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**Th1, Th2, Th17 and T-reg pattern in psoriatic patients: modulation of cytokines and gene targets induced by Etanercept treatment and correlation with the clinical response.**

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## Abstract

**Background.** Psoriasis is sustained by pro-inflammatory CD4-positive T helper cells mainly belonging to the Th1, Th17 and Th22 lineage. **Objective.** The aim of our study was to identify the CD4+ T cell subsets (Th1, Th2, Th17 and Treg) modulations induced by Etanercept in psoriasis patients and to correlate them with the clinical response. **Methods.** We investigated the key transcription factors and related cytokine mRNA expression blood profile by Taqman QRT-PCR and flow cytometry protocols in 19 psoriasis patients treated by Etanercept and 19 healthy subjects. The essential transcription factors and cytokine profile analyzed in this study to identify the four different Th lineages were: Stat4, Tbet, IL12 and INF $\gamma$  for Th1; GATA3, IL4 for Th2; Stat3, RoR $\gamma$ t, IL-23p19 for Th17 and Foxp3, IL2 for T-reg. **Results.** An up-regulation of Th1 and Th17 with a corresponding down-regulation of T-reg subsets was found before treatment in psoriasis patients. Response to Etanercept could be associated to a significant reversal of the Th1/Th17 activation, and a concomitant up-regulation of Th2 and T-reg subsets. **Conclusion.** Our data may contribute to a better understanding of the mechanisms underlying the achievement of clinical response in psoriasis and could be helpful for the identification of early predictive markers of response.

## Introduction

Literature suggest that psoriatic lesions develop through an immune deregulation pathway comprising a dominant type 1 T helper (Th1) cell response and dysfunction of type 2 helper (Th2) cells and regulatory T (T-reg) cell response [1-4], dendritic cells, mast cells, and granulocytes participate in the pathogenesis [5, 6]. Although the real pathway that leads the CD4+ naïve-T cells to differentiate is intricate, the collaboration and cross-regulation among various essential cytokines in the activation/induction of key transcription factors during the process of Th cell differentiation have been well described [7-10]. Following triggering of the TCR by antigen ligation, peripheral Naïve-CD4+ T cells differentiate into effector T-cell that produce high levels of cytokines. Base on the cytokines that produce, effector T cells can be divided into Th1 (INF $\gamma$ , IL-2), Th2 (IL-4, IL-5, IL-9, IL-13), Th17 (IL-17, IL-21, IL-22), Th-9 (IL-9, IL10), Th-22(IL22, TNF, IL-13) and T-reg (TGF- $\beta$ , IL-35) subsets. Briefly CD4+ T cell lineage differentiation is driven by Stat proteins (Signal transducer and activator of transcription) which are activated by cytokines through phosphorylation. Stat proteins interact with specific transcription factors, the so-called Masters regulators (T-bet, GATA-3, ROR $\gamma$ t, Foxp3). The essential transcription factors of CD4+ T lineages are T-bet/Stat-4 (Th1), GATA-3/Stat5 (Th2), Stat3/RoR $\gamma$ t (Th17) and Foxp3/Stat5 (T-reg) therefore

Th1 and Th2 lineages can also be induced, in a different way, through the direct action of the specific Stats (Stat-4 and Stat-3) on cytokine genes [8]. Until recently, the Th17 cell subset, has been potentially involved in autoimmune diseases [11-16]. In humans, commitment to the Th17 lineage is dependent on different cytokines as IL-1 $\beta$  plus IL-6, IL-21, IL-1b, TGF- $\beta$  and IL-23 [17,18]. More recently the new Th-22 cell subset producing high level of IL-22 but not IL-17 or INF $\gamma$  has been described. Differentiation of Naïve-CD4<sup>+</sup> T cells versus Th-22 subset is promoted by IL-6 and TNF or plasmacytoid dendritic cells and is dependent by aryl hydrocarbon receptor (AHR) but independent by RORC [10]. Psoriatic skin lesions are reported to have increase gene expression of IL-23(p19), IL-17 and IL-22, suggesting the potential involvement of Th17 cells in psoriasis [19-22]. In addition, the T-reg dysfunction seem to be coupled with deregulation of Th17 and Th1 cell subsets in psoriasis [23, 24]. Few studies in literature analyze transcription factors gene expression and related cytokines of CD4<sup>+</sup> cell subsets and the modifications induced by treatment [25, 26]. We investigated the key transcription factors and same related cytokine mRNA expression profile in order to correlate them with the clinical response in a cohort of 19 patients with psoriasis during the treatment with Etanercept. The same expression profiles were also investigated in healthy donors as controls.

## Methods

### Patients and treatment

A total of 19 healthy controls and 19 psoriasis patients (13 males and 6 females; median age: 35 years, range: 27-62) referred to the I<sup>st</sup> Dermatologic Clinic of Turin University in the period from Jan 2008 to Jan 2009, were enrolled in this study. Thirteen out of 19 patients were affected by arthropathic psoriasis and 6 by moderate to severe psoriasis vulgaris relapsed or refractory to previous standard systemic treatments (cyclosporin, methotrexate or retinoids). All the patients were treated with anti-TNF- $\alpha$  agent Etanercept. Inclusion criteria for treatment were the following: psoriasis vulgaris involving >10% of body surface area or arthropathic psoriasis; no significant infections or immune suppression; no significant renal, hepatic, or other medical disease. All the patients underwent a wash-out period of 6 weeks before enrolment into this study. Etanercept was given subcutaneously 50 mg twice a week for 12 weeks, then tapered to 50 mg once a week as maintenance therapy [27]. A maximum treatment period of 6 months was given to patients. The severity of psoriasis was assessed by the psoriasis area and severity index (PASI) for each patient; Ritchie Index was applied at baseline to estimate the articular involvement. The mean PASI before treatment was  $19 \pm 6$ , the mean Ritchie index was  $32 \pm 6$ . The assessment of response to treatment

was performed using PASI75 for skin involvement and the American College of Rheumatology (ACR75) criteria for articular involvement. Evaluation was performed in blood samples at the beginning of treatment, T0 (baseline), T1 (after 4 weeks of treatment), T2 (after 8 weeks) and T3 (after 12 weeks). The control group included 19 healthy volunteers with no family history of psoriasis, matched for age and sex with patient group. The present study was performed in compliance with the principles of good clinical practice and according to the Declaration of Helsinki Principles. The study protocol was approved by the Turin University Ethical Committee. All the patients were included after providing their written informed consent.

