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*J Immunol* 2003; 171:4195-4202; ;
doi: 10.4049/jimmunol.171.8.4195
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The Rac-Activating Toxin Cytotoxic Necrotizing Factor 1 Oversees NK Cell-Mediated Activity by Regulating the Actin/Microtubule Interplay

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The cell cytoskeleton is widely acknowledged as a master for NK cell function. Specifically, actin filaments guide the NK cell binding to target cells, engendering the formation of the so-called immunological synapse, while microtubules direct the killer behavior. All these cytoskeleton-dependent activities are competently governed by the Rho GTPases, a family of regulatory molecules encompassing the three different subfamilies, Rho, Rac, and Cdc42. By using a Rac GTPase-activating bacterial protein toxin from Escherichia coli named cytotoxic necrotizing factor 1 (CNF1), we obtained results supporting the activation of Rac GTPase as a booster for effector cell-binding efficiency, recruitment ability, and, consequently, cytotoxicity. In particular, the augmented killer capacity of CNF1-treated NK cells was associated with the increased expression of certain cell adhesion or activation-associated molecules and the reshaping of the actin and microtubule networks. Importantly, CNF1 counteracted the activity exerted by toxins disrupting the cytoskeletal architecture. Hence, the activation of Rho GTPases, particularly Rac, induced by CNF1, appears to orchestrate a dynamic cross talk between microtubules and actin filaments, leading to a fruitful NK cell activity and polarization state. Our findings suggest that protein toxins might be viewed as modulators of NK cell cytotoxic activity and could possibly be regarded as useful pharmacological tools for certain Rho-linked immune diseases in the near future. The Journal of Immunology, 2003, 171: 4195–4202.

Cell-mediated cytotoxicity is part of the immune response involved in the defense against viral infection and cancer. It can be actuated by CTL or NK cells, both capable of switching on a multistage mechanism that allows them to first recognize and bind (conjugate formation) and then to kill (cytotoxicity process) the target cell (TC)2 (1, 2). This chain of events is promoted by the redistribution of killer cell surface structures that polarize toward the TC, an event considered of crucial importance in the NK-TC pairing formation (3). The recruitment and migration of NK cells from blood vessels into target tissues are also in fact piloted by the polarized localization of surface and cytoplasmic molecules (3, 4). This remodeling is supervised by cytoskeletal components, which are able to: 1) drive killer cell migration and locomotion; 2) influence their binding activity toward the TC; 3) regulate conjugate formation; and, finally, 4) modulate the killing efficiency. In particular, the microfilament system (MF) guides the binding process and the junction avidity, allowing the formation of the so-called immunological synapse (IS), a dynamic structure involved in the onset of cell-mediated cytotoxicity (3). The microtubular apparatus (MT), in contrast, plays a key role in the induction of TC lysis (5–7).

MF-targeting toxins, e.g., cytochalasins, impairs NK cell polarization and prevents conjugate formation (6), whereas MT perturbation (e.g., by colchicine) hinders the TC killing process (8). More importantly, some human immune diseases characterized by an impairment of NK cell function also display in parallel alteration in the sequence of the cytoskeletal changes governing an efficient NK cell activity (9).

The actin cytoskeleton that provides the driving force for cell polarization and migration is mainly regulated by small GTPases of the Rho family (5, 10, 11). These regulatory G proteins are molecular switches cycling between a GTP-bound form that activates downstream effectors and an inactive GDP-bound form. They are tightly regulated by upstream factors controlling the exchange of GDP for GTP and the rate of GTP hydrolysis (12). The Rho GTPase family encompasses three main subfamilies, Rac, and Cdc42, all involved in the control of the actin cytoskeleton architecture (13). Rho GTPases can be activated by a variety of extracellular and intracellular stimuli, and recent evidence indicates that MT may act as modulators of Rho GTPase activity, thus indirectly influencing MF (14). Conversely, besides organizing the actin cytoskeleton, Rho GTPases also influence the organization and dynamics of microtubules (14). The new concept, hence, that a strict interplay between MF and MT systems may be surveyed by Rho GTPases, is taking shape. In contrast, it is well established that Rho GTPases act as unique molecular switches at several critical checkpoints in lymphocyte development and function and that they may regulate quite diverse cellular processes in the immune response (15, 16).

