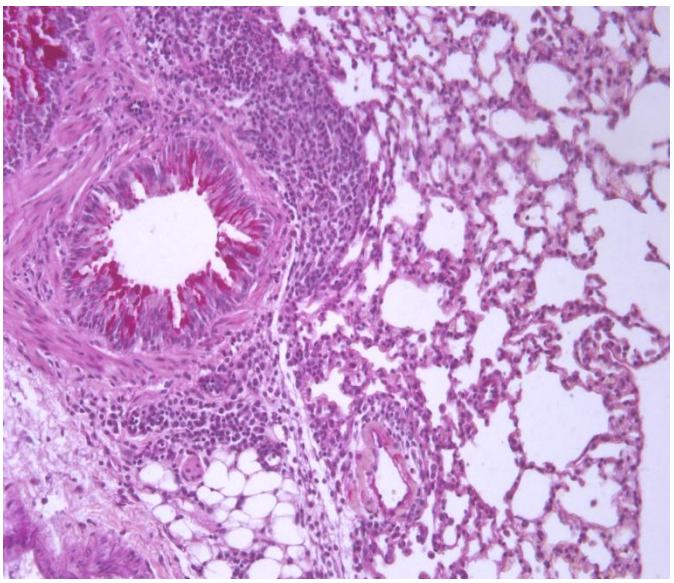
PhD Thesis **June 2010**

STUDY OF THE ROLE OF THE CYTOKINE OSTEOPONTIN IN TYPE-2 ALLERGIC RESPONSES



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Ευχαριστίες……

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Όλα αυτά τα χρόνια κέρδισα μια ξεχωριστη και αγαπημένη φίλη. Ιδιαίτερα ευχαριστώ την Λουκία μου, που από την πρώτη στιγμή με αγκάλιασε και με βοήθησε να εγκλιματιστώ γρήγορα στο εργαστήριο (είμασταν από τις πρώτες φοιτήτριες στο ΙΙΒΕΑΑ)…Μου έμαθε να κάνω cloning και με βοήθησε μαζί με τον Σπυρο Γεωργόπουλο να φτιάξω το construct για το πολύτιμο διαγονιδιακό ποντίκι μας…Πόσες ώρες δεν είχαμε περάσει όλοι μαζί σχεδιάζοντας την στρατηγική μας…Πόσα ξενύχτια στο εργαστήριο μόνες μας εγώ να κάνω FACS και εσύ βακτήρια…

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Τέλος θα ήθελα να ευχαριστήσω την οικογενειά μου και τον Στέλιο μου, που από την αρχή πίστευαν σε εμένα και στήριξαν τις επιλογές μου. Με βοηθήσατε ουσιαστικά στο να μην χάσω τον προσανατολισμό μου και να επικεντρωθώ στην επίτευξη του στόχου μου …

Στον παππού μου, Νικήτα Αλισσάφη

CONTENTS

SUMMARY

Osteopontin (Opn) is important for T helper type 1 (T_H1) immunity and autoimmunity. However, the role of this cytokine in T_H2-mediated allergic disease, as well as its effects on primary versus secondary antigenic encounters and its role in antigenic peripheral tolerance, remain unclear. Here we demonstrate that OPN is expressed in the lungs of asthmatic individuals and Opn-s, the secreted form of Opn, exerts opposing effects on mouse T_H2 effector responses and subsequent allergic airway disease: pro-inflammatory at primary systemic sensitization, and anti-inflammatory during secondary pulmonary antigenic challenge. These opposing effects of Opn-s are mainly mediated by regulation of T_H2-suppressing plasmacytoid dendritic cells (DCs) during primary sensitization and T_H2-promoting conventional DCs during secondary antigenic challenge. Therapeutic administration of recombinant Opn during pulmonary secondary antigenic challenge decreased established T_H2 responses and protected from allergic disease.

Our data demonstrated the anti-inflammatory role of Opn in T_H2-type allergic airway responses. Furthermore Opn is also highly expressed in activated $Foxp3^+$ T_{regs}. Thus, we also investigated whether Opn had an impact on peripheral tolerance. Opn-deficient mice were impaired in mounting antigenic tolerance against a T_H2-driven asthma model disease, while administration of rOpn had the opposite effect. rOpn promoted tolerance by expansion of suppressive plasmacytoid dendritic cells (pDCs) and expansion of $Foxp3^+$ T_{regs}. In fact, rOpn was a pDC survival factor that instructed these cells to generate T_{reg} cells, and also promoted the expansion of $Foxp3^+$ T_{regs}. Overall, these novel effects on T_H2 allergic responses and peripheral tolerance, place Opn as an important therapeutic target and a key cytokine controlling peripheral tolerance, and thus provide new insight into its role in immunity.

ΠΕΡΙΛΗΨΗ

Οι ειδικές ανοσοαπαντήσεις έναντι παθογόνων μεσολαβούνται από Τ βοηθητικά κύτταρα Τύπου 1 (T_H1) ή Τύπου 2 (T_H2) . Η διαφοροποίηση σε T_H1 ή T_H2 σχετίζεται με μια πληθώρα παραγόντων, στους οποίους περιλαμβάνονται οι κυτταροκίνες οι οποίες είναι παρούσες στο μικροπεριβάλλον στο οποίο γίνεται το έναυσμα της ανοσοαπάντησης. Μια από αυτές τις κυτταροκίνες είναι και η οστεοποντίνη (Opn), η οποία παίζει σημαντικό ρόλο κατά τη διαφοροποίηση των Τ βοηθητικών κυττάρων προς τύπου 1 (T_H1). Ωστόσο, δεν έχει ακόμα διευκρινιστεί ο ρόλος της κυτταροκίνης αυτής σε ανοσοαπαντήσεις T_H2, επίσης αδιευκρίνιστη παραμένει ακόμα και η δράση της στην αντιγονο-ειδική περιφερική ανοσοανοχή. Στην διδακτορική αυτή διατριβή παρουσιάζουμε αποτελέσματα για την έκφραση της OPN σε πνεύμονες ασθενών με άσθμα. Επιπρόσθετα τα αποτελεσματά μας δείχνουν ότι η εκκρινόμενη μορφή της Opn (Opn-s), έχει διττό ρόλο στις T_H2 αλλεργικές ανοσοαπαντήσεις: προ-φλεγμονώδη στην φάση της πρωτογενούς ευαισθητοποίησης με το αντιγόνο και αντι-φλεγμονώδη κατά την διάρκεια της δευτερογενούς επανέκθεσης στο αντιγόνο. Η οστεοποντίνη μεσολαβεί αυτές τις διαφορετικές της δράσεις κυρίως με τους εξής μηχανισμούς: Ρυθμίζοντας την προσέλευση των πλασματοκυτταροειδικών δενδριτικών κυττάρων (pDCs), τα οποία έχουν δειχθεί να έχουν ρυθμιστική δράση σε T_H2 ανοσοαπαντήσεις, κατά την φάση της ευαισθητοποίησης, ενώ κατά την φάση της επανέκθεσης η Opn δρα ρυθμίζοντας την προσέλευση των συμβατικών δενδριτικών κυττάρων (cDCs) τα οποία επάγουν τις ανοσοαπαντήσεις τύπου 2. Επιπρόσθετα παραθέτουμε δεδομένα που αποδεικνύουν ότι έπειτα από θεραπευτική χορήγηση της ανασυνδυασμένης Opn (rOpn), κατά την διάρκεια της φάσης επανέκθεσης, παρατηρείται σημαντική μείωση των ανοσοαπαντήσεων T_H2 και προστασία από την ανάπτυξη αλλεργικής ασθένειας.

Τα δεδομένα μας λοιπόν υποδεικνύουν την αντι-φλεγμονώδη δράση της Opn σε καθιερωμένες ανοσοαπαντήσεις Τύπου 2. Επιπρόσθετα, έχει δειχθεί στο παρελθόν ότι η Opn εκφράζεται σε υψηλές ποσότητες από τα ενεργοποιημένα Τ ρυθμιστικά κύτταρα, τα οποία είναι θετικά για τον με ταγραφικό παράγοντα Foxp3 ($Foxp3^+$ T_{regs}). Έχοντας ως δεδομένα τα παραπάνω, μελετήσαμε επίσης των πιθανό ρόλο της Opn στην περιφερική ανοσοανοχή. Ποντίκια τα οποία είχαν έλλειψη της Opn, παρουσίαζαν πρόβληματική ανάπτυξη ανοσοανοχής για αλλεργεία, ενώ η χορήγηση

rOpn παρουσίαζε το αντίθετο αποτέλεσμα. Η rOpn προήγαγε την ανοσοανοχή προκαλώντας άυξηση των ρυθμιστικών κυττάρων pDCs και αύξηση των κυττάρων Foxp3^{+} T_{regs}. Επιπρόσθετα, τα αποτελεσματά μας δείχνουν ότι n rOpn είναι ένας παράγοντας που προωθεί την επιβίωση των κυττάρων pDCs, και οδηγεί αυτά τα κύτταρα να ενισχύσουν την παραγωγή και εξάπλωση των κυττάρων T_{reg}. Συνολικά, η ανακάλυψη αυτών των νέων δράσεων της οστεοποντίνης στις ανοσοαπαντήσεις τύπου 2 και την περιφερική ανοσοανοχή, υποδεικνύει την Opn-s ως έναν πιθανό θεραπευτικό στόχο και ως μια κυτταροκίνη κλειδί για την ανοσοαπάντηση.

INTRODUCTION

Immunology of allergic responses

The basic foundation of the immune system is the capacity to distinguish non-self molecules, with potential to cause harm, from self molecules, a characteristic that exists in a delicate balance between tolerance to self and response/rejection of non-self. Autoimmunity and allergy are disorders in which this balance is disrupted.

Autoimmunity defines a state in which tolerance to self is lost and the immune response is activated against host tissues. On the other hand allergic and hypersensitivity reactions are the result of immune responses to innocuous non-self molecules that are called allergens. This response is mediated by immunoglobulin (Ig) E antibody specific to the allergen. Mast cells and basophils are activated after IgE binding, starting a series of cellular and molecular events that results in the clinical manifestations of allergic disease. IgE-mediated immunity is critical for our defence against parasites, however, the low prevalence of parasitic infections in modern societies has made the role of IgE in allergic disorders of more importance in medical care. Allergic diseases are highly patient specific and include asthma, rhinoconjunctivitis, sinusitis, food allergy, atopic dermatitis, angioedema and urticaria, anaphylaxis, and insect and drug allergy, all of which can occur either alone or in combination. Allergies can affect all age groups and can appear at any time, but it is the marked increase of allergies in children and young adults that is of particular concern.

The increasing prevalence of allergic disorders in urban communities is being intensively studied [1-2]. Some of the possible causes accounting for this observation are related to the environment, such as ambient pollution [3], increased concentration of indoor allergens, diet, and the decrease of childhood infections. The 'hygiene hypothesis', which attempts to explain this increasing prevalence of allergy, is based on the possible immunomodulation induced by bacterial and viral infections early in infancy, modifying the chances of developing an allergic response. However, environmental factors do not fully explain the increase of allergic disease [4-5]. Genetic

predisposition to allergic disorders has also been extensively explored recently, as allergists have known for decades that children of allergic parents are more likely to develop allergic disease. Genetic studies, including linkage analysis of large families, have identified several possible loci containing candidate genes that may confer increased susceptibility to allergic disease [6]. As most allergic disorders express themselves clinically at epidermal or mucosal surfaces, a breakdown of the physical barrier that is normally provided by these surfaces and altered innate immunity is also recognized to be of great importance in allergic reactions. The study of interactions between many susceptibility genes and the environment is revealing new pathophysiological mechanisms and creating unique opportunities for the prevention and treatment of allergy.

The allergic cascade

The allergic inflammatory response is characterized by selection of the T-helper 2 (T_H2)-cell pathway, which is initiated by the uptake of allergens by professional antigen presenting cells (APCs) that present selected peptides on major histo-combatibility (MHC) class II molecules to naive T cells, thereby directing them in favour of a T_H2-cell phenotype in which the transcription factor GATA3 (GATA-binding protein 3) mediates cytokine secretion [7] (**Figure 1**). This is in contrast to the T_H1-cell phenotype that is dominant in autoimmune diseases, in which T-bet controls cytokine secretion — for example, the secretion of interferon-γ (IFNγ)[8]. The crucial role played by dendritic cells (DCs) acting as professional APCs in this sensitization process has recently been explored[9]. B cells are also important for allergen capture and processing, especially in the presence of small amounts of allergen[10]. In the presence of co-stimulation, T cells co-ordinately upregulate expression of a cluster of genes that include the genes encoding interleukin-4 (IL-4), IL-5, IL-9, IL-13 and granulocyte/macrophage colony-stimulating factor (GM-CSF)[11]. These cytokines are involved in the class-switching of B cells to IgE synthesis (IL-4 and IL-13), the recruitment of mast cells (IL-4, IL-9 and IL-13) and the maturation of eosinophils (IL-3, IL-5 and GM-CSF) and basophils (IL-3 and IL-4), which are the main mediator-secreting effector cells of the allergic response.

The imbalance between T_H2-cell and T_H1-cell (that is, IFNγ-producing) responses has formed the basis for our understanding of allergic immune responses for more than two decades. More recently, regulatory T cells (Treg cells) have been discovered as another pivotal subset of CD4+ T cells with implications for allergic disease. These cells are characterized by expression of the transcription factor Foxp3 (Forkhead box P3) and the IL-2 receptor (CD25)[12], but in contrast to activated effector T cells, they express low levels of CD127[13]. Extensive studies in mouse models strongly implicate Treg cells in the suppression of allergic responses[14], and there is emerging evidence that Treg cells also control T_H2-cell responses in humans through the inhibitory cytokines IL-10 and transforming growth factor-β (TGFβ), with atopy resulting from an imbalance between T_H2 cells and Treg cells.

Another newly identified type of CD4+ T cell has been named the T_H17 cell on the basis of secretion of IL-17A and IL-17F, which are associated with neutrophilic inflammation[15]. The transcription factor RORγt (retinoic-acid-receptor-related orphan receptor-γt) identifies T_H17 cells and is selectively activated by IL-1β and IL-6 [16], with IL-23 being responsible for the proliferation of these cells. IL-17A is overexpressed in asthmatic airways in association with neutrophil influx[17] and it induces production of the neutrophil chemoattractant IL-8 (CXCL8) by human airway smooth muscle cells[18].

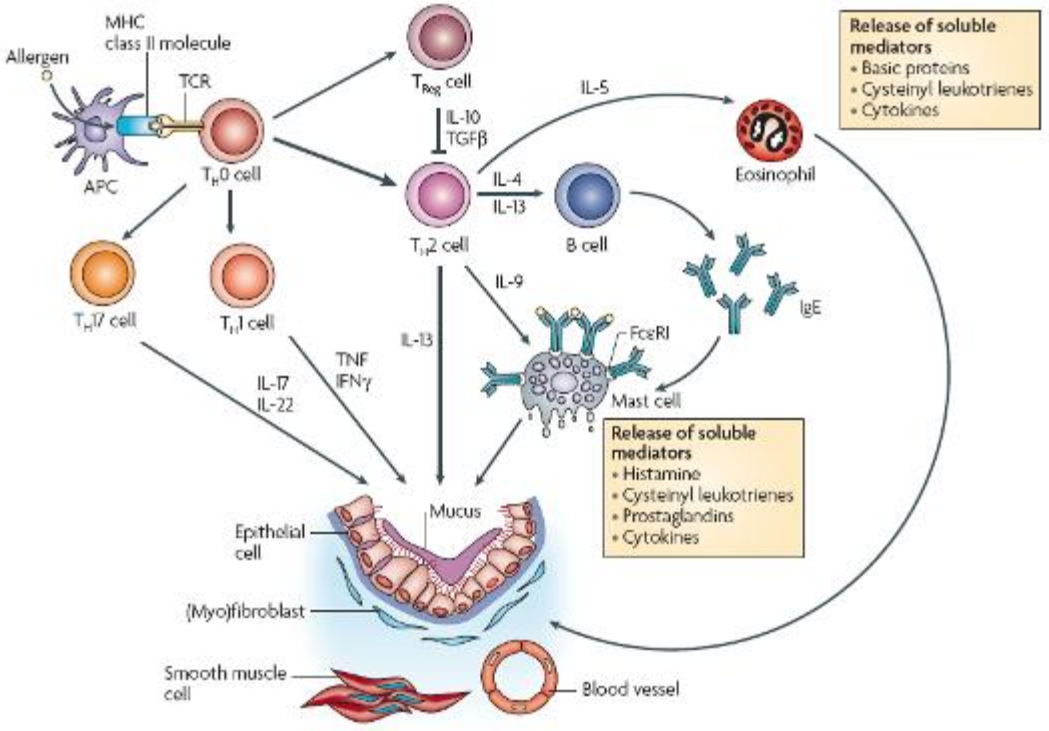


Figure 1 Allergic mechanisms. *In predisposed individuals, initial exposure(s) of professional antigen-presenting cells (APCs) to allergen leads mainly to the activation of allergen-specific T helper 2 (T_H2) cells and IgE synthesis, which is known as allergic sensitization. Subsequent exposures to allergen cause inflammatory-cell recruitment and activation and mediator release, which are responsible for early (acute) allergic responses and late allergic responses. In the early allergic response, within minutes of contact with allergen, IgE-sensitized mast cells degranulate, releasing both pre-formed and newly synthesized mediators in sensitized individuals. These include histamine, cysteinyl leukotrienes and cytokines, which promote vascular permeability, smooth-muscle contraction and mucus production. Chemokines released by mast cells and other cell types direct the recruitment of inflammatory cells that contribute to the late allergic response, which is characterized by an influx of eosinophils and T_H2 cells. Eosinophils release a large number of pro-inflammatory mediators, including cysteinyl leukotrienes and basic proteins (cationic proteins, eosinophil peroxidase, major basic protein and eosinophil-derived neurotoxin), and they might be an important source of pro-inflammatory cytokines such as interleukin-3 (IL-3), IL-5 and IL-13. There is now evidence that T_H1-cell responses might also be responsible for some of the pathogenic*

features in patients suffering from chronic forms of atopy, including epithelial apoptosis and smooth-musclecell activation. Regulatory T (T_{reg}) cells are another important subset of CD4+ T cells with implications for the suppression of T_H2-cell responses in humans involving the inhibitory cytokines IL‑10 and transforming growth factor-β (TGFβ). Another newly identified CD4+ T-cell subset, known as T_H17 cells on the basis of secretion of IL‑17A and IL‑17F, seems to be specifically associated with the neutrophilic inflammatory events that occur during disease exacerbation and in tissue remodelling.

Allergic Asthma

In the past 20 years, the prevalence of asthma has almost doubled, such that asthma now affects approximately 8% to 10% of the population in the United States. It is the leading cause of hospitalization among young children. This epidemic increase in asthma has been attributed to aspects of Western culture, including outdoor and indoor air pollution, childhood immunizations, and cleaner living conditions, but no single cause has been identified. The high prevalence rate has markedly increased the cost of this disease as measured in health care dollars, time away from work and school, and mortality. Asthma has been the focus of media, public health, and research initiatives to improve awareness and compliance with medications and to understand the causes and course of disease.

Asthma is a chronic inflammatory disease of the airways characterized by recurrent episodes of airway obstruction and wheezing. Airway inflammation, reversible airflow obstruction, and an increased sensitivity to nonspecific irritants or bronchoconstricting agents, termed airway hyperresponsiveness (AHR), are the cardinal features of asthma. AHR is defined as an increased bronchoconstrictor response to a nonspecific stimulus [19]. The response can be measured in the laboratory using nonselective stimuli that provoke bronchoconstriction in all asthmatics and is

generally performed using dose-response curves by inhalation of these agents, such as methacholine.

Pathophysiology

In asthma, the airway wall is infiltrated with mononuclear cells, which are mostly CD4 T cells, and with eosinophils. Mast cells, macrophages, plasma cells, and neutrophils are variably increased in the airways of asthmatics compared with those of controls. In the airway lumen, mucus is mixed with activated macrophages, lymphocytes, eosinophils, and sloughed epithelial cells. In some asthmatics, especially severe cases, neutrophils are increased. Structural changes of the airway wall, collectively referred to as airway remodeling, may be a result either of the interaction of inflammatory mediators with stromal cells or of tissue injury. Airway wall thickening ranges from 10% to 300% of normal, leading to a reduction in the airway luminal diameter [20-21]. The small airways (2–4mm) are commonly involved, and in fatal asthma, all the airways except the largest are affected. In addition to inflammatory cells, most of the elements in the airway wall contribute to the increased thickness. Mucous glands hypertrophy, and there is metaplasia of the airway epithelium into mucus-secreting cells. The subepithelial layer, which is 4–5 microns thick in normal subjects, ranges from 7–23 microns in asthmatics as a result of deposition of collagen (Types I, III, and V), fibronectin, and tenascin just below the basement membrane in the lamina reticularis [21-22]. Myofibroblasts, which produce collagens, are hyperplastic. Smooth muscle mass is increased and may occupy up to three times the normal area, predominantly because of cell hyperplasia. There is also vascular dilatation and angiogenesis, increased vascular permeability, and airway wall edema [20]. Airway remodeling and inflammation result in airway hyperresponsiveness (AHR) and airway obstruction, which causes breathlessness and wheezing. Corticosteroid treatment has shown limited, if any, benefit in reducing airway remodelling [23-24].

Mechanisms of Asthma

Sensitization

One of the earliest steps in the establishment of allergic sensitization to an antigen is the generation of an antigen-specific T cell response (**Figure 2a**). Antigens from the environment are constantly introduced into the airways with every breath. Multitudes of airway DCs (dendritic cells) are located under the airway epithelium, where they form an antigen-sampling network in the airway mucosa [25-27]. It is thought that adjuvant signals from airway epithelium such as TSLP (thymic stromal lymphopoietin) and GM-CSF (granulocyte-macrophage colony-stimulating factor), generated in response to inhaled stimuli, influence the activation/maturation state of DCs and help determine whether a particular allergen will trigger a T_H2-type inflammatory response or will lead to tolerance [26,28-31]. Once DCs mature and migrate to the draining lymphoid tissue, they can activate antigen-specific T cells, thus sensitizing the individual to the inhaled antigen. Allergen-specific memory T cells then migrate into the airways where they can reside long term, even during asymptomatic periods [32-35].

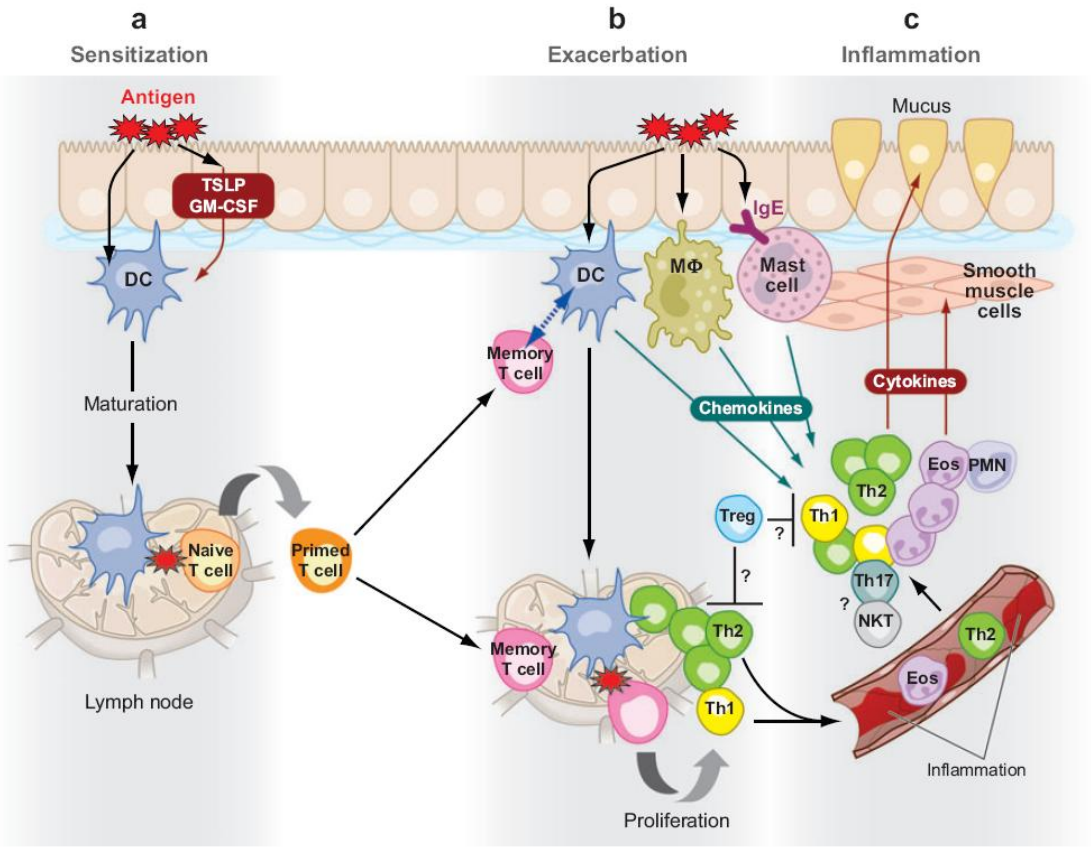


Figure 2 Asthma pathogenesis. *(a) Sensitization to an allergen results from uptake by airway dendritic cells (DCs), maturation of the DCs, migration to lymphoid tissue, and antigen presentation to T cells. Maturation of the DCs requires secondary stimuli generated from epithelial cells following antigen exposure. These primed T cells then reenter the lung, where they provide surveillance for the allergen. (b) Exacerbation of allergic airway inflammation occurs when there is reexposure to allergen and uptake by airway DCs withpresentation of antigen to airway-associated T cells as well as T cells in lymphoid tissue. T cells in the lymph node then proliferate and home to the lung, where they amplify the airway inflammation. (c) The full allergic airway phenotype results from cytokine production from T cells as well as from inflammatory mediators released from recruited eosinophils and other cells in the lung. This results in mucus hypersecretion, smooth muscle cell hyperreactivity, and airway remodeling with chronic inflammation. (PMN, polymorphonuclear cell; Eos, eosinophil; M_, macrophage; Treg, T regulatory cell; NKT, natural killer cell.)*

Exacerbation

Once an individual is sensitized to an antigen, re-exposure in the lung rapidly leads to an exacerbation of allergic airway inflammation (**Figure 2b**). Allergic responses in the airways of sensitized individuals begin with deposition of allergen in the airway mucosa. Once in the airway, the allergen can react with various innate immune cells and can bind to preformed IgE and IgG antibodies in the airways, leading to immediate mediator release from mast cells and causing the so-called early asthmatic response (EAR). These mediators lead to airway edema and bronchoconstriction and initiate the inflammatory infiltrate. Inhaled allergen is also taken up by the airway DCs, which are then stimulated to mature and migrate to local lymphoid tissue [26]. Once in the lymph node, the DCs present processed antigen to both memory and naive T lymphocytes, leading to T cell activation and differentiation. These activated effector T lymphocytes then migrate into the airways, where they secrete cytokines and other mediators, which direct the late asthmatic response (LAR) in the airway.

Asthma Inflammation

In response to cytokines secreted by effector T cells, structural cells as well as other leukocytes in the lung are stimulated to release further inflammatory mediators (**Figure 2c**). This release then stimulates the phenotypic endpoints in asthma: recruitment of eosinophils, mucus hypersecretion by goblet cells in the airway epithelium, smooth muscle cell hyperreactivity, and airway remodelling with chronic inflammation. Eosinophils release further mediators and cytokines into the airways and are critical for the full manifestation of the asthma phenotype [36-39].

CD4 T_H2 cells in asthma

In asthma, CD4 T_H2 cells are believed to initiate and perpetuate disease. Lymphocytes make up a small percentage of total leukocytes in the lung. Yet, CD4 T cells are increased in the airways of asthmatics, and they express activation markers including CD25 and Class II MHC [40-41]. IL-4, IL-5, and IL-13 protein and mRNA levels are

increased in broncho-alveolar lavage (BAL) fluid, BAL cells, and airway biopsies of asthmatics [42-43]. The transcription factor GATA-3 is expressed at high levels in CD4 T cells from asthmatic airways [44], whereas t-bet mRNA is undetectable [45], indicating an unambiguous T_H2 lymphocyte phenotype in the respiratory tract in asthma.

Animal studies confirmed the hypothesis that T_H2 cells promote allergic airways disease and have helped to define the pathways by which T_H2 cells and T_H2 cytokines affect these changes. CD4 T cells are required for antigen-induced allergic airway inflammation and AHR [46]. When T_H2 cells cannot develop in response to antigen, inflammation is not induced. T_H2 cells, when activated in the respiratory tract with inhaled antigen, can stimulate an acute allergic inflammatory response with eosinophils, AHR, and mucus hypersecretion [47-48]. Transgenic mice expressing a dominant-negative mutant of GATA-3 in an inducible and T cell–specific fashion were developed and analyzed in a murine model of allergic inflammation [49]. These studies demonstrated that inhibition of GATA-3 activity after sensitization and T_H2 induction but before inhaled antigen challenge caused blunting of T_H2 effects, including eosinophilic inflammation and AHR.

T_H2 Cell Development

Exposure to foreign protein antigens in the respiratory tract should not induce active inflammation. Mucosal surfaces encounter nontoxic proteins continually, and vigorous immune responses do not generally develop. The respiratory tract must maintain its essential gas exchange function and therefore has evolved to limit access of proteins to the immune system with barriers like the mucus layer and intercellular tight junctions. The respiratory mucosa is not totally impenetrable, and active mechanisms suppress pulmonary immune responses. Inhaled antigens induce immune unresponsiveness in naive T cells. If inhaled protein antigens do not induce tolerance, CD4 T cells are more commonly directed down a T_H2 pathway. Myeloid DCs preferentially skew immune responses toward T_H2 and suppressor T-cell populations [50]. Innate immune responses by mast cells and NKT cells may help to promote T_H2 cell development by their secretion of IL-4 and IL-13 [51-53]. Many aeroallergens possess protease activity that may allow them to overcome the protective mucosal

barrier and induce mast-cell degranulation and IL-4 production [54-55]. The genetics of an individual likely play a key role in biasing CD4 T-cell responses. Asthma traits have been linked strongly with flanking markers of a number of different genes including the human cytokine gene cluster on chromosome 5q31, which contains the genes for IL-4, IL-5, IL-9, IL-13, CD14, and GM-CSF. Polymorphisms in a number of these genes have been associated with a higher incidence of asthma and atopy.

T_H2 Cell Persistence

In asthma, even during periods of quiescent disease, T_H2 cytokines are produced in the airways. In the absence of recent antigen exposure, a typical T_H2 response should die down because normal regulatory functions eliminate effector CD4 T cells after activation. When activation is continual, these mechanisms should also eliminate the responsive cells. T_H2 cell persistence, therefore, must be due to one or a combination of (*a*) increased generation from naive CD4 T cell precursors, (*b*) increased recruitment and/or proliferation of effector/memory CD4 T cells, or (*c*) reduced elimination of effector/memory CD4 T cells.

The remodelled, inflamed airways may promote continued T_H2 cell activation in asthma because they can no longer function as an efficient barrier to limit local immune responses. When exposed to allergens, the damaged airway epithelium may allow more soluble proteins to cross from the luminal to the apical surface, potentially increasing antigen presentation. Injured airway epithelial cells produce GM-CSF, which increases DC maturation. Allergen challenge induces DC progenitors in the bone marrow [56]. Increased vascular permeability should allow rapid migration of inflammatory cells from the vascular space into the airway.

In airway biopsies of asthmatics, DCs were increased in number and activated, expressing more cell-surface Class II MHC molecules [57-58]. DC activation is likely induced by locally secreted mediators such as GM-CSF and by interactions with activated T cells via CD40-CD40 ligand [26,59]. Memory CD4 T_H2 cells that reside in the airways can be activated locally by DC that provide essential co-stimulatory

signals between B7-RP-1-ICOS and OX-40-OX-40 ligands, thereby leading to rapid induction of cytokines and AHR [26,60-62].

Antigen presentation may be prolonged owing to a small population of airway APC that can present antigen for up to eight weeks following inhalational exposure [60]. In this setting, administration of inhaled antigen induces activation rather than tolerance. In mice with active T_H2-induced pulmonary inflammation or in mice previously sensitized to an antigen, inhaled exposure that had induced protective tolerance in naive mice led to T-cell activation [63-66]. Naive T cells may also be activated in LNs to become T_H2 effector cells. Small numbers of activated DCs that present antigen were observed to migrate to the local LN where the environment and features of the DC favor T_H2 generation [26,67].

T_H2 Cytokines

CD4 T_H2 lymphocytes contribute to the inflammatory response and to airway remodelling by producing cytokines. Each cytokine has distinct functional effects in induction of disease, but IL-13 predominates in its contribution to the pathophysiology in asthma. In allergic airway inflammation in mice, IL-13 is required for AHR [68-69]. The precise mechanisms by which IL-13 leads to AHR are unknown, yet IL-13 receptors on the airway epithelium mediate this physiological effect [70]. Mucus hypersecretion in animal models of asthma requires IL-13. IL-13 stimulates the generation of goblet cells, followed by the induction of mucin genes and mucus production [71]. IL-13 and IL-4 both promote eosinophilia by stimulating Stat6-dependent eotaxins [72]. IL-13 also promotes airway remodeling through effects on matrix metalloproteases and activation of TGF-β1 [73]. Airway eosinophilia also depends on IL-5, which controls eosinophil maturation in the bone marrow and recruitment and activation in the respiratory tract [74]. IL-4 remains an essential factor for T_H2 cell differentiation, but compared with IL-13, has a limited role in driving the pulmonary inflammatory response in asthma.

CD4 lymphocytes produce a majority of the T_H2 cytokines in the respiratory tract in asthma. In airway biopsies and BAL cells from asthmatics[75]. Yet, CD8, and NK-T cells, eosinophils, mast cells, basophils, NKcells, and subsets of Class II MHC-expressing accessory cells can produce T_H2 cytokines [75-79]. IL-4, IL-5, and IL-13 produced by non-CD4 T cells may be essential for the development and perpetuation of asthma. The period of cytokine secretion from this array of cells will vary. Thus, as lymphocytes die or cease production of cytokines after their activation, waves of cytokines produced by non-CD4 T cells may follow.

Dendritic Cells in allergic asthma

Naive T cells require mature antigen-presenting cells (APCs) such as dendritic cells (DCs) to proliferate and acquire TH2-cell effector function inresponse to antigen encounter [80]. DCs are crucial in determining the outcome of antigen encounter and integrate signals that are derived from the antigen, its inflammatory context and the host environment into a signal that can be 'read' by naive T cells in the lymphoid tissues[81]. In this perspective, airway DCs are not only crucial for sensitization to inhaled antigens leading to allergy, but also have a crucial role in established inflammation. 'Classical' lung APCs such as alveolar macrophages and B cells seem to be less important in asthma pathogenesis.

Like in other epithelia and skin, the lung is equipped with an elaborate network of DCs that can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes [82]. These cells perform a unique sentinel function in the pulmonary immune response in that they recognize inhaled antigens through expression of ancient pattern-recognition receptors such as Toll-like receptors, NOD-like receptors, and C-type lectin receptors
that will recognize motifs on virtually any inhaled pathogen, allergen, or substance [83]. Additionally, lung DCs express numerous receptors for inflammatory mediators that are released upon damage (so-called damage-associated
molecular patterns like uric acid) to the tissues by pathogens, trauma, vascular damage, or necrosis. By exerting all these direct and indirect sensing mechanisms for

danger in the airways, and at the same time expressing all the machinery to migrate to the regional lung-draining lymph nodes and process antigens on their way to the node [84], DCs are at the nexus of innate and adaptive immunity in the lung.

The Increasing Complexity of Lung DC Subsets

Although lung DCs were originally described in the mouse as a single population of highly dendritic-shaped cells with high degree of expression of CD11c and MHCII [85], it is now clear that at least five different subsets of DCs can be found in the lungs (see **Figure 3**). Most importantly, there is division of labor between these various lung DC subsets, making a closer distinction into subsets almost imperative if one is to understand the biology of lung DCs [82].

The mouse lung is grossly divided into large conducting airways and lung interstitium containing alveloar septa and capillaries where gas exchange is taking place [86]. In steady-state conditions, the conducting airways of all species studied are lined with an intraepithelial highly dendritic network of $MHCII^{hi}CD11c^{hi}$ cells that are mostly $CD11b^{-}$ and at least in the mouse and rat express langerin and the mucosal integrin CD103 (aEb7), and in addition has the propensity of extending dendrites into the airway lumen through formation of tight junctions with bronchial epithelial cells [82,87 88]. Immediately below, the lamina propria of the conducting airways contains $MHCII^{hi}CD11c^{hi}$ cells that are highly expressing CD11b and are a rich source of proinflammatory chemokines [89 90].

A similar broad division into $CD11b^{+}$ and $CD11b^{-}$ can also be applied to lung interstitial DCs. Both $CD11b^{+}$ and $CD11b^{-}$ subsets express high amounts of CD11c, so they can best be denominated as conventional DCs (cDCs), to contrast this with another population of $CD11c^{int}$ plasmacytoid DCs (pDCs) that express Siglec-H, B220 and the bone-marrow stromal antigen-1 (recognized by 120G8 antibody, or PDCA-1) and some markers shared with granulocytes and B cells (**Figure 3**) [82,91-92]. The exact anatomical location of lung pDCs is unclear although they can be found to line alveolar septa in situ and have been recovered from digests of large conducting airways [86,91].

The alveolar space also contains $CD11c^{hi}MHCII^{hi}$ DCs and is easily accessible by bronchoalveolar lavage. At least in the rat and man, alveolar DCs are highly enriched in $CD103^+$ subsets that resemble Langerhans cells in man. One caveat is, however, that lung alveolar macrophages express high amounts of CD11c while lacking CD11b, essentially confusing many analyses of lung DC biology if one does not use autofluorescence to identify macrophages [93].

Under inflammatory conditions, such as viral infection, allergen challenge, or LPS administration, there is recruitment of additional subsets of $CD11b^+$ monocyte-derived DCs that rapidly upregulate CD11c and retain expression of Ly6C as a remnant of their monocytic descent, and are easily confused with resident $CD11b^+$ cDCs [82,94 95].

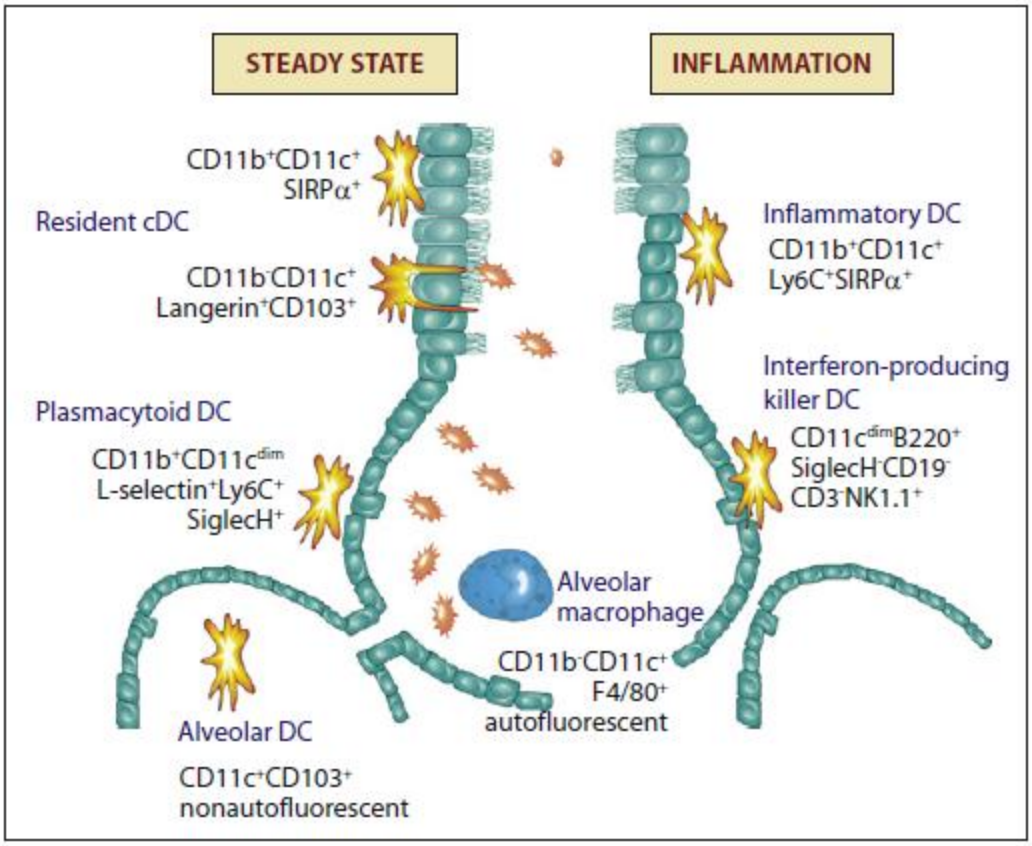


Figure 3. Lung Dendritic Cell Subsets *In steady-state conditions (depicted on the left), conventional DCs (subdivided into $CD11b^+$ and $CD11b^-$ subsets) line the conducting airways. They can also be found back in the deeper interstitial compartments, obtained by enzymatic digestion of peripheral lung. Plasmacytoid DCs are also found in both compartments with a slight preference for the interstitial compartment. Finally, the alveolar space contains DCs that can be easily confused*

with alveolar macrophages if one does not take autofluorescence of the latter into account. Under inflammatory conditions, there is recruitment of CD11b^{+} monocytes to the lungs and these rapidly become DCs. They can still express Ly6C as part of their monocytic descent. In viral infection as well as in some cancers, there is also recruitment of interferon-producing killer DCs, a subset of NK cells that can be mistaken for pDCs in view of their intermediate expression of CD11c and expression of the B cell marker B220. One way of discriminating these is via staining for NK1.1.

Function of airway DCs

Immature DCs are distributed throughout the lungs and are pivotal in controlling the immune response to inhaled antigen. Fluorescent-labelled macromolecules that do not cross the epithelial tight-junction barrier are captured by airway DCs and taken to the T-cell area of draining mediastinal lymph nodes (MLNs) within 12 hours [84,96]. This process occurs continuously under steady state conditions. DCs that take up antigen in the periphery are functionally immature and unable to stimulate T cells [97]. As they arrive in the MLN T-cell area carrying their antigenic cargo, they express an intermediate array of co-stimulatory molecules (somewhere between that of naive and mature DCs) and high levels of MHC class II molecules that can induce the proliferation of naive T cells [84,98]. Studies using the adoptive transfer of T-cell receptor (TCR)-transgenic T cells have shown that airway exposure to harmless lipopolysaccharide (LPS)-free antigens leads to marked T-cell proliferation *in vivo*, mainly in the superficial cervical lymph nodes and MLNs, but not in the non-draining lymph nodes or the lung itself [96,99-100]. The functional outcome of this proliferative T-cell response is, however, tolerance, and this might explain why continuous migration of airway DCs under baseline conditions does not lead to auto- immunity [101]. The most probable explanation is that harmless antigens or self antigens from dying epithelial cells fail to activate the airway DC network fully, such that partially mature DCs induce an abortive proliferative response of 'unfit' T cells that fail to reach the threshold for cytokine survival signals, leading to death by neglect [99-100]. Deletional tolerance is not the only possibility. Studies by Umetsu and colleagues [98,102] have shown that antigen presentation by airway DCs that express the inducible co-

stimulatory molecule ligand (ICOSL) and secrete the immunoregulatory cytokine IL-10 induces the formation of regulatory T cells that can inhibit subsequent inflammatory effector responses [98,102].

Under conditions in which exposure to inhaled antigen is accompanied by an inflammatory stimulus, such as concomitant exposure to LPS or infection of the lungs with virus, naive T-cell clonal expansion is of similar magnitude, but this time generates 'fit' effector T cells [100,103-104]. Airway inflammation induced by virus infection recruits airway DCs and induces their full maturation after arrival in the MLNs [105]. These conditions of immune stimulation can be mimicked by injection of mature bone-marrow-derived DCs into the trachea of naive mice, or by adenovirus-mediated over-expression of the DC-maturation factor granulocyte– macrophage colony-stimulating factor (GMCSF) in the airways of naive mice to activate endogenous DCs [106-107]. As DCs express many pattern recognition receptors (PRRs) and communicate extensively with cells of the innate immune system, microbial stimuli that accompany a particular antigen can markedly change the type of T_H-cell response that is induced[28]. When DC maturation is induced in the absence of strong polarizing signals, the functional outcome is stable T_H2- type immunity, as shown by the occurrence of T_H2-recall responses [67,97,106-108]. However, high doses of LPS or proliferative infection with influenza virus at the time of exposure to ovalbumin (OVA) lead to stable T_H1-cell immunity [97,100,104]. Similarly, bone-marrow-derived DCs that are exposed to high doses of LPS induced an OVA-specific T_H1-cell response after transfer to the lungs of naive mice [109].

When T-cell responses were followed under these conditions, several important features emerged. First, T-cell differentiation followed a cell-division-regulated program of differentiation in which primed $CD44^{hi}CD69^{low}CCR5^{+}$ T cells that had undergone at least three divisions re-circulated to the lungs as cytokine-producing effector T cells [99,109]. After 2 to 4 days, T cells that had divided at least 3–4 times also re-circulated to non-draining lymph nodes and the spleen, therefore most probably representing the central memory T cells that have been described *in vitro* [110] (**Figure 4**). These two different fates of re-circulating T cells have marked implications for how and where memory T cells are stimulated after repeated contact with antigen. The T cells that migrate to the airways are re-stimulated locally by DCs in the airways, without the requirement of further T-cell division [26,111-112]. The few cells that migrate to the lung interstitium are kept in check by the local suppression of DC and T-cell

activation by resident alveolar macrophages [26,113]. By contrast, the resting central memory T cells that re-circulate throughout the lymphoid tissues would again require migratory DCs for their activation, leading to new rounds of cell division and a new wave of effector T cells that migrate to the lungs [111].

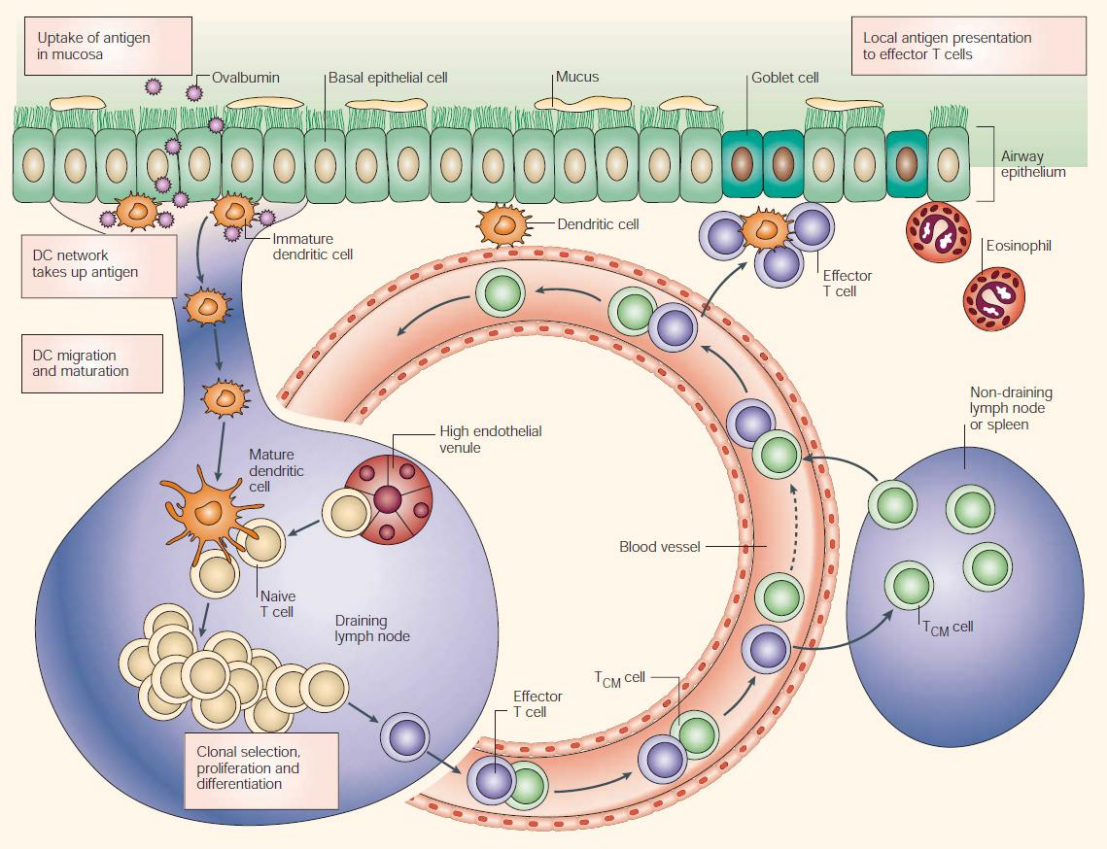


Figure 4 An integrated overview of DCs and CD4+ T-cell migration during primary and secondary immune responses. *Antigen is taken up by dendritic cells (DCs) across the mucosal impermeable barrier. Mucosal DCs continuously migrate from the lungs to the T-cell area of mediastinal lymph nodes (MLNs). In the presence of inflammation, this process is amplified, increasing the possibility that pathogenic substances will be presented to recirculating naive T cells or central memory T (T_{CM}) cells. At the same time, DC maturation will be fully induced. When mature DCs arrive in the MLNs, they select specific T cells from the polyclonal repertoire of cells that migrates through the high endothelial venules and T-cell area. Within 4 days, this will lead to clonal expansion of antigen-specific T cells. When a T cell has acquired a certain threshold number of divisions, it will leave the MLN, to become either a T_{CM} cell or an effector T cell. This is where migration pathways separate, and*

consequently the anatomical requirements for reactivation diverge. The T_{CM} cells will extravasate in other non-draining nodes and spleen, and will eventually accumulate in the spleen over time. Reactivation of these cells will ,therefore, only occur in central lymphoid organs. By contrast, effector T cells will extravasate in peripheral sites of inflammation, including the lung when the original inflammation is still present. In contrast to naive T cells, which are excluded from lung tissues, these effector T cells can be stimulated by local airway DCs to mediate their effector function. In this scenario, alternative antigen-presenting cells might be eosinophils or even epithelial cells, expressing MHC molecules.

