Effect of TNF-α inhibitors on transcriptional levels of pro-inflammatory interleukin-33 and Toll-like receptors-2 and -9 in psoriatic plaques

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Abstract. Tumor necrosis factor (TNF)-α inhibitors are considered to be effective in the treatment of psoriatic plaques, although the precise therapeutic pathway is not clear. Pro-inflammatory molecules, such as Toll-like receptor (TLR)-2 and -9 and interleukin (IL)-33, a member of the IL-1 receptor/TLR superfamily, have been found to be expressed in psoriatic plaques. The aim of the present study was to investigate whether TNF-α inhibitor treatment has an effect on the expression of IL-33 and TLR-2 and -9 in psoriatic plaques. Seventeen patients with psoriatic plaques were treated with a TNF-α inhibitor (etanercept or infliximab) for 12 weeks in an open-label study, and the transcriptional levels of IL-33 and TLR-2 and -9 were determined by reverse transcription-quantitative polymerase chain reaction in paired biopsies of psoriatic plaques obtained at baseline (B) and following the 12 weeks of treatment (P). The psoriasis area severity index (PASI) score was also determined. At B, elevated IL-33 and TLR-2 mRNA levels were observed in all cases, while TLR-9 showed elevated mRNA levels in 76% of cases. At P, reductions in the mRNA levels of IL-33, TLR-2 and TLR-9 were observed, with TLR-2 and -9 levels exhibiting significant reductions ($P<0.0001$, Wilcoxon signed-rank test). PASI scores were significantly reduced by the treatment ($P<0.0001$, Wilcoxon signed-rank test) and the changes in PASI scores exhibited a significant positive Pearson's correlation with the P/B mRNA expression ratios of TLR-2 or -9 in males ($P<0.05$), particularly in the etanercept group ($P<0.0001$). The findings support the efficacy of anti-TNF-α treatment on the innate immune response in psoriatic skin, with a focus on TLR-2 and -9 inhibition, suggesting their role in the pathogenic mechanism of plaque psoriasis, which may be associated with gender.

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Introduction

Plaque psoriasis is the most common form of psoriatic disease, corresponding to a chronic inflammatory skin disorder (1,2). Treatment of severe psoriatic plaques may involve biological agents that block the action of tumor necrosis factor (TNF)-α, such as etanercept and infliximab (2-6). Etanercept is a recombinant human TNF-receptor fusion protein that binds free TNF-α (7,8). Infliximab is a monoclonal anti-TNF-α antibody that can bind both soluble and membrane-bound TNF-α and effectively neutralize its activity (9). The mechanism underlying the action of these drugs as anti-TNF-α agents in psoriasis is not yet clear; however, the application of etanercept and infliximab has been shown to reduce multiple pro-inflammatory pathways in psoriatic plaques (10,11). The efficacy of anti-TNF-α biological treatment supports a role of innate immunity in the pathogenesis of psoriatic disease.

Toll-like receptors (TLRs) have been demonstrated to be essential elements of the innate immune system (12,13). To date, ~10 different types of TLRs (TLR 1-10) have been found in humans. Each TLR is activated by a different microbial component, although they trigger a common myeloid differentiation factor 88 (MyD88)-dependent pathway, leading, via nuclear factor (NF)-κB, to the production of pro-inflammatory cytokines and chemokines, such as TNF-α (12). TLR-2 is a type of TLR that is highly expressed in keratinocytes, and Langerhans and mast cells of psoriatic plaques (14-16) and is activated by various microorganism antigens (17-20) or endogenous heat-shock proteins present at sites of tissue injury and inflammation (21). TLR-9 is another type of TLR that has been observed to be elevated in the keratinocytes of psoriatic skin lesions (22) and is activated by unmethylated DNA sequences (CpG dinucleotides) that are present in bacterial DNA and viruses (17).

