Arteriolar reactivity in lymphocyte-deficient mice

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Leonard S, Croy BA, Murrant CL. Arteriolar reactivity in lymphocyte-deficient mice. Am J Physiol Heart Circ Physiol 301: H1276–H1285, 2011. First published July 8, 2011; doi:10.1152/ajpheart.00346.2011.—Mounting evidence suggests that lymphocytes have the capacity to contribute to the regulation of systemic circulatory control. We postulated that T and natural killer (NK) cells could modify basal microvascular activity under physiologically normal conditions. In situ intravital microscopy of mouse cremaster vasculature was used to evaluate arteriolar reactivities to the vasoconstrictors angiotensin II (ANG II) and phenylephrine (Phe) and the vasodilators acetylcholine (Ach) and adenosine (Ado) in normal [+/+]; wild type (WT) and genetically immunodeficient (T/B +NK- or T+B -NK-) C57BL/6 and BALB/c mice, strain backgrounds with differentially polarized T cell cytokine production. Immunodeficient mice tended to have smaller baseline and maximal diameters of third-order cremaster arterioles than their congenic WT partners. In C57BL/6, baseline diameters were similar in T-B- mice without or with NK cells; in BALB/c, baseline diameters were larger in T-B-NK- than in T-B +NK- mice. Thus, at baseline, lymphocytes tended to promote vasodilation, except BALB/c NK cells, which mediated mild vasoconstriction. The presence of NK cells suppressed dilations to Ado in both strains, to Ach in the C57BL/6 strain, and dilatory responses to ANG II in C57BL/6 and to Phe in BALB/c. In the BALB/c strain, the presence of T and B cells promoted vasodilatory responses to Ado, attenuated dilations to low ACh concentrations, and exaggerated dilation and constriction responses to ANG II. Thus, under agonist challenge, NK cells generally promote constriction, whereas influences of T and B cells depend upon the stimulus. Therefore, lymphocytes or their products have physiological influences on microvascular arteriolar reactivity.

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CARDIOVASCULAR DISEASE IS multifactorial and often associated with inflammatory events. Inflammation affects multiple levels of the vasculature: coronary arterioles (55), conductance arterioles (11, 20, 21), and arterioles of the microcirculation (5, 53). It is suggested that microvascular inflammation, and its resulting alteration of vasomotor responses, precedes manifestations of cardiovascular disease (51). Inflammation is largely mediated by lymphocyte-derived cytokine mixtures that are selectively upregulated to combat specific types of antigenic stimulation (38, 40). Production of type I cytokine mixtures that include interferon γ (IFNγ) and tumor necrosis factor-α (TNF-α) by T helper (Th) lymphocytes (Th1) is implicated in hypertension (21), vascular inflammation, and atherosclerosis (51). Mice lacking T and B cells show a blunted angiotensin (ANG II)-induced hypertension and do not develop the associated abnormalities in vascular function (21). T cells but not B cells were identified as mediating these circulatory effects. Human Th cells have been linked with promotion of hypertension (46, 47) in clinical studies of elderly type 2 diabetic patients (34) and of pregnant women with the hypertensive disorder preeclampsia (46, 47). Thus, there appears to be a close relationship between immune cells, their products, and vascular function under pathological conditions.

Upon antigen stimulation, lymphocytes release a host of secretory products. Th1 cells secrete predominantly IFNγ and interleukin (IL)-2; Th type 2 cells (Th2) predominantly secrete IL-4, -5, -10, and -13 (for review, see Refs. 1 and 40), whereas natural killer (NK) cells release IFNγ among a host of other cytokines, chemokines, mucins, and enzymes (8, 19). Cytokines are vasoactive and modify the responses of other vasoactive compounds (6, 13, 14, 17, 37). There is growing evidence that T cells and NK cell lineages may participate in human and murine vascular regulation under nonpathological conditions. Human T and NK cells possess functional renin-angiotensin systems (29), T and B cells contain all of the elements of the cholinergic signaling pathways, including nitric oxide synthesis (30), and, most recently, NK cells have been shown to produce atrial natriuretic factor (22), indicating the potential for release of the vasoactive molecules besides classic cytokines. Interestingly, in normal healthy mouse and human pregnancy, the uterine vascular bed is strongly influenced by lymphocytes, specifically uterine (u) NK cells. uNK cells trigger structural remodeling of uterine arterioles and alter their responsiveness to vasoactive molecules by midpregnancy (2, 3, 23, 48, 56). Thus, compelling evidence suggests that lymphocytes that have not been activated by antigens may contribute to the physiological regulation of the resistance vasculature in the healthy individuals, but direct experimental evidence for this is missing.

