Published in final edited form as:

Sci Signal. ; 9(433): ra63. doi:10.1126/scisignal.aad6724.

Insulin Receptor Substrate (IRS)-2 negatively regulates alternative macrophage activation and allergic lung inflammation

Preeta Dasgupta^{1,2}, Nicolas J. Dorsey³, Jiaqi Li¹, Xiulan Qi¹, Elizabeth P. Smith¹, Kazuyo Yamaji-Kegan⁵, and Achsah D. Keegan^{1,2,4,*}

¹Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, 800 W. Baltimore St., Baltimore, MD 21201, USA

²Department of Microbiology and Immunology, University of Maryland School of Medicine, 800 W. Baltimore St., Baltimore, MD 21201, USA

³Medical Scientist Training Program, University of Maryland School of Medicine, 800 W. Baltimore St., Baltimore, MD 21201, USA

⁴Research and Development Service, Department of Veterans Affairs, VA Maryland Health Care System, Baltimore, MD

⁵Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institutions, 720 Rutland Ave., Baltimore, MD

Abstract

Insulin Receptor Substrate (IRS)-2 is an adaptor protein that becomes tyrosine phosphorylated in response to IL-4 and IL-13 resulting in activation of the PI-3' kinase/Akt pathway. While the contribution of IL-4 and IL-13 to allergic lung inflammation has been studied extensively, the functional significance of the IRS2 pathway is unclear. To examine the role of IRS2 in allergic disease, we evaluated responses in IRS2-deficient mice. Deficiency of IRS2 resulted in a substantial increase in expression of a subset of genes associated with alternatively activated macrophages (AAM) in response to IL-4 or IL-13 in vitro. Moreover, IRS2^{+/-} and IRS2^{-/-} mice developed enhanced pulmonary inflammation, accumulation of eosinophils and AAM, and airway and vascular remodeling upon allergen stimulation in comparison to IRS2^{+/+} mice; this enhanced response was in part macrophage intrinsic. Loss of IRS2 led to greater phosphorylation of Akt and ribosomal S6 protein in the basal state and upon IL-4 stimulation. Thus, we identify a critical negative regulatory loop downstream of IRS2, demonstrating a previously unrecognized role for IRS2 in suppressing allergic lung inflammation and remodeling.

Keywords

Interleukin-4; Interleukin-13; Insulin Receptor Substrate (IRS)-2; alternatively activated macrophage (AAM); allergic lung inflammation; lung remodeling

* Address correspondence to: Achsah D. Keegan, Center for Vascular and Inflammatory Diseases, 800 W. Baltimore St., Baltimore, MD-21201.

Introduction

The prevalence of asthma and allergic diseases is rapidly increasing across all populations, posing a huge challenge to health care systems around the world. An estimated 300 million (M) people suffer from asthma worldwide, which is projected to reach 400 M by 2025. Interleukin-4 (IL-4) and Interleukin-13 (IL-13) are multifunctional cytokines that play a crucial role in asthma and allergic responses (1–7). While both IL-4 and IL-13 can elicit asthma pathology when provided exogenously, the two cytokines mediate distinct physiologic functions in vivo. IL-4 is required for proliferation and survival of lymphocytes and their differentiation into T_H2 cells (8). In addition, it induces antigen presentation in B cells and also causes antibody class switching from IgM to IgE (reviewed in (9)). IL-4 also preferentially enhances alternative activation of macrophages (10, 11). IL-13 on the other hand is considered to be the effector cytokine, responsible for airway hyperresponsiveness (AHR), excessive mucus production and airway remodeling (4, 5). Due to the important roles played by IL-4 and IL-13, these cytokines and components of their signaling pathways are targets for developing therapeutic strategies to treat allergy and asthma (12, 13). Therefore, it is essential to tease out the precise contributions of each of these pathways to allergic inflammation.

IL-4 and IL-13 trigger allergic responses by engaging two different heterodimeric receptor complexes. Upon ligand binding, either γ_c or IL-13R α 1 dimerizes with IL-4R α to form a functional Type I or Type II receptor respectively. Receptor engagement and heterodimerization activates tyrosine kinases called Janus kinases (Jak) (reviewed in (9)), which phosphorylate tyrosine residues on the cytoplasmic tail of the IL-4R α chain (9, 14–17). These phospho-tyrosine (pY) motifs are docking sites for various proteins and adaptor molecules that contain protein tyrosine binding (PTB) domains or Src homology 2 (SH2) domains such as STAT6, IRS2, SHIP, and SHP1/2.

Insulin Receptor Substrate (IRS)-2 is one of six large adaptor proteins that participate in insulin, IGF-1 and IL-4 receptor signaling (18–20). Since IRS2 plays an important role in insulin-induced responses and pancreatic β cell survival, complete loss of this adaptor protein leads to the eventual development of Type 2 diabetes in mice (21). Conditional deletion of IRS2 in β cells of the pancreas led to reduced β cell mass, obesity and hyperinsulemia (22). The IRS2 pathway also regulates reproductive biology (23). While the role of IRS2 in inducing diabetes has been studied extensively, very little is known about the contribution of IRS2 to asthma pathophysiology.

Previous reports demonstrated that IRS2 is not required for IL-4 induced proliferation of CD4⁺ T cells and secretion of T_H2 cytokines in vitro (24). IRS2 was also not required for the ability of IL-4 to regulate B-cell survival (24), suggesting that IRS2 is not important for B-cell functions. However, we found that serum IgE and IgG1 were elevated in IRS2 transgenic mice immunized with ovalbumin (25). The role of the IRS2 pathway in asthma is unclear. A previous study utilized mice expressing IL-4R α with a mutation (Y500F) in the insulin-IL-4 receptor (I4R) motif of the IL-4R α (26). This mutation prevented docking of PTB-domain containing proteins including IRS2 to the IL-4 receptor and suppressed IRS2 phosphorylation (18, 19). The Y500F mutation impaired T cell proliferation but did not

affect T_H2 cytokine secretion in vitro. Allergen sensitization and challenge in F500 mutant mice resulted in enhanced IgE production, AHR, eosinophilic inflammation and mucus production in vivo when compared to WT Y500 mice, suggesting that pathways dependent on Y500 of the IL-4R α were suppressive.

We previously observed that IL-4 induces greater phosphorylation of IRS2 in macrophages, when compared to IL-13 (10). Absence of the γ_c chain and the Type I receptor reduced the IL-4 induced phosphorylation of IRS2 as well as AAM gene expression, while IL-13-induced responses were unaffected. This suggested that the Type I receptor was more potent in activating IRS2 than the Type II receptor. We hypothesized that enhanced activation of pathways downstream of IRS2 such as PI-3' kinase and Akt, reported to be important for AAM differentiation (27), were responsible for the differential induction of AAM genes by IL-4 and IL-13.

To test this hypothesis, we examined whether IRS2 deficiency would lead to impaired expression of genes important for the AAM phenotype. Since AAMs secrete many proteins that have been shown to enhance allergic responses (28–32), we also evaluated the role of IRS2 in allergic lung inflammation using IRS2^{-/-} mice. Here we report that IRS2^{+/-} and IRS2^{-/-} mice developed enhanced allergic lung inflammation and increased airway and vascular remodeling in comparison to IRS2^{+/+} mice. Additionally, greater AAM gene expression was detected in response to IL-4 or IL-13 in macrophages lacking IRS2 in vitro. These results demonstrate a novel role for IRS2 in negative regulation of allergic lung disease. In addition, loss of IRS2 led to increased phosphorylation of Akt and S6 that likely proceeds via IRS1, revealing a negative feedback loop downstream of IRS2. This work advances our understanding of the regulation of allergic inflammation, paving the way for targeted manipulation of the IL-4/IL-13 pathway in the quest for better therapeutic interventions against asthma.

Results

Expression of genes characteristic of alternatively activated macrophages

We previously demonstrated that IL-4 induced greater phosphorylation of IRS2 and expression of genes associated with the alternatively activated macrophage phenotype in vitro, when compared to IL-13, suggesting that IRS2 may be required for AAM differentiation (10). To determine if enhanced activation of IRS2 by IL-4 was responsible for differences in AAM gene expression induced by IL-4 and IL-13, we analyzed expression of characteristic STAT6-dependent AAM markers in cells deficient in IRS2. Bone marrow derived macrophages from IRS2^{+/+} or IRS2^{-/-} mice were stimulated with a concentration of IL-4 or IL-13 (100 ng/ml) that induces equal phosphorylation of STAT6 (10) for 6 hours or 24 hours. Treatment with either IL-4 or IL-13 increased the amount of *Retnla* mRNA coding for Found in Inflammatory Zone (FIZZ)1 and *Chi3l3* mRNA coding for Ym1 (Fig. 1A) up to 24 hours, while *Arginase (Arg)-1* expression peaked at 6 hours and decreased at 24 hours. As seen earlier with WT macrophages (10), IL-4 was able to induce a more robust induction of *Retnla*, *Chi3l3* and *Arg1* transcripts in IRS2^{+/+} macrophages than IL-13 (Fig. 1A). Deficiency of IRS2, however, did not change this differential AAM gene expression. In fact, in contrast to our prediction, the absence of IRS2 in macrophages led to significantly

enhanced expression of *Retnla*, *Chi3l3* and *Arg1* mRNA by both IL-4 and IL-13 (Fig. 1B). These findings suggest that IRS2 negatively regulates AAM gene expression in vitro and that the differential phenotype induced by IL-4 versus IL-13 may not be due to differential IRS2 phosphorylation.

