Roles of tumor necrosis factor-α receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome

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Tristetraprolin (TTP) is a member of the CCCH tandem zinc-finger class of proteins. It can bind to and destabilize mRNAs encoding tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Conversely, mice deficient in TTP develop a complex syndrome characterized by cachexia, myeloid hyperplasia, and joint and skin inflammation. Studies using anti–TNF-α neutralizing antibodies demonstrated that this syndrome, at least in part, is a consequence of the excess production of TNF-α in the absence of TTP. To evaluate the role played by each TNF-α receptor in the pathogenesis of this syndrome, mice were generated that were deficient in TTP and either or both of the known TNF-α receptors (TNFRs), type 1 (TNFR1) and type 2 (TNFR2). Mice deficient in TTP and TNFR1, or in TTP and both receptors, were protected from developing the TNF-α–induced cachexia and inflammation. In contrast, mice deficient in TNFR2 were more severely affected than mice deficient in TTP alone, suggesting that TNFR2 might play a protective role in the development of the syndrome. In cultured cells derived from these mice, apparent cooperation between the TNFRs was required to achieve normal TNF-α–induced expression of TTP, TNF-α, and GM-CSF mRNAs. Finally, the results showed that TNFR1 plays an important role in mediating TNF-α–induced changes in TNF-α and GM-CSF mRNA stability. (Blood. 2001;98: 2389-2395)

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Introduction

Tristetraprolin (TTP), also known as TIS11, Nup475, or G0S24,[1-4] is the prototype of a recently described family of zinc-finger proteins of the CCCH class.[5] Mice deficient in TTP develop a severe syndrome characterized by growth retardation and cachexia, polyarticular arthritis, dermatitis, autoimmunity, and myeloid hyperplasia accompanied by extramedullary hematopoiesis.[6] The involvement of tumor necrosis factor-α (TNF-α) in the development of this syndrome was initially suggested by the similarity between the phenotype displayed by the TTP-deficient mice and those displayed by several models of excess TNF-α in mice and rats.[7-9] This suggestion was reinforced by the observation that treatment of the TTP-deficient mice with neutralizing anti–TNF-α antibodies resulted in prevention of most of the components of the TTP deficiency–associated phenotype.[6]

Further studies using macrophages derived from either fetal liver or bone marrow showed increased production of TNF-α by cells derived from TTP-deficient mice, and subsequent studies in bone marrow–derived macrophages showed that this increase was due to increased stabilization of TNF-α mRNA in the absence of TTP.[10,11] We then showed that TTP can bind directly to the AU-rich element in the 3′-UTR (untranslated region) of TNF-α mRNA, and, by a still unknown mechanism, promote its destabilization and more rapid degradation.[11,12]

A second aspect of the phenotype was revealed when we found that bone marrow stromal cells (BMSCs) derived from TTP-deficient mice exhibited increased production of the hematopoietic growth factor and proinflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF).[13] As in the case of

TNF-α, this increase was due to the stabilization of GM-CSF mRNA in the absence of TTP. TNF-α has been shown to promote the stabilization of GM-CSF mRNA and thus GM-CSF production.[14] To demonstrate directly that TTP regulates GM-CSF mRNA stabilization, we evaluated this process in mice that were deficient in both TNF receptors (TNFRs). In the absence of TTP and both TNFRs, the half-life of GM-CSF mRNA in BMSCs was still increased,[13] ruling out increased circulating TNF-α as the cause of the increase in GM-CSF production.

TNF-α exerts its actions after binding to 2 receptors, TNF-α receptor 1 (TNFR1, p55, or CD120a) and TNF-α receptor 2 (TNFR2, p75, or CD120b). These 2 receptors share about 28% amino acid identity in their extracellular domains and even less similarity in their intracellular domains, suggesting different signaling roles.[15,16] At present, it appears that TNFR1 mediates most of TNF-α's ability to cause cytotoxicity, endotoxin shock, and activation of gene transcription,[17-20] whereas TNFR2 has been implicated in local inflammatory responses and regulation of early hematopoiesis.[21-24] Notably, TNFR2 has also been implicated in regulation of the responses mediated by TNFR1 by controlling the binding of TNF-α to that receptor.[21,25] Whereas TNFR1 is ubiquitously expressed at fairly constant levels, TNFR2 is expressed mainly in leukocytes and endothelial cells, and its levels are subject to regulation by different stimuli.[26]

In an attempt to determine which aspects of the TTP-associated phenotype are mediated by each receptor, we generated mice deficient in both TNFRs and TTP, and also animals that were deficient in TTP and each individual receptor. We show here that

