

Natural killer cells of Parkinson's disease patients are set up for activation: A possible role for innate immunity in the pathogenesis of this disease

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Received 11 April 2007; received in revised form 25 May 2007; accepted 26 May 2007

Abstract

Neuroinflammation in Parkinson's disease (PD) involves activation of microglia, participation of several inflammatory cytokines, prostaglandins, complement and systemic activation of natural killer (NK) cells, suggesting that innate immunity has a role in the pathogenesis of this disease. In this study, we examined NK activity and the expression of its regulatory molecules in peripheral lymphocytes of PD patients and compared the results with those of healthy controls. Expression of the inhibitory NKG2A receptors was significantly lower in PD, causing PD patients to be susceptible in a condition for NK activation after NK cells bind to target cells via these receptors.

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Keywords: Parkinson's disease; Innate immunity; Natural killer (NK); NKG2A; NKG2D

1. Introduction

The etiology of Parkinson's disease (PD) is considered to involve both genetic and environmental components. Since 1977, monogenetic factors have been found in several familial PD cases that directly support genetic etiology for PD. Mutations in α -synuclein (PARK1) [1–3], parkin (PARK2) [4], UCHL1 (PARK5) [5], PINK1 (PARK6) [6], DJ-1 (PARK7) [7], and LRRK2 (PARK8) [8,9] cause PD with a definite Mendelian inheritance. In these genetic studies, the etiological factors of PD that have received attention are mitochondrial dysfunction, oxidative stress, and protein misfolding.

However, distinct genetic factors are responsible for only a small proportion of all PD cases, and about 90% are

sporadic or have an unknown genetic background [10]. In the sporadic cases, non-genetic or environmental factors are thought to be associated with precise pathogenic mechanisms underlying the selective dopaminergic cell loss seen in PD.

The role of inflammation in the pathogenesis of PD has been judged to be significant, as determined from human and experimental evidence. The findings include elevated levels of antibodies to proteins modified by dopamine oxidation products in PD patients [11], increased levels of cytokines in the CSF and striatum of PD brains [12,13], an increased number of activated microglia in PD brains [14], sustained microglial activity in humans exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) years after drug exposure [15], a decreased risk of developing PD in regular NSAID users [16], and so on. However, whether these immune-mediated inflammatory responses are the cause or rather a consequence of neurodegeneration remains unclear.

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Some epidemiological evidence has suggested a lower incidence of cancer in PD patients [17–21] leads us to think that augmented natural killer (NK) activity act as a first line of defense against tumor cell infiltration.

Several of the findings mentioned above suggest that triggering factors acting on PD-susceptible individuals to PD will utilize several defense mechanisms including microglial activation [14], releasing complement factors [22] and inflammatory cytokines [12,13], upregulated NO [23] and prostaglandin production in neural tissue [24], as well as systemic NK activation [25]. Therefore, we predicted that immune responses in PD patients predominantly shifted to innate immunity. However, little is known about the association between PD and NK, we focused on NK cells that are active members of the innate immunity response system and are activated and proliferated via Th1 cytokines [26,27].

NK activity is modulated by signal transduction through its surface receptors, NKG2A and NKG2D [28,29]. NKG2A receptors recognize MHC class I molecules of HLA-E on target cells, and conduct inhibitory signals [30,31], whereas NKG2D receptors recognize MHC class I analogues of MIC, and conduct excitatory signals [32,33]. In this study, we measured the NK fraction in whole lymphocyte populations, Th1/Th2 cytokines, NK activity and its modulating surface molecules in PD patients and non-PD individuals, in order to clarify the immune response pattern characteristic of PD patients.

2. Materials and methods

2.1. Patients and controls

This study was approved by the Ethics Committee of Fukuoka University School of Medicine and conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. PD patients seen consecutively in 2005 at Fukuoka University Hospital and who satisfied the diagnostic criteria of Litvan et al. [34] were recruited (20 patients, 9 men). Patients with secondary parkinsonism were excluded based on results of clinical examination and by neuroimaging studies. None of the 20 patients had a history of an infectious or immune disorder, at least with the past 3 years, or had experienced any other neurodegenerative disorder. All cases were sporadic. All patients were treated with carbidopa/levodopa in combination with dopamine agonist, monoamine oxidase B inhibitor, and other forms of anti-parkinsonian therapy. None received immunosuppressive therapy. The mean \pm S.D. age was 70.7 ± 7.8 years (range 57–86 years), and the mean \pm S.D. disease duration was 5.4 ± 3.4 years (range 0.5–12 years). The PD stage was evaluated using the Hoehn and Yahr (H&Y) scale during 'on' periods [35]. One PD patient was in stage I, 7 were in stage II, 7 were in stage III, and 5 were in stage IV. The mean H&Y score was 2.9 ± 0.8 .

