The phosphatase CD148 promotes airway hyperresponsiveness through SRC family kinases

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Increased airway smooth muscle (ASM) contractility and the development of airway hyperresponsiveness (AHR) are cardinal features of asthma, but the signaling pathways that promote these changes are poorly understood. Tyrosine phosphorylation is tightly regulated by the opposing actions of protein tyrosine kinases and phosphatases, but little is known about whether tyrosine phosphatases influence AHR. Here, we demonstrate that genetic inactivation of receptor-like protein tyrosine phosphatase J (Ptprj), which encodes CD148, protected mice from the development of increased AHR in two different asthma models. Surprisingly, CD148 deficiency minimally affected the inflammatory response to allergen, but significantly altered baseline pulmonary resistance. Mice specifically lacking CD148 in smooth muscle had decreased AHR, and the frequency of calcium oscillations in CD148-deficient ASM was substantially attenuated, suggesting that signaling pathway alterations may underlie ASM contractility. Biochemical analysis of CD148-deficient ASM revealed hyperphosphorylation of the C-terminal inhibitory tyrosine of SRC family kinases (SFKs), implicating CD148 as a critical positive regulator of SFK signaling in ASM. The effect of CD148 deficiency on ASM contractility could be mimicked by treatment of both mouse trachea and human bronchi with specific SFK inhibitors. Our studies identify CD148 and the SFKs it regulates in ASM as potential targets for the treatment of AHR.

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CD148-deficient mice are protected from AHR in the OVA mouse model of allergic airway disease and have decreased baseline pulmonary resistance. (A) Pulmonary resistance measurements in WT and CD148-deficient (Ptprj\textsuperscript{TM–/TM–}) mice following immunization and intranasal challenge with OVA or saline in C57BL/6 mice (A) or BALB/c mice (B). Data are the mean ± SEM (n = 9–13 animals per group). Statistical significance determined by 2-way ANOVA. *P < 0.05, ***P < 0.001 in the WT versus Ptprj\textsuperscript{TM–/TM–} OVA group; #P < 0.05, ##P < 0.001 comparing the highest dose of ACh in the WT saline group with the Ptprj\textsuperscript{TM–/TM–} saline group.

Figure 1

CD148-deficient mice are protected from AHR in the OVA mouse model of allergic airway disease.
contributions from the Th1 and Th17 immune arms (31, 32). We therefore also examined mediastinal lymph node and lung T cell subsets producing IFN$\gamma$ and IL-17 and found no significant differences in numbers or percentages of these cells between OVA-sensitized and -challenged WT and PtprjTM–/TM– BALB/c mice (Figure 3, F and H–L).

CD148-deficient mice are protected from AHR induced by house dust mite. Although the OVA-induced model of allergic airway disease is one of the most widely used, an important limitation is that OVA is not an antigen that triggers human asthma. Another caveat is that sensitization with OVA through the respiratory tract is not generally successful, thus intraperitoneal sensitization in conjunction with adjuvant is conventionally used (33). Therefore, we opted to extend our studies to the house dust mite (HDM) model, which utilizes a known human antigen and involves sensitization through the respiratory mucosa and thus may better recapitulate human asthma. We found that AHR was similarly attenuated both at baseline and following HDM antigen sensitization and challenge in PtprjTM–/TM– mice (Figure 4A). A slight, but statistically significant, decrease in BAL lymphocytes and macrophages and PAS histologic staining was observed in PtprjTM–/TM– mice compared with control mice, but other inflammatory parameters were not significantly affected (Figure 4, B–D). Collectively, these studies suggest the possibility that the protection afforded to mice by the loss of the CD148 phosphatase in 2 experimental models of asthma and in 2 strains of mice might not reflect a substantial effect of CD148 on the immune responses mediated by hematopoietic cells.

