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Mouse model developed to study the impact of B cell depletion therapy on Atherosclerosis

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نموذج الفأر المطور لدراسة تأثير انخفاض خلايا بي اللمفاوية كعلاج لتصلب الشرايين

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الملخص

الأهداف : مرضى التهاب المفاصل الروماتيزمي لديهم قابليةعالية للإصابة بأمراض القلب. من المثير للإهتمام أن خلايا بي اللمفاوية لديها طريقة معاكسة في التكوين المرضي لمرض التهاب المفاصل الروماتيزمي ومرض تصلب الشرايين .الهدف من هذه الدراسة هو فحص فعالية العلاج بعقار خافض خلايا بي على المجموعات الفرعية للخلايا بي وتصلب الشرايين في الفئران.

الطريقة :قمنا بتحضيرنموذج معملي من الفئران مشابه لمرض تصلب الشرايين من نوع -/- Apo / $huCD20^{+}$ وقد تم علاج الفئران بواسطة دواء مخفض لخلايا بي وهو ريتوكسي ماب وَ قياس نسبة خلايا بي قبل وبعد كل علاج ،وفى نهاية فترة العلاج تم فحص الشرايين التاجية لتقييم حالة التصلب بها.

النتائج : خلال فترة الدراسة , أدى العلاج الأولى بريتوكسي ماب إلى انخفاض كبير (أكبر من 90 %) في نسبة خلايا بي . بينما أظهرت خلايا بي في الطحال والعقد اللمفاوية التى تم حصدها في نهاية الدراسة مقاومة للعلاج بواسطة ريتوكسي ماب. أما بالنسبة للشريان التاجى، فقد تقلص حجم التصلب ضعفين مقارنة بالمجموعة الضابطة والتى تم علاجها بواسطة الاجسام المضادة IgG.

الخلاصة : تم فى هذه الدراسة تجهيز نموذج من الفئران مشابه لمرض تصلب الشرايين، وقد أظهرت المعطيات الأولية تقلصا فى تصلب الشرايين عند استخدام علاج خافض للخلايا بي .

ABSTRACT

Objective
Rheumatoid Arthritis patients have an increased risk of developing co-morbid cardiovascular disease (CVD). Interestingly, B-lymphocytes have an opposite role in the pathogenesis of RA and atherosclerosis. The aim of this study was to investigate the impact of B cell depletion treatment on B cell subsets and atherosclerosis in mice.
Method
We established an experimental model to mimic atherosclerosis and thus raised huCD20^{+}/ApoE$^{-/-}$ mice. These mice were treated with the B cell depleting agent Rituximab, and B cell percentage was examined before and after every treatment. At the termination of the study, aortas were explored for atherosclerotic lesions.
Results
During the course of the study, first treatment with rituximab resulted in a massive reduction (>90%) in B cell percentage. Moreover, B cells of spleen and lymph nodes harvested at the end of study-exhibited resistance to Rituximab treatment. However, the aorta showed almost a 2-fold reduction in lesion size in response to rituximab treatment in comparison with the IgG treated control mice.
Conclusion
This study established a mouse model and the preliminary data is suggestive of reduction in atherosclerotic plaques with B cell depletion therapy.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects the joints and is characterised by bone and cartilage erosion. RA patients show strong susceptibility to cardiovascular diseases leading to morbidity and mortality (1-3). They require sequential immune modulatory therapeutics(4). Patients refractory to treatment with anti TNF can respond effectively to B cell depletion therapy (5).

The only FDA approved B cell depleting agent is Rituximab, a chimeric monoclonal antibody to CD20, which is specifically expressed on human B cells (6-8), which have a transmembrane protein with 4 domains. The antibody started out as a murine antibody and was then engineered to contain parts of the human antibody. This resulted in a chimeric antibody, a combination of a constant region from humans and an antigen binding variable portion of a murine origin. The cardiovascular complications seen in RA patients are the result of atherosclerosis, a gradually advancing, and chronic inflammatory disease seen as asymmetrical intimal thickening of large arteries. (9)
The functional role of B-lymphocytes in atherosclerosis seems very

interesting. In the past, numerous investigators substantiated a protective role for B cells in atherosclerosis (10). Recently, different B cell subsets have been shown to have an opposite role in the course of atherosclerosis. The B2 cells have been shown to have atherogenic character (11), whereas the natural IgM antibody producing serosal B1a cells are atheroprotective (12). The B1a cells produce their atheroprotective effects not only through natural antibodies, but also though the anti-inflammatory cytokine IL-10 (12). This functional dichotomy in B cell subsets in atherosclerosis is intriguing.

