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EXTENDED REPORT

A novel mouse model that develops spontaneous arthritis and is predisposed towards atherosclerosis

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ABSTRACT

Objectives Patients with rheumatoid arthritis (RA) have a reduced life expectancy due to increased cardiovascular disease. The lack of a suitable animal model resembling both RA and atherosclerosis has hindered studies demonstrating a direct link between systemic inflammation in RA and the development of atherosclerosis. Our objective was to overcome this barrier by generating an animal model (K/Bx_A^{g7}) that spontaneously develops both RA-like disease and atherosclerosis.

Methods Arthritis severity was evaluated using clinical indices and immunohistochemical staining of ankle joint specimens. Aortic atherosclerosis was delineated via Sudan IV staining and immunohistochemical analysis. Serum cholesterol and lipoprotein levels were measured using enzymatic assays. Serum levels of cytokines, chemokines and adipokines were determined by Luminex assays.

Results K/Bx_A^{g7} mice developed a destructive arthropathy followed by prominent aortic atherosclerosis. These animals also displayed dyslipidaemia, characterised by reduced serum levels of total cholesterol and high-density lipoprotein, and increased low-density lipoprotein (LDL)/vLDL compared with control mice. Further, there were higher levels of circulating inflammatory mediators, such as interleukin-6, sRANKL and CCL5 in atherosclerotic K/Bx_A^{g7} mice compared with controls. Treatment with etanercept reduced arthritis and atherosclerosis development in K/Bx_A^{g7} mice.

Conclusions K/Bx_A^{g7} mice recapitulate the same sequence of events occurring in patients with RA, namely an erosive, inflammatory arthritis followed by atherosclerosis. These data suggest that the K/Bx_A^{g7} mouse is a novel system for investigating the interplay between systemic inflammation occurring in RA and the development of atherosclerosis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive arthropathy that affects 1% of the population.¹ Patients with RA are at increased risk for premature cardiovascular (CV) events, which contributes greatly to their high mortality.^{2,3} This elevated CV risk is independent of traditional risk factors and may be related to increased systemic inflammation. Further, dyslipidaemia, characterised by elevated serum levels of total cholesterol (TC) and low-density lipoprotein (LDL) and decreased

high-density lipoprotein (HDL), also raises CV risk in RA.⁴ To date, advancement in understanding the relationships among inflammatory arthritis, dyslipidaemia and atherosclerosis in RA has been limited by the lack of a representative animal model.

Mice expressing the KRN T-cell receptor (TCR) in the context of the major histocompatibility complex (MHC) Class II Allele I^{Ak} (A^{g7}) develop a spontaneous, erosive arthritis that resembles RA.⁵ A^{g7} MHC class II alleles present endogenous glucose-6-phosphate isomerase (G6PI) peptides that are recognised by the KRN TCR. Innate and adaptive immune components including B cells, T cells, neutrophils, mast cells, macrophages, complement factors, inflammatory cytokines and Fc receptors have been shown to be instrumental for the development of arthritis in these animals.^{6–8} The arthritis is transferrable to naïve mice through injection of serum containing anti-G6PI antibodies,⁹ with the resulting inflammatory arthritis in recipient mice resembling the effector phase. K/Bx_A^{g7} mice also develop cardiac valvulitis, another feature occurring in RA.¹⁰ Thus, the K/Bx_A^{g7} mouse represents an ideal animal model to examine the influence of inflammatory arthritis on the development of atherosclerosis.

METHODS**Animals**

KRN mice (C57BL/6) were a gift from Drs Diane Mathis and Christophe Benoist. A^{g7} (C57BL/6) mice were provided by Dr Paul Allen. C57BL/6 (Jackson Laboratory, Bar Harbor, Maine, USA), and the NOD mice (Taconic, Germantown, New York, USA) were purchased. Genotyping was confirmed by Transnetyx (Memphis, Tennessee, USA) and flow cytometry. Equal numbers of male and female mice were fed chow or Harlan Teklad atherogenic diet TD.94059 (Harlan, Houston, Texas, USA) containing 15.8% fat and 1.2% cholesterol. A subset of K/Bx_A^{g7} mice was injected subcutaneously with phosphate buffered saline (PBS) or 0.8 mg/kg etanercept twice weekly for 13 weeks. All studies were approved by the IACUC at Northwestern University, and mice were maintained within the Center for Comparative Medicine.

Scoring and induction of arthritis

Ankle width was measured using calipers. Clinical score (total=12) for four paws measured disease severity, scored as 0=normal, 1=swollen wrist/

ankle, 2=swelling extending to forepaw/hindpaw, 3=swelling extending to digits. Clinical damage index (total=40) for four paws was determined by summing the number of irreversibly hyper-extended or flexed joints. Ankle joints were fixed in 10% formalin and decalcified. For the serum transfer-induced arthritis (STIA) experiments, C57BL/6 mice were injected intraperitoneally with 100 μ l of K/BxN serum in 200 μ l PBS.

Immunohistochemistry

Paraffin-embedded ankle sections were stained with H&E. Paraffin-embedded aortic sinus sections were stained with Masson's Trichrome, rat antimouse F4/80 (clone BM8), or rat antimouse CD45 (Caltag) antibodies. Imaging was performed using an Olympus DP40 microscope (Tokyo, Japan) equipped with a DP71 camera.

