

Bacille Calmette Guérin (BCG) can induce Kawasaki disease-like features in *programmed death-1 (PD-1)* gene knockout mice

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Abstract

Objective

Various genetic variants of inhibitory immune signals have been suspected as feasible causes of Kawasaki disease (KD). We investigated the associative role of programmed death-1 (PD-1) gene in the pathogenesis of KD by injecting bacilli Calmette Guérin (BCG) to PD-1 gene knockout (PD-1KO) mice.

Methods

In order to induce KD-like clinical manifestations in young PD-1KO mice, intradermal injection of the bacilli Calmette Guérin (BCG) was performed twice on the abdominal skin with a 4-week interval. For defining the role of BCG, heat shock protein (HSP) 65 was challenged. In addition, *Staphylococcus aureus* was adopted as a microorganism that does not contain HSP65 structure. One month after the second injection, heart, liver, and kidneys were removed and examined.

Results

PD-1KO mice showed KD-like features including prolonged fever for more than 5 days, erythematous swelling on soles, tail skin desquamation, and gallbladder (GB) hydrops. Inflammatory cell aggregation and intimal proliferation in at least more than one coronary artery was found in all PD-1KO mice whereas scanty coronary lesion was found in wild type (WT) mice. When the PD-1KO mice were injected twice with HSP65, coronary arterial lesions similar to those seen after BCG injection were observed. Inflammatory reactions in other organs including hepatic arteries, renal arteries, and biliary arteries were also observed in PD-1KO mice.

Conclusion

Our data suggest that PD-1 gene may be one of the genetic predispositions of KD and antigens containing HSP65 structure could be a triggering factor of KD by our animal model of KD.

Key words

coronary arterial disease, gene, mucocutaneous lymph node syndrome, programmed death-1, inflammation

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Introduction

Currently, Kawasaki disease (KD) is the most common cause of acquired heart disease in childhood in the developed countries (1). This disease is being investigated in relation with sub-clinical atherosclerosis in adolescents (2) and the autoimmune mechanism of KD is important to understand the autoimmune process of atherosclerosis in adulthood (3). Because the coronary arterial lesions in patients with KD are known to be reversible, if the pathogenesis of KD is unveiled, the new exit to treat the atherosclerosis could be developed. Until now, many researchers have searched the pathogenesis of KD on the bridge between infection and autoimmune vasculitis (4, 5). However, the pathogenesis of KD is yet to be elucidated even with the progressive investigations for the past 40 years (4). Several animal models of KD have been investigated to explain the autoimmune mechanisms in the development of KD (4, 6). Previous animal models were focused to evoke coronary arteritis by administering an antigen which has structural homology with cardiac antigen (6). In contrast to previous study, we focused to the immunological switch which is a key factor for turning the immunologic reactions to autoimmune process after infection as the cause of KD.

Although there are fundamental differences between human and animals, an animal model, which can show typical vasculitic features of KD extensively, was developed by adopting *programmed death-1 (PD-1)* gene knockout (KO) (PD-1KO) mice in this study. Among inhibitory immune regulatory genes, the *PD-1* gene was chosen in this study, because we obtained the significant implication about the associative role of *PD-1* gene in the development of KD when we carried out the genetic susceptibility study for KD with *PD-1* gene polymorphism (7).

PD-1: PD-1 ligand 1 (PD-L1) pathway has been studied in autoimmune inflammatory processes of the heart (8, 9). Originally PD-1 KO mice are known for lupus-like phenomena in C57BL/6 background (10) and dilated cardiomyopathy in BALB/c background (11).

We thought that the manifestations of skin desquamation and arthritis mentioned in lupus-like phenomena of PD-1KO mice are much closer to features of KD than those of lupus. The reasons why we adopted the PD-1KO mice in developing animal model of KD are as follows. PD-1KO mice are prone to autoimmune vasculitis in heart, kidneys, skin and joints, which are the target organs of KD. Then, PD-1 molecule is inducible after T cell activation: if there is a defect of *PD-1* gene expression when the host has infection, the activation of T cell would be sustained and the apoptosis of immune cells might be decreased. Therefore we hypothesized that the lack of PD-1 molecule may be an initiation switch for KD after infectious challenges. Finally, PD-1KO mice can exhibit high prolonged fever due to elevated interferon (IFN)- γ deriving from the defect of negative feedback of PD-1: PD-L1 pathway (12). This high prolonged fever is a striking feature of KD and used as a main diagnostic point of KD.

