B-Cell Depletion Promotes Aortic Infiltration of Immunosuppressive Cells and Is Protective of Experimental Aortic Aneurysm

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Objective—B-cell depletion therapy is widely used for treatment of cancers and autoimmune diseases. B cells are abundant in abdominal aortic aneurysms (AAA); however, it is unknown whether B-cell depletion therapy affects AAA growth. Using experimental models of murine AAA, we aim to examine the effect of B-cell depletion on AAA formation.

Approach and Results—Wild-type or apolipoprotein E–knockout mice were treated with mouse monoclonal anti-CD20 or control antibodies and subjected to an elastase perfusion or angiotensin II infusion model to induce AAA, respectively. Anti-CD20 antibody treatment significantly depleted B1 and B2 cells, and strikingly suppressed AAA growth in both models. B-cell depletion resulted in lower circulating IgM levels, but did not affect the levels of IgG or cytokine/chemokine levels. Although the total number of leukocyte remained unchanged in elastase-perfused aortas after anti-CD20 antibody treatment, the number of B-cell subtypes was significantly lower. Interestingly, plasmacytoid dendritic cells expressing the immunomodulatory enzyme indole 2,3-dioxygenase were detected in the aortas of B-cell–depleted mice. In accordance with an increase in indole 2,3-dioxygenase+ plasmacytoid dendritic cells, the number of regulatory T cells was higher, whereas the expression of proinflammatory genes was lower in aortas of B-cell–depleted mice. In a coculture model, the presence of B cells significantly lowered the number of indole 2,3-dioxygenase+ plasmacytoid dendritic cells without affecting total plasmacytoid dendritic cell number.

Conclusions—The present results demonstrate that B-cell depletion protects mice from experimental AAA formation and promotes emergence of an immunosuppressive environment in aorta. (Arterioscler Thromb Vasc Biol. 2016;36:2191-2202. DOI: 10.1161/ATVBAHA.116.307559.)

Key Words: abdominal aortic aneurysm ■ anti-CD20 treatment ■ B1 and B2 cells ■ plasmacytoid dendritic cells ■ regulatory T cell

One of the most commonly clinically utilized B-cell depletion drugs is Rituximab, a chimeric murine/human monoclonal IgG targeting cells expressing CD20. It was first approved for the treatment of non-Hodgkin lymphoma and has demonstrated efficacy in the treatment of multiple CD20-positive B-cell malignancies, including chronic lymphocytic leukemia. After its approval for the treatment of rheumatoid arthritis, clinical use rituximab has been increased. Rituximab specifically depletes pre- and mature B cells but not the pro-B cells, plasma B cells or other cell immune types.2

Afflicting primarily elderly men and smokers, abdominal aortic aneurysms (AAA) carry significant morbidity and mortality remaining the 15th leading cause of death in the United States. Approximately 200,000 patients are diagnosed each year with AAA and nearly 15,000 are in danger of rupture. Infiltration of immune cells has been strongly correlated with AAA formation and rupture.4 Importantly, quantitative analysis of human AAA tissue demonstrated that 41% of infiltrating mononuclear cells are B cells.3 We have previously reported infiltration of both B1 and B2 subtypes in murine experimental AAA.6 The muMT mice, which are genetically deficient in B cells, develop AAA similar to the wild-type (WT) mice, whereas adoptive transfer of B2 cells to muMT mice suppressed aneurysm formation.6 B cells have also been reported to promote mobilization of monocytes from spleen to the AAA in response to angiotensin II (Ang II) in apolipoprotein E–knockout (KO) mice.2 However, it is still unknown whether B-cell depletion is protective or deleterious for experimental murine AAA.

On the basis of our results obtained from muMT mice, we hypothesized that B-cell-depleted mice were prone to AAA formation...
formation. To test the hypothesis, we used anti-CD20 antibody-mediated B-cell depletion strategy in both elastase perfusion and Ang II infusion models of mouse AAA. We used flow cytometry to quantify B-cell depletion and characterize immune cells in AAA, spleen, blood, thymus, and bone marrow; multiplex assay to quantify circulating cytokines/chemokines; and real-time reverse transcription polymerase chain reaction to quantify inflammatory gene expression in aorta.

Materials and Methods

Results

Murine Anti-CD20 Antibody Significantly Depletes Both B1 and B2 Cells and Protects Mice From AAA Formation

To examine effectiveness of the B-cell depletion antibody, WT mice were injected intraperitoneally or intravenously with anti-CD20 or control antibodies, and after 7 days, B1 and B2 cells were examined from blood, spleen, and peritoneal fluid using flow cytometry (Figure 1A). B1 cells were identified as CD19hiB220lo and B2 cells as CD19loB220hi.6,8 B1 cells are known to be predominantly located in the peritoneal cavity in mice.9,10 and our results confirmed these findings (Figure 1B). Notably, both intraperitoneal and intravenous injection of anti-CD20 antibody resulted in a significant decrease in B1 cells. B2 cells were more prominent in spleen and blood and 85% to 95% of B2 cells were depleted at day 7 after both intraperitoneal and intravenous treatments (Figure 1B). However, intraperitoneal treatment depleted B2 cells in peritoneal fluid more efficiently compared with intravenous treatment. Altogether, these results demonstrate anti-CD20 treatment significantly depleted B cells in WT mice after 7 days of treatment.

To examine whether B-cell depletion affects AAA formation, an elastase perfusion model of AAA was first used. A single dose of anti-CD20 treatment maintains depletion of B cells for 3 to 8 weeks.11,12 However, to prevent reappearance of B-cell subtypes, we followed previously published anti-CD20–mediated B-cell depletion strategy in mice,11 in which the WT mice were given 2 doses of anti-CD20 or control antibodies, the first on day 7 before elastase perfusion and the second on day 7 after elastase perfusion of abdominal aorta (Figure 1A). As a negative control, abdominal aortas were perfused with heat-inactivated elastase. At day 14 after elastase perfusion, mice were anesthetized, aortic diameters were measured, and the perfused aorta, blood, peritoneal fluid, bone marrow, thymus, and spleen were collected for the analysis of B-cell depletion. Compared with single dosing, 2 doses anti-CD20 treatment (intraperitoneal or intravenous) depleted >95% of both B1 and B2 cells in various tissues, including spleen and the elastase-perfused aortas (Figure 2A; Figure I in the online-only Data Supplement). This method also depleted B1a, B1b, and B2 cells in peritoneal fluid as determined by 2 methods of B-cell characterization (Figure II in the online-only Data Supplement). Only a midCD19hiB220+ population was preserved in bone marrow of anti-CD20–treated animals representing previously described pro-B or long-lived plasma cells that do not express CD20 (Figure I in the online-only Data Supplement). Aortic perfusion with active elastase induced a significant increase in aortic diameter, that is, AAA formation compared with perfusion of heat-inactivated elastase (Figure 2B). AAA formation was similar in control antibody-treated intraperitoneal and intravenous groups. In contrast to our hypothesis, B-cell depletion strikingly suppressed AAA formation (intraperitoneal: control versus anti-CD20, 97.9±7.4 versus 62.2±4.7%, P<0.01; intravenous: control versus anti-CD20, 97±6.6 versus 55±3.8%, P<0.05; n=8–9; Figure 2B). In accordance with protection from AAA, elastase-perfused aortas of both intraperitoneal and intravenous anti-CD20 antibody-treated groups displayed marked preservation of elastin layers and smaller adventitial area (Figure 2C). Altogether, these results suggest that anti-CD20 antibody treatment significantly depleted B cells in various tissues independent of delivery method of anti-CD20 antibody and protected mice from AAA formation.

