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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 γ for Th1; GATA3, IL4 for Th2; Stat3, RoR γ 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 γ , 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- β , 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 γ t, Foxp3). The essential transcription factors of CD4+ T lineages are T-bet/Stat-4 (Th1), GATA-3/Stat5 (Th2), Stat3/RoR γ 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 β plus IL-6, IL-21, IL-1 β , TGF- β 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 γ has been described. Differentiation of Naïve-CD4 $^+$ 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 $^+$ 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 Ist 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- α 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 ± 6 , the mean Ritchie index was 32 ± 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 μ 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⁺ T cell differentiation (Stat-4 , GATA-3, Stat-3, Foxp3, RoR γ , 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 1.8×10^7 ; 25th 0.18×10^7 - 75th 3×10^7) so normalized data samples were multiplied for 10×10^6 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 ; 25th 0.06×10^4 - 75th 62.3×10^4).

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^{low}) and cells with a highest level of CD25 (defined CD4+CD25^{bright}) that appear as a tail to the right from the major population containing both CD4+CD25^{low} 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 25th and 75th 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 γ t and IL-23p19 mRNAs genes expression. At baseline condition, Stat-3 and RoR γ t expression was significantly higher in psoriatic patients (Stat-3 median: 27.5×10^3 and RoR γ t 0.53×10^3) in comparison to healthy controls (Stat-3 0.017×10^3 ; $p=0.003$ and RoR γ t 0.03×10^3 ; $p<0.0001$). At the same time, although IL-23p19 mRNA median value tended to be higher in psoriatic patients in comparison to healthy controls (4.19×10^3 and 0.616×10^3 , 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: 52.5×10^3) when compared to healthy controls (15.9×10^3 ; $p=0.0001$), while IL-4 median values were higher in psoriatic patients (0.559×10^3 vs 0.17×10^3 ; $p=0.07$). Th1 subset has been identified by Stat-4, Tbet, IL-12P35 and INF γ mRNA expression. At baseline, all were highly significantly over-expressed in psoriasis (Stat4 median: 63.05×10^3 ; Tbet median: 11.3×10^3) than in healthy donors (Stat-4 13.7×10^3 , $p=0.0006$; Tbet 3.5×10^3 ; $p=0.0015$). At the same time, IL-12Pp35 mRNA expression was significantly higher in psoriatic patients (median 0.714×10^3 vs 0.057×10^3 , $p=0.04$) and in INF γ (median: 0.54×10^3 vs 0.15×10^3 $p=0.0008$). 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: 1.91×10^3) compared to healthy controls (11.8×10^3 ; $p=0.0003$). At the same time, no difference in IL-2 median values were found between psoriatic patients and healthy donors (median: 0.285×10^3 vs 0.17×10^3 , respectively $p=0.4$).

Correlation between mark genes and related cytokines expression at baseline

Naïve-CD4⁺ 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 γ p=0.75), Tbet and related cytokines (IL12p35 p=0.5; INF γ p=0.77) or RoR γ 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 γ 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 γ levels was also demonstrated in responders, even if the difference did not achieve a statistical significance (IL4 T0/T3 p=1.0; INF γ 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^{bright}FoxP3 population and clinical response

An up-regulation of the CD4+CD25^{bright}FoxP3⁺ 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^{bright}FoxP3⁺ decrease. The expression trends were therefore analysed separately between responders and non-responders. At baseline, no differences

in the median CD4⁺CD25⁺brightFoxP3⁺ 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⁺ T-cell subsets [9-10]; Th1 (Stat-4 and IL-12p35), Th2 (GATA-3 and IL-4) [28, 29] and Th17 (Stat-3, RoR γ 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 γ t and significant modulation was evident only for Foxp-3 expression level. RoR γ t and Foxp3 can balance Treg and Th-17 differentiation in a concentration-dependent manner and depend also by TGF- β [18]. In addition RoR γ t and Foxp3 can be co-expressed in naïve CD4⁺ T cells so we suggest that mayor studies on purified CD4⁺ were useful for better understand RoR γ 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^{high} IL-10^{low} producing dendritic cell (DC) and, consequently INF γ -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 γ . 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 γ 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 γ 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 γ expression during therapy. Adjunctive studies regarding others competing regulators are needed to better define the role of Tbet and RoR γ 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^{bright}FoxP3⁺ 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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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 TCACCTACGCCACGCTCAT (1206-1225) Reverse ATTGAGTGTCCGCTGCTTCTC (1182-1162) Probe FAM-CTGGGCCATCCTGGA-MGB (1250-1271) |
| ROR γ 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 γ | BC070256.1 | Forward CTAATTATTCGGTAACTGACTTGA (484-507) Reverse ACAGTTCAGCCATCACTTGGA (558-538) Probe FAM-TCCAACGCAAAGCAATACATGAAC-TAMRA (511-534) |

