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Original Article

Tumor Necrosis Factor-α Gene Signature Is Absent in Peripheral Blood Mononuclear Cells of Patients with Granulomatosis Polyangiitis

Maximilian F Koenig[1], Jin Kyun Park[2]

[1]Division of Rheumatology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA, [2]Division of Rheumatology, Department of Internal Medicine, Seoul National University Hospital, Seoul, Korea

Objective. Granulomatosis with polyangiitis (GPA), formally known as Wegener's granulomatosis, is a systemic vasculitis with necrotizing granulomatous inflammation. As treatment directed against tumor necrosis factor (TNF)-α failed in GPA, we investigated whether "TNF-α signature" (i.e. gene expression profile of TNF-α activation) was present in peripheral blood mononuclear cells (PBMCs) of patients with GPA. **Methods.** Gene expression profiling was performed using total RNA from PBMCs of 41 patients with GPA and 23 healthy control subjects using the Illumina microarray technique. Gene set enrichment analysis (GSEA) was performed to detect the presence of TNF-α signature using the curated list C2-V3.0 by the Broad Institute. False discovery rate < 0.05 was considered statistically significant. **Results.** GSEA did not show significant enrichment of any TNF-α associated gene sets in GPA. Expression levels of genes up-regulated by TNF-α did not differ between healthy control subjects, patients in remission (Birmingham Vasculitis Activity Score [BVAS] = 0), and those with active disease (BVAS ≥ 1). In addition, an unsupervised hierarchical clustering of those genes failed to cluster the samples into healthy control subjects and GPA in remission or with active disease. B cell activation signature was enriched in GPA patients. **Conclusion.** Absence of a TNF-α signature in PBMCs may suggest that TNF-α plays a less important role in the pathogenesis of GPA than previously accepted. **(J Rheum Dis 2015;22:293-297)**

Key Words. Tumor necrosis factor-alpha, Granulomatosis with polyangiitis, Gene expression

INTRODUCTION

Granulomatosis with polyangiitis (GPA) is an anti-neutrophil cytoplasmic antibody (ANCA) associated systemic vasculitis that manifests by necrotizing vasculitis affecting medium and small vessels and granulomatous inflammation [1,2]. When primed by tumor necrosis factor-alpha (TNF-α), polymorphic mononuclear cells (PMNs) express proteinase 3 (PR3) on their cell surface. Then, the circulating anti-PR3 antibodies bind to the membranous PR3 and activate PMNs, resulting in degranulation of the free-radical oxygen species and proteases, and subsequently in vascular and tissue injury [3,4]. Further, TNF-α contributes to granuloma formation with multinucleated giant cells [5-7]. As such, TNF-α was expected to play an essential role in GPA pathogenesis. However, Wegener's Granulomatosis Etanercept Trial (WGET) surprisingly failed to show any efficacy of TNF-α inhibitor etanercept for maintenance of disease remission [8], questioning the role of TNF-α in GPA pathogenesis. By contrast, rituximab, anti-CD20 monoclonal antibody that depletes B cells, showed a high efficacy in the treatment of GPA [9].

Cheadle et al. [10] showed that the gene expression profiles of peripheral blood mononuclear cells (PBMCs) varied during active and inactive stage of GPA; expression of myelopoesis gene was upregulated in patients with clinically active GPA. As B cell signaling signature dis-

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Corresponding to : Jin Kyun Park, Division of Rheumatology, Department of Internal Medicine, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea. E-mail : jinkyunpark@gmail.com

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appeared after a successful treatment with rituximab in Sjögren's syndrome, which is characterized by the B cell activation, [11,12], we sought to investigate whether TNF-α signature and B cell activation signaling were present in PBMCs of GPA.

MATERIALS AND METHODS

Patients

As previously described, 41 GPA patients undergoing clinical care in Johns Hopkins University Hospital and 23 healthy control subjects were included in this study [10]. Disease activity scores were measured using a modification of the Birmingham Vasculitis Activity Score for Wegener's granulomatosis (BVAS-WG) considering only factors present on the day of sample collection. Inactive disease was defined as a BVAS-WG score of 0.