Rituximab therapy in patients results in more than 95% reduction of detectable circulating B-lymphocytes (13). Clinical responses suggest a pro-inflammatory role of B cells in RA pathogenesis. Importantly, RA patients are strongly linked with cardiovascular morbidity and mortality and studies suggest that B cells have an anti-inflammatory role in atherosclerosis (14). Keeping this in view, it becomes very important to address the implications of B cell depletion therapy on atherosclerosis, especially when Rituximab is used in refractory cases of RA. This group has in any case a higher than expected incident rate of vascular events and thus subtle risk factor modulation may be of clinical significance. Therefore, it is important to examine the effect of Rituximab therapy on B1a and B2 cells in the circulation and other lymphoid organs, in addition to implications on the course of atherosclerosis.

To investigate this, we decided to use the ApoE-/- murine model of atherosclerosis. These mice develop atherosclerosis as they age; however, if these mice are given a high fat diet, the process of atherosclerosis is accelerated (15-17). Human CD20 transgenic mice express the human CD20 receptor and are, therefore, susceptible to Rituximab-induced B cell depletion (18,19). We crossed these two murine strains to generate the huCD20^{+}/ApoE$^{-/-}$ murine line. These mice were fed on a high fat diet (HFD) and treated with Rituximab to deplete B cell.

The aim of this study was to investigate the impact of B cell depletion therapy on different B cell subsets and, in turn, on plaque formation in atherosclerosis- prone mice.

MATERIAL AND METHODS

A. Modified Mouse Model of Atherosclerosis

Human CD20 (huCD20+) transgenic mice were kindly provided by Professor Mark J Shlomchick (Yale University, New Haven) (18). These mice were crossed with Apolipoprotein E knockout mice (ApoE-/-) (Jackson laboratory) to produce “huCD20+/ApoE-/- mice”. These mice were given 100µl of 100µg intravenous (iv) injections of Rituximab (Rituxan) for the study group, or chromopure human IgG (Jackson Immunochemicals) for the control group, every 4 weeks. To monitor B cell depletion in these mice, they were bled a week before and after each systemic treatment. Mice were weighed every time they were injected or bled. The model was set up to sacrifice mice after 4 treatments around the age of 22 weeks (16 weeks on HFD). At the end of the study, heart and aortas were to be examined for lesions. Spleen, lymph nodes, bone marrow and blood were harvested to examine B cells and their subsets.

B. Peripheral blood mononuclear cells staining (PBMCs)

PBMCs from huCD20+/ApoE-/- were stained as per manufacturer guidelines for different cell surface antigens; CD19-APC (ID3, BD Pharmingen), CD3-PE (145-2c11, BD Pharmingen), B220-PerCP (RA3-6B2, BD Pharmingen), CD21-FITC (7G6, BD Pharmingen), CD23-PE (2G8, Southern Biotech), CD24-PE (BD Pharmingen, M1.69), IgM-PE (BD Pharmingen),

CD11b-PerCP (BD Pharmingen, M1.70), IgM-FITC (BD harmingen, II/41), IgD-FITC (Bioscience, 11-26c), CD43-FITC (BD Pharmingen, S7) and huCD20-FITC (L-27, BD bioscience). Cells were fixed and permeablized using buffer (BD Bioscience). The resulting data were analyzed using Flowjo (Treestar).

C. Flow cytometry

After sacrificing the mice, their organs (spleen, lymph nodes and bone marrow) were harvested and single cell suspensions were stained for different surface antigens; CD19-APC (ID3, BD Pharmingen), CD3-PE (145 2c11, BD Pharmingen), B220-PerCP (RA3-6B2, BD Pharmingen), CD21-FITC (7G6, BD Pharmingen), CD23-PE (2G8, Southern Biotech), CD24-PE (BD pharmingen, M1.69), IgM-PE (BD Pharmingen), IgM-FITC (BD Pharmingen, II/41), IgD-FITC (Bioscience, 11-26c), CD43-FITC (BD Pharmingen, S7) and huCD20-FITC (L-27, BD Bioscience). The staining was performed as per manufacturer guidelines.

