Lack of IκBNS promotes cholate-containing high-fat diet-induced inflammation and atherogenesis in low-density lipoprotein (LDL) receptor-deficient mice

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Background: IκBNS, a nuclear IκB protein, regulates a subset of Toll-like receptor (TLR) dependent genes. A cholate-containing high-fat diet (HFD(CA(+))) induces TLR4 mediated early inflammatory response. The present study aims to clarify the lack of IκBNS promotes atherogenesis in low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice fed HFD(CA(+)) compared with those fed a cholate-free HFD (HFD(CA(-))).

Methods and results: Mice that lacked IκBNS ($IκBNS^{-/-}$) were crossed with $LDLr^{-/-}$ mice and formation of atherosclerotic lesions was analyzed after 6-week consumption of HFD(CA(+)) or HFD(CA(-)). $IκBNS^{-/-}/LDLr^{-/-}$ mice fed HFD(CA(+)) ($IκBNS^{-/-}/LDLr^{-/-}$(CA(+))) showed a 3.5-fold increase of atherosclerotic lesion size in the aorta compared with $LDLr^{-/-}$(CA(+)) mice ($p<0.01$), whereas there was no difference between $LDLr^{-/-}$(CA(-)) and $IκBNS^{-/-}/LDLr^{-/-}$(CA(-)) mice. Immunohistochemical analysis of the aortic root revealed HFD(CA(+)) significantly increased Mac-3 (macrophage)-positive area by 1.5-fold ($p<0.01$) and TLR4, interleukin-6 (IL-6) expression by 1.7-fold ($p<0.05$) and 1.5-fold ($p<0.05$), respectively, in $IκBNS^{-/-}/LDLr^{-/-}$(CA(+)) compared with LDLr-/-(CA(+)) mice. Furthermore, active STAT3 (pSTAT3)-positive cells were significantly increased by 1.7-fold in the lesions of $IκBNS^{-/-}/LDLr^{-/-}$(CA(+)) compared with $LDLr^{-/-}$(CA(+)) mice ($p<0.01$). These findings suggest that IκBNS deficiency and HFD(CA(+)) promote atherogenesis in $LDLr^{-/-}$ mice via TLR4/IL-6/STAT3 pathway. Finally, we showed the monocytes from peripheral blood of $IκBNS^{-/-}/LDLr^{-/-}$(CA(+)) mice were found to contain

the highest proportion of Ly6C^{hi} monocytes among the four groups, suggesting that lack of IκBNS enhanced inflammation in response to HFD(CA(+)) feeding.

Conclusions: The present study is the first to demonstrate that the activation of innate immune system using HFD(CA(+)) induced significant inflammation and atherogenesis in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice.

Keywords: IκBNS, Cholate, Innate immune system, Inflammation, Atherosclerosis.

1. Introduction

Innate immune system, which directs the subsequent development of immune system adaptive immune responses, recognizes the pathogen as non-self by Toll-like receptors (TLRs) and eliminates them while inducing inflammation (1). Activation of the innate immune system via the TLR is negatively regulated by various mechanisms, as overactivity causes various systemic inflammatory diseases. In fact, it has been shown that IκBNS (also known as IκB-δ, or Nfkbid: nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, delta) of nuclear molecule induced by TLR stimulation negatively regulates TLR-dependent subset gene expression by suppressing the activity of nuclear factor-κB (NF-κB) (2, 3). In the unstimulated state, NF-κB (p50/p65 heterodimer) binds to its inhibitory molecule IκBα and exists as an inactive form in the cytoplasm (4-7). However, if inflammatory triggers such as lipopolysaccharide (LPS) are recognized by TLR, degradation of IκBα is induced and it turns into active NF-κB (8, 9). Active NF-κB translocates into the nucleus, binds to the promoter region of various inflammatory mediators, and initiates its transcription (2, 10). IκBNS specifically inhibits the binding of activated NF-κB to the promoter region of interleukin-6 (IL-6) (11). Atherosclerosis has long been recognized as chronic inflammatory disease (12-15). Several TLRs have been shown to be associated with inflammatory activation in human atherosclerotic lesions (16). In particular, TLR4 expression in mouse and human atherosclerotic lesions was observed (16-18), and it was confirmed that

lack of TLR4 has decreased atherosclerotic lesions in apolipoprotein E-deficient (apoE$^{-/-}$) mice (19).

LPS acts as extremely strong stimulator of innate immunity. We tried to investigate whether stimulation of innate immunity could promote atherosclerosis in the IκBNS-deficient atherogenic mice. However all IκBNS-deficient mice died within 4 days of LPS challenge at a dose of which almost all wild-type mice survived over 4days, because IκBNS-deficient mice are highly sensitive to LPS-induced endotoxin shock (3). Then, we decided to use a cholate-containing high fat diet (HFD(CA(+))) such as the Paigen diet, which has been widely used as an atherogenic diet in mice to promote fat and cholesterol absorption (20, 21). Moreover**,** cholate also has a role as a signaling molecule involved in inflammation, indeed a kind of HFD(CA(+)) (Paigen diet) has been shown to induce TLR4 mediated early inflammatory response (22). Furthermore, other kind of HFD(CA(+)) (Paigen diet) increased TLR4 expression in atherosclerotic lesions of apoE$^{-/-}$ mice (23).

