(Fig 5, *B and C*) and airway epithelial cells, but epithelial cells showed very weak immunoreactivity (Fig 5, *D*). This is in agreement with the recent finding[32] that airway epithelial cells of naive animals exhibited little immunoreactivity for amphiregulin, whereas staining of MCs in the peritracheal connective tissues showed prominent amphiregulin immunoreactivity. After antigenic challenge of sensitized, chronically exposed mice, there was transiently increased expression of amphiregulin in the cytoplasm of epithelial cells, but no evidence of staining in other cells such as eosinophils. *In vitro*, the levels of amphiregulin produced by MCs were much higher than those produced by epithelial cells.[30] These results indicate that MCs store amphiregulin in their cytoplasm, and IgE-mediated activation of MCs directly induces mucus production in the human airway.

Antiamphiregulin mAb partially but significantly inhibited mucin gene expression induced by MC supernatants. This may be a result of contributions of other molecules that induce mucin gene expression. Exposure of NCI-H292 cells to TNF-α[33] or IL-1β increased MUC2 expression. In our limited investigation of the expression of the EGF family by human MCs by using GeneChip, MCs expressed epiregulin after cross-linking of FcεRI. Ectodomain shedding of EGFR ligands and EGFR phosphorylation by metalloproteinases such as ADAM17 are implicated in mucin production in airway epithelial cells.[12,34,35] MCs upregulated the expression of TNF-α, IL-1β, and ADAM17 after aggregation of FcεRI (data not shown). These data further support our hypothesis that IgE-mediated MC activation induces mucin production by epithelial cells.

The effect of glucocorticoids on *MUC5AC* expression in human epithelial cells *in vitro* is controversial. Dexamethasone was reported to attenuate steady-state mRNA levels of *MUC5AC*,[36] whereas it was reported to upregulate *MUC5AC* expression.[37] Thus, we examined the effect of 10^{-6} mol/L and 10^{-7} mol/L dexamethasone on steady-state mRNA for *MUC5AC* in NCI-H292 cells by using real-time PCR. We found that these concentrations of dexamethasone did not have a significant effect on steady-state mRNA in *MUC5AC* (data not shown). Furthermore, IL-13–induced MUC5AC overexpression and goblet cell hyperplasia are resistant to glucocorticoid.[38] Treatment of patients with asthma with glucocorticoids has not been significantly effective in relation to overproduction of sputum. Because mucus hypersecretion is an important cause of morbidity and mortality in patients with asthma and no specific treatments are available, further clinical targets and therapeutic strategies are urgently needed. Elucidation of the molecular mechanisms of goblet cell hyperplasia induced by human MC amphiregulin should provide new targets for novel therapeutic interventions.

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ASTHMA

Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma

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Background: The balance between matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) may be critical in extracellular matrix remodelling, a characteristic of asthmatic airways. An excess of TIMP-1 over MMP-9 has been associated with chronic airflow obstruction but the mechanisms underlying this association remain unknown. Recent computed tomographic (CT) studies indicate that airway wall thickening is associated with chronic airflow obstruction.
Methods: Sputum levels of MMP-9, TIMP-1, and their molar ratio were examined in 26 patients with stable asthma and their relationship with pulmonary function and airway wall thickness, assessed by a validated CT technique which measured wall area corrected by body surface area (WA/BSA), the ratio of WA to outer wall area (WA%), and the absolute wall thickness corrected by √BSA of a segmental bronchus (T/√BSA), was examined.
Results: Sputum MMP-9 levels were inversely correlated with WA% and TIMP-1 levels were positively correlated with WA/BSA and T/√BSA. The MMP-9/TIMP-1 molar ratio was inversely correlated with WA% and T/√BSA and positively correlated with post-bronchodilator values of mid-forced expiratory flow and maximum expiratory flow at the quartile of lung volume.
Conclusion: Excess TIMP-1 may have a pathogenetic role in airway wall thickening in asthmatic patients which may result in chronic airflow obstruction.

Inflammation of the airway is a characteristic feature of asthma. Pathological repair of chronic inflammation may result in airway remodelling characterised by hypertrophy and hyperplasia of airway smooth muscle, submucosal gland hyperplasia, goblet cell hyperplasia, and vascular proliferation. Structural changes also include deposition of extracellular matrix (ECM), such as collagen and fibronectin, in the subepithelial basement membrane (SBM) or in the submucosa, around or within airway smooth muscle bundles, and possibly in the adventitia.[1 2]

Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent enzymes, are responsible for the degradation of ECM.[3] Among MMPs, MMP-9 is overexpressed in the serum and/or diseased organs of patients with several disorders characterised by tissue destruction, such as rheumatoid arthritis and pulmonary emphysema.[4 5] MMP-9 is considered to be involved in the pathophysiology of these disorders, presumably by degrading ECM. In patients with asthma, MMP-9 is increased in sputum or bronchoalveolar lavage (BAL) fluid under stable conditions,[6 7] during spontaneous exacerbations,[8] and after local allergen challenge.[9] MMP-9 has also been suggested to enhance the migration of inflammatory cells into the airways[10] through ECM destruction.[11]

As a counterbalance, tissue inhibitor of metalloproteinase-1 (TIMP-1) inhibits the enzymatic activity of MMP-9 by stoichiometric 1:1 binding.[12] TIMP-1 is considered to have fibrogenic properties resulting from inhibition of MMP-9 and promotion of cell growth of fibroblasts or myofibroblasts.[13] In several fibrotic diseases, such as progressive systemic sclerosis[14] and idiopathic pulmonary fibrosis,[15] TIMP-1 is overexpressed and considered to accelerate fibrosis and ECM deposition. In the sputum or BAL fluid of patients with stable asthma, TIMP-1 is increased compared with healthy controls and is overproduced relative to MMP-9.[6 16] Additionally, an excess of TIMP-1 over MMP-9 in the sputum or serum of asthmatic patients has been associated with chronic airflow obstruction.[6 17]

Necroscopic studies indicate that airway wall thickening is involved in the pathophysiology of asthma.[18] We have established a technique of helical computed tomography (CT) to quantify airway wall thickness which has shown that the airways of asthmatic patients are thickened in vivo.[19 20] The degree of thickening may relate to the severity of disease and airflow obstruction.[19–22] Chronic airflow obstruction associated with overproduction of TIMP-1 might therefore theoretically result from deposition of ECM and consequent thickening of the airway wall.[6 17] We therefore investigated the relation of sputum levels of MMP-9 and TIMP-1 and their molar ratio with airway wall thickness as assessed by CT in patients with stable asthma.

METHODS

Subjects

Twenty six patients with stable asthma from our outpatient clinic were studied. Asthma was diagnosed according to the American Thoracic Society criteria. At the time of diagnosis, airway hyperresponsiveness was proven in 15 patients by a continuous inhalation methacholine challenge;[23] the mean (range) Dmin, a marker of airway sensitivity, in these 15

Abbreviations: Ao, outer area of the bronchus; BSA, body surface area; ECM, extracellular matrix; $FEF_{25-75\%}$, mid forced expiratory flow; FEV_1, forced expiratory volume in 1 second; MMP-9, matrix metalloproteinase-9; SBM, subepithelial basement membrane; T, absolute airway thickness; TIMP-1, tissue inhibitor of metalloproteinase-1; WA, airway wall area

patients was 1.96 (0.01–9.53) units. Reversibility of airflow obstruction to 200 µg salbutamol (12% and 200 ml increase in forced expiratory volume in 1 second (FEV_1)) was demonstrated in six patients (mean (SD) pre/post-bronchodilator FEV_1 2.36 (0.85) l/2.81 (1.02) l), and the remaining five patients had peak expiratory flow (PEF) variability of more than 20%. Patients were considered to have stable asthma if the disease had been fully controlled for at least 1 month.[7] Twenty of the 26 patients had never smoked; the remaining six were ex-smokers who had ceased smoking at least 1 year before the study and had smoked a maximum of 10 pack-years. Emphysema was ruled out by CT images in all subjects including the six ex-smokers.

The study was approved by the ethics committee at our institution and written informed consent was obtained from all participants.

Sputum induction and processing

Sputum induction and processing were performed according to the methods of Pin,[24] with slight modification.[25] Briefly, the subjects were premedicated with inhaled salbutamol (200 µg) and then inhaled hypertonic (3%) saline solution from an ultrasonic nebuliser (MU-32, Azwell Inc, Osaka, Japan) for 15 minutes. Adequate plugs of sputum were separated from saliva and first treated with 0.1% dithiothreitol (Sputasol, Oxoid Ltd, Hampshire, UK) followed by Dulbecco's phosphate buffered saline (PBS). After centrifugation, supernatants of sputum were stored at −80°C. Cell differentials were determined by counting at least 400 non-squamous cells stained by the May-Grünwald-Giemsa method.

Zymography

Zymography on SDS-gelatin was used to determine gelatinase activity[3] in sputum according to a method previously described.[7 25] Each supernatant obtained from sputum was diluted (1:3) with PBS and 30 µl of the sample was subjected to electrophoresis on 11% polyacrylamide gels containing 1 mg/ml gelatin in the presence of sodium dodecyl sulfate (SDS-PAGE) under non-reducing conditions. After electrophoresis, gels were washed in Triton X100 for 1 hour, rinsed briefly, and incubated at 37°C for 24 hours in buffer containing 100 mM TRIS HCl pH 7.40 and 10 mM $CaCl_2$. After incubation the gels were stained with Coomassie Brilliant Blue R250 and then destained in a solution of 7.5% acetic acid with 5% methanol. Zones of enzymatic activity were indicated by negative staining: areas of proteolysis appeared as clear bands against a blue background.

Measurement of MMP-9 and TIMP-1

The absolute values of MMP-9 and TIMP-1 levels in sputum were measured using enzyme immunoassay kits (Fuji Chemical Industries Ltd, Toyama, Japan).[25] Assays were performed following the manufacturer's instructions. The MMP-9 assay detects pro (92 kDa) and intermediate forms of MMP-9 with 83 kDa as well as their complexed forms with TIMP-1. The TIMP-1 assay detects free or complexed forms of TIMP-1. This kit does not crossreact with TIMP-2. The detection limits were 3.1 ng/ml for MMP-9 and 1.2 ng/ml for TIMP-1.

CT scans and analysis of airway wall thickness

CT scanning and analysis was performed as reported previously.[19 26] Briefly, using a Toshiba X-Vigor CT scanner (Toshiba, Tokyo, Japan), helical CT scanning was performed at 120 kVp, 50 mA, 3 mm collimation, and pitch 1. Images were reconstructed using the FC 10 algorithm at 2 mm spacings. A targeted reconstruction of the right lung was performed using a subject-specific field of view (FOV) (153–214 mm). These CT data were transferred to a Power PC personal computer via magneto-optical disk and analysed using custom software written in C programming language (Symantec C++, Symantec Corp, CA, USA). For the airway analysis, a cross section of the apical bronchus of the right upper lobe at its origin was selected by a consensus reading of two pulmonologists (HM and AN). The following airway parameters were then measured automatically on the computer: luminal area, short and long radii of the lumen, and absolute airway thickness (T). Outer area of the bronchus (Ao), airway wall area (WA) and percentage wall area (WA%, WA/Ao × 100) were calculated, under the assumptions that the airway was a true circle in the cross sectional plane and that T was constant throughout the wall. Because airway size may be affected by body size, WA and T were normalised using body surface area (BSA).[19 26] Airway wall thickness was estimated as WA/BSA, WA%, and $T/\sqrt{BSA}$. A detailed description of these measures has been published previously.[26]

Pulmonary function

Using a Chestac-65V (Chest MI Corp, Tokyo, Japan), we measured FEV_1, mid forced expiratory flow ($FEF_{25-75\%}$), and maximum expiratory flow at the quartile of FVC ($MEF_{25\%}$) 15 minutes after inhalation of 200 µg salbutamol via metered dose inhaler to assess the degree of chronic airflow obstruction.

Statistical analysis

Stat View software (SAS Institute Inc, Cary, NC, USA) was used. The results are expressed as mean (SD). Relationships between data were analysed using Spearman's rank correlation test. p values of <0.05 were considered significant.

