Interleukin-21 is increased in active systemic lupus erythematosus patients and contributes to the generation of plasma B cells

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Abstract

Objectives

Excessive interleukin- (IL-) 21 production by T cells has been implicated in the pathogenesis of systemic lupus erythematosus (SLE). We explored the expression and function of IL-21 in human SLE.

Methods

IL-21 and IL-21 receptor (IL-21R) expression was assessed by real-time PCR and flow cytometry in peripheral blood mononuclear cells (PBMCs) of SLE patients and healthy controls. PBMCs, purified $CD19^+CD27^-$ naïve and $CD19^+CD27^+$ memory B cells were stimulated with IL-21 and CpG-ODN2006 (TLR-9 agonist) to examine generation of memory and plasma ($CD19^+CD38^{high}IgD^-$) B cells. Apoptosis was assessed by 7AAD staining.

Results

Active SLE patients had 4-fold higher IL-21 mRNA and increased levels of intracellular IL-21 in peripheral blood $CD4^+$ T cells (mean±SD fluorescence intensity, 1.7±0.1 in active versus *0.9±0.3 in inactive SLE and controls, p=0.035). IL-21R mRNA was comparable between SLE and healthy controls. Stimulation of PBMCs with IL-21 increased the proportion of memory and plasma cells; addition of CpG-ODN2006 enhanced these effects. Both naïve and memory B cells responded to IL-21/TLR-9 by increased generation of memory and plasma B cells, respectively; an anti-apoptotic effect was observed. In active SLE, PBMCs stimulation with IL-21 and/or CpG-ODN increased memory and plasma B cells, comparable to healthy controls. Addition of IL-21 to lupus autologous mixed lymphocyte cultures induced significant IgG production, and treatment with IL-21R.Fc to block IL-21/IL-21R interaction reduced the proportion of plasma cells.*

Conclusion

Increased IL-21 may synergise with TLR-9 signalling and contributes to the generation of plasma cells in active SLE patients.

Key words

autoimmunity, cytokines, T-cells, immunoglobulin, plasma cells

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Introduction

Systemic lupus erythematosus (SLE) is characterised by an antigen-driven, T cell-mediated production of autoantibodies by B cells. T-B cell interactions to promote plasma cell (PC) differentiation and immunoglobulin (Ig) production involve cell surface molecules such as CD40/CD40 ligand (CD40L) (1), and T cell-derived soluble factors such as IL-21 (2).

IL-21 is a pleiotropic cytokine produced by $CD4^+$ T cells (including Th17 and follicular $CD4^+$ T cells) that affects the differentiation and function of T, B and NK cells by binding to a receptor consisting of the common γ-chain and the IL-21 receptor (IL-21R). IL-21 has a non-redundant role in promoting B cell activation, survival, and differentiation during humoral immune response (3). In mouse SLE models, IL-21 contributes to disease pathogenesis by inducing PC differentiation (4), and IL-21 inhibition via a soluble inhibitor or IL-21R deletion ameliorates lupus nephritis (4, 5). In humans, Sawalha *et al*. have shown a genetic association between IL-21 polymorphisms and SLE (6).

In addition to B cell receptor crosslinking by antigen and $CD4^+$ T cell help, generation of T cell-dependent B cell responses require Toll-like receptor (TLR)-mediated B cell activation (7). In active SLE, we have reported increased percentage of TLR-9 expressing memory and PCs correlating with the presence of anti-dsDNA antibodies (8). In this paper, we explored the effects of IL-21 and TLR-9 activation in the determination of B cell responses in SLE. To this end, we measured IL-21/IL-21R levels in SLE patients and assessed the effect of IL-21 and TLR-9 stimulation on B cell differentiation.

Materials and methods

Patients and healthy controls

A series of immunological studies were performed in blood specimens obtained from SLE patients (n=45) (Table I) and healthy blood donors (n=16), following informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density-gradient centrifugation. To capture patients with higher disease activity, we used a SLE Disease Activity Index (SLEDAI) cut-off of >7 (8). The study was approved by the Ethics Committee of the University Hospital of Heraklion.

B cell isolation and stimulation

$CD19^+$ B cells were immunomagnetically isolated from PBMCs by negative selection (Miltenyi Biotec, Germany), with purity ranging 82–87%. CD27 beads (Miltenyi Biotec) were used to sort purified B cells into $CD27^+$ (memory) and $CD27^-$ (naïve) cells. PBMCs (10^6/ml) or purified B cells (2.5×10^5 cells/ml) were stimulated for 5–7 days with recombinant IL-21 (100ng/ml, BioSource Int.), CpG-ODN2006 (TLR-9 agonist, 2.5μg/ml, InvivoGen, San Diego, CA, USA), poly I:C (TLR-3 agonist, 25μg/ml), zymosan (TLR-2 agonist, 2.5μg/ml), recombinant CD40L (50μg/ml), IL-10 (25 ng/ml), or IL-6 (100 ng/ml). All cell cultures were performed in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 10μg/ml streptomycin, at 37°C/5% CO_2.

