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Phosphodiesterase 4B is essential for T_H2-cell function and development of airway hyperresponsiveness in allergic asthma

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Abstract

Background—Cyclic AMP (cAMP) signaling modulates functions of inflammatory cells involved in the pathogenesis of asthma, and type 4 cAMP-specific phosphodiesterases (PDE4s) are essential components of this pathway. Induction of the PDE4 isoform PDE4B is necessary for Toll-like receptor signaling in monocytes and macrophages and is associated with T cell receptor/CD3 in T cells; however, its exact physiological function in the development of allergic asthma remains undefined.

Objectives—We investigated the role of PDE4B in the development of allergen-induced airway hyperresponsiveness (AHR) and T_H2-driven inflammatory responses.

Methods—Wild-type and PDE4B$^{-/-}$ mice were sensitized and challenged with ovalbumin and AHR measured in response to inhaled methacholine. Airway inflammation was characterized by analyzing leukocyte infiltration and cytokine accumulation in the airways. Ovalbumin-stimulated cell proliferation and T_H2 cytokine production were determined in cultured bronchial lymph node cells.

Results—Mice deficient in PDE4B do not develop AHR. This protective effect was associated with a significant decrease in eosinophils recruitment to the lungs and decreased T_H2 cytokine levels in the bronchoalveolar lavage fluid. Defects in T-cell replication, T_H2 cytokine production, and dendritic cell migration were evident in cells from the airway-draining lymph nodes. Conversely, accumulation of the T_H1 cytokine IFN-γ was not affected in PDE4B$^{-/-}$ mice. Ablation of the orthologous PDE4 gene PDE4A has no impact on airway inflammation.

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Clinical implications: Inactivation of PDE4B is sufficient to prevent airway inflammation, providing proof of concept that inhibitors with PDE4B selectivity may improve the therapeutic window for the treatment of chronic pulmonary inflammation.

Conclusion—By relieving a cAMP-negative constraint, PDE4B plays an essential role in T_H2-cell activation and dendritic cell recruitment during airway inflammation. These findings provide proof of concept that PDE4 inhibitors with PDE4B selectivity may have efficacy in asthma treatment.

Keywords

Asthma; PDE4B; T_H2 cytokines; airway hyperresponsiveness; airway inflammation; cAMP signaling

Bronchial asthma is a multifactorial, polygenic disorder characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), mucosal edema, and airway obstruction. Its incidence in industrialized countries has steadily increased, resulting in increased morbidity and mortality. T_H2 cells and T_H2 cytokines play central roles in the pathogenesis of allergic asthma[1,2] because they orchestrate airway eosinophilia, AHR, goblet cell hyperplasia, serum IgE elevation, and airway remodeling, all key features of this disease.[3–8] Thus, understanding the regulation of T-cell activation and T_H2 cytokine production in response to allergens is critical for developing useful therapeutic strategies for asthma.

Cyclic AMP (cAMP) signaling is immunomodulatory virtually for all cell types involved in the pathogenesis of asthma.[9] Elevation of cAMP levels leads to airway smooth muscle relaxation and bronchodilation.10 It inhibits a number of immune and inflammatory responses, including T-cell activation and proliferation,11›12 TNF-α production in monocytes and macrophages,[13,14] superoxide anion production in eosinophils,[15] and eosinophil chemotaxis by inflammatory mediators.[16] Given these immunomodulatory effects, increasing intracellular cAMP levels is viewed as a promising strategy for the treatment of pulmonary inflammation, including asthma.

Intracellular cAMP is degraded and inactivated by numerous phosphodiesterases (PDEs), a superfamily of isoenzymes made up of 11 families (PDE1–PDE11). Type 4 cAMP-specific PDEs (PDE4s) are the predominant cAMP-hydrolyzing enzymes in almost all inflammatory cells,9›17 suggesting a role in regulating immune/inflammatory responses. Indeed, inhibition of PDE4 produces a wide range of anti-inflammatory effects.9 PDE4 inhibitors interfere with antigen-induced eosinophil infiltration and development of AHR in animal models of pulmonary inflammation.9 In mammals, the PDE4 family of isozymes includes 4 genes (PDE4A, PDE4B, PDE4C, and PDE4D) with more than 20 PDE4 variants described. Because PDE4s have a highly conserved catalytic domain, most PDE4 inhibitors are nonselective and do not distinguish among isoforms, although 1 recent publication describes compounds with 100-fold specificity for PDE4B over PDE4D.18 Thus, a pharmacologic approach provides little information on the role of individual PDE4 isoforms in the airways and inflammatory cells. During the past decade, several nonselective inhibitors of PDE4, such as Ariflo (GlaxoSmithKline, Brent-ford, United Kingdom) and Roflumilast (Nycomed, Melville, NY), have been developed for their anti-inflammatory properties in the treatment of asthma and chronic obstructive pulmonary disease. 19–21 Although endowed with considerable efficacy, these inhibitors have adverse effects (nausea, emesis, and diarrhea) that limit their dosing and, therefore, their immunomodulatory activity. To improve the therapeutic window of PDE4 inhibitors, we have devised a novel approach to determine whether targeting a single PDE4 isoform retains efficacy while reducing the side effects of nonselective PDE4 inhibitors.

