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Mice lacking alpha2-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock

Abstract

The onset of an acute phase response is one of the initial steps in the defense against an infectious organism. alpha2-macroglobulin (alpha2M), an acute phase protein in most mammalian species, is known to have a broad antiprotease activity, but it can also bind a number of growth factors, cytokines,

ions and lipid factors. We have shown that alpha2M-deficient (MAM-/-) mice are more resistant to a lethal Gram-negative infection compared to control mice. This increased resistance was reflected in significantly higher body temperatures, compared to control mice, during the infection as well as in a prolonged and increased survival. Moreover, the clearance of bacteria in MAM-/- mice was significantly more efficient than in control mice. On the other hand, MAM-/- mice were more susceptible to endotoxin. An LD100 challenge with endotoxin in MAM-/- mice was not lethal for control mice. Our data suggest that alpha2M plays a dual role during an acute phase response. In the establishment of a lethal Gram-negative infection, leading to sepsis and septic shock, it has a mediating role by hampering the efficient clearance of bacteria. During endotoxic shock, however, alpha2M has a rather protective function.

Full text

INTRODUCTION

Septic shock due to a bacterial infection is the main cause of mortality in the intensive care unit [1]. Almost any bacterium can cause septic shock, but *Escherichia coli* and *Klebsiella pneumoniae* are among the most frequent culture isolates [2]. Unfortunately, the emergence of multidrug-resistant bacteria has made the treatment of these patients quite problematic [3]. Septic shock is the result of a very complex sequence of events, which is far from being completely understood. It is characterized by an overwhelming inflammatory response to the infectious microorganism itself or a component of its cell wall, namely endotoxin. This is due to proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1, which initiate a cascade of inflammatory cytokines, leading to the hemodynamic changes and inflammatory events typical of sepsis [4, 5]. However, therapies with an anti-TNF antibody or an IL-1 receptor antagonist have not proven to be helpful for several reasons [6]. First, any cytokine or mediator administered is only part of the puzzle; second, by the time patients reach medical attention, the cascade is in full motion, so that neutralization of an early mediator may be of marginal benefit. Clearly, new and other approaches have to be followed. Therefore, host immune defense plays an increasing role in the final outcome of bacterial invasion. One possibility is to identify the contribution of endogenous molecules in the defense against an infection and to evaluate the therapeutic use. One of the first events following an infection is the acute phase response [7]. It is generally believed that acute phase changes play a major role in adaptation and defense. Acute phase

changes include, amongst others, neuroendocrine and hematological changes, metabolic processes, and changes in nonprotein plasma components. But the most pronounced hallmark of the acute phase response is the change in concentration of a large number of plasma proteins, namely the acute phase proteins. alpha$_2$-macroglobulin (alpha$_2$M) is one of these acute phase proteins [8, 9]. It is a high-molecular weight glycoprotein of 720 kDa, consisting of four identical subunits, and inhibits proteinases from all four major classes [10]. Proteinase binding by alpha$_2$M occurs by a "trap" mechanism in which proteolytic cleavage of the bait region results in a conformational change of the alpha$_2$M tetramer, thereby trapping the proteinase. The alpha$_2$M-proteinase complexes are then rapidly cleared from the circulation by high-affinity receptors on various cell types [11]. Besides proteinases, alpha$_2$M also binds a number of cytokines and growth factors, such as TNF [12], IL-1beta [13], IL-6 [14], IL-10 [15], NGF [16], PDGF [17], TGF-beta [18] and mitogens such as LPS [19]. By binding to its signaling receptor, alpha$_2$M can induce the release of platelet-activating factor [20]. Several groups have described anti-inflammatory effects of alpha$_2$M, for example in D-(+)-galactosamine-induced hepatitis [21], burn trauma [22], anaphylactic shock [23] and inflammation caused by histamin, bradykinin or prostaglandins [24]. Furthermore, alpha$_2$M inhibits PMN chemotaxis [25], influenza virus [26] and *Trypanosoma cruzi* infections [27]. Mouse alpha$_2$M (MAM) has been knocked out in mice, without effects on the viability [28]; this was somewhat surprising, since no alpha$_2$M-deficiency in humans is known, suggesting a vital function [29]. In this paper we investigated the function of alpha$_2$M during shock induced by bacteria and endotoxin.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Harlan Olac (Blackthorn, UK) and were further bred in our animal facility. MAM$^{-/-}$ mice were generated by gene targeting in ES cells. Mutant mice were back-crossed for seven generations in C57BL/6 mice using C57BL/6 mice. After back-crossing, the mice (> 99% C57BL/6) were intercrossed; homozygous knockouts were further bred as brother-sister couples. All mice were kept in a conventional mouse room in 12-hours light/dark cycles, and received food and water ad libitum. Female mice were used at the age of 8 weeks. At that age all mice had a similar body weight.

