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Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8$^+$ T-cell effector function

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Postganglionic sympathetic neurons innervate secondary lymphoid organs and secrete norepinephrine (NE) as the primary neurotransmitter. NE binds and signals through five distinct members of the adrenergic receptor family. In this study, we show elevated expression of the β2-adrenergic receptor (ADRB2) on primary human CD8$^+$ effector memory T cells. Treatment of both human and murine CD8$^+$ T cells with NE decreased IFN-γ and TNF-α secretion and suppressed their cytolytic capacity in response to T-cell receptor (TCR) activation. The effects of NE were specifically reversed by β2-specific antagonists. Adrb2$^{-/-}$ CD8$^+$ T cells were completely resistant to the effects of NE. Further, the ADRB2-specific pharmacological ligand, albuterol, significantly suppressed effector functions in both human and mouse CD8$^+$ T cells. While both TCR activation and stimulation with IL-12 + IL-18 were able to induce inflammatory cytokine secretion, NE failed to suppress IFN-γ secretion in response to IL-12 + IL18. Finally, the long-acting ADRB2-specific agonist, salmeterol, markedly reduced the cytokine secretion capacity of CD8$^+$ T cells in response to infection with vesicular stomatitis virus. This study reveals a novel intrinsic role for ADRB2 signaling in CD8$^+$ T-cell function and underscores the novel role this pathway plays in adaptive T-cell responses to infection.

Keywords: β2-Adrenergic receptor · CD8$^+$ T cells · Cytokine · Cytolysis · Norepinephrine

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Introduction

The sympathetic nervous system regulates several key physiological functions, which are acutely mobilized during the "fight-or-flight" response. These effects are achieved by the release of epinephrine (E) and norepinephrine (NE) from the adrenal gland and NE released by adrenergic nerve endings that terminate in multiple organs such as heart, lungs, and gut. Moreover, sympathetic nerve endings also innervate secondary lymphoid organs [1, 2]. For example, the splenic nerve terminates in adrenergic neurons within the spleen that secrete NE upon stimulation. The stimuli include stress, shock, various cytokines, and importantly infection [3–6].

Adrenergic receptors (ADRs), the family of receptors bound by E and NE, are expressed on cells of the immune system. In human leukocytes, the β-ADRs are expressed with binding sites that vary between 500 and 2000 sites/cell [7, 8]. NK cells express the most binding sites, followed by monocytes, B cells, and CD8$^+$ T cells, with CD4$^+$ T cells expressing the lowest. Functional studies have confirmed that β-ADR signaling regulates various functions in distinct cell subsets. For example, B-cell costimulatory molecules as well as IgE secreted from IgE isotype switched cells are increased [9, 10], while monocytes and macrophages display decreased proinflammatory cytokine production in response to NE

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[11, 12]. In T cells, Th1 cytokine production by $CD4^+$ T cells is decreased with NE [13], and regulatory T-cell function is also enhanced with β2-adrenergic receptor (ADRB2) ligands [14].

Recent studies have begun to focus on the effects of NE on $CD8^+$ T-cell responses. Grebe et al. determined the effect of chemical sympathectomy with 6-hydroxydopamine on anti-influenza $CD8^+$ T-cell responses [5]. In their study, blocking the secretion of NE before influenza infection increased the IFN-γ^+CD8^+ T-cell response, and blocking ADRB2 signaling with ADRB2-specific antagonists paralleled their observations in sympathectomized animals. Kalinichenko et al. demonstrated a role of NE in inhibiting the generation of antitumor $CD8^+$ T cells [15]. The failure to generate $CD8^+$ T cells was mediated in part by suppression of TNF-α from $CD4^+$, $CD8^+$, and $F4/80^+$ cells by NE signaling. This observation was supported by a recent study demonstrating that the long-acting β2-agonist, salmeterol, could inhibit both cytokine secretion and cytotoxic activity in human $CD8^+$ T cells [16]. In contrast, a recent study demonstrated differential sensitivity to NE signaling between naïve and memory $CD8^+$ T cells, and activation of the ADRB2 led to increased inflammatory cytokine secretion, which may reflect differences in various subsets of $CD8^+$ T cells [17]. In some situations, external conditions that stimulate sympathetic signaling can significantly modulate immune responses [18]. For example, thermoregulation through responses to external temperature or internal fever augments cytokine secretion and lytic activity in $CD8^+$ T cells [19]. Further, in mice, housing temperature dramatically influences T-cell-mediated graft-versus-host disease (GVHD) and antitumor responses, which are regulated directly through the ADRB2 [20–23].

In this study, we comprehensively addressed the role of adrenergic signaling on multiple aspects of $CD8^+$ T-cell effector functions that emanate from both T-cell receptor signaling and cytokine-mediated pathways. We found that NE dominantly suppresses inflammatory cytokine secretion and lytic activity by exclusively signaling through the ADRB2. This suppression was specific for TCR-induced functions as stimulation with IL-12 + IL-18 was unaffected by NE to suppress IFN-γ secretion. Taken together, we reveal an important role for ADRB2 signaling to modulate the magnitude of the $CD8^+$ T cell effector function to infection.

Results

NE signaling via ADRB2 inhibits cytokine production and cytolytic activity of human $CD8^+$ T cells

Our previous studies revealed significant gene expression differences between effector memory ($CCR7^{lo}CXCR3^{hi}$; T_{EM}) and central memory/naïve ($CCR7^{hi}CXCR3^{lo}$; $T_{CM/N}$) $CD8^+$ T cells in healthy human donors [24]. These data are available from Gene Expression Omnibus (GEO) under accession number GSE27337. Within this dataset, we found the ADRB2 to be differentially expressed between T_{EM} and $T_{CM/N}$, with T_{EM} cells having higher expression of ADBR2 (Fig. 1A, left panel). This was confirmed using qPCR (Fig. 1A, right panel). Further, other adrenergic