A member of the interleukin-1 receptor (IL-1R)/TLR superfamily, IL-33, has been recognized as a pro-inflammatory molecule that is predominantly expressed in the nucleus of cells with a barrier function, such as endothelial and epithelial cells (23,24). These cells share a common intracellular domain (TIR domain) that may, through MyD88, initiate a signaling cascade, leading to NF-κB translocation (23). IL-33 has been found to be expressed at elevated levels in affected psoriatic skin, compared with healthy skin, and it has also been proposed to represent a novel marker of psoriasis, relative to other inflammatory skin disorders (25-28). Balato *et al* (25) recently demonstrated that inflammatory cytokines, such as TNF-α, induce the secretion of IL-33 from immortalized keratinocytes (24) and support the hypothesis that IL-33 has an important role in the effect of anti-TNF therapy on psoriatic skin (25,29).

In the present study, the aim was to investigate the effect of anti-TNF-α treatment with etanercept or infliximab on pro-inflammatory IL-33, TLR-2 and -9 transcriptional levels in psoriatic plaques. For the purpose of this study the mRNA levels of IL-33, TLR-2 and -9 genes were identified using a precise reverse transcription-quantitative polymerase chain reaction (qPCR) analysis, with biopsies of psoriatic plaques obtained from patients at the start and end of anti-TNF-α therapy.

Subjects and methods

Patients. Seventeen adult patients (mean age ± standard deviation, 46.4±9.6 years; males, 13; and females, 4) with moderate-to-severe psoriasis attending the Department of Dermatology outpatient clinic of the University Hospital of Larissa (Larissa, Greece) were included in an open-label study. Skin biopsy confirmed the diagnosis of plaque psoriasis in these patients. Thirteen of the patients (11 males and 2 females; median age, 48.5 years) were treated for 3 months with etanercept (Enbrel®; Immunex Corp., Thousand Oaks, CA, USA), and 4 of them (2 male and 2 female) were treated with infliximab (Remicade®; Janssen Biotech, Inc., Titusville, NJ, USA). The psoriasis area severity index (PASI) (30) was calculated prior to (PASI-1; range, 10-45.5; median, 20.3) and subsequent to (PASI-2; range, 1.2-20.4; median, 5.2) the treatment.

Tissue collection. Punch skin biopsies (6-mm) were collected from psoriatic plaques of patients prior to initiation of treatment (baseline, B) and following 12 weeks of treatment (post-treatment, P). Biopsies were taken under local anesthesia with 1% lidocaine from one lesion of each patient. The skin biopsies were immediately cryopreserved at -80°C, where they were kept until molecular analysis. Part of the biopsy was used for histological examination. This study was approved by the local Ethics Committee (University Hospital of Larissa), and performed in accordance with the Declaration of Helsinki; all patients gave their informed consent.

Quantitative gene expression analysis. An RNAeasy® Fibrous Tissue Mini kit (Qiagen, Inc., Valencia, CA, USA) was used for total RNA isolation from skin biopsies, and a QuantiFast™ Reverse Transcription kit (Qiagen, Inc.) was used for cDNA synthesis according to the manufacturer's instructions. qPCR analysis of IL-33 (NM_033439), TLR-2 (NM_003264) and -9 (NM_017442) mRNAs was performed using specific primers and dual-labeled probes (QuantiFast® Probe assay; Qiagen, Inc.) by applying Rotor Gene 6.1 (Qiagen, Inc.) according to the manufacturer's instructions. The PCR cycling conditions were as follows: Initial activation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C for 15 sec and extension at 72˚C for 15 sec. The human porphobilinogen deaminase (hPBGD) gene was used as a reference-control, as described previously (31). The quantification of the mRNAs was achieved by creating a standard curve of serial dilutions of hPBGD gene copies. The mRNA expression levels of each IL-33, TLR-2 and -9 target gene were addressed as ratios of target mRNA to control hPBGD mRNAs (target/control mRNA ratios); therefore, target/control mRNA ratios <1.0 or ≥1.0 were assigned as low or elevated mRNA levels, respectively. Additionally, the mRNA expression of each target gene, IL-33, TLR-2 and -9, at P was compared with that at B and expressed as a P/B mRNA ratio (relative expression).