To confirm this protective effect of B-cell depletion in another model, we utilized an Ang II infusion model of murine AAA14 in a small group of male apolipoprotein E–KO mice (n=5–6). The mice were treated with anti-CD20 or control antibodies 7 days before the Ang II infusion (at 1000 ng/kg per minute) via subcutaneous osmotic pumps (Figure IIIA in the online-only Data Supplement). Repeat doses of antibodies were given on days 7 and 21 after pump implantation. As a negative control, a group of apolipoprotein E–KO mice were infused with saline via osmotic pumps and were given control antibodies. The mice were euthanized on day 28 after Ang II infusion, and aortas were harvested. Stages of aneurysm were determined as described by Daugherty et al.14 No aortic aneurysm pathology was observed in any saline-infused mice (Figure IIIAB and IIIC in the online-only Data Supplement). Ang II infusion in control antibody-treated mice resulted in mixed aortic aneurysm, such as no aneurysm (n=1), thoracic aortic aneurysm (n=1, stage I), AAA (stage II, n=1 and stage III, n=2), and ruptured thoracic aorta (n=1, stage intravenous). However, anti-CD20–treated mice were completely protected (n=5; Figure IIIAB and IIIC in the online-only Data Supplement). Successful infusion of Ang II was confirmed via elevated level of plasma aldosterone (Figure IIIID in the online-only Data Supplement). Moreover, the anti-CD20–treated mice demonstrated marked preservation of elastin layers and smaller adventitial area in suprarenal aortas compared with Ang II–infused control antibody-treated mice (Figure IIIID in the online-only Data Supplement). Collectively, our results demonstrate that B-cell depletion protects mice from AAA formation via using 2 unique models of experimental murine AAA.

Circulating Factors in the B-Cell–Depleted Mice Are Not Responsible for the Protection From AAA

B cells regulate inflammation via secreting immunoglobulins (Igs), cytokines, and chemokines or by directly contacting other...
immune cells. Importantly, IgM antibodies have been reported to be anti-inflammatory and protective in the settings of atherosclerosis, whereas, B cell secreted interleukin-2 (IL-2), IL-4, tumor necrosis factor-α, IL-6, interferon-γ (IFN-γ), and IL-12 and tumor necrosis factor-α regulates type 1 and type 2 immune responses. We speculated that B-cell depletion would preserve the IgM level or increase the level of anti-inflammatory cytokines. However, in our study, IgM levels were significantly lower \((P<0.01)\), whereas, as described before by DiLillo et al, IgG levels were similar in the B-cell–depleted mice compared with the control mice (Figures 3A). Moreover, no significant differences were found in the plasma levels of pro- or anti-inflammatory factors, such as IL-1β, IL-6, tumor necrosis factor-α, RANTES (regulated on activation, normal T-cell–expressed and secreted), MIP (macrophage inflammatory protein)-1α, MIP-1β, IP-10, IL-13, Eotaxin, KC (keratinocyte chemoattractant), MIP-2, and MIG (monokine induced by gamma interferon), whereas, the levels of IL-2, IL-4, IL-5, IL-10, IL-17, IL-12-p70, and IFN-γ were below the detection in the control and B-cell–depleted mice (Figures 3B). Altogether, these results suggest that changes in circulating levels of immunoglobulins, cytokines, or chemokines are not the mechanism for attenuation of AAA in the B-cell–depleted mice.

**Anti-CD20 Antibody Treatment Does Not Abrogate Aortic Infiltration of Leukocytes**

Aortic infiltration of immune cells has been strongly associated with AAA formation; therefore, we examined the

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**Figure 1.** Both intraperitoneal and intravenous treatments of anti-CD20 antibody are effective in depleting B cells. A, Schematic representation of study design for B-cell depletion and induction of experimental elastase perfusion model of murine abdominal aortic aneurysm (AAA). B, The proportion of B1 and B2 cells are expressed as percentage of CD45+ hematopoietic cells in wild-type (WT) mice at 7 d after administration of control or anti-CD20 antibody via IP or IV. After 1-way ANOVA, parametric unpaired \(t\) test was applied to determine significant differences between the groups. Values are expressed as means±SEM \((n=3)\) and \(*\), \**, and \***\(\) indicate \(P<0.05, 0.01,\) and \(0.001,\) respectively. n.s. represents not significantly different \((P>0.05)\).
histopathology of aortic cross sections. AAA from B-cell–depleted mice demonstrated infiltration of immune cells, such as macrophages (F 4/80) and neutrophils (Ly6B.2; Figure 4A). Quantification of immunostaining revealed significantly less macrophage content and a trend toward lower neutrophil content in the aortas of B-cell–depleted mice compared with the control antibody-treated mice (Figure 4B). Next, we utilized flow cytometry to define and quantify the immune cells in AAA tissues. For cell counting, specifically the elastase-perfused portion of abdominal aorta was harvested (Figure IV A in the online-only Data Supplement), perfused with heparin-PBS solution ex vivo. Each isolated aorta was digested separately with an enzymatic cocktail, stained with fluorescent-conjugated antibodies, and counting beads were added before running on a flow cytometer. As a control, the number of immune cells in spleen was counted. The gating strategy used for phenotyping immune cells is shown in Figure IVB in the online-only Data Supplement. The results revealed that the number of mononuclear leukocytes (CD45+ cells) was significantly lower in the spleen of anti-CD20–treated mice; however, no

