

学位論文

**「Synergistic enhancement of production of proinflammatory
cytokines of human peripheral blood monocytes by anti-Sm
and anti-RNP antibodies」**

(抗 Sm 抗体と抗 RNP 抗体によるヒト末梢血単球の
炎症性サイトカイン産生の相乗的増加作用)

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著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

背景及び目的

抗 RNP 抗体は、全身性エリテマトーデス(SLE)および混合性結合組織病に出現する自己抗体である。抗 RNP 抗体は、ヒト末梢血単球と反応し炎症性サイトカインの産生を増加させることが明らかになっている。一方、抗 Sm 抗体は、SLE に特異的な自己抗体であり、SLE による精神神経症状の最重症型である急性混迷状態と深く関係していることが知られている。一般的に、抗 Sm 抗体は単独で陽性になることはなく、抗 RNP 抗体と抗 Sm 抗体の両方が陽性となる。しかしながら、抗 Sm 抗体の SLE の病態形成における作用や、単球に与える影響については未だ明らかになっていない。抗 Sm 抗体の認識する抗原は抗 RNP 抗体の認識する抗原と密接に関連していることから、抗 RNP 抗体と同様にヒト末梢血単球に結合し、炎症性サイトカイン産生を増加させる可能性がある。本研究は、単球に対する抗 Sm 抗体の役割を明らかにすることを目的とした。特に抗 Sm 抗体と抗 RNP 抗体による単球の炎症性サイトカイン産生に関する効果に注目した。

方法

健常人ボランティアより末梢血を採取し、Ficoll 比重遠心法にて末梢血単核球を分離し、磁気ビーズ法により単球を単離した。得られた単球を、マウスモノクローナル抗ヒト Sm 抗体 (抗 Sm mAb)、マウスモノクローナル抗ヒト U1 RNP 抗体 (抗 RNP mAb) または対照 IgG1 または IgG3 を用いて様々な条件で染色し、それぞれの抗体の結合をフローサイトメトリーで検討した。また、単球を抗 Sm mAb、抗 RNP mAb または対照 IgG1 または IgG3 の存在下で様々な条件で培養し、培養上清中の IL-6 および TNF α を ELISA により測定した。さらに、単球における IL-6・TNF α および NF κ B の種々の component の mRNA の発現をリアルタイム PCR 法にて測定した。また一部の実験においては、抗 Sm mAb と抗 RNP mAb の代わりにヒト患者血清から分離した抗 Sm 抗体および抗 RNP 抗体の単球に対する効果を検討した。

結果

抗 Sm mAb および抗 RNP mAb の両方が、単離した直後の単球よりも平底プラスチックプレート上で 24 時間培養した活性化単球に、顕著に結合した。注目すべきことに、単球を抗 Sm mAb もしくは抗 RNP mAb の存在下で培養すると、これらの抗体の単球への結合がさらに増加した。

抗 Sm mAb および抗 RNP mAb の両方とも、ヒト末梢血単球による IL-6 産生を用量依存的に増加させたが、後者は前者よりも強力であった。さらに、抗 Sm mAb は、抗 RNP mAb のヒト単球による IL-6 および TNF α 産生を相乗的に増強した。ヒト IgG もしくはヒト IgG F(ab')₂ を培養に添加した両実験において、抗 Sm mAb の抗 RNP mAb のヒト単球による IL-6 産生の相乗的増強は、減弱しなかった。また、Fc 受容体阻害剤を培養に添加した実験において、抗体の単球への結合は著明に低下したが、抗 Sm mAb の抗 RNP mAb のヒト単球による IL-6 産生の相乗的増強は、減弱しなかった。患者血清から分離した抗 Sm 抗体

と抗 RNP 抗体を用いた培養実験においても、抗 Sm 抗体は、抗 RNP 抗体のヒト単球による IL-6 産生を相乗的に増強した。

一方、抗 Sm mAb は、抗 RNP mAb 存在下においてのみ単球の IL-6 および TNF α mRNA の発現を有意に増強した。NF κ B の種々の component の mRNA のなかで、抗 RNP mAb は単球の RelA mRNA の発現のみを有意に増強したが、抗 Sm mAb は全ての component の mRNA 発現に対して有意な効果を示さなかった。

NF κ B 阻害薬である N-acetyl cysteine や pyrrolidine dithiocarbamate の存在下では、抗 Sm mAb 単独の IL-6 産生は完全に抑制されたが、抗 RNP mAb 存在下では抗 Sm mAb は単球 IL-6 産生に対する増強効果を示した。

結論

一般的には、抗 Sm 抗体も抗 RNP 抗体も核タンパク質を認識すると考えられている。本研究では、ヒト末梢血単球の表面に抗 Sm 抗体および抗 RNP 抗体が認識するエピトープが存在することが示された。それらのエピトープの発現は単球の活性化により増加することも明らかになった。

さらに重要なことには、抗 Sm 抗体は、抗 RNP 抗体存在下の単球の炎症性サイトカインの産生を相乗的に増強することが示された。この相乗的増強は、ヒト IgG もしくは Fc 受容体阻害剤の添加により減弱しなかったため、Fc 受容体の作用を必要としない効果であると考えられる。また、抗 Sm 抗体は NF κ B の種々の component の mRNA の発現には全く影響を与えず、NF κ B 阻害薬は抗 Sm 抗体の相乗効果に影響を与えなかったことから、この相乗的増強は NF κ B 経路を介した効果ではないことが示唆された。

本研究の結果より、抗 Sm 抗体は単球の炎症性サイトカインの産生を増強することにより、SLE における脳血液関門の障害にも関与する可能性が強く示唆される。今後は抗 Sm 抗体の単球に対する作用の分子メカニズムの詳細についてさらなる検討が必要である。

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Introduction

Anti-RNP antibodies (anti-RNP) have been found to be expressed in systemic lupus erythematosus (SLE) as well as in mixed connective tissue disease (MCTD) which is frequently associated with pulmonary artery hypertension [1]. Previous studies demonstrated that anti-RNP bound human pulmonary artery endothelial cells (HPAECs) [2]. Accordingly, anti-RNP up-regulated the expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), E-selectin and Class II molecules on HPAECs [3]. In addition, anti-RNP have been shown to enhance the production of proinflammatory cytokines, including IL-6 and TNF- α , by peripheral blood monocytes [4]. It is thus possible that anti-RNP might also bind human peripheral blood monocytes.

Anti-Sm antibodies (anti-Sm) are directed against proteins that constitute the common core of small nuclear ribonucleoprotein (snRNP) particles and are specifically expressed in patients with SLE [5]. Serum anti-Sm have been found to be associated with organic brain syndrome or acute confusional state (ACS) of diffuse neuropsychiatric SLE (NPSLE) [6,7]. Moreover, recent studies have disclosed that Q albumin, an indicator of blood-brain barrier (BBB) damages, was significantly correlated with serum anti-Sm in patients with NPSLE [8]. Of note, previous study showed that BBB damages were strongly linked to elevated levels of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) [9-11]. Thus, human monocytes may contribute to BBB damages through the production of pro-inflammatory cytokines [11]. It is therefore possible that serum anti-Sm might have such proinflammatory effects that result in endothelial dysfunction, leading to BBB dysfunction.

