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ORIGINAL ARTICLE

Time of initial appearance of renal symptoms in the course of systemic lupus erythematosus as a prognostic factor for lupus nephritis

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Abstract The prognosis of lupus nephritis (LN) was studied retrospectively in two LN categories, LN manifested initially at systemic lupus erythematosus (SLE) onset (I-LN) and LN of delayed manifestation after SLE onset (D-LN), based on a chart review (C) of 154 SLE (85 LN) patients with a mean observation of 20.8 ± 9.3 years and a questionnaire study (Q) of 125 LN patients outside our hospital with mean observation of 17.6 ± 9.2 years. In both study groups, half of I-LN patients were relapse-free by Kaplan–Meier analysis after initial therapy, and the relapsed I-LN patients responded to retherapy at higher 5-year relapse-free rates than those of patients receiving initial therapies for D-LN. At last observation, a higher frequency of prolonged remission was shown in I-LN compared with D-LN patients (C: 22/31, 71% versus 14/49, 29%, $P < 0.01$; Q: 65/89, 73% versus 11/33, 33% $P < 0.01$) and also a higher frequency of irreversible renal damage in D-LN compared with I-LN patients (C: 25/49, 51% versus 2/31, 6%, $P < 0.001$; Q: 14/33, 42% versus 6/89, 7%, $P < 0.001$), although class IV pathology was common in patients (C) in both LN categories. Onset time of lupus nephritis in the course of SLE may affect renal prognosis.

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Introduction

Systemic lupus erythematosus (SLE) is a multiple-organ disease, and lupus nephritis (LN) is a major clinical problem because of its high morbidity and mortality rates. In accordance with the chronic nature of SLE, renal symptoms can manifest at various times in the disease course, and a physician cannot predict the future development of LN at the time of SLE onset. Furthermore, it is unclear whether there is a difference in prognosis between LN manifested at the onset of SLE and LN developed later in the course of SLE, because the clinical significance of time of LN onset in the disease course of SLE has not been clearly described in the literature, to our knowledge. Accordingly, in recent clinical trials on therapies for LN including cyclophosphamide and mycophenolate mofetil, mixtures of cases having various time intervals between SLE onset and LN onset have been studied [1, 2]. Although a prognostic impact of renal pathology and a poor prognosis of class IV disease have been established in LN based on the World Health Organization (WHO) classification [3–5] and more recent criteria [3, 6, 7], a later progression or transformation of the pathology cannot necessarily be predicted at the time of the initial biopsy [8, 9].

In our preliminary chart review, we found that numerous cases of remitted SLE had class IV LN at SLE onset. On the other hand, irreversible renal damage was precipitated in patients who initially showed no renal symptoms but later developed LN with various renal pathologies, and the LN of later development was never a case of senile-onset LN. Thus we undertook to study the possible relationship

between renal prognosis and the time of the initial renal manifestation in the course of SLE. The study consisted of two parts: a chart review in our institute and a replication study to reconfirm the results of the chart review using a questionnaire administered to LN patients outside our hospital.

Patients and methods

Chart review for patients with SLE

Hospital records of the International Medical Center of Japan were reviewed for patients with SLE having a disease duration of 5 years or more. SLE was diagnosed according to the classification criteria of the American College of Rheumatology [10]. The clinical information on 154 patients with SLE (139 females and 15 males) with mean age of 48.9 ± 12.6 (median 48) years at the last observation was available for studying the entire disease course, including 436 major therapeutic interventions for SLE and 22 deaths during 3,189 person-years or mean observation period of 20.7 ± 9.3 years. At the time of the present study, 85 patients (80 females and 5 males) had LN, and 28 of these had irreversible renal dysfunction, including 15 patients on hemodialysis therapy.

Onset age, relapse ages, therapeutic doses if available, renal pathology data if any, and disease status after therapy and at the last observation in each patient were serially input in our database for SLE patients. The definition of relapse will be provided in "Results". Minor dose increases during steroid therapy for mild activities of SLE were not analyzed. Chronological profiles of the onsets and relapses of LN or extrarenal SLE flares were analyzed statistically with Stata 9.0 (Stata Corp., College Station, TX, USA).

Questionnaire study for patients with LN

To reconfirm the results of the chart review study in another LN patient group, we undertook a questionnaire study of SLE patients outside our hospital and collected individual data on LN with a SLE duration of 5 years or more. The questionnaire included ages at all of the hospitalizations due to SLE and/or LN from onset to the present time, daily doses of steroid (number of 5 mg prednisolone tablets) before and at the start of therapeutic interventions, combined use of intravenous pulse steroid or cyclophosphamide, and renal status at each hospitalization and at the present time. Renal status, which was based on the information from an attendant doctor to each patient, was expressed in terms of urine protein (negative, positive, nephrotic, or 1+ to 4+), data of serum creatinine levels if available, and clinical categories including no abnormalities, mild persistent renal disease, nephrotic syndrome, renal dysfunction or on hemodialysis.

The ethics committee of our hospital approved the present study. A questionnaire sheet including a description of the aim of our study was inserted once in the *Journal of Patients' Association of Collagen Diseases* in Japan, which was subscribed to by more than 5,000 patients, including approximately 3,000 patients with SLE. An anonymous letter in reply (datasheet) sent to the board of the above Patients' Association with a completed questionnaire was regarded as informed consent to enter the present study. We received a set of the copied datasheets from the board, which preserved the original sheets.

After removing approximately 50 cases with insufficient information in the datasheets, 7 cases having SLE without LN, and 10 cases of SLE with less than 5 years' duration, we recognized 125 datasheets that contained a sufficient description of LN. The filling of a datasheet and voluntary mailing might have resulted in a strong bias toward selecting informative repliers from among thousands of patients. The 125 patients were characterized by excellent medical compliance and were thought to have preserved written records on their own diseases.

The studied patients

Mean age of the 125 patients (121 females and 4 males) in the present study was 46.5 ± 12.5 years (median 46 years). A total of 331 hospitalizations because of therapy for SLE were mentioned during 2,200 person-years, or mean observation period of 17.6 ± 9.2 years. Chronological profiles of the relapses of LN were analyzed statistically with Stata 9.0 similarly to the analysis of the chart review study. Renal pathology in accordance with the WHO classification was mentioned in only 19 datasheets and was not analyzed.

In our SLE database, a whole chronological profile of each patient including all of the hospitalizations from onset to the last observation was specific to one subject like a fingerprint, and no overlapping cases were found by computer analysis.

Results

Definitions of initial-onset LN and delayed-onset LN in the course of SLE

In both the chart review and the questionnaire study, some of the patients had been observed with no or low-dose steroid therapy for their mild SLE conditions during the early disease course, and the initial urine proteins were documented

as negative in most of these patients or unknown in some of the referral patients, except in a small number of patients who received no therapy for their positive renal symptoms.

In the present study, we defined a SLE relapse as hospitalization in order to treat SLE after previous SLE conditions have subsided in response to treatment during hospitalization. In a small number of cases, initial steroid therapy with 20 mg/day or more of prednisolone equivalent was started at an outpatient clinic, and we classified the therapy as "treatment under hospitalization" for simplicity in the present text. We further defined "initial-onset LN" and "delayed-onset LN" as follows.

Initial-onset LN (I-LN)

I-LN was defined as LN diagnosed at the time of onset of SLE, LN diagnosed at the initial treatment under hospitalization for SLE or LN that emerged during the initial course of therapy under hospitalization. Two of the 34 I-LN patients in the chart review and 7 of the 91 I-LN patients in the questionnaire study met one of the latter two definitions of I-LN.

Delayed-onset LN (D-LN)

D-LN was defined as newly developed LN as a SLE relapse after the previous successful therapy under hospitalization. At the time of the present study, 51 patients in the chart review and 34 patients in the questionnaire study were classified into this category of LN.

Mean ages of the patients at SLE onset and D-LN onset in the present study are shown in Table 1. These data were very similar between the two study groups; the mean interval between SLE onset and D-LN onset was approximately 8.9 years in the chart review and 7.3 years in the questionnaire study.

Time course of D-LN developments in the chart review patients is shown in Fig. 1a. The Kaplan–Meier curve showed that D-LN developed in half of the SLE patients of extrarenal onset, and that half of the instances of D-LN occurred after 10 disease-years of SLE. We note that the curve might not represent a natural course of D-LN development among SLE patients because of possible sampling bias by the chart review in our single institute and based on our knowledge of the widely different frequencies of LN among SLE patients noted in the literature [11]. A comparable analysis could not be performed in the questionnaire study, because we collected only cases with a positive history of LN.

Time course of the first-time relapse of SLE after the initial treatment under hospitalization in the chart review patients (Fig. 1b)

A total of 302 SLE relapses were identified in the 154 chart review patients, and these included 110 renal involvements and 192 extrarenal SLE flares. The patients were classified into one of two groups, I-LN ($n = 34$) and others ($n = 120$), at the start point of the analysis. Patients in the "others" group, in whom LN developed later, were further classified into D-LN ($n = 51$), as defined above.

The Kaplan–Meier curve indicating those free from SLE relapse after the initial treatment under hospitalization showed a longer relapse-free period in the I-LN patients compared with the other patients (12 versus 4 years to the first relapse was expected in 50% of each patient group, $P = 0.008$) (Fig. 1b), when patients who received no or low-dose steroid were removed from the analysis.

