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Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation

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Excessive inflammation and tumour-necrosis factor (TNF) synthesis cause morbidity and mortality in diverse human diseases including endotoxaemia, sepsis, rheumatoid arthritis and inflammatory bowel disease^{1–4}. Highly conserved, endogenous mechanisms normally regulate the magnitude of innate immune responses and prevent excessive inflammation. The nervous system, through the vagus nerve, can inhibit significantly and

rapidly the release of macrophage TNF, and attenuate systemic inflammatory responses^{5–7}. This physiological mechanism, termed the 'cholinergic anti-inflammatory pathway'⁵ has major implications in immunology and in therapeutics; however, the identity of the essential macrophage acetylcholine-mediated (cholinergic) receptor that responds to vagus nerve signals was previously unknown. Here we report that the nicotinic acetylcholine receptor α7 subunit is required for acetylcholine inhibition of macrophage TNF release. Electrical stimulation of the vagus nerve inhibits TNF synthesis in wild-type mice, but fails to inhibit TNF synthesis in α7-deficient mice. Thus, the nicotinic acetylcholine receptor α7 subunit is essential for inhibiting cytokine synthesis by the cholinergic anti-inflammatory pathway.

Nicotinic acetylcholine receptors are a family of ligand-gated, pentameric ion channels. In human, 16 different subunits (α1–7, α9–10, β1–4, δ, ε, γ) have been identified that form a large number of homo- and heteropentameric receptors with distinct structural and pharmacological properties^{8–10}. The main function of this receptor family is to transmit signals for the neurotransmitter acetylcholine at neuromuscular junctions and in the central and peripheral nervous systems^{8–12}. Our previous studies have indicated that acetylcholine inhibits the release of TNF and other cytokines through a post-transcriptional mechanism that is dependent on α-bungarotoxin-sensitive nicotinic receptors on primary human macrophages⁵, but the identity of the specific receptor subunit has remained unknown. As a first step towards identifying this macrophage receptor, primary human macrophages were labelled with fluorescein isothiocyanate (FITC)-tagged α-bungarotoxin, a peptide antagonist that binds to a subset of cholinergic receptors^{8,9}. Strong binding of α-bungarotoxin was observed on the macrophage surface (Fig. 1a). Nicotine pretreatment markedly reduced the

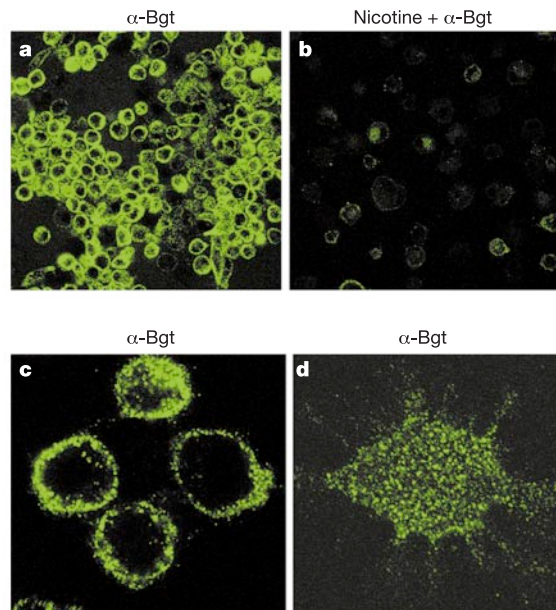


Figure 1 α-Bungarotoxin-binding nicotinic receptors are clustered on the surface of macrophages. Primary human macrophages were stained with fluorescein isothiocyanate (FITC)-labelled α-bungarotoxin (α-Bgt, 1.5 μg ml⁻¹) and viewed by fluorescent confocal microscopy. **a**, Cells were stained with α-bungarotoxin alone. **b**, Nicotine was added to a final concentration of 500 μmol before addition of α-bungarotoxin. **c, d**, Higher magnification reveals receptor clusters. **c**, Focus planes are on the inside layers close to the middle (three lower cells) or close to the surface (upper cell) of cells. **d**, Focus plane is on the surface of the cell. Magnifications: **a, b**, × 50; **c**, × 200; **d**, × 450.

intensity of binding (Fig. 1b). At neuromuscular junctions and at neuronal synapses, nicotinic receptors form receptor aggregates or clusters that facilitate fast signal transmission^{13–15}. Under high magnification, discrete clusters of α -bungarotoxin binding can be seen on the surface of macrophages. These clusters are particularly concentrated on the surface of the cell body (Fig. 1c, d).

