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DNA methylation modulates HRES1/p28 expression in B cells from patients with Lupus

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Abstract

Systemic lupus erythematosus (SLE) disease is an autoimmune disease of unknown aetiology that affects predominantly women of child bearing age. Since previous studies, including ours, have demonstrated that CD4+ T cells and B cells from SLE patients are defective in their ability to methylate their DNA upon antigen stimulation, the aim of this study was to investigate whether DNA demethylation affects the transcription of HRES-1 in B cells. HRES-1 is the prototype of Human Endogenous Retrovirus (HERV) overexpressed in SLE. We have observed that SLE B cells were characterized by their incapacity to methylate the HRES-1 promoter, both in unstimulated and in anti-IgM stimulated B cells. In turn, HRES-1/p28 expression was increased in SLE B cells after B cell receptor engagement, but not in controls. In SLE B cells the Erk/DNMT1 pathway was defective. In addition, blocking the autocrine-loop of IL-6 in SLE B cells with an anti-IL-6 receptor monoclonal antibody restores DNA methylation and control of HRES-1/p28 expression became effective. As a consequence, a better understanding of HERV dysregulation in SLE reinforces our comprehension of the disease and opens new therapeutic perspectives.

Keywords

B cells; DNA methylation; Erk; HRES-1; IL-6; systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) disease is an auto-immune disease with a large spectrum of clinical and immunological manifestations that affects predominantly women of child-bearing age [1]. The aetiology of SLE is multifactorial including genetic, epigenetic, environmental and hormonal factors. DNA methylation, the main epigenetic process, is abnormal in $CD4^+$ T cells and B cells from SLE patients (Reviewed in [2]) leading to the overexpression of DNA methylation sensitive genes such as Human Endogenous Retroviruses (HERV). HERV detection is increased in patients with autoimmune diseases including SLE [3,4], Sjögren's syndrome [5,6], and multiple sclerosis [7]. HERV are not only suspected to be markers of autoimmune diseases they are also suspected of contributing to the development of autoimmune diseases through different mechanisms.

In this way, HERV proteins have been demonstrated to impact immune regulation by producing cross-reactive autoantibodies (Ab) by molecular mimicry [8], or by expressing viral genes with super-antigen properties [9]. Another possibility is that HERV genetic elements interfere with neighboring immune related genes in an abnormal way as described in B cells with the cell surface receptor CD5 [10,11].

HERV were first discovered in the late 1960s, and their involvement since then has been described in all vertebrates [12]. In primates, up to 8% of the genome contains HERV elements, which are 4-fold more than the coding sequences and 7-fold more when considering the number of genes [13]. Phylogenetic analysis has highlighted up to 30 distinct groups ranging from one to many thousand copy numbers [14,15]. HERV chromosomal duplication started over one hundred million years ago and the process is still ongoing. Duplication occurs randomly in a copy-and-paste fashion *via* an intermediate RNA step. Mutations and deletions are frequently observed explaining why full length HERV (~10 kb) are exceptions [16]. HERV are composed of two long terminal repeats (LTR) in the 5′ and 3′ ends and between them three genes *gag*, *pol*, and *env* may be present.

In SLE, the HERV prototype is *HRES-1*, which is located at position 1q42 within chromosome 1 [17]. The link between HRES-1 and SLE started with the observation that anti-HRES-1/p28 *gag* autoAb are detected in up to 50% of SLE patients in contrast to less than 5% in the healthy control groups [18], and that within SLE patients HRES-1/p28 *gag* protein cross-reacts with the autoantigen U1-snRNP [8]. Next, the contribution of HRES-1 in the aetiology of SLE was reinforced by the observation that several *HRES-1* haplotypes were linked with SLE [19,20] and that over-expression of the reverse transcript HRES1-/Rab4 in $CD4^+$ T cells affects TCR signaling [21]. Recently we have suspected that DNA methylation represses HRES-1/p28 expression in healthy control B cells and that this effect can be reversed in the presence of IL-6 [10]. The aim of the present study was to explore whether HRES-1/p28 expression in SLE B cells is related to DNA methylation.