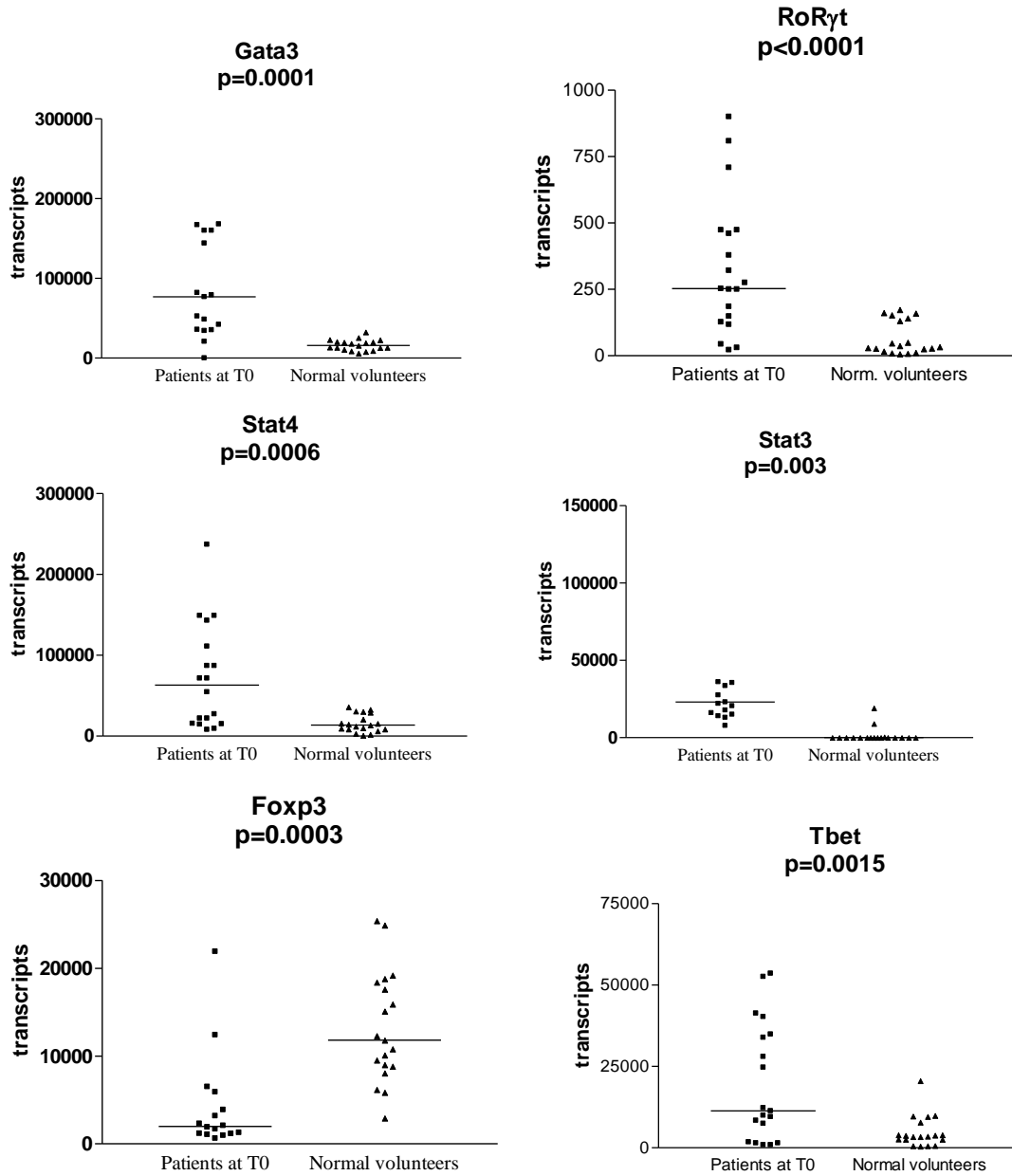


Fig. 1. Expression pattern of six Th cytokines' target genes (Stat-3, Gata-3, Foxp3, Stat-4, RoRyt). Transcripts mRNA/Gapdh relative genes value in psoriasis patients at baseline and in healthy donors. Each point represents a sample; horizontal lines in each column represent median values; P value is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

