

## Inhibition and Genetic Ablation of the B7/CD28 T-Cell Costimulation Axis Prevents Experimental Hypertension

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**Background**—The pathogenesis of hypertension remains poorly understood, and treatment is often unsuccessful. Recent evidence suggests that the adaptive immune response plays an important role in this disease. Various hypertensive stimuli cause T-cell activation and infiltration into target organs such as the vessel and the kidney, which promotes vascular dysfunction and blood pressure elevation. Classically, T-cell activation requires T-cell receptor ligation and costimulation. The latter often involves interaction between B7 ligands (CD80 and CD86) on antigen-presenting cells with the T-cell coreceptor CD28. This study was therefore performed to examine the role of this pathway in hypertension.

**Methods and Results**—Angiotensin II–induced hypertension increased the presence of activated (CD86<sup>+</sup>) dendritic cells in secondary lymphatic tissues. Blockade of B7-dependent costimulation with CTLA4-Ig reduced both angiotensin II– and deoxycorticosterone acetate (DOCA)–salt–induced hypertension. Activation of circulating T cells, T-cell cytokine production, and vascular T-cell accumulation caused by these hypertensive stimuli were abrogated by CTLA4-Ig. Furthermore, in mice lacking B7 ligands, angiotensin II caused minimal blood pressure elevation and vascular inflammation, and these effects were restored by transplantation with wild-type bone marrow.

**Conclusions**—T-cell costimulation via B7 ligands is essential for development of experimental hypertension, and inhibition of this process could have therapeutic benefit in the treatment of this disease. (*Circulation*. 2010;122:2529-2537.)

**Key Words:** hypertension ■ immune system ■ inflammation ■ lymphocytes

Despite extensive study, the cause of most cases of human hypertension remains unknown.<sup>1</sup> Investigation for more than a century has focused on the kidney, the central nervous system, and the vasculature as mediators of this common disease. Increasing evidence suggests that inflammation might contribute to hypertension.<sup>2</sup> Various factors common to the hypertensive milieu, including reactive oxygen species, angiotensin II, and altered physical forces, promote activation of inflammatory cells, including macrophages and T cells, and their infiltration into the vasculature and kidney.<sup>3</sup> Importantly, recent evidence suggests that T cells are essential for development of experimental hypertension. RAG-1<sup>-/-</sup> mice, which lack lymphocytes, have blunted hypertensive responses to angiotensin II infusion and deoxycorticosterone acetate (DOCA)–salt challenge, and adoptive transfer of T cells completely restores hypertension in these animals.<sup>4</sup> Moreover, angiotensin II infusion increases circulating CD44<sup>high</sup>/CCR5<sup>+</sup>/CD69<sup>+</sup> T cells. Such cells mimic the phenotype of activated effector T cells, although the mechanism of how hypertension causes T-cell activation remains undefined.

### Editorial see p 2495 Clinical Perspective on p 2537

Classically, T cells require 2 signals for activation.<sup>5</sup> The first involves interaction of the T-cell receptor with an antigenic peptide presented in the context of major histocompatibility complex on antigen-presenting cells (APCs). The second, referred to as costimulation, involves the simultaneous interaction of receptors in proximity to the T-cell receptor with ligands on the APCs. Among several potential costimulatory interactions, the binding of T-cell CD28 with B7 ligands CD80 and CD86 on APCs is important, particularly for activation of naïve T cells.<sup>6</sup> When T-cell receptor ligation occurs without costimulation of CD28, T-cell activation is prevented and apoptosis is favored.<sup>7</sup> Pharmacological approaches to inhibit B7-dependent costimulation have been used to treat autoimmune diseases and prevent transplant rejection.<sup>8</sup> Immune modulation by this approach might thus inhibit the inflammatory processes that underlie hypertension and vascular dysfunction. The present study was performed to

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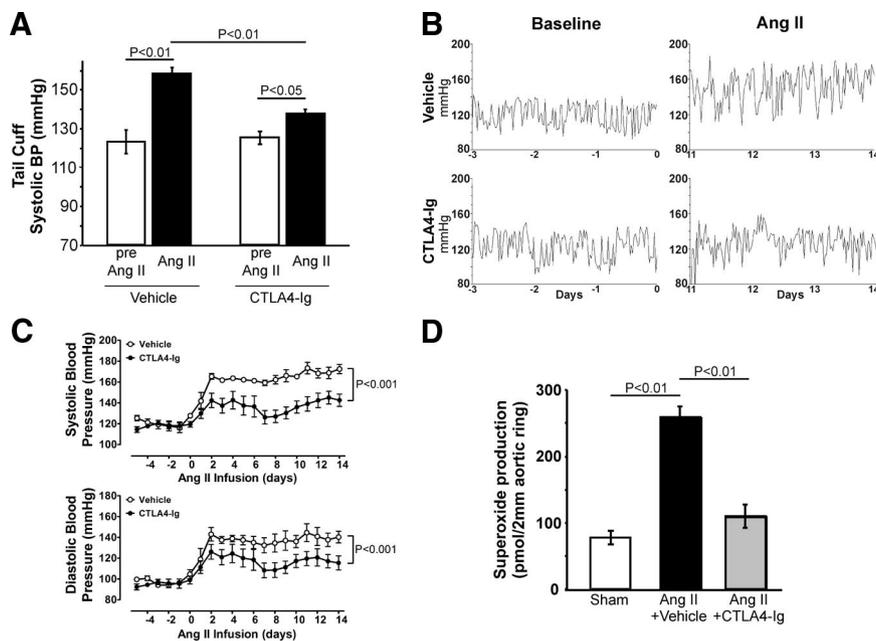
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**Figure 1.** Inhibition of CD80/CD86 costimulation by CTLA4-Ig in angiotensin II (Ang II)-dependent hypertension. C57Bl/6J mice received either Ang II ( $490 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) or buffer (sham) for 14 days via osmotic minipump. Animals were also cotreated with either CTLA4-Ig ( $250 \mu\text{g}$ ) or control intraperitoneal injections 3 days before the onset of Ang II administration and every 3 days thereafter. **A**, Noninvasive blood pressure measurements obtained via the tail-cuff method at days 0 (pre Ang II) and 14 (Ang II) were compared by 2-way ANOVA ( $n=5$  in each group). **B**, Sample traces of telemetric systolic blood pressure recordings obtained in freely moving control C57Bl/6J and CTLA4-Ig-administered mice 3 days before angiotensin II pump implantation (baseline) and during the last 3 days of Ang II infusion. **C**, Average blood pressure values obtained by telemetry at baseline and during Ang II infusion ( $n=6$  for vehicle;  $n=8$  for CTLA4-Ig). Comparisons were made with repeated-measures ANOVA with Scheffé post hoc test. **D**, Aortic superoxide levels measured by monitoring the oxidation of dihydroethidium to 2-hydroxyethidium by high-pressure liquid chromatography after 14 days of either buffer or Ang II infusion ( $n=5$  for sham;  $n=5$  for Ang II+vehicle;  $n=6$  for Ang II+CTLA4-Ig).

determine whether interruption of costimulation, either pharmacologically or by genetic deletion of B7 ligands, would prevent T-cell activation in response to hypertensive stimuli and have antihypertensive effects. Our findings suggest that T-cell activation in the setting of elevated blood pressure requires costimulation and that strategies to prevent this could be useful in special cases of difficult-to-treat hypertension.