Evaluation of atherosclerosis

Peripheral blood was harvested by cardiac puncture, followed by ventricular perfusion with 20 ml of PBS. Aortas were excised, fixed with 10% formalin, and stained with Sudan IV (Sigma-Aldrich, St Louis, Missouri, USA). Atherosclerosis was quantitated by computer-assisted morphometric analysis (ImagePro 6.3 software) of whole aortic (aortic root to iliac bifurcation) specimens. Results were expressed as the percentage of Sudan

IV stained lesion area (calculated by Sudan IV stained area/total aortic area \times 100).

Serum analysis

Serum cytokine and chemokine levels were assessed using Luminex-based (Austin, Texas, USA) 27-plex assays (Affymetrix, Santa Clara, California, USA) according to the manufacturer's instructions. Serum TC, low-density lipoprotein/very low-density lipoprotein (LDL/vLDL) and HDL levels were measured using enzymatic assays according to the manufacturer's instructions (AbCam, Cambridge, Massachusetts, USA).

Statistical analysis

Repeated-measure, two-way analysis of variance with Bonferroni post-tests were used for arthritis analyses. Student's t tests were used to compare Sudan IV staining, lipid and cytokine levels between groups. Linear regression analysis was used with TC, LDL/vLDL or HDL as X variable predictors, and atherosclerotic burden as the Y variable. Statistical significance was established at $p < 0.05$.

RESULTS

K/BxA^{g7} mice develop a severe, destructive arthropathy

In order to understand the interplay between systemic inflammation that occurs in RA-like disease and atherosclerosis,

