Regulation of T-cell function by endogenously produced angiotensin II

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Hoch NE, Guzik TJ, Chen W, Deans T, Maalouf SA, Gratze P, Weyand C, Harrison DG. Regulation of T-cell function by endogenously produced angiotensin II. Am J Physiol Regul Integr Comp Physiol 296: R208 –R216, 2009. First published December 10, 2008; doi:10.1152/ajpregu.90521.2008.—The adaptive immune response, and in particular, T cells have been shown to be important in the genesis of hypertension. In the present study, we sought to determine how the interplay between ANG II, NADPH oxidase, and reactive oxygen species modulates T cell activation and ultimately causes hypertension. We determined that T cells express angiotensinogen, the angiotensin I-converting enzyme, and renin and produce physiological levels of ANG II. AT1 receptors were primarily expressed intracellularly, and endogenously produced ANG II increased T-cell activation, expression of tissue homing markers, and production of the cytokine TNF-α. Inhibition of T-cell ACE reduced TNF-α production, indicating endogenously produced ANG II has a regulatory role in this process. Studies with specific antagonists and T cells from AT1R and AT2R-deficient mice indicated that both receptor subtypes contribute to TNF-α production. We found that superoxide was a critical mediator of T-cell TNF-α production, as this was significantly inhibited by polyethylene glycol (PEG)-SOD, but not PEG-catalase. Thus, T cells contain an endogenous renin-angiotensin system that modulates T-cell function, NADPH oxidase activity, and production of superoxide that, in turn, modulates TNF-α production. These findings contribute to our understanding of how ANG II and T cells enhance inflammation in cardiovascular disease.

cytokines; TNF-α; electron spin resonance; adoptive transfer; NADPH oxidase; superoxide

THE RENIN-ANGIOTENSIN SYSTEM (RAS) is a prominent mediator of hypertension and a key target in the treatment of this disease. ANG II has myriad effects on the cardiovascular system. In many tissues, ANG II activates the NADPH oxidase to produce reactive oxygen species (ROS) (16). In the cardiovascular system, this effect of ANG II has been linked to the induction of cardiac hypertrophy, inflammation, lipid oxidation, endothelial dysfunction, and ultimately increased blood pressure (4).

We have recently demonstrated that the T cell plays an important role in the development of hypertension (18). ANG II increases T-cell activation, production of proinflammatory cytokines, and infiltration into perivascular fat. At this site, the T cell can produce cytokines and release other mediators that affect the smooth muscle cells and endothelium of the adjacent vessel. In this prior study, we demonstrated that the TNF-α antagonist, etanercept prevented ANG II-induced increase in blood pressure and vascular superoxide production, suggesting an important role of this cytokine in hypertension.

In addition to the classical components required for T cell function, T cells express other proteins not typically associated with immune responses, suggesting alternate physiological and pathological roles for the T cell. For example, T cells contain components of the RAS such as the ANG I-converting enzyme (ACE) (6), renin, the renin receptor, and angiotensinogen (Agt) (24). This suggests that T cells may be able to produce ANG II and thus contribute to the local RAS in tissues, however, T-cell production of ANG II has not yet been demonstrated.

Because of the established contributions of ANG II, NADPH oxidase, and ROS to hypertension, we sought to determine whether these mediators were involved in modulation of T cell function. Our findings indicate that T cells have a functional RAS, that they produce ANG II and that the majority of AT1 receptors are expressed within the T cell, suggesting an intracrine RAS. Our results demonstrate that ANG II has direct actions on T cell function, including activation, expression of tissue-homing markers, and production of TNF-α. We further identified a role of the T cell NADPH oxidase and superoxide production in this ANG II-dependent modulation of T cell function.