### **Total mRNA and cDNA preparation**

Total RNA was extracted from  $5-10 \times 10^6$  peripheral blood mononuclear cells (PBMCs) using the Trizol reagent (Invitrogen, Cergy-Pontoise, France) and chloroform/isopropanol precipitation. cDNA was generated by a random cDNA synthesis from 1  $\mu$ g RNA as previously described. [24] .

### **Real-Time RT-PCR amplification of target genes**

Messenger RNAs (mRNA) of critical transcription factors and Stat proteins involved in naïve CD4<sup>+</sup> T cell differentiation (Stat-4 , GATA-3, Stat-3, Foxp3, RoR $\gamma$ , Tbet) and related cytokines (IL-2, IL-4, IL-23p19, IL-12p35) were assessed by Real Time RT-PCR assays using TaqMan Technology (7300 Real-time PCR systems Applied Biosystems). The presence of cellular GAPDH transcript was analyzed to evaluate RNA quality. Primer and probe sequences were designed using the software Primer Express 2.0 (Applied Biosystems) (Table 1). PCR mixtures contained cDNA (10%), forward /reverse primers (900nM), probe (250nM) and Platinum quantitative PCR superMix-UDG with ROX master mix (Invitrogen, Cergy-Pontoise, France). The PCR amplification was carried out under the following conditions: 50°C for 2 min. 95°C for 15 s, 60°C for 1 min. To obtain well-characterized positive controls a clone containing the complete target sequence for genes transcripts was used. Measurement of mRNA level was determined by comparing experimental levels with standard curve generated from serial dilutions of the positive control consisting of single genes PCR amplicons cloned using the pTOPO-TA cloning (Invitrogen), according to the manufacturer's instructions. Data were normalized to GAPDH housekeeping mRNA transcripts. All samples showed a high GAPDH mRNA expression (median  $1.8 \times 10^7$ ; 25<sup>th</sup>  $0.18 \times 10^7$  - 75<sup>th</sup>  $3 \times 10^7$ ) so normalized data samples were multiplied for  $10 \times 10^6$  factor and data expressed as mRNA relative expression value. Samples were run in triplicate for

measurement. RPL27 was analyzed to evaluate RNA quality, samples showed a RPL27 mRNA expression (median  $0.810^4$ ; 25<sup>th</sup>  $0.06 \times 10^4$  - 75<sup>th</sup>  $62.3 \times 10^4$ ).

### **Flow cytometric analysis**

PBL were analysed according to their immunofluorescence reactivity using a FACSCalibur cytometer (Becton Dickinson, San José, CA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a second red diode laser at 635 nm for allophycocyanin antibody detection. Surface markers on blood were performed by three- or four-colour immunofluorescence analyses using simultaneously antibodies conjugated to fluorescein isothiocyanate (FITC), phyco-erythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC). At least 10,000 lymphocytes were collected for each antibody combination. Lymphocyte purity was verified by the usual forward and sideward scattering parameters, by means of a CD45 gating analysis. Isotype-matched negative controls conjugated to each fluorochrome were used to set the location of the cursor for each blood sample. A wide panel of monoclonal antibodies directed against T-cell antigens was routinely tested to screen patients' phenotype. For this study purpose, the following MoAbs were analysed: CD4 PercP (clone SK3, mouse IgG1, BD Biosciences, San José CA), CD25 Pe or APC (clone CD25-3G10, mouse IgG1; Caltag, Burlingame, CA), as previously describe [24] the CD4+CD25+ population can be divided in two different levels of expression: cells that expresses a low level of CD25 (defined CD4+CD25<sup>low</sup>) and cells with a highest level of CD25 (defined CD4+CD25<sup>bright</sup>) that appear as a tail to the right from the major population containing both CD4+CD25<sup>low</sup> and the CD4+CD25- cells. Foxp3 cytometric expression was analysed using mononuclear cells purified from peripheral blood trough Lymphoprep (1.077 g/ml; Axis-Shield Poc As, Oslo, Norway) density gradient centrifugation. Cells were incubated firstly with surface antibodies, anti-CD4 PerCP and anti-CD25 ACP for 15 min at room temperature; then cells were intracellularly stained with anti-Foxp3 PE (clone PCH101, rat IgG2a, eBiosciences; San Diego, CA) according to the manufacturer's protocol, using fixation and permeabilization buffers from the same provider. Evaluation of Treg subpopulations by flow-cytometry was performed before the beginning of treatment (T0 as baseline) and then at T3 time treatment. Baseline values were available in all patients.

### **Statistical analysis**

The results are given as median and 25<sup>th</sup> and 75<sup>th</sup> percentile. Non parametric tests were applied to analyze the differences in sample distribution. For pairwise comparisons, we used a non-parametric 2-tailed Wilcoxon test. P value less  $<0.05$  were considered statistically significant. T-

Test was applied to assess the significance of difference between healthy controls and psoriatic patients at baseline.

