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Regular paper

The *in vitro* modulatory effect of TNFα on the mRNA expression and protein levels of zinc finger protein ZNF334 in CD4+ lymphocytes of healthy people

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We have shown before that the expression of *ZNF334* gene, coding for a newly described zinc finger protein of as yet unknown function, is extremely reduced in CD4+ lymphocytes of rheumatoid arthritis (RA) patients regardless of their age, and thus can be considered a new molecular marker of the disease. Based on the promoter sequence of the gene we speculated that it might be regulated by TNFα. Here we have tested that hypothesis, studying the *in vitro* influence of TNFα on the *ZNF334* gene expression and protein levels in resting and stimulated CD4+ cells of healthy volunteers. We have confirmed that treatment with TNFα modifies the levels of *ZNF334* expression in the CD4+ cells *ex vivo*; however, the effect varied for different individuals and reduction of expression was seen only for those cell samples that initially exhibited high transcriptional activity of the gene, while for those exhibiting initially very low expression, some increase in the transcriptional activity was observed. Incubation with TNFα significantly reduced the amounts of two isoforms of ZNF334 protein (initially high in all subjects) in parallel to the reduced transcription. Finally, the expression of ZNF334 in CD4+ lymphocytes isolated after various periods of anti-CD3 stimulation generally increased with longer culture times, and the effect of TNFα treatment was negligible. Concluding, our results obtained *in vitro* for helper lymphocytes of healthy individuals seem to mimic the regulatory effect of TNFα on the expression of ZNF334 in the cells of RA patients.

Key words: ZNF334, human, CD4+ lymphocytes, TNFα

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INTRODUCTION

We have recently demonstrated that expression of the *ZNF334* gene, encoding one of the recently discovered and as yet very poorly characterized members of the family of zinc finger proteins (ZNFs) of is greatly reduced in peripheral blood CD4+ lymphocytes derived from rheumatoid arthritis (RA) patients (Soroczynska-Cybula *et al.*, 2011). Zinc finger proteins are thought to play an important role as positive or negative regulators of expression of multiple genes, including those involved in the regulation of cell proliferation (Urrutia, 2003; Ghaleb *et al.*, 2005). With the exception of our paper cited above (Soroczynska-Cybula *et al.*, 2011), there are so far (February 2015) no reports about specific function of ZNF334, nor about its disease-associated expression disorders in human T cells.

Tumor necrosis factor (TNFα) is a major proinflammatory cytokine participating in the pathogenesis of RA. Its increased concentrations observed in RA patients exert major modifying effects on their CD4+ lymphocytes, presently considered a major factor in RA pathogenesis (Bryl *et al.*, 2001). It is hypothesized that modified *in vitro* proliferation dynamics of these cells may be associated with their reduced levels of CD28 expression, earlier proven to be due to an indirect effect of TNFα on the *CD28* gene promoter (Bryl *et al.*, 2001). We have demonstrated that the *ZNF334* gene promoter contains a sequence closely homologous to the TNFα-responsive sequence in the promoter of *CD28* gene (Soroczynska-Cybula *et al.*, 2011). These observations led to the hypothesis that also the expression of ZNF334 is controlled by TNFα. As other proinflammatory cytokines, especially IL-1, participate in the RA pathogenesis as well (Arend & Dayer, 1995; Dinarello, 1996; Kay and Calabrese, 2004) and are known to be regulated by TNFα (Kay & Calabrese, 2004), we decided to check here if the effect of the latter, modifying the expression of ZNF334 in CD4+ lymphocytes *in vitro* could be indirect and related to the activities of other TNFα-dependent proinflammatory cytokines, including IL12p70, IL-6, IL-10, IL-1b, and IL-8.

MATERIALS AND METHODS

Subjects. Altogether, six healthy volunteers aged 20-24 years (3 women and 3 men) took part in the study. They were informed about the purpose and method of research and have given their written consent. The project was approved by the Local Commission for Research Ethics at the Medical University of Gdansk.

