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The TORC1-activated Proteins, p70S6K and GRB10, Regulate IL-4 Signaling and M2 Macrophage Polarization by Modulating Phosphorylation of Insulin Receptor Substrate-2*

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Lung M2 macrophages are regulators of airway inflammation, associated with poor lung function in allergic asthma. Previously, we demonstrated that IL-4-induced M2 gene expression correlated with tyrosine phosphorylation of the insulin receptor substrate-2 (IRS-2) in macrophages. We hypothesized that negative regulation of IRS-2 activity after IL-4 stimulation is dependent upon serine phosphorylation of IRS-2. Herein, we describe an inverse relationship between tyrosine phosphorylation (Tyr(P)) and serine phosphorylation (Ser(P)) of IRS-2 after IL-4 stimulation. Inhibiting serine phosphatase activity increased Ser(P)-IRS-2 and decreased Tyr(P)-IRS-2 leading to reduced M2 gene expression (*CD200R*, *CCL22*, *MMP12*, and *TGM2*). We found that inhibition of p70S6K, downstream of TORC1, resulted in diminished Ser(P)-IRS-2 and prolonged Tyr(P)-IRS-2 as well. Inhibition of p70S6K increased expression of *CD200R* and *CCL22* indicating that p70S6K negatively regulates some, but not all, human M2 genes. Knocking down GRB10, another negative regulatory protein downstream of TORC1, enhanced both Tyr(P)-IRS-2 and increased expression of all four M2 genes. Furthermore, GRB10 associated with IRS-2, NEDD4.2 (an E3-ubiquitin ligase), IL-4Rα, and γC after IL-4 stimulation. Both IL-4Rα and γC were ubiquitinated after 30 min of IL-4 treatment, suggesting that GRB10 may regulate degradation of the IL-4 receptor-signaling complex through interactions with NEDD4.2. Taken together, these data highlight two novel regulatory proteins that could be therapeutically manipulated to limit IL-4-induced IRS-2 signaling and polarization of M2 macrophages in allergic inflammation.

Both genetic and environmental factors influence the onset and exacerbation of allergic asthma, which remains one of the most costly, non-communicable diseases of the human population. Allergic inflammation is characterized by airway hyperreactivity, type 2 cytokines (IL-4, -5, and -13), IgE, and the overproduction of mucus in the lung (1–3). Macrophages present at the site of allergic inflammation can be polarized by Th2-cytokines to become "alternatively activated" or M2 macrophages. In addition, the abundance of M2 macrophages in the lungs of asthmatics correlates with symptom severity (4–6). As M2 macrophage polarization is dependent upon IL-4 stimulation, this cytokine and its signaling pathways represent excellent targets for asthma therapies.

Our previous work has identified IL-4 signaling through the type I IL-4 receptor complex and activation of insulin receptor substrate 2 (IRS-2)[2] as important for the degree of polarization of M2 macrophages (7). IL-4 binds first to the IL-4Rα chain with high affinity followed by recruitment of the common γ chain (IL-2Rγ or γC) to create a functional type I receptor. Assembly of this receptor complex leads to recruitment and phosphorylation of the JAK1 and -3 proteins followed by robust, yet transient, tyrosine phosphorylation of IRS-2 and STAT6. Phosphorylated STAT6 homodimerizes and translocates to the nucleus, where it activates transcription of M2 macrophage-specific genes (*ArgI*, *Retnla*, and *Chi3l3* in the mouse and *TGM2*, *MMP12*, *CCL22*, *CD200R* in humans). Activation of the IRS-2 pathway by IL-4 binding the type I IL-4 receptor, but not the type II IL-4 receptor, further enhances the degree of M2 macrophage gene expression (7). Although Shp-1 and SHIP-1 have been implicated in negative regulation of IL-4-induced STAT6 signaling (8), nothing is known of the negative regulatory processes that suppress the IL-4-induced IRS-2 signaling pathway.

Serine phosphorylation of the IRS proteins is one mechanism by which insulin-induced IRS signaling is terminated (9–12). Serine phosphorylation of IRS-1 and IRS-2 prevents p85 binding and PI3K activation (13), promotes IRS degradation, and promotes dissociation of IRS molecules from the insulin receptor (14, 15). A number of different serine/threonine kinases (*e.g.* ERK1/2, TORC1/2, p70S6K, GSK-3α/β, JNK) have been shown to phosphorylate-specific serine residues of IRS-1 to inhibit insulin signaling (11–13). Recent publications, however, highlight the importance of the TOR complexes and TOR-activated proteins in regulating M2 polarization in mouse macrophages in response to IL-4 (16–20). Because our previous work showed that IRS-2 tyrosine phosphorylation correlated with M2 polarization, we sought to determine whether serine phosphoryla-

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[2] The abbreviations used are: IRS-2, insulin receptor substrate 2; IP, immunoprecipitation; qPCR, quantitative PCR; ANOVA, analysis of variance.

tion of IRS-2 and TOR-activated regulatory pathways were responsible for controlling IL-4 signaling through IRS-2 in human macrophages.

Results

IL-4 Had Opposite Effects on Tyrosine and Serine Phosphorylation of IRS-2—Previously we described IL-4-induced tyrosine phosphorylation of IRS-2 correlating with M2 macrophage polarization in both mouse macrophages and human monocytes (7, 21, 22). We hypothesized that IL-4-induced tyrosine phosphorylation of IRS-2 is subject to down-regulation by serine phosphorylation of IRS-2. To test this hypothesis, we simultaneously evaluated tyrosine-phosphorylated (Tyr(P)-) IRS-2 and serine-phosphorylated (Ser(P)-) IRS-2 in human monocytic cells stimulated with IL-4 (10 ng/ml). Maximum tyrosine phosphorylation of IRS-2 occurs between 15 and 30 min of IL-4 stimulation; therefore, we evaluated later time points (time = 60, 90, 120, 150 min) after the peak of IRS-2 activation (7). Accordingly, the amount of Tyr(P)-IRS-2 peaked at 15–30 min of IL-4 stimulation then returned to the level found in unstimulated cells by 90 min (Fig. 1*A*). In contrast, IRS-2 was highly phosphorylated on serine residues before stimulation (Fig. 1*A*, *lower panel*). After IL-4 stimulation, the amount of Ser(P)-IRS-2 decreased dramatically. Ser(P)-IRS-2 returned to the amount seen in unstimulated cells by 150 min of IL-4 stimulation.

