

the BAT³ and when it is either not possible to perform the BAT or the patient has nonresponding basophils.

Apart from its use for diagnostics, the MAT identified patients at risk of severe allergic reactions during OFCs. The sensitivity and negative predictive value of the MAT's optimal cutoff for severity was particularly high, with relatively lower specificity and positive predictive value, indicating that having a MAT result of greater than the cutoff does not necessarily mean the patient will have a severe reaction but that these patients would benefit from more intense educational measures and closer follow-up.

The MAT and the inhibition of MAT results⁹ can facilitate further study of the underlying mechanisms that determine peanut reactivity versus tolerance. This is because the MAT can be used to assess the function of allergen-specific IgE antibodies in their ability to elicit mast cell degranulation and therefore allergic symptoms, as well as the ability of antibodies of other isotypes to interfere with this effect, either by inhibiting, as shown previously for IgG₄,⁹ or contributing to the activation of mast cells and basophils after allergen stimulation. However, this needs to be explored further. Both the BAT and the MAT are useful to test samples with equivocal P-sIgE levels to confirm PA and relay the performance of OFCs that would otherwise have positive results. Because the MAT uses plasma, which can be stored at low temperatures for long periods of time, it allows testing samples collected far from the laboratory or in the past.

The MAT is likely applicable to other food allergens. With the advent of new treatments for food allergy being approved for marketing, the MAT might prove to be a useful *in vitro* assay to monitor treatment response over time and to explore the mechanisms underlying the observed clinical changes during immunomodulatory treatments.

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Dexamethasone induces ω3-derived immunoresolvents driving resolution of allergic airway inflammation



To the Editor:

Resolution of inflammation has long been considered to be a passive process that ensues in an inflammatory response following the dilution of proinflammatory cues. However, this paradigm is shifting as it is becoming increasingly evident that resolution is an active reaction that uses a complex molecular machinery that orderly terminates inflammation. Among the multiple mechanisms involved, specialized proresolving lipid mediators (SPMs) such as resolvins, protectins, and lipoxins have taken center stage.¹ These are derived from ω3 and ω6

