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The CARMA3-Bcl10-MALT1 Signalosome Promotes Angiotensin II-dependent Vascular Inflammation and Atherogenesis*[S]

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The CARMA1, Bcl10, and MALT1 proteins together constitute a signaling complex (CBM signalosome) that mediates antigen-dependent activation of NF-κB in lymphocytes, thereby representing a cornerstone of the adaptive immune response. Although CARMA1 is restricted to cells of the immune system, the analogous CARMA3 protein has a much wider expression pattern. Emerging evidence suggests that CARMA3 can substitute for CARMA1 in non-immune cells to assemble a CARMA3-Bcl10-MALT1 signalosome and mediate G protein-coupled receptor activation of NF-κB. Here we show that one G protein-coupled receptor, the type 1 receptor for angiotensin II, utilizes this mechanism for activation of NF-κB in endothelial and vascular smooth muscle cells, thereby inducing pro-inflammatory signals within the vasculature, a key factor in atherogenesis. Further, we demonstrate that Bcl10-deficient mice are protected from developing angiotensin-dependent atherosclerosis and aortic aneurysms. By uncovering a novel vascular role for the CBM signalosome, these findings illustrate that CBM-dependent signaling has functions outside the realm of adaptive immunity and impacts pathobiology more broadly than previously known.

The Bcl10 and MALT1 proteins were first recognized as targets of specific chromosomal translocations underlying the pathogenesis of mucosa-associated lymphoid tissue (MALT)[2] lymphoma (1). Ultimately, these proteins were found to have a critical function in normal lymphocyte biology where they interact with a third partner, CARMA1, as part of a signaling complex (CBM signalosome) that mediates antigen receptor-dependent NF-κB activation (2–4). Specifically, antigen receptor activation of PKC triggers assembly of the signalosome, wherein CARMA1 and Bcl10 serve as scaffolding/adaptor proteins and MALT1 is a protease that promotes activation of the IκB kinase (IKK) complex. Activated IKK in turn directs the phosphorylation and subsequent degradation of IκB, a protein that sequesters NF-κB in an inactive state (2–4). The consequences of NF-κB activation in lymphocytes include induction of pro-survival signals and elaboration of numerous cytokines, both of which represent cornerstones of the adaptive immune response (2–4). In contrast to this tightly regulated activation, translocations seen in MALT lymphoma result in excessive CBM signaling and concomitant dysregulated NF-κB activity, contributing to an inappropriate survival advantage for affected B cells (1).

For some time, this signaling complex has been considered an exclusive signalosome of the immune system because CARMA1, a protein whose expression is restricted to hematopoietic cells, serves as the scaffold for the complex (2, 5). CARMA3, however, is a close homologue of CARMA1 and has a broader expression profile (6). Recently, we and others demonstrated that an analogous CARMA3-containing CBM signalosome can operate in cells outside the hematopoietic system and mediate NF-κB activation in response to stimulation of a subset of G protein-coupled receptors (6, 7). However, although genetic mutations affecting the CARMA1-containing signalosome of the immune system are well characterized and underlie the pathogenesis of certain immunodeficiencies and lymphoproliferative disorders (3, 4, 8), the potential pathophysiologic impact of similar perturbations of the CARMA3-containing signalosome in non-immune cells is not yet clear.

In this work, we report that one such G protein-coupled receptor, the type 1 receptor for angiotensin II (AT_1R), utilizes the CARMA3-containing CBM signalosome in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) to activate the canonical NF-κB machinery, thereby contributing to pro-inflammatory signaling within the vessel wall. Angiotensin II (Ang II), the ligand for this receptor, plays a key role in the pathogenesis of atherosclerosis, and although this is partly due to its well known capacity to induce hypertension, there is emerging evidence that perhaps the greatest impact of Ang II on atherogenesis lies in its ability to directly induce such pro-inflammatory signals within vascular cells (9). Based on these observations, we hypothesized that disrupting the CBM signalosome would impact atherogenesis, and now we report a new

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[2] The abbreviations used are: MALT, mucosa-associated lymphoid tissue; AT_1R, Ang II type 1 receptor; CARMA, CARD-containing MAGUK protein; CBM, CARMA-Bcl10-MALT1; EC, endothelial cell; IKK, IκB kinase; VSMC, vascular smooth muscle cell; CARD, caspase recruitment domain; MAGUK, membrane-associated guanylate kinase.

