Identification of Pathogenic Cardiac CD11c+ Macrophages in Nod1-Mediated Acute Coronary Arteritis

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Objective—Nod1 is an intracellular pattern recognition receptor for bacterial peptidoglycan fragments. We previously reported that a synthetic Nod1 ligand, FK565, induced acute coronary arteritis in mice similar to that of Kawasaki disease. However, the molecular mechanisms underlying this characteristic inflammation have remained elusive.

Approach and Results—We found that CD11c+MHC class II+ cells accumulated in the heart of FK565-treated mice before arteritis development. Morphological features and gene expression signatures of the cardiac CD11c+MHC class II+ cells suggested that this population is closely related to macrophages, and thus, we designated them cardiac CD11c+ macrophages. Nod1 in nonhematopoietic cells, rather than hematopoietic cells, was required for the increase of cardiac CD11c+ macrophages and arteritis development. Among nonhematopoietic cells, cardiac endothelial cells produced a large amount of chemokines in response to FK565. Endothelial cell–specific blockade of Nod1 signaling suppressed FK565-induced expression of these chemokines, accumulation of cardiac CD11c+ macrophages, and subsequent coronary arteritis development. We also found that CCR2-Ly6C+ inflammatory monocytes in peripheral blood supplied precursors of cardiac CD11c+ macrophages. CCR2-deficient mice or pertussis toxin–treated mice exhibited decreased numbers of cardiac CD11c+ macrophages and reduced arteritis.

Conclusions—These results suggest that Ly6C+ monocytes are recruited to FK565-activated endothelial cells to generate cardiac CD11c+ macrophages, which play a pivotal role in the pathogenesis of acute coronary arteritis. (Arterioscler Thromb Vasc Biol. 2015;35:1423-1433. DOI: 10.1161/ATVBAHA.114.304846.)

Key Words: chemokines ■ endothelial cells ■ macrophages ■ mucocutaneous lymph node syndrome ■ vasculitis

Kawasaki disease (KD) is an acute febrile illness characterized by inflammation that has a striking propensity to damage coronary arteries.1 Although KD is the most common cause of acquired cardiac disease and acute vasculitis in children in the developed world, its cause is largely unknown.2 The epidemiological data suggest that KD has a distinct seasonal occurrence, which implies that pathogens may be involved in the unique inflammation.3 To date, there are a few animal models of KD coronary arteritis using crude extracts from microorganisms, such as Lactobacillus casei or Candida albicans.4,5 However, the molecular and cellular mechanisms underlying this characteristic inflammation remain elusive.

FK565 (heptanoyl-γ-glutamy1(-)-meso-diaminopimelyl(-)-alanine) is a synthetic ligand for nucleotid-binding oligomerization domain-containing protein 1 (Nod1).6 We have recently reported that injection of FK565 in mice induces severe vasculitis at the aortic root similar to that seen in the acute phase of KD.7 Nod1 is a member of the intracellular nucleotide–binding domain and leucine-rich repeat family and recognizes peptidoglycan fragments containing meso-diaminopimelic acid mainly found in Gram-negative bacteria.8 On binding of its ligands, Nod1 oligomerizes and activates nuclear factor-κB, which results in transcription of proinflammatory cytokines and chemokines.9 In contrast to Nod2, which exhibits myeloid cell–specific expression, Nod1 is widely expressed in various tissue types.9 FK565 failed to induce coronary arteritis in Nod1-deficient mice.1 However, it remains unclear what population(s) of cells triggers tissue-specific arteritis.

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1423
Macrophages are phagocytic cells residing in nonlymphoid tissues and are involved in steady-state tissue homeostasis. However, the macrophage population undergoes dynamic alterations under inflammatory conditions, as myeloid cells derived from blood monocytes accumulate in inflamed tissues. Ly6C^hi monocytes are frequently referred to as inflammatory monocytes in a variety of infectious models. After tissue injury or infection, Ly6C^hi monocytes are recruited to the tissues and give rise to effector cells, such as monocyte-derived dendritic cells (DCs), that initiate inflammation. Coronary arteritis in patients with KD is characterized by proliferative granulomatous inflammation and marked accumulation of mononuclear cells at the base of the coronary artery. These cells are implicated in the pathogenesis of the arteritis; however, there is a paucity of data on the origins and functions of these cardiac-infiltrating cells during the course of coronary arteritis in KD.

In this study, we show that, after injection of Nod1 ligand and prior to the onset of arteritis, monocytic-derived CD11c^+ macrophages accumulated in the heart. In vivo depletion of this macrophage population prevented arteritis, thus indicating that the cardiac CD11c^+ macrophages play a pathogenic role in KD-like acute coronary arteritis.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Essential Role of Mononuclear Phagocytes in Nod1-Mediated Coronary Arteritis
Administration of FK565 induces severe vasculitis at the base of coronary artery, which is characterized by granulomatous inflammation and destruction of elastic fiber (Figure 1A). To determine which cell type(s) is responsible for triggering the arteritis, we analyzed several mutant mice in which specific immune cell populations are genetically depleted. FK565 induced typical coronary arteritis in Rag1^-/- mice, indicating that T, B, and natural killer (NK) T cells were dispensable for the development of arteritis (Figure 1B). In addition, FK565 elicited arteritis in IL15^-/- mice, which lack NK cells (Figure 1B). We quantitatively estimated the severity of the arteritis by determining the area of the inflammatory lesion and the number of infiltrating cells. There was no significant difference between wild-type (WT), Rag1^-/-, and IL-15^-/- mice in these parameters (Figure 1C and 1D).