TNFR1 is responsible for the development of arthritis and cachexia in the absence of TTP. These studies confirm the involvement of TNF-α in the etiology of the TTP-deficiency syndrome in mice. However, we also found that TNFR2 might play a protective role in this syndrome because the TTP-deficiency syndrome appeared to be exacerbated in the TTP/TNFR2-deficient mice. Our studies also show a possible cooperative action of both receptors in the TNF-α–stimulated induction of TTP, GM-CSF, and TNF-α itself. Finally, we show that TNFR1 appears to be involved in the stabilization of both GM-CSF and TNF-α mRNAs, which exhibit much longer half-lives in the TTP-deficient animals expressing only that receptor.

Materials and methods

Mice

Mice deficient in both TNFRs were kindly provided by Dr Mark W. Moore (Genentech, South San Francisco, CA) and were interbred with animals that were heterozygous for TTP.[6] Successive interbreeding of the offspring resulted in the following genotypes: TNFR1- and TNFR2-deficient mice (TNFR1/2KO [knockout]), TNFR1-deficient mice (TNFR1KO), TNFR2-deficient mice (TNFR2KO), TTP/TNFR1/2-deficient mice (3KO), TTP/TNFR1-deficient mice (TTP/TNFR1KO), and TTP/TNFR2-deficient mice (TTP/TNFR2KO). Genotyping of the animals was performed as described.[13] All animals were maintained in microisolator cages in a barrier facility. Mouse weights were recorded weekly, and the animals were monitored daily for evidence of clinical deterioration. Animal care and all experiments were in accordance with institutional guidelines for animal use.

Histology

Tissue samples were fixed in 10% neutral buffered formalin and processed according to standard protocols before being stained with hematoxylin-eosin. Bone marrow cells were flushed from the femur and deposited onto glass slides with a cytocentrifuge (Shandon, Pittsburgh, PA). The slides were then stained with the Diff Quick Stain Set (Baxter Health Care, McGraw Park, IL). All tissue sections and cells were analyzed with a Nikon Eclipse 400 microscope (Southern Micro Instruments, Atlanta, GA) and photographed with an Olympus PM-C35B camera (Olympus America, Lake Success, NY).

Cell culture

Bone marrow–derived macrophages (BMMφs) were prepared as described previously.[10] Briefly, marrow cells were flushed from both femurs of individual mice and cultured at 37°C in a 5% CO$_2$ atmosphere. The culture medium was Eagle's modified minimum essential medium, supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 0.2% sodium bicarbonate, 15 mM HEPES, pH 7.4, and 30% (vol/vol) L929 cell-conditioned medium as a source of macrophage colony-stimulating factor.[27] Cells were used after 2 to 3 weeks in culture.

BMSCs were prepared as described previously.[13] Briefly, marrow cells were flushed from both femurs of individual mice, and red blood cells were lysed with 0.15 M ammonium chloride. Cells were cultured at 33°C in a 5% CO$_2$ atmosphere in minimum essential medium-α supplemented with 25% heat-inactivated fetal calf serum (vol/vol), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 0.2% sodium bicarbonate, and 15 mM HEPES, pH 7.4. Cells were grown in T75 cm^2 tissue culture flasks until confluent monolayers were observed (about 4 weeks). After that, monolayers were trypsinized and cells were replated in 60-mm dishes and cultured for another week before use in experiments.

Northern blotting

To study the induction of TTP gene expression by TNF-α, we incubated BMMφs with increasing doses of recombinant mouse TNF-α (rmTNF-α,

0-10 ng/mL; R&D Systems, Minneapolis, MN) for 30 minutes. RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA) according to the directions of the manufacturer. RNA was analyzed by Northern blot on 1.5% (wt/vol) agarose gels, and blots were hybridized with a mouse TTP cDNA.[3]

Similarly, induction of GM-CSF and TNF-α mRNA expression by rmTNF-α was studied in BMSCs after 2 hours of incubation in the presence of increasing doses of rmTNF-α. Blots were sequentially hybridized with a mouse GM-CSF cDNA and a mouse TNF-α cDNA, as described.[10,13]

GM-CSF and TNF-α mRNA stability in BMSCs was studied after induction with lipopolysaccharide (LPS, 1 μg/mL; Sigma, St Louis, MO) for 2 hours, followed by a further incubation in the presence of actinomycin D (5 μg/mL; Sigma) for the times indicated. Blots were hybridized to either a mouse GM-CSF or a mouse TNF-α cDNA probe, as described above. RNA loading was normalized by reprobing all blots with a cDNA probe for cyclophilin or glyceraldehyde 3-phosphate dehydrogenase. All Northern blots were analyzed using a PhosphorImager Typhoon 8600 and Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Results

