Identification of CD245 as myosin 18A, a receptor for surfactant A: A novel pathway for activating human NK lymphocytes

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ABSTRACT
CD245 is a human surface antigen expressed on peripheral blood lymphocytes, initially delineated by two monoclonal antibodies DY12 and DY35. Until now, CD245 molecular and functional characteristics remained largely unknown. We combined immunological and proteomic approaches and identified CD245 as the unconventional myosin 18A, a highly conserved motor enzyme reported as a receptor for the surfactant protein A (SP-A), that plays a critical role in cytokine receptor (CD137) and Golgi budding. We report that the recruitment of CD245 strongly enhanced NK cell cytotoxicity. Further, we show that the enhancement of the NK lymphocytes killing ability toward CD137-ligand expressing target cells could result from the induction of CD137 expression following CD245 engagement. The SP-A receptor could therefore represent a novel and promising target in cancer immunotherapy.

Abbreviations: ADCC, antibody-dependent cell cytotoxicity; EBV, Epstein-Barr Virus; ECL, enhanced chemiluminescence; E/T, effector/target; FCS, fetal calf serum; FAK, Lyn-phokine-activated killer; MHC, major histocompatibility complex; mAb, monoclonal antibody; MS, Mass spectrometry; SP-A, surfactant protein A-receptor; NK, natural killer; NKR, NK receptors; NCR, natural cytotoxicity receptor; NKG2D, NKG2 C-type lectin receptors family D; PBMC, Peripheral blood mononuclear cells; PB-NK, peripheral blood NK cells; PBS, phosphate buffer saline; RPMI, Roswell Park Memorial Institute; SP-A, surfactant protein A; SLAM, signaling lymphocytic activation molecule.

Introduction
Natural killer (NK) cells were identified over 40 y ago as a subset of lymphocytes able to spontaneously kill tumor cells in the absence of pre-stimulation. Present in most mammalian and avian species, NK cells play a critical role in the antitumor and anti-infectious immunity and in reproduction. NK cell cytotoxicity is tightly controlled by a balance between signals delivered through the engagement of activating and inhibitory receptors. Activating NK receptors (NKR) include several members belonging to the natural cytotoxicity receptors (NCR) family (such as NKp46, NKp44 and NKp30), the NKG2 C-type lectin receptors family (NKG2D), the major histocompatibility complex (MHC) class I-specific killer cell Ig-like receptors (KIRs) family, the Ig-like signaling lymphocytic activation molecule (SLAM) family (2B4), as well as unclassified receptors such as CD160. Beside these receptors, costimulatory molecules co-exist at the surface of activated NK cells to potentiate NK cells functions. 4-1BB (CD137) is a costimulatory receptor expressed on T, B and NK cells whose expression is triggered by engagement of the Fc receptors on the NK cell surface, as during antibody-dependent cell cytotoxicity (ADCC). Consequently, the engagement of CD137 increases cetuximab-, rituximab-, and trastuzumab-dependent NK cell cytotoxic function in different cancer models. NK cell activation is dominantly suppressed if the inhibitory NKR bind to MHC class I molecules on target cells. In humans, these receptors mainly belong to C-type lectin receptors, as the NKG2A heterodimer, or to the KIRs family. We previously described CD245 as an unidentified structure on the surface of human peripheral blood lymphocytes that was recognized by the monoclonal antibodies DY12 and DY35. We also reported that CD245 on NK lymphocytes was associated with tyrosine phosphatase activities. To further understand the NK lymphocyte biology, we characterize the molecular and functional properties of CD245 and identify a novel co-activation pathway that potentiates the NK cell cytotoxicity toward virally infected and tumor cells. Our data suggest
that CD245 might be a promising target in the field of human cancer immunotherapy.

**Methods**

**Cells**

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood obtained from healthy donors by density gradient centrifugation over lymphocytes separation medium (PAA Laboratories/GE Healthcare Europe, Vélizy-Villacoublay, France). Fresh peripheral blood NK cells (PB-NK) were isolated by magnetic cell sorting using an NK cell isolation kit according to the manufacturers’ recommendations (MiltenyiBiotec, Bergisch Gladbach, Germany). PB-NK cell purity was shown to be >95% as assessed by flow cytometry. The YT2C2 NK cell line, the murine mastocytoma cell line P815, and the Burkitt-lymphoma B-cell line Raji (all purchased from the ATCC, Manassas, USA), the Epstein-Barr Virus (EBV)-infected B cell line (locally produced) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% penicillin/streptomycin, 2mM L-glutamine, and 10% heat-inactivated fetal calf serum (FCS) (Perbio Science, Villebon-sur-Yvette, France).

