

$\alpha 7$ Nicotinic Acetylcholine Receptor ($\alpha 7$ nAChR) Expression in Bone Marrow-Derived Non-T Cells Is Required for the Inflammatory Reflex

Peder S Olofsson,^{1*} David A Katz,^{1*} Mauricio Rosas-Ballina,¹ Yaakov A Levine,² Mahendar Ochani,¹ Sergio I Valdés-Ferrer,¹ Valentin A Pavlov,¹ Kevin J Tracey,¹ and Sangeeta S Chavan¹

¹The Feinstein Institute for Medical Research, Laboratory of Biomedical Science, Manhasset, New York, United States of America; and

²Setpoint Medical Corporation, Valencia, CA, United States of America.

The immune response to infection or injury coordinates host defense and tissue repair, but also has the capacity to damage host tissues. Recent advances in understanding protective mechanisms have found neural circuits that suppress release of damaging cytokines. Stimulation of the vagus nerve protects from excessive cytokine production and ameliorates experimental inflammatory disease. This mechanism, the inflammatory reflex, requires the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR), a ligand-gated ion channel expressed on macrophages, lymphocytes, neurons and other cells. To investigate cell-specific function of $\alpha 7$ nAChR in the inflammatory reflex, we created chimeric mice by cross-transferring bone marrow between wild-type (WT) and $\alpha 7$ nAChR-deficient mice. Deficiency of $\alpha 7$ nAChR in bone marrow-derived cells significantly impaired vagus nerve-mediated regulation of tumor necrosis factor (TNF), whereas $\alpha 7$ nAChR deficiency in neurons and other cells had no significant effect. In agreement with recent work, the inflammatory reflex was not functional in nude mice, because functional T cells are required for the integrity of the pathway. To investigate the role of T-cell $\alpha 7$ nAChR, we adoptively transferred $\alpha 7$ nAChR-deficient or WT T cells to nude mice. Transfer of WT and $\alpha 7$ nAChR-deficient T cells restored function, indicating that $\alpha 7$ nAChR expression on T cells is not necessary for this pathway. Together, these results indicate that $\alpha 7$ nAChR expression in bone marrow-derived non-T cells is required for the integrity of the inflammatory reflex.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2011.00405

INTRODUCTION

The immune response to infection or injury coordinates host defense and tissue repair, but it also has the inherent capacity to significantly damage host tissues. The release and activity of tumor necrosis factor (TNF), interleukin (IL)-1 and other potentially damaging cytokines is controlled at multiple levels to prevent unrestrained collateral tissue damage that can disable, or even kill, the host (1). Humoral mechanisms that restrain or inhibit these damaging responses include glucocorticoid hor-

mones, soluble cytokine receptors, IL-10, transforming growth factor (TGF)- β and other antiinflammatory cytokines. Activation of cholinergic receptors is also known to regulate immune system activity (2–9). Recent insights in protective mechanisms have revealed that neural circuits suppress release of damaging cytokines and that neural regulation of immune cell activation is an ancient mechanism dating back to nematode worms, a primitive animal with rudimentary nervous and immune systems (10–12).

A prototypical antiinflammatory neural mechanism is the inflammatory reflex (11,13–15). Action potentials arising in the brain stem are transmitted in the cholinergic vagus nerve to terminate in the celiac ganglion, the site of origin of the adrenergic splenic nerve. Signals through the splenic nerve terminate on specialized T cells that respond to norepinephrine by producing acetylcholine, the terminal neurotransmitter in the circuit. Acetylcholine interacts with cytokine producing macrophages in the red pulp and marginal zone to suppress TNF release (15). The cytokine-suppressing mechanism of the inflammatory reflex requires the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR), as evidenced by the observation that the inflammatory reflex is impaired in $\alpha 7$ nAChR-deficient mice (16). Furthermore, deleting $\alpha 7$ nAChR from isolated macrophages impairs the ability of acetylcholine to suppress TNF and other cytokines.

Although these data imply an importance of $\alpha 7$ nAChR in cytokine-producing

*PSO and DAK contributed equally to this work.

Address correspondence to Peder S Olofsson or Sangeeta S Chavan, Laboratory of Biomedical Science, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. Phone: 516-234-1127 (PSO), 516-562-1022 (SSC); Fax: 516-562-2356; E-mail: lolofsson@nshs.edu (PSO), schavan@nshs.edu (SSC).

