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Title page

p38 MAP kinase-γ inhibition by long-acting β_2 adrenergic agonists reversed steroid insensitivity in severe asthma

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Running Title page

Running title: p38 MAP kinase-γ and steroid insensitivity

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Abstract (<250):	231
Introduction (<750):	594
Discussion (<1500):	1342

A list of nonstandard abbreviations

CI, corticosteroid insensitivity; GR, glucocorticoid receptor; HV, healthy volunteers;, JNK, Jun-NH_2-terminal kinase; LABA, long acting β_2-adrenergic agonist; MA, Mild glucocorticoid-sensitive asthma; p38 MAPK-γ, p38 mitogen activated protein kinase gamma; PBMCs, peripheral blood mononuclear cells;

SA, severe asthma; UK, United Kingdom

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Abstract

Corticosteroid insensitivity (CI) is a major barrier to treating severe asthma. Despite intensive research, the molecular mechanism of CI remains uncertain. The aim of this study was to determine abnormality in corticosteroid action in severe asthma, and to identify the molecular mechanism of the long-acting β_2-adrenergic agonists (LABAs), formoterol and salmeterol, on restoration of corticosteroid sensitivity in severe asthma *in vitro*. Peripheral blood mononuclear cells (PBMCs) were obtained from 16 subjects with severe corticosteroid-insensitive asthma, 6 subjects from mild corticosteroid-sensitive asthma and 11 healthy volunteers. Corticosteroid (dexamethasone) sensitivity was determined on TNF-α induced IL-8 production. Glucocorticoid receptor (GR) phosphorylation and kinase phosphorylation were evaluated by immunoprecipitation-western blotting and kinase phosphorylation array in IL-2/IL-4-treated corticosteroid insensitive model in PBMCs. In vitro corticosteroid sensitivity on TNF-α induced IL-8 production was significantly lower in severe asthma than those of healthy volunteers and mild asthmatics. This CI seen in severe asthma was associated with reduced GR nuclear translocation and also with hyper-phosphorylation of GR, which were reversed by LABAs. In IL-2/IL-4- treated PBMCs, LABAs inhibited phosphorylation of JNK and p38MAPK-γ as well as GR phosphorylation. In addition, cells with p38MAPK-γ knock-down by RNA interference did not develop CI in the presence of IL-2/IL-4. Furthermore, p38MAPK-γprotein expression was up-regulated in PBMCs from some patients with severe asthma. In conclusion, p38 MAPK-γ activation impairs corticosteroid action and p38 MAPK-γ inhibition by LABAs has potential for the treatment of severe asthma.

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Introduction

Most patients with asthma are now effectively controlled with inhaled corticosteroids. However, approximately 5% of patients with asthma do not respond well to corticosteroids or require a high dose inhaled or oral corticosteroids to control asthma symptoms, although side effects are still a problem. Thus, corticosteroid insensitivity (CI) presents considerable management problems, accounting for a disproportionate amount of healthcare spending in asthma (Adcock and Ito, 2004;Leung and Szefler, 1998).

The biological actions of corticosteroids are mediated by glucocorticoid receptors (GRs) which are normally located in cell cytoplasm. Corticosteroids cross the cell membrane and bind to GR, which then translocates into the nucleus and its homodimers bind to DNA at glucocorticoid response elements (GRE) in the promoter region of corticosteroid-responsive anti-inflammatory genes, such as secretory leukoprotease inhibitor (SLPI), mitogen-activated kinase phosphatase-1 (MKP-1) and glucocorticoid inducible leucine zipper (GILZ), increasing gene transcription. As well as this GR-GRE binding, GR may directly influence pro-inflammatory signaling by forming inhibitory interactions with pro-inflammatory DNA-binding transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), or by recruitment of co-repressors such as histone deacetylase 2 (Ito et al., 2006). GR nuclear translocation is, therefore, essential and critical step for corticosteroid action. However, as we reported earlier (Matthews et al., 2004b), some of severe asthma patients showed defect of GR nuclear translocation.

Numerous studies demonstrated possible mechanisms of corticosteroid insensitivity, such as overexpression of transcription factors (Adcock et al., 1995), histone deacetylase reduction (Hew et al., 2006;Cosio et al., 2004) and increased

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decoy receptor (Leung et al., 1998). Post-translational modifications of GR, such as phosphorylation, acetylation and ubiquitination, are also important components for the mechanism of corticosteroid resistance (Ito et al., 2006). For example, Rogatsky et al demonstrated that the ability of GR of transcriptional activation was reduced once Ser467 of rat GR (equivalent to Ser226 of human GR) was phosphorylated by JNK (Rogatsky et al., 1998). Irusen et al showed that an inhibitor of p38 mitogen-activate protein kinase (MAPK)-α and β isoforms inhibited Intreleukin (IL)-2/IL-4-induced GR phosphorylation in whole cell extracts (Irusen et al., 2002) though Rogatsky *et al* showed that GR at Ser-246 was not phosphorylated by p38 MAPK (Rogatsky et al., 1998). Thus, GR phosphorylation is reported to be associated with CI but GR phosphorylation has not been detected in clinical samples.

p38 MAPK-γ is one of 4 isoforms of p38MAPKs (Cuenda et al., 1997;Mertens et al., 1996). This kinase is also called stress-activated protein kinase-3 (SAPK3), extracellular signal-regulated kinase 6 (ERK6) or mitogen-activated protein kinase 12 (MAPK12), and is able to phosphorylate PDZ motif containing protein, such as SAP90 and SAP97. p38MAPK-γ is activated by environmental stress, such as oxidative stress and osmotic stress, or pro-inflammatory cytokines, and phosphorylates several downstream targets. p38 MAPK-γ is expressed in T-lymphocytes, macrophages as well as skeletal muscle cells but its function is not certain.

Recently, the combinations of a long-acting β_2-agonist (LABA) with a low dose of inhaled corticosteroid have been reported to achieve better asthma control than either drug alone, or a higher dose of inhaled corticosteroid alone (Miller-Larsson and Selroos, 2006;Reynolds et al., 2005). LABAs alone have been shown to induce GR nuclear translocation in smooth muscle cells and fibroblasts (Eickelberg et

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al., 1999), and also enhance corticosteroid actions *in vitro* and *in vivo* (Pang and Knox, 2000;Usmani et al., 2005;Roth et al., 2002). In this way LABAs may enhance the anti-inflammatory action of corticosteroids, but the molecular mechanism has not been fully elucidated.

Here we show that p38 MAPK-γ causes corticosteroid insensitivity in severe asthma through hyper-phosphorylation of GR, and that this is reversed by LABAs.

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Methods

Materials

Formoterol (*rac*-(R,R)-*N*-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl) propan-2-ylamino]ethyl] phenyl]formamide) and Salmeterol((*RS*)-2-(hydroxymethyl)-4-{1-hydroxy-2-[6-(4-phenylbutoxy) hexylamino]ethyl}phenol) were provided by AstraZeneca (Lund, Sweden) and GlaxoSmithKline (Greenford, UK), respectively. Dexamethasone: (8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9- Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16- trimethyl-6,7,8,9,10,11,12,13,14,15,16,17- dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one) and SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine) were purchased from Sigma-Aldrich (Poole, UK).

Subjects

11 healthy non-smoking subjects (mean age ± SEM 35.8±1.6 yr, 3 females, mean forced expiratory volume in one second (FEV_1±SEM 98.2±2.2 % predicted), 6 mild asthmatic patients 41.5±3.3 years, 3 females, FEV_1 84.5±4.6 % of predicted) and 16 severe asthmatic patients (mean age 35.1 ± 2.6 yr, 11 females, FEV_1 (SEM) 55.0 ± 3.4% predicted) were recruited (Table 1). This study was approved by the Ethics Committee of the Royal Brompton & Harefield Hospitals National Health Service Trust, and all subjects gave written informed consent. 50 ml of blood were taken and PBMCs were separated by Ficoll-Paque gradients.

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Corticosteroid insensitive model:

PBMCs from healthy volunteers were incubated with human recombinant IL-2 (2ng/ml) and IL-4 (10ng/ml) for 48 h.