D. Haematoxylin and Eosin Staining of aortic lesions

Aortic sections stained with hematoxylin and Eosin (H & E) were analysed using a light microscope (Carl Zeiss). Morphometric analysis of the lesions was performed on cross sections through the aortic lesion using an image analysis software (Axio vision 4.4). The aortic lumen and lesion area measurements from 5 sections were then used to calculate the percentage of luminal area obliterated by the lesion.

E. Statistical analysis

Quantitative data were described in the form of mean ± SD and the statistical analysis of the study was done using Graphpad prism version 4 for Mac (Graphpad software).

RESULTS

Rapid depletion of circulating B-lymphocytes with Rituximab treatment

To determine the baseline level of B-lymphocytes in the blood, PBMCs were extracted from the blood at week (0) and were stained for CD19 and human CD20. FACS analysis showed similar B cell percentage, i.e. Rituximab- treated mice (n=2) had a mean of 44.6% ± 0.3 huCD20 positive B cells, while human IgG treated mice (n=2) had a mean of 43.4% ± 7.3.

One week after systemic treatment of all the mice (n=4) they were bled again and the percentage of B cells present in the blood were analysed by FACS. The analysis showed significant depletion of B cells in Rituximab treated mice. In specific, Rituximab-treated mice had 0.45% ± 0.4 (mean ± SD) B cells, representing a 98% reduction (Figure 1, A, B). The huCD20 positive cells were not affected by the treatment (100µg, IgG) and the B cells in the control mice were 45.6% ± 1 (mean ± SD) of the gated lymphocytes (Figure 1, C, D)

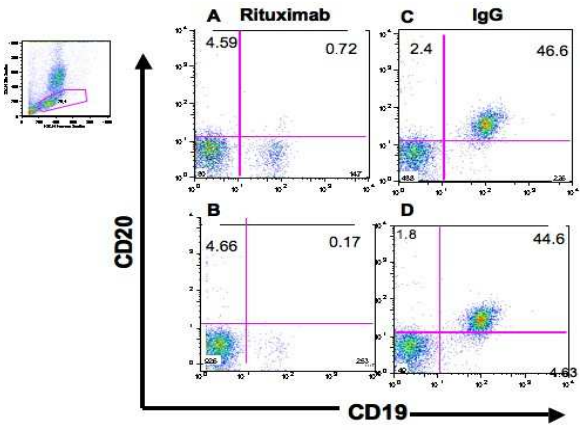

Figure 1. Circulating B cells (huCD20^{+}) one week after systemic treatment

Mice were bled one week after first treatment. (A & B) are Rituximab-treated

mice, (C & D) are the control mice; PBMCs were isolated from the blood and stained for 4-colour flow cytometric analysis. Pseudocolour dot plots were gated on lymphocytes as shown in the smaller panel. (CD19 vs. CD20) showed a 98% reduction in $CD20^+$ B cells in the Rituximab-treated mice, whereasIgG treatment did not affect the CD20 expressing population. The percentages are shown in each quadrant.

Thereafter, the mice were bled again a week before the second treatment to see if the huCD20 positive cells were replenished in the Rituximab-treated mice (n=2) during the two-week time. There was a rapid recovery of huCD20 positive B cells in Rituximab-treated mice as a 77% increase was observed evident from B cell percentages (34.7% ± 5.2). In fact, the mean B cell percentage increased further in the IgG treated mice (n=2), (51.75% ± 4.35).)

However, a week after the second treatment with Rituximab, a modest depletion of 9% was observed in the peripheral blood in these mice; whereas, a week after the second treatment with IgG, the mice had a further 15% increase in there circulating B cells. Moreover, one week after the third treatment, the Rituximab-treated mice had a modest drop of 20% in the circulating B cells, while the IgG-treated mice had steady levels of circulating B cells.