Submitted October 21, 2011; Accepted for publication December 13, 2011; Epub (www.molmed.org) ahead of print December 16, 2011.

cells of the innate immune system (5,8,16,17), $\alpha 7nAChR$ is also expressed by other cells, including neurons, glial cells and T cells. It remained theoretically possible that the impaired inflammatory reflex in the $\alpha 7nAChR$ -deficient mice was attributable to altered neuronal functions. To address this question, we created chimeric mice by cross-transferring bone marrow (BM) derived from wild-type (WT) and $\alpha 7nAChR$ -deficient mice and evaluated the competence of the inflammatory reflex. This study demonstrates that $\alpha 7nAChR$ expression on BM-derived non-T cells, not on neurons, is required for the integrity of the inflammatory reflex.

MATERIALS AND METHODS

Animals

$\alpha 7nAChR$ knockout (KO) B6.129S7-*Chrna7^{tm1Bay}*/J, WT C57BL/6 and WT B6.SJL-*Ptprca^a Pepc^b*/BoyJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and B6.Cg/NTac-*Foxn1tm* NE10 (nude) mice were obtained from Taconic (Albany, NY, USA). Experimental animals were obtained by breeding female heterozygous with male homozygous $\alpha 7nAChR$ KO mice. Animal experiments were approved by the local Institutional Animal Care and Use Committee (IACUC).

Bone Marrow Transfer

BM donor mice, either B6.SJL-*Ptprca^a Pepc^b*/BoyJ (WT mice expressing CD45.1) or B6.129S7-*Chrna7^{tm1Bay}*/J ($\alpha 7nAChR$ -KO mice expressing CD45.2), were euthanized by CO₂ asphyxiation and BM harvested. Harvested BM was passed through a 70- μ m cell strainer (Fisher Scientific, Pittsburgh, PA, USA) into a 50-mL tube and pelleted. Lymphocytes were isolated by using Lympholyte-M (Cedarlane, Burlington, NC, USA) and resuspended in phosphate-buffered saline (PBS) at $1.5\text{--}2.5 \times 10^6$ BM cells per 100 μ L PBS.

Recipient mice were anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (8 mg/kg) and irradiated with 9 Gy by using an AECL 1000 Irradiator (Gammacell, Ot-

Table 1. Designation of chimeras produced by adoptive transfer.

Designation	Transfer setup		Resulting chimera	
	Recipient genotype	Transferred cells	Nervous system	Immune system
WT	WT	—	WT	WT
BM $\alpha 7^0$	WT	$\alpha 7nAChR^{-/-}$ CD45.2 BM	WT	$\alpha 7nAChR^{-/-}$
nBM $\alpha 7^0$	$\alpha 7nAChR^{-/-}$	WT CD45.1 BM	$\alpha 7nAChR^{-/-}$	WT
Nude	Nude	—	Nude	Nude
Twt Nude	Nude	WT CD45.1 CD4 ⁺ T cells	Nude	Nude with WT CD4 ⁺ T cells
T $\alpha 7^0$ Nude	Nude	$\alpha 7nAChR^{-/-}$ CD45.2 CD4 ⁺ T cells	Nude	Nude with $\alpha 7nAChR^{-/-}$ CD4 ⁺ T cells

tawa, ON, Canada). Twenty-four hours later, the mice were anesthetized again and injected with $3\text{--}5 \times 10^6$ BM cells via retro-orbital venous sinus injections. Animals were rested for 10 wks before experiments.

T-Cell Isolation and Transfer

T cells were isolated from spleens of WT and $\alpha 7nAChR$ KO donor mice using the Pan T-Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA, USA) with a purity of approximately 95%. Recipient C57BL/6 nude mice were anesthetized as described above, and $4\text{--}6 \times 10^6$ T cells in 200 μ L PBS were transferred via retro-orbital venous sinus injections. Five days after the transfer, mice were subjected to either vagus nerve stimulation or sham surgery. For coculture experiments, spleen T cells and T cell-depleted splenocytes were enriched by negative selection using the Pan T-cell Isolation Kit (negative selection of T cells) and CD90.2 microbeads (positive selection of T cells for depletion; Miltenyi Biotec), respectively, and were cocultured in 96-well plates in OptiMEM (Invitrogen, Grand Island, NY, USA) at 10^6 cells per well, incubated for 20 h and stimulated with 100 ng/mL lipopolysaccharide (LPS). Supernatants were saved for analysis.

