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Xanomeline suppresses excessive pro-inflammatory cytokine responses through neural signal-mediated pathways and improves survival in lethal inflammation

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Abstract

Inflammatory conditions characterized by excessive immune cell activation and cytokine release, are associated with bidirectional immune system-brain communication, underlying sickness behavior and other physiological responses. The vagus nerve has an important role in this communication by conveying sensory information to the brain, and brain-derived immunoregulatory signals that suppress peripheral cytokine levels and inflammation. Brain muscarinic acetylcholine receptor (mAChR)-mediated cholinergic signaling has been implicated in this regulation. However, the possibility of controlling inflammation by peripheral administration of centrally-acting mAChR agonists is unexplored. To provide insight we used the centrally-acting M1 mAChR agonist xanomeline, previously developed in the context of Alzheimer's disease and schizophrenia. Intraperitoneal administration of xanomeline significantly suppressed serum and splenic TNF levels, alleviated sickness behavior, and increased survival during lethal murine endotoxemia. The anti-inflammatory effects of xanomeline were brain mAChR-mediated and required intact vagus nerve and splenic nerve signaling. The anti-inflammatory efficacy of xanomeline was retained for at least 20h, associated with alterations in splenic lymphocyte, and dendritic cell proportions, and decreased splenocyte responsiveness to endotoxin. These results highlight an important role of the M1 mAChR in a neural circuitry to spleen in which brain cholinergic activation lowers peripheral pro-inflammatory cytokines to levels favoring survival. The therapeutic efficacy of xanomeline was also manifested by significantly improved survival in preclinical settings of severe sepsis. These findings are of interest for strategizing novel therapeutic approaches in inflammatory diseases.

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Keywords

xanomeline; brain muscarinic acetylcholine receptors; vagus nerve; splenic nerve; cytokines; inflammation; sickness behavior; endotoxemia; sepsis

1. Introduction

The release of cytokines is an important constituent of innate immune activation and protective inflammatory responses triggered by infection or tissue damage (Tracey, 2002). However, the overproduction of TNF and other pro-inflammatory cytokines by activated macrophages and other immune cells, and their systemic release can be deleterious and associated with development of inflammatory disorders (Tracey, 2002). Therefore, preventing and neutralizing excessive pro-inflammatory cytokine release is an important therapeutic strategy in inflammatory diseases (Tracey, 2002). Inflammatory conditions are associated with bidirectional immune system-brain communication (Goehler *et al.*, 2000; Kelley and McCusker, 2014; Tracey, 2002; McCusker and Kelley, 2013; Steinman, 2012; Capuron and Miller, 2011; Raison and Miller, 2013). Excessive pro-inflammatory cytokine release frequently results in alterations of brain function, manifested by fever and sickness behavior, and the vagus nerve plays a mediating role in conveying cytokine signals to the brain (Goehler *et al.*, 1999; Goehler *et al.*, 2000; Kelley and McCusker, 2014; McCusker and Kelley, 2013). Brain-derived neural anti-inflammatory mechanisms function to suppress peripheral cytokine responses (Pavlov *et al.*, 2003; Kelley and McCusker, 2014; Tracey, 2009; Pavlov and Tracey, 2004; Olofsson *et al.*, 2012). Efferent vagus nerve-splenic nerve interaction within the cholinergic anti-inflammatory pathway is an essential constituent of these mechanisms during endotoxemia and other inflammatory conditions (Pavlov and Tracey, 2005; Pavlov and Tracey, 2006; Tracey, 2007; Rosas-Ballina *et al.*, 2008; Rosas-Ballina *et al.*, 2011). Accordingly, vagus nerve stimulation and cholinergic modalities have been implicated in the experimental treatment of several inflammatory conditions, including sepsis, inflammatory bowel disease, rheumatoid arthritis, obesity, and type 2 diabetes (Pavlov, 2008; Pavlov and Tracey, 2012; Satapathy *et al.*, 2011; Andersson and Tracey, 2012; Ji *et al.*, 2014).