Analysis of IRS2 and STAT6 phosphorylation in IRS2 deficient mice

To determine whether the STAT6 pathway was amplified in the absence of IRS2, we assessed the extent of IRS2 and STAT6 phosphorylation in the IRS2 deficient mice (fig. S1). As expected, splenocytes and BMM isolated from IRS2^{-/-} mice completely lacked IRS2 protein and its phosphorylated form upon IL-4 stimulation. Interestingly, we found a gene-dosage effect, with the IRS2^{+/-} cells developing reduced IRS2 phosphorylation than the IRS2^{+/+} cells (fig. S1A). It was difficult to detect IRS2 protein in the heterozygous samples, likely because the IRS2 protein quantities were below the detection limit, while the detection of multiple phosphotyrosines on IRS2 was still possible. In contrast, splenocytes and BMM from all three groups of mice demonstrated similar amounts of STAT6 and equal IL-4-induced phosphorylation (fig. S1B). This is consistent with what is known in the literature: disruption of the IRS2 gene does not impair activation of the STAT6 pathway (24).

Role of IRS2 in allergic lung inflammation

Next we examined the contribution of the IRS2 pathway to allergic lung inflammation. IRS2^{+/+}, IRS2^{+/-} or IRS2^{-/-} mice were sensitized and challenged with Ovalbumin (OVA) as depicted in Fig. 2A. Deficiency of IRS2 led to a substantial increase in the total number of cells (T) in the BAL (Fig. 2B). A four-fold increase in cell numbers was observed, from 750,000 cells in OVA-primed and challenged IRS2^{+/+} mice to 2.7 million cells in IRS2^{-/-} mice. This increase in cell numbers was mainly due to an increase in recruitment of macrophages (M) and Eosinophils (E) to the airways. As seen earlier, mice heterozygous for IRS2 showed intermediate recruitment of inflammatory cells into the lungs. However, there was little difference in the percentages of macrophages, eosinophils, lymphocytes (L) and polymorphoneutrophils (P) in each mouse strain.

OVA-sensitization and challenge in IRS2^{+/+} mice led to mild pulmonary inflammation, characterized by the presence of pockets of cellular infiltrates around the airways and blood vessels (Fig. 2C, panels b–d). The increased numbers of BAL cells found in the IRS2^{+/-} and IRS2^{-/-} mice were reflected in the lung tissue as well. Pronounced peribronchial and perivascular cuffing was observed in the lungs of these mice (panels f–h, j–l). As with the BAL data, more eosinophils as well as mononuclear cells were present in the cellular infiltrates. The OVA-induced inflammation seen in IRS2^{+/-} mice was intermediate to that of the IRS2^{+/+} and IRS2^{-/-} mice. The inflammatory response in all groups of mice was allergen dependent, as the alum-primed mice did not show inflammation (panels a, e, and i). These results suggest that IRS2 acts to suppress allergic lung inflammation.

Effect of IRS2 on epithelial cell responses in vivo

However, the loss of IRS2 did not appear to affect the responses of lung epithelial cells. OVA-priming and challenge induced equivalent goblet cell differentiation and mucus production in all three mouse strains as determined by staining with Periodic Acid Schiff

base (fig. S2A). Furthermore, epithelial cells from all three genotypes were able to produce YM1 as well as FIZZ1 (fig. S2B). There was no noticeable difference in the amount of these proteins in epithelial cells in WT mice versus mice deficient in IRS2.

Analysis of IgE and cytokine production

Since IRS2 deficiency caused enhanced allergic inflammation, we examined the role of IRS2 in B- and T-cell responses in vivo (fig. S2, C and D). We found that OVA-priming and challenge induced similar quantities of IgE in all three IRS2 genotypes (fig. S2C). While there was some variability in the amounts of serum IgE detected in each animal, there was no significant difference between the groups. In addition, we found no significant differences in the amount of IL-4, IL-5, or IL-13 present in the BAL fluid in IRS2^{+/+} mice versus IRS2^{+/-} and IRS2^{-/-} mice (fig. S2D).

Role of IRS2 on alternatively activated macrophages in vivo

The AAM gene expression studies in BMM (Fig. 1) in vitro had demonstrated that absence of IRS2 led to increased production of *Chi3l3* and *Retnla* mRNA. Thus, we examined if IRS2 negatively regulates YM1 protein induction in the context of allergic lung inflammation in vivo. We observed greater intensity of YM1 staining in macrophages by IHC in mice lacking IRS2 (Fig. 3A, panel c; dark brown) than macrophages present in WT mice (Fig. 3A, panel a; lighter brown). The differences in YM1 staining intensity in macrophages was quantified using flow cytometry. BAL cells isolated from IRS2^{+/+}, IRS2^{+/-} and IRS2^{-/-} mice were stained with antibodies to CD11b and YM1. In the absence of IRS2, the proportion of CD11b⁺ YM1⁺ cells increased from ~70% to >84% (Fig. 3B, contour plots). More importantly, the MFI of YM1 staining at the single cell level also significantly increased: IRS2^{+/+} macrophages had the least MFI (1047), followed by IRS2^{+/-} macrophages (1388) and macrophages isolated from IRS2^{-/-} mice had the highest staining intensity (1570) (Fig. 3B, histograms and 3C). These results demonstrate that IRS2 negatively regulates AAM protein production in vivo during allergic lung inflammation.

Role of IRS2 in airway and vascular remodeling

It has been reported that both eosinophils and AAM products such as FIZZ1 and YM1 can promote airway and vascular remodeling (31, 33–36). Since IRS2 deficient mice developed enhanced eosinophilia and AAM differentiation, we assessed the extent of collagen deposition in the lungs of these mice. Masson's Trichrome staining of representative lung sections from OVA-sensitized and challenged IRS2^{+/+}, IRS2^{+/-} and IRS2^{-/-} mice revealed that loss of one or both copies of IRS2 led to progressively greater collagen deposition (shown in blue) around the airways (Fig. 4A, panels d–f). When the intensity of collagen staining was quantified using Image J software, it demonstrated that the increase in collagen deposition in IRS2^{-/-} mice was significant (Fig. 4B).

To examine the role of IRS2 in remodeling of small arterioles, we analyzed the intensity of smooth muscle cell actin (SMA) in small peripheral vessels (Fig. 4C) (37). The majority of small (< 80 µm) pulmonary vessels in IRS2^{+/+} mice were non-muscularized (NM) (Fig. 4, C and D). However, mice deficient in IRS2 exhibited a significant decrease in the fraction of NM vessels, with a concomitant increase in the percentage of small vessels with partially-

(PM) or fully-muscularized (FM) phenotypes (Fig. 4, C and D). In addition, $IRS2^{+/-}$ mice showed a gene dosage effect; they developed intermediate levels of pulmonary vascular remodeling. These data suggest that $IRS2$ negatively regulates antigen-induced pulmonary arterial muscularization in vivo.

As shown above, BMM from $IRS2^{-/-}$ mice had significantly higher *Retnla* gene expression in response to IL-4 or IL-13 than did BMM from $IRS2^{+/+}$ mice (Fig. 1). Since FIZZ1 protein can modulate muscularization of pulmonary vessels, we examined the production of FIZZ1 in the lungs by immunofluorescence. There was very little FIZZ1 around the small pulmonary vessels in response to OVA exposure in $IRS2^{+/+}$ mice (Fig. 4E). Similarly, there was little to no FIZZ1 in cells around airways (fig. S2B). However, $IRS2^{+/-}$ and $IRS2^{-/-}$ mice demonstrated striking increases in FIZZ1 protein surrounding the remodeled pulmonary vessels. These data support the previous observation by Daley *et al.* that severely muscularized pulmonary vessels become surrounded by FIZZ1-producing cells in response to antigen exposure (38). Furthermore, we did not observe co-localization of FIZZ1-positive cells and SMA-positive cells (Fig. 4E), indicating that FIZZ1 produced by inflammatory cells lacking $IRS2$ might have a paracrine effect on the muscularization of small pulmonary vessels.