Materials and methods

B-lymphocyte isolation

Peripheral blood was collected from 6 patients with inactive SLE (SLE disease activity index <5) and 6 healthy controls (HC). All patients fulfilled the American College of Rheumatology criteria for SLE [22,23]. Informed consent was obtained from the patients before collecting blood, and the Institutional Review Board at Brest University Medical School approved the study protocol.

Using centrifugation on Ficoll-Hypaque (PAA Laboratories, Linz, Austria), peripheral blood mononuclear cells (PBMC) were isolated and then B cells were negatively purified using the EasySep™ enrichment Kit (Stemcell Technologies Inc., Vancouver, Canada). All purified cells were >98% CD19+.

Cell culture

B cells were suspended in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf sera, 2 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin (Lonza Inc., Allendale, NJ). B cells were seeded at 5×10^5 cells per well, and incubated 24 h with 1 μg/ml of anti-IgM Ab-coated Sepharose beads (Bio-Rad, Hercules, CA) and 10 U/ml IL-2, in the presence or absence of 40 ng/ml anti-IL-6R Ab (R&D Systems, Minneapolis, MN), or 100 ng/ml rhIL-6 (ImmunoTools, Friesoythe, Germany). Inhibition of DNMTs was achieved by incubating the cells 24 h with 20 μM of 5-azacytidine (5-aza) or with 50 μM of the *ras* signal blocker PD98059 (Sigma-Aldrich, St Louis, MO).

Methylation-specific PCR

Genomic DNA was purified using QIAmp 96 DNA blood kit (Qiagen, Carlsbad, CA) and 100 ng template DNA was distributed into three aliquots. The DNA concentration and the 260:280 nm absorbance ratios were calculated using a nanodrop 2000c spectrophotometer (Thermoscientific Nanodrop Technologies, Wilmington, DE). The first aliquot was undigested and used as a positive control. The second aliquot was digested with 20 U of the methylation-insensitive restriction enzyme *Msp*I for 3 h at 37 °C (New England Biolabs, Beverly, MA). The third aliquot was digested with 20 U of *Hpa*II for 3 h at 37 °C. This assay is based on the inability of *Hpa*II restriction enzymes to digest a methylated 5′-CC^mGG-3′ site. Next, the PCR was conducted using primers positioned downstream 5′-GCATATGCACTG GGAAAGGT-3′ and upstream 5′-CCGCCTTTTCAAGTTTC CTC-3′ of the unique *Hpa*II/*Msp*I CCGG site present within the *HRES-1* promoter (Figure 1A). The PCR protocol included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 1 min, and primer extension at 72 °C for 1 min; PCR cycles were followed by final extension at 72 °C for 10 min. The PCR products were separated on an agarose gel and visualized with 0.5 μg/ml ethidium bromide.

mRNA extraction and quantitative RT-PCR

Total mRNA was extracted using the RNAble method (Eurobio, Les-Ullis, France), and cDNA synthesized by reverse transcription in 20 μl volume with Superscript™ II RNase H-RT (Invitrogen Life Sciences, Carlsbad, CA). Quantitative RT-PCR was carried out in 20-μl

mixtures containing 50 ng template cDNA, 1X Sybr® Green PCR Master mix (Applied Biosystems, Foster City, CA), and 500 nM of each primer, HRES-1/p28 sense primer 5′-GGAA GAGGAGATGGGCTACG-3′ and HRES-1/p28 reverse primer 5′-CAGGGAAATCGGGACTCAG-3′. All assays included a negative control, which consisted of the reaction mixture with no template, and a positive control, which consisted of the mixture with 18S rRNA. Comparison of cycle thresholds was completed with the $2^{-\Delta\Delta ct}$ method using 18S as an internal control.

