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Differential Induction of Tumor Necrosis Factor Alpha in Murine and Human Leukocytes by *Mycoplasma arthritidis*-Derived Superantigen

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***Mycoplasma arthritidis*-derived superantigen (MAS) is exclusively produced by *M. arthritidis*, which is the only known mycoplasma to produce a superantigen. As a superantigen, MAS shows properties similar to those of the staphylococcal enterotoxins and related substances, such as binding to major histocompatibility complex (MHC) class II and Vβ-specific stimulation of T cells. In this series of experiments, we demonstrate some differences between MAS and other superantigens. MAS induced the production of tumor necrosis factor alpha (TNF-α) mRNA in human as well as in murine leukocytes. However, only in murine leukocytes was the mRNA adequately translated into the protein. In human peripheral blood mononuclear cells, we found only small amounts of TNF, whereas in murine spleen cells we detected levels more than three times higher. The proliferative response to MAS has been shown to be restricted to I-Eα in the murine MHC. Furthermore, TNF was induced in I-Eα$^+$ bone marrow-derived macrophages by MAS. In these cells, MAS rapidly induced very high levels of TNF and the amounts of mRNA detected correlated to the amount of protein produced. In comparison with other superantigens, including the staphylococcal enterotoxins, toxic shock syndrome toxin 1, and exfoliative toxin A, the failure of MAS to induce TNF-α in human peripheral blood mononuclear cells is specific for MAS and not common to all superantigens. The direct activation of bone marrow-derived macrophages also seems to be specific for MAS. These data suggest that the induction of TNF-α by MAS is dependent on the strength of binding to the MHC class II molecule.**

Mycoplasmas are the smallest autoreplicative microorganisms (20). They are parasites which live associated with the cell membranes of eucaryotic cells (23). Today the genus *Mycoplasma* includes approximately 70 different species and a variety of strains of this species. Mycoplasmas are known to interact with the immune system in different ways (26). *Mycoplasma arthritidis* is a rodent pathogen, inducing an acute inflammatory infection. In mice, this inflammation is followed by chronic joint disease (8). The pathogenic effects of *M. arthritidis* in humans remain to be determined (14). In cultures of *M. arthritidis*, a superantigen (MAS) is produced which was initially described as a mitogen (16). In contrast to other mycoplasma mitogens, MAS is a soluble protein of 26,000 Da (12, 17) and not a component of the cell membrane or a lipid fraction of it (24, 26). Marrack and Kappler (21) included MAS in the group of staphylococcal enterotoxin (SE)-like toxins, which are today called superantigens. Excluding the retroviral superantigens and the not-yet-clarified activity of *Yersinia enterocolitica* (29), MAS is the only member of this group which is not produced by gram-positive bacteria (10). Furthermore, MAS is produced exclusively by *M. arthritidis*. No other mycoplasma tested produced a comparable T-cell stimulus (30). Most superantigens seem to be more specific to human cells (11) than to mouse cells as indicated by superantigen-induced proliferation.

T-cell stimulation by superantigens is dependent on major histocompatibility complex (MHC) class II-bearing accessory cells. MAS showed an MHC class II restriction, and only murine accessory cells expressing an intact I-Eα molecule were able to present MAS (5). Human HLA-DR seems to be the counterpart to murine I-Eα for MAS (22). Like every superantigen, MAS has a specificity for distinct Vβ subfamilies of the T-cell receptor (TCR). To date, we know that the Vβ subfamilies 3.1, 11.1, 12.1, 13.1, and 19.1 in humans and 5.1, 6, 8.1, 8.2, and 8.3 in mice are responsive to MAS (1).

MAS induces interleukin 1 (IL-1), IL-2, IL-4, IL-6, and gamma interferon in human peripheral blood mononuclear cells (PBMC) (25) and gamma interferon and IL-6 in murine cells (12, 18).

Tumor necrosis factor alpha (TNF-α) plays an important role in the pathogenesis of sepsis, different autoimmune diseases, and AIDS (19). In the synovial fluid of patients with rheumatoid arthritis, high levels of IL-1, IL-6, and TNF-α have been detected (2). Furthermore, an intra-articular injection of MAS into rats induced a synovitis (23), and mice suffered from necrosis at the injection point of MAS (6).