Adoptive transfer of $IRS2^{-/-}$ macrophages

To determine whether the enhanced allergen-induced responses observed in the $IRS2^{-/-}$ mice were due to effects in macrophages, we adoptively transferred BMM isolated from $IRS2^{-/-}$ mice or $IRS2^{+/+}$ mice into WT or $IRS2$ -heterozygote recipient mice one day prior to the OVA/alum prime/challenge protocol (Fig. 5A). Adoptive transfer of $IRS2^{-/-}$ BMM to either WT or $IRS2$ -heterozygote recipient mice resulted in an increase in the number of total eosinophils and in the percent eosinophils in the BAL (Fig. 5B). However, the greatest number of eosinophils was present in the primed and challenged $IRS2^{-/-}$ mice that did not receive exogenous macrophages. Moreover, a similar increase in inflammatory infiltrate in the lungs was observed when mice received $IRS2^{-/-}$ BMM compared to $IRS2^{+/+}$ BMM (Fig. 5C). Increased YM1 staining intensity of lung macrophages was also observed. (Fig. 5C, inset). Furthermore, the adoptive transfer of $IRS2^{-/-}$ BMM resulted in increased thickening of the arterial wall of the small pulmonary arteries after allergen priming and challenge (Fig. 5D). Transfer of $IRS2^{-/-}$ BMM lead to a 3-fold increase in the average smooth muscle cell thickness of small pulmonary arteries in WT recipients and a smaller, but significant increase in the heterozygous recipients compared to transfer of $IRS2^{+/+}$ BMM. The smooth muscle cell thickness in the groups receiving $IRS2^{-/-}$ BMM approached the thickness observed in $IRS2^{-/-}$ mice that did not receive exogenous macrophages. However, the adoptive transfer of $IRS2^{-/-}$ BMM did not result in an increase in FIZZ1 staining around small pulmonary vessels above the staining intensity characteristic of each recipient genotype, $IRS2^{+/+}$ (-); $IRS2^{+/-}$ (+); $IRS2^{-/-}$ (++) (fig. S3). These results show that the enhanced inflammation and remodeling observed in allergen challenged $IRS2^{-/-}$ mice is in part intrinsic to the effect of $IRS2$ -deficiency on macrophages.

Effect of IRS2 deficiency on downstream signaling

IRS2 is a key adaptor protein that links the IL-4 receptor to the PI-3' kinase-Akt pathway. Previous studies of IRS2-deficient mice demonstrated that >90% of PI 3' kinase activity induced by IL-4 in splenocytes was eliminated in the absence of IRS2 with a small amount of activity remaining associated with IRS1 (24). Furthermore, stimulation of the AAM phenotype by IL-4 was shown to be PI 3' kinase dependent (27, 39). Because our observations of enhanced AAM responses in the absence of IRS2 represent a paradox, we directly examined how the absence of IRS2 affected activation of the PI-3' kinase/Akt pathway in BMM (Fig. 6). In IRS2^{+/+} cells, IL-4 stimulation led to an increase in the co-precipitation of phosphorylated IRS2 with p85, the regulatory subunit of PI 3' kinase (Fig. 6A). However, loss of one copy of IRS2 was sufficient to reduce this co-precipitation, and in IRS2^{-/-} mice the IL-4 induced co-precipitation with p85 was significantly abrogated (Fig. 6A). We observed a 170 kDa phosphorylated band in anti-p85 immunoprecipitates under basal and IL-4-stimulated conditions; however, we did not observe any other phosphorylated proteins whose co-precipitation with p85 was changed by IL-4. We next examined the pathways further downstream. IL-4 treatment increased the phosphorylation of Akt (Ser⁴⁷³ and Thr³⁰⁸) and ribosomal S6 protein over baseline in IRS2^{+/+} BMM (Fig. 6B). As expected, the PI 3' kinase inhibitor, LY294002, significantly suppressed the basal and IL-4-induced phosphorylation of Akt and S6 (Fig. 6 and fig. S4). Interestingly, both the basal and IL-4-induced phosphorylation of Akt and S6 were significantly greater in IRS2^{+/-} and IRS2^{-/-} macrophages. This increase in Akt activation is consistent with the increased biologic responses in vivo. LY294002 also suppressed the basal and IL-4-induced phosphorylation of Akt and S6 in the IRS2-deficient macrophages (fig. S4). In all 3 IRS2 genotypes, LY294002 inhibited IL-4-induced increase in *Retnla*, but only modestly inhibited *Chi3l3* (Figure 6C), similar to our previous report on the sensitivity of these genes to inhibitors of PI-3' kinase (10). These results suggest that there is an alternate mechanism to activate PI-3' kinase in the absence of IRS2, possibly through low amounts of IRS1 (40), and that there is a negative feed-back loop downstream of IRS2 that becomes dysregulated in the absence of IRS2.

Knock-down of IRS1 in BMM

To determine whether IRS1 could indeed act as an adaptor downstream of IL-4 signaling, we transduced BMM isolated from all 3 IRS2 genotypes with lentiviral vectors containing a shRNA targeting mouse IRS1 (KD) or a non-targeting (NT) shRNA control and evaluated IL-4-induced signaling and gene upregulation (Fig. 7). Transduction of BMM with the IRS1 KD shRNA resulted in a 50–70% decrease in *IRS1* message compared to the NT transduced BMM (Fig. 7A). Analysis of anti-IRS1 immunoprecipitates of the NT transduced BMM revealed that IL-4 stimulated the tyrosine phosphorylation of IRS1 in BMM prepared from all 3 IRS2 genotypes, and that basal tyrosine phosphorylation of IRS1 was enhanced in IRS2^{-/-} BMM (Fig. 7B and fig. S5A). In the KD-transduced BMM the magnitude of tyrosine phosphorylation of IRS1 and the abundance of total IRS1 were greatly reduced as expected. We also found that IRS1 KD resulted in a decrease in the basal and IL-4-induced phosphorylation of Akt and S6 in all 3 genotypes (Fig. 7B and fig. S5A), although the changes did not rise to the level of statistical significance. We further found that IRS1-KD substantially inhibited the IL-4-induced increase in *Retnla* in all 3 IRS2 genotypes with the

greatest effect in the *IRS2*^{-/-} BMM, but only modestly inhibited *Chi3l3* (Fig. 7D). These results suggest that the IRS1 adaptor plays an important role in IL-4-induced signaling and in modulating expression of certain IL-4-induced genes in BMM.

Discussion

In addition to activating the STAT6 signaling pathway, IL-4, and to a lesser extent IL-13, also induce the tyrosine phosphorylation of IRS2. Phosphorylated IRS2 acts as an adapter protein recruiting other signaling proteins such as the p85 subunit of PI 3' kinase and Grb-2. The pathways activated by IRS2 lead to phosphorylation of PI 3' kinase and Akt (41). Although the role of STAT6 in inducing AAM differentiation and allergic lung inflammation has been studied extensively, much less is known about the contribution of IRS2.

Previously, we had found that IL-4 elicited robust phosphorylation of IRS2 and AAM gene expression in macrophages in vitro, while IL-13 induced significantly weaker responses (10). Moreover, IL-4-mediated signaling and gene induction was reduced in macrophages lacking the γ_c chain and the Type I receptor. Thus in this study, we examined whether absence of IRS2 would suppress IL-4-mediated AAM activation. Contrary to our expectations, we found that stimulation of *IRS2*^{-/-} macrophages with IL-4 or IL-13 enhanced expression of *Retnla*, *Chi3l3* and *Arg1* mRNA, when compared to WT macrophages. These findings indicate that IRS2 negatively regulates AAM gene expression.

Moreover, the differential effect of IL-4 versus IL-13 in inducing AAM gene expression was still observed in *IRS2*-deficient cells. Thus, the reduced quantities of AAM transcripts seen in IL-13 stimulated macrophages were not occurring due to reduced IRS2 phosphorylation. It is known that the cytoplasmic tail of IL-13R α 1 contains two binding sites for STAT3 (42). Activation of STAT3 occurs only through Type II R signaling (43). Therefore, it is possible that IL-13 mediated activation of STAT3 is dampening STAT6 responses induced by this cytokine. Alternatively, enhanced recruitment of the protein tyrosine phosphatase, SHP-1, by the Type II receptor may be responsible for the difference in AAM gene expression induced by IL-4 and IL-13. Mutation of the ITIM motif in the IL-4R α chain (Y709F) resulted in increased sensitivity of macrophages to IL-13 mediated AAM activation (44). BMMs expressing IL-4R α Y709 treated with IL-4 led to significantly higher expression of AAM genes (*Arginase1*, *Chi3l3*) and also *Ccl11* in contrast to IL-13. BMM expressing the Y709F mutation, however, demonstrated enhanced STAT6 phosphorylation and led to a dramatic amplification of *Arg1*, *Chi3l3* and *Ccl11* genes in response to IL-13, while leaving the IL-4 induced responses intact or slightly enhanced (44). The authors proposed that differential recruitment of SHP-1 by the Type I and Type II receptors may be the reason for the disproportionate increase in AAM gene expression induced by IL-13.