Statistical analysis. The Wilcoxon signed-rank test was used for the evaluation of changes in IL-33, TLR-2 and -9 mRNA values, as well as in PASI scores, between B and P, which had skewed distributions (P-values reported for one-tailed test). Pearson coefficients were computed in order to investigate associations among IL-33/TLR-2/TLR-9 expression or between the expression of pro-inflammatory molecules and PASI scores (P-values reported for two-tailed test). Analysis of variance was used to investigate changes in PASI score according to the relative expression of IL-33, TLR-2 and -9. All P-values were reported for the two-tailed test. Statistical significance was set at 0.05 and analyses were carried out using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

Results

Clinical response. In all patients, the PASI improved with treatment; the mean ± standard deviation PASI score was reduced from 21.2±8.8 at B to 5.0±4.8 at P (ΔPASI=16.2, P<0.0001; Fig 1A). In patients who received etanercept, the mean PASI score was decreased significantly from 20.3±6.7 at B to 5.2 ±5.3 at P (ΔPASI=15.1, P<0.0001; Wilcoxon signed-rank test; Fig 1A).

TNF-α inhibitors reduce the expression of pro-inflammatory IL-33, TLR-2 and TLR-9 at the transcriptional levels in psoriatic plaques. To investigate the effect of the TNF-α inhibitors etanercept and infliximab on the expression of pro-inflammatory molecules in plaque psoriasis, the transcriptional levels of IL-33, TLR-2 and TLR-9 were assessed in psoriatic lesions before and after treatment. The quantification of IL-33, TLR-2 and TLR-9 expression data revealed that at B, the mRNA levels of IL-33 and TLR-2 were elevated in all psoriatic skin lesions, while those of TLR-9 were elevated in the majority (76%) of cases (Fig 1B). At P, TLR-2 and -9 exhibited significantly lower transcriptional levels compared with those at B (P<0.0001, Wilcoxon signed-rank test), whereas the levels of IL-33 mRNA were reduced, although not significantly (Fig 1C). The etanercept group was the most affected. Paired t-test analysis showed significant reductions of TLR-2 and TLR-9 mRNA levels at the end of etanercept

