Original Article

Effects of Bacillus firmus e volumine ex muris cellulae 6x on cytolytic activity of natural killer cells *in vitro*

Stephan Sudowe [1], Dieter Sonntag [*2]

1- GANZIMMUN Diagnostics AG, Hans-Böckler-Straße 109, 55128 Mainz, Germany; dr.sudowe@ganzimmun.de
2- SANUM-Kehlbeck GmbH & Co. KG, Hasseler Steinweg 9, 27318 Hoya, Germany
*dieter.sonntag@sanum.com - https://orcid.org/0000-0002-1848-4854

Abstract

Natural killer (NK) cells are among the first in defense of the innate immune system by eliminating a variety of abnormal or stressed cells such as cancer cells or virus-infected cells. Individuals who exhibit low cytolytic NK cell activity are believed to be at higher risk of viral infection, tumorigenesis, and various other diseases of the immune system. Therefore, the restoration of impaired NK cell function might be an essential step in immunostimulatory therapy of immunocompromised patients. *Bacillus firmus* is a non-pathogenic gram-positive bacterium of the environment, which possesses various immunomodulatory properties *in vitro* and *in vivo*. This retrospective study reports on the effect of *B. firmus* on the activity of NK cells *in vitro*. Basal cytolytic NK cell activity against tumor cells among peripheral blood mononuclear cells (PBMCs) of routine patients was determined in a standardized NK cell cytotoxicity assay. The impact of the cultivation of PBMCs with *B. firmus* preparation Bacillus firmus e volumine ex muris cellulae (Bacillus firmus (evc)) 6x on tumor cell killing by NK cells was monitored in relation to basal NK cell activity. This study showed that stimulation of PBMCs with Bacillus firmus (evc) 6x *in vitro* led to a significant increase in NK cell function. Substantial improvement in cytolytic NK cell activity (more than 1.3-fold of basal activity) was much more pronounced for compromised NK cell function patients. Due to its immunostimulatory mode of action, Bacillus firmus (evc) may be of particular importance in the therapy of patients with NK cell deficiency.

Keywords: Natural killer (NK) cells, K562 cell line, Bacillus firmus, Immunomodulation

Introduction

The immune system is supposed to protect the human body efficiently and reliably against pathogens and malignancies. It consists of many cell types and effector molecules that interact with each other in a regulated network. The innate immune system contains cells and proteins that, in turn, play an important part in the initiation and subsequent activation of the adaptive immune system. Natural killer (NK) cells are innate immune cells, which account for 5% to 20% of peripheral blood mononuclear lymphocytes. NK cells are found in peripheral blood and the skin, liver, gut, spleen, lung, lymph nodes, thymus, peritoneal cavity, and uterus during gestation [1,2]. NK cells were also detected in mucosal-associated lymphoid tissue like human tonsils, mouse Peyer's patches, and intestinal and lung mucosa [3]. Historically, the name of NK cells is derived from their natural ability to kill tumor cells *in vitro*. Due to their high cytolytic potential, these lymphocytes are specialized in the identification and elimination of virus-infected somatic cells as well as malignant tumor cells [2]. The activity of NK cells regarding recognition and lysis of their target cells is regulated by a complex balance of inhibitory and activating signals derived from receptor molecules on the NK cell surface [4] (see Table 1). However, not all NK cells have high cytotoxic activity. Uterine and vaginal resident NK cells show only low cytotoxic activity, but they can increasingly produce cytokines, e.g., IFN-γ, and

are essential in the early stage of the immune response [5]. Apart from that, NK cells do not require any pre-stimulation to perform their effector functions [6].

Table 1: Activators, inhibitors, and ligands of human NK cells. TLR: toll-like receptor, IL: interleukin, HLA: human leukocyte antigen, TGF: tumor growth factor, MICA: MHC-1 related ligand, KIR: immunoglobin like receptors, Ly49A: type II transmembrane molecule. Table derived from Nouroz et al., 2016 [7].

