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Syk inhibition blocks airway constriction and protects from Th2-induced airway inflammation and remodeling in mice

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Short title: Syk mediates airway constriction and remodeling

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Abstract

Background: Spleen tyrosine kinase (Syk) is an intracellular non-receptor tyrosine kinase, which has been implicated as central immune modulator promoting allergic airway inflammation. Syk inhibition has been proposed as a new therapeutic approach in asthma. However, the direct effects of Syk inhibition on airway constriction independent of allergen sensitization remain elusive.

Methods: Spectral confocal microscopy of human and murine lung tissue was performed to localize Syk expression. The effects of prophylactic or therapeutic Syk inhibition on allergic airway inflammation, hyperresponsiveness and airway remodeling were analyzed in allergen-sensitized and airway-challenged mice. The effects of Syk inhibitors BAY 61-3606 or BI 1002494 on airway function were investigated in isolated lungs of wild-type, *PKC α* -deficient, mast cell-deficient or *eNOS*-deficient mice.

Results: Syk expression was found in human and murine airway smooth muscle cells. Syk inhibition reduced allergic airway inflammation, airway hyperresponsiveness and pulmonary collagen deposition. In naïve mice, Syk inhibition diminished airway responsiveness independently of mast cells, or *PKC α* or *eNOS* expression and rapidly reversed established bronchoconstriction independently of NO. Simultaneous inhibition of Syk and PKC revealed additive dilatory effects, whereas combined inhibition of Syk and rho kinase or Syk and p38 MAPK did not cause additive bronchodilation.

Conclusions: Syk inhibition directly attenuates airway smooth muscle cell contraction independent of its protective immunomodulatory effects on allergic airway inflammation, hyperresponsiveness and airway remodeling. Syk mediates bronchoconstriction in a NO-independent manner, presumably via rho kinase and p38 MAPK, and Syk inhibition might present a promising therapeutic approach in chronic asthma as well as acute asthma attacks.

Keywords

airway remodeling; airway responsiveness; asthma; bronchoconstriction; p38 MAPK

Introduction

Airway inflammation, hyperresponsiveness and remodeling are the main pathophysiological hallmarks of asthma(1). Despite intensive research within the past decades, a large unmet need remains in asthma therapy, in particular for difficult-to-treat asthma patients presenting with frequent exacerbations(2) or acute severe asthma(3). Recently, spleen tyrosine kinase (Syk), a cytoplasmatic non-receptor tyrosine kinase, has emerged as a promising target in asthma therapy(4).

Several lines of evidence support the hypothesis that Syk plays a crucial role in the induction of allergic airway inflammation and airway hyperresponsiveness (AHR)(5-9). Allergen-induced airway inflammation leads to increased pulmonary Syk expression(5), and Syk inhibition results in diminished allergic airway inflammation and/or AHR induced by allergen sensitization and challenge(5,6), or passive sensitization(7). This was specifically shown for the Syk inhibitor BAY 61-3606(6,7), which was used in the present study along with the newly described Syk inhibitor BI 1002494. In further support of this notion, aerosolized Syk antisense oligodeoxynucleotides(8), Syk

siRNA(9) as well as Syk deletion(6) diminished allergic airway inflammation. Syk functions as an important immune modulator and is critical for antibody receptor-mediated activation and/or degranulation of mast cells (MCs) and eosinophils(7). Further, Syk is required for immune complex internalization and antigen presentation in dendritic cells(10).

Interestingly, besides its central role in immune signaling, Syk has also been linked to proliferation of bronchial epithelial(11) and airway smooth muscle cells (ASMCs)(12), suggesting a role for Syk in airway remodeling. Of specific relevance for potentially protective effects of Syk inhibition in agonist-induced airway constriction, Syk knockdown was recently shown to reduce airway contractility in response to methacholine (MCh) independent of leukocyte function(13), implicating that Syk may directly promote smooth muscle cell (SMC) contraction. However, characterization of the bronchodilatory activity of Syk inhibition in lungs of naïve, nonsensitized mice and identification of potential underlying mechanisms of Syk-mediated SMC contraction remain elusive. Here we provide evidence for a previously unrecognized role of Syk as a nitric oxide (NO)-independent modulator of agonist-induced downstream signaling in airway constriction.

Various bronchoconstrictive mediators have been implicated in the pathogenesis of asthma. In asthma patients, elevated plasma levels of serotonin(14) and angiotensin II(15) were reported, and levels of serotonin have been correlated with lung function and disease severity(14). Further, sphingosine-1-phosphate (S1P), which contributes to ASMC growth(16) and contraction(17), was shown to be increased in bronchoalveolar lavage fluid (BALF) of asthmatic patients following antigen challenge(16). Consistently, deficiency of the S1P synthesizing enzyme sphingosine kinase 1 was associated with reduced allergic airway inflammation and airway responsiveness(18). Interestingly, S1P(19) and angiotensin II(20) have been reported to induce Syk phosphorylation in SMCs.

In this study, we first evaluated the effects of the highly selective Syk inhibitor BI 1002494 in allergen-evoked airway inflammation and hyperresponsiveness. Next, we analyzed Syk expression in ASMCs and evaluated the effects of Syk inhibition on bronchoconstriction independent of airway

inflammation. Since Syk-mediated activation of p38 mitogen-activated protein kinase (p38 MAPK) has been repeatedly described(6,9,21,22), we further probed for the mechanistic link between Syk and p38 MAPK signaling in airway constriction. p38 MAPK is a family of serine/threonine protein kinases, which plays a key role in inflammatory processes(23), thereby showing intriguing parallels to Syk: p38 MAPK is activated in allergic lung inflammation and promotes Th2 inflammation, allergen-induced AHR as well as airway remodeling(24,25). Choi and colleagues have recently reported that both Syk and p38 MAPK mediate interleukin (IL)-13 production in human airway epithelial cells in response to house dust mite (*Dermatophagoides farinae*) stimulation(26). Importantly, p38 MAPK is also involved in downstream signaling pathways leading to SMC contraction(22).

Based on these considerations, we hypothesized that Syk may elicit airway constriction via p38 MAPK signaling independent of allergen-induced airway inflammation and AHR.

Methods

An expanded methods section is provided in the online supplement.

