Original Article

A Low-Molecular-Weight Dialysable Leukocyte Extract Selectively Enhances Development of $CD4^+ROR\gamma t^+$ T Cells and IL-17 Production

(cytokine production / IL-17 / low-molecular weight leukocyte extract / *RORgt* / Th17 cells)

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Abstract. A low-molecular-weight (under 10 kDa) dialysable leukocyte extract (called transfer factor, TF) has been shown to be a prospective substance to improve or modulate immune response in autoimmunity, inflammation, infectious diseases or cancers. However, the use of TF has been limited by the absence of any data on the mechanism of its action. Here we show that TF prepared from peripheral blood leukocytes of healthy human donors displays multiple regulatory effects on individual parameters of the immune system. TF decreases proliferation of T and B lymphocytes and partially alters the production of cytokines and nitric oxide by activated macrophages. TF also inhibits production of T helper 1 (Th1) cytokines interleukin 2 (IL-2) and interferon γ, slightly stimulates production of Th2 cytokine IL-10 and considerably enhances the secretion of IL-17 by activated mouse spleen T cells. At the molecular level, TF enhances expression of genes for transcription factor RORγt and for IL-17. The enhanced expression of the *RORgt* gene corresponds with an increase in the number of $ROR\gamma t^+CD4^+$ Th17 cells and with enhanced IL-17 production. In contrast, the expression of the *Foxp3* gene and the proportion of $CD4^+CD25^+Foxp3^+$ regulatory T cells are not significantly changed in the presence of TF. These results suggest that the activation of pro-inflammatory Th17 cells, which have multiple immunoregulatory properties, could be the main mechanism of the immunomodulatory action of a low-molecular-weight leukocyte extract.

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Abbreviations: ConA – concanavalin A, ELISA – enzyme-linked immunosorbent assay, IFN – interferon, IL – interleukin, LPS – lipopolysaccharide, NK – natural killer, NO – nitric oxide, mAb – monoclonal antibody, PBL – peripheral blood leukocyte, PCR – polymerase chain reaction, SD – standard deviation, TF – transfer factor, Th – T helper.

Introduction

A low-molecular weight dialysable leukocyte extract, termed transfer factor (TF), was described for the first time more than a half a century ago as a substance isolated from the leukocytes of immunized individuals and capable of transferring immunity to non-sensitized recipients (Lawrence, 1955). Originally, antigen-specific effects of TF had been demonstrated, and TF was therefore proposed for use in transferring specific immunity (Jeter et al., 1954; Lawrence, 1955). However, Bloom (1973) later suggested that TF can act non-specifically to stimulate the reactivity of a subthreshold number of previously sensitized lymphocytes and thus function as a non-specific adjuvant or immunomodulator.

In spite of promising results demonstrating the therapeutic potential of TF in experimental models in mice (Williams and Kauffman, 1980), rats (Liburd et al., 1972), guinea pigs (Burger et al., 1972), dogs (Simon et al., 1977) and cows (Klesius and Fudenberg, 1977), clinical trials to transfer specific immunity yielded variable results (Lawrence, 1969). The research on TF mostly waned, and only a spare data on therapeutic effects of TF has been recently published in patients with cancers (Franco-Molina et al., 2008; Lara et al., 2010) and varicella zoster virus infections (Estrada-Parra et al., 1998), in experimental pulmonary tuberculosis (Fabre et al., 2004) and in some other infectious diseases, as recently reviewed by Viza et al. (2013). The reason for the decreased research interest was the absence of any *in vitro*

model that would be able to reproducibly prove the efficacy of TF and that would enable characterization of the cellular and molecular mechanism of TF action. There appeared only a spare data showing the inhibitory effects of TF on the cytokine and nitric oxide (NO) production by murine macrophages (Franco-Molina et al., 2007) and on human NK cell activity (Lang et al., 1982). Franco-Molina et al. (2006) and Mendoza-Gamboa et al. (2008) studied the effects of bovine TF on human cancer cell lines *in vitro* and found that TF decreased proliferation of cancer cells, but not normal cells. At the molecular level, TF modulated the AP-1 DNA-binding activity and inhibited expression of several nuclear transcriptional factors in cancer cells.