DNA microarray analysis

Total RNA was extracted from the PBMC using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA). Biotin-labeled complementary RNA (cRNA) was prepared and hybridized to Illumina HumanRef-8 (version 2) Expression BeadChips (Illumina, San Diego, CA, USA), and the signal was detected with streptavidin–Cy3 (National Center for Biotechnology Information Gene Expression Omnibus and GEO Series accession number GSE18885).

Statistical analysis

Differential expression of gene sets was analyzed using gene set enrichment analysis (GSEA) [13]. The Broad Institute JAVA Desktop software (version 2.0) with the Pre-ranked option was utilized for GSEA, which included the Molecular Signatures Database C2 curated gene sets file c2.all.v2.5.symbols.gmt, containing 1,892 gene sets from known pathways and from published studies (http://www.broadinstitute.org/gsea/msigdb/index.jsp). For a higher sensitivity to detect a TNF-α signature, gene sets with an estimated Benjamini-Hochberg false discovery rate of <0.05 instead of <0.01 were considered statistically significant.

Heat maps (and the ordering by hierarchical clustering of the samples and the genes in heat maps) were based on normalization of the expression values in each sample with the use of Z score transformation. Hierarchical clustering was performed using the Cluster and TreeView software program [14].

RESULTS

The mean age of the 41 GPA patients was 50±15 years and 20 (49%) were male. Of them, 73% patients had systemic disease and ANCA was present in 36 patients (88%). The disease activity measured by the BVAS was mild to moderate with BVAS ranging from 0 to 5. Only 1 patient had BVAS >5. At the time of blood collection, 28 patients (68%) were on immunosuppressive drugs [10].

The presence of TNF-α signature in PBMCs was compared between healthy controls and GPA patients. GSEA revealed that out of entire TNF-α downstream signatures, only one signature "TIAN_TNF_SIGNALING_NOT_VIA_NKFB" was enriched in the GPA patients (Table 1) [15]. Next, we compared the expression levels of genes that are significantly up-regulated by TNF-α between healthy controls, GPA in remission (BVAS=0) and GPA with active GPA (BVAS$\geq$1). No gene regulated by TNF-α was significantly differently expressed between the healthy controls vs. GPA or GPA in remission vs. active GPA. Those data support that TNF-α signature was not present in GPA regardless of disease activity. Moreover, an unsupervised hierarchical clustering of those genes failed to cluster the samples into healthy controls and GPA in remission or with active disease (Figure 1).

As B cell depletion treatment is successful in GPA, we investigated as to whether the signature of B cell activation was present [9]. In contrast to TNF-α, numerous gene sets that are involved in B cell activation and survival were enriched in the GPA patients as compared to the healthy controls (Table 2).

DISCUSSION

In the present study GSEA and hierarchical clustering of gene expression profile demonstrated that the PBMCs did not carry the signs of TNF-α activation (i.e. "TNF-α signature") in GPA patients regardless of disease activity.

TNF-α plays a crucial role in inflammatory processes; it is secreted by activated immune cells including T cells and monocyte/macrophages and activates PMNs and promotes granulomatous inflammation with multinucleated giant cells, key histologic hallmark feature of GPA [3,16]. In rheumatoid arthritis (RA), TNF-α plays an essential role and the therapy directed against TNF-α has revolutionized the management and clinical outcome of RA [17,18]. All the more, the result of WGET was surprising that TNF-α inhibitor did not maintain disease re-

Table 1. Absence of TNF-α signature in patients with granulomatosis polyangiitis

Gene set name	Description*	Gene set size	NES	Nominal p	FDR† q
A. Enriched in GPA					
TIAN_TNF_SIGNALING_NOT_VIA_NFKB	Genes modulated in HeLa cells by TNF not via NFKB pathway	15	1.65	0.016	0.049*
ZHANG_RESPONSE_TO_IKK_INHIBITOR_AND_TNF_UP	Genes up-regulated in BxPC3 cells (pancreatic cancer) after treatment with TNFIKI-1, an inhibitor of IkappaB kinase	158	1.26	0.052	0.255
SANA_TNF_SIGNALING_UP	Genes up-regulated in five primary endothelial cell types (lung, aortic, iliac, dermal, and colon) by TNF	55	1.16	0.229	0.365
SANA_TNF_SIGNALING_DN	Genes down-regulated in five primary endothelial cell types (lung, aortic, iliac, dermal, and colon) by TNF	58	0.91	0.622	0.750
TIAN_TNF_SIGNALING_VIA_NFKB	Genes modulated in HeLa cells by TNF	19	0.70	0.884	0.958
BIOCARTA_TNFR1_PATHWAY	TNFR1 Signaling Pathway	28	0.55	0.980	1.000
B. Enriched in healthy controls					
BIOCARTA_TNFR2_PATHWAY	TNFR2 Signaling Pathway	17	0.65	0.930	0.991