Infection model

*K. pneumoniae (*ATCC 43816), a strain which produces lethal sepsis in normal mice [30, 31], was inoculated intraperitoneally (i.p.; 0.5 ml). Survival was scored over a period of at least 10 days.

Injections

LPS from *Salmonella abortus equi* (Sigma Chemical Co., St. Louis, MO) was diluted in pyrogen-free PBS prior to injection and was given i.p. in a volume of 0.5 ml.

Clearance of bacteria

Thirty-four hours after an i.p. injection of *K. pneumoniae*, mice were anesthesized with tribromoethanol. Blood was taken by heart puncture. For preparation of plasma, 450 mul blood was added to 50 mul of sodium citrate (0.1 M). Immediately thereafter, mice were killed by cervical dislocation. Mice were perfused with 10 ml of a 0.9% NaCl solution to wash out the blood. The liver, spleen and kidney were removed aseptically, weighed and homogenized mechanically in sterile saline. For homogenization, the liver was diluted (w/v) 2-fold; spleen and kidney were diluted 10-fold. The suspensions were diluted and plated out on sterile nutrient agar. After overnight incubation at 37° C, CFU numbers were counted.

Blood collections and measurement of body temperatures

Hundred mul blood was withdrawn at the orbital plexus under light ether anesthesia. Rectal body temperatures were measured with an electronic thermometer (model 2001; Comark Electronics, Littlehampton, UK).

Measurement of IL-6

IL-6 was determined as described previously [32]. IL-6-dependent 7TD1 cells were cultured in 96-well microtiter plates (7,000 cells/well) in the presence of medium, serial dilutions of serum, or a murine IL-6 standard. After 3 days of culture, the number of living cells was determined in a hexosaminidase

colorimetric assay; titers were assigned by comparing the dilutions of samples and standard needed to obtain half-maximal growth of 7TD1 cells.

Statistical analysis

Survival was scored and evaluated using a Logrank test. Final lethality was scored using a chi^2 test. The statistical significance of the body temperatures, IL-6 levels and the number of bacterial colonies in blood and organs was determined with a Dunnett Anova test. $P < 0.05$ was considered statistically significant. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

RESULTS

Protection of MAM$^{-/-}$ mice against a lethal bacterial challenge

To test the effect of a lethal Gram-negative infection in MAM$^{-/-}$ mice (compared to control mice) several concentrations of *K. pneumoniae* were injected i.p. When a dose of 10^4 CFU *K. pneumoniae* was injected, no differences in the survival between control and MAM$^{-/-}$ mice were observed (Figure 1A). With a dose of 10^2 CFU, however, MAM$^{-/-}$ mice lived significantly ($p = 0.0038$) longer than control mice (Figure 1B). Mice were observed for 10 days, since after 10 days no further deaths occurred.

