The therapeutic potential of tuftsin-phosphorylcholine in giant cell arteritis

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ABSTRACT

Tuftsin-PhosphorylCholine (TPC) is a novel bi-specific molecule which links tuftsin and phosphorylcholine. TPC has shown immunomodulatory activities in experimental mouse models of autoimmune diseases. We studied herein the effects of TPC *ex vivo* on both peripheral blood mononuclear cells (PBMCs) and temporal artery biopsies (TABs) obtained from patients with giant cell arteritis (GCA) and age-matched disease controls. GCA is an immune-mediated disease affecting large vessels. Levels of 18 cytokines in supernatants, PBMC viability, T helper (Th) cell differentiation of PBMCs and gene expression in TABs were analyzed. Treatment *ex vivo* with TPC decreased the production of IL-1β, IL-2, IL-5, IL-6, IL-9, IL-12(p70), IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IFNγ, TNFα, GM-CSF by CD3/CD28 activated PBMCs whereas it negligibly affected cell viability. It reduced Th1 and Th17 differentiation while did not impact Th22 differentiation in PBMCs stimulated by phorbol 12-myristate 13-acetate plus ionomycin. In inflamed TABs, treatment with TPC down-regulated the production of IL-1β, IL-6, IL-13, IL-17A and CD68 gene expression. The effects of TPC were comparable to the effects of dexamethasone, included as the standard of care, with the exception of a greater reduction of IL-2, IL-18, IFNγ in CD3/CD28 activated PBMCs and CD68 gene in inflamed TABs. In conclusion our results warrant further investigations regarding TPC as an immunotherapeutic agent in GCA and potentially other autoimmune and inflammatory diseases.

1. Introduction

Giant cell arteritis (GCA) is an inflammatory disease of large- and medium-sized arteries, mainly the branches of the proximal aorta [1,2]. It is the most common vasculitis in Western countries in individuals older than 50 years of age, with an annual incidence of 1/3000–1/25,000 adults over 50 years old. It is characterized by immune cells infiltrating the arteries with production of cytokines, chemokines, growth factors, proteases which can lead to tissue remodeling and intimal thickening with subsequent stenoses or occlusions resulting in ischemic events (e.g. vision loss, aortic aneurysms, stroke and organ infarcts) [3]. The therapeutic protocol consists of high-dose corticosteroids maintained for the first month with subsequent slow tapering [1,4,5]. Despite treatment more than 35% of patients have at least one relapse during the disease course and some patients are unable to reach sustained remission with corticosteroids [6–9]. Moreover, more than

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Fig. 1. TPC modulates cytokine production by CD3/CD28 activated PBMCs. PBMCs were activated with anti-CD3/CD28 beads in presence of different doses of TPC or dexamethasone for 48 h. PBMCs were obtained from 5 patients with GCA and 5 control patients. Levels of 18 cytokines were simultaneously detected in culture supernatants with the Procarta Plex Th1, Th2, Th9, Th17, Th22, Treg cytokine MagPix assay. Graphs display cytokine concentrations relative to untreated PBMCs stimulated with CD3/CD28 set to 100% (mean ± SEM). Paired one-way ANOVA was applied then setting the FDR < 5% through the Benjamini-Hochberg method. [#] = $P < 0.05$, * = $P < 0.01$ *versus* untreated PBMCs; [§] = $P < 0.05$ TPC- *versus* dexamethasone-treated PBMCs.

80% of patients experience corticosteroid-related adverse events and two or more events occur in 58% of patients [10]. Novel therapies to save or replace corticosteroids are thus needed. To date, only tocilizumab (a biologic agent inhibiting interleukin-6 receptor) has shown a clear beneficial evidence in randomized controlled trials of a corticosteroid-saving effect [11,12].

Tuftsin-PhosphorylCholine (TPC) is a novel bi-specific small molecule with immunomodulatory activities which links tuftsin and phosphorylcholine. Tuftsin (Thr-Lys-Pro-Arg) is a self natural immunomodulating peptide produced by enzymatic cleavage of the Fc-domain of the heavy chain of IgG in the spleen [13]. Phosphorylcholine (PC) is a small zwitterionic molecule present in those helminths secretory molecules which permit helminths to survive in the host

inducing a situation of immune tolerance as well as on the surface of apoptotic cells and of several bacteria regulating adhesion to epithelial cells and immune recognition [14]. Subcutaneous (5 μg/mouse) [15–18] and oral (50 μg/mouse and 250 μg/mouse) [16,19] administration of TPC has shown immunomodulatory effects in three experimental mouse models of autoimmune diseases. Administration of TPC delayed glomerulonephritis onset in lupus prone mice [15,17], reduced colitis in mice with dextran sodium sulfate induced colitis [19] and prevented joint damage in mice with collagen-induced arthritis [16,18]. In the three models, TPC inhibited proinflammatory cytokine expression such as IL-6, IL-17, TNFα, IFNγ, increased anti-inflammatory IL-10, enhanced expansion of T and B regulatory cells, overall resulting in a reduction of disease severity and longer survival of mice.

Table 1

TPC half maximal inhibitory concentration (IC50) of cytokine production by CD3/CD28 activated PBMCs.

