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IL-2 and IL-4 Stimulate MEK1 Expression and Contribute to T Cell Resistance against Suppression by TGF-β and IL-10 in Asthma

Qiaoling Liang,1 Lei Guo,1 Shaila Gogate,1 Zunayet Karim, Arezoo Hanifi, Donald Y. Leung, Magdalena M. Gorska, and Rafeul Alam

The T cell-driven airway inflammation in chronic asthma is uninhibited and sustained. We examined the resistance of T cells from asthmatic patients against suppression by TGF-β, IL-10 and glucocorticoids and explored its signaling mechanism. CD4⁺CD25² T cells from allergic asthmatic subjects demonstrated increased TCR-stimulated proliferation as compared with healthy and chronic obstructive pulmonary disease controls. This proliferation was resistant to inhibition by TGF-β, IL-10, and dexamethasone and to anergy induction. CD4 T cells from asthmatic patients, but not chronic obstructive pulmonary disease, allergic rhinitis, and healthy subjects, showed increased expression of MEK1, heightened phosphorylation of ERK1/2, and increased levels of c-Fos. IL-2 and IL-4 stimulated the expression of MEK1 and c-Fos and induced T cell resistance. The inhibition of MEK1 reversed, whereas induced expression of c-Fos and JunB promoted T cell resistance against TGF-β and IL-10-mediated suppression. We have uncovered an IL-2- and IL-4-driven MEK1 induction mechanism that results in heightened ERK1/2 activation in asthmatic T cells and make them resistant to certain inhibitory mechanisms. The Journal of Immunology, 2010, 185: 5704–5713.

Asthma is characterized by the persistence of Th2-type inflammation leading to airway hyperresponsiveness, increased mucus production, and airway remodeling (1, 2). T cells play an essential role in this process. T cell activation is tightly controlled by a variety of processes including activation-induced cell death, induction of tolerance by dendritic cells and cytokines, T cell anergy, and active suppression by regulatory T cells (Tregs) (3). Tregs are effective in turning off inflammatory processes in autoimmune (4) and allergic diseases in animal models (5–7). These studies led to the hypothesis that allergic diseases result from failure of Tregs to inhibit T cells (8, 9).

Studies of natural Tregs in human asthma are inconclusive. The number of CD4⁺CD25² T cells have shown that these cells are unable to suppress either T effector cell proliferation and/or Th2 cytokine production, especially when studied in the allergy season (17, 18). These studies lead to difficulties in interpreting human natural Treg data. The interpretation of the function of natural Tregs is further complicated by the fact that the assay involves three different cell types: CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells and irradiated APCs. An abnormal outcome from this assay may result from the dysfunction of any or all of the three cell types.

T cell function is also regulated by certain inhibitory cytokines such as TGF-β and IL-10. Both TGF-β and IL-10 are produced by a variety of nonlymphoid and lymphoid cell types including inducible Tregs (19, 20). Both cytokines inhibit experimental asthma in the mouse model. The level of IL-10 increases in patients after allergen immunotherapy. In this study, we asked if the T cell response to TGF-β, IL-10, and glucocorticoids is altered in allergic asthma. Anergy is a self-regulatory mechanism that ensures that TCR stimulation in the absence of costimulation does not lead to T cell proliferation and an immune response (21). We examined anergy induction in T cells from allergic asthmatic patients. We also studied the signaling mechanism of abnormal T cell function in asthma. We show that T cells from allergic asthmatic patients express increased levels of MEK1, which drives ERK1/2 activation. ERK1/2 induces c-Fos and JunB, which confer resistance to T cells against suppression by TGF-β and IL-10 and against anergy induction.

Materials and Methods

Human subjects

The protocol for human blood draw and T cell signaling studies was approved by the Institutional Review Board of National Jewish Health (Denver, CO). Patients were recruited from the Asthma and Allergy Clinic of National Jewish Health. Allergic asthma was diagnosed if a study subject had a positive skin test to environmental allergens and met the National Asthma Education Expert panel criteria for diagnosis of asthma (12%) reversibility in forced expiratory volume in the first second [FEV₁] or a positive methacholine test) with and without the presence of allergic rhinitis. Allergic rhinitis was diagnosed if a study subject had a positive

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Abbreviations used in this paper: ATS, American Thoracic Society; COPD, chronic obstructive pulmonary disease; DUSP, dual-specific phosphatase; FEV₁, forced expiratory volume in the first second; HSD, honestly significant difference; MFI, mean fluorescence intensity; MKP, MAPK phosphatase; NA, not applicable; PC₂₀, concentration of methacholine inducing a 20% drop in airflow; RV, retroviral vector; Treg, regulatory T cell.