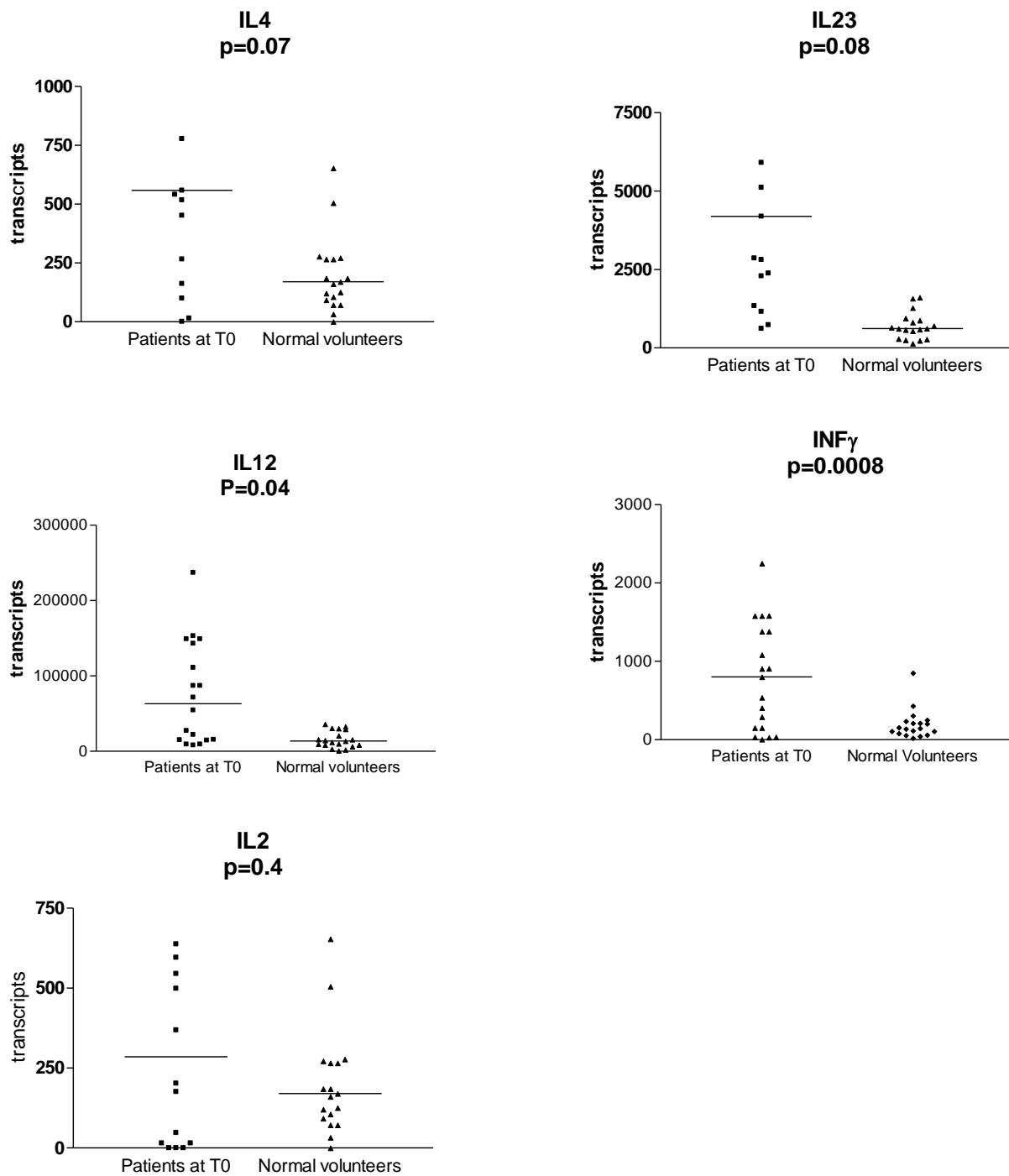


Fig. 2. Expression pattern of four circulating Th cytokines (IL-23, IL-4, IL-2, IL-12p35, INF γ). Transcripts mRNA/Gapdh relative genes value in psoriasis patients at baseline and in healthy donors. Each point represents a sample; horizontal lines in each column represent median values; P value is the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