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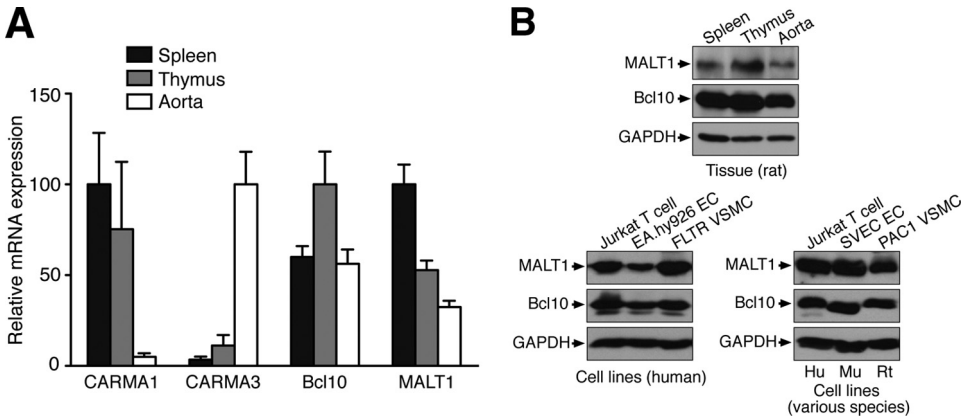

FIGURE 1. **Expression of CBM signalosome components in the vasculature.** *A*, levels of mRNA transcript corresponding to the indicated genes were measured by quantitative RT-PCR. For each transcript, a level of 100% was assigned for the tissue with highest expression. Data are expressed as average ± S.E. for at least three determinations. *B*, extracts were prepared from whole spleen, thymus, and aorta, and immunoblots were performed to compare levels of Bcl10 and MALT1 within the three tissues. Similarly, extracts were prepared from cell lines derived from T lymphocytes (Jurkat), endothelial cells (EA.hy926 or SVEC4–10), and vascular smooth muscle cells (FLTR or PAC1-AR) to compare Bcl10 and MALT1 levels in vascular cells with those seen in lymphocytes. These studies could not be performed for CARMA3 due to the lack of a sensitive and specific antibody. *Hu*, human; *Mu*, mouse; *Rt*, rat.

phenotype for Bcl10-deficient mice that is revealed under conditions of chronic Ang II administration. Specifically, we show that *Bcl10*$^{-/-}$ mice are dramatically protected from developing both Ang II-dependent atherosclerotic lesions and Ang II-dependent abdominal aortic aneurysms. These findings reveal a novel role for Bcl10 in atherogenesis and indicate that the CBM signalosome may represent a new target for pharmaceutical intervention in this disease process.

RESULTS

To determine whether a CARMA3-containing CBM signalosome might play a role in mediating the inflammatory effects of Ang II in the vasculature, we first tested whether the CARMA3, Bcl10, and MALT1 proteins are expressed within vascular cells. Using quantitative RT-PCR, we found that CARMA3 is expressed in aorta at a much higher level than in the lymphocyte-rich spleen and thymus, whereas the opposite is true for CARMA1 (Fig. 1*A*). In contrast, levels of Bcl10 and MALT1 mRNA are more even across the three tissues (Fig. 1*A*), a finding that is reflected at the protein level, as demonstrated by Western blotting (Fig. 1*B*). In addition, Western analyses showed that the Bcl10 and MALT1 proteins are expressed at comparable levels in cultured cell lines derived from ECs, VSMCs, and T lymphocytes, regardless of their origin from human, mouse, or rat (Fig. 1*B*).

We next established models for studying NF-κB activation in these two vascular cell types and found that immortalized PAC1-AR cells, derived from adult rat arterial vascular smooth muscle, show reproducible NF-κB activation following treatment with Ang II, which can be demonstrated by measuring phosphorylation of endogenous IκB (Fig. 2*A*). siRNA-mediated knockdown of CARMA3, however, almost completely ablated the response to Ang II while having no effect on either Ang II-dependent ERK activation or TNFα-dependent p-IκB generation (Fig. 2*A*).

