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CD8+ T cells contribute to the development of coronary arteritis in the *Lactobacillus casei* extract-induced murine model of Kawasaki Disease

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Abstract

Objective—Kawasaki disease (KD) is the leading cause of acquired heart disease among children in developed countries. Human KD coronary lesions are characterized by increased presence of infiltrating $CD3^+$ T cells, however the specific contributions of the different T cell subpopulations in coronary arteritis development remains unknown. Therefore, we sought to investigate the function of $CD4^+$, $CD8^+$, Regulatory T cells (T_{Reg}), and NK T cells in the pathogenesis of the KD.

Methods and Results—T cell subsets function in KD development was addressed by using a well-established murine model of *Lactobacillus casei* cell wall extract (LCWE)-induced KD vasculitis. LCWE-injected mice developed coronary lesions characterized by the presence of inflammatory cell infiltrations. Frequently, this chronic inflammation resulted in complete occlusion of the coronaries due to luminal myofibroblast proliferation (LMP) as well as the development of coronary arteritis and aortitis. In this study we demonstrate the requirement of $CD8^+$ T cells but not $CD4^+$, NK T cells or T_{Reg} cells in the development of KD vasculitis by using several Knockout (KO) murine strains and depleting monoclonal antibodies.

Conclusions—The LCWE-induced KD vasculitis murine model mimics many histological features of the human disease such as the presence of $CD8^+$ T cells and LMP in the coronary

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artery lesions as well as epicardial coronary arteritis. Moreover, $CD8^+$ T cells functionally contribute to the development of KD vasculitis in this KD murine model. Therapeutic strategies targeting infiltrating $CD8^+$ T cells might be useful in the management of human KD.

INTRODUCTION

Kawasaki disease (KD) is an acute systemic vasculitis of unknown etiology affecting predominantly children from 6 months to 5 years of age (1). KD represents the leading cause of acquired heart disease among children in the United States and other developed countries and is associated with the development of acute and subacute coronary arteritis and myocarditis (2–4). The etiology of KD remains unknown, although the current paradigm is that KD could be triggered by an infectious agent that elicits inflammatory responses directed at cardiovascular tissues in genetically susceptible hosts (3). The limited understanding of the etiologic agent(s) and the cellular and molecular immune mechanisms involved in KD pathogenesis continue to thwart the development of more efficacious treatments or cure (5,6). In addition, the very limited availability of KD patients tissue samples has significantly impeded our progress in understanding KD etiology and pathogenesis, making the availability of a relevant animal model extremely valuable. KD involves systemic inflammation with a distinct predilection for the coronary arteries. KD, once thought of as an acute self-limiting disease, is now being increasingly recognized to induce long-term cardiovascular complications, including vascular changes and ongoing remodeling such as luminal myofibroblast proliferation (LMP), leading to coronary artery (CA) stenosis with both cardiovascular and myocardial complications (7–9).

The *Lactobacillus casei* Cell Wall Extract (LCWE) murine model of KD vasculitis closely phenocopies the important histological as well as the immune and pathological features of the human disease (i.e. coronary arteritis, coronary stenosis, aortitis, myocarditis, aneurysms) (10–13). A single i.p. injection of LCWE into wild type (WT) mice reproducibly induces aortitis, proximal coronary arteritis, myocarditis as well as other systemic artery abnormalities, including abdominal aorta dilatations and even aneurysms which are histopathological features similar to the cardiovascular pathologies observed in human KD (10,12–15). This LCWE-induced KD experimental murine model reliably predicts efficacy of treatment options in children with KD (11,16,17). While no animal model can fully mimic human disease, the LCWE-induced KD murine model has been widely accepted as a reliable experimental model able to provide novel insights of KD immunopathology and potential leads for the development therapeutics interventions aiming to treat and prevent the cardiovascular complications associated with KD. The translational value of this animal model has recently been shown again when the discovery of the key role of IL-1 signaling in this experimental murine model of KD vasculitis, has recently led to the initiation of three Phase II clinical trials with the IL-1R antagonist (anakinra) or anti-IL-1β (canakinumab) in KD patients (14,15,18).

Although the mechanism of KD induced cardiovascular lesion development is unclear, strong evidences indicate that the pathology is immune mediated (19–22). Immunohistological analysis of tissues collected from KD patients demonstrate the presence of dendritic cells (DCs) in the coronary lesions as well as their co-localization with $CD3^+$ T

cells (19). Circulating $CD4^+$ and $CD8^+$ T cells are also increased in KD patients with coronary lesions and $CD8^+$ T cells are the dominant cell type present in those lesions (23,24). Several studies have demonstrated that KD acute phase is also associated with decreased numbers and compromised functions of circulating $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (T_{Reg}) cells (25,26). Intravenous Immunoglobulin (IVIG) treatment results in increased proportion and suppressive activities of T_{Reg} cells (25,27).

In this study, we demonstrate that the LCWE-murine KD vasculitis model phenocopies many pathological features of human KD, including the development of epicardial coronary arteritis, as well as progressive LMP and scarring/stenosis of the CA. We also observe the presence of both $CD4^+$ and $CD8^+$ T cells in the LCWE-induced coronary lesions. Although the absence of either T_{Reg} cells or NK T cells did not impact the development of LCWE-induced KD vasculitis, $CD8^+$ T cells play a critical role in KD development since their depletion resulted in decreased KD vasculitis and lesion formation. Finally, adoptive transfer of $CD8^+$ T cells promotes the development of vasculitis in Rag-$1^{-/-}$ mice, a strain of mice known to be resistant to LCWE-induced KD (13). The present report underlines the key function of $CD8^+$ T cells in in the LCWE-induced KD lesions, similar to human KD, and targeting this T cell subset during KD development could potentially lead to the generation of novel therapeutics.

MATERIALS AND METHODS

Mice

C57BL/6, CD45.1^+, $CD4^{-/-}$ ($CD4^{tm1Mak}$), Rag-$1^{-/-}$ ($Rag1^{tm1Mom}$) and $Cd1d^{-/-}$ ($Cd1^{tm1Gru}$) mice, all on C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME). $Foxp3^{DTR}$ transgenic mice were a kind gift of Alexander Rudensky (Sloan-Kettering Institute, New York, NY). All animals were housed under specific pathogen-free conditions at the animal center of the Cedars-Sinai Medical Center and experiments conducted under approved IACUC protocols.

In vivo cell depletion

The following antibodies were given i.p. at 200μg per injection: anti-CD8α (Clone: 2.43; BioXcell), anti- CD8β (Clone: 53-5.8; BioXcell), anti-CD25 (Clone: PC61; eBioscience), as well as rat IgG2B (Clone: LTF-2; BioXcell) and rat IgG1 (Clone: HRPN; BioXcell) isotype controls. For *in vivo* T_{Reg} cell depletion with Diphteria Toxin (DT; Sigma-Aldrich), $Foxp3^{DTR}$ mice were given 50μg/kg of DT i.p. every other day as previously described (28,29).