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded PB-NK cells were analyzed for CD245 expression using a standard peroxidase method. Mouse anti-human CD245 (DY12), or granzyme B (clone GrB-7, DAKO) monoclonal antibody (mAb) was used as primary antibody, followed by biotin-conjugated anti-mouse Ig antibody and revealed with streptavidin-peroxidase method. Mouse anti-human CD245 (DY12), or granzyme B mAb and protein G-Sepharose beads. The precipitated proteins were separated by SDS-8% PAGE and transferred onto a nitrocellulose membrane (Millipore, Bedford, USA). Immunoblot analyses were performed using rabbit anti-myosin 18A antibodies (Protein Tech Group, Manchester, UK) or anti-SHP-1, anti-SHP-2, anti-SHIP and anti-PAK2 polyclonal Abs (Cell Signaling Technologies, Beverly, USA), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit Iggs secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA) and revealed with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, GE Healthcare Europe). For the biotinylation experiments, HRP-coupled-streptavidin was used.

**Mass spectrometry (MS)**

After immunoprecipitation with DY12 mAb or control IgG, the area of interest was cut out with a scalpel from the nitrocellulose and processed for MS analysis without chemical treatment as previously described. Proteins were digested with trypsin and MS analysis was carried out using a MALDI TOF/TOF ABI 4800 apparatus (Applied Biosystems, Foster City, USA). The masses obtained by MS-MALDI were analyzed using the Expasy database and software (http://www.expasy.org) and a local Visual Basic for Applications (VBA) software (Microsoft Excel, Microsoft, Redmond, USA).

**Cytotoxicity assays**

Cytotoxicity assays were performed according to a standard 51Cr-release method. Target cells were labeled with 100 μCi of Na51CrO4 for 90 min at 37°C, washed three times in culture medium and plated in 96-well V-bottom microtiter plates (Greiner BioOne, Courttaboeuf, France).

In redirected cytotoxicity assays on P815 cells, PB-NK cells were left untreated or pre-activated with recombinant human IL-2 (100 IU/mL, Peprotech) for 24 h. 51Cr-labeled P815 cells were incubated with DY12 or isotype control mAb, alone or in combination with anti-CD2335 (NKP46) or anti-CD337 (NKP30) mAb (10 μg/mL each).

In lymphokine-activated killer (LAK) assays against an EBV-infected B cell line, PB-NK lymphocytes were left untreated or preactivated with recombinant human IL-2 for 24 h and then treated with F(ab’)2 fragments of DY12 or isotype control mAb (10 μg/mL) or recombinant surfactant protein A (SP-A, 100 ng/mL) (USCNK, Houston, USA) for 1 h at room temperature.

For all assays, a fixed number of 10⁹ target cells/well was used and NK cells were added at various E/T cell ratios, as indicated. All conditions were done in triplicate.

After 4 h of culture at 37°C, the plates were spun down and 100 μL of the cell supernatant were collected from each well. The 51Cr release was quantified using a gamma-counter (Packard Instrument Company, Meriden, USA). The percentage of specific lysis was calculated as follows: % Specific lysis = [(Sample cpm - Spontaneous Lysis Control cpm)/(Maximum Lysis Control cpm - Spontaneous Lysis Control cpm)] × 100.

The specific lysis was considered significant if >10%.

**Lymphocytes activation**

For the study of NK cell activating receptors expression, freshly isolated PB-NK cells were first in vitro stimulated for
1 h at 37°C with DY12 or control IgG1 mAb (10 μg/mL), washed and incubated with rabbit anti-mouse IgG antibodies to allow mAb cross-linking (Jackson ImmunoResearch Laboratories) (10 μg/mL). To activate T lymphocytes, freshly isolated PBMC were cultured in flat bottom 96-wells plate coated with 0.3 μg/well of anti-CD3 mAb. For B lymphocytes activation, PBMC were cultured in round bottom 96-wells plate in complete RPMI medium with 10 μg/mL of polyclonal goat anti-human anti-IgM Ab. After 72 h of culture in complete RPMI medium, cells were harvested and washed with PBS before staining.

