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Impaired dual-specificity protein phosphatase DUSP4 reduces corticosteroid sensitivity

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Abbreviations

Ab; antibody

Dex; dexamethasone

DUSP; dual-specificity phosphatase

FKBP51; FK506-binding protein 51

FM; formoterol

GAPDH; glyceraldehyde 3-phosphate dehydrogenase

GR; glucocorticoid receptor

JNK; c-Jun N-terminal kinase

KD; knockdown

LABA; long-acting β_2 -adrenergic agonist

MAPK; mitogen-activated protein kinase

MKP; mitogen-activated protein kinase phosphatase

NT; non-treatment control

PBMC; peripheral blood mononuclear cells

siRNA; small interfering RNA

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Abstract

We have reported that phosphorylation of glucocorticoid receptor (GR) at Ser²²⁶ reduces GR nuclear translocation resulting in corticosteroid insensitivity in patients with severe asthmas. A serine/threonine protein phosphatase, PP2A, which regulates JNK1 and GR-Ser²²⁶ signaling, is involved in this mechanism. Here, we further explored dual-specificity protein kinase phosphatases (DUSPs) with the ability to dephosphorylate JNK, and identified DUSP4 as a phosphatase involved in the regulation of corticosteroid sensitivity. The effects of knocking down DUSPs (DUSP1, 4, 8, 16 and 22) by siRNA were evaluated in a monocytic cell line (U937). Corticosteroid sensitivity was determined by dexamethasone enhancement of FKBP51 or inhibition of TNF α -induced IFN γ and IL-8 expression and to translocate GR from cell cytoplasm to nucleus. The nuclear/cytoplasmic GR, phosphorylation levels of GR-Ser²²⁶ and JNK1, co-immunoprecipitated GR-JNK1-DUSP4, and DUSP4 expression were analyzed by Western-blotting and/or Imaging Flow Cytometry. Phosphatase activity of immunoprecipitated DUSP4 was measured by fluorescence-based assay. Knockdown of DUSP4 enhanced phosphorylation of GR-Ser²²⁶ and JNK1 and reduced GR nuclear translocation and corticosteroid sensitivity. Co-immunoprecipitation experiments showed that DUSP4 is

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associated with GR and JNK1. In PBMCs from severe asthmatics, DUSP4 expression was reduced versus healthy subjects and negatively correlated with phosphorylation levels of GR-Ser²²⁶ and JNK1. Formoterol enhanced DUSP4 activity and restored corticosteroid sensitivity reduced by DUSP4 siRNA. In conclusion, DUSP4 regulates corticosteroid sensitivity via dephosphorylation of JNK1 and GR-Ser²²⁶. DUSP4 activation by formoterol restores impaired corticosteroid sensitivity, indicating that DUSP4 is crucial in regulating corticosteroid sensitivity and therefore might be a novel therapeutic target in severe asthma.

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Introduction

Most patients with bronchial asthma, one of major inflammatory airway diseases, are now well controlled by inhaled corticosteroids (Barnes, 2010). However, some severe asthmatics have uncontrolled disease despite treatment with high doses of inhaled corticosteroids. A major unmet need is the development of more effective treatment for these patients with corticosteroid-insensitive asthma.

The c-Jun N-terminal kinases (JNKs), members of stress-activated protein kinases, are activated by many environmental stimuli such as proinflammatory cytokines, radiation, osmotic stress, and oxidative stress (Shen and Liu, 2006). Previously, Sousa *et al.* reported that JNK was hyperphosphorylated in peripheral blood mononuclear cells (PBMCs) from patients with corticosteroid-insensitive asthma (Sousa *et al.*, 1999). We also confirmed that phosphorylation levels of JNK1 are enhanced in PBMCs from patients with severe asthma and with IL-2/IL-4-induced steroid-insensitivity in PBMCs (Kobayashi *et al.*, 2011). Thus, JNK1 phosphorylation is a key factor of corticosteroid insensitivity, at least in severe asthma.

Multiple factors can be involved in corticosteroid insensitivity in severe asthma; such as decreased glucocorticoid receptor (GR) expression, defective GR-ligand binding, reduced

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GR nuclear translocation and GR-glucocorticoid response element (GRE) binding (Ito and Mercado, 2009). Consistent with previous findings (Itoh et al., 2002; Rogatsky et al., 1998), we found that GR-Ser²²⁶ phosphorylation via JNK1 activation might inhibit GR nuclear translocation leading to impairment of corticosteroid sensitivity (Kobayashi et al., 2011).

Budziszewska et al. showed that the serine/threonine phosphatase PP2A amplifies GR action through dephosphorylation of JNK (Budziszewska et al., 2010). In support of this, we also found that PP2A regulates GR nuclear translocation concomitant with dephosphorylation of GR-Ser²²⁶ via JNK1 (Kobayashi et al., 2011). Besides PP2A, it has been reported that activation of JNKs is regulated by dual-specificity protein phosphatases (DUSPs), which belong to the superfamily of protein tyrosine phosphatases and recognize phospho-serine/threonine and phospho-tyrosine residues within one substrate (Alonso et al., 2004; Tonks, 2006). Based on sequence similarity, DUSPs are further classified into six subgroups, which include mitogen-activated protein kinase phosphatases (MKPs), slingshot protein phosphatases, phosphatases of regenerating liver (PRLs), Cdc14 phosphatases, phosphatase and tensin homolog deleted on chromosome 10 (PTENs), myotubularins, and atypical DUSPs (Patterson et al., 2009).

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MKPs contain a highly conserved C-terminal catalytic domain and an N-terminal CH2 domain (for Cdc25 homology 2) including critical KIMs (kinase-interacting motifs) which confer specific mitogen-activated protein kinase (MAPK) substrate specificity. The activation of MAPKs requires phosphorylation of both the threonine (Thr) and tyrosine (Tyr) residues located within the TXY (Thr-Xaa-Tyr) motif by dual specificity MAPK kinases (Davis, 2000), whereas MKPs dephosphorylate MAPKs at both phospho-Thr and phospho-Tyr residues within the MAPK TXY activation motif (Patterson et al., 2009).

Here we have investigated the role of MKPs in corticosteroid sensitivity, especially via regulation of JNK1-GR-Ser²²⁶ pathway. Amongst MKPs, we focused on DUSP1, DUSP4, DUSP8 and DUSP16, which are predominantly involved in dephosphorylation of JNK (Chen et al., 2001; Chu et al., 1996; Liu et al., 1995; Tanoue et al., 2001). In addition, as another target that can dephosphorylate JNK, we also explored DUSP22, an atypical DUSP, which share some characteristics of the MKPs with the consensus DUSP catalytic domain but lack of the N-terminal CH2 domain (Aoyama et al., 2001).

In this study performed in the U937 monocytic cell line and in PBMCs from healthy and severe asthmatic subjects, we found that DUSP4, one of the MKPs, regulates corticosteroid

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sensitivity by inhibition of JNK1 and GR-Ser²²⁶ phosphorylation.