KD vasculitis murine model and heart pathology assessment

Lactobacillus casei (ATCC 11578) cell wall extract was prepared as previously described (10,12). 5 weeks old male mice were injected i.p. with either 500μg of LCWE or PBS. At the mentioned days post-injection, mice were euthanized, hearts removed and embedded in Optimal Cutting Temperature (OCT) compound for histological examination. Serial cryosections (7μm) of heart tissue, were stained with hematoxilyn and eosin (H&E) and histopathological scoring of coronary arteritis, aortic root vasculitis, and myocarditis were

performed by a pathologist blinded to the experimental set up. Trichrome staining and KD lesions assessment were performed as previously described (15). Severity of heart vessels inflammation was scored as follow: 0, no inflammation; 1, rare inflammatory cells; 2, scattered inflammatory cells; 3, diffuse infiltrate of inflammatory cells; and 4, dense cluster of inflammatory cells. Myocardial inflammation was scored as followed: 0, no myocardial fibrosis; 1, very minimal focal subepicardial interstitial fibrosis just infiltrating beneath epicardial fat; 2, mild subepicardial interstitial fibrosis infiltrating deeper into subepicardial myocardium; 3, multifocal subepicardial interstitial fibrosis; and 4, replacement fibrosis. Imaging of the sections was performed on a Biorevo BZ-9000 (Keyence). The study and identification of the cells present in the coronary lesions was performed by transmission or scanning electron microscopy (TEM or SEM). Hearts at 14 days post-LCWE injection were removed, dissected into 1 mm specimens and fixed in 4% glutaraldehyde. Ultrathin sections (70nm) were cut and examined under TEM or SEM.

Immunofluorescence

Frozen heart sections of PBS and LCWE-injected mice were stained with the following antibodies: anti-mouse CD8 (Clone 53-6.7; Tonbo Bioscience), anti-Ly6G (Clone: RBC-8C5; eBioscience), anti-CD11b (Clone: M1/70; eBioscience), anti-F4/80 (Clone: BM8; eBioscience), anti-mouse CD4 (Clone: GK1.5; Biolegend) and anti-mouse Foxp3 (Clone: FJK-16s; eBioscience). Isotype controls were used as negative controls. Before imaging, the nuclei were counterstained with ProLong Gold Antifade Reagent containing DAPI (Invitrogen). Images were obtained using a Biorevo BZ-9000 (Keyence) fluorescent microscope.

RNA-sequencing

Heart tissues were collected from PBS (n=5) and LCWE-injected (n=5) WT mice 7 day after injection. RNA was extracted by using a RNeasy Mini Kit (Qiagen). RNA quality was further determined by using an Bioanalyzer (Agilent); only samples with a RIN score higher that 7 were used. RNA-Sequencing and subsequent analysis were then performed as previously described (14).

Flow cytometry

The following murine antibodies were used: anti-mouse CD3 (145–2C11), anti mouse CD4 (RM4-5), anti-mouse CD8 (53–6.7), anti-mouse CD25 (PC61), anti-mouse Foxp3 (FJK-16S), anti-mouse CD45.1 (A20). Dead cells were routinely excluded based on the staining of eFluor 780 Fixable Viability dye (eBioscience). Stained cells were analyzed on a LSRII (BD Biosciences) and the data were processed using Flowjo (Tree Star Inc.).

$CD8^+$ T cells reconstitution of Rag-$1^{-/-}$ mice

$CD8^+$ T cells were negatively isolated from the spleens of $CD45.1^+$ WT C57BL/6 mice using magnetic beads according to the manufacturer protocol (STEMCELL Technologies). The purity of isolated $CD8^+$ T lymphocytes was >94%. On day 0, $4x10^6$ $CD8^+$ T cells suspended in 100μl of PBS were injected retro-orbitally into Rag-$1^{-/-}$ mice. One day after,

non-reconstituted and reconstituted Rag-$1^{-/-}$ mice received either PBS or LCWE. KD vasculitis and heart pathology was assessed as described earlier.

Statistical Analysis

Results are reported as mean ± SEM. Statistical analysis were performed with Prism GraphPad version 4.03 (GraphPad Software). Mann Whitney unpaired two-tailed t tests were used for comparisons of two groups. Fisher's exact tests were used to compare the incidence of coronary lesion formation. For experiments involving more than 2 groups, one-way ANOVA with Tukey's post-tests analysis were used. A p-value of $p<0.05$ was considered statistically significant. *: $p<0.05$, ** : $p<0.01$, *** : $p<0.001$.

RESULTS

Proximal and epicardial coronary arteritis and luminal myofibroblast proliferation development in the LCWE-induced murine model of KD vasculitis

The LCWE-induced murine model of KD vasculitis has been developed and used to decipher the immunopathological mechanisms underlying KD development with, ultimately, the goal of establishing novel and more effective KD therapeutics. The resulting coronary arteritis in LCWE-induced murine KD model is characterized by infiltration of inflammatory cells and destruction of the extracellular matrix, especially elastic tissue in vascular media, with resultant coronary artery aneurysm (CAAs) formation (10–12,17). A recent review questioned if the LCWE-induced murine model of KD vasculitis may exactly replicate human KD pathology as it mostly affects elastic arteries, such as the aorta and the coronary ostium, and necessarily the primary target of human KD, the epicardial muscular coronary arteries (9,30). Here we now show that one week after LCWE injection, WT mice develop CA lesions characterized by the presence of inflammatory cells infiltration and the destruction of elastic tissue in the vascular media resulting in CAAs formation (Figure 1A). Furthermore, development of arteritis in epicardial muscular CA with absence of damage to the aortic wall is also observed (Figure 1B). The recently reported three linked pathological processes leading to human KD arteriopathy; acute or necrotizing arteritis, sub-acute or chronic arteritis and LMP (8) are also replicated in the LCWE-induced murine KD vasculitis model (Figure 1C). The proliferation of medial smooth muscle cells inside the CA lumen can potentially lead to complete CA stenosis (Figure 1C). The inflammation that develops in human histological KD vasculitis is characterized by the accumulation of monocytes/macrophages and plasma cells in the CA lesions (21,31). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) of murine heart sections 14 days after LCWE-injection also revealed the presence of monocytes (Figure 1D, E), plasma cells (Figure 1F) and fibroblast (Figure 1G) in close contact with the CA endothelium of the KD mice. The identification of these cells were confirmed with specific cell stains obtained in serial sections obtained from the same blocks (data not shown). Therefore, these additional pathological findings add to the overwhelming evidence of published data further supporting that the LCWE-induced experimental KD murine model strongly mimics the main pathological features of human KD, including the presence of epicardial muscular coronary arteritis and development of LMP in the coronary arteries.