METHODS

Animals. Mice were maintained in the Emory Animal Facility under standard conditions (12:12-h light-dark cycle, 20°C room temperature), and were given Purina Rodent Chow 5001 diet and water ad libitum. The Institutional Animal Care and Use Committee at Emory University approved all experimental protocols. C57BL/6 and RAG1−/− mice were obtained from Jackson Laboratories. The p47phox−/− mice and their appropriate controls were obtained from Taconic. AT1R−/− mice were bred from animals provided by Dr. Tadashi Inagami (Vanderbilt University). All mice were on a C57BL/6 background. ANG II (490 ng·min−1·kg−1) was infused, and blood pressure was measured noninvasively as described previously (44). For adoptive transfer, mice were anesthetized with xylazine/ketamine, and cells were injected via tail vein. ANG II infusion, and blood pressure monitoring was begun 3 wk after adoptive transfer.

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T-cell isolation and culture. CD4+, CD8+, or all T cells were isolated from peripheral blood mononuclear cells (PBMC) and spleens as previously described (18). Briefly, spleens were removed and disrupted using forceps to release a single cell suspension that was passed through a 70-μm sterile strainer. Total blood leukocytes were isolated from whole heparinized blood, following osmotic lysis of excess red blood cells. Cells were centrifuged (800 g) and washed twice with PBS. For purified T-cell separation, splenocytes or PBMC were isolated from donor mice and were purified using auto-MACS and either CD4+, CD8+, or Pan T cell isolation kits (Miltenyi Biotech, Auburn, CA). Cell purity was confirmed by FACS analysis to be ≥ 99% by staining a fraction of the isolated T cells with a fluorescent anti-CD3 antibody. T cells were cultured as previously described (18). Briefly, 2 × 10^6 cells in 100 μl of media were seeded on 96-well plates coated with anti-CD3 antibodies (BD Biosciences, San Jose, CA), with or without additional treatments, and cultured for various times as indicated for each experiment.

Quantitative real-time and standard PCR. RNA was extracted from either T cells or indicated tissues using TriZOL (Invitrogen, Carlsbad, CA), and purified using procedure with the Qiagen RNeasy Kit. Following purification, 1 μg was reverse-transcribed using random hexamers and Superscript III (Invitrogen). The resulting cDNA was purified with the PCR Purification Kit (Qiagen). For receptor expression using a fixation/permeabilization kit Santa Cruz) for 60 min at 4°C. Intracellular staining was also performed for receptor expression using a fixation/permeabilization kit (eBioscience, San Diego, CA). For receptor expression, a secondary antibody incubation was performed with a FITC-conjugated secondary goat-anti-rabbit antibody (Santa Cruz, 1 μg/ml) for 60 min at 4°C, followed by two washes with FACS buffer. Following extracellular and intracellular immunostaining procedures, cells were resuspended in FACS buffer and analyzed immediately on an LSR-II flow cytometer with DIVA software (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed with Flowjo software (Treestar, Ashland, OR).

Measurement of ANG II and cytokine production. ANG II production was measured in media from cultured cells using the ANG II enzyme immunoassay kit (SPL-Bio, Cayman Chemicals, Ann Arbor, MI). A standard curve, negative controls (blank wells with Ellman’s developing reagent), negative binding wells that only received anti-angiotensin II Fab’ monoclonal antibody labeled with acetyloleines-terase (wells treated with culturing media), and positive controls (wells containing 9.5 pg/myocardial ANG II) were run in each experiment. Samples were loaded in duplicate and quantified by absorbance at 405 nm. The picogram per milliliter value for each sample was calculated by using the A405corr readings in the linear formula obtained from the standard curve. Cytokine production of T cells was measured using the cytometric bead array (BD Biosciences, San Jose, CA), as previously described (18). Briefly, T cells were cultured 48 h, and the media samples were collected for analysis of the amount of secreted TNF-α, IFN-γ, IL-2, IL-4, and IL-5.