Mice

Animal procedures were approved by government and institutional authorities. Wild-type mice: Female BALB/c mice aged 6-8 weeks were used for analyses of allergic airway inflammation and female C57BL/6N mice aged 8-10 weeks or male C57BL/6J mice aged 16-19 weeks for *ex vivo* analyses. Transgenic mice: 129/SV mice deficient in protein kinase C alpha (*PKCα*^{-/-}), mast cell-deficient mice (*MC*^{-/-}; C57BL/6-Cpa3-Cre;Mcl-1^{fl/fl}), and NMRI/C57BL/6 mice deficient in endothelial nitric oxide synthase (*eNOS*^{-/-}), were analyzed *ex vivo*.

Allergen-evoked airway inflammation

Acute ovalbumin (OVA) model: Mice were sensitized by intraperitoneal applications of 20 µg OVA in 100 µL PBS and 100 µL Al(OH)₃ on day 0, 14 and 21, and challenged by 20 min inhalation of 1% aerosolized OVA in PBS on day 26 and 27.

Chronic triple allergen model: Mice were sensitized by intraperitoneal injections of 20 µg OVA, 2.5 µg cockroach extract and 2.5 µg house dust mite in 100 µL PBS and 100 µL Al(OH)₃ on day 0, 14 and 21, and challenged by intratracheal application of 20 µg allergen in 50 µL PBS in the following pattern: OVA on day 26, 27, 47 and 48, cockroach extract on day 33, 34, 54 and 55, house dust mite on day 40, 41, 61 and 62 (Figure 5A)(27).

BI 1002494 (10, 30 or 100 mg/kg) in 0.5% Natrosol hydroxyethylcellulose was dosed orally b.i.d. on day 26 and 27 (acute model) or on day 47-51, 54-58 and 61-62 (chronic model). Analyses were performed 24 hours after the final challenge.

Pulmonary and humoral immune response

BAL was performed twice and BALF cells were quantified(27). Chemokines and cytokines were assessed in BALF supernatant via multiplex assay(27). Total serum IgE was quantified by ELISA.

AHR *in vivo*

The airway obstruction index enhanced pause (Penh) was measured in response to increasing concentrations of MCh using whole-body-plethysmography(27).

Airway remodeling

Sections of paraformaldehyde-fixed and paraffin-embedded lungs were incubated with α -smooth muscle actin (α -SMA) antibody and airway smooth muscle thickening was quantified by automated analyses(27). Collagen I was assessed in lung homogenate supernatant via ELISA(27).

Human lung explants

After approval by the ethics committee and obtaining written informed consent from each patient (EA2/050/08, EA2/023/07), lung tissue explants were freshly obtained from patients mainly suffering from bronchial carcinoma. Small tissue cylinders were stamped out of tumor-free peripheral lung tissue(28).

Immunofluorescence

Paraformaldehyde-fixed and paraffin-embedded lung tissue was deparaffinized. After antigen retrieval, permeabilization and blocking, sections were incubated with Syk (N-19) antibody, followed by Alexa Fluor 488-conjugated secondary antibody, Cy3™-labeled anti- α -SMA antibody and nuclear counterstaining. Spectral confocal microscopy was performed(28).

Airway responsiveness in isolated mouse lungs

Murine lungs were isolated(18), perfused with buffer and ventilated with negative pressure ventilation. Increasing concentrations of MCh, serotonin, S1P, angiotensin II or potassium chloride (KCl) were administered to the perfusate. The respective inhibitor, BAY 61-3606 (1 μ mol/L), BI 1002494 (0.1, 0.3, 1 or 3 μ mol/L), NO synthesis inhibitor L-NAME (1 mmol/L), p38 MAPK inhibitor SB 203580 (10 μ mol/L), rho kinase (RhoK) inhibitor Y-27632 (5 μ mol/L), or protein kinase C (PKC) inhibitor bisindolylmaleimide I hydrochloride (BIM; 10 μ mol/L), or the corresponding solvent (H₂O or DMSO) were administered to the perfusate ≥ 10 min before stimulus application. The change in res_{aw} was expressed as factor of baseline res_{aw} (fold res_{aw}).

Human Airway smooth muscle cells (HASMCs)

PDGF-BB-induced proliferation and TNF-induced cytokine/chemokine release in cultured HASMCs in the presence or absence of BI 1002494 were analyzed via flow cytometry (Supplementary Figure 1) or ELISA, respectively.

Statistical analysis

Mann-Whitney *U* test for comparison between groups, two-way ANOVA for comparison of dose-response curves obtained in isolated mouse lungs, paired *t*-test for comparison of measured values before and after treatment and one-way ANOVA with Turkey's multiple comparisons test for analysis of HASMC function were performed.

Results

BI 1002494 reduces allergic airway inflammation and AHR

First, we evaluated the effects of the novel, highly specific Syk inhibitor BI 1002494 in pulmonary Th2 inflammation. To this end, dose-dependent effects of oral BI 1002494 treatment on allergic airway inflammation and AHR were analyzed in mice. Systemic sensitization with ovalbumin and repetitive airway challenge led to a pulmonary influx of eosinophils (Fig. 1A), elevated levels of the Th2 cytokine IL-4 (Fig. 1B), and AHR to MCh (Fig. 1C) as compared to sham-sensitized control mice. BI 1002494 dose-dependently reduced allergic airway inflammation (Fig. 1A-B) and AHR (Fig. 1C). Interestingly, the BI 1002494 concentration of 10 mg/kg had no effects on pulmonary eosinophil counts (Fig. 1A) or on AHR (Fig. 1C) but was sufficient to decrease IL-4 levels in the BALF supernatant (Fig. 1B). The BI 1002494 concentration of 100 mg/kg had strong protective effects on airway inflammation (Fig. 1A-B) and further reduced AHR (Fig. 1C).

The reduction in AHR following Syk inhibition has traditionally been considered as a result of attenuated airway inflammation. Alternatively, effects of Syk inhibition on AHR may be directly related to Syk-mediated smooth muscle function.

Syk is expressed in ASMCs

To characterize the role of Syk in airway smooth muscle, we first probed for Syk expression by spectral confocal microscopy. We detected Syk expression in ASMCs by demonstrating co-localization of Syk and α -SMA in freshly harvested human (Fig. 2A) and murine (Fig. 2B) lung tissue. Furthermore, Syk was shown to be expressed in human (Fig. 2A) and murine (Fig. 2B) airway epithelial cells in accordance with recent findings(5).

