

p38 α Protein Negatively Regulates T Helper Type 2 Responses by Orchestrating Multiple T Cell Receptor-associated Signals*

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Background: The MAP kinase p38 α is a critical regulator of innate and adaptive immunity.

Results: p38 α -deficient T helper cells produce more IL-4 in response to antigen exposure and preferentially differentiate into T helper type 2 (Th2) cells.

Conclusion: p38 α negatively regulates Th2 responses *in vitro* and *in vivo* through modulating multiple TCR-associated signals.

Significance: Interfering with p38 α pathway may present new therapeutic strategy for Th2 immunity-mediated diseases.

Mitogen-activated protein kinase p38 α is a critical regulator of certain inflammatory diseases. However, its role in T helper type 2 (Th2) responses and allergic inflammation remains unknown. Here we show an increase in the production of interleukin-4 (IL-4) in p38 α ^{-/-} CD4⁺ T cells in response to antigen stimulation. p38 α -deficient naïve CD4⁺ T cells preferentially differentiate into Th2 cells through increased endogenous production of IL-4. Consistent with those results, we also observed decreased expression of p38 α during T helper cell differentiation. Furthermore, deficiency of p38 α alters the balance in the expression of NFATc1 and NFATc2 under steady-state conditions and enhances the expression and nuclear translocation of NFATc1 in CD4⁺ T cells upon antigen stimulation. Knockdown of NFATc1 significantly inhibits Th2 differentiation in p38 α ^{-/-} T cells but not in p38 α ^{+/-} T cells. p38 α deficiency also inhibits the activation of Akt but enhances the activation of ERK in response to T cell receptor engagement without impacting IL-2/Stat5 signaling. In a model of ovalbumin-induced acute allergic airway inflammation, mice with induced deletion of p38 α show elevated serum ovalbumin-specific IgE level, increased infiltration of eosinophils, and higher concentrations of Th2 cytokines including IL-4 and IL-5 in the bronchoalveolar lavage fluid relative to control mice. Taken together, p38 α regulates multiple T cell receptor-associated signals and negatively influences Th2 differentiation and allergic inflammation.

p38 along with ERK and JNK belong to the family of mitogen-activated protein kinases (MAPKs), which are key intracellular signaling molecules involved in regulating a wide spectrum of extracellular signals. MAPKs are critical modulators of immune cell development, activation, differentiation, and survival. p38 α

was first discovered and cloned as an evolutionarily conserved protein kinase whose activity is induced by lipopolysaccharide and interleukin 1 (IL-1) (1–3). p38 α regulates the production of key inflammatory mediators by cells of the innate immune system, including TNF- α , IL-1- β , and cox-2 (4). In addition, p38 also acts downstream of cytokines such as TNF- α and mediates some of its effects. The p38 pathway plays a central role in inflammatory diseases such as rheumatoid arthritis and Crohn disease (5). There are four closely related isoforms of p38 including, p38 α , p38 β , p38 γ , and p38 δ (6). In CD4⁺ T cells, p38 is activated in response to stimulation via the TCR² and costimulatory molecules (7–9), and p38 mediates production of cytokines (10, 11). However, the role of p38 α in adaptive immunity and T cell functions remains poorly understood.

CD4⁺ T cells play multiple and essential roles in the immune system. They help B cells generate antibody, regulate responses of CD8⁺ T cells, modulate macrophage functions and coordinate immune responses against pathogenic microorganisms. These functions are largely achieved by the differentiation of naïve CD4⁺ T cells into effector cells after stimulation by antigen presentation and the cytokine environment (12). There are at least four types of T helper cells, Th1, Th2, Th17, and T_{regs}. Although the specific role of p38 α in T cells is not known, previous studies using mice expressing a dominant negative form of p38 have demonstrated reduced expression of IFN- γ in Th1 cells (13). In constitutively activated MKK6 transgenic mice, increased IFN- γ production and reduced IL-4 production were observed (13). These results suggest that inhibition of p38 may promote IL-4 production and Th2 responses. However, TCR can activate p38 via alternate a mechanism(s) that is independent of MKK3/6 activation (7, 8). Therefore, to understand the physiologic role of p38 α in T cell-mediated immune responses, we have utilized a newly established p38 α conditional deletion system.

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² The abbreviations used are: TCR, T cell receptor; OVA, ovalbumin; BAL, bronchoalveolar lavage; Th1, T helper type 1; NFAT, nuclear factor of activated T cell; BAL, bronchoalveolar lavage.

Our studies demonstrate that disruption of p38 α in CD4⁺ T cells promotes IL-4 production in response to antigen stimulation. p38 α -deficient CD4⁺ T cells preferentially differentiate into Th2 phenotype through increased endogenous production of IL-4. During T helper cell activation, mRNA and protein levels of p38 α are decreased. p38 α deficiency alters the balance in the expression of NFATc1 and NFATc2 and increases protein level and nuclear translocation of NFATc1 in CD4⁺ T cells upon TCR stimulation. Down-regulation of NFATc1 reverses enhanced Th2 differentiation due to absence of p38 α . Deletion of p38 α also impairs TCR-induced activation of Akt and enhances the activation of ERK. In ovalbumin-induced acute allergic airway inflammation, p38 α ^{-/-} mice show increased infiltration of eosinophils and a higher concentration of Th2 cytokines IL-4 and IL-5 in bronchoalveolar lavage fluid relative to control mice. We conclude that p38 α is a negative regulator of Th2 responses *in vitro* and *in vivo* by affecting multiple TCR-associated signals.

EXPERIMENTAL PROCEDURES

Mice—Mice carrying the floxed p38 α allele (p38 α ^{fl/fl}) have been previously described (14, 15). Mx-Cre mice were purchased from The Jackson Laboratory (Bar Harbor, ME). p38 α ^{fl/fl} mice were crossed to Mx-Cre mice. Heterozygous Mx-Cre⁺p38 α ^{fl/+} (p38 α ^{+/-}) mice were crossed to p38 α ^{fl/fl} mice to obtain Mx-Cre⁺p38 α ^{fl/fl} (p38 α ^{-/-}) mice. To activate the interferon- γ -inducible Mx-Cre recombinase, 6–8-week old mice were injected intraperitoneally 3 times with polyIC. All experiments were performed at least 8 weeks after polyIC treatment. All studies were performed in a C57Bl/6 genetic background. All mice were maintained in specific-pathogen-free conditions, and experiments were approved by the Indiana University Laboratory Animal Research Center.

Antibodies, Cytokines, and Reagents—Anti-CD3, anti-CD28, anti-NFATc1, anti-NFATc2, phosphatidylethanolamine (PE)-anti-IFN- γ , PE-anti-IL17A, PE-anti-ROR γ t, FITC-anti-CD4, annexin V, and 7-Aminoactinomycin D antibodies were purchased from BD Biosciences. PEcy7-anti-IL-4 was purchased from eBioscience (San Diego, CA). Akt, phospho-Akt, ERK, phospho-ERK, Stat5, phospho-Stat5, I κ B α , and p38 α antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody was purchased from Sigma. Murine IL-4, IL-2, IL-12, and IL-6 were purchased from Peprotech (Rocky Hill, NJ). TGF- β was purchased from R&D.

Immunoblotting—Cells were lysed in a lysis buffer solution that consisted of 20 mM Tris-HCl, 150 mM NaCl, 1 mM β -glycerol phosphate, 1 mM EDTA, 1% Triton X-100, 1 μ g/ml leupeptin (cell signaling). The lysates were obtained by centrifugation at 10,000 $\times$ g for 30 min. Protein concentration was quantified by bicinchoninic acid kit (Pierce). Cell lysates were boiled in sample buffer (187.5 mM Tris-HCl, 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromphenol blue), and equal amounts of protein were resolved by gel electrophoresis on precast SDS-PAGE (Invitrogen) in running buffer as described by the manufacturer and transferred to nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk diluted in Tris-buffered saline containing Tween 20, then incubated with the indicated antibodies. Supersignal West Dura Extend Duration

Detection System (Pierce) was used to expose the membrane to film.