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Materials and Methods

Reagents

Formoterol fumarate dihydrate (formoterol), salmeterol xinafoate (salmeterol), salbutamol/albuterol, ICI-118551, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Brij[®]35 and the rabbit polyclonal antibody (Ab) to phospho-GR-Ser²²⁶ were purchased from Sigma-Aldrich (Poole, UK). The rabbit polyclonal Abs to phospho-GR-Ser²²⁶, DUSP16 and DUSP22, and the mouse monoclonal Abs to β -actin and TATA binding protein TBP were obtained from Abcam (Cambridge, UK). The rabbit polyclonal Abs to GR, MKP-1 (DUSP1) and MKP-2 (DUSP4), the mouse monoclonal Abs to MKP-2 (DUSP4) and α -tubulin, the goat polyclonal anti-DUSP8 Ab, and protein A/G plus-agarose immunoprecipitation reagent were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The rabbit polyclonal anti-phospho-SAPK/JNK and anti-SAPK/JNK Abs were obtained from Cell Signaling Technology (Danvers, MA). The mouse monoclonal anti-MKP2 Ab and 7-AAD were obtained from BD Biosciences (San Jose, CA). The chicken polyclonal Ab to phospho-JNK/SAPK was obtained from GeneTex (San Antonio, Tx). As immunoprecipitation reagents, TrueBlot[®] anti-rabbit Ig IP beads were purchased from

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eBioscience (Hatfield, UK).

Subjects

PBMCs were obtained from 22 patients with severe asthma (as defined by GINA guideline (FitzGerald, 2015)) and 16 age-matched healthy volunteers and separated by Ficoll-Paque PLUS (GE-Healthcare, Uppsala, Sweden). The characteristics of subjects are shown in the Table 1 and 2. This study was approved by the local ethics committee of Royal Brompton and Harefield NHS Trust (07/Q0404/31) and the local ethics committee of Kansai Medical University (KanIRin1313), and written informed consent was obtained from each patient or volunteer.

Cells

The human monocytic cell line U937 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in complete growth medium (PRMI 1640; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine at 37°C in a humidified atmosphere with 5% CO₂. Cell viability was assessed microscopically by trypan

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blue staining. Cell toxicity was determined by MTT assay when needed.

Cell Lysis, Immunoprecipitation, and Western Blotting

Cell protein extracts were prepared using modified RIPA buffer (50 mM Tris HCL pH 7.4, 0.5-1.0% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl with freshly added complete protease (Roche, Mannheim, Germany)), as described previously (Ito et al., 2000; Ito et al., 2001). Phosphatase inhibitor (Active Motif, Rixensart, Belgium) was used when needed. Nuclear extraction was performed using Active Motif kit. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Immunoprecipitation was conducted with anti-DUSP4 Ab or anti-GR Ab. Protein extracts (40 µg protein per well, and 60 µg protein for phosphoprotein detection) or immunoprecipitates were analyzed by SDS-PAGE (Invitrogen, Paisley, UK) and detected with Western blot analysis by chemiluminescence (ECL Plus; GE Healthcare, Chalfont St. Giles, UK). For co-immunoprecipitation study, 2 g of whole cell protein extracts immunoprecipitated with primary Ab (the rabbit anti-GR Ab or anti-DUSP4 Ab) and TrueBlot anti-rabbit Ig IP Beads (Rockland Immunochemicals Inc., Limerick, PA) were detected with the mouse monoclonal Abs to MKP-2 (DUSP4) or the rabbit monoclonal Abs to

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JNK1, and the rabbit polyclonal GR Ab.

Quantitative RT-PCR

Total RNA extraction and reverse transcription were performed using a NucleoSpin RNA (MACHEREY-NAGEL, Duren, Germany) and a PrimeScript RT MasterMix (Perfect Real Time) (TAKARA BIO, Shiga, Japan). Gene transcript level of FK506-binding protein 51 (FKBP51), IFN γ , IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified by real-time PCR using a Rotor-Gene SYBR Green PCR kit (QIAGEN, Hilden, Germany) on a Rotor-Gene Q HRM (Corbett Research, Cambridge, UK). Amplification primers (5'-3') were: FKBP51 (NM_004117), forward (F) –CAG CTG CTC ATG AAC GAG TTT G, reverse (R) –GCT TTA TTG GCC TCT TCC TTG G; IL-8 (NM_000584.3), forward (F) –ACT GAG AGT GAT TGA GAG TGG AC, reverse (R) –AAC CCT CTG CAC CCA GTT TTC; IFN γ (NM_000619.2), forward (F) –TTC AGC TCT GCA TCG TTT TG, reverse (R) –TCT TTT GGA TGC TCT GGT CA; GAPDH (NM_002046), F –TTC ACC ACC ATG GAG AAG GC, R –AGG AGG CAT TGC TGA TGA TCT.

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Corticosteroid sensitivity

Cells were treated with dexamethasone (Dex) for 45 min, followed by TNF α -stimulation (10 ng/ml overnight or for 4 h). Dex-induced FKBP51 and TNF α -induced IFN γ and IL-8 mRNA expression was evaluated by RT-PCR as mentioned above. In addition, TNF α -induced IL-8 concentrations were determined by sandwich ELISA according to the manufacturer's instructions (R&D Systems Europe). IC₅₀ values for Dex on IL-8 production (Dex-IC₅₀) calculated using the computer program Prism 4.0 (GraphPad Software Inc., San Diego, CA) or the ability of Dex to enhance FKBP51 and inhibit TNF α -induced IFN γ and IL-8 levels were used as markers for corticosteroid sensitivity.

Glucocorticoid receptor nuclear translocation

Cells were treated with Dex (10^{-7} M) for 1 h. Nuclear and cytoplasmic GR were measured by Western blot. TBP (for nuclear protein) or α tubulin (for cytoplasmic protein) expression was used as a control for protein loading. As an index of GR nuclear translocation, the ratio of nuclear GR to TBP was calculated.

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Protein phosphatase activity

Phosphatase activity was assayed by using the SensoLyte™ MFP Protein Phosphatase Assay system (AnaSpec, San Jose, CA) (Kobayashi et al., 2011). Cell lysates were immunoprecipitated with anti-DUSP4 antibody. Immunoprecipitated DUSP4 with assay buffer (50 mM Tris HCl pH 7.0, 1 mM EDTA, 5 mM DTT, 0.01% Brij®35, 1 mM BSA) was transferred to a 96-well plate, and then the same volume of 3-O-methylfluorescein phosphate (MFP) reaction solution in 1M DTT in assay buffer was added. DUSP4-induced dephosphorylation was monitored by measuring the fluorescence of MFP product. Phosphatase activity was calculated as the slope of fluorescence recordings and expressed as arbitrary fluorescence units per microgram of protein.

RNA interference

DUSP1, DUSP4, DUSP8, DUSP16 and DUSP22 siRNAs and non-silencing scrambled control siRNA (AllStars Negative Control siRNA) were purchased from QIAGEN (Crawley, UK). For U937 cells, the siRNA sequences (0.5 µM) were transfected using an HVJ Envelope (HVJ-E) Vector Kit GenomONE-Neo (Ishikawa Sangyo Kaisha Ltd., Osaka, Japan) as described

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previously (Kobayashi et al., 2011). For PBMCs, 0.2 μ M of siRNA was transfected by Nucleofection[®] (Lonza, Basel, Switzerland), according to the manufacturer's instructions.

Imaging Flow Cytometer

Purified PBMCs were fixed and permeabilized by BD Cytofix/Cytoperm solution (BD Biosciences), followed by incubation with DUSP4, phospho-JNK or phospho-GR-Ser²²⁶ antibodies. DUSP4, phospho-JNK or phospho-GR-Ser²²⁶ were further labeled with APC-, AF488- or PE-conjugated secondary antibodies, respectively. The expression levels of these molecules were measured using Amnis[®] ImageStream[®]X Mark II and mean fluorescence intensities (MFI) were analyzed using IDEAS version 6.0 (Merck Millipore, Darmstadt, Germany) as previously described (Marangon et al., 2013).