FITC-dexamethasone incorporation

PBMCs were incubated with FITC-conjugated dexamethasone (FITC-Dex, 10^{-6}M) for 30 min at 37ºC. Non-specific FITC diffusion was determined in the presence of 10^{-5}M non-conjugated Dex, and subtracted from the total FITC fluorescence value. The nuclear fraction was prepared by hypotonic buffer (Active Motif, Rixensart, Belgium) for 10 min-incubation, followed by pulse voltexingwith 0.1% NP-40 containing PBS. FITC-dexamethasone in nuclei was extracted with 0.5% NP-40 containing PBS on ice for 20 min. The concentration of FITC-Dex was determined using a standard curve to different concentrations of FITC-Dex. FITC was detected at 488nm in fluorescent plate reader.

Detection of GR and phosphorylated GR

Whole cell extracts were prepared with modified RIPA buffer (Ito et al., 2000). For the detection of GR, whole cell extracts were separated by Tris-Glycine SDS/PAGE and transferred to nitrocellulose membrane. The GR level was normalized to β-actin expression. For the detection of phosphorylated GR, GR was immunopurified using anti-GR antibody conjugated Agarose A/G (P-20, Santa Cruz Biotechnology Inc., CA) and separated by SDS-PAGE/Western-blotting. Phosphorylation level was determined with anti-pan-phosphoserine antibody (Santa Cruz Biotechnology Inc., CA) and was normalized to GR expression. In addition, GR phosphorylated at Ser226 was determined using anti-phosphorylated (S226) GR antibody (New England

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Biolabs (UK) Ltd., Hertfordshire, UK). The band density was calculated by densitometry (UVP Bioimaging Systems, Cambridge, UK) using Labworks software (Ultra-Violet Products, Cambridge, UK).

RT-PCR

Cells were harvested for total RNA isolation. Commercially available kits were used to extract total cellular RNA (RNeasy, Qiagen, Crawley, U.K.) and to perform reverse transcription (Omniscript RT, Qiagen). Gene transcript level of p38 MAP-α, -γ and -δ and the housekeeping gene GNB2L1 or GAPDH were quantified by real-time PCR using a Taqman PCR kit (Applied Biosystems, Warrington, UK) on a Rotor-Gene 3000 PCR apparatus (Corbett Research, N.S.W., Australia).

ELISA

Cells were treated with dexamethasone (10^{-12} to 10^{-6}M) for 30 min in the presence or absence of LABA, and then stimulated with either TNF-α (1 ng/ml) or a combination of anti-human CD3 (10μg/ml) and CD28 antibodies (8μg/ml) (BD Biosciences, Oxford, UK) overnight. IL-8 and IL-2 levels in supernatant were determined by sandwich ELISA (Duoset® ELISA for human IL-8, R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

Kinase profiling

The phosphorylation of 19 different kinases was evaluated using the Human Phospho-MAPK Array Kit Proteome Profiler™ (R&D Systems, R&D Systems Europe,

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Abingdon, UK) according to the manufacturer's instructions. HSP27 (phosphorylated and total) and p38MAPKγ(phosphorylated p38MAPK/SAPK and total) were detected by western blotting. All antibodies were purchased from R&D Systems Europe (Abingdon, UK).

Measurement of phosphorylated and total p38MAPKγ in cells

Phosphorylated p38MAPKγ and total p38MAPKγ were detected in PBMCs obtained from healthy subjects using p38MAPKγ (T183/Y185) phosphorylation and total cell-based ELISA (Duoset IC ELISA, R&D Systems Europe, Abingdon, UK). Briefly, cells are stimulated with human recombinant IL-2 (2ng/ml) and IL-4 (10ng/ml) for 48 h and then treated with formoterol, salmeterol or salbutamol for 20min. Cells were collected and lysed using lysis buffer according to the manufacturer's instructions.

RNA interference

Short interference (si) RNA of the p38 MAPK-δ (MAPK13) and p38 MAPK-γ (MAPKK 12) were purchased from Dharmacon Inc.(Colorado Springs, CO, USA) and transfected by nucleofection using AMAXA®Nucreofector® (Lonza GmbH, Cologne, Germany) according to the manufacturer's instructions (100 nM each). Cells were incubated for 24 h and then stimulated with IL-2/IL-4 for further 48 h. Non-specific control duplex (scrambled oligonucleotide:sc, 47% GC content) were also purchased from Dharmacon Inc. (Colorado Springs, CO, USA).

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Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Analysis of variance was done by Kruskal-Wallis analysis and when significant, comparisons were made by Mann Whitney U-test using the PC analysis package SPSS 10.0 (SPSS Inc., Chicago, IL) or Graph Pad Prism 4 Software (Graph Pad Prism, San Diego, CA). The differences between treatment groups in the *in vitro* data were analyzed by Welch's t-test. The correlation between two parameters was determined by Spearman methods. A p value < 0.05 was considered statistically significant.

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Results

PBMCs from severe asthma were corticosteroid insensitive due to defect of GR nuclear translocation

As shown in Fig.1A, PBMCs produced IL-8 when stimulated with TNF-α in severe asthma patients (SA, 1430±286 pg/ml), to a similar level as seen in healthy volunteers (HV, 1650±304 pg/ml) although the IL-8 production was significantly higher in mild asthmatics (MA, 2160±94.9 pg/ml) than that in healthy volunteers. In contrast, when 50% inhibitory activity of dexamethasone (Dex-IC_{50}) on TNF-α-induced IL-8 release was calculated as an index of corticosteroid sensitivity, the Dex-IC_{50} values in PBMCs from SA (181 ± 28.7 nM) were significantly higher than those of HV (15.5 ± 4.2 nM; $p<0.01$) and MA (20.0 ± 3.8 nM; $p<0.01$) (Fig.1B).

In PBMCs, there were no significant differences in GRα mRNA expression (GRα/GNB2L1: 70.4±17.8 in SA, 102.4±34.4 in HV, 89.3±24.3 in MA), GRβ mRNA expression (GRβ/GNB2L1: 0.00042±0.00015 in SA, 0.000002±0.0000005 in HV, 0.00043±0.00029 in MA) or in GRα protein expression (GRα/β–actin: 1681±205 in SA, 4914±763 in HV, 3050±670 in MA).

As a marker of GR nuclear translocation after ligand binding, we determined the amount of FITC-conjugated Dex (FITC-Dex) in nuclei. As the anti-inflammatory efficacy of FITC-Dex was 10 times lower than unlabelled Dex (IC_{50} values: FITC-Dex, 5.6 x 10^{-8}M vs. unlabelled Dex, 4.3 x 10^{-9}M on IL-1β-induced IL-8 production in A549 cells), we used a relatively high concentration (10^{-6}M) of FITC-Dex for the assay. As shown in Fig. 1C, GR nuclear translocation was significantly impaired in SA cells [FITC-Dex in nuclei, 4.8±0.9 nM, $p<0.01$ *vs.* HV (11.0±1.5 nM), $p<0.05$ *vs.* MA (9.7±1.4 nM)]. In addition, there was a significant, negative correlation between

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the IC_{50}-Dex value on TNF-α-induced IL-8 release and the amount of FITC-Dex in nuclei (Spearman r=-0.55 p=0.0035), suggesting less Dex efficacy was associated with defect of GR nuclear translocation.