The neural regulation of inflammation is integrated in the brain, and brain muscarinic acetylcholine receptors (mAChRs) have been implicated in the neural control of peripheral pro-inflammatory cytokine levels (Pavlov *et al.*, 2006; Ji *et al.*, 2014). However, the anti-inflammatory efficacy of peripheral administration of centrally-acting mAChR agonists is not evaluated. Here we studied the anti-inflammatory effects of the centrally-acting M1 mAChR agonist xanomeline (Shannon *et al.*, 1994; Farde *et al.*, 1996). This compound has previously been developed as an experimental therapeutic for the treatment of Alzheimer's disease (Shannon *et al.*, 1994; Bodick *et al.*, 1997a; Frederick *et al.*, 2002). In addition, the efficacy of xanomeline in improving positive and negative syndromes, as well as cognitive symptom clusters in patients with schizophrenia is considered a breakthrough (Shekhar *et al.*, 2008; McKinzie and Bymaster, 2012; Jones *et al.*, 2012). We show that peripheral administration of xanomeline reduces pro-inflammatory cytokine levels, increases survival, and attenuates sickness behavior in lethal murine endotoxemia. The anti-inflammatory

effects of xanomeline are brain mAChR-mediated and require intact vagus nerve and splenic nerve signaling. Xanomeline administration also provides a relatively long-lasting (at least 20h) anti-inflammatory “priming”, associated with alterations in splenic lymphocyte and dendritic cell proportions, and decreased splenocyte responsiveness to endotoxin. In addition, xanomeline treatment in a clinically relevant time frame also improves survival in a murine model of polymicrobial sepsis. These findings extend and advance our understanding of the neural cholinergic regulation of cytokine responses and inflammation.

2. Materials and Methods

2.1. Animals

Male BALB/c mice (24–28g, Taconic) and male Sprague–Dawley rats (300–380g, Charles River) were used in experiments. Animals were allowed to acclimate for at least two weeks prior to the corresponding experiment. All animals were housed in standard conditions (room temperature 22°C with a 12h light–dark cycle) with access to regular chow and water. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Feinstein Institute for Medical Research, Manhasset, NY.

2.2. Chemicals

The M1 mAChR agonists xanomeline and AF267B (Fisher *et al.*, 2002; Caccamo *et al.*, 2006) were synthesized in the laboratory of Medicinal Chemistry at the Feinstein Institute for Medical Research by following previously published methodology (Supplementary Figures 1 and 2) and oxalate salt forms of the compounds were used in experiments. Atropine sulfate and atropine methyl nitrate were purchased from Sigma. Galantamine (Galantamine hydrobromide) was purchased from Calbiochem.

2.3. Endotoxemia and drug treatment

Endotoxemia in mice and rats was induced by administering LPS (endotoxin, Sigma L4130 0111:B4, 6 mg/kg, i.p.). Groups of animals were treated i.p. with sterile saline (controls), or different doses of xanomeline or AF267B, 1h prior to endotoxin administration. In other experiments sterile saline, xanomeline (20 mg/kg) or galantamine (4 mg/kg) were injected i.p. 20h or 30h prior to endotoxin. Animals were euthanized by CO₂ asphyxiation 1.5h after endotoxin administration, and blood was collected via cardiac puncture for cytokine determination. In other sets of survival experiments, groups of mice were treated with sterile saline, xanomeline (i.p.) or AF267B (i.p.) 1h prior to endotoxin (6 mg/kg, i.p.) injection. Where indicated in cytokine analysis and survival experiments, mice were pretreated with atropine sulfate or atropine methyl nitrate (4 mg/kg, i.p.) 15 min prior to xanomeline treatment. Mice were monitored for survival and clinical manifestations of endotoxemia, including lethargy, piloerection, and huddling twice daily for the first 7 days, and then daily for the remainder of the 14 day experiments.

2.4. Vagotomy

Cervical unilateral (right side) vagotomy was performed as previously described (Pavlov *et al.*, 2009). Mice were anesthetized by isoflurane inhalation and the right cervical vagus nerve was exposed, ligated with a 4-0 silk suture, and divided. In sham-operated animals, the cervical vagus nerve was visualized, but neither was isolated from the surrounding tissues nor transected. All animals were permitted to recover for 7 days following the surgical procedure and before their inclusion in endotoxemic experiments.

2.5. Splenic nerve transection

Splenic nerve transection was performed as previously described (Rosas-Ballina *et al.*, 2008). Rats were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and the peritoneal cavity was accessed through a midline abdominal incision. After the main branches of the splenic vessels were identified and isolated, the splenic nerve, which traverses along the splenic artery, was incised at several points with forceps. In sham-operated rats, the splenic vessels were isolated, but the nerve was left intact. Animals were permitted to recover for 7 days following the surgical procedure and before their inclusion in endotoxemic experiments. To assess the efficacy of splenic nerve transection, spleens were treated with the glyoxylic acid to visualize the catecholaminergic fibers (de la Torre, 1980; Rosas-Ballina *et al.*, 2008). Only animals whose spleens showed complete absence of catecholamines were included in the analysis.