RNA Analysis by Reverse Transcription and Quantitative Real-time PCR—Total RNA was isolated. First strand cDNA was synthesized from 1 μ g of total RNA using the SuperScript system (Invitrogen). Gene expression was examined with the 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA) using the Stratagene Brilliant II SYBR Green qPCR kit. Data were normalized to the expression of β -actin. Primer set for p38 α detection was purchased from RealTimePrimers (Elkins Park, PA).

Proliferation Assay—Splenocytes were mechanically disrupted to obtain single cell suspensions. Splenocytes were stimulated with plate-bound anti-CD3 and anti-CD28 antibody. Cells were pulsed with 1.0 μ Ci of [³H]thymidine (PerkinElmer Life Sciences) during the last 6 h of a 48-h culture period. Cells were then harvested with an automated 96-well cell harvester (Brandel, Gaithersburg, MD), and thymidine incorporation was determined as counts per minute.

Splenocyte Th2 Response—Splenocytes from OVA-sensitized mice were stimulated with OVA (100 μ g/ml) for 72 h, cell-free supernatants were collected, and the concentrations of IL-4, IL-5, and IFN- γ were measured by ELISA.

Isolation of Cells and T Helper Cell Differentiation—Naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells (1 $\times$ 10⁶ cells/ml) were purified from spleen via magnetic isolation (Miltenyi Biotec, Auburn, CA). For Th0 differentiation, purified naïve T cells in complete RPMI 1640 medium with IL-2 (25 units/ml) were stimulated with plate-bound anti-CD3 (2 μ g/ml) and soluble anti-CD28 (1 μ g/ml) antibodies. Cells were expanded on day 3. After 5 days of culture, differentiated cells were re-stimulated with plate-bound anti-CD3 and stained for IL-4 and IFN- γ or re-stimulated to prepare supernatants, and cytokine production was measured by ELISA. For Th1 differentiation, IL-12 (5 ng/ml) and anti-IL-4 (10 μ g/ml; BD Bioscience) were added to primary cultures. For Th2 differentiation, IL-4 (5 ng/ml) and anti-IFN- γ (10 μ g/ml; BD Bioscience) were added to primary cultures. For Th17 differentiation, IL-6 (20 ng/ml), TGF- β (2 ng/ml), anti-IL-4 (10 μ g/ml), and anti-IFN- γ (10 μ g/ml) were used.

Preparation of Nuclear Extracts—CD4⁺ T cells were either left unstimulated or stimulated with anti-CD3 and anti-CD28 antibodies. Nuclear extracts were prepared by first incubating the cells with a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors for 15 min, then 0.4% Nonidet P-40 was added, and cells were vortexed. Cells were then spun to pellet at 6000 rpm for 5 min in a benchtop centrifuge. The nuclear pellets were resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 0.63 M NaCl, 25% glycerol, 0.5 mM DTT, and protease inhibitors, then vigorously vortexed for 30 min in the cold room. The lysed nuclei were centrifuged at maximum speed for 5 min. For nuclear translocation assay, the supernatants were immunoblotted with the indicated antibodies.

Retroviral Transduction and T Cell Differentiation—The pMKO.1 GFP retroviral vector (Addgene plasmid 10676, Cambridge, MA) was kindly provided by Dr. William Hahn. Mouse

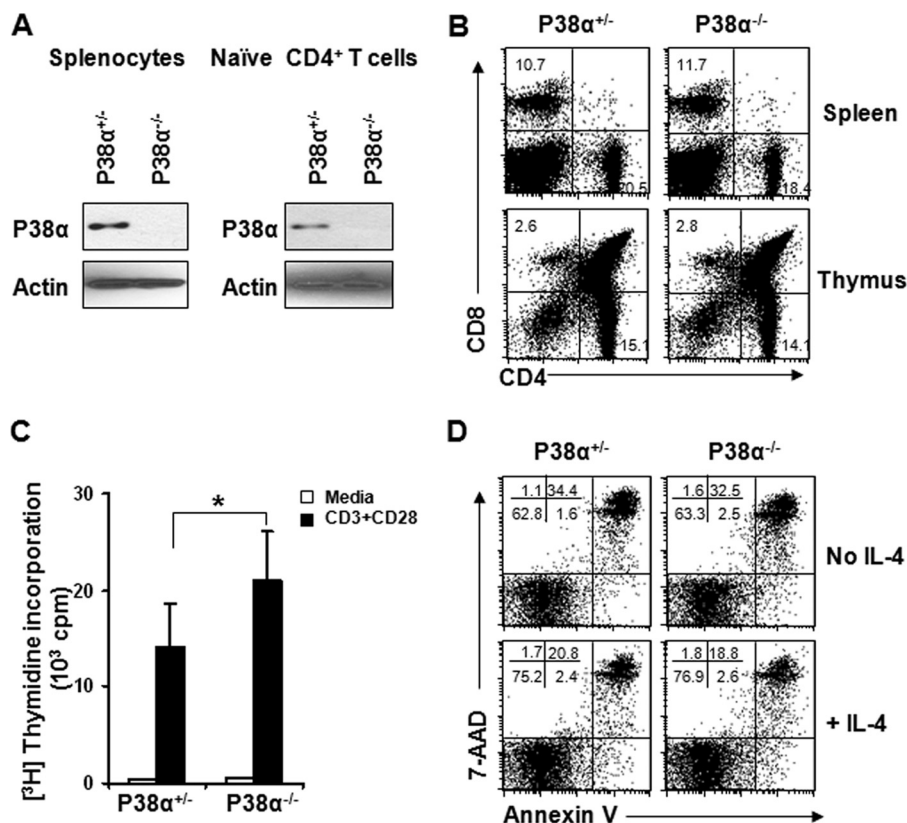


FIGURE 1. Effects of p38 α deletion on T cell development and biological functions. *A*, total cell lysates from spleen and naïve CD4⁺CD25[−]CD62L^{hi}CD44^{lo} T cells from p38 α ^{+/−} and p38 α ^{−/−} mice were prepared, and the protein level of p38 α was detected by immunoblotting. Data are representative of at least three independent experiments. *B*, thymocytes and splenocytes were harvested from p38 α ^{+/−} and p38 α ^{−/−} mice. Populations of CD4⁺ and CD8⁺ cells were analyzed by flow cytometry. Data are representative of at least five independent experiments. *C*, splenocytes from p38 α ^{+/−} and p38 α ^{−/−} mice were stimulated with immobilized anti-CD3 and anti-CD28 antibody for 48 h. Cell proliferation was measured by [³H]thymidine incorporation. *, $p < 0.05$. Data are the mean $\pm$ S.D.; $n = 6$. *D*, naïve CD4⁺ T cells from p38 α ^{+/−} and p38 α ^{−/−} mice were cultured in the presence or absence of IL-4. Apoptotic cells were detected by flow cytometry through annexin v and aminoactinomycin D (7-AAD) staining. Data are representative of three independent experiments.

NFATc1 shRNA sequences were obtained from Origene and were cloned into pMKO.1 GFP vector. Recombinant retrovirus was generated as described (16). For retroviral transduction, purified naïve CD4⁺ T cells were activated by plate-bound anti-CD3 and soluble anti-CD28 and were “spininfected” with fresh retroviral supernatant containing Polybrene. After viral transduction, GFP-positive cells were sorted and cultured under Th0 condition for further analysis.