**NK cell degranulation assay and blocking of the CD137/CD137L interaction**

Freshly isolated PB-NK cells were activated as described above. Raji target cells were then added to a final volume of 150 μL/well at various E/T ratios. After 4 h of culture at 37°C in the presence of PE-Cy7-conjugated anti-CD107a (Becton Dickinson), cells were washed and prepared for flow cytometry analysis. In some experiments, human 4-1BB-Ligand/TNFFSF9 affinity purified polyclonal Ab (R&D systems, Minneapolis, USA) was added to the culture at a final concentration of 10 μg/mL to block the CD137/CD137L interaction.

**Flow cytometry analysis**

The mAbs used were the following: anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD20, anti-CD56, anti-CD197 (C-C chemokine receptor type 7 (CCR7)), anti-γδ T-cell receptor mAb (MiltenyiBiotec), and anti-CD245 mAb (DY12, mouse IgG1, locally produced). Irrelevant isotype-matched mAbs were used as negative controls. Fluorescein isothiocyanate (FITC), allophycocyanin (APC)- or R-phycoerythrin (RPE)-conjugated goat anti-mouse IgG or IgM antibodies (Beckman Coulter, Brea, USA) were used as secondary reagents. Brieﬂy, cells were incubated with the speciﬁc mAb for 30 min at 4°C, washed twice in phosphate buffer saline (PBS) (Life Technologies, Carlsbad, USA), and further incubated with the appropriate secondary Abs. Cells were washed and analyzed by flow cytometry on a FC500 analyzer (Beckman Coulter). In some experiments, PBMC were activated with anti-CD3 or anti-IgM antibodies for 72 h before labeling.

To characterize the expression of NK cell activating receptors after CD245 engagement, NK cells were activated as described in the “Activation of NK cells” section, washed and labeled with Fixable Viability Stain 450 (Becton Dickinson, Franklin Lakes, USA) and the following antibodies to human cell surface antigens: APC-conjugated anti-CD137, PE-conjugated anti-NKG2D, FITC-conjugated anti-DNAX Accessory Molecule-1 (DNAM-1, CD226), PE-conjugated anti-CD160 (Becton Dickinson), PE-conjugated anti-NKp30 (CD337), anti-NKp44 (CD336), and anti-NKp46 (CD335) (Beckman Coulter).

To study CD137L expression on Raji cells, Raji cell lines were cultured and treated as described above, washed and stained with Fixable Viability Stain 450 (Becton Dickinson) and PE-conjugated anti-CD137L (Becton Dickinson) for flow cytometry analysis. Cells were washed and analyzed on a Canto II Flow-Cytometer (Becton Dickinson).

**Analysis**

Flow cytometry analysis was carried out using the FlowJo software version X. All values are expressed as means of fluorescence intensity (MFI). Values are plotted with their mean and standard deviation and compared between groups with Prism software (Graph Pad version 6) by two-tailed Mann–Whitney U test or ANOVA (for cytotoxicity tests) to compare continuous variables. p ≤ 0.05 was considered as statistically significant.

**Results**

**Human NK cells express the long (α) and short (β) isoforms of myosin 18A (CD245)**

By using the two mAbs DY12 and DY35, we previously described CD245 as a surface protein with an apparent molecular weight of approximately 220 kDa expressed by a large panel of normal and malignant human hematopoietic cells. In order to identify CD245 protein sequence, YT2C2 cells (the leukemic NK cell line used in the original immunization program leading to the selection of the anti-CD245 mAbs) were biotinylated and cell lysates were subjected to immunoprecipitation with DY12 or a control IgG1 mAb. As shown in Fig. 1A, after migration of the immunoprecipitates on SDS-PAGE and immunoblot analysis with HRP-conjugated streptavidin, we confirmed the detection of CD245 molecules in the 220–240 kDa area. This area was cut out from the nitrocellulose, subjected to trypsin digestion and then processed for mass spectrometry (MS) analysis.