We hypothesized that T cells and NK cells influence the resting arteriolar vasculature and influence agonist-stimulated responses in the absence of antigen simulation. To address these hypotheses, microvascular responses to vasoconstrictors and vasodilators were examined in normal mice and in mice genetically deleted in T and B cells or T, B, and NK cells. The choice of mouse strain for these experiments is critically important. Distinct mouse strains have well-defined differences in T cell subsets. T cells are described as helper or effector subtypes as defined by surface expression of CD4+ or CD8+, respectively. CD4+ T cells are further subclassified as Th1 and Th2 cells. These differ in their production of specific cytokines, and both subtypes are distinct from CD8+ effector T cells (10, 32, 41, 45). The C57BL/6 strain is biased toward Th1 anti-inflammatory responses, whereas the BALB/c strain is biased toward Th2 anti-inflammatory responses (10, 36, 39, 55). Both Th1 and Th2 are relevant to the healthy human state. The typical cytokine pattern of the adult, nonpregnant human is...
Th1 dominance (7, 43), whereas there is a shift toward Th2 dominance during healthy human pregnancy (7) and during aging (16). We quantified third-order cremaster arteriolar responses to vasoactive agonists in both C57BL/6 and BALB/c mice to determine whether lymphocytes make contributions to microvessel homeostasis and reactivity across strain backgrounds. The intravital cremaster preparation is a well-established model ideal for visualizing the microcirculation. The resistance arteriolar vasculature was chosen as the focus of this study because of its relevance in determining mean arterial pressure.

**MATERIALS AND METHODS**

**Animals.** Adult male C57BL/6/NCrI−/− [C57BL/6 wild type (WT); 26 mice] and BALB/cAnNcrI−/− (BALB/c WT; 20 mice) were used. Severe combined immune deficient [Prkdcscid (SCID)] mice on C57BL/6 (19 mice) and BALB/c (16 mice) backgrounds were used as T−B−NK+ mice. Rag2−/− Il2rg−/− double knockout mice on C57BL/6 (20 mice) and BALB/c (17 mice) backgrounds were used as T−B−NK− lymphoid (ALY) mice. WT and SCID mice were purchased from Charles River (St. Constant, Quebec, Canada). BALB/cALY mice were bred at University of Guelph from pairs generously provided by Dr. James P. Di Santo, Paris, France. BALB/cALY mice were bred at Queen’s University from pairs generously provided by Dr. Mamoru Ito, Kawasaki, Japan. WT mice received standard husbandry; immunodeficient mice received barrier husbandry using similar bedding and food. Both husbandry units were pathogen free during the course of these studies as determined by negative serology in sentinel animals. All animals were acclimatized for at least 1 week at Guelph before use in protocols. Mice were 8–14 wk of age when used in protocols approved by the University of Guelph Animal Care Committee.

**Surgical preparation for cremaster intravitral microscopy.** Surgeries were based upon the model developed by Baez (4) as modified by Kim and Sarelius (31). Mice were anesthetized with pentobarbital (70 mg/kg ip). A tracheotomy was performed to maintain airway patency. Polyethylene catheters (internal diameter ~35 μm) were inserted in the right jugular vein for anesthetic infusion (70 mg/kg given in 0.025-ml doses as needed) and in the left carotid artery to monitor mean arterial pressure. Esophageal temperature was maintained at 37°C with a heated water coil. The cremaster muscle was exposed, continuously superfused [36°C bicarbonate-buffered PSS composed of (mmol/l): 131.9 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, 30 NaHCO3 (all from Fisher Scientific, Ottawa, Ontario, Canada), and 0.3 mg/l d-tubocurarine (curare) (Sigma Aldrich, Oakville, Ontario, Canada)], and equilibrated with 5% CO2-95% N2 to a pH of 7.4 ± 0.05. The cremaster muscle was spread over a silicone pedestal (Silgard; WPI, Sarasota, FL) and secured with pins. The mouse was then transferred to the microscope stage, and its vasculature was allowed to equilibrate for 45–60 min before study.

