

## Arabinoxylan rice bran (MGN-3/Biobran) enhances natural killer cell-mediated cytotoxicity against neuroblastoma *in vitro* and *in vivo*

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### Abstract

**Background aims.** Natural killer cell (NK) cytotoxic activity plays a major role in natural immunologic defences against malignancies. NK cells are emerging as a tool for adoptive cancer immunotherapies. Arabinoxylan rice bran (MGN-3/Biobran) has been described as a biological response modifier that can enhance the cytotoxic activity of NK cells. This study evaluated the effect of MGN-3/Biobran on NK cell activation, expansion and cytotoxicity against neuroblastoma cells. **Methods.** NK cells were enriched with magnetic beads and stimulated with MGN-3/Biobran. NK cell activation was evaluated via analysis of their phenotype, and their expansion capability was tracked. The *in vitro* cytotoxic ability of the activated NK cells was tested against K562, Jurkat, A673, NB1691, A-204, RD and RH-30 cell lines and the *in vivo* cytotoxic ability against the NB1691 cell line. **Results.** MGN-3/Biobran stimulation of NK cells induced a higher expression of the activation-associated receptors CD25 and CD69 than in unstimulated cells ( $P < 0.05$ ). The expression of NKG2D, DNAM, NCRs and TLRs remained unchanged. Overnight MGN-3/Biobran stimulation increased NK cell cytotoxic activity against all cell lines tested *in vitro* and decelerated neuroblastoma growth *in vivo*. The mechanism is not mediated by lipopolysaccharide contamination in MGN-3/Biobran. Furthermore, the addition of MGN-3/Biobran promoted NK cell expansion and decreased T cells *in vitro*. **Conclusions.** Our data show that MGN-3/Biobran upregulates NK cell activation markers, stimulates NK cell cytotoxic activity against neuroblastoma *in vitro* and *in vivo* and selectively augments the expansion of NK cells. These results may be useful for future NK cell therapeutic strategies of the treatment of neuroblastoma.

**Key Words:** arabinoxylan rice bran (MGN-3/Biobran), cytotoxic activity, natural killer (NK) cells, neuroblastoma

### Introduction

Natural killer cell (NK) cytotoxic activity plays a major role in our natural immunologic defences against the development of malignancies, as evidenced by the fact that decreased NK cell cytotoxic activity is associated with a higher risk of tumor development in healthy people [1]. Additionally, after hematopoietic stem cell transplantation, high NK cell cytotoxic activity is associated with a decreased

risk of relapse in patients [2]. The cytotoxic activity of NK cells can be increased through healthy lifestyle practices [3–5], biological response modifiers [6,7], growth hormone [8] and cytokines [9–12]. Malignant cells can decrease NK cell cytotoxic activity through the release of suppressive cytokines and/or the reduction of activating receptors on NK cells [13,14]. NK cell activity can also be suppressed by antibodies [15,16] and chemotherapeutic drugs [17].

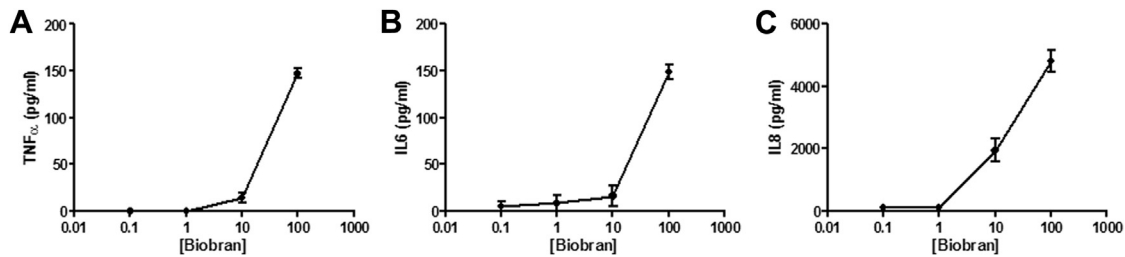


Figure 1. Cytokine levels in the culture supernatants were determined using the Cytometric Bead Array Flex Set (BD Biosciences) and analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). (A) TNF- $\alpha$  levels, (B) IL-6 levels and (C) IL-8 levels.

Therefore, maintaining high NK cell cytotoxic activity should be targeted in both cancer patients and the healthy population.

MGN-3/Biobran is an arabinoxyylan from rice bran that has been modified by carbohydrate hydrolysing enzymes from shiitake mushrooms [18]. This food supplement that has been reported to enhance NK cell cytotoxic activity against tumors in adult patients, *in vitro* and *in vivo* [19,20]. Furthermore, it has been described as having a synergistic anti-tumor effect with conventional treatment for some cancers, such as breast cancer and hepatocellular carcinoma [21–25]. These data have brought to light the possibility of using MGN-3/Biobran as a supplemental treatment for cancer in adult patients. However, no data have been reported for pediatric tumors. Our goal was to explore the role of MGN-3/Biobran as a NK cell stimulator against pediatric tumors *in vitro* and *in vivo* as well as the role of MGN-3/Biobran in NK cell expansion using various cytokine combinations and stimulator cell lines.

## Methods

### Cell preparation

Our local institutional ethics committee approved this study. peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation from blood samples taken from healthy volunteers. Blood was gently layered onto an equal volume of Ficoll-Paque

Plus (GE Healthcare) and centrifuged at 400 *g* for 20 min at room temperature. The PBMCs were harvested from the interface and washed twice with phosphate-buffered isotonic saline (PBS) and centrifuged at 400 *g* for 10 min. NK cells were then enriched by magnetic bead selection (NK cell isolation KIT or CD56 microbeads; Miltenyi Biotec) (see online supplementary Figure 1 for reviewers). Whole blood was layered on top of a Ficoll cushion and centrifuged at 1800 rpm for 30 min at room temperature. The lymphocyte/monocytic fraction was isolated, washed with PBS and subjected to red blood cell lysis (ammonium chloride solution; Stem Cell Technologies) for 5 min at room temperature, and following an additional wash with PBS, monocytes were cultured under adherent conditions in RPMI 1640 medium (Gibco-BRL, Life Technologies Ltd) supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Adherent monocytes were cultured for 7–10 days to allow for differentiation into macrophages. Macrophages were used as biosensors to identify the optimal dose of MGN-3/Biobran to stimulate NK cells without stimulate macrophages.