To unravel the role played by Rho-dependent pathways in the NK cell activity, effector cells were challenged with Escherichia coli cytotoxic necrotizing factor 1 (CNF1), a bacterial protein toxin able to directly activate the Rho GTPases (17, 18). Very recently, Rac has emerged as the preferential target of the toxin (19). In our case, CNF1 favored both binding and killing activities in NK cells.

Abbreviations used in this paper: TC, target cell; CD, cytochalasin D; CdB, Clostridium difficile toxin B; CNF1, cytotoxic necrotizing factor 1; DEM, demecolcine; EC, effector cell; IS, immunological synapse; IVM, intensified video microscopy; MF, microfilament; MT microtubule; O/N, overnight; SEM, scanning electron microscopy; WASP, Wiskott-Aldrich syndrome protein.
as well as dynamic processes supporting the cytotoxic ability, such as NK cell recycling and recruitment. This was most probably achieved by switching on/off the Rac GTPase that governs the interplay between the actin cytoskeleton and microtubules.

Materials and Methods

Cells

PBMC. Human PBMC were isolated by Ficoll-Hypaque (Flow Laboratories, Irvine, U.K.) gradient separation of buffy coats obtained from healthy volunteer blood donors, by the Transfusion Center of the University of Rome “La Sapienza” (Italy). PBMC were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 5 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and used as effector cells (EC), as described below.

NK cell purification. Highly purified (>95%) human NK cells (CD56+ I) were obtained from PBMC by depletion of T cells, B cells, and myeloid cells using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. NK cells were then resuspended in the medium, as above.

TC. The human erythroleukemic cell line K562 (TC) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 5 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were subcultured in 25-cm² Falcon plastic flasks at a density of ~10⁶ cells/ml. Subseeding were performed every 48 h. Flasks were placed in a 37°C incubator containing a 5% CO₂ humidified atmosphere.

Toxins and treatments

Toxins. E. coli CNF1, used to activate the Rac GTPase (19), was obtained from the 392 ISS strain (kindly provided by V. Falbo, Rome, Italy) and purified, as previously described (20). As negative controls, two different compounds were used: 1) a heat-inactivated (98°C for 10 min) CNF1 protein toxin (preparation, and 2) a nontoxic mutant of CNF1 that completely lacked the enzymatic activity. This mutant, in which the catalytic cysteine residue (cys 866) was converted to serine (CNF1 C866S), was kindly provided by E. Lemichez (Nice, France) and prepared, as previously described by Schmidt et al. (21). As a positive control, IL-2 (100 U/ml) was challenged overnight (O/N) (18 h) with PBMC or purified NK cells. Clostridium difficile toxin B (CdB), an inhibitor of Rho GTPases (22), was generously provided by M. Popoff (Paris, France). Cytochalaizin D (CD), an inhibitor of actin polymerization, and demecolcine (DEM), a microtubule-depolymerizing agent, were purchased from Sigma-Aldrich (St. Louis, MO).

Treatments. In all experiments, before the analysis of NK cell-mediated activities, effector cells were exposed to CNF1 (10⁻¹¹, 10⁻¹⁰, 10⁻⁹ M), a heat-inactivated CNF1, or mutated CNF1 (CNF1 C866S) for 18 h (O/N). CdB (5 ng/ml), CD (6 μg/ml), and DEM (1, 10, 50, and 100 μM) were either preincubated for 2 h and then exposed to CNF1 O/N at 37°C or incubated O/N when used as controls in the absence of CNF1.