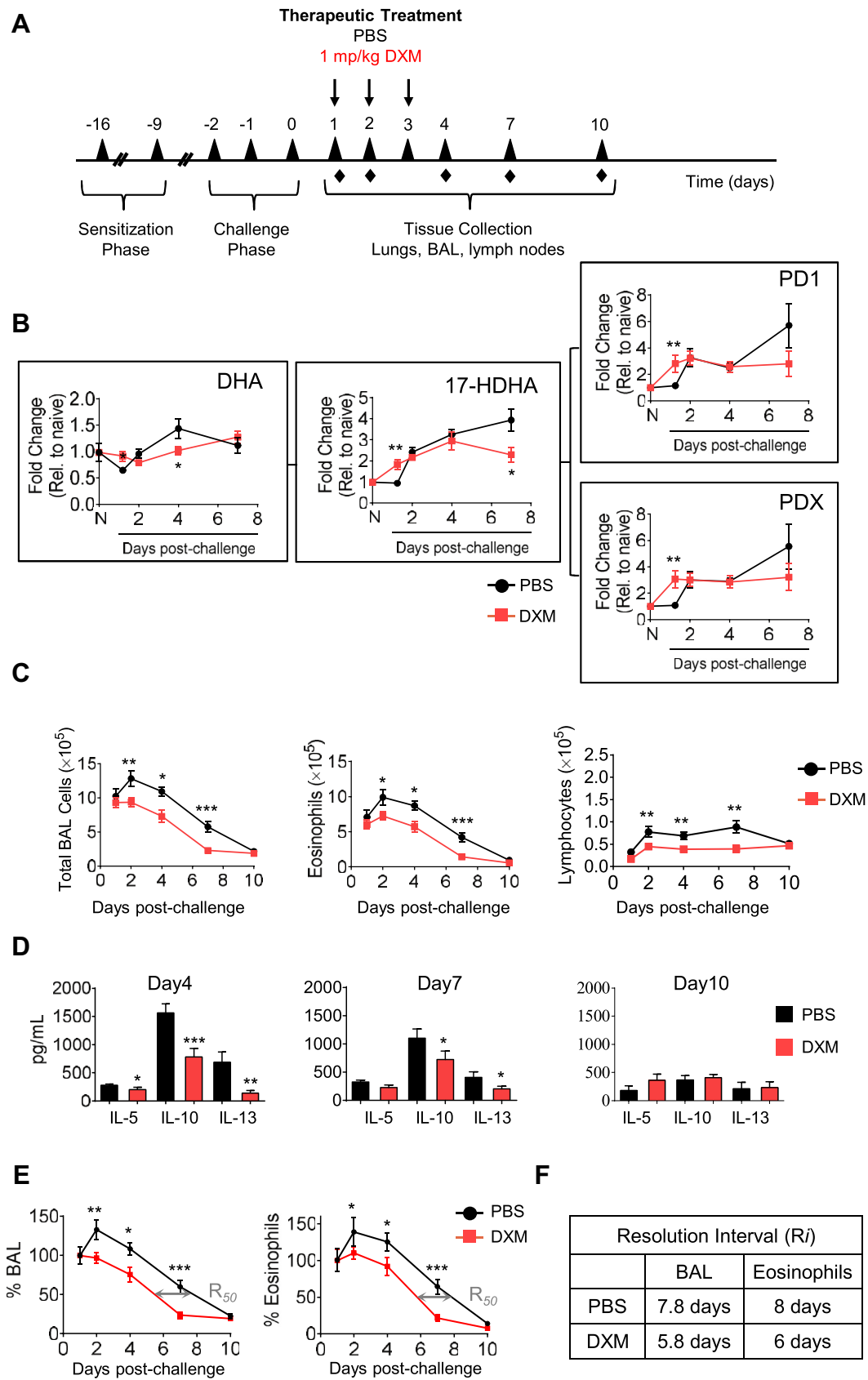


FIG 1. Effect of DXM on the biosynthesis of DHA-derived proresolving lipid mediators and the resolution of allergic airway inflammation. **A**, Schematic of the experimental protocol. DXM treatment is depicted in red. Rhombus indicates timepoints of collection. **B**, Tissue lipidomic profiling over a time course of 7 days postchallenge showing early activation of the DHA-17-HDHA-PD1/PDX pathway triggered by DXM. Data are expressed as fold induction relative to naive non-inflamed (N) mice. For levels below the limit of quantification (LOQ), half LOQ values were used. **C**, Total leukocyte, eosinophil, and lymphocyte numbers in BAL.

