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Boysenberry ingestion supports fibrolytic macrophages with the capacity to ameliorate chronic lung remodeling

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Shaw OM, Hurst RD, Harper JL. Boysenberry ingestion supports fibrolytic macrophages with the capacity to ameliorate chronic lung remodeling. *Am J Physiol Lung Cell Mol Physiol* 311: L628–L638, 2016. First published July 1, 2016; doi:10.1152/ajplung.00309.2015.—Lung fibrosis negatively impacts on lung function in chronic asthma and is linked to the development of profibrotic macrophage phenotypes. Epidemiological studies have found that lung function benefits from increased consumption of fruit high in polyphenols. We investigated the effect of boysenberry consumption, in both therapeutic and prophylactic treatment strategies in a mouse model of chronic antigen-induced airway inflammation. Boysenberry consumption reduced collagen deposition and ameliorated tissue remodeling alongside an increase in the presence of CD68+CD206+arginase+ alternatively activated macrophages in the lung tissue. The decrease in tissue remodeling was associated with increased expression of profibrolytic matrix metalloproteinase-9 protein in total lung tissue. We identified alternatively activated macrophages in the mice that consumed boysenberry as a source of the matrix metalloproteinase-9. Oral boysenberry treatment may moderate chronic tissue remodeling by supporting the development of profibrolytic alternatively activated macrophages expressing matrix metalloproteinase-9. Regular boysenberry consumption therefore has the potential to moderate chronic lung remodeling and fibrosis in asthma and other chronic pulmonary diseases.

airway remodeling; chronic lung fibrosis; fibrolytic macrophages; fruit consumption

IT IS ESTIMATED THAT 150 MILLION people are affected by asthma worldwide, with a 5–15% prevalence in children (61). In the case of chronic asthma there is evidence of cumulative tissue remodeling, fibrosis, and consequent loss of lung function (45, 59). Current asthma treatments are designed to manage inflammation, symptoms, and the severity of asthma attacks (30, 43). However, there are no current treatments available that are known to modulate aberrant tissue remodeling.

Asthma pathogenesis and lung tissue remodeling have been linked to an increase in profibrotic, arginase-positive, alternatively activated macrophages (AAMs) in the lung (27, 29, 34). However, temporal depletion of macrophage populations in a model of bleomycin-induced pulmonary fibrosis illustrates that lung macrophages may also develop fibrolytic functions that contribute toward the resolution of fibrosis (14).

Mediators of tissue remodeling, such as the matrix metalloproteinases (MMPs), play an important role in regulating fibrosis (5, 7, 8, 10, 38). Of these, MMP-9 is widely reported to increase in conditions of lung inflammation and fibrosis and is associated with improved symptoms in asthma sufferers (25, 32, 33). MMP-9, in concert with other MMPs, exerts fibrolytic activity that leads to the breakdown of denatured collagens that could moderate inappropriate lung remodeling (5, 60). As such MMP-9 may represent a possible therapeutic target to limit lung damage in chronic asthma as well as other pulmonary diseases.

Large epidemiological studies have found that increased fruit and vegetable consumption correlates with reduced asthma symptoms (39, 46, 47). These population studies have identified foods high in polyphenols such as apples, pears (13, 51, 62), carrots, tomatoes (46–48), and citrus (11) as having inverse correlations with frequency and severity of reported asthma symptoms, in particular wheeze and cough symptoms (11, 13, 46, 47). However, the effect of fruits high in polyphenols on lung fibrosis and tissue remodeling is unknown. In this study we tested the hypothesis that boysenberry consumption would reduce allergen-induced lung remodeling in a chronic model of asthma. We investigated the effect of boysenberry consumption on lung fibrosis, lung macrophage phenotype, and MMP-9 expression in a chronic model of allergic airway inflammation. We present evidence that oral boysenberry treatment supports the development of lung macrophages that express a mixed antifibrotic, AAM phenotype with the capacity to ameliorate fibrosis and promote balanced lung repair.

METHODS

Materials. Anti-β-actin (clone AC-15), ovalbumin (OVA), 4% formalin, Tween 20, *trans*-hydroxyproline, 3,3′-diaminobenzidine (DAB) substrate, ketamine/xylazine, and all other chemicals were from Sigma (Auckland, NZ). Alum was from Serya (Heidelberg, Germany). The boysenberry juice was New Zealand 65 Brix boysenberry juice concentrate kindly provided by Berryfruit Export NZ. Anti-mouse polyclonal inducible nitric oxide synthase (iNOS) (ab3523), arginase, TIMP-1 (ab38978), and MMP-9 (ab38898) were from Abcam (Cambridge, UK). Antibodies against mouse CD68 (clone FA-11) CD3e, CD8a, CD4, CD11b, CD11c, and Gr-1 were from BioLegend (San Diego, CA) and anti-CD206 (clone MR5D3) was from AbDSerotec (Oxford, UK). Anti-mouse SiglecF, MHCII, and CD45 were from BD Biosciences (San Jose, CA). TGFβ ELISA kit was from R&D Systems (Minneapolis, MN). Vectastain Elite ABC staining kit was from Vector Laboratories (Burlingame, CA). Bio-Plex multiplex cytokine assays for IL-4, IL-5, IL-6, IL-13, and IFNγ, DC Lowry protein assay kit, and PVDF membrane were from Bio-Rad (Hercules, CA). BSA, NuPage 4–12% gels, MES running buffer, sample loading buffer, Novex sharp prestained, and MagicMark XP protein standards and all other buffers were from Life Technologies (Auckland, NZ).

Mice. C57BL/6J male mice were bred and group housed (5 per cage) in conventional polycarbonate cages with a filter top, in a specific pathogen-free animal facility at the Malaghan Institute of

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Medical Research, Wellington, New Zealand. All experimental procedures were approved by the Victoria University of Wellington Animal Ethics Committee (approval number 2011R3M). Mice were maintained on a 12-h light-dark cycle, at 21 ± 2°C ambient temperature with freely available irradiated standard laboratory rodent chow (Specialty Feeds, Glen Forrest, WA, Australia) and acidified water.

OVA-induced airway inflammation. Six-week-old mice were randomized into experimental groups ($n = 10$ per group) and primed intraperitoneally (ip) with 100 μg OVA in 200 μl alum adjuvant on *day 0*. On day +7 mice were challenged intranasally (i.n.) with 100 μg OVA or PBS. To establish chronic disease the i.n. challenge was repeated weekly (Figs. 1*A* and 6*A*). Four days following the last i.n. OVA challenge mice were euthanized (ketamine/xylazine overdose) and bronchial-alveolar lavage fluid (BALF), serum, mediastinal lymph nodes and lung tissue were collected.

Oral boysenberry treatment. For the treatment studies mice were fasted overnight before being orally gavaged with 250 μl of boysenberry juice (10 mg/kg of total anthocyanins) or sterile water on the day of OVA challenge and again 2 days post-OVA challenge (Figs. 1*A* and 6*A*).

