46</sup> Following T cell activation, Crk binds phosphorylated Cbl and p85 (a regulatory subunit of PI3-K) with the Crk-SH2 domain and SH3N domain, respectively.<sup>47</sup> In NIH-3T3 fibroblasts, endogenous Crk migrates similarly to a monomeric, non-complexed protein and is tyrosine-phosphorylated.<sup>27</sup> It will be of interest to understand the state of endogenous Crk in resting primary lymphocytes. CrkL, but not Crk, is constitutively associated with C3G (Crk SH3-binding guanine nucleotide-releasing protein) in thymocytes. The association of C3G with CrkL is unaffected by CD3 stimulation of thymocytes and is not induced to associate with Crk.<sup>48</sup> Interestingly, other studies have shown that binding of Cbl-b with CrkL interferes with its efficacy to interact with the other binding partner-C3G. C3G is also a guanine exchange factor of Rap-1 (Ras-related GTP-binding protein 1).<sup>49</sup> Therefore, Cbl-b functions as a negative regulator of CrkL-C3G-mediated Rap1 (a member of the Ras family of small GTPases) activation in TCR-stimulated T cells.<sup>50</sup> In both Jurkat and normal human peripheral blood-derived T cells, TCR stimulation induces the association of Cbl with all three forms of the human Crk protein, most prominently with CrkL.<sup>43</sup> In anti-CD3-stimulated Jurkat T cells, TCR stimulation does not induce the phosphorylation of Crk or CrkL.<sup>43</sup> In summary, Crk family proteins involve TCR signaling by forming multi-complex with other signal molecules such as C3G. However, how phosphorylated Crk regulates TCR signaling and whether TCR stimulation modulates the ratio of Crk and phosphorylated Crk remain unclear. Additional *in vivo* studies in primary T lymphocytes will be required to confirm the physiological function of Crk in TCR signaling and associations between Crk and other signal molecules.

### Crk in T cell co-stimulatory receptor signaling

In Jurkat cells, co-receptor CD2 engagement, not by CD3 engagement, induces the association of tyrosine-phosphorylated p62, dok1 (downstream of Tyrosine Kinase a member of the Dok family of adaptor molecules) to CrkL.<sup>51</sup> CrkL also plays an important role in lymphocyte function-associated antigen 1 (LFA-1) integrin ( $\alpha$ L $\beta$ 2) adhesion in T

lymphocytes.<sup>52</sup> In T cells, stimulation of TCR and beta1 integrin induces the association of p105CasL, a p130Cas homologous protein, with the SH2 domain of Crk or CrkL.<sup>53–55</sup> In primary T lymphocytes, LFA-1 does not bind to its ligand ICAM-1 without TCR engagement that delivers ‘inside-out’ signals to LFA-1. The CrkL-C3G complex is required for TCR-stimulated activation of Rap1, leading to integrin adhesion.<sup>52</sup> Similar to the roles of Crk in TCR signaling, the majority of work on the roles of Crk in T cells co-stimulatory receptors has relied on classical biochemical approaches to identify the binding partners such as p130Cas. However, the signaling pathways initiated by TCR that result in the role of Crk in integrin adhesion or other co-stimulatory receptors remain relatively obscure.

### Crk in T cell cytokine signaling

Crk not only modulates the TCR and co-receptors signaling, but also plays a critical role in the cytokine secretion. Crk proteins interact with the hematopoietic progenitor kinase 1 (HPK1), a serine-threonine kinase, which plays a critical role in IL-2 secretion.<sup>56</sup> Interestingly, stimulation by IL-2 alone induces the specific phosphorylation of CrkL, but not of Crk, in human IL-2-dependent Kit 225 T lymphocytes.<sup>57</sup> How IL-2 alone induces the specific phosphorylation of CrkL remains unclear. It will be of interest to investigate the roles of Crk family proteins in cytokine signaling.