Using a gene targeting strategy, we have previously demonstrated that PDE4 genes are nonredundant, each with specific functions. Ablation of PDE4D, for instance, affects a wide spectrum of physiological functions,[22–27] a finding consistent with the wide expression of

PDE4D in tissues and cells. Ablation of PDE4B selectively blocks LPS-induced TNF-α production and other inflammatory responses.[26,28,29]

It is well established that TCR activation in T cells signals through tyrosine phosphorylation, protein kinase C activation, and Ca^{2+} mobilization, whereas the role of cAMP signaling in these cells is less well defined. Several studies have shown that T-cell activation is associated with a transient increase in cAMP,[30] but the significance of this finding is unclear. Conversely, it is well documented that T-cell proliferation does not occur if the increase in cAMP is sustained rather than transient.[30–32] Abrahamsen et al[33] recently reported that on TCR and CD28 stimulation in human peripheral T cells, PDE4 is recruited to lipid rafts where cAMP is degraded locally, thereby potentiating T-cell activation. Moreover, PDE4B2 may be associated with the CD3ε chain of the TCR complex in peripheral T cells and phosphorylated at a unique tyrosine residue after CD3 ligation.[34] When stably transfected in Jurkat cells, PDE4B2 localizes to the immunologic synapse on activation, and the increase in PDE4B2 activity correlates with increased IL-2 production.[35] All these findings point to a potential role of PDE4, particularly PDE4B, in regulation of T cells. Finally, a link between the 1p32 region where the PDE4B gene is located and asthma susceptibility was recently proposed.[36] Nevertheless, the exact physiological role of PDE4B in T_H2 effector cells as well as their underlying mechanisms in the pathogenesis of allergic asthma remains to be explored.

Although protected from AHR, PDE4D-deficient mice developed normal eosinophil infiltration in the lungs when sensitized and challenged with allergen.[23] In addition, $PDE4D^{-/-}$ splenic cells cultured in the presence of allergen proliferate and produce IL-4 normally.[23] Given the established effect of PDE4 inhibitors on inflammatory responses[9] as well as the unique role of PDE4B in TCR[34,35] and TLR[28,29] signaling, we hypothesized that PDE4B, rather than PDE4D or PDE4A, plays a major role in the control of inflammation. Here, we investigated the development of allergen-induced AHR and T_H2-driven inflammatory responses in $PDE4B^{-/-}$ mice. We demonstrate that $PDE4B^{-/-}$ mice do not develop AHR when immunized and challenged in the lung with ovalbumin (OVA). T_H2 effector responses were also affected after PDE4B ablation. These results demonstrate a crucial role of PDE4B in the control of T_H2-driven airway inflammatory responses and the development of AHR.

METHODS

Mice

Generation of PDE4B and PDE4A homozygous null mice was described previously.[26,28] Mice used in this study were 2 to 5 months of age and on a mixed C57BL/6-129/Ola background. In addition, the $PDE4B^{-/-}$ allele was transferred to a pure C57BL/6 background by 12 generations of backcrossing and purity confirmed by single nucleotide polymorphism analysis. The results reported in this article's Figs E1, E6, and E7 in the Online Repository at www.jacionline.org were derived from mice on this pure C57BL/6 background. All experimental procedures involving animals were approved by the Administrative Panel on Laboratory Animal Care at Stanford and University of California, San Francisco.

Immunization protocols

Mice were immunized and challenged with OVA as described previously.[23] Briefly, 50 μg OVA complexed with aluminum potassium sulfate (alum) was administered intraperitoneally on days 0 and 14, and 50 μg OVA in 50 μL saline intranasally on days 14, 25, 26, and 27. Control mice received alum intraperitoneally and saline intranasally. AHR to

inhaled methacholine or intravenous injection of acetylcholine was measured 24 hours after the last intranasal dose of OVA (day 28). On day 29, mice were bled by cardiac puncture, followed by either bronchoalveolar lavage (BAL) and bronchial lymph node dissection or lung fixation. In some experiments, the sensitization protocol was modified by omitting the last intranasal administration of OVA. These mice were killed on day 27, and BAL was performed to evaluate the leukocyte accumulation. For experiments in Fig 1, mice were primed with a single dose of OVA emulsified in alum or incomplete Freund adjuvant. For non T_H2-biased responses, 50 μg OVA in alum was given intraperitoneally, or 75 μg (50 μL) OVA in incomplete Freund adjuvant was injected in each footpad. After 9 days, spleens or popliteal lymph nodes, respectively, were removed for *in vitro* proliferation assay.

Noninvasive and invasive measurement of airway responsiveness

Noninvasive measurement of AHR was performed in conscious mice placed in a whole-body plethysmograph (model PLY 3211; Buxco Electronics Inc, Troy, NY) as described previously.[23] Lung resistance (R_L) and dynamic compliance (C_{dyn}) to increasing concentrations of methacholine were also measured. Briefly, after anesthesia and intubation, mice were connected to plethysmograph chambers (model PLY 3111; Buxco Research Systems,Wilmington, NC), and respiration was maintained by a ventilator (type 845; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Ventilation was achieved at 150 breaths/min and a tidal volume of 0.2 mL. Aerosolized methacholine (20 μL) was administered for 5 seconds with a tidal volume of 0.2 mL. The data for R_L and C_{dyn} were continuously recorded for 3 minutes after each aerosol challenge and analyzed by Buxco BioSystem XA software (Buxco,Wilmington, NC). Data are expressed as the percent of PBS baseline values.

Bronchoalveolar lavage, lung histology, and histochemistry

Mice were killed by CO_2 asphyxiation. Airways were lavaged via a tracheal tube, and total and differential cells in the BAL fluid were counted as described. 23 BAL fluid (BALF) was centrifuged and supernatants collected and stored at −20°C for cytokine measurements. In some mice, no BAL was performed, but lungs were removed, fixed, processed for embedding, and sectioned and stained as detailed previously.[23] Mucin-containing goblet cells were detected by periodic acid-Schiff (PAS) stain. Infiltration was quantified as detailed in the Methods section, Histologic evaluation in this article's Online Repository at www.jacionline.org.

Stimulation of bronchial and popliteal lymph node cells and spleen cells *in vitro*

Cells isolated from bronchial and popliteal lymph nodes were cultured at 5×10^5 per well in 96-well plates and stimulated *in vitro* in the absence or presence of increasing concentrations of OVA. Splenic cells were depleted of B cells by adherence to goat antimouse immunoglobulin-coated plates and then cultured at 4×10^5 per well in 96-well plates and stimulated with OVA as described. For proliferation measurement, cells were incubated for 3 days and pulsed with either 1 μCi [^{3}H]thymidine for the last 16 hours or 10 μmol/L bromodeoxyuridine for the last 8 hours. For ELISA, supernatants of the culture medium were collected after 4 days of incubation.

