Immune System

Obligatory Role for B Cells in the Development of Angiotensin II–Dependent Hypertension

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Abstract—Clinical hypertension is associated with raised serum IgG antibodies. However, whether antibodies are causative agents in hypertension remains unknown. We investigated whether hypertension in mice is associated with B-cell activation and IgG production and moreover whether B-cell/IgG deficiency affords protection against hypertension and vascular remodeling. Angiotensin II (Ang II) infusion (0.7 mg/kg per day; 28 days) was associated with (1) a 25% increase in the proportion of splenic B cells expressing the activation marker CD86, (2) an 80% increase in splenic plasma cell numbers, (3) a 500% increase in circulating IgG, and (4) marked IgG accumulation in the aortic adventitia. In B-cell–activating factor receptor–deficient (BAFF-R−/−) mice, which lack mature B cells, there was no evidence of Ang II–induced increases in serum IgG. Furthermore, the hypertensive response to Ang II was attenuated in BAFF-R+ (Δ30±4 mm Hg) relative to wild-type (41±5 mm Hg) mice, and this response was rescued by B-cell transfer. BAFF-R−/− mice displayed reduced IgG accumulation in the aorta, which was associated with 80% fewer aortic macrophages and a 70% reduction in transforming growth factor-β expression. BAFF-R−/− mice were also protected from Ang II–induced collagen deposition and aortic stiffening (assessed by pulse wave velocity analysis). Finally, like BAFF-R deficiency, pharmacological depletion of B cells with an anti-CD20 antibody attenuated Ang II–induced hypertension by ≈35%. Hence, these studies demonstrate that B cells/IgGs are crucial for the development of Ang II–induced hypertension and vessel remodeling in mice. Thus, B-cell–targeted therapies—currently used for autoimmune diseases—may hold promise as future treatments for hypertension. (Hypertension. 2015;66:1023-1033. DOI: 10.1161/HYPERTENSIONAHA.115.05779.)

Key Words: hypertension • immune system • immunology • inflammation • lymphocytes

Studies during the past decade have highlighted the important role of the adaptive immune system in the renal and vascular inflammation that contributes to hypertension and disease sequelae, such as kidney failure, atherosclerosis, and cardiac dysfunction. Specifically, these studies describe an imbalance between the levels of proinflammatory effector T lymphocytes (eg, T helper cells and cytotoxic T cells)1,2 and immune-suppressing T regulatory cells,3,4 which ultimately promote inflammation and oxidative stress in key blood pressure (BP) regulating organs and tissues. However, to date, the role of other (non–T cell) components of the adaptive immune system has remained largely unexplored.

B cells play a crucial role in mammalian adaptive immunity via their ability to detect and process antigens, terminally differentiate into plasma cells, and produce antibodies.5 Antibodies bind with high specificity to antigens derived either from invading pathogens or the host’s own cells to elicit immune or autoimmune responses, respectively. When bound to antigens, antibodies elicit (auto)immune responses via interactions of their Fc region with various elements of

Received July 14, 2015; first decision July 28, 2015; revision accepted August 7, 2015.
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This paper was sent to Toshiro Fujita, Consulting editor, for review by expert referees, editorial decision, and final disposition.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.115.05779/-/DC1.

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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.115.05779
the immune system. For example, antibody–antigen complexes can interact with Fc receptors expressed on leucocytes, such as natural killer cells and granulocytes. This stimulates the release of death-inducing molecules or cytotoxic granule contents, which ultimately causes the destruction of antibody-bound target cells through a process known as antibody-dependent cellular cytotoxicity. Antibody–antigen complexes can also interact with Fc receptors expressed on macrophages. Antibody binding to macrophages leads to changes in their polarization state, increased cytokine production, or activation of phagocytotic pathways, which ultimately aids in the clearance of the target antigen. Finally, antibody–antigen complexes can interact with C1q, leading to activation of the complement cascade and in turn opsonization of pathogenic particles, chemoaattraction of leucocytes, and the formation of membrane attack complexes on antibody-tagged target cells.

It has been known for several decades that essential hypertension in humans is associated with elevated serum levels of antibodies. Thus, after the report by Ebringer and Doyle in 1970 about elevated IgG titers in a cohort of severely hypertensive patients, there have been several reports that hypertension in human is strongly associated with increased serum levels of IgG, IgA, or IgM antibodies. Furthermore, studies in animal models, such as the spontaneously hypertensive rat, have also provided evidence for an association between hypertension and raised antibody levels. However, to our knowledge, no studies have examined directly whether these antibodies or the B cells that generate them actually contribute to the disease process.

In this study, we investigated the role of B cells in angiotensin II (Ang II)–induced hypertension in mice. We found that Ang II–induced hypertension was associated with increases in the proportions of activated B cells and plasma cells in lymphoid tissues, as well as changes in splenic architecture indicative of B-cell activation. Ang II infusion also resulted in an increase in both serum and aortic antibody deposits of IgG2b and IgG3. Importantly, mice either genetically deficient of B cells (B-cell–activating factor receptor–deficient [BAFF-R−/−] mice) or pharmacologically depleted of B cells (anti-CD20 antibody treatment) were protected from the chronic pressor response of Ang II in BAFF-R−/− mice. Importantly, mice either genetically deficient of B cells (BAFF-R−/− mice) or pharmacologically depleted of B cells (anti-CD20 antibody treatment) were protected from the chronic pressor response of Ang II in BAFF-R−/− mice. Importantly, mice either genetically deficient of B cells (BAFF-R−/− mice) or pharmacologically depleted of B cells (anti-CD20 antibody treatment) were protected from the chronic pressor response.