We further interrogated the role of Ser(P)-IRS-2 by inhibiting total serine phosphatase activity with calyculin A. IL-4 signaling was initiated, and calyculin A was added after 30 min (Fig. 1*B*). Tyrosine phosphorylation of IRS-2 was significantly reduced after the addition of calyculin A (Fig. 1*B*, *top panel*). Concomitantly, we detected a significant increase in Ser(P)-IRS-2 (Fig. 1*B*, *middle panel*) with a corresponding increase in the molecular weight of IRS-2 as a result (Fig. 1*B*, *bottom panel*). Calyculin A did not increase STAT6 phosphorylation; however, phosphorylation of AKT (Ser-473) and mTOR (Ser-2448) were modulated downstream of IRS-2 (Fig. 1*C*) (23, 24). As anticipated after the calyculin A-induced ablation of Tyr(P)-IRS-2, we detected significant decreases in M2 gene expression of *CD200R* (*, $p < 0.05$) and *TGM-2* and *CCL22* (**, $p < 0.01$, Fig. 1*D*). These results confirmed an inverse correlation between serine and tyrosine phosphorylation of IRS-2 following IL-4 stimulation.

TORC1-specific Inhibitors Altered IRS-2 Phosphorylation and M2 Gene Expression—Activation of 44 serine/threonine kinases, including serine/threonine kinases of the AKT/TOR pathway, were screened and evaluated using a phosphokinase array (Fig. 2*A*). As we observed in the previous experiment (Fig. 1*C*), IL-4 induced phosphorylation of TOR (Ser-2448) and AKT (Ser-473) (Fig. 2*A*, *left* and *middle panel*). P70S6K, downstream of TOR, also showed changes in phosphorylation with IL-4 stimulation. Phosphorylation of AKT and p70S6K in response to IL-4 was confirmed by Western blot (Fig. 2*A*, *right panel*, DMSO-treated cells).

Next, we used inhibitors of TORC1/2 (PP242), TORC1 (rapamycin), and p70S6K (PF4708671) to determine their role in serine phosphorylation of IRS-2 after IL-4 stimulation. We first validated the specificity of each inhibitor in the U937 cells (Fig. 2*A*, *right side*) (25–27). Phosphorylation of serine 473 of AKT, a TORC2 phosphorylation site, was inhibited by PP242 but not rapamycin (5 nM) (Fig. 2*A*, *right top panel*). Rapamycin and PP242 both inhibited TORC1 activity, as demonstrated by diminished phosphorylation of the TORC1 substrate, p70S6K (Thr-389) (Fig. 2*A*, *right middle panel*). PF-4708671 inhibited p70S6K, as shown by diminished phosphorylation of its substrate, S6 (Fig. 2*A*, *right bottom panel*).

We pretreated U937 cells for 1 h with each serine kinase inhibitor to determine the duration of IRS-2 signaling and phosphorylation of AKT, p70S6K, and TOR as before (Fig. 1, *A* and *B*). Rapamycin and p70S6K inhibitor treatments prolonged Tyr(P)-IRS-2 compared with DMSO treatment (Fig. 2*B*, *top panel of blots* and *upper panel of graphs*). The amount of serine phosphorylation of IRS-2 significantly decreased in cells treated with the p70S6K inhibitor (Fig. 3*A*, *middle panel of blots* and *lower panel of graphs*). Treatment with PP242 did not change the amount of Tyr(P)- or Ser(P)-IRS-2. STAT6 phosphorylation at Tyr-641 was not changed by any of the inhibitors (Fig. 2*B*, *right side of panel*).

Next, we assessed the induction of M2 macrophage-specific genes in U937 cells treated with the various inhibitors. IL-4 induced expression of *TGM-2*, *MMP12*, *CCL22*, and *CD200R* in DMSO control-treated cells. Inhibition of TORC1 with rapamycin (Fig. 2*C*, *open bars*) modestly increased expression of *CCL22* at 24 h (+, $p < 0.1$) but not the other M2 genes. PP242 treatment did not significantly alter expression of any of the M2 genes. However, inhibition of p70S6K significantly increased expression of *CD200R* and *CCL22* above that of DMSO control at 24 h (**, $p < 0.01$; *, $p < 0.05$; Fig. 2*C*, *black bars*). This finding matched the prolonged Tyr(P)-IRS-2 and diminished Ser(P)-IRS-2 signaling we observed with this inhibitor. Expression of *MMP12* and *TGM2* was not increased by p70S6K inhibition. These data suggest that p70S6K is involved in negatively regulating IRS-2 signaling and expression of some, but not all, human M2 genes.

GRB10 Was Activated and Transiently Associated with IL-4 Receptor Subunits after IL-4 Stimulation—Because not all M2 genes were augmented by p70S6K inhibition, we evaluated other negative regulatory proteins downstream of TORC1. Other studies have shown that the small adaptor protein, GRB10, is also activated by TORC1 (28, 29) and negatively regulates IRS-1 signaling after insulin and IGF-I stimulation (28–31). We hypothesized that GRB10 would regulate IL-4-activated IRS-2 signaling. First, we confirmed that GRB10 was activated in response to IL-4 by evaluating the phosphorylation of target residues of TORC1 activity. We observed GRB10 (Ser-501/503) phosphorylation at 30 min of IL-4 stimulation. Phosphorylation of GRB10 declined at 90 and 120 min of IL-4 stimulation (Fig. 3*A*). This kinetic of phosphorylation of GRB10 (Ser-501/503) correlated with tyrosine and serine phosphorylation of IRS-2.