PD-1KO mice show features of vasculitis spontaneously when they are aged. Because this feature does not represent the characteristics of KD which develop in children, we assumed that an appropriate infectious challenge in young PD-1KO mice could express KD-like features, which can be a good candidate for an animal model of KD. Then which infectious challenge would be proper? Clinical observation of erythematous change in bacilli Calmette Guérin (BCG) vaccination sites in patients with KD and some reports on evoking coronary arteritis with BCG (13-16) gave us a hint as to the most feasible infectious challenge in making our animal model of KD.

Hence our study was focused on inducing KD-like features in young PD-1 KO mice with BCG stimuli and showing that the defect in PD-1 expression after infectious stimuli can be related with the development of KD.

Materials and methods

Experimental groups

PD-1^{-/-} mice on a C57BL/6 background (PD-1KOB6) and PD-1^{-/-} mice on a BALB/c background (PD-1KOBALB)

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 Competing interests: none declared.

Table I. Grading criteria for evaluating the degree of inflammation in the heart lesions adopted in the experiment (20).

Inflammatory lesion	Grade	Criteria
Coronary arteritis	0	No inflammation
	1	Inflammation only in adventitia
	2	Inflammation in the adventitia and focal areas of the myointima
	3	Inflammation in the adventitia and diffuse area of the myointima
Myocarditis	0	No inflammation
	1	Single focus of inflammation
	2	Multifocal inflammation
	3	Diffuse inflammation

(RIKEN, Ibaraki, Japan) were used in this study (10). Three week- (wk) old mice were used with equal sex distribution within the group. Wild-type (WT) C57BL/6 (WTB6) and WT BALB/c (WTBALB) (Japan SLC Inc., Shizuoka, Japan) at the same age as the PD-1KO mice were used as control mice. Each group was divided into 3 treatment groups which were injected with BCG, heat shock protein 65 (HSP65), or saline (S). BCG was selected as a main infectious stimulus and HSP65 was selected for defining the role of BCG in provoking vasculitis according to the literature (13, 17, 18).

All the mice were maintained in barrier-filtered cages with individual ventilating system under specific pathogen-free (SPF) conditions and fed a normal mouse chow diet and tap water *ad libitum*. Experimental protocols were approved by the Institutional Animal Care at Yonsei University Medical Research Centre.

Induction of KD-like phenomena

Immunisation was performed twice at a 4-week (wk) interval. The first immunisation (day 0) was performed when the mice reached 3 wks of age. They received the second injection 4 wks after the first injection. BCG (Pasteur strain 11740) was kindly donated by Dr Cho's laboratory (19) in the Department of Microbiology, Yonsei University College of Medicine. Before injection, thawed BCG was sonicated and diluted with phosphate-buffered saline (PBS) to reach the desired concentration (1×10^7 CFU/mL). BCG in dose of 4×10^5 CFU (0.04 mL) was injected intradermally on the abdominal skin of each mouse.

The pups were subcutaneously immunised with 1 μ g of endotoxin-free recombinant mycobacterial HSP65 (Stressgen, Victoria, British Columbia, Canada) emulsified with incomplete Freund's adjuvant (IFA) (Sigma, St Louis, MO, USA) in a final volume of 0.1 mL. As the control group of another infection, *Staphylococcus aureus* (ATCC no.25923, American Type Culture Collection, VA, USA) was used. *S. aureus* titrated to 4×10^5 CFU, the same dose of BCG, was injected intradermally on the abdominal surface with same protocol. This experiment was performed only in PD-1KOBALB.

Staining of arterial lesions

The mice were euthanised at 4 wks, 8 wks, 12 wks, or 24 wks after the second injection and their heart, kidneys, and liver were removed. Paraffin blocks for haematoxylin-eosin (H-E) staining and immunohistochemical staining were prepared. Coronary arteries were identified from aortic root adjacent to coronary sinus. Serial 5 μ m-thick sections were collected. The lesion of coronary arteries or myocardium was scored according to the scoring method of Freeman *et al.* (20) (Table I).

Immunohistochemical staining

Antibodies for immunohistochemical (IHS) staining included rat anti-mouse CD3 antibody (Ab), rat anti-mouse CD4 Ab, and rat anti-mouse CD8 Ab for T cells (eBioscience, San Diego, CA, USA), rat anti-mouse CD19 Ab for B cells (eBioscience) and rat anti-mouse CD 31 Ab (eBioscience) for macrophages, fibroblasts, or neutrophils. As secondary Abs, biotinylated anti-rat IgG (eBioscience) was used.

