direct sequencing. Oligonucleotide sequences and PCR conditions are available on request.

Northern and *in situ* hybridizations

Northern and *in situ* hybridization to sectioned tissue were done as described[3,11]. The 738-nucleotide *edaradd* probe contained the entire 627-nucleotide ORF plus 76 nucleotides of the 5′ and 35 nucleotides of the 3′ untranslated region. For whole-mount *in situ* hybridization[3], we used full-length *edar* and *Shh* riboprobes.

Protein interaction and NF-κB reporter assays

GST fusion constructs were prepared by cloning cDNAs into pGEX4T-1 (Amersham Pharmacia). The intracellular portion of Edar (EdarIC) contained amino acids 212–448; the Edar death domain deletion (EdarICΔDD) contained amino acids 212–322; Edar-addΔDD contained amino acids 1–124. Deletion of the seven amino acids Tyr-Pro-Ile-Gln-Asp-Thr-Gly from wild-type Edaradd created EdaraddΔ34-40. RANKIC is amino acids 235–625 and p75NTRIC amino acids 264–417 of mouse RANK and mouse p75NTR, respectively. We carried out *in vitro* transcription/translation using the TNT Coupled Reticulocyte Lysate system (Promega) in the presence of [^{35}S]methionine (Amersham Pharmacia). We then expressed the GST fusion proteins in *Escherichia coli* strain BL21. Bacteria were lysed by sonication in PBS plus 0.5% Triton X-100. We used 2 μl of *in vitro* transcription/translation reaction for each pull-down assay. Protein mixtures were incubated at 4 °C for 2 h with 15 μl of glutathione–sepharose beads (Amersham Pharmacia). The beads were washed four times with PBS plus 0.1% Triton X-100 and bound proteins were eluted with 20 μl of 50 mM glutathione.

For immunoprecipitations, individual cDNAs were cloned into pSG5 (Stratagene) downstream of two copies of a Flag or haemagglutinin A (HA) epitope tag. HEK293T cells grown in 25-cm^2 flasks were transfected using Lipofectamine2000 (Lifetech). Whole-cell extracts were prepared 30 h after transfection by adding 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5 % NP-40, protease inhibitors). Cell lysates (850 μl) were added to 40 μl of anti-Flag M2 affinity gel (Sigma) and rotated at 4 °C overnight. The affinity gels were washed with lysis buffer five times and eluted with 20 μl of 0.1 M glycine (pH 3.1); the eluted proteins were assayed on western blots using a 1:1,250 dilution of rat anti-HA primary antibody (3F10; Roche) followed by a 1:3,500 dilution of HRP conjugated rabbit anti-rat secondary antibody (Zymed) and detection by enhanced chemiluminescence (Amersham).

For NF-κB activation assays, HEK293T cells grown in 24-well plates were transfected using Lipofectamine2000 (Lifetech). Each well received 0.2 μg of pNF-κBLuc luciferase reporter (Clontech), cDNA(s) for expression in pSG5–HA, and empty pSG5–HA vector to give 1.0 μg of DNA per well. We lysed the cells 28 h after transfection and measured luciferase activities (Tropix; Promega) using a luminometer. Each transfection was performed in quadruplicate. To compare relative protein expression levels, transfections were done as in the reporter assays. After 28 h, each well of cells was lysed in 100 μl of SDS–PAGE buffer, and 20 μl of lysate was used for anti-HA western blotting as described above.

Received 31 May; accepted 11 October 2001.

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank the families and physicians who assisted in gathering clinical information and samples, in particular G. Lestringant. We also thank D. Moore and D. Dowhan for technical advice and reagents. This work was supported by grants from the NIH and the National Foundation for Ectodermal Dysplasias.