CARMA proteins appear to act as scaffolds in recruiting not only Bcl10 and MALT1 but also IKKγ, the regulatory subunit of the IKK complex (10). In this way, IKKγ is brought into proximity with MALT1 and becomes available as a substrate for MALT1-directed, K63-linked polyubiquitination, which is thought to represent a principal mechanism by which the CBM signalosome stimulates IKK activity (4, 11, 12). Thus, we reasoned that if the CBM complex mediates the response to Ang II in VSMCs, we should be able to detect Ang II-dependent polyubiquitination of IKKγ. Indeed, treatment of PAC1-AR cells with Ang II, but not TNFα, which utilizes a distinct pathway for IKK activation, did result in robust IKKγ polyubiquitination (Fig. 2*B*).

We then used an RNA interference-based approach to test the involvement of the Bcl10 and MALT1 proteins in a more direct fashion. Similar to what we had observed with CARMA3 knockdown, siRNA-mediated knockdown of either Bcl10 or MALT1 resulted in complete ablation of Ang II-dependent IκB phosphorylation (Fig. 2*C*, *left*). The effect was again specific in that IκB phosphorylation remained intact when cells were stimulated with IL-1β (Fig. 2*C*, *right*). In addition, we found that the Bcl10 and MALT1 proteins are selectively used for stimulation of the NF-κB pathway because Ang II-dependent phosphorylation of ERK was unaffected in their absence (supplemental Fig. 1, *A* and *B*).

Further support for the role of the CBM signalosome in mediating Ang II-dependent NF-κB activation was obtained with the use of a reporter construct designed to express luciferase under the control of a promoter with multiple tandem copies of the canonical (p50/p65-responsive) NF-κB-response element. We found that co-expression of a CARMA3 dominant-negative mutant (CARMA3-ΔCARD), which lacks the caspase recruitment domain (CARD) and therefore cannot recruit Bcl10 (13), resulted in specific impairment of Ang II-dependent luciferase expression in PAC1-AR cells while not affecting luciferase expression induced by TNFα (Fig. 2*D*). Similarly, siRNA-mediated Bcl10 knockdown inhibited Ang II-dependent luciferase expression, without impairing the response to either TNFα or IL-1β (Fig. 2*E*).

Having definitively shown the critical importance of the CBM signalosome in mediating Ang II-responsive canonical NF-κB activation in cultured VSMCs, we then sought to determine whether the same mechanism operates in ECs, specifically in context of primary cell cultures. To this end, we prepared separate cultures of primary VSMCs and ECs from fresh rat aorta and analyzed each using the NF-κB-luciferase reporter assay system. Interestingly, we found that ECs prepared in this fashion were even more responsive to Ang II than were VSMCs but similarly activated NF-κB through AT_1R (supplemental Fig. 2, *A* and *B*). Further, the ECs responded to Ang II with induction of endogenous NF-κB-responsive genes relevant to atherogenesis, such as *IL-6* and the scavenger receptor for oxidized LDL, *LOX-1* (supplemental Fig. 2*C*). We then

