

Protein Tyrosine Phosphatases Regulate Asthma Development in a Murine Asthma Model¹

Philippe Pouliot,^{*†} Pierre Camateros,[†] Danuta Radzioch,[†] Bart N. Lambrecht,[‡] and Martin Olivier^{2*†}

Allergic asthma is a chronic inflammatory disease characterized by Th2-type inflammation. Although the cellular interactions are now well studied, the intracellular signaling involved in asthma development is still a developing field. Protein tyrosine kinases are one focus of such research and their inhibition shows improvement of asthmatic features. Interestingly, very little attention was given to protein tyrosine phosphatases (PTPs), the counterparts to protein tyrosine kinases, in the development of asthma. Previous studies from our laboratory showed that pharmacological inhibition of PTPs induced a transient Th1 response in the spleen. Therefore, we hypothesized that modulation of PTPs could influence asthma development. To assess PTP functions, we used the PTP inhibitor bis-*peroxovanadium* bpV(phen) in a murine model of asthma during either allergen sensitization or challenge. Inhibition of PTPs during allergen sensitization resulted in the reduction of key features of allergic asthma: serum IgE levels, lung tissue inflammation, eosinophilia, and airway hyperresponsiveness. Of utmost interest, PTP inhibition at allergen challenge resulted in a very similar improvement of asthmatic features. Of further importance, we observed that bpV(phen) treatment modulated cytokine expression in the spleen and, more specifically, favored Th1 cytokines while inhibiting Th2 cytokines. Collectively, we show for the first time that intact activity of PTPs is required for a complete induction of asthma in a mouse model. This clearly suggests that PTPs have a pivotal regulatory role in the development of asthmatic diseases, which opens the possibility of new therapeutic avenues. *The Journal of Immunology*, 2009, 182: 1334–1340.

Allergic asthma is a chronic inflammatory disease characterized by sustained Th2-type immune response (1). Development of this disease relies on the sensitization to an allergen and subsequently on the IgE-mediated response to that allergen (2). Mast cell degranulation following allergen recognition by membrane-bound IgEs leads to bronchoconstriction and recruitment of inflammatory cells. Recruited eosinophils and Th2 cells further promote the allergic inflammation and favor tissue damage, which can lead to lung remodeling (2). Although the various cell interactions leading to asthma development are now better understood, little is known regarding the intracellular events regulating cellular responses involved in asthma.

The importance of intracellular signaling in cell regulation is indisputable: alterations in signaling pathways can result in severe dysfunction of the immune response. Of the events involved in the transmission of a signal from the membrane to the nucleus, tyrosine phosphorylation is a critical step that is often obligatory for numerous cell receptors related to immunity (3–6). For example, tyrosine phosphorylation in the TCR complex is the first detectable event occurring after receptor ligation (5, 6). Consequently, the

regulation of tyrosine phosphorylation must be tightly regulated and this is achieved by a balance between the protein tyrosine kinases (PTKs),³ which add a phosphate group, and the protein tyrosine phosphatases (PTPs), which remove it (7, 8). In addition to its role in TCR signaling, tyrosine phosphorylation is also critical to other receptors such as IL-4, IL-5, IL-13, and IFN- γ receptors (9) as well as the Fc ϵ R1 receptor (10), which all exert a potent role in the modulation of immune response. Since PTKs were characterized 10 years before PTPs (11–13), their role in asthma development is now better understood (14) and previous studies show that inhibition of various PTKs successfully reduced asthmatic symptoms in animal models (reviewed in Ref. 14).

In contrast, very little is known about the role of PTPs in asthma. Upon allergen challenge, phosphatase and tensin homolog (PTEN) protein expression was shown to be down-regulated, which favors development of the disease in a murine model (15). Conversely, overexpression of PTEN by an adenoviral vector reduced asthma development. The PTP Src homology 2 domain-containing phosphatase (SHP) 1 was also shown to play a role in asthma development: if SHP-1 activity is reduced (as in *Ptpn6*^{mc/+} mice), investigators noted an exacerbated development of asthma (16). However, there are no reports regarding the pharmacological modulation of PTPs in asthma. We previously reported (17, 18) that PTP inhibitors of the *peroxovanadium* class alone or in combination with various stimuli (e.g., IFN- γ) can significantly augment signaling pathways and transcriptional events, which result in the promotion of specific cellular functions. In mouse experiments, i.p. injections of the *peroxovanadium* PTP inhibitor bpV(phen)

*Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada; [†]Centre for the Study of Host Resistance, The Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada; and [‡]Department of Respiratory Diseases, University Hospital Ghent, Ghent, Belgium

Received for publication June 19, 2008. Accepted for publication November 16, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Funding was provided by the Canadian Institute of Health Research through an operating grant (to M.O.). P.P. is the recipient of a Canada Graduate Scholarships-Doctoral Award from the Canadian Institute of Health Research.

² Address correspondence and reprint requests to Dr. Martin Olivier, Department of Microbiology and Immunology, McGill University, Lyman Duff Medical Building (Room 610), 3775 University Street, Montreal, Quebec, Canada H9S 3W6. E-mail address: martin.olivier@mcgill.ca

³ Abbreviations used in this paper: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PTEN, phosphatase and tensin homolog; SHP, Src homology 2 domain-containing phosphatase; AHR, airway hyperresponsiveness; BALf, bronchoalveolar lavage fluid; DAPI, 4',6-diamidino-2-phenylindole; Penh, enhanced pause; pNPP, *p*-nitrophenyl phosphate; pV, bpV(phen); Sal, saline.

triggered a preferential expression of Th1-type, but not Th2-type, cytokines in the spleen (18). Therefore, we hypothesized that PTP inhibition could favor a Th1 immune response and alter asthma development.