Creation of Chimera by Adoptive Transfer of Bone Marrow

Chimeric mice with $\alpha 7nAChR$ deficiency in BM-derived cells (BM $\alpha 7^0$ mice) or non-BM-derived cells (nBM $\alpha 7^0$ mice)

were created by adoptive transfer of WT or $\alpha 7nAChR$ -deficient BM cells to $\alpha 7nAChR$ -deficient or WT mice (Table 1). In addition, WT or $\alpha 7nAChR$ -deficient CD4⁺ T cells were transferred to nude mice to create Twt and T $\alpha 7^0$ nude mice, respectively (Table 1). For BM transplantation, CD45.1⁺ WT and CD45.2⁺ $\alpha 7nAChR$ -deficient mice were used as donors.

Vagus Nerve Stimulation

Mice were anesthetized as described above. A midline cervical incision was made and the left carotid sheath, which contains the left cervical branch of the vagus nerve, was isolated. A bipolar cuff electrode (Microprobes, Gaithersburg, MD, USA) was secured around the carotid sheath, and 1 mA was applied at 10 Hz for 1 min (Setpoint Medical Stimulator, SetPoint Medical, Valencia, CA, USA). In sham-operated animals, a cervical incision was made, but the electrode was not applied. Subsequently, the incision was stapled closed, and mice recovered in a cage on top of a heating pad. Three hours later, 5 mg/kg endotoxin (LPS from *Escherichia coli*, 0111:B4; Sigma, St. Louis, MO, USA) was injected intraperitoneally, and mice were euthanized via CO₂ asphyxiation 90 min thereafter. Blood was obtained via cardiac puncture and spleens were removed.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum and supernatant TNF were measured using the TNF ELISA kit (R&D Systems).

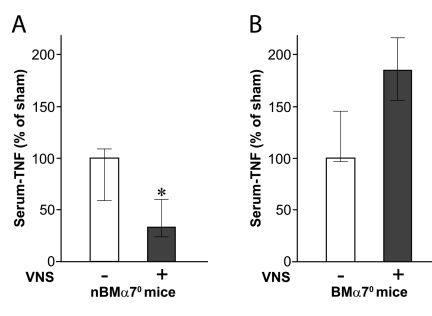


Figure 1. Effect of $\alpha 7$ nAChR deficiency in non-BM-derived cells and BM-derived cells on vagus nerve-mediated TNF suppression in endotoxemia. Mice with $\alpha 7$ nAChR deficiency either in non-BM-derived (nBM $\alpha 7^0$) (n = 6) (A) or BM-derived cells (BM $\alpha 7^0$) (n = 5) (B) were subjected to vagus nerve stimulation followed by intraperitoneal endotoxin injection. Serum TNF was measured 90 min after endotoxin administration. Median TNF levels in pg/mL (lower quartile–upper quartile) were Sham, 476 (279–521), and vagus nerve stimulation (VNS), 159 (114–287), in nBM $\alpha 7^0$ mice and Sham, 275 (265–400), and VNS, 508 (428–594), in BM $\alpha 7^0$ mice. Results in diagrams are expressed as the median TNF as percent of sham (lower quartile–upper quartile). * $P < 0.05$.

Flow Cytometry

Splenocytes were isolated as previously described (15) and stained with rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC, USA), rat anti-mouse CD3 (eBioscience, San Diego, CA, USA), mouse anti-mouse CD45.1 (BD Pharmingen, San Diego, CA, USA) and mouse anti-mouse CD45.2 (BD Pharmingen) antibodies. Cells were then washed and resuspended in PBS containing 2% fetal bovine serum and 0.09% sodium azide, acquired in a

BD LSR II flow cytometer and analyzed using FlowJo v9.3.1. Purity of T-cell and non-T-cell isolates was determined by using an anti-T cell receptor β (anti-TCR β) antibody (BD Pharmingen).

Statistics

Data are expressed as the median (lower to upper quartile) because of the asymmetric distribution. Differences between groups were analyzed using the Mann-Whitney U test. $P < 0.05$ was considered significant.

