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INTERLEUKIN-10 BLOCKADE CORRECTS IMPAIRED IN VITRO CELLULAR IMMUNE RESPONSES OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS

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Objective. **Many systemic lupus erythematosus (SLE) patients display impaired cellular immune responses against allo- or recall antigens. Given the down-regulating properties of interleukin-10 (IL-10) on antigen-presenting cell functions, this study was undertaken to investigate whether the well-known overproduction of IL-10 by SLE peripheral blood mononuclear cells (PBMC) was involved in this process.**

Methods. **We measured the proliferation of SLE or control PBMC against irradiated allogeneic dendritic cells in the absence or presence of antibodies blocking IL-10 activity, or in the absence or presence of IL-12.**

Results. **As a group, SLE PBMC proliferated against allogeneic targets less than control PBMC. However, SLE patients could be categorized as good responders or poor responders according to the amplitude of their allogeneic response. Interestingly, serum IL-10 concentrations were significantly higher in the poor responders than in the good responders or in the controls, and addition of antibodies blocking IL-10 activity significantly increased the proliferative responses of the group. We confirmed the role of IL-10 in the impaired allogeneic responses displayed by SLE PBMC by demonstrating that addition of IL-10–containing SLE PBMC supernatants inhibited a normal allogeneic response between unrelated healthy controls, and by showing that this inhibitory effect was commensurate with the concentrations of IL-10 measured in the supernatants. In this experimental setting, we also demonstrated that IL-10–containing SLE PBMC supernatants inhibited IL-12 p35 and IL-12 p40 gene expression. Consistent with the last observation, we found that addition of exogenous IL-12 restored the proliferation of poor-responder SLE patients' PBMC.**

Conclusion. **Taken together, these results indicate that dysregulation of the IL-10/IL-12 balance plays a critical role in the impaired cellular immune responses observed in SLE patients.**

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by overt B cell activation that results in both polyclonal hypergammaglobulinemia and specific autoantibody production (1). Contrasting with these inappropriate humoral immune responses, many SLE patients have impaired cellular immune reactions (2,3). Thus, Tsokos et al demonstrated that SLE peripheral blood mononuclear cells (PBMC) proliferated less in response to allogeneic targets than did control PBMC, and that their in vitro production of interferon-γ (IFNγ) upon exposure to recall antigens was decreased (4). Moreover, those investigators provided experimental evidence that antigen-presenting cell (APC) function was abnormal in SLE patients by showing that IFNγ-induced B7-1 (CD80) expression on lupus APC was significantly reduced compared with controls. Accordingly, the impaired responses to recall antigens could be corrected in the presence of CD80-transfected murine cells, thereby indicating that low CD80 expression on APC accounted for the defective cellular immune responses observed in

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SLE. While the clinical significance of these observations is still largely unknown, impaired cellular immunity might contribute to the increased susceptibility to infection observed in SLE patients (5,6).

Interleukin-10 (IL-10) is a pleiotropic cytokine that plays a critical role in human SLE. The cytokine is spontaneously produced in huge amounts by SLE PBMC (7), and serum IL-10 titers correlate with clinical and serologic disease activity indices (8). More importantly, spontaneous in vitro immunoglobulin production by SLE PBMC is dramatically inhibited by adding blocking anti–IL-10 antibodies. Similarly, anti-DNA antibody titers are reduced by IL-10 blockade in SCID mice reconstituted with SLE PBMC (9). However, IL-10 is a potent inhibitor of dendritic cell (DC) function, as shown by its down-regulation of both IL-12 production and expression of HLA class II and costimulatory molecules (10–13). Taken together, these observations led us to consider the possibility that the impaired cellular immunity observed in SLE patients might be related to IL-10 overexpression.

Here we confirm that SLE PBMC display impaired cellular immune responses against allogeneic stimuli, and we show that this defect can be corrected in vitro by IL-10 blockade or IL-12 supplementation.

PATIENTS AND METHODS

Patients. Fourteen consecutive SLE patients fulfilling the American College of Rheumatology classification criteria (14) were selected for this study. All had active disease, defined as a Safety of Estrogens in Lupus Erythematosus: National Assessment (trial)–SLE Disease Activity Index (SLEDAI [15]) score of ≥1, and most had flares of their disease by the time of blood sampling. Their clinical and treatment data are summarized in Table 1.