Isotype- matched Abs, rat IgG₁ and rat IgG_{2b} were used as controls.

Analysis of double negative T cell

subset and double positive T cell subset
Cells were stained with Per CP-conjugated rat anti-mouse CD3, FITC-conjugated rat anti-mouse CD4, APC-conjugated rat anti-mouse CD8, and PE-conjugated rat anti-mouse CD25 (eBioscience). The fraction of positive-staining cells was determined relative to isotype control-stained cells. The percentage of double negative (DN) (CD4⁻CD8⁻) T cells and the percentage of double positive (CD4⁺CD25⁺) T cells were obtained. The data were analysed by LSRII (BD Biosciences, Franklin Lakes, NJ, USA) with DIVA™ software (BD Biosciences).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software Inc., San Diego, CA, USA). The data of two groups, PD-1KO mice and WT mice were analysed using the Mann-Whitney test. $P < 0.05$ was considered to be statistically significant.

Results

Clinical similarity

Prolonged fever, GB hydrops, palmar erythema, and skin desquamation, which are observed in KD patients, were detected in both backgrounds of PD-1KO mice after second BCG injection (Fig. 1). Endotoxin-free recombinant *Mycobacterium bovis* HSP65 induced the same phenomena.

After the first BCG injection, rectal body temperature was increased up to 37.5°C ~ 38.5°C on days 10–14 for 1–2 days and then subsided spontaneously. Booster BCG immunisation provoked high body temperature of 38°C ~ 39.5°C (data not shown). All PD-1KO mice showed at least a 3 day fever on days 7–10 after the second injection of BCG while control groups showed transient fever.

Similar to palmar erythema observed in the patients with KD, erythematous changes of soles were seen in all twenty PD-1KO mice injected with BCG or HSP65 twice during the febrile period,

while no change of skin in WT mice was observed (Fig. 1A and 1B). In addition, skin desquamation which can be observed in patients with KD was observed in PD-1KO mice (Fig. 1C). Histologic confirmation of skin changes were performed in both WT and PD-1KO mice (Fig. 1D). Inflammatory cells were aggregated in whole dermis layer and perivascular area, which were proved to be not only CD4⁺ T cells but also CD8⁺ cytotoxic T cells and B cells by immunohistochemical staining (Fig. 1E and 1F). Subsequent desquamation was observed in subepidermal layers where the inflammatory cells were aggregated.

Pathology of coronary arteritis

Compared with WT mice, PD-1KO mice of both backgrounds (PD-1KOBALB and PD-1KOB6) with the second injection of BCG or HSP65 showed heart lesions including coronary arteritis, pericarditis and myocarditis similar to KD (Fig. 2). Infiltrations of inflammatory cells in the adventitial layer, aggregation of inflammatory cells on the endothelial cell layer, proliferation of the intimal layer, edematous change of arterial wall, and absorption of elastic tissue and fibrosis were found. All PD-1KOBALB and PD-1KOB6 mice showed grade 2–3 coronary arteritis 1 month after the second injection while WT mice showed scanty inflammatory changes in heart (Table II). Coronary arterial lesions remained and seemed to be aggravated after the second injection when we dissected the coronary lesions serially with at a one-month interval for 6 months (data not shown). Similar degrees of coronary arteritis (more than grade 2) were observed in PD-1KO mice after second injection of HSP65 as well as BCG (Fig. 2D–F). However, no coronary arterial lesion was found in PD-1KO mice injected with *S. aureus* (Fig. 2G).

When we examined the heart lesions by IHC staining, infiltrated inflammatory cells were CD3, CD8, CD19, and CD31 positive cells. These cells were observed not only in the adventitial layer but also in the endothelial layer (Fig. 2H–K). Various inflammatory cells were found among myocytes and in pericardium as well.