Since TNF showed cytotoxic and destructive effects, we investigated whether MAS-induced effects in mice and rats can be correlated with TNF induction by MAS. Furthermore, in this series of experiments, we examine the capacity of MAS and other superantigens to induce TNF in human leukocytes.

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MATERIALS AND METHODS

Reagents. MAS was obtained from cultures of *M. arthritidis* Jasmin (ATCC 14124; American Type Culture Collection,

Rockville, Md.), and one batch was used throughout the study. Uninoculated mycoplasma broth, partially purified MAS, and uninoculated mycoplasma broth treated by identical procedures were prepared with the same batches of horse serum (GIBCO/BRL, Berlin, Germany), fresh yeast extract, and pleuropneumonialike organism broth. Partial purification was achieved by a modification of a previously published procedure (12). Briefly, mycoplasma-free supernatants were adjusted to pH 8.5 and dialyzed against polyethylene glycol-EDTA. The concentrate was loaded on a Q-Sepharose column (Pharmacia, Uppsala, Sweden), and the nonbinding fraction was pooled and concentrated 10 times. All preparations were tested for freedom from lipopolysaccharide (LPS; described in Results) and mycoplasmas (as indicated by enhancement broth and final cultivation on solid media). LPS-free preparations of SEs A, B, C1, C2, C3, D, and E (SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE, respectively), toxic shock syndrome toxin 1 (TSST-1), and exfoliative toxin A (ExFTA) were obtained from Toxin Technology, Inc. (Sarasota, Fla.).

Cell preparations. Buffy coats were obtained from healthy donors and separated over a Ficoll-Hypaque (Biochrom, Berlin, Germany) gradient as described previously (28). The PBMC of the interphase were collected and washed twice with phosphate-buffered saline.

Murine spleens and human spleens from organ donors were cut into pieces before they were mashed in a petri dish with the plunger of a syringe. All cellular debris was removed by filtration through gauze. Subsequently, the cells were processed in the same way as buffy coat cells.

For processing of bone marrow, the mice were killed and both femurs in each mouse were dissected. Bones were separated from the legs under sterile conditions, and the bone marrow was flushed out by using a syringe. The bone marrow was mashed, filtered, and washed like spleen cells.

Mouse strains C3H/HeJ and C57BL/6 were obtained from Zentralinstitut für Versuchstierkunde (Hannover, Germany). Mice were received at the age of 4 to 6 weeks and used within 8 to 10 weeks.

Culture conditions. Human cells and murine spleen cells were used at a final concentration of 3×10^6 cells per ml of culture medium. They were cultured in RPMI 1640 (Biochrom) supplemented with 10% myoclone quality heat-inactivated fetal calf serum (GIBCO/BRL), 1% penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml; Biochrom), and 1% 200 mM L-glutamine (Biochrom).

Bone marrow cells were stimulated to differentiate into macrophages by macrophage colony-stimulating factor-containing medium over a period of 8 to 10 days. After separation, bone marrow cells were cultured at 3×10^5 cells per ml in RPMI 1640 (Biochrom) supplemented with 12% myoclone quality heat-inactivated fetal calf serum (GIBCO/BRL), 8% heat-inactivated horse serum (GIBCO/BRL), 1% 100× essential and nonessential amino acids (Biochrom), 1% penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml; Biochrom), and 1% 200 mM L-glutamine (Biochrom) and subsequently with 20% of the L-cell supernatant containing macrophage colony-stimulating factor. The supernatant was taken from a 5-day-old confluent L929 cell culture, sterile filtered, and stored frozen. Primary cultures of bone marrow cells were fed with 50% fresh medium after 4 days. Cells were then incubated for an additional 3 days before the whole medium was changed and nonadherent cells were discarded. Adherent cells were incubated for 1 to 3 days longer, until all cells were differentiated. All cultures were proven to be 95% purified macrophages and free of T cells. All cell cultures were induced by using amounts of superantigens determined to be optimal in previous studies or 5% MAS, uninoculated myoplasma broth, or partially purified preparations of it.