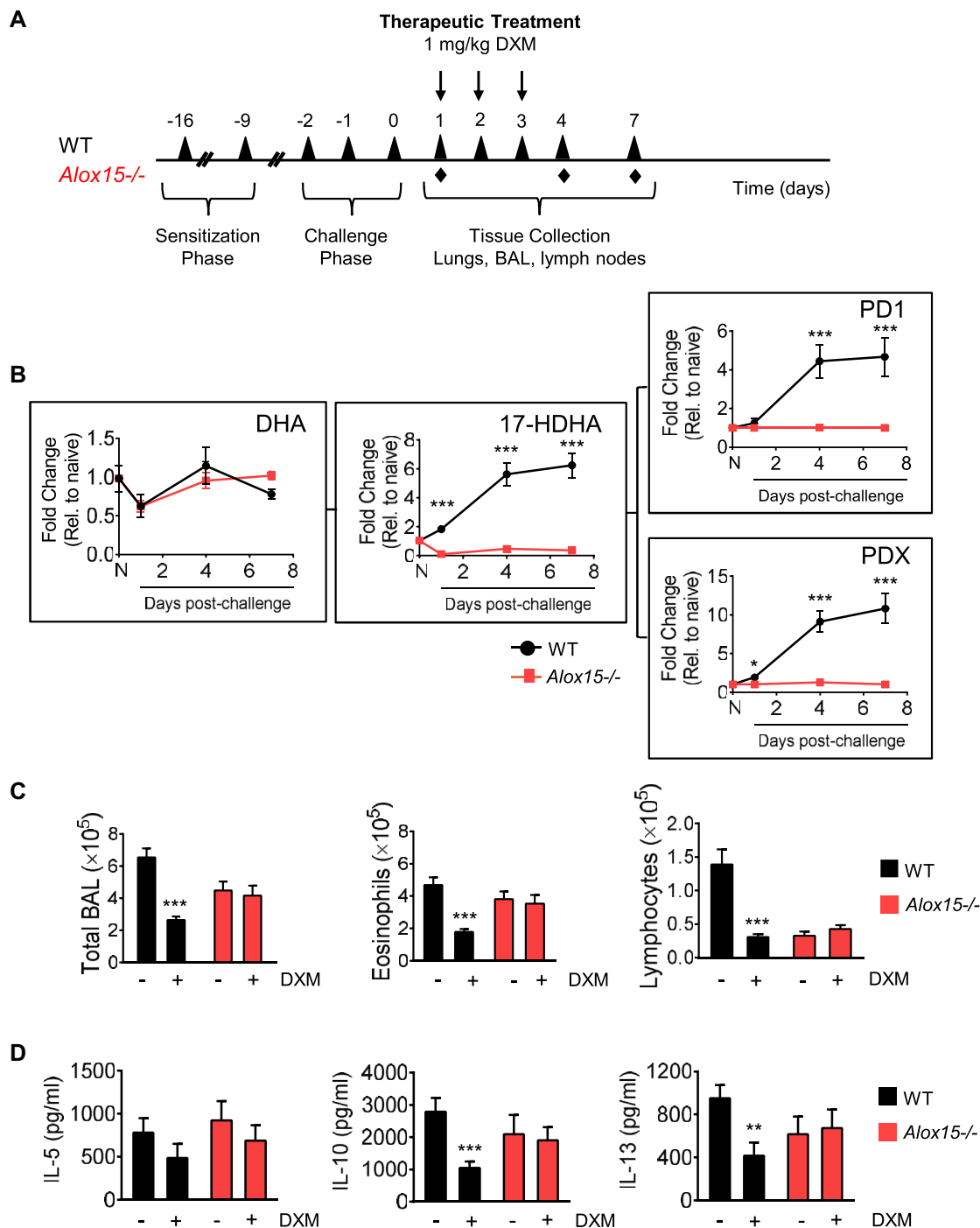


FIG 2. Dependence of DXM proresolving capacity on the activation of the DHA-17-HDHA-PD1/PDX pathway. **A**, Schematic of the experimental protocol using wild-type (WT) and *Alox15*-deficient (*Alox15^{-/-}*) mice depicted in red. Rhombus indicates timepoints of collection. **B**, Tissue lipidomics analysis of non-treated *Alox15^{-/-}* mice compared with WT mice. Data are expressed as fold induction relative to naive (N) mice. For levels below the limit of quantification (LOQ), half LOQ values were used. **C**, Effect of DXM administration on total leukocyte, eosinophil, and lymphocyte numbers in BAL at day 4 postchallenge. **D**, Effect of DXM administration on T_H2 -cell responses at day 4 postchallenge. Data represent mean $\pm$ SEM of $n = 6$ to 23 mice/group pooled from at least 3 independent experiments. *** $P < .001$, ** $P < .01$, and * $P < .05$ compared with WT mice.

D, T_H2 cytokine responses in mediastinal lymph nodes. **E**, Resolution curves for total leukocyte and eosinophil numbers in BAL. **F**, Calculated resolution intervals (Ri) based on total leukocyte and eosinophil resolution curves. Data represent mean $\pm$ SEM of $n = 9$ to 23 mice/group pooled from at least 3 independent experiments. *** $P < .001$, ** $P < .01$, and * $P < .05$ compared with PBS-treated controls.

polyunsaturated fatty acids through complex intracellular and transcellular biosynthetic pathways, and promote resolution of inflammation by their manifold and concerted functions inhibiting leukocyte trafficking, dampening proinflammatory signaling, inducing apoptosis, and promoting anti-inflammatory M2-like macrophage phenotypes, efferocytosis, and tissue restitution.¹

Impaired induction of SPMs has been associated with defective resolution of inflammation and chronic diseases including asthma. Thus, reduced lipoxin A4 (LXA4) levels have been detected in blood, bronchoalveolar lavage fluid (BAL), sputum, and exhaled breath condensates of patients with severe asthma, correlating with disease severity.²⁻⁴ Moreover, impaired protectin D1 (PD1) production has been described in patients with asthma compared with healthy individuals.^{5,6} These observations are functionally relevant because administration of synthetic forms of SPMs in animal models of asthma has been notably effective in resolving inflammation.⁷ Still, little is known about the effect of known drugs in the production of proresolving lipids, hampering the exploitation of this emerging new knowledge for therapy.

Here, we have investigated the role of dexamethasone (DXM), a prototypical glucocorticosteroid, in the generation of SPMs and the resolution of inflammation. We used an established model of allergic airway disease based on ovalbumin sensitization and challenge (see Fig E1, A, in this article's Online Repository at www.jacionline.org), which triggers a T_H2-driven inflammatory response in the airways peaking at day 1 postchallenge and gradually resolving thereafter (Fig E1, B-D).⁷ This is accompanied by the production of docosahexaenoic acid (DHA)-derived protectins such as PD1 and protectin DX (PDX), and their intermediate metabolite 17-hydroxydocosahexaenoic acid (17-HDHA), in the lung, which starts at day 1 and peaks at day 4 postchallenge (Fig E1, E), in line with their proresolving activity. In contrast, vehicle control (PBS) challenge does not trigger any SPMs (Fig E1, E). Prostaglandins PGD2 and PGE2, which are required for the activation of SPM pathways,⁸ are also induced, whereas the eicosapentaenoic acid–derived metabolite resolvin E1 (RvE1) is not detectable (data not shown). The induction of SPMs is typical because none of these mediators is upregulated in serum (see Fig E2 in this article's Online Repository at www.jacionline.org).

