Nicotinamide mononucleotide augments the cytotoxic activity of natural killer cells in young and elderly mice

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ABSTRACT

Nicotinamide mononucleotide (NMN), a key nicotinamide adenine dinucleotide (NAD⁺) intermediate, has been shown to ameliorate various pathologies in elderly mouse disease models. Natural killer (NK) cells are important innate immune cells; however, their functions decline with aging. In this study, we examined the effect of NMN treatment on NK cells in mice. Intraperitoneal administration of NMN augmented NK cell cytotoxic activity in both young and elderly B6 mice as well as young BALB/c mice. Oral administration of NMN also increased NK cell cytotoxicity in elderly B6 and BALB/c mice. However, the NK cell population was not increased in the mice whose NK cell cytotoxic activity was activated by NMN. Interestingly, NMN administration did not augment NK cell cytotoxic activity in IFN-γ deficient mice. These results suggest that NMN administration augments NK cell cytotoxic activity, but not cell number, in a manner dependent on IFN-γ in both young and elderly mice.

INTRODUCTION

Aging is frequently associated with the development of serious chronic diseases, so called age-associated diseases, such as cancer, cardiovascular diseases, type 2 diabetes, obesity, and hypertension (Belikov 2019). Due to the worldwide increase in the average of human lifespan, the prevention of these age-associated diseases is now a critical social issue. “Inflammaging” describes the low-grade, chronic and systemic inflammation without overt infection (“sterile” inflammation) that occurs with aging and is a highly significant risk factor for age-associated diseases, morbidity, and mortality (Franceschi et al. 2000). It has been demonstrated epidemiologically that a state of mild inflammation, revealed by elevated levels of inflammatory biomarkers such as C-reactive protein and interleukin-6 (IL-6), is associated and predictive of many aging phenotypes.

Cell damage induced by oxidative stress is the other critical factor involved in most age-associated diseases. Recent studies have shown that decreases in nicotinamide adenine dinucleotide (NAD⁺) levels in multiple tissues contribute to the pathogenesis of age-associated functional disorders (Imai and Guarente 2014). Moreover, it was reported that the administration of nicotinamide mononucleotide (NMN), a key NAD⁺ intermediate in mammals, restores tissue NAD⁺ levels and ameliorates obesity, insulin resistance, muscle mitochondrial dysfunction, renal failure, and retinal degeneration in mice (Hasegawa et al. 2013; Lin et al. 2016; Mills et al. 2016; Yoshino et al. 2011). Thus, preventive and therapeutic application of key NAD⁺ intermediates, such as NMN, which can replenish cellular NAD⁺ levels, is attractive as an evidence-based anti-aging intervention (Yoshino et al. 2018).

Immune activity is attenuated by aging (Nikolich-Zugich 2018). In particular, natural killer (NK) cells, innate immune cytotoxic cells that lyse cancer cells
or virally infected cells (Trinchieri 1989), are affected by multiple factors including aging, mental stress, and lifestyle choices including ingestion of certain foods (Kusaka et al. 1992; Borella et al. 1999; Di Lorenzo et al. 1999; Bennett et al. 2003). An epidemiological study showed that a human population with low NK cell activity has a significantly higher risk of developing cancer compared with populations with intermediate or high NK cell activity (Imai et al. 2000). Moreover, for infectious diseases, the mortality rate of elderly subjects with low NK cell activity is significantly higher than that of those with high NK cell activity (Ogata et al. 2001). It is possible that reduced NK cell activity in the elderly is involved in the pathogenesis of age-associated diseases and the increased mortality. Further, anti-aging drugs might augment NK cell activity. In this study, we examined the effect of NMN administration on NK cell activity in mice.

MATERIALS AND METHODS

Reagents. Nicotinamide mononucleotide (NMN) was kindly provided by the Oriental Yeast Co. (Tokyo, Japan). The dose of NMN (mg/kg/day) administered was selected according to the effective dose used in the previous mouse and human studies (Stromsdorfer et al. 2016). The NMN was dissolved in PBS (−) at the required concentrations and administered intraperitoneally or orally at 200 μL/mouse/day.