Autologous mixed lymphocyte cultures

PBMC $CD3^+$ T cells were isolated from SLE patients and healthy controls using a magnetic bead positive selection system (Pan T Cell Isolation kit, Miltenyi Biotec), with purity ranging 92–95%. Autologous T-B cell co-cultures (both at 2.5×10^5/ml) were set up in 96-well U-bottom plates (200 μl/well), in presence of suboptimal amounts of soluble anti-CD3 mAb (100 ng/ml, OKT3, eBioscience). Recombinant IL-21R. Fc, consisting of the extracellular portion of human IL-21R and the Fc portion of human IgG1 (R&D Systems), was used at a concentration 10 μg/ml to block IL-21/IL-21R interaction (9). In some experiments, cells were stimulated with recombinant IL-21 and/or CpG-ODN2006. On day 5, cells were harvested to evaluate for plasma cell induction (by flow cytometry), and supernatants were collected for IgG measurement.

Real Time PCR

RNA was extracted from PBMCs and purified B cells using the RNeasy kit

Table I. Clinical and demographic characteristics of SLE patients.

Characteristic	n or mean ±SD[1]
Gender (female/male)	42/3
Age (years)	40 ± 12
SLE duration (years)	5 ± 6
ACR classification criteria (number)	5 ± 1
Malar rash	32
Discoid rash	14
Photosensitivity	35
Oral ulcers	15
Non-erosive arthritis	34
Serositis	7
Renal disorder	6
Neurologic disorder	2
Haematologic disorder	13
Immunologic disorder	14
Positive antinuclear antibody	37
SLE activity (inactive/active[2])	29/16
Inactive SLE (SLEDAI)	3.9 ± 2.3
Active SLE (SLEDAI)	9.3 ± 1.3
Medications	
Glucocorticoids	20
Hydroxychloroquine	24
Methotrexate	4
Immunosuppressive[3]	11
None	9

[1]Standard deviation.
[2]Active disease was defined as SLE disease activity index (SELENA-SLEDAI) >7.
[3]Includes azathioprine, mycophenolate mofetil or cyclophosphamide.

(Qiagen). One μg total RNA was reverse transcribed and diluted 1:5 in RNase-free water. Human IL-21 and IL-21R cDNA-specific primers were as follows: IL-21 FW=GAG-TGG-TCA-GCT-TTT-TCC-TGT-T; IL-21 REV=AGG-AAT-TCT-TTG-GGT-GGT-TTT-T; IL-21R FW= CTT-ACC-TGG-CAA-GAC-CAG-TAT-GA, and IL-21R REV= GTA-GAA-GGC-AGG-GTC-TTC-GTA-AT. A 20μl reaction consisted of 5μl diluted cDNA, 1× iTaq SYBR Green master mix (Biorad), and IL-21 or IL-21R primers (0.6 μM each). All reactions were run in triplicates on ABI-prism7000 for 40 cycles (95°C 15 sec, 60°C 1 min) after an initial denaturation step (95°C, 10 min). A relative quantification method using a standard curve was used to quantify gene expression after normalisation to the GAPDH reference gene.

Flow cytometry

B cells were stained for CD19, CD80, CD86, CD268 (BAFF-receptor), CD27, CD38, and IgD (Immunotech, France). In brief, cells were incubated with fluorochrome-conjugated monoclonal antibodies for 30 minutes on ice. Isotype-matched IgG controls were used. Naïve B cells were defined as $CD19^+ CD27^-$; memory B cells as $CD19^+ CD27^+$; PCs as $CD19^+ CD38^{high} IgD^-$. (2) B cell apoptosis was assessed by 7-aminoactinomycin D (7-AAD) immunostaining according to the manufacturer's protocol (Invitrogen). In some experiments, staining for annexin V and propidium iodide (BD Pharmingen) was also performed. All flow cytometric analyses were performed with a FACS Calibur dual laser cytometer (BD Biosciences, San Jose, CA, USA) using CellQuest (BD Biosciences) acquisition and Flowjo (Treestar, Ashland, OR, USA) software.

Intracellular IL-21 and TLR-9 staining

For IL-21 staining, PBMCs were stimulated in RPMI-1640 medium with soluble anti-CD3 mAb (100 ng/ml) for 5 hours. Cytokine release was blocked by brefeldin A (10 ng/ml, Sigma). Cells were treated with BD Cytofix-Cytoperm for 15 min on ice, and were incubated with PE-anti-IL21 and FITC-anti-CD4 antibodies (all from BD Pharmingen). For TLR-9 staining, PBMCs were fixed and permeabilised using BD Cytofix/Cytoperm buffer, followed by staining with PE-anti-TLR9 (eBioscience) and PC5-anti-CD19 antibodies.

ELISA

IL-21 was measured in -70ºC stored serum samples from SLE patients (n=12 with active disease, n=11 with inactive disease) and healthy controls (n=11). Briefly, 96-well plates were coated with 2 μg/mL capture mouse anti-human IL-21 (J148-1134, BD Pharmingen) at 4°C overnight. Plates were washed 4 times with PBS with 0.1% Tween-20 and blocked with PBS containing 3% BSA. Serum aliquots and IL-21 standards were incubated for 4 hours at 37°C. Plates were washed with PBS-Tween and a biotinylated detection antibody (I76-539, BD Pharmingen) was added at 1μg/ml and incubated for 1 hour at 37°C. Plates were again washed and incubated with horseradish peroxidase–conjugated streptavidin (BD Pharmingen) for 30 minutes at room temperature, washed, and developed with TMB substrate (eBioscience), and optical densities were read. IL-21 concentrations were derived from the standard curve. Total IgG was measured in T–B cell co-culture supernatants using the Easy-Titer® IgG Assay Kit (Thermo-Scientific) according to manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software. Data are presented as mean±standard deviation (SD). The non-parametric Kruskal-Wallis test was used to compare more than two independent samples, followed by the Dunn's test for pairwise comparisons. The Mann-Whitney U-test was used to compare two independent samples, and the Wilcoxon signed rank test for paired samples. *p*-values (two-tailed) <0.05 were considered as statistically significant.