2.6. Spleen and blood sample preparations for flow cytometry analysis

Spleen and whole blood were collected immediately after euthanasia. Whole blood was obtained by cardiac puncture in EDTA-coated tubes (Capiject®, Terumo Medical, Summerset, NJ) and centrifuged (1500rpm for 5min); supernatants were collected and transferred to dry microcentrifuge tubes and spun again (3000rpm for 5min). Supernatants were frozen immediately at -80°C until analysis. Spleens were collected under aseptic technique and processed as described previously (Valdes-Ferrer *et al.*, 2013b). In brief, spleens were flushed with 1ml of sterile PBS+0.5mM EDTA (PBE) and cells extracted by gentle pressure between glass slides. The obtained material was then resuspended in 10ml of PBE and passed through a 40µm filter, and centrifuged at 1500rpm for 5 min. In order to eliminate RBCs, the cell pellet was resuspended in 10ml of lysis buffer (5PRIME, Hamburg, Germany) for 10min on ice. Cells were washed once with PBS and counted. For cell analysis, 10⁵ cells were incubated for 15min in 50µl of staining buffer with Fc block (BD biosciences, San Jose, CA). Cells were stained for flow cytometry using various combinations of the following antibodies: FITC-conjugated Rat anti-Mouse CD3 (BD Biosciences, San Jose, CA); PE-Cy7-conjugated Rat anti-Mouse CD19 (BD Biosciences, San Jose, CA); FITC-conjugated Rat anti-Mouse Ly-6C (eBioscience, San Diego, CA); Pacific Blue-conjugated Rat anti-Mouse CD11b (BD Biosciences, San Jose, CA); PE-conjugated Rat anti-Mouse CD11b (BD Biosciences, San Jose, CA); PE -conjugated Rat anti-Mouse Ly-6G (BD Biosciences, San Jose, CA); APC-conjugated CD11c (BD Bioscience, San Jose, CA); and PE-Cy7-conjugated Rat anti-Mouse F4/80 (eBioscience, San Diego, CA). Data were acquired using a LSRII flow cytometer (BD Biosciences, San Jose, CA), and analyzed with FlowJo version 9.0 (Tree Star, Inc). Five cell groups were

characterized as previously described (Valdes-Ferrer *et al.*, 2013b; Swirski *et al.*, 2009; Eberlein *et al.*, 2010): B-cells (CD3^{neg}, CD19⁺); T-cells (CD3⁺, CD19^{neg}); dendritic (DC) cells (CD11b⁺, F4/80⁺, Ly-6G⁺, CD11c⁺) cells; polymorphonuclear (PMN) cells (CD3^{neg}, CD19^{neg}, CD11b⁺, Ly-6G⁺); and monocytes (CD3^{neg}, CD19^{neg}, CD11b⁺, Ly-6G^{neg}, Ly-6C⁺)/macrophages (CD11b⁺ F480⁺)

2.7. Ex-vivo endotoxin challenge

Splenocytes were isolated as previously described (Valdes-Ferrer *et al.*, 2013a). Ex-vivo challenge was performed in RPMI supplemented with 10% FCS, penicillin 100 U/mL, and streptomycin (100 µg/mL; Gibco). 2×10^5 spleen cells were incubated for 24 h in 200 µL of medium alone, or medium containing endotoxin (25 ng/ml) in flat-bottom sterile 96 well plates for 24h at 37°C, 5% CO₂. After 24h, supernatants were collected and preserved immediately at -80°C until analysis.

2.8. Cytokine determination

Blood, collected via cardiac puncture immediately after euthanasia was allowed to clot for 1h and 15 min and then centrifuged at 5,000 rpm (1,500×g) for 10 min. Supernatants (sera) were subsequently collected and kept at -80 °C prior to serum TNF or IL-6 determination by ELISA (R&D Systems) according to the manufacturer's recommendations. The *ex vivo* endotoxin-induced splenocyte production of TNF, IL-6, IL-10, IL-12p70, IFN-γ, monocyte chemotactic protein 1 (MCP1, also known as CCL2) was measured by flow cytometry-based bead assay using the Mouse Inflammation Kit (BD Biosciences, San Jose, CA) and analyzed using a FACSArray instrument (BD Biosciences, San Jose, CA).

