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Wiskott-Aldrich syndrome protein (WASp) and N-WASp are involved in the regulation of NK-cell migration upon NKG2D activation

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NKG2D is a transmembrane receptor mainly expressed on CD8$^+$ T cells and NK cells. Engagement of NKG2D with its ligands can trigger a cytotoxic response. It has been shown that tumor cells deliver soluble NKG2D ligands as a mechanism of immune evasion through the downregulation of surface-expressed NKG2D. These ligands may be also secreted in microvesicles and regulate NK-cell function, but the existence of alternative mechanisms has not been explored. In this study, we describe that NKG2D activation inhibits NK-cell chemotaxis toward a CXCL12 gradient. Costimulation of the inhibitory receptor NKG2A rescues NK-cell migration rates. Thus, the balance of NKG2D/NKG2A activation may determine the migratory ability of NK cells. Furthermore, our data indicated that NKG2D cross-linking induces the activation of the Rho GTPases Rac1 and Cdc42, while RhoA activity is decreased. Pharmacological inhibition of the Cdc42 effectors Wiskott-Aldrich syndrome protein (WASp)/N-WASp, and the reduction of their levels using RNA interference partially abolished NKG2D-mediated impairment of cell migration, suggesting a pivotal role of Cdc42 in the regulation of NK-cell migration by NKG2D activation. Therefore, our results provide a new mechanism that may contribute to the immune response or evasion in tumors.

Keywords: Chemotaxis · Migration · NKG2D · Rho GTPases · WASp

Introduction

NK cells are the first host defense against viral infections and tumors. Degranulation and secretion of cytokines and cytotoxic molecules are the main NK-cell functions. The outcome of NK-cell activity is regulated by a balance between activating and inhibitory signals delivered by a repertoire of surface receptors. In human NK cells, these receptors may be classified in killer cell immunoglobulin-like receptors (KIR), natural cytotoxic receptors (NCRs), or C-type lectin receptors [1–3]. NKG2D is a C-type lectin activating receptor which is expressed not only in NK cells, acting as an activating receptor itself, but also in $\gamma\delta$ T, CD8$^+$ $\alpha\beta$ T lymphocytes and NKT cells in humans, where it acts as a costimulatory molecule [4, 5].

In humans, the ligands for NKG2D (NKG2DL) are major histocompatibility class I-related chain A/B (MICA/B) and UL-16 binding proteins 1-5 (ULBP1-5) [6]. NKG2D surface levels are regulated by these ligands. In fact, NKG2DL expression may result in immune activation or immune evasion. Although ligand expression is normally restricted under physiological conditions,

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it is increased in a broad variety of malignant diseases. High expression of MICA and ULBP2 is detected in ovarian cancer tissue and related to a poor prognosis [7]. Expression of NKG2DL on the cell surface of leukemia cells has also been described [8]. Furthermore, it has been described that the soluble forms of NKG2DL may be released from tumor cells [9, 10] and that elevated levels are found in multiple pathologies, such as epithelial tumors or hematopoietic malignancies [11]. In addition, NKG2D ligands may be secreted via exosomes maintaining their ability to modulate NKG2D cell surface levels [12]. Cancer-derived exosomes may downregulate receptor expression and reduce NK and $CD8^+$ T cells functional responses, as reported recently [13, 14]. Thus, the NKG2D recognition system has been postulated as a relevant mechanism in the immune response to cancer or in the tumor evasion from immunosurveillance [15, 16].

The possible role of NKG2D in regulation of other processes that may be involved in these responses is not fully explored. NK-cell recruitment during an immune response through migratory signals is essential to properly reach the target tissues. Chemokines play a critical role in this process, regulating NK-cell extravasation. Therefore, we analyzed the effect of NKG2D activation on NK-cell chemotaxis and the potential involvement of the Rho family of small GTP-binding proteins (Rho-GTPases), as they play a pivotal role in multiple processes, including the cytoskeleton reorganization during cell migration and the immunological synapse (IS) formation.

Results

NKG2D activation inhibits NK-cell migration

We evaluated the regulation of NK-cell migration upon NKG2D activation in human NK cells cultured with complete medium (resting) or activated with 50 U/mL IL-2 for 48 h. NK cells were stimulated with plate-bound mAb against NKG2D or IgG as a control for 20 min and then assayed for their ability to migrate through fibronectin-coated transwells toward an SDF-1α (stromal cell derived factor-1α/CXCL12) gradient. Number of migrating cells was initially monitored every 2 h for up to 16 h and then counted. As shown in Fig. 1A, NKG2D activation inhibited cell migration of IL-2-activated NK cells ($^{**}p < 0.005$) compared with IgG, while no effect was observed in nonstimulated (resting) cells. These results were confirmed using the IL-2-dependent natural killer leukemia (NKL) cell line. Migration assays were performed through fibronectin in response to CXCL12 or CX3CL1/fractalkine, both chemokines that are known to act as chemoattractants for NK cells [17]. Our results indicated that NKG2D activation significantly decreased cell motility in random, CXCL12- or CX3CL1-induced migration ($^{*}p < 0.05$; Fig. 1B). Moreover, inhibition of NK-cell migration is not exclusive of the fibronectin/alpha4 integrin axis, as NKG2D stimulation inhibited NKL chemotaxis and random migration on ICAM-1 (data not shown). As NKG2D is expressed in the majority of $CD8^+$ T cells, we performed similar experiments with human $CD8^+$ T cells activated with IL-2 for 48 h.