$CD4^+$ and $CD8^+$ T cells accumulate in CA lesions of LCWE-induced murine KD vasculitis

We previously have identified the presence of various immune cells type (DCs, plasmacytoid DCs, macrophages, and $CD3^+$ T cells) in the CA of KD patients as well as in the CA of LCWE-injected mice (13,19). In an effort to better understand the immune-cellular components of the lesions over time we performed immunofluorescence analyses of murine heart sections collected from PBS control and LCWE-injected mice 7 and 35 days post-injection. As shown in Figure 2, $CD8^+$ T cells, $Ly6G^+$ (neutrophils), $CD11b^+$ and $F4/80^+$ (macrophages) as well as double positive $CD4^+$ $Foxp3^+$ T_{Reg} cells were present in the CA lesion of LCWE-injected mice (Figure 2B, C). Isotype control did not show any positive staining and T cells were not observed in the CA of naïve animals (Figure 2B, C) These results confirm the presence and infiltration of T cells, $CD4^+$, $CD8^+$ and T_{Reg} cells in the CA during LCWE-induced KD vasculitis.

$CD4^+$ $Foxp3^+$ T_{Reg} and NK T cells are not involved in the development of LCWE-induced KD vasculitis

Since the presence of $CD4^+$ $Foxp3^+$ T_{Reg} cells in the CA lesions during LCWE-induced KD murine vasculitis was demonstrated by immunofluorescence and because human KD development is associated with reduced numbers and decreased functionality of circulating T_{Reg} cells (25,26), we hypothesized that $CD4^+$ $Foxp3^+$ T_{Reg} cells may play a role in controlling KD vasculitis in this murine model. To demonstrate the regulation of LCWE-induced KD vasculitis by T_{Reg} cells, we employed two different models of acute transient specific T_{Reg} cell depletion; the *Foxp3*^{DTR} mice and the administration of anti-CD25 monoclonal antibody (mAb) (Supplementary Figure 1A-C). WT mice were treated with either Rat IgG isotype or anti-CD25 mAb and the hearts harvested at day 7 post LCWE-injection (Supplementary Figure 1A). The efficiency of the anti-CD25 mAb treatment was assessed by flow cytometry and resulted in a 2 fold T_{Reg} cells depletion in the spleen of treated mice (Supplementary Figure 1D, E). Compared to LCWE-injected control mice treated with Rat IgG isotype, T_{Reg} cell depletion by anti-CD25 mAb treatment did not demonstrate significant differences in the development of coronary arteritis and overall cardiovascular disease incidence (Figure 3A–D). Alternatively, we used *Foxp3*^{DTR} mice that express the Diphtheria Toxin Receptor (DTR) under the control of the *Foxp3* promoter (32). *Foxp3*^{DTR} mice were treated with Diphtheria Toxin (DT) for one or two weeks (Supplementary Figure 1B, C). Effective T_{Reg} cell depletion was assessed in the spleen by flow cytometry of DT-treated *Foxp3*^{DTR} mice one week after LCWE–injection (Supplementary Figure 1 F, G). T_{Reg} cell depletion with DT treatment for 1 or 2 weeks did not impact the development of LCWE-induced KD vasculitis as demonstrated by identical occurrence of heart vessels inflammation, disease incidence and myocardium inflammation between the control and DT-treated T_{Reg} depleted groups (Figure 3 E–I).

We next assessed the potential involvement of NK T cells in LCWE-induced KD vasculitis as these cells are known to respond to carbohydrate moieties (33), which make up the LCWE. Since murine KD vasculitis is induced by cell wall extract of *Lactobacillus casei*, we hypothesize that cell-wall derived carbohydrate moiety recognition by NK T cells may be involved in the development of KD vasculitis. *Cd1d*$^{-/-}$ mice were injected with LCWE and the incidence of KD vasculitis was assessed 2 weeks after injection. Absence of NK T cells

did not protect from LCWE-induced vasculitis since no significant difference was observed in incidence and heart vessels inflammation development between *Cd1d*$^{-/-}$ and WT mice (Supplementary Figure 2A–C). These data demonstrate that T_{Reg} cells do not confer protection and that NK T cells do not have a pathogenic function at least in this murine model of KD vasculitis.

CD8$^+$ T but not CD4$^+$ T cells are required in LCWE-induced vasculitis and myocarditis

Human and murine KD vasculitis are characterized by the accumulation of a large number of dendritic cells (DCs) and CD3$^+$ T cells in the coronary lesions (13,19). By acting as antigen presenting cells (APCs), DCs induce and support the adaptive immune response. Although human KD lesions are characterized by an influx of CD3$^+$ T cells and infiltration in the coronary arterial wall, studies addressing the specific functions of CD4$^+$ and CD8$^+$ T cells subsets in KD vasculitis development are lacking (13,19,23). In order to characterize the role of CD4$^+$ T cells in KD vasculitis development, we injected WT and CD4$^{-/-}$ mice with LCWE and assessed the incidence, development and severity of KD vasculitis one week after injection (Figure 4A, B). Absence of CD4$^+$ T cells did not prevent the development of LCWE-induced KD vasculitis as CD4$^{-/-}$ mice developed vessels inflammation and myocarditis at identical frequency and same pathological degree of severity, indicating that CD4$^+$ T cells were not required for LCWE-induced KD (Figure 4 A–C).

Immunohistochemical staining of CAAs samples collected from human KD patients demonstrated that CD8$^+$ T cells were more predominant in the lesion than CD4$^+$ T cells (23). Interestingly, gene expression analysis showed significant upregulation of genes associated with CD8$^+$ T cell cytotoxic functions such as CD8a, CD8b1, granzymes A and B, but not Perforin-1, in heart tissues of LCWE-injected mice developing KD when compared to control naïve PBS-injected mice (Figure 4 D, E). Therefore, we next investigated the function of CD8$^+$ T cells in the LCWE-induced murine model of KD vasculitis by using an antibody depletion approach. WT mice were treated with either anti-mouse CD8α or control Rat IgG isotype on days -2, 0, 3, and 5, were injected with LCWE on day 0 and analyzed for KD vasculitis development at day 7 (Supplementary Figure 3A). Since CD8α is also expressed by a subpopulation of splenic DCs (34), this depletion treatment could also potentially affect the DCs compartment which is required for the LCWE-induced KD vasculitis model (35). Therefore, CD8$^+$ T cell depletion was also performed using an anti-mouse mAb targeting CD8β, only expressed by CD8$^+$ T cells (Supplementary Figure 3A). Both anti-CD8α and anti-CD8β depletion treatment were efficient as demonstrated by a complete depletion of the splenic CD8$^+$ T cell population in treated mice compared to control (Supplementary Figure 3B, C). Compared to LCWE-injected mice, CD8$^+$ T cells depleted mice were protected and developed significantly lower LCWE-induced vasculitic inflammation (Figure 4F, G) as well as significantly decreased myocardial inflammation (Figure 4H).