Statistical analysis

Comparisons of two groups of data were performed using Mann-Whitney U test or paired t-test as appropriate. Correlation coefficients were calculated with the use of Pearson's rank method.

Other data were analyzed by ANOVA with post *hoc* test adjusted for multiple comparisons

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(Bonferroni's test), as appropriate. The difference was considered statistically significant if $p <$

0.05. Descriptive statistics were expressed as the mean \pm SEM.

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Results

Reduced DUSP4 causes corticosteroid insensitivity and decreases GR nuclear translocation

In U937 cells, DUSPs (DUSP1, 4, 8, 16 and 22) were knocked-down (KD) using siRNA.

Western blotting analysis confirmed more than 30% knockdown (KD) of DUSPs, at least to the

same extent (Supplemental Figure 1). DUSP1 and DUSP4 KDs reduced the inhibitory effect of

Dex on TNF α -induced IL-8 production, so that Dex-IC₅₀ increased significantly from 108.2 (\pm

10.5) for scrambled control to 185.8 (\pm 2.8) for DUSP1 and to 271.0 (\pm 9.1) for DUSP4.

Notably, DUSP4 siRNA increased the Dex-IC₅₀ values on IL-8 production to significantly

greater extent than DUSP1 siRNA (Fig. 1A). In addition, DUSP4 KD significantly reduced the

ability of Dex to enhance FKBP51 and inhibit TNF α -induced IFN γ and IL-8 mRNA

expressions (Fig. 1B).

We next examined whether DUSP4 is associated with JNK1 and GR phosphorylation

which might be a key factor in the regulation of GR nuclear translocation (Kobayashi et al.,

2011). Although both DUSP1 and DUSP4 KDs enhanced JNK1 phosphorylation (Fig. 1C), only

DUSP4 KD increased the phosphorylation levels of GR-Ser²²⁶ (Fig. 1D).

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Furthermore, co-immunoprecipitation experiments revealed that DUSP4 is located in the same complex with JNK1 and GR (Fig. 1E), suggesting that DUSP4 may control both JNK1 and GR phosphorylation levels. Importantly, as GR is mainly located in the cell cytoplasm but not in the cell nucleus under normal condition, it can be speculated that DUSP4 associates with JNK1 and GR in the cell cytoplasm. In line with these findings, DUSP4 knockdown reduced GR translocation from cell cytoplasm into nucleus (Fig. 1F).

DUSP4 is reduced in PBMCs from severe asthma

We confirmed that in PBMCs from severe asthmatics, DUSP4 protein expression was significantly reduced compared with those from healthy volunteers (DUSP4: 0.33 ± 0.02 in healthy volunteers, 0.22 ± 0.02 in severe asthmatics) (Fig. 2A).

Analysis using Imaging Flow Cytometer visually showed reduced DUSP4 expression level which was negatively correlated with phosphorylation levels of GR-Ser²²⁶ and JNK (Fig. 2B and C). These findings suggest that impaired DUSP4 might fail to dephosphorylate JNK and GR-Ser²²⁶ resulting in corticosteroid insensitivity observed in severe asthma.

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DUSP4 is a therapeutic target for restoration of corticosteroid sensitivity

In order to confirm that DUSP4 may be a therapeutic target, we next examined the effect of DUSP4 activation on restoration of corticosteroid sensitivity. Formoterol, a long-acting β_2 -adrenergic agonist (LABA), increased activity of immunoprecipitated (IP)-DUSP4 in whole cell extracts of U937 cells in a dose dependent manner with maximum effect at 10^{-9} M (1.6 fold for 10^{-9} M vs. non-treatment) (Fig. 3A). Salmeterol (10^{-7} M), another LABA, also activated IP-DUSP4 but to a lesser extent than formoterol while albuterol (10^{-7} M), a short-acting β_2 -adrenergic agonist, had no effect. In addition, the LABA-dependent increase in IP-DUSP4 activity was antagonized by ICI-118551, a selective β_2 -adrenergic receptor antagonist (Fig. 3B), suggesting that DUSP4 is activated by LABAs through β_2 -adrenergic receptors.

As observed in U937 cells (Fig. 1 A and B), in PBMCs from healthy volunteers, DUSP4 KD significantly reduced the ability of Dex to enhance FKBP51 and inhibit TNF α -induced IFN γ and IL-8 mRNA expressions (Fig. 3C). DUSP4 KD also significantly reduced IP-DUSP4 activity (Fig. 3D). Formoterol significantly restored IP-DUSP4 activity in DUSP4 KD PBMCs (Fig. 3D) and concomitantly restored corticosteroid sensitivity improving

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the efficacy of Dex up to 80% (Fig. 3C). In addition, we confirmed that formoterol-mediated DUSP4 activation restored corticosteroid sensitivity possibly via dephosphorylation of JNK1 and GR-Ser²²⁶ in the IL-2/IL-4-induced corticosteroid-insensitive model (Kobayashi et al., 2011; Mercado et al., 2012) (Supplemental Figure 2). These results indicate that DUSP4 may be a therapeutic target for restoration of corticosteroid sensitivity.

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Discussion

In this study, we examined the involvement of DUSPs on regulation of corticosteroid sensitivity in U937 monocytic cell line and PBMCs from healthy subjects and severe asthmatic patients.

We revealed that DUSP4 is associated with JNK1 and GR in the cytoplasmic complex and that knockdown of DUSP4 reduces GR nuclear translocation most likely via hyperphosphorylation of JNK1 and GR-Ser²²⁶. This contributes to corticosteroid insensitivity, measured as increased IC₅₀ value for the inhibitory effect of Dex on TNF α -induced IL-8 secretion. We demonstrated that DUSP4 is reduced in PBMCs from severe asthmatics and that DUSP4 expression negatively correlates with enhanced phosphorylation levels of JNK1 and GR-Ser²²⁶ in these cells. Furthermore, we showed that DUSP4 was activated by formoterol, and that formoterol restored corticosteroid sensitivity in PBMCs in which DUSP4 activity was reduced. Thus, DUSP4 appears to regulate corticosteroid sensitivity via dephosphorylation of JNK1 and GR-Ser²²⁶.

DUSP4 is a member of MKP family of phosphatases known to dephosphorylate mainly ERK1/2 and JNK (Camps et al., 2000). DUSP4 has been shown to play roles in cellular functions such as cell apoptosis and senescence (CadAlbert et al., 2005; Tresini et al., 2007).

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DUSP4 is also involved in regulation of immune system and inflammatory responses (Al-Mutairi et al., 2010; Lubos et al., 2011; McCoy et al., 2008). However, the role of DUSP4 in corticosteroid sensitivity via regulation of GR phosphorylation has not previously been studied. It has been reported that DUSP1, also known as MKP-1, inhibits airway inflammatory responses induced by MAPKs probably in NF- κ B-dependent manner (King et al., 2009) and that corticosteroids and formoterol enhance DUSP1 expression (Manetsch et al., 2012). Consistent with the previous report in severe asthma (Bhavsar et al., 2008), we have also found that reduced expression of DUSP1 caused corticosteroid insensitivity, but to a significantly lesser extent than reduced expression of DUSP4. DUSP4 (but also DUSP1, 8, 10 and 16) deletion by siRNA enhances JNK phosphorylation under oxidative stress and suggests that these DUSPs coordinate to control JNK activation (Teng et al., 2007). Similarly, it seems that DUSP1, 4, 8, 16 and 22 are involved in regulation of JNK phosphorylation in our study (data shown only for DUSP1 and DUSP4) but only reduced expression of DUSP1 and DUSP4 enhanced corticosteroid sensitivity. However, in contrast to DUSP4, reduced expression of DUSP1 had no effect on the phosphorylation of GR-Ser²²⁶, suggesting that DUSP4 might regulate corticosteroid sensitivity in a different way to DUSP1. Although DUSP4 can

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dephosphorylate MAPKs in both cytoplasm and cell nucleus to (Sloss et al., 2005), our co-immunoprecipitation study showed that DUSP4 associates with JNK1 and GR mainly located in the cytoplasm but not in the nucleus under baseline conditions. Thus, DUSP4 can dephosphorylate JNK1 and GR-Ser²²⁶ in the cytoplasm, resulting in increased GR nuclear translocation.