Flow cytometry

Intracellular phosphorylated mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/pErk1/2) staining (Dako, Carpinteria, CA) and DNA methyl transferase 1 (DNMT1) staining (Abcam, Cambridge, UK) were performed, respectively, after permeabilization using the cytofix/cytoperm intracellular staining kit (BD Biosciences, San Jose, CA) or permeabilization using 70% methanol in purified B-cells stimulated or not with 1 μg/ml of anti-IgM Ab (Bio-Rad). Next, anti-pErk1/2 binding was revealed after 10 min anti-IgM stimulation and DNMT1 binding after 24 h anti-IgM stimulation using a $F(ab)'_2$ goat FITC anti-murine IgG (Jackson Immunosearch, West Grove, MO). Results were expressed in mean fluorescence intensity (MFI).

Statistical analysis

The results are expressed in arithmetic means with standard deviations (SD). Data were compared using the Mann–Whitney *U*-test for unpaired data. Significance was assessed as $p<0.05$.

Results

Impaired HRES-1 promoter methylation in SLE B cells explains HRES-1/p28 overexpression in anti-IgM stimulated B cells

Analysis of the 713 pb 5′ U3-R-U5 promoter sequence of *HRES-1* (Genbank X16660) reveals 9 CpG motifs including a *Hpa*II/*Msp*I 5′-CCGG-3′ motif at position +559 (Figure 1A). Next, in order to test *HRES-1* promoter methylation status, a MS-PCR was developed using specific primers flanking the *Hpa*II/*Msp*I motif. The PCR was conducted using either undigested DNA (Figure 1B lane 1, referred to as 100% methylation), DNA digested with the methylation-insensitive enzyme *Msp*I (lane 2, 0% methylation), and DNA digested with the methylation-sensitive enzyme *Hpa*II (lane 3).

The *Hpa*II MS-PCR analysis of *HRES-1* promoter methylation status in unstimulated and anti-IgM stimulated B cells from 6 SLE patients and 6 healthy controls (HC) revealed that *HRES-1* promoter was hypomethylated in SLE B cells both when analyzing unstimulated B cells (*HRES-1* methylation status: 10.2% ± 2.3% in SLE B cells *versus* 35.1% ± 10.9% in HC B cells, $p<0.05$) and when analyzing anti-IgM stimulated B cells (6.9% ± 5.3% in SLE B cells *versus* 62.8% ± 12.1% in HC B cells, $p<0.01$) (Figure 1C). To determine the functional consequences of DNA methylation changes in *HRES-1* promoter (Figure 1D), we performed a gene expression analysis. HRES-1/p28 mRNA levels were not different when considering unstimulated B cells between SLE patients and HC. After B cell receptor (BCR)

engagement, HRES-1/p28 mRNA expression was increased 3.2-fold in SLE B cells and repressed 2.1-fold in HC B cells. These results indicate that induction of HRES-1/p28 following BCR engagement is regulated at the epigenetic level.

The Erk/DNMT1 pathway is defective in anti-IgM stimulated SLE B cells

As described in SLE T cells [24,25], the incapacity of SLE B cells to methylate their DNA may result from a defective Erk/DNMT1 pathway. To test this hypothesis, SLE B cells and HC B cells were stimulated with an anti-IgM Ab and the intracellular phosphorylation status of the MAPK Erk was evaluated after 10 min by flow cytometry. As shown in Figure 2(A) in a representative experiment, the MAPK Erk was strongly phosphorylated in HC B cells after BCR engagement (pErk1/2: 7.5 ± 3.8% *versus* 73.5 ± 19.9 with anti-IgM, $p<0.05$), but not in SLE B cells (pErk1/2: 24.1 ± 4.4% *versus* 27.5 ± 5.6 with anti-IgM, non significant). Next, the role of the MAPK Erk pathway on DNMT1 induction was examined. In HC B cells, but not in SLE B cells, DNMT1 expression was induced after 24 h anti-IgM stimulation (MFI DNMT1: 22.7 ± 2.7% *versus* 32.6 ± 6.5 with anti-IgM in HC, $p<0.05$) and this induction was repressed in the presence of the MEK/Erk inhibitor PD98059 (Figure 2B).