The reason for enhanced induction of AAM genes in the absence of IRS2 is unclear; proximal STAT6 activation was normal in *IRS2*^{+/-} and *IRS2*^{-/-} mice. We and others have shown that STAT6 signaling is essential for induction of AAM genes (45). The promoter regions of *Retnla*, *Chi3l3* and *Arg1* all have critical STAT6 binding sites in close proximity to C/EBP binding sites (46–48). Moreover, STAT6 and C/EBP cooperate to induce maximal *Retnla* and *Arg1* gene expression (47, 48). It has been reported that insulin signaling through

the IRS1/2-PI-3K-Akt pathway leads to suppression of C/EBP β -mediated transactivation of genes (49). Insulin-induced activation of Akt disrupted interactions between the C/EBP β transactivation domain and certain coactivator proteins (such as p300/CBP), abrogating C/EBP-mediated activation of genes. Since IL-4 and to some extent IL-13 also activate the IRS2-PI-3K-Akt pathway, it is possible that IL-4/IL-13 suppresses C/EBP as well.

Another possible explanation for enhanced gene expression is the loss of negative feedback mechanisms. Weisser *et al.* have demonstrated that deficiency of SH2-domain-containing inositol-5'-phosphatase (SHIP) results in increased IL-4-induced alternatively activated macrophage differentiation (27). Conversely, macrophages stimulated with IL-4 produced less SHIP and increased production of YM1 and Arg1. Furthermore, loss of SHIP and induction of the AAM phenotype was dependent on PI-3' kinase activity. Here we have shown that in absence of IRS2, the IL-4-stimulated co-precipitation of IRS2 with p85 was abrogated. In spite of this loss, both the basal and IL-4-induced phosphorylation of Akt and S6 were greater in IRS2^{+/-} and IRS2^{-/-} macrophages and were reduced by the PI-3' kinase inhibitor, LY294002. These results suggest that there is an alternate mechanism to activate the PI-3' kinase-Akt axis in response to IL-4 or IL-13 in the absence of IRS2. Our studies have identified IRS1 as an alternate adaptor utilized by IL-4 for signaling via the PI-3' kinase/Akt pathway and modulate gene induction in BMM.

Another possible negative feedback mechanism could be mediated through the serine phosphorylation of IRS1 or even IRS2 itself. A recent study defined a role for mTOR Complex 1 (mTORC1) in attenuating IL-4 induced AAM polarization (50). Macrophage specific deletion of Tsc1 resulted in constitutive activation of mTORC1, which led to reduced IL-4-induced Akt phosphorylation and *Arg1* and *Retnla* gene expression. It has been proposed that serine phosphorylation of IRS1 or IRS2 by mTORC1 or other kinases negatively regulates their ability to link to the PI 3' kinase/Akt pathway (51). In this scenario, IL-4-induced activation of Akt and mTORC1 in macrophages could then act as a negative regulator of the PI-3' kinase-Akt signaling pathway by the serine phosphorylation of IRS1 or IRS2. However, in the absence of IRS2, this major negative feedback loop would be absent (IRS2^{-/-}) or impaired (IRS2^{+/-}), perhaps explaining the enhanced AAM phenotype and allergic lung inflammation responses. A number of recent studies evaluating the role of mTORC1 in AAM differentiation in mice and humans have reported conflicting results (reviewed in(52)), suggesting that mTORC1 likely regulates both positive and negative signals downstream of IL-4 in a complex fashion. Regardless of the mechanism, our findings of enhanced basal and IL-4-induced activation of the Akt pathway in macrophages from IRS2-deficient mice support a regulatory role for this adaptor protein. Consistent with our findings, enhanced basal activity of PI 3' kinase in liver and skeletal muscle of IRS2-deficient mice was noted previously, but not analyzed further (21).

Evaluation of the role of IRS2 in allergic lung inflammation in vivo also indicated that this signaling protein suppresses pulmonary inflammation, eosinophilia and induction of YM1 in macrophages. Allergen sensitization and challenge in IRS2^{+/-} and IRS2^{-/-} mice led to a significant increase in the numbers of eosinophils and macrophages present in the BAL compared to IRS2^{+/+} mice. Examination of H&E stained lung sections from IRS2^{+/-} and IRS2^{-/-} also showed increased inflammation with mononuclear cells and eosinophils around

the airways and blood vessels. Adoptive transfer of macrophages revealed that this increase in inflammation is in part due to the effect of IRS2-deficiency on the macrophage itself. The presence of increased inflammation in the IRS2^{+/-} mice, which show no signs of peripheral insulin resistance, indicates that our findings are not simply due to global metabolic changes in the mice.

No significant differences in IL-4, IL-5 and IL-13, however, were detected in the BAL fluid between IRS2^{+/+}, IRS2^{+/-} and IRS2^{-/-} mice. A slight reduction in IL-5 was seen in IRS2^{-/-} mice, which may have been due to increased consumption of this cytokine by the elevated numbers of eosinophils. In vitro studies by Wurster and colleagues indicated that IRS2 was not required for T_H2 differentiation but modestly influenced the quantity of IL-4 and IL-5 produced (24). Our results show that in vivo T_H2 cytokine production and allergic inflammation can occur independently of IRS2. We previously published that transgenic overexpression of IRS2 led to enhanced IgE production by B cells upon OVA stimulation (25). However, our present results demonstrate that IRS2 is not required for IgE production.

The enhanced allergic lung inflammation phenotype we observed in mice deficient in IRS2 is consistent with the findings reported by Blaeser *et al.* They showed that mutation of a critical tyrosine residue in the I4R region to phenylalanine (Y500F) led to abrogated IRS2 phosphorylation and Akt signaling (26). The F500 mice also developed severe inflammation, eosinophilia, elevated serum IgE, and increased goblet cell metaplasia and AHR in comparison to the Y500 mice. Both groups of mice produced similar amounts of T_H2 cytokines. Unlike the Y500F mutation, IRS2 deficiency did not affect serum IgE or epithelial cell responses such as goblet cell metaplasia. This difference in responses may be because the mutant receptor disrupted other signaling pathways in addition to the IRS2 pathway. Furthermore, the IL-4R α is present in all cell types, whereas the activation pattern and functional role of IRS2 is limited to certain cell types.

It has been reported that SHIP negatively regulates T_H2 differentiation and allergic lung inflammation (53). SHIP^{-/-} mice spontaneously develop pulmonary inflammation, with recruitment of macrophages and eosinophils. Loss of SHIP1 also led to increased abundance of chitinase gene expression and enhanced lung remodeling. Since the phenotype observed in IRS2^{-/-} mice in this study is similar to SHIP1 deficient animals, it is conceivable that loss of IRS2 results in decreased negative regulation of SHIP1 in vivo.

Similar to the in vitro AAM gene expression data, we found that YM1 protein intensity in macrophages was increased in mice lacking either one or both alleles of IRS-2 in vivo. Several reports suggest a link between elevated quantities of AAM products seen in mice deficient in IRS2 and the severity of pulmonary inflammation although no single product has been accepted as the critical agent. YM1 can act as a chemoattractant and recruit eosinophils into the lung (30), and it may also be involved in tissue remodeling and fibrosis (31). In addition to YM1, AAM can secrete many other factors such as IGF-1, AMCase, BRP-39 and various chemokines. IL-13 mediated induction of AMCase in macrophages and epithelial cells caused T_H2 inflammation and AHR, which was abrogated when AMCase was neutralized (54). Alveolar macrophages were reported to synthesize large quantities of BRP-39, a chitinase-like protein, during allergic lung inflammation and antigen-specific T_H2

responses, tissue inflammation and fibrosis were significantly reduced in mice deficient in BRP-39 (32).

Daley and colleagues demonstrated that severe pulmonary arterial muscularization in mice in response to prolonged exposure to *Aspergillus* or OVA antigen is under the control of the immune system (38). In that study, the authors showed that the triggering of a T_H2-skewed immune response results in pulmonary arterial muscularization. Notably, their study also provided evidence that antigen-induced pulmonary vascular remodeling is significantly suppressed in IL-4^{-/-} mice, suggesting that an IL-4-dependent T_H2 response induces severe pulmonary vascular remodeling. In addition, IL-4 was necessary for the development of severely remodeled pulmonary arteries and for the gathering of FIZZ1-positive cells around them (38).

In the absence of IRS2, we observed a striking increase in muscularization of small vessels in an acute allergen sensitization/challenge model; this muscularization was accompanied by increased production of FIZZ1 by cells surrounding the blood vessels. These results suggest a novel, critical role for IRS2 in controlling pulmonary arterial remodeling induced by a T_H2 immune response. These findings are consistent with a report demonstrating that IRS2 deficient mice develop increased neointima formation in a murine vessel injury-induced atherosclerosis model (55). Injury of the femoral artery induced significantly increased intima thickness in IRS2^{-/-} mice when compared to IRS2^{+/+} mice. In another study, Mita *et al.* demonstrated that transfer of IRS2-deficient bone marrow into WT mice led to enhanced accumulation of IRS2^{-/-} macrophages in the vascular wall together with increased expression of proinflammatory cytokines such as MCP-1 (56). Using an adoptive transfer approach, we determined that the negative regulation of pulmonary arterial muscularization by IRS2 was at least in part intrinsic to the macrophage. However, additional effects of IRS2 in vascular endothelial cells and/or smooth muscle cells may also participate in complex cross-talk between macrophages and structural cells in vivo, since we did not observe a further increase in FIZZ1 staining of perivascular macrophages when IRS2^{-/-} BMM were transferred to IRS2^{+/+} or IRS2^{+/-} recipient mice. While our in vitro signaling studies demonstrated a link between control of PI-3' kinase/Akt pathway and *Retnla* by IRS2 in MCSF-dependent macrophages, these in vivo results strongly suggest that control of signaling pathways and protein production during inflammatory responses is more complex.