### Crk in T cell development

In 1999, Imaizumi *et al.*<sup>58</sup> used the gene trap insertional mutagenesis approach to generate a mutant mouse line that is considered a Crk-II-specific disrupted mouse line as these mice express the truncated Crk proteins (similar to CrkI). Mice with CrkII-specific disruption (still expressing CrkL and CrkI) do not exhibit any obvious abnormalities, suggesting either that Crk-II is not essential for embryonic development or that CrkL may play a redundant and complementary role in embryonic development.<sup>58</sup> Park *et al.*<sup>59</sup> generated a complete null allele of Crk mice (still expressing CrkL) by using the Cre-loxP recombination approach. Crk-deficient mice die perinatally.<sup>59</sup> No Crk-null mice can survive to adulthood.<sup>59</sup> The Crk<sup>-/-</sup> embryonic data show that Crk is involved in cardiac and craniofacial development and plays an essential role in embryonic development,<sup>59</sup> which is reminiscent of the phenotype of CrkL<sup>-/-</sup> mice.<sup>60</sup> In 2001, Guris *et al.*<sup>60</sup> generated CrkL<sup>-/-</sup> mice. However, the majority of homozygous CrkL<sup>-/-</sup> mice die at late stages of embryonic development.<sup>60</sup> Only a small number of CrkL<sup>-/-</sup> mice (< 2%) survive, which suggests that *CrkL* is also an essential gene for embryonic development.<sup>60</sup> The phenotypes of Crk- and CrkL-deficient mice are summarized in Table 3. No data report T cell development in Crk<sup>-/-</sup> mice due to the lack of Crk<sup>-/-</sup> adult mice.

In 2003, Peterson *et al.*<sup>61</sup> investigated the role of CrkL in T-cell development and function. In CrkL<sup>-/-</sup> mice, the peripheral blood counts and functions are normal, whereas immunization induces comparable levels of IgG2a and IgG1 antibodies.<sup>61</sup> Transferring CrkL<sup>-/-</sup> fetal liver cells into irradiated Rag2<sup>-/-</sup> recipients resulted in the functions of T cells from these chimeric mice being normal, revealing that CrkL is not essential for T cell development or function.<sup>61</sup> Interestingly, the steady-state level of the Crk protein is not altered in the CrkL<sup>-/-</sup> mice, which indicates that Crk does not compensate for CrkL function

*in vivo*. Taken together, both *Crk* and *CrkL* genes are required for embryonic development in mice.

Intriguingly, *CrkL*<sup>-/-</sup> mice also show a phenotype characteristic of human DiGeorge/velocardiofacial syndrome (DGS/VCFS).<sup>60, 62, 63</sup> Three genes (*Tbx1*, *CrkL*, and *Erk2*) in chromosome 22q11.2 are responsible for the phenotypic features of DGS/VCFS.<sup>60, 63–66</sup> *Tbx1* and *CrkL* are two main genes for DGS/VCFS.<sup>64</sup> Heterozygous deletions within human chromosome 22q11 are the genetic basis of DGS/VCFS.<sup>67</sup> More than 80% of cases of DGS/VCFS are associated with a deletion within chromosome 22q11.<sup>64</sup> Not only multiple candidate genes (30–50 genes) located within the region,<sup>68</sup> but also dose-sensitive interaction of these genes, such as *Tbx1* and *CrkL* in mice, play roles in DGS/VCFS,<sup>63</sup> which adds an additional layer of complexity to the role of *CrkL* in DGS/VCFS. In general, decreased numbers of T cells with variable functional deficits (such as reduced proliferation capability to phytohemagglutinin) and normal production of immunoglobulin are observed in DGS/VCFS.<sup>69, 70</sup> Therefore, DGS/VCFS may provide a tool to study the role of *Crk* in human immunobiology and immunodeficiency disease.