Therefore, we examined to clarify the stimulation of innate immunity using HFD(CA(+)) promotes atherogenesis in the IκBNS-deficient LDLr$^{-/-}$ mice compared with those fed a cholate-free HFD (HFD(CA (-))).

2. Materials and methods

2.1. Animals

The generation of LDLr$^{-/-}$ mice that lacked IκBNS (IκBNS$^{-/-}$/LDLr$^{-/-}$) used in this study has been described previously (24). Details of IκBNS-deficient mice were described in the previous report (3). We used 8 to 12 weeks old male LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice. We investigated atherosclerotic lesions in both mice after 6-week consumption of HFD(CA(+)), which included 16% fats in the form soy bean oil, cocoa butter, and coconut oil, 1.25% cholesterol, and 0.5% sodium cholate (D12336, Research Diets, New Brunswick, NJ) or HFD(CA(-)) (99020201, Research Diets). The mice were randomly divided into 4 groups ((1) HFD (CA (-))-fed LDLr$^{-/-}$ (LDLr$^{-/-}$(CA(-))) mice; (2) HFD (CA (-))-fed IκBNS$^{-/-}$/LDLr$^{-/-}$ (IκBNS$^{-/-}$/LDLr$^{-/-}$ (CA(-))) mice; (3) HFD (CA (+))-fed LDLr$^{-/-}$ (LDLr$^{-/-}$ (CA(+))) mice; (4) HFD (CA (+))-fed IκBNS$^{-/-}$/LDLr$^{-/-}$ (IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+))) mice. This study was performed according to the protocols approved by the Juntendo University Board for Studies in Experimental Animals.

2.2. Quantification of atherosclerotic lesions

After measuring systolic blood pressure, mice were euthanized by pentobarbital injection, and the heart and aorta were flushed with 0.9% NaCl followed by 4% paraformaldehyde. After perfusion procedure, the aorta was harvested and fixed with 10% neutral buffered

formalin for 48 hours, embedded in paraffin, and sectioned from just above the aortic valve throughout the aortic sinus (each 6 μm thickness). We used equally spaced 5 cross sections (100 μm interval) from the initial appearance of the aortic valves to quantify atherosclerotic lesions in the aortic sinus for each mouse. The samples were stained with Elastica van Gieson, and then photographed using a BX53 microscope (OLYMPUS, Tokyo, Japan). The luminal, atherosclerotic lesions and vascular area were calculated using ImageJ 1.51j8 (National Institute of Health, Bethesda, MD). Quantification of the atherosclerotic lesions was performed by two blinded observers.

The whole aortas were also stained with Sudan IV. The surface atherosclerotic lesions were expressed as the percent of the lesion area extending from the ascending aorta to the iliac bifurcation.

2.3. Immunohistochemistry

Immunohistochemical detection was done with a Discovery XT stainer (Ventana Medical Systems, Tucson, AZ). After blocking endogenous peroxidase activity and revitalizing the tissue antigens with CC1 buffer, the following primary antibodies were applied: monoclonal rat anti-mouse Mac-3 (BD Biosciences, San Jose, CA), monoclonal rat anti-mouse TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-mouse IL-6 (Abcam, Cambridge, MA) and monoclonal rabbit anti-mouse phospho-STAT3 (pSTAT3) (Cell

Signaling Technology, Danvers, MA). Antigens were visualized with the staining system, iView DAB Detection Kit (Ventana Medical Systems), and with hematoxylin counterstaining. The ratio of the positive staining area for Mac-3, TLR4 and IL-6 to vascular area were quantified using the KS400 Carl Zeiss image analysis system (Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany). The percentage of pSTAT3 positive nuclei in the vascular wall of the aortic root were quantified using the ImageJ 1.51j8.

2.4. Plasma cytokine measurement

IL-6 levels in plasma were measured by enzyme-linked immunosorbent assay (ELISA) (BioLegend, San Diego, CA) following the manufacturer's instructions.

2.5. Blood cell analysis

Blood was collected from the tail vein, lysed using red blood cell lysis buffer (BioLegend). Cells were washed in FACS buffer (0.2%FBS in PBS) and non-specific binding sites were blocked by incubating 15 minutes at 4°C with a Fc-blocking antibody (anti-CD16/32, clone 2.4G2, BD Biosciences). Next, cell suspensions were stained for 30 minutes at 4°C with the following fluorescent conjugated antibodies: CD45 PE-Cy7 (clone 30-F11, BD Biosciences), CD11b FITC (clone M1/70, BioLegend), CD115 APC (clone AFS98, BioLegend), Ly6C PE (clone HK1.4, BioLegend). Following washing with FACS buffer they were analyzed on a

FACS CantoII flow cytometer (BD Biosciences) and data was processed using FACS Diva and FlowJo software (BD Biosciences).