Materials and Methods

Mice

B6;129-*IRS2^{tm1Mfw}*/J mice were cryo-recovered at Jackson labs (Bar Harbor, ME) under contract. Two pairs of male and female IRS2^{+/-} mice were shipped to University of Maryland, Baltimore and bred in house. C57BL/6 NTac mice were purchased from Taconic (Germantown, NY). The IRS2^{+/-} mice were backcrossed to the C57BL/6 NTac background for 10 generations, before being used to establish breeding colonies. Since IRS2^{-/-} female mice are infertile, this line was maintained by breeding IRS2^{+/-} littermates. IRS2^{+/+}, IRS2^{+/-} and IRS2^{-/-} mice in each litter were identified by genotyping, using primers and protocols recommended by Jackson Labs. In addition to genotyping IRS2 mice, blood glucose was measured at the time of weaning pups (3 weeks) and monitored every few days

until used in experiments. As previously described (21) IRS2^{+/+} and IRS2^{+/-} mice utilize glucose normally and maintain normal blood glucose throughout their lives. The IRS2^{-/-} mice show mild insulin resistance, develop increasing glucose quantities over time (10–12 weeks of age), and eventually develop diabetes (21). Therefore, in all our experiments, we used mice less than 8 weeks of age with blood glucose less than 250 mg/dL (non-diabetic). Blood glucose quantities measured in the 3 genotypes (IRS2^{+/+}, IRS2^{+/-}, and IRS2^{-/-}) were not significantly different from one another at this age. All experimental procedures mentioned here were performed in accordance to the guidelines issued by the Institutional Animal Care and Use Committee at UMB.

Cell Culture

Singe cell suspensions were prepared from spleens by mechanical disruption. For generation of bone marrow macrophages (BMM), bone marrow was harvested from femurs and tibias of mice. Adherent stromal cells were depleted by culturing the bone marrow overnight in complete α -MEM medium. Non-adherent cells were collected and subjected to red blood cell lysis. The remaining mononuclear cells were plated and cultured in complete α -MEM medium supplemented with rmM-CSF (20 ng/ml; R&D Systems). This media was replenished every other day for 4–6 days. Following this, cells were stimulated in the presence or absence of the indicated amount of IL-4 or IL-13 (R&D Systems Minneapolis, MN) for various time points in the presence or absence of LY294002 (1 μ M).

IRS1 knock-down

To knock down IRS1 expression in BMM, shRNA-IRS1-PLKO.1-puro and control non-targeting (NT) lentiviral vectors were obtained (Sigma-Aldrich Mission shRNA SHCLNG-NM_010570 and SHC002V, respectively) and used to transfect HEK293T cells along with PsPax2 and PMD.2G (developed by Dr. Didier Trono, EIA, provided by Dr. Ricardo Feldman from University of Maryland) using Fugene6 reagent (Roche) as described previously (57). After 24 hours, transfection medium was replaced with fresh medium containing sodium pyruvate (1 mM) and HEPES (25 mM). Viral supernatant was collected at 48, 72, and 96 hours after infection, filtered through a 0.45 μ m filter, and added the same day to primary BMM cultures in the presence of polybrene (20 ng/ml) at a ratio of 1:3 viral supernatant to medium. One day after the third addition of viral supernatant, the cells were rested in complete medium with MCSF for one day before selection with puromycin (1 μ g/ml) for one day. Subsequently, the medium was replaced and the cells treated with IL-4 for various times. Total mRNA and cell lysates were prepared for RNA and protein analysis respectively.

Immunoprecipitation and western blot analysis

Total cell lysates were immunoprecipitated with antibodies against IRS2, IRS1, p85 (Upstate/Millipore) or STAT6 (M-20, Santa Cruz) and Protein G-agarose beads (Life Technologies). Samples were run on a 7.5% polyacrylamide gel and transferred onto an Immobilon membrane (Millipore, Billerica, MA). The blots were blocked with BSA (Sigma) and incubated with antibodies against phosphotyrosine (PY-20, BD Biosciences) and pSTAT6 (STAT6-pTyr⁶⁴¹, Cell Signaling Technologies, Danvers, MA) as well as total IRS2, IRS1, or p85 (Upstate/Millipore) or STAT6 (M-20, Santa Cruz). Total cell lysates

were evaluated by western blotting using antibodies to pAKT-Ser⁴⁷³, pAkt-Thr³⁰⁸, Akt, phosphorylated ribosomal S6 protein (pS6^{235,236}) or S6 (Cell Signaling Technologies, Danvers, MA). Anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibody was used (GE Healthcare). Protein bands were detected using a chemiluminescence reagent (ECL; Denville Scientific Inc., Metuchen, NJ).

Densitometric Analysis

Shorter exposures of films were chosen for densitometric analysis to ensure the intensities of bands were in the linear range of film. The films were scanned on a flatbed scanner and the integrated density of the band was calculated using NIH Image J Software. The relative abundance of phosphorylation was calculated as a ratio of the density of the phosphorylated band divided by the density of the total protein band. To compare results from a minimum of 3 independent experiments, the ratio of phosphoprotein/total protein in response to a certain stimulus (such as IL-4) was set to 100% (maximal) and the ratio in response to other stimuli was compared to that maximal value.

Quantitative real-time PCR

Real-Time PCR was performed as described in (10). Briefly, total RNA was isolated from BMM cultures using the RNeasy kit (Qiagen, Valencia, CA) and DNA contamination was removed by DNase treatment. Complementary DNA (cDNA) was generated using the SuperScriptTM III First Strand Synthesis System (Life Technologies). Real-Time PCR was performed with specific primer sets (Life Technologies) by methods previously described (58) on an Applied Biosystems Inc. 7900HT machine. The relative abundance of mRNA for specific genes was calculated as fold induction over background levels detected in control samples, with hypoxanthine guanine phosphoribosyl transferase (*HPRT*) as the internal reference gene (2^{-C_t} method (59)). To compare results from a minimum of 3 independent experiments, the fold induction of mRNA in response to a certain stimulus (such as IL-4) was set to 100% (maximal) and the fold induction in response to other stimuli was compared to that maximal value.

Allergic lung inflammation protocol

The ovalbumin (OVA)/alum model of allergic lung inflammation was used. Mice were immunized twice with either 100 µg of OVA/alum or alum alone 5 days apart. One week later, on day 12 and 14, mice were challenged with aerosolized 1% OVA in PBS for 40 mins. Bronchial lavage was performed 48 hours after the last challenge by cannulating the trachea and flushing the airways with 1 ml of PBS. The samples were centrifuged; the cellular component was used for cytospin preparation and differential counts or FACS, while the supernatant was used for cytokine analysis.

Adoptive Cell Transfer

The adoptive transfer of macrophages was performed as described previously (60). MCSF-dependent bone-marrow derived macrophages (BMM) were prepared from 4-week-old IRS2^{+/+} or IRS2^{-/-} mice as described above. After 6 days in culture, the adherent cells were isolated using a cell scraper and collected by centrifugation. These cells were routinely

>95% F4/80⁺/CD11b⁺ macrophages. BMM were washed and transferred to IRS2^{+/+} or IRS2^{+/-} recipient mice by intraperitoneal (IP) injection at 5×10⁶ cells per mouse. Priming with OVA was initiated the next day.

Lung histology and immunohistochemistry

Lung histology sections were prepared as described previously (45). Briefly, mouse heart and lungs were perfused with 10–15 ml of PBS and immediately fixed with 10% formalin. The tissues were embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E) or Periodic acid Schiff (PAS). For immunohistochemistry, lung sections were stained with a 1:100 dilution of rabbit anti-mouse FIZZ1 (Abcam, Cambridge, MA) or 1:100 dilution of rabbit anti-mouse YM1 (Stem Cell Technologies, Vancouver, Canada), followed by biotinylated anti-rabbit antibodies (1:200; Vector Laboratories Inc.). Slides were then incubated with ABC Elite reagent (Vector Laboratories) and developed in 3,3'-diaminobenzidine chromogen and counterstained with Mayer's Hematoxylin.

