Commensal Microbiota Contributes to Chronic Endocarditis in \textit{TAX1BP1} Deficient Mice

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Abstract

Tax1-binding protein 1 (Tax1bp1) negatively regulates NF-κB by editing the ubiquitylation of target molecules by its catalytic partner A20. Genetically engineered \textit{TAX1BP1}\textsuperscript{-}deficient (KO) mice develop age-dependent inflammatory constitutions in multiple organs manifested as valvulitis or dermatitis and succumb to premature death. Laser capture dissection and gene expression microarray analysis on the mitral valves of \textit{TAX1BP1-KO} mice (8 and 16 week old) revealed 588 gene transcription alterations from the wild type. SAA3 (serum amyloid A3), \textit{CH13L1}, \textit{HP}, \textit{IL1B} and \textit{SPP1/OPN} were induced 1,180-, 361-, 187-, 122- and 101-fold respectively. \textit{WIF1} (Wnt inhibitory factor 1) exhibited 11-fold reduction. Intense Saa3 staining and significant iκBα reduction were reconfirmed and massive infiltration of inflammatory lymphocytes and edema formation were seen in the area. Antibiotics-induced ‘germ free’ status or the additional \textit{TAX1BP1}\textsuperscript{-}deficient (KO) mice’s inflammatory lesions. These pathological conditions, as we named ‘pseudo-inf ective endocarditis’ were boosted by the commensal microbiota who are usually harmless by their nature. This experimental outcome raises a novel mechanistic linkage between endothelial inflammation caused by the ubiquitin remodeling immune regulators and fatal cardiac dysfunction.

Introduction

The transcription factor NF-κB is essential for the regulation of the innate and adaptive immune responses. NF-κB is activated in response to a wide variety of stimuli, such as inflammation, DNA damage, or nociception \cite{1,2}, and is involved in embryogenesis and multiple tissue development \cite{3}. The NF-κB family comprises five proteins including RelA (p65), RelB, c-Rel, NF-κB1, and NF-κB2, and their transcriptional activities are tightly controlled to ensure their transient signaling in response to specific stimuli. The NF-κB signaling cascade is usually triggered by sensor molecules, such as toll-like receptor (TLR) family proteins. These proteins can identify the presence of a wide range of microorganisms and then transmit that information through phosphorylation relays to downstream kinases, which eventually culminate at the I-κB kinase (IKK). IKK activates NF-κB via phosphorylation of inhibitory I-κB proteins (primarily I-κBα), which leads to its ubiquitylation and degradation by the 26S proteasome complex and allows NF-κB to enter the nucleus. I-κB is induced by NF-κB to function in a negative feedback loop that terminates NF-κB signaling. Aberrant activation of NF-κB has been linked to several pathological features such as allergic responses, autoimmune diseases, septic shock, and carcinogenesis in a variety of organs \cite{4}.

In addition to I-κB, deubiquitinase A20 (also referred to as TNFα-induced protein 3 or TNFAIP3) targets important signaling intermediates upstream of I-κB to terminate NF-κB activation \cite{5,6}. A20 cleaves Lys63 (K63)-linked polyubiquitin chains on downstream kinases, which eventually culminate at the I-κB kinase (IKK). IKK activates NF-κB via phosphorylation of inhibitory I-κB proteins (primarily I-κBα), which leads to its ubiquitylation and degradation by the 26S proteasome complex and allows NF-κB to enter the nucleus. I-κB is induced by NF-κB to function in a negative feedback loop that terminates NF-κB signaling. Aberrant activation of NF-κB has been linked to several pathological features such as allergic responses, autoimmune diseases, septic shock, and carcinogenesis in a variety of organs \cite{4}.

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overlapping substrates, such as E3 ubiquitin ligase TRAF6 and adaptor molecule RIP1, with the help of the substrate-specific adaptor Tax1-binding protein 1 (TAX1BP1) [7,8]. TAX1BP1 intrinsically regulates NF-κB by recruiting A20 to the target molecules to remove their polyubiquitin chains, which play important roles in their assembly into the IKK complex [9,9]. Deficiencies in A20 or TAX1BP1 lead to uncontrolled and spontaneous systemic inflammation in mice as a result of unchecked NF-κB signaling [8,10].

TAX1BP1 was originally identified as a host cell factor that binds to the encoded protein of human T-lymphotropic virus type 1 (HTLV-1), known as Tax1 [7]. Tax1 is a potent activator of NF-κB and a major pathogenic factor in HTLV-1 associated diseases (HADs), such as HTLV-1 associated myelopathy (HAM) or HTLV-1 uveitis (HU [11]), and adult T-cell leukemia (ATL [12]). Tax1 interrupts the ability of Tax1bp1 to connect to and recruit A20 to target molecules and thus evokes persistent NF-κB activation [13,14]. Tax1 also activates NF-κB by binding to the NF-κB essential modulator (NEMO), a regulatory subunit of IKK [15]. The aberrant activation of NF-κB in HADs can therefore be attributed to Tax1, which leads to Tax1bp1 dysfunction, over-activation of IKK, or both. Epidemiological studies provide support for a close link between HTLV-1 infection and HAD or other inflammatory diseases such as Sjögren’s syndrome [16], vascular dementia [17], and atherosclerosis [18]. Moreover, recent accumulating evidence strongly suggests that several mutations in the A20 locus are primarily responsible for the development of Crohn’s disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes [19].

