Redox-Regulated Suppression of Splenic T-Lymphocyte Activation in a Model of Sympathoexcitation

Adam J. Case, Matthew C. Zimmerman

Abstract—Sympathoexcitation, increased circulating norepinephrine, and elevated levels of reactive oxygen species are driving forces underlying numerous cardiovascular diseases, including hypertension. However, the effects of elevated norepinephrine and subsequent reactive oxygen species production in splenic T-lymphocytes during hypertension are not currently understood. We hypothesized that increased systemic levels of norepinephrine inhibits the activation of splenic T-lymphocytes via redox signaling. To address this hypothesis, we examined the status of T-lymphocyte activation in spleens of a mouse model of sympathoexcitation-driven hypertension (ie, norepinephrine infusion). Splenic T-lymphocytes from norepinephrine-infused mice demonstrated decreased proliferation accompanied by a reduction in interferon gamma and tumor necrosis factor-\(\alpha\) production as compared with T-lymphocytes from saline-infused mice. Additionally, norepinephrine directly inhibited splenic T-lymphocyte proliferation and cytokine production ex vivo in a dose-dependent manner. Furthermore, norepinephrine caused an increase in G1 arrest in norepinephrine-treated T-lymphocytes, and this was accompanied by a decrease in pro-growth cyclin D3, E1, and E2 mRNA expression. Interestingly, norepinephrine caused an increase in cellular superoxide, which was shown to be partially causal to the inhibitory effects of norepinephrine, as antioxidant supplementation (ie, Tempol) to norepinephrine-infused mice moderately restored T-lymphocyte growth and proinflammatory cytokine production. Our findings indicate that suppression of splenic T-lymphocyte activation occurs in a norepinephrine-driven model of hypertension due to, at least in part, an increase in superoxide. We speculate that further understanding of how norepinephrine mediates its inhibitory effects on splenic T-lymphocytes may elucidate novel pathways for therapeutic mimicry to suppress T-lymphocyte-mediated inflammation in an array of diseases. (Hypertension. 2015;65:916-923. DOI: 10.1161/HYPERTENSIONAHA.114.05075.) • Online Data Supplement

Key Words: cardiovascular disease • hypertension • immunosuppression • inflammation • norepinephrine • reactive oxygen species • superoxide

O ver-activation of the sympathetic nervous system, or sympathoexcitation, is a hallmark of cardiovascular and cerebrovascular diseases, such as heart failure, stroke, and hypertension.\(^1-4\) Norepinephrine is the primary neurotransmitter of the sympathetic nervous system, and during times of chronic sympathoexcitation, circulating levels of norepinephrine may increase 2- to 6-fold over respective controls.\(^4-6\) Surges in both systemic circulating as well as localized norepinephrine can potentiate damage and stimulate reactive oxygen species (ROS) production in peripheral organs, such as heart, vasculature, and kidneys.\(^7\) However, although the immune system has been implicated as a potential contributor to cardiovascular diseases, such as hypertension,\(^8,9\) it remains unclear how increased sympathetic outflow affects the cell types that constitute this functional organ system.

The immune system, specifically T-lymphocytes, has been demonstrated to be a strong contributor to the complete hypertensive response.\(^8,9\) Intriguingly, T-lymphocytes express both \(\alpha\) and \(\beta\) adrenergic receptors, which suggest these cells may be subject to sympathetic control by norepinephrine\(^10\). In the current study, we address the effect of chronically elevated norepinephrine on T-lymphocytes in a model of sympathoexcitation-driven hypertension (ie, norepinephrine infusion). This model was selected to examine the effects of solely increased norepinephrine on splenic T-lymphocyte function and to eliminate the potential for confounding factors (eg, baroreflex suppression, salt disturbances, neurogenic feedback) that may be observed in other models of hypertension. Additionally, we focused specifically on splenic T-lymphocytes because of the unique property of the spleen being innervated by only catecholaminergic efferent nerve fibers.\(^11\) This specific and restricted innervation of the spleen has shown to be critical in limiting splenic-derived inflammation.
during a systemic immune response and further supports the potential for significant sympathetic regulation of splenic-derived T-lymphocytes. To date, the majority of studies examining T-lymphocyte activation in hypertension specifically focus their attention in cardiovascular organs (e.g., vasculature, kidney), which leaves the status of splenic T-lymphocyte activation unknown. Furthermore, the spleen is home to a substantial proportion of resting naïve T-lymphocytes that may not be actively contributing to the hypertensive phenotype, but would be essential in the immune response toward a secondary infection. Recent evidence suggests that chronic sympathoexcitation in the context of cardiovascular disease may be a predisposition to immunodeficiency, which warrants further examination into the effects of increased norepinephrine on this specific population of T-lymphocytes.

Herein, we tested the hypothesis that increased systemic levels of norepinephrine and consequent ROS production inhibits splenic T-lymphocytes from normal activation. Indeed, we show norepinephrine suppresses growth and cytokine production of splenic T-lymphocytes treated with norepinephrine both in vivo and ex vivo. Furthermore, we demonstrate the novel observation that these inhibitory effects are partially facilitated through the specific ROS, superoxide (O$_2^-$), after norepinephrine stimulation. Overall, this work suggests splenic T-lymphocytes are inhibited by norepinephrine during hypertension, and this suppression may have significant consequences on normal immune responses to secondary infections or insults.

**Methods**

A detailed description of the materials and methods can be found in the online-only Data Supplement.