Hematopoietic and endothelial lineage deletion of CD148 does not affect AHR. Since CD148 is expressed on hematopoietic as well as on nonhematopoietic cells, and the contribution of the Th2 immune response in asthma is very well documented (34), we wanted to further verify that the protection afforded by CD148 deficiency was indeed independent of its function in hematopoietic lineage cells. Using mice harboring a floxed allele of CD148 (which also eliminates the membrane-encoding exon) that allows for the lineage-specific inactivation, CD148 function was eliminated from hematopoietic and endothelial cells using a Vav1-Cre transgene (PtprjTM–fl/TM–; Vav-Cre). B cells (Figure 5A) and monocytes (data not shown) from PtprjTM–fl/TM–; Vav-Cre mice verified CD148 deletion efficiency comparable to Ptprj TM–/TM– constitutively deleted cells. When these mice were challenged with OVA, there was no statistically significant difference in the development of AHR when CD148 was deleted from hematopoietic and endothelial cells (PtprjTM–fl/TM–; Vav-Cre mice) as compared with control mice (Figure 5B). Additionally, baseline pulmonary airway resistance was unaffected by deletion of CD148 from these lineages. The inflammatory response revealed an increase in total BAL cell counts in PtprjTM–fl/TM–; Vav-Cre mice, which was largely driven by an increase in neutrophils, but other cell types were comparable to control groups (Figure 5C). Likewise, histologic scores and serum IgE did not differ in the Vav-Cre–targeted mice compared with the responses of control mice (Figure 5, D and E). Collectively, these results support the notion that CD148 protection from induced AHR is independent of its expression in hematopoietic lineage cells.
Partial deletion of CD148 from smooth muscle leads to attenuation of AHR. Since CD148 is expressed in smooth muscle, we wondered whether CD148 phosphatase function in ASM could be contributing to AHR. We therefore generated mice specifically lacking CD148 in smooth muscle by crossing our PtprjTM–/TM–; SMA-rTTA Cre mice with mice expressing the reverse tetracycline transactivator under the control of the α-smooth muscle actin promoter (α-SMA-rTTA) and (tetO)7-Cre (35). The tissue specificity of deletion in smooth muscle has been previously confirmed (36). Mice carrying all 4 alleles will be referred to as PtprjTM–/TM–; SMA-rTTA Cre mice and PtprjTM–/TM– Cre mice (data not shown). A caveat of this strategy is that deletion of the floxed CD148 allele is not specific to ASM, as it would also occur in vascular smooth muscle.

Mice with the deletion of CD148 in smooth muscle (PtprjTM–/TM–; SMA-rTTA Cre) demonstrated diminished AHR compared with PtprjTM–/TM– Cre mice, however the magnitude of diminution was less profound than in constitutively deleted mice (Figure 6B). The inflammatory response was comparable between PtprjTM–/TM–; SMA-rTTA Cre and control PtprjTM–/TM– Cre mice, as measured by BAL cell accumulation (Figure 6C), mucus production (Figure 6D), and OVA-specific IgE production (Figure 6E). The less robust protection from AHR could be a consequence of incomplete deletion of CD148 from bronchial ASM, as well as the possibility that CD148 acts on other cell types, such as airway epithelium that could be mediating AHR. Nonetheless, the statistically significant reduction in AHR in PtprjTM–/TM–; SMA Cre mice illustrates an impact of CD148 on signaling pathways in ASM, cells that play a critical role in bronchoconstriction.

Absence of CD148 phosphatase activity impairs tracheal contractility and SFK activation. ASM contraction occurs via GPCR activation, such as through Gq-coupled muscarinic, histamine, endothelin, thromboxane, and cysteinyl leukotriene receptors, and Gq-coupled 5-HT and adenosine receptors (37). Stimulation of the Gq receptor in ASM leads to activation of phospholipase C beta (PLCβ), which hydrolyzes phosphoinositol 4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Increased IP3 and DAG levels stimulate an increase in intracellular calcium through release from internal stores and influx from membrane-bound channels and activation of PKC. The rise in intracellular calcium promotes calcium binding to calmodulin, which complexes with and activates myosin light chain kinase (MLCK). MLCK phosphorylates regulatory myosin light chains (MLCs) and enables actin to activate the myosin ATPase activity required for cross-bridge cycling and contraction (38).