To confirm the contribution of the MAPK Erk/DNMT1 pathway to the control of DNA methylation, anti-IgM stimulation was repeated in HC B cells either in the presence of PD98059, or in the presence of the DNMT1 inhibitor 5-azacytidine. As shown in Figure 2(C and D), Erk and DNMT1 inhibitors affect the anti-IgM-dependent *HRES-1* promoter DNA methylation process and as a consequence this inhibition contribute to the enhancement of HRES-1/p28 mRNA expression.

The role of IL-6 in demetlylation and HRES-1/p28 expression

Finally, based on the control of DNMT1 by IL-6 in B cells [10], and to test the effect of the SLE B cell autocrine loop of IL-6 [26] on HRES-1/p28 expression, a blocking anti-IL6 receptor was used in anti-IgM stimulated SLE B cells. As reported in Figure 3(A–B), blocking IL-6 in SLE B cells restores anti-IgM-dependent *HRES-1* promoter methylation, which represses HRES-1/p28 expression. To further highlight the importance of IL-6 in the control of DNA methylation, HC B cells were anti-IgM-stimulated in the presence of rIL-6 (Figure 3C–D). The addition of rIL-6 affects the ability of the anti-IgM stimulation to methylate the *HRES-1* promoter, thus permitting HRES-1/p28 expression in HC B cells.

Discussion

In this report we have observed that (i) DNA methylation is impaired in resting and anti-IgM stimulated SLE B cells, that (ii) HRES-1/p28 expression is regulated by DNA methylation through the Erk/DNMT1 pathway, and that (iii) this defect is reversible when blocking the autocrine loop of IL-6.

Results generated from our previous studies and others indicated that demethylation of DNA can be related to the inhibition or lack of DNMTs, to an increase of demethylating activity, or from a combination of both mechanisms [10,27]. No association between DNMT1 polymorphism and SLE was observed [28]. In SLE, several DNA demethylating external

factors such as hydralazine used as primary drug for treating hypertension [24], ultraviolet B irradiation [29], and gender [30] have been associated with SLE susceptibility and flare severity in genetically predisposed individuals. In addition, internal DNA demethylating factors may be also suspected such as cell cycle arrest [10], viral infection [31], and in response to cellular communication. We have recently observed that DNA demethylation in salivary glands from patients with primary Sjögren's syndrome is related to the infiltrating B cells [32]. Last but not least, several microRNA have been reported in SLE to target DNMT1 directly or indirectly [33,34].

In $CD4^+$, $CD8^+$ and $CD19^+$ B cells isolated from SLE patients DNA demethylation is associated with disease activity, leucopenia, lymphopenia and UVB irradiation with differences observed according to the lymphocyte subset [35,36]. The genome-wide methylation analysis has been recently explored, from these studies several genes relevant to SLE including type I interferon genes were highlighted [37–40]. Interestingly, most of the DNA methylation dysregulated genes were previously identified by genome-wide association studies (GWAS) [41].

As a consequence, altered methylation influences T cells in SLE causing up-regulation of co-stimulating molecules (CD70, CD40 ligand), integrins (LFA-1) and cytokines (IL-1, IL-10, IL-13) [42]. In SLE, we proposed that B cell dysfunctions [43,44] are also altered by DNA demethylation [45] and the contribution of DNA demethylated B cells in the autoimmune process has been demonstrated in mice [46].