Figure 2. Both intraperitoneal (IP) and intravenous (IV) treatments of anti-CD20 antibody suppress elastase perfusion-induced abdominal aortic aneurysm (AAA) formation with marked depletion of B cells and preservation of elastin layers. A, Representative flow cytometry plots showing depletion of B cells in spleen and aorta of wild-type (WT) mice administered 2 doses of control or anti-CD20 antibody via IV. The plots have been gated for singlets, live CD45+ cells. B, Increase in aorta diameter in WT mice treated with 2 doses of control or anti-CD20 antibody via IP or IV. ∆ Elastase and Elastase indicate aortic perfusion with heat-inactivated elastase and elastase, respectively (n=8–9). After 1-way ANOVA, parametric unpaired t test was applied to determine significant differences between the groups. C, Representative images showing AAA sections stained for Verhoeff–Van Gieson (VVG; elastic fibers, black). A small segment of images acquired in 4× is shown in ×20 magnification. Scale bar in 4× images is 500 µm and in 20× images is 50 µm. Elastic fiber area (presented as percentage of total aortic area) and adventitial area were determined using ImageJ. After 1-way ANOVA, nonparametric t test (Mann–Whitney test) was applied to determine significant differences between the groups. Values are expressed as means±SEM and *, **, and *** indicate P<0.05, 0.01, and 0.001, respectively.
significant difference was observed in the aortas (Figure 4C). Furthermore, the spleen and aorta from control antibody-treated mice demonstrated the presence of B1 and B2 cells, as well as IgM+IgD+ B1 and B2 cells, the numbers of which were significantly lower or absent in anti-CD20–treated mice (Figure 4D; Figure V in the online-only Data Supplement). These results together suggest that immune cells accumulating in aorta may be creating an immunosuppressive microenvironment protecting the B-cell–depleted mice from AAA.

**B-Cell Depletion Increases Aortic Infiltration of Immunosuppressive Cells**

As the number of infiltrated leukocytes in the aorta was not affected after B-cell depletion, and B-cell–depleted mice were protected from AAA formation, we speculated that the absence of B cells created an immunosuppressive environment in aorta.

To investigate the presence of immunosuppressive cells, we first stained aortic cross sections from control and anti-CD20 antibody-treated mice for the presence of T and B cells using anti-CD3ε and anti-B220 antibodies, respectively (Figure 5A). CD3ε+ and B220+ cells stained in proximity to each other in AAAs from control antibody-treated mice, which was in accordance with our previously published literature.6 CD3ε+ and, unexpectedly, B220+ cells were found in the AAAs of B-cell–depleted mice (Figure 5A). To confirm whether these B220+ cells were B cells, the aortic cross sections were stained with anti-B220 and anti-CD20 antibodies. Interestingly, 2 types of B220+ cells, that is, B200+CD20+ and B220+CD20− were identified in AAA of control antibody-treated mice, whereas, only B220+CD20− cells, although lesser in number, were present in the aorta of anti-CD20 antibody-treated mice (Figure 5B). To further characterize and quantify these B220+CD20− cells, we performed flow cytometry. The number of B220+ cells was significantly lower in the aortas of anti-CD20−treated mice compared with control antibody-treated mice (not shown). Furthermore, these B220+ cells in B-cell–depleted mice were identified as plasmacytoid dendritic cells (pDCs) expressing both CD317/plasmacytoid dendritic cell antigen-1 and B220. The pDCs were populated as hiCD317+B220+ and midCD317+B220+ in aorta (Figure 5C) and also in blood (Figure VIA in the online-only Data Supplement) and spleen (not shown). In blood, the midCD317+B220+ population was a well-defined; however, in aorta, a large portion of the cell population (40.2±1.6% of mononuclear live CD45+CD4− cells, n=7) was CD317+ irrespective of control or anti-CD20 antibody treatment. A small population of pDC is known to express surface CD19 and intracellular immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) and plays a strong immunosuppressive role.18–20 Therefore, we included CD19 and IDO antibodies in our flow cytometry antibody panel. Specifically, in the entire population of
CD45+CD317+B220+ cells, only the midpopulation was CD19+ and strongly expressed IDO. CD19+IDO+ cells were undetected in hiCD317+B220+ and the number of midCD19+IDO+ cells was significantly higher in AAA (Figure 5D) and blood (Figure VIB in the online-only Data Supplement) of anti-CD20 antibody-treated mice. These results suggest that B-cell depletion increased aortic content of IDO-expressing pDCs.

As the number of circulating IDO+ pDC was increased, we hypothesized that tryptophan catabolism would be increased systemically, leading to global immunosuppression after B-cell depletion. However, we did not find significant differences in the level of tryptophan and its metabolite kynurenine, or in the kynurenine:tryptophan ratio in circulation (Figure VIC in the online-only Data Supplement), suggesting a recessive role of IDO-expressing pDCs in circulation.

IDO-expressing pDCs maintain the immunosuppressive environment by promoting regulatory T cell (Treg) generation in tissues. Therefore, we quantified Tregs in aorta and spleen using flow cytometry. As reported before, absolute Treg number, but not the proportion of Treg (expressed as percentage

Figure 4. Infiltration of immune cells in the aortas of control and anti-CD20 antibody treated mice. A, Representative images showing abdominal aortic aneurysm (AAA) sections stained for F4/80 (macrophages, brown), Ly-6B.2 (neutrophils, brown), and nuclei (hematoxylin); scale bar, 50 µm. B, The immunostained area in A is determined using ImageJ and expressed as percentage of total aortic area. C and D, Flow cytometry quantification of CD45+ immune cells, and B1 and B2 cells in spleen and AAA of control or anti-CD20 antibody-treated wild-type mice via intravenous (n=4–5). Nonparametric t test (Mann–Whitney test) was applied to determine significant differences between the groups. Values are expressed as means±SEM and * and *** indicate P<0.05 and 0.001, respectively. P values >0.05 are indicated on the top of the bars.
Figure 5. Plasmacytoid dendritic cells (pDCs) are present in mouse abdominal aortic aneurysms (AAAs). A, Representative images acquired on a light microscope showing AAA sections stained for B220+ (brown) cells and CD3ε+ (black) cells; scale bar, 10 µm. Black arrows indicate CD3+ cells and red arrows indicate B220+ cells. * indicates lumen. B, Immunofluorescent images acquired on (Continued)
of CD4+ T cells), was significantly lower in spleen following anti-CD20–mediated B-cell depletion (Figure 6A). Staining of aortic cross sections with CD3ε and forkhead box P3 antibodies demonstrated presence of Tregs in aortas of control antibody and anti-CD20 antibody-treated mice (Figure 6B). Furthermore, flow cytometry revealed significantly higher Treg number and percentage (% of CD4+ T cells) in the aortas of anti-CD20–treated mice (Figure 6C). Altogether, these results suggest that B-cell depletion created an immunosuppressive microenvironment in aorta.