Next we showed that GRB10 associated with subunits of the IL-4 receptor complex. We observed a transient association of phosphorylated GRB10 with the IL-4Rα chain and γC by co-IP after IL-4 stimulation (Fig. 3, *B* and *C*). We also detected GRB10 and the IL-4Rα and γC together, leading us to hypothesize that GRB10 may regulate IL-4-induced responses by mediating internalization and degradation the type I IL-4 receptor signal-

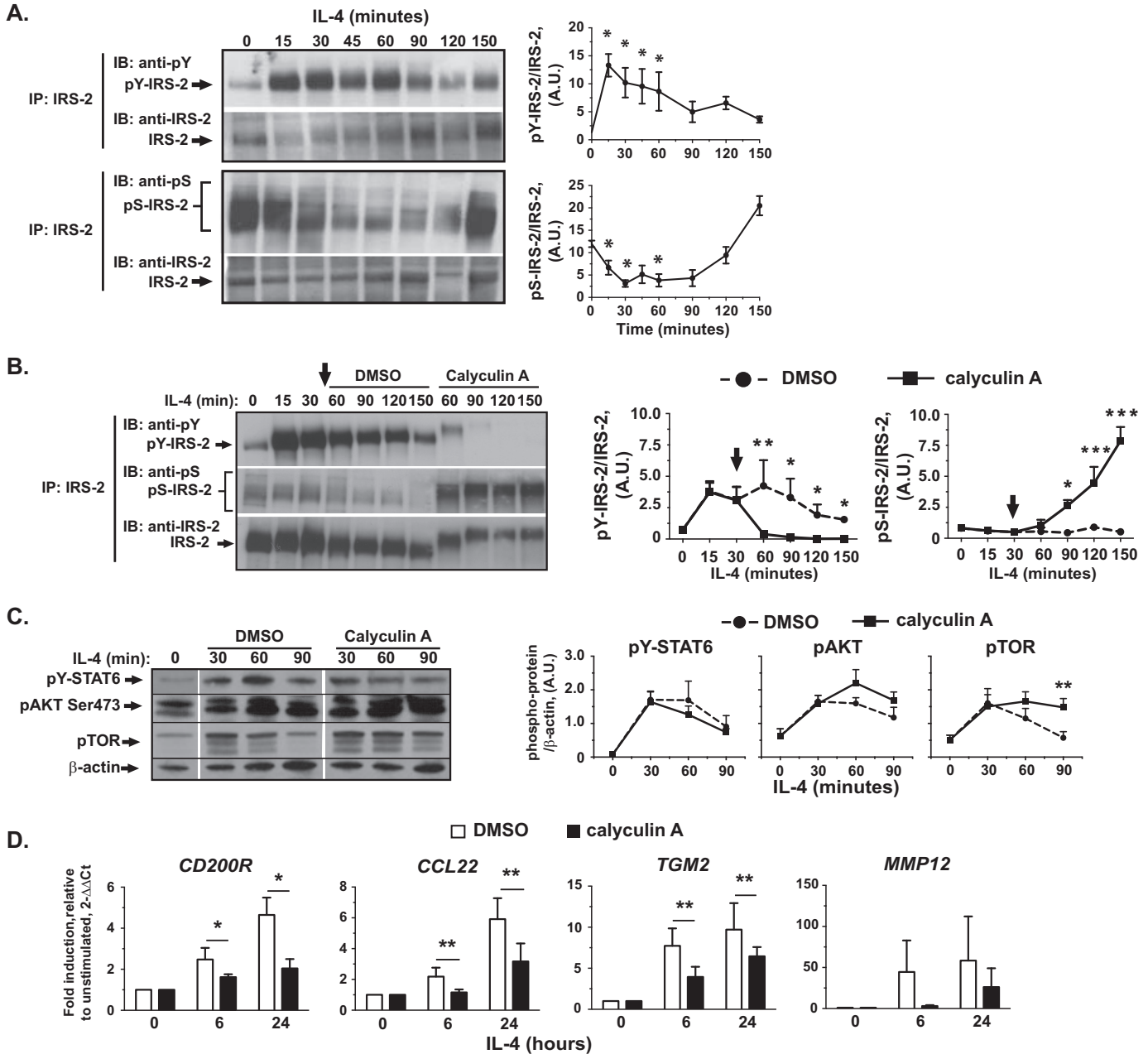

FIGURE 1. **IL-4 has opposite effects on tyrosine and serine phosphorylation of IRS-2.** Human monocytic (U937) cells were serum-starved for 2 h then stimulated with IL-4 (10 ng/ml) for the indicated times. *A*, IRS-2 protein was immunoprecipitated from cell lysates, and Western blot (*IB*) membranes were probed with anti-phosphotyrosine (*pY*), anti-phosphoserine (*pS*), and anti-IRS-2 antibodies. Representative images of the films (*left*) are shown. Films were scanned, and densitometry was performed on the bands. The amount of Tyr(P)-IRS-2 normalized to the total IRS-2 is graphed from three separate experiments (*right*). Mean ± S.E. are represented. *, $p < 0.05$; *A.U.*, arbitrary units. *B*, U937 cells stimulated with IL-4 for 30 min then treated with 25 nM calyculin A or DMSO vehicle control. Tyr(P)- and Ser(P)-IRS-2 was assessed as in *A*. A representative Western blot film and densitometry for three separate experiments are shown. Mean ± S.E. is represented. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, phospho-TOR (Ser-2448), -AKT (Ser-473), and -STAT6 (Tyr-641) were assessed at the indicated time points in the DMSO- or calyculin A-treated cells. A representative Western blot image is shown. The *white vertical bar* indicates that the bands were from non-adjacent lanes. The representative bands are from the same gel/membrane. Densitometry is representative of two separate experiments, and statistical significance was determined using multiple Student's *t* tests. **, $p < 0.01$. *D*, human M2 gene expression was determined by quantitative PCR at 6 and 24 h of IL-4 stimulation with or without calyculin A, as described in *B*. Relative -fold induction of mRNA is expressed using the standard $2^{-\Delta\Delta Ct}$ calculation, compared with unstimulated cells. Mean ± S.E. is graphed ($n = 3$; statistical analysis was performed using two-way ANOVAs with Bonferroni post-tests. *, $p < 0.05$; **, $p < 0.01$; +, $p < 0.1$).

ing complex. We detected NEDD4.2 and ubiquitination of γC and IL-4Rα with IL-4 stimulation (Fig. 3, *A* and *B*). These findings are consistent with reports of NEDD4.2 binding to γC, and γC ubiquitination and degradation in T-cells (32). To our knowledge we are the first to show GRB10 binding to subunits of the type I IL-4 receptor complex after IL-4-induced activation. We also demonstrated that GRB10, phosphorylated TOR, and phospho-p70S6K could be detected in the IRS-2 immunoprecipitates after IL-4 stimulation (Fig. 3*D*).