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Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage

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Inappropriate or prolonged inflammation is the main cause of many diseases[1]; for this reason it is important to understand the physiological mechanisms that terminate inflammation *in vivo*[2]. Agonists for several G_s-protein-coupled receptors[3], including cell-surface adenosine purinergic receptors[4–7], can increase levels of immunosuppressive cyclic AMP in immune cells[8–15]; however, it was unknown whether any of these receptors regulates inflammation *in vivo*. Here we show that A2a adenosine receptors have a non-redundant role in the attenuation of inflammation and tissue damage *in vivo*. Sub-threshold doses of an inflammatory stimulus[16,17] that caused minimal tissue damage in wild-type mice were sufficient to induce extensive tissue damage, more prolonged and higher levels of pro-inflammatory cytokines, and death of male animals deficient in the A2a adenosine receptor. Similar observations were made in studies of three different models of inflammation and liver damage as well as during bacterial endotoxin-induced septic shock. We suggest that A2a adenosine receptors are a critical part of the physiological

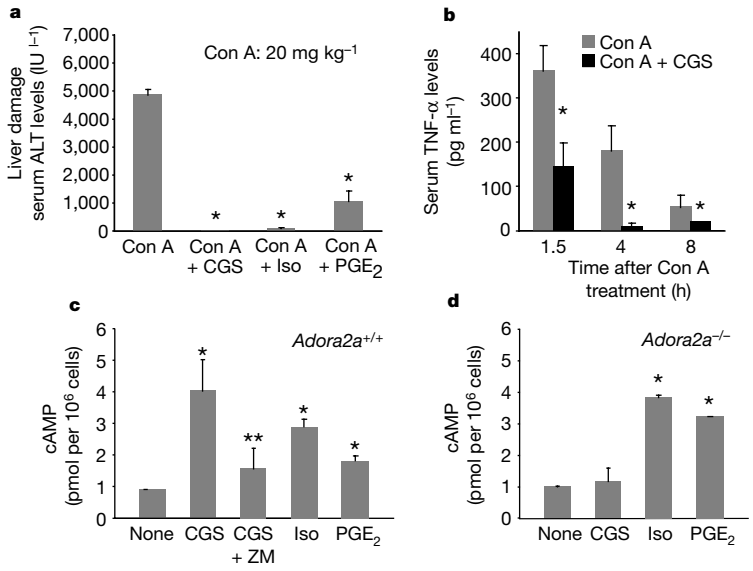

Figure 1 Pharmacological activation of Adora2a or other G_s-protein-coupled receptors *in vivo* prevents Con A-induced liver damage and pro-inflammatory TNF-α accumulation *in vivo*. **a**, Activation of any of several cAMP-elevating G_s-coupled receptors can block Con A-induced liver injury. Female C57BL/6 mice ($n = 5$) were first injected with Adora2a agonist CGS21680 (CGS) (2 mg kg^{-1}), 100 mg kg^{-1} isoproterenol (Iso), or 5 mg kg^{-1} PGE_2, and then with Con A (20 mg kg^{-1}). The Con A-induced liver damage was evaluated after 8 h. The differences between treated and untreated mice are statistically significant as indicated by the asterisk ($P < 0.05$). **b**, CGS21680 (2 mg kg^{-1}) inhibits Con A-induced elevation of serum TNF-α. **c**, **d**, Adora2a agonist CGS21680 does not increase cAMP in mononuclear cells from *Adora2a*$^{-/-}$ mice livers. Adora2a antagonist ZM241385 inhibits CGS21680-induced cAMP in mononuclear cells from *Adora2a*$^{+/+}$ mice livers. Lymphoid cells from *Adora2a*$^{-/-}$ mice retain the cAMP response to ligands of other G_s-protein-coupled receptors. Cells were treated *in vitro* with 10 μM CGS21680, 1 μM ZM241385 (ZM), 100 μM isoproterenol, or 1 μM PGE_2 for 30 min at 37 °C. Data shown represent the mean ± standard error of three preparations. Asterisk, $P < 0.05$ compared with controls; double asterisk, $P < 0.05$ compared with cells incubated with CGS21680. Units on the *y* axis of Figs 1a, 2a, c and 3 are International Units of ALT activity.