Cytokines and antibodies. Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF; molgramostim) was purchased from Novartis (East Hanover, NJ), while rhIL-4 and rhIL-12 were kind gifts from Schering-Plough Research Institute (Kenilworth, NJ) and M. Gately (Hoffmann-LaRoche, Nutley, NJ), respectively. A caprine polyclonal antibody blocking hIL-10 (anti–hIL-10) and a murine monoclonal antibody blocking hIL-10 receptor (anti–hIL-10R) were obtained from R&D Systems (Minneapolis, MN). A caprine polyclonal antibody (anti-MAGE3) and a murine monoclonal antibody (antitrinitrophenol) (both obtained from J. Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium) were used as control antibodies.

Cell cultures and allogeneic reactions. *DC*. PBMC from healthy donors were isolated by Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation, stored at −80°C in DMSO, thawed in the presence of DNase (5 IU/ml; Sigma, St. Louis, MO) to avoid excessive cell clumping during the thawing procedure, and left to adhere for 2 hours at 37°C at a density of 5×10^6/ml. Nonadherent cells were discarded, and adherent cells were cultured in the presence of IL-4 (100 IU/ml) and GM-CSF (100 ng/ml) in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-arginine (0.55 m*M*), L-asparagine (0.24 m*M*), and L-glutamine (1.5 m*M*) (referred to as complete RPMI medium). Cultures were fed on days 2 and 4 by replacing half of the medium with fresh medium supplemented with cytokines. On day 7, the nonadherent cell population contained >95% DC, as assessed by flow cytometry (CD11c+,CD14− cells).

PBMC. PBMC from healthy donors or SLE patients were isolated by Lymphoprep (Nycomed Pharma) density gradient centrifugation, stored at −80°C in DMSO, and thawed in the presence of DNase before being used in allogeneic reactions. For some experiments, thawed PBMC were seeded (3×10^6/well) in 24-well plates in complete RPMI medium, and culture supernatants were harvested after 24 hours.

Allogeneic reactions. Control or SLE PBMC (2.5×10^5/well) were cocultured in quadruplicate, flat-bottomed microtiter plates with allogeneic, irradiated (5,000 rads) DC (5×10^3/well) in the presence or absence of neutralizing polyclonal anti–hIL-10 or monoclonal anti–hIL-10R or their respective control antibodies (25 μg/ml). Alternatively, exogenous IL-12 was added at a concentration of 5 ng/ml. On day 4, supernatants were harvested for determination of cytokine concentrations, and proliferations were measured after an overnight pulse with 0.5 μCi ^{3}H-thymidine (Amersham Life Science, Little Chalfont, UK). In some experiments, PBMC (2.5×10^5/well) from a healthy donor were cultured in the presence of irradiated DC (5×10^3/well) from another healthy donor in the presence of SLE PBMC culture supernatants. On day 1, half of the cells were lysed in TriPur (Boehringer, Mannheim, Germany), and total RNA was purified by chloroform extraction. On day 4, proliferative responses of the remaining cells were measured in quadruplicate after an overnight pulse with 0.5 μCi ^{3}H-thymidine.

Table 1. Patient characteristics and treatment*

Patient/ age/sex	SELENA–SLEDAI score	Anti-DNA titer (Farr assay), IU/ml	Treatment (mg/day)
1/31/F	32	62	None
2/38/F	6	72	Pred. (5), HCQ (200)
3/36/F	8	137	Pred. (5), HCQ (400)
4/25/F	24	738	Naproxen (1,000)
5/22/F	6	62	CQ (100)
6/46/M	14	14	HCQ (50)
7/39/F	19	1,750	Pred. (7.5)
8/32/F	9	210	CQ (50)
9/14/F	27	4,352	None
10/36/F	10	13	AZA (125), Pred. (30)
11/29/F	2	5	Pred. (5), CQ (100)
12/19/F	31	2,280	None
13/54/F	10	374	None
14/42/F	16	25	Pred. (25), HCQ (200)

* SELENA-SLEDAI = Safety of Estrogens in Lupus Erythematosus: National Assessment (trial)–Systemic Lupus Erythematosus Disease Activity Index; Pred. = prednisolone; HCQ = hydroxychloroquine; CQ = chloroquine; AZA = azathioprine.

Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analyses. DNA was synthesized from total RNA by using oligo(dT) primers (Boehringer) and murine Moloney leukemia virus RT (Life Technologies, Grand Island, NY). Serially diluted complementary DNA (cDNA) was amplified by PCR using recombinant *Taq* DNA polymerase (Takara Shuzo, Shiga, Japan) and specific primers for human β-actin (5′-GCTGGAAGGTGGACAGCGAG-3′; 3′-TGGCATCGTGATGGACTCCG-5′), IL-12 p35 (5′-CTTCACCACTCCCAAAACCTG-3′; 3′-AGCTCGTCACTCTGTCAATAG-5′), IL-12 p40 (5′-TCACCTGGACCTTGGACCAG-3′; 3′-GCATGAAGAAGTATGCAGAG-5′), CD80 (5′-GTCCAAATTGTTGGCTTTCA-3′; 3′-CCCTAGTACTCCGTAAGAAG-5′), and CD86 (5′-TGATTCGGACAGTTGGACCCTGACAC-3′; 3′-CGCCGAAAATAGAAGTGGAA-5′).

PCR was performed as follows: 1 minute at 94°C; then 1 minute at 56°C for β-actin, 52°C for IL-12 p35 and IL-12 p40, 48°C for CD80, and 50°C for CD86; and then 2 minutes at 72°C for 20 cycles (β-actin) or 30 cycles (IL-12 p35, IL-12 p40, CD80, and CD86). The PCR products were blotted on nylon filters and hybridized with ^{32}P-labeled internal oligonucleotides specific for β-actin (5′-TTCACCACCACGGCCGAGCGG-3′), IL-12 p35 (5′-TTAGTAGTATTTATGAAGAC-3′), IL-12 p40 (5′-GGCTCTGGCAAAACCCTGAC-3′), CD80 (5′-TTCAAGCGGGAACACCTGG-3′), or CD86 (5′-GTATCAATGTATCATCCATCAC-3′). Net counts were measured on a phosphoscreen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA) for each cDNA dilution, and ratios of the IL-12 p35, IL-12 p40, CD80, or CD86 counts to the β-actin counts were calculated for dilutions where amplification was linear.

Determination of cytokine concentrations. Serum IL-10 titers were measured in SLE patients and controls using a commercial enzyme-linked immunosorbent assay (ELISA) (Cytoscreen US; Biosource International, Camarillo, CA). For SLE patients, serum was sampled on the same day as PBMC. IL-10 titers were also measured in SLE or control PBMC supernatants using the same ELISA.

Statistical analysis. Data were analyzed using Mann-Whitney or Student *t*-tests.

RESULTS

In a first set of experiments, we investigated whether SLE PBMC displayed impaired cellular immune responses against allogeneic stimuli. We compared the proliferation of SLE or control PBMC in response to irradiated allogeneic APC prepared from randomly selected healthy donors. As a group, SLE PBMC proliferated less than control cells (Figure 1A). However, as indicated in Figure 1B, patients could be classified as poor responders or good responders according to the intensity of the proliferation of their PBMC against allogeneic targets. Patients were categorized as poor responders if proliferation of their PBMC was more than 2 SD below the mean response observed in controls. The mean SLEDAI score did not differ between patients classified as poor responders or good responders. Although the mean anti-DNA antibody titers were considerably more elevated in poor responders than in good responders (mean ± SEM 1,126 ± 554 IU/ml versus 181 ± 112 IU/ml), the difference was not statistically significant ($P = 0.5$).

Given the well-known inhibitory effect of IL-10 on cellular immune reactions and the well-described

