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REDOX-REGULATED SUPPRESSION OF SPLENIC T-LYMPHOCYTE ACTIVATION IN A MODEL OF SYMPATHOEXCITATION

Adam J. Case, PhD[1] and **Matthew C. Zimmerman, PhD**[1,2]

[1]Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE

[2]Redox Biology Center, University of Nebraska Lincoln, Lincoln, NE

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 (*i.e.* 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 to 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 (*i.e.* Tempol) to norepinephrine-infused mice moderately restored T-lymphocyte growth and pro-inflammatory 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.

Mail Correspondence To: Matthew C. Zimmerman, Ph.D., Associate Professor, Director, Free Radicals in Medicine Program, Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5850, Phone: 402-559-7842, Fax: 402-559-4438, mczimmerman@unmc.edu.

Conflict of Interest/Disclosures

None

Keywords

Hypertension; Reactive Oxygen Species; Superoxide; Norepinephrine; Immunosuppression; Cardiovascular Disease; Inflammation

Introduction

Over-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 (NE) is the primary neurotransmitter of the sympathetic nervous system, and during times of chronic sympathoexcitation circulating levels of NE may increase 2–6 fold over respective controls[4–6]. Surges in both systemic circulating as well as localized NE can potentiate damage and stimulate reactive oxygen species (ROS) production in peripheral organs such as heart, vasculature, and kidneys[7]. However, while 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 impacts the cell types that constitute this functional organ system.

The immune system, specifically T-lymphocytes, have been demonstrated to be a strong contributor to the complete hypertensive response[8, 9]. Intriguingly, T-lymphocytes express both α and β adrenergic receptors, which suggest these cells may be subject to sympathetic control by NE[10]. In the current study, we address the effect of chronically elevated NE on T-lymphocytes in a model of sympathoexcitation-driven hypertension (*i.e.* NE infusion). This model was selected to examine the effects of solely increased NE on splenic T-lymphocyte function, and to eliminate the potential for confounding factors (*e.g.* baroreflex suppression, salt disturbances, neurogenic feedback) that may be observed in other models of hypertension. Additionally, we focused specifically on splenic T-lymphocytes due to 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[12]. 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 towards a secondary infection. Recent evidence suggests that chronic sympathoexcitation in the context of cardiovascular disease may be a predisposition to immunodeficiency[4, 13–15], which warrants further examination into the effects of increased NE on this specific population of T-lymphocytes.

Herein, we tested the hypothesis that increased systemic levels of NE and consequent ROS production inhibits splenic T-lymphocytes from normal activation. Indeed, we show NE suppresses growth and cytokine production of splenic T-lymphocytes treated with NE both *in vivo* and *ex vivo*. Furthermore, we demonstrate the novel observation that these inhibitory

effects are partially facilitated through the specific ROS, superoxide ($O_2^{\bullet -}$), after NE stimulation. Overall, this work suggests splenic T-lymphocytes are inhibited by NE 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 Data Supplement.

Mice

All experiments were performed using male wild-type C57BL/6 inbred mice. Hypertension was induced by the subcutaneous infusion of NE (3.8 μg/kg/min)[16] using osmotic mini-pumps for 14 days. Mean arterial pressure and heart rate were recorded using intra-arterial 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 NE 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 NE previously demonstrated to lead to the reported elevated levels of circulating NE during sympathoexcitation[16]. After two weeks of NE infusion, urine NE levels increased approximately two orders of magnitude from 4.4 ± 1.5 ng/mL in saline-infused mice to 385.4 ± 32.4 ng/mL in NE-infused mice, while steady-state plasma levels increased roughly 6-fold (saline: 0.8 ± 0.1 ng/mL; NE: 4.6 ± 1.0 ng/mL) (Figure 1A). As previously reported in this animal model, NE infusion produced a significant, rapid, and consistent rise in mean arterial pressure (MAP) that averaged 20.6 ± 0.6 mmHg on day 14 with no change in heart rate (Figure 1B). Because of the significant rise in systemic levels of NE and evident hypertension (hypertension defined by the Eighth Joint National Committee as a rise in MAP ≥15 mmHg[17]) in our model, we examined splenic T-lymphocytes on day 14 of NE infusion. T-lymphocytes were cultured *ex vivo* and activated by CD3 stimulation (10 μg/mL; optimal activation dose identified in Figure S1A–D) to understand the effects of NE infusion on early stages of T-lymphocyte activation. Total splenic T-lymphocytes isolated from mice on day 14 of NE 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 pro-inflammatory cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) at every time point beyond 24-hour post-plating (Figure 1D). Of note, T-lymphocyte CD28 co-stimulation has demonstrated importance in the perpetuation of hypertension[18]. Due to this, we replicated these *ex vivo* experiments with the addition of 2 μg/mL soluble anti-CD28 antibody (optimal dose identified in Figure S1A–D) in addition to plate-bound anti-CD3. We observed similar decreases in cell numbers and pro-inflammatory cytokine levels from T-lymphocytes isolated from NE-infused animals independent of CD28 stimulation (Figure