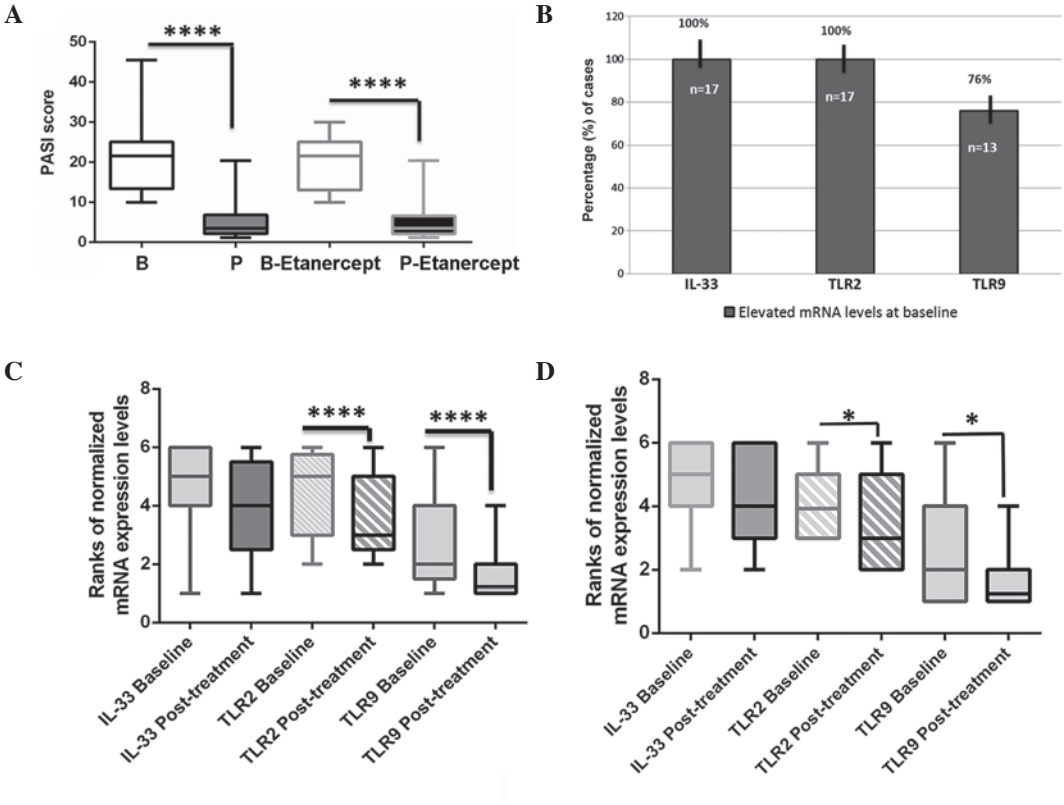

Figure 1. Transcriptional changes of the pro-inflammatory molecules IL-33, TLR-2 and TLR-9, and changes in PASI scores, of psoriatic plaques prior to (baseline, B) and at the end (post-treatment, P) of anti-TNF-α treatment. (A) Statistically significant differences of PASI scores post-treatment vs. baseline in psoriatic skin lesions treated with TNF-α inhibitors, and particularly by etanercept (Wilcoxon matched-pairs signed rank test; one-tailed). (B) Percentages of plaque psoriatic cases (%) presenting elevated (target/hPBGD ≥1) mRNA levels of IL-33, TLR-2 and TLR-9 at baseline in psoriatic skin. Differences in the expression levels of IL-33, TLR-2 and TLR-9 mRNA post-treatment vs. baseline in (C) the whole treatment group and (D) in male patients treated with etanercept (Wilcoxon matched-pairs signed rank test; one-tailed. Graphs were created using Graph Pad Prism 6 software. The boxplots represent the mean ranks of mRNAs evaluated by quantitative polymerase chain reaction. The upper line indicates the highest value, the lower line the lowest value and the middle line the mean of normalized quantities of each variable. *P<0.01; ****P<0.0001. IL, interleukin; TLR, Toll-like receptor; PASI, psoriasis area severity index; TNF, tumor necrosis factor; hPBGD, human porphobilinogen deaminase.

therapy compared with those at B (P=0.017 and P=0.0239, respectively; Fig 1D), particularly in males (P=0.0210 and P=0.0415, respectively).

Transcriptional levels of pro-inflammatory IL-33, TLR-2 and TLR-9 show significant linear Pearson's correlations. Significant linear Pearson's correlations were observed between the transcriptional levels of IL-33 and TLR-2 at B and P (r=0.953814 and r=0.782228, respectively; P<0.0001). Moreover, significant positive correlations were observed at B between IL-33 and TLR-9 and between TLR-2 and TLR-9 (r=0.914635 and r=0.763433, respectively; P<0.0001), which were was less strong at the end of anti-TNF-α treatment (r=0.534675 and r=0.668582, respectively; P<0.05).

Additionally, strong linear Pearson's correlations were identified between the relative (P/B) mRNA expression ratios of IL-33 and TLR-2, IL-33 and TLR-9 (Fig 2A-a) or TLR-2 and TLR-9 (Fig. 2A-b; r=0.804390, r=0.876705 and r=0.916274, respectively; P<0.0001), particularly in male patients who received etanercept therapy (r=0.803771, r=0.886865 and r=0.92, respectively; P<0.0003).

ΔPASI scores present significant linear Pearson's correlations with changes in TLR-2 or TLR-9 mRNA expression levels. Pearson's correlation analysis revealed a significant linear correlation between the change in PASI score (ΔPASI) and the relative (P/B) mRNA expression ratios of TLR-2 and TLR-9 in the psoriatic plaques of males treated with TNF-α inhibitors (r=0.556958 and r=0.555675, P<0.05; respectively), particularly in the etanercept group (r=0.764774 or r=0.725006, respectively; P<0.0001; Fig 2B). No significant correlation was found between ΔPASI and changes in IL-33 mRNA in the etanercept group by Pearson's analysis.