Activator	Inhibitor	Receptors	Ligands	Cytotoxicity
TLR ligand	IL-10	NKG2A	HLA	IL-2
IFN-α/β	TGF-β	NKG2D	MICA	IL-12
IL-2	MHC-I	CD16	IgG	IL-15
IL-12	KIR	NKp30	Tumor	IL-18
IL-15	Ly49A	NKp44	Tumor	IL-25
IL-18	Tumor	NKp46	Tumor	IFN-γ

NK cells are mainly known for the defense against viral infections and the detection and destruction of tumor cells. The protective role of NK cells in several virus infections, such as influenza virus, HIV-1, and viral hepatitis, is well documented [8-10]. However, NK cells are also involved in other immunological processes. Especially mucosal resident NK cells are the first to defend against infections with bacteria, viruses, fungi, and protozoa [5,11]. There is increasing evidence that NK cells can affect the cellular and humoral adaptive immune response and are involved in autoimmunity processes [12]. Likewise, accumulating data highlight the importance of NK cells in host immune response against cancer and therapy-induced antitumor response. Individuals who exhibit lower NK cell activity are believed to be at higher risk of infection and tumorigenesis [13]. An 11-year follow-up study of the general Japanese population indicated that low natural cytotoxic activity was associated with increased cancer risk [13]. Evidence of NK cell deficiency has been observed in numerous studies of patients with cancer [14-17]. High tumoricidal NK cell activity has been shown to correlate with a good prognosis in several malignant diseases [14,16,17]. NK cell cytotoxicity against tumor cells was significantly related to the overall survival and progression-free survival of cancer patients and response rate to antitumor therapy [17]. There is an increasing number of NK cells deficient-people and/or in their functions [18]. During tumor progression, tumor cells develop multiple mechanisms to escape immune surveillance by manipulating immune cells to induce dysfunctional NK cells. Negative regulatory immune cells in the tumor microenvironment, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), may help tumors to further suppress NK cells [19]. In addition, primary cancer treatments like chemotherapy and ionizing radiation can compromise antitumor immune responses by their immunosuppressive side effects on NK cells [19]. Moreover, the deterioration of the immune system in aging, a process is known as 'immunosenescence', is thought to contribute to increased morbidity and mortality from infections and cancer in older individuals through a reduction in NK cell activity [20,21].

For the reasons outlined above, preserving or restoring NK cell functionality could be a promising curative strategy and should be a primary therapeutic objective in cancer patients and those with a low NK cell activity. NK cells are feasible targets of stimulation in immunotherapeutic approaches such as antibody-based strategies and adoptive cell transfer [22]. Moreover, NK cell activation has

been shown in preclinical and clinical studies to be one of the critical mechanisms for the biological effects induced by various natural health products used as immunomodulators in complementary and alternative medicine [23]. *Bacillus (B.) firmus*, a non-pathogenic and non-toxigenic gram-positive bacterium of the environment, possessed different immunomodulatory properties *in vitro* and *in vivo* [24-29]. *B. firmus* showed immunostimulating effects on human [27] and murine B lymphocytes [25] and macrophages [28]. All IgG subclasses and IgA have increased *in vitro* following incubation of splenocytes with inactivated *B. firmus*. Furthermore, the formation of IL-10 and IFN-γ is induced [25]. Immunization with *B. firmus* showed a marked systemic and mucosal increase of IgG and IgA antibodies in mice [29]. So far, however, it has not been investigated whether *B. firmus* can also exert an immunomodulatory effect on NK cells. Therefore, the effects of *B. firmus* preparation Bacillus firmus e volumine ex muris cellulae 6x (Bacillus firmus (evc) 6x) on the activity of NK cells *in vitro* was investigated in a retrospective study. Basal cytolytic NK cell activity against tumor cells among peripheral blood mononuclear cells (PBMCs) of routine patients was determined in a standardized NK cell cytotoxicity assay. The impact of the cultivation of PBMCs with *B. firmus* preparation Bacillus firmus (evc) 6x (Recarcin® 6x) on tumor cell killing by NK cells was monitored in relation to basal NK cell activity.