Adoptive transfer of B cells into BAFF-R−/− mice recapitulated the Ang II–induced pressor response. BAFF-R−/− mice were also protected from Ang II–induced aortic macrophage and CD4+ T cell accumulation, extracellular matrix (ECM) remodeling, and aortic stiffening. Hence, these findings reveal a hitherto unidentified role for B cells in the pathogenesis of Ang II–induced hypertension. Adoptive Transfer of B Cells Into BAFF-R−/− Mice Restores the Pressor Response to Ang II Continuous BP monitoring via radiotelemetry confirmed the findings from tail-cuff plethysmography that, relative to wild-type mice, BAFF-R−/− mice were severely deficient (<10%) in B cells in their spleens and other organs, such as the aorta and kidneys (Figure S1A, S1C, and S1D in the online-only Data Supplement). Although BAFF-R−/− mice also seemed to have ≈40% fewer T cells in their spleens compared with wild-type mice, T-cell numbers were not affected in the other organs examined (Figure S1B and S1E; Figure S2A and S2B).

To determine whether B-cell deficiency might afford protection against hypertension, we compared pressor responses to Ang II in BAFF-R−/− versus wild-type mice. In wild-type mice, Ang II infusion caused a rapid rise in systolic BP from 117±3 mm Hg at baseline to a maximum level of 165±5 mm Hg by day 21 (Figure 1A). BAFF-R−/− mice exhibited a similar baseline BP to that of wild-type mice of 112±3 mm Hg. However, the pressor response to Ang II was attenuated in BAFF-R−/− mice with systolic BP reaching a maximum of only 149±4 mm Hg at day 21 and remaining at this level until day 28 (Figure 1A). BP remained largely unchanged for the 28-day study period in saline-infused animals, and there were no differences in BP between BAFF-R−/− and wild-type mice at any timepoint (Figure 1A).

Cardiac hypertrophy is a major consequence of sustained elevations in BP. Cardiac hypertrophy whether assessed by heart weight/body weight ratio (Figure 1B) or by heart weight alone (Figure S3) was apparent after Ang II infusion in both wild-type and BAFF-R−/− mice. However, the magnitude of Ang II–induced hypertrophy was less in BAFF-R−/− mice than that in wild-type mice (Figure 1B; Figure S3).

As an alternative approach to investigating the role of B cells in Ang II–induced hypertension, a cohort of wild-type mice were pretreated with a B-cell–depleting, anti-mouse CD20 antibody. The control group for these experiments comprised mice pretreated with an antibody against human CD20, which does not cross-react with the mouse protein. Mice that received the mouse-specific CD20 antibody had far fewer (>97%) B cells in their spleens than mice treated with the human-specific antibody (Figure S1F). Furthermore, although baseline BPs (ie, pre–Ang II) were similar between the 2 groups, the maximum pressor response after 28 days of Ang II infusion was reduced in mice treated with the mouse-specific (155±8 mm Hg) versus the human-specific (172±5 mm Hg) CD20 antibody (Figure 1C).
cells into BAFF-R−/− mice was sufficient to fully recapitulate the pressor response to Ang II to levels observed in wild-type mice (Figure 2A).

**Ang II Infusion Results in B-Cell Activation, Increased Plasma Cell Numbers in Lymphoid Tissues, and Elevated Serum IgG**

Flow cytometric analysis of cell suspensions derived from mouse spleens and para-aortic lymph nodes revealed that after 28 days of Ang II infusion, there was no change in the number of B cells (CD45+CD19+; Figure 3A–3C). Nonetheless, in spleens and lymph nodes from Ang II–infused animals, a higher proportion of the B cells expressed the costimulatory molecule, CD86 (Figure 3D–3F). CD86 is expressed at low levels in resting B cells but is upregulated by stimuli, such as cytokines and bacterial lipopolysaccharide.16,17 Hence, our data suggest that Ang II infusion results in activation of B cells in peripheral lymphoid organs.

After activation, B cells often undergo affinity maturation whereby they differentiate into antibody-producing plasma cells.18 Additional analyses of the cell populations present in the spleen revealed that there were approximately twice as many plasmablasts (CD19+CD138+ cells) and plasma cells (CD19−CD138+) in Ang II–infused mice relative to saline-infused animals (Figure 3G–3I). Furthermore, analysis of spleen architecture revealed follicular hyperplasia in Ang II–versus saline-treated mice (Figure S5A and S5B). There was also a trend for an increase in spleen weight in Ang II–treated mice, although this difference failed to reach statistical significance (Figure S5C).

As the primary function of plasmablasts and plasma cells is to produce antibodies, we next investigated whether Ang II infusion altered circulating immunoglobulin titers. Ang II–infused mice displayed elevated serum levels of IgG (Figure 4A) but not of IgA, IgE, or IgM, compared with saline-infused animals (Figure S6A–S6C). Further analyses of the specific subclasses of IgG revealed IgG2b and IgG3 to be elevated in Ang II–versus saline-infused mice, with levels of IgG1 and IgG2a remaining unaffected (Figure 4B–4E).

Relative to wild-type mice, BAFF-R−/− mice seemed to display modest reductions in certain antibody isotypes under physiological (ie, normotensive) conditions, including IgA, IgE, IgM (Figure S6A–S6C), IgG1, and IgG2a (Figure 4B and 4C). However, these differences were only statistically significant for IgG2a (Figure 4C). Importantly, in BAFF-R−/− mice, Ang II infusion did not cause an increase in circulating levels of any of the antibody isotypes, including those that were elevated by Ang II in wild-type mice (ie, IgG2b and IgG3; Figure 4D and 4E).