We hypothesized that an intrinsic difference in ASM contractility in PtprjTM–/TM– mice accounted for the attenuated AHR observed. We measured the contractility of tracheal rings from PtprjTM–/TM– and control mice following methacholine (MCh) treatment. Diminished contractility was observed following MCh stimulation of tracheal rings harvested from PtprjTM–/TM– mice (Figure 7A). We speculated that positive regulation of SFKs in ASM by thromboxane, and cysteinyl leukotriene receptors, and Gq-coupled 5-HT and adenosine receptors (37). Stimulation of the Gq receptor in ASM leads to activation of phospholipase C beta (PLCβ), which hydrolyzes phosphoinositol 4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Increased IP3 and DAG levels stimulate an increase in intracellular calcium through release from internal stores and influx from membrane-bound channels and activation of PKC. The rise in intracellular calcium promotes calcium binding to calmodulin, which complexes with and activates myosin light chain kinase (MLCK). MLCK phosphorylates regulatory myosin light chains (MLCs) and enables actin to activate the myosin ATPase activity required for cross-bridge cycling and contraction (38).

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ulotary tyrosine strongly suggests that it serves as a substrate for the CD148 phosphatase, though it does not exclude the existence of other CD148 substrates in ASM. Stimulation of agarose-embedded lung slices with MCh induces circumferential calcium oscillations along the ASM, the frequency of which determines airway contractility (39). We observed a striking 2-fold decrease in calcium oscillation frequency in MCh-stimulated lung slices from PtprjTM–/TM– mice compared with WT mice (Figure 7, D and E, and Supplemental Videos 1 and 2), further substantiating the positive regulatory effect of CD148 on ASM contractility. We wondered whether treatment with an SFK inhibitor might recapitulate the genetic loss of the CD148 phosphatase, though it does not exclude the existence of other CD148 substrates in ASM. Stimulation of agarose-embedded lung slices from PtprjTM–/TM– mice compared with WT mice (Figure 7, D and E, and Supplemental Videos 1 and 2), further substantiating the positive regulatory effect of CD148 on ASM contractility.

Pharmacologic inhibition of SFKs attenuates mouse tracheal contractility and human bronchial contractility. We wondered whether treatment with an SFK inhibitor might recapitulate the genetic loss of CD148 phosphatase activity. SU6656 is an SFK-specific inhibitor targeting SRC, YES, FYN, and LYN (40), the primary SFKs found in ASM (41). Mouse tracheal rings pretreated with SU6656 had significantly diminished contractility following stimulation with MCh (Figure 8A) compared with vehicle treatment. To further substantiate that this inhibitory effect was a consequence of SFK inhibition, we used a chemically distinct and highly specific SFK inhibitor, AZD0530, which targets multiple SFK members with high potency. Treatment of WT tracheal rings with AZD0530 led to a significant decrease in tracheal contractility (Figure 8B). PtprjTM–/TM– tracheal rings exhibited diminished baseline contractility, as previously observed, but importantly, AZD0530 treatment of PtprjTM–/TM– tracheal rings did not further impair tracheal contractility, strongly arguing that the genetic loss of Ptprj and the inhibition of SFK were in a similar pathway (Figure 8C). Human bronchial rings pretreated with SU6656 similarly demonstrated diminished contractile force in response to MCh (Figure 8D). This observation supports the contribution of SFKs to human ASM contractility and suggests relevance to AHR in human asthma.

Discussion

Dysregulated ASM function underlies the pathogenesis of asthma and AHR (6, 42). The contraction and relaxation of ASM is primarily mediated through various GPCRs, the aberrant regulation of which can lead to AHR (37, 43, 44). Cytokines, growth factors, integrins, and other mediators further modulate ASM function. The control of ASM contractility by tyrosine phosphatases has not been characterized. Our work provides evidence that the RPTP CD148 is a critical positive regulator of SFKs in ASM. We observed marked attenuation of AHR in mice lacking CD148 that is not primarily attributable to a dampened immune response. In addition, we report that CD148-deficient mice manifest diminished baseline pulmonary resistance in the absence of allergen sensitization and challenge, implicating decreased intrinsic ASM contractility. We provide compelling genetic, functional, and biochemical evidence that CD148 positively regulates ASM contractility via SFKs. Pharmacologic inhibition of SFKs diminishes baseline pulmonary contractility, the frequency of which determines airway contractility, but does not exclude the possibility that other cell types, such as airway epithelium, may also be contributing to the attenuation. We show that CD148 deficiency impairs SFK activation, leading to decreased murine tracheal contractility that is recapitulated by chemical inhibition of SFKs in both murine trachea and human bronchi. Our data suggest that inhibition of CD148 phosphatase activity or specific inhibition of SFKs may be attractive therapeutic strategies for the treatment of AHR.