## RESULTS

### QRT-PCR

#### **T cells subsets distribution in peripheral blood of normal and psoriatic population.**

Messenger-RNA levels of Th1, Th2, TH17 and Treg genes target and related cytokines have been evaluated in healthy controls and psoriatic patients at baseline (Fig.1 and 2). Th17 subset is identified by Stat-3, RoR $\gamma$ t and IL-23p19 mRNAs genes expression. At baseline condition, Stat-3 and RoR $\gamma$ t expression was significantly higher in psoriatic patients (Stat-3 median:  $27.5 \times 10^3$  and RoR $\gamma$ t  $0.53 \times 10^3$ ) in comparison to healthy controls (Stat-3  $0.017 \times 10^3$ ;  $p=0.003$  and RoR $\gamma$ t  $0.03 \times 10^3$ ;  $p<0.0001$ ). At the same time, although IL-23p19 mRNA median value tended to be higher in psoriatic patients in comparison to healthy controls ( $4.19 \times 10^3$  and  $0.616 \times 10^3$ , respectively), the difference was not statistically significant ( $p=0.08$ ). Th2 subset is identified by GATA-3 gene and IL-4 mRNA expression. At baseline, a highly significant increase in GATA-3 expression was observed in psoriatic patients (median:  $52.5 \times 10^3$ ) when compared to healthy controls ( $15.9 \times 10^3$ ;  $p=0.0001$ ), while IL-4 median values were higher in psoriatic patients ( $0.559 \times 10^3$  vs  $0.17 \times 10^3$ ;  $p=0.07$ ). Th1 subset has been identified by Stat-4, Tbet, IL-12P35 and INF $\gamma$  mRNA expression. At baseline, all were highly significantly over-expressed in psoriasis (Stat4 median:  $63.05 \times 10^3$ ; Tbet median:  $11.3 \times 10^3$ ) than in healthy donors (Stat-4  $13.7 \times 10^3$ ,  $p=0.0006$ ; Tbet  $3.5 \times 10^3$ ;  $p=0.0015$ ). At the same time, IL-12Pp35 mRNA expression was significantly higher in psoriatic patients (median  $0.714 \times 10^3$  vs  $0.057 \times 10^3$ ,  $p=0.04$ ) and in INF $\gamma$  (median:  $0.54 \times 10^3$  vs  $0.15 \times 10^3$   $p=0.0008$ ). The T-reg subset has been identified by Foxp3 and IL-2 mRNA expression. At baseline, psoriatic patients showed a statistically significant down-regulation of Foxp3 expression (median:  $1.91 \times 10^3$ ) compared to healthy controls ( $11.8 \times 10^3$ ;  $p=0.0003$ ). At the same time, no difference in IL-2 median values were found between psoriatic patients and healthy donors (median:  $0.285 \times 10^3$  vs  $0.17 \times 10^3$ , respectively  $p=0.4$ ).

#### **Correlation between mark genes and related cytokines expression at baseline**

Naïve-CD4<sup>+</sup> T cell lineage differentiation depends on the activation of key-genes determined by specific cytokine signalling patterns that T cells receive during initial interaction with antigen, therefore we investigated the correlation between gene-target and related cytokine mRNA expression profile. A significant direct correlation was found between Stat-3 and IL-23p19 (Spearman test:  $p=0.0002$ ), Gata-3 and IL-4 ( $p=0.04$ ) and Foxp3 and IL-2 ( $p=0.0007$ ). No

statistically significant correlation has been found between Stat4 and related cytokines (IL12 p=0.48; INF $\gamma$  p=0.75), Tbet and related cytokines (IL12p35 p=0.5; INF $\gamma$  p=0.77) or RoR $\gamma$  and IL-23 (p=0.5). No protein data are available .

### **Messenger-RNAs Genes expression and clinical response**

A clinical response (complete or partial) was achieved in 15/19 patients during 12 weeks of treatment. Modulation of the key transcription factors and cytokines mRNAs at different time treatments (T1,T2 and T3) in responder patients are shown in figure 3. Median values and significance at T0 and T3 time treatment are reported in Tables 2 and 3. We observed a progressive down-modulation of Stat-3 and Stat-4 genes during the treatment in the responder group, with a significant difference from baseline (T0) since the second month of treatment (T2: p=0.04; p=0.03 respectively); The decreasing trend was maintained at T3 (p=0.05; p=0.02 respectively). Also RoR $\gamma$ t genes showed a down-modulation in the responder group although a not significant difference was found (p=0.84). On the other hand, an up-regulation was observed in Foxp3 (T2:p=0.45; T3:p=0.04), and GATA-3 (p=0.002; p=0.02 at T2 and T3, respectively). No significant difference in Tbet expression was observed during treatment (T1, T2 and T3 p $\geq$ 0.1). No difference in baseline values was evidenced between responder and non-responder patients. Non-responder patients had transcription factors values superimposables at T0 and T1,T2 and T3. Related cytokine mRNAs showed a similar trend of modulation, although a significant difference was found only in T3 in three out of five cytokines in responder group (Table 3 ). Briefly Wilcoxon analysis (Wco) showed a significant down-regulation in responders for IL-23p19 and IL-12p35 (p=0.039, p=0.02 respectively) and an up-regulation for IL-2 (T2 p=0.06, T3 p=0.019); An increase in IL-4 and INF $\gamma$  levels was also demonstrated in responders, even if the difference did not achieve a statistical significance (IL4 T0/T3 p=1.0; INF $\gamma$  T0/T3 p=0.13). No significant differences in cytokines levels was demonstrated at baseline between responders and non responders and during treatment in non responder patients.

### **Flow cytometric analysis**

#### **Modulation of CD4+CD25<sup>bright</sup>FoxP3 population and clinical response**

An up-regulation of the CD4+CD25<sup>bright</sup>FoxP3<sup>+</sup> subset occurred during treatment in all 15 responder patients, with a median percentage increase from 15.1% to 81.8%, whereas the remaining showed reduction (percentage decrease between 20% and 57.7%). None response was achieved in patient who showed a CD4+CD25<sup>bright</sup>FoxP3<sup>+</sup> decrease. The expression trends were therefore analysed separately between responders and non-responders. At baseline, no differences

in the median CD4<sup>+</sup>CD25<sup>+</sup>brightFoxP3<sup>+</sup> values were found between these two groups (median 2.2%, 25th-75th percentile: 1.4%-2.7% vs 3.05%, 25th-75th Mann-Whitney test: 0.08). After 12 weeks of treatment, a significant difference compared to baseline condition was evident in responder patients (median 2.2%, 25th-75th percentile: 1.4%-2.7% vs 3.3%, 25th-75th percentile: 2.1%-4%; Mann-Whitney test: 0.038). No significant difference was visible between baseline condition (T0) and T3 time treatment in non responder patients (median 3.05% vs 2.0%, Mann-Whitney test: 0.34).