Finally, in order to assess the possibility of a functional redundancy between CD4$^+$ and CD8$^+$ T cells in the LCWE-induced coronary vasculitis development, we performed simultaneous depletion of CD4$^+$ and CD8$^+$ T cells. CD4$^{-/-}$ mice were treated with anti-

CD8α depleting mAb and injected them with LCWE to induced KD. Acute vasculitis with intense heart vessels and myocardium inflammation developed in $CD4^{-/-}$ mice treated with the isotype control whereas CD8 depletion led to a significant protection from KD vasculitis development in $CD4^{-/-}$ mice (Supplementary Figure 4A, B). These data indicate that $CD4^{+}$ T cells do not play a role in LCWE-induced vasculitis and myocarditis, while $CD8^{+}$ T cells are a critical factor in determining the severity of the lesions.

Immunofluorescent staining of heart sections from LCWE-injected Rag-$1^{-/-}$ mice demonstrate the presence of innate inflammatory cells, such as neutrophils ($Ly6G^{+}$) and macrophages ($F4/80^{+}$) close to the coronary artery, although at decreased levels than LCWE-injected WT mice (Supplementary Figure 5 A–C and Figure 2B). It has been already published that T cells, but not B cells, are required for the development of LCWE-induced KD vasculitis and LCWE-injected Rag-$1^{-/-}$ mice demonstrate reduced heart lesions incidence (13). Therefore, we reasoned that reconstitution of the $CD8^{+}$ T cell compartment might recapitulate LCWE-induced KD vasculitis in resistant Rag-$1^{-/-}$ mice. Rag-$1^{-/-}$ mice were reconstituted with $CD45.1^{+}$ $CD8^{+}$ T cells isolated from the spleen of WT mice, injected with LCWE and analyzed 7 days post-injection. Adoptively transferred $CD8^{+}$ T cells were retrieved by flow cytometry 7 days after transfer in the spleen and draining lymph nodes (LNs) of the recipient mice (Supplementary Figure 6A, B). Compared to non-reconstituted LCWE-injected Rag-$1^{-/-}$ mice, mice reconstituted with $CD8^{+}$ T cells developed KD vasculitis as evidenced by intense inflammatory cell infiltration around the coronary artery and the aorta (Figure 5A). Quantification of heart vessels and myocardium inflammation demonstrated a significant increased incidence and severity of inflammation of coronary arteritis and myocardium inflammation in $CD8^{+}$ T cells reconstitute Rag-$1^{-/-}$ mice (Figure 5A, B). These results reveal the importance and the pathogenic role of $CD8^{+}$ T cells in the LCWE-induced KD vasculitis murine model.

DISCUSSION

In this study, we demonstrate that the LCWE-induced KD murine vasculitis model replicates additional features of the human KD pathology, including the development of epicardial coronary arteritis and LMP progression, key histological processes observed in human KD pathology. Recently, it has been postulated that 3 distinct, however linked, pathological processes are involved in the development of human KD vasculopathy (8). The initial process, necrotizing arteritis, triggers the 2 others, the sub-acute and acute chronic vasculitis as well as LMP in the CA potentially and ultimately leading to stenosis (8). In the current study, we now demonstrate for the first time that those 3 key histological human KD features are also recapitulated in LCWE-injected mice developing KD vasculitis. We are also reporting the development of epicardial coronary arteritis and the presence of LMP in the CA of LCWE-injected mice. By electronic microscopy, we have also identify monocytes, histiocytes and plasma cells in CA lesions of LCWE-injected mice, cells subsets also present in CA from human KD patients (8,21,31).

Increased evidence suggest that KD pathogenesis results from abnormal and imbalanced innate and adaptive immune responses, however the contribution of the different immunological cell subsets involved in the development of CA lesions, such as monocytes/

macrophages (31), $CD8^+$ T cells (23) and oligoclonal IgA^+ plasma cells (21,22), still remain obscure. Strategies aiming to down-modulate the activation of those immune cells and to induce regulatory mechanisms could potentially lead to the development of novel therapeutics. Several studies demonstrate abnormalities in the T_{Reg} cell compartment during human KD. Compared to healthy controls or febrile patients with an active non-KD infection, febrile patients in the acute KD phase exhibit decreased proportions of circulating $CD4^+$ $CD25^+$ $Foxp3^+$ T_{Reg} cells (36,37). *In vitro*, IVIG treatment enhances the suppressive functions of $CD4^+$ $CD25^+$ T_{Reg} cells and increases the intracellular expression of Foxp3, IL-10 and TGF-β (27). Thereby, the proportion of circulating T_{Reg} cells in KD patients returns to the same and normal levels observed in healthy controls between 2 to 3 days after IVIG treatment, however in IVIG-resistant children the percentages and absolute numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{Reg} cells remain significantly lower (25,36,38). We have observed the presence of $CD4^+$ $Foxp3^+$ T_{Reg} cells near the CA lesions of LCWE-injected mice, nevertheless their depletion by two different methodologies did not affect the development of KD vasculitis coronary arteritis and myocarditis in this murine model of KD vasculitis. While depletion of T_{Reg} cells did not lead to an increase in severity of inflammation, it would be interesting to see if strategies resulting in expanding T_{Reg} cell numbers, such as T_{Reg} cell adoptive transfer, could lead to decreased disease development and incidence.

Human KD vasculitis is characterized by intense $CD3^+$ T cell infiltration in the arterial layers of KD coronary aneurysm (19,39). Among the inflammatory infiltrations, the $CD3^+$ $CD8^+$ T cell population is more prevalent than $CD4^+$ T cell in KD patients as compared to healthy controls (23,24,39). Furthermore, RNA sequencing of CA tissues collected from either KD and non-KD control patients demonstrated a significant upregulation of pathways associated with activated cytotoxic T lymphocytes function, antigen presentation, toll like receptor (TLR) signaling and type I interferon response in KD arteritis (40).