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skin test to environmental allergens, had symptoms of rhinitis (seasonal or perennial) with or without conjunctivitis but no lower respiratory symptoms, and no airflow limitation by spirometry. Severe asthma was defined according to the American Thoracic Society (ATS) major criteria of either continuous or near-continuous oral glucocorticoid or high-dose inhaled glucocorticoid or both to achieve a level of mild to moderate persistent asthma and by the presence of two or more minor criteria of asthma control (22). Chronic obstructive pulmonary disease (COPD) was diagnosed based upon the Global Initiative for Chronic Obstructive Lung Disease criteria. Healthy control subjects were skin test negative and did not have any acute or chronic health problems. Demographic, clinical, and laboratory data about the three study populations (asthma, COPD, and healthy controls) are shown in Table I. Blood was drawn from patients and healthy subjects upon written consent. Blood was anticoagulated with EDTA.

Source of the Abs

Rabbit anti-ERK1/2, mouse monoclonal anti–p-ERK1/2 (clone E4), mouse monoclonal anti-MEK1 (clone H8), rabbit anti-MAPK phosphatase 1 (MKP1), rabbit anti-MAPK phosphatase 3 (MKP3), rabbit anti-p-Akt were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal anti-MEK1/2 (clone 47E6) and mouse monoclonal anti–p-ERK (clone E10) were from Cell Signaling Technology (Danvers, MA). PE-labeled mouse monoclonal anti-CD45RA and CD45RO were from eBioscience (San Diego, CA). PE-labeled mouse monoclonal anti–FOXP3 was from BD Biosciences (San Jose, CA). CCRT Ab was from R&D Systems (Minneapolis, MN).

Blood processing and isolation of CD4 T cell subpopulation

PBMCs were prepared by centrifugation over Ficoll-Hypaque (Histopaque) gradients. Untouched CD4 T cells or CD4+CD25+ Tregs were isolated by CD4 T cell isolation kit-II or CD4+CD25+ regulatory isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instruction.

Flow cytometry

Cells were fixed with 2% paraformaldehyde and followed by incubation in 90% methanol. Following washing, they were treated with 10% goat serum to block IgG receptors. After washing, primary Ab against the target signaling molecule was added at 1:500 to 1:1000 dilution according to the supplier’s recommendation and incubated for 30 min. Following further washing, cells were incubated with an FITC- or allophycocyanin-labeled secondary Ab against the primary Ab (1:1000) in 5% goat serum for another 30 min, washed, and analyzed by flow cytometry. Most of the flow cytometric experiments were performed using CYAN (Beckman-Coulter, Fullerton, CA) unless otherwise stated. Data were analyzed by the software FlowJo (Tree Star, Ashland, OR). The increased expression of p-ERK1/2 in CD4 T cells was confirmed using separated CD4+CD25+ T cells from asthmatic patients (not shown). We examined the number of CD4+CD25+ T cells that were FOXP3 positive (not shown). The number of CD4+CD25+ T cells was 7 ± 0.8 and 11 ± 3.3% (p = 0.2) in healthy controls and allergic asthmatic patients, respectively.

Results

CD4+CD25+ T cells from allergic asthmatic patients are hyperproliferative

We examined the proliferative function of CD4 T cells in 20 patients with allergic asthma and compared them with that from 20 healthy subjects and 10 patients with COPD. The patient characteristics are shown in Table I. Asthmatic and COPD subjects were allowed to continue their routine medications, which included inhaled β-agonists and inhaled steroids. CD4 T cells represent a functionally heterogeneous population and contain various Treg subpopulations including CD4+CD25+ natural Tregs. The latter cell type is known to inhibit the proliferation of CD4+CD25+ T cells. For this reason, we performed the proliferation experiments with separated CD4+CD25+ T cells. CD4+CD25+ T cells from asthmatic patients showed basally increased proliferation without any stimulation (Fig. 1A). Stimulation with anti-CD3 and anti-CD28 Abs induced proliferation of CD4+CD25+ T cells from all study groups. However, CD4+CD25+ T cells from asthmatic patients proliferated at a significantly higher rate. We also studied CD4+CD25+ T cell proliferation by flow cytometric analysis of CFSE dilution on a limited number of patients. The result showed a similar increase in CD4 T cells proliferation from allergic asthmatic patients (not shown). We examined the number of CD4+CD25+ T cells using the Miltenyi purification kit (Miltenyi Biotec). Flow cytometry showed that 88% of these CD4+CD25+ T cells were FOXP3 positive (not shown). The number of CD4+CD25+ T cells was 7 ± 0.8 and 11 ± 3.3% (p = 0.2) in healthy controls and allergic asthmatic patients, respectively.