Notably, the expression of anti-Sm is always associated with anti-RNP in patients with SLE, although its mechanism remains uncertain [12]. Since anti-RNP bind HPAECs as well as human peripheral blood monocytes [2-4], it is also possible that anti-Sm might also bind these cells and influence the production of proinflammatory cytokines thereof. The present study was therefore designed in order to explore the effects of anti-Sm on the production of proinflammatory cytokines by human peripheral blood monocytes. Special attention was directed to the interactions of anti-Sm and anti-RNP in the effects on monocytes.

Materials and methods

Informed consents of the participants

Written informed consents were obtained from the participants of the study. This study was approved by the institutional ethical committee of Kitasato University School of Medicine (Ref. No. B09-55).

Cell preparation

Human peripheral blood monocytes were purified by the same method as previously described [13]. Thus, peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers who gave written informed consent by centrifugation of heparinized venous blood over sodium diatrizoate-Ficoll gradients. Monocytes were purified from PBMCs using Monocyte Isolation Kit II (Miltenyi Biotec, Tokyo, Japan). The monocytes contained >95% CD14⁺ monocytes, <0.1% CD3⁺ T cells, and <0.1% CD19⁺ B cells, as determined by analysis with flow cytometry [13].

Antibodies

Monoclonal anti-Sm antibody (murine IgG3) (anti-Sm mAb), which recognizes BB' and D proteins of Sm, was purchased from Thermo Scientific, Fremont, CA. Monoclonal anti-RNP antibody (murine IgG1) (anti-RNP mAb), which recognizes 68-kd protein of U1-RNP, was purchased from Synaptic systems, Gottingen, Germany. Murine control IgG1 and IgG3 were purchased from Cappel, West Chester, PA, and from Abcam, Cambridge, UK, respectively.

Preparation of human IgG

Sera were obtained from 4 patients with SLE or MCTD or from 2 healthy individuals who gave written informed consent. IgG fractions were purified from the sera using a protein G-Sepharose 4FF column (Amersham Pharmacia Biotech, Uppsala, Sweden). The concentrations of anti-DNA antibodies (anti-DNA), anti-Sm and anti-RNP in the purified IgG were determined by ELISA using MESACUP (MBL, Nagoya, Japan). Since it has been shown that anti-ribosomal P antibodies enhance the IL-6 production of monocytes [13], the concentrations of anti-ribosomal P antibodies activity were determined by ELISA as previously described [14]. All the IgG samples had no detectable anti-ribosomal P antibodies. IgG fractions from patients or from healthy individuals were appropriately mixed to generate IgG preparations which contain varying concentrations of anti-Sm with the constant concentration of anti-RNP in the absence of anti-ribosomal P antibodies (Table 1).

Reagents

Human IgG F(ab')₂ fragments (Gamma Venin P) were purchased from Sanofi, Paris, France. N-acetyl cysteine (NAC) (Sigma, St Louis, MO), pyrrolidine dithiocarbamate (PDTC) (Abcam), TPCA-1 (Abcam), methyl- β -cyclodextrin (Sigma) and cytochalasin D (Wako, Osaka, Japan) were also purchased. Human BD Fc Block was purchased from BD Biosciences, Franklin Lakes, NJ.

Culture medium

RPMI 1640 medium (Nikken, Kyoto, Japan) supplemented with penicillin G (Life Technologies, Gaithersburg, MD) (100 U/ml), streptomycin (Life Technologies) (100 μ g/ml),

L-glutamine (Sigma) (0.3 mg/ml), and 10% fetal bovine serum (FBS) (Life Technologies) was used throughout the cultures.

Flow cytometry

Fresh or cultured monocytes (5×10^5 /sample) were stained with anti-RNP mAb, anti-Sm mAb, IgG1 or IgG3 (5 μ g/ml), followed by counterstaining with fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse IgG (Cappel), as previously described [13]. The staining procedures were carried out at 4°C with staining buffer containing 2% normal human serum to block Fc gamma receptor [13]. After staining, the cells were treated in saline with 50 μ g/ml propidium iodide (PI; Sigma) for more than 5 minutes at room temperature, followed by analysis using Cell Lab Quanta SC (Beckman Coulter, Miami, FL). The gating threshold for PI staining to identify viable cells was determined using live cells without PI staining. The density of staining was expressed as the change in mean fluorescence intensity (MFI) for staining of all the cells with anti-RNP or anti-Sm, which was calculated by subtracting the MFI of staining of all the cells with control IgG1 or IgG3, respectively. Also differences of MFI for staining with anti-RNP mAb from staining with control IgG1 and those of MFI for staining with anti-Sm mAb from staining with control IgG3 were statistically analyzed with Paired t test.

In some experiments, purified monocytes (1×10^6 /well) were cultured in 24-well microtiter plates (Nunc, Roskilde, Denmark) in culture medium at 37°C in 5% CO₂ with the presence of anti-RNP mAb, anti-Sm mAb, IgG1 or IgG3 (5 μ g/ml) for 24 hours, after which the cells were harvested and stained with FITC-conjugated goat F(ab')₂ anti-mouse IgG, followed by analysis on flowcytometry.

Cell cultures

Purified monocytes (1×10^6 /well) were cultured in 24-well microtiter plates (Nunc) with anti-RNP mAb, anti-Sm mAb, control IgG1, or control IgG3 at various concentrations. In some experiments, purified monocytes were cultured in the presence of 100 μ g/ml of human IgG preparations containing various concentrations of anti-Sm and anti-RNP (Table 1). After 48 or 96 hours of incubation, the supernatants were harvested and assayed for IL-6 and TNF- α using the Human IL-6 ELISA Kit (Affymetrix, San Diego, CA) and the TNF- α Human ELISA Kit High Sensitivity (Thermo Scientific), respectively.

RNA isolation and real-time quantitative polymerase chain reaction (PCR)

After 4 hours of incubation, monocytes were harvested for RNA extraction. Total RNA was isolated from cultured cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's directions. cDNA was prepared from 1 μ g of total RNA using PrimeScript II RTase (Takara Bio, Shiga, Japan) with Oligo dT primers (Takara Bio), and was subjected to analysis with real-time PCR using LightCycler 4.1 (Roche Diagnostics, Lewes, UK). Real-time PCR for IL-6, TNF- α and β -actin was performed using SYBR Premix Ex Taq II (Takara Bio) as previously described [13,15]. Amplification was performed according to the standard protocol recommended by the manufacturer. All results for the copy number of IL-6 mRNA or TNF- α mRNA were calibrated to the copy number of β -actin obtained from the same cDNA samples. Real-time PCR for NFkB1 (p50), NFkB2 (p52) and RelA (p65) in cultured monocytes was also performed as previously described [16]. The expression of

mRNA for NF κ B1 (p50), and RelA (p65) is shown as the ratio of the copy numbers to those of β -actin mRNA.