Surprisingly little is understood about the physiologic functions of the RPTP CD148. Most studies have been based on overexpression in cell lines or knockdown approaches, which may not accurately reflect in vivo biology. Overexpression may lead to loss of phosphatase substrate specificity, and incomplete knockdown may result in variable effects, given the complex positive and negative regulatory properties of phosphatases (45). Here, we have used a mouse carrying a targeted deletion of the CD148 transmembrane domain such that phosphatase activity was abrogated. We have previously shown that CD148, in conjunction with another RPTP, CD45, positively regulates SFKs in B cell and macrophage immunoreceptor signaling by dephosphorylating the C-terminal inhibitory tyrosine of SFKs (19). In ASM, which does not express CD45, we have discovered that the CD148 phosphatase plays a prominent role in the positive regulation of SFKs. We speculate...
that the C-terminal tyrosine of SFKs may serve as the direct substrate for CD148, given that it was found to be hyperphosphorylated in CD148 phosphatase–deficient ASM tissue, though this finding does not exclude the possible existence of other CD148 substrates. Of the predominant SFKs expressed in human ASM (SRC, YES, FYN, and LYN) (41), it is unclear which of these individual SFK members most prominently contributes to the generation of AHR. Future investigation will be needed to define which SFKs are specifically targeted by CD148 in ASM. For instance, our group has recently shown that in neutrophils, CD45 and CD148 play contrasting roles in chemoattractant G protein–coupled receptor signaling, which is in part mediated by the ability of CD45 and CD148 to preferentially regulate different SFK members. Therefore, CD148 may dephosphorylate the C-terminal inhibitory tyrosine residue of specific SFKs that are critical positive regulators of ASM contractility.

Networks of cytokines, growth factors, chemokines, and neurotransmitters work in concert, exerting effects on several different cell types implicated in asthma pathogenesis (46). Given this complexity, targeting one specific mediator may have limited benefit. Various receptor and nonreceptor tyrosine kinase pathways, in both hematopoietic and structural cells, contribute to asthma pathogenesis. SRC family kinases are enzymes that proximally regulate several pathways relevant to asthma, including antigen receptors, receptor tyrosine kinases, cytokine receptors, GPCRs, and integrins (16, 47). Classical GPCRs, which are directly implicated in ASM contractile responses, activate serine/threonine kinases or ion channels regulated by second messengers leading to rapid short-term responses. Mounting evidence also implicates GPCR signaling in the control of cell growth, proliferation, and differentiation by activating tyrosine phosphorylation cascades, including SFKs (48). SFKs can integrate the crosstalk between GPCR and RTK signaling pathways, and many mechanisms have been implicated, including the direct association of GPCRs with SFKs or other receptor-associated proteins, as well as the transactivation of RTKs and focal adhesion complexes by GPCR stimulation (16, 47, 48).