Table 1 Characteristics and outcomes of study patients (n=26)

Age (years)	53.8 (16.3)
Sex (F/M)	11/15
Disease duration (years)	8.6 (8.3)
Disease severity (steps 1:2:3:4)*	1:9:13:3
Atopic status (yes/no)†	16/10
FEV_1 (%pred)‡	95.8 (17.2)
FEV_1 (%pred)§	102.1 (17.8)
FEV_1/FVC (%)§	78.3 (9.7)
$FEF_{25-75\%}$ (%pred)§	73.2 (30.7)
$MEF_{25\%}$ (%pred)§	54.8 (27.2)
Inhaled steroid (µg/day)¶	816 (270)
WA/BSA (mm^2/m^2)	17.9 (3.8)
WA% (%)	66.7 (5.6)
$T/\sqrt{BSA}$ (mm/m)	1.2 (0.1)
Sputum MMP-9 levels (ng/ml)	598.2 (388.6)
Sputum TIMP-1 levels (ng/ml)	855.0 (497.3)
Molar ratio of MMP-9/TIMP-1	0.22 (0.13)

$FEF_{25-75\%}$, mid-forced expiratory flow; $MEF_{25\%}$, maximum expiratory flow at the quartile of lung volume; WA/BSA, wall area/body surface area; WA%, 100 × WA/outer area of the bronchus; T, absolute airway thickness; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.
Values are given as mean (SD).
*Severity of the disease was determined based on the Global Initiative for Asthma.
†Patients were considered atopic when one or more specific IgE antibodies against cat dander, dog dander, weed, grass pollen, mold and house dust mite were positive.
‡Pre-bronchodilator and §post-bronchodilator values.
¶Data from 25 patients taking inhaled steroids (expressed in equivalent doses of beclomethasone dipropionate).

Table 2 Correlation coefficients (*r* values) between sputum markers and airway wall thickness or post-bronchodilator pulmonary function

	MMP-9 levels	TIMP-1 levels	Molar ratio of MMP-9/TIMP-1
WA/BSA (mm^2/m^2)	0.24	0.42*	−0.12
WA%	−0.47†	0.15	−0.64‡
T/√BSA (mm/m)	−0.15	0.41*	−0.54§
FEV_1 (%pred)	0.24	0.12	0.25
$FEF_{25-75\%}$ (%pred)	0.11	−0.26	0.41*
$MEF_{25\%}$ (%pred)	0.20	−0.21	0.42*

WA/BSA, wall area/body surface area; WA%, 100×WA/outer area of the bronchus; T, absolute airway thickness; $FEF_{25-75\%}$, mid-forced expiratory flow; $MEF_{25\%}$, maximum expiratory flow at the quartile of lung volume; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.
*p<0.05, †p<0.03, ‡p<0.003, §p<0.01.

RESULTS

The clinical characteristics and outcomes of the 26 patients are shown in table 1. One patient regarded as of step 1 severity was treated only with a short acting inhaled β agonist as required. Others were treated with inhaled corticosteroids for at least 3 months before the study. Two of the 26 patients were examined for MMP-9 or TIMP-1 levels alone because the sputum samples of these patients were too small to analyse both. As reported previously,[6 25] zymographic analysis of sputum samples from the patients showed a major band of enzymatic activity at 92 kDa which corresponds to pro-MMP-9 (data not shown).

Table 2 shows correlation coefficients between the sputum indices and the measures of airway wall thickness or airflow obstruction. Sputum MMP-9 levels were inversely correlated with WA% and TIMP-1 levels were positively correlated with WA/BSA and T/√BSA. The MMP-9/TIMP-1 molar ratio was inversely correlated with WA% (fig 1) and T/√BSA and positively correlated with post-bronchodilator values of $FEF_{25-75\%}$ and $MEF_{25\%}$ but not with FEV_1 (table 2). No significant correlation was observed between disease duration or severity and the sputum indices (data not shown).

The MMP-9 levels were significantly correlated with the absolute number of sputum neutrophils ($r = 0.47$, $p = 0.036$) and marginally with that of macrophages ($r = 0.43$, $p = 0.058$). The TIMP-1 levels tended to correlate with the absolute number of neutrophils ($r = 0.38$, $p = 0.099$). No significant correlation was found between sputum eosinophil number and either MMP-9 or TIMP-1 levels (data not shown).

DISCUSSION

This is the first study to evaluate the relationship between airway wall thickness (assessed by CT scanning) and sputum levels of MMP-9, TIMP-1, and their molar ratio in asthmatic patients. We have shown that an absolute increase in TIMP-1 or its relative excess over MMP-9 in the asthmatic airway may be associated with airway wall thickening, as well as with chronic airflow obstruction.

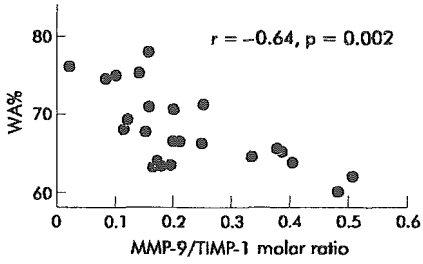

Figure 1 Correlation between WA% (100 × wall area/outer area of the bronchus) and MMP-9/TIMP-1 molar ratio.

Vignola and colleagues have shown that the molar ratio between MMP-9 and TIMP-1 in the sputum of asthmatic patients is positively correlated with FEV_1. The patients had not received any form of corticosteroids during the 2 months before the study. The authors indicated that the excess of TIMP-1 may lead to airflow obstruction, possibly by a role in the pathogenesis of ECM remodelling.[6] Bossé and colleagues have evaluated the relationship between the responsiveness of FEV_1 to oral corticosteroid treatment (methylprednisolone 40 mg/day for 2 weeks) and the pretreatment serum molar ratio of MMP-9/TIMP-1 in severe asthmatic patients requiring high doses of inhaled corticosteroids.[17] The baseline serum MMP-9/TIMP-1 ratio strongly correlated with the degree of increase in FEV_1 after oral corticosteroid treatment. The authors concluded that the excess of TIMP-1 may lead to irreversible airflow obstruction, possibly through airway fibrosis. Our findings that the sputum MMP-9/TIMP-1 ratio correlated with $FEF_{25-75\%}$ and $MEF_{25\%}$ are consistent with those of Vignola *et al*[6] and Bossé *et al*,[17] both showing an association between the excess TIMP-1 and airflow obstruction. Unlike these studies, we used post-bronchodilator values of pulmonary function which are more indicative of "irreversible" functional changes. We also used sputum samples which provide a more direct measure of airway pathophysiology than serum samples as used by Bossé *et al*.[17] The reason why the molar ratio was only associated with $FEF_{25-75\%}$ and $MEF_{25\%}$ but not with FEV_1 in our study may be that a fixed airflow obstruction was limited to the peripheral airways only because our patients may have had milder disease than those in the previous studies.[6 17] Correlations between the sputum indices and peripheral airflow obstruction are relevant because induced sputum is considered to originate not only from the central but also from the small airways and alveoli.[27]

We have shown, for the first time to our knowledge, an association between excess TIMP-1 and whole airway wall thickening. Specifically, the sputum TIMP-1 levels were positively correlated with—and the molar ratio of MMP-9/TIMP-1 inversely correlated with—airway wall thickness as assessed by CT scanning. Although CT scanning cannot identify the pathological details that may contribute to airway wall thickening, ECM deposition in the airways through excess TIMP-1 probably contributed to the observed thickening. A recent study of a murine model of asthma employing chronic ovalbumin challenge shows that the TIMP-1 level in BAL fluid is increased after 12 weeks of repeated antigen challenge and that this increase is sustained during the following 20 weeks with the same treatment. In parallel, excessive collagen deposition in the lung emerges at 16 weeks of treatment and persists thereafter. The authors

deduce a possible association between the increase in TIMP-1 and ECM deposition in their model.[28] In the airways of patients with asthma, ECM deposition is seen not only in the SBM but also around smooth muscle cells and possibly in the adventitial layer,[2] which may gather volume and contribute to the whole airway wall thickening. In fact, the degree of SBM thickening correlates with the degree of airway wall thickening as assessed histologically[29] and by CT scanning.[22] TIMP-1 excess may therefore be associated with ECM deposition and consequent whole airway wall thickening. The latter may be related to chronic airway obstruction as suggested by previous CT studies.[19–22]

In our study, sputum MMP-9 levels correlated significantly with the number of neutrophils and marginally with the number of macrophages. Our results are consistent with those of previous human[6 10] and murine studies.[28] These cells might therefore be potential sources of MMP-9 in the asthmatic airways, although eosinophils are also considered as candidate cells by other investigators.[30]

Uncontrolled airway inflammation or uncontrolled disease status may potentially affect airway wall thickness. All 26 patients in our study had experienced full symptom control for at least 1 month, and all but one had been treated with inhaled corticosteroids for at least 3 months. We selected these patients for our study to reduce the effect of inflammation as much as possible in order to better address the "irreversible" component of airway wall thickening using CT.[21 22] Although the levels of sputum indices might or might not have been modified by corticosteroid treatment,[31–33] the association we found between the sputum indices and pulmonary function is consistent with those of a previous study[6] conducted in patients not treated with corticosteroids.

The three measures of airway wall thickness used in our study are distinct from each other. WA/BSA may not be affected by bronchoconstriction, unlike WA% or T/√BSA; WA% is a corrected index of airway wall thickness by airway size, whereas T/√BSA indicates an absolute thickness of the wall.[34]

One possible limitation of our study is that sputum levels of MMP-9 and TIMP-1 might not reflect the actual behaviour of the enzymes within bronchial tissue. However, both mucosal expression[35] and sputum levels[6] of MMP-9 as well as TIMP-1 are consistently increased in patients with asthma compared with healthy controls. Increases in TIMP-1 relative to MMP-9 in induced sputum[33] and increased expression of TIMP-1 in bronchial tissue[31] are consistently observed following systemic or inhaled corticosteroid treatment. It is therefore reasonable to assume that sputum levels of MMP-9 and TIMP-1 may indirectly reflect their levels in the airway mucosa.

In conclusion, the data from the present study suggest the involvement of excess TIMP-1 in the development of airway wall thickening and chronic airflow obstruction in asthmatic patients. The regulatory mechanisms responsible for the balance between MMP-9 and TIMP-1, and its possible pharmacological modulation, need to be addressed in future studies.

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The authors have no competing interests.

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LUNG ALERT

Absence of C/EBPα may cause bronchial smooth muscle proliferation in asthma

▲ Roth M, Johnson PRA, Borger P, *et al.* Dysfunctional interaction of C/EBPα and the glucocorticoid receptor in asthmatic bronchial smooth muscle cells. *N Engl J Med* 2004;**351**:560–74

Proliferation of bronchial smooth muscle (BSM) cells occurs in the airways of asthmatic patients. Glucocorticoids exert antiproliferative effects via CCAAT/enhancer binding protein α (C/EBPα), an antiproliferative transcription factor. This paper studies signalling pathways in BSM cell proliferation from 20 asthmatic and 26 non-asthmatic subjects (lung cancer/CF/emphysema).

A series of elegant experiments (using immunoblotting/electrophoresis) first established that, in response to glucocorticoid, asthmatic and non-asthmatic BSM cells display similar glucocorticoid receptor (GR) characteristics, similar anti-inflammatory effects (interleukin (IL)-6 suppression), and a similar reduction in IL-6 suppression with a GR inhibitor. However, whereas glucocorticoid significantly suppresses cell proliferation in control BSM cells (by 24–28%), no such suppression is seen in asthmatic BSM cells. None of the asthmatic cell lines expressed the C/EBPα transcription factor whereas all controls did (tissue specific to BSM). Transfection of asthmatic cells with a C/EBPα expression vector significantly slowed (and allowed steroids to suppress) proliferation.

The authors conclude that asthmatic BSM cell proliferation results from absence of C/EBPα, which also explains the lack of steroid suppression. Steroid treatment suppresses IL-6 production in both groups, therefore demonstrating two distinct signalling pathways: anti-inflammatory (cytokine mediated) and antiproliferative (transcription factor mediated).

Why asthmatic BSM cells lack C/EBPα remains unclear (previous steroid use and treatment length were often unknown, perhaps influencing in vitro response) and whether CF or emphysema subjects can be used as controls is debatable. The implication that steroids do not suppress BSM proliferation in asthma is clearly important and the demonstration of two distinct signalling pathways merits further investigation.