Cells. Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque™ flotation from fasting venous blood, counted and their viability (accepted at least 95%) was estimated with trypan blue exclusion test. CD4+ lymphocytes were immuno-magnetically purified from resting or mitogen-stimulated and cytokine-treated PBMC with the CD4+ Negative Isolation Kit™ (Dynal Biotech) according to the manufacturer's protocol. Purity

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Abbreviations: PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; RFU, relative fluorescence units

of the CD4+ cells obtained that way assessed by FACS analysis always exceeded 97%. Enriched CD4+ cells were flash-frozen in liquid nitrogen and stored at –80°C until further processing.

Samples of purified, resting CD4+ T cells suspended at 1×10^6 or 2×10^6 in 2 ml of complete culture medium (RPMI (Sigma) with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) were incubated in the wells of a 24-well culture plate with or without 10 ng/ml TNFα (R&D Systems, Minneapolis, MN) for 6 hours at 37°C in 5% CO_2. The optimal concentration of TNFα and the stimulation time had previously been established in pilot experiments. The cells were harvested after six hours of cytokine treatment and assayed for the expression of ZNF334. In order to confirm further the role of TNFα in the regulation of ZNF334 expression, some cell samples pretreated with the cytokine were then washed to remove free TNFα and cultured for additional 6 or 12 hours before the assessment of ZNF334 expression.

In order to assess the expression of ZNF334 in the CD4+ cells proliferating *in vitro*, and the possible effect of TNFα in these conditions, PBMC were stimulated with immobilized monoclonal anti-CD3 (125 ng/ml) for 72 or 120 hours (Bryl & Witkowski, 2004), without or in the presence of 10 ng/ml TNFα. At the end of the culture period, CD4+ lymphocytes were isolated as above and processed for ZNF334 determination.

Quantitative real-time PCR estimation of *ZNF334* gene expression. Total RNA was isolated from enriched CD4+ cells using TriReagent™ (Sigma Aldrich, USA) and manufacturer's protocol. cDNA was prepared using oligo-dT as starters and AMV reverse transcriptase (Promega, USA). The following pairs of primers were used for detection of the mRNAs of interest:
ZNF334 — sense: 5'-AGGAAAGCCAGCAATCAAAA-3', antisense: 5'-TCCAGTGAGGCTTGTCTTCA-3', and
GAPDH — sense: 5'-GGCGTCTTCACCACCATGG-3', antisense: 5'-TGCTGATGATCTTGAGGCTG-3'.

The products of *ZNF334* and *GAPDH* genes were amplified by PCR in Eppendorf Personal Mastercycler™ using the same pairs of primers in order to prepare the standard curves for the quantitations. The following reaction conditions were applied: initial denaturation at 94°C, 10 min; 30 amplification cycles including melting for 30 s at 94°C, annealing 30 s at 55°C, and elongation 30 s at 72°C; after last cycle termination 10 min. at 72°C followed by cooling and storage at 4°C. LightCycler™ and FastStart DNA Master SYBR Green I Kit (both from Roche Diagnostics) were used for real-time PCR. The reaction was performed using 10 minutes' activation at 95°C, followed with 40 cycles including 10 s at 95°C; 5 s at 55°C and 10 s at 72°C each, followed by 30 s cooling at 40°C. Results were calculated on the basis of the standard curve using LightCycler Software 4.05 (Roche Diagnostics, Germany) and expressed as relative fluorescence units (RFU).

Western blot analysis of amount of ZNF334 protein in isolated CD4+ lymphocytes. Proteins from lysates from 250 000 immunomagnetically purified CD4+ lymphocytes per sample were resolved using standard SDS-PAGE according to (Laemmli, 1970). Proteins were transferred to nitrocellulose membrane using a Trans-Blot SD SemiDry Transfer Cell (Bio-Rad), the membrane was blocked with 5% no fat milk and probed for ZNF334 using Rabbit anti-human ZNF334 polyclonal antibody (Abcam, 1:300), or for actin (gel loading control) using mouse mAb to β-actin (Abcam, 1:300). Appropriate peroxidase-conjugated anti-Ig Abs and ECL system (Super Signal West Pico Chemiluminescent Substrate; Pierce) were used to visualize the proteins of interest. The bands were recorded on X-ray film, digitized using GDS-8000 instrument and quantified using the LabWorks software (both from Ultra-Violet Products).