Measurement of temperatures and cytokines

During the challenge, body temperatures and serum IL-6 levels of the mice were measured. Thirty-two and 56 hours after a challenge with 10^4 CFU *K. pneumoniae*, MAM$^{-/-}$ mice had a significantly higher temperature than the control mice ($p = 0.027$ and 0.0072, respectively). When a dose of 10^2 CFU *K. pneumoniae* was used, MAM$^{-/-}$ mice also had a significantly higher temperature than the control mice (32 hours after the challenge; $p = 0.0273$) (Table 1). IL-6 levels in the serum of MAM$^{-/-}$ mice were significantly lower ($p = 0.0017$) than control mice, measured 34 hours after a challenge with 10^4 CFU *K. pneumoniae* (Table 1).

Clearance of K. pneumoniae *in blood and different organs*

In order to unravel the mechanism of protection against a lethal challenge with *K. pneumoniae*, we studied the number of bacteria in different organs and blood. Thirty-four hours after a challenge with 10^2 CFU of *K. pneumoniae*, $MAM^{-/-}$ mice showed significantly less bacteria in the lung, spleen ($p < 0.01$), liver and blood ($p < 0.05$) than control mice (Figure 2). These data suggest that the mechanism of protection is probably due to a faster clearance of bacteria in $MAM^{-/-}$ mice.

Sensitization of $MAM^{-/-}$ mice to an endotoxin challenge

To test whether $alpha_2M$ plays a role in LPS-mediated sepsis, $MAM^{-/-}$ mice were injected with different doses of endotoxin in order to determine the LD_{100} (data not shown). In a second experiment, $MAM^{-/-}$ and control mice were injected with 100 mug of endotoxin (= LD_{100} for $MAM^{-/-}$ mice) and survival was scored (Figure 3). The results of these experiments show that $MAM^{-/-}$ mice are more susceptible to endotoxic shock than control mice.

DISCUSSION

Septic shock is the culmination of a series of events initiated by the presence of microorganisms (and their proteins or lipid products) in the circulation and is mediated by the host's own immune system [1]. Shock is the result of the combined action of several proinflammatory cytokines induced in macrophages and other cells [5]. Septic shock and sepsis, leading to cardiovascular depression and multiple organ failure, are causing more than 100,000 casualties per year in the United States [33]. Unfortunately, the emergence of multidrug-resistant bacteria has made the treatment of these patients quite problematic [3]. Moreover, therapies with an anti-TNF antibody or IL-1 receptor antagonist have not proven to be helpful [6]. Clearly, new and other approaches are needed. Therefore it is necessary to understand the function of the different aspects of the host's defense system against a challenge with living bacteria and/or their immune stimulating cell-wall components. One of the first reactions following an infection is the onset of an acute phase response. The main characteristic is the change in concentration of a large number of plasma proteins, the acute phase proteins [7]. It is widely held that components of the acute phase response influence the inflammatory response or enhance the adaptation to noxious stimuli. Although this is probably true most of the time, it is not invariably true. The host response may be either protective or destructive; an example of the latter is septic shock [8]. $alpha_2M$ is an acute phase reactant

in most mammalian species, but not in humans [9, 11]. It is a high molecular weight glycoprotein (720 kDa) consisting of four identical subunits, and it is able to inhibit proteinases from all four major classes by using a so-called "trap mechanism" [10]. The trapped proteinase is removed from the circulation by high affinity receptor binding of the complex [11]. alpha$_2$M is also able to bind a number of cytokines and growth factors. The binding to alpha$_2$M does not necessarily lead to a reduced activity of the cytokine or growth factor, but it may have effects on the pharmacokinetics and bioavailability of the hormones [29]. Several groups have described the anti-inflammatory effects of alpha$_2$M *in vivo* [21-27]. alpha$_2$M has been knocked out in mice, without effects on the viability [28].

In the present study, we investigated the possible function of alpha$_2$M during septic shock induced by *K. pneumoniae* [30, 31] and in LPS-induced shock using MAM$^{-/-}$ mice. This work is an extension of previous research on the role of acute phase proteins in TNF-induced and septic shock [34-36]. We found that MAM$^{-/-}$ mice did not significantly live longer when given a high dose of bacteria i.p. (10^4 CFU), although MAM$^{-/-}$ mice had higher temperatures and lower IL-6 levels after the bacterial infection. Survival of MAM$^{-/-}$ mice was 54.5%, compared to 7.6% for control mice, when a lower dose of bacteria (10^2 CFU) was administered. Protection of MAM$^{-/-}$ mice was also reflected in significantly higher body temperatures 32 hours after the infection.

