Cutting Edge: Activation of NK Cell-Mediated Cytotoxicity by a SAP-Independent Receptor of the CD2 Family

Axel Bouchon, Marina Cella, Helen L. Grierson, Jeffrey I. Cohen and Marco Colonna

*J Immunol* 2001; 167:5517-5521; doi: 10.4049/jimmunol.167.10.5517
http://www.jimmunol.org/content/167/10/5517

References

This article cites 20 articles, 7 of which you can access for free at:
http://www.jimmunol.org/content/167/10/5517.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Activation of NK Cell-Mediated Cytotoxicity by a SAP-Independent Receptor of the CD2 Family

Axel Bouchon,* Marina Cella,† Helen L. Grierson,‡ Jeffrey I. Cohen,§ and Marco Colonna2*†

Some CD2 family receptors stimulate NK cell-mediated cytotoxicity through a signaling pathway, which is dependent on the recruitment of an adapter protein called SLAM-associated protein (SAP). In this work we identify a novel leukocyte cell surface receptor of the CD2 family called CD2-like receptor activating cytotoxic cells (CRACC). CRACC is expressed on cytotoxic lymphocytes, activated B cells, and mature dendritic cells, and activates NK cell-mediated cytotoxicity. Remarkably, although CRACC displays cytoplasmic motifs similar to those recruiting SAP, CRACC-mediated cytotoxicity occurs in the absence of SAP and requires activation of extracellular signal-regulated kinases-1/2. Thus, CRACC is a unique CD2-like receptor which mediates NK cell activation through a SAP-independent extracellular signal-regulated kinase-mediated pathway. The Journal of Immunology, 2001, 167: 5517–5521.

N atural killer cells contribute to early, nonadaptive host responses against pathogens by granule exocytosis-mediated cytotoxicity and IFN-γ release. These effector responses are initiated by multiple NK cell receptors, which activate signaling pathways involving protein tyrosine kinases as well as mitogen-activated protein kinases (MAPK).1 One emerging group of activating NK cell receptors encompasses cell surface molecules of the Ig superfamily homologous to CD2. The prototype of these receptors, known as 2B4/CD244 (3–7), stimulates cytotoxicity through a signaling pathway, which is strictly dependent on the recruitment of an adapter protein called SLAM-associated protein (SAP) or SH2D1A (2, 7). Thus, NK cells derived from SAP-deficient individuals are no longer activated through 2B4 (2, 8–12). SAP is also essential for the signal transduction of other CD2 family receptors, such as SLAM/CD150, CD84, and Ly-9, which are differentially expressed on cytotoxic lymphocytes, Th cells, B cells, and myeloid cells (2, 13, 14). The lack of function of all these receptors in SAP-deficient individuals results in a complex deficit of NK, T, and B cell responses, which leads to uncontrolled EBV infections and, ultimately, to the X-linked lymphoproliferative disease (XLPD) (2, 13, 14).

In an attempt to identify novel cell surface receptors potentially involved in controlling EBV infections, we searched the expressed sequence tag database for CD2-like molecules. By this approach we have identified a novel human receptor called CD2-like receptor activating cytotoxic cells (CRACC). Functional characterization revealed that CRACC triggers NK cell-mediated cytotoxicity through a unique SAP-independent extracellular signal-regulated kinase (ERK)-dependent signaling pathway.

Materials and Methods

Cloning of CRACC cDNA
GenBank expressed sequence tagged database revealed that CRACC is a cell surface molecule homologous to CD2 on the basis of amino acid sequence homology. We performed an open reading frame encoding CRACC. CRACC cDNA was amplified from NK and CD8 T cell RNA by RT-PCR, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and sequenced. PCR primers were: 5′-ATGCTGGTTCCAACAT and 3′-ATTAATAGGAATACTTCTAA.

Production of CRACC-HulG fusion protein and anti-CRACC mAb
To produce soluble CRACC, J558L mouse myeloma cells were transfected with a chimeric gene encoding the CRACC extracellular domain fused with human IgG1 constant regions (CRACC-HulG). Anti-CRACC mAb 162 (mouse IgG2b, κ) was raised by immunizing BALB/c mice against CRACC-HulG. F(ab′)2 of mAb 162 were prepared using the F(ab′)2 Kit (Pierce, Rockford, IL).