Histopathological studies of the LCWE-induced KD vasculitis murine model revealed lesions similar to human KD with the presence of both T cells and DCs in the coronary arteries (13). While B cell deficient mice retained normal LCWE-induced KD lesions, Rag-$1^{-/-}$ mice were protected, indicating an important role for T cells in this model of KD (13). In this study we have shown that both $CD4^+$ and $CD8^+$ T cells were present in the LCWE-induced coronary lesions and that $CD8^+$ T cells play a critical role in LCWE-induced KD vasculitis. Our RNA sequencing data also demonstrate increased expression of genes associated with $CD8^+$ T cell cytotoxic functions, such as granzymes A and B, in heart tissues of LCWE-injected mice compared to PBS-injected mice. Guzman-Cottrill *et al.* also reported the predominant presence of $CD8^+$ T cells in inflammatory cell infiltrations during acute human KD coronary arteritis; however and surprisingly those $CD8^+$ T cells do not express the cytotoxic proteins granzyme B and perforin (39). Cytotoxic $CD8^+$ T cells also produce IFN-γ, however this cytokine does not appear to play a key function in LCWE-induced murine KD vasculitis since LCWE-injected IFN-$\gamma^{-/-}$ mice demonstrate similar disease incidence and coronary arteritis than WT mice (15,41).

We have previously shown that Caspase-1/IL-1α and IL-1β pathways are important for the development of coronary arteritis, myocarditis and abdominal aorta aneurysm in the LCWE-induced KD murine model (14,15,35). The body of evidence supporting the importance of

the IL-1 pathway in KD pathogenesis is now compelling enough for the launch of three different clinical trials of IL-1 blockade for treatment of acute KD (18). Of interest, IL-1 was shown to expand and enhance the differentiation of antigen-specific $CD8^+$ T cells *in vitro* and *in vivo* (42,43). IL-1 also increases $CD4^+$ T cells antigen-driven proliferation however IL-1 effect on $CD8^+$ T cells has been reported to be independent of the presence of $CD4^+$ T cells (43,44). In the present study, we report that the absence of $CD4^+$ T cell does not impact the development and incidence of LCWE-induced KD vasculitis. On the other hand, $CD8^+$ T cells had a dramatic effect on the development of LCWE-induced KD vasculitis; their depletion significantly decreased coronary arteritis and myocarditis whereas their adoptive transfer enforced vasculitis development in Rag-$1^{-/-}$ mice, a strain known to be resistant to LCWE-induced vasculitis. Still, the mechanisms by which IL-1 promotes $CD8^+$ T cells expansion remain unknown, appear to be direct and require the expression of IL-1R1 by $CD8^+$ T cells (43). It has been suggested that IL-1 could block $CD8^+$ T cells activation-induced cell death by decreasing their FasL expression (45). Because KD is thought to have an autoimmune component to its pathogenesis, perhaps $CD8^+$ T cells may act differently than usual cytotoxic cells and promote coronary arteritis by inducing apoptosis in otherwise normal healthy cells associated with the coronary. $CD8^+$ T-cells have also been found to secrete various chemokines which could lead to the recruitment of immune cells to the lesions. Characterization of the precise mechanisms by which $CD8^+$ T cells are effective and participate to the pathology of the LCWE-induced model of vasculitis would require further studies.

A possible sequential model of KD pathology development is that LCWE activates macrophages and DCs through the TLR2/MyD88 pathway (12) and induces their production of inflammatory cytokine, including IL-1β and IL-1α (15). The IL-1 released in the environment further acts on stromal cells near the sites of lesion development to promote additional release of pro-inflammatory cytokines, chemokines, and boost the influx of inflammatory cells (35). At the same time, DCs that co-localized in the CA lesions near the $CD3^+$ T cells present the antigen and stimulate an autoimmune response mediated by the activated $CD8^+$ T cells. The T cells migrate to the sites of inflammation, along with neutrophils, DCs, and macrophages to fully institute the vasculitic lesion development.

While the etiology of KD remains elusive, new treatment modalities are still needed, especially for children that do not respond to IVIG treatment and are at increased risk for coronary artery abnormalities. While the LCWE murine model of KD vasculitis only serves as a proxy for human KD, it has allowed us to model new therapeutics and predicted the use of IVIG and anti-TNFα in the clinical setting, as well as a two clinical trials (NCT02390596, NCT02179853) investigating the use of anakinra in KD patients and one using anti-IL-1β (EUCTR 2015-003763-11). Our current studies identified $CD8^+$ T cells as playing an important role in lesion development and thus place these cells a potential target for therapy. Until the exact etiology and immunopathology of human KD is known, continued research with the mouse model of KD vasculitis remains an important endeavor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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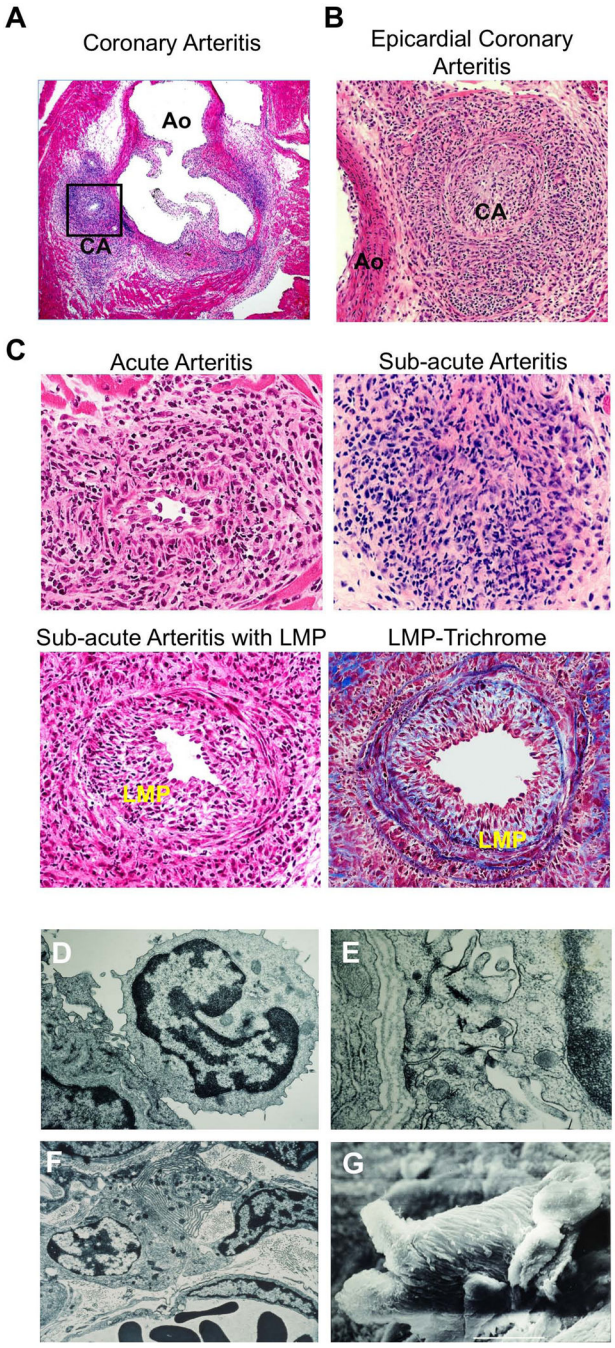