To clarify the mechanism of protection, we studied the clearance of bacteria in blood and different organs. We found that the number of bacteria in blood, liver, kidney and spleen, 34 hours after infection with a lethal dose of *K. pneumoniae*, was significantly lower in MAM$^{-/-}$ mice than in control mice. Since early bacteremia has been observed in animals infected with *K. pneumoniae*, it is postulated that mice are dying due to dissemination of the invading organism and associated sepsis [31]. Therefore, the survival benefits conferred by the absence of alpha$_2$M may be a result of enhanced initial clearance of *K. pneumoniae*, as evidenced by the significant reduction in CFU noted in blood and liver, kidney and lung homogenates. This initial clearance of bacteria likely leads to a more effective containment of the infection and to an improved outcome. The absence of alpha$_2$M can contribute to an enhanced clearance of bacteria in different ways. First, the enhanced clearance of bacteria can be the result of an enhanced neutrophil recruitment. It has already been demonstrated, both *in vitro* and *in vivo*, that alpha$_2$M shows strong PMN chemotaxis- and

phagocytosis-inhibiting properties [19, 25]. Since alpha$_2$M is not the only acute phase protein inhibiting the chemotactic response, we cannot ascribe the enhanced clearance of bacteria in MAM$^{-/-}$ mice to the PMN-inhibiting property of alpha$_2$M only. Other mechanisms are clearly also involved. Second, binding of receptor-recognized forms of alpha$_2$M to macrophage alpha$_2$M-signaling receptor induces synthesis of platelet activating factor, a well-known mediator of endotoxic shock in rodents [20]. Third, alpha$_2$M is also known to bind and inhibit a number of proteases. Neutrophil elastase (NE), one of the proteases that bind to alpha$_2$M [19], plays an important role in the defense against a Gram-negative infection. It was shown that NE$^{-/-}$ mice are more susceptible than their normal littermates to sepsis and death following an i.p. infection with *K. pneumoniae*. NE is also directly cytotoxic to *K. pneumoniae* [31]. TNF has been demonstrated to be an essential cytokine mediator of bacterial clearance [37]. alpha$_2$M suppresses the secretion of TNF by human peri-pheral blood mononuclear cells in a concentration-dependent manner [38]. Since the alpha$_2$M concentration rises during an acute phase response caused by a bacterial challenge, it is possible that less TNF is released from mononuclear cells than needed to provide a sufficient clearance of bacteria. Finally, as alpha$_2$M binds a wide variety of molecules, we cannot exclude other mechanisms contributing to the clearance of bacteria affected by the absence of alpha$_2$M.

In contrast to the potentially mediating role of alpha$_2$M during a Gram-negative infection, we showed that alpha$_2$M plays a rather protective role during endotoxic shock, since MAM$^{-/-}$ mice are more susceptible to an endotoxin challenge than control mice. In line with these results, alpha$_2$M has been reported to protect against endotoxic shock, possibly by binding to the vasodilating agent PGE$_2$, thereby reducing vascular permeability [39]. MAM$^{-/-}$ mice, challenged in the footpad with LPS from *Escherichia coli* (not from *Salmonella abortus equi*), have been shown to be less susceptible to such a challenge than control mice [28]. It should be noted that the latter experiments were done in mice with a mixed genetic background, since they had not been backcrossed. In our experiments, we used MAM$^{-/-}$ mice that had been backcrossed for seven generations in a C57BL/6 background. Since it has been shown that alpha$_2$M can bind LPS, this opposite effect may be due to a differential binding of alpha$_2$M to different types of LPS.