## Methods

### Animal Models

C57Bl/6J mice and mice with genetic deletion of both CD80 and CD86 ( $B7^{-/-}$  mice) on a C57Bl/6J background were obtained from Jackson Laboratories (Bar Harbor, Maine) and were fed regular chow. Diet and water were provided ad libitum. The Emory University Animal Care and Use Committee approved the protocol for animal use. Mice at 12 weeks of age were selected at random from their cages for surgical and treatment interventions. In 1 series of experiments, we used a  $2 \times 2$  design to compare the effect of coadministration of CTLA4-Ig on the hypertensive response to angiotensin II. Angiotensin II was administered subcutaneously at a rate of  $490 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 14 days with osmotic minipumps as described previously.<sup>9</sup> Sham-operated animals underwent an identical surgical procedure, except the diluent without angiotensin II was infused. The investigator was blinded as to which mice received sham or angiotensin II infusions. The fusion protein CTLA4-Ig ( $250 \mu\text{g}$ ), which inhibits T-cell costimulation, was administered intraperitoneally every 3 days beginning 3 days before minipump implantation. As a control, either saline or an isotype control antibody was administered to mice not receiving CTLA4-Ig.

In additional experiments, we used a  $2 \times 2$  design to determine whether CTLA4-Ig could prevent a separate form of hypertension caused by administration of DOCA and salt (DOCA-salt hypertension). For creation of DOCA-salt hypertension, mice underwent uninephrec-

tomy and subcutaneous placement of a DOCA pellet and then received 0.9% NaCl in the drinking water. The sham surgery involved a surgical incision and placement of a placebo pellet. Animals were treated with either CTLA4-Ig or its placebo as described above beginning 3 days before surgery and every 3 days thereafter.

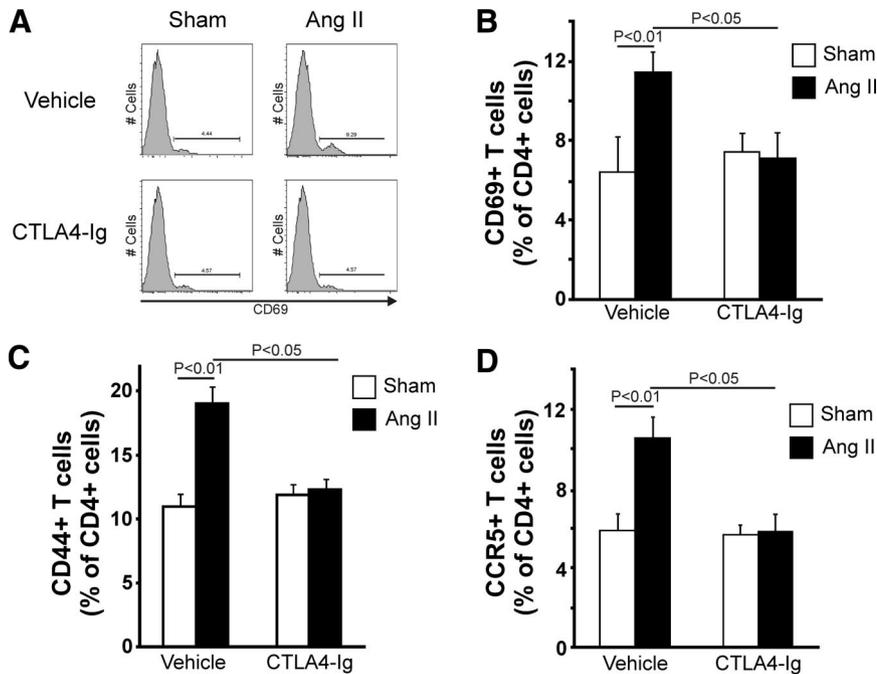
In 2 separate experiments, we sought to determine whether CTLA4-Ig would reverse established hypertension. To accomplish this, we performed 2 additional experiments using  $2 \times 2$  designs in which CTLA4-Ig treatment was initiated 2 weeks after either angiotensin II infusion (Sham/Ang II + Vehicle/CTLA4-Ig) or the DOCA-salt challenge (Sham/DOCA + Vehicle/CTLA4-Ig). CTLA4-Ig was administered every 3 days during the 2- or 4-week treatment regimens, respectively.

### Bone Marrow Engraftment

In a separate experiment, bone marrow was obtained from 8-week-old wild-type (WT) donor mice by flushing femurs and tibiae with RPMI 1640 medium. Nucleated cells were counted, and bone marrow was resuspended at a concentration of  $2 \times 10^7/\text{mL}$ . Eight-week-old recipient  $B7^{-/-}$  mice were irradiated with 1100 rad and then were immediately reconstituted with  $2 \times 10^6$  WT bone marrow cells via retro-orbital injection. After 6 weeks of engraftment, the recipient mice were randomized to receive either sham or angiotensin II osmotic minipump surgery. After 14 days of infusion, spleen-derived dendritic cells (DCs) from recipient mice were analyzed by flow cytometry for CD80<sup>+</sup> or CD86<sup>+</sup> expression to confirm successful engraftment.

### Blood Pressure Measurements

Blood pressure was measured by the tail-cuff method at days 0, 7, and 14 (and day 21 for the DOCA-salt model) with the MC4000 Multichannel System for mice (Hatteras Instruments, Cary, NC). In studies examining the effect of CTLA4-Ig on angiotensin II-induced hypertension only, we also measured intra-arterial blood pressure in freely moving mice using radiotelemetry. For reversal studies, blood



**Figure 2.** CTLA4-Ig inhibits T-cell activation in angiotensin II (Ang II)-dependent hypertension. Representative histograms (A) and mean data (B) are shown comparing the effects of vehicle vs CTLA4-Ig treatment on CD4<sup>+</sup> T-cell surface expression of the early-activation antigen CD69 during Ang II-induced hypertension. C and D, Effect of CTLA4-Ig on the percentage of circulating CD4<sup>+</sup>/CD44<sup>high</sup> and CD4<sup>+</sup>/CCR5<sup>+</sup> cells in Ang II-induced hypertension (n=6 for sham+vehicle; n=9 for Ang II+vehicle; n=6 for sham+CTLA4-Ig; n=10 for Ang II+CTLA4-Ig). Comparisons were made with 2-way ANOVA, and statistical values reflect the Bonferroni correction.

pressures were measured weekly for 4 to 6 weeks during the course of angiotensin II or DOCA-salt challenge.