**Experimental protocols.** The cremaster vasculature was transilluminated with a tungsten lamp and visualized using an Olympus BX51WI microscope (×20 objective; ×1.6 magnification changer). Images were captured with a Sony DXC-390 3CCD color camera, displayed on a Sony Trinitron color video monitor, and recorded to VHS tape (Sony SVO-9600 MD video recorder). Final vascular magnification was times ~2,000.

Third-order arterioles (40–50 μm) were identified by retrograde counting up the arteriolar tree from a capillary bed. Vasoactive agonists were applied topically to selected arterioles. Each preparation was tested with one or two agonists. For the latter protocol, one vasodilator and one vasoconstrictor were randomly selected, as was agonist application order. ANG II (10−8 to 10−6 mol/l) and phenylephrine (Phe; 10−7 to 10−5 mol/l) were used to test vasoconstriction; acetylcholine (ACH; 10−7 to 10−5 mol/l) and adenosine (Ado; 10−8 to 10−6 mol/l) were used to test vasodilation. The different stimuli were chosen such that there were two vasodilators and two constrictors, agonists to represent nervous (Phe), hormonal (ANG II), and local (ACh and Ado) regulating pathways and to have both endothelial cell-dependent (ACH) and -independent (Phe) stimuli represented. Agonists were purchased from Sigma-Aldrich (St. Louis, MO); concentrations were chosen to cover a range of vascular responses.

After equilibration, a 1-min baseline diameter recording was made before applying the lowest agonist dose. Agonists were topically applied via the superfusate (agonist + PSS) for 5 min (10 min for ANG II), in increasing concentrations. Vessels were recorded continuously until completion of all agonist applications. Next, video recording ceased for a washout by PSS superfusion (30–45 min). Upon return of the vessel to baseline diameter, the second agonist, expected to induce a response in the opposite direction, was applied, and the protocol was repeated. At the end of each experiment, maximum diameter was recorded by superfusing 10−4 mol/l sodium nitroprusside (a nitric oxide donor) to induce maximal dilation.

**Data and statistical analyses.** Digitized images were captured online every 10 s from baseline through ACh, Ado, or Phe exposure and every 20 s for ANG II. Internal diameters were measured from digitized images using Image Pro software. Data are expressed as means ± SE and are presented as either change in diameter (μm) from baseline (diameter − baseline diameter) or change in diameter expressed as a percentage of maximum vessel diameter [(diameter − baseline diameter)/maximum diameter × 100]. Only one arteriole was studied per animal preparation; n indicates the number of arterioles observed per treatment. Group means were first compared with a two-way ANOVA to determine interactions between drug concentrations and genotypes, when applicable. Next, a repeated-measures ANOVA was performed for each variable. When significance reached P ≤ 0.05, a protected least-squares difference test was used post hoc to test group means. Because of differences observed in baseline and maximum diameters (Table 1), linear regression analysis was used to determine potential correlations between either the initial baseline diameter or the dilatory potential (maximum diameter − baseline diameter) of arterioles and response to the lowest dose of each drug.

**Table 1. Mouse weights and third-order cremaster arteriole baseline and maximum diameters for all genotypes tested**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mouse Wt, g</th>
<th>Baseline Diameter, μm</th>
<th>Maximum Diameter, μm</th>
<th>Baseline Diameter, %maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6ALY</td>
<td>32.9 ± 1.2*</td>
<td>13.7 ± 1.4*</td>
<td>38.4 ± 2.4*</td>
<td>36.5 ± 3.2*</td>
</tr>
<tr>
<td>C57BL/6SCID</td>
<td>23.4 ± 0.3</td>
<td>14.1 ± 1.5*</td>
<td>37.0 ± 2.2*</td>
<td>37.6 ± 2.9*</td>
</tr>
<tr>
<td>C57BL/6WT</td>
<td>32.7 ± 1.0*</td>
<td>24.2 ± 2.7</td>
<td>44.4 ± 1.9</td>
<td>55.3 ± 5.5</td>
</tr>
<tr>
<td>BALB/c ALY</td>
<td>35.6 ± 0.8*</td>
<td>17.5 ± 1.9</td>
<td>41.9 ± 2.1*</td>
<td>42.4 ± 4.7</td>
</tr>
<tr>
<td>BALB/c SCID</td>
<td>27.3 ± 0.4</td>
<td>13.8 ± 1.3*</td>
<td>35.6 ± 1.9*</td>
<td>39.7 ± 4.0</td>
</tr>
<tr>
<td>BALB/c WT</td>
<td>26.6 ± 0.4*</td>
<td>18.5 ± 1.5*</td>
<td>41.7 ± 1.4</td>
<td>44.7 ± 3.9</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. ALY, lymphoid; SCID, severe combined immune deficient; WT, wild type. *Significantly different from WT within the same strain at P ≤ 0.05. #Significantly different from SCID within the same strain. Significance when genotype is compared across strain.
RESULTS