Cytokine	PBMCs from GCA patients		PBMCs from control patients	
	IC50	CI	IC50	CI
IL-1β	2.3	(1.3–4.0)	5.4	(1.4–20.3)
IL-2	21.9	(9.6–50.2)	24.1	(7.2–80.8)
IL-5	5.2	(2.1–12.9)	3.0	(1.1–8.2)
IL-6	6.7	(2.6–17.3)	70.8	(6.0–835.6)
IL-9	1.6	(0.1–18.5)	1.6	(0.1–22.7)
IL-10	NA		NA	
IL-12	2.6	(1.4–5.1)	1.8	(0.9–3.6)
IL-13	2.5	(1.3–5.0)	1.9	(0.9–3.8)
IL-17A	28.1	(8.2–96.9)	NA	
IL-18	250.2	(123.8–505.5)	165.4	(30.2–904.7)
IL-21	14.19	(2.4–85.1)	3.3	(1.3–8.3)
IL-22	85.2	(20.1–361.4)	40.2	(7.2–225.8)
IL-23	2.7	(1.3–5.5)	1.5	(0.3–8.1)
IFNγ	51.4	(30.0–88.0)	100.1	(15.5–646.6)
TNFα	4.9	(2.3–10.2)	3.2	(1.5–7.1)
GM-CSF	3.6	(1.4–9.1)	3.4	(1.1–10.8)

IC50 (ng/ml) were calculated drawing dose-response curves with GraphPad Prism 7 software. The 95% confidence intervals (CI) are shown in brackets. NA = not applicable.

Data on mouse models indicate that TPC could be an immunotherapeutic agent for immune-mediated diseases. However experience with human samples is currently lacking and is needed to validate the therapeutic potential of TPC and proceed towards the clinical experimentation in patients with autoimmune and inflammatory diseases. In the present paper we thus investigated the effects of TPC *ex vivo* on human samples: peripheral blood mononuclear cells (PBMCs) and temporal artery biopsies (TABs) obtained from patients with a suspicion of GCA.

2. Materials and methods

2.1. Patients

Ten patients suspected to have GCA were included in the study. All patients were naïve from corticosteroids. Patients underwent temporal artery biopsy (TAB) to analyze inflammation and confirm the diagnosis. Five patients had TABs with a transmural inflammatory infiltrate (biopsy-proven GCA patients, 2 women and 3 men, median age: 80 years, range: 65–83 years) while five patients had normal, non-inflamed

TABs and received a different diagnosis (control patients, 3 women and 2 men, median age: 75, range: 63–89 years). All patients with GCA satisfied the American College of Rheumatology (ACR) criteria for GCA [20] and none of the control patients. The diagnosis of GCA was also excluded during the follow-up in the 5 controls. The study was approved by the Local Ethics Committee (Reggio Emilia, Italy; protocol number 1276 dated 7th September 2016) in compliance with the Declaration of Helsinki and written informed consent was obtained from all patients.
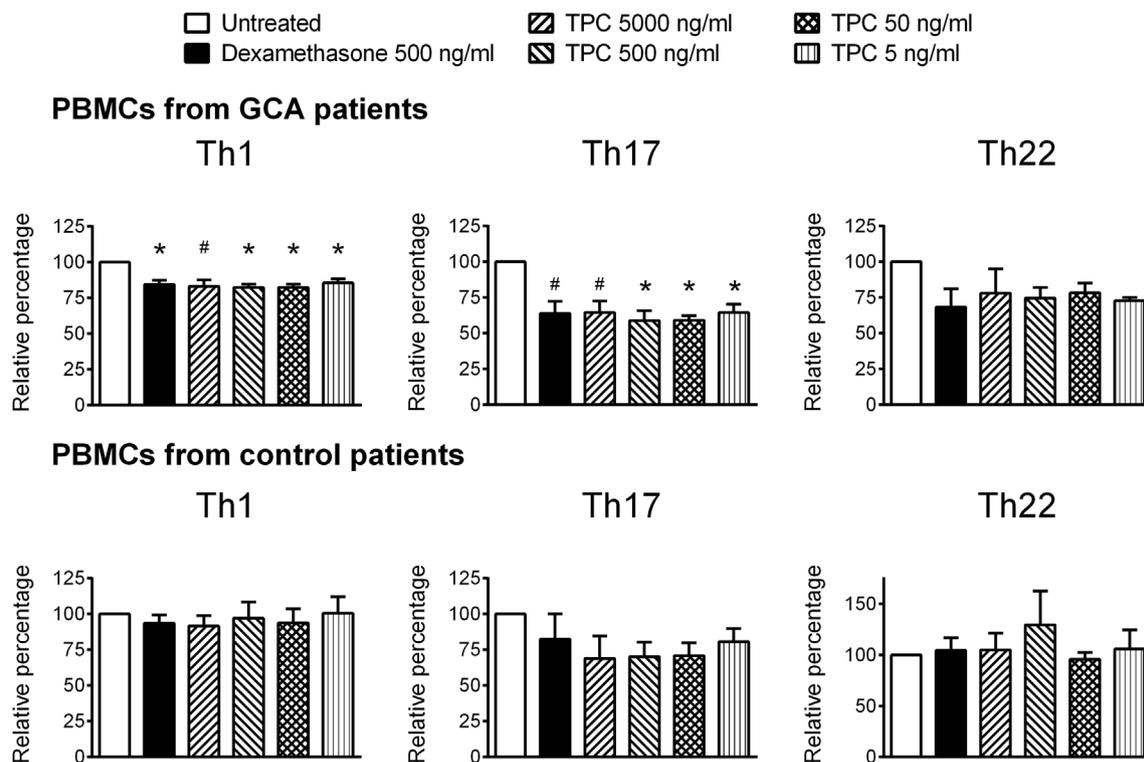
2.2. Biological samples

Peripheral blood mononuclear cells (PBMCs) were isolated by histopaque-1077 density gradient centrifugation (Sigma) and stored frozen in liquid nitrogen in 90% heat inactivated fetal bovine serum (FBS, Gibco, ThermoFisher) 10% dimethyl sulfoxide (Sigma) until use. TABs were in part fixed in formalin and embedded in paraffin for diagnosis, in part placed in RPMI +10% FBS (ThermoFisher) containing 50 μg/ml gentamicin and 2 mM glutamine (Sigma) for TPC treatment *ex vivo*.