**Mice**

All experiments were performed using male wild-type C57BL/6 inbred mice. Hypertension was induced by the subcutaneous infusion of norepinephrine (3.8 μg/kg/min) using osmotic mini-pumps for 14 days. Mean arterial pressure and heart rate were recorded using intraarterial telemetry devices in conscious unrestrained animals. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

**Results**

**Increased Circulating Norepinephrine In Vivo Inhibits Activation of Splenic T-Lymphocytes**

To model the effects of increased sympathetic drive, we used a mouse model subcutaneously infused with a dose of norepinephrine previously demonstrated to lead to the reported elevated levels of circulating norepinephrine during sympathoexcitation. After 2 weeks of norepinephrine infusion, urine norepinephrine levels increased ≈2 orders of magnitude from 4.4±1.5 ng/mL in saline-infused mice to 385.4±32.4 ng/mL in norepinephrine-infused mice, whereas steady-state plasma levels increased roughly 6-fold (saline, 0.8±0.1 ng/mL; norepinephrine, 4.6±1.0 ng/mL; Figure 1A). As previously reported in this animal model, norepinephrine infusion produced a significant, rapid, and consistent rise in mean arterial pressure that averaged 20.6±0.6 mm Hg on day 14 with no change in heart rate (Figure 1B). Because of the significant rise in systemic levels of norepinephrine and evident hypertension (hypertension defined by the Eighth Joint National Committee as a rise in mean arterial pressure ≥15 mm Hg) in our model, we examined splenic T-lymphocytes on day 14 of norepinephrine infusion. T-lymphocytes were cultured ex vivo and activated by CD3 stimulation (10 μg/mL; optimal activation dose identified in Figure S1A–S1D in Figure 1.

**Figure 1.** Norepinephrine (NE)-induced hypertension suppresses splenic T-lymphocyte activation. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. **A**, Urine and plasma NE levels 14 days after saline or NE infusion. N=5. **B**, Mean arterial pressure (MAP) and heart rate (HR) during 14 days of saline or NE infusion. Arrow indicates start of NE infusion. N=8. **C**, T-lymphocyte numbers at 0 to 48 hours of ex vivo culture with CD3 stimulation. T-lymphocytes were isolated on day 14 after the start of saline or NE infusion and plated for 48 hours with CD3 stimulation. N=4. **D**, Interferon gamma (IFNγ) and tumor necrosis factor-α (TNFα) levels in media at 0 to 48 hours of T-lymphocyte ex vivo culture. ND indicates nondetectable. N=4. *P<0.05 vs saline-infused.
the online-only Data Supplement) to understand the effects of norepinephrine infusion on early stages of T-lymphocyte activation. Total splenic T-lymphocytes isolated from mice on day 14 of norepinephrine infusion and cultured ex vivo under optimized CD3 stimulation demonstrated a 20%±5% decrease in cell numbers after 48 hours (Figure 1C). Moreover, we observed significant decreases in the proinflammatory cytokines interferon gamma and tumor necrosis factor-α at every time point beyond 24-hour post-plating (Figure 1D). Of note, T-lymphocyte CD28 costimulation has demonstrated importance in the perpetuation of hypertension. Because of this, we replicated these ex vivo experiments with the addition of 2 μg/mL soluble anti-CD28 antibody (optimal dose identified in Figure S1A–S1D) in addition to plate-bound anti-CD3. We observed similar decreases in cell numbers and proinflammatory cytokine levels from T-lymphocytes isolated from norepinephrine-infused animals independent of CD28 stimulation (Figure S2A and S2B). Last, we observed no change in the expression level of α or β adrenergic receptors with saline or norepinephrine-infusion (Figure S3). Overall, these data suggest that increased circulating norepinephrine in vivo reprograms splenic T-lymphocytes to an inhibitory state, which leads to a blunted response during canonical (ie, CD3±CD28) activation.

**Norepinephrine Directly Inactivates Naïve T-Lymphocytes**

To address the direct effects of norepinephrine on T-lymphocytes, we isolated splenic T-lymphocytes from unchallenged mice and cultured the cells as described above with increasing doses of norepinephrine. Similar to what we observed with T-lymphocytes from norepinephrine-infused animals, we detected a norepinephrine dose-dependent decrease in numbers of T-lymphocytes after 48 hours of culture with CD3±CD28 stimulation (Figure 2A; Figure S4B). Interferon gamma and tumor necrosis factor-α production per cell were also decreased by increasing doses of norepinephrine, with 10 μM norepinephrine producing similar inhibitory effects to what was observed with in vivo norepinephrine infusion (Figure 2B; Figure S4A). As such, our subsequent ex vivo experiments were performed using 10 μM norepinephrine. It should be noted that although the exact concentration of norepinephrine that splenic T-lymphocytes are exposed to is unknown, we posit that because of the synaptic terminals of catecholaminergic nerves terminating directly on the white pulp centers of the spleen that these T-lymphocytes are exposed to a significant amount of norepinephrine.

**T<sub>H</sub>2 Lymphocytes Are Significantly Increased With Norepinephrine Stimulation**

The decrease in excreted cytokines (ie, tumor necrosis factor-α and interferon gamma) from norepinephrine-treated T-lymphocytes could be as a result of an alteration in cellular function or polarization. To examine this, splenic T-lymphocytes from norepinephrine-infused animals were immunophenotyped. Total number of splenocytes was unchanged when comparing norepinephrine-infused and saline-infused spleens (Figure S5A), which demonstrates that increased circulating norepinephrine does not cause significant atrophy of the spleen. Furthermore, total spleen immunophenotyping showed no significant change in percentage of CD3+, CD4+, or CD8+ lymphocytes (Figures S5B, S6A, and S6B). Moreover, screening CD4+ T-lymphocytes for intracellular markers of polarization displayed no significant changes between norepinephrine and saline-infused spleens (Figures S5C and S6C). These findings strongly suggest that norepinephrine alters the internal function of T-lymphocytes before activation and polarization. Because of this, we examined the same immunophenotyping parameters on purified T-lymphocytes activated ex vivo via CD3 stimulation in the presence of norepinephrine. It was first observed that ex vivo culture of T-lymphocytes increased the percentage of CD8+ relative to CD4+ cells, but norepinephrine did not significantly affect this distribution (Figures S5D, S7A, and S7B). Additionally, although norepinephrine had no effect on the early polarization of T<sub>R<sub>reg</sub>, T<sub>H1</sub>, or T<sub>H17</sub> cells, it did significantly increase the proportion of T<sub>H2</sub> cells (Figures S5E and S7C), which are known to limit proinflammatory T<sub>H1</sub> differentiation. Taken together, these data indicate that...
norepinephrine does not affect T-lymphocyte polarization in vivo before activation and that ex vivo exposure to norepinephrine during initial (ie, 48 hours) CD3 activation may drive T<sub>P2</sub> differentiation. In addition, these results suggest that norepinephrine reprograms naïve splenic T-lymphocytes predisposing them to inhibited canonical activation and, as such, may influence their ability to function properly in the event of a secondary infection.