Baseline and maximum diameters. Baseline diameters and maximum diameters of third-order cremaster arterioles were significantly smaller in C57BL/6SCID and ALY than in C56Bl/6WT mice (Table 1). There were also differences in mouse weight whereby C57BL/6SCID was significantly smaller than either C56Bl/6ALY or WT. Because the trends in diameter and weight among the genotypes were not similar, the differences in vessel size could not be attributed solely to differences in animal size. Differences in baseline diameter between the C57BL/6 genotypes could result in differences in vascular responsiveness. Therefore, potential correlations between vessel responses and both 1) baseline diameter (i.e., vasomotor tone) and 2) dilatory potentials were determined by genotype. Average $r^2$ values for the C57BL/6 genotypes were: 1) $0.23 \pm 0.06$ for baseline diameter vs. response and 2) $0.31 \pm 0.07$ for dilatory potential vs. response. The lack of correlation between the baseline diameter, the dilatory potential, and the reactivity of the blood vessels indicates that neither the differences in baseline diameter nor dilatory potential affected comparisons of C57BL/6 responses across genotypes. Baseline expressed as a percent of maximum diameter showed that SCID and ALY were significantly smaller than WT, indicating that their baseline diameter was more constricted than WT even when the differences in maximum diameter were taken into account.

BALB/cSCID arterioles had significantly smaller baseline and maximal diameters than BALB/cWT (Table 1). There were also differences in mouse weight whereby BALB/cALY was significantly smaller than both BALB/cSCID or WT, confirming in this second inbred strain that the differences in vessel size were not solely determined by animal size. Because of differences in vessel diameter, correlations between peak response at the lowest dose of agonist used and either 1) baseline diameter or 2) dilatory potential were calculated. Average $r^2$ values were: 1) $0.28 \pm 0.16$ for baseline diameter vs. response and 2) $0.31 \pm 0.15$ for dilatory potential vs. response. Therefore, neither differences in baseline diameter nor dilatory potential affected comparisons of BALB/c vascular responses across genotypes. When BALB/c SCID baseline diameters were expressed as a percent of maximum diameter, there was no significant difference between BALB/c SCID and BALB/c WT baselines, indicating that the baseline diameters are proportionally similar given the absolute vessel size.

Cross-strain comparisons (i.e., C57BL/6WT vs. BALB/cWT) of baseline and maximum diameters showed that BALB/cWT arteriolar baseline diameters were significantly smaller than C57BL/6WT baseline diameters (Table 1), whereas maximum diameters did not differ. BALB/cWT mice weighed significantly less than aged-matched C57BL/6WT mice, and therefore we would expect that the maximum diameter of the BALB/cWT would be smaller than C57BL/6WT but they were not. Mouse size should not impact baseline diameter independently of maximal diameter; therefore, mouse size does not account for cross-species differences in baseline diameter.

Response to ANG II. To elucidate effects of NK cells on the vasculature, we compared responses of the ALY genotype lacking NK and T and B cells with the SCID genotype having only NK cell lymphocytes. ANG II caused a vasoconstriction at $10^{-8}$ mol/l in the C57BL/6ALY genotype and a vasoconstriction at $10^{-7}$ and $10^{-6}$ mol/l. The presence of NK cells in the SCID genotype attenuated the vasodilation of $10^{-8}$ mol/l ANG II (Figs. 1A and 2A). There were no significant differences between BALB/cALY and BALB/cSCID in response to any ANG II concentration (Figs. 1B and 2B).