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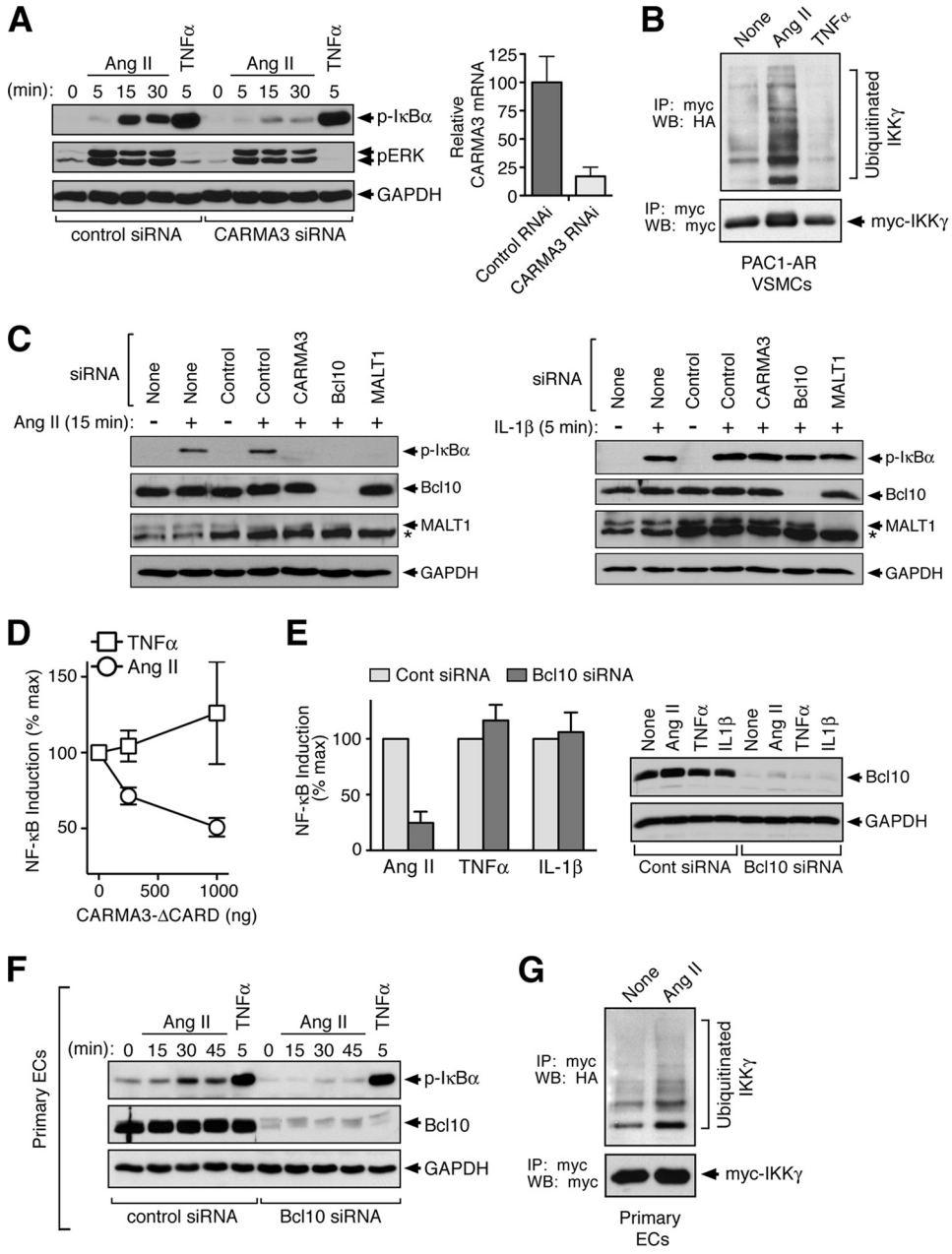

FIGURE 2. **The CARMA3-Bcl10-MALT1 signalosome mediates Ang II-dependent NF-κB activation in vascular cells.** *A*, PAC1-AR smooth muscle cells were transiently transfected with either control siRNA or siRNA targeting CARMA3. After 48 h, cells were treated with Ang II for varying periods of time (or with TNFα for 5 min) before harvesting and analyzing by Western blot. Knockdown of CARMA3 mRNA was assessed by quantitative RT-PCR (*right*). Within 15 min, Ang II treatment induces the phosphorylation of IκB, a marker of canonical NF-κB signaling. *p-IκBα*, phosphorylated IκBα; *pERK*, phosphorylated ERK. Data are mean ± S.E. *B*, PAC1-AR cells were transfected with Myc-tagged IKKγ and HA-tagged ubiquitin prior to treatment with either Ang II or TNFα. Cellular extracts were prepared, and IKKγ was immunoprecipitated (*IP*) with anti-Myc. Precipitates were then analyzed by immunoblotting with anti-HA to reveal evidence of IKKγ polyubiquitination. *WB*, Western blot. *C*, PAC1-AR cells were transfected with siRNAs targeting CARMA3, Bcl10, or MALT1 or with a control siRNA. After 48 h, cells were treated with Ang II for 15 min (*left*) or with IL-1β for 5 min (*right*) before analyzing by Western blot. Effective knockdown of Bcl10 and MALT1 was monitored by Western blotting, whereas knockdown of CARMA3 was confirmed by quantitative RT-PCR as in *panel A* (*not shown*). *, non-specific band. *D*, the dominant-negative mutant, CARMA3-ΔCARD, was expressed in PAC1-AR cells and the effect on Ang II- or TNFα-dependent NF-κB activation was assessed by NF-κB luciferase reporter assay. Results are expressed as percent of maximal induction (luciferase/control *Renilla*) achieved in the absence of the CARMA3 dominant-negative mutant. Data (mean ± S.E.) are from at least three separate determinations. *E*, PAC1-AR cells were transiently transfected with siRNA targeting Bcl10 or control siRNA. After 24 h, cells were transfected a second time with the NF-κB-luciferase and control *Renilla* plasmids, and the response to Ang II, TNFα, and IL-1β was assessed the next day. Knockdown was confirmed by immunoblot as shown. NF-κB induction was determined as above using the dual luciferase assay system. Data (mean ± S.E.) are from at least three separate determinations. *F*, primary rat aortic ECs were transiently transfected with Bcl10 siRNA as in previous panels, and Ang II/TNFα-dependent p-IκB generation was assayed as above. *G*, Ang II-dependent IKKγ polyubiquitination in primary ECs was assayed as described in the legend for *panel B*.