Aside from CpG-rich sequences within the genes, CpG are present within "junk DNA" to control DNA repetitive transposons and retroelements, respectively 2.8% and 41.5% of the human genome [47]. Retroelements can further be divided in three groups: long interspersed nuclear elements (LINEs, 20.1% of the human genome), short interspersed nuclear elements (ALU, 10.6%; MIR 2.5%), and HERVs (8.3%). In lymphocytes from SLE patients, the DNA demethylation process is selective, since ALU sequences are not concerned [37,48], while LINE-1 sequences are demethylated and among HERV elements the HERV-E family is particularly sensitive to DNA methylation [3,49].

Consistent with this observation is the finding that the HERV-E element *HERV-CD5* is deregulated at the epigenetic level in SLE B cells [10]. *HRES-1* and *HERV-CD5* share several caracteristics (i) chromosomal integration occurs at the stage of old word primate divergence [16], (ii) they are both class1 HERVs and related to the C-type retroviruses [50], (iii) they are silenced by DNA methylation, and (iiii) they interfere with immune genes, respectively, CD4 and CD5 [51,52]. Divergences between *HRES-1* and *HERV-CD5* are related (i) to the HERV family sub-class, (ii) to the absence of *HRES-1* 3′LTR, and (iii) to the B cell specificity for *HERV-CD5* [53].

The proposed involvement of IL-6 in promoting auto-reactivity is supported by *in vitro* and *in vivo* studies leading to the development of an anti-IL6 receptor blocking mAb, tocilizumab, which is effective in SLE [54]. In patients treated with tocilizumab, IL-6 repression is associated with the decrease of lymphocyte activation and the normalization of the B and T cell subsets [55]. The precise mechanism, by which IL-6 interferes with

lymphocyte homeostasis, remains to be elucidated. According to our report, part of the effect may be related to the action of IL-6 on DNA methylation. Interestingly, mutations of DNMT3b as observed in immuno-deficiency, centromere instability and facial anomalies (ICF) syndrome lead to major dysregulation of lymphogenesis [56,57].

In conclusion, the finding that DNA methylation controls HERV transcription is relevant for understanding mechanisms that control autoreactivity. In addition, the data provide further arguments to consider in the development of epigenetic based therapies in SLE.

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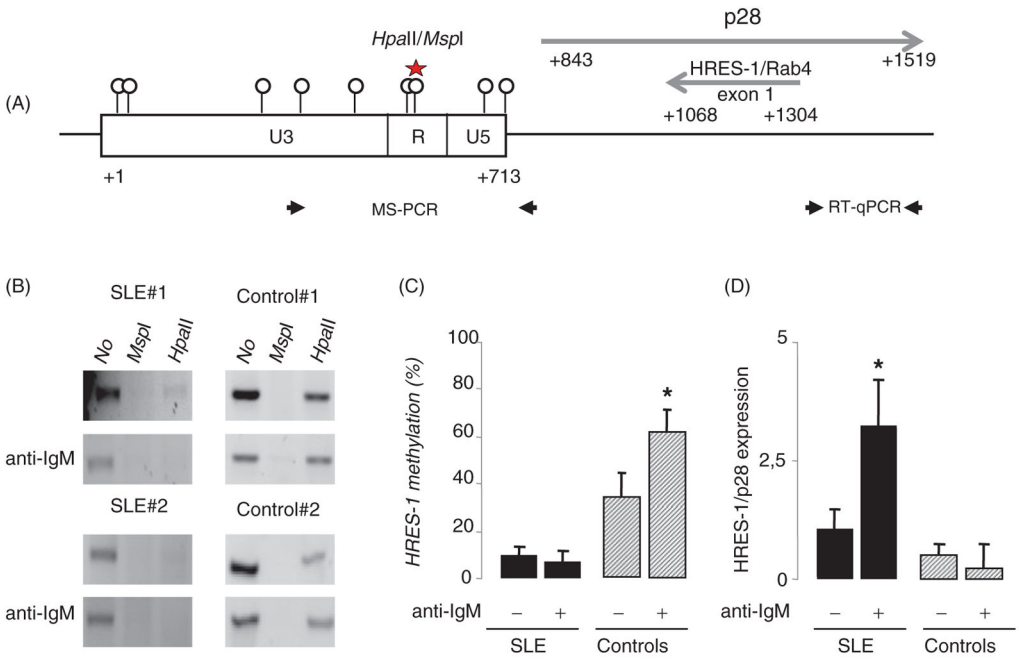

Figure 1.