Measurement of superoxide using electron spin resonance. For detection of O2·− the spin probe 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine (TMH) was used as previously described (43). T cells were cultured 3 h with or without 1 μmol/l perindopril in 24-well dishes in 1 × 10⁶ cells/ml of media. Cells were pelleted, and following removal of the media, they were resuspended in Krebs-HEPES buffer (pH 7.35) containing 50 μM DF and 5 μM DETC to a final concentration of 1.5 × 10⁶ cells per 100 μl. The TMH spin probe was dissolved to 0.5 mmol/l in 0.9% NaCl with Chelex containing 50 μM DF and 5 μM DETC and used at a final concentration of 50 μmol/l. The dissolved spin probe was continuously bubbled with argon gas prior to use. ESR spectra were recorded using a Bruker EMX spectrometer using the following settings: field sweep, 80 G; microwave frequency, 9.39 GHz; microwave power, 2 mW; modulation amplitude, 5 G; conversion time, 327.68 ms; time constant, 5,242.88 ms; 512 points resolution; and receiver gain, 1 × 10⁴.

AT₃R−/− adoptive transfer, ANG II infusion, and measurement of blood pressure. RAG-1−/− mice underwent adoptive transfer of 10⁷ T cells from either C57BL/6 or AT₂−/− mice via tail vein injection as previously described (18). After allowing 3 wk for T-cell engraftment, subcutaneous osmotic pumps were then implanted for administration of 490 ng·min⁻¹·kg⁻¹ ANG II, after which T cells from spleens were harvested and stained for T cells markers to verify reconstitution of the T-cell population using flow cytometry.

Blood pressure was measured using the tail cuff approach with a device specifically designed for mice (Hatters Instruments, Cary, NC). All tail-cuff measurements were acquired between 8 and 10 AM each day. Animals were accustomed to the device for 5 days before data were acquired. Baseline (prepump implantation) measurements were obtained for two consecutive days, and measurements were averaged. Day 1 was counted as the day pumps were implanted. Values were again obtained on days 6 and 7 and their average presented as day 7 measurements. Finally, pressures were again measured on days 13 and 14 and presented as day 14 measurements. Blood pressure measurements on RAG-1−/− mice following adoptive transfer of T cells from either C57BL/6 or AT₂−/− mice via tail vein injection as previously described (18). After allowing 3 wk for T-cell engraftment, subcutaneous osmotic pumps were then implanted for administration of 490 ng·min⁻¹·kg⁻¹ ANG II, after which T cells from spleens were harvested and stained for T cells markers to verify reconstitution of the T-cell population using flow cytometry.

Statistical methods. For data involving comparisons of two groups, a Student’s one-tailed t-test was used. When comparing the effect of an intervention, paired analysis was performed, as T cells taken from each mouse were divided into untreated and treated groups. For comparisons between more than two groups, one-way or two-way ANOVA was employed. P values <0.05 were accepted as significantly different. When ANOVA indicated significant differences, the Student-Newman-Keuls post hoc test was used for comparison of multiple groups, the Bonferroni test was used for selected comparisons, and the Dunnett’s test was used for repeated-measures analysis.
RESULTS

T-cell response to exogenous and endogenous ANG II—variations in spleen vs. blood-derived cells. To examine the effect of ANG II on T cells, T cells were isolated from the spleen and peripheral blood, purified and cultured in plates containing anti-CD3 in the presence or absence of ANG II. In preliminary studies of spleen-derived cells, we found dose-dependent, albeit small increases in the acute surface marker CD69 after a 3-h exposure to ANG II (10–500 nmol/l, Fig. 1A), while CD25 was only minimally affected by 500 nmol/l ANG II in these cells (Fig. 1B). A slight increase in the cell surface homing marker, CCR5, was observed after 12-h exposure to 500 nmol/l ANG II (Fig. 1C). Likewise, TNF-α was modestly increased by ANG II in spleen-derived T cells (Fig. 1D), while IFN-γ, IL-5, IL-4, and IL-2 either did not change in response to ANG II or levels were too low to be detected. In the absence of anti-CD3, ANG II had no stimulatory effect, in keeping with the concept that this hormone acts as a costimulatory molecule for T cell activation (30).