We next investigated the contributions of the myeloid cell population using antibody-mediated cell depletion. Administration of anti-Ly6G (1A8) in mice efficiently depleted neutrophils (Figure 1E) but had no effect on FK565-induced arteritis (Figure 1F–1H). Intravenous injection of clodronate liposomes (Clod-liposome), widely used for systemic depletion of mononuclear phagocytes, including monocytes, macrophages, and DCs in vivo, rapidly eliminated blood monocytes but not neutrophils (Figure 1E). In Clod-liposome-treated mice, FK565-induced coronary arteritis was dramatically reduced (Figure 1F–1H).

Collectively, these results reveal that mononuclear phagocytes, but not neutrophils and lymphocytes, play a role in the pathogenesis of FK565-induced arteritis.

Accumulation of CD11c^+ MHC Class II^+ Cells at the Site of Inflammation
To further characterize the cell types involved in arteritis, we analyzed heart-infiltrating cells in FK565-treated mice. We observed a significant accumulation of CD45^- hematopoietic cells in the heart after FK565 treatment (Figure 2A). The cell infiltration was Nod1-dependent, as this was not observed in Nod1^-/- mice (Figure 2A). When we divided cardiac CD45^- cells into myeloid cells (CD11b^+Ly6G^-NK1.1^-), neutrophils (CD11b^+Ly6G^-), NK cells (CD11b^-Ly6G^-NK1.1^-), and a CD11b^- population that included lymphocytes, we found that the number of myeloid CD11b^-Ly6G^-NK1.1^- cells was increased in FK565-treated mice (Figure 2A and 2B). Within this population, the frequency of CD11c^- MHC II^+ cells were markedly increased (Figure 2A and 2C), and immunohistochemical analyses revealed that CD11c^- mononuclear cells accumulated around the coronary artery (Figure 2D). The CD11c^- MHC II^+ population also expressed macrophage markers (CD64, F4/80, and SIRPα) and costimulatory molecules (CD40 and CD80; Figure 2E) but lacked the expression of markers for alveolar macrophages (Siglec F) and DC subsets (CD8α, B220, and CCR7) (Figure 2E).

We next investigated the morphological features of the cardiac CD11c^- MHC II^+ cells. Transmission electron microscopic analysis revealed that this population had mononuclear nuclei, electron-dense lysosomes, and dendrites (Figure 2F). To further characterize the identity of this population, we compared the gene expression signature of the cardiac CD11c^- MHC II^+ cells with those of various hematopoietic cells. Cluster analysis (Figure 1A in the online-only Data Supplement) and principal component analysis (Figure 1B in the online-only Data Supplement) of the microarray data indicated that this population clustered together with macrophages. This subset constitutively expressed unique sets of genes, such as MerTK and CD64 (Figure 2F) but lacked the expression of markers for alveolar macrophages (Siglec F) and DC subsets (CD8α, B220, and CCR7) (Figure 2E).

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9, before the recovery of the coronary artery, CD11c-MHC II+ cells were diminished in the heart. The coronary arteritis rapidly dissipated by day 14. These kinetics suggest that the cardiac CD11c+ macrophages may be closely related to the initiation of arteritis.

Depletion of CD11c+ Cells Compromised Nod1-Mediated Arteritis

To assess whether cardiac CD11c+ macrophages are required for FK565-induced arteritis, we used 2 approaches to deplete this population in vivo. As shown in Figure 1F, Clod-liposome treatment efficiently prevented arteritis. In Clod-liposome–treated mice, the number of cardiac CD11c+ macrophages was found to be dramatically reduced compared with untreated mice (Figure 3A).

We next performed specific depletion of CD11c+ cells using CD11c-diphtheria toxin receptor (DTR) transgenic (Tg) mice. As administration of DT in CD11c-DTR mice is known to be lethal, we reconstituted WT mice with bone marrow (BM) cells from CD11c-DTR mice on the Ly5.1 background. Blood CD11c+ cells were decreased to one third of normal levels on DT treatment. These mice showed significantly a lower number of cardiac CD11c+ macrophages compared with control mice (Figure 3C). Importantly, the severity of coronary arteritis was also significantly reduced in CD11c+ cell–depleted mice (Figure 3D–3F). Collectively,
these results suggest that cardiac CD11c+ macrophages promote FK565-induced coronary artery disorder.