The mechanisms by which SFKs are regulated in ASM, and in particular the phosphatases that control their activation status, remain obscure (48–50). Much of our knowledge is based on the use of chemical SFK inhibitors, which not only lack selectivity for specific SFK members, but also target other kinases. Our understanding of SFKs is further complicated by the existence of multiple SFK members that may have contrasting functions as well as differing patterns of expression in various tissues. One example is the SFK LYN, which is known to play both positive and negative regulatory roles (51). Mice genetically deficient for LYN demonstrated an enhanced Th2 response and more severe asthma, likely related to the negative regulatory effects of LYN in the hematopoietic lineage (52). Therefore, in considering SFK inhibition as a potential asthma therapy, an optimal SFK inhibitor should not target LYN or the negative regulatory effects of LYN in the hematopoietic lineage (52).
Figure 6
Partial protection from AHR in mice with deletion of CD148 from smooth muscle cells. (A) Immunofluorescence staining of mouse tracheal smooth muscle with primary hamster antibody to CD148, secondary goat anti-hamster Alexa 488 (green), and α-SMA (SMA, red) as indicated. Right column shows quantitative ImageJ analysis of line scans (areas indicated by white boxes). Mice of the indicated genotypes were immunized and intranasally challenged with OVA or saline (B–E). (B) Pulmonary resistance measurements after intravenous administration of increasing doses of ACh in Ptprj<sup>TM-<i>fl/fl</i></sup>;TetO-Cre and Ptprj<sup>TM-<i>fl/fl</i></sup>;SMA-rTTA;TetO-Cre mice of the C57BL/6 strain. *<i>P</i> < 0.05 for Ptprj<sup>TM-<i>fl/fl</i></sup>;TetO-Cre versus Ptprj<sup>TM-<i>fl/fl</i></sup>;SMA-rTTA;TetO-Cre at the highest dose of ACh, 2-way ANOVA. (C) BAL cell counts of total cells, macrophages, eosinophils, lymphocytes, and neutrophils of Ptprj<sup>TM-<i>fl/fl</i></sup>;TetO-Cre and Ptprj<sup>TM-<i>fl/fl</i></sup>;SMA-rTTA;TetO-Cre mice. (D) Histologic scoring by a blinded observer of H&E-stained sections for degree of inflammation around airways (left panel) and PAS staining for PAS-positive mucus-producing goblet cells (right panel) in WT, Ptprj<sup>TM-<i>fl/fl</i></sup>;TetO-Cre and Ptprj<sup>TM-<i>fl/fl</i></sup>;SMA-rTTA;TetO-Cre mice. (E) Relative OVA-specific serum IgE levels measured by ELISA in Ptprj<sup>TM-<i>fl/fl</i></sup>;TetO-Cre and Ptprj<sup>TM-<i>fl/fl</i></sup>;SMA-rTTA;TetO-Cre mice. Data for all panels show the mean ± SEM with 8 to 15 animals per group. Scale bar: 50 μm (A). ***<i>P</i> < 0.001, unpaired 2-tailed Student’s t test (D).
SFK inhibitor PP1, which is not highly selective, reduced the migration of human ASM cells and diminished the contractility of rat tracheal smooth muscle (50, 53, 54). SRC has been implicated in ASM proliferation and migration in response to various GPCR and receptor tyrosine kinase agonists (55). Interestingly, angiotensin II–augmented contractility of the rat left main bronchus following carbachol stimulation was attenuated by pretreatment with the SFK inhibitor SU6656 (56). However, in contrast to our studies, no inhibition of baseline rat bronchial contractility was seen with SU6656 pretreatment, which we observed in both mouse tracheal and human bronchial contractility. Perhaps species differences or contrasting technical approaches may underlie this discrepancy. In rat pulmonary artery vascular smooth muscle, the selective SRC family kinase inhibitors SU6656 and PP2 inhibited PGF₂α–induced contraction and MLC phosphorylation, implicating a positive regulatory role for SFKs in GPCR-mediated vascular smooth muscle contraction (57). We found that CD148-deficient ASM demonstrated more inhibition of SFKs than WT ASM, and diminished the frequency of calcium oscillations and contraction following stimulation with muscarinic agonists, underscoring a critical and perhaps proximal role for SFKs in the activation of this contractile pathway.