The *HRES-1* promoter region is demethylated in B cells from SLE patients and DNA demethylation modifies HRES-1/p28 expression. A: Schematic representation of the organization of *HRES-1* on chromosome 1q42 region (GenBank X16660). Circles identify the 9 CpG motifs within the 5′ U3-R-U5 LTR region of *HRES-1*. Of note, the 3′ LTR region of *HRES-1* is lacking. The *Hpa*II/*Msp*I motif present in the 5′U3-R-U5 LTR region is represented (star) as well as HRES-1/p28 and HRES-1/Rab4 transcript positions, and primers used in the MS-PCR and quantitative RT-PCR (RT-qPCR). B: Analysis of *HRES-1* promoter methylation by amplification of genomic DNA digested with the methylation sensitive *Hpa*II, or with the methylation insensitive *Msp*I enzyme. The 502 bp *HRES-1* amplicon contains one *Hpa*II/*Msp*I site. C: Histograms representing *HRES-1* promoter methylation status in 6 SLE patients and 6 healthy controls, the percentage of methylation was quantified by calculating the ratio of *Hpa*II-digested to undigested bands. D: Quantitative RT-PCR results presenting as histograms revealing that a 24-h stimulation of B cells with an anti-IgM increases HRES-1/p28 transcription in B cells from SLE patients, but not from healthy controls. The symbol * represented $p<0.05$.

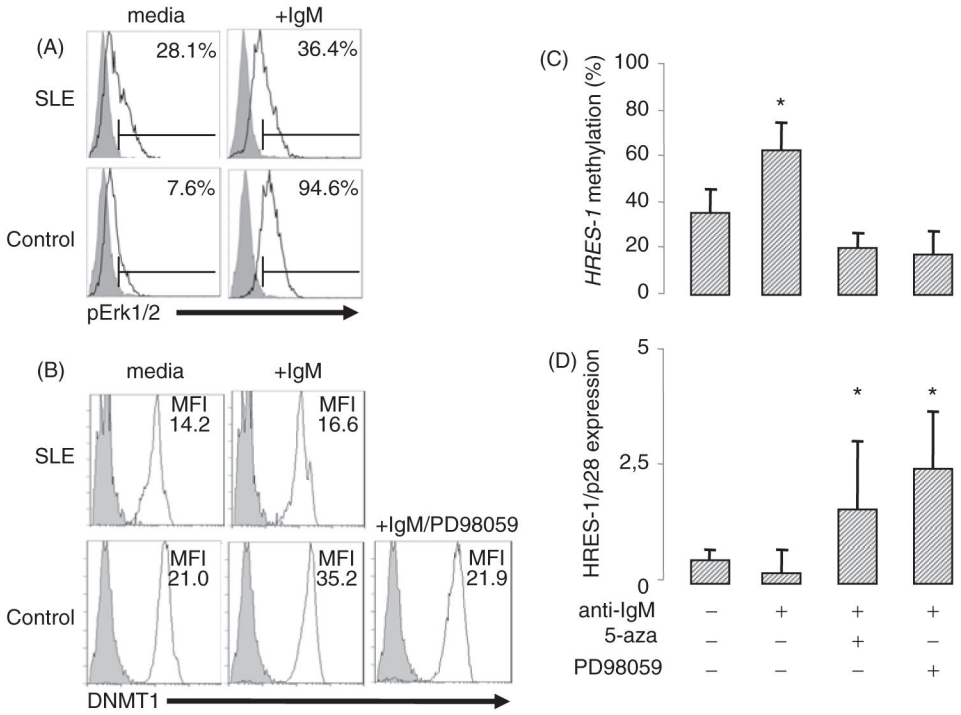

Figure 2.