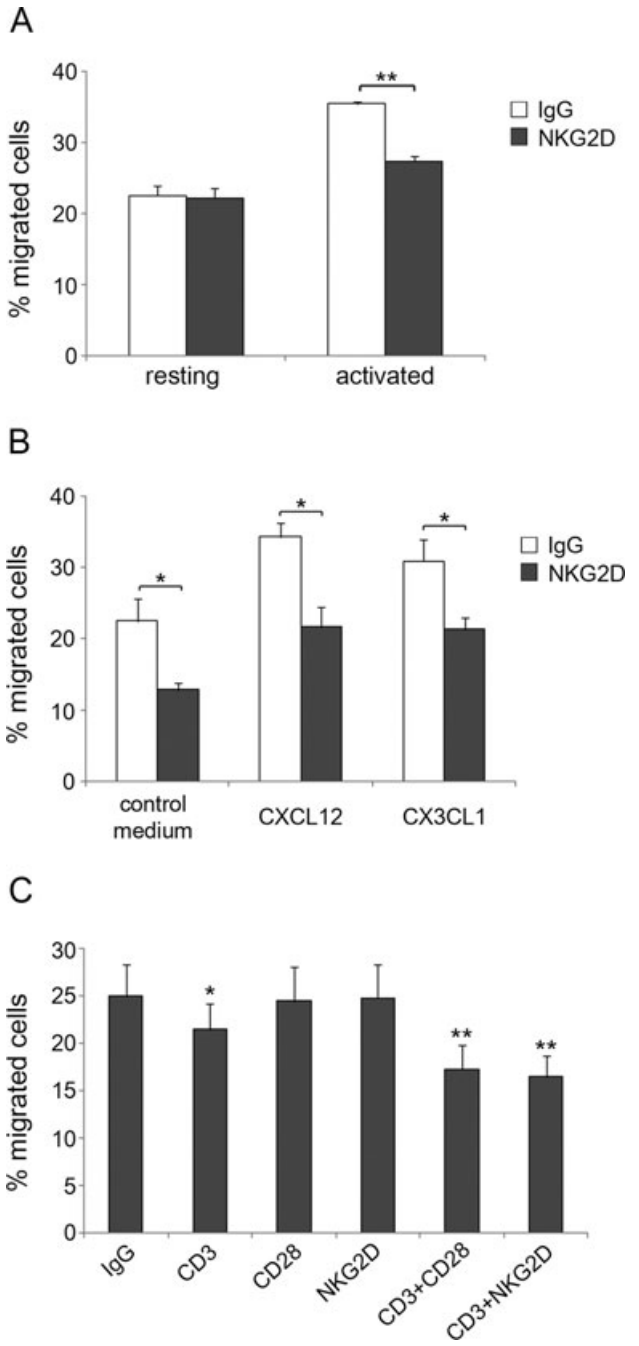

Figure 1. NKG2D activation inhibits NK-cell migration. (A) Isolated human resting or IL-2-activated NK cells were assayed for their ability to migrate through fibronectin-coated inserts (5 μg/mL), using CXCL12/SDF-1 (50 ng/mL) as a chemoattractant. After incubation with plate-bound antibodies against NKG2D or IgG as a control, cells were allowed to migrate for 16 h. Results shown are the mean + SEM of two replicates pooled from four independent experiments. $^{**}p < 0.005$, Student's *t*-test. (B) NKL cells were incubated with plate-bound antibodies, added to fibronectin-coated inserts (5μg/mL) and assessed in random, CXCL12- and CX3CL1-directed migration assays. Mean + SEM of two replicates pooled from three independent experiments are shown. $^{*}p < 0.05$, Student's *t*-test. (C) Primary $CD8^+$ T cells activated with IL-2 for 48 h were stimulated with the indicated antibodies and then added to transwell filters coated with fibronectin. Results represent the mean + SEM of two replicates pooled from four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.005$, Student's *t*-test.

Cells were stimulated with plate-bound anti-CD3, anti-CD28, and anti-NKG2D mAb alone or in combinations in comparison with control IgG-coated plates. Migration rates differed significantly between the different treatments (Fig. 1C). Thus, engagement of the TCR inhibited T-cell migration ($^*p < 0.05$), as described in previous reports [18, 19]. Migration of NKG2D- or CD28-activated cells was unchanged, whereas costimulation via CD28 and NKG2D further significantly decreased migration rates ($^{**}p < 0.005$) compared with the control condition.

To gain insight into the mechanism involved in the inhibition of NK migration upon NKG2D stimulation, we determined the surface levels of integrin CD49d, CXCR4 (CD184), and NKG2D after activation. Expression of NKG2D was not altered compared with the control condition after 20 min of NKG2D cross-linking. Neither expression of CXCR4 or CD49d was significantly different (Fig. 2A). Moreover, adhesion of primary NK cells (Fig. 2B) or NKL cells (Fig. 2C) to fibronectin was not altered. Adhesion of $CD8^+$ T cells to fibronectin was not affected by the treatments, except in cells activated with anti-CD3 + anti-NKG2D, in which adhesion of $CD8^+$ T cells decreased (Fig. 2D). Therefore differences on cell migration are not attributable to an increase in cell adhesion to the substrate. Changes in F-actin content upon NKG2D activation were then explored, since cell migration involves a complex rearrangement of the actin cytoskeleton. We found that F-actin content increased after receptor stimulation ($^*p < 0.05$; Fig. 2E), suggesting that Rho GTPases may be implicated in NKG2D-mediated responses.