Migration of dendritic cells from lungs to the draining lymph nodes

Naive PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice were administered fluorescein isothiocyanate (FITC)–OVA by nasal instillation, and the FITC$^+$ dendritic cells in the bronchial lymph nodes were analyzed by flow cytometry as described in the Methods section, Migration of dendritic cells in the Online Repository.

Additional methods

The isolation of lymph node and spleen cells from PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice, purification of CD4^{+} T cells, preparation of T cell–depleted, mitomycin C–treated splenocytes, and differentiation of purified CD4^{+} T cells to T_H1 or T_H2 cells *in vitro*, as well as the PDE activity measurements, are described in the Methods section, Immunoprecipitation and PDE assay in the Online Repository.

RESULTS

PDE4B$^{-/-}$ mice do not develop AHR

To investigate the effect of PDE4B ablation on the development of AHR, C57BL6/129Ola PDE4B$^{-/-}$ mice and wild-type littermates were sensitized intraperitoneally and challenged intranasally with OVA (see Methods). Twenty-four hours after the final allergen exposure, airway responsiveness to increasing concentrations of methacholine was measured by either whole-body plethysmography or invasive methods. Wild-type littermates sensitized and challenged with OVA developed significant AHR (Fig 1, *A*). In contrast, OVA-sensitized PDE4B$^{-/-}$ mice exhibited no increase in airway responsiveness compared with alum-sensitized and saline-challenged mice. Using invasive measurements, lung resistance and compliance were not changed in PDE4B$^{-/-}$ mice (Fig 1, *B and C*). Protection from development of AHR was also present when the PDE4B$^{-/-}$ allele was tested on a pure C57BL/6 background (Fig E1, *A*). These findings demonstrate that PDE4B is required for the development of allergic AHR.

When C57BL6/129Ola PDE4A$^{-/-}$ mice were used to measure AHR, no increased responses were observed after sensitization ($P < .001$; see this article's Fig E2 in the Online Repository at www.jacionline.org). However, these data should be interpreted with some caution because the response to methacholine of wild-type (PDE4A$^{+/+}$) littermates was reduced compared with PDE4B$^{+/+}$ mice on the same background. The cause of this difference is unclear, perhaps dependent on genetic variations among mouse lines.

PDE4B$^{-/-}$ mice display decreased eosinophil infiltration but develop normal goblet cell hyperplasia

To examine whether protection from AHR is associated with decreased inflammation, total leukocyte number in the BALF and its cellular composition were assessed in C57BL6/129Ola PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. As shown in Fig 2, *A*, cell count was moderately but significantly reduced ($P < .05$) in PDE4B$^{-/-}$ BALF compared with the wild-type counterparts. This reduction was mainly a result of a decrease in eosinophils infiltration. Unlike PDE4B$^{-/-}$ mice, mice deficient in PDE4A showed normal total cell number and eosinophil in the BALF (see this article's Fig E3, *A*, in the Online Repository at www.jacionline.org). Together with our previous observations that PDE4D$^{-/-}$ mice develop normal airway inflammation after allergen sensitization,[23] these findings indicated that, of the 3 PDE4 genes studied, only PDE4B is involved in the allergen-induced airway inflammation.

Although eosinophil infiltration is decreased in airways, the effect was not as profound as that reported with PDE4 inhibitors, 37–40 possibly a consequence of differences in sensitization protocol, species used, or genetic backgrounds. To test how different protocols affect the PDE4B phenotype, the number of intranasal administrations of OVA was reduced from 4 to 3 (see Methods). Under these conditions, the difference of BAL cell accumulation between wild-type and PDE4B$^{-/-}$ mice became highly significant (Fig 2, *B*), with total cell number and eosinophil count reduced by approximately 70% ($P < .01$).

To quantify further the T-cell response in PDE4B$^{-/-}$ lungs, T_H1 and T_H2 cytokines were measured in the BALF of sensitized PDE4B$^{-/-}$ and PDE4A$^{-/-}$ mice and their corresponding wild-type littermates. IFN-γ accumulation was comparable in wild-type and PDE4B$^{-/-}$ mice, documenting the successful sensitization and the normal T_H1 response (Fig 2, *C*). Conversely, a decrease in T_H2 cytokines was observed in the BALF derived from PDE4B$^{-/-}$ mice (Fig 2, *C*). After intranasal exposure of OVA, TNF-α accumulation was also significantly decreased in the PDE4B$^{-/-}$ mice (data not shown). Conversely, cytokine accumulation in the PDE4A$^{-/-}$ mice was not affected (Fig E3, *B*).

Histology of the lungs from sensitized wild-type mice showed extensive leukocyte infiltration in the perivascular and peribronchial spaces, sometimes with inflammatory cells infiltrating the alveoli (Fig 3, *A*). In addition, the epithelium of the bronchi showed a large increase in PAS-stained cells, indicating massive hyperplasia of goblet cells. Quantitative analysis of the degree of inflammation confirmed a significant ($P < .05$) reduction in the PDE4B null lungs (Fig 3, *B*). Goblet hyperplasia was clearly evident in the sections of sensitized PDE4B$^{-/-}$ mice (Fig 3, *A*).

Circulating OVA-specific IgG_1 and IgE were indistinguishable in wild-type, PDE4B$^{-/-}$, and PDE4A$^{-/-}$ mice (see this article's Table E1 in the Online Repository at www.jacionline.org). Together with our previous observations that PDE4D$^{-/-}$ mice have normal serum levels of OVA-specific IgE,[23] these results suggest that ablation of any of 3 PDE4 genes does not disrupt B-cell responses.