Cytokine measurements. Human TNF-α was measured with an enzyme-linked immunosorbent assay (ELISA) obtained from Biermann (Bad Nauheim, Germany), and human TNF receptors (p55 and p75) were measured with an ELISA from Hoffman-La Roche (Basel, Switzerland). Murine TNF-α was measured with an ELISA obtained from Genzyme (Ismaning, Germany). All ELISAs were quantified with an ELISA-Reader (Anthos Labotec, Salzburg, Austria).

RNA isolation and blot analysis. Total cellular RNA was isolated by a procedure modified from that described by Chomczynski and Sacchi (4). Briefly, 5×10^7 cells were lysed in 5 ml of a 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol (GTC solution) and squeezed through the 25-gauge needle of a syringe three times. Lysed cells were frozen and stored at −20°C. Samples were thawed simultaneously and mixed with 0.5 ml of 0.2 M sodium acetate (pH 4), 5 ml of water-saturated phenol, and 1 ml of chloroform-isoamyl alcohol (49:1), mixed thoroughly, and incubated for 15 min on ice. Phenol extraction was performed by 20 min of centrifugation at 10,000 × *g*, after which the water phase was mixed with 5 ml of 2-propanol and stored for a minimum of 1 h at −20°C. The RNA was then pelleted and resuspended in 300 ml of GTC solution, mixed with the same volume of 2-propanol, and stored again for a minimum of 1 h at −20°C. The RNA was pelleted again, washed with 80% RNase-free ethanol, dried in a vacuum, and dissolved in RNase-free water. Ten micrograms of isolated total RNA was separated by agarose gel electrophoresis, blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), hybridized under stringent conditions with ^{32}P-labelled probes, and detected by autoradiography (Kodak X-Omat AR films). TNF probes were kindly provided by Daniela Männel (Regensburg, Germany). As a control, blots were washed and hybridized again with β-actin.

RESULTS

Induction of TNF-α in human cells. We have previously shown that MAS induces IL-1, IL-2, IL-4, and IL-6 in human cells and that monokines are produced faster and in larger amounts by PBMC than spleen cells (25). For this reason, we also present investigations of these two cell types as sources here.

There is a difference in the amounts of TNF-α production in PBMC and spleen cells after MAS induction. Like the other two monokines, TNF is also released faster by PBMC. However, in comparison with the other MAS-induced monokines, TNF is produced only in small amounts after stimulation with MAS. In PBMC cultures, we observed a peak in TNF production after 3 h, whereas in spleen cells there was no significant TNF production within the first 24 h (Fig. 1). TNF production in spleen cell cultures ($n = 4$) reached a maximum after 72 h at 104 pg/ml, whereas in PBMC cultures ($n = 10$), 158 pg/ml was produced after 3 h. TNF levels decreased after the first 3 h of cultivation. Uninoculated mycoplasma broth showed no significant TNF production. Data from independent experiments were averaged, and only samples from blood donors showing no significant spontaneous TNF release were included.

The small amounts of TNF measured indicate that synthesis of TNF is not induced and that the released TNF was preformed in the cells. We looked for TNF mRNA expression after MAS induction. In contrast to the small amounts of TNF protein in the culture, we detected a strong TNF mRNA signal

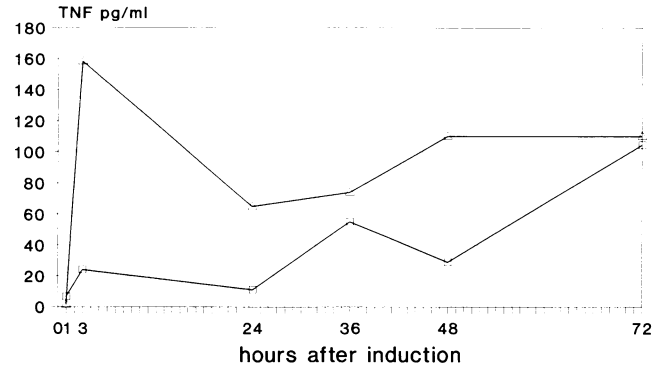

FIG. 1. Differential induction of TNF-α in human PBMC (△) and spleen cells (□). Cells were cultured at a density of 3×10^6/ml and induced with 5% MAS or 5% uninoculated mycoplasma broth. Blood samples with a spontaneous TNF release higher than 20 pg/ml were not considered in the study. Leukocytes lacking or with low spontaneous activity were not stimulated by uninoculated mycoplasma broth. Measured maxima of spontaneous activity and after treatment with uninoculated mycoplasma broth were 2 pg of TNF per ml.