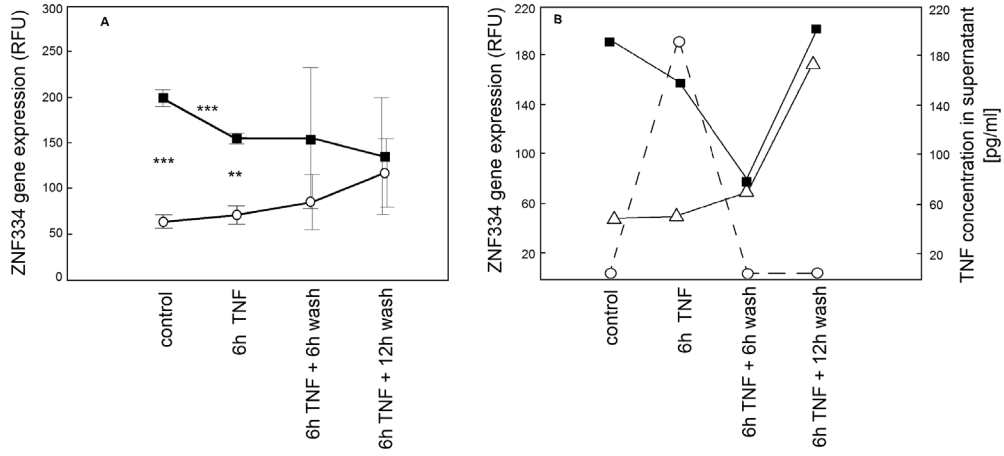

Figure 1. (A) Bidirectional effect of TNFα on *ZNF334* expression in human CD4+ cells *in vitro*.
Expression of *ZNF334* was estimated in untreated, isolated CD4+ lymphocytes (**control**), in the cells treated with 10 ng/ml TNFα for 6 hours (**6 h TNF**), then the cytokine was washed out and the cells incubated for another 6 (**6 h TNF + 6 h wash**) or 12 hours (**6 h TNF + 12 h wash**) prior to *ZNF334* expression analysis by real-time PCR. Results are expressed as relative fluorescence units (RFU) as in Materials and Methods. Groups of individuals exhibiting initially high and low levels of *ZNF334* expression were distinguished by K-means cluster analysis and their levels of expression of ZNF334 differed significantly at rest (***p = 0.001) and after 6-hour treatment with the cytokine (**p=0.007). Data are shown as means ±S.D. (**B)** Effect of TNFα and its removal on expression of *ZNF334* in resting CD4+ lymphocytes of representative high ZNF334 expresser (black squares) and low expresser (open triangles). Concentrations of TNFα in the supernatants were measured by CBA technique and a representative result is shown (open circles; TNF concentration range is shown on the right vertical axis). The *ZNF334* expression was measured as in A. The figure shows results of one representative experiment out of three giving similar results.

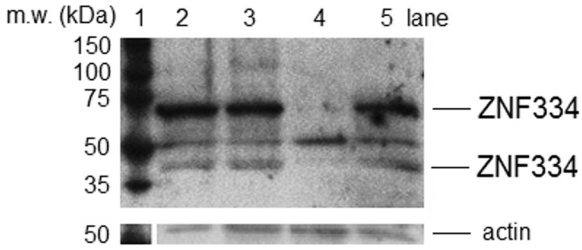

Figure 2. Treatment with TNFα transiently reduces *ZNF334* protein levels in CD4+ lymphocytes *in vitro*.
Isolated CD4+ cells were incubated for 6 hours with 10 ng/ml TNF*α* and immediately lysed (lane 3), or incubated with TNF*α* for 6 hours and then without the cytokine for subsequent 6 (lane 4) or 12 hours (lane 5) prior to lysis and Western blot processing. Control cells (lane 2) were incubated for 6 hours without the cytokine. Lane 1 contains molecular weight marker. Marked positions of ZNF334 correspond to those described in the data sheet provided by the antibody supplier. The Western blot shown is representative for three experiments giving similar results and the CD4+ cells were obtained from the 'high ZNF334 expresser' illustrated in Fig. 1B.