Phenotypes of the TTP/TNFR-deficient mice

Development. Mice deficient in TNFR1, TNFR2, or both have been described previously,[20,22] and all of them appear to develop normally. Their weights are comparable to those of wild-type (WT) mice, and they do not show any obvious signs of disease. We generated mice that were deficient in TTP and both TNFRs (3KO) and also mice deficient in TTP and each individual receptor (TTP/TNFR1KO or TTP/TNFR2KO). The TTPKO mice used in this study had been back-crossed into C57Bl/6 mice for an average of 11 generations (range, 10-12 generations). Controls were mice deficient in either or both TNFRs. At 5 weeks of age, growth retardation was already apparent in the TTPKO mice (average body weight, 7.8 ± 2.1 g, n = 4, versus 18.1 ± 1.4 g in the WT mice, n = 7) (mean ± SD) ($P < .0001$). Mice of the 3KO and the TTP/TNFR1KO genotypes exhibited the same rate of growth as the WT animals (18.3 ± 2.2 g, n = 16, and 18.1 ± 1.7 g, n = 14, respectively). On the other hand, the average weight of the TTP/TNFR2KO mice at 5 weeks was 9 ± 2.7 g (n = 5) ($P < .03$ compared with WT). After 12 weeks, the pattern remained the same: WT, 23.7 ± 2.2 g (n = 7); TTPKO, 9.3 ± 2.1 g (n = 4); 3KO, 23.7 ± 2.2 g (n = 16); TTP/TNFR1KO, 23.8 ± 3.1 g (n = 14); and TTP/TNFR2KO, 12.4 ± 0.5 g (n = 5). However, at 20 weeks of age, mice of the 3KO and TTP/TNFR1KO genotypes appeared to slow in their weight gain: WT, 33.1 ± 0.1 g (n = 4); 3KO, 25.2 ± 2.9 g (n = 16); and TTP/TNFR1KO, 26.3 ± 4 g (n = 14).

At 5 weeks of age, there were no obvious physical abnormalities in the 3KO or TTP/TNFR1KO mice. However, by this age the TTP/TNFR2KO mice already displayed early signs of joint inflammation, characterized by swelling and discoloration of the paws (Figure 1). This onset of inflammation occurred earlier than in the TTPKO mice, which did not show signs of paw joint inflammation until 8 to 10 weeks of age. These paw changes were not seen in the other genotypes, even after several months of observation (data not shown). Over time, the TTP/TNFR2 mice rapidly deteriorated, as did the TTPKO mice, whereas the TTP/TNFR1KO and 3KO mice continued to develop normally and without signs of disease (except for the leveling off of the weight gain that occurs after 5 months of age). Both of these genotypes were fertile and able to carry gestation to term, and both had normal litter sizes and reared the
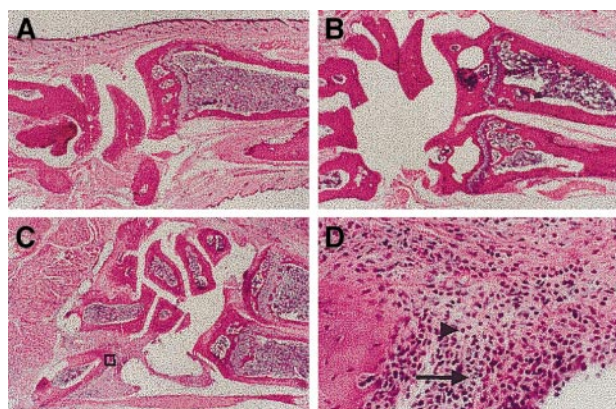
Figure 1. Early development of inflammatory arthritis in the TTP/TNFR2KO mice. In this 5-week-old TTP/TNFR2KO mouse, the swelling and discoloration of the front paws are already obvious. This same phenomenon was observed in animals of this genotype as young as 3 to 4 weeks of age.

pups normally. Breeding was not attempted with the homozygous TTP/TNFR2KO mice.

Histology. Carpal joints of adult mice were examined histologically at approximately 6 months of age (Figure 2). The 3KO (Figure 2A) and the TTP/TNFR1KO mice (Figure 2B) exhibited no signs of joint inflammation, in agreement with the external examination of the animals. However, in the TTP/TNFR2KO mice (Figure 2C), the histology of the joint was markedly abnormal, with severe erosion of the articular surfaces, invasion of the joint cavities by inflammatory pannus, and dramatic infiltration of the surrounding soft tissues by mononuclear and polymorphonuclear inflammatory cells (Figure 2D). This pathology was very similar to that described in the TTPKO mice,[6] but appeared to be more aggressive in both extent and earlier age at onset.