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Two-way ANOVA tests were used to evaluate statistically significant differences between multiple groups, after which Tukey tests were performed for paired comparisons if the multiple group comparison indicated a difference between groups. Results were considered statistically significant at $p<0.05$.

3. Results

3.1. HFD(CA(+)) significantly promotes atherosclerosis in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice

We investigated atherosclerotic lesions in LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice after 6-week consumption of HFD(CA(+)) or HFD(CA(-)). Systolic blood pressure was similar among the four groups (Data not shown). The extent of atherosclerosis in the aorta (en face) was significantly increased in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) mice compared with others after 6-week consumption of HFD ($p<0.01$) (**Fig. 1A**). Interestingly, HFD(CA(-)) did not induce significant atherosclerotic lesions in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice after 6-week consumption (**Fig. 1A**).

Aortic root atherosclerotic lesions of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) mice were also significantly larger than those in others after 6-week consumption of HFD ($p<0.05$) (**Fig. 1B**). HFD(CA(-)) also did not induce significant atherosclerotic lesions in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice after 6-week consumption (**Fig. 1B**). These results show that only IκBNS$^{-/-}$/LDLr$^{-/-}$ mice induces significant atherosclerotic lesions after 6-week consumption of HFD(CA(+)).

3.2. HFD(CA(+)) significantly promotes macrophage accumulation in the aorta of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice

To examine the accumulation of inflammatory cells of the aortic root after 6-week

consumption of HFD(CA(+)) or HFD(CA(-)), we performed immunohistochemistry for macrophages (Mac-3). Mac-3-positive area of the vascular wall was significantly increased in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) compared with LDLr$^{-/-}$(CA(+)) mice (27.8±1.7 (n=8) vs. 18.1±2.4% (n=7); $p<0.01$), while there were no significant differences between IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(-)) and LDLr$^{-/-}$(CA(-)) mice (21.7±1.5 (n=10) vs. 21.2±2.1% (n=7); not significant) (**Fig. 3**). These results indicate that HFD(CA(+)) also promotes accumulation of macrophages in IκBNS-deficient LDLr$^{-/-}$ mice.

3.3. HFD(CA(+)) induces TLR4-dependent IL-6 expression and activates STAT3 in the aorta of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice

To evaluate the expressions of TLR4, IL-6, and pSTAT3 of the aortic root after 6-week consumption of HFD(CA(+)) or HFD(CA(-)), we performed immunohistochemistry for these proteins. After 6-week consumption of HFD(CA(-)), we detected no significant differences in the expressions of these three proteins between LDLr$^{-/-}$(CA(-)) and IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(-)) mice (**Fig. 3A-C**). However, TLR4 expression in the aorta of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) was significantly stronger than that of LDLr$^{-/-}$(CA(+)) mice (22.8±1.6 (n=8) vs. 15.3±2.7% (n=7); $p<0.05$) (**Fig. 3A**). IL-6 protein expression in the aorta of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) was also significantly stronger than that of LDLr$^{-/-}$(CA(+)) mice (22.4±1.5 (n=8) vs. 13.1±2.2% (n=7); $p<0.05$) (**Fig. 3B**). Furthermore, the percentage of pSTAT3 positive cells in

the aorta of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) were significantly higher than those of LDLr$^{-/-}$(CA(+)) mice (31.8±1.3 (n=8) vs. 19.0±2.5% (n=7); $p<0.01$) (**Fig. 3C**). These findings indicate that lack of IκBNS up-regulates TLR4 expression after consumption of HFD(CA(+)) and activates IL-6/STAT3 signaling pathway, resulting in the significant development of atherosclerosis in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) mice.

3.4. HFD(CA(+)) significantly increases plasma levels of IL-6 in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice

To examine the plasma levels of IL-6 after 6-week consumption of HFD(CA(+)) or HFD(CA(-)), we analyzed blood samples from all four groups. The plasma IL-6 levels in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) were significantly higher than those in LDLr$^{-/-}$(CA(+)) mice (15.2±1.9 (n=12) vs. 8.3±1.5 pg/mL (n=14); $p<0.01$), whereas no significant differences were observed between IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(-)) and LDLr$^{-/-}$(CA(-)) mice (5.6±1.5 (n=8) vs. 5.8±0.8 pg/mL (n=12); not significant) (**Fig. 3D**). These findings suggest that the high plasma IL-6 levels in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice should be induced by HFD(CA(+)).