Relative amounts of ZNF334 protein were expressed as arbitrary densitometric units after standardization versus actin content.

Analysis of proinflammatory cytokine levels. The levels of proinflammatory cytokines in the sera of subjects and in culture supernatants were evaluated using flow cytometry and the Human Th1/Th2 Cytokine Kit (CBA, Becton Dickinson, USA) according to the manufacturer's instructions. In addition to TNFα, the following cytokines were analyzed: IL12p70, IL-6, IL-10, IL-1b, and IL-8.

Statistical analysis was performed using the Statistica 10.0 (StatSoft, Poland) package. The K-means cluster approach was applied to distinguish between high and low expressers of ZNF334.

RESULTS

Effect of TNFα on ZNF334 gene expression in resting CD4+ lymphocytes

While assessing the levels of expression of the *ZNF334* gene in resting CD4+ lymphocytes, we found that even in the relatively small group of individuals tested the expression was either high or low, with no intermediates, which allowed us to separate our subjects into 'high-' and 'low-expressers'. Interestingly, treatment with 10 ng/ml TNFα seems to have opposite "homeostatic" effects depending on the initial levels of ZNF334 expression. Thus, initially high expression levels were significantly reduced, while those initially low — increased after a 6-hour incubation with the cytokine and at least a 6-hour-long cytokine-free post-incubation period, so eventually the levels of expression of the *ZNF334* gene were similar in all samples, regardless of their initial values (Fig. 1). The *ZNF334* expression changes in resting CD4+ cells treated with TNFα showed relatively large inter-individual variability, seen mostly after 12 hours into treatment (6 hours of TNFα exposure plus 6 hours in cytokine-free medium; Fig. 1A). It seems worth noting that the reduction of *ZNF334* transcription due to TNFα action could be transient as shown for a representative 'high expresser' (Fig. 1B), which could explain the apparent high variability of the effect of treatment with the cytokine.

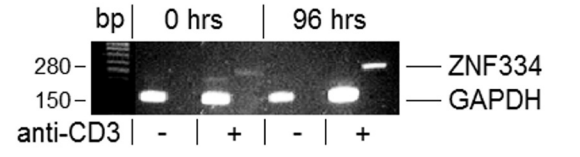

Figure 3. Four-day *in vitro* PBMC stimulation induces *ZNF334* expression in CD4+ lymphocytes.
Details of PBMC stimulation, separation of CD4+ cells and RT-PCR experiment design are described in the Materials and Methods. Representative result of three experiments showing similarly increased expression of ZNF334 after 4-day anti-CD3 stimulation of PBMC.

The data presented above had shown substantial individual variability of the effect of exposure to TNFα on the expression of ZNF334 gene in CD4+ lymphocytes. As the effect (especially reduced expression in 'high expressers') was only seen at least 6 hours into the treatment (Fig. 1), it was possible that it was not related to a direct action of TNFα, but to some other cytokine(s) stimulated by the TNFα treatment. In order to check for such possibility, we attempted to assess the levels of TNFα and proinflammatory cytokines IL-1β, IL-6, IL-8, IL-10 and IL-12p70, in supernatants from the incubation of cells with TNFα and to search for correlations between the actual levels of those cytokines and the expression of *ZNF334*. The TNFα levels in the supernatants after 6 h of incubation with TNFα (10 ng/ml) had expectedly shown an increase above the control values (the latter ranging from 0 to 4.8 pg/ml); however, a large inter-individual scatter of the cytokine concentrations (ranging from 44.4 to 204.5 pg/ml, not shown) could be observed, possibly due to inter-individual difference in binding and endocytosis of the exogenous cytokine. We did not observe significant changes in the concentrations of other cytokines studied over the whole time of the experiment; in fact, all these levels were near-zero throughout (not shown). These result can be interpreted as a lack of any influence of the above-mentioned cytokines on expression of *ZNF334*.