Results

Increased IL-21 expression in patients with active SLE

We first examined whether IL-21 and IL-21R are overexpressed in lupus, and we measured their mRNA levels in PBMCs from SLE patients and healthy individuals. Active SLE patients (n=10) had 4-fold increased IL-21 mRNA compared to inactive SLE patients (n=10) and healthy individuals (n=10) (Kruskal-Wallis p=0.016; p<0.05 for both comparisons) (Fig. 1A). Both in total PBMCs and in purified $CD19^+$ B cells, we found no differential expression of IL-21R mRNA in active *versus* inactive SLE patients and healthy controls (Fig. 1B). Since T-helper cells represent a major source of IL-21, we performed flow cytometry in total PBMCs and assessed for intracellular IL-21 staining in $CD4^+$-gated T cells. A very low proportion (<5%) of freshly isolated T cells were $IL\text{-}21^+$, and therefore we quantified IL-21 expression by measuring the relative mean fluorescence intensity (ΔMFI=MFI of IL-21 *minus* MFI of IgG isotype control). IL-21 ΔMFI was increased in active SLE (1.7±0.1, n=4) compared to healthy controls (1.0±0.3, n=3, p=0.035) or inactive SLE (0.9±0.5,

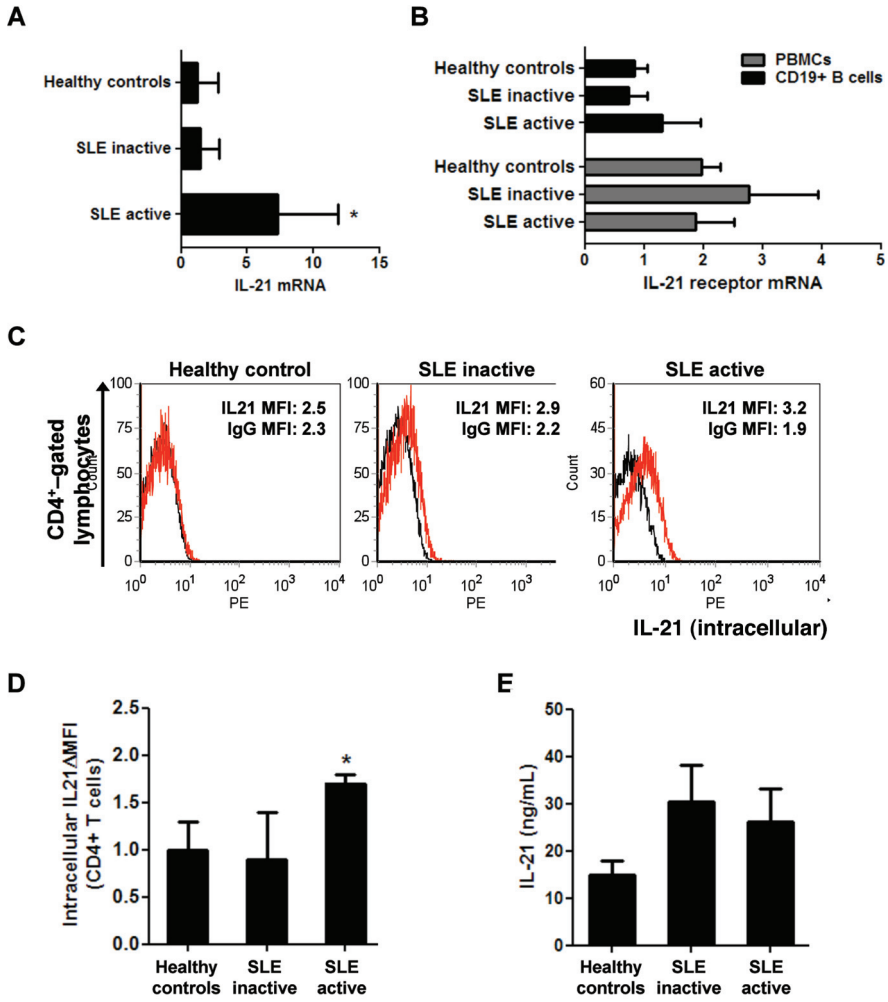

Fig. 1. Increased IL-21 – but not IL-21R – expression in patients with active SLE.
(A) PBMCs from SLE patients and healthy controls were used for total RNA extraction and IL-21 mRNA was assessed by quantitative real-time PCR. Active SLE patients (n=10) exhibit 4-fold increased IL-21 mRNA compared to inactive SLE (n=10) and healthy controls (n=10) (**p<0.05 for both comparisons). **(B)** PBMCs and purified CD19$^+$ B cells from patients and controls were used for total RNA extraction and IL-21R mRNA was assessed by quantitative real-time PCR. We found comparable expression of IL-21R in SLE patients with active (n=8 for PBMCs, n=3 for B cells) and inactive (n=7 for PBMCs, n=4 for B cells) disease and healthy controls (n=9 for PBMCs, n=3 for B cells). **(C)** Intracellular flow cytometry staining for IL-21 was performed in total PBMCs as described in *Materials and Methods*. Isotype (Ig) control markers were used in all experiments and intracellular IL-21 expression was quantified in CD4$^+$-gated T cells as follows: ΔMFI (mean fluorescence intensity) = MFI of IL-21 minus MFI of Ig control. **(D)** Increased mean IL-21 ΔMFI in active SLE (1.7±0.1, n=4) compared to inactive SLE (0.9±0.5, n=4) and healthy controls (1.0±0.3, n=3, p=0.035) (*p<0.05: active SLE *versus* healthy controls). **(E)** No significant variation in serum IL-21 concentrations in healthy controls (n=11) and *versus* patients with inactive (n=12) or active (n=11) SLE.