**Decreased T-Lymphocyte Numbers Are Caused by Cell Cycle Arrest**

The decrease in T-lymphocyte numbers observed with norepinephrine stimulation could be caused by either increased cell death or decreased cell proliferation. We first examined apoptosis using annexin V and propidium iodide staining. After 48 hours of norepinephrine treatment ex vivo with CD3±CD28 costimulation, no significant changes in annexin V or propidium iodide–positive T-lymphocytes were identified (Figure S8). These data suggest cell death is not a major contributor to the decrease in T-lymphocytes after norepinephrine stimulation. Next, we assessed the status of cell cycle progression in T-lymphocytes using the Krishan propidium iodide method.<sup>19</sup> Norepinephrine treatment led to an ≈20% increase in T-lymphocytes in G1 phase, while proportionally decreasing the number of cells in both S and G2 phase (Figure S9A and S9B). We also investigated cellular proliferation by carboxyfluorescein succinimidyl ester staining. Norepinephrine treatment ex vivo (±CD28 costimulation) significantly decreased the proportion of dividing cells and the proliferative index in both CD4+ and CD8+ lymphocytes (Figure S10A and S10B). To elucidate a possible mechanism behind the G1 arrested T-lymphocytes, we examined the mRNA expression level of the cyclin D and E families. These transcriptionally regulated proteins have been shown to be critical in the progression of the cell cycle from G1 to S phase.<sup>20</sup>

In T-lymphocytes from norepinephrine-infused animals, cyclin D3, E1, and E2 mRNA was significantly lower than saline-infused T-lymphocytes, with cyclin D1 and D2 remaining unchanged (Figure S11A). Moreover, steady-state mRNA levels of cyclins D3, E1, and E2 were also significantly reduced with direct stimulation of norepinephrine on splenic T-lymphocytes during ex vivo culture (Figure S11B). In summary, norepinephrine does not increase T-lymphocyte death, but limits cellular proliferation and arrests cell cycle progression at the G1–S checkpoint through a possible downregulation of specific cyclins.

**Norepinephrine Increases Steady-State Superoxide (O<sub>2</sub><sup>−</sup>) levels in T-Lymphocytes**

O<sub>2</sub><sup>−</sup> has been implicated as a primary signaling intermediate during norepinephrine stimulation in an array of cell types,<sup>20–23</sup> but its presence in norepinephrine-treated T-lymphocytes has not been explored. Using the O<sub>2</sub><sup>−</sup>-sensitive dye dihydroethidium and flow cytometry, we observed an approximate 70% increase in cellular O<sub>2</sub><sup>−</sup> levels in both CD4+ and CD8+ norepinephrine-treated T-lymphocytes ex vivo compared with control (Figure 3A and 3B). When examining T-lymphocytes from the in vivo sympathoexcitation model before culture, we observed a slight but nonsignificant increase (P=0.07) in cellular O<sub>2</sub><sup>−</sup> in T-lymphocytes from norepinephrine-infused mice compared with saline-infused (Figure 3C). However, once cultured ex vivo with CD3±CD28 stimulation, the T-lymphocytes from the norepinephrine-infused animals demonstrated a significant increase in steady-state O<sub>2</sub><sup>−</sup> levels compared with saline-infused, and this response was independent of T-lymphocyte subtype (Figure 3C; Figure S12A and S12B). Interestingly, although long-term infusion of norepinephrine did not significantly increase steady-state levels of O<sub>2</sub><sup>−</sup> in freshly isolated T-lymphocytes, the acute treatment (30 minutes) of splenic T-lymphocytes with norepinephrine did produce a significant increase in O<sub>2</sub><sup>−</sup> (Figure S12C). This observation of variable O<sub>2</sub><sup>−</sup> levels at different time points of norepinephrine-treatment suggests the potential for temporal control of steady-state O<sub>2</sub><sup>−</sup> flux or even ROS-induced ROS production in T-lymphocytes with norepinephrine stimulation.<sup>24</sup> Overall, norepinephrine causes an elevation in T-lymphocyte cellular O<sub>2</sub><sup>−</sup> levels, and these increases are correlated with a suppression of T-lymphocyte growth and proinflammatory cytokine production.

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**Figure 3.** O<sub>2</sub><sup>−</sup> is increased in norepinephrine (NE)-stimulated T-lymphocytes. T-lymphocytes were isolated from unchallenged (A and B) or saline/NE-infused (C; 3.8 µg/kg/min, day 14) mice and plated for 48 hours with CD3 stimulation. A, Representative dihydroethidium (DHE) flow cytometry analysis of CD4+ and CD8+ T-lymphocytes after 48 hours in ex vivo culture with 0 or 10 µM NE. B, Quantification of DHE oxidation in T-lymphocytes 48 hours after ex vivo culture with 0 or 10 µM NE. N=5. C, Quantification of DHE oxidation in T-lymphocytes from saline or NE-infused animals before and 48 hours after ex vivo culture. N=5. *P<0.05 vs 0 µM NE.
Superoxide-Specific Antioxidant Supplementation Rescues T-Lymphocytes From Norepinephrine-Mediated Inhibition

To identify a potential mechanistic role for $O_2^{\bullet-}$ in the norepinephrine-driven inhibition of T-lymphocytes, we treated mice with Tempol, a $O_2^{\bullet-}$-scavenging antioxidant, concurrently with norepinephrine infusion. T-lymphocytes isolated from mice administered Tempol demonstrated a complete rescue in growth when cultured ex vivo with CD3εC28 stimulation (Figure 4A; Figure S13B). In addition, levels of the proinflammatory cytokines interferon gamma and tumor necrosis factor-$\alpha$ were significantly increased with Tempol compared with norepinephrine infusion alone (Figure 4B; Figure S13A). In contrast, Tempol did not affect mean arterial pressure, suggesting that the observed partial rescue in the splenic T-lymphocytes was as a result of a potential redox mechanism as opposed to alleviation of the induced hypertension (Figure 4C). Indeed, although norepinephrine infusion increased dihydroethidium oxidation in T-lymphocytes after ex vivo culture, T-lymphocytes from Tempol-treated mice had attenuated dihydroethidium oxidation, suggesting decreased levels of cellular $O_2^{\bullet-}$ (Figure 4D; Figure S13C). Taken together, these data infer a partially causal role for increased $O_2^{\bullet-}$ in inducing the inhibitory effect of norepinephrine on splenic T-lymphocyte proliferation and cytokine production.