Microscopic examination of the bone marrow cavities from mice of all 3 TTP-deficient genotypes (Figure 2) revealed the hypercellular marrow that is typical of the TTPKO mice.[6] In the present study, this marrow hypercellularity was present even in the absence of both TNFRs. Cell preparations of bone marrow from

animals expressing TTP (Figure 3, panels WT and TNFR1/2KO) showed a heterogeneous population of cells, including members of the 3 hematopoietic lineages (lymphoid, myeloid, and erythroid). In contrast, whenever TTP was absent (Figure 3, panels TTPKO, 3KO, TTP/TNFR1KO, and TTP/TNFR2KO), there was a marked increase in the proportion of myeloid cells at all stages of differentiation, with almost complete disappearance of the lymphoid and erythroid lineages. The percentages of myeloid cells in these genotypes were as follows: TTPKO, 77% $\pm$ 2.1% (n = 3); 3KO, 68.3% $\pm$ 2.6% (n = 6); TTP/TNFR1KO, 64.7% $\pm$ 4% (n = 3); and TTP/TNFR2KO, 72.2% $\pm$ 5% (n = 4) (mean $\pm$ SEM). In each case, this represents at least a 2-fold increase in the proportion of myeloid cells compared with marrow from WT mice (30.4% $\pm$ 3% [n = 7]).

These results were somewhat unexpected because TTP-deficient mice treated with neutralizing antibodies to TNF-α for 8 weeks did not exhibit significant myeloid hyperplasia at 73 days of age compared with control animals (33.7% $\pm$ 3.1% marrow myeloid cells in the antibody-treated KO mice versus 30.4% $\pm$ 3.0% in control mice).[6] Because these mice were considerably younger than the 3KO mice evaluated in the present study, we analyzed marrow from 3KO mice at 82 days of age. Marrow from these mice contained 37.5% $\pm$ 2.5% myeloid cells (n = 4), suggesting strongly that the severe myeloid hyperplasia seen in the older 3KO mice was an age-related effect of the abnormal genotype that was not apparent either in the younger mice treated with TNF-α antibodies or in the younger 3KO mice.

In the TTPKO mouse, myeloid hyperplasia in the bone marrow is always accompanied by extramedullary hematopoiesis, particularly in the spleen, which is almost always significantly enlarged.[6] This phenomenon was also seen in the 3KO and TTP/TNFR1KO mice older than 6 months of age; the spleens of these animals weighed up to 700 mg (normal, less than 100 mg). Even when splenomegaly was not as obvious, the normal architecture of the spleen was altered in the 3 genotypes lacking TTP. Spleens from TNFR1/2KO mice (Figure 4) appeared in all respects to be similar to those of the WT mice (not shown), with normal architecture of the white and red pulps and a population of predominantly lymphoid cells, with the occasional mature granulocyte. However, spleens from animals of the other 3 genotypes, in which TTP was absent, were strikingly similar to the spleens of the TTPKO mice,[6]
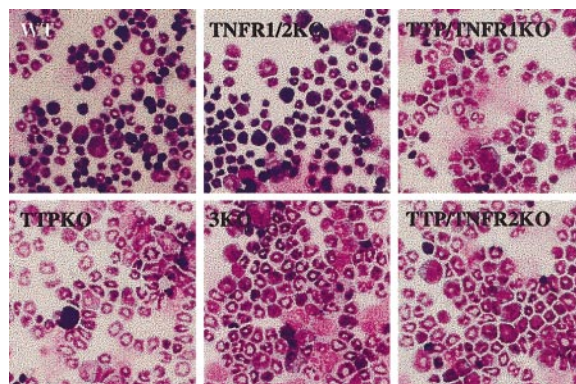

Figure 2. Histology of the carpal joint in TTP/TNFR-deficient mice. (A-C) Hematoxylin-eosin–stained sections of the carpal joint of 6-month-old mice at $\times$ 10 magnification, with the metacarpal bones to the left and the radius and ulna to the right; the radial head is on top in each section. (A) Carpal joint of a mouse deficient in TTP and both TNFRs. (B) Carpal joint from a mouse deficient in TTP and TNFR1. (C) Carpal joint from a mouse deficient in TTP and TNFR2. Both (A) and (B) demonstrate a clean articular cavity, smooth articular surfaces, and absence of inflammation or pannus formation. In contrast, (C) demonstrates the presence of severe inflammation, with erosion of the articular surfaces and invasion of the articular cavity by the prominent pannus. All 3 genotypes (A-C) exhibit marked intramedullary hypercellularity. (D) Higher-power view ($\times$ 100 magnification) of the area delimited by the box in (C). The cellular component of the inflammatory infiltrate is of both polymorphonuclear (arrowhead) and mononuclear (arrow) lineages.