n=4, p=0.078) (Fig. 1C-1D). Serum IL-21 concentrations were not significantly different in active SLE (n=11) *versus* inactive SLE (n=12) or healthy controls (n=11) (Fig. 1E).

Stimulation of PBMCs with IL-21 increases the proportion of memory and plasma B cells

T-cell-derived IL-21 may exert positive or negative effects on B cell differentiation and survival depending on the concomitant presence of other stimuli, particularly TLR agonists. (10) Based on our result of increased IL-21 in active SLE, we examined the effect of IL-21 alone or in combination with TLR agonists on generation of memory B cells (CD19$^+$ CD27$^+$) and PCs (CD19$^+$ CD38^{high} IgD$^-$), assessed by flow cytometry. Stimulation of PBMCs with recombinant IL-21 and CpG-ODN2006 (TLR-9 agonist) resulted in increased proportion of memory and plasma B cells (Fig. 2A-2B); the combination of both stimuli was more effective than the combination of IL-21 with TLR-2 or TLR-3 agonists, or the combination of TLR-9 with other B-cell growth factors such as IL-6, IL-10, or recombinant CD40-ligand (Fig. 2A-2B and data not shown).

We further explored the effect of IL-21 in separated CD19$^+$ CD27$^-$ naïve and CD19$^+$ CD27$^+$ memory B cells from healthy individuals. Stimulation of naïve B cells with recombinant IL-21 for 5 days induced memory cells as indicated by upregulated surface CD27 expression (Fig. 3A, representative figure from n=3 independent experiments). Addition of CpG-ODN2006 modestly enhanced this effect (right panel). In CD27$^+$ memory-enriched B cells, IL-21 stimulation resulted in increased proportion of CD38^{high} IgD$^-$ PCs (Fig. 3B, representative figure from n=3 independent experiments). IL-21 and TLR-9 activation caused a reduction in the proportion of apoptotic 7AAD$^+$ cells (Fig. 3C-D), and this effect was observed in both CD27$^-$ naïve and CD27$^+$ memory B cell subsets (data not shown). Preliminary experiments show that this effect is accompanied by an increase in levels of activated (phosphorylated) extracellular signal-regulated kinase (ERK) in B cells (data not shown).

Induction of plasma B cells in active SLE patients following stimulation with IL-21

Autoantibody-producing PCs play an important role in lupus pathogenesis. Based on our results of increased IL-21 in active SLE and enhanced B cell differentiation following activation with IL-21 and CpG-ODN2006, we examined the effect of IL-21/TLR-9 activation on generation of memory and plasma B cells in SLE patients. We found that PBMCs from patients with active disease (n=7) and healthy controls (n=4) displayed comparable induction of CD19$^+$ CD27$^+$ memory B cells upon activation with IL-21 and/or TLR-9 (active SLE: 49±4% in IL-21/TLR-9 *versus* 27±4% in untreated; controls: 46±4% in IL-21/TLR-9 *versus* 26±3% in untreated) (Fig. 4A). Combined IL-21 and TLR-9 activation significantly increased the proportion of CD19$^+$ CD38^{high} IgD$^-$ PCs in both active