Syk inhibition leads to diminished airway responsiveness in isolated mouse lungs

Next, we determined the functional role of Syk in airway responsiveness to different bronchoconstrictive stimuli by use of the Syk inhibitor BAY 61-3606 in isolated perfused and ventilated mouse lungs. Basal airway resistance was unaffected by Syk inhibition (Fig. 2C), yet MCh-evoked airway constriction was largely diminished following Syk inhibition (Fig. 2D). Similarly, airway responsiveness to serotonin (Fig. 2E), S1P (Fig. 2F), angiotensin II (Fig. 2G) and KCl (Fig. 2H) was blunted by BAY 61-3606 treatment, suggesting a central role for Syk in the signaling cascade mediating agonist-induced bronchoconstriction.

BAY 61-3606 reduces bronchoconstriction independently of MCs, or PKC α or eNOS expression

BAY 61-3606 was previously reported to reduce PKC α activity(10). To prove that the observed effects of BAY 61-3606 on airway responsiveness are not related to PKC α inhibition, effects of BAY 61-3606 were re-evaluated in serotonin-induced bronchoconstriction in the absence of PKC α . Consistent with the findings in wild-type mice, BAY 61-3606 showed no effect on basal airway resistance in PKC α ^{-/-} mice (1.22 ± 0.03 cmH₂O/mL/s, mean $\pm$ SEM) as compared to solvent controls

(1.18 ± 0.03), whereas serotonin-induced bronchoconstriction was largely diminished following BAY 61-3606 treatment (Fig. 3A), as demonstrated before in wild-type mice (Fig. 2E). Thus, the reduction of airway responsiveness induced by BAY 61-3606 does not seem to be related to inhibition of PKC α .

Moreover, Syk-mediated bronchoconstriction was not attributable to MC function, as Syk inhibition also reduced airway responsiveness in MC-deficient mice (Fig. 3B).

Since Syk has been linked to NO production in airway epithelial cells(21) and eNOS is a known regulator of bronchoconstriction(29), we tested whether the attenuating effects of Syk inhibition on bronchoconstriction may depend on eNOS-derived NO. However, in *eNOS*^{-/-} lungs serotonin-induced bronchoconstriction was again greatly reduced following Syk inhibition (Fig. 3C), suggesting that these attenuating effects are independent of eNOS.

Syk inhibition rapidly reverses airway constriction independent of NO signaling

To prove efficacy of Syk inhibition in the context of sustained bronchoconstriction, BAY 61-3606 was applied in isolated mouse lungs 28 minutes after initiation of continuous MCh-evoked airway constriction. BAY 61-3606 administration to the perfusate reduced the sustained bronchoconstriction in the presence of MCh by approximately 50% (Fig. 3D, Supplementary Figure 2). When BAY 61-3606 treatment was stopped, MCh-evoked airway constriction was rapidly re-established. Since NO is known to mediate its dilatory effects on SMCs in a rapidly reversible manner, we analyzed whether inhibition of endogenous NO synthesis by L-NAME may attenuate BAY 61-3606-evoked bronchodilation. However, no relevant differences in the degree of bronchodilation with or without NO inhibition were detected (Fig. 3D, Supplementary Figure 2), suggesting that Syk regulates the airway tone independent of NO signaling.

Syk promotes airway responsiveness via RhoK and p38 MAPK signaling

Airway constriction depends on RhoK(17,30) and PKC signaling(17). To determine the mechanisms underlying Syk-dependent bronchoconstriction, we analyzed the effects of separate or simultaneous inhibition of Syk and/or RhoK as well as of Syk and/or PKC on serotonin-evoked airway responsiveness.

None of these inhibitor treatments affected basal airway resistance. However, as expected, inhibition of Syk, RhoK or PKC distinctly reduced serotonin-evoked airway constriction compared to solvent treatment ($p \leq 0.001$, respectively; Fig. 3E). Interestingly, no additive inhibitory effects on airway constriction as compared to RhoK inhibition (with Y-27632) were detected when simultaneously targeting Syk and RhoK, suggesting that Syk-mediated bronchoconstrictive effects at least partly depend on RhoK signaling. In contrast, PKC-mediated bronchoconstriction seems to be Syk-independent, since simultaneous Syk and PKC inhibition (with BIM) resulted in additive inhibitory effects as compared to PKC inhibition only (Fig. 3E).

p38 MAPK has been shown to be activated by RhoA/RhoK signaling(31) and to contribute to bronchoconstriction(24,25). Since Syk-induced p38 MAPK activation has been repeatedly described(6,9,21,22), we further analyzed the effects of separate or simultaneous inhibition of Syk and/or p38 MAPK (with SB203580) on serotonin-evoked airway responsiveness. Basal airway resistance was unaltered following Syk and/or p38 MAPK inhibition. However, both p38 MAPK and Syk inhibition reduced airway responsiveness to serotonin ($p=0.0178$ and $p=0.0006$, respectively; Fig. 3F). Intriguingly, simultaneous inhibition of Syk and p38 MAPK had no additive inhibitory effects on airway constriction as compared to Syk inhibition alone, suggesting that Syk exerts its bronchoconstrictive effects via p38 MAPK (Fig. 3F). Nevertheless, the fact that the effects on bronchoconstriction due to Syk inhibition or p38 MAPK inhibition significantly differ ($p=0.0045$) suggests that Syk does not exclusively act via p38 MAPK.

BI 1002494 reduces airway responsiveness in naïve mice

The attenuating effects of Syk inhibition on bronchoconstriction were confirmed by experiments using the highly selective Syk inhibitor BI 1002494. Analogous to our findings with BAY 61-3606, BI 1002494 did not affect basal airway tone (Fig. 4A), whereas airway responsiveness to serotonin was profoundly and dose-dependently reduced (Fig. 4B). As an exposure simulation of the BI 1002494 doses used in the *in vivo* experiments (100 mg/kg b.i.d.) yielded a BI 1002494 plasma concentration of 978 nmol/L after 24 hours (Supplementary Figure 3), we continued our *ex vivo* experiments with the BI 1002494 concentration of 1 μ mol/L, which again attenuated airway constriction induced by angiotensin II (Fig. 4C) or KCl (Fig. 4D).

Therapeutic Syk inhibition reduces airway remodeling in a chronic triple allergen asthma model

Mechanical forces in the airways as a result of bronchoconstriction are believed to substantially contribute to airway remodeling(32). Syk, as a central mediator of bronchoconstriction, may thereby promote structural changes in the asthmatic airways. In further support of this notion, Syk has been linked to ASMC(12) and epithelial cell proliferation(11) as well as to collagen deposition in the kidney(33). We therefore investigated the potential protective effects of Syk inhibition on airway remodeling in a chronic, severe model of pulmonary Th2 inflammation using a triple allergen combination(27).