In the list of the 239 masses of tryptic peptides obtained, 59 corresponded to those of myosin 18A, with a difference lower than 36 ppm from the corresponding theoretical mass (Fig. 1B). To further confirm that the CD245 molecule expressed by the YT2C2 cell line was indeed the unconventional myosin 18A, YT2C2 cell lysates were immunoprecipitated using DY12 mAb or an IgG1 control isotype and the immunoprecipitates were subjected to immunoblotting using polyclonal anti-myosin 18A antibodies. This led to the specific detection of the α (230 kDa) and β (190 kDa) isoforms of myosin 18A in DY12 immunoprecipitate (Fig. 1C). Thus, CD245 expressed at the cell surface of human YT2C2 NK cell line is the bona fide myosin 18A. Of note, both α and β isoforms were expressed in YT2C2 cells, whereas only the α isoform was found in biotinylated YT2C2 immunoprecipitate, suggesting that only the α isoform is expressed at the cell surface of YT2C2 cells. These data are consistent with previous studies in mice that showed that myosin 18Aζ (p230) and β (p190) had different subcellular localizations, the former colocalizing with the endoplasmic reticulum and Golgi structures. Myosin 18A was reported as a receptor for the surfactant protein A (SP-A), a collectin notably present in human lung. Thus, we performed immunoprecipitation with DY12 mAb using fresh human lung extracts. The results show the recognition of both myosin 18A isoforms by DY12 mAb in these cellular extracts (Fig. 1D).
CD245 expression at the cell surface of peripheral blood lymphocytes is constitutive and increased by activation

To investigate the expression of CD245 on distinct subsets of PBMCs, freshly isolated PBMCs from healthy subjects were analyzed by flow cytometry. As shown in Fig. 2A, all investigated peripheral blood lymphocyte subsets expressed significantly CD245 although at various levels. Most CD3+ T cells including γδ lymphocytes, CD56bright+ as well as CD56dim+ NK cells, and half of the CD20+ B cells expressed CD245. This expression was also associated with the one of CCR7, a chemokine receptor expressed in T, B and CD56bright+ NK cells, involved in lymph node homing. In addition, the expression of CD245 is higher on monocytes as compared to lymphocytes (Fig. 2B) and was found increased after 3 d of activation of NK cells with IL-2 (Fig. 2C), and of T and B cells with anti-CD3 or anti-IgM antibodies, respectively (Fig. 2D and E).

Recruitment of CD245 on peripheral blood NK cells enhances the CD335/NKp46 and CD337/NKp30-mediated NK cell redirected cytotoxicity toward the mastocytoma cell line P815

SP-A, a ligand for myosin 18A/CD245, has previously been shown to stimulate the anti-tumor immunity in vivo in a xenograft mouse model. This antitumor effect of SP-A was dependent on NK cells in vivo although the exact mechanisms remained unknown. To address this issue, we investigated whether the engagement of CD245 was able to modulate the NK cell redirected cytotoxicity against the murine mastocytoma cell line P815. Prior to functional investigations of CD245 role on human PB-NK cells, its expression was confirmed by immunohistochemistry using the DY12 mAb. Anti-granzyme B antibodies were used as positive control. The resulting data clearly show the presence of myosin 18A at the cell membrane and in the cytoplasm of PB-NK lymphocytes (Fig. 3A). As shown in Fig. 3B (upper panels), NK cells stimulated with DY12 mAb alone exhibited a poor cytotoxic activity against P815 cell line (specific lysis of 6% at an E/T ratio of 10/1). In contrast, engagement of CD335 and CD337 induced the cellular cytotoxicity of PB-NK cells that was significantly enhanced by the coengagement of CD245. Similar results were obtained when using IL-2-activated NK cells (Fig. 3B, lower panel). These data identify CD245 as a co-activator of NK cell cytotoxic activity triggered by the NCR CD335 and CD337, and support the hypothesis that the antitumor effect of SP-A in vivo might be mediated by its interaction with CD245 on NK cells.
CD245 engagement by DY12 mAb or by its physiological ligand SP-A increases the IL-2 activated NK cell cytotoxicity toward EBV-infected B cells