NKG2D and NKG2A ligation rescues impaired migration

Since NK-cell function is regulated by a balance of activating and inhibitory signals, we next investigated whether an inhibitory receptor could rescue the cell motility. To address this issue, NKL cells were incubated with anti-NKG2D, anti-NKG2A, or both together and then were assayed for their ability to migrate. NKG2D activation leads to a decrease in the migration rate ($^*p < 0.05$; Fig. 3A). This effect was abrogated by costimulation of the NKG2A receptor, while the activation of this receptor alone did not affect cell migration. These results are in keeping with the study of Culley et al. [20], which describes that activating signals in NK cells, such as interaction of NKG2D with MICA promotes a stop signal to form the IS, while inhibitory signals may reverse this effect.

Recent works have shown the remarkable role of ULBP2 as a marker for disease progression [8, 21]. In addition, it has been reported that proteasome inhibitor treatment leads to a higher sensitivity to NK-cell-mediated killing by upregulation of ULBP2 expression [22], supporting its potential relevance in the development of anticancer therapy. We next evaluate whether NKG2D/ULBP2 interaction exerted similar effects than NKG2D cross-linking and found that activation of NKG2D receptor with plate-bound ULBP2 efficiently inhibited NK-cell migration ($^*p < 0.05$; Fig. 3B).

NKG2D regulates the activity of Rho GTPases Rac1, RhoA, and Cdc42

Since the Rho GTPase family of proteins plays a pivotal role in the regulation of actin cytoskeleton organization and microtubule dynamics during cell polarization and migration [23, 24], regulation of the best characterized members, Rac1, Cdc42, and RhoA upon NKG2D activation was analyzed. Cells treated as indicate were lysed and the activity of these Rho GTPases determined in pull down assays as described in methods. Rac1 and Cdc42 showed activation, whereas we observed a decrease in RhoA activity ($^*p < 0.05$; Fig. 4A).

Prompted by these results, we studied the involvement of Rac1 and Cdc42 together with an F-actin increase in response to NKG2D crosslinking. Human NK cells were cultured with IL-2 for 48 h and pretreated for 30 min with the Rac inhibitor NSC23766 (0.1 μM) or wiskostatin (1 μM), a selective inhibitor of neural Wiskott-Aldrich syndrome (WAS) protein (N-WASp) [25], the effector of Cdc42 [26]. Cell viability after treatment with NSC23766 and wiskostatin was greater than 94%. Then cells were incubated with anti-NKG2D antibody and tested for their chemotactic response to CXCL12. Activation of the receptor inhibited cell migration in control- and NSC23766-treated cells ($^*p < 0.05$; Fig. 4B). Thus, inhibition of Rac1 was not sufficient to rescue NK-cell migration. Pretreatment of NK cells with wiskostatin impaired NK-cell motility under control conditions, while anti-NKG2D-treated cells showed a partial rescue of the migratory ability ($^*p < 0.05$). Similar results were found in the NKL cell line. NSC23766 pretreatment increased NKL migration in control conditions, suggesting that a basal overactivation of Rac1 may be impairing cell motility, but the Rac1 inhibitor did not rescue the NKG2D-induced inhibition of NK-cell migration (Fig. 4C; $^*p < 0.05$). Regarding wiskostatin, cells pretreated with this agent exhibited decreased chemotaxis in control conditions ($^*p < 0.05$; Fig. 4D), whereas triggering of the NKG2D receptor with wiskostatin pretreatment showed a recovery of cell migration ($^*p < 0.05$). These results suggest that the Cdc42/WASp axis is involved in the regulation of NK-cell migration upon NKG2D activation.

N-WASp involvement in NKG2D-mediated migration

WASp-family proteins activate the Arp2/3 complex, leading to the formation of F-actin structures that are necessary in several cellular processes including cell migration. Because wiskostatin selectively inhibits N-WASp, we therefore investigated whether N-WASp is involved in the inhibition of NKG2D-mediated migration. N-WASp was knocked down using siRNA and chemotaxis assays were performed. Depletion of N-WASp in NKL cells partially rescued CXCL12-induced migration, as the percentage of inhibition decreased from 27% to 12% (Fig. 5A). We performed a similar approach with an N-WASp homolog, WASp, and confirmed that depletion of the protein was not affecting N-WASp and vice versa (Fig. 5A and B). In human NK cells, WASp is required for cytotoxic activity and the formation of the IS [27]. Under