CD4 T cells from asthmatic patients are partially resistant to TGF-β1-, IL-10-, and glucocorticoid-mediated suppression and anergy induction

Next, we examined the response of CD4+CD25+ T cells from allergic asthmatic patients to the inhibitory effect of IL-10 and TGF-β1. Both cytokines induced a dose-dependent inhibition of T cell proliferation in both asthmatic and control subjects (Fig. 1B, 1C). However, this inhibition was significantly lower in asthmatic patients. Glucocorticoids are endogenous immunoregulatory hormones and also therapeutic agents. They inhibit T cell proliferation. We asked if the glucocorticoid inhibition of T cell proliferation is altered in asthmatic subjects. For these experiments, we used the total CD4 population. Dexamethasone inhibited CD4 T cell proliferation in both study populations (Fig. 1D). The IC50 for dexamethasone in asthmatic patients was in the range of 10 nM, which is similar to what has previously been reported (26).
The IC50 was ~2 orders of magnitude lower in healthy controls, indicating a relative resistance in asthmatic patients. In addition to immunoregulatory cytokines and endogenous hormones, the induction of anergy plays an important role in downregulating an immune response. According to a classic anergy induction protocol (27), preincubation with ionomycin led to reduced CD4 T cell proliferation in healthy controls when stimulated with anti-CD3 and anti-CD28 Abs (Fig. 1E). This proliferation was unaffected in T cells from patients with allergic asthma. As a matter of fact, we have observed a trend toward higher proliferation. The results in aggregate suggest that CD4+ T cells from asthmatic subjects are partially resistant to the inhibitory effect of TGF-β, IL-10, and glucocorticoids and to anergy induction.

The MAPK family members play an important role in T cell proliferation (28). We determined the contribution of ERK1/2, JNK, and p38 signaling pathways to CD4 T cell proliferation

### Table I. Demographic and clinical characteristics of study subjects

<table>
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<th>Age (y)</th>
<th>Mean Range</th>
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<td>12–60</td>
<td>5</td>
<td>0–9</td>
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<tr>
<td>PC20 for methacholine (mg/ml)</td>
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<td>&lt;0.31–8</td>
<td>NA</td>
<td>NA</td>
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<td>Daily topical glucocorticoid dose (inhaled for asthma and COPD, nasal for rhinitis) (µg)</td>
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<td>0–1000</td>
<td>418</td>
<td>250–1000</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>No. on systemic steroids</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>270</td>
<td>40–458</td>
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<tr>
<td>Total IgE (kU/l)</td>
<td>424</td>
<td>13–1865</td>
<td>79</td>
<td>12–209</td>
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</table>

NA, not applicable; PC20, concentration of methacholine inducing a 20% drop in airflow.

The MAPK family members play an important role in T cell proliferation (28). We determined the contribution of ERK1/2, JNK, and p38 signaling pathways to CD4 T cell proliferation.
using pharmacological inhibitors. The MEK1/2 inhibitor U0126 but not the JNK inhibitor SP600125 and the p38 inhibitor SB202190 blocked T cell proliferation in response to anti-CD3/CD28 stimulation (Fig. 2A). SP600125 and SB202190 were active in these experiments, as they inhibited the phosphorylation of c-Jun and activating transcription factor (not shown) as described previously (29). Our results point to an important role of ERK1/2 in human T cell proliferation.

Next, we studied ERK1/2 signaling in CD4 T cells from patients with allergic asthma, healthy controls, and COPD. Fig. 2B shows a representative flow cytogram for p-ERK1/2 by CD4 T cells from an asthmatic patient and a healthy control. Fig. 2C shows a scatter plot of (geometric) mean fluorescence intensity (MFI) of p-ERK1/2 from individual study subjects. p-ERK1/2 was significantly increased in the asthmatic group as compared with the healthy and COPD control groups. p-ERK1/2 was also increased in the COPD