α 1, α 7 and α 9 had been described as potential α -bungarotoxin-binding nicotinic receptor subunits in mammalian cells^{8,9}. α 1, together with β 1, δ and either ϵ (adult) or γ (fetal) subunits, can form heteropentameric nicotinic receptors that regulate muscle contraction; α 7 and α 9 can each form homopentameric nicotinic receptors^{8,9}. To determine whether these receptor subunits are expressed in macrophages, we isolated RNA from primary human macrophages differentiated *in vitro* from peripheral blood mononuclear cells (PBMCs), and performed polymerase chain reaction with reverse transcription (RT-PCR) analyses. To increase the sensitivity and specificity of the experiments, we conducted two rounds of PCR using nested primers specific to each subunit. The identities of the PCR products were confirmed by sequencing. Expression of α 1, α 10 (data not shown) and α 7 (Fig. 2a) messenger RNA was detected in human macrophages derived from unrelated blood donors. The same RT-PCR strategy did not detect the expression of the δ -subunit, a necessary component of the α 1 heteropentameric nicotinic acetylcholine receptor, or mRNA of the α 9 subunit in human macrophages (data not shown).

The protein expression of α 1 and α 7 subunits was then examined by western blotting. An antibody specific for the nicotinic acetylcholine receptor α 7 subunit (hereafter referred to as the α 7 subunit) recognized a clear band with an apparent relative molecular mass of 55,000 (M_r 55K) (similar to the published molecular mass for α 7 protein^{16,17}) from both differentiated primary macrophages and from undifferentiated PBMCs (data not shown). α 1 protein expression was downregulated to undetectable levels during *in vitro* differentiation of PBMCs to macrophages (data not shown). To confirm that the positive signals in macrophages represented the α 7 subunit that binds α -bungarotoxin, we used α -bungarotoxin-conjugated beads to pull-down proteins prepared

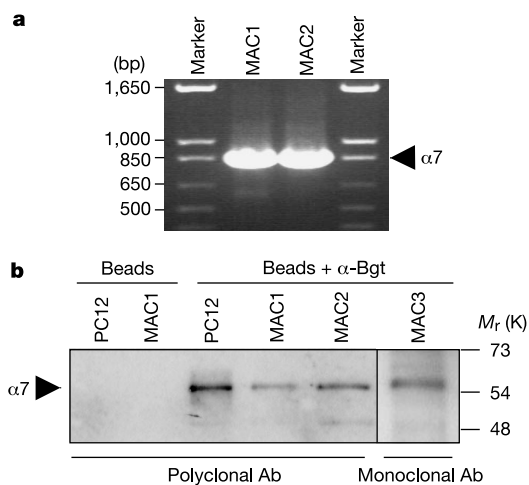


Figure 2 Messenger RNA and protein expression of nicotinic acetylcholine receptor α 7 subunit in primary human macrophages. **a**, RT-PCR analysis. RT-PCR with primers specific for the α 7 subunit generated an 843-base pair (bp) α 7 band. PCR products were verified by sequencing (data not shown). MAC1 and MAC2 are macrophages derived from two unrelated donors. **b**, Western blots. Cell lysates from PC12 cells or human macrophages (MAC) were incubated with either control Sepharose beads or Sepharose beads conjugated with α -bungarotoxin. The bound proteins were analysed by α 7-specific polyclonal and monoclonal antibodies as indicated.

from either human macrophages or PC12 cells (rat pheochromocytoma cells, which express α 7 homopentamer¹⁷). Retained proteins were analysed by western blotting using polyclonal or monoclonal α 7-specific antibodies that recognize both the human and rat α 7 subunit (the human and rat α 7 subunits contain the same number of amino acids and are 94% identical^{16,18}). We found that human macrophages express the α -bungarotoxin-binding α 7 subunit with an apparent molecular mass similar to that of the α 7 subunit isolated from PC12 cells (Fig. 2b). The identity of the macrophage α 7 subunit was confirmed by cloning of the full-length complementary DNA of the macrophage-expressed α 7 subunit by RT-PCR methods. The full-length cDNA of the α 7 subunit in macrophages contains exons 1 to 10, identical to the α 7 subunit expressed in neurons¹⁹. Together, these data identify the α 7 subunit as the α -bungarotoxin-binding receptor expressed on the surface of human macrophages.