in a Northern (RNA) blot which was comparable to that induced by SEB (Fig. 2). TNF mRNA was expressed 1 h after induction and increased for up to 3 h thereafter, whereas controls showed no TNF mRNA. The large amounts of TNF mRNA did not correlate with the amounts of measured TNF protein. To study the delay between release of TNF mRNA and protein, we measured the soluble TNF receptors p55 and p75 to rule out the described blocking in the ELISA detection of TNF by the soluble receptors. Surprisingly, we found no changes in the levels of soluble TNF receptors after induction with MAS (data not shown).

TNF induction in murine cells. In previous studies, we have shown that murine cells produce large amounts of IL-6 (12) comparable to those induced in human cells (25). To determine whether the delay between the production of IL-6 and TNF is founded on the induction pathway of MAS or whether it is species specific, we investigated the induction of TNF in different murine cell systems. After MAS induction, human

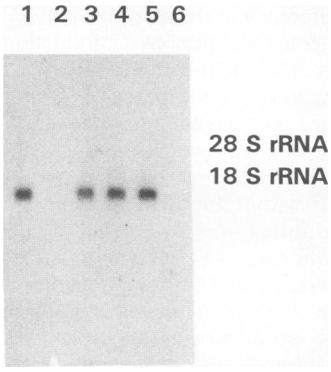

FIG. 2. TNF-α mRNA expression in PBMC after MAS induction. Cells were cultured at a density of 3×10^6/ml and induced with 500 ng of SEB per ml, 5% MAS, or 5% uninoculated mycoplasma broth. The RNA and blot were prepared as described in Materials and Methods. Control hybridization with β-actin showed equal amounts of mRNA in all samples. Lanes: 1, 3 h, SEB; 2, 3 h, control; 3, 1 h, MAS; 4, 2 h, MAS; 5, 3 h, MAS; 6, 3 h, mycoplasma broth.

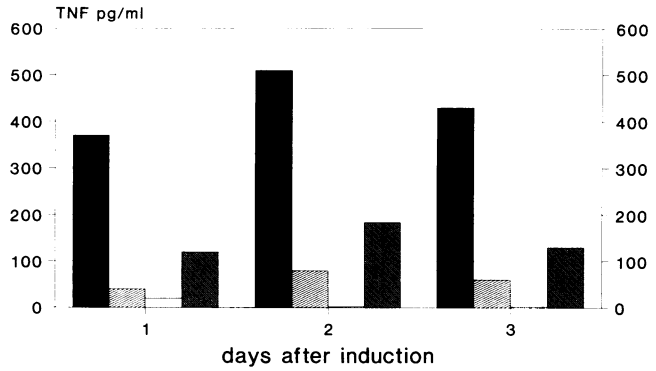

FIG. 3. TNF induction by MAS in murine spleen cells. Cells from C3H/HeJ mice were cultured at a density of 3×10^6/ml and induced with 500 ng of SEB per ml, 5% MAS, or 5% uninoculated mycoplasma broth. Symbols: ■, MAS; ▨, mycoplasma broth; □, control; ▧, SEB.

cells produced a maximum of 158 pg of TNF per ml, whereas murine spleen cells produced up to 510 pg/ml.