**Flow Cytometry**

At the end of each treatment period, mice were killed, and blood and tissue were harvested for immediate flow cytometric analysis. Flow cytometry was used to characterize circulating T-cell activation phenotypes and vascular T-cell infiltration. DC maturation was examined in spleens and lymph nodes of sham and angiotensin II-treated mice. Please refer to the online-only Data Supplement for a detailed description of tissue digestion and flow cytometry.

**Cytokine Detection**

Spleen or lymph nodes were digested as described above and were purified with an autoMACS and a pan T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell purity was confirmed to be >95%. Splenic and lymph node T cells were plated at a density of 2x10<sup>5</sup> per well in 96-well plates coated with anti-CD3 antibody (BD Pharmingen, San Diego, CA) and cultured for 48 hours. A cytometric bead array (BD Pharmingen, San Diego, CA) was used to determine levels of tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , interleukin (IL)-2, IL-4, and IL-5 that had been released into the media.

**Quantification of Vascular Superoxide Production**

In a separate set of mice (n=5 to 6), aortic superoxide production was determined by monitoring conversion of dihydroethidium to 2-hydroxyethidium as described previously.<sup>10</sup>

**Statistical Analyses**

All data are expressed as mean  $\pm$  SEM. For blood pressure measurements over time, repeated-measures ANOVA with a Scheffé post hoc test was performed with PASW Statistics 18.0 for Mac. For measurements obtained at 1 point in time, comparisons of animals or treatments were made with 2-way ANOVA using GraphPad Prism 5.0. When significance was indicated, specific comparisons were made based on predesignated hypotheses with Bonferroni post hoc 2-tailed analysis. To ensure that the overall risk of a type 1 error for multiple comparisons did not exceed 0.05, a Bonferroni correction was applied by dividing 0.05 by the number of comparisons. The reported level of significance indicated herein for these comparisons represents values after the Bonferroni correction. When 2 comparisons were made, a 2-tailed *t* test was used.

**Results**

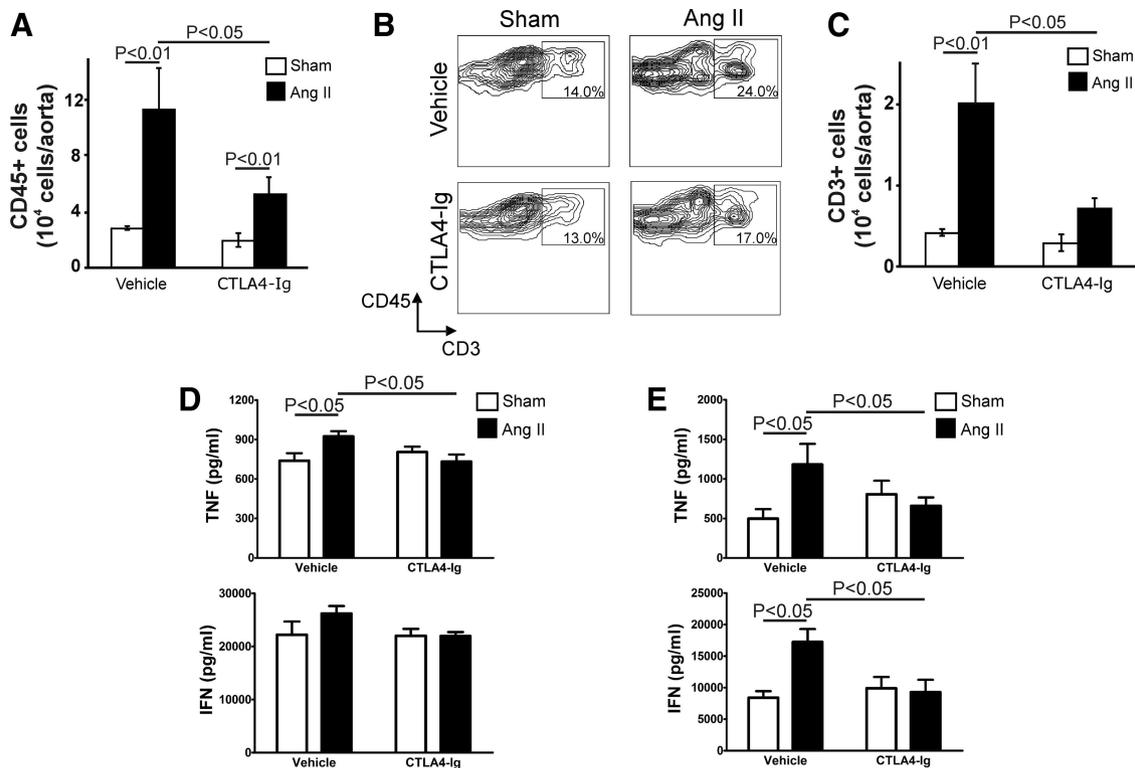
**Pharmacological Inhibition of Costimulation Reduces Angiotensin II-Induced Hypertension**

Using both noninvasive tail-cuff measurements (Figure 1A) and invasive monitoring of blood pressure by radiotelemetry (Figure 1B and 1C), we found that cotreatment with the fusion protein CTLA4-Ig, which blocks CD28 interactions with B7 ligands, significantly attenuated the hypertension induced by angiotensin II (Figure 1A through 1C; online-only Data Supplement Table I). Radiotelemetry demonstrated that systolic pressure averaged 168  $\pm$  1.5 mm Hg in mice cotreated with isotype control antibodies and 139  $\pm$  2.8 mm Hg in CTLA4-Ig-treated mice during the last week of angiotensin II infusion (*P*<0.001). Likewise, diastolic pressure was reduced from 139  $\pm$  1.3 to 116  $\pm$  1.6 mm Hg (*P*<0.001) by CTLA4-Ig cotreatment (Figure 1C).

A hallmark of hypertension is an increase in vascular superoxide production, which in turn alters vascular tone and promotes vascular disease.<sup>11</sup> Angiotensin II-induced hypertension was associated with a 3-fold increase in aortic superoxide production, and this was not observed in aortas obtained from mice treated with CTLA4-Ig (Figure 1D).

**Inhibition of T-Cell Activation and Vascular Inflammation by CTLA4-Ig in Angiotensin II-Induced Hypertension**

As described previously,<sup>4</sup> sustained angiotensin II infusion caused a modest but significant increase in the percentage of T cells expressing the early-activation marker CD69 (Figure 2A and 2B). The percentages of circulating T cells expressing CD44<sup>high</sup> (Figure 2C) and the surface marker CCR5 (Figure 2D), which plays a critical role in tissue homing, were also increased by angiotensin II. These responses were eliminated by cotreatment with CTLA4-Ig (Figure 2).