Formoterol reversed corticosteroid insensitivity in PBMCs of severe asthma by enhancing GR nuclear translocation

Treatment with formoterol (FOR, 1nM) decreased the Dex-IC_{50} value for TNF-α induced IL-8 release in PBMCs from SA patients (Dex-IC_{50} with FOR 42.8±21.0 nM *vs*. without FOR 182.5±28.2 nM, n=6, $p<0.05$, Fig.2A) and a similar change but with a lower effect was found with salmeterol (SAL, 100nM) (Dex-IC_{50} with SAL 83.7±16.7 nM *vs*. without SAL 180.7±28.7 nM, n= 15, $p<0.05$) (Table 2). In SA patients, treatment with FOR 1nM showed better improvement index than SAL (for severe asthma: 4.3 for FOR vs 2.2 for SAL, Table 2) but the efficacy was not significantly different between FOR and SAL. Neither FOR nor SAL significantly changed corticosteroid sensitivity in HV (Dex-IC_{50} with FOR 21.5±4.8 nM, and with SAL 31.0±8.3 nM *vs*. 15.5±4.2 nM in non-treatment; Table 2) or in MA patients (Dex-IC_{50} with SAL 14.7±3.5 nM *vs*. 20.0±3.8 nM in non-treatment; FOR was not tested in MA patients). As well as reduced corticosteroid sensitivity to TNFα-induced IL-8 release, corticosteroid sensitivity to CD3/CD28-induced IL-2 release from PBMC was also decreased in SA (Dex-IC_{50} 77.6±25.4 nM in SA n=8, vs.11.5±3.3 nM in HV n=7 and 40.1±16.4 nM in MA n=6) and reversed by SAL (100nM) (Dex-IC_{50} with SAL 30.4±14.4 nM, p=0.0065; FOR was not tested), suggesting SAL increased Dex sensitivity. In addition, in SA, FOR (1nM) enhanced GR nuclear translocation evaluated with FITC-Dex (Nuclear FITC-Dex with FOR 9.5±1.5 nM *vs*. Nuclear FITC-Dex without FOR 4.8±0.9 nM, $p<0.05$, Fig.2B). Similar results were found with

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SAL (100nM) (Nuclear FITC-Dex with SAL 14.2±2.7 nM *vs*. Nuclear FITC-Dex without SAL 4.8±0.9 nM, $p<0.05$, n=16). The improvement in Dex-IC_{50} by FOR (increased ratio of Dex-IC_{50} without FOR vs. Dex-IC_{50} with FOR) correlated well with the improvement of FITC-Dex accumulation in nuclei by FOR (decreased ratio of FITC-Dex in nuclei without FOR vs. FITC-Dex in nuclei with FOR; Spearman r=-0.77, p=0.042). In addition, the improvement in Dex-IC_{50} by FOR was also negatively correlated with FITC-Dex values in nuclei (Spearman r=-0.73, p=0.0013, Fig.2C), suggesting that PBMCs with defect of GR nuclear translocation is more sensitive to FOR treatment.

GRs were highly phosphorylated in PBMCs of severe asthma and dephosphorylated by formoterol

As shown in Fig.3A and B, GR in cytoplasm of PBMCs was highly phosphorylated at serine residues in SA (ratio of phospho-GR/GR: 0.48±0.065, Fig.3B) compared with those of HV (0.22±0.083, Fig.3B) and MA (0.25±0.050, not shown). FOR significantly inhibited phosphorylation of GR after 20 min incubation (ratio of phospho-GR/GR 0.58±0.093 in SA, 0.22±0.037 with 10nM FOR, 0.25±0.050 with 1M FOR, $p<0.05$ vs.non-treated SA, Fig.3C).

IL-2/IL-4 treatment of PBMCs from HVs induced corticosteroid insensitivity with impaired GR nuclear translocation in PBMCs (FITC-Dex in nuclei: 2.3±1.1 nM with IL-2/IL-4 *vs*. 10.7±1.3 nM without IL-2/IL-4, $p<0.05$). IL-2/IL-4 treatment of PBMCs also induced phosphorylation of GR (ratio of phospho-GR/GR 0.42±0.047 with IL-2/IL-4 *vs*. 0.11±0.024 without IL-2/IL-4, $p<0.05$ Fig. 3D, 3E) and FOR significantly inhibited this effect (ratio of phospho-GR/GR 0.17±0.044 with 10nM FOR and 0.17±0.048 with 1nM FOR, for both $p<0.05$ *vs*. 0.42±0.047 without FOR;

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Fig.3D, 3E). In addition, the GR phosphorylated at Serine 226 were determined in same samples. As shown in Fig.3F, GR phosphorylation at Ser 226 was also significantly ($p<0.05$) elevated in PBMCs from SA.

p38 MAPK-γ activation caused corticosteroid insensitivity and was inhibited by formoterol

To determine the kinase activated by 48 h cell incubation with IL-2 and IL-4, which may be involved in the GR phosphorylation (directly or indirectly), kinase phosphorylation array analysis was performed in PBMCs from healthy volunteers. The 48 h exposure to IL-2/IL-4 up-regulated phosphorylation of all 4 isoforms of p38 MAPK (α, β, γ, δ), RSK1 and 2, Akt2 (and pan-Akt), JNK2, JNK3 (and pan-JNK), MSK1, HSP27, GSK-α and -β, and p70S6 (Fig. 4A and 4B). In this experiment, higher concentration of FOR (10nM) was used to maximize the effect. FOR significantly inhibited only phosphorylation of pan-JNK and p38 MAPK-γ (Fig. 4A, 4B). Phosphorylation of p38 MAPK-α and –β was up-regulated by IL-2/IL-4, but was not inhibited by FOR. Western-blotting analysis also showed that FM (1 and 10nM) decreased p38MAPKγ phosphorylation but not p38α phosphorylation (Fig. 4C). In contrast, although p38MAPK α/β inhibitor SB203580 inhibited phosphprylation of HSP27, a p38MAPKα downstream molecule, it did not inhibit p38MAPKγ phosphorylation (Fig. 4C). These results were also quantified and shown in supplementary Figure 1. Furthermore, inhibitory effects of β-adrenoceptor agonists on phosphorylation of p38MAPKγ was evaluated by cell based ELISA. As shown in Fig. 4D, FOR and SAL concentration-dependently inhibited phosphorylation of

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p38MAPKγ, and the IC_{50} values were 0.97nM and 26nM, respectively although salbutamol showed partial inhibition at 100nM.

Short interference RNAs (siRNA) against p38 MAPK-γ and -δ were transfected to PBMCs from healthy volunteers to obtain knock-downs (KD). After 24 h, mRNA levels of both p38MAPKγ and δ were reduced by more than 75%. KD cells (and normal cells) were treated with IL-2 and IL-4 for 48 h and stimulated with CD3/CD28 in the presence or absence of Dex to determine Dex-IC_{50} on IL-2 production. The expected IL2/IL-4-induced corticosteroid resistance to CD3/CD28-induced IL-2 release was not seen in cells with p38 MAPK-γ knocked-down (KD) by RNA interference (Fig. 5A). By contrast, p38 MAPK-δ KD did not prevent corticosteroid insensitivity (Fig. 5A). Although FOR (1nM) reversed IL-2/IL-4 dependent corticosteroid insensitivity, SB203580, a selective p38 MAPK-α and -β inhibitor, did not restore corticosteroid sensitivity in IL-2/IL-4 treated PBMCs (Fig. 5A). Improvement of Dex-IC_{50} on CD3/CD28-induced IL-2 release by FOR correlated well with the improvement by p38 MAPK-γ KD ($r=0.53$, $p=0.0079$, Fig. 5B) in the same subjects.

U937 cells (a monocytic cell line) were transfected with siRNAs against p38 MAPK-γ or scrambled oligonucleotides as control for 24 h. As shown in Fig. 5C, p38MAPKγ was clearly knocked down in this condition. Cells were then treated with IL-2/IL-4 for 48 h and stimulated with TNF-α (1 ng/ml) in the presence or absence of Dex to determine Dex-IC_{50} values. Dex inhibited TNFα-induced IL-8 production with an IC_{50} of 4.6nM, but Dex potency was decreased in the presence of IL-2/IL-4 (Dex-IC_{50}: 36nM). However, p38 MAPK-γ KD shifted dose-response curve of Dex leftward (Dex-IC_{50} 9.6nM *vs*. 36nM with scrambled oligonucleotide treatment as a control, all cells were treated with IL-2/IL-4,

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p<0.05), suggesting that Dex counteracts the development of IL-2/IL-4-induced corticosteroid insensitivity (Fig. 5D). In contrast, KD of p38 MAPK-δ did not affect IL-2/IL-4-induced corticosteroid insensitivity (Dex-IC_{50} 21nM *vs*. 36nM with scrambled oligonucleotides treatment, not significant, data not shown). Furthermore, p38 MAPK-γ KD, but not p38 MAPK-δ KD, also inhibited GR phosphorylation by IL-2/IL-4 (Fig. 5E) where FOR, but not SB203580, inhibited GR phosphorylation in this U937 system (Fig. 5F). When mRNAs of p38MAPKs were determined in PBMCs, there was no significant difference in mRNA encoding p38 MAPK-α, β and -δ between HV and SA (data not shown) as well as p38MAPKγ (Fig. 5G). However, there was a good correlation between mRNA expression of p38 MAPK-γ and GR nuclear translocation (nuclear FITC-Dex) (p<0.05, date not shown).) in SA patients, suggesting higher p38MAPKγ causes defect of GR function. More importantly, when p38MAPKγ protein expression was analysed and the level was normalized to α-tublin expression (a house keeping gene) in PBMCs from 3 HV and 6 SA (supplementary Figure 2, there was significant correlation between GR phosphorylation and p38MAPKγ protein expression (Fig. 5H). For further confirmation of role of p38MAPKγ in corticosteroid insensitivity, p38MAPKγ was also overexpressed in U937 cells, and Dex-IC_{50} was determined. The U937 cells with p38MAPKγ overexpression showed significantly higher Dex-IC_{50} value on TNFα-induced IL-8 release after IL-2/IL-4 treatment. (Supplementary Figure 3).