The Erk/DNMT pathway is defective in SLE B cells. A–B: Cytoplasmic staining of pErk (A) and DNMT1 (B) in permeabilized B cells stimulated with anti-IgM or not for 10 min (pErk) and 24 h (DNMT1). Representative flow cytometric profiles are represented. C: *HRES-1* promoter analysis by MS-PCR reveals that the Erk/DNMT1 pathway was involved in *HRES-1* promoter methylation when control B cells were BCR stimulated with an anti-IgM Ab. Inhibition of pErk and DNMTs was respectively achieved by incubating the cells 24 h with 50 μM of the *ras* signal blocker PD98059, and with 20 μM of 5-azacytidine (5-aza). D: Quantitative PCR measurement of HRES-1/P28 mRNA levels. The symbol * represented $p<0.05$.

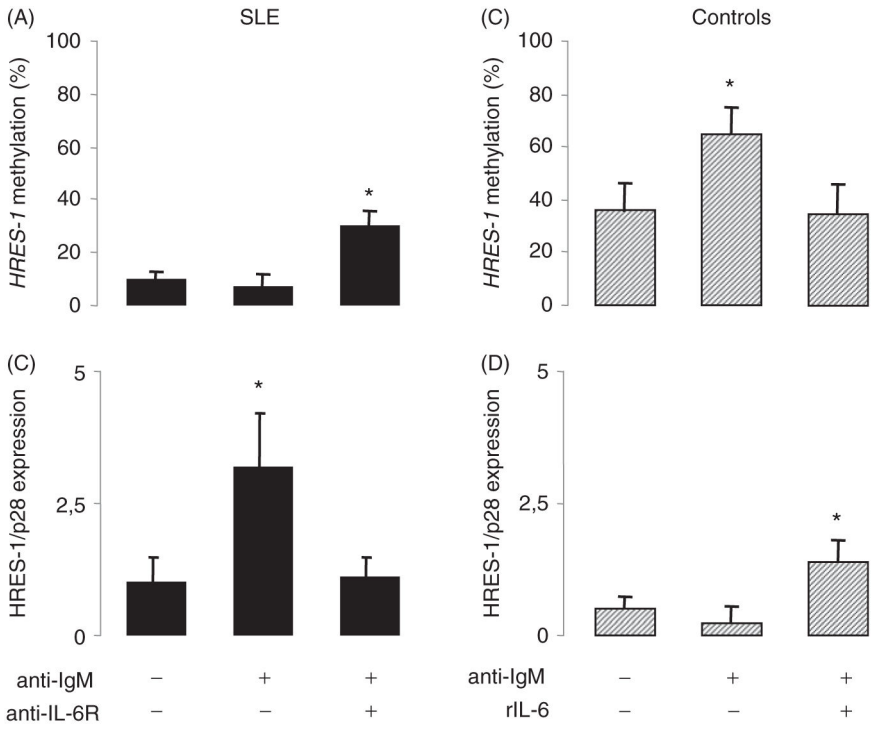

Figure 3.

The effect of IL-6 on HRES-1/P28 expression and *HRES-1* promoter methylation status. A/C: *HRES-1* promoter analysis by MS-PCR in SLE B cells (A) and healthy control B cells (C) after BCR engagement using an anti-IgM Ab. Based on our previous observation that anti-IgM stimulated SLE B cells, but not the controls, express high amounts of IL-6 [26], the influence of the autocrine loop of IL-6 was tested by adding 40 ng/ml of a blocking anti-IL-6 receptor Ab in SLE B, or by adding 100 ng/ml of rIL-6 in healthy control B cells. B/D: Quantitative PCR measurement of HRES-1/P28.