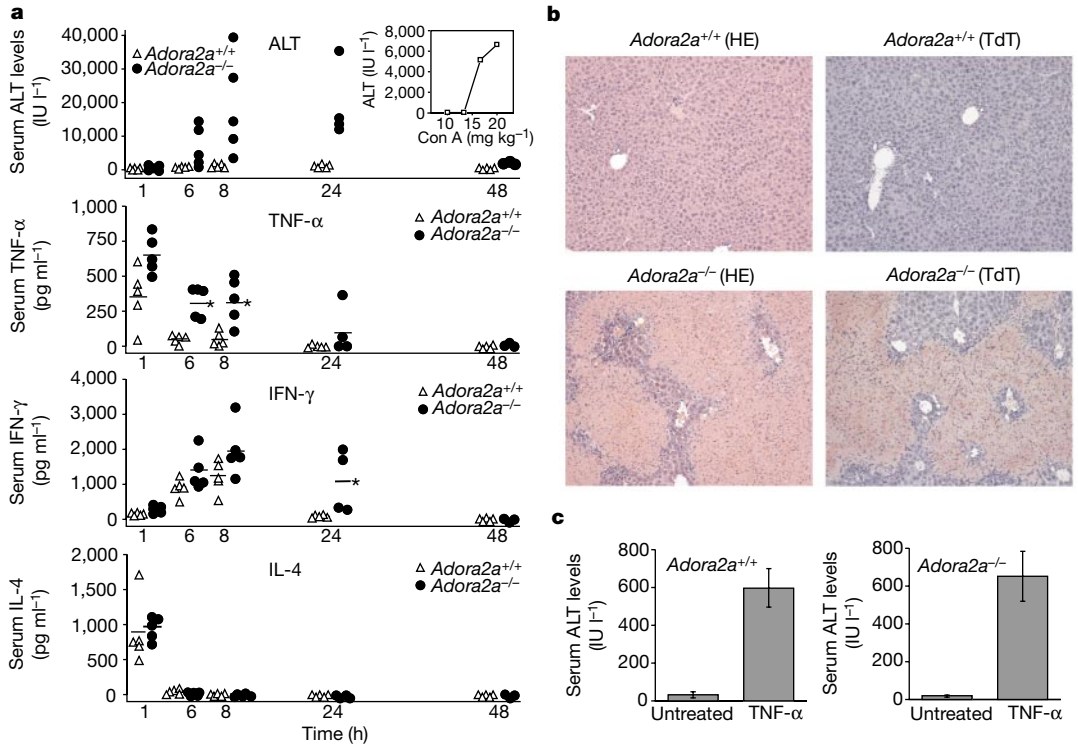

Figure 2 Enhanced and prolonged accumulation of pro-inflammatory cytokines and exaggerated liver damage in A2a-receptor-deficient mice. **a**, *Adora2a*$^{+/+}$ ($n = 5$) and *Adora2a*$^{-/-}$ ($n = 5$) mice were injected intravenously with a sub-optimal dose (12.5 mg kg^{-1}) of Con A (inset, ALT graph). Serum levels of cytokines were measured at indicated times. Data shown are representative of four separate experiments. Asterisk, $P < 0.05$ compared with *Adora2a*$^{+/+}$ mice. **b**, Increased inflammation and liver damage in *Adora2a*$^{-/-}$ mice as evidenced by accumulation of dead cells and leukocytes using TdT apoptosis test and haematoxylin and eosin (HE) stain. **c**, A deficiency in A2a receptors does not affect the susceptibility of hepatocytes to *in vivo* damage by TNF-α. *Adora2a*$^{+/+}$ ($n = 5$) and *Adora2a*$^{-/-}$ ($n = 5$) mice were treated with a combination of D-galactosamine (700 mg kg^{-1}) and TNF-α (15 μg kg^{-1}). Liver damage was evaluated 6 h later by measuring serum ALT levels.

negative feedback mechanism for limitation and termination of both tissue-specific and systemic inflammatory responses.

The accumulation of extracellular adenosine in inflamed and damaged tissues[18–22] and the immunosuppressive properties of cAMP-elevating adenosine receptors[8–11,14,15,23] indicate that signalling by A2a adenosine receptors on immune cells is a possible natural mechanism of inhibition and/or termination of inflammation. To test whether the absence of functional A2a adenosine receptors results in increased inflammation and exacerbated tissue damage, we used mice deficient in the adenosine A2a receptor gene (*Adora2a*$^{-/-}$)[24,25], which had been characterized previously in studies of immune cells[26,27] and cardiovascular and neurological systems[24,25].