Sensitization and Challenge Protocol—To induce acute allergic airway inflammation, mice were sensitized on day 0 and day 7 by intraperitoneal injection of 25 μ g of OVA emulsified in aluminum hydroxide gel (Sigma). On days 14, 15, and 16, anesthetized mice were challenged intranasal with 100 μ g of OVA in PBS. Mice were analyzed after the last challenge.

BAL Fluid and Histology—Mice were anesthetized and the tracheas were cannulated. BAL fluid was collected with 1.0 ml of ice-cold PBS. BAL fluid was centrifuged at 1400 $\times$ g, and the supernatant was used to measure cytokine levels. The pellet was resuspended, the total cell count was measured, and cytospin was prepared. Lungs were fixed (10% formaldehyde) overnight. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, toluidine blue, and Giemsa.

ELISA—Supernatants were collected for cytokine detection. Cytokines were detected with ELISA kit from Biolegend (IL-4, IL-5, IFN- γ , and IL-17A) according to the instructions. Serum

OVA-specific IgE was quantified with ELISA kit from SHI-BAYGI (Japan) according to the manufacturer's protocol.

Statistical Analysis—Two-group comparisons were done with Student's t tests. Intergroup differences were considered significant at $p < 0.05$.

RESULTS

Effects of p38 α Deletion on the Development, Proliferation, and Apoptosis of T Cells—To address the genetic role of p38 α in regulation of immune response, a newly established p38 α conditional deletion system was utilized (14, 15). Previous studies have used Mx-Cre mice to efficiently delete the targeted gene to study its effect on CD4⁺ T helper cells (17, 18). By crossing mice bearing floxed p38 α alleles with Mx-Cre bearing mice, p38 α protein in splenocytes and purified CD4⁺CD25[−]CD62L^{hi}CD44^{lo} naïve T cells from p38 α ^{−/−} mice was completely deleted after three injections of polyIC (Fig. 1A). Based on previous reports and our observations, there was no significant phenotypic difference between wild type and p38 α ^{+/−} mice. Thus, in this study, we used p38 α ^{+/−} mice as the control mice. Flow cytometric analysis revealed that CD4:CD8 ratios were similar in the thymus and spleen of p38 α ^{+/−} and p38 α ^{−/−} mice (Fig. 1B). These results suggest that inactivation of p38 α does not perturb the development of CD4⁺ and CD8⁺ cells. We next investigated T cell function in the absence of p38 α . Inhibition of

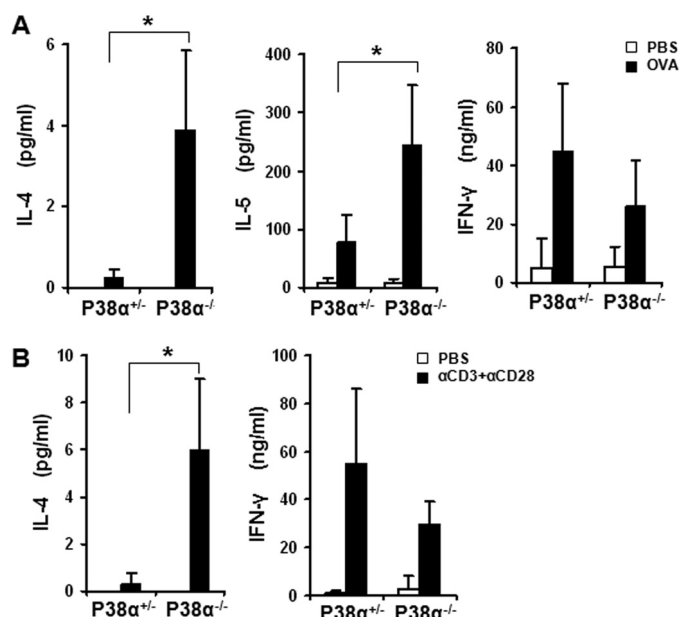


FIGURE 2. Absence of p38 α enhances IL-4 production when exposed to antigen. A, cultured splenocytes from control or OVA-sensitized p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were re-stimulated with OVA (100 μ g/ml) for 72 h. Concentration of IL-4, IL-5, and IFN- γ in the supernatants was measured by ELISA ($n = 5$ per group). *, $p < 0.05$. Data are the mean $\pm$ S.D. B, naive CD4 $^{+}$ CD25 $^{-}$ CD62L ^{hi} CD44 ^{lo} T cells from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were stimulated with immobilized anti-CD3 and anti-CD28. After 48 h, the concentration of IL-4 and IFN- γ in the supernatants was measured as panel A. *, $p < 0.05$. Data are the mean $\pm$ S.D.; $n = 5$.

the p38 pathway promotes cell cycle progression in multiple cell types (14, 19). To determine whether p38 α affects T cell proliferation in response to antigen exposure, splenocytes from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were stimulated with α CD3 and α CD28 antibodies. [^{3} H]Thymidine incorporation assay showed that p38 $\alpha^{-/-}$ T cells proliferated modestly but significantly more than p38 $\alpha^{+/+}$ T cells (Fig. 1C). Studies have shown that IL-4 prevents the apoptosis of CD4 $^{+}$ T cells in the absence of TCR activation (20). We, therefore, questioned whether deletion of p38 α imposed more resistance in T cells to apoptotic stimuli. Under culture conditions lacking IL-4, apoptotic CD4 $^{+}$ T cells detected by annexin V and aminoactinomycin D staining were not altered as a result of p38 α deficiency (Fig. 1D). These results suggest that inactivation of p38 α is dispensable for T cell development and apoptosis but modestly influences T cell proliferation.

p38 α -deficient T Helper Cells Produce More Th2 Cytokines in Response to Antigen—CD4 $^{+}$ helper T cells are the essential regulator of adaptive immunity. After being activated, naïve CD4 $^{+}$ precursor cells differentiate into cytokine-secreting effector cells. Th1 produce interferon- γ , and Th2 cells make IL-4, IL-5, and IL-13 (21). Next, we examined the cytokine profile of CD4 $^{+}$ T cells from p38 $\alpha^{-/-}$ mice after TCR stimulation. Control and p38 $\alpha^{-/-}$ mice were immunized with OVA plus aluminum hydroxide, and total splenocytes were isolated and re-stimulated with OVA to determine the antigen-specific T cell response. As shown in Fig. 2A, enhanced production of IL-4 and IL-5 was detected by ELISA from p38 $\alpha^{-/-}$ splenocytes compared with control splenocytes. However, the production of IFN- γ , a key Th1 cytokine, was lower in p38 $\alpha^{-/-}$ splenocytes

relative to control splenocytes. These results suggest that deletion of p38 α causes a systemic increase in Th2 immunity. To further determine whether the enhanced Th2 cytokine production is due to an intrinsic T cell hyperresponsiveness to antigen stimulation, purified CD4 $^{+}$ CD25 $^{-}$ CD62L ^{hi} CD44 ^{lo} naïve T cells from control and p38 $\alpha^{-/-}$ mice were stimulated with plate-bound antibody to CD3 and anti-(α)CD28 for 48 h. The absence of p38 α triggered greater production of IL-4. Meanwhile, the production of IFN- γ moderately decreased in p38 $\alpha^{-/-}$ T cells compared with control T cells (Fig. 2B). These results demonstrate that inactivation of p38 promotes the production of Th2 cytokines in T cells when exposed to antigen stimulation.

