

Expression Profiling of PBMC-based Diagnostic Gene Markers Isolated from Vasculitis Patients

Shigeto KOBAYASHI¹, Akihiko ITO², Daisuke OKUZAKI^{3,4}, Hiroaki ONDA^{3,5}, Norikazu YABUTA³, Ippei NAGAMORI³, Kazuo SUZUKI^{6,7}, Hiroshi HASHIMOTO¹, and Hiroshi NOJIMA^{3,4,5,*}

Department of Rheumatology and Internal Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan¹; Division of Molecular Pathology, Department of Cancer Biology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan²; Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 562-0031, Japan³; DNA-chip Development Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 562-0031, Japan⁴; Innovation Plaza Osaka, Izumi, Osaka 594-1144, Japan⁵; Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan⁶ and Inflammation Program, Department of Immunology, Chiba University, Graduate School of Medicine, Inohana 1-8-1 Chuo-ku, Chiba, 260-8670, Japan⁷

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Abstract

Vasculitis (angiitis) is a systemic autoimmune disease that often causes fatal symptoms. We aimed to isolate cDNA markers that would be useful for diagnosing not only vasculitis but also other autoimmune diseases. For this purpose, we used stepwise subtractive hybridization and cDNA microarray analyses to comprehensively isolate the genes whose expressions are augmented in peripheral blood mononuclear cells (PBMCs) pooled from vasculitis patients. Subsequently, we used quantitative real-time polymerase chain reaction (qRT-PCR) to examine the mRNA levels of each candidate gene in individual patients. These analyses indicated that seven genes exhibit remarkably augmented expression in many vasculitis patients. Of these genes, we analyzed G0/G1 switch gene 2 (G0S2) further because G0S2 expression is also enhanced in the PBMCs of patients with systemic lupus erythematoses (SLE). We generated G0S2 transgenic mice that ubiquitously overexpress human G0S2. Although we did not observe any obvious vasculitis-related histopathologic findings in these mice, these mice are unhealthy as they produce only few offspring and showed elevated serum levels of two autoimmunity-related antibodies, anti-nuclear antibody, and anti-double strand DNA antibody. Thus, our large-scale gene profiling study may help finding sensitive and specific DNA markers for diagnosing autoimmune diseases including vasculitis and SLE.

Key words: vasculitis; angiitis; G0S2; EGR1; amphiregulin; hemoglobin delta

1. Introduction

There are ~10 different disorders that are classified as vasculitis (also known as angiitis). Vasculitis is a

systemic autoimmune disease characterized by the chronic inflammation of systemic blood vessels, veins, and arteries of all types and sizes. This often causes symptoms that can rapidly induce death, such as the formation of blood clots (thrombosis), restriction of oxygenated blood supply (ischemia), and irreversible injury to affected organs.¹ Although immune system disturbances appear to be the main cause of the disease, the pathogenesis of vasculitis is not fully understood. The diagnosis and treatment of vasculitis was

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* To whom correspondence should be addressed.
Tel. + 81 6-6875-3980. Fax. + 81 6-6875-5192.
E-mail: snj-0212@biken.osaka-u.ac.jp

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recently greatly aided by the observation that three vasculitis disorders, namely Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and Churg–Strauss syndrome (CSS), are characterized by the presence of circulating antineutrophil cytoplasmic antibodies (ANCA).^{2,3} Two different types of ANCA have been identified. C-ANCA targets proteinase-3 and is most frequently found in WG, whereas P-ANCA, which targets myeloperoxidase, is most frequently detected in MPA and CSS. Consequently, enzyme immunoassays (EIA) that examine the ability of patient serum to bind to these enzymes can be used to diagnose WG, MPA, and CSS. However, diagnoses made on the basis of positive EIAs will still need to be confirmed by biopsies. Moreover, many other vasculitis disorders are not characterized by the presence of ANCA. Thus, the diagnosis of vasculitis disorders requires the identification of more convenient and generally applicable diagnostic markers. The identification of these markers may also provide insights into the pathogenesis behind this disorder and improve the therapy of vasculitis.

We have isolated putative gene markers that are characteristic to the autoimmune diseases, such as systemic lupus erythematoses (SLE)⁴ and rheumatoid arthritis (RA)⁵, using peripheral blood mononuclear cells (PBMCs). Here, we sought to identify new gene markers that can distinguish the autoimmune disease, in particular vasculitis, from other diseases with high specificity when PBMCs serve as the sample. For this purpose, we used the stepwise subtraction technique⁶ and high-density oligonucleotide microarrays to isolate those genes that show dramatically upregulated expression commonly in the pooled PBMC mRNAs of varieties of vasculitis patients regardless of the patient's symptom, active, or inactive phase when compared with normal volunteer PBMC mRNAs. To identify the genes that are upregulated in many vasculitis patients, we selected and mixed the PBMC mRNAs from patients with one of the following seven vasculitis disorders: WG, MPA, Takayasu's arteritis (TA), allergic granulomatous angiitis (AGA), malignant RA (MRA = rheumatoid vasculitis), giant cell arteritis (GCA), and polyarteritis nodosa (PN). We found that the following seven genes are commonly upregulated in the PBMCs of many of these vasculitis patients regardless of their symptoms: early growth response 1 (*EGR1*), G0/G1 switch gene 2 (*GOS2*), hemoglobin delta (*HBD*), amphiregulin (also known as *AREG*), interleukin-1 receptor type II (*IL1R2*), *calgranulin C*, and a novel gene named *TVAS10*. Of these genes, we selected *GOS2* for further analysis because its physiological functions are poorly understood. We prepared anti-*GOS2* antibodies and then generated *GOS2* transgenic mice and examined their phenotype. We propose that

the vasculitis gene markers we identified here may be useful for the future diagnosis of vasculitis.

2. Materials and methods

2.1. Human subjects and ethical considerations

All systemic vasculitis patients used in this study were diagnosed according to a previously documented proposal (the ACR criteria and the CHCC criteria).¹ This study was reviewed and approved by the Internal Review Board of the Research Institute for Microbial Diseases, Osaka University. Accordingly, written informed consent was obtained from all participants before their PBMCs were obtained. Serum samples were consecutively obtained regardless of the patient's symptom, active, or inactive phase.

2.2. Statistical analysis

Significant differences were determined by using Mann–Whitney *U*-test (Figs. 1 and 2B, Supplementary Fig. S1). The data are expressed as means \pm SE. *P*-value of <0.05 or <0.01 was considered to be statistically significant.