GRB10 Knockdown Increased Tyrosine Phosphorylation of IRS-2 and M2 Macrophage Gene Expression—To determine whether GRB10 was involved in modulating Tyr(P)-IRS-2 and

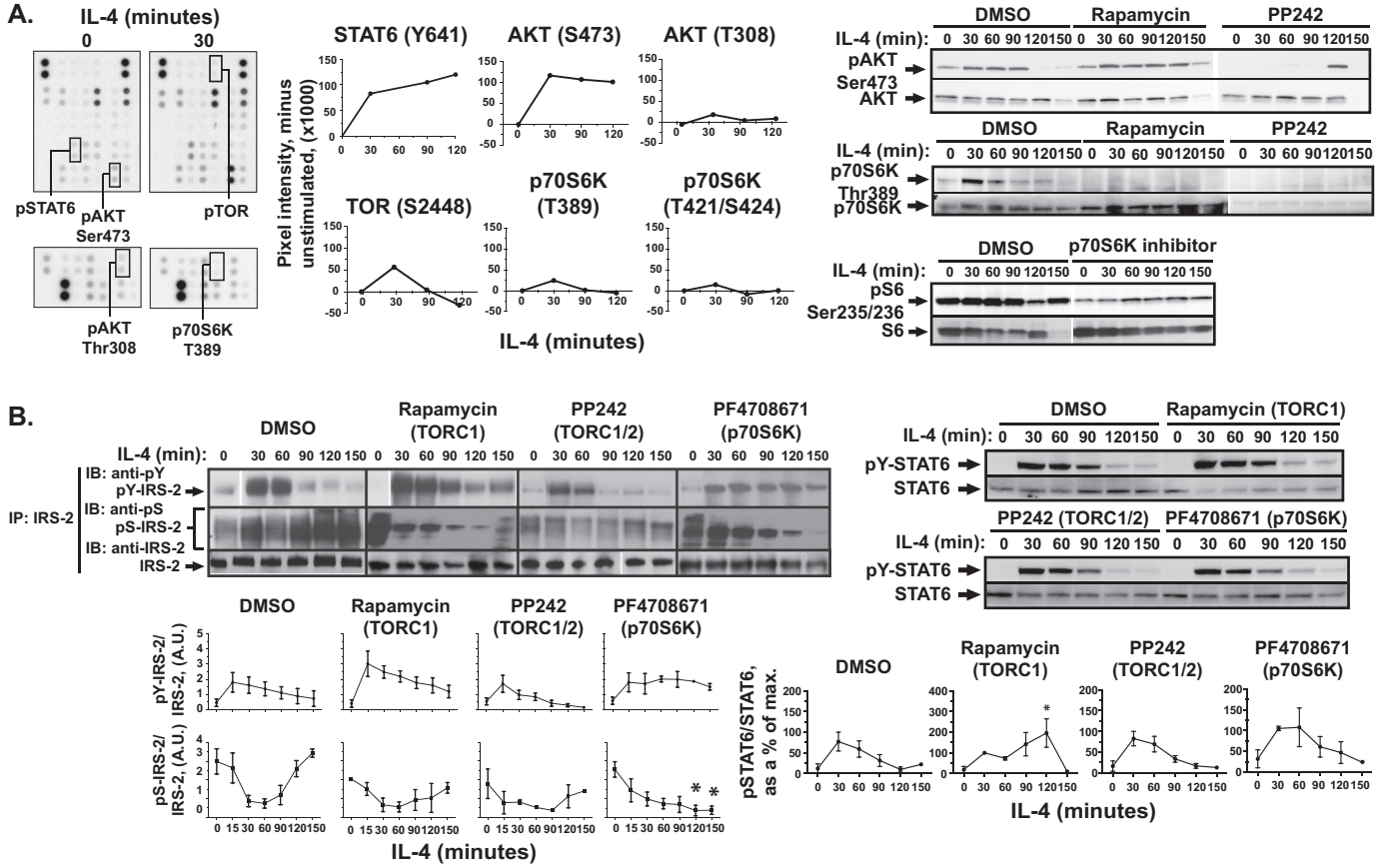

FIGURE 2. **TORC1-specific inhibitors altered IL-4 signaling and M2 gene expression.** *A*, *left*, cell lysates from IL-4-stimulated U937 were incubated with kinase array membranes. A representative Western blot image of the membranes (*top left*), a 10× image of the area on the array membrane corresponding to phosphorylated STAT6 (Tyr-641), and quantitative densitometry (*bottom left*) are shown. Quantitative densitometry for phosphorylated AKT (Ser-473, and Thr-308), TOR (Ser-2448), p70S6K (Thr-389), and p70S6K (Thr-421/Ser-424) are shown (*right*). Mean pixel intensity (1000×) for each phosphoprotein is shown. *A*, *right*, validation of serine kinase inhibition with rapamycin (5 nM), PP242 (500 nM), and PF4708671 (20 μM). Cells were pretreated for 1 h with the different inhibitors and stimulated with IL-4 for the indicated times. Phosphorylation of AKT (Ser-473), p70S6K (Thr-389), S6 (Ser-235/236), and the total target protein was determined by Western blot (*IB*). *B*, IRS-2 was immunoprecipitated as in Fig. 1*A* after serine kinase inhibition. Tyr(P)-IRS-2 and Ser(P)-IRS-2 were determined in three separate experiments. Tyr(P)-STAT6 was determined and quantitated relative to total STAT 6 protein. *C*, human M2 gene expression was determined by quantitative PCR at 6 and 24t h of IL-4 stimulation with or without TORC1, TORC1/2 and p70S6K inhibition, as described in *B*. Relative -fold induction of mRNA was determined. Results were normalized across experiments using the maximum expression vehicle control to determine the % of maximum with indicated inhibitor. Mean ± S.E. are graphed ($n = 3$; statistical analysis was performed using two-way ANOVA with Bonferroni post-tests where appropriate and simple Student's *t* test. **, $p < 0.01$; *, $p < 0.05$; +, $p < 0.1$).

