Variable Increased Expression of Program Death-1 and Program Death-1 Ligands on Peripheral Mononuclear Cells Is Not Impaired in Patients with Systemic Lupus Erythematosus

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Programmed death-1 (PD-1) was shown to deliver an inhibitory signal after binding to its ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC). Recently, up-regulated expression of PD-1 molecule and/or its ligands was demonstrated in human diseases including rheumatoid arthritis and inflammatory colitis. The study aimed to investigate the expression and function of PD-1 and PD-1 ligands on circulating T cells, B cells and monocytes from patient with systemic lupus erythematosus (SLE). The results showed that patients with SLE had significantly increased percentages of PD-1-expressing CD3⁺T cells and CD19⁺B cells, PD-L1-expressing CD19⁺B cells and PD-L2-expressing CD14⁺B monocytes. In selected SLE patients and normal subjects, functional study of PD-1/PD-L pathway on the production of cytokines by stimulated PBMC was examined. Blockages of PD-1 or PD-1 ligands substantially increased the production of IL-2, IFN-γ and IL-10, the amplitude of increase roughly ranged from one to three times. There were no significant differences of the enhancing effects on cytokine production by blockage of PD-1/PD-L pathway between SLE patients and normal subjects. The study indicates that there are no intrinsically defective expression and function of PD-1 and PD-1 ligands on PBMC in patients with SLE.

1. Introduction

Programmed death-1 (PD-1) is a novel member of CD28 family. PD-1 was shown to deliver a negative signal after binding to its ligands, PD-L1 (B7-H1) or PD-L2 (B7-DC). Recently, up-regulated expression of PD-1 molecule and/or its ligands was demonstrated in human diseases including rheumatoid arthritis and inflammatory colitis. The study aimed to investigate the expression and function of PD-1 and PD-1 ligands on circulating T cells, B cells and monocytes from patient with systemic lupus erythematosus (SLE). The results showed that patients with SLE had significantly increased percentages of PD-1-expressing CD3⁺T cells and CD19⁺B cells, PD-L1-expressing CD19⁺B cells and PD-L2-expressing CD14⁺B monocytes. In selected SLE patients and normal subjects, functional study of PD-1/PD-L pathway on the production of cytokines by stimulated PBMC was examined. Blockages of PD-1 or PD-1 ligands substantially increased the production of IL-2, IFN-γ and IL-10, the amplitude of increase roughly ranged from one to three times. There were no significant differences of the enhancing effects on cytokine production by blockage of PD-1/PD-L pathway between SLE patients and normal subjects. The study indicates that there are no intrinsically defective expression and function of PD-1 and PD-1 ligands on PBMC in patients with SLE.

2. Patients and Methods

2.1. Patients. Twenty-eight patients who fulfilled at least 4 criteria for the diagnosis of SLE set by the American College of Rheumatology [12] were enrolled in the study. They include 26 females and 2 males. All enrolled patients were not taking corticosteroid and immunosuppressant agents for
more than 4 weeks prior to the study. 26 young healthy females were used as a control group. Their peripheral blood was drawn, and PBMCs were immediately isolated for flow cytometric analysis and in vitro culture.

2.2. Measurement of PD-1 and PD-1 Ligands Expression by Flowcytometry. Flowcytometry with dual staining was performed using fluorescece-conjugated MABs including FITC-conjugated anti-CD3, anti-CD19, and anti-CD14 (BD Biosciences, Sna Diego CA, USA) and PE-conjugated anti-PD-1, anti-PD-L1, and anti-PD-L2 (MIH clones, e Bioscience, Sna Diego, CA, USA). Briefly, PBMCs were prepared from fresh heparinized blood using Ficoll-Hypaque density gradient separation and adjusted to 5 x 10^6 cells/mL. Two hundred microliters of cell suspension were incubated simultaneously with 20 μL FITC-conjugated MAB and 20 μL PE-conjugated MABs in different combinations on ice in the dark for 30 minutes. Cells incubated with FITC- and PE-conjugated mouse IgG were used as isotype controls. The stained cells were analyzed with a FACSCalibur (Becton Dickinson) flow cytometer and associated software programs (CELLQuest).