To understand the role of PTPs in asthma pathogenesis, we used a murine model of asthma where PTPs are inhibited either during the allergen sensitization phase or during allergen challenge when the lung disease is developing. Our data indicate that PTP inhibition during allergen sensitization leads to a significant reduction of asthma-related features including reduced serum IgE titers (total and allergen specific), recruitment of lung inflammatory cells, and lung eosinophilia, which substantially prevented the development of airway hyperresponsiveness (AHR). These results are paralleled by an increased level of IFN- γ in the bronchoalveolar lavage fluid (BALf). Inhibition of PTPs during allergen challenge resulted in similar observations with the exception of IgE titers that are not modulated at this stage. Levels of IFN- γ found in BALf are also increased upon treatment with bpV(phen) during allergen challenge. Collectively, our data indicate a role for PTPs in the regulation of the Th1/Th2 response in a mouse model of allergic asthma.

Materials and Methods

Chemicals and reagents

OVA grade V and aluminum hydroxide gel were purchased from Sigma-Aldrich. Bis-peroxovanadium bpV(phen) was synthesized as described previously (19). PCR primers were ordered from IDT anti-IL-4R α was obtained from BD Biosciences, anti-IL-2R α from eBioscience, and 4',6-diamidino-2-phenylindole (DAPI) from Invitrogen.

Animals and sensitization protocol

Six- to 8-wk-old BALB/c mice were purchased from Charles Rivers Canada and housed in the McGill University animal facility in accordance with the Canadian Council on Animal Care guidelines. Mice were injected i.p. on days 0 and 7 with 40 μ g of OVA and 2.6 mg of aluminum hydroxide in a total of a 200- μ l injection of saline. Allergen challenges were performed on days 21, 22, and 23, by nebulization of 5% OVA in saline for 5 min. Inhibition with bis-peroxovanadium bpV(phen) was performed daily by s.c. injection of 2.5 μ mol/30 g body weight in a total of 250 μ l of saline (or saline only for control group). Inhibition was performed from day -1 to day 19 (inclusive) for inhibition during sensitization or from days 20 to 23 (inclusive) for inhibition at allergen challenge. For the dose-response experiment with bpV(phen), all animals were sensitized with 10 μ g of OVA and 1 mg of alum (Thermo Scientific) in 500 μ l on day 0. Then, bpV(phen) was administered at either 0.1, 1 or 10 μ M in saline, s.c. on days 10, 11, 12, and 13. OVA challenges (1% OVA grade 3 in PBS, 30 min. nebulized; Sigma-Aldrich) were performed on days 11, 12, and 13, 6 h after bpV(phen) injection. Mice were sacrificed on day 14 and bronchoalveolar lavage was performed to evaluate lung eosinophilia.

Animal AHR measurement

Forty-eight hours after the last allergen challenge, mice were placed in a whole-body plethysmograph chamber (Buxco Research Systems). Enhanced pause (Penh) was measured after each nebulization with increasing doses of methacholine and this measure was used to evaluate AHR. Animals were sacrificed after analysis for evaluation of other parameters.

Serum IgE measurement

The level of IgE Abs was measured in serum obtained from mice. Total IgEs were measured using the ELISA technique according to the manufacturer's protocol (BD Pharmingen; capture Ab clone R35-72 and detection Ab clone R35-118). Specific IgE titers were measured using the same capture Ab as above, but the detection Ab was replaced with 10 μ g/ml biotinylated OVA. OVA grade 5 was obtained from Sigma-Aldrich and conjugated to biotin using a biotin conjugation kit from Sigma-Aldrich. A ratio of 4 biotin/OVA was achieved as calculated by the extinction coefficient of avidin-2-(4'-hydroxyazobenzene) benzoic acid.

Bronchoalveolar lavage procedure

Lungs were lavaged with 1 ml of saline. BALf was spun down and the cell pellet was resuspended in 100 μ l of PBS, counted, and applied on a glass

microscope slide using a cytospin apparatus. Slides were stained using the Diff-Quik stain and blind differential cell counts were performed. After the bronchoalveolar lavage procedure, the lung was inflated with paraformaldehyde at a pressure of 25 cm H₂O and incubated in paraformaldehyde for 48 h to achieve tissue fixation. After fixation, the lung was processed in paraffin, cut into 5- μ m sections, and mounted on slides and then stained with H&E following standard procedures. Inflammation was evaluated on a scale of 0–4 for perialveolar/peribronchial or perivascular recruitment.

Cytokine mRNA expression analysis

At sacrifice, mouse organs were retrieved and flash-frozen. RNA was extracted using a tissue homogenizer and TRIzol reagent according to the manufacturer's protocol (Invitrogen Canada). Reverse transcriptase was performed using oligo(dT) primers as previously described (20). Quantitative relative PCR was performed using Invitrogen Platinum qPCR Super-Mixes and 0.4 μ M primer in 25 μ l. Quantitative PCR parameters are as follows: 50°C for 2 min and 95°C for 3 min (95°C for 20 s, 60°C for 30 s, 72°C for 20 s, 80°C (reading step) for 20 s) for 40 cycles followed by a melting curve. Annealing temperatures of all primers was 60°C. Primer sequences are as follows: GAPDH, 5'-CGG ATT TGG CCG TAT TGG GCG CCT-3' and 3'-ACA TAC TCA GCA CCG GCC TCA CCC-5'; IL-4, 5'-AACATGGGAAAACCTCCATGC-5' and 3'-TTGCATGATGC TCTTTAGGC-5'; IL-10, 5'-GGT TGC CAA GCC TTA TCG GA-3' and 3'-ACC TGC TCC ACT GCC TTG CT-5'; IL-12, 5'-GGA AGC ACG GCA GCA GAA TA-3' and 3'-AAC TTG AGG GAG AAG TAG GAA TGG-5'; and IFN- γ , 5'-GCGTCATTGAATCACACCTG-3' and 3'-TGAGCTCATTGAATGCTTGG-5'.