2.3. TPC and dexamethasone treatments

TPC was provided by TPCera (Givat Ram Campus of Hebrew University, High-Tech Village building 2.3, Jerusalem, Israel) at 5 mg/ml in PBS and stored frozen in aliquots at −20 °C. A material transfer agreement was signed between the Arcispedale Santa Maria Nuova-IRCCS, Reggio Emilia, Italy and TPCera. No data are currently available on the systemic concentration of TPC reached in the mouse models. Therefore, we chose to test *ex vivo* the effects of 10-fold serial dilutions of TPC starting from the quantity administered subcutaneously in mice: 5000 ng/ml, 500 ng/ml, 50 ng/ml, 5 ng/ml. In all the experiments, treatment with dexamethasone (Hospira) at 500 ng/ml was included as reference for the standard therapy. We selected the 500 ng/ml dexamethasone dose to have results comparable to those reported by Maria Cinta Cid and coworkers [21]. Untreated PBMCs and TABs were used as baseline reference.

PBMCs were thawed, suspended in RPMI +10% FBS and counted with a Fuchs-Rosenthal hemocytometer with trypan blue dye to evaluate dead cells. PBMCs were seeded at 500,000 cells/500 μl in 24 well plates in RPMI +10% FBS + CD3/CD28 beads (bead-to-cell ratio of 1:1) (Invitrogen, Dynabeads human T-activator CD3/CD28) in presence of TPC or dexamethasone. After 48 h treatment supernatants were collected.

For functional assays on TABs the protocol developed and validated

Table 2

Cytokine concentrations in supernatants of untreated CD3/CD28 activated PBMCs and TABs from GCA and control patients.

Cytokine	CD3/CD28 activated PBMCs from GCA patients	CD3/CD28 activated PBMCs from control patients	Inflamed TABs from GCA patients	Normal TABs from control patients
IL-1β	325.4 ± 199.3	220.5 ± 166.5	47.2 ± 22.7	not detected
IL-2	3494.0 ± 332.0	3172.0 ± 881.6	18.4 ± 2.9	15.9 ± 3.0
IL-5	153.1 ± 48.3	157.0 ± 54.1	15.6 ± 4.0	13.6 ± 3.8
IL-6	13830.0 ± 7318.0	8155.0 ± 5264.0	47135.0 ± 10756.0	46401.0 ± 20674.0
IL-9	2162.0 ± 874.3	1974.0 ± 793.4	not detected	not detected
IL-10	1095.0 ± 320.1	994.7 ± 405.6	10.7 ± 5.6	4.6 ± 3.5
IL-12	21.9 ± 6.3	26.9 ± 10.7	not detected	not detected
IL-13	1025.0 ± 342.7	890.1 ± 286.0	1.7 ± 0.4	not detected
IL-17A	656.2 ± 155.5	572.8 ± 209.4	2.6 ± 0.8	not detected
IL-18	669.2 ± 239.6	505.7 ± 179.4	17.6 ± 7.6	not detected
IL-21	906.0 ± 138.9	705.1 ± 223.8	not detected	not detected
IL-22	697.8 ± 104.4	646.7 ± 220.2	not detected	not detected
IL-23	61.8 ± 19.0	39.5 ± 18.0	not detected	not detected
IFNγ	9273.0 ± 3369.0	4886.0 ± 1441.0	not detected	not detected
TNFα	2442.0 ± 359.1	2347.0 ± 623.8	9.7 ± 1.8	5.8 ± 1.9
GM-CSF	1789.0 ± 460.7	1424.0 ± 485.3	not detected	not detected

Mean cytokine concentrations ± standard error of the untreated samples are reported (pg/ml). Supernatants were conditioned by CD3/CD28 activated PBMCs for 48 h and by TABs for 5 days.

Fig. 2. TPC effects on T helper differentiation induced by PMA plus ionomycin. PBMCs were treated for 48 h with different doses of TPC or dexamethasone then stimulated with PMA plus ionomycin in presence of brefeldin A for 4 h maintaining the treatment. PBMCs were obtained from 5 patients with GCA and 5 control patients. T helper (Th) subsets were defined by flow cytometry in the viable $CD4^+$ lymphocyte gate and are shown relative to untreated PBMCs stimulated with PMA plus ionomycin set to 100% (mean ± SEM). Paired one-way ANOVA was applied then setting the FDR < 5% through the Benjamini-Hochberg method. # = P < 0.05, * = P < 0.01 *versus* untreated PBMCs.

by Maria Cinta Cid and coworkers was followed [21]. TABs were cut in fragments of similar volume, embedded in 25 μl matrigel (BD Biosciences) drops and incubated in 1 ml RPMI + 10% FBS, 2 mM glutamine, 50 μg/ml gentamicin plus TPC or dexamethasone in 24 well plates at 37 °C, 5% CO_2. After 5 days of treatment supernatants and TAB sections were collected.