2.3. Cell Culture and Stimulation. In selected SLE patients and normal individuals, their PBMCs (300 μL) at the concentration of 1 x 10^6 cells/mL were cultured in 96-well microtiter plates under the stimulation with PMA (5 ng/mL) plus Ionomycin (1 μg/mL). For blocking PD-1/PD-1 ligands interaction, we added MAB against PD-1 (MIH4 clone) or PD-L1 (MIH1 clone) or PD-L2 (MIH18 clone) in the cultured cell system. These were functional grade purified antihuman MAbs. Their endotoxin levels were all less than 0.001 ng/μg antibody as determined by the LAL assay. The different groups include addition of anti-PD-1 MAB (1 μg/well), anti-PD-L1 MAB (1 μg/well), anti-PD-L2 MAB (0.5 μg/well), or anti-PD-L1 plus anti-PD-L2. After 48 hours’ culture, the supernatants were collected, and cytokines including IFN-γ, IL-2, IL-4, and IL-10 were assayed by ELISA kits.

2.4. Assay of Cytokines by ELISA. The concentrations of different cytokines in cultured supernatants were measured by commercially available ELISA kits (Bender MedSystems, Vienna, Austria). The assay employed the quantitative sandwich enzyme immunoassay technique and was performed as manufacturer’s instruction. The principle of the test was briefly described below. Standard or samples were added to react with specific anticytokine MAB precoated onto a microplate for 2 hours at room temperature. After washing, horseradish peroxidase-conjugated polyclonal antibodies against cytokines were added to react with the bound cytokines for 1 hour. Then, substrate solution was added and incubated for 20 minutes, followed by addition of stop solution. The optical density of each well was immediately determined by microplate reader set to wavelength 450 nm. The concentrations of cytokines in each sample were calculated from the standard curves.

2.5. Statistics. Fisher’s LSD method was applied to compare the levels of PD-1 and PD-1 ligands-expressing cells between SLE and normal controls. Wilcoxon rank sum test was used to compare the increase percentages of cytokine production between patients with SLE and normal subjects.

3. Results

3.1. Expression of PD-1/PD-1 Ligands on PBMC. The expression of PD-1 and its two ligands on freshly prepared PBMC from 26 normal controls and 28 patients with SLE were shown in Table 1. One representative FACS Dot-plot data of PD-1 and PD-1 ligands on T cells, B cells, and monocytes was depicted in Figure 1. In normal controls, PD-1 was barely detectable on T cells (<1%). Only a very minority of B cells (mean 2.14%) and monocytes (mean 5.78%) expressed a low level of PD-1. In contrast, PD-L1 expression was readily detected on T cells, B cells, and monocytes. Almost all monocytes (mean 96.66%) expressed PD-L1. Different to PD-L1, PD-L2 expression was mainly noted on monocytes (mean 72.12%) with only a minority of T cells and B cells (mean <4%) expressed PD-L2. Similar expression pattern of PD-1 and PD-1 ligands on each mononuclear cell subset was also observed in patients with SLE.

Compared with normal controls, patients with SLE had significantly increased percentages of PD-1-expressing CD3^+^T cells (1.51 ± 1.12% versus 0.64 ± 0.53%, P < .001) and PD-1-expressing CD19^+^B cells (5.11 ± 3.91% versus 2.14 ± 1.67%, P < .005). Regarding PD-1 ligands, SLE patients had more PD-L1-expressing CD19^+^B cells (20.59 ± 10.24% versus 13.21 ± 1.67%, P < .005) and PD-L2-expressing CD14^+^monocytes (84.78 ± 12.82% versus 72.12 ± 26.92%, P < .005) than normal controls. Despite slightly increased frequencies of PD-1 and PD-1 ligands expression in some cell populations from SLE patients, the mean fluorescence intensities of PD-1 and PD-1 ligands expression on the positive cells were not significantly different between patients with SLE and normal controls (data not shown). The result suggests that the expression of PD-1/PD-1 ligands was not impaired in human SLE.