We have previously shown that protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is activated by formoterol and that activated PP2A could restore corticosteroid sensitivity through dephosphorylation of JNK1 and GR-Ser²²⁶ (Kobayashi et al., 2011). In addition, formoterol has been shown to restore corticosteroid sensitivity by dephosphorylation of GR in PBMC from severe asthma and in IL-2/IL-4-induced corticosteroid insensitive PBMCs (Mercado et al., 2011). This is the reason why we investigated formoterol as a possible activator of DUSP4. In fact, our present findings indicate that formoterol activates DUSP4 and suggest that DUSP4 activation may be a therapeutic target for restoration of corticosteroid sensitivity. It remains unclear how formoterol activates DUSP4. Nevertheless, this study shows that DUSP4 may be activated via β_2 -adrenergic receptor pathway while PP2A has been reported to be activated by formoterol in β_2 -adrenergic receptor-independent pathway (Kobayashi et al., 2012).

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This difference between DUSP4 and PP2A may be due to the difference in localization of these phosphatases. DUSP4 is mainly located in the cell nucleus and to a lesser degree in the cytoplasm (Guan and Butch, 1995; Sloss et al., 2005), while the catalytic subunit of PP2A with enzymatic activity is located mainly in the cytoplasm and to a lesser degree in the cell membrane (Janssens and Goris, 2001; Kobayashi et al., 2012). Addition of formoterol to an inhaled corticosteroid has a potential for improving asthma control compared with increasing the dose of inhaled corticosteroid in patients with uncontrolled asthma (O'Byrne et al., 2008), which supports previous findings that formoterol restores corticosteroid sensitivity in severe asthmatics (Mercado et al., 2011). Restoration of DUSP4 activity and corticosteroid sensitivity by formoterol, as demonstrated in this study, may contribute to a better control of severe asthma by inhaled combination therapy with a corticosteroid and formoterol than therapy with an inhaled corticosteroid alone.

GR-Ser²²⁶ phosphorylation, which links to inactivation of GR via JNK signaling, has been reported to be regulated also by protein phosphatase 5 (PP5), another serine/threonine phosphatase. PP5 is associated with GR-heat shock protein 90 complex and dephosphorylates GR-Ser²²⁶ (Wang et al., 2007). PP5 also has the potential to control GR ability to translocate into

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the nucleus and to bind glucocorticosteroids (Hinds and Sanchez, 2008). However, in our study PP5 reduction by siRNA did not induce corticosteroid insensitivity (data not shown). Protein tyrosine phosphatase PTP-RR, which can regulate PP2A activity, is also involved in regulation of corticosteroid sensitivity (Kobayashi et al., 2016). Thus, several phosphatases might regulate corticosteroid sensitivity in collaboration. As shown in Supplemental Figure 1, knockdown of DUSP4 using siRNA in our system was limited (~30% reduction). Taken together, in this study DUSP4 knockdown reduced corticosteroid sensitivity to a small but significant extent.

In conclusion, we examined the role of DUSPs in the regulation of corticosteroid sensitivity. We showed that DUSP4 is associated with GR and JNK1 in the cell cytoplasm and regulates GR nuclear translocation, most likely via dephosphorylation of JNK1 and GR-Ser²²⁶. Reduced DUSP4 expression is associated with hyperphosphorylation of GR-Ser²²⁶ in PBMCs from severe asthmatics and may contribute to corticosteroid insensitivity in severe asthma. Indeed, DUSP4 activation by formoterol restores impaired corticosteroid sensitivity, indicating that DUSP4 is crucial in regulation of corticosteroid sensitivity and therefore might be a novel therapeutic target in patients with severe asthma.

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Authorship Contributions

Participated in research design: Yoshiki Kobayashi, Kazuhiro Ito, and Peter J. Barnes.

Conducted experiments: Yoshiki Kobayashi, Akira Kanda, and Nicolas Mercado.

Performed data analysis: Yoshiki Kobayashi, Akira Kanda, and Nicolas Mercado.

Wrote or contributed to the writing of the manuscript: Yoshiki Kobayashi, Kazuhiro Ito, Koichi

Tomoda, and Peter J. Barnes.

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Footnotes

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KI is currently an employee of Pulmocide Ltd and has honorary contract with Imperial College.

AML is an employee of AstraZeneca. PB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GlaxoSmithKline, Novartis, Nycomed, Pfizer, RespiVert, Teva and UCB and has received research funding from Aquinox Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GlaxoSmithKline, Novartis, Nycomed, Pfizer, and Prosonix. The other authors have declared no competing interests for this research.

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Figure Legends

Fig. 1: Effect of DUSP4 reduction by siRNA on steroid sensitivity.

U937 cells were transfected with scrambled control (SC) and DUSP1 (D1), DUSP4 (D4), DUSP8, DUSP16 and DUSP22 siRNAs. (A) The ability of dexamethasone (Dex) to inhibit TNF α -induced IL-8 production was evaluated in U937 cells and expressed as % inhibition by Dex with Dex-IC₅₀ value. (B) The ability of Dex (10⁻⁷ M) to enhance FKBP5 (i) and inhibit TNF α -induced IFN γ (ii) and IL-8 (iii) mRNA levels were evaluated and expressed relative to GAPDH mRNA levels. (C) Phosphorylation levels of JNK1 (D) phosphorylation of GR-Ser²²⁶ measured by Western blot. (E) DUSP4 association with JNK1 and GR in the whole cell extract obtained from U937 cells. GR (upper lane), DUSP4 (middle lane) and JNK1 (lower lane) expressions in GR-immunoprecipitates (IP-GR) and DUSP4-immunoprecipitates (IP-D4) were detected by Western blot. Immunobeads without protein and protein without immunoprecipitation were used as controls. (F) U937 cells were treated with Dex (10⁻⁷ M) for 1 h. Nuclear and cytoplasmic GR were measured by Western blot. TBP (for nuclear protein) or α tubulin (for cytoplasmic protein) expression was used as a control for protein loading. As an index of GR nuclear translocation, the ratio of nuclear GR to TBP was calculated. Values

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represent means of three experiments \pm SEM; # $P < 0.05$, ## $P < 0.01$, Bonferroni (vs. the cells with same treatment in SC siRNA group); \$ $P < 0.01$, Bonferroni (vs. DUSP1); * $P < 0.05$, ** $P < 0.01$, Bonferroni (as shown between two groups).

