Title

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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(5)

ISSN

0027-8424

Authors

Lee, Jihyung
Kim, Tae Hoon
Murray, Fiona
et al.

Publication Date

2015-02-03

DOI

10.1073/pnas.1417972112

Peer reviewed

Cyclic AMP concentrations in dendritic cells induce and regulate Th2 immunity and allergic asthma

Jihyung Lee[a,1], Tae Hoon Kim[a,b,1], Fiona Murray[a,c], Xiangli Li[a], Sara S. Choi[a], David H. Broide[a], Maripat Corr[a], Jongdae Lee[a], Nicholas J. G. Webster[a,d], Paul A. Insel[a,e], and Eyal Raz[a,2]

Departments of [a]Medicine and [e]Pharmacology, University of California, San Diego, La Jolla, CA 92093; [b]Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Korea University, Seoul 136-705, Republic of Korea; [c]School of Medical Science, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland; and [d]Medical Research Service, Veteran Affairs San Diego Healthcare System, San Diego, CA 92161

Edited* by Dennis A. Carson, University of California, San Diego, La Jolla, CA, and approved January 1, 2015 (received for review September 17, 2014)

The inductive role of dendritic cells (DC) in Th2 differentiation has not been fully defined. We addressed this gap in knowledge by focusing on signaling events mediated by the heterotrimeric GTP binding proteins Gαs, and Gαi, which respectively stimulate and inhibit the activation of adenylyl cyclases and the synthesis of cAMP. We show here that deletion of *Gnas*, the gene that encodes Gαs in mouse CD11c$^+$ cells (*Gnas*$^{\Delta CD11c}$ mice), and the accompanying decrease in cAMP provoke Th2 polarization and yields a prominent allergic phenotype, whereas increases in cAMP inhibit these responses. The effects of cAMP on DC can be demonstrated in vitro and in vivo and are mediated via PKA. Certain gene products made by *Gnas*$^{\Delta CD11c}$ DC affect the Th2 bias. These findings imply that G protein-coupled receptors, the physiological regulators of Gαs and Gαi activation and cAMP formation, act via PKA to regulate Th bias in DC and in turn, Th2-mediated immunopathologies.

cAMP | dendritic cells | Th2 | PKA | asthma

The induction of Th cell response requires antigen-presenting cells (APC), especially dendritic cells (DC), but the mechanisms for this induction have not been fully elucidated (1, 2). Because DC do not produce IL-4, which is mandatory for GATA3 induction and Th2 cell differentiation (3, 4), other cell types may be involved in Th2 responses (1, 5, 6), including basophils (7), epithelial cells (8), and innate lymphoid cells (ILC), especially ILC group 2 (ILC2) (9). ILCs are a multifunctional group of cells that are able to secrete immunoregulatory cytokines that affect immune responses. They reside at mucosal surfaces, where they are exposed to microbial or environmental stimulation (10). Such cells can secrete IL-4 or alarmins, including IL-25, IL-33, and TSLP (epithelial cells), which support Th2 differentiation (11). Other DC-derived inducers of Th2-differentiation are the Notch ligand Jagged 1 (12) and the interaction between OX40 and OX40L (13). However, little is known regarding the signaling networks that provoke DC to induce Th2 immunity.

Pharmacological inhibition of cyclic nucleotide phosphodiesterase 4 (PDE4), which is highly expressed in DC, can produce improvement in animal models of inflammation and autoimmunity and can suppress human Th1-polarizing capacity by increasing cAMP concentrations (14, 15). Based on these findings and our previous work that identified a role for cAMP in DC in Th17 induction (16), we hypothesized that cAMP regulates DC and affects Th differentiation bias. To test this hypothesis, we studied the regulation of DC activity by heterotrimeric (αβγ) GTP binding proteins that regulate cAMP synthesis through their modulation of the activity of adenylyl cyclases (ACs): Gαs, which stimulates AC activity, and Gαi, which inhibits membrane AC activity.

In the current studies, we engineered mice that have a CD11c-specific deletion of *Gnas* (CD11c-Cre *Gnas*$^{fl/fl}$, i.e., *Gnas*$^{\Delta CD11c}$), the gene that encodes Gαs (17). We found that cAMP accumulation is much less in response to Gαs activation of CD11c$^+$ cells from these mice than from littermate (fl/fl) controls. Unexpectedly, the *Gnas*$^{\Delta CD11c}$ mice display a striking phenotype: They develop spontaneous Th2 immunity even though these mice have a C57BL/6 genetic background, which is biased to Th1 immunity (18). Consequently, these mice develop later airway inflammation that is consistent with allergic asthma (19). Bone marrow (BM)-derived DC (BMDC) from the *Gnas*$^{\Delta CD11c}$ mice display a pro-Th2 phenotype (i.e., induce a Th2 response when cocultured with CD4 T cells), which can be prevented by treatment with a cAMP analog. Additional studies show that the cAMP effector protein kinase A (PKA) and molecules whose expression is regulated by cAMP play a key role in the induction of the Th2-biased phenotype of DC. The current results thus identify a previously unappreciated role for Gαs-regulated cAMP synthesis and cAMP/PKA in DC in determining Th subset differentiation and resultant responses.