Most of the patients without I-LN had mild SLE conditions at the time of the initial treatment under hospitalization, because neuropsychiatric involvement was not common as the initial manifestation (data not shown).

Table 1 Onset age and initial therapy for LN and/or SLE

		Onset age, years (mean ± SD)	Dose of therapy (PSL[a], mg/day)	Patient ratio that received pulse steroid[b] or IVCY[c]	
Chart review ($n = 85$)					
I-LN ($n = 34$)	SLE + LN	29.1 ± 14.8	51.3 ± 15.2 ($n = 31$)	26% (8/31)	10% (3/31)
D-LN ($n = 51$)	SLE	25.0 ± 10.9	35.0 ± 13.1 ($n = 42$)	#	
	LN	33.9 ± 12.2	41.3 ± 14.4 ($n = 45$)	64% (29/45)	2% (1/45)
Questionnaire study ($n = 125$)					
I-LN ($n = 91$)	SLE + LN	30.1 ± 12.1	44.7 ± 14.7 ($n = 84$)	29% (26/91)	12% (11/91)
D-LN ($n = 34$)	SLE	27.1 ± 11.8	39.1 ± 16.7 ($n = 32$)	#	
	LN	34.4 ± 13.5	43.0 ± 15.2 ($n = 27$)	32% (11/34)	15% (5/34)

[a] PSL, prednisolone equivalent

[b] Intravenous methylprednisolone pulse therapy

[c] Intravenous cyclophosphamide pulse therapy

Pulse steroid or IVCY was rarely used for SLE of D-LN patients at the onset of SLE

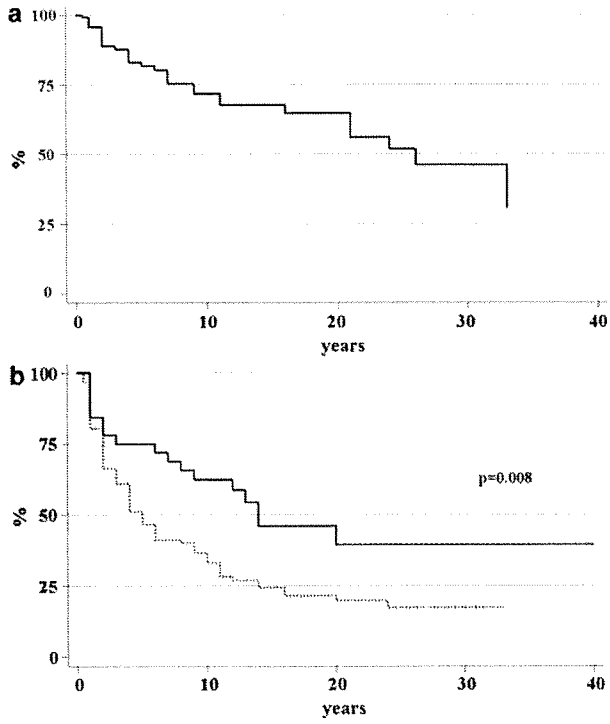

Fig. 1 **a** Kaplan–Meier curve indicating those free from LN development, for patients without initial renal involvement at onset of SLE in the chart review study. Analysis starts at the onset of SLE, and the result shows a time course of developing delayed-onset LN (D-LN). **b** Kaplan–Meier curve indicating those free from SLE relapse after the initial therapy for SLE in the chart review study. Analysis starts at the initial treatment under hospitalization for SLE. *Solid line* patients in whom the initial therapy was targeted at LN (I-LN) ($n = 31$). *Dotted line* patients in whom the initial therapy was targeted at SLE without renal involvement ($n = 95$). The log-rank test was used for statistical analysis

Accordingly, the mean initial steroid dose tended to be higher in the I-LN patients (prednisolone equivalent: 51.3 ± 15.2 mg/day, $n = 31$ versus 36.4 ± 14.1 mg/day, $n = 52$ in the non-I-LN patients of identified steroid dose). High-dose pulse steroid or cyclophosphamide was combined in a quarter of or a small number of I-LN patients (Table 1), respectively, for treating the LN, and was rarely used in non-I-LN patients. Because I-LN and non-I-LN have different organ involvement, the difference in relapse rates after therapy between the two disease categories may not be attributed to the difference in therapeutic intensities. If renal involvement at the onset of SLE indicates a severe form of SLE, a longer relapse-free period in the I-LN patients than in the non-I-LN patients (Fig. 1b) may be paradoxical, or it may be attributed to D-LN development in the non-I-LN patients. Thus, we further studied therapeutic responses in the I-LN patients and the D-LN patients.

Renal relapse-free rates in the I-LN patients (Fig. 2)

The Kaplan–Meier curve indicating freedom from LN relapse after the initial treatment under hospitalization for the I-LN is shown in Fig. 2 for the chart review patients and the questionnaire study patients. A total of five patients who received no therapeutic intervention for LN were removed from the calculation. The results in the two study groups showed consistently that half of the I-LN patients were expected to be renal-relapse-free after the initial therapy.

In the chart review, 18 (58%) of 31 treated patients had no LN relapse throughout the observation period, and all of these patients achieved complete renal remission, although extrarenal SLE flares were observed in 5 patients. Renal relapse was defined as rehospitalization in order to treat SLE accompanied by emerging or increasing proteinuria. Renal remission was defined by normal serum creatinine levels and no urine abnormalities

In the questionnaire study, 52 (58%) out of 89 treated patients had no LN relapse throughout the observation. Of these, 90% (47/52) of the patients achieved complete renal

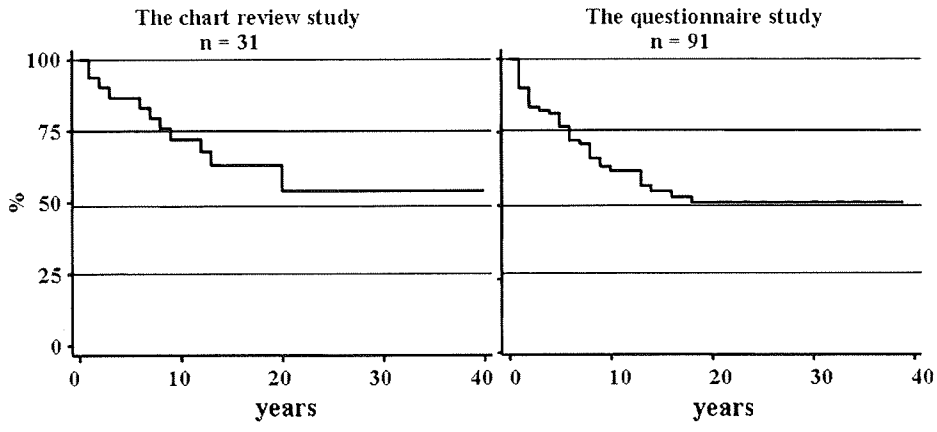

Fig. 2 Kaplan–Meier curve indicating patients free from LN relapse after the initial therapy for I-LN in the two study groups. Analysis starts at the initial treatment under hospitalization for I-LN

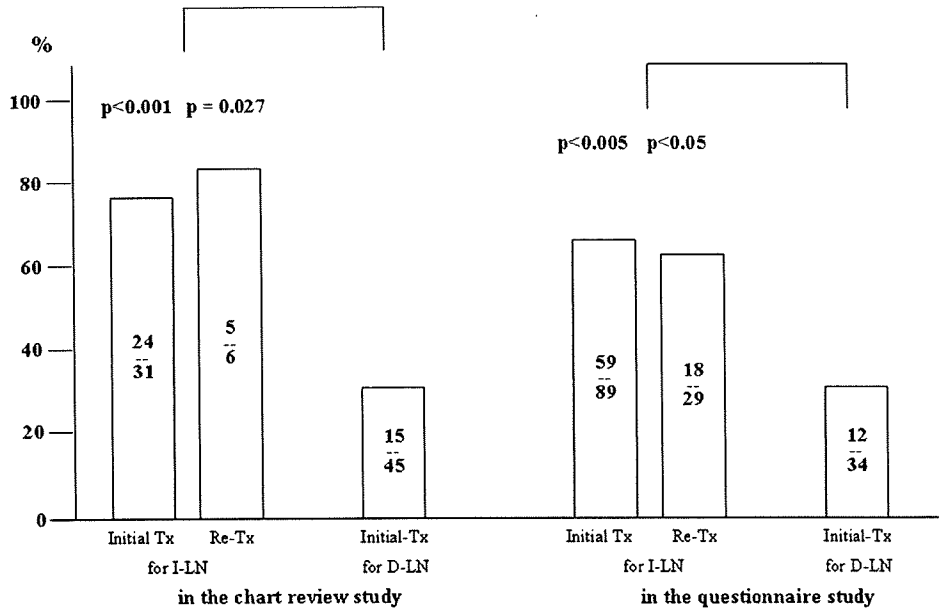

Fig. 3 Response rate to therapy for LN. Initial therapy for I-LN, retherapy for relapsed I-LN, and initial therapy for D-LN were studied by historical follow-up for 5 years; ratios of patients who responded to therapy for LN are shown. Definition of therapeutic response is given in the text. Chi-squared or Fisher's exact probability test was used for statistical analysis

remission. Of the remaining five patients with no relapse but no remission, three patients had massive urine protein and elevated serum creatinine levels at SLE onset that did not respond to initial therapies, and renal deterioration progressed without further therapeutic interventions, and the other two patients had mild persistent urine protein at the last observations.