For research purposes, we established TAX1BP1-deficient (-KO) mice, which display exacerbated of inflammation (characterized as valvulitis and dermatitis) in an age-dependent manner in addition to functional inadequacies manifested in growth retardation and premature death [8]. To elucidate the molecular mechanisms underlying the manifestation of inflammatory symptoms and their link to premature or possible cardiac abnormalities induced by TAX1BP1-deficiency, we performed a series of pathological evaluations using TAX1BP1-KO mice: (1) laser capture microdissection (LCM) and gene expression microarray-based profiling of the mitral valves, which was reevaluated using real-time polymerase chain reaction (RT-PCR); (2) multiplex cytokine and chemokine quantitation in sera on systemic inflammatory constitution; (3) histochemical and electron microscopic analyses of multiple pathogenic foci; and (4) antibiotic treatments and cross experimentation with MyD88-deficient mice [20] to examine the role of commensal microbiota in the pathogenesis of TAX1BP1-KO mice.

From our experimental data, we conclude that systemic inflammation and cardiac structural abnormalities in TAX1BP1-KO mice originated from commensal microbiota, which are usually harmless in nature. Furthermore, these results indicate a potential risk to asymptomatic HTLV-1 carriers, which should be addressed by further clinical research.

Materials and Methods

Animals

TAX1BP1-KO mice having replaced their exon 17 region with CMV-driven NEOF gene in reverse orientation [8] and their wild-type (WT) littermates as controls were analyzed throughout the experiment. These strains are maintained as F9 or advanced generations of C57BL/6CrSlc or the original 129/+ Ter/SvJcl. MyD88 deficient mice are kind gifts from professor Hitoshi Nakashima from Fukuoka University [21]. Homozygous TAX1BP1-KO mice were crossbred with homozygous MyD88KO background to generate MyD88/TAX1BP1-KO mutants. Each of the targeted loci was evaluated by PCR. These mice were bred and maintained under specific pathogen-free (SPF) conditions at the animal facility of Oita University Faculty of Medicine. All the mice related manipulations were performed with protocols approved by the animal ethics committee at the Oita University (Justified numbers, daily care, treatment and euthanasia procedures).

Laser capture microdissection

Three mitral valves from 8 or 16 week old (-wk) male TAX1BP1-KO and their WT littermates were collected by Arcturus XT laser capture microdissection system according to a manufacture’s directions.

RNA isolation and gene expression microarray analysis

Total RNAs were purified from the mitral valves using RNaseasy mini kit (Qiagen). RNA quantity and purity were evaluated using a NanoDrop 2000 (NanoDrop Technologies). All RNA samples were labeled, linearly amplified by Low Input Quick Amp Labeling Kit and RNA Spike-In Kit then analyzed with Whole Mouse Genome Microarray Kit (Agilent). Signal intensities were quantitated with laser confocal scanner and analyzed with Feature Extraction software (Version 10.7.3.1, Agilent) and R statistical package (Version 2.13.1). Probe set data were median-normalized per chip. Empirical Bayesian method controlling for false discovery rate (FDR: <3% and logFC >1.0 [22]) for comparison of differentially expressed between TAX1BP1-KO mice and their WT. Principal Component Analysis (PCA) for the systematic trend examination, heatmaps by R Software and volcano plot analysis were applied to identify the single mRNA differentially expressed in TAX1BP1-KO mice (log2-fold expression change on the x-axis and t test p values on the y-axis, negative log). Each dot represents a single probe. The complete gene expression dataset can be viewed in the Gene Expression Omnibus (GEO) repository accession number GSE43932 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43932).

Quantitative real-time-polymerase chain reaction (RT-PCR)

Taqman quantitative RT-PCR was performed to validate a subset of genes. Random hexamer-primed cDNA templates were

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**Table 1.** Primer sequences.

| SAA3   | acagcccttctgggaatctgctg | atgctcgggggaactatcag #26 |
|--------|--------------------------|----------------------------|
| TAX1BP1| ataaaagttgaagacacccgac  | cactcagaaatgggttg  #56    |
| EFCAB2 | tgtgcctgtgctgttagctg   | cctgtccttcaccttctg  #80   |
| GAPDH  | tcgacatcgtaaactaacta  | tgcagcctctctcactgctg #89  |

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**Table 2.**

| IL6       | gctaccaaactggataaatcagga | ccaggtgcagctgtagtcaga #6 |
|-----------|---------------------------|--------------------------|
| CXCL1     | agacctcgccccacctccattc  | tgcacgctcgctcttgctg #83 |
| GAPDH     | tgcagctttctctcactgctg #89 |

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The output of RT-PCR reactions were quantitated with LightCycler 480 System (Roche). Primer sequences were listed in Table 1. Each reaction was run in triplicate with endogenous control GAPDH on the same reaction plate.