3.5. HFD(CA(+)) induces monocyte phenotypic switch towards Ly6C^{hi} subset in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice

Since atherosclerosis and inflammation were increased in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) compared with LDLr$^{-/-}$(CA(+)) mice, we investigated whether IκBNS might be involved in

the regulation of monocyte phenotypic switch after consumption of HFD(CA (+)). To test this, we have determined the monocyte subsets in the peripheral blood from the four groups. We found that the percentages of Ly6C^{hi} monocytes were substantially high in the peripheral blood of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) compared with LDLr$^{-/-}$(CA(+)) mice (**Fig. 4**). In contrast, percentages of Ly6C^{lo} monocytes were found to be substantially low in the peripheral blood of IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) compared with LDLr$^{-/-}$(CA(+)) mice (**Fig. 4**). These findings reveal that lack of IκBNS enhances inflammation in response to HFD(CA(+)) feeding and thereby influence atherogenesis in IκBNS$^{-/-}$/LDLr$^{-/-}$(CA(+)) mice.

4. Discussion

Although NF-κB protein induces the expression of different inflammatory cytokines in macrophages, IκBNS selectively suppresses the expression of LPS-induced IL-6 in macrophages (11). IκBNS deficient mice are highly susceptible to LPS-induced endotoxin shock and inflammatory bowel disease, which was mediated by TLR signaling pathway (3). Taken together, IκBNS regulates the expression of TLR-related genes via regulation of NF-κB activity (3). Furthermore, IL-6/STAT pathway modulates LPS/TLR4-driven inflammatory responses, overactivation of STAT3 upregulates IL-6 production directly and via TLR4 signaling (25). These findings indicate that stimulation of innate immunity should promote TLR4-induced inflammation in the IκBNS-deficient mice. However all IκBNS-deficient mice died within 4 days of LPS challenge at a dose of which almost all wild-type mice survived over 4 days (3), and we decided to use HFD(CA(+)) which has been shown to induce TLR4 mediated early inflammatory response (22). Other report demonstrated that expression of TLR4 in atherosclerotic plaques of apoE-/- mice fed HFD(CA(+)) (Paigen diet) increases (23). We, thus, tried to see if the stimulation of innate immunity using HFD(CA(+)) could promote inflammation and development of atherosclerosis in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice. In this study, HFD(CA(+)) significantly increased atherosclerotic lesions in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice at 6 weeks after consumption of the diet, although there was no difference in atherosclerotic lesions between LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice fed

HFD(CA(-)) for 6 weeks which is consistent with the results of our previous study (24). Furthermore, immunohistochemical analysis revealed that HFD(CA(+)) significantly increased the expression levels of TLR4, IL-6, and pSTAT3 in atherosclerotic plaques in IκBNS$^{-/-}$/LDLr$^{-/-}$ compared with LDLr$^{-/-}$ mice at 6-week consumption of the diet, while there was no difference in the expression levels of these proteins in atherosclerotic plaques between LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice fed HFD(CA(-)) for 6 weeks. These findings suggest that effect of activation of TLR4 signaling by cholate and activation of IL-6/STAT3 pathway by IκBNS deficiency synergistically induced early inflammatory response and resulted in significant atherogenesis at only 6 weeks after consumption of HFD(CA(+)).

We also showed that plasma levels of IL-6 in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice fed HFD(CA(+)) were significantly increased compared with LDLr$^{-/-}$ mice fed the diet after 6 weeks, whereas plasma levels of IL-6 were not different between LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice after consumption of HFD(CA(-)) for 6 weeks which is consistent with our preliminary study. Previous study showed IκBNS$^{-/-}$ mice injected intraperitoneally with LPS increased serum levels of IL-6 compared with wild-type mice (3). These findings suggest that HFD(CA(+)) induces systemic inflammation in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice similarly to LPS injection through the TLR4 signaling pathway. As a result, HFD(CA(+)) induces not only the local increase of IL-6 protein expression in atherosclerotic lesions but also plasma levels of IL-6. We think the increase in IL-6 must also contribute to the development of atherosclerotic lesions. Indeed,

previous study demonstrated that injection of IL-6 accelerated atherosclerosis in both apoE$^{-/-}$ and wild-type mice, indicating that IL-6 has a significant atherogenic effects (26).