Among CD11c+ macrophage populations, we have recently shown that CD169+ macrophages are involved in vasculitis.13 We thus investigated the role of this subset in Nod1-induced coronary arteritis using CD169-DTR mice.14,15 However, specific depletion of CD169+ macrophages did not alter the frequency of cardiac CD11c+ macrophages or subsequent arteritis (Figure 3G–3J), suggesting that this subset plays a minor role in the arteritis.

Nod1 Expression in Nonhematopoietic Cells Was Required for Arteritis

Since Nod1 is ubiquitously expressed, we next established which Nod1-expressing cells are responsible for triggering the accumulation of cardiac CD11c+ macrophages and the coronary arteritis. To determine whether Nod1-expressing hematopoietic or nonhematopoietic cells are required for these events, we established reciprocal BM chimera mice using WT mice (Ly5.1+ background) and Nod1–/– mice (Ly5.2+ background). BM cells from WT or Nod1–/– mice were transferred into Nod1–/– or WT recipient mice (and vice versa) that had been lethally irradiated. In WT mice reconstituted with Nod1–/– BM cells, the severity of coronary arteritis was not altered (Figure 4A–4C). Furthermore, a significant accumulation of Nod1-deficient CD11c+ MHC II+ macrophages was evident in WT hosts (Figure 4D and 4E), indicating that these macrophages do not need to express Nod1 on their own to accumulate in the heart. In contrast, Nod1+ mice reconstituted with WT BM cells did not show any signs of arteritis or accumulation of CD11c+ macrophages (Figure 4A–4D). These results indicate that Nod1 expression in nonhematopoietic cells, but
not in hematopoietic cells, is required for coronary arteritis induced by FK565.

**Blockade of Nuclear Factor-κB Signaling in Endothelial Cells Compromised Arteritis**

We next determined the Nod1-expressing nonhematopoietic cells responsible for the pathogenesis of the arteritis. Endothelial cells (ECs) are a strong candidate, as they are known to regulate cardiovascular homeostasis in various physiological and pathological processes. To block Nod1 signaling in an endothelium-specific manner, we used Tg mice that express dominant-negative IκBα in ECs (E-DNIκBα Tg; Figure IV in the online-only Data Supplement). FK565-induced arteritis and the number of CD11c+ macrophages in the heart were both significantly reduced in the E-DNIκBα Tg mice (Figure 4F–4I). Collectively, these results suggested that nuclear factor-κB–activating receptors, including Nod1 in ECs, play a crucial role in the accumulation of CD11c+ macrophages and the pathogenesis of coronary arteritis.

**Ly6C⁺ Blood Monocytes Migrated to the Heart in Response to FK565**

From the above observations, we hypothesized that cardiac ECs may recruit precursors of CD11c+ macrophages from peripheral blood to heart tissue. We therefore examined the cellularity of peripheral blood after FK565 treatment. The proportion of blood CD11b+Ly6G−NK1.1− cells increased before the accumulation of cardiac CD11c+ macrophages (Figure 5A). The majority of the CD11b+Ly6G NK1.1− cells were CD115+ and F4/80+, thus being characterized as monocytes (Figure 5A). These monocytes consisted of 2 populations, Ly6C⁺CD11c⁺ and Ly6C⁻CD11c− cells (Figure 5A),
both of which were increased in the periphery on FK565 treatment in a Nod1-dependent manner (Figure 5B). Ly6C<sup>hi</sup> monocytes are referred to as inflammatory monocytes and are known to cause inflammation after migration into tissues. To determine whether this population contains a precursor of cardiac CD11c<sup>+</sup> macrophages, Ly6C<sup>hi</sup> monocytes were selectively labeled using FITC-conjugated beads (Figure 5C). After depletion of blood monocytes using Clod-liposome, Ly6C<sup>hi</sup> monocytes rapidly recover and incorporate fluorescence beads. When these mice were further treated with FK565, a substantial population of fluorescence-positive cardiac CD11c<sup>+</sup> macrophages appeared (Figure 5C). In contrast, residential cardiac DCs (CD11c<sup>+</sup>CD103<sup>+</sup> cells) were negative for fluorescence.

**Figure 4.** Contribution of Nod1 in endothelial cells to the development of coronary arteritis. A–C, Reciprocal bone marrow (BM) chimeras using wild-type (WT) mice (Ly5.1<sup>+</sup> background) and Nod1<sup>−/−</sup> mice (Ly5.2<sup>+</sup> background) were established. Histological evaluation of coronary arteritis in the indicated BM chimeras after administration of FK565. Evaluation was performed as in Figure 1B–1D. D, Numbers of cardiac CD11c<sup>+</sup> macrophages in BM chimeras after administration of FK565. E, Donor chimerism of cardiac CD11c<sup>+</sup> macrophages (cardiac CD11c<sup>+</sup> macs) in Ly5.1<sup>+</sup> WT mice reconstituted with Ly5.2<sup>+</sup> Nod1<sup>−/−</sup> BM (WT≤Nod1<sup>−/−</sup>). The percentage of each gated subpopulation is indicated. F–H, Histological evaluation of coronary arteritis in WT littermates (WT) and E-DNikB<sup>Tg</sup> mice (Tg) after administration of FK565. I, Number of cardiac CD11c<sup>+</sup> macs in WT littermates and E-DNikB<sup>Tg</sup> mice treated with FK565. In B, C, G, and H, each circle represents an individual mouse. Data are presented as means±SD (D and I) and are representative of 2 independent experiments with 3 to 5 mice per group; *P<0.05 and **P<0.01.
Collectively, these results suggest that the cardiac CD11c+ macrophages mainly originate from blood Ly6C\textsuperscript{hi} monocytes.