Clodronate liposome depletion of lung macrophages. Clodronate liposomes were prepared as previously described (58). Chronic OVA-induced tissue damage was established over 5 wk. Mice were then treated intranasally with 100 μl clodronate liposomes the day prior to each oral gavage with 250 μl of boysenberry juice (10 mg/kg of total anthocyanins) or sterile water (Fig. 6*A*). Two days following the last oral gavage mice were euthanized (ketamine/xylazine overdose) and BALF, serum, mediastinal lymph nodes, and lung tissue were collected.

Flow cytometric, histological, and immunohistological tissue analysis. Cells isolated from the BALF were stained for key surface markers to identify monocytes/macrophages (CD45+/CD11b+/Cd11c+/MHCIIlow) and eosinophils (CD45+/CD11b+/siglecF+) by flow cytometry as previously described (52). TGFβ ELISA and Bio-Plex multiplex cytokine assays were performed on lung tissue supernatants following the manufacturer's instructions. Lung tissue was fixed in 4% formalin, sectioned, and stained with hematoxylin and eosin (H&E), Masson's Trichrome or Alcian blue-periodic acid-Schiff (AB-PAS) stains (Dept. of Pathology, Wellington School of Medicine, University of Otago, Wellington, NZ). Further sections were cut for immunological labeling. Lung sections were incubated with biotin-conjugated MMP-9, then labeled with DAB and counterstained with hematoxylin. Other tissue sections were incubated with fluorescently labeled CD68 (31), CD206 (57), and arginase or MMP-9 (44), then counterlabeled with DAPI-containing mounting medium. All sections were imaged on an Olympus BX51 compound microscope and captured by using cellSens (Olympus NZ) software, bright light in color and fluorescence in grayscale. Fluorescence images were processed (cropped, false colored, and merged) in Pixelmator image software (Vilnius, Lithuania).

Fluorescently labeled cells were quantified by four independent, blinded observers. Cells were counted in random fields from multiple animals and scored as negative, single positive, or double positive for CD68, CD206, arginase, or MMP-9. Data were expressed as a percentage of total cells counted.

Biochemical and molecular biological tissue analysis. Lung tissue was snap frozen and stored at −70°C. Lung collagen was quantified by the hydroxyproline assay as previously described (2).

For Western blotting, tissue was homogenized in protein lysis buffer (Tris·HCl, NaCl, 10% Nonidet P-40, 10% sodium deoxycholate, 100 nM ETDA, pH 7.4 with protease and phosphatase inhibitors). Protein concentration was quantified by a Lowry protein assay as per the manufacturer's instruction. Samples (30 μg protein) were separated by SDS-PAGE gel electrophoresis under reducing conditions and transferred onto PVDF membrane. Nonspecific protein binding was blocked with 3% BSA (10 mM PBS with 0.2% Tween 20) and the membranes were probed overnight with primary antibodies specific to iNOS (64), arginase (53), MMP-9 (44), and TIMP-1 (55), or β-actin (12) loading control (4°C). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence on a Carestream Gel Logic Pro 6000 imager. Protein expression was densitometrically quantified and normalized to β-actin with ImageJ's Gel analysis tool (50). Images were processed and cropped in Pixelmator image software.

Statistical analysis. Data were analyzed by one-tailed Student's *t*-test for comparisons between two groups or one-way ANOVA with Tukey's post hoc test for comparisons between three or more groups as indicated (Prism, GraphPad, San Diego, CA). $P < 0.05$ or less was considered statistically significant.

RESULTS

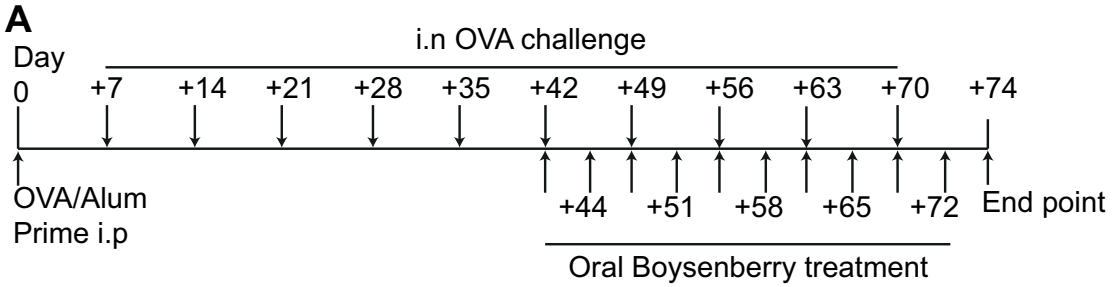
Boysenberry consumption ameliorates OVA-induced airway inflammation. To investigate the effect of boysenberry treatment on established lung remodeling, mice were challenged weekly with intranasal OVA for 5 wk, then challenged weekly with OVA for an additional 5 wk alongside weekly oral treatment with boysenberry (Fig. 1*A*).

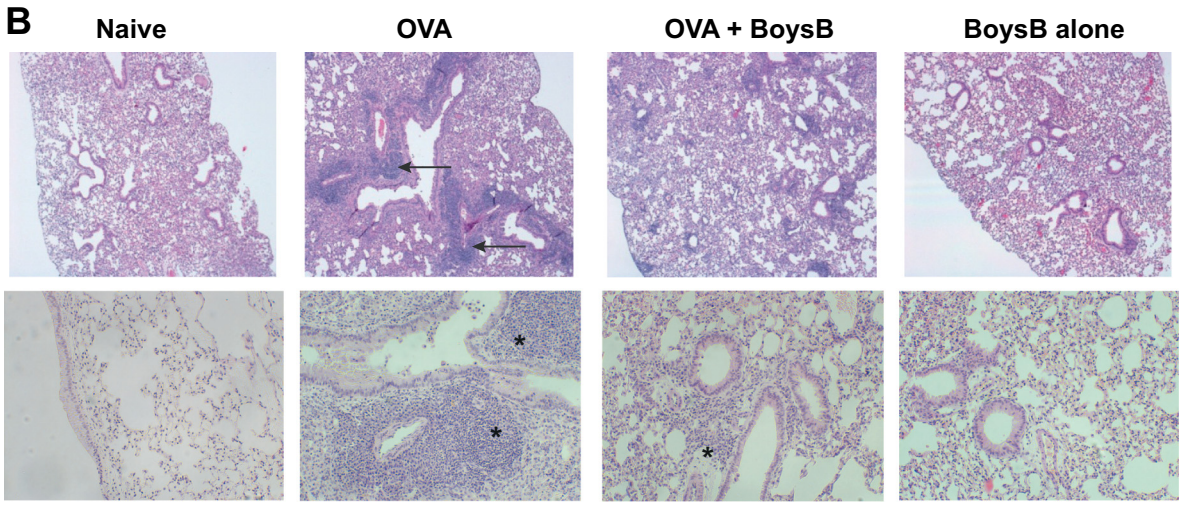
As shown in Fig. 1*B*, lung tissue from OVA-challenged mice exhibited increased cellular infiltrate and loss of lung structure. OVA-induced cellular infiltrate and lung damage were decreased in boysenberry-treated mice (Fig. 1*B*). Staining of lung tissue for mucus production identified fewer mucus-positive cells in OVA-challenged mice receiving boysenberry treatment compared with OVA only-challenged mice (Fig. 1*C*). Boysenberry treatment alone had no effect on cellular infiltration, lung structure, or mucus production.