Figure 3. Cytology of the bone marrow in TTP/TNFR-deficient mice. Bone marrow was flushed from the femurs of adult mice, and cytopreparations were stained with a modified May-Grünwald-Giemsa stain. Magnification was $\times$ 100. In both the WT and TNFR1/2KO mice, the marrow contains a normal mixture of cells of the myeloid, erythroid, and lymphoid lineages. However, in the TTP-deficient cells, regardless of the presence or absence of TNF-α receptors (TTPKO, TTP/TNFR1KO, TTP/TNFR2KO, or 3KO), the marrow cellularity appears to be strikingly shifted to the myeloid lineage.
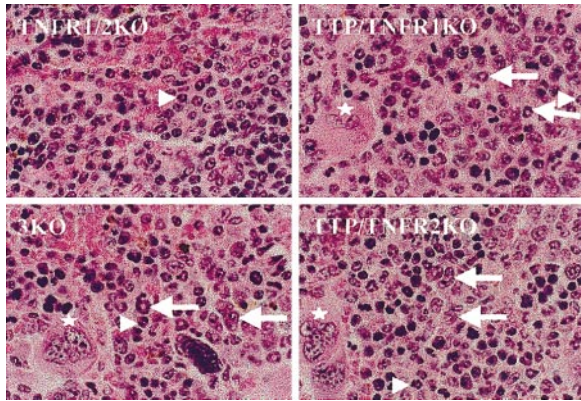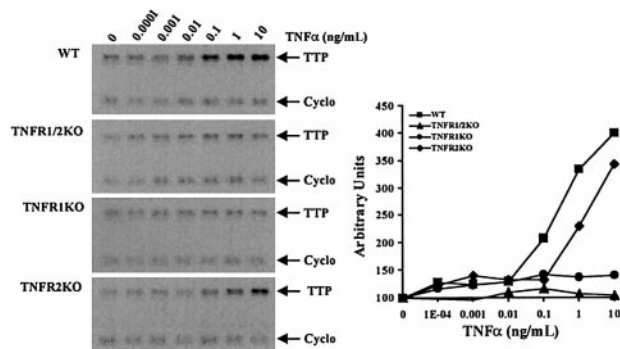
Figure 4. Histology of the spleen in TTP/TNFR-deficient mice. The figure shows hematoxylin-eosin–stained sections of spleen at × 250 magnification. In the TNFR1/2KO mice, the cellularity of the spleen is essentially identical to that of WT mice. The prominent cell type is lymphoid, but the occasional mature granulocyte is present (arrowhead). However, in the remaining panels from TTP-deficient mice (3KO, TTP/TNFR1KO, and TTP/TNFR2KO), myeloid cells at different stages of differentiation have largely replaced the lymphoid cells (arrowheads, mature granulocytes; arrows, immature myeloid precursors). Note also the presence of increased numbers of megakaryocytes (asterisk), a phenomenon also observed in spleens from the TTPKO mice.

with increased numbers of myeloid precursors at different stages of differentiation (Figure 4, panels 3KO, TTP/TNFR1KO, and TTP/TNFR2KO). In addition, increased numbers of megakaryocytes were observed, as in the original TTPKO mice (E.C. and P.J.B., unpublished observations, 1998-2001).

Involvement of TNFR1 and TNFR2 in the expression of TTP

To evaluate the role played by each TNFR in the induction of TTP expression by TNF-α, we incubated BMMϕs for 30 minutes in the presence of increasing concentrations of rmTNF-α, and then isolated and analyzed RNA by Northern blot. In WT BMMϕs, TTP mRNA accumulation was first detected after exposure to 0.1 ng/mL rmTNF-α for 30 minutes (Figure 5). In the absence of both TNFR1

and TNFR2, TNF-α caused no increase in TTP mRNA accumulation. The absence of TNFR1 alone also completely prevented rmTNF-α–induced TTP mRNA accumulation. When TNFR1 alone was present, rmTNF-α induced TTP mRNA accumulation. However, there was a clear shift to the right in the rmTNF-α concentration needed to trigger this response. In the cells of this genotype, no increase in TTP mRNA accumulation was seen after exposure to 0.1 ng/mL rmTNF-α, and even 10 ng/mL rmTNF-α stimulated less TTP mRNA accumulation than that seen in the cells from the WT animals.