Transfer of Ly6C\textsuperscript{hi} Monocytes Generated Cardiac CD11c\textsuperscript{+} Macrophages

We next asked whether adoptive transfer of Ly6C\textsuperscript{hi} monocytes is sufficient to generate cardiac CD11c\textsuperscript{+} macrophages. Consistent with the results from Clod-liposome treatment (Figure 1F–1H; Figure 3A), systemic depletion of radiosensitive hematopoietic cells by whole-body irradiation prevented both appearance of cardiac CD11c\textsuperscript{+} macrophages and arteritis development (Figure 5D, 5F, and 5G). However, adoptive transfer of Ly6C\textsuperscript{hi} BM monocytes into these mice restored the accumulation of the cardiac CD11c\textsuperscript{+} macrophages (Figure 5D and 5E). Notably, substantial arteritis, characterized by a thickening of the coronary artery wall and cell infiltration, was detected in these mice after transfer (Figure 5F and 5G). Taken together with the above results, we concluded that Ly6C\textsuperscript{hi} monocytes are strong candidates for the precursors of pathogenic CD11c\textsuperscript{+} macrophages in the heart.

ECs Produced Chemokines Through Nod1

We next examined whether cardiac ECs produce chemotactic factors to recruit blood monocytes. To assess this possibility, CD31\textsuperscript{+} ECs were purified from murine hearts and stimulated with FK565 in vitro. FK565 induced the expression...
of CCL2, CCL5, CXCL2, CXCL5, and CXCL10 in ECs in a Nod1-dependent manner (Figure 6A). Of note, these chemokines were preferentially induced in ECs rather than BM-derived macrophages (Figure 6A). Likewise, primary human ECs preferentially responded to FK565 to produce CCL2 compared with smooth muscle cells or adventitial fibroblast (Figure V in the online-only Data Supplement). This is in contrast to the inflammatory cytokines tumor necrosis factor and IL-1α, which were mainly produced by BM-derived macrophages (Figure VIA in the online-only Data Supplement). In addition, these chemokines were not induced in ECs from E-DNIκB Tg mice (Figure VIB in the online-only Data Supplement). These results indicate that cardiac ECs respond to Nod1 ligand by producing chemokines, which may recruit the precursors of cardiac CD11c+ macrophages. Indeed, the increase of CD11c+ macrophages was not due to the local proliferation of tissue resident cells (Figure VII in the online-only Data Supplement).
Inhibition of G-Protein Coupled Receptor
Attenuated Coronary Arteritis

The above observations led us to examine the role of EC-derived chemokines in the accumulation of cardiac CD11c+ macrophages and the pathogenesis of arteritis. To this end, we treated mice with pertussis toxin, which blocks G protein–coupled receptors, including chemokine receptors. Figure 6B–6E show that pertussis toxin treatment almost completely suppressed the accumulation of cardiac CD11c+ macrophages and aorta destruction. These results suggest that G protein–coupled receptors, including chemokine receptors, are involved in the accumulation of cardiac CD11c+ macrophages and arteritis development.

**Discussion**

In this study, we show that the accumulation of cardiac CD11c+ macrophages plays a central role in acute coronary arteritis.

CD11c antigen has been a commonly used marker for conventional DCs, although recent studies have revealed that it is expressed by various myeloid subsets. Yet, the cardiac CD11c+ macrophages characterized in this study seem to be distinct from such previously reported myeloid subsets. For example, alveolar CD11c+ macrophages express Siglec F19 and CD169/CD11c+ macrophages residing in lymph nodes express CD8α,18 whereas the cardiac CD11c+ cells did not express these markers (Figure 2E). Furthermore, depletion of CD169+ macrophages using CD169-DTR mice did not alter the frequency of cardiac CD11c+ macrophages or subsequent arteritis (Figure 3G–3J). Several other CD11c+ tissue macrophages have been recently reported in tissues, such as lung, kidney, intestine, and adipose tissues. At present, the relationship between these tissue-specific macrophages and the cardiac CD11c+ macrophages has not been rigorously investigated. Monocyte-derived inflammatory DCs are specialized in triggering immune responses at sites of inflammation and originate from circulating CCR2+Ly6C+ monocytes. Hence, monocyte-derived DCs share some similarities with cardiac CD11c+ macrophages with respect to their origin and inflammatory potential, although they differ from each other in their expression level of Ly6C. To the best of our knowledge, the most similar population to cardiac CD11c+ macrophages would be monocyte-derived inflammatory macrophages in lamina propria, as they acquire CD11c and major histocompatibility complex (MHC) II expression with decreasing Ly6C expression during differentiation in gut.