To study whether the α 7 subunit is required for cholinergic inhibition of TNF release, we synthesized phosphorothioate antisense oligonucleotides surrounding the translation-initiation codon of the human α 7 subunit gene. Antisense oligonucleotides to similar regions of the α 1 and α 10 subunit genes were synthesized as controls. Macrophages exposed to the antisense oligonucleotides specific for α 7 (AS α 7) were significantly less responsive to the TNF-inhibitory action of nicotine (Fig. 3a), and antisense oligonucleotides to the α 7 subunit restored macrophage TNF release in the presence of nicotine. Exposure of macrophages to AS α 7 did not stimulate TNF synthesis in the absence of lipopolysaccharide (LPS) and nicotine. Antisense oligonucleotides to α 1 (AS α 1) and α 10

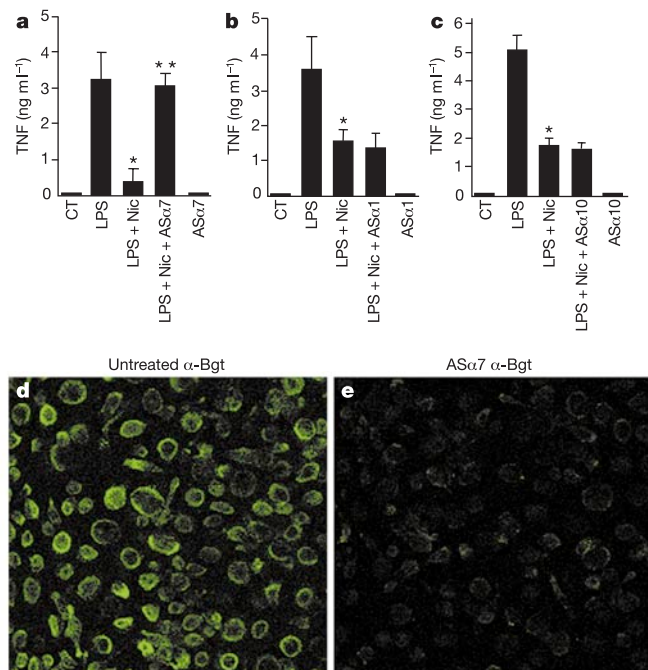


Figure 3 Antisense oligonucleotides to the α 7 subunit inhibit the effect of nicotine on TNF release. **a–c**, TNF release from lipopolysaccharide (LPS)-stimulated primary human macrophages pretreated with antisense oligonucleotides to different subunits of nicotinic receptors. Where indicated, nicotine (Nic, 1 μ M) was added 5–10 min before LPS induction (100 ng ml⁻¹). TNF levels in the cell culture medium were determined by L929 assays. CT, control (unstimulated) macrophage cultures. AS α 7, AS α 1 and AS α 10 are antisense oligonucleotides to α 7, α 1 and α 10 subunits, respectively. Asterisk, $P < 0.05$ versus LPS; double asterisk, $P < 0.05$ versus LPS + Nic. **d, e**, FITC-labelled α -bungarotoxin staining of primary human macrophages treated (**e**) or untreated (**d**) with AS α 7 and viewed by fluorescent confocal microscopy.

(AS α 10) subunits, under similar conditions, did not significantly change the effect of nicotine on LPS-induced TNF release (Fig. 3b, c), indicating that the suppression of TNF by nicotine is specific to the α 7 subunit. Further sets of antisense oligonucleotides to α 7, α 1 and α 10 subunits gave similar results (data not shown). Addition of AS α 7 to macrophage cultures decreased the surface binding of FITC-labelled α -bungarotoxin (Fig. 3d, e). Together these data indicate that the α 7 subunit is necessary for cholinergic inhibition of TNF release in macrophages.