Involvement of TNFR1 and TNFR2 in the expression and stability of GM-CSF mRNA

We also examined the role played by each TNFR in the induction of GM-CSF mRNA. In WT BMSCs, GM-CSF mRNA was detected after 2 hours of exposure to 0.1 ng/mL rmTNF-α (Figure 6). The absence of TNFR1 resulted in the complete lack of expression of GM-CSF mRNA, even after the highest dose of rmTNF-α was used (10 ng/mL). However, although in the absence of TNFR2 there was TNF-α induction of GM-CSF mRNA accumulation, there was a shift to the right in the concentration of rmTNF-α required for the first detectable expression of GM-CSF mRNA. In the absence of TNFR2, GM-CSF mRNA was first detected only after a 2-hour incubation in the presence of 1 ng/mL rmTNF-α, in contrast to 0.1 ng/mL in the WT cells.

We also evaluated which TNFR was involved in the regulation of GM-CSF mRNA stability. To do this, we compared the half-life
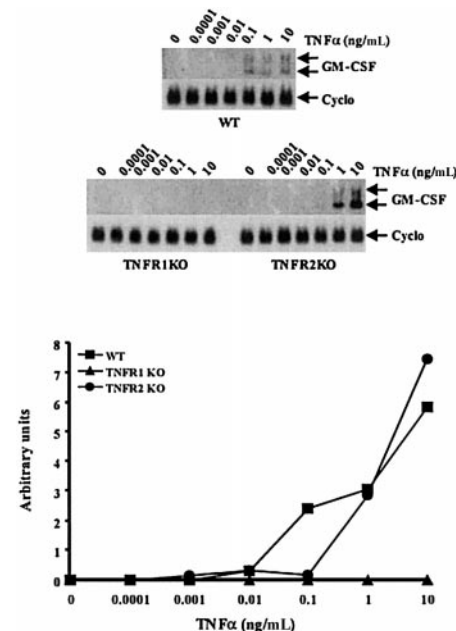

Figure 5. Differential induction of TTP mRNA accumulation mediated by TNFR1 and TNFR2. BMMϕs were prepared as described in "Materials and methods" and were stimulated with increasing concentrations of rmTNF-α for 30 minutes. Northern blots from these experiments are shown on the left; the TTP and cyclophilin (Cyclo) mRNAs are indicated. On the right is the PhosphorImager quantitation of these blots after the TTP mRNA levels had been normalized for cyclophilin mRNA levels. WT macrophages exhibited detectable expression of TTP mRNA after stimulation with as little as 0.1 ng/mL rmTNF-α. However, in the absence of both TNFRs (TNFR1/2KO), there was no induction of TTP mRNA expression, even at the highest concentration of rmTNF-α used, 10 ng/mL. In the absence of TNFR1 (TNFR1KO), there was also no induction of TTP mRNA expression. However, in the absence of TNFR2 (TNFR2KO), rmTNF-α induced the expression of TTP mRNA, but there was a shift to the right of one order of magnitude in the concentration-dependence curve compared with that seen in WT macrophages. The experiment shown here is representative of 3 independent experiments using cells derived from different groups of animals.

Figure 6. Differential induction of GM-CSF mRNA accumulation mediated by TNFR1 and TNFR2. BMSCs were prepared as described in "Materials and methods" and were stimulated with increasing concentrations of rmTNF-α for 2 hours. Northern blots from these experiments are shown on the top; the GM-CSF and cyclophilin (Cyclo) mRNAs are indicated. On the bottom is the PhosphorImager quantitation of these blots after the GM-CSF mRNA levels had been normalized for cyclophilin mRNA levels. WT BMSCs exhibited readily detectable expression of GM-CSF mRNA after stimulation with as little as 0.1 ng/mL rmTNF-α. However, in the absence of TNFR1 (TNFR1KO), there was no induction of GM-CSF mRNA expression, even at 10 ng/mL rmTNF-α. In the absence of TNFR2 (TNFR2KO), rmTNF-α induced the expression of GM-CSF mRNA, but there was a shift to the right of one order of magnitude in the concentration-dependence curve compared with that seen in WT macrophages. The experiment shown here is representative of 2 independent experiments with cells derived from different groups of animals.

of GM-CSF mRNA after actinomycin D treatment of BMSCs stimulated for 2 hours with 1 μg/mL LPS. In the TNFR1KO and TNFR2KO cells, the larger (polyadenylated) form of GM-CSF mRNA decayed very quickly after actinomycin D treatment, whereas the smaller (deadenylated) form[13] decreased with a somewhat longer half-life (Figure 7). The estimated half-life of both mRNA species combined was 35 minutes and 39 minutes for the 2 genotypes, respectively. However, when the BMSCs were derived from the TTP/TNFR1KO cells, the decay of the polyadenylated form of the mRNA was somewhat delayed, with an estimated half-life of 49 minutes. In the absence of both TTP and TNFR2, there was little detectable deadenylated GM-CSF mRNA, and the half-life of the total mRNA was greatly increased (415 minutes).