Previously, we showed that $MAM^{-/-}$ mice are protected against TNF-induced lethality [36]. This is, however, not in contradiction with the present results demonstrating that $MAM^{-/-}$ mice are more susceptible to LPS-induced lethality, since LPS-induced toxicity does not necessarily depend on TNF induction [40-42].

CONCLUSION

In conclusion, we demonstrate that $MAM^{-/-}$ mice are resistant to a lethal Gram-negative infection. $MAM^{-/-}$ mice have significantly higher temperatures during the infection and live significantly longer than control mice. Moreover, $MAM^{-/-}$ mice have significantly less bacteria in the blood and in different organs, suggesting a more efficient clearing mechanism in the absence of $alpha_2M$. We think that the capacity of $alpha_2M$ to bind and to inhibit a number of molecules, able to contribute to an efficient defense against the bacterial challenge, might be a reasonable explanation for protection. $MAM^{-/-}$ mice were, however, more susceptible to an endotoxin challenge, possibly due to lack of binding of PGE_2 by $alpha_2M$ in the course of endotoxic shock, resulting in an increased vascular permeability (compared to control animals).

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Figures

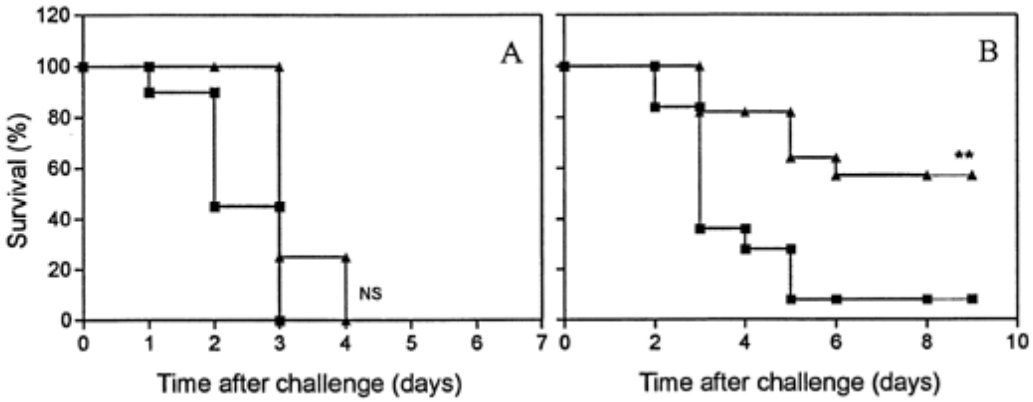


Figure 1

A. Lethal effect of a dose of 10^4 CFU of *K. pneumoniae* given i.p. in MAM$^{-/-}$ (triangle ; n = 8) and control mice (square ; n = 20). **B.** Lethal effect of a dose of 10^2 CFU of *K. pneumoniae* in MAM$^{-/-}$ (triangle ; n = 11) and control mice (square ; n = 13). ** $p < 0.01$.