ASM calcium oscillations induced by muscarinic agonists are, in part, mediated by the binding of IP3 to the IP3 receptor (IP3R), calcium release from the sarcoplasmic reticulum, and complex feedback circuitry (58). We provide strong evidence that the tyrosine phosphatase CD148 modulates this pathway, although the exact mechanisms at play remain unclear. CD148 may regulate GPCR sensitization and/or desensitization through its effects on SFKs or other substrates. Another possibility could be modification of the IP3 receptor itself via tyrosine phosphorylation, which was shown to occur in T cells by the SFK FYN (59). Oscillation frequency is also regulated by sarcoplasmic reticulum (SR) calcium content and is dependent on the refilling of SR stores via the sarcoplasmic/endo-sacoplasmic reticulum Ca²⁺ ATPase (SERCA) pumps and via calcium influx from extracellular sources. How CD148 influences these pathways remains an important area for future investigation, and, notably, the intersection of tyrosine phosphorylation pathways with pathways regulating calcium oscillations has not been well characterized. One exciting possibility is that the positive regulatory role of CD148 on SFKs may regulate transient receptor potential (TRP) channels, which are phosphorylated by SRC (60–62), and by this mechanism, CD148 may regulate calcium oscillation frequency. This is certainly a high priority for future investigation.

SFKs are proximally involved in modulating several receptor tyrosine kinase (RTK) growth factors that have been implicated in asthma pathogenesis such as EGFR, VEGFR, PDGFR, and c-KIT (2, 10). There exists evidence for both negative and positive regulation of RTK signaling by CD148 (63–65), suggesting that the role of CD148 may be context dependent. Consistent with our studies, other groups have reported that CD148 is a positive regulator of SFKs via dephosphorylation of the C-terminal inhibitory tyrosine of SFKs in thyroid carcinoma (66) as well as in endothelial cells (65). Although we did not directly evaluate the effect of the CD148 phosphatase on RTK signaling pathways in ASM, it is conceivable that CD148 could be playing an important regulatory role through its influence on SFKs.
SFKs are critical regulators of integrins, which transmit signals via focal adhesion kinase (FAK) and focal adhesion signaling complexes that affect the actin cytoskeleton, an important determinant of ASM contractility (67). CD148 has been shown to play a positive regulatory role in platelet integrin function via SFK regulation (25). Future studies will more closely examine the role of CD148 in ASM integrin function and activation of focal adhesion complexes, other mechanisms by which CD148 could impact ASM contractility. CD148 function in ASM contractility could potentially be regulated by binding its recently identified ligands, thrombospondin-1 (TSP1) and syndecan-2, proteins that modulate interactions with extracellular matrix components (14, 15). For example, TSP1 stimulates vascular smooth muscle cell migration through FAK, so it is plausible that TSP1 interactions with CD148 could regulate ASM contractility (68).

Although the exact mechanisms by which the CD148 phosphatase and SFKs are involved in AHR remain incompletely defined, our data strongly suggest that the CD148 phosphatase is a critical positive regulator of SFK activity in ASM. These data present exciting new strategies for asthma treatment, focusing on the inhibition of ASM contractility. We propose two potential therapeutic approaches for asthma, directed at ASM: (a) inhibition of CD148 phosphatase activity through targeting the CD148 extracellular domain and/or modulating interactions with its ligands; or (b) inhibition of SFKs. Strategically designed inhaled inhibitors of the CD148 phosphatase or of specific SFKs could directly impact ASM function and AHR with minimal systemic side effects.

Methods

Mice

Mice constitutively lacking the CD148 (Ptpn1) transmembrane domain were generated as previously described (19) on C57BL/6 and BALB/c backgrounds, both of which were backcrossed at least 10 generations. Mice with a floxed CD148 transmembrane allele were generated by inserting loxP recombination sites flanking the CD148 transmembrane exon 18. Conditional deletion of the CD148 floxed allele on hematopoietic and endothelial cells was achieved by crossing Ptpn1<sup>Tm1/Tm1<sup>cre</sup></sup> mice with Vav1-Cre (Vav-Cre) transgenic mice, as previously described (69). α-SMA-rTtta mice were provided by M. Shipley (Washington University School of Medicine, St. Louis, Missouri, USA) and have been previously described (36). The (TetO)<sub>r</sub>-Cre mouse line has been previously described (35). All animals used were between 8 and 16 weeks of age. Sex-matched littermate controls were used in all experiments for AHR studies. For tracheal ring contractility measurements and lung slice calcium oscillation studies, BALB/c strains were used.