In vitro responses of T cells derived from the PDE4B$^{-/-}$ mice are disrupted

To determine whether T_H2-cell responses are PDE4B-dependent, bronchial or mediastinal lymph nodes were dissected from C57BL6/129Ola or pure C56BL/6 wild-type, PDE4B$^{-/-}$, and PDE4A$^{-/-}$ mice, and cells were cultured in the absence or presence of OVA at different concentrations. After sensitization, CD4$^+$ and CD8$^+$ cells as well as total T cells (CD3$^+$) retrieved from bronchial lymph nodes of wild-type and PDE4B$^{-/-}$ mice were comparable (see this article's Fig E4 in the Online Repository at www.jacionline.org). A decreased response of OVA-specific T cells in the PDE4B$^{-/-}$ mice was demonstrated by [^{3}H]thymidine incorporation. The replication of PDE4B$^{-/-}$ cells in response to increasing concentrations of OVA was reduced by more than 50% compared to wild-type cells regardless of the genetic background (Figs 4, *A*, and E1, *B*), whereas the response of PDE4A$^{-/-}$ cells was undistinguishable from controls (see this article's Fig E5, *A*, in the Online Repository at www.jacionline.org). Attempts to measure the number of T_H1 and T_H2 cells present in the draining lymph nodes by flow cytometry were unsuccessful because of the limited number of cells available in the OVA model used. However, a major decrease in T_H2, but not T_H1, cytokines was observed in the culture medium of PDE4B$^{-/-}$ cells. The levels of IL-4, IL-5, and IL-13 were reduced more than 95% ($P < .001$; Figs 4, *B*, and E1, *C*). Cytokine production by PDE4A$^{-/-}$ cells was instead normal (Fig E5, *B*).

To determine whether the effect of PDE4B ablation is reproduced by acute inhibition of PDE4, sensitized wild-type and PDE4B$^{-/-}$ cells from bronchial lymph nodes were cultured in the presence of OVA with or without rolipram, a PDE4-selective inhibitor, and OVA-stimulated cell proliferation was measured by thymidine or BrdU incorporation. Rolipram produced a significant decrease in cell replication in wild-type cells (Figs 4, *C*, and E6, *A*), confirming that acute inhibition of PDE4 decreases antigen-stimulated proliferation. Conversely, rolipram did not significantly reduce proliferation of PDE4B$^{-/-}$ cells in all background tested (Figs 4, *C*, and E6, *A*), strongly suggesting that the effect of the PDE4 inhibitor requires PDE4B but not PDE4A or PDE4D, although all 3 isoforms are expressed in these cells.[41] When purified CD4$^+$ T cells from bronchial lymph nodes were cocultured with splenic antigen-presenting cells (APCs) derived from wild-type mice, the OVA-

induced PDE4B$^{-/-}$ T-cell proliferation could not be restored (Fig E7), confirming that a defect intrinsic to T cells is associated with PDE4B ablation.

In a complementary experiment, cells were derived from spleens of mice primed intraperitoneally with OVA, B cells removed by panning and the resulting cell populations were challenged with OVA *in vitro*. An increase in cell replication was observed in wild-type cells in response to increasing concentrations of allergen (Figs 5, *A*, and E6, *C*), but proliferation was not affected in PDE4B$^{-/-}$ cells on either genetic background (Figs 5, *A*, and E6, *C*). To assess the responsiveness of T_H1 cells, mice were immunized with OVA emulsified with incomplete Freund adjuvant by footpad injection, and proliferation of cells derived from popliteal lymph nodes was assessed *in vitro*. In this non T_H2-biased paradigm, wild-type and PDE4B$^{-/-}$ cells behaved in an indistinguishable fashion (Fig 5, *B*).

Given the altered macrophage function of the PDE4B$^{-/-}$ mice,[28,29] a defect in APCs may be associated with the major decrease in T_H2 cytokine secreting cells in bronchial lymph nodes. To test this possibility, the migration of FITC-OVA–positive cells from the airways to the draining lymph nodes was monitored. A consistent and significant decrease in MHC II^{hi} CD11c^{+} cells was observed with PDE4B$^{-/-}$ mice (Fig 6).

To determine whether the pattern of PDE4B expression contributes to the major differences in T_H1/T_H2 responses in the PDE4B$^{-/-}$ mice, T_H1 and T_H2 cells were differentiated *in vitro* from lymph node CD4^{+} T cells of naive PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. PDE activity analyses revealed that both cells express similar high levels of PDE4B (see this article's Fig E8 in the Online Repository at www.jacionline.org). Thus, the differences in response observed *in vivo* and *in vitro* cannot be attributed to restricted expression of PDE4B to T_H2 cells.

DISCUSSION

The current studies demonstrate that PDE4B is essential for development of AHR and induction of normal T_H2-cell functions in allergen-sensitized mice. Unlike PDE4A or PDE4D, ablation of PDE4B protects mice from developing AHR and leukocyte infiltration to the lungs. Thus, inhibition of a single PDE4 isotype is sufficient to ameliorate the airway inflammation and hyperreactivity associated with allergic asthma.

It is accepted that the T_H2 cytokines IL-4, IL-5, and IL-13 are the principal mediators in induction of airway inflammation and AHR. Using gene-targeted mice and experimental models of asthma, AHR can be dissociated from airway inflammation, [23,42,43] because the presence of pulmonary leukocyte infiltration and IgE production is not sufficient for AHR development. This is true also for the PDE4 knockout mice. In PDE4D$^{-/-}$ mice, allergen sensitization induces exaggerated pulmonary eosinophilia and serum IgE but does not provoke AHR (see this article's Table E2 in the Online Repository at www.jacionline.org). In contrast, together with protection from AHR, allergen-sensitized PDE4B$^{-/-}$ mice have reduced eosinophil infiltration and T_H2 cytokine accumulation in the airways. *Ex vivo* tracheal ring contractility shows that the absence of AHR in PDE4D$^{-/-}$ mice is a result of a disruption of the muscarinic-mediated bron-choconstriction. [25] Conversely, PDE4A$^{-/-}$ and PDE4B$^{-/-}$ tracheas respond to muscarinic cholinergic stimulation normally,[25] indicating an intact airway smooth muscle function. Thus, the mechanisms by which PDE4D and PDE4B contribute to the development of AHR are clearly distinct.