Results

Generation of CD11c-Cre *Gnas*$^{fl/fl}$ (*Gnas*$^{\Delta CD11c}$) Mice. GPCR-mediated increase in intracellular cAMP requires the activation of AC by Gαs (20). To obtain mice with DC deficient in this response, we used the Cre-loxP system to generate mice (C57BL/6 background) with a targeted deletion of *Gnas* in CD11c$^+$ cells, as we described for CD4$^+$ T cells (21). Splenic CD11c$^+$/CD11b$^-$ cells from *Gnas*$^{\Delta CD11c}$ mice express low levels of *Gnas* mRNA and accumulate much less cAMP than do splenic CD11b$^+$/CD11c$^-$ cells (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1 *A* and *B*). *Gnas*$^{\Delta CD11c}$ mice develop normally and have a similar percentage of CD11c$^+$, CD4$^+$, effector memory (CD44^{high}CD62L^{low}), naïve (CD44^{low}CD62L^{high}) CD4$^+$ and CD8$^+$ T cells, as do

Significance

Allergic asthma is characterized by Th2 type inflammation, leading to airway hyperresponsiveness and remodeling. However, the mechanisms by which DC promote Th2 differentiation remain unclear. Herein we demonstrate that low cAMP levels in DC induce Th2-biased responses in vitro and in vivo. Furthermore, mice with conditional deletion of *Gnas* in DC (*Gnas*$^{\Delta CD11c}$ mice) develop spontaneous bronchial asthma that shares multiple similarities with human asthma. In contrast, increasing cAMP levels inhibit these responses. Thus, regulators of cAMP levels in DC such as G-protein-coupled receptors are non-pattern recognition receptors that play a significant role in CD4 T cell differentiation.

Author contributions: Jihyung Lee, T.H.K., P.A.I., and E.R. designed research; Jihyung Lee, T.H.K., F.M., X.L., S.S.C., and Jongdae Lee performed research; F.M., D.H.B., M.C., N.J.G.W., and P.A.I. contributed new reagents/analytic tools; Jihyung Lee, T.H.K., F.M., X.L., D.H.B., M.C., Jongdae Lee, N.J.G.W., P.A.I., and E.R. analyzed data; and Jihyung Lee, T.H.K., and E.R. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]Jihyung Lee and T.H.K. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: eraz@ucsd.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1417972112/-/DCSupplemental.

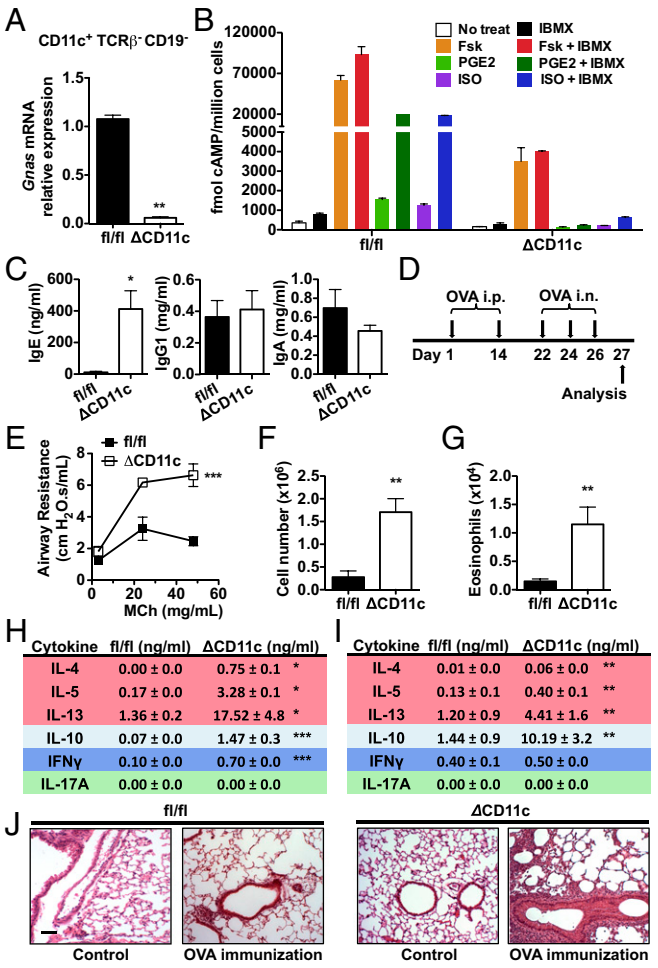

Fig. 1. *Gnas*$^{\Delta CD11c}$ mice are atopic and are predisposed toward Th2 immunity. (*A*) CD11c-specific deletion of *Gnas* was confirmed by qPCR. Total mRNA was prepared from FACS-sorted splenic cells (CD11c$^+$ TCRβ$^-$ CD19$^-$). (*B*) cAMP level was determined by RIA in CD11c$^+$ cells treated with vehicle, forskolin (Fsk, 10 μM), isoproterenol (Iso, 10 μM), or prostaglandin E_2 (PGE_2, 1 μM) in the presence of the PDE inhibitor isobutylmethylxanthine (IBMX, 200 μM). $n = 3$ per group, representative of three independent experiments. (*C*) Serum IgE, IgG1, and IgA levels in the 2-mo-old fl/fl and *Gnas*$^{\Delta CD11c}$ mice (ELISA). IgG2a levels were below the detection level. (*D*) OVA immunization protocol and challenge. (*E*) Mean values ± SEM of airway resistance for fl/fl and *Gnas*$^{\Delta CD11c}$ mice after intranasal (i.n.) OVA instillation and methacholine (MCh) challenge. (*F* and *G*) Total cell (*F*) and eosinophil (*G*) counts in bronchoalveolar lavage (BAL) fluid. Cytokine response of CD4$^+$ T cells from the bronchial lymph nodes (*H*) and spleen (*I*). (*J*) H&E staining of the lung. (Magnification: 100×; scale bar: 100 μm.) Data are mean ± SEM of three independent assays in each group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

littermate (fl/fl) control mice (*SI Appendix*, Fig. S1*C*). Thus, the loss of *Gnas* does not alter the number of peripheral DC or T cells.