Recent advances in the methods of cellular and molecular immunology, as well as the recognition of individual immunoregulatory cytokines and the description of phenotypically and functionally distinct cell populations, have rekindled attempts to explore the cellular and molecular targets of TF and to characterize the mechanism of its action. For this purpose, we evaluated the immunomodulatory effects of a low-molecular-weight (< 10 kDa) dialysable extract prepared from peripheral blood leukocytes of healthy human donors. We were able to detect a wide range of functional activities of TF with both positive and negative impacts on individual parameters of the immune response. Among these functional properties of TF, a promoting effect on the development of $CD4^+ROR\gamma t^+$ Th17 cells and on IL-17 production by activated T cells has been found to be the most prominent property. Since IL-17 is a potent proinflammatory cytokine with multiple immunoregulatory effects in inflammation, autoimmunity, infectious diseases, transplantation immunity and cancer (Miossec and Kolls, 2012; Zhu and Qian, 2012), the recognized effects of TF can explain the mechanisms of its actions and represent a rationale for the use of TF in immunomodulation.

Material and Methods

Preparation of transfer factor

The substance under the commercial name Immodin was obtained from Sevapharma (Prague, Czech Republic). The method for TF preparation was adopted from the original procedure described by Lawrence (1974). In brief, peripheral blood leukocytes (buffy coat) were isolated from healthy human blood donors and were adjusted to a concentration of 2×10^8 cells/ml in 0.9% saline (0.9% water solution of NaCl). The cell suspension was frozen to -70 °C in a freezing device and defrosted in water bath at 37 °C. This procedure was repeated six times. The substance was dialysed for 24 h in an excess of sterile water using a peristaltic pump. Subsequently, the material was concentrated by lyophilization. The concentrate was dissolved in sterile distilled water, ultrafiltered with the use of a Pall TFF System through a 10 kDa Centramate TFF membrane 10 kDa (Pall Austria Filter GmbH, Vienna, Austria) and heated at 60 °C. The material (molecular weight < 10,000) was filtered through a 0.2 μm sterilization filter (Pall Austria Filter), divided into ampoules and lyophilized. After standard dissolution in serum-free culture medium, 1 ml of the TF solution corresponded to the extract from 50×10^6 leukocytes.

Cell proliferation

A single-cell suspension of spleen cells from 7-8 week-old BALB/c mice, obtained from the breeding colony of the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, was prepared in RPMI 1640 medium (Sigma-Aldrich., St. Louis, MO) containing 10 % heat-inactivated foetal calf serum (Sigma), antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin), 10 mM HEPES buffer and 5×10^{-5} M 2-mercaptoethanol (hereafter referred to as complete RPMI 1640 medium). The cells at a concentration 0.6×10^6 cells/ml were cultured in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in a volume of 200 μl of complete RPMI 1640 medium at 37 °C in an atmosphere of 5% of CO_2 unstimulated or stimulated with 5 μg/ml of bacterial lipopolysaccharide (LPS, Difco Laboratories, Detroit, MI) or 1.5 μg/ml of concanavalin A (ConA, Sigma). Cell proliferation was determined by adding [^{3}H]thymidine (1 μCi/well, MP Biomedicals, Santa Ana, CA) for the last 6 h of the 72-h incubation period.

To evaluate the effects of TF on cell proliferation, freshly dissolved lyophilized material (dissolved in serum-free RPMI 1640 medium to a concentration corresponding to the extract from 50×10^6 leukocytes in 1 ml) was added to cultures of stimulated spleen cells to make 25, 8.3 and 2.8 percent of the culture volume. This standard dilution represents the amount of TF corresponding to the extract from 12.5, 4.2 and 1.4×10^6 PBL/ml.

Cytokine production by T cells

Spleen cells (0.6×10^6/ml) from BALB/c mice were cultured in a volume of 1.0 ml of complete RPMI 1640 medium in 48-well tissue culture plates (Corning Inc., Corning, NY) unstimulated or stimulated with 1.5 μg/ml of ConA (Sigma). Dissolved TF (1 ml of solution corresponding to the extract from 50×10^6 leukocytes) was added to the cultures to make up 25, 8.3 and 2.8 percent of the culture volume. Supernatants were harvested after 24-h (IL-2 determination), 48-h (IFN-γ) or 72-h (IL-10 and IL-17) incubation period. The concentrations of cytokines in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

Nitric oxide (NO) and cytokine production by macrophages

Peritoneal exudate cells (as a source of macrophages) were obtained by washing the peritoneal cavity of BALB/c mice with 10 ml of culture medium. The cells at a concentration of 0.9×10^6 cells/ml were cultured in 48-well tissue culture plates (Corning) unstimulated or stimulated with LPS (5 μg/ml) and IFN-γ (5 ng/ml,

PeproTech, Rocky Hill, NJ) in the absence or presence of TF (25, 8.3 and 2.8 percent of the culture volume). The supernatants were harvested after 48-h (NO determination) or 72-h (IL-1β, IL-6 and IL-12 determination) incubation period.