Effect of TNFα on the levels of ZNF334 protein in CD4+ lymphocytes

After confirming that the expression of *ZNF334* gene at mRNA level could be affected by TNFα, we attempted to check if the cytokine treatment affects also the levels of ZNF334 protein in the CD4+ lymphocytes. Two forms of ZNF334 protein, with relative masses of roughly 70 and 45 kDa, were detected as described by the anti-ZNF334 antibody manufacturer (Fig. 2). Additionally, a third band reacting with the anti-ZNF334 antibody used, of apparent molecular weight around 50 kDa, could also be seen. Following 6 hours of *in vitro* TNFα treatment and subsequent 6 hours of washout, the amount of the 70-kDa and 45-kDa forms had dropped significantly, while that of the 50-kDa form had increased (Fig. 2, lane 4). Interestingly, the effect of TNFα was transient and after another 6 hours without the cytokine the levels of all three forms of the ZNF334 protein returned to control values (Fig. 2, lane 5). We never saw an increase of the ZNF334 protein level above the resting value, regardless the concomitant results of the assessment of the effect of TNFα on ZNF334 gene transcription (i.e., also in 'low expressers' the levels of ZNF334 protein behaved as described).

Effect of *in vitro* stimulation on the expression of *ZNF334* gene

Stimulation of PBMC *in vitro* increases the secretion of cytokines, including TNFα (Bryl *et al.*, 2001; 2005) which could result in the modulation of expression of *ZNF334*. In order to assess this possibility, we stimulated the PBMC *in vitro* using immobilized anti-CD3 for 1 to 4 days. This stimulation resulted in a noticeable increase in the expression of *ZNF334* gene in purified $CD4^+$ lymphocytes in relation to the unstimulated cells, but only after 4 days in culture (Fig. 3). Additional treatment of anti-CD3-stimulated PBMC with 10 ng/ml TNFα for 72 or 120 hours did not further change the expression of *ZNF334* (not shown).

DISCUSSION

$CD4^+$ T cells in RA patients are characterized by phenotypic abnormalities including higher percentage of immune memory cells, the increase in the percentage of cells showing no or greatly reduced expression of CD28 receptor (Bryl *et al.*, 2001), Klotho (Bryl *et al.*, 2001; Witkowski *et al.*, 2007), telomere shortening (Koetz *et al.*, 2000) and by changed function, including abnormal proliferation, increased apoptosis and impaired production of cytokines (Pawelec *et al.*, 2001; Fulop *et al.*, 2003). The chronically increased levels of proinflammatory TNFα observed in RA patients (Bryl *et al.*, 2005) are recognized as a major cause of these changes.

We have recently shown that also the expression of a gene coding for a new, still poorly characterized zinc finger protein ZNF334 (the gene of which contains a motif in the promoter region homologous to those found in the *CD28* and *Klotho* genes and thought to be responsive to TNFα) is greatly reduced in $CD4^+CD28^+$ lymphocytes of RA patients (Witkowski *et al.*, 2007). Here we tested *in vitro* the hypothesis that the chronically elevated levels of TNFα observed in RA patients are responsible for the down-regulation of the *ZNF334* gene expression. Our results confirmed that *in vitro* treatment of human PBMC from healthy volunteers with TNFα regulates the expression of *ZNF334* at both the mRNA and protein levels. One has to bear in mind that even in a healthy person the inflammatory processes (as opposite to inflammatory diseases) occur all the time and some TNFα is always present in extracellular compartments; thus, the demonstrated regulation of *ZNF334* by TNFα may have some physiological relevancy. Despite this circumstantial evidence, a direct proof of the TNFα effect on *ZNF334* expression in the cells of RA patients is still to be demonstrated. Interestingly, the effect of TNFα seems to be that of a homeostat, reducing an initially high expression of the gene and rising its relatively low expression. As the function of ZNF334 is as yet unknown, it is virtually impossible to predict the biological role of such a homeostatic regulation of its expression by the cytokine. As we were unable to show any secretion of proinflammatory cytokines (IL-1, IL-6 and others tested) in the supernatants from TNFα-treated cells, we could exclude their participation in the control over ZNF334 expression thereby strengthening the assumption about the direct role of TNFα. Still, the relatively slow, bidirectional 'homeostatic' effects of TNFα on the expression of ZNF334 gene may suggest that other mechanisms, apparently differently reacting to TNFα treatment, intervene and decide whether the gene's transcription will be reduced or augmented.