Concanavalin A (Con A)-induced liver injury in mice is mediated by T cells, macrophages and cytokines tumour-necrosis factor (TNF)-α, interleukin (IL)-4 and interferon (IFN)-γ, and represents a well described *in vivo* inflammation model of viral and autoimmune hepatitis[16,17]. The pharmacological activation of A2a receptors by a selective A2a receptor agonist[26], CGS21680, prevented liver damage (Fig. 1a and Supplementary Information) and pro-inflammatory TNF-α accumulation *in vivo* (Fig. 1b). CGS21680 also inhibited secretion of TNF-α, IL-12 and IFN-γ by activated macrophages and T cells *in vitro* (data not shown). These data, however, do not show conclusively that adenosine receptors actually serve *in vivo* as physiological regulators of inflammation; other G_s-coupled receptors (for example, prostaglandin E_2 (PGE_2), histamine H2 and β-adrenergic receptors[12,13]) could also function in a similar capacity, as they trigger cAMP accumulation (Fig. 1c, d), inhibit cytokine secretion by immune cells *in vitro* and prevent liver damage *in vivo* (Fig. 1a).

We next investigated whether genetic inactivation of A2a receptors increases the intensity and prolongs duration of T-cell- and macrophage-dependent pro-inflammatory cytokine accumulation and liver damage *in vivo* in *Adora2a*$^{-/-}$ mice treated with Con A. We confirmed that A2a receptor signalling (cAMP accumulation) is abolished in liver mononuclear cells (Fig. 1c, d) and macrophages (data not shown) from *Adora2a*$^{-/-}$ mice as compared with cells from *Adora2a*$^{+/+}$ littermates. The function of other G_s-coupled receptors is not affected in *Adora2a*$^{-/-}$ mice, as their ligands have similar effects on cells of both *Adora2a*$^{+/+}$ and *Adora2a*$^{-/-}$ mice (Fig. 1c, d). The Con-A-induced inflammatory damage was so severe in *Adora2a*$^{-/-}$ mice that two out of four male mice in the group died within 8 h, thereby precluding time-course studies. The same stimuli given to control wild-type *Adora2a*$^{+/+}$ mice did not cause death. Even a sub-optimal dose of an inflammatory stimulus (see inset to Fig. 2a) resulted in the death of two out of five male *Adora2a*$^{-/-}$ mice within 48 h, whereas all *Adora2a*$^{+/+}$ controls survived. Low doses of Con A, which caused only minimal or no liver damage in control *Adora2a*$^{+/+}$ mice, were sufficient to induce extensive liver damage in *Adora2a*$^{-/-}$ mice (Fig. 2b). Exacerbated inflammation and tissue damage in *Adora2a*$^{-/-}$ mice could not be explained by increased susceptibility of *Adora2a*$^{-/-}$ hepatocytes to TNF-α, as TNF-α was equally efficient in directly destroying hepatocytes in both *Adora2a*$^{-/-}$ and *Adora2a*$^{+/+}$ mice *in vivo* (Fig. 2c and data not shown). Furthermore, excessive and prolonged pro-inflammatory TNF-α accumulation was observed in the serum of *Adora2a*$^{-/-}$ mice compared with low or undetectable TNF-α levels in *Adora2a*$^{+/+}$ mice (Fig. 2a). IFN-γ was also present at higher concentrations and for a longer duration in *Adora2a*$^{-/-}$ mice, although levels of IL-4 were not different between *Adora2a*$^{-/-}$ and