Because (i) NK cells play a critical and first-line role in the antiviral immune defense,20 (ii) human NK cells express high levels of CD245, (iii) CD245 cell surface expression is increased in the presence of IL-2 in NK cells and (iv) CD245 corresponds to myosin 18A that has been shown to be a receptor for SP-A,17 a protein involved in viral clearance in the human lung,21 we asked whether engagement of CD245 on the NK cell surface was able to regulate their IL-2-activated killer activity against virally infected cells. As expected, freshly isolated PB-NK lymphocytes failed to kill an EBV-infected B cell line and engagement of CD245 with DY12 mAb was unable to overcome the resistance to NK cell cytotoxicity of these highly MHC class I molecules expressing target cells (Fig. 4A). In contrast, we observed that short-term IL-2-activated PB-NK lymphocytes exhibited a weak but significant cytotoxicity (specific lysis of 28% at the 50/1 E/T ratio) toward the EBV-infected B cells (Fig. 4D). These results clearly indicate that DY12 mAb as well as the physiological ligand SP-A trigger the enhancement of the NK lymphocyte cytolytic function through CD245 targeting.

The increased NK cell degranulation mediated through the engagement of CD245 could be 4-1BB (CD137) dependent

To further understand the mechanism by which recruitment of CD245 increases the NK cell cytotoxicity, we studied the expression of activating NK cell receptors after engagement of CD245 by DY12. As shown in Fig. 5A, CD245 targeting did not induce any significant change in the expression of Nkp30, Nkp44, Nkp46 or NK2D. It also showed no significant impact on the expression of DNAM1 and CD160 (data not shown). In contrast, CD245 engagement increased the membrane expression of CD137 (4-1BB) (Fig. 5A). Next, we asked whether NK cell cytotoxicity toward CD137L-expressing cells could depend on the CD137/CD137L interaction.
CD137L has been shown to be expressed by human B cells and dendritic cells.\textsuperscript{22} As Raji is a well-characterized Burkitt B-cell lymphoma cell line, and because Burkitt lymphoma has been shown to express CD137L,\textsuperscript{23} we wondered whether the NK cell degranulation is increased following incubation with DY12 mAb and in the presence of Raji cells. Prior to using the Raji cell line as target, we confirmed its CD137L expression (Fig. 5B). Consequently, in cytotoxic assays, the engagement of CD245 with DY12 mAb increased the NK cell degranulation in the presence of Raji cells, as assessed by the enhanced level of CD107a (Fig. 5C). Blocking the CD137-CD137L interaction with an anti-human CD137L Ab completely abrogated the CD245-induced NK cell degranulation in the presence of Raji cells. Note that peripheral blood lymphocytes from some healthy individuals could degranulate in the presence of the so-called NK-cell-resistant target cell line Raji without being activated with IL-2. Altogether, these data suggest that the NK cell cytotoxicity induced by the recruitment of myosin 18A in the presence of CD137L-expressing target cells is CD137 dependent.

**Myosin 18A interacts with PAK-2 and SHP-2, two key signal transducers of the NK cell activation and degranulation processes**

As shown previously, recruitment of myosin 18A enhanced NK cell cytotoxicity against tumor cells or virally infected cell lines (Figs. 3–4). NK cell cytotoxicity is dependent on cytoskeleton reorganization, to create the NK immune synapse first\textsuperscript{24} and further allow the polarization and exocytosis of the cytolytic granules.\textsuperscript{25,26} Myosin 18A has been shown to play a role in the cytoskeleton organization and to interact with PAK-2 in epithelial cell lines.\textsuperscript{27} To further elucidate the mechanisms by which CD245 stimulation increased NK cell degranulation and lysis of target cells that failed to express CD137L, immunoprecipitates were prepared on YT2C2 NK cell line lysates with DY12 or control IgG1 isotype and subjected to electrophoresis and immunoblotting using anti-PAK2 antibody. Whole YT2C2 cell lysate was used as positive control. As shown in Fig. 6A, a weak but significant amount of PAK2 was specifically recovered in DY12 immunoprecipitate. This data is in favor of an interaction between myosin 18A and PAK2 in NK cells, therefore identifying PAK2 as a potential signal transducer of the myosin 18A mediated NK cell cytotoxicity.