2.9. Cecal ligation and puncture surgery and drug treatment

A standardized model of cecal ligation and puncture (CLP)-induced severe polymicrobial sepsis was used (Pavlov *et al.*, 2007; Parrish *et al.*, 2008). Mice were anesthetized using ketamine 100 mg/kg and xylazine 8 mg/kg, administered intramuscularly. Abdominal access was gained via a midline incision. The cecum was isolated and ligated with a 6-0 silk ligature below the ileocecal valve and then punctured once with a 22G needle. Stool (approximately 1 mm) was extruded from the hole, and the cecum placed back into the abdominal cavity. The abdomen was closed with two layers of 6-0 Ethilon sutures. An antibiotic (Imipenem-Cilastatin, 0.5 mg/kg, subcutaneously, in a total volume of 0.5 ml/mouse) was administered immediately after CLP as part of the resuscitation fluid. In some experiments mice were injected i.p. with sterile saline or xanomeline (2, 5 or 20 mg/kg) 20h prior to CLP. In other sets of experiments mice were randomized 24h after CLP and then two therapeutic regimens of drug treatments were used. In the first regimen, mice were injected i.p. with either sterile saline or xanomeline (1, 2, 5, or 20 mg/kg, i.p.) twice daily for 3 consecutive days. In the second regimen, mice were injected i.p. with sterile saline or xanomeline (1 or 2 mg/kg) at 24h, 30h and 46h following CLP. Mice were monitored for survival and sepsis-associated clinical signs twice daily for the first 7 days, and then daily for the remaining of 14 day experiments.

2.10. Statistical analysis

Values are presented as mean $\pm$ SEM. ANOVA, followed by a post-hoc analysis was used for multiple comparisons. Student's *t*-test was used for two groups of data comparison. Survival experiments were analyzed by Log-Rank test. All tests were performed using GraphPad Prism. *P* values equal or below 0.05 were considered significant

3. Results

3.1. Peripheral administration of centrally-acting M1 mAChR agonists suppresses systemic TNF levels and improves survival in endotoxemia through a central mAChR-dependent mechanism

We first examined whether intraperitoneal (i.p.) administration of xanomeline alters systemic pro-inflammatory cytokine responses. Xanomeline was administered 1h prior to a lethal endotoxin dose (6 mg/kg, i.p.) in mice and serum TNF analyzed 1.5h later. Xanomeline significantly and dose-dependently reduced serum TNF levels as compared to control saline-administered mice (Figure 1A). In addition to decreasing serum TNF, xanomeline dose-dependently lowered serum IL-6 in endotoxemic mice, thus indicating the broader scope of xanomeline effects on cytokine responses (Supplementary Figure 3). We next examined whether these effects were associated with improved survival. Xanomeline i.p. administration 1h prior to endotoxin (6 mg/kg, i.p.) significantly (up to 100%) and dose-dependently improved survival as compared to saline administration (Figure 1B). This improvement of the survival rate by xanomeline treatment was accompanied by marked alleviation of decreased locomotor activity, lethargy, piloerection, and huddling – components of sickness behavior, preceding mortality. Furthermore, i.p. treatment with AF267B, a structurally-different (Supplementary Figure 2) centrally-acting M1 mAChR agonist (Fisher *et al.*, 2002; Caccamo *et al.*, 2006) 1h prior to endotoxin (6 mg/kg, i.p.), also resulted in a dose-dependent suppression of serum TNF levels, improved survival (Supplementary Figure 4A,B), and alleviated sickness behavior in endotoxemic animals. To evaluate the role of CNS versus peripheral mAChRs in mediating the anti-inflammatory effects of xanomeline we used atropine sulfate, a mAChR antagonist that crosses the blood-brain barrier and atropine methyl nitrate, a mAChR antagonist that does not penetrate it. Pre-treatment with atropine sulfate (4 mg/kg, i.p.) abolished the suppressing effect of xanomeline on serum TNF in endotoxemic mice (Figure 1C). Pretreatment with the same dose atropine methyl nitrate did not significantly alter the magnitude of this anti-inflammatory effect (Figure 1C). By using this approach we also found that brain mAChR mediate the survival-improving effect of xanomeline (Figure 1D). Neither atropine sulfate nor atropine methyl nitrate administration alone altered significantly survival in endotoxemia (Supplementary Figure 5). Together these results show the anti-inflammatory efficacy of a peripherally administered centrally-acting M1 mAChR agonist through a CNS mAChRs-mediated mechanism.