Cytotoxicity assay

K562 TC (1 × 10⁵) were labeled with ⁵¹Cr by incubation with 100 μCi of Na₂⁵¹CrO₄ (NEN, Boston, MA) for 1 h at 37°C (23). EC were admixed with TC (1 × 10⁵ cells/well) at several ratios ranging from 3:1 up to 100:1. All experiments were performed in triplicate. Cell mixtures were incubated in bottom 96-well microtiter plates at 37°C for 4 h in an incubator containing a 5% CO₂ humidified atmosphere and then centrifuged at 800 rpm for 5 min. One hundred microliters of the supernatant were collected from each well and counted in a gamma counter (Wallac, Turku, Finland). Spontaneous release and percentage of specific ⁵¹Cr release were determined, as described (24).

Activated Rac GTPase pull down

Human NK cells, purified as described above, were treated for different lengths of time with CNF1 at 37°C. For each duration, cells were resuspended in buffer A (HEPES, 50 mM, pH 7.3; NaCl, 0.1 M; MgCl₂, 10 mM; glycerol, 5%; Nonidet P-40, 1%; NaF, 10 mM; PMSF, 1 mM; leupeptin, 10 μg/ml; pepstatin, 10 μg/ml; and aprotinin, 10 μg/ml), and activated Rac was precipitated by adding 80 μg of the Rac effector GST-p21-activated kinase-CD to 400 μg of total cell lysate, as previously described (25). Briefly, cell lysates/GST-p21-activated kinase mixture were gently rocked for 40 min at 4°C and, following pulse centrifugation, washed three times in buffer B (composed as buffer A, but with Nonidet P-40, 0.5%). Proteins were resolved on 12% SDS-PAGE and transferred on polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Activated Rac GTPase was revealed by immunoblotting using an anti-Rac mAb (Transduction Laboratories, Lexington, KY). In parallel, total cell lysates were immunoblotted with the same anti-Rac Ab to verify the equal amount of Rac proteins engaged in the pull-down assays.

Cell surface molecule analyses

The surface expression of molecules associated with effector cell activation (CD69, the IL-2R CD25, and HLA-DR), cell-cell interaction (CD2, CD11a/CD18 (LFA-1), CD54 (ICAM-1)), and that specifically mark NK cells (CD56), was defined by flow cytometry in toxin-treated or untreated cells. In particular, cells that were first washed in PBS were double stained with PE-conjugated Abs to CD56 and FITC-conjugated Abs to CD69, CD25, HLA-DR, CD2, CD11a, CD18, and CD54 (all from BD Biosciences, San Diego, CA) for at least 30 min at 4°C. Samples were then washed twice with PBS and fixed in 1% Formalin in PBS. Stained cells were then examined by flow cytometry using a FACScan (BD Biosciences), and the data were analyzed with CellQuest software (BD Biosciences) equipped with a 488 argon laser.

Morphometric analyses

Binding evaluation. EC were admixed with TC at three different E:T ratios (3:1, 6:1, and 12:1) in conical tube and centrifuged at 600 rpm for 5 min at room temperature. EC were subsequently incubated for 1, 3, and 4 h at 37°C in 5% CO₂. The binding ability of EC to bind to TC (number of cell pairs) was evaluated by phase-contrast light microscopy (minimum of 50 diverse fields, final magnification ×500). Parallel evaluation of nonpaired EC was also performed. Additional experiments were also conducted to define NK cell/NK cell aggregation state in absence of TC by using hereby flow cytometry (analyzing physical parameters: high forward and side scatter light) and phase-contrast microscopy (50 diverse fields, final magnification ×500). Each experiment was performed in triplicate.

Effector cell recruitment evaluation. Scanning electron microscopy (SEM) appraisal of effector cell recruitment was obtained by counting the number of conjugates with more than two effector cells bound to the same TC after 2 h of E:T coculture. This was assessed in at least 50 different microscopic fields scored at the same magnification (×500) at 6:1 E:T ratio. Each experiment was performed in triplicate.