Deletion of p38 α Promotes Th2 Cell Commitment through Enhanced Endogenous Production of IL-4—Because p38 $\alpha^{-/-}$ CD4 $^{+}$ T cells showed enhanced production of IL-4 and IL-4 is required for the initiation of Th2 cell differentiation (21), we examined if p38 α regulates the differentiation of Th2 cells. To assess whether deficiency of p38 α in T cells interfered with their ability to differentiate into IL-4-producing Th2 cells, IFN- γ -producing Th1 cells, and IL-17-producing Th17 cells, naïve CD4 $^{+}$ CD25 $^{-}$ CD62L ^{hi} CD44 ^{lo} T cells were purified from control and p38 $\alpha^{-/-}$ spleens and cultured under neutral (Th0), Th1, Th2, or Th17 polarizing conditions. In the Th0 condition, the frequency of IL-4-producing cells increased almost 2-fold as measured by flow cytometry (Fig. 3A), and a more potent production of IL-4 and IL-5 was detected by ELISA in supernatants derived from p38 $\alpha^{-/-}$ cultures (Fig. 3B). In contrast, p38 α deficiency resulted in fewer IFN- γ -producing cells, and reduced production of IFN- γ was seen by ELISA in supernatants from cultured cells (Fig. 3, A and B). Both p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ CD4 $^{+}$ T cells barely differentiated into Th17 cells or produced IL-17A under Th0 conditions (data not shown).

In Th2 conditions, with excess exogenous IL-4, p38 $\alpha^{-/-}$ T cells developed into IL-4 $^{+}$ cells as efficiently as control T cells, and no significant difference in the secretion of IL-4 was observed between p38 $\alpha^{-/-}$ and control T cells (Fig. 3, C and D). Th1-polarizing conditions induced a moderate decrease in the percentage of IFN- γ -producing cells and less IFN- γ production in p38 α -deficient populations than in control populations (Fig. 3, C and D).

Noubade *et al.* (22) reported that p38 controls Th17 development in a T cell-intrinsic manner. However, Huang *et al.* (23) recently reported that p38 α plays a selective role in dendritic cell-mediated Th17 differentiation but not in T cell-intrinsic Th17 differentiation. In our experiments we detected no significant difference in the differentiation of Th17 cells between p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ CD4 $^{+}$ T cells under Th17 skewing conditions (Fig. 3, C and D). These results suggest that p38 α may not regulate Th17 differentiation directly. The differences in the conclusions among different research groups could be a result of differences in the specificity of the mouse models used.

We further wondered whether p38 α modulates the expression of the master transcription factors involved in T helper cell differentiation, including T-bet (Th1), Gata-3 (Th2), and ROR γ t (Th17). Consistent with the differentiation data, deletion of p38 α slightly increased the expression of Gata-3 and inhibited the expression of T-bet. At the same time, p38 α defi-

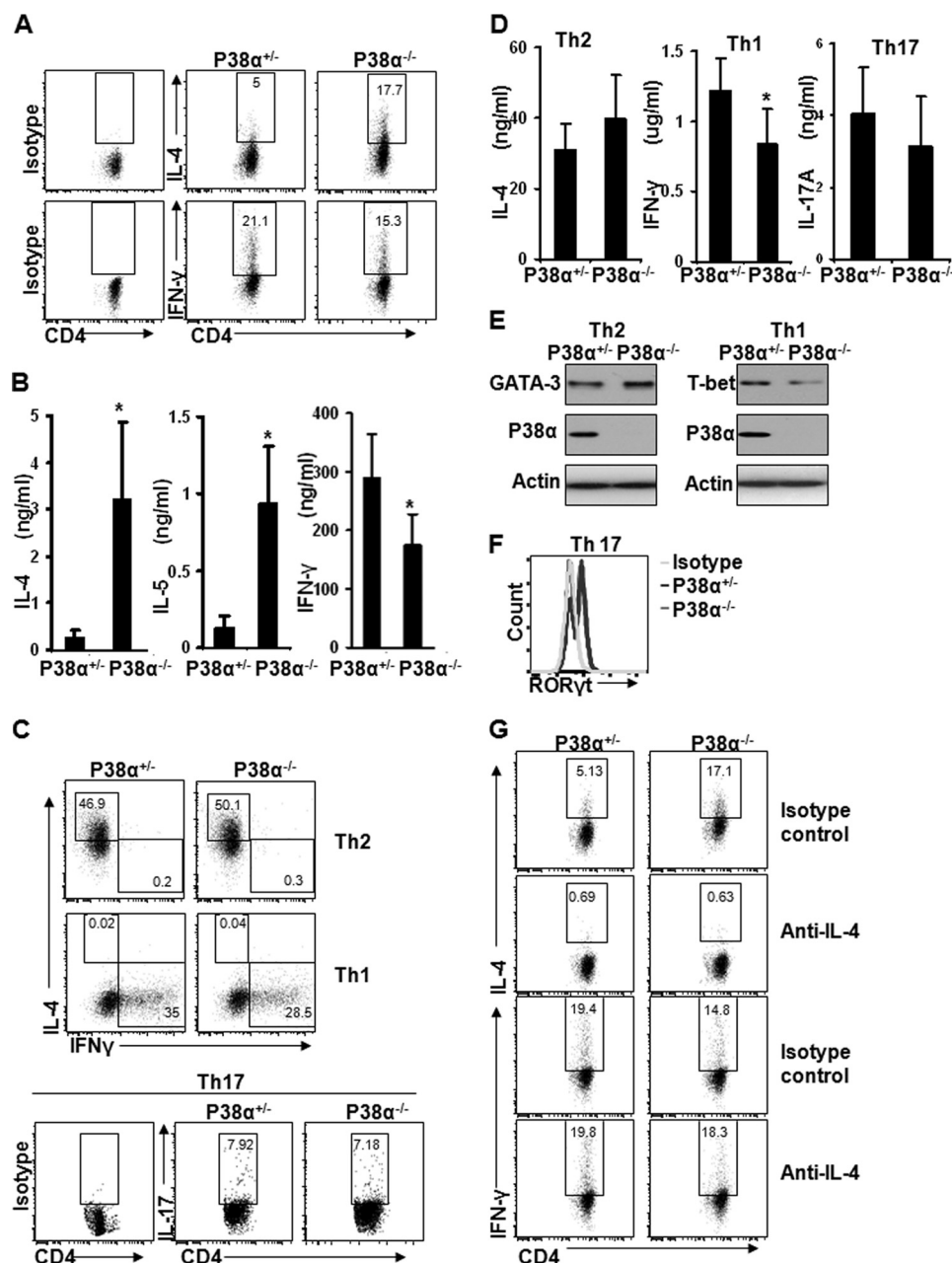


FIGURE 3. Effect of p38 α inactivation on T helper cell lineage commitment. Purified naïve CD4⁺ T cells from p38 α ^{+/−} and p38 α ^{−/−} mice were cultured under non-polarizing conditions (Th0) for 5 days and analyzed by flow cytometry for intracellular cytokines (A) and ELISA for production of IL-4, IL-5 and IFN- γ in the supernatants (B). *, $p < 0.05$. Data in B are the mean $\pm$ S.D.; $n = 5$. Naïve CD4⁺ p38 α ^{+/−} and p38 α ^{−/−} T cells were polarized in Th1, Th2, or Th17 conditions for 5 days. Percentages of IL-4⁺, IFN- γ ⁺, and IL-17A⁺ cells were analyzed by flow cytometry (C), and production of IL-4, IFN- γ , and IL-17A were measured by ELISA (D). *, $p < 0.05$. Data in D are the mean $\pm$ S.D.; $n = 5$ –6. Naïve CD4⁺ p38 α ^{+/−} and p38 α ^{−/−} T cells were cultured under Th1, Th2, or Th17 conditions, expression of T-bet and Gata-3 was detected by immunoblotting (E), and intracellular ROR γ t was measured with fluorescent anti-mouse-ROR γ t by flow cytometry (F). G, naïve CD4⁺ p38 α ^{+/−} and p38 α ^{−/−} T cells were cultured as in panel A in the presence of isotype control or anti-IL-4 antibody. Percentages of IL-4⁺ and IFN- γ ⁺ cells were assessed as in panel A. Data in E, F, and G are representative of three independent experiments.

ciency did not significantly alter ROR γ t expression (Fig. 3, E and F).