***Gnas*$^{\Delta CD11c}$ Mice Are Atopic and Develop Spontaneous Th2-Mediated Inflammation.** Two-month-old *Gnas*$^{\Delta CD11c}$ mice have increased serum IgE levels but a similar cytokine profile of CD4$^+$ T cells and lung histology compared with cohoused littermate fl/fl mice (Fig. 1*C* and *SI Appendix*, Fig. S1 *D* and *E*). We reasoned that if *Gnas*$^{\Delta CD11c}$ mice were immunized with a conventional antigen without an adjuvant and challenged (22), they would develop Th2-mediated lung inflammation. Indeed, ovalbumin (OVA) immunization (Fig. 1*D*) provoked strong airway hyperreactivity (AHR), and increased the number of eosinophils in the bronchoalveolar lavage (BAL) fluid, Th2 cytokine response and airway inflammation in the *Gnas*$^{\Delta CD11c}$, but not littermate fl/fl, mice (Fig. 1 *E*–*J*). Moreover, 5- to 6-mo-old *Gnas*$^{\Delta CD11c}$ (but not fl/fl) mice develop "spontaneous" Th2 response, i.e., without immunization (Fig. 2*A*), and display features of severe Th2-mediated lung inflammation (i.e., similar to those developed in experimental allergic asthma by OVA/alum immunization and OVA challenge), including AHR (Fig. 2*B*), increased number of eosinophils in the BAL fluid (Fig. 2*C*), increased serum IgE, IgG1 levels (Fig. 2*D*), along with airway inflammation and remodeling (Fig. 2*E*). However, 6-mo-old *Rag1*$^{-/-}$ or *Rag1*/*Gnas*$^{\Delta CD11c}$ double KO (DKO) mice did not demonstrate airway inflammation (Fig. 2*F*). Despite their higher IgE serum levels, *Gnas*$^{\Delta CD11c}$ mice housed in the barrier room (where air is filtered and food is sterilized) did not develop Th2 bias or histologic lung abnormalities at 5–6 mo of age (*SI Appendix*, Fig. S2). Interestingly and in contrast to the pathology observed in the airways, we did not identify any overt G-I immunopathology. Collectively, these data indicate that the *Gnas*$^{\Delta CD11c}$ mice are atopic; they are poised to mount "spontaneous" Th2 bias

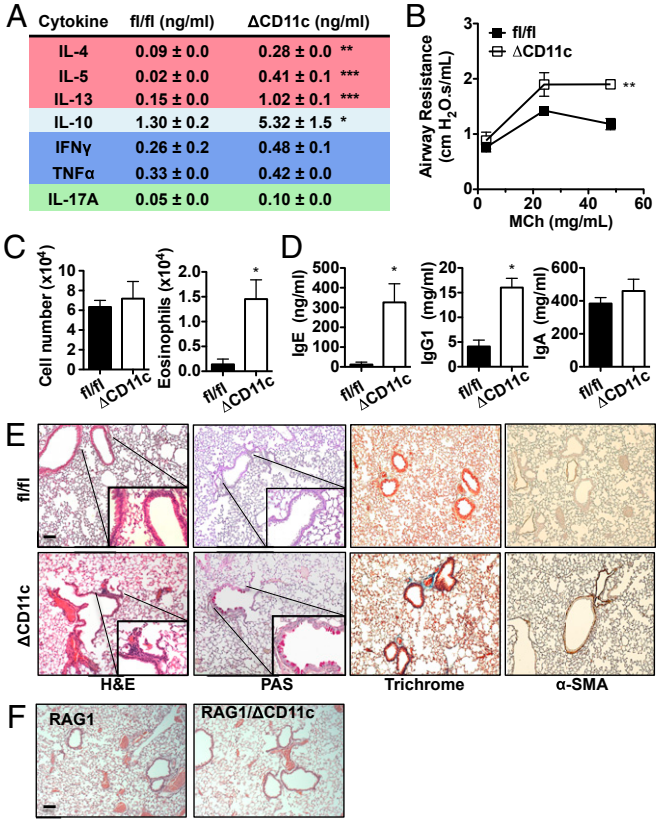

Fig. 2. Spontaneous Th2 responses in 6-mo-old *Gnas*$^{\Delta CD11c}$ mice. (*A*) Cytokine profile of anti-CD3/28 Ab-stimulated CD4$^+$ T cells (spleen) from 6-mo-old fl/fl and *Gnas*$^{\Delta CD11c}$ mice (ELISA). (*B*) Mean values ± SEM of airway resistance after MCh challenge. (*C*) Total cell and eosinophil counts in the BAL fluid. (*D*) Serum IgE, IgG1, and IgA levels (ELISA). (*E*) Histologic lung tissue analysis: H&E, PAS (red–purple), Trichrome (blue) and anti-SMA (brown) in the lung tissues. (Magnification: 100×; scale bar: 100 μm.) (*F*) Histological analysis of lung tissue in 6-mo-old *Rag1*$^{-/-}$ or *Rag1*/*Gnas*$^{\Delta CD11c}$ double KO (DKO) mice does not show airway inflammation. No difference between the two groups was identified. H&E staining are shown. (Magnification: 100×; scale bar: 100 μm.) Data are mean ± SEM, $n = 4$–6 in mice per experiment in each group and are representative of the results obtained in two different experiments with similar results; $^*P < 0.05$, $^{**}P < 0.01$.

responses and airway inflammation most likely in response to environmental stimuli.

BMDC and Lung DC from *Gnas*$^{\Delta CD11c}$ Mice Induce a Th2 Differentiation. We used in vitro bone marrow-derived dendritic cells (BMDC) and naïve splenic OT2 CD4$^+$ T cells to characterize the intrinsic role of BMDC from *Gnas*$^{\Delta CD11c}$ mice in Th2 bias. We first cultured BM cells with GM-CSF and found similar % of CD11c$^+$, B220$^-$ and CD11b$^+$ cells derived from *Gnas*$^{\Delta CD11c}$ and fl/fl mice (*SI Appendix*, Fig. S3*A*). We then isolated CD11c$^+$/Flt3$^+$ double-positive cells by FACS (*SI Appendix*, Fig. S4*A*) (23, 24) and cocultured these cells with FACS-sorted naïve OT2 splenic CD4$^+$ T cells for 3 d. BMDC derived from *Gnas*$^{\Delta CD11c}$ mice (but not from fl/fl controls) induced high (seven- to ninefold increased) levels of IL-4 in the cocultured OT2 CD4$^+$ T cells (Fig. 3 *A* and *B*).