Statistical analysis

Comparisons among 4 groups and between 2 groups were carried out by Repeated –Measures one-way ANOVA with multiple comparison and by Paired t test, respectively, using GraphPad Prism 7.03 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Binding of anti-RNP mAb and anti-Sm mAb to human peripheral blood monocytes

Initial experiments were carried out to explore whether anti-RNP and anti-Sm might bind peripheral blood monocytes. As shown in Fig 1A, anti-RNP mAb bound to resting monocytes and more prominently to activated monocytes that had been cultured on flat-bottomed plastic plates for 24 hours [17]. Thus, the MFI for anti-RNP was significantly higher than that for IgG1(Fig 1B). Although the binding of anti-Sm mAb on the surface of resting monocytes were very modest compared with that of anti-RNP mAb, the MFI for anti-Sm was significantly higher than that for IgG3 (Fig 1B). However, after activation through adherence on flat-bottomed plastic plates for 24 hours, the binding of anti-Sm mAb on the surface of monocytes was significantly up-regulated. These results indicate that both anti-Sm and anti-RNP bind to the surface of viable human monocytes and that such binding is upregulated upon activation of human monocytes. Of note, further increased binding of anti-Sm and anti-RNP was found when monocytes were cultured with the presence of these antibodies (Fig 1A). Therefore, the data confirm that anti-Sm and anti-RNP bind monocytes to influence their functions.

Enhancement of the production of IL-6 and TNF- α of human peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb

Next experiments examined whether anti-Sm and anti-RNP might influence the function of human monocytes. Because the production of inflammatory cytokines is an important feature

of monocytes, the effects of anti-Sm mAb and anti-RNP mAb on the production of IL-6 and TNF- α were explored. As shown in Fig 2A, the production of IL-6 of human monocytes increased in a time-dependent manner from 48 hours to 96 hours. More importantly, anti-Sm mAb (3 μ g/ml) as well as anti-RNP mAb (3 μ g/ml) enhanced the production of IL-6 at each time point compared with that in control cultures with IgG1+IgG3, although the effect of the former was less marked than that of the latter. In addition, anti-Sm mAb further increased the production of IL-6 in the presence of anti-RNP mAb.

As shown in Fig 2B, anti-Sm mAb and anti-RNP mAb enhanced the production of IL-6 of monocytes in a dose-response manner. It appears that anti-Sm mAb further increased the production of IL-6 even in the presence of the higher concentration of anti-RNP mAb (9 μ g/ml). These results suggest that the effects of anti-Sm might be synergistic with those of anti-RNP.

To further explore the interactions between anti-Sm mAb and anti-RNP mAb in the regulation of the production of IL-6 and TNF- α of human monocytes, highly purified monocytes were cultured with various concentrations of anti-Sm (0-2 μ g/ml) in the presence of the constant concentration of anti-RNP mAb or control IgG1 (1 μ g/ml). As shown in Fig 3, anti-Sm mAb enhanced the production of IL-6 and TNF- α of human monocytes in a dose-dependent manner in the presence or absence of anti-RNP mAb. More importantly, it was evident that the effects of anti-Sm mAb were synergistic with those of anti-RNP mAb. Thus, the production of IL-6 and TNF- α in the presence of both anti-Sm mAb and anti-RNP mAb (solid line) apparently exceeded the sum of the production of IL-6 and TNF- α in the presence of anti-Sm mAb alone plus that in the presence anti-RNP mAb alone (broken line). The results confirm that anti-Sm

mAb and anti-RNP mAb exert synergistic enhancing effects on the production of IL-6 and TNF- α of human monocytes.

Enhancement of the production of IL-6 of human peripheral blood monocytes by human anti-Sm and anti-RNP

Next experiments were performed to confirm that human anti-Sm and human anti-RNP exert comparable effects on the production of IL-6 to those by murine anti-Sm mAb and murine anti-RNP mAb. As shown in Fig 4, the production of IL-6 was increased along with the increase in concentrations of anti-Sm in the presence of the constant concentration of anti-RNP. Again, the IL-6 production in the presence of both human anti-Sm and human anti-RNP was significantly higher than the sum of the IL-6 production in the presence of anti-RNP (0.9 U/ml) alone plus that in the presence of anti-Sm (1.1 U/ml) alone (solid bar in Fig 4). The results confirm that human anti-Sm and human anti-RNP synergistically enhance the production of IL-6 of human peripheral blood monocytes.

Effects of Fc receptor blocking on the synergistic enhancement of IL-6 production of peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb

In order to examine whether Fc portions are required for the effects of anti-Sm mAb and anti-RNP mAb, experiments were carried out in which the effects of addition of whole molecule human IgG and human IgG F(ab')₂ fragments were explored. As shown in Fig 5, the synergistic effects of anti-Sm mAb and anti-RNP mAb on the IL-6 production of monocytes in cultures with whole molecule human IgG (100 μ g/ml) were comparable to those in cultures

with human IgG F(ab')₂ fragments (100 µg/ml). The results therefore suggest that Fc portions might not be required for the synergistic effects of anti-Sm mAb and anti-RNP mAb.

Further experiments were carried out in which the effects of a purified recombinant Fc protein (Human BD Fc Block), which minimizes non-specific binding of immunoglobulins to Fc receptors, was examined. As shown in Fig 6A, addition of the human Fc blocker markedly decreased the binding of anti-Sm mAb and anti-RNP mAb on human monocytes. However, the synergistic effects of anti-Sm mAb and anti-RNP mAb on the IL-6 production of human monocytes were preserved in the presence of the human Fc blocker (Fig 6B). The results thus confirm that Fc portions are not required for the synergistic effects of anti-Sm mAb and anti-RNP mAb on the IL-6 production of human monocytes.

Effects of anti-Sm mAb and anti-RNP mAb on the expression of mRNA for IL-6 and TNF- α in human peripheral blood monocytes

Next experiments were carried out to explore whether the synergistic enhancing effects of anti-Sm mAb and anti-RNP mAb might be observed at mRNA levels. As shown in Fig 7, anti-Sm mAb (3 µg/ml) had no significant effect on the expression of mRNA for IL-6 and TNF- α in monocytes in the absence of anti-RNP mAb. However, anti-Sm mAb (3 µg/ml) significantly increased the expression of mRNA for IL-6 and TNF- α in monocytes in the presence of anti-RNP mAb (3 µg/ml). The results indicate that the synergistic enhancing effects on the production of IL-6 and TNF- α of human monocytes by anti-Sm mAb and anti-RNP mAb are accounted for by their effects on the expression of mRNA for IL-6 and TNF- α .

Effect of anti-Sm mAb and anti-RNP mAb on the expression of mRNAs for various components of NFkB in human peripheral blood monocytes

Previous studies have demonstrated that the enhancement of the expression of mRNAs for various components of NFkB results in the up-regulation of activity of NFkB [18,19]. Next experiments were carried out to explore the effects of anti-Sm mAb and anti-RNP mAb on the expression of mRNAs for various components of NFkB. Highly purified peripheral blood from healthy individuals were cultured in the presence of anti-Sm mAb and anti-RNP mAb for 4 hours, after which the expression of mRNA for various components of NFkB was examined. As shown in Fig 8, anti-RNP mAb, but not anti-Sm mAb, significantly enhanced the expression of RelA (p65) mRNA, whereas neither mAb significantly influenced the expression of NFkB1 (p50) or NFkB2 (p52). The results indicate that anti-RNP mAb, but not anti-Sm mAb, enhances the expression of RelA (p65) mRNA. In addition, it is suggested that the action of anti-Sm mAb in the synergism with anti-RNP mAb might involve such mechanisms other than p65 mRNA up-regulation.