These results regarding renal relapse suggested that renal remission was common in I-LN only in response to the initial therapy. We further studied prognoses of relapsed I-LN patients in comparison with those of D-LN patients, because first-time relapse of I-LN and onset of D-LN are similarly defined under "relapse of SLE."

Comparison of renal response to therapy between I-LN and D-LN (Fig. 3)

Initial therapies for I-LN or D-LN, respectively, were begun based on renal biopsy in most of the patients of the chart review study, as described later, and positive urine protein was found to be a major reason for the therapeutic intervention in all of the chart review and the questionnaire study patients. Retherapy for relapsed I-LN was begun at the time of rehospitalization in order to treat SLE accompanied by emerging or increasing proteinuria.

Response criteria

Responses to therapy for LN were estimated by 5-year historical follow-up after the initial therapy for I-LN or D-LN, and after the retherapy for I-LN of the first-time relapse. Positive renal response was defined as no relapse, no progressive renal dysfunction, and no nephrotic levels of urine protein during 5-year observation after therapy. The patients in the chart review study who responded to therapy also met response criteria similar to those in the literature [12], i.e., serum creatinine not exceeding the lowest level during treatment under hospitalization, proteinuria less than 3+ or a urine-protein/creatinine ratio less than 1, and no nephritic findings in the urine sediment for at least 6 months after therapy. In the questionnaire study patients, the responses to therapy were identified based on the mention of no renal dysfunction and no or less than 3+ proteinuria after therapy.

Patients who received no therapeutic intervention for renal deterioration were removed from the calculation. In the descriptions below, we classified a patient who showed both renal abnormalities and renal relapse after therapy into a category of renal relapse.

Chart review study

A better renal response to initial therapy was observed in the I-LN patients compared with the D-LN patients (Fig. 3). Initial steroid therapies for LN in the I-LN and the D-LN are shown in Table 1. The difference in the therapeutic doses did not seem large enough to explain the different response rates between the I-LN and the D-LN patients.

WHO class IV histology was documented at the renal biopsy before therapy for LN with similar frequencies (I-LN: 14/21, 64% versus D-LN: 22/39, 56%) in the two LN categories, and the chronic lesions were each identified in only a small number of patients. The total results of

histology identified in 21 I-LN versus 39 D-LN were: II, 1 versus 5; III, 0 versus 1; IVa or b, 6 versus 10; IVc, 2 versus 4; IVd, 0 versus 0; IV of unknown subclass, 6 versus 8; V, 6 versus 11; and there was no significant difference in the distribution of the histology between I-LN and D-LN.

Serological data before therapy (the number of patients) were identified in most of the patients treated for I-LN (31) or D-LN (45). Serum anti-DNA antibody levels were elevated in 89% (24/27) of I-LN patients and 93% (37/40) of D-LN patients; the mean titer in I-LN patients was 100.5 ± 20.1 IU/ml ($n = 11$) by enzyme-linked immunosorbent assay (ELISA) for anti-double-stranded DNA IgG antibodies (normal range <20), 10 ± 2 IU/ml ($n = 10$) by radioimmunoassay (RIA) for anti-DNA antibodies (normal range <6), or unknown except positive results ($n = 3$), whereas that in D-LN patients was 56.7 ± 11.2 IU/ml ($n = 12$) by ELISA, 12 ± 2 IU/ml ($n = 15$) by RIA, or unknown except positive results ($n = 10$). Hypocomplementemia was observed in 83% (20/24) of I-LN patients and 93% (38/41) of D-LN patients based on the data of serum C3 and/or CH50. Of these, the comparable assays for C3 (normal range 60–95 mg/dl) showed mean levels 40.2 ± 5.6 mg/dl ($n = 13$) in I-LN patients and 43.5 ± 9.9 mg/dl ($n = 27$) in D-LN patients, respectively. The above data showed that the two patient groups I-LN and D-LN had similar serological abnormalities before initial therapy for LN.

Renal status (number of patients) during 5 years after initial therapy for LN

The 31 I-LN patients showed one of the following: 5-year remission (22), mild persistent proteinuria (2) including accompanied renal dysfunction (1) with serum creatinine >1 mg/dl after rapidly progressing glomerulonephritis (RPGN), or renal relapse (6). A negative response was found in seven patients.

The 45 D-LN patients showed one of the following: 5-year remission (10), mild persistent proteinuria (5), or persistent proteinuria of nephrotic levels (5), including accompanying renal dysfunction (3) with serum creatinine >1 mg/dl after RPGN, or renal relapse (25). A negative response was found in 30 patients.

Renal status during 5 years after retherapy for the first relapse of I-LN

LN relapse occurred in 11 of the 31 I-LN patients after the initial therapy. Of these, nine patients received therapeutic interventions at the mean prednisolone-equivalent dose of 44.4 ± 15.1 mg/day, and a methylprednisolone pulse was added in two patients. By the time of the present study, six patients had been followed for more than 5 years after the retherapy for LN; 5-year remission (three), mild persistent proteinuria (two) or a second renal relapse (one) during 5 years were found in the six patients. A negative response was found in one patient. The renal response to the retherapy was again better than that following the initial therapy for D-LN (Fig. 3).

Serum anti-DNA antibody levels before therapy were elevated in 89% (8/9) of the patients treated for relapsed I-LN, and the mean titer was 59.5 ± 22.1 IU/ml ($n = 5$) by ELISA or 8 ± 2 IU/ml ($n = 3$) by RIA. Hypocomplementemia was observed in 78% (7/9) of the patients, and the mean serum C3 level was 48.1 ± 5.1 mg/dl ($n = 7$).

The questionnaire study

A better renal response to initial therapy was observed in the I-LN patients compared with the D-LN patients (Fig. 3). Initial steroid therapies for LN in the I-LN and the D-LN are shown in Table 1. The difference in the therapeutic doses did not seem large enough to explain the different response rates between the I-LN and the D-LN patients.

Renal status (the number of patients) during 5 years after initial therapy for LN

The 89 I-LN patients showed one of the following: 5-year remission (32), probable remission or mild proteinuria (16), mild persistent proteinuria (11), chronic proteinuria of nephrotic levels accompanying renal dysfunction (3) after RPGN, or renal relapse (27). "Probable remission or mild proteinuria" was defined based on no subsequent relapse and the mention of no renal abnormalities at the last observation but no mention of the early renal status after therapy. A negative response was found in 30 patients.

The 34 D-LN patients showed one of the following: 5-year remission (9), mild persistent proteinuria (3), persistent proteinuria of nephrotic levels and/or renal dysfunction (12), or renal relapse (10). At least five patients had RPGN before therapy. A negative response was found in 22 patients.

Renal status during 5 years after retherapy for the first relapse of I-LN

LN relapse occurred in 39 of the 91 I-LN patients, and 36 patients received therapeutic interventions. The identified mean prednisolone-equivalent dose was 45.5 ± 13.3 mg/day ($n = 30$), and the combination use of steroid pulse was mentioned by ten patients, and that of cyclophosphamide by six patients. The 29 patients who received retherapy and 5-year follow-up showed one of the following: 5-year remission (8), mild persistent proteinuria

Table 2 Renal outcomes of I-LN or D-LN in the two study groups: renal status over 3 years at time of the last observation

	n	Remission	MPD	N + CRF + HD	Flare	Observation period of LN (years)
Chart review						
I-LN	31	22 (71%)*	7	0 + 0 + 2 (6%)	0	19.6 ± 9.2
D-LN	49	14 (29%)	8	4 + 11 + 10 (51%)**	2	13.0 ± 8.1
Questionnaire study						
I-LN	89	65 (73%)*	8	0 + 4 + 2 (7%)	10	17.5 ± 9.1
D-LN	33	11 (33%)	5	6 + 4 + 4 (42%)**	3	12.1 ± 7.8

Remission: no renal abnormalities but including SLE conditions of serologically active clinically quiescent (SACQ) disease for at least 3 years. MPD (mild persistent disease; positive urine proteins less than nephrotic levels). N (nephrotic levels of urine proteins): prolonged massive urine proteins without apparent renal dysfunction. CRF (chronic renal failure but no uremia): prolonged elevation of serum creatinine levels (>1 mg/dl) or mention of renal dysfunction in the questionnaire based on information from attendant doctors. HD (hemodialysis): on maintenance HD or having a history of receiving renal implantation irrelevant to the present renal status. Flare: flare of SLE and/or LN within 3 years by the last observation

* $P < 0.01$, ** $P < 0.001$ (chi-squared test)

(3), probable remission or mild proteinuria (7), persistent proteinuria of nephrotic levels and/or renal dysfunction (2), or a second renal relapse (9). A negative response was found in 11 patients. The renal response to the retherapy was again better than that following the initial therapy for D-LN (Fig. 3).