A group of non-PD individuals (non-PD) was recruited over time and consisted of medical staff of Fukuoka University and spouses of PD patients without any neurologic or immunologic disorder on clinical and neurologic examinations. The age of the control group (20 subjects, 11 men) was matched to the age of the PD group to within 5 years; and the mean \pm S.D. age of the controls was 67.6 ± 9.4 years (range 51–85 years).

2.2. Blood sampling

All blood samples were collected between 9:00 and 11:00 a.m. to avoid any influence from circadian variation on lymphocyte subsets or from

cytokine fluctuations, and all were subjected to further treatment within 2 h. Heparinized peripheral venous blood samples were diluted with an equal volume of a wash buffer (1 \times phosphate buffered saline, 3% fetal bovine serum, 2 mM EDTA-2Na) and were gently overlaid onto a half volume of LSM Lymphocyte Separation Medium (ICN Biomedicals Inc., Aurora, OH). The solutions were then centrifuged at 24 $^{\circ}$ C for 30 min at 500g. Fractions of peripheral blood mononuclear cells (PBMCs) were collected, resuspended, washed four times in a wash buffer. Collected cells were used for the lactate dehydrogenase (LDH) cytotoxic assay and flow cytometry. Blood samples collected in EDTA-2K-containing tubes were used for white blood cell counts and the hemograms were examined at the Fukuoka University Hospital Laboratory. Sera were also collected for determination of total protein and its fractions including complement components, IgE and IgG1-3. Sera were also used for Th1/Th2 cytokine measurements using enzyme-linked immunosorbent assay (ELISA).

2.3. Th1/Th2 cytokines measured by ELISA

Aliquots of sera were used to measure Th1/Th2 cytokines with the Human Th1/Th2 ELISA Ready-SET-Go! System (eBiosciences, San Diego, CA) according to the manufacturer's protocol. The system includes monoclonal antibodies against human interferon (IFN)- γ , interleukin (IL)-2, IL-4, and IL-10 with corresponding recombinant human standard proteins.

The assays were performed in duplicate for each blood sample.

2.4. NK activity measured by the LDH cytotoxic assay

K562 cells (American Type Culture Collection, CCL 243) at $3.3 \times 10^5 \text{ mL}^{-1}$ in RPMI 1640 without phenol red (Invitrogen, Carlsbad, CA) were challenged with the collected PBMCs at $2.0 \times 10^6 \text{ mL}^{-1}$. Then, the LDH cytotoxic assay was performed with an LDH cytotoxic test (Wako, Osaka, Japan) according to the manufacturer's protocol. Control experiments were also performed using either K562 cells or PBMCs, and K562 cells with 2% Triton X-100 were used as a positive control. The experiments were performed in triplicate for each blood sample.

2.5. NK receptors detected by flow cytometry

Blood samples contained 2×10^5 PBMCs in 50 μ L of a wash buffer with 0.1% sodium azide (NaN_3). Each sample was stained with 5–10 μ L of selected monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) (Beckman Coulter, Fullerton, CA) or phycoerythrin (PE) (BD Biosciences, Franklin Lakes, NJ). The staining combinations included anti-human CD3-PC5 (Beckman Coulter)/anti-human CD56-FITC (eBiosciences)/anti-human NKG2A-PE (R&D systems, Minneapolis, MN), anti-human CD3-PC5/anti-human CD56-FITC/anti-human NKG2D-PE (R&D systems). Mouse IgG1-FITC, mouse IgG1-PE (BD Biosciences), and PC5 (Beckman Coulter) were used for control staining.

After a 30 min incubation on ice in the dark, each sample was washed three times with wash buffer containing NaN_3 , and analyzed using an Excel flow cytometer (Beckman Coulter). Usually, 10,000 cells were scanned for each sample, and the findings were expressed as the percentage of cells yielding a specific fluorescence in a gated lymphocyte region. The assays were performed in triplicate for each blood sample.

2.6. Statistical analysis

Data from PD and non-PD subjects obtained from each experiment were analyzed and compared using the statistical software program SAS 9.1.3 (SAS Institute Inc., Cary, NC) under a site-license agreement with Fukuoka University, or with Stat View-J 5.0.1 (Hulinks, Tokyo, Japan). A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. General laboratory data

No statistically significant difference between PD and non-PD groups was found in total white blood cell counts and their fractions, in total proteins and their respective fractions including complement proteins, or in total IgG and its subfractions IgG1, IgG2, IgG3, and IgE ($P > 0.05$ by the Mann–Whitney U -test, data not shown).