Effects of N-acetyl cysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) on the synergistic enhancement of the production of IL-6 of peripheral blood monocytes by anti-Sm mAb and anti-RNP

Activation of NFkB transcription factor is critical for the expression of proinflammatory cytokines in human monocytes [20,21]. To investigate the mechanism of synergistic enhancement of expression of IL-6 by anti-Sm mAb and anti-RNP mAb, next experiments

examined the effects of NAC, an inhibitor of NFkB [22]. As shown in Fig 9, the enhancement of production of IL-6 of monocytes by anti-Sm mAb and anti-RNP mAb was markedly decreased by addition of NAC (1-3 mM). Thus, NAC markedly suppressed the baseline IL-6 production and almost completely abrogated the enhancing effect of anti-Sm mAb alone on IL-6 production in the absence of anti-RNP mAb. However, anti-Sm mAb still markedly up-regulated the IL-6 production in the presence of anti-RNP under the influences of NAC. It has been shown that the antioxidant PDTC specifically inhibits the transcription of IL-6, IL-8, and GM-CSF genes through the inhibition of the NFkB activation, while increasing the expression of AP-1 [23]. In the presence of PDTC (50 μ M), which suppressed the baseline IL-6 production, anti-Sm mAb still enhanced the IL-6 production in the presence of anti-RNP mAb (Fig 10). These results strongly suggest that the synergistic effects of anti-Sm mAb on the IL-6 production of monocytes in the presence of anti-RNP mAb might not involve the activation of NFkB.

Discussion

Although anti-Sm and anti-RNP recognize nuclear proteins, several studies have shown that they bound the surface of certain types of cells. Thus, it has been shown that the epitopes recognized by anti-Sm existed on the surface of neuronal cells [7], while anti-RNP were found to bind endothelial cells [2]. The results in the current studies confirm that the epitopes recognized by anti-Sm and anti-RNP exist on the surface of human peripheral blood monocytes. Of note, the results also demonstrate that the expression of epitopes for anti-Sm and anti-RNP on the surface of human monocytes are upregulated upon activation. In this regard, the regulation of the expression of the epitopes for anti-Sm and anti-RNP was comparable to that of the expression of the epitopes for anti-ribosomal P antibodies in human monocytes [13].

The data in the present study have demonstrated that anti-Sm and anti-RNP enhanced the production of IL-6 and TNF- α of human monocytes at protein and mRNA levels, as is also the case in anti-ribosomal P antibodies [13]. The enhancing effect of anti-Sm alone was modest and much smaller than that of anti-RNP alone. However, anti-Sm enhanced the production of IL-6 and TNF- α of human monocytes much more markedly in the presence of anti-RNP. Apparently, the effects of anti-Sm and anti-RNP on the production of IL-6 and TNF- α were synergistic rather than additive.

The synergistic effects suggest that the signal delivered by anti-Sm might be different from that delivered by anti-RNP. In this regard, the data in the present study revealed that anti-RNP, but not anti-Sm, up-regulated the expression of RelA (p65) mRNA. Notably, the enhanced expression of RelA (p65) has been found to result in the activation of NF κ B signaling [18,19].

Therefore, it is also possible that anti-RNP might also promote the activation of NFkB. Accordingly, NAC and PDTC markedly decreased the production of IL-6 in the presence of anti-RNP alone. However, anti-Sm still enhanced the IL-6 production in the presence of anti-RNP under the influences of NAC or PDTC which almost completely abrogated the IL-6 production by anti-Sm alone in the absence of anti-RNP. The data therefore indicate that the signals delivered by anti-Sm in the synergism with anti-RNP do not involve NFkB. Further studies are required to identify the nature of signals delivered by anti-Sm.

Anti-Sm are directed against proteins that constitute the common core of small nuclear ribonucleoprotein (snRNP) particles [5]. Of note, all patients with positive serum anti-Sm express also serum anti-RNP in clinical practice [5]. Therefore, the synergistic effects of anti-Sm and anti-RNP demonstrated in the present study are considered to take place always in vivo and play a role in the development of inflammatory reactions in SLE. Anti-RNP react with proteins that are associated with U1-RNA and form U1snRNP, including A protein, C protein and 68-kd protein [5]. Anti-RNP mAb used in the present studies reacts only with 68-kd protein. It should be noted, however, that all human sera containing anti-U1-RNP antibodies reacted the cloned 68-kd RNP protein [12]. In fact, IgG fractions containing anti-RNP purified from the patients' sera enhanced the production of IL-6 of monocytes in the present study. However, it remains to be elucidated whether antibodies to A protein or to C protein might have similar effects.

Previous studies demonstrated that serum anti-Sm were significantly higher in acute confusional state (ACS) than that in non-ACS diffuse neuropsychiatric SLE (NPSLE) or focal NPSLE [6,7]. Of note, serum IL-6 was also significantly higher in ACS than that in the other

2 groups of NPSLE [24]. Moreover, it has been found that serum anti-Sm as well as serum IL-6 levels were significantly correlated with Q albumin in patients with diffuse NPSLE, including ACS and non-ACS [8,24]. Notably, it was disclosed that the increased production of IL-6 in the CNS could influence the function of BBB [25]. On the other hand, it should be emphasized that TNF- α has been found to result in the damages of BBB [10,11]. Therefore, the results in the current studies support the hypothesis that anti-Sm might cause BBB damages through upregulation of the production of IL-6 and TNF- α .

Conclusions

The present study has confirmed that anti-Sm as well as anti-RNP bind on the surface of human monocytes. More importantly, it has been demonstrated that anti-Sm and anti-RNP synergistically enhance the production of IL-6 by human monocytes. Although the present study partially disclosed the mechanism of synergistic effects by anti-Sm and anti-RNP, further studies would be important for a complete understanding of the roles of these antibodies in the pathogenesis of SLE.

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Table 1. Preparation of mixtures of IgG containing varying concentrations of anti-Sm and anti-RNP.

No.	<u>Contents of various IgG samples^a</u>						<u>Concentrations</u>			
	PtA (μ l)	PtB (μ l)	PtC (μ l)	PtD (μ l)	HE (μ l)	HF (μ l)	PBS (μ l)	Anti- RNP (U/ml)	Anti- Sm (U/ml)	IgG (mg/ml)
1	0	0	0	50	100	100	150	9	0	1.08
2	40	0	0	0	150	150	60	9	3	1.06
3	0	40	0	0	133	133	100	9	7	0.98
4	40	0	200	0	50	50	60	9	9	1.03
5	0	40	133	0	67	67	100	9	11	1.04
6	0	0	133	0	133	133	0	0	11	0.97
7	0	0	0	0	200	200	0	0	0	1.01

^aIgG fractions from 4 patients (PtA, PtB, PtC, PtD) and from 2 healthy individuals (HE, HF) were mixed with or without phosphate buffered saline (PBS) as indicated. The concentrations of IgG, anti-Sm and anti-RNP in each IgG sample:

PtA (Patient A) (IgG; 2.69 mg/dl, Anti-Sm; 32 IU/ml, Anti-RNP; 96 IU/ml)

PtB (Patient B) (IgG; 3.58 mg/dl, Anti-Sm; 75 IU/ml, Anti-RNP; 96 IU/ml)

PtC (Patient C) (IgG; 1.18 mg/dl, Anti-Sm; 38 IU/ml, Anti-RNP; 0 IU/ml)

PtD (Patient D) (IgG; 4.28 mg/dl, Anti-Sm; 0 IU/ml, Anti-RNP; 77 IU/ml)

HE (Healthy E) (IgG; 1.10 mg/dl), HF (Healthy F) (IgG; 1.08 mg/dl).