Using this setting, we administered DXM therapeutically, at days 1, 2, and 3 postchallenge, and assessed its effects (Fig 1, A). Surprisingly, we observed rapid and robust activation of the DHA•17-HDHA pathway within 6 hours of DXM administration (day 1 postchallenge), resulting in over 3-fold higher concentrations of PD1 and PDX compared to naive animals whereas vehicle (PBS)-treated controls exhibited no difference (Fig 1, B). This may even be an underestimation as in naive and most vehicle-control treated animals PD1 and PDX are undetectable but a value of half the limit of quantification was still used for comparisons. On the contrary, RvE1 and LXA4 were not detected, whereas PGD2 and PGE2, although present, were not differentially altered (data not shown). This early boost in SPM production was not anymore detectable 24 or 72 hours after DXM administration (day 2 or 4 postchallenge), as levels in vehicle-treated control animals were also increased, while at 144 hours (day 7 postchallenge) higher, yet not significant, levels of PD1 and PDX were observed in the control group (Fig 1, B). These findings demonstrate that DXM acutely induces the

mobilization of the DHA•17-HDHA•PD1/PDX pathway during allergic airway disease in mice.

We then examined whether the ability of DXM to upregulate PD1 and PDX could also modulate resolution of allergic airway disease. DXM was unable to generate a notable anti-inflammatory or proresolving effect within 6 hours of treatment, a very short time interval (Fig 1, C and D). However, at 24 hours (day 2 postchallenge) and onward, DXM reduced total leukocyte and eosinophil numbers in the BAL more rapidly than vehicle control, and decreased ovalbumin-specific IL-5 and IL-13 levels in mediastinal lymph nodes. Accordingly, DXM shortened the resolution interval (Ri), the time interval from the peak of inflammation at day 1 postchallenge to the 50% reduction point, by 2 days, a very significant effect for this model (Fig 1, E and F). To confirm that DXM accelerates the resolution of inflammation through the induction of DHA-derived SPMs, rather than other effects on the immune system, we used 12,15-lipoxygenase deficient (*Alox15*^{-/-}) mice, which cannot metabolize DHA to downstream bioactive mediators such as protectins (Fig 2, A and B). Abrogation of PD1 and PDX generation completely reversed DXM-mediated resolution of allergic airway inflammation including total leukocyte and eosinophil infiltration in the BAL, and T_H2 cytokine production in mediastinal lymph nodes (Fig 2, C and D; see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). Although this does not exclude the involvement of other bioactive lipids requiring 12, 15-lipoxygenase for their generation, the potent proresolving activity of synthetic PD1 administration in similar settings^{5,7} indicates that at least PD1 and its functionally similar isomer PDX are principally involved.

In conclusion, our study describes a novel function of DXM, a prototypical glucocorticosteroid, in the activation of SPM networks. It reveals that DXM enhances the metabolism of DHA to its bioactive derivatives PD1 and PDX. This effect is rapid, inducing at 3-fold higher levels than vehicle within 6 hours of treatment, providing an early boost to the process of resolution, markedly reducing both the duration and magnitude of inflammation. This effect is also transient because no differences between DXM and vehicle-treated animals were observed later on, possibly reflecting the induction of counter-regulatory pathways suppressing SPM networks, and lack of need for additional SPMs in an effectively resolving situation. Notably, the effect of DXM is specific for the D-series SPMs because there was no observed mobilization of other proresolving pathways such as lipoxin A4 or RvE1 nor PGD2 and PGE2.

At present, very few pharmaceutical substances have a documented ability to activate SPM networks. Aspirin and statins provoke conformational changes on cyclooxygenases, altering their enzymatic activity and leading to the production of epimeric forms of lipoxins, resolvins, and protectins, depending on the substrate.⁹ Resiquimod, a TLR7/8 agonist, induces in turn DHA-derived SPMs in human macrophages and experimental settings of airway disease.⁷ Synthetic forms of SPMs such as RvE1 and LXA4 are also being explored although their short half-life and chemical stability constitute major hurdles toward their clinical application. Our study therefore adds DXM as a new agonist of resolution, expanding the short list of clinically used drugs that are able to actively induce SPM pathways. It also provides novel insight into the mechanism of action of DXM, a well-known and widely used drug, with important implications for glucocorticosteroid use in the clinic.

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Matrix protein tenascin-C expands and reversibly blocks maturation of murine eosinophil progenitors



To the Editor:

Eosinophils are effector cells in allergy that are both recruited from circulation and develop via *in situ* hematopoiesis in the local tissue environment.^{E1-E3} Although eosinophil depletion in tissues is a highly sought-after therapeutic strategy in treating allergic disease, little is known about the role of tissue factors in promoting eosinophilia. In disease, tissue eosinophils typically encounter provisional extracellular matrix (ECM) glycoproteins, such as periostin, versican, thrombospondins, and tenascins.¹ Particularly, eosinophil expansion in tissues strongly correlates

