

Expression profiles of regulatory and helper T-cell-associated genes in nasal polyposis

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To cite this article: Li CW, Zhang KK, Li TY, Lin ZB, Li YY, Curotto de Lafaille MA, Shi L, Wang DY. Expression profiles of regulatory and helper T-cell-associated genes in nasal polyposis. *Allergy* 2012; DOI:10.1111/j.1398-9995.2012.02811.x.

Keywords

airway inflammation; FOXP3; nasal polyposis; T-helper cells; T-regulatory cells.

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Accepted for publication 04 February 2012

DOI:10.1111/j.1398-9995.2012.02811.x

Edited by: Marek Sanak

Abstract

Background: Nasal polyposis (NP) is a Th2-skewed inflammatory disorder, but it is unclear what role regulatory T cells (T-reg) play in disease pathology. We investigated the expression profiles of T-reg and T-helper-cell-associated genes and their response to glucocorticosteroid (GC) treatment in Chinese patients with NP.

Methods: Biopsies were obtained from 29 non-treated NP patients for comparison with inferior turbinates collected from healthy controls. In 13 patients, NP samples were collected both before and after short-term oral GC treatment. Levels of mRNA for T-cell markers were determined by microarray and quantitative PCR. Cellular infiltrates were assessed by histo- and immunohistochemistry.

Results: FOXP3⁺ T-reg were increased in GC-naïve NP, and numbers were negatively correlated with eosinophil infiltration. Helios staining was not detected, suggesting that FOXP3⁺ cells in NP are not thymus-derived T-reg. Compared with controls, mRNA levels corresponding to T-reg genes were significantly increased in NP (FOXP3, TGFB1, IL10, SMAD3, IL2RA, and JAK3), but transcription factors associated with Th2 (GATA3) or Th17 responses (RORc) were significantly reduced. FOXP3 mRNA levels positively correlated with other T-reg cell markers. Microarray analysis showed that most Th2-related markers (e.g., Eotaxin-1, CCL13, and CCL18) were upregulated in GC-naïve NP vs controls. GC therapy significantly suppressed eosinophilic inflammation in NP, but did not significantly alter the expression levels of T-reg/Th2-associated genes.

Conclusions: Upregulation of FOXP3⁺-inducible T-reg cells and downregulation of Th2 and Th17 markers in NP indicate a regulatory response occurring at a site of persistent mucosal inflammation. However, immune regulation fails to control the underlying tissue pathology. Expression of T-reg/Th2 markers after GC treatment was unaltered, suggesting that T-cell-driving NP inflammatory mediators are GC resistant.

Nasal polyposis (NP) is a common, chronic inflammatory disorder of the upper airways, which is often associated with asthma and other airway diseases (1). Eosinophil-dominated inflammation appears to be a hallmark of NP in Caucasians (1), while our previous data indicate that Chinese NP is

characterized by the combined infiltration of eosinophils, neutrophils, and CD4⁺/CD8⁺ T lymphocytes (2).

T-reg cells have been shown to suppress Th1/Th2 responses and allergic inflammation in the airways (3, 4). Forkhead box P3 (FOXP3) is currently regarded as the most specific marker for T-reg cells, because this transcription factor is essential for T-reg development, survival, and function (5, 6). While most FOXP3⁺ T-regs are natural T-regs (nT-regs) that originate in the thymus, FOXP3⁺ T-reg differentiation can also be

Abbreviations

NP nasal polyposis; GC glucocorticosteroid; T-reg T-regulatory.

induced in peripheral lymphoid organs and tissues under both tolerizing and inflammatory conditions (7). In mice, the transcription factor Helios is expressed in nT-regs but not in induced T-regs (iT-regs), and corresponding Helios⁺ and Helios⁻ populations of FOXP3⁺ T-regs also exist in humans, suggesting that Helios may delineate human nT-regs (8).

Recent research has demonstrated reduced expression of FOXP3 and TGFB1 in NP in both Caucasian and Chinese patients compared with nasal mucosa from healthy controls, indicating that impaired T-reg function in NP may be responsible for the uncontrolled Th2 response associated with this disorder (9, 10). However, the hypothesis that T-regs are decreased in chronic airway inflammation has been challenged by several studies that report an increased frequency of T-reg in moderate/severe asthma and allergic rhinitis (AR) that were not affected by glucocorticosteroid (GC) treatment (11–13). These conflicting data indicate that the regulation of T-reg frequency in T-cell-mediated airway inflammation (including NP, AR, and asthma) has yet to be fully elucidated. To clarify the role played by T-reg cells in NP, we systematically analyzed the expression levels of a panel of T-reg and T-helper cell signature genes by DNA microarray and real-time RT-PCR analysis and determined how their expression was affected by GC treatment.

Methods

Study subjects

Patients with NP ($n = 29$) and non-NP controls ($n = 19$) were recruited from the Department of Otolaryngology in the First Affiliated Hospital, Sun Yat-Sen University, and the Department of Otolaryngology in the Qilu Hospital, Shandong University. The clinical characteristics of the subjects are shown in Table 1. Six patients had a history of physician-diagnosed asthma, but all were in a stable condition without any active treatment during the previous 3 months. All patients with NP were bilateral with Grade-3 NP that had completely obstructed the nasal cavity (1). Biopsies were obtained during functional endoscopic sinus surgery. In 13 patients with NP, polyp biopsies were taken both prior to the initiation of therapy (GC-naïve) and after oral prednisone treatment (GC-treated; 10 mg thrice per day for 10 days). Biopsies of inferior turbinate (IT) mucosa were obtained from 19 non-NP control patients with septal deviation, who had been scheduled for septal plastic surgery. None of the study subjects had concurrent upper respiratory infections or had taken any form of GC or antibiotic treatment within the 3 months preceding the study. Approval to conduct the study was obtained from the Institutional Review Boards of the Sun Yet-Sen University, Shan Dong University, and National University of Singapore.

Histology

Paraffin sections of nasal tissues were stained with hematoxylin and eosin (for evaluation of eosinophils) and immunohistochemical staining (for evaluation of neutrophils, CD4⁺ T cells, FOXP3⁺ T-reg, and Helios⁺ T-reg). Human tonsils

Table 1 Patient characteristics and cellular infiltration

	Healthy controls	Patients with NP*	<i>P</i> -value†
Sample size (number)	19	29	N/A
Age (years), median (1st–3rd quartile)	33 (26–40)	45 (32–50)	N/A
Gender (M/F)	13/6	20/9	N/A
Atopy	2	4	N/A
Asthma	0	6	N/A
Epithelial hyperplasia‡	2	21	<0.001
Cellular infiltration (%) median (1st–3rd quartile)			
Eosinophils	4 (3–5)	13 (5–56)	<0.001
Neutrophils	5 (3–8)	11 (6–21)	0.002
CD4 ⁺ cells	10 (4–14)	12 (6–32)	NS
FOXP3 ⁺ T-reg cells	0.7 (0–0.9)	6.5 (3.1–10.5)	<0.001

*Two NP paraffin sections were of poor quality and were not evaluated by immunohistochemistry.

†*P*-value was calculated by Mann–Whitney two-tailed test. *P*-values < 0.05 were considered significant. N/A, not applicable; NS, not significant.

‡Epithelium with more than four layers was defined as ‘epithelial hyperplasia’.

from patient with tonsillitis served as positive control tissues for FOXP3 and Helios staining. Detail of the immunohistochemistry procedure and antibodies used is provided in the Supporting Information.

The proportions of eosinophils, neutrophils, CD4⁺ T cells, FOXP3⁺ T-reg, and Helios⁺ T-reg were calculated among 200 leukocytes counted at 400× magnification in every five fields with high intensity of positive cells (formula: positive staining cells/200 leukocytes × 100%). Eosinophilia or neutrophilia was defined as eosinophil or neutrophil frequency ≥ 10%.

Microarray

Total cellular RNA was isolated from nasal tissues using RiboPure Kit (Applied Biosystems, Foster City, CA, USA). Nine GC-naïve NP tissue samples and five controls were selected for microarray analysis using the Human Gene 1.0 ST Array System (Affymetrix, Santa Clara, CA, USA). The microarray data were processed according to the manufacturer's instructions. Details of microarray analyses are provided in the Supporting Information.

Quantitative PCR

Total RNA was reverse-transcribed, and real-time RT-PCR analysis was performed using TaqMan gene expression assays (Table S1). Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_t}$ method (14), with GAPDH as a reference.

Statistical analysis

All data were analyzed using SPSS statistical software V16.0 (SPSS Inc., Chicago, IL, USA). Differences in mRNA levels

and cell counts between GC-naïve NP vs control groups were assessed by Mann–Whitney two-tailed test. Wilcoxon matched-pairs signed-rank test was performed to compare the gene expression differences between GC-treated and GC-naïve NP samples. Spearman's rank analysis was used to assess the correlation of gene expression levels for FOXP3 with other gene markers and cell numbers. P -values < 0.05 were considered significant.