Recently, Epelman et al demonstrated that a small number of Ly6C+ monocyte-derived CD11c+MHC II+ macrophages were observed in the inflamed heart. This population might be closely related to cardiac CD11c+ macrophages, and this issue warrants further investigation. Collectively, these recent observations define Ly6C+ monocytes as one of the most important potential precursors of cardiac macrophages during inflammation.

As cardiac CD11c+ macrophages express costimulatory molecules and MHC II, they might participate in tissue inflammation as antigen-presenting cells. However, this seems unlikely because T cell-deficient Rag1−/− mice develop coronary arteritis in response to Nod1 ligand (Figure 1B). Furthermore, we confirmed that MHC molecules were dispensable for these events, as FK565 induced coronary arteritis in MHC-deficient (TAP1−/−CIITA−/−) mice (data not shown).

Although cardiac CD11c+ macrophages are responsible for FK565-induced arteritis, the key effector molecule(s) that participates in the progression of the inflammation is still unclear. The gene expression profile of cardiac CD11c+ macrophages showed that they express classical M1-like genes rather than M2-like genes (data not shown).27 Notably, matrix metalloproteinase (MMP)-2, MMP-9, MMP-13, and MMP-24 were highly expressed in the cardiac CD11c+ macrophages (Figure II A in the online-only Data Supplement). These proteases may exacerbate arteritis by directly targeting elastic fibers, as recent studies demonstrate the genetic association between MMPs and KD.28,29 Given that the control of progression during the destruction phase is important for the prognosis of KD, more detailed studies are needed to clarify the molecular mechanisms acting at this critical stage. On the other hand, the characteristics of cardiac CD11c+ macrophages might suggest a physiological role for this population during infection or noninfectious insults, such as mediation of antimicrobial activity or tissue remodeling/repair.

It has been demonstrated that crude microbial components induce coronary arteritis similar to KD. In Lactobacillus casei cell wall extract–induced coronary arteritis, toll-like receptor-2 and Dectin-1 are reported to trigger the pathogenesis. Dectin-2 might be responsible for Candida albicans water-soluble fraction–induced KD, as Dectin-2 mediates the production of proinflammatory cytokines in response to Candida albicans water-soluble fraction. It will be intriguing to investigate whether cardiac CD11c+ macrophages also contribute to pathogenesis in other KD models that are triggered through these pattern recognition receptors.

Nod1 ligand induced increase in blood Ly6C+ monocytes (Figure 5A and 5B). One of the possible origins of these blood monocytes may be BM monocytes, as BM monocytes egress
into peripheral blood in response to inflammatory stimuli.\(^3\),\(^4\)
In fact, the frequency of Ly6C\(^{hi}\) monocytes in BM was slightly decreased on FK565 injection (data not shown). Thus, Ly6C\(^{hi}\) monocytes in BM might migrate into the periphery in response to Nod1 stimulation and may thus provide a progenitor pool of heart-infiltrating CD11c\(^{+}\) macrophages.

Our study illustrated the importance of Nod1 in nonhematopoietic cells. Recently, the role of Nod1 in nonhematopoietic cells has been demonstrated in numerous in vivo models.\(^3\),\(^5\),\(^6\)
Thus, Nod1 seems to be a principal pattern recognition receptor that shapes nonhematopoietic environments for the function of hematopoietic cells in various physiological and pathological settings.

Since there is no specific diagnostic tests for KD, more definite criteria are needed for early therapeutic interventions. We showed here that the increase of Ly6C\(^{hi}\) monocytes in peripheral blood occurred before arteritis. In this respect, there was an association between increased numbers of CD14\(^{+}\) monocytes in peripheral blood and the occurrence of coronary artery lesions in KD.\(^3\)
A monocyte marker CD64 was also significantly upregulated in peripheral blood mononuclear cell transcriptomes of patients with acute KD.\(^9\)
Thus, monitoring the precursor monocytes in patients may be a novel diagnostic option for KD.

The complete suppression of arteritis by pertussis toxin in this study suggests a critical role for G-protein–coupled receptors in the progression of KD-like coronary arteritis. Given this study suggests a critical role for G-protein–coupled receptors in the partial suppression of arteritis in CCR2\(^{-/-}\) mice, it is possible that other chemokines or G-protein–coupled receptors–mediated pathways may also contribute to this process.\(^4\)
Intravenous γ-globulin is a definitive and successful first-line therapy for KD, whereas its mechanisms of action are not fully understood. It is reported that one possible mechanism of intravenous γ-globulin is neutralization or downregulation of γ-globulin is a definitive and successful first-line therapy for KD, whereas its mechanisms of action are not fully understood. It is reported that one possible mechanism of intravenous γ-globulin is neutralization or downregulation of serum chemokines.\(^4\),\(^5\)
Together with accumulating evidence, this study proposes that blockades of key chemokine receptors could represent a novel therapeutic option for KD.

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**Disclosures**

None.