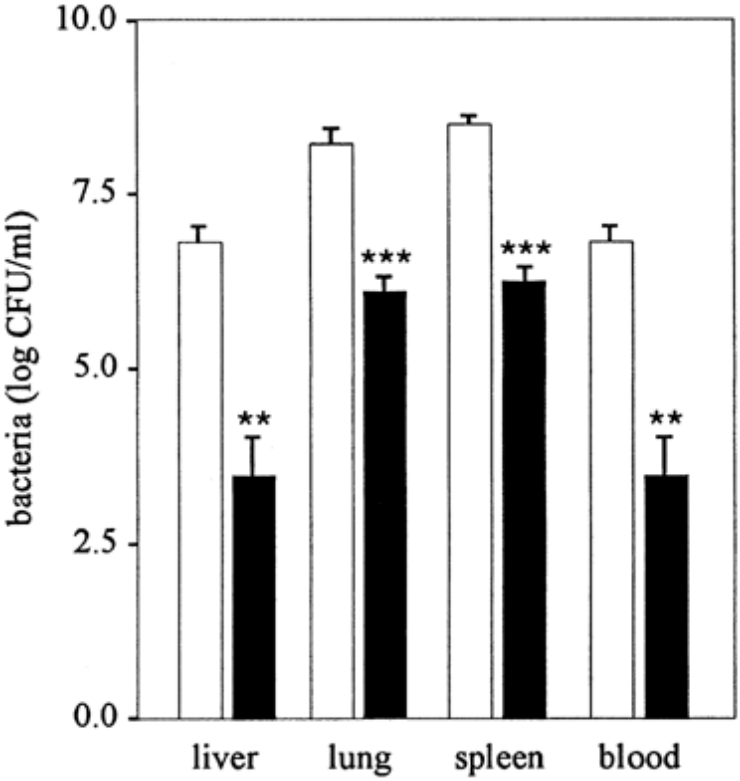


Figure 2
Counts of *K. pneumoniae* colonies in organs after 34 hours of a challenge with 10^2 CFU of *K. pneumoniae*.
Each bar represents the mean ± SD of log CFU/ml of homogenized tissue from three mice (open bars, control mice; black bars, $MAM^{-/-}$ mice). ** $p < 0.01$; *** $p < 0.001$.

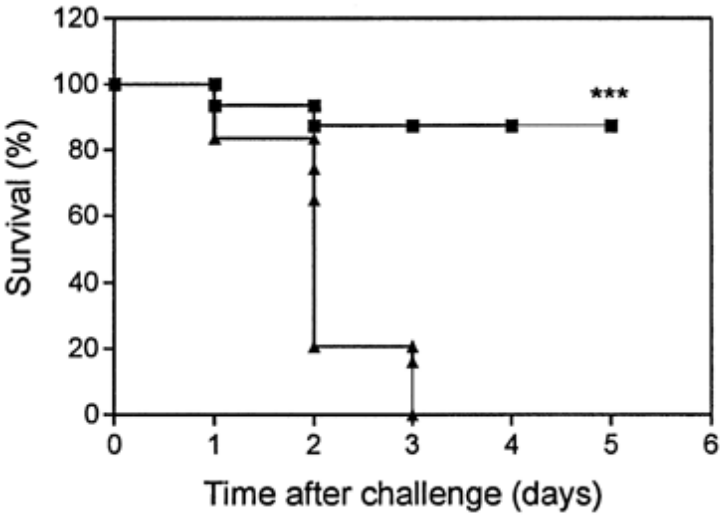


Figure 3
Survival following an i.p. challenge with 100 mug of endotoxin in MAM$^{-/-}$ (triangle ; n = 43) and control mice (square ; n = 16). *** p < 0.001.

Table 1
Temperatures and IL-6 levels after i.p. challenge with *K. pneumoniae* of MAM$^{-/-}$ mice and controls

Challenge	Genotype	After 32 hours Temperature (° C)	After 56 hours Temperature (° C)	After 34 hours IL-6 (pg/ml)
10^4 CFU	Controls	35.7 ± 1.8 (n = 10)	27.5 ± 4.1 (n = 3)	4,860 ± 918 (n = 9)
	MAM$^{-/-}$	37.4 ± 0.3 (n = 7)*	36.4 ± 0.8 (n = 4)**	2,327 ± 80 (n = 3)**
10^3 CFU	Controls	34.9 ± 3.9 (n = 13)	ND	ND
	MAM$^{-/-}$	37.8 ± 0.5 (n = 11)*	ND	ND

* p < 0.05; ** p < 0.01 (statistical significance compared to the values of wild-type mice at the same timepoint).

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Moshe Hacham, Shmuel Argov, Rosalyn M. White, Shraga Segal, Ron N. Apte
IL-1alpha, IL-1beta, organ cytokine expression, young/old age, homeostasis.

The combination of the interleukin-1alpha (IL-1alpha-889) genotype and the interleukin-10 (IL-10 ATA) haplotype is associated with increased interleukin-10 (IL-10) plasma levels in healthy individuals

Sanna Kilpinen, Heini Huhtala, Mikko Hurme
interleukin-1, interleukin-10, gene polymorphism, plasma.