Scanning electron microscopy (SEM)

Conjugated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose at room temperature for 20 min. After three washes in the same buffer, the cells were plated on poly(t-lysine)-coated coverslips for 20 min at room temperature. Cells were postfixed with 1% osmium tetroxide for 1 h, dehydrated through graded ethanol 70%, 80%, and 95%, and gold coated with a Balzers Union SCD 040 apparatus (Balzers, Liechtenstein). The samples were then examined with a Cambridge 360 scanning electron microscope.

Static cytometry analysis

For static cytometry analysis of E:T conjugates, cells were fixed with 3.7% (w/v) formaldehyde in PBS, pH 7.4, for 15 min at room temperature. After washing in the same buffer, cells were plated on poly(t-lysine)-coated coverslips for 20 min at room temperature. Cells were permeabilized with 0.5% (w/v) Triton X-100 (Sigma-Aldrich) in PBS for 5 min at room temperature. For cytosekelsk analyses, conjugated cells were stained with fluorescein-phalloidin (Sigma-Aldrich) or with anti-tubulin Abs (Sigma-Aldrich) at 37°C for 30 min. The first was a toxin capable of directly binding F-actin and was usually linked to a fluorescent marker. The second was a mixture of a and β (1:1) anti-tubulin mAbs capable of reacting with the cellular microtubular network. For the detection of tubulin, cells were subsequently incubated with anti-mouse IgG fluorescein-linked whole Ab (Sigma-Aldrich) at 37°C for 30 min. Finally, after washing, all the samples were mounted with glycerol/PBS (2:1) and observed with a Nikon Microphot fluorescence microscope by using an intensified video microscopy (IVM) equipment (charge couple device camera by Carl Zeiss Oberkochen, Germany). Pictures were obtained and processed by using specific software by Deltasisstem (Milan, Italy).

Data analysis and statistics

Statistical analyses were performed by using Student’s t test. Regarding flow cytometry studies, at least 10,000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest software, and the statistical significance was calculated by using the parametric Kolmogorov-Smirnov (K/S) test. As a general rule, p values of less than 0.01 were considered significant.
Results

\textbf{CNF1 augments cell-mediated cytotoxicity and induces Rac activation in NK cells}

Human effector cells exposed to CNF1 toxin were tested for their binding and killing aptness. NK cell-mediated cytotoxicity, defined as the percentage of cells releasing $^{51}$Cr (24), was evaluated at different E:T ratios ranging from 100:1 to 3:1 and by using three different CNF1 concentrations ($10^{-11}$ M, $10^{-10}$ M, $10^{-9}$ M). As shown in Fig. 1A, a significant increase in cytotoxicity was scored at all the CNF1 concentrations tested. Therefore, for all the ensuing experiments, according to previous reports dealing with the toxin (20), the concentration of CNF1 $10^{-10}$ M was chosen. The lowest E:T ratios analyzed were the more significantly modified ($p < 0.001$), being cytotoxicity values duplicated in some instances (see ratios 12:1 and 6:1). Notably, at higher ratios (50:1, 100:1), CNF1 failed in inducing significant changes in NK cell activity (data not shown). The significance of morphological changes is that they direct the IS formation, and the consequence of this is the establishment of the conjugate (2, 26). We consequently performed a SEM study to analyze the cell surface features of NK/TC pairs (Fig. 1B). The three different E:T ratios (12:1, 6:1, 3:1) used furnished overlapping results, at least from a morphological point of view. Hence, in Fig. 1B (and Fig. 1C; see below), conjugates at an E:T ratio of 6:1 after 2 h of conjugation are shown, in accordance with what was obtained with binding studies (Fig. 2A; see below). Of interest, CNF1 caused an impressive remodeling of the binding features, the conjugates forming an intimate contact region between effector and TC (Fig. 1B, right panel), more intertwined of that occurring in untreated pairs where a punctuate contact region was detectable (Fig. 1B, left panel). Based on the assumption that such a morphological remodeling may depend upon cytoskeleton rearrangement, an IVM analysis aimed at evaluating the actin microfilament involvement was performed. F-actin polarization was augmented in conjugates formed by NK cells exposed to CNF1 (Fig. 1C, right panel) if compared with the actin state in control pairs (Fig. 1C, left panel). In addition, two or more effectors were often visible as small cells with a typical polarized actin ring adhering on the surface of a single TC (Fig. 1C, right panel) (for the analyses of these multiple pairs, see below).