Protein phosphatase activity measurement by p-nitrophenyl phosphate (pNPP) hydrolysis

Analysis of phosphatase activity was performed using a variation of our previously reported method (21). Tissue samples were collected first and flash-frozen in liquid nitrogen. Then, tissue samples were homogenized in PTP lysis buffer (50 mM Tris (pH 7), 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-ME, 25 μ g/ml aprotinin, and 25 μ g/ml leupeptin) using a PRO 200 tissue homogenizer (Pro Scientific). Igepal (1%) was added after homogenization. Samples were then kept on ice for 45 min with agitation each 10 min. Tissue lysates were then centrifuged and the supernatant was used. Protein concentration was determined by the Bradford method following the manufacturer's protocol (Bio-Rad). Twenty micrograms of protein was used to evaluate phosphatase activity. Incubation was performed in phosphatase reaction buffer (50 mM HEPES (pH 7.5), 0.1% 2-ME, and 10 mM pNPP) at 37°C and absorbance at 405 nm was acquired every 15 min using the automated Powerwave 340 absorbance reader (Bio-Tek Instruments).

IFN- γ and IL-4 measurement in the BALf

After sacrifice, supernatant from BALf were frozen at -80°C until measurement of cytokines by ELISA. IFN- γ ELISA (BD Pharmingen) and IL-4 ELISA (eBioscience) were performed as specified by the manufacturer; each kit had a detection limit of 10 and 4 pg/ml, respectively.

Flow cytometry

Pooled spleen and lymph nodes cells were treated with bpV(phen) (0.1, 1, or 10 μ M) for 1 h before the addition of 10 ng/ml IL-4 for 18 h. The cells were then analyzed by flow cytometry for expression of IL-4R α as well as IL-2R α and DAPI. Analysis was performed on a FACSARIA II (BD Biosciences).

Statistical analysis

Statistically significant differences were identified using the ANOVA module of StatView from the SAS Institute (version 5). Values of $p \leq 0.05$ were considered statistically significant. All data are presented as mean $\pm$ SEM.

Results

Inhibition of PTP activity during allergen sensitization

From previous experiments, we knew that a transient inhibition of PTPs could be maintained by daily injections of bpV(phen) (Ref. 18 and data not shown). bpV(phen) was therefore administered daily during the sensitization protocol starting on day -1, to abolish PTP activity before sensitization, until treatment was terminated on day 19 (48 h before allergen challenge). This was to avoid PTPs inhibition during allergen challenge (days 21–23).

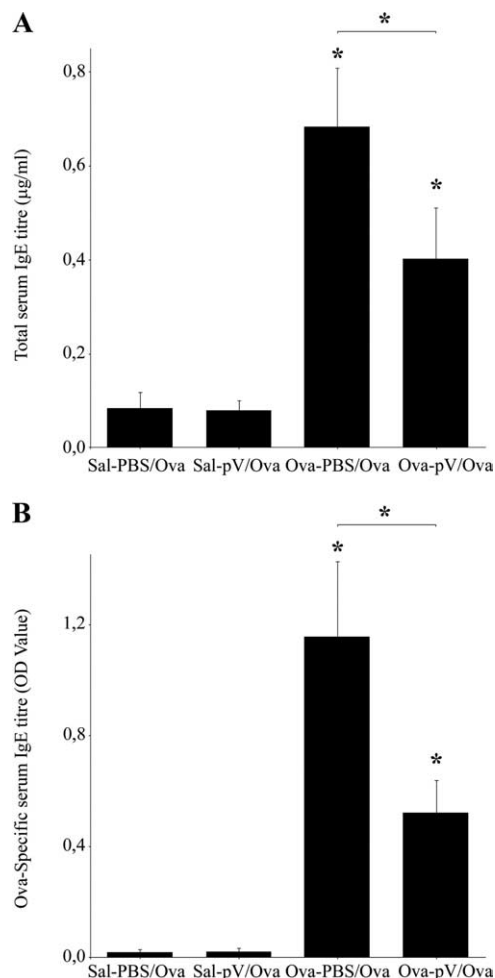


FIGURE 1. Levels of serum IgEs in mice receiving PTP inhibition during allergen sensitization. Blood was collected at sacrifice and serum obtained. Total IgE (A) and OVA-specific (B) IgE levels were measured by a sandwich ELISA. Data shown represent the average of 13–14 animals/group. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$). Group identifications are as following: sensitization treatment/challenge.

An effective OVA sensitization was confirmed by measurement of serum IgE levels. As shown in Fig. 1, OVA sensitization (OVA-PBS/OVA group) induces an increase in total and specific IgE levels when compared with saline-sensitized animals (Sal-PBS/OVA), as expected in this model. Animals treated with bpV(phen) during sensitization to saline (Sal-pV/OVA) showed no difference with PBS-treated/saline-sensitized controls (Sal-PBS/OVA). But interestingly, animals sensitized to OVA, treated with bpV(phen) (OVA-pV/OVA) had lower levels of both total and OVA-specific IgEs; this effect was slightly more pronounced with OVA-specific IgE titers. Thus, PTP inhibition during allergen sensitization prevented the usual production of IgEs observed in this model.

Next, we evaluated recruitment of inflammatory cells to the lung by analyzing the cells found in the BALf. As reported in Fig. 2A, OVA sensitization before OVA challenge increased the total number of cells found in the BALf and, as expected in this model, recruited lymphocytes and eosinophils to the lung. Treatment with bpV(phen) during sensitization did not affect the basal cellular populations of non-OVA-sensitized animals (Sal-pV/OVA), but significantly reduced total cell infiltration in the BALf of OVA-sensitized animals (OVA-pV/OVA). The classical eosinophilic infiltration is also reduced to a third of the OVA control (OVA-PBS/

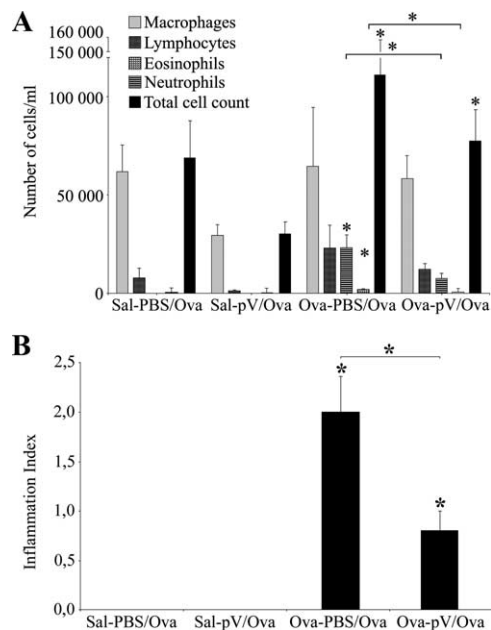


FIGURE 2. Lung inflammation in mice receiving PTP inhibition during allergen sensitization. Inflammatory cell recruitment to the lung was evaluated. A, Recruitment of inflammatory cells to the BALf compartment was evaluated by differential counts and total cell counts. Data shown represent the average of 13–14 animals/group. B, Local tissue inflammation was evaluated on histological sections. Observations were made at $\times 400$ on five to six animals per group and graded from 0 (no infiltration) to 4 (maximal infiltration). *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