Transient transfections
CRACC cDNA was subcloned into pCMV-1-FLAG (Kodak, Rochester, NY) and expressed as amino-terminal FLAG peptide fusion protein (CRACC<sub>FLAG</sub>) in 293 cells. Cell surface expression of CRACC<sub>FLAG</sub> was determined by flow cytometry with mAb M2 (anti-FLAG; Kodak).

Cells
PBMC and NK cell lines from normal controls and XLPD patients were obtained as previously described (8). NK92 is a human NK cell line which
lacks the Fc receptor CD16. PR15 is a murine mastocytoma cell line. Peripheral B cells and monocyte-derived dendritic cells (DC) were activated by incubation with CD40L-expressing cells and influenza virus strain PR8, respectively.

**Flow cytometry**

In four-color flow cytometric analysis, PBMCs were sequentially incubated with PBS-20% human serum, anti-CRACC mAbs 162, PE-conjugated human-adsorbed goat anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL) and PBS-20% normal mouse serum. One aliquot of cells was further incubated with anti-CD3-PC5, anti-CD19-FITC, and anti-CD56-APC mAbs (Immunotech, Marseille, France). A second aliquot was incubated with anti-CD3-PC5, anti-CD4-FITC, and anti-CD8-APC mAbs (Immunotech).

**51Cr release assay**

NK cell cytotoxicity was tested against [51Cr]-labeled PR15 cells in the presence of 10 μg/ml of either mAb 162, mAb IC7 (anti-2B4, IgG1; Refs. 6 and 7), mAb 9E2 (anti-NKp46, IgG1 (Ref. 8), mAb (anti-CD16, IgG1 (DAKO, Carpinteria, CA) or a control mouse IgG (Immunotech). F(ab′)2 of mAb 162 were used as indicated. In some experiments, NK92 cells were preincubated for 1 h with the mitogen-activated protein/ERK (MEK) inhibitor PD98059 (20 μM) (Calbiochem, San Diego, CA) before coincubation with [51Cr]-labeled PR15 target cells. Control NK92 cells were incubated with DMSO, which was used as a solvent for PD98059.

**Surface biotinylation, pervanadate treatment, and immunoprecipitations**

Immunoprecipitations with mAb 162 or control IgG from biotinylated NK92 cells were performed and analyzed as previously described (4). Lytesates from endosome-treated cells were subjected to immunoprecipitation with mAbs 162, IC7, Z199 (anti-NKG2A; Immunotech), or control IgG1. Western blot analyses of immunoprecipitates were performed with anti-phosphotyrosine PY20-HRP (BD Transduction Laboratories, Lexington, KY), anti-SAP (kindly provided by S. Tangye, University of Sidney, Sydney, Australia, and H. Nakajima, National Institute for Longevity Sciences, Aichi, Japan), anti-SHP-1 (BD Transduction Laboratories), anti-SHP-2 (BD Transduction Laboratories), anti-SHIP (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-EAT2 rabbit antibodies. Anti-EAT2 antisera was generated by immunizing rabbits with the keyhole limpet hemocyanin-conjugated peptide DLPYYHGRLTKQDCETL. Western blot analysis with anti-phospho-ERK1/2 and anti-ERK1/2 Abs (New England Biolabs, Beverly, MA) was performed on NK92 cells following stimulation with mAb 162 or a control IgG mAb in the presence of a cross-linking Ab (goat anti-mouse IgG, F(ab′)2; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1.5 and 10 min. In some experiments, NK92 cells were preincubated for 1 h with the MEK inhibitor PD98059 (20 μM).