Multiple cytokine & chemokine quantification

The 3-, 8-, 16- and 32-wk male TAX1BP1-KO and their WT littermates were anesthetized and an aliquot of serum (12.5 μl) from heart blood were collected (n = 5/groups). Quantitation of 23 cytokines and chemokines was performed by a multiplex ELISA system (Bio-Plex, BioRad) and analyzed by the Bio-Plex Manager Software 6.1 (Bio-Rad) with a five-parameter curve-fitting algorithm for standard curve calculations.

Immunohistochemistry

A standard avidin-biotin-peroxidase technique or hematoxylin and eosin (HE) staining were employed for Saa3 and IκBα staining or morphological observation of heart, liver and skin tissues of 8- or 16-wk male TAX1BP1-KO and their WT littermates (n = 5/group). Rabbit polyclonal anti-Saa3 antibody (ab59736, abcam), rabbit monoclonal anti-IκBα antibody (ab32518, abcam) or control antibody for visualization of antigens with EnVision + System-HRP Labelled Polymer Anti-Rabbit (Dako). DAB + Liquid (Dako) for positive staining and Mayer’s hematoxylin solution for counterstaining. Images were captured with BZ-9000 (KEYENCE). Mice whole eye sections were examined with anti-T6BP antibody (ab22049, abcam). Anti-IgG (H+L), rabbit, goat-poly, DyLight 649 (KPL) was used as secondary antibodies.

Electron microscopy

For transmission electron microscopy (TEM), mitral valve, atrioventricular node, sinoatrial node and papillary muscles of the left ventricle of 8-, 16-, 60-wk male TAX1BP1-KO and their WT littermates (n = 3/groups) were fixed with 2.5% glutaraldehyde/2% paraformaldehyde in a 0.1 M cacodylate buffer (pH7.4) for 3 hr or longer at 4°C. After a washing in the cacodylate buffer, specimens were postfixed in 2% osmium tetroxide in cacodylate buffer for 2 hr, washed with cacodylate buffer, dehydrated with ethanol and embedded in epoxy resin. Thin section specimens (80–90 nm) were then stained with uranyl acetate and lead citrate and examined with TEM H-7650 (at 80 kV, HITACHI).

Western blotting

Tissues from liver, heart, spleen, muscle, lung, skin, stomach and brain from WT BL6 were lysed with Co-IP buffer [23] and equal amounts of protein solutions (20 μg/lane) were separated by
Table 3. Gene symbol, gene description, fold change and p-value for all genes up-regulated by >20-fold in TAX1BP1-KO mice.

| SYMBOL | DESCRIPTION | Fold activation | adj.P. Val |
|--------|-------------|----------------|-----------|
| SAA3  | Serum amyloid A 3 | 1179.5 | 0.006 |
| CHI3L1 | Chitinase 3-like 1 | 361.0 | 0.006 |
| HP     | Haptoglobin     | 187.2 | 0.007 |
| IL18   | Interleukin 1 beta | 121.9 | 0.007 |
| SPP1/OPN | Secreted phosphoprotein 1/Osteopontin | 100.7 | 0.006 |
| CCL2/MCP1 | Chemokine (C-C motif ligand 2/Monocyte chemotactic protein-1 | 81.7 | 0.021 |
| CLEC7A/DECTIN1 | C-type lectin domain family 7, member a/Decin-1 | 81.0 | 0.005 |
| SERPINA3G | Serine (or cysteine) peptidase inhibitor, clade A, member 3G | 73.0 | 0.006 |
| LCN2   | Lipocalin 2     | 65.3  | 0.024 |
| SAA1   | Serum amyloid A 1 | 61.1  | 0.024 |
| CXCL13/BLC | Chemokine (C-X-C motif ligand 13/8 lymphocyte chemo-attractant | 52.9 | 0.0001 |
| SLPI   | Secretory leukocyte peptidase inhibitor | 39.8 | 0.009 |
| CLEC4D/DECTIN2 | C-type lectin domain family 4, member d | 39.6 | 0.006 |
| TIMP1  | Tissue inhibitor of metalloproteinase 1 | 37.4 | 0.024 |
| CCL17/TARC | Chemokine (C-C motif ligand 17/Thymus and activation regulated chemokine | 35.2 | 0.020 |
| CCL7   | Chemokine (C-C motif ligand 7 | 33.8 | 0.025 |
| LGA53/GALECTIN3 | Lectin, galactose binding, soluble 3/Galectin-3 | 33.5 | 0.008 |
| SIRPB1A | Signal-regulatory protein beta 1A | 33.3 | 0.006 |
| CHL1   | Cell adhesion molecule with homology to L1CAM | 32.4 | 0.027 |
| CCL8   | Chemokine (C-C motif ligand 8 | 31.4 | 0.006 |
| BCL2A1B | B-cell leukemia/lymphoma 2 related protein A1b | 27.0 | 0.006 |
| MEFV   | Mediterranean fever | 26.7 | 0.006 |
| PLCB8  | Placenta-specific 8 | 21.7 | 0.008 |
| ZMYND15 | Zinc finger, MYND-type containing 15 | 20.6 | 0.007 |
| ITGA5  | Integrin alpha X | 20.0 | 0.006 |