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Murine Plasmacytoid Dendritic Cells Produce IFN-γ upon IL-4 Stimulation[1]

Akira Suto, Hiroshi Nakajima,[2] Naoki Tokumasa, Hiroaki Takatori, Shin-ichiro Kagami, Kotaro Suzuki, and Itsuo Iwamoto

IL-4 plays a key role in inducing IL-4 production in $CD4^+$ T cells, functioning as an important determinant for Th2 cell differentiation. We show here that IL-4 induces IFN-γ production in $B220^+$ plasmacytoid dendritic cells (PDCs). By searching for cell populations that produce IFN-γ upon IL-4 stimulation, we found that PDCs were a major IFN-γ-producing cell upon IL-4 stimulation in wild-type and Rag-$2^{-/-}$ splenocytes. Isolated PDCs, but not $CD11b^+$ DCs or $CD8^+$ DCs, produced IFN-γ upon IL-4 stimulation. In vivo, the depletion of PDCs by anti-Ly6G/C Ab prevented IFN-γ production induced by IL-4 administration. We also found that IL-4 induced IFN-γ production, but not IL-12 or IFN-α production, in PDCs and also strongly enhanced CpG oligodeoxynucleotide-induced IFN-γ production, but not CpG oligodeoxynucleotide-induced IL-12 or IFN-α production. However, IL-4 did not induce IFN-γ production in Stat$6^{-/-}$ PDCs. Moreover, IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism, and only the Stat4-expressing PDCs produced IFN-γ. Furthermore, IL-4 did not induce IFN-γ production in Stat$4^{-/-}$ PDCs. These results indicate that PDCs preferentially produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. *The Journal of Immunology,* **2005, 175: 5681–5689.**

Cytokine environment is critical for the differentiation and commitment of immune cells. For example, IL-4, a representative Th2 cytokine, induces further Th2 cell differentiation, whereas a Th1 cytokine IFN-γ in coordination with IL-12 induces Th1 cell differentiation (1–3). Although these positive feedback mechanisms are essential for the profound differentiation of Th cells, the immune system also has a number of intrinsic and extrinsic machinery to antagonize the excessive differentiation of immune cells (4, 5).

Dendritic cells (DCs)[3] are a migratory group of bone marrow-derived leukocytes with at least three subtypes in mouse spleen: $CD8^+$ DCs, $CD11b^+$ DCs, and $B220^+$ DCs ("plasmacytoid DCs") (PDCs) (6, 7). Although $CD8^+$ DCs and $CD11b^+$ DCs express high levels of MHC class II molecules and costimulatory molecules such as CD80 and induce T cell proliferation, PDCs express MHC class II molecules at very low levels, do not express CD80, and fail to stimulate T cell proliferation (8, 9). These findings suggest that PDCs are immature DCs with a weak ability as APCs. On the other hand, PDCs localize in the T cell zone of lymphoid tissues and produce a large amount of type I IFNs upon bacterial or viral infection (10–12). Therefore, it is suggested that PDCs play a key role in innate immune responses.

Recently, a number of experiments have suggested that innate immune responses contribute significant polarizing influences on Th differentiation (13). The global view is that TLR activation of APCs such as DCs induces cytokine production that favors Th1-type immune responses and prevents the development of deleterious Th2 responses (13). On the other hand, a recent study has shown that PDCs inhibit Th2 responses even in the absence of TLR signaling (14). However, the role of PDCs in Th differentiation is still largely unknown.

In this study, by searching for cell populations that produce IFN-γ upon IL-4 stimulation, we found that PDCs were a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induced IFN-γ production in PDCs by a Stat6-dependent mechanism. We also found that IL-4 induced Stat4 expression in PDCs through a Stat6-dependent mechanism and that only the Stat4-expressing PDCs produced IFN-γ. Furthermore, we found that Stat4-deficient PDCs did not produce IFN-γ upon IL-4 stimulation. Our results highlight a unique function of IL-4-induced IFN-γ production in PDCs in the immune regulation of cytokine networks.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories. Stat6-deficient (Stat$6^{-/-}$) mice (15) and Rag-$2^{-/-}$ mice were backcrossed for more than eight generations onto BALB/c mice. Stat$4^{-/-}$ mice on a BALB/c background were purchased from The Jackson Laboratory. OVA-specific DO11.10 TCR transgenic (DO11.10^+) mice (16) were backcrossed over 10 generations onto BALB/c mice. All mice were housed in microisolator cages under specific pathogen-free conditions and all experiments were performed according to the guidelines of Chiba University.

Reagents

Mouse IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 were purchased from PeproTech. Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN) 1668 (TCCATGACGTTCCTGATGCT) was purchased from Hokkaido System Science.

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[3] Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; ODN, oligodeoxynucleotide; WT, wild type.

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Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4 FITC, PE (H129.19), anti-CD8 FITC, PE (53-6.7), anti-B220 FITC, PE, allophycocyanin, PerCP, biotin (RA3-6B2), anti-CD3 PE (145-2C11), anti-CD19 PE (ID3), anti-CD11b (Mac-1) PE (M1/70), anti-CD11c FITC (HL3), anti-Ly6G/C PE (RB6-8C5), anti-erythroid PE (TER-119), anti-pan NK PE (DX5), anti-CD80 PE (16-10A1), anti-CD86 PE (GL-1), and anti-I-A^d PE (AMS-32.1). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Isolation of DC subtypes

Splenic DCs were prepared using OptiPrep (Axis Shield) according to the manufacturer's instructions. In brief, spleens were cut into small fragments and then digested with collagenase A (0.5 mg/ml; Roche) for 10 min at 37°C with continuous agitation. Digested fragments were filtered through a stainless steel sieve, and cells were resuspended in 3 ml of HBSS and then mixed with 1 ml of OptiPrep to make 15% iodixanol solution (density 1.085 g/ml). Cell suspension was overlaid with 5 ml of 12% iodixanol solution (density 1.069 g/ml) and subsequently with 3 ml of HBSS. Low-density cells were collected by centrifugation at 600 × *g* for 15 min at room temperature. Low-density cells were stained with anti-CD11c FITC and FITC-stained cells were positively collected using anti-FITC microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The resultant cells were routinely >95% pure $CD11c^+$ cells by FACS analysis.

To isolate PDCs, low-density cells were prepared from wild-type (WT), $Stat6^{-/-}$ splenocytes, or $Stat4^{-/-}$ splenocytes and then stained with a mixture of PE-labeled Abs to CD3, CD19, CD11b, DX5, and TER-119. After PE-stained cells were depleted using anti-PE microbeads (Miltenyi Biotec), cells in flow-through were stained with anti-B220 biotin and subsequently $B220^+$ cells were positively collected using streptavidin microbeads (Miltenyi Biotec). Alternatively, PDCs were purified using a PDC isolation kit according to the manufacturer's instructions (Miltenyi Biotec). In both cases, the resultant cells were >95% pure $B220^+CD19^-$ cells by FACS analysis.

For $CD11b^+$ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs to CD3, B220, DX5, TER-119, and CD8. After PE-stained cells were depleted using anti-PE microbeads, cells in flow-through were stained with anti-CD11c FITC and $CD11c^+$ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure $CD11b^+CD8^-CD11c^+$ cells by FACS analysis.

For $CD8^+$ DCs purification, low-density cells were prepared from WT splenocytes and stained with a mixture of PE-labeled Abs against CD3, B220, DX5, TER-119, and CD11b and PE-stained cells were depleted using anti-PE microbeads. Cells in flow-through were stained with anti-CD11c FITC and $CD11c^+$ cells were positively collected using anti-FITC microbeads. The resultant cells were routinely >95% pure $CD11b^-CD8^+CD11c^+$ cells by FACS analysis.

Cell culture

Isolated PDCs, $CD11b^+$ DCs, or $CD8^+$ DCs were cultured (5×10^5/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics (complete RPMI 1640 medium) at 37°C for 72 h in the presence or in the absence of IL-4 (20 ng/ml). In some experiments, PDCs were stimulated with CpG ODN (10 μg/ml) for 48 or 72 h. PDCs were also stimulated with IL-2, IL-7, IL-9, IL-13, or IL-15 (20 ng/ml each) for 72 h to determine whether these cytokines induce IFN-γ production from PDCs. In other experiments, anti-IL-12 (p40/p70) Ab (10 μg/ml, clone C17.8; BD Pharmingen), anti-IL-2R β-chain Ab (10 μg/ml, clone TM-β1; BD Pharmingen), or anti-IL-18 Ab (5 μg/ml, clone 93-10C; MBL) was added to neutralize IL-12, IL-2 and IL-15, or IL-18, respectively. A mixture of anti-murine IFN-α Ab (20 μg/ml, clone 4EA1) (17), anti-murine IFN-β Ab (20 μg/ml, clone 7DF3) (17), and anti-type I IFN receptor antisera (10 μg/ml, R&D Systems) was used to neutralize type I IFNs.

ELISAs

The amounts of IFN-γ and IL-12 in the culture supernatant were measured by the enzyme immunoassay using murine IFN-γ and IL-12 (p70) ELISA kits from BD Pharmingen. The amounts of IFN-α in the culture supernatant were measured by an IFN-α ELISA kit from PBL. The assays were performed in duplicate according to the manufacturers' instruction. The minimum significant values of these assays were 31.3 pg/ml IFN-γ, 62.5 pg/ml IL-12, and 12.5 pg/ml IFN-α.

Intracellular staining for IFN-γ

Cells were stimulated with IL-4 (20 ng/ml) in the complete RPMI 1640 medium for the indicated periods (48 or 72 h). Monensin (2 μM; Sigma-Aldrich) was added for final 4 h to prevent cytokine release. After surface staining, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IFN-γ allophycocyanin (XMG1.2; BD Pharmingen) as described previously (18).

Intracellular Stat4 staining

Intracellular staining for Stat4 was performed as described elsewhere (19) with minor modifications. In brief, isolated PDCs from WT splenocytes or $Stat6^{-/-}$ splenocytes were cultured for 48 h in the presence or in the absence of IL-4 (20 ng/ml). Cells were harvested, washed with PBS, fixed with IC FIX, and permeabilized with 90% methanol and subsequently with IC PERM. Cells were then incubated with anti-Stat4 Ab (Zymed) or control rabbit IgG (Serotec) for 30 min at room temperature. After washing, cells were incubated with Alexa Fluor 647-conjugated anti-rabbit IgG Ab (Molecular Probes) and analyzed on a FACSCalibur. In the case of double

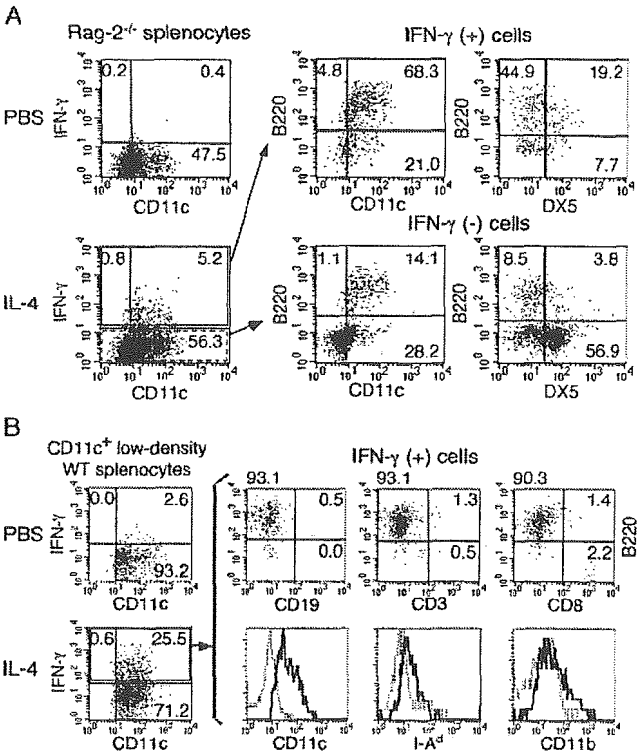

FIGURE 1. $B220^+$ PDCs produce IFN-γ upon IL-4 stimulation. *A*, Splenocytes from Rag-$2^{-/-}$ mice were cultured with or without IL-4 (20 ng/ml) for 3 days with monensin added for the final 4 h. After cells were stained with anti-B220 and either anti-CD11c or anti-DX5, intracellular staining for IFN-γ was performed. Representative FACS profiles of anti-CD11c vs anti-IFN-γ staining (*left panels*) and anti-CD11c vs anti-B220 or anti-DX5 vs anti-B220 staining gating on either $IFN\text{-}\gamma^+$ cells or $IFN\text{-}\gamma^-$ cells (*right panels*) are shown. *B*, $CD11c^+$ low-density splenocytes were isolated from WT mice as described in the *Materials and Methods*. Cells were then cultured with or without IL-4 for 3 days and analyzed for the expression of CD11c, B220, CD19, CD8, CD11b, Ly6G/C, and I-A^d together with the intracellular IFN-γ. Shown are representative FACS profiles of anti-CD11c vs anti-IFN-γ staining from four independent experiments (*left panels*). Representative FACS profiles of anti-CD19 vs anti-B220, anti-CD3 vs anti-B220, and anti-CD8 vs anti-B220 staining on $IFN\text{-}\gamma^+$ cells, as well as histograms for anti-Ly6G/C, anti-CD11b, and anti-I-A^d staining on $IFN\text{-}\gamma^+$ cells are shown in the *right panels*. Dashed lines indicate the staining with isotype-matched control Abs.