OVA), while lymphocytic infiltration is reduced by half, showing that PTP inhibition can prevent inflammatory cell recruitment to the BALf. We further studied these observations on lung tissue by microscopically monitoring histological lung preparations stained with H&E. We observed an important perivascular and peribronchial inflammation in the OVA control (OVA-PBS/OVA) but observed a significant reduction this inflammation upon bpV(phen) treatment (Fig. 2B). Therefore, it suggests a role for PTPs in the full development of allergic asthma disease.

We were then interested to assess whether PTP inhibition can also reduce the severity of physiological asthmatic features such as AHR. Two days after allergen challenge, AHR of mice was measured with the Penh value. Treatment with bpV(phen) did not affect the basal level Penh in unsensitized animals (Fig. 3). In contrast, PTP inhibition in sensitized animals resulted in a dramatic reduction of AHR. Thus, showing that bpV(phen) treatment during allergen sensitization can modulate both immunological and physiological features of asthma in our mouse model.

To assess how lung homeostasis is modified by our treatment, we investigated the production of cytokines in the BALf, namely, IFN- γ and IL-4, to evaluate the status of the Th1/Th2 balance. As shown in Fig. 4, levels of IFN- γ in BALf are similar in unsensitized controls, but are completely absent in OVA-sensitized and OVA-challenged (OVA-PBS/OVA) mice. The treatment with bpV(phen) during allergen sensitization restores IFN- γ in the BALf to normal levels after allergen challenge. These results clearly illustrate that bpV(phen) can alter the balance of the Th1/Th2 responses in this model.

Inhibition of PTP activity during allergen challenge

We previously reported that bpV(phen) is able to inhibit PTP activity in the spleen (18). In this study, we wanted to confirm that

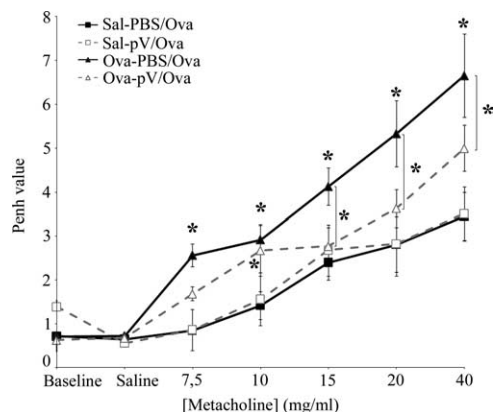


FIGURE 3. AHR of mice receiving PTP inhibition during allergen sensitization. Mice were evaluated for AHR to methacholine in a whole-body plethysmograph. After recording the values of baseline breathing and the values of PBS only, increasing doses of methacholine were nebulized to the chamber and respiratory pattern was recorded. Penh value was used as a measure of AHR. Data shown represent the average of 9–14 mice/group. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

bpV(phen) is able to inhibit PTP activity in the lung, and to investigate the role of PTPs during allergen challenge. After s.c. injection of bpV(phen), we evaluated total phosphatase activity in both the spleen and lung. Fig. 5 shows that bpV(phen) injections substantially diminished total lung phosphatase activity by 50% over a 24-h period, while in the spleen activity was reduced by 40%. We also observed that injections of bpV(phen) reduced phosphatase activity in the kidney and liver (data not shown). Therefore, bpV(phen) is able to inhibit PTP activity in the lung and can be used s.c. during allergen challenge.

If PTP inhibition favored a Th1-type response, as we hypothesized, then inhibition during allergic sensitization would alter asthma development because it may reduce the extent of the Th2 response that develops. In this study, we wanted to verify whether inhibition of PTP activity is able to affect local lung allergic dis-

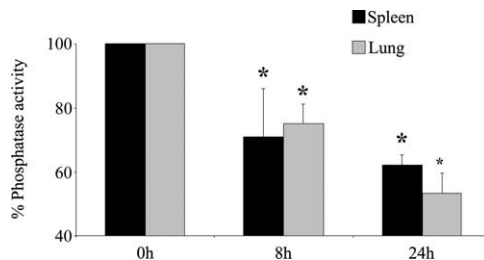


FIGURE 5. Modulation of phosphatase activity in the spleen and lung. Phosphatase activity was measured in spleen and lung tissue by cleavage of the pNPP substrate in a time-dependant manner. Data represent the average of three animals per time point. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

ease development after allergen challenge (in previously sensitized mice). We inhibited PTP activity by daily s.c. injections of bpV(phen) beginning 1 day before the first allergen challenge until the last day (days 20–23). When mice received both bpV(phen) treatment and OVA aerosol (days 21–23), bpV(phen) was administered 6 h before the allergen challenge. Mice were sacrificed 48 h after the last allergen challenge.

Allergic sensitization of these mice was confirmed by the detection of elevated levels of IgE. However, although mice in the OVA-challenged groups showed a trend in increased production of total IgEs, this did not reach statistical significance (data not shown). Consequently, bpV(phen) treatment did not significantly affect levels of IgE (neither total nor OVA specific), which were previously induced by i.p. sensitization.