Statistical significance (p<0.03) was calculated using the Empirical Bayesian method controlling for false discovery rate (FDR) <3% and logFC >1.0 on R statistical package (Version 2.15.1). Fold change represents a comparison between mean normalized signal intensity for control (n = 6) versus TAX1BP1-KO mice (n = 6). doi:10.1371/journal.pone.0073205.t003

SDS-PAGE and transferred to immobilon membranes (Millipore) and incubated with primary antibodies, T6BP Antibody (sc-15274, Santa Cruz) or anti-Tubulin antibody (ab6160, abcam) and secondary antibodies, donkey anti-goat IgG-HRP (sc-2033, Santa Cruz) or ZyMAX™ Goat anti-Rat IgG(H+L) HRP conjugate (81–9520, invitrogen) and visualized with ECL Western Blotting Detection System (GE Healthcare Lifesciences) and high-performance chemiluminescence film.

Evaluation of physiological responses to LPS-stimulation

200 μg of Salmonella typhimurium lipopolysaccharide (LPS, Sigma) in 100 μl sterile pyrogen-free saline were injected into the footpads of TAX1BP1-KO or WT littermates (n = 4/groups). Tissue lysates were prepared from eyeball and the expression of Tax1bp1, IκBα (anti-IκBα rabbit mAb, #4812, Cell Signaling Technology) and Tubulin were evaluated by western blotting. Total RNAs were prepared from eyeballs of TAX1BP1-KO or WT littermates (n = 4/groups). Taqman quantitative RT-PCR was performed as described above (See Table 2).

Sera from peripheral blood samples were collected 0, 6 and 12 hr after LPS injection and quantitated with Bio-Plex Pro™ Mouse Cytokine 23-plex kit.

Enzyme-linked immunosorbant assay (ELISA)

The amounts of Saa3 and Cxcl13 Sera from 16-wk mice (n = 5/group) were measured with MOUSE SAA-3 ELISA KIT (Millipore) and Mouse CXCL13/BLC/BGA-1 Quantikine ELISA Kit (R&D Systems).

Endocarditis in TAX1BP1 Deficient Mice
Table 4. Gene symbol, gene description, fold change and p-value for all genes down-regulated by >5 fold in TAX1BP1-KO mice.

| SYMBOL    | DESCRIPTION                                                                 | Fold suppression | adj.P. Val  |
|------------|------------------------------------------------------------------------------|------------------|-------------|
| TAX1BP1    | Tax1 (human T-cell leukemia virus type 1) binding protein 1                    | 56.7             | 0.0000001   |
| WIFI       | Wnt inhibitory factor 1                                                      | 11.1             | 0.0205      |
| UCMA       | Upper zone of growth plate and cartilage matrix associated                   | 9.1              | 0.0173      |
| EFCAB2     | EF-hand calcium binding domain 2                                              | 8.0              | 0.0001      |
| FAM107A/DRR1| Family with sequence similarity 107, member A/down-regulated in renal cell carcinoma 1 | 7.6              | 0.0219      |
| TSC22D3    | TSC22 domain family, member 3                                                | 7.3              | 0.0197      |
| TAX1BP1    | Tax1 (human T-cell leukemia virus type 1) binding protein 1                    | 7.2              | 0.0004      |
| MAP3K6/ASK2| Mitogen-activated protein kinase kinase kinase 6                               | 7.1              | 0.0212      |
| 6030422H21RIK | RIKEN cDNA 6030422H21 gene                                                  | 6.8              | 0.0124      |
| TSC22D3    | TSC22 domain family, member 3                                                | 5.9              | 0.0240      |
| PENK       | Preproenkephalin                                                             | 5.7              | 0.0119      |
| CNTFR      | Ciliary neurotrophic factor receptor                                          | 5.3              | 0.0104      |
| COL11A2    | Collagen, type XI, alpha 2                                                    | 5.3              | 0.0069      |
| RXFP3      | Relaxin family peptide receptor 3                                             | 5.2              | 0.0197      |
| NRXN1      | Neurexin I                                                                    | 5.1              | 0.0110      |
| CYTL1      | Cytokine-like 1                                                               | 5.0              | 0.0099      |

Statistical significance (p<0.03) was calculated using the Empirical Bayesian method controlling for false discovery rate (FDR) <3% and logFC >1.0 on R statistical package (Version 2.15.1). Fold change represents a comparison between mean normalized signal intensity for control (n = 6) versus TAX1BP1-KO mice (n = 6).

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Figure 2. Validation of genes and proteins identified their expression alteration in the mitral valves of TAX1BP1-KO mice. RT-PCR validation of genes identified their expression alteration in the mitral valves of TAX1BP1-KO mice, A) SAA3 B) EFCAB2 respectively. Gray bar: TAX1BP1-KO, black bar: WT. Mitral valve specimens were prepared from 16-wk TAX1BP1-KO mice or their WT littermates and stained by anti-Saa3 antibody (C and D) or anti-I-κBa antibody respectively (E and F).

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Telemetric electrocardiogram (ECG)

Sixteen week old male $TAX1BP1$-KO or WT littermates with or without antibiotic treatment ($n = 5$/group) were monitored with telemetric electrocardiogram. Telemetric transmitter was implanted into the back of mice under aseptic conditions and the muscle layers and the skin were closed with resorbable sutures. Data were acquired at least 72 hour after the implantation with a receiver placed under the cage and a full-disclosure 72 hour recordings were analyzed off-line and the P-Q intervals were evaluated.