## DISCUSSION

In this prospective study, we evaluated the profile expression of selected cytokines and their transcription factors known as genes required for the balance in differentiation of the most known functional CD4<sup>+</sup> T-cell subsets [9-10]; Th1 (Stat-4 and IL-12p35), Th2 (GATA-3 and IL-4) [28, 29] and Th17 (Stat-3, RoR $\gamma$ t and IL-23p19)[ 18, 30-33] and of the regulatory T cell lineage (Foxp3 and IL-2) [10,31]. The aim of our study was to ascertain in blood the differences between psoriasis patients and healthy donors and to evaluate if their expression level could be coupled with clinical response. The first main result of our study is the identification of an immune activation pattern in peripheral blood lymphocytes characteristic of psoriatic patients, consisting in an up-regulation of Th1 and Th17 and down-regulation of T-reg subsets compare to healthy controls. The differences between psoriasis and healthy donors were much more evident when considering mRNA levels of transcriptional factor genes. We notice that Foxp3 showed higher expression level than RoR $\gamma$ t and significant modulation was evident only for Foxp-3 expression level. RoR $\gamma$ t and Foxp3 can balance Treg and Th-17 differentiation in a concentration-dependent manner and depend also by TGF- $\beta$  [18]. In addition RoR $\gamma$ t and Foxp3 can be co-expressed in naïve CD4<sup>+</sup> T cells so we suggest that mayor studies on purified CD4<sup>+</sup> were useful for better understand RoR $\gamma$ t function in psoriatic patients. The cytokine expression followed similar trends even if did not always reach statistical significant differences when compared to healthy donors. This could be due to different reasons: in general, more that one cytokine is required for differentiation to any particular phenotype, cross-regulation among the cytokines may occur at transcriptional level when a transcriptional repressor is induced. Finally, Th cytokines produced by each differentiated cell potentially provide a powerful positive or negative feedback loop. Furthermore the lack of protein data is a limitation of the study. These data confirm that the psoriatic model system could be due to an autoimmune inflammation sustained by an up-regulation of Th17 and Th1 cells and the concomitant inhibition of T-reg population. Indeed, Th1 cytokines were found in high levels both in lesional skin and in the

peripheral blood of psoriatic patients [17, 25, 26]. These data confirm our previous study on circulating T-reg showing that a higher post-treatment expression of mRNA Foxp3 was observed in responders compared to non-responders. In accordance with Antonelli *et al.*[34] that found high circulating serum levels of the Th1 and Th2-related chemokine in long lasting psoriasis we found a high baseline levels of IL-4 expression. IL-4 is known to be a classical Th2 driving cytokine and a hallmark cytokine of autoimmune and atopic diseases [35,36] and Guenova *et al* [37] recently demonstrate that IL-4 indirectly drives human Th1 differentiation. In fact base on a regulatory balance between IL-12 and IL-10, high concentrations of IL-4 orchestrates the development of IL12<sup>high</sup> IL-10<sup>low</sup> producing dendritic cell (DC) and, consequently INF $\gamma$ -dominated Th1 immune response. DC are known to be involved in the development of chronic inflammatory diseases as psoriasis by inducing Th1 cells through the secretion of INF $\gamma$ . In the light of these evidence we suggest that Th1 immune pattern in patients with a long disease duration can be sustained by IL-4 high level conditioning IL-12 producing DC differentiation . The role of IL-4 in the Th1 to Th2 shift during treatment in responder patients remain ambiguous in our data and need of adjunctive protein study and DC evaluation. Anyway Ghoreschi *et al.* [38] reported that IL-4 therapy in psoriatic patient can induce increase in IL-4 producing CD4+ T cell and markedly improved psoriasis. Always in accordance with Ghoreschi and Bosè [38, 26] we observed that the induction of Th2 (Gata-3) responses was associated with increase in INF $\gamma$  but without reach statistical significant difference. This surprising trend is in contrast with *in vitro* studies [39, 40] but by contrast *in vivo* the induction of Th2 responses has been reported regularly associated with simultaneous increase in INF $\gamma$  producing T cells [41]. New insights into the actions of IL-4 are essential to explain this phenomenon .

Regarding to modification induced by therapy, our data show that the achievement of clinical response is associated to specific modulation of the immune activation pattern. In fact, responding patients were characterized by a reversal of the Th1/Th17 activation, and by an up-regulation of Th2 and T-reg subsets. Indeed, our data showed a down-modulation in the mRNA expression of hallmark target gene and cytokines Stat-3/IL-23p19, Stat-4/IL-12p35, and conversely an up-regulation of GATA-3 and Foxp3/IL-2; Differently from Stat4 and Stat3 we didn't found significant down-modulation in Tbet and RoR $\gamma$  expression during therapy. Adjunctive studies regarding others competing regulators are needed to better define the role of Tbet and RoR $\gamma$  in the immune response to therapy. The up-regulation of Treg has been confirmed by phenotypical data that show an increased of the CD4+CD25<sup>bright</sup>FoxP3<sup>+</sup> subset in all responsive patients. Even if circulating Treg cells represent always a small subset, the median 43.0% increase after treatment with respect to baseline well demonstrate the biological drug effect.

The finding that these modifications occur in responding patients only suggest a relationship with the mechanisms involved in response induction triggered by Etanercept. However, the low number of non responding patients and lack of serum protein data in our study undoubtedly limits this evidence which need to be confirmed by larger series of patients. Moreover, it is important to note that the majority of these modifications occurred early after Etanercept beginning and achieved statistical significance already since the second month of treatment. In conclusion this finding confirms the potential involvement of CD4+ T-cells modulation in the mechanisms of response induction. It is interesting to note that this phenomenon appears to be characteristics of biological treatment, as much as neither cyclosporin nor methotrexate affects the balance between Th1 and Th2 cells [42].