Results

Patient characteristics and cellular infiltration

Clinical characteristics and histopathologic profiles of the untreated patients with NP are summarized in Table 1. The infiltration of eosinophils ($P < 0.001$) and neutrophils ($P = 0.002$) was significantly increased in GC-naïve NP compared with controls, and 56% (15/27) of patients with NP displayed evidence of airway eosinophilia. In contrast, CD4+ T-cell numbers were not significantly different between GC-naïve NP and healthy controls. FOXP3⁺ T-regs were detected in all samples of patients with NP, but these cells were only present in 42% (8/19) of the control samples. Among control nasal tissues that did exhibit FOXP3⁺ cells, this population was present at a reduced frequency compared with GC-naïve patients with NP. Representative sections of FOXP3 staining in NP and control tissues are shown in Fig. 1. In NP mucosa, FOXP3⁺ cells were mainly localized around the small blood vessels in the lamina propria, while some were present in the epithelium. Few or no FOXP3⁺ cells were found in nasal mucosa samples from control subjects. Helios staining was readily detected in tonsil tissues (Fig. S1), but was scarce in nasal tissues from patients with

NP and control samples, indicating that most T-regs in NPs were iT-regs. After GC treatment of patients with NP, eosinophil numbers were significantly decreased, but the frequency of other inflammatory cells (neutrophils and CD4+ T cells) and FOXP3⁺ T-reg cells was not affected (Fig. S2).

In GC-naïve NP, the percentage of FOXP3⁺ cells was negatively correlated with eosinophil frequency ($r = -0.406$, $P = 0.03$) but positively correlated with CD4+ T-cell numbers ($r = 0.568$, $P = 0.002$) (Fig. S3). Interestingly, the eosinophil cell count was negatively correlated with neutrophil numbers ($r = -0.458$, $P = 0.01$) and with CD4+ T-cell infiltration ($r = -0.476$, $P = 0.01$) (Fig. S3).

Transcriptional profile of T-cell-associated genes

Table 2 summarizes the expression change of the selected T-cell- and eosinophil-associated genes of interest. Th2-related genes were increased, while Th1 signature gene IFNG was decreased in GC-naïve NP compared with controls. The microarray results were described in detail in the Supporting Information.

Quantitative PCR evaluation

To evaluate the microarray results, the T-reg-cell-associated markers (FOXP3, TGFB1, IL10, SMAD3, IL2RA, and JAK3), Th1/Th2/Th17 transcription factors (T-bet, GATA3, and RORc, respectively), and eosinophil-related genes (CCL13 and CCL18) were further analyzed in a larger sample size (29 GC-naïve NPs and 19 controls) by real-time RT-PCR. The PCR data for TGFB1, SMAD3, IL2RA, JAK3, T-bet, CCL13, and CCL18 were comparable to the results obtained by microarray analysis. No significant differences

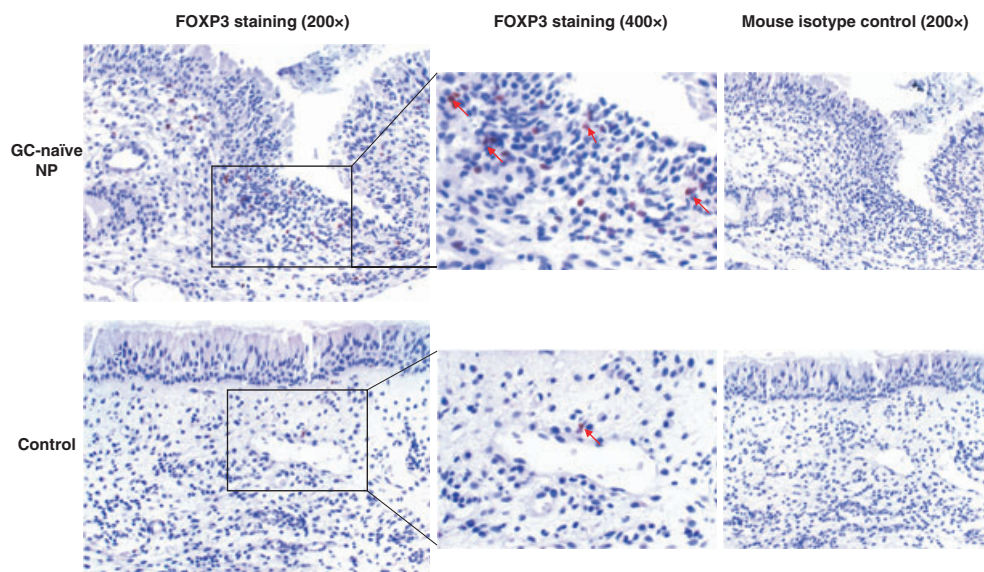


Figure 1 Expression of FOXP3 protein determined by means of immunohistochemistry in the representative tissues from a patient

with NP (before GC treatment) and control. Arrows (red color) indicate FOXP3-positive cells. Original magnification: 200 $\times$ or 400 $\times$.

Table 2 Modulated expression of T cell and eosinophil-associated genes

Gene symbol	Description	Fold*	FDR (%)
Genes important for T-reg cell development and function			
IL2RA [†]	Interleukin 2 receptor, alpha	2.53	0.19
JAK3 [†]	Janus kinase 3	1.84	0.97
TGFB1 [†]	Transforming growth factor, beta 1	1.73	0.75
SMAD3 [†]	SMAD family member 3	1.51	0.76
FOXP3 [†]	Forkhead box P3	1.06	37.11
IL10 [†]	Interleukin 10	1.30	8.70
Th-specific transcription factors			
RORc [†]	RAR-related orphan receptor C	0.68	1.81
GATA3 [†]	GATA-binding protein 3	0.77	0.48
T-bet [†]	T-box 21	0.97	5.48
Markers related to Th2 response and eosinophil recruitment			
CCL18 [†]	Chemokine (C-C motif) ligand 18	57.90	0.76
CCL13 [†]	Chemokine (C-C motif) ligand 13	6.52	0.76
IL5RA	Interleukin 5 receptor, alpha	3.20	0.77
IL1RL1	Interleukin 1 receptor-like 1 (IL33 receptor)	3.07	1.62
CCL11	Chemokine (C-C motif) ligand 11 (Eotaxin 1)	2.89	1.60
CCL24	Chemokine (C-C motif) ligand 24 (Eotaxin 2)	2.72	1.62
CCR3	Chemokine (C-C motif) receptor 3	2.41	0.94
IL4R	Interleukin 4 receptor	1.96	0.76
Markers related to Th1 response			
IL18	Interleukin 18	1.92	3.09
IL18R1	Interleukin 18 receptor 1	1.89	0.76
IFNG	Interferon, gamma	0.61	0.77

FDR, false discovery rate.

*Fold change was calculated by GC-naïve NP vs control.

[†]Genes were evaluated in real-time RT-PCR.

in the levels of FOXP3, IL10, GATA3, and RORc were detected by microarray analysis, but changes in the expression of these gene factors were instead revealed by real-time RT-PCR. This discrepancy may be a consequence of the low sensitivity of the microarray technique and the small sample number.

The expression levels of T-reg genes were significantly higher in GC-naïve patients with NP than in controls (FOXP3, 6.51-fold; TGFB1, 1.93-fold; IL10, 1.98-fold; SMAD3, 1.63-fold; IL2RA, 3.96-fold; and JAK3, 2.25-fold; Fig. 2). GATA3 and RORc expression was significantly decreased (2.05-fold and 1.98-fold, respectively) in GC-naïve NP vs controls (Fig. 2), while T-bet expression was unchanged. After GC treatment, an increase in RORc expression was observed (2.61-fold), while no significant changes

were detected in any other T-reg-or T-helper-cell-associated genes (Fig. S4). The two eosinophil-related genes (CCL13, 45.5-fold; CCL18, 144-fold) were highly upregulated in GC-naïve NPs (Fig. 2), but only CCL13 (3.27-fold) was significantly suppressed in NPs after GC therapy (Fig. S3).

We further investigated the relationships between these T-cell-associated genes (Fig. 3). The expression of FOXP3 was positively correlated with other T-reg-cell-associated genes TGFB1 ($r = 0.757$, $P < 0.001$), IL10 ($r = 0.312$, $P = 0.03$), SMAD3 ($r = 0.528$, $P = 0.004$), IL2RA ($r = 0.618$, $P < 0.001$), and JAK3 ($r = 0.682$, $P < 0.001$) in GC-naïve NP and controls. FOXP3 was also negatively correlated with GATA3 ($r = -0.372$, $P = 0.009$) and RORc ($r = -0.471$, $P < 0.001$), while no significant correlation with T-bet was evident ($r = 0.092$, $P = 0.191$).