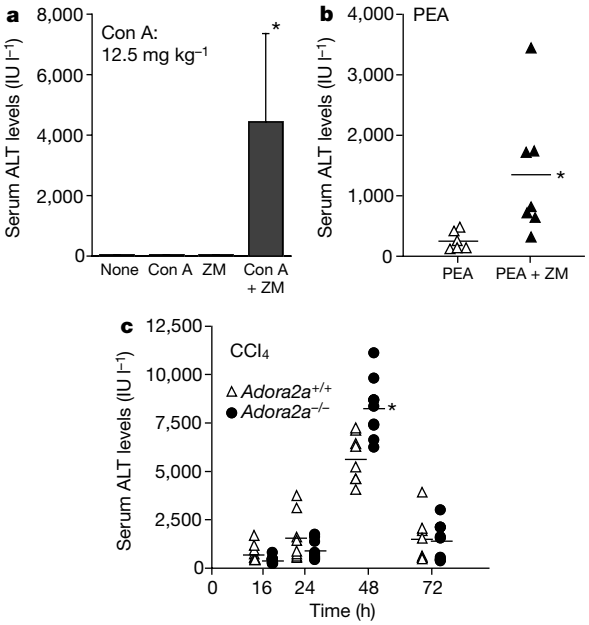

Figure 3 Pharmacological inactivation of A2a receptors *in vivo* by antagonist ZM241385 exacerbates Con A- (**a**) and PEA-induced (**b**) liver damage. Genetic Adora2a deficiency enhances carbon tetrachloride (CCl_4) hepatotoxicity (**c**). B6 mice ($n = 5$) were injected with 12.5 mg kg^{-1} Con A alone or in combination with Adora2a antagonist ZM241385 (2 mg kg^{-1}) followed by ALT measurements and histological evaluation. Exotoxin A (PEA, 100 μg kg^{-1}) was injected alone ($n = 6$) or in combination with ZM241385 ($n = 7$), and liver damage was evaluated at 24 h. *Adora2a*$^{+/+}$ ($n = 7$) and *Adora2a*$^{-/-}$ mice ($n = 7$) were injected intraperitoneally with 0.5 ml kg^{-1} CCl_4. Data shown are representative of three separate experiments. Asterisk, $P < 0.05$ compared with *Adora2a*$^{+/+}$ mice.

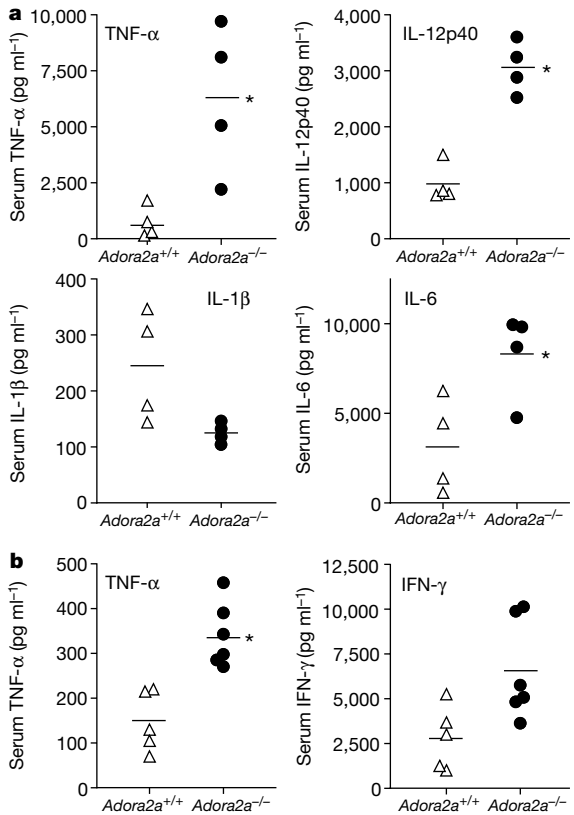

Figure 4 Enhanced accumulation of pro-inflammatory cytokines and tissue damage in A2a-receptor-deficient mice treated with endotoxin. **a**, LPS (100 μg kg^{-1}) was injected into the dorsal air pouch of *Adora2a*$^{-/-}$ and *Adora2a*$^{+/+}$ mice ($n = 4$), and serum cytokines levels were estimated at different times after LPS injection. Data shown are TNF-α levels at 1 h, IL-12p40 levels at 3 h, IL-1β levels at 3 h and IL-6 levels at 1 h. Asterisk, $P < 0.05$ compared with *Adora2a*$^{+/+}$ mice. **b**, *Adora2a*$^{+/+}$ and *Adora2a*$^{-/-}$ mice ($n = 6$) were injected intravenously with LPS (5 mg kg^{-1}). Serum cytokine levels after 16 h are shown. Data shown are representative of three separate experiments. Asterisk, $P < 0.05$ compared with *Adora2a*$^{+/+}$ mice.