To elucidate the effects of T and B cells on the vasculature, responses of the SCID genotype were compared with WT. In C57BL/6 mice, $10^{-8}$ mol/l ANG II induced little response in SCID but significant vasodilation in WT arterioles, indicating that the presence of T and B cells promoted an ANG II vasodilation (Figs. 1A and 2A). ANG II at $10^{-7}$ and $10^{-6}$ mol/l caused vasoconstriction in both C57BL/6SCID and WT that did not differ significantly. Within the BALB/c strain, $10^{-8}$ mol/l ANG II produced a variable response with no significant effect on vascular diameter in SCID genotype and a nonsignificant, highly variable response (peak diameter change 15.5 $\pm$ 13.8 $\mu$m) in BALB/cWT. Further investigation of the data showed two distinct patterns in WT (Figs. 1B and 2B). One-half of the arterioles ($n = 6$) responded with a maximal dilation (group 1). The remainder ($n = 5$) responded with significant vasoconstriction (group 2). No experimental factor could account for this divergence. Divergent responses were present within animal groups shipped at the same time and within animal groups shipped and tested months apart. Furthermore, the order of drug application did not account for the two patterns. We reviewed order of drug exposure as a potential contributor to the divergent directional responses of BALB/cWT to $10^{-8}$ mol/l ANG II and found that constrictor and dilatory responses were independent of whether ANG II was applied in the first or second trial and were independent of the vasodilator paired with ANG II.

To determine whether T cell cytokine bias was a factor in determining the vascular response to ANG II, arteriolar responses of C57BL/6WT were compared with BALB/cWT (Fig. 2C). The response to $10^{-8}$ mol/l ANG II in C57BL/6WT produced a significant vasodilation that was not significantly different from the nonsignificant dilation with very large error (peak change in diameter 15.5 $\pm$ 13.8 $\mu$m) produced by $10^{-8}$ mol/l ANG II in BALB/cWT. Once separated into the two response patterns, BALB/cWT group 1 showed a large, significant dilation, whereas BALB/cWT group 2 showed a significant constriction. Arteriolar diameters in response to $10^{-7}$ and $10^{-6}$ mol/l ANG II were not significantly different between C57BL/6WT and either group of BALB/cWT.

Response to Phe. There were no differences in the response to Phe between the C57BL/6ALY and SCID vessels (Figs. 3A and 4A). Of note, at the highest concentration of Phe ($10^{-5}$ mol/l), all vessels were considered closed. Thus, the change in diameter from $10^{-6}$ to $10^{-5}$ mol/l Phe may not accurately reflect the constrictor capacity of $10^{-5}$ mol/l Phe, but rather that the vessels were closed and could not constrict further. BALB/cALY arterioles had divergent response patterns to $10^{-7}$ mol/l Phe ($n = 10$; Figs. 3B and 4B). One-half of the arterioles ($n = 5$) maximally dilated to $10^{-7}$ mol/l Phe (group 1). The remainder ($n = 5$; group 2) significantly vasoconstricted. Again, no experimental factors could account for the divergent patterns. This divergent pattern of response was not present in the BALB/cSCID strain, which showed a singular, vasoconstrictor response pattern indicating that the presence of NK cells in the
SCID genotype suppressed the dilatory response of group 1, resulting in a constriction at all Phe concentrations.

There were no differences in either strain in the responses of SCID genotype compared with the WT (Figs. 3 and 4), indicating a lack of affect of T and B cells on the ability of Phe to cause vasoconstriction. Cross-strain WT comparisons also showed no significant differences between arteriolar diameters in response to any Phe concentration (Fig. 4C).

Response to ACh. In the C57BL/6 strain, we observed a vasodilation in response to 10^{-5} and 10^{-6} mol/l ACh in ALY that was attenuated in the SCID genotype (Fig. 5A), implicating NK cells in suppression of the dilation. The same was not true in the BALB/c strain where there were no significant differences between the arteriolar responses of ALY and SCID genotypes (Fig. 5B). When comparing SCID with WT responses to ACh, vasodilation was inhibited at 10^{-5} mol/l ACh in C57BL/6 and at 10^{-7} mol/l ACh in BALB/c. Thus, presence of T and B cells attenuated ACh-induced vasodilation. Cross-strain WT comparisons showed no significant differences between arteriolar diameters in response to any ACh concentration (Fig. 5C).

Response to Ado. Within the C57BL/6 strain, the dilatory affect of Ado in the ALY genotype was attenuated at 10^{-7} mol/l in SCID arterioles (Fig. 6A). Similarly, in the BALB/c
strain, the dilatory affect of Ado in the ALY genotype was attenuated at $10^{-8}$ mol/l in SCID arterioles (Fig. 6B). Thus, the presence of NK cells attenuated vasodilation at low Ado concentrations. When comparing SCID with WT, only the BALB/c strain differed in response to Ado (Fig. 6B). BALB/c WT showed amplified dilation in response to $10^{-8}$ mol/l Ado compared with SCID, indicating the presence of T and B cells amplified Ado’s ability to cause dilation at low concentrations. Cross-strain WT comparisons showed no significant differences between arteriolar diameters in response to any Ado concentration (Fig. 4C).