Circulating monocytes in mice can be classified into Ly6C^{hi} and Ly6C^{lo} subsets based on the expression levels of Ly6C (27-29). Previous reports showed that Ly6C^{hi} monocytes mobilizes more rapidly to sites of inflammation such as atherosclerotic plaques than Ly6C^{lo} monocytes does (29-32). In both LDLr$^{-/-}$ and IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, the proportion of Ly6C^{hi} monocytes were higher and the proportion of Ly6C^{lo} monocytes were lower in HFD(CA(+)) than in HFD(CA(-)) groups. Furthermore, the deficiency of IκBNS caused the proportion of Ly6C^{hi} monocytes to be higher and the proportion of Ly6C^{lo} monocytes to be lower. These findings reveal that IκBNS deficiency enhances inflammation in response to HFD(CA(+)) feeding and thereby influence atherogenesis, as Ly6C^{hi} monocytes are linked to disease progression and Ly6C^{lo} monocytes are associated with disease regression (32, 33)

Fig 5 summarizes the present findings. (i) HFD(CA(+)) induces an early inflammatory response via TLR4 (22). (ii) Lack of IκBNS up-regulates TLR4 expression and NF-κB activity, and promotes induction of TLR4-dependent genes including IL-6 after HFD(CA(+)) feeding. (iii) STAT3 activation has been in atherosclerotic lesions (34), and its activation is involved in the development of atherosclerosis (35). In the present study, IL-6 expression and STAT3 activation were increased in the foam cell rich-atherosclerotic lesions of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice at only 6 weeks after consumption of HFD(CA(+)), indicating

deficiency of IκBNS promoted both inflammation and development of atherosclerotic lesions via the TLR4/IL-6/STAT3 signaling pathway.

NF-κB and its regulator IκB proteins participate importantly in the regulation of host immune responses. In this study, we elucidated the role of IκBNS that participates in HFD(CA(+))-induced inflammation. In conclusion, IκBNS$^{-/-}$/LDLr$^{-/-}$ mice showed significant development of atherosclerosis at only 6 weeks after consumption of HFD(CA(+)). We revealed the effect of overactivation of inflammatory pathway such as TLR4/IL-6/STAT3 pathway caused by disruption of innate immune suppression on atherogenesis. These findings may lead to identification of new therapeutic targets in the treatment of atherosclerosis.

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Conflict of interest

None declared.

Author contributions

Kenichi Kitamura: Conception and design of the work, data collection, data analysis and interpretation, drafting the article.

Kikuo Isoda: Conception and design of the work, critical revision of the article, final approval of the version to be published.

Koji Akita: Data collection.

Katsutoshi Miyosawa: Data collection.

Tomoyasu Kadoguchi: Data collection.

Kazunori Shimada: Critical revision of the article, final approval of the version to be published.

Hiroyuki Daida: Conception and design of the work, critical revision of the article, final approval of the version to be published.

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Legends for Figures

Fig. 1. HFD(CA(+)) significantly promotes atherosclerosis in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice.

(A) Sudan IV staining of aortas from the four groups after 6-week consumption of HFD(CA(-)) or HFD(CA(+)). (bars=2 mm) (upper panels). Quantitative analysis of the relative surface area of the atherosclerotic lesions in aortas (lower panel). Data are expressed as means±SEM (n=9-10 for per group). **p<0.01, ***p<0.001.

(B) Elastica van Gieson staining of the aortic root from the four groups after 6-week consumption of HFD(CA(-)) or HFD(CA(+)). (bars=500 μm) (upper panels). Quantitative comparison of atherosclerotic lesions in the aortic root (lower panel). Data are expressed as means±SEM (n=7-10 for per group). *p<0.05, **p<0.01. DKO: IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, CA(-): cholate-free HFD, CA(+): cholate-containing HFD.

Fig. 2. HFD(CA(+)) significantly promotes accumulation of macrophages in the aortic root atherosclerotic lesions of IκBNS$^{-/-}$/LDLr$^{-/-}$ mice.

(A) Immunohistochemical staining for Mac-3 of the aortic root from the four groups at 6 weeks after consumption of HFD(CA(-)) or HFD(CA(+)).

(B) Quantitative analysis of Mac-3-positive area in the aortic root. Data are expressed as means±SEM (n=7-10 for per group). **p<0.01. DKO: IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, CA(-): cholate-free HFD, CA(+): cholate-containing HFD.

Fig. 3. HFD(CA(+)) induces TLR4-dependent IL-6 expression and activates STAT3 in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice.

(A): Immunohistochemical staining for TLR4 of the aortic root from the four groups at 6 weeks after consumption of HFD(CA(-)) or HFD(CA(+)). (bars=100 μm) (upper panels). Quantitative analysis of TLR4-positive area in the aortic root (lower panel). Data are expressed as means±SEM (n=7-10 for per group). *p<0.05.

(B) Immunohistochemical staining for IL-6 of the aortic root from the four groups at 6 weeks after consumption of HFD(CA(-)) or HFD(CA(+)). (bars=100 μm) (upper panels). Quantitative analysis of IL-6-positive area in the aortic root (lower panel). Data are expressed as means±SEM (n=7-10 for per group). *p<0.05.