B cell directed therapy did not affect lymphocytes of spleen and lymph nodes

Having established that depletion of circulating B cells was massive (98%) after the first treatment and later on they resisted depletion, even as small as 9% depletion was seen in these mice as compared to their B cell levels that were replenished in 3 weeks' time prior to treatment. Mice were sacrificed and aorta, spleen, bone marrow, lymph nodes and blood were all harvested from the mice. Aortas were examined for atherosclerotic lesions. Bone marrow and secondary lymphoid organs were assessed for B cell depletion.

The ratios of B to T lymphocytes in the spleen of Rituximab- vs. IgG-treated mice were 1.85 and 1.6, respectively. On the other hand, the ratios of lymphocytes (B and T) in the lymph nodes of Rituximab- and IgG-treated mice were 0.58 and 0.68, respectively. This showed that both spleen and lymph nodes had similar ratios of lymphocytes, so the depletion therapy did not affect the huCD20 positive B cells in these organs (Figure 2).

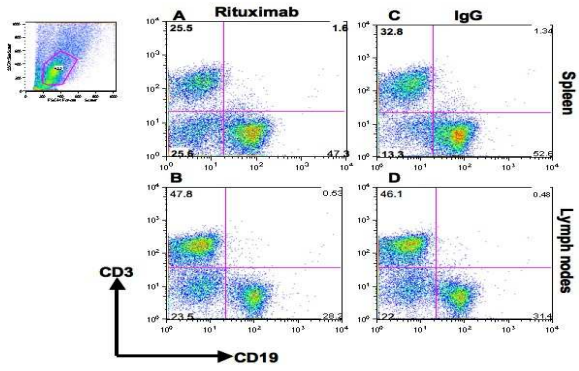

Figure 2. Depletion therapy and lymphocytes in spleen and lymph nodes

Single cell suspensions of lymphocytes were extracted from spleen (A,C) and lymph nodes (B,D). Pseudocolour dot plots were gated on lymphocytes as shown in the smaller panel in the left corner. B and T lymphocyte percentages in spleen of Rituximab-treated mice were 47.3% and 25.5%, and in lymph nodes 28.2% and 47.8%, respectively. On the other hand, B and T lymphocyte percentages in spleen of IgG- treated mice were 52.6% and 32.8%, and in lymph nodes 31.4% and 46.1%, respectively. The percentages are indicated in corner of each quadrant.

Effect of Rituximab on B cell subsets in spleen

Rituximab treatment in $huCD20^+/ApoE^{-/-}$ mice demonstrated no depletion of B cells in spleen and lymph nodes. We also wanted to validate if the treatment affected different B cells subsets in secondary

lymphoid organs. Harvested spleen cells were stained for different B cells subsets. The identification of transitional (T2), marginal zone (Mz) and follicular (Fo) B cells in the spleen was possible through combination of several markers, which are expressed during development. The combination included CD19, CD21 and CD23; a plot of forward scatter (FSC) vs. CD19 can be used to separate ($CD21^+CD23^+$) cells. This population was subdivided into Fo ($CD21^+CD23^+$lo), T2 ($CD21^+CD23^+$hi) and Mz ($CD21^+CD23^+$dull) B cells. There was a difference noted in marginal zone B cells between Rituximab-treated (4.18%) and control mice (2.84%). This altered representation of B cell subset percentage could be a response of decreased follicular B cells (Figure 3).

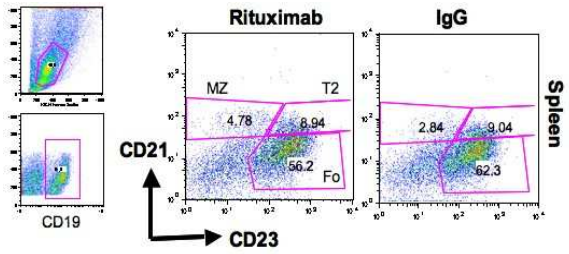

Figure 3. Marginal zone (MZ), follicular (Fo) and transitional type 2 (T2) B cells