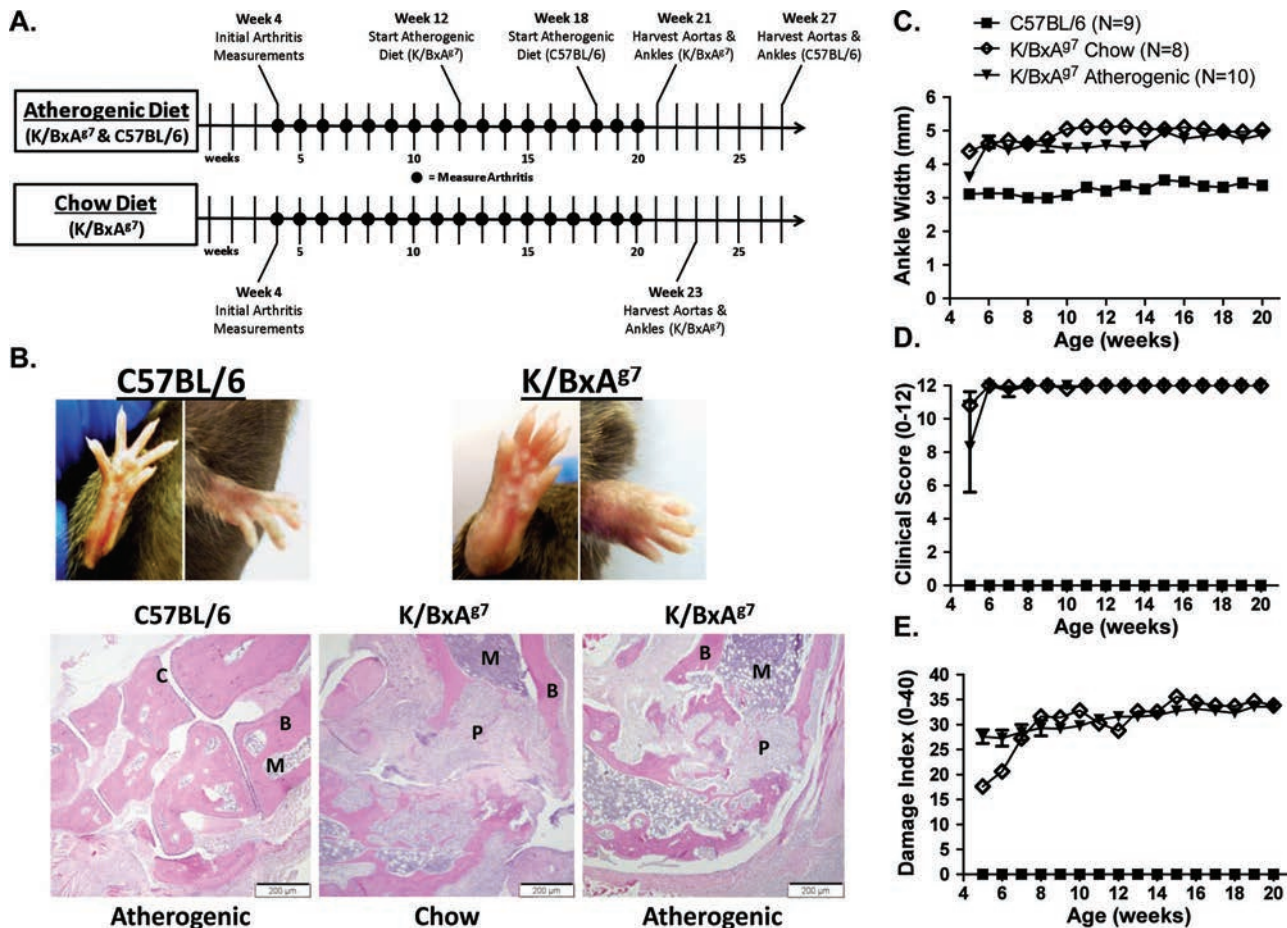


Figure 1 K/BxA^{g7} mice develop a severe, destructive arthropathy. (A) Experimental timeline. (B) Forepaw and hindpaw images (upper panel) from 6-week-old C57BL/6 and K/BxA^{g7} mice. H&E staining of representative ankle joint specimens (lower panel) from C57BL/6 and K/BxA^{g7} mice fed an atherogenic diet, or K/BxA^{g7} mice fed chow. (P)=pannus (B)=bone (C)=cartilage (M)=bone marrow. Mean \pm SEM (C) ankle width, (D) clinical score and (E) damage index in C57BL/6 (filled squares), and K/BxA^{g7} (filled triangles) mice fed an atherogenic diet or K/BxA^{g7} (open diamonds) mice fed chow. Statistically significant differences ($p < 0.0001$) were observed between K/BxA^{g7} (atherogenic or chow diet) and C57BL/6 mice at all time points.