Fig.2: DUSP4 expression in PBMCs from severe asthma

(A) DUSP4 protein expression in PBMCs from healthy volunteers (HV) and severe asthmatics (SA) by Western-blotting analysis. (B)(C) DUSP4 (APC-labeled), phospho-GR-Ser²²⁶ (PE-labeled) and phospho-JNK (AF488-labeled) protein expression in PBMCs from HV and SA by Imaging Flow Cytometric analysis. (B) DUSP4 expression (MFI; mean fluorescence intensity corrected by isotype control) and its correlation with phosphorylation levels of GR-Ser²²⁶ (pGR-Ser²²⁶) and JNK (pJNK) are shown. (C) Sample images of single cell from each subject are shown. # $P < 0.05$, ## $P < 0.01$, Mann-Whitney (vs. HV).

Fig. 3: Effects of formoterol on DUSP4 .

The activity of immunopurified DUSP4 (IP-DUSP4) was analyzed in U937 cells. (A) Cells were incubated with formoterol (FM: 10^{-11} to 10^{-7} M), salmeterol (SM; 10^{-7} M) or

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salbutamol/albuterol (SB; 10^{-7} M) for 20 min before cell lysis. (B) Cells were preincubated with or without β_2 -adrenergic antagonist, ICI-118551 (10^{-5} M) for 30 min, followed by the treatment of FM (10^{-9} M) or SM (10^{-7} M) for 20 min. Data are expressed as fold changes against non-treatment (NT). PBMCs from healthy volunteers were transfected with scrambled control (SC) or DUSP4 siRNAs. At 24 h after transfection, cells were treated with FM (10^{-9} M) or SM (10^{-7} M) for 20 min. (C) The ability of dexamethasone (Dex: 10^{-7} M) to enhance FKBP5 (i) and inhibit TNF α -induced IFN γ (ii) and IL-8 (iii) mRNA levels were evaluated and expressed relative to GAPDH mRNA levels. (D) Immunoprecipitated DUSP4 (IP-DUSP4) activity was analyzed. Values represent means of four (A and B) or three (C and D) experiments \pm SEM. ^f $P < 0.01$, Bonferroni (vs. NT); ^s $P < 0.01$, Bonferroni (as shown between two groups); [#] $P < 0.01$, t-test (between DUSP4 and SC siRNAs groups in vehicle-treated cells); * $P < 0.05$, ** $P < 0.01$, t-test (between formoterol- and vehicle-treated cells in DUSP4 siRNA group).

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Table 1: Characteristics of subjects (for Western-blotting analysis)

	Healthy volunteers (n=9)	Severe asthmatics (n=11)
Gender (M:F)	2:7	5:6
Age (years)	49.9 ± 8.4	52.6 ± 14.9
FEV _{1.0} %pred.	90.7 ± 8.5	72.5 ± 16.9*
FEV _{1.0} /FVC	78.8 ± 3.7	68.3 ± 8.1*
ICS	none	11/11 [655 ± 220 µg] ¹⁾
OCS	none	5/11 [17.5 ± 4.1 mg] ²⁾
LABA	none	11/11

ICS: inhaled corticosteroid, ¹⁾ fluticasone propionate equivalent dose; OCS: oral corticosteroid,

²⁾ prednisolone equivalent dose; LABA: long-acting β₂-adrenergic agonist; * *P* < 0.05,

Mann-Whitney (vs. healthy volunteers). Data are the number of subjects and mean ± SD.

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Table 2 Characteristics of subjects (for Imaging Flow Cytometric analysis)

	Healthy volunteers (n=7)	Severe asthmatics (n=11)
Gender (M:F)	4:3	6:5
Age (years)	53.5 ± 9.0	60.2 ± 9.8
FEV _{1.0} %pred.	90.3 ± 9.8	67.7 ± 21.6*
FEV _{1.0} /FVC	82.6 ± 5.0	66.1 ± 14.3*
ICS	none	11/11 [882 ± 325 µg] ¹⁾
OCS	none	3/11 [5.4 ± 4.0 mg] ²⁾
LABA	none	10/11
LTRA	none	8/11
Omalizumab	none	1/11

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ICS: inhaled corticosteroid, ¹⁾ fluticasone propionate equivalent dose; OCS: oral corticosteroid,

²⁾ prednisolone equivalent dose; LABA: long-acting β_2 -adrenergic agonist; LTRA: leukotriene

receptor antagonist; * $P < 0.05$, Mann-Whitney (vs. healthy volunteers). Data are the number of

subjects and mean \pm SD.