Next, we evaluated the cells found in the BALf. As shown in Fig. 6A, OVA challenge in OVA-sensitized control animals (OVA/OVA-PBS) results in a significant recruitment of total inflammatory cells, with a typical increase in lymphocyte and eosinophil populations. Treatment of these animals with bpV(phen) markedly diminished total inflammatory cell recruitment to the BALf; this occurred with a dramatic decrease in the lymphocytic population and an almost complete disappearance of eosinophils. We subsequently confirmed this decrease in inflammatory cell infiltration on histological slides of lung tissue. As seen in Fig. 6B, the prominent perivascular and peribronchiolar infiltration observed in OVA-sensitized/OVA-challenged (OVA/OVA-PBS) animals was reduced when compared with their bpV(phen)-treated counterparts (OVA/OVA-pV). This reveals the potency of PTP inhibition on this feature of asthma.

To complete our evaluation of the effect of bpV(phen) on allergic asthma development, we measured the AHR of these animals. Fig. 7 shows that OVA-sensitized/OVA-challenged (OVA/OVA-PBS) animals exhibit strong AHR, while OVA-sensitized/saline-challenged (with or without bpV(phen)) exhibit no AHR. PTP inhibition in OVA-sensitized/OVA-challenged animals (OVA/OVA-pV) resulted in the normalization of AHR in these animals; they no longer showed significant AHR.

We also investigated the production of cytokines in the BALf (IFN- γ and IL-4) to assess any affect on the lung homeostasis. In this context, Fig. 8 shows that bpV(phen) treatment in OVA-challenged (OVA/OVA-pV) animals slightly increased their IFN- γ production as compared with their nontreated controls (OVA/OVA-PBS). The overall IFN- γ levels were lower in this case than in Fig. 4. In contrast, IL-4 protein remained below the limit of detection for our assay (data not shown).

To better characterize the effect of bpV(phen) at allergen challenge, we were interested in performing a dose-response experiment. Our experience with bpV(phen) established that it is safe for

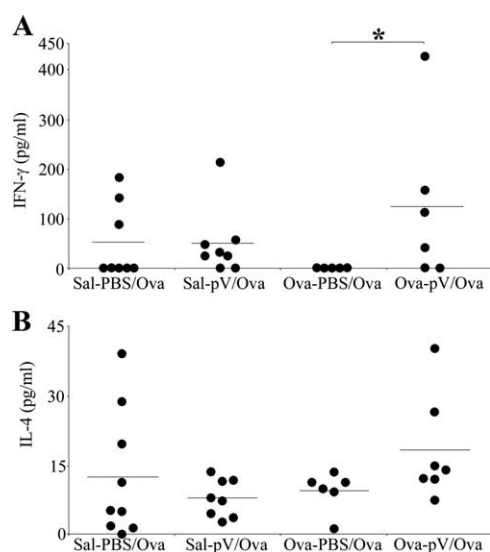


FIGURE 4. Cytokines present in the BALf of mice receiving PTP inhibition during allergen sensitization. BALf supernatants were evaluated for the presence of cytokines by ELISA. Levels of IFN- γ (A) and IL-4 (B) were measured. Data represent the average of six to nine animals per group. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

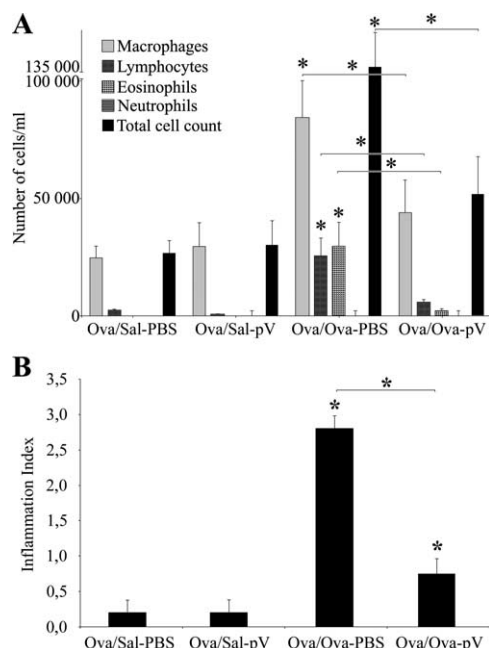


FIGURE 6. Lung inflammation of mice receiving PTP inhibition during allergen challenge. Inflammatory cell recruitment to the lung was evaluated. *A*, Recruitment of inflammatory cells to the BALf compartment was evaluated by differential count and total cell count. Data shown represent the average of six to seven animals per group. *B*, Local tissue inflammation was evaluated on histological sections. Observations were made at $\times 400$ on five animals per group and graded from 0 (no infiltration) to 4 (maximal infiltration). *, Significant difference with appropriate control or identified sample ($p \leq 0.05$). Group identifications are as following: sensitization/challenge treatment.

daily injections in animals up to 6 wk at doses up to $1 \mu\text{M}$ (17). We therefore established our dose-response experiment with bpV(phen) ranging from 0.1 to $10 \mu\text{M}$. As shown in Fig. 9, bpV(phen) reduced recruitment of eosinophils to the BALf compartment in a dose-dependent manner, with almost a complete reduction at a dose of $10 \mu\text{M}$.

Effects of PTP inhibition on cellular processes

To precisely examine the cellular mechanisms that are critically affected by bpV(phen)-induced PTP inhibition, we first inves-

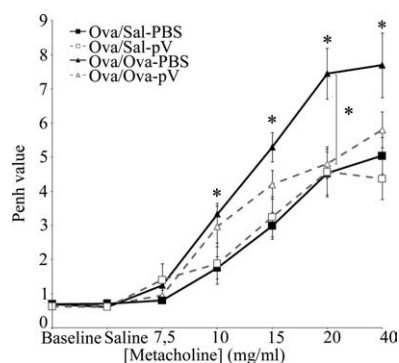


FIGURE 7. AHR of mice receiving PTP inhibition during allergen challenge. Mice were evaluated for AHR to methacholine in a whole-body plethysmograph. After recording the values of baseline breathing and the values of PBS only, increasing doses of methacholine were nebulized to the chamber and respiratory pattern was recorded. Penh value was then used as a measure of AHR. Data shown represent the average of 10–11 mice/group. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