2.4. Cytokine quantification

Supernatants were centrifuged at 10,000 x g for 1 min to remove cell debris then stored at −80 °C. Levels of 18 cytokines were quantified with the Procarta Plex Th1, Th2, Th9, Th17, Th22, Treg cytokine panel (eBioscience) using the MagPix instrument (Bio-Rad). The experimental lower limits of detection of the cytokines were: IL-1β: 0.9 pg/ml, IL-2: 5.1 pg/ml, IL-4: 9.1 pg/ml, IL-5: 6.2 pg/ml, IL-6: 7.6 pg/ml, IL-9: 7.5 pg/ml, IL-10: 1.6 pg/ml, IL-12(p70): 0.9 pg/ml, IL-13: 2.0 pg/ml, IL-17A: 2.0 pg/ml, IL-18: 6.5 pg/ml, IL-21: 8.3 pg/ml, IL-22: 95.7 pg/ml, IL-23: 14.6 pg/ml, IL-27: 22.0 pg/ml, IFNγ: 9.3 pg/ml, TNFα: 3.4 pg/ml, GM-CSF: 11.7 pg/ml. Values extrapolated from the standard curves were considered not reliable thus a concentration = 0 pg/ml was assigned. Due to technical issues data regarding IL-4 and IL-27 were considered not reliable.

2.5. Flow cytometry

PBMCs were seeded at 500,000 cells/500 μl in 24 well plates in RPMI +10% FBS plus TPC or dexamethasone. After 48 h treatment, PBMCs were collected and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin (Sigma) at 37 °C 5% CO_2 maintaining the treatment with TPC or dexamethasone. After 30 min, brefeldin A (Sigma) was added at 10 μg/ml. After further 3 h and 30 min, cells were collected and stained first with 100 μl Live/Dead

Fixable Dead Cell Stain kit near-IR-fluorescent reactive dye (Molecular Probes) at 0.1% in PBS for 10 min at room temperature to exclude dead cells from analysis, then with antibodies against CD4 for 20 min at room temperature (PerCP-Cy5.5 mouse anti-human CD4, clone RPA-T4, BD Biosciences). After fixation and permeabilization with 250 μl BD Cytofix/Cytoperm Fixation/Permeabilization solution at 4 °C for 20 min, cells were stained with antibodies against intracellular cytokines: anti-Human IL-17A PE-Cyanine 7 (clone eBio64DEC17), anti-Human/Mouse IL-22 APC (clone IL22JOP), anti-Human IFN gamma Alexa Fluor 488 (clone 4S.B3, all from eBioscience) in 100 μl perm/wash buffer for 30 min at 4 °C as previously reported [22]. PBMCs were suspended in PBS +1% FBS, acquired with the FACSCanto II flow cytometer and analyzed with the FACSDiva software (BD Biosciences). Th1 cells were defined as $IFNγ^+IL-17^{neg}IL-22^{neg}$ $CD4^+$ lymphocytes; Th22 cells were defined as $IL-22^+IFNγ^{neg}IL-17^{neg}$ $CD4^+$ lymphocytes; Th17 cells were defined as $IL-17^+IFNγ^{neg}IL-22^{neg}$ $CD4^+$ lymphocytes.

2.6. Cell viability

PBMCs were seeded at 100,000 cells/100 μl in 96 well plates in RPMI +10% FBS and stimulated with CD3/CD28 beads plus/minus TPC or dexamethasone. Cell viability was determined after 48 h treatment by adding 10 μl of WST-1 proliferation reagent (Sigma) into each well. After 4 h incubation at 37 °C, 5% CO_2 optical density was measured at 450 nm and 600 nm.