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## REFERENCES

1. Yawalkar N, Karlen S, Hunger R, Brand CU, Braathen LR.. Expression of interleukin-12 is increased in psoriatic skin. *J Invest Dermatol.* 1998 Dec;111(6):1053-7.
2. Brian JN. Cracking the cytokines code in psoriasis. *Nature Medicine.* Vol. 13 n. 3 Mar 2007: 242-244.
3. Nicolas JF, Chamchick N, Thivolet J, Wijdenes J, Morel P, Revillard JP. CD4 antibody treatment of severe psoriasis. *Lancet* 1991;338:321.
4. Gottlieb AB, Lebwohl M, Shirin S, Sherr A, Gilleaudeau P, Singer G, et al. Anti-CD4 monoclonal antibody treatment of moderate to severe psoriasis vulgaris: results of a pilot, multicenter, multiple-dose, placebo-controlled study. *J Am Acad Dermatol* 2000;43:595–604.
5. Bak RO, Mikkelsen JG. Regulation of cytokines by small RNAs during skin inflammation. *J Biomed Sci.* 2010 Jul 1;17:53. Review
6. Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosisio D, Madonna S., et al. Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med.* 2009 January 19; 206(1): 249–258.
7. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood.* 2008 Sep 1;112(5):1557-69. Review
8. Zhu J, Paul WE. Peripheral CD4 T cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev.* 2010 Nov; 238 (1):247-262
9. Zhu K, Ye J, Wu M, Cheng H Expression of Th1 and Th2 cytokine-associated transcription factors, T-bet and GATA-3, in peripheral blood mononuclear cells and skin lesions of patients with psoriasis vulgaris. *Arch Dermatol Res.* 2010 Sep;302(7):517-23.
10. Annunziato F and Romagnani S. Heterogeneity of human effector CD4+ T cells. *Arthritis Res Ther.* 2009;11(6):257.
11. Hueber AJ, McInnes IB. Immune regulation in psoriasis and psoriasis arthritis-recent development. *Immunology Letters* 114 (2007)59-65.
12. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;6:1123–1132.
13. Harrington LE, Mangan PR, Weaver CT. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol* 2006; 18:349–356.
14. Wraith DC, Nicolson KS, Whitley NT. Regulatory CD4+ T cells and the control of autoimmune disease. *Curr Opin Immunol* 2004; 16:695-701.

15. De Boer OJ, van der Meer JJ, Teeling P, van der Loos CM, van der Wal AC. Low numbers of FOXP3 positive regulatory T cells are present in all developmental stages of human atherosclerotic lesions. *PLoS One*. 2007 Aug 22;2(1):e779.
16. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD et al., Development cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007;8:950-7.
17. Korn T, Bettelli E, Oukka M, Kuchroo VK IL-17 and Th17 Cells. *Annu Rev Immunol*. 2009;27:485-517.
18. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgamma function. *Nature*. 2008 May 8;453(7192):236-40.
19. Nograles KE, Zaba LC, Guttman-Yassky E, Fuentes-Duculan J, Suárez-Fariñas M, Cardinale I, et al. Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways *Br J Dermatol*. 2008 November ; 159(5): 1092–1102.
20. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235–238.
21. Zaba LC, Cardinale I, Gilleaudeau P, Sullivan-Whalen M, Suárez-Fariñas M, Fuentes-Duculan J, et al. Amelioration of epidermal hyperplasia by TNF inhibition is associated with reduced Th17 responses. *J Exp Med* 2007 December; 204(13) :3183-94.
22. Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum* 2007; 56:2936–2946.
23. Lowes MA, Kikuchi T, Fuentes-Duculan J, Cardinale I, Zaba LC, Haider AS, et al. Psoriasis Vulgaris Lesions Contain Discrete Populations of Th1 and Th17 T Cells. *Journal of Investigative Dermatology* (2008) 128, 1207–1211.
24. Quaglino P, Ortoncelli M, Comessatti A, Ponti R, Novelli M, Bergallo M, et al. Circulating CD4+CD25 bright FOXP3+ T cells are up-regulated by biological therapies and correlate with the clinical response in psoriasis patients. *Dermatology*. 2009;219(3):250-8.
25. Zaba LC, Suárez-Fariñas M, Fuentes-Duculan J, Nograles KE, Guttman-Yassky E, Cardinale I, Lowes MA, Krueger JG. Effective treatment of psoriasis with etanercept is linked to suppression of IL-17 signaling, not immediate response TNF genes. *J Allergy Clin Immunol*. 2009 Nov;124(5):1022-10.

26. Bosè F, Raeli L, Garutti C, Frigerio E, Cozzi A, Crimi M, Caprioli F, Scavelli R, Altomare G, Geginat J, Abrignani S, Reali E. Dual role of anti-TNF therapy: Enhancement of TCR-mediated T cell activation in peripheral blood and inhibition of inflammation in target tissues. *Clin Immunol.* 2011 May;139(2):164-76.
27. Sterry W, Ortonne JP, Kirkham B, Brocq O, Robertson D, Pedersen RD, et al. Comparison of two Etanercept regimens for treatment of psoriasis and psoriatic arthritis: PRESTA randomised double blind multicentric trial *BMJ* 2010 Feb 2;340:c147
28. Ho IC, Tai TS, Pai SY. Gata3 and T cell lineage: essential functions before and after T-helper-2- cell differentiation. *Nat Rev Immunol* 2009, Feb;9(2):125-35. Review.
29. Wang L et al Distinct function for the transcription factor GATA-3 and ThPOK during intratymic differentiation of CD4+ T cells. *Nature Immunol* (2008) 9, 1122-130
30. Dong C TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol.* 2008 May;8(5):337-48. Review.
31. Ozer A, Murat Aral SS and Pinar C. Serum Levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-8, IL-12, IL-17, and IL-18 in Patients with Active Psoriasis and Correlation with Disease Severity. *Mediators of Inflammation* 2005: 5 (2005) 273–279.
32. Caproni M, Antiga E, Melani L, Volpi W, Del Bianco E, Fabbri P. Serum levels of IL-17 and IL-22 are reduced by etanercept, but not by acitretin, in patients with psoriasis: a randomized-controlled trial. *J Clin Immunol.* 2009 Mar;29(2):210-4.
33. Kagami S, Rizzo HL, Lee JJ, Koguchi Y, Blauvelt A. Circulating Th17, Th22, and Th1 Cells Are Increased in Psoriasis. *J Invest Dermatol.* 2009 May;130(5):1373-83. Epub 2009 Dec 24.
34. Antonelli A, Fallahi P, Delle Sedie A, Ferrari SM, Maccheroni M, Bombardieri S, et al. High values of Th1 (CXCL10) and Th2 (CCL2) chemokines in patients with psoriatic arthritis. *Clin Exp Rheumatol.* 2009 Jan-Feb;27(1):22-7.
35. Grewe M, Bruijnzeel-Koomen CA, Schöpf E, Thepen T, Langeveld-Wildschut AG, Ruzicka T, Krutmann J. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today.* 1998 Aug;19(8):359-61
36. Elbe-Bürger A, Egyed A, Olt S, Klubal R, Mann U, Rappersberger K, Rot A, Stingl G Overexpression of IL-4 alters the homeostasis in the skin. *J Invest Dermatol.* 2002 May;118(5):767-78.
37. Guenova E, Volz T, Sauer K, Kaesler S, Müller MR, Wölbing F, Chen K, Schwärzler C, Brossart P, Röcken M, Biedermann T. IL-4-mediated fine tuning of IL-12p70 production by human DC. *Eur J Immunol.* 2008 Nov;38(11):3138-49.