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Discussion

Severe asthma is characterized by corticosteroid insensitive inflammation. We showed here that IC_{50} value of Dex on TNF-α stimulated IL-8 release in PBMCs of SA was higher by approximately 10 fold than those of HV or MA, suggesting PBMCs from SA were also steroid insensitive *in vitro*.

At a molecular level, the reduction in corticosteroid responsiveness observed in cells from SA patients has been ascribed to a reduced number of GR, altered affinity of the ligand for GR, reduced ability of the GR to bind to DNA, increased expression of inflammatory transcription factors, such as AP-1, that compete for DNA binding, or reduction of histone deacetylase-2 (Adcock et al., 2006). In this study, there were no significant differences in GRα mRNA and protein expression. GRβ mRNA expression likely increased in SA, but not significant. It is possibly due to lack of power, however, several reports have shown that GRβ overexpression was not critical to corticosteroid insensitivity in SA (Torrego et al., 2004;Irusen et al., 2002). Irusen also demonstrated that GR affinity in nuclei was decreased in SA, although we did not analyse the GR function in nuclei in present study (Irusen et al., 2002). At least we found the defect of GR in cytoplasm.

An increase of activated GR in nuclei is critical for GR action. For the detection of ligand bound GR in nuclei, we determined the amount of FITC-conjugated Dex in nuclei instead of using classical immunocytochemistry or Western blotting method, which are not quantitative, but time-consuming and requires a large number of cells. As the anti-inflammatory efficacy of FITC-conjugated Dex is 10 fold weaker than that of unlabelled Dex on IL-1β-induced IL-8 production in A549 cells, we used a relatively high concentration (10^{-6}M) of FITC-Dex for the assay. There was a good correlation between % of GR positive nuclei in immunocytochemistry assay

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and the absolute value of FITC-Dex in nuclei in PBMC of healthy volunteers (data not shown, r=0.65, $p<0.05$), suggesting that this FITC-Dex method is useful for quantification of GR nuclear translocation in a small number of cells. We demonstrated that GR nuclear translocation was significantly impaired in SA PBMCs (Fig. 1C), which is supported by previous report (Matthews et al., 2004). In addition, there was a significant, negative correlation between the IC_{50}-Dex value on TNF-α-induced IL-8 release and the amount of FITC-Dex in nuclei, indicating that patients with less GR nuclear translocation are more corticosteroid insensitive (Spearman r=-0.55 p=0.0035). LABAs are reported to be able to enhance corticosteroid sensitivity in several *in vitro* systems and clinical trial, even in our PBMC systems, we also confirmed that add-on-treatment with FOR (1nM) and SAL (100nM) decreased the Dex-IC_{50} value for TNF-α induced IL-8 release in PBMCs from SA patients(Fig. 2A), although the efficacy of SAL on restoration of corticosteroid sensitivity was weaker than that of FOR. Neither FOR nor SAL changed corticosteroid sensitivity in HV and MA (Table 2). In addition, FOR (1nM) enhanced GR nuclear translocation evaluated with FITC-Dex in SA (Fig.2B). The levels of corticosteroid sensitivity and restoration by FOR or SAL were not affected by current medication (oral steroid or inhaled steroid therapy; data not shown). The improvement in Dex-IC_{50} with FOR (ratio of Dex-IC_{50} with FOR vs. Dex-IC_{50} without FOR) correlated well with the improvement of FITC-Dex accumulation in nuclei by FOR (Spearman r=-0.77, p=0.042), suggesting that FOR reversed corticosteroid insensitivity by enhancement of GR nuclear translocation. The improvement in Dex-IC_{50} with FOR was negatively correlated with basal FITC-Dex values in nuclei (Spearman r=-0.73, p=0.0013, Fig.2C), suggesting that patients with less GR nuclear translocation are more sensitive to FOR-dependent reversal of corticosteroid resistance.

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GR are reported to be phospho-proteins, and phosphorylation of inactive GR may block subsequent hormone binding, affect GR subcellular localization and GR nuclear cytoplasmic trafficking through the nuclear pore complex (Ismaili and Garabedian, 2004). We demonstrated that GR in cytoplasm was highly phosphorylated at serine residues in SA compared with those of HV and MA (Fig.3A and B), and there was a good correlation between GR phosphorylation and FITC-Dex nuclear translocation (as an index of capability of GR on nuclear translocation). Phosphorylation of Ser^{226} is reported to cause defect of GR mediated transcriptional activation (Rogatsky et al., 1998) or enhancement of nuclear export (Itoh et al., 2002b). Very interestingly, the level of GR phosphorylation at Ser226 was higher in SA compared with HV(Fig.3F). Even more importantly, FOR significantly inhibited phosphorylation of GR (Fig.3C). IL-2/IL-4 treatment, which is known to mimics corticosteroid insensitivity seen in SA (Irusen et al., 2002;Larsson et al., 1997;Kam et al., 1993),also induced phosphorylation of GR (Fig. 3D, 3E) and significantly inhibited by FOR (Fig.3D, 3E).

Several kinases such as mitogen protein kinases (MAPK), cyclin-dependent kinase (CDK), glycogen synthase kinase-3 (GSK-3) and c-Jun N-terminal kinases (JNK) are reported to phosphorylate GR, each of them having distinct specificities for potential phosphorylation sites (Ito et al., 2006b). Kinase phosphorylation array analysis demonstrated that IL-2/IL-4 treatment up-regulated phosphorylation of several stress kinases, including p38 MAPK (α, β, γ, δ) and JNK (Fig. 4A and 4B). Very interestingly FOR (10^{-8}M) significantly inhibited only phosphorylation of pan-JNK and p38 MAPK-γ (Fig. 4A, 4B). GR Ser^{226} phosphorylation is reported to be catalyzed by JNK and to inactivate GR (Rogatsky et al., 1998). The leucine-rich sequences flanking Ser^{246} in rat GR (Ser^{226} in human GR) is also reported to be

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involved in nuclear export (Itoh et al., 2002), and phosphorylation of this site may increase GR nuclear export as the means of inactivating GR transcriptional enhancement, leading eventually to accumulation of phosphorylated GR in the cytoplasm. Thus, JNK will be a key kinase on GR phosphorylation in this finding, but the roles of p38 MAPK-γ on GR phosphorylation and corticosteroid effects have not previously been reported.

In U937 cells and PBMCs where p38 MAPK-γ was knocked-down (KD) by RNA interference, IL2/IL-4 exposure did not induce corticosteroid insensitivity (Fig. 5A and 5D) . Furthermore, p38 MAPK-γ KD, but not p38 MAPK-δ KD, inhibited GR phosphorylation by IL-2/IL-4 (Fig. 5E). Thus, p38 MAPK-γ appears to be a key kinase regulating corticosteroid sensitivity, probably by phosphorylation of cytoplasmic GR. In fact, PBMCs with higher level of GR phosphorylation at Ser^{226} showed higher level of mRNA expression of p38 MAPK-γ (Fig. 5H). . The p38MAPKγ protein expression was also higher in SAthan healthy subjects (supplementary Fig.2). Further analysis with p38MAPKγ overexpression in U937 cells (web-depository Fig 3) supported the finding that p38MAPKγ overexpression in SA is likely one of molecular mechanisms of steroid insensitivity.