Fig 1. Flow cytometric analysis of binding of anti-Sm mAb and anti-RNP mAb to human peripheral blood monocytes. (A) Bindings of anti-Sm mAb and anti-RNP mAb on resting monocytes and monocytes activated by adhesion (stained in tubes), and binding of anti-Sm mAb and anti-RNP mAb during cultures. The mean fluorescence intensity (MFI) for specific anti-Sm mAb staining and anti-RNP mAb staining is indicated. Representative of 6 different experiments with similar results. (B) Differences of the mean fluorescence intensity (MFI) for control IgG1 vs anti-RNP mAb and control IgG3 vs anti-Sm mAb, in staining on resting monocytes or on monocytes activated by adhesion. Statistical significance was determined by paired t test.

Fig 2. Effects of anti-RNP mAb and anti-Sm mAb on the production of IL-6 of peripheral blood monocytes. (A) Time kinetics of the effects of anti-RNP mAb and anti-Sm mAb. Highly purified monocytes were cultured in the presence of various antibodies (3 µg/ml). Mean values with standard deviation (Error bars) of 6 different experiments are shown. Statistical significance was examined by Paired t test. *Significant at $p \leq 0.05$ compared with 48 hours of culture. (B) Dose responses of anti-RNP mAb and anti-Sm mAb on the production of IL-6. Highly purified monocytes were cultured in the presence of various concentrations of anti-Sm mAb, anti-RNP mAb, control IgG1 or control IgG3. After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (Error bars) of 6 different experiments are shown.

Fig 3. Synergistic effects of anti-Sm mAb and anti-RNP mAb on the production of IL-6 (A) and TNF- α (B) of peripheral blood monocytes. Highly purified monocytes were cultured with varying concentrations of anti-Sm mAb in the presence of anti-RNP mAb (1

µg/ml) or control IgG1 (1 µg/ml). After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (Error bars) of 5 different experiments are shown. The broken line indicate the sum of the IL-6 and TNF-α production in the presence of anti-RNP mAb alone (1 µg/ml) plus that in the presence of IgG1 (1 µg/ml) with various concentrations of anti-Sm mAb. Statistical significance was analyzed using Paired t test.

* Significantly higher ($p \leq 0.05$) than the point of anti-Sm of 1 µg/ml in the broken line.

** Significantly higher ($p \leq 0.005$) than the point of anti-Sm of 2 µg/ml in the broken line.

Fig 4. Effects of human anti-Sm and human anti-RNP on the production of IL-6 of peripheral blood monocytes. Highly purified monocytes (1×10^6 /ml) from 4 healthy individuals were cultured in the presence of various IgG fractions containing varying concentrations of human anti-Sm and human anti-RNP at a final concentration of 100 µg/ml IgG. Thus, each IgG preparation shown in Table 1 was added at 1:10 [volume/volume]. After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (Error bars) of 4 different experiments are shown. The solid bar indicate the sum of IL-6 production in the presence of anti-RNP (0.9 U/ml) alone plus that in the presence of anti-Sm (1.1 U/ml) alone. Statistical significance was analyzed using Paired t test.

Fig 5. Effects of human IgG or human IgG F(ab')₂ fragments on the synergistic enhancement of the production of IL-6 of peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb. Highly purified monocytes were cultured in the presence of whole molecule human IgG (100 µg/ml) or human IgG F(ab')₂ fragments (100 µg/ml) with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml). After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation

(error bars) of 3 different experiments are shown. Statistical significance was evaluated by paired t test.

Fig 6. Effects of human recombinant Fc protein on the synergistic enhancement of the production of IL-6 of peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb.

Highly purified monocytes were cultured with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml) in the presence or absence of the human Fc blocker (Human BD Fc Block) (50 µg/ml). (A) After 24 hours the cells were harvested and stained with FITC-conjugated goat F(ab')₂ anti-mouse IgG, followed by analysis on flowcytometry. The mean fluorescence intensity (MFI) for specific anti-Sm mAb staining and anti-RNP mAb staining is indicated. Representative of 3 different experiments with similar results. (B) After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (error bars) of 3 different experiments are shown. Statistical significance was evaluated by paired t test.

Fig 7. Effects of anti-Sm mAb and anti-RNP mAb on the expression of mRNA for IL-6 (A) and TNF-α (B) in peripheral blood monocytes.

Highly purified monocytes from 8 healthy individuals were cultured with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml). After 4 hours of incubation, total RNA was isolated, and real-time quantitative polymerase chain reaction was performed as described in Materials and Methods. All results for IL-6 and TNF-α mRNA copy numbers were calibrated to the copy numbers of β-actin from the same cDNA sample. Statistical significance was evaluated by Repeated-Measures one-way ANOVA with multiple comparison.

Fig 8. Effects of anti-Sm mAb and anti-RNP mAb on the expression of mRNAs for

various components of NFkB. Highly purified monocytes from 6 healthy individuals were cultured with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml). After 4 hours of incubation, total RNA was isolated, and real-time quantitative polymerase chain reaction was performed as described in Materials and Methods. All results for NFkB1 (p50), RelA (p65) and NFkB2 (p52) mRNA copy numbers were calibrated to the copy numbers of β-actin from the same cDNA sample. Statistical significance was evaluated by Repeated-Measures one-way ANOVA with multiple comparison.

Fig 9. Effects of N-acetyl cysteine (NAC) on the synergistic enhancement of the production of IL-6 of peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb.

Highly purified monocytes were cultured in the presence or absence of NAC (1 mM or 3 mM) with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml). After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (error bars) of 3 different experiments are shown. Statistical significance was evaluated by paired t test.

Fig 10. Effects of pyrrolidine dithiocarbamate (PDTC) on the synergistic enhancement of the production of IL-6 of peripheral blood monocytes by anti-Sm mAb and anti-RNP mAb. Highly purified monocytes were cultured in the presence or absence of PDTC (50 µM) with various combination of anti-Sm mAb, anti-RNP mAb, control IgG1 or IgG3 (3 µg/ml). After 48 hours of incubation, the supernatants were assayed for IL-6. Mean values with standard deviation (error bars) of 2 different experiments with reproducible results are shown.