NO determination

Nitrite concentrations in culture supernatants were measured using the Griess reaction (Green et al., 1982). In brief, 100 µl of the tested supernatants were incubated with 50 µl of 1% sulfanilamide (in 3% H_3PO_4) and 50 µl of 0.3% *N*-1-naphthylethylenediamine dihydrochloride (in 3% H_3PO_4). Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

ELISA

The production of IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17 and IFN-γ was quantified by ELISA. Cytokine-specific capture and detection monoclonal antibodies (mAb) purchased from PharMingen (San Jose, CA) were used for the detection of IL-2, IL-6, and IFN-γ. IL-1 β, IL-10, IL-12 and IL-17 were measured using ELISA kits purchased from R&D Systems (Minneapolis, WN), following the instructions of the manufacturer. The reaction was quantified by spectrophotometry using a Sunrise Remote ELISA Reader (Gröding, Austria).

Flow cytometry

Spleen cells were stimulated for 72 h with ConA (1.5 µg/ml) in the absence or presence of TF, as described in the case of cytokine production. The expression of cell surface molecules or the intracellular expression of transcriptional factors RORγt and Foxp3 was determined by flow cytometry as previously described (Svobodova et al., 2012). For intracellular staining, the cells were first incubated for 30 min with Alexa Fluor 700-labelled anti-CD4 mAb (clone GK1.5, BioLegend, San Diego, CA) and/or FITC-labelled anti-CD25 mAb (clone PC61, BioLegend). A Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular Probes, Eugene, OR) was used for the staining of dead cells. Cells were permeabilized using the eBioscience Foxp3 buffer staining set according to the manufacturer's instructions. For intracellular staining the cells were incubated for 30 min with PE-labelled anti-mouse/rat Foxp3 mAb (clone FJK-16s, eBioscience, San Diego, CA), or PE-conjugated anti-mouse/human RORγt mAb (clone AFKJS-9, eBioscience). Data were collected using an LSRII cytometer (BD Biosciences) and analysed using FlowJo software (Tree Star, Ashland, OR).

Detection of RORgt and IL17 gene expression by real-time PCR

Spleen cells (0.6×10^6 cells/ml) from BALB/c mice were cultured in a volume of 1.0 ml of complete RPMI 1640 medium unstimulated or stimulated with ConA (1.5 µg/ml) in the absence or presence of TF (at the same concentrations in the case of cytokine production). After 72-h incubation, the cells were harvested and total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One µg of total RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 µl using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in an iCycler (BioRad, Hercules, CA) as previously described (Holan et al., 2010). The primers used for amplification included: *GAPDH*: sense - AGAACATCATCCCTGCATCC, nonsense - ACATTGGGGGTAGGAACAC, *RORgt*: sense - ACCTCTTTTCACGGGAGGA, nonsense - TCCCACATCTCCCACATTG, *Foxp3*: sense - AGAAGCTGGGAGCTATGCAG, nonsense - GCTACGATGCAGCAAGAGC, and *IL17F*: sense - CCCAGGAAGACATA CTTAGAAGAAA, nonsense - CAACAGTAGCAAAGACTTGACCAT. Each individual experiment was performed in triplicate and the reaction efficiency for each gene was estimated by the dilution curve method. Fluorescence data were collected at each cycle after an elongation step at 80 °C for 5 s and the data were analysed with an iCycler Detection system, Version 3.1.

Statistical analysis

The results are expressed as the mean ± SD. Comparisons between two groups were analysed by Student's *t*-test, and multiple comparisons were analysed by ANOVA. A value of $P < 0.05$ was considered statistically significant. For all statistical evaluations the null hypothesis was considered.

Results

The effect of TF on the proliferation of T and B lymphocytes

Spleen cells were stimulated for 72 h with T-cell mitogen ConA or B-cell mitogen LPS in the absence or presence of the indicated concentrations of TF and the cell proliferation was determined. As demonstrated in Fig. 1, only at the highest concentration (corresponding to the extract from 12.5×10^6 leukocytes/ml) TF decreased cell proliferation.