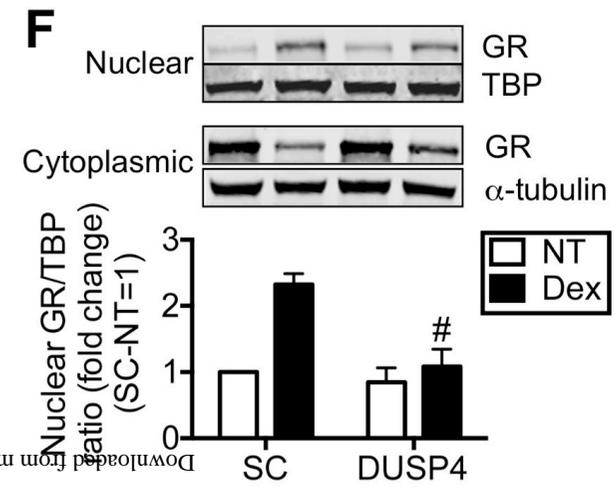
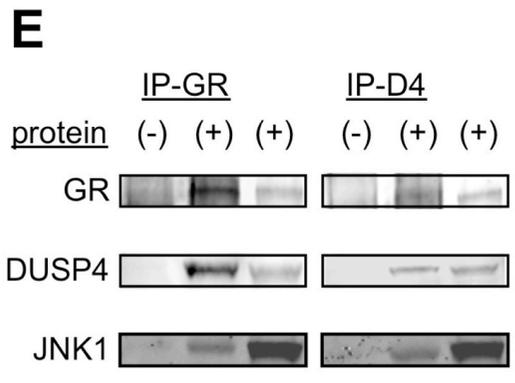
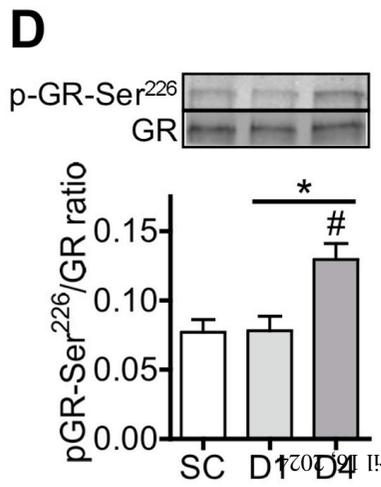
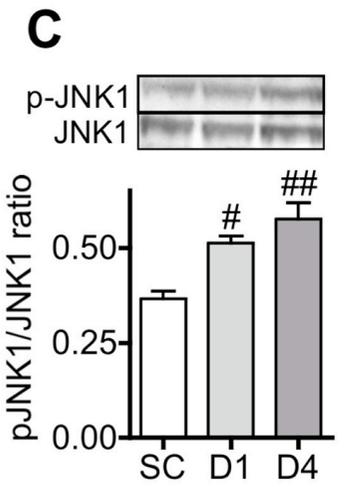
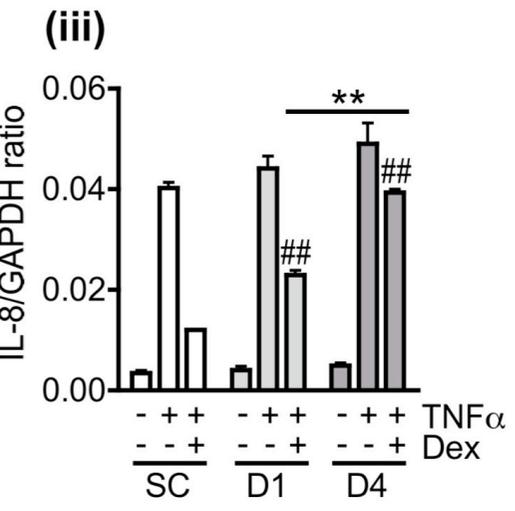
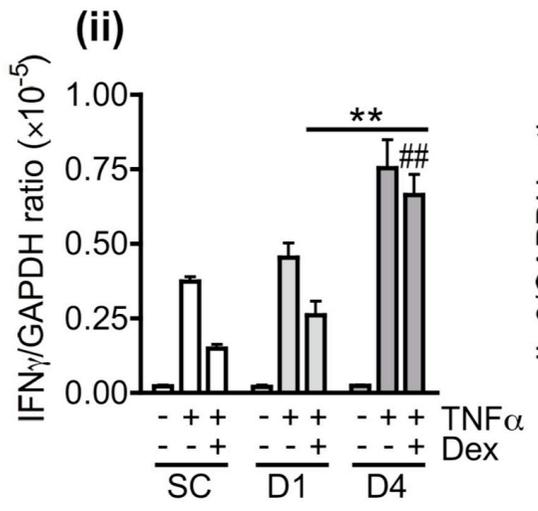
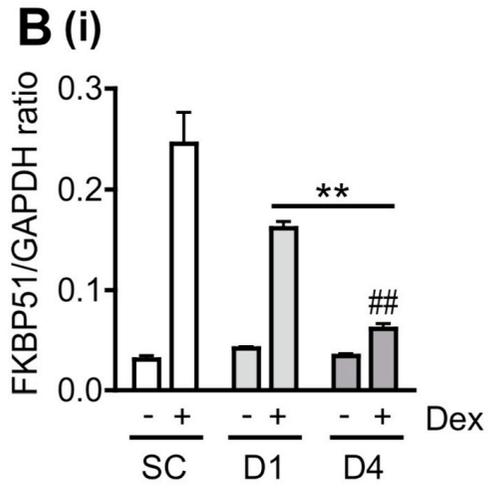
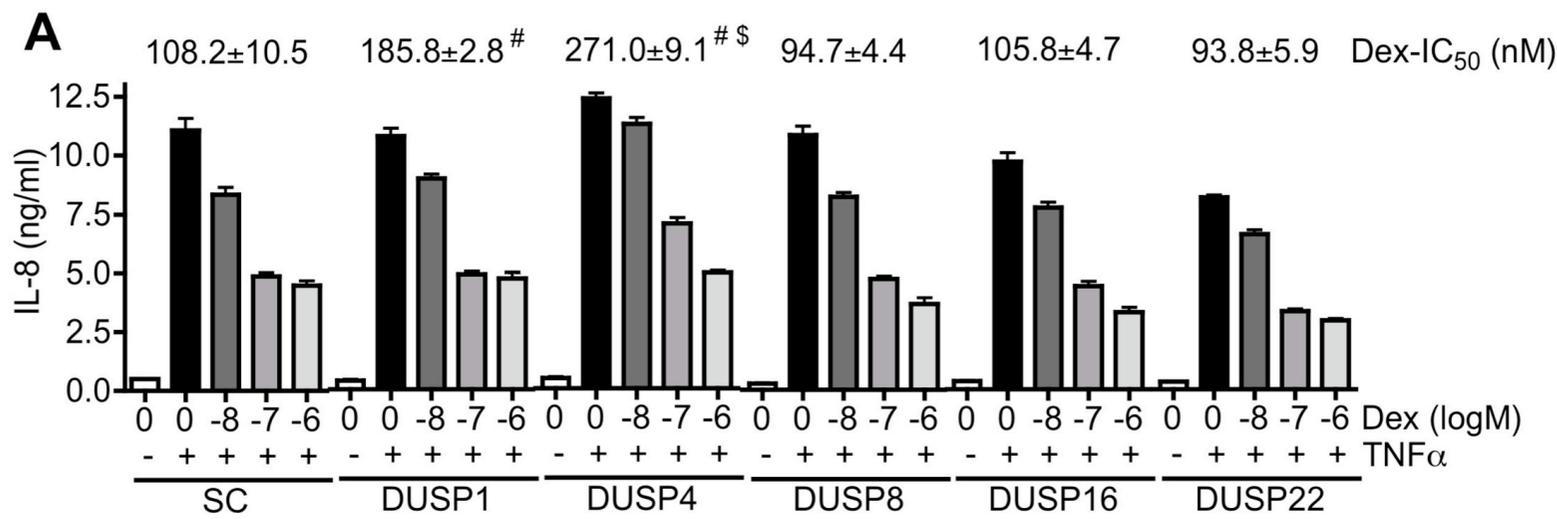