In all GC-naïve NP tissues, the expression level of FOXP3 mRNA was negatively correlated with eosinophil frequency ($r = -0.607$, $P < 0.001$), but positively correlated with CD4⁺ cell counts ($r = 0.432$, $P = 0.02$) (Fig. 4). FOXP3 expression level was not significantly correlated with neutrophil counts (data not shown). CCL13 and CCL18 mRNA levels were positively correlated with eosinophil counts, but they were negatively correlated with FOXP3 expression level (Fig. 4).

Discussion

The immunologic nature of the inflammatory infiltrate in NP and the role T-reg play in NP inflammation remain unclear. In the current study, we investigated the presence of T-reg and T-helper cells, as well as their related genes in biopsies obtained from patients with NP. NP samples were collected from two separate cities, one in eastern China and one in southern China, but no significant differences were observed in histomorphology or molecular characterization of these tissues. All tested molecular and cellular markers were also not significantly different between the six asthmatic patients and non-asthmatic NP patients.

FOXP3 mRNA levels were significantly upregulated together with other T-reg-associated genes (TGFB1, IL10, SMAD3, IL2RA, and JAK3) in GC-naïve NP relative to controls. An increased frequency of FOXP3⁺ cells (considered as Helios⁺ i-Tregs) was also found in biopsies of NP vs controls. In addition, there was a negative relationship between the expression levels (both at the cellular and mRNA levels) of FOXP3 and eosinophil counts, indicating that the data are consistent with reports that demonstrated an increase in FOXP3⁺ T-reg in moderate/severe asthma and AR tissues (12, 13) and with experimental data describing the role of T-reg in animal models of chronic asthma (15).

FOXP3 is widely accepted as a unique factor of the T-reg cell lineage. FOXP3 not only is essential in T-reg development but also performs a suppressive function in controlling the transcription of pro-inflammatory genes (including T-helper-cell-derived cytokines) (5, 16). The expression of FOXP3 is regulated by a variety of gene molecules produced by both inflammatory cells and structural cells. TGFB1 is a critical factor in T-reg cell growth and differen-

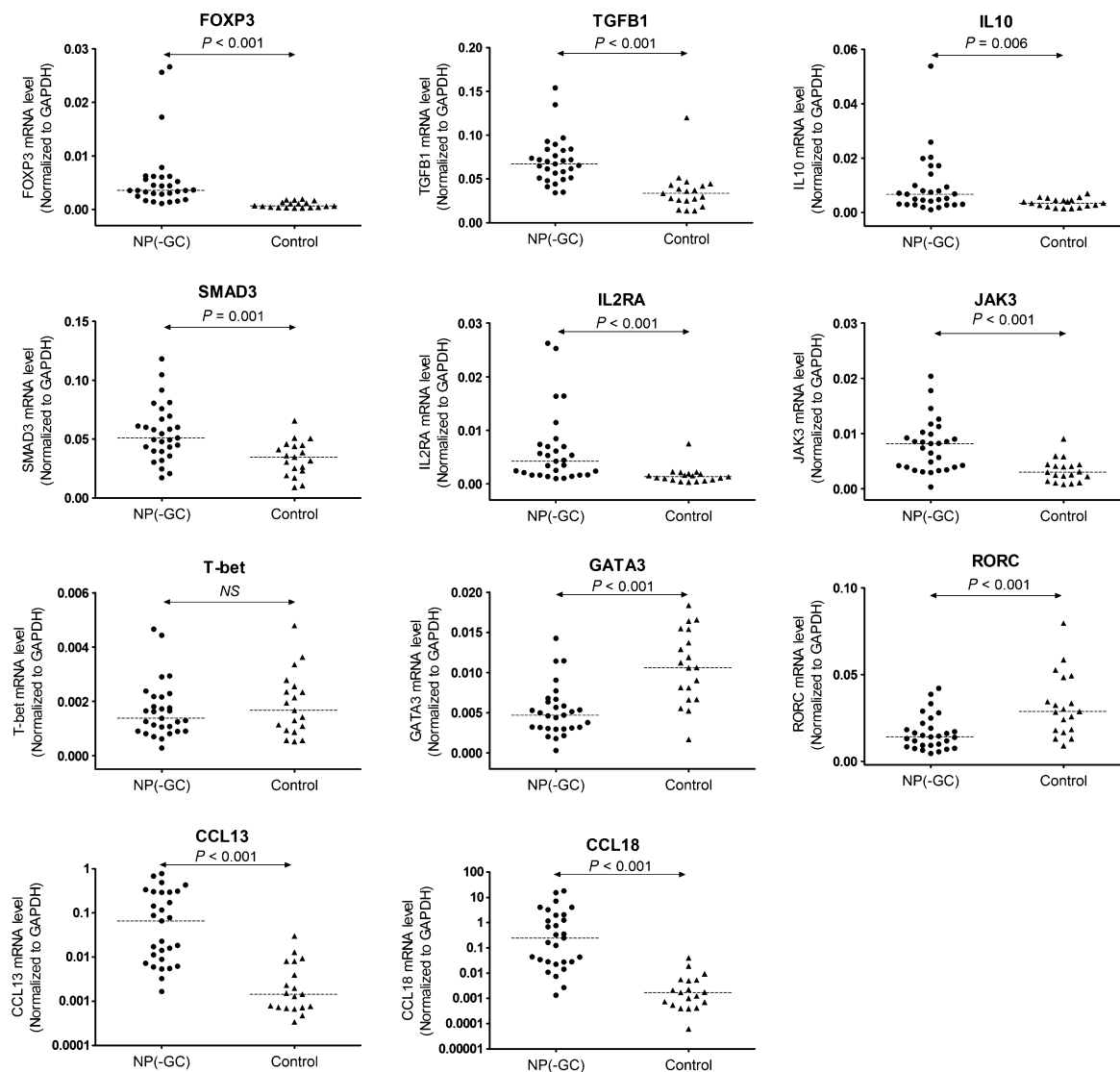


Figure 2 Quantitative determination of mRNA by real-time RT-PCR for FOXP3, TGFB1, IL10, SMAD3, IL2RA, JAK3, T-bet, GATA3, RORC, CCL13, and CCL18 in NP tissues from GC-naïve NP (GC) patients (solid circles) and inferior turbinate from the controls (solid triangles). All target mRNA relative expression levels are displayed

as ratios of GAPDH transcript levels in the same RNA sample. In graphs of CCL13 and CCL18, Y-axis scale is logarithmic (base 10)-transformed. Medians are indicated by horizontal dashed lines. Statistical analyses were performed by Mann-Whitney two-tailed test. 'NS' indicates not significantly different.

tiation that can activate either classic SMAD signaling pathways in T cells or SMAD-independent pathways (e.g., IL2RA-JAK3) and can induce the expression of FOXP3 (5, 16). The major cellular sources for TGFB1 are epithelial cells and eosinophils in airway tissues (17). Accordingly, we found that epithelial hyperplasia and infiltration of eosinophils in the lamina propria were two major histopathologic changes in NP tissues, which is in line with our previous studies (18, 19). With a close correlation between mRNA levels of FOXP3 and TGFB1, as well as with other activation signaling factors (SMAD3, IL2RA, and JAK3), our data suggest that both TGFB1-and TGFB1-signaling

activities are involved in the upregulation of FOXP3 mRNA in the inflammatory site of NP. In addition, we have also found the upregulation of IL10 in NP samples, which is the important cytokine produced by FOXP3⁺ T-reg cells and contribute to immune homeostasis (20). These data further support the increase in T-reg immune activity in NP inflammation.