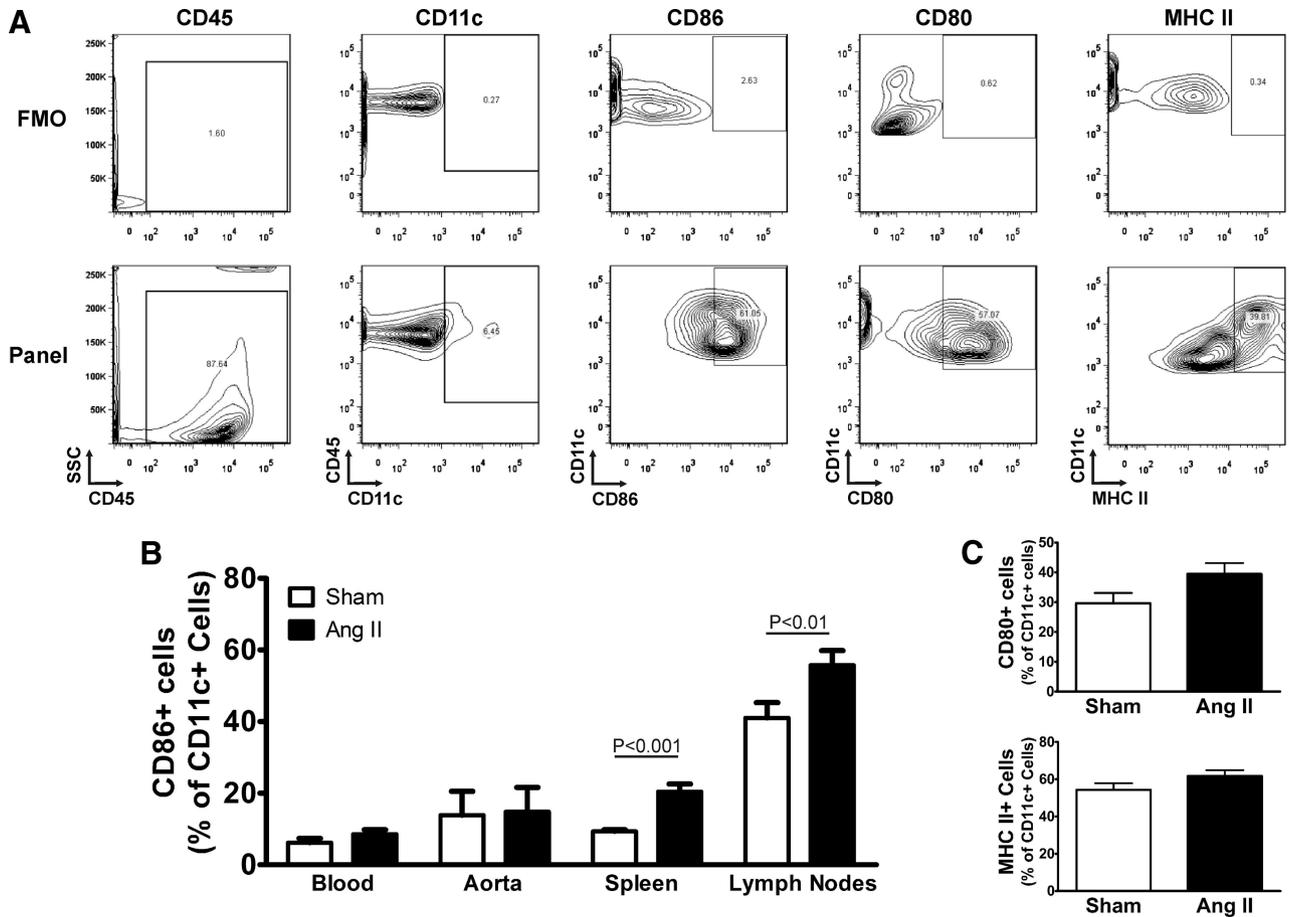
**Figure 3.** CTLA4-Ig inhibits vascular leukocyte and T-cell infiltration in angiotensin II (Ang II)-dependent hypertension. A, Absolute numbers of total leukocytes (CD45<sup>+</sup> cells) in the aorta of sham- and Ang II-infused mice treated with vehicle or CTLA4-Ig (n=6 for sham+vehicle; n=6 for Ang II+vehicle; n=6 for sham+CTLA4-Ig; n=8 for Ang II+CTLA4-Ig). B, Flow cytometry showing T cells (CD45<sup>+</sup>CD3<sup>+</sup>) in aortic samples from sham- and Ang II-infused mice treated with vehicle or CTLA4-Ig. C, Mean values for total aortic T cells (CD3<sup>+</sup> cells) in all treatment groups (n=6 for sham+vehicle; n=6 for Ang II+vehicle; n=6 for sham+CTLA4-Ig; n=8 for Ang II+CTLA4-Ig). D and E, Production of the inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  by T cells isolated from (D) spleen and (E) lymph nodes. Cells were stimulated for 48 hours on an anti-CD3 plate (n=5 for sham+vehicle; n=5 for Ang II+vehicle; n=5 for sham+CTLA4-Ig; n=6 for Ang II+CTLA4-Ig). Comparisons were made with 2-way ANOVA, and statistical values reflect the Bonferroni correction.

Previously, we have shown that sustained angiotensin II infusion increases infiltration of total leukocytes and CD3<sup>+</sup> cells into the aorta and that the predominant site of infiltration is the perivascular adipose tissue.<sup>4</sup> In the present study, we confirmed these findings. Analysis of aortic homogenates revealed that angiotensin II infusion increased total leukocytes by 4-fold (Figure 3A). CTLA4-Ig cotreatment attenuated aortic accumulation of total leukocytes (Figure 3A), largely due to a decrease in CD3<sup>+</sup> cells (Figure 3C). Consistent with our previous study, angiotensin II increased aortic infiltration of CD4<sup>+</sup>, CD8<sup>+</sup>, and double-negative T-cell subsets, and this was reduced by CTLA4-Ig treatment (online-only Data Supplement Figure I). The increase in aortic macrophages caused by angiotensin II was not altered significantly by CTLA4-Ig cotreatment (online-only Data Supplement Figure IIA). CTLA4-Ig had no effect on the aortic content of B cells, DCs, or natural killer cells (online-only Data Supplement Figure IIB). Angiotensin II-induced hypertension was associated with a modest increase in tumor necrosis factor- $\alpha$  and interferon- $\gamma$  by T cells isolated from the spleen but a marked increase in production of these cytokines in cells isolated from lymph nodes (Figure 3D and 3E). Importantly, CTLA4-Ig prevented these increases in T-cell cytokine production caused by angiotensin II. T-cell production of IL-4 and IL-5 was below the limit of detection in all treatment groups (data not shown).