In our system, FOR converted phosphorylated GR and p38 MAPK-γ to non-phosphorylated forms within 20 min after IL-2/IL-4 treatment for 48 h, suggesting that FOR might dephosphorylate GR via dephosphorylation of p38 MAPK-γ rather than by inhibiting p38 MAPK-γ directly. In fact, FOR (or SAL) (100nM, 1000nM) did not directly inhibit p38 MAPK-γ kinase activity (Millipore kinase profiler assay, data not shown). That is, FOR might enhance a specific phosphatase to dephosphorylate p38 MAPK-γ. The phosphatase which specifically dephosphorylates

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p38 MAPK-γ has not yet been identified. However, cAMP-PKA signals are reported to enhance activity of protein phosphatase (PP)2A (Feschenko et al., 2002). PP2C is also known as cAMP coupled phosphatase (Yokoyama et al., 1995), and PP1 and PP5 are reported to be involved in GR localization (DeFranco et al., 1991;Hinds, Jr. and Sanchez, 2007;Dean et al., 2001). Further studies will be required to clarify the molecular mechanism of LABA on dephosphorylation of p38MAPKγ (and GR).

Thus, LABAs restored corticosteroid sensitivity defected by phosphorylation (Fig. 6). Corticosteroid itself is reported to increase beta-adrenoceptor expression(Aksoy et al., 2002) and also LABAs are reported to enhance GR nuclear translocation(Usmani et al., 2005;Eickelberg et al., 1999). This should be a self-enforcing loop induced by the combination therapy of LABA and inhaled corticosteroid. Corticosteroid-insensitive severe diseases are heterogeneous, but our study demonstrated that at least a sub-population of SA patients is characterized by defective GR nuclear translocation and GR hyper-phosphorylation which were reversed by LABA via p38 MAPK-γ-dependent mechanism (Fig.6). Our studies provide new insights into the regulation of inflammation and raise the prospects of new classes of compounds to treat SAand other inflammatory diseases.

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Authors' contributions

Participated in research design: Mercado, To, Adcock, Ito

Conducted experiments: Mercado, To, Kobayashi, Ito

Performed data analysis: Mercado, To, Kobayashi, Ito

Wrote or contributed to the writing of the manuscript: Ito, Barnes

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Footnote

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Figure Legends

FIGURE 1.

TNF-α-induced IL-8 production (A), dexamethasone sensitivity to inhibit this response (IC_{50}-Dex, B), and GR nuclear translocation by FITC-Dex incorporation assay (C) were determined in PBMCs obtained from healthy volunteers (HV), mild asthmatics (MA) and severe asthma patients (SA). NS=not significant.

FIGURE 2.

Effects of formoterol (FOR) (1nM) on dexamethasone sensitivity to inhibit TNF-α-induced IL-8 production (IC_{50}-Dex, A), and GR nuclear translocation determined by FITC-Dex incorporation assay (B) were evaluated in PBMCs obtained from severe asthma patients. (C) Correlation between FITC-Dex in nuclei and ratio of Dex-IC_{50} without vs. with FOR, as an index of the improvement of corticosteroid sensitivity with FOR.

FIGURE 3.

GR phosphorylation in severe asthma and restoration by formoterol

Serine phosphorylation of immunoprecipitated GR in PBMCs of severe asthma patients (SA) and healthy volunteers (HV) (A, B). Effects of formoterol (FOR) on GR phosphorylation in PBMCs obtained from SA (C) and in IL-2/IL-4-treated PBMCs from HV (D, E). pGR: phosphorylated GR. *$p<0.05$ vs. severe asthma PBMCs (C) or vs. IL-2/IL-4 treated PBMCs without FOR (E) by Wilcoxon ranked-pair test. (F) GR phosphorylation at S^{226} was also detected in PBMCs from HV (n=4) and SA (n=6); #$p<0.05$ for SA vs. HV.

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FIGURE 4.

Kinase profiling in IL-2/IL-4-treated PBMCs from healthy volunteers**.** Representative image (A) and densitometric analysis (B) of kinase phosphorylation array. PBMCs were incubated with IL-2/IL-4 for 48 h and then incubated with formoterol (FOR, 10nM) for 20 min. Density of each dot was calculated and % increase over non-treatment was determined. Open bars indicate treatment with IL-2/IL-4 alone and closed bars indicate IL-2/IL-4 treatment with FOR. Significant induction or reduction by FOR are indicated by the bold arrows. Data were plotted as means ± SEM of $n = 3$ independent experiments. C: Western-blotting analysis of phosphorylation of p38MAPKα, γ and HSP27. Cells were treated as shown in panel A. SB: SB203580, D: Cell based ELISA of p38MAPKγ phosphorylation. Formoterol, Salmeterol and Salbutamol were treated 20 min after 48hr-treatment of IL-2/IL-4, and inhibitory effects vs. IL-2/IL-4 control were calculated and plotted.

FIGURE 5.

p38 MAPK-γ causes corticosteroid insensitivity. A: Effects of p38 MAPK-γ or –δ knockdown (KD) by RNA interference on IL2/IL-4-induced dexamethasone resistance to CD3/CD28-induced IL-2 release sensitivity in PBMCs from healthy volunteers. Formoterol (FOR, 1nM) or SB-203580 (SB, 1μM) were also preincubated for 20 min before CD3/CD28 treatment. B: Spearman correlation analysis between formoterol efficacy and p38 MAPK-γ KD efficacy in each sample from healthy volunteer. C: Representative image of p38MAPKγ knockdown in U937 cells. SC (scrambled oligonucleotides). E: Effect of p38 MAPK-γ on anti-inflammatory action of dexamethasone in U937 cells on TNF-α-induced IL-8 (D) and GR phosphorylation

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by IL-2/IL-4. F: Effects of FOR (1 or 10nM) or SB-203580 (SB, 1μM) on GR phosphorylation in U937 cells. G**:** p38 MAP-γ mRNA expression in PBMCs from severe asthma patients (SA), healthy volunteers (HV) and mild asthmatic patients (MA). H. The relationship between phosphorylated GR (p-GR) corrected to total GR expression and p38MAPKγ protein expression corrected to α-tublin, evaluated by western blotting in PBMCs from 3 healthy volunteers and 6 SA patients (supplementary Fig. 2).

FIGURE 6.

Precise mechanism of formoterol on restoration of corticosteroid sensitivity in severe asthma. Severe allergic inflammation associated with production of IL-2 and IL-4 induced corticosteroid insensitivity via GR phosphorylation by p38MAPKγ activation. Formoterol inhibits p38MAPKγ phosphorylation possibly via phosphatase activation, and inhibits GR phosphorylation.

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Table 1. Characteristics of the subjects recruited

	Healthy Volunteers	Mild asthma	Severe asthma
N	11	6	16
Gender (Male/Female)	8/3	3/3	5/11
Age (yr)	35.8 ± 1.6	41.5 ± 3.3	35.1 ± 2.6
Asthma duration (yr)	na	nd	16.3 ± 2.6
Atopy	2/11	2/6	6/15
FEV_1 (% pred.)	98.2 ± 2.2	84.5 ± 4.6	55.0 ± 3.4
FEV_1/FVC (%)	96.8 ± 3.1	79.9 ± 3.2	46.6 ± 12.6
Oral steroid (mg)	0	0	16.8 ± 4.0
Inhaled steroid (μg)	0	0	1860 ± 206
Others	na	Albuterol on demand	Albuterol on demand

na: not applicable, nd: not determined

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Table 2. Effect of treatment with formoterol and salmeterol on corticosteroid sensitivity

	No.Patients studied	Non-treated	Formoterol (1nM)	Salmeterol (100nM)
TNF-α-induced IL-8 release: Dex-IC_{50} values (nM): mean ± SEM [ ]: Dex-IC_{50}without treatment/Dex-IC_{50} with LABAs				
Healthy	7	15.5 ± 4.2	21.5 ± 4.8 [0.72]	31.0 ± 8.3 [0.5]
Mild asthma	6	20.0 ± 3.8	nd	14.7 ± 3.5 [1.4]
Severe asthma	6	182.5 ± 28.2	42.8 ± 21.0 [4.3]	
	15	180.7 ± 28.7		83.7 ± 16.7 [2.2]

The IC_{50} values (nM) of dexamethasone (Dex-IC_{50}) are shown in this table (Mean ± SEM).

nd: not determined.