### Reagents

Anti-human monoclonal antibodies (mAbs) used in the study were CD3PE-Cy7, CD45-FITC, CD69-FITC and CD314 (NKG2D)-APC (all from Becton Dickinson); CD56-APC, CD25-PE, CD336

Table I. Biobran and IL-15 overnight stimulation effect on activating NK receptors.

	Resting		Biobran		IL-15		Biobran/resting Ratio	IL-15/resting Ratio
	MFI	SD	MFI	SD	MFI	SD		
CD69	508.3	889.2	1591.7	741.1	18032.6	14136.6	<b>3.13</b>	<b>35.48</b>
CD25	481	448.9	1537	520.3	1864	2843.8	<b>3.2</b>	<b>3.88</b>
NKG2D	4634.2	5762	5074.3	4761	9914.9	11491	1.09	<b>2.14</b>
DNAM	1960.6	2529	2501.1	1174	3344.8	5391	1.28	1.71
NKp44	1017.7	1473	1808	2780	886.3	2110	1.78	0.87
NKp30	1300.7	1990	1519.8	2508	4971.5	1567	1.17	<b>3.82</b>
NKp46	1134.4	1044	1159.4	1695	1568.8	1187	1.02	1.38
TLR4	2654	2858	1348	2533	1963	2150	0.51	0.74
TLR9	5854	6284	5200	6448	8779	6231	0.89	1.50

Data express MFI, SD, and ratios from 3 healthy controls. Bold indicates statistical significance.

(NKp44)-PE and CD335 (NKp46)-PE (all from Beckman Coulter); CD337 (NKp30)-PE (Miltenyi Biotec). Fluorochrome-labeled mAbs against TLR-4 and TLR-9 were obtained from Enzo Life Sciences AG.

Interleukin (IL)-15 was obtained from CellGenix. IL-2 (Proleukin) was obtained from Novartis. MGN-3/Biobran was provided by Daiwa Pharmaceuticals Co Ltd. Lipopolysaccharide (LPS; Sigma 0127:B8) was used as toll-like receptor-4 (TLR-4) ligand, and polymyxin B (InvivoGen) was used as an inhibitor of LPS-induced activation of TLR-4.

#### Cell lines

K562 erythroleukaemia, Jurkat T lymphoid leukaemia, A673 Ewing sarcoma (all from ATCC), NB1691 neuroblastoma cell line (kindly provided by Dr. A. Davidoff of St. Jude's Children's Research Hospital), A-204 embryonic rhabdomyosarcoma, RD embryonic rhabdomyosarcoma and RH-30 alveolar rhabdomyosarcoma (all from DSZM) cell lines were used as targets for NK cell natural cytotoxicity assays *in vitro*. The luciferase-transduced neuroblastoma cell line (NB1691luc) was kindly provided by Dr A. Davidoff and was used *in vitro* and in a quantitative *in vivo* mouse model [26,27]. Irradiated K562 and K562 with expression of cell membrane-bound IL-15 and 4-1BBL (K562-mb15-41BBL, kindly provided by Dr. D. Campana, National University of Singapore) were used as feeder cells for NK cell activation and expansion [28].

#### Phenotypic analysis

The surface phenotype of overnight MGN-3/Biobran (100 µg/mL)-stimulated NK cells, overnight IL-15 (10 ng/mL)-stimulated NK cells, unstimulated NK cells and expanded NK cells from 3 healthy adult volunteers was determined using 6-color immunofluorescent staining. We stained  $5 \times 10^5$  fresh NK cells from various conditions with appropriate mouse anti-human monoclonal antibodies for 30 min in the dark at 4°C. The cells were washed twice with cold PBS, resuspended in 0.5 mL of PBS and analyzed using a FACSCanto II flow cytometer (Becton Dickinson). The percentage of positive cells and mean fluorescence intensity (MFI) ratios were determined for each cell surface antigen. Controls were applied using appropriate isotype control antibodies.

#### Cytotoxicity assays and NK cell stimulation

The natural cytotoxicity of NK cells was monitored in a conventional 2-hour europium-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid release assay

(Perkin-Elmer Wallac) as described previously [29]. K562, Jurkat, A673, NB1691, A-204, RD and RH-30 cell lines were used as the target cells. In brief, target cells were labeled with a fluorescence-enhancing ligand (bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate). This hydrophobic ligand quickly penetrates the cell membrane. Within the cell, the hydrolysis of ester bonds results in the ligand becoming hydrophilic and therefore unable to pass through the cell membrane. Cytolysis, however, results in the release of the ligand and ultimately a reaction of the ligand with the europium to form a stable, fluorescing chelate, which is evaluated fluorometrically (Infinite F200 reader TECAN Group Ltd). The following formulas were used to calculate spontaneous and specific cytotoxicity:

% Specific release

$$= (\text{Experimental release} - \text{spontaneous release}) / (\text{Maximum release} - \text{spontaneous release}) \times 100$$

% Spontaneous release

$$= (\text{Spontaneous release} - \text{background}) / (\text{Maximum release} - \text{background}) \times 100$$

NK cells from healthy volunteers were stimulated overnight with 100 µg/mL MGN-3/BioBran, 10 ng/mL IL-15, 40 IU/mL or 1000 IU/mL IL-2 or with a combination of MGN-3/Biobran and 40 IU/ml IL-2. Cultures were performed in complete culture medium (RPMI 1640 supplemented with 10% of heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 ng/mL streptomycin, and 2 mmol/L glutamine) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cytotoxic activity was assessed as described earlier.