Next, to confirm the presence of an immunosuppressive environment, we examined local inflammatory gene expression in aortas using real-time reverse transcription polymerase chain reaction. The results demonstrated significant decrease in the expression of IFN-γ, IL-1β, and a trend in the reductions IL-6 and IL-10 in anti-CD20–treated mice; however, TGF-β expression was similar to the control antibody-treated mice (Figure 6D) confirming emergence of an immunosuppressive environment in aorta after B-cell depletion.

**Presence of B Cells Lowers the Number of IDO+ pDCs**

Chen et al.21 and Baban et al.23 have reported that the immunosuppressive IDO+ pDCs differentiate naive CD4 T cells to Tregs. However, it is unknown whether the presence of B cells affect the number of IDO+ pDCs. As the number of IDO+ pDC is increased in the B-cell–depleted mice, we hypothesized that B cells suppress IDO+ pDCs. To test this, we developed a coculture model. We used synthetic oligonucleotides containing unmethylated CpG oligodeoxynucleotide motifs to induce IDO expression in pDCs.21,23 First, the pDC-enriched cell fraction was collected from the spleen of WT mice using MACS column, and IDO expression was induced via treatment with class B CpG oligodeoxynucleotide 1826 in a 96-well culture plate for 48 hours (detail experimental method is described in the online Supplemental Material). As a control, pDCs were treated with control oligodeoxynucleotide 1826. The expression of pDC markers CD317 and B220 was found to be significantly increased in oligodeoxynucleotide 1826–treated pDCs (Figure 7A through 7C). Again, only the midCD317+B220+ population was dominated with a high number of immunosuppressive CD19+IDO+ cells. Next, the entire B-cell population was isolated (via MACS column) from WT spleen, and various concentrations of B cells were cultured with IDO+ pDCs for 24 hours. Addition of increasing concentrations of B cells did not affect the number of pDCs; however, it significantly decreased the number of IDO+ pDC (Figure 7D). These results suggest that the presence of B cells suppresses the emergence of immunosuppressive IDO+ pDCs.

**Discussion**

Herein, we demonstrate that anti-CD20 treatment, whether injected by intraperitoneal or intravenous, strongly suppressed AAA formation in WT mice. The rationale for selecting 2 methods of delivery is that B1 cells are predominant in the peritoneal cavity, whereas B2 cells are in the spleen and blood of mouse. Patients, however, receive anti-CD20 treatment intravenously. We hypothesized that intravenous treatment would deplete more B2 cells in the spleen sparing the B1 cells in the peritoneal cavity, whereas intraperitoneal treatment would deplete more B1 cells in the peritoneal cavity sparing B2 cells in the spleen. After 7 days of anti-CD20 treatment, a significant depletion of circulating and splenic B1 and B2 cells was achieved; however, peritoneal B1 and B2 cells were resistant to depletion. Hamaguchi et al.24 reported a similar finding using a different clone of monoclonal anti-CD20 antibody. Interestingly, after 2 doses of anti-CD20 treatment, B1 and B2 cells were almost depleted in peritoneal cavity, spleen, blood, and bone marrow. In the same line, IgM level was significantly lower in the B-cell–depleted mice, which is primarily produced by B1 cells. We did not find a decrease in IgG levels, potentially because long-lived plasma B cells, which do not express CD20, are preserved in bone marrow after B-cell depletion and maintain circulating IgG levels.16 In fact, our results demonstrated the presence of a midCD19+B220+ population in bone marrow after 2 doses of anti-CD20 antibody treatment. Although, further flow cytometry experiments are required for confirmation, the midCD19+B220+ population are most likely long-lived plasma cells.

pDCs are known to recognize viral- and bacterial-derived products and induce synthesis of type I interferon genes, such as IFN-α and IFN-β, and promote activation of a proinflammatory response via activating effector T cell, cytotoxic T cells, and natural killer cells.25 On the contrary, tolerogenic pDCs express IDO and have potent immunomodulatory properties including the induction of Treg differentiation from naive CD4+ T cells.26,27 Such tolerogenic pDCs seem to be representing a subset of pDCs and are tissue specific.28,29 Increased expression of IDO degrades tryptophan promoting immune tolerance or suppressing immune activation of neighboring cells.30 Daissormont et al.31 have reported that pDCs are scarcely present in atherosclerosis and blocking IDO activity in pDCs exacerbates atherosclerosis. Our results provide the first evidence of emergence of IDO producing pDCs after the depletion of B cell in aorta using anti-CD20 antibody. In the context of aortic aneurysms, Tregs have been shown to suppress experimental murine AAA.32 Importantly, patients with AAAs do not have functional circulating Tregs.33 In our study, depletion of B cells increased the number of Tregs and IDO-expressing pDCs in aorta. In aortas of B-cell–depleted mice,
the IDO-expressing pDCs may create an immunosuppressive environment leading to a decrease in pro-inflammatory gene expression. It is unknown whether IDO-expressing pDCs would differentiate naive CD4 T cells to Treg or recruit Treg to the aorta after elastase perfusion. We found a significant increase in circulating IDO+ pDCs after B-cell depletion; however, this was not correlated with an increase in tryptophan degradation or increase in kynurenine synthesis. Although
other tryptophan metabolites, such as anthranilic acid, were not measured, it is likely that the concentration of tryptophan metabolites is higher in AAA tissues compared with the circulating level underscoring tissue-specific role of tolerogenic pDCs. Moreover, possible role of IL-10–producing dendritic cell or T cell in the protection cannot be ruled out. However, our IDO+ pDC:B-cell coculture demonstrated that the presence of B cells significantly lowered the number of CD19+IDO+ pDCs. Our data are in consistent with a model that B cells control immunosuppressive effects of pDC by limiting IDO+ pDC population and regulate inflammation and AAA as shown in the graphic abstract. In support of this model, recently, Yun et al.\textsuperscript{34} demonstrate that aortic pDCs-expressing IDO protects against atherosclerosis via generation of Tregs.

We have previously reported that the muMT mice, genetically deficient in B cells, develop AAA similar to the WT mice, and adoptive transfer of B2 cells suppresses AAA formation in muMT mice, suggesting AAA formation is dependent on B-cell subsets. In this study, we created B-cell deficiency in mature WT mice by using anti-CD20 antibody, which affected the homeostasis of T cells, primarily by reducing T-cell number including Tregs in spleen as demonstrated by us and also reported previously by Lykken et al.\textsuperscript{22} Interestingly, IDO-expressing pDC number was significantly increased in various tissues of B-cell–depleted mice. Moreover, B-cell depletion has also been shown to improve endothelial function and reduce systemic inflammation in patients with rheumatoid arthritis.\textsuperscript{35} Therefore, further studies are needed to understand the paradox immune response in muMT and anti-CD20 antibody-mediated B-cell–depleted mice.