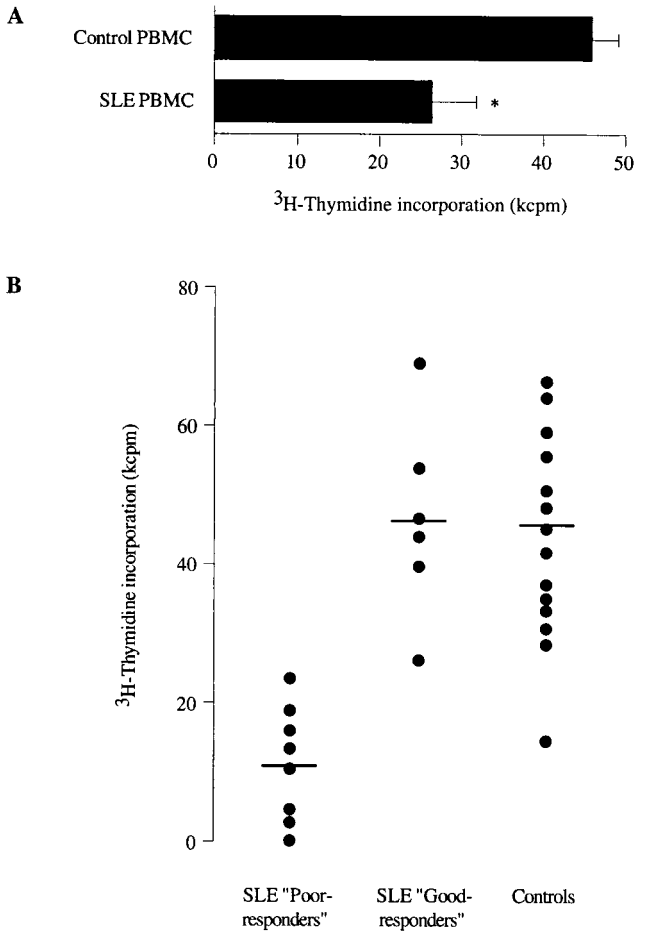

Figure 1. Proliferative responses of systemic lupus erythematosus (SLE) or control peripheral blood mononuclear cells (PBMC) versus allogeneic dendritic cells (DC). PBMC (2.5×10^5/well) were cultured in the presence of irradiated allogeneic DC (5×10^3/well) in microtiter plates. After 5 days, proliferations were measured in quadruplicate cultures by ^{3}H-thymidine incorporation. **A,** Results are expressed as the mean and SEM proliferation observed with control (n = 14) or SLE (n = 14) PBMC. **B,** Using a cutoff value defined as the mean proliferative response of the controls − 2 SD, SLE patients' PBMC could be divided into "Good responders" (n = 6) or "Poor responders" (n = 8), corresponding to patients 1–6 and patients 7–14, respectively (see Table 1). * = $P < 0.05$.

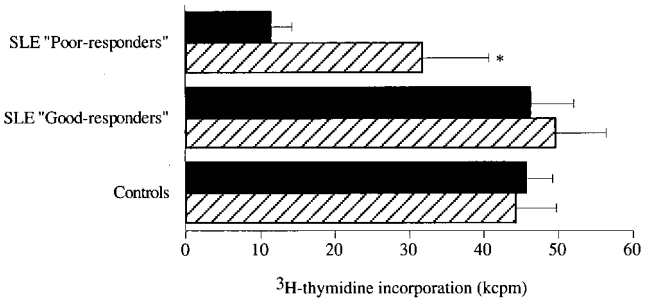

Figure 2. Effect of interleukin-10 (IL-10) blockade on allogeneic responses of SLE or control PBMC. PBMC (2.5×10^5/well) were cultured in the presence of irradiated allogeneic DC (5×10^3/well) with (hatched bars) or without (closed bars) addition of a blocking anti–IL-10 or anti–IL-10 receptor (anti–IL-10R) antibody. Proliferations were measured in quadruplicate cultures after 5 days by ^{3}H-thymidine incorporation. Results are expressed as the mean and SEM proliferation observed in SLE "Poor responders" (n = 8), SLE "Good responders" (n = 6), and controls (n = 14). In 4 controls and 4 SLE patients (2 Good responders and 2 Poor responders), an anti–IL-10R monoclonal antibody was used instead of an anti–IL-10 polyclonal antibody. The results were pooled, since they did not differ according to the antibody used. For instance, addition of the anti–IL-10 antibody increased the mean ± SEM allogeneic proliferation of 6 Poor responder SLE patients' PBMC from 12,164 ± 3,669 cpm to 35,728 ± 11,686 cpm, while addition of the anti–IL-10R antibody increased the proliferation of 2 additional Poor responder SLE patients' PBMC from 9,045 ± 4,364 cpm to 19,975 ± 4,070 cpm. Control antibodies had no effect on ^{3}H-thymidine incorporation. * = $P < 0.01$. See Figure 1 for other definitions.