Immune tolerance and reguatory T cells

The defensive capacity of the immune system needs a mechanism to counterbalance its power and minimize unnecessary tissue damage. Several processes ensure that the different immune effector cells are not activated against host tissues, innocuous substance or for prolonged periods of time, after the threat is resolved. Together, these processes constitute immune tolerance, and could be classified as central, if occurring in primary lymphoid organs, or peripheral, if occurring in other tissues [114]. An example of central tolerance is the deletion of T cells in the thymus, or B cells in the bone marrow, if these cells express antigen receptors with specificity for self-antigens. The protein AutoImmune REgulator, or AIRE, is a transcription factor that promotes in the thymus the expression of genes which are characteristically expressed in other organs, presumably to present self-antigens to newly formed T cells, and facilitates the elimination of self-reactive T cells, as part of the immune tolerance process. AIRE-deficient patients develop severe autoimmunity, involving multiple endocrine organs, in a syndrome named Autoimmune PolyEndocrinopathy Candidiasis, Ectodermal Dystrophy (APECED). Because not all self-antigens are expressed during the central induction of tolerance, other self-reactive T cells may need to be inactivated in the periphery. In lymphoid organs, lymphocytes with high affinity are deleted upon encounter with self-antigens. In other peripheral tissues, immune tolerance occurs by the induction of anergy. It was observed in experimental models that a T cell subset with the capacity of inhibiting autoimmune responses emerged during tolerance induction. In addition, it was noted that IL-2 knockout mice

developed significant autoimmunity. Recent studies have provided evidence of a subset of T cells characterized by high levels of CD25 expression (IL-2R-α-chain), named regulatory T cells or 'T_{regs}', which suppress the function of other T cells when present in the same site (**Figure 5**) [115]. The modulatory function of these cells appears to require the expression of the forkhead protein Foxp3 [116]. T cell proliferation and cytokine responses are blunted in the presence of T_{regs}. In experimental models, Tregs can function through cell–cell contact and in other models through the secretion of IL-10 and/or TGF-β, inducing activation-induced cell death or anergy. A rare human disorder caused by mutations in the FoxP3 gene, the Immunodeficiency PolyEndocrinopathy, Enteropathy X-linked (IPEX), is characterized by a deficiency of $CD4^+CD25^+$ T regulatory cells, and subsequent development of severe autoimmunity, including insulin-dependent diabetes mellitus and inflammatory colitis, as well as atopic dermatitis and asthma [117]. Other types of regulatory T cells have been described that are not $Foxp3^+$. T_H3 cells produce IL-4, IL-10 and TGF-β and are predominantly located in the mucosa. T_r1 are cells that produce TGF-β only, and are induced by high concentrations of IL-10.

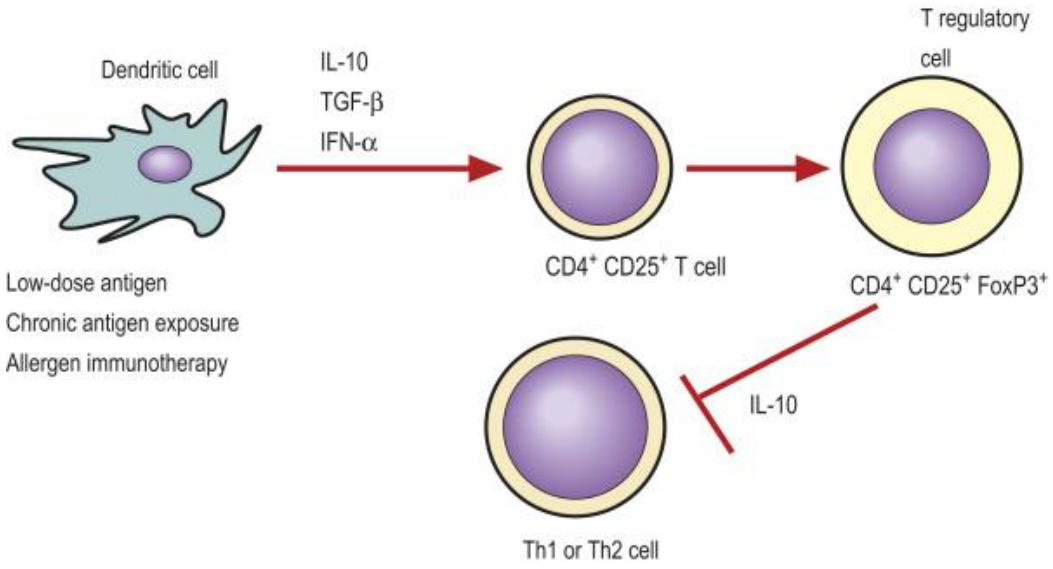


Figure 5. Regulatory T cells are generated by the interaction of antigen-presenting cells and T cells, mediated by the cytokines IL-10, TGF-β, and IFN-α. *These*

cytokines are secreted when the antigen is presented under certain conditions, such as when administering allergen immunotherapy at very low concentration. Regulatory T cells secrete IL-10 and inhibit effector T cells that share similar antigen specificity.

Antigen-induced Immunosuppression of allergic airway disease

It is evident from both murine and human studies that allergic airway disease can be regulated by active mechanisms of immunosuppression and cytokines play an integral role in this process. Not only do cytokines play a critical role in shaping T helper differentiation along different pathways, T_H1, T_H2, or T_H17, they also serve equally important functions in inhibiting the development of the T_H subsets. For example, T cells engineered to secrete TGF-β were found to inhibit airway inflammation and AHR [118]. Like TGF-β, IL-10 was also shown to inhibit the development of the asthma phenotype [119]. The importance of TGF-β as a key immunoregulatory molecule in mucosal inflammation was first described in the form of TGF-β-secreting T_H3 cells in studies of oral tolerance in mice [120]. Later, both TGF-β and IL-10 were implicated in regulatory function of T cells in a murine model of allergic airways disease [121]. TGF-β has been shown to induce Foxp3 mRNA expression in activated $CD4^+CD25^-$ T cells resulting in the accumulation of $CD4^+CD25^+$ T_{regs} [122-123]. When adoptively transferred to mice, these Tregs block antigen-induced airway inflammation Chen, 2003 #958}. In a reciprocal fashion, expression of a negative regulator of TGF-β signaling, Smad7, in T cells enhanced airway inflammation and AHR [124].

Murine models of airway tolerance have been developed using inhaled or intranasally (i.n.) delivered antigen. In a model of tolerance, involving delivery of ovalbumin (OVA) i.n. consecutively for 3 days, regulatory T cells secreting IL-10 were implicated in the development of tolerance in the respiratory tract [102]. In the same system, ICOS–ICOS ligand interaction has been shown to be important for the generation of IL-10-secreting Tregs [98]. In a different study, when antigen-pulsed APCs overexpressing the Notch ligand Jagged-1 were introduced into mice, subsequent challenge with the same antigen profoundly inhibited immune responses as measured in vitro [125]. Adoptive transfer of $CD4^+$ T cells, but not $CD8^+$ T cells, transferred tolerance to recipient mice and the tolerance was shown to be antigen-

specific [125]. Using repeated exposure to inhaled antigen as the model of tolerance induction [126], a role for $CD4^+CD25^+$ T regulatory cells (Tregs) has been identified [127-128]. In these studies, $CD4^+CD25^+$ T_{regs} expressing Foxp3 and membrane-bound TGF-β were shown to be induced by inhaled antigen and mediate immunosuppressive functions by engaging the Notch1-Hes1 axis in target T helper cells.

Tolerance induction by DCs

Airway DCs form a dense network in the lung where they are ideally placed to sample inhaled antigens and these cells migrate to draining lymph nodes (LNs) to stimulate naive T cells [84]. As most allergens are immunologically inert proteins, the usual outcome of their inhalation is tolerance and inflammation does not develop upon chronic exposure [91]. This is best shown for the model antigen ovalbumin (OVA). When given to the airways of naive mice via aerosolization, nasal droplet aspiration or intratracheal injection, it renders mice tolerant to a subsequent immunization with OVA in adjuvant, and effectively inhibits the development of airway inflammation, a feature of true immunological tolerance [91]. It was therefore long enigmatic how sensitization to natural allergens occurred. An important discovery was the fact that most clinically important allergens, such as the major Der p 1 allergen from HDM, are proteolytic enzymes that can directly activate DCs or epithelial cells to break the process of tolerance and promote T_H2 responses [129-130]. However, other allergens such as the experimental allergen OVA do not have any intrinsic activating properties. For these antigens, contaminating molecules or environmental exposures (respiratory viruses, air pollution) might pull the trigger on DC activation. Eisenbarth showed that low-level TLR4 agonists admixed with harmless OVA prime DCs to induce a T_H2 response, by inducing their full maturation, yet not their production of IL-12. This process has been recently described as being dependent on the activation of the adapter molecule MyD88 in pulmonary DCs [31]. This is clinically important information as most natural allergens, such as HDM, cockroach, and animal dander, contain endotoxin and undoubtedly other TLR agonists.

From the above discussion, is seems that the decision between tolerance or immunity (in the lungs) is controlled by the degree of maturity of cDCs interacting with naive T cells, a process driven by signals from the innate immune system [131]. It has indeed

been shown that immature cDCs induce abortive T-cell proliferation in responding T cells and induce regulatory T cells (T_{regs}) [102]. Another level of complexity arose when it was shown that (respiratory) tolerance might be a function of a subset of plasmacytoid DCs [91,132]. Removal of pDCs from mice using depleting antibodies led to a break in inhalational tolerance to OVA and the development of 'asthmatic' inflammation [91]. The precise mechanisms by which pDCs promote tolerance are unknown, but in the absence of pDCs, cDCs become more immunogenic and induce the formation of effector cytokines from dividing T cells [131]. The negative signal that is delivered by pDCs has not been elucidated, but could be the high-level expression of programmed death ligand (PDL)-1, delivering a negative signal to T cells or to cDCs directly [91,133]. Additionally, pDCs can produce the tryptophan-metabolizing enzyme indoleamine 2,3-dioxygenase (IDO), which has a strong inhibitory activity on T-cell proliferation [134], and inhibits inflammatory airway disease. Interestingly, IDO expression has been demonstrated recently in pulmonary $CD11c^+$ cells although the exact cell type involved has not clearly been identified. Another explanation to the tolerogenic properties of pDCs is related to their immature phenotype, as it has been demonstrated that immature DCs can induce regulatory T cells. Ex vivo at least, lung-derived pDCs promoted formation of Treg cells that were specific for OVA [91].

Osteopontin

Osteopontin (Opn) is an *O*-glycosylated phosphoprotein that contains the arginine-glycine-aspartic acid (RGD) integrin-binding domain commonly found in matrix proteins [135]. Opn was initially identified in 1979 as a secreted protein associated with malignant transformation but has been independently discovered by investigators from diverse scientific disciplines and has been associated
with a remarkable range of pathological processes. Its role as an important adhesive bone matrix protein has been well described and reviewed, yet its role as a regulator of immune events and pathology is less well recognized. In the 14 years since Opn was first discussed by Harvey Cantor in *Cytokine and Growth Factor Reviews* there has been remarkable progress in our understanding of the immunology of this protein [136]. It is now recognized as a key cytokine involved in immune cell recruitment and type-1 (Th1) cytokine expression at sites of inflammation [137-138]. It also appears to regulate important aspects of tissue repair and remodeling [139-140]. This juxtaposition between cytokines and matrix proteins has led to the classification of Opn as a matricellular protein, though other have suggested that it be reclassified as a member of the interleukin family [135].

Osteopontin has pleiotropic effects based on its capacity to interact with multiple binding partners and the fact that it is subjected to various posttranslational modifications and enzymatic reactions. A brief history of the discovery and naming of osteopontin helps to explain the multifaceted characteristics of a molecule that has a surprising name. The prefix of its name (from the Greek osteo, meaning bone) suggests that its function is related to bones. The naming of osteopontin was prophetic: when they cloned osteopontin in 1986, Oldberg and colleagues [141] wrote that "we suggest that the protein is named osteopontin, denoting that it is a product of cells in the osteoid matrix and that it can form a bridge (in latin, pons) between cells and the mineral in the matrix". The concept that osteopontin forms bridges is remarkably perceptive: it clearly binds integrins and CD44 as well as several extracellular matrix proteins, including fibronectin andvitronectin. Osteopontin therefore interacts with various biological processes that range from cell adhesion to coagulation.

Osteopontin was first called transformation-specific phosphoprotein in a study on proteins induced in transformed cells by Senger, Wirth and Hynes in 1979 and 1980 [142]. When osteopontin was first described, its function was unknown. Cantor, who first cloned what turned out to be osteopontin 10 years later, proceeded to name it early T cell activation 1 (Eta-1) [143]. When he cloned the gene that encodes osteopontin (Opn), Cantor had discussed the importance of the RGD (arginine, glycine and aspartic acid) integrin-binding domain and its potential interaction with other bridging proteins. Nowadays, the protein is more formally named secreted phosphoprotein 1 (SPP1) and is also known as osteopontin, BNSP, BSPI, Eta-1 and MGC110940. The name osteopontin came into wider use as its biological roles were delineated [144].

Molecular biology and protein structure

The Opn gene is located on human chromosome 4q13 (mouse chromosome 5 at the ricr locus) [145-146]. Although human Opn protein contains 314 amino acids with a predicted molecular mass of 32 kDa, there is substantial post-translational modification, including phosphorylation and N-linked glycosylation which results in a protein with electrophoretic mobility between 44 and 75 kDa (**Figure 6**) [141,144,147]. The protein is acidic, hydrophilic, and highly negatively charged with features of a secreted protein lacking a membrane anchoring domain [147]. Human Opn has two integrin-binding motifs: a typical RGD domain and a second integrin-binding site SVVYGLR (for serine-valine-valine-tyrosine-glycine-leucine-arginine) [141,148]. There is a calcium binding site, two consensus heparin binding domains, and run of 9–10 aspartate residues which are thought to represent an hydroxyapatite binding sequence [141,145]. There is substantial variation of Opn protein structure [149].

The Opn gene itself is multiallelic with at least two human and three murine alleles [149]. The function of allelic variants in humans is unknown, while in mice, consistent with mapping to the ricr locus, the alleles correlate with resistance to rickettsial infection [145]. There is also evidence of alternative RNA splicing of the human Opn gene with three Opn cDNAs identified [149]. The function of these splice variants is unknown. Opn isoforms that differ in phosphorylation patterns have also been detected and these isoforms appear to be functionally distinct [137,150].

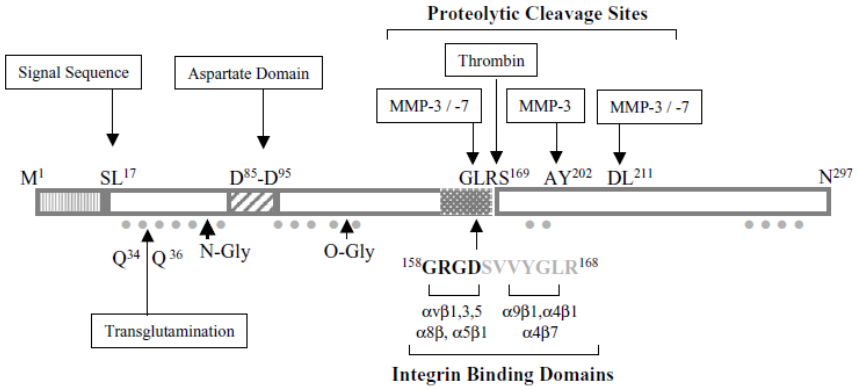


Figure 6. *Schematic representation of protein structure showing binding motifs, cleavage sites, and putative phosphorylation and glycosylation sites. N-gly-, N-glycosylation; MMP, matrix metalloproteinase; , phosphorylation sites.*

Opn is structurally and functionally modulated by proteolytic processing (**Fig. 6**). Opn contains a biologically active thrombin cleavage site, which lies within six amino acids of the RGD sequence [151]. There is *in vitro* and *in vivo* evidence that thrombin cleavage of Opn at this site modulates integrin binding and protein function [151]. Most of the recognized biological activity of Opn resides in the N-terminal thrombin cleaved fragment (Fig. 1). Opn can also be specifically cleaved by certain matrix metalloproteinases (MMP) [152].

Opn is also a member of the recently described SIBLING (for *s*mall *i*ntegrin-*b*inding *l*igand, *N*-linked *g*lycoproteins) family of proteins which includes bone sialoprotein and dentin matrix protein-1 [153]. Rather than being linked in primary protein sequence this family of proteins share a common genetic locus on human chromosome 4 (mouse chromosome 5). They also share a non-coding first exon, similar splice variants (type-0 introns) and the presence several conserved regions including casein kinase II phosphorylation sites, RGD integrin-binding domains, and polyacidic stretches. SIBLINGS have a high affinity for hydroxy apatite and can support cell attachment in vitro through their RGD integrin-binding sites. Importantly SIBLING

proteins are essentially unstructured in solution [153]. The resultant flexibility appears to facilitate rapid sequential interactions with various binding partners and thus SIBLINGs can bridge two distinct proteins into a biologically active complex. For instance, Opn can simultaneously bind cell surface receptors such as αvβ3 integrin and complement Factor H [154].

Osteopontin receptors

Osteopontin binds a number of integrins via two contiguous but distinct motifs within the sequence GRGDSVVYGLR (amino acid residues 158–168 on human protein, see **Fig. 6**). RGD binding integrins include several of the αv class (αvβ3, αvβ1, αvβ5) as well as α8β1 and α5β1 [155-157]. A subfamily of integrins α4β1, α4β7, and α9β1, linked by sequence homology and their ligand-binding repertoire, can bind to Opn via the SVVYGLR motif [148,158-160]. Opn integrin interactions, in particular involving SVVYGLR, are often cryptic requiring liberation of the binding domain by thrombin cleavage just downstream at Arg 168 [159-160]. Opn can also interact with a non-integrin cell surface receptor, CD44 [161]. Rather than the standard CD44 receptor alone, v6 and/or the v7 splice variants appear to be required for interaction with Opn [161-163]. It has been suggested that the interaction of Opn with CD44 involves primarily binding to β1 integrins rather than directly with CD44 [164]. Signal pathways that may be activated by Opn-receptor interactions have been recently reviewed and include focal adhesion kinases, nuclear factor- (NF-) kB, RhoA, and gelsolin associated phosphatidyl-inositol 3- (PI 3-) kinase [165]. The promiscuous nature of Opn receptor binding is more typical of ECM proteins and presumably accounts for many of the diverse functions of this matricellular protein.

Isoforms

Two isoforms of Osteopontin have been reported, secreted Opn (Opn-s) and intracellular (Opn-i), that represent alternative translational products of a single full-length *Opn* mRNA [166]. This mechanism, which does not involve alternative mRNA transcription initiation or splicing, generates a full-length secreted Opn protein (Opn-

s) and a smaller intracellular product (Opn-i) from a single full-length mRNA species. Translation of Opn-s is initiated from the 5' canonical AUG start site, whereas translation of the Opn-i isoform is initiated from a downstream non-AUG codon (**Figure 7**). Downstream translation of Opn-i is accompanied by deletion of the N-terminal 16-aa signal sequence, allowing the shortened protein product to localize in cytoplasm but not secretory vesicles [166]. Expression of the two isoforms differs depending on cell type: DCs express high levels of iOpn but low levels of sOpn, whereas the reverse is true of activated T cells [167]. It is possible that cell-type-specific factors may regulate the alternative translation of *Opn* mRNA. Analysis of the subcellular localization of iOPN in DCs by confocal microscopy and gradient separation has confirmed that it is localized in the cytoplasm and dendrites [166], but not in the Golgi apparatus. This expression pattern reflects the direct entry of nascent iOPN protein into the cytoplasm following its synthesis [166]. The functional properties of iOPN suggest that it may function as an adaptor protein rather than as a catalytic molecule.

It colocalizes with myeloid differentiation primary-response gene 88 (MyD88) and Toll-like receptor 9 (TLR9) in DCs, and with ezrin (a component of the ezrin–radixin–moesin complex), polo-like kinase 1 and the cytoplasmic domain of CD44 in other cell types [168-171]. iOpn is a member of the SIBLING (small integrin-binding ligand N-linked glycoproteins) family, the members

of which display extended folding in solution and have structural features that enable them to interact with multiple binding partners and to facilitate the organization of specialized macromolecular clusters [172].

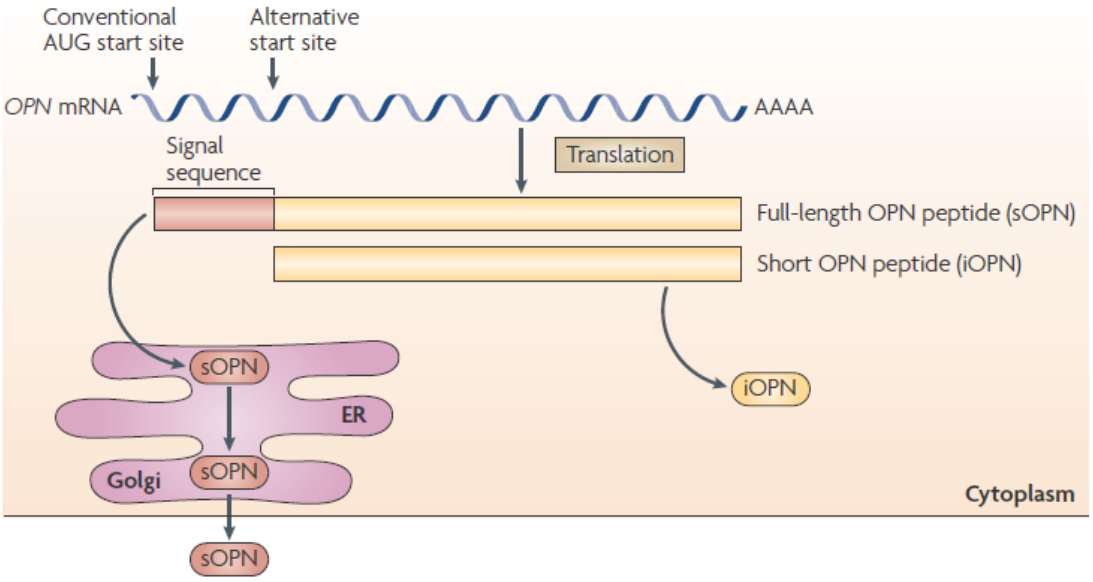


Figure 7. ***Alternative translational mechanism that generates sOpn and iOpn.*** *Following transcription and processing, mature osteopontin (Opn) mRNA has a conventional AUG translation start site and a downstream alternative translation start site. Translation from the conventional start site results in the generation of the full-length Opn peptide, which includes a signal sequence that targets the nascent peptide to secretory vesicles, resulting in its secretion from the cell (sOpn). Translation from the alternative translation start site results in a short Opn peptide product that lacks a signal sequence and remains inside the cell (iOpn). ER, endoplasmic reticulum.*

Signaling pathways

Opn signaling through integrins can modulate (via activation of Ras and Src) the phosphorylation of kinases (NIK, IKKb) involved in NFkB activation [173-174], this results in the degradation of IkB, an inhibitor of NFkB [175]. NFkB regulates expression of many inflammatory cytokines. Consequently, Opn may modulate immune responses through activation of the NFkB pathway, and the absence of Opn may be associated with diminished cytokine functionality. In a study of the role of Opn in the exacerbation of EAE it was observed that Opn appeared to enhance the survival of myelin-reactive T cells through the regulation of the transcription factors Foxo3a and NFkB and the expression of genes involve in apoptosis [176]. Several reports have

concluded that Opn expression is increased by PI3K/Akt signaling. Opn signaling through CD44 engagement promotes cell survival by activating the PI3K/Akt pathway [177]. A genetic profiling study documented that Opn is a downstream effector of the PI3K/Akt pathway, which is antagonized by PTEN, melanoma lines defective in PTEN expression exhibited increased Opn expression [178].

Intracellular Opn is found to be localized together with the MyD88 and TLR9 complex near the inner cytoplasmic membrane, it activates nuclear translocation of transcription factor IRF7 to induce robust IFN-a production [170].

Pathways for Opn signaling are depicted in **Fig. 8**. Opn expression is responsive to many transcription factors [179-180]. The Opn promoter can be activated by TGF-β through Smad signaling pathways [181]. An activator protein-1 (AP-1) consensus site in the Opn promoter has been identified that supports Opn transcription in macrophages [182]. Liver X receptor agonists inhibit cytokine-induced Opn expression in macrophages through interference with AP-1 signaling pathways. AP-1 regulation is further demonstrated in that Opn transcription is suppressed by PPAR-a agonists through repression of AP-1-dependent transactivation of the Opn promoter [183].

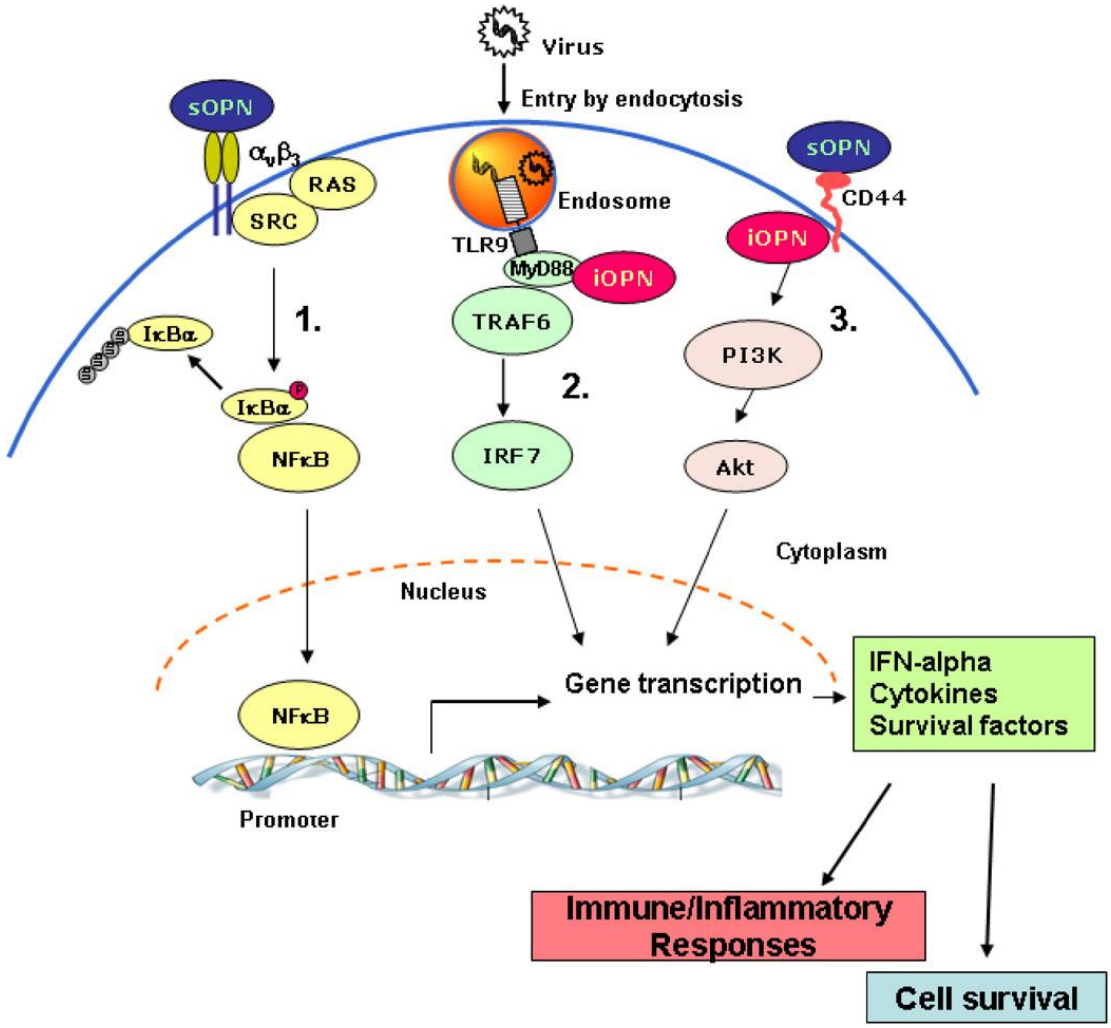


Figure 8. ***Opn interacts with different signaling pathways to mediate immune responses.*** *(1) Engagement of extracellular Opn (sOpn in blue) to (for example) integrin avb3 signals through the Src and FAK tyrosine kinases to activate transcription factor NFkB. Opn promotes phosphorylation of IkBa, which leads to the degradation of IkBa and nuclear translocation of NFkB which regulates the transcription of a variety of pro-inflammatory cytokines and mediators* [175]*. (2) In plasmacytoid DCs, intracellular Opn (iOpn in red) is associated with MyD88 during TLR9 engagement with viral DNA in the endosome, promoting TLR9 signaling toward IRF7 rather than IRF-5/NFkB, which would allow for robust IFN-g production* [170]*. (3) Opn interaction with CD44 activates PI3k/Akt signaling, which can mediate cell survival* [178]*.*

Cellular and tissue expression

Early studies of Opn focused almost exclusively on its association with bone metabolism. A survey of the expression and distribution of Opn in normal human tissue revealed widespread expression in non-bony sites and in particular at luminal epithelial surfaces [184]. In particular, in the lung at baseline Opn is expressed by bronchial epithelial cells and scattered alveolar macrophages [184]]. During pathological processes Opn is highly expressed by injured epithelium, alveolar and interstitial macrophages, T cells, and pulmonary vascular endothelium [185-186]. Fibroblasts have not been shown to express Opn in human lung disease, though cardiac rat fibroblasts are a prominent source of the protein [187]. Although Opn has been identified in bone matrix there is no evidence to suggest that Opn forms part of non-bony tissue matrix in vivo. Significant levels of Opn have been detected in plasma, urine, and breast milk [188-189].

The above pattern of tissue expression of Opn protein reflects the known cellular sources of Opn. Studies from both cell lines and in most cases primary cells have demonstrated that a variety of immune and non-immune cells express Opn. Immune cellular sources include macrophages (including macrophage-derived cells such as osteoclasts, kuppfer cells, and epithelioid cells), dendritic cells, T cells, B cells, and NK cells (including NK-derived granulated metrial cells located in the endometrium) [185-186,190-194].

Principal "non-immune" sources of the protein are smooth muscle cells, epithelial cells and endothelial cells as well as fibroblasts, osteoclasts, osteoblasts, and certain tumor cells [184,187,192,195-198]. In epithelium and some macrophages Opn protein appears to be constitutively expressed but in all cells studied it is rapidly up-regulated soon after cellular activation. The Opn promoter is remarkably responsive to many agents including cytokines, growth factors and hormones, especially those that activate protein kinase C[165].

Opn expression is induced by a variety of factors ranging from growth factors and cytokines (including IL-1, IL-2,IL-3, GM-CSF, TNF-α, TGF-β, Bone morphogenic protein, angiotensin II) to direct activation by bacteria and bacterial products (LPS, mycobacteria, CFA) [135,145,165,186,194,199].

Opn and innate immunity

Most infectious agents induce inflammatory responses by activating innate immunity in processes often involving macrophages and neutrophils. These cells are the professional phagocytes that provide the first line of defense of the immune system. Epithelial damage causes release of the cytokines IL-1 and IL-8, which recruit macrophages and neutrophils to the site of injury. Macrophages express toll like receptors that recognize pathogen-associated molecular patterns (PAMPs) and engulf the pathogen, leading to the secretion of cytokines/chemokines that attract cells such as neutrophils and monocytes to sites of injury or infection.

Opn's role in innate immunity is reflected in its protective function in infectious diseases. It contributes to the mucosal defense against viral pathogens. Significant up-regulation of Opn mRNA in murine intestine and epithelial cells was observed in rotavirus infection, and the disease was prolonged in Opn-deficient mice[200]. Likewise, Opn deficient mice are impaired in their ability to clear Listeria monocytogenes after systemic infection[200]. **Fig. 9** illustrates aspects of the involvement of OPN in immune responses.

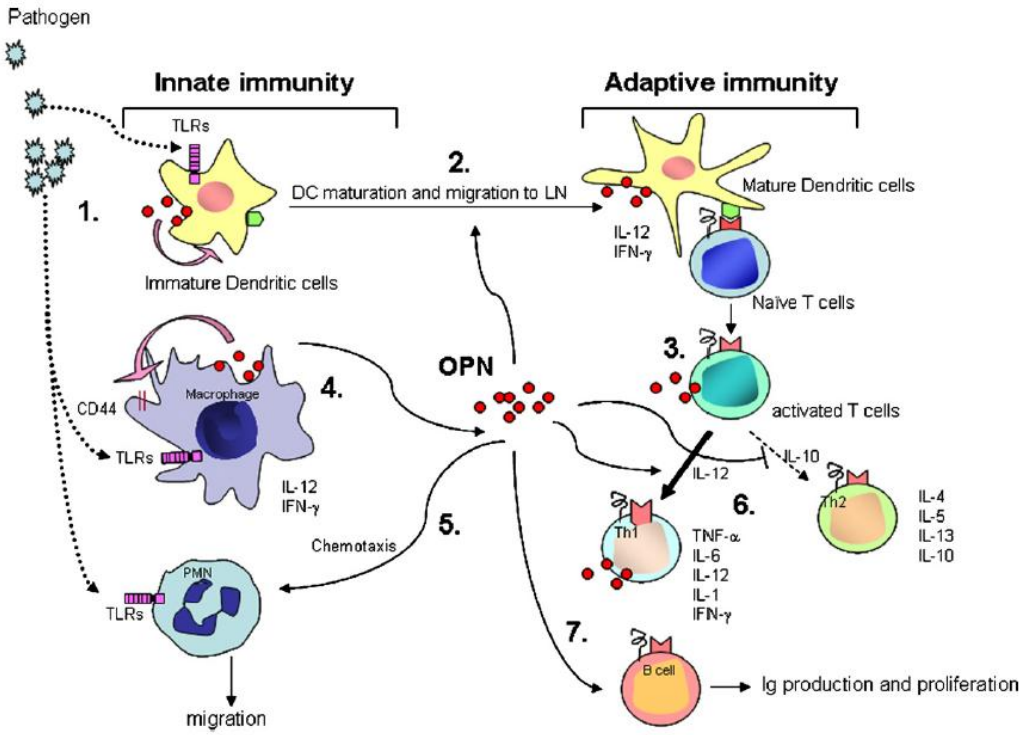


Figure 9. ***Opn's role in the regulation of innate and adaptive immunity.*** *(1) Exogenous pathogens activate TLRs on the surface of cells, including macrophages, neutrophils and immature dendritic cells. OPN is secreted by macrophages and dendritic cells when challenged by foreign antigens and enhances the activation and functions of these cells. OPN promotes neutrophil migration towards the site of injury. (2) OPN promotes immature dendritic cells to mature and migrate to draining lymph nodes, where they present processed antigens through the MHC to naı̈ve T cells and initiate a cell-mediated immune response. (3) Signals from DCs activate naı̈ve T cells and determine the polarization of T cells to Th1 or Th2 type cytokine responses. (4) Macrophages produce large amounts of OPN, which in an autocrine/paracrine manner contributes to the migration of macrophages and the expression of the pro-inflammatory cytokine IL-12. (5) OPN produced by various immune cells at inflammatory sites promotes infiltration of neutrophils. (6) Activated T cells are promoted by IL-12 to differentiate towards the Th1 type, producing Th1 cytokines (IL-12, IFNg). OPN inhibits production of the Th2 cytokine IL-10, which leads to an enhanced Th1 response. (7) OPN promotes B lymphocyte proliferation and immunoglobulin production.*

OPN and antimicrobial responses

The contribution of OPN to protective immunity against microbial infection was first suggested by findings that the Opn gene mapped to a locus (Ric) on mouse chromosome 5 that confers resistance to infection by Rickettsia tsutsugamushi, an obligate intracellular bacterium that causes human scrub typhus [147 201]. Subsequent studies suggested that Opn expression also contributes to protective immunity against several additional intracellular pathogens, including viruses, bacteria and protozoa [161,200,202-205]. Further evidence of the contribution of Opn to the protection against intracellular pathogens came from a study on Opn deficient mice, which were more susceptible to infection with Mycobacterium bovis than Opn-sufficient mice [202]. Although the expression of sOpn may also promote antimicrobial immunity through the suppression of the anti-inflammatory cytokine IL-10 [137], control of mycobacterial infection depends mainly on the induction of an IL-12 response [206]. Moreover, analysis of plasma from patients with tuberculosis revealed that high levels of sOpn correlated with increased levels of IL-12 [204-205], perhaps reflecting an interaction between sOpn and its receptor αvβ3 integrin, which can upregulate IL-12 secretion by APCs [161]. Opn expression (detected by immunohistochemistry) in patients with tuberculosis was also associated with efficient granuloma formation and inversely correlated with mycobacterial dissemination and disease mortality [203].

Analysis of Opn-deficient mice suggested that this protein also contributes to protection against other intracellular pathogens, including rotavirus [200], herpes simplex virus type 1 [137,170], Listeria monocytogenes [137] and Plasmodium falciparum [207].

Although protection against intracellular pathogens has generally been attributed to the expression of sOpn, protection against some of these microorganisms may also be the result of iOpn expression. Several studies using immunohistochemistry or flow cytometry showed that Opn was present in pathogen-infected cells [203,207]. However, additional investigation is required to determine whether these studies reflect the presence of iOpn, sOpn or both isoforms. The protective role of Opn against infection does not extend to all types of pathogen. For example, Opn deficiency does not attenuate resistance to some viruses, including influenza virus [208], or to the extracellular bacterium Borrelia burgdorferi in mice [209].

In the study examining the effect of Opn deficiency on the protection against viral infections [208], Opn-independent production of antiviral cytokines, including interferon-γ (IFN-γ), could have bypassed the need for Opn-dependent protection [210]. In the case of B. burgdorferi infection [209], the pathogen inocula that were used in this study might have been insufficient to induce Opn-dependent protection, as suppression of IL-10 by Opn, which results in enhanced resistance to B. burgdorferi [211], may require higher titres of this bacterium. In addition to sOpn-dependent induction of the pro-inflammatory cytokine IL-12 [137], iOPN expression by plasmacytoid DCs (pDCs) can enhance T_H1-cell responses through the upregulation of IFNγ expression by these cells following viral infection [170]. Recent studies also showed that iOpn expression by conventional DCs (cDCs) induces the development of T_H17 cells [212] (see below). However, studies on T_H17-cell protective immune responses that are mediated by Opn have not yet been conducted.

Opn and macrophages

Monocytes express a low level of Opn, but as they differentiate into macrophages Opn expression is increased; it becomes constitutively expressed in macrophages and can be further up-regulated by LPS stimulation [213]. Opn has been shown to regulate macrophage functions including migration [169], activation [214], phagocytosis [215], proinflammatory cytokine production [216] and nitric oxide synthesis [217] in response to various inflammatory challenges. In the absence of Opn expression, macrophage migration and cytokine production are impaired [218]. In macrophages Opn both regulates the distribution of CD44 [169] and, as iOpn, co-localizes with CD44 at the inner surface of the plasma membrane (**Fig. 8**). Opn-deficient macrophages have a more diffuse distribution of CD44, suggesting that CD44 cooperates with Opn in regulating macrophage migration. At sites of tissue injury, Opn produced by macrophages fosters cell adhesion and may act as an opsonin facilitating phagocytosis of foreign bodies [215]. PDGF released by platelets and macrophages (or mast cells) at sites of injury stimulates mouse skin fibroblasts to produce Opn, which hinders wound repair and enhances scarring [219]. It has been demonstrated in macrophages that a phosphorylation dependent interaction between the amino-terminal thrombin fragment of Opn and its integrin receptor stimulated IL-12 expression, whereas a

phosphorylation-independent interaction of the C-terminal half with CD44 inhibited IL-10 expression [137]. The implication is that the interaction of Opn with integrins and CD44(v) stimulates via different signal transduction pathways distinct patterns of cytokine/chemokine expression and the specific immune response.

Opn and neutrophils

In contrast to macrophages, few studies have reported on Opn expression by neutrophils. Neutrophils (the majority of the polymorphonuclear leukocytes) are the primary responders to exogenous and endogenous stimuli and rapidly infiltrate the injured tissue to initiate an innate host defense. Opn is a chemoattractant for neutrophils [220-222].

Activated neutrophils release proteases, reactive oxygen and nitrogen species, and cytotoxic mediators that in combination with phagocytosis eliminate invading infectious agents. However, cytotoxic products released by neutrophils may also cause host tissue destruction such as observed in inflammatory bowel disease [223]. Opn has been found to play an important role in neutrophil recruitment in alcoholic liver disease [220,224]. The expression and localization of CD44 in neutrophils is independent of Opn, although CD44 is required for the polarization and chemotaxis of neutrophils [225]. Opn is important for the migration of neutrophils *in vitro* [222]. However, Opn does not appear to be involved in neutrophil activities such as phagocytosis, superoxide release, or the production of cytokines and proteases.

Osteopontin and dendritic cells

Opn is highly expressed in immature DCs but production decreases during maturation, because treatment with an anti-Opn antibody blocked LPS induced human DC maturation it was suggested that Opn acts in an autocrine and/or paracrine manner to induce DC maturation [226]. As it is for other inflammatory cells, Opn is also a survival factor for DCs. Blockade of Opn with anti-Opn antibody reduced expression of co-stimulatory and class II molecules by human monocyte-derived DCs and promoted apoptosis of mature DCs [226]. Opn was implicated in DC migration in studies

demonstrating that Opn initiates Langerhans cell/DC emigration from the epidermis and attracts them to draining lymph nodes by interacting with CD44 and av integrin [221]. Opn-deficient mice exhibit a significantly reduced contact hypersensitivity reaction possibly due to an impaired ability to attract Langerhans cells/DCs to draining lymph nodes. Opn-activated DCs produce IL-12 and TNF-a, and when incubated with naive T cells, can induce naive T cells into T_H1-polarizing, IFN-g-producing T cells [227]. Opn's presence in T cell-DC interactions may decisively influence T-cell polarization. This concept is emphasized by the deficiency of T_H1 immunity in Opn deficient mice, which leads to a compromised host defense against bacterial and viral infections [200,216] and ameliorated autoimmunity [138].

Opn and cell-mediated immunity

Elevated Opn expression is found in various immunological disorders. Up-regulation of Opn is implicated in the formation of granulomas in tuberculosis, sarcoidosis and silicosis, all of which involve cell-mediated immune reactions [149,186]. Studies of $Opn^{-/-}$ mice revealed that Opn's main immune-modulatory function is the enhancement of T_H1 over T_H2 immunity through two general mechanisms: regulation of cytokine production via 1) interaction with the avb3 integrin to induce pro-inflammatory IL-12 and 2) ligation of CD44v to suppress anti-inflammatory cytokine IL-10 production [137]. Autocrine amplification of cytokine production and cross-inhibition ensure progressive polarization of the T-helper cells towards either T_H1 or T_H2 cells [228]. Opn is produced by activated T cells and is classified as a T_H1 cytokine because it modulates cell-mediated immunity by promoting the T_H1 response. In $CD4^+$ T-cells, Opn mRNA is expressed in T_H1, but not T_H2, polarized cells [229].
Moreover, soluble Opn may modulate the differentiation and proliferation of $CD4^+$ and $CD8^+$ T cells [230]. Opn gene expression in activated T cells, but not macrophages, is regulated by T-bet, a transcription factor that controls $CD4^+$ T_H1 cell lineage commitment. T-bet-dependent expression of Opn in T cells is essential for efficient skewing of $CD4^+$ and $CD8^+$ T cells toward the T_H1 and type 1 $CD8^+$ T cells (Tc1) pathway [230]. Opn can also stimulate human peripheral blood T cells to express IFN-g and CD40L, which subsequently induce IL-12 expression from monocytes. Enhanced expression of CD40L by T cells may explain the ability of Opn to induce B-cell

proliferation and antibody production [231]. At low concentrations, Opn promotes chemotaxis but not chemokinesis of T cells, while activated T-cell adhesion is enhanced at high Opn concentrations, especially following cleavage of Opn by thrombin [185]. The formation of granulation tissue and the intensity of inflammatory reactions are dramatically reduced in the absence of Opn expression [232].

Recent evidence indicates that plasma Opn levels increase in active Crohn's disease compared with quiescent disease and declined after treatment with the anti-TNF-a monoclonal antibody infliximab [233]. Furthermore, the increase of Opn levels was inversely correlated with in vitro T-cell IL-10 production. Opn's reciprocal effects on IL-12 and IL-10 expression result in a two-phase action in regulating immune responses in several immune disorders [176,234]. Opn regulates cell mediated immunity by promoting T cell proliferation and migration as demonstrated in transgenic mice over-expressing Opn. These mice also exhibited an increase in the percentage of $CD4^+$ cells in lymph nodes [235]. Upon stimulation with 2,4-dinitrofluorobenzene (DNFB, a substance that evokes delayed-type hypersensitivity in mice) increased $CD8^+$ T cell infiltration and enhanced contact hypersensitivity were observed in the Opn transgenic mice.

While Opn is generally classified as a pro-inflammatory cytokine, it appears to have anti-inflammatory effects in certain pathological contexts. The multifunctional nature of Opn may reflect expression of specific isoforms, levels or timing of production and tissue distribution. A two-phase role of Opn in immune regulation has been revealed in several studies exploring Opn's action in various immune diseases. Upregulated Opn expression in the early phase of lpr disease (a mutation in the lpr gene leads to a phenotype resembling systemic lupus erythematosus) elicited polyclonal B-cell activation that was dependent on Opn-stimulated IL-12 and IFN-g production [236]. However, during the late stage Opn limited further exacerbation through inhibition of T_H2 cytokine dependent polyclonal B cell activation.

Dual (pro- vs. anti-inflammatory stage) effects of Opn in colitis have been reported. Crohn's disease is characterized as a T_H1-directed immune response with increased $CD4^+$ T cell production of IFN-g and activation of macrophages that secrete TNF-a and IL-12. In contrast, ulcerative colitis is associated with an atypical T_H2 response mediated by a distinct subset of NKT cells that produce IL-13 and are cytotoxic for epithelial cells. Compared to control mice, $Opn^{-/-}$ mice are more susceptible to acute colitis but resistant to chronic colitis. Elevated plasma Opn levels mediate sustained

inflammation in active disease whereas reduced plasma Opn results in less inflammation and stimulates mucosal repair [237]]. Opn plasma concentrations correlate with the severity of Crohn's disease, consistent with a role for Opn in macrophage/dendritic and T_H1-mediated immune responses [237-238]. Opn is anti-inflammatory in the acute phase but pro-inflammatory in the chronic phase of colitis. The detrimental effects of Opn associated with a protracted T_H1 immune response contrasts with the protective effects as shown in dextran sodium sulfate-induced acute colitis in $OPN^{-/-}$ mice, which show an exacerbated disease [223]. The inflammatory destruction seen in the acute colitis in $Opn^{-/-}$ mice is a result of a prolonged innate immune response (neutrophils), which compensates for the impaired adaptive and specific T_H1 immune defense.

Although there is mounting evidence demonstrating Opn's impact on T_H1 immunity against viral, bacterial and self-antigens, contradictory reports on the role of Opn have been reported. Elevation of Opn levels is believed to contribute to mononuclear cell infiltration and renal injury in murine anti-glomerular basement membrane nephritis; however, it does not significantly contribute to the glomerular and tubulointerstitial mononuclear cell infiltration in an *in vivo* murine model of glomerulonephritis [239]. Opn has been found to suppress bacterial antigen-specific T cell proliferation and DC migration when Opn is supplied together with heat-killed Listeria monocytogenes [240]. In another study, when $Opn^{-/-}$ mice were analyzed after influenza infection in the lung, viral clearance, lung inflammation, and recruitment of effector T cells to the lung were comparable to Opn+/+ mice [208]. These discrepancies may be due to variations in the genetic background of knockout mice used in different studies or to the pleiotropic nature of Opn, which can not only interact with multiple receptors but also activate different and sometimes conflicting, signaling pathways.

Osteopontin and autoimmunity

It has been suggested that Opn is an autoantigen for certain autoimmune diseases such as osteoarthritis and rheumatoid arthritis [241-242]. As demonstrated in the collagen-induced arthritis model of rheumatoid arthritis, Opn recruits inflammatory cells to arthritic joints. The levels of thrombin-cleaved Opn are significantly increased in the

plasma and synovial fluid of rheumatoid arthritis patients compared with plasma from healthy controls and from patients with osteoarthritis; this may be important in that the cleaved Opn is able to engage additional integrins [243]. Interestingly, splenic monocytes expressing a4 and a9 integrins from arthritic mice demonstrated enhanced migration toward thrombin-cleaved Opn compared with splenic monocytes from non-arthritic mice. Furthermore, treatment of arthritic mice with an antibody (M5 Ab) directed against the sequence SLAYGLR, exposed by thrombin cleavage of murine Opn, inhibited synovitis and inflammatory cell infiltration into the joints of treated mice compared with those of arthritic control mice.

Opn's association with multiple sclerosis has been studied intensively. Opn has been identified as the most prominent cytokine expressed within multiple sclerosis (MS) lesions. The plaques found in human multiple sclerosis patients have a fivefold increase in Opn transcripts [138]. Significantly increased Opn protein levels were found in the plasma of relapsing-remitting MS patients whereas the Opn protein levels in primary progressive and secondary progressive MS patients were similar to healthy control levels [244]. In the mouse experimental autoimmune encephalomyelitis (EAE) model, administration of Opn to $Opn^{-/-}$ mice induced recurrent relapses, worsening paralysis and neurological deficits. Reactive astrocytes as well as inflammatory cells are major sources of Opn in rat EAE and its effect seems to be mediated by CD44 [245]. It has been suggested that the mechanism of action of Opn involves promoting the survival of activated myelin-reactive T-cells, which leads to relapse and progression of MS [176]. Increased levels of Opn transcripts were reported in both the brain tissue of MS patients and the spinal cords of an EAE rat model [138]. In contrast, in autoimmune myocarditis it appears that neither Opn nor CD44v6/v7 play any role in the development of the disease [246].