The results in the two study groups were consistent, and suggested that renal response to both the initial therapy for I-LN and the retherapy for relapsed I-LN were better than that to the initial therapy for D-LN.

Renal outcomes: renal status over 3 years at the time of the last observation (Table 2)

Renal status was classified according to definitions described in the footnotes of Table 2, and we classified a patient that showed both of chronic renal damage and recent renal flare into a category of chronic renal damage but not flare. A higher frequency of renal remission was observed in I-LN compared with D-LN, and a higher frequency of irreversible renal damage including nephrotic levels of prolonged urine proteins or chronic renal failure was observed in D-LN compared with I-LN.

The above results were consistent in the two study groups and suggested a good renal prognosis of I-LN patients and a poor renal prognosis of D-LN patients. In most of the patients with irreversible renal damage in the present study, frequent relapse resulted in renal deterioration.

Patients whose irreversible renal damage was established before the initial therapy were removed from the estimation of renal outcomes shown in Table 2. This included three I-LN patients and two D-LN patients in the chart review and two I-LN patients and one D-LN patient in the questionnaire study. Some of the remaining patients in the chart review did not receive an appropriate therapeutic intervention for renal deterioration that developed in the course of SLE for various reasons, including a referral delay, poor medical compliance, and accompanying chronic infection.

Except in the case of receiving insufficient therapy as described above, most of the patients in the chart review and the questionnaire study were treated for LN appropriately with 30–60 mg/day prednisolone-equivalent doses of steroid at the onset of LN and at the first LN relapse. For repeated SLE relapses, however, intensities of therapeutic interventions were widely different from case to case.

Renal outcomes in reference to the renal pathology before therapy in the chart review study

Renal pathology before initial therapy for LN (the first-time biopsy; Fig. 4)

Renal outcomes in the chart review patients were collated according to the renal pathology data at biopsy before initial therapy (Fig. 4), which were available in 61 out of 85 LN patients based on the reports by pathologists in our hospital or the referral description of other hospitals. In addition, one I-LN and three D-LN patients manifested RPGN at the onset of LN and had probable class IV-LN.

The descriptions of renal pathology found by chart review were mostly in accordance with the WHO classification (3, 4), although the information on subclass was not comprehensive. In the present study (Fig. 4), we classified "a description of membranous LN" or "class V without description of combined III or IV" into "V", and "diffuse proliferative LN without subclass-description" into "IV-" (Fig. 4). In addition, "IV + V" documented in some of the case records was also denoted as "IV-" but not Vd. Thus, "IV-" in Fig. 4 might contain various types of class IV.

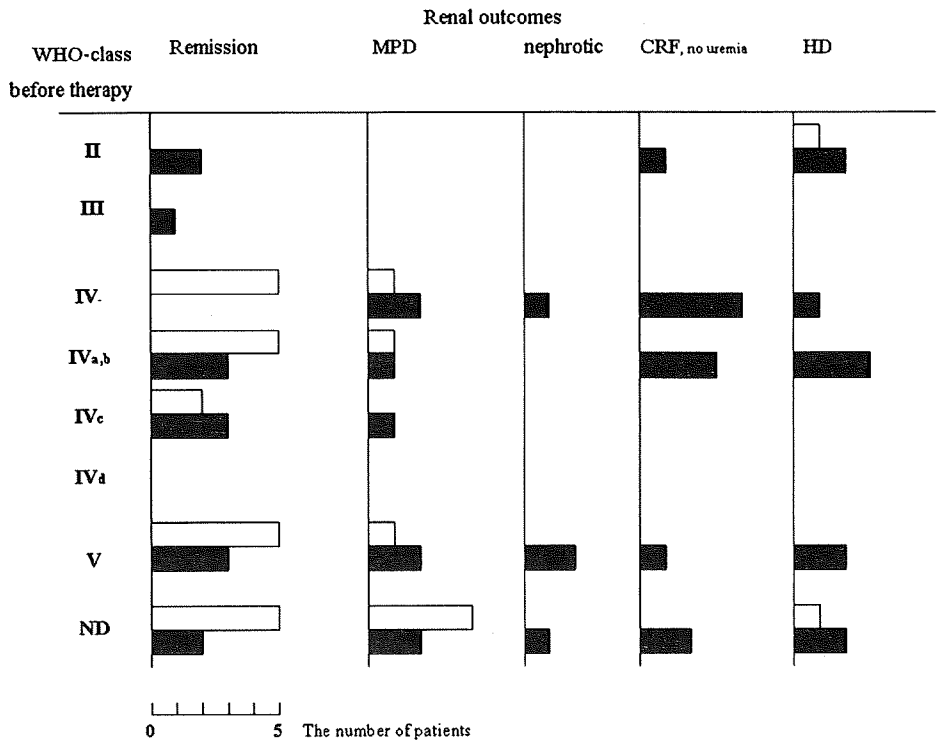

Fig. 4 Renal outcomes in 31 I-LN patients and 47 D-LN patients in the chart review study in reference to renal pathology at biopsy before initial therapy for LN. Each *bar* denotes the number of patients (*white bar* I-LN, *black bar* D-LN). "IV-" includes diffuse proliferative LN when lacking information regarding activity, necrosis or sclerosis findings. The definition of "V" is given in the text. *ND* not done, *MPD* mild persistent disease, *CRF* chronic renal failure without uremia, *HD* hemodyalysis

Patients with I-LN Class IV was common in these patients but rarely associated with the irreversible renal damage at the time of the last observation. Two patients had end-stage renal disease: a patient with RPGN at SLE onset followed by repeated renal relapses, and another patient with class II-LN at SLE onset and suffering from later developed thrombotic thrombocytopenic purpura (TTP) that caused uremia.

Patients with D-LN Irreversible renal damage was common in these patients having various renal pathology classes including class IV before initial therapy for the LN. TTP was involved in two patients and directly caused chronic renal failure in at least one of the patients.

Renal pathology before therapy for relapsed LN (change of the histology)

The renal pathology at time of the LN relapse was documented in five I-LN patients and nine D-LN patients. These findings are described below in reference to those at first biopsy (in parentheses) and clinical renal outcomes in terms defined in Table 2.

Patients with I-LN In four patients, retherapy for relapsed LN of class II (V), IVa (V), IVa (ND) or IVc (V) led to a clinical remission at the last observation. In another patient, LN of class IVc + V (IVa) manifested after self-discontinuation of maintenance therapy, and responded to retherapy and led to mild persistent disease (MPD) that was relapse-free for 16 years by the present time.

Patients with D-LN In two patients, each of the retherapies for relapsed LN of class IVc (V) using combined cyclophosphamide improved nephrotic syndrome to MPD. In another three patients, relapsed LN of class IVa (II), IVd (IVb) or IVd (ND) resulted in CRF without uremia despite retherapy. In the remaining four patients, relapsed LN of class IVd (V), IVd (II), IVc (IVa) or Va (V) accompanied by intractable nephrotic syndrome resulted in CRF on hemodialysis despite retherapy.

As described above, class IV with sclerosing lesions was commonly uncovered at the second biopsy in the D-LN patients who resulted in CRF. On the other hand, a transformation of renal histology observed in most of the examined cases of relapsed I-LN showed a small impact on clinical prognosis.

Discussion

The present chart review study and questionnaire study consistently showed a relatively better prognosis of I-LN patients compared with D-LN patients. Half of the I-LN patients were expected to be relapse-free after the initial therapy (Fig. 2) and most of the relapse-free patients

achieved renal remission throughout the observation period. In the I-LN cases of relapsed LN, most patients responded to retherapy (Fig. 3). Consistent with these findings, more than 70% of the I-LN patients had obtained prolonged renal remission at the last observation (Table 2). In contrast, a poor prognosis of D-LN patients was shown, and irreversible real damage was precipitated in this category of LN. The resulting data (Figs. 2, 3 and Table 2) in the two study groups were surprisingly similar, and strongly suggested the prognostic impact of the difference between the two chronological categories I-LN and D-LN. A pathological transformation towards class IV with sclerosing lesions was found at the second biopsy in most of the examined D-LN cases in the chart review.

Despite a similarity in the patients' demographics between the present two study groups (Table 1), there was a large difference in the ratio of D-LN to I-LN patients: 51/34 (1.5) in the chart review and 34/91 (0.37) in the questionnaire study ($P < 0.00001$). The chart review study in our hospital included all of the deceased patients and numerous numbers of referral patients because of intractable disease, and thus patients with severe forms of SLE may be overrepresented in the chart review study. The result of the higher D-LN/I-LN ratio in the chart review than that in the questionnaire study may be consistent with the putative poorer prognosis of D-LN compared with I-LN.

The present study suggested that D-LN tended to progress in renal damage despite steroid therapy, in contrast to the good therapeutic response of I-LN even having renal pathology class IV. Cyclophosphamide, which has been included recently in a standard regimen for treating LN in our hospital, had not been widely used for the cases in the present study that included only therapies occurring more than 5 years ago. Therapy using steroid and combined cyclophosphamide may improve the prognosis of D-LN patients, and the efficacy of therapy for I-LN and that for D-LN should be estimated separately because of a probable difference in therapeutic response between the two LN categories.

Conclusions

The time of the initial appearance of renal symptoms in the course of SLE may have a prognostic impact on lupus nephritis.