Macrophages are an important source of TNF produced in response to bacterial endotoxin *in vivo*^{20,21}. To investigate whether the α 7 subunit is essential for the cholinergic anti-inflammatory pathway *in vivo*, we measured TNF production in mice deficient in the α 7 subunit gene originally generated by Orr-Urtreger and colleagues using genetic knockout technology²². In agreement with the original description of these mice²², mice lacking the α 7 subunit develop normally and show no gross anatomical defects^{22,23}. The serum TNF level in α 7-deficient mice after administration of endotoxin was significantly higher than wild-type endotoxaemic mice (wild-type serum TNF, 8.0 ± 2.4 ng ml⁻¹; α 7 knockout serum TNF, 18.1 ± 3.7 ng ml⁻¹, $P < 0.05$ (two-tailed *t*-test)) (Fig. 4a). TNF production in liver and spleen was also significantly higher in knockout mice (Fig. 4b, c), indicating a critical function of the α 7 subunit in the normal regulation of systemic inflammatory responses *in vivo*. Endotoxaemic α 7 knockout mice also produce significantly higher levels of interleukin (IL)-1 β (Fig. 4d) and IL-6 (Fig. 4e) as compared with wild-type mice. Macrophages derived from α 7 knockout mice were refractory to cholinergic agonists, and produced TNF normally in the presence of nicotine or acetylcholine (Table 1). Thus, α 7 subunit expression in macrophages is essential for cholinergic suppression of TNF.

To determine whether the α 7 subunit is required for vagus-nerve-dependent inhibition of systemic TNF, we applied electrical stimulation⁵ to the vagus nerve of endotoxaemic wild-type or

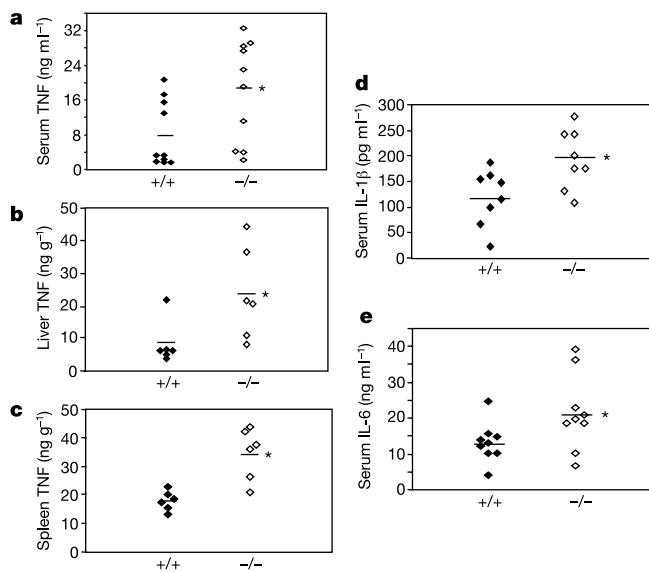


Figure 4 Increased cytokine production in α 7-subunit-deficient mice during endotoxaemia. α 7-deficient mice (-/-) or age- and sex-matched wild-type mice (+/+) were injected with LPS (0.1 mg kg⁻¹, intraperitoneally). Blood and organs were obtained either 1 h (for TNF) or 4 h (for IL-1 β and IL-6) after LPS administration. Levels of TNF, IL-1 β and IL-6 in serum or organs were measured with enzyme-linked immunosorbent assay (ELISA). **a**, TNF levels in serum; $n = 10$ per group; **b**, TNF levels in liver; $n = 6$ per group; **c**, TNF levels in spleen; $n = 6$ per group; **d**, IL-1 β levels in serum; $n = 8$ per group; **e**, IL-6 levels in serum; $n = 9$ per group. Asterisk, $P < 0.05$ versus wild-type controls. Horizontal line, the mean of the group.

Table 1 TNF production by wild-type and α 7-deficient peritoneal macrophages

	TNF (ng ml ⁻¹) wild type	TNF (ng ml ⁻¹) α 7 knockout
Control	0.004 ± 0.0005	0.004 ± 0.0005
LPS	16.8 ± 2.3	18.1 ± 4.9
LPS + nicotine (1 μ M)	$5.2 \pm 0.9^*$	17.8 ± 0.6
LPS + nicotine (10 μ M)	$7.3 \pm 1.0^*$	17.4 ± 2.9
LPS + Ach (1 μ M)	10.3 ± 1.1	20.4 ± 3.8
LPS + Ach (10 μ M)	$5.7 \pm 0.9^*$	21.4 ± 2.4

Thioglycollate-elicited peritoneal macrophages isolated from wild-type mice or nicotinic acetylcholine receptor α 7 subunit knockout mice were stimulated with LPS (100 ng ml⁻¹) for 4 h in culture. Control indicates unstimulated macrophage cultures. Where indicated, nicotine or acetylcholine (Ach) were added 5–10 min before LPS administration. TNF levels were measured by ELISA. Results are mean \pm s.e.m.; $n = 8$ per group. Asterisk, $P < 0.05$ versus LPS.