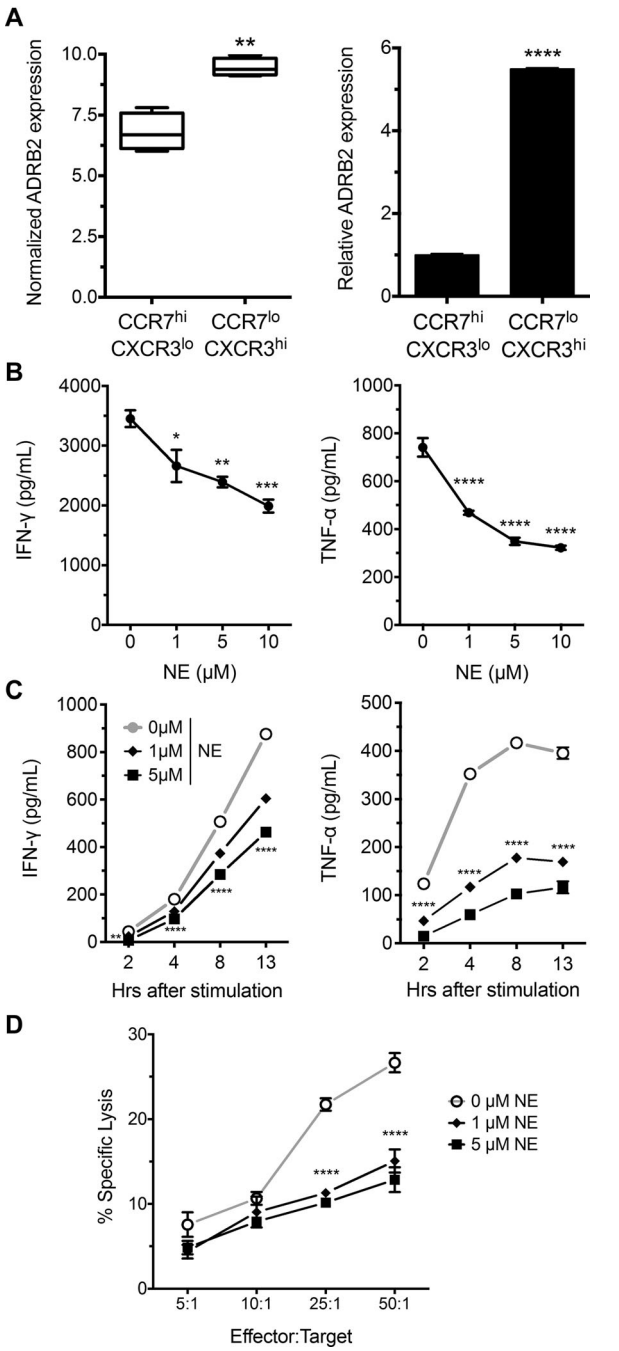

Figure 1. NE suppresses human $CD8^+$ T-cell cytokine secretion and lytic activity. (A) ADRB2 mRNA expression was assessed by microarray (left, [24]) and qPCR (right) in $CD8^+CCR7^{hi}CXCR3^{lo}$ and $CD8^+CCR7^{lo}CXCR3^{hi}$ cells from peripheral blood of healthy human donors. (B and C) $CD8^+$ T cells were isolated and activated (B) for 24 h with increasing concentrations of NE or (C) with anti-CD3 and anti-CD28 antibodies for the indicated time points. IFN-γ and TNF-α in the supernatants were measured by ELISA. (D) $CD8^+$ T cells were isolated and incubated with ^{51}Cr-labeled and anti-CD3-coated target cells at increasing effector to target ratios. ^{51}Cr release was measured by scintillation counting 12 h later. (B and D) Data are shown as mean ± SEM of three replicates and are representative of three experiments performed with similar results. (C) The kinetic experiment was performed once. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to $CCR7^{hi}$ or to 0 μM NE, by Student's t-test or two-way ANOVA with Bonferroni posttest, respectively.

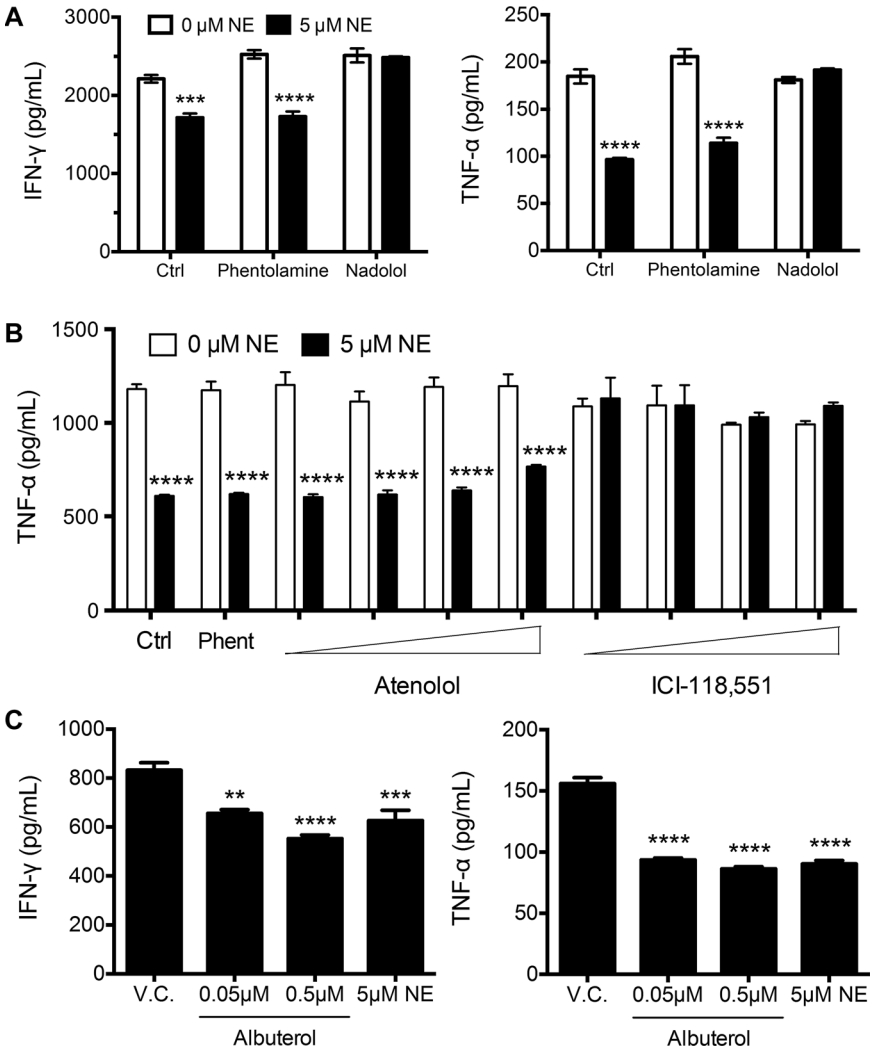

Figure 2. NE suppresses human cytokine secretion in $CD8^+$ T cells through the ADRB2. (A) Human $CD8^+$ T cells were isolated and activated with anti-CD3 and anti-CD28 antibodies and NE for 24 h in the presence of Milli-Q water (Ctrl), or a pan-α (phentolamine), or a pan-β (nadolol) adrenergic receptor antagonist. IFN-γ and TNF-α in the supernatants were measured by ELISA. (B) $CD8^+$ T cells were isolated and activated as in (A), in the presence of phentolamine (10 μM), or increasing concentrations of a β1-specific (atenolol, 0.1–5 μM), or a β2-specific (ICI-118, 551, 0.1–5 μM) adrenergic receptor antagonist. TNF-α production was measured at 24 h by ELISA. (C) $CD8^+$ T cells were isolated and activated as in (A), in the presence of methanol (V.C.) or increasing concentrations of albuterol or NE, and IFN-γ and TNF-α production measured at 24 h. Data shown are presented as mean ± SEM of three replicates and are representative of (A and C) two experiments and (B) three experiments, all from individual donors. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to 0 μM NE or V.C., by two-way (A and B) or one-way (C) ANOVA with Bonferroni posttest.