Assessment of Airway and Pulmonary vascular remodeling

Lung sections were stained with Masson's Trichrome to detect collagen deposition. The collagen content around the airways was quantified using NIH Image J software (National Institutes of Health, Bethesda, MD) (45). Pulmonary vascular remodeling of the mice was assessed as previously published (37). Briefly, lung sections were dual labeled with antibodies to von Willebrand factor (vWF; Dako, Glostrup, Denmark) and α -smooth muscle actin (SMA; Dako) to stain endothelium and vascular smooth muscle, respectively; then they were counterstained with hematoxylin (37). To assess remodeling of the lung arteries and arterioles, an investigator blinded to treatment group examined 100 arteries per lung section at random under 40× objectives using an Olympus-BHS microscope (Olympus, Tokyo, Japan) attached to a QImaging Retiga 4000RV digital camera (QImaging, British Columbia, Canada). Small arteries with an internal diameter $\leq 80 \mu\text{m}$ were then classified as non-muscular (NM), partially muscular (PM), or fully muscular (FM), according to SMA staining. Negative control sections for the immunohistochemical experiments received identical treatments but were not exposed to the primary antibody; they showed no specific staining. In some cases, the SMC thickness of multiple individual small pulmonary arteries from multiple lungs per group was measured as described (45).

Immunofluorescence and confocal microscopy

Immunofluorescence staining was carried out as described previously (37). Briefly, the paraffin sections were blocked with appropriate blocking serum (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature and then treated with anti-FIZZ1 (Abcam, Cambridge, UK) and anti-SMA (Dako) antibodies. Then the sections were incubated with the appropriate fluorochrome-coupled secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Finally, the sections were washed in PBS, mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen, Grand Island, NY), and covered and sealed with a glass coverslip. Negative control sections for the immunohistochemical experiments received identical treatments but were not exposed to the primary antibody; they showed no specific staining. Staining was imaged with a Zeiss 510 Meta confocal microscope (Carl

Zeiss Microscopy, Thornwood, NY) at the Johns Hopkins School of Medicine Microscope Facility.

Cytokine and IgE analysis

Cytokines in the BAL fluid were analyzed by using ELISA kits for IL-4 (BioLegend, San Diego, CA), IL-5 and IL-13 (all from R&D Systems, Minneapolis, MN). Concentration of IgE in the serum was measured using the BD OptEIA kit (BD Biosciences).

Flow cytometry

Single cell suspensions of BAL cells were incubated with Fc Block (2.4G2, BD Biosciences) followed by staining with fluorochrome-conjugated antibodies to surface markers (CD11b, F4/80 and Siglec F, BD Biosciences). After washing twice, cells were fixed with 4% paraformaldehyde and permeabilized using a BD Cytotfix/Cytoperm kit (BD Biosciences). Intracellular cytokine staining for YM1 was performed. Cells were treated with an unconjugated anti-YM1 antibody (Stem Cell Signalling) followed by a fluorochrome conjugated secondary antibody. Samples were acquired using a FACS Calibur machine (Becton Dickinson, Franklin Lakes, NJ) and data was analyzed using FlowJo software (Treestar, CostaMesa, CA). Macrophages were gated by forward and side scatter and then on CD11b+, F4/80+, SiglecF-cells.

Statistical Analysis

Student two-tailed *t* test was used to compare the differences between two groups and to calculate the significance values following an initial F-test query. *p* values of 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by PHS grants AI038985 and HL110111 and VA-MERIT I01 BX001850 to ADK, PHA/ATS/Pfizer Research Fellowship in Pulmonary Arterial Hypertension to KY-K, and PHS grants T32HL06798 and T32AI007540, and UNCF/Merck Science Initiative to NJD. We acknowledge Dr. William E. Paul (NIAID, NIH) for his insightful advice and longstanding support over the course of this work and Dr. Nicola M. Heller (Johns Hopkins University Medical School) for helpful discussions and suggestions for the preparation of this manuscript.

References

1. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med*. 1998; 187:939–948. [PubMed: 9500796]
2. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, Locksley RM. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol Med*. 1998; 4:344–355. [PubMed: 9642684]
3. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med*. 1997; 186:1737–1747. [PubMed: 9362533]

4. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science*. 1998; 282:2261–2263. [PubMed: 9856950]
5. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science*. 1998; 282:2258–2261. [PubMed: 9856949]
6. Mathew A, MacLean JA, DeHaan E, Tager AM, Green FH, Luster AD. Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation. *J Exp Med*. 2001; 193:1087–1096. [PubMed: 11342593]
7. Kelly-Welch AE, Melo ME, Smith E, Ford AQ, Haudenschild C, Noben-Trauth N, Keegan AD. Complex role of the IL-4 receptor alpha in a murine model of airway inflammation: expression of the IL-4 receptor alpha on nonlymphoid cells of bone marrow origin contributes to severity of inflammation. *J Immunol*. 2004; 172:4545–4555. [PubMed: 15034072]
8. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 1996; 4:313–319. [PubMed: 8624821]
9. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol*. 1999; 17:701–738. [PubMed: 10358772]
10. Heller NM, Qi X, Junttila IS, Shirey KA, Vogel SN, Paul WE, Keegan AD. Type I IL-4Rs selectively activate IRS-2 to induce target gene expression in macrophages. *Sci Signal*. 2008; 1:ra17. [PubMed: 19109239]
11. Dasgupta P, Qi X, Smith EP, Keegan AD. Absence of the common gamma chain (gamma(c)), a critical component of the Type I IL-4 receptor, increases the severity of allergic lung inflammation. *PLoS One*. 2013; 8:e71344. [PubMed: 23940740]
12. Paul WE. History of interleukin-4. *Cytokine*. 2015; 75:3–7. [PubMed: 25814340]
13. May RD, Fung M. Strategies targeting the IL-4/IL-13 axes in disease. *Cytokine*. 2015
14. Kelly-Welch AE, Hanson EM, Boothby MR, Keegan AD. Interleukin-4 and interleukin-13 signaling connections maps. *Science*. 2003; 300:1527–1528. [PubMed: 12791978]
15. Pernis AB, Rothman PB. JAK-STAT signaling in asthma. *J Clin Invest*. 2002; 109:1279–1283. [PubMed: 12021241]
16. Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD. Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. *J Immunol*. 1998; 160:3502–3512. [PubMed: 9531312]
17. Jiang H, Harris MB, Rothman P. IL-4/IL-13 signaling beyond JAK/STAT. *J Allergy Clin Immunol*. 2000; 105:1063–1070. [PubMed: 10856136]
18. Keegan AD, Nelms K, White M, Wang LM, Pierce JH, Paul WE. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. *Cell*. 1994; 76:811–820. [PubMed: 8124718]
19. Wang HY, Zamorano J, Keegan AD. A role for the insulin-interleukin (IL)-4 receptor motif of the IL-4 receptor alpha-chain in regulating activation of the insulin receptor substrate 2 and signal transducer and activator of transcription 6 pathways. Analysis by mutagenesis. *J Biol Chem*. 1998; 273:9898–9905. [PubMed: 9545332]
20. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG Jr, Glasheen E, Lane WS, Pierce JH, White MF. Role of IRS-2 in insulin and cytokine signalling. *Nature*. 1995; 377:173–177. [PubMed: 7675087]
21. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*. 1998; 391:900–904. [PubMed: 9495343]
22. Choudhury AI, Heffron H, Smith MA, Al-Qassab H, Xu AW, Selman C, Simmgen M, Clements M, Claret M, Maccoll G, Bedford DC, Hisadome K, Diakonov I, Moosajee V, Bell JD, Speakman JR, Batterham RL, Barsh GS, Ashford ML, Withers DJ. The role of insulin receptor substrate 2 in hypothalamic and beta cell function. *J Clin Invest*. 2005; 115:940–950. [PubMed: 15841180]
23. Burks DJ, Font de Mora J, Schubert M, Withers DJ, Myers MG, Towery HH, Altamuro SL, Flint CL, White MF. IRS-2 pathways integrate female reproduction and energy homeostasis. *Nature*. 2000; 407:377–382. [PubMed: 11014193]