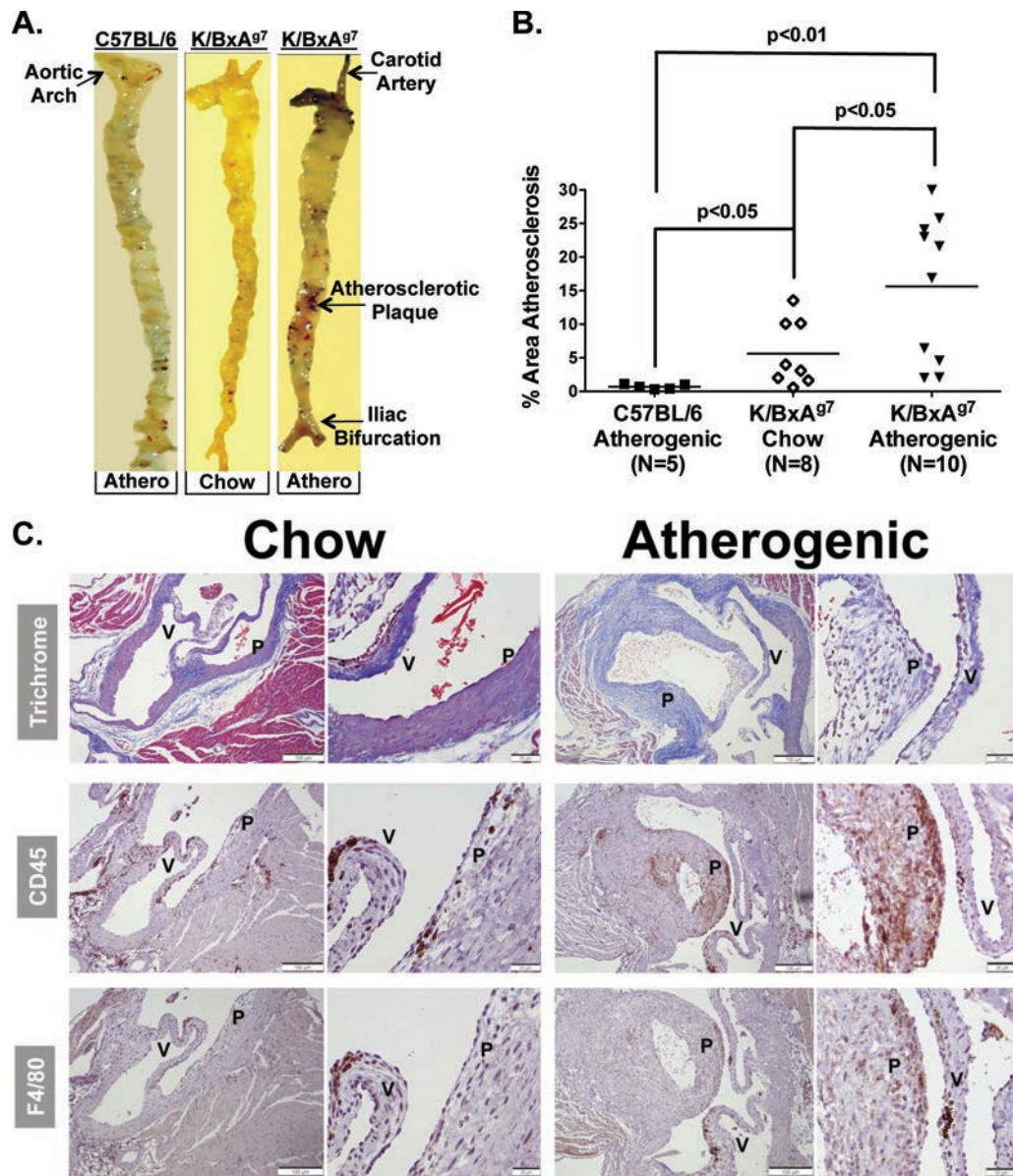


Figure 2 K/BxA⁹⁷ mice are susceptible to atherosclerosis. (A) Sudan IV staining of representative aortic specimens from C57BL/6 or K/BxA⁹⁷ mice fed an atherogenic diet ('athero') or a K/BxA⁹⁷ mouse fed chow. (B) Mean \pm SEM percentage atherosclerotic area of Sudan IV stained aortic specimens from K/BxA⁹⁷ (filled triangles) and C57BL/6 (filled squares) mice fed an atherogenic diet, or K/BxA⁹⁷ (open diamonds) mice fed chow. (C) Masson's trichrome, anti-CD45 antibody or anti-F4/80 antibody staining of aortic sinus specimens from representative K/BxA⁹⁷ mice fed chow, or an atherogenic diet. 10 \times (left) and 40 \times (right) magnification, are depicted for aortic specimens. P=atherosclerotic plaque, V=aortic valve.

we generated mice (K/BxA⁹⁷) that express the KRN TCR transgene (K), and the MHC class II Allele H-2k (A⁹⁷), which were backcrossed for several generations onto a C57BL/6 (H-2b) background.¹¹ While K/BxN mice¹² have been used extensively to model RA, the non-obese diabetic (NOD (N)) background, which predisposes K/BxN animals toward diabetes, may confound the atherosclerosis studies. At 4–5 weeks of age (figure 1A), K/BxA⁹⁷ mice developed a severe arthropathy, as measured by histologic ankle joint analysis (figure 1B), ankle width (figure 1C), clinical score (figure 1D) and damage index (figure 1E). In contrast, no arthritis was observed in control animals (C57BL/6) on a mixed H-2b and H-2k (A⁹⁷), or a pure H-2b background (unpublished data). There were also no gender differences identified between mice. Our data suggest that K/BxA⁹⁷ mice develop a spontaneous, destructive arthritis that mimics RA, which is in agreement with previous findings.¹³

K/BxA⁹⁷ mice are susceptible to atherosclerosis

Patients with RA are predisposed toward atherosclerotic disease.^{2,3} To determine whether K/BxA⁹⁷ animals are susceptible to atherosclerosis, subgroups of mice were fed chow for 12 weeks followed by an atherogenic diet, or continued on the chow diet (figure 1A). K/BxA⁹⁷ mice displayed a 22-fold increase in aortic atherosclerosis compared with control mice, which exhibited negligible lesion formation even on an atherogenic diet (figures 2A,B). Further, plaque area was 2.3-fold greater in K/BxA⁹⁷ mice fed an atherogenic diet compared with chow (figures 2A,B). Gender-specific differences between mice were not observed. Leucocytes and macrophages were highly present in atherosclerotic plaques from K/BxA⁹⁷ mice (figure 2C), similar to RA patients.¹⁴ These data suggest that K/BxA⁹⁷ arthritic/atherosclerotic mice develop a histological and cellular plaque composition that is reminiscent of patients with RA.