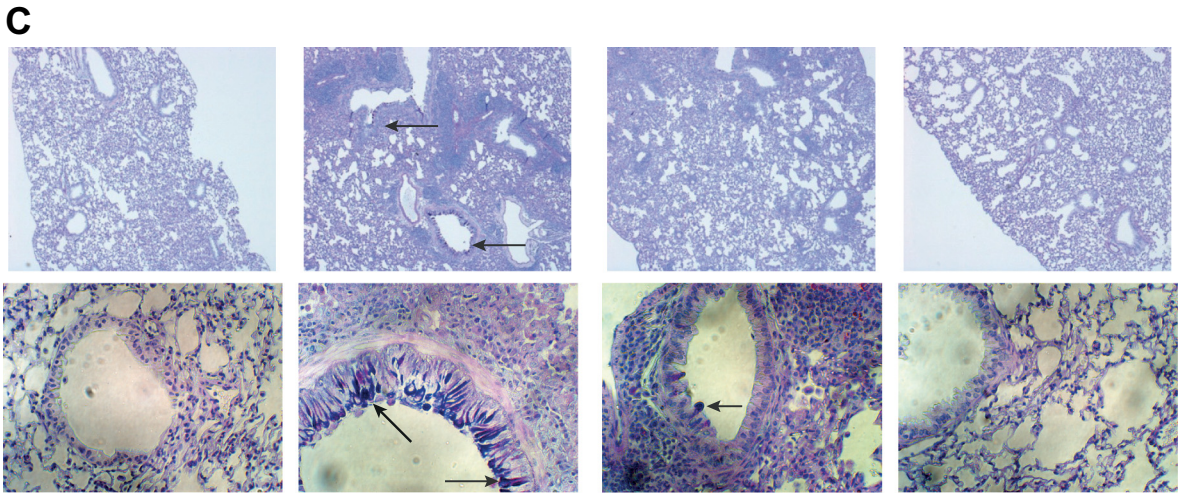
Boysenberry treatment increases AAMs in the lung of OVA-challenged mice. H&E-stained lung tissue sections showed more macrophages in OVA/boysenberry-treated mice compared with OVA mice (Fig. 2*A*). Immunoblot analysis of lung tissue identified a decrease in iNOS expression in the lung tissue of OVA/boysenberry-treated mice compared with OVA challenge alone (Fig. 2, *B* and *C*). At the same time we observed an increase in arginase expression in OVA-challenged mice (Fig. 2, *B* and *D*). that was further enhanced in OVA-challenged mice treated with boysenberry. Arginase expression was not affected by boysenberry treatment alone.

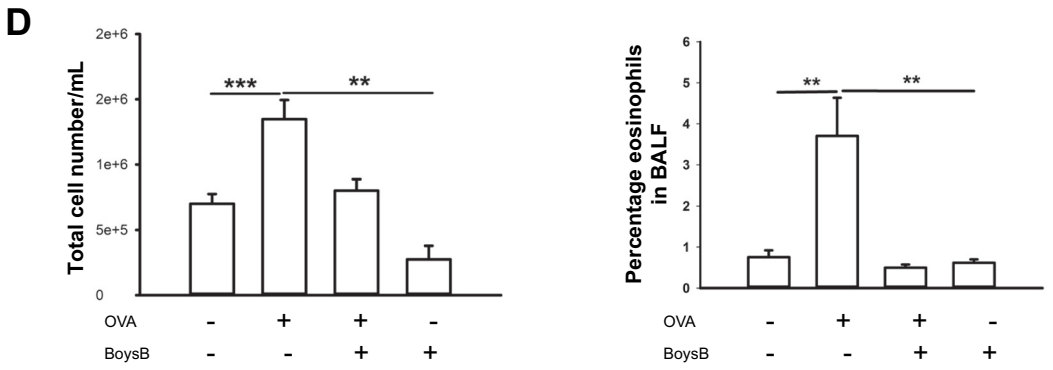
AAMs expressing arginase are closely associated with lung remodeling (29). To determine whether the observed lung macrophages were alternatively activated, lung tissue was stained with fluorescently labeled antibodies for the macrophage marker CD68 and the AAM markers CD206 and arginase. Lung tissue from OVA/boysenberry-treated mice showed an increase in CD68+CD206+arginase+ macrophages compared with OVA-challenged mice (Fig. 3). Quantitative analysis of the CD68+CD206+arginase+ macrophages further confirmed a significant increase in the percentage of CD68+CD206+arginase+ macrophages in the lung tissue of OVA/boysenberry-treated mice compared with OVA-challenged mice (60.00 ± 3.54% compared with 23.47 ± 5.61%, $P < 0.001$, one-tailed Student's *t*-test). Together these data identify an increase in the number of lung macrophages expressing an alternatively activated phenotype in OVA-challenged mice receiving boysenberry treatment.

Boysenberry treatment decreases OVA-induced collagen deposition and increases MMP-9 expression in the lung. Increased AAMs and arginase expression are commonly associ-