Figure 1

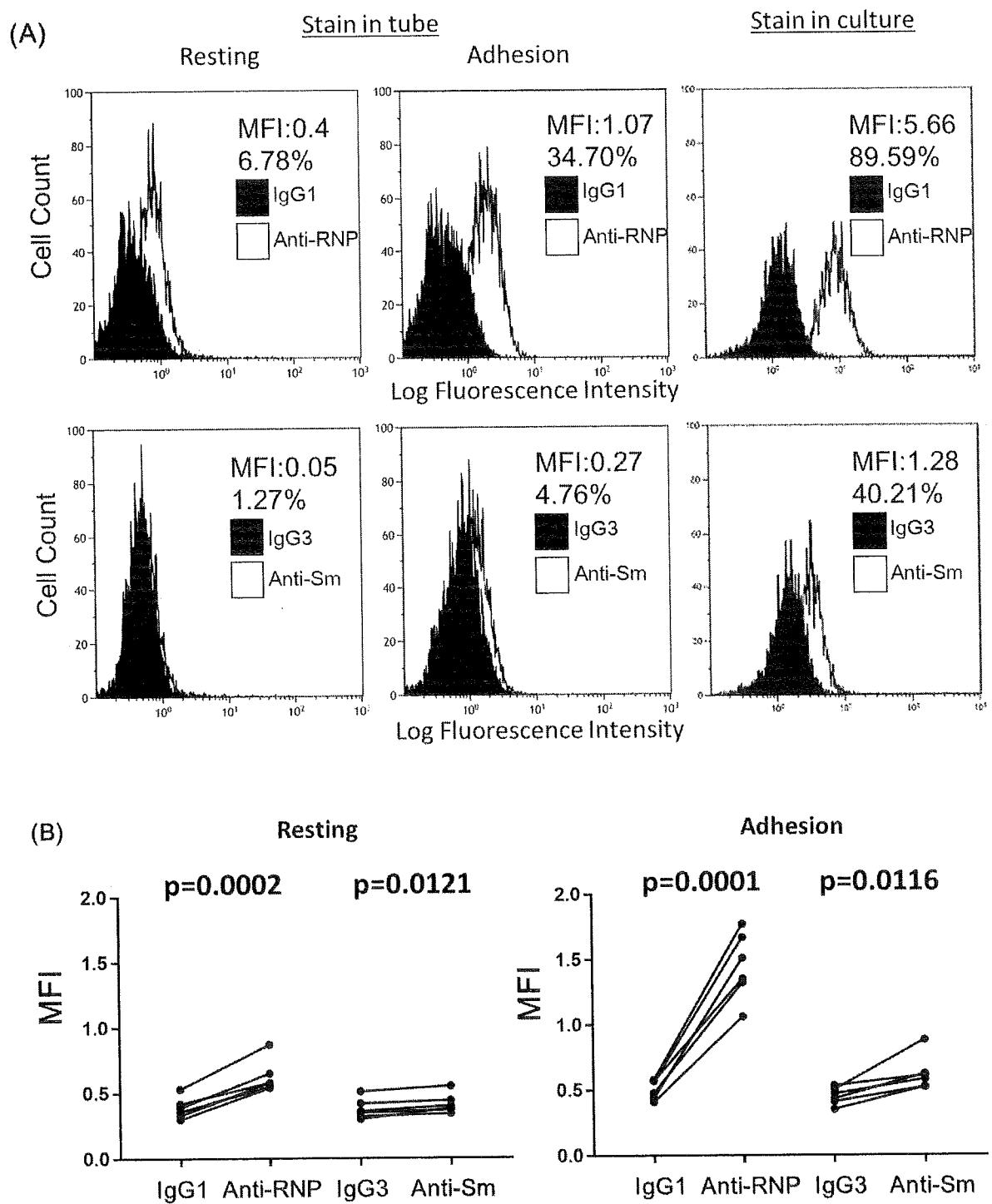


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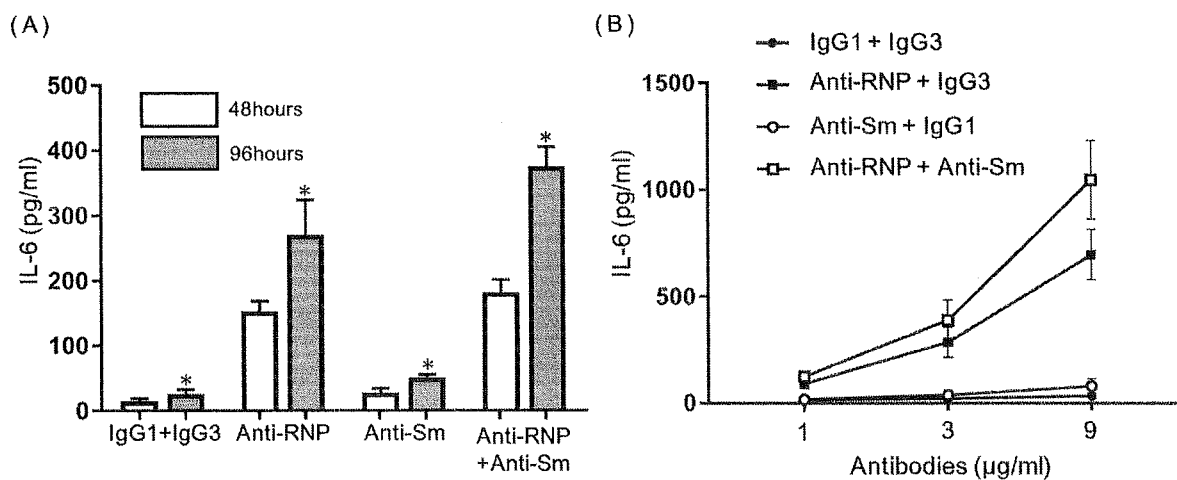


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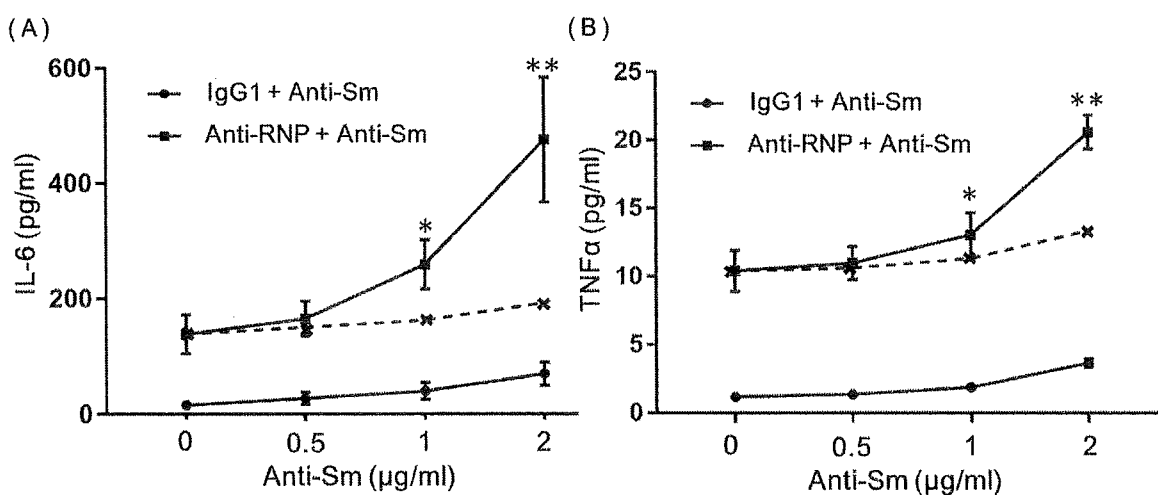