The interleukin-6 G(-174)C polymorphism and the ex vivo interleukin-6 response to endotoxin in severely injured

blunt trauma patients

Michael Heesen, Udo Obertacke, F. Ulrich Schade, Brunhilde Bloemeke, Matthias Majetschak
interleukin-6, endotoxin, genetic polymorphism, polymerase chain reaction, whole blood, critical illness.

Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals

Daniël Broekaert, Sven Eyckerman, Delphine Lavens, Annick Verhee, Wim Waelput, Joël Vandekerckhove, Jan Tavernier
forskolin, IL-6, leptin, rPAP/Reg gene family, signal transduction.

Mice lacking alpha2-macroglobulin show an increased host defense against Gram-negative bacterial sepsis, but are more susceptible to endotoxic shock

Tino Hochepied, Fred Van Leuven, Claude Libert
TNF, shock, acute phase, inflammation, alpha2-macroglobulin.

Tumor necrosis factor-alpha mRNA stability in human peripheral blood cells after lipopolysaccharide stimulation

Lucile Baseggio, Carole Charlot, Jacques Bienvenu, Pascale Felman, Gilles Salles
tumor necrosis factor, mRNA stability, lipopolysaccharide, tristetraprolin.

Caspase-1 levels in biological fluids from patients with multiple sclerosis and from patients with other neurological and non-neurological diseases

Diego Franciotta, Gianvito Martino, Elisabetta Zardini, Roberto Furlan, Roberto Bergamaschi, Maira Gironi, Alessandra Bergami, Giovanna Angelini, Fabrizio De Benedetti, Patrizia Pignatti, Gianna Moscato, Vittorio Cosi
asthma, cerebrospinal fluid, juvenile idiopathic arthritis, sputum, synovial fluid, THP-1 cells.

Divergent effects of tumor necrosis factor-alpha and lymphotoxin-alpha on lethal endotoxemia and infection with live Salmonella typhimurium in mice

Edi Dharmana, Monique Keuter, Mihai G. Netea, Ineke C.M.M. Verschueren, Bart Jan Kullberg
tumor necrosis factor, lymphotoxin, salmonella typhimunium, knock-out mice.

Th1/Th2 cytokine balance and nitric oxide in cerebrospinal fluid and serum from patients with multiple sclerosis

María del Carmen Rodríguez-Sáinz, Silvia Sánchez-Ramón, Clara de Andrés, Margarita Rodríguez-Mahou, M Angeles Muñoz-Fernández
cytokine, nitric oxide, multiple sclerosis, cerebrospinal fluid, oligoclonal bands, Igs.

Retinoids, cisplatin and interferon-alpha in recurrent or metastatic cervical squamous cell carcinoma: clinical results of 2 phase II trials

Anne-Chantal Braud, Laurence Gonzague, François Bertucci, Dominique Genre, Jacques Camerlo, Gwenaelle Gravis, Anthony Goncalves, Vincent Moutardier, Frédéric Viret, Dominique Maraninchi, Patrice Viens
all-trans-retinoic acid, 13 Cis retinoic acid, interferon-alpha, cervical squamous cell carcinoma.

Circulating macrophage colony stimulating factor as a marker of tumour progression

Raymond S. McDermott, Laurent Deneux, Véronique Mosseri, Jacques Védrenne, Krishna Clough, Alain Fourquet, José Rodriguez, Jean-Marc Cosset, Xavier Sastre, Philippe Beuzeboc, Pierre Pouillart, Susy M. Scholl
CSF-1, tumour progression, metastasis, de-differentiation.

The effect of interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) on granulocyte macrophage-colony stimulating factor (GM-CSF) production by neuronal precursor cells

J. Benjamin Dame, Nasser Chegini, Robert D. Christensen, Sandra E. Juul
IL-1beta, TNF-alpha, GM-CSF, neuron, mRNA, protein.

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