M2 gene expression, we used a small interfering RNA approach to specifically target GRB10 for knockdown. U937 cells were nucleofected with siGRB10 or control siRNA and stimulated with IL-4. We confirmed GRB10 knockdown by Western blot and PCR (65–95% knockdown) (Fig. 4*A*). Tyrosine phosphorylation of IRS-2 and STAT6 were determined after IL-4 treatment (Fig. 4*B*). We detected a significant increase in Tyr(P)-IRS-2 in cells transfected with siGRB10 compared with siRNA-control cells (*, $p < 0.05$; Fig. 4*B*, *upper two panels*). Phosphorylation of STAT6 was not different between siGRB10 and control siRNA (Fig. 4*B*, *lower two panels*).

We next examined the effect of GRB10 knockdown on IL-4-induced M2 gene expression; cells lacking GRB10 expressed significantly more mRNA for *TGM-2*, *CCL22*, and *MMP12* at 24 and 48 h of IL-4 stimulation (*, $p < 0.05$, Fig. 4*C*). CD200R expression was also modestly increased in siGRB10-treated U937 cells. Expression of another hallmark human M2 protein, CD206, was significantly increased on the surface of cells trans-

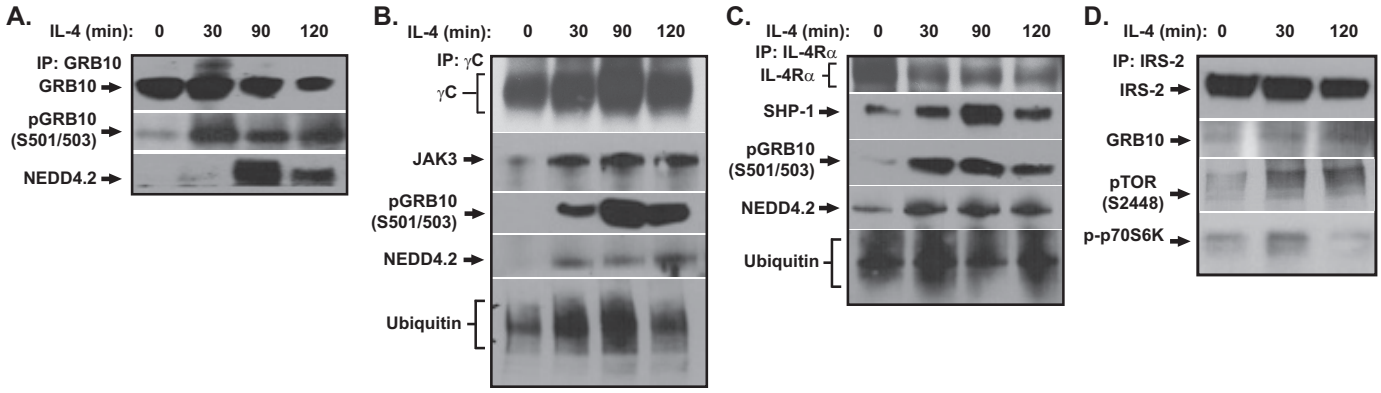

FIGURE 3. **GRB10 was activated and transiently associated with IL-4 receptor subunits after IL-4 stimulation.** *A*, GRB10. *B*, γC. *C*, IL-4Rα. *D*, IRS-2 were immunoprecipitated from U937 cells stimulated with IL-4. Immunoprecipitation was confirmed by Western blot for the indicated proteins (GRB10, γC, IL-4Rα, and IRS-2). We detected SHP-1 with IL-4Rα and JAK3 with γC as the positive control. Representative blotting images are shown (γC IP with GRB10 ($n = 4$); GRB10 IP with pGRB10 ($n = 5$); GRB10 with NEDD4.2 ($n = 3$); γC with NEDD4.2 ($n = 2$); γC and IL-4Rα with ubiquitin ($n = 2$); IRS-2 with all targets ($n = 2$)).