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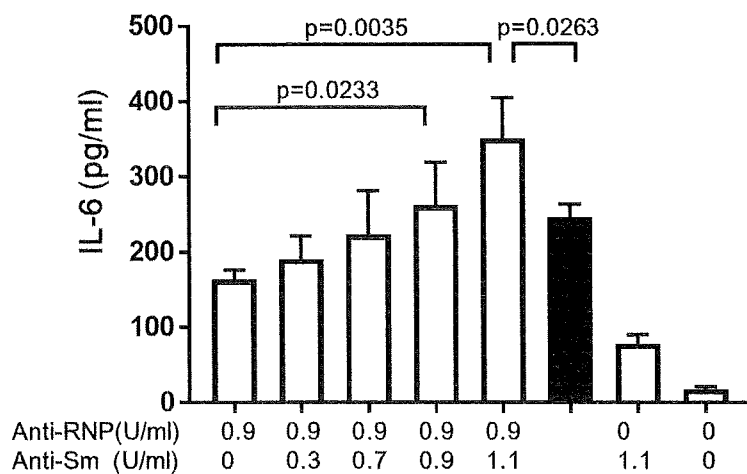


Figure 5

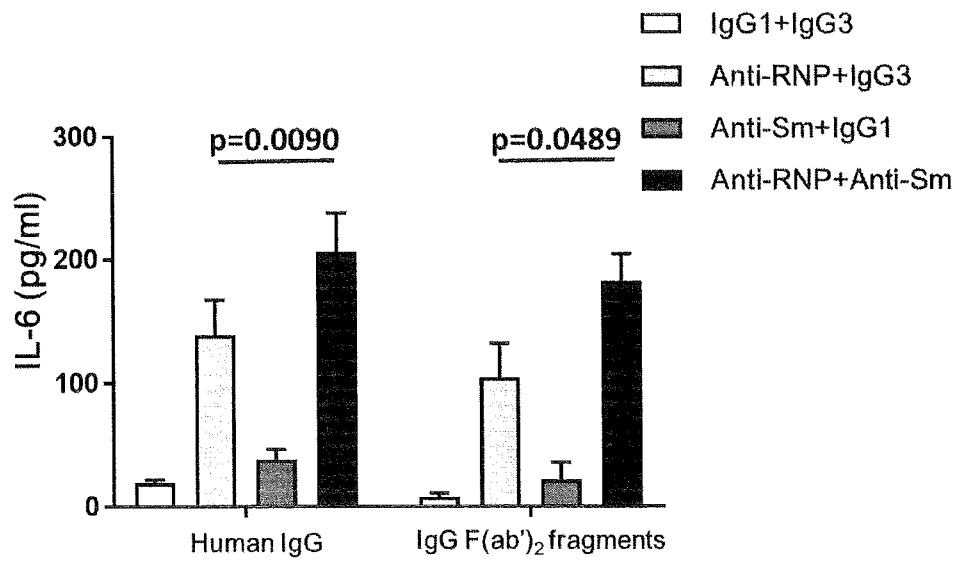
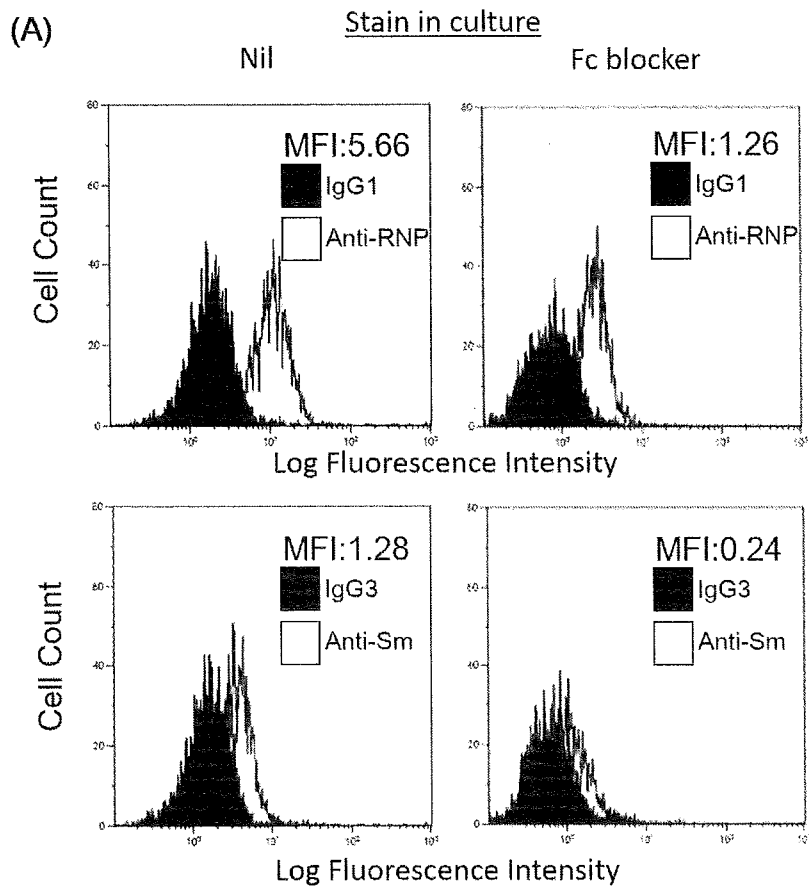