In the current report, expression levels of Th1/Th2/Th17 transcription factors T-bet/GATA3/RORC were also analyzed. The mRNAs of GATA3 and RORC were downregulated in GC-naïve NP vs controls, indicating the reduction of Th2 and Th17 effector cells in NP. GATA3 and RORC have

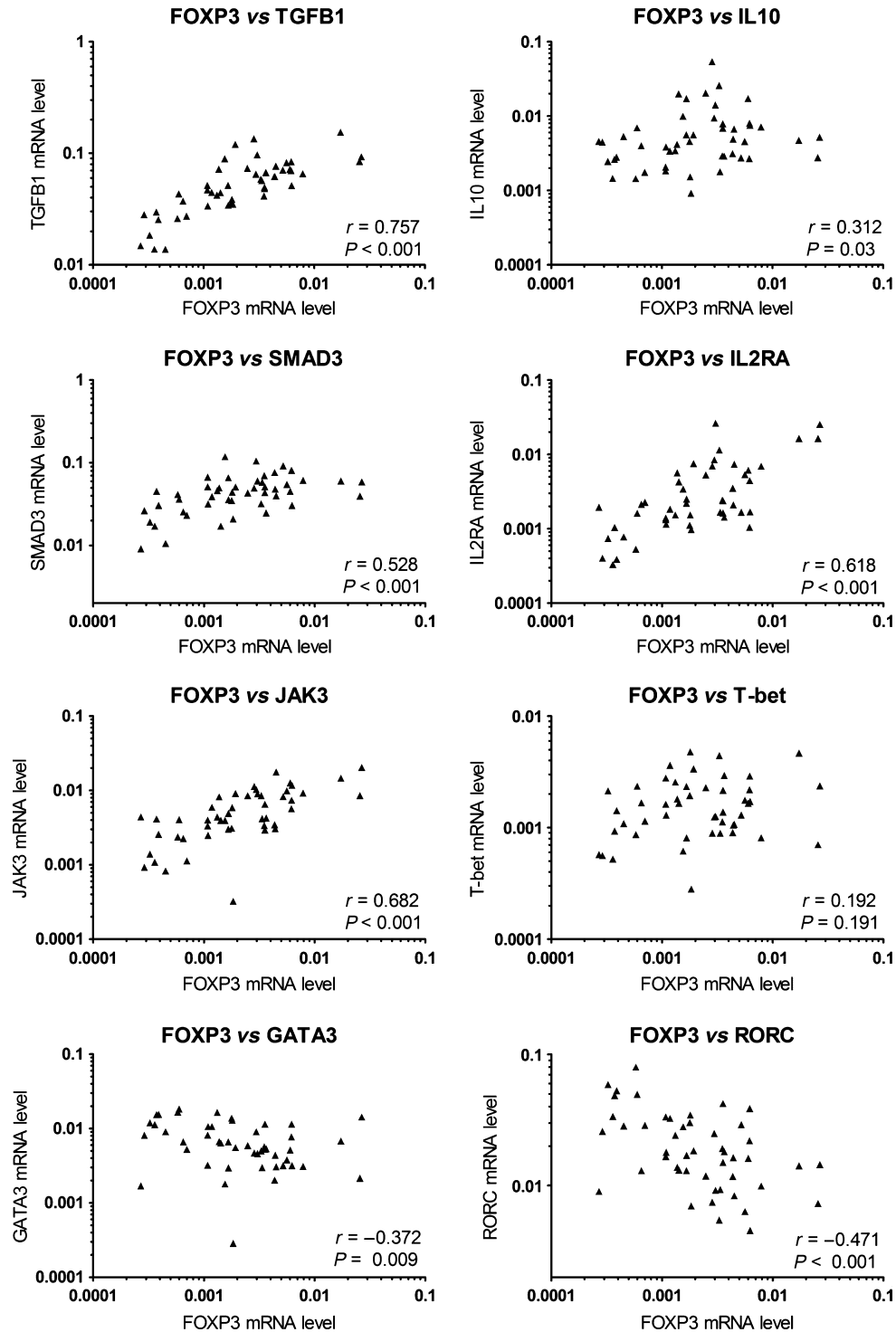


Figure 3 Correlation between mRNA level of FOXP3 and TGFB1, IL10, SMAD3, IL2RA, JAK3, T-bet, GATA3, and RORc was illustrated in GC-naïve NP and control tissues (solid triangles) by Spear-

man's rank analysis. Y-axis scale is logarithmic (base 10)-transformed. r , Spearman's rank correlation coefficient.

been reported to inhibit the expression of FOXP3 via the binding of its promoter region (21, 22). Moreover, the mRNAs of GATA3 and RORc were negatively correlated

with the FOXP3 mRNA in our analyses. These results suggest that the increase in FOXP3 may be associated with the decrease in GATA3 and RORc in NP.

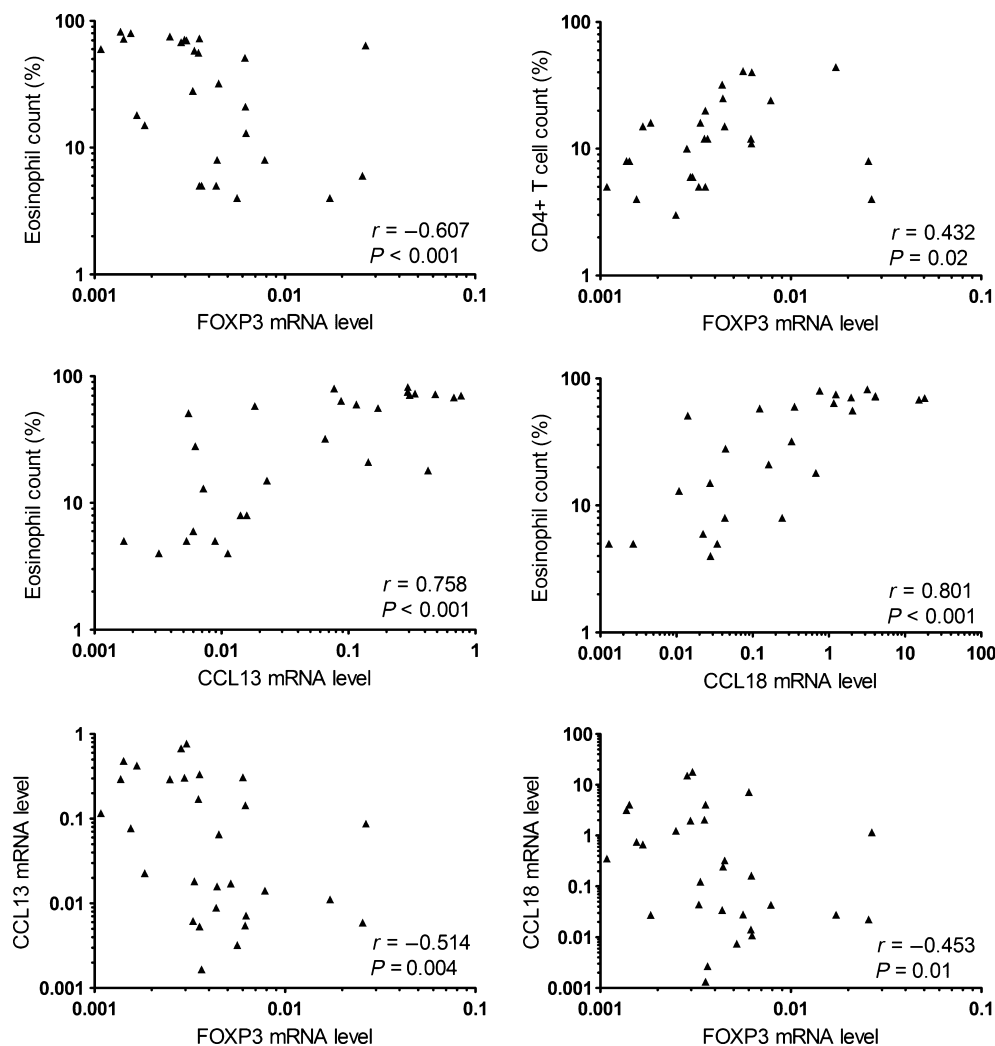


Figure 4 Correlation between mRNA level of FOXP3 and eosinophils and CD4 cells, CCL13/CCL18 mRNA levels and eosinophils and FOXP3 expression was described in GC-naïve NPs (solid triangles)

by Spearman's rank analysis. Y-axis scale is logarithmic (base 10)-transformed. r , Spearman's rank correlation coefficient.

Our data further indicated that many Th2-related cytokines/receptors (Eotaxin-1, CCL13, CCL18, IL5R, and CCR3, etc.) are upregulated in GC-naïve NP, while archetypal Th1 cytokine IFNG was downregulated. The cellular sources of the chemokines could be lymphocytes, epithelial cells, eosinophils, and macrophages, as they have been found to contribute to the chemoattraction of Th2 cells and eosinophils both directly and indirectly (23, 24). In particular, CCL18 and CCL13 were the top two Th-2-related chemokines with significant increase in NPs. These two genes have been reported to contribute to the eosinophil recruitments in NP (25, 26). Our data are able to confirm a positive correlation between CCL13/CCL18 and eosinophils, but a negative correlation between the expression levels of FOXP3 and CCL13/CCL18 in NP. The gene expression pattern uncovered in our study suggests that the immune-pathologic mechanism underlying NP is characterized by a Th2-polarized

milieu and severe eosinophilia. The enhanced levels of Th2 markers and eosinophil infiltration detected imply that the increase in T-reg frequency failed to curtail Th2 inflammatory responses in GC-naïve NP.