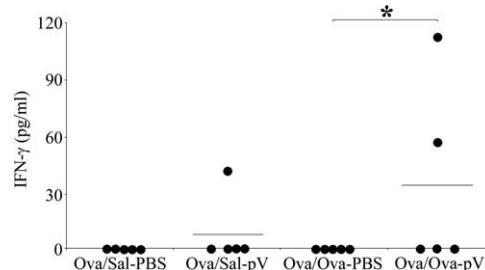


FIGURE 8. Cytokines present in the BALf of mice receiving PTP inhibition during allergen challenge. BALf supernatants were evaluated for the presence of cytokines by ELISA. Levels of IFN- γ were evaluated. Data represent the average of five to nine animals per group. Levels of IL-4 remained below the detection limit of the assay. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

tigated the degranulation and cytokine expression of RBL-2H3 cells as a model for Fc ϵ R1-mediated activation of mast cells. Although many doses and experimental conditions were assessed, our results did not show any variation in the degranulation or cytokine expression of these cells following Fc ϵ R1 activation by OVA-DNP and bpV(phen) treatment (data not shown). This suggested that mast cell Fc ϵ R1-mediated degranulation may not be a critical mechanism affected by bpV(phen) in vivo. Next, we verified whether T cell proliferation was affected by the bpV(phen) treatment. Using MF2.2D9 MHC class II-restricted T-T hybridoma and DC2.4 dendritic cell lines (22), we found that treatment of cells with subcytotoxic doses of bpV(phen) did not affect cell proliferation (data not shown). Even with the use of CFSE-labeled OT-II T cells transferred in vivo into C57BL/6 mouse before OVA injection, we did not observe a modulation of T cell proliferation by bpV(phen) (data not shown).

We also wanted to extend our previous findings regarding the modulation of Th1 and Th2 cytokine expression in the spleen following treatment with bpV(phen) (18). In this study, we evaluated by quantitative RT-PCR the expression of IFN- γ , IL-12, IL-4, and IL-10 in the spleen after bpV(phen) treatment. As seen in Fig. 10A, IL-12 expression is increased after injection with the PTP inhibitor and this increase is sustained for 24 h. The same observation was made with IFN- γ , which supports induction of a Th1 response. Very interestingly, IL-4, as well as IL-10, expression in the spleen was markedly reduced by PTP inhibitor treatment; these effects

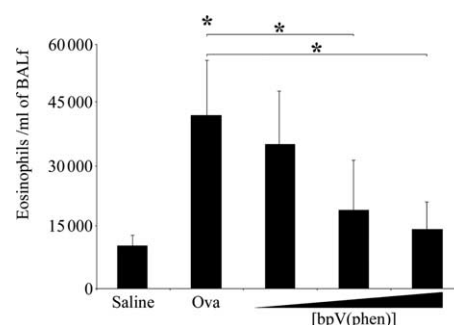


FIGURE 9. Dose-response effect of bpV(phen) in mice receiving PTP inhibition during allergen challenge. Mice were all sensitized to OVA and were challenged with OVA, except for the saline control group. Mice were treated with bpV(phen) at 0.1, 1, or $10 \mu\text{M}$ 1 day before the first OVA challenge and 6 h before each subsequent OVA challenge. Then recruitment of eosinophils in the BALf were evaluated. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$).

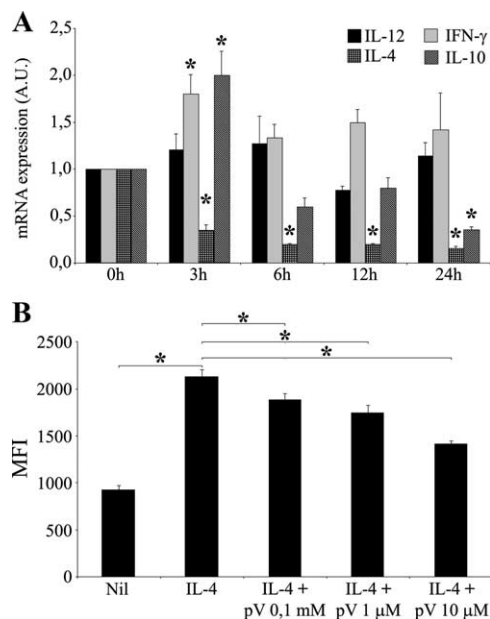


FIGURE 10. Effect of bpV(phen) on cytokine and IL-4R α expression in the spleen. **A**, Modulation of cytokine mRNA expression following treatment with bpV(phen) was evaluated in the spleen. IFN- γ and IL-12 were assessed as Th1 cytokines while IL-4 and IL-10 were used as Th2 cytokines. Data represent the average of six animals per time point. *, Significant difference with appropriate control or identified sample ($p \leq 0.05$). **B**, Pooled spleen and lymph nodes cells were given bpV(phen) at 0.1, 1, or 10 μ M 1 h before being stimulated with or without 10 ng/ml IL-4. Expression of IL-4R α was assessed by flow cytometry after 24 h. Expression of IL-2R α was also assessed (data not shown). Cell viability remained above 95% among all of the groups as assessed by DAPI staining (data not shown). *, Significant difference with appropriate control or identified sample ($p \leq 0.05$). MFI, Mean fluorescence intensity.

also support the Th1 polarization observed. Since the balance between these mediators is crucial for the establishment of proper allergic inflammation, these early acting events reinforce our hypothesis and provide a consistent mechanism for the reduction of the asthmatic phenotype in these mice.

It appears that bpV(phen) treatment reduces the Th2 response. In this regard, it was reported that the IL-4R α chain harbors an ITIM that could allow the binding of the Src homology 2-containing phosphatases SHP-1, SHP-2, and/or SHIP (23). It was also shown that an intact tyrosine phosphatase activity is required for the IL-4-induced increased expression of the IL-4R α chain (24). Indeed, inhibition of tyrosine phosphatase activity by vanadate prevented an increase in IL-4R α expression on pooled cells from spleen and lymph nodes. This suggests a role for phosphatases in the regulation of IL-4R α expression. In our model, preventing T cells from sensing an increase in IL-4 could play a role in the reduction of the Th2 response while consequently enhancing/sustaining the Th1 response. To confirm this, pooled spleen and lymph node cells were stimulated with IL-4 after treatment with or without bpV(phen). Fig. 10B shows that the mean fluorescence index of IL-4R α -expressing cells was increased upon overnight treatment with IL-4, as previously reported (24). Treatment with increasing doses of bpV(phen) reduced this IL-4-driven increase in IL-4R α expression. This reveals that bpV(phen) was indeed able to prevent IL-4R α expression and possibly reduced the ability to sense IL-4 in these cells. IL-2R α expression, on the other hand, remained constant with all treatments (data not shown), therefore showing the specificity of IL-4R α modulation by treatment with both IL-4 and bpV(phen).