24. Wurster AL, Withers DJ, Uchida T, White MF, Grusby MJ. Stat6 and IRS-2 cooperate in interleukin 4 (IL-4)-induced proliferation and differentiation but are dispensable for IL-4-dependent rescue from apoptosis. *Mol Cell Biol.* 2002; 22:117–126. [PubMed: 11739727]
25. Kelly-Welch AE, Wang HY, Wang LM, Pierce JH, Jay G, Finkelman F, Keegan AD. Transgenic expression of insulin receptor substrate 2 in murine B cells alters the cell density-dependence of IgE production in vitro and enhances IgE production in vivo. *J Immunol.* 2004; 172:2803–2810. [PubMed: 14978080]
26. Blaeser F, Bryce PJ, Ho N, Raman V, Dedeoglu F, Donaldson DD, Geha RS, Oettgen HC, Chatila TA. Targeted inactivation of the IL-4 receptor alpha chain I4R motif promotes allergic airway inflammation. *J Exp Med.* 2003; 198:1189–1200. [PubMed: 14557412]
27. Weisser SB, McLaren KW, Voglmaier N, van Netten-Thomas CJ, Antov A, Flavell RA, Sly LM. Alternative activation of macrophages by IL-4 requires SHIP degradation. *Eur J Immunol.* 2011; 41:1742–1753. [PubMed: 21469115]
28. Maarsingh H, Dekkers BG, Zuidhof AB, Bos IS, Menzen MH, Klein T, Flik G, Zaagsma J, Meurs H. Increased arginase activity contributes to airway remodelling in chronic allergic asthma. *Eur Respir J.* 2011; 38:318–328. [PubMed: 21310883]
29. North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. *Am J Physiol Lung Cell Mol Physiol.* 2009; 296:L911–920. [PubMed: 19286931]
30. Owhashi M, Maruyama H, Nawa Y. Kinetic study of eosinophil chemotactic factor production with reference to eosinophilia and granuloma formation in mice infected with *Schistosoma japonicum*. *Int J Parasitol.* 1996; 26:705–711. [PubMed: 8894761]
31. Chang NC, Hung SI, Hwa KY, Kato I, Chen JE, Liu CH, Chang AC. A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J Biol Chem.* 2001; 276:17497–17506. [PubMed: 11297523]
32. Lee CG, Hartl D, Lee GR, Koller B, Matsuura H, Da Silva CA, Sohn MH, Cohn L, Homer RJ, Kozhich AA, Humbles A, Kearley J, Coyle A, Chupp G, Reed J, Flavell RA, Elias JA. Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. *J Exp Med.* 2009; 206:1149–1166. [PubMed: 19414556]
33. Yamaji-Kegan K, Su Q, Angelini DJ, Myers AC, Cheadle C, Johns RA. Hypoxia-Induced Mitogenic Factor (HIMF/FIZZ1/RELM{alpha}) Increases Lung Inflammation and Activates Pulmonary Microvascular Endothelial Cells via an IL-4-Dependent Mechanism. *J Immunol.* 2010
34. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C. A critical role for eosinophils in allergic airways remodeling. *Science.* 2004; 305:1776–1779. [PubMed: 15375268]
35. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, McElwain K, McElwain S, Friedman S, Broide DH. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest.* 2004; 113:551–560. [PubMed: 14966564]
36. Liu T, Dhanasekaran SM, Jin H, Hu B, Tomlins SA, Chinnaiyan AM, Phan SH. FIZZ1 stimulation of myofibroblast differentiation. *Am J Pathol.* 2004; 164:1315–1326. [PubMed: 15039219]
37. Yamaji-Kegan K, Takimoto E, Zhang A, Weiner NC, Meuchel LW, Berger AE, Cheadle C, Johns RA. Hypoxia-induced mitogenic factor (FIZZ1/RELMalpha) induces endothelial cell apoptosis and subsequent interleukin-4-dependent pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2014; 306:L1090–1103. [PubMed: 24793164]
38. Daley E, Emson C, Guignabert C, de Waal Malefyt R, Louten J, Kurup VP, Hogaboam C, Taraseviciene-Stewart L, Voelkel NF, Rabinovitch M, Grunig E, Grunig G. Pulmonary arterial remodeling induced by a Th2 immune response. *J Exp Med.* 2008; 205:361–372. [PubMed: 18227220]
39. Rauh MJ, Ho V, Pereira C, Sham A, Sly LM, Lam V, Huxham L, Minchinton AI, Mui A, Krystal G. SHIP represses the generation of alternatively activated macrophages. *Immunity.* 2005; 23:361–374. [PubMed: 16226502]
40. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, Collins M, Grusby MJ. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of

naive Th cells into interferon gamma-producing Th1 cells. *J Exp Med*. 2002; 196:969–977. [PubMed: 12370258]

41. Wang LM, Keegan AD, Li W, Lienhard GE, Pacini S, Gutkind JS, Myers MG Jr, Sun XJ, White MF, Aaronson SA, et al. Common elements in interleukin 4 and insulin signaling pathways in factor-dependent hematopoietic cells. *Proc Natl Acad Sci U S A*. 1993; 90:4032–4036. [PubMed: 7683417]
42. Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE Jr, Yancopoulos GD. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science*. 1995; 267:1349–1353. [PubMed: 7871433]
43. Orchansky PL, Kwan R, Lee F, Schrader JW. Characterization of the cytoplasmic domain of interleukin-13 receptor-alpha. *J Biol Chem*. 1999; 274:20818–20825. [PubMed: 10409622]
44. Tachdjian R, Al Khatib S, Schwinglshackl A, Kim HS, Chen A, Blasioli J, Mathias C, Kim HY, Umetsu DT, Oettgen HC, Chatila TA. In vivo regulation of the allergic response by the IL-4 receptor alpha chain immunoreceptor tyrosine-based inhibitory motif. *J Allergy Clin Immunol*. 2010; 125:1128–1136 e1128. [PubMed: 20392476]
45. Dasgupta P, Chapoval SP, Smith EP, Keegan AD. Transfer of in vivo primed transgenic T cells supports allergic lung inflammation and FIZZ1 and Ym1 production in an IL-4Ralpha and STAT6 dependent manner. *BMC Immunol*. 2011; 12:60. [PubMed: 22014099]
46. Welch JS, Escoubet-Lozach L, Sykes DB, Liddiard K, Greaves DR, Glass CK. TH2 cytokines and allergic challenge induce Ym1 expression in macrophages by a STAT6-dependent mechanism. *J Biol Chem*. 2002; 277:42821–42829. [PubMed: 12215441]
47. Stutz AM, Pickart LA, Trifilieff A, Baumruker T, Prieschl-Strassmayr E, Woisetschlager M. The Th2 cell cytokines IL-4 and IL-13 regulate found in inflammatory zone 1/resistin-like molecule alpha gene expression by a STAT6 and CCAAT/enhancer-binding protein-dependent mechanism. *J Immunol*. 2003; 170:1789–1796. [PubMed: 12574343]
48. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. *Gene*. 2005; 353:98–106. [PubMed: 15922518]
49. Guo S, Cichy SB, He X, Yang Q, Ragland M, Ghosh AK, Johnson PF, Unterman TG. Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta. *J Biol Chem*. 2001; 276:8516–8523. [PubMed: 11116148]
50. Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun*. 2013; 4:2834. [PubMed: 24280772]
51. Gibson SL, Ma Z, Shaw LM. Divergent roles for IRS-1 and IRS-2 in breast cancer metastasis. *Cell Cycle*. 2007; 6:631–637. [PubMed: 17361103]
52. McCormick SM, Heller NM. Commentary: IL-4 and IL-13 receptors and signaling. *Cytokine*. 2015; 75:38–50. [PubMed: 26187331]
53. Oh SY, Zheng T, Bailey ML, Barber DL, Schroeder JT, Kim YK, Zhu Z. Src homology 2 domain-containing inositol 5-phosphatase 1 deficiency leads to a spontaneous allergic inflammation in the murine lung. *J Allergy Clin Immunol*. 2007; 119:123–131. [PubMed: 17208593]
54. Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elias JA. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science*. 2004; 304:1678–1682. [PubMed: 15192232]
55. Kubota T, Kubota N, Moroi M, Terauchi Y, Kobayashi T, Kamata K, Suzuki R, Tobe K, Namiki A, Aizawa S, Nagai R, Kadowaki T, Yamaguchi T. Lack of insulin receptor substrate-2 causes progressive neointima formation in response to vessel injury. *Circulation*. 2003; 107:3073–3080. [PubMed: 12810606]
56. Mita T, Azuma K, Goto H, Jin WL, Arakawa M, Nomiyama T, Suzuki R, Kubota N, Tobe K, Kadowaki T, Fujitani Y, Hirose T, Kawamori R, Watada H. IRS-2 deficiency in macrophages promotes their accumulation in the vascular wall. *Biochem Biophys Res Commun*. 2011; 415:545–550. [PubMed: 22074825]

57. Yu M, Qi X, Moreno JL, Farber DL, Keegan AD. NF-kappaB signaling participates in both RANKL-and IL-4-induced macrophage fusion: receptor cross-talk leads to alterations in NF-kappaB pathways. *J Immunol.* 2011; 187:1797–1806. [PubMed: 21734075]
58. Cuesta N, Salkowski CA, Thomas KE, Vogel SN. Regulation of lipopolysaccharide sensitivity by IFN regulatory factor-2. *J Immunol.* 2003; 170:5739–5747. [PubMed: 12759457]
59. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
60. Ford AQ, Dasgupta P, Mikhailenko I, Smith EM, Noben-Trauth N, Keegan AD. Adoptive transfer of IL-4Ralpha+ macrophages is sufficient to enhance eosinophilic inflammation in a mouse model of allergic lung inflammation. *BMC Immunol.* 2012; 13:6. [PubMed: 22292924]

One sentence summary

IRS2 negatively regulates IL-4-induced signaling in vitro and suppresses allergic lung inflammation and remodeling in vivo, advancing our understanding of a key pathway involved in the regulation of allergic inflammation.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Analysis of AAM gene expression in *IRS2*^{-/-} mice

(A) BMM were prepared from *IRS2*^{+/+} or *IRS2*^{-/-} mice as described in Materials and Methods. Cells were stimulated with either IL-4 or IL-13 (100 ng/ml) for 6 or 24 hours as indicated. Cells lysates were prepared, total RNA isolated and converted into cDNA. The relative abundance of mRNA encoding *Arg1*, *Retnla*, and *Chi3L3* was analyzed using quantitative real time PCR as described (10). *HPRT* was used as the housekeeping gene. Data are represented as fold induction (2^{-Ct}) relative to unstimulated and *HPRT* controls. Representative data from one of three experiments is shown. (B) The average data from three independent experiments were expressed as a percentage of maximal induction (IL-4-stimulated value = 100%) at 6 hours *p<0.05.