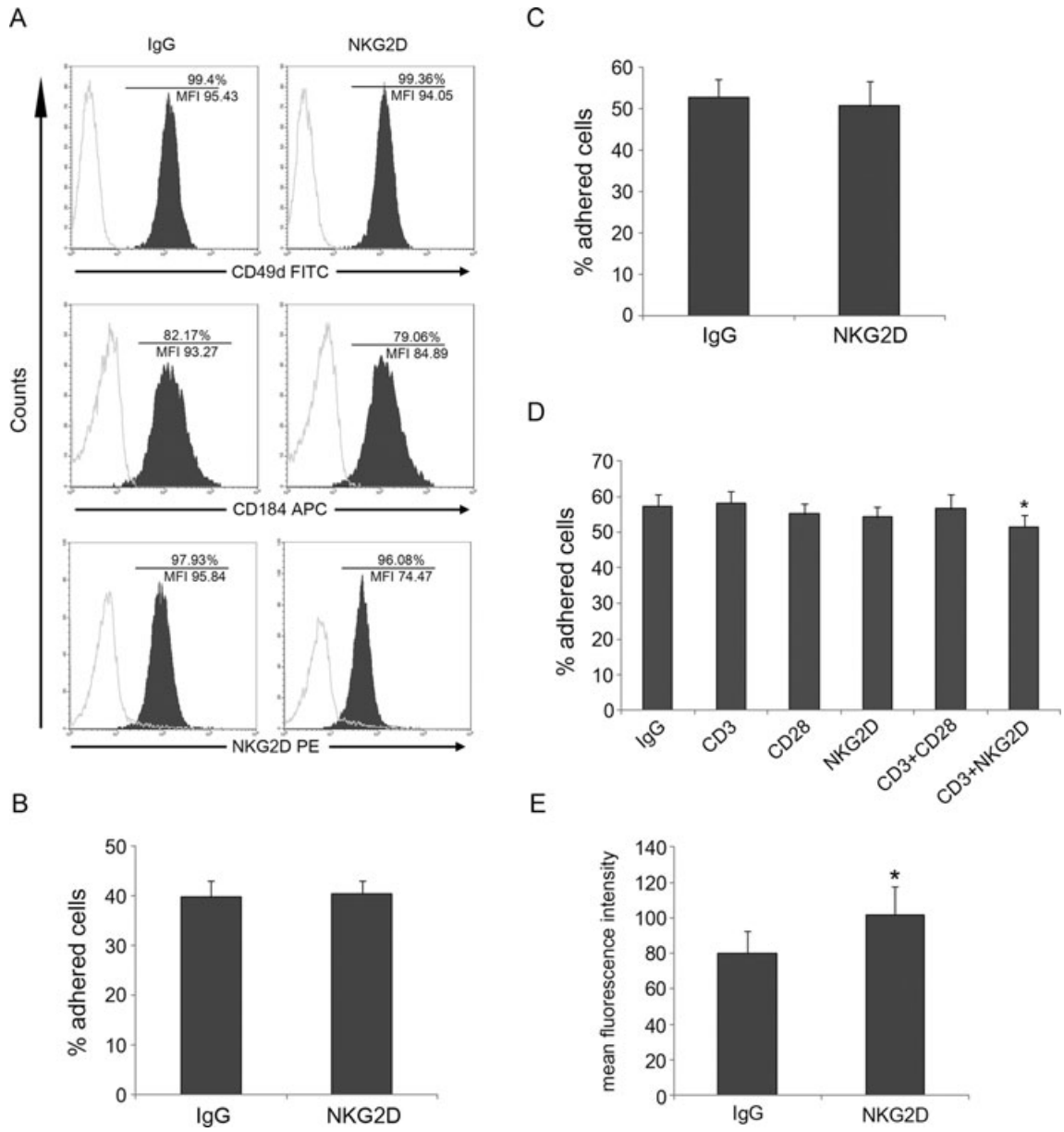

Figure 2. NKG2D effect on cell migration is independent of cell adhesion. (A) CD49d, CXCR4 (CD184), and NKG2D expression was measured after 20 min NKG2D cross-linking. NKL cells were incubated with plate-bound anti-NKG2D or IgG as a control, stained with the indicated mAbs or conjugated isotypic mAbs for each fluorochrome and analyzed by flow cytometry. A representative histogram for each condition is shown, with the percentage of cells expressing the indicated molecules and the median of the fluorescence intensity (MFI). Data shown are representative of four independent experiments. BCECF-labeled human (B) NK cells, (C) NKL cells, or (D) $CD8^+$ T cells were plated on fibronectin-coated wells and adhesion was determined after 1.5 h. Percentage of adhesion was estimated relative to the total number of plated cells. Results shown represent the mean + SEM of three replicates pooled from four independent experiments. $^*p < 0.05$, Student's *t*-test. (E) NKL cells were incubated with the indicated antibodies, fixed, permeabilized, and stained with phalloidin-TRITC. The mean fluorescence intensity was determined. Shown are the mean + SEM of four independent experiments. $^*p < 0.05$, Student's *t*-test.

control conditions, cells transfected with siRNA for WASp showed a dramatic reduction in CXCL12-induced chemotaxis ($^*p < 0.05$; Fig. 5B), in agreement with Stabile et al. [28]. However, activation of NKG2D in transfected cells did not impair cell migration compared with cells transfected with scrambled siRNA (2% of inhibition), indicating that both N-WASp and WASp proteins are involved in the regulation of NK-cell migration upon NKG2D stimulation. Changes in F-actin content were also determined by flow cytometry in the presence of the inhibitors and in siRNA knockdown experiments (Fig. 5C). Wiskostatin pretreatment of NK cells reduced F-actin levels in comparison with untreated cells ($^*p < 0.05$). F-actin levels also declined in cells transfected with siRNA for N-WASp, while NKG2D activation still increased the amount of F-actin ($^*p < 0.05$). Knockdown of WASp showed a decrease in F-actin levels in NKG2D-treated cells, although it does not reach statistical significance. Therefore, a lack of correlation between the

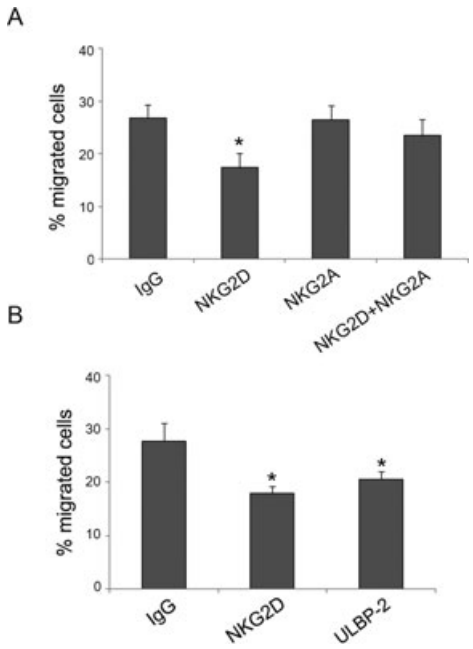

Figure 3. Activating and inhibitory signals in NK-cell chemotaxis. (A) NKL cells were activated with anti-NKG2D, anti-NKG2A or both mAbs and assessed for migration ability on fibronectin-coated inserts. The mean rates + SEM of three experiments with duplicate samples are shown. $^*p < 0.05$, Student's *t*-test. (B) Migration rates of pretreated NKL cells with plate-bound IgG, anti-NKG2D or recombinant ULBP-2. Results are the mean + SEM of two replicates pooled from four independent experiments. $^*p < 0.05$, Student's *t*-test.