As previously demonstrated, CD245 was found associated with a phosphatase activity in the YT2C2 NK cell line.\textsuperscript{36} Among the main phosphatases involved in NKR-mediated signaling pathways are the Src-homology domain-containing phosphatases (SHP) such as the SH2 domain-containing inositol 5’-phosphatase (SHIP), SHP-1 and SHP-2. These phosphatases interact with phosphorylated tyrosine residues on other proteins through their SH2 domains.\textsuperscript{28–31} We thus investigated whether myosin 18A was able to recruit SHP-1, SHP-2 or SHIP. As demonstrated in Fig. 6B, immunoprecipitation
of YT2C2 cell lysates with DY12 mAb and further immunoblotting of the resulting immunoprecipitates using anti-SHP-1, -SHP-2 or -SHIP antibodies revealed the presence of the phosphatase SHP-2, but not of SHP-1 or SHIP within myosin 18A immunoprecipitates. Thus, SHP-2 may be involved in the regulation of myosin 18A dependent activating signals.

**Discussion**

In the present work, we identified CD245, a human cell surface antigen expressed on peripheral blood lymphocytes, as the unconventional myosin 18A, a highly conserved motor enzyme involved in cytoskeleton organization and Golgi budding. It is worthy to mention that by using a polyclonal antibody against surfactant protein A-receptor (SP-R)-210 that was previously shown to detect myosin 18A,17 Samten et al. found a reactivity only with a very small fraction of peripheral blood CD3⁺ lymphocytes, and that this staining increased 5- to 10-fold and was extended to the whole lymphocyte population after stimulation with Mycobacterium tuberculosis.33 Using the DY12 mAb, we found that CD245/myosin 18A was constitutively expressed by most lymphocytes including CD3⁺ lymphocytes, and that its membrane expression was enhanced on NK, T and B lymphocytes following activation. In addition, monocytes were found to also express CD245/myosin18A, although at higher level than lymphocytes. This discrepancy with the previously published data could be explained by a better specificity or affinity of the DY12 mAb for myosin 18A than the polyclonal antibody against the SP-R-210. Last, Myo18A was found to co-precipitate with SHP-2, a phosphatase with a key role in NK cell receptors-mediated signaling pathways,31 and with PAK-2, a serine/threonine kinase that controls the cytoskeletal organization.27 Of note, SHP-2 has previously been shown to negatively regulate NK cell function,31 although in other settings, it mediated a positive proliferation signal and activated the IL-1/Erk-mediated cytoskeleton reorganization.35

Our novel molecular and functional data on a newly described NK cell co-activating receptor have broad potential applications. The unconventional myosin 18A is a member of the myosin superfamily of motor enzymes. Myosins generally contain a conserved catalytic head that catalyzes ATP

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**Figure 4.** Engagement of CD245 with DY12 mAb or its physiological ligand SP-A increases the NK lymphocytes lymphokine-activated killer activity toward EBV-infected B cells. Cytotoxicity assays were performed according to a standard ⁵¹Cr-release method. Effector cells were untreated (A and C) or IL-2 activated (B and D) PB-NK lymphocytes from healthy donors and target cell was an Epstein-Barr virus (EBV)-infected B cell line. (A and B) Target cells were preincubated with DY12 control F(ab’)2 or control F(ab’)2, mAb fragment at 10 μg/mL prior to contact with the NK cells. (C and D) The assay was performed in medium alone or the presence of recombinant SP-A at 100 ng/mL as indicated. Assays were performed in triplicate at various E/T cell ratios, as indicated. Results are shown as mean percentages of specific lysis ± SD. Statistics were calculated using the ANOVA test, *p < 0.05.
hydrolysis and binds F-actin, thus promoting motility. The first myosin, M2, was discovered in muscle extracts and is referred to as conventional myosin, whereas other classes, including class 18, are called unconventional myosins. Myosin 2A is required for cytolytic granule exocytosis in human NK cells.36 Class 18 myosins have been involved in fundamental tissular processes in mammalians, including epithelial cell migration,37 stromal cell differentiation38 and tumor suppression.39,40 In humans and mice, myosin 18A is expressed in hematopoietic cells as two splice variants, referred to as α (230 kDa) and β (190 kDa). At the cell level, myosin 18A participates in cytoskeleton organization,41 Golgi budding32 and