Next, we showed that the presence of anti-IL-4 abolished the ability of p38 α ^{−/−} naïve T cells to preferentially commit to the Th2 phenotype and mildly increased Th1 differentiation in p38 α ^{−/−} T cells (Fig. 3G). This observation is consistent with the concept of inhibitory action of IL-4 on Th1 development (24, 25). Together, these results demonstrate that in the absence of polarizing cytokine, TCR-induced IL-4 production by CD4⁺ T helper cells is enhanced due to the absence of p38 α ,

and p38 α -deficient naïve T cells preferentially differentiate into Th2 cells due to increased endogenous production of IL-4.

p38 α Expression Is Reduced during T Helper Cell Differentiation—In T helper cells, short-term stimulation of TCR (less than an hour) can activate p38 through alternative mechanisms that are independent of MKK3/6 (7, 8). However, the expression of p38 α during a time span required for T helper cell differentiation remains unknown. We collected protein samples from wild type naïve CD4⁺ T cells that were induced to differentiate under un-polarized conditions (Th0). Western

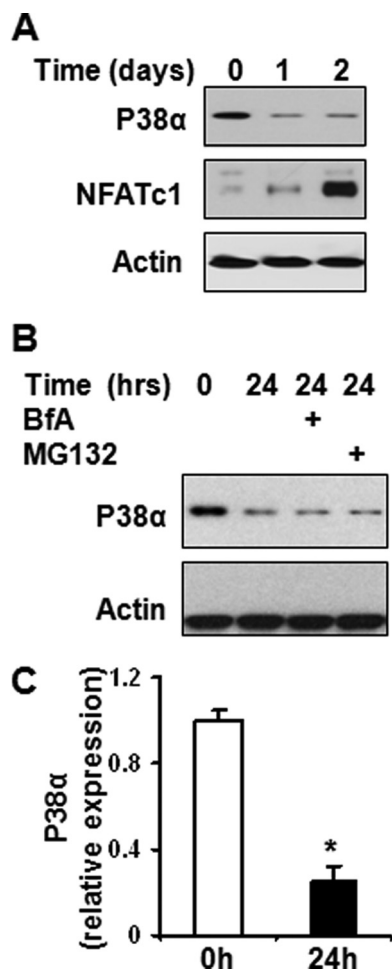


FIGURE 4. p38 α expression is compromised during T helper cell differentiation. A, naïve CD4⁺ T cells were activated with immobilized anti-CD3 (2 μ g/ml) and anti-CD28 (1 μ g/ml) and cultured under Th0 conditions for the indicated times. Protein levels of p38 α and NFATc1 were detected by immunoblotting. B, naïve CD4⁺ T cells were pretreated with bafilomycin A1 (100 ng/ml) and MG132 (20 μ M), respectively, then activated by anti-CD3 and anti-CD28. p38 α levels were measured by immunoblotting. C, naïve CD4⁺ T cells were cultured as panel A. Total RNA was extracted, and mRNA levels of p38 α were detected by real-time PCR. p38 α expression relative to β -actin is shown as the mean $\pm$ S.D. from triplicate wells. *, $p < 0.05$. Data are representative of three independent experiments.

blotting results showed that compared with naïve CD4⁺ T cells, p38 α levels were significantly reduced during T cell differentiation (Fig. 4A), whereas the expression of NFATc1, a positive regulator of IL-4 production and Th2 differentiation (26), was increased (Fig. 4A). The inverse expression pattern between p38 α and NFATc1 further suggests that repression of p38 α pathway may be necessary for IL-4 secretion and Th2 development. We further examined the mechanism underlying down-regulation of p38 α during T cell commitment. Treatment of cells with bafilomycin A1, a lysosome inhibitor, and MG132, a proteasome inhibitor, failed to prevent reduction of p38 α expression (Fig. 4B). Thus, it is unlikely that decreased p38 α expression is mediated via the degradation of p38 α protein. In contrast, real-time PCR analysis revealed that mRNA levels of p38 α were significantly reduced in differentiating CD4⁺ T cells relative to naïve CD4⁺ T cells (Fig. 4C), suggesting T helper cell activation negatively impacts the expression of p38 α at the level

of mRNA. These results further support that p38 α is a negative regulator of Th2 development.

p38 α Is Required to Maintain Homeostasis of NFAT Transcription Factors in T Helper Cells and Down-regulation of NFATc1 Partially Reverses the Enhanced Th2 Development Due to p38 α Deficiency—Next, we further investigated the mechanisms underlying negative regulation of p38 α on IL-4 production and Th2 responses. Downstream of TCR, Ca²⁺-dependent NFAT transcription factors play critical roles in regulating T cell functions. T cells express NFAT1/NFATc2, NFAT2/NFATc1, and NFAT4/NFATc3 (27). The balance among NFAT family members is required for proper generation of Th2 cells. NFATc1-deficient lymphocytes show decreased IL-4 production (26). However, NFAT1/NFATc2 knock-out mice show enhanced allergic immune response (28), and NFAT1/NFATc2-deficient T cells produce more IL-4 and preferentially differentiate into Th2 cells (29). T cells that lack both NFATc2 and NFATc3 differentiate into Th2 cells (30). We, therefore, assessed whether p38 α modulates the expression of NFAT transcription factors in CD4⁺ T helper cells. First, we detected basal protein levels of NFATc1 and NFATc2 in control and p38 α ^{-/-} CD4⁺ T cells. As shown in Fig. 5A, ratio of NFATc1: NFATc2 was higher in p38 α ^{-/-} T cells relative to control T cells, whereas the protein level of JunB, another transcription factor involved in Th2 development (31), and Fyn, a tyrosine kinase downstream of TCR but upstream of PKC- θ /NF- κ B involved in modulating Th2 cytokine secretion (32), was comparable between control and p38 α ^{-/-} T cells. These results suggest that deletion of p38 α skews the balance of NFATc1 and NFATc2 expression under steady-state conditions. Consistently, we found that inhibition of the p38 pathway by SB203580 in CD4⁺ T cells also impairs the expression of NFATc2 but not NFATc1 (Fig. 5B). These results suggest that expression and activity of p38 α plays an important role in maintaining basal NFATc2 expression compared with NFATc1 expression.

We next examined whether p38 α regulates NFATc1 expression after T helper cell activation. As shown in Fig. 5C, TCR stimulation induced higher expression of NFATc1 in p38 α ^{-/-} T cells compared with control T cells. TCR activation also increased the expression of NFATc2; however, its level was significantly lower in p38 α ^{-/-} T cells relative to control T cells. Consistent with the results derived using whole cell lysates, p38 α inactivation also resulted in increased nuclear NFATc1 (Fig. 5D). In contrast, nuclear localization of p65 RelA protein, a subunit of NF- κ B complex, in p38 α ^{-/-} T cells was comparable to control T cells after TCR stimulation. However, we observed the presence of nuclear P65 in p38 α ^{-/-} T cells but not in control T cells under steady-state conditions, suggesting increased basal NF- κ B activity in these cells due to p38 α inactivation.