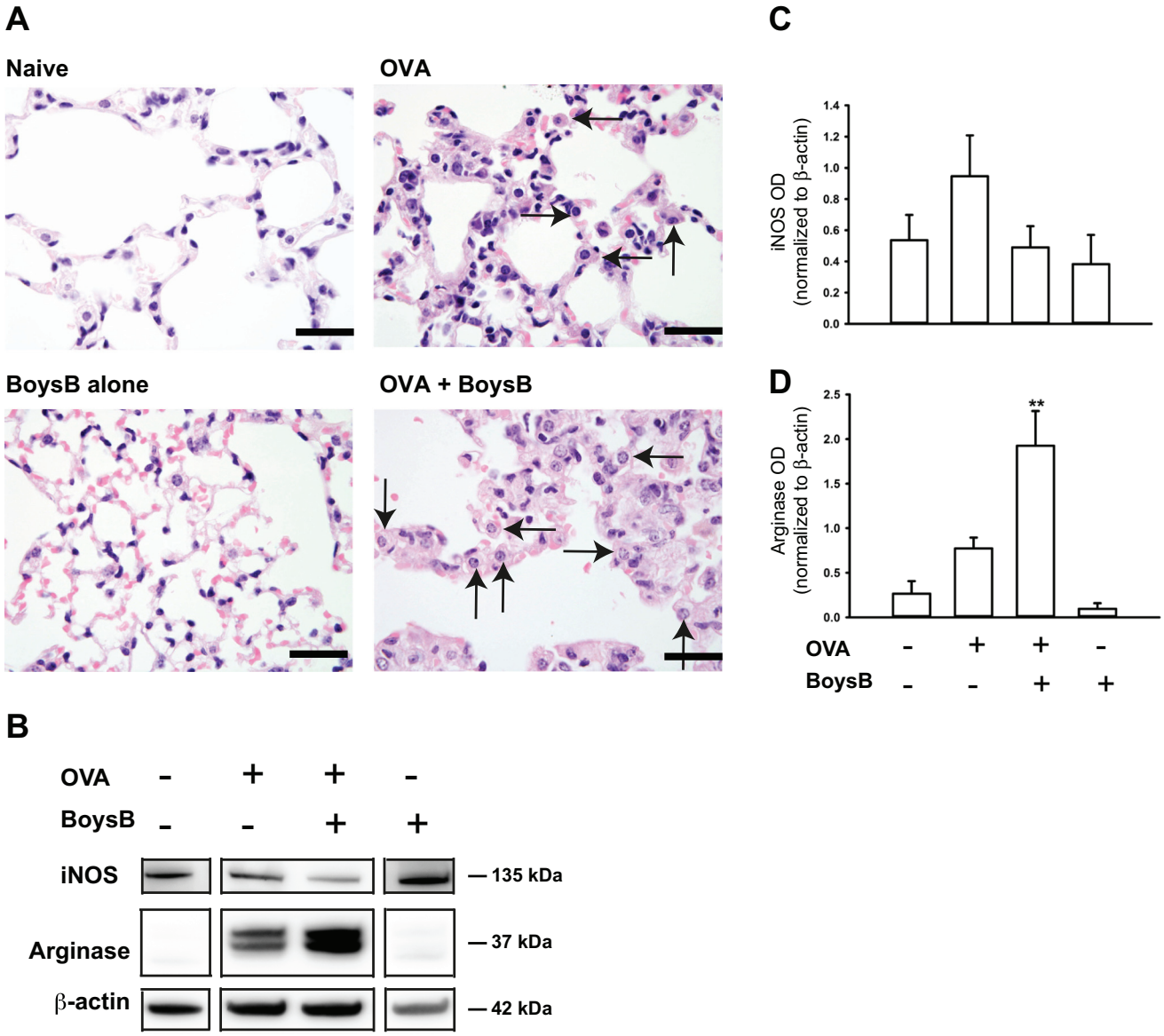


Fig. 2. Boysenberry treatment increases arginase expression and macrophage accumulation in lung tissue during OVA-induced chronic lung inflammation. *A*: representative H&E staining of lung tissue from 10-wk OVA-challenged mice, with and without boysenberry treatment. Arrows indicate macrophages. Magnification ×100, scale 200 μm. *B*: representative Western blot analysis of iNOS (135 kDa) and arginase (37 kDa) expression in lung tissue. Noncontiguous bands from the same Western blot are shown. *C* and *D*: quantification of iNOS and arginase Western blot signals normalized to β-actin signal. $^{**}P < 0.01$ (n = 10 per group) one-way ANOVA with Tukey's post hoc test.

ated with tissue fibrosis (14, 27, 66); therefore we looked at the effect of boysenberry treatment on OVA-induced collagen deposition in the lung. OVA challenge alone resulted in abnormal collagen deposition in the airways with signs of collagen invasion throughout the lung tissue that was abrogated in the lungs of OVA/boysenberry-treated mice (Fig. 4). Next we measured the levels of hydroxyproline in the lung tissue as a surrogate marker of collagen deposition (2, 63). There was a significant drop in the levels of hydroxyproline in the lungs of OVA-challenged mice treated with boysenberry, confirming that boysenberry treatment ameliorated OVA-induced collagen deposition (Fig. 4*B*). Boysenberry restored the OVA-induced

Fig. 1. Therapeutic oral boysenberry treatment reduces OVA-induced chronic lung inflammation. *A*: 6-wk-old male C57Bl/6 mice (n = 10 per group) were primed ip with OVA/alum (*day 0*) then challenged i.n. with OVA every 7 days for 10 wk. From *weeks 6* to *10* boysenberry juice was administered orally (gavage) 1 h prior to, and 2 days after, each i.n. OVA challenge. *B*: representative H&E staining of lung tissue from naive, 10-wk OVA challenge only (OVA), 10-wk OVA challenge with therapeutic boysenberry (OVA+BoysB) treatment, and boysenberry alone (BoysB)-treated mice. Arrows and * indicate immune cell infiltrate. Magnification ×4 (*top*) and ×10 (*bottom*). *C*: representative AB-PAS staining of lung tissue. Arrows indicate dark purple mucus-positive bronchioles. Magnification ×4 (*top*) and ×20 (*bottom*). *D*: total cells per ml BALF and flow cytometric quantification of percentage of eosinophils in BALF following final OVA challenge. $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 10 per group) one-way ANOVA with Tukey's post hoc test compared with naive and OVA challenge with therapeutic boysenberry treatment and boysenberry alone-treated mice.