RESULTS

$\alpha 7$ nAChR in BM-Derived Cells Is Required for Vagus Nerve-Mediated Inhibition of TNF

nBM $\alpha 7^0$ mice were created by adoptive transfer of WT BM to irradiated $\alpha 7$ nAChR KO mice to obtain mice that express $\alpha 7$ nAChR in BM-derived cells, including T cells and macrophages, but not in neurons and other tissues (Table 1). Vagus nerve stimulation of these chimeric animals significantly reduced serum levels of endotoxin-induced TNF (Figure 1A). Because the BM transfer does not restore $\alpha 7$ nAChR expression in neurons, these observations indicate that neural expression of $\alpha 7$ nAChR is not required for the integrity of the inflammatory reflex. Subsequently, BM $\alpha 7^0$ mice were created by adoptive transfer of $\alpha 7$ nAChR-deficient BM to irradiated WT mice to obtain animals with WT expression of $\alpha 7$ nAChR in neurons and all other tissues, but not in BM-derived cells (Table 1). Vagus nerve stimulation failed to inhibit serum TNF

levels in these BM $\alpha 7^0$ animals (Figure 1B). Flow cytometry analysis of splenocytes from chimeric mice 10 wks after transfer showed that $\geq 84\%$ of macrophages and $\geq 70\%$ of T cells were derived from donor mice and cell fractions were similar between the chimeras (Table 2). Together, these results indicate that $\alpha 7$ nAChR expression in BM-derived cells, but not neurons, is required for the functional integrity of the inflammatory reflex.

$\alpha 7$ nAChR Expression on T Cells Does Not Affect Endotoxin-Induced TNF Production

We recently discovered that acetylcholine producing T cells are necessary for the functional integrity of the inflammatory reflex (15). An abundance of data implicates β adrenergic receptors expressed on these T cells as pivotal for their activation to produce acetylcholine, but it remained theoretically possible that T-cell $\alpha 7$ nAChR expression (18) is also required for the integrity of the inflammatory reflex. To address this question, we adoptively transferred WT and $\alpha 7$ nAChR-deficient T cells to nude mice. Comparable numbers of T cells were observed in the spleen after transfers (Table 3). As expected from previous work (15), vagus nerve stimulation failed to reduce endotoxin-induced serum TNF levels in nude mice, indicating that the inflammatory reflex is functionally impaired in this strain (Figure 2A). Adoptive transfer of WT T cells into nude mice (to create Twt nude mice) restored the ability of vagus nerve stimulation to significantly inhibit TNF (Figure 2B), confirming that T cells are necessary for the inhibition of TNF mediated by vagus nerve stimulation. We then transferred T cells isolated from $\alpha 7$ nAChR-deficient mice into nude mice

Table 2. Repopulation after adoptive transfer of BM.

	Macrophages			T cells		
	Sham	VNS	P	Sham	VNS	P
BM $\alpha 7^0$ CD45.1 ⁺	11 \pm 0.9	13 \pm 2.4	0.42	30 \pm 1.6	29 \pm 2.9	0.80
BM $\alpha 7^0$ CD45.2 ⁺	86 \pm 1.1	84 \pm 2.9	0.83	70 \pm 1.5	71 \pm 2.7	0.49
nBM $\alpha 7^0$ CD45.1 ⁺	92 \pm 0.8	93 \pm 0.6	0.23	82 \pm 1.0	82 \pm 2.5	0.82
nBM $\alpha 7^0$ CD45.2 ⁺	4.0 \pm 0.6	5.0 \pm 0.7	0.55	18 \pm 1.0	17 \pm 2.4	0.66

Splenocytes from recipient mice were analyzed 10 wks after transfer. Numbers are mean percent \pm SEM of CD45.1⁺ (WT) and CD45.2⁺ ($\alpha 7$ nAChR-deficient) cells of macrophages or T cells as indicated.

Table 3. Transfer of T cells to nude mice.

	Sham	VNS	P
Twt nude mice	3.6 \pm 0.2	3.0 \pm 0.3	0.13
T $\alpha 7^0$ nude mice	4.8 \pm 0.5	5.0 \pm 0.3	0.77

Numbers are mean percent T cells of total splenocytes \pm SEM.