Highly elevated expression of Opn at the site of disease and in plasma is observed in autoimmune diseases. Importantly, the resistance to spontaneous remission (clinical relapse) and the attenuated disease phenotype in $Opn^{-/-}$ mice suggests a role for Opn as a potentially critical factor in the pathogenesis of autoimmunity. Opn deficiency is linked to a reduced T_H1 immune response in autoimmunity. The $Opn^{-/-}$ mice are resistant to progressive EAE and have less frequent remissions [247]. Additionally, $Opn^{-/-}$ mice exhibit slower progression of experimental autoimmune uveoretinitis [248]. Polymorphisms in the gene encoding Opn have been linked to the development of systemic lupus erythematosus and multiple sclerosis [249-250]. Opn plays a two-phased-

role in lupus. Early in the disease, Opn enhances the pathology by inducing polyclonal B cell activation and isotype switching [251-252] that substantially elevate IgG, IgM and IgA levels. During the late phase, lupus is characterized by T_H2 cytokine-induced pathogenic autoantibody production. Opn limits further exacerbation by inhibiting T_H2-induced IL-10/IL-4 production [236].

NKT cells are critical regulators of autoimmune responses. Opn is secreted by NKT and augments NKT cell activation, triggering neutrophil infiltration and activation. Both Opn-null mice and NKT deficient mice are resistant to concanavalin A-induced hepatitis [253]. Furthermore, a neutralizing antibody specific for a cryptic integrin-binding epitope of Opn exposed by thrombin cleavage ameliorated hepatitis, indicating that the thrombin cleaved form of Opn is involved in inflammatory process through NKT cells and neutrophil infiltration and leads to tissue damage in Con-A-induced hepatitis.

iOpn in pDCs and T_H1-cell responses.

Although all nucleated cells can produce type I IFNs, pDCs are specialized cells that rapidly secrete high levels of type I IFNs following viral stimulation. Binding of type I IFNs to the type I IFN receptor (IFNAR) [254] upregulates IFNα production. Therefore, this pathway favours the induction and maintenance of T_H1-cell responses [255-256].

Engagement of TLR9 results in T-betdependent expression of iOpn in pDCs [170]. Association of nascent iOpn with MyD88 results in the activation of the transcription factor IFN-regulatory factor 7 (IRF7) and induction of Ifna expression [170]. Although TLR9-dependent activation of MyD88 can also activate the transcription factor nuclear factor-kB (NF-kB) and induce the expression of pro-inflammatory cytokines, including IL-6 and tumour-necrosis factor (TNF), iOpn has been shown to selectively couple TLR9 activation with that of IRF7 instead of NF-kB [170].

Some of the functional consequences of this iOpn-dependent pathway for IFNα production in pDCs have been delineated. iOpn expression by pDCs is essential for IFNα-dependent antigen cross-presentation to CD8+ T cells, resistance to infection by

herpes simplex virus type 1 and induction of natural-killer cell cytotoxicity that protects mice from the lethal effects of B16 melanoma [170]. These findings are consistent with the view that iOpn expression by pDCs is essential for IFNα-dependent immunity and T_H1-cell responses in vivo.

iOpn in cDCs and T_H17-cell responses.

T_H1 and T_H2 cells enhance the clearance of intracellular bacteria and viruses or parasites, respectively. A recently defined third subset of T_H cells that produce IL-17 — termed T_H17 cells — can protect the host from infection by extracellular bacteria and fungi, including clinically important pathogens such as B. burgdorferi and Candida albicans [257]. However, IL-17 can also promote tissue damage, and uncontrolled T_H17-cell responses are thought to contribute to several human autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, Crohn's disease, ulcerative colitis and SLE [258-263].

Recent studies have shown that iOpn expression by cDCs has a key role in promoting T_H17-cell responses. iOpn suppresses the expression of IL-27, which is known to inhibit the development of T_H17 cells [212]. In addition, Opn-deficient cDCs secrete high levels of IL-27 and therefore attenuate the T_H17-cell response. This suggests that iOpn releases the 'brake' on the T_H17-cell responses by suppressing IL-27 [263-264]. Whether sOpn expression by activated T cells also participates in the generation or maintenance of the T_H17-cell response is currently being investigated.

Furthermore, engagement of IFNAR has been shown to suppress the expression of iOpn, which leads to enhanced secretion of IL-27 and, consequently, to attenuated T_H17-cell responses11,53. In addition, engagement of IFNAR following viral infection results in an IL-27-dependent blockade of T_H17-cell responses [212]. This effect may prevent unwanted or excessive inflammatory responses during viral infection, without compromising the protective antiviral immunity that is mediated by pDCs and T_H1 cells. T_H17-cell responses may be further inhibited by the increased iOpn-dependent secretion of type I IFNs by pDCs following TLR7 or TLR9 activation by viral RNA or DNA, which would activate IFNAR on the surface of

cDCs (**Fig. 10**). Therefore, this interaction between the two main DC subsets may regulate the level of activation of T_H-cell subsets.

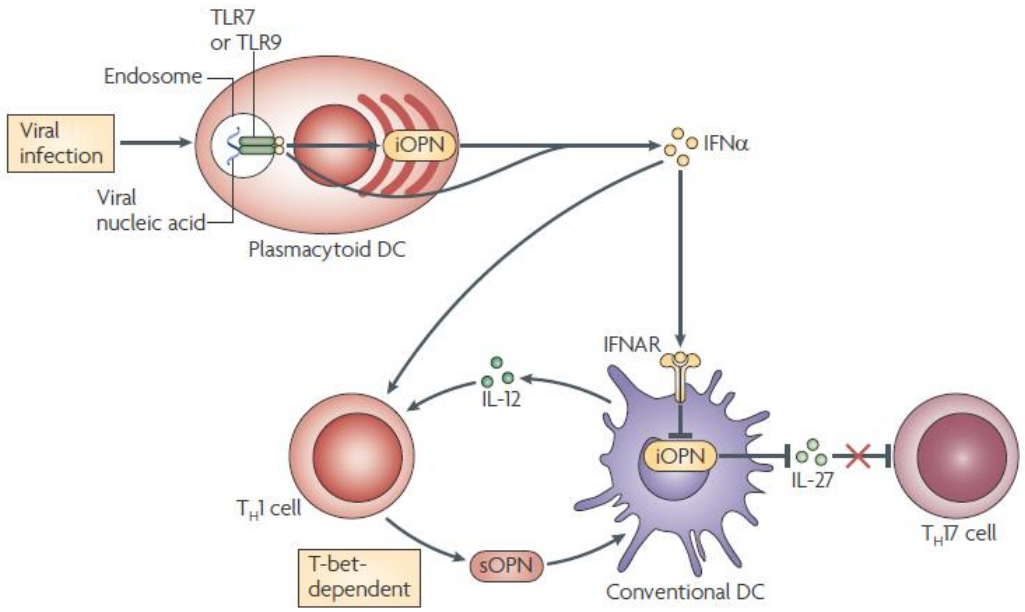


Figure 10. ***T-helper-cell polarization by osteopontin isoforms expressed by different cell types.*** *The expression of the intracellular isoform of osteopontin (iOpn) is induced in plasmacytoid dendritic cells (pDCs) following ligation of Toll-like receptor 7 (TLR7) or TLR9, for example, during viral infection, and results in enhanced production of interferon-α (IFNα)*[170]*. This production of IFNα by pDCs contributes to T helper 1 (T_H1)-cell responses during viral infection. Activated T cells produce high levels of the secreted isoform of osteopontin (sOpn), which is under the control of the transcription factor T-bet* [230]*. This triggers T_H1-cell responses through the induction of secretion of pro-inflammatory cytokines, including interleukin-12 (IL-12), by antigen-presenting cells* [137]*. iOpn expressed by conventional DCs induces T_H17-cell responses by blocking the expression of IL-27, which suppresses the development of T_H17 cells. Type I IFN receptor (IFNAR) engagement suppresses the expression of iOpn, thereby removing the 'brake' on IL-27 expression and, consequently, on attenuated T_H17-cell responses* [212]*.*

Opn in the central nervous system

The identification of a potential role for Opn in the pathogenesis of multiple sclerosis came from findings that plaques from the brain and spinal cords of rats with experimental autoimmune encephalomyelitis (EAE) contained high levels of Opn transcripts [138] and that Opn-deficient mice showed decreased development of the disease [138,247]. The initial concern that other genes linked to the *Opn* locus might be responsible for the second finding proved to be unfounded [265], as administration of recombinant Opn to wild-type mice exacerbated disease [176], and the attenuated phenotype that was observed in Opn deficient mice was still apparent after 16 generations of backcrossing [230].

Both sOpn and iOpn have been shown to regulate EAE development. T-cell-derived sOpn can inhibit apoptosis of pathogenic T cells, thereby sustaining T-cell pathogenicity during the relapse and progression of EAE [176]. In addition, the expression of iOpn by APCs, rather than T cells, regulates the onset of EAE[212] and the generation of proinflammatory T_H17 cells, as determined by immunization with antigen-pulsed DCs that differ in their expression of iOpn [212]. These findings suggest that iOpn expression by DCs exacerbates EAE development by promoting the T_H17-cell response, whereas sOpn may sustain disease by dampening apoptosis of pathogenic T cells (**Fig. 11**).

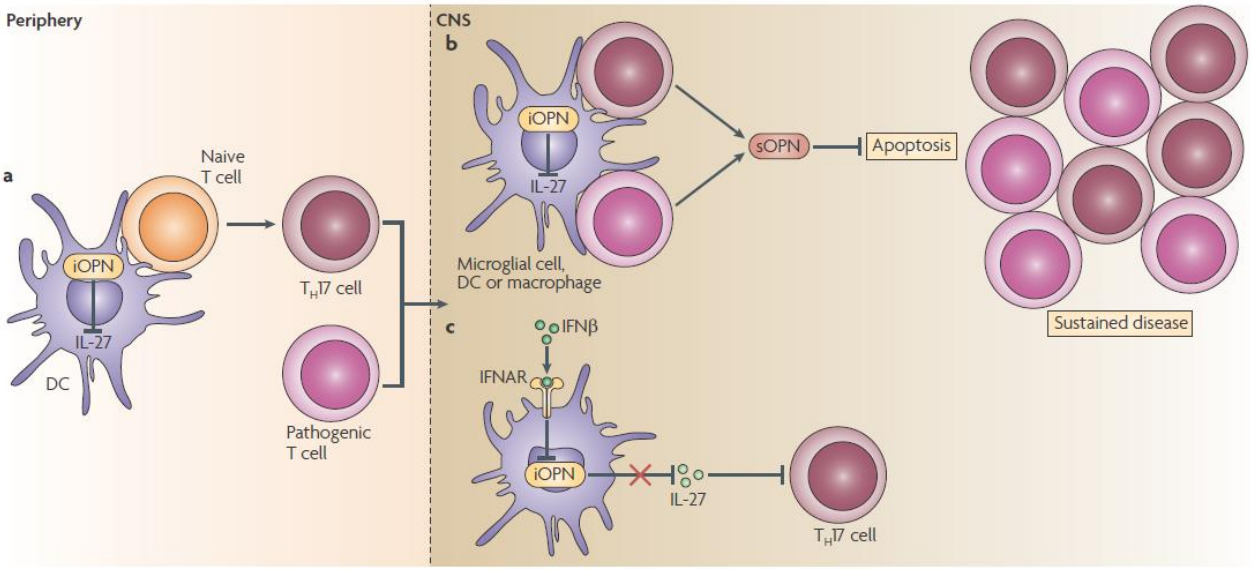


Figure 11. ***Osteopontin-mediated regulation of pathogenic CD4$^+$ T cells in experimental autoimmune encephalomyelitis.*** *a) Dendritic cells (DCs) activate naive CD4$^+$ T cells following the presentation of myelin antigen in peripheral lymphoid tissues. Inhibition of the expression of interleukin-27 (IL-27) by the intracellular isoform of osteopontin (iOpn) releases the 'brake' on T helper 17 (T_H17)-cell development, resulting in a robust T_H17-cell response [212]. b) Activated T cells that migrate to the central nervous system (CNS) are re-stimulated by microglial cells, DCs and macrophages. Expression of iOpn by these antigen-presenting cells further enhances the T_H17-cell response. At this stage, activated T cells produce high levels of the secreted Opn isoform (sOpn) [230], which promotes the survival of pathogenic T_H1 and T_H17 cells through the inhibition of apoptosis, resulting in sustained disease [176]. c) Induction of type I interferon receptor (IFNAR)-mediated signalling in APCs following the administration of interferon-β (IFNβ) could result in the suppression of iOpn expression and upregulation of IL-27, which attenuates T_H17-cell responses and, therefore, could inhibit the development of inflammatory disease in the CNS.*

Objective of the PhD thesis

Activation and differentiation of T_H immunity depends on interactions of T_H cells with antigen presenting cells, such as dendritic cells (DCs), and cytokines play a critical role in this process. Opn is a cytokine originally identified as the predominant transcript expressed by activated T cells. Previous studies were focused on the role of Opn during T_H1 viral and autoimmune processes where responses were ongoing via repetitive antigenic encounter [137,247]. However, the effect of this cytokine during primary versus secondary antigenic encounters remains unclear. Moreover, the role of Opn in T_H2-mediated allergic responses, a rising health issue in industrialized countries [266], has not been elucidated. Thus, we investigated the *in vivo* effects of Opn-s in distinct phases of a T_H2 immune response and subsequent disease development, using an established mouse model of ovalbumin (OVA)-induced allergic airway inflammation [36]. We also examined whether the role of Opn-s was mediated by effects on DC subsets. By comparing the results obtained upon neutralization of Opn-s with those from *Opn*$^{-/-}$ mice, we studied the immuno-regulatory activity of the Opn isoforms in T_H2 allergic responses and disease phenotype.

As the incidence of allergic disease has risen dramatically, much effort has been put in determining the control mechanisms of peripheral tolerance to allergens. Regulatory T cells and dendritic cells (DC) are important suppressors of dysregulated T_H2 responses to inhaled antigens. Although, recent data suggest that Opn is highly expressed in activated Foxp3$^+$ T_{reg} cell, and is important for regulating aspects of DC biology, it has not been addressed if and how Opn impacts on peripheral tolerance. Thus, we investigated the *in vivo* role of Opn in peripheral tolerance induction to the model antigen ovalbumin (OVA), using an established OVA-mouse model of asthma as the readout system.

RESULTS

CHAPTER 1

A critical role for osteopontin in allergic airway disease: regulation of dendritic cell subsets

SUMMARY

Osteopontin (Opn) is important for T helper type 1 (T_H1) immunity and autoimmunity. However, the role of this cytokine in T_H2-mediated allergic disease, as well as its effects on primary versus secondary antigenic encounters, remain unclear. Here we demonstrate that OPN is expressed in the lungs of asthmatic individuals and Opn-s, the secreted form of Opn, exerts opposing effects on mouse T_H2 effector responses and subsequent allergic airway disease: pro-inflammatory at primary systemic sensitization, and anti-inflammatory during secondary pulmonary antigenic challenge. These opposing effects of Opn-s are mainly mediated by regulation of T_H2-suppressing plasmacytoid dendritic cells (DCs) during primary sensitization and T_H2-promoting conventional DCs during secondary antigenic challenge. Therapeutic administration of recombinant Opn during pulmonary secondary antigenic challenge decreased established T_H2 responses and protected from allergic disease. These novel effects on T_H2 allergic responses place Opn-s as an important therapeutic target and provide new insight into its role in immunity.

INTRODUCTION

Immunity against pathogens is mediated through induction of antigen-specific T helper (T_H)1 and T_H2 lymphocytes. T_H1 immunity confers protection against intracellular pathogens and when excessive can lead to autoimmunity [267-268]. Aberrant T_H2-cell activation against environmental antigens may induce allergy and asthma [269]. Activation and differentiation of T_H immunity depends on interactions of T_H cells with antigen presenting cells, such as dendritic cells (DCs), and cytokines play a critical role in this process.

Opn is a cytokine originally identified as the predominant transcript expressed by activated T cells [136,270]. Opn-deficient (*Opn*$^{-/-}$) mice exhibit reduced immunity to viruses [137] and other microorganisms [271], develop milder experimental autoimmune encephalomyelitis [138,176,247] and are resistant to development of autoimmune keratitis [137], all T_H1-linked responses. Increased OPN expression was also shown in affected tissues from individuals with rheumatoid arthritis, Crohn disease, and multiple sclerosis [176,272-273]. Also, *OPN* polymorphisms have been linked to the development of systemic lupus erythematosus and multiple sclerosis [249-250], suggesting a role in autoimmunity.

An important recent study has demonstrated that the intracellular form of Opn (Opn-i) is essential for IFN-α production by plasmacytoid DCs (pDCs) upon viral infection or CpG-oligonucleotide administration [170]. Additionally, recombinant Opn (rOpn) induces maturation of T_H1-polarizing human DCs *in vitro* [227], and blockade of Opn-s reduces co-stimulatory and Class II molecule expression on human monocyte-derived DCs [226]. Moreover, *Opn*$^{-/-}$ mice exhibit reduced trinitro-chloro-benzene-induced migration of DCs to draining lymph nodes (DLNs) [221]. In contrast, rOpn administration inhibits bacterial-induced DC migration [240]. Opn-i and Opn-s can therefore affect DC functions, crucial in determining the outcome of adaptive immunity.

Previous studies were focused on the role of Opn during T_H1 viral and autoimmune processes where responses were ongoing via repetitive antigenic encounter [137,247]. However, the effect of this cytokine during primary versus secondary antigenic

encounters remains unclear. Moreover, the role of Opn in T_H2-mediated allergic responses, a rising health issue in industrialized countries [266], has not been elucidated. Thus, we investigated the *in vivo* effects of Opn-s in distinct phases of a T_H2 immune response and subsequent disease development, using an established mouse model of ovalbumin (OVA)-induced allergic airway inflammation [36]. We also examined whether the role of Opn-s was mediated by effects on DC subsets. By comparing the results obtained upon neutralization of Opn-s with those from $Opn^{-/-}$ mice, we studied the immuno-regulatory activity of the Opn isoforms in T_H2 allergic responses and disease phenotype.

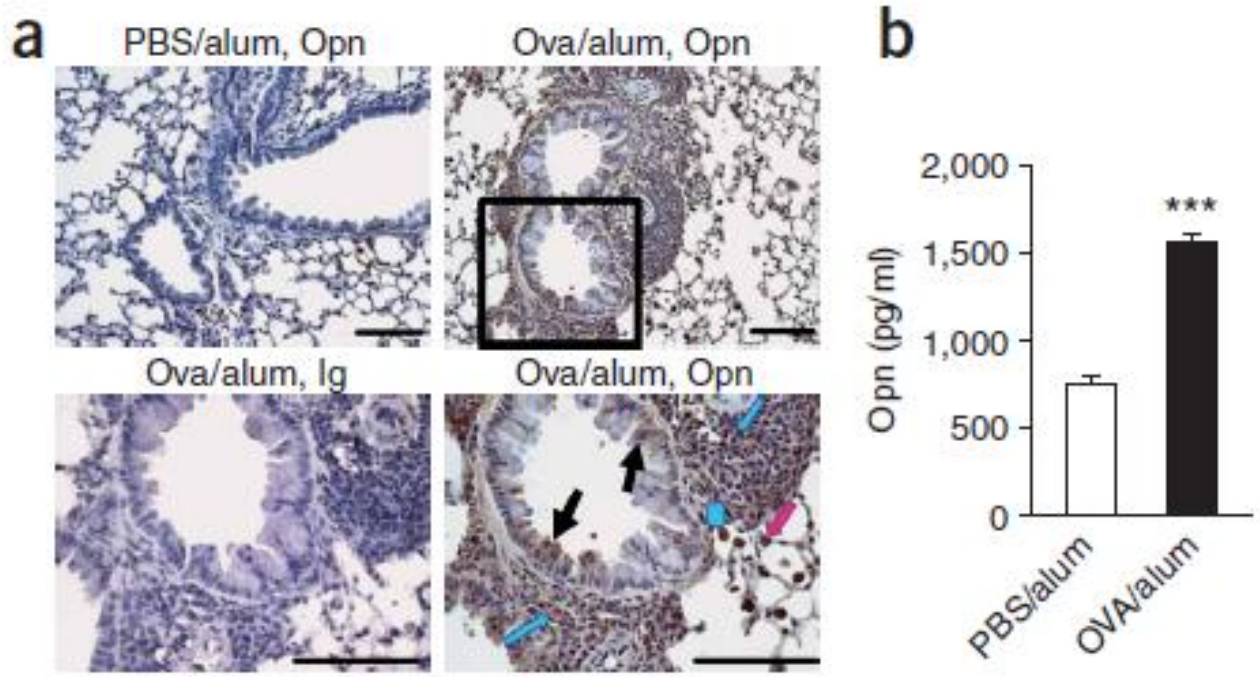
RESULTS

Increased lung Opn expression in allergic disease

We investigated Opn expression during allergic T_H2 responses, using a mouse model of airway inflammation induced by OVA-alum sensitization followed by airway OVA challenges. There was upregulation of lung Opn expression in OVA-alum sensitized as compared to PBS-alum sensitized mice (alum controls) (**Fig. 1a**), localized mainly at sites of leukocytic infiltration and in bronchial and alveolar epithelial cells. Opn was also increased in lung homogenates from OVA-sensitized mice (**Fig. 1b**).

Lung biopsies from asthmatics had increased OPN expression by bronchial epithelial cells (ciliated epithelium) and inflammatory cells underneath the subepithelial membrane as compared to healthy subjects (**Fig. 1c**). There were increased percentages of OPN-positive epithelial and subepithelial cells (**Fig. 1d**)

Figure 1



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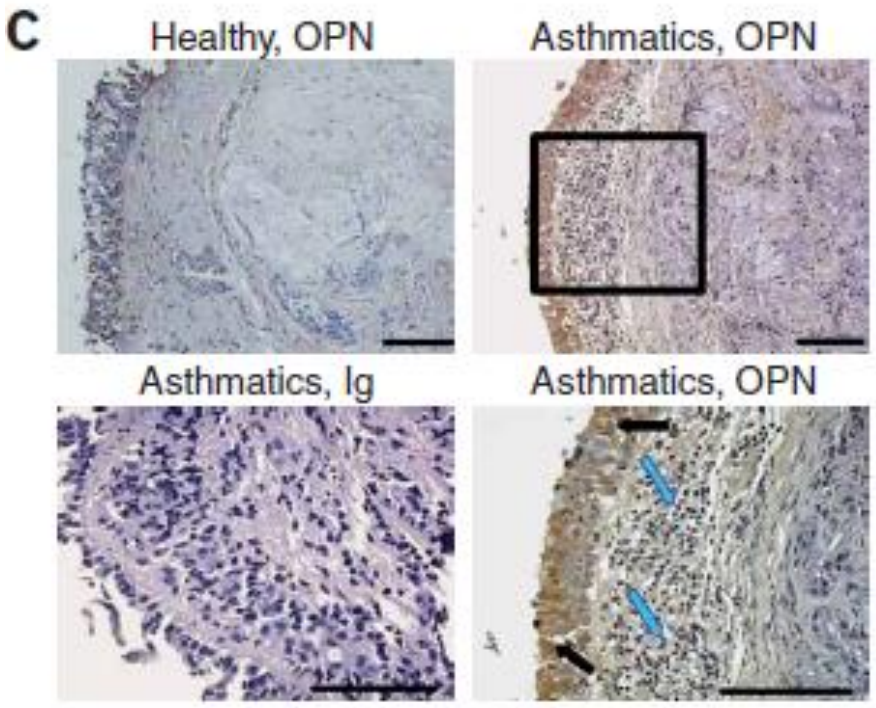


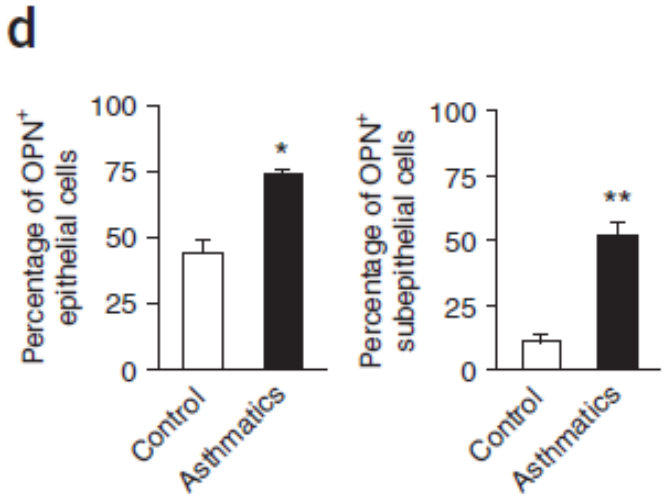
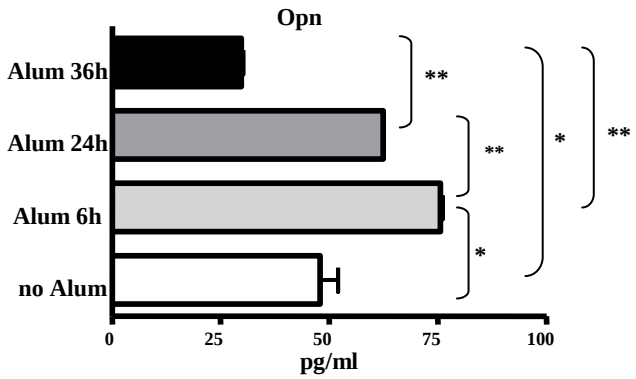


Figure 1 Opn lung expression in allergic airway disease (**a**) Photomicrograph of lung sections from PBS/alum-sensitized (alum controls, upper left panel) and OVA/alum-

sensitized BALB/c mice (upper right panel) stained with Opn-specific antibody. Immunized mice received also three challenges with aerosolized OVA. Opn was expressed by infiltrating leukocytes (white arrows), including macrophages (arrowhead), by bronchial epithelial cells (black arrows) and alveolar epithelial cells (grey arrow) (lower right panel). Ig control staining of a section from OVA/alum-sensitized mice is shown (lower left panel). Specific staining is depicted in brown while the nuclei are stained blue with haematoxylin. Bar represents 100μm. (**b**) Opn levels in OVA/alum-sensitized (black bars) or alum controls (white bars) lung homogenates are shown. Data are expressed as mean ± SEM; n = 6–8 mice/group in two independent experiments (***P = 0.0025). (**c**) Photomicrograph of OPN expression in bronchial biopsies from healthy individuals (upper left panel) and asthmatics (upper right panel) stained with Opn-specific antibody. OPN expression in the asthmatics was localized in bronchial epithelial cells (black arrows) and subepithelial infiltrating leukocytes (white arrows) (lower right panel). Ig control staining of a biopsy from an asthmatic is shown (lower left panel). Bar represents 100μm. (**d**) Percentages of OPN^+ epithelial and subepithelial cells from lung biopsies from asthmatics (black bars) or healthy individuals (white bars) are shown. Cell counts are expressed as mean ± SEM and were obtained from three high power fields (x 400) from each biopsy. Statistical significance was obtained by unpaired Student's t-test, (**P = 0.0033, epithelial cells) (**P = 0.0071, subepithelial cells).

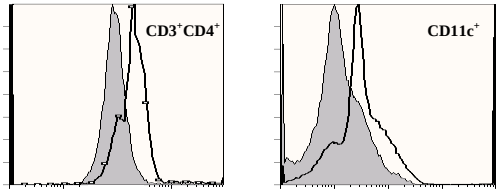
In order to address whether alum induces Opn's expression in the draining lymph nodes (LNs), we immunized BALB/c mice with alum alone (i.p.), isolated DLNs (inguinal, axillary) at different time points (6h, 24h, 36h) and measured Opn expression in homogenates by ELISA and also in single cell suspension by FACS intracellular staining. Our data showed an increase in Opn expression 6h after alum immunization as compared to naïve mice. Opn levels at 24h and 36h, dropped to those of the naïve mice, as seen below.



Alum immunization induces Opn expression in LNs from BALB/c mice. Opn levels were measured by ELISA in DLN homogenates of BALB/c mice at different time points following Alum i.p. immunization (no alum vs 6h *p = 0.0220, no alum vs 36h *p = 0.0491, no alum vs 24h p = 0.0728, alum 6h vs alum 24h **p = 0.0014, alum 6h vs alum 36h **p = 0.0002, alum 24h vs alum 36h **p = 0.0002).

To adrees which cells in the draining LNs awere expressing Opn we performed intracellular FACS staining. This experiment showed that Opn expression was slightly upregulated in $CD3^+CD4^+$ T cells and $CD11c^+$ cells in alum only immunized (i.p.) BALB/c mice.

Furthermore, OVA airway challenge of sensitized mice induced an increase (approximately 2.5-fold) in Opn expression in the lungs (and DLNs) as compared to alum-only sensitized mice (**Fig. 1**). Thus, Opn expression is upregulated in Th2-inducing protocols of immunization and is further increased during antigen-specific recall immune responses and allergic disease.



Representative FACS plots showing Opn expression by $CD3^+CD4^+$ T cells and $CD11c^+$ DCs from LNs of naïve BALB/c mice (filled histogram) and BALB/c mice 6h following Alum (i.p.) immunization (open histogram).

Endogenous Opn-s is pro-inflammatory at sensitization

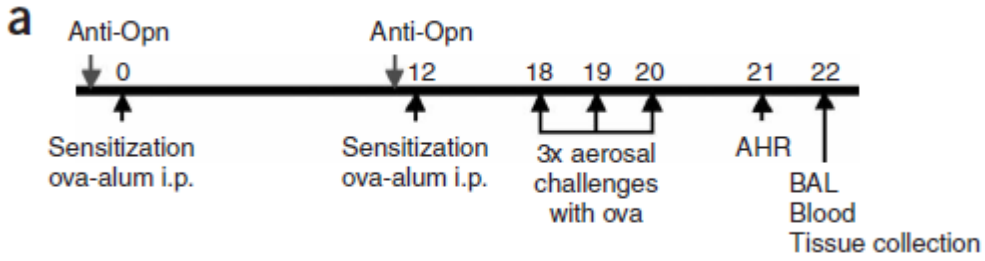
To investigate whether Opn-s participates during the induction of a T_H2 response, we administered a neutralizing antibody to Opn (or isotype (Ig) control) before OVA-alum sensitization (**Fig. 2a**). Following subsequent challenge through the airways with OVA, these mice exhibited decreased BAL eosinophils, lymphomononuclear cells (**Fig. 2b**) and airway hyper-responsiveness (AHR), as compared to Ig-treated mice, reaching levels similar to those of alum controls (**Fig. 2c**). Lung leukocytic infiltration and mucus secretion were also decreased (**Fig. 2d**), accompanied by a decrease in the eosinophil-specific chemokine, CCL11, in lungs (**Fig. 2e**).

Lung IL-4, IL-13 and IL-10 levels were decreased in mice treated with Opn-specific antibody (**Fig. 2e**). IL-12 -a T_H1 cytokine produced by DCs, macrophages and airway epithelial cells [274-275]- levels were also decreased (**Fig. 2e**). We attribute these decreases to the overall decreased pulmonary inflammation. Cytokine levels in BAL exhibited similar patterns (data not shown).

We examined OVA-specific T_H2 responses by measuring cytokine levels in supernatants of draining lymph node (DLN) cell cultures stimulated *ex vivo* with OVA. Treatment with Opn-specific antibody resulted in decreased IL-4, IL-13 and IL-10 levels (**Fig. 2f**). OVA-specific -IgG1, -IgG2a and -IgE levels were decreased in Opn-specific antibody-treated mice (**Fig. 2f**).

We observed decreased percentages of T1/ST2$^+$ T_H2 cells in lung DLNs of Opn-specific antibody-treated mice, right after the first OVA challenge (**Fig. 2g**), and after three challenges (data not shown). Blockade of Opn-s resulted in decreased pulmonary levels of the T_H2 cell-specific chemokine, CCL22 (**Fig. 2g**).
Thus, antibody-mediated depletion of endogenous Opn-s at the phase of antigenic sensitization resulted in reduction of T_H2 allergic responses and consequent suppression of disease.

Figure 2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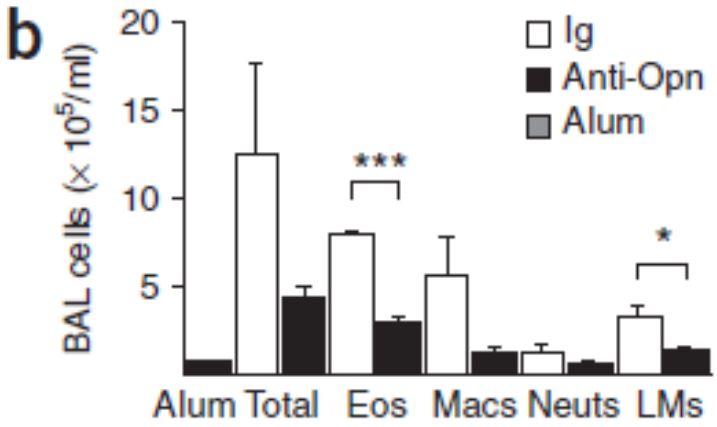
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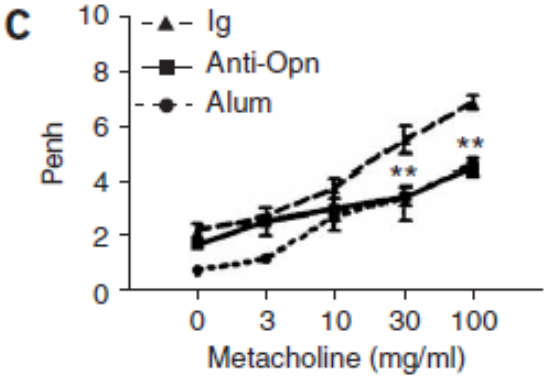
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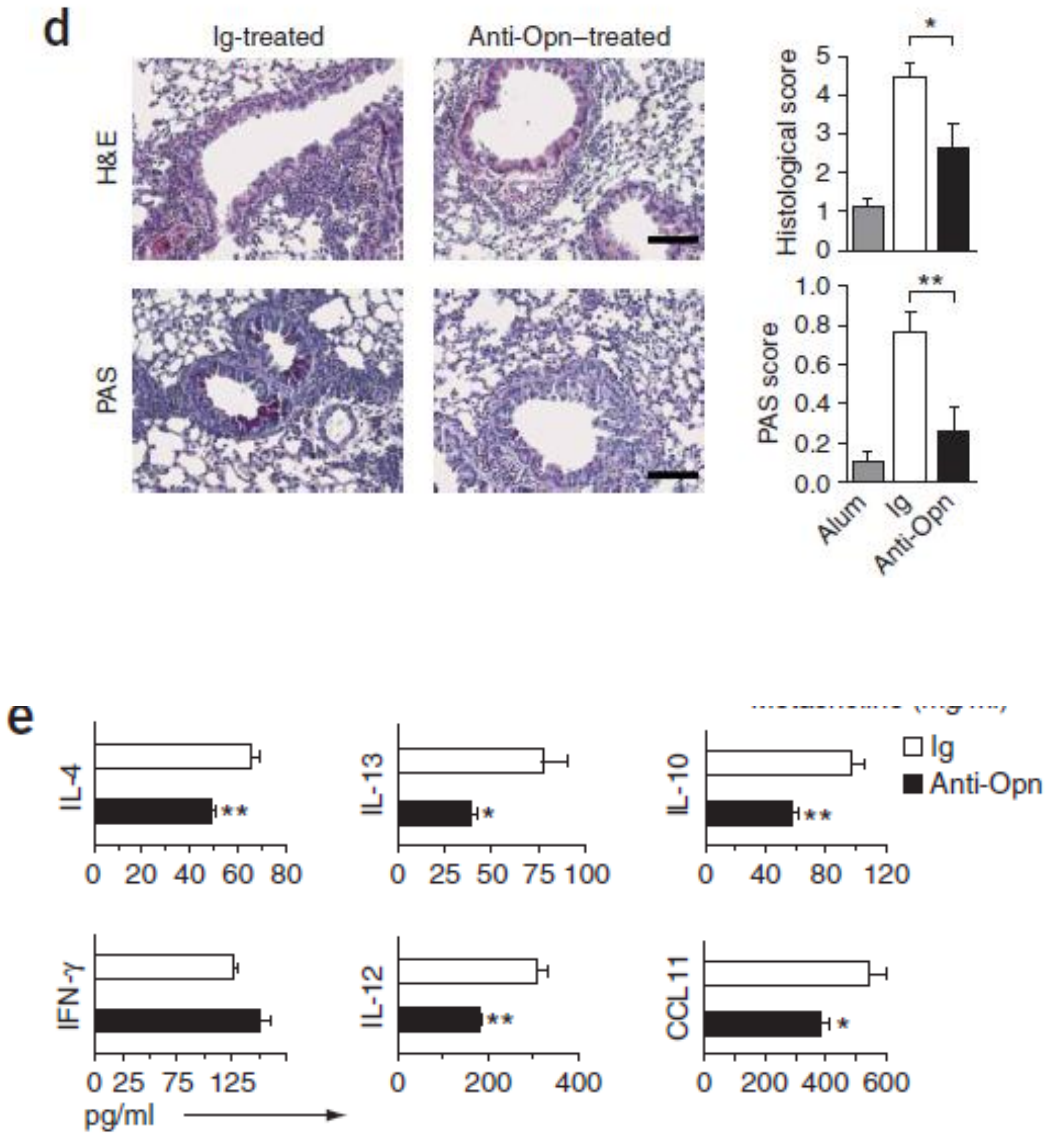

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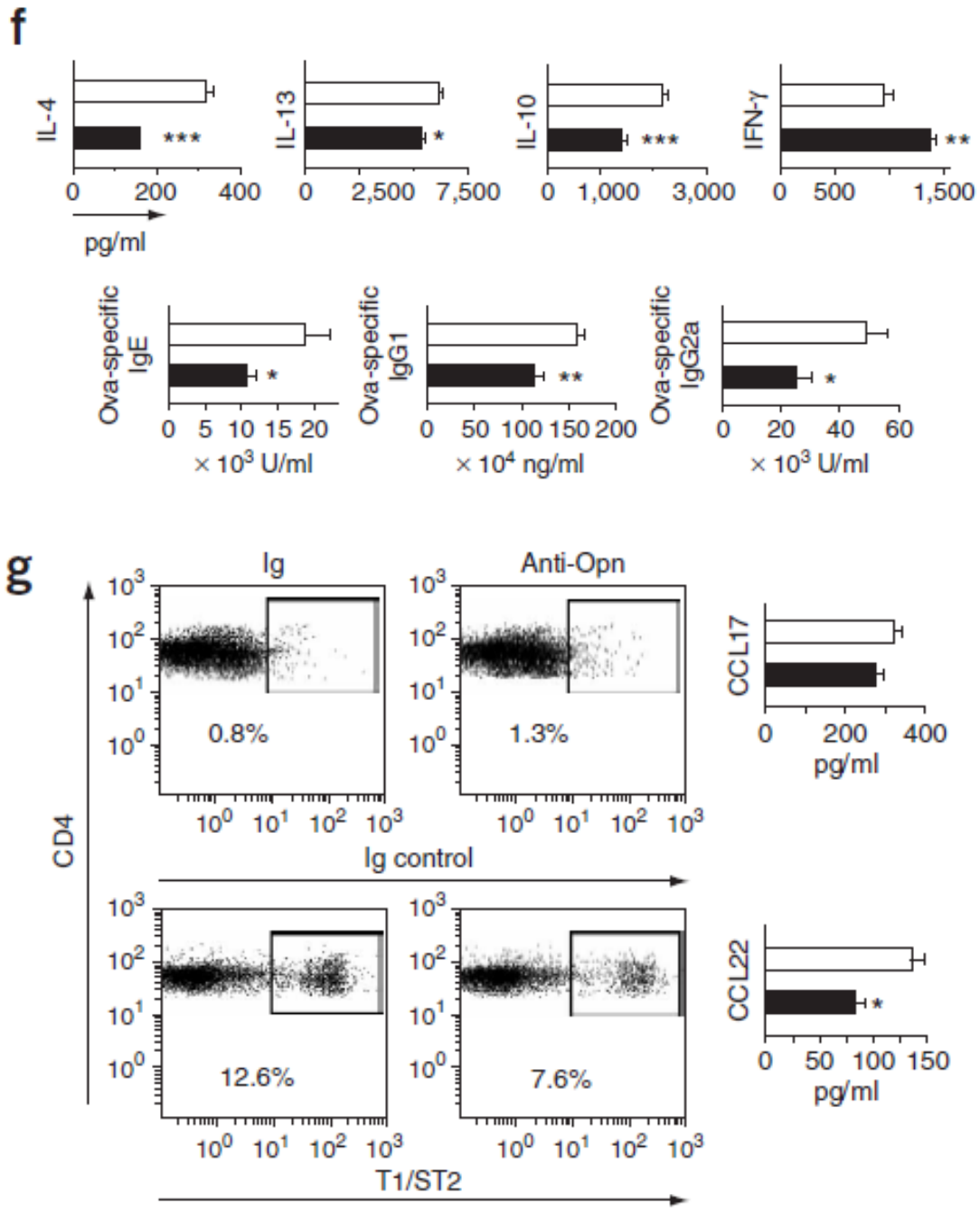


Figure 2 Opn-s blockade at priming reduces allergic disease. (**a**) Experimental protocol used to neutralize endogenous Opn-s during sensitization. BALB/c mice received two doses of anti-Opn or Ig control before each OVA/alum sensitization. (**b**) BAL differentials are expressed as mean ± SEM; *n* = 6–8 mice/group, three independent experiments. Unpaired Student's *t*-test was performed (eosinophils, ****P* = 0.0008), (lymphomononuclear (LMs) **P* = 0.0209). (**c**) AHR responses for PenH were analyzed (for anti-Opn and Ig-treated mice) by two-way ANOVA for repeated

measures and unpaired Student's *t*-test, ***P* = 0.010. (**d**) Lung inflammation (upper panels) and mucus secretion (lower panels) are shown. Unpaired Student's *t*-test was used in histological scores, **P* = 0.0269 (H&E), **P* = 0.0156 (PAS). Bar represents 100μm. (**e**) Lung levels of IL-4 (***P* = 0.0015), IL-13 (**P* = 0.0255), IL-10 (***P* = 0.007), IFN-γ (*P* = 0.068), IL-12 (***P* = 0.0026) and CCL11 (**P* = 0.0377). (**f**) Levels of IL-4 (****P* < 0.0001), IL-13 (**P* = 0.0121), IL-10 (****P* = 0.0002), and IFN-γ (***P* = 0.0014) in supernatants of OVA-stimulated DLNs. Serum levels of OVA-specific-IgE (**P* = 0.0328), -IgG1 (***P* = 0.0072), and -IgG2a (**P* = 0.0437). (**g**) BALB/c mice were sensitized as above and OVA-challenged on d 18. Anti-Opn or Ig control were administered before each sensitization. Percentages of T1/ST2$^+$ DLN cells gated on CD3$^+$CD4$^+$ T cells are shown. Isotype control staining for the T1/ST2 marker is also shown. One representative experiment of three, *n* = 3–5 mice/group. CCL17 (*P* = 0.1749) and CCL22 (**P* = 0.0115) lung levels are shown.

Endogenous Opn-s is anti-inflammatory during challenge

We investigated the role of endogenous Opn-s in secondary allergic responses by administering neutralizing antibody to Opn (or Ig control) before each OVA challenge in sensitized mice (**Fig. 3a**). Opn-s neutralization increased BAL total infiltrating cells, eosinophils (**Fig. 3b**), AHR responses (**Fig. 3c**), pulmonary inflammation (**Fig. 3d**) and mucus secretion (**Fig. 3d**). Alum controls had lower inflammation and AHR (**Fig. 3b**-**d**).

Lung IL-4, IL-13, IL-10, IFN-γ and CCL11 levels were increased in Opn-specific antibody-treated mice (**Fig. 3e**). Increased pulmonary IFN-γ levels have been suggested to play a pathologic role in allergic airway disease [276-279]. BAL cytokine levels were similarly increased in Opn-specific antibody-treated mice (data not shown).

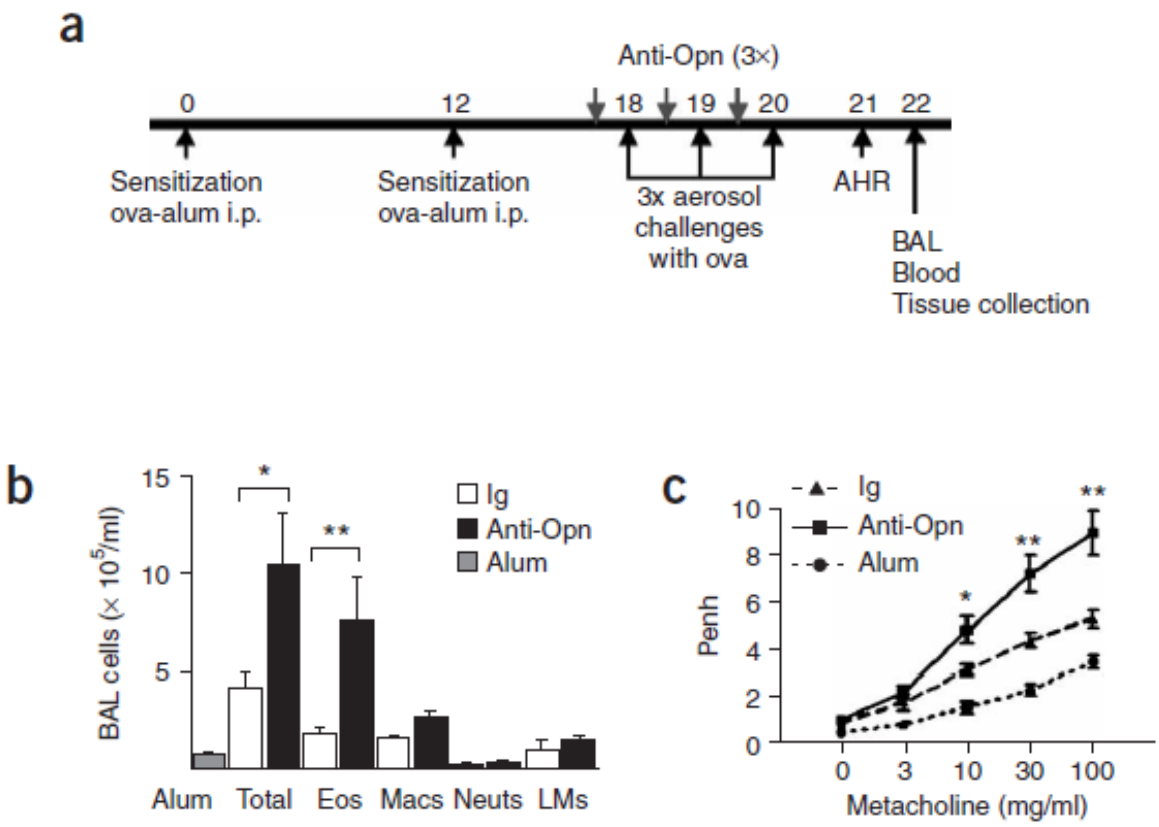
In OVA-stimulated DLNs, blockade of Opn-s during challenge increased IL-13 and IL-10 and decreased IFN-γ levels (**Fig. 3f**). OVA-specific-IgG1 levels were increased

while OVA-specific-IgG2a responses were decreased, indicative of a T_H2 shift (**Fig. 3f**).

We observed increased percentages of DLN T1/ST2$^+$ T_H2 cells following Opn-s neutralization, after the first intranasal OVA challenge (**Fig. 3g**), as well as after three OVA challenges [(antibody to Opn, 23.8–34,5% T1/ST2$^+$ of T cells) (Ig control 4,8–13,8% T1/ST2$^+$ of T cells)]. In both cases, we observed increased lung levels of CCL22 and CCL17 (**Fig. 3g**).

Overall, and in contrast to its effect at sensitization, blockade of endogenous Opn-s during antigenic challenge enhanced T_H2 allergic recall responses and exacerbated disease phenotype.