Mice. Twenty, twenty-two, thirty-four and thirty week-old C57BL/6 (B6) and six and thirty week-old BALB/c wild-type (WT) mice were purchased from Charles River Japan Inc. (Yokohama, Japan). BALB/c IFN-γ-deficient (Ifng−/−) mice were derived as described previously (Yuminamochi et al. 2007). All mice were maintained under specific pathogen-free (SPF) conditions and used in accordance with the experimental animal guidelines of Juntendo University following the guidelines of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Flow cytometry. Mice mononuclear cells (MNCs) were prepared from liver and spleen as previously described (Takeda et al. 2018), stained with FITC-conjugated anti-mouse CD3 monoclonal antibody (mAb) (145-2C11) and PE-conjugated anti-NK1.1 mAb (PK136) or anti-CD49b mAb (DX5) following pre-incubation with anti-mouse CD16/32 mAb (2.4G2) to avoid non-specific binding of mAbs to Fcγ receptors, and analyzed using a FacsCaliber (BD Biosciences, San Jose, CA). All reagents were purchased from eBioscience (San Diego, CA). Data were analyzed by BD CellQuest Pro (BD Biosciences). Anti-CD49b mAb (DX5) was used to define NK cells in BALB/c mice that lack NK1.1 gene expression.

Analysis of NK cell cytotoxicity. The cytotoxic activity of NK cells in mice MNCs was examined using YAC-1 cells, moloney murine leukemia virus induced lymphoma derived from A/Sn mouse, as target cells using a standard 4 h 51Cr release assay as previously described (Yuminamochi et al. 2007). Briefly, mice liver or spleen MNCs were adjusted to the appropriate cell concentrations in 10% fetal calf serum (FCS)/RPMI 1640 and 100 μL of the cell suspension was put into each well of a 96-well round-bottomed microtiter plate (NUNC, Roskilde, Denmark). Thereafter, 100 μL of 51Cr-labeled YAC-1 cell suspension was added (1 × 105 cells/well). The cells were incubated in 5% CO2 in humidified air at 37°C for 4 h. The percentage of specific lysis was calculated according to the following formula: % specific lysis = (experimental release – spontaneous release) / (maximal release – spontaneous release) × 100. Data are presented as the mean ± SD of triplicate samples.

Statistical analysis. Data were analyzed with a two-tailed Student’s t test. P values of less than 0.05 or 0.005 are considered significant.

RESULTS

Augmentation of NK cell cytotoxic activity following intraperitoneal treatment of mice with NMN

We first examined the effect of intraperitoneal administration of NMN on NK cell activity using adult (22 or 25 week-old) B6 mice and young adult (8 week-old) BALB/c mice. When NMN was administered at 625 mg/kg for 8 days into adult B6 mice, NK cell cytotoxic activity of liver, but not spleen, MNCs was significantly augmented (Fig. 1A). When NMN was administered into adult B6 mice for 4 days, NK cell cytotoxicity of liver, but not spleen, MNCs was significantly augmented similarly at both 625 and 1250 mg/kg (Fig. 1B). Moreover, intraperitoneal administration of NMN at 625 mg/kg into young adult BALB/c mice also significantly augmented NK cell cytotoxicity in the liver, but not spleen, MNCs (Fig. 1C). Interestingly, the NK cell population did not significantly increase in either liver or spleen MNCs of NMN-treated mice (Fig. 2A–
Fig. 1  Activation of NK cell cytotoxicity following intraperitoneal administration of NMN in adult B6 and young adult BALB/c mice. Twenty-two week-old B6 mice were intraperitoneally administered 625 mg/kg/day of NMN (■) or PBS (−) (□) for 8 days (A). Twenty-five week-old B6 mice were intraperitoneally administered 625 (■) or 1250 (●) mg/kg/day of NMN or PBS (−) (□) for 4 days (B). Eight week-old BALB/c mice were intraperitoneally administered 625 mg/kg/day of NMN (■) or PBS (−) (□) for 4 days (C). Then, liver and spleen PBMCs were prepared and the cytotoxic activity was analyzed using NK cell-sensitive target cells, YAC-1, at the indicated effector/target ratios. Data are presented as mean ± SD of triplicate samples. *P < 0.05 compared with control. **P < 0.005 compared with control. Similar results were obtained in three independent experiments.