3.2. Anti-inflammatory effects of xanomeline are mediated through neural signaling to spleen

A role for the vagus nerve-based cholinergic anti-inflammatory pathway as a communication circuit translating CNS mAChR-mediated signaling into suppression of

excessive peripheral inflammation has been previously suggested (Pavlov *et al.*, 2006; Pavlov *et al.*, 2009). Suppression of splenic TNF by activation of this cholinergic anti-inflammatory pathway has a major contribution to alleviation of systemic inflammatory responses in endotoxemia (Huston *et al.*, 2006). Therefore, we examined the effect of xanomeline on splenic TNF levels. Xanomeline (20 mg/kg, i.p.) administered 1h prior to LPS (6 mg/kg, i.p.) significantly reduced splenic TNF levels as compared to saline administration (Figure 2A). We next examined whether surgical transection of the vagus nerve (vagotomy) alters this xanomeline effect. As shown on Figure 2B, in contrast to sham-operated controls, xanomeline administration in animals with cervical unilateral vagotomy failed to reduce splenic TNF significantly (Figure 2B). Functional interaction between vagus nerve and splenic nerve signaling is required for the cholinergic anti-inflammatory pathway in endotoxemic rats (Rosas-Ballina *et al.*, 2008). Therefore, we next studied whether an intact splenic nerve is required for xanomeline anti-inflammatory effects. Dose dependency of xanomeline effects in suppressing rat serum and splenic TNF levels was achieved by administering lower (1–5 mg/kg) drug doses (as compared to doses in mice) prior to endotoxin (Figure 2C,D). Xanomeline (20 or 10 mg/kg, i.p.) administration resulted in further dramatic suppression of serum TNF levels (Supplementary Figure 6). While xanomeline significantly reduced serum TNF levels in sham-operated endotoxemic rats, it failed to exert this effect in animals with splenic nerve transection (Figure 2E). Accordingly, in contrast to suppression of splenic TNF in sham-operated endotoxemic animals, xanomeline failed to significantly lower splenic TNF in rats with splenic nerve transection (Figure 2F). These results indicate a role of vagus and splenic nerve signaling in mediating the anti-inflammatory effects of xanomeline.

3.3. Xanomeline administration provides long-term anti-inflammatory “priming”

To provide insight into the time window of anti-inflammatory protection by xanomeline we examined whether administration of xanomeline as early as 20h prior to endotoxin alters cytokine responses. Xanomeline (20mg/kg, i.p.) administered at this early time point significantly suppressed serum TNF levels (Figure 3A). Administration of this compound (20 mg/kg, i.p.) 20h before endotoxin also significantly improved survival (Figure 3B). When xanomeline (20 mg/kg, i.p.) was administered at 30h prior to endotoxin, it failed to significantly alter the survival rate (Figure 3C). We next tested whether the anti-inflammatory efficacy of another approach of brain cholinergic and mAChR-mediated activation is also retained within comparable time frame. We used galantamine, a centrally-acting cholinesterase inhibitor, whose anti-inflammatory effects require signaling through brain mAChRs and the vagus nerve (Pavlov *et al.*, 2009). Galantamine (4 mg/kg, i.p.) injected 20h prior to LPS in mice significantly suppressed serum TNF levels and improved survival, but failed to affect survival when administered 30h before LPS (Supplementary Figure 7). We further examined the contribution of the spleen – a major source of TNF and a target of the cholinergic anti-inflammatory pathway during endotoxemia (Huston *et al.*, 2006; Rosas-Ballina *et al.*, 2008; Rosas-Ballina *et al.*, 2011) in the anti-inflammatory “priming” by xanomeline. Xanomeline early (–20h) administration in mice resulted in a significant increase in the proportion of T cells and a decrease in the proportion of B cells and dendritic cells in spleen (Figure 3D). In addition, this xanomeline treatment rendered splenocytes significantly less sensitive to inflammatory activation as demonstrated by lower

TNF, IFN- γ , MCP1, IL-6 and IL-10 release from *ex vivo* endotoxin-stimulated total spleen cells (Figure 3E). Together these results demonstrate a previously unrecognized priming anti-inflammatory effect of xanomeline associated with altered splenic leukocyte subpopulations and reduced splenocyte inflammatory activation.

