Therapy with belimumab may suppress the response of peripheral blood mononuclear cells to apoptotic cells.

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Abstract

Belimumab, on top of background Systemic Lupus Erythematosus (SLE) standard therapy, is effective in reducing SLE disease activity and preventing lupus flares, which was confirmed in "BLISS trials". The mechanisms involved in preventing lupus flare by belimumab remain largely unknown. We aimed to explore the response of PBMCs from lupus patients under belimumab treatment to the apoptotic cells. PBMCs, obtained from five lupus patients with disease flare treated with belimumab, were co-incubated with the apoptotic cells induced from Jurkat cells treatment. After incubation, the cells were harvested for surface markers analysis by flow cytometry and the supernatants were tested for cytokines assayed by ELISA kits. Before belimumab treatment, Peripheral Blood Mononuclear Cells (PBMCs) from lupus patients showed markedly secretion of Interferon-α (IFNα) and Interleukin-6 (IL-6) in response to the apoptotic cells. After belimumab treatment, lupus PBMCs were characterized by significant reduction of IFNα secretion (P=0.0103) upon recognition of the apoptotic cells. Reduced activation was also observed in lupus monocytes and T lymphocyte. Belimumab treatment suppressed response of lupus PBMCs to the apoptotic cells, which provides a fresh clue to understand mechanism other than B-cell depletion in the prevention of lupus flare.

Introduction

Systemic Lupus Erythematosus (SLE) has a relapsing-remitting course with disease activity flares over time. Flare is common, occurring in 65-70% of patients with SLE within 1 y even when they are receiving standard SLE therapy [1]. The environmental triggers for lupus flare, for example ultraviolet light and infection, were suggested to mediate the apoptotic and necrotic cells production [2]. SLE is characterized by a myriad of immunological abnormalities involving defective clearance of apoptotic materials [3]. The accumulated cellular remnants, including nuclear autoantigens and nucleosomes, might consequently change tolerance and lead to flare.

B-lymphocyte activity plays a pivotal role in the development and course of SLE. B-Cell-Activation Factor (BAFF) is an essential factor for B cell maturation, survival, proliferation and immunoglobulin class switching. Belimumab is a fully humanized monoclonal antibody that specifically binds to the soluble BAFF and prevents BAFF interaction with its receptors.

Belimumab was approved by the US FDA for lupus therapy in 2011. Supported by the randomized controlled trials, the addition of belimumab to standard therapy was confirmed to be effective in reducing SLE disease activity and delaying the time to lupus flares [4]. The longer term efficacy of belimumab as maintenance treatment to prevent lupus flares has to be established with extended observational studies [5,6]. As for the mechanism, previous study showed belimumab therapy reduced the number of circulating naive B cells, activated B cells and plasma cells, but not the number of circulating memory B cells [7]. However, the mechanisms other than B-cell depletion remain an open question.

Given PBMC's availability from SLE patients and its extensive use in auto-immune disorders, we used PBMCs in the study. Here, we revealed suppressed responses of Peripheral Blood Mononuclear Cells (PBMCs) from lupus patients under belimumab treatment to the apoptotic cells. We also observed: (1) IFNα secretion was significantly decreased; (2) The deactivation of monocytes and T lymphocytes.

Patients and Methods

Patients

Five SLE patients enrolled in the present study visited department of rheumatology every 1 to 6 months in the West China hospital. Ethics approval for this study was granted by the Medical Ethics Review Board of West China Hospital, Sichuan University School. Informed consent was obtained from all study subjects. All studies were performed according to the Declaration of Helsinki.

All patients fulfil ≥ 4 of the 1997 American College of Rheumatology classification criteria for SLE. Flare was defined as an increase in SLE Disease Activity Index 2000 update (SLEDAI-2K) with score of ≥ 4 from the previous visit [8,9]. On top of background SLE therapy, they received belimumab at the dosage of 10 mg/kg to be given intravenously at 2 week intervals for the first three doses, followed by 4 week intervals.