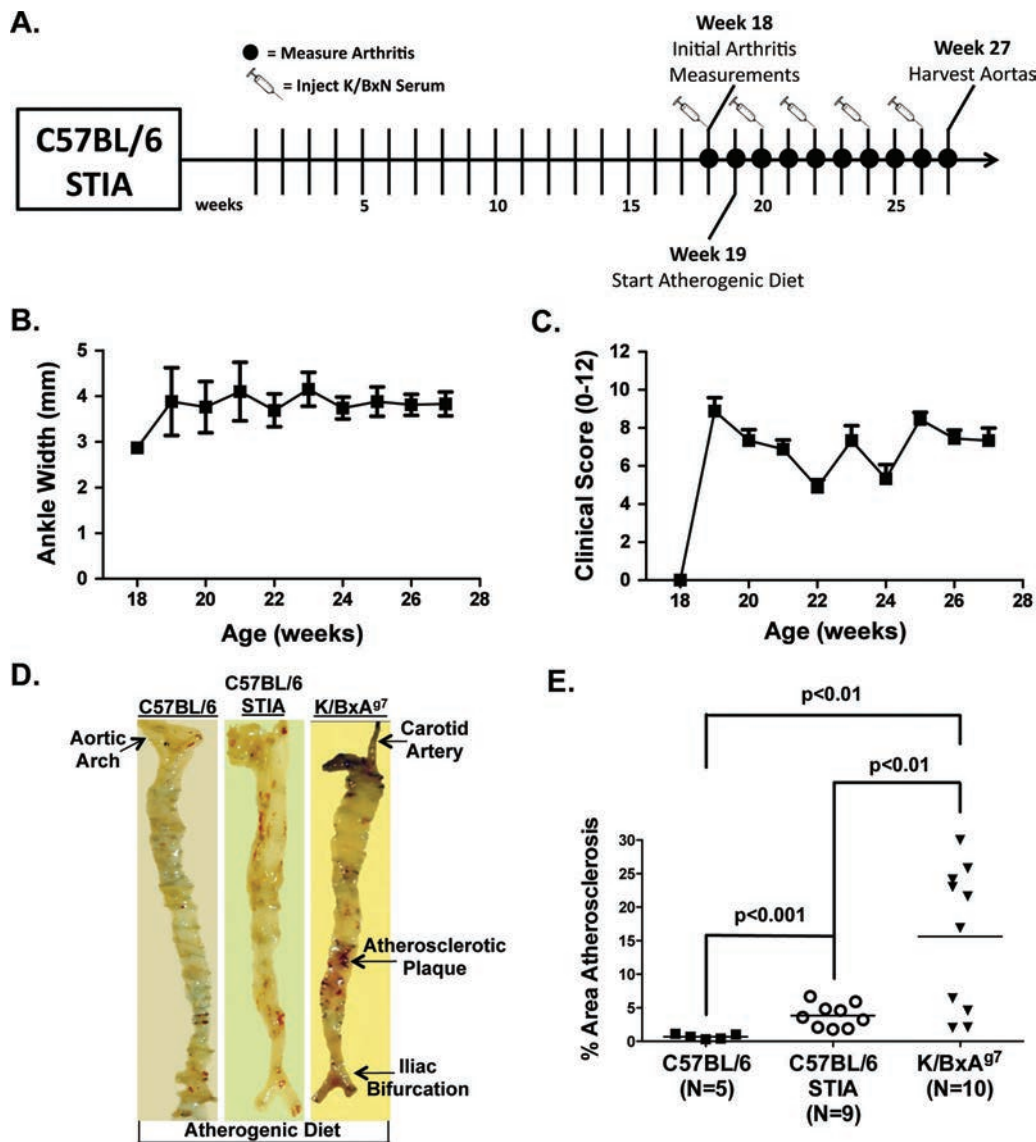


Figure 3 Induction of arthritis triggers the development of atherosclerosis in C57BL/6 mice. (A) Experimental timeline. C57BL/6 serum transfer-induced arthritis (STIA) mice were intraperitoneally injected with K/BxN serum bimonthly, and (B) joint width and (C) clinical score were assessed weekly, thereafter (mean±SEM). (D) Sudan IV staining of a representative aortic specimen from a C57BL/6 STIA mouse. C57BL/6 and K/BxA^{g7} mice treated as in (A) are included for comparison. (E) Percentage (mean±SEM) atherosclerotic area of Sudan IV stained aortic specimens from C57BL/6 (filled squares), C57BL/6 STIA (open circles) and K/BxA^{g7} (filled triangles) mice.

The K/BxN STIA model⁹ resembles the effector phase of RA. Eighteen-week-old C57BL/6 mice were injected bimonthly with K/BxN serum, and were fed an atherogenic diet for 9 weeks (figures 3A–C). Atherosclerosis levels were 5.4-fold higher ($p<0.001$) in STIA mice than in controls fed an atherogenic diet (figures 3D,E). However, plaque area was still 4.1-fold greater in K/BxA^{g7} compared with STIA mice (figures 3D,E). Together, these data indicate that induction of inflammatory arthritis via endogenous (K/BxA^{g7}) or transferred (K/BxN) arthritogenic serum provokes the development of atherosclerosis. Furthermore, arthritis and atherosclerosis in the STIA model does not require specific T-cell receptors or MHC class II alleles.

K/BxA^{g7} mice are dyslipidaemic

Dyslipidaemia may be an important contributor to increased CV mortality in RA.⁴ K/BxA^{g7} mice exhibited marked dyslipidaemia, characterised by reduced serum levels of TC (figure 4A, $p<0.01$), increased LDL/vLDL (figure 4B, $p<0.01$), and diminished HDL

(figure 4C, $p<0.01$) compared with non-arthritic controls on an atherogenic diet. However, LDL/vLDL levels were increased by 3.2-fold ($p<0.0001$) in K/BxA^{g7} mice fed an atherogenic diet versus chow, while TC and HDL levels were unaffected by diet (figure 4). Lower HDL levels in K/BxA^{g7} mice were associated with greater amounts of atherosclerosis ($R^2=0.60$, $p<0.05$, figure 3F) whereas, TC (figure 3D) and LDL/vLDL (figure 3E) were not significantly related to atherosclerotic burden. Similar to K/BxA^{g7} mice, STIA mice demonstrated reduced levels of TC (figure 3A, $p<0.01$) and HDL (figure 3C, $p<0.01$), and elevated LDL/vLDL (figure 3B, $p=0.12$) compared with non-arthritic controls. Altogether, these findings show that dyslipidaemia may contribute to the elevated atherosclerosis observed in K/BxA^{g7} mice, and that induction of arthritis elicits dyslipidaemia in normal animals.