effect of NKG2D activation on cell migration and F-actin content was observed in our knock-down experiments. Altogether, these data indicated that WASp proteins are involved in the inhibition of NK-cell migration upon NKG2D activation. Furthermore, these effects were not dependent on the regulation of actin dynamics by the Cdc42/WASp/Arp2/3 pathway.

Discussion

In this study, we describe that both N-WASp and WASp participate in the inhibition of NK-cell chemotaxis in response to NKG2D WASp engagement, and that this effect is not dependent on the regulation of F-actin dynamics.

NKG2D initiated NK-cell cytotoxicity and cytokine secretion are regulated by a balance of activating and inhibitory signals on the NK cell, which eventually determines the contact with the target cell [1]. The regulation of cell migration by NKG2D signaling takes place in the context of an immune response. This effect may be disrupted when inhibitory signals dominate. While CD94/NKG2A signaling disrupts synapse formation [29], NKG2D engagement is known to promote a stop signal [20]. Our results are consistent with these studies, as we analyzed how this stop signal affects NK-cell motility after stimulating NKG2D, while activation of an inhibitory receptor such as NKG2A reverses this effect. The ability to migrate of NK cells also decreased when NKG2D was stimulated with ULBP2, a natural ligand of this receptor. NKG2D is also expressed in $CD8^+$ T lymphocytes and acts as a costimulatory molecule [4, 5]. NKG2D-mediated costimulation of $CD8^+$ T cells impaired migration, supporting a role of this receptor in CXCL12-induced chemotaxis of NK cells and cytotoxic lymphocytes.

It is well known that the CXCR4-CXCL12 axis is not only implicated in the migration of tumor cells, but directs trafficking of leukocytes to different organs and tissues as well [30, 31]. NKG2D activation altered neither CXCR4 nor CD49d surface levels, and cells adhere normally to fibronectin, the substrate used in our assays, indicating that the differences observed are not due to a defect on chemokine engagement or an increase in cell adhesion. Immune cell responses involve a complex regulation of the cytoskeleton dynamics to migrate to the tissues and contact the target cell, among other functions [32]. NKG2D cross-linking correlated with an increase in F-actin content suggesting a role for the Rho GTPase family of proteins. Rho GTPases play an important role in NK activity, as they regulate cell polarization, adhesion, and vesicle trafficking of the cytotoxic granules toward the target cell [23, 24]. Rac1 is activated by NKG2D ligation, while RhoA activity decreased. This antagonistic activity has been previously described in fibroblast cells [33], and in cell types of the immune system, such as T cells [19] and neutrophils [34]. A balance between Rac and Rho activities is necessary for migratory polarity, with Rac regulating actin polymerization and leading edge formation while RhoA activity is responsible for rear-end organization. Cdc42 activity is also increased compared with the control condition, which regulates microtubule organizing center (MTOC) localization in the IS [35] and together with Rac1 controls cytoskeletal reorganization at the leading edge of the migrating cell and is essential for the establishment of cell polarity [24, 36]. It has been described that constitutively activated mutants of RhoA, Rac1, and Cdc42 impair T-cell migration. Noteworthy is that mutant of Cdc42 has more inhibitory effect on CXCL12-mediated migration [37]. Here, we report that treatment with wiskostatin, a selective chemical inhibitor of N-WASp, partially rescued NK-cell chemotaxis upon NKG2D activation while the Rac inhibitor NSC23766 had no effect, supporting a prominent role of Cdc42 on the regulation of NK-cell migration in response to NKG2D activation. Cdc42 interacts with both N-WASp and WASp effectors. The major findings of the involvement of their interaction are described in lymphocytes of patients suffering WAS. WASp function is crucial in T-cell chemotaxis [38–40] and is required to correctly form the IS in NK cells, as mutations in WASp impaired NK activity [27]. However, implication of N-WASp in NKG2D-mediated signaling and NK-cell motility has not been explored. Knocking down N-WASp expression with siRNA partially reverted NKG2D effect on cell migration in keeping with wiskostatin effect. Regarding WASp, the inhibition of its expression led to a defect in NK-cell motility in IgG-treated cells, as described recently [28] and NKG2D activation failed to decrease migration rates. These results indicate that both proteins, N-WASp and WASp, act downstream Cdc42 for the regulation of NK-cell migration in our experimental conditions. However, the possibility of a defective NKG2D-induced response or