Antibiotic treatment

$TAX1BP1$-KO or WT littermate male mice were first raised with the normal diets and water for 4 weeks, and then, antibiotic group ($n = 5$/groups) received ampicillin (1 g/L; Wako), vancomycin hydrochloride (500 mg/L; Wako), neomycin trisulfate salt hydrate (1 g/L; Sigma-Aldrich), and metronidazole (1 g/L; Wako) in drinking water for 12 weeks [24]. The non-antibiotic controls were equally raised and maintained except for antibiotics treatment. Both groups of mice were maintained in flexible film isolators under a strict 12-hour light cycle and fed an autoclaved chow diet and tap water ad libitum. Germ free status was verified regularly by ensuring negative cultures from mouse feces in three media types: nutrient agar (Nissui), pourmedia sheep blood agar M70 (Eiken), and Sabouraud agar (Nissui). Microbial colonies were counted after incubation at 37°C for 48 hour (aerobes) or 72 hour (anaerobes). Both groups of mice were anesthetized and sacrificed at the end of 16 weeks experimental period. Daily fluid consumption, body weight, liver function (ALT, AST), renal...

**Figure 3.** Inflammatory properties in the multiple organs of $TAX1BP1$-KO mice. The morphologic and functional alterations of the environments of liver (A and B) and skin (C and D) were also examined with HE-staining. Red and white triangles indicate accumulated lymphocytes and Councilman bodies respectively. doi:10.1371/journal.pone.0073205.g003

**Figure 4.** Massive infiltration of inflammatory cells causes severe tissue lesion in the mitral valves of $TAX1BP1$-KO mice. Electron microscopy examinations on the mitral valves of 8-, 16- and 60-wk $TAX1BP1$-KO mice (A: 8wkKO, and C: 60wkKO) and their WT littermates (B: 8wWT and D: 60wWT). See Figure S1 for details. Each panel was duplicated with colorized areas in specific cell types and abbreviated descriptions (Fig. 4A’ to 4D’). Abbreviations, CL: Collagen layer; EC: Endothelial cell; ED: Edema; FB: Fibroblast; FC: Fibrocyte; GD: Granule deposition; MΦ: Macrophage; NP: Neutrophil; PC: Plasma cell; TC: T cell. doi:10.1371/journal.pone.0073205.g004
function (BUN), nutritional status (TG, GLU, TP) and spleen weight (After 10% formalin fixation) were examined. Caecum surface area was measured with Image J (NIH). In general, there were no particular adverse effects on mice through antibiotic treatment.

Statistical analysis
All numerical data are expressed as means ± SD. Statistical significance was assessed by Student’s two-tailed t-test. In the case of ELISA, Statistical analyses were performed by one-way analysis of variance and Steel-Dwass test. Data were considered significant when P < 0.05.

Results
LCM- and gene expression microarray array-based profiling of the mitral valves in TAX1BP1-KO mice and reevaluation by RT-PCR and immunostaining
We have previously observed that the mRNA expression level for several inflammatory cytokines, including IL-1β and TNFα, increases in the cardiac and skin tissues of TAX1BP1-KO mice; more importantly, these mice showed mitral valvulitis and premature death compared to their wild-type (WT) littermates.

However, the underlying mechanisms involved in these processes remain unknown [8]. To date, information on variations in the levels of gene expression in regions of the heart (more specifically, the mitral valves) showing inflammation in TAX1BP1-KO mice is still lacking. This pathologic event is thought to be linked to premature death, which might be brought on by cardiac failure. In the current study, we employed LCM- and gene expression microarray-based techniques to obtain detailed information on the levels of gene expression in organs showing pathological changes. Total RNA was extracted from three independent tissue samples obtained from the mitral valves of 8- or 16-week-old male (WT and TAX1BP1-KO) mice by using LCM, which was followed by total RNA extraction. Then, global mRNA expression profiles were identified using unsupervised hierarchical clustering.

Figure 5. Enhanced expression of inflammatory genes after the LPS-stimulation to TAX1BP1-KO mice. 200 µg of Salmonella typhimurium lipopolysaccharide (LPS) in 100 µl sterile pyrogen-free saline were injected into a right footpads of TAX1BP1-KO or WT littermate mice. At the time 2, 6, 12, 24 and 48 hour post-injection (PT), each group of mouse were euthanized and tissues including serum, lymphocytes and eyes were collected. A) LPS-triggered induction of Tax1bp1 in eye tissue was monitored. Ten µg of cell lysates from WT BL6 mice at 2, 6, 12, 24 and 48 hour PT of LPS were probed with anti-Tax1bp1, -IκBα and -Tubulin antibodies. B, C) Total RNAs of eye tissues from at 6, 12 and 48 hour PT of LPS to TAX1BP1-KO or WT littermates and their untreated controls were prepared and the expressions of IL-6 and CXCL1 were quantitated with RT-PCR. D, E) Sera from at 6, 12 and 48 hour PT of LPS to TAX1BP1-KO or WT littermates and their untreated controls were collected and the amount of IL-6 and Cxcl1 were quantitated with multiplex ELISA system (BioRad). Gray bar: TAX1BP1-KO, black bar: WT littermate.