receptor family member mRNAs were below the limit of detection through the microarray analysis. In order to determine the role that the ADRB2 may play in effector function, $CD8^+$ T cells were isolated from PBMCs of healthy human donors and acutely activated in the absence or presence of NE with plate-bound anti-CD3 and anti-CD28 Abs. IFN-γ and TNF-α secretion was assessed 24 h later and found to be suppressed by NE (Fig. 1B). Of note, we consistently observed that the magnitude of suppression was much greater for TNF-α than for IFN-γ at all concentrations of NE. The kinetics of this suppression was rapid, as NE decreased both IFN-γ and TNF-α cytokine secretion as early as 2 h poststimulation (Fig. 1C). As part of the $CD8^+$ T-cell functional assessment, we tested the ability of NE to modulate cytolytic activity using a redirected lysis assay. We observed significant killing activity at all effector to target ratios that was significantly suppressed by NE (Fig. 1D).

Although the other ADR family member mRNAs were not detected as assessed by microarray (GSE27337 [24]), it was possible that NE was suppressing $CD8^+$ T-cell effector functions through multiple receptors. Specific adrenergic receptor antagonists were used to determine which receptor was involved in the suppression of $CD8^+$ T-cell function. Phentolamine, a pan α-ADR antagonist, failed to reverse the inhibition of cytokine secretion by NE, indicating that the α-ADR family members were not involved in this pathway (Fig. 2A). In contrast, nadolol, a pan β-ADR antagonist, completely reversed the effects of NE (Fig. 2A). The β-ADR family consists of three members: β1 (ADRB1), β2 (ADRB2), and β3 (ADRB3). As such, we found that atenolol, an ADRB1-specific antagonist, failed to reverse the effects of NE, even at the highest concentration tested (Fig. 2B). However, the ADRB2-specific antagonist ICI-118, 551 completely reversed the suppressive effects of NE with concentrations as low as 100 nM, confirming an exclusive role for the ADRB2 in suppressing $CD8^+$ T-cell effector function. In order to further confirm this hypothesis, we utilized the ADRB2-specific ligand albuterol. Similar to the effect seen with NE, albuterol significantly suppressed cytokine secretion in human $CD8^+$ T cells at 100-fold lower concentrations than NE due to differences in binding affinities (Fig. 2C).