S2A–B). Last, we observed no change in the expression level of α or β adrenergic receptors with saline or NE-infusion (Figure S3). Overall, these data suggest that increased circulating NE *in vivo* reprograms splenic T-lymphocytes to an inhibitory state, which leads to a blunted response during canonical (*i.e.* CD3 ± CD28) activation.

NE directly inactivates naïve T-lymphocytes

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

T_H2 lymphocytes are significantly increased with NE stimulation

The decrease in excreted cytokines (*i.e.* TNFα and IFNγ) from NE-treated T-lymphocytes could be due to an alteration in cellular function or polarization. To examine this, splenic T-lymphocytes from NE-infused animals were immunophenotyped. Total number of splenocytes was unchanged when comparing NE-infused and saline-infused spleens (Figure S5A), which demonstrates that increased circulating NE does not cause significant atrophy of the spleen. Furthermore, total spleen immunophenotyping showed no significant change in percentage of CD3+, CD4+, or CD8+ lymphocytes (Figure S5B, Figure S6A–B). Moreover, screening CD4+ T-lymphocytes for intracellular markers of polarization displayed no significant changes between NE and saline-infused spleens (Figure S5C, Figure S6C). These findings strongly suggest that NE alters the internal function of T-lymphocytes prior to activation and polarization. Due to this, we examined the same immunophenotyping parameters on purified T-lymphocytes activated *ex vivo* via CD3 stimulation in the presence of NE. It was first observed that *ex vivo* culture of T-lymphocytes increased the percentage of CD8+ relative to CD4+ cells, but NE did not significantly affect this distribution (Figure S5D, Figure S7A–B). Additionally, while NE had no impact on the early polarization of T_{Reg}, T_H1, or T_H17 cells, it did significantly increase the proportion of T_H2 cells (Figure S5E, Figure S7C), which are known to limit pro-inflammatory T_H1 differentiation. Taken together, these data indicate that NE does not appear to affect T-lymphocyte polarization *in vivo* prior to activation and that *ex vivo* exposure to NE during initial (*i.e.* 48 hours) CD3 activation may drive T_H2 differentiation. In addition, these results suggest that NE 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 due to cell cycle arrest

The decrease in T-lymphocyte numbers observed with NE stimulation could be due to either increased cell death or decreased cell proliferation. We first examined apoptosis using annexin V and propidium iodide (PI) staining. After 48 hours of NE treatment *ex vivo* with CD3 ± CD28 co-stimulation, no significant changes in annexin V or PI positive T-lymphocytes were identified (Figure S8). These data suggest cell death is not a major contributor to the decrease in T-lymphocytes following NE stimulation. Next, we assessed the status of cell cycle progression in T-lymphocytes using the Krishan PI method[19]. NE treatment led to an approximate 20% increase in T-lymphocytes in G1 phase, while proportionally decreasing the number of cells in both S and G2 phase (Figure S9A–B). We also investigated cellular proliferation by carboxyfluorescein succinimidyl ester (CFSE) staining. NE treatment *ex vivo* (±CD28 co-stimulation) significantly decreased the proportion of dividing cells and the proliferative index in both CD4+ and CD8+ lymphocytes (Figure S10A–B). 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[20]. In T-lymphocytes from NE-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 NE on splenic T-lymphocytes during *ex vivo* culture (Figure S11B). In summary, NE does not increase T-lymphocyte death, but limits cellular proliferation and arrests cell cycle progression at the G1-S checkpoint through a possible down-regulation of specific cyclins.