Discussion

Herein, we describe the potent inhibitory effect of norepinephrine on splenic T-lymphocytes in a model of sympathoexcitation-driven hypertension. Additionally, we show norepinephrine has a direct consequence in modulating the canonical activation of splenic T-lymphocytes. This direct effect is important in the context of hypertension because it has been demonstrated that angiotensin II, a prohypertensive peptide, does not have a significant direct effect on the modulation of T-lymphocyte activity.25 Norepinephrine effects on T-lymphocytes have been examined previously in other models of disease, but the consensus is conflicted as to the exact function of norepinephrine on T-lymphocyte development and activation.25 For example, our work confirms and extends that of others who show norepinephrine acts to suppress activation of naïve T-lymphocytes in unfractionated populations.26,27 Additionally, work using specific T-lymphocyte subsets, such as naïve CD4+ or CD8+ cells, has shown norepinephrine-mediated suppression of immune activity primarily mediated through $\beta_2$-adrenergic signaling.28,29 However, under different conditions, such as specific systemic infections, stress, or targeted T-lymphocyte differentiation, ex vivo norepinephrine has been shown to enhance the proinflammatory activation of T-lymphocytes.30–32 Although this evidence is inconsistent, numerous variables are at play in these studies that may explain the differences in conclusions.

First, although T-lymphocytes express both $\alpha$ and $\beta$ adrenergic receptors, specific isoforms of these receptors have been found in different quantities on T-lymphocyte subsets. For example, naïve T-lymphocytes highly express the

Figure 4. Scavenging of $O_2^{\bullet-}$ rescues the inhibitory phenotype of T-lymphocytes from norepinephrine (NE)-infused animals. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. Drinking water was supplemented with 1 mmol/L Tempol (Temp) 5 days prior and throughout the entire infusion. T-lymphocytes were isolated on day 14 after the start of saline or NE infusion and cultured for 48 hours with CD3ε stimulation. A, T-lymphocyte numbers at 0 to 48 hours of ex vivo culture. N=4. B, Interferon gamma (IFN-$\gamma$) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) levels in media at 0 to 48 hours of T-lymphocyte ex vivo culture. ND indicates nondetectable. N=4. C, Mean arterial pressure (MAP) during 14 days of saline or NE infusion with Tempol supplementation. Arrow indicates start of NE infusion. N=4. D, Dihydroethidium (DHE) oxidation in T-lymphocytes before and after 48 hours ex vivo culture. N=4. *$P<0.05$ vs saline-infused. **$P<0.05$ vs NE-infused.
β2-adrenergic receptor, and our data suggest that stimulation with norepinephrine during T-lymphocyte activation predisposes differentiation to the TH2 lineage. Notably, it has been observed that T_h2-differentiated, but not T_h1-differentiated, CD4+ T-lymphocytes lack the β2-adrenergic receptor and, thus, may be a mechanism to become resistant to this catecholamine once polarized. Understanding that norepinephrine demonstrates differential immunomodulatory effects on different subclasses, mixtures, and stages of differentiated T-lymphocytes increases the likelihood of conflicting results between different experimental setups. Second, a temporal and developmental component of norepinephrine stimulation may be critical in the phenotype rendered in T-lymphocytes. In one clinical study, short-term administration of norepinephrine augmented the number of CD8+ circulating T-lymphocytes, but long-term administration resulted in decreased T-lymphocyte numbers. Additionally, the majority of evidence, including our data presented herein, suggests that naïve unchallenged T-lymphocytes are suppressed by norepinephrine stimulation, but the functionality of preexisting activated T-lymphocytes (eg, during infection) is exacerbated. Finally, a spatial component of norepinephrine-mediated effects on T-lymphocytes may also be at play. We demonstrate that T-lymphocytes in the spleen, a catecholaminergic-innervated lymphoid organ, are suppressed by norepinephrine infusion in vivo. In contrast, Marvar et al demonstrated increased T-lymphocyte numbers and activation in the aorta of norepinephrine-infused mice. Although appearing conflicting, both situations of T-lymphocyte activation and inhibition are most likely occurring concurrently, but in different locations. This hypothesis would be supported if specific T-lymphocytes (ie, vasculature or renal positioned) were activated before the increased sympathoexcitation and norepinephrine outflow associated with hypertension. Under these circumstances, norepinephrine would potentiate the effects of the activated T-lymphocytes in these cardiovascular-related organs, but suppress the inactivated naïve T-lymphocytes located in the spleen. As such, it is tempting to speculate that the norepinephrine-mediated inhibition of splenic T-lymphocytes, as we observed, is a compensatory mechanism attempting to inhibit resting T-lymphocytes so that they cannot further add to the inflammation contributing to the hypertension. Overall, further research examining more detailed parameters, such as specific T-lymphocyte subsets, timing of norepinephrine administration, and organ-specific effects of norepinephrine on T-lymphocytes, are highly warranted in the context of various models of hypertension.

Current dogma suggests that hypertension is a systemic inflammatory disease, whereas our data implies that not all peripheral organs demonstrate increased inflammation. That is, we demonstrate that T-lymphocytes in the spleens of norepinephrine-driven hypertensive mice show no signs of increased inflammatory parameters and, in fact, are significantly suppressed. These findings are consistent with the observation that hypertensive mice and humans do not exhibit constitutional symptoms (eg, fever, malaise, myalgia) associated with increased systemic immune activation and circulating proinflammatory cytokines. These observations further support the notion that hypertension leads to a site-specific (eg, vascular or renal) and localized activation of T-lymphocyte inflammation as opposed to systemic. In this manner, although administration of systemic immunosuppressants may be indicated as a possible therapy for hypertension, it may further the sympathoexcitation-mediated immunocompromised state in lymphoid organs, such as the spleen, and predispose hypertensive patients to secondary infections. In summary, we present evidence that T-lymphocytes are not uniformly activated during hypertension, and this finding may preclude systemic targeting of the immune system for hypertension therapy.