Figure 3



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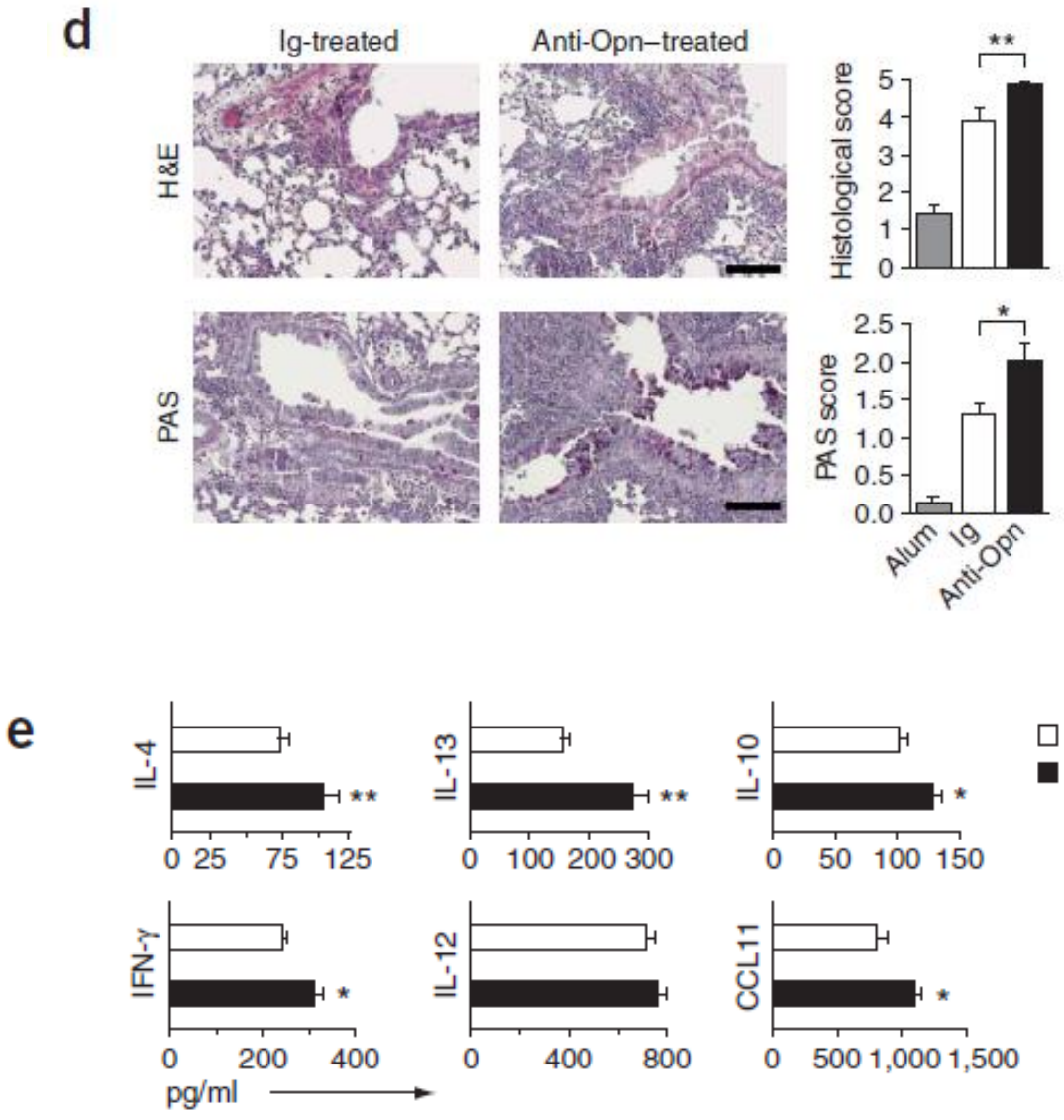

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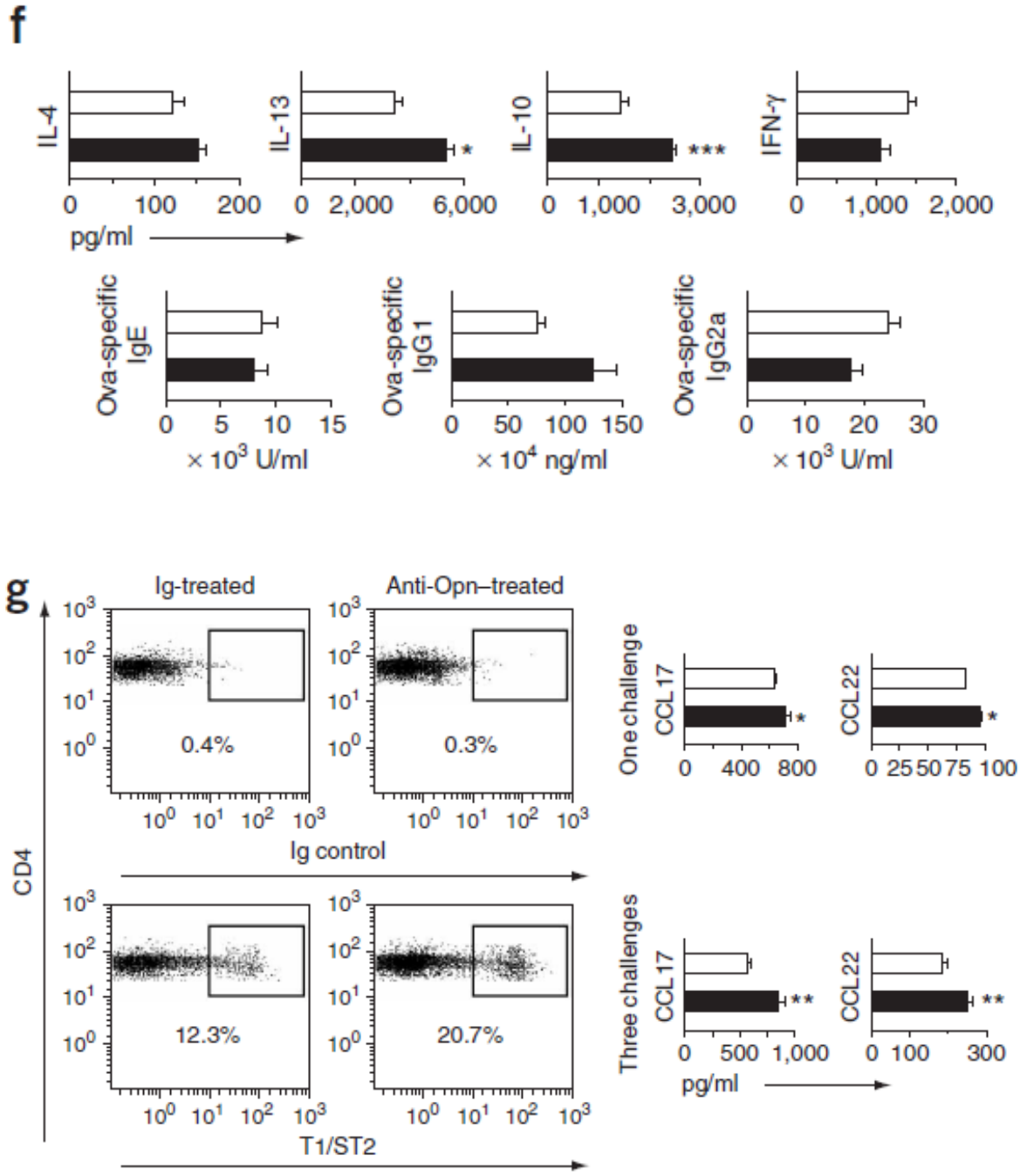


Figure 3 Opn-s blockade at challenge enhances allergic disease. (**a**) Experimental protocol used to neutralize endogenous Opn-s during challenge. BALB/c mice received three doses of anti-Opn or Ig control before challenge. (**b**) BAL differentials are expressed as mean ± SEM; *n* = 5–8 mice/group, five independent experiments. Unpaired Student's *t*-test was performed (total cell number, **P* = 0.0410), (eosinophils **P* = 0.0276). (**c**) AHR responses for PenH were analyzed as in **Fig. 2**,

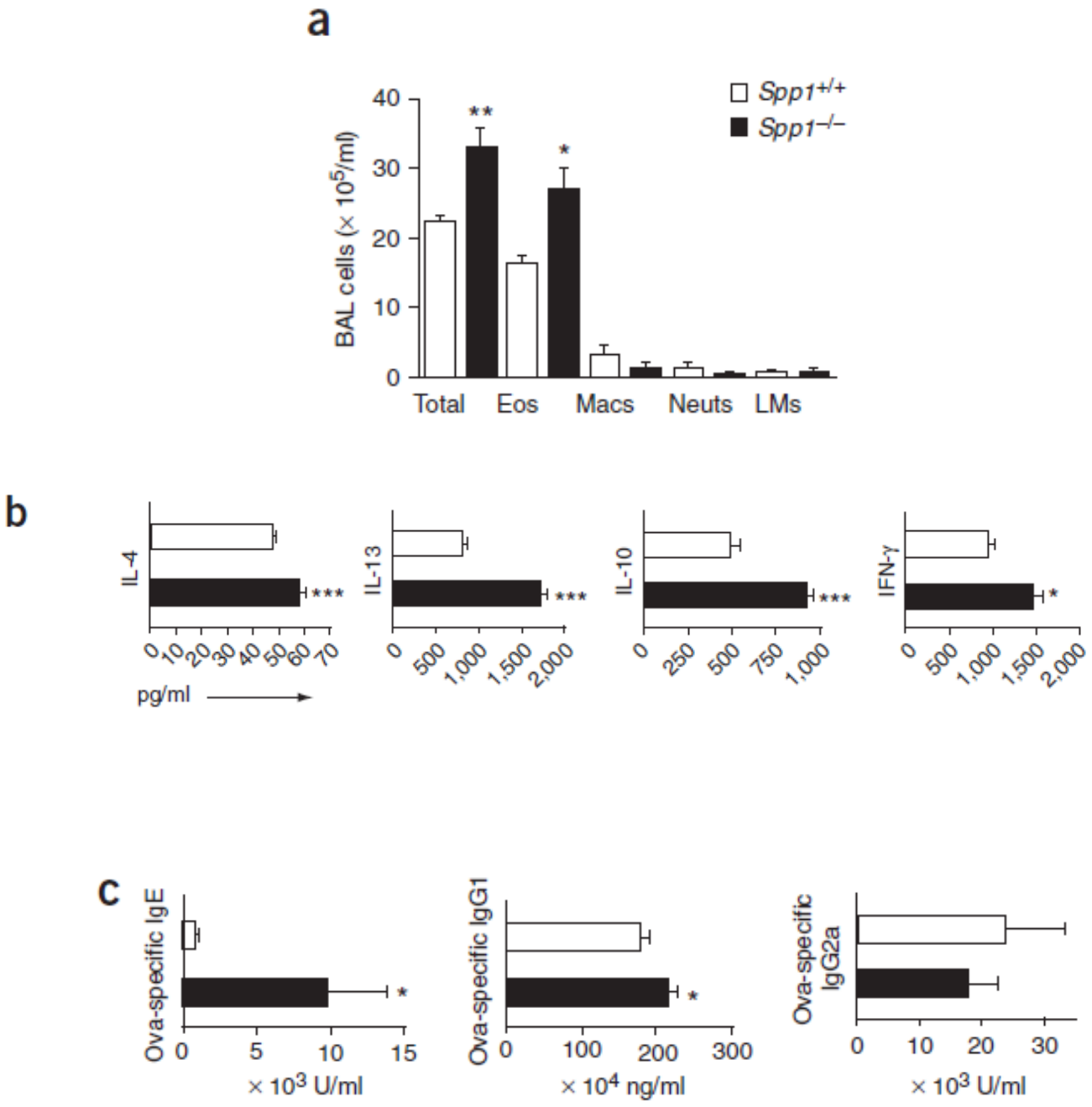
(*P = 0.027, **P = 0.010). (**d**) Lung inflammation and mucus secretion are shown as in **Fig. 2**. Statistical significance was obtained as in **Fig. 2**, **P = 0.0052 (H&E), *P = 0.0355 (PAS). Bar represents 100μm. (**e**) Lung levels of IL-4 (**P = 0.0067), IL-13 (**P = 0.0022), IL-10 (*P = 0.0122), IFN-γ (*P = 0.0186), IL-12 (P = 0.4268) and CCL11 (*P = 0.0163). (**f**) Levels of IL-4 (P = 0.0838), IL-13 (*P = 0.0118), IL-10 (***P < 0.0001), IFN-γ (P = 0.0794) in supernatants of OVA-stimulated DLN cells. Serum levels of OVA-specific-IgE (P = 0.7173), -IgG1 (P = 0.1299) and -IgG2a (P = 0.1012). (**g**) Percentages of T1/ST2$^+$ cells gated on CD3$^+$CD4$^+$ T cells. Isotype control staining for the T1/ST2 marker is shown. One representative experiment of three, n = 3–5 mice/group. Lung levels of CCL17 (*P = 0.0396) and CCL22 (*P = 0.0126) for mice which received one OVA challenge (and antibody treatment) (upper panels). Lung levels of CCL17 (**P = 0.0053) and CCL22 (**P = 0.0036) for mice which received three OVA challenges (and antibody treatments) (lower panels).

Opn$^{-/-}$ mice have enhanced Th2-mediated responses

Opn$^{-/-}$ mice had increased numbers of BAL inflammatory cells and eosinophils compared to *Opn*$^{+/+}$ (**Fig. 4a**). Differences in lung T_H2 cytokine and chemokine levels were not statistically significant (data not shown). However, *Opn*$^{-/-}$ mice have a predominantly C57BL/6 genetic background, considered resistant to allergic inflammation and are subjected to possible compensatory mechanisms.

OVA-stimulated DLN cells from *Opn*$^{-/-}$ mice produced increased IL-4, IL-13, IL-10 and IFN-γ, as compared to *wt* (**Fig. 4b**). OVA-specific-IgG1 levels were increased, while OVA-specific-IgG2a responses were decreased in *Opn*$^{-/-}$ mice, suggestive of a T_H2 shift (**Fig. 4c**). We also observed increased levels of OVA-specific-IgE in *Opn*$^{-/-}$ mice (**Fig. 4c**). OVA-specific-IgE was increased in BALB/c mice treated with antibody to Opn during both sensitization and challenge phases (**Fig. 4d**), indicating no involvement of Opn-i.

Figure 4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Spp1+/+
Spp1−/−
BAL cells (× 10^5/ml)
40
30
20
10
0
**
*
Total
Eos
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Neuts
LMs
b
IL-4
***
0 10 20 30 40 50 60 70
pg/ml
IL-13
***
0 500 1,000 1,500 2,000
IL-10
***
0 250 500 750 1,000
IFN-γ
*
0 500 1,000 1,500 2,000
c
Ova-specific IgE
*
0 5 10 15
× 10^3 U/ml
Ova-specific IgG1
*
0 100 200 300
× 10^4 ng/ml
Ova-specific IgG2a
0 10 20 30
× 10^3 U/ml

Figure 4 cont.

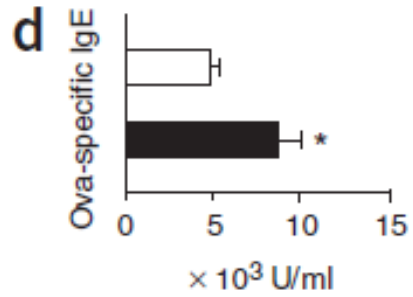


Figure 4 *Opn*$^{-/-}$ mice exhibit enhanced Th2 responses. (**a**) *Opn*$^{+/+}$ and *Opn*$^{-/-}$ mice were immunized with OVA/alum i.p., on d 0 and 12 and challenged through the airways with aerosolized OVA between d 18–23. Differential cell counts in BAL from *Opn*$^{+/+}$ or *Opn*$^{-/-}$ mice (total cell number, ***P* = 0.0073), (eosinophils **P* = 0.0320) are shown. Data are expressed as mean ± SEM; *n* = 4–6 mice/group, three independent experiments. Statistical significance was obtained by unpaired Student's *t*-test. (**b**) IL-4 (****P* < 0.0001), IL-13 (****P* < 0.0001), IL-10 (****P* < 0.0001) and IFN-γ (**P* = 0.0455) levels in supernatants from OVA-stimulated DLN cells. (**c**) Serum OVA-specific-IgE levels (**P* = 0.0415), -IgG1 (**P* = 0.0378) and -IgG2a (*P* = 0.5660) from *Opn*$^{+/+}$ or *Opn*$^{-/-}$ mice. Statistical significance was obtained as before. (**d**) BALB/c mice were immunized with OVA/alum, i.p., on d 0 and 12 and challenged with aerosolized OVA between d 18–20. Anti-Opn or Ig control was administered during both OVA sensitization and challenge phases. OVA-specific-IgE levels in the sera (**P* = 0.0236) are shown. Values are expressed as mean ± SEM; *n* = 4–6 mice/group, two independent experiments. Statistical significance was obtained by unpaired Student's *t*-test.

Opn-s blockade at sensitization affects pDC numbers

In order to explore the effect of Opn-s neutralization at sensitization on final disease outcome, we examined early T_H2 responses. BALB/c mice were treated with Opn-

specific antibody or Ig control before sensitization with Alexa-Fluor-OVA in alum, and $CD11c^+$ cell-driven responses were examined. Co-cultures of DLN $CD11c^+$ cells from Opn-s-neutralized mice with DO11.10 responder T cells produced lower levels of IL-4, IL-13 and IFN-γ, as compared to those from Ig-treated mice (**Fig. 5a**), suggestive of reduced priming effect. We obtained similar results from OVA-stimulated whole DLNs (Opn-specific antibody 37.33 ± 2.46 vs Ig-treated 96.67 ± 7.92 pg/ml of IL-4, and Opn-specific antibody 717.3 ± 25.00 vs Ig-treated 962.4 ± 38.07 pg/ml of IFN-γ).

Two subtypes of DCs have been mainly demonstrated to participate in immune responses: conventional DCs ($CD11c^+B220^-$ or $CD11c^+B220^-Gr1^-$ cDCs), considered immunogenic, and pDCs, considered mainly regulatory [91,132,280-281]. $CD11c^+$PDCA-1^+/$120G8^+Gr\text{-}1^+$ cells have been described as pDCs in allergic airway inflammation, exhibiting suppressing effects on Th2 responses [91,133,282].

Opn-specific antibody-treated mice had increased percentages and total numbers of DLN $CD11c^+$PDCA-$1^+Gr\text{-}1^+$ pDCs (characterized also as $CD11c^+Gr\text{-}1^+B220^+$) and of Ag-loaded (Alexa-Fluor-OVA^+) pDCs (**Fig. 5b**). OVA uptake was not influenced, since percentages of OVA^+ cells among pDCs were similar (approximately 52 ± 5% for Opn-s neutralization versus 47 ± 5% for Ig). No differences were observed in the percentages and numbers of cDCs ($CD11c^+B220^-Gr\text{-}1^-$) or Alexa-Fluor-$OVA^+$ cDCs (**Fig. 5b**). The percentages of OVA^+ cells among cDCs were similar (approximately 45 ± 5% for Opn-s neutralization versus 51 ± 5% for Ig). Numbers of $CD11c^+$ cells within DLNs were similar among groups (65,420 ± 2,289 cells/mouse for Opn-s neutralization versus 65,250 ± 6,284 for Ig). Purified DLN $CD11c^+$cells from Opn-s-neutralized mice stimulated with CpG-oligodeoxynucleotides produced increased levels of IFN-α, a defining characteristic of pDCs [91,283] (**Fig. 5c**).

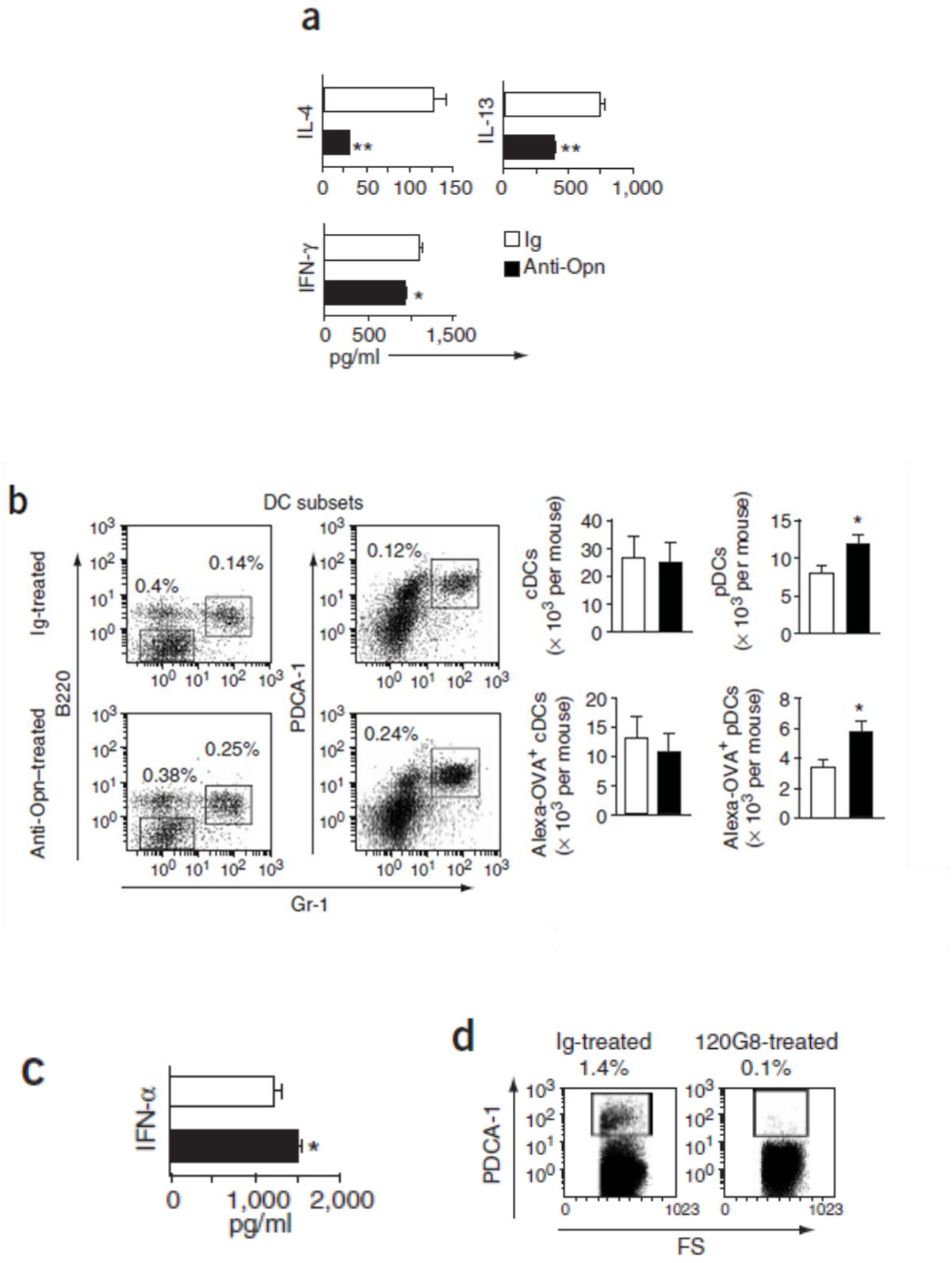
$Opn^{-/-}$ mice exhibited no significant enhancement of pDC-recruitment in DLNs as compared to $Opn^{+/+}$ during priming (8,732 ± 2,900 versus 6,518 ± 2,100 cells/mouse). No differences were observed in $CD11c^+$ cell-recruitment (39,800 ± 4,800 versus 35,800 ± 9,200 cells/mouse). Studies using a substantially different sensitization protocol involving trinitro-chloro-benzene administration demonstrated decreased migration of $CD11c^+$ cells to skin and DLNs in $Opn^{-/-}$ mice [221]. The discrepancies

between the latter report and our findings may be attributed to different innate mechanisms.

Recent studies have demonstrated that pDCs suppressed T_H2 responses [91]. To address whether the effects of Opn-s blockade during sensitization were mediated by the pDC population, we depleted pDCs (using the 120G8 antibody [284]) prior to Ova/alum sensitization and Opn-s blockade in naïve BALB/c mice. pDC-depletion was successful as shown by flow cytometric analysis of PDCA-1^+ cells (**Fig. 5d**). The Opn-specific antibody treatment had no effect on primary Th2 responses in pDC-depleted mice and these responses were now similar to those of Ig-treated, pDC-depleted mice. This was depicted by IL-4, IL-13 and IFN-γ levels and OVA-specific proliferative responses (**Fig. 5e**). In both groups, pDC-depleted mice exhibited increased IL-4, IL-13 and IFN-γ levels (**Fig. 5e**), suggestive of a regulatory role for pDCs as previously described [91]. Isolated pDCs from DLNs in co-cultures with DO11.10 T cells did not induce measurable cytokine levels, while cDCs induced cytokine release (IL-4 levels: 92 ± 10 pg/ml and IFN-γ levels: 476 ± 20 pg/ml), suggestive of immunogenic potential in these cells.

In conclusion, the decrease in T_H2 priming observed after Opn-s neutralization is mediated by increased numbers of regulatory pDCs in DLNs.

Figure 5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IL-4
IL-13
IFN-γ
Ig
Anti-Opn
pg/ml
b
DC subsets
Ig-treated
Anti-Opn–treated
B220
PDCA-1
Gr-1
0.4%
0.14%
0.38%
0.25%
0.12%
0.24%
cDCs (× 10³ per mouse)
pDCs (× 10³ per mouse)
Alexa-OVA⁺ cDCs (× 10³ per mouse)
Alexa-OVA⁺ pDCs (× 10³ per mouse)
c
IFN-α
pg/ml
d
Ig-treated
1.4%
120G8-treated
0.1%
PDCA-1
FS

Figure 5 cont.

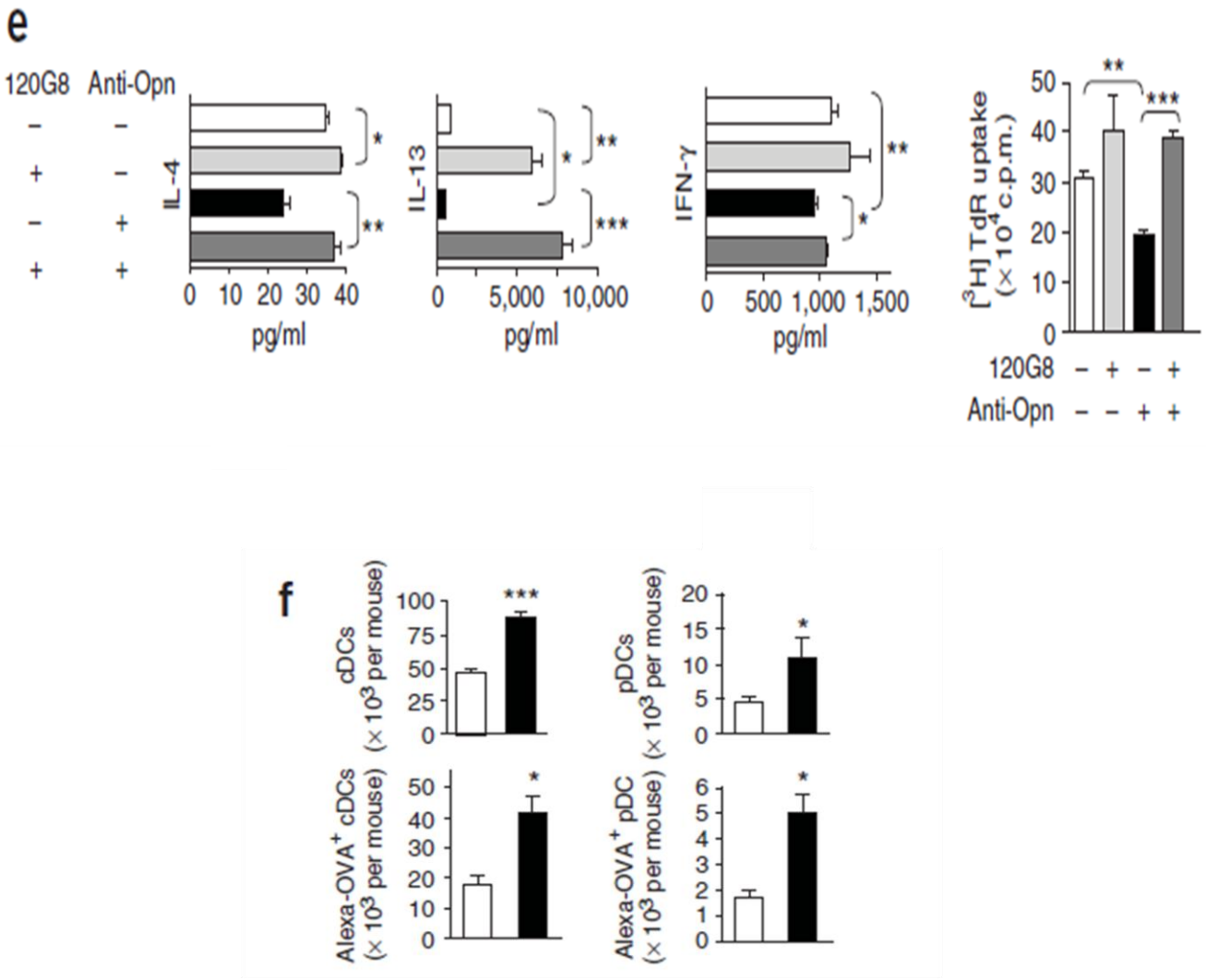


Figure 5 cont.

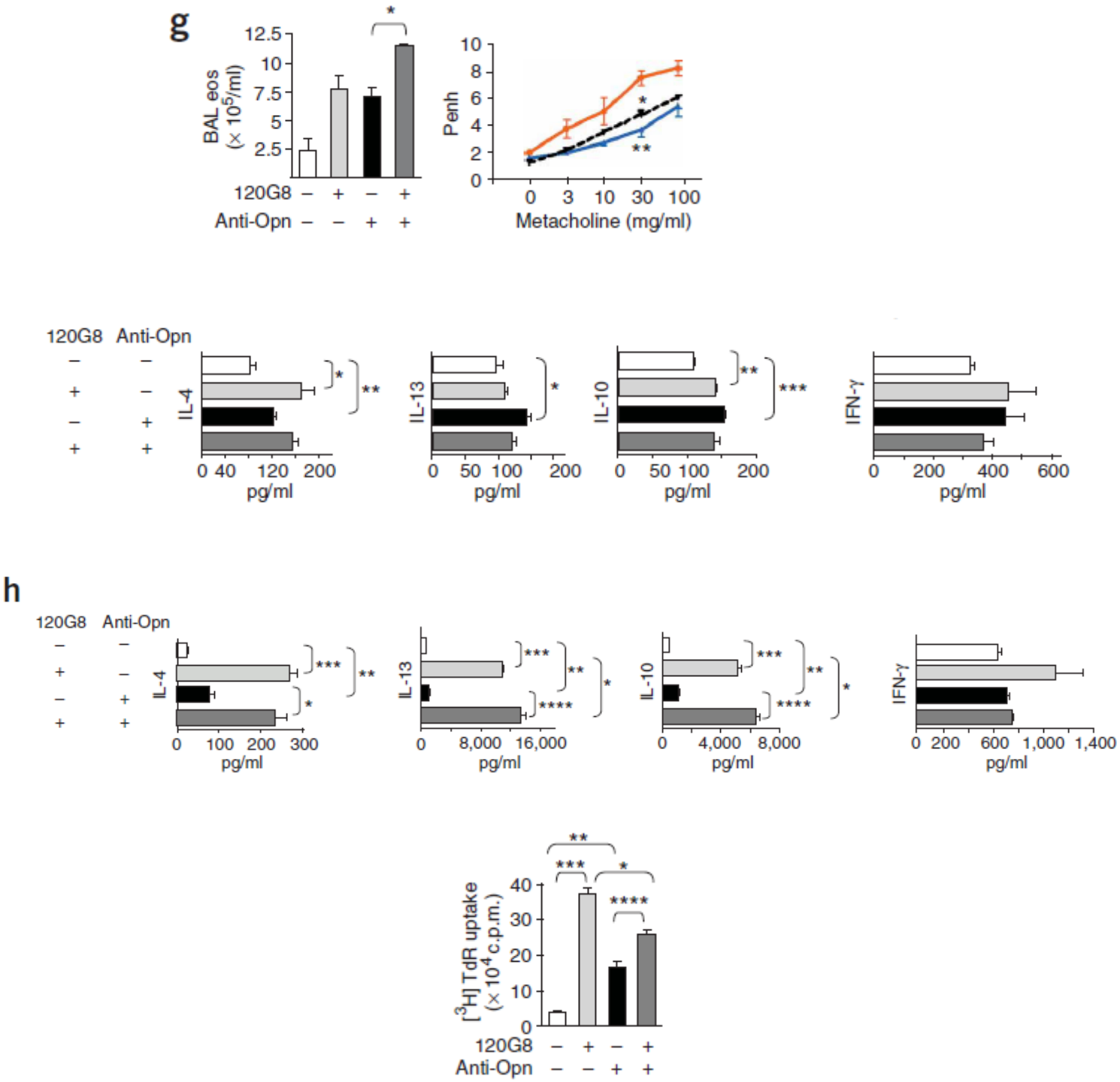


Figure 5 Opn-s blockade affects Th2 responses via DC recruitment. (**a**) IL-4 (**P = 0.0024), IL-13 (**P = 0.0017) and IFN-γ (*P = 0.0365) from OVA-stimulated co-cultures of DLN $CD11c^+$ with DO11.10 Th cells. Data expressed as mean ± SEM; n = 4–6 mice/group, three independent experiments. (**b**) Percentages of DLN $7AAD^-$ $CD11c^+B220^-Gr\text{-}1^-$cDCs, $7AAD^-CD11c^+B220^+Gr\text{-}1^+$pDCs and $7AAD^-$

$CD11c^+PDCA\text{-}1^+Gr\text{-}1^+$ pDCs. Numbers of cDCs, pDCs (*P = 0.0166) and Alexa-Fluor-OVA^+-cDCs and -pDCs (*P = 0.0221). (**c**) IFN-α (*P = 0.0143) from CpG-stimulated $CD11c^+$ cells. (**d**) Percentages of $7AAD^-PDCA\text{-}1^+$ cells. (**e**) IL-4 [(Ig vs Ig+120G8, *P = 0.0461), (anti-Opn vs anti-Opn+120G8, *P = 0.0176)], IL-13 [*P = 0.016, **P = 0.0014, ***P = 0.0003] and IFN-γ [(anti-Opn vs anti-Opn+120G8, *P = 0.0283), (Ig vs anti-Opn, *P = 0.0365)] from OVA-stimulated DLNs. [^{3}H]-thymidine incorporation of OVA-stimulated DLNs [**P = 0.0013, ***P = 0.0004]; n = 4 mice/group, three experiments. (**f**) Numbers of cDCs (***P = 0.0001), pDCs (*P = 0.0375) and Alexa-Fluor-OVA^+ -cDCs (*P = 0.0202) and -pDCs (*P = 0.0116); n = 5–7 mice/group, three independent experiments. (**g**) BAL differentials (*P = 0.0351) and AHR [(anti-Opn (solid line), Ig+120G8 (dashed line) or anti-Opn+120G8 (solid line with circles)]; [*P = 0.0132, **P = 0.0034]; n = 5–8 mice/group, two experiments. Lung IL-4 [(Ig vs Ig+120G8, *P = 0.0349), (Ig vs anti-Opn, *P = 0.0491)], IL-13 [*P = 0.0299], IL-10 [**P = 0.0064, ***P = 0.0006] and IFN-γ. (**h**) IL-4 [***P = 0.0002, (anti-Opn vs anti-Opn+120G8, **P = 0.0074), (Ig vs anti-Opn, **P = 0.0076)], IL-13 [(Ig+120G8 vs anti-Opn+120G8, *P = 0.0142), **P = 0.0016, (Ig vs Ig+120G8, ***P < 0.0001), (anti-Opn vs anti-Opn+120G8, ***P < 0.0001)], IL-10 [*P = 0.0112, **P = 0.0025, (Ig vs Ig+120G8,***P < 0.0001), (anti-Opn vs anti-Opn+120G8, ***P < 0.0001)] and IFN-γ from OVA-stimulated DLNs. [^{3}H]-thymidine incorporation of OVA-stimulated DLNs [(anti-Opn vs anti-Opn+120G8, *P = 0.0108), (Ig+anti-120G8 vs anti-Opn+120G8, *P = 0.0116), **P = 0.0016, ***P = 0.0001]. Unpaired Student's t-test was performed throughout.

Opn-s blockade at challenge affects cDC numbers

We investigated cDC and pDC recruitment when Opn-s was neutralized during challenge (**Fig. 3a**, protocol). There was an increase in total and Alexa-Fluor-OVA^+ cDCs and pDCs in DLNs of Opn-specific antibody-treated mice (**Fig. 5f**). OVA^+ cells among cDCs and pDCs were similar (approximately 47 ± 6% and 43 ± 5% for Opn-s neutralization versus 53 ± 3% and 49 ± 6% for Ig). Opn-s blockade increased total numbers of $CD11c^+$ cells (data not shown). Similar results on DC subsets were obtained following one, instead of three, intranasal challenges (data not shown). Of

note, both triple and single challenges of Opn-specific antibody-treated mice enhanced AHR, increased percentages of DLN T1/ST2$^+$ T_H2 cells and IL-4 in OVA-stimulated DLNs (**Fig. 3g** and data not shown). Overall, we observed increased recruitment of cDCs and pDCs in lung DLNs with the increase of cDCs being greater than that of pDCs.

In order to examine the role of pDCs in the above settings, we used pDC-depleted mice. These exhibited increased allergic responses in comparison to their respective non-pDC-depleted mice (**Fig. 5g**, **h**), indicating a regulatory role for pDCs during secondary responses. Importantly, in pDC-depleted mice, treatment with Opn-specific antibody increased total BAL cell (not shown) and eosinophil numbers, enhanced AHR, IL-13, and IL-10 levels in OVA-specific DLN responses, as compared to pDC-depleted, Ig-treated mice (**Fig. 5g**, **h**), suggesting no involvement of pDCs in the pro-allergic effect of Opn-s neutralization during challenge.

Co-cultures of cDCs with DO11.10 T cells produced increased IL-4 and IL-13 levels, showing T_H2-promoting potential (data not shown). Similar increases in cDC numbers have been linked to dramatically enhanced inflammation [133] and T_H2 proliferation [84]. Overall, enhancement of T_H2 responses due to Opn-s blockade at challenge was influenced by increased recruitment of immunogenic cDCs.

Administration of rOpn is protective at challenge

rOpn administered along with OVA/alum during sensitization increased IL-13 and IFN-γ levels in OVA-stimulated DLNs (**Fig. 6a**), suggesting an Opn-s pro-inflammatory role during T_H2 priming.

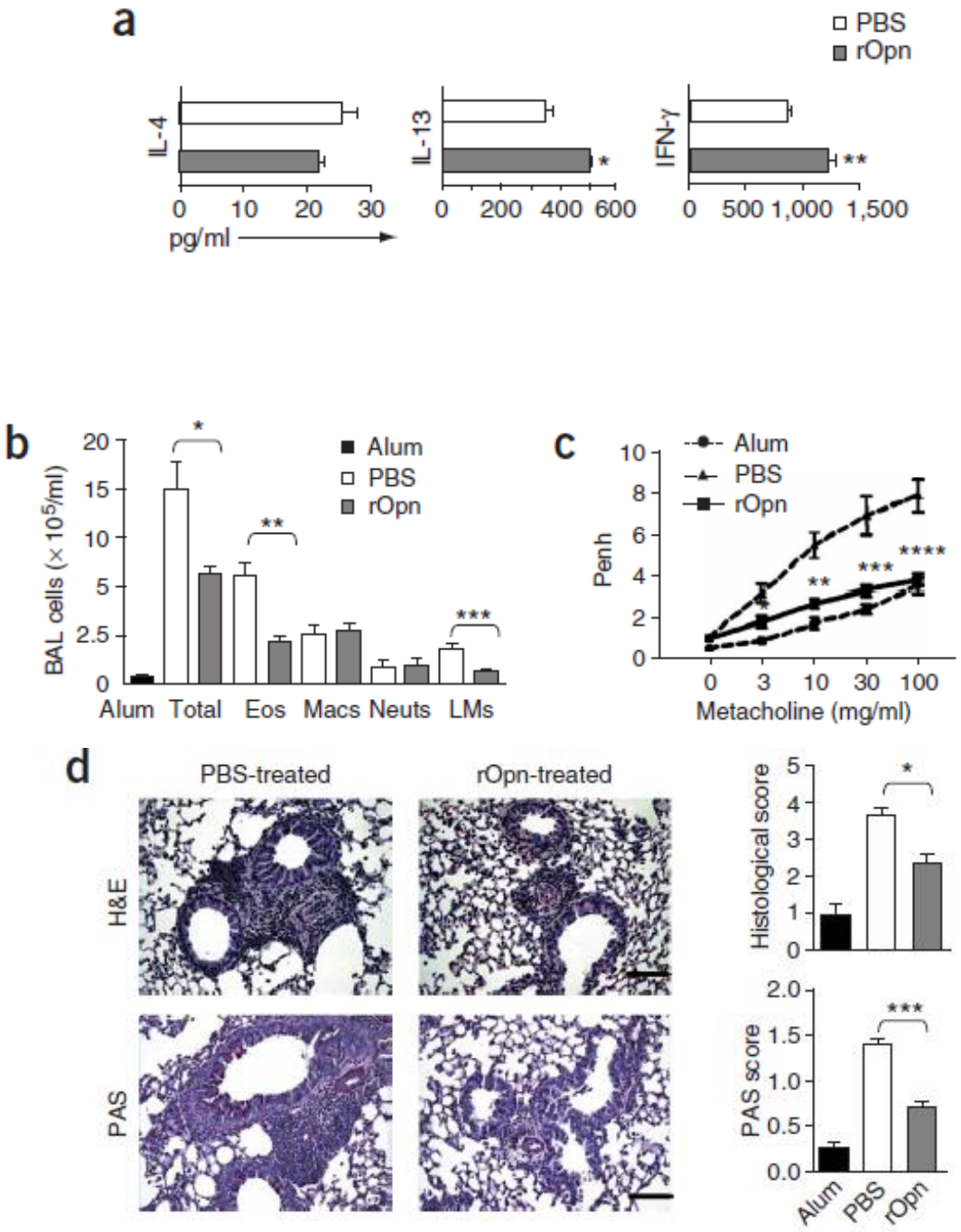
Intranasal administration of rOpn before OVA challenge decreased BAL total cell numbers, eosinophils and mononuclear cells (**Fig. 6b**) and AHR responses, to levels of controls (**Fig. 6c**). Lung leukocytic infiltration, mucus secretion (**Fig. 6d**) and lung IL-4, IL-13, IL-10, IFN-γ, CCL11, CCL17 and CCL22 levels were also decreased, while

IL-12 levels were increased (**Fig. 6e**). BAL cytokines exhibited a similar pattern (data not shown).

OVA-stimulated DLN cells from rOpn-treated mice produced decreased IL-4, IL-13 and IFN-γ levels (**Fig. 6f**). OVA-specific-IgG1 and –IgE levels were decreased, while –IgG2a levels were increased (**Fig. 6g**).

These results point to a suppressive role for endogenous Opn-s during secondary allergic airway responses.

Figure 6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IL-4
IL-13
IFN-γ
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pg/ml
0 200 400 600
0 500 1,000 1,500
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BAL cells (×10⁵/ml)
Alum
PBS
rOpn
Alum Total Eos Macs Neuts LMs
c
Penh
Metacholine (mg/ml)
d
PBS-treated
rOpn-treated
H&E
PAS
Histological score
PAS score
Alum PBS rOpn

Figure 6 cont.

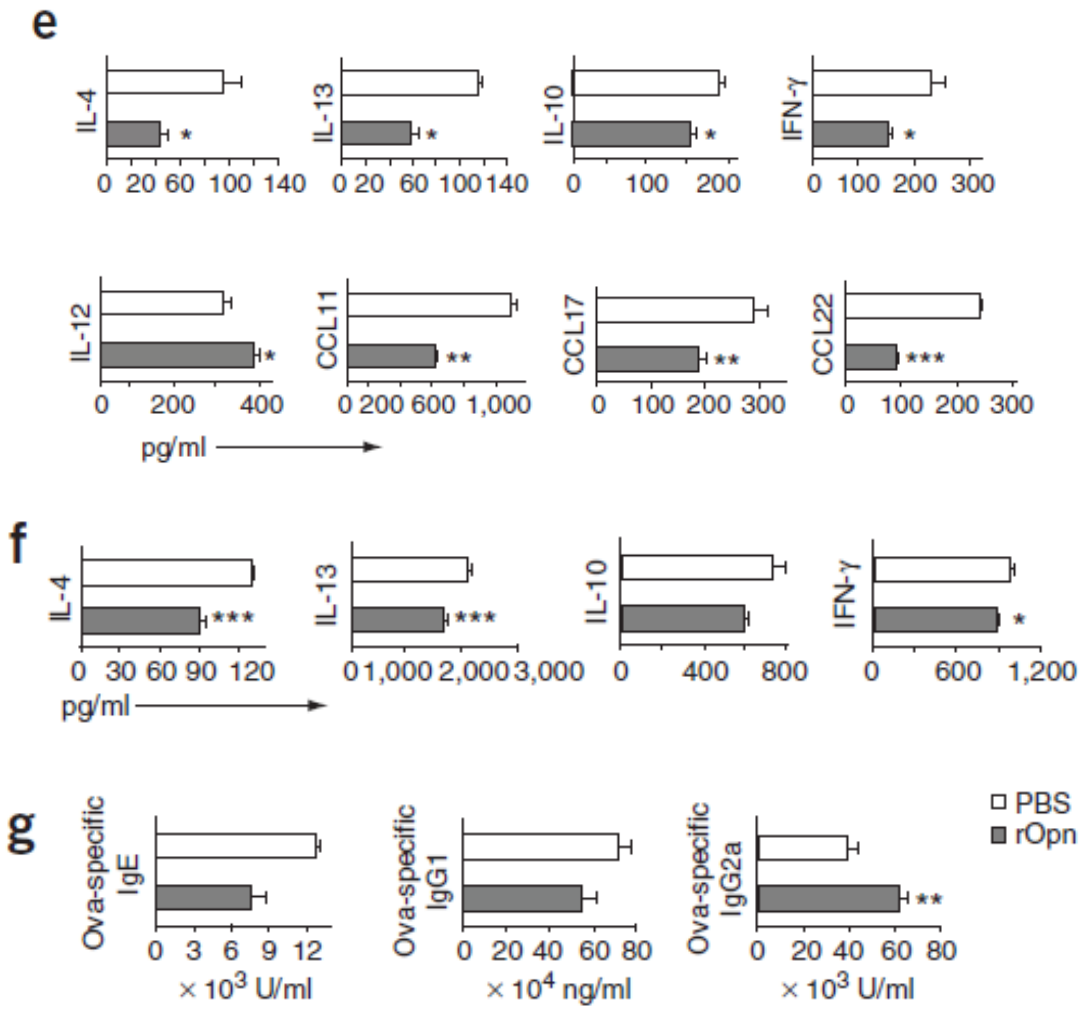


Figure 6 rOpn is protective during pulmonary challenge. (**a**) BALB/c mice were treated as described in Methods. Levels of IL-4 ($P = 0.2266$), IL-13 ($^{*}P = 0.0113$) and IFN-γ ($^{**}P = 0.0079$) in supernatants of OVA-stimulated DLN cells. (**b**) BALB/c mice were treated as described in Methods. BAL differentials are shown; (total cells $^{*}P = 0.0430$), (eosinophils $^{**}P = 0.0099$), (LMs $^{**}P = 0.0067$). (**c**) AHR responses for PenH were analyzed as in **Fig. 2**; [(Met (3), $^{*}P = 0.032$), (Met (10), $^{**}P = 0.0038$), Met (30), $^{**}P = 0.0083$), Met (100), $^{**}P = 0.0017$)]. (**d**) Lung inflammation (upper

panels) and mucus secretion (lower panels); Histological scores for H&E (*P = 0.03) and PAS (***P = 0.0002). Bar represents 100μm. (**e**) Lung levels of IL-4 (*P = 0.0378), IL-13 (*P = 0.0141), IL-10 (*P = 0.04), IFN-γ (*P = 0.037), IL-12 (*P = 0.0271), CCL11 (**P = 0.0022), CCL17 (**P = 0.005) and CCL22 (***P < 0.0001). **(f)** IL-4 (***P < 0.0001), IL-13 (***P = 0.0003), IL-10 (P = 0.1322), IFN-γ (*P = 0.0229) levels in supernatants of OVA-stimulated DLN cells. **(g)** Serum levels of OVA-specific-IgE (P = 0.0589), -IgG1 (P = 0.0703) and –IgG2a (**P = 0.0045). Data are expressed as mean ± SEM; n = 6–8 mice/group in three independent experiments. Unpaired Student's t-test was performed throughout.

rOpn suppresses allergic disease through enhanced recruitment of pDCs and Tregs.

Our previous results showed that therapeutic intranasal administration of recombinant Opn (rOpn) during pulmonary allergen challenge, protected mice from airway disease. However the exact mechanism for this rOpn-mediated protection was not known. In order to examine this mechanism, we used a well-established mouse model of allergic airway inflammation induced by two OVA/alum intraperitonially (i.p.) sensitizations followed by three airway OVA challenges and administered intranasally (i.n.) rOpn before each allergen challenge (**Fig. 7a**). Our studies have revealed that rOpn reduced T_H2 responses, to investigate if this reduction was due to increased recruitment of T_H2 cells, we checked T1/ST2, a specific T_H2 cell marker. We observed that rOpn decreased the percentages of T_H cells positive for T1/ST2 in lung DLNs (**Fig. 7b**) and also in lung (data not shown). In order to determine if this reduction was due to a

regulatory population, suppressing T_H2 cells we examined the recruitment of T regulatory cells and pDCs. rOpn administration during pulmonary allergen challenge induced enhanced DLN recruitment of $CD3^+CD4^+CD25^+FoxP3^+$ Treg cells (**Fig. 7c**) as well as $CD3^-CD19^-CD11c^+PDCA\text{-}1^+Gr\text{-}1^+$ regulatory pDCs (**Fig. 7d**), we also obtained similar results for $CD3^-CD19^-CD11c^+PDCA\text{-}1^+Gr\text{-}1^+$ pDCs (data not shown). No differences were observed in numbers of $CD3^-CD19^-CD11c^+B220^-Gr\text{-}1^-$ cDCs (**Fig. 7d**).

Thus, rOpn suppresses allergic disease through enhanced recruitment of regulatory pDCs and Tregs.