### Evidence for Maturation of APCs in Response to Sustained Angiotensin II Infusion and Role for B7 Ligands in Hypertension

After antigen uptake, DCs and other APCs undergo a maturation process characterized by increased surface expression of major histocompatibility complex II and the B7 ligand CD86.<sup>12</sup> Flow cytometry was therefore used to detect CD11c<sup>+</sup> cells circulating in blood and in single-cell isolates of the aorta, spleen, and lymph nodes (Figure 4A). The percentage of these cells expressing CD80 and major histocompatibility complex II was unchanged by sustained angiotensin II infusion in all of these compartments (Figure 4C). In contrast, angiotensin II significantly increased the percentage of CD11c cells that expressed CD86 in the spleen and lymph nodes (Figure 4B).

These findings show that DC expression of CD86 is selectively increased by hypertension in secondary lymphoid organs, in keeping with a classic pathway for T-cell activation, and are compatible with the antihypertensive effects of CTLA4-Ig shown above. To further examine the role of B7 ligands in hypertension, we performed additional studies in mice deficient in CD80 and CD86 (B7<sup>-/-</sup> mice). The hypertensive response to angiotensin II was markedly reduced in these mice (Figure 5A). Moreover, the increase in circulating CD69<sup>+</sup> and CD44<sup>high</sup> T cells caused by angiotensin II did not occur in B7<sup>-/-</sup> mice (Figure 5B). Finally, the absence



**Figure 4.** Angiotensin II (Ang II) increases CD86 expression on CD11c<sup>+</sup> cells in secondary lymphoid organs. A, Examples of staining of lymph node–derived CD11c<sup>+</sup> cells. Fluorescence minus 1 (FMO) controls were used to unequivocally identify CD45<sup>+</sup>, CD11c<sup>+</sup>, CD80<sup>+</sup>, CD86<sup>+</sup>, and major histocompatibility complex (MHC) II<sup>+</sup> DCs. CD11c<sup>+</sup> cells were gated within CD45<sup>+</sup> gate. Subsequently, CD80<sup>+</sup>, CD86<sup>+</sup>, and MHC II<sup>+</sup> cells were identified. B, Bar graph showing frequencies of CD11c<sup>+</sup>CD86<sup>+</sup> cells from blood, aorta, spleen, and lymph nodes (n=10, 10, 4, and 16 for both sham and Ang II, respectively). C, Percentages of CD11c<sup>+</sup>CD80<sup>+</sup> and CD11c<sup>+</sup>MHC II<sup>+</sup> cells in lymph nodes from vehicle and Ang II–treated wild-type mice (n=6 for both groups).

of B7 ligands protected against the vascular infiltration of CD3<sup>+</sup> cells during angiotensin II infusion (Figure 5C and 5D).

B7 molecules are expressed not only on DCs but also on nonhemopoietic cells such as endothelial cells.<sup>13</sup> To directly examine the role of B7 on bone marrow–derived cells, we performed bone marrow transplant experiments. B7<sup>-/-</sup> mice were lethally irradiated and then given bone marrow from WT C57Bl/6J mice. Six weeks later, these animals were treated with sustained infusions of either angiotensin II or vehicle for the ensuing 2 weeks. To confirm successful engraftment of WT bone marrow, we used flow cytometry to analyze B7 ligand surface expression on spleen-derived DCs. Recipient B7<sup>-/-</sup> mice displayed an expression profile very similar to WT mice, which indicates restoration of B7 ligand expression on DCs (Figure 6B). Engrafting WT bone marrow into B7<sup>-/-</sup> mice completely restored angiotensin II–induced hypertension (Figure 6A) and aortic infiltration of leukocytes and T cells (Figure 6C and 6D).

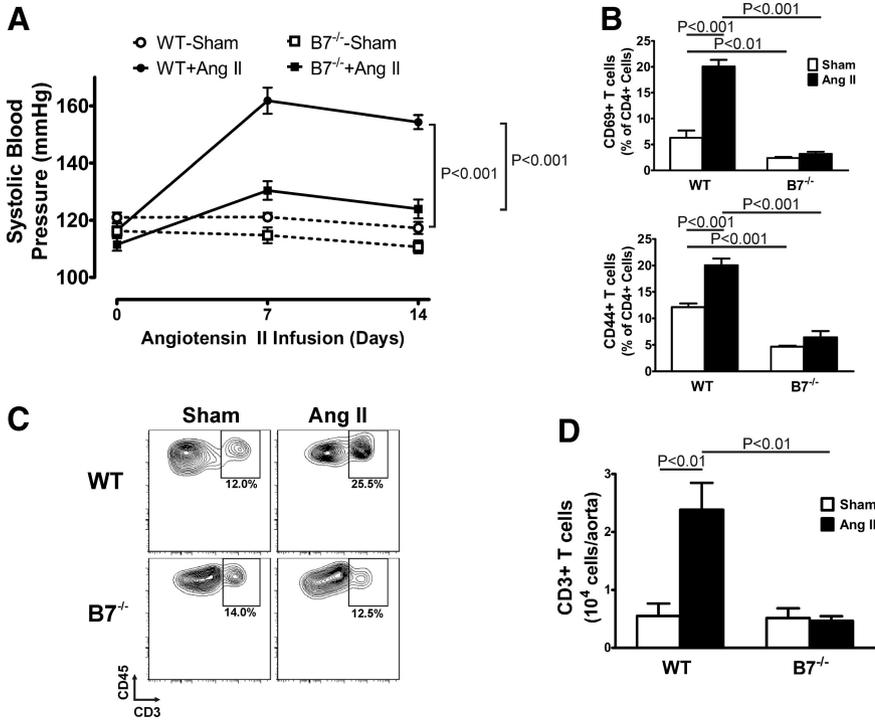
### CTLA4-Ig Prevents DOCA-Salt-Induced Hypertension

It is conceivable that costimulation might affect only angiotensin II–induced hypertension and not be effective in other

forms of hypertension. We therefore performed additional studies in mice with DOCA-salt hypertension. This model of hypertension is associated with suppression of the renin-angiotensin system and is sodium and volume dependent, but similar to angiotensin II–induced hypertension, it is associated with inflammation and oxidative tissue damage.<sup>14</sup> We have previously shown that T cells are also critical in this model.<sup>4</sup> As was the case with angiotensin II, CTLA4-Ig reduced the hypertensive response to the DOCA-salt challenge (Figure 7A). DOCA-salt hypertension also promoted T-cell activation, as evidenced by an increase in circulating CD69<sup>+</sup> and CD44<sup>high</sup> cells. Cotreatment with CTLA4-Ig prevented these changes in T-cell activation markers (Figure 7B). The aortic infiltration of total leukocytes and T cells was increased significantly by DOCA-salt hypertension, and this was prevented by CTLA4-Ig cotreatment (Figure 7C through 7E).