Finally, we observe the novel finding that O_2^- is increased with norepinephrine stimulation of T-lymphocytes and that increased O_2^- scavenging via Tempol significantly restores the original proinflammatory potential of the cells, but does not decrease the elevated blood pressure in norepinephrine-infused mice. We interpret these findings to mean that O_2^- is a partial mediator of the inhibitory phenotype in T-lymphocytes exposed to norepinephrine, and this inhibition is not due solely to changes in blood pressure. ROS have become well accepted as intracellular signaling molecules and play a primary role in various cell types during hypertension. In fact, alterations in the redox environment have been directly linked to changes in cell cycle regulation similar to what we have observed in our norepinephrine-stimulated T-lymphocytes. Furthermore, norepinephrine has been shown to increase levels of ROS in an array of cell types, but until now had not been examined in T-lymphocytes. Studies are currently underway in our laboratory, which are designed to aid in the further understanding of specific redox-sensitive intracellular signaling pathways affected by norepinephrine-mediated ROS production. Additionally, we observed that a norepinephrine-induced increase in O_2^- leads to a suppression of immune function, whereas others have observed angiotensin II-induced escalations in O_2^- causing enhanced lymphocyte inflammation. These findings fully support the notion that not all ROS-inducing events are created equal and, in fact, may have specific intracellular signaling pathways and subcellular molecules in which they target. Together, these observations may explain why antioxidant therapy has demonstrated minimal clinical success for diseases, such as hypertension, as global targeting of ROS may inhibit both pro- and anti-inflammatory pathways throughout the body.

Perspectives

In recent years, research examining how the immune system contributes to hypertension has grown exponentially, and it has become mostly accepted that the immune system systemically is contributing to the hypertensive phenotype. However, our findings support a hypertension model of site-specific and localized inflammation as opposed to systemic. More specifically, we have elucidated that increased systemic levels of norepinephrine in a mouse model of sympathoexcitation-driven hypertension directly desensitizes splenic T-lymphocytes to canonical activation, whereas in this same model, it has previously been shown that vascular and renal T-lymphocytes are activated. Furthermore,
we report that the inhibitory effects of norepinephrine on T-lymphocytes are mediated in part by increased steady-state $\cdot O_2^*$ flux, as increased $\cdot O_2^*$-scavenging significantly restores the original proinflammatory potential of the cells. We speculate that further understanding of how norepinephrine mediates its effects on organ-specific localized T-lymphocytes may elucidate novel pathways for therapeutic mimicry to modulate T-lymphocyte-mediated inflammation in various pathologies, including hypertension.

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**Novelty and Significance**

**What Is New?**
- The majority of recent research examining inflammation in hypertension has primarily focused on how T-lymphocytes contribute to elevated blood pressure and end organ damage. Herein, we present new data showing that increased levels of norepinephrine in a model of sympathoexcitation-driven hypertension inhibit splenic-derived T-lymphocytes activity and the redox-mediated regulation of this inhibition.

**What Is Relevant?**
- T-lymphocytes localized to the vasculature and kidneys have been demonstrated to have proinflammatory effects that exacerbate the hypertensive phenotype, but the status of splenic T-lymphocytes in hypertension remains unclear. We demonstrate that splenic T-lymphocytes exposed to elevated levels of circulating norepinephrine are blunted in growth and cytokine production upon canonical activation. Furthermore, norepinephrine increases levels of splenic T-lymphocyte superoxide, which we show is mechanistic in the inhibitory phenotype of these immune cells during sympathoexcitation.

**Summary**

T-lymphocytes from the spleens of hypertensive animals are subject to activation-suppression by increased circulating levels of norepinephrine in a redox-dependent manner.
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REDOX-REGULATED SUPPRESSION OF SPLENIC T-LYMPHOCYTE ACTIVATION
IN A MODEL OF SYMPATHOEXCITATION

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Materials and Methods

Mice. All experiments were performed using male wild-type C57BL/6 inbred mice (C57BL/6NHsd, age 8-12 weeks, weight 20-25 g) purchased from Harlan Laboratories (Indianapolis, IN). For infusion studies, mice were implanted subcutaneously with osmotic minipumps (Alzet #1002, Durect Corporation, Cupertino, CA) delivering either norepinephrine (NE; 3.8 μg/kg/min; Sigma #A7256, St. Louis, MO) or saline for 14 days. Anesthetized mice were shaved on the left midclavicular line, and surgical site was sterilized using iodine solution (Fisher Thermo Scientific #NC9833401, Waltham, MA) followed by 70% ethanol. A superficial incision was made and the subcutaneous tissue expanded for pump implantation. Following pump implantation, incision site was sutured using 6-0 prolene sutures (Ethicon #8697G, New Brunswick, NJ) and cleaned with iodine solution. Blood pressure recordings were performed using intra-carotid arterial catheters attached to radiotelemeters for direct measurement of mean arterial pressure and heart rate in conscious unrestrained animals. Briefly, for this surgery anesthetized mice were shaved in the right supraclavicular region, and surgical site sterilized using iodine solution followed by 70% ethanol. A neck incision was made to isolate the left carotid artery, which was clamped prior to severing. The artery was tied off using 7-0 silk sutures (Braintree Scientific #SUT-S 103, Braintree, MA) superior to the incision, while the inferior portion was fitted and secured with a sterile catheter of the telemetry recording device (Data Sciences International #PA-C10, Minneapolis, MN). Catheters and recording devices were secured subcutaneously, and incision site was sutured using 6-0 prolene sutures. Mice were allowed one-week recovery after telemeter implantation prior to any further experimentation. Hemodynamic recordings were performed for 20 seconds every minute for 2 hours daily for the duration of the experiment. Averages of mean arterial pressure and heart rate were calculated daily over the 2 hour period when the mice displayed minimal activity. For antioxidant supplementation, 1 mM Tempol (Enzo Life Sciences #ALX-430-081, Farmingdale, NY) was added to the drinking water (ad libitum) of mice. Several reports have demonstrated this dose shows increased O₂*-scavenging in various cardiovascular disease-related systems²-⁶. Sucrose (4 g/100 mL; Fisher Thermo Scientific #S25590A, Waltham, MA) was added to both control and Tempol water bottles to offset the taste of Tempol, and water bottles were made fresh every 3 days. Tempol was added to drinking water 5 days prior to minipump implantation. Mice were given access to standard chow (Teklad Laboratory Diet #8656, Harlan Laboratories, Madison, WI) ad libitum. For non-Tempol experiments, mice were given access to untreated water ad libitum. For all surgical procedures, mice were anesthetized using 0.5-2% isoflurane supplemented with 1 liter/min oxygen. Bupivacaine (0.5% solution) was used as postsurgical anesthetic, and mice were monitored daily post-operation for signs of illness or infection. Mice were euthanized by pentobarbital overdose (5.85 mg bolus, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) administered intraperitoneally. All procedures were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.
It should be noted we chose our model of NE-infusion (3.8 μg/kg/min) to mimic increased sympathoexcitation, which occurs in an array of diseases related to cardiovascular disease (e.g. stroke, heart failure, and hypertension) as well as other diseases (e.g. stress, psychological disorders, or cancer). In both animal models and human disease, sympathoexcitation may increase plasma NE levels anywhere from 2-6 fold\textsuperscript{7-9}. In our model, we observe an approximate 6-fold increase in plasma levels of NE which is in the range of observed values in models of sympathoexcitation.