Adora2a$^{+/+}$ mice (Fig. 2a). Pharmacological inactivation of A2a receptors in *Adora2a*$^{+/+}$ wild-type mice using a selective A2 receptor antagonist, ZM2413856 (refs 26, 28), also resulted in an increased inflammatory tissue damage in *Adora2a*$^{-/-}$ mice (Fig. 3a; see also Supplementary Information).

Tissue-protecting properties of A2 adenosine receptors were further confirmed in other models of inflammatory liver injury and systemic inflammation (Figs 3b, c and 4). Inactivation of A2 adenosine receptors in *Adora2a*$^{+/+}$ mice by an antagonist, ZM241385, exacerbated (Fig. 3b) the T-cell- and TNF-α-dependent acute hepatotoxicity of *Pseudomonas aeruginosa* exotoxin A (PEA)[29]. The increased liver injury was observed in *Adora2a*$^{-/-}$ mice during chemically induced[30] (carbon tetrachloride, CCl_4) acute hepatotoxicity (Fig. 3c). The A2a adenosine receptors are also shown to be crucial in downregulating pro-inflammatory cytokine accumulation and tissue damage (see Supplementary Information) in an *in vivo* septic shock model[1] after subcutaneous (Fig. 4a) and intravenous (Fig. 4b) bacterial endotoxin (lipopolysaccharide, LPS) injection.

The striking phenotype of markedly enhanced liver damage and sepsis in Adora2a-deficient mice suggests that no other mechanism of downregulation of inflammation *in vivo* is able to fully compensate for the lack of adenosine receptors in T cells and cells of the innate immune system. The uniqueness of the adenosine receptors may lie in the physiology of accumulation of abundant and ubiquitous purine nucleotides[5,6] in the local inflammatory environment[18–22]. Finally, the marked effects of A2a receptor deficiency on functions of immune cells *in vivo* point to the possible role of A2a receptors and other purinergic receptors in the regulation of different stages of immune responses, including antigen presentation, T-cell activation, expansion, survival and memory. □

Methods

Mice

The C57BL/6 A2a-receptor-deficient mice (Adora2a$^{-/-}$ N7) were described previously[25–27]. We determined the A2a receptor genotypes of mice by Southern blot analysis[25]. Littermates or age-matched wild-type (*Adora2a*$^{+/+}$) and homozygous (*Adora2a*$^{-/-}$) mice were used in experiments for better reproducibility of results.

Reagents

Concanavalin A (type IV), D-galactosamine, isoproterenol, PGE_2, LPS (*Escherichia coli* 0111:B4), PEA, CCl_4 and a serum transaminase ALT determination kit were purchased from Sigma. We purchased CGS21680 and ZM241385 from Tocris. Recombinant mouse TNF-α was purchased from Pharmingen.

Measurements of cAMP

Liver mononuclear cells were separated from parenchymal hepatocytes and cell debris by centrifugation using 33% Percoll (see Supplementary Information for detailed methods). Stimulation of cells and the measurement of cAMP levels were executed as described previously[26]. Liver mononuclear cells (1×10^5 cells per 200 μl) were incubated at 37 °C for 30 min in the presence of 10 μM CGS21680, 1 μM ZM241385, 100 μM isoproterenol, or 1 μM PGE_2. Levels of cAMP were determined using a cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech).

Induction of liver injury

Mice were injected intravenously with Con A (12.5 or 20 mg kg^{-1}) in sterile PBS, and serum samples were taken or mice were killed at indicated time points. Some mice were coinjected intraperitoneally with CGS21680 (2 mg kg^{-1}), ZM241385 (2 mg kg^{-1}), isoproterenol (100 mg kg^{-1}), or PGE_2 (5 mg kg^{-1}) just before treatment with Con A. The magnitude of liver damage was evaluated by serum alanine aminotransferase (ALT) levels and liver tissue histology. Effects of TNF-α on liver injury of *Adora2a*$^{+/+}$ and *Adora2a*$^{-/-}$ mice were compared by injecting mice with a combination of D-galactosamine and TNF-α. D-galactosamine (700 mg per kg) was injected intraperitoneally 30 min before intravenous injection of recombinant mouse TNF-α (4–15 μg kg^{-1}). After 6 h, mice were killed and the liver damage was evaluated as described.