confirmed the role of the CBM signalosome in ECs by showing that siRNA-mediated Bcl10 knockdown was effective at specifically blocking Ang II-dependent IκB phosphorylation (Fig. 2*F*), just as was seen in VSMCs. Also similar to what we had observed in VSMCs, Ang II treatment induced IKKγ polyubiquitination in primary ECs, consistent with the known mechanism for CBM-dependent activation of the IKK complex (Fig. 2*G*). Taken together, our results in immortalized VSMCs and primary ECs demonstrate that the CBM signalosome mediates Ang II-dependent canonical NF-κB signaling in vascular cells.

We next used a genetic approach to investigate the role of the CBM signalosome in mediating pathophysiologic consequences of Ang II-dependent pro-inflammatory signaling. Specifically, we evaluated the effect of Bcl0 deficiency on Ang II-induced atherogenesis in $ApoE^{-/-}$ mice. These mice suffer from premature atherosclerosis due to hyperlipidemia. However, chronic infusion of Ang II, which mimics the relatively common clinical problem of renin-angiotensin system dysfunction, substantially accelerates the process (14). We crossed $ApoE^{-/-}$ mice with $Bcl10^{-/-}$ mice and then compared the degree of atherosclerosis in $ApoE^{-/-}$ *versus* $ApoE^{-/-}Bcl10^{-/-}$ mice infused for 4 weeks with Ang II. Although there was no difference in the pressor response between the two strains (supplemental Fig. 3*A*), we observed a dramatic decrease in the extent of atherosclerosis in $ApoE^{-/-}Bcl10^{-/-}$ mice as compared with $ApoE^{-/-}$ mice (Fig. 3, *A–C*). In addition, whereas 8 of 22 $ApoE^{-/-}$ mice (36%) developed abdominal aortic aneurysms, only a single $ApoE^{-/-}Bcl10^{-/-}$ mouse (8%) developed an abdominal aortic aneurysm, and this aneurysm was significantly less severe than the aneurysms observed in the presence of Bcl10 (supplemental Fig. 3*B*).

Consistent with the decrease in the rate of atherosclerosis in $ApoE^{-/-}Bcl10^{-/-}$ mice, we found

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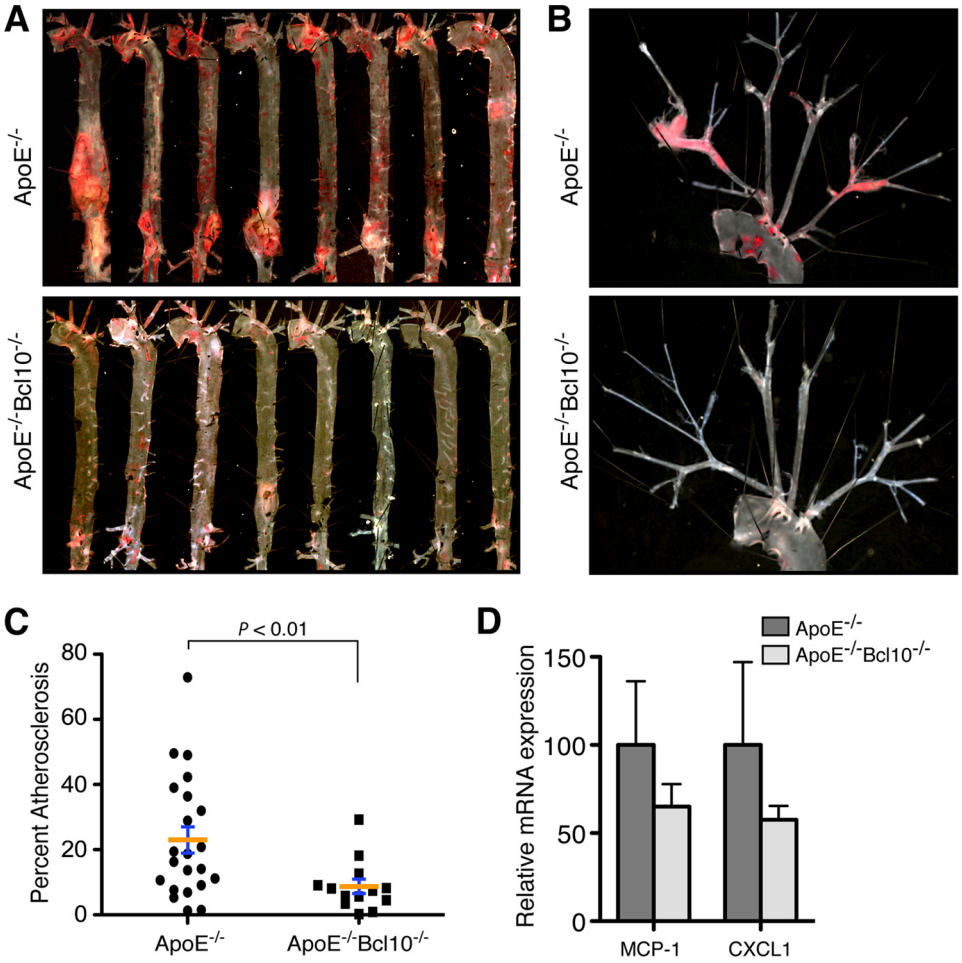