Monoclonal antibodies

For immunostaining and analysis by Fluorescence-Activated Cell Sorting (FACS), we used fluorochrome-conjugated mouse Monoclonal Antibodies (mAb) against human CD14-PE, CD80-PE-Cy7, CD86-APC, CD3-PE, CD69-PE-Cy7, CD25-APC, and appropriate isotype controls (all from BD PharMingen, SanDiego, CA).

Preparation for apoptotic Jurkat cells

For induction of apoptosis, Jurkat cells (5×10^6 cells/mL) were treated with 100 nM staurosporine (Beyotime, CN) for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Apoptotic and necrotic cell death were confirmed by staining with propidium iodide/annexin V-FITC (Figure 1).
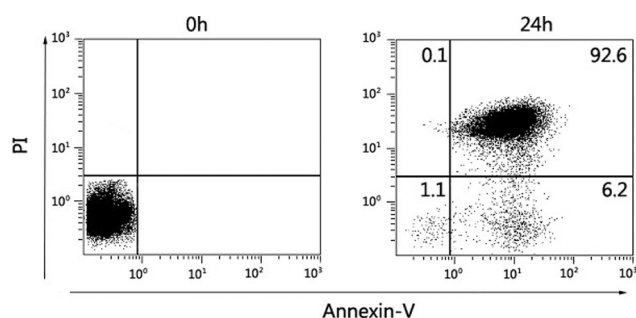

Figure 1. The apoptotic cells induced from Jurkat cells were confirmed by staining with propidium iodide and annexin-V.

Assay for PBMCs response to apoptotic Jurkat cells

Human PBMCs were freshly isolated by Ficoll density-gradient centrifugation. Labelled with 2 μM CFSE (Invitrogen), PBMCs (5×10^6 cells) were co-incubated with apoptotic Jurkat cells (5×10^6 cells) in 24-well tissue culture plates for 24 h at 37°C. After incubation, cells were harvested for flow cytometry analysis using FC500 (Beckman Coulter, USA). Data were analyzed using CXP software (Beckman).

The supernatants were tested for cytokines. Levels of TNFα, IFNα, IFNβ, IFNγ, IL-6, IL-17 and IL-21 were determined by commercially available ELISA kits (R and D Systems).

Statistical analysis

Comparisons between the two groups (T0 and T12w) were performed by paired student's t test. Statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA). P values less than 0.05 were considered significant.

Results

Baseline characteristics

Baseline demographics and disease characteristics of SLE patients were shown in (Table 1). Treatment with belimumab for 12 weeks reduced the disease activity.

Altered cytokines secretion from lupus PBMCs in response to apoptotic cells after belimumab treatment

To determine whether PBMCs from lupus patients under belimumab treatment have a distinct pattern of cytokines secretion in response to apoptotic cells co-incubation, we measured the production of several cytokines using an assay, in which lupus PBMCs were co-incubated with the apoptotic cells induced from Jurkat cells. Before belimumab treatment, prominent IFNα and IL-6 secretions (mean ± SD: 494.6 ± 166.5 pg/mL *vs.* 1465 ± 198.3 pg/mL) were detected in the supernatants when lupus PBMCs were exposed to the apoptotic cells. After belimumab treatment, lupus PBMCs significantly reduced IFNα secretion, but not IL-6 secretion, in response to the apoptotic cells co-incubation (Figure 2A). It is noted that PBMCs from these five lupus patients showed the unanimous declined tendency of IFNα secretion other than IL-6 secretion in the presence of the apoptotic cells (Figures 2B and 2C).
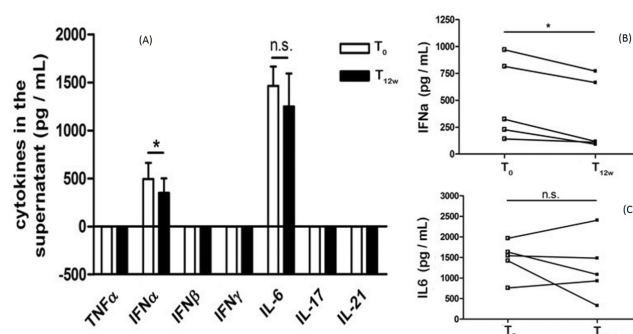