3.2. Th1/Th2 cytokine patterns

Blood sampling between 9:00 and 11:00 a.m. yielded relatively stable values of human cytokines even though there has been shown to be dynamic diurnal rhythmicity in the cytokine secretion pattern [36]. Serum levels of IFN- γ and IL-2 as Th1 cytokines, and IL-4 and IL-10 as Th2 cytokines were statistically equivalent in the PD and non-PD groups (no significant differences by the Mann–Whitney U -test, data not shown).

3.3. NK activity

In determining NK activity with the LDH cytotoxic assay using K562 cells, the cytotoxic effect on K562 cells

induced by 2% Triton-X was taken as 100%, and relative percentages were calculated (Fig. 1). We found equivalent activities in the PD and non-PD groups (no significant difference by the Mann–Whitney U -test, Fig. 1A).

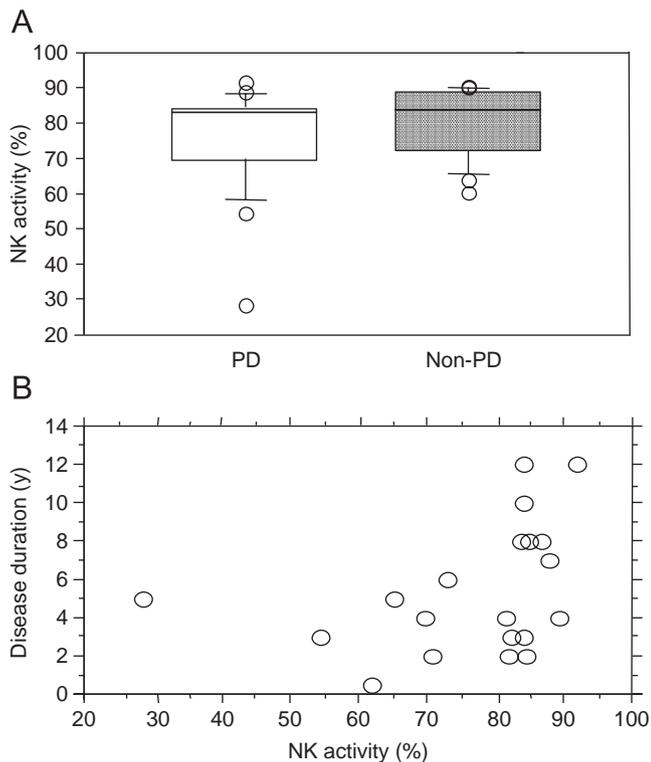


Fig. 1. (A) NK activity measured by its cytotoxic effect on K562 cells detected by LDH release of lysed cells. Values are shown as a % relative to the control cytotoxicity with K562 cells with 2% Triton-X as 100%. PD, Parkinson's disease patients; non-PD, non-Parkinson's disease individuals. No significant difference by the Mann–Whitney U -test. (B) Correlation between NK activity and disease duration in PD patients, with a statistical significance of $P < 0.05$ by Spearman's rank test.