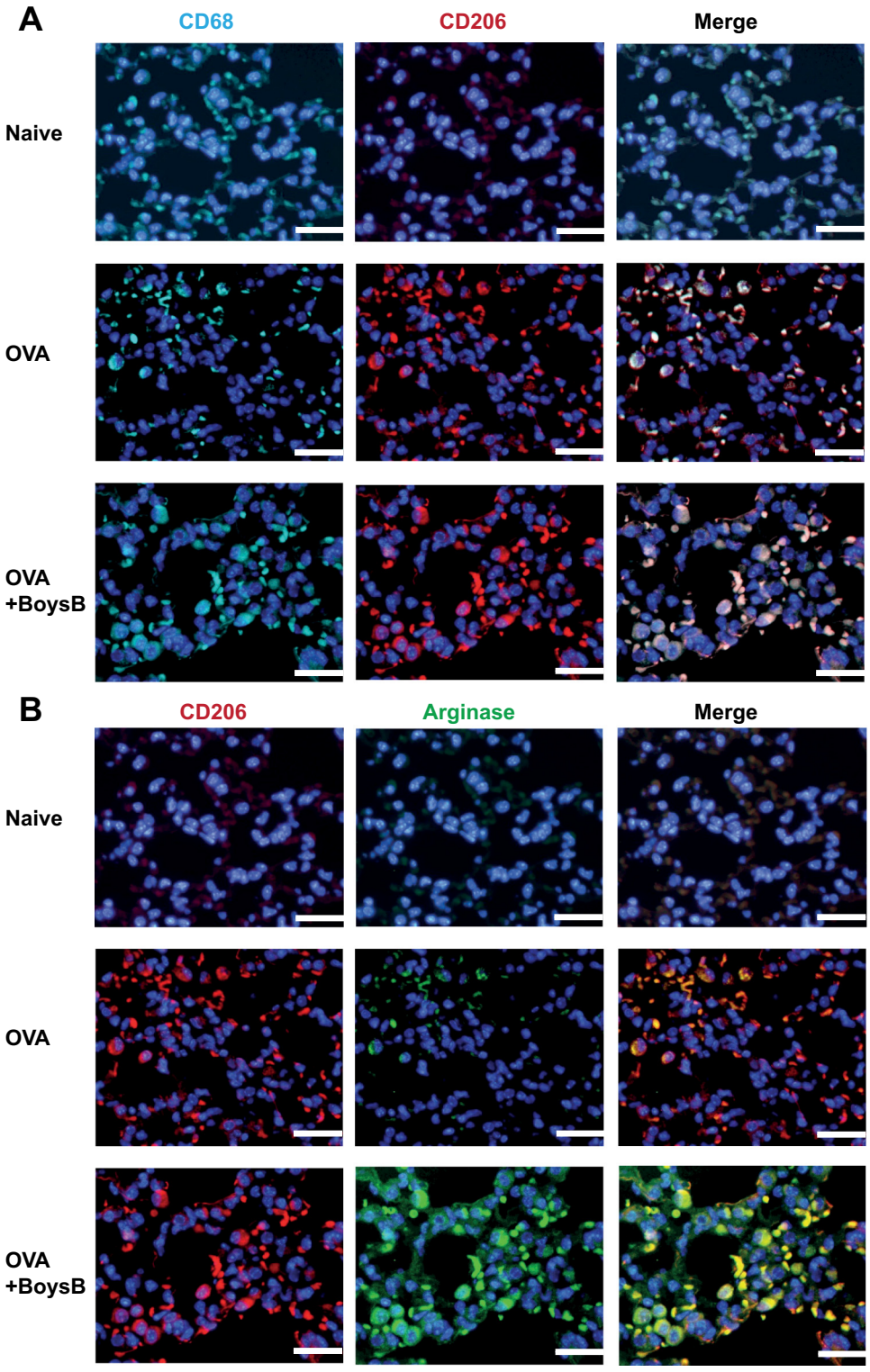


Fig. 3. Boysenberry treatment increases the accumulation of arginase+ alternatively activated macrophages. Representative immunofluorescent labeling of lung tissue from 10-wk OVA-challenged mice with and without boysenberry treatment. *A*: CD68+ CD206+ macrophages identified by *. *B*: CD206+ arginase+ macrophages identified by *. DAPI nuclear stain (dark blue). Magnification ×40, scale 200 μm.

decrease in the levels of TGFβ in the lung (Fig. 4*C*) but did not affect the levels of IL-4, IL-5, IL-6, IL-13, or IFNγ (data not shown).

To determine how boysenberry treatment could be moderating lung fibrosis we measured the expression of MMP-9 in the lung tissue by immunoblot. We found that MMP-9 expression was increased in OVA-challenged mice treated with boysenberry compared with mice challenged with OVA alone (Fig. 4*D*). Boysenberry treatment alone did not affect MMP-9 levels in the lung. Tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) is the endogenous inhibitor of MMP-9 (49). The ratio of TIMP-1/MMP-9 expression significantly increased in the lung tissue of chronic OVA-challenged mice and this increase was reversed with boysenberry treatment (Fig. 4*E*).

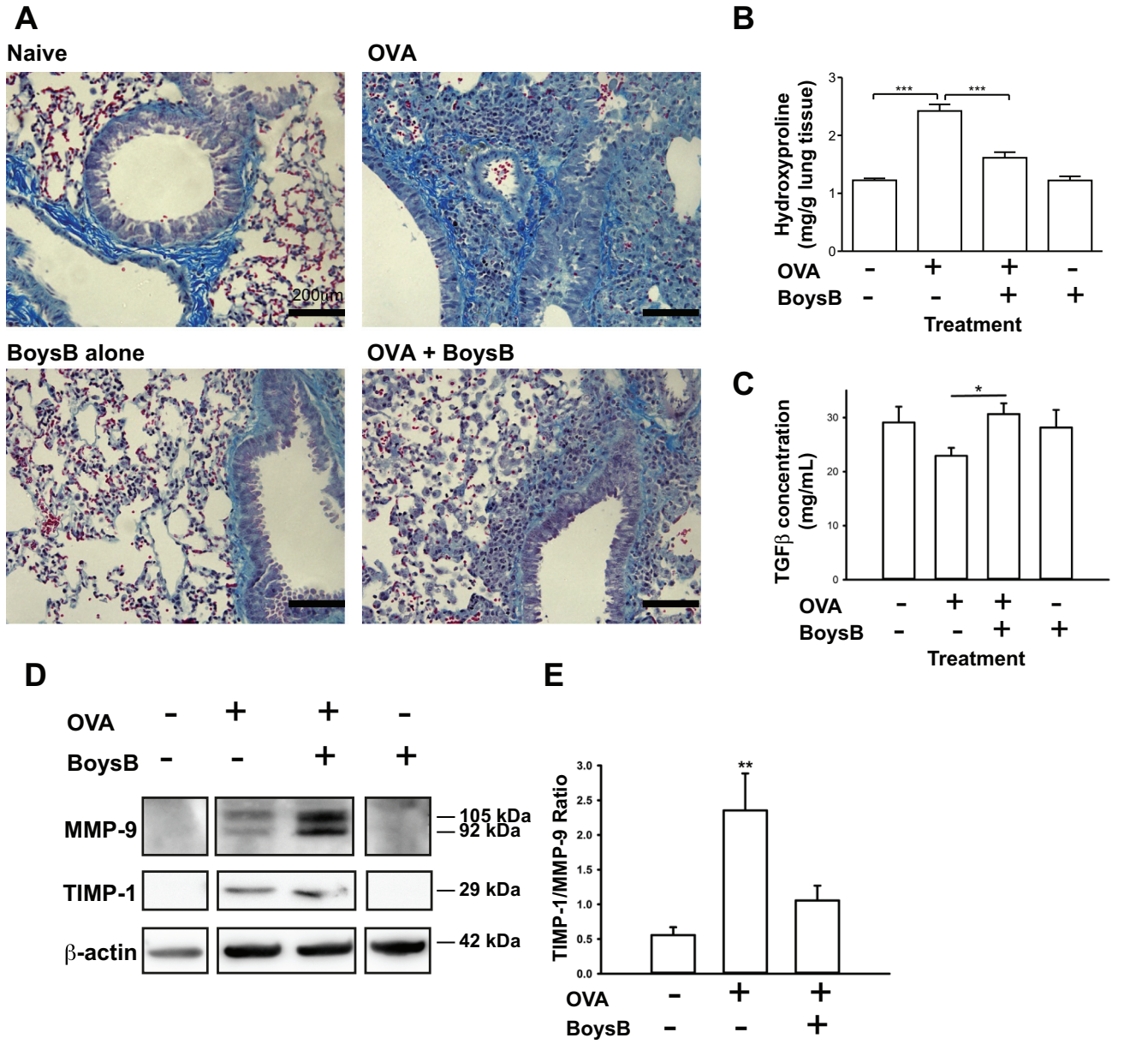


Fig. 4. Boysenberry treatment decreases collagen deposition and increases MMP-9 protein expression in lung tissue during OVA-induced chronic lung inflammation. *A*: representative Masson's trichrome staining. Magnification ×40, scale 200 μm. *B*: hydroxyproline levels (mg/g lung tissue); ***$P < 0.001$ ($n = 10$) one-way ANOVA with Tukey's post hoc test. *C*: lung TGFβ concentration as determined by ELISA; *$P < 0.05$ ($n = 10$ per group) one-way ANOVA with Tukey's post hoc test. *D*: Western blot analysis of MMP-9 (pro 105 kDa; active 92 kDa) and TIMP-1 (29 kDa) expression (noncontiguous bands from the same Western blot are shown) in lung tissue from 10-wk OVA-challenged mice with and without boysenberry treatment. *E*: ratio of TIMP-1/MMP-9 protein expression normalized to β-actin loading control; **$P < 0.01$ ($n = 10$) one-way ANOVA with Tukey's post hoc test compared with naive and OVA plus boysenberry treatment.