38. Ghoreschi K, Thomas P, Breit S, Dugas M, Mailhammer R, van Eden W, van der Zee R, Biedermann T, Prinz J, Mack M, Mrowietz U, Christophers E, Schlöndorff D, Plewig G, Sander CA, Röcken M. Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease. *Nat Med.* 2003 Jan;9(1):40-6
39. Abbas AK, Murphy KM, Sher A Functional diversity of helper T lymphocytes. *Nature.* 1996 Oct 31;383(6603):787-93.
40. Paul WE, Seder RA. Lymphocyte responses and cytokines. *Cell.* 1994 Jan 28;76(2):241-51.
41. Biedermann T, Zimmermann S, Himmelrich H, Gummy A, Egeter O, Sakrauski AK, Seegmüller I, Voigt H, Launois P, Levine AD, Wagner H, Heeg K, Louis JA, Röcken M. IL-4 instructs TH1 responses and resistance to *Leishmania major* in susceptible BALB/c mice. *Nat Immunol.* 2001 Nov;2(11):1054-60.
42. Rentenaar RJ, Heydendael VM, van Diepen FN, de Rie MA, ten Berge IJ. Systemic treatment with either cyclosporin A or methotrexate does not influence the T helper 1/T helper 2 balance in psoriatic patients. *J Clin Immunol.* 2004 Jul;24(4):361-9.

Tab.1 Primers and probe sequence genes

Gene	Accession number	Sequence 5'-3' (position)
STAT3	AB451232.1	Forward TCCTGGTGTCTCCACTGGTCTA (1994-2015) Reverse TTCCGAATGCCTCCTCCTT (2053-2035) Probe FAM-CTCTATCCTGACATTCC-MGB (2017-2033)
IL23	BC067511.1	Forward TCAGTGCCAGCAGCTTTCAC (126-145) Reverse TCTCTTAGATCCATGTGTCCCAC (206-184) Probe FAM-CTCTGCACACTGGCCTGGAGTGCA-TAMRA (151-174)
IL2	DQ861285.1	Forward GCTGATGAGACAGCAACCATTG (384-405) Reverse TTGAGATGATGCTTTGACAAAAGG (453-430) Probe FAM-AGAATTTCTGAACAGATGGAT-MGB (407-427)
IL4	AM937235.1	Forward CGGCTCGACAGGAACCTCT (365-383) Reverse TCCAAGAAGTTTCCAACGTA CTCT (453-429) Probe FAM-CGGGCTTGAATTCCTGTCCTGTGAAG-TAMRA (393-418)
IL12p35	AF101062.1	Forward GAGGGCCGTCAGCAACAT (237-254) Reverse ATGATCAATCTTTCAGAAGTGCAA (315-291) Probe FAM-CTCCAGAAGGCCAGACAACTCTAGAATTTACC-TAMRA (256-289)
STAT4	NM_003151	Forward GGCATCAATTGACAAGAATGTTTC (1333-1357) Reverse GACATGGCTTTGACATTAGTTCCA (1393-1370) Probe FAM-ACTCTAAGCAACAGAAGAT-MGB (1387-1411)
GATA3	NM_002051	Forward TTCCCAAGAACAGCTCGTT (1709-1729) Reverse GGCTCAGGGAGGACATGTGT (1750-1770) Probe FAM-AACCCCGCCGCC CT-MGB (1730-1744)
FOXP3	NM_014009.3	Forward TCACCTACGCCAGCTCAT (1206-1225) Reverse ATTGAGTGTCCGCTGCTTCTC (1182-1162) Probe FAM-CTGGGCCATCCTGGA-MGB (1250-1271)
ROR $\gamma$ t	U16997.1	Forward CGGGCCTACAATGCTGACA (1168-1186) Reverse GCCACCGTATTTGCCTCAA (1221-1202) Probe FAM-CCGCACGGTCTTT-MGB
TBET	AF241243.2	Forward ACACGCATATCTTTACTTTCCAAGAA (909-934) Reverse TCAGCTGAGTAATCTCGGCATTC (986-964) Probe CCCAGTTCATTGCCGTGACTGCC (936-958)
IFN $\gamma$	BC070256.1	Forward CTAATTATTCGGTAACTGACTTGA (484-507) Reverse ACAGTTCAGCCATCACTTGGA (558-538) Probe FAM-TCCAACGCAAAGCAATACATGAAC-TAMRA (511-534)