The data showed that the results for all samples from TAX1BP1-KO mice clearly deviated from those for control mice, indicating detectable differences in the gene transcription patterns of the two genetic backgrounds. A gene list was compiled on the basis of normalization and statistical analysis (P < 0.03, logFC > 1.0). Using these criteria, alterations in 588 gene expression profiles were identified. Unsupervised hierarchical clustering...
analysis (Cluster 3.0; Stanford University) of the 588 genes resulted in the separation of all \( \text{TAX1BP1} \)-KO from their paired WT controls. In total, 428 probes were upregulated and 160 were downregulated for a total of 24,000 genes (Fig. 1B). We then applied volcano plot analysis to identify the differences in mitral valve mRNA expression in \( \text{TAX1BP1} \)-KO mice and the controls.

Figure 6. Amelioration of inflammatory valvulitis and conduction disturbance after the antibiotics treatment on \( \text{TAX1BP1} \)-KO mice. \( \text{TAX1BP1} \)-KO or WT littermate mice (male) were first raised with the normal diets and water for 4 weeks, and then, antibiotic treatment group (C, D, G and H, \( n = 5 \)/group) provided ampicillin (1 g/L; Wako), vancomycin Hydrochloride (500 mg/L; Wako), neomycin trisulfate salt hydrate (1 g/L; Sigma-Aldrich), and metronidazole (1 g/L; Wako) in drinking water for 12 weeks based on a protocol of the commensal depletion (Rakoff-Nahoum S., Cell 2004). The non-antibiotics controls (A, B, E and F, \( n = 5 \)/group) were equally raised and maintained except for antibiotics treatment. Each group of mice were anesthetized and sacrificed at the end of 16 weeks experimental period and histochemical representatives of each group were displayed with HE-staining (A to D) or anti-Saa3 immuno-staining (IS, E to H). I. Heart rhythms of 16-week-old \( \text{TAX1BP1} \)-KO treated with antibiotics over 12 weeks (male, \( n = 5 \)/group) were monitored with telemetric electrocardiogram (12-lead ECG). J) The average values of PQ-intervals were compared with those of untreated \( \text{TAX1BP1} \)-KO mice and their WT littermates. doi:10.1371/journal.pone.0073205.g006

Figure 7. Reduction of the Saa3 and Cxcl1 expression in the sera of \( \text{TAX1BP1} \)-KO mice after the antibiotics treatment. ELISA quantitation of Saa3 (A) or Cxcl13 (B) of the sera on four groups were performed. Gray bar: \( \text{TAX1BP1} \)-KO mice, black bar: WT littermates (\( n = 5 \)/group). doi:10.1371/journal.pone.0073205.g007
The plot showed a log2-fold change in mRNA expression between the two groups on the x-axis and the negative log of the t-test p-values on the y-axis. Each gene was represented by a single dot. Using the plot, we identified 588 probes that showed a more than 2-fold differential expression of mRNA when compared to the controls (p<0.03, Fig. 1C).

Tables 3 and 4 list the gene symbols, gene descriptions, fold changes, and p-value for all genes upregulated by more than 20-2-fold differential expression of mRNA when compared to the controls (p<0.03, Fig. 1C).

Figure 8. Splenic hypertrophy of *TAX1BP1*-KO mice and its cancellation by antibiotics treatment. Examinations on the spleen volume (A) and the area of cecum (B) were performed. The average values of spleen volumes (C) and cecum areas (D) were displayed (n=5/group). doi:10.1371/journal.pone.0073205.g008

Figure 9. Cancelation of valvulitis in the *MyD88/TAX1BP1* double-KO mice. The HE-staining (A, B) and immunostaining of Saa3 (C, D) and I-κBα (E, F) were compared between *TAX1BP1*-KO and *MyD88/TAX1BP1*-KO mice. ELISA quantification of Saa3 (I) and Cxcl13 on the sera of both genetic background. doi:10.1371/journal.pone.0073205.g009
fold or downregulated by more than 5-fold. Most of the upregulated genes were primarily involved in inflammation. The gene showing the highest level of induction, SAA3, (i.e., 1,180 fold induction) along with SAA1 (i.e., 61 fold, 10th induction) are well-known inflammatory markers in patients with autoimmune disease, chronic infection and cancer [25]. SAA3 is also hyper-induced at the site of injury [26], inflammation [27] in mice experimental models. Additionally, genes related to immune modulation, including pathogen recognition, inflammation, chemotaxis [28–30], or tissue adhesion, degeneration and rearrangement [31,32] were induced in the mitral valves of TAX1BP1-KO mice. The characteristics of the downregulated genes also suggested the link between inflammation and tissue degeneration (Table S1); for example, such as WIF1, a Wnt signaling suppressor; UCMA, a gene associated with cartilage development [33–35]. EFCAB2 is a functional partner of the voltage-gated Ca2+ channel [36]. TSC22D3 (also known as GILZ, a Glucocorticoid Induced Leucine Zipper) is an IL-10-inducible immune suppressor [37].