2.7. Gene expression

After treatment TABs were immediately snap-frozen in liquid nitrogen then stored at −80 °C. RNA was extracted with the RNA/DNA/Protein Purification Plus Kit (Norgen Biotek). Tissue homogenization was made effective by the use of pestles. RNA was quantified with the
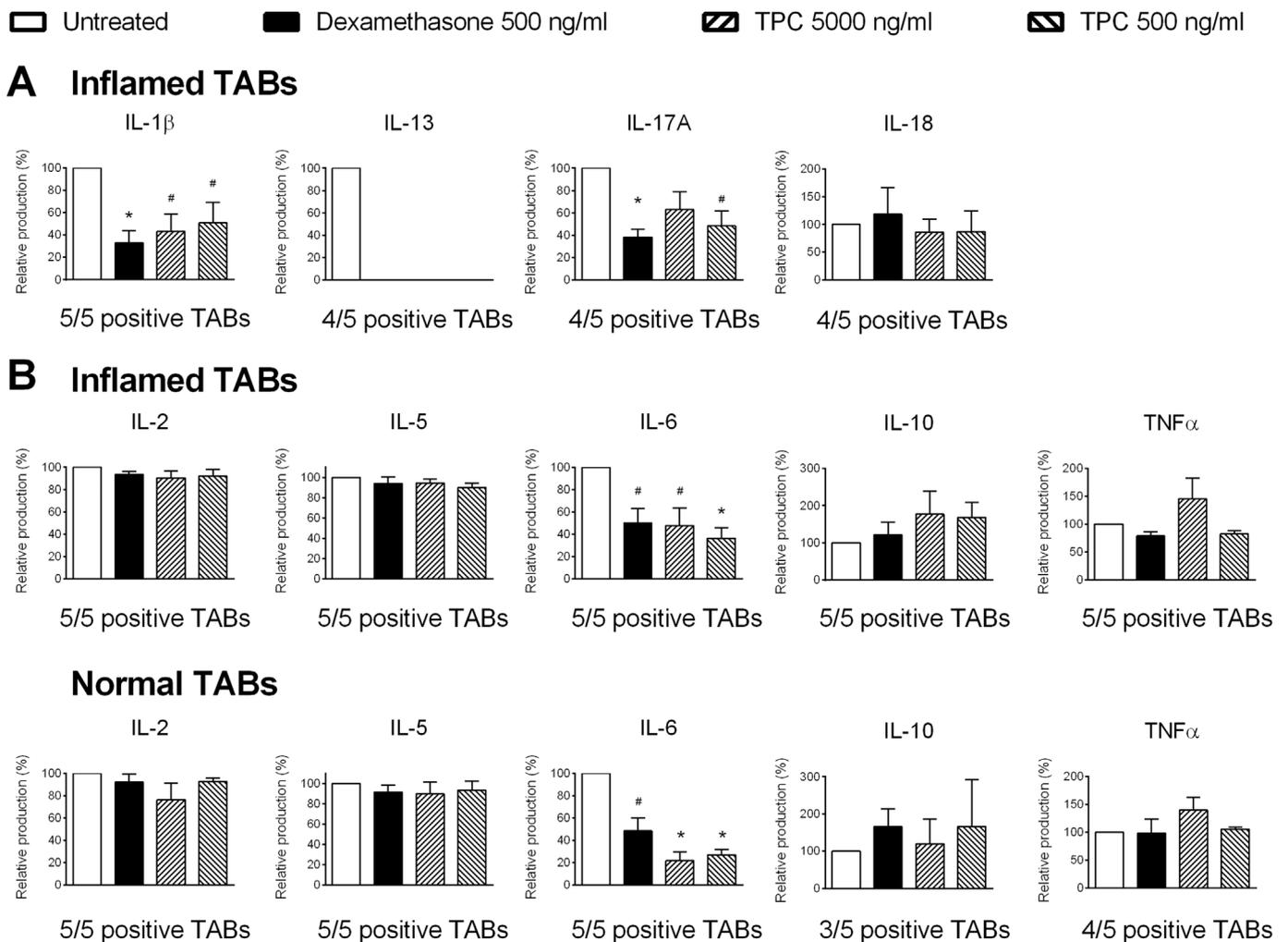
Fig. 3. TPC modulates cytokine production by TABs. TAB fragments were cultured *ex vivo* in presence of TPC or dexamethasone for 5 days. TABs were obtained from 5 patients with GCA and 5 control patients. Levels of 18 cytokines were simultaneously detected in culture supernatants with the Procarta Plex Th1, Th2, Th9, Th17, Th22, Treg cytokine MagPix assay. (**A**) Cytokines detected only in the inflamed TABs. (**B**) Cytokines detected both in the inflamed and normal TABs. Levels are shown relative to untreated TABs set to 100% (mean ± SEM). Paired one-way ANOVA was applied then setting the FDR < 5% through the Benjamini-Hochberg method. # = P < 0.05, * = P < 0.01 *versus* untreated TABs. The fraction of untreated TABs in which cytokines were detected is reported below the x-axis.

NanoDrop instrument (Thermo Scientific) then 62.5 ng of RNA were reverse transcribed with the PrimeScrip RT Reagent Kit with gDNA Eraser (Takara). Real-time PCR was performed with the SYBR Premix Ex Taq II (Tli RNaseH Plus) containing the ROX Reference Dye (Takara) and QuantiTect primer assays (Qiagen) as previously reported [23]. GAPDH was used as reference to normalize gene expression data.

2.8. Statistical analysis

Effects of TPC and dexamethasone were calculated relative to the untreated PBMC and TAB samples included in each assay and presented as mean ± SEM. Data were analyzed with paired one-way ANOVA then correcting for multiple comparisons by the Benjamini-Hochberg method setting the false discovery rate (FDR) < 5%. To determine the TPC half maximal inhibitory concentration (IC50) dose-response curves were drawn applying nonlinear regression plotting the logarithm of TPC concentrations *versus* the relative responses. Statistical analyses were performed with GraphPad Prism 7 software.
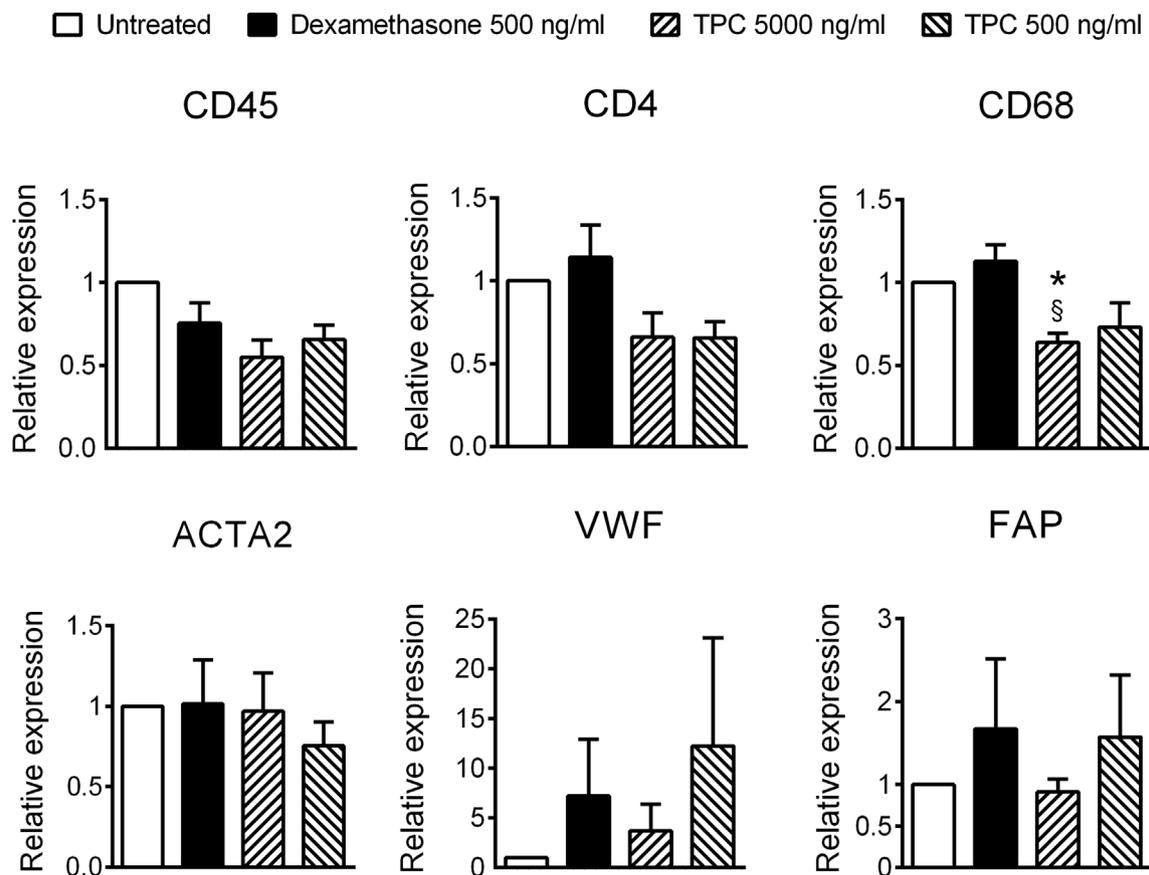
3. Results