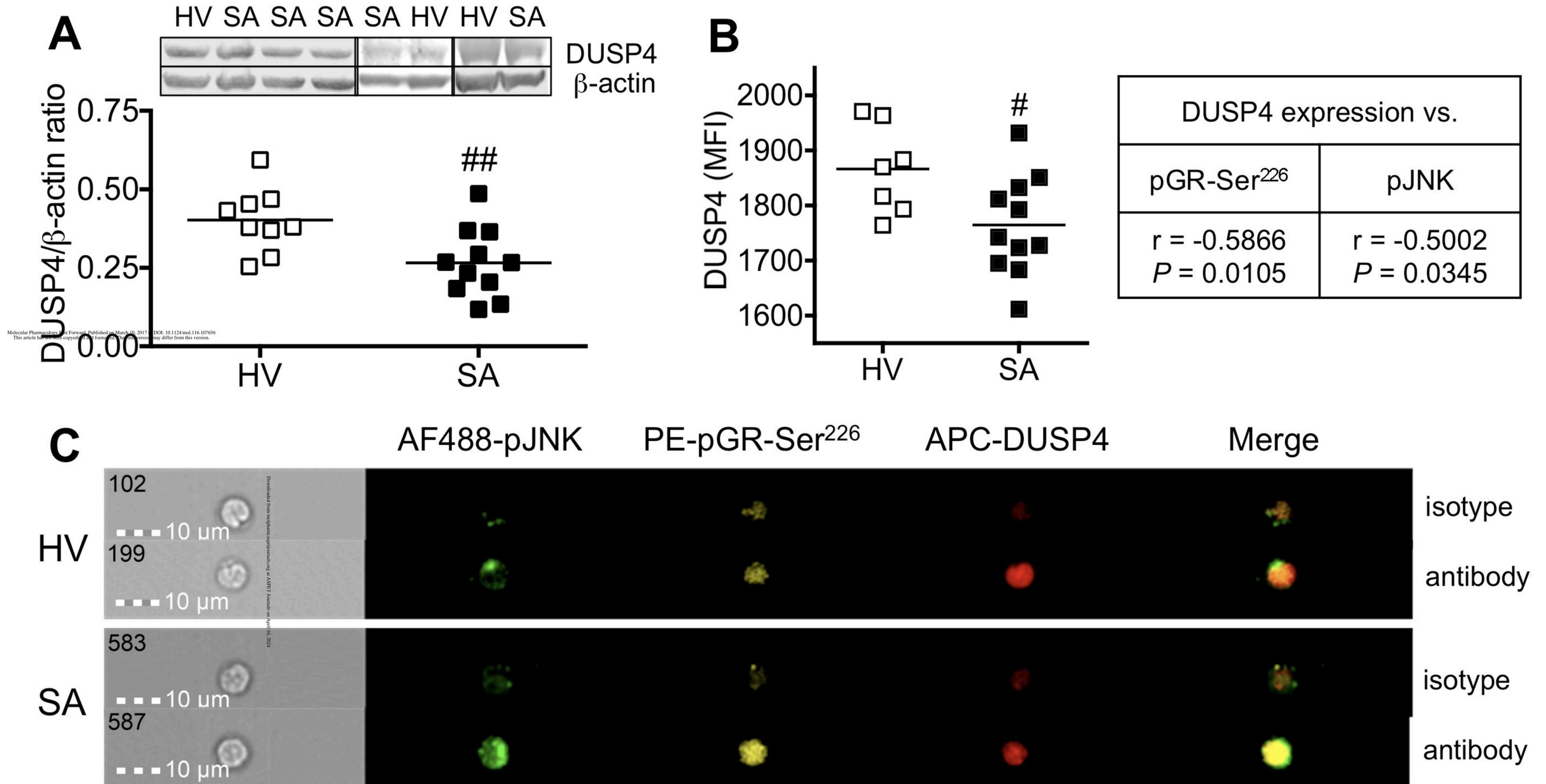
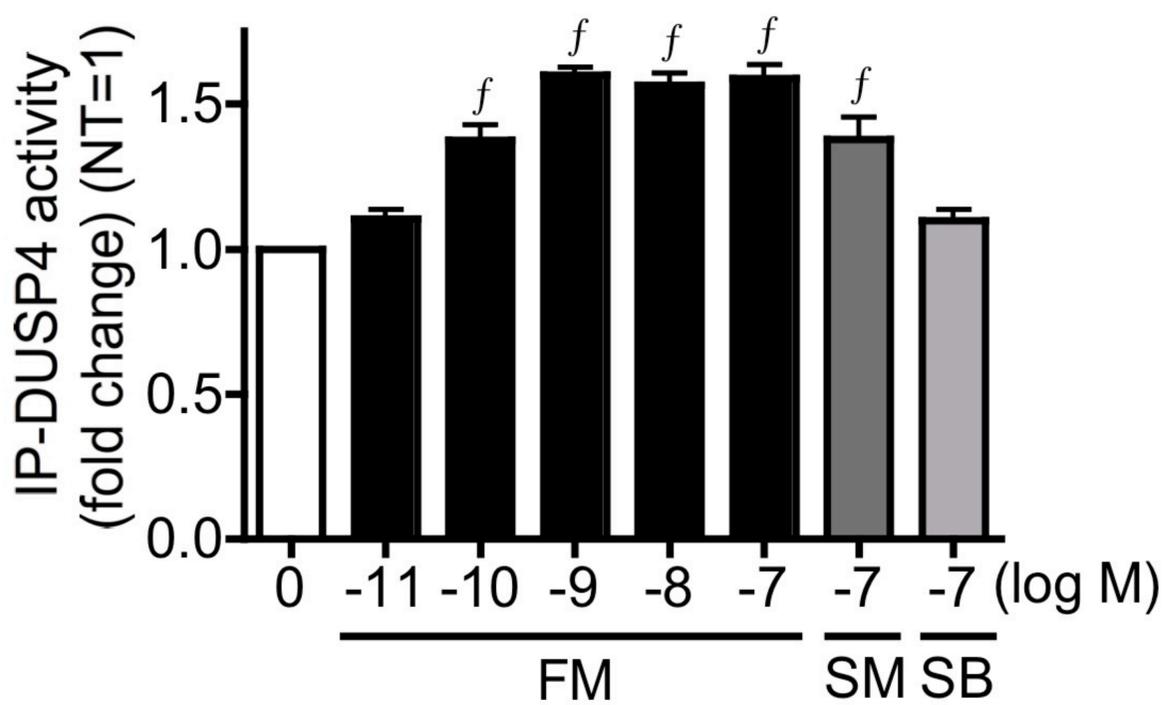
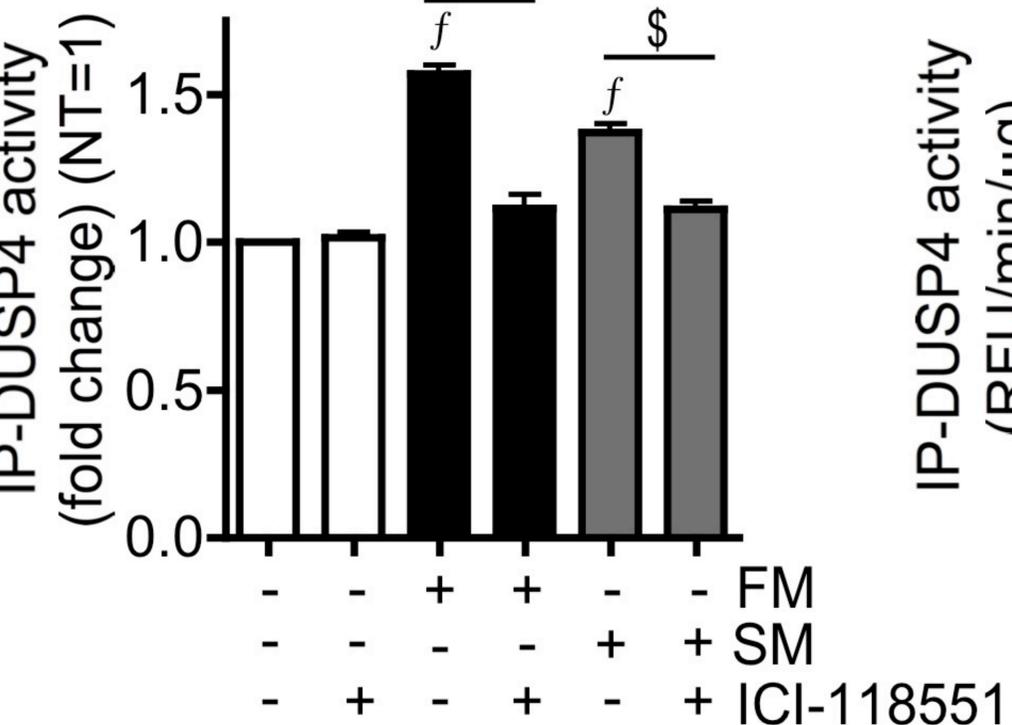
Fig. 2