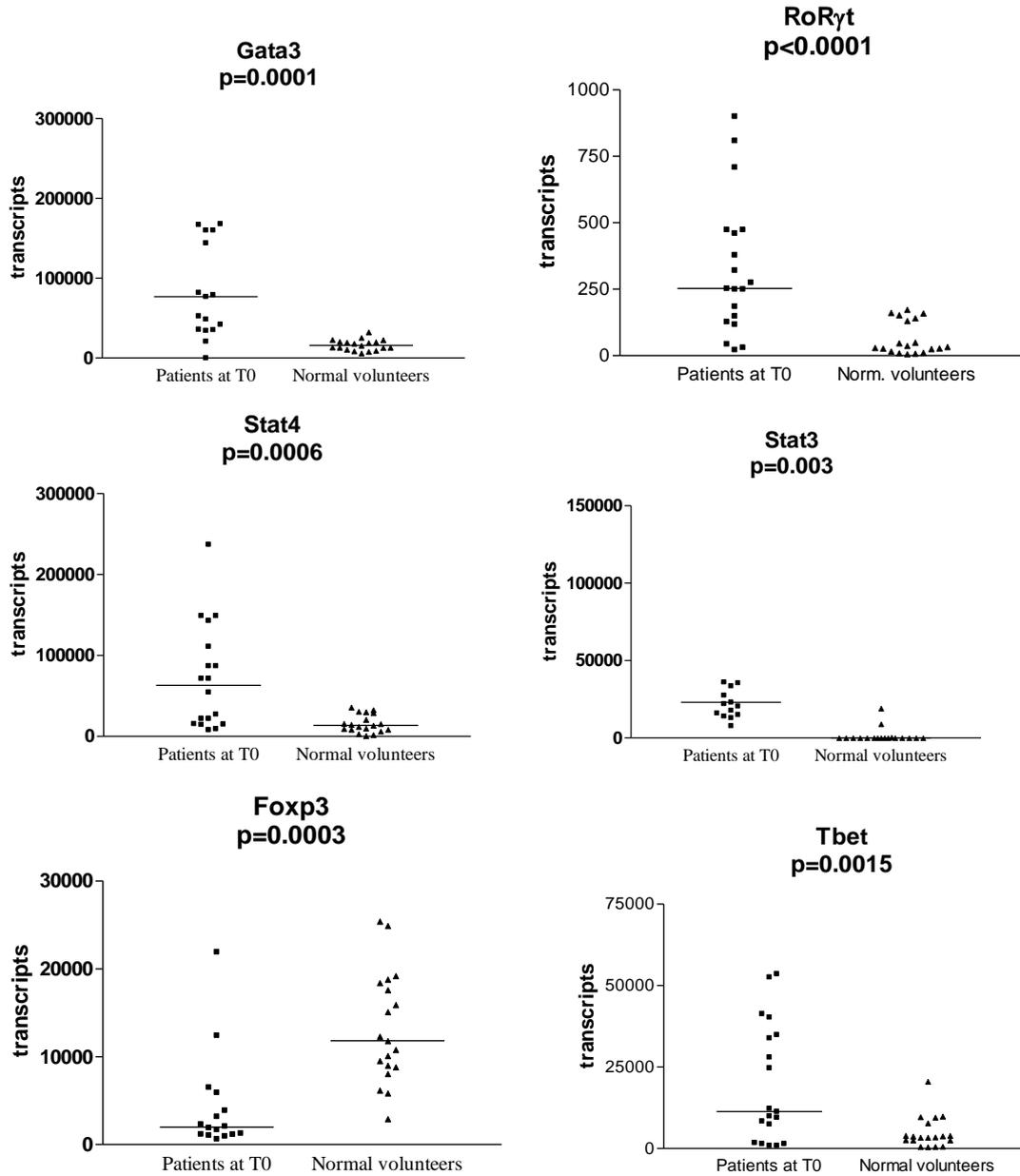


Fig. 1. Expression pattern of six Th cytokines' target genes (Stat-3, Gata-3, Foxp3, Stat-4, RoRyt). Transcripts mRNA/Gapdh relative genes value in psoriasis patients at baseline and in healthy donors. Each point represents a sample; horizontal lines in each column represent median values; P value is the statistical significance of T-test between two group.