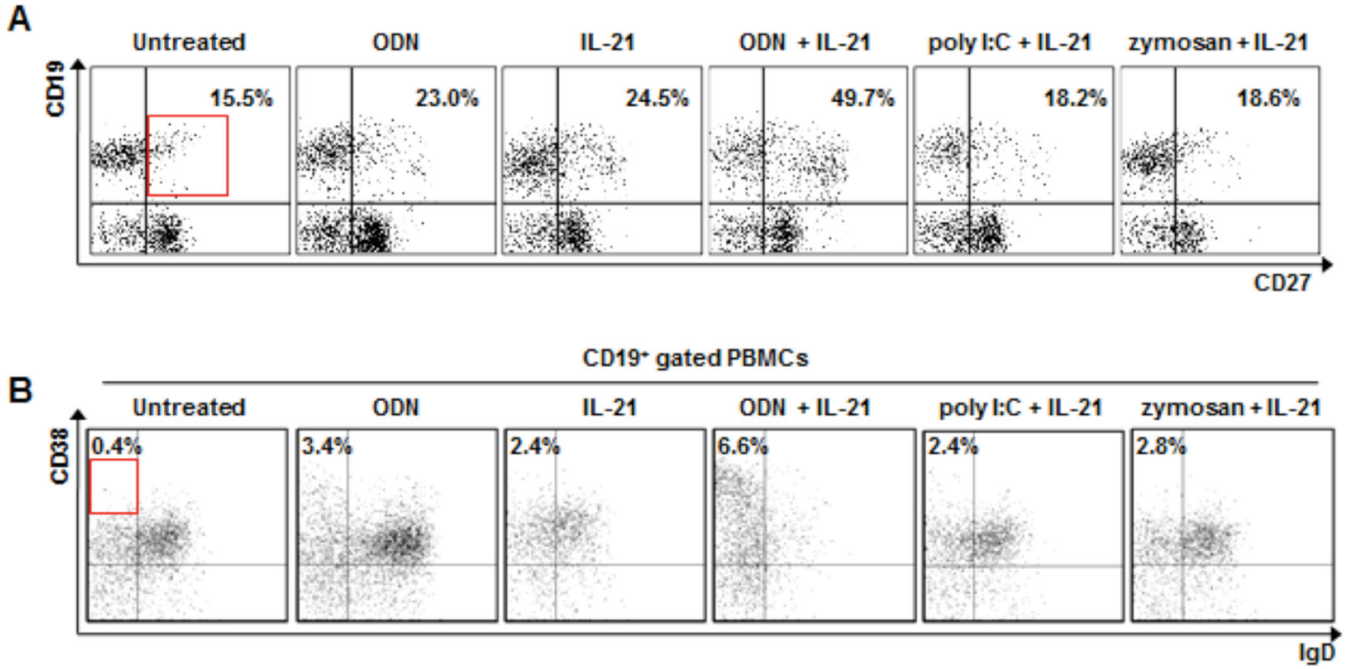

Fig. 2. Stimulation of PBMCs with IL-21 and TLR agonists increases the proportion of memory and plasma B cells.
(A) Healthy control PBMCs (10^6/mL) were left untreated or were stimulated with recombinant IL-21, CpG-ODN2006 (TLR-9 agonist), poly I:C (TLR-3 agonist), zymosan (TLR-2 agonist), IL-6, IL-10, or CD40-ligand as described in *Materials and Methods*. The proportion of memory B cells ($CD19^+CD27^+$) was determined by flow cytometry on day 7. Combined IL-21/TLR-9 activation was more potent than IL-21/TLR-3 and IL-21/TLR-2 activation in inducing the generation of memory B cells. **(B)** Healthy PBMCs were stimulated with IL-21/TLR-9, IL-21/TLR-3, and IL-21/TLR-2 as described in (A), and the proportion of PCs ($CD19^+CD38^{high}IgD^-$) was assessed on day 7. Representative flow cytometry dot plots from n=3 independent experiments. Percentages in panels A and B were calculated using total $CD19^+$ cells as denominator.

SLE patients (8.3±2.0% *versus* 0.3±0.1% in untreated, p=0.006) and healthy controls (8.9±3.4% *versus* 1.3±0.6% in untreated, p=0.05) (Fig. 4A).

To demonstrate the role of IL-21 in the generation of Ig-secreting PCs in SLE patients, we performed autologous mixed lymphocyte cultures using sub-optimally anti-CD3-activated SLE $CD3^+$ T cells to interact with autologous $CD19^+$ B cells. Addition of exogenous IL-21 and/or CpG-ODN2006 in the culture caused a significant increase in secreted IgG (859±287 ng/ml for IL-21, 169±8 ng/ml for ODN2006, 796±528 ng/ml for their combination, results from n=3 patients) (Fig. 4B). In another set of mixed lymphocyte cultures, addition of IL-21R.Fc to block IL-21/IL-21R interaction caused a consistent reduction in the proportion of $CD19^+$ PCs (8.8±1.7% *versus* 6.7±1.7%, p=0.004) (Fig. 4C). Together, these results suggest a role for IL-21 in the generation of Ig-secreting PCs in patients with active SLE.

Discussion

This study shows that IL-21 is upregulated in active SLE patients and may contribute to the generation of memory and plasma B cells. High IL-21 levels have been detected in the BSXB-*Yaa* lupus mouse model, and neutralisation of IL-21 in late disease improved survival, presumably by dampening the humoral immune responses (4). Moreover, IL-21 has a pathogenic role in the MLR-Fas(*lpr*) murine model of SLE by affecting B cell function and regulating the production of pathogenic autoantibodies (5). Although the main source of IL-21 are activated $CD4^+$ T cells, there is evidence to support that subsets of follicular germinal centre and extra-follicular T helper cells also produce high amounts of IL-21, thereby promoting PC differentiation and production of high-affinity, isotype-switched autoantibodies in systemic autoimmunity (11, 12). To this end, Simpson *et al*. (13). have reported expansion of circulating T cells resembling follicular helper T cells in severe human SLE, but they found no correlation with serum IL-21 levels.

IL-21 can have both positive and negative effects on B cell survival depending on presence or absence of other stimuli. In presence of innate signals such as LPS (TLR-4 agonist) or CpG DNA (TLR-9 agonist), addition of IL-21 inhibits proliferation and survival of murine follicular and splenic B cells (14, 15). In contrast, other investigators have reported that IL-21 augmented the proliferation and survival of CD40L-stimulated human naïve and memory primary B cells and B cell lines (16, 17). We found that activation of B cells with IL-21 and CpG-ODN2006 increased the proportion of memory and plasma cells, and this was followed by reduced ratio of apoptotic cells. A similar less pronounced result was observed in response to poly I:C (TLR-3 agonist) and IL-21 activation, suggesting that IL-21 may enhance the growth effects of TLR signalling in human B cells. One cannot rule out any additional B cell proliferative or differentiation effects of IL-21 and/or TLR-9 signalling, not assessed in our study.