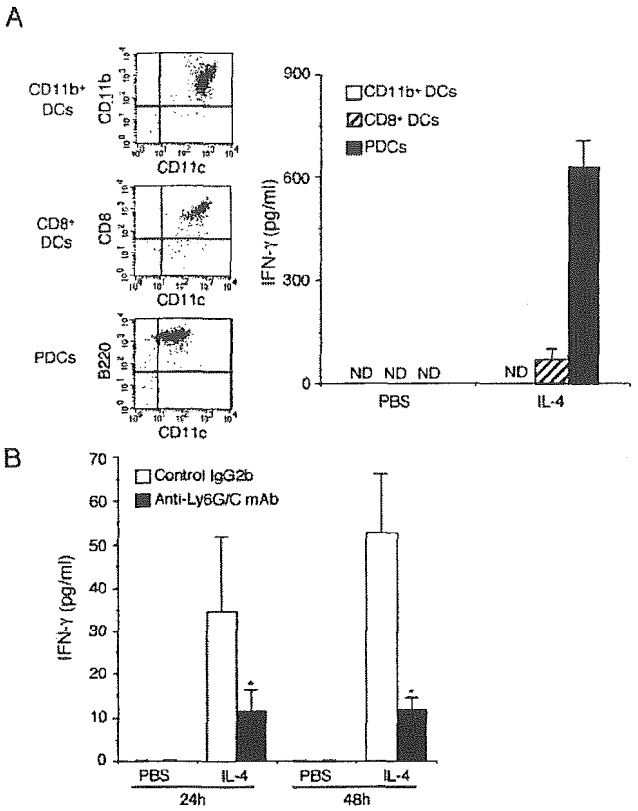

FIGURE 2. PDCs but not $CD11b^+$ or $CD8^+$ DCs produce IFN-γ upon IL-4 stimulation. *A*, PDCs, $CD11b^+$ DCs, and $CD8^+$ DCs were prepared from WT splenocytes as described in *Materials and Methods*. Each DC subtype was cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Representative FACS profiles of isolated each DC subtype are shown in the *left panels*. Data are means ± SD from four independent experiments. ND = not detectable. *B*, PDCs produce IFN-γ upon IL-4 stimulation in vivo. Rag-$2^{-/-}$ mice were injected i.p. with anti-Ly6G/C Ab (500 μg/mouse) or rat IgG2b (as a control). Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of mice. The levels of IFN-γ in the serum were determined by ELISA at 24 and 48 h after IL-4 injection. Data are means ± SD for four mice in each group. ND, not detectable. *, Significantly different from the mean value of the corresponding control response (control IgG2b); $p < 0.01$.

intracellular staining for Stat4 and IFN-γ, FITC-conjugated anti-rabbit IgG Ab (Zymed) was used as a second Ab.

RT-PCR analysis

Total cellular RNA was prepared and RT-PCR analysis was performed as described previously (20). The primer pairs for Stat4 were 5′-CTTGGGT GGACCAATCTGAA-3′ and 5′-TGGTCTTGAGACTTCGCACG-3′. The primer pairs for GATA3 and T-bet were described elsewhere (21). RT-PCR for β-actin was performed as a control. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs.

Taqman PCR analysis

The expression levels of Stat4 mRNA were determined by real-time PCR using a standard protocol on ABI PRISM 7000 instrument (Applied Biosystems). PCR primers and fluorogenic probes for Stat4, T-bet, and GATA3 were described previously (22). The levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (Applied Biosystems).

Effect of in vivo depletion of PDCs on IFN-γ production induced by IL-4 administration

To deplete PDCs in vivo, anti-Ly6G/C Ab (500 μg/mouse; BD Pharmingen) was injected i.p. to Rag-$2^{-/-}$ mice as described previously (12). As a control, purified rat IgG2b (BD Pharmingen) was injected to Rag-$2^{-/-}$ mice. In some experiments, 120G8 Ab (500 μg/mouse; a gift from Drs. G. Trinchieri and D. La Face, Schering-Plough Research Institute, Dardilly, France) (23) was injected to Rag-$2^{-/-}$ mice to deplete PDCs. Twenty-four hours later, rIL-4 (10 μg/mouse) or saline (as a control) was injected i.v. in the retro-orbital vein of the mice. The levels of IFN-γ in sera were determined by ELISA using a highly sensitive mouse IFN-γ ELISA kit (AN-18; BD Pharmingen) at 24 and 48 h after IL-4 injection. The minimum significant value of this assay was 3 pg/ml IFN-γ.

Th1 and Th2 cell differentiation

Splenic $CD4^+$ T cells from DO11.10^+ mice were purified (>90% pure by flow cytometry) using T cell enrichment columns (R&D Systems) and stimulated with plate-bound anti-CD3ε mAb (5 μg/ml, clone 145-2C11; BD Pharmingen) plus anti-CD28 mAb (5 μg/ml, clone 37.51; BD Pharmingen) at 37°C for 48 h in the presence of IL-12 (7.5 ng/ml; R&D Systems) (Th1 condition) or IL-4 (15 ng/ml; R&D Systems) and anti-IFN-γ mAb (15 μg/ml, clone XMG1.2; BD Pharmingen) (Th2 condition) as described previously (18).

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired *t* test. Values of $p < 0.05$ were considered significant.

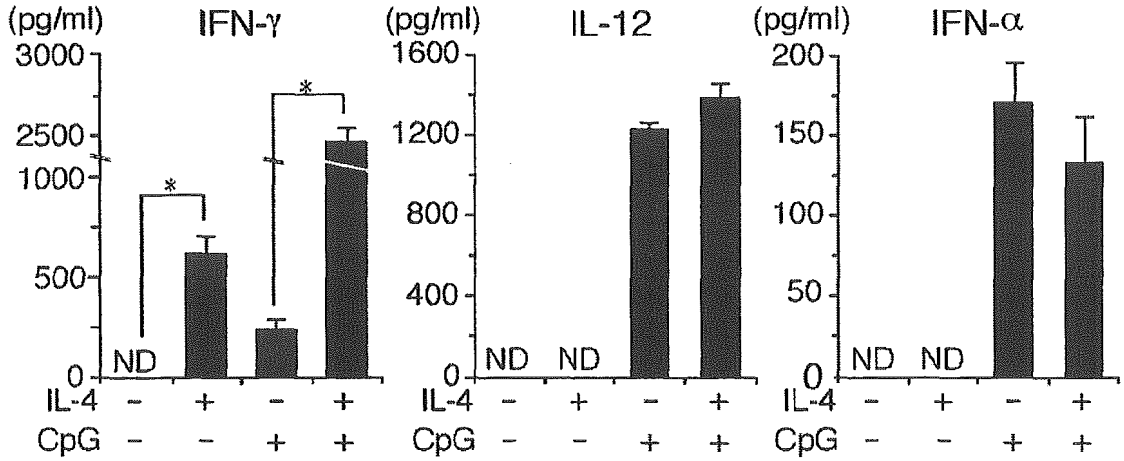

FIGURE 3. IL-4 induces IFN-γ but not IL-12 or IFN-α production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 (20 ng/ml) and/or CpG ODN (10 μg/ml) for 3 days, and the amounts of IFN-γ, IL-12, and IFN-α in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. *, $p < 0.001$.

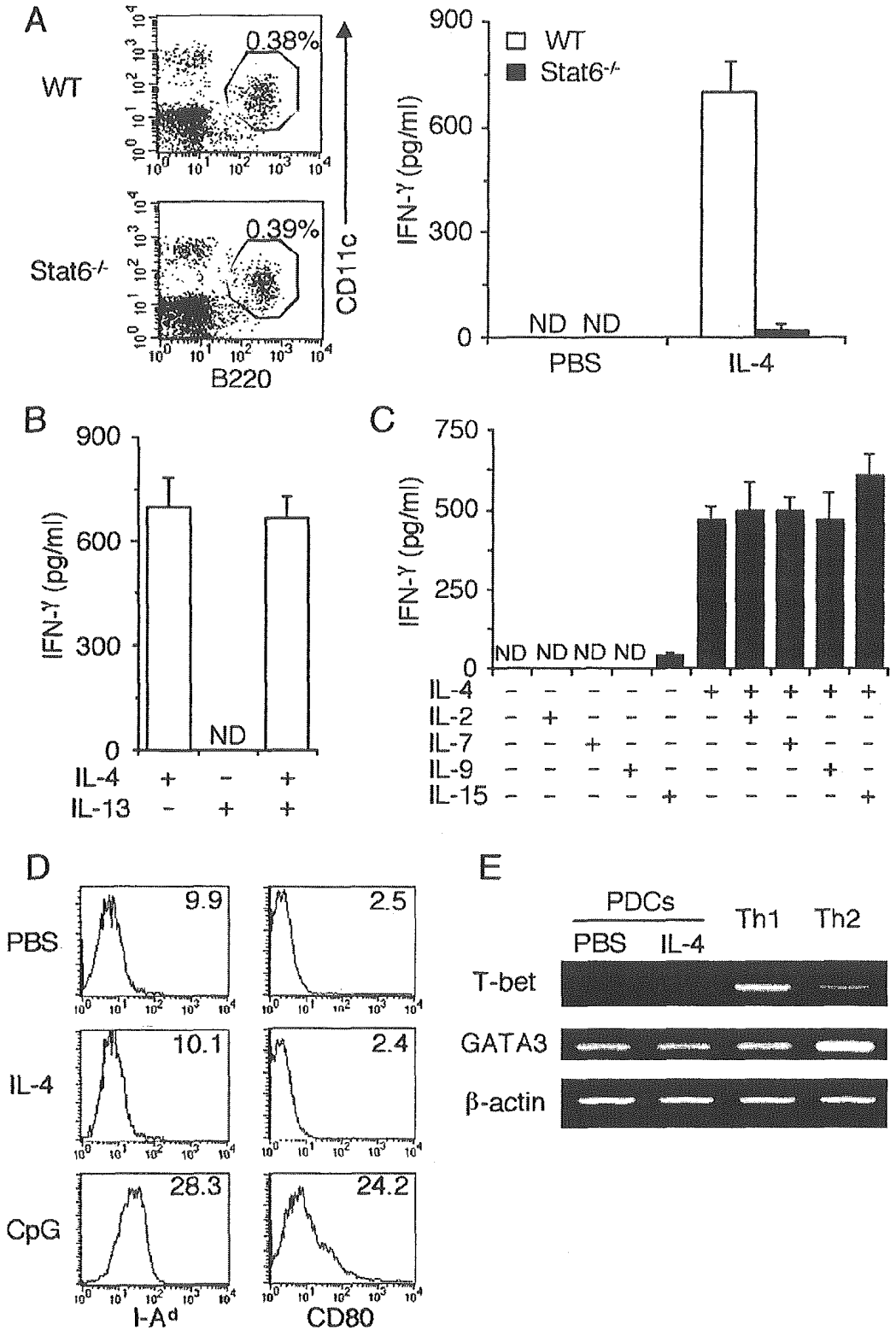

FIGURE 4. IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism. *A*, Stat6 is essential for IL-4-induced IFN-γ production in PDCs. Representative anti-B220 vs anti-CD11c staining on $CD3^-CD11b^-CD19^-DX5^-TER\text{-}119^-$ splenocytes from WT mice and $Stat6^{-/-}$ mice are shown in the *left panels* (n = 6 mice for each genotype), indicating normal development of PDCs in $Stat6^{-/-}$ mice. Isolated PDCs from WT splenocytes or $Stat6^{-/-}$ splenocytes were cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. *B*, IL-13 does not induce IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 and/or IL-13 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent experiments. ND, not detectable. *C*, Other γc-dependent cytokines do not enhance IL-4-induced IFN-γ production from PDCs. Isolated PDCs from WT splenocytes were cultured with IL-2 (20 ng/ml), IL-7 (20 ng/ml), IL-9 (20 ng/ml), or IL-15 (20 ng/ml) in the presence or in the absence of IL-4 (20 ng/ml) for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. Data are means ± SD from four independent (*Figure legend continues*)

Results

$B220^+$ PDCs produce IFN-γ upon IL-4 stimulation

To examine the negative-feedback regulation of cytokine networks, we searched for cell populations that produce IFN-γ upon IL-4 stimulation. We found that ~5% of IL-4-stimulated Rag-$2^{-/-}$ splenocytes became positive for intracellular IFN-γ staining (Fig. 1*A*, *left panels*). Multicolor FACS analyses revealed that the majority of IL-4-induced, IFN-γ-producing cells expressed CD11c at low levels, expressed B220 at high levels, but lacked the expression of DX5 (Fig. 1*A*, *right panels*). In contrast, the majority of IFN-γ-nonproducing cells in IL-4-stimulated Rag-$2^{-/-}$ splenocytes were positive for DX5 but negative for B220 (Fig. 1*A*), suggesting that these IFN-γ-nonproducing cells are NK cells.