**References**

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Pathogenic Macrophages in Acute Coronary Arteritis


Significance

Kawasaki disease is a fatal cardiac disease with acute coronary arteritis. However, the actual cause is still unknown. Here, we demonstrate that a unique cardiac CD11c+ macrophages play a pathogenic role in coronary arteritis induced by the Nod ligand. This population originates from Ly6C+ blood monocytes after Nod1 ligand stimulation. We therefore propose that Nod1 ligand induces coronary arteritis via sequential processes: (1) Nod1 ligand activates cardiac endothelial cells to produce chemokines, (2) these chemokines recruit Ly6C+ monocytes from the blood into the heart thereby generating cardiac CD11c+ macrophages, and (3) cardiac CD11c+ macrophages expressing inflammatory genes induce coronary arteritis. Since there are currently no specific diagnostic tests for Kawasaki disease, monitoring the precursor monocytes in patients may be a novel promising diagnostic tool for Kawasaki disease. Furthermore, the arteritis score was ameliorated in mice lacking CCR2, suggesting that blockade of key chemokine receptors could represent a novel therapeutic option for Kawasaki disease.
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Materials and Methods

Mice
C57BL/6J mice were obtained from Japan Clea. Congenic mice (Ly5.1) were from Sankyo Laboratory. Nod1\(^{-/-}\) mice in C57BL/6 background \(^1\) were provided through the courtesy of Tak Mak. IL15\(^{-/-}\) mice were purchased from Taconic. CCR2\(^{-/-}\) mice and CCR5\(^{-/-}\) mice were purchased from Jackson laboratory. Transgenic mice expressing dominant-negative IkB under the Tie2 promoter/enhancer (E-DNI\(_{κB}\) Tg mice) and CD169-DTR mice were previously described \(^2,3\). CD11c-DTR mice \(^4\) were crossed with C57BL/6-Ly5.1 congenic mice. All mice were maintained on a C57BL/6 background and in a filtered-air laminar-flow enclosure and given standard laboratory food and water ad libitum. Animal protocols were approved by the committee of Ethics on Animal Experiment, Faculty of Medical Science, Kyushu University. For the generation of BM chimeras, recipient mice were lethally irradiated (8 Gy) using Gamma cell 40 (MDS Nordion) and were reconstituted with 1 \(\times 10^7\) BM cells from donor mice. The chimeric mice were analyzed 7-8 weeks after BM reconstitution.

Coronary arteritis
Mice were administered 500 µg of FK565 (provided by Astellas Pharma) subcutaneously at day 0 and day 3 \(^5\). After mice were euthanized, the hearts were perfused with phosphate buffered saline (PBS) and dissected at day6, unless otherwise indicated. The hearts were 4% paraformaldehyde-fixed and paraffin-embedded or embedded in Optimal Cutting Temperature compound (Sakura Finetechical) and quickly frozen in liquid nitrogen for histological and immunohistological analyses. Cross sections of the aortic root were prepared where the three aortic valve cusps and coronary arteritis was assessed by hematoxylin and eosin (HE) stain and Verhoeff’s elastic fiber stain of the section as previously described \(^5\). For scoring inflammatory cell infiltration, the lesion area and cell infiltration around the coronary arteries were measured by Dynamic Cell Count software (BZ-H1C, Keyence). Immunohistochemical staining for cardiac CD11c\(^+\) cells were performed on frozen aortic sections using anti-CD11c (N418). Horseradish peroxidase activity was visualized with Peroxidase Stain DAB Kit (Nacalai Tesque) to give the reaction product a brown
color, and then the sections were counterstained with hematoxylin. All images were taken with a BZ-9000 microscope (×20) and reconstructed using BZII-analyzer (Keyence).

**Preparation of cardiac cells**

Heart tissue digestion and cell isolation was as previously described \(^6,7\) with minor modification. Briefly, murine heart was rapidly removed and perfused with 10 ml cold RPMI-1640 containing 2% FCS. Tissue digestion was preformed by perfusion with the RPMI-1640 containing collagenase II (Worthington). Hearts were weighted, removed non-ventrical tissue and homogenized with a glass homogenizer. The cell suspension centrifuged at 60 × g to separate cardiomyocytes from non-cardiomyocytes. Supernatant containing the non-cardiomyocyte fraction was passed through a 40 µm mesh filter. Non-cardiomyocytes were preincubated with Fc blocker (BD Biosciences) and stained with the aforementioned antibodies and analyzed by Gallios Flow Cytometer (Beckman Coulter). To evaluate proliferation, 2 mg of Bromodeoxyuridine (BrdU, Sigma) was injected i.p. 2 hr before harvest. For morphological and transcriptome analyses, cardiac \(\text{CD11c}^+\text{MHC II}^+\) cells were sorted as \(\text{PI}^−\text{CD45}^−\text{Ly6G}^−\text{NK1.1}^−\text{CD11b}^+\text{CD11c}^+\text{MHC II}^+\) using FACS Aria cell sorter (BD Biosciences). Sorted cells were subjected to RNA preparation and transmission electron microscopy (TEM).