FIGURE 3. **Bcl10 deficiency protects *ApoE*$^{-/-}$ mice from Ang II-dependent atherogenesis.** *A*, aortas from the *ApoE*$^{-/-}$ ($n = 22$) and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ ($n = 13$) groups following 4 weeks of chronic Ang II infusion (500 ng/kg/min) and Oil Red O staining. The most significantly affected aortic segments from both groups are shown. *B*, the aortic arch and major arterial branches extending from the arch for a representative mouse in each group are also shown. *C*, quantification of atherosclerotic lesional area for all mice is shown, presented as the percentage of the surface area of the entire aorta (ascending, arch, and descending to point of the femoral artery bifurcation) stained positively with Oil Red O. The average percentage of area covered by lesion is indicated by an *orange bar*, and the S.E. is indicated by *blue error bars*. *D*, male *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice ($n = 14$ and 13, respectively) were infused with 500 ng/kg/min Ang II for 3 days, after which MCP-1 and CXCL1 mRNA levels were quantified from excised aortas. The level of each of these mRNAs in aortas from *ApoE*$^{-/-}$ mice, normalized to GAPDH and TATA-binding protein mRNA, was set arbitrarily at 100.

that serum levels of several pro-inflammatory mediators, all implicated in atherogenesis (15), were also lower in *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice following the period of Ang II infusion (supplemental Fig. 4). These included CD40 ligand, SCF/c-Kit ligand, IL-1α, CXCL6, and Eotaxin/CCL11. We next used quantitative RT-PCR to directly evaluate whether Ang II induction of critical NF-κB target genes in the aortic wall is impacted by Bcl10 deficiency. Interestingly, although the absence of Bcl10 did not result in a reduction in the Ang II-dependent induction of *IL-6 in vivo* (supplemental Fig. 5), we did observe a trend toward reduction of both *MCP-1* and *CXCL-1* expression in the aortas (Fig. 3*D*).

DISCUSSION

Ang II is now recognized for its powerful pro-inflammatory effects on muscular arteries and its key pressor-independent contribution to atherogenesis (9). Many of these pro-inflammatory effects are mediated by activation of NF-κB within both ECs and VSMCs. Despite this understanding, there remains uncertainty regarding the precise molecular mechanisms by which Ang II can activate NF-κB within the vessel wall. Many researchers have shown canonical NF-κB activation in response to Ang II, but using distinct models, others have suggested that alternative routes are critical, for example via AT_1R-dependent serine kinase activation and subsequent Ser^{536} phosphorylation of the p65 subunit of NF-κB (17). Thus, it is likely that there are significant cell type- and context-dependent factors that influence the degree of canonical activation (17).