**FIGURE 2.** Expression of p-ERK1/2 in CD4 T cells. A, Effect of MAPK inhibitor on T cell proliferation. CD4 T cells from healthy donors were stimulated with anti-CD3/CD28 Abs in the presence of the MEK1/2 inhibitor U0126, the p38 inhibitor SB202190, and the JNK inhibitor SP600125, and [3H]Thymidine uptake was measured at 72 h. *p = 0.04; **p = 0.03 compared with DMSO. B, Blood CD4 T cells were stained with mouse mAb against the phosphorylated (activating phosphorylation) form of ERK1/2 (p-ERK1/2) or mouse IgG1 isotype control, followed by an Alexa 488-labeled anti-mouse secondary Ab. One sample of CD4 T cells was stimulated with PMA prestaining (positive control). A representative flow cytogram is shown. y-axis, Cell counts at each level of fluorescence intensity; x-axis, Fluorescence intensity. C, p-ERK1/2 immunostaining of purified CD4 T cells as described above from 20 allergic asthmatic patients, 10 healthy, and 10 COPD controls. Each symbol represents a single study subject, and the same color indicates paired samples. Data are presented as geometric MFI. p < 0.0001 (Kruskal-Wallis ANOVA); *p < 0.01 asthma versus other groups, and COPD versus allergy and healthy (Tukey HSD test). D, Western blotting of CD4 T cells for p-ERK1/2 following stimulation of the cells with anti-CD3/CD28 (n = 3). The membranes were reprobed for ERK1/2 as a loading control. Density of the bands was measured by the National Institutes of Health Image J software (Bethesda, MD). E, Immunofluorescent staining of CD4 T cells from an allergic asthmatic patient and healthy control for basal expression of p-ERK1/2 (green) and nuclear counterstaining with DAPI (blue) (n = 3). Original magnification ×100. F, Correlation between p-ERK1/2 and FEV1. G, Effect of signaling inhibitors on TGF-β–induced inhibition of T cell proliferation. CD4+ T cells were stimulated with anti-CD3/CD28 in the presence or absence of TGF-β. U0126 or SP600125 was added to the indicated cultures at 5 μM concentration. [3H]Thymidine uptake during the last 16 h of culture was measured (n = 5). p values (paired t test) for the difference are as follows: 1, 0.02; 2, 0.01 (both were compared with the TGF-β+ and inhibitor− samples); 3, 0.01 compared with the TGF-β+ and MEK inhibitor− samples; 4, 0.1 compared with the TGF-β+ sample and 0.01 compared with the MEK inhibitor− sample.
group as compared with the healthy group. We measured total ERK1/2 in asthmatic, COPD, and healthy control subjects (n = 8). The ERK1/2 level was $519 \pm 92, 476 \pm 102$, and $458 \pm 89$ (MFI) in the asthma, COPD, and healthy groups, respectively, and the differences were not significant. The ratio of p-ERK1/2 to total ERK1/2 under basal conditions in these study subjects was $10.6 \pm 2.1, 4.1 \pm 1.3$, and $4.0 \pm 1.1\%$, respectively. We were concerned that in vitro processing might alter p-ERK1/2 expression. To address this concern, we examined CD4 T cell expression of p-ERK1/2 in WBCs (buffy coat) isolated by heta-starch sedimentation in mononuclear cells isolated by Histopaque gradient centrifugation and in purified CD4 T cells negatively isolated by magnetic beads. There was no difference in the p-ERK1/2 expression level in CD4 cells isolated by these three methods (not shown). We confirmed the increased expression of p-ERK1/2 by Western blotting (Fig. 2D) and immunofluorescent staining (Fig. 2E). On Western blotting, the anti-CD3/C28 Ab stimulation caused increased phosphorylation of ERK1/2 at early time points, which persisted longer in the cell sample from the asthmatic patient as compared with the healthy control (Fig. 2D, compare the upper band of p-ERK1 at 30 and 60 min). The basal ERK1/2 expression level was similar in the asthmatic patient and healthy control. Immunofluorescence for p-ERK1/2 was mainly observed in T cells from asthmatic patients. The immunostaining was primarily cytosolic with modest nuclear localization. There was a modest increase in basal p-ERK1/2 in cells from the asthmatic patient. The p-ERK1/2 expression level (MFI from the flow cytometric studies) negatively correlated ($r = -0.77, p = 0.001$) with the basal airflow measure FEV1 in asthmatic patients (Fig. 2F).

Next, we examined the role of the ERK1/2 signaling pathway in T cell resistance against TGF-β in asthma. We chose TGF-β and not IL-10 for these experiments because the former induced a higher level of inhibition of T cell proliferation (compare the inhibition level in Fig. 1B and 1C). Because ERK1/2 is partially activated in asthmatic subjects under basal conditions, we reasoned that a partial inhibition of ERK1/2 would eliminate the basal difference in the activation state of T cells between asthma and normal subjects and allow TGF-β to exert its maximal inhibitory effect. To this end, we cultured CD4⁺CD25⁻ T cells in the presence of a suboptimal concentration (5 μM) of U0126 and SP600125 (as a control). This suboptimal concentration was chosen based upon the dose-response curve of inhibition of T cell proliferation, as shown in Fig. 2A. As expected, we observed reduced inhibition of T cell proliferation with U0126 (Fig. 2G) as compared with Fig. 2A. At this level of MEK1/2 inhibition, TGF-β was far more effective and inhibited T cell proliferation.

**FIGURE 3.** Expression of MAPK regulators in T cells. A–C, Flow cytometric analyses of the expression of MKP1, MKP3, and MEK1. Purified CD4 T cells from the indicated study groups were stained for MKP1, MKP3, and MEK1 with Alexa 488-labeled Abs. A, n = 9, 6, and 6 for asthma, healthy, and COPD, respectively. B, n = 11, 9, and 6 for asthma, healthy, and COPD, respectively. C, n = 29, 22, and 10 for asthma, healthy, and COPD, respectively. Each symbol represents a single study subject. p = 0.0001 (Kruskal-Wallis ANOVA). *p < 0.01 asthma versus healthy and COPD (Tukey HSD test). D, Western blotting for MEK1 expression. Purified CD4 T cells from five randomly selected allergic asthmatic (A1–5) patients and four healthy normal (N1–4) controls were Western blotted for MEK1. The membrane was reprobed for actin to demonstrate protein loading. E, Densitometric analysis of MEK1 expression in the Western blot membrane from D. *p = 0.001 (Mann–Whitney U test). F, Correlation between the T cell MEK1 level and FEV1 in asthmatic patients (Spearman’s rank correlation test). G, Comparison of MEK1 and p-ERK1/2 expression among severe and mild asthmatic patients (ATS criteria, see Ref. 22) and allergic rhinitis patients without asthma. p < 0.0001 Kruskal-Wallis ANOVA; *p < 0.05 compared with the corresponding data from the allergic rhinitis group (Tukey-Kramer test); **p < 0.05 compared with the corresponding data from the mild asthma and allergic rhinitis groups.
Increased expression of MEK1 in asthma