IL-4 eGFP reporter/OT2 (4Get/OT2) CD4$^+$ T cells cocultured with *Gnas*$^{\Delta CD11c}$ BMDC had a 17.9-fold increase in eGFP expression (Fig. 3*C*). Cocultured OT2 CD4$^+$ T cells displayed very high expression levels of Th2 cell marker T1/ST2 (25) (45-fold increase, Fig. 3*D*). Analysis of the Th lineage commitment factors of these cocultured OT2 CD4$^+$ T cells revealed higher GATA3 levels (2.6-fold increase, Fig. 3*E*), indicating that BMDC from *Gnas*$^{\Delta CD11c}$ mice have a pro-Th2 phenotype, i.e., they induce Th2 differentiation. However, these BMDC did not display altered expression of Th2-promoting cognate molecules, such as Jagged 1 (12), OX40L (13) or FcεR1 (26) (Fig. 3*F*). CD11c$^+$ single-positive cells from *Gnas*$^{\Delta CD11c}$ mice provoked a similar response (*SI Appendix*, Fig. S4 *A*-*C*). Because GM-CSF-derived BMDC enhance development of inflammatory DC (27), we also stimulated BM cultures with Flt3 ligand, which promotes development of both plasmacytoid and conventional DC (23). Incubation with Flt3 ligand promoted an increase in CD11c$^+$ expression in BM cultures similar to that produced by GM-CSF (*SI Appendix*, Fig. S3*B*). Flt3-derived CD11c$^+$ BM cells from *Gnas*$^{\Delta CD11c}$ (but not fl/fl) mice provoked a Th2 bias in the CD4$^+$ T-cell differentiation assay (*SI Appendix*, Fig. S4*D*). CD11c$^+$/Flt3$^+$ BM cells are a small fraction of the CD11c$^+$ BM cells (*SI Appendix*, Fig. S4*A*), and because double-positive and the single-positive BM cells displayed a similar pro-Th2 phenotype, we undertook further in vitro analyses using CD11c$^+$ BM cells (i.e., single-positive BMDC).

We also analyzed the function of FACS-isolated lung DC subsets on the OT2 Th2 cell differentiation. Resident lung DC from *Gnas*$^{\Delta CD11c}$ mice (Fig. 3*G*), but not alveolar macrophages (Fig. 3*H*), induced a Th2 bias ex vivo. Another lung DC subset, lung inflammatory DC, isolated from OVA/alum-immunized and OVA-challenged (22), *Gnas*$^{\Delta CD11c}$ mice also displayed an increase in their pro-Th2 phenotype (Fig. 3*I*). Collectively, these data indicate that interaction of CD11c$^+$ DC and CD4$^+$ T cells is sufficient to provoke Th2 differentiation in this coculture system in vitro and ex vivo.

To assess Th2 differentiation in vivo, we transferred naïve 4Get CD4$^+$ T cells (28, 29) into *Rag1*$^{-/-}$ or *Rag1*/*Gnas*$^{\Delta CD11c}$ DKO mice and 3 wk later analyzed eGFP fluorescence in splenic T cells. We found that >20% of the CD4$^+$ T cells isolated from the DKO mice were eGFP-positive compared with those from the *Rag1*$^{-/-}$ mice (Fig. 3*J*). Together with the findings from the ex vivo studies, these results show the crucial role of CD11c$^+$ DC-derived from *Gnas*$^{\Delta CD11c}$ mice in the induction of Th2 bias.