The effect of TF on the production of cytokines and NO by macrophages

Mouse peritoneal macrophages were stimulated with LPS/IFN-γ in the absence or presence of TF and the levels of cytokines and NO in the supernatants were determined. Fig. 2 shows that the production of IL-1β (Fig 2B) was significantly increased in the presence of TF; the production of IL-12 (Fig 2A) and IL-6 (Fig 2C) was not significantly altered in the presence of TF. Similarly, the production of NO was only slightly decreased in the cultures containing the highest concentration of TF (Fig. 2D).

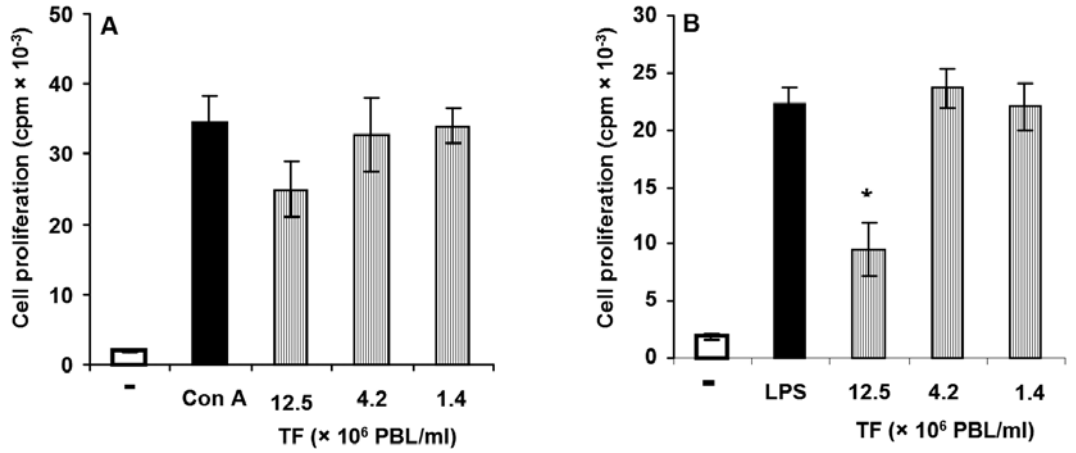

Fig. 1. The effects of TF on the proliferation of T and B lymphocytes. Spleen cells from BALB/c mice were cultured unstimulated (-) or stimulated with (A) T-cell mitogen ConA (1.5 μg/ml) or (B) B-cell mitogen LPS (5 μg/ml) in the absence or presence of the indicated concentrations of TF (the concentrations of TF corresponded to the extract from 12.5, 4.2 or 1.4 × 10^6 PBL/ml). Cell proliferation was determined by adding [^{3}H]thymidine for the last 6 h of the 72-h incubation period. Each bar represents the mean ± SD from four independent experiments. The value with the asterisk is significantly (*P < 0.05) different from the control (cells stimulated without TF).