**B-Cell Deficiency Prevents Ang II–Induced Accumulation of IgG Deposits and Macrophages in the Vessel Wall**

Macrophages accumulate in the vascular wall during Ang II–dependent hypertension and likely contribute to chronically elevated BP by promoting collagen deposition and vessel remodeling.19,20 Macrophages are often found localized to sites of IgG deposition, and it is thought that interactions between IgG and the Fc γ-receptor on macrophages may
promote their survival and polarization toward alternatively activated (M2) phenotypes. Thus, to determine whether the aorta might represent a site of Ig accumulation during Ang II infusion, immunohistochemistry was performed using antibodies against various Ig subclasses. Staining for both IgG2b and IgG3 was evident in the adventitia of aortas from saline-infused wild-type mice and was markedly greater in Ang II–infused mice (Figure 5A and 5B). Staining of IgG2b and IgG3 was also detected in aortas from BAFF-R−/− mice; however, there was no evidence that Ang II increased staining in the B-cell–deficient animals. Finally, we could find no evidence of IgG2a or IgM deposits in aortas from saline or Ang II–infused mice of either strain (data not shown).

Next, we examined whether the Ang II–induced increases in IgG2b and IgG3 deposition in the vascular wall were accompanied by changes in macrophage numbers or activation state. The total number of macrophages (CD45+CD11b+F4/80+) present in the aortic wall of Ang II–infused wild-type mice was almost 3× greater than that in saline-infused mice (Figure 6A). Macrophage numbers in aortas from saline-infused BAFF-R−/− mice were similar to those in saline-infused wild-types; however, in BAFF-R−/− mice, Ang II infusion did not cause macrophage accumulation (Figure 6B). The polarization state of macrophages can be classified on the basis of relative expression levels of CD206 and major histocompatibility complex II (MHCII)21 with M1 macrophages being defined as CD206−MHCIIhi and M2 macrophages as CD206+MHCIIlo (Figure 6C). On the basis of these criteria, the majority (>75%) of macrophages present in the aorta of both wild-type and BAFF-R−/− mice were in a nonpolarized or transitional state (ie, either CD206−MHCIIlo or CD206+MHCIIlo; Figure 6D). Infusion of Ang II increased the number of transitional macrophages present in the aortas of wild-type mice but had no effect on this macrophage population in BAFF-R−/− mice (Figure 6D). In both mouse strains, the proportion of aortic macrophages that could be defined as either M1 or M2 polarized was low (8%–10%) under basal conditions (Figure 6E and 6F). However, although Ang II infusion in wild-type mice caused a 3-fold increase in M2 macrophage numbers, it had little effect on the number of M1 macrophages present in the aorta (Figure 6E and 6F). Ang II infusion did not affect either the M1 or M2 macrophage populations in BAFF-R−/− mice (Figure 6E and 6F). A previous study showed that IgG immune complexes can induce interleukin (IL)-1β production from M2 macrophages.22 In this study, we not only observed increased IL-1β expression in the vessel wall of wild-type mice after Ang II infusion (Figure S7A) but also showed that stimulation of cultured RAW264.7 cells (a murine immortalized macrophage cell line) with IgG isolated from the serum of wild-type mice promoted IL-1β production (Figure S7B). Thus, our findings are consistent with the idea that IgG accumulates in the vessel wall during hypertension to promote polarization of macrophages toward an IL-1β–producing M2 phenotype.

Previous studies have shown that in addition to increasing the number of macrophages in the vessel wall, infusion of mice with Ang II also induces an accumulation of T cells in the adventitia and periaortic adipose tissue.12,23 In this study, we demonstrated that 28 days of Ang II infusion in wild-type mice...
tended to increase total T-cell (ie, CD45+CD3+) numbers in the aortic wall (Figure S2A and S2B). Further analysis of T-cell subtype(s) revealed that Ang II had minimal effects on the number of CD8+ (cytotoxic) T cells but increased the number of CD4+ (T helper/T regulatory) cells in the aortic wall of wild-type mice (Figure S2C–S2E). Again, there was no evidence of any effect of Ang II on any T-cell subsets in BAFF-R−/− mice (Figure S2D and S2E). To provide some insight into the polarization state of the CD4+ T cells in the aorta, we measured expression of various T helper (TH) 1-, TH2-, and TH17-related genes. There were detectable levels of some TH1 (tumor necrosis factor-α and interferons-γ)–, TH2 (IL-10)–, and TH17 (IL-6)–related genes in aortas of normotensive wild-type mice. However, Ang II had no significant effect on expression of any of these genes (Figure S8). Similar findings were obtained in BAFF-R−/− mice (Figure S8). Thus, on the basis of these findings, it would seem that 28 days of Ang II infusion is not associated with any clear polarization of T helper cells present within the aorta.

B-Cell Deficiency Prevents Ang II–Induced ECM Remodeling and Stiffening of the Aorta

Aortic stiffening is an important consequence of hypertension that not only exacerbates elevations in systolic BP but also contributes to end points relevant to cardiovascular morbidity and mortality, including cardiac hypertrophy, coronary ischemia, and end-organ damage.24 A major underlying cause of aortic stiffening is ECM remodeling, characterized primarily by an increase in collagen content.24 Real-time polymerase chain reaction revealed that mRNA levels of collagens I, III, and V were upregulated in aortas of Ang II– versus saline-infused wild-type mice (Figure 7A–7C). In contrast, in BAFF-R−/− mice, Ang II had no effect on aortic expression levels of the different collagen subtypes (Figure 7A–7C). Reflecting these changes in mRNA expression, histochemical analysis using either Masson trichrome (Figure 7D and 7E) or picrosirius red (Figure S9A and S9B) staining showed that Ang II promoted a marked increase in the amount of collagen in aortas of wild-type but not of BAFF-R−/− mice and that this was mainly localized to the adventitia.