with tenascin-C (TNC) deposition.^{2,E4} Although highly restricted in healthy adult tissues, TNC is strongly expressed *de novo* during wound healing and pathologic tissue remodeling. TNC is a hexabrachion featuring epidermal growth factor-like repeats, fibronectin type III (FN3)-like repeats, and a fibrinogen-like globe that interface integrins and other matrix components in tissues.^{3,E5,E6} Importantly, TNC is a known hematopoietic niche component in the bone marrow stroma.^{4,E7} Moreover, exposing naive murine eosinophils to TNC-enriched provisional matrices significantly upregulates the gene expression of immaturity markers (*Ly6a* [Sca-1], *CD34*) and suppresses *IL-5Rα* (*IL5ra* [CD125]) expression, as determined by RNA-Seq (our unpublished data, 2018). This led us to hypothesize that TNC represents a factor in the provisional allergic tissue microenvironment that could potentially support *in situ* eosinophil progenitor expansion. In support, we show that in murine bone marrow-derived cultures, TNC (1) 3-fold expanded the available Lin[−]Sca1⁺ early precursor pool; (2) significantly downregulated *IL-5Rα* expression on Lin[−]CD45⁺c-kit⁺ cells during the eosinophil lineage commitment phase; and (3) served as a reversible block of eosinophil maturation, as TNC withdrawal from cultures rescued maturation and increased the final eosinophil yield. Furthermore, we show that TNC knockout mice lack *in situ* lung expansion of Lin[−]c-kit⁺CD34⁺ common myeloid progenitors and Lin[−]Siglec-F⁺Sca-1⁺ cells in an allergic inflammation model, while exhibiting accelerated maturation of lung eosinophils *ex vivo*. Adding TNC to lung homogenates cultured in IL-5 *ex vivo* suppressed eosinophil maturation in a manner consistent with the TNC eosinophil maturation block in bone marrow-derived cultures. Experimental procedures and additional references can be found in this article's Online Repository at www.jacionline.org.

Bone marrow-derived eosinophil cultures supplemented with TNC significantly expanded the Lin[−]Sca-1⁺ early precursor pool compared with controls (Fig 1, B-D; see Fig E1 in this article's Online Repository at www.jacionline.org). Maturation suppression was evidenced by constitutive Sca-1 expression (hematopoietic progenitor/stem cell marker) at all time points in TNC culture (Fig 1, D). Terminal IL-5-driven eosinophil differentiation and maturation was significantly suppressed by TNC addition, as determined by flow cytometry (Fig 1, B and C). In comparison with control samples expressing only Siglec-F, TNC-treated cultures on day 15 had a substantial proportion of eosinophils expressing both Siglec-F (eosinophil marker) and Sca-1 (Fig 1, C). Compared with controls, differential staining at TNC culture end points revealed morphology characteristic for class III eosinophil progenitor cells,^{E12} featuring a lack of distinct eosin staining and nonsegmented nuclei (Fig 1, B). TNC may have a direct effect on maturing eosinophils via the binding of integrin-binding and fibronectin (and other ECM)-binding repeats within its FN3 domain,³ which was specifically presented to eosinophils in our cultures. Among integrins, α9β1 integrin specifically binds the AEIDGIEL sequence in the FN3 TNC domain,^{5,E13} and this integrin expression on human hematopoietic stem and progenitor cells was found to regulate cell proliferation and differentiation.⁶ These findings are consistent with reports of TNC and other provisional ECM proteins regulating hematopoiesis.^{E9-E11} TNC sustains hematopoietic regeneration after bone marrow ablation, which is dependent on FN3 domain binding.⁴ Mice lacking TNC show reduced bone marrow colony-forming capacity and hematopoietic cell production. Within

METHODS

Mice

Wild-type C57BL/6J and *Alox15^{-/-}* mice on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, Me). Mice were fed a normal chow global diet 2918 containing 18.5% protein and 5.5% fat (Harlan Mucedola, Milan, Italy) and housed in individually ventilated cages under specific pathogen-free conditions in full compliance with Federation for Laboratory Animal Science Associations (FELASA) recommendations at the Animal House Facility of the Biomedical Research Foundation of the Academy of Athens. All procedures had received prior approval from the institutions and regional ethical review boards and were in accordance with the US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736-01) and with the European Union Directive 86/609/EEC for animal research.

Preclinical model of ovalbumin-induced allergic airway inflammation

Induction of allergic airway inflammation in C57BL/6 mice required 2 systemic sensitization injections with 7.5 μ g ovalbumin (OVA) (Albumin, from chicken egg white, grade V, Sigma-Aldrich, St Louis, Mo) emulsified in aluminum salts ($\text{Al}(\text{OH})_3$) and subsequent exposure after 7 days to 5% w/v aerosolized OVA (30-minute challenge for 3 sequential days). Mice that were sensitized and challenged with PBS were used as nondiseased controls. Untreated (nonsensitized, nonchallenged) mice were used as naive controls. All mice were aged 8 to 10 weeks at the time of experimentation.

BAL and differential cell counts

BAL of the whole lung was performed twice with 0.5 mL saline. A total of 100,000 cells were subjected to cytopspin centrifugation at 600 rpm for 3 minutes. Cytopspins were stained with May-Grumwald-Giemsa and analyzed by differential cell counting.

Edema assessment

Tissues were weighed before and after drying at 60°C for 2 days. The weight difference reflected fluid accumulation in the tissue.