Serum cytokines/chemokines are altered in K/BxA^{g7} mice

Cytokines, chemokines and adipokines are central to the development of atherosclerosis.^{15 16} Serum levels of interleukin-6,

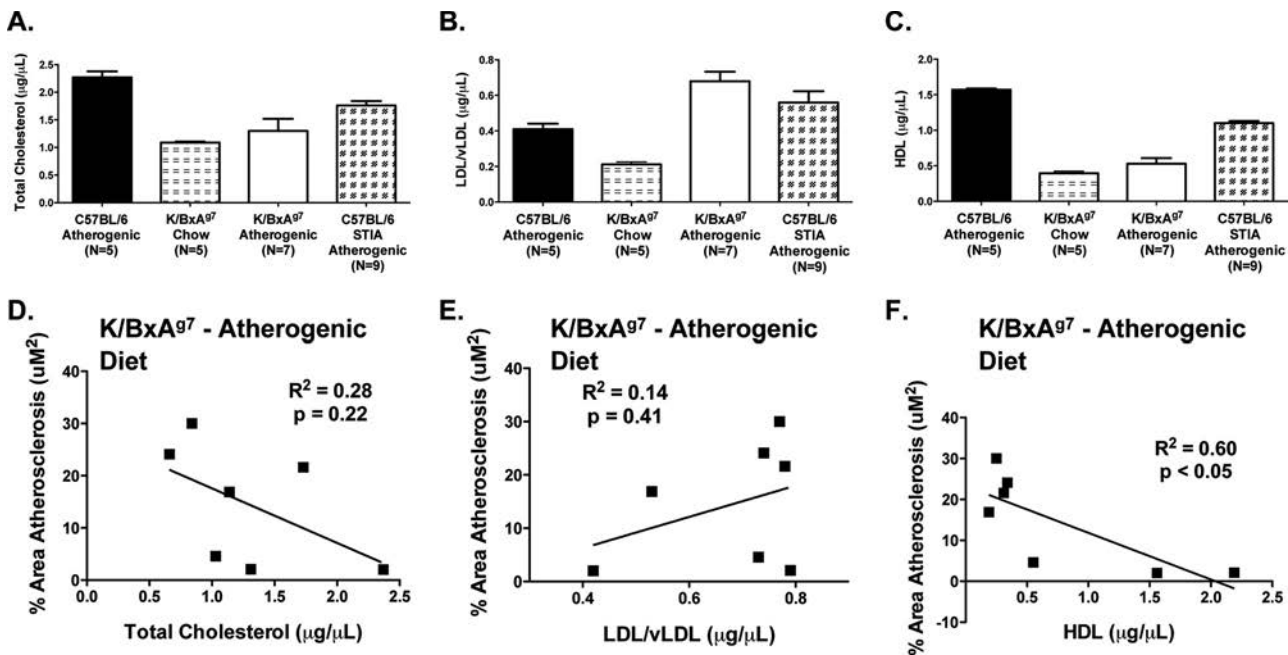


Figure 4 K/BxA⁹⁷ mice develop dyslipidaemia. Serum was harvested from 27-week-old C57BL/6 (filled bars), 27-week-old C57BL/6 serum transfer-induced arthritis (STIA) (chequered bars), and 21-week-old K/BxA⁹⁷ (open bars) mice fed an atherogenic diet for 9 weeks, or 23-week-old K/BxA⁹⁷ mice maintained on chow (horizontal bars). Mean ± SEM (A) total cholesterol (TC), (B) low-density lipoprotein (LDL)/vLDL and (C) high-density lipoprotein (HDL) are depicted. All t test comparisons between groups were statistically significant ($p < 0.05$) with the following exceptions: TC – K/BxA⁹⁷ atherogenic versus chow, LDL/vLDL–K/BxA⁹⁷ and C57BL/6 atherogenic versus C57BL/6 STIA atherogenic, and HDL–K/BxA⁹⁷ atherogenic versus chow. Linear regression analysis of (D) TC, (E) LDL/vLDL and (F) HDL versus atherosclerosis.