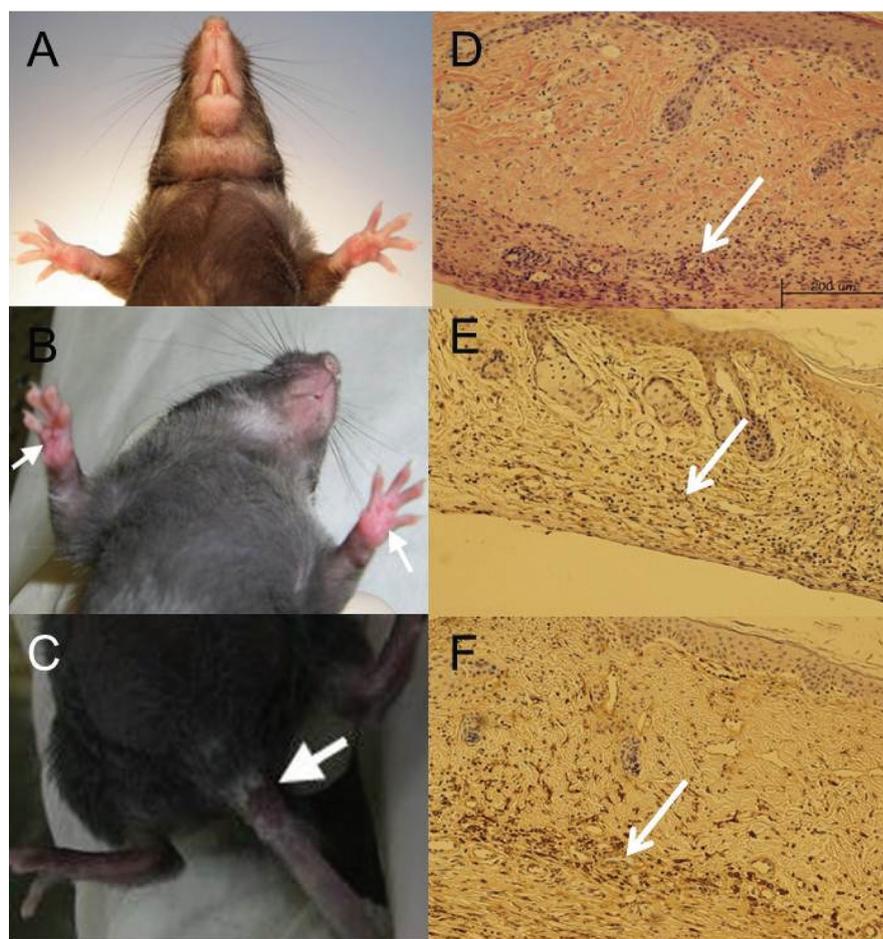


Fig. 1. Erythematous inflammatory changes of sole skin in *PD-1* gene knockout (PD-1KO) mice after the second BCG injection. **A:** PD-1KO mice with no antigenic challenge. **B:** Oedematous and reddened skin (white arrows) in PD-1KO mice. **C:** Skin desquamation of proximal portion of tail. **D:** Microscopic findings of abdominal skin stained with Haematoxylin & Eosin (x200). **E:** Immunohistochemical staining with antibodies (Abs) specific for CD3 T cells (x200). **F:** Immunohistochemical staining with Abs specific for CD19 B cells (x200).

Pathology of renal arteries, hepatic arteries, and biliary arteries

PD-1KO mice of both background injected with BCG or HSP65 twice showed systemic vasculitis including hepatic arteries, biliary arteries and renal arteries while WT mice injected with same antigens showed few inflammatory changes in arteries mentioned above. GB hydrops and multicystic kidneys were found in PD-1KO mice injected with BCG or HSP65 (Fig. 3). Histological findings of GB hydrops were the inflammatory changes of biliary ducts and biliary arteries. Microscopic findings of multicystic kidneys were arterial obliterans or severe inflammations of renal arteries. Mononuclear inflammatory cells were also aggregated in portal triad and renal arteries of PD-1KO mice.

The differences of splenic T cell population

In PD-1KO mice, the peripheral proportion of CD4⁺CD8⁻ (DN) T cells was increased significantly (mean \pm standard deviation, 21.3 \pm 3.1%) on day 14 compared with that of WT mice (9.07 \pm 1.04%) ($p < 0.01$). CD4⁺CD25⁺ T cells in PD-1KO mice were decreased significantly (1.69 \pm 0.52%) compared with WT mice (4.08 \pm 0.28%) ($p < 0.05$) (Fig. 4).