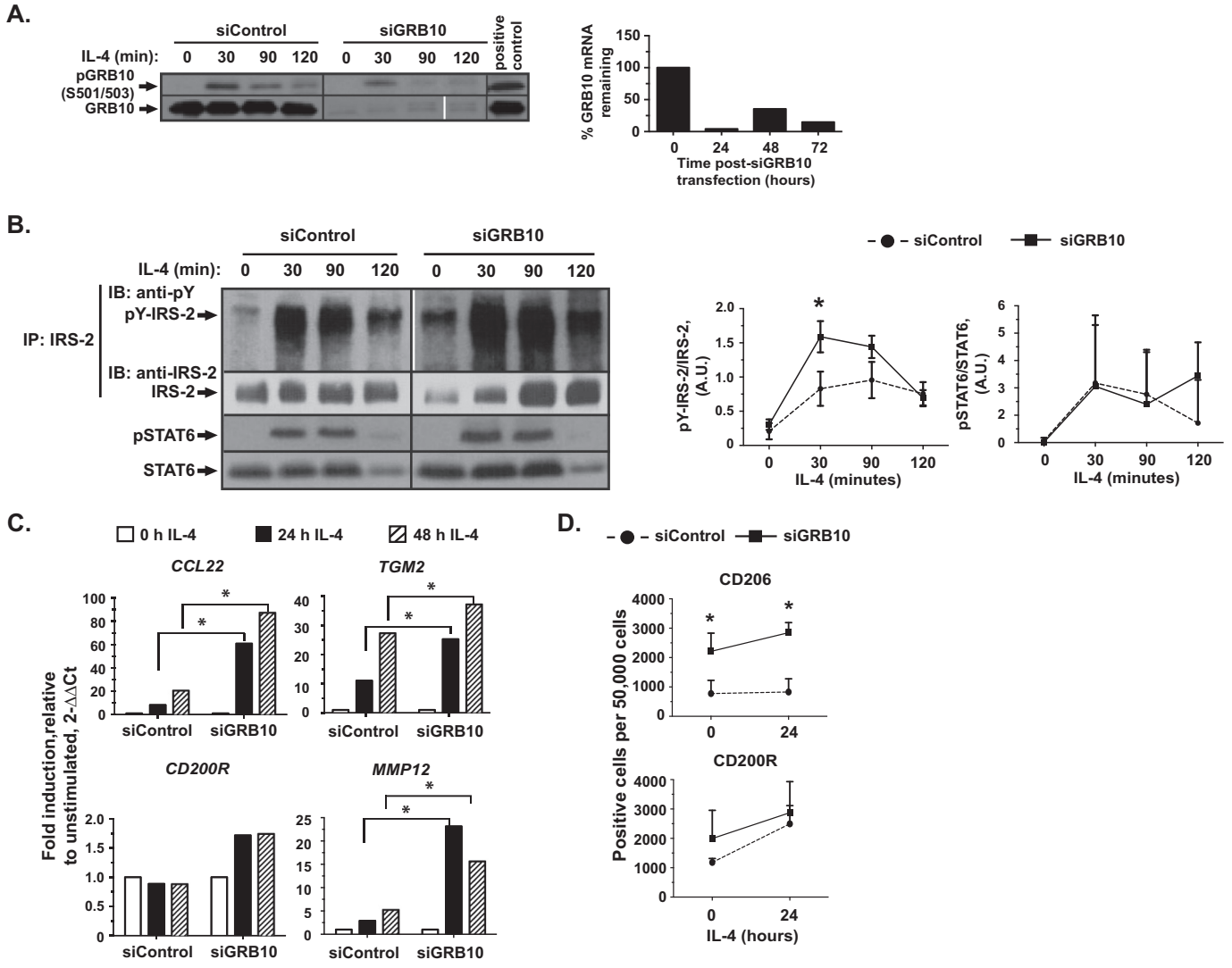

FIGURE 4. **GRB10 knockdown increased tyrosine phosphorylation of IRS-2 and M2 macrophage gene expression.** *A*, U937 cells were nucleofected with siRNA to GRB10 or negative control siRNA (50 nM). 24 h later, siRNA-transfected cells were stimulated with 10 ng/ml IL-4. Phosphorylation of GRB10 and total GRB10 was determined by Western blot (*left*) and by qPCR in unstimulated cells (*right*). *B*, tyrosine phosphorylation of IRS-2 and STAT6 were analyzed as in Fig. 1*A* and 2. *IB*, Western blot. *A.U.*, absorbance units. *C*, human M2 gene expression was determined by quantitative PCR after 24 and 48 h of IL-4 stimulation as described in Fig. 1*C*. Mean ± S.E. are graphed ($n = 3$ independent experiments). *, $p < 0.05$. *D*, cell surface CD200R and CD206 were detected by flow cytometry in U937 cells stimulated with IL-4 for 24 h. Data are representative of two-three separate experiments. Statistical significance was determined by Student's *t* test; *, indicates $p < 0.05$.