## THE ROLE OF CRK IN REGULATING B CELL FUNCTIONS

### Crk in B-cell antigen receptor (BCR) signaling

In the DT40 B cell line, B cell antigen receptor (BCR) ligation induces the formation of the Cbl-CrkII complex.<sup>71</sup> The association of Cbl and CrkII is transient, depends on ligand-induced signaling, and does not require tyrosine phosphorylation of CrkII but does require the tyrosine phosphorylation of Cbl. The dissociation of Cbl-CrkII over time is due to the dephosphorylation of Cbl.<sup>71</sup> CrkII also plays a role in BCR endocytosis.<sup>72</sup> BCR ligation reduces the phosphorylation of CrkII in the DT40 B cell line. At least, the phosphorylation of CrkII did not increase after BCR ligation, which may be related to the constitutive phosphorylation of CrkII in resting the DT40 B cell line. Similarly, *CrkL* is not phosphorylated upon engagement of the BCR in the human Burkitt lymphoma lines Ramos and Daudi.<sup>73</sup> Generally, stimulation of activation receptors, such as BCR and TCR, does not induce the phosphorylation of *Crk*. In the Ramos B cell line, BCR ligation induces the SH2 domains of *CrkL* and *CrkII* to bind to Cas and Cbl. In addition to the formation of *Crk*-Cas-Cbl, *Crk* also interacts with C3G in Ramos cells via its SH3 domains, which can translocate C3G from the cytosol to the plasma membrane, where Rap is located upon BCR ligation.<sup>74</sup> In human tonsillar B cells and Nalm-6/ARH-77 B cell lines, ligation of either beta1 integrin or BCR induces tyrosine phosphorylation of human enhancer of filamentation-1 (HEF1), which forms a complex with *CrkL*.<sup>75</sup> BCR stimulation also induces the interaction of Src homology 2 domain containing (Shc)/growth factor receptor-bound protein 2 (Grb2) complex and *Crk*.<sup>76</sup> In human primary tonsillar B cells, BCR stimulation induces the association of Vav with *Crk*.<sup>73</sup> BCR stimulation cannot induce the tyrosine phosphorylation of either *Crk* or *CrkL*.<sup>73</sup> Overall, one of the main efforts directed toward *Crk* in BCR signaling is finding the binding partners of *Crk* in B cell lines. In summary, the association of Vav and *Crk* in human primary tonsillar B cells upon BCR stimulation is very intriguing, given the multiple roles of Vav in immune responses. Similar to the signal pathways of *Crk*

in TCR signaling, Cbl and C3G are the major players in BCR signaling among Crk binding partners.

### Crk in B cells co-stimulatory receptors signaling

Stimulation of BCR and LFA-1 induces the tyrosine phosphorylation and association of p130Cas with the Crk/CrkL SH2 domain.<sup>26</sup> In CD21-expressing Raji cells, a Burkitt B lymphoma cell line, activation of CD21 induces interaction of Cbl with the SH2 domain of CrkL.<sup>77</sup> Whether Crk also plays a role in the development of B cells remains unclear. In the 22q11.2 deletion syndrome (DiGeorge syndrome), in which the total B cell numbers are unaffected, some patients shows a significantly decreased proportion of memory B cells,<sup>78</sup> which indicates a possible role of CrkL in B cell development and memory. In summary, Crk adaptor proteins play a role in BCR signaling, BCR endocytosis, and co-stimulatory receptor signaling by interacting with other signal molecules such as Cbl. The spatial-temporal details of Crk signal and physiological signaling events in primary B cells need to be elucidated.

## THE ROLE OF CRK IN NK CELL FUNCTIONS

### Crk in NK cells inhibitory receptors signaling

NK cell inhibitory receptors block activating signaling by recruiting phosphatases that dephosphorylate critical molecules such as Vav1 involved in activation.<sup>79</sup> The detail of inhibitory receptor signaling has been described previously.<sup>79, 80</sup> In 2008, Peterson and Long<sup>35</sup> demonstrated that engagement of inhibitory receptors, including CD94-NKG2A and KIR, induces phosphorylation of Crk in both NK cell lines and primary NK cells. Crk phosphorylation leads to disassociation of the c-Cbl-Crk-p130CAS-C3G complex in NK cells in conjugates with their target cells expressing human leukocyte antigen class I. This c-Cbl-Crk-p130CAS-C3G complex occurs during NK cells activation induced by NK cells susceptible target cells such as 721.221 cells (an EBV transfected B cell line), which is similar to the complex formation between Crk and Cbl upon TCR and BCR ligation in T and B cells.<sup>15</sup> Interestingly, during inhibition of NK cells by engagement of inhibitory receptors killer-cell immunoglobulin-like receptor (e.g. KIR2DL1) and CD94-NKG2A, phosphorylated Crk dissociates from other signaling molecules such as C3G and p130cas, which is reminiscent of the status of auto-inhibition of chicken CrkII.<sup>39</sup> However, the status of Crk and ratio of Crk/pCrk in resting NK cells and the similarity in structure and function between cis conformation of Crk and phosphorylated Crk remain unclear.