Discussion

Animal models of KD have been developed with various animals because of their advantage of a direct approach to the target system, coronary arteries (21, 22). Most animal models were made to induce coronary arteritis (Table III). An ideal animal model of KD may

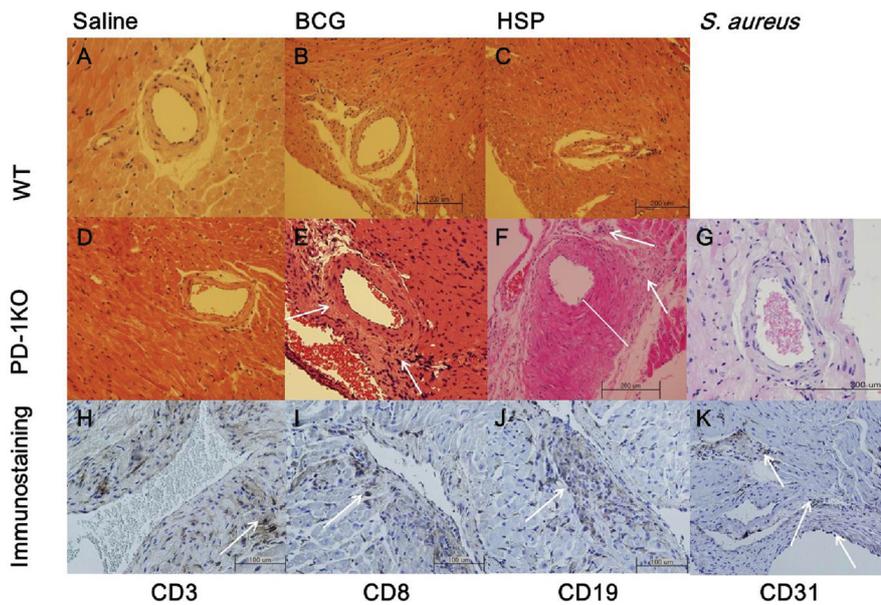


Fig. 2. Coronary arteritis in PD-1knockout (KO) mice compared with that in wild-type (WT) mice. Three groups of WT mice showed no changes of coronary arteries (A-C). PD-1KO mice injected with BCG or HSP twice showed severe inflammatory changes of coronary arteries and myocytes compared with PD-1KO mice injected with saline or *Staphylococcus aureus* (D-G). The severe intimal proliferation, more than 3-fold increase of normal thickness, was found in coronary arteries of PD-1 KO mice injected with HSP65. CD3⁺ T cells, CD8⁺ T cells, CD19⁺ B cells, CD31⁺ cells were found in coronary lesions and among myocytes (H-K).

Table II. Comparison of the occurrence of coronary arteritis and myocarditis between PD-1KO mice and WT mice of either C57BL/6 or BALB/c background.

Genotype	C57BL/6						BALB/c						
	WTB6		PD-1KOB6		WTBALB		PD-1KOBALB						
Groups	S	B	H	S	B	H	S	B	H	S	B	H	
Immunisation* (No. of mice)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	
Coronary arteritis													
Grade	0	5	3	3	5	0	0	4	4	4	5	0	0
	1	0	2	2	0	1	0	1	1	1	0	0	0
	2	0	0	0	0	2	2	0	0	0	0	3	2
	3	0	0	0	0	2	3	0	0	0	0	2	3
Myocarditis													
Grade	0	5	3	3	5	0	0	5	4	4	4	0	0
	1	0	2	2	0	0	0	0	1	1	1	0	0
	2	0	0	0	0	1	2	0	0	0	0	2	2
	3	0	0	0	0	4	3	0	0	0	0	3	3

*S: saline, B: BCG, H: HSP65; **The data from PD-1KO mice injected with *Staphylococcus aureus* were not included in this table.

be able to show not only coronary arteritis but also other clinical findings originating from systemic vasculitis and aberrant immunologic profiles of KD with subsequent pathologic tissue changes. The animal model of KD with PD-1KO mice in this experiment sufficiently satisfied our expectation. Vasculitis of skin, heart, liver, gallbladder, and kidneys were successfully induced in young PD-1KO mice by BCG

or HSP65 injection as much as fully depicted in the murine models of KD using *Candida* antigens by Takahashi *et al.* (22).

In addition to induction of arteritis, the animal models of KD have been attempted to prove the genetic predispositions of KD, so various genetic backgrounds of mice have been adopted to demonstrate the differences in the incidence of lesions depending on the

background of mice (Table III). We also speculated the defect of *PD-1* gene as one of genetic predispositions of KD and observed a definite difference in the incidence of coronary arteritis between the PD-1KO mice and control mice. As well reviewed by Yeung (23, 24), genetic defects of co-stimulatory molecules involved in T cell activation, TLR2, CD40, CD40L, proinflammatory cytokines such as tumour necrosis factor (TNF)- α or IFN- γ , and matrix metalloproteinases have been tested through animal models of KD.