Interestingly, the resting levels of ZNF334 protein in $CD4^+$ lymphocytes of healthy people seem to be uniformly high; this may signify some important homeostatic role for the protein in these lymphocytes, requiring further studies. The only *in vitro*-observable effect of TNFα on ZNF334 protein level is its transient reduction (Fig. 2). In our experiments this transiency (in both the reduction of ZNF334 mRNA and protein levels) was apparently related to the removal of free cytokine after 6-hour treatment. This supports our hypothesis of the permanent reduction of ZNF334 transcription and protein in the RA patents' cells chronically exposed to TNFα.

Notably, the expression of ZNF334 was increased in the $CD4^+$ cells stimulated *in vitro* with anti-CD3, once again bringing up the possibility of a crucial, homeostatic role of the protein also in responding T cells.

The similar timing of both effects (reduced transcription and protein levels of ZNF334 upon TNFα treatment) suggests that they are related, but the length of the period from initial cytokine treatment to an observable change of transcription or protein amount suggests additional intervening mechanisms. One explanation of the effect of TNFα on ZNF334 protein level could be stimulation of limited proteolysis of the latter, corroborated by simultaneous appearance of its clipped (50 kDa) form alongside with the disappearance of the native, 65-kDa protein (Fig. 2, lane 4), as well as the disappearance of the 45-kDa form of ZNF334 after 12 hours of TNFα treatment; in the latter case the amount of the putative clipped form (with an expected m.w. of around 30 kDa) seems to be below the detection limit. A possible intracellular proteolytic enzyme responsible for this effect could be μ-calpain, as at least some effects of TNFα have been shown to depend on the enzyme activity, known to be stimulated by the cytokine (Sorimachi *et al.*, 1997; Goll *et al.*, 2003). The amino acid sequence of ZNF334 is relatively rich in proline, glutamine, serine and threonine, known to be enriched in the protein targets of calpains as so called PEST motif (Tompa *et al.*, 2004); this makes ZNF334 a potential target for calpain-dependent proteolysis. Our pilot experiments indicate that calpain may be hyperactive in the $CD4^+$ lymphocytes of RA patients (JMW, unpublished), which gives some substance to the abovementioned speculation, still requiring an experimental proof.

Zinc finger proteins are thought to function as either positive or negative regulators of expression of other genes (Urrutia, 2003; Soroczynska-Cybula *et al.*, 2011). Recently, a novel function has been proposed for two related zinc finger proteins, ZNF91 and ZNF93, suggested to have evolved to suppress retrotransposons (Jacobs *et al.*, 2014). Currently, no data are available on physiological functions of ZNF334 in T cells or, as a matter of fact, in any other cell type. Interestingly, our analyses indicate that ZNF91 shows some sequence homology with ZNF334 (JMW, unpublished data). A decreased activity of ZNF334 could be partly responsible for the abnormal proliferation of $CD4^+$ cells derived from patients with RA by decreasing the activity of genes controlling the cell cycle, such as cyclins and cyclin-dependent kinases. Recently it has been demonstrated that cyclin D1 is controlled by proteins belonging to the family of zinc finger protein KLF13 (Nemer & Horb, 2007). A reduced ZNF334 activity could also mediate the phenotypic abnormalities (especially loss of CD28) observed in the $CD4^+$ cells of RA patients. At this time, however, all these possibilities are purely speculative pending further detailed studies.

Author contributions

IH — performed the experiments; JMW, MS-C, EB — designed the study; IH, MS-C wrote the draft, and JMW wrote the final version of the paper.

Conflict of interest

The authors declare no conflict of interest.

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