Glucocorticosteroid is the first-choice pharmacological treatment for NP, and although the suppressive function of GC in inflammatory diseases is marked, we could not find any change in FOXP3 expression or any difference in T-reg-related markers in response to oral GC therapy in NP. Our data are in agreement with previous reports showing that topical GC is unable to alter the number of FOXP3⁺ T-reg in AR (12), suggesting that GC attenuation of inflammation may not depend on T-reg modulation.

Our data appear inconsistent with the rationale that severe inflammatory disease is directly associated with inadequate expression of FOXP3. Indeed, other investigators have reported a reduction in FOXP3 mRNA accompanied by a

reduction in FOXP3⁺ cells in NP and have suggested that deficient T-reg function contributes to disease (9, 27). These contrasting findings can perhaps be attributed to the heterogeneity of NP in different ethnic groups and between disease profiles. Although the role of T-reg in controlling T-helper-cell-mediated inflammatory responses is increasingly apparent, the presence of large numbers of FOXP3⁺ T-reg cells in severe inflammation remains complicated. It is unclear whether the function of T-reg cells is impaired in chronic inflammation, or whether T-reg are simply overwhelmed by pro-inflammatory signals, or whether T-reg themselves could even contribute to inflammatory pathology in different subtypes of disease.

In conclusion, we report the increased expression of FOXP3 mRNA/protein and functionally related genes in NP tissues and that expression of these genes were not altered by short-term oral GC therapy. Our data confirm the common pathological features of eosinophilia and Th2-polarized inflammation in Chinese NP and demonstrate that FOXP3⁺ iT-reg cells preferentially accumulate in the nasal mucosa during inflammation. Increased T-reg frequency may not suffice to completely suppress the Th2 response in NP, but may perhaps succeed in limiting the severity of this inflammation.

Acknowledgments

This study was supported by grants (NMRC/1162/2008 and NMRC IRG10may086) from the National Medical Research Council (NMRC) of Singapore. The authors thank Neil McCarthy for proof reading the manuscript.

Author contributions

The study was designed by Wang, De Yun; Shi, Li; Li, Chun Wei; Zhang, Ke Ke; Li, Tian Ying; Lin, Zhi Bin; and Curotto de Lafaille, Maria Alicia. Samples were collected by Wang, De Yun; Shi, Li; Li, Chun Wei; Zhang, Ke Ke; Li, Tian Ying; and Lin, Zhi Bin, and experimental work was performed by Li, Chun Wei; Zhang, Ke Ke; Li, Ying Ying;

and Lin, Zhi Bin. Data were analyzed by Wang, De Yun; Shi, Li; Li, Chun Wei; Zhang, Ke Ke; Li, Tian Ying; and Curotto de Lafaille, Maria Alicia. Manuscript was written by Wang, De Yun; Shi, Li; Li, Chun Wei; and Curotto de Lafaille, Maria Alicia.

Conflict of interest

The authors declared that no conflict of interest exists.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

Figure S1. Expression of Helios and FOXP3 protein determined by means of immunohistochemistry in the tonsil tissue from tonsillitis patient.

Figure S2. Cell count (in percentage) of eosinophil, neutrophil, CD4⁺ T cell, and FoxP3⁺ Treg cell in NPs in response to GC treatment.

Figure S3. Correlation between FoxP3⁺ T-reg cells vs eosinophils and CD4⁺ T cells, and eosinophils vs neutrophils and CD4 T cells were described in GC-naïve NP (solid triangles) by Spearman rank analysis.

Figure S4. mRNA expression levels of FOXP3, TGFB1, SMAD3, IL2RA, JAK3, T-bet, GATA3, and RORc in NP in response to GC treatment.

Table S1. Identity for Taqman Gene Expression Assays-On-DemandTM.

Table S2. Expression change of Treg cells related genes and Th cells transcription factors (by microarray analysis).

Table S3. Significant expression change of Cytokines/chemokines/receptors and CD molecules (by microarray analysis).

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Online Supplement

Expression profiles of regulatory and helper T cell-associated genes in nasal polyposis

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Material and Methods

Histological experiment and evaluation

Nasal biopsies were fixed in formalin, embedded in paraffin and sectioned at 4µm with a Leica microtome (Leica, Wetzlar, Germany). Briefly, (i) slides were processed with Target Retrieval Buffer (Dako A/S, Glostrup, Denmark) and endogenous peroxidase activity was blocked with 3% H₂O₂; (ii) slides were stained with mouse anti-human neutrophil elastase monoclonal antibody [Clone NP57] (Dako A/S), mouse anti-human CD4 monoclonal antibody [Clone 4B12] (Dako A/S), mouse anti-FOXP3 monoclonal antibody [Clone 236A/E7] (Abcam, Cambridge, UK), and rabbit anti-Helios polyclonal antibody (Abcam) at dilutions of 1:500 and 1:400, 1:200, and 1:50

respectively; (iii) species- and subtype-matched antibodies were used as negative controls [N-Universal Negative Control for mouse IgG] (Dako A/S); (iv) slides stained with primary antibodies and negative controls were incubated at 4°C overnight and were then incubated with DAKO EnVision+System-HRP (Dako A/S) at room temperature for 30min; (v) daminobenzidine was used as substrate for color development. All slides were counterstained with hematoxylin.

Microarray data analysis

Affymetrix probe-level data set in CEL files were generated by Human Gene 1.0 ST Array System (Affymetrix): GC-naïve NP tissue (9 chips) versus controls (5 chips). The raw data were normalized by the Robust Multichip Average (RMA) method, available in RMAexpress software version 0.4.1 Release (Free version available in <http://rmaexpress.bmbolstad.com/>) (1). RMA consists of a background adjustment, quantile normalization, and the median-polish summary method, producing a single normalized expression set for each comparison. RMA transformed data were on the log base 2 scale.

The RMA normalized data was imported to Microsoft Excel spreadsheets and formatted for analysis by the Significant Analysis of Micorarray (SAM) software, version 3.00 (Free version available in <http://www-stat.stanford.edu/~tibs/SAM/>) (2). Differentially expressed genes in were identified using the SAM method, which accepts normalized expression data sets and identifies statistically significant changes in gene expression by assigning each gene a score (called “*d* score”) based on its change in expression relative to the standard deviation of repeated measurements. Genes with scores greater than a threshold are deemed potentially

significant. The percentage of such genes identified by chance is the false discovery rate (FDR). SAM uses permutations of empirical measurements to estimate the FDR for the called list in the form of a 90% confidence interval. FDR controls the expected proportion of incorrectly rejected null hypotheses (type I errors). In addition, fold change has also been indicated in each probe ID in SAM generated gene list. The options selected for the SAM analysis: (1). Response type: two-class, unpaired data (comparison of GC-naïve NP versus control); (2). Data logged: logged (base 2); (3). Number of permutations: 5000. Hence, genes identified with FDR of 0.06 in the dataset of GC-naïve NP versus control were deemed significant if they passed a 1.5-fold change filter. Hence, all the transcripts (n=28,869) were issued a fold change with FDR.

Gene annotations of the analyzed transcripts were verified by means of web-based program Netaffx™ Analysis Center (<http://www.affymetric.com/analysis/index.affx>). This resource contains probe sequences and up to date gene annotations and allows researchers to quickly search for gene annotation information, compare and refine results, and export data into Excel-friendly formats. Two filtration steps were performed: Firstly, those transcripts which did not contain identified gene symbols were filtered. Secondly, those genes with redundant probe identities and those transcripts with unknown gene ontology (such as those annotated with “chromosome open reading frame”, “hypothetical protein”, “KIAAs” and “family with sequence similarity”) were filtered. Finally, totally 2,111 genes (supervised gene list) with well-known annotation were determined.

To identify changes in inflammatory genes which are related to T-cell functions, two strategies were used to analyse the array data: (i) Screening the T-reg-associated genes and the T-helper

cell-specific transcription factors via the unsupervised whole gene list (no threshold, with specific FDR value) set for the significant level of the genes; (ii) Summarizing cytokines/receptors, chemokine/receptors, and CD molecules via the supervised gene list (threshold set for the significant genes).

Results

Transcriptional profile of T-cell-associated genes

Microarray analysis was performed in 9 GC-naïve NP and in 5 controls. From the unsupervised whole gene list, a group of T-reg and T-helper cell-specific markers were selected based on published data (3-5) and their fold changes (GC-naïve NP relative to controls) are listed in **Table E2**. TGFB1, SMAD3, IL2RA, JAK3, and RUNX1 were up-regulated greater than 1.5 fold in GC-naïve NP compared with controls. However, the expression of T-reg and Th1/Th2/Th17 transcription factors FOXP3, T-bet, GATA3, and RORc as assessed by microarray were not altered (less than 1.5 fold change in GC-naïve NP versus controls).