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Research article

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Interactions among type I and type II interferon, tumor necrosis factor, and β-estradiol in the regulation of immune response-related gene expressions in systemic lupus erythematosus

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Abstract

Introduction Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by various clinical manifestations. Several cytokines interact and play pathological roles in SLE, although the etiopathology is still obscure. In the present study we investigated the network of immune response-related molecules expressed in the peripheral blood of SLE patients, and the effects of cytokine interactions on the regulation of these molecules.

Methods Gene expression profiles of peripheral blood from SLE patients and from healthy women were analyzed using DNA microarray analysis. Differentially expressed genes classified into the immune response category were selected and analyzed using bioinformatics tools. Since interactions among TNF, IFNγ, β-estradiol (E2), and IFNα may regulate the expression of interferon-inducible (IFI) genes, stimulating and co-stimulating experiments were carried out on peripheral blood mononuclear cells followed by analysis using quantitative RT-PCR.

Results Thirty-eight downregulated genes and 68 upregulated genes were identified in the functional category of immune response. Overexpressed IFI genes were confirmed in SLE patient peripheral bloods. Using network-based analysis on these genes, several networks including cytokines – such as TNF and IFNγ – and E2 were constructed. TNF-regulated genes were dominant in these networks, but *in vitro* TNF stimulation on peripheral blood mononuclear cells showed no differences in the above gene expressions between SLE and healthy individuals. Co-stimulating with IFNα and one of TNF, IFNγ, or E2 revealed that TNF has repressive effects while IFNγ essentially has synergistic effects on IFI gene expressions *in vitro*. E2 showed variable effects on IFI gene expressions among three individuals.

Conclusions TNF may repress the abnormal regulation by IFNα in SLE while IFNγ may have a synergistic effect. Interactions between IFNα and one of TNF, IFNγ, or E2 appear to be involved in the pathogenesis of SLE.

Introduction

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by multiple organ damage, high titers of autoantibodies, and various clinical manifestations [1]. Numerous disorders in the immune system and abnormalities in cytokine productions have been described in patients with SLE. The exact pathological mechanisms are still obscure, however, and the roles of the cytokines are not well understood. High levels of TNF, type I interferon, and type II interferon in the sera of patients with SLE have been reported [2-4]. On the other hand, an impaired production of IL-12 by T lymphocytes from SLE patients *in vitro* has also been

aRNA: amino allyl RNA; Ct: cycle threshold; E2: β-estradiol; FcγR: Fcγ receptor; GBP: guanylate binding protein; HLA: human leukocyte antigen; IFI: interferon-inducible; IFIT: interferon-induced protein with tetratricopeptide repeats; IFN: interferon; IL: interleukin; IRF7: interferon regulatory factor 7; ISG15: interferon-stimulated gene, 15 kDa; MAPK: mitogen-activated protein kinase; MHC: major histocompatibility complex; NFκB: nuclear factor of kappa light polypeptide; OAS1: 2',5'-oligoadenylate synthetase 1; OASL: 2',5'-oligoadenylate synthetase-like; PBMC: peripheral blood mononuclear cell; PCR: polymerase chain reaction; RT: reverse transcription; SLE: systemic lupus erythematosus; TLR: Toll-like receptor; TNF: tumor necrosis factor.

observed [5,6]. Cytokines are pleiotropic in their biological activity, and it is known that our immunity is regulated by highly sophisticated cytokine networks. Comprehending the pathological roles of these abnormally induced cytokines and immunoregulatory networks of cytokines in SLE patients is therefore important so that appropriate treatment can be offered.

The microarray is a powerful tool to exhaustively investigate the gene expressions of autoimmune diseases that have complex pathogenesis and heterogeneous manifestations, such as SLE. So too are the various databases and bioinformatics tools such as gene ontology analysis, which can functionally categorize genes, or network-based analysis to investigate molecule interactions [7]. These tools have proven useful to further analyze the enormous data from microarray analysis, providing several new findings [8].

Most microarray analyses in SLE have been performed using peripheral blood mononuclear cells (PBMCs) while recent studies provide strong evidence that IFN-related genes are overexpressed in SLE patients [9-13]. In the present study, to investigate the abnormal immune system in SLE, we focused on genes in the functional category of immune response differentially expressed in SLE patients compared with healthy individuals. Our results using SLE whole blood showed definite overexpression of IFN-regulated genes in this category. As molecules in the immune response category are always communicating with each other, we performed a network-based analysis to identify aberrant regulations or interactions among differentially expressed molecules observed in this study. We also investigated the effect of interactions between IFNα and one of TNF, IFNγ, or β-estradiol (E2) on the expression of these molecules.

Materials and methods

Patients and healthy individuals

Eleven patients (all women, median age 35 years, range 27 to 72 years) with SLE fulfilled by the diagnostic criteria of the American College of Rheumatology [14] and six healthy women were enrolled in the present study after obtaining their written informed consent. The study was approved by the Ethical Committee of Osaka University Medical School for clinical studies on human subjects.

The majority of the SLE patients (n = 10) were treated with <20 mg/day prednisolone. Three of these 10 patients were treated with one of cyclosporine, azathioprine, or methotrexate in combination with prednisolone, respectively. The remaining patient was treated with >20 mg/day prednisolone.

The median disease activity score of SLE patients based on the SLE Disease Activity Index 2000 instrument [15] was 10 (range 6 to 24). Two patients had very active states (SLE Disease Activity Index 2000 score >12) while the other patients had active states (SLE Disease Activity Index 2000 score = 4 to 12). The median of the assessment based on the BILAG index [16] was 4 (range 1 to 13).

Meanwhile, the median of the total white blood cells for the patients was 6,160 (range 4,840 to 12,230). The median of the total number (proportion) of neutrophils was 4,919 (80.0%) (range 3,640 to 9,674, 75.2% to 90.1%), and that of lymphocytes was 838 (11.8%) (range 480 to 1,517, 6.6% to 20.5%).

GeneChip microarray and data analysis

Peripheral blood was collected directly into PAXGene tubes (Qiagen, Valencia, CA, USA). Total RNA was extracted using the PAXGene Blood RNA kit with the optimal on-column DNase digestion (Qiagen). Amino allyl RNA (aRNA) was synthesized from 1 μg total RNA using the Amino Allyl MessageAmp™ aRNA kit (Ambion, Austin, TX, USA). Five micrograms of aRNA from each sample (11 SLE patients and six healthy control individuals) and the equivalent quantity of reference aRNA from a mixture of RNA extracted from peripheral blood of 12 healthy women were subjected to Cy3 and Cy5 labeling, respectively. Both labeled aRNAs were mixed in equal amounts and were hybridized with the oligonucleotide-based DNA microarray AceGene (HumanOligoChip30K; DNA Chip Research, Yokohama, Japan), which contained about 30,000 human genes.

The microarrays were scanned using ScanArray Lite (PerkinElmer, Boston, MA, USA) and the signal values were calculated using the DNASIS Array (Hitachi Software Engineering, Tokyo, Japan) according to the manufacturer's instructions. The intensities of no-probe spots were used as the background. The median and standard deviation of background levels were calculated. Genes whose intensities were less than the median plus two standard deviations of the background level were identified as null. The Cy3/Cy5 ratios of all spots on the DNA microarray were normalized by the global ratio median. Genes with at least 80% good data across each group of samples were selected for further analysis. The microarray data have been deposited in NCBIs Gene Expression Omnibus [GEO:GSE12374].

Gene ontology and network-based analysis

Genes identified to be differentially expressed by >10% according to the microarray analysis with a median signal intensity difference of at least 100 between the SLE patient and healthy individual groups (in order to reduce errors pertaining to low-level expression at close to noise level) were functionally categorized using Expression Analysis Systematic Explorer version 2.0 bioinformatics software [17,18]. Interactions among the differentially expressed genes in the functional category of immune response were investigated through the use of Ingenuity Pathway Analysis version 5.5 [19]. Networks generated by less than five uploaded genes were excluded from the analysis.

Stimulation of peripheral blood mononuclear cells

To assess TNF signaling, PBMCs from six patients diagnosed with SLE and from three healthy individuals were utilized. All PBMCs used in the experiments were isolated from heparinized whole blood using a Ficoll-Paque™ Plus (GE Healthcare Biosciences, Uppsala, Sweden) gradient centrifugation according to the manufacturer's recommendations. The cells were incubated in RPMI 1640 with 10% heat-inactivated fetal bovine serum and TNF (20 ng/ml) in a carbon dioxide incubator at 37°C for 24 hours.

To examine the effects of interactions between IFNα and one of TNF, IFNγ, or E2 on interferon-inducible (IFI) genes, we performed co-stimulating experiments on PBMCs. The PBMCs isolated from three healthy women were cultured with 20 ng/ml TNF, 15 ng/ml IFNγ, 2 ng/ml E2, and 500 U/ml IFNα or null, and were co-stimulated with TNF and IFNα, with IFNγ and IFNα, or with E2 and IFNα. PBMCs were cultured at a final concentration of 1.5×10^6 cells/ml.