3.4. Xanomeline improves survival in mice with severe sepsis

We further evaluated the efficacy of xanomeline in preclinical settings of sepsis, a lethal syndrome characterized by dysregulated immune responses and a number one killer in the intensive care units (Vincent *et al.*, 2013). Cecal ligation and puncture (CLP) is a widely used preclinical model of severe polymicrobial sepsis (Rittirsch *et al.*, 2009). Based on the long-lasting anti-inflammatory “priming” effect of xanomeline in the context of endotoxemia we first examined whether xanomeline administration at 20h prior to CLP alters sepsis severity. Single administration of xanomeline (20mg/kg, i.p.) 20h prior to CLP resulted in worsened survival rate as compared to the saline-treated group (Figure 4A). Administration of lower xanomeline doses (5 or 2mg/kg) at the same time point (20h) prior to CLP did not result in significant survival alterations (Figure 4B). Next, we examined the therapeutic efficacy of xanomeline in the treatment of mice with already developed severe sepsis and associated sickness behavior, manifested by lethargy, piloerection, huddling and malaise. Mice were randomized 24h after CLP and treated with xanomeline or saline. The highest drug dose (20 mg/kg) tested in the context of endotoxemia did not alter survival in severe sepsis as compared to saline administration (data not shown). However, treatments with lower drug doses (2 or 5 mg/kg) initiated 24h after CLP surgery and continued twice daily for 3 consecutive days significantly improved survival (Figure 4C) and attenuated sepsis-associated clinical signs. The lack of significant effect of 1 mg/kg xanomeline indicated the dose-dependency of these drug effects (Figure 4C). Furthermore, drug administration within a shorter time window, at 24h, 30h and 46h after the CLP surgery, was sufficient to improve survival dose-dependently (Figure 4D). The improved survival rate correlated with attenuation of sickness behavior. Together these results indicate that while single xanomeline administration as a preventive approach is inefficient in alleviating sepsis severity, drug treatments initiated in a clinically-relevant time frame significantly improve survival of mice with CLP-induced sepsis.

4. Discussion

Here, we show that peripheral administration of xanomeline, a centrally-acting M1 mAChR agonist triggers neuro-immune interaction that results in a significant anti-inflammatory protection. In this interaction CNS cholinergic activation through mAChRs is associated with vagus and splenic nerve signaling, resulting in suppression of excessive cytokine responses, alleviated sickness behavior, and improved survival.

These results, together with data indicating the anti-inflammatory efficacy of AF267B – a structurally-different, centrally-acting M1 mAChR agonist, validate targeting brain mAChRs by a peripherally administered compound as an approach in treating inflammatory conditions. The “net” effects of peripheral administration of xanomeline, a M1 mAChR agonist with extensive CNS penetration, in non-anesthetized rodents, are: lower pro-

inflammatory cytokine levels, improved survival, and mitigated sickness behavior during lethal inflammation. Previous preclinical studies have reported a transient decrease in animal locomotor activity following xanomeline administration, mediated through brain mAChRs (Maehara *et al.*, 2011). This xanomeline effect was also observed in our experiments, especially after injecting higher drug doses. Interestingly, this temporary xanomeline effect correlated with attenuation of endotoxin-induced decreased locomotor activity and lethargy. Xanomeline, as a M1 mAChR agonist, has also been previously shown not to alter body temperature and cause hypothermia (Shannon *et al.*, 1994), indicating no effect on CNS temperature regulation, in which the M2 subtype of mAChRs has been implicated (Gomez *et al.*, 1999). These findings suggest that xanomeline treatments would not exacerbate endotoxemia- and sepsis-associated hypothermia (Saito *et al.*, 2003). Our results point to brain mAChRs as a mediating component of xanomeline anti-inflammatory effects. In addition to the CNS, mAChRs are expressed in the periphery and on immune cells (Kawashima *et al.*, 2012). However, immune cell M1/M5 mAChR deficiency has been associated with decreased TNF and IL-6 secretion (Kawashima *et al.*, 2012), suggesting a tonic pro-inflammatory role of these receptors.

Our results also indicate that the anti-inflammatory effects of xanomeline in endotoxemia require signaling through the vagus nerve and the splenic nerve. Previous studies have revealed that the vagus nerve is functionally associated with the splenic nerve within the cholinergic anti-inflammatory pathway, which suppresses splenic TNF during endotoxemia (Rosas-Ballina *et al.*, 2008; Rosas-Ballina *et al.*, 2011). The right vagus nerve, through abdominal posterior celiac branches, contributes innervations to the celiac and superior mesenteric ganglia (Berthoud and Powley, 1993), where the splenic nerve arises (Bellinger *et al.*, 1989). Accordingly, unilateral (right side) vagotomy has been used in studying the anti-inflammatory role of the vagus nerve (Pavlov *et al.*, 2009; Su *et al.*, 2010; Mirakaj *et al.*, 2014) to avoid potential lethality associated with cervical bilateral vagotomy. Here we show that xanomeline significantly suppresses splenic TNF levels, and vagus nerve signaling is a mediating component because cervical unilateral vagotomy significantly attenuated this effect. Furthermore, splenic nerve transection also resulted in reduced anti-inflammatory efficacy of xanomeline. These results indicate that xanomeline can be regarded as a brain activator of the cholinergic anti-inflammatory pathway.