Material and Methods

The following methods were performed in routine diagnostics at a diagnostic laboratory (GANZIMMUN Diagnostics AG, Mainz, Germany). Blood samples were routinely examined by performing a cytotoxicity assay on PBMCs to determine NK cell activity. Additional testing of various remedies, including Bacillus firmus (evc) 6x (Recarcin® 6x) (SANUM-Kehlbeck GmbH & Co. KG, Hoya, Germany) was performed. This testing allowed a retrospective data analysis of 100 blood samples. Data collection was performed anonymously, the use of human leukocytes was in full accordance with the German laws of bioethics.

Bacillus firmus preparation

Bacillus firmus (Type strain DSM 4816) is used to produce the remedy Recarcin® 6x (SANUM-Kehlbeck GmbH & Co. KG, Hoya, Germany). Preparation of Bacillus firmus (evc) 6x is according to the method of "German Homeopathic Pharmacopoeia (HAB) draft monograph Prescription A: Mother tinctures made of bacteria fungi or yeasts (isopathics)". Bacillus firmus (evc) 6x is the active ingredient of the remedy Recarcin® 6x and contains cellular compartments of *B. firmus*. Preparation of the remedy is without alcohol. One capsule of Recarcin® 6x contains 330 mg of Bacillus firmus e volumine ex muris cellulae (lyophil., steril.) 6x.

Preparation of effector cells

Fresh heparinized peripheral blood samples were collected from 100 individuals, 33 to 87 years of age, including males and females between 2012 and 2015. PBMCs were isolated from 20 mL of heparinized blood by density gradient centrifugation using Biocoll separating solution (Biochrom GmbH, Berlin, Germany). PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in complete RPMI 1640 culture medium (Biochrom GmbH), supplemented with 1% penicillin/streptomycin (Biochrom GmbH; final concentrations 100 U/mL and 100 µg/mL, respectively) and 10% fetal calf serum (Biochrom GmbH). PBMCs were adjusted to 1x106 cells/mL. Measurement of NK cell function was performed in cell culture laboratories of GANZIMMUN Diagnostics AG (Mainz, Germany).

K562 leukemia target cells

MHC class I-negative K562 leukemia cells [30] were used as target cells in the NK cell cytotoxicity assay. K562 tumor cells were stained with fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) by incubation for 8 min with 1.75 µM/L CFSE (Invitrogen/Molecular Probes, Eugene, OR, USA) at room temperature and protected from light. After that, cells were washed twice with a complete RPMI 1640 medium to remove excessive CFSE and incubated overnight at 37 °C and 5% CO_2 until use.

NK cell cytotoxicity assay

To determine basal cytolytic NK cell activity, flow cytometric NK cell cytotoxicity assay was performed [31]. CFSE-labelled K562 tumor cells (8x103 cells/well) were cocultured in triplicates for 20 hours with PBMCs (2x105 cells/well) in 96-well flat-bottomed, polystyrene TC cell culture plates (Sarstedt, Nümbrecht, Germany) in a final volume of 250 µL complete RPMI 1640 medium at 37 °C and 5% CO2 (effector: target ratio = 25:1). In parallel cocultures, 20 IU/mL human recombinant interleukin-2 (IL-2) (Proleukin® S; Novartis, Nürnberg, Germany) as a stimulator of NK cell activity or Bacillus firmus (evc) 6x (Recarcin® 6x; SANUM-Kehlbeck GmbH & Co. KG, Hoya, Germany) (final dilution 1:25) were added. To estimate spontaneous cell death of leukemia cells, K562 cells were cultured in separate wells without PBMCs in a final volume of 250 µL complete RPMI 1640 medium.