TNF [GenBank:CAA26669] and IFNγ [GenBank:AAB59534] were purchased from R&D Systems (Minneapolis, MN, USA). IFNα [GenBank:NP_000596] and E2 were purchased from PBL Biomedical Laboratories (Piscataway, NJ, USA) and Sigma (St Louis, MO, USA), respectively.

Preparation of cDNA and quantitative RT-PCR

Total RNA from the PBMCs was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using 2.5 μM random hexamers and 125 units MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) in a 100 μl reaction mixture. Four microliters of the twofold-diluted cDNA products were amplified in a 25 μl reaction mixture containing TaqMan Universal Master Mix and each TaqMan probes (Applied Biosystems). The assay identification numbers for the probes are presented in Table 1.

The real-time PCR was performed in a 96-well optical plate with the Applied Biosystems 7500 real-time PCR system under the following cycling conditions: 2 minutes at 50°C (one cycle), 10 minutes at 95°C (one cycle), 15 seconds at 95°C and 1 minute at 60°C (40 cycles). For each gene (performed in duplicate for each sample), cycle threshold (Ct) values were determined from the linear region of the amplification plot and were normalized by subtracting the Ct value of GAPDH (generating a ΔCt value). The response to the cytokines or E2 was determined by subtracting the ΔCt value for the time-matched control from the ΔCt value for the stimulated sample (ΔΔCt value). The fold change was subsequently calculated using the formula $2^{-\Delta\Delta Ct}$ (where ΔΔCt was converted to an absolute value).

Statistical analysis

The unpaired Mann-Whitney test was used to determine statistically significant differences in the mRNA expression levels between the SLE patient and healthy individual groups. The criterion for the statistical significance was $P < 0.05$.

Results

Immune response-related genes identified by gene ontology analysis

Thirty-eight downregulated genes and 68 upregulated genes were categorized into the functional category of immune response. Most of the 68 upregulated genes were interferon regulated – including 17 IFI genes such as interferon-induced protein with tetratricopeptide repeats (IFIT) 1, 2',5'-oligoadenylate synthetase 1 (OAS1), 2',5'-oligoadenylate synthetase-like (OASL), interferon-stimulated gene, 15 kDa (ISG15), and interferon regulatory factor 7 (IRF7) that have been reported as overexpressed in the PBMCs of SLE.

Network-based analysis on the downregulated or upregulated genes in the functional category of immune response

There were two networks represented by the downregulated genes. Twenty-three out of the 38 downregulated genes were included in the first network, including p38 mitogen-activated protein kinase (MAPK) complex and NFκB complex depicted at the center of Figure 1a. p38 MAPK is phosphorylated in response to inflammatory cytokines including IL-1β [20] and TNF. Phosphorylated p38 MAPK contributes to the activation of NFκB, which regulates the gene expression of various cytokines, chemokines and adhesion molecules [21]. Although TNF was not identified in this network, we found that most of the molecules were TNF-regulated – including cell surface antigens (CD40, CD14, CD1C), chemokine (C-C motif) receptor 7, and acute phase proteins such as serum amyloid A_1 and apelin. These data, together with a previous report of increased TNF levels in the serum of SLE patients [2], suggested that an abnormality in TNF signaling might exist. Meanwhile, a cluster of MHC class II genes consisting of HLA-DRA, HLA-DQA1, HLA-DQB1, and CD74 (also known as HLA-DRG) were also identified in this network. The second network, composed of nine downregulated genes, implied that there were interactions among TNF, IFNγ, IL-2, IL-4, and E2 (Figure 1b).

Our analysis found only four networks represented by the upregulated molecules. The first network, constructed by 25 upregulated molecules, was the network with p38 MAPK, NFκB, and TNF receptor depicted at the center of Figure 2a. A cluster of the Toll-like receptor (TLR) family (that is, TLR1, TLR2, TLR4, and TLR5) and another cluster of Fcγ receptors (FcγRs) were identified in this network. The two clusters were indirectly connected through p38 MAPK and NFκB, suggesting there may be functional interactions among these molecules through this pathway. This network was overlapped with

Table 1

Assay identification numbers for probes

Probe	Identification number
CD40	Hs01002913_m1
CD1C	Hs00233509_m1
CD14	Hs00169122_g1
Chemokine (C-C motif) receptor 7 (CCR7)	Hs00171054_m1
IL12B	Hs00233688_m1
IL-4 receptor (IL4R)	Hs00166237_m1
Prostaglandin E synthase (PTGES)	Hs00610420_m1
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	Hs01911452_m1
Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3)	Hs00382744_m1
Interferon-induced protein with tetratricopeptide repeats 5 (IFIT5)	Hs00202721_m1
Interferon, alpha-inducible protein 6 (IFI6)	Hs00242571_m1
Interferon, gamma-inducible protein 16 (IFI16)	Hs00194261_m1
Interferon, alpha-inducible protein 27 (IFI27)	Hs00271467_m1
Interferon, gamma-inducible protein 30 (IFI30)	Hs00173838_m1
Interferon-induced protein 35 (IFI35)	Hs00413458_m1
Interferon induced transmembrane protein 1 (IFITM1)	Hs01652522_g1
Interferon-stimulated gene, 15 kDa (ISG15)	Hs00192713_m1
Interferon regulatory factor 7 (IRF7)	Hs00242190_g1
2',5'-oligoadenylate synthetase 1 (OAS1)	Hs00242943_m1
2',5'-oligoadenylate synthetase-like (OASL)	Hs00388714_m1
Guanylate binding protein 1 (GBP1)	Hs00266717_m1
Guanylate binding protein 2 (GBP2)	Hs00269759_m1
IL8RA	Hs00174146_m1
C-type lectin domain family 4, member E (CLEC4E)	Hs00372017_m1
TNFα-induced protein 6 (TNFAIP6)	Hs00200180_m1

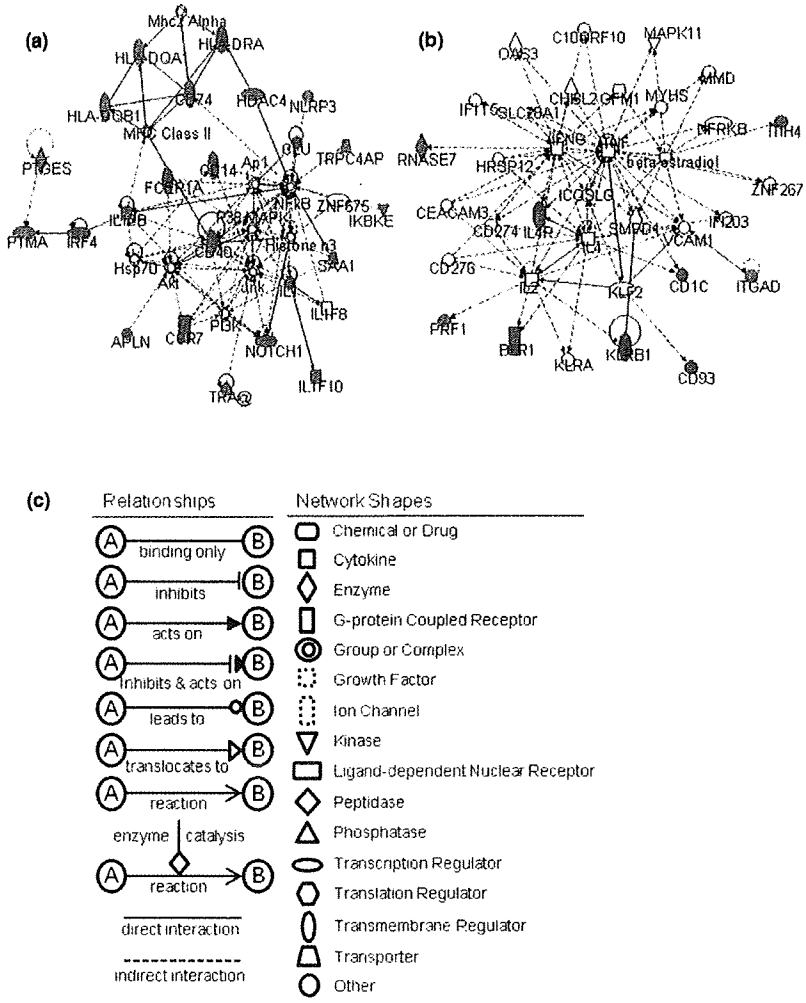
the fourth network, whose central molecules were IFNγ and E2 (Figure 2d). There were nine IFI molecules found in the first and fourth networks. The second network was represented by Akt and a calcium ion at the center (Figure 2b), while the third network was mainly attributed to TNF (Figure 2c). We found that two IFI molecules were included in the second network, and that seven out of the 14 upregulated molecules that constructed the third network were IFI molecules.

Gathering the above results, TNF, IFNγ, and E2 were depicted by both downregulated and upregulated molecules in the networks. As most of the genes in the immune response were TNF regulated, we performed stimulating experiments on the PBMCs of SLE patients and healthy individuals to assess the TNF regulation on the immune response-related molecules in SLE. On the other hand, although the expression of IFNα was not upregulated and was not depicted in networks related to TNF, IFNγ, or E2, IFI molecules were found ranging over the four networks. Furthermore, it has been reported that there exist elevated levels of type I interferon in the SLE serum. Type I interferon therefore appears to have complicated interactions with various cytokines and E2. This encouraged us to further examine the effects of interactions between IFNα and one of TNF, IFNγ, or E2 on IFI gene expression.