Previous studies have shown a complex interaction between xanomeline and the M1 mAChR (Christopoulos *et al.*, 1998; Grant *et al.*, 2010). This interaction is characterized by binding of xanomeline to the orthosteric and an allosteric site of the receptor molecule, which results in wash-resistant long-term M1 mAChR modulation (Christopoulos *et al.*, 1998; Grant *et al.*, 2010). A recent study has shown that the longterm modulation of the M1 AChR by xanomeline is abolished by blockade of the orthosteric site of the receptor molecule by atropine, without affecting the persistent binding of xanomeline to an allosteric site (Grant *et al.*, 2010). Here we report that the anti-inflammatory activity of xanomeline is retained for as long as 20h. The role of the previously described unique modulation of the M1 mAChR by xanomeline in this long-term anti-inflammatory “priming” by xanomeline remains to be elucidated. However, our findings demonstrating anti-inflammatory priming within the same time frame with the centrally-acting cholinesterase inhibitor galantamine

suggests that this is not an effect unique for xanomeline. Here, we also show that the long-term anti-inflammatory effects of xanomeline are associated with increased percentage of T cells in spleen. This finding is in line with the important role of splenic T cells as a necessary cellular mediator of the cholinergic anti-inflammatory pathway (Rosas-Ballina *et al.*, 2011). Therefore, increased splenic T cell subpopulation might be regarded as a peripheral point for retention of long-term xanomeline anti-inflammatory signaling, which ultimately correlates with lower pro-inflammatory cytokine production as indicated in this present study.

While endotoxemia is a model of robust systemic release of TNF with deleterious consequences, TNF production at early phases of CLP-induced sepsis has been associated with beneficial effects, including facilitated phagocytosis of bacteria by immune cells and confinement of the inflammatory process (Echtenacher *et al.*, 1990). Therefore, whereas suppressing TNF release in endotoxemia is beneficial, targeted upregulation or suppression of this cytokine at early stages of CLP-induced sepsis has been related to decreased or increased mortality respectively (Echtenacher *et al.*, 1990; Echtenacher *et al.*, 1995; Echtenacher and Mannel, 2002). In contrast to improving survival in endotoxemia, the early (–20h) administration of xanomeline (20 mg/kg) failed to improve survival in CLP-induced sepsis and even worsened it. Although the effect of xanomeline on cytokine release in this model was not studied, one could relate this finding to a TNF suppressing effect of xanomeline as shown in endotoxemia. While preventing sepsis is important, treating sepsis is undoubtedly one of the most challenging tasks in the intensive care units, and specific pharmacotherapy is currently lacking. Accordingly, mortality of sepsis remains high. Here we show that xanomeline treatments of mice with established CLP-induced sepsis, manifested by peritonitis and characteristic sickness behavior, significantly improve survival and attenuate behavioral manifestations of sepsis. The previously indicated demonstrated lack of xanomeline effect on body temperature The lack of These observations indicate the therapeutic advantage that a centrally-acting mAChR agonist administration provides in preclinical settings of sepsis. Xanomeline administration within 24h–46h after the onset of sepsis resulted in similar protection against mortality as the extended 3 day treatment, which suggests the importance of this time window. These findings add xanomeline to other cholinergic modalities which improve survival in preclinical sepsis in a clinically-relevant time frame (Pavlov *et al.*, 2007; Huston *et al.*, 2007; Parrish *et al.*, 2008). In this study, lower doses of xanomeline were required to significantly improve survival in severe sepsis as compared to endotoxemia. A plausible explanation might be that the drug was administered in mice with already developed sepsis and underlying pathophysiological alterations, including compromised blood-brain barrier (Hofer *et al.*, 2008; Flierl *et al.*, 2009).

Clinical studies with xanomeline report alleviation of behavioral disturbances and improvement of some cognitive aspects in patients with Alzheimer's disease (Bodick *et al.*, 1997b), and improvement of positive and negative syndromes, verbal learning and short-term memory in patients with schizophrenia (Shekhar *et al.*, 2008). Interestingly, schizophrenia, a disease in which xanomeline has therapeutic effects, is associated with low-grade peripheral inflammation with a proposed pathogenic role (Meyer *et al.*, 2011). In light

of our current results one may speculate that anti-inflammatory effects of xanomeline might contribute to its efficacy in schizophrenia.