Lymph node isolation and culture

Mediastinal lymph nodes were isolated from mice. Single-cell suspensions were generated with the use of a 70- μ m pore size cell strainer. A total of 0.5 million cells/well were plated in triplicates in U-shaped 96-well plates. Cells were *in vitro* restimulated with 100 μ g/mL OVA. Culture supernatants were collected after 48 hours and stored at -20°C.

T-cell responses

The concentrations of $\text{T}_\text{H}2$ cytokines were determined by ELISA (Ready-SET-Go! Kits, eBioscience, San Diego, Calif). The immunoassay was performed on mediastinal lymph node culture supernatants.

DXM dosing

DXM was systemically administered intraperitoneally in the form of water-soluble DXM-21-phosphate disodium salt (Sigma-Aldrich) at 1 mg/kg (mpk) dose per day, for 3 sequential days.

Chemical reagents

PGE2-d4 (9-oxo-11 α ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic-3,3,4,4-d4 acid) and PDX (10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-

docosahexaenoic acid) were purchased from Cayman Chem (Ann Arbor, Mich). All solvents and eluent additives used were of LC-MS grade. Acetonitrile (ACN) (Riedel-de Haen), water (Sigma), ammonium acetate (Fluka), and formic acid (Fluka) were purchased from Sigma-Aldrich.

Calibration curve

Lung tissue and serum were isolated from naive C57BL/6 mice. Lungs were homogenized in 4 mL ice-cold 70% methanol/1 g of wet tissue. Lung and serum calibration curves ranged from 2.5 ng/g to 50 ng/g for PDX and from 1 ng/mL to 10 ng/mL for resolvin D1, RvE1, LXA4, PDX, PGD2, PGE2, EXC4, leukotriene B4, 17-HDHA, and 7-hydroxy-docosahexaenoic acid, respectively.

Sample preparation

After homogenization, 200 μ L of lung homogenate was spiked with internal standard (IS: PGE2-d4). Samples were cleaned-up by centrifugation and supernatants were collected. Solid precipitates were washed twice with 100 μ L ice-cold 50% MeOH (v/v). Mouse serum was collected at designated time points. For LC-MS/MS with multiple reaction monitoring analyses, 200 μ L of mouse serum was spiked with 10 μ L 1 μ g/mL IS.

Solid-phase extraction

The resulting samples were acidified with 1 mL aqueous solution of 0.1% formic acid (FA) (v/v) and were immediately subjected to solid-phase extraction (Oasis HLB Extraction plate, C8, 30 μ m, 10 mg of sorbent, Waters Corporation, Milford, Mass). Ninety-six-well solid-phase extraction cartridges were preconditioned with 500 μ L MeOH and equilibrated with 500 μ L H_2O . The cartridges were then washed sequentially with 500 μ L H_2O and 500 μ L 15% MeOH (v/v). Finally, the analytes were eluted with 200 μ L 90% ACN (v/v). Samples were evaporated to dryness in a SpeedVac Savant SPD1010 (ThermoFisher, New York, NY) at room temperature, under vacuum. The dry residue was dissolved in 100 μ L of 50:50 mobile phase A:B, followed by vortex and sonication.