Therapeutic application of BI 1002494 was performed after systemic sensitization and establishment of allergic airway inflammation via repetitive airway challenge with ovalbumin, cockroach extract and/or house dust mite (Fig. 5A). Syk inhibition marginally affected the humoral immune response (Fig. 5B). This modest effect, however, is not surprising, as the application of BI 1002494 was initiated at a late stage of the protocol, after sensitization and 3 weeks of airway challenge (Fig. 5A). Nevertheless, allergen-induced eosinophilic airway inflammation (Fig. 5C) and BALF cytokine levels

of IL-4, IL-10 and IL-1 β (Fig. 5D) were strongly reduced due to Syk inhibition, which is in accordance with the effects of Syk inhibition on acute Th2-induced airway inflammation (Fig. 1A-B).

Importantly, Syk inhibition partially protected from allergen-induced airway remodeling (Fig. 5E-F). Both the increase in pulmonary collagen I deposition (Fig. 5E) and the increase in airway smooth muscle mass (Fig. 5F) were reduced following BI 1002494 treatment by ~55% and ~61%, respectively. Although the attenuating effect of BI 1002494 on ASMC thickening, assessed by automated analyses of α -smooth muscle actin stained lung slides, did not reach the level of statistical significance, the prevention is an interesting finding as it may indicate a functional role of Syk in Th2-induced SMC proliferation.

Syk promotes proliferation and proinflammatory mediator release in HASMCs

To further characterize Syk function in ASMCs, the effects of BI 1002494 treatment on platelet-derived growth factor-induced proliferation and tumor necrosis factor-induced cytokine/chemokine release of HASMCs were assessed. Both proliferation (Fig. 6A) and proinflammatory function reflected by the secretion of eotaxin-1 and IL-6 (Fig. 6B) were markedly reduced following Syk inhibition pointing to a central proliferative and immunomodulatory role of Syk in ASMCs.

Discussion

In the present study, we detected Syk expression in ASMCs of freshly harvested human and murine lung tissue and identified Syk as an important NO-independent mediator of airway constriction in naïve lungs. Syk inhibitor BAY 61-3606 rapidly reversed established bronchoconstriction of airways. Syk contributes in parallel to Th2 inflammation, AHR and airway remodeling, and Syk inhibitors have already been proven to be generally well tolerated in humans(34). Hence, Syk may present a promising target for both long-term therapy of asthma and management of acute asthmatic attacks.

In isolated murine lungs, Syk inhibition remarkably reduced airway responsiveness towards clinically relevant stimuli in line with a recent report by Wang and colleagues showing that Syk inhibitor NVP-QAB-205 reduced airway contractility of *ex vivo* murine lung slices evoked by particulate matter air pollutants(13). Treatment with BAY 61-3606 and/or BI 1002494 not only diminished airway contraction induced by activators of G protein-coupled receptors, namely methacholine, serotonin, sphingosine-1-phosphate and angiotensin II, but also blunted KCl-evoked bronchoconstriction. Interestingly, all of these stimuli induce airway contraction in a RhoK-dependent manner(17,30,35,36) and are known activators of p38 MAPK(37-41).

RhoK(17,30) and p38 MAPK(24,25) are important regulators of agonist-induced airway contraction. In SMCs, the GTP-binding protein RhoA activates RhoK, which itself inactivates myosin light-chain (MLC) phosphatase and subsequently promotes MLC phosphorylation leading to calcium sensitization and contraction(42). In lung microvascular endothelial cells, p38 MAPK was shown to act downstream of RhoK(31). Our results imply for the first time that Syk promotes airway constriction at least partly via activation of RhoK and p38 MAPK, possibly acting as an intermediary between RhoK and p38 MAPK, as Syk inhibition was less efficient to attenuate airway constriction than RhoK inhibition yet more efficient than p38 MAPK inhibition. However, cross signaling between RhoK, Syk and p38 MAPK is also possible and the exact molecular interactions between Syk, RhoK and p38 MAPK that mediate airway constriction remain to be dissected.

NO is known to relax airway smooth muscle(29). So far, three NO synthase isoforms have been identified, namely endothelial (eNOS), neuronal (nNOS) and inducible NO synthase (iNOS). Particularly the constitutively expressed isoforms eNOS and nNOS, which are able to generate NO within seconds, may have dilatory properties in acute airway responsiveness(29). Interestingly, Syk has been linked to NO synthesis in airway epithelial cells(21). However, the bronchodilatory effects induced by Syk inhibition in this study were similarly present in *eNOS*^{-/-} lungs and following inhibition

of all three NO synthase isoforms, suggesting that NO signaling does not contribute critically to the reduced bronchoconstriction following Syk inhibition.

IgE-dependent MC degranulation, an important feature of asthma pathobiology, is known to critically depend on Syk(7). Notably, antibody-independent MC activation e.g. via activation of the serotonin receptor 5-HT_{1A} has also been proposed(43), suggesting that Syk inhibition may alter the antibody-independent release of bronchoactive mediators from MCs. However, analogously to wild-type mice, Syk inhibition reduced bronchoconstriction in MC-deficient mice indicating MC-independent signaling.

BAY 61-3606 has been shown to inhibit PKC α (10). However, the functional effects of BAY 61-3606 on airways in the present study are most likely Syk-specific and not related to PKC α inhibition, since BAY 61-3606 attenuated airway constriction in wild-type as well as in PKC α -deficient mice. In further support of this notion, simultaneous Syk and PKC inhibition induced additive bronchodilatory effects as compared to PKC inhibition alone, suggesting signalling through distinct, separate pathways. Finally, we are able to rule out compound-specific effects, since the attenuating effects on bronchoconstriction could be recapitulated with the highly selective Syk inhibitor BI 1002494.

ASMCs are increasingly recognized as immunomodulatory cells, which may exert pro-inflammatory effects via p38 MAPK, thereby promoting airway remodeling(44). Interestingly, Syk has been shown to essentially promote IgE-induced transcriptional regulation of thymic stromal lymphopoietin in ASMCs(45). Syk has further been linked to proliferation of ASMCs(12) and bronchial epithelial cells(11) as well as to collagen deposition in the kidney(33). The role of Syk in allergen-induced airway remodeling, however, remained elusive. The currently detected protective effects of therapeutic Syk inhibition on airway remodeling support the hypothesis of Syk-mediated airway remodeling and further point to a potential clinical benefit of Syk inhibition in asthmatic patients. Mechanistically, Syk may mediate airway remodeling in various ways, e.g. by promoting Th2 inflammation, by promoting pro-inflammatory functions of ASMCs(44,45) and by promoting

mechanical forces in the airways independently of inflammation(32). Further studies are thus necessary to clarify the exact role of Syk in airway remodeling.