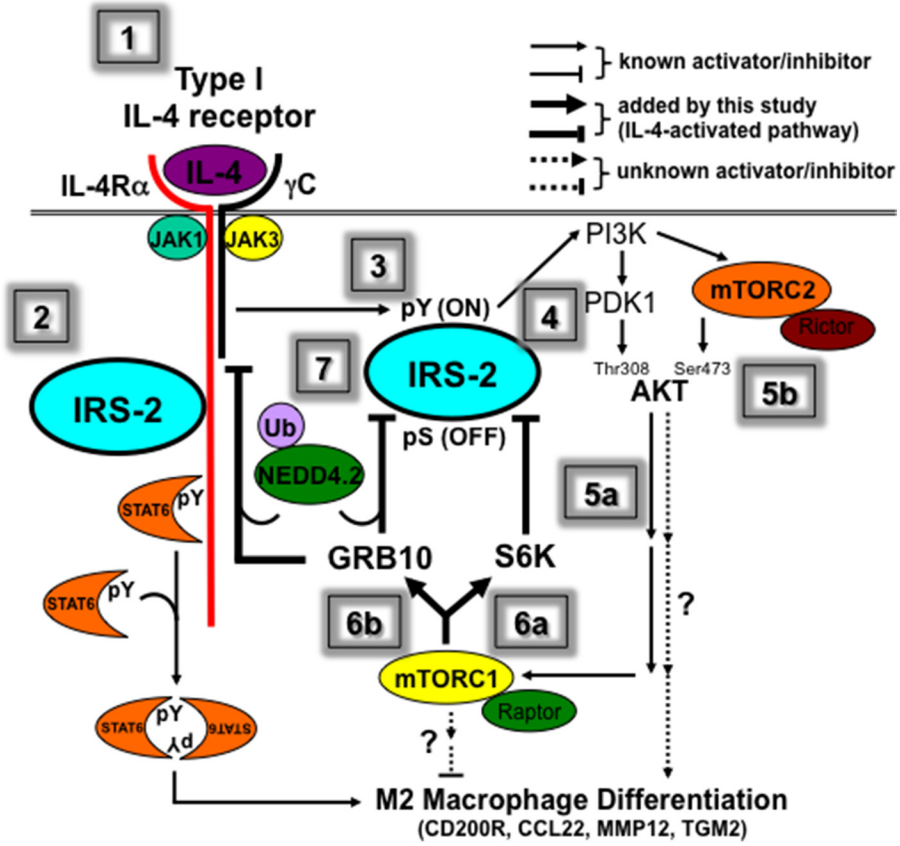

FIGURE 5. **Regulatory pathways of IL-4 signaling and M2 macrophage differentiation.** IL-4 stimulation is a potent inducer of M2 macrophage polarization. *1*, IL-4 can bind two kinds of IL-4 receptor complexes on monocyte-macrophages, type I (shown here) and type II. *2*, both STAT6 and IRS-2 are recruited to the IL-4 receptor α chain after JAK phosphorylation of key tyrosine residues in the cytoplasmic domain. *3*, phosphorylation of tyrosine residues of IRS-2 occurs, presumably by proximity to the activated JAKs. Tyrosine phosphorylation of IRS-2 occurs most potently as a result of engagement of IL-4 with type I receptor complexes. Engagement of type II receptor complexes by either IL-4 or IL-13 does not elicit robust tyrosine phosphorylation of IRS-2. *4*, PI3K is activated through docking and activation of p85α with tyrosine phosphorylated IRS-2. PIP3 activation of PDK1 leads to phosphorylation of AKT Thr308. *5a*, AKT activates mTORC1 as well as possibly other pathways downstream of AKT that directly contribute to M2 gene expression. 5b. PIP3-activated mTORC2 fully activates AKT function by phosphorylation of Ser-473. *6a*, mTORC1 phosphorylates p70S6K, which interacts with IRS-2 to phosphorylate serine residues and thereby limit IL-4 signaling. *6b*, GRB10 is directly activated and stabilized by mTORC1. *7*, GRB10 interacts with IRS-2, common γ chain (γC), IL-4Rα and NEDD4.2 to target these proteins for ubiquitination (*Ub*) and, most likely, proteasomal degradation. Our current study establishes that the mTORC-1 activated proteins p70S6K and GRB10 limit signaling of tyrosine-phosphorylated IRS-2 through serine phosphorylation of IRS-2 and ubiquitination, respectively. The effect of these two negative regulatory mechanisms is a reduction in M2 polarization of human macrophages.

fected with siGRB10 and stimulated with IL-4 as compared with control siRNA cells (*, $p < 0.05$; Fig. 4*D*). There was also a trend toward elevated cell surface CD200R in the GRB10 siRNA-transfected cells after IL-4 stimulation. Taken together with our co-IP and FACS data, these results demonstrate that GRB10 negatively regulates IL-4-induced IRS-2 signaling and M2 macrophage gene expression.

Discussion

IRS-2 has been described as a key regulator of M2 macrophage polarization (7, 33). Given the correlation between the presence of M2 macrophages in the asthmatic lung and asthma severity in humans (4–6), understanding the regulation of IL-4-activated signaling pathways that lead to M2 polarization is critical for interrupting asthma progression.

The goal of this study was to determine mechanisms that negatively regulate the activity of IRS-2 in response to IL-4. Our current results show that down-regulation of IRS-2 signaling by serine phosphorylation follows a paradigm previously established for insulin responses (34–37). Various well defined mechanisms, including serine/threonine kinase activation, steric hindrance, and ubiquitin-mediated degradation of IRS proteins (38), are known to limit insulin-induced IRS-1 activation. Our findings similarly show an on-to-off tyrosine/serine phosphorylation switch for IRS-2 in response to IL-4 (summarized in Fig. 5). IL-4 activates tyrosine phosphorylation and M2 macrophage polarization, whereas serine phosphorylation inhibits IL-4-activated IRS-2 signaling. Using pharmacological and siRNA knockdown approaches, we found that two TORC1-activated proteins, p70S6K and GRB10, were important negative regulators of IL-4-induced IRS-2 signaling and M2 gene expression in human monocytes.

Based upon the results of the Ser(P)- and Tyr(P)-IRS-2 studies, we expected induction of the human M2 gene panel after amplifying Tyr(P)-IRS-2 tyrosine with p70S6K inhibition. Indeed, two of four M2 genes were enhanced with p70S6K inhibition, indicating a greater complexity to regulation of M2 gene expression than anticipated. Many M2 genes share common activating and inhibitory pathways (39, 40) and can be AKT-dependent or -independent (41). For example, studies of macrophage polarization using macrophages derived from Tsc1 (16, 17)-, Raptor (42)-, and Rictor (16, 42)-deficient mice have con-

cluded that TORC1 down-regulates IL-4 signaling/M2 polarization (16, 17) and TORC2 promotes M2 polarization (42). However, TORC1 serves as a signaling "node" that can activate multiple downstream signaling pathways (*e.g.* p70S6K, GRB10, 4E-BP, and others) that may also influence macrophage polarization (43, 44). It is clear from our study that control of IL-4 signaling and M2 gene expression downstream of TORC1 is not all mediated via p70S6K. To that end, we demonstrated that another TORC1-activated protein, GRB10, could regulate IL-4-induced IRS-2 signaling to promote human M2 gene expression. From our co-immunoprecipitation experiments we suggested a second mechanism by which GRB10 bound to receptor subunits that make up the type I IL-4 receptor complex (IL-4Rα, γC) mediates interaction with NEDD4.2. In this way IL-4 signaling could be suppressed by GRB10 by reducing cell surface availability of the type I IL-4 receptor. Future work will address the precise role of GRB10 in regulating this pathway.

In summary, our work shows for the first time that serine phosphorylation of IRS-2 is an important mechanism for down-regulating activation of IRS-2 and M2 gene expression in response to IL-4. We show that tyrosine phosphorylation of IRS-2 and M2 gene expression is controlled by p70S6K and by GRB10, two proteins activated by TORC1 after IL-4 stimulation. Specifically, p70S6K regulated serine phosphorylation of IRS-2 in response to IL-4 and expression of some, but not all, human M2 macrophage genes. GRB10 also negatively regulated the IL-4-activated IRS-2 pathway and M2 gene expression in human monocytes, likely by regulating cell surface expression of the type I IL-4 receptor. To our knowledge we are the first to show GRB10 binding to the components of the IL-4 receptor complex and its effect on IL-4-induced signaling responses and human M2 macrophage gene expression. Our work may provide new therapeutic avenues where p70S6K or GRB10 activity could be enhanced to prevent M2 macrophage polarization in diseases like asthma.