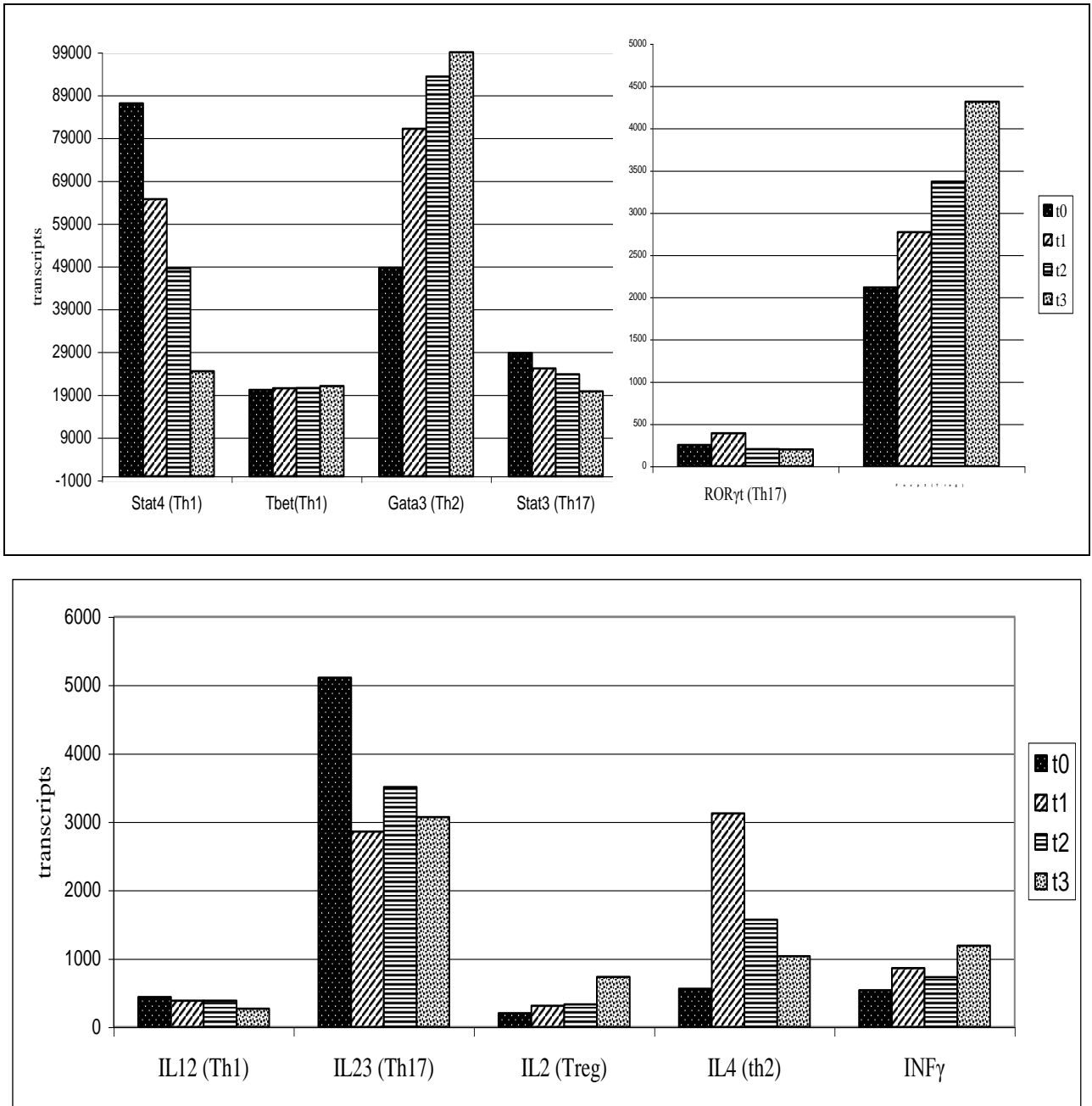


Fig. 3. Kinetics of cytokines and related transcriptional factor genes (mRNA/Gapdh relative values) in responder patients during treatment.