#### Murine model

NB-1691luc  $2 \times 10^5$  neuroblastoma cells were injected intravenously into 12-week-old NOD-scid IL-2Rgnull mice. For the isolation of NK cells, we used PBMCs from healthy volunteers. NK cells were then enriched by magnetic bead selection (NK cell isolation KIT, Miltenyi Biotec). NK cells obtained were >90% CD3-CD56+. Fresh NK cells or NK cells activated with 100 µg/mL MGN-3/BioBran overnight were used. Intravenous NK cellular therapy began 7 days after the injection of tumor cells and was performed twice a week for 4 weeks. In 2 independent experiments (4 mice per group), we compared an untreated cohort (control group) with a cohort receiving  $1 \times 10^6$  unstimulated NK cells (NK group) and a cohort receiving  $1 \times 10^6$  NK cells

stimulated overnight with 100 µg/mL MGN-3/Biobran (NK-Biobran group). Bioluminescence imaging was performed after the initiation of NK cell therapy on days 7, 14, 28 and 42 after intraperitoneal injection of 100 µL of luciferin dissolved in PBS at a concentration of 15 mg/mL.

Five minutes after the administration of substrate, the animals were anesthetized using iso-fluorane (induction of anaesthesia at 3% and then maintained at 1.5%) and transferred to the Xenogen IVIS Lumina II (Quantitative Fluorescent and Bioluminescent Imaging, Xenogen Corporation). Images were captured at varied exposures and the analysis was performed using Xenogen Living Image Software (version 3.2). For bioluminescence imaging plots, a rectangular region of interest encompassing the entire thorax and abdomen was applied for each mouse and total flux (photons/s) calculated in ventral and prone positions at 180 s exposure. This value was scaled to a comparable background value (from a nontumor bearing, luciferin-injected control mouse). All experiments were conducted following the guidelines of the Institutional Animal Care and Use Committees according to criteria outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

#### *NK cell activation and expansion*

Expansion was achieved by 14 days of culture with or without 100 µg/mL MGN-3/Biobran and cytokines (100 IU/mL IL-2 or 100 IU/mL IL-2 plus 10 ng/ml IL-15) or additional coculture with irradiated feeder cells consisting of K562 cells or K562-mb15-41BBL [28]. In brief, PBMCs were obtained from 5 healthy adult volunteers by density gradient centrifugation (Ficoll). PBMCs were incubated in a 6-well flat-bottom plate with or without MGN-3/Biobran and human cytokines (IL-2, IL-2 + IL-15) or cocultured at 1:1.5 ratio with sublethal irradiated K562 or K562-mb15-41BBL feeder cells. The culture medium was RPMI 1640 supplemented with 10% AB fresh frozen human plasma, L-glutamine and penicillin-streptomycin (Biochrom). Fresh medium was added every 2 days. After 14 days, cells were collected and analyzed for phenotype and *in vitro* NK cell cytotoxicity.

#### *Cytometric bead array and flow cytometer analysis to determine TLR agonist contamination in MGN-3/Biobran*

The release of tumor necrosis factor (TNF)- $\alpha$ , IL-6 and IL-8 in human macrophages after exposure to LPS (10 ng/mL) or MGN-3/Biobran (at 10, 100, 1000 and 10,000 µg/mL) was detected by cytometric

bead array technique Flex Set (BD Biosciences) following the manufacturer's protocol and then analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). MGN-3/Biobran concentration (100 µg/mL) was determined to be the highest concentration that did not induce inflammation (elevation of TNF- $\alpha$ , IL-6 and IL-8, Figure 1). MGN-3/Biobran was screened for its potential agonistic effect on TLR-2, -3, -4, -5, -7, -8 and 9 by InvivoGen. Because traces of LPS in MGN-3/Biobran could increase NK cell cytotoxicity by TLR-4 signaling, the determination of contaminating lipopolysaccharide/endotoxin, the TLR-4 ligand, in MGN-3/Biobran (100 µg/mL) was carried out by BioChem GmbH. In addition, we quantified LPS/endotoxin by chromogenic assay (ToxinSensor Chromogenic LAL Endotoxin Assay Kit, GenScript). Functional *in vitro* cytotoxicity assays were performed against K562 and NB1691 cell lines as targets using LPS (10 ng/mL) as an NK cell stimulus and polymyxin B (100 µg/mL) as an inhibitor of LPS-induced activation of TLR-4. Finally, we performed cytotoxic assays against the NB1691 cell line using MGN-3/Biobran-stimulated NK cells with polymyxin B inhibition.

#### *Statistical analysis*

Results are shown as means  $\pm$  SD. Non-parametric Wilcoxon tests were used to compare MGN-3/Biobran effect on NK cell phenotype, cytotoxicity and expansion rate. In the mouse model, survival was estimated by the univariate Kaplan-Meier method and compared using the log-rank test. Statistical significance was defined as  $P < 0.05$ .

## **Results**

#### *NK phenotyping*

The addition of MGN-3/Biobran-stimulated NK cells resulted in an increase in CD69 and CD25 expression from a median of 9%–88% and 6%–90%, respectively (MFI ratio increased 3.1-fold and 3.2-fold, respectively). The percentages and MFI of the other receptors studied was unchanged. IL-15-stimulated NK cells, used as a positive control, increased the median expression of CD25 significantly (6%–92%, MFI ratio increased 3.9-fold), CD69 (9%–98%, MFI ratio increased 35.5-fold), NKG2D (92%–97%, MFI ratio increased 2.1 fold), DNAM (81% to 96%, MFI ratio increased 1.7 fold) and Nkp30 (54 to 81, MFI increased 3.8 fold). Table I and Figure 2A and B show the response of activating receptors on NK cells to overnight MGN-3/Biobran and IL-15 stimulation.