Figure 2. (A) Altered cytokines production of PBMCs from SLE patients under belimumab treatment in response to apoptotic cells; (B and C) The changes of IFNα and IL6 from the five patients were illustrated individually. Lupus PBMCs were co-incubated with the apoptotic cells induced from Jurkat cells. Cytokine levels in the supernatants were tested by enzyme-linked immunosorbent assay. Values are the mean ± SEM of 5 lupus patients. P values were determined by paired Student's t test. *P<0.05 and NS: No Significant.

Reduced activation of lupus monocytes and T lymphocytes in response to apoptotic cells co-incubation after belimumab treatment

Monocytes from SLE patient's blood were found to function as antigen-presenting cells. With an increased expression of costimulatory molecules CD80 and CD86, lupus monocytes are efficient stimulators of naive allogeneic CD4+ T cell proliferation [10]. We detected these two surface markers of lupus monocytes and compared their expression levels between before and after belimumab treatments. As shown in Figures 3A and 3C, the expression levels of CD80 and CD86 on the surface of lupus monocytes decreased after belimumab treatment. Furthermore, when exposed to the apoptotic cells, the monocytes from lupus patients under belimumab treatment still showed much lower expression levels of CD80 and CD86 compared to those from the patients prior to the belimumab treatment (Figure 3C).

As for the responses of T lymphocytes to the apoptotic cells co-incubation, CD69, the early activation marker of T lymphocytes, did not show the differentiated expression between before and after belimumab therapy (Figure 3B). However, CD25 expression, the late activation marker, remarkably decreased on T lymphocytes from lupus patients after they received belimumab therapy (Figure 3D).
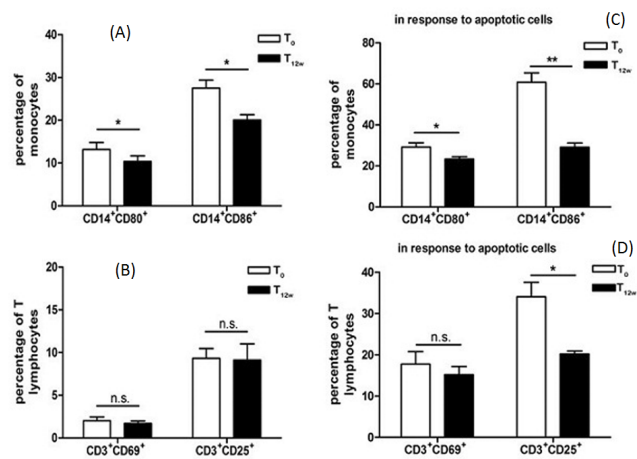

Figure 3. *The expression levels of activated surface markers on lupus monocytes and T lymphocytes before (T0) and after (T12w) belimumab treatment. (A and B) Lupus PBMCs were co-incubated without the apoptotic cells induced from Jurkat cells; (C and D) Lupus PBMCs were co-incubated with the apoptotic cells induced from Jurkat cells. The expression levels of surface markers on lupus monocytes and T lymphocytes were analyzed by flow cytometry. Values are the mean ± SEM of 5 lupus patients. P values were determined by paired Student's t test. **P<0.01, *P<0.05 and NS: No Significant.*

Table 1. *Baseline demographics and disease characteristics of SLE patients.*

Patients	Sex	Age (y)	Disease duration (y)	Flare episodes	SLEDAI-2K score		Background medication
					T_0	T_{12W}	
No. 1	M	26	3	Cutaneous vasculitis	12	4	Prednisone 10 mg qd, HCQ 200 mg bid, Aza 50 mg qd
No. 2	F	22	1.5	Rash, arthritis, cutaneous vasculitis	20	6	Prednisone 10 mg qd, HCQ 200 mg bid
No. 3	F	45	3	Proteinuria, thrombcytopenia	9	4	Prednisone 15 mg qd, HCQ 200 mg bid, CTX 50 mg qd
No. 4	F	25	0.5	Rash, arthritis, proteinuria	10	4	Prednisone 50 mg qd, HCQ 200 mg bid, MMF 500 mg bid
No. 5	F	19	3	Cutaneous vasculitis	12	4	Prednisone 7.5 mg qd, HCQ 200 mg bid, Aza 50 mg qd