GSEA showed that only one of the downstream genes of TNF-α activation was enriched in patents with GPA as compared to healthy controls. FDR: false discovery rate, GPA: granulomatosis with polyangiitis, GSEA: gene set enrichment analysis, NES: normalized enrichment score, TNF: tumor necrosis factor, TNFR: tumor necrosis fator receptor. *Description of gene sets are available on http://www.broadinstitute. org/gsea. †FDR < 0.05 is considered significant.

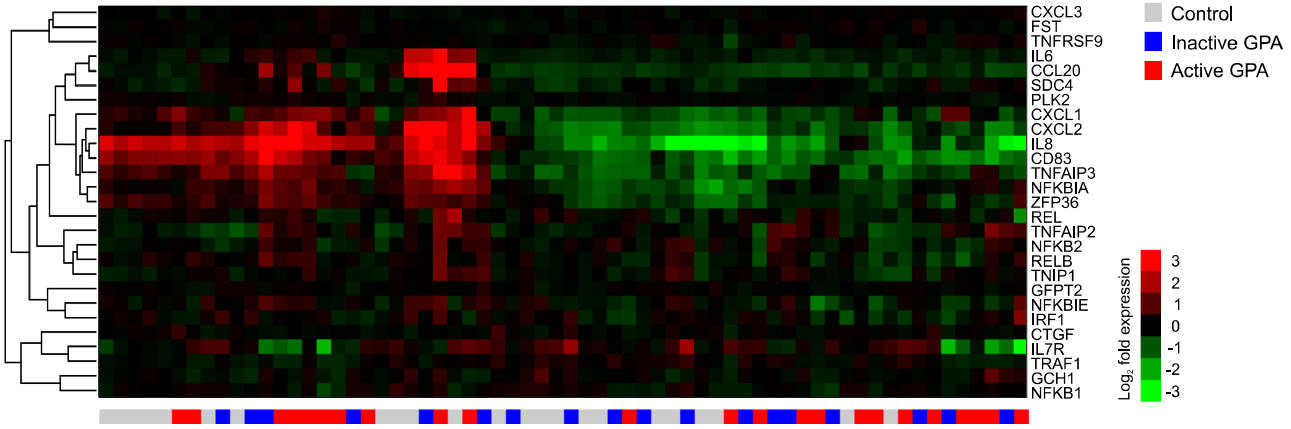

Figure 1. Unsupervised hierarchical clustering of the TNF-α downstream genes failed to cluster the samples into healthy and GPA subjects. Expression pattern of TNF-α downstream genes of controls, inactive, and active GPA did not cluster. GPA: granulomatosis with polyangiitis, TNF: tumor necrosis factor.

mission [8], questioning the critical of TNF-α in GPA pathogenesis. In our study, the absence of "TNF-α signature" regardless of disease activity may deliver a possible explanation for the absent efficacy of TNF-α inhibitor in GPA.

The absence of TNF-α signature does not necessarily implicate its absence in the sites of active inflammation such as lung granulomas or vasculitic lesions. Although the periphery continuously communicate with inflammatory sites via blood stream transporting cellular and humoral components, the immune cells that are activated by TNF-α might be captured at the site of inflammation and are, therefore, less abundant in the blood. Indeed, activated cells move to the inflammatory sites and remain there following chemokines and adhesion molecules [19]. Monocytes migrate, differentiate into macrophages and remain at the site of inflammation, orchestrating the inflammatory processes including gran-