2.3. Transgene vector construction and production of *GOS2* transgenic mice

To construct the transgene vector pCX-*GOS2*, the human *GOS2* ORF was cloned from a SLE cDNA library⁴ by PCR with the *EcoRI* site-bearing primers *GOS2-EcoRI-5'F* (5'-TATGAATTCGCCACCATGGAAACGGTCCAGGAGCTGATC-3') and *GOS2-EcoRI-3'R* (5'-ATAGAATTCCTAGGAGGCGTGCTGCCGTTGGAC-3'). The *GOS2-EcoRI-5'F* primer contains the Kozak sequence ahead of the start codon of *GOS2* cDNA, whereas the *GOS2-EcoRI-3'R* primer contains the stop codon of *GOS2* cDNA. The sequence of the amplified product was confirmed by DNA sequencing. As previously described,⁷ the *EcoRI*-digested fragment of the amplified *GOS2* ORF was inserted into the *EcoRI* site of the pCAGGS expression vector, which possesses a chicken β -actin promoter and a cytomegalovirus enhancer (CMV-IE enhancer). The resulting construct was designated pCX-*GOS2*. To prepare the *GOS2* fragment bearing the chicken β -actin promoter and the CMV-IE enhancer for injection into fertilized eggs, the *Sall/HindIII* fragment of pCX-*GOS2* was purified by electrophoresis and agarose gel extraction. The *GOS2* transgenic embryos and mice were generated according to previously described protocols.⁷ Briefly, the purified DNA fragments were injected into B6D2F1 \times B6D2F1 fertilized eggs. The incorporation of the transgene was examined by the genomic PCR analysis using DNA extracted from the tail and the pCX-*GOS2*-1685F (5'-GCTGGTTGTTGTGCTGTCTCATCA-3') and pCX-*GOS2*-2161R (5'-GCCAG

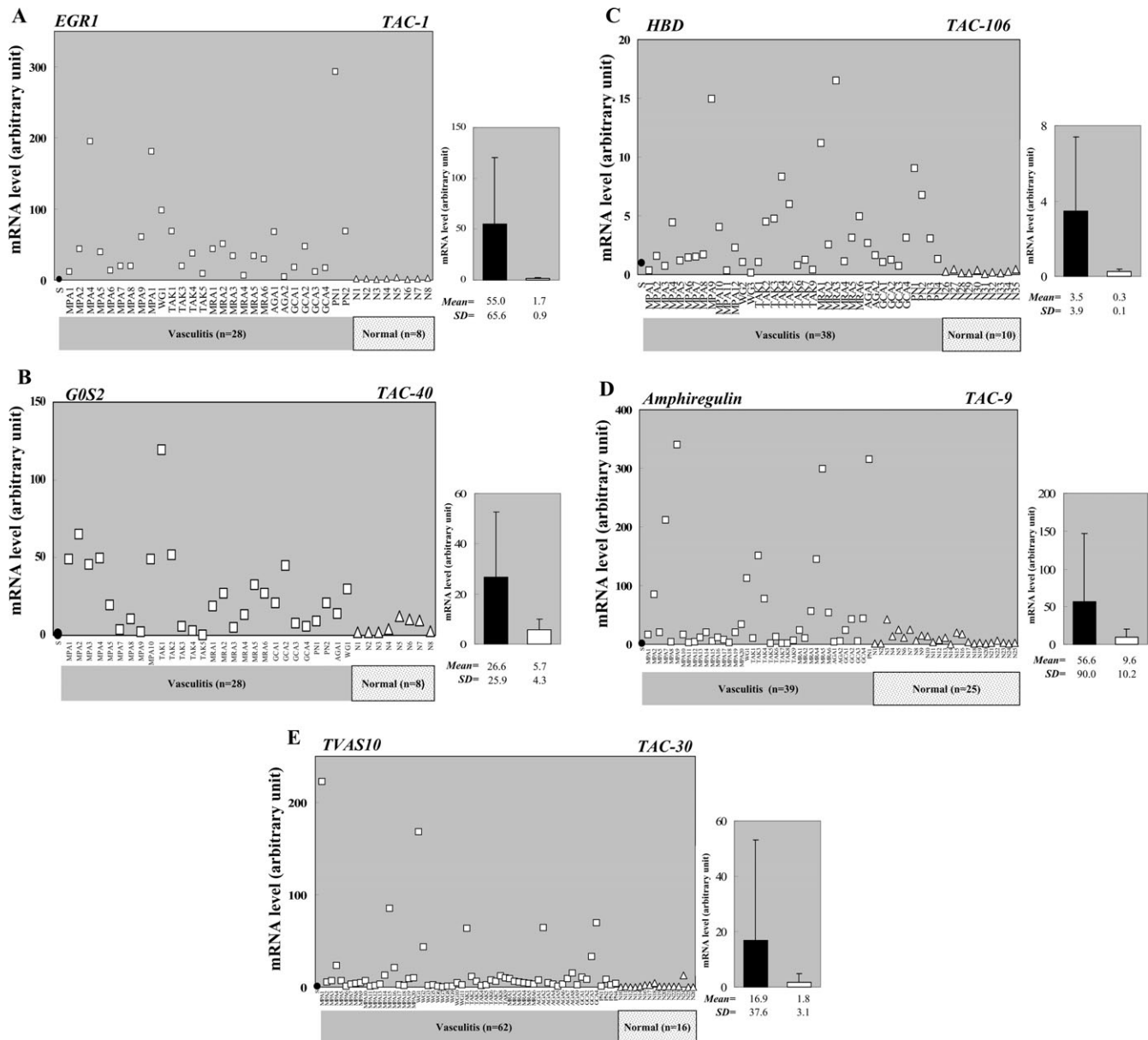


Figure 1. mRNA expression of selected *TAS/TAC* genes in the PBMCs from individual vasculitis patients and normal volunteers. qRT-PCR analyses show that (A) *EGR1* (*TAC-1*), (B) *GOS2* (*TAC-40*), (C) *HBD* (*TAC-106*), (D) *Amphiregulin* (*TAC-9*), and (E) *TVAS10* (*TAC-30*) are generally upregulated in the PBMCs of vasculitis patient (circles) but not in the PBMCs of normal volunteers (triangles). Each symbol denotes the mean value of a sample analyzed in triplicate. The leftmost filled circle signifies an RNA sample from the PBMCs of a healthy volunteer (male, age 52), which was used as a standard sample and whose expression level was set to 1.0. It allowed us to compare all of the expression profiles tested in this study. Average \pm SE values of the vasculitis patient and normal volunteer groups are shown in the right-hand graphs and differ significantly ($P < 0.01$).

AAGTCAGATGCTCAAGGGCTTCA-3') primers. The PCR conditions involved TaKaRa Ex *Taq* polymerase (Takara, Shiga, Japan) and a pre-heating step (95°C for 2 min), 30 reaction step cycles (95°C for 30 s, 58°C for 30 s, 72°C for 1 min), and a final elongation step (72°C for 5 min). The founder mice were mated with C57BL/6 mice and both the transmittance of the transgene and the successful expression of human *GOS2* protein were examined by western blot analysis of total cell extracts of mouse tails

using one of the anti-*GOS2* monoclonal antibodies (clone #3-1) we generated (see section 3.4 and Supplementary Fig. S2).