*T-lymphocyte isolation and ex vivo culture.* Spleens from mice were dissected, physically disrupted into single cell suspensions using ground glass slides (Fisher Thermo Scientific #6684h61, Waltham, MA), and run through 70 μM nylon mesh filters (Fisher Thermo Scientific #352350, Waltham, MA) to remove large debris. Red blood cell lysis buffer (15.5 mM NH₄Cl, 1 mM KHCO₃, 10 μM EDTA) was used to remove contaminating erythrocytes. After a second filtering through 70 μM nylon mesh, T-lymphocytes were sorted and collected using the Pan T-cell Isolation Kit II (Miltenyi Biotec #130-095-130, San Diego, CA) and an AutoMACS magnetic cell separator (Miltenyi Biotec, San Diego, CA). For ex vivo culture, T-lymphocytes were seeded on tissue culture plates that had been coated using a solution of 0, 0.1, 1.0, or 10 μg/mL anti-CD3ε antibody (eBioscience clone 145-2C11, #16-0031-086, San Diego, CA) overnight at 4°C. In specific wells, 0, 1, or 2 μg/mL anti-CD28 antibody (eBioscience clone 37.51, #16-0281-85, San Diego, CA) was added to media. NE or saline (vehicle) were added at time of plating, and 24 hours after plating. T-lymphocytes were cultured using RPMI Medium 1640 without phenol red (Gibco #11835-030, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals #S11150, Lawrenceville, GA), 2 mM L-glutamine (Hyclone #SH30034.02, Waltham, MA), 10 mM HEPES (Fisher Thermo Scientific #BP299, Waltham, MA), 1% Penicillin/Streptomycin (Gibco #15140-122, Grand Island, NY), and 50 μM β-mercaptoethanol (Sigma-Aldrich #M6250, St. Louis, MO). At time of analysis, cells were scrape harvested, removed by centrifugation, and counted using size exclusion on a Beckman Coulter counter. Media was used for cytokine analysis.

*Cytokine and NE quantification.* On-plate ELISA’s utilizing respective standard curves were used for quantification of cytokines and NE. All ELISA’s were purchased as premade kits, and were followed as per manufacturers’ instructions. The following kits were used: tumor necrosis factor alpha (TNFα; eBioscience #88-7324, San Diego, CA), interferon gamma (IFNγ; eBioscience #88-7314, San Diego, CA), and NE (Rocky Mountain Diagnostics #BA E-6200, Colorado Springs, CO).

*T-lymphocyte immunophenotyping.* T-lymphocytes were resuspended in ice cold staining buffer (Phosphate buffered saline + 10% FBS). Cells were blocked using anti-CD16/CD32 antibody (BD Biosciences clone 2.4G2, #553141, San Jose, CA) prior to staining. A dose of 1.0 μg/mL of FITC-CD3 (BD Biosciences clone 17A2, #561798, San Jose, CA), PE-CD4 (BD Biosciences clone GK1.5, #557308, San Jose, CA), and APC-CD8a (BD Biosciences clone 53-6.7, #553035, San Jose, CA) were added and incubated at 4°C for 30 min. Cells were washed thrice using ice cold staining buffer.
followed by fixation in 1% paraformaldehyde (Sigma-Aldrich #P6148, St. Louis, MO). For intracellular staining, T-lymphocytes were first blocked with anti-CD16/CD32 antibody and then stained with AF488-CD4 (eBioscience clone GK1.5, #53-0041-82, San Diego, CA) in cold staining buffer. Cells were then fixed and permeabilized using a standard kit (eBioscience #00-5523-00, San Diego, CA). Normal rat serum (eBioscience #24-5555-94, San Diego, CA) was used as a secondary blocking agent prior to intracellular staining. A dose of 1.0 μg/mL of AF647-Foxp3 (BD Biosciences clone R16-715, #563486, San Jose, CA), PECF594-IFNγ (BD Biosciences clone XMG1.2, #562333, San Jose, CA), BV421-IL-4 (BD Biosciences clone 11B11, #562915, San Jose, CA), and AF700-IL-17a (BD Biosciences clone TC11-18H10, #560820, San Jose, CA) were added and incubated at 4°C for 30 min. Cells were washed thrice using ice cold staining buffer prior to analysis. T-lymphocytes were analyzed by flow cytometry on a FACSCalibur or LSRII Green Laser (Becton Dickinson, Franklin Lakes, NJ) and quantified using FlowJo cytometric analysis software (Tree Star, Ashland, OR).

Apoptosis, proliferation, cell cycle, and superoxide analysis. For apoptosis analysis, the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies #V13241, Grand Island, NY) was used as per manufacturer’s instructions on cells 48 hours post-plating. For proliferation, carboxyfluorescein succinimidyl ester (CFSE) was added to cells at time of isolation prior to plating for activation. Cells were harvested at 48 hours post-plating, labeled with appropriate CD4 and CD8 antibodies, and analyzed. For cell cycle analysis, an adapted Krishan protocol was applied. Briefly, T-lymphocytes were resuspended for 20 minutes in Krishan cell cycle buffer, which consists of 0.05 mg/mL propidium iodide (PI; Sigma-Aldrich #P4170, St. Louis, MO), 0.10% sodium citrate (Fisher Thermo Scientific #BP327, Waltham, MA), 0.03% nonidet P-40 (NP-40; Amresco #E109, Solon, OH), and 0.02 mg/mL RNaseA (Invitrogen #12091-021, Grand Island, NY). For superoxide measurement, T-lymphocytes were resuspended in serum-free, phenol red-free culture media (see aforementioned media recipe) with 10 μM dihydroethidium (DHE; VWR #101447-534, Chicago, IL) and appropriate CD4 and CD8 antibodies for 30 min at 37°C. All analyses were run on a FACSCalibur or LSRII Green Laser, and quantified using FlowJo or ModFit cytometric analysis software.