Figure 7

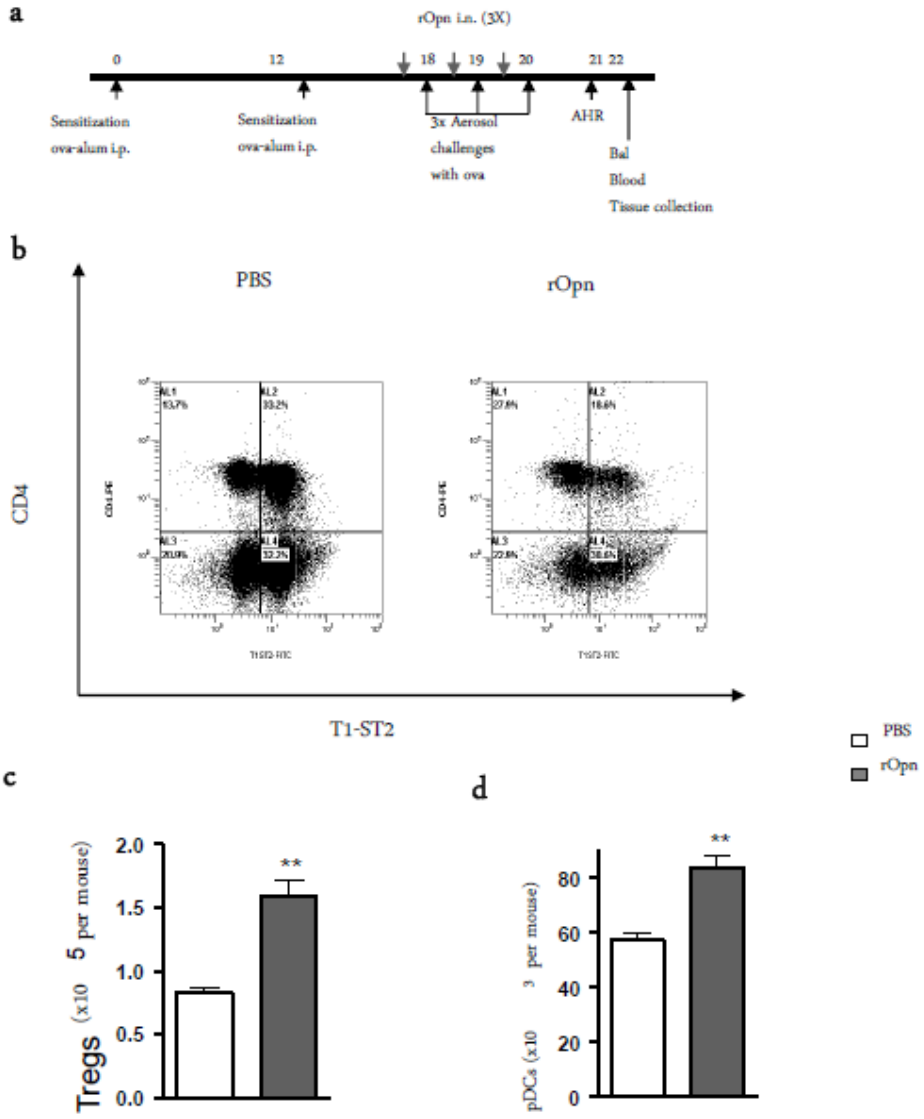


Figure 7 rOpn suppresses allergic disease through enhanced recruitment of pDCs and Tregs. (**a**) Experimental protocol used to administrate rOpn during challenge. BALB/c mice were sensitized with OVA/alum i.p. on days 0 and 12 and then challenged with OVA between days 18-20. rOpn (2.5µg/mouse) or PBS were administered i.p. 2-3 hours before each OVA challenge. (**b**) Percentages of T1/ST2+ DLN cells gated on CD3+CD4+ T cells are shown for PBS-treated (left panel) or rOpn-treated (right panel) mice are shown. One representative experiment of three, n = 3–5 mice per group. (**c**) Numbers of $CD3^{+}CD4^{+}CD25^{+}FoxP3^{+}$ Tregs. (**d**) Numbers of DLN $7AAD^{-}$ $CD3^{-}CD19^{-}CD11c^{+}B220^{+}Gr\text{-}1^{+}$ pDCs.

Opn exerts a proinflammatory role during priming of disease in HDM model

Furthermore in order to further evaluate the effect of Opn in allergic airway disease we used a clinically relevant allergen such as house dust mite (HDM). We performed experiments using *Opn-/-* mice and administered rOpn during HDM i.n. sensitization. Our data showed significantly enhanced lung inflammation and mucus secretion (**Figure 8b**), as well as increaed infiltrations in the lung as denoted by the increased numbers of eosinophils present in the BAL of these mic (**Figure 8a**). Furthermore, we noted a significant increase in T_H2 responses in *ex vivo* cultures of DLN cells from rOpn treated mice, in the presence of HDM, indicating a priming effect of rOpn in this model as well (**Figure 8e**). There were no significant differences in the T_H2 responses in the BAL and the lung among rOpn treated and PBS treated mice (**Figure 8c,d**). However, we believe that the exact mechanisms involved in the effect of rOpn in the HDM model deserve further experimentation. It will certainly be of great interest to study the immunoregulatory effects of both Opn-s and Opn-i in this model and this will be a subject of future studies.

Figure 8

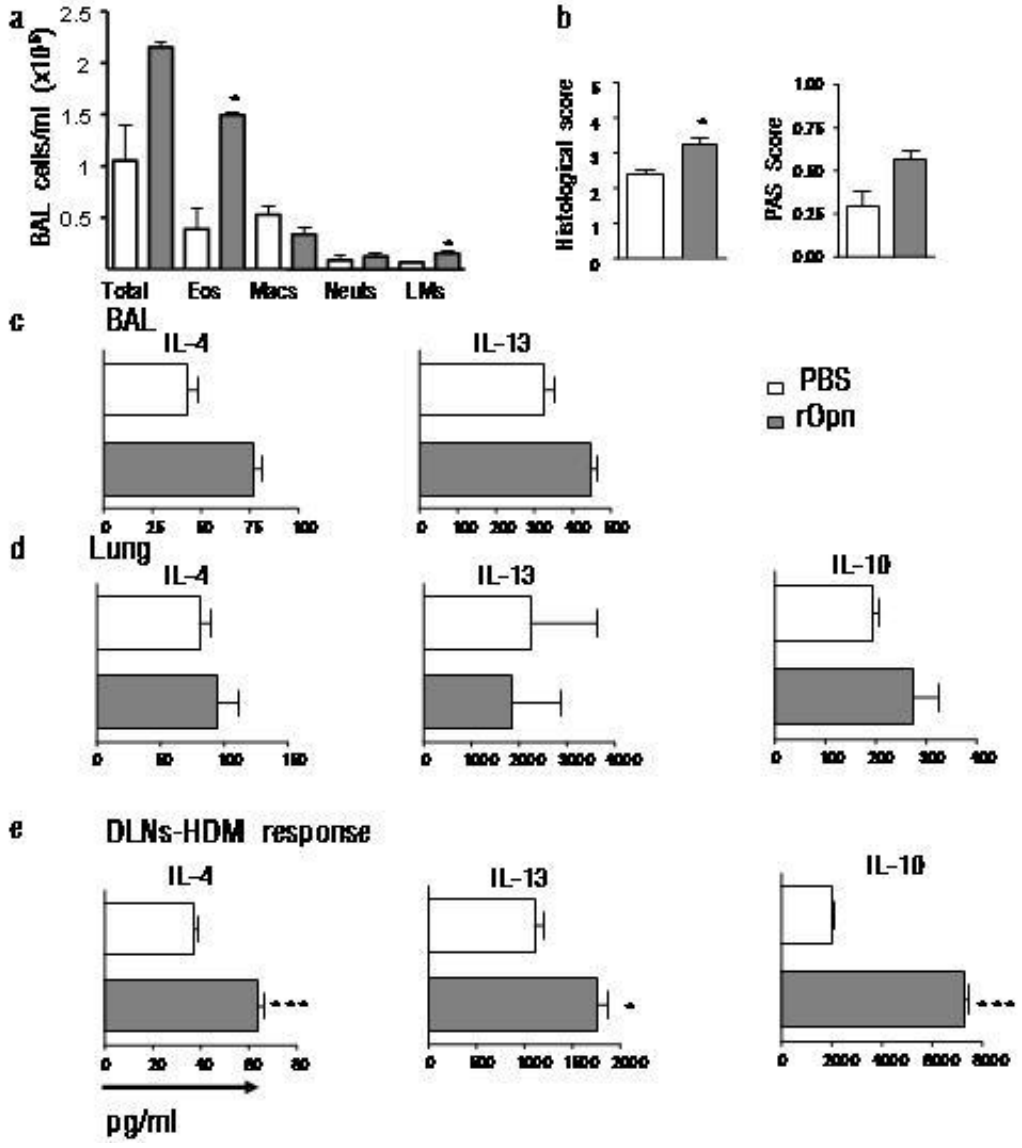


Figure legend. rOpn administration during priming enhanced Th2 responses to HDM *Opn-/-* or *wt* mice were sensitized i.n. with 150μg of HDM on day 0 and 50μg of HDM on day 7. On day 7 mice received one dose of rOpn (4 μg) or PBS i.n.. Mice were sacrificed 24h later. a) Differential cell counts in bronchoalveolar lavage (BAL) from *Opn-/-* mice treated with rOpn (grey bars) or with PBS (white bars) are shown

(eos, *p = 0.0335, L/Ms, *p = 0.0446). b) H&E (*p = 0.0232) and PAS histological scores, for mice which received rOpn (grey bars) or PBS (white bars) are also shown. c) IL-4 and IL-13 levels were measured in BAL supernatants by ELISA. d) IL-4, IL-13 and IL-10 levels were measured in lung homogenates by ELISA. e) IL-4 (*** p = 0.0005), IL-13 (* p = 0.0114), IL-10 (*** p < 0.0001) levels were measured by ELISA in the supernatants of DLN cells collected from mice treated with PBS or rOpn, pooled for each group and stimulated *in vitro* with 5μg/ml HDM for 48h. Data are expressed as mean ± SEM; n = 4-6 mice per group in two independent experiments. Statistical significance (between rOpn and PBS-treated mice) was obtained by unpaired Student's *t*-test.

DISCUSSION

Previous studies have demonstrated the impact of Opn on T_H1-associated immunity during ongoing immune responses against viral, bacterial and self antigens [137,170,271]. Our results point to dual and opposing effects of Opn-s on T_H2-mediated allergic airway disease: pro-inflammatory at primary systemic sensitization, and anti-inflammatory during pulmonary secondary antigenic challenge. Neutralization of Opn-s during initial antigenic encounter increased recruitment of regulatory PDCA-1^+Gr-1^+ pDCs in DLNs, which mediated decreased primary T_H2 responses. In contrast, Opn-s blockade during challenge enhanced T_H2 effector responses, mainly mediated by increased recruitment of T_H2-promoting cDCs in DLNs. Importantly, intranasal administration of rOpn during antigenic challenge reversed established T_H2 responses and protected from allergic disease.

In agreement with previous studies [91], our experiments revealed that pDCs were immuno-suppressive for T_H2 responses. pDC depletion, prior to Opn-s neutralization, restored OVA/alum-driven responses, revealing that the dampening effect of Opn-s neutralization during priming was mainly mediated by pDCs. This initial pDC-

mediated dampening in priming provided explanation for the subsequent decreased T_H2-mediated pathology following pulmonary challenge. Opn-blockade was also accompanied by decreased IFN-γ production while rOpn administration enhanced T_H2 priming accompanied by increased IFN-γ production. IFN-γ may participate in the Opn-s-mediated effect, particularly as decreased IFN-γ production during OVA/alum sensitization reduces priming [276]. Curiously, Opn-s neutralization at sensitization resulted in increased lung IFN-γ levels following challenge. In this setting, IFN-γ may exert an immuno-regulatory role, associated with the increased pDC numbers at priming. In support, adoptive transfer of pDCs during sensitization enhanced IFN-γ levels and protected from allergic airway disease [280], and induction of IFN-γ-producing regulatory T cells reduced allergic airway inflammation [285].

The implicit pro-inflammatory effect of Opn-s during priming was surprising, as one would expect that blocking a T_H1-inducer [230] at the initial point of T_H differentiation would upregulate T_H2 responses. However, it was rather Opn-s blockade during recall responses that resulted in enhanced allergic pulmonary inflammation and disease. The same effect was observed in mice treated with Opn-specific antibody during both sensitization and challenge phases (data not shown and **Fig. 4d**) and in *Opn*$^{-/-}$ mice which developed increased T_H2 responses. Previous studies have demonstrated that *Opn*$^{-/-}$ mice, during repetitive antigenic encounters, have decreased T_H1 immunity [136-137] and autoimmunity [138,176,247]. Our data imply that the previously-demonstrated effect of Opn-s in T_H1/T_H2 balance is operating predominantly during recall responses.

Opn-s neutralization during challenge increased DLN cDC and pDC numbers. In allergic airway disease, the most powerful immunogenic potential of $CD11c^+$ cells [90] stems from cDCs [91,133]. For example, blockade of the C5a receptor during allergic airway inflammation increased recruitment of cDCs, enhancing T_H2 responses [133]. Conversely, pDCs were suppressive during antigenic challenge, as revealed by our experiments. Importantly, in the absence of pDCs, Opn-s blockade still enhanced T_H2 responses and allergic disease. Therefore, the increased induction of cDCs upon Opn-s neutralization provides explanation for the exacerbation of T_H2-mediated disease. It is also likely that Opn-s neutralization induces a stronger T_H2 response due to the known effect of Opn-s on antigen-presenting-cells influencing T_H1/T_H2 balance [137]. In support,

local rOpn administration before challenge decreased T_H2 responses and increased IL-12 production.

To examine whether pDCs mediate the effect of Opn-s blockade, we used the 120G8 monoclonal antibody, which has been described as pDC specific and pDC depleting [91,133,284,286-287]. We found by flow cytometry that 120G8 strongly bound all pDCs from naive and OVA/alum-sensitized mice (data not shown). A recent study indicated that 120G8 binds to an epitope of the bone marrow stromal antigen-2 [288]. This study also showed that bone marrow stromal antigen-2 is primarily expressed on all pDCs and to a lesser degree on some immune (plasma) cells, following activation by IFN or virus [288]. Thus, in addition to pDCs, we cannot exclude the contribution of other cell types to the Opn-mediated effect on T_H2 responses.

Comparing results obtained from Opn-s neutralization to those from *Opn*$^{-/-}$ mice, we showed that Opn-s plays a predominant role in allergic airway inflammation. However, considering the critical role of Opn-i in CpG-mediated pDCs signaling [170], its involvement in T_H2 regulation is probable. Administration of CpG, alone or in conjunction with allergens, in lungs of allergic mice reversed established inflammation, possibly through an effect on IFN-α production by pDCs [289-290]. Remarkably, both isoforms affect pDCs: Opn-s regulates pDC recruitment in allergic response, as described here, while Opn-i is essential for pDC functions in viral immunity [170].

Increased Opn expression in allergic airway disease may be part of an inherent protective mechanism, as suggested by disease exacerbation following Opn-s blockade at challenge. In fact, *OPN* was recently demonstrated as a critical gene upregulated during bee-venom immunotherapy [291]. In our experiments, administration of rOpn at challenge protected from allergic disease. This was mainly mediated via a shift towards an anti-allergic Th1, as shown by increased IL-12 and OVA-specific-IgG2a levels. IL-12 intranasal administration during challenge suppresses airway disease [292]. Our data show for the first time that as with IL-12, rOpn is an effective regulator of allergic airway disease.

The variable effect of Opn-s on Th2 immunity points once more to cytokines playing opposing roles depending on the phase and milieu of the immune response. Opn-s

effects on pDC biology as well as its contribution to autoimmunity remain to be elucidated.

METHODS

Mice. BALB/c and OVA-specific T-cell receptor-transgenic DO11.10 (*Tcr*-tg-DO11.10) mice were purchased from The Jackson Laboratory. *Opn*$^{-/-}$ mice were backcrossed onto the C57BL/6 background for seven generations. Mice were housed at the Animal Facility of IIBEAA, fully accredited by the European Union Association for Assessment and Accreditation of Laboratory Animal Care and the NIH Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736–01). Animal protocols were performed according to guidelines of the European Union for animal research.

***In vivo* experimental protocols.** BALB/c mice were sensitized with 0.01 mg/mouse OVA (Sigma-Aldrich) in 0.2 ml alum (Serva) intraperitoneally (i.p.) on d 0 and 12. Control mice received PBS/alum. Mice received aerosolized OVA (5%, for 20 min) on d 18–20. Mice received (20 μg/mouse) affinity-purified neutralizing antibody to Opn (AF-808, R&D Systems) or Ig control (R&D Systems) i.p., 2–3 h before sensitization or challenge. OVA/alum-sensitized *Opn*$^{-/-}$ and *Opn*$^{+/+}$ littermate mice received six OVA challenges. In **Fig. 5a-c**, BALB/c or *Opn*$^{-/-}$ and *Opn*$^{+/+}$ mice received 40 μg/mouse of Opn-specific antibody or Ig control i.p. and 2–3 h later, were sensitized i.p. with 0.1 mg Alexa-Fluor-conjugated OVA/alum (LPS-low, Molecular Probes). CD11c^{+} cell-driven responses and DC subsets were examined 40 h later when these cells traffic to DLNs [91]. In **Fig. 5e**, BALB/c mice received 225 μg/mouse/d of 120G8 pDC-depleting antibody or Ig control (rat IgG1/κ, BD Biosciences) i.p., between d –3 and 0. On d 0, mice received 40 μg/mouse of Opn-specific antibody or Ig control i.p., and 2–3 h later, were sensitized i.p. with 0.1 mg Alexa-Fluor-OVA/alum. Mice were sacrificed 40 h following sensitization. In **Fig. 5f**, BALB/c mice were sensitized with OVA/alum i.p. on d 0 and 12 and challenged intranasally (i.n.) with one or three doses of 0.5 mg Alexa-Fluor-OVA starting on d 18. Opn-specific antibody or Ig control (40 μg/mouse) were administered i.p. 2–3 h before challenge. In **Fig. 5g**, **h**, BALB/c mice were sensitized with 10 μg of OVA/alum i.p. on d 0 and 12 and received 225 μg/mouse/d of 120G8 pDC-depleting antibody or Ig control (i.p.), between d 17–20. Mice also received 20 μg/mouse/d of Opn-specific antibody or Ig control

i.p., 2–3 h before OVA challenge, between d 18–20. Mice were sacrificed 40 h after the final challenge. In **Fig. 6a**, BALB/c mice received 4 μg/mouse of mouse rOpn (R&D Systems) or PBS i.p. and 2–3 h later, were sensitized i.p. with 0.1mg Alexa-Fluor-OVA/alum. In **Fig. 6b-g**, BALB/c mice were sensitized with OVA/alum i.p. on d 0 and 12 and challenged i.n. with 0.5 mg Alexa-Fluor-OVA between d 18–20. rOpn (3.5 μg/mouse) or PBS were administered i.p. 2–3 h before challenge. Mice were sacrificed 40 h after the final challenge.

Analysis of BAL. BAL harvesting was performed as described previously [293]. Briefly, inflammatory cells were obtained by cannulation of the trachea and lavage of the airway lumen with PBS. Cytospin slides were prepared by Wright-Giemsa staining. All differential counts were performed blind and in a randomized order at the end of the study.

AHR. AHR, a clinical measurement of asthma, and BAL were performed, as previously described [293]. Paraffin-embedded sections were stained with haematoxylin & eosin (H&E) or Periodic-Acid-Schiff (PAS), as previously described [36].

Human Subjects. Flexible bronchoscopy was performed on asthmatics, classified and treated according to the Global Initiative for Asthma guidelines (one mild intermittent, one moderate and four severe), and nine healthy volunteers. Biopsies were taken as previously described [294]. The study was approved by the Sotiria Hospital Ethics Committee and individuals signed an informed consent form.

Immunohistochemistry. Paraffin-embedded sections were immuno-stained as previously described [293]. Antibodies to human OPN (MAB-1433, R&D Systems) and to mouse Opn (AF-808, R&D Systems) were used. Matched isotype IgG (R&D Systems) were used as controls.

Cell culture, proliferation and cytokine analysis. Lung homogenates were obtained as previously described [293]. DLN (mediastinal, following i.n. sensitization or challenge and inguinal and axillary during i.p. sensitization) cells were isolated, as previously described [295]. DLN cells, alone or with $CD4^+$ T cells (Dynal) from DO11.10 mice, were cultured with 125 μg/ml OVA for 48 h. $CD11c^+$ cells purified

from DLNs (Myltenyi Biotec) were co-cultured with Th cells from DO11.10 mice and 125 μg/ml OVA for 48 h. For pDC and cDC isolation, a combination of the above-described method with the pDC isolation kit (Myltenyi Biotec) was used. Proliferation assays were performed as previously described [295]. Cytokines and chemokines were measured using ELISA kits for IL-4, IL-10, IFN-γ, IL-12 (BD Biosciences) and IL-13, Opn, CCL11, CCL22 and CCL17 (R&D Systems). A newer kit was used for IL-13 in pDC depletion experiments (R&D Systems). $CD11c^+$ cells were cultured with 0.2 μg/ml CpG-oligodeoxynucleotides (5'-TCCATGACGTTCCTGATGCT-3') or control GpC (5'-TCCATGAGCTTCCTGATGCT-3') (MWG, Biotech), synthesized as described [281]. IFN-α was measured 24 h later, by ELISA (PBL Biomedical Laboratories).

Serum antibody concentration. OVA-specific IgE, -IgG1 and -IgG2a antibodies were measured as described [296].

Flow-cytometric analysis. Live DLN cells ($7AAD^-$, BD Biosciences) were stained with conjugated antibodies to CD4, CD3, CD11c, B220, CD11b, Gr-1, PDCA-1 (BD Biosciences) and T1/ST2 (MD Biosciences). FACS analysis was performed using a Coulter cytometer (Cytomics, FC 500).

CHAPTER 2

A key role for osteopontin in promoting peripheral tolerance

ABSTRACT

Osteopontin (Opn) is mainly known as an effector cytokine of T_H1- and T_H17-mediated diseases. However, Opn is also highly expressed in activated $Foxp3^+$ T_{regs}, and has an anti-inflammatory effect in T_H2-type allergic airway responses. Thus, we investigated whether Opn has an impact on peripheral tolerance. Opn-deficient mice were impaired in mounting antigenic tolerance against a T_H2-driven asthma model disease, while administration of rOpn had the opposite effect. rOpn promoted tolerance by expansion of suppressive plasmacytoid dendritic cells (pDCs) and expansion of $Foxp3^+$ T_{regs}. In fact, rOpn was a pDC survival factor that instructed these cells to generate T_{reg} cells, and also promoted the expansion of $Foxp3^+$ T_{regs}. Overall, these data identify Opn as a key cytokine controlling peripheral tolerance.

INTRODUCTION

The mechanisms of central and peripheral tolerance are crucial for maintaining homeostasis in the immune system and prevent exaggerated immune responses to intrinsically harmless self or foreign antigens. For example, adequate peripheral tolerance prevents healthy individuals from mounting pathogenic immune responses to inhaled allergens. Failure of this tolerance mechanism could be at the basis of allergic asthma, a disease caused by aberrant T_H2 immune responses to inhaled allergens and leading to eosinophilic airway inflammation, mucus hyper-secretion and variable airway obstruction[11,297].

As the incidence of allergic disease has risen dramatically, much effort has been put in determining the control mechanisms of peripheral tolerance to allergens in the hope of finding a treatment or prevention strategy for allergic disease. Regulatory T cells (T_{reg}) that are either Foxp3$^+$ or Foxp3$^-$ are important suppressors of dysregulated T_H2 responses to inhaled antigens, as constitutive or induced deficiency of these cells leads to severe asthmatic reactions[98,127,285,298-299]. Successful immunotherapy for allergy as well as natural desensitization is accompanied by a rise in antigen-specific T_{reg} cells[300-301]. Likewise, several groups have demonstrated that both conventional (c) as well as plasmacytoid (p) dendritic cells (DC) are key regulators of T_H2 responses in allergic airway inflammation[91,98,132,285,302-303]. As in many processes of immunoregulation, cytokines like TGFβ and IL-10 are also important regulators of tolerance to inhaled antigens, that could be exploited to treat allergy in the future [304-305].

Osteopontin (Opn) is a cytokine originally reported as essential for efficient development of T_H1-mediated immune responses and is expressed by activated T cells, DCs and macrophages[137-138]. Dysregulated expression of Opn, has been associated with several types of autoimmune diseases, where T_H17 cells play also an important role[273,306-309]. Our group has demonstrated that Opn is also implicated in the regulation of T_H2-type allergic airway inflammation. Opn is highly expressed in the lungs of asthmatic patients and in the lungs of mice with allergic airway inflammation[310]. Recent data suggest that Opn might also have immunoregulatory effects, as it is highly expressed in activated Foxp3$^+$ T_{reg} cells[311]. Several reports have

described that Opn is an important cytokine for regulating aspects of DC biology, and might therefore indirectly impact on many adaptive immune responses[170,226-227,310,312].

Although both DCs and T_{regs} are crucial controllers of peripheral tolerance, it has not been addressed if and how Opn impacts on peripheral tolerance. Thus, we investigated the *in vivo* role of Opn in peripheral tolerance induction to the model antigen ovalbumin (OVA), using an established OVA-mouse model of asthma as the readout system.

RESULTS

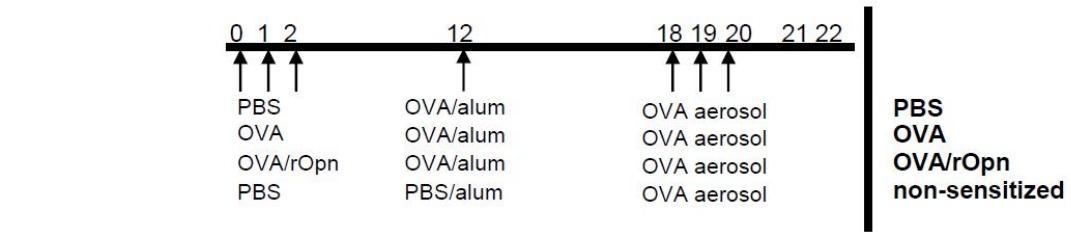
rOpn promotes antigenic tolerance and enhances protection from allergic airway disease.

To address the effects of Opn on peripheral tolerance induction, we used a protocol in which tolerizing endotoxin-free chicken ovalbumin (OVA) was injected intraperitoneally (i.p.) together with rOpn or PBS. Tolerization was assessed by subsequently immunizing mice with OVA in the T_H2 adjuvant alum, followed by a series of OVA aerosol exposures, a classical model of asthma in the mouse[36] (**Fig. 1a**). As expected, mice treated with PBS prior to disease induction exhibited significantly elevated allergic responses compared to the non-sensitized group, as indicated by enhanced pause (Penh) measurement (**Fig. 1b**), inflammation in the bronchoalveolar lavage (BAL) and the lung (**Fig. 1c and d**). Mice receiving tolerizing OVA prior to disease induction, had significantly decreased BAL and lung tissue eosinophilia and lymphocytosis, as well as goblet cell hyperplasia (**Fig. 1c and d)**, compared to mice treated with PBS. Moreover, OVA-treated mice had significantly reduced Penh (**Fig. 1b**).

In OVA/rOpn-treated mice, an additional 2-fold decrease in the numbers of BAL eosinophils (**Fig. 1c**) and in Penh (**Fig. 1b**) was noted, as compared to OVA-treated mice, reaching levels similar to those of the non-sensitized group (**Fig. 1b**). Lung leukocytic infiltration and mucus secretion were also further decreased (**Fig. 1d**). Most importantly, OVA/rOpn treatment, resulted in significantly decreased levels of interleukin (IL)-4, IL-13, IFNγ and IL-10 in the BAL as well as in supernatants of draining mediastinal lymph nodes (MLN) cell cultures stimulated *ex vivo* with OVA, compared to OVA-treated mice (**Fig. 1e and f**). Moreover, OVA/rOpn administration resulted in a 2-fold reduction of OVA-specific T_H cell proliferation to the levels of non-sensitized responders (**Fig. 1g**).

These results indicate that rOpn administration promotes peripheral tolerance to OVA resulting in suppressed T_H2 allergic responses and airway disease.

Figure 1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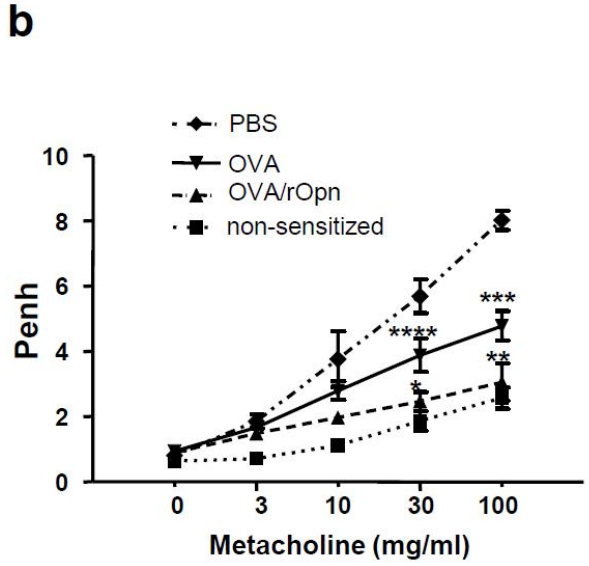
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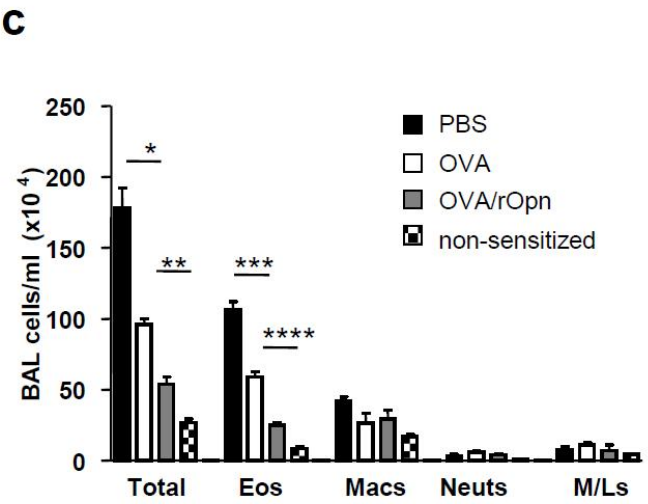
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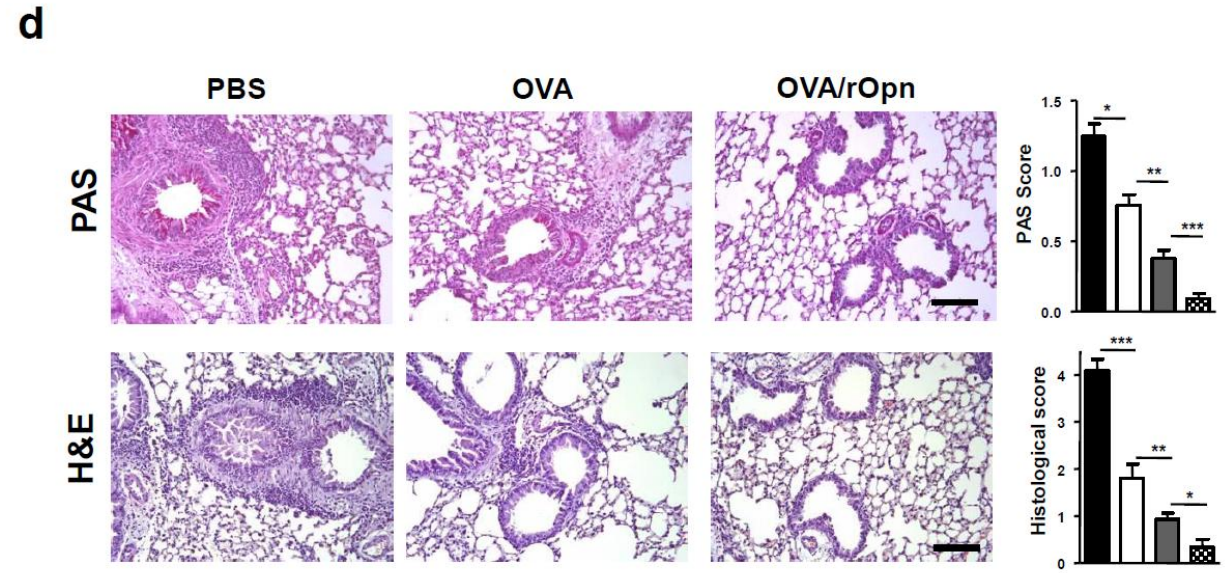

d
PBS
OVA
OVA/rOpn
PAS
H&E
PAS Score
Histological score

Figure 1 cont.

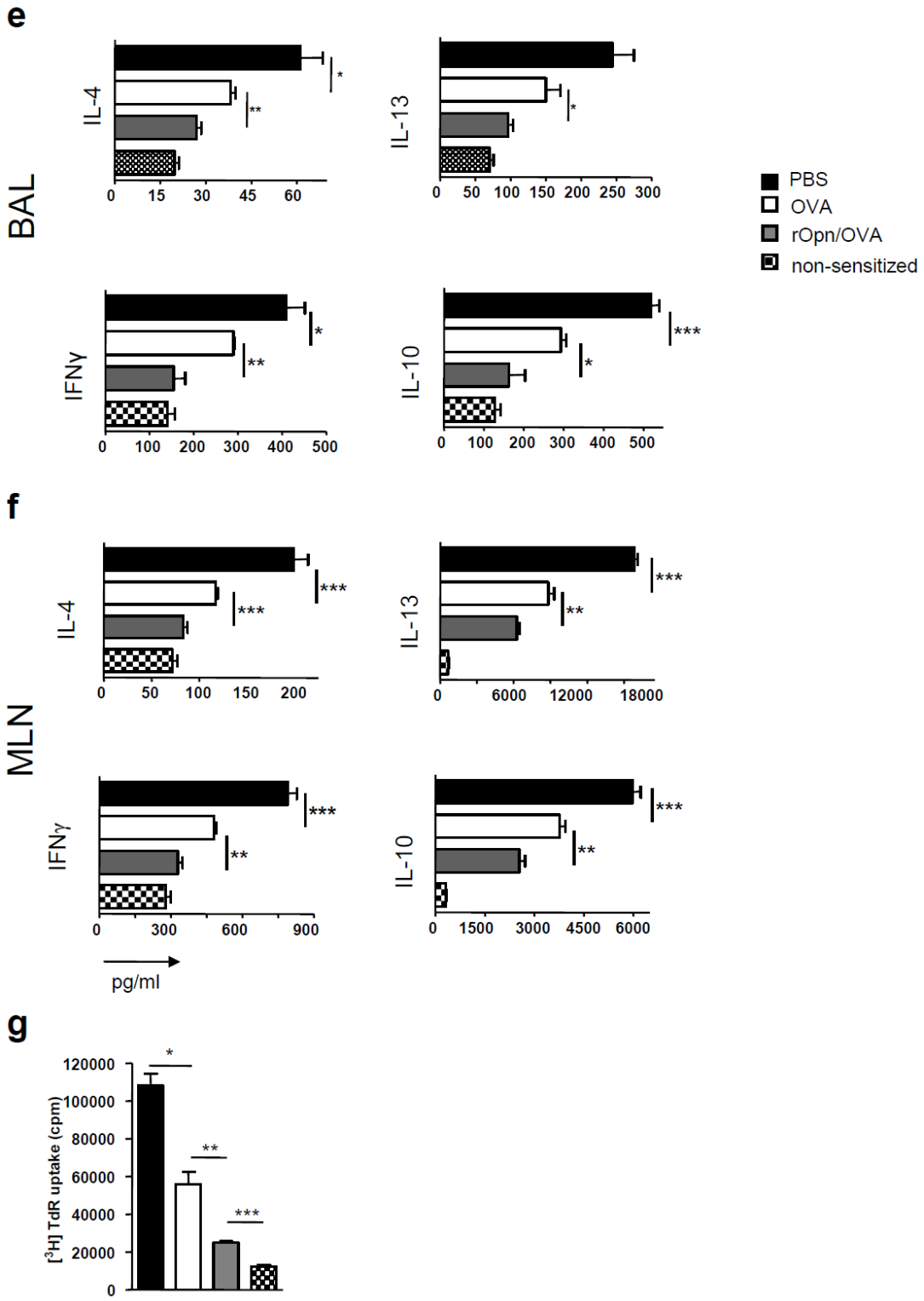


Figure 1 rOpn boosts antigenic tolerance and enhances protection from allergic airway disease. (a) Experimental protocol used for tolerance induction. (b) AHR responses for pause-enhanced (PenH). (**P*=0.003, ***P*=0.002, ****P*<0.0001,

****P=0.001, two-way repeated-measures analysis of variance (ANOVA), and unpaired Student's t-test). One representative experiment of three. (c) Differential cell counts in BAL from PBS-treated (black bars), OVA-treated (white bars), OVA-rOpn treated (grey bars), and non-sensitized (pattern bars) mice are shown. (*P=0.0006, **P=0.0002, ***P<0.0001, ****P=0.0002). Data are expressed as mean ± SEM; n = 8 mice per group, three independent experiments. Statistical significance was studied by unpaired Student's t-test. (d) Lung inflammation (top) (*P=0.0021, **P=0.0042, ***P=0.0017) and mucus secretion (bottom) (***P=0.0002, **P=0.0141, *P=0.0157, unpaired Student's t-test). Scale bar, 100 μm. (e) BAL levels of IL-4 (*P=0.0241, **P=0.0015), IL-13 (*P=0.0405), IFNγ (*P=0.02, **P=0.0019) and IL-10 (*P=0.0182, ***P=0.0002). Statistical significance was studied by unpaired Student's t-test, three independent experiments. (f) Levels of IL-4 (***P=0.0003, IL-13 ***P<0.0001, **P=0.0036), IFNγ (***P<0.0001, **P=0.0005) and IL-10 (***P<0.0001, **P=0.0021) in supernatants of OVA-stimulated DLN cells. (g) [^{3}H]-thymidine incorporation of OVA-stimulated DLN cells (*P=0.0025, **P=0.0011, ***P<0.0001). Statistical significance was studied by unpaired Student's t-test, three independent experiments.

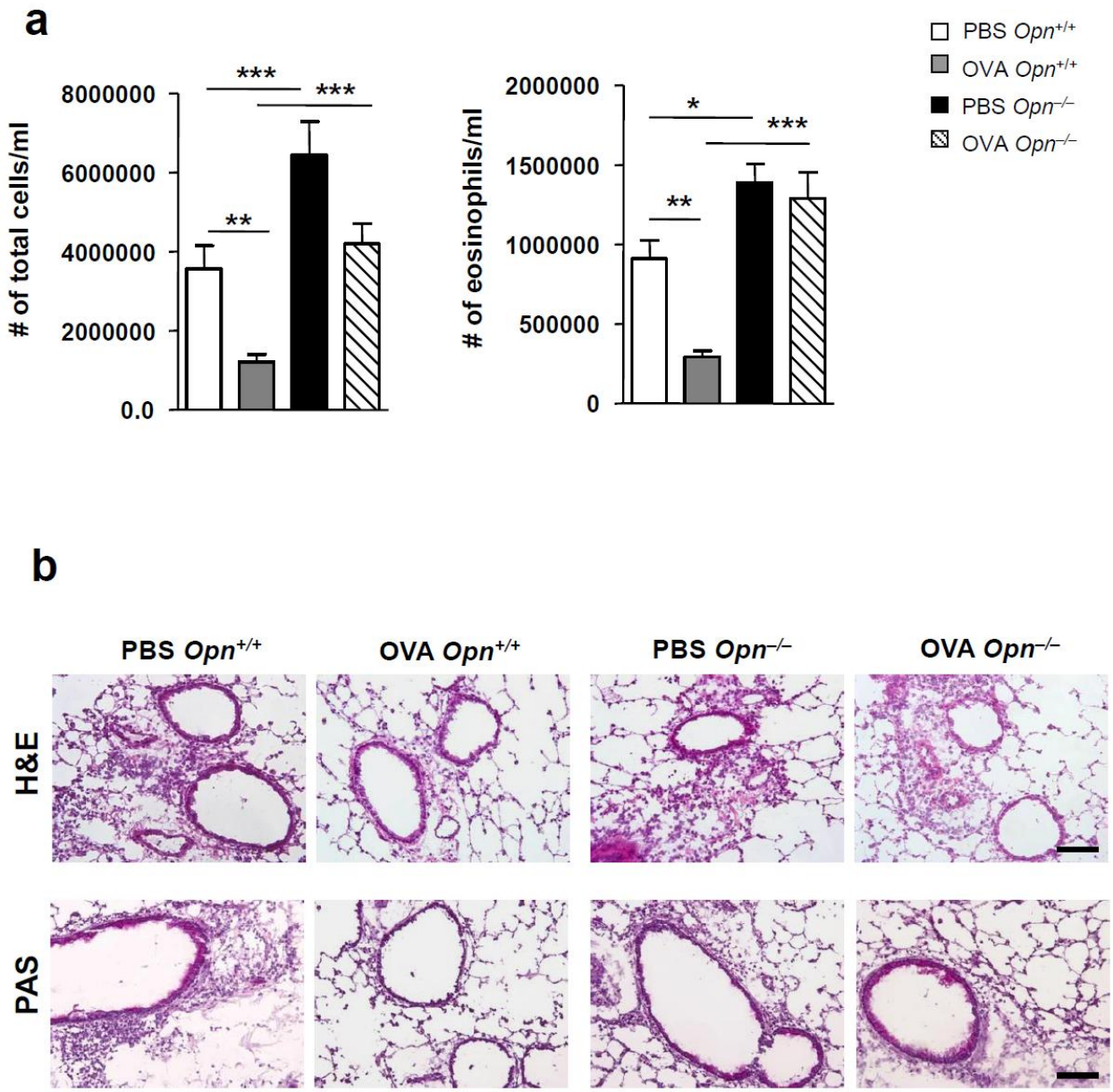
Opn$^{-/-}$ mice are resistant to tolerance induction.

We next investigated whether deficiency of endogenous Opn had any effect on tolerance induction. For this we subjected *Opn*$^{-/-}$ and *Opn*$^{+/+}$ mice to the protocol described in Figure 1. As expected, treatment of *Opn*$^{+/+}$ mice with OVA prior to OVA/alum sensitization resulted in 66% and 68% reduction of total cells and eosinophils respectively, in the BAL fluid, compared to PBS-treated *Opn*$^{+/+}$ mice (**Fig. 2a**). However, when OVA was administered to *Opn*$^{-/-}$ mice, the reduction of inflammatory cells in the BAL fluid, was far less pronounced compared to the one observed following OVA administration in *Opn*$^{+/+}$ mice (35% reduction in total cells and 8% reduction in eosinophils) (**Fig. 2a**). Furthermore, OVA administration in *Opn*$^{-/-}$ mice could not dampen the inflammation and mucus secretion in the lung as efficiently as when administered in *Opn*$^{+/+}$ mice (**Fig. 2b**).

OVA–stimulated MLN cells from OVA-treated *Opn*$^{+/+}$ mice produced approximately 50% lower levels of T_H2 cytokines, compared to cells from PBS-treated *Opn*$^{+/+}$ mice, whereas administration of OVA to *Opn*$^{-/-}$ mice resulted in a smaller reduction (reduction of cytokine levels following OVA-treatment in *Opn*$^{+/+}$ vs *Opn*$^{-/-}$ respectively IL-4: 51% vs 31%, IL-5: 49% vs 14%, IL-13: 57% vs 24%, IL-10: 41% vs 20%) (**Fig. 2c**). The decrease in levels of IFNγ was the same among groups (26% vs 28%) (**Fig. 2c**).

Finally, OVA administration to *Opn*$^{-/-}$ mice resulted in smaller decrease in the percentages of $CD3^+CD4^+$ proliferating cells in cultures of MLN cells, compared to cultures of MLN cells from OVA-treated wild-type mice (65% in *Opn*$^{+/+}$ vs 28% in *Opn*$^{-/-}$ mice) (**Fig. 2d**). The above results demonstrate that *Opn* deficient mice are less competent to develop tolerance in this setting, however tolerance was not fully abrogated. The levels of T_H2 responses in OVA-treated *Opn*$^{-/-}$ mice were still lower than those of the PBS-treated *Opn*$^{-/-}$ mice. Notably however, *Opn*$^{-/-}$ mice develop exaggerated T_H2 responses following OVA/alum sensitization, as previously reported[310].

Figure 2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PBS Opn+/+
OVA Opn+/+
PBS Opn–/–
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Figure 2 cont.

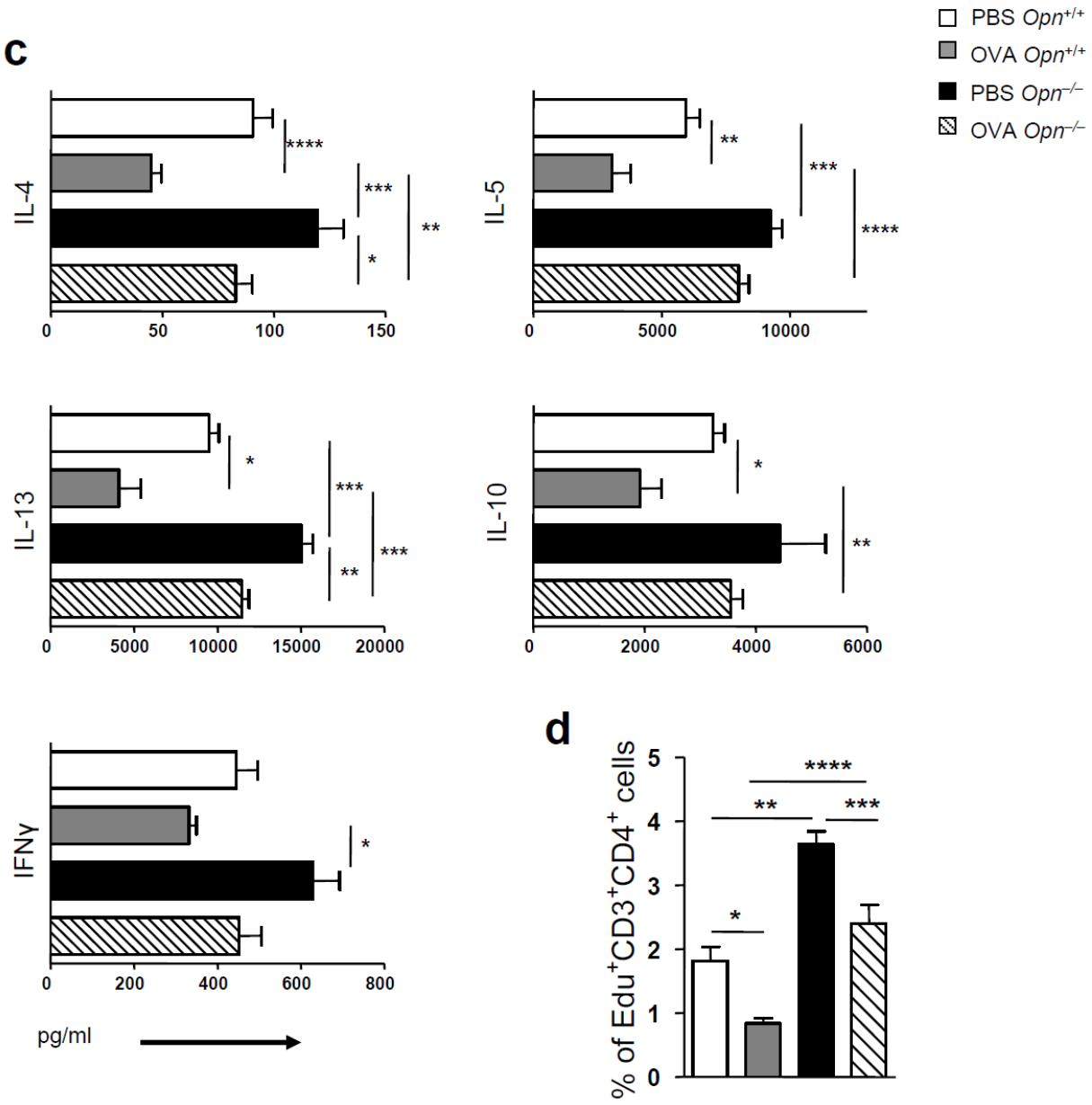


Figure 2 *Opn–/–* mice are more resistant to tolerance induction. OVA-treated *Opn–/–* or *Opn*+/+ mice received three doses of endotoxin-free OVA i.p. on days 0, 1 and 2. On day 12, mice were sensitized i.p. with OVA in alum and were subsequently challenged through the airways with aerosolized OVA between days 18-20. PBStreated *Opn–/–* or *Opn*+/+ mice received one i.p. injection with OVA in alum on

day 12 and were then challenged with aerolized OVA between days 18-20. (a) Differential cell counts in BAL from PBS-treated *Opn*+/+ mice (white bars), OVA treated *Opn*+/+ (grey bars), PBS-treated *Opn*–/– (black bar), and OVA-treated *Opn*–/– (striped bar) mice are shown (total cells: ***P<0.0001, **P=0.0021, eosinophils: *P=0.0154 **P=0.0028, ***P=0.0011). Data are expressed as mean ± SEM; n = 8 mice per group. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (b) Lung inflammation (top) and mucus secretion (bottom) (unpaired Student's t-test). Scale bar, 100 μm. (c) Levels of IL-4 (****P=0.0008, ***P=0.0002, **P=0.0018, *P=0.0164), IL-5 (**P=0.0053, ***P=0.0001, ****P=<0.0001), IL-13 (*P=0.0003, **P=0.0002, ***P<0.0001), IL-10 (*P=0.0058, **P=0.0012) and IFNγ (*P=0.0170) in supernatants of OVA-stimulated DLN cells. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (d) Percentages of Edu+CD3+CD4+ OVA-stimulated DLN cells (*P=0.0143, **P=0.0037, ***P=0.0066, ****P=0.00250). Statistical significance was studied by unpaired Student's t-test, three independent experiments.

rOpn promotes tolerance through Foxp3$^+$ T$_{reg}$ cells.

To better understand how rOpn promotes tolerance leading to inhibition of T_H2 responses, we investigated the events occurring early (1 week) following the administration of rOpn and prior to disease induction. In fact, we examined the presence of Foxp3$^+$ T regulatory (T_{reg}) cells, considered an important cell subset suppressing immune responses[313-315], in the DLNs of the peritoneal cavity[316]. The protocol used for these studies involved administration of endotoxin-free OVA along with either rOpn or PBS for three constitutive days. One week after the last treatment, we examined the numbers of Foxp3$^+$ T_{regs} in the DLNs. Mice treated with OVA/rOpn had a 2-fold increase in percentages and total numbers of CD4$^+$Foxp3$^+$ T_{reg} cells (per mouse: 354,800 ± 78,540 cells for OVA/rOpn versus 150,300 ± 16,580 cells for OVA, P = 0.0290) compared to OVA-treated mice (**Fig. 3a**). We could not track any significant differences among groups in the numbers of Foxp3$^+$ T_{regs} in non-DLNs

(data not shown). We also examined the numbers of Foxp3$^+$ T_{reg} cells in the MLNs after OVA administration followed by sensitization with OVA/alum and airway challenges with OVA (protocol described in figure 1). Once again we noticed that, even following disease induction, we could still find increased numbers of CD4$^+$ Foxp3$^+$ T_{reg} cells in mice tolerized with OVA/rOpn compared to OVA-treated group (data not shown). Our results demonstrate for the first time that rOpn leads to Foxp3$^+$ T_{reg} accumulation in the LN draining the site of peripheral tolerance induction.

We next investigated whether DLN cells from OVA/rOpn-tolerized mice, containing higher numbers of T_{reg} cells, exerted increased suppressive activity. For this we co-cultured DLN cells from either OVA/rOpn or OVA-tolerized mice with naïve responders obtained from OVA-specific TCR Tg DO11.10 mice. DLN cells from OVA/rOpn-treated mice suppressed T_H2 cytokines IL-4 and IL-13 and enhanced IL-10 production, as compared to OVA-treated DLN cells (**Fig. 3b**). IFNγ levels where similar among groups (**Fig. 3b**). Importantly, DLN cells from OVA/rOpn-treated mice were able to induce a 2-fold inhibition of the OVA-specific proliferation of T cells compared to DLN cells from OVA-treated mice (**Fig. 3c**), suggesting that these cells have suppressive activity.