2.4. Histological examination

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Mouse tissues were fixed immediately after removal with 4% paraformaldehyde, then embedded in paraffin, and cut into sections (4 μ m

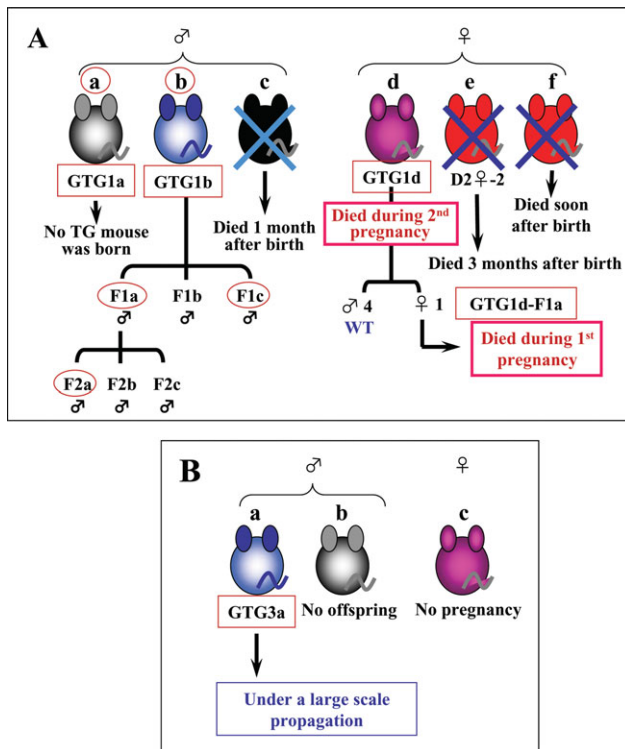


Figure 2. Family tree of *GOS2* transgenic mice. **(A)** Of the 61 mice tested in the first trial, six *GOS2* transgenic mouse lines were generated. They all died except for the GTG1b line, which was not useful for further analysis because the human *GOS2* gene was introduced on the Y chromosome; consequently, this mouse only produced male *GOS2* transgenic mice. **(B)** Of the 22 mice tested in the third trial, three *GOS2* transgenic mouse lines were generated. The only surviving GTG3a line is now under large scale propagation to establish a strain.

thick). Some sections were stained with hematoxylin and eosin according to standard procedures, whereas others were stained with the clone #3-1 monoclonal anti-*GOS2* antibody according to the previously described procedure.⁸ To evaluate the immunostain, sections of the same organs from *GOS2*-TG and C57BL/6 mice were processed at the same time. When the immunoreactive signals in the former sections were substantially stronger than those in the latter, they were considered to indicate the exogenous *GOS2* proteins produced from the transgene.

2.5. *In situ* hybridization

Sections were processed in the Genostaff laboratory (Tokyo, Japan) by using the DIG RNA labeling and detection kits (Roche Diagnostics, Mannheim, Germany). Briefly, *GOS2* antisense and sense (negative control) RNA probes were prepared by *in vitro* transcription of the pBluescript vector containing the full-length human *GOS2* cDNA according to the manufacturer's instructions. Hybridized signals were colored blue with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as a substrate of alkaline

phosphatase. The sense probe did not yield any significant stains. Further information concerning the *in situ* hybridization (ISH) method is available on request.

2.6. Serologic examination

Approximately 200 μ l of peripheral blood per mouse were collected from the orbital plexus under anesthesia and were left for 1 h to coagulate. After centrifugation, \sim 20 μ l sera were obtained. The sera were frozen at -20°C and sent to the Mitsubishi Kagaku BCL laboratory (Tokyo, Japan), where each serum was diluted 500 times and analyzed for the levels of anti-nuclear and anti-double strand (ds) DNA antibodies by fluorescent antibody tests or enzyme immunoassays.

3. Results

3.1. Identification of vasculitis-specific genes by stepwise subtraction and DNA microarray analysis

To isolate the putative vasculitis-specific genes that are commonly upregulated in the PBMCs of vasculitis patients, we first used our stepwise subtractive hybridization method. PBMC samples were obtained from vasculitis patients (50 patients in total) regardless of the patient's symptom, active, or inactive phase, and a cDNA library was prepared from their pooled mRNAs by the linker-primer method using a pAP3neo vector.⁹ Stepwise subtractive hybridization was then performed using the biotinylated pooled mRNAs from the PBMCs of eight normal volunteers, as described previously.⁶ This generated a library of genes that are upregulated in only vasculitis PBMCs. To examine whether these genes are actually upregulated in vasculitis but not normal PBMCs, we performed northern blot analysis using the pooled mRNAs from the vasculitis and normal PBMCs (Fig. 3A). For the cDNA clones whose expression levels were too low for detection by northern blot analysis, we examined their expression in vasculitis and normal PBMCs by RT-PCR (Fig. 3B). The 29 genes that were confirmed to be upregulated in vasculitis patients were called *TAS* after transcript augmented in vasculitis isolated by stepwise-subtraction. The *TAS* genes and their accession numbers are summarized in Table 1.

To increase the number of putative vasculitis-specific genes, we also performed a genome-wide complementary DNA microarray analysis using the Agilent Hu44K array with the same pooled vasculitis and normal PBMC RNA samples. When we tested the top 402 genes from the microarray list of vasculitis-upregulated genes (data not shown) by RT-PCR, we identified 63 genes whose expression levels are dramatically upregulated in vasculitis patient PBMCs

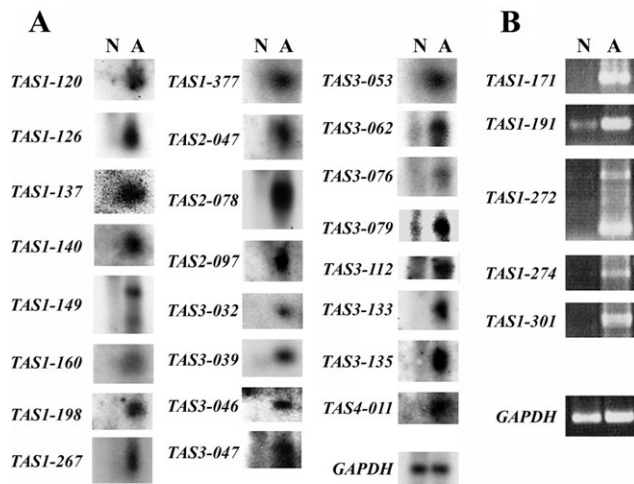


Figure 3. mRNA expression levels of the *TAS* genes. Individual *TAS* genes were subjected to northern blot (A) or RT-PCR (B) analysis to confirm they are upregulated in the PBMCs of 50 vasculitis patients (A) but not in the PBMCs of eight normal volunteers (N). Table 1 provides the names of the *TAS* genes. *GAPDH* was also analyzed as a loading control. The annealing temperature for RT-PCR was always 58°C and amplification occurred over 30 cycles. The sequences of the primers used to identify the *TAS* genes are presented in Supplementary Table S1.

(Fig. 4). We called them *TAC* after transcript augmented in vasculitis isolated by cDNA-microarray. The *TAC* genes and their accession numbers are summarized in Table 2. Dual specificity phosphatase 1 (*DUSP1*) and *IL1R2* were identified by both methods. Of the 92 *TAS* and *TAC* genes, 30 genes are uncharacterized genes. These are designated as *TVAS* after transcript increased in vasculitis.

3.2. Expression profiles of vasculitis-upregulated genes in individual patients

To determine whether these vasculitis-specific candidate genes are indeed upregulated in patients suffering from WG, MPA, TA, AGA, MRA, GCA, or PN and thus are general markers of vasculitis, we subjected 25 of the *TAS* and *TAC* genes (underlined in Tables 1 and 2) to quantitative RT-PCR (qRT-PCR) using PBMC RNA samples from indicated numbers of individual vasculitis patients and normal volunteers as negative controls (Fig. 1 and Supplementary Fig. S1). In every qRT-PCR, a standard RNA (denoted as normal with a relative intensity of 1.0) from the PBMCs of a healthy volunteer (male, age 52) was used (see the leftmost filled circle in Fig. 1A–E); this allowed us to compare the expression profiles of the genes tested in this study. It also allowed us to compare the expression profiles of the genes in this study with those of other genes tested in our previous studies on SLE⁴ and RA.⁵ With this experiment, we expected to isolate those genes that are highly

expressed not only in many vasculitis patients but also in patients with SLE⁴ and RA;⁵ the identification of such marker genes may be useful as sensitive and specific diagnostic tools.