Flow cytometry

Cultures were transferred to 5 mL-FACS (fluorescence-activated cell sorting) tubes (polystyrene, 12x75 mm; Sarstedt) the next day. 100 µL of PBS and 50 µL of propidium iodide (PI; Sigma-Aldrich, Taufkirchen, Germany) (final concentration 10 µg/mL) were added to the cell suspension and mixed. All samples were analyzed within 1 hour on a Navios™ EX flow cytometer (Beckman Coulter, Krefeld, Germany), using Navios™ EX Cytometer 2.0 software for data acquisition and analysis. Dead K562 leukemia cells were identified and gated as CFSE (FL1 channel) and PI (FL3 channel) double-positive cells. Basal cytolytic NK cell activity was calculated as the percentage of dead K562 leukemia cells, adjusted for spontaneous K562 cell lysis. The immunostimulatory capacity of IL-2 or Bacillus firmus (evc) 6x for NK cell-mediated tumor cell killing was assessed individually concerning basal cytolytic NK cell activity (set as 1).

Statistical analysis

Paired two-tailed Student's t-test performed statistical comparisons between two groups. Significant differences were determined according to a threshold of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Statistical analysis and graphical illustrations were performed with GraphPad Prism version 8.

Results

The immunostimulatory capacity of IL-2 or Bacillus firmus (evc) 6x for NK cell-mediated tumor cell killing was assessed individually concerning basal cytolytic NK cell activity (set as 1). Basal NK cell activity and NK cell activity after stimulation with IL-2 or Bacillus firmus (evc) 6x were determined in a NK cell cytotoxicity assay for a total of 100 samples of different individuals. Additional testing of various remedies, including Bacillus firmus (evc) 6x (Recarcin® 6x), was performed. This testing allowed a retrospective data analysis. The data of 100 samples were divided into three groups depending on the cytolytic NK cell activity of different individuals. 32 individuals showed a basal cytolytic NK cell activity lower than 5% regarding to K562 cell lysis (group A) (Figure 1-II), which represents a strongly decreased activity and which denotes an increased risk of tumor development