Before further proceeding into the investigation, an important aspect ascertained was the ability of CNF1 to activate the Rho GTPases in NK cells. Because it was very recently reported that the Rac GTPase represents the main protein target for CNF1 (19), we assessed by using pull-down experiments whether CNF1 was capable of activating the Rac GTPase in a purified population of NK cells (CD16-positive cells; refer to Materials and Methods). We observed that CNF1 induced a transient activation of Rac in CD16$^+$ NK cells, reaching a maximum after 4 h of toxin exposure and dramatically dropping after 5 h, when the GTP resulted unbound (Fig. 1D), in the same manner as that occurring in other cell types (19).

\textbf{CNF1 favors NK cell-mediated binding activity}

The results mentioned above indicated a modulatory activity of CNF1 on E:T conjugate formation first, and, consequently, on EC cytotoxicity. Hence, the ability of CNF1-treated EC to bind the TC was quantified at the three different E:T ratios (12:1, 6:1, 3:1), the same ratios tested for the morphological analyses (see above). Fig. 2A, which illustrates the results obtained after 1 h of killer/prey interaction, clearly showed remarkable differences between CNF1-treated and control samples. For instance, the percentage of E:T pairs at 12:1, 6:1, and 3:1 ratios was increased by 35 $\pm$ 4% (median value) in CNF1-treated samples with respect to control samples (Fig. 2A). The three E:T ratios tested, however, behaved in a very similar way. Therefore, all the subsequent experiments were performed with an E:T ratio of 6:1. Furthermore, a time course performed to define the binding behavior of NK cells in the presence of CNF1 clearly indicated a difference in trend between control and treated samples in their ability to form pairs (Fig. 2B). In

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\caption{The Rac-activating toxin CNF1 strengthens NK cell-mediated cytotoxicity. A. Cytotoxicity. Different concentrations of CNF1 induce a significant increase in cytotoxicity at all E:T ratios. Note, however, that a more significant increase in cytotoxicity is detectable at lower ratios. B and C. Analytical cytology. B. Scanning electron micrographs of E:T pairs in the presence or absence of CNF1. The ultrastructural analysis gives evidence that the presence of CNF1 modifies the IS, arousing surface ruffles toward the target (compare right with left panel). C. IVM fluorescence analysis of the actin network in E:T pairs in the presence or absence of CNF1 reveals an intense actin polarization toward the target in control pairs (left panel) even more pronounced in CNF1-treated cells (right panel). Note as the toxin can favor the adhesion of several NK cells to the TC surface. Magnification of SEM micrographs: $\times3000$ magnification of fluorescence micrographs $\times2500$. D. Immunoblot showing the transient activation of Rac due to CNF1 in NK-purified cells. In parallel, total cell lysates are used to verify the equal amount of the Rac GTPase subjected to pull-down experiments.}
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such a percentage was significantly (p < 0.01) augmented by toxin exposure at all E:T ratios considered. B. Time course (recycling). Time course experiments indicating the percentage of conjugated cells in control and CNF1-treated cells. Note in the latter the high percentage of cell pairs after 1 h of conjugation, a value reached by control samples only after 2 h. In the presence of CNF1, however, the binding rate remains above 30% along with the conjugation time (up to 4 h), while dropping to very low values in control samples. C. Multiple conjugates (recruitment). Scanning electron micrograph showing several effector cells surrounding a TC (left panel). The quantitative morphometric analysis indicates a highly significant increase of these multiple E:T pairs in CNF1-exposed samples with respect to controls (right panel). D. Binding. The decreased binding activity due to CdB or CD administration is significantly counteracted by CNF1. Mean values ± SD are shown.