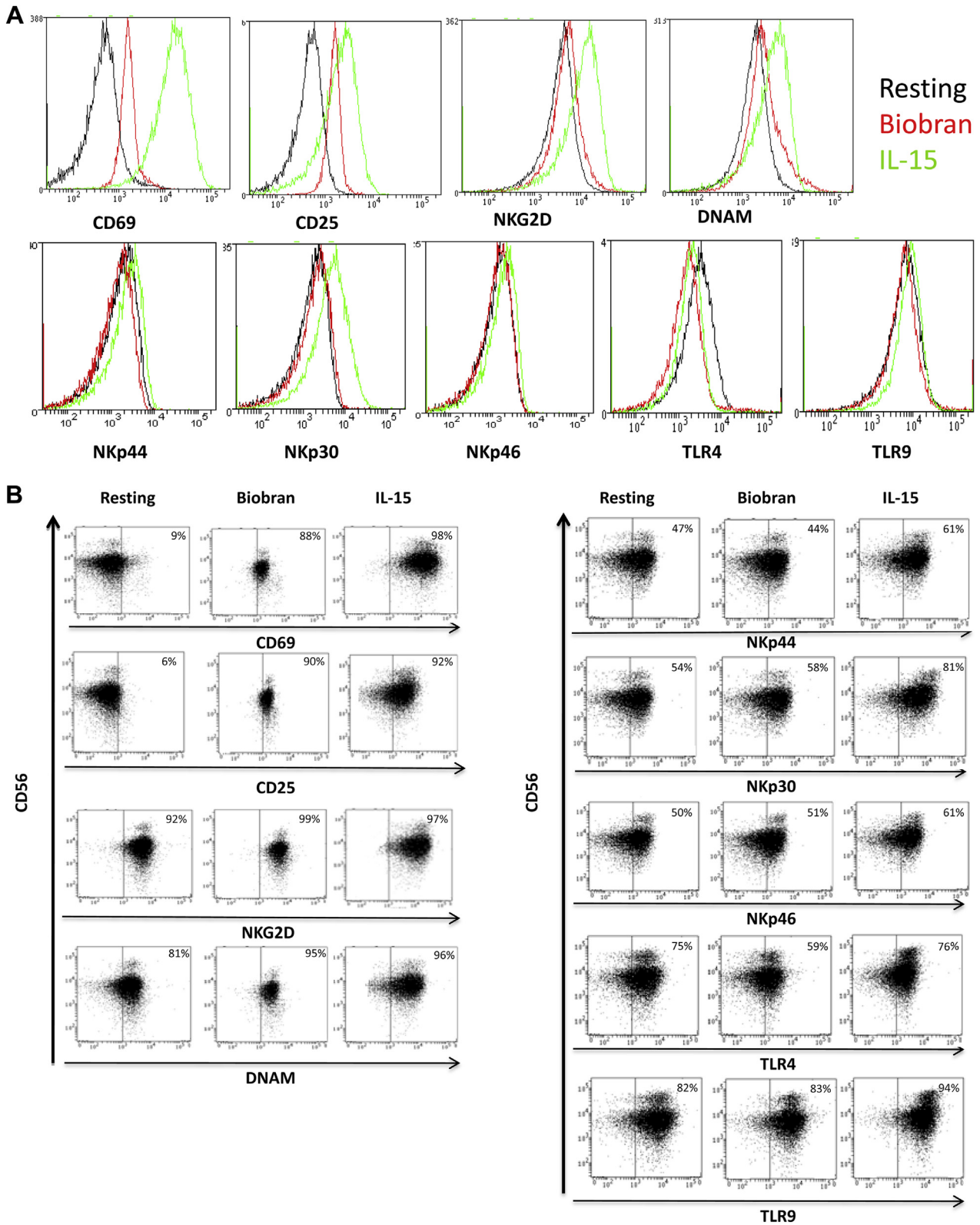


Figure 2. (A) Mean fluorescence intensity from activating NK cell receptors: at rest (black), MGN-3/Biobran (red) and IL-15 stimulated (green) in 3 healthy controls. (B) Percentages of activation markers expression on resting, MGN-3/Biobran- and IL-15-stimulated NK cells.

*In vitro* cytotoxicity assays

Overnight stimulation with MGN-3/Biobran resulted in a significant increase in NK cell cytotoxicity against all tested cell lines at an E/T ratio of 8:1 (K562, NB1691, Jurkat, A673) or 10:1 (A-204, RD, RH-30) compared with resting NK cells (Figure 3A, K562 80% vs. 69%,  $P = 0.03$ , NB1691 41% vs. 23%,  $P = 0.03$ , Jurkat 40% vs. 19%,  $P = 0.03$ , A673 34% vs. 13%,  $P = 0.02$ , A204 34% vs. 18%,  $P = 0.03$ , RD 45% vs. 22%,  $P = 0.002$ , RH-30 34% vs. 18%,  $P = 0.02$ ). Stimulation with IL-15 led to even higher percentages of lysis of the K562 (100%), NB1691 (61%), Jurkat (60%) and A673 (58%) cell lines (Figure 3B). To test the synergistic effect of IL-2 and MGN-3/Biobran, we compared stimulation with high-dose IL-2 (1000 IU/mL) with low dose IL-2 (40 IU/mL) and low dose IL-2 + MGN-3/Biobran. Adding MGN-3/Biobran to low dose IL-2 further enhanced the stimulatory effect of 40 IU/mL IL-2 and resulted in comparable cytotoxicity to that obtained with 1000 IU/mL IL-2 (Figure 3C). To test the safety profile of MGN-3/Biobran-stimulated NK cells, we performed cytotoxicity assays on negative controls (autologous CD56 negative cells), which revealed an absence of cytotoxicity (supplementary Figure 2 and supplementary Table I for reviewers).