F: Female; M: Male; SLEDAI: Systemic Lupus Erythematosus Activity Index; T_0: Before belimumab treatment; T_{12W}: Treatment with belimumab for 12 weeks; HCQ: Hydroxychloroquine; Aza: Azathioprine; CTX: Cyclophosphamide; MMF: Mycophenolate Mofetil.

Discussion

These five lupus patients with disease flare presented the improvement in disease activity and declined SLEDAI scores when they received belimumab therapy for 12 weeks. Interestingly, our data revealed that lupus PBMCs showed the suppressed response to the apoptotic cells co-incubation after belimumab treatment, especially IFNα secretion. This may provide a new perspective for understanding the mechanism of preventing lupus flare by belimumab.

Apoptosis plays a key role in pathophysiology of SLE [11]. When autoimmune B cells attack the body's own tissues, they are normally undergoing apoptosis. In SLE, B cells acquired the ability to survive and proliferate, which protect them from cell death. Particularly, B-cell Activating Factor (BAFF) is required for the development and survival of B cells and BAFF is overexpressed in SLE patients [12]. Type I interferon's (IFNs) plays an important role in the pathogenesis of SLE. There is cross-talk among type I IFNs, Toll-Like Receptors (TLR) and BAFF [13]. Several studies indicated that the

presence of IFNα could influence the expression of BAFF. For instance, induction of BAFF expression was described in myeloid cells, such as Dendritic Cells (DCs) and macrophages, after *in vitro* stimulation with IFNα [14,15]. A recent study revealed that IFNα-treated SLE monocytes exhibited higher intracellular levels of BAFF, which was mobilized to the membrane rapidly and subsequently released [16]. However, excess BAFF could increase auto-reactive B-cell survival and proliferation, followed by elevating auto-antibodies production and forming immune complexes (ICs). SLE-ICs, especially interferonic ICs, may contribute to unwanted production of IFNα through TLR7/9 in plasmacytoid DCs. Thus, IFNα and BAFF are acting in a vicious circle. Treatment of SLE patients with anti-IFNα mAb could reduce BAFF expression in whole blood [17]. Our study further suggested that treatment of SLE patients with belimumab significantly reduced IFNα secretion in PBMCs upon recognition of apoptotic cells. Given expression of BAFF is controlled by IRF transcription factors and BAFF expression is directly induced by type I IFNs *via* IRF1 and IRF2, reduced IFN-α secretion may suggest there's a feedback loop in IRF-IFNs-BAFF signalling [18]. IRF regulates BAFF expression *via* type 1 IFNs while IFN-α may also be regulated by BAFF when belimumab is present. In addition, levels of CD80 and CD86 dramatically decreased in lupus monocytes in response to co-incubation with apoptotic cells, after treatment with belimumab. The activation status of T lymphocytes showed the same tendency as well as monocytes. These two phenomena may be associated with the decreased level of IFNα.

In summary, our study indicated that PBMCs from the five SLE patients under belimumab treatment showed the suppressed response to the apoptotic cells. The samples size in this study is limited, but it provides clue of the possible mechanism of belimumab's role in preventing lupus flare (other than B-cell depletion): antagonizing effects on the response to co-incubation with apoptotic cells, such as inhibiting IFNα secretion. Thus, further study with larger sample size is required to deepen our understanding of belimumab's phamalogical actions. It's also intriguing to explore whether other SLE drugs (such as hydroxychloroquine, azathioprine and cyclophosphamide) have similar actions to regulate response of cytokine secretion under apoptotic stimuli.

Acknowledgment

Conflict of Interest

The authors declare they have no conflicts of interest.

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