**Cells**

Cardiac endothelial cells were isolated from murine heart using a CD31 microbeads (Miltenyi Biotec) and cultured as previously described \(^2\) with modification. Briefly, diced heart tissues were treated with collagenase II (Worthington) for 1hr at 37°C. Cell suspensions were incubated with rat anti-mouse CD31 and CD31-positive cells were sorted using MACS (Miltenyi). Sorted cells were further cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing Endothelial cell growth supplement (Sigma) for 7 days and used for subsequent analyses. Bone marrow-derived macrophages (BMDM) were prepared as previously described \(^8\). Hematopoietic stem cells (HSC) were sorted as \(\text{Lin}^−\text{Sca-1}^{hi}\text{c-kit}^−\) using MACS. Bone marrow-derived mast cells
(BMMC) were prepared as previously described. For stimulation, BMDMs and cardiac endothelial cells were stimulated with 10 µg/ml FK565 for 6 hr.

**Human primary cells**
The normal human aortic endothelial cell, the normal human aortic smooth muscle cells, and the normal human aortic adventitial fibroblasts were purchased from Lonza. Each primary cells in 2nd passages were stimulated with FK565 (10 µg/mL). After 6 hr incubation, the supernatant was collected and measured cytokine level by Cytometric beads array (BD).

**Antibodies**
Anti-CD11c (N418), anti-CD64 (X54-5/7.1), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD103 (2E7) anti-CD115 (AFS98), anti-F4/80 (BM8), anti-I-A/I-E (M5/114.15.2), anti-CD45.1 (Ly5.1, A20) and anti-CD45.2 (Ly5.2, 104) were purchased from BioLegend. Anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-CD8α (53-6.7), anti-CD40 (3/23) and anti-CD11b (M1/70), anti-Ly6C (AL-21), anti-Ly6G (1A8), anti-NK1.1 (PK136) and anti-SIRPα (P84) were from BD Bioscience. Anti-CCR2 (475301) was from R&D.

**In vivo cell depletion**
For depletion of neutrophils, 100 µg anti-Ly6G mAb (1A8) was administered by i.p. injection daily. For phagocyte depletion, 200 µl Clodronate liposome (Katayama Chemical) was injected i.v. on day 0 and day 3. For specific depletion of CD11c+ cells, irradiated WT (Ly5.2) mice were reconstituted with BM cells of CD11c-DTR mice on a Ly5.1 background. At 7 weeks after reconstruction, 50 ng/g DT (Sigma) was injected i.p. before administration of FK565 on day 0 and day 3.

**Fluorescence labeling of blood monocytes**
0.5 µm Fluoresbrite FITC-dyed plain microspheres (Polysciences Inc.) were diluted in PBS, and the solution was injected into mice in order to selectively label Ly6Clo monocytes. For selective labeling of Ly6Chi monocytes, Clodronate
lipsosome was injected i.v. in order to transiently deplete monocytes, followed by fluorescent microspheres i.v. 16-18 hours later.  

**Adoptive transfer of BM monocytes**

BM monocytes were sorted from Lin (CD3, CD4, CD8, B220, Ly6G, NK1.1, c-Kit, TER119)− and CD11b+ cells using MACS (Miltenyi). These sorted monocytes were positive for CD115 and Ly6C. WT mice were lethally irradiated and were injected $2 \times 10^7$ BM monocytes from Ly5.1 mice. 1hr after adoptive transfer, mice were administered FK565 on day0 and day3. Their hearts were analyzed at day 6 as described above.

**Microarray analysis**

The cRNA was amplified, labeled using GeneChip® WT Terminal Labeling and Control Kit, and hybridized to an Affymetrix Mouse Genome 430 2.0 array according to the manufacturer’s instructions. All hybridized microarrays were scanned by an Affymetrix scanner. Relative hybridization intensities and background hybridization values were calculated using Affymetrix Power Tool. To compare differentially expressed genes between sorted cells and other cell subsets, gene array data from GSE10246 in GEO datasets were imported. The raw signal intensities of all samples were log2-transformed and normalized by RMA and quantile algorithm [P] with Affymetrix Power Tool software version 1.15.0. The heat map was generated by R software [R]. We used a hierarchical clustering method to sort the genes and samples. The color indicated the distance from the median of each row. Principal component analysis (PCA) was computed with the MeV software version 4.8.1 across 33 macrophage core genes, using median centered method and 10 neighbors for KNN imputation. The 3D plots were generated using rgl package, in the R statistical environment. The GEO accession number for the array data set is GSE60506.

**Real-time PCR**

Total RNA was isolated using Trizol Reagent (Life Technologies). RNA expression was quantified by real-time PCR using SYBR qPCR Mix (TOYOBO), and values were normalized to GAPDH expression. Primer sequences were
available upon request.