Discussion

The present findings have provided evidence of the efficacy of anti-TNF-α therapy in reducing the innate immune response, indicating that the pro-inflammatory factors TLR-2, -9 and IL-33 play a role in the pathogenic mechanism of plaque psoriasis. The results support the involvement of innate immune response elements in the pathophysiology of psoriasis, in line with previous studies (12,17,22-24,26,27,32-36), although some previous studies have reported conflicting results regarding TLR-9 expression in psoriasis (22,34). The activation of IL-33, TLR-2 and TLR-9 in psoriatic plaques may be important since such activation has been previously associated with the activation of NF-κB (12,23,37).

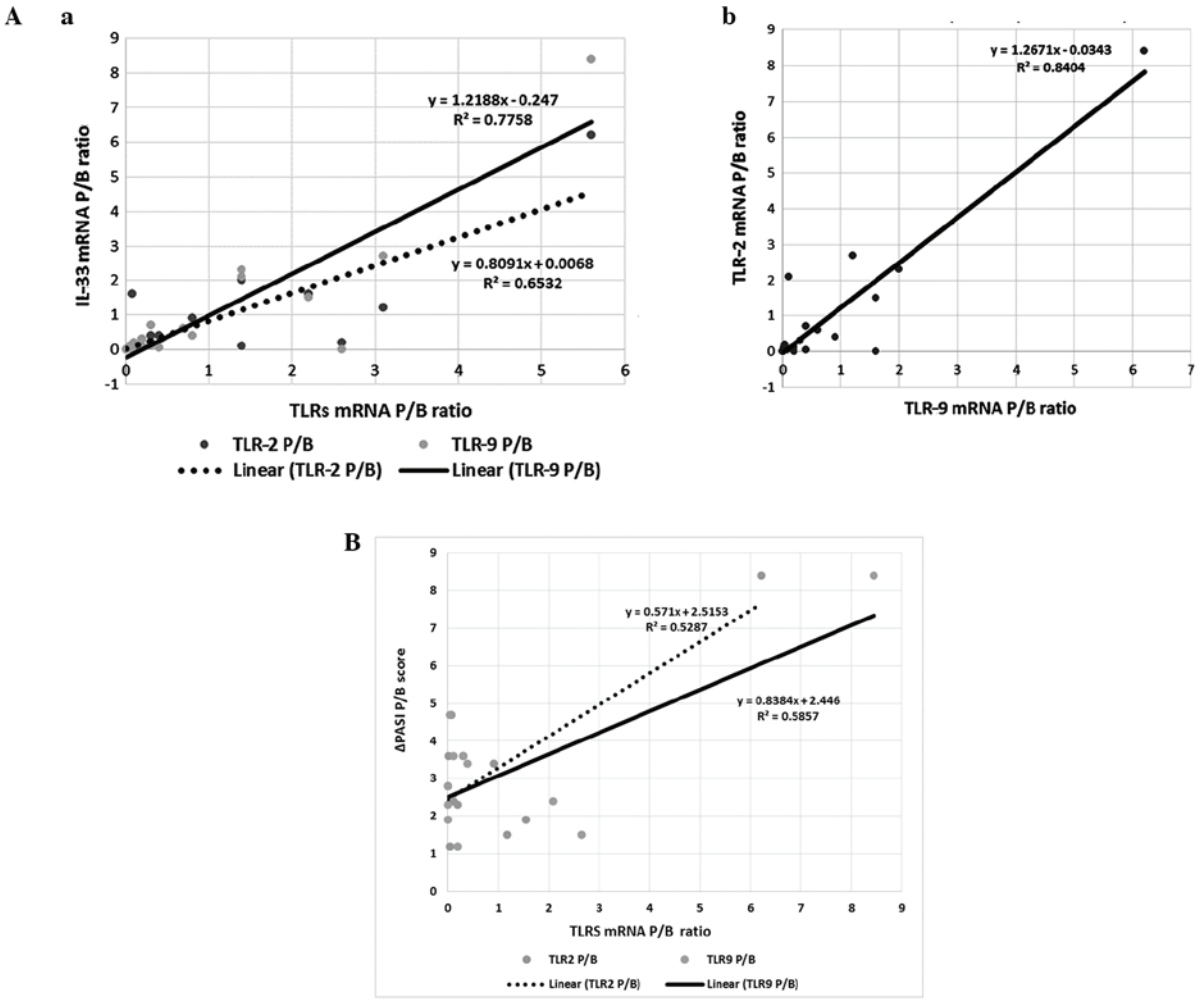

Figure 2. Correlations among mRNA alterations [post-treatment (P)/baseline (B) ratios] of pro-inflammatory IL-33, TLR-2 and TLR-9 and/or PASI score changes (ΔPASI), under anti-TNF-α treatment of psoriatic plaques. (A) Linear regression coefficients (R2) determined by Pearson's correlation analysis of P/B mRNA alterations between (a) IL-33 and TLR-2 (r=0.8082) or TLR-9 (r=0.8808), and (b) TLR-2 and TLR-9 (r=0.916713) (P<0.0001, two-tailed), in psoriatic plaques, treated with TNF-α inhibitors (etanercept or infliximab). (B) Linear regression coefficients (R2) determined by Pearson's correlation analysis between ΔPASI scores and relative (P/B) mRNA expression ratios of the pro-inflammatory molecules TLR-2 (r= 0.76528) and TLR-9 (r= 0.72714), in psoriatic plaques of male patients following etanercept treatment (P<0.05, two-tailed).

The present findings showed that TLR-2 and -9 were most affected by anti-TNF-α therapy, indicating that they play a critical role in the psoriatic innate immune response. IL-33 can act as both a pro- and anti-inflammatory factor (38). It is currently considered that biologically active IL-33 is released during necrosis as an endogenous danger or 'alarm' signal; during apoptosis, IL-33 is cleaved and inactivated (3,27). The present results indicate that the pro-inflammatory function of IL-33 in psoriatic skin can be inhibited by TNF-α blockers, in agreement with previous studies by Balato *et al* (25) and Li *et al* (39) which have described the regulation of IL33 by TNF-α.