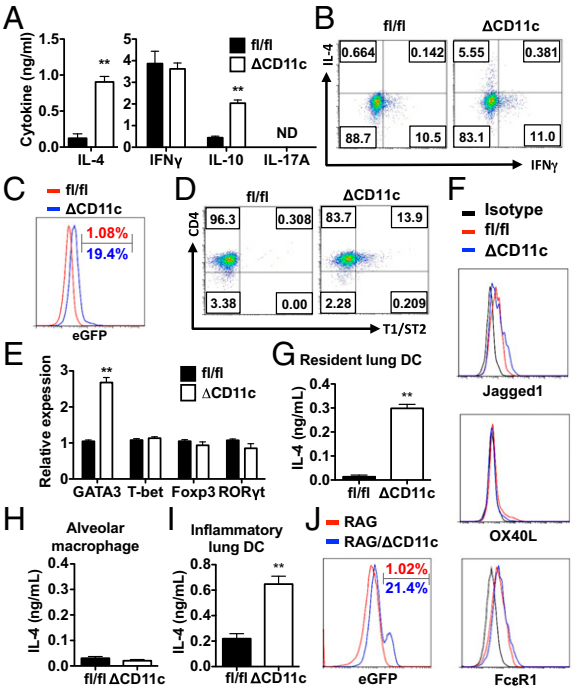

Fig. 3. Bone marrow and lung DC from *Gnas*$^{\Delta CD11c}$ mice induce a Th2 bias. (*A* and *B*) Cytokine levels (ELISA; *A*) and intracellular cytokine staining (*B*) in the isolated OT2 CD4$^+$ T cells cocultured with BMDC from fl/fl and *Gnas*$^{\Delta CD11c}$ mice. (*C*) Naïve 4Get/OT2 CD4$^+$ T cells were cocultured with BMDC from fl/fl or *Gnas*$^{\Delta CD11c}$ mice for 3 d and the eGFP fluorescence intensity was analyzed after PMA/ionomycin stimulation (FACS). (*D*) FACS staining of CD4 and T1/ST2 markers in isolated OT2 CD4$^+$ T cells cocultured with BMDC from fl/fl and *Gnas*$^{\Delta CD11c}$ mice. (*E*) qPCR analysis of lineage commitment factors in the isolated OT2 CD4$^+$ T cells cocultured with BMDC from fl/fl and *Gnas*$^{\Delta CD11c}$ mice. (*F*) Expression levels of Th2-driving molecules in BMDC from fl/fl and *Gnas*$^{\Delta CD11c}$ mice (FACS). (*G* and *H*) IL-4 levels of anti-CD3/28 Ab-stimulated OT2 CD4$^+$ T cells cocultured with resident lung DC (CD45$^+$, CD11c$^+$, Siglec F^{low}, AF^{low}; *G*) or alveolar macrophages (CD45$^+$, CD11c$^+$, Siglec F^{hi}, AF^{hi}; *H*) pooled from four mice and inflammatory lung DC (CD45$^+$, CD11c$^+$, CD11b$^+$, Sirp1α$^+$; *I*) from fl/fl and *Gnas*$^{\Delta CD11c}$ individual mice. (*J*) Naïve 4Get CD4$^+$ T cells (2×10^6 cells per mouse) were i.v. transferred into RAG KO (red) or RAG/*Gnas*$^{\Delta CD11c}$ DKO (blue) mice. The eGFP fluorescence intensity of the splenic TCRβ$^+$ cells was recorded (using FACS). Data are mean ± SEM, $n = 3$–4 mice in each group and are representative of results obtained in three independent experiments. $^{**}P < 0.01$.

PKA and Gαi Signaling Regulate the Induction of the Pro-Th2 Phenotype of BMDC. The results shown thus far provide evidence for the pro-Th2 phenotype of BMDC and lung DC from *Gnas*$^{\Delta CD11c}$. To explore the possible inhibitory role of cAMP on Th2-mediated immunity, we incubated BMDC with the cell-permeable cAMP analog, 8-CPT-cAMP (CPT). Incubation with CPT abolished the subsequent production of IL-4 by cocultured OT2 CD4$^+$ T cells (Fig. 4*A*).

We analyzed the two main cAMP signaling effectors, PKA and Exchange protein activated by cAMP (EPAC), for their role in the pro-Th2 phenotype of BMDC of *Gnas*$^{\Delta CD11c}$ mice. Treatment with N6, a PKA-selective cAMP agonist, but not with 8ME, an EPAC agonist, abolished the pro-Th2 phenotype of *Gnas*$^{\Delta CD11c}$ BMDC (Fig. 4*B*). Moreover, treatment of wild-type (WT)-BMDC with a PKA inhibitor (H-89), but not an EPAC inhibitor (CE3F4), promoted their pro-Th2 phenotype (Fig. 4*C*). These data implicate cAMP-PKA signaling in the inhibition of induction of the pro-Th2 phenotype of DC.

The deletion of *Gnas* in CD11c$^+$ cells alters the balance between Gαs and Gαi in terms of cAMP synthesis and action in favor of Gαi signaling. To assess the role of Gαi, we incubated WT-BMDC with the Gαi activator mastoparan, a peptide toxin from wasp venom (30). Incubation with mastoparan 7 (MP7) (31) induced a pro-Th2 phenotype in WT-BMDC (Fig. 4*D*). Moreover, incubation of H-89-treated or MP7-treated WT-BMDC with pertussis toxin (PTX), which blocks Gαi activation, inhibited this pro-Th2 phenotype (Fig. 4 *C* and *D*). Additionally, inhibition of Gαi signaling in *Gnas*$^{\Delta CD11c}$ BMDC with PTX

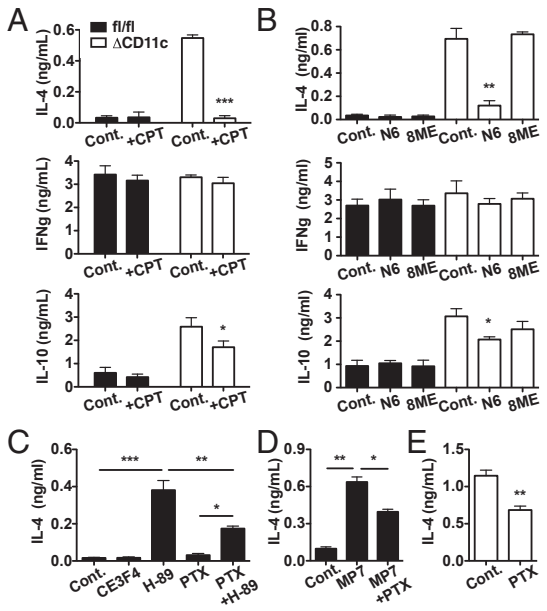

Fig. 4. Analysis of cAMP signaling and Gαs/Gαi balance involved in the pro-Th2 DC phenotype. (*A* and *B*) Cytokine levels of anti-CD3/28 Ab-stimulated OT2 CD4$^+$ T cells cocultured with BMDC from fl/fl and *Gnas*$^{\Delta CD11c}$ mice treated with CPT (*A*) or N6 (a PKA-specific cAMP analog, 50 μM) or 8ME (an EPAC-specific cAMP analog, 50 μM) (ELISA) (*B*). (*C*) WT (B6) BMDC treated with EPAC inhibitor (CE3F4, 50 μM) or PKA inhibitor (H-89, 10 μM) with or without PTX (100 ng/mL) (ELISA). (*D*) WT BMDC treated with MP7 (1 μM) with or without PTX (100 ng/mL) (ELISA). (*E*) *Gnas*$^{\Delta CD11c}$ BMDC treated with PTX (100 ng/mL). Data are mean ± SEM, $n = 3$ in each group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data shown are representative of results obtained in three independent experiments.

suppressed their pro-Th2 phenotype (Fig. 4*E*). Thus, cAMP-PKA signaling inhibits the pro-Th2 phenotype of both WT and *Gnas*$^{\Delta CD11c}$ BMDC and activation of Gαi contributes to the pro-Th2 phenotype of the latter cells. This result implies that the pro-Th2 phenotype in the *Gnas*-depleted CD11c$^+$ cells reflects an altered Gαs/Gαi balance in the activation of AC, and derives from a decrease in cAMP concentration and reduced PKA activation in DC.