The phosphorylation of the MAPK is regulated by dual-specific (30) as well as nonspecific phosphatases. We examined two dual-specific phosphatases (DUSPs) that dephosphorylate ERK1/2 as well as other MAPKs. The expression level of MKP1 (DUSP1) and MKP3 (DUSP6) was similar (Fig. 3A, 3B) in asthmatic patients and control groups (COPD and healthy subjects). Next, we examined the expression of MEK1, the upstream activator of ERK1/2. Interestingly, the expression level of MEK1 was significantly increased in allergic asthmatic patients as compared with the healthy and COPD groups (Fig. 3C). The increased MEK1 expression was further confirmed by Western blotting of CD4 T cells from asthmatic patients and healthy controls (Fig. 3D, 3E). We asked if MEK1 expression correlated with the clinical severity of asthma. The increased expression of MEK1 in T cells negatively correlated \( r = -0.64, p = 0.02 \) with the airflow measurement FEV1 in asthmatic patients (Fig. 3F). Because decreased FEV1 is associated with severe asthma, we classified asthmatic patients into mild and severe asthma according to the ATS criteria (22), which incorporate not only FEV1 but also symptoms and medications. We also examined a group of allergic rhinitis patients without asthma (no lower respiratory symptoms and normal spirometry). There was a significant difference (multigroup comparison) in MEK1 and p-ERK1/2 expression among these three groups: severe asthma, mild asthma, and allergic rhinitis (Fig. 3G). There was also a significant difference (two group comparison) between severe and mild asthma and mild asthma and allergic rhinitis. Increasing severity of asthma is manifested by the need for higher doses of inhaled and/or systemic steroids (22). So, we examined the correlation between dexamethasone inhibition of T cell proliferation and MEK1 expression. There was a low level but statistically significant negative correlation between these two parameters (Fig. 4A). MEK1 drives ERK1/2 activation and T cell proliferation. Accordingly, we observed a strong correlation between MEK1 and pERK1/2 in T cells \( (r = 0.827, p = 0.0001) \). We also observed a good correlation between MEK1 and CD4 T cell proliferation (Fig. 4B).

IL-2 and IL-4 induce T cell resistance against TGF-β- and IL-10-mediated suppression

To delineate the mechanism of T cell resistance against TGF-β and IL-10 in asthma, we examined the effect of select cytokines. Previous mouse studies have indicated a role for IL-2, IL-6, and TNF-α in T cell resistance in autoimmune animal models (31, 32). Further, a role for IL-4 and IL-9 has been demonstrated using human Th2 clones (33). However, there are no studies with primary human T cells in asthma. We examined the effect of IL-2, IL-4, IL-6, IL-9, and IL-13 on T cell proliferation in the presence of TGF-β or IL-10. T cells do not express IL-13R, so this cytokine served as a negative control. IL-2 and IL-4 but not IL-6, IL-9, or IL-13, rendered protection from TGF-β- and IL-10-mediated suppression of T cell proliferation (Fig. 4C).

TCR, IL-2, and IL-4 stimulation induces MEK1

The induction of T cell resistance by IL-2 and IL-4 prompted us to investigate its mechanism. We asked if these cytokines could increase the expression of MEK1 in T cells. To this goal, we stimulated separately CD4 T cells and mononuclear cells with the cytokines and without stimulation with anti-CD3 and anti-CD28 Abs. The stimulated CD4 T cell culture supernatant was measured by ELISA.