**Results**

**CRACC is a novel transmembrane protein of the CD2 superfamily**

CRACC cDNA encodes a protein of 335 amino acids with a predicted molecular mass of ~37 kDa (Fig. 1). A putative hydrophobic signal peptide is followed by an extracellular region composed of two Ig superfamily domains containing seven potential N-glycosylation sites. The membrane-distal V-type Ig fold lacks the inter-β sheets disulfide bridge. This feature is a hallmark of the CD2 family members (15). The membrane-proximal Ig fold is of the C2 type. The hydrophobic transmembrane domain is followed by a cytoplasmic domain, which contains four tyrosine-based motifs. Some of these motifs closely resemble those recruiting the adapter protein SAP (2), which is essential for 2B4-mediated activation (Fig. 1) (2, 7–14). CRACC cDNA was amplified by RT-PCR from human NK cells and CD8+ T cells (data not shown). Therefore, we designated this molecule CD2-like receptor activating cytotoxic cells, CRACC. An alignment of the extracellular domains of CD2 family members showed that CRACC is most closely related to Ly-9 and CD84 (~28% identity) (Fig. 1 and data not shown). The gene encoding CRACC was identified within the human chromosome 1 genomic sequence performed by the Sanger Center (Cambridge, U.K.; accession no. AL121985, tentative gene designation LOC57823, tentative protein designation 19A24). It maps on human chromosome 1q23–24, telomeric of CD48, CD150, and CD84, and centromeric of Ly-9 (CD229) and 2B4. A cDNA corresponding to CRACC was also recently cloned from NK cells by Boles and Mathew (16) (protein designation CS1, accession no. AF291815).

CRACC is an ~66-kDa cell-surface glycoprotein selectively expressed on NK cells, a subset of cytotoxic T cells, and activated B cells and DCs

To investigate the cellular distribution of CRACC, we produced an anti-CRACC mAb, which specifically stained CRACC-transfected 293 cells, as compared with control transfectants (Fig. 2A). In human peripheral blood, CRACC was expressed on virtually all NK cells, a large subset of CD8+ T cells, and an average maximum of peripheral CD4+ T cells (Fig. 2B). CRACC was also detectable on a small subset of peripheral B cells (Fig. 2B) and became strongly expressed on all B cells upon activation through CD40 (Fig. 2C). CRACC was not expressed on monocytes and immature DC derived in vitro from monocytes, but was up-regulated upon DC maturation induced by influenza virus (Fig. 2D), lipopolysaccharide, and CD40L (data not shown). To determine CRACC biochemical characteristics, we immunoprecipitated CRACC from the NK cell line NK92, detecting a broad band of ~66 kDa under reducing conditions (Fig. 3). After deglycosylation, the immunoprecipitate appeared as a sharp band of ~37 kDa, which corresponds to the predicted molecular mass of CRACC polypeptide (Fig. 3). Together, these results identify CRACC as an ~66-kDa glycoprotein preferentially expressed on cytotoxic lymphocytes, activated B cells, and mature DCs.
CRACC triggers cytotoxicity of NK cells derived from both normal donors and XLPD patients

Expression of CRACC on NK cells and CD8+ T lymphocytes suggested it was involved in the activation of cell-mediated cytotoxicity. This hypothesis was investigated by reverse Ab-dependent cell-mediated cytotoxicity (rADCC). In these experiments, the Fc receptor (FcR) murine mastocytoma cell line P815 was incubated with NK cells in the presence of different mAbs which bind the FcR on target cells and triggering receptors on NK cells, thereby mimicking the stimulatory ligands. Anti-CRACC mAb activated lysis of P815 cells by NK92, whereas the F(ab')2 of the same Ab had no effect (Fig. 4A). The lysis triggered by simultaneous engagement of CRACC and CD16 or CRACC and NKp46 was approximately equivalent to the sum of the lyses induced by the same Ab had no effect (Fig. 4A). Thus, CRACC-mediated pathway does not synergize with those initiated by CD16 or NKp46.

The structural similarity between the cytoplasmic tyrosine-based motifs of CRACC, CD150, 2B4, and CD84 suggested that CRACC-mediated activation might require recruitment of SAP. Thus, we tested the function of CRACC by rADCC using NK cells from SAP-deficient XLPD patients. NK cells derived from both XLPD patients and controls revealed normal cell surface expression of CRACC as well as NKp46 and 2B4 (data not shown).

Remarkably, the anti-CRACC Ab triggered lysis of P815 by both SAP-deficient and normal NK cells (Fig. 4, C–F), as did the anti-NKp46 Ab. In contrast, the anti-2B4 mAb triggered lysis of P815 only by normal NK cells. Thus, CRACC-mediated activation of NK cells is SAP-independent.
CRACC recruits 19- and 39-kDa phosphoproteins upon tyrosine phosphorylation and does not associate with SAP, EAT-2, or protein tyrosine phosphatases