To further assess the role of NFATc1 in mediating the enhanced Th2 differentiation due to p38 α deficiency, we knocked down NFATc1 expression in CD4⁺ T cells by retroviral transduction of shRNAs as indicated (Fig. 5E). Down-regulation of NFATc1 did not significantly inhibit Th2 differentiation among p38 α ^{+/+} T cells. However, knockdown of NFATc1 in p38 α ^{-/-} T cells significantly reduced IL-4⁺ population,

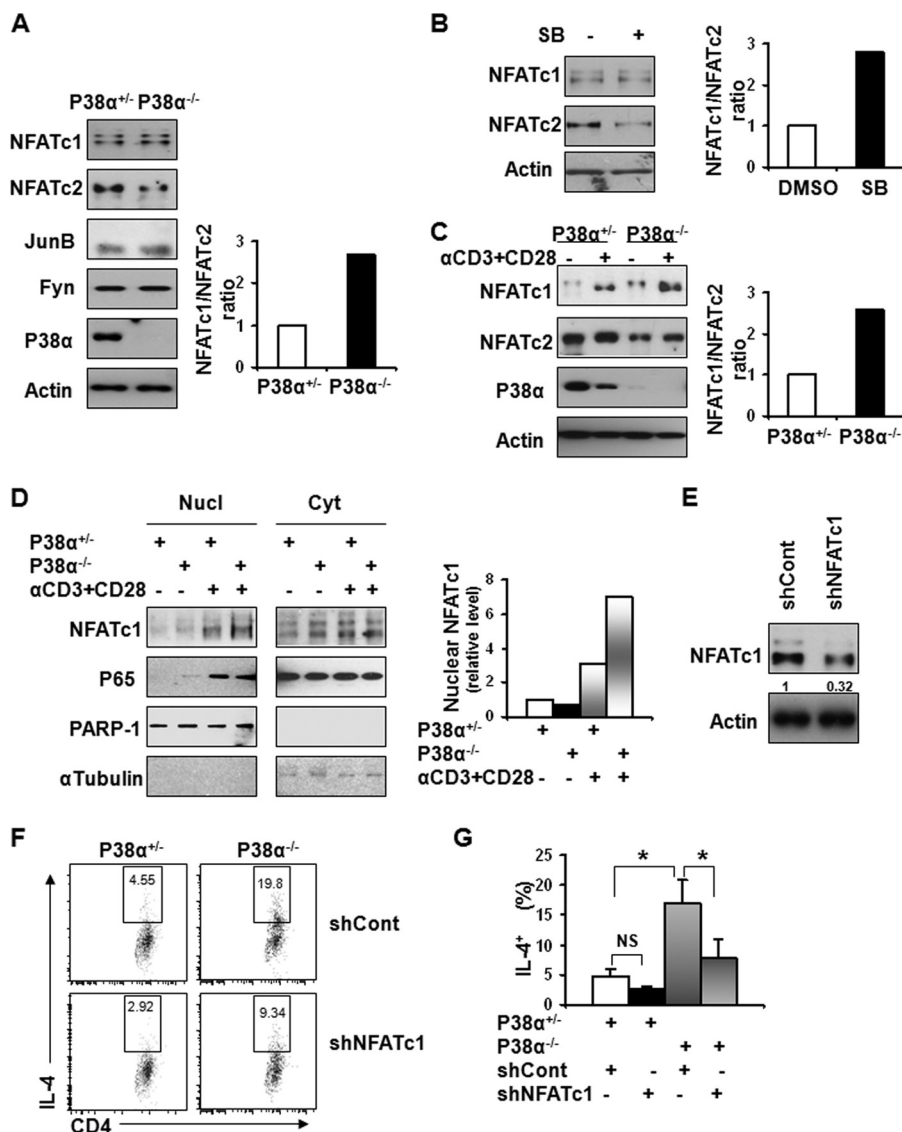


FIGURE 5. Altered NFAT homeostasis is required for enhanced Th2 differentiation due to p38 α deficiency. *A*, naive CD4 $^{+}$ T cells were collected from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice. Total cell lysates were prepared and analyzed by immunoblotting for NFATc1, NFATc2, JunB, Fyn, and p38 α expression. *B*, CD4 $^{+}$ T cells were treated with SB203580 (SB; 10 μ M), and protein levels of NFATc1 and NFATc2 were detected by immunoblotting. *C*, naive CD4 $^{+}$ T cells from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 for 48 h. Total protein was prepared, and expression of NFATc1 and NFATc2 was measured by immunoblotting. *D*, CD4 $^{+}$ T cells from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were treated as in *panel C*. Nuclear protein was extracted, and protein levels of NFATc1 and P65 RelA were detected by immunoblotting. *E*, CD4 $^{+}$ T cells were infected with control or NFATc1 shRNA retrovirus. Protein levels of sorted GFP $^{+}$ cells were measured by immunoblotting. *F*, CD4 $^{+}$ T cells from p38 $\alpha^{+/+}$ and p38 $\alpha^{-/-}$ mice were "spininfected" with control or NFATc1 shRNA retrovirus as *panel E*. Sorted GFP $^{+}$ cells were further cultured under Th0 conditions, and percentages of IL-4 $^{+}$ cells were analyzed by flow cytometry. *G*, shown is a quantitative analysis of the average percentage of IL-4 $^{+}$ cells ($n = 3-4$). *, $p < 0.05$. NS, not significant. Data are the mean $\pm$ S.D. Signals in *A-E* were quantified by densitometric scanning.

although it was still higher than the p38 $\alpha^{+/+}$ counterpart, indicating that in addition to NFATc1, other signaling molecules are likely involved (Fig. 5, *F* and *G*). Together, these data demonstrate that deletion of p38 α alters the homeostasis of NFAT transcription factors, which may promote Th2 differentiation.

Effects of the Absence of p38 α on Downstream Pathways of TCR and IL-2—TCR, IL-4, IL-2, and Notch signaling pathways play critical roles in IL-4 production and Th2 development (21, 33). Our observation that deletion of p38 α has no significant influence on Th2 differentiation in the presence of exogenous IL-4 suggests that p38 α may not affect IL-4/Stat6 signaling. We wondered whether inactivation of p38 α alters downstream pathways from TCR and IL-2. Ca $^{2+}$ /calcineurin/NFATs, PI3K,

and ERK can be activated by TCR and CD28 signaling (34–36). PKC- θ is also a critical downstream target of both TCR and CD28 and can activate NF- κ B (37, 38). Studies have shown that PI-3 kinase/Akt, ERK, and NF- κ B all mediate T cell differentiation. Activation of Akt promotes Th1 commitment (20). ERK is required for Th2 differentiation (39, 40). PKC- θ /NF- κ B mediates Th2 development (20, 32). We consistently observed reduced basal phosphorylation of Akt in p38 $\alpha^{-/-}$ T cells *versus* control T cells (Fig. 6*A*), suggesting impaired PI3K/Akt signaling due to the absence of p38 α in these cells. After stimulation by α CD3 and α CD28, the absence of p38 α markedly impaired the phosphorylation of Akt (Fig. 6*A*). These biochemical findings are consistent with our results demonstrating that deletion