Transforming growth factor-β (TGF-β) is a powerful stimulus of collagen production by fibroblasts and vascular smooth muscle cells, which can be produced by various cell types, such as macrophages.25 In this study, we showed that Ang II infusion caused an approximate 2-fold increase in TGF-β expression in aortas of wild-type mice but had no effect on mRNA levels of the cytokine in BAFF-R−/− mice (Figure 7F). Interestingly, the level of expression of TGF-β in the vessel wall (irrespective of treatment or mouse strain) was positively correlated with that of the M2 macrophage marker, CD206 (Figure 7G) but not with an M1 marker, inducible nitric oxide synthase (Figure S10). In further support of the concept that IgG can promote TGF-β production from macrophages, we demonstrated that 24-hour incubation of cultured RAW264.7

Figure 3. Angiotensin II (Ang II) increases B-cell activation and plasma cell numbers in lymphoid organs of wild-type mice. Total B-cell (CD45+CD19+) numbers (A–C) and the percentage of B cells that express the activation marker CD86 (D–F) in spleens and para-aortic lymph nodes of saline- versus Ang II–infused mice. Also shown are total plasmablast numbers (CD45+CD19+CD138+) and plasma cell (CD45+CD19−CD138+) numbers in the spleen (G–I). A, D, and G, Representative flow cytometric plots. B, C, E, F, H, and I, Group data (mean±SEM) from n≥7 experiments. SSC-A indicates side scatter area. *P<0.05 or **P<0.01 for Student unpaired t test.
macrophages with IgG purified from serum of Ang II–treated mice enhanced their capacity to produce TGF-β, whereas IgG from saline-treated mice had no effect (Figure 7H).

To determine whether the changes in collagen content translated to alterations in the mechanical properties of the vessel wall, noninvasive ultrasound measurements of aortic distensibility (elasticity) and pulse wave velocity (a measure of stiffening) were used. Under basal conditions, there were no obvious differences in either aortic distensibility or pulse wave velocity between wild-type and BAFF-R−/− mice (Figure 8A and 8B). However, after Ang II infusion, clear strain differences in mechanical properties were revealed. Thus, Ang II reduced aortic distensibility and increased pulse wave velocity in wild-type mice, whereas it had no effect on either measure in BAFF-R−/− mice (Figure 8A and 8B).

Discussion

This study provides the first direct evidence that B cells are essential in the pathogenesis of Ang II–dependent hypertension. First, we showed that Ang II–dependent hypertension in mice is associated with B-cell activation in various lymphoid organs, along with an increase in IgG levels in the serum and aortic wall. Furthermore, we found that B-cell–deficient BAFF-R−/− mice were largely protected from the chronicpressor effects of Ang II and several measures of vascular inflammation and dysfunction, including leukocyte accumulation, ECM remodeling, and aortic stiffening. Finally, as proof of concept that B cells could be targeted therapeutically to reduce hypertension, we demonstrated that an anti–B-cell antibody—alogous to that already used in the clinic to treat autoimmune diseases—was effective at preventing BP increases in response to Ang II infusion.

B-cell activation can occur via T-cell–dependent or T-cell–independent processes. The former involves direct interactions between the T-cell receptors and antigens presented by B cells via MHCII. The formation of this immune synapse allows the delivery of cytokines to the B cells and their subsequent activation. Alternatively, B cells can be activated independently of T cells by stimuli, such as danger-associated and pathogen-associated molecular patterns. Moreover, B cells are known to express Ang II type 1 receptors, and we have recently shown that Ang II itself may be able to enhance B-cell activity. Irrespective of the mechanism, an early consequence of B-cell activation is the upregulation of costimulatory molecules, such as CD40, CD80, and CD86, and this serves to enhance B-cell interactions, not only with T cells but also with other leukocytes, such as macrophages. Indeed, in this study, we showed that in lymphoid tissues of Ang II–infused mice, a greater proportion of B cells expressed CD86. We also found evidence of increased germinal center numbers in spleens of Ang II–treated mice, further suggesting that the model is associated with increased B-cell activation.

One of the major outcomes of B-cell activation is that the cells become terminally differentiated into antibody-producing plasma cells. This involves a loss of certain B-cell markers, such as B220, CD19, and CD20, and simultaneous upregulation of costimulatory molecules, such as CD40, CD80, and CD86, and this serves to enhance B-cell interactions, not only with T cells but also with other leukocytes, such as macrophages. Indeed, in this study, we showed that in lymphoid tissues of Ang II–infused mice, a greater proportion of B cells expressed CD86. We also found evidence of increased germinal center numbers in spleens of Ang II–treated mice, further suggesting that the model is associated with increased B-cell activation.

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and IgE) specific antibodies. In further support of the concept that hypertension is associated with B-cell activation, we showed that in Ang II– versus saline-infused mice, there were increased numbers of both plasmablasts and plasma cells in the spleen. Moreover, we detected elevated amounts of IgG—and in particular those IgG subclasses (2b and 3) that display

**Figure 5.** Angiotensin II (Ang II)–induced hypertension is associated with increases in IgG2b and IgG3 deposits in the aorta. Paraffin-embedded aortic sections from saline- and Ang II–infused wild-type and B-cell–activating factor receptor-deficient (BAFF-R−/−) mice were stained with hematoxylin and antibodies against IgG2b (A) and IgG3 (B). Top, Representative sections. Bottom, Group data (mean±SEM) from n≥6 experiments. Scale bar, 200 μm. **P<0.01 or ***P<0.001 for 2-way ANOVA with Tukey corrections. n.s. indicates nonsignificant.

**Figure 6.** B-cell–activating factor receptor–deficient (BAFF-R−/−) mice are protected from angiotensin II (Ang II)–induced macrophage accumulation in the vessel wall. Numbers of total macrophages (A and B) and macrophage subsets (C) defined as transitional (ie, CD206−MHCh or CD206−MHClo; D), M1 (CD206−MHClo; E), or M2 (CD206+MHClo; F) in aortas from wild-type and BAFF-R−/− mice infused with either saline or Ang II. A and C, Representative flow cytometric plots. B and D–F, Group data (mean±SEM) from n≥10 experiments. **P<0.01 or ***P<0.001 for 2-way ANOVA with Tukey corrections.
the highest activity in terms of opsonophagocytosis and cell destruction—in both the serum and aortic wall, providing further evidence for B-cell activation in the model.