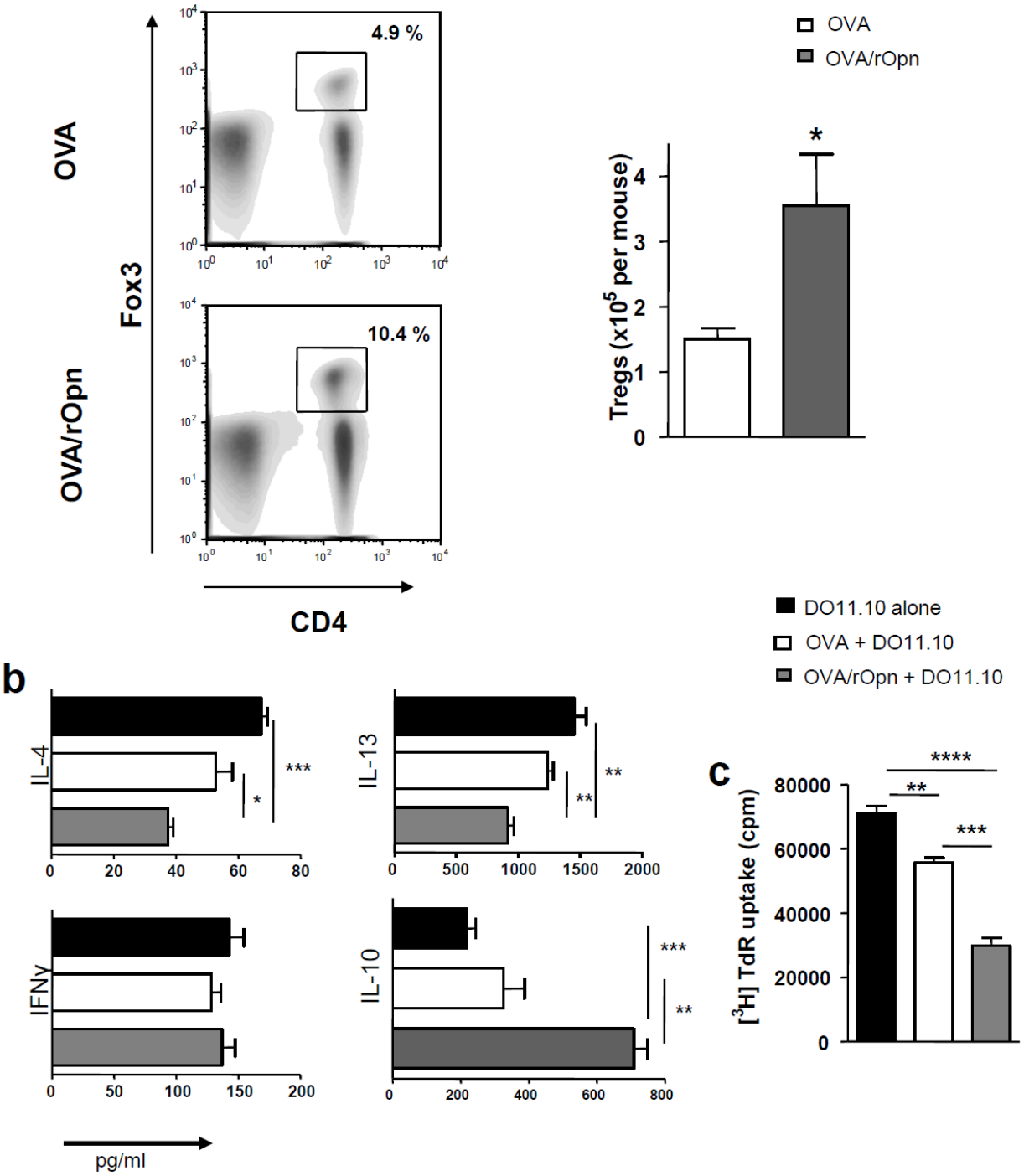
To test whether induction or expansion of Foxp3$^+$ T_{regs} was indeed the mechanism by which rOpn promotes tolerance, we depleted Foxp3$^+$ T_{regs} using "depletion of regulatory T cell" (DEREG) mice[317]. These mice express a diphtheria toxin (DT) receptor-enhanced green fluorescent fusion protein under the control of the *foxp3* gene locus, allowing selective and efficient depletion of Foxp3$^+$ T_{reg} cells after DT injection. In order to deplete Foxp3$^+$ T_{regs}, Tg mice were given two DT or PBS injections prior to PBS/rOpn-OVA administration (**Fig. 3d**). Administration of OVA prior to OVA/alum sensitization was able to induce tolerance and dampen T_H2 responses in DEREG mice (**Fig. 3e-h**). Furthermore, when OVA was mixed with rOpn, there was additional suppression of the T_H2 responses, compared to the one induced by administration of OVA alone (**Fig. 3e-h**).

Of great importance, T_{reg} depletion in DEREG mice abolished tolerance induction as OVA-treated T_{reg}-depleted mice exhibited significantly increased BAL and lung inflammation (**Fig. 3e,f**), and T_H2 specific responses to OVA (**Fig. 3g,h**), in

comparison to their respective OVA treated non-T_{reg}-depleted mice, reaching levels similar to PBS-treated group (**Fig. 3e-h**). Thus, selective depletion of $Foxp3^+$ T_{reg} cells abrogated antigenic tolerance induction, providing for the first time direct evidence that these cells are essential for antigenic tolerance.

Notably, depletion of T_{regs} in DEREG mice also abolished the potential of OVA/rOpn administration to reduce total numbers of BAL cells and eosinophils, or to decrease inflammation and mucus cell hyperplasia in the lung (**Fig. 3e,f**). The levels of IL-5, IL-13, IL-10 cytokines and the percentages of proliferating cells in OVA-specific DLN responses were significantly enhanced (approximately 5-6-fold) following rOpn treatment in T_{reg} depleted mice (**Fig. 3g,h**), indicating that rOpn promotes tolerance through T_{reg} cells.

Figure 3


a
OVA
OVA/rOpn
Fox3
CD4
4.9 %
10.4 %
OVA
OVA/rOpn
Tregs (x10^5 per mouse)
*
b
DO11.10 alone
OVA + DO11.10
OVA/rOpn + DO11.10
IL-4
IL-13
IFNγ
IL-10
pg/ml
c
[3H] TdR uptake (cpm)
****
**
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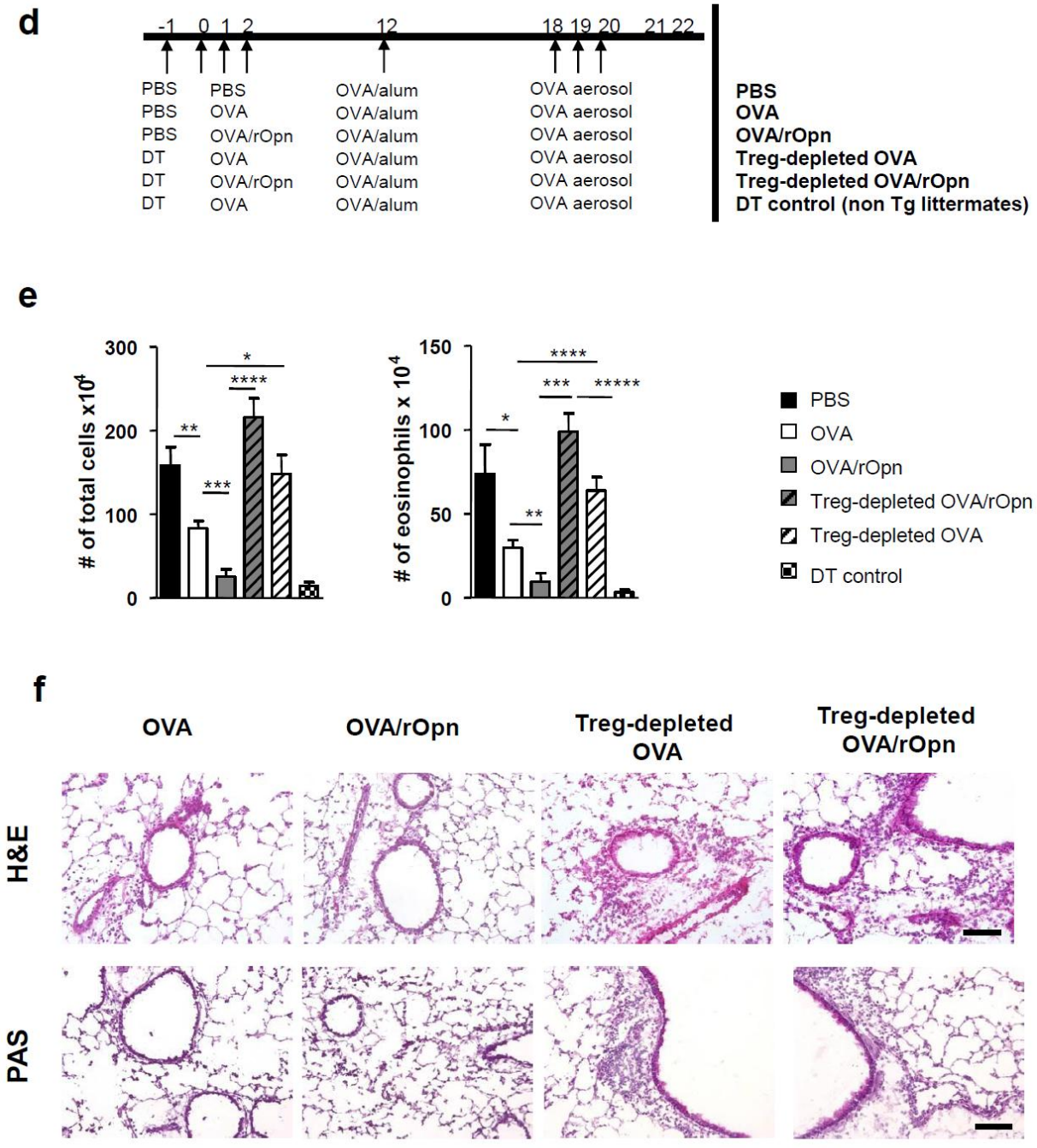
d
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PBS OVA OVA/alum OVA aerosol
PBS OVA/rOpn OVA/alum OVA aerosol
DT OVA OVA/alum OVA aerosol
DT OVA/rOpn OVA/alum OVA aerosol
DT OVA OVA/alum OVA aerosol
PBS
OVA
OVA/rOpn
Treg-depleted OVA
Treg-depleted OVA/rOpn
DT control (non Tg littermates)
e
# of total cells x10^4
300
200
100
0
# of eosinophils x 10^4
150
100
50
0
PBS
OVA
OVA/rOpn
Treg-depleted OVA/rOpn
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PAS

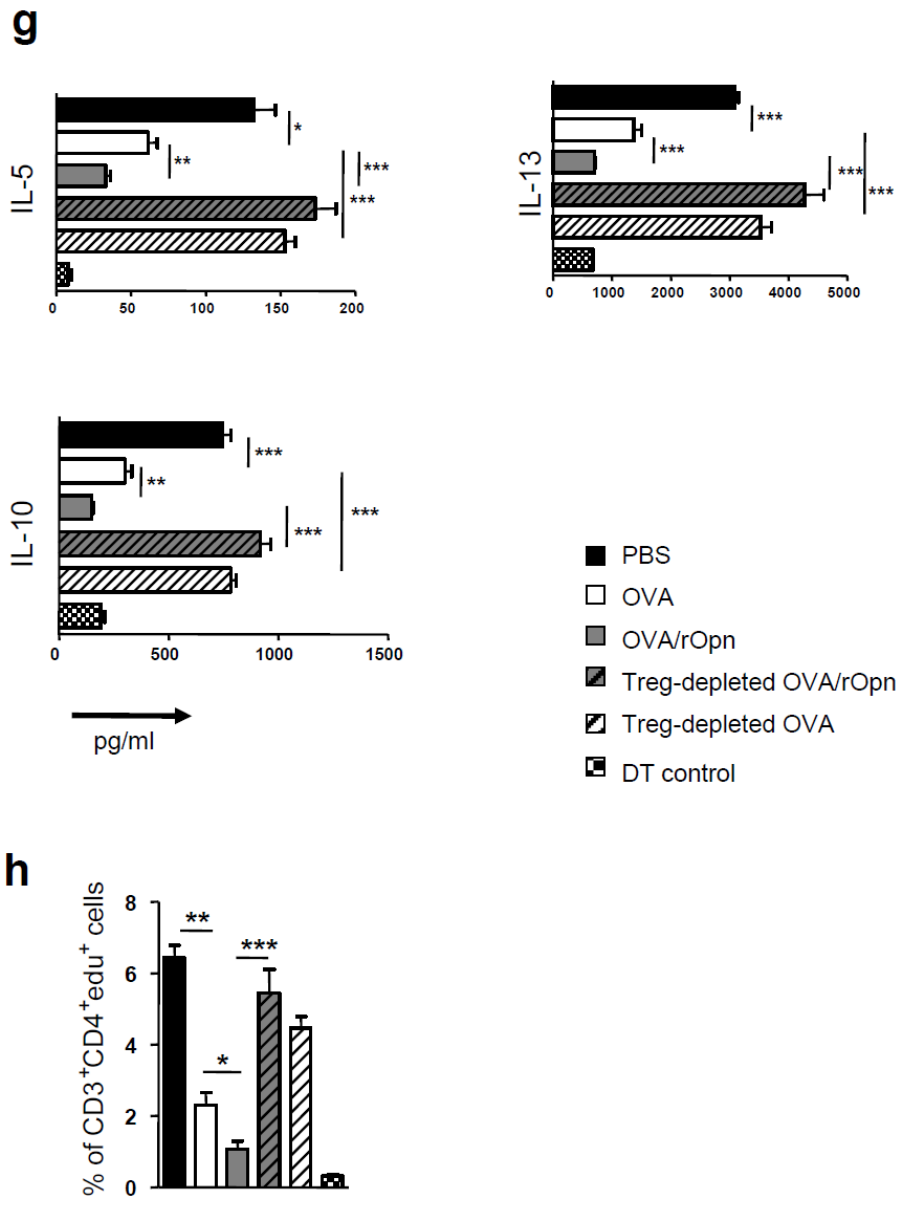


Figure 3 rOpn boosts tolerance through Treg cells. (**a**) Mice received endotoxin-free OVA i.p. on days 0, 1 and 2 along with rOpn or PBS and were euthanized 1 week later. Percentages of CD4+Foxp3+ cells from OVA-treated mice (upper left) or OVA/rOpn-treated mice (bottom left), one representative experiment of three.

Numbers of CD4+Foxp3+ cells from OVA-treated mice (white bar) or OVA/rOpntreated mice (grey bar) (*P=0.004). Results are expressed as mean ± s.e.m. n = 4–6 mice per group, three independent experiments. Statistical significance was studied by unpaired Student's t-test. (**b**) IL-4 (*P=0.0279, ***P<0.0001), IL-13 (**P=0.0017), IFNγ and IL-10 (**P=0.0017, ***P<0.0001), levels from co-cultures of OVAstimulated DLN cells with DO11.10 cells. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (**c**) [3H]-thymidine incorporation (TdR) of co-cultured OVA-stimulated DLNs cells with DO11.10 cells (**P=0.0047, ***P=0.0001, ****P<0.0001). n = 4-6 mice per group, three experiments. c.p.m.: counts per min. (d) Protocol used for tolerance induction in DEREG mice. (e) Numbers of total cells in the BAL (left panel) (**P=0.0069, ***P=0.0007, ****P<0.0001, *P=0.0297) and numbers of eosinophils in the BAL (right panel) (*P=0.0280, **P=0.0157, ***P<0.0001, ****P=0.0274, *****P=0.0041) of PBS-treated (black bar), OVA-treated (white bar), OVA/rOpntreated (grey bar), Treg-depleted OVA-treated (white striped bar) and Treg-depleted OVA/rOpn-treated (grey striped bar) DEREG mice and DT administered as control to non DEREG Tg mice (white blocked bar). Statistical significance was studied by unpaired Student's t-test, three independent experiments. (**f**) Lung inflammation (top) and mucus secretion (bottom) (unpaired Student's t-test). Scale bar, 100 μm. (**g**) Levels of IL-5 (*P=0.001, **P=0.0015, ***P<0.0001), IL-13 (***P<0.0001) and IL-10 (**P=0.0009, ***P<0.0001) in supernatants of OVA-stimulated DLN cells (**h**) Percentages of Edu+CD3+CD4+ of OVA-stimulated DLN cells (*P=0.0437, **P=0.0032, ***P=0.0012). Statistical significance was studied by unpaired Student's t-test, three independent experiments.

rOpn promotes tolerance through pDCs.

pDCs have consistently been described to suppress immune responses to antigens [91,132,287,310]. Due to known effects of Opn on DC subsets[212,310,318 319], and in an attempt to further delineate the mechanism of rOpn action during tolerance, we examined the effect of Opn on pDCs and cDCs early (40hrs) following tolerance induction. Mice treated with OVA/rOpn had an approximately 2-fold increase in the percentages and total numbers of $7AAD^{-}CD3^{-}CD19^{-}CD11c^{+}Gr\text{-}1^{+}PDCA\text{-}1^{+}$ pDCs in the DLNs (23,230 ± 844.8 cells per mouse for OVA/rOpn versus 15,360 ± 590.4 cells for OVA $P < 0.0001$) (**Fig. 4a**) compared to treatment with OVA alone, while there were no differences in the numbers of immunogenic cDCs in the DLNs ($7AAD^{-}CD3^{-}CD19^{-}$ $CD11c^{+}B220^{-}Gr\text{-}1^{-}$) of OVA and OVA/rOpn-treated groups (19,700 ± 608.3 cells per mouse for OVA/rOpn versus 19,110 ± 757.2 cells for OVA, $P = 0.5763$) (**Fig. 4a**). Further characterization of pDCs, revealed that OVA/rOpn administration resulted in significantly increased expression of CD86 on this cell subset (data not shown).

To test whether pDCs mediate enhancement of tolerance by Opn, we depleted this subset prior to tolerance induction. For this, mice were injected with the 120G8 antibody, specific for efficient pDC depletion[284], before induction of tolerance. Immune parameters were analyzed one week later. In order to probe for induction of a suppressive activity in OVA-specific responses, we co-cultured DLN cells from treated mice with naïve responders from DO11.10 mice. DLN cells from OVA-treated pDC-depleted mice had notably lower suppressive activity on DO11.10 cells, compared to cells obtained from OVA-treated non-pDC-depleted mice, demonstrating the suppressive function of pDCs in the context of antigenic tolerance induction (**Fig. 4b-c**).

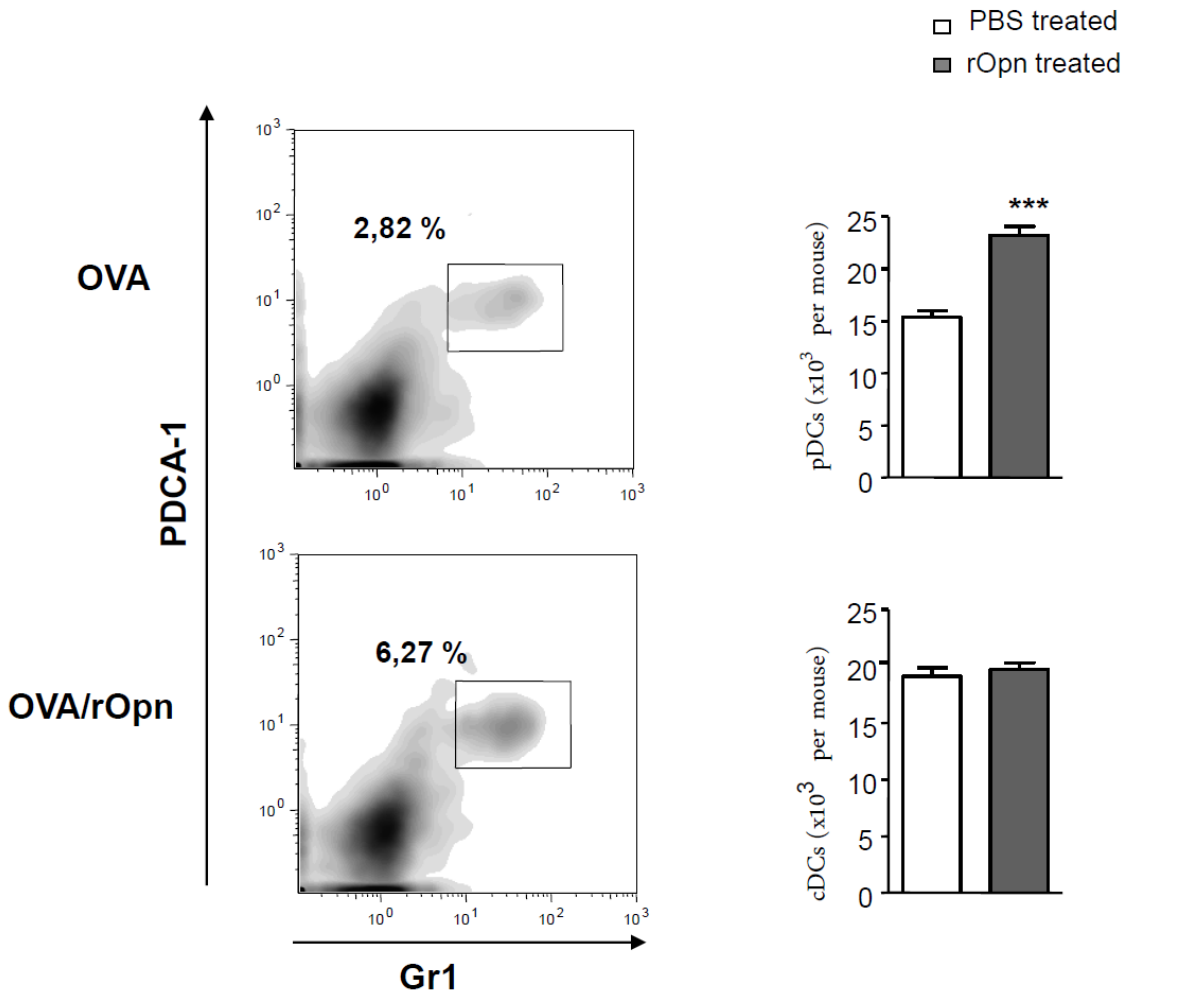
DLN cells from OVA/rOpn-treated pDC-depleted mice were significantly less suppressive, as shown by the notably enhanced IL-4, IL-13, IFNγ and IL-10 production, compared to DLN cells from non-pDC-depleted mice, reaching levels similar to those of OVA-treated pDC-depleted DLN cells (**Fig. 4b**). Importantly, DLN cells from OVA/rOpn-treated pDC-depleted mice were significantly less capable of suppressing OVA-specific T cell proliferation compared to OVA-treated pDC-depleted cells (**Fig. 4c**).

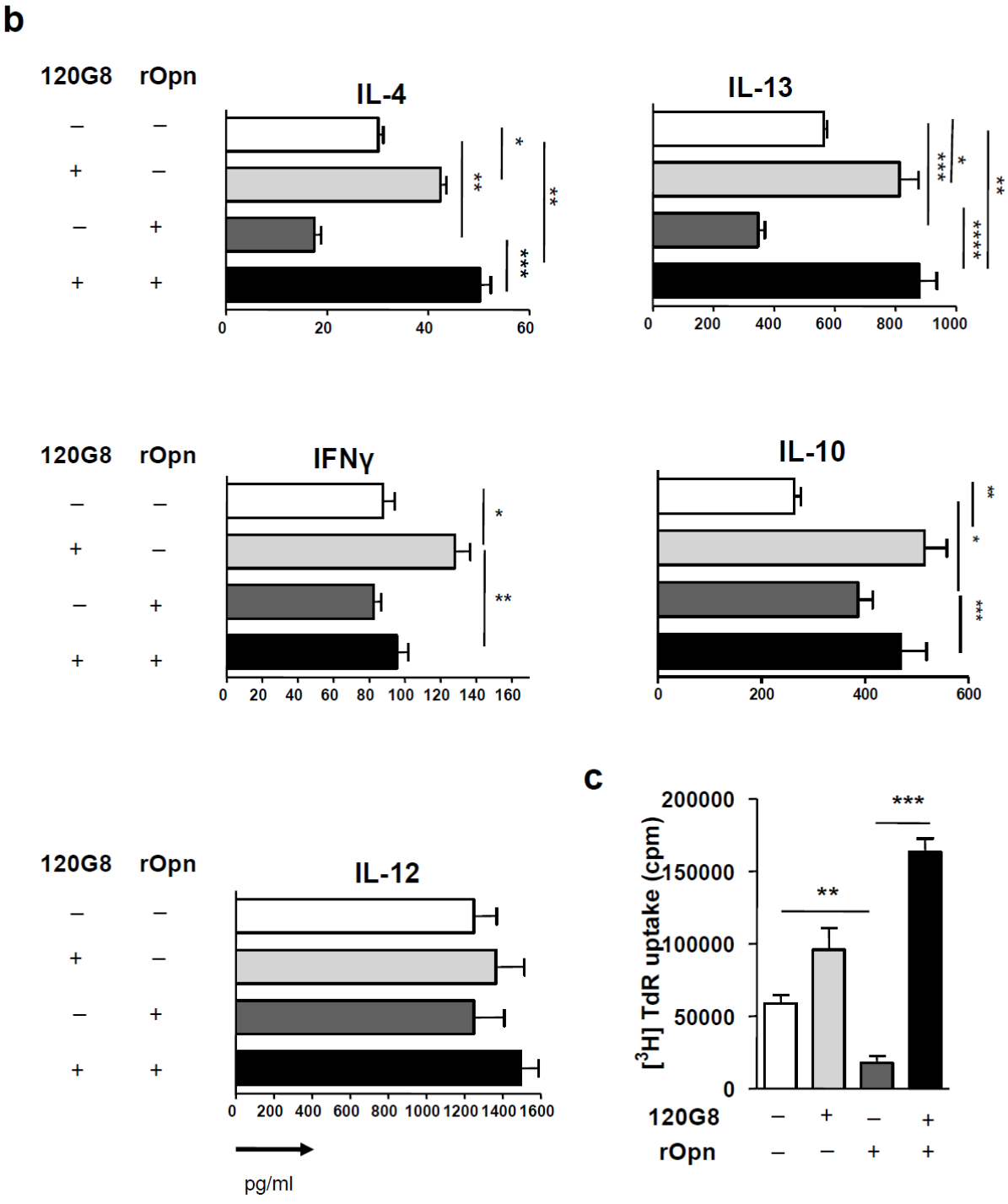
We verified that OVA/rOpn-treated mice had increased numbers of T_{regs} in the DLNs (**Fig. 3a**). To investigate whether the increased pDC numbers induced by rOpn had an effect on T_{reg} cells, we examined the numbers of $Foxp3^+$ T_{reg} cells in the DLNs of pDC-depleted mice. Again, OVA/rOpn treatment significantly increased the numbers of $CD4^+Foxp3^+$ T_{regs} (2-fold) in comparison to OVA treatment (**Fig. 4d**). Depletion of pDCs prior to OVA/rOpn administration resulted in a 2-fold decrease in accumulation of $Foxp3^+$ T cells as evident by percentages and numbers of $CD4^+Foxp3^+$ T_{reg} cells (**Fig. 4d**) in comparison to non-depleted OVA/rOpn-treated mice (239,300 ± 20,900 cells per mouse for OVA/rOpn pDC-depleted versus 371,100 ± 35,120 cells for OVA/rOpn non-pDC-depleted, P = 0.0070). Thus, the enhancement of tolerance observed by rOpn administration was mediated by increased numbers of suppressive pDCs in DLNs that regulate T_H2 responses, possibly via T_{reg} cell induction.

We then examined whether adoptive transfer of *ex vivo* cultured pDCs treated with rOpn would affect their tolerogenic ability. One week prior to OVA/alum sensitization, mice were injected with bone marrow-derived pDCs that were pre-treated with OVA and rOpn or control. As expected, in mice that were not injected with pDCs, OVA-sensitization and OVA-challenge resulted in eosinophilia in the BAL fluid (**Fig. 4e**). However, a significant decrease of BAL fluid eosinophils was observed in pDC-injected mice (**Fig. 4e**). Importantly, the decrease in eosinophilia was even greater (significantly) when pDCs were pre-treated with rOpn (**Fig. 4e**). Overall, our findings demonstrate that pDCs mediate Opn enhancement of tolerance.
Add ranges/numbers as before

Figure 4

a


PBS treated
rOpn treated
OVA
OVA/rOpn
PDCA-1
Gr1
2,82 %
6,27 %
***
pDCs (x10³ per mouse)
cDCs (x10³ per mouse)


b
120G8
rOpn
IL-4
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IFNγ
IL-10
IL-12
pg/ml
c
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120G8
rOpn

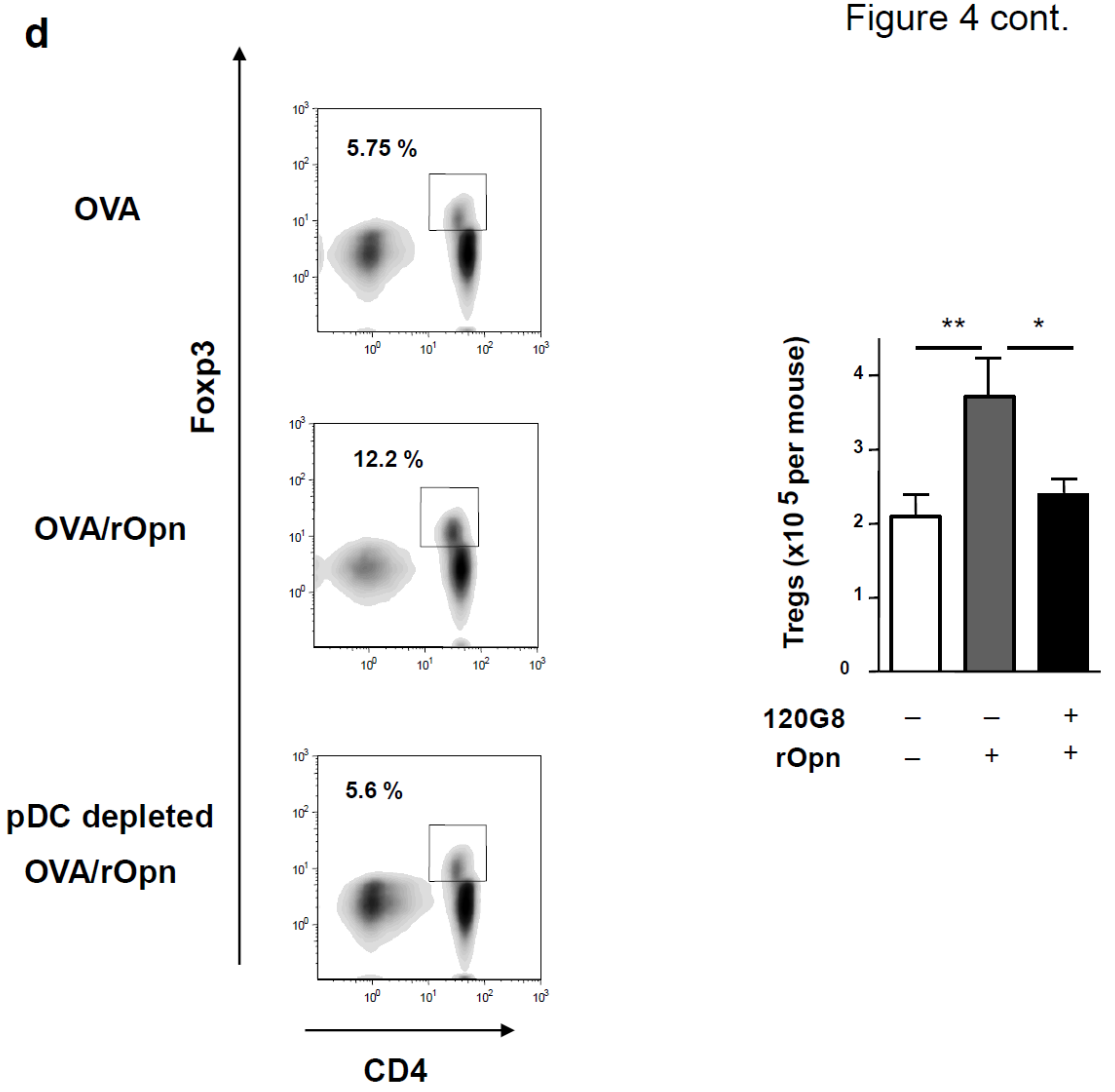


Figure 4 rOpn enhances tolerance through pDCs (a) Mice received endotoxin-free OVA i.p. on days 0, 1 and 2 along with rOpn or PBS, 40 hrs later were euthanized. Percentages of DLN 7AAD–CD11c+PDCA-1+GR-1+ pDCs from PBS-treated

tolerized (upper left) or rOpn-treated tolerized mice (bottom left), one representative experiment of three. Numbers of DLN 7AAD–CD11c+B220+Gr-1+ pDCs (left panel) (***P<0.0001) and 7AAD–CD11c+B220–Gr-1– cDCs (right panel) are shown. Results are expressed as mean ± SEM; n = 6 mice per group. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (b) Mice received four doses of 120G8 pDC-depleting antibody or Ig control i.p. on days 0, 1, 2 and 3, followed by three doses of endotoxin-free OVA i.p. on days 4, 5 and 6. Tolerized mice also received three doses of rOpn or PBS, i.p. together with endotoxin-free OVA. One week later mice were euthanized. Levels of IL-4 (*P=0.0009, **P=0.0003, ***P<0.0001), IL-13 (*P=0.0205, **P=0.0066, ***P=0.0009, ****P=0.0007), IFNγ (*P=0.0133, **P=0.0276), IL-10 (*P=0.0276, **P=0.0048, ***P=0.0068) and IL-12 in supernatants of co-cultures of OVA-stimulated DLN cells with DO11.10 cells from OVA-treated mice (white bars), OVA-treated pDC-depleted mice (light grey bars), OVA/rOpn-treated mice (dark grey bars) and pDC-depleted OVA/rOpn-treated mice (black bars). Data are expressed as mean ± SEM; n = 6 mice per group. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (c) [3H]-thymidine incorporation (**P=0.0016, ***P<0.0001). Data are expressed as mean ± SEM; n = 6 mice per group. Statistical significance was studied by unpaired Student's t-test, three independent experiments. (d) Percentages of CD4+Foxp3+ DLN cells are shown for OVA-treated (upper left panel), OVA/rOpn-treated (middle left panel) or pDC-depleted OVA/rOpn-treated mice (bottom left panel). One representative experiment of three, n =5 mice per group. Numbers of CD3+CD4+Foxp3+ Tregs (right panel) (*P=0.007, **P=0.0021). Statistical significance was studied by unpaired Student's t-test, three independent experiments. (**e**) Mice received 1 x10^6 pDCs i.v. One week later mice were sensitized with OVA/alum followed by OVA aerosols. Numbers of total cells in the BAL (left) (**P=0.0069, ***P=0.0007, ****P<0.0001, *P=0.0297) and numbers of eosinophils in the BAL (right) (*P=0.0280, **P=0.0157, ***P<0.0001, ****P=0.0274, *****P=0.0041) of PBS-treated mice (black bar), mice that received OVA-treated pDCs (white bar), and mice that received OVA/rOpn-treated pDCs (grey bar). Statistical significance was studied by unpaired Student's t-test, results pooled two independent experiments.

Opn promotes regulatory T cell induction *in vitro*.

Our previous results (**Figure 4**) demonstrated that rOpn was no longer able to induce increased accumulation of $Foxp3^+$ T_{regs} in the DLNs when administered in the absence of pDCs. To test the effect of rOpn on the ability of pDCs to endow tolerogenic potential to naïve T cells *in vitro*, we isolated $7AAD^-CD11c^+CD11b^-B220^+$ pDCs pulsed with OVA, treated with PBS or rOpn, and cultured them with naive DO11.10 $CD4^+$ T cells for 4 days. Suppressive activity was assayed on freshly purified CFSE-labelled DO11.10 $CD4^+$ T cells stimulated with irradiated splenocytes and $OVA_{323-339}$ peptide, for 7 days, in the presence or absence of pDC-stimulated T cells. DO11.10 $CD4^+$ T cells cultured in the absence of pDC-stimulated T cells, had the highest T cell divisions in response to OVA peptide, whereas the addition of OVA-treated pDC-stimulated T cells suppressed the proliferation of DO11.10 cells, as also reported in the past by other studies[320]. Of note, the addition of T cells generated by rOpn/OVA-stimulated pDCs further significantly dampened the divisions of DO11.10 T cells (**Fig. 5a**). These results demonstrate that rOpn-treated pDCs are able to induce regulatory T cells with more potent suppressive activities against T_H2 effector responses.

In fact, using an established protocol for the generation of $Foxp3^+$ T_{regs} by TGFβ [321], we observed that rOpn treatment could significantly enhance $Foxp3^+$ T_{reg} cell formation (**Fig. 5b**). As expected, TGFβ alone was able to induce high numbers of $Foxp3^+$ T cells in cultures of isolated $CD4^+CD62L^+CD25^-$ T cells from DO11.10 mice stimulated with $OVA_{323-339}$ peptide (**Fig. 5b**). rOpn added along with TGFβ to the same cultures resulted in increased percentages of $Foxp3^+$ T cells in comparison to TGFβ alone (19.77 ± 1.027 % for rOpn along with TGFβ versus 13.70 ± 1.778 % for TGFβ alone, $P = 0.0418$) (**Fig. 5b**). Thus, Opn enhances $Foxp3^+$ T_{reg} induction.

Figure 5

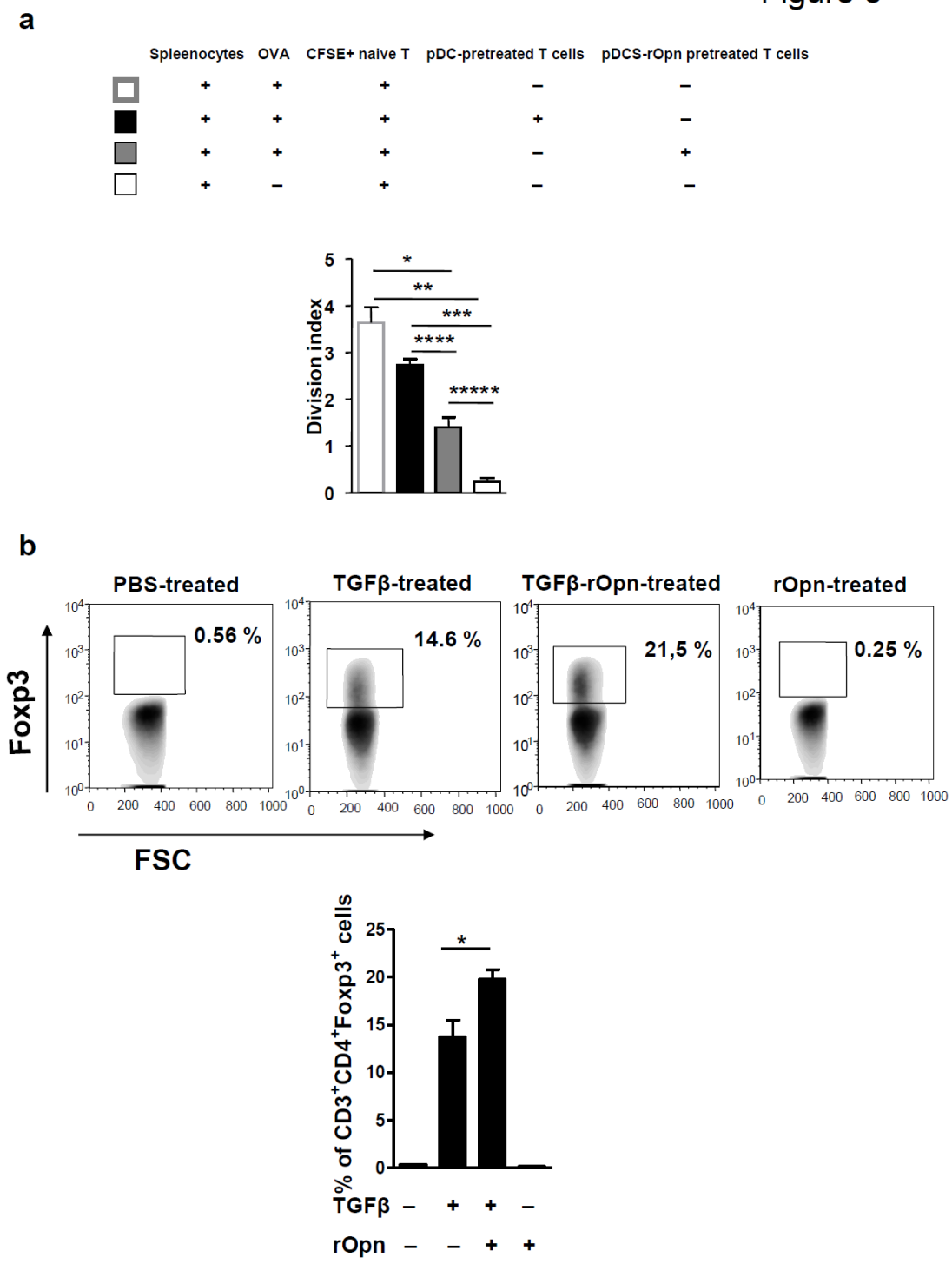


Figure 5 Opn promotes regulatory T cell induction *in vitro* (a) Isolated pDCs (from Flt3L cultures) pulsed with OVA, treated with vehicle or with rOpn, were cultured with naive DO11.10 CD4+ T cells for 3 d. T cells were harvested and cultured without OVA in the presence of recombinant mouse IL-2 for an additional 7 d. Suppressive activity was assayed by culturing freshly purified CFSE labelled DO11.10 CD4+ T cells with irradiated BALB/c splenocytes, with OVA323-339

peptide, in the presence or absence of DC-stimulated T cells. CFSE uptake was assayed 7 days later. Division index (**P*=0.0045, ***P*=0.0006, ****P*=0.0052, *****P*<0.0001, ******P*=0.0067). One representative experiment of three. (b) CD4+CD62L+CD25– T cells were isolated from DO11.10 mice, cultured with bone marrow derived DCs and treated with OVA in the presence or absence of TGFβ along with rOpn or PBS. Foxp3 expression was assayed 4 days later. Percentages of Foxp3+ cells from PBS-treated (upper left panel), TGFβ-treated (upper middle left panel), TGFβ/rOpn-treated (upper middle right panel), rOpn-treated (upper right panel) cultures. One representative experiment of three. Percentages of CD3+CD4+Foxp3+ cells (**P*=0.0139). Statistical significance was studied by unpaired Student's *t*-test.

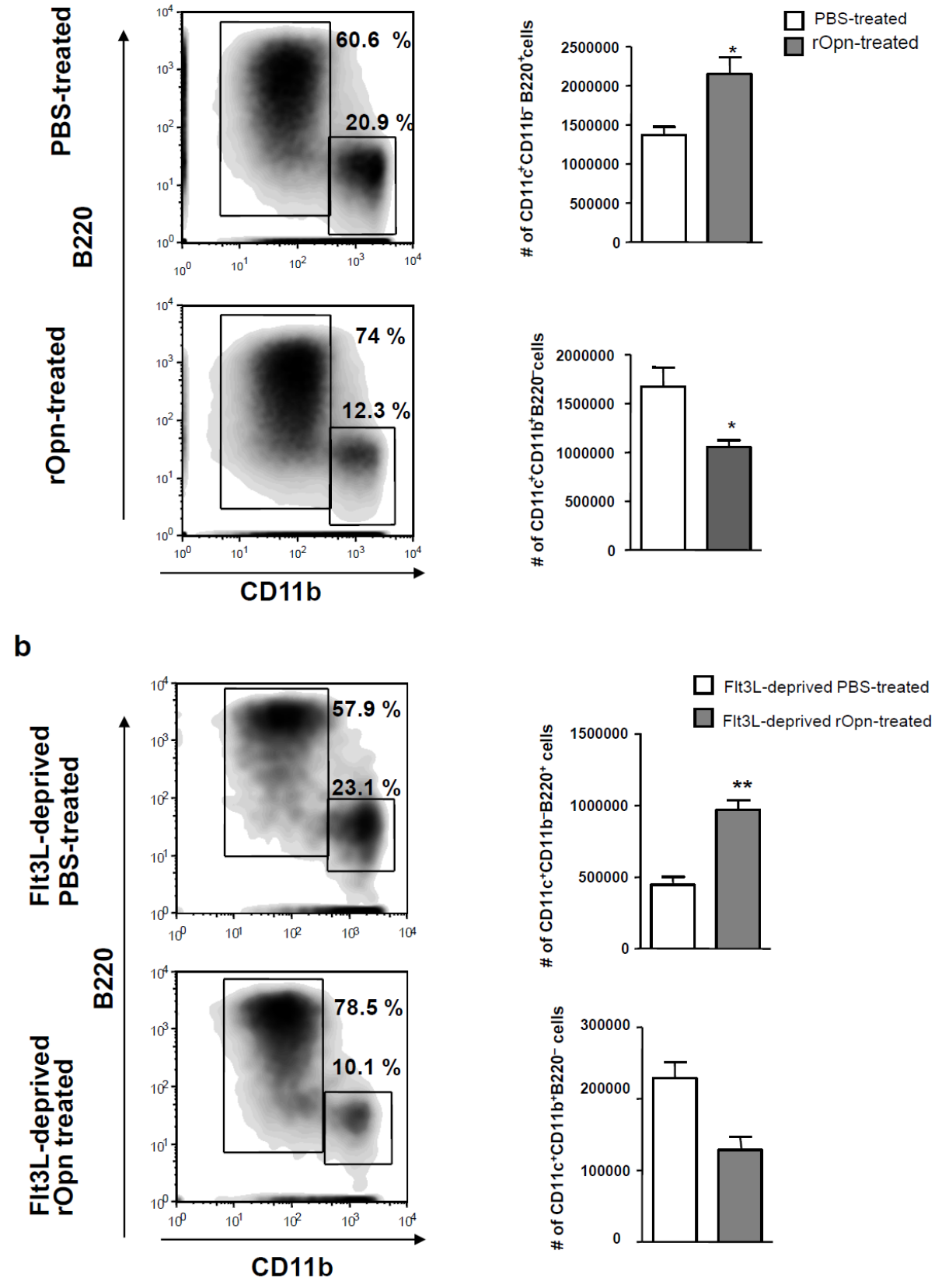
Opn affects the development of DCs.

In several studies, Opn has been demonstrated to be affecting function of DCs[170,212,227,310,319]. We were very intrigued on whether Opn could affect DC differentiation and development. Thus, we evaluated the effect of Opn on development of pDCs and cDCs[322-324]. Treatment of Flt3L bone marrow cultures with rOpn resulted in a significant increase in the numbers of $7AAD^{-}CD11c^{+}CD11b^{-}B220^{+}$ pDCs, compared to PBS treatment (2,149,000 ± 214,100 cells for rOpn and 1,367,000 ± 105,900 cells for PBS, P = 0.0451) (**Fig. 6a**). Moreover, rOpn addition resulted in a significant reduction in the numbers of $7AAD^{-}CD11c^{+}CD11b^{+}B220^{-}$ cDCs compared to PBS treatment (1,118,000 ± 82,860 cells for rOpn versus 1,624,000 ± 178,800 cells for PBS, P = 0.0332) (**Fig. 6a**). Further characterization of cDCs, proved that rOpn treatment resulted in lower levels of both $CD24^{+}$ and $CD24^{-}$ subpopulations of cDCs (data not shown). Most importantly, under Flt3L deprivation conditions, rOpn lead to preferential survival of pDCs (969,400 ± 69,210 cells for rOpn versus 445,500 ± 55,970 for PBS, P = 0.0042) over cDCs (128,400 ± 18,560 cells for rOpn versus 228,900 ± 22,070 for PBS, P = 0.0734) (**Fig. 6b**). Thus, Opn is a survival factor for pDCs. Of importance, Opn can affect the development of DC

subsets and more specifically can switch Flt3L-driven cultures towards enhanced pDC polarization.

To evaluate the effect of absence of Opn in the generation of pDCs and cDCs, we cultured bone marrow from *Opn*$^{-/-}$ or wild type mice with Flt3L in the presence or absence of rOpn. *Opn*$^{-/-}$ cultures had significantly lower numbers of 7AAD^{-} CD11c^{+}CD11b^{-}B220^{+} pDCs (1,209,600 ± 74,760 cells for *Opn*$^{-/-}$ versus 1,613,350 ± 95,900 cells for *Opn*$^{+/+}$, $P < 0.002$) and correspondingly higher numbers of 7AAD^{-} CD11c^{+}CD11b^{+}B220^{-} cDCs (620,550 ± 44,760 cells for *Opn*$^{-/-}$ versus 477,400 ± 55,700 cells for *Opn*$^{+/+}$, $P = 0.0185$), compared to cultures from *Opn*$^{+/+}$ cells (**Fig. 6c**). Addition of rOpn in the *Opn*$^{-/-}$ cultures restored the numbers of pDCs to levels similar to *Opn*$^{+/+}$ cultures (1,494,500 ± 84,370 cells for rOpn *Opn*$^{-/-}$ versus 1,209,600 ± 74,760 cells for PBS *Opn*$^{-/-}$, $P = 0.009$), and decreased the numbers of cDCs, compared to PBS-treated *Opn*$^{-/-}$ cultures (515,000 ± 91,670 cells for rOpn *Opn*$^{-/-}$ versus 620,550 ± 44,760 cells for PBS *Opn*$^{-/-}$, $P = 0.0515$) (**Fig. 6c**). Again, addition of rOpn to *Opn*$^{+/+}$ cultures resulted in further enhancement of pDC generation (**Fig. 6c**). Our results demonstrate that Opn enhances pDC and suppresses cDC development.