In 2012, Liu *et al.* demonstrated that inhibitory receptor CD94-NKG2A alone induces phosphorylation of Crk.<sup>81</sup> In this study, the authors used a reductionist model of lipid bilayers containing ligand for activating receptor CD16 (Fc) and ligand for inhibitory receptor CD94-NKG2A (HLA-E) to selectively induce activation and inhibitory signaling, respectively.<sup>81</sup> Combining live cell imaging with lipid bilayers, the authors demonstrated the distinct roles of un-phosphorylation and phosphorylation of Crk in the control of actin polymerization in the inhibitory synapse induced by HLA-E and Fc containing lipid bilayer. The discovery of the ability of NK inhibitory receptor alone to induce phosphorylation of the adaptor proteins-Crk/CrkL marks a true paradigm shift in which inhibitory receptor



alone is sufficient to induce tyrosine phosphorylation, an event usually indicating cellular activation.<sup>81</sup> Therefore, a new model for inhibitory receptor function is proposed (Figure 4): Crk phosphorylation prevents Crk-dependent activation signals from their intended remodeling of the filamentous-actin (F-actin) network.<sup>81</sup> However, whether Crk can interact directly with the cytoplasmic domain of the inhibitory receptor, CD94-NKG2A/KIR, and how un-phosphorylated Crk and phosphorylated Crk affect actin dynamics at immunological synapses remain unclear. Overall, studies on the role of Crk in NK cell function have just begun. The role of the Crk family proteins in inhibitory receptors signaling and their roles in the cell biology of NK cells such as education/memory remain unclear.

### Crk in NK cell activating receptor signaling

NK cell activation is controlled by integration of signals from activation and inhibition.<sup>82</sup> Crk not only plays a role in NK cells inhibitory receptor signaling as described above, but also modulates the signaling of activating receptors. During NK cell activation induced by susceptible target cells, Crk forms a complex with C3G (presumably via the SH3N domain) and p130cas (presumably via the SH2 domain). During activation, the majority of Crk or CrkL is in the un-phosphorylated state. The un-phosphorylated CrkL may constitutively associate with endogenous Abl via the fully accessible SH3N domain, which indicates that the SH3N domain in un-phosphorylated CrkL may not be available during NK cell activation.<sup>35</sup> The un-phosphorylated CrkII forms a very different structure, compared to CrkL.<sup>22</sup> It is not clear which isoform of Crk is dominant in activated NK cells. In 2009, Segovis *et al.*<sup>83</sup> found that CrkL is important in NK cell-target cell conjugate formation, microtubule organization center (MTOC) polarization, NKG2D (activating receptor) - dependent cellular cytotoxicity, and antibody-stimulated granule release. In the conjugation between human NK clones with target cells expressing MICA (a ligand for NKG2D), CrkL was recruited to cytotoxic immunological synapses in an NKG2D-dependent manner. However, how CrkL is engaged with activating receptors signaling is not clear, and whether CrkL serves a functionally redundant role with CrkII in NK cellular cytotoxicity remains to be determined. In the recent study by Liu *et al.*<sup>81</sup>, Crk also is involved in CD16 (activating receptor)-induced degranulation. Silencing of Crk family of proteins expression with siRNAs targeted to both Crk and CrkL in human primary NK cells has profound effects on NK cells activation by CD16 signaling. Imaging of NK cells on lipid bilayers carrying Fc alone reveals that movement of Fc microclusters, phosphorylation of Vav1, and accumulation of F-actin are greatly reduced.<sup>81</sup> In a human NK cell line, NK3.3, stimulation of NK3.3 with IL-2 or crosslinking of CD2 or CD16 induces the interactions between Crk and Cbl upon silencing of Crk family of proteins.<sup>84</sup> The combination of IL-2 with CD2 crosslinking further increases tyrosine phosphorylation and association of Cbl with Crk.<sup>84, 85</sup> Taken together, Crk family proteins not only contribute to NKG2D mediated natural cytotoxicity signaling for adhesion, granulation polarization, and degranulation, but also play a critical role in the CD16 mediated cytotoxicity, also known as antibody-dependent cellular cytotoxicity.

## THE ROLE OF CRK IN OTHER IMMUNE CELL FUNCTIONS

CrkL is a major specific substrate for Abl in neutrophils. CrkL is heavily tyrosine phosphorylated in neutrophils of patients with Philadelphia chromosome positive chronic myelogenous leukemia (CML).<sup>21</sup> This finding is similar to the observation from v-Crk-transformed 3Y1 cells.<sup>27</sup> In Philadelphia chromosome negative peripheral blood cells, CrkL is present only in the non-phosphorylated form.<sup>86</sup> In contrast, all Bcr/Abl<sup>+</sup> CML and acute lymphoblastic leukemia patient samples examined show clear tyrosine-phosphorylation of CrkL.<sup>20, 86</sup> What role Crk plays in CML remains unclear. In summary, the role of Crk in other immune cells such as dendritic cells and macrophages remains to be determined.