Of the 25 *TAS/TAC* genes tested, *TAC-1* [early growth responses 1 (*EGR1*)] was one of the most strongly upregulated genes commonly in the PBMCs of many vasculitis patients, as 96% of patients expressed this gene at 3–300-fold higher levels than healthy volunteers (Fig. 1A). *EGR1* encodes a zinc finger transcription factor whose expression is induced within minutes upon the reception of growth stimuli; its expression then decays within a few hours. It promotes cellular differentiation along a macrophage lineage.¹⁰ It also plays a critical role in the response to both acute and chronic vascular stress by inducing the expression of several gene products linked to cellular perturbation. This is particularly true for the cellular perturbation in the vasculature that is induced by hypoxia, ischemia/reperfusion, atherosclerosis, and acute vascular injury.¹¹ It remains unclear whether the induction of *EGR1* in vasculitis is a pathogenic response or merely results from vascular stress. Nonetheless, *EGR1* may serve as a gene marker for the diagnosis of vasculitis.

TAC-40 (*GOS2*) is a lymphocyte G0/G1 switch gene that encodes a putative cell cycle inhibitor whose expression may be required before cells become committed to enter the G1 phase of the cell cycle.¹² Nearly, half of the vasculitis patients showed >20-fold increased expression, whereas the expression of most of the normal volunteers was much lower (Fig. 1B). Notably, relative to healthy controls, *GOS2* expression is also enhanced in the PBMCs from patients with other autoimmune diseases, namely SLE⁴ and RA.⁵ Microarray and qRT-PCR analyses also showed that *GOS2* is upregulated in the PBMCs from psoriasis patients suffering from severe generalized disease.¹³ Thus, it may be interesting to analyze *GOS2* further, as it may shed light on the pathogenesis of vasculitis at the molecular level (see below).

Nearly 77% of the vasculitis patients expressed 3–16-fold more *TAC-106* (*HBD*) mRNA than the normal volunteers (Fig. 1C). *HBD* is a minor type of hemoglobin (it makes up only 2–3% of adult hemoglobin) that forms a complex with α -hemoglobin called hemoglobin A₂ ($\alpha_2\delta_2$).¹⁴ Although high HbA₂ levels are diagnostic for the β -thalassemia trait, its relevance to vasculitis pathology is unknown. Nonetheless, it may be useful as a diagnostic gene marker for vasculitis.

Amphiregulin is one of the epidermal growth factor (EGF)-like growth factors that stimulate cell growth by activating the EGF receptor signaling of target cells in an autocrine/juxtacrine fashion.¹⁵ We previously reported that *Amphiregulin* expression is enhanced in the PBMCs of some patients with SLE⁴ and RA.⁵

Table 1. List of stepwise subtraction identified *TAS* genes that show upregulated expression in vasculitis patient PBMCs

Gene name	Accession number	Sequence description
<i>TAS1-120</i>	AK091533	Unknown (sapiens cDNA FLJ34214) = <i>TVAS1</i>
<i>TAS1-126</i>	NM_006013	Ribosomal protein L10 (RPL10)
<i>TAS1-137</i>	AC007318	Unknown (AL049356) = <i>TVAS2</i>
<i>TAS1-140</i>	BC033089	Lipocalin 2 (oncogene 24p3)
<i>TAS1-149</i>	BC008684	Similar to splicing factor, arginine/serine-rich (MGC:9742)
<i>TAS1-160</i>	NM_016304	Unknown (C15orf15) = <i>TVAS3</i>
<i>TAS1-198</i>	BC010878	ASF1 anti-silencing function 1 homolog A
<i>TAS1-267</i>	BC042436	Unknown (IMAGE:4471726) = <i>TVAS4</i>
<i>TAS1-377</i>	AY275537	Unknown (isolate 183 mitochondrion) = <i>TVAS5</i>
<i>TAS2-047</i>	BC047681	S100 calcium binding protein A9 (calgranulin B)
<i>TAS2-078</i>	NM_004417	Dual specificity phosphatase 1 (DUSP1)
<i>TAS2-097</i>	D89974	Glycosylphosphatidyl inositol-anchored protein GPI-80
<i>TAS3-032</i>	NM_015384	Nipped-B homolog (<i>Drosophila</i>) (NIPBL)
<i>TAS3-039</i>	NM_001030	Ribosomal protein S27 (metalloproteinase 1) (RPS27)
<i>TAS3-046</i>	BC000927	Poly(A) polymerase alpha
<i>TAS3-047</i>	NM_000517	Hemoglobin, alpha 2 (HBA2)
<i>TAS3-053</i>	NM_004633	Interleukin 1 receptor, type II (IL1R2)(AY124010)
<i>TAS3-062</i>	BC000163	Vimentin
<i>TAS3-076</i>	BC064910	Beta-2-microglobulin
<i>TAS3-079</i>	BC001429	Annexin A5
<i>TAS3-112</i>	AY195792	Unknown (haplotype As9Y mitochondrion) = <i>TVAS6</i>
<i>TAS3-133</i>	NM_005870	Sin3-associated polypeptide, 18kDa (SAP18)
<i>TAS3-135</i>	BC018183	NADH dehydrogenase (ubiquinone) 1 beta subcomplex
<i>TAS4-011</i>	CR611844	Unknown (CS0DL007YO12 of B cells) = <i>TVAS7</i>
<i>TAS1-171</i>	AY260957	Insulin-like growth factor 1 (somatomedin C) (IGF1)
<i>TAS1-191</i>	NM_005621	S100 calcium-binding protein A12 (calgranulin C)
<i>TAS1-272</i>	NM_003805	CASP2 and RIPK1 domain containing adaptor with death domain (CRADD)
<i>TAS1-274</i>	NM_004592	Splicing factor, arginine/serine-rich 8 (SFRS8)
<i>TAS1-301</i>	BC062616	Protein serine kinase H1

The expression levels of the *TAS* genes were examined by northern blot (Fig. 1A) and/or RT-PCR (Fig. 1B). The underlined genes were subjected to qRT-PCR to determine their generality of expression in vasculitis patients but not normal volunteers (Fig. 3).

However, compared with SLE and RA patients, a far smaller proportion of vasculitis patients show enhanced *Amphiregulin* expression (Fig. 1D). Thus, *Amphiregulin* may be more significant as a DNA marker for SLE and RA than for vasculitis.

TAC30 (= *TVAS10*) encodes an uncharacterized novel protein that is composed primarily of an ankyrin repeat, which is one of the most frequently observed amino-acid motifs and is important for protein-protein interactions.¹⁶ About a half of the vasculitis patients showed 10–200-fold increased expression of *TAC30*, whereas healthy volunteers showed very low expression except for one case (Fig. 1E).

Interleukin-1 (IL1), a principal macrophage-derived cytokine, plays a pivotal role in the immunoinflammatory process by triggering cell activation *via*

its type I receptor (IL1R1); in contrast, its type II receptor (IL1R2) lacks the intracellular domain for IL1 signaling and functions as a potent, specific, and natural inhibitor of IL1 by acting as a decoy receptor.¹⁷ About half of the vasculitis patients showed 20–50-fold *IL1R2* expression (one patient showed ~300-fold expression), whereas healthy volunteers showed very low expression except for one case (Supplementary Fig. S1A). This increased expression of *IL1R2* may be related to the vascular inflammation of the vasculitis patients.