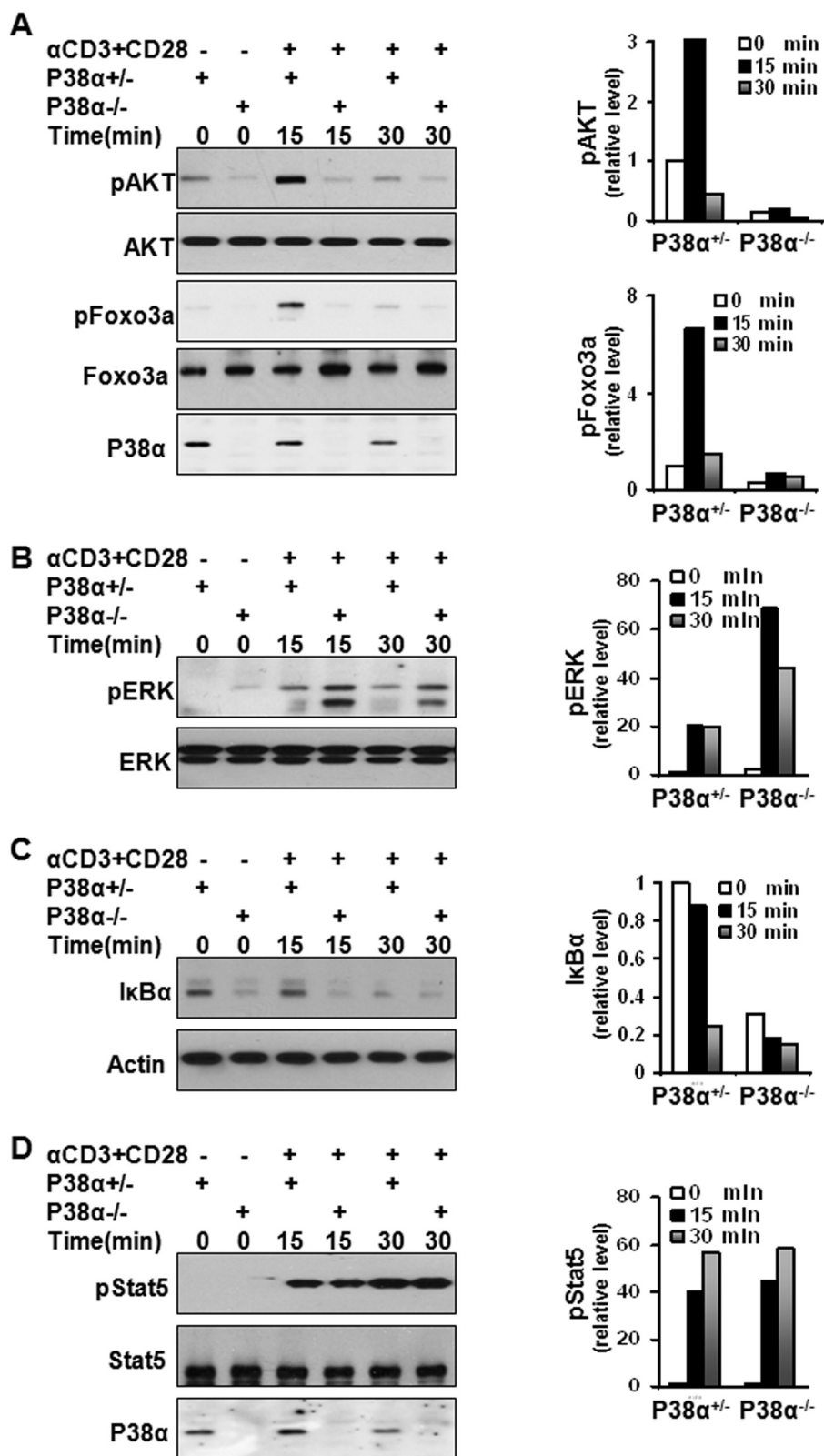


FIGURE 6. **p38 α modulates TCR and IL-2 signaling.** A, naïve CD4⁺ T cells from p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 for indicated times. Total and phosphorylated Akt and Foxo3a were detected by immunoblotting. B, CD4⁺ T cells from p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice were treated as in panel A, and total and phosphorylated ERK was detected by immunoblotting. C, after exposure to antigen, the protein level of I κ B α was measured in p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ T cells by immunoblotting. D, *in vitro*-expanded CD4⁺ T cells from p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice were stimulated with IL-2, and total and phosphorylated Stat5 was detected by immunoblotting. Data are representative of two or three independent experiments. All signals were quantified by densitometric scanning.

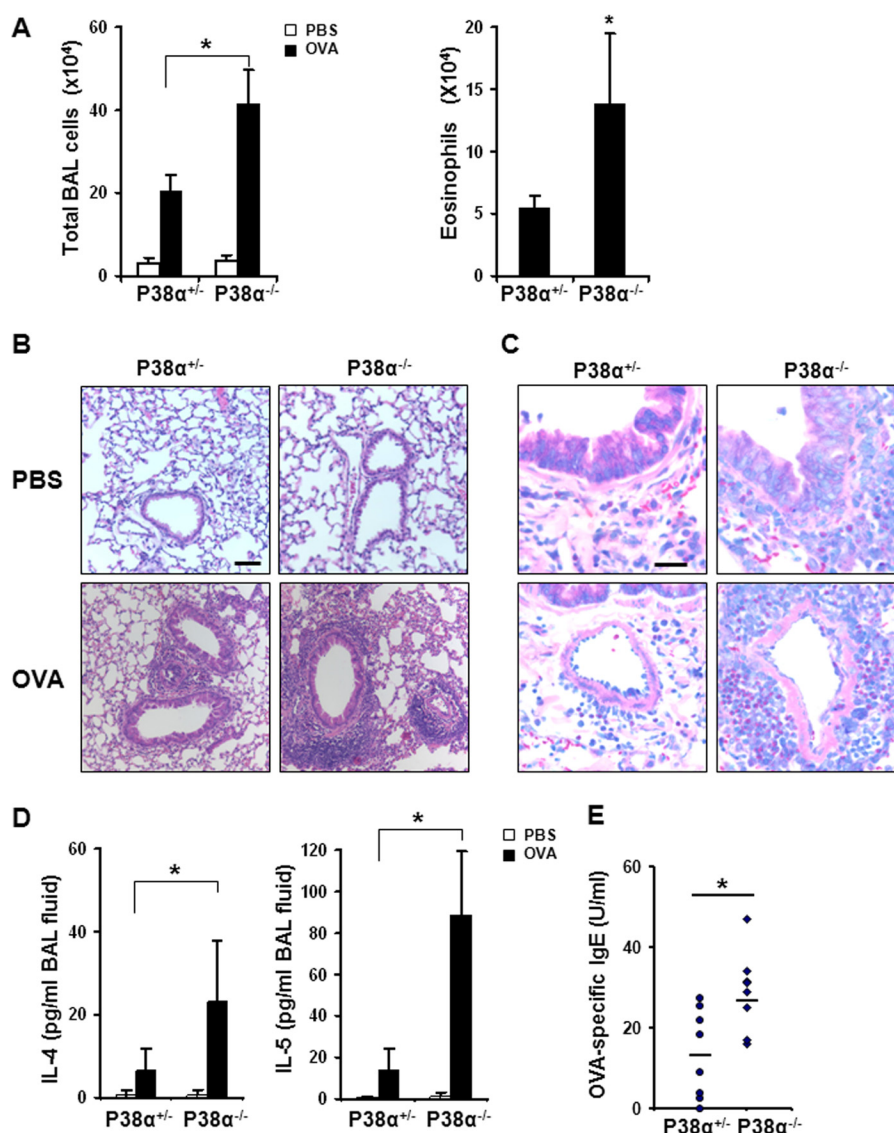


FIGURE 7. p38 $\alpha^{-/-}$ mice display enhanced allergic airway inflammation. *A*, total BAL fluid cells from OVA-sensitized/challenged p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice and number of eosinophils in BAL fluid were counted. *B*, lung histopathology of OVA-sensitized mice after the final challenge is shown. Lung sections stained with hematoxylin and eosin show increased perivascular and peribronchial infiltration of leukocytes in p38 $\alpha^{-/-}$ mice. Scale bar, 50 μ m. *C*, lung histopathology demonstrates perivascular and peribronchial infiltration of eosinophils by Giemsa staining. Scale bar, 12.5 μ m. *D*, concentration of IL-4 and IL-5 in BAL fluid from OVA-sensitized/challenged p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice was detected by ELISA. *E*, serum OVA-specific IgE concentration in OVA-sensitized/challenged p38 $\alpha^{+/-}$ and p38 $\alpha^{-/-}$ mice was measured by ELISA. The number of mice per group in panels *A*–*E* was between four and eight. *, $p < 0.05$, data in *A* and *D* are mean $\pm$ S.D.; horizontal bars in *E* denote the mean.