3.2. Effects of Blocking PD-1/PD-1 Ligands Pathway on Cytokines Production by Stimulated PBMC In Vitro. Cytokines are considered to play an important role in the pathogenesis of SLE. We thus designed to investigate the functional effects of blocking PD-1 or PD-1 ligands on the production of various cytokines produced by in vitro cultured PBMC. The cytokines examined in the study included Th1-derived cytokines (IL-2 and IFN-γ) and Th2-derived cytokines (IL-4, IL-10). In selected patients and normal individuals, 8 in each, their freshly prepared PBMCs were cultured and stimulated with PMA plus ionomycin in the absence or presence of anti-PD-1 MAb, anti-PD-L1 MAb, anti-PD-L2 MAb, or anti-PD-L1 plus anti-PD-L2 MAbs. We found that blockage of either PD-1 or PD-1 ligands substantially increased the production of IL-2, IFN-γ, and IL-10 in all cases of PBMC from both SLE and normal individuals but has no any effect on IL-4. As expected, the
concentration of cytokines produced by stimulated PBMC in vitro varied widely among different SLE patients and normal controls. For further comparison between SLE and normal subjects, the amplitudes of enhancement in cytokine production were expressed as percentages of increase, which was calculated by the increased amount of cytokine in the presence of blocking Ab divided by baseline cytokine concentration in isotype IgG control. Among the experiments, a few samples of stimulated PBMC had no detectable baseline cytokine. They were omitted in comparison due to difficulty in calculation of percentages of increase. As shown in Table 2, blockage of PD-1/PD-1 ligands pathway greatly increased production of IFN-γ, which ranged averagely from 134% to 160% in normal controls and from 153% to 203% in SLE. It seemed that the enhancement of IFN-γ production was stronger in SLE. However, the differences did not reach statistical significance. Similar to IFN-γ, IL-2 levels were much increased, in which the amplitudes of increased percentages ranged averagely from 156% to 243% in normal control and from 189% to 397% in patient with SLE (Table 3). The enhancing effect on IL-10 was readily seen too, which ranged averagely from 97% to 126% in normal controls and from 73% to 132% in SLE (Table 4). There were also no significant differences in the increased production of IL-2 and IL-10 between SLE patients and normal subjects. In addition, it appeared that anti-PD-1 possessed similar effects as anti-PD-L1 or anti-PD-L2. Meanwhile, there were no synergic effects by anti-PD-L1.
plus anti-PD-L2 compared with either alone. There was no any effect on IL-4 effect by blockage of PD-1/PD-1 ligands pathway (data not shown).

4. Discussion

The expression of PD-1 and its two ligands on PBMC of normal individuals and patients with SLE in the present study was in concordance with previous observation [13–15]. PD-1 molecule was scarcely expressed on resting T cells. On the contrary, PD-L1 is constitutively expressed on T cells, B cells macrophages, and dentritic cells (DCs) and further upregulated upon activation. In contrast, the expression of PD-L2 was regulated more tightly and observed at sites of immune privilege including placenta and eye [16–18]. The expression of PD-L1 on nonlymphoid tissue was shown to play a crucial role in the control and maintenance of peripheral T cell tolerance [19].