*In vivo* model

To examine whether the stimulated effect of MGN-3/Biobran on NK cells *in vitro* has clinical significance, we then extended our investigation to an *in vivo* xenograft model of luciferase-transfected neuroblastoma. Figure 4A shows ventral and dorsal bio-images of 3 representative mice receiving PBS (control),  $1 \times 10^6$  unstimulated NK cells and  $1 \times 10^6$  MGN-3/Biobran-stimulated NK cells. There was a dramatic progression of the NB1691 tumors in the control group and unstimulated NK cell group, whereas significant neuroblastoma growth inhibition was observed in the cohort that received  $1 \times 10^6$  MGN-3/Biobran-stimulated NK cells (Figure 4B and supplementary Table II for reviewers). We also observed that MGN-3/Biobran-stimulated NK cells significantly increased survival in the NOD/scid/IL-2R $\gamma$ null-hu model ( $P < 0.05$ ; Figure 4C).

*Role of MGN-3/Biobran in expansion of NK cells*

After 2 weeks of culture, NK cells expanded more strongly when MGN-3/Biobran was added to the culture medium (supplementary Table III). In contrast, T-cell expansion was decreased when MGN-3/Biobran was added to the culture medium (Figure 5A). MGN-3/Biobran addition to IL-2

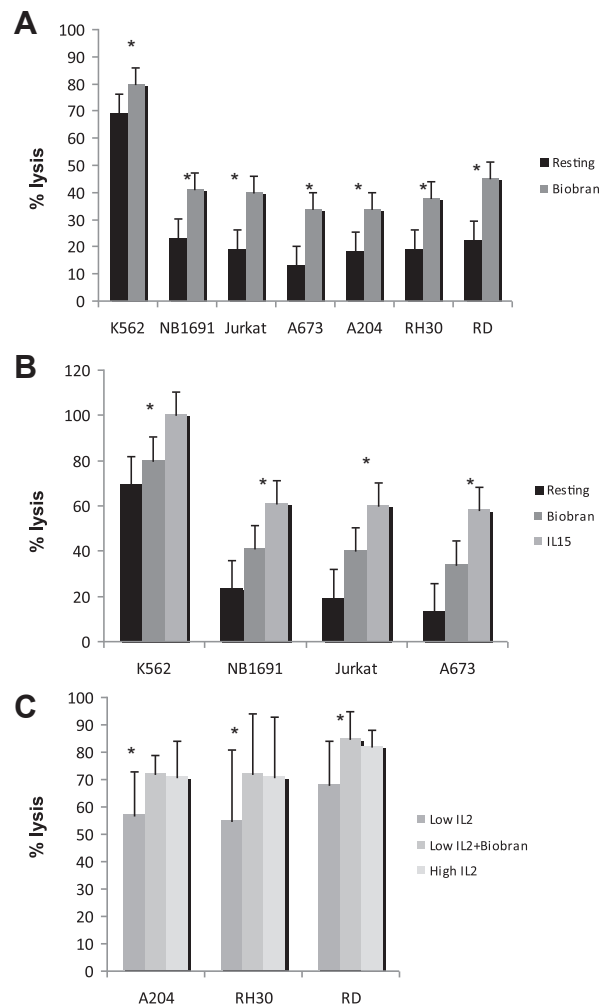


Figure 3. (A) MGN-3-stimulated NK cell cytotoxic activity against K562, NB1691, Jurkat and A673 cell lines (effector/target ratio 8:1), A-204, RD and RH-30 (effector/target 10:1). (B) IL-15 and MGN-3-stimulated NK cell cytotoxic activity against K562, NB1691, Jurkat and A673 cell lines (effector/target ratio 8:1). (C) IL-2- and MGN-3-stimulated NK cell cytotoxic activity against A-204, RD and RH-30 (effector/target 10:1). Data include results from 3 healthy volunteers in 3 independent experiments. \*Statistically significant.

and IL-2 + IL-15 cultures did not produce a statistically significant difference in NKT cells and B cells. The cytotoxic activity of expanded NK cells did not significantly change when MGN-3/Biobran was added to the culture medium (Figure 5B). In contrast, the addition of IL-15 enhanced cytotoxicity compared with IL-2 alone, even when using transfected K562 cell line.

*Mechanisms of MGN-3/Biobran stimulation on NK cells*

Because human NK cells can be stimulated by TLRs, we tested TLR triggering by MGN-3/Biobran using human macrophages as biosensors to identify the optimal dose of MGN-3/Biobran to stimulate NK cells without stimulate macrophages. Only high