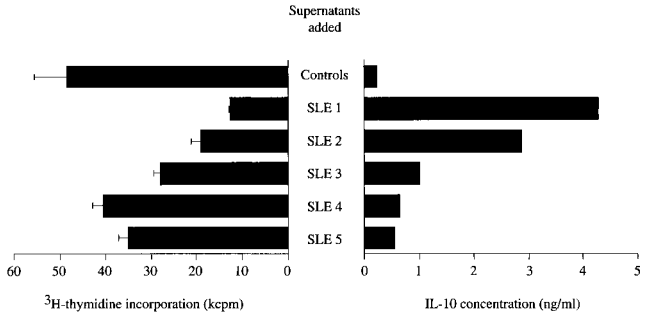

Figure 3. Effect of SLE or control supernatants on a normal allogeneic response. Control (n = 5) or SLE (n = 5) PBMC supernatants, obtained as described in Patients and Methods, were added (at a 3:4 dilution) to an allogeneic reaction between PBMC and irradiated DC obtained from 2 unrelated healthy donors. Proliferative responses (left panel, mean and SEM of quadruplicate cultures) were measured after 5 days by ^{3}H-thymidine incorporation. Interleukin-10 (IL-10) concentrations (right panel) were measured by enzyme-linked immunosorbent assay in the same supernatants. Data from the 5 control supernatants were pooled. See Figure 1 for other definitions.

overexpression of this cytokine in SLE, we investigated whether the impaired allogeneic response observed with poor responders' SLE PBMC was related to increased IL-10 production. Accordingly, serum IL-10 titers were found to be significantly higher in the poor responders (mean ± SEM 132 ± 78 pg/ml; n = 7) than in the good responders (mean ± SEM 11 ± 4 pg/ml; n = 6) or in the controls (mean ± SEM 11 ± 2 pg/ml; n = 14) ($P < 0.05$ for both). Moreover, addition of neutralizing anti–hIL-10 or anti–hIL-10R antibodies significantly increased the level of proliferation of poor responders' PBMC, while it had no effect on good responders' PBMC or on control PBMC (Figure 2).

We indirectly confirmed the role of IL-10 on impaired cellular immune responses displayed by SLE PBMC using a different experimental setting. We tested whether culture supernatants from SLE PBMC inhibited a normal allogeneic response between 2 unrelated controls. Compared with control PBMC supernatants (n = 5), addition of SLE PBMC supernatants (n = 5) significantly reduced the proliferation rates (mean ± SEM 26,948 ± 5,071 counts per minute versus 48,430 ± 7,293 cpm; $P < 0.05$). For SLE PBMC supernatants, this inhibitory effect was commensurate with the concentrations of IL-10 measured in the supernatants (r = 0.94) (Figure 3).

To investigate the mechanisms underlying the inhibitory effects of SLE PBMC supernatants, we tested whether their addition to a standard allogeneic reaction inhibited IL-12 p35, IL-12 p40, CD80, and CD86 gene expression. As indicated by the semiquantitative PCR analyses shown in Figure 4, IL-12 p35 and IL-12 p40 messages were down-regulated by IL-10–containing

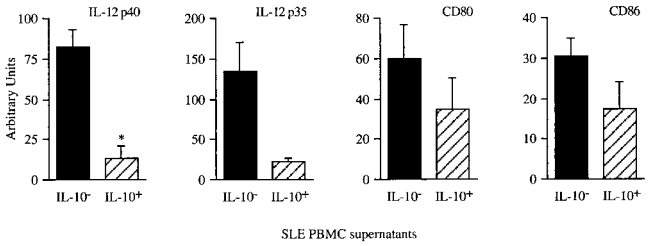

Figure 4. Effect of SLE PBMC supernatants on interleukin-12 (IL-12), CD80, and CD86 gene expression. PBMC from healthy donors were cultured with irradiated allogeneic DC in the presence of SLE PBMC supernatants (at a 3:4 dilution) that contained 2.8 ng/ml and 3.6 ng/ml IL-10 (IL-10$^+$, hatched bars; n = 2) or 0.3 ng/ml and 0.2 ng/ml IL-10 (IL-10$^-$, closed bars; n = 2). Semiquantitative reverse transcriptase–polymerase chain reaction analyses were performed as described in Patients and Methods. Results are expressed as mean and SEM arbitrary units, calculated as the ratio (× 100) of the IL-12 p40, IL-12 p35, CD80, or CD86 counts to the β-actin counts. * = $P < 0.05$. See Figure 1 for other definitions.