Involvement of TNFR1 and TNFR2 in the expression and stability of TNF-α mRNA

Using the same cultures of BMSCs, we studied the induction and stability of TNF-α mRNA. TNFR2 alone failed to mediate the rmTNF-α–mediated expression of TNF-α in BMSCs (Figure 8). In contrast, TNFR1 was able to mediate the rmTNF-α–stimulated expression of TNF-α, but higher concentrations of rmTNF-α were required to achieve the same response as that seen in the WT cells. Accumulation of TNF-α mRNA was readily detectable after exposure of the WT BMSCs to 0.01 ng/mL rmTNF-α, whereas it was not detectable until a concentration of 0.1 ng/mL was used in the TNFR2KO BMSCs.

Finally, because TNF-α promotes GM-CSF mRNA stability, we
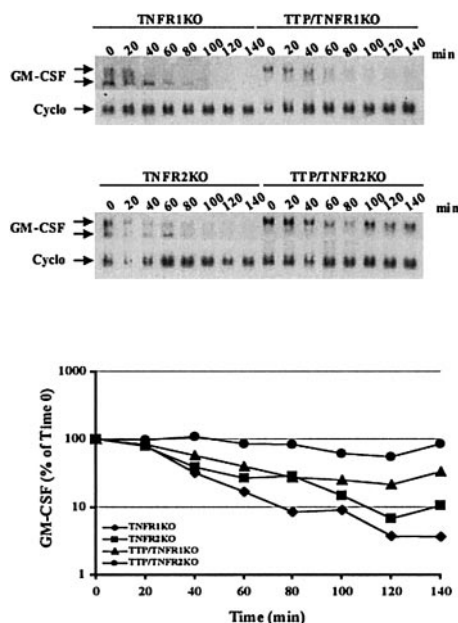
Figure 7. Effect of TNFR1 and TNFR2 on the stability of GM-CSF mRNA. BMSCs were prepared as described in "Materials and methods" and were stimulated with 1 μg/mL LPS for 2 hours. After that, actinomycin D (5 μg/mL) was added, and the cells were harvested for RNA at the indicated times. Northern blots from these experiments are shown at the top, with the 2 species of GM-CSF mRNA and the single species of cyclophilin indicated. On the bottom is the PhosphorImager quantitation of the GM-CSF mRNA levels (total hybridizable mRNA, ie, both species together) after normalization to cyclophilin mRNA levels. The data are expressed as a percentage of the value at time 0, which was assigned the value of 100%. In the presence of TTP (TNFR1KO, TNFR2KO), GM-CSF mRNA appeared as a typical doublet of 0.8 and 1 kb. However, when TTP was absent (TTP/TNFR1KO, TTP/TNFR2KO), the larger species appeared to be predominant. Note also that in the absence of TTP, the stability of the mRNA was increased, particularly when both TTP and TNFR2 were absent. The experiment shown here is representative of 2 independent experiments with cells derived from different groups of animals.
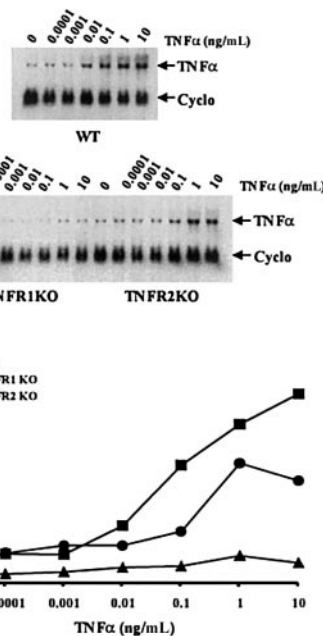
Figure 8. Differential induction of TNF-α mRNA accumulation mediated by TNFR1 and TNFR2. BMSCs were prepared as described in "Materials and methods" and were stimulated with increasing concentrations of rmTNF-α for 2 hours. Northern blots from these experiments are shown on the top; the TNF-α and cyclophilin (Cyclo) mRNAs are indicated. On the bottom is the PhosphorImager quantitation of these blots after the TNF-α mRNA levels had been normalized for cyclophilin mRNA levels. WT BMSCs exhibited detectable expression of TNF-α mRNA after stimulation with as little as 0.01 ng/mL rmTNF-α. In the absence of TNFR1 (TNFR1KO), there was no induction of TNF-α mRNA expression, even at 10 ng/mL rmTNF-α. However, in the absence of TNFR2 (TNFR2KO), rmTNF-α induced the expression of TNF-α mRNA, but there was a shift to the right of one order of magnitude in the concentration-dependence curve compared with that seen in WT macrophages. The experiment shown here is representative of 2 independent experiments with cells derived from different groups of animals.