To determine whether the activation of B cells was a cause or a merely a consequence of hypertension, we examined hypertension in BAFF-R−/− mice. The BAFF-R is expressed primarily on B cells, and the binding of the ligand BAFF to this receptor is crucial for the survival and maturation of B cells. There is also some evidence that a subset of CD4+ T cells in the spleen express the BAFF-R; however, these cells constituted <3% of the total CD4+ T cell population. Thus, BAFF-R−/− mice have markedly reduced numbers of B cells but an otherwise relatively intact immune system. Confirming this, we showed that under physiological (ie, normotensive) conditions, BAFF-R−/− mice were virtually devoid of B cells in both lymphoid and nonlymphoid (eg, aorta and kidney) tissues. BAFF-R−/− mice displayed lower levels of some IgG antibodies in the circulation. By contrast, T-cell and macrophage numbers were largely unaffected in most of the tissues except for the spleen, where BAFF-R−/− mice had ≈40% fewer T cells than wild-type mice. Importantly BAFF-R−/− mice were protected from the chronic pressor effects of Ang II, whereas adoptive transfer of B cells into these animals recapitulated the hypertensive response. Together with the observation that the adoptive transfers only increased B-cell numbers in the spleen (ie, without affecting T cells), these findings highlight for the first time the obligatory role of B cells in the development of Ang II–dependent hypertension.

Previously, Guzik et al1 showed that the blunted hypertensive response to Ang II in recombinase-activating gene-1 enzyme knockout (RAG1−/−) mice—which are deficient in all
lymphocytes—was restored after adoptive transfer of T cells but not of B cells. Although the latter finding might seem to be at odds with our suggestion of a crucial role for B cells in the development of hypertension, there are at least 2 possible explanations to account for the apparent differences between these 2 studies.\(^29\)

First, it is known that retroviral-mediated reintroduction of the RAG1 gene only causes a modest increase in B-cell numbers in RAG1\(^{−/−}\) mice but restores T-cell numbers back to control levels.\(^37\) This suggests that B-cell (but not T cell) survival may be fundamentally impaired in RAG1\(^{−/−}\) mice, which if true might preclude any possibility of adoptively transferred B cells engrafting and surviving in RAG1\(^{−/−}\) mice to mediate an effect. Furthermore, even if the adoptively transferred B cells can survive in RAG1\(^{−/−}\) mice, it is entirely possible that they would never become activated. As mentioned previously, B-cell activation is normally dependent on highly specific interactions with T cells,\(^18\) which were obviously still lacking in the RAG1\(^{−/−}\) mice administered with B cells.

There are several potential mechanisms by which B cells and the antibodies they produce could participate in the development of hypertension. For example, it has been suggested that the antibodies expressed in hypertensive individuals display agonistic actions on receptors and ion channels involved in the regulation of BP, including Ang II type 1 receptors, \(\alpha\)1-adrenoreceptors, \(\beta\)1-adrenoreceptors, and L-type voltage-operated calcium channels.\(^29,38-41\) In addition, under conditions, such as systemic lupus erythematosus, IgG antibodies form immune complexes with soluble antigens in the circulation.\(^42\) Deposition of these immune complexes within the kidney promotes inflammation and hypertension.\(^42\) There is evidence for immune complex deposition in the kidneys in spontaneously hypertensive rats treated with deoxycorticosterone acetate/salt.\(^46\) The findings from this study suggest that antibodies might additionally play a role in the functional and structural changes that occur in the vascular wall during Ang II–induced hypertension. We present evidence that Ang II–dependent hypertension is associated with the deposition of IgG in the adventitia. IgG complexes are detected by Fc \(\gamma\)-receptors expressed on several leukocyte subsets, including B cells, natural killer cells, and dendritic cells.\(^41\) Macrophages are another cell type that expresses Fc \(\gamma\)-receptors, and it is known that stimulation of these receptors by IgG promotes cell survival and polarization toward an M2-like activation state.\(^5,22\) Although M2 macrophages are generally regarded as anti-inflammatory, the M2 macrophage phenotype arising from IgG stimulation is unusual in that it seems to display certain proinflammatory properties, including production of IL-1\(\beta\).\(^22\) Indeed, in this study, we showed that expression of IL-1\(\beta\) was increased in aortas from Ang II–treated mice. Moreover, stimulation of cultured murine macrophages with IgG isolated from mouse serum markedly increased IL-1\(\beta\) expression. There is limited information on the role of IL-1\(\beta\) in the vascular remodeling/pathophysiology associated with hypertension. However, ex vivo studies on isolated arteries have shown that IL-1\(\beta\) can promote reactive oxygen species production, vasoconstriction, and endothelial dysfunction,\(^49\) all of which are consistent with a prohypertensive role for IL-1\(\beta\). Indeed, we have recently shown that inhibition of inflammasome activity (which is necessary for IL-1\(\beta\) production) blunted hypertensive responses to both Ang II and deoxycorticosterone acetate/salt in mice.\(^46\)

Previous studies have shown that Ang II–dependent hypertension is associated with collagen accumulation in the adventitia and that this is a major contributor to vessel stiffening.\(^1,47\) Stiffening of the large arteries is a feature of hypertension and most likely occurs initially as an adaptive mechanism to bolster vessel wall strength in the face of increased transmural pressures.\(^24,40\) However, over time vascular stiffening likely contributes to the elevated cardiovascular risk associated with hypertension by promoting further increases in systolic pressure, left ventricular hypertrophy, cardiac ischemia, increased pulse pressures, and end-organ damage.\(^48\) A major function of M2 macrophages is to promote ECM remodeling (including collagen deposition) and tissue growth as part of wound healing.\(^49\) An important mediator of the ECM remodeling functions of macrophages is TGF-\(\beta\).\(^23,50\) Indeed, we showed that TGF-\(\beta\) expression was elevated in aortas of wild-type mice after Ang II infusion and that its expression was strongly positively correlated with that of the classical M2 macrophage marker CD206, consistent with the idea that these cells were an important source of TGF-\(\beta\) in the vessel wall. The fact that Ang II–induced increases in TGF-\(\beta\) expression, M2 macrophage numbers, and IgG deposition were all attenuated in BAFF-R\(^{−/−}\) mice is consistent with a role for B cells/IgGs in promoting TGF-\(\beta\) production by macrophages. In further support of this, IgG purified from Ang II– but not saline-treated mice induced the production of TGF-\(\beta\) from cultured murine macrophages. When considered in light of our observation that BAFF-R\(^{−/−}\) mice were also protected from Ang II–induced increases in aortic pulse wave velocity, these point to a crucial role for B cells and IgG in the vascular fibrosis and stiffening that accompanies chronic hypertension.