## PERSPECTIVES

In this review, the roles of the Crk family of proteins in T, B, and NK cells receptor function have been briefly summarized. Since the identification of Crk in 1980s, great progress has been made in addressing the physiological and pathological roles of Crk in normal and transformed fibroblasts. Most research on Crk has focused on the cell apoptosis, proliferation, transformation processes, and identification of Crk binding partners.<sup>7, 8</sup> Although some of the signaling properties of Crk have been identified in the Jurkat cell line or other transformed lymphocyte cell lines by traditional biochemical and cell biological approaches, the role of Crk in immune response, including immune cytokine secretion, cytotoxicity, immunological synapse formation, and inhibitory receptor signaling on primary immune cells, as well as in infectious and primary immunodeficiency diseases, is poorly understood. Four important and significant questions about Crk in immune cells are listed as follows:

### **Do different isoforms of Crk family of proteins play different roles in immune cells?**

Given the different isoforms of Crk that exist in immune cells, an obvious question that remains to be answered is which isoform in particular types of immune cells, such as T, B, and NK cells, plays a role in different immune responses. Whether these isoforms play a complementary or redundant role in immunoreceptor signaling remains unclear. Addressing these questions will be helpful to design a specific target to harness the immune cells during infection and to induce the effective immune responses against cancer.

### **What is the role of phosphorylated Crk in NK cell licensing?**

In human primary NK cells, the phosphorylation of Crk induced by inhibitory receptor CD94-NKG2A alone is unexpected, considering that phosphorylation events usually indicate cell activation.<sup>81</sup> Given that the MHC class I-specific inhibitory receptors not only control NK cell cytotoxicity but also have a role in tuning intrinsic responsiveness of NK cells to subsequent activation signaling (a process referred to as 'licensing', 'arming', or 'education'<sup>87-90</sup>), this finding indicates that the phosphorylated Crk may play a role in NK cell licensing. However, how phosphorylated Crk family proteins function in NK cell education or NK licensing remains unclear, as does the status/amount of Crk (phosphorylated Crk, un-phosphorylated Crk, or de-phosphorylated Crk) in resting immune cells, IL-2 activated NK cells, and activating or inhibitory receptors-stimulated NK cells.

### What is the role of Crk in NK memory?

NK memory is an emerging topic in the field of immunology.<sup>91, 92</sup> One of the immunological features of the 22q11.2 deletion syndrome (DiGeorge syndrome) is the significantly reduced number of CD27<sup>+</sup> IgM<sup>+</sup> IgD<sup>+</sup> memory B-lymphocytes, which suggests that Crk family of proteins may play a role in B cell memory.<sup>67, 93, 94</sup> Therefore, it will be of interest to determine if Crk also plays a role in NK cell memory.

### How do Crk family proteins impact cytoskeletal organization?

Cytoskeletal remodeling plays a critical role in controlling cytotoxicity of cytotoxic T lymphocytes and NK cells.<sup>95, 96</sup> During activation of human primary NK cells induced by activating receptor CD16, Crk silencing has profound effects on actin organization, of which F-actin appears as disorganized patches at cytotoxic immunological synapses.<sup>81</sup> During inhibition of NK cells induced by inhibitory receptor CD94/NKG2A, F-actin is disrupted through Crk phosphorylation.<sup>81</sup> The molecular mechanism of cytoskeletal actin remodeling by Crk or phosphorylated Crk is not clear. Crk may regulate the F-actin dynamics via two distinct pathways. One is that Crk interacts with several downstream signal molecules such as Paxillin, C3G/Rap-1, and p130Cas/DOCK180/Rac-1 to regulate F-actin.<sup>14</sup> The second pathway could be the interaction between Crk and pVav-1, as shown in Figure 4. Vav-1 is a well-known molecule that can regulate actin cytoskeletal reorganization in both T and B cells.<sup>97-99</sup> Therefore, it will be of interest to investigate the possible mechanisms of Crk regulating actin dynamics in human NK cells, which will greatly enhance our understanding of how Crk functions as a master regulatory switch operating upstream of Vav-1 which in turn directly influences actin dynamics.