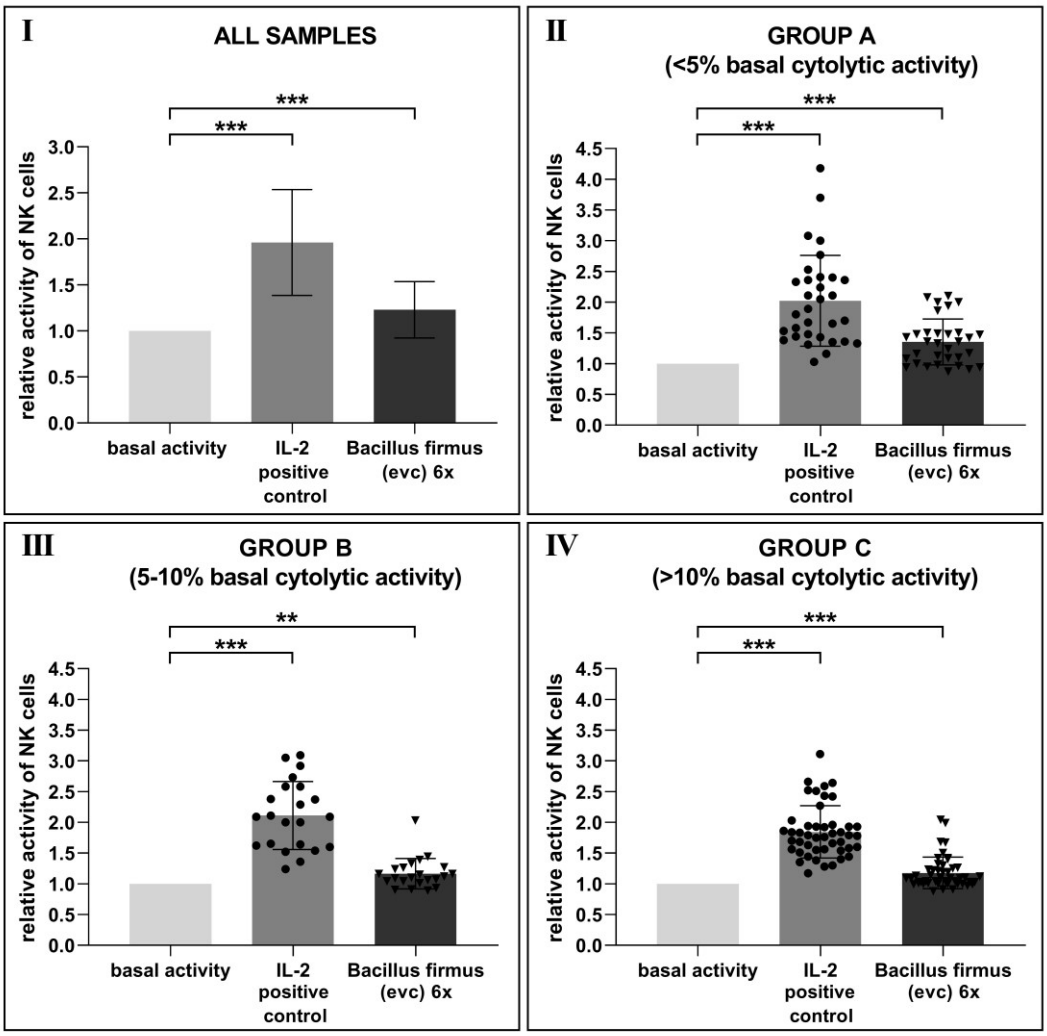

Figure 1: NK cell activity after stimulation with IL-2 (Proleukin®) or Bacillus firmus e volumine ex muris cellulae 6x (Recarcin® 6x). (**I**) All samples, n = 100; (**II**) Group A: Samples of individuals with strongly decreased NK cell activity (<5% basal cytolytic activity), n = 32; (**III**) Group B: Samples of individuals with slightly decreased NK cell activity (5–10% basal cytolytic activity), n = 22; (**IV**) Group C: Samples of individuals with normal NK cell activity (>10% basal cytolytic activity), n = 46. The basal activity was set to 1, respectively. Bacillus firmus (evc) 6x = Bacillus firmus e volumine ex muris cellulae 6x. The difference to basal activity was analyzed by paired two-tailed Student's *t* test: **p < 0.01, ***p < 0.001. Each circle or triangle represent one sample. The data are shown as means. Error bars represent the SD.

and serious or consecutive viral infections [13,18], respectively. 22 individuals exhibited a basal NK cell activity between 5% and 10%, indicating a slightly decreased cytolytic activity (group B) (Figure 1-III). The other 46 individuals had regular basal cytolytic NK cell activity higher than 10% (group C) (Figure 1-IV). Stimulation of NK cells with recombinant interleukin-2 (IL-2) in the form of the drug Proleukin®, which is known as a potent activator of NK cells [32], led to a substantial increase in the lysis rate in general (1.96 ± 0.57 in relation to basal activity) (Figure 1-I). In comparison, the enhancement of NK cell activity in cultures of all samples stimulated with Bacillus firmus (evc) 6x was lower but statistically significant (1.23 ± 0.31) (Figure 1-I). The lysis rate of K562 cells after addition of Bacillus firmus (evc) 6x in cultures without PBMCs was below 1% (data not shown),