Here we find that both VSMCs and ECs can respond to Ang II with significant canonical NF-κB activation and identify for the first time a defined molecular pathway in vascular cells linking AT_1R ligation to the canonical NF-κB machinery. This pathway requires assembly of a signaling module composed of three proteins, CARMA3, Bcl10, and MALT1. Importantly, disruption of the complex *in vivo*, through genetic deletion of *Bcl10*, dramatically protects *ApoE*$^{-/-}$ mice from developing both Ang II-dependent atherosclerotic lesions and Ang II-dependent aortic aneurysms. This protection correlated with decreased *in vivo* levels of several pro-inflammatory cytokines and chemokines that have been strongly implicated in the pathogenesis of atherosclerosis. Of these, the factor that showed the most significant reduction ($p < 0.05$) was CD40 ligand (CD40L), which is well known as an inflammatory serum biomarker for increased cardiovascular disease risk (18). CD40L promotes atherothrombosis by stimulating platelet aggregation and by inducing expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) on ECs as well as the secretion of pro-inflammatory chemokines and matrix metalloproteinases from both ECs and VSMCs (19). Importantly, *in vivo* studies have demonstrated that blockade of CD40L in mice, through administration of neutralizing antibodies or through genetic deletion of the *CD40L* gene, prevents atherosclerosis (20).

The decreased rate of atherosclerosis observed in Bcl10-deficient mice also correlated with a trend toward decreased expression of key NF-κB-responsive genes in the vessel wall proper. Specifically, mRNAs for GROα/CXCL1 and MCP-1/CCL2, two Ang II-induced chemokines (21), were reduced in

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aortic tissue of $ApoE^{-/-}Bcl10^{-/-}$ mice, as compared with $ApoE^{-/-}$ mice, following infusion of Ang II. CXCL1, expressed on the luminal surface of ECs, promotes monocyte arrest via interaction with the CXCR2 receptor (22). MCP-1 is expressed by both ECs and VSMCs and binds to the CCR2 receptor, present on circulating monocytes, thereby promoting entry of monocytes into the intima (15). Thus, our findings suggest that the CBM signalosome may be required for Ang II to effectively promote monocyte/macrophage recruitment to the atherosclerosis-prone vessel wall. However, the current data showed only a trend toward reduction in the levels of these chemokines, and further work will be required to explore this phenomenon more fully. In contrast to *CXCL1* and *MCP-1*, Ang II-dependent induction of *IL-6* was clearly unaffected by the absence of Bcl10, suggesting that other pathways known to mediate induction of *IL-6*, such as the ERK pathway, can compensate *in vivo* for the loss of Ang II-responsive canonical NF-κB activation. The above observations underscore the complexity of identifying the specific alterations in NF-κB-dependent gene expression that are responsible for the observed phenotypic protection from atherogenesis seen in Bcl10-deficient mice. In fact, the dramatic reduction in atherosclerosis likely reflects the cumulative effect of only subtle alterations in expression of numerous genes, each of which may be difficult to quantify in isolation.

Although not a major focus of the current work, we also noted the striking protection from aortic aneurysm formation afforded by Bcl10 deficiency. As is the case for atherogenesis, considerable data point to the importance of local NF-κB activation in the development of aneurysms, and a number of NF-κB-responsive genes, including those encoding for matrix metalloproteinases, have been implicated as participating in various ways to the destruction of vascular wall integrity, leading to focal weakening and expansion of the aortic wall (23). Thus, future work will be required to address the role of the CBM signalosome in regulating these matrix proteinases, in addition to the pro-inflammatory mediators discussed above.

Although we have focused on the direct pro-inflammatory effects of Ang II on cells of the vessel wall proper, an open question remains as to whether Ang II-responsive CBM signaling might also be active in non-resident cells that contribute to atherogenesis once recruited to a developing atherosclerotic lesion. In particular, Ang II is known to activate NF-κB in monocytes/macrophages, an integral component of such lesions (24). Studies designed to restore functional Bcl10 in the macrophages of $ApoE^{-/-}Bcl10^{-/-}$ mice, via bone marrow transplantation or other approaches, will help clarify this issue.

In summary, we conclude that the CBM signalosome has a major pathophysiologic role that extends beyond its known role in lymphocytes and adaptive immunity. These findings are of particular practical importance in light of the recent discovery of an inhibitor of the MALT1 protease (25), the enzymatically active component of the signalosome that communicates with the downstream NF-κB machinery. Thus, the CBM signalosome is a "drugable" target, and potential exists for exploring pharmaceutical inhibitors of MALT1 as a novel approach to the treatment of cardiovascular disease.

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