Identification of Th2 Biasing Molecules in *Gnas*$^{\Delta CD11c}$ BMDC. Using DNA microarray analysis, we found 1,910 genes differentially expressed in BMDC of the *Gnas*$^{\Delta CD11c}$ mice compared with fl/fl mice (Dataset S1 and Fig. 5*A*). An enrichment analysis of the 670 genes that had >twofold difference in expression revealed that there was enrichment in immune response and cell cycle genes, suggesting that the decrease in Gαs expression and cAMP concentration may alter the proliferation of BMDC in *Gnas*$^{\Delta CD11c}$ mice (*SI Appendix*, Tables S1 and S2). Network analysis of transcription factors driving the expression profile identified cAMP response element-binding protein-1 (CREB1) as the most important transcriptional regulator (*SI Appendix*, Table S3) with 29% of the differentially regulated genes being CREB targets (*SI Appendix*, Fig. S5).

The transcriptomic analysis revealed gene products that may induce Th2 differentiation. Hematopoietic prostaglandin D synthase (Hpgds) was increased in *Gnas*$^{\Delta CD11c}$ BMDC (Dataset S1). Its product, PGD_2, binds to CRTH2 on CD4 T cells (32) and induces Th2 differentiation. We confirmed the increased transcript levels of Hpgds in *Gnas*$^{\Delta CD11c}$ BMDC by qPCR and its inhibition by CPT (i.e., increase in cellular cAMP levels) (Fig. 5*B*). Moreover, DC transfected with Hpgds siRNA (*SI Appendix*, Fig. S6*A*) inhibited expression of the pro-Th2 phenotype (Fig. 5*C*). The transcript of CCL2 (MCP-1) (Dataset S1 and Fig. 5*D*), a chemokine that activates the Gαi-linked GPCR, CCR2, is highly increased in *Gnas*$^{\Delta CD11c}$ BMDC. CCL2 can regulate Th2 commitment and IL-4 expression (33). Treatment with CPT inhibited CCL2 expression (Fig. 5*D*) and addition of a CCL2 neutralizing Ab inhibited the pro-Th2 phenotype of *Gnas*$^{\Delta CD11c}$ BMDC (Fig. 5*E*). Furthermore, CCR2 blockade of OT2 T cells, but not of BMDC, reduced IL-4 production in coculture with *Gnas*$^{\Delta CD11c}$ BMDCs; thus, CCL2 secreted from DCs also induces the pro-Th2 phenotype (Fig. 5*F*). Hgpds and CCL2 are each regulated by cAMP but their regulation is independent of one another (*SI Appendix*, Fig. S6). Taken together, these results imply that multiple cAMP-regulated genes in DC may contribute to the Th2 bias observed in *Gnas*$^{\Delta CD11c}$ mice and help determine the propensity for Th2 in WT mice.

Adoptive Transfer of BMDC from *Gnas*$^{\Delta CD11c}$ Mice Induces a Th2 Bias in WT Recipients and Increasing cAMP Concentration in Those Cells Blocks It. The cAMP agonist CPT inhibits the pro-Th2 phenotype of *Gnas*$^{\Delta CD11c}$ BMDC (Fig. 4*A*). We used adoptive transfer (34) to assess for the in vivo relevance of this finding (Fig. 6*A*). Intranasal transfer of OVA-loaded BMDC from *Gnas*$^{\Delta CD11c}$ mice induced OVA-specific IL-4 by splenic CD4$^+$ T cells (Fig. 6*B*), higher levels of IgE (Fig. 6*C*), and airway inflammation (Fig. 6*D*) in WT and *Gnas*$^{\Delta CD11c}$ recipients. However, treatment with CPT of *Gnas*$^{\Delta CD11c}$ BMDC before transfer to recipient mice inhibited