To exclude LPS artifacts, we used C57BL/6 and C3H/HeJ mice. C3H/HeJ mice are I-Eα^+ MAS responders but known to be LPS nonresponders, and C57BL/6 mice are I-Eα^- and, therefore, MAS nonresponders. However, C57BL/6 mice respond to LPS. C3H/HeJ mice produced high levels of TNF-α after induction with MAS, whereas C57BL/6 mice were not stimulated by MAS. In mouse spleen cell cultures, we saw a maximum in TNF production 2 days after stimulation with MAS, and the uninoculated mycoplasma broth showed no significant side effects (Fig. 3). The TNF titer increased continuously through the first 24 h. SEB induced less TNF than MAS in murine splenocytes. The maximum amounts of TNF induced by MAS in the murine spleen cell culture were three times higher than that produced in human PBMC and nearly five times higher than that produced in human spleen cells.

Previously, we have shown that MAS induced IL-6 in bone marrow-derived macrophages (BMM) (12). With respect to these experiments, we investigated whether MAS also induces TNF in this cellular system. Interestingly, we found that TNF is produced quickly and in very large amounts by BMM (Fig. 4). We detected significant levels of TNF 6 h and a maximum of 1,230 pg/ml 12 h after stimulation with MAS, whereas all

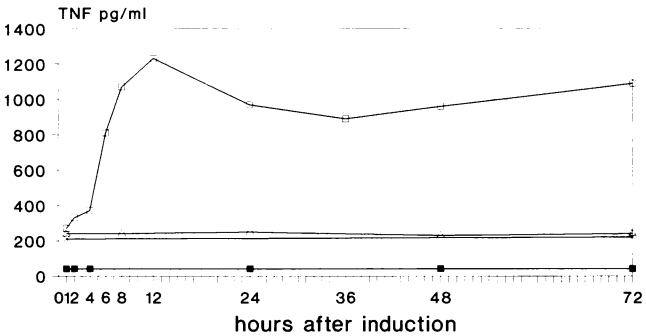

FIG. 4. MAS induction of BMM. Bone marrow cells were stimulated to differentiate into macrophages as described in Materials and Methods. BMM from C3H/HeJ or C57BL/6 mice were induced with 5% MAS or 5% uninoculated mycoplasma broth. Symbols: □, C3H BMM + MAS; X, C3H BMM control; △, C3H BMM + mycoplasma broth; ■, C57BL/6 BMM + MAS.

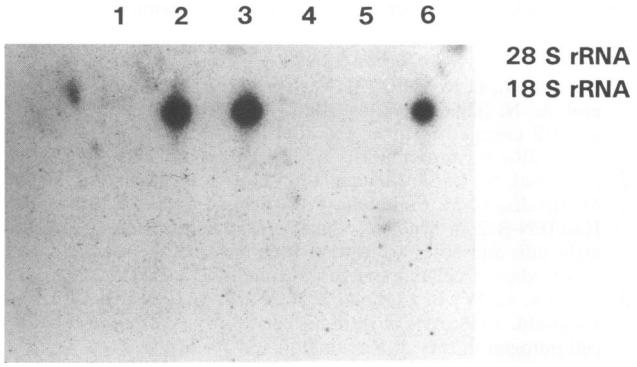

FIG. 5. TNF-α mRNA expression in BMM. Bone marrow cells were stimulated to differentiate into macrophages as described in Materials and Methods. Cells were induced with 5% MAS or 5% uninoculated mycoplasma broth. The RNA and blot were prepared as described in Materials and Methods. Control hybridization with β-actin showed equal amounts of mRNA in all samples. Lanes: 1, control; 2, 1 h, MAS; 3, 4 h, MAS; 4, 4 h, mycoplasma broth; 5, 8 h, mycoplasma broth; 6, 8 h, MAS.