indicating that the preparation exerted no direct cytotoxic effects on the tumor cells. With regard to each group separately the enhancement of NK cell activity in cultures stimulated with Bacillus firmus (evc) 6x was consequently lower than with IL-2, but statistically significant (Figure 1-II-IV). If the lysis rate is increased by more than 1.3-fold, it is accepted to be a substantial improvement in NK cell activity. To analyze the immunomodulatory properties of Bacillus firmus (evc) 6x, the proportion of samples in which the lysis rate increased more than 1.3-fold was determined separately for each group, shown in Figure 2. 94% of the samples showed a lysis rate increased by more than 1.3-fold after IL-2 treatment (Figure 2-II) and 30% of samples after Bacillus firmus (evc) 6x treatment (Figure 2-I). Of note is the fact that the group with strongly decreased NK cell activity has largest proportion of samples which were activated substantially (group A: 17/32, 53.1%) compared to the groups with slightly decreased activity (group B: 4/22, 18.2%) or normal activity (group C: 9/46, 19.6%) after Bacillus firmus (evc) 6x treatment (Figure 2-I). In conclusion, Bacillus firmus (evc) 6x stimulated NK cell activity in each group differently, whereas IL-2 stimulated the NK cell activity in all three groups in same manner (group A: 30/32, 93.8%; group B: 21/22, 95.5%; group C: 43/46, 93.5%) (Figure 2-II).

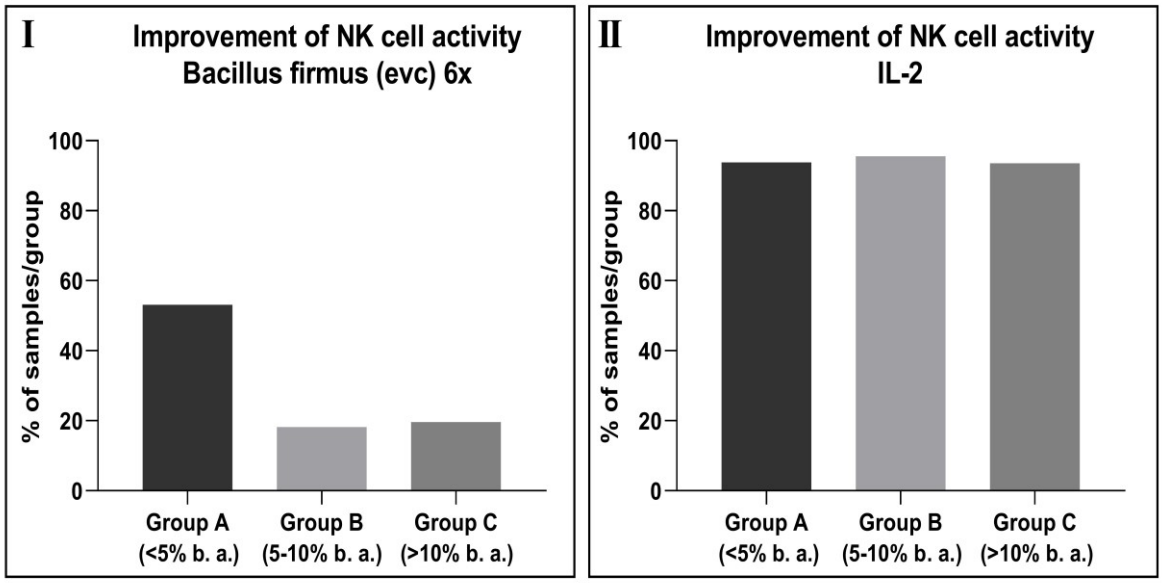

Figure 2: Percentage of samples per group with substantial improvement of NK cell activity. (**I**) Bacillus firmus e volumine ex muris cellulae 6x (Recarcin® 6x) stimulates the NK cell activity in each group differently. 30/100 samples showed substantial improvement. (**II**) IL-2 (positive control) (Proleukin®) stimulates the NK cell activity in each group in same manner. 94/100 samples showed substantial improvement. Group A: Samples of individuals with strongly decreased NK cell activity (<5% basal cytolytic activity), n = 22, Group B: Samples of individuals with slightly decreased NK cell activity (5–10% basal cytolytic activity), n = 32, Group C: Samples of individuals with normal NK cell activity (>10% basal cytolytic activity), n = 46, (total: n = 100). Substantial improvement of NK cell activity is defined as an improvement of the lysis rate by more than 1.3-fold. b. a.= basal cytolytic activity. Percentages were calculated separately in each group.