The effects of TPC were determined *ex vivo* on activated PBMCs (viability, production of cytokines in culture supernatants and

polarization towards T helper subsets) and TABs (production of cytokines in culture supernatants and gene expression) obtained from 5 biopsy-proven GCA patients and 5 biopsy-negative non-GCA, control patients. Effects of TPC were compared to the effects of dexamethasone as the standard of care.

3.1. Effects of TPC on peripheral blood mononuclear cells

To model *ex vivo* the inflammatory state, PBMCs were activated through CD3/CD28 ligation (bead-to-cell ratio of 1:1) and PMA (50 ng/ml) plus ionomicin (1 µg/ml) which stimulate T lymphocytes. PBMCs activated through CD3/CD28 ligation and treated with 5000 ng/ml TPC for 48 h showed a statistically significant lower concentration in supernatants of almost all the investigated cytokines compared to PBMCs stimulated only with anti-CD3/CD28 beads (Fig. 1). In particular IL-1β, IL-9, IL-12(p70), IL-13, IL-23 and TNFα showed a mean decrease greater than 80%. IL-2, IL-5, IL-6, IL-17A, IL-18, IL-21, IL-22, IFNγ and GM-CSF showed a mean decrease between 50% and 80%. Instead IL-10 showed a different trend (Fig. 1). TPC at 5000 and 500 ng/ml had comparable effects on cytokine production by CD3/CD28 activated PBMCs. Instead TPC at 5 ng/ml had less effects on IL-1β, IL-2, IL-5, IL-6, IL-12(p70), IL-13, IL-17A, IL-18, IL-21, IL-23, IFNγ and TNFα compared with the other TPC doses (P < 0.05). A dose-response of TPC (500, 50,

☐ Untreated   ■ Dexamethasone 500 ng/ml   ▨ TPC 5000 ng/ml   ◩ TPC 500 ng/ml

Fig. 4. TPC modulates gene expression in inflamed TABs. RNA was extracted from TAB fragments cultured *ex vivo* in presence of TPC or dexamethasone for 5 days. TABs were obtained from 5 patients with GCA. Gene expression was determined by real-time PCR relative to untreated TABs through the ΔΔCt method (mean ± SEM). Paired one-way ANOVA was applied then setting the FDR < 5% through the Benjamini-Hochberg method. * = P < 0.01 *versus* untreated TABs; [§] = P < 0.01 TPC- *versus* dexamethasone-treated TABs.

5 ng/ml doses) was found regarding the production of IL-2, IL-18 and IFNγ. The IC50 of TPC on cytokine production by CD3/CD28 activated PBMCs are reported in Table 1. Treatment with TPC had mainly similar effects on CD3/CD28 activated PBMCs from GCA and controls patients (Fig. 1). Untreated samples from GCA and control patients had comparable concentrations of cytokines in supernatants (Table 2). Although treatment with TPC had often similar effects to the treatment with dexamethasone, TPC at 5000 ng/ml decreased the levels of IL-2, IL-18 and IFNγ in supernatants more than dexamethasone (P < 0.05, Fig. 1). Paired values obtained from samples from each patients are shown in Supplementary Fig. 1.

Both TPC at 5000, 500, 50 ng/ml and dexamethasone slightly reduced viability of CD3/CD28 activated PBMCs (mean reduction of 10%) but results were not statistically significant (data not shown).