IL-5 is known to be the primary signal for eosinophil activation, recruitment, growth, differentiation, and survival in T_H2-driven inflammation. A significant decrease in IL-5 accumulation in PDE4B$^{-/-}$ airways was indeed accompanied by a decrease in eosinophil infiltration, particularly after reduced exposure to antigen. The reduction of eosinophilia by

PDE4B ablation is not as profound as that seen with PDE4 inhibitors.[37–40] This is not a result of concomitant suppression of PDE4A and PDE4D isozymes by the PDE4 inhibitor, because both PDE4A$^{-/-}$ and PDE4D$^{-/-}$ mice develop normal airway inflammation after OVA sensitization. Disruption of antigen-induced IL-5 production,[44] inhibition of chemoattractant-mediated and cytokine-mediated adhesion,[45] and suppression of eosinophil chemotaxis[46] may be the basis of the PDE4B phenotype. Although we have confirmed most of the phenotypes associated with PDE4B ablation on a C57Bl/6 background, it cannot be excluded that some of the effects may not be present in all mouse strains.

IL-13 is a critical mediator for the development of AHR and mucus generation. Numerous studies using IL-13–deficient or transgenic mice, as well as administration of recombinant IL-13 and IL-13 neutralization, demonstrated an essential role of IL-13 in the induction of AHR.[5,6,43] Moreover, mice deficient in IL-13 do not develop AHR despite the presence of high levels of IL-4 and IL-5 and significant airway inflammation, further indicating that IL-13 is indispensable.[43] In view of these findings and given our observations that mice deficient in PDE4B display a significant decrease in IL-13 levels both in the BALF and in the cultured lymph node cells, we propose that the loss of AHR in the PDE4B$^{-/-}$ mice is in part a result of disrupted IL-13 production. The decreased IL-13 did not affect mucus generation, whereas an effect has been reported in the IL-13$^{-/-}$ mice. This discordant effect may result from the fact that a complete absence of IL-13 is necessary to block goblet cell hyperplasia and mucus production.

IL-4 is known to promote isotype switching of B cells to IgE production.[47] Mice deficient in IL-4 fail to produce measurable levels of allergen-specific IgE.[48] Conversely, a report has indicated that cAMP-elevating agents, such as cAMP analogs, PDE inhibitors, β_2-agonists, and E-series prostaglandins, augment IgE production,[49] which is consistent with our results that ablation of PDE4B, and therefore an environment of increased cAMP, results in IgE production in spite of low IL-4 in these mice. Moreover, it has been shown that potentiation of IL-4– induced IgE production by cAMP occurs only when B cells were stimulated at a suboptimal concentration of IL-4.[50] This provides a further explanation for the normal immunoglobulin isotype switch observed in PDE4B$^{-/-}$ mice.

The characterization of PDE4B null mice demonstrates that inflammatory responses are disrupted at the levels of both innate and acquired immunity. PDE4B is involved in TLR signaling, and its expression is induced on stimulation of cells with LPS.[28,29] Ablation of PDE4B produces a decrease in LPS-induced TNF-α production in monocytes and macrophages *in vitro*. From these data and the *in vivo* observation that TNF-α is decreased in BALF after OVA sensitization, we surmise that inflammatory responses in the PDE4B$^{-/-}$ mice develop in an environment of decreased TNF- α levels. This defectmay have an impact on the recruitment of inflammatory cells to the lung, on activation of dendritic cells, and on chemokine production by the airways. *In vitro* and *in vivo* data demonstrate that T-cell responses, such as T-cell replication and T_H2 cytokine production, are compromised in the PDE4B$^{-/-}$ mice. In view of these multiple defects and the findings that removal of PDE4A or PDE4D does not disrupt inflammatory responses, we propose that PDE inhibitors with PDE4B selectivity should improve the therapeutic window of nonselective PDE4 inhibitors. Given the reports that themost significant gastrointestinal side effects of nonselective PDE4 inhibitors have been associated with PDE4D,[18,24] it is hypothesized that compounds with PDE4B selectivity may be better tolerated, as already suggested by the first PDE4B-selective inhibitors synthesized.[18]

In conclusion, these data demonstrate that PDE4B plays a specific, nonredundant function in inflammatory cells involved in T_H2-cell responses and AHR development on allergen sensitization. A defect in inflammatory cells, mainly T_H2 cells, is most likely the cause of

absence of AHR in PDE4B null mice. Additional studies are necessary to elucidate the exact contribution of dendritic cells to the phenotype and the role of PDE4B in the signaling pathways involved in T_H2-cell differentiation and effector actions. Understanding function of PDE4B and the underlying mechanisms will provide a rational basis for the development of PDE4B-selective inhibitors for the treatment of asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AHR	Airway hyperresponsiveness
alum	Aluminum potassium sulfate
APC	Antigen-presenting cell
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
cAMP	Cyclic AMP
C_{dyn}	Dynamic compliance
FITC	Fluorescein isothiocyanate
OVA	Ovalbumin
PAS	Periodic acid-Schiff
PDE	Cyclic nucleotide phosphodiesterase
PDE4	Type 4 cyclic AMP–specific phosphodiesterase
R_L	Lung resistance