The single cell suspension was isolated from Rituximab- and IgG-treated mice. These cells were stained for FACS analysis. A plot of forward scatter (FSC) was used to separate out lymphocytes as shown in the top small panel on the left. These cells were further gated on to $CD19^+$ cells to separate $CD21^+CD23^+$ B cells. The $CD21^+CD23^+$ fraction was further subdivided into $CD21^+CD23^+$lo Fo cells and $CD21^+CD23^+$hi T2 and Mz B cells. The percentages of Fo, T2 and Mz cells are written beside the gated factions**.**

Rituximab seems to increase transitional type 1 B cells in spleen

Harvested cells from spleen and lymph nodes were stained for T1, T2, Mz and Fo B cells. This was possible through combination of several markers, which are expressed during development. The combination included CD19, CD21 and CD24; a plot of forward scatter (FSC) vs. CD19 was used to separate $CD21^+$ and $CD24^+$ cells. This population was subdivided into T 1 ($CD21^+CD24^+$lo), T2, Mz ($CD21^+CD24^+$hi), but this did not allow the discrimination of Mz and T2 cells, while Fo cells were an intermediate population ($CD21^+CD24^+$). The response of Rituximab treatment on splenic B cells (T1, Mz and Fo) was 31.5%, 8.01%, 49.6% vs. IgG treatment 16.3%, 5.56%, 63.6%, respectively. These demonstrate that Rituximab resulted in an increase in the T1 subset (31.5%) compared to IgG treatment, which resulted in a 16.3% increase in the same subset (Figure 4).

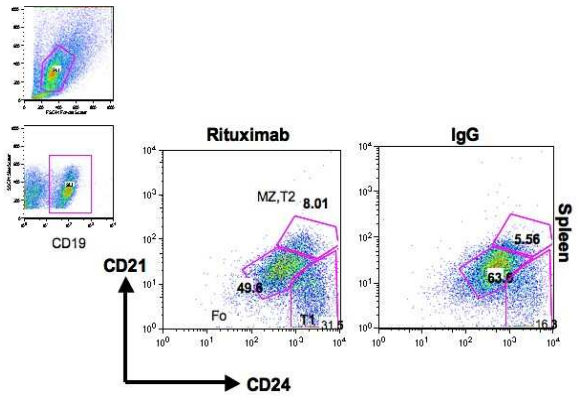

Figure 4. B cells subsets in spleen

Single cell suspensions of spleen and lymph node was isolated from Rituximab- and IgG-treated mice. A plot of forward scatter (FSC) was used to separate out lymphocytes as shown in the top small panel on the left. A plot of forward scatter vs. CD19 was used to separate ($CD21^+CD24^+$) B cells. The $CD21^+CD24^+$ fraction can be further subdivided into T 1 ($CD21^+CD24^+$lo), the T2+Mz ($CD21^+CD24^+$hi) and Fo ($CD21^+CD24^+$ intermediate population). The CD21 and CD24 staining did not allow discrimination of MZ & T2 cells. The percentage in Rituximab treated mice in spleen was compared to control mice.

B1a cells in spleen and lymph nodes resist depletion

Previous studies have shown that generation of natural antibodies IgM (T15) can be atheroprotective, which implies a role for B1 cells in atherosclerosis (10,12,20). Therefore we estimated the percentages of B1 cells in spleen and lymph nodes of treated mice (n=3). Harvested cells from spleen and lymph nodes were stained for flow cytometric analysis of the B cell subset (B1a). A plot of forward scatter (FSC) vs. side scatter was used to gate lymphocytes. This was then used to gate $CD19^{+}IgD^{+}$ intermediate population. This population was used to separate ($CD5^{+}CD43^{+}$ Hi) population recognised as B1a cells. The response of the Rituximab treatment on the percentage of the gated B cell population, i.e. B1a cells was 4.51% whereas in the IgG-treated mice it was 6.64%, with no significant difference in the statistical form. In fact, a similar observation was made for the percentage of B1a subset in the lymph nodes of Rituximab-treated mice (14.1%) compared to IgG-treated mice (11.6%) as shown (Figure 5).