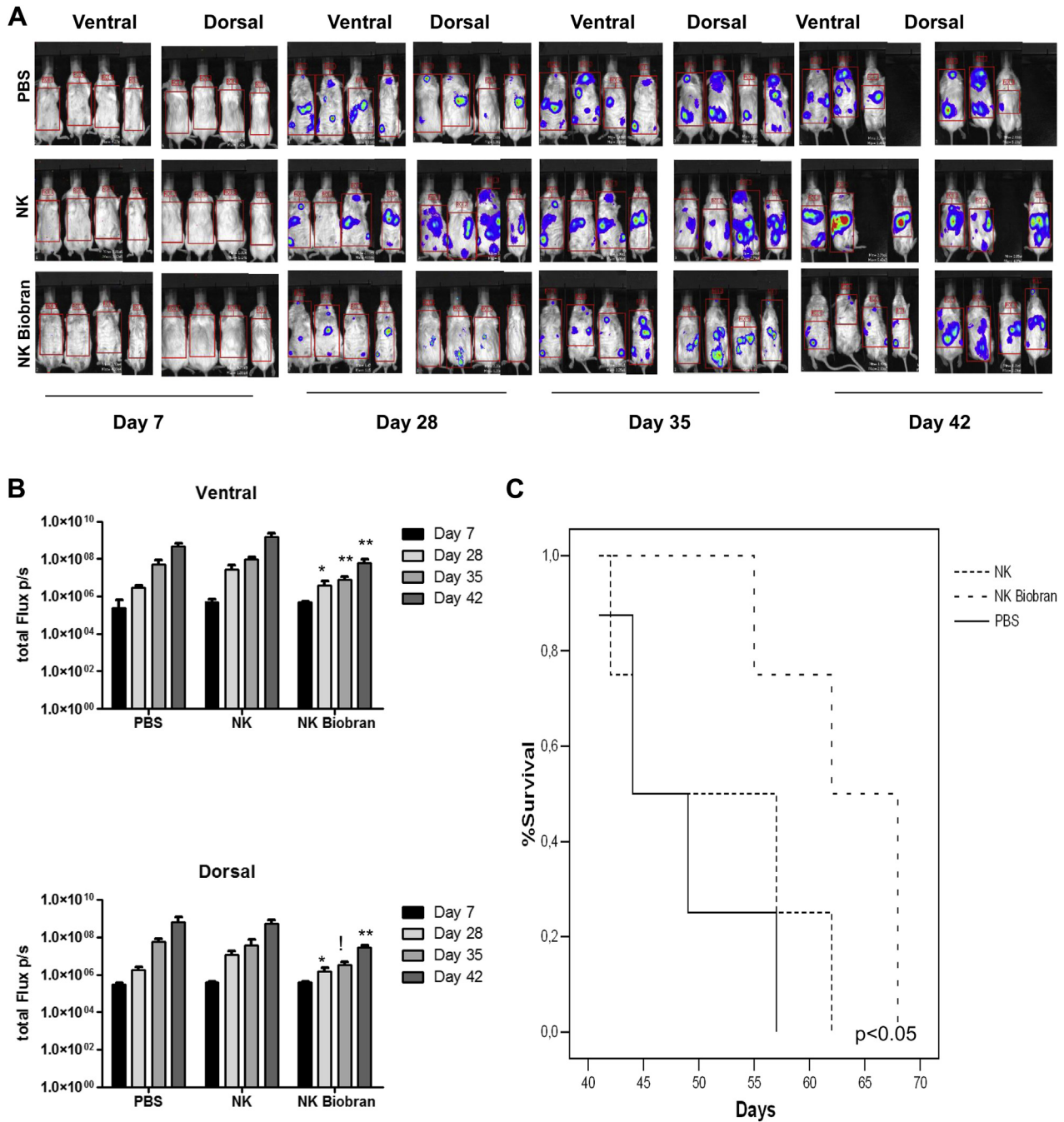


Figure 4. (A) Imaging of NB1691 tumors expressing luciferase illustrates the neuroblastoma burden in mice receiving PBS (control),  $1 \times 10^6$  freshly isolated unstimulated NK cells and  $1 \times 10^6$  MGN-3-stimulated NK cells (given by 8 intravenous injections, twice a week for 4 consecutive weeks). Three representative mice, ventral and dorsal, from each group are shown. ROI, region of interest. (B) The fold tumor volume relative to baseline values was significantly lower in the cohort of mice that received MGN-3-stimulated NK cells than in either the control group or the group that received resting NK cells. (C) Kaplan-Meier curves indicate the survival of each group of mice. †Statistically significant compared with control group. \*Statistically significant compared with unstimulated NK cells group. \*\*Statistically significant compared with both groups.

levels of MGN-3/Biobran (10 mg/mL) resulted in the release of IL-8, IL-6 and TNF- $\alpha$  (4776, 164 and 132 and pg/ml, respectively), see Figure 1. These measurements were significantly lower than those observed with LPS (10 ng/mL) stimulation (7487, 362 and 208 pg/mL, respectively).

We observed traces (Eu/mL = 1.68) of LPS contamination in MGN-3 Biobran in a limulus amoebocyte lysate (LAL) assay. To investigate the role of LPS contamination of MGN-3/Biobran as a mechanism of stimulation, we determined *in vitro* cytotoxicity assays against NB1691. These assays