These results indicate that boysenberry-mediated reduction in collagen deposition and tissue remodeling was associated with elevated production of fibrolytic MMP-9 and a subsequent rebalance in the ratio of TIMP-1/MMP-9.

Alternatively activated macrophages are a source of MMP-9 protein in the lungs of OVA/boysenberry-treated mice. Lung tissue slides were analyzed to identify potential cellular sources of MMP-9. DAB-MMP-9 staining identified a high degree of MMP+ cells exhibiting macrophage morphology in OVA/boysenberry-treated mice compared with OVA-treated controls (Fig. 5*A*). Immunofluorescent staining (Fig. 5*B*) and quantitative analysis of the lung tissue confirmed that there were more MMP-9+/CD206+/CD68+ cells present in OVA/boysenberry-treated lungs than those challenged with OVA alone (39.30 ± 6.39 vs. 21.07 ± 5.82%; $P < 0.05$, one-tailed Student's *t*-test). These results identify CD206+/CD68+ AAMs as a source of the increased MMP-9 protein levels.

Depletion of lung macrophages reduces the beneficial effect of boysenberry consumption on tissue remodeling in established chronic lung inflammation. Next, we looked at the effect of depleting lung macrophages on the beneficial effects of boysenberry on chronic lung inflammation. Macrophages were

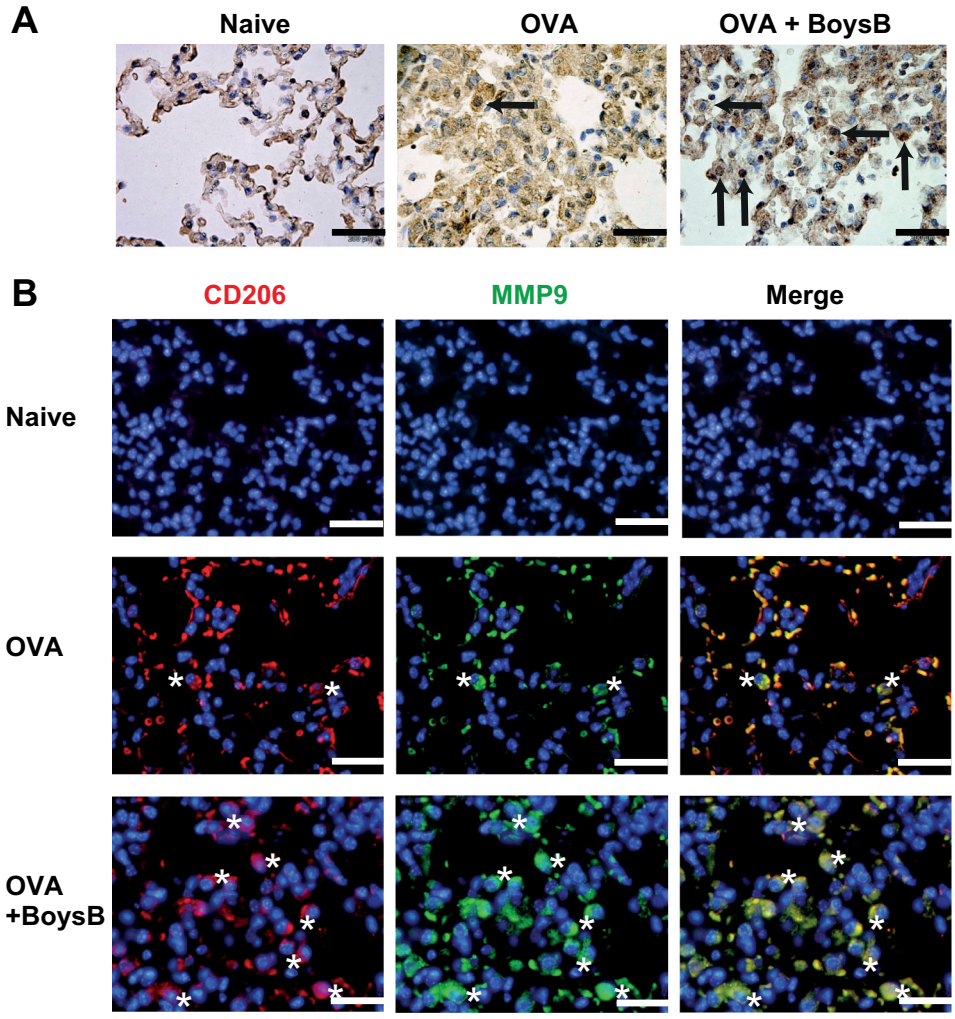


Fig. 5. Boysenberry treatment increases MMP-9 expression by alternatively activated macrophages in lung tissue during OVA-induced chronic lung inflammation. *A*: DAB labeling of MMP-9+ macrophages (arrows). *B*: immunofluorescent labeling of CD206+ MMP-9+ macrophages (*). DAPI nuclear stain (dark blue). Magnification ×40, scale 200 μm.

depleted by administration of clodronate liposomes after establishing chronic lung inflammation and remodeling, and prior to administration of each boysenberry treatment (Fig. 6*A*). We confirmed that we had significantly depleted the lung macrophages by flow cytometry (Fig. 6*B*) and that this was associated with a significant reduction in hydroxyproline levels in the lung of OVA-challenged mice treated with boysenberry (Fig. 6*C*). These data indicate that boysenberry requires macrophages to mediate its beneficial effects on lung tissue remodeling.

Boysenberry treatment prophylactically prevents OVA-induced airway inflammation. Finally, we tested the effect of boysenberry treatment using a prophylactic dosing regimen (Fig. 7*A*). Again, boysenberry treatment resulted in abrogation of OVA-induced tissue remodeling and significantly reduced cells in the lung lavage fluid (Fig. 7, *B–D*), This was associated with lower levels of hydroxyproline in the lung tissue and a decrease in the ratio of TIMP-1/MMP-9 expression (Fig. 7, *E–G*).