NE increases steady-state superoxide ($O_2^{\bullet-}$) levels in T-lymphocytes

$O_2^{\bullet-}$ has been implicated as a primary signaling intermediate during NE-stimulation in an array of cell types[21–23], but its presence in NE-treated T-lymphocytes has not been explored. Using the $O_2^{\bullet-}$ sensitive dye dihydroethidium (DHE) and flow cytometry, we observed an approximate 70% increase in cellular $O_2^{\bullet-}$ levels in both CD4+ and CD8+ NE-treated T-lymphocytes *ex vivo* compared to control (Figure 3A–B). When examining T-lymphocytes from the *in vivo* sympathoexcitation model before culture, we observed a slight but non-significant increase ($p=0.07$) in cellular $O_2^{\bullet-}$ in T-lymphocytes from NE-infused mice compared with saline-infused (Figure 3C). However, once cultured *ex vivo* with CD3 ± CD28 stimulation the T-lymphocytes from the NE-infused animals demonstrated a significant increase in steady-state $O_2^{\bullet-}$ levels compared to saline-infused, and this response was independent of T-lymphocyte subtype (Figure 3C, S12A–B). Interestingly, while long-term infusion of NE did not significantly increase steady-state levels of $O_2^{\bullet-}$ in freshly isolated T-lymphocytes, the acute treatment (30 minutes) of splenic T-lymphocytes with NE did produce a significant increase in $O_2^{\bullet-}$ (Figure S12C). This observation of variable $O_2^{\bullet-}$ levels at different time points of NE-treatment suggests the potential for temporal control of steady-state $O_2^{\bullet-}$ flux or even ROS-induced-ROS production in T-lymphocytes with NE-stimulation[24]. Overall, NE causes an elevation in T-lymphocyte cellular $O_2^{\bullet-}$ levels, and these increases are correlated with a suppression of T-lymphocyte growth and pro-inflammatory cytokine production.

Superoxide-specific antioxidant supplementation rescues T-lymphocytes from NE-mediated inhibition

To identify a potential mechanistic role for $O_2^{\bullet-}$ in the NE-driven inhibition of T-lymphocytes, we treated mice with Tempol, a $O_2^{\bullet-}$-scavenging antioxidant, concurrently with NE infusion. T-lymphocytes isolated from mice administered Tempol demonstrated a complete rescue in growth when cultured *ex vivo* with CD3 ± C28 stimulation (Figure 4A, S13B). In addition, levels of the pro-inflammatory cytokines IFNγ and TNFα were significantly increased with Tempol compared to NE infusion alone (Figure 4B, S13A). In contrast, Tempol did not affect MAP suggesting the observed partial rescue in the splenic T-lymphocytes was due to a potential redox mechanism as opposed to alleviation of the induced hypertension (Figure 4C). Indeed, while NE infusion increased DHE oxidation in T-lymphocytes after *ex vivo* culture, T-lymphocytes from Tempol treated mice had attenuated DHE 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 NE on splenic T-lymphocyte proliferation and cytokine production.

Discussion

Herein, we describe the potent inhibitory effect of NE on splenic T-lymphocytes in a model of sympathoexcitation-driven hypertension. Additionally, we show NE 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 pro-hypertensive peptide, does not have a significant direct effect on the modulation of T-lymphocyte activity[16]. NE effects on T-lymphocytes have been examined previously in other models of disease, but the consensus is conflicted as to the exact function of NE on T-lymphocyte development and activation[25]. For example, our work confirms and extends that of others who show NE acts to suppress activation of naïve T-lymphocytes in unfractionated populations[26, 27]. Additionally, work using specific T-lymphocytes subsets such as naïve CD4+ or CD8+ cells have shown NE-mediated suppression of immune activity primarily mediated through β2-adrenergic signaling[28, 29]. However, under different conditions such as specific systemic infections, stress, or targeted T-lymphocyte differentiation *ex vivo* NE has been shown to enhance the pro-inflammatory activation of T-lymphocytes[30–32]. While this evidence is inconsistent, numerous variables are at play in these studies that may explain the differences in conclusions.