We then followed a second experimental setting based on the stimulation of PBMCs with PMA plus ionomycin for 4 h in presence of TPC or dexamethasone followed by intracellular flow cytometry to determine T helper (Th) cell polarization. Stimulation with PMA plus ionomycin induced a comparable differentiation towards Th1, Th17 and Th22 in PBMCs from GCA and control patients (Th1: 12.8 ± 2.2% in GCA *versus* 12.1 ± 1.3% in controls; Th17: 0.59 ± 0.16% in GCA *versus* 0.60 ± 0.15% in controls; Th22: 0.64 ± 0.10% in GCA *versus* 0.59 ± 0.14% in controls). Percentages were calculated in the CD4^{+} viable lymphocyte gates). In this experimental setting, TPC treatment reduced the percentage of Th1 lymphocytes (about 15%) and Th17 lymphocytes (about 35%) in PBMCs from GCA patients while did not modify the percentage of Th22 lymphocytes with respect to PBMCs stimulated with PMA plus ionomycin alone (Fig. 2). The effects of dexamethasone were similar to TPC (Fig. 2). Paired values obtained

from samples from each patients are shown in Supplementary Fig. 2. Neither TPC nor dexamethasone treatments modified the percentages of CD4^{+} viable lymphocytes evaluated by the live/dead cell reactive dye (data not shown).

3.2. Effects of TPC on temporal artery biopsies

To analyze the effects of TPC treatment at the tissue level, TABs were immediately placed in culture medium, cut in fragments of similar volume, then treated *ex vivo* with TPC for 5 days in comparison to dexamethasone. Due to the TAB length (0.5–1 cm), the effects of only two different doses of TPC could be analyzed. Not all the investigated cytokines were detected in culture supernatants of TABs. IL-1β, IL-13, IL-17A, IL-18 were detected only in the supernatants from the inflamed TABs (Table 2) while IL-2, IL-5, IL-6, IL-10, TNFα were detected in the supernatants from both the inflamed and normal TABs, at comparable concentrations (Table 2). Inflamed TABs treated with TPC showed lower levels of IL-1β, IL-13 and IL-17A in supernatants compared to untreated TABs (Fig. 3A). In particular IL-13 was detected in the supernatants from the untreated TABs and not detected in the supernatants from the TPC-treated TABs. In addition, both inflamed and normal TABs treated with TPC showed a decreased level of IL-6 in supernatants (Fig. 3B). Overall, TPC at 5000 and 500 ng/ml produced comparable results, almost halving IL-1β, IL-6 and IL-17A concentrations in supernatants from TABs. TPC treatment did not modify IL-2, IL-5, IL-10, IL-18 and TNFα production (Fig. 3). Treatment with TPC had similar effects to treatment with dexamethasone (Fig. 3). Paired results obtained from individual samples are shown in Supplementary Fig. 3.

To determine the effects of TPC treatment on the load of infiltrating

immune cells as well as arterial resident cells, mRNA expression of CD45, CD4, CD68 which reflects total leukocytes, T helper cells and macrophages, ACTA2 (alpha smooth muscle actin), VWF (Von Willebrand factor) and FAP (fibroblast activation protein alpha) which reflects smooth muscle cells, endothelial cells and fibroblasts were determined in the inflamed TABs. After treatment with TPC at 5000 ng/ml, inflamed TABs showed a lower expression of CD45, CD4 and CD68 ($P < 0.05$, CD45 mean decrease of 45%; CD4 mean decrease of 34%; CD68 mean decrease of 36%) but following correction for multiple testing only the reduction of CD68 was considered a discovery with a FDR < 5% (Fig. 4). CD45, CD4 and CD68 mRNA expression were not modified by treatment with dexamethasone (Fig. 4). The effects of TPC treatment on CD68 were different from the effects of dexamethasone ($P < 0.01$). Neither TPC nor dexamethasone treatments consistently modified the expression of ACTA2, VWF, FAP (Fig. 4). Paired results obtained from individual samples are shown in Supplementary Fig. 4.

4. Discussion

In the present study we showed immunomodulatory effects of TPC on human samples. We focused on samples from patients subjected to a biopsy of temporal artery for a suspicion of GCA, comparing patients with and without GCA. In addition we compared the effects of TPC with those of the corticosteroid dexamethasone, the standard of care in GCA. Treatment with TPC of PBMCs activated *ex vivo* through CD3/CD28 led to a decrease, greater than 50%, in the production of several pro-inflammatory cytokines whereas cell viability was negligibly affected. In addition, it led to a decrease of Th1 and Th17 differentiation induced in PBMCs by PMA plus ionomycin. At the tissue level, treatment with TPC led to a lower secretion of IL-1β, IL-6, IL-13 and IL-17A and a reduced expression of CD68 by inflamed TABs.

Various cytokines modulated by TPC have been reported deregulated in GCA: IL-1β, IL-2, IL-6, IL-9, IL-12, IL-17, IL-18, IL-21, IL-22, IL-23, TNFα, IFNγ [22,24–26] as well as Th1 and Th17 lymphocytes [24–26]. Functional studies *in vitro*, mouse models of vasculitis and randomized controlled trials with targeted therapies in patients with GCA demonstrated that IFNγ, IL-6, IL-12, IL-17, IL-22, IL-23 have a role in the pathogenesis of the disease [22,27,28]. IFNγ can activate macrophages and vascular smooth muscle cells and contribute to the recruitment and maintenance of infiltrating inflammatory cells. IL-17 can act on macrophages, neutrophils, endothelial cells and fibroblasts. IL-6 can induce the acute phase responses, modulate T and B cell responses and promote arterial remodeling. IL-22 can promote B cell responses and arterial remodeling. IL-12 and IL-23 can favor leukocyte transendothelial infiltration and myofibroblast migration. Through the down-regulation of the above mentioned cytokines, TPC may thus reduce the systemic and vascular manifestations in patients with GCA.