In contrast to the modest effect of exogenous ANG II on TNF-α production from spleen-derived T cells, we found that the addition of ANG II to blood-derived T cells in culture had no effect on this cytokine’s production (Fig. 2A). Because T cells have been reported to express multiple components of the RAS, we considered the possibility that blood-derived T cells produce endogenous ANG II, which could self-activate the cells, and that exogenous ANG II might therefore have minimal additional effects. The addition of the ANG I-converting enzyme inhibitor perindopril (1 μmol/l) caused a significant decrease in TNF-α levels (Fig. 2B) in cells exposed to anti-CD3 without ANG II for 48 h. This decrease in TNF-α was reversed by the addition of ANG II (100 nmol/l) (Fig. 2B). Perindopril did not affect levels of other cytokines including IFN-γ, IL-2, IL-4, and IL-5 (data not shown).

Production of ANG II by T cells—confirmation of an endogenous RAS. To confirm that T cells produce ANG II, we used ELISA to detect this octapeptide in the media of cultured T cells. Following 48 h of exposure to anti-CD3, blood-derived T cells released 10 ± 1.02 pg/ml/10⁶ cells (Fig. 3A). Media alone contained no detectable ANG II. Moreover, T cell production of ANG II was completely prevented by perindopril, confirming specificity of this assay. Among blood-derived T cells, the CD4 subset produced slightly more ANG II than CD8+ cells. Interestingly, T cells from the spleen produced significantly less ANG II compared with circulating T cells. Of interest, when we added 100 nmol/l of exogenous ANG II to...
cultured T cells, the amount of ANG II that was detected after 48 h in culture was 30 pg/ml. This is markedly less than the calculated amount that should have been present given the amount added and volume of the culture medium (1 μg/ml), indicating that there is likely marked degradation of ANG II in the culture.

It has been previously reported that T cells produce and possess all components of the RAS. Using real-time PCR, we were able to confirm that T cells express modest quantities of the ANG I-converting enzyme, equivalent to that observed in the liver (Fig. 3B). T cells also expressed small amounts of renin mRNA, which was much less than that of the kidney but greater than that found in the liver or lung (Fig. 3C). T cells also produced angiotensinogen, which again was less than that produced by the liver but greater than that produced by the lung and equivalent to that produced by the kidney (Fig. 3D).

Angiotensin receptors in T cells. We also found that T cells express both AT1a and AT1b receptors using real-time PCR (Fig. 4A). While we were unable to detect the AT2 receptor using this approach, conventional PCR techniques identified mRNA for this receptor (Fig. 4B). We further examined the presence of AT1 and AT2 receptors using fluorescent cell sorting. Conventional surface staining resulted in barely detectable signals (−0.06% expression of AT1R, 0.03% for isotype control, Fig. 4C). In contrast, intracellular staining revealed that within the CD4+ population of PBMCs, 5% of cells express the AT1a receptor, while 3.5% of the CD8+ population expressed this receptor (Fig. 4D). The amount of AT2 receptor detected by FACS analysis using either surface or intracellular staining was minimal and barely above the staining of the isotype control. In addition control experiments, we found that AT1R−/− T cells had no intracellular staining for AT1 receptors (Fig. 4E).

Role of ANG II receptor activation on T-cell production of TNF-α. To gain further insight into the role of the AT1 receptor in T-cell function, peripheral blood T cells were cultured on anti-CD3 plates for 48 h in the presence or absence of the AT1 antagonist losartan (1 μmol/l). Losartan decreased production of TNF-α by 30% (Fig. 5A). Surprisingly, the AT2 antagonist PD123319 also decreased TNF-α production by a similar extent, and the combination of these antagonists had an additional effect compared with losartan alone (Fig. 5A). To avoid nonspecific pharmacological effects of losartan and PD123319, we performed additional experiments using T cells from both AT1R−/− and AT2R−/− mice (Fig. 5B). Cells from both of these animals produced 60% less TNF-α than cells from wild-type C57BL/6 mice. These findings confirm a role of ANG II receptors in modulating T cell production of TNF-α.