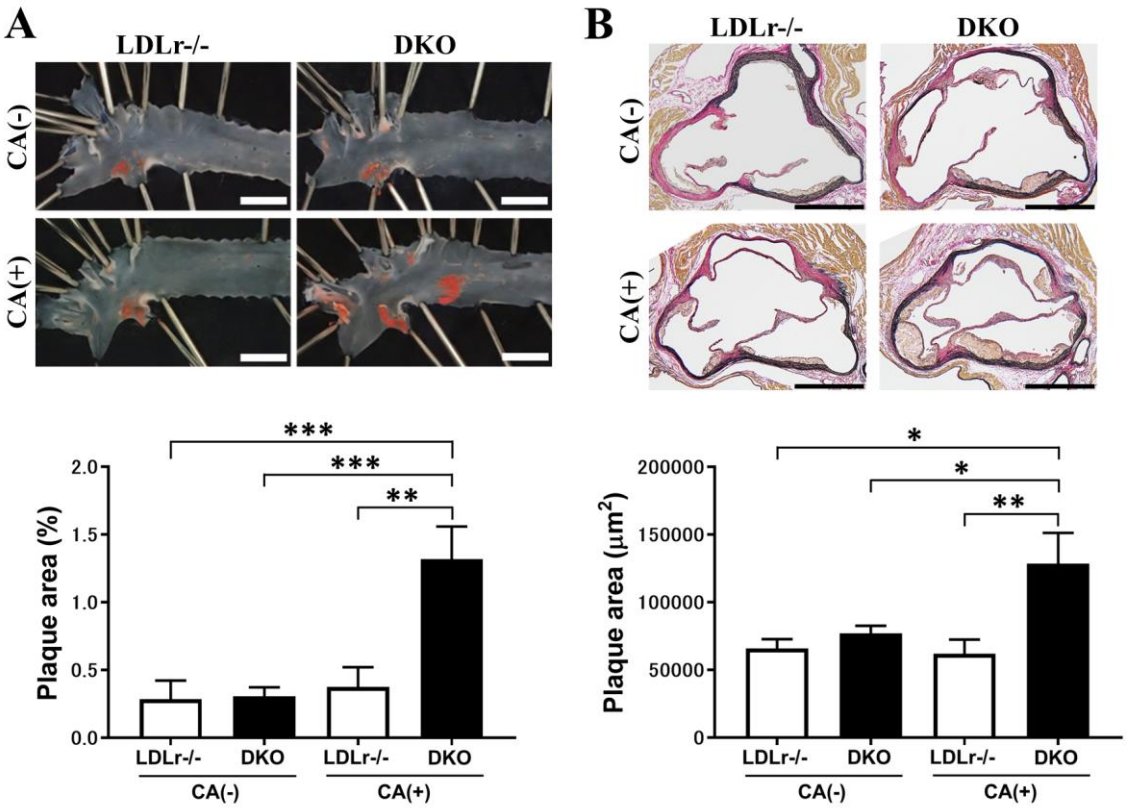
(C) Immunohistochemical staining for phospho-STAT3 (pSTAT3) of the aortic root from the four groups at 6 weeks after consumption of HFD(CA(-)) or HFD(CA(+)). (bars=100 μm) (upper panels). Quantitative analysis of pSTAT3 positive cells in the vascular wall of the aortic root (lower panel). Data are expressed as means±SEM (n=7-10 for per group). **p<0.01.

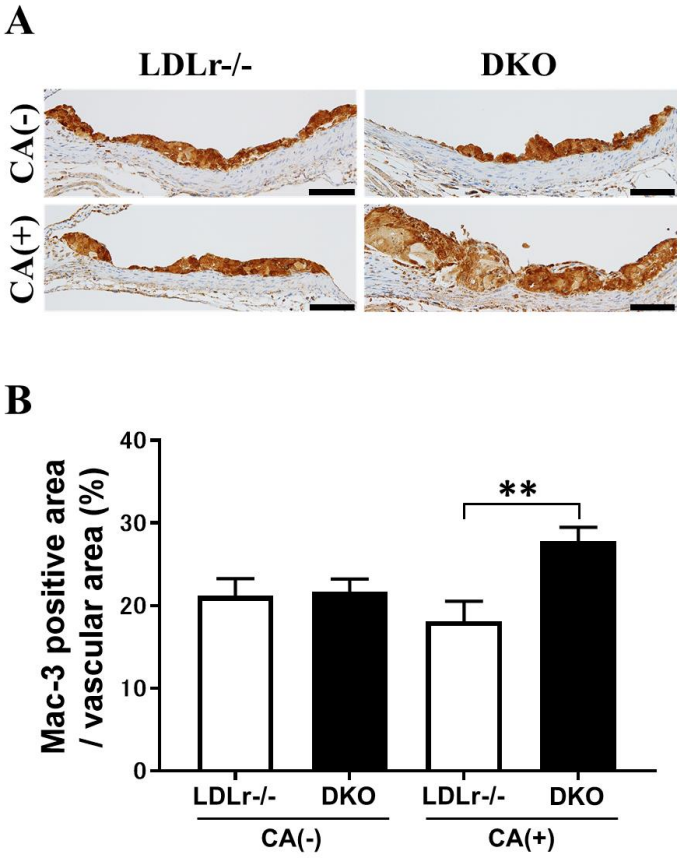
(D) Plasma levels of IL-6 of the four groups after 6-week consumption of HFD(CA(-)) or HFD(CA(+)). Data are expressed as means±SEM (n=8-14 for per group). **p<0.01, ***p<0.001. DKO: IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, CA(-): cholate-free HFD, CA(+): cholate-containing HFD.