In human SLE, PC differentiation and Ig production correlates with disease activity and involves both cell surface molecules (CD40L) and T cell-derived soluble factors (18-20). The role of TLR-9 overexpressed in active lupus B cells in promoting autoantibodies pro-

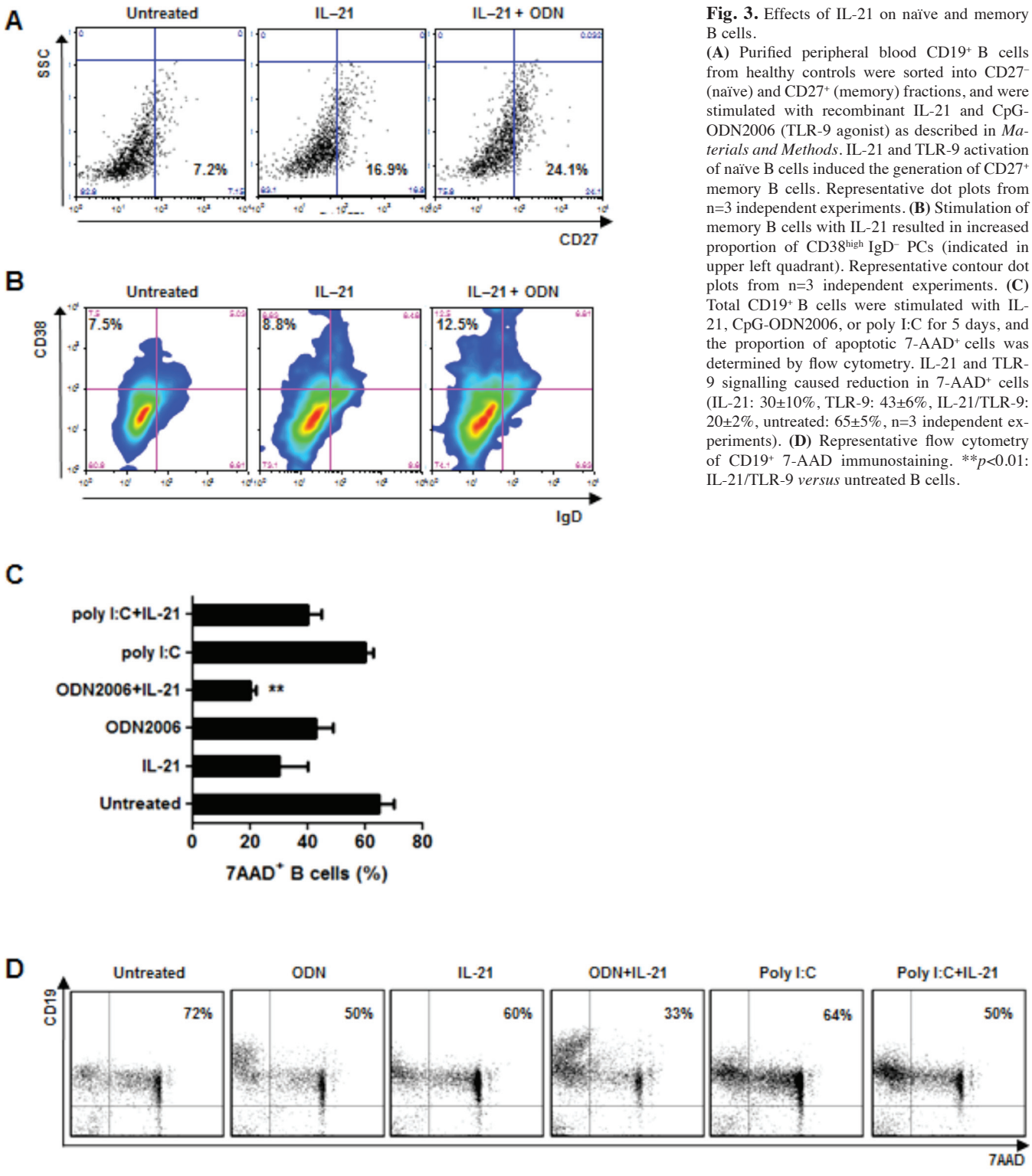

Fig. 3. Effects of IL-21 on naïve and memory B cells.
(A) Purified peripheral blood CD19$^+$ B cells from healthy controls were sorted into CD27$^-$ (naïve) and CD27$^+$ (memory) fractions, and were stimulated with recombinant IL-21 and CpG-ODN2006 (TLR-9 agonist) as described in *Materials and Methods*. IL-21 and TLR-9 activation of naïve B cells induced the generation of CD27$^+$ memory B cells. Representative dot plots from n=3 independent experiments. **(B)** Stimulation of memory B cells with IL-21 resulted in increased proportion of CD38^{high} IgD$^-$ PCs (indicated in upper left quadrant). Representative contour dot plots from n=3 independent experiments. **(C)** Total CD19$^+$ B cells were stimulated with IL-21, CpG-ODN2006, or poly I:C for 5 days, and the proportion of apoptotic 7-AAD$^+$ cells was determined by flow cytometry. IL-21 and TLR-9 signalling caused reduction in 7-AAD$^+$ cells (IL-21: 30±10%, TLR-9: 43±6%, IL-21/TLR-9: 20±2%, untreated: 65±5%, n=3 independent experiments). **(D)** Representative flow cytometry of CD19$^+$ 7-AAD immunostaining. $^{**}p<0.01$: IL-21/TLR-9 *versus* untreated B cells.