Fig. 3

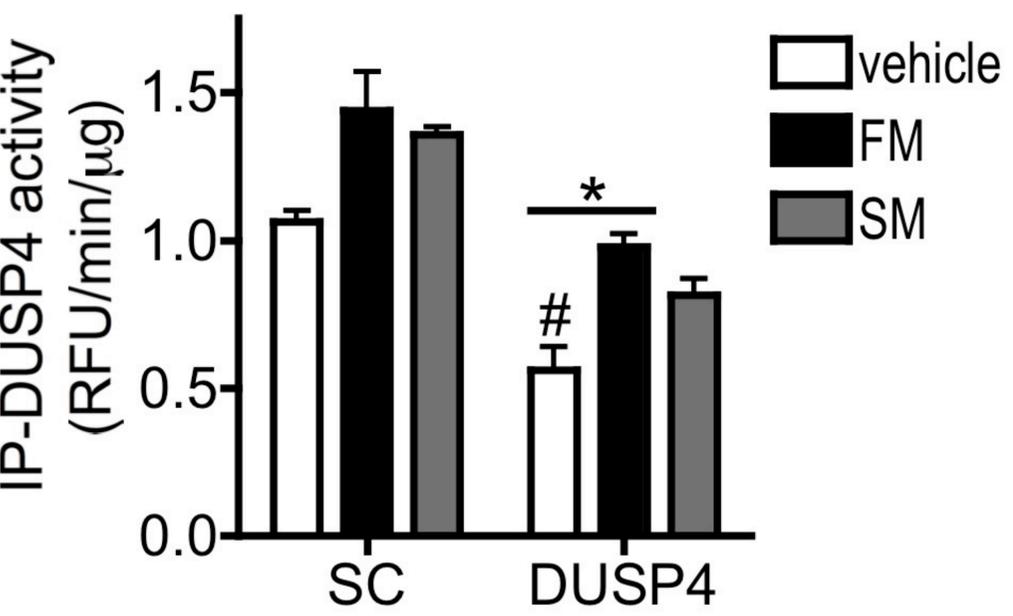
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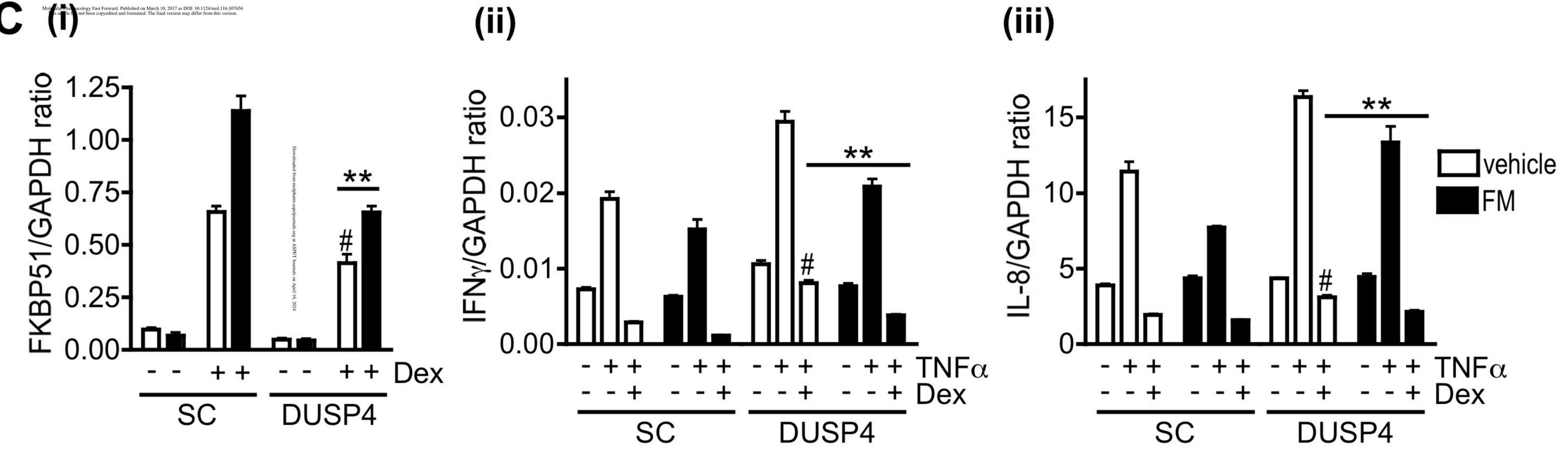
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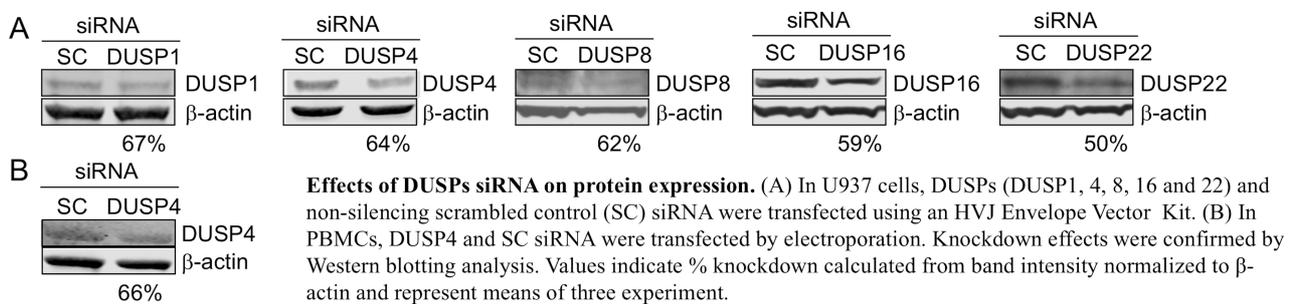
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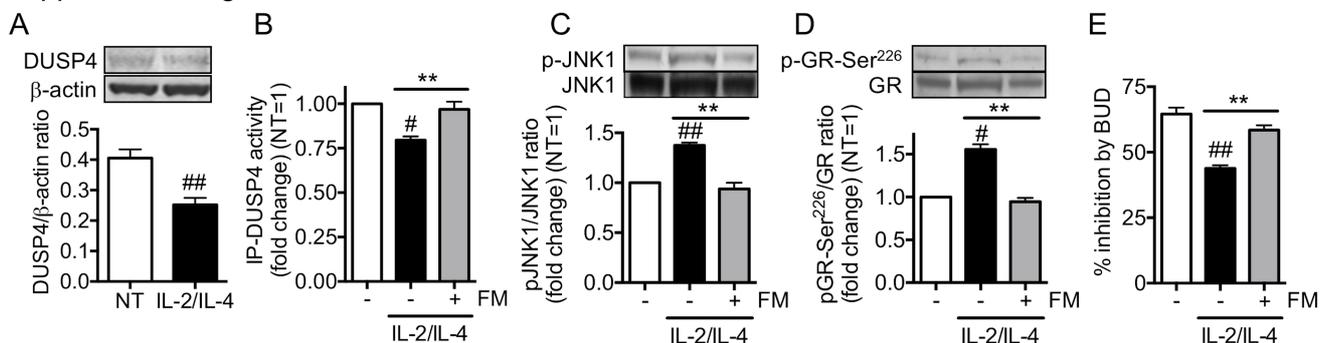
C



Supplemental Figure 1



Supplemental Figure 2



Effect of formoterol (FM)-mediated DUSP4 activation on IL-2/IL-4-induced corticosteroid insensitive model. U937 cells were co-stimulated with IL-2 (20ng/ml) and IL-4 (10ng/ml) for 48 h, followed by the treatment with formoterol (FM; 10^{-9} M) for 20 min. (A) DUSP4 protein expression. (B) Activities of immunopurified DUSP4 (IP-DUSP4). (C), (D) Effect of FM on IL-2/4-induced JNK1 (C) and GR-Ser²²⁶ (D) phosphorylation levels. (E) The ability of budesonide (BUD; 10^{-9} M) to inhibit TNF α -induced IL-8 production was evaluated and expressed as % inhibition by BUD. Values represent means of four (A, E) or three (B, C, D) experiments \pm SEM. # $P < 0.05$, ## $P < 0.01$, t-test (vs. non-treatment control; NT), and ** $P < 0.01$, Boneferroni (as shown between two groups).