Discussion

Asthma is known to be a chronic inflammatory disease sustained by a Th2 response (2). Since we previously observed that inhibition of PTPs (by the inhibitor bpV(phen)) favored the expression of Th1 cytokines in the spleen (18), we hypothesized that PTP inhibition could antagonize the development of asthma by preventing the establishment of the required Th2 response. In the present study, we report that PTP inhibition, either during allergen sensitization or allergen challenge, prevented the establishment of an allergic asthmatic disease in a mouse model. PTP inhibition during allergen sensitization reduced IgE levels, which could account for the reduction of the allergic status after subsequent allergen challenge. PTP inhibition at allergen challenge, on the other hand, did not affect serum IgE titers. Although it is known that allergen exposure can increase levels of IgE (25), the short time period between allergen challenge and IgE measurement in our model probably precluded the observation of a significant modulation. Nevertheless, as both experimental procedures result in a reduced asthmatic phenotype while effecting the IgE titers differently, this clearly states that bpV(phen) modulates other processes. As we previously showed, Th1/Th2 balance is affected by bpV(phen) treatment (18); therefore, we speculated that a more pronounced Th1 reaction was created during sensitization, resulting in a weaker asthma phenotype at the allergen challenge afterward. We observed that expression of Th1 cytokines (IFN- γ and IL-12) was increased while expression of Th2 cytokines (IL-4 and IL-10) was dramatically reduced in the spleen upon bpV(phen) treatment, thus supporting the hypothesis.

The quantities of cytokines found in the BALf also support our hypothesis since the presence of IFN- γ is increased in the BALf of mice treated with bpV(phen) in comparison to the allergic nontreated group. Although this observation was made in both experimental sets (PTP inhibition during allergen sensitization or challenge), the effect is more potent and therefore more convincing when the inhibition is made during allergen sensitization. This might reflect either a deeper effect of bpV(phen) on the immune response if the inhibition occurs during the sensitization phase or it might reflect the effect of cumulative injections, as more injections of bpV(phen) are performed in the sensitization-treated group.

During allergen challenge, it was important to confirm that our inhibitor reached the lung. We verified that phosphatase activity was inhibited in the lung upon s.c. injection of bpV(phen) and observed a substantial decrease in phosphatase activity over the next 24-h period. In this experimental set, all mice were originally sensitized toward a Th2 response with the OVA-alum complex (26). Therefore, the fact that PTP inhibition only at allergen challenge can prevent the development of asthma features strongly suggests that some PTPs are involved in the acute events of the disease and thus can prevent the unfolding of a preprogrammed reaction. It is interesting to consider that Th1 and Th2 clones do not exhibit the same pattern of tyrosine phosphorylation in the early events of TCR activation (27); Th1 clones have a stronger phosphorylation profile than Th2 clones. Therefore, it is tempting to suggest that the use of PTP inhibitors can increase the basal phosphorylation level of all clones, hence, preventing the Th2 clones from being activated with their classically minimal level of phosphorylation. Although this requires more investigation, such a mechanism could explain the prevalence of a Th1 response, without implicating a quantitative modulation of the populations or the complete depletion of Th2 cells.

Although very few studies were performed on the role of PTPs in asthma, it was reported that inhibition of SHP-1 or PTEN favored asthma development (15, 16). The partial absence of

SHP-1 in moth-eaten heterozygous (*Ptpn6^{me/+}*) mice resulted in a marked increased of allergic inflammation, which was reflected by increased eosinophilia and AHR (16). In a different study, PTEN protein expression was first down-regulated in the lung upon allergen challenge (15). In the study by Kwak et al. (15), if PTEN levels were restored or if PI3K inhibitors were used, asthma development was prevented, therefore showing that PTEN is necessary to counteract PI3K and prevent asthma development. Although these results diverge from ours, many facts might explain this discrepancy. First, it is possible that the effect of SHP-1 or PTEN inhibition is unnoticed in our model as global PTP inhibition will inhibit the pathways leading to their activation. Second, many phosphatases have a positive role in signaling pathways. Their inhibition might hamper certain signaling pathways and this effect could be more important than the specific inhibition of SHP-1 or PTEN. For example, CD45 is known to dephosphorylate an inhibitory phosphotyrosine on Src family kinases (28) and these kinases are necessary for TCR signaling. SHP2 is another PTP known to exert a positive effect on signaling pathways because it also stimulates activation of Src family kinases (29). The individual roles of PTPs in the allergic asthma are still to be explored and we are currently investigating the implication of various PTPs of immunological importance.

Our results regarding FcεR1-mediated degranulation and T cell proliferation have limitations regarding their reflection of what happens in vivo. In the case of FcεR1-mediated degranulation, we cannot speculate that inhibition of PTPs in mast cells has no effect as mast cells can be activated by other mechanisms than their FcεR1 receptor. However, it strongly suggests that mast cell activation through their high-affinity IgE receptor is probably not involved in our in vivo results. In a similar manner, although T cell proliferation was not significantly affected, T cells in vivo might be differently polarized or their activation might be hampered by PTP inhibition, as suggested by the different patterns of phosphorylation observed in Th1 and Th2 clones. These issues will be studied with more scrutiny in a successive project.

In contrast, our results regarding the inhibition of IL-4Rα expression upon bpV(phen) treatment are promising. Indeed, a reduced recognition of IL-4 could reduce the biological effect of this cytokine on the target cells and might dampen the intensity of the Th2 response induced, as we observed. Although interesting, the in vivo relevance of this observation needs to be confirmed, but this is a promising avenue.