Figure 6



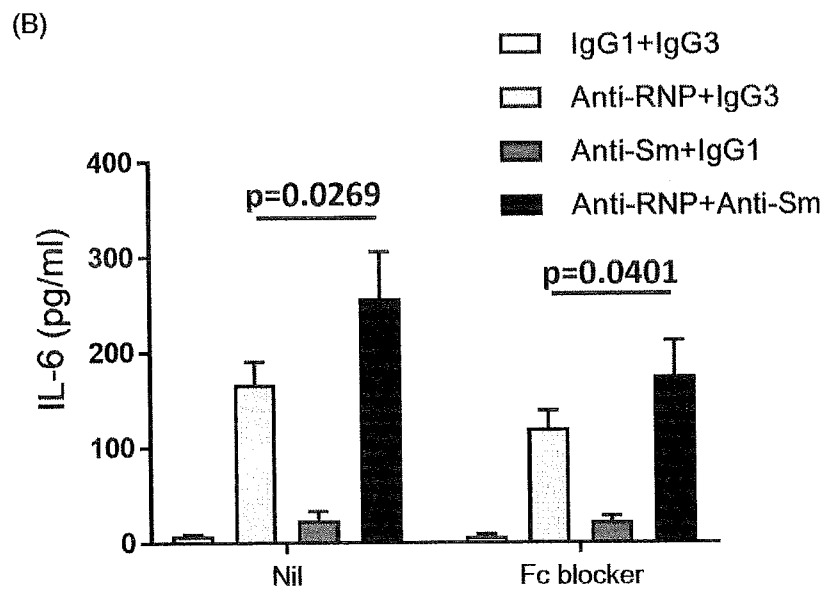


Figure 7

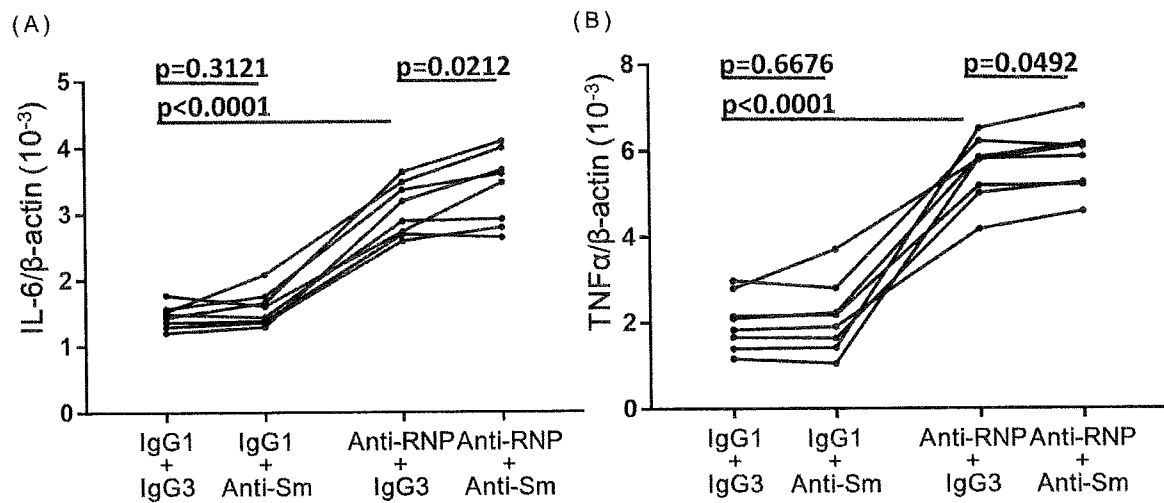


Figure 8

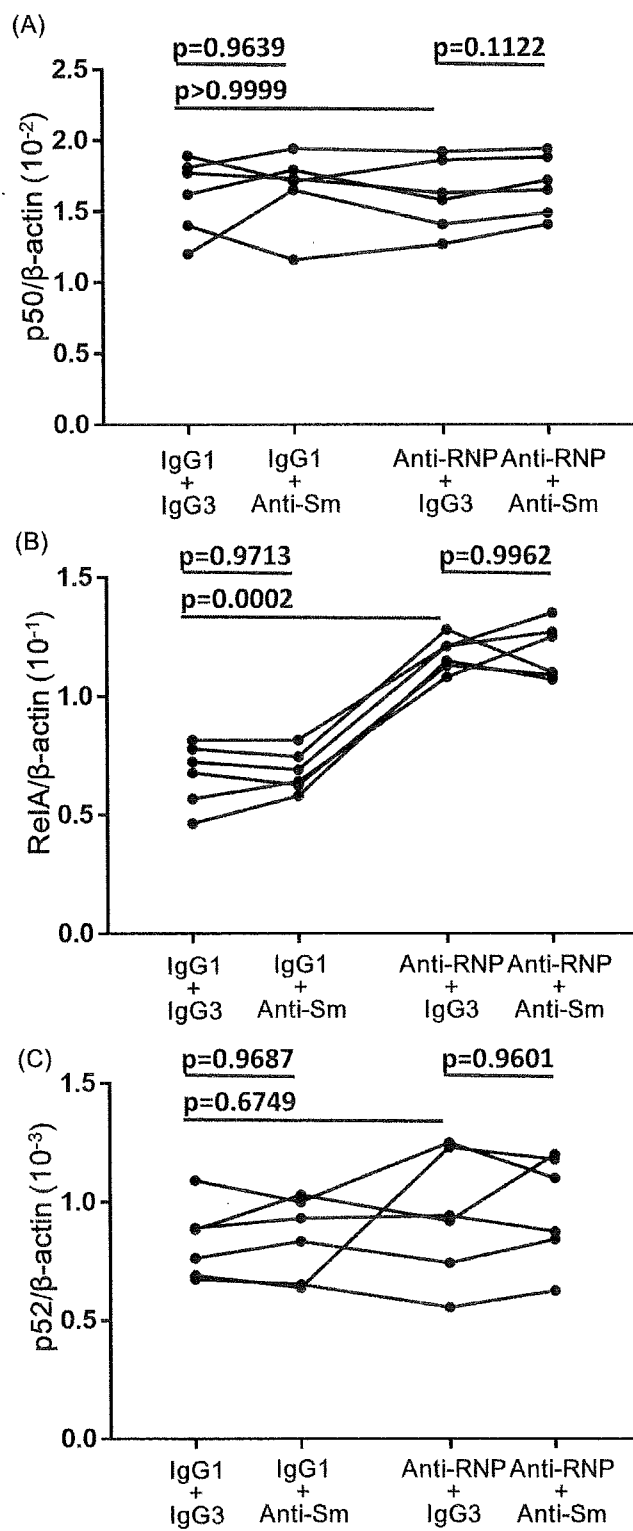


Figure 9

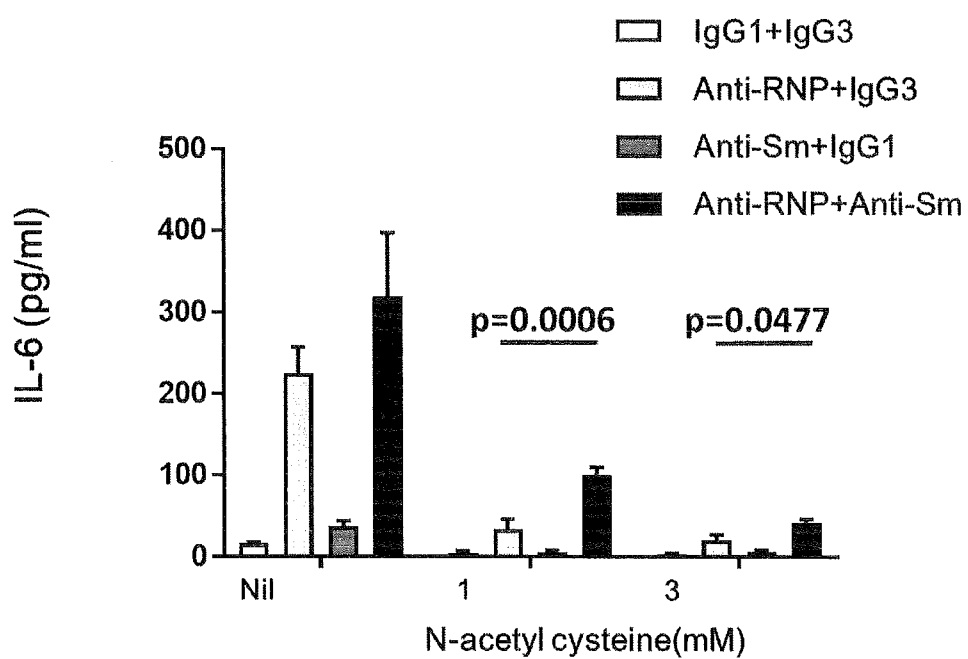


Figure 10