DISCUSSION

Fruit consumption has been linked with improved lung function in asthma sufferers and the amelioration of acute airway inflammation in experimental models (16, 19, 40). Herein we report that consumption of boysenberry juice has the potential to moderate chronic lung remodeling and fibrosis in both a therapeutic and a prophylactic setting. Furthermore, our data indicate that macrophages play an important role in boysenberry-mediated protection and that this protection may result from modulation of AAMs and increased MMP-9 activity.

An increase in both arginase activity (26, 27, 41) and AAMs (4, 9) is often linked with asthma pathogenesis. However, there is evidence that the presence of AAMs does not specifically underpin the development of allergic asthma (37), which indicates that AAMs may play an alternative role. We found that the boysenberry treatment increased the population of arginase-positive AAMs alongside a drop in iNOS expression in the lung tissue of chronic OVA-challenged mice. Arginase and iNOS play an interactive role in regulating lung inflammation and repair (34, 35, 66). Where iNOS activity is associated with active inflammation, arginase expression is indicative of a switch toward inflammatory resolution (35, 63). Boysenberry consumption therefore appears to rebalance the lung environment, supporting inflammation resolution by modulating the functional phenotype of AAMs in the lung.

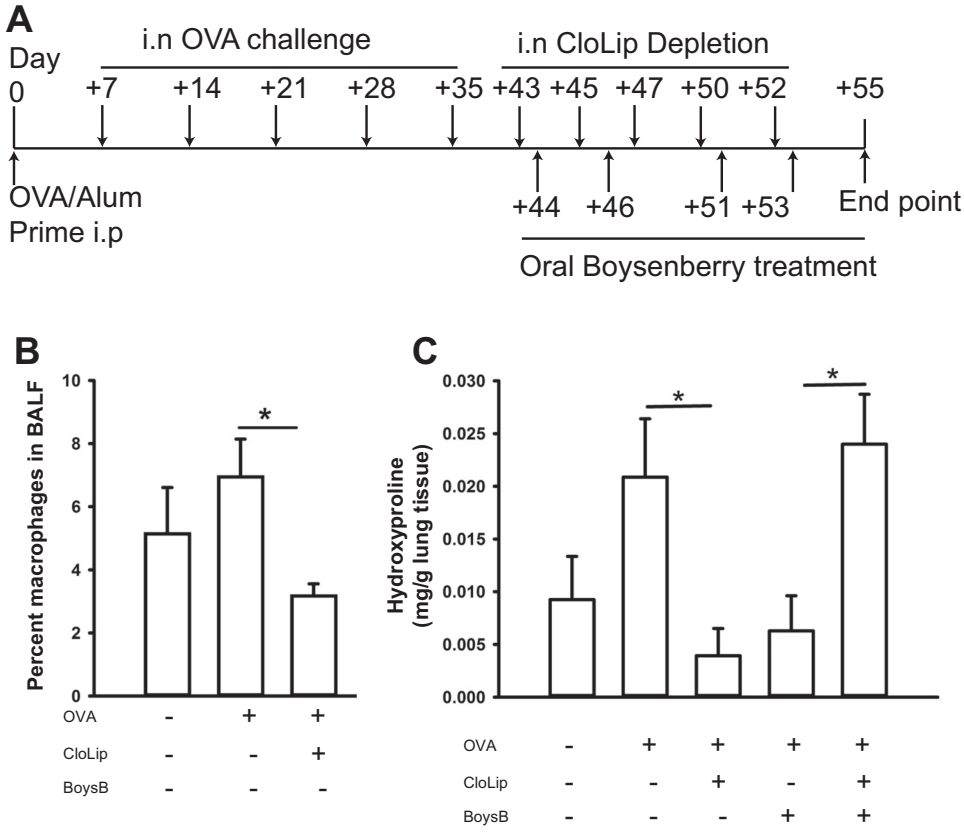


Fig. 6. Depletion of lung macrophages reduced the effect of oral boysenberry treatment on OVA-induced chronic lung inflammation. *A*: 6-wk-old male C57Bl/6 mice (n = 10 per group) were primed ip with OVA/alum (*day 0*) then challenged i.n. with OVA every 7 days for 5 wk. From *weeks 6* to *7* macrophages were depleted using clodronate liposomes (CloLip) the day before boysenberry juice was administered orally (gavage). *B*: flow cytometric quantification of percentage of macrophages in BALF following final clodronate macrophage depletion; $*P < 0.05$ (n = 10 per group) one-way ANOVA with Tukey's post hoc test. *C*: hydroxyproline levels (mg/g lung tissue) in the lung; $*P < 0.05$ (n = 10 per group) one-way ANOVA with Tukey's post hoc test.

The presence of AAMs has been associated with decreased Th-2 cytokine production in lung inflammation (36, 42). However, we found no changes in the levels of Th-2 cytokines IL-4, IL-5, and IL-13 with boysenberry consumption following OVA challenge. This indicated that inhibition of proinflammatory Th-2 cytokine production by AAMs was not contributing to the protective effect of boysenberry treatment.

Clinical and animal data indicate that the role of MMP-9 in asthma is multifaceted. Lung macrophages producing MMP-9 have been identified in both experimental and clinical settings (1, 5, 49). Elevated levels of active MMP-9 have been found in plasma and sputum samples from patients with asthma, compared with healthy controls (3, 23). Increased MMP-9 expression has been correlated with acute asthma exacerbation, including increased lung eosinophilia (6, 23). Conversely, an increase in MMP-9 levels has been associated with improved lung function in airway disease (25, 65). MMP-9 overexpression has also been shown to have beneficial effects in a model of pulmonary fibrosis (5). In contrast, data from MMP-9 knockout mice show a partial reduction in the development of asthma symptoms and reduced remodeling but, in some cases, a lack of MMP-9 has been shown to exacerbate disease (15, 24, 32).

MMP-9 exerts many downstream effects on different immune parameters, including the activation of both pro- and anti-inflammatory cytokines (15). Nevertheless, our data indicate that boysenberry-induced protection of lung tissue from chronic collagen deposition and fibrosis is orchestrated, in part, through the generation of fibrolytic AAM producing MMP-9. Consistent with this, our data show that depletion of macrophages during the resolution phase of inflammation leads to increased collagen deposition with boysenberry consumption. A similar resolution-promoting role for macrophages has been illustrated in bleomycin-induced pulmonary fibrosis (14).

Matrix metalloproteinases are regulated by their natural inhibitors TIMPs, and high TIMP-1/MMP-9 ratios are proposed to favor collagen deposition and lung remodeling (21, 28, 38). Here we observed a significant increase in the ratio of expression of TIMP-1/MMP-9 in the lung tissue of chronic OVA-challenged mice that was reversed by boysenberry treatment. The drop in the ratio of TIMP-1/MMP-9 in boysenberry-treated mice therefore represents a potentially beneficial readjustment in the regulation of collagen deposition and breakdown.