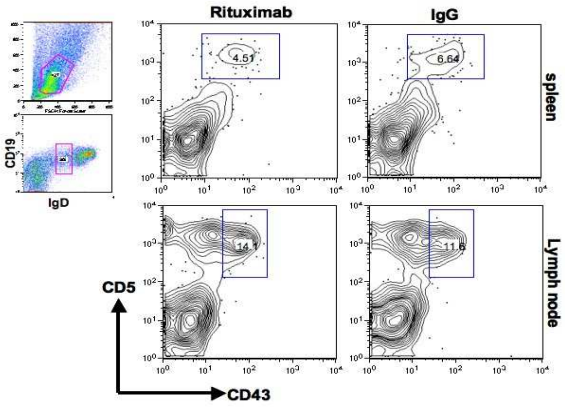

Figure 5. B1a cells in spleen and lymph nodes

Single cell suspensions of spleen and lymph nodes were isolated from Rituximab- and IgG-treated mice. A plot of forward scatter (FSC) was used to separate out lymphocytes as shown in the top small panel on the left. These cells were further gated on to ($CD19^{+}$ IgD^{+}) intermediate population to separate ($CD43^{+}CD5^{+}$)^{Hi} B1a cells.

Rituximab treatment reduced atherosclerotic lesions

The H and E stained sections demonstrate atherosclerotic lesions in the aorta of the tested mice. The lesions seen in the Rituximab-treated mice were small as compared to the lesions in control mice. The cholesterol cleft can be seen as white holes or clefts in the areas of maximum elevation of the lesion into the lumen (Figure 6 A & B).

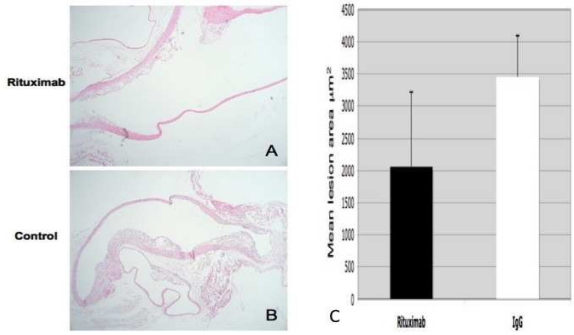

Figure 6. Aortic lesions in huCD20^{+}/ApoE$^{-/-}$ mice

Mice were given a high fat diet (HFD), treated every four weeks with IV Rituximab or IgG (control), and were sacrificed around 9 weeks. The aortic lesions were examined by H & E staining. As shown in figure 6, the Rituximab-treated mouse (A) had small lesions, whereas the IgG-treated mouse (B) had much larger lesions that covered a larger area of the lining layer. The average lesion size in cross section through the aortic origin is indicated in μm^2. The Rituximab-treated mice(black bar) had almost half the lesion area as compared to IgG-treated mice (white bar) (C).

The aortic lesions and the luminal area were measured in μm^2 using the imaging software (Axiovision version 4). The mean lesion area was calculated for 5 sections, every section 7µm thick and each section 7µm apart. The average lesion size was

measured for both Rituximab-treated mice and IgG-treated controls as shown in table 1. This showed an almost 2-fold reduction in lesion area in Rituximab-treated mice. As the sections used for measuring the lesions size were from 5 consecutive slides, this means the lesions extended longitudinally over 70μm from the proximal aorta.

Table 1 Effect of treatment on atherosclerotic lesions in huCD20^{+}/ApoE$^{-/-}$ mice

	Rituximab 1		Rituximab 2		IgG 1	IgG 2
Average lesion size (μm^2 ± SD)	2058 1158.5	±	1893 1146	±	3982 ± 638	2932 ± 694.3
Average aortic lumen	28522 11858	±	30939 7440	±	20772 ± 1336	1611 ± 2549
Percentage occlusion of lumen	5.58 2.88%	±	5.57 2.68%	±	19.3 ± 5.15 %	18.17 ± 3.91 %

We observed a variation in size of the aorta between these mice, so to ensure that the results reflected the absolute lesion area compared to vessel size, we determined the percentage area of the lesion relative to luminal area. We calculated the percentage of aortic lumen occluded for all 5 sections per mouse by (lesion area / luminal area × 100) for each section of the tested mice (n=4) as previously described (21), which revealed the percentage of aortic lumen obliterated with lesion. There was reduction in the percentage area of aortic lumen obliterated with lesion in the Rituximab-treated compared to IgG treated control mice (n=2) as shown in table 1. The lesion of the Rituximab- treated mouse had small plaques on 1-3 sections. In fact, the last 2 sections had no lesions at all. On the other hand, the IgG- treated mice had lesions bigger than the Rituximab-treated mouse.