For all of the agonists tested, there were no significant differences in time to reach peak change in diameter, rate of dilatory response, or extent of recovery from peak diameter change (data not shown).

DISCUSSION

We used a genetic approach to address whether resting lymphocytes contribute to regulation of arteriolar reactivity in the intact mouse cremaster model. Although the C57BL/6 WT mouse, a Th1-biased strain, is routinely used for microcirculatory studies, this is the first set of cremaster arteriolar reactivity studies reported for BALB/c WT, a Th2 cytokine-biased strain, and for lymphocyte-deficient mice. Genotype differences in baseline diameter indicate a constrictor influence of NK cells and a dilatory influence of T and B cells on the resting diameter of unstimulated arterioles. On agonist-stimulated arterioles, NK cells consistently attenuated vasodilatory effects. The effects of T and B cells were less consistent and had agonist specificity. T and B cells enhanced the dilatory effect of ANG II in C57BL/6 and Ado in BALB/c while inhibiting vasodilation to ACh in C57BL/6 and BALB/c arterioles. The Th1/Th2 cell bias was important with respect to ANG II responsiveness. Our data show that NK cells and T and B cells can act as circulating paracrine mediators that can influence the microvasculature under healthy, nonantigen activated conditions.

The effect of lymphocytes on maximal and baseline arteriolar diameter. In this study, C57BL/6 WT mice had maximum arteriolar diameters similar to those reported by others (26) and were significantly larger than maximal diameters of both C57BL/6 SCID and ALY genotypes. BALB/cSCID maximal diameters were significantly smaller than WT and ALY of the same strain. Differences in maximum diameters of both strains could not be attributed to the differences in animal weights and therefore animal size. High concentrations of the nitric oxide donor sodium nitroprusside were used to determine maximal diameter. Therefore, differences in maximum diameters between genotypes could indicate that C57BL/6 SCID and ALY as well as BALB/c SCID arterioles were less responsive to nitric oxide. However, there was no decrease in responsiveness of arterioles of these genotypes to ACh, a nitric oxide-dependent agonist (Fig. 5), refuting this possibility. Not assessed in this study was the role that small differences in blood pressure play between groups, which might also contribute to structural changes that will influence maximal diameter. Using 24-h continuous radiotelemetry in female mice, we measured mean arterial pressure in C57BL/6 WT (107 ± 2 mmHg), BALB/c WT (113 ± 1 mmHg), BALB/c ALY (113 ± 1 mmHg), and BALB/c SCID (118 ± 1 mmHg) (Burke S, Adamson M, and
Croy BA, unpublished observations). We have not assessed males. Therefore, the differences in absolute maximal diameters within strains implies developmental differences in arteriolar structure that may be influenced by mean arterial pressure or by the presence of lymphocytes; the contribution of each will require further investigation.

The differences in baseline diameters within and between strains provide support for the hypothesis that lymphocytes influence the microvasculature. The baseline arteriolar diameter of C57BL/6ALY was not altered by the presence of NK cells (SCID); C57BL/6 SCID arterioles had significantly smaller arteriolar diameters than when B, T, and NK cells were present (WT), indicating that T and B cells have basal dilatory effects in the absence of arteriolar stimulation. The baseline diameter of BALB/cSCID arterioles was significantly smaller in the presence of T and B cells (WT). These data indicate a dilatory influence of T and B cells on the resting diameter of unstimulated arterioles. Baseline diameters of the BALB/WT mice were significantly smaller than C57BL/6WT, suggesting that T cell cytokine bias may influence the resting diameter of unstimulated arterioles. Therefore, at rest, vascular tone is influenced by T and B cells in C57BL/6 and BALB/c mice, and there is an influence of the T cell subtype. These data indicate that vasoactive cytokines or other mediators may be released from naïve, unstimulated cells and promote changes that contribute to the intracellular environment of endothelium or vascular smooth muscle, which sets the baseline diameter of the vessel. Unstimulated lymphocytes may release products that act directly on endothelial or smooth muscle cells or act indirectly by affecting other contributors to baseline tone such as local matrix, hormonal, and neuronal influences.