The administration of TPC *in vivo* in mouse models of autoimmune diseases decreased the production of the pro-inflammatory cytokines: IL-1β, IL-17, TNFα, IFNγ and increased the production of the anti-inflammatory cytokines: IL-10 and TGFβ by splenocytes and in the colon [15–19]. TPC treatment *ex vivo* of samples from patients with GCA similarly decreased the production of IL-1β, IL-17, TNFα, IFNγ by CD3/CD28 activated PBMCs and of IL-1β and IL-17 by inflamed TABs. Importantly, TPC did not induce a suppression of all the investigated cytokines: the secretion of IL-10 was either unaffected or slightly increased by TPC treatment strengthening the results.

The analysis of the effects of TPC on samples from patients with a suspicion of GCA but who received a different diagnosis allowed to check for GCA-specific effects of TPC and effects of TPC on normal TABs. The fact that TPC had mainly similar effects on PBMCs from patients with and without GCA when activated *ex vivo* by anti-CD3/CD28 beads and PMA plus ionomycin which mimic a T cell-driven inflammation, suggests that TPC could have anti-inflammatory activities not restricted to GCA. Therefore we can hypothesize that TPC could be also evaluated for the therapy of other autoimmune and inflammatory

diseases characterized by T cell activation. Besides, it has to be considered that the control patients enrolled in the study had a suspicion of GCA which was afterwards not confirmed. Therefore they are disease controls, not healthy subject controls.

The effects of TPC treatment on inflamed TABs resulted from the modulation of immune and stromal cells: fibroblasts, smooth muscle cells and endothelial cells. IL-1β, IL-13, IL-17A and IL-18 were detected only in supernatants from inflamed TABs indicating that they were mainly produced by infiltrating immune cells. IL-2, IL-5, IL-6, IL-10 and TNFα were detected in supernatants from both inflamed and normal TABs indicating that they were likely produced by stromal cells in addition to infiltrating immune cells. Production of IL-6 was affected by TPC treatment both in inflamed and normal TABs thus we can speculate that TPC modulated IL-6 expression not only in immune cells but also in arterial stromal cells.

TPC at 5000 and 500 ng/ml had comparable effects indicating a saturation of signaling pathways and/or receptors. The mechanisms of actions of TPC are currently under investigation [18]. TPC effects can derive from both the tuftsin and the phosphorylcholine parts. Tuftsin in itself can bind the receptor neuropilin-1 (NRP1) [18,29] which is mainly expressed by monocytes, macrophages, neutrophils but also by vascular endothelial cells and vascular smooth muscle cells, thus ranking inflammatory diseases of vessels (e.g. GCA) at the first position as candidate which could benefit from a therapy with TPC. In addition, NRP1 can form receptor complexes for vascular endothelial growth factors (VEGFs), class 3 semaphorins and platelet-derived growth factor (PDGF) and can regulate VEGF- and PDGF-induced activities and neointimal hyperplasia [30–32]. The tuftsin part of TPC might thus drive a NRP1-mediated endocytosis of TPC in monocytes, macrophages, neutrophils and arterial cells and might also antagonize the activities of VEGF and PDGF which are involved in GCA pathogenesis [33,34]. Phosphorylcholine in itself can bind toll like receptor 4 (TLR4) [18] and platelet activating factor (PAF) receptor on antigen presenting cells, macrophages, T and B lymphocytes competing with the respective ligands and dampening inflammation. The activation of TLR4 has been reported in TABs from patients with GCA and can induce transmural inflammation in human temporal artery-SCID chimeras [35]. Phosphorylcholine can also bind C-reactive protein. Moreover, in mouse models, phosphorylcholine has been shown to polarize immune response towards Th2 while reducing Th1, desensitize T and B cell receptor signaling, reduce T and B cell proliferation and dampen the ability of macrophages to produce pro-inflammatory cytokines [14,36].

Immunological effects of TPC treatment were mainly comparable to the effects of dexamethasone. However, TPC treatment led to a higher down-regulation of IL-2, IL-18 and IFNγ in CD3/CD28 activated PBMCs and CD68 mRNA in inflamed TABs compared to dexamethasone treatment, indicating a higher impact on Th1 lymphocytes and a potential reduction of infiltrating macrophages in arteries. It is known that the corticosteroid therapy cannot effectively dampen arterial infiltrating leukocytes [37,38], cannot suppress the Th1 cell response in patients with GCA and the chronic phase of GCA can persist as a Th1-dependent disease [3,24]. Therefore TPC might be not only as beneficial as corticosteroids but even more beneficial than corticosteroids in patients with GCA. We can speculate that it might be used in association to corticosteroids (e.g. to spare corticosteroids) or as an alternative therapy to corticosteroids.

The major limit of the present study is the small patients' cohort. However, it is intended as a pilot study which set the basis for the potential use of TPC in human beings affected by autoimmune and inflammatory diseases.

5. Conclusions

Overall the results indicate that TPC can have immunomodulatory effects on human samples at least equal to those of corticosteroids. In patients with GCA, treatment with TPC might reduce the production of

pro-inflammatory cytokines by T cells and Th1 and Th17 polarization. In addition TPC might target arterial cells, macrophages and dendritic cells through NRP1 and TLR4. This might allow to control not only the systemic component of the disease but also arterial remodeling and inflammation. The results warrant further investigations on TPC for the application of this compound in the therapy of GCA and possibly other autoimmune and inflammatory diseases.

Conflicts of interest

Yehuda Shoenfeld and Miri Blank are shareholders in TPCera. The other authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Croci had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: S.C., Y.S., C.S. Sample collection, acquisition and analysis of data: S.C., M.B., F.M., A.C., A.F., A.S., A.C., L.C., L.B. Interpretation of data. S.C., M.B., L.B., O.P., M.F., M.P., M.B., Y.S., C.S.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.01.002.

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