HPLC-MS/MS analysis

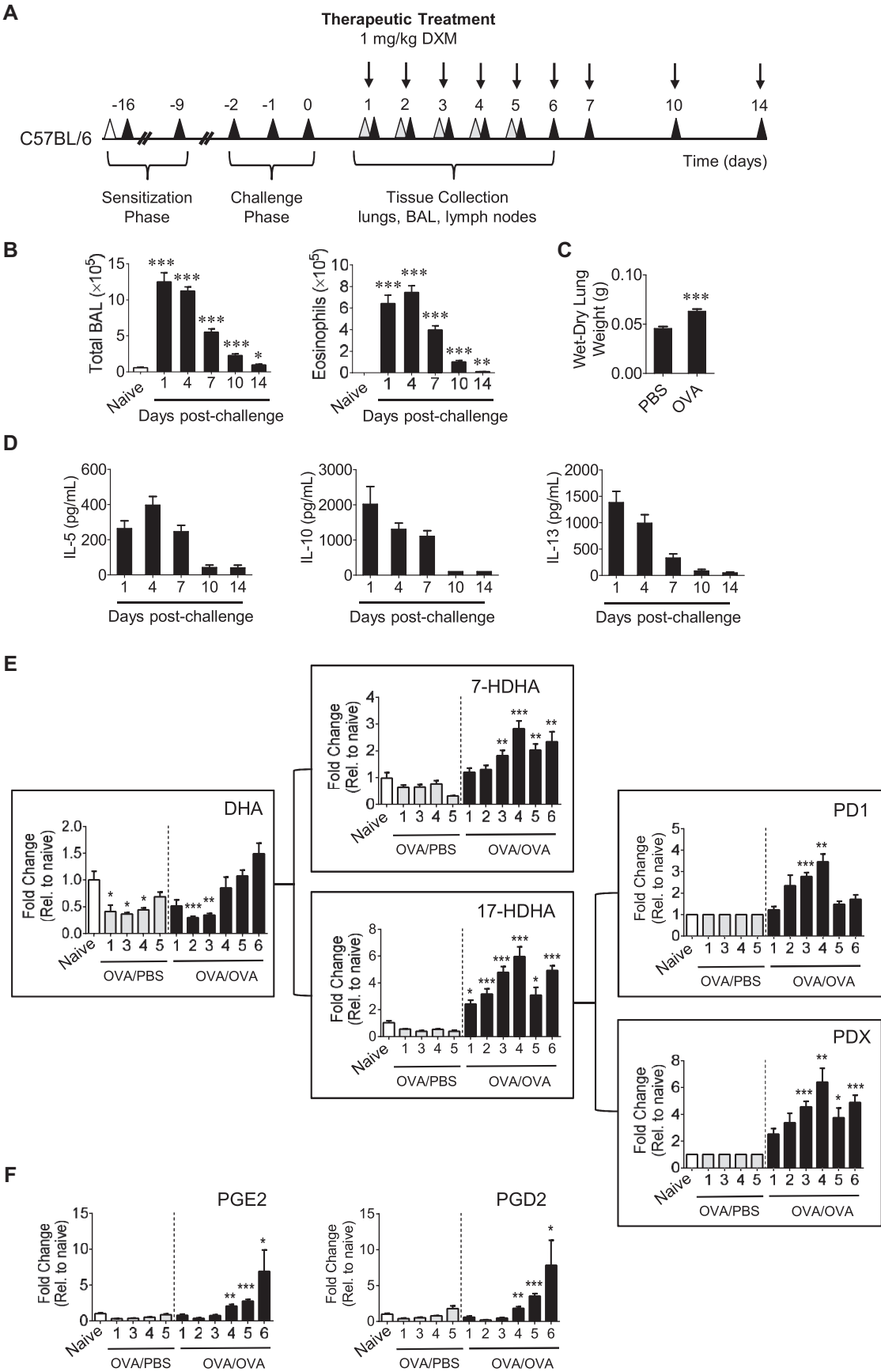
HPLC was performed with an Ultimate 3000 Series system (Dionex Corporation, Sunnyvale, Calif). The mobile phase consisted of solvents A (10% ACN, 90% water, 2 mM ammonium acetate, 0.1% FA) and B (90% ACN, 10% water, 2 mM ammonium acetate, 0.1% FA). A multilinear gradient was performed with a flow rate of 300 μ L/min. A Waters Symmetry C8 column (2.1 $\times$ 50 mm, 3.5 μ m) was used at stable 25°C. The injection volume was 10 μ L. Mass spectrometry was performed on an API 4000 QTRAP MS/MS system fitted with a TurboIonSpray source and a hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). The instrument was operated in the negative ion mode.

Absolute and relative quantification of lipid mediators in biological matrices

Quantification was performed in multiple reaction monitoring mode of operation. Values of lipid mediators are expressed as fold changes relative to the levels of the respective analytes in naive mice. In the case of PD1 and PDX, wherever levels were below the limit of quantification, half limit of quantification values were used.

Statistical analysis

Statistical significance of differences was assessed by a 2-tailed Student *t* test for parametric data and a Mann-Whitney *U* test for nonparametric data. *P* values of less than .05 were considered statistically significant.