To further characterize cell populations that produce IFN-γ upon IL-4 stimulation, $CD11c^+$ low-density splenocytes were isolated from WT mice and then stimulated with IL-4. Again, the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and expressed CD11c at low levels (Fig. 1*B*). IFN-γ-producing cells were also positive for anti-Ly6G/C staining (Fig. 1*B*). Moreover, IFN-γ-producing cells expressed class II MHC molecules ($I\text{-}A^d$) at very low levels but lacked the expression of CD19, CD3, CD8, and CD11b (Fig. 1*B*). These results suggest that the IL-4-induced, IFN-γ-producing cells are very similar to type I IFN-producing PDCs (10–12).

PDCs but not $CD11b^+$ DC or $CD8^+$ DCs produce IFN-γ upon IL-4 stimulation

To determine whether PDCs specifically produce IFN-γ upon IL-4 stimulation, isolated PDCs, $CD11b^+$ DCs, and $CD8^+$ DCs were examined for their ability of IFN-γ production upon IL-4 stimulation. Consistent with the data obtained by intracellular IFN-γ staining (Fig. 1), isolated PDCs produced a considerable amount of IFN-γ upon IL-4 stimulation (625.5 ± 79.9 pg/ml, means ± SD, $n = 4$) (Fig. 2*A*). On the other hand, IL-4-stimulated $CD8^+$ DCs produced little IFN-γ (74.3 ± 32.2 pg/ml, $n = 4$) and IL-4-stimulated $CD11b^+$ DCs did not produce IFN-γ (Fig. 2*A*). Together with the data shown in Fig. 1, these results indicate that among DC subtypes, PDCs specifically produce IFN-γ upon IL-4 stimulation.

We also examined whether PDCs produced IFN-γ upon IL-4 stimulation in vivo. As shown in Fig. 2*B*, when rIL-4 was administered i.v. to Rag-$2^{-/-}$ mice, a considerable amount of IFN-γ was detected in the serum after 24 and 48 h. Importantly, the depletion of PDCs with preinjection of anti-Ly6G/C Ab significantly decreased the IL-4-induced IFN-γ production ($n = 4$ mice, $p < 0.01$) (Fig. 2*B*). A similar trend was observed with 120G8 Ab, which depletes PDCs more specifically (23), although statistical significance was not achieved due to the limited number of mice examined (data not shown). These results suggest that PDCs produce IFN-γ upon IL-4 stimulation in vivo.

IL-4 preferentially induces IFN-γ production in PDCs

PDCs have been identified as a potent producer of IFN-α and IL-12 upon viral or bacterial infection (8–12). Therefore, we examined whether IL-4-induced IFN-α and IL-12 production in isolated PDCs. However, IL-4 did not induce the production of IFN-α or IL-12 (p70) in PDCs ($n = 4$) (Fig. 3). In contrast, PDCs produced considerable amounts of IFN-γ, IFN-α, and IL-12 upon CpG ODN stimulation, a potent stimulator of PDCs through TLR9 (24, 25) (Fig. 3). Furthermore, IL-4 strikingly enhanced CpG ODN-induced IFN-γ production ~10-fold but not CpG ODN-induced IFN-α or IL-12 production in PDCs ($n = 4, p < 0.001$) (Fig. 3). These results indicate that IL-4 preferentially induces IFN-γ production in PDCs.

IL-4 induces IFN-γ production in PDCs by a Stat6-dependent mechanism

It is well established that IL-4 uses Stat6 as a signaling molecule (26). Therefore, we next studied whether Stat6 was required for IL-4-induced IFN-γ production in PDCs using Stat6-deficient ($Stat6^{-/-}$) mice. The number of PDCs ($CD19^-B220^+CD11c^{low}$ cells) in spleen was similar between $Stat6^{-/-}$ mice and WT mice (Fig. 4*A*), suggesting that Stat6 is not essential for the development of PDCs. However, when isolated PDCs were stimulated with IL-4, WT PDCs but not $Stat6^{-/-}$ PDCs produced IFN-γ (Fig. 4*A*). On the other hand, CpG ODN-induced IFN-γ production was similarly observed between WT PDCs and $Stat6^{-/-}$ PDCs (data not shown). These results indicate that among signaling molecules under IL-4, Stat6 is essential for IFN-γ production in PDCs. We also examined the effect of IL-13, which shares type II IL-4R with IL-4 and activates Stat6 (26), on IFN-γ production in PDCs. However, IL-13 did not induce IFN-γ production in PDCs (Fig. 4*B*) nor enhance IL-4-induced IFN-γ production in PDCs (Fig. 4*B*), suggesting that type I IL-4R but not type II IL-4R is involved in IL-4-induced IFN-γ production in PDCs. Moreover, another representative Th2 cytokine, IL-5, did not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs (data not shown).

Other γc-dependent cytokines do not induce IFN-γ production nor enhance IL-4-induced IFN-γ production in PDCs

To determine whether other γc-dependent cytokines induce IFN-γ production in PDCs, isolated PDCs were stimulated with IL-2, IL-7, IL-9, and IL-15 in the presence or in the absence of IL-4 for 3 days. As shown in Fig. 4*C*, none of γc-dependent cytokines, except for IL-4 induced IFN-γ production in PDCs (Fig. 4*C*). In addition, none of them significantly enhanced IFN-γ production in IL-4-stimulated PDCs (Fig. 4*C*).

IL-4 does not alter the maturation state of PDCs

It has been shown that the ability of DCs for cytokine production depends on their maturation state (27, 28). We then examined whether IL-4 changed the maturation state of PDCs and thus induced IFN-γ-producing ability. Consistent with previous reports (8–12), isolated PDCs expressed $I\text{-}A^d$ at very low levels, and lacked the expression of CD80 (Fig. 4*D*) and CD86 (data not shown). IL-4 did not alter the expression levels of $I\text{-}A^d$, CD80, and CD86 of PDCs (Fig. 4*D* and data not shown). In contrast, when PDCs were stimulated with CpG ODN, the expression levels of $I\text{-}A^d$ and CD80 were significantly increased (Fig. 4*D*). In addition,

experiments. ND, not detectable. *D*, IL-4 does not alter the maturation state of PDCs. Isolated PDCs from WT splenocytes were cultured with IL-4 (20 ng/ml) or CpG ODN (10 μg/ml) for 48 h, and the levels of $I\text{-}A^d$ and CD80 on PDCs were analyzed by FACS. Shown are representative histograms, and the mean fluorescent intensities for anti-$I\text{-}A^d$ and anti-CD80 staining on live cells (propidium iodide $(PI)^-$ cells) from four independent experiments. Forty-three percent of PDCs could survive in the presence of IL-4, whereas 69% of PDCs could survive in the presence of CpG ODN (data not shown). *E*, T-bet is not induced by IL-4 in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 16 h. As controls, Th1-polarized cells or Th2-polarized cells were prepared from DO11.10 TCR transgenic mice as described in the *Materials and Methods*. Shown are representative data of RT-PCR analysis for T-bet, GATA3, and β-actin mRNA from four independent experiments.

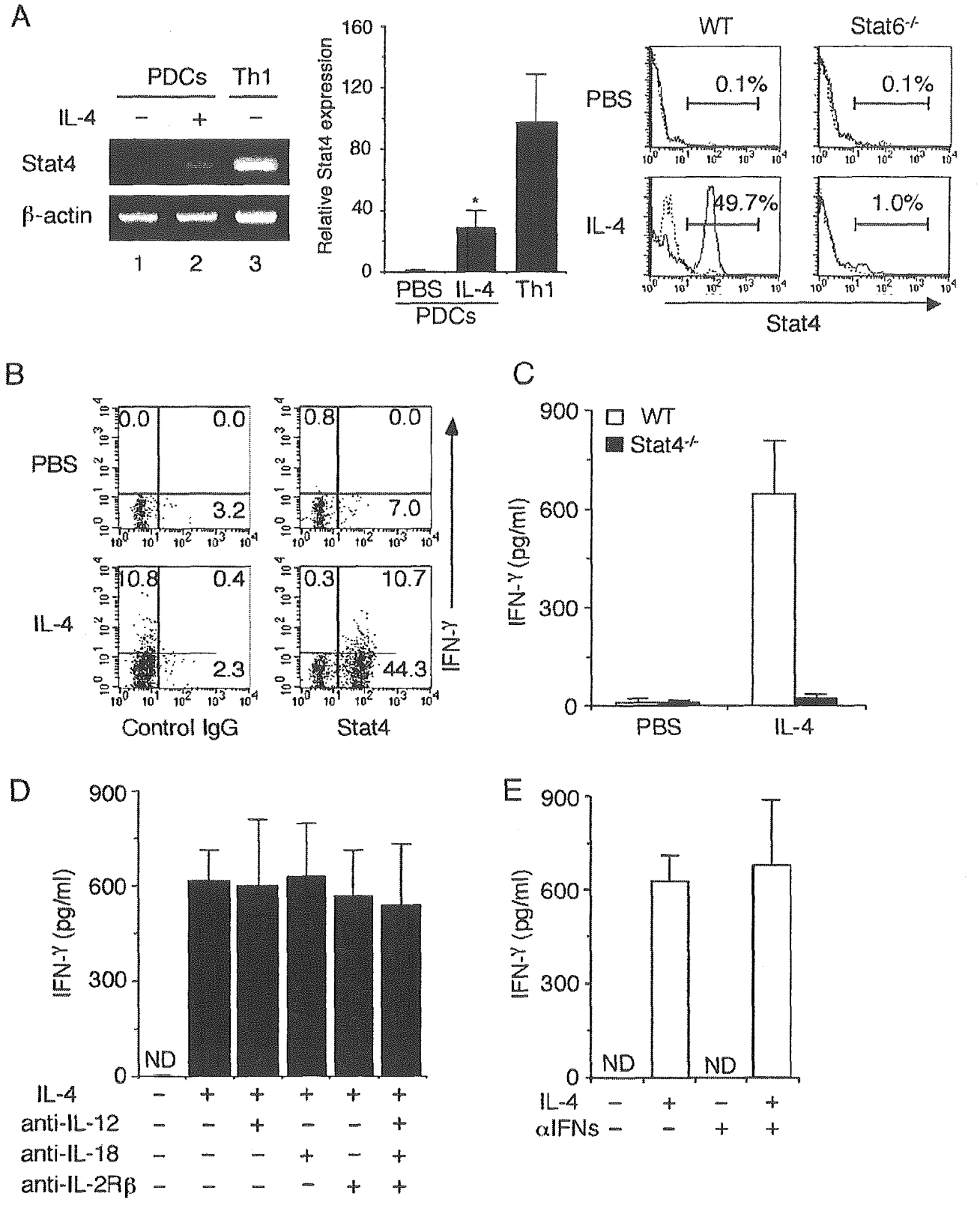

FIGURE 5. IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism. *A*, Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 16 h. As s control for Stat4-expressing cells, Th1-polarized cells were prepared from DO10$^+$ mice as described previously (15). Shown are representative data of RT-PCR analysis for Stat4 and β-actin mRNA from four independent experiments (*left panels*). Taqman PCR analysis for Stat4 and GAPDH (as a control) mRNA was performed, and the levels of Stat4 mRNA were normalized to the levels of GAPDH mRNA (*middle panel*). Data are means ± SD from four independent experiments. *, Significantly different from the mean value of control response (PBS); $p < 0.01$. Isolated PDCs from WT splenocytes or Stat6$^{-/-}$ splenocytes were stimulated with or without IL-4 (20 ng/ml) for 48 h, and the expression levels of Stat4 were evaluated by intracellular staining. Shown are representative FACS profiles from four independent experiments (*right panels*). *B*, IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 48 h. Intracellular (*Figure legend continues*)

IL-4 did not change the morphology of PDCs, whereas CpG ODN changed PDCs to dendritic morphology (data not shown). These results suggest that IL-4 does not change the maturation state of PDCs.