Figure 1. Histologic and morphological findings in the LCWE-induced KD murine model

WT mice were injected with LCWE i.p., hearts were harvested 14 days later, H&E and Trichrome staining as well as TEM and SEM were performed on heart sections. Ao and CA indicates the lumen of the aorta and the coronary artery respectively. (**A**) Lesions of the aortic route and inflammatory cell infiltration around the CA with an almost complete obliteration of the ostium of the coronary artery (magnification X40). (**B**) Heart section showing epicardial coronary arteritis and absence of inflammatory cells in the aortic wall (magnification X100). (**C**) Acute arteritis with an obliterated artery, a sub-acute and sub-

acute arteritis characterized by LMP. LMP and non-specific neointimal proliferation injury to the arterial wall as demonstrated by trichrome staining (magnification X200). (**D**) TEM micrograph showing a monocyte adhering into coronary endothelium (magnification X21,000). (**E**) TEM micrograph of a monocyte fusion with an endothelial cell cytoplasm (magnification X66,000). (**F**) TEM micrograph of two plasma cells and an histiocyte adjacent to a capillary in the CA (magnification X18,000). (**G**) SEM micrograph of an adhering fibroblast to the coronary artery wall (magnification X6000).

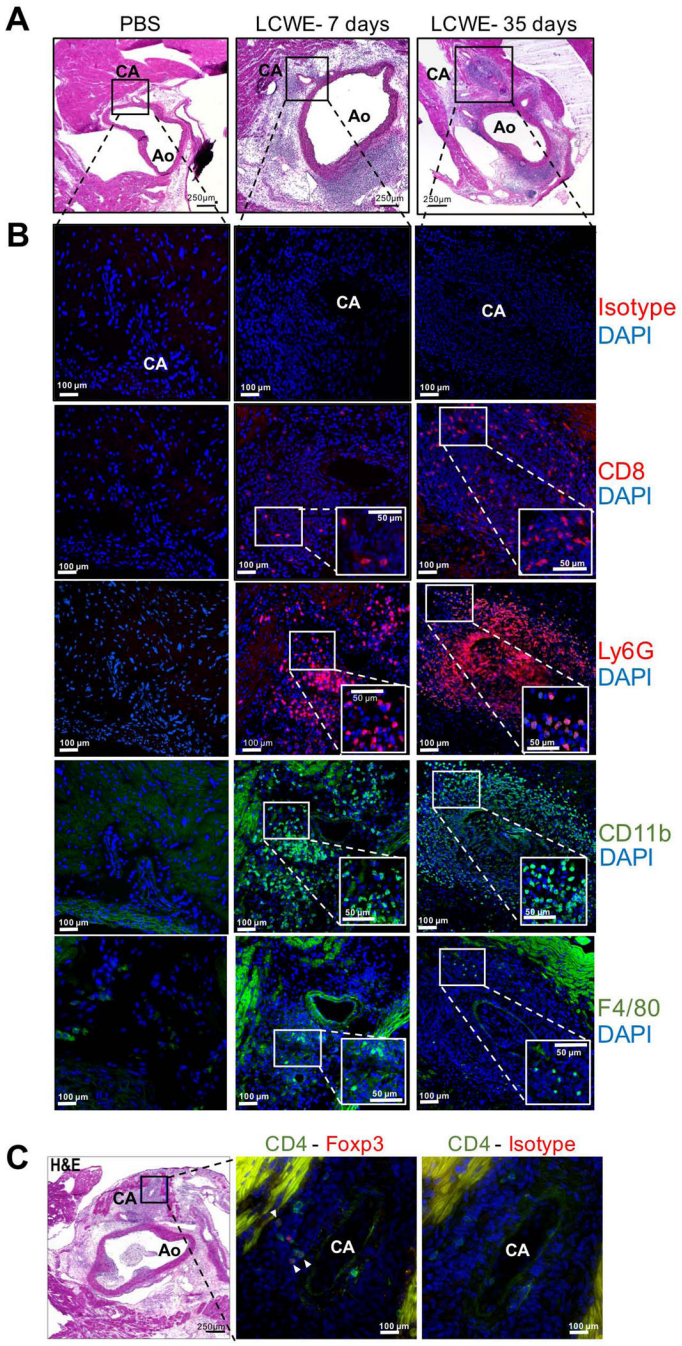


Figure 2. CD4$^+$, CD8$^+$ and T_{Reg} cells infiltrate the coronary artery in the KD murine model

Hearts of PBS or LCWE-injected WT mice were harvested at days 7 and 35 post-injection. (**A**) H&E staining from frozen heart serial sections. (**B**) Immunofluorescent co-staining from serial frozen heart sections for isotype control (red color), anti-CD8 (red), anti-Ly6G (red), anti-CD11b (green) or anti-F4/80 (green) and DAPI (Blue). (**C**) H&E and immunofluorescent co-staining for anti-CD4 (Green), anti-Foxp3 or isotype control (Red) and DAPI (Blue) from frozen heart serial sections 7 days post LCWE injection. The white arrows indicate CD4$^+$ Foxp3$^+$ T_{Reg} cells. CA stands for coronary artery and Ao for the aorta.