We further confirmed the microarray results for SAA3 and EFCAB2 by using RT-PCR (Fig. 2AB and Figure S1) and for Saa3 (induction) or I-kBα (reduction) by using immunostaining for mitral valve samples from 16-wk TAX1BP1-KO mice (Fig. 2C to F). In addition to these microenvironmental changes, broad-spectrum inflammatory effects, such as lymphocyte accumulation, apoptotic Councilman body formation, and Kupffer cell hyper-spectrum inflammatory effects, such as lymphocyte accumulation, were observed in 16-wk apopotic Councilman body formation, and Kupffer cell hyper-spectrum inflammatory effects, such as lymphocyte accumulation, F). In addition to these microenvironmental changes, broad-spectrum inflammatory effects, such as lymphocyte accumulation, apoptotic Councilman body formation, and Kupffer cell hyper-spectrum inflammatory effects, such as lymphocyte accumulation, were observed in 16-wk apopotic Councilman body formation, and Kupffer cell hyper-spectrum inflammatory effects, such as lymphocyte accumulation, F).

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Amelioration of the inflammatory symptoms and the cardiac conduction defect of TAX1BP1-KO mice by antibiotic treatment and simultaneous MyD88 deficiency

Microbial infections spontaneously cause severe endothelial inflammatory diseases such as rheumatic fever and Kawasaki disease [38]. At the subcellular level, modulation of the threshold of immune cell activation, differentiation, and immune cell activity in response to non-self or self antigens in TAX1BP1-KO mice (Fig. 1 and Tables 3 and 4) might evoke autoimmune profiles and heart dysfunction. To test this hypothesis, we examined the link between the commensal microbiota and mitral valvulitis and endocarditis in TAX1BP1-KO mice. When the mice were 4 weeks old, antibiotics were orally administered to all subjects over a 12-week period. The telemetric electrocardiogram profiles then sacrificed for the pathologic examination. Inflammatory hyper-trophy (Fig. 6A) and extensive Saa3 staining (Fig. 6E) of the mitral valves in TAX1BP1-KO mice were abolished with antibiotic treatments (Fig. 6C and G); no changes were observed in their WT littersates (Fig. 6B, D, F and H). Extended PQ-intervals observed by telemetric electrocardiogram in TAX1BP1-KO mice (Fig. 6I, middle panel) were alleviated with the administration of antibiotics (Fig. 6I, bottom panel). The statistical significance of the differences in the PQ-intervals was tested (Fig. 6J). The antibiotic regimen also reduced the secretion of Saa3 and Cxcl11 in the sera of TAX1BP1-KO mice (Fig. 7A, B), and splenic hypertrophy of TAX1BP1-KO mice was almost nonexistent (Fig. 8A). Typical cecum thickening due to antibiotic treatment was also confirmed (Fig. 8B), and fecal microbes were completely disappeared under these conditions (data not shown). If the eradication of microbiota is the main reason for the amelioration of the symptoms in TAX1BP1-KO mice, we hypothesized that the disruption of the innate immune cascade could bring about similar results. We crossed TAX1BP1-KO mice with MyD88-KO mice [20] and examined the morphological features or immunostaining profiles of marker proteins in the mitral valves of 16-week-old TAX1BP1-KO and MyD88/TAX1BP1-KO mice. MyD88/TAX1BP1-double knockout canceled hyperplasia (Fig. 9A, B), Saa3 induction (Fig. 9C, D) and I-kBα degradation (Fig. 9E, F). Comparisons of ELISA values for TAX1BP1-KO and MyD88/TAX1BP1-KO mice also indicated amelioration of the inflammatory response in MyD88/TAX1BP1-KO mice (Fig. 9G, H).

Discussion

Chronic infection with a retrovirus can have a significant impact on the host immune system. In the case of HTLV-1 infection, the pathological features of the disease are influenced by multiple factors. While HIV causes immune deficiency in the host, HTLV-1 causes a wide range of inflammatory symptoms (HAM and HU) and, in some cases, immunosuppressive ATL, a malignant growth of regulatory T-lymphocytes [39,40]. Furthermore, HAD patients frequently display impaired immune response such as an ineffective interferon response in HAM patients [41] and frequent development of dermatitis in ATL patients [42].

Multiple inflammatory symptoms, including cardiac valvulitis, dermatitis, and a hypersensitive response to endotoxins and inflammatory cytokines, were noted in our preclinical model involving TAX1BP1-KO mice. More importantly, TAX1BP1-KO mice died prematurely because of unknown mechanisms [8]. In this study, we discovered the hyper-induction of multiple inflammation-related genes including SAA3, CHE11, HP, IL1B, SPP1/OPN, and the significant reduction of TSC22D3/GILZ in the mitral valves and microenvironment deterioration in a progressive age-dependent manner for TAX1BP1-KO mice [43–47], the
significant reduction of EFCAB2 expression was highly implicated in functional defects of the heart [36].