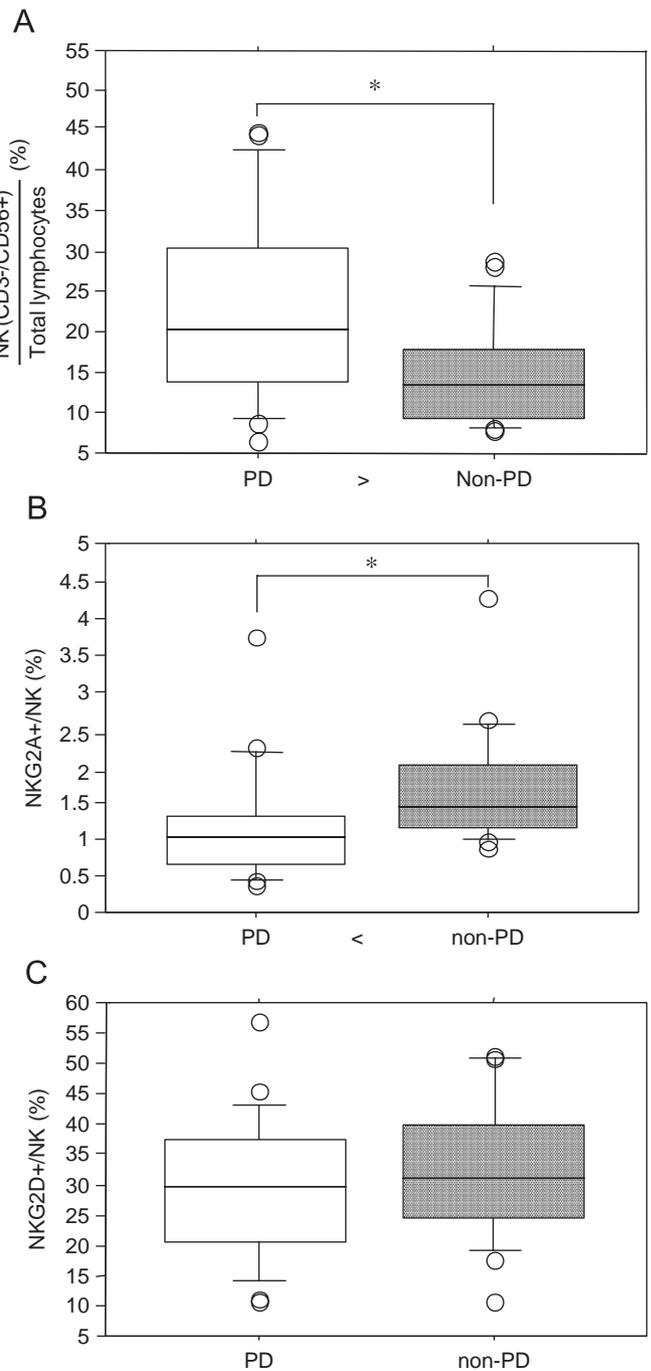


Fig. 2. Flow cytometry of peripheral blood lymphocytes from PD patients and non-PD individuals. (A) Ratios of CD3–CD56+ NK cells relative to total lymphocytes are shown as a %. PD, Parkinson's disease control; non-PD, non-PD individuals. $*P < 0.05$ by the Mann–Whitney U -test. (B) Ratios of NKG2A+ cells relative to CD3–CD56+ NK cells are shown as a %. PD, Parkinson's disease patients; non-PD, non-Parkinson's disease individuals. $*P < 0.05$ by the Mann–Whitney U -test. (C) Ratios of NKG2D+ cells relative to CD3–CD56+ NK cells are shown as a %. PD, Parkinson's disease patients; non-PD, non-Parkinson's disease individuals. No significant difference by the Mann–Whitney U -test.

When the NK activity of each PD patient was plotted in relation to disease duration, the correlation was positive, suggesting that NK activity increases as the disease advances ($P < 0.05$ by Spearman's rank test, Fig. 1B).

3.4. NK cells and their surface markers

Flow cytometry revealed the NK cells to be CD3–CD56+. The percentage of NK cells among the total lymphocytes of the PD group was higher than that of non-PD group, statistically significant ($P < 0.05$ by the Mann–Whitney *U*-test, Fig. 2A). When we compared inhibitory receptor expression on NK cells, the percentage of NKG2A+ cells among CD3–CD56+ NK cells in the PD group was statistically lower than that of the non-PD group ($P < 0.05$ by the Mann–Whitney *U*-test, Fig. 2B). In contrast, there was no significant difference between both groups in the percentage of NKG2D+ cells among CD3–CD56+ NK cells ($P > 0.05$ by the Mann–Whitney *U*-test, Fig. 2C).

4. Discussion

As measured by its cytotoxic effect on K562 cells, NK activity was not significantly different in the PD and non-PD groups. K562 cells lack HLA-E ligands, and inhibitory signal transduction through HLA-E/NKG2A could not be estimated with this assay system [37]. As for the NKG2A/

NKG2D regulatory system for target-cell-mediated NK activation, the LDH cytotoxic assay with K562 cells can be a useful method for observing independent contribution of the excitatory MIC/NKG2D pathway in the absence of the inhibitory HLA-E/NKG2A pathway (Fig. 3B). When the assay was performed, both groups showed a similar level of NK activation. Results of flow cytometry showed that inhibitory NKG2A expression was significantly low in the PD group. Taken together with these findings, we concluded that target-cell-dependent NK activation could be augmented in PD patients compared to non-PD individuals under intact conditions of the HLA-E/NKG2A and MIC/NKG2D pathways (Fig. 3A). This difference between the two groups is considered to result from set-up conditions dependent on target-cell-binding to NK cells. In fact, looking upstream to assess levels of IFN- γ , IL-2, IL-4, and IL-10, we could not observe a Th-1 predominant cytokine pattern in PD patients, nor any other laboratory data suggest any deviation toward Th-1 predominance (data not shown). This subtle but consistent deviation may have significance in the long-term pathological process in the brain; a hypothesis that could be confirmed if a larger number of samples from different PD case settings were available to give reproducible results in future studies.