duction has recently been documented (8, 21). Based on our findings of increased IL-21, we studied the effect of IL-21 and TLR-9 on B cell differentiation and found that the combination of these two signals resulted in significant induction of PCs in active SLE patients that was comparable to the induction of PCs in healthy controls. Of note, IL-21 stimulation caused no induction of intracellular TLR-9 levels in B cells (data not shown). Although these results need further confirmation in experimental mouse studies and using different combinations and dosages of stimuli, they suggest that enhanced TLR-9 signalling and excessive amounts of T cell-derived IL-21 could contribute to increased percentage of circulating PCs at least in some SLE patients. Although IL-21R expression was comparable between SLE patients and controls, aberrant IL-21R signalling could also augment IL-21 responses in these patients. Inter-

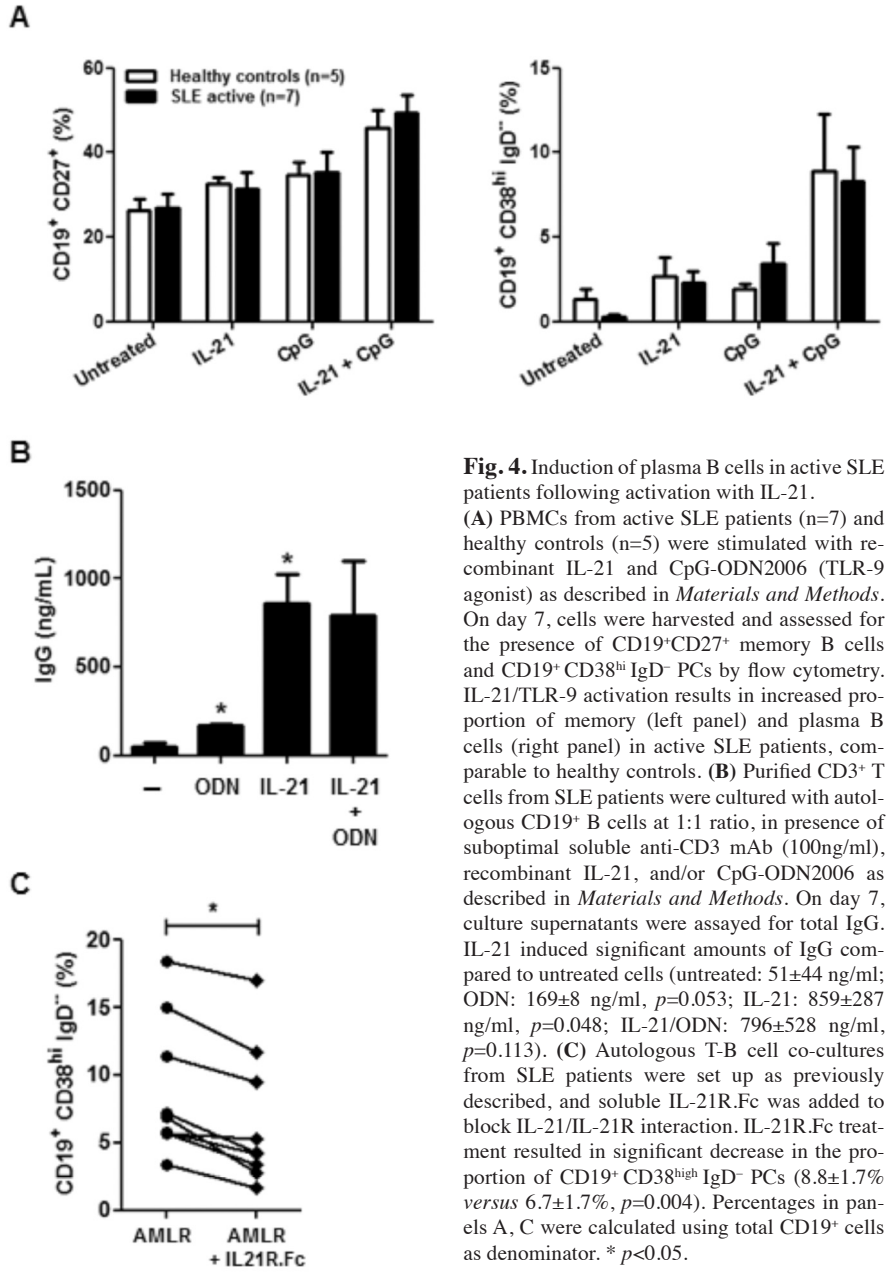

Fig. 4. Induction of plasma B cells in active SLE patients following activation with IL-21.
(A) PBMCs from active SLE patients (n=7) and healthy controls (n=5) were stimulated with recombinant IL-21 and CpG-ODN2006 (TLR-9 agonist) as described in *Materials and Methods*. On day 7, cells were harvested and assessed for the presence of $CD19^+CD27^+$ memory B cells and $CD19^+ CD38^{hi} IgD^-$ PCs by flow cytometry. IL-21/TLR-9 activation results in increased proportion of memory (left panel) and plasma B cells (right panel) in active SLE patients, comparable to healthy controls. **(B)** Purified $CD3^+$ T cells from SLE patients were cultured with autologous $CD19^+$ B cells at 1:1 ratio, in presence of suboptimal soluble anti-CD3 mAb (100ng/ml), recombinant IL-21, and/or CpG-ODN2006 as described in *Materials and Methods*. On day 7, culture supernatants were assayed for total IgG. IL-21 induced significant amounts of IgG compared to untreated cells (untreated: 51±44 ng/ml; ODN: 169±8 ng/ml, p=0.053; IL-21: 859±287 ng/ml, p=0.048; IL-21/ODN: 796±528 ng/ml, p=0.113). **(C)** Autologous T-B cell co-cultures from SLE patients were set up as previously described, and soluble IL-21R.Fc was added to block IL-21/IL-21R interaction. IL-21R.Fc treatment resulted in significant decrease in the proportion of $CD19^+ CD38^{high} IgD^-$ PCs (8.8±1.7% *versus* 6.7±1.7%, p=0.004). Percentages in panels A, C were calculated using total $CD19^+$ cells as denominator. * p<0.05.

estingly, a single nucleotide polymorphism within the IL-21R gene has been reported to confer risk for SLE (22).

A variety of signals act in concert to promote the breaching of tolerance and the production of autoantibodies in lupus patients. Among other, these signals include TLR-9 and IL-21. Integration of these signals may induce functional properties on lupus B cells that none of these signals alone could induce, thus contributing to the generation of PCs. The pioneering work from several groups on the role of IL-21 on human B cell biology (23) and in murine lupus nephritis (4, 5) suggests that IL-21 may be a therapeutic target in lupus. Inhibition of B cell differentiation via inhibitors of IL-21 or their depletion by the use of proteasome inhibitors (24) deserves further investigation for the treatment of this disease.

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References