Among various genetic studies of KD in human (25), Onouchi *et al.* reported the genetic polymorphism of inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) which is negative regulator of activated T cells (26). ITPKC is also involved in the inflammation after PD-1 signal pathway (12). The target gene of our study, *PD-1* gene is known as important inhibitory switch in T cell activation as well as *ITPKC* gene (12). Therefore, PD-1KO mice may be feasible for revealing the host genetic susceptibility and relationship between KD and T cell function when the triggering factor is infection.

Coronary arterial lesions were induced in our animal model with the same incidence as experiments performed with *Lactobacillus* cell wall extracts (LCWE) demonstrated by Lau *et al.* (24). Instead of LCWE, which is the most frequently used immunogen in animal model of KD, BCG was used in this study for the following reasons. First, clinically BCG erythema was detected in 70–88% of patients with KD who had received BCG vaccination within 6 months (27, 28). Therefore the authors thought that percutaneous injection or intradermal injection site of BCG would be easily detectable lesions of vasculitis and BCG might play a role in initiating vasculitis. Second, according to the literature, BCG has been widely used in evoking chronic inflammation (17) such as atherosclerosis (13, 16) or rheumatoid arthritis (18, 29). Thus, we used BCG as a potent antigen for inducing inflammation. As a result, BCG challenge in PD-1KO mice was as effective as LCWE in evoking autoimmune coronary arteri-

tis. We assumed that the BCG effect on autoimmune vasculitis may be due to HSP65 in the same line of research by Yokota *et al.* (30). Hence we chose HSP65 directly as another inducer instead of any other microorganisms. The data from HSP65 group were compatible with those from BCG group. When we performed the same experiments using *S. aureus*, no coronary arterial lesion was observed except for a few pericarditis or endocarditis. Because *S. aureus* does not contain the structure of HSP65, this result can support the special role of HSP65 for the developing autoimmune inflammation in coronary arteries. In another animal model of KD developed by Nakamura *et al.* (28), BCG was used also in T cell priming in evoking autoimmune coronary arteritis and they used *M. intracellulare*

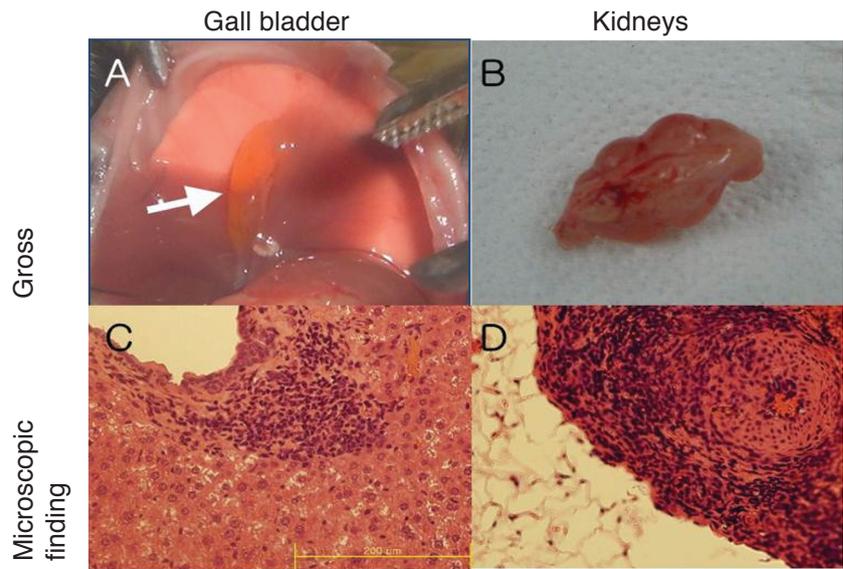


Fig. 3. The vasculitis of portal triads and renal arteries in PD-1-KO mice injected with BCG. **A,** GB hydrops. **B.** Multicystic kidney. **C.** Mononuclear inflammatory cells were infiltrated in portal triad of PD-1-KO mice H&E stain (x200). **D,** Arteriosclerosis obliterans of a renal artery (x200).