Experimental Procedures

Reagents and Antibodies—The serine kinase inhibitors used in this study were as follows: rapamycin (LC Laboratories, Woburn, MA), PP242 (Millipore, Billerica, MA), PF4708671 (Sigma). The antibodies used for immunoprecipitation and Western blotting were as follows: rabbit anti-human NEDD4.2 (Cell Signaling Technologies, Boston, MA), rabbit anti-human phospho-NEDD4.2 (Ser-448, clone EPR8269) (2), Abcam, Cambridge, MA), rabbit anti-mouse GRB10 (Millipore), rabbit anti-human phospho-GRB10 (Ser-501/Ser-503, Millipore), mouse anti-phosphotyrosine-HRP (clone 4G10, Millipore), goat anti-human IRS-2 (M-19, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-human STAT6 (Santa Cruz Biotechnology), rabbit anti-human IL-4Rα (clone C-20), rabbit anti-human γC (clone C-20), goat anti-human JAK3 (clone: N15), and mouse anti-human β-actin (clone C4, Santa Cruz Technologies), rabbit anti-human phospho-TOR (Ser-2448), rabbit anti-human p70S6K, rabbit anti-human phospho-p70S6K (Thr-389), rabbit anti-human AKT, rabbit anti-human phospho-AKT (Ser-473), rabbit anti-human S6, and rabbit anti-human phospho-S6 (Ser-235/236, Cell Signaling Technologies), anti-ubiquitin-HRP (Enzo, Farmingdale, NY), mouse anti-human phospho-serine (BD Biosciences), and mouse anti-human SHP-1 (clone 52/PTP1C/SHP1) and mouse anti-human phospho-STAT6 (Tyr-641) (Cell Signaling Technologies). The antibodies for flow cytometry were as follows: mouse anti-human CD200R-PE (clone OX-108, Biolegend, San Diego, CA), mouse anti-human IL-4Rα-APC (clone 25463, R&D Systems, Minneapolis, MN), and mouse anti-human CD206-APC (clone 19.2), rat anti-human γC-PE (clone TUGh4), and mouse anti-human phospho-STAT6 (Tyr-641, clone 18/P-Stat6, BD Pharmingen).

Cell Culture—Human monocytic cells, U937, were maintained in RPMI (Life Technologies) media supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Gene Knockdown, Overexpression, and Nucleofection—siRNA against GRB10, GAPDH, and HPRT (hypoxanthine-guanine phosphoribosyltransferase) (Sigma) were transfected using an Amaxa Nucleofector Kit C (Lonza, Allendale, NJ) at concentrations up to 500 nM according to the manufacturer's protocol. Protein knockdown was confirmed by Western blotting for the target protein, and gene knockdown was confirmed using quantitative PCR.

Cell Signaling Experiments—Cell signaling was carried out as previously described (7, 33). Briefly, cells were serum-starved for a minimum of 2 h, up to 12 h, then pretreated for 1 h with serine kinase inhibitors at the concentrations indicated in the figure legend before stimulation with IL-4 (10 ng/ml). For the calyculin A experiments, cells were stimulated with IL-4 (10 ng/ml) for 30 min, then calyculin A (25–100 nM) was added for the duration of the experiment. A separate signaling experiment with DMSO treatment was included in all experiments as a vehicle control. Cells were collected and lysed using 1% Nonidet P-40 lysis buffer (described below) at different time points as indicated.

Immunoprecipitation and Western Blotting—Cells were lysed with buffer containing 1% Nonidet P-40 detergent, protease inhibitors, 1 mM PMSF, 1 mM sodium orthovanadate, and 25 nM calyculin A (previously described Refs. 7 and 33). Immunoprecipitating antibodies were incubated with cell lysates for a minimum of 2 h up to 12 h. Antibodies were rotated with protein G-agarose beads, collected by centrifugation, and washed three times before Western blotting. A Proteome Profiler Human Phospho-Kinase Array (R&D Systems) was used to detect serine kinases phosphorylated in response to IL-4 stimulation. Briefly, 1×10^7 cells were stimulated with 10 ng/ml of IL-4 for 0–120 min and lysed in 1 ml of lysis buffer as described above. Detection membranes were incubated with 375 μg of protein from cell lysates. Detection of bound protein was determined according to the manufacturer's protocol. All films were developed and digitally scanned, then analyzed using the NIH ImageJ software to determine pixel density of each spot or band. All protein data is normalized to the loading control (GAPDH or β-actin) or unstimulated control as indicated.

Quantitative PCR—U937 cells were pretreated with calyculin A or serine kinase inhibitors (concentrations described under "Results") for 1 h then stimulated with IL-4 (10 ng/ml) for up to 48 h. RNA isolation was carried out using an RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instruc-

TABLE 1
Primer sequences for mRNA detection by PCR

Target gene	Sequence (5′ → 3′)
GRB10 forward	CAA ACA GGA CGC GTG ATA GA
GRB10 reverse	GAG GAC ATC TGC GGT CAT AAG
CD200R forward	CAT CGT GGA TAT CAC CTC CAA G
CD200R reverse	CTT GCT TAG TGG CAC AAT CGC
MMP12 forward	AAC CAA CGC TTG CCA AAT CC
MMP12 reverse	CCT TCA GCC AGA AGA ACC TGT
CCL22 forward	GCG TGG TGT TGC TAA CCT TC
CCL22 reverse	GAG AGT TGG CAC AGG CTT CT
TGM2 forward	CAG GAG AAG AGC GAA GGG AC
TGM2 reverse	GAG GTT GGA CTC CGT AAG GC

tions. cDNA was synthesized from 500 ng of purified RNA, and qPCR was performed. The qPCR primer sets used are as described previously (7, 33) and in Table 1.

Statistical Analyses—All statistical analysis was carried out using GraphPad Prism Version 6. One-way analysis of variance (ANOVA) was used to establish differences among treatment groups in each experiment. A two-way ANOVA with repeated measures was used where appropriate to determine interaction between time and treatment (*i.e.* serine kinase inhibition, phosphatase inhibition) on cell signaling outcomes over time. Bonferroni post-tests were completed for every experiment to determine statistical differences between each treatment group compared with non-stimulated control. Statistical trend levels were set at $p < 0.1$, and statistical significance levels for all tests were set at $p < 0.05$.

Author Contributions—K. J. W. conceptualized the experiments presented in Figs. 2–4 and provided data acquisition in Figs. 1–4, statistical analysis throughout, and manuscript preparation. X. F. provided data acquisition and statistical analysis for Figs. 2 and 3. N. M. G. conceptualized the experiments and provided data acquisition in Figs. 1 and 2. J. J. T. provided data acquisition of Fig. 2. N. M. G. provided funding support, conceptualization of experiments throughout, figure design, and manuscript preparation.

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