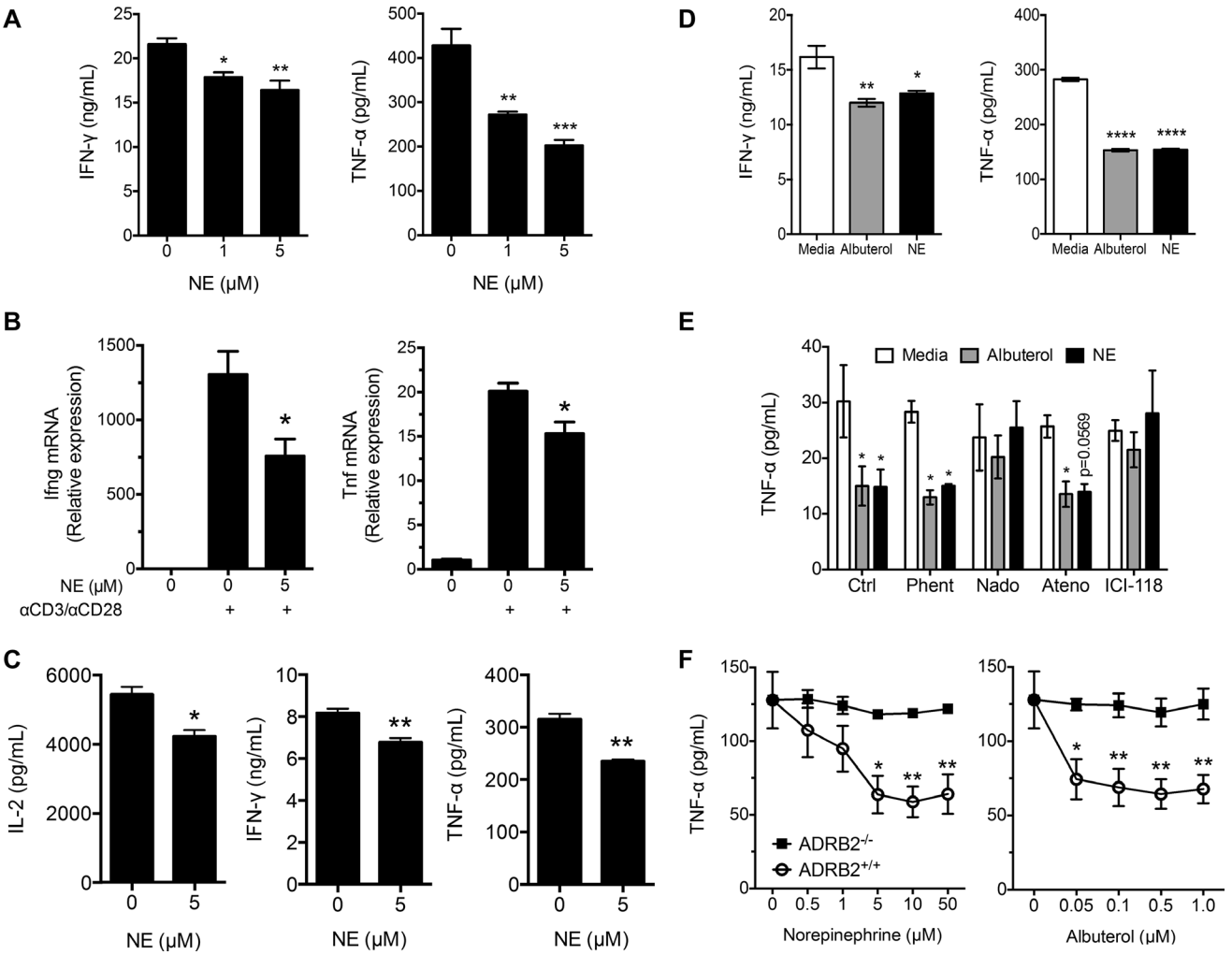

Figure 3. ADRB2 signaling modulates mouse CD8$^+$ T-cell function. (A) Purified CD8$^+$ T cells from C57Bl/6 mice were activated with anti-CD3 and anti-CD28 antibodies and increasing concentrations of NE for 24 h. IFN-γ and TNF-α in the supernatants were measured by ELISA. (B) CD8$^+$ T cells from Cl4 mice were activated with anti-CD3 and anti-CD28 antibodies in the presence or absence of NE for 12 h. RNA was harvested and *Ifng* and *Tnf* transcripts were quantified by qPCR. (C) Splenocytes from Cl4 mice were incubated overnight with the HA peptide IYSTVASSL in the absence of exogenous IL-2 and in the presence or absence of NE. IL-2, IFN-γ, and TNF-α were measured from the supernatants at 24 h. (D) Splenocytes from Cl4 mice were incubated with the HA peptide IYSTVASSL in the presence or absence of albuterol or NE. IFN-γ and TNF-α in the supernatants were measured at 18 h. (E) CD8$^+$ T cells from Balb/cJ mice were activated as in (A), in the presence of albuterol or NE and in the presence of Milli-Q water (Ctrl), or a pan-α (phentolamine), a pan-β (nadolol), a β1-specific (atenolol), or a β2-specific (ICI-118, 551) adrenergic receptor antagonist (100 nM each). IFN-γ (not shown) and TNF-α in the supernatants were measured. (F) CD8$^+$ T cells from ADRB2$^{+/+}$ or ADRB2$^{-/-}$ Balb/cJ mice were isolated and activated as in (A), in the presence of increasing concentrations of NE or albuterol. IFN-γ (not shown) and TNF-α in the supernatants were measured. Data are presented as mean ± SEM of triplicate determinations and are representative of (A and D) three experiments, (B, E, and F) two experiments, and (C) one experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to 0 μM NE or media by Student's *t*-test (C), or one-way (A, B, and D) or two-way (E and F) ANOVA with Bonferroni posttest.

NE signaling inhibits cytokine production of murine CD8$^+$ T cells in response to CD3 stimulation

Although few studies have addressed the effect of NE on CD8$^+$ T-cell responses in mice [5, 15], there remains little understanding of the specificity for ADRB2 signaling and its intrinsic effects on CD8$^+$ T-cell function. Therefore, we decided to expand our studies to address whether the effects of ADRB2 signaling were conserved in murine CD8$^+$ T cells, as suggested by studies of chemical sympathectomy during viral infections [5]. Mouse CD8$^+$ T cells from spleen and lymph nodes (LNs) of naïve C57Bl/6 mice were activated with plate-bound anti-CD3/anti-CD28 Abs for 24 h with increasing concentrations of NE. As observed in human CD8$^+$ T cells, NE suppressed IFN-γ and TNF-α secretion in a dose-dependent manner in mouse CD8$^+$ T cells (Fig. 3A). Likewise, the effects of NE were found to reduce IFN-γ and TNF-α mRNA levels in response to anti-CD3 stimulation (Fig. 3B). In order to determine if ADRB2 signaling could interfere with antigen-specific

cytokine secretion, CD8$^+$ T cells from TCR transgenic clone 4 (Cl4) mice were tested, which have a TCR specific for the influenza HA peptide (IYSTVASSL). Spleen cells from Cl4 mice were incubated for 24 h with IYSTVASSL in the presence or absence of NE. Both IFN-γ and TNF-α as well as IL-2 secretion were suppressed by NE (Fig. 3C). Further, both NE and the β2-specific agonist albuterol suppressed cytokine secretion from mouse Cl4 CD8$^+$ T cells (Fig. 3D), suggesting that the suppression was mediated through ADRB2-dependent signaling. To confirm this, we activated purified CD8$^+$ T cells from Balb/cJ mice with plate-bound anti-CD3/anti-CD28 Abs in the presence or absence of NE or albuterol, and in the presence of different ADR antagonists. As expected, only nadolol and ICI-118, 551 were able to revert the effect of NE and albuterol on mouse CD8$^+$ T-cell cytokine secretion (Fig. 3E). This result was confirmed with purified CD8$^+$ T cells from ADRB2$^{-/-}$ animals as neither NE nor albuterol were able to suppress cytokine secretion in genetically ADRB2-deficient CD8$^+$ T cells (Fig. 3E). Notably, the effect of NE on IFN-γ secretion was dependent upon the strength of stimulation, while TNF-α was responsive to NE regardless of stimulation strength (Supporting Information Fig. 1).

NE suppresses CD8$^+$ T-cell function independently of nicotinic acetylcholine receptor signaling

Although ADRB2 signaling was required for cytokine suppression by NE, it was possible that the effects were indirect through the induction of a negative regulatory pathway. Indeed, Rosas-Ballina et al. demonstrated that memory-like CD4$^+$ T cells could secrete acetylcholine in response to high concentrations of NE [25]. Acetylcholine can decrease proinflammatory cytokine secretion of macrophages by signaling through the α7 nicotinic acetylcholine receptor [26–28]. To test whether NE was driving acetylcholine-mediated suppression of cytokine secretion, OT-I spleen cell suspensions were activated with the CD8$^+$ T-cell-specific ovalbumin peptide (SIINFEKL) in the presence or absence of NE and increasing concentrations of methyllycaconitine (an α7 nicotinic acetylcholine receptor antagonist). As shown, methyllycaconitine failed to revert the effect of NE, even at concentrations as high as 10 μM (Fig. 4A). The ADRB2 signals through the adenylyl cyclase pathway in many cell types, and elevated cAMP levels have been shown to inhibit cytokine expression in CD4$^+$ T cells [29, 30]. As expected, forskolin markedly suppressed TNF-α secretion in CD8$^+$ T cells in response to TCR stimulation (Fig. 4B). However, attempts to block this suppression with two distinct adenylyl cyclase inhibitors, 2′5′-dideoxyadenosine and KH7, failed to reverse the inhibitory effects of NE on TNF-α secretion. These results indicate that while cAMP mobilization profoundly blocks TCR-mediated cytokine expression, additional pathways may interface with adenylyl cyclase activation by NE to suppress CD8$^+$ T-cell effector function. Nonetheless, that data support a direct role for ADRB2 signaling that does not require autocrine responses to acetylcholine.

Long-acting β2 agonists suppress cytokine secretion capacity of CD8$^+$ CTLs in vivo

Upon responding to infection, naïve CD8$^+$ T cells divide in response to both antigen and cytokine signals delivered by antigen presenting cells. IL-12 potently drives their differentiation into primary effector cells, which later contract into a small pool of memory cells as the infection resolves. While NE acutely inhibited TCR-mediated effector activity (described above), it was possible that ADRB2 signaling could also influence their development into effector cells in response to IL-12, either by altering their proliferative capacity or by regulating their capacity to secrete inflammatory cytokines upon restimulation. To address the role of NE in altering proliferation, VPD450-labeled Cl4 cells were activated with HA peptide in the absence or presence of NE over a period of 3 days. Dividing cells were tracked based upon VPD450 dilution. NE did not significantly alter the proportion of cells that expanded, nor was there a delay in their progression from one cell division to the next (Fig. 5A). Further, no differences were observed in the rate of cell division between WT and ADRB2-deficient cells (Fig. 5A). To assess the effects of NE during effector cell differentiation, human CD8$^+$ T cells were activated with anti-CD3/anti-CD28 in the absence or presence of IL-12 for 7 days, and NE was added to these cultures at the initiation of priming. IL-12 significantly enhanced the ability of these cells to secrete IFN-γ upon restimulation, while TNF-α secretion remained unaffected. Further, addition of NE to these cultures during priming did not impact their ability to secrete either IFN-γ or TNF-α upon recall stimulation.