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**FIGURE 4.** A, Correlation between T cell MEK1 expression and dexamethasone inhibition of T cell proliferation. B, Correlation between MEK1 expression and CD4 T cell proliferation in response to anti-CD3/CD28 Abs. The \( r \) and \( p \) values were analyzed by the Spearman’s rank correlation test. C, Role of cytokines in T cell resistance against cytokines. CD4+ T cells from healthy controls were cultured without (control, open white bars) or with IL-10 or TGF-β in the absence (No IL) or presence of IL-2, IL-4, IL-6, IL-9, and IL-13 (10 ng/ml). All samples were stimulated with anti-CD3/CD28 Abs and [3H]thymidine uptake was measured at 72 h \( (n = 5) \). *p < 0.01. D, Role of cytokines in MEK1 expression. Mononuclear cells from asthmatic subjects were cultured alone (medium) or with IL-2, IL4, IL-6, IL-12, IL-25 (all at 10 ng/ml), and anti-CD3/CD28 Abs (5 μg/ml) for 48 h. MEK1 expression was assessed by flow cytometry following double staining for CD4 and MEK1. \*p < 0.01 \( (n = 6) \), paired \( t \) test. E and F, The production of IL-2 and IL-4 by CD4 T cells. E, Intracellular IL-4+ CD4 T cells were measured by flow cytometry after stimulation with and without CD3/CD28 Abs. F, IL-2 production in the stimulated CD4 T cell culture supernatant was measured by ELISA.

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in asthmatic patients at a level that was comparable to that observed in normal subjects. The JNK inhibitor had no effects. The result from the culture of the cells with the vehicle control (DMSO) was similar to that with the JNK inhibitor (not shown).
CD28 Abs. The expression of MEK1 and ERK1/2 in CD4 T cells was examined by flow cytometry. Direct stimulation of CD4 T cells with the cytokines or with anti-CD3/28 did not have any effects on MEK1 expression (not shown). In contrast, stimulation of mononuclear cells with IL-2, IL-4, and anti-CD3/CD28 but not with IL-6, IL-12, and IL-25 significantly induced MEK1. IL-2, IL-4, and anti-CD3/CD28 induced a 1.9-, 1.5-, and 2.2-fold increase in MEK1 expression, respectively, in CD4 T cells (Fig. 4D). Anti-CD3/CD28 stimulation had a modest effect (1.2-fold increase) on ERK1/2 expression, and the cytokine stimulation had no effects. We also examined the combined effect of anti-CD3/CD28 and IL-2 or IL-4. The anti-CD3/CD28–induced MEK1 expression was further augmented by IL-2 and IL-4 (Fig. 5). The differences between anti-CD3/CD28 and cytokine stimulation were statistically significant ($p = 0.005$ for IL-2; $p = 0.01$ for IL-4). MEK1 induction by IL-2, IL-4, and anti-CD3/CD28 in mononuclear cell cultures but not in CD4 T cell cultures suggests that CD4 T cells require additional costimulation (secreted or membrane expressed) from non-T cells. Because anti-CD3/CD28 stimulation induces MEK1 expression, we asked if the expression of CD3ε on CD4 T cells was different in asthmatic patients. The MFI of CD3ε was $2995 \pm 383$ and $3094 \pm 308$ in healthy controls ($n = 7$) and asthmatic patients ($n = 6$), respectively, and the difference was not statistically significant.

Next, we examined basal and stimulated expression of IL-2 and IL-4 in CD4 T cells by flow cytometry. A very low number of IL-4–producing cells was detectable in both healthy controls and asthmatic patients, and their number was increased after anti-CD3/CD28 stimulation. The number of IL-4+ CD4 T cells at the baseline and poststimulation was higher in asthmatic patients than in healthy controls (Fig. 4E). CD4 T cells from both healthy controls and asthmatic patients showed intracellular IL-2 under basal conditions by flow cytometry. However, we are unable to detect IL-2 in the culture supernatant (48 h culture) under basal conditions by ELISA. IL-2 was readily detectable in the culture supernatant upon stimulation with anti-CD3/CD28 Abs. Stimulated

**FIGURE 5.** MEK1 induction by cytokine and TCR stimulation. Mononuclear cells from an asthmatic subject were cultured alone (medium) or stimulated with anti-CD3 and CD28 Abs with or without IL-2 or IL-4 (10 ng/ml) for 48 h. MEK1 expression was assessed by double staining for CD4 and MEK1. A, Isotype control Ab staining of mononuclear cells. Cultured mononuclear cells were stained with mouse IgG1 (control for p-ERK1/2) and rabbit IgG (control for MEK1). Note that anti-CD3/CD28 stimulation (right panel) shifted the cell population slightly to the right likely due to the increased cell size. The gate was adjusted in all anti-CD3/CD28–treated samples (B) to reflect this change. B, MEK1 expression in CD4 T cells upon culture with medium, anti-CD3/CD28, anti-CD3/CD28 + IL-2, and anti-CD3/CD28 + IL-4. The number in the quadrant represents percent of the total cells. The bottom number in the upper right quadrant represents geometric mean of fluorescence intensity.
IL-2 production was significantly higher in asthmatic patients than in healthy controls (Fig. 4F).