TGFβ is associated with both normal (20) and pathological (17, 22, 56) tissue repair processes through its role in extracellular matrix production. In this study we observed that chronic OVA challenge led to a decrease in TGFβ levels that was reversed by boysenberry consumption. There is evidence that TGFβ lowers the TIMP-1/MMP-9 ratio, thus favoring a more fibrolytic environment (18, 54, 56). As such the increase in TGFβ levels observed in the lungs of OVA-challenged mice following boysenberry treatment could serve to limit excessive tissue fibrosis and inappropriate remodeling during lung repair by lowering the TIMP-1/MMP-9 ratio. TGFβ is also known to stimulate fibroblast contraction for normal tissue repair (20), which could likewise contribute toward the beneficial effects of boysenberry treatment. As such the elevation of TGFβ has the potential to promote an anti-inflammatory, proresolution environment within the lung via multiple mechanisms.

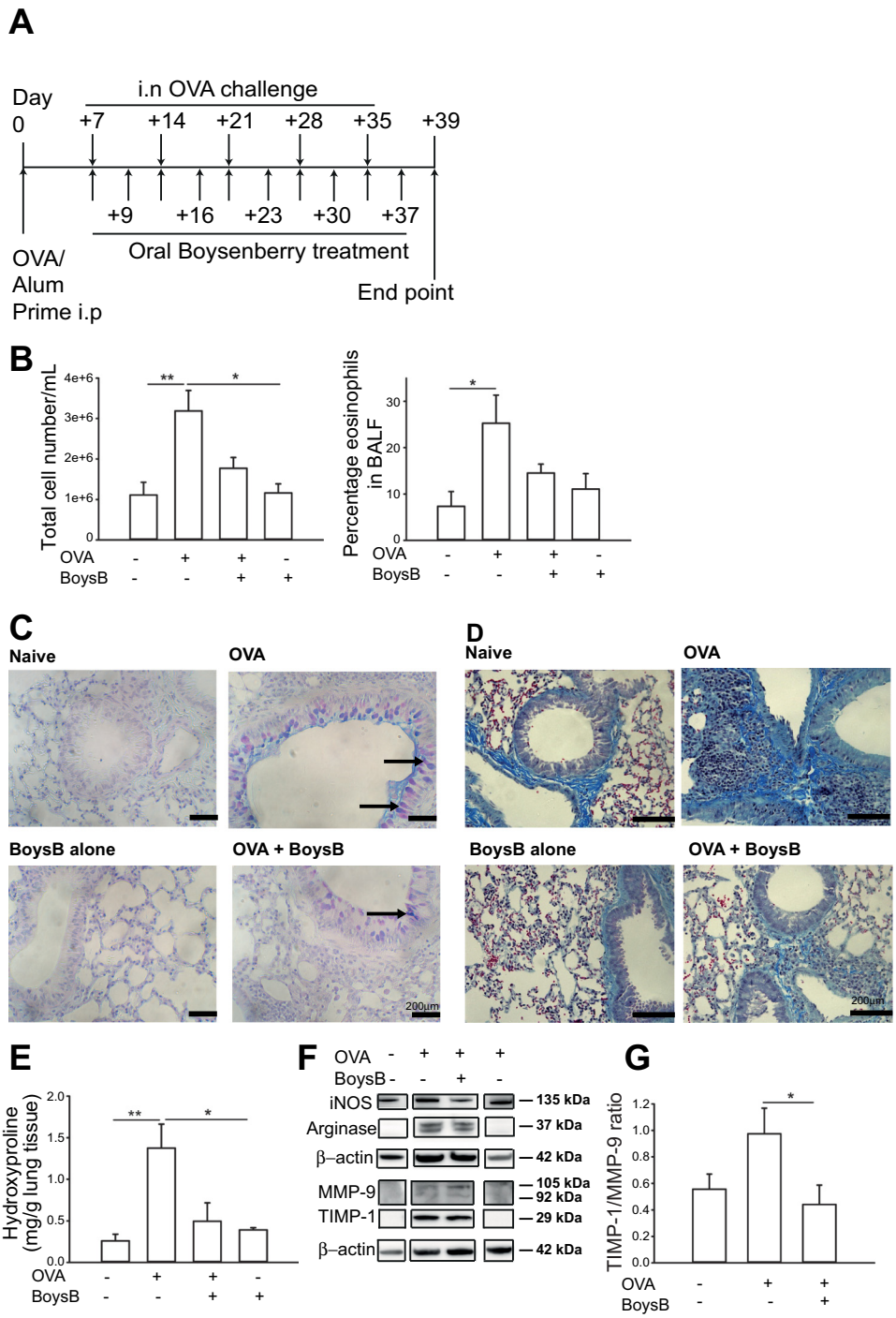


Fig. 7. Prophylactic oral boysenberry treatment reduces OVA-induced chronic lung inflammation and collagen deposition. *A*: 6-wk-old male C57Bl/6 mice ($n = 10$ per group) were primed ip with OVA/alum then challenged i.n. with OVA every 7 days for 5 wk. Boysenberry juice was administered orally (gavage) 1 h prior and 2 days after each i.n. OVA challenge. *B*: lung tissue was stained with total cells per ml BALF and flow cytometric quantification of percentage of eosinophils in BALF following final OVA challenge; $*P < 0.05$, $**P < 0.01$ ($n = 10$ per group) one-way ANOVA with Tukey's post hoc test. *C*: AB-PAS, dark purple mucus-positive bronchioles (arrows); magnification ×20, scale 200 μm. *D*: Masson's trichrome; magnification ×40, scale 200 μm. *E*: hydroxyproline levels (mg/g lung tissue) in the lung. $*P < 0.05$, $**P < 0.01$ ($n = 10$ per group) one-way ANOVA with Tukey's post hoc test. *F*: Western blot analysis of iNOS, arginase, MMP-9, and TIMP-1 lung tissue. Noncontiguous bands from the same Western blot are shown. *G*: ratio of TIMP-1/MMP-9 protein levels normalized to β-actin loading control. $*P < 0.05$, ($n = 10$ per group) one-way ANOVA with Tukey's post hoc test.

The results from our studies show that boysenberry administration exhibits a beneficial effect on chronic lung fibrosis in both a therapeutic and a prophylactic setting. This indicates that boysenberry consumption may help avoid inappropriate fibrotic remodeling in cases of both poorly controlled and well-controlled asthma. Finally, our findings provide the first evidence that boysenberry consumption could be used to support the development of fibrolytic AAMs with the potential to regulate appropriate lung remodeling in asthma and other lung conditions exhibiting fibrotic pathologies.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

O.M.S., R.D.H., and J.L.H. conception and design of research; O.M.S. performed experiments; O.M.S. and J.L.H. analyzed data; O.M.S., R.D.H., and J.L.H. interpreted results of experiments; O.M.S. prepared figures; O.M.S.,

R.D.H., and J.L.H. drafted manuscript; O.M.S., R.D.H., and J.L.H. edited and revised manuscript; O.M.S., R.D.H., and J.L.H. approved final version of manuscript.

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