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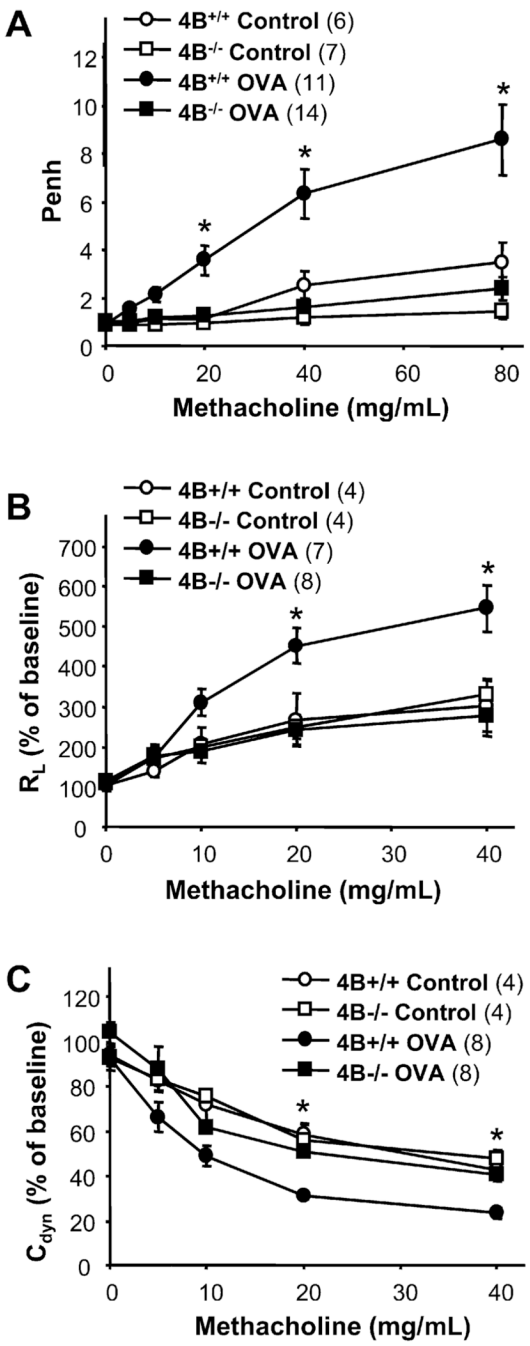

FIG 1.

AHR in OVA-sensitized PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. C57BL6/129Ola PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice were immunized and challenged with OVA (50 μg) in alum intraperitoneally on days 0 and 14, and intranasally (50 μg OVA in 50 μL saline) on days 14, 25, 26, and 27. Control mice received alum intraperitoneally and normal saline intranasally. On day 28, AHR in response to increasing concentrations of methacholine was measured either from conscious mice using a whole-body plethysmograph and expressed as enhanced pause (*Penh;* **A**) or from anesthetized and c-annulated mice **(B and C)**. In the latter case, R_L and C_{dyn} are reported. Data are the means ± SEMs with the number of mice

in each group in parentheses. *$P < .005$ (compared with the OVA-sensitized wild-type littermates).

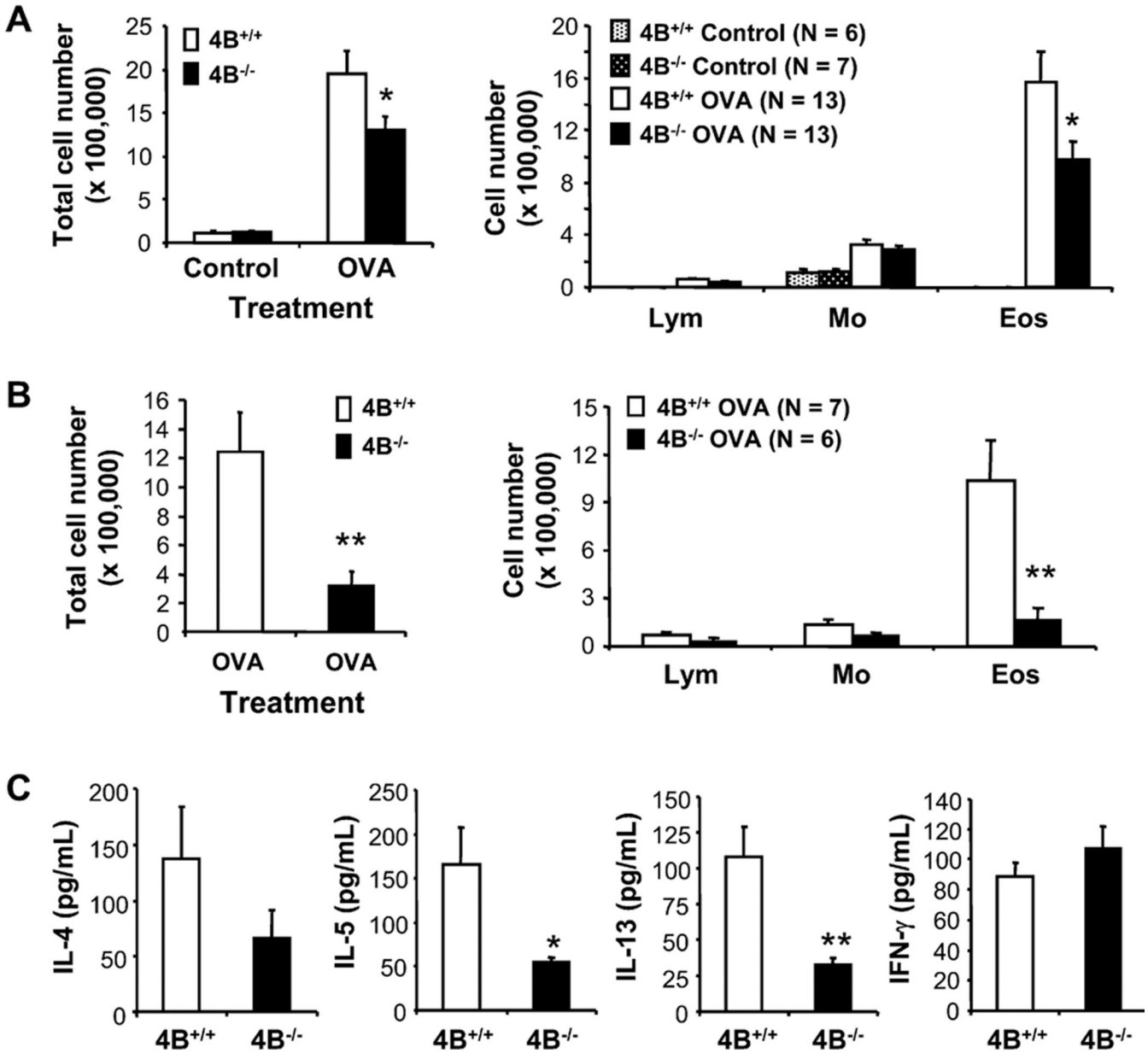

FIG 2.