Discussion

NK cells are among the first in defense of the innate immune system. They support the defense of microbes and can eliminate a variety of abnormal or stressed cells, like cancer cells and virus-infected cells [2]. There are various causes for a reduced NK cell activity, for example, psychological stress

[33], sleep deprivation [34], and diet [35]. NK cell activity is also reduced in older people, making them more susceptible to viral infections [36]. Low NK cell titers in the blood are also often associated with various immune system diseases [37]. In addition, NK cells are usually reduced in patients with rheumatoid arthritis and AOSD (Adult-onset Still's disease), a rare type of inflammatory arthritis [38,39]. Decreased NK cell activity generally increases the susceptibility to cancer and infections, e.g., for infections with herpes viruses [18]. Therefore, activation of NK cells might be an essential step in therapy, especially in cases of NK cell deficiency such as malignant diseases [40]. *B. firmus*, a non-pathogenic bacterium of the environment, possessed different immunomodulatory properties *in vitro* and *in vivo* [24-29], including immunostimulating effects on human [27] and murine B lymphocytes [25] and macrophages [28]. Furthermore, a study from Lomakova and colleagues showed the ability of inactivated *B. firmus* to activate murine peritoneal cells and enhanced production of several important immunomodulatory cytokines *in vitro* [41]. In this context, the use of *B. firmus* in human medicine has already been discussed. However, in previous studies, it has not yet been investigated whether *B. firmus* can activate NK cells. Our data analysis of the cytotoxicity assay of NK cells revealed that Bacillus firmus (evc) 6x (Recarcin® 6x) had a stimulatory effect on human NK cells, especially in samples of individuals with strongly decreased NK cell activity. In over 50% of samples in the group with strongly decreased NK cell activity, the activity was increased by more than 1.3-fold (substantial improvement). This is by far the highest proportion of samples compared to the other two groups (samples with slightly decreased activity and normal activity of NK cells) (Figure 2-I). These results show that Bacillus firmus (evc) 6x can stimulate the cytolytic NK cell activity in each group differently and displayed the greatest stimulating effect in a group with strongly decreased NK cell activity. In comparison, IL-2 stimulated the NK cell activity in each group equally. This suggests an immunomodulatory mechanism of action of Bacillus firmus e volumine ex muris cellulae 6x. The different modes of action of IL-2 and *B. firmus* preparation (Bacillus firmus (evc) 6x) could be due to the fact that Bacillus firmus (evc) 6x, unlike IL-2, is not a single substance, as it consists of different cellular compartments and can therefore act at different cellular levels. It is known that *B. firmus* stimulates immune cells to release cytokines [25,26,41] and that even some cellular fractions of *B. firmus* alone are capable of interacting with immune cells [24,28]. Cellular components of bacterial preparation Bacillus firmus (evc) 6x probably caused a differential cytokine release leading to adapted activation of NK cells in different samples. Therefore, this might explain the immunomodulatory mode of action of Bacillus firmus (evc) 6x. Further investigation is required to verify this mechanism.

Conclusion

Our results display the ability of the remedy Bacillus firmus e volumine ex muris cellulae 6x (Recarcin® 6x) to stimulate cytolytic NK cell activity to varying degrees, depending on the basal cytolytic activity of NK cells of different individuals. Due to its immunomodulatory mechanism of action, this remedy may be of particular importance in therapy in patients with NK cell deficiency. Further investigations *in vitro*, and especially in clinical trials, are required.

Statement of conflict of interest and financial support

" The authors declare a conflict of interest; the result relates to a product produced by SANUM-Kehlbeck GmbH & Co. KG, Germany, with which the correspondent author is linked. "

" The authors declare that all material necessary for the conduct of this research was provided by GANZIMMUN Diagnostics AG, Mainz, Germany. "

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