First, while T-lymphocytes express both α and β 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 β2-adrenergic receptor[25], and our data suggest stimulation with NE during T-lymphocyte activation predisposes differentiation to the T_H2 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[33]. Understanding that NE 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

NE-stimulation may be critical in the phenotype rendered in T-lymphocytes. In one clinical study, short-term administration of NE augmented the number of CD8+ circulating T-lymphocytes, but long-term administration resulted in decreased T-lymphocyte numbers[34]. Additionally, the majority of evidence, including our data presented herein, suggests that naïve unchallenged T-lymphocytes are suppressed by NE-stimulation, but the functionality of pre-existing activated T-lymphocytes (*e.g.* during infection) is exacerbated[30]. Last, a spatial component of NE-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 NE infusion *in vivo*. In contrast, Marvar *et al*. demonstrated increased T-lymphocyte numbers and activation in the aorta of NE-infused mice[16]. While 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 (*i.e.* vasculature or renal positioned) were activated prior to the increased sympathoexcitation and NE outflow associated with hypertension[35]. Under these circumstances, NE 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 NE-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 NE-administration, and organ-specific effects of NE 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 NE-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 (*e.g.* fever, malaise, myalgia) associated with increased systemic immune activation and circulating pro-inflammatory cytokines. These observations further support the notion that hypertension leads to a site-specific (*e.g.* vascular or renal) and localized activation of T-lymphocyte inflammation as opposed to systemic. In this manner, while 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 $O_2^{\bullet-}$ is increased with NE stimulation of T-lymphocytes, and that increased $O_2^{\bullet-}$-scavenging via Tempol significantly restores the original pro-inflammatory potential of the cells, but does not decrease the elevated blood pressure in NE-infused mice. We interpret these findings to mean that $O_2^{\bullet-}$ is a partial mediator of the inhibitory phenotype in T-lymphocytes exposed to NE 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 NE-stimulated T-lymphocytes[36]. Furthermore, NE 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 NE-mediated ROS production. Additionally, we observed that a NE-induced increase in $O_2^{\bullet-}$ leads to a suppression of immune function, while others have observed angiotensin II-induced escalations in $O_2^{\bullet-}$ causing enhanced lymphocyte inflammation[9]. These findings fully support the notion that not all ROS-inducing events are created equal, and in fact, may have very 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[37], 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 NE in a mouse model of sympathoexcitation-driven hypertension directly desensitizes splenic T-lymphocytes to canonical activation, while in this same model it has previously been shown that vascular and renal T-lymphocytes are activated[16]. Furthermore, we report that the inhibitory effects of NE on T-lymphocytes are mediated in part by increased steady-state $O_2^{\bullet-}$ flux, as increased $O_2^{\bullet-}$-scavenging significantly restores the original pro-inflammatory potential of the cells. We speculate that further understanding of how NE 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

1) 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 inhibits splenic derived T-lymphocytes activity and the redox-mediated regulation of this inhibition.

2) What is relevant?

T-lymphocytes localized to the vasculature and kidneys have been demonstrated to have pro-inflammatory 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 inhibitor phenotype of these immune cells during sympathoexcitation.

3) 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.