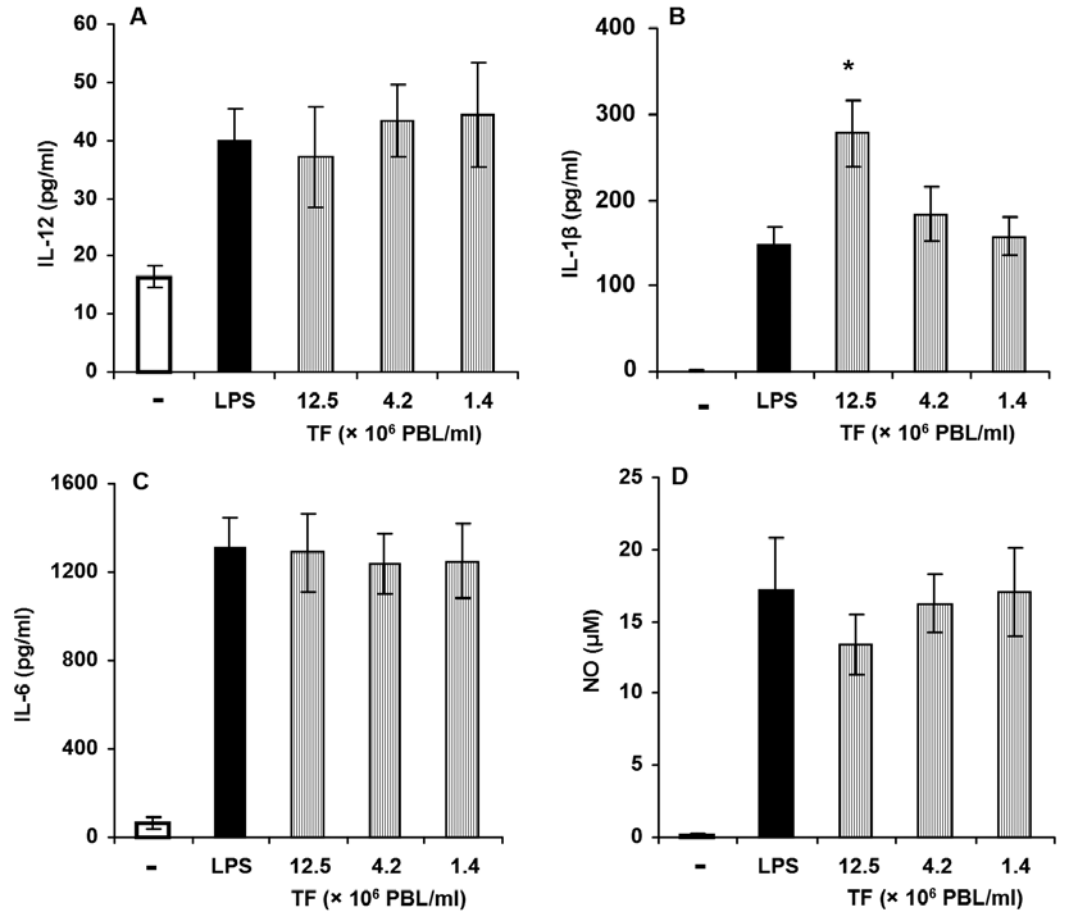

Fig. 2. The effect of TF on cytokine and NO production by activated macrophages. Mouse peritoneal macrophages were stimulated with LPS (5 μg/ml)/IFN-γ (5 ng/ml) with or without the indicated concentrations of TF. After a 72-h incubation period, the concentrations of IL-12 (A), IL-1β (B) and IL-6 (C) in the supernatants were determined by ELISA. The concentration of NO (D) in the supernatants was measured by spectrophotometry. Each bar represents the mean ± SD from four independent experiments. The value with the asterisk is significantly (*P < 0.05) different from the control (cells stimulated without TF).

The effect of TF on the production of cytokines by activated T cells

Spleen cells stimulated with ConA produced significant levels of cytokines. The production of the Th1 cytokines IL-2 and IFN-γ was slightly inhibited in cultures containing the highest concentration of TF (Fig. 3A, B). In contrast, the production of the Th2 cytokine IL-10 was enhanced in cultures supplemented with TF (Fig. 3C). However, the production of IL-17 was increased considerably (more than three times, $P < 0.00l$) in the cultures containing TF (Fig. 3D). This enhancement was also statistically significant at a lower dilution of TF (corresponding to the extract from 4.2×10^6 leukocytes/ml).

The effect of TF on the expression of genes for RORγt and IL-17 and on the proportion of $CD4^+RORγt^+$ cells

The effect of TF on IL-17 production was confirmed at the level of gene expression for Th17 cell lineage-specific transcription factor RORγt and for IL-17. As shown in Fig. 4, the expression of *RORgt* and *IL17* genes was significantly enhanced in the cultures of spleen cells stimulated with ConA and containing TF in comparison with the cultures stimulated without TF. In addition, using flow cytometry, a significant increase in the proportion of cells expressing the Th17 cell lineage-specific marker RORγt ($CD4^+RORγt^+$ Th17 cells) was observed in the cultures stimulated in the presence of TF (Fig. 4).

The expression of the Foxp3 gene and the development of regulatory T cells (Tregs) in TF-containing cultures

Spleen cells from BALB/c mice were stimulated with ConA in the presence of TF, and the expression of the *Foxp3* gene and the proportion of cells with the regulatory T-cell phenotype ($CD4^+CD25^+Foxp3^+$ cells) were determined. As demonstrated in Fig. 5A, the expression of the *Foxp3* gene was not significantly altered in the cultures containing TF (while the expression of the *RORgt* gene in the same cultures was significantly enhanced –