In additional studies, we found that losartan had no effect on TNF-α production by T cells from AT1R−/− mice, and PD123319 had no effect on TNF-α production from cells of AT2R−/− mice (data not shown), indicating that the effects of these drugs on wild-type cells were unlikely nonspecific.

Role of the NADPH oxidase and ROS in modulation of T-cell production of TNF-α. ANG II potently activates the NADPH oxidase in other cell types, and ROS from this source are known to activate inflammatory signals, in part, by activating NF-κB-mediated gene transcription. NF-κB, in turn, has been implicated in T-cell production of cytokines such as TNF-α. We, therefore, performed additional experiments to determine whether endogenously produced ANG II could modulate T-cell ROS production and whether this could impact TNF-α production. T cells were exposed to perindopril for 4 h, and superoxide production from these cells was measured using electron spin resonance and the spin probe TMH. T cell production of superoxide was reduced by 30% by perindopril (Fig. 6A). To determine whether the NADPH oxidase participates in TNF-α production, T cells from p47phox−/− mice were cultured on anti-CD3 plates for 48 h, and the release of TNF-α into the media was measured. Compared with cells from wild-type C57BL/6 mice, T cells from p47phox−/− mice produced 40% less TNF-α (Fig. 6B).

The above data strongly support a role of ANG II in activation of the T cell NADPH oxidase, which then produces...
ROS to stimulate T-cell TNF-α production. To gain insight into the specific ROS involved in this process, additional experiments were performed in which T cells were preincubated with either PEG-SOD or PEG-catalase (100 U/ml for each) for 4 h before adding cells to anti-CD3 plates. PEG-SOD and PEG-catalase treatment was maintained during exposure to anti-CD3. Control cells were exposed to PEG alone. PEG-SOD decreased T cell production of TNF-α by 50% compared with PEG alone, while PEG-catalase was without effect (Fig. 6C).

Taken together, these data indicate that superoxide produced by the NADPH oxidase contributes to T-cell TNF-α production.

Role of the T-cell AT2 receptor in vivo regulation of blood pressure. We have previously shown that the T-cell AT1 receptor is important for modulation of blood pressure, in that adoptive transfer of AT1R−/− cells to RAG-1−/− mice resulted in a blunted hypertensive response to ANG II compared with adoptive transfer of wild-type T cells. Because we observed reduced T-cell production of TNF-α in T cells from AT2R−/− mice, we sought to determine whether these cells would display reduced effectiveness in vivo. To accomplish this, mice underwent adoptive transfer of 10⁷ T cells from either C57BL/6 or

Fig. 4. ANG II receptors in T cells. Real-time PCR with Taqman primers and probe sets was used to detect AT1α, AT1β, and AT2 receptors in T cells (A). Because AT2 receptors were undetectable using Taqman primers, standard PCR was used to identify AT2 receptor and 18S mRNA in spleen-derived total T cells (B). Data shown are representative of four repeated experiments. Fluorescent cell sorting (FACS) was used to detect the AT1 receptor protein on CD4+ and CD8+ T cells (C; n = 6). Because minimal surface AT1 receptor protein was detected, additional experiments were performed using cell permeabilization to detect intracellular AT1 receptors (D; n = 3). E: comparison of intracellular staining for AT1R in WT and AT1R−/− T cells (n = 3).

Fig. 5. Role of AT1 and AT2 receptors in modulation of T cell TNF-α production. T cells were isolated by negative selection from blood, and 2 × 10⁵ cells were cultured on anti-CD3 plates for 48 h. Following this, TNF-α production was determined using ELISA in untreated cells, cells treated with the AT1R antagonist losartan, the AT2R antagonist PD 123319, or the combination of these antagonists (A; n = 6). Additional experiments were performed comparing the production of TNF-α between wild-type T cells, T cells from AT1R−/−, and AT2R−/− mice (B; n = 4–7).