**Figure 5.** CD245-enhanced NK cell degranulation in the presence of 4-1BBL/CD137L-expressing target cells is 4-1BB/CD137-dependent. (A) The expression of the activating NK cell receptors Nkp30, Nkp44, Nkp46 and NKG2D and of CD137 was monitored by flow cytometry on human NK cells triggered with an isotype control or DY12 mAb. FMO was used to determine positivity thresholds. The NK cells from five healthy donors were tested. Shown are the mean percentages ± SD of positive cells for each marker. Statistics were calculated with the Mann–Whitney U-test, “p < 0.01. (B) Raji cells were assessed for CD137L expression by flow cytometry. Unstained control was used to determine the positivity threshold. (C) PB-NK lymphocytes from healthy donors (n = 2) were incubated with DY12 or control IgG mAb (10 μg/mL), followed by cross-linking with rabbit anti-mouse IgG antibodies. The target cells were then added with or without anti-CD137L antibodies (10 μg/mL). CD107a expression was measured on CD3−CD56+ NK cells by flow cytometry. Results shown are mean percentages ± SD of CD107a MFI. Statistics were calculated using the ANOVA test, *p < 0.05.

**Figure 6.** Myosin 18A co-precipitates with PAK-2 and SHP-2, two key signal transducers in NK cell activation and degranulation processes. Immunoprecipitates were performed on YT2C2 cell lysates with DY12 or control IgG1 mAb and subjected to electrophoresis and immunoblotting using anti-PAK2 (A), SHIP, SHP-1 or SHP-2 (B) antibodies. Total YT2C2 cell lysates were used as positive controls.
DNA-damaged-induced Golgi dispersion by its association with F-actin and Golgi Phosphoprotein 3 (GOLPH3) in epithelial cells, but its specific role in NK cells was not shown yet. Myosin 18A was also reported as a receptor for the surfactant protein A (SP-A), a collectin present in human lung, blood, intestinal tract, and skin that participates in the elimination of pathogens. SP-A has also been shown to strongly stimulate the anti-tumor immunity in a xenograft mouse model. Tumor cells transduced with SP-A grew slower than those transduced with the vector alone. This anti-tumor effect of SP-A was entirely dependent on NK cells in vivo, although the exact mechanism remained unknown. We showed that myosin 18A/CD245 is a potent human NK cell co-activating receptor, whose cell surface expression is increased by IL-2. CD245 stimulation by DY12 mAb or its physiological ligand SP-A was able to increase NK cell degranulation and lymphokine-activated killer activity toward tumor B cells and EBV-infected B cells, respectively. Our data support the hypothesis that this major antitumor effect of SP-A in vivo is mediated by its interaction with myosin 18A on NK cells.

We also found that myosin 18A stimulation was able to induce CD137 expression at the NK cell surface and that the myosin 18A induced NK cell cytotoxicity toward CD137L-expressing tumor cells was dependent on the CD137/CD137L interaction. The use of monoclonal antibodies modulating the NK cell antitumor function is a fast growing field of research. On one side, monoclonal antibodies able to induce ADCC by targeting both the cancer cell and the FcγRIIIA/CD16-activating receptor present on NK cells have revolutionized the management of lymphoma and human cancer. On the other side, not all malignancies have an identified target and some malignancies with identified targets escape to therapeutic antibodies. In such immunotherapeutic fields, strategies that aim at increasing the efficacy of monoclonal antibodies are promising. Stimulation of the CD137 (4-1BB) receptor present on NK cells has been shown to increase the efficacy of cetuximab, trastuzumab and rituximab in both in vitro and in vivo models of human cancer. The use of monoclonal antibodies that modulate the expression of CD137 at the NK cell surface, such as DY12 mAb (Fig. 5), could be interesting in such application. In conclusion, the NK cell activating receptor CD245 appears as a very promising target in the field of the immunotherapy of human cancer and hematological malignancies. Further in vivo studies are needed to determine the optimal conditions for the use of agonistic antibodies to myosin 18A/CD245 as immunotherapeutic agents in this context.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

AdM and JG performed the experiments and analysis. AMC, ND and AB designed the research. AdM, JG, AMC, ND and AB wrote the manuscript. ND, JDB, DG, PV, AT, CG, MB, YM and AB critically reviewed the manuscript.

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