Three phagocyte-specific S100 proteins called calgranulin-A, -B, and -C act separately during calcium-dependent signaling. In particular, *TAS1-191* (*calgranulin C*) secretion by tumor necrosis factor (TNF)-stimulated granulocytes causes target cell activation that results in the upregulated expression of proinflammatory

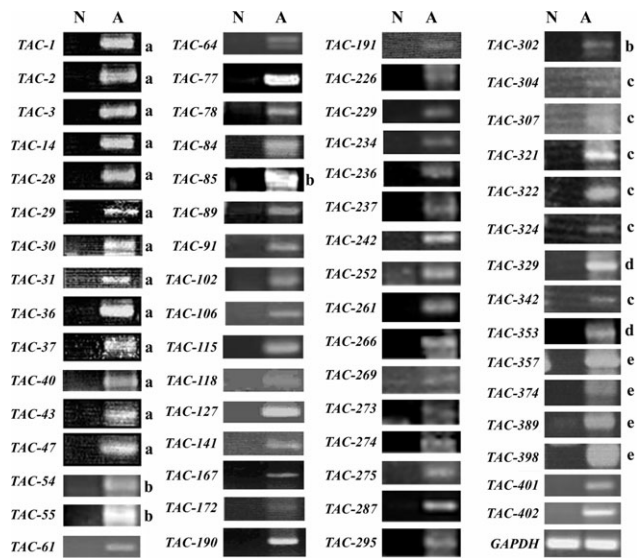


Figure 4. mRNA expression levels of the TAC genes. Individual TAC genes were subjected to RT-PCR analysis to confirm they are upregulated in the PBMCs of 50 vasculitis patients (A) but not in the PBMCs of eight normal volunteers (N). GAPDH was also analyzed as a loading control. The annealing temperature and amplification cycles for RT-PCR were always 50°C and 35 cycles, respectively, with the exception of the reactions denoted by a (55°C, 40 cycles), b (50°C, 30 cycles), c (58°C, 27 cycles), d (50°C, 40 cycles), and e (58°C, 30 cycles). The sequences of the primers used to identify the TAC genes are presented in Supplementary Table S1.

cytokines such as TNF and IL1 β ; the TNF that is released may then stimulate granulocytes to secrete more calgranulin C, thereby establishing a self-amplifying positive feedback loop.¹⁸ About 30% of vasculitis patients showed 10–50-fold *calgranulin C* expression (one patient showed ~150-fold expression), whereas most of the healthy volunteers showed low expression (Supplementary Fig. S1B). In contrast, the vasculitis patients and normal volunteers did not differ in their expression of *calgranulin B* (TAS2-047) (data not shown).

3.3. Expression pattern of TAS/TAC genes in PBMC

We previously reported that *GOS2* was primarily expressed in monocytes, whereas *Amphiregulin* was expressed in both monocytes, T cells and B cells by performing RT-PCR on multiple-tissue cDNA panels (MTC) from Clontech (Palo Alto, CA).⁵ To determine whether *EGR1*, *HBD*, *TVAS10*, *IL1R2*, and *calgranulin C* are also expressed in particular human blood cells, we conducted similar experiments. As shown in Fig. 5, *EGR1* and *IL1R2* were expressed ubiquitously in all lanes, albeit with varying intensities. cDNA from placenta, which also contains many blood cells, served as a control. All genes except *calgranulin C* were strongly expressed in activated CD4⁺ T cells (lane 8) and weakly expressed in activated mononuclear cells (aMNCs) (lane 7). Only *EGR1* and *TVAS10* were expressed in activated CD8⁺ T (lane 9)

cells. *EGR1*, *TVAS10*, *IL1R2*, and *calgranulin C* were expressed in resting CD8⁺ T cells (T-suppressor/cytotoxic; lane 2) and monocytes (lane 4). These observations together indicate that these vasculitis-upregulated genes have similar expression profiles. Unfortunately, we could not perform this analysis on vasculitis patients because of the low numbers of PBMCs that we could obtain from the patients.

3.4. Preparation of transgenic mice that overexpress *GOS2*

We selected *GOS2* for further analysis because little is known about its physiological significance and function. To explore the physiological significance of *GOS2*, we prepared transgenic mice that overexpress the human gene for *GOS2*. For this purpose, we constructed plasmid DNA in which human *GOS2* cDNA lies directly downstream of the beta-actin promoter, as described previously.⁷ This construct was used to generate transgenic mouse lines that express human *GOS2* protein in all tissues. The successful generation of these transgenic mice was confirmed by PCR and/or western blot analysis using DNA or cell extracts obtained from the tail. Expression of *GOS2* protein was confirmed by the monoclonal antibody (#3-1) we prepared here (see Supplementary Result and Fig. S2). Although the transgenic mice did not exhibit an obvious phenotype, they seemed to be unhealthy as they did not produce large numbers of offspring. This made it very difficult to establish this transgenic mouse line. For example, in the first attempt to generate this line, 61 mice were tested by PCR for expression of human *GOS2*. Of these, three female and three male mice were found to bear the transgene (GTG1a-f, see Fig. 2). However, the GTG1f female mouse died soon after birth and the GTG1e female and GTG1c male mice died 3 and 1 months after birth, respectively (Supplementary Fig. S4A). Moreover, while the surviving female transgenic mouse (GTG1d) produced one female transgenic mouse, which safely grew to adulthood, the mother died during her next pregnancy. Her daughter also died during her first pregnancy. In addition, the surviving male mouse GTG1b only produced male transgenic mice when mated with wild-type B6 female mice because the human *GOS2* gene was introduced into the Y chromosome. Thus, this mouse could not be used further. Furthermore, the remaining surviving male mouse, GTG1a, did not produce transgenic mouse when mated with wild-type B6 female mice. Thus, we failed to establish a human *GOS2*-expressing transgenic strain in this trial. The second trial did not generate any transgenic mice. However, in the third trial, of the 22 mice examined by PCR (Fig. 6A) and western blot analysis (Fig. 6B), we found three

Table 2. List of microarray identified TAC genes that show upregulated expression in the PBMCs of vasculitis patients