The MEK1/ERK-regulated c-Fos expression is increased in asthma

The immediate early genes of the AP-1 complex play an essential role in T cell proliferation by inducing many genes, including IL-2 (34). Next, we studied the role of the MEK1/ERK pathway in the induction of select AP-1 complex proteins. The inhibition of MEK1 by U0126 blocked the expression of c-Fos and JunB but not c-Jun or JunD (Fig. 6A), suggesting that the foregoing two molecules could potentially contribute to T cell resistance. We also studied the effect of IL-4 on the expression of JunB and c-Fos. The expression of both c-Fos and JunB was augmented by IL-4 (Fig. 6B). To further confirm the relationship between the MEK1/ERK pathway and JunB induction, we studied the co-expression of p-ERK1/2 and JunB in CD4 T cells. We observed JunB expression only in those CD4 T cells that simultaneously expressed p-ERK1/2 (Fig. 6C, 6D).

c-Fos and JunB promote T cell resistance

Because the MEK1/ERK pathway regulates expression of c-Fos and JunB, we asked if their expression was increased in T cells from asthma. Flow cytometric analysis and Western blotting showed increased expression of c-Fos in CD4 T cells from asthma (Fig. 7A, 7B). We were unable to detect any increase in basal JunB expression in CD4 T cells by Western blot. Next, we asked if transient overexpression of c-Fos and JunB would make T cells resistant to IL-10– and TGF-β. We transiently overexpressed both genes using a GFP-expressing bicistronic RV. The GFP-expressing cells were sorted and checked for increased expression of the genes by Western blotting (Fig. 7C, 7D). Note that the level of overexpression of JunB is largely similar to that observed under physiological conditions poststimulation of cells with anti-CD3/28 (Fig. 6B). We also compared anti-CD3/CD28 stimulated proliferation of normal noninfected T cells and GFP-RV–infected T cells to ensure that the retroviral infection-related T cell processing does not alter T cell proliferation. The proliferation was comparable (25,868 ± 1813 cpm in normal T cells versus 27,678 ± 2499 cpm in GFP-RV–treated cells). The overexpression of c-Fos and JunB genes in normal T cells caused increased proliferation upon stimulation with anti-CD3/CD28 Abs (Fig. 7E). This treatment also made normal T cells resistant to IL-10– (Fig. 7F) and TGF-β–mediated suppression (Fig. 7G).

Discussion

We have shown that T cells from allergic asthmatic patients are partially resistant to TGF-β–, IL-10–, and glucocorticoid-mediated inhibition of proliferation. Infected and sorted CD4 T cells from healthy donors (as described above) were cultured with anti-CD3/CD28 Abs, and [3H]thymidine uptake was measured at 72 h (Fig. 6F). The proliferation was comparable (25,868 ± 1813 cpm in normal T cells versus 27,678 ± 2499 cpm in GFP-RV–treated cells). The overexpression of c-Fos and JunB genes in normal T cells caused increased proliferation upon stimulation with anti-CD3/CD28 Abs (Fig. 7E). This treatment also made normal T cells resistant to IL-10– (Fig. 7F) and TGF-β–mediated suppression (Fig. 7G).

FIGURE 6. A, Effect of MEK1/2 inhibition on select AP-1 transcription factors. CD4 T cells from a healthy control were cultured with anti-CD3/CD28 in the presence of U0126 or vehicle (DMSO) and then Western blotted for the expression of c-Jun, JunB, and c-Fos at 4 h and JunD at 24 h. The JunB membrane was reprobed for tubulin to demonstrate protein loading (n = 3). B, Effect of IL-4 on c-Fos and JunB expression. CD4 T cells from an asthmatic donor was stimulated with an anti-CD3 Ab, IL-4, or in combination for 4 h and then Western blotted for JunB or c-Fos (n = 3). The JunB membrane was reprobed for tubulin. C, Coexpression of p-ERK1/2 and JunB. CD4 T cells from an asthmatic donor were stimulated with an anti-CD3 Ab, IL-4, and then processed for double immunostaining using a mouse monoclonal anti-p-ERK1/2 and rabbit anti-JunB Ab. Species- and isotype-specific secondary Abs were labeled with Alexa 549 (red) and Alexa 488 (green), respectively. Nuclei were stained blue with DAPI (n = 3). D, Control immunostaining with rabbit IgG. Isotype control immunostaining for the mouse monoclonal anti-p-ERK1/2 Ab is shown in Fig. 2E. C and D, Original magnification ×100.