T-bet is not induced by IL-4 in PDCs

T-bet plays an important role in inducing IFN-γ production in $CD4^+$ T cells (29). Recent findings using T-bet-deficient mice have also suggested that T-bet is vital for IFN-γ production from $CD11c^+$ DCs upon IL-12 stimulation (30). To examine the possible involvement of T-bet in IL-4-induced IFN-γ production in PDCs, we examined the expression of T-bet mRNA in PDCs in the presence or in the absence of IL-4 stimulation. The expression of GATA3, an important negative regulator of IFN-γ production (4), was also examined in parallel. As shown in Fig. 4*E*, unstimulated PDCs expressed GATA3 mRNA but not T-bet mRNA. IL-4 did not induce the expression of T-bet mRNA nor alter the expression levels of GATA3 mRNA (Fig. 4*E*). T-bet mRNA was not detected by Taqman PCR analysis even after IL-4 stimulation (data not shown). These results suggest that T-bet may not be involved in IL-4-induced IFN-γ production in PDCs.

IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and Stat4-expressing PDCs produce IFN-γ

It has been demonstrated that Stat4 is required for IFN-γ production in many cell types (31, 32). Stat4 expression has also been demonstrated to be correlated with IFN-γ-producing ability in $CD8^+$ DCs (33). Therefore, we next examined the expression levels of Stat4 in IL-4-stimulated PDCs. As shown in Fig. 5*A*, in the absence of IL-4 stimulation, isolated WT PDCs did not express Stat4 mRNA (*lane 1*). However, Stat4 mRNA was significantly up-regulated in WT PDCs upon IL-4 stimulation (*lane 2*), although the expression level was still lower than that in Th1-polarized cells (*lane 3*) (Fig. 5A, *left panel*). Stat4 mRNA induction by IL-4 stimulation was confirmed by real-time PCR analysis (Fig. 5*A*, *middle panel*). We also examined the expression levels of Stat4 at protein levels using intracellular staining in WT PDCs and $Stat6^{-/-}$ PDCs and found that IL-4 significantly induced Stat4 expression in ~50% of WT PDCs but not in $Stat6^{-/-}$ PDCs (Fig. 5*A*, *right panel*). These results indicate that Stat6 is essential for IL-4-induced Stat4 expression in PDCs. In addition, although IFN-γ has been shown to induce Stat4 expression in some cell types (34), anti-IFN-γ Ab did not affect IL-4-induced Stat4 expression in PDCs (data not shown).

We then examined the correlation between Stat4 expression and IFN-γ production at single-cell levels by double intracellular staining. Interestingly, Stat4-expressing PDCs but not Stat4-nonexpressing PDCs produced IFN-γ upon IL-4 stimulation (Fig. 5*B*). We also examined whether Stat4 was essential for IL-4-induced IFN-γ production in PDCs using $Stat4^{-/-}$ mice. Although PDCs normally developed in $Stat4^{-/-}$ mice (data not shown), IL-4 did not induce IFN-γ production in $Stat4^{-/-}$ PDCs (Fig. 5*C*). Taken together, these results indicate that the induction of Stat4 by IL-4-Stat6 signaling is required for IFN-γ production in PDCs.

IL-4-induced IFN-γ production does not depend on IL-12 or type I IFNs

To determine whether endogenously produced cytokines from PDCs are involved in IL-4-induced IFN-γ production, we examined the effect of blocking Abs against IL-12 (p70), IL-2R β-chain (a shared receptor for IL-2 and IL-15), and IL-18 on IL-4-induced IFN-γ production in PDCs. However, none of the Abs inhibited IL-4-induced IFN-γ production in PDCs even when these Abs were added altogether (Fig. 5*D*). To determine the possible involvement of type I IFNs in IL-4-induced IFN-γ production in PDCs, we also examined the effect of a mixture of neutralizing Abs to IFN-α, IFN-β, and type I IFN receptor on IL-4-induced IFN-γ production in PDCs. However, these Abs did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*E*). As expected, the addition of anti-IL-4 mAb canceled IL-4-induced IFN-γ production in PDCs (data not shown). These results indicate that none of IL-12, IL-2, IL-15, IL-18, or type I IFNs is required for IL-4-induced IFN-γ production in PDCs.

Discussion

In this study, we show that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs by a Stat6-dependent mechanism. Moreover, IL-4 induces Stat4 expression in PDCs through a Stat6-dependent mechanism and the IL-4-induced, Stat4-expressing PDCs produce IFN-γ. Furthermore, $Stat4^{-/-}$ PDCs do not produce IFN-γ upon IL-4 stimulation. These results suggest that PDCs produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms.

We demonstrate that PDCs are a major IFN-γ-producing cell upon IL-4 stimulation. By searching for $Rag\text{-}2^{-/-}$ splenocyte populations that produce IFN-γ upon IL-4 stimulation, we found that the majority of IL-4-induced, IFN-γ-producing cells expressed B220 at high levels and CD11c and Ly6G/C at low levels (Fig. 1). We also found that IL-4 induced IFN-γ production from isolated $B220^+$ PDCs but not from $CD11b^+$ DCs or $CD8^+$ DCs (Fig. 2*A*) and that the depletion of PDCs by anti-Ly6G/C Ab prevented IL-4-induced IFN-γ production in vivo (Fig. 2*B*). Inhibition of IL-4-induced IFN-γ production was similarly observed with the administration of 120G8 Ab, although statistical significance was not achieved due to the limited number of mice examined. On the other hand, IL-4 did not induce IFN-α or IL-12 production in PDCs (Fig. 3). IL-4 also strongly enhanced CpG ODN-induced IFN-γ production but not CpG ODN-induced IFN-α or IL-12 production in PDCs (Fig. 3). Taken together, these results indicate that PDCs are a major IFN-γ producer upon IL-4 stimulation and that IL-4 preferentially induces IFN-γ production in PDCs.

It is well recognized that Stat6 plays a critical role in the production of IL-4 in $CD4^+$ T cells upon IL-4 stimulation through the induction of GATA3, a master regulator of Th2 cell differentiation (4). In contrast, we show here that IL-4 induces IFN-γ production

staining for IFN-γ together with Stat4 staining was performed as described in *Materials and Methods*. Control IgG was used as a negative control of anti-Stat4 Ab. Representative FACS profiles from four independent experiments are shown. *C*, Isolated PDCs from WT splenocytes or $Stat4^{-/-}$ splenocytes were cultured with or without IL-4 for 3 days, and the amounts of IFN-γ in the supernatants were measured by ELISA. FACS analysis performed as described in Fig. 4*A* indicates normal development of PDCs in $Stat4^{-/-}$ mice (data not shown). Data are means ± SD from three independent experiments. *D*, None of IL-2, IL-12, IL-15, or IL-18 is required for IL-4-induced IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 3 days in the presence of Abs against IL-12, IL-18, or IL-2Rβ. The amount of IFN-γ in the supernatant was measured by ELISA. Data are means ± SD from four independent experiments. *E*, Type I IFNs are not required for IL-4-induced IFN-γ production in PDCs. Isolated PDCs from WT splenocytes were cultured with or without IL-4 (20 ng/ml) for 3 days in the presence or in the absence of a mixture of neutralizing Abs to IFN-α, IFN-β, and type I IFN receptor. The amount of IFN-γ in the supernatant was measured by ELISA. Data are means ± SD from four independent experiments.

in PDCs by a Stat6-dependent mechanism (Fig. 4*A*). We also show that IL-4 does not alter the expression levels of GATA3 (Fig. 4*E*) nor induce the expression of T-bet, a key molecule for IFN-γ production in CD4 T cells (29), in PDCs (Fig. 4*E*). Therefore, in contrast to $CD4^+$ T cells, the expression levels of T-bet and GATA3 may not be causatively associated with the production of IFN-γ in PDCs.

Our results show that IL-4, but not other γc-dependent cytokines, induces IFN-γ production from PDCs (Fig. 4*C*). In contrast, it has been demonstrated recently that IL-4 synergistically enhances IL-2-induced IFN-γ production from NK cells, but IL-4 itself does not induce IFN-γ production from NK cells (35). It has also been shown that although IL-4 enhances IL-12-induced IFN-γ production from $CD8^+$ DCs, IL-4 itself does not induce IFN-γ production from $CD8^+$ DCs (36). Moreover, we found that bone marrow-derived PDCs generated with fms-like tyrosine kinase-3 ligand did not produce IFN-γ upon IL-4 stimulation (data not shown). Thus, the IL-4 signaling pathway for IFN-γ production may differ depending not only on cell types but also on the maturation state of the cells.

We also show that IL-4 induces Stat4 expression in PDCs by a Stat6-dependent mechanism (Fig. 5*A*), that only the Stat4-expressing PDCs produce IFN-γ at single-cell levels (Fig. 5*B*), and that $Stat4^{-/-}$ PDCs do not produce IFN-γ upon IL-4 stimulation (Fig. 5*C*). Therefore, it is indicated that Stat4 is required for IL-4-induced IFN-γ production in PDCs. Interestingly, we also found that when PDCs were stimulated with CpG ODN for 48 h, Stat4 induction was detected by intracellular FACS analysis (data not shown). This finding may account for the synergistic effect of CpG ODN on IL-4-induced IFN-γ production in PDCs (Fig. 3).

The mechanisms leading to Stat4 activation could not be yet identified. Indeed, the phosphorylation status of Stat4 could not be clearly defined in IL-4-stimulated PDCs presumably for technical reasons (data not shown). However, as tyrosine phosphorylation is required for the activity of STAT proteins (37), a Stat4-activating cytokine seems to be involved in IL-4-induced IFN-γ production in PDCs. Because IL-12 is a representative cytokine that activates Stat4 (38) and because it has been reported that IL-4 enhances IL-12 production from $CD11c^+$ DCs (39) or $CD8\alpha^+$ DCs (40) in some situations, it was suggested that IL-12 might be responsible for activating Stat4 in PDCs. However, we found that IL-4 by itself does not induce IL-12 production from PDCs (Fig. 3) and that a neutralizing Ab against IL-12 did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*D*), suggesting that IL-12 is not responsible for the activation of Stat4 in PDCs.

Recently, it has also been demonstrated that in addition to IL-12, type I IFNs activate Stat4 and induce IFN-γ production in some cell types such as $CD8^+$ T cells (41). However, again, we found that IL-4 by itself did not induce IFN-α production from PDCs (Fig. 3) and that neutralizing Abs against type I IFNs did not inhibit IL-4-induced IFN-γ production in PDCs (Fig. 5*E*). These findings suggest that type I IFNs are not responsible for the activation of Stat4 in PDCs. Recent studies have also demonstrated that IL-23 (42) and IL-21 (43) use Stat4 as a signaling molecule in some cell types. Therefore, IL-23, IL-21, or an undefined Stat4-activating cytokine may function as a Stat4-activating cytokine and then may contribute to IL-4-induced IFN-γ production in PDCs. Further studies that identify the cytokine responsible for Stat4 activation are required for the understanding of the mechanism leading to IL-4 induced IFN-γ production in PDCs.

The effect of IL-4 on the expression of Stat4 in DCs seems different depending on the subtypes of DCs, as well as the maturation state of each DC subtype. Recently, Fukao et al. (33) have shown that when IL-4 is present during the maturation of $CD11c^+$ DCs, IL-4 suppresses Stat4 induction and subsequent IL-12-induced IFN-γ production in $CD11c^+$ DCs. On the other hand, the same group has shown that IL-4 does not alter the expression levels of Stat4 in mature $CD8^+$ DCs (36). However, we showed that IL-4-Stat6 signaling induced Stat4 expression in PDCs (Fig. 5). Moreover, we found that the maturation state of PDCs, assessed by the expression levels of CD80 and $I\text{-}A^d$, was similar between Stat4-expressing PDCs and Stat4-nonexpressing PDCs (data not shown). Therefore, our results indicate that IL-4 specifically induces Stat4 expression and IFN-γ-producing ability in PDCs without affecting their maturation state.

In the present study, we showed that a typical Th2 cytokine IL-4 induced the production of a typical Th1 cytokine IFN-γ in PDCs in BALB/c mice. IL-4-induced IFN-γ production in PDCs was also observed in C57BL/6 mice (data not shown), suggesting that this phenomenon is a general one observed beyond strain differences. Because IL-4 is produced in an early phase in immune responses by NK T cells (44) and/or basophils (45, 46), the IL-4-induced IFN-γ production by PDCs may function in the negative-feedback regulation against a Th2-type deviation in an early phase of immune responses. In this regard, de Heer et al. (14) have demonstrated recently that PDCs inhibit typical Th2 responses such as IgE production and allergic airway inflammation. Although the authors indicated the induction of regulatory T cells as the mechanism underlying the PDC-mediated Th2 cell suppression (14), IL-4-induced IFN-γ production in PDCs may also contribute to the PDC-mediated inhibition of allergic airway inflammation because IFN-γ inhibits Ag-induced Th2 cell differentiation (1–3) and allergic airway inflammation (47).