α 7-deficient mice. Electrical stimulation of the vagus nerve significantly attenuated endotoxin-induced serum TNF levels in wild-type mice (Fig. 5). Vagus nerve stimulation using this protocol in α 7-deficient mice, however, failed to reduce serum TNF levels during endotoxaemia (Fig. 5). Thus, vagus nerve inhibition of TNF *in vivo* is dependent on the α 7 subunit.

These observations have several implications related both to understanding how the nervous system can regulate innate inflammatory responses in real time, and to designing experimental therapeutics that function via this physiological mechanism. Previous data indicate that the α 7 subunit forms homopentameric receptors that are involved in fast chemical signalling between cells^{8–10}. Neuronal α 7 receptors are highly permeable to calcium^{24,25}, and we have observed that nicotine induces transient calcium influx in macrophages (data not shown). Disruption of α 7 subunit expression *in vivo* significantly increases endotoxin-induced TNF release, indicating that the activity of this cholinergic anti-inflammatory pathway normally regulates the release of cytokines from macrophages and perhaps other cytokine-producing cells. It now seems that inactivation of this pathway can contribute to excessive systemic release of cytokines during endotoxaemia, and perhaps other states of infection or injury.

Deficiency of the α 7 subunit rendered the vagus nerve ineffective as a physiological pathway to inhibit TNF release, indicating that α 7 is essential for vagus nerve regulation of acute TNF release during the systemic inflammatory response to endotoxaemia. Thus, acetylcholine released from vagus nerve endings, or perhaps from other sources (for example, lymphocytes or epithelial cells) can specifically inhibit macrophage activation. It is quite possible that other primary immune cells (for example, mast cells, microglia cells, Kupffer cells, splenocytes) that produce cytokines during the acute, local response to infection or injury might express α 7 subunits, and

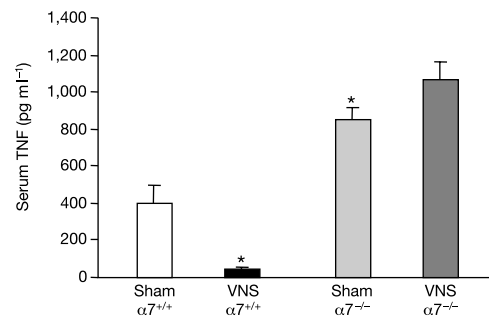


Figure 5 Vagus nerve stimulation does not inhibit TNF production in α 7-subunit-deficient mice. α 7-subunit-deficient mice (-/-) or age- and sex-matched wild-type mice (+/+) were subjected to either sham operation or vagus nerve stimulation (VNS, left vagus; 1 V, 2 ms, 1 Hz); blood was collected 2 h after LPS administration. Serum TNF levels were determined by ELISA. $n = 10$ (sham α 7^{+/+}); $n = 11$ (VNS α 7^{+/+}, sham α 7^{-/-}, VNS α 7^{-/-}). Asterisk, $P < 0.05$ versus sham α 7^{+/+}.

that these cells might be sensitive to the anti-inflammatory effects of acetylcholine. Thus, there is significant potential for developing cholinergic agonists that target $\alpha 7$ subunits on peripheral immune cells for use as anti-inflammatory agents to inhibit release of TNF and other proinflammatory cytokines (for example, high mobility group box-1 protein)^{2,27}. Vagus nerve stimulators can enhance the anti-inflammatory activity of the cholinergic anti-inflammatory pathway in animals⁵⁻⁷; furthermore, vagus nerve stimulators have already been used safely in humans with seizure disorders. As TNF is already a clinically validated drug target for rheumatoid arthritis and Crohn's disease, it is now reasonable to consider the therapeutic potential for targeting the nicotinic acetylcholine receptor $\alpha 7$ subunit to inhibit TNF, either by direct pharmacological approaches, or through increasing activity in the vagus nerve. □

Methods

α -Bungarotoxin staining and confocal microscopy

Isolation and culture of human macrophages was performed as described previously⁵. Cells were differentiated for seven days in the presence of macrophage colony stimulating factor (MCSF; 2 ng ml⁻¹) in complete culture medium (RPMI 1640 with 10% heat-inactivated human serum). Differentiated macrophages were incubated with FITC-labelled α -bungarotoxin at 1.5 μ g ml⁻¹ (Sigma) in the cell culture medium at 4 °C for 15 min. Where indicated, nicotine was added to a final concentration of 500 μ M before the addition of α -bungarotoxin. Cells were washed three times with RPMI medium (Gibco) and then fixed for 15 min at room temperature in 4% paraformaldehyde-PBS solution (pH 7.2). After fixation, cells were washed with PBS once and mounted for viewing by fluorescent confocal microscopy.