Two main pathways regulate the acute expression of cytokine genes in effector T cells. In the context of antigen recognition, TCR signaling drives rapid transcription of IFN-γ and TNF-α. However, CD8$^+$ T cells can respond innately by secreting inflammatory cytokines in response to combined stimulation with IL-12 and IL-18 in the absence of cognate antigen [31, 32]. To test the effects of ADRB2 signaling on IL-12/IL-18-induced cytokine secretion, Cl4 T cells were stimulated with IL-12, IL-18, or both in the absence or presence of NE. Surprisingly, in contrast to TCR activation, IL-12/IL-18 stimulation selectively induced IFN-γ, but not TNF-α secretion. However, NE did not significantly inhibit IFN-γ secretion from these cultures (Supporting Information Fig. 2). Collectively, these data suggest that NE signaling selectively suppresses acute TCR-driven IFN-γ and TNF-α secretion from CD8$^+$ T cells, but does not influence either their proliferation or their development into effectors in response to IL-12.

Although the in vitro priming experiments described above suggested that NE did not directly block effector cell development, it was possible that ADRB2 signaling could influence their development in vivo during pathogen responses. Utilizing an adoptive cell transfer model followed by viral infection, we tested this indirect mechanism by treating recipient mice with the long-acting β2-specific agonist salmeterol (Fig. 6A). OT-I cells were transferred i.v. into naïve C57Bl/6 hosts, and the next day mice were infected i.v. with VSV-OVA (vesicular stomatitis virus expressing chicken ovalbumin). Salmeterol was administered i.p. for three

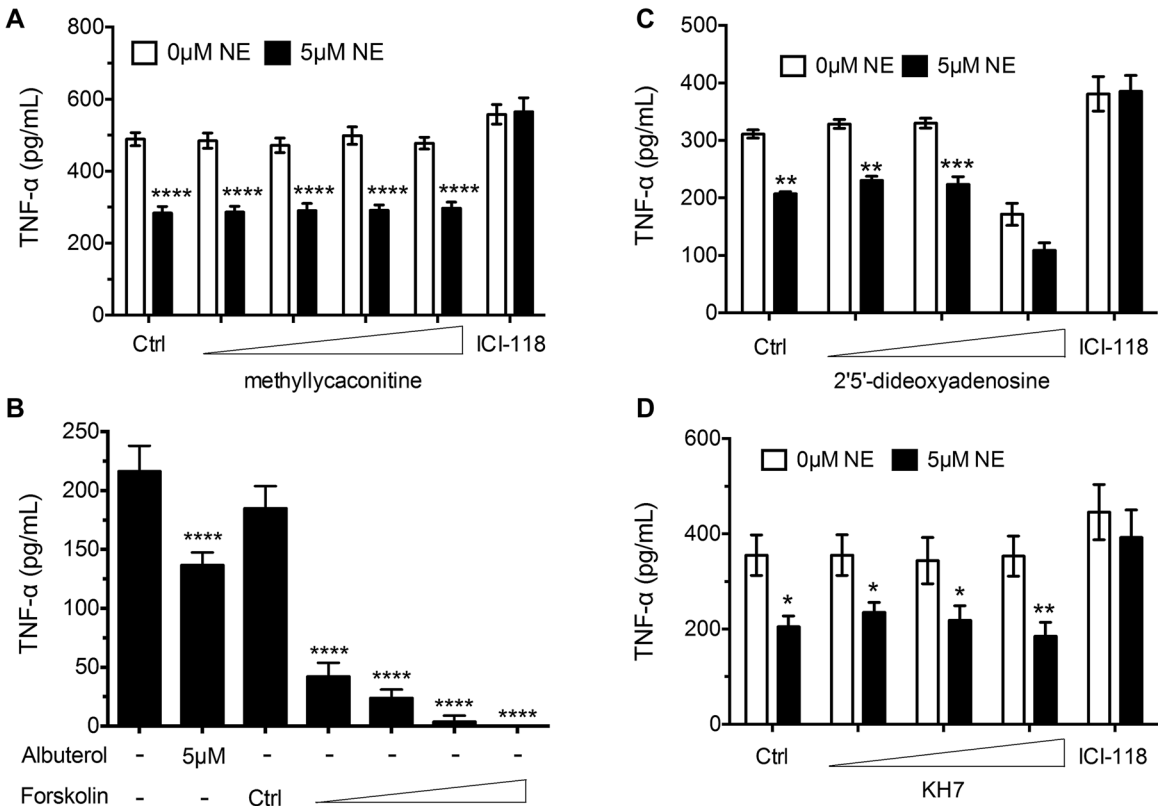

Figure 4. ADRB2 signaling suppresses mouse CD8$^+$ T-cell cytokine secretion independent of acetylcholine or cAMP. (A) Splenocytes from OT-I mice were isolated and incubated 23 h with the OVA peptide SIINFEKL and NE with increasing concentrations of an α7 nicotinic receptor antagonist (methyllycaconitine) or a β2 adrenergic receptor antagonist (ICI-118, 551). TNF-α in the supernatants was measured. (B) Splenocytes from Cl4 mice were incubated overnight with the HA peptide IYSTVASSL and in the presence or absence of albuterol or increasing concentrations of the cAMP stimulator forskolin. TNF-α in the supernatant was measured. (C) Splenocytes from Cl4 mice were incubated with HA peptide in the presence or absence of NE, and with increasing concentrations of an adenylyl cyclase inhibitor (2′5′-dideoxyadenosine) or a β2 adrenergic receptor antagonist (ICI-118, 551). TNF-α in the supernatants was measured. (D) CD8$^+$ T cells from Balb/cJ mice were isolated and stimulated with anti-CD3 and anti-CD28 Abs (3 μg/mL each) for 24 h in the presence or absence of NE and increasing concentrations of a soluble adenylyl cyclase inhibitor (KH7) or a β2 adrenergic receptor antagonist (ICI-118, 551). TNF-α in the supernatants was measured. Data are presented as mean ± SEM of triplicate determinations and are representative of (A and D) a single experiment with pooled data from three mice measured separately, (B and C) two experiments with pooled data from three mice measured separately in each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to 0 μM NE, no albuterol, or Ctrl, two-way (A, C, and D) or one-way (B) ANOVA with Bonferroni posttest.

consecutive days, beginning on the day of infection. Spleens were then harvested on day 7 postinfection to assess OT-I cell expansion and cytokine secretion upon restimulation. As expected, OT-I cells expanded upon VSV-OVA infection (Fig. 6B). Salmeterol did not affect either the percentage of antigen-specific cells, or the total number of expanded cells present at day 7 (Fig. 6B), consistent with results from in vitro proliferation assays (Fig. 5A). Although the expansion of antigen-specific cells was unaffected by treatment with salmeterol, their programmed cytokine secretion capacity was significantly reduced by approximately 25% (Fig. 6C). Considering that ADRB2 signaling by stimulation with NE in vitro did not alter effector cell development directly (Fig. 5B), it was likely that the effect of salmeterol on cytokine secretion capacity was mediated by an indirect cell-extrinsic mechanism. Thus, the primary role of ADRB2 signaling during CD8$^+$ T-cell priming in vivo may reside within innate cells perhaps through decreasing IL-12 secretion [33], while the intrinsic role of this pathway directly suppresses cytokine secretion and lytic activity once cells have developed into primary effectors.