DISCUSSION

This result defines an approach that now requires to be expanded to yield truly informative data. Initial observations demonstrate proof of principle that the methodologies are sound and can generate data of a meaningful nature. RA patients are vulnerable to cardiovascular morbidity and mortality and we hypothesised that depletion of B-lymphocytes (atheroprotective cells) (14) in the experimental model of atherosclerosis might result in aggravation of lesion formation in the mice. However, this preliminary data from a limited number of mice (n=4) gives us the clue what to expect from a full study with a reasonable number of huCD20^{+}/ApoE$^{-/-}$ mice and is in fact reassuring.

The current data show a 2-fold reduction in lesion size in response to Rituximab treatment in comparison with the IgG-treated control mice. Variation was observed in size of aortic lumen and arterial wall thickness in these genetically engineered mice. Therefore not only was the aortic lesion area compared, but we also calculated the percentage area of the aortic lumen obliterated. Importantly, the measures of aortic lesion area and percentage area of the aortic lumen obliterated have both shown that B cell depletion resulted in at least 2-fold reduction in atherosclerotic lesions. Importantly, these lesions extended vertically from root of aorta towards the ascending aorta. However, the overall reduction in arterial wall thickness of these huCD20^{+}/ApoE$^{-/-}$ mice needs to be validated in future studies.

B cell depletion every four weeks with Rituximab (100μg) was monitored throughout the course of the study, and a massive (>90%) reduction was observed in the B cell percentage after the first treatment, which was followed by rapid recovery of the B-lymphocytes in a three weeks' time. Interestingly, the systemic

treatments (second and third) resulted in modest depletion of B cells (9-20%). In one study, Rituximab (type I) has been shown to be 5 times less potent then tositumomab (type II). The return of B cells was reported 30-35 days following treatment (16). In addition, comparison of B cell depletion with a single dose of Rituximab (250µg) in two different strains of mice was performed, and it was demonstrated that C57BL/6 mice were more resistant than BALB/c. In fact, the analysis of B cell depletion in peripheral lymphoid organs (bone marrow and lymph nodes) was resistant to Rituximab therapy (22). Although in the present study the Rituximab dose (100µg) is less than half the dose used in the study mentioned, a massive depletion was confirmed in our mice on C57BL/6 background. However, the spleen and lymph nodes harvested at the end of the study did show resistance to rituximab therapy.

Mz and B1 B cells are potent responders to TLR activation, which has resulted in them being referred to as innate B cells (23-25). Moreover, pneumococcal vaccination in $Ldlr^{-/-}$ mouse has been demonstrated to reduce the atherosclerotic burden in mice, which was shown to be a result of oxLDL specific IgM antibodies produced by splenic B cells (26). In the present study, we observed an increase in the T1 B cells in the spleens of Rituximab-treated mice. Even though this subpopulation is small, but there is a fold increase in this population, which might be because they escape depletion or because of some stimulatory response. Moreover, the B1 B cells are mainly found in the peritoneal cavity (27). A modest population of B1 B cells was identified in the spleen and lymph nodes, which was not affected by Rituximab treatment. It is very important to confirm if this $CD20^{+}$/$ApoE^{-/-}$ mouse model has a normal lipid profile to confirm that the effects observed were of B cell-directed therapy (Rituximab) rather than as a result of changes in serum lipids.

CONCLUSIONS

The present preliminary study demonstrates that this is a feasible mouse model to estimate the effects of Rituximab treatment on vascular disease, since it is unlikely that sufficient numbers of RA patients will be treated in the short to medium term to generate reliable vascular outcome data. Therefore, as this current study is extended, it will likely generate helpful information for future translation to the clinic. Moreover, the model will offer a rich potential for mechanistic testing and to evaluate formally the role for B cells in atherosclerosis progression.

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