In conclusion, we have shown that PDCs preferentially produce IFN-γ upon IL-4 stimulation by Stat6- and Stat4-dependent mechanisms. Although further studies are required to address the physiological importance of IL-4-induced IFN-γ production in PDCs, our results would give a new insight into PDC-mediated immune regulation of cytokine network.

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Disclosures

The authors have no financial conflict of interest.

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Indispensable Role of Stat5a in Stat6-Independent Th2 Cell Differentiation and Allergic Airway Inflammation[1]

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It is well-recognized that Stat6 plays a critical role in Th2 cell differentiation and the induction of allergic inflammation. We have previously shown that Stat5a is also required for Th2 cell differentiation and allergic airway inflammation. However, it is the relative importance and redundancy of Stat6 and Stat5a in Th2 cell differentiation and allergic airway inflammation are unknown. In this study we addressed these issues by comparing Stat5a-deficient ($Stat5a^{-/-}$) mice, $Stat6^{-/-}$ mice, and Stat5a- and Stat6 double-deficient ($Stat5a^{-/-}$ $Stat6^{-/-}$) mice on the same genetic background. Th2 cell differentiation was severely decreased in $Stat6^{-/-}CD4^+$ T cells, but Stat6-independent Th2 cell differentiation was still significantly observed in $Stat6^{-/-}CD4^+$ T cells. However, even in the Th2-polarizing condition (IL-4 plus anti-IFN-γ mAb), no Th2 cells developed in $Stat5a^{-/-}Stat6^{-/-}$ $CD4^+$ T cells. Moreover, Ag-induced eosinophil and lymphocyte recruitment in the airways was severely decreased in $Stat5a^{-/-}Stat6^{-/-}$ mice compared with that in $Stat6^{-/-}$ mice. These results indicate that Stat5a plays an indispensable role in Stat6-independent Th2 cell differentiation and subsequent Th2 cell-mediated allergic airway inflammation. ***The Journal of Immunology*****, 2005, 174: 3734–3740.**

Newly activated $CD4^+$ T cells differentiate into at least two functionally distinct subsets, Th1 and Th2 cells, as defined by their patterns of cytokine production (1, 2). Th1 cells produce IFN-γ and lymphotoxin and are responsible for delayed-type hypersensitivity reactions, promoting control of intracellular pathogens (1, 2). Th2 cells produce IL-4, IL-5, and IL-13 and provide an excellent helper function for Ab production, particularly of IgE (1, 2). Th2 cells are essential for promoting host defense against helminths, but uncontrolled Th2 cell activation to noninvasive Ags (allergen) causes atopic disorders, including asthma (3, 4).

Over the last several years, significant progress has been made in the molecular mechanisms for Th2 cell differentiation (5–7). Although early studies have indicated that Stat6 (8–10), a cytosolic latent transcription factor that is rapidly activated after cellular exposure to IL-4 and IL-13, is essential for Th2 cell differentiation through the induction of GATA3 (5–7), recent studies have revealed that Stat6-deficient ($Stat6^{-/-}$) $CD4^+$ T cells make a considerable amount of IL-4 upon stimulation with TCR (11). In addition, it has been demonstrated that Th2 cell-mediated allergic airway inflammation is still observed in $Stat6^{-/-}$ mice (12–15). Therefore, in addition to the Stat6-dependent pathway, the Stat6-independent pathway participates in in vitro Th2 cell differentiation as well as in vivo Th2 cell-mediated immune responses.

In contrast, we have shown that Ag-induced IL-5 production and eosinophil recruitment in the airways are decreased in $Stat5a^{-/-}$ mice (16). In addition, we have shown that Th cell differentiation in $Stat5a^{-/-}$ mice is biased toward the Th1 type at single cell levels and that retrovirus-mediated expression of Stat5a restores the impaired Th2 cell differentiation of $Stat5a^{-/-}CD4^+$ T cells (17). Consistent with these findings, it has recently been shown that the enforced expression of a constitutively active form of Stat5a induces IL-4 production in $CD4^+$ T cells by enhancing the accessibility of the IL-4 gene (18). These findings suggest that the intrinsic expression of Stat5a in $CD4^+$ T cells plays an important role in Th2 cell differentiation and the induction of allergic airway inflammation. However, the relative importance and redundancy of Stat5a-mediated Th2 cell differentiation and Stat6-mediated Th2 cell differentiation are still unclear.

In the present study we addressed these issues by comparing Th2 cell differentiation in $Stat5a^{-/-}$ mice, $Stat6^{-/-}$ mice, and Stat5a and Stat6 double-deficient ($Stat5a^{-/-}Stat6^{-/-}$) mice in the same genetic background. We also examined allergic airway inflammation in these mice as a model of in vivo Th2 cell-mediated immune responses. We found that Th2 cell differentiation and allergic airway inflammation were severely decreased in $Stat5a^{-/-}$ $Stat6^{-/-}$ mice compared with those in $Stat5a^{-/-}$ mice or $Stat6^{-/-}$ mice. Our results suggest that Stat5a is essential for Th2 cell differentiation in the absence of Stat6 activation and vice versa.

Materials and Methods

Mice

Stat5a-deficient ($Stat5a^{-/-}$) mice (19) and Stat6-deficient ($Stat6^{-/-}$) mice (8) were backcrossed to BALB/c mice (Charles River Laboratories) for eight generations. $Stat5a^{+/-}Stat6^{+/-}$ male mice were mated with $Stat5a^{+/-}$ $Stat6^{+/-}$ female mice to obtain $Stat5a^{+/+}Stat6^{+/+}$ mice (wild-type (WT)[3] mice), $Stat5a^{-/-}Stat6^{+/+}$ mice ($Stat5a^{-/-}$ mice), $Stat5a^{+/+}Stat6^{-/-}$ mice ($Stat6^{-/-}$ mice), and $Stat5a^{-/-}Stat6^{-/-}$ mice within the litter. All experiments were performed according to the guidelines of Chiba University.

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[3] Abbreviations used in this paper: WT, wild type; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff.

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Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4-FITC, -PE, -allophycocyanin, and -PerCP (H129.19); anti-CD8-FITC and -PE (53-6.7); anti-B220-allophycocyanin (RA3-6B2); anti-IgM-FITC (R6-60.2); anti-CD69-FITC (H1.3F3); anti-CD62L-FITC (MEL-14); anti-TCR Vβ8.1,2-FITC (MR5-2); and anti-pan-NK-PE (DX5). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Cell culture

Splenocytes (2×10^6 cells/ml) from WT mice, Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (mAb) (5 μg/ml; clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics in a 24-well microtiter plate at 37°C for 48 h. Where indicated, IL-12 (15 ng/ml; PeproTech EC) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech EC) and anti-IFN-γ mAb (15 μg/ml; clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (17). Cells were washed with PBS, then cultured for another 3 days in Th0 (no exogenous cytokines), Th1, or Th2 conditions in the presence of IL-2 (20 U/ml; PeproTech).

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4 vs IFN-γ was performed as described previously (17). In brief, cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 6 h, with monensin (2 μM) (Sigma-Aldrich) added for the final 4 h. After being stained with anti-CD4-PerCP, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IL-4-PE (BVD4-1D11; BD Pharmingen) and anti-IFN-γ-allophycocyanin (XMG1.2; BD Pharmingen) for 30 min at 4°C. The cytokine profile (IL-4 vs IFN-γ) of CD4$^+$ cells was analyzed on a FACSCalibur using CellQuest software.

Ag-induced allergic inflammation in the airways

Allergic airway inflammation was induced by the inhalation of OVA (Sigma-Aldrich) in sensitized mice as described previously (20). Briefly, mice (aged 7–8 wk) were immunized i.p. twice with 4 μg of OVA in 4 mg of aluminum hydroxide at a 2-wk interval. Twelve to 14 days after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer three times, for 20 min each time, at 24-h intervals. As a control, 0.9% saline alone was administered by the nebulizer. Forty-eight hours after the last inhalation, trachea and lung were excised, fixed in 10% buffered-formalin, and embedded in paraffin. The specimens (3 μm thick) of the trachea were stained with Luna and H&E solutions. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per length of the basement membrane of trachea, which was measured with a digital curvimeter.

Lung sections were stained with H&E and periodic acid-Schiff (PAS) according to standard protocols. The magnitude of inflammatory cell infiltration in the perivascular and peribronchial spaces on H&E-stained lung sections was evaluated by a semiquantitative scoring system as described previously (21): +5 signified a large (more than three cells deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signified a small number of inflammatory foci. The H&E-stained sections were coded and then examined by two observers in a blind manner, the sum of the scores from each lung was divided by the number of airways examined for the score, and the average of the two determinations for each section was used for subsequent calculations. PAS-stained lung sections were also categorized according to the abundance of PAS$^+$ goblet cells and assigned numerical scores as described previously (22): 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%.

The numbers of eosinophils, lymphocytes, and macrophages recovered in the bronchoalveolar lavage fluid (BALF) were evaluated as described previously (16). In short, after bronchoalveolar lavage was performed with 2 ml of PBS, BALF was centrifuged at 400 $\times$ g for 5 min at 4°C, and differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution.

ELISA

Cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 12 h. The amounts of IL-4, IL-5, IL-10, and IFN-γ in the culture supernatant were measured by enzyme immunoassay using murine IL-4, IL-5, IL-10, and IFN-γ ELISA kits (BD Pharmingen). The amount of IL-13 in the culture supernatant was measured using an ELISA kit from R&D Systems. The assays were performed in duplicate according to the manufacturer's instructions. The minimum significant values of these assays were 15 pg/ml IL-4 and IL-5 and 30 pg/ml IFN-γ, IL-10, and IL-13.

Data analysis

Data are summarized as the mean $\pm$ SD. The statistical analysis of the results was performed by unpaired t test. A value of $p < 0.05$ was considered significant.

Results

Normal CD4$^+$ T cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice

It has been shown that not only Stat6 (8–10), but also Stat5a (16–18), play critical roles in Th2 cell differentiation. To investigate the relative importance of Stat5a- and Stat6-mediated signaling in Th2 cell differentiation in detail, we generated Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice on the same genetic background and compared the development and differentiation of CD4$^+$ T cells among these mice. Consistent with the previous reports (16, 23), the number of splenocytes in Stat5a$^{-/-}$ mice was modestly, but significantly, decreased compared with that in WT mice (Fig. 1*A*). The number of splenocytes in Stat5a$^{-/-}$Stat6$^{-/-}$ mice was also decreased compared with that in Stat6$^{-/-}$ mice (Fig. 1*A*). However, FACS analysis revealed that the frequencies of CD4$^+$ T cells and CD8$^+$ T cells were similar among WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 1*B*). The expression of CD69 and CD62L on CD4$^+$ T cells was also similar among these mice (data not shown). Based on B220 vs IgM staining, B cells in the spleen exhibited normal maturation in these mice (Fig. 1*B*). These results indicate that T and B cells can develop even in the absence of Stat5a and Stat6.

Stat6-independent Th2 cell differentiation depends on Stat5a

We then examined cytokine production from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ T cells. Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0 (no exogenous cytokines), Th1 (in the presence of IL-12), or Th2 (in the presence of

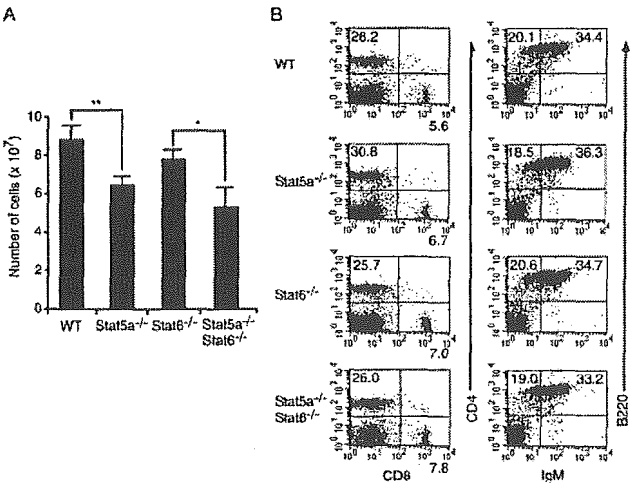

FIGURE 1. Normal T cell and B cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, Number of splenocytes in WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Data are the mean $\pm$ SD from eight mice for each genotype. *, $p < 0.05$; **, $p < 0.01$. *B*, Flow cytometric analysis of splenocytes from 6-wk-old mice. Cells were stained with anti-CD4-PE vs anti-CD8-FITC or anti-B220-allophycocyanin vs anti-IgM-FITC. Shown are representative FACS profiles from five mice in each group.