Table 2. Effect of addition of interleukin-12 (IL-12) on allogeneic responses of systemic lupus erythematosus (SLE) or control peripheral blood mononuclear cells (PBMC)*

	Allogeneic proliferation, cpm	
	No additive	IL-12
SLE patients		
Poor responders (n = 5)	15,255 ± 2,477	35,038 ± 3,554†
Good responders (n = 5)	43,000 ± 5,549	53,413 ± 8,604
Controls (n = 7)	39,743 ± 3,903	49,414 ± 5,033

* Values are the mean ± SEM. PBMC (2.5×10^5/well) were cultured in the presence of irradiated dendritic cells (5×10^3/well) with or without 5 ng/ml IL-12. Proliferations were measured in quadruplicate cultures after 5 days by ^{3}H-thymidine incorporation.
† $P < 0.01$ versus no additive.

SLE PBMC supernatants, but not by SLE PBMC supernatants that contained very low amounts of IL-10. No similar effect was observed on CD80 or CD86 expression.

Finally, the observation that IL-10–containing SLE PBMC supernatants inhibited IL-12 expression suggested that the impaired allogeneic responses displayed by poor responders' SLE PBMC could be corrected by exogenously added IL-12. Accordingly, as shown in Table 2, the allogeneic response of PBMC obtained from SLE patients classified as poor responders was increased by IL-12, while addition of the cytokine had no effect on the allogeneic response of PBMC isolated from controls or good-responder SLE patients.

DISCUSSION

The data presented here indicate that the impaired in vitro allogeneic response observed in a subset of SLE patients can be corrected by IL-10 blockade or by IL-12 supplementation. These results, together with those obtained with IL-10–containing SLE PBMC supernatants added to a normal allogeneic reaction, suggest a causal relationship between overexpression of IL-10, down-regulation of IL-12, and impaired cellular immune responses.

Imbalance between IL-10 and IL-12 is a well-known immunologic feature of SLE (16), but has never been shown to be involved in the impaired in vitro cellular immune responses of SLE patients. Thus, Liu et al demonstrated that SLE PBMC and monocytes produce less IL-12 in vitro (17–19). Moreover, they showed that addition of a neutralizing anti–IL-10 antibody significantly reversed this impaired production of IL-12. Since IL-12 triggers type 1 immune responses, it was tempting to speculate that IL-10–induced down-regulation of IL-12 was involved in the impaired cellular immunity observed in SLE.

In a first set of experiments, we confirmed that a subset of SLE PBMC from patients referred to as poor responders displayed impaired proliferative responses against irradiated allogeneic APC. Next, we demonstrated that serum from these patients contained much more IL-10 than that from patients whose PBMC displayed normal allogeneic responses. Surprisingly, IL-10 titers measured in supernatants of PBMC responding against allogeneic APC did not differ between good- and poor-responder SLE patients (data not shown), a discrepancy that might be explained by IL-10 consumption.

Two lines of evidence further indicated that IL-10 production by SLE PBMC was involved in inhibiting their proliferative responses against allogeneic APC. First, the addition of antibodies blocking IL-10 activity specifically restored the proliferation of the poor responders' PBMC. Second, we found that the addition of SLE PBMC supernatants inhibited the proliferation of normal PBMC against allogeneic APC compared with control PBMC supernatants. This inhibitory effect correlated positively with the amounts of IL-10 measured in the supernatants.

IL-10 is known to display inhibitory effects on APC functions (20). It inhibits DC maturation and cell surface expression of HLA class II or costimulatory (CD86) molecules (10). De Smedt et al (13) further demonstrated that IL-10 inhibits IL-12 production by DC derived from adherent PBMC. We therefore evaluated whether the inhibitory effects of IL-10 on the proliferative responses of SLE PBMC against allogeneic APC were mediated by down-regulation of IL-12 or B7 expression. Using semiquantitative RT-PCR analyses, we could demonstrate that addition of IL-10–containing SLE PBMC culture supernatants clearly inhibited IL-12 p35 and IL-12 p40 expression in a standard allogeneic reaction compared with SLE culture supernatants that contained very low amounts of the cytokine. No effect on CD80 or CD86 expression could be detected, although we could not rule out the possibility that growth factors present in SLE PBMC culture supernatants or in the FCS might have obscured an inhibitory effect. Interestingly, we found that addition of exogenous IL-12 restored the proliferation of poor responders' PBMC against allogeneic APC, while it had no significant effect on the proliferation of good responders' SLE PBMC or control PBMC.

Taken together, our results indicate that IL-10

inhibits the proliferative responses of SLE PBMC against allogeneic APC, and suggest that correction of the IL-10/IL-12 imbalance may restore normal cellular immune responses in this condition.

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