Gene expression profiles of peripheral blood mononuclear cells by TNF stimulation for SLE patients and healthy individuals

Seven downregulated genes (CD40, CD1C, CD14, chemokine (C-C motif) receptor 7, IL12B, IL-4 receptor, and prostaglandin E synthase) and 12 upregulated genes (IFIT1, IFIT3, IFIT5, ISG15, IRF7, OASL, OAS1, guanylate binding protein

Figure 1

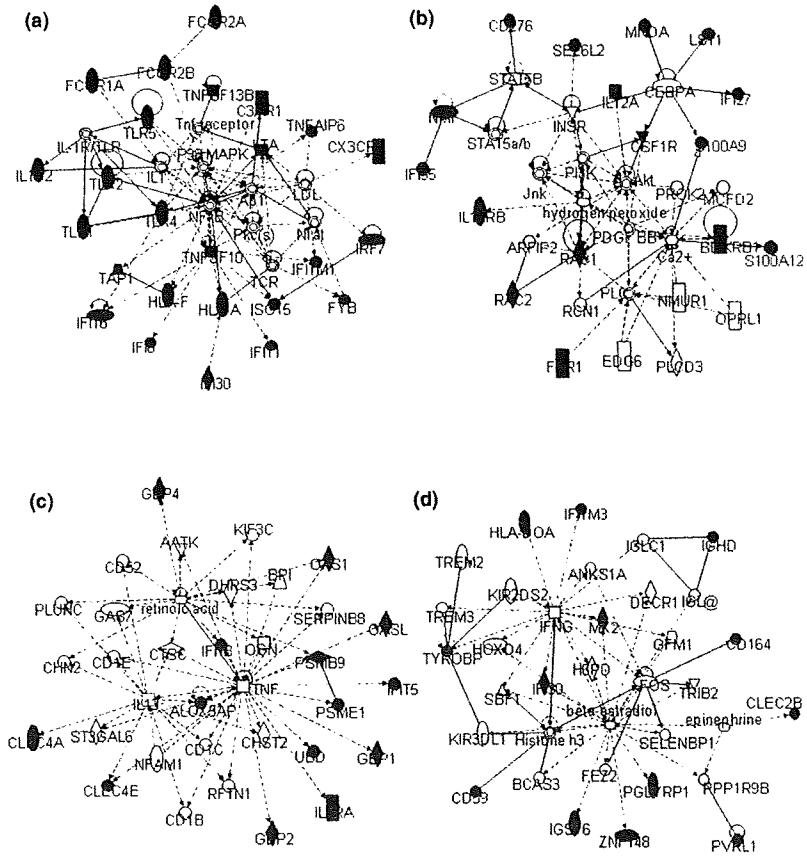


Network-based analysis of downregulated genes in the functional category of immune response. (a) Network 1 and **(b)** Network 2 constructed by downregulated genes. **(c)** Network graphical representation. Genes or gene products are represented as individual nodes whose shapes represent the functional class of gene products. The biological relationship between the two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature stored in the Ingenuity Pathways Knowledge Base (IPKB). Genes in colored nodes were found over-represented in the functional category of immune response. Genes in uncolored nodes were not found over-represented but were depicted by the computationally generated networks on the basis of evidence stored in the IPKB indicating a strong biologic relevance to that network.

(GBP) 1, GBP2, IL8RA, C-type lectin domain family 4 member E, and TNFα-induced protein 6), all of which were TNF regulated, were selected and their mRNA expressions upon TNF stimulation were measured by quantitative RT-PCR. All of the genes selected showed essentially the same responses to TNF stimulation on PBMCs independent of the individual (Figure 3). CD40, IL12B, prostaglandin E synthase, C-type lectin domain family 4 member E, and TNFα-induced protein 6 were upregulated, while CD1C, IFIT1, IFIT3, OAS1, and IL8RA were downregulated upon TNF stimulation in both SLE patients and healthy individuals.

The *in vivo* gene expression profiles of SLE, however, were different from the results of *in vitro* PBMC stimulation by TNF. For example, CD40 was downregulated *in vivo* but was upregulated upon TNF stimulation *in vitro*. Meanwhile, IFI genes such as IFIT1, IFIT3, OAS1, ISG15 and IRF7, and IL8RA were upregulated *in vivo*, but IFIT1, IFIT3, OAS1 and IL8RA were downregulated, while ISG15 and IRF7 showed almost no response to TNF *in vitro*. These data suggest that other soluble factors might be involved in the regulation on the gene expression. Indeed, high levels of interferon in SLE serum have been suggested to cause overexpression of IFI genes [22]. Interestingly, we not only found that TNF had repressive

Figure 2



Network-based analysis of upregulated genes in the functional category of immune response. (a) Network 1, **(b)** Network 2, **(c)** Network 3, and **(d)** Network 4 constructed by upregulated genes.

effects on IFI genes IFIT1, IFIT3, IFIT5, ISG15, and IRF7 expression, but that the effect was significantly stronger on SLE patients' PBMCs than those of healthy individuals. This result may be caused by the differences in the baseline expressions where IFI genes were overexpressed *in vivo* in SLE patients.

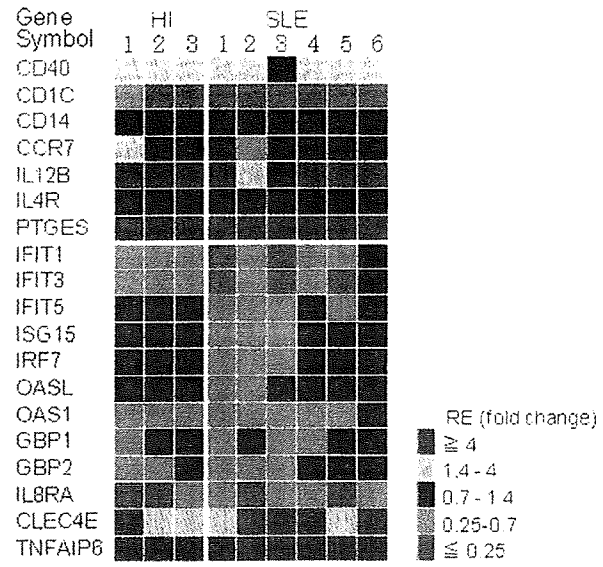
Repressive effect of TNF on interferon-inducible gene expressions in peripheral blood mononuclear cells *in vitro*

The expression of 15 IFI genes (IFIT1, IFIT3, IFIT5, IFI6, IFI16, IFI27, IFI30, IFI35, interferon-induced transmembrane protein 1, ISG15, IRF7, OAS1, OASL, GBP1, and GBP2) in PBMCs upon stimulation were measured. All of these genes were upregulated upon IFNα stimulation, while only some were upregulated by IFNγ (data not shown). On the other hand, TNF also showed a repressive effect on the expressions of most IFI genes in PBMCs *in vitro* in this experiment.

The relative expressions of three of the representative genes (that is, IFIT1, IFIT3, and IFI27) from three women are shown in Figure 4. A remarkable suppression was observed through the TNF and IFNα co-stimulating experiment (Figure 4a). On the other hand, there was synergism between IFNγ and IFNα on IFI gene expressions, although with some exceptions like IFIT1 (Figure 4b). IFIT1 was downregulated upon IFNγ and IFNα co-stimulation, unlike stimulation with IFNα alone. E2 showed no significant or consistent interaction with IFNα for most of the IFI genes. Inconsistent responses to E2 stimulation, however, were observed among the three healthy donors on IFI27. E2 tended to downregulate IFI27 expression in one donor but upregulated expression in the other two donors (Figure 4c).

To test a hypothesis that TNF decreases IFI gene expression through suppressing IFNα production, we examined the effect of TNF or IFNα on IFNα mRNA expression. Its expression was too low to be measured and there were no significant changes in TNF, IFNα, or TNF + IFNα 24-hour-stimulated PBMCs.

Figure 3



Effect of TNF stimulation on gene expression in healthy individuals and systemic lupus erythematosus patients. Peripheral blood mononuclear cells (PBMCs) from six systemic lupus erythematosus (SLE) patients and three healthy individuals (HI) were isolated and stimulated for 24 hours in the absence and presence of 20 ng/ml TNF. The relative mRNA expressions (RE) compared between TNF-stimulated and non-stimulated control individuals were measured using quantitative RT-PCR. The RE of seven downregulated genes (highlighted in green) and 12 upregulated genes (highlighted in red) are designated by five colors as shown. See Table 1 for gene identification.