To characterize the CRACC signaling pathway, CRACC was immunoprecipitated from NK92, which was either unstimulated or stimulated with sodium pervanadate. Anti-phosphotyrosine blot of CRACC immunoprecipitates showed a substantial tyrosine phosphorylation of CRACC in pervanadate-treated cells together with the association of a 19-kDa tyrosine phosphorylated protein (Fig. 5A). A weak 39-kDa phosphoprotein was also observed which was reminiscent of the linker for activation of T cells (LAT) previously shown to associate to 2B4 (17). However, LAT was not detectable by Western blot analysis of CRACC immunoprecipitates (data not shown). Anti-SAP immunoblotting demonstrated lack of SAP association, in agreement with the results obtained in rADCC experiments (Fig. 5B). We also investigated the potential recruitment of other proteins previously found to be associated with CD2-like receptors, such as SHP-1 (11), SHP-2 (2, 7), SHIP (18), or EAT-2, which is a SAP-homologous adapter protein encoded on human chromosome 1 (19). However, we could not detect association of CRACC with any of these proteins by specific immunoblot analysis (Fig. 5B). Thus, in pervanadate-treated NK cells, CRACC is tyrosine phosphorylated, is associated with 19- and 39-kDa phosphophorylated proteins, and does not recruit LAT, SAP, EAT-2, SHP-1, SHP-2, or SHIP.

CRACC triggers ERK activation while pharmacological inhibition of ERK blocks CRACC-mediated cytotoxicity

Recent evidence indicates that spontaneous cytotoxicity of NK cells against target cells requires activation of ERK (1, 20). Thus, we asked whether CRACC activates ERK and, if this is the case, whether ERK activation is essential for CRACC-mediated cytotoxicity. Ab-mediated cross-linking of CRACC in NK92 induced tyrosine phosphorylation of ERK1/2, as demonstrated by anti-phospho-ERK1/2 immunoblotting (Fig. 6A). In addition, CRACC-mediated rADCC was partially inhibited by pretreatment of NK92 with PD98059, a specific inhibitor of ERK phosphorylation (Fig. 6B). Thus, CRACC-mediated cytotoxicity occurs through an ERK-mediated pathway.

Discussion

Our study identifies CRACC as a cell surface glycoprotein of the CD2 family, which activates NK cell-mediated cytotoxicity through an ERK-mediated pathway which is SAP-independent. The lack of recruitment of SAP or EAT-2 adapters by CRACC is a unique feature among CD2-like receptors (2). Because CRACC is functional in SAP-deficient XLPD patients, it may be crucial in host responses against viruses other than EBV. We have demonstrated that ligation of CRACC induces phosphorylation and activation of ERK and that pharmacological inhibition of ERK reduces CRACC-mediated cytotoxicity. It was shown that spontaneous and Ab-dependent NK cell-mediated cytotoxicity are also ERK-dependent and that ERK activation occurs through Ras-independent and Ras-dependent pathways, respectively (1, 20). It will be important to define the sequence of signal transducers, which is initiated by engagement of CRACC and leads to ERK activation. The 19- and 39-kDa phosphorylated proteins recruited by CRACC upon tyrosine phosphorylation may be critical intermediates along this pathway.

The characterization of CRACC is a further demonstration that NK cell-target cell recognition is highly complex and involves multiple interactions at the NK cell/target cell interface. Like other receptors of the CD2 family, CRACC may mediate homotypic interaction or bind to other members of the same receptor family on target cells (3). We detected no interaction of soluble CRACC-HuIgG fusion protein with 293 cells expressing each of the known CD2 family members by flow cytometry (data not shown). The binding affinity of CRACC for its ligand may be too low to be detected by this assay. Alternatively, CRACC may bind to new
members of the CD2 family yet to be characterized, such as BLAME, which was very recently discovered (21). The expression of CRACC on CTLs is noteworthy. CRACC induced no CTL-mediated cytotoxicity in rADCC (data not shown). Thus, CRACC may instead costimulate CD8⁺ T cells by interacting with ligands expressed on target cells. The expression of CRACC in activated B cells and mature DC further supports the idea that CRACC may be involved in modulating not only innate responses but also Ag-specific responses to pathogens. This dual role may be important in controlling infections by pathogens other than EBV.

Acknowledgments
We thank Jacqueline Samaridis and Lena Angman for excellent technical assistance, Rachel Ettinger and Susan Gilfillan for reviewing the manuscript, and Hideo Nakajima and Stuart Tangye for anti-SAP Ab.