Discussion

The interface between the nervous and immune systems has recently gained significant interest, and the connection between these systems is quite intimate given that secondary lymphoid organs are innervated by postganglionic neurons [1, 2]. These innervations end in adrenergic neurons, which secrete NE upon stimulation [3, 4]. The majority of studies have focused on the effects of nervous system cues on innate cells, and ADRB2 signaling has been shown to decrease proinflammatory cytokine secretion in macrophages [12]. Dendritic cells are also affected by ADRB2 signaling, which inhibits antigen cross-presentation [33]. And mast cells fail to degranulate upon stimulus in the presence of

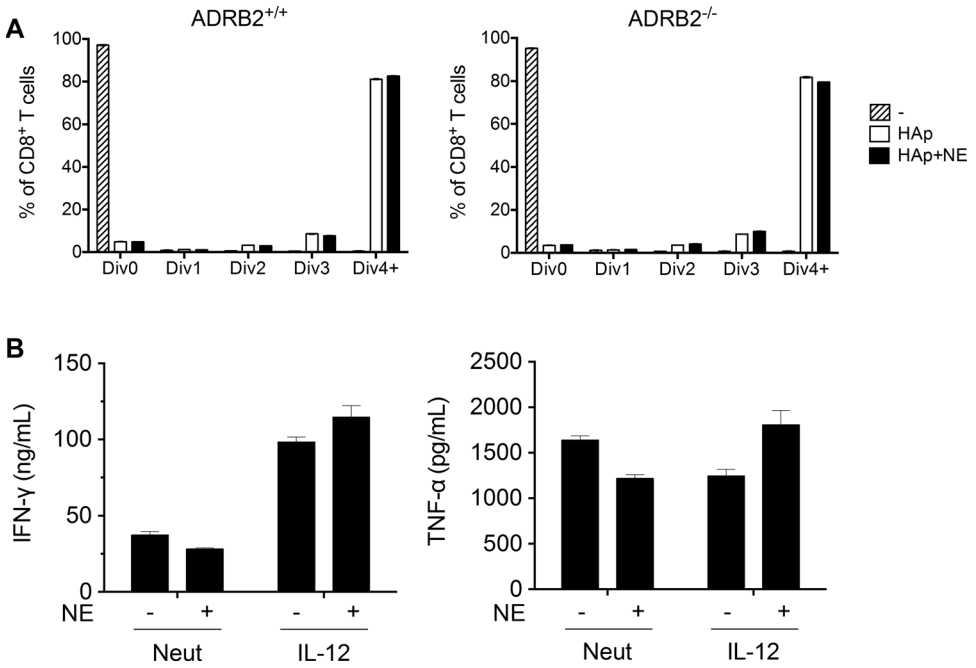

Figure 5. ADRB2 signaling does not affect $CD8^+$ T-cell proliferation or programming. (A) Splenocytes from ADRB2-sufficient or ADRB2-deficient Cl4 mice were isolated and incubated for 3 days in the presence or absence of the HA peptide IYSTVASSL and NE. Proliferation was measured by dilution of a proliferation dye by flow cytometry. (B) Human $CD8^+CD45RA^+$ cells were purified from peripheral blood and activated with plate-bound anti-CD3 and anti-CD28 antibodies, either neutralizing cytokines (Neut) or in the presence of rhIL-12 (IL-12) and in the presence or absence of NE for 7 days. On day 8, cells were restimulated with plate-bound anti-CD3 for 24 h. IFN-γ and TNF-α in the supernatant were measured by ELISA. Data are presented as mean ± SEM of triplicate determinations and are representative of (A) three experiments with at least one mouse in each experiment, and (B) two independent experiments from separate donors. Comparisons of NE treatments to controls were not significant as assessed by two-way ANOVA with Bonferroni posttest.

ADRB2 ligands [34–37]. Overall, the ADRB2 acts to dampen the innate immune response by decreasing both cytokine secretion and other components of cell-mediated functions.

However, not all reports show a negative regulation of immune system function by adrenergic signals. Additional studies have demonstrated that B cells increase CD86 expression and secrete more IgE, without affecting IgE isotype switching, when ADRB2 ligands are present [9, 10]. As for $CD4^+$ T cells responding to ADRB2 signaling, the effects depend on the population in question. High concentrations of NE have been shown to induce memory-like $CD4^+$ T cells to secrete acetylcholine [25]. Furthermore, regulatory $CD4^+$ T cells respond positively to ADRB2 ligands by increasing their suppressive activity, as well as their induction of iTregs from naïve $CD4^+$ T cells [14]. In contrast, NE acutely decreases cytokine production from Th1 cells, while leaving cytokine secretion from Th2 cells unaffected [13, 38]. This is due to chromatin modifications within the ADRB2 promoter that allow gene expression in Th1 cells while silencing it in Th2 cells [39]. In contrast, polarization of naïve $CD4^+$ T cells under Th1 conditions in the presence of NE increased their capacity to secrete IFN-γ [40].

Several studies have examined the effects of ADRB2 signaling on $CD8^+$ T cells. One study showed adrenergic signaling, specifically through the ADRB2, inhibited the production of $CD8^+$IFN-γ^+ cells upon influenza A infection [5]. However, the intrinsic effect of ADRB2 signaling on $CD8^+$ T cells was not addressed in this study. A second study examined the effect of NE on cytokine and gene expression in different subsets of memory $CD8^+$ T cells [17]. NE was shown to decrease IFN-γ and IL-2 expression, consistent with results from our data presented here, as well as other previous studies [15, 16]. Importantly, Zalli et al. recently demonstrated that relatively high concentrations (50 μM) of the long-acting β2-agonist salmeterol could inhibit human $CD8^+$ T-cell effector functions [16], which was consistent with our in vivo findings. In contrast, Slota et al. recently reported an increase in other cytokines, namely, TNF-α, IL-1α, IL-6, and CCL-2 [17]. The observation that TNF-α was increased by ADRB2 signaling was inconsistent with the results shown in our current study, as well as in others [15]. The biological relevance of IL-1α and IL-6 gene and cytokine expression on $CD8^+$ T cells remains to be elucidated. Nonetheless, it is important to point out that the memory populations studied by Slota et al. do not behave the way we currently understand these populations. Mainly, effector memory and central memory $CD8^+$ T cells were shown to secrete similar levels of cytokines in their study. This is opposite to the currently understood notion that effector memory cells retain acute and increased effector functions, while central memory cells have much lower capacity for immediate effector function but retain the ability to rapidly proliferate to give rise to new effector cells. The way that memory cell populations were identified and isolated in their study could account for some of the discrepancies seen between their studies and others that are in agreement with our current findings.