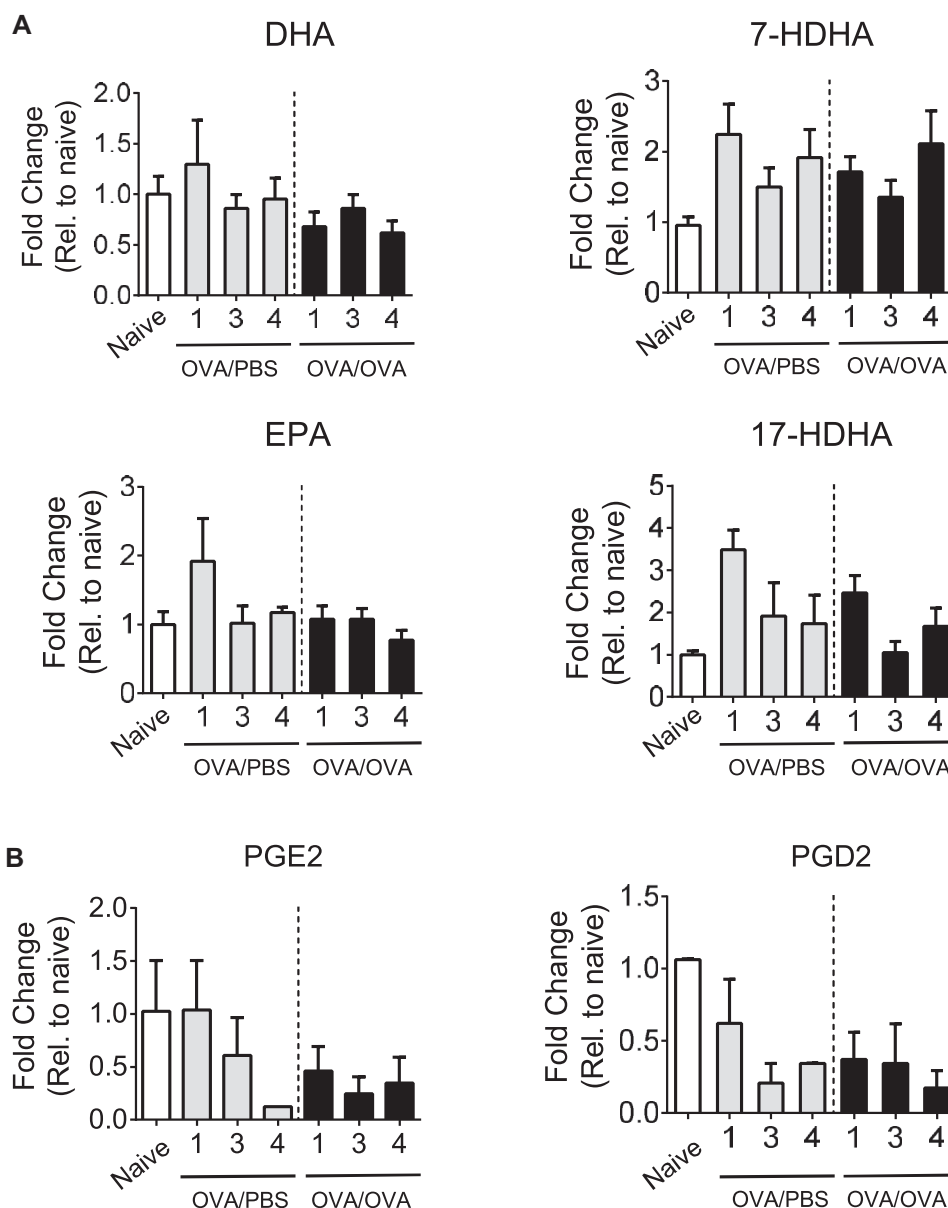


FIG E2. Production of ω 3-derived proresolving lipid mediators in the serum of mice with experimental allergic airway inflammation. Naive (*white*), OVA- sensitized, PBS-challenged (*gray*), and OVA-sensitized, OVA-challenged (*black*) mice were studied. **A**, Quantification of DHA, its monohydroxy metabolites 7-HDHA and 17-HDHA, and EPA in mouse serum. No other DHA- or EPA-metabolites were identified. **B**, Presence of the arachidonic acid-derived prostaglandins PGE2 and PGD2 in mouse serum. Data represent mean $\pm$ SEM of $n = 2$ -9 mice/group pooled from 2 independent experiments. Differences were not statistically significant when compared with naive mice. EPA, Eicosapentaenoic acid; 7-HDHA, 7-hydroxy-docosahexaenoic acid.

FIG E1. Production of ω 3-derived proresolving lipid mediators in the lung of mice with experimental allergic airway inflammation. **A**, Schematic of the experimental protocol. Naive (*white*), OVA-sensitized, PBS-challenged (*gray*), and OVA-sensitized, OVA-challenged (*black*) mice were studied. **B**, Total leukocyte and eosinophil presence in BAL. **C**, Edema formation assessed by wet-dry lung weight. **D**, T_H2 -cell responses in mediastinal lymph nodes. **E**, Induction of the DHA-17-HDHA-PD1/PDX pathway in the lung assessed by targeted lipidomics. **F**, Presence of the prostaglandins PGE2 and PGD2 in the lung assessed by targeted lipidomics. Data represent mean $\pm$ SEM of $n = 5$ -43 mice/group pooled from at least 3 independent experiments. *** $P < .001$, ** $P < .01$, and * $P < .05$ compared with naive mice.

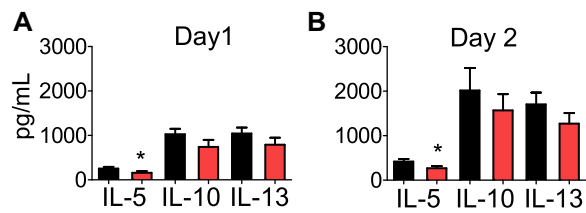


FIG E3. DXM accelerates resolution of airway inflammation. Monitoring of T_H2 cytokine release at day 1 (**A**) and day 2 (**B**) post-DXM (*red*) administration compared with PBS-treated controls (*black*). Data represent mean $\pm$ SEM of $n = 9$ to 23 mice/group pooled from at least 3 independent experiments. * $P < .05$ compared with PBS-treated controls.