In summary, finding and identifying the proteins associated with Crk by classical biochemical approaches has been a major effort in the field during the past several decades. The time has come for immunologists to implement new cutting-edge technologies to explore the roles of Crk in immune response. The emerging super resolution imaging of live cells such as stimulated emission depletion microscopy and photoactivated localization microscopy approaches should elucidate the spatiotemporal details of Crk in the immune responses.

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### Abbreviations

<b>Abl</b>	Abelson murine leukemia kinase
<b>Bcr</b>	Breakpoint cluster region
<b>BCR</b>	B-cell antigen receptor
<b>Cas</b>	Crk-associated substrate

<b>Cbl</b>	Casitas B-lineage lymphoma
<b>Crk</b>	CT10 (chicken. tumor virus no. 10) regulator of kinase
<b>CrkL</b>	CT10 (chicken. tumor virus no. 10) regulator of kinase like
<b>C3G</b>	Crk SH3-binding GNRP (guanine nucleotide-releasing protein)
<b>CypA</b>	cyclophilin A
<b>DOCK180</b>	180-kDa protein downstream of Crk
<b>Dok1</b>	downstream of tyrosine kinases protein 1
<b>Erk</b>	Extracellular signal-regulated kinase
<b>F-actin</b>	filamentous –actin
<b>Gab1</b>	growth factor receptor-bound protein 2 (GRB2)-associated binding protein 1
<b>HEF1</b>	human enhancer of filamentation 1
<b>HPK1</b>	hematopoietic progenitor kinase 1
<b>KIR</b>	killer-cell immunoglobulin-like receptor
<b>PTB</b>	phosphotyrosine-binding domain
<b>PTKs</b>	protein tyrosine kinases
<b>PI3-K</b>	phosphoinositide 3-kinase
<b>Rac-1</b>	Ras-related C3 botulinum toxin substrate 1
<b>Rap-1</b>	Ras-related GTP-binding protein 1
<b>RT-loop</b>	loop in SH3 structure named after arginine, R, and threonine, T residues commonly found in it
<b>SH2 and SH3</b>	Src homology 2 and 3
<b>SHP-1</b>	SH2 domain-containing phosphatase 1
<b>TCR</b>	T cell receptor

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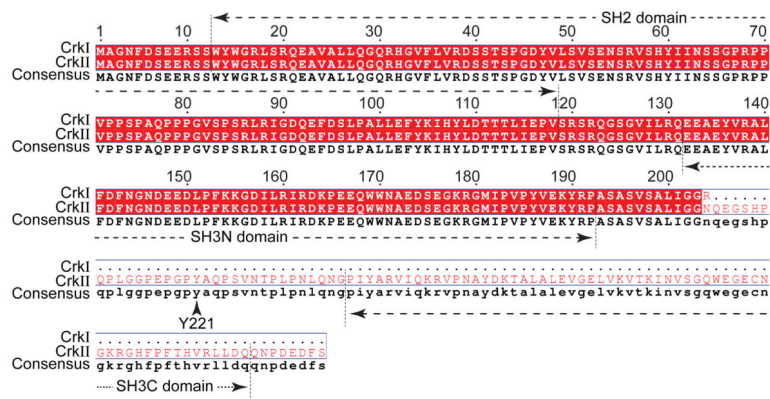
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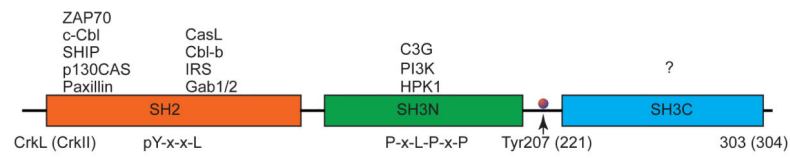


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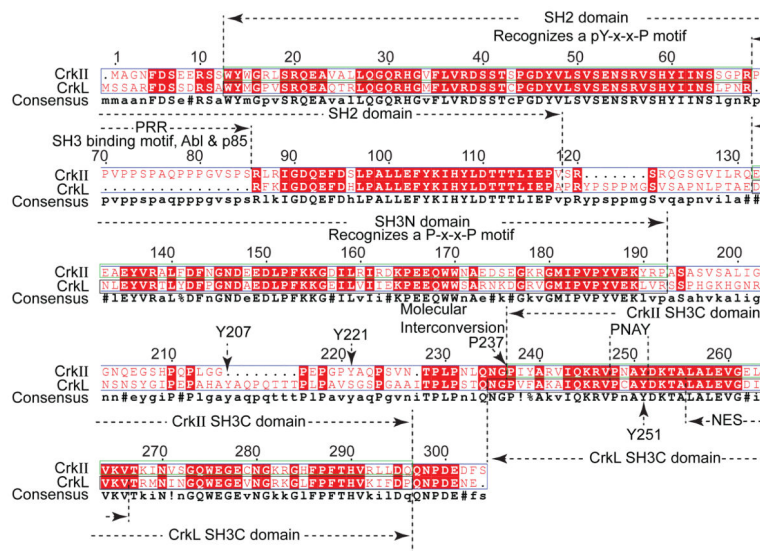
**Figure 1. Comparison of CrkI and CrkII in sequence**