1. KOSHY M, BERGER D, CROW MK: Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes. *J Clin Invest* 1996; 98: 826-37.
2. ETTINGER R, SIMS GP, ROBBINS R *et al*.: IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells. *J Immunol* 2007; 178: 2872-82.
3. ETTINGER R, KUCHEN S, LIPSKY PE: The role of IL-21 in regulating B-cell function in health and disease. *Immunol Rev* 2008; 223: 60-86.
4. BUBIER JA, SPROULE TJ, FOREMAN O *et al*.: A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci U S A* 2009; 106: 1518-23.
5. HERBER D, BROWN TP, LIANG S, YOUNG DA, COLLINS M, DUNUSSI-JOANNOPOULOS K: IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R. Fc reduces disease progression. *J Immunol* 2007; 178: 3822-30.
6. SAWALHA AH, KAUFMAN KM, KELLY JA *et al*.: Genetic association of interleukin-21 polymorphisms with systemic lupus erythematosus. *Ann Rheum Dis* 2008; 67: 458-61.
7. PASARE C, MEDZHITOV R: Control of B-cell responses by Toll-like receptors. *Nature* 2005; 438: 364-8.
8. PAPADIMITRAKI ED, CHOULAKI C, KOUTALA E *et al*.: Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process. *Arthritis Rheum* 2006; 54: 3601-11.
9. KUCHEN S, ROBBINS R, SIMS GP *et al*.: Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during $CD4^+$ T cell-B cell collaboration. *J Immunol* 2007; 179: 5886-96.
10. SPOLSKI R, LEONARD WJ: Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol* 2008; 26: 57-79.
11. BRYANT VL, MA CS, AVERY DT *et al*.: Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by $CXCR5^+$ T follicular helper cells. *J Immunol* 2007; 179: 8180-90.
12. ODEGARD JM, MARKS BR, DIPLACIDO LD *et al*.: ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* 2008; 205: 2873-86.
13. SIMPSON N, GATENBY PA, WILSON A *et al*.: Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* 2010; 62: 234-44.
14. MEHTA DS, WURSTER AL, WHITTERS MJ, YOUNG DA, COLLINS M, GRUSBY MJ: IL-21 induces the apoptosis of resting and activated primary B cells. *J Immunol* 2003; 170: 4111-8.
15. JIN H, CARRIO R, YU A, MALEK TR: Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest,

or Bim-dependent apoptosis. *J Immunol* 2004; 173: 657-65.
16. GOOD KL, BRYANT VL, TANGYE SG: Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21. *J Immunol* 2006; 177: 5236-47.
17. KONFORTE D, PAIGE CJ: Identification of cellular intermediates and molecular pathways induced by IL-21 in human B cells. *J Immunol* 2006; 177: 8381-92.
18. JACOBI AM, ODENDAHL M, REITER K *et al*.:Correlation between circulating CD27 high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 2003; 48: 1332-42.
19. JACOBI AM, REITER K, MACKAY M *et al*.: Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis Rheum* 2008; 58: 1762-73.
20. ODENDAHL M, JACOBI A, HANSEN A *et al*.: Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol* 2000; 165: 5970-9.
21. NAKANO S, MORIMOTO S, SUZUKI J *et al*.: Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus. *Rheumatology* (Oxford) 2008; 47: 145-9.
22. WEBB R, MERRILL JT, KELLY JA *et al*.: A polymorphism within IL21R confers risk for systemic lupus erythematosus. *Arthritis Rheum* 2009; 60: 2402-7.
23. MEHTA DS, WURSTER AL, GRUSBY MJ: Biology of IL-21 and the IL-21 receptor. *Immunol Rev* 2004; 202: 84-95.
24. NEUBERT K, MEISTER S, MOSER K *et al*.: The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. *Nat Med* 2008; 14: 748-55.