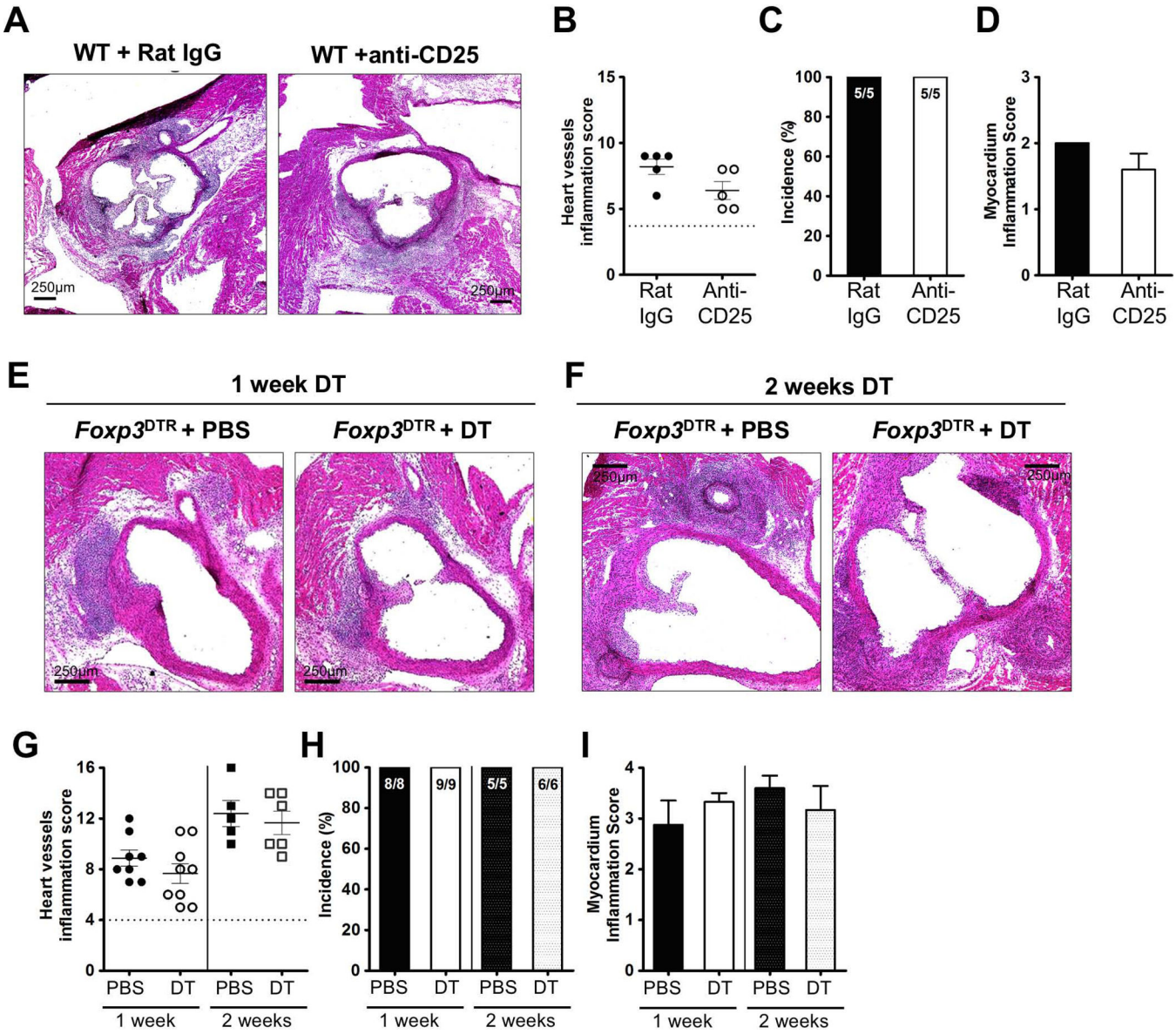
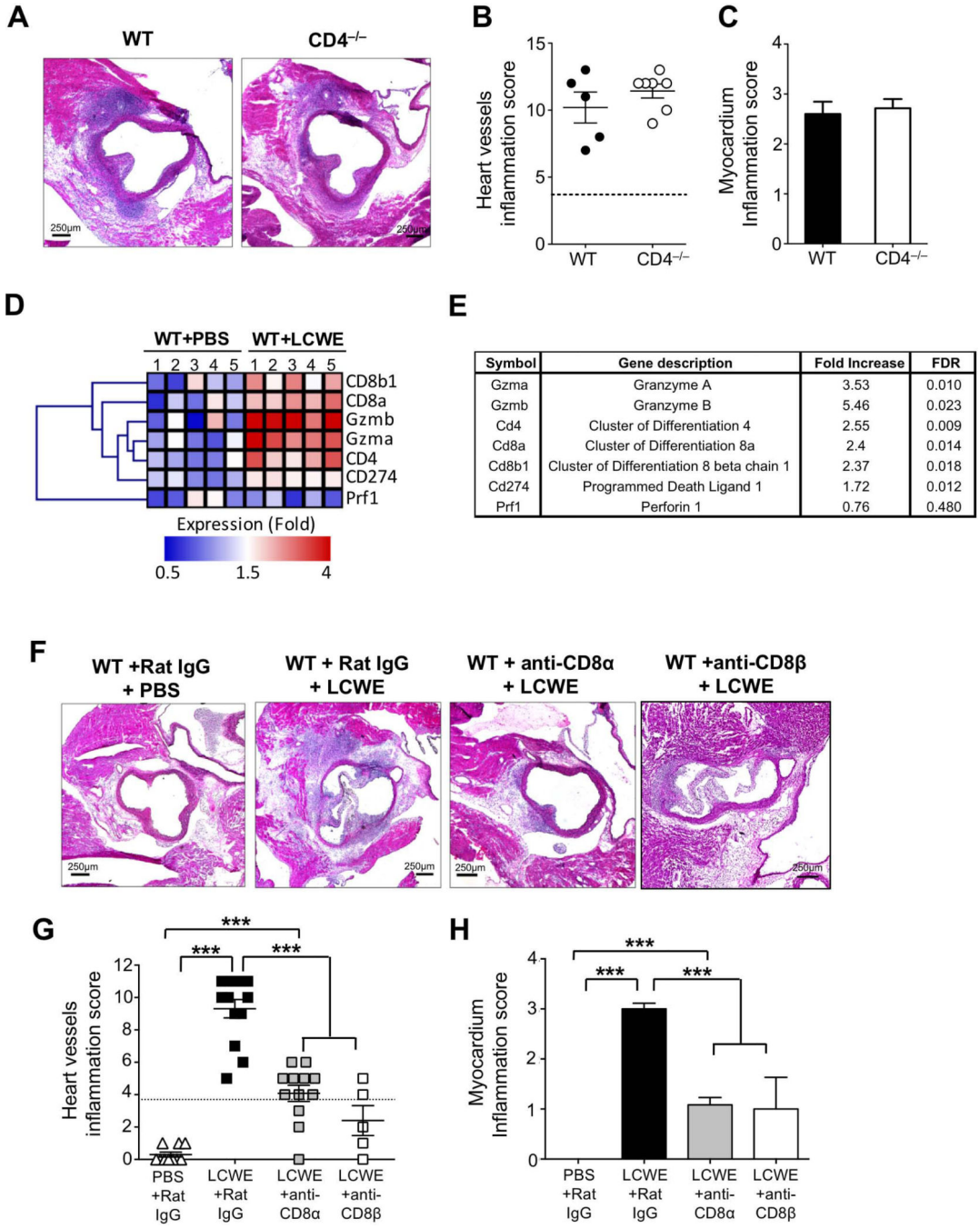


Figure 3. T_{Reg} depletion does not protect from LCWE-induced KD vasculitis and myocarditis

(**A**) H&E staining of frozen heart sections from LCWE-injected WT mice treated with anti-CD25 mAb or a Rat IgG isotype control 7 days after LCWE injection. (**B–D**) Heart vessels inflammation score (B), vasculitis incidence (C) and myocardial inflammation score (D) of anti-CD25 or Rat IgG treated LCWE-injected WT mice. (**E–F**) H&E staining from frozen heart section of PBS or DT treated *Foxp3*^{DTR} mice 7 (E) and 14 days (F) after LCWE injection. (**G–I)** Heart vessels inflammation score (G), vasculitis incidence (H) and myocardial inflammation score (I) of PBS or DT treated *Foxp3*^{DTR} mice 7 and 14 days after LCWE injection. Results presented as mean ± SEM, n=5–9 mice/group. Statistics were performed by Mann-Whitney unpaired t test (B, D, F, H) and Fisher exact test for incidence (C and G).