Figure 6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PBS-treated
rOpn-treated
B220
CD11b
60.6 %
20.9 %
74 %
12.3 %
PBS-treated
rOpn-treated
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# of CD11c+CD11b+B220- cells
b
Flt3L-deprived PBS-treated
Flt3L-deprived rOpn treated
57.9 %
23.1 %
78.5 %
10.1 %
Flt3L-deprived PBS-treated
Flt3L-deprived rOpn-treated
# of CD11c+CD11b-B220+ cells
# of CD11c+CD11b+B220- cells

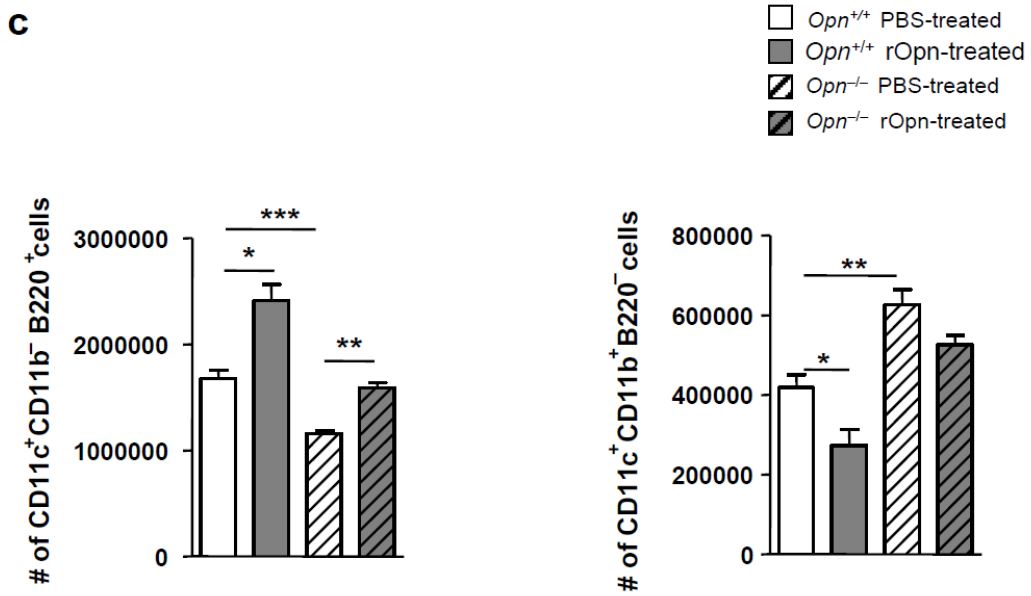

Figure 6 Opn affects the generation of DCs. Bone marrow cells were isolated and cultured with Flt3-L in the presence of rOpn or PBS. (a) Percentages of 7AAD–CD11c+B220+CD11b– pDCs (upper left gates) and 7AAD–CD11c+B220–CD11b+ cDCs (bottom left gates) from PBS-treated (upper left panel) or rOpn-treated cultures (bottom left panel). One representative experiment of three. Numbers of 7AAD–CD11c+B220+CD11b– pDCs (upper right pannel) (**P*=0.0451) and 7AAD–CD11c+B220–CD11b+ cDCs (bottom right panel) (**P*=0.0262) from PBS-treated (white bar) or rOpn-treated (grey bar) cultures. Statistical significance was studied by unpaired Student's *t*-test, three independent experiments. (b) Bone marrow cells were isolated and cultured under Flt3L deprivation conditions in the presence of rOpn or PBS. Percentages of 7AAD–CD11c+B220+CD11b– pDCs (upper left gates) and 7AAD–CD11c+B220–CD11b+ cDCs (bottom left gates) from PBS-treated (upper left panel) or rOpn-treated cultures (bottom left panel). One representative experiment of three. Numbers of 7AAD–CD11c+B220+CD11b– pDCs (upper right pannel) (***P*=0.0042) and 7AAD–CD11c+B220–CD11b+ cDCs (bottom right panel) from

PBS-treated (white bars) or rOpn-treated (grey bars) cultures. Statistical significance was studied by unpaired Student's *t*-test, three independent experiments. (c) Numbers of 7AAD–CD11c+B220+CD11b– pDCs (left panel) (**P*=0.0127, ***P*=0.0022, ****P*=0.003) and 7AAD–CD11c+B220–CD11b+ cDCs (right panel) (**P*=0.0478, ***P*=0.0139) from PBS-treated *Opn*+/+ (white bars), rOpn-treated *Opn*+/+ (grey bars), PBS-treated *Opn*–/– (white striped bar) or rOpn-treated *Opn*–/– (grey striped bar) cultures. Statistical significance was studied by unpaired Student's *t*-test, three independent experiments.

DISCUSSION

Previous studies by our group revealed an important anti-inflammatory role for Opn in ongoing allergic airway disease[310]. In the current report, we unveil a key role for Opn in prevention from allergic airway disease. Our experiments demonstrate that Opn can facilitate and promote antigenic tolerance induction. Importantly, we provide evidence that Opn-mediated enhancement of tolerance operates via $Foxp3^+$ T_{reg} cells, a cell subset that we demonstrate to be essential for tolerance induction, and via suppressive pDCs[91,310,325]. Moreover, our results place Opn as an important factor for specific development and survival of pDCs.

rOpn administration together with antigen (OVA) promoted tolerance and suppressed subsequent allergic immune responses, whereas *Opn* deficiency led to resistance in the development of antigenic tolerance. In our effort to delineate the mechanism of action of Opn, we found that administration of rOpn during tolerance induction significantly enhanced the numbers of $Foxp3^+$ T_{reg} cells in the DLNs. Furthermore, in $Foxp3^+$ T_{reg}-depleted (DEREG) mice, rOpn was unable to enhance tolerance, indicating that its effect is mediated by $Foxp3^+$ T_{reg} cells. To our knowledge, this is the first report showing that Opn has an effect on the $Foxp3^+$ T_{reg} population and on T_{regs} in general. In an effort to test the effects of Opn on $Foxp3^+$ T_{regs}, we used an *in vitro* system for induction of $Foxp3^+$ T_{regs} by TGFβ[321]. When rOpn was added to cultures along with TGFβ, there was a significant enhancement in the development of

Foxp3$^+$ T cells compared to cultures treated with TGFβ alone. Thus, rOpn acts synergistically with TGFβ to promote induction of Foxp3$^+$ T_{regs}. It is possible that Opn is important for the homeostasis/development of Foxp3$^+$ T_{regs} as indicated by a study demonstrating Opn to be one of the most up-regulated genes in stimulated Foxp3$^+$ T_{regs}[311].

Mice depleted of Foxp3$^+$ T_{regs} and tolerized with OVA were no longer able to develop antigenic tolerance, leading to enhanced T_H2 responses to levels similar or greater to those of PBS-treated mice. As stated above, we depleted Foxp3$^+$ T_{reg} cells using the DEREG mice. The depletion was performed prior to antigenic tolerance induction, indicating that naturally-occurring Foxp3$^+$ T_{reg} cells as opposed to induced T_{regs} were mediating the induction of tolerance as well as the rOpn enhancing effect. Thus, our results indicate that naturally-occurring Foxp3$^+$ T_{reg} cells (nT_{regs}) are essential for the development of antigenic tolerance in general. Concerning the involvement of nT_{regs} in induction of tolerance, studies so far had conflicting results. One of the studies, using anti-CD25 for T_{reg} depletion, concludes that nT_{regs} are necessary for tolerance to be established[326]. However, the possibility that induced T_{regs} were also depleted cannot be excluded, because administration of the anti-CD25 antibody was continued even after tolerance induction. In another study, the authors argue that nT_{regs} are not necessary for antigenic tolerance induction[327]. However, the authors did not exclude the possibility that orally administered OVA in RAG$^{-/-}$DO11.10 Tg mice makes its way to the thymus and promotes the development of immature cells into nT_{regs}. Nevertheless, it is clear that none of the studies mentioned above used the direct approach of depleting Foxp3$^+$ T_{regs} using a genetic targeting approach.

In allergic airway disease, cDCs are considered pro-allergic[90,328], whereas pDCs are crucial for preventing asthmatic reactions and suppressing T_H2 responses[91,310]. Shortly after administration of rOpn during antigenic tolerance induction we observed significantly increased numbers of pDCs in the DLN whereas there was no effect on cDC numbers. To examine whether pDCs mediate the effect of rOpn on tolerance, we used to separate approaches. The first approach involved depleting pDCs prior to tolerance induction using the 120G8 antibody[284]. We found by flow cytometry that 120G8 strongly bound all pDCs but we did not track expression of this marker on any other cell subset (including T cells) (data not shown). In the absence of pDCs, DLN

cells were no longer able to suppress T_H2 cytokines produced by naïve responder DO11.10 cells, demonstrating that pDCs are very important for tolerance induction in general as previously reported[91,287,325]. Furthermore, pDC depletion prior to OVA/rOpn administration, abolished suppressive activity of DLN cells on effector cells. The second approach involved adoptive transfer of pDCs prior to OVA/alum sensitization. Transfer of OVA-treated pDCs, as reported by previous studies [91], reduced eosinophilia. A further reduction in eosinophilia was noticed when OVA/rOpn-treated pDCs where transferred into recipients, revealing that the promoting effect of rOpn during tolerance is not only mediated by $Foxp3^+$ T_{regs} as mentioned above, but also by pDCs.

In the absence of pDCs, rOpn administration was no longer able to induce increased numbers of $Foxp3^+$ T_{regs} in the DLNs, indicating that rOpn can also indirectly affect T_{reg} numbers through its effects on pDCs. Taking into consideration that pDCs are able to induce the generation of suppressive T cells *in vitro*[320,329], we studied the effect of rOpn on the ability of pDCs to endow tolerogenic potential to naïve T cells. Indeed, T cells that were cultured in the presence of rOpn-treated pDCs were more potent immunosuppressors. Overall, it appears that rOpn influences T_{regs} both directly and indirectly through its effects on pDCs. One possible explanation could be that Opn might affect the potential of pDCs to attract T_{regs} through enhanced production of chemokines. Alternatively, Opn could induce pDCs to stimulate T_{reg} cells by regulating PDL-1 expression[330] or by enhancing their TGFβ production thus leading to increased induction of Treg cells[331].

In several studies, Opn has been demonstrated to be crucial in affecting DC functions [170,227,310,312]. However, none of these studies focused on the direct effect of Opn on the development of DC subsets. Here we show that Opn-s has an important impact on pDC/cDC balance and, in fact, directs DC development in favour of pDCs. Addition of rOpn in Flt3L bone marrow cultures, significantly increased pDC generation, whereas suppressed cDC development. Our experiments with bone marrow cells under Flt3L deprivation conditions also revealed that Opn-s is a specific survival factor for pDCs. Importantly, Flt3L bone marrow cultures from $Opn^{-/-}$ mice had reduced numbers of pDCs and increased numbers of cDCs, compared to $Opn^{+/+}$

cultures. Our experiments demonstrate for the first time, that Opn is critical for the development of DCs.

Overall, our results place Opn as a critical component of immune regulation machinery and tolerance induction to antigens. Furthermore, these data point to novel effects of Opn on Foxp3^{+} T_{reg} cells and the developmental pathway of DC subset generation. The molecular mechanisms underlying these important effects of Opn remain to be explored.

METHODS

Mice. BALB/c and OVA-specific T-cell receptor–transgenic DO11.10 mice (*Tcr-Tg*-DO11.10) purchased from the Jackson Laboratory, $Opn^{-/-}$ mice (backcrossed to BALB/c background for 9 generations) were housed at the Animal Facility of the Biomedical Research Foundation of the Academy of Athens and at the University Hospital Gent (Gent, Belgium). DEREG (C57BL/6 background) mice were housed at the University Hospital Gent (Gent, Belgium). All procedures 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, and approved by the animal ethics committee at Gent University. All mice used in the experiments were 8-10 weeks old.

***In vivo* experimental protocols**. For the data depicted in Figures 1 and 2, mice received 200 μg of endograde OVA (Profos) or PBS i.p on days 0, 1 and 2. A total of 2.5 μg rOpn (R&D Systems) or PBS was administered i.p. along with OVA on days 0, 1 and 2. On day 12, mice were sensitized i.p. with OVA or PBS (50 μg) in 0.2 ml alum (Serva) and were subsequently challenged through the airways with 5% aerosolized OVA Grade V (Sigma-Aldrich) between days 18-20. Mice were euthanized on day 22. In Figure 3 a-c mice received 200 μg of endograde OVA (Profos) or PBS i.p on days 0, 1 and 2. A total of 2.5 μg rOpn (R&D Systems) or PBS was administered i.p. along with OVA on days 0, 1 and 2. Mice were euthanized one week later. In Figure 3 d-h DEREG mice received 1μg of DT (Sigma-Aldrich) or PBS, i.p. on days -1 and 0 (6 hours prior to OVA injection). On days 0, 1 and 2 mice received 200 μg of endograde OVA or PBS i.p. A total of 2.5 μg rOpn or PBS was administered i.p. along with OVA on days 0, 1 and 2. On day 12, mice were sensitized i.p. with OVA in alum and were subsequently challenged through the airways with 5% aerosolized OVA between days 18-20. Mice were euthanized on day 22. For DT control group, non Tg littermates were administered DT on days -1 and 0 (6 hours prior to OVA injection). On days 0, 1 and 2 mice received 200 μg of endograde OVA i.p. On day 12, mice were sensitized i.p. with OVA in alum and were subsequently challenged through the airways with 5% aerosolized OVA between days 18-20. Mice were euthanized on day 22. In Figure 4a mice were analyzed on day 4, 40hrs after the last OVA i.p. injection. For depletion of pDCs mice received 225 μg of

120G8 pDC-depleting antibody or Ig control (rat IgG1/κ, BD Biosciences) i.p., daily, between days –3 and 0. Then on days 0, 1 and 2 mice received i.p. OVA along with rOpn or PBS. Mice were euthanized one week later.

Penh. Lung function was measured in mice 24h after the final OVA challenge (day 21) by whole body plethysmography (Buxco Technologies) in order to calculate enhanced pause (Penh). Responses to inhaled methacholine (Sigma Aldrich) at concentrations of 3–100 mg/ml were measured for 1m, as described previously[310].

Analysis of BAL. BAL harvesting was performed as described previously[332]. Briefly, inflammatory cells were obtained by cannulation of the trachea and lavage of the airway lumen with PBS. In Figure 1 cytospin slides were prepared by Wright-Giemsa staining. All differential counts were performed blind and in a randomized order at the end of the study. In Figures 2, 3 BAL cells were stained for FACS as described previously[90].

Lung histology. In Figure 1 paraffin-embedded sections (4μm) were stained with haematoxylin & eosin (H & E) to evaluate lung infiltration, as described previously[36]. Goblet cells were counted on Periodic-Acid-Schiff (PAS)-stained lung sections[310]. In Figures 2, 3 lungs were frozen and 5μm sections were prepared and stained with H&E and PAS.

Cell culture, proliferation and cytokine analysis. We used a previously described method[295] to isolate cells from DLNs (mediastinal for Fig. 1, 2, 3g-h and inguinal for Fig. 3a-c, 4). We cultured DLN cells with 125 μg/ml endograde OVA for 48 h. In some experiments, DLN cells were co-cultured with $CD4^+$ T cells (isolated with Dynal beads as previously described[90]) from TCR-Tg-DO11.10 mice at a 2:1 ratio in the presence of 0.25μg/ml $OVA_{323-339}$ peptide for 48 h. We performed proliferation assays for Fig 1, 3c, 4 as previously described[295]. For Fig. 2, 3h proliferation of cells was measured as % of Edu^+ cells by FACS, using a kit from Molecular Probes. To measure cytokines we used ELISA kits for IL-10, IFNγ and IL-12 (BD Biosciences) and IL-4, IL-13, (R&D Systems).

Flow-cytometric analysis. We stained live DLN cells ($7AAD^-$, BD Biosciences) with conjugated antibodies to CD4, CD3, CD19, CD11c, CD11b, CD24, B220, Gr-1, CD86, MHCII (BD Biosciences), PDCA-1 (Miltenyi-Biotech). For intra-nuclear staining of FoxP3 we used permeabilization kit and antibody (e-biosciences). To perform the flow cytometry, in some cases we used a Coulter cytometer (Cytomics, FC 500), whereas elsewhere we used FACS ARIAII (BD). Analysis of data was performed by Flow-Jo (Tree Star Inc.)

Generation of BM-DCs. For generation of pDCs and cDCs, bone marrow was cultured for 4 days with culture medium supplemented with 200ng/ml rhFlt3-L (Amgen) and 50 ng/ml rmSCF (Peprotech) in the presence or absence of 500ng/ml rmOpn in a 6 well plate, at a concentration of $1x10^6$ cells/ml. At day 4 of the culture, all non-adherent cells were collected and placed back in the culture with 200ng/ml rhFlt3-L, with or without 500ng/ml rmOpn. On day 8 extra medium was added to the culture with 200ng/ml rhFlt3-L, with or without 500ng/ml rmOpn. On day 11 of the culture cells were analysed by flow cytometry. For Flt3-L deprivation experiments cells were placed in the culture on day 0 with 200ng/ml rhFlt3-L (Amgen) and 50 ng/ml rmSCF (Peprotech) in the presence or absence of 500ng/ml rmOpn. On day 4, all non-adherent cells were collected and placed back in the culture in the absence of Flt3-L with or without 500ng/ml rmOpn. On day 8 extra medium was added to the culture with or without 500ng/ml rmOpn. On day 11 of the culture cells were analysed by flow cytometry.
For GMCSF cultures, bone marrow was cultured with 20 ng/ml rmGMCSF (a kind gift from Prof. K. Thielemans, Free University Brussel, Brussels, Belgium), in 6 well plate, at a concentration of $5x10^5$ cells/ml. At day 3 of the culture, extra medium with 20 ng/ml rmGMCSF was added. At days 6 and 8 culture was refreshed and cells were collected at day 10.

Suppressive assay, T_{reg} induction *in vitro*. Bone marrow was cultured with Flt3-L as described above. On day 10 cells were pulsed with 100μg/ml OVA (Worthington) in the presence or absence of 500ng/ml rmOpn. On day 11 pDCs were sorted based on their expression of CD11c, PDCA-1, B220, CD11b using a FACS Aria II flow cytometer (BD). A purity of ≥98% was obtained. pDCs pulsed with OVA, treated with vehicle or with rOpn, were cultured with naive DO11.10 $CD4^+$ T cells for 3 d at

a 1:5 ratio. T cells were harvested and cultured without OVA in the presence of 1ng/ml recombinant mouse IL-2 (R&D) for additional 7 d. Suppressive activity was assayed on 10^5 freshly purified CFSE labelled DO11.10 $CD4^+$ T cells stimulated with 10^4 irradiated BALB/c splenocytes, with 1μg/ml $OVA_{323-339}$ peptide, in the presence or absence of 10^5 DC-stimulated T cells. CFSE uptake was assayed 7 days later.
For *in vitro* T_{reg} induction, we sorted T cells from naïve DO11.10 mice as $CD4^+CD62L^+CD25^-$. $2x10^5$ sorted T cells were cultured with 10^4 bone marrow derived DCs from GMCSF cultures (described above) and treated with 0.01μg/ml $OVA_{323-339}$ peptide in the presence or absence of 20ng/ml rhTGF-b (R&D Systems) along with 500ng/ml rOpn or PBS. Foxp3 expression was assayed 4 days later.

***In vivo* adoptive transfer of pDCs.** For data depicted in Fig.4e bone marrow was cultured with Flt3L as described above. On day 11, pDCs were sorted based on their expression of PDCA-1, B220, CD11c, and CD11b using a FACSAria flow cytometer (BD Biosciences). A purity of _96% was obtained. Sorted pDCs were cultured with 100 μg/ml endograde OVA and 200 ng/ml FlT3L in the presence or absence of 200 ng/ml rOpn for one day. 1x106 pDCs were injected i.v. via the tail vein. One week later mice were immunized with OVA/Alum followed by 3 OVA aerosols. Mice were sacrificed 2 days after the last aerosol.

CHAPTER 3

Generation of Opn conditional transgenic mice

Introduction

All our studies so far, focused on the effect of secreted osteopontin on T_H2 responses. Our results pointed towards a novel and significant role of Opn, depending on the phase of the allergic airway disease that was studied. Thus, Opn was proinflammatory during the onset of the disease and anti-inflammatory at the secondary response. Although we showed that Opn mediated its effects by differentially regulating the recruitment of pDCs and cDCs, we still don't know which cell type is responsible for the production of Opn that causes the recruitment of DC subsets that are responsible for the T_H2 responses.

To study the cellular source of Opn we need to have special tools that can help us denineate our question. One useful tool would be the generation of conditional transgenic mice that could either contiuously or inducibly overexpress Opn in a tissue specific manner.

Production of mouse Opn cDNA

In order to clone the mouse Opn cDNA, we isolated $CD4^+$ T cells from spleens of C57BL/6J mice, using microbeads. As Opn is known to be the predominant transcript expressed by activated T cells [136,270], we activated $CD4^+$ T cells, by culturing them in the presence of concanavalin-A (Con-A). 24 hours later we isolated RNA and produced cDNA by RT-PCR.

The full length mouse Opn cDNA was generated by PCR using primers designed for the mRNA NM_009263 (forward primer binding position 211-232 bp: 5'CTAACTACGACCATGAGATTGG3', reverse primer binding position 1442-1661 bp 5'GGGTATAGTGATATAGACTG3') (**Figure 1**). The cDNA was then ligated to pGEM-Teasy vector (Promega). To confirm correct 5'-3' orientation of the Opn cDNA we performed digestions with restriction enzymes (**Figure 2**) The correct cDNA sequence was confirmed by sequencing both strands of the cDNA.

FIGURE 1

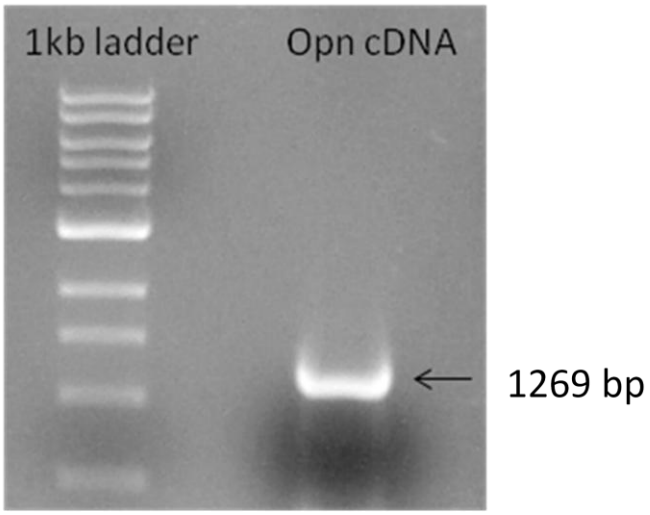

Figure 1 Opn cDNA generated by PCR from cDNA NM_009263

FIGURE 2

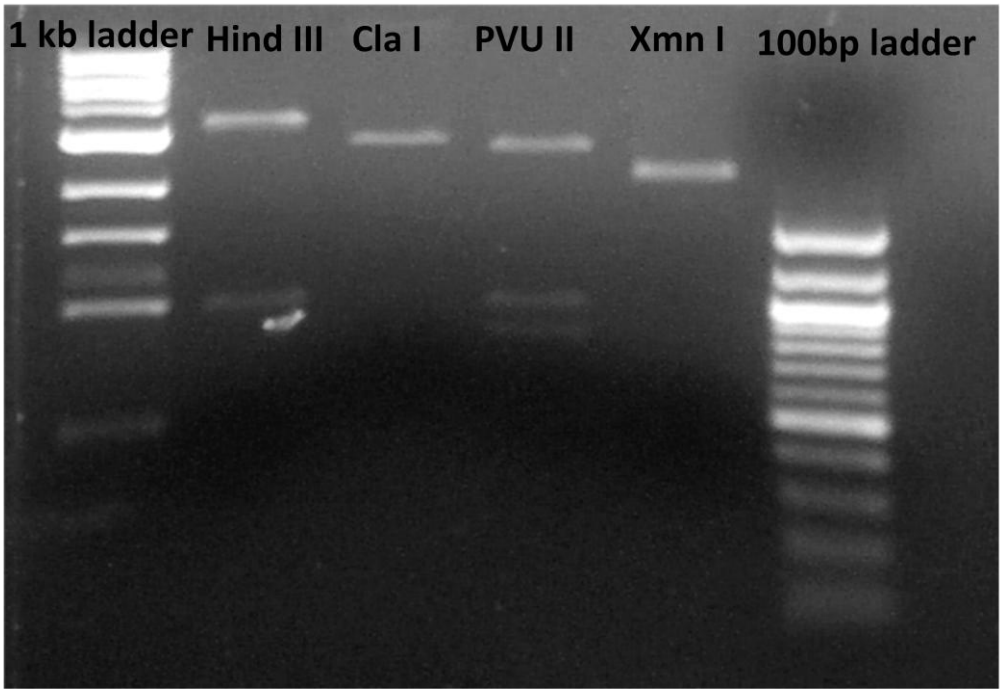

Figure 2 Confirmation of correct Opn cDNA orientation. Digestion of PGEM-Teasy containing Opn cDNA with restriction enzymes.

Generation of construct containing actin promoter and Opn cDNA

The 1269bp Opn fragment was isolated from the PGEM-Teasy vector with the use of the restriction enzyme EcoRI (**Figure 3 right**).

FIGURE 3

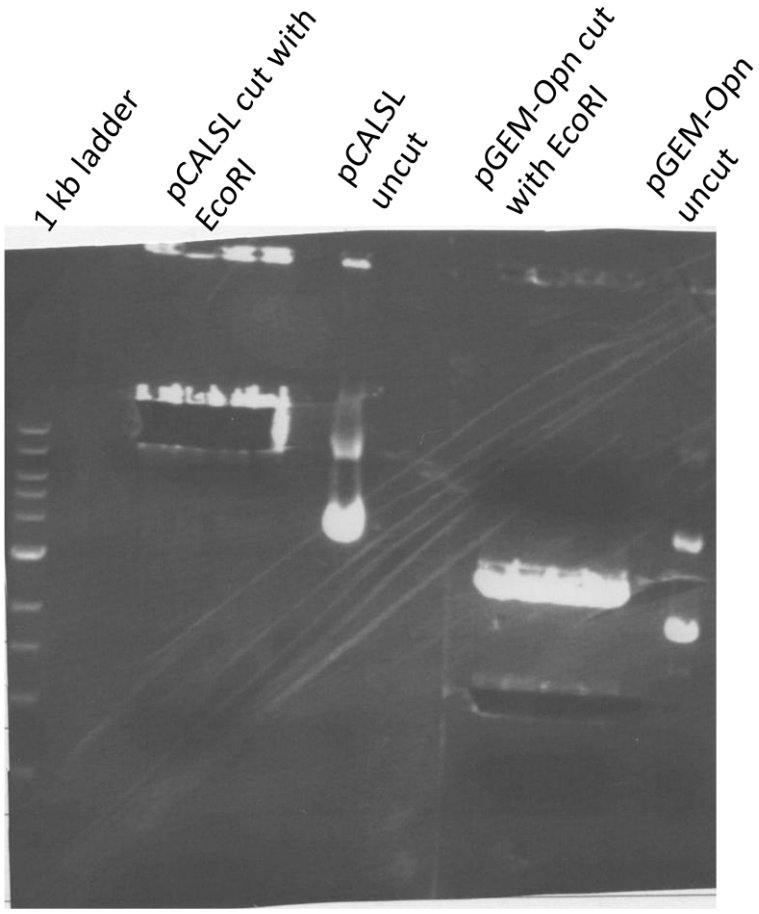

Figure 3 Digestion of pCALSL and pGEM-Opn with EcoRI and isolation of the digested fragments for ligation

Next, Opn cDNA was cloned in plasmid pCALSL (plasmid Chicken Actin LoxP STOP LoxP) at EcoRI restriction site (**Figure 3 left**), using T4 ligase. The pCALSL plasmid contains a general eukaryotic promoter of chicken b-actin and a downstream intron that is used for enhancing the cDNA transcription. Downstream of the intron there is a STOP cassette and polyA tails, used for the end of stop of the transcription,

and that is flanked by 2 loxP sites. The cDNA is cloned downstream of the last loxP site, so it cannot be trancripted due to the STOP cassette. In the presence of the enzyme Cre recombinase the loxP sites are recognized and are recombined, leading to the deletion of the STOP cassette and thus the proper transcription of the gene.

Taking advantage of this system, Opn wil be expressed tissue specifically, as long as transgenic mice containing the pCALSL + Opn gene will be crossed to mice that express tissue specific Cre recombinase (**Figure 4**).

FIGURE 4

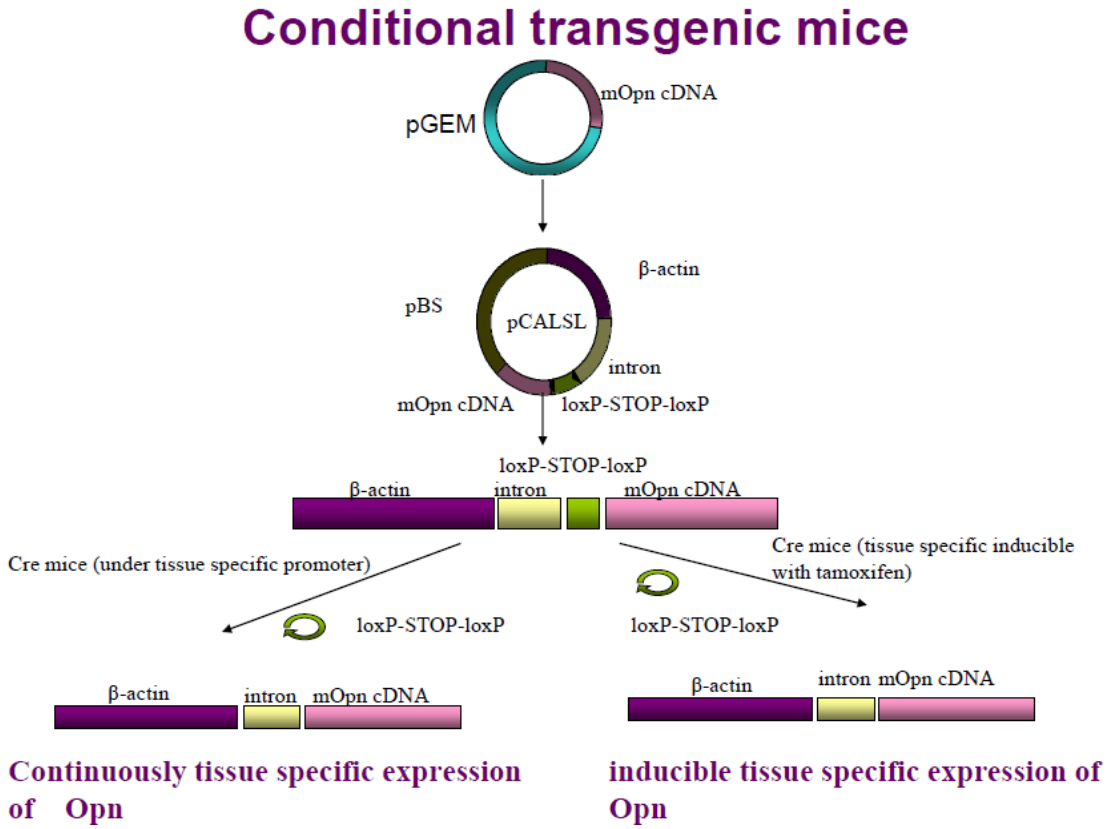

Figure 4 schematic representation of the pCALSL + mouse Opn. Deletion of the prokaryotic elements of the plasmid (in order to be injected to mice) and recombination of loxp sites leads to tissue specific expression of Opn.

The cDNA, was cloned into the restriction sites following the floxed ‘‘stop’’ cassette of the chicken h-actin-loxP-stoploxP (pCALSL) vector (gift of Dr. R. Kelleher).

After transformation of DH5a E.Coli with the ligation product we confirmed the insertion by digasting with EcoRI (**Figure 5**). Indeed all of the colonies contained the correct insertion.

FIGURE 5

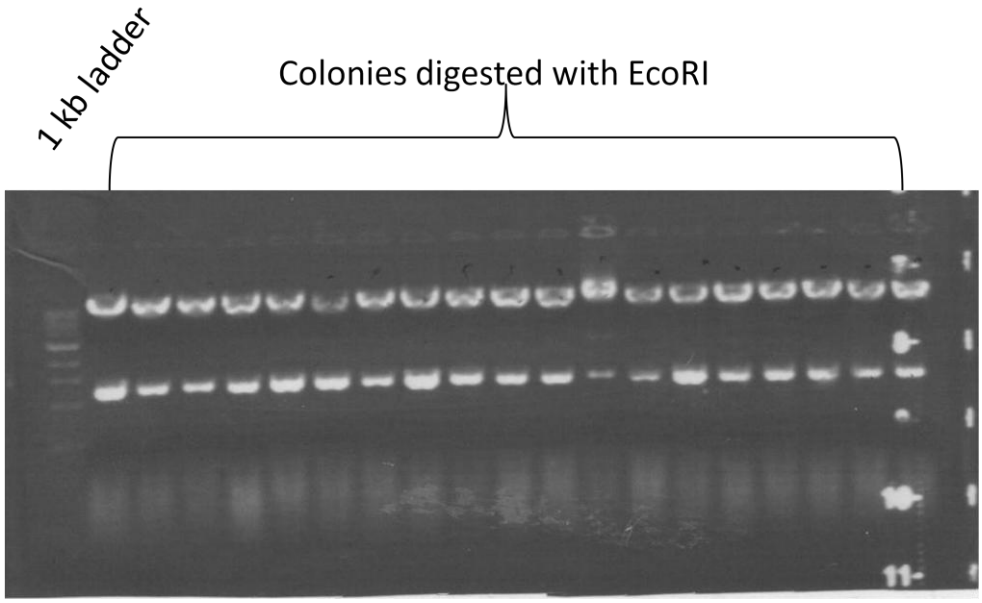

Figure 5 Digestion with EcoRI of DNA from different colonies in order to confirm liation of pCALSL and Opn.

In order to confirm the correct 5'-3'orientation of Opn cDNA in pCALSL plasmid, we performed digestions of the DNA from the collonies with with HincII and NotI restriction enzymes (**Figure 6**).

FIGURE 6

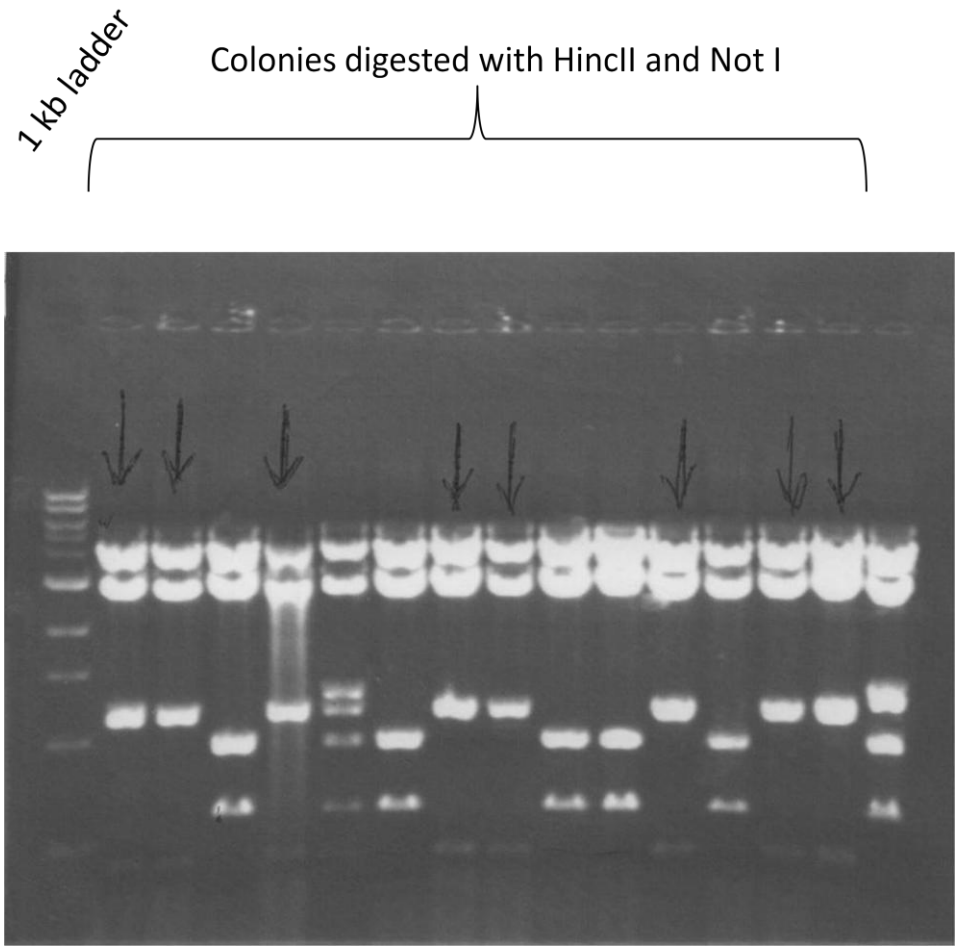

Figure 6 Digestions of colonies with HincII and NotI for verifying the 5'-3' correct orientation of Opn cDNA. Colonies pointed with arrows contain the correct orientation.

The next step was to add an SV40 late polyadenylation signal downstream of Opn cDNA. To verify the inserion we digested the product with XbaI and SalI. The SV40 polyA tail was 400 bp (**Figure 7**).

FIGURE 7

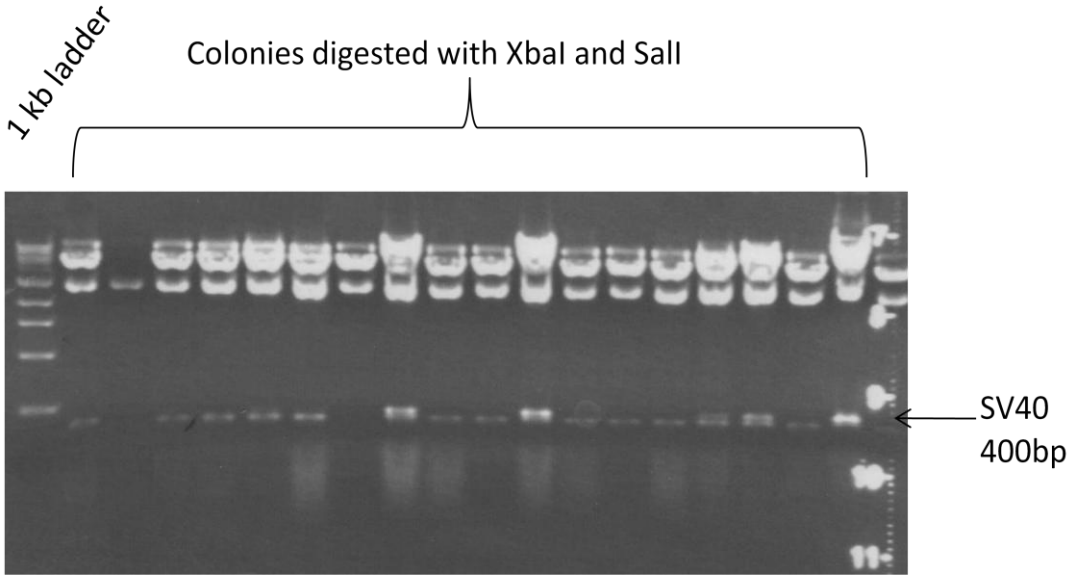

Figure 7 Digestions with XbaI and SalI for the verification of SV40 insertion

Finally, the transgene was digested with SalI in order to delete the prokaryotic backbone elements and to be injected into the pronucleus of C57BL/6J embryos to obtain Opn transgenic mice (**Figure 8**). Southern analysis was performed on six independent lines of transgenic mice to determine the number of the transgene insertion sites.

FIGURE 8

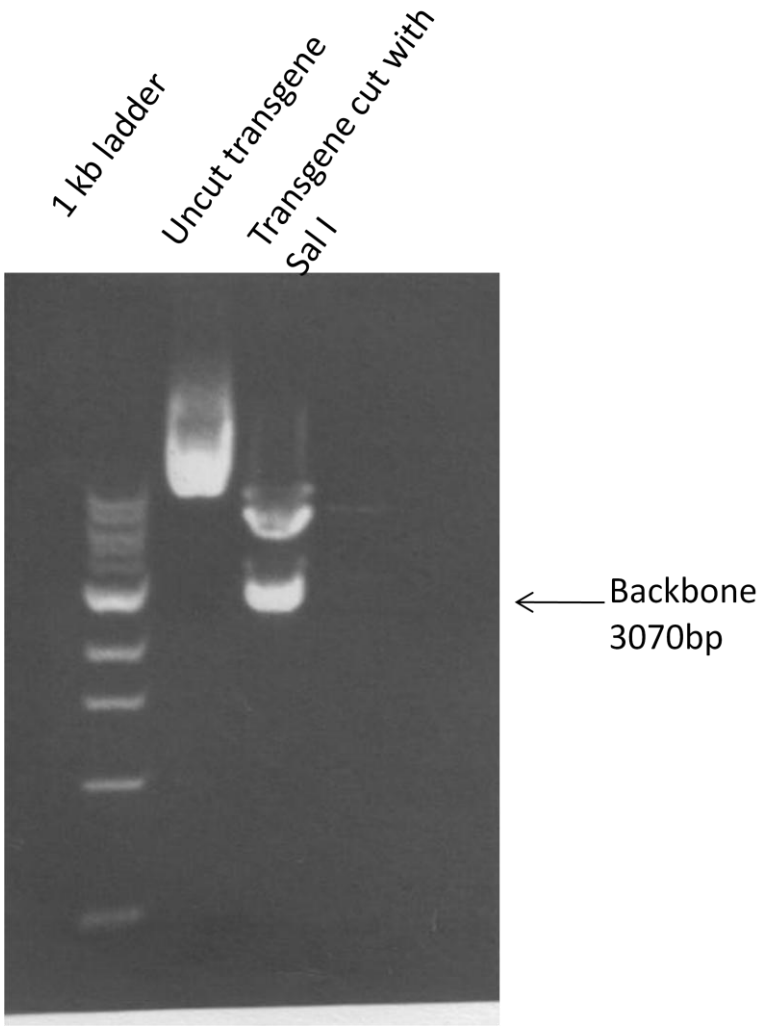

Figure 8 Digestion of the final transgene containing with SalI in order to remove the unwanted prokaryotic backbone sequence.

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Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets

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Osteopontin (Opn) is important for T helper type 1 (T_H1) immunity and autoimmunity. However, the role of this cytokine in T_H2-mediated allergic disease as well as its effects on primary versus secondary antigenic encounters remain unclear. Here we demonstrate that OPN is expressed in the lungs of asthmatic individuals and that Opn-s, the secreted form of Opn, exerts opposing effects on mouse T_H2 effector responses and subsequent allergic airway disease: pro-inflammatory at primary systemic sensitization, and anti-inflammatory during secondary pulmonary antigenic challenge. These effects of Opn-s are mainly mediated by the regulation of T_H2-suppressing plasmacytoid dendritic cells (DCs) during primary sensitization and T_H2-promoting conventional DCs during secondary antigenic challenge. Therapeutic administration of recombinant Opn during pulmonary secondary antigenic challenge decreased established T_H2 responses and protected mice from allergic disease. These effects on T_H2 allergic responses suggest that Opn-s is an important therapeutic target and provide new insight into its role in immunity.

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Immunity against pathogens is mediated through the induction of antigen-specific T helper (T_H) type 1 and type 2 lymphocytes. T_H1 immunity confers protection against intracellular pathogens and, when excessive, can lead to autoimmunity[1,2]. Aberrant T_H2 cell activation against environmental antigens may induce allergy and asthma[3]. Activation and differentiation of T_H immunity depends on interactions of T_H cells with antigen-presenting cells, such as dendritic cells (DCs), and cytokines play a crucial role in this process.

Opn is a cytokine originally identified as the predominant transcript expressed by activated T cells[4,5]. Opn-deficient (*Spp1*$^{-/-}$, also known as *Opn*$^{-/-}$) mice exhibit reduced immunity to viruses[6] and other microorganisms[7], develop milder experimental autoimmune encephalomyelitis[8–10] and are resistant to the development of autoimmune keratitis[6], all T_H1-linked responses. Increased OPN expression has also been shown in affected tissues from individuals with rheumatoid arthritis, Crohn disease and multiple sclerosis[10–12]. Also, polymorphisms in the gene encoding OPN have been linked to the development of systemic lupus erythematosus and multiple sclerosis[13,14], suggesting a role in autoimmunity.

An important recent study has demonstrated that the intracellular form of Opn (Opn-i) is essential for interferon (IFN)-α production by plasmacytoid DCs (pDCs) upon viral infection or CpG oligonucleotide administration[15]. Additionally, recombinant OPN (rOPN) induces maturation of T_H1-polarizing human DCs *in vitro*[16], and blockade of Opn-s reduces costimulatory molecule and class II molecule expression on human monocyte–derived DCs[17]. Moreover, *Spp1*$^{-/-}$ mice exhibit reduced trinitrochlorobenzene–induced migration of DCs to draining lymph nodes (DLNs)[18]. In contrast, rOpn administration inhibits bacterially induced DC migration[19]. Opn-i and Opn-s can therefore affect DC functions, which are crucial in determining the outcome of adaptive immunity.

Previous studies have focused on the role of Opn during T_H1 viral and autoimmune processes in which responses were ongoing by means of repetitive antigenic encounter[6,8]. However, the effect of this cytokine during primary versus secondary antigenic encounters remains unclear. Moreover, the role of Opn in T_H2-mediated allergic responses, a rising health issue in industrialized countries[20], has not been elucidated. Therefore, we investigated the *in vivo* effects of Opn-s in distinct phases of a T_H2 immune response and subsequent disease development, using an established mouse model of ovalbumin (OVA)-induced allergic airway inflammation[21]. We also examined whether the role of Opn-s was mediated by effects on DC subsets. By comparing the results obtained upon neutralization of Opn-s with those from *Spp1*$^{-/-}$ mice, we studied the immunoregulatory activity of the Opn isoforms in T_H2 allergic responses and the disease phenotype.

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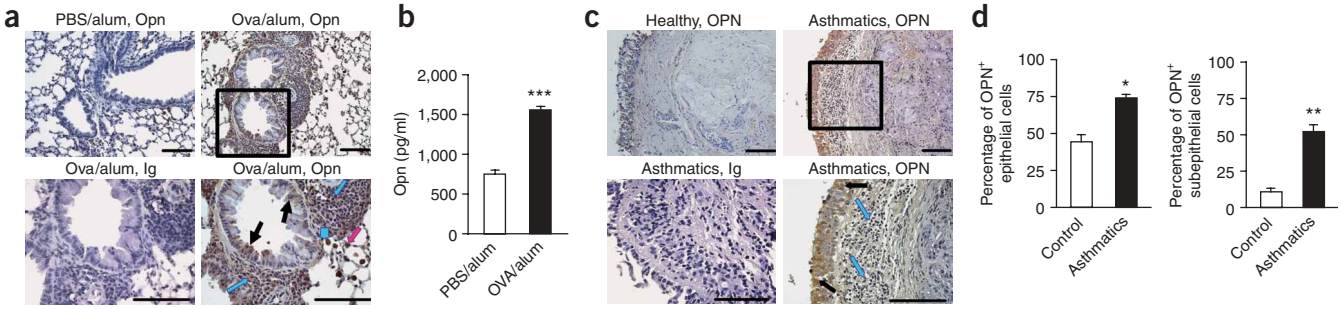

Figure 1 Expression of Opn in the lung in allergic airway disease. (**a**) Photomicrograph of lung sections from PBS/alum-sensitized BALB/c mice (alum controls) and OVA/alum-sensitized BALB/c mice stained with Opn-specific antibody. Immunized mice also received three challenges with aerosolized OVA. In OVA/alum mice, Opn was expressed by infiltrating leukocytes (blue arrows), including macrophages (blue arrowhead), by bronchial epithelial cells (black arrows) and by alveolar epithelial cells (pink arrow). Black rectangle corresponds to magnified version shown in bottom right panel. Ig control staining of a section from OVA/alum-sensitized mice is also shown. Specific staining is depicted in brown; nuclei are stained blue with hematoxylin. (**b**) Opn levels in lung homogenates of OVA/alum-sensitized mice and alum controls. Data are expressed as mean ± s.e.m. $n = 6$–8 mice per group in two independent experiments. $^{***}P = 0.0025$. (**c**) Photomicrograph of OPN expression in bronchial biopsies from healthy individuals and asthmatics, stained with OPN-specific antibody. OPN expression in the asthmatics was localized in bronchial epithelial cells (black arrows) and subepithelial infiltrating leukocytes (blue arrows). Ig control staining of a biopsy from an asthmatic is shown. (**d**) Percentages of OPN^+ epithelial and subepithelial cells from lung biopsies of asthmatics and healthy individuals. Cell counts are expressed as mean ± s.e.m. For each biopsy, data were obtained using three high-power fields (×400). $^{*}P = 0.0033$, $^{**}P = 0.0071$ (unpaired Student's *t*-test). Scale bars, 100 μm.

RESULTS

Increased lung Opn expression in allergic disease

We investigated Opn expression during allergic T_H2 responses, using a mouse model of airway inflammation induced by OVA/alum sensitization followed by airway OVA challenges. There was upregulation of lung Opn expression in mice sensitized with OVA/alum as compared to PBS/alum (alum controls) (**Fig. 1a**), localized mainly at sites of leukocytic infiltration and in bronchial and alveolar epithelial cells. Opn was also increased in lung homogenates from OVA-sensitized mice (**Fig. 1b**).

In humans, lung biopsies from asthmatics had increased OPN expression in bronchial epithelial cells (ciliated epithelium) and inflammatory cells underneath the subepithelial membrane, as compared to healthy subjects (**Fig. 1c**). The percentage of OPN-positive epithelial and subepithelial cells was also increased in asthmatic individuals compared to controls (**Fig. 1d**).