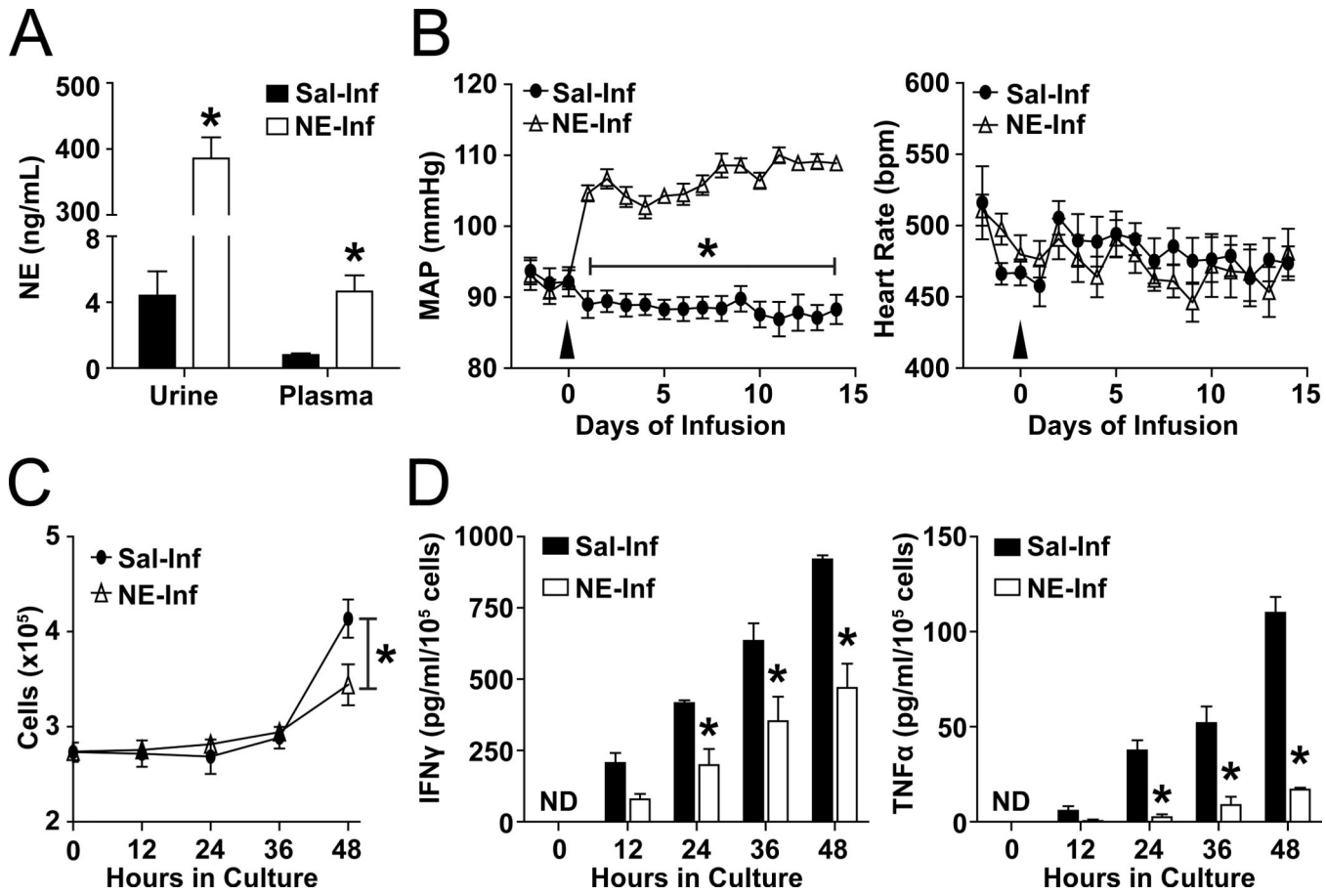

Figure 1. NE-induced hypertension suppresses splenic T-lymphocyte activation

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 0–48 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**. IFNγ and TNFα levels in media at 0–48 hours of T-lymphocyte *ex vivo* culture. ND indicates non-detectable. N=4. *p<0.05 vs. saline-infused.