CrkI and CrkII sequences are aligned by Multalin version 5.4.1 software (<http://multalin.toulouse.inra.fr/multalin/>). The consensus sequences are marked in Red.

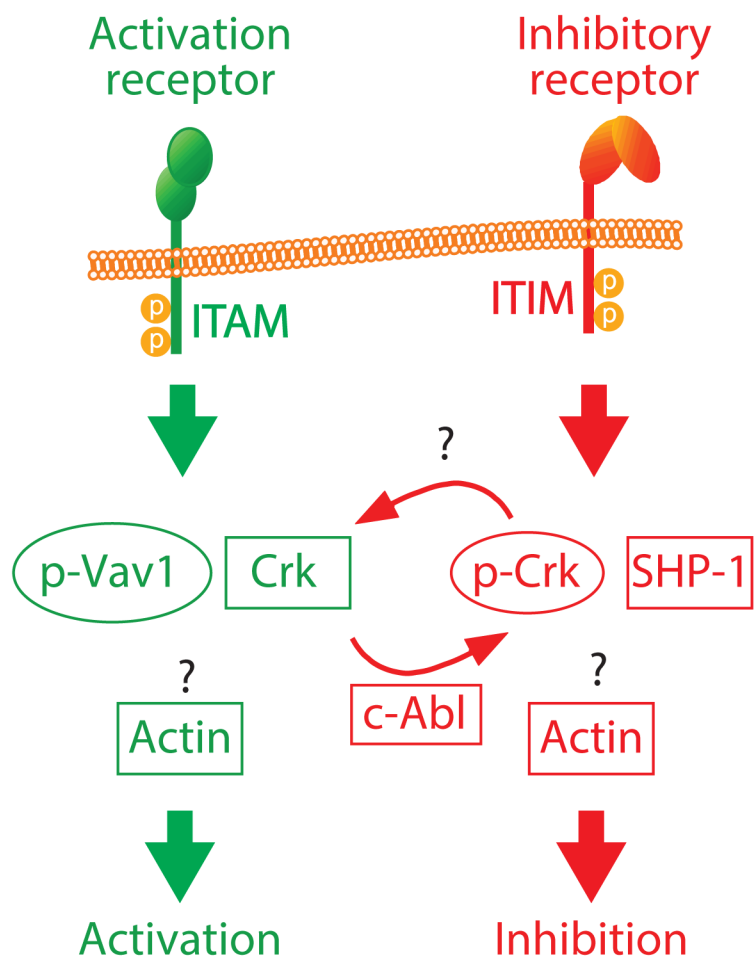


**Figure 2. Domains of Crk and its binding partners**

Schematic diagram of the domains of CrkL (303 aa) and CrkII (304 aa). The binding partners of SH2 and SH3 domains are listed on the top.



**Figure 3. Domain Comparison of CrkL and CrkII**  
 Sequence alignment of CrkL and CrkII by Multalin version 5.4.1 (<http://multalin.toulouse.inra.fr/multalin/>). The consensus symbols are as follows: ! is anyone of IV. % is anyone of FY. # is anyone of NDQE<sub>B</sub>Z.  
 NES = nuclear export signal. PRR = proline-rich region.



**Figure 4. Signaling mediated by Crk in human NK cell**

Phosphorylation of Crk induced by inhibitory receptors blocks NK cell activation. At activating synapses, Crk is required for the movement of activation receptor microclusters and their ability to trigger activation signals (not shown). During inhibition triggered by inhibitory receptor, Crk is phosphorylated and associates with the tyrosine kinase c-Abl. Interconversion between un-phosphorylated Crk (Green) and phosphorylated Crk (Red) functions as a molecular switch in the control of NK cell activation via interaction between Crk and pVav-1 or interaction between pCrk and SHP-1. Green indicates receptors and signal molecules that contribute to activation. Red indicates receptors and signal molecules that contribute to inhibition.