Figure 1

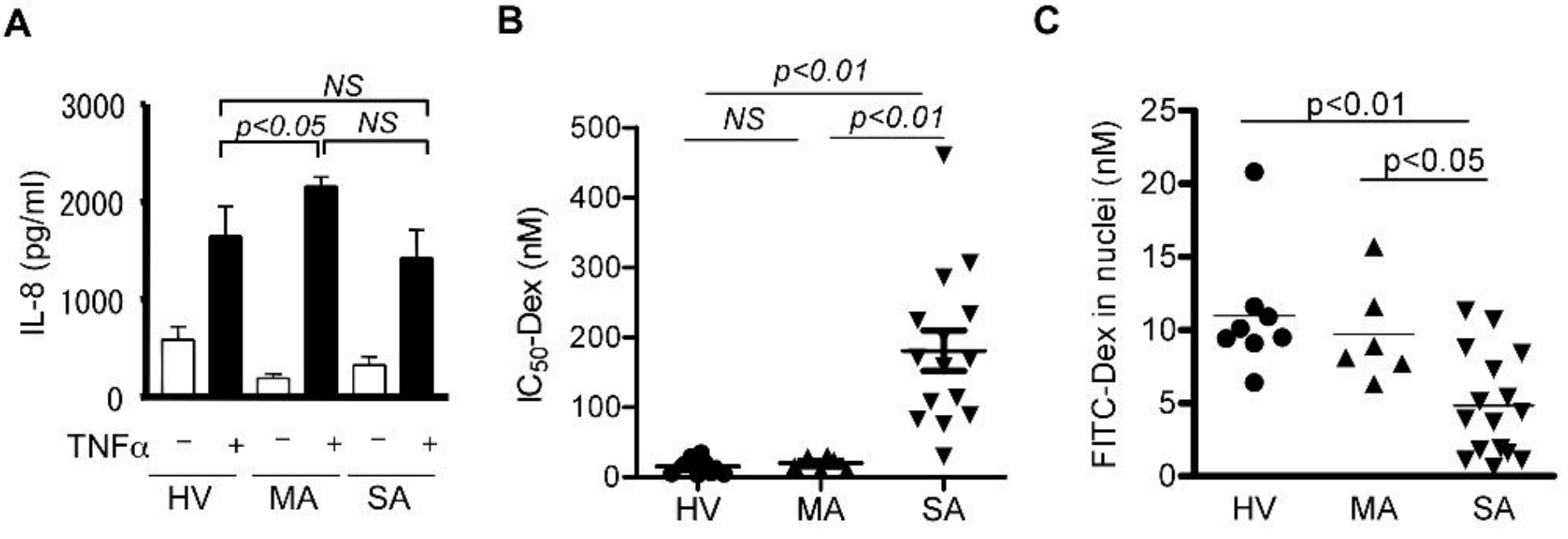

A
IL-8 (pg/ml)
3000
2000
1000
0
NS
p<0.05
NS
TNFα − + − + − +
HV
MA
SA
B
IC50-Dex (nM)
500
400
300
200
100
0
p<0.01
NS
p<0.01
HV
MA
SA
C
FITC-Dex in nuclei (nM)
25
20
15
10
5
0
p<0.01
p<0.05
HV
MA
SA

Figure 2

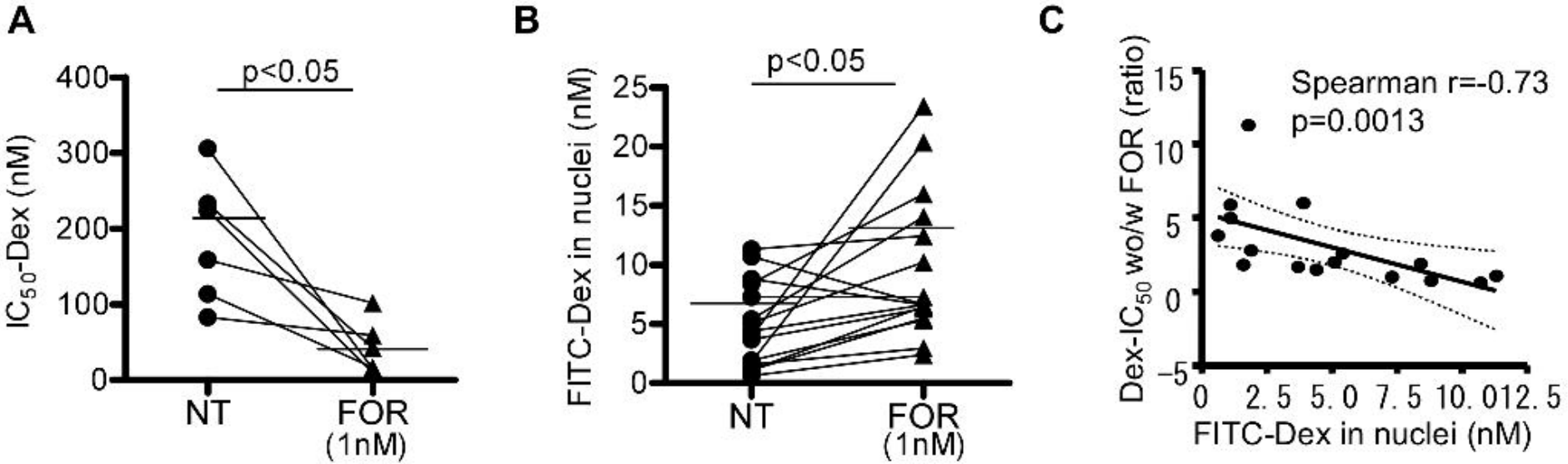

A
p<0.05
400
300
200
100
0
IC50-Dex (nM)
NT
FOR
(1nM)
B
p<0.05
25
20
15
10
5
0
FITC-Dex in nuclei (nM)
NT
FOR
(1nM)
C
Spearman r=-0.73
p=0.0013
15
10
5
0
−5
Dex-IC50 wo/w FOR (ratio)
0
2.5
5.0
7.5
10.0
12.5
FITC-Dex in nuclei (nM)