RNA extraction, cDNA production, and quantitative real-time RT-PCR. Total RNA was extracted from purified T-lymphocytes using the RNAeasy mini kit (Qiagen # 74104, Valencia, CA) according to the manufacturer’s protocol. Concentration of RNA was determined spectrophotometrically using a Nanodrop 2000 Spectrophotometer (Fisher Thermo Scientific, Waltham, MA). The high capacity cDNA archive kit (Applied Biosystems #4368813, Grand Island, NY) was used to obtain cDNA from total RNA. Generated cDNA was then subjected to SYBR green (Applied Biosystems #4385612, Grand Island, NY) quantitative real-time PCR with primers specific to the coding sequence of the respective genes. PCR product specificity was determined by thermal dissociation. A threshold in the linear range of PCR amplification was selected and the cycle threshold (Ct) determined. Levels of transcripts were then normalized to the 18s loading control and compared relative to the control sample (saline-infused or treated T-lymphocytes) using the 2^ΔΔCt method. Experiments were run in triplicate and data
expressed as percent change of control. Primer sequences for specific genes were as follows: 18s forward, 5'-gccgaaggcttactttga-3'; 18s reverse, 5'-catgcctccagttccgaa-3'; Cyclin D1 forward, 5'-catccatgcggaaatcg-3'; Cyclin D1 reverse, 5'-cagggcgtctttcataa-3'; Cyclin D2 forward, 5'-gtgcaagaagcacatccaaacc-3'; Cyclin D2 reverse, 5'-ctcagacatcttacagcag-3'; Cyclin D3 forward, 5'-acttgatgtggagttgttg-3'; Cyclin D3 reverse, 5'-ccagggagtccatagcagagg-3'; Cyclin E1 forward, 5'-caagtggcttacagtaac-3'; Cyclin E1 reverse, 5'-gacgcacaggtcagatcagtc-3'; Cyclin E2 forward, 5'-cacccccataagataagaaaca-3'; Cyclin E2 reverse, 5'-ctgtgaacaggtcagatcagtc-3'; α1a forward, 5'-gtgggaagttcgtcctc-3'; α1a reverse, 5'-ggcttgaaattcgggaaga-3'; α1b forward, 5'-taaagtcaacggtgctcctcact-3'; α1b reverse, 5'-gggtgagagcagttgttg-3'; α1d forward, 5'-gttttcgctcttggtttggt-3'; α1d reverse, 5'-ttggaagacgccctctgtg-3'; α2a forward, 5'-ttggaagacgcccttctctc-3'; α2a reverse, 5'-cttcctcctgtggcctc-3'; α2b forward, 5'-taaagtcaacggtcagatcagtc-3'; α2b reverse, 5'-gggtgagagcagttgttg-3'; α2c forward, 5'-gtgggaagttcgtcctcact-3'; α2c reverse, 5'-ggcttgaaattcgggaaga-3'; β1 forward, 5'-ccattggtggtattcagtc-3'; β1 reverse, 5'-gggtgagagcagttgttg-3'; β2 forward, 5'-tgctatcataacagggctcttc-3'; β2 reverse, 5'-accctcgggcctttctttc-3'; β3 forward, 5'-ctcctcctgtccttctgtg-3'; β3 reverse, 5'-ttcataacagggctctttc-3'.

Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM). Assessments of blood pressure and heart rate were performed using 1-way ANOVA followed by Newman-Keuls post hoc test when significance was observed. For two group comparisons, Student's t-test was used. For multiple comparisons, 2-way ANOVA followed by Bonferroni correction was used. GraphPad Prism 5.0 statistical and graphing software was used for all analyses. Differences were considered significant at p<0.05.
References