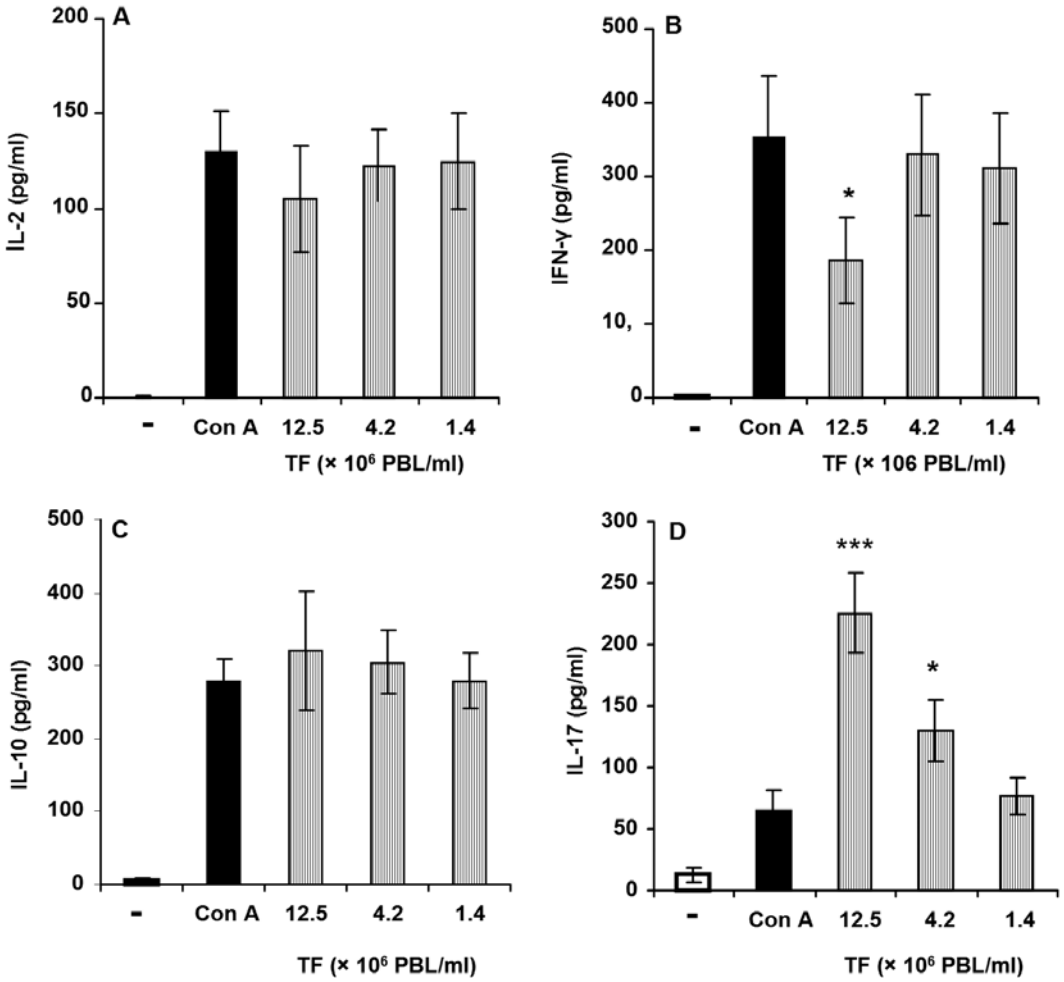

Fig. 3. The effect of TF on cytokine production by activated T cells. Spleen cells were cultured unstimulated (-) or stimulated with ConA (1.5 μg/ml) in the absence or presence of indicated concentrations of TF. The levels of IL-2 (A), IFN-γ (B), IL-10 (C) and IL-17 (D) in the supernatants were determined by ELISA. Each bar represents the mean ± SD from five independent experiments. Values with asterisks are significantly (*$P < 0.05$, ***$P < 0.001$) different from the control (cells stimulated without TF).