Fig. 6. Role of the NADPH oxidase and superoxide in T-cell production of TNF-α. T cells (2 × 10⁵) were cultured on anti-CD3 plates in the presence or absence of the indicated agents. Superoxide was measured using the 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine spin-probe with electron spin resonance in the absence and presence of the ANG I-converting enzyme inhibitor (A; n = 5). In other studies, T cells from wild-type and p47phox−/− mice were cultured on anti-CD3 plates for 48 h, and the release of TNF-α into the media determined by ELISA (B; n = 6–8). Studies to determine the role of hydrogen peroxide vs. superoxide production by the NADPH oxidase were also performed by treating T cells with either polyethylene glycol-linked catalase (PEG-catalase, 100 U/ml) or scavenger hydrogen peroxide or PEG-superoxide dismutase (PEG-SOD). PEG alone was used as a control (C; n = 8).
AT$_2$R$^{-/-}$ mice into RAG-1$^{-/-}$ mice. Three weeks following adoptive transfer, baseline blood pressure was measured using tail-cuff, and osmotic minipumps were then placed for infusion of ANG II. Adoptive transfer of wild-type T cells into RAG-1$^{-/-}$ mice restored the hypertensive response to ANG II to a level observed in ANG II-infused C57BL/6 mice. In contrast, adoptive transfer of the AT$_2$R$^{-/-}$ T cells resulted in a blunted hypertensive response to ANG II compared with that observed in either C57BL/6 mice or RAG-1$^{-/-}$ mice following adoptive transfer of wild-type T cells (Fig. 7A), despite achieving similar degrees of T-cell reconstitution (Fig. 7B).

**DISCUSSION**

In the present study, we found that T cells produce ANG II, which then exerts an autocrine action to stimulate production of superoxide. Superoxide, in turn, promotes T cell production of the cytokine TNF-α. The NADPH oxidase is important in this process, because T cells from p47$^{phox}^{-/-}$ mice demonstrated reduced production of TNF-α. These actions of endogenously produced ANG II require T cell receptor ligation with anti-CD3, suggesting that the octapeptide has a role in augmenting ongoing immune responses. Finally, the effects of ANG II on T cells seem to be mediated by both AT$_1$ and AT$_2$ receptors, in that both AT$_1$ and AT$_2$ receptor antagonists inhibited T-cell production of TNF-α, and the production of this cytokine was reduced to an equal extent in T cells from mice lacking these receptors. These data are consistent with the scheme presented in Fig. 8.

Our findings are in keeping with prior studies, demonstrating that T cells possess components of the RAS (24). The angiotensin-converting enzyme has also been colocalized with T cells in atherosclerotic lesions (7). ANG II has also been detected in the supernatant of mixed mononuclear cell preparations, although the precise contribution of T cells was not demonstrated (23). To our knowledge, therefore, the current study is the first to definitively document production of ANG II by T cells. Of interest, the amount of ANG II produced by these cells was substantial, on the order of what we could detect after the addition of 100 nM of ANG II to the tissue culture. There was also obvious degradation of ANG II 2 days following addition of 100 nM (30 pg/ml) was substantially less than the calculated amount that should have been present in the absence of degradation (1 μg/ml). The circulating levels of ANG II have been estimated to be ~60 pg/ml in humans (22) and to range from 10 to 300 pg/ml in rats based on the level of hydration (26). Thus, the levels of ANG II released in the media of these cells are clearly
within a physiological range. Moreover, the media substantially diluted the values that we measured such that the intracellular levels of ANG II are clearly much higher than the values in the media.