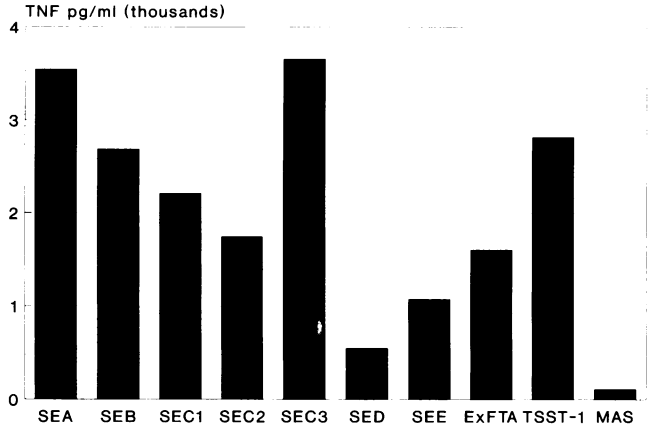

FIG. 6. TNF induction by superantigens. PBMC were cultured at a density of 3×10^6/ml, and PBMC from the same blood donors were used to compare the superantigens. For stimulation, superantigens were titrated in twofold dilution steps. Samples were harvested 48 h after induction with the appropriate superantigen. Levels of TNF shown are always the production maximum independent of the superantigen dose. Optimal doses were as follows: SEA, SEC1, and SEC2, 1.25 μg/ml; SEB, 2.5 μg/ml; SED and ExFTA, 39 ng/ml; SEE and TSST-1, 20 ng/ml; SEC3, 312 pg/ml; MAS, small amounts similar to those for SEC3.

other superantigens tested (SEA, SEB, SED, and SEE) failed to induce TNF in BMM. Furthermore, we saw no TNF induction in BMM of C57BL/6 mice. Compared with spleen cells, this system of in vitro-derived and highly active macrophages produced a higher background, but the background was equal to that of controls and after treatment with uninoculated mycoplasma broth. However, in Northern blot analysis of BMM, we saw induction of TNF mRNA neither in the control nor after treatment with the uninoculated mycoplasma broth. Furthermore, as opposed to the human cell cultures, the levels of mRNA detected in BMM correlated with the amounts of TNF produced (Fig. 5). TNF mRNA was first detectable 1 h after induction, reached a maximum after 3 h and decreased after 8 h. These kinetics of mRNA expression correlated to the time-delayed release of measured TNF protein.

Since we were using C3H/HeJ mice, we could rule out contaminating LPS. However, to show that TNF production is induced specifically by MAS, we used C57BL/6 mice as a control. As postulated, the measured amounts of TNF in C57BL/6 mouse cell cultures were always on the order of the background amounts. Therefore, the MAS preparations, also used for human cells, were free of LPS.

Comparison between MAS and other superantigens. In view of the differences seen between MAS-induced TNF in human and murine cells, we determined whether the low level of induction of TNF in PBMC is specific to stimulation by superantigens or whether it is unique for MAS. We titrated all available superantigens in twofold dilution steps to be sure that they were used in optimal concentrations for TNF stimulation. Individual assays with different superantigens were performed with identical cell preparations from the same blood donors, and TNF was measured 2 days after induction. The 2-day time period was chosen because all kinetics at this time of production were comparable. Major differences in kinetics are found within the first 12 h. Comparable to the described observations on proliferation, we saw that the SEs, TSST-1, and ExFTA induced higher levels of TNF in PBMC than MAS did (Fig. 6). Interestingly, we saw that the amounts of superantigens which induce maximum production of TNF divided the superantigens tested into two groups. SEA, SEC1, and SEC2 stimulated maximum production of TNF at a concentration of 1.25 μg/ml and SEB did so at 2.5 μg/ml, whereas only 39 ng of SED or ExFTA per ml, 20 ng of SEE or TSST-1 per ml, or 312 pg of SEC3 per ml or a similarly low concentration of MAS was needed to stimulate maximum production of TNF. However, all other superantigens tested showed 5 to 32 times higher levels of induced TNF than MAS. Therefore, in fact, TNF is induced by low doses of MAS, but the induction is definitely less than that of the other superantigens tested.

DISCUSSION

In this study, we showed that TNF-α is also induced by MAS. In contrast to other cytokines in human cells, as we have previously shown (25), TNF protein is induced only in small amounts by MAS. Furthermore, TNF showed many differences in induction from that of other MAS-induced cytokines. IL-1 and IL-6 were produced in higher levels by PBMC than by human spleen cells, whereas TNF showed nearly the same maximal amounts but with different kinetics. The previously tested monokines are only comparable on the basis of their kinetics in PBMC with our present findings. However, we detected a strong signal of TNF-α mRNA in human cells which did not correlate with TNF synthesis. The differences between TNF mRNA produced and measured TNF protein were not caused by the effect of the soluble TNF receptors p55 and p75. We detected no changes between the levels of soluble TNF receptors in the controls and the MAS-stimulated probes. For this reason, the discrepancy between the mRNA produced and protein measured cannot be explained by the inhibition of soluble TNF receptors for TNF detection by ELISA. We cannot explain this difference, but there must be a missing signal or capacity to translate the mRNA.