particular, while a high percentage of conjugates (78%) was already found after 1 h of binding assay in CNF1-treated samples, such a percentage was significantly lower in control samples (25%). Curiously, in 2 h, the trend was quite the opposite (75% in control samples vs 28% in CNF1-treated samples), although the percentage of E:T pairs reverted to being significantly higher in CNF1-treated cells with respect to controls at later time points of coculture indicated in Fig. 2B, between 3 and 4 h (Fig. 2B). This was an indication of a recycling of effector cells once in the presence of CNF1. Besides promoting effector cell recycling, what was extremely interesting was that CNF1 also furthered effector cell recruitment (Fig. 2C). CNF1, in fact, promoted the recruitment of a higher number of effector cells on the same TC (48 ± 3%) in comparison with what occurred in untreated samples (18 ± 1.5%) (Fig. 2C). These results, based on morphometrical analyses conducted by SEM, showed a number of multiple pairs (more than two effectors per TC). A typical SEM micrograph depicting multiple pairing of extremely polarized EC is shown in Fig. 2C (left panel). Conversely, the number of free effectors was also evaluated. In fact, according to the above results, a significantly lower percentage of unpaired EC was detected in CNF1-treated samples (9 ± 2% after 1 h of E:T pairing) with respect to control samples (59.3 ± 8%). Moreover, given the role of the actin cytoskeleton in the NK cell-mediated binding activity, effector cells were challenged with IL-2 or with agents that differently perturb the actin architecture, namely CdB, a Rho GTPase inhibitory toxin, and CD, a drug that causes the actin breakdown without coming into contact with the Rho GTPases. O/N exposure to IL-2 was found to be irrelevant both in binding activity and cytotoxicity (data not shown). In contrast, as shown in Fig. 2D, CNF1 partially, but significantly reverted the inhibitory activity of CdB and CD. This suggested that in cells challenged with CNF1, mechanisms other than actin reorganization are crucial for the NK-mediated cell-binding activity.

**CNF1 influences NK-mediated adhesion properties**

The ability of CNF1 to reinforce the NK-mediated binding activity without apparently interfering with the actin network prompted us to investigate the expression of some surface molecules that are largely acknowledged as crucial in NK cell activation and function (1). Flow cytometry analysis revealed the ability of CNF1 to significantly increase (p < 0.01) the activation markers CD69 and HLA-DR expression as well as some adhesion molecules involved in NK/TC pairing, e.g., CD18 and ICAM-1 (CD54) and, to a lesser but significant extent (p < 0.05), IL-2R (CD25). CD2 and CD11a molecules remained unchanged (data not shown). The results achieved on CD69, CD25, HLA-DR, and CD18 molecules are shown in Fig. 3A. O/N exposure to IL-2 did not onset a similar response (data not shown).

We next performed checks aimed at evaluating: 1) the involvement of the enzymatic activity of CNF1 in the responses triggered by the toxin in purified NK cells, and 2) the ability of the toxin to modulate NK/NK aggregation. Thus, to verify whether the enzymatic activity of CNF1 could specifically influence the actin polarization at one pole of NK cells, we used a nontoxic mutant of CNF1 (CNF1 C866S) that completely lacks its enzymatic activity (21). As shown in Fig. 3B, the mutant CNF1 was not capable of triggering actin polarization at one pole of NK cells, as did CNF1 and the positive control IL-2. Furthermore, CNF1 C866S did not induce either marker activation or increased cytotoxicity (in terms of both 51Cr release and percentage of binding) after 18 h of treatment (data not shown). These data demonstrated the importance of the enzymatic activity of CNF1 in the NK-mediated responses. In addition, a heat-inactivated CNF1 (98°C for 10 min) that was used as an additional control failed in inducing measurable effects either in terms of binding activity or in terms of cytotoxicity (data not shown). Finally, to rule out the possibility that NK cells could form self aggregates and subsequently bind TC, experiments were conducted in the same conditions as above, i.e., by using mutant CNF1 or IL-2. The morphometrical analyses performed as stated in **Materials and Methods** revealed no significant difference in promoting NK/NK aggregation among the various samples (Fig. 3C).