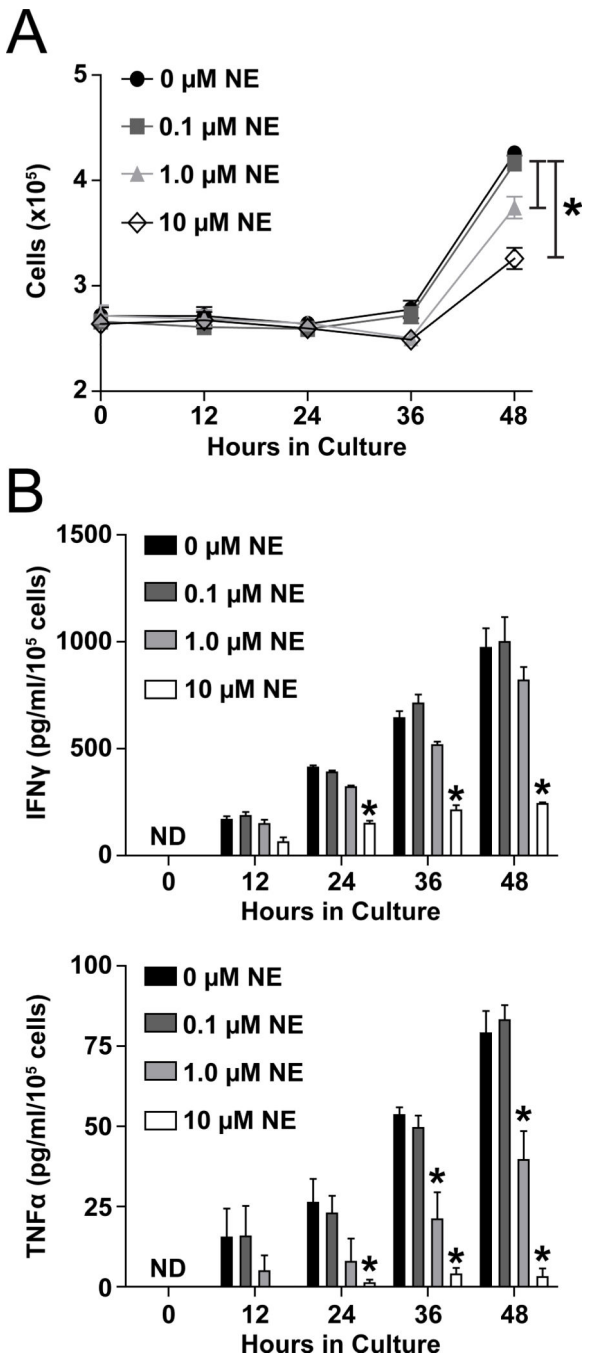

Figure 2. NE has direct inhibitory effects on T-lymphocytes

T-lymphocytes were isolated from unchallenged mice and plated for 48 hours with CD3 stimulation with increasing doses of NE. **A**. T-lymphocyte cell counts 0–48 hours of *ex vivo* culture. N=4. **B**. IFNγ and TNFα levels in media at 0–48 hours of T-lymphocyte *ex vivo* culture. ND indicates non-detectable. N=4. *p<0.05 vs. 0 μM NE.

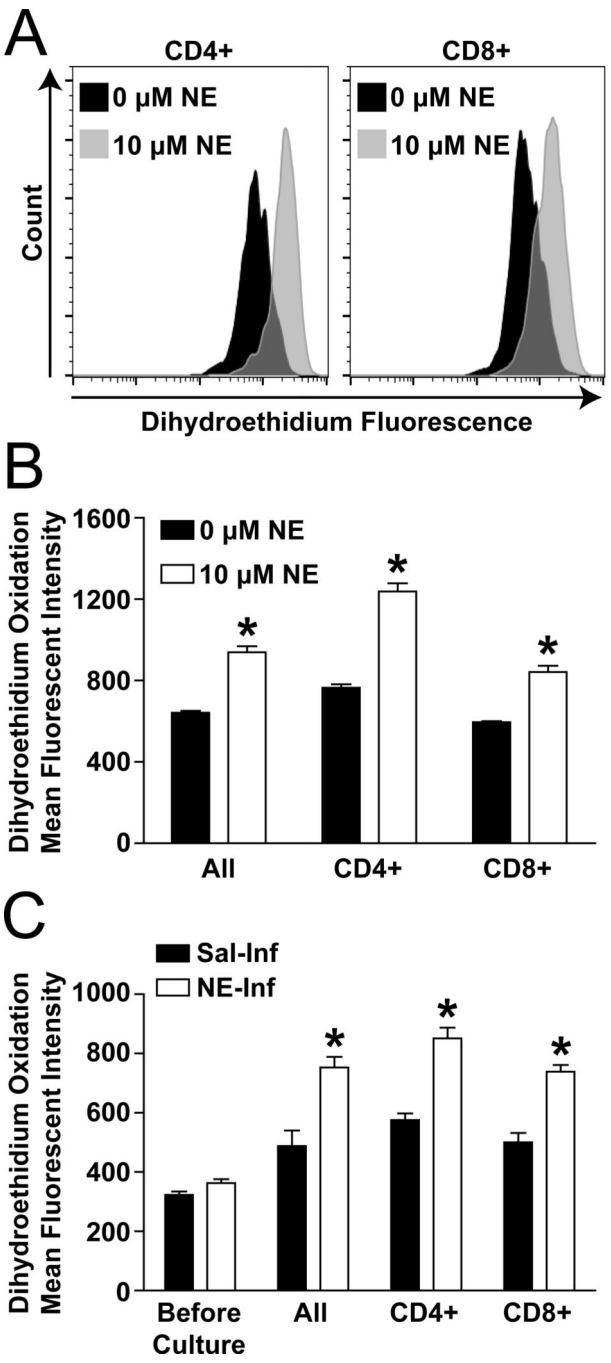

Figure 3. $O_2^{\bullet-}$ is increased in NE-stimulated T-lymphocytes

T-lymphocytes were isolated from unchallenged **(A–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.