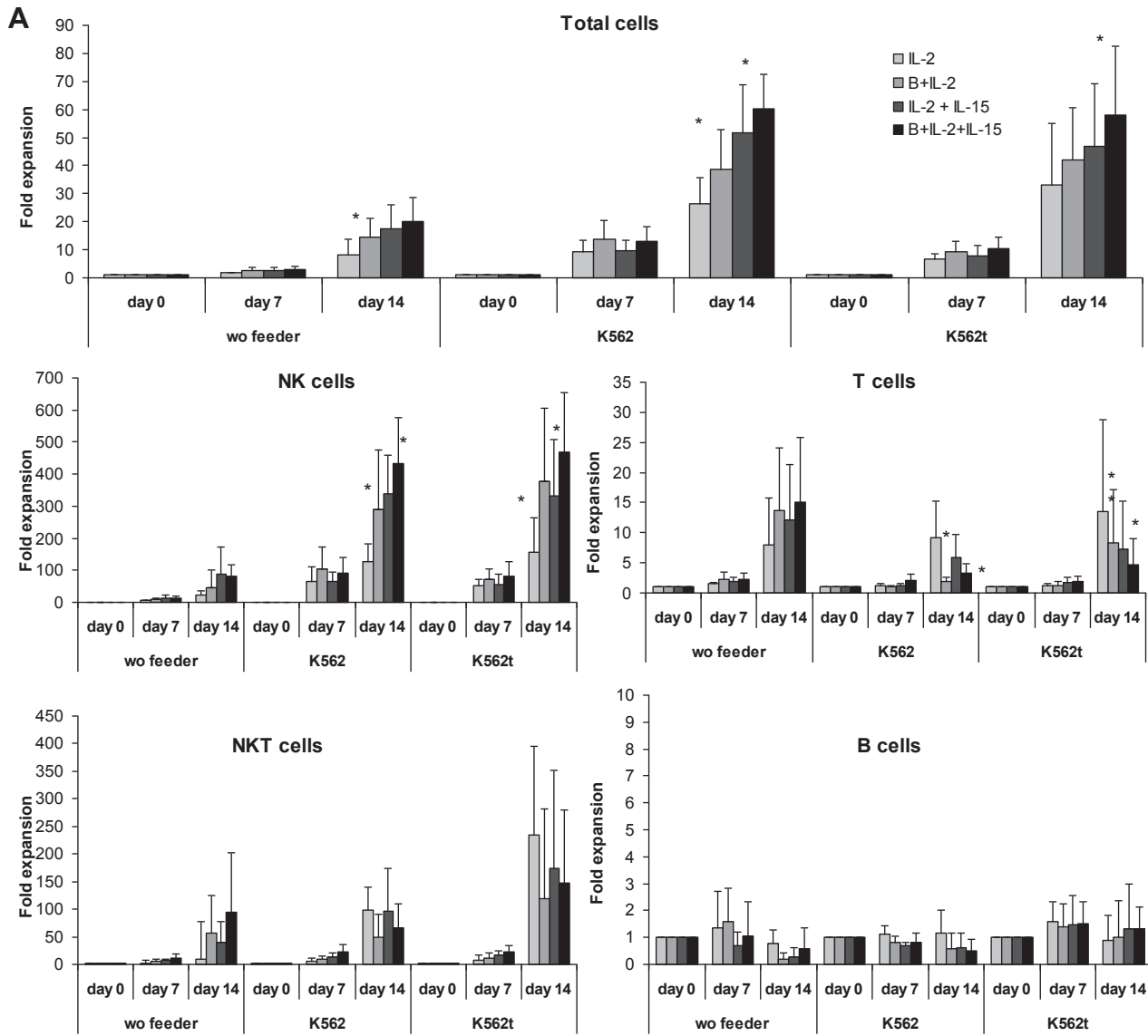


Figure 5. (A) Kinetics of total cells, NK cells, T cells, NKT cells and B cells after 14 days of culture expansion from 5 healthy donors with and without (wo) MGN-3/Biobran and cytokines (IL-2 or IL-2 + IL-15) or co-culture with irradiated feeder cells consisting of K562 cells or K562-mb15-41BBL. \*Statistically significant. (B) Cytotoxic activity of expanded NK cells adding MGN-3/Biobran to the culture medium.

showed increased cytotoxic activity of LPS-stimulated NK cells compared with resting NK cells, whereas polymyxin B abrogated the effect of LPS stimulation (Figure 6A). In contrast, the stimulating effect of MGN-3/Biobran on NK activity against NB1691 could not be antagonized with polymyxin B (Figure 6B). The mechanism of NK stimulation is not mediated by LPS contamination in MGN-3/Biobran.

## Discussion

Published findings have shown that the use of MGN-3/Biobran in cancer therapy can improve outcomes in some adult cancer patients [30,31]. A clinical trial of adult patients with hepatocellular carcinoma

showed that the addition of MGN-3/Biobran to interventional therapies including transarterial chemoembolization, percutaneous ethanol injection, radiofrequency ablation and cryoablation improved overall survival [21]. It has also been reported that the addition of MGN-3 stimulated innate immunity in multiple myeloma patients by increasing NK cell cytotoxic activity, levels of myeloid DCs and concentrations of T helper cell type 1-related cytokines [32]. There is no reported data regarding the use of MGN-3/Biobran with pediatric tumors.

Our study shows that MGN-3/Biobran stimulation of NK cells improved both *in vitro* and *in vivo* cytotoxic activity against various pediatric tumor cell lines. We demonstrated increased NK cell mediated killing



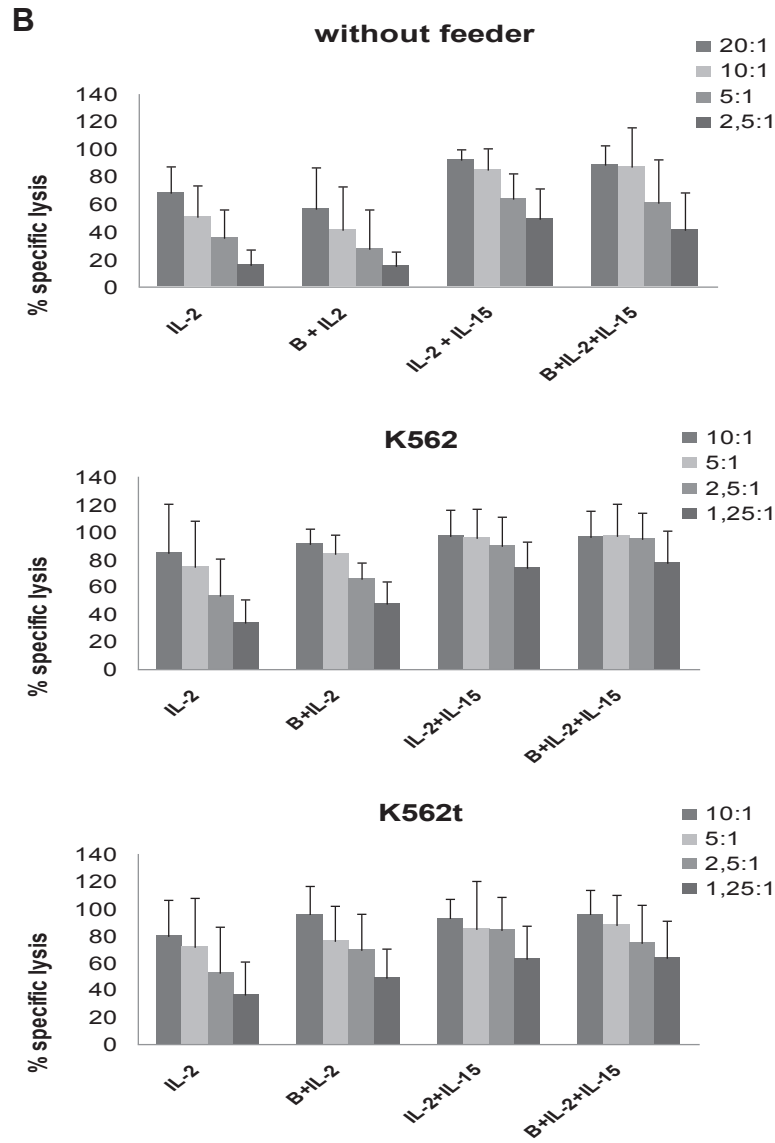


Figure 5. (continued).

of acute leukaemia, neuroblastoma, Ewing sarcoma, embryonic rhabdomyosarcoma and alveolar rhabdomyosarcoma cell lines *in vitro* after stimulation with MGN-3/Biobran. We also observed a significant inhibition of neuroblastoma growth and a significant improvement in survival in a NOD/scid/IL-2R $\gamma$ null neuroblastoma model when using MGN-3/Biobran-stimulated NK cells. These data are in agreement with previous data published on adult malignancies [20–25].