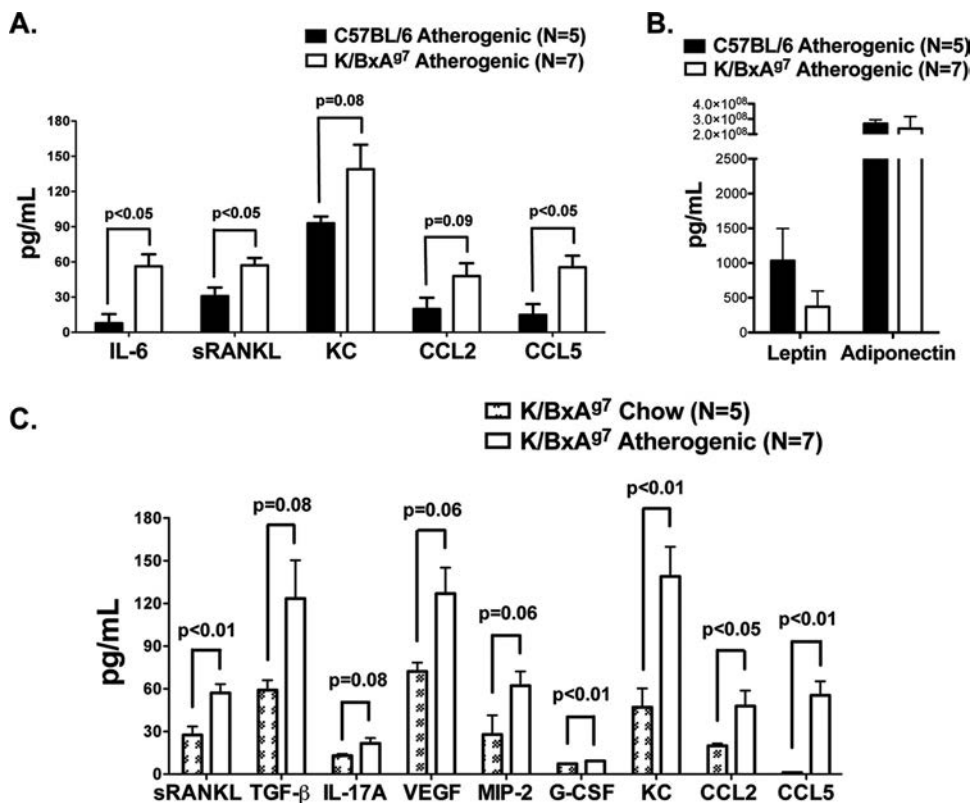


Figure 5 Altered serum cytokine/chemokine profiles in K/BxA⁹⁷ mice. (A) Serum cytokines and chemokines and (B) adipokines (mean ± SEM) in C57BL/6 (filled bars) and K/BxA⁹⁷ (open bars) mice fed an atherogenic diet were measured via Luminex. (C) Serum cytokines and chemokines were measured via Luminex in 23-week-old K/BxA⁹⁷ mice fed chow (chequered bars), or 21-week-old K/BxA⁹⁷ mice fed an atherogenic diet for 9 weeks (open bars).

sRANKL and CCL5 were significantly greater ($p < 0.05$), while KC and CCL-2 trended toward significance in K/BxA^{g7} mice compared with C57BL/6 controls fed an atherogenic diet (figure 5A). Elevations in IL-21, IL-12/23 p40, tumour necrosis factor (TNF α), MIP-2, CXCL10 and GM-CSF were also detected, but were not significant in K/BxA^{g7} mice compared with controls (online figure 1). In contrast, VEGF, TGF- β , IL-4, IL-13, IL-1 α , IL-1 β , IL-17A, G-CSF, CCL3, leptin and adiponectin were expressed at similar levels (figure 5B and online figure 1) and IL-10, interferon γ , IL-2, IL-12 p70 and IL-23 p19 were undetectable (unpublished data). Further, sRANKL, G-CSF, KC, CCL2 and CCL5 were increased in K/BxA^{g7} mice fed an atherogenic diet compared with chow (figure 5C), while levels of the

remaining cytokines and chemokines were unaffected by diet (online figure 1). Together, these data suggest that higher levels of circulating cytokines and chemokines, but not adipokines, may contribute to increased atherosclerosis in K/BxA^{g7} mice.

TNF blockade improves arthritis and atherosclerosis in K/BxA^{g7} mice

Etanercept is a TNF antagonist commonly employed to treat RA.¹⁷ K/BxA^{g7} mice on an atherogenic diet were treated biweekly with PBS or etanercept for 13 weeks. Administration of etanercept reduced arthritis severity (figure 6B), as indicated by a 0.2–0.4 mm decrease in ankle width, and by a 2.0-fold reduction in joint clinical score, as compared with PBS-treated