**CNF1 instructs NK cell-mediated killing and microtubule organization**

To define the role played by CNF1 in NK cell-mediated cytotoxicity, effector cells were challenged with agents that perturb either the actin cytoskeleton (CdB or CD) or the microtubular apparatus.
(the colchicine analog DEM). EC treatment with cytoskeleton perturbing agents was followed by an O/N treatment with CNF1, and cytotoxicity was evaluated at the 6:1 EC:TC ratio. In accordance with data on binding activity (Fig. 2D), both CdB and CD diminished the NK cell-mediated cytotoxic activity with respect to untreated samples (−45% with CdB and −55% with CD; open columns in Fig. 4A), an effect that was abolished when CNF1 was added to the culture medium (Fig. 4A). In contrast, the microtubular apparatus, which is critically involved in the killing phase of the cytotoxicity process (8), was also perturbed by CNF1. In fact, the decreased TC cytotoxicity found after exposure to the highest DEM concentrations (50 and 100 μM) was significantly restored by the subsequent O/N challenge with CNF1 (Fig. 4B). Therefore, this pointed to the fact that the toxin acts on the microtubules and hence on the related killing machinery. Qualitative IVM analyses clearly supported these results (Fig. 4C), the microtubular apparatus being highly polarized in control as well as in CNF1-exposed EC (Fig. 4C, upper panels). Depolymerization of microtubules by DEM contributed to the lack of typical microtubule marginalization with the consequent failure of cell-cell polarization in EC (Fig. 4C, bottom left panel). This was fully counteracted by CNF1 that allowed the reconstitution of polarized cell pairs (Fig. 4C, bottom right panel).

Discussion
The key point of this work is the proposal that a protein toxin (CNF1) can act as a powerful tool in the field of cellular immunology, in the same vein of other toxins largely used in such a context over the years, such as botulinum toxin (27). In our view, CNF1, because of its peculiar activating property on the Rac
GTPase, can actually represent an invaluable modulator and supervisor of the immune cell activity. The novelty of these findings also resides on the evidence that the Rac GTPase orchestrates the cytoskeleton assembly and arrangement in natural immunity, such as that represented by natural cytotoxicity. CTL or NK cells are the master of natural cytotoxicity, and their engagement with TC guides the onset of the immunological synapse (3, 28). It is worth noting that IS, whose formation involves dramatic changes in cell polarity, clustering of molecules at the contact area of cell conjugates, and a dynamic redistribution of cell membrane receptors (29), is instructed by the cell cytoskeleton (28). As supported by a plethora of studies, dissection of the cell cytoskeleton into the main components (actin microfilaments and microtubules) and associated molecules gives evidence of a specificity in the role played by each cytoskeletal system in NK cell activities (6, 28, 29). In particular, the integrity of actin microfilaments appears to be mandatory for both binding and killing activities, whereas microtubules have been heeded as master of the NK killing efficiency. Besides directing IS formation and cytotoxicity in NK cells, the cytoskeleton also governs cell locomotion and migration, because its extreme versatility enables immune cells to migrate under conditions of flow through narrow spaces and onto target tissues to perform their specific tasks. Conceivably, polarization of actin in NK cells challenged with CNF1 as well as with IL-2 may account for an increased locomotion of effector cells.