In order to identify the significant Th-related genes, supervised gene list was determined based on a fold change threshold (1.5-fold) with FDR (0.06). Accordingly, 2,111 transcripts (1,241 up-regulated and 870 down-regulated) were differentially expressed in GC-naïve NP and controls. Among them, mRNA levels for 19 cytokines/receptors, 13 chemokines/receptors, and 29 CD molecules were significantly different between GC-naïve NPs and controls (**Table E3**).

Table 2 summarizes the expression change of the selected T-cell and eosinophil-associated genes of interest (based on the gene lists of **Table E2** and **Table E3**). Th2-related genes were increased, while Th1 signature gene IFNG was decreased in GC-naïve NP compared with controls.

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5. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 2009;**30**(5):616-625.

Table E1 Identity for Taqman Gene Expression Assays-On-Demand™

Gene Symbol	Description	Assay ID
FOXP3	Forkhead box P3	Hs01085834_m1
TGFB1	Transforming growth factor, beta 1	Hs00998133_m1
IL10	Interleukin 10	Hs00961622_m1
SMAD3	SMAD family member 3	Hs00969210_m1

IL2RA	Interleukin 2 receptor, alpha	Hs00907777_m1
JAK3	Janus Kinase 3	Hs00169663_m1
T-bet	T-box 21	Hs00203436_m1
GATA3	GATA binding protein 3	Hs00231122_m1
RORc	RAR-related orphan receptor C	Hs01076112_m1
CCL13	Chemokine (C-C motif) ligand 13	Hs00234646_m1
CCL18	Chemokine (C-C motif) ligand 18	Hs00268113_m1

Table E2 Expression change of Treg cells related genes and Th cells transcription factors (by microarray analysis)

Gene Symbol	Description	Fold*	FDR(%)#
<i>Genes important for Treg differentiation, survival and function</i>			
IL2RA [†]	interleukin 2 receptor, alpha	2.53	0.19
JAK3 [†]	Janus kinase 3	1.84	0.97
TGFB1 [†]	transforming growth factor, beta 1	1.73	0.75
SMAD3 [†]	SMAD family member 3	1.61	0.76
ITGB8	integrin, beta 8	1.40	8.10
STAT5A	signal transducer and activator of transcription 5A	1.26	6.22
TGFB1	transforming growth factor, beta receptor 1	1.02	40.12
FOXP3	forkhead box P3	1.06	37.11
IL10	Interleukin 10	1.30	8.70
IL2	interleukin 2	0.85	3.52
PRKCQ	protein kinase C, theta (also PKC-θ)	1.42	7.13
CARD11	caspase recruitment domain family, member 11	1.33	5.35
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.17	7.13

BCL10	B-cell CLL/lymphoma 10	1.02	35.43
MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.99	40.12
RUNX1 [†]	runt-related transcription factor 1	1.53	0.15
CTLA4	cytotoxic T-lymphocyte-associated protein 4	1.20	26.83
NRP1	neuropilin 1	1.03	38.67
ITGAE	integrin, alpha E	1.01	20.44
IRF4	interferon regulatory factor 4	0.85	13.37
ICOS	inducible T-cell co-stimulator	0.98	40.12
<i>Th-specific transcription factors</i>			
RORc	RAR-related orphan receptor C	0.68	1.81
GATA3	GATA binding protein 3	0.77	0.48
T-bet	T-box 21	0.97	5.48

* Fold change was calculated by GC-naïve NP versus control.

FDR, false discovery rate.

[†] Genes with fold change more than 1.5.

Table S3 Significant expression change of Cytokines/chemokines/receptors and CD molecules (by microarray analysis)

Gene Symbol	Description	Fold [*]	FDR(%) [#]
<i>Interleukins</i>			

IL5RA	interleukin 5 receptor, alpha	3.2	0.766
IL1RL1	interleukin 1 receptor-like 1 (IL33 receptor)	3.07	1.623
IL8RA	interleukin 8 receptor, alpha	2.96	1.623
IL8RB	interleukin 8 receptor, beta	2.71	1.623
IL2RA	interleukin 2 receptor, alpha (CD25)	2.53	0.766
IL1RAP	interleukin 1 receptor accessory protein	2.23	0.766
IL1R2	interleukin 1 receptor, type II	2.09	4.027
IL4R	interleukin 4 receptor	1.96	0.766
IL18	interleukin 18	1.92	3.086
IL20RB	interleukin 20 receptor beta	1.89	4.027
IL18R1	interleukin 18 receptor 1	1.89	0.766
IL3RA	interleukin 3 receptor, alpha	1.72	0.766
IL1RN	interleukin 1 receptor antagonist	1.7	5.24
IL2RG	interleukin 2 receptor, gamma	1.68	3.086
IL2RB	interleukin 2 receptor, beta	1.52	0.939
IL6 [†]	interleukin 6 (interferon, beta 2)	0.37	1.602
IFNG	interferon, gamma	0.61	0.766
IL11RA	interleukin 11 receptor, alpha	0.61	0.263

Chemokines

CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	57.9	0.766
CCL13	chemokine (C-C motif) ligand 13	6.52	0.766
CCL8	chemokine (C-C motif) ligand 8	3.87	4.027
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	3.02	5.906

CCL11	chemokine (C-C motif) ligand 11 (Eotaxin 1)	2.89	1.602
CCL24	chemokine (C-C motif) ligand 24 (Eotaxin 2)	2.72	1.623
CCR1	chemokine (C-C motif) receptor 1	2.53	0.766
CCR3	chemokine (C-C motif) receptor 3	2.41	0.939
CCR4	chemokine (C-C motif) receptor 4	2.24	0.939
CCL2 [†]	chemokine (C-C motif) ligand 2	2.08	5.24
CXCL16	chemokine (C-X-C motif) ligand 16	1.73	0.766
CCL22	chemokine (C-C motif) ligand 22	1.55	0.766
CCL28	chemokine (C-C motif) ligand 28	0.17	0.263
CXCL2	chemokine (C-X-C motif) ligand 2	0.5	0.263
CCL14	chemokine (C-C motif) ligand 14	0.54	0.263
CCL16	chemokine (C-C motif) ligand 16	0.62	0.263
CXCL14	chemokine (C-X-C motif) ligand 14	0.63	1.164
<i>CD molecules</i>			
CD163	CD163 molecule	3.62	1.623
CD36	CD36 molecule (thrombospondin receptor)	3.54	1.602
CD180	CD180 molecule	3.06	0.766
CD1C	CD1c molecule	3.05	1.602
CD1B	CD1b molecule	2.54	1.602
CD52	CD52 molecule	2.53	0.766
CD68	CD68 molecule	2.5	0.766
CD84	CD84 molecule	2.44	0.766
CD86	CD86 molecule	2.36	3.086
CD209	CD209 molecule	2.37	0.766

CD177	CD177 molecule	2.3	0.766
CD53	CD53 molecule	2.19	2.335
CD274	CD274 molecule	2.03	1.623
CD37	CD37 molecule	1.8	0.939
CD48	CD48 molecule	1.77	4.027
CD163L1	CD163 molecule-like 1	1.75	1.623
CD200R1	CD200 receptor 1	1.74	5.24
CD1E	CD1e molecule	1.69	0.766
CD300A	CD300a molecule	1.69	1.602
CD300LF	CD300 molecule-like family member f	1.69	0.939
CD244	CD244 molecule, natural killer cell receptor 2B4	1.68	0.766
CD177	CD177 molecule	1.67	0.766
CD300LB	CD300 molecule-like family member b	1.64	1.623
CD44	CD44 molecule (Indian blood group)	1.63	1.623
CD3E	CD3e molecule, epsilon (CD3-TCR complex)	1.57	2.335
CD28	CD28 molecule	1.55	2.335
CD3G	CD3g molecule, gamma (CD3-TCR complex)	1.53	4.027
CD22	CD22 molecule	1.52	3.086

* Fold change was calculated by GC-naïve NP versus control.

FDR, false discovery rate.

† IL6 and CXCL2 were significantly up-regulated at more than 1.5-fold in NP after GC treatment.

(unpublished data in previous microarray study)

Figure legends

Figure S1 Expression of Helios and FOXP3 protein determined by means of immunohistochemistry in the tonsil tissue from tonsillitis patient. Arrows indicate Helios or FOXP3 positive cells. Original magnification: 200 × or 400 ×.

Figure S2 Cell count (in percentage) of eosinophil, neutrophil, CD4⁺ T cell, and FoxP3⁺ Treg cell in NPs in response to GC treatment. Connecting lines indicate data obtained in the same subject before (-GC) and after GC (+GC) treatment. Statistical analyses were performed by Wilcoxon matched pairs sign rank test. “NS” indicates no significant difference.