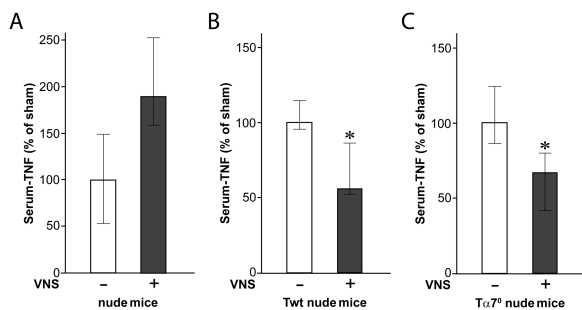


Figure 2. Effect of transfer of $\alpha 7nAChR$ -deficient T cells to nude mice on vagus nerve-mediated TNF suppression in endotoxemia. Nude mice were subjected to vagus nerve stimulation (VNS) followed by endotoxemia in the presence or absence of functional T cells. TNF was measured in serum 90 min after endotoxin administration. (A) Nude mice ($n = 4$). Median TNF levels in pg/mL (lower quartile–upper quartile) were Sham, 324 (170–483), and VNS, 621 (512–816) ($P = 0.08$). (B) Nude mice after transfer of WT T cells (Twt) ($n = 5$ sham and $n = 6$ VNS). TNF levels were Sham, 1,120 (1,070–1,290), and VNS, 630 (589–974) ($P = 0.03$). (C) Nude mice after transfer of $\alpha 7nAChR$ -deficient T cells (T $\alpha 7^0$) ($n = 6$). TNF levels were Sham, 831 (715–1,030), and VNS, 556 (346–665) ($P = 0.04$). Results in diagrams are expressed as median TNF as percent of sham (lower quartile–upper quartile). * $P < 0.05$.

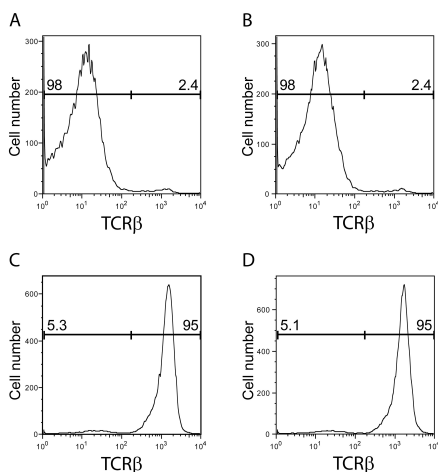


Figure 3. Purity of isolated murine splenocyte fractions. Cells were stained with an anti-TCR β antibody and analyzed by flow cytometry. T-cell-depleted splenocytes from WT (A) and $\alpha 7nAChR$ KO mice (B) are shown. T-cell fraction from WT mice (C) and $\alpha 7nAChR$ KO mice (D) are shown. The numbers in the graph show fractions of TCR β ⁻ and TCR β ⁺ cells.

to create T $\alpha 7^0$ nude mice. Vagus nerve stimulation inhibited TNF production to a similar degree also in T $\alpha 7^0$ nude mice (Figure 2C). Together, these data demonstrate that $\alpha 7nAChR$ expression on T cells is not necessary for vagus-nerve mediated

icantly inhibited endotoxin-induced TNF production ($P < 0.05$, Figure 4). The magnitude of inhibition was similar after addition of either group of T cells. Collectively, these findings indicate that $\alpha 7nAChR$ expression on T cells is not required for T cell-mediated inhibition of TNF.

DISCUSSION

This study reveals that $\alpha 7nAChR$ expression in BM-derived non-T cells is necessary for the function of the inflammatory reflex. $\alpha 7nAChR$ in neurons and other non-BM derived cells and on T cells is expendable in vagus nerve stimulation-mediated inhibition of cytokine production. The results are consistent with earlier pharmacological data on cytokine producing cells and implicate a role for $\alpha 7nAChR$ signaling in mediating inhibition of cell activation.

The efferent arc of the inflammatory reflex, termed the “cholinergic antiinflammatory pathway,” can be stimulated using electrical vagus nerve stimulators to prevent or reverse damage in experimental endotoxemia, sepsis, pancreatitis, arthritis, colitis and other inflammatory syndromes (21). The spleen is a major organ target for the antiinflammatory effects of efferent vagus nerve signals in endotoxemia because the spleen is the major source of TNF during endotoxemia and efferent vagus signals control production of TNF there (22). Administration of $\alpha 7nAChR$ agonists also suppresses cytokine release and attenuates tissue damage during inflammation (5,8,17). Deficiency or impairment of $\alpha 7nAChR$ signaling, or the cholinergic antiinflammatory pathway, leads to overproduction of cytokines and enhances tissue damage (16,17).