### Reversal of Hypertension by CTLA4-Ig

We performed additional experiments to determine whether CTLA4-Ig could reverse established angiotensin II and DOCA-salt–induced hypertension. Two weeks of angiotensin II infusion or DOCA-salt challenge raised blood pressures to 175±2.4 and 153±4.1 mm Hg, respectively. As we have



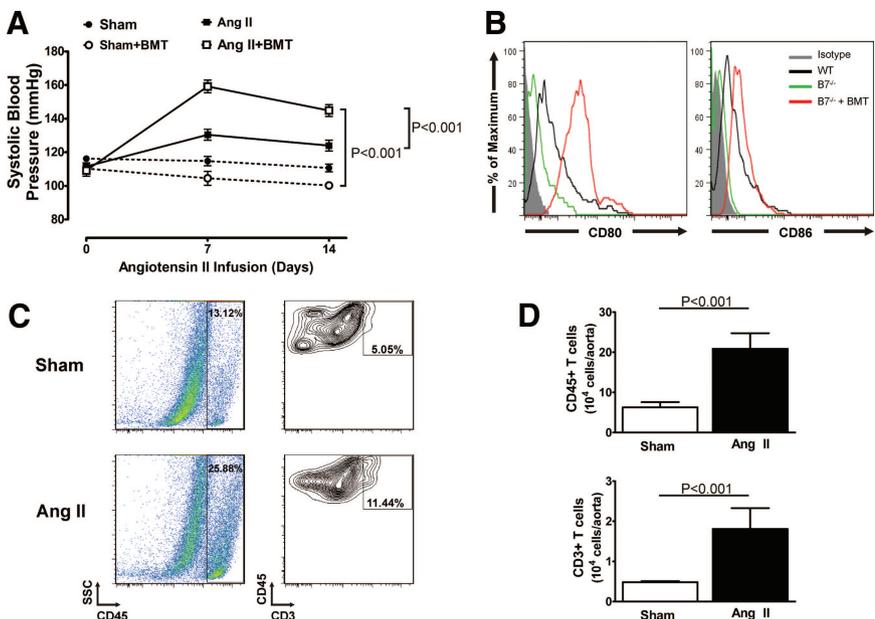
**Figure 5.** Role of CD80/CD86 costimulation on development of angiotensin II (Ang II)-dependent hypertension. Ang II ( $490 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) was infused for 14 days in either wild-type C57Bl/6J (WT) or  $B7^{-/-}$  mice. A, Repeated-measures ANOVA with Scheffé post hoc test was used to compare noninvasive blood pressure measurements obtained via tail cuff ( $n=7$  for WT-sham;  $n=6$  for WT-Ang II;  $n=11$  for  $B7^{-/-}$ -sham;  $n=12$  for  $B7^{-/-}$ -Ang II). B, Average percentage of circulating  $CD4^{+}/CD69^{+}$  and  $CD4^{+}/CD44^{\text{high}}$  cells as determined by flow cytometry in blood from vehicle- or Ang II-treated WT or  $B7^{-/-}$  mice ( $n=7$  for WT-sham;  $n=6$  for WT-Ang II;  $n=11$  for  $B7^{-/-}$ -sham;  $n=11$  for  $B7^{-/-}$ -Ang II). C and D, Representative contour plots acquired by flow cytometry (C) and average aortic  $CD45^{+}CD3^{+}$  cells in sham and Ang II-treated WT or  $B7^{-/-}$  mice (D;  $n=7$  for WT-sham;  $n=6$  for WT-Ang II;  $n=9$  for  $B7^{-/-}$ -sham;  $n=9$  for  $B7^{-/-}$ -Ang II). Comparisons of flow cytometry data were made with 2-way ANOVA, and statistical values reflect the Bonferroni correction for multiple comparisons.

reported in the present study and a previous study, 2 weeks of angiotensin II infusion significantly increased activation of circulating T cells and infiltration of these cells. In the present study, we found that 2 weeks of DOCA-salt-induced hypertension had similar effects (online-only Data Supplement Figure III). At this time point, we began treatment with CTLA4-Ig ( $250 \mu\text{g}$  per mouse) delivered intraperitoneally every 3 days as described above for 2 or 4 weeks. Treatment with CTLA4-Ig significantly lowered blood pressure in mice made hypertensive by either angiotensin II (Figure 8A) or DOCA-salt treatment (Figure 8B). Of note, in DOCA-salt hypertensive mice, 4 weeks of CTLA4-Ig treatment lowered blood pressure to values observed in mice that had received

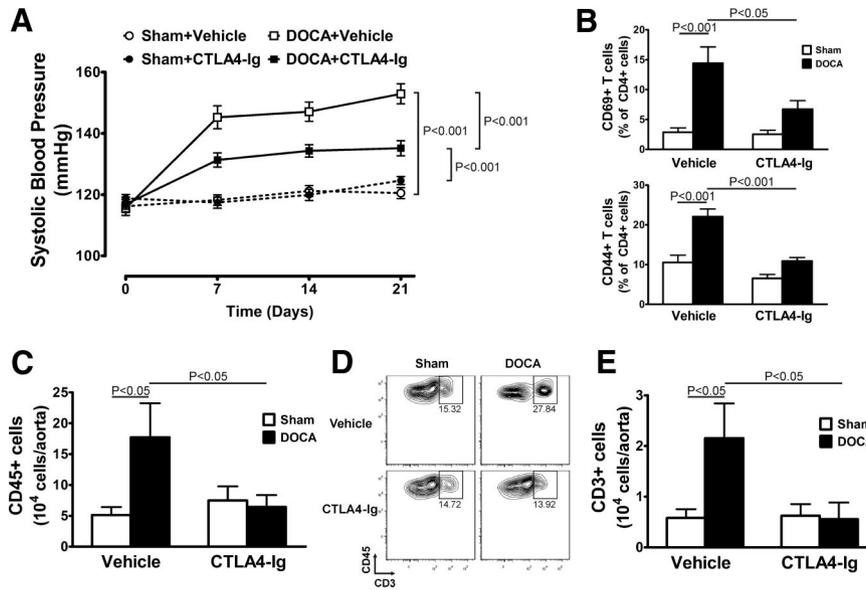
sham surgery. These data indicate that blockade of costimulation not only prevents hypertension but also lowers established hypertension.