Gene name	Accession number	Sequence description
<u>TAC-1</u>	NM_001964	Early growth response 1 (EGR1) gene product
<u>TAC-2</u>	AK025198	Unknown (FLJ21545) = TVAS8
<u>TAC-3</u>	NM_000584	Interleukin 8 (IL8)
<u>TAC-9</u>	NM_001657	Amphiregulin
<u>TAC-14</u>	NM_003596	Tyrosylprotein sulfotransferase 1 (TPST1)
<u>TAC-19</u>	NM_004633	Interleukin 1 receptor, type II (IL1R2)
<u>TAC-28</u>	NM_000715	Complement component 4 binding protein, alpha (C4BPA)
<u>TAC-29</u>	NM_017762	Unknown (FLJ20313) = TVAS9
<u>TAC-30</u>	NM_144590	Unknown (ankyrin repeat domain 22: MGC22805) = TVAS10
<u>TAC-31</u>	BC013734	Prostaglandin-endoperoxide synthase 2
<u>TAC-36</u>	AB044805	6-phosphofructo-2-kinase heart isoform
<u>TAC-37</u>	NM_001511	Chemokine (C-X-C motif) ligand 1 (CXCL1)
<u>TAC-40</u>	NM_015714	G0/G1 switch gene 2 (G0S2)
<u>TAC-43</u>	NM_004545	NADH dehydrogenase (ubiquinone) 1 beta (NDUFB1)
<u>TAC-47</u>	NM_080657	Viperin (cig5)
<u>TAC-54</u>	BC029495	Unknown (MGC:33104) = TVAS11
<u>TAC-55</u>	AF249277	Cervical cancer suppressor-1
<u>TAC-61</u>	BC065737	Unknown (IMAGE:30404477) = TVAS12
<u>TAC-64</u>	NM_000032	Aminolevulinate, delta-, synthase 2 (ALAS2)
<u>TAC-77</u>	NM_000661	Ribosomal protein L9 (RPL9)
<u>TAC-78</u>	NM_004233	CD83 antigen (immunoglobulin superfamily)
<u>TAC-84</u>	X60364	ALAS mRNA for 5-aminolevulinate synthase precursor
<u>TAC-85</u>	AK098605	Unknown (FLJ25739) = TVAS13
<u>TAC-89</u>	NM_004833	Absent in melanoma 2 (AIM2)
<u>TAC-91</u>	NM_001738	Carbonic anhydrase I (CA1)
<u>TAC-102</u>	NM_005143	Haptoglobin (HP)
<u>TAC-106</u>	BQ446275	Hemoglobin delta (HBD)
<u>TAC-115</u>	NM_182522	TFAFA4 protein (TFAFA4)
<u>TAC-118</u>	L03419	Fc-gamma receptor I B1
<u>TAC-127</u>	NM_007115	Tumor necrosis factor, alpha-induced protein 6 (TNFAIP6)
<u>TAC-141</u>	BX537874	Unknown (DKFZp313P036) = TVAS14
<u>TAC-167</u>	BQ674642	Unknown = TVAS15
<u>TAC-172</u>	AL390162	Unknown (DKFZp761A1916) = TVAS16
<u>TAC-190</u>	NR_001459	Unknown (C14orf62) = TVAS17
<u>TAC-191</u>	NM_002983	Chemokine (C-C motif) ligand 3 (CCL3)
<u>TAC-226</u>	BC031359	Unknown (IMAGE:4778855) = TVAS18
<u>TAC-229</u>	NM_002064	Glutaredoxin (thioltransferase) (GLRX)
<u>TAC-234</u>	NM_000963	Prostaglandin G/H synthase and cyclooxygenase (PTGS2)
<u>TAC-236</u>	NM_001124	Adrenomedullin (ADM)
<u>TAC-237</u>	NM_014320	Heme-binding protein 2 (HEBP2)
<u>TAC-242</u>	AW901958	Unknown = TVAS19
<u>TAC-252</u>	NM_003096	Small nuclear ribonucleoprotein polypeptide G (SNRPG)
<u>TAC-261</u>	NM_014879	G protein-coupled receptor 105 (GPR105)
<u>TAC-266</u>	BC032663	Phorbol-12-myristate-13-acetate-induced protein 1
<u>TAC-269</u>	BC032480	Unknown (IMAGE:5214272) = TVAS20
<u>TAC-273</u>	NM_182619	Secretory protein LOC348174 (LOC348174)
<u>TAC-274</u>	NM_012472	Testis specific leucine rich repeat protein (TSLRP)

Continued

Table 2. Continued

Gene name	Accession number	Sequence description
TAC-275	AX721252	Unknown (Patent WO0220754) = TVAS21
TAC-287	NM_032412	Putative nuclear protein (ORF1-FL49)
TAC-295	NM_024850	Butyrophilin-like 8 (BTNL8)
TAC-302	BC005984	Unknown (IMAGE:4247211) = TVAS22
TAC-304	NM_033655	Unknown = TVAS23
TAC-307	BC042517	Unknown (IMAGE:4822953) = TVAS24
TAC-321	AL831953	Unknown (DKFZp667P0410) = TVAS25
TAC-322	AK128746	Unknown (FLJ44672) = TVAS26
TAC-324	AF067801	Unknown (HDCGC21P) = TVAS27
TAC-330	D86962	Unknown (KIAA0207) = TVAS28
<u>TAC-343</u>	NM_004417	Dual specificity phosphatase 1 (DUSP1)
TAC-353	M64109	Udulin 2
TAC-357	BC053669	Unknown (IMAGE:6146402) = TVAS29
TAC-374	NM_016220	Zinc finger protein (ZFD25)
TAC-389	NM_001955	Endothelin 1 (EDN1)
TAC-398	NM_020995	Haptoglobin-related protein (HPR)
TAC-401	NM_001161	Nudix (nucleoside diphosphate linked moiety X)-type motif 2 (NUDT2)
TAC-402	AY358224	Unknown (UNQ9368) = TVAS30

The expression levels were examined by RT-PCR (Fig. 2). The underlined genes were subjected to qRT-PCR to determine their generality of expression in vasculitis patients but not normal volunteers (Fig. 3).

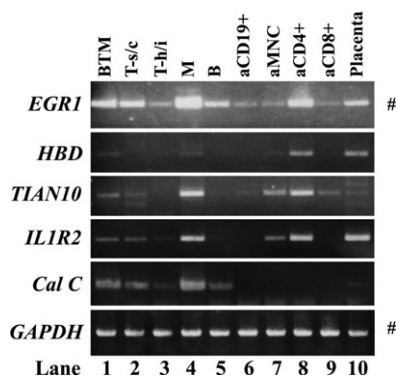


Figure 5. Determination of the human blood cells that express *EGR1*, *HBD*, *TVAS10*, *IL1R2*, and *calgranulin C* (*Cal C*). RT-PCR was performed by using the multiple-tissue cDNA panel for human blood fractions (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications were conducted at 50°C over 33 cycles except as indicated at the right of the panels by # (50°C over 30 cycles). Lane 1, mononuclear cells (B, T cells, and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated CD19+ cells (aCD10+). Lane 7, activated mononuclear cells (aMNC). Lane 8, activated CD4+ cells (aCD8+). Lane 9, activated CD8+ cells. Lane 10, human placenta, which also includes many blood cells. The latter lane served as a control.

transgenic mice, of which one (GTG3a) produced offspring (see Supplementary Fig. S4B). These mice appear to be healthier than the other transgenic

mice and consequently we have been able to maintain this line probably due to lower *GOS2* levels than other transgenic mice. Thus, the exogenous and ubiquitous overexpression of human *GOS2* seems to make the transgenic mice unhealthy and inhibits their production of offspring.

3.5. Human *GOS2* expression patterns in transgenic mice

To examine the expression patterns of exogenous human *GOS2* in various transgenic mouse tissues, we first performed ISH analysis. As shown in some representative pictures (Fig. 6C), high-level expression of *GOS2* mRNA was detected in various organs and tissues. The signals in the urinary tubule, gastric foveola, and esophageal mucosa were distributed in epithelial clumps (Fig. 6Ci–iii). In contrast, scattered or diffusely distributed signals were detected in the cardiac ventricle, gastric smooth muscle, and seminiferous tubule (Fig. 6Civ–vi).