FIGURE 7. Effect of c-Fos and JunB overexpression. A and B, Expression of c-Fos in T cells from asthma. Purified CD4 T cells from allergic asthmatic patients and control subjects (n = 6) were stained for c-Fos and analyzed by flow cytometry (A). Each symbol represents a single donor. Cell lysates from select donors were Western blotted for c-Fos (B) (n = 3). C and D, Overexpression of c-Fos and JunB. CD4 T cells from a healthy donor were infected with a GFP (RV), GFP-cFos (Fos), or GFP-JunB (JunB)-expressing bicistronic RV. The GFP-expressing cells were sorted and Western blotted for expression of c-Fos (C) and JunB (D). JunB-expressing cells were also stimulated with anti-CD3 and anti-CD28 Abs to determine the effect of stimulation. The membrane was reprobed for actin to demonstrate protein loading (n = 3). Effect of c-Fos and JunB overexpression on proliferation (E) and IL-10– (F) and TGF-β–mediated (G) inhibition of proliferation. Infected and sorted CD4 T cells from healthy donors (as described above) were cultured with anti-CD3/28 Abs, and [3H]thymidine uptake was measured at 72 h (E). *p < 0.02 (paired t test); n = 3. F, Infected and sorted CD4 T cells were cultured as per the IL-10–mediated suppression protocol. *p = 0.04 (t test); n = 3. G, Infected and sorted CD4 T cells were cultured as per the TGF-β–mediated suppression assay protocol (n = 3). *p = 0.01 (t test); n = 4.
Steroid resistance has been studied well in the past (reviewed in Ref. 40). Many asthmatic patients, especially those with severe asthma, manifest steroid insensitivity, requiring higher doses of the medication (22). Steroid resistance can be induced in vitro with microbial superantigens, and this form of resistance is mediated by ERK1/2 (41). An important role for MEK–ERK but not STAT5 signaling in steroid resistance of T cells was independently reported by another group (42). Our results are in agreement with both studies. We further show that the ERK1/2-regulated transcription factors c-Fos and JunB contribute to the T cell resistance against suppression by the inhibitory cytokines TGF-β and IL-10.

One of the important discoveries of this paper is the increased MEK1 level in CD4 T cells from asthmatic patients. Patients with allergic rhinitis did not express high levels of MEK1 or p-ERK1/2. This suggests that the Th2 phenotype alone does not lead to increased MEK1. Allergen stimulation is unlikely to be a contributor, as the frequency of allergen-specific T cells is very low:

\[ \text{ERK1/2} \overset{\text{activation}}{\rightarrow} \text{MAPKs} \]

We identified IL-2 and IL-4 as two critical cytokines that confer MEK1 and p-ERK1/2 expression cannot be ruled out. Thus, our results could be an underestimation and should be interpreted with caution. It would be important to conduct a study with mild asthmatic and allergic rhinitis patients who do not take topical steroids or other controller medications daily and compare their MEK1 level. The increase in MEK1 is biologically significant, as T cells from asthmatic patients show higher levels of c-Fos expression. The expression of c-Fos has previously been shown to be increased in cells conditionally overexpressing MEK1 (43).

We identified IL-2 and IL-4 as two critical cytokines that confer resistance to T cells, in part, by inducing MEK1 expression. IL-4 has previously been shown to increase MEK1 expression in mast cells (44). The effect of IL-2 on MEK1 induction has not been previously studied. IL-2 is considered a Th1 cytokine. Interestingly, inhibition of IL-2 induces asthma-like symptoms in humans (45) and aggravates airway inflammation in a mouse model of asthma (46). IL-2 along with IL-4 induces steroid resistance in T cells (47). IL-2 and IL-4 also reverse the tolerance phenotype of T cells from allergic subjects who underwent specific immunotherapy (48). Finally, bioinformatics approaches of microarray data from allergic subjects have identified IL-2 and IL-4 as two crucial atopic network hubs that coordinate the induction of other atopy-related genes (49, 50). A clinical validation of the importance of IL-2 comes from a recent study in which daclizumab, an anti–IL-2R Ab, improved lung function and disease symptoms in human asthma (51).

One of the immediate actions of ERK1/2 is the transcription of AP-1 complex proteins. We show that c-Fos and JunB are specifically induced by ERK1/2. AP-1 is formed through hetero or homodimerization of proteins belonging to three families: Fos, Jun, and activating transcription factor. FOXP3 and AP-1 occupy the same DNA region on the il2 promoter and interact with overlapping, but not identical, residues of NFAT (52). As a result, AP-1 antagonizes FOXP3. JunB is important for Th2 differentiation (53). In its absence, the differentiation of Th2 is impaired.

The MEK1–ERK1/2 signaling pathway is one of the most critical signaling pathways that regulate growth, proliferation, survival, movement, and secretory processes of cells (28). Because inflammation is characterized by all of the foregoing processes, ERK1/2 has the potential to play an important role in asthma. The ERK1/2-regulated transcription factors ESE-2 and -3 have been identified as asthma susceptibility genes (54, 55). Inhibition of MEK1 blocks airway inflammation in the mouse model (56).

Our data suggest that this pathway contributes to T cell resistance against homeostatic inhibitory mechanisms. We know that a Th2 response to allergens alone is not sufficient to induce chronic asthma because many allergic rhinitis patients do not have asthma. There must be an additional biochemical process that allows inflammation to persist and induce chronic asthma. We believe that the development of T cells resistance in asthma but not in allergic rhinitis represents this additional process, and it plays an important role in establishing chronic inflammation in asthma.

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Disclosures

The authors have no financial conflicts of interest.

References