Discussion

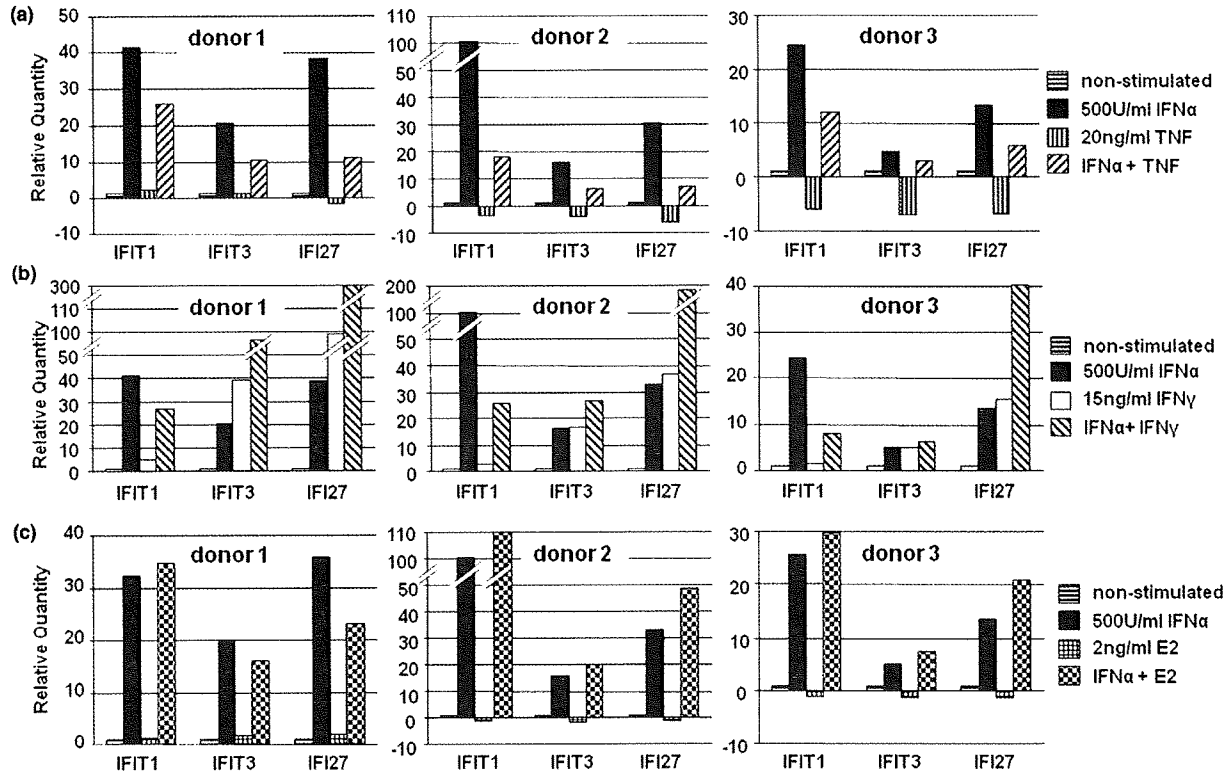
To identify the molecules involved in the aberrant immune system of SLE, we compared the gene expression profiles of peripheral blood between SLE patients and healthy individuals using microarray technology followed by gene ontology analysis. Most previously reported SLE studies utilizing microarray analysis have used PBMCs, but in the present study we used whole blood from SLE patients to exhaustively analyze the gene expression profiles of immune response-related molecules *in vivo*. Despite an additional proportion of granulocytes (mainly neutrophils), our results showed that there was an overexpression of several interferon-regulated genes. This result was in agreement with a previous report showing that peripheral blood from SLE patients had remarkably homogeneous gene expression patterns with an overexpression of IFI genes [10], and confirms the involvement of interferon in SLE.

Since the immune system is regulated by an elaborate network, interactions among the downregulated genes and the upregulated genes of the immune response category were further investigated by utilizing network-based analysis. A cluster of the TLR family (that is, TLR1, TLR2, TLR4, and TLR5) and another cluster of FcγRs were upregulated and depicted in the same network, which had p38 MAPK and NFκB at the center. Our finding that FcγR genes were overexpressed in the peripheral blood of SLE patients is novel, although the overexpression of TLR genes has been recently reported [23]. Furthermore, this is the first report showing that these clusters possibly interact with each other through p38 MAPK and NFκB signaling pathways in a network, and consequently contribute to SLE. Indeed, it has been shown that FcγRIIb is a gene susceptible to SLE both in humans and mice [24]. Means and Luster have reported that a functional interaction between TLR9 and CD32 (also known as FcγRIIa) may be involved in the pathogenesis of SLE, and they also have suggested the possibility that TLR7 may activate cells through similar pathways [25]. Although in our study overexpression of DNA-recognizing TLR9, which has been suggested to be triggered by immune complexes containing DNA in SLE [26,27], was not statistically significant according to the rank test, seven out of the 11 SLE patients showed upregulated expressions of TLR9. In addition, TLR1, TLR2, TLR4, and TLR5 – which serve to recognize bacterial components such as lipopolysaccharide or lipopeptides [28,29] – were also upregulated. Our network-based analysis therefore suggested the hypothesis that the interaction between TLRs and FcγRs is involved in the pathogenesis of SLE.

We additionally found that networks whose central molecule was TNF, IFNγ, or E2 were represented by both the downregulated genes and the upregulated genes in the functional category of immune response. This observation suggested that TNF, IFNγ, or E2 may be involved in the abnormal expressions of both downregulated and upregulated genes in the immune response. Indeed, the elevated level of some cytokines such as TNF and interferon in the sera of SLE patients has been reported [2,4,30,31]. Although our data did not show a significant increase in the gene expressions of TNF, IFNγ, or IFNα in themselves according to rank test, more than one-half of the SLE patients' individual data showed an increase in the TNF gene expression in our study (data not shown). For IFNα, the expression was not increased in the peripheral blood but it may be produced at the other site. Siegal and colleagues have demonstrated that purified interferon-producing cells were $CD4^+CD11c^-$ type 2 dendritic cell precursors, which produce 200 to 1,000 times more IFNα than other blood cells after a microbial challenge [32]. E2 is enzymatically synthesized in the ovary, and therefore does not transcript and cannot be detected in peripheral blood in the present study. There is, however, a 10 to 15 times higher frequency of SLE in women during childbearing years, probably due to an estrogen hormonal effect [33]. We therefore believe these results are a good reason to further investigate E2 involvement in SLE pathogenesis.

Concerning the interaction between cytokines, to our knowledge this is the first report showing that TNF has a repressive effect on IFI genes *in vitro*. Although the exact mechanisms of

Figure 4



Effect of cytokines or β-estradiol on the expressions of interferon-inducible genes. Peripheral blood mononuclear cells from three healthy donors were cultured with the indicated cytokines for 24 hours. RNA was analyzed by quantitative RT-PCR as described in Materials and methods. Relative expression of the indicated genes – interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), and interferon alpha-inducible protein 27 (IFI27) – compared with their nonstimulated cultures is shown. Each bar represents the mean value of duplicate wells as compared with the nonstimulated control. Downregulated genes were arbitrarily assigned a negative value.

the IFI gene product involvement in SLE pathogenesis are still poorly understood, we suspect that the elevated expression of TNF in SLE reduces the overexpression of IFI genes. Since serum levels of both TNF and IFNα were reportedly elevated in SLE, as mentioned above, it is possible that the increased serum TNF level in SLE is an outcome to compensate the immune system balance altered by IFNα in SLE. Consider that patients with rheumatoid arthritis or Crohn's disease under TNF-blocking therapies can develop autoantibodies to nuclear antigens [34]; therapeutic TNF blockades could thus lead to an exacerbation of certain autoimmune diseases such as SLE and to provoke lupus-like manifestations. Palucka and colleagues reported recently that blocking TNF signaling increases the production of IFNα by plasmacytoid dendritic cells and induces an IFN signature in the blood of arthritis patients [35]. This may be another mechanism for TNF inhibitor to induce the IFN signature. We confirmed that there was no significant effect, however, of TNF on IFNα gene expression in the PBMCs in our experiment. Furthermore, the 500 units/ml IFNα we used for stimulation is obviously a higher amount than endogenously produced IFNα. TNF therefore appeared to directly suppress IFI gene expression in PBMCs. We suggest that the direct suppressive effect of TNF on the IFN signature induced by IFNα, at least, exists in the network regulation of cytokines *in vivo*.

The results of the co-stimulating experiments did not show any strong evidence of a functional interaction between E2 and IFNα on the expression of IFI genes. Inconsistent gene expression patterns were observed in the co-stimulating experiments, possibly due to the hormonal effects of the women donors. The modulation of estrogens on humoral immune response seems to be greatly dependent on its physiological concentration, and E2 is a versatile hormone that plays a wide variety of roles in our body [36]. We therefore cannot exclude the possibility that E2 also plays a significant role in the pathophysiology of SLE.

Conclusion

TNF may have a counter effect on the abnormal regulation of IFNα on the immune response-related gene expressions, while IFNγ may have a synergistic effect with IFNα in SLE. Interactions between IFNα and one of TNF, IFNγ, or E2 had a suggested involvement in the pathogenesis of SLE.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

H-ML and TM contributed equally to this work. H-ML performed data analysis, interpretation of the microarray studies, sample preparation, stimulating and co-stimulating experiments, RNA purification, quantitative RT-PCR assays, and drafted of manuscript. TM performed data analysis, interpretation of the microarray studies, and patient recruitment. HS assisted with data analysis. CA performed labeling and scanning of the microarrays. YA assisted with data analysis. NY-H assisted with data analysis. KM assisted in microarray data acquirement. NN designed the study, enrolled patients, and assisted with data analysis and interpretation. All authors read and approved the final manuscript.

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