Endogenous Opn-s is pro-inflammatory at sensitization

To investigate whether Opn-s participates during the induction of a T_H2 response, we administered a neutralizing antibody to Opn (or an isotype (Ig) control) before OVA/alum sensitization (**Fig. 2a**). Following subsequent challenge through the airways with OVA, mice treated with the Opn antibody exhibited decreased numbers of bronchoalveolar lavage (BAL) eosinophils, lymphomononuclear cells (**Fig. 2b**) and decreased airway hyper-responsiveness (AHR), as compared to Ig-treated mice, reaching levels similar to those of the alum controls (**Fig. 2c**). Lung leukocytic infiltration and mucus secretion were also decreased (**Fig. 2d**), accompanied by a decrease in the eosinophil-specific chemokine CCL11 in the lungs (**Fig. 2e**).

Lung interleukin (IL)-4, IL-13 and IL-10 levels were decreased in mice treated with Opn-specific antibody (**Fig. 2e**). Levels of IL-12, a T_H1 cytokine produced by DCs, macrophages and airway epithelial cells[22,23], were also decreased (**Fig. 2e**). We attribute these decreases to the overall decrease in pulmonary inflammation. Cytokine levels in BAL exhibited similar patterns (data not shown).

We examined OVA-specific T_H2 responses by measuring cytokine levels in supernatants of DLN cell cultures stimulated *ex vivo* with OVA. Treatment with Opn-specific antibody resulted in decreased IL-4, IL-13 and IL-10 levels (**Fig. 2f**). Levels of OVA-specific IgG1, IgG2a and IgE were decreased in mice treated with Opn-specific antibody (**Fig. 2f**).

We observed decreased percentages of T_H cells positive for T1/ST2, a T_H2 cell marker, in lung DLNs of mice treated with Opn-specific antibody, right after the first OVA challenge (**Fig. 2g**) and after three challenges (data not shown). Blockade of Opn-s resulted in decreased pulmonary levels of the T_H2 cell–specific chemokine CCL22 (**Fig. 2g**).

Thus, antibody-mediated depletion of endogenous Opn-s during antigenic sensitization resulted in a reduction of T_H2 allergic responses and the consequent suppression of disease.

Endogenous Opn-s is anti-inflammatory during challenge

We investigated the role of endogenous Opn-s in secondary allergic responses by administering neutralizing antibody to Opn (or Ig control) before each OVA challenge in sensitized mice (**Fig. 3a**). Opn-s neutralization increased the total number of infiltrating cells and eosinophils measured in the BAL (**Fig. 3b**), AHR responses (**Fig. 3c**), pulmonary inflammation (**Fig. 3d**) and mucus secretion (**Fig. 3d**). Alum controls had lower inflammation and AHR (**Fig. 3b–d**).

Levels of IL-4, IL-13, IL-10, IFN-γ and CCL11 in the lung were increased in mice treated with Opn-specific antibody (**Fig. 3e**). It has been suggested that increased pulmonary IFN-γ levels play a pathologic role in allergic airway disease[24–27]. BAL cytokine levels were similarly increased in mice treated with Opn-specific antibody (data not shown).

In OVA-stimulated DLNs, blockade of Opn-s during challenge increased IL-13 and IL-10 and decreased IFN-γ (**Fig. 3f**). Levels of OVA-specific IgG1 were increased whereas OVA-specific IgG2a responses were decreased, indicative of a T_H2 shift (**Fig. 3f**).

We observed increased percentages of DLN $T1/ST2^+$ T_H2 cells following Opn-s neutralization, after the first intranasal OVA challenge (**Fig. 3g**) as well as after three OVA challenges (percentages of $T1/ST2^+$ cells among gated T_H cells: with antibody to Opn, 23.8–34.5%; with Ig control, 4.8–13.8%). In both cases, we observed increased levels of CCL22 and CCL17 in the lungs (**Fig. 3g**).

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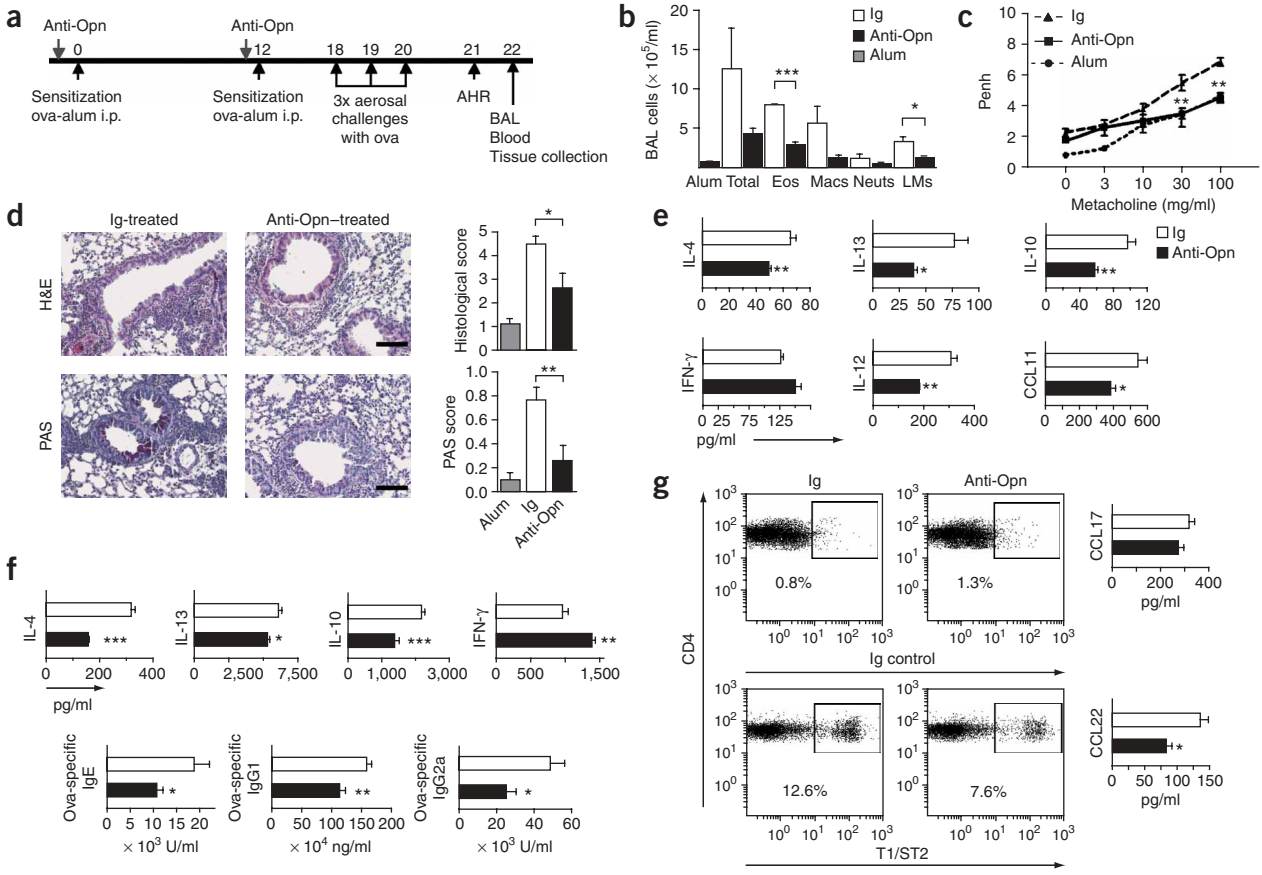

Figure 2 Opn-s blockade at priming reduces allergic disease. (**a**) Experimental protocol used to neutralize endogenous Opn-s during sensitization. BALB/c mice received two doses of anti-Opn or Ig control before each OVA/alum sensitization. (**b**) BAL differentials are expressed as mean ± s.e.m. $n = 6$–8 mice per group, three independent experiments. $^{***}P = 0.0008$, $^{*}P = 0.0209$ (unpaired Student's *t*-test). (**c**) AHR responses for enhanced pause (Penh) in mice treated with either anti-Opn or Ig. $^{**}P = 0.010$ (two-way repeated-measures analysis of variance (ANOVA), and unpaired Student's *t*-test). (**d**) Lung inflammation (top) and mucus secretion (bottom). $^{*}P = 0.0269$, $^{**}P = 0.0156$ (unpaired Student's *t*-test). Scale bar, 100 μm. (**e**) Lung levels of IL-4 ($^{**}P = 0.0015$), IL-13 ($^{*}P = 0.0255$), IL-10 ($^{**}P = 0.007$), IFN-γ ($P = 0.068$), IL-12 ($^{**}P = 0.0026$) and CCL11 ($^{*}P = 0.0377$). (**f**) Levels of IL-4 ($^{***}P < 0.0001$), IL-13 ($^{*}P = 0.0121$), IL-10 ($^{***}P = 0.0002$) and IFN-γ ($^{**}P = 0.0014$) in supernatants of OVA-stimulated DLNs. Serum levels of OVA-specific IgE ($^{*}P = 0.0328$), IgG1 ($^{**}P = 0.0072$) and IgG2a ($^{*}P = 0.0437$). (**g**) BALB/c mice were sensitized as above and challenged with OVA on day 18. Anti-Opn or the Ig control were administered before each sensitization. Percentages of T1/ST2^{+} DLN cells gated on CD3^{+}CD4^{+} T cells are shown, along with isotype control staining for the T1/ST2 marker. One representative experiment of three. $n = 3$–5 mice per group. Lung levels of CCL17 ($P = 0.1749$) and CCL22 ($^{*}P = 0.0115$) are shown.

Overall, and in contrast to its effect at sensitization, blockade of endogenous Opn-s during antigenic challenge enhanced T_H2 allergic recall responses and exacerbated the disease phenotype.

Spp1$^{-/-}$ mice have enhanced T_H2-mediated responses

Spp1$^{-/-}$ mice had increased numbers of BAL inflammatory cells and eosinophils compared to *Spp1*$^{+/+}$ (**Fig. 4a**). Lung T_H2 cytokine and chemokine levels were similar (data not shown). However, *Spp1*$^{-/-}$ mice have a predominantly C57BL/6 genetic background, which is thought to confer resistance to allergic inflammation, and deficiency in Opn may involve possible compensatory mechanisms.

OVA-stimulated DLN cells from *Spp1*$^{-/-}$ mice produced increased IL-4, IL-13, IL-10 and IFN-γ, as compared to cells from the wild-type mice (**Fig. 4b**). In *Spp1*$^{-/-}$ mice, OVA-specific IgG1 levels were increased whereas OVA-specific IgG2a responses were decreased, suggestive of a T_H2 shift (**Fig. 4c**). We also observed increased levels of OVA-specific IgE in these mice (**Fig. 4c**). OVA-specific IgE was increased in BALB/c mice treated with antibody to Opn during both the sensitization and challenge phases (**Fig. 4d**), indicating no involvement of Opn-i.

Opn-s blockade at sensitization affects pDC numbers

To explore the effect of Opn-s neutralization at sensitization on final disease outcome, we examined early T_H2 responses. We treated BALB/c mice with Opn-specific antibody or Ig control before sensitization with Alexa Fluor–OVA in alum, and examined CD11c^{+} cell–driven responses. Cocultures of DLN CD11c^{+} cells from Opn-s–neutralized mice with DO11.10 responder T cells produced lower levels of IL-4, IL-13 and IFN-γ, as compared to those from Ig-treated mice (**Fig. 5a**), suggestive of a reduced priming effect. We obtained similar results from OVA-stimulated whole DLNs (Opn-specific antibody versus Ig: 37.33 ± 2.46 versus 96.67 ± 7.92 pg/ml of IL-4; 717.3 ± 25.00 versus 962.4 ± 38.07 pg/ml of IFN-γ).

It has been shown that two main subtypes of DCs participate in immune responses: conventional DCs (CD11c^{+}B220^{-} or

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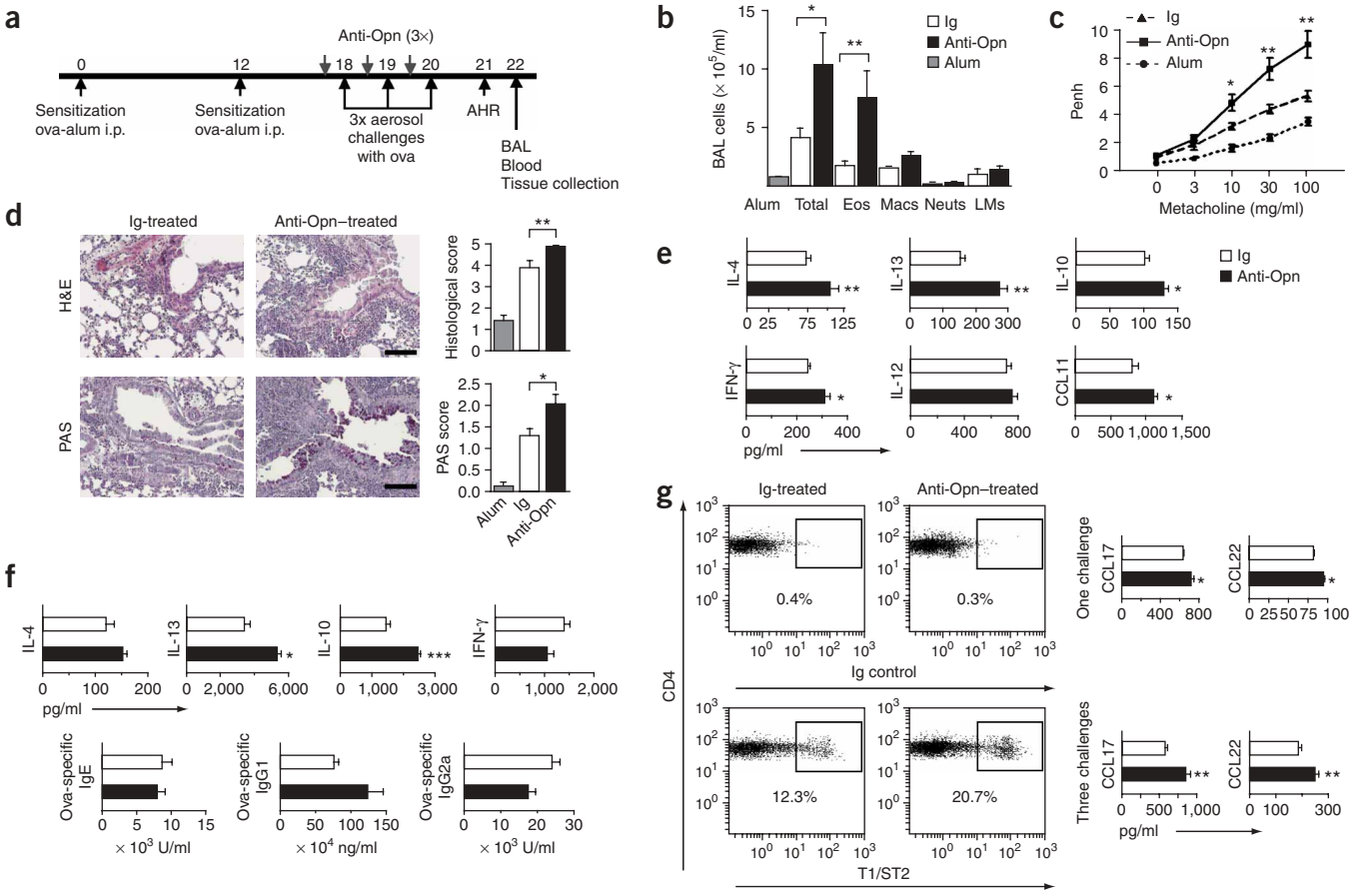

Figure 3 Opn-s blockade at challenge enhances allergic disease. (**a**) Experimental protocol used to neutralize endogenous Opn-s during challenge. BALB/c mice received three doses of anti-Opn or Ig control before challenge. (**b**) BAL differentials are expressed as mean ± s.e.m. $n = 5$–8 mice per group, five independent experiments. $^{*}P = 0.0410$, $^{**}P = 0.0276$ (unpaired Student's *t*-test). (**c**) AHR responses for Penh were analyzed as in **Figure 2c**. $^{*}P = 0.027$, $^{**}P = 0.010$ (*t*-test and two-way ANOVA). (**d**) Lung inflammation and mucus secretion. $^{**}P = 0.0052$, $^{*}P = 0.0355$ (obtained as in **Figure 2d**). Scale bar, 100 μm. (**e**) Lung levels of IL-4 ($^{**}P = 0.0067$), IL-13 ($^{**}P = 0.0022$), IL-10 ($^{*}P = 0.0122$), IFN-γ ($^{*}P = 0.0186$), IL-12 ($P = 0.4268$) and CCL11 ($^{*}P = 0.0163$). (**f**) Levels of IL-4 ($P = 0.0838$), IL-13 ($^{*}P = 0.0118$), IL-10 ($^{***}P < 0.0001$), IFN-γ ($P = 0.0794$) in supernatants of OVA-stimulated DLN cells. Serum levels of OVA-specific IgE ($P = 0.7173$), IgG1 ($P = 0.1299$) and IgG2a ($P = 0.1012$). (**g**) Percentages of T1/ST2^{+} cells gated on CD3^{+}CD4^{+} T cells. Isotype control staining for the T1/ST2 marker is shown. One representative experiment of three. $n = 3$–5 mice per group. Top, lung levels of CCL17 ($^{*}P = 0.0396$) and CCL22 ($^{*}P = 0.0126$) for mice that received one OVA challenge (and antibody treatment). Bottom, lung levels of CCL17 ($^{**}P = 0.0053$) and CCL22 ($^{**}P = 0.0036$) for mice that received three OVA challenges (and antibody treatment).

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CD11c^{+}B220^{-}Gr1^{-} cDCs), considered immunogenic, and pDCs, considered mainly regulatory[28–31]. CD11c^{+}PDCA-1^{+}/120G8^{+}Gr-1^{+} cells have been described as pDCs in allergic airway inflammation, exhibiting suppressive effects on T_H2 responses[28,32,33].

Mice treated with Opn-specific antibody had increased percentages and total numbers of DLN CD11c^{+}PDCA-1^{+}Gr-1^{+} pDCs (characterized also as CD11c^{+}Gr-1^{+}B220^{+}) and of Ag-loaded (Alexa Fluor–OVA^{+}) pDCs (**Fig. 5b**). OVA uptake was not influenced, as the percentages of OVA^{+} cells among pDCs were similar (approximately 52 ± 5% for Opn-s neutralization versus 47 ± 5% for Ig). We observed no differences in the percentages and numbers of cDCs (CD11c^{+}B220^{-}Gr-1^{-}) or Alexa Fluor–OVA^{+} cDCs (**Fig. 5b**). The percentages of OVA^{+} cells among cDCs were similar (approximately 45 ± 5% for Opn-s neutralization versus 51 ± 5% for Ig). The numbers of CD11c^{+} cells within DLNs were similar among groups (per mouse: 65,420 ± 2,289 cells for Opn-s neutralization versus 65,250 ± 6,284 for Ig). Purified DLN CD11c^{+}cells from Opn-s–neutralized mice stimulated with CpG oligodeoxynucleotides produced increased levels of IFN-α, a defining characteristic of pDCs (refs. 28,34 and **Fig. 5c**).

Spp1$^{-/-}$ mice exhibited no significant enhancement of pDC recruitment in DLNs during priming, as compared to *Spp1*$^{+/+}$ mice (8,732 ± 2,900 versus 6,518 ± 2,100 cells per mouse, $P = 0.7170$). We observed no differences in CD11c^{+} cell recruitment (39,800 ± 4,800 versus 35,800 ± 9,200 cells per mouse). A study using a substantially different sensitization protocol, involving trinitrochlorobenzene administration, has demonstrated decreased migration of CD11c^{+} cells to skin and DLNs in *Spp1*$^{-/-}$ mice[18]. The discrepancies between this report and our findings might be due to different innate mechanisms.

A recent study has shown that pDCs suppress T_H2 responses[28]. To address whether the effects of Opn-s blockade during sensitization were mediated by the pDC population, we depleted pDCs (using the 120G8 antibody[35]) before OVA/alum sensitization and Opn-s blockade in naive BALB/c mice. pDC depletion was successful, as shown by flow cytometric analysis of PDCA-1^{+} cells (**Fig. 5d**). The Opn-specific antibody treatment had no effect on primary T_H2 responses in

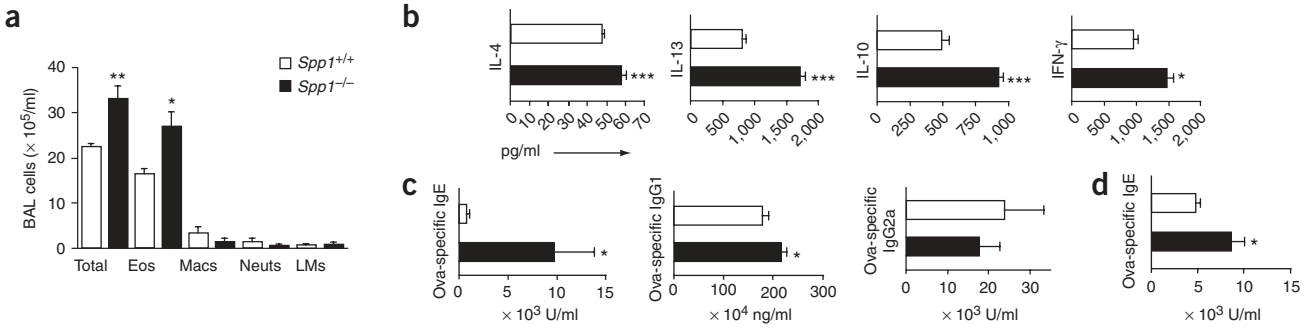

Figure 4 *Spp1*$^{-/-}$ mice exhibit enhanced T_H2 responses. (**a**) *Spp1*$^{+/+}$ and *Spp1*$^{-/-}$ mice were immunized with OVA/alum i.p. on days 0 and 12, and challenged through the airways with aerosolized OVA from day 18 to day 23. Differential cell counts in BAL from *Spp1*$^{+/+}$ and *Spp1*$^{-/-}$ mice. $^{**}P = 0.0073$ for total cell number, $^{*}P = 0.0320$ for eosinophils. Data are expressed as mean ± s.e.m. $n = 4$–6 mice per group, three independent experiments. (**b**) IL-4 ($^{***}P < 0.0001$), IL-13 ($^{***}P < 0.0001$), IL-10 ($^{***}P < 0.0001$) and IFN-γ ($^{*}P = 0.0455$) levels in supernatants from OVA-stimulated DLN cells. (**c**) Levels of serum OVA-specific IgE ($^{*}P = 0.0415$), IgG1 ($^{*}P = 0.0378$) and IgG2a ($P = 0.5660$) from *Spp1*$^{+/+}$ and *Spp1*$^{-/-}$ mice. (**d**) BALB/c mice were immunized with OVA/alum i.p. on days 0 and 12, and challenged through the airways with aerosolized OVA from day 18 to day 20. Either anti-Opn or Ig control was administered during both the OVA sensitization and challenge phases. OVA-specific IgE levels in the sera ($^{*}P = 0.0236$) are shown. Values are expressed as mean ± s.e.m. $n = 4$–6 mice per group, two independent experiments. All *P*-values were obtained by unpaired Student's *t*-test.

pDC-depleted mice, and after treatment, these responses were similar to those in Ig-treated, pDC-depleted mice. This was indicated by the IL-4, IL-13 and IFN-γ levels and the OVA-specific proliferative responses (**Fig. 5e**). In both groups, pDC-depleted mice exhibited increased IL-4, IL-13 and IFN-γ levels (**Fig. 5e**), suggestive of a regulatory role for pDCs, as previously described[28]. Isolated pDCs from DLNs in cocultures with DO11.10 T cells did not induce measurable cytokine levels, whereas cDCs induced cytokine release (IL-4 levels: 92 ± 10 pg/ml; IFN-γ levels: 476 ± 20 pg/ml), suggesting that these cells might have immunogenic potential.

We therefore concluded that the decrease in T_H2 priming observed after Opn-s neutralization was mediated by increased numbers of regulatory pDCs in DLNs.

Opn-s blockade at challenge affects cDC numbers

We investigated cDC and pDC recruitment when Opn-s was neutralized during challenge (**Fig. 3a**, protocol). There was an increase in total and Alexa Fluor–OVA$^+$ cDCs and pDCs in DLNs of mice treated with Opn-specific antibody (**Fig. 5f**). OVA$^+$ cells among cDCs and pDCs were similar (approximately 47 ± 6% and 43 ± 5% for Opn-s neutralization, versus 53 ± 3% and 49 ± 6% for Ig). Opn-s blockade increased total numbers of CD11c$^+$ cells (data not shown). We obtained similar results on DC subsets following one, instead of three, intranasal challenges (data not shown). Of note, both triple and single challenges of mice treated with Opn-specific antibody enhanced AHR, increased the percentage of DLN T1/ST2$^+$ T_H2 cells and IL-4 in OVA-stimulated DLNs (**Fig. 3g** and data not shown). Overall, we observed increased recruitment of cDCs and pDCs in lung DLNs, with the increase for cDCs being greater than that for pDCs.

To examine the role of pDCs in the above settings, we used pDC-depleted mice. These exhibited increased allergic responses in comparison to their respective non–pDC-depleted mice (**Fig. 5g**,**h**), indicating a regulatory role for pDCs during secondary responses. Notably, in pDC-depleted mice, treatment with Opn-specific antibody, as compared to treatment with control Ig, increased total numbers of BAL cells (data not shown) and eosinophils as well as the levels of AHR, IL-13 and IL-10 in OVA-specific DLN responses (**Fig. 5g**,**h**), suggesting that pDCs are not involved in the proallergic effect of Opn-s neutralization during challenge.

Cocultures of cDCs with DO11.10 T cells produced increased IL-4 and IL-13 levels, showing the T_H2-promoting potential of the cDCs (data not shown). Similar increases in cDC numbers have been linked to markedly enhanced inflammation[32] and T_H2 proliferation[36]. Overall, enhancement of T_H2 responses due to Opn-s blockade at challenge was influenced by the increased recruitment of immunogenic cDCs.

Administration of rOpn is protective at challenge

rOpn administered along with OVA/alum during sensitization increased IL-13 and IFN-γ levels in OVA-stimulated DLNs (**Fig. 6a**), suggesting a pro-inflammatory role for Opn-s during T_H2 priming.

Intranasal administration of rOpn before OVA challenge decreased the total numbers of BAL cells, eosinophils and mononuclear cells (**Fig. 6b**) and AHR responses, to the levels seen in the controls (**Fig. 6c**). Lung leukocytic infiltration, mucus secretion (**Fig. 6d**) and levels of IL-4, IL-13, IL-10, IFN-γ, CCL11, CCL17 and CCL22 were also decreased, whereas IL-12 levels were increased (**Fig. 6e**). BAL cytokines exhibited a similar pattern (data not shown).

OVA-stimulated DLN cells from rOpn-treated mice produced decreased IL-4, IL-13 and IFN-γ levels (**Fig. 6f**). OVA-specific IgG1 and IgE levels were decreased, whereas IgG2a levels were increased (**Fig. 6g**). These results point to a suppressive role for endogenous Opn-s during secondary allergic airway responses.

DISCUSSION

Previous studies have demonstrated the impact of Opn on T_H1-associated immunity during ongoing immune responses against viral, bacterial and self antigens[6,7,15]. Our results point to dual and opposing effects of Opn-s on T_H2-mediated allergic airway disease: pro-inflammatory at primary systemic sensitization, and anti-inflammatory during pulmonary secondary antigenic challenge. Neutralization of Opn-s during initial antigenic encounter increased the recruitment of regulatory PDCA-1$^+$Gr-1$^+$ pDCs in DLNs, which mediated a decrease in primary T_H2 responses. In contrast, Opn-s blockade during challenge enhanced T_H2 effector responses, mainly mediated by increased recruitment of T_H2-promoting cDCs in DLNs. Intranasal administration of rOpn during antigenic challenge reversed established T_H2 responses and conferred protection from allergic disease.

In agreement with a previous study[28], our experiments revealed that pDCs were immunosuppressive for T_H2 responses. pDC depletion,

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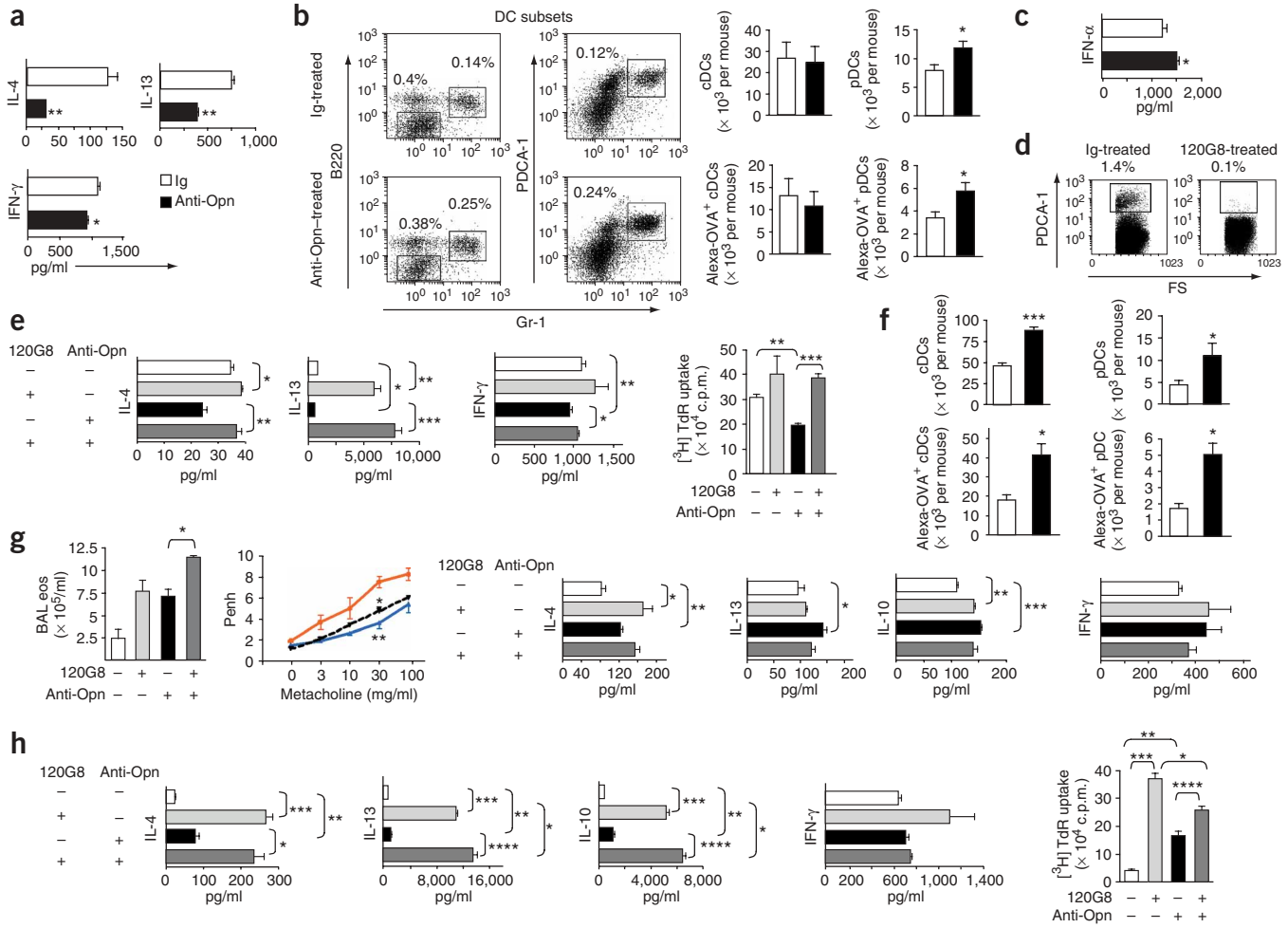

Figure 5 Opn-s blockade affects T_H2 responses through DC recruitment. (**a**) IL-4 (**$P = 0.0024$), IL-13 (**$P = 0.0017$) and IFN-γ (*$P = 0.0365$) from OVA-stimulated cocultures of DLN CD11c$^+$ cells with DO11.10 T_H cells. Data are expressed as mean ± s.e.m. $n = 4$–6 mice per group, three independent experiments. (**b**) Percentages of DLN 7AAD$^-$CD11c$^+$B220$^-$Gr-1$^-$ cDCs (left, bottom), 7AAD$^-$CD11c$^+$B220$^+$Gr-1$^+$ pDCs (left, top) and 7AAD$^-$CD11c$^+$PDCA-1$^+$Gr-1$^+$ pDCs (right). Numbers of cDCs and pDCs (*$P = 0.0166$), and Alexa Fluor–OVA$^+$ cDCs and pDCs (*$P = 0.0221$). (**c**) IFN-α (*$P = 0.0143$) from CpG-stimulated CD11c$^+$ cells. (**d**) Percentages of 7AAD$^-$PDCA-1$^+$ cells. (**e**) IL-4 (*$P = 0.0461$, **$P = 0.0176$), IL-13 (*$P = 0.016$, **$P = 0.0014$, ***$P = 0.0003$) and IFN-γ (*$P = 0.0283$, **$P = 0.0365$) from OVA-stimulated DLNs. [^{3}H]thymidine incorporation (TdR) of OVA-stimulated DLNs (**$P = 0.0013$, ***$P = 0.0004$). $n = 4$ mice per group, three experiments. c.p.m., counts per min. (**f**) Numbers of cDCs (***$P = 0.0001$) and pDCs (*$P = 0.0375$), and Alexa Fluor–OVA$^+$ cDCs (*$P = 0.0202$) and pDCs (*$P = 0.0116$). $n = 5$–7 mice per group, three independent experiments. (**g**) BAL eosinophils (*$P = 0.0351$) and AHR (anti-Opn, blue line; Ig + 120G8, dashed line; anti-Opn + 120G8, orange line). *$P = 0.0132$, **$P = 0.0034$. $n = 5$–8 mice per group, two experiments. Lung IL-4 (*$P = 0.0349$, **$P = 0.0491$), IL-13 (*$P = 0.0299$), IL-10 (**$P = 0.0064$, ***$P = 0.0006$) and IFN-γ. (**h**) Results from OVA-stimulated DLNs. IL-4 (***$P = 0.0002$, *$P = 0.0074$, **$P = 0.0076$), IL-13 (*$P = 0.0142$), **$P = 0.0016$, ***$P < 0.0001$, ****$P < 0.0001$), IL-10 (*$P = 0.0112$, **$P = 0.0025$, ***$P < 0.0001$, ****$P < 0.0001$) and IFN-γ. [^{3}H]thymidine incorporation of OVA-stimulated DLNs (****$P = 0.0108$, *$P = 0.0116$, **$P = 0.0016$, ***$P = 0.0001$). Unpaired Student's *t*-test for all statistical analyses.

before Opn-s neutralization, restored OVA/alum-driven responses, revealing that the dampening effect of Opn-s neutralization during priming was mainly mediated by pDCs. This initial pDC-mediated dampening in priming provided an explanation for the subsequent decrease in T_H2-mediated pathology following pulmonary challenge. Opn blockade was also accompanied by decreased IFN-γ production whereas rOpn administration enhanced T_H2 priming and was accompanied by increased IFN-γ production. IFN-γ may participate in the Opn-s–mediated effect, particularly as decreased IFN-γ production during OVA/alum sensitization reduces priming[24]. Opn-s neutralization at sensitization resulted in increased lung IFN-γ levels following challenge. In this setting, IFN-γ may exert an immunoregulatory role, associated with the increased number of pDCs at priming. In support of this idea, adoptive transfer of pDCs during sensitization enhances IFN-γ levels and confers protection from allergic airway disease[29], and induction of IFN-γ–producing regulatory T cells reduces allergic airway inflammation[37].

We were surprised to note the implicit pro-inflammatory effect of Opn-s during priming, as one would expect that blocking a T_H1 inducer[38] at the initial point of T_H differentiation would upregulate T_H2 responses. However, it was rather Opn-s blockade during recall responses that resulted in enhanced allergic pulmonary inflammation and disease. We observed the same effect in mice treated with Opn-specific antibody during both the sensitization and challenge phases (data not shown and **Fig. 4d**) and in *Spp1*$^{-/-}$ mice, which developed increased T_H2 responses. Previous studies have demonstrated that

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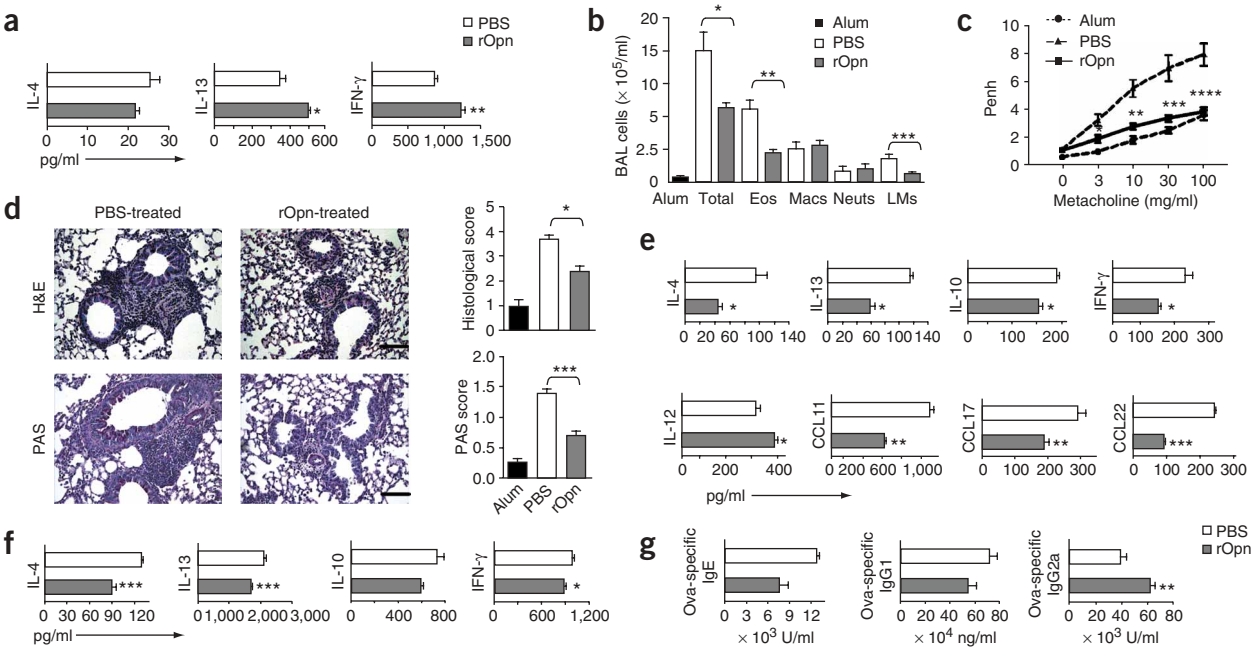

Figure 6 rOpn is protective during pulmonary challenge. BALB/c mice were treated as described in Methods. (**a**) Mice received rOpn before OVA/alum sensitization. Levels of IL-4 ($P = 0.2266$), IL-13 ($^*P = 0.0113$) and IFN-γ ($^{**}P = 0.0079$) in supernatants of OVA-stimulated DLN cells. (**b**–**g**) BALB/c mice received rOpn before OVA aerosol challenges. In **b**, BAL differentials ($^*P = 0.0430$, $^{**}P = 0.0099$, $^{***}P = 0.0067$). In **c**, AHR responses for Penh, analyzed as in **Figure 2c** ($^*P = 0.032$, $^{**}P = 0.0038$, $^{***}P = 0.0083$, $^{****}P = 0.0017$). In **d**, lung inflammation (top) and mucus secretion (bottom). Histological scores for H&E ($^*P = 0.03$) and PAS ($^{***}P = 0.0002$). Scale bar, 100 μm. In **e**, lung levels of IL-4 ($^*P = 0.0378$), IL-13 ($^*P = 0.0141$), IL-10 ($^*P = 0.04$), IFN-γ ($^*P = 0.037$), IL-12 ($^*P = 0.0271$), CCL11 ($^{**}P = 0.0022$), CCL17 ($^{**}P = 0.005$) and CCL22 ($^{***}P < 0.0001$). In **f**, results from supernatants of OVA-stimulated DLN cells. Levels of IL-4 ($^{***}P < 0.0001$), IL-13 ($^{***}P = 0.0003$), IL-10 ($P = 0.1322$) and IFN-γ ($^*P = 0.0229$). In **g**, serum levels of OVA-specific-IgE ($P = 0.0589$), IgG1 ($P = 0.0703$) and IgG2a ($^{**}P = 0.0045$). Data are expressed as mean ± s.e.m. $n = 6$–8 mice per group in three independent experiments. Unpaired Student's *t*-test for all statistical analyses.

during repetitive antigenic encounters, *Spp1*$^{-/-}$ mice have decreased T_H1 immunity[4,6] and autoimmunity[8–10]. Our data imply that the previously demonstrated effect of Opn-s in T_H1/T_H2 balance operates predominantly during recall responses.

Opn-s neutralization during challenge increased DLN cDC and pDC numbers. In allergic airway disease, the most powerful immunogenic potential of CD11c$^+$ cells[39] stems from cDCs (refs. 28,32). For example, blockade of the C5a receptor during allergic airway inflammation increases the recruitment of cDCs, enhancing T_H2 responses[32]. However, we found that pDCs were suppressive during antigenic challenge. In the absence of pDCs, Opn-s blockade still enhanced T_H2 responses and allergic disease. Therefore, the increased induction of cDCs upon Opn-s neutralization provides an explanation for the exacerbation of T_H2-mediated disease. It is also likely that Opn-s neutralization induces a stronger T_H2 response, as Opn-s is known to affect antigen-presenting cells and thus influence the T_H1/T_H2 balance[6]. In support of this idea, local rOpn administration before challenge decreased T_H2 responses and increased IL-12 production.

To examine whether pDCs mediate the effect of Opn-s blockade, we used the 120G8 monoclonal antibody, which has been described as pDC specific and pDC depleting[28,32,35,40,41]. We found by flow cytometry that 120G8 strongly bound all pDCs from naive and OVA/alum-sensitized mice (data not shown). A recent study indicated that 120G8 binds to an epitope of the bone marrow stromal antigen-2 (ref. 42). This study also showed that bone marrow stromal antigen-2 is primarily expressed on all pDCs and to a lesser degree on some immune (plasma) cells, following activation by IFN or virus[42]. Thus, in addition to pDCs, we cannot exclude the contribution of other cell types to the Opn-mediated effect on T_H2 responses.

Comparing the results obtained from Opn-s neutralization to those from knockout of *Spp1*, we found that Opn-s plays a predominant role in allergic airway inflammation. However, considering the critical role of Opn-i in CpG-mediated pDC signaling[15], its involvement in T_H2 regulation is probable. Administration of CpG, alone or in conjunction with allergens, in the lungs of allergic mice reversed established inflammation, possibly through an effect on IFN-α production by pDCs (refs. 43,44). Notably, both isoforms affect pDCs: Opn-s regulates pDC recruitment in allergic response, as described here, whereas Opn-i is essential for functions of pDCs in viral immunity[15].

Increased Opn expression in allergic airway disease may be part of an inherent protective mechanism, as suggested by the fact that the disease was exacerbated following Opn-s blockade at challenge. In fact, it was recently shown that the gene encoding OPN is critically upregulated during bee-venom immunotherapy[45]. In our experiments, administration of rOpn at challenge provided protection from allergic disease. This was mainly mediated through a shift toward an antiallergic T_H1, as shown by increased levels of IL-12 and OVA-specific IgG2a. Intranasal administration of IL-12 during challenge suppresses airway disease[46]. Our data show that, as with IL-12, rOpn is an effective regulator of allergic airway disease.

The variable effect of Opn-s on T_H2 immunity points once more to cytokines playing opposing roles depending on the phase and milieu of the immune response. The effects of Opn-s on pDC biology as well as their contribution to autoimmunity remain to be elucidated.

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METHODS

Mice. We purchased BALB/c and OVA-specific T-cell receptor–transgenic DO11.10 (*Tcr*-TG-DO11.10) mice from the Jackson Laboratory. We backcrossed *Spp1*$^{-/-}$ mice onto the C57BL/6 background for seven generations. Mice were housed at the Animal Facility of the Foundation for Biomedical Research of the Academy of Athens. All procedures 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.

***In vivo* experimental protocols.** We sensitized BALB/c mice with 0.01 mg mouse OVA (Sigma-Aldrich) in 0.2 ml alum (Serva) intraperitoneally (i.p.) on days 0 and 12. Control mice received PBS/alum. We administered aerosolized OVA (5%, for 20 min) on days 18–20. Mice received 20 μg of affinity-purified neutralizing antibody to Opn (AF-808, R&D Systems) or Ig control (R&D Systems) i.p., 2–3 h before sensitization or challenge. OVA/alum-sensitized *Spp1*$^{-/-}$ and *Spp1*$^{+/+}$ littermate mice received six OVA challenges on days 18–23. For the data depicted in **Figure 5a–c**, BALB/c mice received 40 μg of Opn-specific antibody or Ig control i.p.; 2–3 h later, BALB/c (or *Spp1*$^{-/-}$ and *Spp1*$^{+/+}$) mice were sensitized i.p. with 0.1 mg Alexa Fluor-conjugated OVA/alum (LPS-low, Molecular Probes). We examined CD11c^{+} cell–driven responses and DC subsets 40 h later, which is when these cells traffic to DLNs (ref. 28). For the results shown in **Figure 5e**, BALB/c mice received 225 μg of 120G8 pDC-depleting antibody or Ig control (rat IgG1/κ, BD Biosciences) i.p. daily, for 4 d before sensitization. Then (day 0), mice received 40 μg of Opn-specific antibody or Ig control i.p.; 2–3 h later, they were sensitized i.p. with 0.1 mg Alexa Fluor–OVA/alum. Mice were killed 40 h following sensitization. For the data shown in **Figure 5f**, BALB/c mice were sensitized with OVA/alum i.p. on days 0 and 12, and, starting on day 18, were challenged intranasally with one or three doses of 0.5 mg Alexa Fluor–OVA. We administered Opn-specific antibody or Ig control (40 μg per mouse) i.p. 2–3 h before challenge. The data depicted in **Figure 5g,h** are from BALB/c mice sensitized with 10 μg of OVA/alum i.p. on days 0 and 12 and then given 225 μg of 120G8 pDC-depleting antibody or Ig control (i.p.) daily from days 17 to 20. Mice also received 20 μg of Opn-specific antibody or Ig control i.p. daily, 2–3 h before OVA challenge, from days 18 to 20. Mice were killed 40 h after the final challenge. In **Figure 6a**, the data are from BALB/c mice given 4 μg of mouse rOpn (R&D Systems) or PBS i.p., and then, 2–3 h later, sensitized i.p. with 0.1mg Alexa Fluor–OVA/alum. For the data in **Figure 6b–g**, we sensitized BALB/c mice with OVA/alum i.p. on days 0 and 12 and then challenged them intranasally with 0.5 mg Alexa Fluor–OVA from days 18 to 20. We administered rOpn (2.5 μg per mouse) or PBS i.p. 2–3 h before challenge. Mice were killed 40 h after the final challenge.

AHR and airway inflammation. We measured AHR, a clinical measurement of asthma, as enhanced pause (Penh) and BAL inflammatory cells, as previously described[47]. We stained paraffin-embedded sections with hematoxylin & eosin (H&E) or Periodic-Acid-Schiff (PAS), as previously described[21].

Human subjects. We performed flexible bronchoscopy on asthmatics, classified and treated according to the Global Initiative for Asthma guidelines (one mild intermittent, one moderate and four severe), and nine healthy volunteers. We took biopsies as previously described[48]. The study was approved by the Sotiria Hospital Ethics Committee, and individuals signed an informed-consent form.

Immunohistochemistry. We immunostained paraffin-embedded sections as previously described[47]. We used antibodies to human OPN (MAB-1433, R&D Systems) and mouse Opn (AF-808, R&D Systems). For a control, we used matched isotype IgG (R&D Systems).

Cell culture, proliferation and cytokine analysis. We obtained lung homogenates as previously described[47]. We used a previously described method[49] to isolate cells from DLNs (mediastinal, following intranasal treatment, or inguinal and axillary following i.p. treatment). We cultured DLN cells, alone or with CD4^{+} T cells (Dynal) from DO11.10 mice, with 125 μg/ml OVA for 48 h. We cocultured CD11c^{+} cells purified from DLNs (Miltenyi Biotec) with T_H cells from DO11.10 mice and 125 μg/ml OVA, for 48 h. For pDC and cDC isolation, a combination of the above-described method with the pDC isolation kit (Miltenyi Biotec) was used. We performed proliferation assays as previously described[49]. To measure cytokines and chemokines, we used ELISA kits for IL-4, IL-10, IFN-γ and IL-12 (BD Biosciences) and IL-13, Opn, CCL11, CCL22 and CCL17 (R&D Systems). We used a newer kit for IL-13 in pDC depletion experiments (R&D Systems). We cultured CD11c^{+} cells with 0.2 μg/ml CpG oligodeoxynucleotides (5′-TCCATGACGTTCCTGATGCT-3′) or control GpC (5′-TCCATGAGCTTCCTGATGCT-3′) (MWG, Biotech), synthesized as described[31]. After 24 h, we measured IFN-α, by ELISA (PBL Biomedical Laboratories).

Serum antibody concentration. We measured OVA-specific IgE, IgG1 and IgG2a antibodies as described[50].

Flow-cytometric analysis. We stained live DLN cells (7AAD^{-}, BD Biosciences) with conjugated antibodies to CD4, CD3, CD11c, B220, CD11b, Gr-1, PDCA-1 (BD Biosciences) and T1/ST2 (MD Biosciences). To perform the FACS analysis, we used a Coulter cytometer (Cytomics, FC 500).

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AUTHOR CONTRIBUTIONS

G.X. designed experiments, performed animal studies and immunohistochemistry, generated figures, analyzed data and wrote the manuscript. T.A. performed animal studies, tissue-culture experiments, generated figures and performed flow cytometry. M.S. assisted with the animal studies and tissue-culture experiments. D.C.M.S. assisted with the animal studies and image analysis. E.E. and M.G. performed bronchoscopies, and provided human lung biopsies and clinical characteristics of the individuals. B.N.L. provided antibodies, assisted with the design of experiments and participated in discussions. C.M.L. assisted with experimental design, writing and critical editing of the manuscript. V.P. provided crucial ideas, designed experiments, analyzed data, supervised the study and wrote the manuscript with G.X.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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