Our study is the first to reveal the important role that PTPs play in the development of allergic asthma. We also identify PTPs as potential therapeutic targets since bpV(phen) can prevent lung allergic disease in presensitized individuals before allergen challenge. In terms of fundamental research, this study also highlights the importance of PTPs in the regulation of immune responses. We are currently pursuing investigations on various PTP-deficient mice to identify the roles of specific PTPs, which could provide new therapeutic targets.

Acknowledgments

We acknowledge the help of Karen K. Yam for careful revision of this manuscript. Acknowledgments are also made to Evridiki Sgouroudis and Ekaterina Yurchenko for their technical help.

Disclosures

The authors have no financial conflict of interest.

References

- van Rij, L. S., and B. N. Lambrecht. 2005. Dendritic cells in asthma: a function beyond sensitization. *Clin. Exp. Allergy* 35: 1125–1134.
- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144: 1591–1599.
- June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA* 87: 7722–7726.
- Mustelin, T., K. M. Coggeshall, N. Isakov, and A. Altman. 1990. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* 247: 1584–1587.
- Stanley, J. B., R. Gorczynski, C. K. Huang, J. Love, and G. B. Mills. 1990. Tyrosine phosphorylation is an obligatory event in IL-2 secretion. *J. Immunol.* 145: 2189–2198.
- Tonks, N. K. 2006. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat. Rev. Mol. Cell Biol.* 7: 833–846.
- Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell* 103: 211–225.
- Aaronson, D. S., and C. M. Horvath. 2002. A road map for those who don't know JAK-STAT. *Science* 296: 1653–1655.
- Siraganian, R. P. 2003. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* 15: 639–646.
- Guan, K. L., R. S. Haun, S. J. Watson, R. L. Geahlen, and J. E. Dixon. 1990. Cloning and expression of a protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* 87: 1501–1505.
- Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischler, and H. M. Goodman. 1980. Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product. *Nature* 287: 198–203.
- Charbonneau, H., N. K. Tonks, S. Kumar, C. D. Diltz, M. Harrylock, D. E. Cool, E. G. Krebs, E. H. Fischer, and K. A. Walsh. 1989. Human placenta protein-tyrosine-phosphatase: amino acid sequence and relationship to a family of receptor-like proteins. *Proc. Natl. Acad. Sci. USA* 86: 5252–5256.
- Wong, W. S. 2005. Inhibitors of the tyrosine kinase signaling cascade for asthma. *Curr. Opin. Pharmacol.* 5: 264–271.
- Kwak, Y. G., C. H. Song, H. K. Yi, P. H. Hwang, J. S. Kim, K. S. Lee, and Y. C. Lee. 2003. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J. Clin. Invest.* 111: 1083–1092.
- Kamata, T., M. Yamashita, M. Kimura, K. Murata, M. Inami, C. Shimizu, K. Sugaya, C. R. Wang, M. Taniguchi, and T. Nakayama. 2003. Src homology 2 domain-containing tyrosine phosphatase SHP-1 controls the development of allergic airway inflammation. *J. Clin. Invest.* 111: 109–119.
- Olivier, M., B. J. Romero-Gallo, C. Matte, J. Blanchette, B. I. Posner, M. J. Tremblay, and R. Faure. 1998. Modulation of interferon-γ-induced macrophage activation by phosphotyrosine phosphatases inhibition: effect on murine *Leishmaniasis* progression. *J. Biol. Chem.* 273: 13944–13949.
- Matte, C., J. F. Marquis, J. Blanchette, P. Gros, R. Faure, B. I. Posner, and M. Olivier. 2000. Peroxovanadium-mediated protection against murine leishmaniasis: role of the modulation of nitric oxide. *Eur. J. Immunol.* 30: 2555–2564.
- Posner, B. I., R. Faure, J. W. Burgess, A. P. Bevan, D. Lachance, G. Zhang-Sun, I. G. Fantus, J. B. Ng, D. A. Hall, B. S. Lum, et al. 1994. Peroxovanadium compounds: a new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. *J. Biol. Chem.* 269: 4596–4604.
- Pouliot, P., V. Turmel, E. Gelinas, M. Laviolette, and E. Y. Bissonnette. 2005. Interleukin-4 production by human alveolar macrophages. *Clin. Exp. Allergy* 35: 804–810.
- Gomez, M. A., S. Li, M. L. Tremblay, and M. Olivier. 2007. NRAMP-1 expression modulates protein-tyrosine phosphatase activity in macrophages: impact on host cell signaling and functions. *J. Biol. Chem.* 282: 36190–36198.
- Shen, Z., G. Reznikoff, G. Dranoff, and K. L. Rock. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J. Immunol.* 158: 2723–2730.
- Kashiwada, M., C. C. Giallourakis, P. Y. Pan, and P. B. Rothman. 2001. Immunoreceptor tyrosine-based inhibitory motif of the IL-4 receptor associates with SH2-containing phosphatases and regulates IL-4-induced proliferation. *J. Immunol.* 167: 6382–6387.
- Huang, H., and W. E. Paul. 2000. Protein tyrosine phosphatase activity is required for IL-4 induction of IL-4 receptor α-chain. *J. Immunol.* 164: 1211–1215.
- Henderson, L. L., J. B. Larson, and G. J. Gleich. 1975. Maximal rise in IgE antibody following ragweed pollination season. *J. Allergy Clin. Immunol.* 55: 10–15.
- Petrovsky, N., and J. C. Aguilar. 2004. Vaccine adjuvants: current state and future trends. *Immunol. Cell Biol.* 82: 488–496.
- Hannier, S., C. Bitegye, and S. Demotz. 2002. Early events of TCR signaling are distinct in human Th1 and Th2 cells. *J. Immunol.* 169: 1904–1911.
- Sieh, M., J. B. Bolen, and A. Weiss. 1993. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. *EMBO J.* 12: 315–321.
- Zhang, S. Q., W. Yang, M. I. Kontaridis, T. G. Bivona, G. Wen, T. Araki, J. Luo, J. A. Thompson, B. L. Schraven, M. R. Philips, and B. G. Neel. 2004. Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. *Mol. Cell* 13: 341–355.