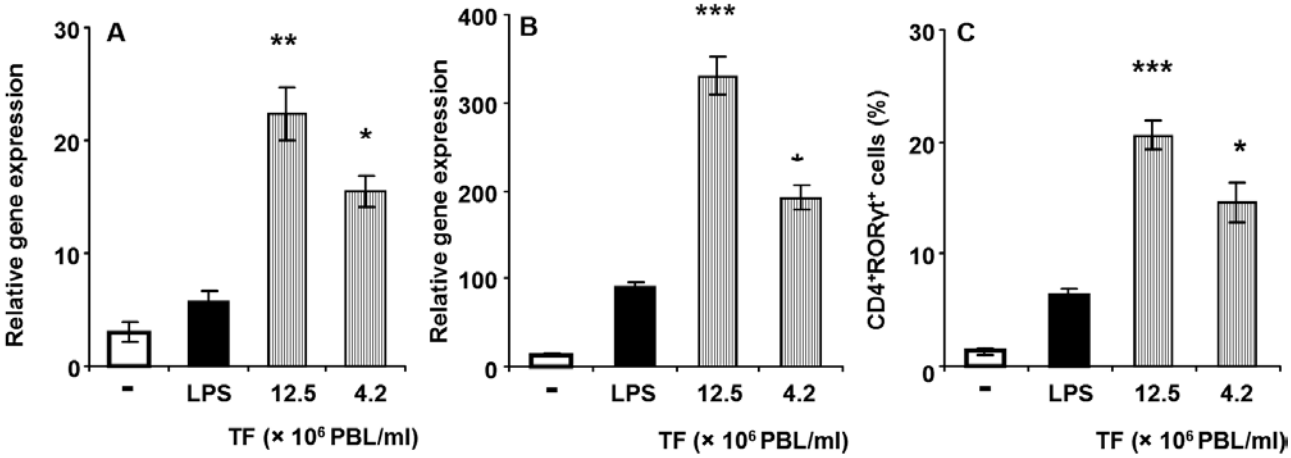

Fig. 4. The effect of TF on the *RORgt* and *IL17* gene expression and on the development of $CD4^+ROR\gamma t^+$ Th17 cells. Spleen cells were cultured unstimulated (-) or were stimulated with ConA (1.5 μg/ml) in the absence or presence of the indicated concentrations of TF. After a 72-h incubation period, the cells were harvested and the expression of genes for RORγt (A) and IL-17 (B) was determined by real-time PCR. In addition, the proportion of $CD4^+ROR\gamma t^+$ cells (C) was determined by flow cytometry. Each bar represents the mean ± SD from four independent experiments. Values with asterisks are significantly (*P < 0.05, **P < 0.01, ***P < 0.001) different from the control (cells stimulated without TF).

see Fig. 4). Similarly, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was not significantly altered in the cultures stimulated in the presence of TF (Fig. 5B).

Discussion

The appropriate functioning of the immune system is a basic requirement for the healthy life. When the immune system reacts insufficiently or incorrectly, various therapeutic approaches have been proposed and tested to improve or modulate its reactivity. One of the substances with immunomodulatory effects is a low-molecular weight dialysable leukocyte extract, originally called transfer factor. This extract was proposed to transfer or support specific immunity (Lawrence, 1955), but subsequently more general immunomodulatory properties of TF were detected and TF was used to improve or balance immunological reactivity in different models and in various species, including humans (Jeter et al., 1954; Lawrence, 1955, 1969; Burger et al., 1972; Liburd et al., 1972; Bloom, 1973; Klesius and Fudenberg, 1977; Simon et al., 1977; Williams and Kauffman, 1980; reviewed in Viza et al., 2013).

The mechanism underlying the immunomodulatory action of TF has remained unexplained. One of the reasons for this gap in our knowledge was the absence of appropriate *in vitro* tests and the lack of knowledge about cytokines and other immunoregulatory molecules at the time when TF was initially tested. To identify the cell populations responsive to TF and to characterize the mechanism of TF action, we tested a low-molecular weight extract prepared from the peripheral blood leukocytes of healthy human donors. This substance was evaluated for its effects on gene expression, lymphocyte