Symbol	Gene description	Fold Increase	FDR
Gzma	Granzyme A	3.53	0.010
Gzmb	Granzyme B	5.46	0.023
Cd4	Cluster of Differentiation 4	2.55	0.009
Cd8a	Cluster of Differentiation 8a	2.4	0.014
Cd8b1	Cluster of Differentiation 8 beta chain 1	2.37	0.018
Cd274	Programmed Death Ligand 1	1.72	0.012
Prf1	Perforin 1	0.76	0.480

Figure 4. CD8$^+$ T cells depletion protects against KD vasculitis and myocarditis

(**A**) H&E staining of frozen heart sections of WT and CD4$^{-/-}$ mice 7 days after LCWE injection. (**B–C**) Heart vessels inflammation (B) and myocardial inflammation (C) scores of LCWE-injected WT and CD4$^{-/-}$ mice 7 days post-injection. (**D–E**) Heat map (D) and list (E) of CD8$^+$ cytotoxic T cell functions related genes expression in heart tissues collected from LCWE-injected WT mice (n=5) relative to the average expression of PBS-injected control mice (n=5). (**F**) H&E staining of frozen heart sections from PBS and LCWE-injected WT mice treated either with anti-CD8α, anti-CD8β or Rat IgG isotype control 7 days after

LCWE injection. (**G, H**) Heart vessels inflammation (**G**) and myocardial inflammation (**H**) scores of PBS or LCWE-injected WT mice treated with either anti-CD8α, anti-CD8β or Rat IgG isotype 7 days post-LCWE injection. Results presented as mean ± SEM, n=5–12 mice/group. *** $p < 0.001$ by one way ANOVA with Tukey's post-test analysis.

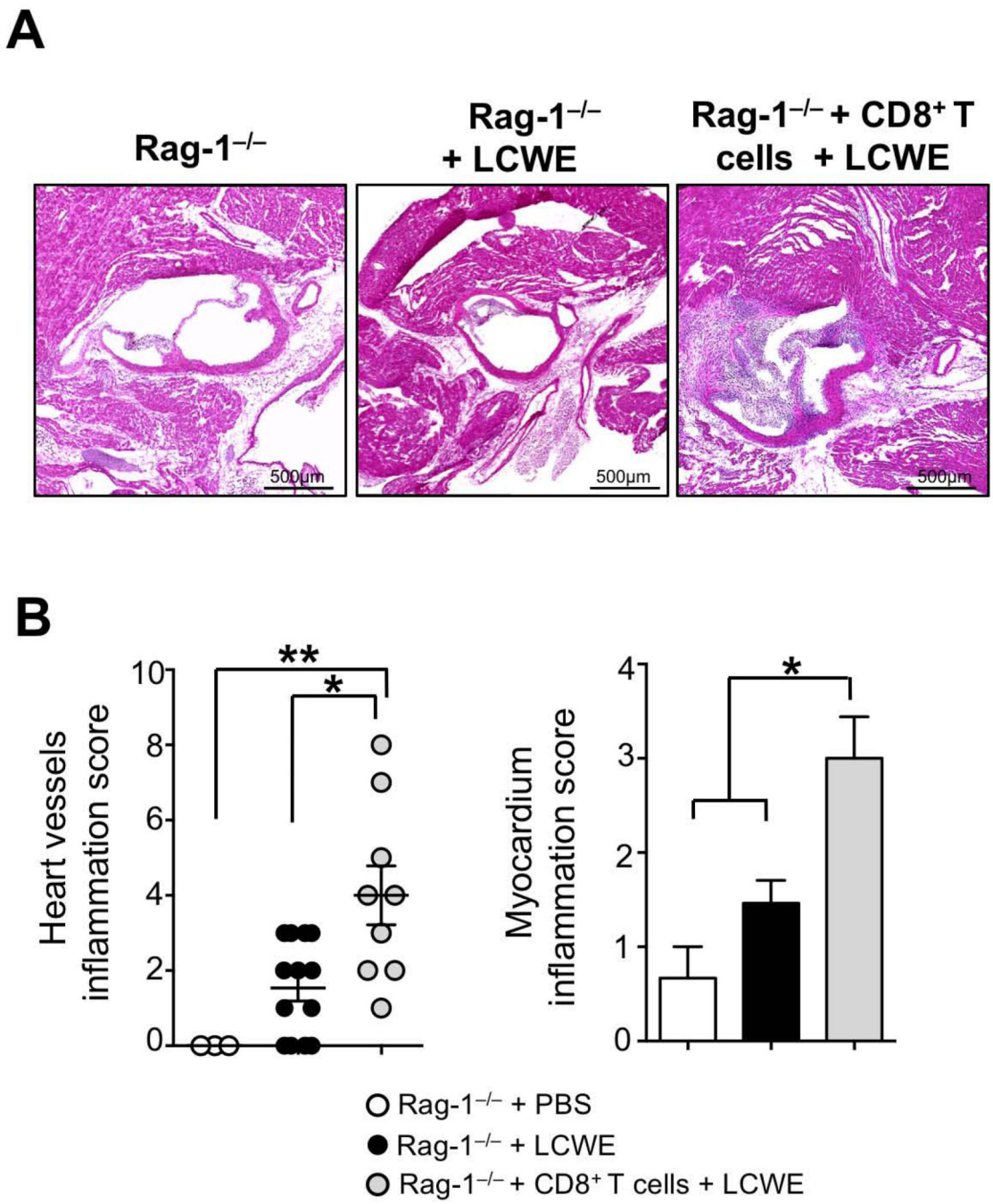


Figure 5. Adoptive transfer of CD8$^+$ T cells restores KD vasculitis development in Rag-1$^{-/-}$ mice

(**A–B**) CD8$^+$ T cells were purified from naïve CD45.1$^+$ WT C57BL/6 mice and i.v. transferred into Rag-1$^{-/-}$ mice one day before LCWE injection. Hearts were harvested 7 days post-LCWE injection. (**A**) H&E staining of frozen heart section from PBS-injected Rag-1$^{-/-}$ mice, Rag-1$^{-/-}$ mice and CD8$^+$ T cells reconstituted Rag-1$^{-/-}$ mice injected with LCWE. (**B**) Heart vessels and myocardial inflammation score from the mouse groups in (A).

Results presented as mean ± SEM, n=3–12 mice/group. *$p < 0.05$ and ** $p < 0.01$ by one way ANOVA with Tukey's post-test analysis.