Our study has not addressed the nature of the antigen target(s) of the IgG antibodies present in hypertensive mice, information that could be valuable for the development of new antihypertensive therapies. Currently, it is thought that 12% to 25% of hypertensive individuals are resistant to conventional drug therapies, even when these therapies are used in combination.\(^51\) These patients could potentially benefit from
strategies involving immune tolerization to such antigen targets. However, even without knowledge of the antigen targets, our findings may already be relevant from a translational perspective. B-cell depletion is routinely used in the clinic for treatment of autoimmune diseases, such as systemic lupus erythematosus.22 We have shown that B-cell depletion with an anti-CD20 monoclonal antibody (analogous to clinically used drugs, such as rituximab) protects against hypertension induced by Ang II in mice, providing proof-of-concept that this could be a useful antihypertensive therapy in the future, particularly in those patients with refractory hypertension where the benefits of BP lowering may outweigh the risks associated with potential suppression of immune function.

Perspectives

We have provided the first evidence for an obligatory role for B cells in the development of Ang II–induced hypertension and vessel stiffening in mice. When considered alongside previous findings of crucial roles for other leukocyte subsets, including T cells and macrophages,4,19 this study further highlights that hypertension is dependent on a coordinated response of both the innate and adaptive arms of the immune system. As such, it reinforces the emerging paradigm that the cause of hypertension is akin to autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, opening up possibilities for novel treatments involving immuno-modulating or anti-inflammatory drugs.

Acknowledgments

We thank Genentech Inc for their generous donation of the anti-CD20 antibodies used in these experiments.

Sources of Funding

This work was supported by grants from the National Health and Medical Research Council of Australia (APP1041326). Drs Drummond, Sobey, Samuel, and Peter were supported by Senior Research Fellowships from the National Health and Medical Research Council of Australia (nos APP1006017, APP1079467, APP101766, and APP1079492, respectively). C.T. Chan was supported by an Australian Postgraduate Award (no. 5131432).

Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- Here, we provide definitive evidence that B cells are required for the development of angiotensin II-dependent hypertension in mice.

**What Is Relevant?**

- These findings add to the growing body of evidence that hypertension is an autoimmune condition involving the coordinated activation of multiple immune cell types that work in concert to promote vascular inflammation and fibrosis.

**Summary**

The recognition that hypertension is an immune disorder is important from a clinical perspective as it opens up new possibilities for treatments (eg, B-cell depletion therapy) akin to those already used for diseases mellitus, such as rheumatoid arthritis and systemic lupus erythematosus.
Obligatory Role for B Cells in the Development of Angiotensin II–Dependent Hypertension

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*Hypertension*. 2015;66:1023-1033; originally published online September 8, 2015; doi: 10.1161/HYPERTENSIONAHA.115.05779

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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

http://hyper.ahajournals.org/content/66/5/1023

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2015/09/08/HYPERTENSIONAHA.115.05779.DC1

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SUPPLEMENTAL MATERIAL

Manuscript Title: An Obligatory Role for B Cells in the Development of Angiotensin II-dependent Hypertension

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Supplemental Detailed Materials and Methods

Animals

This study was approved by the Monash University Animal Research Platform Animal Ethics Committee (Ethics No MARP/2012/156) and was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition (2013). Male C57BL6/J (wild-type) mice (10-12 weeks old) were obtained from Monash Animal Services (Clayton, Australia). B cell activating factor-receptor deficient (BAFF-R⁻/⁻) mice on a C57BL6/J background were bred and housed at Animal Research Laboratories (Clayton, Australia). Hypertension was induced by the implantation of a subcutaneous osmotic minipump (Alzet, USA) which delivered angiotensin II (Ang II; 0.7 mg/kg/day) for 28 days. Normotensive control mice were implanted with a minipump containing 0.9% saline (vehicle). At the end of the treatment period, hearts and spleens were harvested from wild-type and BAFF-R⁻/⁻ mice and weighed.

Blood pressure (BP) was monitored via tail-cuff plethysmography or radiotelemetry. For tail-cuff plethysmography, systolic blood BP was measured immediately before, and 3, 7, 10, 14, 17, 21, 24 and 28 days after minipump implantation using the MC4000 Multichannel system (Hatteras Instruments, USA). For mice allocated to the radiotelemetry group, animals were surgically implanted with the telemeter probe (Model TA11PA-C10, Data Sciences International, USA) as outlined previously and allowed at least 10 days to recover before the induction of hypertension. BP, heart rate, and activity were monitored over 24 hours in freely moving animals for at least 3 days prior to and throughout the treatment period.