Figure 2. Effect of IRS2 on allergic lung inflammation

(A) The asthma protocol used in this study is described in detail in Materials and Methods (B) IRS2^{+/+}, IRS2^{+/-} or IRS2^{-/-} mice were primed and boosted with OVA/alum and challenged with 1% OVA in PBS. Bronchoalveolar lavage (BAL) cells were harvested and subjected to cytopspin and differential counting. The total number of BAL cells (T) and numbers and percentages of macrophages (M), eosinophils (E), lymphocytes (L) and polymorphoneutrophils (P) present in the BAL were counted. * $p < 0.05$. $n = 3-4$ mice in each group. (C) Mice were primed and boosted with alum or OVA/alum as indicated and

challenged with 1% OVA in PBS. Representative photomicrographs of H&E (10×, 20× and 40×) stained lung sections of groups are shown. Arrows point to areas of inflammation around airways (AW) and blood vessels (BV). Representative data from one of three independent experiments is shown.

Figure 3. YM1 expression in macrophages in vivo

IRS2^{+/+}, IRS2^{+/-} and IRS2^{-/-} mice were subjected to the allergic lung inflammation protocol described in Figure 2. (A) YM1 protein expression in lung sections from the above mice was detected using IHC. Photomicrographs (100x) of YM1⁺ macrophages (indicated by arrows) from representative lung sections are shown. (B) BAL cells were isolated from the three groups of mice and analyzed by flow cytometry. Percentages of CD11b⁺ YM1⁺ cells shown as contour plots and mean fluorescence intensity (MFI) of YM1 staining (bold line) represented in the form of histograms. Staining with secondary antibody was used as control. (C) MFI of YM1 staining in macrophages in the three groups of mice is graphed. * $p < 0.05$. $n = 3-4$ mice in each group. Representative data from one of two independent experiments is shown.

Figure 4. $IRS2$ deficient mice developed increased airway and vascular remodeling
 $IRS2^{+/+}$, $IRS2^{+/-}$ and $IRS2^{-/-}$ mice were subjected to the asthma protocol as described in Figure 2. (A) Lung sections of mice were stained with Masson's Trichrome. Photomicrographs of collagen deposition around the airways in mice treated with Alum (panels a–c) or OVA/alum (panels d–f) are shown. (B) Collagen deposition in the lung was quantified using NIH Image J software. Data is represented as area of collagen (blue stain) $\pm$ SEM. $n=3-4$ mice/group. An average of 10 airways was analyzed per mouse. * $p<0.05$. (C): Lung sections were double-stained with antibodies to vWF (brown) and SMA (red) (400x). (D) Muscularization of small pulmonary arteries in mouse lungs from the 6 different groups of mice was evaluated as described in Materials and Methods. Bar graph shows the percentage of small pulmonary arteries in mice that were nonmuscular (NM), partially

muscular (PM), or fully muscular (FM). (Means $\pm$ SEM; * $p < 0.01$, ** $p < 0.0001$ vs. IRS2^{+/+}, Ova or IRS2^{+/-}, Ova). More than 500 vessels were counted in each group. n=3–4 mice in each group. E. Antigen-induced pulmonary vascular muscularization (SMA, red) and FIZZ1 production (green) were analyzed in each genotype by confocal microscopy (Scale bar: 50 μ m). Representative photomicrographs of lung sections from 3–4 mice per group are shown. Representative data from one of two independent experiments is shown.

Figure 5. Adoptive transfer of $IRS2^{-/-}$ BMM leads to enhanced features of allergic lung inflammation and remodeling

(A) BMM were prepared from $IRS2^{+/+}$ or $IRS2^{-/-}$ mice as described in Materials and Methods. The cells were washed and adoptively transferred to untreated $IRS2^{+/+}$ or $IRS2^{+/-}$ mice by IP injection as indicated. One day after transfer, the OVA prime/challenge protocol was initiated in the recipient mice and in $IRS2^{-/-}$ mice. (B) BAL cells were harvested and subjected to cytopspin and differential counting. The total number of BAL eosinophils and the percentage of eosinophils in the BAL were quantified. * $p<0.05$. $n=3-4$ mice in each group. (C) Representative photomicrographs of H&E (20 $\times$ and 40 $\times$) and anti-YM1 (40 $\times$ and high

power inset) stained lung sections of groups are shown. (D) Lung sections were stained with anti-vWF and SMA and thickness of the arterial wall of the small pulmonary arteries was measured. Left; Representative photomicrographs of small pulmonary vessels. Right; average SMC layer thickness (n=50/group) $\pm$ SEM is graphed. * $p < 0.05$, ** $p < 0.0005$; *** $p < 0.00001$

Figure 6. Enhanced phosphorylation of Akt and S6 in the absence of IRS2

(A) Left. BMM isolated from IRS2^{+/+}, IRS2^{+/-} or IRS2^{-/-} mice were deprived of serum and MCSF for 2 hours before treatment with or without 20 ng/ml IL-4 for 30 mins. Whole cell lysates were prepared followed by immunoprecipitation with anti-p85. Western blots were probed with anti-phosphotyrosine (PY-20) and the region of the blot with tyrosine phosphorylated IRS1/IRS2 is shown. The blots were stripped and re-probed with anti-p85. Right. Densitometry was performed and the average data from three independent experiments with PY-20 and p85 signal was calculated as a percentage of maximal induction (IL-4-stimulated value = 100%) $\pm$ SEM * p <0.05. (B) Left. BMM isolated from IRS2^{+/+}, IRS2^{+/-} or IRS2^{-/-} mice were deprived of serum and MCSF for 2 hours before treatment

with or without 20 ng/ml IL-4 for 30 mins in the presence or absence of LY294002 (1 μ M). Whole cell lysates were prepared followed by western blotting. Blots were probed with anti-pSer⁴⁷³Akt, anti-pThr³⁰⁸Akt, or anti-pSer^{235, 236}S6 as indicated. The blots were stripped and re-probed with anti-Akt or anti-S6 as appropriate. Right. Densitometric analysis. Representative data from one of three independent experiments is shown. (C) BMM were prepared as described above and were stimulated with IL-4 (100 ng/ml) for 6 hours in the presence or absence of LY294002 (1 μ M). Cells lysates were prepared, total RNA isolated and converted into cDNA. The relative abundance of mRNA *Retnla* and *Chi3l3* in IL-4 treated samples relative to unstimulated and *HPRT* was analyzed using quantitative real time PCR as described. The ability of LY294002 to suppress the IL-4-induced increase in mRNA was calculated as % inhibition. The average % inhibition from 3 independent experiments $\pm$ SEM is shown graphically.

Figure 7. Contribution of IRS1 to signaling pathways and gene expression

(A) BMM isolated from IRS2^{+/+}, IRS2^{+/-} or IRS2^{-/-} mice were cultured in the presence of lentivirus containing non-targeting (NT) shRNA or IRS1-targeting (KD) shRNA constructs as described in Materials and Methods. Cells were lysed and total RNA was prepared. The relative abundance of *IRS1* mRNA was analyzed by RT-PCR followed by densitometry. The average (n=3) $\pm$ SEM is shown. *p<0.05. (B) BMM treated as in (A) were deprived of serum and MCSF for 2 hours before treatment with or without 20 ng/ml IL-4 for 30 mins. Whole cell lysates were prepared followed by western blotting. Blots were probed with anti-pSer⁴⁷³ Akt, anti-pThr³⁰⁸ Akt, or anti-pSer^{235, 236} S6 as indicated. The blots were stripped and re-probed with anti-Akt or anti-S6 as appropriate. (C–D) BMM treated as in (A) were stimulated with IL-4 (100 ng/ml) for 6 hours. Cells lysates were prepared, total RNA isolated and converted into cDNA. The relative abundance of *Retnla* and *Chi3L3* in IL-4 treated samples relative to unstimulated was analyzed using quantitative real time PCR as described. (C) Representative data from one experiment showing IL-4-induced induction (fold induction) of *Retnla* in NT-versus IRS1-KD shRNA treated BMM. (D) The ability of IRS1-targeting shRNA to suppress the IL-4-induced increase in mRNA relative to the NT control was calculated as % inhibition [1-(fold induction by IL-4 in KD/fold induction by

IL-4 in NT)] $\times 100$. The average % inhibition from 3 independent experiments $\pm$ SEM is shown graphically. * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript