

The adaptor protein insulin receptor substrate 2 inhibits alternative macrophage activation and allergic lung inflammation

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Insulin receptor substrate 2 (IRS2) is an adaptor protein that becomes tyrosine-phosphorylated in response to the cytokines interleukin-4 (IL-4) and IL-13, which results in activation of the phosphoinositide 3-kinase (PI3K)–Akt pathway. IL-4 and IL-13 contribute to allergic lung inflammation. To examine the role of IRS2 in allergic disease, we evaluated the responses of IRS2-deficient (*IRS2*^{−/−}) mice. Unexpectedly, loss of IRS2 resulted in a substantial increase in the expression of a subset of genes associated with the generation of alternatively activated macrophages (AAMs) in response to IL-4 or IL-13 *in vitro*. AAMs secrete factors that enhance allergic responses and promote airway remodeling. Moreover, compared to *IRS2*^{+/+} mice, *IRS2*^{+/−} and *IRS2*^{−/−} mice developed enhanced pulmonary inflammation, accumulated eosinophils and AAMs, and exhibited airway and vascular remodeling upon allergen stimulation, responses that partially depended on macrophage-intrinsic IRS2 signaling. Both in unstimulated and IL-4-stimulated macrophages, lack of IRS2 enhanced phosphorylation of Akt and ribosomal S6 protein. Thus, we identified a critical inhibitory loop downstream of IRS2, demonstrating an unanticipated and previously unrecognized role for IRS2 in suppressing allergic lung inflammation and remodeling.

INTRODUCTION

The prevalence of asthma and allergic diseases is rapidly increasing across all populations, which poses a huge challenge to health care systems around the world. An estimated 300 million people suffer from asthma worldwide, which is projected to reach 400 million by 2025. Interleukin-4 (IL-4) and IL-13 are multifunctional cytokines that play a crucial role in asthma and allergic responses (1–7). Although both IL-4 and IL-13 elicit asthma pathology when provided exogenously, the two cytokines mediate distinct physiologic functions *in vivo*. IL-4 is required for the proliferation and survival of lymphocytes and for their differentiation into T helper 2 (T_H2) cells (8). In addition, IL-4 induces antigen presentation by B cells and causes antibody class switching from immunoglobulin M (IgM) to IgE (9). IL-4 also preferentially enhances the alternative activation of macrophages (10, 11). IL-13, on the other hand, is considered to be the effector cytokine, being responsible for airway hyperresponsiveness (AHR), excessive mucus production, and airway remodeling (4, 5). Because of 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 stimulate allergic responses by engaging two different heterodimeric receptor complexes. Upon ligand binding, either the common γ subunit (γ_c) or the IL-13 receptor $\alpha 1$ subunit (IL-13R $\alpha 1$)

dimerizes with IL-4R α to form a functional type I or type II receptor, respectively. Receptor engagement and heterodimerization activate tyrosine kinases of the Janus kinase (Jak) family (9), which phosphorylate tyrosine residues in the cytoplasmic tail of the IL-4R α chain (9, 14–17). These phosphorylated 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 signal transducer and activator of transcription 6 (STAT6), insulin receptor substrate 2 (IRS2), SH2 domain-containing inositol-5-phosphatase (SHIP), and SH2 domain-containing protein tyrosine phosphatases 1 and 2 (SHP1/2).

IRS2 is one of six large adaptor proteins that participate in insulin, insulin-like growth factor 1 (IGF-1), and IL-4R signaling (18–20). Because IRS2 plays an important role in insulin-induced responses and the survival of pancreatic β cells, complete loss of this adaptor protein leads to the eventual development of type 2 diabetes in mice (21). Conditional deletion of the gene encoding IRS2 in pancreatic β cells leads to reduced β cell mass, increased obesity, and hyperinsulinemia (22). The IRS2 pathway is also required for oocyte survival and fertility (23). Whereas 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 the IL-4-induced proliferation of CD4⁺ T cells or the secretion of T_H2-type cytokines *in vitro* (24). IRS2 is also not required for the ability of IL-4 to promote B cell survival (24), which suggests that IRS2 is not important for B cell functions. However, we found that the serum concentrations of IgE and IgG1 are increased in *IRS2* transgenic mice immunized with ovalbumin (OVA) compared to those in similarly treated wild-type mice (25). The role of the IRS2 pathway in asthma is unclear. A previous study used mice expressing IL-4R α with a mutation [Tyr⁵⁰⁰→Phe (Y500F)] in the insulin–IL-4 receptor (I4R) motif of IL-4R α (26). This mutation prevents the docking of PTB domain-containing proteins, including IRS2, to IL-4R and suppresses IRS2 phosphorylation (18, 19). The Y500F mutation impairs T cell proliferation but does not affect T_H2-type

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cytokine secretion in vitro. Allergen sensitization and challenge in F500 mutant mice result in enhanced IgE production, AHR, eosinophilic inflammation, and mucus production in vivo when compared to wild-type (Y^{500}) mice, which suggests that pathways dependent on Tyr^{500} of IL-4R α are immunosuppressive.

We previously observed that IL-4 induces greater phosphorylation of IRS2 in macrophages than does IL-13 (10). Experiments with cells deficient in the γ_c chain and the type I receptor showed that the IL-4-stimulated phosphorylation of IRS2 was reduced, as was the expression of alternatively activated macrophage (AAM)-associated genes; however, IL-13-stimulated responses were unaffected. This suggests that the type I receptor is more potent in activating IRS2 than is the type II receptor. We hypothesized that enhanced activation of pathways downstream of IRS2 such as phosphoinositide 3-kinase (PI3K) and Akt, which are important for the generation of AAMs (27), was responsible for the differential induction of AAM-associated 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. Because AAMs secrete many proteins that enhance allergic responses (28–32), we also evaluated the role of IRS2 in allergic lung inflammation in experiments with IRS2-deficient ($IRS2^{-/-}$) mice. However, we found that $IRS2^{+/-}$ and $IRS2^{-/-}$ mice developed enhanced allergic lung inflammation and increased airway and vascular remodeling in comparison to $IRS2^{+/+}$ mice. In addition, AAM-associated gene expression was enhanced in response to IL-4 or IL-13 in IRS2-deficient macrophages in vitro compared to that in wild-type cells. These results suggest a previously uncharacterized role for IRS2 in the negative regulation of allergic lung disease. Furthermore, loss of IRS2 led to increased phosphorylation of Akt and S6, which was likely IRS1-dependent, 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

Absence of IRS2 in macrophages leads to enhanced expression of *Retnla*, *Chi3l3*, and *Arg1*

We previously demonstrated that, compared to IL-13, IL-4 induced greater phosphorylation of IRS2 and expression of genes associated with the AAM phenotype in vitro, suggesting that IRS2 may be required for AAM differentiation (10). To determine whether enhanced activation of IRS2 by IL-4 was responsible for this difference, we analyzed the relative expression of characteristic, STAT6-dependent AAM-associated genes in IRS2-deficient cells. Bone marrow-derived macrophages (BMMs) from $IRS2^{+/+}$ and $IRS2^{-/-}$ mice were incubated for 6 or 24 hours with con-

centrations of IL-4 or IL-13 (100 ng/ml) that stimulated equivalent phosphorylation of STAT6 (10). IL-4 or IL-13 increased the amount of *Retnla* mRNA, which encodes FIZZ1 (found in inflammatory zone 1), and *Chi3l3* mRNA, which encodes YM1 (Fig. 1A) up to 24 hours, whereas *Arg1* mRNA abundance was maximal at 6 hours and decreased at 24 hours. As was seen previously with wild-type macrophages (10), IL-4 induced the expression of *Retnla*, *Chi3l3*, and *Arg1* in $IRS2^{+/+}$ macrophages to a greater extent than did IL-13 (Fig. 1A), and IRS2 deficiency did not abrogate this differential AAM-associated gene expression. In contrast to our prediction, the absence of IRS2 in macrophages led to enhanced increases in *Retnla*, *Chi3l3*, and *Arg1* mRNA abundances by both IL-4 and IL-13 (Fig. 1B). These findings suggest that IRS2 inhibits the expression of AAM-associated genes in cells in vitro and that the differential phenotypes induced by IL-4 and IL-13 may not be due to differential phosphorylation of IRS2.

STAT6 phosphorylation is unaffected by IRS2 deficiency

To determine whether the absence of IRS2 affected the STAT6 pathway, we assessed the extent of IRS2 and STAT6 phosphorylation in the IRS2-deficient mice (fig. S1). As expected, splenocytes and BMMs from $IRS2^{-/-}$ mice completely lacked IRS2 protein and its phosphorylated form in response to IL-4. We found evidence of a gene dosage effect, with the $IRS2^{+/-}$

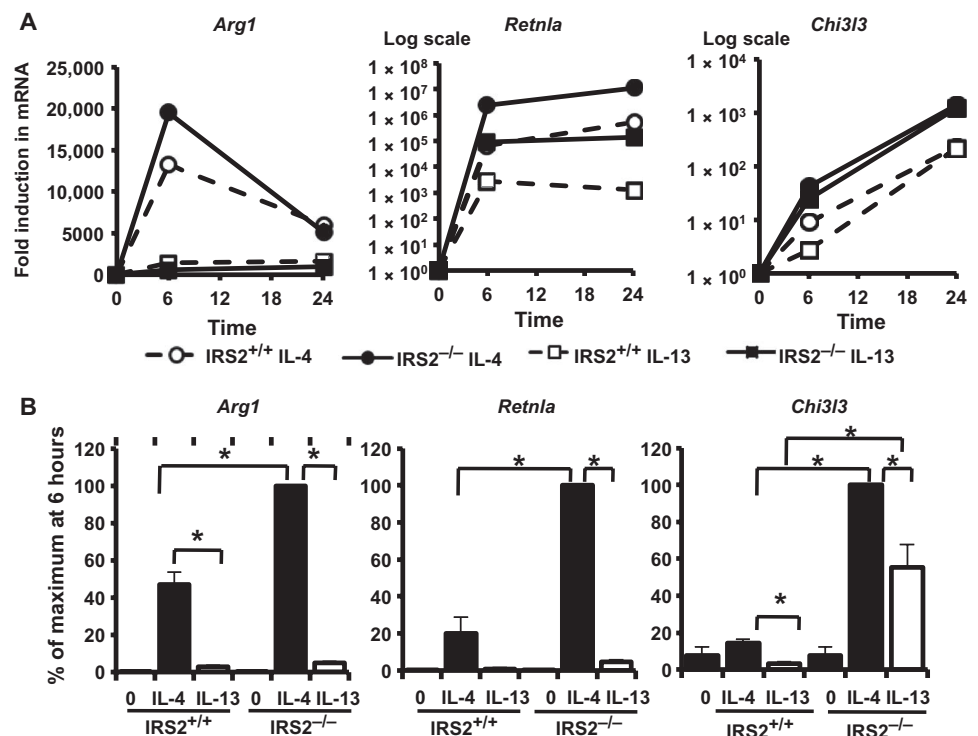


Fig. 1. Analysis of AAM-associated gene expression in $IRS2^{-/-}$ mice. (A) BMMs from $IRS2^{+/+}$ or $IRS2^{-/-}$ mice were left unstimulated (0) or were stimulated with either IL-4 or IL-13 (100 ng/ml) for 6 or 24 hours as indicated. The relative abundances of *Arg1*, *Retnla*, and *Chi3l3* mRNAs normalized to that of *HPRT* mRNA were determined by quantitative real-time polymerase chain reaction (qRT-PCR) analysis as described in Materials and Methods. Data are represented as the fold increase ($2^{-\Delta\Delta Ct}$) in mRNA in stimulated cells relative to that in unstimulated cells. Data are from one experiment and are representative of three experiments. (B) The average data from all three independent experiments are expressed as a percentage of maximal induction in the indicated mRNAs at 6 hours. The indicated mRNA abundances in IL-4-stimulated $IRS2^{-/-}$ cells at 6 hours were set at 100%. Data are means $\pm$ SEM. * $P < 0.05$.

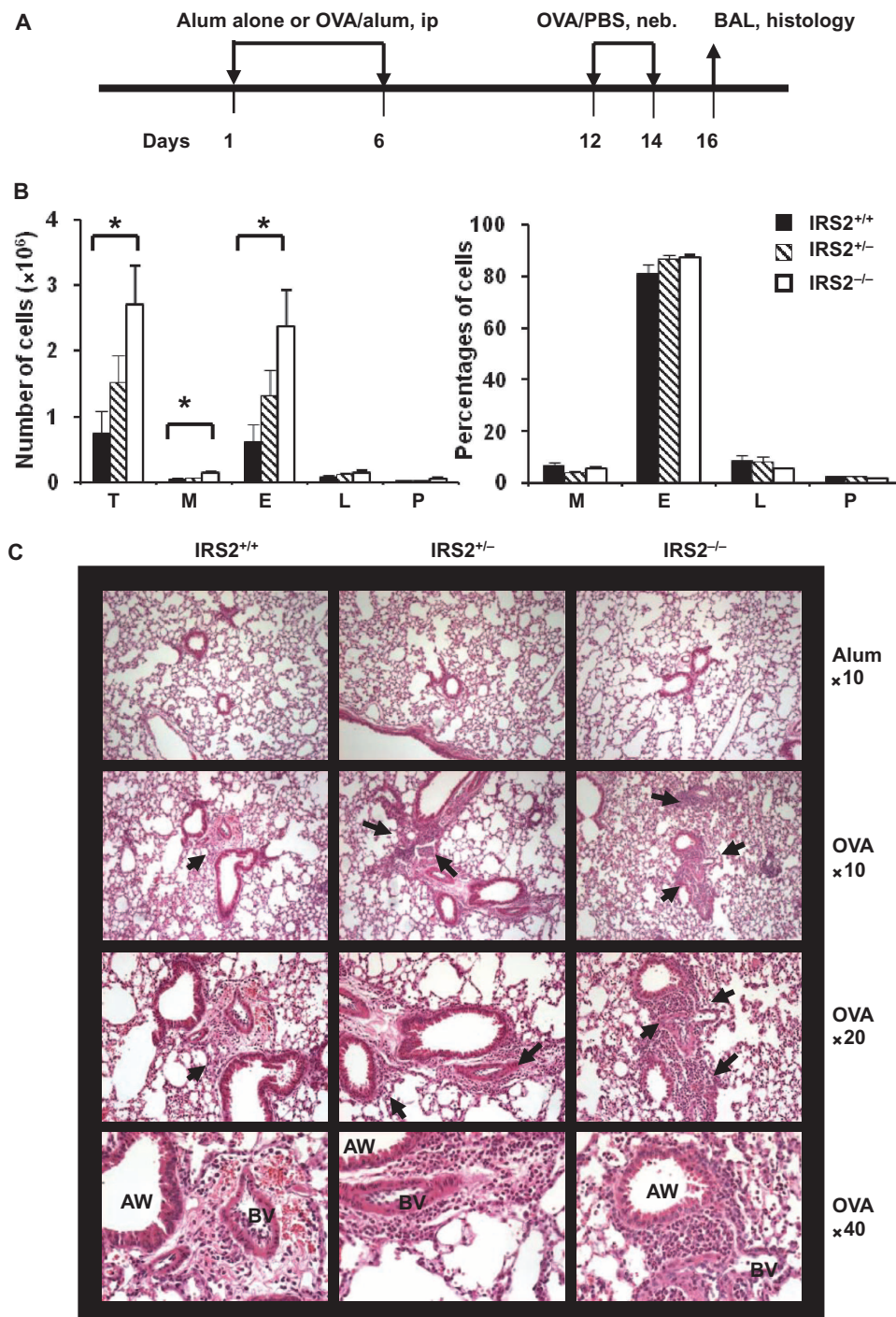
cells showing reduced IRS2 phosphorylation compared to that of $IRS2^{+/+}$ cells (fig. S1A). It was difficult to detect the IRS2 protein in the heterozygous samples, likely because the IRS2 protein quantities were below the detection limit, whereas the detection of multiple phosphotyrosines on IRS2 was still possible (fig. S1A). In contrast, splenocytes and BMMs from all three groups of mice exhibited robust phosphorylation of STAT6 in the presence of IL-4 (fig. S1B), which is consistent with a previous study showing that disruption of the *IRS2* gene does not impair activation of the STAT6 pathway (24).

IRS2 deficiency enhances allergic lung inflammation

Next, we examined the contribution of the IRS2 pathway to allergic lung inflammation. $IRS2^{+/+}$, $IRS2^{+/-}$, and $IRS2^{-/-}$ mice were sensitized and challenged with OVA peptide (Fig. 2A). Deficiency in IRS2 led to a substantial increase in the total number of T cells in the bronchoalveolar lavage (BAL) fluid, from 750,000 cells in OVA-primed and challenged $IRS2^{+/+}$ mice to 2.7 million cells in similarly treated $IRS2^{-/-}$ mice (Fig. 2B). This increase in cell numbers was mainly due to an increase in recruitment

Fig. 2. Effect of IRS2 on allergic lung inflammation.

(A) Outline of the asthma protocol used in this study, which is described in detail in Materials and Methods. ip, intraperitoneal; neb., nebulized. (B) $IRS2^{+/+}$, $IRS2^{+/-}$, and $IRS2^{-/-}$ mice were primed and boosted with OVA and alum and then were challenged with 1% OVA in phosphate-buffered saline (PBS). BAL cells were harvested and subjected to cytospin and differential counting. The total number of BAL cells (T, left) and the numbers (left) and percentages (right) of macrophages (M), eosinophils (E), lymphocytes (L), and polymorphonuclear neutrophils (P) present in the BAL were determined. Data are means $\pm$ SEM of three or four mice of each group. $*P < 0.05$. (C) Mice of the indicated genotypes were primed and boosted with alum alone or with OVA and alum and were challenged with 1% OVA in PBS. Representative photomicrographs of hematoxylin and eosin (H&E)-stained lung sections with magnifications at $\times 10$, $\times 20$, and $\times 40$ are shown. Arrows point to areas of inflammation around airways (AW) and blood vessels (BV). Data are from one experiment and are representative of three independent experiments.



of eosinophils to the airways. Consistent with earlier results, *IRS2*^{+/-} mice showed intermediate recruitment of inflammatory cells into the lungs (Fig. 2B); however, under these conditions, there was little difference in the percentages of macrophages, eosinophils, lymphocytes, and polymorphonuclear neutrophils in the BAL fluid from each mouse strain.

The sensitization and challenge of *IRS2*^{+/-} mice with OVA led to mild pulmonary inflammation, which was characterized by the presence of pockets of cellular infiltrates around the airways and blood vessels (Fig. 2C). The increased numbers of cells found in the BAL fluid of *IRS2*^{+/-} and *IRS2*^{-/-} mice were reflected in our observations of lung histology. Pronounced peribronchial and perivascular cuffing of eosinophils was observed in the lungs of these mice (Fig. 2C). The OVA-induced inflammation seen in *IRS2*^{+/-} mice appeared to be intermediate between that of the *IRS2*^{+/+} and that of the *IRS2*^{-/-} mice. The inflammatory response in all groups of mice was allergen-dependent, because the alum-primed mice did not show inflammation (Fig. 2C). These results suggest that IRS2 acts to suppress allergic lung inflammation.

IRS2 deficiency does not affect epithelial cell responses, serum IgE concentration, or cytokine production in vivo

We found that the loss of IRS2 did not appear to affect the responses of lung epithelial cells. Priming and challenge of mice with OVA 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 mice of all three genotypes produced both YM1 and FIZZ1 (fig. S2B). There was no noticeable difference in the amounts of these proteins in epithelial cells in wild-type mice and those in mice deficient in IRS2. Because 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 priming and challenge with OVA induced similar quantities of IgE in all three genotypes of mice (fig. S2C). In addition, we found no statistically significant differences in the amounts of IL-4, IL-5, or IL-13 present in the BAL fluid of each of the mouse strains (fig. S2D).

IRS2 deficiency increases YM1 protein abundance in AAMs in vivo

Our study of AAM-associated gene expression in BMMs in vitro (Fig. 1) demonstrated that the absence of IRS2 led to increased production of *Chi3l3* and *Retnla* mRNAs. Thus, we examined whether IRS2 inhibited YM1 protein production in the context of allergic lung inflammation in vivo. Through immunohistochemical analysis, we observed a greater intensity of YM1 signal in macrophages from IRS2-deficient mice compared to that in macrophages from wild-type mice (Fig. 3A). The differences in YM1 staining intensity in macrophages was quantified by flow cytometric analysis. Cells isolated from the BAL fluid of *IRS2*^{+/+}, *IRS2*^{+/-}, and *IRS2*^{-/-} mice were stained with antibodies specific for CD11b and YM1. In the absence of IRS2, the percentage of CD11b⁺YM1⁺ cells increased from the ~70% observed in wild-type mice to >84% (Fig. 3B, left). Furthermore, the mean fluorescence intensity (MFI) of YM1 staining at the single-cell level was substantially increased: *IRS2*^{+/+} macrophages had the smallest MFI (1047), which was followed by *IRS2*^{+/-} macrophages (MFI = 1388), with macrophages from *IRS2*^{-/-} mice having the greatest staining intensity (MFI = 1570) (Fig. 3, B and C). These results suggest that IRS2 inhibits AAM-associated protein production in vivo during allergic lung inflammation.

IRS2 deficiency enhances airway and vascular remodeling

Eosinophils and AAM products, such as FIZZ1 and YM1, promote airway and vascular remodeling (31, 33–36). Because IRS2-deficient mice

developed enhanced eosinophilia and the generation of AAMs, 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 OVA-challenged *IRS2*^{+/+}, *IRS2*^{+/-}, and *IRS2*^{-/-} mice revealed that the loss of one or both copies of *IRS2* led to progressively

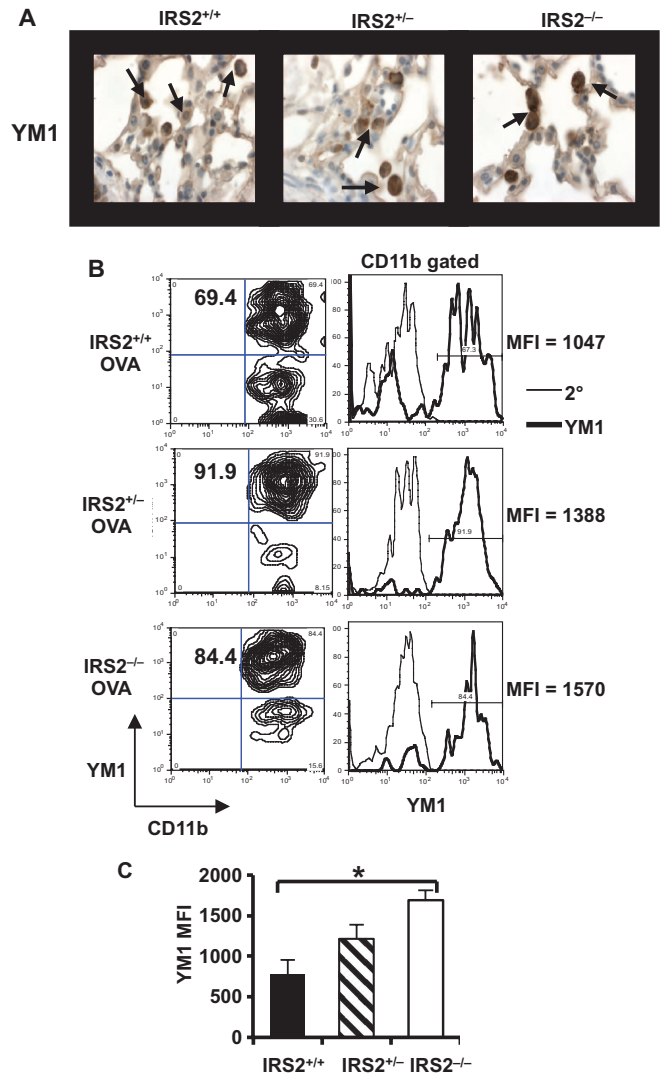


Fig. 3. Analysis of YM1 abundance in macrophages in vivo. (A to C) *IRS2*^{+/+}, *IRS2*^{+/-}, and *IRS2*^{-/-} mice were subjected to the allergic lung inflammation protocol described in Fig. 2. (A) YM1 protein abundance in lung sections from the indicated mice was detected by immunohistochemistry. Photomicrographs (magnification, ×100) of YM1⁺ macrophages (indicated by arrows) from representative lung sections are shown. (B) BAL cells were isolated from the indicated groups of mice and analyzed by flow cytometry. The percentages of CD11b⁺YM1⁺ cells are shown in contour plots (left), whereas the MFI of YM1 staining (bold line) is represented in the form of histograms (right). Staining with secondary antibody (2°, nonbold line) was used as control. (C) MFIs of YM1 staining in macrophages in the three groups of mice. Data are means ± SEM of three or four mice of each group from one experiment and are representative of two independent experiments. **P* < 0.05.

greater collagen deposition around the airways (Fig. 4A). When the intensity of collagen staining was quantified, we found that the increase in collagen deposition in *IRS2*^{-/-} mice was statistically significant (Fig. 4B). To examine the role of *IRS2* in the remodeling of small arterioles, we analyzed the intensity of smooth muscle cell actin (SMA) in small peripheral vessels (Fig. 4C) (37). Most of the small (≤ 80 μ m in diameter) pulmonary vessels in *IRS2*^{+/+} mice were nonmuscularized (Fig. 4, C and D). However, mice deficient in *IRS2* exhibited a statistically significant decrease in the percentage of nonmuscularized vessels, with concomitant increases in the percentages of small vessels that were partially or fully muscularized (Fig. 4, C and D). In addition, *IRS2*^{+/-} mice showed a gene dosage effect in that they had pulmonary vascular remodeling of intermediate extent. These data suggest that *IRS2* inhibits antigen-induced pulmonary arterial muscularization in vivo.

As shown earlier, BMMs from *IRS2*^{-/-} mice exhibited significantly greater *Retnla* expression in response to IL-4 or IL-13 than did BMMs from *IRS2*^{+/+} mice (Fig. 1). Because FIZZ1 protein modulates the muscularization of pulmonary vessels, we used immunofluorescence staining to examine the production of FIZZ1 in the lungs. There was very little FIZZ1 around the small pulmonary vessels in response to OVA in *IRS2*^{+/+} mice (Fig. 4E). Similarly, there was little or no FIZZ1 in cells around the airways (fig. S2B). However, *IRS2*^{+/-} and *IRS2*^{-/-} mice demonstrated marked increases in FIZZ1 protein abundance surrounding the remodeled pulmonary vessels (Fig. 4E). These data support the previous observation by Daley *et al.* that substantially muscularized pulmonary vessels become surrounded by FIZZ1-producing cells in response to antigen (38). Furthermore, we did not observe colocalization of FIZZ1- and SMA-positive cells (Fig. 4E), suggesting that the FIZZ1 produced by *IRS2*-deficient inflammatory cells might have a paracrine effect on the muscularization of small pulmonary vessels.

Adoptive transfer of *IRS2*^{-/-} macrophages enhances inflammation and vascular remodeling in recipient mice

To determine whether the enhanced allergen-induced responses observed in the *IRS2*^{-/-} mice were due to the effects in macrophages, we adoptively transferred BMMs isolated from *IRS2*^{-/-} mice or *IRS2*^{+/+} mice into wild-type or *IRS2*^{+/-} recipient mice 1 day

before they were subjected to the OVA/alum prime/challenge protocol (Fig. 5A). Adoptive transfer of *IRS2*^{-/-} BMMs to either wild-type or *IRS2*^{+/-} recipient mice resulted in an increase in the number of total

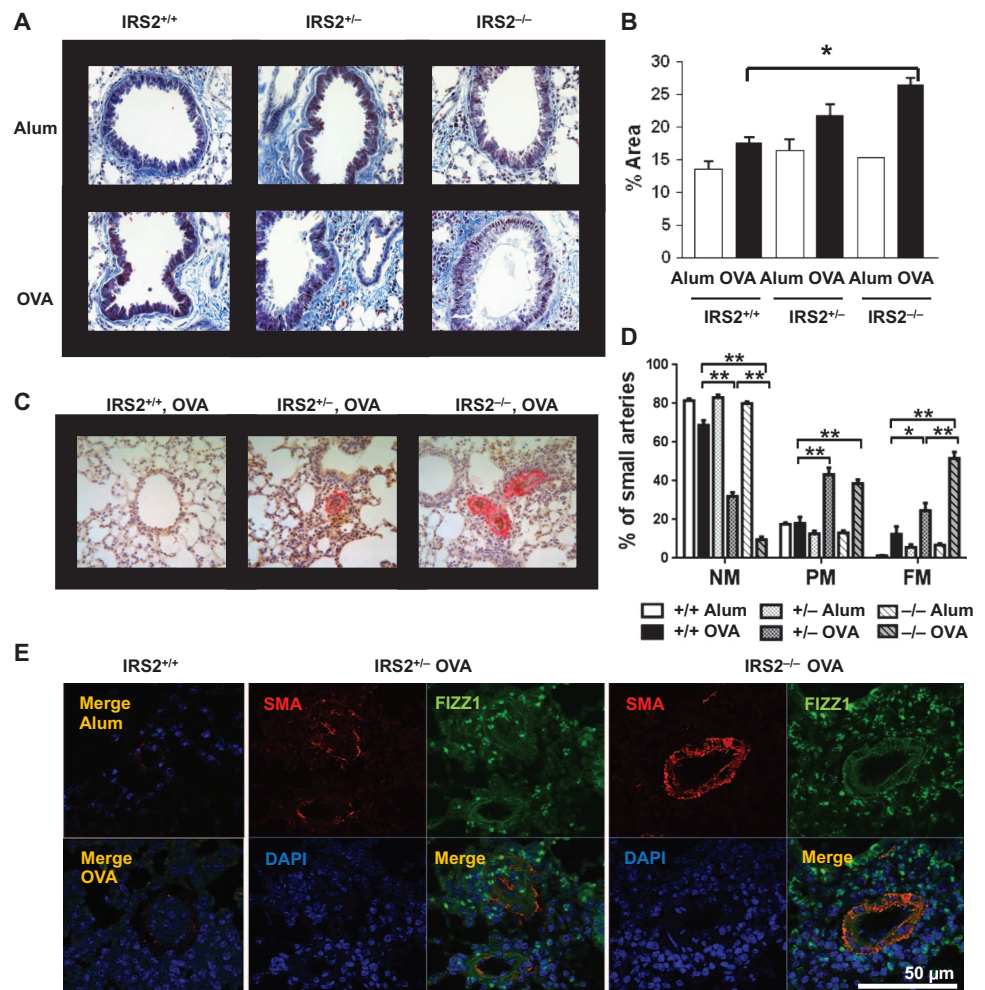
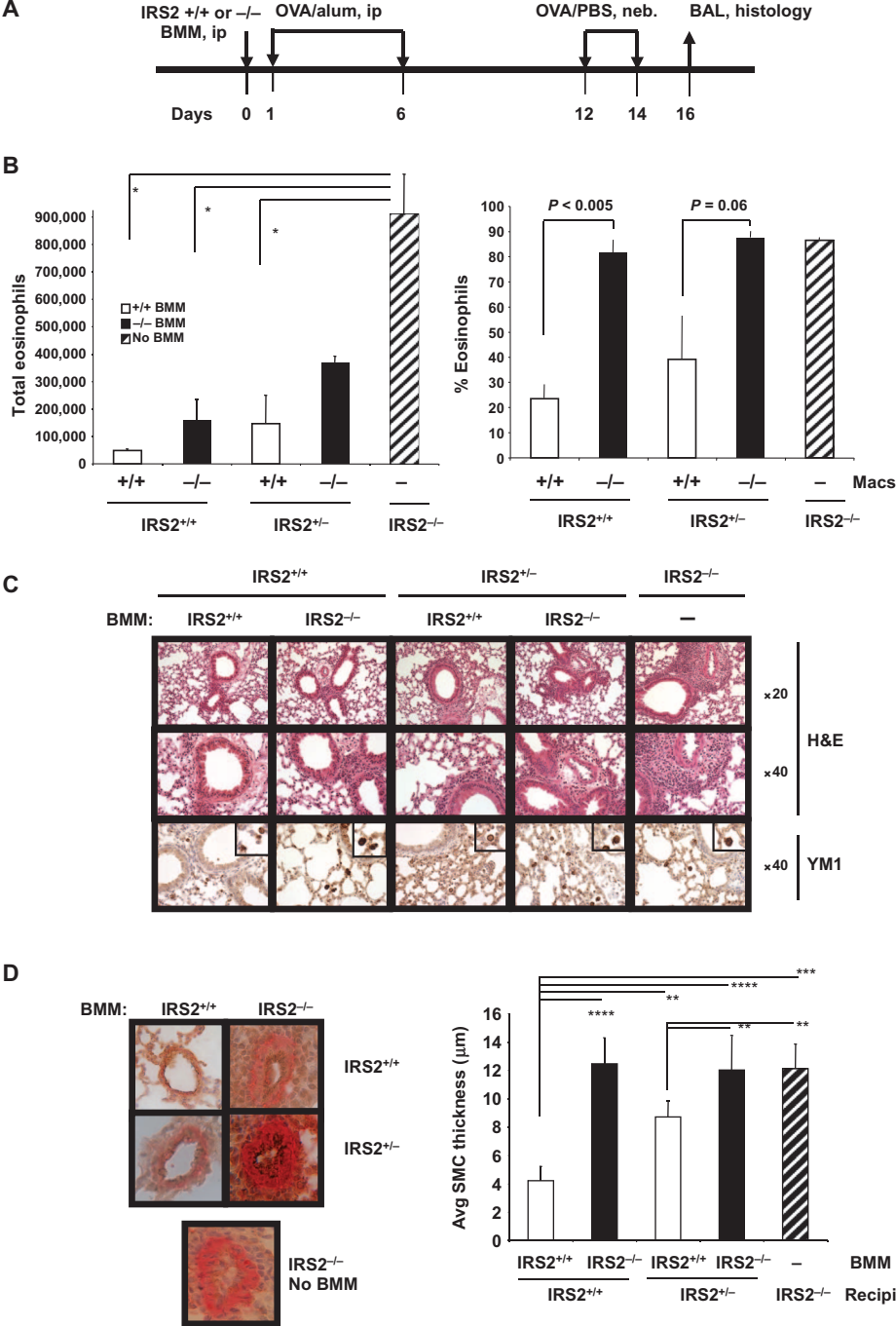


Fig. 4. *IRS2*-deficient mice develop increased airway and vascular remodeling. (A to E) *IRS2*^{+/+}, *IRS2*^{+/-}, and *IRS2*^{-/-} mice were subjected to the asthma protocol as described in Fig. 2. (A) Lung sections of the indicated mice were stained with Masson's trichrome. Photomicrographs of collagen deposition around the airways in mice treated with alum alone (top) or with OVA and alum (bottom) are shown. Images are representative of three experiments. (B) Collagen deposition in the lung was quantified with National Institutes of Health (NIH) ImageJ software. Data are the mean percentage area of collagen (blue stain) $\pm$ SEM of three or four mice per group. An average of 10 airways per mouse was analyzed. **P* < 0.05. (C) Lung sections from the indicated mice were double-stained with antibodies against von Willebrand factor (vWF; brown) and SMA (red) and are shown at $\times 400$ magnification. Images are representative of three experiments. (D) Muscularization of small pulmonary arteries in mouse lungs from the six 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 nonmuscularized (NM), partially muscularized (PM), or fully muscularized (FM). Data are means $\pm$ SEM of three or four mice per group. **P* < 0.01, ***P* < 0.0001 versus OVA-treated *IRS2*^{+/+} or OVA-treated *IRS2*^{+/-}. More than 500 vessels from each group were counted. (E) Antigen-induced pulmonary vascular muscularization (SMA, red) and FIZZ1 production (green) were analyzed in mice of the indicated groups by confocal microscopy. DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclei. Scale bar, 50 μ m. Photomicrographs of lung sections from three or four mice per group from a single experiment are shown and are representative of two independent experiments.

eosinophils and, in contrast to earlier results (Fig. 2B), in the percentage of eosinophils in the BAL fluid (Fig. 5B). However, the greatest number of eosinophils was present in the primed and challenged *IRS2*^{-/-} mice that did not receive exogenous macrophages (Fig. 5B). Moreover, a similar increase in inflammatory infiltrate in the lungs was observed when mice received *IRS2*^{-/-} BMMs rather than *IRS2*^{+/+} BMMs (Fig. 5C). We also observed increased YM1 staining intensity of lung macrophages. (Fig. 5C, inset). Furthermore, the adoptive transfer of *IRS2*^{-/-} BMMs to wild-type or *IRS2*^{+/-} mice resulted in

increased thickening of the arterial wall of the small pulmonary arteries after allergen priming and challenge (Fig. 5D). Transfer of *IRS2*^{-/-} BMMs led to a threefold increase in the average smooth muscle cell thickness of small pulmonary arteries in wild-type recipients and a smaller, but statistically significant, increase in the heterozygous recipients compared to that in wild-type mice that received *IRS2*^{+/+} BMMs. The smooth muscle cell thickness in the small pulmonary arteries of the mice that received *IRS2*^{-/-} BMMs was similar to that observed in *IRS2*^{-/-} mice that did not receive exogenous macrophages.

Fig. 5. Adoptive transfer of *IRS2*^{-/-} BMMs leads to increased allergic lung inflammation and remodeling. (A to D) BMMs from *IRS2*^{+/+} and *IRS2*^{-/-} mice were prepared as described in Materials and Methods, washed, and adoptively transferred to untreated *IRS2*^{+/+} or *IRS2*^{+/-} mice by intraperitoneal injection as indicated (A). One day after transfer, the OVA prime/challenge protocol was initiated in the recipient mice and, as a control, in *IRS2*^{-/-} mice that had not received any cells. (B) BAL cells were harvested from the indicated groups of mice and subjected to cytospin and differential counting. The total number of BAL eosinophils (left) and the percentage of eosinophils (right) in the BAL fluid were quantified. Data are means ± SEM of three or four mice per group. **P* < 0.05. (C) Representative photomicrographs of lung sections of the indicated groups of mice stained by H&E (magnification, ×20 and ×40) and anti-YM1 antibody (×40 and ×100 high-power inset) are shown. Images are representative of three or four mice from two experiments. (D) Lung sections from the indicated mice were stained with anti- α -vWF and anti-SMA antibodies, and the thickness of the arterial wall of the small pulmonary arteries was measured. Left: Representative photomicrographs of small pulmonary vessels. Right: Average smooth muscle cell (SMC) layer thickness of 20 vessels per mouse. Data are means ± SEM of three or four mice per group from two experiments. **P* < 0.05, ***P* < 0.001, ****P* < 0.0005, *****P* < 0.0001.



These results suggest that the enhanced inflammation and remodeling observed in allergen-challenged *IRS2*^{-/-} mice are, in part, intrinsic to the effect of IRS2 deficiency on macrophages. However, the adoptive transfer of *IRS2*^{-/-} BMMs did not result in any increase in FIZZ1 staining around the small pulmonary vessels that was greater than the staining intensity characteristic of each recipient genotype (fig. S3). Thus, the macrophage-intrinsic activity may be independent of FIZZ1.

IRS2 deficiency enhances the downstream Akt signaling pathway

IRS2 is a key adaptor protein that links IL-4R to the PI3K-Akt pathway. Previous studies of IRS2-deficient mice demonstrated that >90% of the PI3K activity in splenocytes stimulated by IL-4 is eliminated in the absence of IRS2, with a small amount of remaining activity associated with IRS1 (24). Furthermore, stimulation of the generation of AAMs by IL-4 is PI3K-dependent (27, 39). Because our observations of enhanced expression of AAM-associated genes in the absence of IRS2 are paradoxical, we directly examined how the absence of IRS2 affected activation of the PI3K-Akt pathway in BMMs (Fig. 6). In *IRS2*^{+/+} cells, IL-4 stimulated an increase in the amount of phosphorylated IRS2 (pIRS2) that coimmunoprecipitated with p85, the regulatory subunit of PI3K (Fig. 6A). Loss of one copy of *IRS2* was sufficient to reduce this association, whereas in *IRS2*^{-/-} mice, the IL-4-stimulated coimmunoprecipitation of pIRS2 with p85 was substantially abrogated as expected (Fig. 6A). We also observed a 170-kD phosphorylated band in samples immunoprecipitated with anti-p85 antibody from unstimulated and IL-4-stimulated cells; however, we did not observe any other phosphorylated proteins whose coimmunoprecipitation with p85 was changed by IL-4.

We next examined the pathways further downstream. IL-4 increased the extent of phosphorylation of Akt (at Ser⁴⁷³ and Thr³⁰⁸) and ribosomal S6 protein compared to that under unstimulated conditions in *IRS2*^{+/+} BMMs (Fig. 6B). As expected, the PI3K inhibitor LY294002 substantially suppressed the basal and IL-4-induced phosphorylation of Akt, with the most marked effect being on Akt-Ser⁴⁷³, as well as of S6 (Fig. 6B and fig. S4). Furthermore, both the basal and IL-4-induced phosphorylation of Akt and S6 were substantially increased in *IRS2*^{-/-} macrophages compared to that in *IRS2*^{+/+} macrophages. This increase in Akt activation was consistent

with increased biological responses in vivo (Figs. 2 to 5). LY294002 also suppressed the basal and IL-4-induced phosphorylation of Akt and S6 in IRS2-deficient macrophages (fig. S4). In BMMs from mice of all three

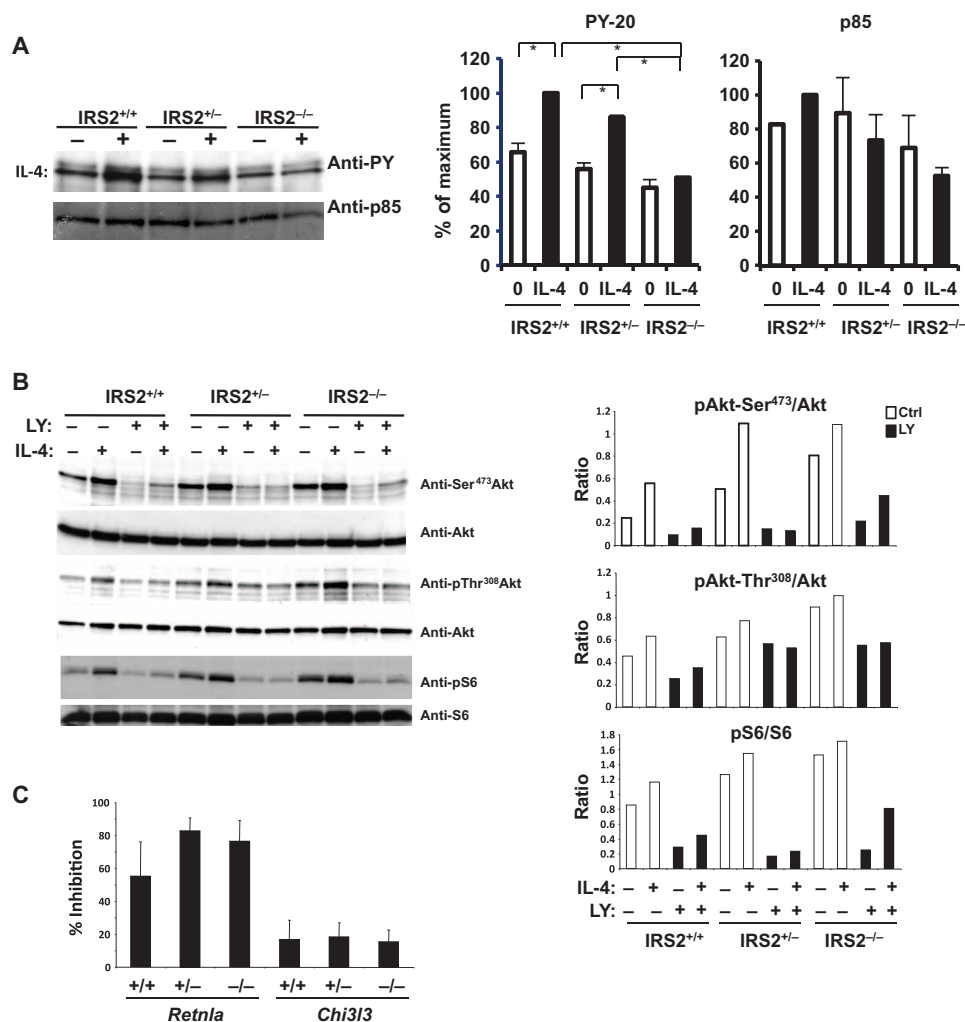


Fig. 6. Loss of IRS2 in macrophages leads to the enhanced phosphorylation of Akt and S6. (A) Left: BMMs isolated from the indicated mice were deprived of serum and macrophage colony-stimulating factor (MCSF) for 2 hours before being treated with or without IL-4 (20 ng/ml) for 30 min. Whole-cell lysates were subjected to immunoprecipitation with anti-p85 antibody and Western blotting analysis with the indicated antibodies, and the region of the blot containing tyrosine-phosphorylated IRS1/IRS2 is shown. Right: Densitometric analysis of the indicated band intensities. The intensities of the phosphotyrosine (PY-20) and p85 bands were calculated as percentages of those in IL-4-treated *IRS2*^{+/+} cells, which were set at 100%. Data are means $\pm$ SEM of three independent experiments. **P* < 0.05. (B) Left: BMMs isolated from the indicated mice were deprived of serum and MCSF for 2 hours before being treated with or without IL-4 (20 ng/ml) for 30 min in the presence or absence of 1 μ M LY294002 (LY). Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. Right: Densitometric analysis of the indicated band intensities. Data are from one experiment and are representative of three independent experiments. Pooled densitometric data from all experiments are shown in fig. S4. (C) BMMs from mice of the indicated *IRS2* genotypes were left unstimulated or were stimulated with IL-4 (100 ng/ml) for 6 hours in the presence or absence of 1 μ M LY294002. The relative abundances of *Retnla* and *Chi3l3* mRNAs in IL-4-treated samples relative to those in unstimulated were analyzed by qRT-PCR and normalized to that of *HPRT* mRNA. The ability of LY294002 to suppress the IL-4-induced increase in mRNA was calculated as percentage inhibition. Data are the mean percentage inhibition $\pm$ SEM of three independent experiments.

genotypes, LY294002 inhibited the IL-4-induced increase in *Retnla* expression, but it only modestly inhibited the expression of *Chi3l3* (Fig. 6C), consistent with our previous report on the sensitivity of these genes to PI3K inhibitors (10). These results suggest that there is an alternative mechanism to activate PI3K in the absence of IRS2, possibly through low amounts of IRS1 (40), and that there is a negative feedback loop downstream of IRS2 that becomes dysregulated in the absence of IRS2.

Knockdown of IRS1 in BMMs suppresses the Akt pathway and the induction of *Retnla* expression by IL-4

To determine whether IRS1 could indeed act as an adaptor downstream of IL-4 signaling, we transduced BMMs isolated from mice of all three genotypes with lentiviruses encoding a short hairpin RNA (shRNA) targeting mouse *IRS1* (IRS1 KD shRNA) or a nontargeting (NT) shRNA control, and then we evaluated IL-4-stimulated signaling and gene expression (Fig. 7). Expression of the IRS1 KD shRNA in BMMs resulted in a 50 to 70% decrease in *IRS1* mRNA abundance compared to that in BMMs expressing the NT shRNA control (Fig. 7A). Analysis of anti-IRS1 immunoprecipitates from the BMMs expressing NT shRNA revealed that IL-4 stimulated the tyrosine phosphorylation of IRS1 in BMMs prepared from mice of all three genotypes and that the basal extent of tyrosine phosphorylation of IRS1 was enhanced in the *IRS2*^{-/-} BMMs (Fig. 7B and fig. S5A). In the BMMs expressing IRS1 KD shRNA, the magnitude of tyrosine phosphorylation of IRS1 and the abundance of total IRS1 were greatly reduced as expected (Fig. 7B and fig. S5A). We also found that the expression of the IRS1 KD shRNA resulted in a decrease in the basal and IL-4-induced phosphorylation of Akt and S6 in total lysates prepared from BMMs of all three genotypes (Fig. 7B and fig. S5B), although the changes were not statistically significant. The knockdown approach required additional time in culture in the presence of MCSF and lentivirus-containing supernatant followed by selection in puromycin before the addition of cytokine. This requirement likely explains the increase in experimental variability in independent Western blotting analyses. We further found that knockdown of IRS1 substantially inhibited the IL-4-induced increase in *Retnla* expression in BMMs from all three mouse genotypes (Fig. 7C), with the greatest effect in the *IRS2*^{-/-} BMMs, but that *IRS2* knockdown only modestly inhibited *Chi3l3* expression (Fig. 7D). These results suggest that IRS1 plays an important role in IL-4-stimulated signaling and in modulating the expression of certain IL-4-induced genes in BMMs.

DISCUSSION

In addition to activating the STAT6 signaling pathway, IL-4, and to a lesser extent IL-13, also stimulates the tyrosine phosphorylation of IRS2. Phosphorylated IRS2 acts as an adaptor protein to recruit other signaling proteins, such as the p85 subunit of PI3K and Grb-2, to IL-4R α . The pathways activated by IRS2 lead to the phosphorylation of PI3K and Akt (41). Although the role of STAT6 in inducing the differentiation of AAMs and allergic lung inflammation has been studied extensively, much less is known about the contribution of IRS2.

Previously, we found that IL-4 elicited robust phosphorylation of IRS2 and AAM-associated gene expression in macrophages in vitro, whereas IL-13 induced substantially weaker responses (10). Moreover, IL-4-dependent signaling and gene expression are reduced in macrophages lacking the γ_c chain and the type I receptor. Thus, in this study, we examined whether the absence of IRS2 could suppress the IL-4-dependent

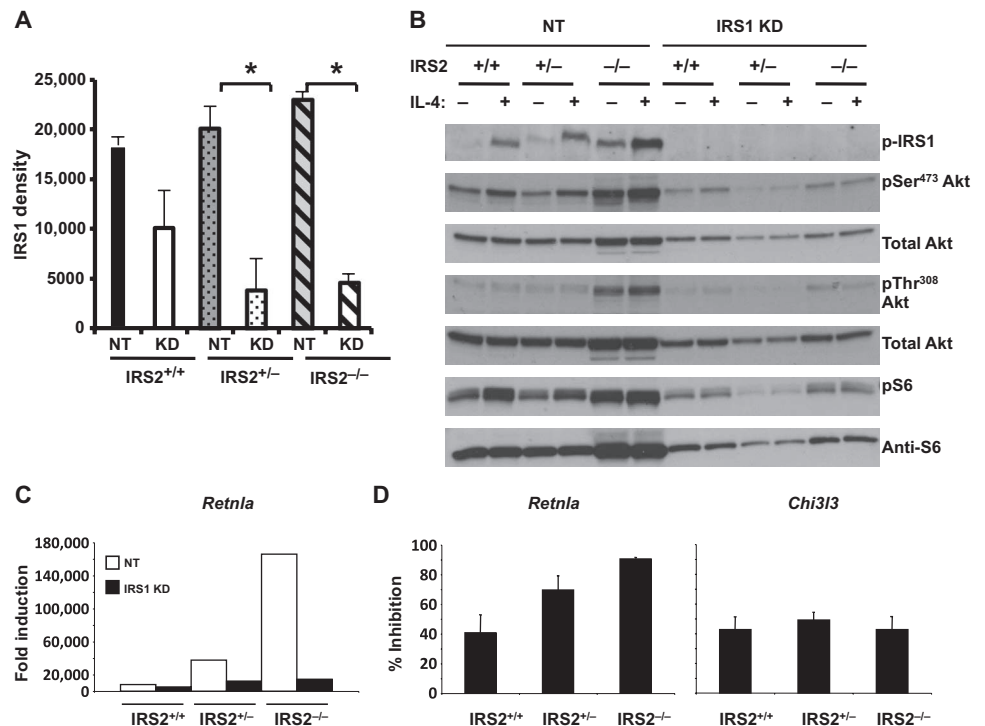


Fig. 7. Contribution of IRS1 to signaling pathways and gene expression. (A) BMMs isolated from the indicated mice were transduced with lentiviruses encoding NT shRNA or IRS1 KD shRNA. Seventy-two hours later, the relative abundance of *IRS1* mRNA was analyzed by qRT-PCR analysis and densitometry. Data are means $\pm$ SEM of three independent experiments. $*P < 0.05$. (B) BMMs from the indicated mice transduced with the indicated lentiviruses as described in (A) were deprived of serum and MCSF for 2 hours before being treated with or without IL-4 (20 ng/ml) for 30 min. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. Western blots are representative of three experiments. Pooled densitometric data from all experiments are shown in fig. S5. (C and D) BMMs from the indicated mice and treated as described in (A) were left unstimulated or were stimulated with IL-4 (100 ng/ml) for 6 hours. The relative abundances of *Retnla* and *Chi3l3* mRNAs in IL-4-treated samples relative to those in unstimulated cells were analyzed by qRT-PCR. (C) Representative data from one experiment showing the IL-4-induced fold induction in *Retnla* mRNA in BMMs expressing NT compared to that in BMMs expressing IRS1 KD shRNA. (D) The ability of the IRS1-targeting shRNA to suppress the IL-4-induced increase in the abundances of the indicated mRNAs relative to those in cells expressing the NT shRNA control was calculated as percentage inhibition: $[1 - (\text{fold induction by IL-4 in IRS1 KD} / \text{fold induction by IL-4 in NT})] \times 100$. Data are the mean percentage inhibition $\pm$ SEM of three independent experiments. $*P < 0.05$.

activation of AAMs. Contrary to our expectations, we found that the stimulation of *IRS2*^{-/-} macrophages with IL-4 or IL-13 enhanced the expression of *Retnla*, *Chi3l3*, and *Arg1* compared to that in wild-type macrophages. These findings suggest that *IRS2* inhibits the expression of AAM-associated genes. Moreover, the differential effects of IL-4 and IL-13 in the induction of AAM-associated gene expression were also observed in *IRS2*-deficient cells. Thus, the reduced quantities of AAM-associated transcripts that we observed in IL-13-stimulated macrophages were not due to reduced *IRS2* phosphorylation. The cytoplasmic tail of IL-13Rα1 contains two STAT3-binding sites (42). STAT3 activation occurs only through type II receptor signaling (43). Therefore, it is possible that the IL-13-mediated activation of STAT3 dampens the ability of STAT6 to increase gene transcription in response to this cytokine. Alternatively, the enhanced recruitment of the protein tyrosine phosphatase SHP1 by the type II receptor may be responsible for the difference in AAM-associated gene expression induced by IL-4 and IL-13. Mutation of the ITIM (immunoreceptor tyrosine-based inhibition motif) in the IL-4Rα chain (Y709F) results in the increased sensitivity of macrophages to IL-13-mediated generation of AAMs (44). In BMMs expressing IL-4Rα Y⁷⁰⁹, the IL-4-stimulated expression of the AAM-associated genes *Arg1*, *Chi3l3*, and *Ccl11* is greater than that in response to IL-13. However, BMMs expressing IL-4Rα F⁷⁰⁹ demonstrate enhanced STAT6 phosphorylation and substantially increased expression of *Arg1*, *Chi3l3*, and *Ccl11* in response to IL-13, whereas IL-4-stimulated responses are intact or slightly enhanced (44). The authors of that study proposed that differential recruitment of SHP1 by the type I and type II receptors might be responsible for the disproportionate increase in AAM-associated gene expression induced by IL-13.

The reason for the enhanced induction of AAM-associated genes in the absence of *IRS2* is unclear because we found that proximal STAT6 activation was normal in *IRS2*^{+/-} and *IRS2*^{-/-} mice. We and others showed that STAT6 signaling is essential for the induction of AAM-associated genes (45). The promoter regions of *Retnla*, *Chi3l3*, and *Arg1* all have critical STAT6-binding sites in close proximity to CCAAT/enhancer binding protein (C/EBP)-binding sites (46–48). Moreover, STAT6 and C/EBP cooperate to induce maximal expression of *Retnla* and *Arg1* (47, 48). Insulin signaling through the *IRS1*/2-PI3K-Akt pathway suppresses the C/EBPβ-mediated transactivation of genes (49). The insulin-induced activation of Akt disrupts interactions between the C/EBPβ transactivation domain and certain coactivator proteins (such as p300/CBP), abrogating the C/EBP-mediated activation of gene expression. Because IL-4 (and, to some extent, IL-13) also activates the *IRS2*-PI3K-Akt pathway, it is possible that IL-4, IL-13, or both suppress C/EBP as well.

Another possible explanation for the enhanced gene expression is the loss of negative feedback mechanisms. Weisser *et al.* demonstrated that a deficiency in SHIP results in the increased IL-4-induced differentiation of AAMs (27). Conversely, macrophages stimulated with IL-4 produce less SHIP but more YM1 and *Arg1*. Furthermore, degradation of SHIP and induction of the AAM phenotype depend on the kinase activity of PI3K. Here, we showed that in the absence of *IRS2*, the IL-4-stimulated coimmunoprecipitation of *IRS2* with p85 was abrogated. Despite this loss, both the basal and IL-4-induced phosphorylation of Akt and S6 were greater in *IRS2*^{+/-} and *IRS2*^{-/-} macrophages than in *IRS2*^{+/+} macrophages and were reduced in extent by the PI3K inhibitor LY294002. These results suggest that there is an alternate mechanism to activate the PI3K-Akt axis in response to IL-4 or IL-13 in the absence of *IRS2*. Our studies have identified *IRS1* as an alternative adaptor protein that is used by IL-4 for signaling through the PI3K-Akt pathway and the modulation of gene expression in BMMs.

Another possible negative feedback mechanism could be mediated through the serine phosphorylation of *IRS1* or even of *IRS2* itself. A study

defined a role for mammalian target of rapamycin complex 1 (mTORC1) in attenuating the IL-4-induced differentiation of AAMs (50). The macrophage-specific deletion of *TSC1* resulted in constitutive activation of mTORC1, which led to reduced IL-4-dependent phosphorylation of Akt and expression of *Arg1* and *Retnla*. It has been proposed that the serine phosphorylation of *IRS1* or *IRS2* by mTORC1 or other kinases inhibits their ability to link to the PI3K-Akt pathway (51). In this scenario, the IL-4-dependent activation of Akt and mTORC1 in macrophages could then act as a negative regulator of the PI3K-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*^{+/-}), which perhaps explains the enhanced AAM phenotype and allergic lung inflammation responses in these mice. A number of studies evaluating the role of mTORC1 in AAM differentiation in mice and humans have reported conflicting results (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-stimulated activation of the Akt pathway in macrophages from *IRS2*-deficient mice support a regulatory role for this adaptor protein. Consistent with our findings, the enhanced basal activity of PI3K in the liver and skeletal muscle of *IRS2*-deficient mice was noted previously but not analyzed further (21).

Our evaluation of the role of *IRS2* in allergic lung inflammation *in vivo* also indicated that this signaling protein suppressed pulmonary inflammation, eosinophilia, and YM1 production in macrophages. Allergen sensitization and challenge in *IRS2*^{+/-} and *IRS2*^{-/-} mice led to a substantial increase in the numbers of eosinophils present in the BAL fluid compared to those in the BAL fluid of *IRS2*^{+/+} mice. Examination of H&E-stained lung sections from *IRS2*^{+/-} and *IRS2*^{-/-} mice also showed increased inflammation, with mononuclear cells and eosinophils surrounding the airways and blood vessels. The presence of increased inflammation in the *IRS2*^{+/-} mice, which showed no signs of peripheral insulin resistance, indicates that our findings were not simply due to global metabolic changes in the mice. Adoptive transfer experiments with macrophages revealed that this increase in inflammation was, in part, due to the effect of *IRS2* deficiency in the macrophage itself. The effect of *IRS2* deficiency in the absence of transferred exogenous macrophages (Fig. 2B) was more substantial on the total number, rather than the percentage, of eosinophils, whereas the transfer of large numbers of *IRS2*-deficient macrophages increased the percentages of eosinophils in both *IRS2*^{+/-} and *IRS2*^{-/-} recipients (Fig. 5B). It is possible that large numbers of *IRS2*^{-/-} macrophages acted to specifically increase the percentages of eosinophils and the total number of cells recruited by secreting chemotactic factors such as YM1 and eotaxin-1.

No statistically significant differences in the amounts of IL-4, IL-5, or IL-13 were detected in the BAL fluid of *IRS2*^{+/+}, *IRS2*^{+/-}, and *IRS2*^{-/-} mice. A slight reduction in the amount of IL-5 was seen in *IRS2*^{-/-} mice, which may have been due to the increased consumption of this cytokine by the increased numbers of eosinophils. *In vitro* studies by Wurster *et al.* indicated that *IRS2* was not required for the differentiation of naïve T cells into T_H2 cells but that it modestly influenced the quantities of IL-4 and IL-5 produced (24). Our results showed that T_H2-type cytokine production and allergic inflammation *in vivo* occurred independently of *IRS2*. We previously found that the transgenic overexpression of *IRS2* led to enhanced IgE production by B cells upon OVA stimulation (25); however, the results of our current study demonstrated that *IRS2* was not required for IgE production.

The enhanced allergic lung inflammation phenotype that we observed in mice deficient in *IRS2* is consistent with the findings reported by Blaeser *et al.*, who 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, increased serum IgE, increased goblet cell metaplasia, and AHR in comparison to the Y⁵⁰⁰ mice. Both groups of mice produced similar amounts of T_H2-type cytokines. Unlike the Y500F mutation, IRS2 deficiency did not affect serum IgE amounts 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, IL-4R α is present in all cell types, whereas the activation pattern and functional role of IRS2 are more limited. SHIP1 inhibits the differentiation of naïve cells into T_H2 cells and allergic lung inflammation (53). *SHIP1*^{-/-} mice spontaneously develop pulmonary inflammation, with the recruitment of macrophages and eosinophils to the lung. Loss of SHIP1 also leads to the increased expression of the gene encoding chitinase and enhanced lung remodeling. Because the phenotype observed in *IRS2*^{-/-} mice in this study is similar to that of SHIP1-deficient animals, it is possible that the loss of IRS2 results in decreased negative regulation by SHIP1 in vivo.

Similar to the in vitro AAM-associated gene expression data, we found that YM1 protein intensity in macrophages was increased in mice lacking either one or both alleles of *IRS2* in vivo. Several reports suggest a link between the increased quantities of AAM-secreted proteins seen in *IRS2*-deficient mice and the severity of pulmonary inflammation; however, no single product has been accepted as the critical agent. YM1 can act as a chemoattractant to recruit eosinophils into the lung (30), and it may also be involved in tissue remodeling and fibrosis (31). In addition to secreting YM1, AAMs can secrete many other factors, such as IGF-1, acidic mammalian chitinase (AMCase), breast regression protein-39 (BRP-39), and various chemokines. The IL-13-mediated production of AMCase in macrophages and epithelial cells causes T_H2 cell-dependent inflammation and AHR, which are abrogated when AMCase is neutralized (54). Alveolar macrophages synthesize large quantities of BRP-39, a chitinase-like protein, during allergic lung inflammation, and antigen-specific T_H2-type responses, tissue inflammation, and fibrosis are substantially reduced in mice deficient in BRP-39 (32).

Daley *et al.* 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. Furthermore, their study also provided evidence that antigen-induced pulmonary vascular remodeling is substantially suppressed in *IL-4*^{-/-} mice, suggesting that an IL-4-dependent, T_H2-type response induces severe pulmonary vascular remodeling. In addition, IL-4 is necessary for the development of severely remodeled pulmonary arteries and for the recruitment of FIZZ1-positive cells around them (38). In the absence of IRS2, we observed a marked increase in the muscularization of small vessels in an acute allergen sensitization/challenge model, which was accompanied by increased production of FIZZ1 by cells surrounding the blood vessels. These results suggest a previously uncharacterized critical role for IRS2 in controlling pulmonary arterial remodeling induced by a T_H2-type immune response. These findings are consistent with a report demonstrating that *IRS2*-deficient mice develop increased neointima formation in a vessel injury-induced mouse model of atherosclerosis (55). Injury of the femoral artery induces substantially increased intima thickness in *IRS2*^{-/-} mice compared to that in *IRS2*^{+/-} mice. In another study, Mita *et al.* demonstrated that the transfer of *IRS2*-deficient bone marrow cells into wild-type mice leads to the enhanced accumulation of *IRS2*^{-/-} macrophages in the vascular wall together with the increased production of proinflammatory cytokines such as MCP-1 (56). Through an adoptive transfer approach, we determined that

the inhibition of pulmonary arterial muscularization by IRS2 was at least, in part, macrophage-intrinsic. However, additional effects of IRS2 in vascular endothelial cells, smooth muscle cells, or both may also participate in the complex crosstalk between macrophages and structural cells in vivo because we did not observe a further increase in the intensity of FIZZ1 staining in perivascular macrophages when *IRS2*^{-/-} BMMs were transferred to *IRS2*^{+/-} or *IRS2*^{+/-} recipient mice. Whereas our in vitro signaling studies demonstrated a link between control of the PI3K-Akt pathway and *Retnla* expression by IRS2 in MCSF-dependent macrophages, these in vivo results suggest that the control of signaling pathways and protein production during inflammatory responses is more complex.

MATERIALS AND METHODS

Mice

B6;129-*IRS2*^{tm1Mfw}/J mice were cryorecovered at The Jackson Laboratory under contract. Two pairs of male and female *IRS2*^{+/-} mice were shipped to the University of Maryland, Baltimore, and bred in house. C57BL/6 NTac mice were purchased from Taconic. The *IRS2*^{+/-} mice were backcrossed to the C57BL/6 NTac background for 10 generations before being used to establish breeding colonies. Because *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 with primers and protocols recommended by The Jackson Laboratory. In addition to the genotyping of *IRS2* mice, blood glucose was measured at the time of weaning pups (3 weeks) and monitored every few days until the mice were used in experiments. As previously described (21), *IRS2*^{+/-} and *IRS2*^{+/-} mice use glucose normally and maintain normal blood glucose concentrations throughout their lives. The *IRS2*^{-/-} mice show mild insulin resistance, develop increasing glucose quantities over time (10 to 12 weeks of age), and eventually develop diabetes (21). Therefore, in all of our experiments, we used mice that were less than 8 weeks of age and had blood glucose concentrations less than 250 mg/dl (nondiabetic). Blood glucose quantities measured in mice of all three genotypes (*IRS2*^{+/-}, *IRS2*^{+/-}, and *IRS2*^{-/-}) were not statistically significantly different from one another at this age. All experimental procedures mentioned here were performed in accordance with the guidelines issued by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore.