Flow Cytometry

At the end of the treatment period, mice were killed and perfused with PBS containing 5% heparin (400 U/mL) to remove blood from vascular organs. Thoracic aortas (with perivascular fat intact), kidneys, spleen and para-aortic lymph nodes were harvested and enzymatically digested in PBS containing collagenase type IX (125 U/mL), collagenase type IS (450 U/mL) and hyaluronidase IS (60 U/mL) for 30 min at 37°C. Cell suspensions were passed through a 70 μm sterile cell strainer and washed in 0.5% bovine serum albumin dissolved in PBS (“FACS buffer”). Cells were resuspended in FACS buffer and then incubated with a cocktail of antibodies including APC anti-F4/80 (BM8) or APC anti-CD3ε (UCHT1), APC-Cy7 anti-CD45 (30-F11), PE anti-CD19 (1D3) or PE anti-CD206 (15-2), FITC anti-CD4 (OKT4) or FITC anti-MHC II (M5/114), BV605 anti-CD8 (53-6.7), PE-Cy5 anti-CD86 (GL-1) and Pacific Blue anti-CD11b (M1/70). Following cell staining, cell populations were analysed via flow cytometry using an LSRII Flow Cytometer and DIVA software (Becton Dickinson, USA). Data were analysed with FlowJo software (version 9.8.2, Tree Star Inc., USA). The numbers of total leukocyte and leukocyte subsets in the vessel wall were normalised to CountBright counting beads (Invitrogen, USA) and expressed as cells per organ.
Measurement of Serum Antibodies

Serum samples were collected from saline- or Ang II-treated wild-type and BAFF-R−/− mice. Antibody isotypes (IgG1, IgG2a, IgG2b, IgG3, IgA, IgE and IgM) were quantitatively measured using ProcartaPlex Mouse Antibody Isotyping Panel (eBioscience, USA) according to the manufacturer’s instructions. The antibody assay was run on a Bio-Plex® MAGPIX® Multi-Plex Reader (BioRad, USA). Data were analysed using Procartaplex Analyst (eBioscience, USA).

Pharmacological Depletion of B Cells

In some experiments, wild-type mice assigned to receive angiotensin II-infusion were randomly allocated to undergo further treatment with either an anti-mouse CD20 antibody previously shown to ablate B cells for up to three weeks in mice, or an anti-human CD20 antibody (to serve as a control). Mice received bolus doses of 5 mg/kg antibody (i.v.) one day prior to and then again 14 days after the induction of hypertension. The anti-CD20 antibodies were received as a generous gift from Genentech (USA).

Adoptive Transfer of Purified B Cells

BAFF-R−/− mice that had undergone surgical implantation of radiotelemetry probes were randomly assigned to receive two injections of either purified B cells or vehicle (PBS), the first 21 days prior and the second 10 days prior to the induction of hypertension with angiotensin II. B cells for adoptive transfer were obtained from spleens of 10-12 week-old male C56BL6/J mice. Spleens were passed through a 70 μm cell strainer and red blood cells were subjected to osmotic lysis, yielding a single cell suspension. B cells were then isolated from this suspension using a B cell-specific magnetic sorting, negative isolation kit (Miltenyi Biotech, USA). The purity of the resulting B cell preparation was confirmed to be >95% using flow cytometry. 20 million B cells were suspended in 200 μL of sterile PBS and immediately injected into the tail vein of BAFF-R−/− mice. At the conclusion of the experiment, the spleens and lymph nodes of recipient mice were analyzed by flow cytometry to confirm successful B cell engraftment.

IgG purification

IgG was purified from the serum of saline- and Ang II- treated wild-type mice using the Ab SpinTrap (GE Healthcare Life Sciences) and quantified on a Nanodrop 1000D spectrophotometer.

Stimulation of RAW 264.7 cells with IgG

A murine immortalized macrophage cell line (RAW 246.7) was cultured in 6-well plates in Dulbecco’s Modified Eagles Medium supplemented with 10% fetal bovine serum. Cells were stimulated for 24 h with either purified IgG (100 μg/mL) or vehicle. At the end of the treatment period, the conditioned media was collected for ELISA, while the cells themselves were harvested for RNA extraction.

Measurement of TGF-β

TGF-β was measured in the conditioned media of IgG-treated RAW 264.7 cells by ELISA according to the manufacturer’s instructions (eBioscience, USA).
Real-time PCR

Thoracic aortas were harvested from saline- and Ang II- treated wild-type and BAFF-R<sup>−/−</sup> mice, snap frozen in liquid nitrogen and pulverized. RNA was extracted from aortas or RAW 264.7 cells using the RNeasy Micro Kit-RNA (Qiagen, USA) and the amount of RNA in each sample was quantified using the Nanodrop 1000D spectrophotometer (Thermo Scientific, USA). RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA), which was in turn used as a template in real-time PCR with Taqman<sup>®</sup> primers and probes for collagens I, III and V, TGF-β, CD206, iNOS or IL-1β (Applied Biosystems, USA). GAPDH was used as the housekeeping gene. Real-time PCR was performed on the CFX96 Touch™ Real-Time PCR Detection Machine (BioRad, USA). Gene expression was quantified (relative to a reference control value) using the comparative Ct Method. 6

(Immunohistochemistry

Freshly isolated spleens and thoracic aortas from saline- and Ang II- treated wild-type and BAFF-R<sup>−/−</sup> mice were formalin-fixed, paraffin-embedded and sectioned (5 μm). Aortic adventitial collagen content was quantified via Masson’s trichrome or picrosirius red staining. Antibodies specific for IgG2a and IgM (BD Biosciences, USA), IgG2b and IgG3 (Life Technologies, USA) were used to stain for antibody deposits in the vessel wall. Spleen architecture was evaluated after haematoxylin and eosin staining. Percentage area of IgG and collagen staining, and numbers of germinal centers were assessed in a blinded fashion using ImageJ software (NIH Image, USA).