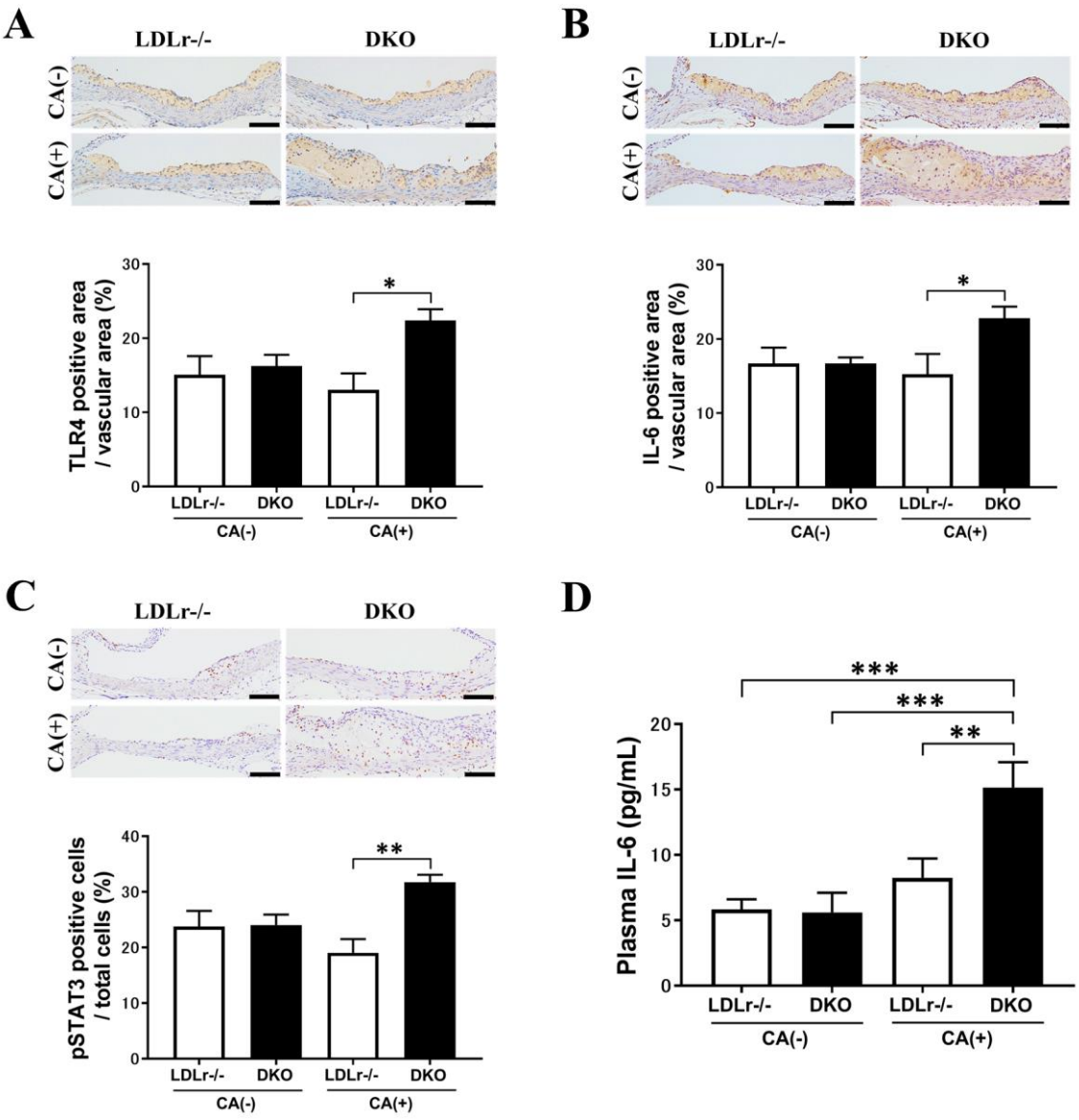
Fig. 4. HFD(CA(+)) induces monocyte phenotypic switch towards Ly6C^{hi} subset in IκBNS$^{-/-}$/LDLr$^{-/-}$ mice.