Table 2. Presence of B-cell activation signature in granulomatosis polyangiitis

Gene set name	Description*	Gene set size	NES	Nominal p	FDR† q
A. Enriched in GPA					
HUTTMANN_B_CLL_POOR_SURVIVAL_UP	Up-regulated genes in B-CLL patients expressing high levels of ZAP70 and CD38, which are associated with poor survival	203	2.0	<0.001	0.004
FAELT_B_CLL_WITH_VH3_21_UP	Genes up-regulated in samples from B-CLL with the immunoglobulin heavy chain VH3-21 gene	39	1.71	0.009	0.038
MOREAUX_MULTIPLE_MYELOMA_BY_TACI_UP	Up-regulated genes distinguishing in multiple myeloma samples with higher expression of TACI	230	1.69	0.009	0.041
TARTE_PLASMA_CELL_VS_PLASMABLAST_UP	Genes up-regulated in mature plasma cells compared with plasmablastic B lymphocytes	255	1.73	<0.001	0.031
TARTE_PLASMA_CELL_VS_B_LYMPHOCYTE_UP	Genes up-regulated in plasma cells compared with B lymphocytes	74	1.68	<0.001	0.043
B. Not enriched in GPA					
SA_B_CELL_RECEPTOR_COMPLEXES	Antigen binding to B cell receptors activates protein tyrosine kinases, such as the Src family, which ultimate activate MAP kinases	22	1.26	0.168	0.257
ST_B_CELL_ANTIGEN_RECEPTOR	B Cell Antigen Receptor	37	1.03	0.406	0.557
MOREAUX_B_LYMPHOCYTE_MATURATION_BY_TACI_UP	Genes up-regulated in normal bone marrow plasma cells compared to polyclonal plasmablasts that also distinguished multiple myeloma samples by expression of levels of TACI	50	0.86	0.710	0.813
C. Enriched in healthy controls					
HUTTMANN_B_CLL_POOR_SURVIVAL_DN	Down-regulated genes in B-CLL patients expressing high levels of ZAP70 and CD38, which are associated with poor survival	49	2.25	<0.001	0.010
FAELT_B_CLL_WITH_VH_REARRANGEMENTS_DN	Genes down-regulated in B-CLL patients with mutated immunoglobulin variable heavy chain genes	44	1.76	0.002	0.033

GSEA showed that the genes involved in B cell activation and survival were enriched in patents with GPA as compared to healthy controls. B-CLL: B-cell chronic lymphocytic leukemia, FDR: false discovery rate, GPA: granulomatosis with polyangiitis, GSEA: gene set enrichment analysis, NES: normalized enrichment score, MAP: mitogen-activated protein, TACI: transmembrane activator and calcium modulator and cyclophylin ligand interactor. *Description of gene sets are available on http://www.broadinstitute.org/gsea. †FDR<0.05 is considered significant.

uloma formation and neutrophil activation leading to necrotizing granulomatous inflammation [20-22]. Activated T cells migrate and remain there as well [23]. In short, TNF-α activated cells may redistribute to the site of active inflammation. However, we recently reported that circulating and lesional monocytes and macrophages in GPA were alternatively activated with decreased TNF-α production [24].

In contrast to TNF-α signature, there was a robust correlation between GPA disease activity and myelopoesis gene expression in the same cohort as previously reported, suggesting that gene expression signature is associated with disease activity [10]. In addition, B cell activation signature was present in GPA (Table 2). This is striking since rituximab, CD20 antibody that depletes B cells effectively, is highly effective in the treatment of GPA and the presence of target signature was predictive of therapeutic response to treatment as well [9,12,25]. Similarly, in Sjögren's syndrome, which is also characterized by chronic B cell activation, the presence of signature 10 of B cell activation signaling predicts a positive treatment response to rituximab and it disappears after a successful treatment. However, it is not clear as to whether the gene signatures in the PBMCs can reflect those in the active inflammatory lesions such as in lung or kidney of GPA. In short, the gene signature profile of B cell activation but not that of TNF-α is present in GPA. Further research is warranted to investigate the gene expression

profile in the active GPA lesions.

CONCLUSION

Our study suggests the absent TNF-α signature in the PBMC might explain the absent efficacy of TNF-α inhibitors in GPA. TNF-α might play a less important role in GPA pathogenesis than previously expected.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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