Antibodies and reagents

Anti-murine CD148 mAb (8A-1) was generated as previously described (12). Secondary goat anti-hamster FITC antibody was from Invitrogen, and secondary goat anti-hamster Alexa 488 antibody was from Molecular Probes (Invitrogen). The following antibodies were from BD Biosciences—Pharmpingen: anti-CD4 (clone GK1.5); anti-CD8a (clone 53-6.7); anti-γδ TCR (clone GL-3); anti-CD45R/B220 (clone RA3-6B2); and anti-IFNγ. The following antibodies were from eBioscience: anti–IL-13 (clone eBio13A) and anti–IL-17A (clone eBio17B7). Antibodies against phospho-SRC (Tyr527) and total SRC were from Cell Signaling Technology.

Murine models of allergic airway disease

OVA-alum model. Six- to 8-week-old sex-matched Ptpn1<sup>Tm1/Tm1<sup>cre</sup></sup> and littermate control mice were sensitized on days 0, 7, and 14 by intraperitoneal injection of 50 μg OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum potassium sulfate. Control animals received an equal volume of emulsified saline/aluminum potassium sulfate. Subsequently, mice were anesthetized with isoflurane and intranasally challenged on 3 consecutive days (days 21, 22, and 23) by aspiration of 100 μl saline.

HDM model. Mice were anesthetized with isoflurane prior to intranasal aspiration of 40 μl of dust mite fecal pellet preparation (2.5 mg/ml; Greer Laboratories) or saline on days 0, 21, 22, and 23.

Twenty-four hours after the last challenge, mice were anesthetized with ketamine (100 mg/kg of body weight), xylazine (10 mg/kg), and acepromazine (3 mg/kg). Pulmonary resistance was determined using invasive cannulation of the trachea as previously described (36).
Assessment of pulmonary inflammation and mucus production
Lungs were subjected to 5 consecutive lavages with 0.8 ml of PBS. After lysing red blood cells, the total cells were counted with a hemocytometer. Cytospin preparations were stained with a HEMA 3 stain set (Fisher Scientific), and cell differential percentages were determined based on light microscopic evaluation of greater than 300 cells per slide. Lavaged lungs were inflated with 10% buffered formalin to 25 cm H2O of pressure. Multiple paraffin-embedded 5-μm sections of the entire mouse lung were prepared and stained with H&E and PAS to evaluate mucus production.

Assessment of serum IgE levels
Sera were obtained from blood collected by cardiac puncture from antigen- or vehicle-treated mice after airway responsiveness measurements. Serum IgE levels were measured by enzyme-linked immunosorbent assay using microplates coated with OVA (OVA-specific IgE) or anti-mouse IgE (total IgE, R35-72; BD Biosciences — Pharmingen). Diluted serum samples were added to each well, and the bound IgE was detected with biotinylated anti-mouse IgE (R35-118; BD Biosciences — Pharmingen). Color development was achieved using streptavidin-conjugated HRP (BD Biosciences — Pharmingen) followed by the addition of HRP substrate (TMB; BD Biosciences — Pharmingen).

Intracellular cytokine staining
Mediastinal lymph nodes and lung were isolated, minced, and dispersed through 70-μm nylon filters and washed. After red blood cell lysis, cells were incubated for 4 hours at 37°C in RPMI containing 50 ng/ml PMA (Sigma-Aldrich), 1 μM ionomycin (Sigma-Aldrich), and 10 μg/ml brefeldin A (Epicentre). Cells were then resuspended in PBS/2% FCS and stained with the indicated antibodies for cell surface markers and intracellular cytokines. DAPI exclusion identified live cells, and samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences), Gallios (Beckman Coulter), or LSR II (BD Biosciences). Data were analyzed with FlowJo software (TreeStar).

Tracheal and bronchial ring contractility assays
Tracheal ring contractility assays were performed as previously described (70). In some experiments, tracheal rings were preincubated with SU6656 (Sigma-Aldrich) at 10 μM or AZD0530 at 5 μM (Selleck Chemicals) in a 37°C incubator with 5% CO2 overnight. Lung slices were labeled by Oregon Green 488 BAPTA-AM from Molecular Probes (Invitrogen; 20 μM in HBSS containing 0.1% Pluronic F-127 from Molecular Probes and 100 μM sulfobromophthalein) for 45 minutes at 30°C and de-esterified for 30 minutes at 30°C in HBSS containing 100 μM sulfobromophthalein. Lung slices were placed in coverglass chambers (Labtek) immobilized with a slice anchor. Fluorescence imaging was performed with a NIKON spinning disk confocal microscope at 20 frames per second using a x10 Nikon objective. Changes in fluorescence intensity from selected regions of interest (5 × 5 pixels) were analyzed using ImageJ software (NIH).