Airway inflammation in OVA-sensitized PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. Mice were sensitized as described in Fig 2 **(A)** or by reducing the intranasal challenge to 3 (on days 14, 25, and 26; **B**). After sensitization, the mice were anesthetized, and BAL was performed. Total and differential cell counts in the BALF were determined as detailed in the Methods. Data are the means ± SEMs (n = 13–19 mice/genotype in *A*, and N = 6–7 mice/genotype in B). **P* < .05; ***P* < .01 (compared with OVA-sensitized PDE4B$^{+/+}$ mice). **C,** Levels of T_H2 cytokines (IL-4, IL-5, and IL-13) and IFN-γ in the BALF supernatants were determined by ELISA. Data are the means ± SEMs (5–6 mice/genotype) **P* < .05; ***P* < .005. *C*, Control; *Eos*, eosinophils; *Lym*, lymphocytes; *Mo*, monocytes and macrophages.

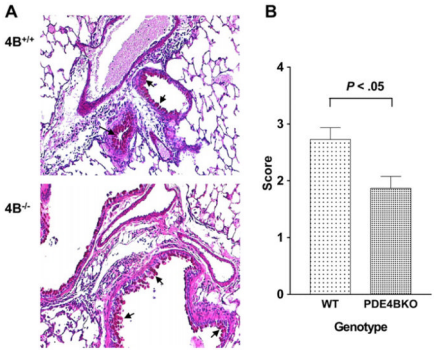

FIG 3.

Inflammatory cell infiltration and PAS-stained mucus-producing cells in the lungs of PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. Mice were immunized and challenged with OVA as described in Fig 2. One day after the last OVA challenge, lungs were processed for staining with PAS. *Arrows* point to goblet cells *(magenta)* in the airway epithelium **(A)**. The quantitation of the degree of inflammation is reported in **B** ($P < .05$; N = 6–7).

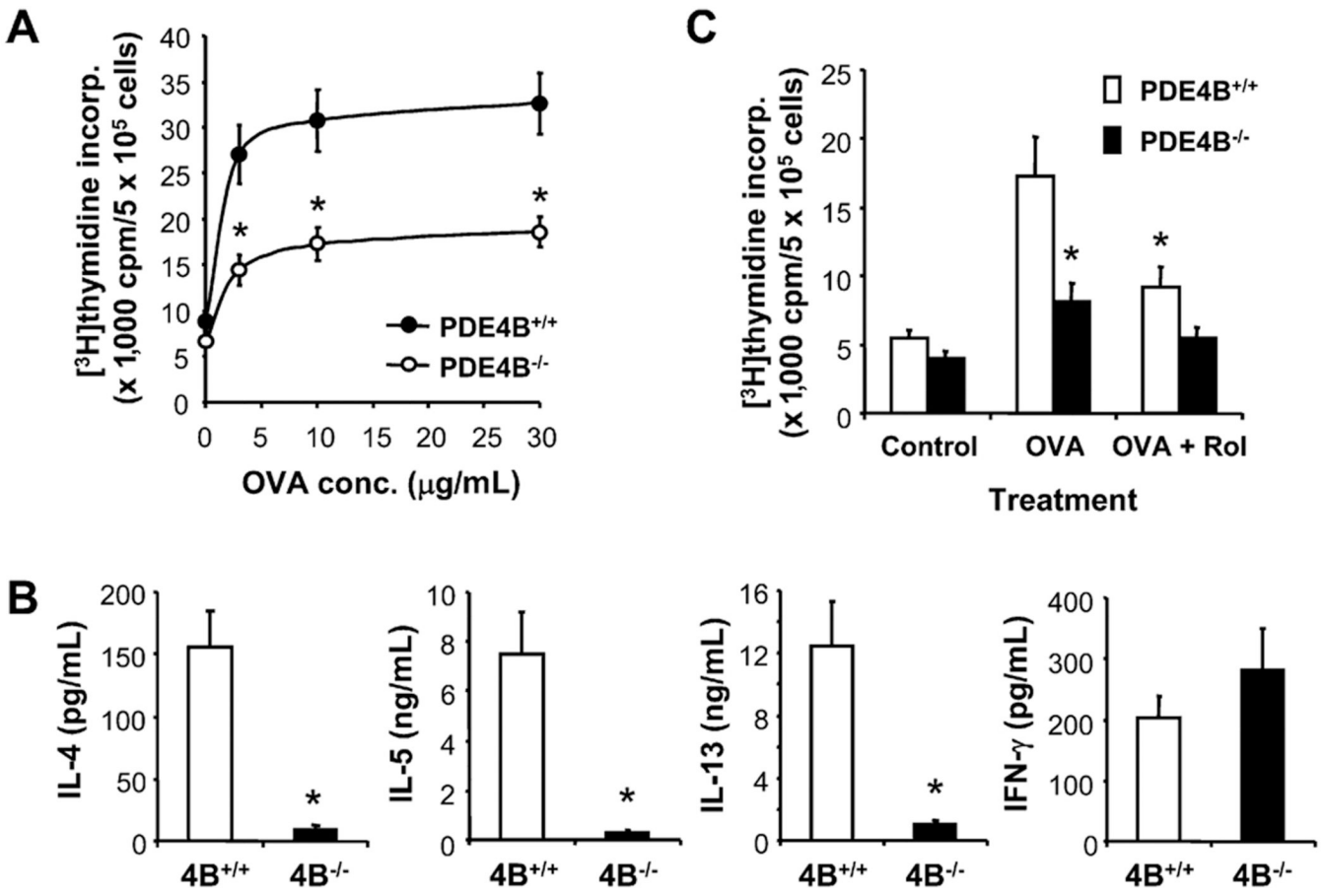

FIG 4.