Measurement of vessel stiffness

A high-resolution ultrasound imaging system (Vevo 2100, FUJIFILM Visualsonics Inc., Canada) was used to assess aortic stiffness in saline- and Ang II- treated wild-type and BAFF-R<sup>−/−</sup> mice. Briefly, mice were anaesthetised with 1.5% isoflurane, placed on a heated platform and their abdomen was shaved and coated with ultrasound transmission gel (Aquasonic, USA). Pulsed-wave Doppler images and EKV™ retrospective acquired B-Mode images were obtained from Longitudinal sections of abdominal aortas of mice using a MS-400 ultrasound transducer. To measure both aortic distensibility and aortic pulse wave velocity, data were exported and analysed using the VevoLab and VevoVasc software (FUJIFILM Visualsonics Inc., Canada) and the lnD-V loop method. 7

Statistical Analysis

Data are presented as mean ± S.E.M. Data were analysed either by Student’s unpaired t-test, one-way or two-way analysis of variance (ANOVA) with Tukey’s post-hoc tests, or by linear regression analysis as appropriate. P<0.05 was considered statistically significant. All analyses were performed using GraphPad Prism v6.0 (GraphPad Software Inc., USA).
Supplemental References


**Supplemental Figures and Figure Legends**

**Supplemental Figure S1.** Effect of BAFF-R-deficiency and an anti-mouse CD20 antibody on B and T cell numbers in various organs from mice. Total B cell (CD45^+CD19^+) numbers in the spleen (A), aorta (C) and kidney (D), and total T cell (CD45^+CD3^+) numbers in the spleen (B) and kidney (E) of wild type and BAFF-R^-/- mice treated with saline or Ang II. Total B cell (CD45^+CD19^+) numbers in the spleens of wild-type mice treated with a mouse- or human- specific CD20 antibody (F). Values are presented as mean ± S.E.M. from n≥9 experiments. **P<0.01 or ***P<0.001 for 2-way ANOVA with Tukey’s corrections (A-E) or 1-way ANOVA with Tukey’s corrections (F).
Supplemental Figure S2. BAFF-R−/− mice are protected from Ang II-induced T cell accumulation in the vessel wall. Numbers of total T cells (A and B) and T cell subsets (C to E) in aortas from wild type and BAFF-R−/− mice treated with saline or Ang II. Panels A and C are representative flow cytometric plots, while panels B, D, and E show group data (mean ± S.E.M.) from n≥11 experiments. *P<0.05 for 2-way ANOVA with Tukey’s corrections.
Supplemental Figure S3. Effect of Ang II on heart and body weights in mice. Heart weights (A) and body weights (B) measured at the end of 28 days of saline- or Ang II-treatment in wild-type and BAFF-R^{-/-} mice. Values are presented as mean ± S.E.M. from n≥11 experiments. *P<0.05 or ***P<0.001 for 2-way ANOVA with Tukey’s corrections.
Supplemental Figure S4. Adoptive transfer of B cells into BAFF-R−/− mice does not affect T cell numbers. Total T cell (CD45+CD3+) numbers in the spleens of Ang II-treated wild-type mice, and Ang II-treated BAFF-R−/− mice injected with either PBS (vehicle) or 40 million B cells. Values are presented as mean ± S.E.M. from n≥4 experiments.
Supplemental Figure S5. Ang II-treatment is associated with changes in splenic architecture indicative of B cell activation. Spleen sections from Ang II- and saline-infused mice were stained with haematoxylin and eosin (A) and numbers of germinal centers (B) were quantified. Spleen weight to body weight ratios (C) from Ang II- and saline- treated wild-type mice were also assessed. In panel A, the scale bar indicates 300 µm. Values are presented as mean ± S.E.M. from n≥6 experiments. *P≤0.05 for Student’s unpaired t-test.
Supplemental Figure S6. Ang II does not change serum concentrations of IgA, IgE or IgM in wild-type or BAFF-R\(^{-/-}\) mice. Concentrations of total IgA (A), IgE (B) and IgM (C) in serum from wild-type and BAFF-R\(^{-/-}\) mice treated with Ang II or saline. Values are presented as mean ± S.E.M. from \(n \geq 8\) experiments.
Supplemental Figure S7. IL-1β expression in aortas of hypertensive mice and in cultured macrophages stimulated with purified mouse IgG. Messenger RNA expression of IL-1β as measured by real-time PCR from (A) aortas of wild-type and BAFF-R−/− mice treated with saline or Ang II, and (B) RAW 264.7 macrophages stimulated for 24 h with purified IgG (100µg/mL) from saline- and Ang II-treated wild-type mice. Values are presented as mean ± S.E.M. from n≥6 experiments. *P<0.05 for 2-way ANOVA (A) or 1-way ANOVA (B) with Tukey’s corrections.
Supplemental Figure S8. Effect of Ang II-infusion in mice on genes associated with \( T_H \) cell polarization in the aorta. Messenger RNA expression as measured by real-time PCR of \( T_H^1 \)-associated genes TNF-\( \alpha \) and IFN-\( \gamma \), \( T_H^{17} \)-associated genes IL-6 and IL-17, or \( T_H^2 \)-associated genes IL-10, IL-4, IL-5 and IL-13 in aortas of wild type and BAFF-R\(^{-/-}\) mice treated with saline or Ang II. Values are presented as mean ± S.E.M. from \( n \geq 6 \) experiments. For genes IL-4, IL-5 and IL-13, \( N.D. \) = not detected.
Supplemental Figure S9. BAFF-R⁻/⁻ mice are protected from Ang II-induced increases in aortic collagen. Protein expression of total collagen as measured by Picrosirius staining in aortas from wild type and BAFF-R⁻/⁻ mice treated with saline or Ang II. Panel A shows representative images while panel B shows the group data (mean ± S.E.M.) from n≥6 experiments. In panel A, the scale bar indicates 200 μm. *P<0.05 for 2-way ANOVA with Tukey’s corrections.
Supplemental Figure S10. No evidence of a positive association between expression of TGF-β and the M1 macrophage marker, iNOS. Messenger RNA expression of TGF-β and iNOS in aortas of wild-type and BAFF-R<sup>-/-</sup> mice treated with saline or Ang II was quantified using real-time PCR and analysed by linear regression.