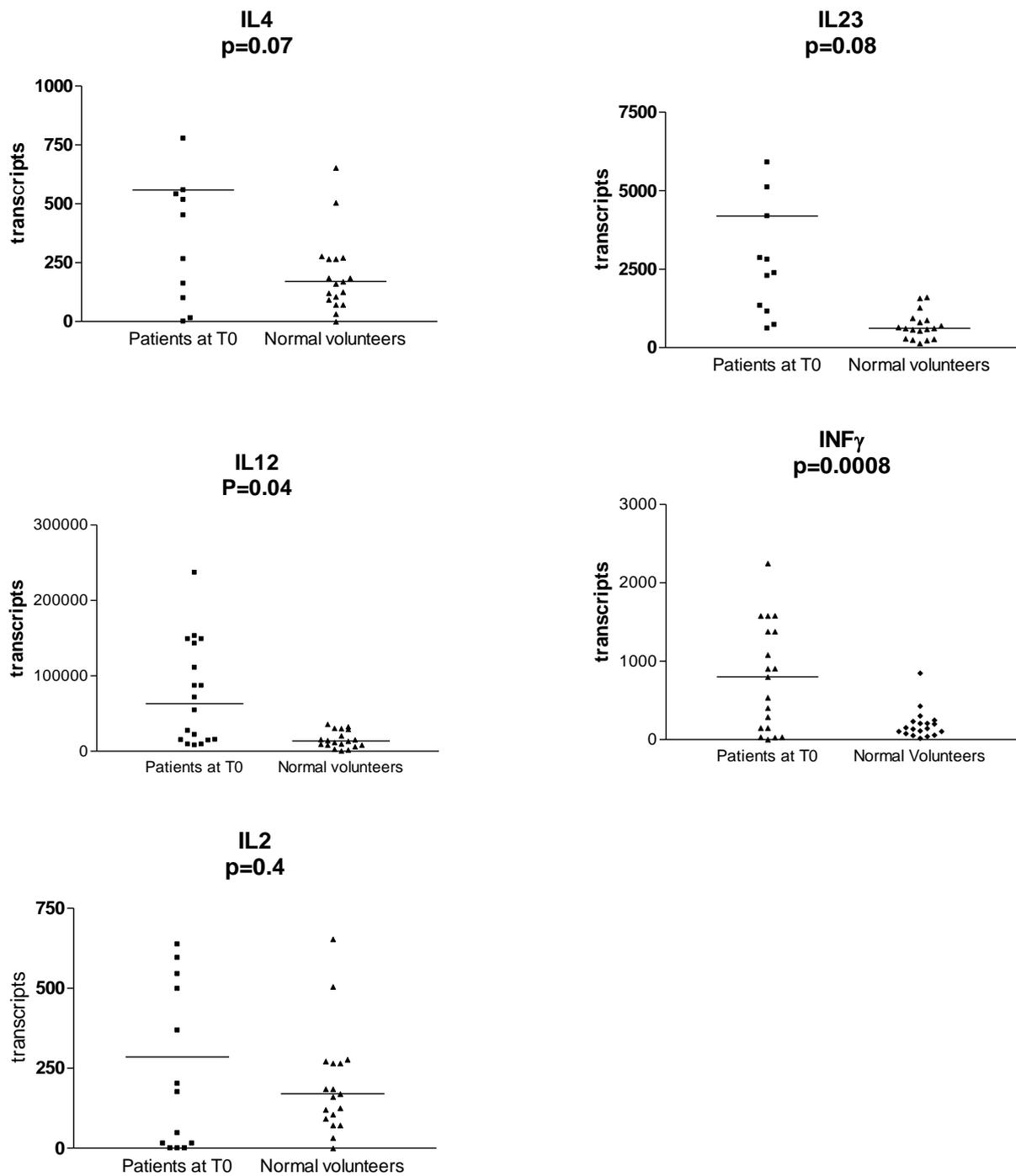


Fig. 2. Expression pattern of four circulating Th cytokines (IL-23, IL-4, IL-2, IL-12p35,  $\text{INF}\gamma$ ). Transcripts mRNA/Gapdh relative genes value in psoriasis patients at baseline and in healthy donors. Each point represents a sample; horizontal lines in each column represent median values; P value is the statistical significance of T-test between two group.

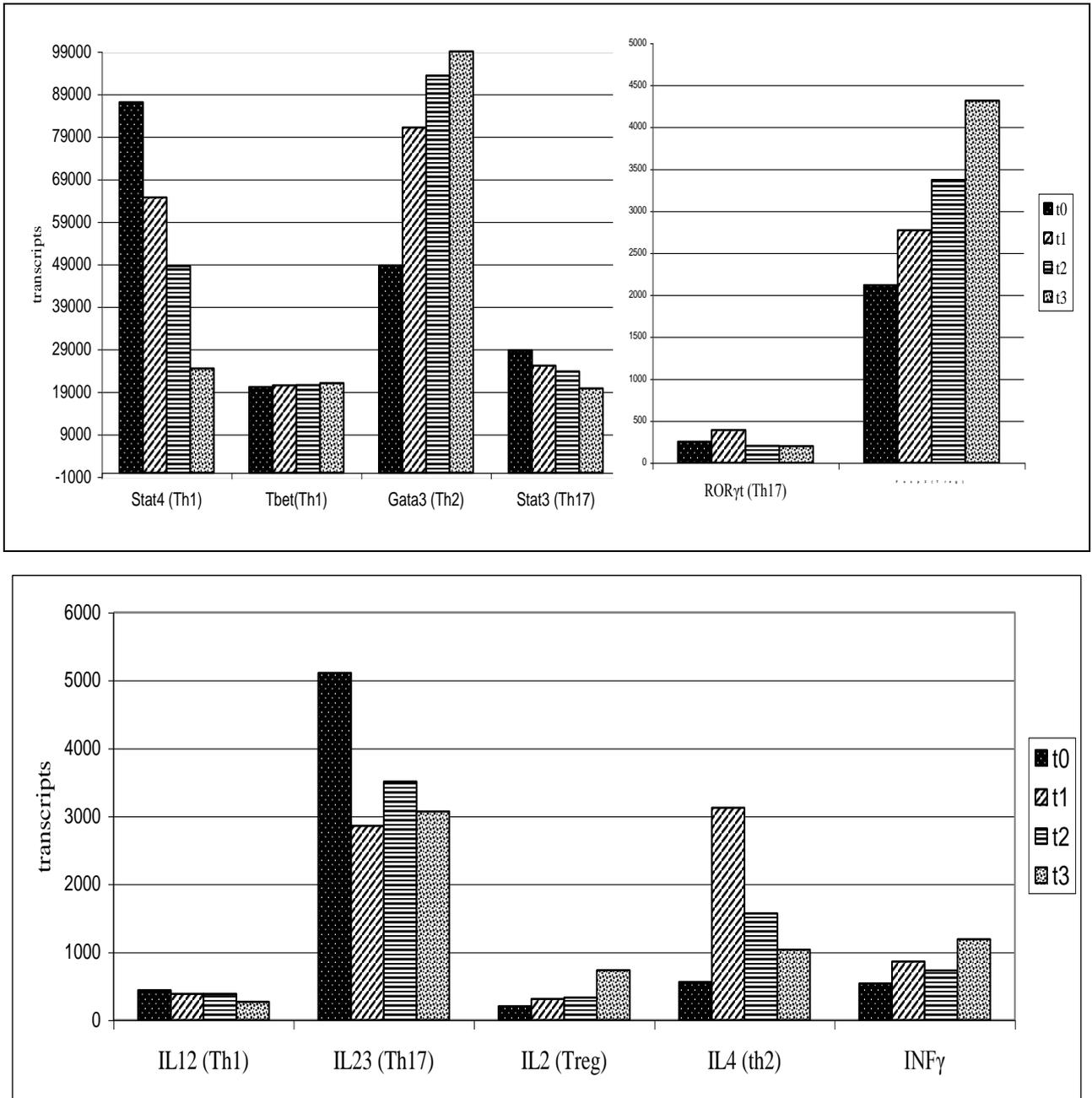


Fig. 3. Kinetics of cytokines and related transcriptional factor genes (mRNA/Gapdh relative values) in responder patients during treatment.