Immunohistochemical analysis using the monoclonal antibody (#3-1) also detected exogenously expressed human *GOS2* proteins in various organs and tissues of the transgenic mice. As shown in some representative pictures (Fig. 6D), the cellular distribution pattern of these proteins was similar to that of exogenous *GOS2* mRNA (Fig. 6C). In the kidney, although most urinary tubule epithelia were

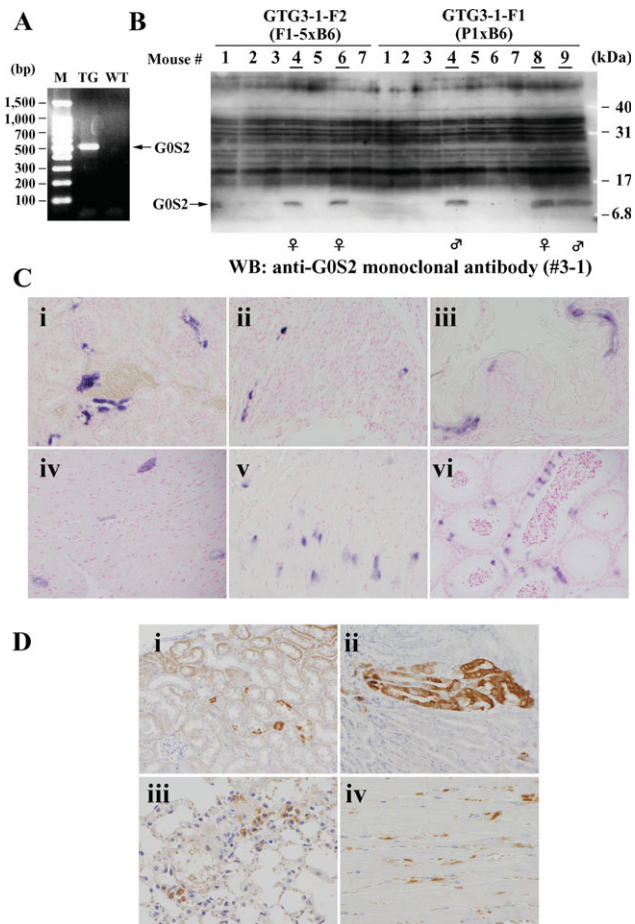


Figure 6. Establishment of *GOS2* transgenic mice. **(A)** An example of the RT-PCR analyses used to identify *GOS2* transgenic mice. **(B)** Western blot analysis to identify *GOS2* transgenic mice. The panel for ATG3-F1 and -F2 mice is shown as an example. The transgenic mouse numbers are underlined. **(C)** ISH analyses of *GOS2*-TG mice. Sections from the kidney (i), stomach (ii, mucosa; v, smooth muscle), esophagus (iii), heart (iv), and epididymis (vi) of adult *GOS2*-TG mice (GTG1b is shown as an example) were hybridized with the human *GOS2* antisense probe and colored blue by using the alkaline phosphatase reaction. The nuclei were counterstained with nuclear fast red. Original magnification: $\times 200$. **(D)** Immunological detection of exogenous *GOS2* proteins in various organs of *GOS2*-TG mice (GTG1b is shown as an example). Sections from the kidney (i), stomach (ii), lung (iii), and heart (iv) of the adult *GOS2*-TG mice were incubated with the mouse monoclonal anti-*GOS2* antibody (#3-1) and stained with diaminobenzidine. The nuclei were counterstained with hematoxylin. Original magnification: **A** and **B**, $\times 200$; **C** and **D**, $\times 400$.

weakly positive for *GOS2*, other epithelia expressed this protein at high levels; the latter epithelia also existed in clumps (Fig. 6Di). A similar patchy distribution of strong *GOS2* signals was detected in the foveolar epithelia of the stomach (Fig. 6Dii). In the lung and heart, there was a scattered distribution of exogenous *GOS2* signals in the alveolar epithelium and a diffuse distribution in cardiac ventricular muscle, respectively (Fig. 6Diii and iv).

3.6. Phenotypes of *GOS2* transgenic mice

To explore the phenotypes of the *GOS2* transgenic mouse, we first subjected various organs of *GOS2* transgenic mice and wild-type (C57BL/6) mice to histological examination. Obvious histopathological findings that related directly to vasculitis were not observed. Notably, microabscess-like panniculitis lesions were detected in the dermis and subcutaneous fat tissue (Fig. 7A). Of the eight TG mice examined, such lesions were detected in three. We also measured the serum levels of two autoimmunity-related antibodies, namely, anti-nuclear antibody (ANA) and anti-dsDNA antibody. We found both were increased in *GOS2* TG mice: the OD490 for ANA in the transgenic mice was 0.359 ± 0.170 [compared with 0.219 ± 0.044 for wild-type (B6) mice], whereas the OD490 for anti-dsDNA antibody was 0.294 ± 0.090 (compared with 0.188 ± 0.038 in wild-type mice) (Fig. 7B). However, these differences did not reach statistical significance,

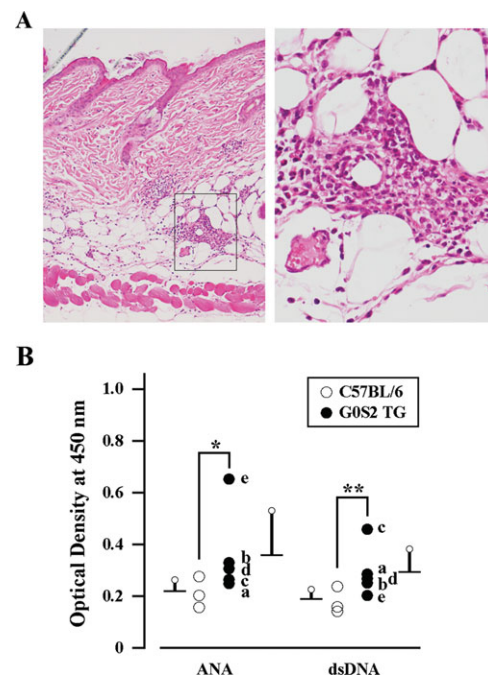


Figure 7. Histological and serological phenotypes of *GOS2*-TG mice. **(A)** Histological phenotype of *GOS2*-TG mice (GTG1b is shown as an example). Sections of the back skin of adult *GOS2*-TG mice were stained with hematoxylin and eosin. A boxed area in the left panel is enlarged in the right to show microabscess lesions containing numerous neutrophils. Original magnification: left, $\times 100$; right, $\times 400$. **(B)** Serological phenotypes of *GOS2*-TG mice (GTG1a is shown as an example). The sera from adult C57BL/6 (open circle) or *GOS2*-TG (closed circle) mice were examined for the levels of ANA and anti-dsDNA antibody. Asterisk and double asterisks indicate 'not significant' because $P = 0.1011$ and $P = 0.0526$, respectively, as determined by the Mann-Whitney *U*-test of differences between *GOS2*-TG and wild-type mice. The *GOS2* TG mice examined are designated as follows: a (GTG1a), b (GTG1b), c (GTG1b-F1a), d (GTG1b-F2a), and e (GTG1b-F1c). The values of individual mice are plotted.

in part, because of the limited number of *GOS2* TG mice we could use for these measurements. Taken together, it appears that *GOS2* may participate in the pathogenesis of autoimmune diseases in general, and in particular, it may be related to inflammation.