In our study, the B cells were depleted before the induction of AAA. It is unknown whether B-cell depletion would

Figure 7. Presence of B cells affects the number of indole 2,3-dioxygenase (IDO)+ plasmacytoid dendritic cells (pDCs). A, Schematic representation of study design of IDO-expressing pDC:B-cell coculture. pDCs isolated from spleen of wild-type mice were treated with 0.2 µmol/L of oligodeoxynucleotide (ODN) 1826 or control ODN (Co-ODN 1826) for 2 d. Subsequently, the culture medium was removed and medium containing indicated concentrations of isolated splenic B cells were added. The coculture was continued for 24 h and expression of B220, CD317, CD19, and IDO was determined via flow cytometry. B, Representative flow plots showing midCD317+B220+ cells, which are further gated from the CD19+IDO+ population. C, Quantification of midCD317+B220+ cells (significant difference between co-ODN and each of the ODN-treated group is shown) and (D) quantification of CD19+IDO+ pDCs. Parametric unpaired t test was applied to determine significant differences between the groups. Values are expressed as means±SD and *, **, and *** indicate \(P<0.05, 0.01,\) and 0.001, respectively.
suppress established experimental AAA in mice. It is also unknown whether AAA formation will still be suppressed after a long-term depletion of B cells. In support of our finding, Mellak et al1 have reported that Ang II mobilizes monocytes from spleen to aorta in a B cell–dependent manner and promote AAA formation in the apolipoprotein E–KO mice. As far as atherosclerosis is concerned, there are reports supporting4–6 and contradicting7 the protective role of IDO-expressing pDCs. Therefore, generation of pDC-specific IDO-KO mouse is required to confirm that IDO-expressing pDCs protect B-cell–depleted mice from AAA.

In conclusion, using 2 experimental models of murine AAA, we demonstrated that anti-CD20 antibody-mediated B-cell depletion increased the number of IDO-expressing pDCs and created an immunosuppressive environment that attenuated inflammatory gene expression and suppressed AAA growth. Although further mechanistic studies are needed, these findings have the potential to lead to the development of nonsurgical strategies to prevent aneurysm formation. Furthermore, this evidence suggests that patients undergoing B-cell depletion therapy may be protective from AAA formation.

Acknowledgments
We acknowledge the assistance of Flow Cytometry Core Facility, Advanced Microscopy Facility, CVRC Histology Core, and Research Histology Core at University of Virginia.

Sources of Funding
This work was supported by National Scientist Development Grant 1R01 DK080044 from the American Heart Association (to A.K. Meher). K08HL098560 from the National Heart, Lung, and Blood Institute (to G. Ailawadi), NIH R01 5K01HL124131 to G.R. Upchurch Jr, NIH R01 DK096076 and a Grant in Aid from the American Heart Association (to N. Leitinger). V. Serbulea was supported by an American Heart Association (AHA) predoctoral grant.

Disclosures
None.

References


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**Highlights**

- Both intraperitoneal and intravenous treatments of anti-CD20 antibody significantly depleted B cells and protected mice from abdominal aortic aneurysm.
- Despite significant depletion of aortic B-cell content, anti-CD20 antibody treatment did not affect the number of aortic infiltrated immune cells.
- A distinct population of indole 2,3-dioxygenase–expressing plasmacytoid dendritic cells appeared in aorta after B-cell depletion.
- In vivo, infiltration of indole 2,3-dioxygenase+ plasmacytoid dendritic cells was associated with increase in the number of regulatory T cells and decrease in the expression of proinflammatory genes in aorta, suggesting emergence of an immunosuppressive environment after B-cell depletion. In vitro, presence of B cells significantly lowered the number of indole 2,3-dioxygenase+ plasmacytoid dendritic cells.
- B-cell depletion therapy may be beneficial for patients with abdominal aortic aneurysm.
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Arterioscler Thromb Vasc Biol. 2016;36:2191-2202; originally published online September 15, 2016;
doi: 10.1161/ATVBAHA.116.307559

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

Materials and Methods

Mice
Seven weeks old male C57BL/6 (stock # 000664) and apolipoprotein A knockout mice (stock # 002052) mice were obtained from The Jackson Laboratory (US). All mice were given water and a minimal phytoestrogen diet (2016 Teklad Diet; Harlan Laboratories, Indianapolis, IN) ad libitum. The mice were used for various studies at 8 weeks of age. All protocols were approved by University of Virginia Animal Care and Use Committee.

B cell depletion and experimental model of mouse AAA
The B cell depleting antibody clone 18B12 (IgG2a isotype) and the isotype control antibody (clone 2B8) are obtained from Biogen Idec, Inc, (Cambridge, MA). The antibodies were diluted in PBS at 1 mg/ml concentration and 250 µl of the diluted antibody was injected to the mice intraperitoneally (IP) or intravenously (IV). Seven days following antibody treatment, the mice were sacrificed and B cell depletion in various tissues was examined using flow cytometry. To find out the effect of B cell depletion on AAA formation, control or anti-CD20 antibodies were injected IP or IV at seven days before and seven days after the induction of AAA using the elastase perfusion model. Similarly, in the AngII model, mice were injected with control or anti-CD20 antibodies via IV at 7 days before the osmotic pump implantation, and 7 and 21 days after the pump implantation.

Elastase perfusion model of murine AAA:
The elastase perfusion model was performed as described by Johnston et al. Following adequate anesthesia with inhaled isoflurane, a midline laparotomy was performed with reflection of the peritoneal contents superiorly. The abdominal aorta was isolated from the level of the left renal vein to the iliac artery bifurcation. Once the lateral tissue was dissected from the aorta branches were cauterized or ligated with 10-0 nylon suture. The maximal aortic diameter was measured via video micrometry (Leica Microsystems, Heerbrugg, Switzerland). The aorta was occluded with a 5-0 silk suture distally followed by proximal occlusion below the level of the renal veins. An aortotomy was made with a 30-gauge needle, using external compression the residual blood was evacuated from the aorta via the aortotomy. The aorta was cannulated with 0.033” polyethylene catheter (Braintree Scientific, Braintree, MA) and perfused with purified porcine elastase solution (Sigma-Aldrich, St. Louis, MO) diluted in 0.9% sodium chloride for 5 minutes. An infusion pump (Kent Scientific, Torrington, CT) was utilized to provide uniform flow delivery. Aortic turgor and aortic wall digestion were evaluated via visual inspection and ballotability. Following perfusion, the catheter was removed, the elastase solution was evacuated, and the aortotomy was closed with a single simple stitch using 10-0 nylon suture. The aortic ligation sutures were then untied. Typically, after the perfusion, we record about 50% expansion in aorta diameter. The distal extremities were inspected for adequate perfusion and the aorta was examined for the presence of thrombus. The peritoneal contents were returned to their anatomical position and the skin was closed in two layers: a running 6-0 polyglactin for the abdominal fascia followed by interrupted 6-0 polypropylene sutures for skin closure. Mice received buprenorphine IP for analgesia.