RT-PCR

Total RNA was prepared from *in vitro* differentiated human macrophages using TRIzol reagent. Reverse transcription and the first round of PCR were performed using Titan One Tube RT-PCR Kit (Roche) according to the manufacturer's protocol. The second round of nested PCR was conducted using Promega PCR master mix. The PCR products from nested PCRs were run on agarose gels, recovered using the Gene Clean III Kit (BioLab Inc.) and sent for sequencing to confirm the results. The primer sets for reverse transcription and the first round of PCR were: $\alpha 1$, sense primer 5'-CCAGACCTGAGCAACTTCATGG-3', antisense primer 5'-AATGAGTCGACCTGCAACACG-3'; $\alpha 7$, sense primer 5'-GACTGTTCCGTTCCAGATGG-3', antisense primer 5'-ACGAAGTTGGGAGCCGACA TCA-3'; $\alpha 9$, sense primer 5'-CGAGATCAGTACGATGGCCTAG-3', antisense primer 5'-TCTGTGACTAATCCGCTCTTGC-3'. The primer sets for nested PCRs were: $\alpha 1$, sense primer 5'-ATCACCTA-CCACTTCGTCATGC-3', antisense primer 5'-GTAATGGTCCCA TCACCATTGC-3'; $\alpha 7$, sense primer 5'-CCCGCAAGAGGAGTGAAGGT-3', antisense primer 5'-TGCAGATGATGGTGAAGACC-3'; $\alpha 9$, sense primer 5'-AGAGCCT GTGAACACCAATGTGG-3', antisense primer 5'-ATGACTTTCGCCACCTTCTTCC-3'. For cloning of the full-length $\alpha 7$ cDNA, the following primers were used: 5'-AGGTGC CTCTGTGGCGCA-3' with 5'-GACTACTCAGTGGCCCTG-3'; 5'-CGACACGGAGAGC GTGGAG-3' with 5'-GGTACGGATGTGCCAAGGAGT-3'; 5'-CAAGGATCCGGACTC AACATGCGCTGCTCG-3' with 5'-CGGCTCGAGTCCACAGTGTGGTTACGCAA GTC-3'.

Western blotting and α -bungarotoxin pull-down assay

Cell lysates were prepared by incubating PC12 cells or primary human macrophages with lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02% Na₃S, 1% Triton X-100 and protease inhibitor cocktail) on ice for 90 min. Equal amounts of total protein were loaded on SDS-polyacrylamide gel electrophoresis (PAGE) for western blotting with either $\alpha 7$ -specific antibody (Santa Cruz sc-1447) or $\alpha 1$ monoclonal antibody (Oncogene). For the α -bungarotoxin pull-down assay, α -bungarotoxin (Sigma) was conjugated to CNBr-activated Sepharose beads (Pharmacia) and then incubated with cell lysates at 4 °C overnight. The beads and bound proteins were washed four times with lysis buffer and analysed by western blotting with $\alpha 7$ -specific antibodies (polyclonal, Santa Cruz H-302; monoclonal, Sigma M-220).

Antisense oligonucleotide experiments

Phosphorothioate antisense oligonucleotides were synthesized and purified by Genosys. The sequences for the oligonucleotides were: AS $\alpha 7$, 5'-GCAGCGCATGTTGAGTCCCG-3'; AS $\alpha 1$, 5'-GGGCTCCATGGGCTACCGGA-3'; AS $\alpha 10$, 5'-CCCCATGGCCCTGGCACTGC-3'. These sequences cover the divergent translation-initiation regions of $\alpha 7$, $\alpha 1$ and $\alpha 10$ genes. Delivery of the antisense oligonucleotides was carried out as in ref. 26 at 1 μ M concentration of the oligonucleotides for 24 h. For cell culture experiments, the oligonucleotide-pretreated macrophage cultures were washed with fresh medium and stimulated with 100 ng ml⁻¹ LPS with or without nicotine (1 μ M, added 5-10 min before LPS). At 4 h after LPS administration, the amounts of TNF released were measured by L929 assay and then verified by TNF enzyme-linked immunosorbent assay (ELISA). For α -bungarotoxin staining, pretreated cells were washed and processed for FITC- α -bungarotoxin staining as described above. Nicotine and other nicotinic acetylcholine receptor $\alpha 7$ subunit agonists also significantly inhibit LPS-induced TNF release in the murine macrophage-like cell line RAW264.7 (data not shown).