IL-4 and anti-IFN-γ mAb) conditions for 2 days, then cultured for another 3 days in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells were restimulated with plate-bound anti-CD3 mAb for 12 h, and the amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined. In the Th0 condition, IL-4 and IL-5 production was significantly decreased in Stat5a$^{-/-}$ splenocytes compared with that in WT splenocytes (Fig. 2), consistent with our previous report (17). IL-4 and IL-5 production was more severely decreased in Stat6$^{-/-}$ splenocytes (Fig. 2). However, significant IL-4 and IL-5 production was still detected in Stat6$^{-/-}$ splenocytes (Fig. 2). In contrast, almost no IL-4 or IL-5 was detected in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes in the Th0 condition (Fig. 2). Furthermore, even when Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes were stimulated with anti-CD3 Ab in Th2 condition, they did not significantly produce IL-4 and IL-5 ($n = 5$; $p < 0.01$; Fig. 2). Similarly, IL-10 and IL-13 production was significantly decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes compared with that in Stat5a$^{-/-}$ or Stat6$^{-/-}$ splenocytes in the Th2 condition (Fig. 2). By contrast, IFN-γ production did not change in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes in the Th0 condition and, instead, was increased in the Th1 condition compared with that in WT splenocytes or Stat6$^{-/-}$ splenocytes ($n = 5$; $p < 0.01$; Fig. 2). In contrast, no significant differences were observed in the proliferative responses of T cells among these mice in Th0, Th1, and Th2 conditions (data not shown), suggesting that the impaired Th2 cytokine production in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes does not result from possible defects in cell proliferation.

Next, we examined Th1/Th2 cell differentiation at single-cell levels (Fig. 3). Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0, Th1, or Th2 conditions, and the cytokine profile (IL-4 vs IFN-γ) of CD4$^+$ T cells was evaluated by intracellular cytokine analysis. In the Th0 condition, CD4$^+$ T cells that produced IL-4, but not IFN-γ, were significantly decreased in Stat5a$^{-/-}$ mice compared with those in WT mice (Fig. 3, *a* vs *b*). IL-4-producing CD4$^+$ cells were more severely decreased in Stat6$^{-/-}$ mice but IL-4-producing CD4$^+$ cells still developed in

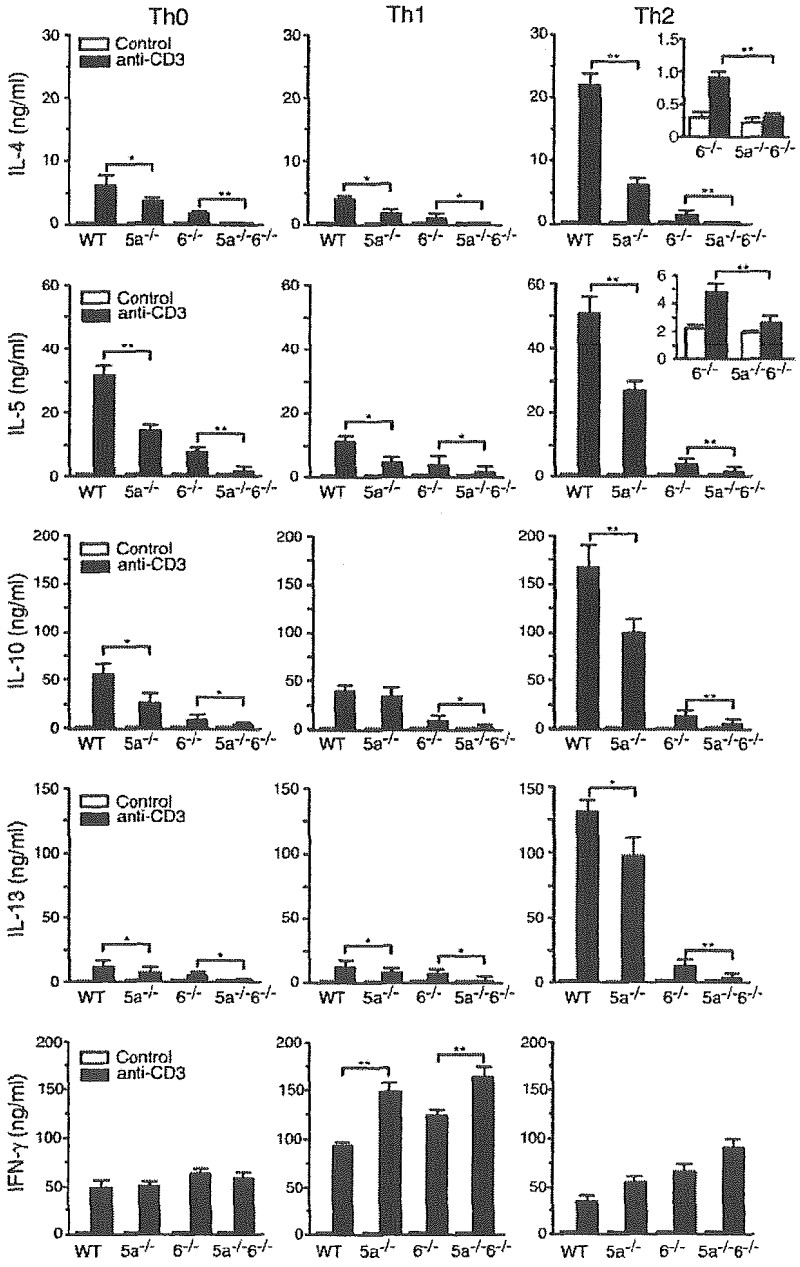

FIGURE 2. Th2 cytokine production is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$ (5a$^{-/-}$), Stat6$^{-/-}$ (6$^{-/-}$), or Stat5a$^{-/-}$ Stat6$^{-/-}$ (5a$^{-/-}$ 6$^{-/-}$) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 condition (no exogenous cytokines), the Th1 condition (in the presence of IL-12), or the Th2 condition (in the presence of IL-4 and anti-IFN-γ mAb) for 48 h, then cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells (1×10^6/ml) were restimulated with plate-bound anti-CD3 mAb for 12 h in the absence of exogenous cytokines. The amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined by ELISA. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$.

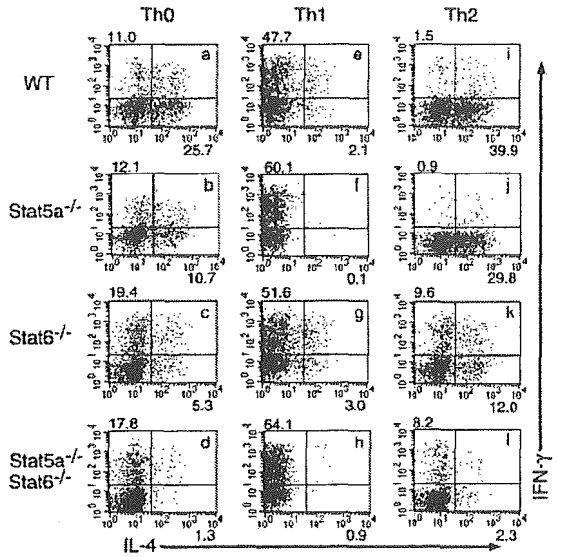

FIGURE 3. Th2 cell differentiation is severely decreased in Stat5a$^{-/-}$ Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, or Stat5a$^{-/-}$ Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th0, Th1, or Th2 conditions and cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. Cells were washed and restimulated with plate-bound anti-CD3 mAb for 6 h. Intracellular cytokine profiles for IL-4 vs IFN-γ were determined on CD4$^+$ T cells. Shown are representative FACS profiles from five mice in each group.

Stat6$^{-/-}$ mice (Fig. 3*c*). Consistent with a previous report (11), IL-4-producing CD4$^+$ cells in Stat6$^{-/-}$ mice lacked the expression of DX5, and the frequency of TCR Vβ8$^+$ cells was not significantly increased in these cells (data not shown), suggesting that the majority of IL-4-producing CD4$^+$ cells in Stat6$^{-/-}$ mice were conventional Th2 cells, but not NK T cells. Importantly, Th2 cells were hardly detected in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*d*). The frequency of Th2 cells in the Th0 condition was as follows: WT mice, 24.7 ± 3.4%; Stat5a$^{-/-}$ mice, 10.2 ± 2.6%; Stat6$^{-/-}$ mice, 5.5 ± 1.1%; and Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 1.2 ± 0.3% (mean ± SD; n = 5 experiments in each group).

When splenocytes were cultured in Th2-polarizing conditions, the frequency of Th2 cells increased in Stat5a$^{-/-}$ mice and Stat6$^{-/-}$ mice, although the frequency of Th2 cells was still significantly lower in Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice than that in WT mice (Fig. 3). However, even in the Th2 condition, the frequency of Th2 cells did not significantly increase in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*l*). These results indicate that Stat5a is essential for Stat6-independent Th2 cell differentiation and vice versa.

In contrast, in the Th1 condition, CD4$^+$ T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in Stat5a$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT and Stat6$^{-/-}$ mice, respectively (WT mice, 44.9 ± 8.2%; Stat5a$^{-/-}$ mice, 62.3 ± 11.9% ($p < 0.05$); Stat6$^{-/-}$ mice, 50.8 ± 12.9%; Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 66.4 ± 12.3% ($p < 0.05$); n = 5; Fig. 3). In contrast, in the Th0 or Th2 condition, Th1 cells were significantly increased in Stat6$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT mice and Stat5a$^{-/-}$ mice, respectively (Fig. 3). These results suggest that Stat5a and Stat6 are differently involved in the suppression of Th1 cell differentiation, depending on the cytokine environment.

Interestingly, CD4$^+$ T cells that produced both IFN-γ and IL-4 tended to be increased in Stat6$^{-/-}$ mice, but not in Stat5a$^{-/-}$ mice (Fig. 3). These results suggest that Stat6 may also play a role in the suppression of IFN-γ production in developing Th2 cells; this idea is consistent with the previous finding that Stat6 induces the expression of GATA3 (24), a master regulator of Th2 cells that induces Th2 cytokine production and inhibits IFN-γ production in T cells (5–7).

Stat5a-dependent, Stat6-independent Th2 cell differentiation participates in Ag-induced eosinophil and lymphocyte recruitment into the airways

To clarify the in vivo role of Stat5a-dependent, Stat6-independent Th2 cell differentiation, we examined Ag-induced airway inflammation as a model of Th2 cell-mediated in vivo immune responses. Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and control WT mice were immunized twice with OVA; 2 wk later, these mice were challenged with aerosolized OVA three times at 24-h intervals. Forty-eight hours after the last Ag challenge, airway inflammation

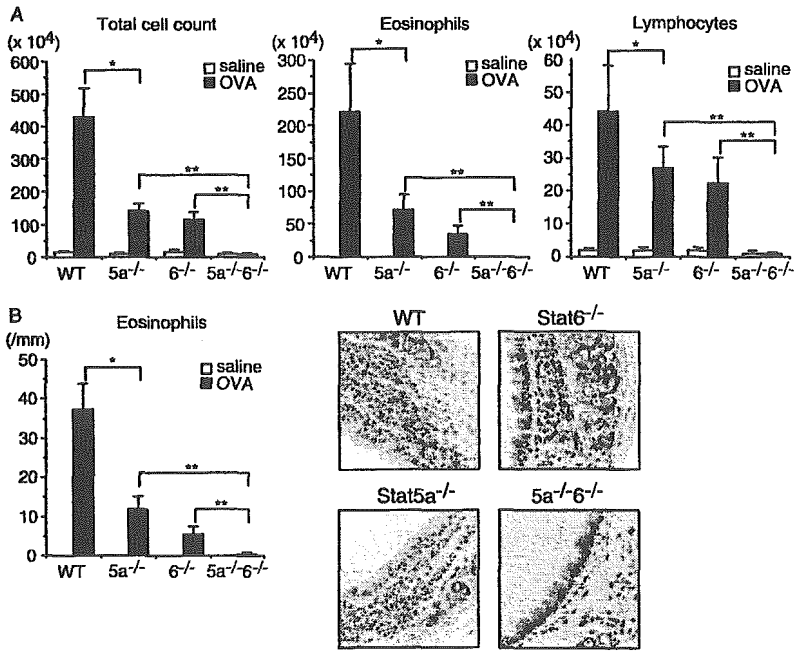

FIGURE 4. Ag-induced eosinophil and lymphocyte recruitment into the airways is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, OVA-sensitized Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and littermate WT mice were challenged with the inhalation of OVA or saline (as a control) three times at 24-h intervals. The numbers of total cells, eosinophils, and lymphocytes in BALF were evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. *B*, Similar to *A*, OVA-sensitized mice were challenged with inhaled OVA or saline, and the number of eosinophils infiltrating the submucosal tissue of trachea was evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. Representative photomicrographs of trachea sections stained with Luma solution are also shown (×100).