Whereas it seemed likely that functional expression of $\alpha 7nAChR$ by macrophages and cytokine-producing cells accounted for the mechanism, it could not be ruled out that $\alpha 7nAChR$ expressed in the brain and autonomic ganglia (23,24) was required to complete the cholinergic antiinflammatory circuit. For instance, vagus nerve stimulation in an intact animal elicits both efferent and afferent signals, and it would be conceivable that $\alpha 7nAChRs$ in

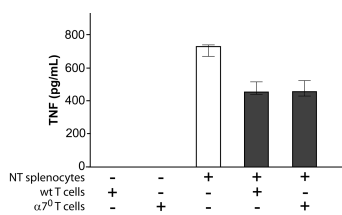


Figure 4. Effect of T-cell $\alpha 7nAChR$ deficiency on suppression of endotoxin-induced TNF production *in vitro*. T cells from WT or $\alpha 7nAChR$ -deficient mice were cocultured with T cell-depleted WT splenocytes, stimulated with endotoxin and TNF measured in the supernatant. Results are shown as median TNF in pg/mL (lower quartile–upper quartile). NT, T cell depleted; $\alpha 7^0$, $\alpha 7nAChR$ KO.

TNF suppression *in vivo*. Furthermore, T cells have been implicated in suppressing innate immune responses (19,20), and it is conceivable that $\alpha 7nAChR$ would be required for the effect. To investigate this, T cells were isolated from total splenocytes of $\alpha 7nAChR$ -deficient mice and then cocultured with T cell-depleted WT splenocytes. T-cell fractions were $\geq 94\%$ pure and non-T-cell fractions were $\geq 97\%$ pure, as assessed by fluorescence-activated cell sorting (Figure 3). Addition of either WT or $\alpha 7nAChR$ -deficient T cells to cultures of T cell-depleted splenocytes signif-

the central nervous system are involved in processing of afferent signals that ultimately result in efferent activity in the contralateral vagus or other nerves. In addition, $\alpha 7$ nAChR expressed in neurons in the celiac ganglion might receive acetylcholine signals released from descending vagus or sympathetic nerve endings that terminate there. Further, the splenic nerve is adrenergic, not cholinergic (25), and it has been unclear how signals from the cholinergic vagus nerve could be sensed by $\alpha 7$ nAChR in the spleen. We recently demonstrated that vagus nerve stimulation indeed increases splenic acetylcholine levels and that a subset of acetylcholine-producing T cells are required for the efferent signals in the inflammatory reflex (15). Thus, we resolved the question of how signals from the cholinergic vagus nerve could be sensed by $\alpha 7$ nAChR on cytokine-producing splenic macrophages. The present study advances mechanistic understanding and shows that $\alpha 7$ nAChR expression in immune cells, but not in neurons or T cells, is required for the functional integrity of the inflammatory reflex.

T cells can inhibit innate immune responses (19,20), as confirmed by the coculture experiments in this study. A subset of splenic acetylcholine-producing T cells respond to adrenergic neural signals (15), and it had been suggested that T-cell $\alpha 7$ nAChR expression might be important for the cholinergic antiinflammatory pathway (26,27). The present findings indicate that the integrity of the efferent arm of the inflammatory reflex and the antiinflammatory effects of T cells are independent of T-cell $\alpha 7$ nAChR. Because norepinephrine can increase production of acetylcholine in select T cells, it is conceivable that activation of β -adrenergic or other neurotransmitter receptors other than $\alpha 7$ nAChR contributes to the antiinflammatory effects of T cells (15,18).

CONCLUSION

Thus, the inflammatory reflex requires $\alpha 7$ nAChR expression on non-T cell, BM-derived immune cells. Together with our recent findings on splenic acetylcholine-producing T cells, these data re-

solve the mechanism for signal transfer from the vagus nerve to TNF-producing cells in the spleen.

ACKNOWLEDGMENTS

This work was supported in part by grants from National Institute of General Medical Sciences, National Institutes of Health (NIGMS, NIH; GM57226 and 3R01GM057226-10S1) to KJ Tracey and from the Wenner-Gren Foundations in Stockholm to PS Olofsson.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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