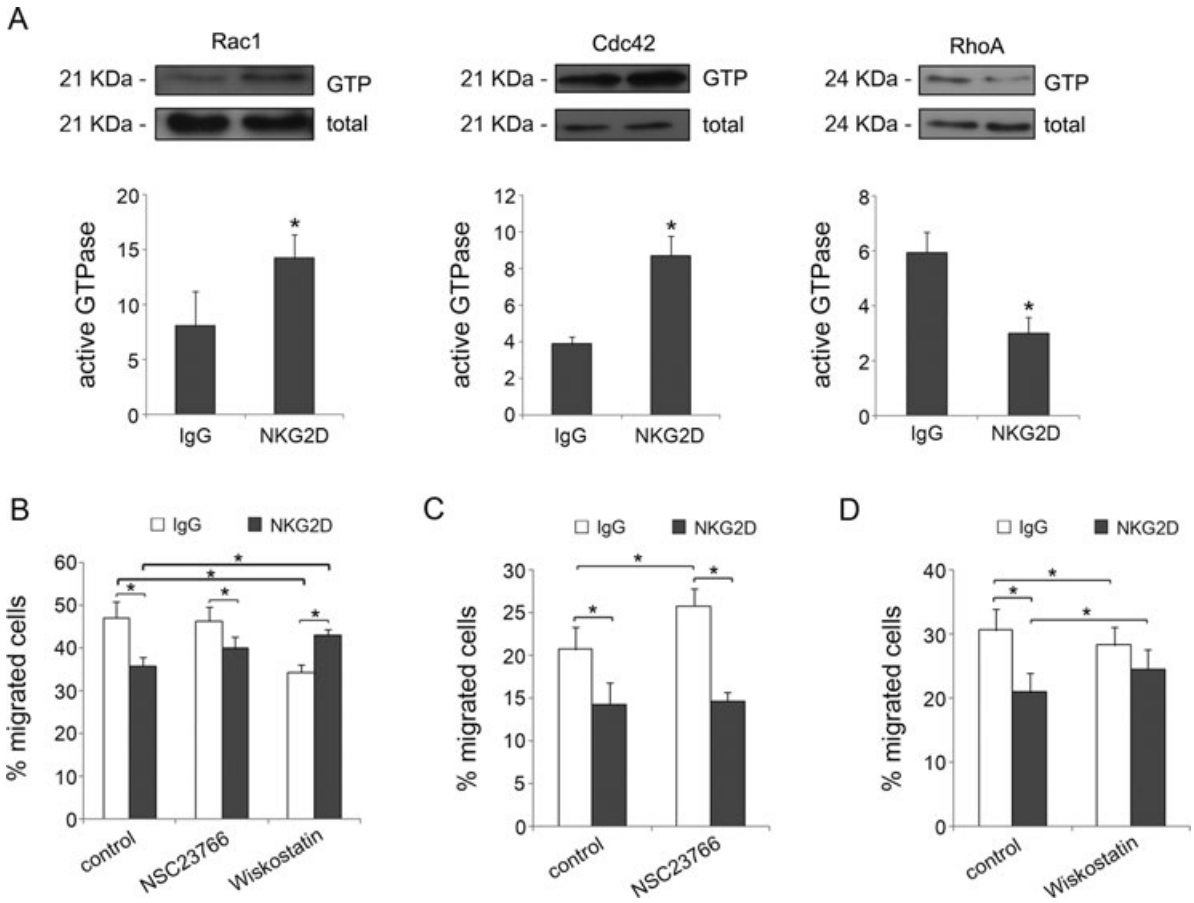

Figure 4. Regulation of Rho GTPases activity by NKG2D activation. (A) NKL cells were incubated with plate-bound anti-NKG2D or control IgG. After activation, cells were lysed and pull down assays were performed to analyze Rac1 (left), Cdc42 (middle), and RhoA (right) activity. A representative experiment for each Rho-GTPase is shown. Results indicate the mean active GTPase/total GTPase ratio of five independent experiments + SEM. $^*p < 0.05$, Student's *t*-test. (B) Human NK cells were pretreated with the Rac1 inhibitor NSC23766 (0.1 μM) and the inhibitor of N-WASp activity, wiskostatin (1 μM) or vehicle (DMSO). Cells were incubated with IgG or anti-NKG2D. Results shown are the mean + SEM of two replicates pooled from three experiments. $^*p < 0.05$, Student's *t*-test. A similar procedure was used with NKL cells treated with (C) NSC23766 and (D) wiskostatin. Data shown are the mean + SEM of two replicates pooled from four independent experiments. $p^* < 0.05$, Student's *t*-test.

chemokine-induced inside-out signaling in WASp knocked down cells cannot be ruled out, as this protein is necessary for the IS formation and it has been shown that its function is not compensated by N-WASp in NK cells [27, 41].

Cell migration involves important changes in the actin cytoskeleton and microtubules network. NK cells treated with wiskostatin or transfected with N-WASp-specific siRNA showed no significant decrease in F-actin upon NKG2D crosslinking compared with control cells or control siRNA transfected cells, respectively. A recent study describes that IL-2 may promote actin polymerization via the induction of WAVE2 in a WASp-independent manner [42]. WAVE proteins, which are other WASp family members, mediate cytoskeletal effects of active Rac [43]. Thus, different pathways regulate cytoskeletal rearrangement and may compensate for the WASp defect. In addition to actin dynamics, Cdc42/WASp pathway regulates accumulation of raft patches at the cell surface in different cell lines [44]. These domains act as microtubule stabilizers through IQGAP1 association and promote sustained directional motility. In NK cells, Cdc42, WASp, and Cdc42-interactin protein-4 (CIP4) regulate MTOC positioning upon cell activation, and WASp deficiency results in reduced F-actin accumulation and MTOC polarization [45]. Therefore a balance between both actin and microtubule dynamics is required for cell polarization and migration. We can hypothesize that overactivation of Cdc42 and its downstream effectors may alter the balance between the microtubules' growth and catastrophe and F-actin accumulation inhibiting cell migration.