In studies of PEA-induced (100 μg kg^{-1} intravenously) liver injury[29], some of the mice were also treated with intraperitoneal injections of ZM241385 before and 12 h after PEA injection. Studies of CCl_4-induced hepatotoxicity[30] were conducted after intraperitoneal injections of *Adora2a*$^{+/+}$ and *Adora2a*$^{-/-}$ mice with CCl_4 (0.5 ml kg^{-1}) dissolved in olive oil.

Treatment with endotoxin (LPS)

Endotoxic shock in male *Adora2a*$^{-/-}$ mice and age-matched *Adora2a*$^{+/+}$ mice was induced by intravenous injection of 5 mg kg^{-1} LPS. At 1 and 16 h after injection, samples of blood were taken by retro-orbital bleeding. LPS was injected (100 μg kg^{-1}) into a dorsal air pouch, which was prepared using sterile air essentially as described[2].

Histological examination and apoptosis assays

Apoptosis assays (the detection of apoptotic cells by *in situ* staining of single-strand breaks in nuclear DNA) were performed by molecular histology. The histological evaluation of tissues after intravenous injections of mice with endotoxin (LPS) was performed by D. Haines. See Supplementary Information for detailed methods.

Measurements of cytokine levels

TNF-α, IFN-γ, IL-4, IL-6, IL-1β and IL-12p40 levels in the sera and culture supernatant were determined using enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D systems according to the manufacturer's suggestions.

Statistical analysis

Data are expressed as mean ± s.e.m. Differences between groups were evaluated using Student's *t*-test.

Received 17 September; accepted 8 October 2001.

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank J. Fan Chen, S. Fink and M. A. Schwartzschild for providing A2a receptor gene deficient mice; W. E. Paul, R. N. Germain, S. Apasov, P. Smith and P. M. Murphy for support, discussions and help; and J. Kinsel and S. Starnes for help in preparation of the manuscript.

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MIF regulates innate immune responses through modulation of Toll-like receptor 4

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Macrophages are pivotal effector cells of the innate immune system, which is vital for recognizing and eliminating invasive microbial pathogens[1,2]. When microbial products bind to pathogen-recognition receptors, macrophages become activated and release a broad array of cytokines[3] that orchestrate the host innate and adaptive immune responses. Initially identified as a T-cell cytokine[4,5], macrophage migration inhibitory factor (MIF) is also a macrophage cytokine and an important mediator of inflammation and sepsis[6–12]. Here we report that MIF is an essential regulator of macrophage responses to endotoxin (lipopolysaccharide) and Gram-negative bacteria. Compared with wild-type cells, MIF-deficient macrophages are hyporesponsive to lipopolysaccharide and Gram-negative bacteria, as shown by a profound reduction in the activity of NF-κB and the production of tumour-necrosis factor-α. This reduction is due to a downregulation of Toll-like receptor 4 (TLR4), the signal-transducing molecule of the lipopolysaccharide receptor complex, and is associated with decreased activity of transcription factor PU.1, which is required for optimal expression of the *Tlr4* gene in myeloid cells. These findings identify an important role for MIF in innate immunity and provide a molecular basis for the resistance of MIF-deficient mice to endotoxic shock.

Monocytes and macrophages constitutively express copious amounts of MIF; such expression is an unusual feature for a cytokine[7]. Given that MIF has been shown to exert intracellular activity[13], we reasoned that reducing the endogenous content of MIF in macrophages might help to unravel the function of MIF in the innate immune system.

RAW 264.7 mouse macrophages were stably transfected with a plasmid encoding an antisense MIF messenger RNA. Two clones (designated as AS 2.8 and AS 2.23) exhibiting a marked reduction in MIF protein were compared with control macrophages transfected with the empty plasmid (Fig. 1a). In contrast to control macrophages, antisense MIF macrophages were hyporesponsive to endotoxin (*Escherichia coli* O111:B4 lipopolysaccharide; LPS) stimulation, as shown by a profound reduction in tumour-necrosis factor-α (TNF-α) and interleukin 6 (IL-6) (Fig. 1b; and data not shown). Similar results were obtained when antisense MIF macrophages were exposed to heat-killed *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*—the three most frequent causes of Gram-negative sepsis in humans (Fig. 1c).