In conclusion, these results highlight a xanomeline-activated anti-inflammatory mechanism involving brain mAChRs and peripheral neural signaling within the cholinergic anti-inflammatory pathway that function to suppress lethal inflammation. Our findings are of interest for further studying centrally-acting M1 mAChR agonists as experimental therapeutic agents in a broader spectrum of inflammatory conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Xanomeline suppresses serum TNF levels and improves survival in endotoxemia through a CNS mAChR-mediated signaling

(A) Xanomeline (X) (5, 10, 20 mg/kg, i.p.) treatment 1h prior to endotoxin (6 mg/kg, i.p.) administration dose-dependently reduces serum TNF as compare to saline (S) treated controls ($n=5-7$ per group, $*P<0.001$, $**P<0.0001$) (B) Xanomeline (X) (5, 20 mg/kg, i.p.) dose-dependently improves survival in murine endotoxemia ($n=17-20$ per group, $*P<0.0001$) (C) Atropine sulfate (AS), but not atropine methyl nitrate (AMN) abolishes xanomeline (X) suppressive effect on serum TNF ($n=8-10$ per group, $*P<0.0001$) (D) Atropine sulfate (AS), but not atropine methyl nitrate (AMN) abolishes survival improvement by xanomeline (X) in endotoxemia ($n=10$ per group, $*P<0.0001$). Mice were pre-treated with AS (4 mg/kg, i.p.) or AMN (4 mg/kg, i.p.) 15 min prior to xanomeline (20 mg/kg, i.p.).

Figure 2. Anti-inflammatory effects of xanomeline during endotoxemia are vagus nerve- and splenic nerve-dependent

(A) Xanomeline (X) reduces splenic TNF ($n=8-10$ per group, $*P<0.003$) in endotoxemic mice as compared to saline (S) treated controls. (B) Xanomeline reduces splenic TNF in sham-operated mice, but not in mice with vagotomy ($n=8-10$ per group, $*P<0.001$) (C) Xanomeline i.p. administration in rats suppresses serum TNF levels during endotoxemia ($n=5-7$ per group, $*P<0.03$, $**P<0.003$). (D) Xanomeline i.p. administration in rats suppresses splenic TNF levels ($n=5-7$ per group, $*P<0.03$). (E) Xanomeline (X) i.p.

administration suppresses serum TNF in sham-operated animals, but not in rats with splenic nerve transection (SNT) (n=4–6 per group, $*P<0.02$) (F) Xanomeline (X) i.p. administration suppresses splenic TNF in shamoperated animals, but not in rats with splenic nerve transection (SNT) (n=4–6 per group, $*P<0.03$).

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Figure 3. Xanomeline treatment provides a long-lasting anti-inflammatory protection associated with specific changes in the proportions of spleen cell subpopulations and cytokine responsiveness

(A) Xanomeline (X, 20 mg/kg) i.p. injection in mice 20h prior to endotoxin (6 mg/kg, i.p.) lowers serum TNF levels as compared to saline (S) (n=8 per group, * $P<0.005$) (B) Xanomeline (X, 20 mg/kg) i.p. injection in mice 20h prior to endotoxin (6 mg/kg, i.p.) improves survival as compared to saline (S) (n=10 per group, * $P<0.005$) (C) Xanomeline (X, 20 mg/kg) i.p. injection in mice 30h prior to endotoxin (6 mg/kg, i.p.) does not alter survival as compared to saline (S) (n=10 per group) (D) Xanomeline (X, 20 mg/kg) i.p. injection in mice 20h prior to euthanasia alters the proportions of splenocyte subpopulations as compared to saline (S) injection (n=6 per group, * $P<0.04$, ** $P<0.02$, *** $P<0.01$) (E) Xanomeline (X, 20 mg/kg) i.p. injection in mice 20h prior to euthanasia alters splenocyte cytokine production in response to endotoxin (n=6 per group, * $P<0.03$, ** $P<0.003$, *** $P<0.0005$, **** $P<0.0001$)

Figure 4. Delayed xanomeline treatment improves survival in CLP-induced severe sepsis
 (A) Xanomeline (X, 20 mg/kg) i.p. injection in mice 20h prior to cecal ligation and puncture (CLP) worsens survival as compared to saline (S) (n=15 per group, $*P<0.05$) (B) Xanomeline (X, 2 or 5 mg/kg) i.p. injection in mice 20h prior to CLP does not alter survival as compared to saline (S) (n=22–24 per group) (C) Xanomeline (X, 1, 2, or 3 mg/kg) i.p. injection, initiated 24h following CLP and continued twice daily for 3 consecutive days improves survival as compared to saline (n=26–28 per group, $*P<0.04$) (S) (D) Xanomeline (X, 1 or 2 mg/kg) injected i.p. at 24h, 30h and 46h after CLP dose-dependently improves survival as compared to saline (n=27 per group, $*P<0.04$)