The mechanism and dose by which MGN-3/Biobran increases NK cell activity remains unknown. We suggest that a variety of immune mechanisms may be involved in the beneficial effect observed with MGN-3/Biobran treatment of NK cells. Because high doses of MGN-3/Biobran resulted in modifying macrophages from M0 to M1

releasing IL-6, IL-8 and TNF- $\alpha$ , we considered a low dose of MGN-3/Biobran to remove the NK cell activation caused by an inflammatory background. Because TLR agonists can stimulate human NK cells, we hypothesized that LPS contamination in MGN-3/Biobran could increase NK cell cytotoxicity by TLR-4 signaling. In our study, a small amount of LPS contamination was observed. However, neutralizing LPS with polymyxin B did not abrogate the stimulating effect of MGN-3/Biobran on NK activity, suggesting that LPS contamination is not the mechanism through which MGN-3/Biobran stimulates NK cells. According to our data, MGN-3/Biobran appears to activate resting NK cells, but it is unable to further activate cells undergoing expansion with IL-15, despite augmenting expansion

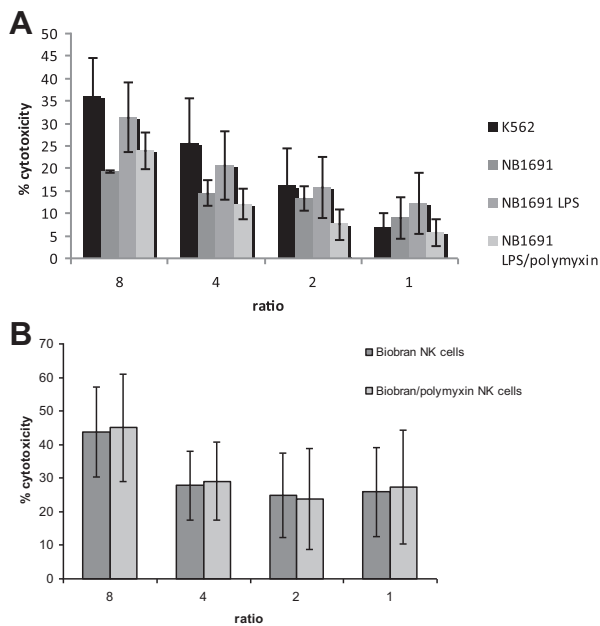


Figure 6. (A) LPS-stimulated NK cells increase resting NK cell cytotoxicity against NB1691 and polymyxin B abrogates LPS stimulation to a resting state. (B) MGN-3/Biobran stimulates NK cells against NB1691 that is not antagonized by polymyxin B.

during this period. This outcome suggests a mechanism that partially overlaps with IL-15. Another theory is an apoptotic effect mediated by activation of NK cells releasing TNF- $\alpha$  and IFN- $\gamma$  [30,33]. This theory is supported by recently reported data that the addition of MGN-3/Biobran to chemotherapy had a synergistic effect, as evidenced by enhanced apoptosis and cell proliferation inhibition in breast cancer cells [34]. Another possible mechanism could be the augmentation of activating receptors on NK cells stimulated with MGN-3/Biobran. We observed an increase of the activation-associated receptors CD69 and CD25 on MGN-3/Biobran-stimulated NK cells of healthy donors. CD69 elevation on NK cells correlates with an increase in NK cell cytotoxicity [35–37]. In addition, proliferative potential is indicated by CD25 expression elevation on NK cells [38]. Lastly, MGN-3/Biobran interaction with other immune cells has been also reported [39,40].

The adoptive transfer of *in vitro*–activated NK cells is currently used for cancer therapy. Recent studies have demonstrated that NK cells can be expanded to large numbers *ex vivo* using various methods, including using K562-mb15-41BBL as feeder cells [28]. These expanded NK cells exerted antitumor activity *in vitro* on a variety of cell lines and malignancies including adult and pediatric cancers [41–43]. When we added MGN-3/Biobran in various expansion protocols, we observed an improvement in the expansion of NK cells, a retention of cytotoxic activity and a reduction in T-cell proliferation. These data could be important for

large-scale expansion of highly cytotoxic clinical-grade NK cells, especially in an allogeneic setting where T cells should be removed to avoid graft versus host disease. Additionally, using MGN-3/Biobran in combination with low-dose IL-2 increased NK cell cytotoxic activity to the same level as high dose IL-2. These data are in accordance with earlier studies [44]. Therefore, MGN-3/Biobran and low dose IL-2 act synergistically and this approach can avoid toxicities related to high dose IL-2 treatment *in vivo*.

Data from adult patient studies have suggested that the use of MGN-3/Biobran as an alternative or adjuvant treatment to various immunotherapeutic approaches may be beneficial in the treatment of malignancy [20,22,24]. Our results extend to the pediatric patient population, as demonstrated by an increase in NK cell cytotoxic activity with the addition of MGN-3/Biobran against a variety of pediatric tumors *in vitro* and neuroblastoma *in vivo*. We also observed that the addition of MGN-3/Biobran increased NK cell expansion/activation and, in combination with a low-dose of IL-2, has a beneficial effect on activating NK cells for the purpose of immunotherapy against neuroblastoma. Further studies are warranted in the pediatric clinical setting to elucidate the role of MGN-3/Biobran in combination with chemo-immune protocols.

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### Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jcyt.2014.11.001>.