of p38 α inhibits the production of IFN- γ and Th1 differentiation. As a downstream target of Akt, phosphorylation of Foxo3a was also reduced in p38 $\alpha^{-/-}$ T cells relative to control T cells (Fig. 6A), suggesting that the absence of p38 α induces higher Foxo3a activity in activated T helper cells. In contrast to Akt, inactivation of p38 α significantly enhanced TCR-induced activation of ERK (Fig. 6B). In addition, we also observed reduced basal levels of I κ B α protein in p38 $\alpha^{-/-}$ T cells (Fig. 6C), suggesting a higher basal IKK α / β activity in p38 α -deficient T cells. However, after antigen stimulation the degradation of I κ B α in control T cells was comparable to p38 $\alpha^{-/-}$ T cells, indicating that p38 α is dispensable for the PKC- θ /NF- κ B pathway in T helper cells (Fig. 6C), which is consistent with our p65 nuclear translocation findings observed in Fig. 5D. IL-2/Stat5 is required for optimal IL-4 production during T cell activation

(21). To explore whether p38 α modulates IL-2/Stat5 activation, T cells were subjected to IL-2 stimulation, and the level of tyrosine phosphorylation of Stat5 was assessed. Stat5 phosphorylation was similar in p38 $\alpha^{-/-}$ T cells and control T cells (Fig. 6D). These results suggest that the absence of p38 α affects downstream output of TCR but not IL-2 signaling.

Disruption of p38 α Enhances the Susceptibility to Acute Allergic Airway Inflammation—To further assess the effects of p38 α deletion on Th2 responses *in vivo*, OVA-induced airway allergic inflammation, which is mediated by Th2 immunity, was studied. We immunized mice intraperitoneally with OVA and the adjuvant aluminum hydroxide. After the final OVA challenge, we found that in p38 $\alpha^{-/-}$ mice, the total number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid were almost 2.0-fold higher than control counterparts, and the

number of eosinophils in the BAL fluid was similarly increased (Fig. 7A). Lung histology showed higher numbers of inflammatory cells around blood vessels and airways of p38 α ^{-/-} mice relative to controls (Fig. 7B). Giemsa staining showed less eosinophils around airways and the blood vessels of control mice compared with p38 α ^{-/-} mice (Fig. 7C). Th2 cytokines IL-4 and IL-5 in the BAL fluid from p38 α ^{-/-} mice were higher compared with control mice based on ELISA analysis (Fig. 7D). Consistent with this result, deletion of p38 α significantly elevated the OVA-specific IgE level in the serum (Fig. 7E). Thus, inactivation of p38 α imposes hyper-responsiveness of Th2 immunity during acute allergic airway inflammation.

DISCUSSION

p38 is a master regulator of critical inflammatory mediators, including TNF- α , IL-1, and COX2, and activation of p38 is believed to play an essential role in the pathogenesis of diseases such as rheumatoid arthritis and Crohn disease. However, its role in other types of inflammatory responses such as allergic inflammation is unclear. Here, we provide evidence to demonstrate that p38 α negatively regulates Th2 immunity. p38 α deficiency elevates TCR-induced IL-4 expression and promotes T helper cell differentiation into Th2 phenotype. During T helper cell activation, mRNA and protein levels of p38 α are decreased. p38 α ^{-/-} mice display more severe allergic airway inflammation relative to control mice. The absence of p38 α alters the homeostasis of NFAT transcription factors and affects pathways downstream from TCR. Interfering with p38 α may present a potential therapeutic strategy for Th2 immunity-mediated diseases.

Based on our results, we propose a model for p38 α in the regulation of NFAT transcription factors, IL-4 production, and Th2 differentiation in response to T cell activation. In control T helper cells, the presence of p38 α maintains NFAT (NFATc1 and NFATc2) homeostasis. Upon TCR stimulation, down-regulation of p38 α shifts the balance toward NFATc1 from NFATc2, and increased expression of NFATc1 promotes IL-4 production and Th2 differentiation. However, in p38 α ^{-/-} T helper cells, the absence of p38 α not only alters NFAT homeostasis under steady-state conditions, but once activated, these cells respond more rapidly, as they were already “primed.” After TCR stimulation, the absence of p38 α inhibits Akt activation and enhances ERK activation, which may result in decreased Th1 and increased Th2 differentiation (20, 39) but may not affect IL-4/Stat6 and IL-2/Stat5 pathways. These data suggest that p38 α utilizes multiple TCR-associated signals to influence Th2 commitment.

Prior studies involving TCR-mediated p38 activation have primarily focused on its phosphorylation by upstream kinases such as MKK3/6 as well as alternative pathways (7, 8). Here, we provide evidence to suggest that during T helper cell differentiation, expression of p38 α is compromised. This may represent a novel mechanism to modulate p38 α MAP kinase signaling. In T helper cells, this form of regulation is not restricted to the p38 pathway. Previous studies have shown that during T cell activation, TCR also induces JNK expression (41). Although it is known that p38 α mediates stress response and regulates apoptosis in many cell types including in CD8 T cells (42), in CD4 T

cells the PKC- θ /NF- κ B pathway is a more important regulator of apoptosis. It is curious that T helper cells restrain prolonged activation of p38 during T cell activation. Although it is not clear how exactly this is achieved; temporospatial regulation of p38 α could explain the diverse functions p38 α is able to mediate in distinct cell types. We propose that during T cell differentiation, T helper cells utilize at least two mechanisms to regulate the p38 α pathway. If antigen exposure is temporary and short-lived, TCR activates p38 α through phosphorylation. In contrast, if antigen persists, prolonged process of differentiation inhibits the expression of p38 α . Thus, the duration of antigen presence may regulate T helper cell functions through differential regulation of the p38 α pathway, and p38 α may play an important role in balancing Th1 and Th2 response.

Recently, studies utilizing mice deficient in the expression of MK2 have shown increased OVA-induced airway inflammation (43). In a skin inflammation model, conditional knock-out of p38 α in myeloid cells also enhances inflammatory response (44). Hyper-inflammatory phenotype is also observed in mice lacking both MSK1 and MSK2 (45). Thus, the divergence of pro-inflammatory and anti-inflammatory signaling in the p38 pathway highlights the importance to selectively target the inflammatory processes.

Because Mx-cre can disrupt p38 α in hematopoietic cells, other immune cells in addition to T cells could contribute to the *in vivo* phenotype of allergic airway inflammation. Of note, we did not observe the phenotypic difference of B cell development between control and p38 α ^{-/-} mice. And a previous report has shown that B cells are not required for OVA-induced allergic airway inflammation (46). There is evidence that mast cells do not play an essential role in OVA-induced acute airway allergic inflammation (47). The amount of blood eosinophils in p38 α ^{-/-} mice is comparable to control mice.³ Most of our *in vitro* experiments are conducted in purified CD4⁺ T cells, and the results are consistent with the altered Th2 response *in vivo*. Thus, we believe that enhanced allergic airway inflammation in p38 α ^{-/-} mice is due to altered T cell function due to p38 α deficiency in T cells.

Th2 immunity regulates both anti-parasitic and allergic responses; inhibition of p38 may enhance expulsion of intestinal parasites. However, in the case of allergic inflammation, inhibition of p38 may accelerate disease pathogenesis, and therefore, inhibition of p38 should be considered cautiously. Taken together, it will be helpful to further identify downstream regulators of p38 during modulation of Th2 immunity. These regulators may represent attractive targets for therapeutic intervention in asthma and other allergic inflammatory diseases.

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