**The effects of lymphocytes on responses to applied agonists.** The most dramatic effects of the lymphocytes were on the vasoactive response to ANG II. We observed that both NK cells and T and B cells attenuated an ANG II vasodilatory response and caused a vasoconstriction in the C57BL/6 strain. Similar results were observed for T and B cells but not NK cells in the BALB/c strain. There was also an effect of Th cell...

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**Fig. 3.** Change in diameter of arterioles in response to topically applied phenylephrine (Phe) in increasing concentrations ($10^{-7}$ to $10^{-5}$ mol/l) in 5-min intervals to 3 genotypes of the C57BL/6 strain (A) and BALB/c strain (B). The arrows represent time of Phe dose application. For BALB/cALY, 5 animals showed vasodilatory responses (group 1), whereas 5 others showed vasoconstrictive responses (group 2).
bias shown by the disparate dilatory and constriction responses observed in the BALB/cWT mouse compared with the moderate dilatory response in C57BL/6WT.

Products released by lymphocytes may be influencing the ANG II intracellular signaling pathways or the membrane...
receptor population. In C57BL/6WT, ANG II receptor type 2 (AT2) and AT1 receptors are expressed in skeletal muscle microvessels and promote vasodilation and vasoconstriction, respectively (9). The distribution of these receptors in microvessels of immune deficient mice has not been defined. The shift from vasodilation to vasoconstriction could indicate a lymphocyte influence on the membrane receptor population promoting a shift from AT2 to AT1. The cytokine IL-6 has been shown to induce endothelial cell dysfunction by promoting overexpression of AT1 (54). ANG II via AT1 has been implicated in the cardiovascular complications of obesity and diabetes (for reviews, see Refs. 12 and 24) and the hypertensive pregnancy complication preeclampsia (27). Therefore, a switch in membrane receptor dominance because of alterations in relative numbers of NK cells, T and B cells, or Th cell bias may exacerbate such cardiovascular complications. This would be particularly true in healthy states such as pregnancy and aging where there is a normal shift from the Th1 to the Th2 dominant state (7, 16) and in pathological states where a similar Th1/Th2 shift has been observed [hypercholesteremia (57)]. Therefore, to understand how ANG II functions in health and disease, it is critical that we understand further how lymphocytes alter ANG II reactivity.

There was little impact of lymphocytes on the effects of Phe in either mouse strain with the exception of the BALB/cALY where the dilatory response produced in one-half of the arterioles was suppressed in the presence of NK cells. Dilatory responses to an adrenergic agonist could be produced through β-adrenergic membrane receptors, and constriction would result from α-adrenergic membrane receptor stimulation. Because Phe only engages the latter receptor population, the mechanism for NK cell involvement in a Phe dilatory response may be indirect. The overall lack of effect of lymphocytes on Phe-induced vasoconstrictions indicates that neuronal or hormonal α-adrenergic control over the microvasculature is not influenced by the presence of lymphocytes.

Lymphocytes did not systematically affect ACh responses in either strain, although in the C57BL/6 strain NK cells attenuated dilation at higher doses of ACh and T and B cells further attenuated dilation. T and B cells also attenuated vasodilation at low concentrations of ACh in the BALB/c strain. Endothelial cells stimulated by ACh produce dilatory responses that are dependent on both nitric oxide and prostaglandins as signaling components (25, 26, 33, 49, 50). Our data indicate that these signaling pathways may be suppressed in the presence of lymphocytes. Endothelial cell function is an important component of local blood flow control within tissues through its responses to the chemical and physical (wall shear stress) properties of blood. Similarly, in regard to local regulation of blood flow, Ado has been shown to be an important paracrine regulator of vascular function, especially Ado production by tissue in response to metabolic need for blood flow (15, 35, 42, 44). The Ado response was also unaltered by lymphocytes except at a single dose of the single Ado concentration where NK cells suppressed dilation in the C57BL/6 strain. In the BALB/c strain, the responses to 10^{-8} mol/l Ado were attenuated by NK cells and amplified by T and B cells. Both the effects of lymphocytes on endothelial cell responses to ACh and the responses to Ado indicate that the local tissue control of the microvasculature is not consistently affected by the presence of lymphocytes but may be modified under specific conditions.