The mRNA levels of the various components of RAS were compared with tissues known to express these proteins. Not surprisingly, T-cell expression of the ANG I-converting enzyme was substantially less than that in the lung, but as much as that in the liver. Likewise, T cells expressed substantially less renin than did the kidney, but more than the liver or lung. In preliminary studies, we found it impossible to maintain T cells in culture without serum and therefore cannot exclude a role of ACE and renin present in T-cell production of ANG II. This situation, however, is not unlike that encountered in vivo, where resident T cells in tissue could produce angiotensinogen, which could serve as a substrate for renin and subsequently ACE in the interstitial fluid. Of interest, the levels of renin, angiotensinogen, and ACE were much lower in spleen-derived cells compared with circulating T cells, in keeping with our finding that spleen-derived cells produce less ANG II than circulating T cells. This might explain the ability of exogenous ANG II to stimulate spleen-derived cells and its lack of effect in blood-derived cells. These differences in RAS components are in keeping with other properties of central lymphoid vs. blood T cells (28).

Numerous cells involved in inflammatory responses, including macrophages (14), B cells (41), neutrophils (42), and resident cells in tissues (32) can produce TNF-α. One might, therefore, question the significance of T-cell production of this cytokine in an inflammatory milieu that contains other cells. There is evidence that TNF-α has important influences on T cells, such as modulating proliferation of the human immunodeficiency virus (29) and regulation of helper activity in B cell activation (20). Effector CD8+ cells rapidly produce TNF-α in response to T cell receptor ligation, which, in turn, decreases viability of adjacent antigen-presenting cells (3). Naïve T cells produce large amounts of TNF-α upon activation, at a time when they are migrating to outer T cell zones in lymph nodes (31). TNF-α can down regulate CD4+ cells and modulate the effector phase of CD8+ cells during viral infection (36, 37). A recent elegant study compared the effects of specific deletion of T-cell TNF-α using Cre-Lox technology in macrophages and T cells and showed that T cell-derived TNF-α plays a critical role in the response to intracellular infectious agents and autoimmune hepatitis (17). In our previous study, we found that hypertension was associated with a marked infiltration of T cells in the perivascular fat, and that TNF-α inhibition with etanercept could prevent the increase in blood pressure and vascular superoxide caused by ANG II (18). It is, therefore, likely that TNF-α production by T cells plays an important role in their function and also modulates adjacent cells. In the setting of hypertension, these effects of T cell-derived TNF-α likely involve stimulation of the vascular NADPH oxidase and superoxide production.

In cell culture, ANG II can directly stimulate NADPH oxidase activity in many cell types, including vascular smooth muscle cells and endothelial cells (8, 15). Our current findings, and those of our previous studies, however, indicate that the levels of ANG II achieved during chronic infusión are insufficient to directly achieve this effect. It is likely that ANG II, TNF-α, and other cytokines act in concert to amplify the inflammatory process in hypertension. In vascular smooth muscle cells, ANG II stimulates TNF-α production via activation of the NADPH oxidase and subsequent ROS production, thereby promoting a feed-forward response (40).

A surprising finding of the present study was that both AT$_1$R and AT$_2$R receptor blockade reduced T-cell production of TNF-α. This conclusion was based not only on pharmacological inhibition with losartan and PD123319 but also on studies of T cells from AT$_1$R- and AT$_2$R-deficient mice. In keeping with a role of T cell AT$_2$R in increasing blood pressure, we also found that the hypertensive response to ANG II was modestly blunted in RAG1$^{-/-}$ mice after adoptive transfer of AT$_2$R$^{-/-}$ T cells. These data are similar to those we previously published showing that the hypertensive response to ANG II was blunted following adoptive transfer of AT$_1$R$^{-/-}$ T cells compared with that observed following adoptive transfer of wild-type cells (18). In general, the AT$_1$R and AT$_2$R have been considered to signal opposite effects, such that the AT$_1$R promotes vascular smooth muscle growth, vasoconstriction, and superoxide production, while AT$_2$R inhibits these events (5). There are, however, instances in which the AT$_2$R has proinflammatory roles. As an example, expression of either the AT$_1$R or AT$_2$R led to similar degrees of NF-κB activation in COS7 cells (45). In keeping with this, blockade of both AT$_1$R and AT$_2$R was required to reduce the inflammation and NF-κB activation caused by unilateral ureteral obstruction (10). In human preadipocytes, the ANG II-stimulated release of IL-6 and IL-8 is inhibited by AT$_1$R blockade and to a lesser extent by AT$_2$R inhibition, suggesting that both receptors signal a proinflammatory response in these cells (39). AT$_2$R receptors are up-regulated in the kidney during ANG II infusion at sites of renal inflammation and tubular cell apoptosis, suggesting a role in this pathological response (35). Our findings that both receptors promote T-cell production of TNF-α and contribute to hypertension in vivo are in accord with these prior studies.