Interestingly, the activated forms of Rho GTPases that guide the formation of complex actin-rich structures (13) have been reported to likewise efficiently modulate IS formation (30, 31) and to competently influence the structure and dynamics of microtubules (12). The mentioned results give support to the activation of Rho GTPases, particularly Rac, as a prerequisite for the cytotoxic activity in NK cells. In this respect, the influence exerted by Rac GTPase-acting toxins on MF and MT interplay can be regarded as a novel insight. In fact, CNF1-induced Rac activation augmented NK cell-mediated efficiency, in terms of binding activity and NK cell recruitment, while the inhibitory toxin CdB produced the opposite effect. This was probably due to an increase in cell contractility and movement, which, in turn, favors the NK/TC binding and NK cell recruitment. In our case, the Rac-activating toxin CNF1 also directed MT polarization, in agreement with the current understanding of microtubules as an additional target of Rho GTPases (11, 12, 32, 33). Now largely recognized is the existence of a dynamic cross talk between microtubules and the actin cytoskeleton that is chiefly supervised by Rho GTPases. For instance, Cdc42 activity might have an effect on microtubule organization that parallels its role in the regulation of cell polarity. This was first demonstrated in T cells, in which Cdc42 is required for the reorientation of the centrosome toward APCs (34). Although the mechanism of centrosome reorientation is still undefined, the requirement of microtubules suggests the existence of a microtubule...
motor-driven mechanism analogous to the dynein-dynactin-dependent movements of the spindle poles during mitosis (14). Moreover, RhoA, another GTPase belonging to the Rho family, activates myosin through a Rho-kinase-induced phosphorylation of myosin L chain (35, 36), ensuring the intriguing possibility of a Rho-dependent dual regulation of both microtubule- and actin-based motor proteins. Based on the findings mentioned in this work, it is conceivable to hypothesize that the Rac GTPase can in turn act as a regulator of microfilament/microtubule interplay. Interestingly, cytoskeletal components of the ERM family (ezrin, radixin, moesin), which also are governed by Rho GTPases, localize to the activating NK cell IS (37, 38).

Besides being strictly controlled by the cell cytoktoskeleton, NK/Tc interaction is also regulated by a large array of triggering and inhibitory receptors whose balance determines the outgrowth of cytotoxicity. The expression pattern of such receptors relies upon the NK cell activation state and instructs new recognition capability to NK cells (39). CNF1, which in our instance reinforced the cytotoxic activity of NK cells, also augmented the expression of certain surface molecules, e.g., cell adhesion molecules such as CD2, or activation-associated molecules such as CD69. In particular, the cross-linking of the triggering receptor CD69 is known to induce a cytotoxic activity in activated NK cells by triggering tyrosine phosphorylation and consequently activation of an array of molecules comprising the Rho family-specific exchange factor Vav1 (40, 41). Of interest, CD69 expression was significantly augmented in NK cells challenged with CNF1. In contrast, it is conceivable that CNF1, as in other cell types (42), can up-regulate the expression of certain cell adhesion molecules on the NK cell surface. Therefore, a hypothetical scenario for CNF1 action would foresee in the toxin-dependent Rac activation the driving force promoting both binding and killing in NK cells. By switching on the Rac GTPase, in fact, CNF1 promotes presumably transcription and certainly the expression of surface molecules that play a key role in NK cell activity. At the same time, Rac-GTP supervises the reshape of both actin and tubulin networks, governing their conveyance to one pole of the effector cell, most likely including the surface adhesion and signaling-related molecules. Possibly, Rac also instructs movement in NK cells. Thus, CNF1 precisely designs the architecture of NK cells in the same vein of findings mentioned in this work, establishing and solidifying a connection.

Interestingly, a link with the Wiskott-Aldrich disease, a human pathology characterized by a defect in a protein controlled by the Rho family member Cdc42 (Wiskott-Aldrich syndrome protein (WASP)), exhibits an impaired NK cell function (43). Stimulatory signals lead to Vav activation that switches on Rac and Cdc42 GTPases. WASP, a substrate for Cdc42, together with Rac promotes actin polymerization that in turn drives the clustering of signaling molecules, thus leading to the onset of an immune response (44). The mutation in WASP observed in NK cells of patients bearing the Wiskott-Aldrich syndrome resulted in a defective actin polymerization (43, 44). This was most likely the cause of the specific impairment of both IS formation (binding) and cytotoxicity (killing) that contributed to the progression of the disease. Hence, as a general rule, it is conceivable to regard Rho-acting protein toxins as boosters or depressors of NK cell cytotoxic activity. In the long run, the possible implication of such a hypothesis could be a reappraisal of the role of Rho GTPases in some immune diseases as well as of the immune pharmacological activity of protein toxins.

References

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