References
1. Perussia, B. Signaling for cytotoxicity. 2000. Nat. Immunol. 1:372.
2. Morra, M., D. Howie, M. S. Grande, J. Sayos, N. Wang, C. Wu, P. Engel, and C. Terhorst. 2001. X-linked lymphoproliferative disease: a progressive immunodeficiency. Annu. Rev. Immunol. 19:657.
3. Mathew, P. A., B. A. Garni-Wagner, K. Land, A. Takashima, E. Stoneman, M. Bennett, and V. Kumar. 1993. Cloning and characterisation of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated NK cells and T cells. J. Immunol. 151:5528.
4. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. Eur. J. Immunol. 29:1676.
5. Boles, K. S., H. Nakajima, M. Colonna, S. S. Chuang, S. E. Stepp, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. Tissue Antigens 54:27.
6. Kubin, M. Z., D. L. Parshley, W. Din, J. Y. Waugh, T. Davis-Smith, C. A. Smith, B. M. Macduff, R. J. Armitage, W. Chin, L. Cassiano, et al. 1999. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. Eur. J. Immunol. 29:3466.
7. Tangye, S. G., S. Lazetic, E. Woollatt, G. R. Sutherland, L. L. Lanier, and J. H. Phillips. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. J. Immunol. 162:6981.
8. Nakajima, H., M. Cella, A. Bouchon, H. L. Grierson, J. Lewis, C. S. Duckett, J. I. Cohen, and M. Colonna. 2000. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. Eur. J. Immunol. 30:3309.
9. Tangye, S. G., J. H. Phillips, L. L. Lanier, and K. E. Nichols. 2000. Functional requirement for SAP in 2B4-mediated activation of human NK cells as revealed by the X-linked lymphoproliferative syndrome. J. Immunol. 165:2932.
10. Benoist, L., X. Wang, H. F. Pabst, J. Dutz, and R. Tan. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. J. Immunol. 165:1549.
11. Parolini, S., C. Bottino, M. Falco, R. Augugliaro, S. Giliani, R. Franceschini, H. D. Ochs, H. Wolf, J. Y. Bonnefoy, R. Biassoni, et al. 2000. X-linked lymphoproliferative disease: 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. J. Exp. Med. 192:337.
12. Sayos, J., K. B. Nguyen, C. Wu, S. E. Stepp, D. Howie, J. D. Schatzle, V. Kumar, C. A. Biron, and C. Terhorst. 2000. Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene products interact with SLAM and 2B4. Int. Immunol. 12:1749.
13. Nichols, K. E. 2000. X-linked lymphoproliferative disease: genetics and biochemistry. Rev. Immunogenet. 2:256.
14. Schuster, V., H. W. Kruth. 2000. X-linked lymphoproliferative disease is caused by deficiency of a novel SH2 domain-containing signal transduction adaptor protein. Immunol. Rev. 178:21.
15. Barclay, A. N., M. H. Brown, S. K. A. Law, A. J. McKechnie, M. G. Tomlinson, and P. A. van der Merwe. 1997. CD2. In The Leukocyte Antigen Facts Book. Academic Press, San Diego, p. 134.
16. Boles, K. S., and P. A. Mathew. 2001. Molecular cloning of CS1, a novel human natural killer cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. Immunogenetics 52:302.
17. Bottino, C., R. Augugliaro, R. Castriconi, M. Nanni, R. Biassoni, L. Moretta, and A. Moretta. 2000. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. Eur. J. Immunol. 30:3718.
18. Shlapatska, L. M., S. V. Mikhalkov, A. G. Berdova, O. M. Zelensky, T. J. Yun, K. E. Nichols, E. A. Clark, and S. P. Sidorenko. 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J. Immunol. 166:5480.
19. Thompson, A. D., B. S. Braun, A. Arvand, S. D. Stewart, W. A. May, E. Chen, J. Korenberg, and C. Denny. 1996. EAT-2 is a novel SH2 domain containing protein that is upregulated by Ewing’s sarcoma EWS/FLI1 fusion gene. Oncogene 13:2649.
20. Jiang, K., B. Zhong, D. L. Gilvary, B. C. Corliss, E. Hong-Geller, S. Wei, and J. I. Cohen. 2000. Functional requirement for SAP in 2B4-mediated activation of human NK cells as revealed by the X-linked lymphoproliferative syndrome. J. Immunol. 165:2932.