$\alpha 7$ -deficient mice

Mice deficient for the $\alpha 7$ nicotinic receptor (C57BL/6 background), and wild-type littermates were purchased from The Jackson Laboratory (B6.129S7-Chrna^{7tm1Bay}, number 003232). Breeding of homozygous knockout mice or wild-type mice was established to obtain progenies. Male or female mice about 8-12 weeks old (together with age- and sex-matched wild-type controls) were used for endotoxin experiments. Mice were weighed individually and 0.1 mg kg⁻¹ LPS was administered s.c. (intraperitoneal injection). For TNF experiments, blood, liver and spleen were collected 1 h after LPS administration. For IL-1 β and IL-6 experiments, blood samples were collected 4 h after LPS administration. The amounts of TNF, IL-1 β and IL-6 were measured by ELISA. We confirmed the genotypes of the mice by genomic PCR strategies. Peritoneal macrophages were isolated 48 h after intraperitoneal administration of thioglycollate (9%) from $\alpha 7$ knockout and wild-type male and female mice at 8 weeks of age (*n* = 8 per group). Macrophages were pooled for each group and cultured overnight. Nicotine and acetylcholine were added 5-10 min before LPS administration (100 ng ml⁻¹). Pyridostigmine bromide (100 μ M) was added with acetylcholine. Four hours after LPS administration, we measured TNF levels by ELISA.

Vagus nerve stimulation

$\alpha 7$ -deficient mice (C57BL/6 background, male and female) and age- and sex-matched wild-type C57BL/6 mice were anaesthetized with ketamine (100 mg kg⁻¹, intramuscularly) and xylazine (10 mg kg⁻¹, intramuscularly). Mice were either subjected to sham operation or vagus nerve stimulation (left cervical vagus, 1 V, 2 ms, 1 Hz) with an electrical stimulation module (STM100A, Harvard Apparatus). Stimulation was performed for 20 min (10 min before and 10 min after LPS administration). LPS was given at a lethal dose (75 mg kg⁻¹, intraperitoneally). Blood was collected 2 h after LPS administration. TNF levels were measured by ELISA.

Statistical analysis

Statistical analysis was performed using a two-tailed *t*-test where indicated; *P* < 0.05 is considered significant. Experiments were performed in duplicate or triplicate; for *in vivo* and *ex vivo* experiments, *n* refers to the number of animals under each condition.

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Activation of human CD4⁺ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype

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The immune system must distinguish not only between self and non-self, but also between innocuous and pathological foreign antigens to prevent unnecessary or self-destructive immune responses. Unresponsiveness to harmless antigens is established through central and peripheral processes¹. Whereas clonal deletion and anergy are mechanisms of peripheral tolerance^{2,3}, active suppression by T-regulatory 1 (Tr1) cells has emerged as an essential factor in the control of autoreactive cells⁴. Tr1 cells are CD4⁺ T lymphocytes that are defined by their production of interleukin 10 (IL-10)⁵ and suppression of T-helper cells⁶; however, the physiological conditions underlying Tr1 differentiation are unknown. Here we show that co-engagement of CD3 and the complement regulator CD46 in the presence of IL-2 induces a Tr1-specific cytokine phenotype in human CD4⁺ T cells. These CD3/CD46-stimulated IL-10-producing CD4⁺ cells proliferate strongly, suppress activation of bystander T cells and acquire a memory phenotype. Our findings identify an endogenous receptor-mediated event that drives Tr1 differentiation and suggest that the complement system has a previously unappreciated role in T-cell-mediated immunity and tolerance.