These mechanisms may account not only for NKG2D-induced stop signal responsible for NK-target cell interaction [20], but also could participate in tumor evasion through the interaction with NKG2DL present on the surface of microvesicles.

Material and methods

Cell culture and receptor activation

PBMC were obtained from buffy coats by gradient centrifugation using Lymphoprep separation. NK cells were isolated by

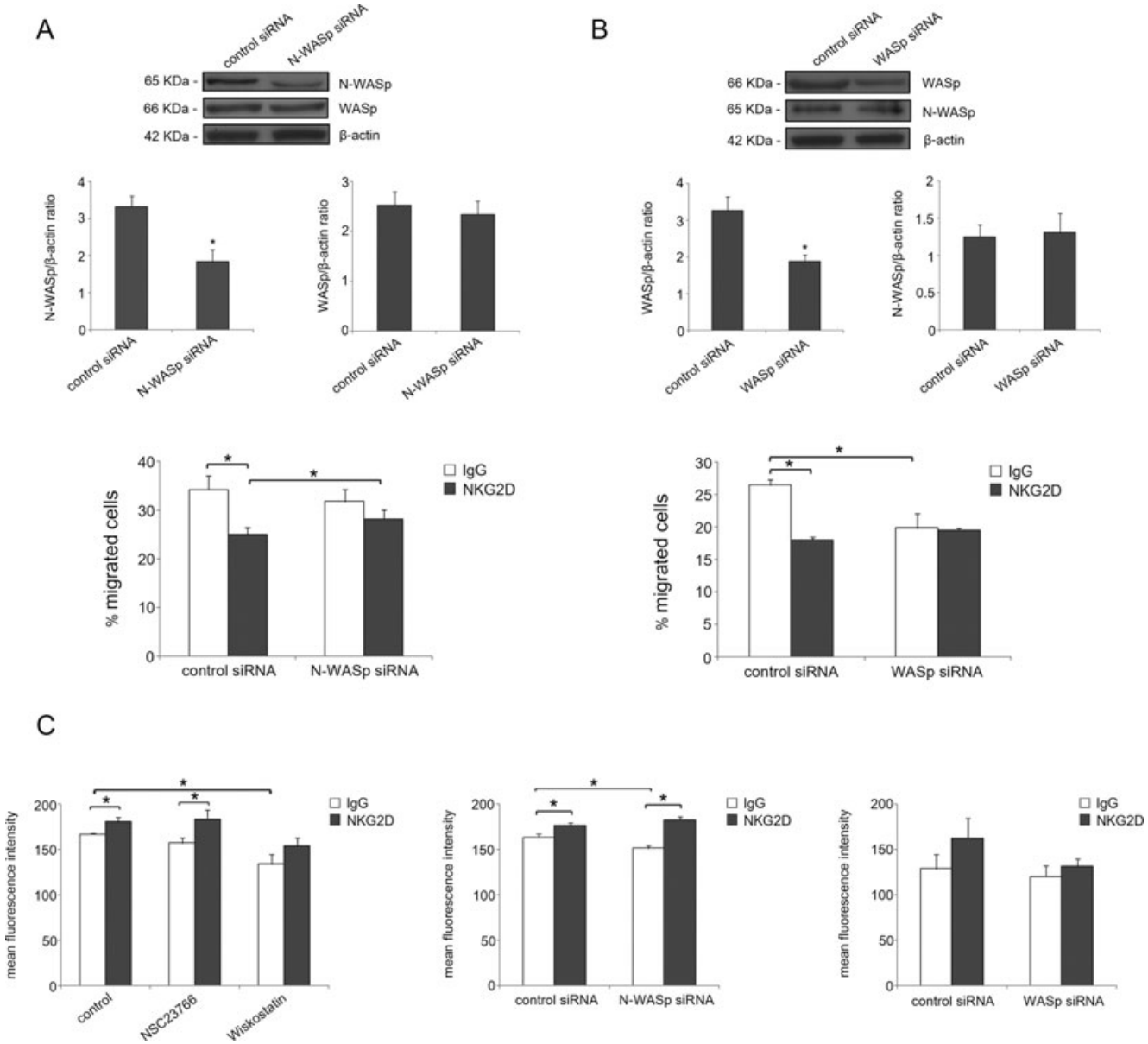

Figure 5. Role of N-WASp and WASp in NK-cell migration. NKL cells were transfected either with siRNA targeting (A) N-WASp, (B) WASp or a siRNA control. Cells were lysed and N-WASp and WASp expression levels were analyzed by Western blotting. Anti-β-actin blots were included as protein loading controls. Twenty four hours after transfection, cells were incubated with anti-NKG2D or IgG and their ability to migrate was assayed and shown as the percent inhibition of migration. Data shown are the mean + SEM of two replicates pooled from three independent experiments. $^*p < 0.05$, Student's *t*-test. (C) F-actin content was measured in human NK cells or in NKL cells transfected with siRNA for N-WASp and WASp. Cells were treated with the indicated agents and then incubated with anti-NKG2D or IgG. Cells were fixed, permeabilized, stained with phalloidin-TRITC and the mean fluorescence intensity was determined. Results are shown as the mean of three independent experiments. $^*p < 0.05$, Student's *t*-test.

negative selection using Dynabeads NK-cell kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Isolation of $CD3^+$ $CD8^+$ T cells was done by magnetic bead sorting with directly conjugated CD8 microbeads, according to the manufacturer´s instructions (MACS, Miltenyi Biotec). The purity of isolated NK cells and $CD8^+$ T cells was tested by flow cytometry. Cells were used when purity was more than 90%. The human NK leukemia cell line (NKL) was kindly donated by Dr. Jerome Ritz (Dana-Farber Cancer Institute, Boston, MA, USA) and cultured as described [46].