Cell culture

Single-cell suspensions were prepared from mouse spleens by mechanical disruption. To generate BMMs, bone marrow was harvested from the femurs and tibias of mice. Adherent stromal cells were depleted by culturing the bone marrow overnight in complete α -minimum essential medium (α -MEM). Nonadherent 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 recombinant mouse MCSF (20 ng/ml; R&D Systems). This medium was replenished every other day for 4 to 6 days. The cells were then stimulated in the presence or absence of the amounts of IL-4 or IL-13 (R&D Systems) indicated in the figure legends for various times in the presence or absence of 1 μ M LY294002.

IRS1 knockdown

To knock down IRS1 in BMMs, shRNA-IRS1-PLKO.1-puro and control NT lentiviral vectors were obtained (MISSION shRNA SHCLNG-NM_010570 and SHC002V, respectively; Sigma-Aldrich) and were used to transfect human embryonic kidney (HEK) 293T cells together with psPax2 and pMD2.G (developed by D. Trono, EIA, and provided by

R. Feldman from the University of Maryland) with FuGENE6 reagent (Roche) as described previously (57). After 24 hours, the transfection medium was replaced with fresh medium containing 1 mM sodium pyruvate and 25 mM Hepes. Viral supernatant was collected at 48, 72, and 96 hours after infection, filtered through a 0.45- μ m filter, and added on the same day to primary BMM cultures in the presence of Polybrene (20 ng/ml) at a viral supernatant-to-medium ratio of 1:3. One day after the third addition of viral supernatant, the cells were rested in complete medium with MCSF for 1 day before undergoing selection with puromycin (1 μ g/ml) for 1 day. The medium was subsequently replaced, and the cells were treated with IL-4 for various times. Total mRNA and cell lysates were prepared for RNA and protein analysis, respectively.

Immunoprecipitations and Western blotting analysis

Total cell lysates were subjected to immunoprecipitation with antibodies against IRS2, IRS1, p85 (Upstate/Millipore), or STAT6 (M-20, Santa Cruz) and Protein G-agarose beads (Life Technologies). Samples were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon membrane (Millipore). The blots were blocked with bovine serum albumin (Sigma-Aldrich) and incubated with antibodies against phosphotyrosine residues (PY-20; BD Biosciences) and pSTAT6 (STAT6-pTyr⁶⁴¹; Cell Signaling Technologies); total IRS2, IRS1, or p85 (Upstate/Millipore); or STAT6 (M-20; Santa Cruz Biotechnology) as appropriate. Total cell lysates were evaluated by Western blotting with antibodies against pAkt-Ser⁴⁷³, pAkt-Thr³⁰⁸, total Akt, phosphorylated ribosomal S6 protein (pS6-Ser^{235/236}), or total S6 protein (Cell Signaling Technologies). Horseradish peroxidase-linked secondary anti-mouse or anti-rabbit antibodies were used (GE Healthcare). Protein bands were detected with a chemiluminescence reagent (enhanced chemiluminescence; Denville Scientific Inc.).

Densitometric analysis

Shorter exposures of films were chosen for densitometric analysis to ensure that the intensities of bands were in the linear range of the film. The films were scanned on a flatbed scanner, and the integrated densities of bands of interest were calculated with NIH ImageJ Software. The relative abundance of phosphorylated protein 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 three independent experiments, the ratio of phosphoprotein to 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.

qRT-PCR analysis

qRT-PCR analysis was performed as described previously (10). Briefly, total RNA was isolated from BMMs with the RNeasy kit (Qiagen), and DNA contamination was removed by deoxyribonuclease treatment. Complementary DNA (cDNA) was generated with the SuperScript III First-Strand Synthesis System (Life Technologies). RT-PCR was performed with specific primer sets (Life Technologies) by methods previously described (58) on an Applied Biosystems 7900HT machine. The relative abundance of a given mRNA was calculated as fold induction over the amounts detected in control samples, with hypoxanthine guanine phosphoribosyl transferase (*HPRT*) used as the internal reference gene [$2^{-\Delta\Delta C_t}$ method (59)]. To compare results from a minimum of three independent experiments, the fold induction in the abundance of a given 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 OVA/alum model of allergic lung inflammation was used. Mice were immunized twice either with 100 μ g of OVA and alum or with alum alone 5 days apart. One week later, on days 12 and 14, the mice were challenged with aerosolized 1% OVA in PBS for 40 min. BAL 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, and the cellular component was used for cytospin preparation and differential counts or flow cytometric analysis, whereas the supernatant was used for cytokine analysis.

Adoptive cell transfer

The adoptive transfer of macrophages was performed as described previously (60). MCSF-dependent BMMs were prepared from 4-week-old *IRS2*^{+/+} or *IRS2*^{-/-} mice as described earlier. After 6 days in culture, the adherent cells were isolated with a cell scraper and collected by centrifugation. These cells were routinely >95% F4/80⁺CD11b⁺ macrophages. BMMs were washed and transferred to *IRS2*^{+/+} or *IRS2*^{-/-} recipient mice by intraperitoneal injection at 5×10^6 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 to 15 ml of PBS and immediately fixed with 10% formalin. The tissues were embedded in paraffin, sectioned, and stained with H&E or periodic acid-Schiff. For immunohistochemistry, lung sections were stained with a 1:100 dilution of rabbit anti-mouse FIZZ1 antibody (Abcam) or a 1:100 dilution of rabbit anti-mouse YM1 antibody (Stem Cell Technologies), followed by biotinylated anti-rabbit antibodies (1:200; Vector Laboratories). Slides were then incubated with Elite ABC 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 with NIH ImageJ software (45). Pulmonary vascular remodeling of the mice was assessed as previously described (37). Briefly, lung sections were dual-labeled with antibodies to vWF (Dako) and α -SMA (Dako) to stain the endothelium and vascular smooth muscle, respectively. The sections were then counterstained with hematoxylin (37). To assess remodeling of the lung arteries and arterioles, an investigator blinded to the treatment group examined 100 arteries per lung section at random under a 40 $\times$ objective lens with an Olympus BHS microscope (Olympus) attached to a QImaging Retiga-4000RV digital camera (QImaging). Small arteries with an internal diameter ≤ 80 μ m were then classified as nonmuscular, partially muscular, or fully muscular according to SMA staining. Negative control sections for the immunohistochemical experiments received identical treatments but were not exposed to the primary antibody; hence, they showed no specific staining. In some cases, the smooth muscle cell thickness of multiple individual small pulmonary arteries from multiple lungs per group was measured as described previously (45).

Immunofluorescence and confocal microscopy

Immunofluorescence staining was performed as described previously (37). Briefly, the paraffin sections were blocked with an appropriate blocking serum (Vector Laboratories) for 1 hour at room temperature and then treated with anti-FIZZ1 (Abcam) and anti-SMA (Dako) antibodies. The sections were then incubated with the appropriate fluorochrome-coupled

secondary antibodies (Jackson ImmunoResearch). Finally, the sections were washed in PBS, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), 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; hence, they showed no specific staining. Staining was imaged with a Zeiss 510 Meta confocal microscope (Carl Zeiss Microscopy) at The Johns Hopkins School of Medicine Microscope Facility.

Cytokine and IgE analysis

Cytokines in the BAL fluid were analyzed by enzyme-linked immunosorbent assay kits for IL-4 (BioLegend), IL-5, and IL-13 (both from R&D Systems). The concentration of IgE in the serum was measured with the BD OptEIA kit (BD Biosciences).

Flow cytometric analysis

Single-cell suspensions of BAL fluid cells were incubated with Fc Block (2.4G2, BD Biosciences) and then were incubated with fluorochrome-conjugated antibodies to surface markers (CD11b, F4/80, and Siglec F; BD Biosciences). After being washed twice, the cells were fixed with 4% paraformaldehyde and permeabilized with a BD Cytofix/Cytoperm kit (BD Biosciences). Intracellular cytokine staining for YM1 was performed. Cells were treated with an unconjugated anti-YM1 antibody (Stem Cell Signaling), followed by a fluorochrome-conjugated secondary antibody. Samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed by FlowJo software (Tree Star). Macrophages were gated by forward scatter and side scatter and then based on the presence of CD11b and F4/80 and the absence of Siglec F.

Statistical analysis

To assess the statistical significance of differences between the means of multiple groups, we used a global *F* test based on one-way analysis of variance (ANOVA). If *P* < 0.05, we performed post hoc pairwise tests with Tukey's multiple comparison approach. Similarly, repeated-measures ANOVA followed by post hoc Tukey's test was used for the analysis of normalized data derived from repeat experiments performed independently (for example, Western blots and qRT-PCR analyses). Analyses were performed with GraphPad Prism software.

SUPPLEMENTARY MATERIALS

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Fig. S1. The STAT6 pathway is unaffected by the loss of IRS2.

Fig. S2. Loss of IRS2 has no effect on epithelial responses or cytokine and IgE production.

Fig. S3. Analysis of FIZZ1 abundance in mice after the adoptive transfer of macrophages.

Fig. S4. Quantitation of the effects of *IRS2* genotype and LY294002 on the IL-4-stimulated activation of the Akt pathway.

Fig. S5. Quantitation of the effects of IRS1 knockdown.

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