Figure 3

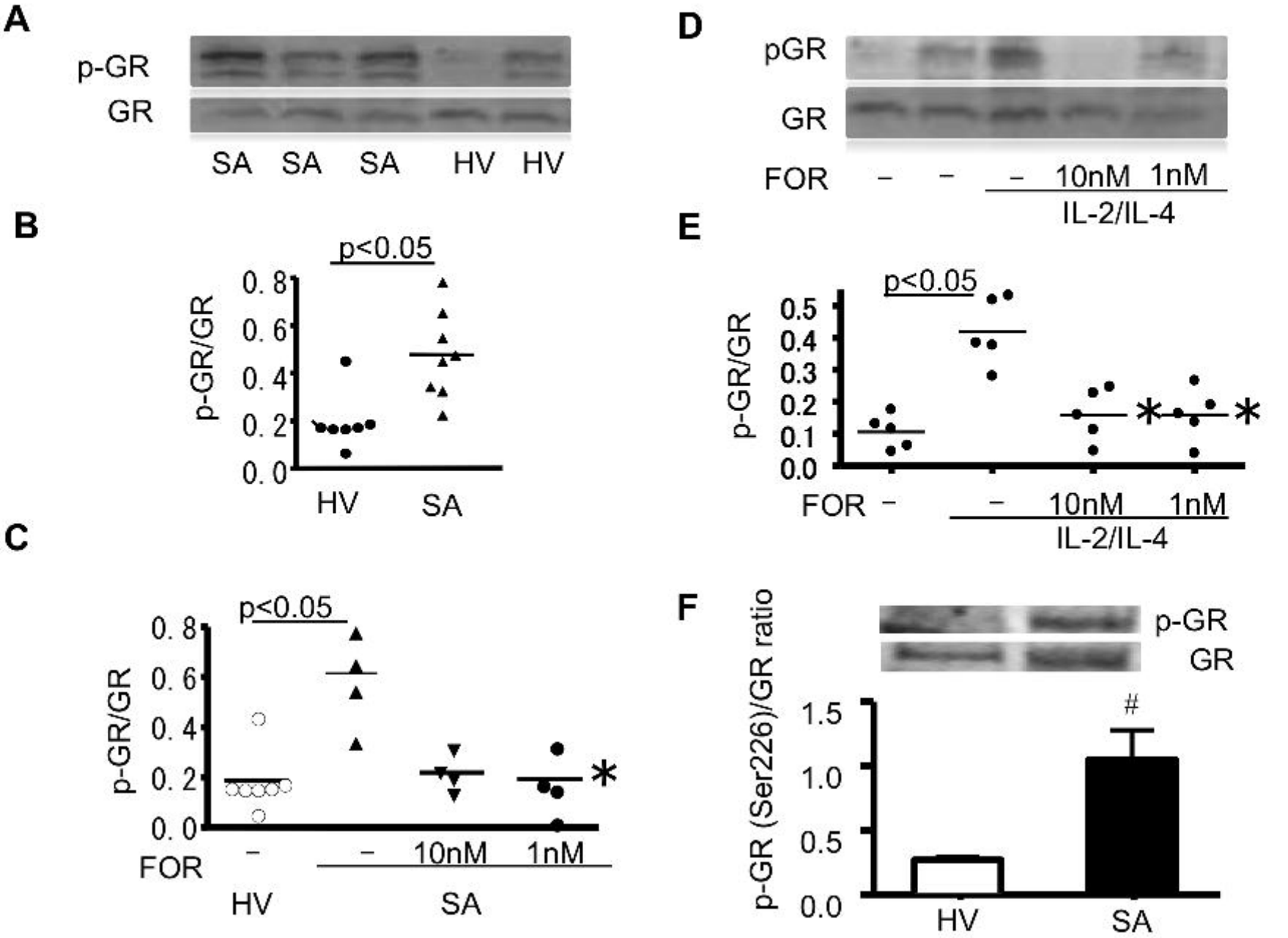

A
p-GR
GR
SA SA SA HV HV
B
p<0.05
p-GR/GR
0.8
0.6
0.4
0.2
0.0
HV SA
C
p<0.05
p-GR/GR
0.8
0.6
0.4
0.2
0.0
FOR – – 10nM 1nM
HV SA
D
pGR
GR
FOR – – – 10nM 1nM
IL-2/IL-4
E
p<0.05
p-GR/GR
0.5
0.4
0.3
0.2
0.1
0.0
FOR – – 10nM 1nM
IL-2/IL-4
F
p-GR
GR
p-GR (Ser226)/GR ratio
1.5
1.0
0.5
0.0
#
HV SA

Figure 4

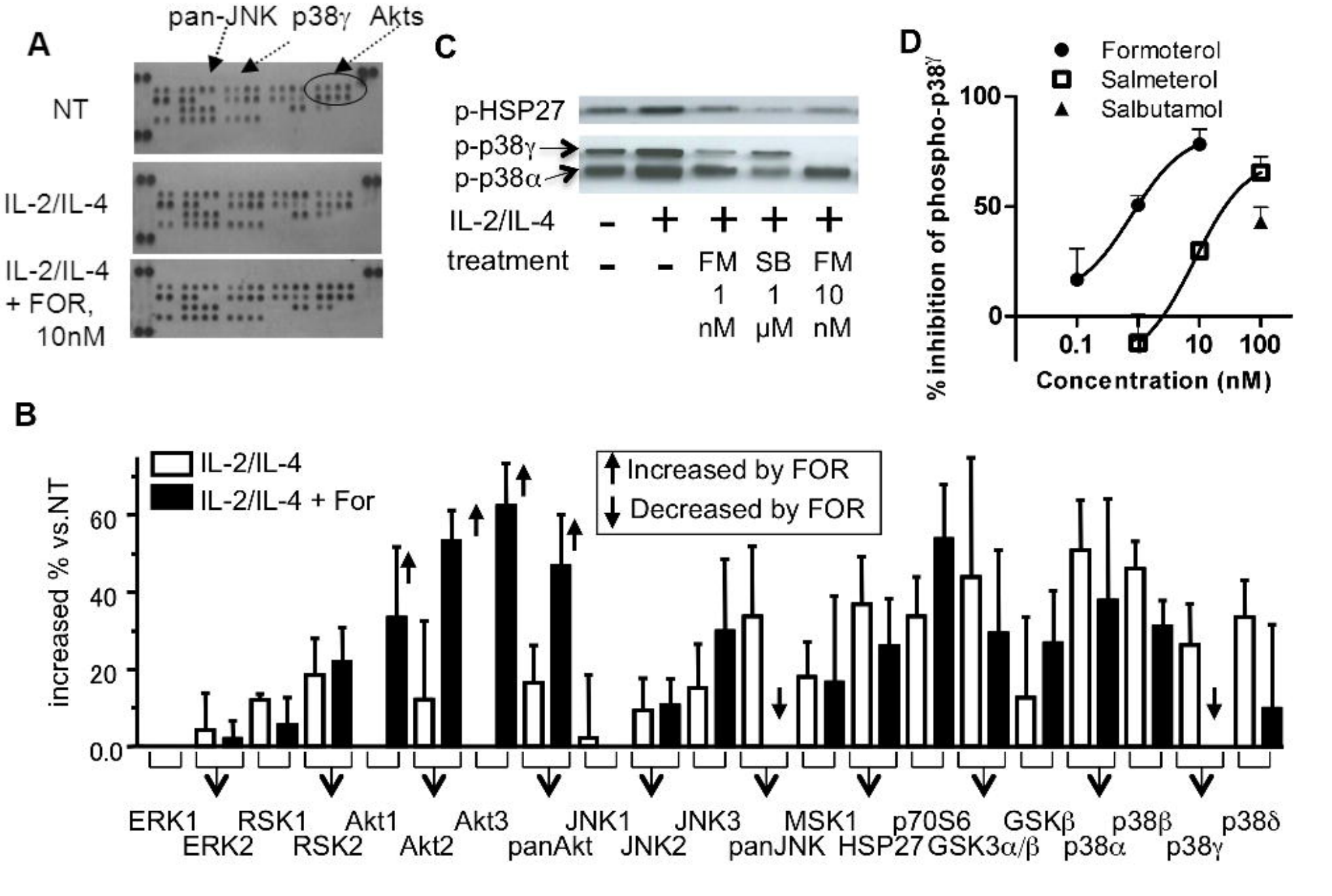

A
pan-JNK
p38γ
Akts
NT
IL-2/IL-4
IL-2/IL-4 + FOR, 10nM
C
p-HSP27
p-p38γ
p-p38α
IL-2/IL-4
treatment
FM 1 nM
SB 1 μM
FM 10 nM
D
Formoterol
Salmeterol
Salbutamol
% inhibition of phospho-p38γ
100
50
0
0.1
10
100
Concentration (nM)
B
IL-2/IL-4
IL-2/IL-4 + For
Increased by FOR
Decreased by FOR
increased % vs.NT
60
40
20
0.0
ERK1
ERK2
RSK1
RSK2
Akt1
Akt2
Akt3
panAkt
JNK1
JNK2
JNK3
panJNK
MSK1
HSP27
p70S6
GSK3α/β
GSKβ
p38α
p38β
p38γ
p38δ