Using fluorescent cell sorting, we were unable to demonstrate the presence of AT$_2$ receptor protein on either the cell surface or intracellularly in T cells despite the above considerations. It is possible that the antibody that we employed does not work well for FACS. It is also likely that the AT$_2$ receptor is present in very low levels that cannot be detected by FACS. In keeping with this, we were only able to detect AT$_2$R mRNA using conventional PCR. The fact that PD123319 reduced TNF-α production and that T cells from AT$_2$R$^{-/-}$ mice produced reduced amounts of TNF-α strongly suggests that these cells indeed contain a functioning AT$_2$ receptor. This conclusion is further strengthened by our finding that adoptive transfer of AT$_2$R$^{-/-}$ T cells in RAG1$^{-/-}$ mice led to reduced hypertension compared with adoptive transfer of cells from wild-type mice.

An interesting finding in our present study is that the majority of T cell AT$_1$R receptors are intracellular and therefore would be particularly prone to activation by endogenously produced ANG II. This result is in keeping with the concept that ANG II and its receptors can exist as an intracrine system, in which signaling occurs within cells (25, 34). A similar role of intracellular ANG II has been demonstrated in the kidney (46), myocardial cells (38), the central nervous system (12, 21), and hepatic cells (9). In the case of myocytes, intracrine ANG II has been implicated in hypertrophy (2) and is increased by hyperglycemia (38). It will be of substantial interest to study...
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factors, such as glucose, that could modulate T cell ANG II or its receptors.

It should be emphasized that the pathway outlined in Fig. 8 only partly explains TNF-α and superoxide production by T cells. Blockade of the angiotensin-converting enzyme, angiotensin receptors, and scavenging of superoxide reduced TNF-α production by only about 30 to 60%, indicating that a substantial portion of this is independent of this pathway. Nevertheless, a potentially important portion of T-cell TNF-α production is linked to this pathway. Given these considerations, our present data support a role of T-cell production of ANG II in autocrine stimulation of this cell. Further studies are needed to understand the role of T cell endogenously produced ANG II in diseases such as these and in cardiovascular pathology.

Perspectives and Significance

The current study has defined a new pathway in T cells that involves production of ANG II, superoxide, and the inflammatory cytokine TNF-α (Fig. 8). We have previously shown that ANG II-induced hypertension is associated with accumulation of effector-like T cells in the perivascular fat (18), and T cells are also known to accumulate in and to promote atherosclerotic lesions (19). It is, therefore, likely that this pathway contributes to the progression of vascular disease in these conditions. We believe that T cells are unlikely a major source of superoxide in hypertensive vessels, but that their production of TNF-α and other cytokines promotes a pro-oxidant milieu, which together with ANG II markedly stimulates the production of ROS by adjacent vascular cells. It is also likely that T cell production of ANG II, superoxide, and TNF-α could contribute to inflammation in other settings where T cells accumulate. These findings could explain why interruption of the RAS improves T cell-mediated diseases such as arthritis (11, 27, 33), transplant rejection (1), and experimental myocarditis (13) and also findings could explain why interruption of the RAS improves ANG II, superoxide, and TNF-α-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circ Res 102: 488–496, 2008.

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