The vascular responses to agonists were surprisingly similar between strains given their different T cell complements and biases towards production of different cytokines (52). The only difference in responses between strains was in response to low

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Fig. 6. Average peak diameter changes of arterioles in response to topically applied adenosine (Ado) in increasing concentrations to 3 genotypes in the C57BL/6 strain (A), 3 genotypes in the BALB/c strain (B), and the WT genotype of C57BL/6 and BALB/c strains (C). All changes in diameter are significantly different from 0 except where indicated by +. *Significant difference in the change in diameter between ALY and SCID within the same strain and the same drug concentration; α indicates a significant difference between SCID and WT within the same strain and the same drug concentration.
doses of ANG II whereby the C57BL/6WT strain showed a moderate vasodilatory response, whereas the BALB/cWT strain showed two opposing responses, one WT group that constricted and one that dilated in response to ANG II. The mechanism behind the divergent response within a single mouse strain is unknown and will require further investigation. Between the two mouse strains we investigated, female BALB/c mice have been shown to have more regulatory T cells than female C57BL/6 (10). In males, numerical differences in CD4+ and CD8+ T cells are present (Mouse Phenome Database; Jackson Laboratory, Bar Harbor, ME). C57BL/6 mice produce more IL-17, a proinflammatory cytokine, in response to allografts than BALB/c, suggesting a relatively larger Th17 cell population in C57BL/6. Although differences in lymphocyte numbers and subset heterogeneity may account for the differing responses to ANG II, we did not observe any altered responsiveness to Phe, Ado, or ACh, indicating that Th1 and Th2 bias may have limited impact on T cell modification of vascular reactivity. How might lymphocytes alter vascular responsiveness? Our data suggest that lymphocytes can act as circulating cells that can affect vasculature function similar to the way red blood cells circulate and release vasoactive factors (18, 28). In pathological situations, secretion of vasoactive cytokines and paracrine factors is the primary mechanism by which lymphocytes are thought to affect vascular reactivity. Cytokines are known to be both vasoactive and to modify responses to vasoactive compounds. Chronic IL-2 infusion inhibited ACh-induced vasodilations and reduced reactivity to hypercapnic constriction (17). A 20-h exposure to IFNγ reduced bradykinin-induced vasodilations and altered the bradykinin membrane receptor population (13, 14). TNF-α, IL-6, and IL-1 have effects on baseline diameter as well as affects on NE- and Phe-induced constrictions but under varying and different experimental conditions (6, 37). In contrast to these findings that mimick inflammatory responses, our data support the idea that lymphocyte-derived molecules have physiological actions on stable microvessels under non-antigen-stimulated conditions, similar to how uNK cell-derived IFNγ has a physiological role in promoting gestational structural change in implantation site arterioles (3). NK cells and T cells have been shown to have an intact renin-angiotensin system (29), T and B cells have an intact cholinergic system (30), and, recently, NK cells have been shown to produce atrial natriuretic factor (22). Further work is needed to determine whether these and other vasoactive products are released from lymphocytes under homeostatic conditions. Perspectives. The baseline diameter of unstimulated arterioles results from many inputs, nervous, humoral, and paracrine. Our data indicate that there is also input from lymphocytes. Lymphocytes are rich sources of cytokines and other mediators. These may be released from naïve cells and promote changes that contribute to the intracellular environment of endothelium or vascular smooth muscle, which sets the baseline from which the blood vessel responds to agonists. This may be the basis from which lymphocytes modify responses of the microvasculature, a responsiveness that can be altered by lymphocyte population shifts. Under non-agonist-stimulated conditions, lymphocytes tended to promote vasodilation. Under agonist challenge, NK cells generally promote constriction, whereas influences of T and B cells depend upon the stimulus. Therefore, our data provide evidence that lymphocytes or their products can physiologically influence the arteriolar microvascular and are important factors in determining vascular responsiveness. Given that the arteriolar resistance vasculature is a primary determinant of total peripheral resistance and therefore a determinant of mean arterial pressure, our data indicate that naïve lymphocytes may contribute, in part, to the regulation of blood pressure. GRANTS This research was funded by the Natural Sciences and Engineering Research Council of Canada, Ontario Ministry of Agriculture, Food and Rural Affairs, Canadian Institutes of Health Research (Grant no. 67956), and the Canada Research Chairs Program. DISCLOSURES No conflicts of interest are declared by the authors. REFERENCES 1. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 383: 787–793, 1996. 2. Ashkar AA, Croy BA. Interferon-gamma contributes to the normalcy of murine pregnancy. 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