In summary, Syk promotes antigen-induced lung inflammation, AHR and airway remodeling. In addition, we here identify Syk as a direct NO-independent regulator of airway constriction possibly via modulation of RhoK and p38 MAPK signaling. Syk inhibition showed protective effects against several-bronchoconstrictors and rapidly reversed established bronchoconstriction, suggesting a promising perspective for pharmacological Syk inhibition in both chronic therapy and acute interventions in asthma patients.

Abbreviations

AHR, airway hyperresponsiveness

Ang II, angiotensin II

ASMC, airway smooth muscle cell

b.i.d., twice daily

BIM, bisindolylmaleimide I hydrochloride

eNOS, endothelial nitric oxide synthase

HASMC, human airway smooth muscle cell

iNOS, inducible nitric oxide synthase

KCl, potassium chloride

MC, mast cell

MCh, methacholine

MLC, myosin light chain

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

p38 MAPK, p38 mitogen-activated protein kinase

PKC, protein kinase C

PKC α , protein kinase C alpha

RhoK, rho kinase

S1P, sphingosine-1-phosphate

SMC, smooth muscle cell

Syk, spleen tyrosine kinase

Competing interests

David Lamb, Lutz Wollin and Klaus Erb are employees of Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. None of the other authors have financial or non-financial competing interests.

Authors' contributions

CT and MW designed the study. All authors contributed to sample acquisition and analysis. CT, JH, ACH, DL, LW, KE, EB, HM, ASG, WMK, NS and MW interpreted the data. CT, DL and MW drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Spleen tyrosine kinase (Syk) inhibition dose-dependently reduced allergen-induced airway inflammation and airway hyperresponsiveness (AHR). For induction of pulmonary Th2 inflammation, mice were systemically sensitized by intraperitoneal application of ovalbumin (OVA) (day 0, 14 and 21) and repetitively challenged by intratracheal OVA application (day 26, 27). Control mice were sham-sensitized and sham-challenged with PBS, respectively (PBS ctrl). Analyses were performed 24 hours after the last airway challenge. Oral treatment with Syk inhibitor BI 1002494 (10, 30 or 100 mg/kg b.i.d. on day 26 and 27) dose-dependently reduced eosinophil numbers in the bronchoalveolar lavage fluid (BALF) (A), BALF supernatant interleukin (IL)-4 levels (B) as well as AHR to methacholine (MCh) (C), which was assessed *in vivo* using whole-body-plethysmography. Values are given as mean and SEM; n=7-8 per group. For A and B: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. OVA ctrl group. For C: * $p \leq 0.05$ 100 mg/kg BI 1002494 vs. OVA ctrl group.

Figure 2. Syk is expressed in airway smooth muscle cells and mediates bronchoconstriction. Syk staining (green) was observed in the cytosol of human (A) and murine (B) airway epithelial cells (asterisks) and, as revealed by co-localization (merge; orange) with α -smooth muscle actin (α -SMA; red), in airway smooth muscle cells (arrowheads). Nuclei were counterstained with DAPI (blue). Merged images were combined with differential interference contrast to reveal lung morphology (grey). In isolated perfused and ventilated mouse lungs, basal airway resistance (res_{aw}), assessed ~20 min after solvent or Syk inhibitor BAY 61-3606 administration to the perfusate, was unaffected by Syk inhibition (C). Airway responsiveness (fold res_{aw}) to MCh (D), serotonin (E), sphingosine-1-phosphate (S1P) (F), angiotensin II (Ang II) (G), or potassium chloride (KCl) (H) was profoundly diminished following Syk inhibition. For A and B: The scale bar represents 10 μ m and is valid for all photomicrographs. Representative images of both species (n=3 each) are shown. For C-H: Values are given as mean and SEM; n $\geq$ 29 (C) or n=5-7 (D-H) per group. The figure legends in D are valid for D-H. ** $p \leq 0.01$, *** $p < 0.001$ vs. indicated group.

Figure 3. Syk promotes airway responsiveness possibly via rho kinase and p38 mitogen-activated protein kinase (p38 MAPK), independently of mast cells, or PKC α or eNOS expression. In isolated perfused and ventilated lungs deficient for protein kinase C alpha (*PKC α ^{-/-}*) (A), mast cells (*MC^{-/-}*) (B) or endothelial nitric oxide synthase (*eNOS^{-/-}*) (C), Syk inhibitor BAY 61-3606 largely decreased serotonin-induced bronchoconstriction. (D) Isolated wild-type lungs were constantly perfused with methacholine (MCh; 30 μ mol/L) to induce sustained bronchoconstriction. After 28 min of sustained airway constriction, Syk inhibitor BAY 61-3606 was added to the perfusate for 16 min. Syk inhibition rapidly reduced the sustained bronchoconstriction (D). In separate experiments, L-NAME was added to the perfusate to inhibit endogenous nitric oxide synthesis, which did not alter BAY 61-3606-induced bronchodilation (D). Combined administration of Syk inhibitor BAY 61-3606 and protein kinase C (PKC) inhibitor BIM revealed additive attenuating effects on serotonin-evoked bronchoconstriction as compared to exclusive PKC inhibition (E), whereas combined treatment with BAY 61-3606 and rho kinase inhibitor Y-27632 (E) or BAY 61-3606 and p38 MAPK inhibitor SB 203580 (F) showed no additive dilatory effects. The airway responsiveness of the respective solvent group to 100 μ mol/L serotonin was set to 100% (E, F). Values are given as mean and SEM; n=5-6 (A), n=4 (B), n=9 (C), n=6-7 (D), n=4-6 (E) or 5-7 (F) per group. The figure legends in A are valid for A-C. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. indicated group (A-C, E, F); *** $p \leq 0.001$ vs. indicated time point (control group) (D); ## $p \leq 0.01$, ### $p \leq 0.001$ vs. indicated time point (L-NAME group) (D); ns = not significant.

Figure 4. Syk inhibitor BI 1002494 dose-dependently reduced airway responsiveness. Application of Syk inhibitor BI 1002494 did not affect basal airway resistance (A) in isolated perfused and ventilated mouse lungs. Airway responsiveness to serotonin (B), Ang II (C), or KCl (D) was profoundly diminished by Syk inhibition. Values are given as mean and SEM; n=4-7 (A, B) or n=6-7 (C, D) per group. * $p \leq 0.05$ vs. indicated group.