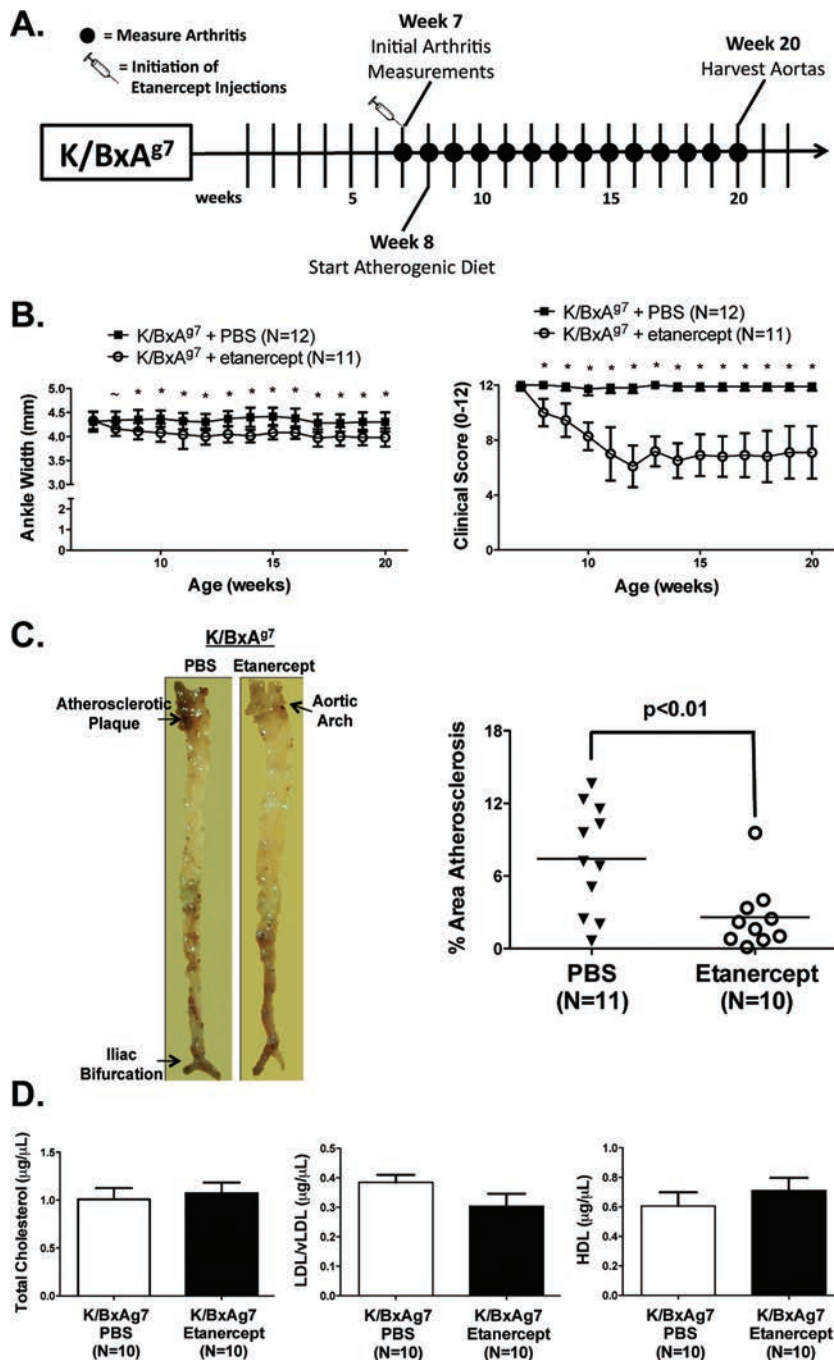


Figure 6 Improved arthritis, atherosclerosis and dyslipidaemia in K/BxA^{g7} mice treated with etanercept. (A) Experimental timeline. (B) Arthritis severity, (C) mean \pm SEM % area aortic atherosclerosis and (D) TC, LDL/VLDL and HDL levels in K/BxA^{g7} animals treated with phosphate buffered saline (open) or etanercept (filled). ~ denotes statistical significance at $p < 0.05$, * at $p < 0.01$.

controls. Atherosclerotic burden was reduced by 3.0-fold in etanercept-treated mice compared with controls (figure 6C). Further, etanercept therapy in K/BxA^{g7} mice reversed aortic valvulitis thickening (figure 3),¹⁰ and impaired valvular function (online Videos 1–4). TC, LDL/vLDL and HDL levels were unaffected by etanercept treatment (figure 6D). Together, these data demonstrate a link between arthritis severity and the development of atherosclerosis in K/BxA^{g7} mice.

DISCUSSION

Here, we characterised K/BxA^{g7} mice as a unique model of arthritis/atherosclerosis that recapitulates the same sequence of events occurring in RA. Even passive transfer of arthritis triggers the development of atherosclerosis, suggesting that both disease states occur independently of the unique TCR (KRN) and MHC class II allele (A^{g7}) present in K/BxA^{g7} mice. While previous studies have shown that mice lacking Fas¹⁸ or FasL,¹⁹ or that are on a genetically predisposed background (BWF1),²⁰ develop SLE-like disease and enhanced atherosclerosis when fed a high fat diet, we are the first to generate a murine model of RA-like disease that is also susceptible to atherosclerosis. K/BxA^{g7} mice also develop dyslipidaemia, and display increased serum levels of pro-inflammatory cytokines and chemokines. TNF blockade diminishes arthritis and atherosclerosis in K/BxA^{g7} mice. Collectively, these data link arthritis and atherosclerosis as interdependent disease processes, and establish the K/BxA^{g7} mouse as a unique model for preclinical testing of therapeutics for these disorders.

K/BxA^{g7} mice develop a destructive inflammatory arthritis mirroring RA patients with severe or untreated disease. Unlike RA, there was no evidence of calcified aortic lesions or thrombotic events despite extensive atherosclerosis in K/BxA^{g7} animals (unpublished data). Yet, K/BxA^{g7} mice demonstrated a lipid paradox (diminished TC and HDL) that resembles the dyslipidaemia seen in RA patients with the greatest inflammatory burden.²¹ There was a significant inverse association between HDL levels and plaque area in K/BxA^{g7} mice, consistent with previous studies showing an atheroprotective role of HDL in RA.⁴ However, treatment with etanercept improves arthritis and atherosclerosis without affecting lipid levels in K/BxA^{g7} mice, suggesting a non-causative relationship between dyslipidaemia and atherosclerosis in these animals. Future studies will determine whether altering dyslipidaemia impacts the development of atherosclerosis in K/BxA^{g7} mice.

Since cytokines and chemokines are potent orchestrators of inflammation, altered levels of these factors may contribute to the development of arthritis and vascular disease in K/BxA^{g7} mice. Indeed, elevated levels of several inflammatory mediators were evident in K/BxA^{g7} mice and TNF blockade led to improved arthritis and atherosclerosis. However, epidemiologic studies have yielded conflicting data on the effects of disease-modifying treatments on RA disease activity and CV disease. Thus, dissecting the disease-specific effects of individual mediators will be critical to understanding the relationships among inflammatory arthritis, dyslipidaemia and atherosclerosis in K/BxA^{g7} mice as a model for RA-like disease.

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