Control of self-reactive T lymphocytes by regulatory T cells has been proposed to mediate anergy and thereby peripheral tolerance and prevention of autoimmunity⁷. Such cells suppress immune responses through either direct cell–cell interactions or the release of inhibitory cytokines such as IL-10 and transforming growth factor-β (TGF-β)^{8–11}. The differentiation of CD4⁺ lymphocytes into Tr1 cells is poorly defined, in part because of difficulties in inducing and culturing such cells. Only after the stimulation of

human CD4⁺ T cells with allogeneic monocytes or murine CD4⁺ T lymphocytes with antigen were some Tr1 clones generated; however, these cells had weak proliferative capacities and required the presence of IL-10 (ref. 5). Tr1 cell differentiation has also been induced by incubating CD4⁺ T cells with dexamethasone and vitamin D3 (ref. 12).

Defining the stimuli that drive differentiation of these cells would significantly enhance our understanding of their ontogeny and capabilities. Here we identify the complement regulatory protein CD46 as a physiological inducer of Tr1 cell development. CD46 is a widely expressed transmembrane glycoprotein that inhibits complement activation on host cells^{13,14}. CD46 is also used as a receptor by several human pathogens, including measles virus, herpesvirus 6 and two groups of pathogenic bacteria^{15–19}. Crosslinking CD46 through its physiological or pathogenic ligands initiates signalling events in several types of human cell^{20–23} and in cells derived from CD46 transgenic mice²⁴.

We stimulated purified human CD4⁺ lymphocytes with immobilized monoclonal antibodies and assessed their cytokine profile (Fig. 1). Cells stimulated with CD3 and CD46 (CD3/CD46) synthesized small amounts of IL-2, large amounts of IL-10 and intermediate amounts of TGF-β. By contrast, CD3/CD28 activation induced large amounts of IL-2, no IL-10 and barely detectable amounts of TGF-β. Neither stimulatory condition induced IL-4, but both led to the expression of similar quantities of IL-12 and interferon-γ (IFN-γ). Thus, CD3/CD46-stimulated cells have a cytokine profile that is distinct from that induced by stimulation with antibodies to CD3 and CD28 but similar to that of Tr1 cells⁵. IL-12 in the cultures suggests the presence of antigen-presenting

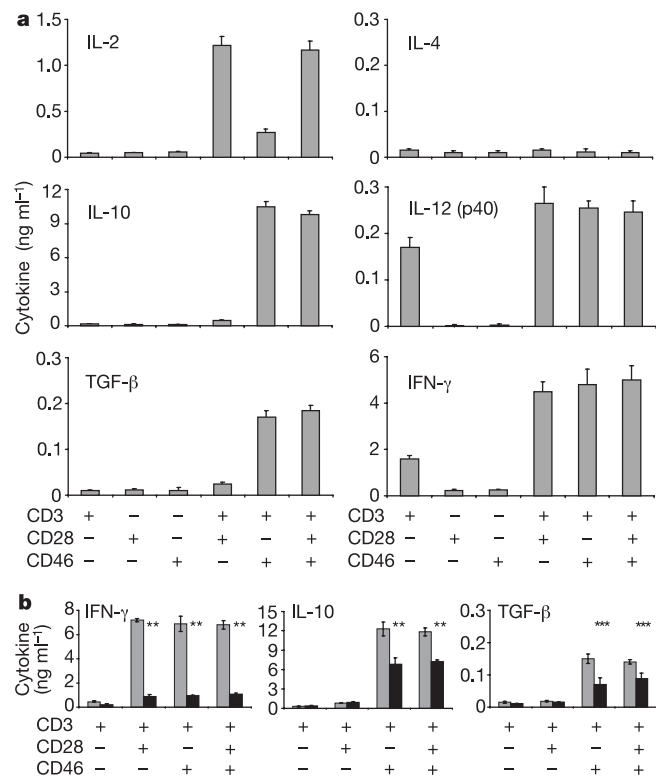


Figure 1 CD3/CD46 stimulation induces IL-10 production in human peripheral blood CD4⁺ T lymphocytes. **a**, Cytokine profile of stimulated T cells. Supernatants were collected at day 3. The same cytokine profile was observed with a second monoclonal antibody to CD46 (TRA-2-10). **b**, Effect of IL-12 neutralization. Cells were incubated with the indicated monoclonal antibodies in the presence (black bars) or absence (grey bars) of a neutralizing monoclonal antibody to IL-12. In all figures, values shown are the mean ± s.d. of at least three experiments. ***P* < 0.005, ****P* < 0.01.

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