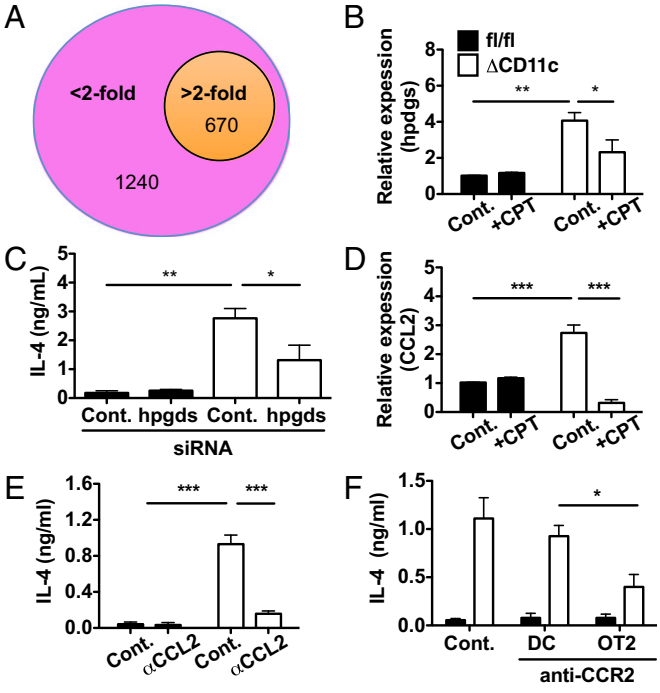

Fig. 5. Differentially expressed molecules regulate the pro-Th2 DC phenotype. (*A*) Venn diagram showing differentially expressed genes (Bonf. $\alpha < 0.05$). Pink circle contains all mouse differentially expressed genes, orange circle contains genes with >twofold change. (*B* and *D*) The mRNA levels (qPCR) of Hpgds (*B*) and CCL2 (*D*) in fl/fl and *Gnas*$^{\Delta CD11c}$ BMDC incubated without or with 8-CPT-cAMP (50 μM). (*C* and *E*) IL-4 levels of anti-CD3/28 Ab-stimulated OT2 CD4$^+$ T cells cocultured with Hpgds (*C*) siRNA-transfected BMDC or treated with anti-CCL2 neutralizing Abs (*E*). (*F*) BMDC or OT2 CD4$^+$ T cells were treated with CCR2 antagonist (RS 504393, 10 μM) for 30 min, washed in PBS, and then cocultured for 3 d. T cells were then isolated and IL-4 levels in the supernatants of anti-CD3/28 Ab-stimulated OT2 cells were analyzed (ELISA). Data are mean ± SEM, $n = 3$ assays in each group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The data shown are representative of results obtained in three independent experiments.

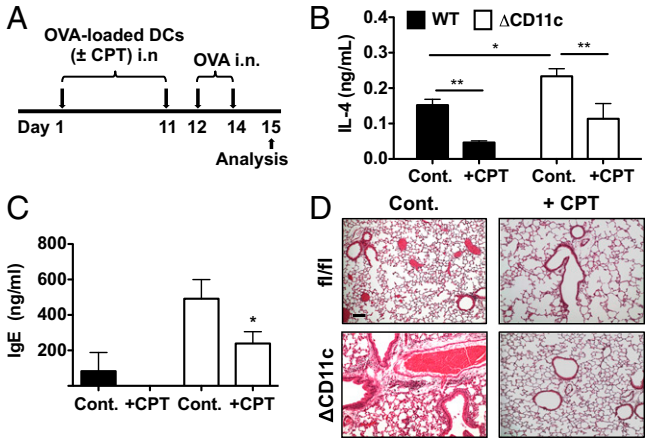

Fig. 6. Adoptive transfer of BMDC from *Gnas*$^{\Delta CD11c}$ mice induces a Th2 bias in vivo, a response that is inhibited by CPT, a cell-permeable cAMP analog. (*A*) Protocol of the adoptive transfer. OVA-loaded *Gnas*$^{\Delta CD11c}$ BMDC were incubated in the absence and presence of 50 μM 8-CPT-cAMP (CPT) before i.n. transfer to WT (B6 mice) and *Gnas*$^{\Delta CD11c}$ recipients (2×10^5 cells per recipient). (*B*) IL-4 levels of anti-CD3/28 Ab-stimulated CD4$^+$ T cells (spleen) from WT or *Gnas*$^{\Delta CD11c}$ recipients (ELISA). (*C*) Serum levels of IgE and IgG1 from WT and *Gnas*$^{\Delta CD11c}$ mice that received *Gnas*$^{\Delta CD11c}$ BMDC loaded with OVA with/without CPT. (*D*) Lung histology from WT and *Gnas*$^{\Delta CD11c}$ recipients. (Magnification: 100×; scale bar: 100 μm.) Data are mean ± SEM, $n = 4$ recipient mice in each group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

development of Th2 bias and airway inflammation in the recipients (Fig. 6 *B–D*). Thus, increased cAMP concentration and signaling inhibits the pro-Th2 phenotype of BMDC from *Gnas*$^{\Delta CD11c}$ mice both in vitro and in vivo.

Discussion

Advances in the studies of innate and adaptive immunity have revealed the molecular basis of Th1, Th17, and Treg induction by DC (35). Such studies also showed the important role of activation of pattern recognition receptors (PRR) by microbial products in the differentiation of the Th1/Th17 subsets (36, 37). In contrast, the mechanisms by which DC induce a Th2 response remain obscure, and thus, the involvement of other cell types has been proposed (7, 8), especially ILC2 (9). The data presented here indicate that a Gαs/Gαi signaling imbalance that favors a low cAMP concentration in BMDC and lung CD11c$^+$ DC provokes a Th2 response (Fig. 3), which is reversed by increasing cAMP content and PKA activation in DC (Fig. 4). These data, combined with our previous observations that show induction of Th17 response (16) by high levels of cAMP in DC mostly via Gαs activation, indicate that in addition to PRR, signaling via regulators of cAMP levels, Gαs, and Gαi in CD11c$^+$ DC (and not in CD11c$^-$ ILC2 cells) (38) contributes to Th subset differentiation. Furthermore, the current findings help explain certain previous data, for example evidence that CGRP, a neuronally derived peptide that binds to a Gs-coupled receptor, can be an antiinflammatory mediator in the setting of allergic airway inflammation (39).