Figure 5. Therapeutic Syk inhibition reduced allergen-induced airway remodeling. Mice were treated as illustrated (A) and analyses were performed 24 hours after the last challenge. Systemic sensitization by intraperitoneal application of the triple allergen combination (TAC) of OVA, cockroach extract (CRA) and house dust mite (HDM) (day 0, 14 and 21) and repetitive intratracheal airway challenge with OVA (day 26, 27, 47, 48), CRA (day 33, 34, 54, 55) or HDM (day 40, 41, 61, 62) induced a humoral immune response (B), an allergic lung inflammation (C, D) and profound airway remodeling (E, F). Oral treatment with Syk inhibitor BI 1002494 (100 mg/kg; b.i.d. on day 47-51, 54-58, 61-62) did not significantly reduce total serum immunoglobulin E (IgE) (B), but strongly diminished BALF eosinophil numbers (C) and BALF supernatant levels of IL-4, IL-10 and IL-1 β (D). BALF cytokine levels of IL-5, KC and IL-12p70 showed a trend towards reduction following BI 1002494 application (D). Airway remodeling was characterized by increased collagen I levels (E) and airway smooth muscle mass (F) in the lung. The elevation in pulmonary collagen I was less pronounced following BI 1002494 treatment (E). Automated quantification of airway smooth muscle thickness revealed that the allergen-evoked thickening was by trend reduced due to Syk inhibition (F). The PBS control group was set to 100% (dotted line; E, F). Values are given as mean and SEM; n=6-9 per group. * $p \leq 0.05$, ** $p \leq 0.01$ vs. untreated sensitized and challenged group (TAC).

Figure 6. Syk inhibitor BI 1002494 reduced proliferation and cytokine/chemokine release in human airway smooth muscle cells (HASMCs). HASMCs were cultured and stimulated with platelet-derived growth factor-BB (PDGF-BB; 10 ng/mL), tumor necrosis factor (TNF; 10 ng/mL) or medium alone in the presence or absence of Syk inhibitor BI 1002494. PDGF-induced HASMC proliferation, as reflected by the proportion of EdU-positive cells assessed via flow cytometry, was fully blocked by BI 1002494 treatment (A). TNF evoked an increase in eotaxin-1, IL-6 and IL-8 release in HASMC supernatant (B). Levels of eotaxin-1 and IL-6 were reduced following Syk inhibition (B). Supernatant IL-8 levels showed a trend towards reduction following BI 1002494 treatment (B). Values are given as mean and SEM; n= 3 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. indicated group.