At the time of harvest (14 days of elastase perfusion), mice were anesthetized with inhaled isoflurane, and a midline laparotomy was made with dissection of the infrarenal abdominal aorta from the level of the left renal vein to the iliac bifurcation. Once dissected, the aorta was photographed, and the maximal aortic diameter was measured with video micrometry (Leica Microsystems, Heerbrugg, Switzerland). Blood was collected with a 25g needle from the inferior vena cava. The aorta was then harvested from below the level of the left renal vein to
the aortotomy closure for tissue analysis. Generally, at the time of harvest, any extra connective tissue present was removed from the abdominal aorta, and the elastase perfused segment of aorta was harvested excluding the sites of temporary ligatures and aortotomy. For flow cytometry and gene expression analysis, care was taken not to include any small lymph nodes present near to the aorta.

AngII infusion-induced AAA:
Calculation and dissolution of AngII, osmotic pump filling and AngII infusion-induced AAA was performed as described by Daugherty and Lu et al[1, 2]. In this well-established model of experimental aneurysm induction, 8 weeks old male ApoE KO were infused with AngII (1000 ng/kg/min) via Alzet® osmotic pumps (Model 2004) for 4 weeks. Control animals were undergone sham surgery and placement of saline carrying osmotic pumps. Implantation was completed following injection of intraperitoneal anesthesia (ketamine (60 mg/kg)/xylazine (8 mg/kg)), followed by removing hair nape of the animal's neck, disinfecting and drying, making a small incision, minimal tunneling posteriorly to accommodate osmotic pumps. All incisions were undergone staple closer, and the animals were allowed to recover for 4 weeks. Following the end of the study period, animals were sacrificed with CO2 inhalation. Next, a small cut was made in the right atrium and the mice were perfused with 50 ml of 1x PBS solution containing 5 mM EDTA through the left ventricle. Subsequently, the whole aorta was excised from the root to the iliac bifurcation, fixed in 4% paraformaldehyde and imaged to study of abdominal aortic aneurysms. In this study, three groups of mice were used: (1) Control antibody treated and infused with saline, n= 5, (2) Control antibody treated and infused with AngII, n=6 and (3) Anti-CD20 antibody treated and infused with AngII, n=5.

Immunohistochemistry
The aortas were perfused with saline before harvesting, and stored in Zinc Formalin for 24 hours, followed by storing in 70% Ethanol for another 24 hours. Subsequently, the aortic tissues were imbedded in paraffin and 10 µm sections were cut. Verhoeff-Van Gieson (VVG) staining on aortic sections was performed to stain the elastin layers. For immunohistochemistry, following antibodies were used: F4/80 Antibody (clone Cl:A3-1, AbD serotec, Raleigh, NC); rat anti-mouse Ly-6B.2 antibody (clone 7/4, AbD serotec, Raleigh, NC); Rat anti-mouse CD45R (clone RA3-6B2, BD Pharmingen, San Jose, CA); anti-CD3 antibody (sc-1127, Santa Cruz Biotechnology, Inc., Dallas, TX); Anti-FOXP3 antibody (ab54501, Abcam) and CD20 antibody (sc-7735, Santa Cruz Biotechnology, Inc.). Whole molecule control primary antibodies of Rat IgG (31933), Goat IgG (31245) and Rabbit IgG (31235) were from ThermoFisher Scientific (Waltham, MA).

Multiple antigen labeling was performed using ImmPACT DAB Peroxidase (HRP) Substrate, (SK-4105) and ImmPACT SG Peroxidase (HRP) Substrate (SK-4705) obtained from Vector Laboratories, Burlingame, CA. Briefly, after antigen retrieval using Citric Acid based antigen unmasking solution (Vector Laboratories, Burlingame, CA), aortic sections were blocked using Donkey serum and Avidin-Biotin kit (SP-2001, Vector Laboratories), primary antibody was applied and incubated overnight at 4 °C. After washing the primary antibody, biotinylated secondary antibody was applied and incubated at room temperature for 1 hour, then washed and incubated with diluted ABC (PK-6100, Vector Laboratories). After washing again, the sections were stained with ImmPACT DAB for 10 to 15 minutes and excess DAB was removed by washing the sections in water. Subsequently, the sections were blocked and stained with secondary primary antibody similar to the first one. Finally, the sections were mounted on VectaMount AQ Aqueous Mounting Medium (H-5501, Vector Laboratories) and images were acquired on Nikon Eclipse Ti –U inverted microscope and NIS-Elements Br microscope imaging software. Immunofluorescence staining of aortic sections was performed using standard protocol. The stained sections were mounted using VECTASHIELD Mounting Medium with
DAPI (H-1200, Vector Laboratories) and confocal images were acquired on Zeiss LSM 700 using 405, 488 and 633 nm lasers and ZEN software.

**Quantification plasma IgM and IgG**
Plasma samples from WT mice were collected 14 days after inducing AAA via elastase perfusion. Total IgM and IgG levels in plasma collected from mice were quantified using Mouse IgM ELISA Ready-SET-Go (Cat# 88-50470-22) and Mouse IgG total ELISA Ready-SET-Go (Cat# 88-50400-22) from eBioscience (San Diego, CA), respectively.

**Quantification of plasma cytokines and chemokines**
Plasma samples from WT mice were collected 14 days after inducing AAA via elastase perfusion. MILLIPLEX® Multiplex Assay Kit for quantification of mouse plasma IL-2, IL-4, IL-5, IL-10, IL-17, IL12-p70, IFNγ, IL-1β, IL-6, TNFα, RANTES, MIP-1α, MIP-1β, IP-10, IL-13, Eotaxin, KC, MIP-1, MIP-2 and MIG were obtained from EMD Millipore (Massachusetts, US) and run on a Luminex 100 IS System as described by the manufacturer.

**Flow cytometry**
The aortic tissues were harvested at 14 days following elastase perfusion, digested with an enzymatic cocktail and stained with fluorescent dye-conjugated antibodies as described before by Meher et al. Briefly, elastase perfused aortas were carefully collected from mice under a dissection microscope. Extra connective tissues were removed from the abdominal aorta, and the elastase perfused segment of aorta was harvested excluding the sites of temporary ligatures and aortotomy. Care was taken not to include any small lymph nodes present near to the aorta. The isolated aortas were individually digested with an enzymatic cocktail of collagenase type I (1000 U/ml), collagenase type XI (400 U/ml), Hyaluronidase type I-s (125 U/ml) and DNase (60 U/ml) and stained with flow antibodies. Thus, one aorta constitute one flow sample. Following antibodies were used: Purified anti-mouse CD16/32 (clone 93, Biolegend), PE/Cy7 anti-mouse CD3ε (clone 145-2C11, Biolegend), Brilliant Violet 785™ anti-mouse CD4 Antibody (clone RM4-5, Biolegend), PE rat anti-mouse Foxp3 (clone MF23, BD Pharmingen), APC/Cy7 anti-mouse CD45 (clone 30-F11, Biolegend), Alexa Fluor 488 anti-mouse/human CD45R/B220 (clone RA3-6B2, Biolegend), PerCP/Cy5.5 anti-mouse CD19 (clone 6D5, Biolegend), Alexa Fluor 647 anti-mouse CD5 (clone 53-7.3, Biolegend), PE/Cy7 anti-mouse IgM Antibody (clone RMM-1, Biolegend), Brilliant Violet 650™ anti-mouse IgD Antibody (clone 11-26C.2a, Biolegend), Brilliant Violet 650™ anti-mouse CD317 (B72, PDCA-1) Antibody (clone 927, Biolegend), Alexa Fluor® 647 anti-IDO1 Antibody and clone 2E2/IDO1, Biolegend). Dead cells were discriminated using LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Cat# L34955, Invitrogen). After staining cell suspensions with live/dead stain and cell surface antibodies, the cells were fixed and permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Cat# 554715, BD Biosciences, San Jose, CA) or fixed with 4% paraformaldehyde for 15 min at room temperature, washed with FACS buffer (PBS containing 2% FBS and 0.05% Sodium Azide) and permeabilized using BD Permeabilization Solution. The cells were stained with intracellular antibodies before adding CountBright Absolute Counting Beads (Molecular Probes) and running on the 16 color Flow cytometer machine Becton Dickinson LSFRFortessa (equipped with laser lines 488nm, 405nm, 561nm, and 640nm) located in the Flow Cytometry Core Facility at University of Virginia. Fluorescent minus one (FMO) controls were used for each antibody in the experiment. Since the number of CD45+ lymphocyte is quite variable among the groups, while acquiring events in flow cytometer, we acquired 50,000 live cells. For counting of cells, 5100 CountBright absolute counting beads were added to 400 µl of cell suspension, and identified in Forward and Side-scatter gating.

**Measurement of tryptophan and kynurenine via Mass Spectrometry**
Plasma Tryptophan (Trp) and Kynurenine (Kyn) were quantified as described before. Fifty microliters of water and 20 µl of d5-Tryptophan (d5-Trp, internal standard) were added to 50 µl of mouse plasma. The plasma proteins were removed by precipitation with 25 µl of 5-Sulfosalicylic acid and centrifugation at 17,000 x g for 5 min. The resulting clear supernatant was used for mass spectrometry analysis. Trp, Kyn and d5-Trp were profiled in positive ion mode a Prominence UFLC Liquid Chromatography System (Shimadzu) coupled to a 4000 QTRAP Mass Spectrometer (AB Sciex) fitted with an electrospray ionization source. The ion spray voltage was set to 5.5kJ and the source temperature was set to 100°C. The mass spectrometer was tuned for each individual analyte for optimal detection, and establishment of multiple reaction monitoring (MRM). The first quadrupole selected the protonated ions at mass to charge ratios (m/z): 205.082 (Trp), 209.101 (Kyn), and 210.089 (d5-Trp). Nitrogen was used as the standard collision gas to produce the highest intensity fragments selected by the third quadrupole (m/z): 188.1 (Trp), 191.0 (Kyn), and 150.1 (d5-Trp). Standard curves of Trp, Kyn, and d5-Trp were created over the range of 10nM-100µM using dilutions of each of the purified compounds (Sigma). The column used to separate the metabolites was a 50 x 2.1mm 5µm Discovery C18 (Supelco). The column was loaded and eluted with a binary solvent system comprised of mobile phase A (5mM ammonium formate in water, pH 3.5) and mobile phase B (100% acetonitrile). The mobile phase was pumped at 0.2 mL/min. The loading and elution of the column started with a linear gradient of 5% solvent B to 80% B over 2.5 minutes. 80% solvent B was held for 1.5 minutes, followed by a 10 second linear gradient to 5% solvent B, where it held for another 2 minutes to the end of the program. Analyst software (version 1.6, AB Sciex) was used to process the raw LC-MS data and calculate the peak integration of each highest intensity ion.

Real-time RT PCR
The aortas were harvested as described in 'Flow cytometry'. After harvest, aortas were perfused with PBS-EDTA and frozen in liquid nitrogen and stored in -80 °C. At the time of preparation, the aortas were grinded using a sterile mortar pestle and transferred to TRIzol Reagent (Cat# 15596-026, ThermoFisher Scientific, Grand Island, NY). Subsequently, cDNA was synthesized and gene expression was quantified as described previously by Meher et al.

Coculture of pDC and B cell
pDCs and B cells were isolated from spleens of 8 to 10 weeks old male C57BL/6 mice using MACS column and Plasmacytoid Dendritic Cell Isolation Kit (Cat. No. 130-107-093) and Pan B Cell Isolation Kit (Cat. No. 130-095-813) from Miltenyi Biotech (San Diego, CA). ODN 1826 and Co-ODN 1826 were obtained from Invivogen (San Diego, CA). Soon after isolation, pDCs were cultured for 2 days in with 0.2 µM of ODN 1826 or co-ODN 1826 in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1x Antibiotic-Antimycotic (Cat. No. 15240062), 1x MEM Non-Essential Amino Acids Solution (Cat. No. 11140050), 1x Sodium Pyruvate (Cat. No. 11360070) and 55 µM 2-Mercaptoethanol (Cat. No. 21985023) from ThermoFisher Scientific (Waltham, MA) at 37 °C in a humidified incubator supplied with 5% CO2. After 2 days of incubation, the culture medium was completely removed and splenic B cells were added at various concentrations. The coculture was continued for 1 days, and thereafter, the cells were collected, washed in PBS, stained with fixable live/dead marker and stained for CD317, B220, CD19 and IDO, run on a flow cytometer and analyzed as described in ‘Flow cytometry’ section of the Supplemental material. Separately, ODN 1826 stimulated mouse splenocytes were used to prepare FMO controls such as CD317 FMO, B220 FMO, CD19 FMO and IDO FMO to develop gates for flow data analysis.