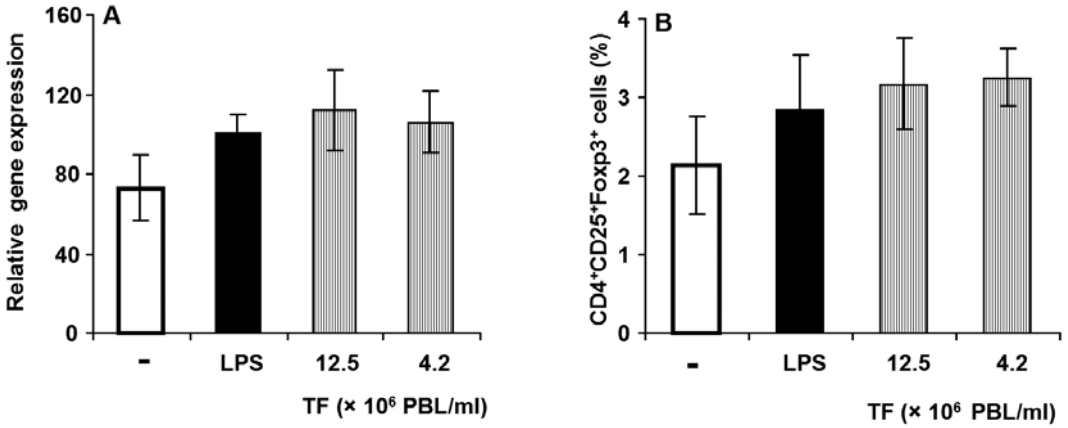

Fig. 5. The effect of TF on the *Foxp3* gene expression and the development of $CD4^+CD25^+Foxp3^+$ Tregs. Spleen cells from BALB/c mice were cultured unstimulated (-) or stimulated with ConA (1.5 μg/ml) in the absence or presence of the indicated concentrations of TF. The expression of the *Foxp3* gene in cultured cells was determined by real-time PCR (A). The proportion of $CD4^+CD25^+Foxp3^+$ cells in cell cultures was determined by flow cytometry (B). Each bar represents the mean ± SD from four independent experiments.

proliferation and cytokine production by ConA-stimulated spleen cells and LPS-activated peritoneal macrophages. We found that TF only slightly inhibited proliferation of spleen cells stimulated with T- and B-cell mitogens and did not significantly influence the production of IL-10, IL-6, IL-12 or NO by LPS-activated peritoneal macrophages. Only the production of IL-1β by the activated macrophages was increased. More interesting results were obtained when the effects of TF were tested on cytokine production by ConA-activated spleen T cells. TF slightly inhibited production of IL-2, significantly reduced production of another Th1 cytokine, IFN-γ, slightly increased production of the Th2 cytokine IL-10, but considerably and reproducibly enhanced the secretion of IL-17, a proinflammatory cytokine produced by Th17 cells and having multiple immunomodulatory properties.

To analyse the enhancing effect of TF on IL-17 production, we determined the effect of TF on the expression of the transcription factor RORγt, which is required for the development of Th17 cells and for the expression of the *IL17* gene (Ivanov et al., 2006; Manel et al., 2008). Using real-time PCR we found a significant enhancement of the expression of the *RORgt* gene in spleen cell cultures containing TF. Simultaneously, a significant increase in the proportion of $CD4^+ROR\gamma t^+$ cells was observed. In parallel, an increase in the expression of the *IL17* gene was detected in the cultures containing TF. Taken together, these results indicate that TF stimulates expression of transcription factor RORγt and enhances the proportion of $CD4^+ROR\gamma t^+$ cells, which in turn leads to the enhanced expression of the gene for IL-17 and increased production of IL-17. In contrast, the expression of the gene for the transcription factor Foxp3, a marker of Tregs, or the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was not significantly changed in cultures of spleen cells stimulated in the presence of TF.

The results thus suggest that TF can display multiple regulatory effects on individual facets of the immune system. However, the major effect can be detected in the pathway of Th17 cell development and IL-17 production. IL-17 is a highly pleiotropic cytokine having multiple effects in various immunological situations, including inflammation, autoimmunity, transplantation reactions, asthma and anti-tumour immunity (Aggarwal and Gurney, 2002; Kolls and Lindén, 2004). It has been shown that IL-17 contributes to immune reactions by activation of a number of genes, including genes for chemokines, nitric oxide synthase, metalloproteases and other cytokines (Jovanovic et al., 1998; Awane et al., 1999). In addition, IL-17 cooperates with other cytokines to enhance local inflammatory reactions or to modulate functions of the immune system (Kawaguchi et al., 2001; Jones and Chan, 2002). Our observation thus offer an explanation of the mechanism responsible for the therapeutic effects of TF observed in different models (Lawrence, 1969, 1974; Williams and Kauffman, 1980).

In the present study we did not characterize the active substance in the crude TF preparation. We intended to test the effects of the same material that is used in the experimental and clinical studies. The previous analyses have indicated that the biological activity is carried out by molecules of ca 3.5–5 kDa molecular weight which are oligoribonucleopeptides in their nature (Kirkpatrick, 2000; Ojeda et al., 2005; Viza et al., 2013). Since such small molecules usually act over an interspecies barrier, it enabled us to use human TF and a mouse detection system.

Even though the nature of active substance(s) in the low-molecular-weight leukocyte extract has not been exactly elucidated, our data clearly show that TF enhances expression of the genes for RORγt and IL-17. This pathway may represent the main mechanism of immunomodulatory and immunotherapeutic actions of TF described in various models.

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