T_H2-cell responses in OVA-sensitized PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. Mice were sensitized as described in Fig 2. Single-cell suspensions of the bronchial lymph nodes were prepared and cultured in the presence of increasing OVA concentrations **(A)** or in the presence of 30 μg/mL OVA alone, a combination of OVA and 10 μmol/L rolipram, or vehicle (control; **C**). Proliferation of OVA-specific cells was assessed by incorporation of [^{3}H]thymidine over the last 16 hours of the 3-day culture. Data are the means ± SEMs (n = 12 mice/genotype in A and 4–7 mice/genotype in C). *P < .05 (compared with the wild-type cells treated with OVA alone). **B,** Cytokine levels in the culture medium collected after 4 days of incubation in the presence of 30 μg/mL OVA were determined by ELISA. Data are the means ± SEMs (n = 7–13 mice/genotype). *P < .01. *Conc.*, Concentration; *Rol*, rolipram.

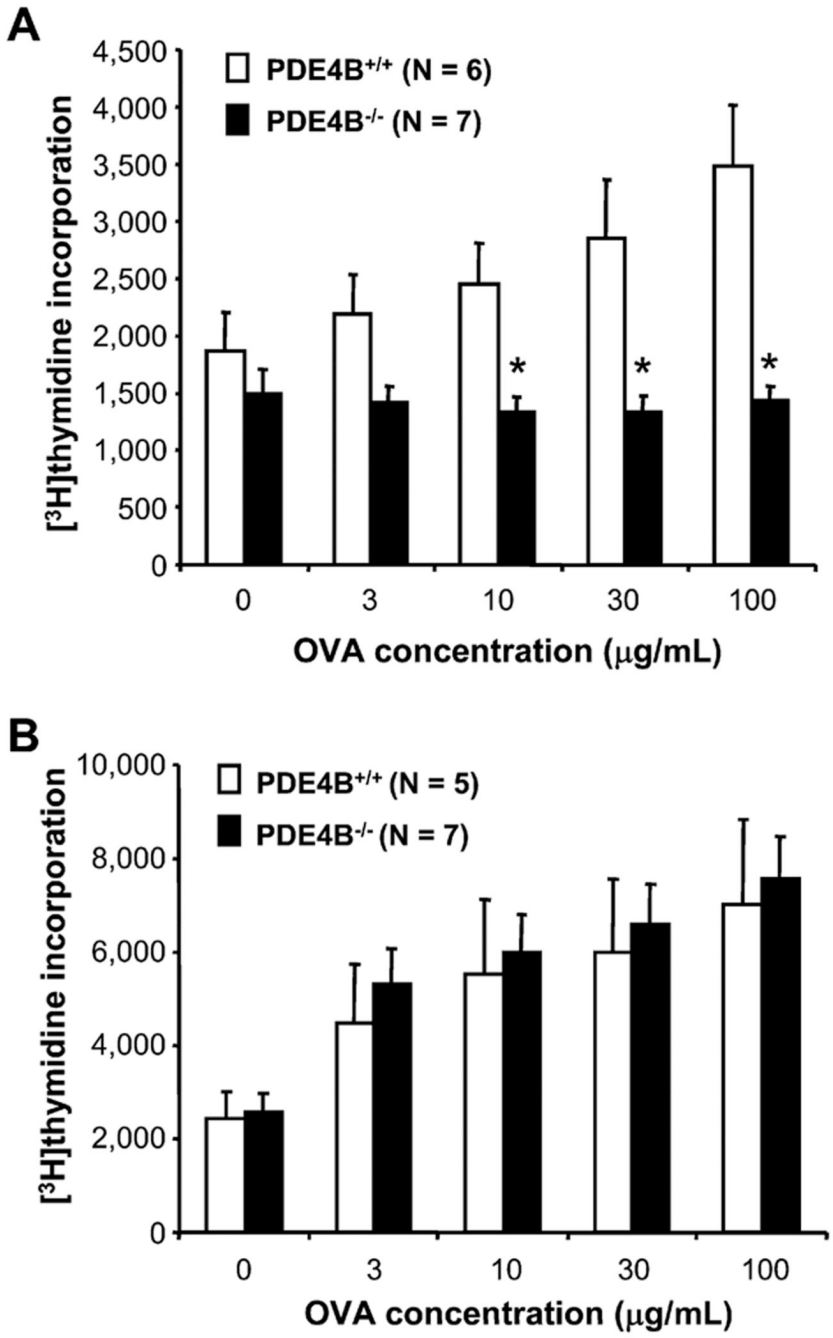

FIG 5.

Proliferation of splenocytes and popliteal lymph node cells from OVA-primed PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice. **A,** Mice were given 1 dose of OVA (50 μg) in alum intraperitoneally. Nine days later, the spleen was excised, and cells were cultured in the presence of increasing OVA concentrations. **B**, Mice were given OVA conjugated with incomplete Freund adjuvant by injection into hind footpads intradermally (75 μg OVA each footpad). Nine days later, popliteal lymph nodes were excised, and cells were cultured in the presence increasing OVA. Proliferation of OVA-specific cells was assessed by incorporation of [^{3}H]thymidine over the last 16 hours of 3-day culture. Data are the means ± SEMs. *P < .05 (compared with the corresponding wild-type cells).

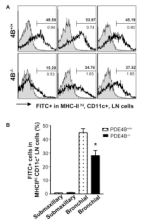

FIG 6.

Dendritic cell migration from the lung to draining lymph nodes (*LNs*). Naive PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice were administered FITC-OVA. After 24 hours, the bronchial and submaxillary lymph nodes were excised and a single-cell suspension prepared. Cells were stained with phycoerythrin-conjugated antimouse MHC II and APC-conjugated antimouse CD11c mAbs followed by fluorescence-activated cell sorting analysis. Representative histograms from 3 PDE4B$^{+/+}$ and PDE4B$^{-/-}$ mice are shown in **A**. The percentage of FITC^{+} cells in a population of MHC-IIh^{i}, CD11c^{+} cells is summarized in **B**. Cells from submaxillary LNs were used as a negative control. **P* < .05 compared with wild-type mice (N = 8 mice/group).