In the present study, the efficacy of the TNF-α inhibitors etanercept and infliximab in plaque psoriasis therapy has been clearly demonstrated, in agreement with previous studies (2-6). Furthermore, concerning the small number of infliximab-treated cases or female patients, this study revealed notable observations regarding correlations among pro-inflammatory genes, anti-TNF-α treatment type and gender. It was observed that etanercept and infliximab exhibited similar effects on the expression of the three innate immune response factors, IL-33, TLR-2 and -9, in psoriatic skin lesions; however, the present data indicate that TLR-2 and IL-33 may share a common stimulation pattern in psoriatic plaques, in line with a previous report on inflamed skin (39). Male patients may exhibit a distinct biological response to etanercept compared to females, implying a different pathophysiological mechanism of psoriatic plaques. This finding is consistent with a previous report, which suggested that males exhibit more severe psoriasis, as compared with females (40). Additionally, TLR-2 and -9 may play a role as indices of severe psoriasis, since their mRNA alterations showed significant positive correlations with ΔPASI-2 in male patients that received etanercept.

In conclusion, the transcriptional levels of the three pro-inflammatory factors, IL-33, TLR-2 and -9, were examined prior to and subsequent to 3 months of anti-TNF-α treatment for psoriatic plaques. Despite the small number of study cases, the results support the efficacy of the TNF-α inhibitors etanercept or infliximab in reducing the innate immune response and indicate that the pro-inflammatory factors IL-33, TLR-2 and -9 play a role in psoriatic biology. Etanercept tended to be more

effective in innate immune response inhibition in males. The present findings support the instigation of further investigations into innate immune response elements, particularly TLR-2 and -9, under anti-TNF-α treatment, including a more extensive group of patients, in order to clarify the possible pathophysiological mechanisms of psoriatic plaques and TNF-α therapy mechanisms according to their action and efficacy, as well as the associations with gender.

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