Compared with normal controls, generally patients with SLE had increased expression of PD-1 and PD-1 ligands on T cells, B cells, and monocytes, some of which reached statistically significant differences, because PD-1 could be induced on T cells after activation, and increased activated T cells have already been well documented in patients with SLE. Thus the increased numbers of PD-1-expressing CD3+ T cells in SLE patients could be due to increased numbers of activated T cells in the periphery of SLE patients. Increased expression of PD-1 and/or PD-1 ligands had also been reported in other human autoimmune diseases. Hatachi et al. demonstrated that PD-1+ T cells were enriched in RA synovial fluid, and phenotypic analysis suggested that these cells a unique anergic cell subset [20]. Kobayashi et al. showed that the expression of PD-1 in salivary lymphocytes and PD-L1 on ductal and acinar epithelial cells in salivary glands was enhanced in patients with Sjogren's syndrome [21]. However, decreased PD-1 expression on peripheral CD4+ T cells was found in patients with type-1 diabetes in one study [22]. Probably, PD-1/PD-1 ligands pathway plays different roles in systemic versus organ-specific autoimmune diseases. In addition, one study in Lichen planus, a T cell-mediated chronic inflammatory mucocutaneous disease, revealed abundant expression of PD-1 and PD-L1 in infiltrating T cells and macrophages in the subepithelium and substantial PD-L1 on keratinocytes [23].

The inhibitory function of PD-1 molecule was initially suggested by the studies on PD-1 deficient mice, which exhibited hyperactivation of the immune system and subsequently developed autoimmune diseases. Interestingly, PD-1 deficient mice developed different kinds of autoimmune diseases depending on the genetic background [5–7]. On C57BL/6 background, PD-1 deficient mice developed lupus-like glomerulonephritis and arthritis with deposition of IgG, and C3 in the glomeruli, whereas PD-1 deficient mice suffered a fatal dilated cardiomyopathy on BALB/c background.

The result of our functional study was in concordance with the concept of the immunosuppressive role of PD-1/PD-1 ligands. We showed that blockage of PD-1/PD-1 ligands pathway augmented the production of cytokines in stimulated PBMC in vitro, with predominant effects on Th-1 derived cytokines (IL-2 and IFN-γ). Regarding Th-2 cytokines, we also observed enhancing effect on IL-10 though weaker compared with those on IL-2 and IFN-γ. However, there was no effect on IL-4 production. Blockage PD-1 molecule or either of the two PD-1 ligands demonstrated similar effects on the production of various cytokines. As previously reported, blockage with both anti-PD-L1 and anti-PD-L2 did not show a significantly synergic effect in our study. The functional study also revealed similar results between SLE patients and normal controls. Conflicting...
results had been reported in some studies. Ansari et al. reported that blockade of PD-1 or PD-L1 but not PD-L2 exaggerated diabetes in prediabetic NOD mice [24]. They observed the expression of PD-L1 but not PD-L2 on the β cells, which might explain the reason why the anti-PD-L1 but not anti-PD-L2 antibody exaggerated diabetes, whereas, Salama et al. observed the acceleration of EAE by the blockade of PD-1 and PD-L2 but not PD-L1 [25]. Since PD-L1 but not PD-L2 was highly expressed in the central nervous system, the expression of ligands in target organs might not determine the efficient of each blocking antibody. Blockade of PD-1/PD-L pathway might have an opposite effect on autoimmune diseases in certain condition. Kanai et al. examined the effect of blocking antibodies against PD-L1 and PD-L2 on the colitis model [26]. Antibodies against PD-L1 but not PD-L2 suppressed the development of colitis, indicating the costimulatory function of PD-L1 in the inflammatory response in the gut. According to the report by Loke and Allison [27], the expression of PD-L1 and PD-L2 was differentially regulated by Th1 and Th2 cytokines. Since the recent analyses using blocking antibodies against PD-L1 and PD-L2 revealed nonoverlapping functions of these ligands, the differential regulation of PD-L1 and PD-L2 expression might have some biological significance.

In conclusion, the present study found that there were no intrinsically defective expression of PD-1 and PD-1 ligands on PBMC in patients with SLE. On the contrary, we noted increased expression of PD-1 and/or its ligands in some subsets of PBMC from patients with SLE. Since PD-1 and PD-1 ligands play a role in regulation of both activation and tolerance of lymphocytes, the findings of our study most likely reflect the activation status of PBMC in lupus patients and they do not rule out a defect in tolerance mediated by PD-1. The exact function of increased PD-1 and PD-1 ligands in some subsets of PBMC in the pathogenesis of human SLE needs further study.

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