However, there seems to be a major difference between the induction of TNF in human cells and that in murine cells. MAS induced large amounts of TNF-α in murine cells. This effect is specific for MAS because the C57BL/6 mice, which are I-Eα$^-$ and, therefore, MAS nonresponders and LPS responders, did not produce TNF after MAS stimulation. In contrast, C3H/

HeJ mice, LPS nonresponders and I-Eα$^+$ MAS responders, produced high levels of TNF after induction with MAS. The induced TNF could cause the destructive effects in *M. arthritidis*-induced arthritis and synovitis in intra-articularly MAS-treated rats (3). The infection and the resulting experimentally induced arthritis are accompanied by toxic shock syndrome. The toxic shock syndrome following intravenous injection possibly resulted from MAS-induced TNF. Furthermore, TNF could be the reason for the described effect that mice suffer from necrosis at the injection site of MAS (6). At least, the turmoricidal effect shown by the murine macrophage cell line J774.1 after contact with *M. arthritidis* (9) could be caused by induced TNF.

The produced TNF seems to be directly induced in the monocytes because BMM also produced TNF after MAS induction. This is similar to the previously reported induction of IL-6 in BMM (12). The induction in BMM is also dependent on I-Eα in the MHC, since only the I-Eα$^+$ C3H/HeJ mouse BMM responded to MAS whereas the I-Eα$^-$ C57BL/6 mouse BMM did not. For this reason, an artifact by the in vitro-derived macrophages could be ruled out. The fact that BMM produced more TNF seems to be dependent on the number of macrophages in the culture. Furthermore, the BMM seem to be more reactive than the spleen cells because they have a higher spontaneous activity. In contrast to human cells, the amounts of TNF mRNA correlated with the produced TNF protein in BMM. Therefore, the difference in TNF induction in human and murine cells seems to be a missing translational signal in humans. To date we have not identified the nature of the missing signal in human cells; however, MAS definitely has the capability to induce TNF. We have some evidence that the missing signal is related to the strength of MAS binding to the MHC class II molecule. I-Eα seems to be bound to MAS more strongly than HLA-DR because transfected cells more effectively presented MAS to human T cells than HLA-DR (22). This may be the reason why *M. arthritidis* induces an arthritis only in MAS-responsive mouse strains (7). Furthermore, this could explain why the role of *M. arthritidis* in humans has not been clarified. It is possible that the HLA types with a higher risk of rheumatoid arthritis have a higher affinity for MAS. TNF has been found in the synovial fluid of patients with rheumatoid arthritis (2). Other investigators described a divergence from the normal distribution of Vβ specificities in the synovial fluid of patients with rheumatoid arthritis (13). The amplified Vβ subfamilies of the TCR were Vβ 3, Vβ 14, and Vβ 17 (alternatively called Vβ 19). The subfamilies Vβ 3 and Vβ 19 were also described as being stimulated by MAS (1). Collectively, this provides evidence that a superantigen, possibly MAS, plays a pathogenic role in rheumatoid arthritis. The linkage to HLA-DR specificities has to be proven by further investigations.

In comparison with other superantigens, MAS induced less TNF-α in human PBMC. However, in murine BMM, MAS is the only reactive superantigen. This underlines the presumptions that MAS has a higher affinity for murine I-Eα and that MAS is the only superantigen that directly induces macrophages without connecting macrophages with T cells by the superantigen bridge. The role of TSST-1-induced TNF in the pathogenesis of toxic shock syndrome is well known (15, 27). However, the enterotoxins could play a pathological role in gram-positive sepsis by inducing TNF. By inducing TNF, the enterotoxins act like LPS in gram-negative sepsis.

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