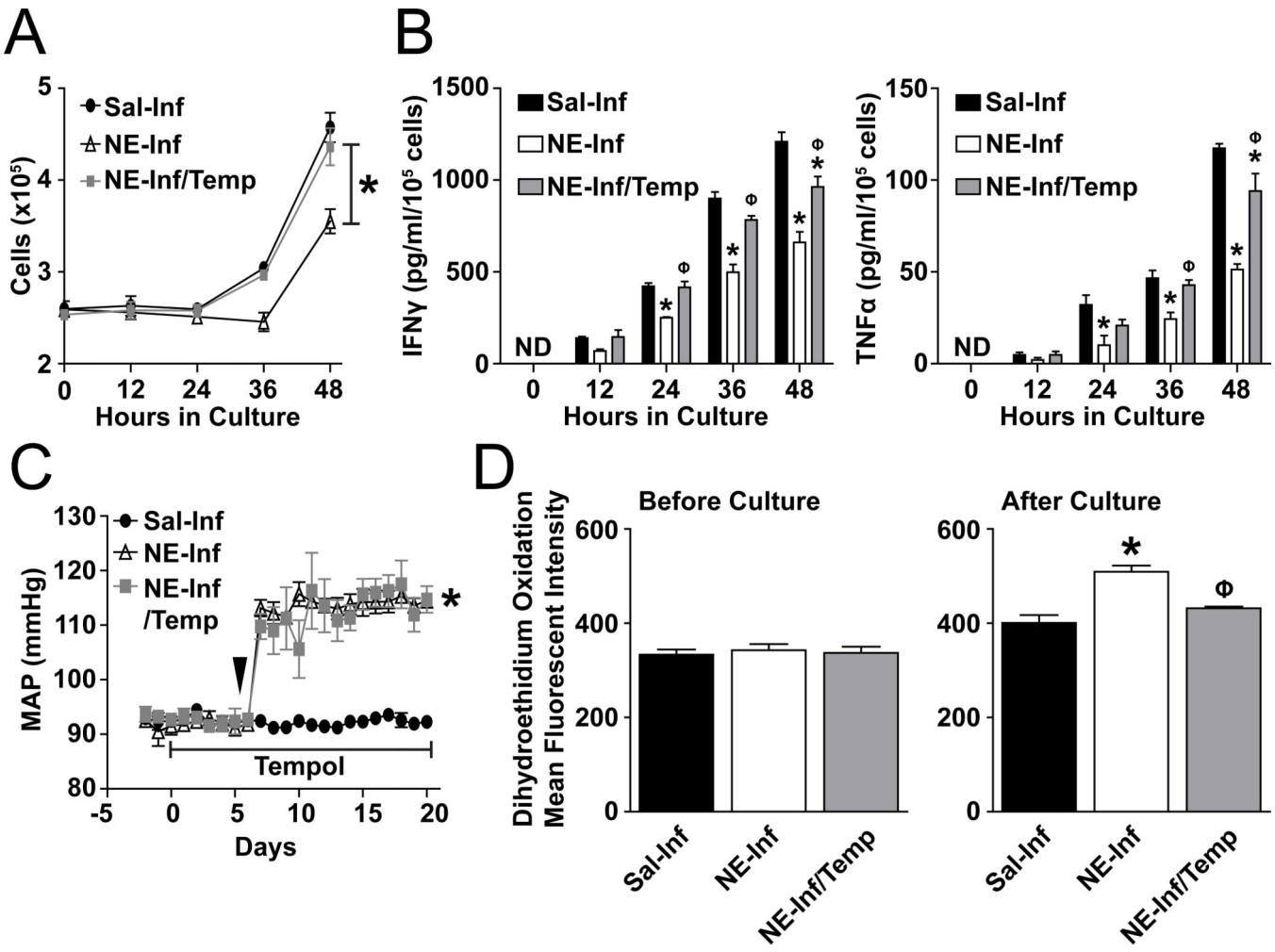

Figure 4. Scavenging of $O_2^{\bullet-}$ rescues the inhibitory phenotype of T-lymphocytes from NE-infused animals

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 stimulation. **A**. T-lymphocyte numbers at 0–48 hours of *ex vivo* culture. N=4. **B**. IFNγ and TNFα levels in media at 0–48 hours of T-lymphocyte *ex vivo* culture. ND indicates non-detectable. 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. $^{\Phi}$p<0.05 vs. NE-infused.