Figure 5

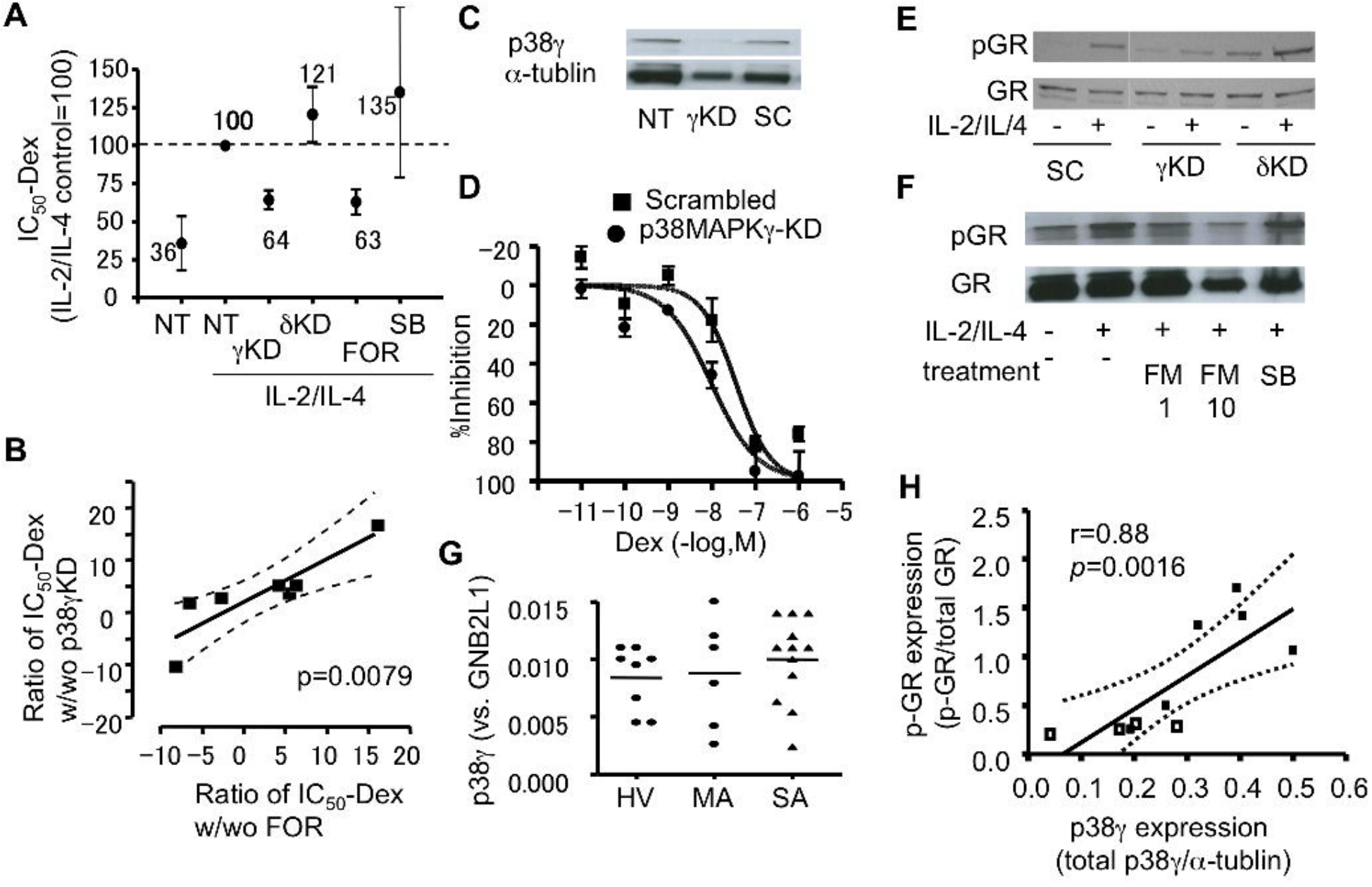
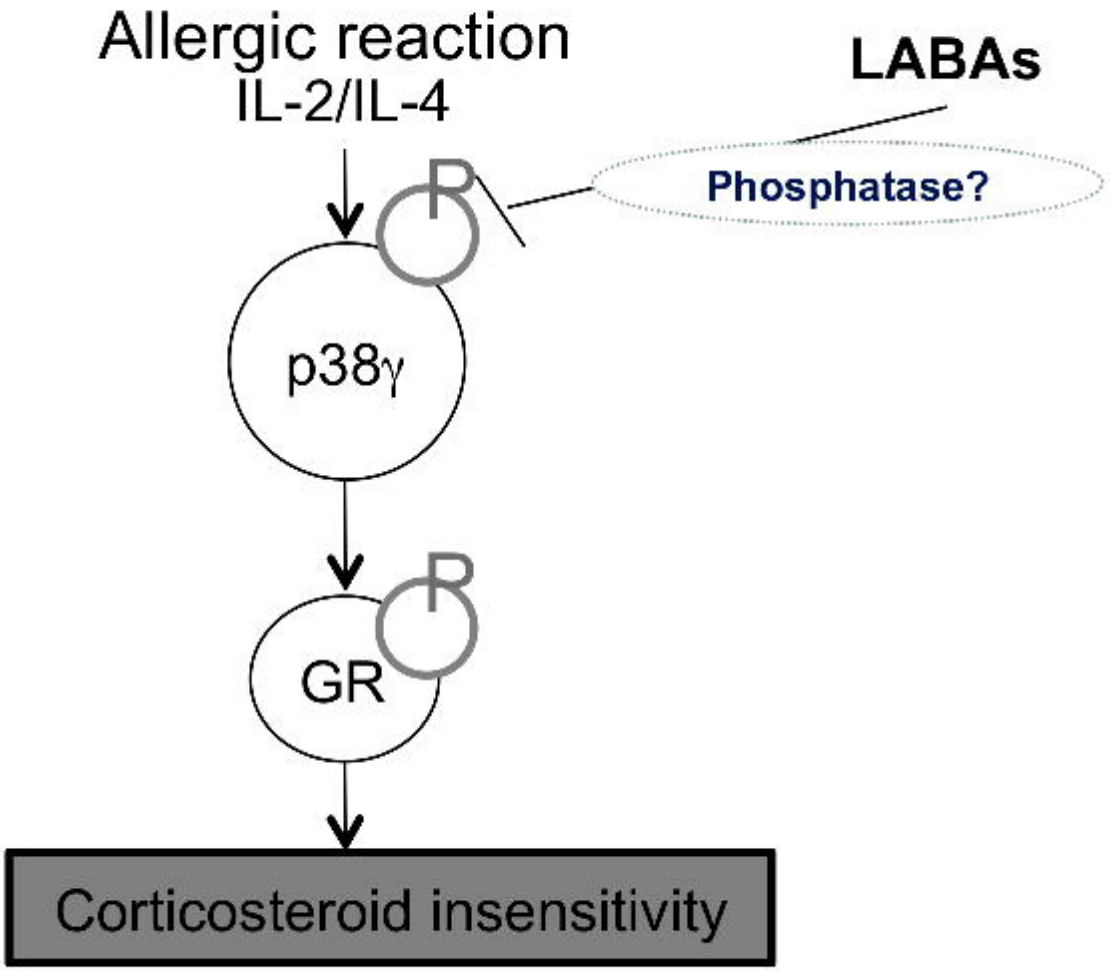

A
IC50-Dex
(IL-2/IL-4 control=100)
150
125
100
75
50
25
0
100
121
135
36
64
63
NT
NT
γKD
δKD
FOR
SB
IL-2/IL-4
B
Ratio of IC50-Dex
w/wo p38γKD
20
10
0
−10
−20
p=0.0079
−10 −5 0 5 10 15 20
Ratio of IC50-Dex
w/wo FOR
C
p38γ
α-tublin
NT γKD SC
D
Scrambled
p38MAPKγ-KD
%Inhibition
−20
0
20
40
60
80
100
−11 −10 −9 −8 −7 −6 −5
Dex (-log,M)
E
pGR
GR
IL-2/IL4 - + - + - +
SC
γKD
δKD
F
pGR
GR
IL-2/IL-4 - + + + +
treatment - - FM 1 FM 10 SB
G
p38γ (vs. GNB2L1)
0.015
0.010
0.005
0.000
HV
MA
SA
H
p-GR expression
(p-GR/total GR)
2.5
2.0
1.5
1.0
0.5
0.0
r=0.88
p=0.0016
0.0 0.1 0.2 0.3 0.4 0.5 0.6
p38γ expression
(total p38γ/α-tublin)

Figure 6


Allergic reaction
IL-2/IL-4
LABAs
Phosphatase?
P
p38γ
P
GR
Corticosteroid insensitivity