Although *Gnas*$^{\Delta CD11c}$ mice are atopic from an early age (Fig. 1), conditions that influence the microbiota have been proposed to affect a Th2 bias and induction of Th2-mediated airway inflammation (40). Indeed, high serum IgE levels were found in germ-free mice (41). We question whether the microbiome of our *Gnas*$^{\Delta CD11c}$ mice has a substantial impact on their Th2 bias for the following reasons: (*i*) Our mice were raised under specific pathogen-free (SPF) and not under germ-free conditions. (*ii*) BM cells from *Gnas*$^{\Delta CD11c}$, but not fl/fl, mice that were cultured and differentiated for 7 d in vitro under sterile conditions induce Th2 bias of OT2 CD4$^+$ T cells (Fig. 3 *A* and *B*), which was abolished by cell permeable cAMP (Fig. 4 *A* and *B*). (*iii*) Blocking PKA signaling (H89) or activating Gai (mastoparan) in WT BMDC provokes a Th2 bias, results that are consistent with the signaling pathway that induces the Th2 bias by *Gnas*$^{\Delta CD11c}$ BMDC (Fig. 4 *C* and *D*). (*iv*) Our breeding strategy provides both fl/fl and *Gnas*$^{\Delta CD11c}$ littermates and their cohousing for 6 mo did not induce Th2 bias or any other change in the CD4$^+$ T-cell response in the fl/fl mice nor did it affect development of Th2 bias in the *Gnas*$^{\Delta CD11c}$ mice (*SI Appendix*, Fig. S2). (*v*) Injection of sterile antigen (OVA) was sufficient to provoke an OVA-specific Th2 response in young *Gnas*$^{\Delta CD11c}$, but not fl/fl, mice (Fig. 1 *E–H*). (*vi*) The *Gnas*$^{\Delta CD11c}$ mice are atopic at an early age and develop Th2 bias in a time-dependent manner (by age 5–6 mo) under SPF conditions (Fig. 2). Collectively, although our results do not rule out alterations in the microbioata composition in the *Gnas*$^{\Delta CD11c}$ mice, these findings argue for gene–environment interactions other than with the microbiome as the main drivers of the Th2 bias and the development of Th2-mediated murine asthma (42).

We found that the wasp venom-derived Gαi agonist mastoparan induces the pro-Th2 DC phenotype in WT-BMDC and that inhibition of Gαi signaling (i.e., treatment with PTX) suppresses this phenotype in WT-BMDC and the pro-Th2 phenotype of *Gnas*$^{\Delta CD11c}$ mice (Fig. 4). Mastoparan is derived from yellow jackets (*Vespula vulgaris*) and shares certain activities (43) with melittin, the principal active component of bee venom; activities of mellitin include Gαi activation and Gαs inhibition (44). It is intriguing to speculate that a decrease in cAMP concentrations in DC at the sting areas of affected individuals may be a mechanism by which *Hymenoptera* venoms induce Th2 bias and allergy in humans.

Our results indicate that the activation of PKA inhibits the pro-Th2 phenotype of *Gnas*$^{\Delta CD11c}$ BMDC and suggest that the inhibition of PKA induces a pro-Th2 phenotype in WT-BMDC (Fig. 4). Transcriptomic analysis of *Gnas*$^{\Delta CD11c}$ BMDC identified 2 cAMP-regulated gene products (Hpgds and CCL2) that affect the pro-Th2 phenotype of BMDC ex vivo (Dataset S1 and Fig. 5) and have been reported to contribute to Th2 immunity and asthma in vivo (45, 46). Those genes and perhaps others (Dataset S1) are predicted to mediate the induction of Th2 by *Gnas*$^{\Delta CD11c}$ BMDC. Overall, our data indicate that a balance between Gαs and Gαi signaling appears to determine the pro-Th2 phenotype in both WT and *Gnas*$^{\Delta CD11c}$ BMDC. Consistent with this idea, transcriptomic analysis points to a role for CREB in mediating cAMP effects that determine the pro-Th2 phenotype of DC (*SI Appendix*, Fig. S5).

Numerous animal models have been used to explore the pathogenesis of allergic disorders (47, 48). However, the failure to translate drug candidates identified in such models to humans with those diseases raises questions regarding the utility of those models and emphasizes a need for new models that more accurately reflect human immunology and genetics (49). The increasing prevalence of allergic diseases during recent decades imposes significant public health challenges and has substantial health care-related costs (50–52). The pathophysiology of *Gnas*$^{\Delta CD11c}$ mice mimics that observed in allergic/asthmatic patients: *Gnas*$^{\Delta CD11c}$ mice are atopic, develop spontaneous Th2 response and a progressive chronic allergic phenotype that is akin to what occurs in patients with allergic asthma. Thus, *Gnas*$^{\Delta CD11c}$ mice may be a unique platform to identify novel molecular effectors of Th2 differentiation, their role in the induction of the allergic phenotype and the related airway inflammation.

Gnas$^{\Delta CD11c}$ mice may facilitate the discovery and testing of new therapeutics to prevent and treat allergy and asthma in humans. Based on the results shown here, we propose that targeting of DC-expressed GPCRs, the physiological activators of Gαs and Gαi (and thus the regulators of cAMP formation) may

provide such a therapeutic approach. Alternative means of influencing cAMP/PKA signaling can be envisaged but the wide utility and safety of drugs directed at GPCRs, including in the treatment of clinical features of allergic disorders, favor such receptors as attractive targets for developing DC-directed therapy that will influence Th2 immunity and its consequent immunopathologies.

Materials and Methods

C57BL/6 (B6) mice were purchased from Harlan Laboratories. CD11c-Cre transgenic mice and OT2 (B6) were purchased from The Jackson Laboratory. To generate Gαs-deficient dendritic cells, lox-flanked G*nas* (23) (a kind gift from Lee Weinstein, NIH) were crossed to CD11c-Cre mice. The $CD11c^{+}$ cells in the $Cre^{+}Gnas^{\Delta CD11c}$ mice were determined to be $Gnas^{\Delta CD11c}$. The fl/fl littermates ($Cre^{-}Gnas^{fl/fl}$) or B6 were used as control. IL4-eGFP reporter (4Get) mice originally made by Dr. Richard Locksley (University of California, San Francisco) were a gift from Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA). 4Get mice were bred to OT2 mice to yield 4Get/OT2 mice. As outlined in *Results*, these animals were used in selected experiments. All mice were kept in a SPF facility.

For details of experimental conditions and analysis, see *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. The study was funded by NIH Grants HD12303, CA23100, and DK063491 (to N.J.G.W.); NIH Grants AI 38425, AI 70535, and AI 72115 (to D.B.); NIH Grants AI095623 and AI077989 (to E.R.); Veterans Affairs Grant I01BX000130 (to N.J.G.W.); and a grant from the Crohn's and Colitis Foundation of America (to E.R.).

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