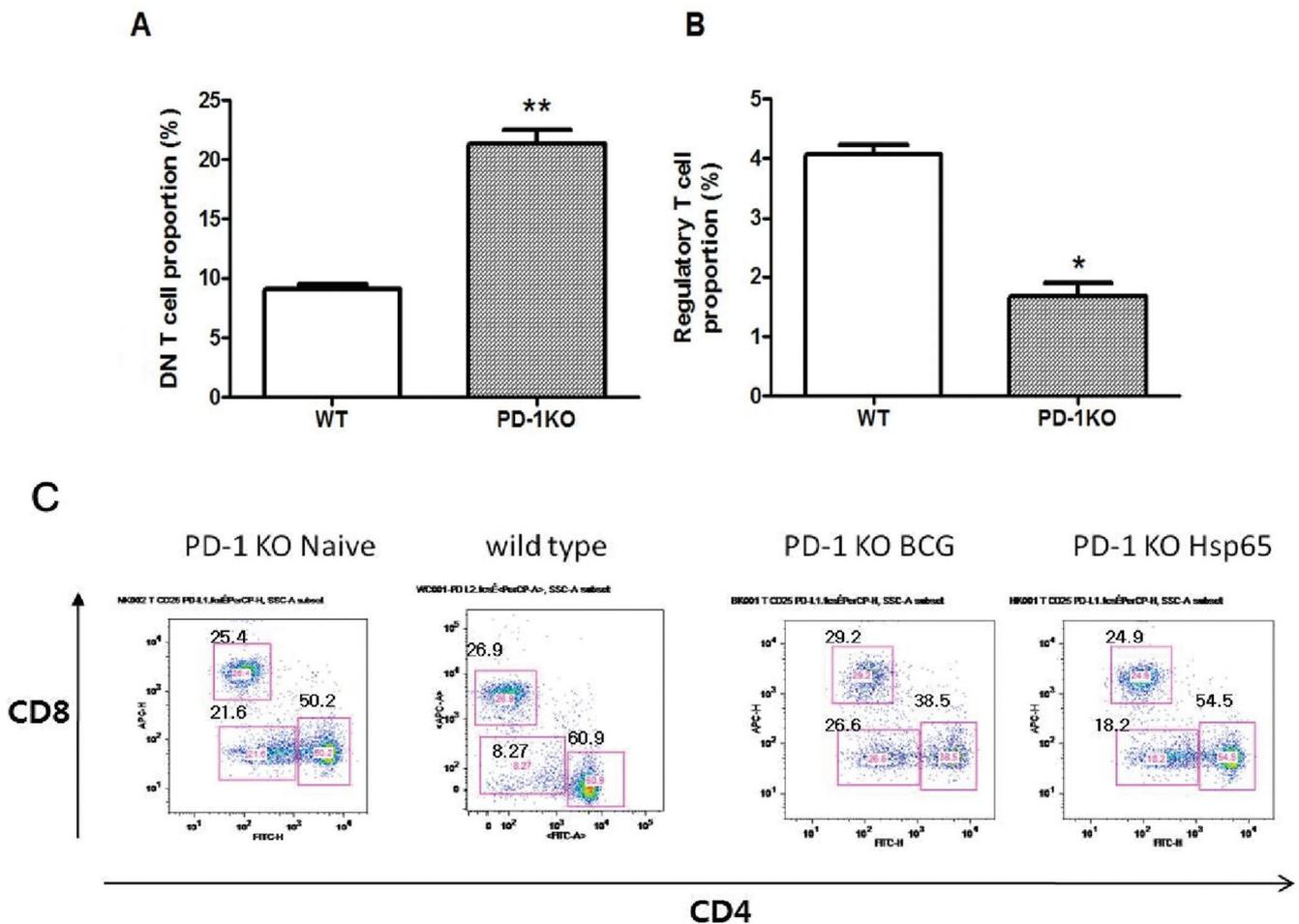


Fig. 4. The proportion of CD4⁻CD8⁻ double negative (DN) T cells and CD4⁺CD25⁺ regulatory T cells in spleens of PD-1 knockout (PD-1KO) mice compared with those of wild-type (WT) mice.
* $p < 0.05$, ** $p < 0.01$

Table III. The history of animal models of Kawasaki disease (KD).