In contrast, control and antisense MIF macrophages responded equally well to stimulation with heat-killed Gram-positive bacteria (*Staphylococcus aureus*, group A streptococci), peptidoglycan or the yeast particle zymosan, and to stimulation with phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore (Fig. 1d; and data not shown). Thus, antisense MIF macrophages displayed a defect in response to LPS and Gram-negative bacteria, but not to other microbial stimuli, indicating that MIF might have a role in the signalling pathways activated by LPS in the macrophage.

To explore the molecular mechanism by which MIF regulates the responses of macrophages to LPS and Gram-negative bacteria, we examined the intracellular events that control expression of the TNF-α gene. The DNA-binding activity of nuclear factor kappa B (NF-κB), the luciferase reporter activity driven by NF-κB, and the concentrations of TNF-α mRNA (Fig. 1e; and Supplementary Information) were all markedly decreased in antisense MIF macrophages stimulated with LPS. These results indicate that reducing the endogenous concentrations of MIF in the macrophage may impede LPS signal transduction at a point positioned between the binding of LPS to its receptor complex and the activation of NF-κB.

Recognition of LPS and Gram-negative bacteria by the host requires cooperative interplay between the LPS-binding protein (LBP)[14], CD14 (ref. 15) and Toll-like receptor 4 (TLR4)[16]. LBP binds and transfers LPS-containing particles to a receptor complex

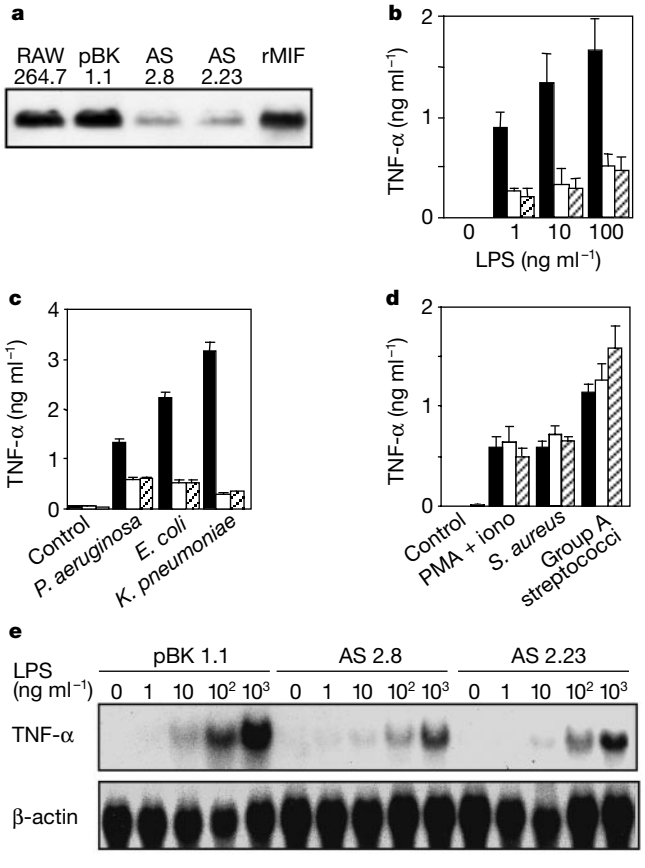

Figure 1 Hyporesponsiveness of antisense MIF macrophages to activation by LPS and Gram-negative bacteria. **a**, Western blot of intracellular MIF content of RAW 264.7 macrophages stably transfected with an empty plasmid (pBK 1.1) or with an antisense MIF cDNA plasmid (AS 2.8 and AS 2.23). rMIF, recombinant MIF. **b**–**d**, TNF-α production by pBK 1.1 (black bars), AS 2.8 (white bars) and AS 2.23 (hatched bars) macrophages stimulated with LPS, 10^8 c.f.u. of heat-killed bacteria, or PMA plus ionomycin (PMA+iono). Data are the mean ± s.d. of triplicate samples from 1 experiment and are representative of 3–9 independent experiments. **e**, Northern blots of TNF-α and β-actin mRNA in pBK 1.1, AS 2.8 and AS 2.23 macrophages stimulated for 2 h with LPS. Data are representative of four separate experiments.