NK cells of the innate immune system not only act as a first-line defense or as non-specific effectors, but also act as a go-between the innate and adaptive immune systems [38,39]. Therefore, it is difficult to accept that each system could accomplish its function independently. When we

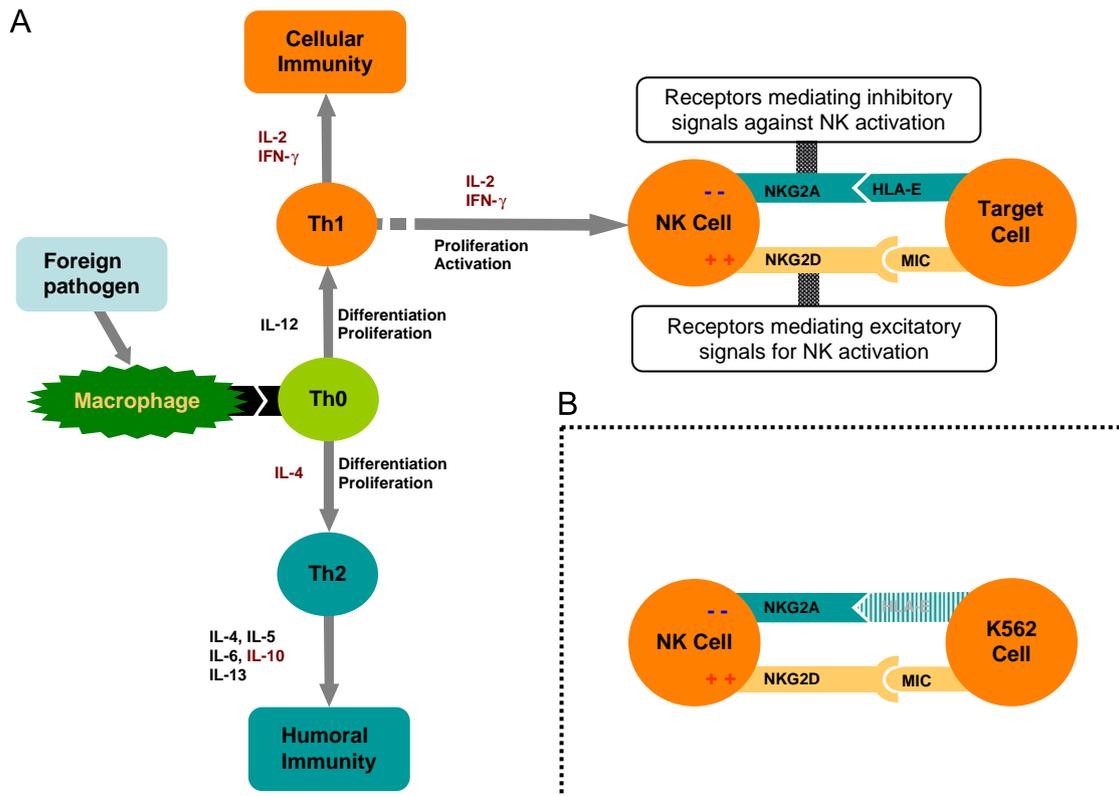


Fig. 3. (A) Schematic drawing of innate and adaptive immunity in relation to NK activation through the HLA-E/NKG2A and MIC/NKG2D pathways. (B) K562 cells lack HLA-E on NK-activating modulation.

address predominant innate immunity, or the Th-1 prone immune response, we may be referring to the compensatory predominance of innate immunity due to a lack of cross talk between innate and adaptive immune systems. NK activation could be the result of these phenomena.

In experimental parkinsonism induced by MPTP, the severity and duration of symptoms after treatment depends on the genetic background of the animal strain [40,41]. C57BL/6 mice are prone to exhibit a Th-1 immune response, while BALB/c mice are Th-2-prone responders [42–44]. MPTP-induced selective nigral neurotoxicity is best demonstrated in the Th-1-prone responder type of C57BL/6 mice, in which Th-1-predominant immune responses are considered to be essential.

As PD lacks any overt clinical phenotypes indicative of Th-1 or Th-2 disorders, a very subtle deviation in the immune response might account for its pathogenesis [45]. PD is thought to be a multi-factorial disease triggered by genetic and environmental causes. Any environmental factor that augments NK activation in PD would serve as a clue for further probing of the pathogenic mechanism of sporadic PD cases.

Acknowledgments

We are grateful to all patients and their families, and to the healthy volunteers who gave their informed consent to enroll in this study. We also thank Ms Akiko Mori for her technical assistance and Dr. William Campbell for English editing of the manuscript.

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