Fluorescence microscopy
To evaluate tissue-specific deletion of Pparg in the airway smooth muscle of PpargTM/Δg-Tg/Δg-SMA-rtTA TetO-Cre mice, lung tissue sections were stained for CD148 and α-SMA. The hamster mAb8A1 (recognizing CD148) was used at a 1:200 dilution, followed by goat anti-hamster secondary antibody conjugated to Alexa488 (Molecular Probes; Invitrogen) at a 1:200 dilution. A directly conjugated anti-α-SMA Cy3 antibody (Sigma-Aldrich) was used at a 1:500 dilution.

Slides were visualized using a Zeiss Axiovert 200M microscope with a PCO Sensicam, and images were captured with Slidebook 5.0 software. Tracheal images were taken using a ×40 oil objective. Bronchial airway images were taken using a ×100 oil objective. To evaluate CD148 colocalization with α-SMA, a linear area transecting the smooth muscle strip was analyzed using ImageJ software (NIH).

Western blotting
Mouse tracheas were isolated and epithelial cells were removed by scraping the inner epithelial lining with a cotton swab. The posterior tracheal strip consisting primarily of smooth muscle was isolated under a dissecting microscope. Tracheal strips were stimulated for 5 minutes at 37°C with carbachol (10 μM; Sigma-Aldrich) and then homogenized in ice-cold lysis buffer (150 mM NaCl; 50 mM Tris pH 8; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; and a cocktail of protease and phosphatase inhibitors). Lysates were centrifuged at 434,902 g for 30 minutes at 4°C and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology). Samples were separated by a gradient gel (Life Technologies), transferred to a PVDF membrane (Millipore), and blocked for 30 minutes in Tris-buffered saline containing 5% nonfat milk. Membranes were incubated overnight with the indicated antibodies. After washing, membranes were incubated with a peroxidase-conjugated secondary antibody for 45 minutes, washed, and then developed with Plus-ECL reagent (PerkinElmer) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Blots were developed using the Kodak Image Station 440CF and Kodak Molecular Imaging software (version 4.0.5).

Statistics
Two-way ANOVA was used for comparison of multiple groups using Prism (GraphPad Software), and when differences were statistically significant, this was followed with a Bonferroni t test for subsequent pairwise analysis. Differences with a P value of less than 0.05 were considered statistically significant. P values for comparisons of 2 different groups of mice were calculated with an unpaired 2-tailed Student’s t test, and error bars were calculated as the SEM unless otherwise stated.

Study approval
All mice were housed in a specific pathogen-free animal facility at the University of California San Francisco (UCSF). Animals were treated according to protocols that were approved by the university animal care

CO2 overnight. Lung slices were labeled by Oregon Green 488 BAPTA-AM from Molecular Probes (Invitrogen; 20 μM in HBSS containing 0.1% Pluronic F-127 from Molecular Probes and 100 μM sulfobromophthalein) for 45 minutes at 30°C and de-esterified for 30 minutes at 30°C in HBSS containing 100 μM sulfobromophthalein. Lung slices were placed in coverglass chambers (Labtek) immobilized with a slice anchor. Fluorescence imaging was performed with a NIKON spinning disk confocal microscope at 20 frames per second using a x10 Nikon objective. Changes in fluorescence intensity from selected regions of interest (5 × 5 pixels) were analyzed using ImageJ software (NIH).

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ethics and veterinary committees in accordance with NIH guidelines. For human bronchial ring contractility experiments, cadaveric human lungs were obtained from brain-dead donors whose lungs could not be used for transplantation, and as with all cadaveric tissue, did not require approval by the UCSF Committee on Human Research.

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