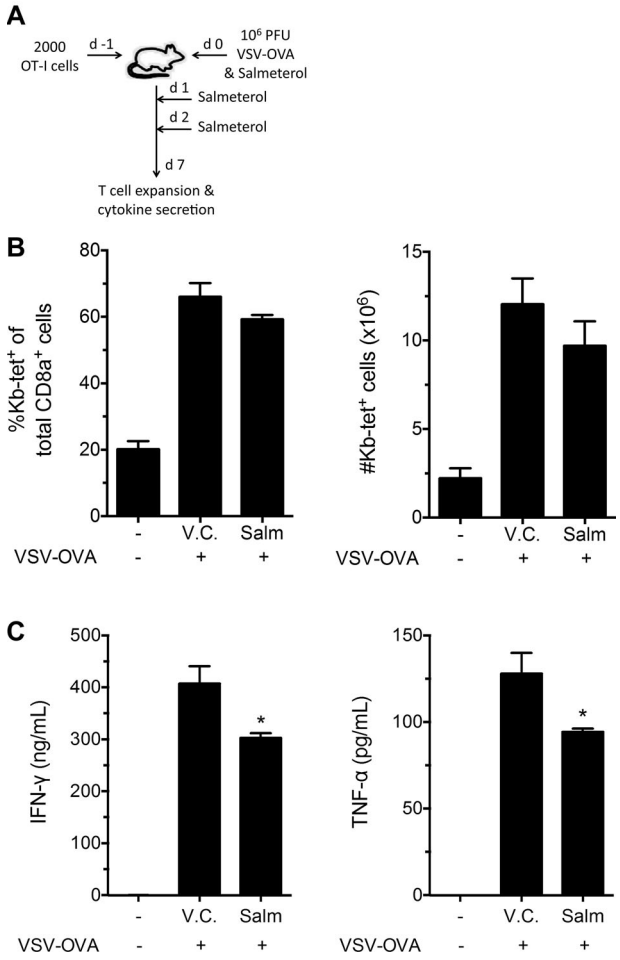

Figure 6. ADRB2 signaling modulates cytokine secretion programming in mouse CD8$^+$ T cells in vivo. (A) OT-I T cells were transferred (2000/recipient) into naïve C57Bl/6 mice followed by infection with 10^6 PFU VSV-OVA the next day (day 0). DMSO (V.C.) or salmeterol was given i.p. on days 0, 1, and 2. (B) Spleens were harvested on day 7, and OT-I-specific cellular expansion was determined by flow cytometry. Data display percent of Kb-tet$^+$ cells of the total CD8$^+$ T-cell pool (left) and the total numbers of Kb-tet$^+$ cells within the spleen (right). (C) IFN-γ and TNF-α secretion were measured from supernatants by ELISA after 24 h restimulation with SIINFEKL peptide. Data are presented as mean ± SEM of triplicate determinations and are representative of two independent experiments with four mice per treatment group in each experiment. $^*p < 0.05$ compared to V.C., one-way ANOVA with Bonferroni posttest.

Despite the possibility that high concentrations of NE can elicit acetylcholine production from T cells, we found no role for this pathway in mediating cytokine suppression in primary CD8$^+$ T cells. Rather, we found that mimicking the canonical signaling pathway of ADRB2 via the cAMP stimulator forskolin was able to downregulate cytokine secretion from CD8$^+$ T cells, which has been shown before for CD4$^+$ T cells [29, 30]. However, despite testing different adenylyl cyclase inhibitors, we were unable to reverse the inhibition of cytokine secretion by NE. This could be due to ineffective activity by the inhibitors and the toxic effects of the inhibitors on cytokine secretion when used at higher concentrations. Finally, salmeterol was able to reduce cytokine secretion programming of CD8$^+$ T cells in vivo, in the context of a viral infection. This is in agreement with the results from a chemical sympathectomy model with viral infection [5, 6]. However, this effect is more likely due to indirect effects on antigen presenting cells since we showed no effect of NE on CD8$^+$ T-cell programming, nor proliferation, in vitro.

In summary, we have described an intrinsic effect of ADRB2 signaling on human and mouse CD8$^+$ T-cell effector functions. NE, the natural ligand for ADRB2 in immune organs, as well as pharmacological ADRB2 ligands, were able to suppress IFN-γ and TNF-α cytokine secretion acutely when cells were stimulated through their TCR. This is the first report to demonstrate a differential suppression for TNF-α modulation, which was considerably more responsive to NE, while the effect of NE on IFN-γ depends more on T-cell receptor signal strength, as evidenced by titration of TCR stimulation and IL-12/IL-18-induced IFN-γ secretion. Additionally, NE was able to suppress cytolytic capacity of human CD8$^+$ T cells ex vivo.

Materials and methods

Mice

C57Bl/6, OT-I, Balb/cJ, Clone4-Tg (Cl4), and Adrb2$^{-/-}$ mice were housed in specific pathogen-free conditions at the University of Texas Southwestern Medical Center Animal Research Center facilities. Adrb2$^{-/-}$ mice [41] were a kind gift from Dr. Virginia Sanders (Ohio State University), and Clone4-Tg (Cl4) mice [42] were purchased from Jax mice (Jackson laboratory). All experiments involving mice in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Human subjects

Informed consent was obtained from adult healthy donors according to the guidelines established by the Internal Review Board of the University of Texas Southwestern Medical Center and the Declaration of Helsinki. Peripheral blood was then collected by venipuncture.

CD8$^+$ T-cell isolation and culture

For the isolation of human CD8$^+$ T cells, peripheral blood mononuclear cells (PBMCs) were obtained by ficoll density centrifugation of whole blood from healthy human donors. CD8$^+$ T cells were purified using a negative isolation kit (BD Biosciences). Alternatively, cells were isolated using a FACSAria sorter (BD Biosciences). For mouse, CD8$^+$ T cells were purified from spleen and LNs using a negative isolation kit (Invitrogen) or by sorting. For cytokine secretion, human purified CD8$^+$ T cells were cultured