### Discussion

In previous studies, we found that T cells play an important role in the genesis of hypertension.<sup>4,15</sup> These studies showed that hypertensive stimuli are associated with T-cell activation and the entry of effector-like T cells into the perivascular adipose tissue. We hypothesized that these cells release cytokines that affect function of the adjacent vessel, stimulating superoxide production and altering vasomotor tone. In the present study, we show that prevention of T-cell costimu-



**Figure 6.** Engrafting wild-type (WT) bone marrow into irradiated  $B7^{-/-}$  mice restored angiotensin II (Ang II)-induced hypertension and vascular infiltration. A, Repeated-measures ANOVA with Scheffé post hoc test was used to compare non-invasive blood pressure measurements obtained via tail cuff ( $n=4$  for sham;  $n=6$  for Ang II;  $n=4$  for sham+BMT;  $n=6$  for Ang II+BMT). B, Histograms showing CD80 and CD86 expression on DCs derived from WT and  $B7^{-/-}$  mice and  $B7^{-/-}$  mice engrafted with WT bone marrow. C, Representative flow cytometry gated for aortic  $CD45^{+}$  and  $CD3^{+}$  cells from vehicle and Ang II-treated  $B7^{-/-}$  mice engrafted with WT bone marrow. D, Numbers of  $CD45^{+}$  and  $CD3^{+}$  cells in aortas of sham and Ang II-treated  $B7^{-/-}$  mice engrafted with WT bone marrow ( $n=6$  for sham;  $n=6$  for Ang II). Statistical values shown in D were obtained by 2-tailed *t* test.

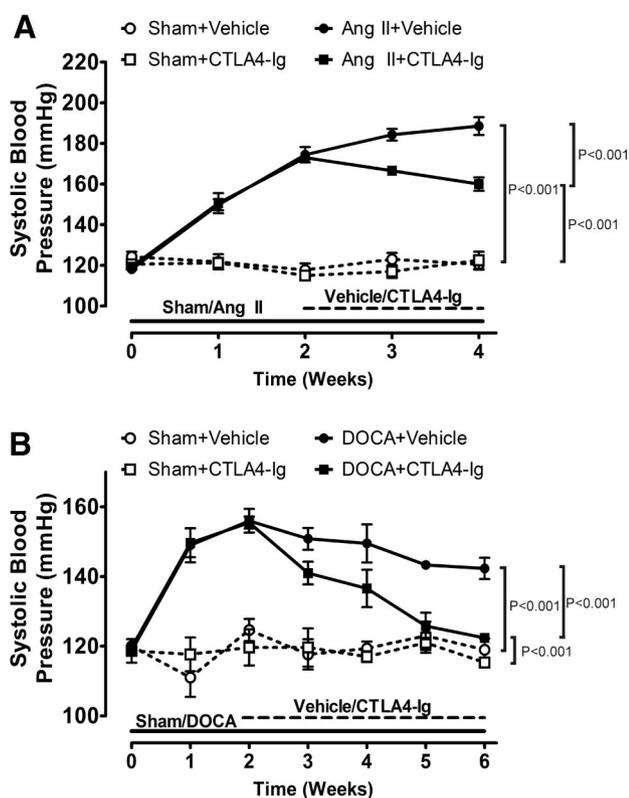


**Figure 7.** CTLA4-Ig attenuates DOCA-salt hypertension and its related vascular inflammation. DOCA-salt hypertension was induced in C57Bl/6J mice by uninephrectomy, subcutaneous implantation of a pellet containing DOCA, and addition of 0.9% NaCl to the drinking water. Vehicle or CTLA4-Ig (250  $\mu$ g) was administered intraperitoneally 3 days before uninephrectomy and every 3 days thereafter. A, Repeated-measures ANOVA with Scheffé post hoc test was used to compare noninvasive blood pressure measurements obtained via the tail-cuff method (n=10 for sham+vehicle; n=10 for sham+CTLA4-Ig; n=12 for DOCA+vehicle; n=13 for DOCA+CTLA4-Ig). B, Average percentage of circulating CD4<sup>+</sup> lymphocytes expressing CD69<sup>+</sup> and CD44<sup>high</sup> as determined by flow cytometry (n=10 for sham+vehicle; n=8 for sham+CTLA4-Ig; n=11 for DOCA+vehicle; n=9 for DOCA+CTLA4-Ig). C, Absolute numbers of total leukocytes (CD45<sup>+</sup> cells) in aortas of sham- and DOCA-salt mice treated with vehicle or CTLA4-Ig (n=10 for sham+vehicle; n=10 for sham+CTLA4-Ig; n=11 for DOCA+vehicle; n=11 for DOCA+CTLA4-Ig). D and E, Representative flow cytometric (D) and average aortic T-cell accumulation (E; CD3<sup>+</sup>/CD45<sup>+</sup>) in control mice and mice with DOCA-salt hypertension treated with either vehicle or CTLA4-Ig (n=10 for sham+vehicle group; n=10 for sham+CTLA4-Ig; n=11 for DOCA+vehicle; n=11 for DOCA+CTLA4-Ig). Comparisons of flow cytometry data were made with 2-way ANOVA, and statistical values reflect the Bonferroni correction for multiple comparisons.

lation by treatment with CTLA4-Ig or by genetic deletion of B7 ligands prevents T-cell activation and vascular inflammation caused by hypertension. This was associated with a reduction in the hypertensive response and vascular superoxide production. In keeping with an antiinflammatory effect, CTLA4-Ig prevented the increase in tumor necrosis factor- $\alpha$  and interferon- $\gamma$  production by T cells isolated from the spleen and lymph nodes of hypertensive mice. We further showed that the effect of costimulation blockade was not limited to angiotensin II-induced hypertension but also occurred in mice with DOCA-salt hypertension. Furthermore, we demonstrated that blockade of costimulation could also reverse established hypertension. The present study therefore provides unequivocal evidence that inhibition of the adaptive immune response can ameliorate high blood pressure and that modulation of costimulation might be effective in the treatment of otherwise refractory or malignant hypertension.

A previously unanswered question is how hypertensive stimuli such as angiotensin II and high salt activate T cells. It is possible that hypertensive stimuli could mobilize T cells activated by other nonspecific stimuli. Another possibility is that the major effect of angiotensin II is to increase adhesion and chemoattractant molecules such as intercellular adhesion molecule-1 and RANTES that attract T cells previously activated by other unrelated stimuli. These “passive” mechanisms are possible because stimuli such as angiotensin II increase vascular expression of adhesion molecules and chemokines, largely via redox-mediated mechanisms.<sup>16</sup> Fur-

thermore, even when mice are kept in clean conditions, modest T-cell activation by exogenous antigens is unavoidable. Given that costimulation occurs simultaneously with T-cell receptor activation, the present findings suggest that T cells are activated via a classic mechanism in the setting of hypertension. Our findings are therefore consistent with a scenario in which hypertensive stimuli prompt formation of neoantigens that are presented to T cells via APCs, which leads to T-cell activation. The increase in circulating cells bearing markers such as CD69, CD44<sup>high</sup>, and CCR5 in response to angiotensin II is compatible with this hypothesis. Moreover, our finding that angiotensin II increased the level of CD86<sup>+</sup>CD11c<sup>+</sup> cells in secondary lymphoid organs is compatible with maturation of DCs, which occurs on antigen presentation.<sup>12</sup> The nature of neoantigens involved in hypertension remains undefined; however, this situation is analogous to the setting of atherosclerosis, in which molecules such as altered oxidized lipoproteins, heat shock proteins, and bacterial and viral proteins have been implicated as neoantigens.<sup>17</sup> Importantly, blockade of costimulation was effective not only in angiotensin II-induced hypertension but also in DOCA-salt hypertension. This form of hypertension is associated with suppressed circulating renin levels,<sup>18</sup> and the present findings therefore indicate that costimulation contributes to hypertension that is independent of angiotensin II. These data are compatible with our previous findings that RAG-1<sup>-/-</sup> mice are resistant to DOCA-salt hypertension<sup>4</sup> and that high salt promotes T-cell recruitment into the kidney.<sup>3</sup>