Fig. 2  Effect of intraperitoneal administration of NMN on the NK cell population in hepatic and splenic MNCs. The proportion of NK cells in MNCs from livers and spleens of the mice presented in Fig. 1 A and B. The percentage of CD3−NK1.1+ cells (NK cells), CD3+ NK1.1− cells (NKT cells), and CD3+ NK1.1+ cells (T cells) in B6 mice presented in Fig. 1A and B is indicated on each panel. C. The percentage of CD3−CD49b+ cells (NK cells), CD3+CD49b+ cells (NKT cells), and CD3+CD49b− (T cells) cells in BALB/c mice presented in Fig. 1C is indicated on each panel. Data are shown as mean ± SD of triplicate samples. Similar results were obtained in two independent experiments.
These results suggest that NK cell activity, but not NK cell number, in the liver MNCs was augmented by the intraperitoneal administration of NMN in the mice.

**Augmentation of NK cell cytotoxic activity following intraperitoneal treatment of elderly B6 mice with NMN**

It has been reported that NK cell activity is reduced by aging (Di Lorenzo et al. 1999). Thus, it is possible that the effects of NMN administration on NK cell cytotoxicity are reduced in the aging mice. Thus, we performed a dose escalation trial of NMN administration in elderly (36 week-old) B6 mice. When the mice were intraperitoneally administered 1250, 625 or 313 mg/kg of NMN for 4 days, NK cell activity was significantly augmented in liver MNCs at effector/target ratios of 10 and 5 as well as in spleen MNCs at an effector/target ratio of 100 when compared with the control (Fig. 3A). Notably, the proportion of NK cells was not significantly increased even in the mice with significantly augmented NK cell activity (Fig. 3B). These results demonstrated that intraperitoneal administration of more than 313 mg/kg of NMN for 4 days augments NK cell cytotoxicity in liver and spleen MNCs, but not the number of NK cells, even in aging mice.

**Oral administration of NMN augments NK cell cytotoxicity**

NMN has been broadly used as the dietary supplement around the world. Therefore, we orally administered NMN into elderly (32 week-old) B6 and BALB/c mice and examined NK cell cytotoxicity. When the mice were orally treated with 625 mg/kg of NMN, NK cell cytotoxicity was augmented in liver MNCs and also in spleen at an effector/target ratio of 200 in both B6 and BALB/c mice (Fig. 4A). Again, the proportion of NK cells was not significantly increased by NMN treatment (Fig. 3B). Thus, both intraperitoneal and oral administration of NMN increased NK cell cytotoxicity, but not cell number, in the elderly mice.

**Requirement for IFN-γ in NMN-induced activation of NK cell cytotoxicity**

We have reported that IFN-γ plays a critical role in the activation of NK cell cytotoxicity following treatment with *Lactobacillus casei Shirota* (LeS), *Panax ginseng*, as well as *Agaricus blazei* Murill and propolis (Takeda et al. 2006; Yuminamochi et al. 2007; Takeda and Okumura 2015; Takeda et al. 2018). Thus, we investigated the contribution of IFN-γ in the NMN-induced activation of NK cell cytotoxicity.

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**Fig. 3** Effect of intraperitoneal administration of NMN on NK cells in hepatic and splenic MNCs of elderly B6 mice. Thirty-six week-old B6 mice were intraperitoneally administered 125 (▼), 313 (▲), 625 (■) or 1250 (●) mg/kg/day of NMN or PBS (□) for 4 days. Then, liver and spleen MNCs were prepared and the cytotoxic activity was analyzed using NK cell-sensitive target cells, YAC-1, at the indicated effector/target ratios (A). The proportion of NK cells was examined by flow cytometry as described in the Materials and Methods (B). The percentage of CD3− NK1.1+ cells (NK cells), CD3+ NK1.1+ cells (NKT cells), and CD3+ NK1.1− cells (T cells) is indicated on each panel. Data are presented as mean ± SD of triplicate samples. *P < 0.05 compared with control. Similar results were obtained in three independent experiments.
NK cell activation by NMN

The proportion of liver MNCs compared with spleen MNCs. On the other hand, we reported that liver NK cells contain an immature NK cell population (Takeda et al. 2005), and it was also reported that the phenotype of NK cells varies among organs (Hayakawa and Smyth 2006). Thus, the magnitude of response to the same stimulants might be varied amongst NK cells depending on their resident organs. Further studies to explore functional differences of NK cells within resident organs will be needed.