**Table 1**

Comparison of human CrkII and CrkL

	<b>CrkII</b>	<b>CrkL</b>
<b>Gene</b>	<i>CRK</i>	<i>CRKL</i>
<b>Gene location</b>	Chromosome 17p13.3	Chromosome 22q11.21
<b>Structure of un-phosphorylated Crk</b>	Binding to Bcr-Abl in a repressed structure Full accessible SH2 binding site Masked SH3N binding site SH3C interacts extensively with other SH2	Constitutively associates with Bcr-Abl Partially masked SH2 binding site Fully accessible SH3N binding site Independent SH3C domain
<b>Bcr-Abl binding to phosphorylated Crk</b>	Abrogated	Not abrogated
<b>SH3N accessibility in phosphorylated Crk</b>	Masked	Fully accessible
<b>Function in transforming fibroblasts</b>	does not transform fibroblasts.	Transforms fibroblasts.
<b>Number of amine acid (aa)</b>	304	303

**Similarities:**

- 1 Both are considered to be adaptor proteins.
- 2 Both have a role in the malignancy of various human cancers.
- 3 Both contain SH2-SH3-SH3 domain.
- 4 Both SH2 domains of CrkL and CrkII recognize phosphorylated tyrosine containing pYxxP domain.
- 5 Both SH3N domains associate with effector proteins containing PxxP motifs.
- 6 Both contain a regulatory tyrosine site.
- 7 Both carry SH3C domain.

**Table 2**

Proteins interact with Crk in T, B, and NK cells.

<b>Protein</b>	<b>Molecular Weight (kDa)</b>	<b>Stimulation</b>	<b>Cell Type</b>	<b>Biological function</b>
<b>p130cas</b>	130	Stimulated with 721.221	NK Cell	NK cell activation
		BCR cross-linking	Ramos B Cell	B cell activation
<b>p120-C-Cbl</b>	120	NIH-3T3 fibroblasts transfected with HLA-DR7	Human T cell clones	T cell anergy
		SDF-1 (Stromal cell-derived factor SDF-1)	Jurkat Cell	Chemokine receptor signaling
<b>Cbl-b</b>	120	Anti-CD3	Jurkat Cell	Negative regulator of TCR
<b>Gab3</b>	97	Interleukin-2	T, NK cell	IL-2 signaling
<b>ZAP70</b>	70	Anti-CD3	Jurkat Cell	T lymphocytes activation
<b>HPK1</b>	97	Anti-CD3	Jurkat Cell	T lymphocytes activation
<b>PI3K</b>	85	Anti-CD3	Jurkat Cell	T lymphocytes activation
<b>C3G</b>	120	Stimulated with 721.221	NK Cell	NK cell activation
		BCR and integrin ligation	B Cell	B cell activation

**Table 3**

Phenotypes of Crk and CrkL-deficient mice

	CrkI $-/-$	CrkII mutant mice lacking CrkII by the Gene Trap strategy	Crk $-/-$	CrkL $-/-$
<b>General phenotypes</b>	ND*	No obvious abnormalities	<ol style="list-style-type: none"> <li>1 Die at embryonic development.</li> <li>2 Defect in blood vessel, vascular smooth muscle cells, nasal development, and cleft palate.</li> </ol>	<ol style="list-style-type: none"> <li>1 Cardiovascular, craniofacial, and glandular features with embryonic death, a similar phenotype characteristic of DiGeorge Syndrome in humans</li> <li>2 Fewer than 2% of the expected proportion of homozygous mutant mice surviving to birth.</li> </ol>
<b>Viability</b>	ND	Normal	95% of mice die. The remainder (5%) die shortly after birth.	98% of mice die.
<b>B cell development</b>	ND	ND	ND	ND
<b>T cell development</b>	ND	ND	ND	Normal
<b>T cell function</b>	ND	ND	ND	<ol style="list-style-type: none"> <li>1 Peripheral blood counts are normal.</li> <li>2 Thymus has 40% fewer cells.</li> <li>3 No alteration in T cell function as measured by T cell numbers, expression of memory markers, IL-2 production, proliferation, and differentiation into Th1/Th2 phenotypes.</li> </ol>
<b>NK cell development</b>	ND	ND	ND	ND

\* no data