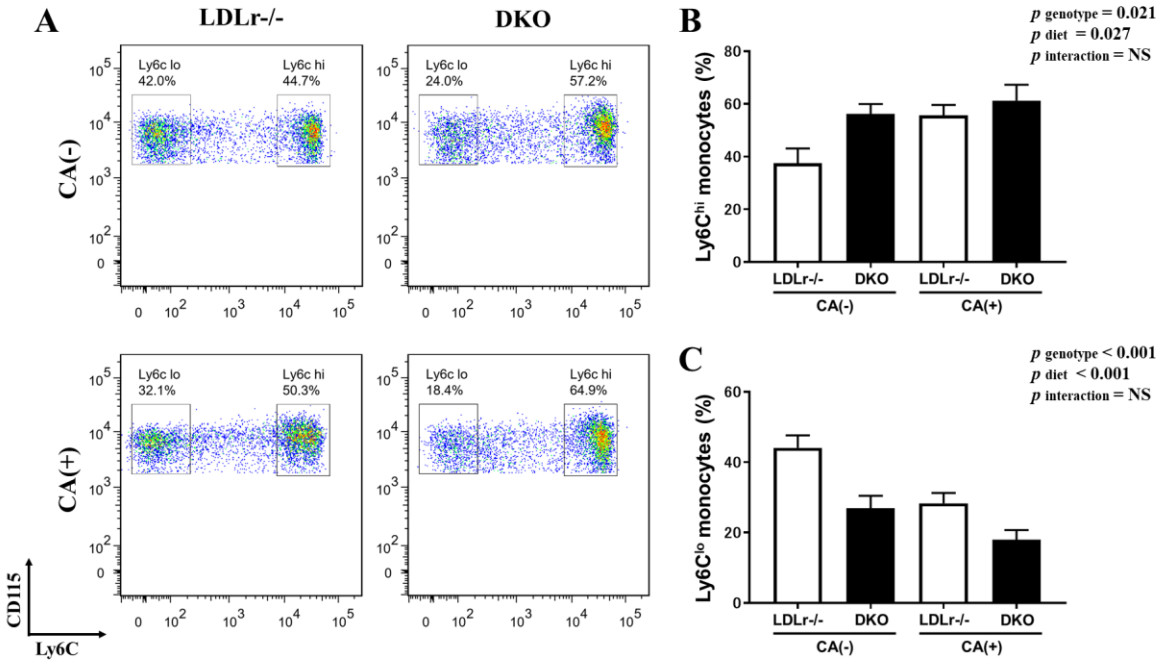
Blood cells were collected from the four groups after 6-week consumption of HFD(CA(-)) or HFD(CA(+)). (A) Ly6C was detected on monocytes (CD11b+ CD 115+) by flow cytometry. The proportions of Ly6C^{hi} (B) and Ly6C^{lo} (C) monocytes to all monocytes were quantitatively compared using Flowjo software. Data are expressed as means±SEM (n=7 for per group). Two-way ANOVA analysis revealed a significant difference between the genotype (Ly6C^{hi}: *p<0.05, and Ly6C^{lo}: ***p<0.001) and the diet (Ly6C^{hi}: *p<0.05, and Ly6C^{lo}: ***p<0.001). DKO: IκBNS$^{-/-}$/LDLr$^{-/-}$ mice, CA(-): cholate-free HFD, CA(+): cholate-containing HFD.

Fig.5. A model depicting the influence of IκBNS deficiency in HFD(CA(+)) induced inflammation and atherosclerosis.


A
LDLr-/-
DKO
CA(-)
CA(+)
B
LDLr-/-
DKO
CA(-)
CA(+)
***
***
**
Plaque area (%)
2.0
1.5
1.0
0.5
0.0
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)
*
*
**
Plaque area (μm2)
200000
150000
100000
50000
0
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)


A
LDLr-/-
DKO
CA(-)
CA(+)
B
Mac-3 positive area / vascular area (%)
40
30
20
10
0
**
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)


A
LDLr-/-
DKO
CA(-)
CA(+)
TLR4 positive area / vascular area (%)
30
20
10
0
*
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)
B
LDLr-/-
DKO
CA(-)
CA(+)
IL-6 positive area / vascular area (%)
30
20
10
0
*
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)
C
LDLr-/-
DKO
CA(-)
CA(+)
pSTAT3 positive cells / total cells (%)
40
30
20
10
0
**
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)
D
***
***
**
Plasma IL-6 (pg/mL)
20
15
10
5
0
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)


A
LDLr-/-
DKO
CA(-)
CA(+)
Ly6c lo
42.0%
Ly6c hi
44.7%
Ly6c lo
24.0%
Ly6c hi
57.2%
Ly6c lo
32.1%
Ly6c hi
50.3%
Ly6c lo
18.4%
Ly6c hi
64.9%
CD115
Ly6C
B
p genotype = 0.021
p diet = 0.027
p interaction = NS
Ly6Chi monocytes (%)
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)
C
p genotype < 0.001
p diet < 0.001
p interaction = NS
Ly6Clo monocytes (%)
LDLr-/-
DKO
LDLr-/-
DKO
CA(-)
CA(+)