Statistical analysis
The programs GraphPad Prism 5 and Excel were used for data analysis and preparing graphs. Two groups are compared using t-test, whereas, multiple groups were compared using one-way ANOVA and multiple t-tests were used to determine the significant difference in aneurysm pathology between two groups. Typically, before determining significant differences between or among the groups, column statistics was applied on the data points of each group in the GraphPad Prism. As recommended by Prism, D'Agostino-Pearson normality test was performed. If the P value is not significant (>0.05), a parametric test (unpaired t-test assuming both populations have the same SD) was applied to determine significant differences between the groups. If the P value is significant (<0.05), non-parametric t-test (Mann-Whitney test) was applied. In the multiple comparisons, if a significant difference was found among the groups, pair of groups compared using a parametric or non-parametric t-test which was again based on the values obtained from the normality test and indicated in figure legends. Statistical analyses are provided in each figure legend. Differences between the groups were considered significant when p-value is <0.05. p-values >0.05 were indicated in the graphs.

References
Supplemental Figure I: Anti-CD20 antibody-mediated depletion of B cells in mice. Representative flow cytometry plots showing depletion of B1 and B2 cells in blood and bone marrow of WT mice administered two doses of anti-CD20 antibody either via IP or IV. WT mice were also injected with control antibody via IP as negative control.
Supplemental Figure II: Anti-CD20 antibody-mediated depletion of B cells in peritoneal cavity. Representative flow cytometry plots showing depletion of B1a and B1b cells via two methods of characterization (A and B) in peritoneal fluid of WT mice administered two doses of anti-CD20 antibody either via IP or IV. WT mice were also injected with control antibody via IP as negative control. A schematic presentation of the gating is also shown.
Supplemental Figure III

A. Study design for B cell depletion and induction of AngII-induced aortic aneurysm

- Day -7: Control Ab Anti-CD20 Ab
- Day 0: Saline or AngII infusion
- Day 7: Control Ab Anti-CD20 Ab
- Day 21: Control Ab Anti-CD20 Ab
- Day 28: Harvest & Examine B Cell Depletion

B. Aneurysm phenotype of aortas

- Control Ab Saline
- Control Ab AngII
- Anti-CD20 Ab AngII

C. AAA stage (%)

- Control AB (Saline), n=5
- Control AB (AngII), n=6
- Anti-CD20 AB (AngII), n=5

D. Plasma aldosterone (pg/ml)

E. VVG staining of suprarenal aorta showing elastin degradation

- Control Ab (Saline)
- Control Ab (AngII)
- Anti-CD20 Ab (AngII)

Continued
Supplemental Figure III: Figure 3: B cell depletion suppresses AngII infusion-induced aortic aneurysm in ApoE KO mice. A, Schematic representation of study design for B cell depletion and induction of experimental AngII infusion model of murine AAA. B, Photograph showing aneurysm pathology. Arrows indicate the site of aneurysm. An enlarged image of ruptured thoracic aneurysm is shown in the right panel. C, Quantification of stages of aneurysm: Control antibody treated and infused with saline, n= 5; Control antibody treated and infused with AngII, n=6 and Anti-CD20 antibody treated and infused with AngII, n=5. To determine the significant difference in aneurysm pathology between two groups, multiple t-test was used, in which, aneurysm pathology score of each mouse was entered into side-by-side subcolumns all in a row for a group and unpaired t test with fewer assumptions was applied. D, Quantification of plasma aldosterone as a measure of AngII infusion (n=5-6). Following one-way ANOVA, parametric unpaired t-test was applied to determine significant differences between the groups. E, Representative images showing AAA sections stained for Verhoeff-Van Gieson or VVG (elastic fibers, black). A small segment of images acquired in 4x is shown in 20x magnification. Scale bar in 4x images is 500 µm and in 20x images is 50 µm. Elastic fiber area (presented as percentage of total aortic area) and adventitial area were determined using ImageJ. Following one-way ANOVA, non-parametric t-test (Mann-Whitney test) was applied to determine significant differences between the groups. Values are expressed as means ± SEM, “**” and “***” indicate p< 0.01 and 0.001, respectively.
Supplemental Figure IV: Schematic presentation of aorta harvested and gating strategy of flow cytometry method. **A**, Schematic presentation showing specific segment of the aorta (region within the dotted line) was harvested for flow cytometry, gene expressing analysis and immunohistochemistry. **B**, Representative flow cytometry plots from mouse splenocytes showing gating strategy used in the current study. Based on scattering properties, mononuclear cells were gated, followed by gating singlets and live cells. The live cells were further gated for CD45+, followed by B1 and B2 cells and IgM+IgD+ populations.
Supplemental Figure V: IgM and IgD populations in aorta and spleen. Quantification of IgM+IgD+ populations in B1 and B2 cells from AAA and spleen of WT mice administered two doses of control or anti-CD20 antibody via IV. Following one-way ANOVA, non-parametric t-test (Mann-Whitney test) was applied to determine significant differences between the groups. Values are expressed as means ± SEM (n=3-5) and ***, **** indicate p<0.05, 0.01 and 0.001, respectively. ‘p’ values >0.05 are indicated.
Supplemental Figure VI: The number of IDO expressing pDC is increased in the blood of B cell depleted mice. 

A, Representative Flow cytometry plots showing gating and increase in concentration of mid B220+/CD317+CD19+IDO+ cells in blood (100 µl) following anti-CD20 antibody treatment via IV. The cells have been gated for singlets, live, CD45+ and CD4- mononuclear cells. B, Increase in number of mid CD317+B220+ and mid CD19+IDO+ cells following anti-CD20 antibody treatment (n=3-4). C, Quantification of Tryptophan and Kynurenine, and Kynurenine-Tryptophan ratio following anti-CD20 antibody treatment (n=4-5). Non-parametric t-test (Mann-Whitney test) was applied to determine significant differences between the groups. ** indicates p<0.05. *p’ values >0.05 are indicated.
**Graphic Abstract**

**Presence of B cell**

- **B**
- **IDO+ pDC**
- **pDC**
- **CD4**

**Absence of B cell**

- **IDO+ pDC**
- **CD4**
- **Treg**

- **IFNγ, IL-1β & IL-6**
  - **↑ AAA**
  - **↓ AAA**