Figure 1

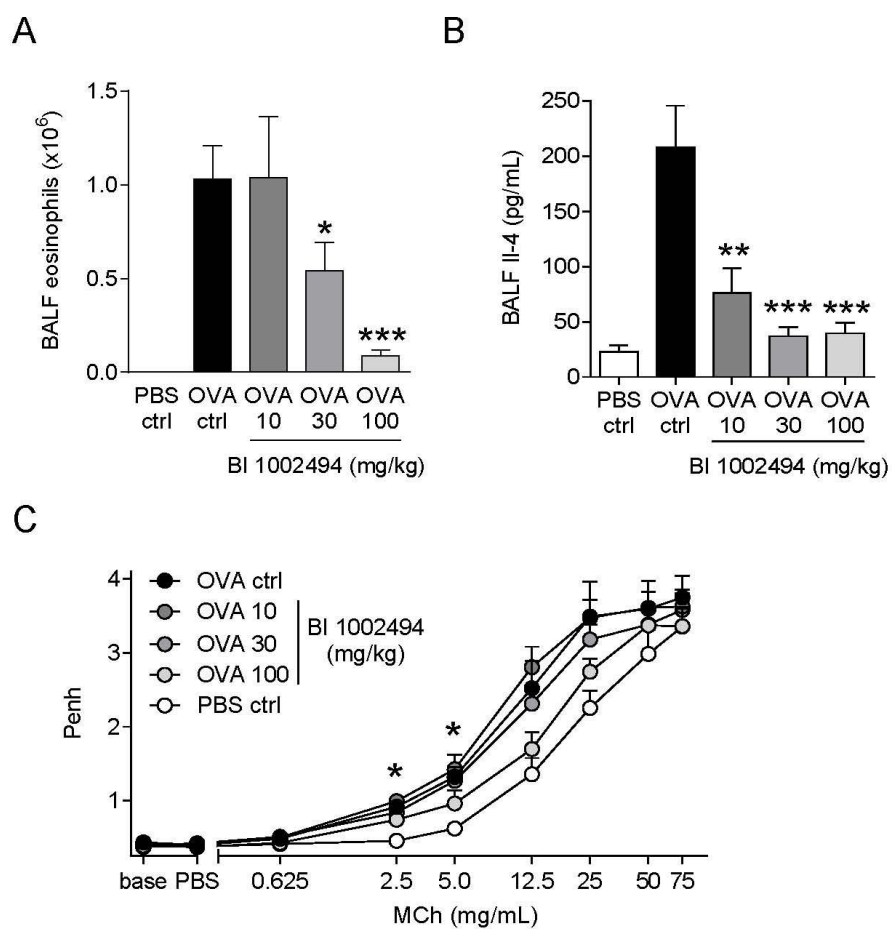


Figure 2

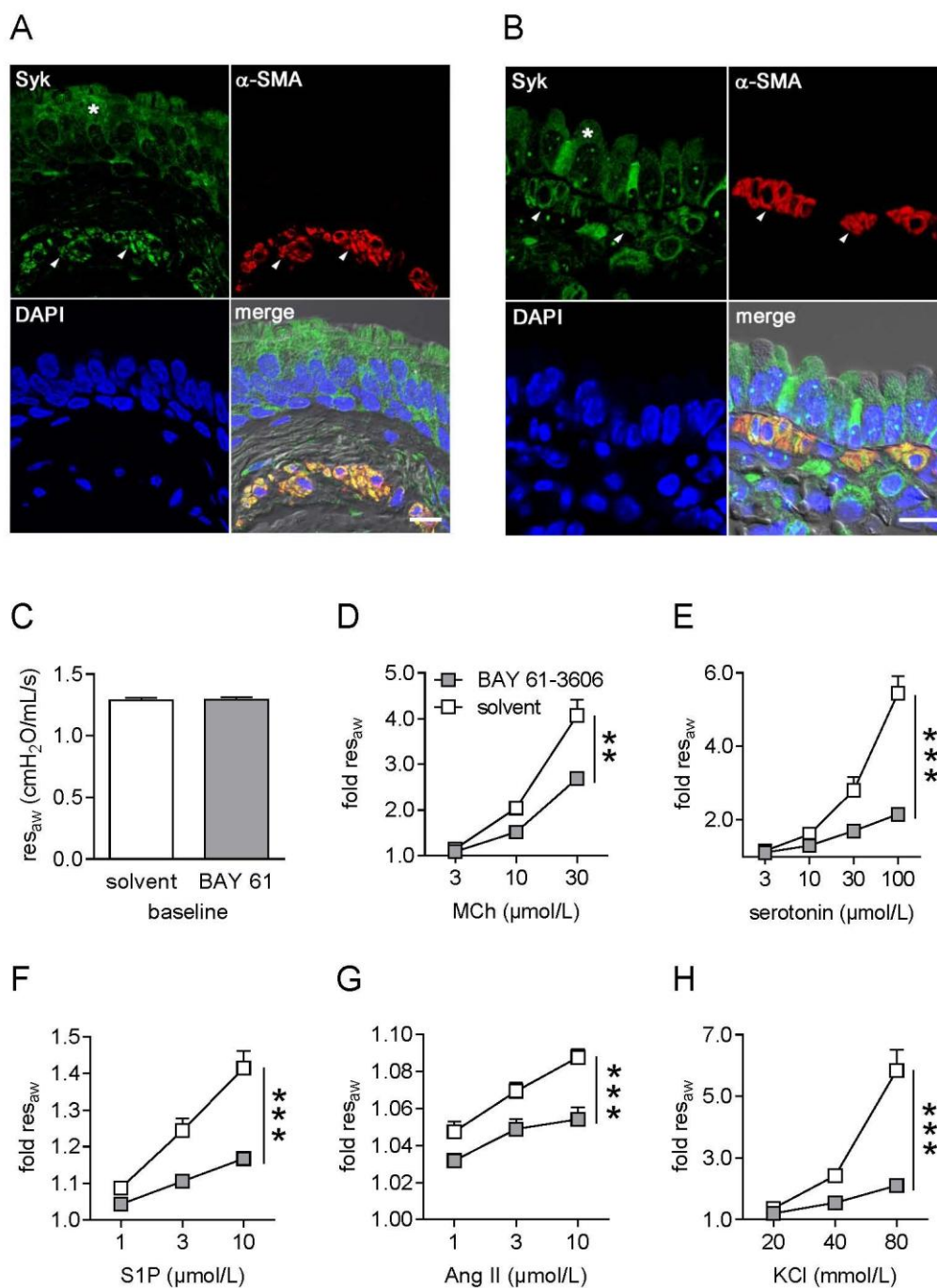


Figure 3

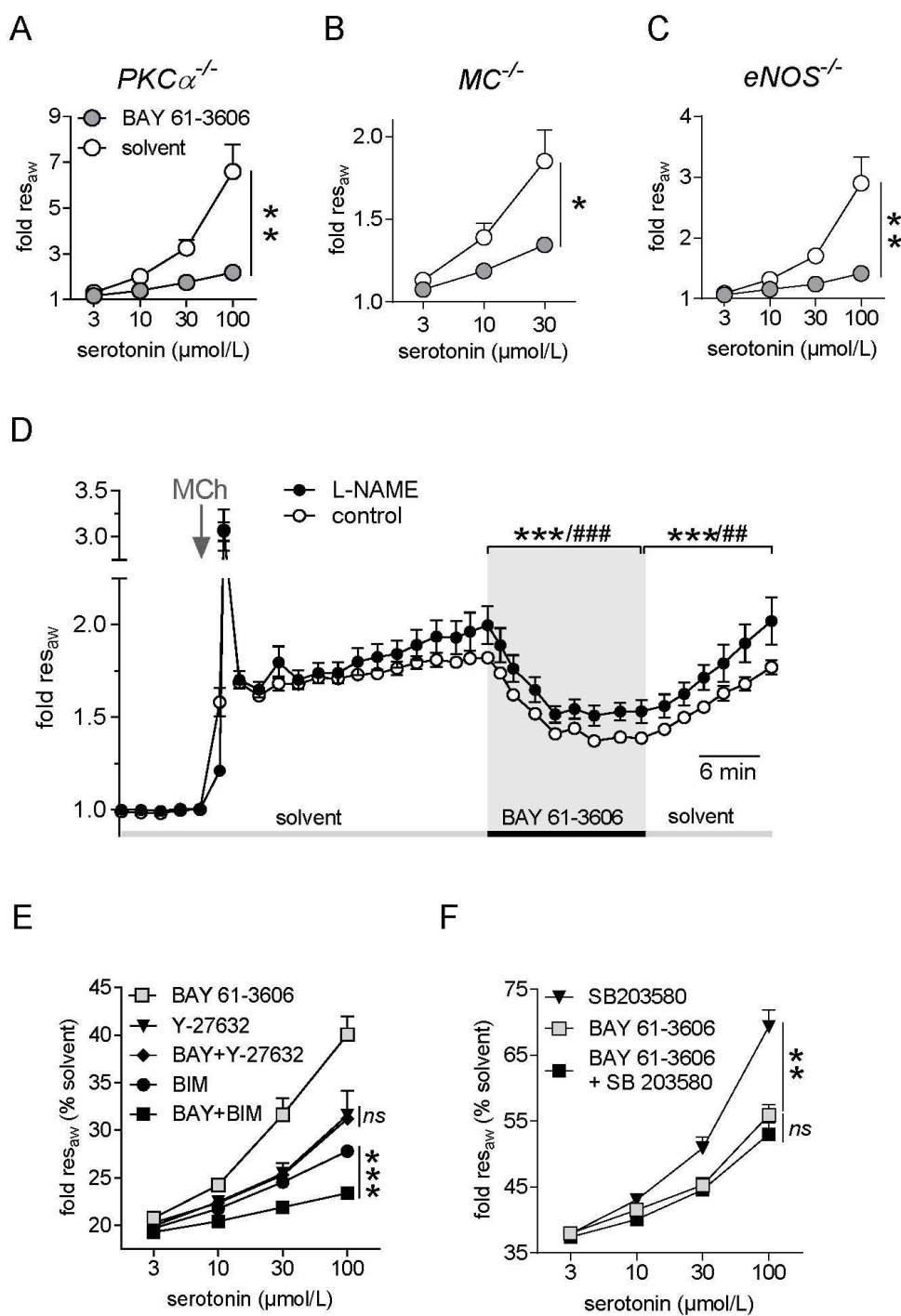