We have previously reported that LcS or Agaricus blazei Murill stimulates macrophages or dendritic cells to produce IL-12 and may be the underlying mechanism of augmented NK cell activity following oral intake of LcS or A. blazei (Takeda et al. 2006; Yuminamochi et al. 2007). IL-12 is one of the typical cytokines that activates cytotoxicity and IFN-γ production, but does not substantially induce proliferation of NK cells (Brunda 1994; Trinchieri 1995). Thus, IL-12 might also contribute to the activation of NK cell cytotoxicity by NMN. Further experiments will be required to explore the mechanisms underlying the augmentation of NK cell cytotoxicity by NMN.

DISCUSSION

We have herein demonstrated that intraperitoneal or oral administration of NMN, a key NAD⁺ intermediate in mammals, augments the cytotoxicity of NK cells in a manner dependent on IFN-γ in mice. Of note, the proportion of NK cells did not significantly increase following the administration of NMN. Thus, augmentation of NK cell activity by NMN appears to be mainly due to the augmentation of cytotoxic activity in individual cells.

Interestingly, augmentation of NK cell cytotoxicity was observed in liver MNCs, but not spleen MNCs, in some mice administered NMN. Notably, the proportions of the NK cell populations in liver or spleen were not affected even in mice whose NK cell cytotoxicity was activated by NMN treatment. It is well known that NK cells are more abundant in liver MNCs compared with spleen MNCs of mice as presented in our results. Thus, the selective increase in NK cell cytotoxicity in liver MNCs might be due to the fact that NK cells make up a larger proportion of liver MNCs compared with spleen MNCs. On the other hand, we reported that liver NK cells contain an immature NK cell population (Takeda et al. 2005), and it was also reported that the phenotype of NK cells varies among organs (Hayakawa and Smyth 2006). Thus, the magnitude of response to the same stimulants might be varied amongst NK cells depending on their resident organs. Further studies to explore functional differences of NK cells within resident organs will be needed.

We have previously reported that LeS or Agaricus blazei Murill stimulates macrophages or dendritic cells to produce IL-12 and may be the underlying mechanism of augmented NK cell activity following oral intake of LeS or A. blazei (Takeda et al. 2006; Yuminamochi et al. 2007). IL-12 is one of the typical cytokines that activates cytotoxicity and IFN-γ production, but does not substantially induce proliferation of NK cells (Brunda 1994; Trinchieri 1995). Thus, IL-12 might also contribute to the activation of NK cell cytotoxicity by NMN. Further experiments will be required to explore the mechanisms underlying the augmentation of NK cell cytotoxicity by NMN.

Notably, oral administration of NMN at 625 mg/kg augmented NK cell cytotoxicity similarly to the intraperitoneal administration of same amount of NMN. It was reported that NMN is rapidly absorbed through a transporter in the small intestine and orally administered NMN is quickly utilized to synthesize NAD⁺ in tissues (Grozio et al. 2019). Thus,
Thus, modulation of gut microbiota by oral administration of NMN might contribute to NK cell activation; however it should be noted that only 4 days of oral treatment with NMN was sufficient to induce NK cell activation. Nonetheless, further experiments to examine gut microbiota after NMN ingestion might provide additional insight into the effector mechanisms of NMN.

NMN is one of the most attractive anti-aging dietary supplements. Moreover, the use of dietary supplements among older people has increased rapidly due to the desire to reduce the risk of development of chronic diseases (Walrand 2018). Attenuation of immune activity by aging is expected to be involved in the pathogenesis of such age-associated diseases (Nikolich-Zugich 2018). Thus, maintenance of immune function, including NK cell activity, might be preventative for some diseases and improve the health condition of older people. Further studies will be required to examine whether long-term treatment with NMN can restore NK cell activity lost with aging, and would underscore the role of NK cells in wellness.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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