References	Animals used in inducing KD	Inducers	Implications for understanding the mechanism of KD
Murata H. Microbiol Immunol 1979; 23: 825-31	DD and DDY strain of 3-week old mice	<i>Candida albicans</i> water soluble fraction (CAWS)	Successfully induced coronary arteritis by an extract of <i>Candida albicans</i> isolated from a patients with KD
Lehman TJ <i>et al.</i> Arthritis Rheum 1985; 28: 652-9	C57BL/6, A/J, Balb/c, C3H/HeJ, Nude mice A/J background Nude mice C57BL/6 background	Group B <i>Lactobacillus casei</i> cell wall extracts (LCWE)	Around 50% success for inducing coronary arteritis in various inbred mouse strains except C3H/HeJ. Macrophages may play an essential role in the pathogenesis of coronary arteritis
Felsburg PG <i>et al.</i> Clin Immunol Immunopathol 1992; 65: 110-8	Young dogs	Naturally occurred	Necrotising vasculitis which combines immunoregulatory abnormalities
Onouchi Z <i>et al.</i> Angiology 1995; 46: 679-87	Weanling rabbits	Serum sickness	The mechanism of coronary arterial dilatation is dependent on age factors irrespective of underlying mechanism
Yeung RS <i>et al.</i> Int Immunol 2003; 15: 79-89	C57BL/6 and Balb/c hCD4 ⁺ DQ6 ⁺ mCD4 ⁻ mCD8 ⁻ (MHC class II DQ6 transgenic mice)	Group B <i>Lactobacillus casei</i> cell wall extracts (LCWE)	The superantigenic effects of LCWE is responsible for induction of coronary arteritis
Takahashi K <i>et al.</i> Inflamm Res 2004; 53: 72-7	CD-1, C57BL/6N, C3H/HeN, Balb/c, DBA/2N, CBA/JN	<i>Candida albicans</i> water soluble fraction (CAWS)	Mouse model showing systemic vasculitis as similar as vasculitis in patients with Kawasaki disease
Ohno N Jpn J Infect Dis 2004; 57: S9-10	DBA/2, C3H/HeN	<i>Candida albicans</i> water soluble fraction (CAWS)	The genetic background of the immune response to CAWS is involved in the occurrence of coronary arteritis
Nakamura T <i>et al.</i> FEMS Immunol med Microbiol 2007; 49: 391-7	C57BL/6 3 weeks old	Bacille Calmette-Guérin (BCG) and crude extract from <i>Mycobacterium intracellulare</i> (eMI)	Immune response to mycobacteria in young mice induced autoimmune vasculitis and atypical mycobacterial infection boosts the development of coronary arteritis
Schulte DJ <i>et al.</i> J Immunol 2009; 183: 5311-8	C57BL/6, RAG1 ^{-/-} B6 background, B-null mice	Group B <i>Lactobacillus casei</i> cell wall extracts (LCWE)	T cells but not B cells are required for coronary lesion formation. Macrophage and dendritic cells may collaborate with T cells in the pathogenesis of coronary arteritis

as an immune booster having similar structure.

Interestingly, as early as 1993, Yokota *et al.* suggested that anti-HSP65 antibody in the serum of patients in the convalescent phase of KD can recognise epitopes of both mycobacterial HSP65 and human origin HSP, whereas anti-HSP65 antibody obtained from the subject in normal immune state only recognises that HSP65 originated from bacteria (30).

Therefore, host immunologic response to HSP65 was considered more important than exposure to HSP65 itself. In this study, we demonstrated that PD-1 deficiency is an important host factor by showing that few inflammatory cells were found in coronary arteries of WT mice injected with same amount and frequency of BCG or HSP65.

Regarding the T cell subpopulation, PD-1KO mice showed increased CD3⁺CD4⁺CD8⁻ DN T cell subset compared with WT mice ($p < 0.01$) regardless of the antigenic challenge. DN T cell has been known to be increased in autoimmune vasculitis and to inhibit the expansion of normal T cell subset (31). It is reported that lacking of PD-1 might be related with autoimmune vasculitis not only by lowering peripheral threshold of T cell receptor (32) but also by alteration of T cell development in the thymus (33). Therefore PD-1 deficiency has dual genetic advantages in inducing autoimmune vasculitis.

The clinical implication of this study may be the importance of individual genetic susceptibility of KD after infectious stimuli. The genetic susceptibility provides a hint for early diagnosis

and early intervention of KD beyond young childhood (2, 25). Until now, 10-20% of KD is recurrent or resistant to high dose intravenous immunoglobulin therapy (5). For this group of patients, a new treatment modality for inducing T cell apoptosis against persistent T cell activation could be attempted.

In conclusion, through the animal model of KD adopting PD-1KO mice with BCG injection, we suggest that KD may develop by infectious microorganisms containing HSP65 molecule in the genetically susceptible population and *PD-1* gene may be one of the most important genetic predispositions of KD.

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