4. Discussion

In the present study, we report candidate gene markers for vasculitis that may be useful for diagnosing and understanding the basic pathogenesis of vasculitis and other autoimmune diseases such as SLE. These genes were identified by stepwise subtraction and cDNA microarray techniques, which permitted us to comprehensively isolate the genes that show augmented mRNA expression in the PBMCs of vasculitis patients when compared with normal volunteers. The vasculitis-specific upregulation of these genes was validated with northern blot and/or RT-PCR analyses (Figs 3 and 4, Tables 1 and 2). We then showed by RT-PCR analysis that *EGR1*, *GOS2*, *HBD*, *TVAS10*, *IL1R2*, *Amphiregulin*, and *calgranulin C* are frequently upregulated in the PBMCs of vasculitis patients (Fig. 1 and Supplementary Fig. S1). MTC analysis revealed that in normal PBMCs, these genes are expressed primarily by monocytes and/or activated CD4+ cells (Fig. 5). We also showed previously using different primers and PCR conditions that *GOS2* is predominantly expressed by monocytes.⁵ These results suggest that monocytes, including macrophages, express these vasculitis-upregulated genes and thus may play a role in the pathogenesis of vasculitis.

We noticed that several putative vasculitis-upregulated genes also show upregulated expression in

other autoimmune diseases, namely, SLE and RA (Table 3). Of these, *EGR1* is particularly interesting as the degree to which it is upregulated in most vasculitis patients is remarkable (Fig. 1A). *EGR1* is induced by a variety of cellular stresses, including hypoxia, and may function as a master switch that triggers the expression of numerous key inflammatory mediators. In particular, *EGR1* induces a number of gene products that have been linked to cellular perturbation, especially in the vasculature. Moreover, many of these genes are often induced in the PBMCs of patients afflicted with a variety of autoimmune diseases. For example, we showed previously that *EGR1* is markedly upregulated in the PBMCs of SLE patients.⁴ It also plays a critical role in promoting cellular differentiation along a macrophage lineage.¹⁰ Nonetheless, *EGR1*-null mice are viable and develop and grow normally.¹⁹ Rigorous analyses will be needed to determine whether the upregulation of *EGR1* mRNA in many vasculitis patients is pathogenically significant or merely an associated phenomenon.

GOS2 also displayed enhanced expression in many vasculitis patients (Fig. 1B). *GOS2* was first identified as one of the G0/G1 switch (*GOS*) genes that are differentially expressed in lymphocytes during their lectin-induced switch from the G0 to the G1 phases of the cell cycle.²⁰ *GOS2* is one of the genes that is upregulated during normal implantation but its expression is significantly lower in women with endometriosis that is associated with pelvic pain and infertility with implantation failure.²¹ In a replicative senescence model employing human dermal fibroblasts (HDF), *GOS2* expression was upregulated in old HDF cells, which suggests that it participates in senescence.²² Microarray and qRT-PCR analyses of

Table 3. List of the *TAC/TAS* genes that also show upregulated expression in PBMCs from SLE and RA patients

Name	Vasculitis	SLE	RA	Sequence description
<i>TAC-1</i>	42.0	44.7	nc	Early growth response 1 (<i>EGR1</i>)
<i>TAC-2</i>	41.7	24.1	nc	Unknown (FLJ21545) = <i>TVAS8</i>
<i>TAC-3</i>	29.7	23.2	2.4	Interleukin 8 (<i>IL8</i>)
<i>TAC-9</i>	12.3	12.3	27.4	Amphiregulin
<i>TAC-13</i>	9.7	11.3	1.8	CD69 antigen (early T-cell activation antigen)
<i>TAC-14</i>	9.4	7.2	3.9	Tyrosylprotein sulfotransferase 1 (<i>TPST1</i>)
<i>TAC-19</i>	9.1	5.3	9.4	Interleukin 1 receptor, type II (<i>IL1R2</i>)
<i>TAC-20</i>	8.8	18.0	1.4	Similar to interferon alpha-inducible protein 27
<i>TAC-34</i>	6.9	11.5	2.4	Sin3-associated polypeptide, 30kDa (<i>SAP30</i>)
<i>TAC-37</i>	6.8	3.9	2.4	Chemokine (C-X-C motif) ligand 1 (<i>CXCL1</i>)
<i>TAC-40</i>	6.7	14.8	4.0	G0/G1 switch gene 2 (<i>GOS2</i>)
<i>TAC-47</i>	6.4	20.7	nc	Viperin (<i>cig5</i>)
<i>TAC-51</i>	6.2	10.7	4.4	Nuclear factor of kappa B (<i>NFκB</i>)
<i>TAC-78</i>	5.5	11.4	1.7	CD83 antigen (immunoglobulin superfamily)

The numbers represent fold change compared with normal volunteers; nc, no conspicuous change between RA and control.

PBMCs from psoriasis patients suffering from severe generalized disease also revealed the upregulation of *GOS2*.¹³ Moreover, we showed previously that *GOS2* mRNA levels are markedly increased in the PBMCs from patients with the autoimmune diseases SLE⁴ and RA.⁵ *GOS2* is a putative target gene of peroxisome-proliferator-activated receptor (PPAR) alpha, which belongs to a group of transcription factors that are involved in numerous processes, including lipid metabolism and adipogenesis. Moreover, it was shown that *GOS2* is upregulated after PPAR δ activation and that it may be involved in the PPAR δ -mediated mitigation of cardiac fibroblast proliferation.²³

We here generated *GOS2*-TG mice that ubiquitously overexpress *GOS2* proteins (Fig. 6). We showed that these mice are unhealthy as they do not produce many offspring (Fig. 2) and frequently display formations of microabscess-like panniculitis lesions in the dermis and subcutaneous fat tissue (Fig. 7A). The development of these microabscess-like panniculitis lesions may reflect the dysregulation of the murine immune system in these mice. Supporting this is that some of the *GOS2*-TG mice had augmented levels of immunodeficiency-related anti-nuclear and anti-dsDNA antibodies in their sera (Fig. 7B), although these phenotypes are related not to vasculitis but to SLE. It should be noted, however, that these serum differences did not achieve statistical significance, largely because of the shortage of *GOS2*-TG mice. It will be necessary to study larger numbers of *GOS2*-TG mice to confirm this association between *GOS2* overexpression and the development of autoimmune features. Should this association be confirmed, the high-quality polyclonal and monoclonal anti-*GOS2* antibodies that we produced may be useful as tools (perhaps in enzyme-linked EIA) for diagnosing not just vasculitis but also other autoimmune diseases.

We performed this study on a highly heterogeneous cohort of patients, including those with systemic vasculitis. All of these diseases share the inflammatory involvement of blood vessels. However, their target organs, pathogenic mechanisms, clinical findings, and outcomes are quite different. Moreover, although the disease activity status in these patients was heterogeneous and some were receiving corticosteroid or immunosuppressive therapy, these factors were ignored in our patient recruitment protocol. Nonetheless, despite these marked heterogeneous features of our patient population, we could isolate putative common genes that are upregulated, which was our original goal. We are currently performing in parallel the expression profiling of the genes that are up- or downregulated in each individual disease and analyzing these profiles in terms of the symptoms, disease activity status, and clinical treatment of the patients. However, the results obtained from

such studies are highly complex, which has hampered the identification of candidate gene markers that can be used for diagnosis.

Taken together, we conclude that the putative gene markers we identified here, in particular *EGR1* and *GOS2*, may be useful for diagnosing not only vasculitis but also other autoimmune diseases, perhaps by qRT-PCR and antibody-based methods. Other, as yet uncharacterized, genes that we detected as being upregulated in vasculitis may also be useful candidate gene markers and thus are worth further detailed analysis. In particular, the identification of the functions of these genes may reveal the hitherto unknown mechanism(s) that underlie the pathogenesis of vasculitis. This in turn may shed light on therapeutic avenues for treating vasculitis.

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Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

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