NKL or NK cells were stimulated with 10 μg/mL of plate-bound mAb against NKG2D (R&D Systems; MN, USA), anti-NKG2A (R&D), IgG (Jackson ImmunoResearch; Suffolk, UK) as control or 5 μg/mL recombinant human ULBP-2/Fc chimera (R&D). $CD8^+$ T cells were stimulated with plate-bound antibodies to CD3 (OKT-3; 1 μg/mL; eBioscience; San Diego, CA, USA),

CD28 (CD28.2; 5 μg/mL; eBioscience) and NKG2D alone or in combination.

Migration assays

All assays were performed in transwell chambers (6.5 mm diameter, 3.0 or 5 μm pore size; Costar Corporation). NKL, NK cells, or $CD8^+$ T cells (3×10^5–5×10^5) were incubated with plate-bound antibodies, then removed from the plates and allowed to migrate through Transwell membranes coated with ICAM-1/Fc chimera (5 μg/mL; R&D Systems) or fibronectin (5 μg/mL; Sigma-Aldrich; St Louis, MO, USA) in starving medium (random migration) or medium containing 50 ng/mL CXCL12/stromal cell-derived factor-1 (SDF-1) or 1 nM CX3CL1/fractalkine (Peprotech; London, UK). Experiments were performed as duplicates. Migrated NK cells and $CD8^+$ T cells were counted after 16 h and in case of NKL cells after 3.5 h in a Casy Counter (Roche Innovatis AG; Reutlingen, Germany). Where indicated, cells were pretreated for 30 min with chemical inhibitors of Rac1 (NSC23766; Calbiochem EMD Chemicals, San Diego, CA, USA) and N-WASp/WASp (wiskostatin; Enzo Life Sciences; Lausen, Switzerland).

Adhesion assays

NKL, NK cells, and $CD8^+$ T cells were washed and adjusted to 10^6 cells/mL. Then 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM; Molecular Probes) was added to the cell suspension and the mixture was incubated for 20 min. Labeled cells were washed twice with medium containing 1% BSA and activated with plate-bound antibodies. Then, cells were added to ICAM-1- or fibronectin-coated wells (5 μg/mL) at approximately 10^5 cells/well and incubated for 1.5 h at 37°C with 5% CO_2. Nonadherent cells were removed by washing twice with PBS. Experiments were performed as triplicates. Fluorescence was quantified using an LS 55 Luminescence Spectrometer (PerkinElmer).

Flow cytometry

For surface staining, the following mAbs were used: anti-CD3-PerCP, anti-CD49d-FITC, anti-CD11a-FITC, anti-CD8-allophycocyanin (Immunostep; Spain); anti-NKG2D-PE, anti-NKG2D-allophycocyanin (BioLegend; San Diego, CA, USA); anti-CD16-PE, anti-CD56-PE (BD Pharmingen; Erembodegem, Belgium); anti-CD184-allophycocyanin (eBioscience) NKL cells were incubated with the indicated mAbs and their respective isotype controls according to the manufacturer's instructions and analyzed on a FACSCalibur (Becton Dickinson). Viability of the cells after activation with plate-bound antibodies was determined using 7-amino-actinomycin D (7-AAD; Immunostep) and was greater than 95%.

Determination of F-actin content

F-actin levels were determined by flow cytometry using TRITC-phalloidin as previously described [47].

Rho GTPase activity assays

Activation of members of the Rho GTPase family of proteins Rac1, RhoA, and Cdc42 was analyzed as previously described [19].

RNA interference

NKL were transfected using specific ON-TARGETplus SMARTpool siRNA for N-WASp (L-006444-00-0005), for WASp (L-028294-00-0005), or ON-TARGETplus nontargeting pool (Dharmacon; Thermo Fisher Scientific., Lafayette, CO, USA) according to the manufacturer's instructions. Twenty four hours after transfection, cells were stimulated with anti-NKG2D or IgG and transwell migration assays were performed as described above. Viability of the cells was determined by flow cytometry using 7-AAD and dead cells were removed by centrifugation before each experiment. The effect of siRNA on levels of N-WASp and WASp was analyzed by western blotting.

Western blotting

Cell lysates were separated in SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk in TBST for 1 h at room temperature, and then incubated with the indicated primary antibodies overnight at 4°C with gently rocking. Membranes were washed and HRP-conjugated secondary antibodies were added for 1 h at room temperature. Blots were developed with the ECL detection system (Amersham; GE Healthcare, Munich, Germany). The following antibodies were used: mouse monoclonal anti-Rac1 (Upstate; Millipore Corporation, Billerica, MA, USA); rabbit polyclonal anti-Cdc42 (Cell Signaling Technology; Danvers, MA, USA); mouse monoclonal anti-RhoA, mouse monoclonal anti-WASp and rabbit polyclonal anti-N-WASp (SantaCruz Biotech; Santa Cruz, CA, USA); mouse monoclonal anti-β-actin (Sigma). Secondary antibodies HRP-conjugated were from Dako (Glostrup, Denmark).

Statistical analysis

Differences in the mean observations between groups were analyzed with Student's *t*-test. Statistical significance was set at $p < 0.05$. Values are expressed as mean + SEM.

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Abbreviations: **IS**: immunological synapse · **MTOC**: microtubule-organizing center · **NKG2DL**: NKG2D ligands · **N-WASp**: neural-WASp · **ULBP2**: UL-16 binding protein 2 · **WASp**: Wiskott-Aldrich syndrome protein

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