HTLV-1-transgenic mice develop autoimmune immune response closely related to those observed for rheumatoid arthritis [40] or Sjögren’s syndrome [49]. A rat model, infected with the HTLV-1 producing cell line, is known to develop HAM-like myelopathies in seronegative carrier rats [50]. A Tax1-transgenic mouse model, which specifically expresses Tax1 in T-lymphocytes, illustrates the development of aggressive ATL-like lymphoma with continuous invasion of lymphomatous cells into multiple organs such as the skin, liver and spleen [51,52]. Subcutaneous inoculation of HTLV-1 transformed cells into NOG mice also results in ATL-like symptoms [53]. These transgenic/transplant models show symptoms similar to those found in human clinical cases. Furthermore, HTLV-1-driven inflammatory symptoms tend to occur in patients with HAD under normal host immune response conditions, while ATL-like symptoms develop under immunosuppressive conditions [54].

TAX1BP1−KO mice displayed invasive growth of lymphocytes into multiple organs (Fig. 3) and splenic hypertrophy (Fig. 8). We previously observed that transplantation of TAX1BP1−KO bone marrow to γ-irradiated normal mice resulted in the same inflammatory responses [8]. These results imply that TAX1BP1−KO model may be correlated with inflammatory HAD. The novelty of this system is identification of possible risk factors associated with vascular disease in HTLV-1 carriers [17,18]. Preliminary electrocardiogram experiments using TAX1BP1−KO mice showed an abnormal prolongation of PQ intervals and/or atrioventricular conduction defects (Fig. 6I, J), which might cause fatal cardiac failure. Since the PQ interval and atrioventricular conduction highly depend on the functioning of voltage-dependent L-type Ca2+ channels, L-type Ca2+ channel function may deteriorate in the heart of TAX1BP1−KO mice. Of note, EFCAB2, a functional partner in the voltage-gated Ca2+ channel, was significantly downregulated in the cardiac tissue of TAX1BP1−KO mice (Table 4). Further studies are required to elucidate these defects caused in TAX1BP1−KO mice.

Intensive antibiotic treatment [24] for TAX1BP1−KO mice significantly ameliorated inflammatory symptoms (Fig. 6). TAX1BP1−KO mice crossedbreed with MyD88−KO mice showed similar results. Since the intrinsic role of Tax1bp1 is to inhibit unnecessarily activated innate immunity responses [8], a functional deficiency of Tax1bp1 through HTLV-1 infection can lead to similar symptoms in humans; that is, commensal microbiota can cause pseudo-Infecetogenic endocarditis symptoms [55]. The extent of the deficiency, however, is much more moderate than that of typical infective endocarditis (IE) [56].

A large population-based epidemiological study revealed that the prevalence of heart valve disease in the entire population of the United States is 2.5% [53]. IE is thought to result from the following sequence of events: (1) the formation of nonbacterial thrombotic endocarditis on the surface of a cardiac valve or elsewhere that endothelial damage occurs; (2) bacteremia; and (3) the adherence of the bacteria in the bloodstream to nonbacterial thrombotic endocarditis and proliferation of bacteria within a vegetation [57]. Viridans group streptococci are a part of normal skin, oral, respiratory, and gastrointestinal tract flora, and are responsible for ≥50% of community-acquired native valve IE cases [58]. Another review reported that 20% of IE cases originated from culture-negative or Enterococci [59]. Each of these epidemiological surveys clearly indicates the importance of prevention and control measures with regard to microbial infection and vegetation. However, it is still not known why IE is developed in limited population and it is not clear whether there are any differences in the frequencies of allelic polymorphisms in the immune response genes for IE patients?

In summary, HTLV-1 induces diverse forms of inflammatory disorders [60,61], which may originate from the functional dysregulation of Tax1bp1. Single-nucleotide polymorphisms (SNPs) in A20 or RNF11, catalytic partners of Tax1bp1, has been linked to many inflammatory diseases [19,62,63]. However, in the case of TAX1BP1 SNPs, only one study has linked them to the head and neck cancer [64]. The genetic variations in TAX1BP1 and its partners would provide novel insights on the pathogenic machinery of HADs.

Supplementary Information

Figure S1 Validation of genes identified their expression alteration in the mitral valves of TAX1BP1−KO mice. RT-PCR validation of genes identified their expression alteration in the mitral valves of TAX1BP1−KO mice, A CCL2 B CHBEL1 respectively. Gray bar: TAX1BP1−KO, black bar: WT. Mitral valve specimens were prepared as described in Fig. 2A. Primers and probes were as indicated. (PDF)

Table S1 Age-dependent induction of pro-inflammatory proteins in the sera of TAX1BP1−KO mice. Sera from four different weeks of age (3, 8, 16 and 32) of TAX1BP1 homozygous knockout (Homo-KO), heterozygous knockout (Hetero-KO) or their WT littermates were collected and examined with multiplex ELISA quantitation kit (Bio-Plex ProTM Mouse Cytokine 23-plex Assay, BioRad). Each value is an average of four different samples. (PDF)

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Author Contributions

Conceived and designed the experiments: HI KO AN. Performed the experiments: SN EI YT YW T. Matsumoto T. Mitsui TY K. Inoue HK. Analyzed the data: KTJ MH MM TK. Wrote the paper: SN KTJ KO AN HI. Conducted bioinformatics: SN YT WF HI.

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