**Figure 8.** Treatment with CTLA4-Ig reverses angiotensin II (Ang II) and DOCA-salt-induced hypertension. **A**, Two weeks post-sham or Ang II treatment (4-week pump), mice were randomly assigned to receive either vehicle or CTLA4-Ig for the remaining 2 weeks of Ang II infusion ( $n=5$  for all groups). **B**, Similarly, after 2 weeks of sham/DOCA-salt challenge, mice were randomly assigned to receive either vehicle or CTLA4-Ig for 4 weeks ( $n=5$  for all groups). Noninvasive blood pressure measurements were obtained weekly via tail cuff. Repeated-measures ANOVA with Scheffé post hoc test was used to compare blood pressure responses during the vehicle/CTLA4-Ig treatment phase only.

Agents that prevent costimulation, such as CTLA4-Ig, have been developed to treat autoimmune diseases such as rheumatoid arthritis and transplant rejection.<sup>19</sup> There is some debate that these agents might also affect the immune response by outside-in signaling and modulation of APC function. As an example, it has been shown recently that CTLA4-Ig promotes formation and activation of T regulatory cells.<sup>20,21</sup> These actions have been attributed in part to activation of indoleamine 2,3-dioxygenase (IDO) in DCs.<sup>22,23</sup> IDO-competent cells in turn are potent suppressors of T-cell function and can inhibit activation of these cells in an inflammatory environment.<sup>24</sup> It is therefore possible that CTLA4-Ig exerts its antihypertensive effect via promotion of T regulatory cell function. In preliminary studies, however, we have not found an increase in circulating CD4<sup>+</sup>/CD25<sup>+</sup>/FoxP3<sup>+</sup> cells in CTLA4-Ig-treated hypertensive mice (data not shown), which indicates that an increase in T regulatory cells is probably not responsible for the effects of CTLA4-Ig in this setting. Moreover, these effects of CTLA4-Ig to increase immunoregulatory T cells are dependent on B7 signaling. Our finding that B7<sup>-/-</sup> mice are also resistant to the hypertension and vascular inflammation caused by angiotensin II indicates that APC signaling via B7 is unlikely to

be the only mechanism by which CTLA4-Ig reduces blood pressure, but it does emphasize the role of B7-mediated costimulation in mediating hypertension. Importantly, engrafting WT bone marrow into B7<sup>-/-</sup> mice restored angiotensin II-induced hypertension and vascular inflammation, which further supports a role for the B7/CD28 axis in hypertension.

In keeping with the present studies, the T-cell-suppressing agent mycophenolate mofetil reduces blood pressure in experimental animals.<sup>3</sup> Moreover, mycophenolate mofetil and the antiinflammatory agent dexamethasone prevent renal damage in hypertensive transgenic rats expressing human renin and angiotensinogen.<sup>25</sup> Levels of DCs in the kidney were elevated in these animals and were reduced by dexamethasone. These findings support a role for T-cell activation via interaction with DCs in the setting of hypertension. Mycophenolate mofetil has also been shown to lower blood pressure in humans with psoriasis and rheumatoid arthritis.<sup>26</sup> This agent interferes with purine metabolism and therefore could have nonspecific effects on other rapidly dividing cells; however, when taken together with our present study, this supports a role for T-cell activation in hypertension.

It is of interest that the number of vascular macrophages was not affected by CTLA4-Ig. Despite this, this agent had antihypertensive effects and lowered vascular superoxide levels. This result is similar to the previously reported effect of T-cell inhibition with an antibody against very late antigen-4 (anti-VLA4) on autoimmune encephalitis in rats.<sup>27</sup> VLA4 inhibition suppressed disease and prevented T-cell infiltration into the central nervous system but did not alter macrophage accumulation. Studies such as these should not be interpreted as indicating that macrophages have no role but rather that T cells and macrophages engage in substantial cross talk to promote inflammation. The present data would indicate that the ingress of macrophages in vessels in the absence of T-cell accumulation has minimal effect on blood pressure and vascular superoxide production.

These findings might have clinical relevance. Although numerous agents are available for treatment of hypertension, a sizable proportion of the hypertensive population is either refractory to treatment or requires multiple agents to achieve blood pressure control.<sup>28</sup> Moreover, patients with accelerated or malignant hypertension are often difficult to treat and have target-organ damage that could be mediated by underlying inflammation.<sup>29</sup> Costimulation blockade might provide an alternative form of therapy targeted toward reducing inflammatory-mediated tissue damage. Thus, although costimulation blockade might not be used routinely to treat all individuals with hypertension, this therapy might be used in cases that are more difficult to control, particularly for brief periods during initiation of treatment.

In summary, these studies indicate that the hypertension caused by various stimuli such as angiotensin II, mineralocorticoids, and high salt requires T-cell costimulation and that costimulation is essential for the vascular inflammation that occurs in response to these common stressors. Future studies are needed to define the triggering mechanisms that activate T cells and APCs in hypertension. Moreover, treatment with agents that suppress costimulation might have a role in

certain cases of either difficult-to-treat or malignant hypertension.

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### CLINICAL PERSPECTIVE

Recent evidence indicates that inflammation, and in particular the adaptive immune response, contributes to hypertension. Hypertensive stimuli such as angiotensin II and mineralocorticoids promote T-cell activation and infiltration into vessels and the kidney. Prior studies have also shown that mice lacking T cells are resistant to hypertensive stimuli. In this study, additional evidence supporting a role of T cells in the genesis of hypertension is provided. Inhibition of T-cell costimulation with the agent CTLA4-Ig or by genetic deletion of the costimulatory molecules CD80 and CD86 markedly lowered the hypertensive response to angiotensin II or DOCA-salt challenge in mice. Moreover, treatment with CTLA4-Ig had blood pressure-lowering effects in experimental animals with established hypertension. These experiments support an immune mechanism for hypertension and point to new treatment strategies for difficult-to-treat cases of hypertension. In particular, CTLA4-Ig might prove beneficial in patients with malignant hypertension, which is often associated with end-organ damage that might be immune mediated.