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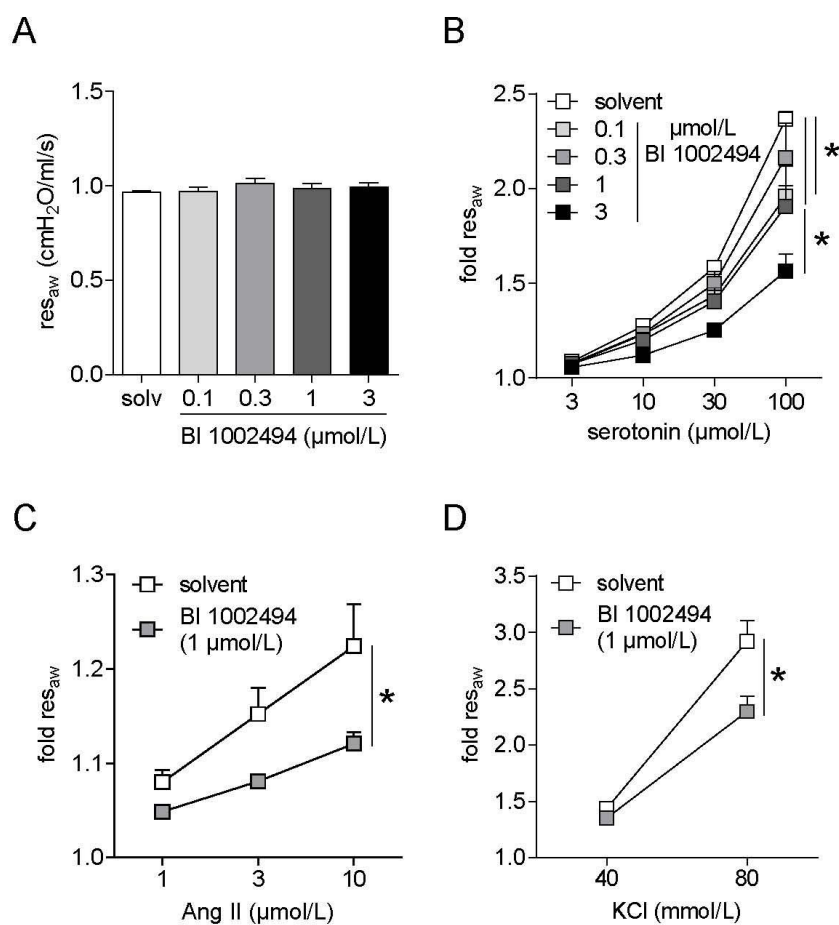


Figure 5

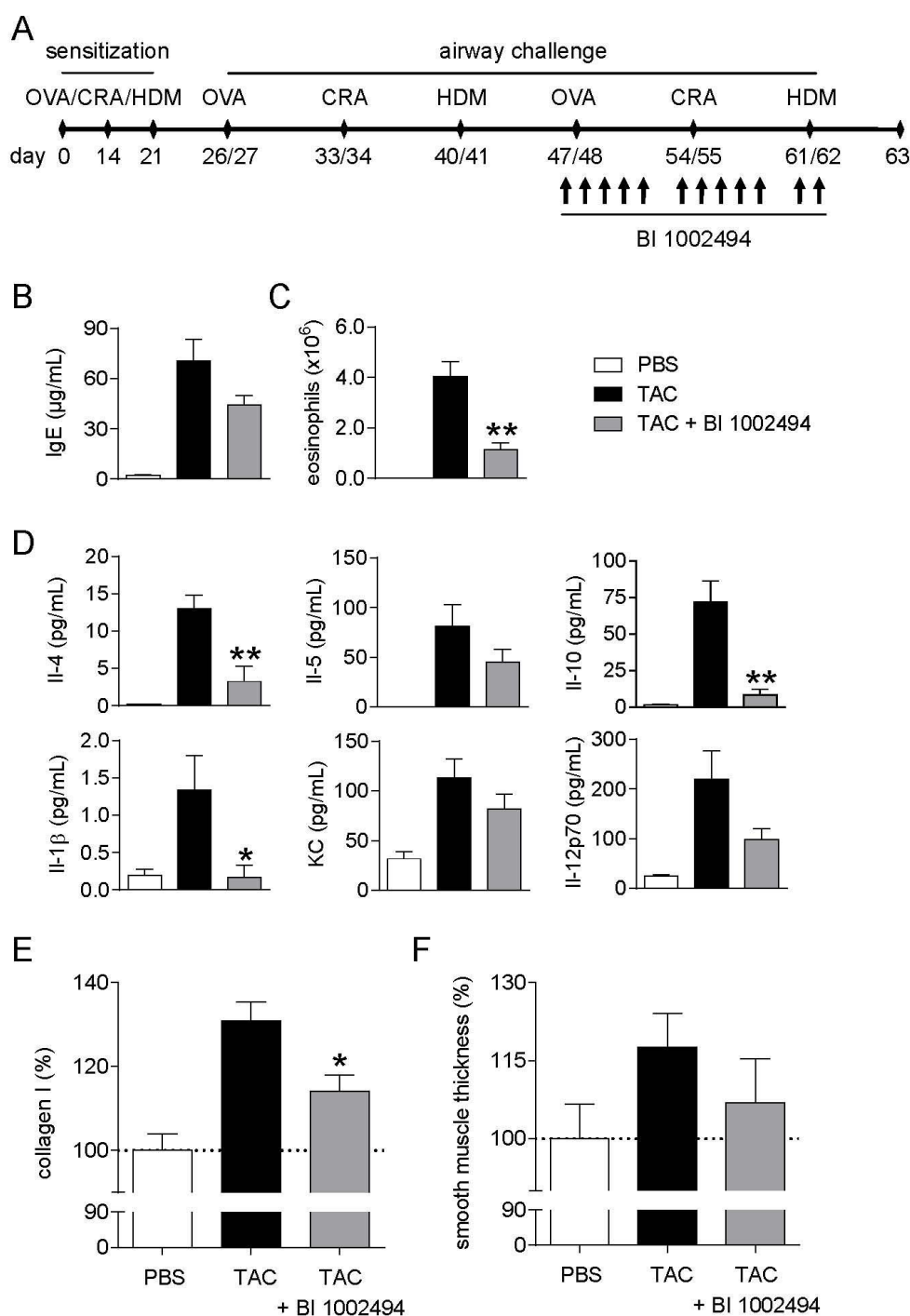


Figure 6