with plate-bound anti-CD3/anti-CD28 (clones OKT3/CD28.2, 3 μg/mL each) in complete IMDM supplemented with 10% FBS. Cells were cultured at 1×10^6/mL and supplemented with rhIL2 (200 U/mL). For differentiation, human $CD8^+CD45RA^+$ cells were isolated by sorting and stimulated as above for 7 days, with a 1:10 split on day 3, under neutralized or IL-12 polarization conditions as previously described [24, 43]. Cells were then rested overnight without rhIL2 and restimulated with plate-bound anti-CD3 (OKT3, 3 μg/mL). Mouse-purified $CD8^+$ T cells were cultured at 1×10^6/mL with plate-bound anti-CD3/anti-CD28 (clones 2C11/37.51, 3 μg/mL each for ELISA, 2 μg/mL each for RNA gene expression). Cl4 and OT-I spleen cell suspensions were cultured at 2×10^6/mL, or as indicated, with the $CD8^+$ T-cell-specific influenza A hemagglutinin (HA) peptide (IYSTVASSL, 0.5 μM, or as indicated), or the ovalbumin (OVA) peptide (SIINFEKL, 10 nM), in supplemented media as indicated above. For proliferation assays, Cl4 splenocytes were labeled with VPD450 according to manufacturer's instructions and incubated with IYSTVASSL (0.05 μM) for 3 days. For restimulation of spleen single cell suspensions from infection experiments, cells were incubated at 2×10^6/mL, and supplemented with rhIL2 (200 U/mL), in the presence or absence of the $CD8^+$ T-cell-specific OVA peptide (SIINFEKL, 10 nM).

Antagonists and ADRB2 ligands

Phentolamine hydrochloride (Sigma, P7547), nadolol (Sigma, N1892), atenolol (Sigma, A7655), and ICI-118, 551 hydrochloride (SelleckChem, 0821) were used in this study as antagonists for different adrenergic receptors and were dissolved in Milli-Q water and filter-sterilized. Three different ADRB2 agonists were used. Norepinephrine bitartrate (Sigma, A0937) was dissolved in complete IMDM supplemented with 10% FBS. Albuterol (salbutamol sulfate, SelleckChem, S2507) was dissolved in either methanol (Fig. 2C) or complete IMDM supplemented with 10% FBS (all others). Salmeterol (Tocris, 1660) was dissolved in DMSO. The α7 nicotinic acetylcholine receptor antagonist, methyllycaconitine, was dissolved in Milli-Q water. Forskolin was dissolved in DMSO and used to stimulate cAMP production. The corresponding vehicle control was used for each experiment.

qPCR

Total RNA was isolated 12 h after stimulation using the RNeasy® Mini Kit with DNaseI treatment (Qiagen), according to manufacturer's instructions. Reverse transcription was then performed with the ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers directed against *Ppia* were used for reference of mouse gene expression. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [44].

Mouse primer sequences: *Ppia*: For 5′-TTATTCCAGGATTCAT GTGCCAGGG-3′, Rev 5′-TCATGCCTTCTTTCACCTTCCCAA-3′; *Ifng*: For 5′-ACAATCAGGCCATCAGCAACAAC-3′, Rev 5′-CAGCG ACTCCTTTTCCGCTTC-3′; *Tnf*: For 5′-CTGTAGCCCACGTC GTAGCA-3′, Rev 5′-AGCAAATCGGCTGACGGTGT-3′.

ELISA

Human IFN-γ and TNF-α cytokine secretion was determined 24 h after cell activation (with anti-CD3/anti-CD28 Abs), unless otherwise stated. ELISA MAX kits (BioLegend) were used according to manufacturer's instructions. For mouse cytokine secretion assays, supernatants were harvested 24 h after stimulation (with anti-CD3/anti-CD28 Abs, or IYSTVASSL) or 21–24 h after restimulation with SIINFEKL. ELISA MAX kit (BioLegend) was used for mouse TNF-α analysis, according to manufacturer's instructions. The mouse IFN-γ ELISA used coating antibody (BD Biosciences Rat anti-Mouse IFN-γ, Clone R4-6A2, 551216; used at 3 μg/mL), standards (eBioscience rmIFN-γ, 14-8311-63; top standard at 8 ng/mL), detection antibody (BioLegend biotin-Rat anti-Mouse IFN-γ, Clone XMG1.2, 505804; used at 0.5 μg/mL), and streptavidin (BioLegend, 405210; used 1:1000) following BioLegend's ELISA MAX mouse TNF-α protocol.

Flow cytometry

PMBCs from healthy human donors were labeled with anti-CD8α-PE (BD Biosciences, 555635) and anti-CD45RA-FITC (BD Biosciences, 555488), or anti-CCR7-PE (BD Biosciences, 552176), anti-CXCR3-AlexaFluor488 (BD Biosciences, 558047), and anti-CD8α-AlexaFluor647 (BD Biosciences, 557708) antibodies for cell sorting. Mouse spleen and LNs single-cell suspensions were stained with anti-CD8α-PE (BioLegend, 100708) for cell sorting. For proliferation experiments, cell suspensions were stained with anti-CD8α-APC (BioLegend, 100712), AnnexinV-FITC (BioLegend, 640906), and Ghost Dye-Red780 (Tonbo, 13–0865), and Violet Proliferation Dye 450 (VPD450, BD Biosciences, 562158).

Antibody redirected lysis assay

A redirected killing assay [45] was performed by labeling THP-1 target cells with 100–500 μCi of ^{51}Cr ($Na_2[^{51}Cr]O_4$) for 90 min. Cells were then washed and left unlabeled or labeled with anti-CD3 Ab (OKT3; 1.5μg/mL) for 20 min to provide antigen-receptor-dependent stimulation. Cells were then washed twice and resuspended at 10 000 cells/mL. One thousand labeled THP-1 target cells were incubated with human $CD8^+$ T cells at increasing effector:target (E:T) ratios for 12–16 h at 37°C in complete IMDM with 10% FBS and supplemented with rhIL2 (200 U/mL) in the presence or absence of NE. Cytolytic capacity was determined by scintillation counting of ^{51}Cr release into the supernatant. Percent specific lysis was calculated with the formula: (Sample – Spontaneous) ÷ (Maximum – Spontaneous) × 100. Where "Spontaneous" is determined by incubating labeled THP-1 cells alone, and

"Maximum" is determined by adding detergent to labeled THP-1 cells incubated alone.

Vesicular stomatitis virus infections

For infections, C57Bl/6 mice received 2000 OT-I cells i.v. 1 day before i.v. infection with 10^6 plaque-forming units (PFUs) of VSV-OVA. Salmeterol, a long-acting ADRB2 agonist, was given at a dose of 40 μg/mouse i.p. on the day of infection, as well as days 1 and 2 postinfection. Control mice received sterile DMSO i.p. Spleens were harvested on day 7 postinfection and single-cell suspensions made using frosted glass slides for analysis.

Statistical analysis

Two different statistical tests were performed using GraphPad Prism 6 software. For simple pairwise comparisons, a Student's two-tailed *t*-test was used. Otherwise, a one-way or two-way ANOVA was used followed by a Bonferroni posttest for pairwise comparisons within the groups, as indicated in the figure legends. Differences were considered significant when $p \leq 0.05$.

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Abbreviations: **ADR**: adrenergic receptor · **ADRB2**: β2-adrenergic receptor · **NE**: norepinephrine · **CTLs**: cytotoxic T lymphocytes

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