Figure S3 Correlation between FoxP3⁺ T-reg cells versus eosinophils and CD4⁺ T cells, and eosinophils versus neutrophils and CD4 T cells were described in GC-naïve NP (solid triangles) by Spearman rank analysis. Y-axis scale is logarithmic (base 10) transformed. *r*, Spearman's rank correlation coefficient.

Figure S4 mRNA expression levels of FOXP3, TGFB1, SMAD3, IL2RA, JAK3, T-bet, GATA3, and RORc in NP in response to GC treatment. Connecting lines indicate data obtained in the same subject before (-GC) and after GC (+GC) treatment. Y-axis scale is logarithmic (base 10) transformed. Statistical analyses were performed by Wilcoxon matched pairs sign rank test. “NS” indicates no significant difference.

Figure E1

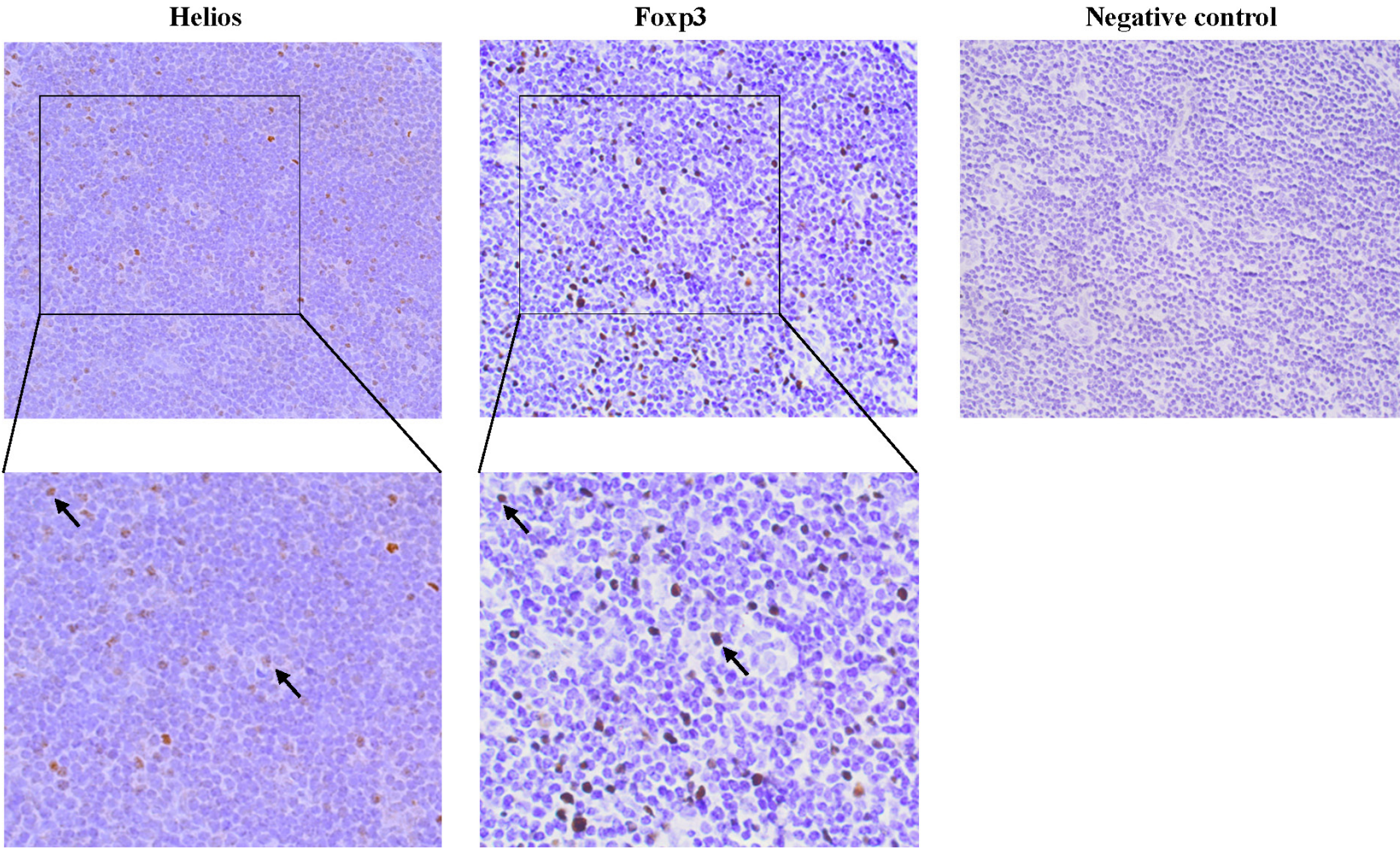


Figure E2

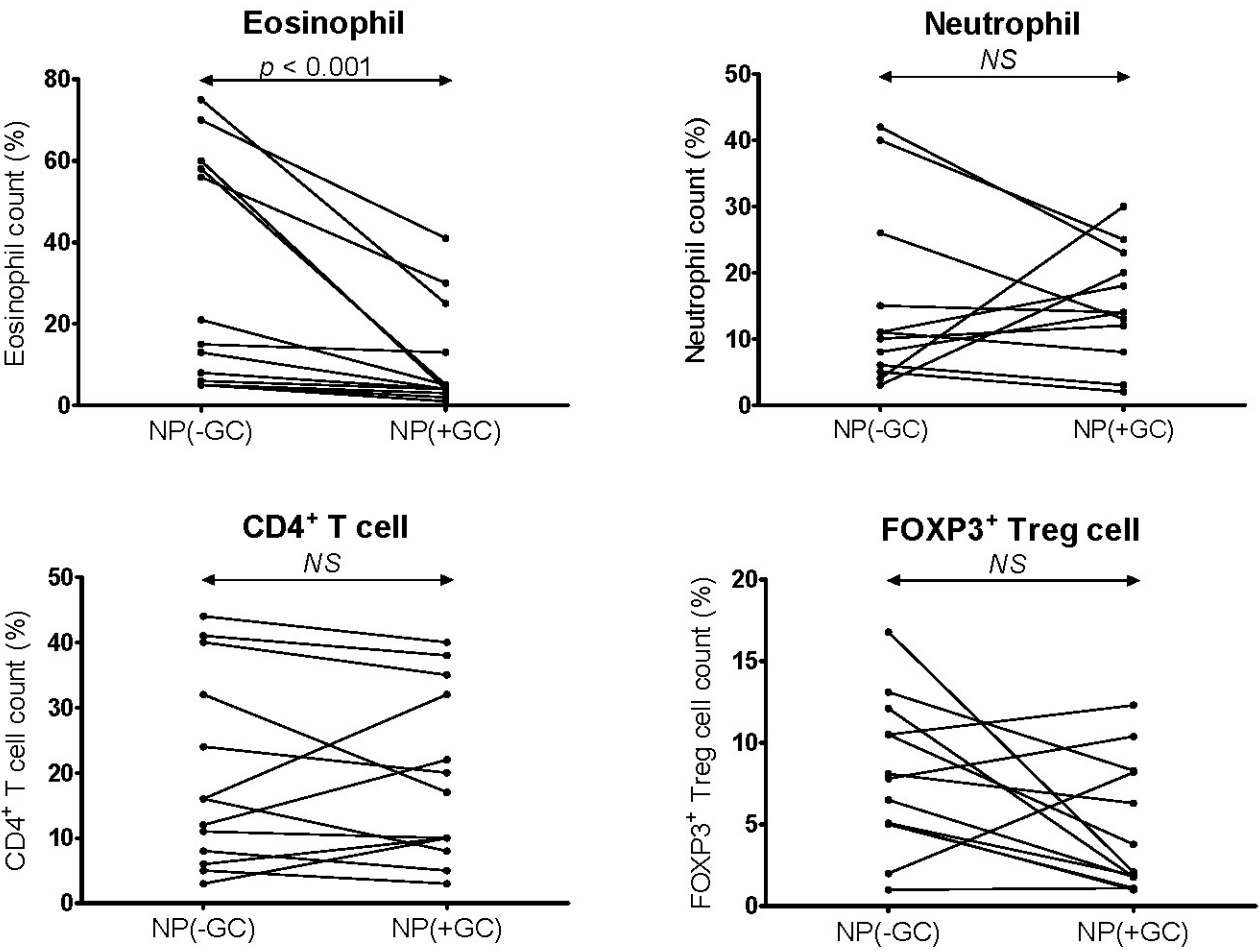


Figure E3

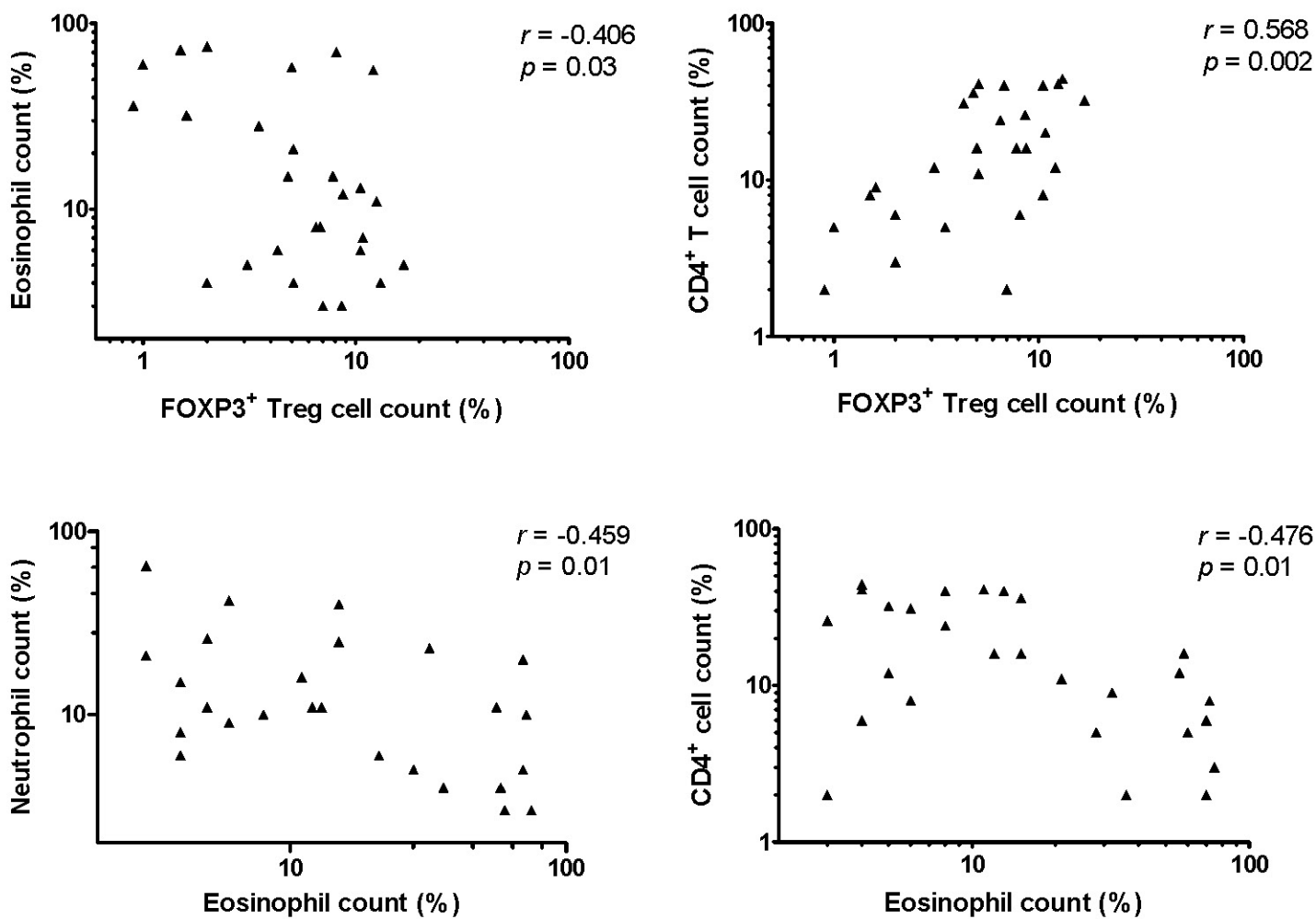


Figure E4

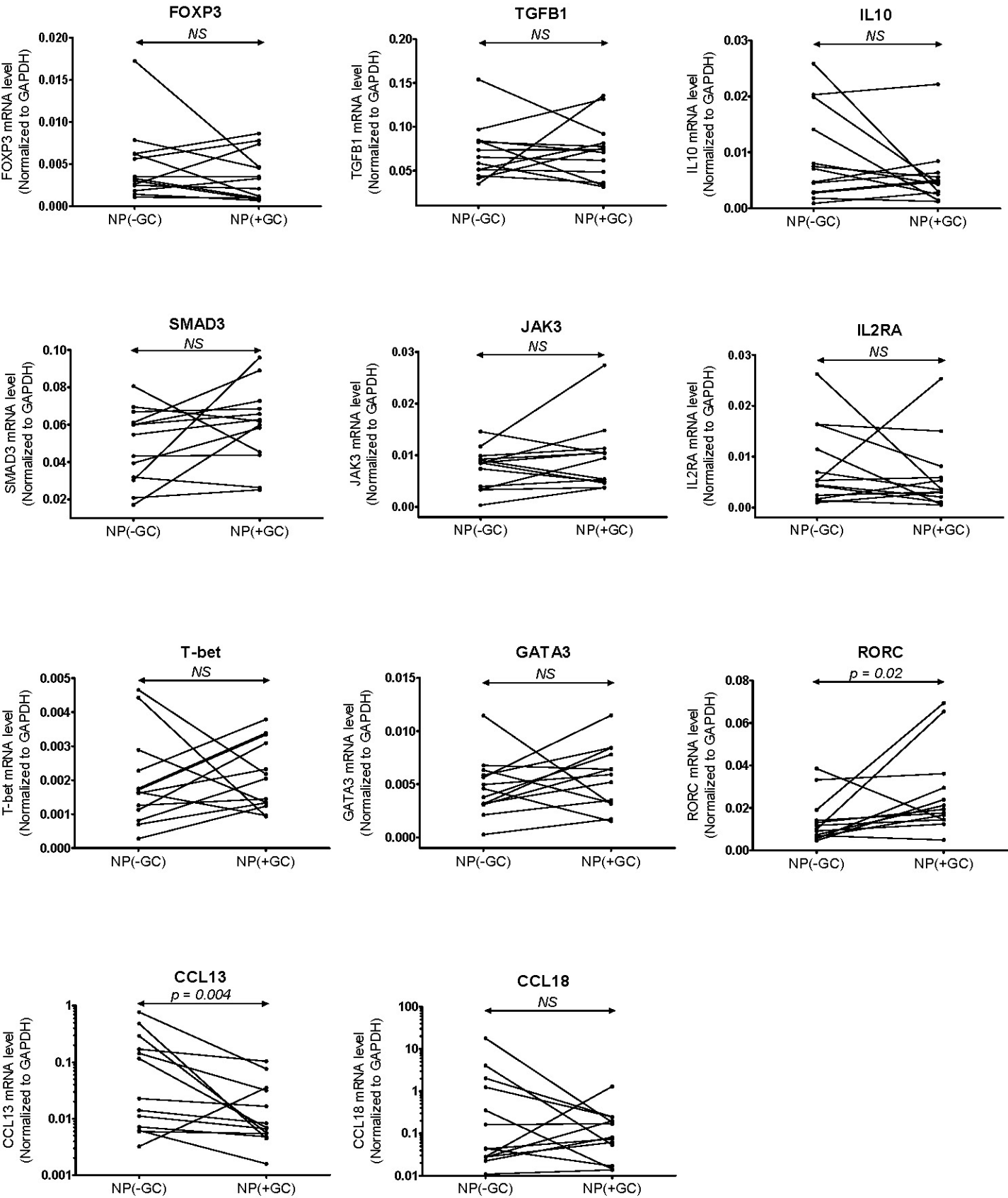


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CD48	CD48 molecule	1.77	4.027
CD163L1	CD163 molecule-like 1	1.75	1.623
CD200R1	CD200 receptor 1	1.74	5.24
CD1E	CD1e molecule	1.69	0.766
CD300A	CD300a molecule	1.69	1.602
CD300LF	CD300 molecule-like family member f	1.69	0.939
CD244	CD244 molecule, natural killer cell receptor 2B4	1.68	0.766
CD177	CD177 molecule	1.67	0.766
CD300LB	CD300 molecule-like family member b	1.64	1.623
CD44	CD44 molecule (Indian blood group)	1.63	1.623
CD3E	CD3e molecule, epsilon (CD3-TCR complex)	1.57	2.335
CD28	CD28 molecule	1.55	2.335
CD3G	CD3g molecule, gamma (CD3-TCR complex)	1.53	4.027
CD22	CD22 molecule	1.52	3.086

* Fold change was calculated by GC-naïve NP versus control.

FDR, false discovery rate.

[†] IL6 and CXCL2 were significantly up-regulated at more than 1.5-fold in NP after GC treatment.

(unpublished data in previous microarray study)