Figure S1. NE suppression occurs during sub-optimal and optimal CD3 and CD28 T-lymphocyte activation. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with increasing doses of CD3 and CD28 stimulation with 0 or 10 μM NE. A. T-lymphocyte cell counts 0-48 hours with 0 μg/mL CD3. N=4. B. T-lymphocyte cell counts 0-48 hours with 0.1 μg/mL CD3. N=4. C. T-lymphocyte cell counts 0-48 hours with 1.0 μg/mL CD3. N=4. D. T-lymphocyte cell counts 0-48 hours with 10 μg/mL CD3. N=4. *p<0.05 vs. 0 μM NE.
Figure S2. *In vivo* NE-induced suppression of T-lymphocytes persists with CD28 co-stimulation. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. T-lymphocytes were isolated on day 14 after the start of saline or NE infusion and cultured for 48 hours with CD3 and CD28 stimulation. A. T-lymphocyte numbers at 0-48 hours of *ex vivo* culture with CD3 and CD28 stimulation. N=4. B. IFNγ and TNFα levels in media at 0-48 hours of T-lymphocyte *ex vivo* culture with CD3 and CD28 stimulation. ND indicates non-detectable. N=4. *p<0.05 vs. saline-infused.
Figure S3. NE-infusion does not alter T-lymphocyte adrenergic receptor expression. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. T-lymphocytes were isolated on day 14, and RNA was immediately isolated. Data show quantitative real-time RT-PCR analysis of various adrenergic receptor mRNA. Data are normalized to saline-infused within a respective adrenergic receptor mRNA. ND indicates non-detectable.
Figure S4. CD28 co-stimulation does not abrogate the direct NE inhibitory effects on T-lymphocytes. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 and CD28 stimulation with increasing doses of NE. A. IFNγ and TNFα levels in media at 0-48 hours of T-lymphocyte ex vivo culture with CD3 and CD28 stimulation. ND indicates non-detectable. N=4. B. T-lymphocyte numbers at 0-48 hours of ex vivo culture with CD3 and CD28 stimulation. N=4. *p<0.05 vs. 0 μM NE.
Figure S5. NE increases T<sub>h</sub>2 polarization during early activation. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days (A-C) or T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 stimulation with 0 or 10 μM NE (D-E). A. Total splenocytes 14 days after saline or NE infusion. N=7. B. Immunophenotype of surface markers for major T-lymphocyte subclasses from splenocytes isolated on day 14 of saline or NE infusion. N=6. C. Immunophenotype of intracellular markers of specific CD4<sup>+</sup> T-lymphocyte subsets on day 14 of saline or NE infusion. N=6. D. Immunophenotype of surface markers for major T-lymphocyte subclasses before and 48 hours after ex vivo culture. E. Immunophenotype of intracellular markers of specific CD4<sup>+</sup> T-lymphocyte subsets 48 hours after ex vivo culture. N=6. *p<0.05 vs. 0 μM NE. **p<0.05 vs. Before Culture T-lymphocytes.
Figure S6. NE infusion does not alter T-lymphocyte polarization in vivo. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. T-lymphocytes were isolated on day 14, and immediately analyzed for T-lymphocyte markers. A. Representative forward and side scatter distribution of saline or NE-infused splenocytes. B. Representative CD4+ distribution of saline or NE-infused splenocytes. C. Representative T_{reg} (CD4+, Foxp3+), T_{h1} (CD4+, IFNγ+), T_{h2} (CD4+, IL-4+), and T_{h17} (CD4+, IL-17a+) distributions of saline or NE-infused splenocytes. Fluorescence minus one (FMO) indicates appropriate gating control in which all fluorescent antibodies are added minus the antibody being queried.
Figure S7. NE initiates TH2 polarization in early activated T-lymphocytes. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 stimulation and 0 or 10 μM NE. A. Representative forward and side scatter distribution of 0 or 10 μM NE-treated T-lymphocytes after 48 hours. B. Representative CD4+ distribution of 0 or 10 μM NE-treated T-lymphocytes after 48 hours. C. Representative T_reg (CD4+, Foxp3+), T_H1 (CD4+, IFNγ+), T_H2 (CD4+, IL-4+), and T_H17 (CD4+, IL-17a+) distributions of 0 or 10 μM NE-treated CD4+ T-lymphocytes after 48 hours. Fluorescence minus one (FMO) indicates appropriate gating control in which all fluorescent antibodies are added minus the antibody being queried.
Figure S8. NE does not increase T-lymphocyte apoptosis. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 (±CD28) stimulation and 0 or 10 μM NE. A. Representative annexin V (AV) and propidium iodide (PI) scatter plot of T-lymphocytes after 48 hours in ex vivo culture. B. Quantification of apoptosis assay from T-lymphocytes after 48 hours in ex vivo culture with CD3 (±CD28) stimulation. N=5.
Figure S9. NE inhibits cell cycle progression. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 (±CD28) stimulation and 0 or 10 μM NE. **A.** Representative Krishan PI cell cycle analysis of T-lymphocytes after 48 hours in *ex vivo* culture. **B.** Quantification of cell cycle assay from T-lymphocytes after 48 hours in *ex vivo* culture with CD3 (±CD28) stimulation. N=5. *p<0.05 vs. 0 μM NE.*
Figure S10. NE inhibits T-lymphocyte proliferation. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 (±CD28) stimulation and 0 or 10 μM NE. A. Representative CFSE modeling of T-lymphocytes after 48 hours in ex vivo culture. P0-P4 represents population doublings as CFSE fluorescence intensity diminishes with cell replication, while grey histogram displays unstimulated (freshly isolated T-lymphocytes) P0 control. B. Quantification of CFSE assay from T-lymphocytes after 48 hours in ex vivo culture with CD3 (±CD28) stimulation. N=4. *p<0.05 vs. 0 μM NE.
Figure S11. NE decreases cyclin expression. A. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. T-lymphocytes were isolated on day 14, and RNA was immediately isolated. Quantitative real-time RT-PCR analysis of various cyclin mRNA in T-lymphocytes isolated on day 14 after the start of saline or NE infusion. Data are normalized to saline-infused within a respective cyclin mRNA. N=5. B. T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 (±CD28) stimulation and 0 or 10 μM NE. Quantitative real-time RT-PCR analysis of various cyclin mRNA in T-lymphocytes after 48 hours ex vivo culture. Data are normalized to freshly isolated T-lymphocytes prior to ex vivo culture (Fresh) within a respective cyclin mRNA. N=5. *p<0.05 vs. 0 μM NE. Ψp<0.05 vs. Fresh.
Figure S12. Superoxide (O2−) is increased with NE-stimulation in T-lymphocytes. T-lymphocytes were isolated from unchallenged (A) or saline/NE-infused (B; 3.8 μg/kg/min – day 14) mice and plated for 48 hours with CD3 and CD28 stimulation. A. Quantification of dihydroethidium (DHE) oxidation in T-lymphocytes 48 hours after ex vivo culture with 0 or 10 μM NE. N=5. B. Quantification of DHE oxidation in T-lymphocytes from saline or NE-infused animals before and 48 hours after ex vivo culture. N=5. C. T-lymphocytes were isolated from unchallenged mice and were immediately treated with 0 or 10 μM NE for 30 minutes in the presence of DHE. Quantification of DHE oxidation in freshly isolated T-lymphocytes acutely treated with 0 or 10 μM NE. N=5. *p<0.05 vs. 0 μM NE.
Figure S13. Anti-oxidant supplementation rescues the NE-inhibitory effect with CD28 co-activation. Mice were infused with saline or NE (3.8 μg/kg/min) for 14 days. Drinking water was supplemented with 1 mM Tempol (Temp) 5 days prior and throughout the entire infusion. T-lymphocytes were isolated on day 14 after the start of saline or NE infusion and cultured for 48 hours with CD3 and CD28 stimulation. A. IFNγ and TNFα levels in media at 0-48 hours of T-lymphocyte ex vivo culture. ND indicates non-detectable. N=4. B. T-lymphocyte numbers at 0-48 hours of ex vivo culture. N=4. C. Dihydroethidium (DHE) oxidation in T-lymphocytes before and after 48 hours ex vivo culture. N=4. *p<0.05 vs. saline-infused. Φp<0.05 vs. NE-infused.