**Electron microscopic analysis**  
Transmission electron microscopy (TEM) of sorted cells was performed as previously described \(^{13}\) with minor modifications. Briefly, cells were fixed with a fix buffer (2.5% glutaraldehyde, 0.1 M sucrose and 0.1 M sodium cacodylate, pH 7.4) for over night, followed by rinse 15 min at room temperature in 0.1 M phosphate buffer. Then cells were postfixed for 1 hr with 1% OsO4, dehydrated in ethanol and propylene oxide, and embedded in the Epon 812 resin. Thin sections were stained for 5 min with uranyl acetate, and for 10 min with lead acetate. Sections were analyzed on a Tecnai-20 transmission electron microscope (FEI Corp.), and images were collected with an Eagle 2k HR digital camera (FEI Corp.).

**Statistical analysis**  
Data were analyzed by Student’s t-test, Dunnett’s test or Tukey-Kramer honestly significant difference (HSD) test using a statistical software, JMP version 9.0 (SAS Institute). Values of p < 0.05 were considered statistically significant.
Reference


10. Dunay IR, Fuchs A and Sibley LD. Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. *Infection and immunity*. 2010;78:1564-70.


Supplementary Figure I. Gene expression signature of cardiac CD11c^+MHC II^+ cells isolated from hearts of FK565-treated mice.

A, Hierarchical clustering analysis of gene array data between sorted cells and hematopoietic subsets from GSE10246. The color indicated the distance from the median of each row. B, Principal component analysis (PCA) of the microarray data. PDC: plasmacytoid dendritic cells, FoB: follicular B cells, MZB: marginal zone B cells, Th: thymocytes, DP: double positive, SP: single positive, HSC: hematopoietic stem cells, CMP: common myeloid progenitor, GMP: granulocyte-macrophage progenitor, MEP: megakaryo-erythroid progenitor. Results from two independent experiments are shown.
Supplementary Figure II. Gene expression profiles in cardiac CD11c+MHC II+ cells. Hierarchical clustering analysis of gene array data between sorted cardiac CD11c+MHC II+ cells and all subsets from GSE10246. Heat maps of the genes related with MMPs (A), immunology (B), and inflammation (C). Results from two independent experiments for cardiac CD11c+MHC II+ cells were appeared as x1 and x2. The color indicated the distance from the median of each row.
**Supplementary Figure III.** Rapid accumulation of cardiac CD11c⁺ macrophages in heart after FK565 injection.

WT mice were administered FK565 and their hearts were analyzed at indicated days. FACS analysis of heart CD45⁺CD11b⁺Ly6G⁻NK1.1⁻myeloid cells (left) and histological findings in heart. HE stain (middle) and Verhoeff’s elastic fiber stain (right). The numbers indicated the cell numbers (means ± SD) of cardiac CD11c⁺ macrophages (cardiac 11c⁺ macs) and leukocytes around the coronary arteries (cell infiltration). Scale bars represent 100 μm. N.T., not tested. Data are representative for three independent mice per group.
Supplementary Figure IV. Human IkBα was preferentially expressed in endothelial cells from E-DNIκB Tg mice.

Relative mRNA expression of human IkBα in murine cardiac endothelial cells (EC), Lin−Sca-1hi−c-kit− hematopoietic stem cells (HSC), bone marrow-derived mast cells (BMMC) and bone marrow-derived macrophages (BMDM) from WT and E-DNIκB Tg mice. Levels of mRNA expression are presented relative to those of GAPDH. Data shown are mean ± SD of triplicate PCRs.
**Supplementary Figure V.** Human primary endothelial cells preferentially responded to Nod1 stimulation. Human primary endothelial cells, smooth muscle cells and adventitial fibroblasts (Lonza) were stimulated with FK565 (10 μg/ml). Supernatant CCL2 was measured by cytometric bead array (BD). Data shown are mean of triplicates ± SD. **, p < 0.01.
Supplementary Figure VI. FK565 stimulates EC and BMDM in a NFκB-dependent manner.  

A, Relative mRNA expression of the indicated chemokines in murine cardiac endothelial cells (EC) and bone marrow-derived macrophages (BMDM) from WT and Nod1−/− mice in the presence and absence of FK565 (10 μg/ml). N.D., not detected.  

B, Relative expression of chemokines in EC from WT and E-DNIκB Tg (Tg) mice. Levels of mRNA expression are presented relative to those in unstimulated cells. Data shown are mean ± SD of triplicate PCRs.
Supplementary Figure VII. Cardiac CD11c⁺ macrophages did not proliferate after FK565 treatment. Percentage of proliferating cells was evaluated by means of whole body labeling with bromodeoxyuridine (BrdU) in cardiac CD11c⁺ macrophages (cardiac 11c⁺ macs). CD25⁻CD44⁻ DN4 thymocytes were used as positive control. Data shown are mean ± SD of three mice.
Supplementary Figure VIII. CCR5 is not involved in FK565-mediated coronary arteritis.

A-C, Histological evaluation of coronary arteritis in WT littermate and CCR5−/− mice after administration of FK565. Evaluation was performed as Figure 1B-1D. D, Number of cardiac CD11c+ macrophages (cardiac 11c+ macs) in WT littermate and CCR5−/− mice treated with FK565. Three mice per group per experiment. In B and C, each circle represents an individual mouse. NS, not significant.