examined whether TNF-α could influence the stability of its own mRNA (Figure 9). TNFR1KO cells exhibited the shortest TNF-α mRNA half-life (40 minutes), with a similar value in the absence of TNFR2 (48 minutes). These values are similar to those observed in WT cells (35-40 minutes).[11,13] However, the absence of both TTP and TNFR1 resulted in an increased half-life of TNF-α mRNA (63 minutes), and this was 149 minutes when TNFR2 absence was combined with the absence of TTP.

Discussion

The main goal of the present study was to establish the role played by TNF-α in the development of the chronic inflammatory syndrome resulting from TTP deficiency. This syndrome includes cachexia, polyarticular arthritis, systemic autoimmunity, myeloid hyperplasia, and extramedullary hematopoiesis.[6] Treatment of newborn TTPKO mice with a monoclonal hamster anti-mouse neutralizing antibody[28] for 9 weeks resulted in normal growth rates and no detectable myeloid hyperplasia or arthritis.[6] However, there are potential problems with the long-term use of neutralizing antibodies raised in a different species, including inadequate neutralization, the development of "anti-antibodies," and others. To avoid the potential problems associated with exogenous antibody treatment, we interbred the TTPKO mice with the TNFR1/2KO mice. This genetic approach should prevent all actions of TNF-α from the moment of conception. Our preliminary studies on the
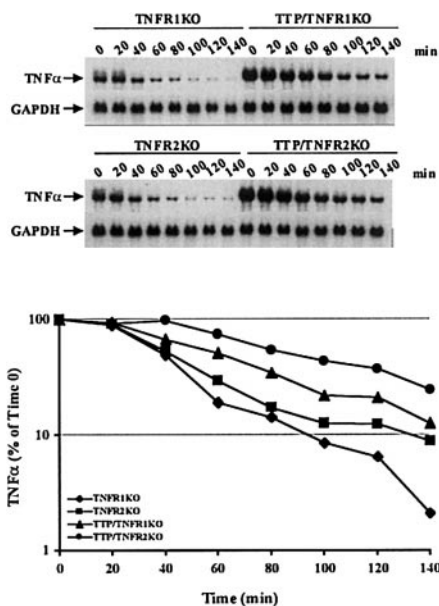
Figure 9. Effect of TNFR1 and TNFR2 on the stability of TNF-α mRNA. BMSCs were prepared as described in "Materials and methods" and were stimulated with 1 μg/mL LPS for 2 hours. After that, actinomycin D (5 μg/mL) was added and cells were harvested for RNA at the indicated times. Northern blots from these experiments are shown at the top, with the TNF-α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs indicated. On the bottom is the PhosphorImager quantitation of the TNF-α mRNA levels after normalization to GAPDH mRNA levels. The absence of either TNFR1 (TNFR1KO) or TNFR2 (TNFR2KO) did not affect the half-life of TNF-α mRNA. However, when TTP was also absent (TTP/TNFR1KO, TTP/TNFR2KO), the stability of TNF-α mRNA was increased, particularly when both TTP and TNFR2 were absent. The experiment shown here is representative of 2 independent experiments with cells derived from different groups of animals.

3KO mice[13] confirmed our initial observations with the neutralizing antibodies. That is, these mice developed normally and did not develop cachexia or arthritis at any age, establishing the primary pathogenetic role of TNF-α in these aspects of the TTP-deficiency syndrome. However, as the animals aged, they developed both medullary and extramedullary myeloid hyperplasia, and their growth rate slowed modestly. We conclude that these late-onset phenomena may be due to the excess production of GM-CSF that occurs in the absence of TTP.[13]

A second goal of these studies was to explore the involvement of each TNFR in the development of the TTP-deficiency phenotype. Previous studies have demonstrated that each TNFR appears to be responsible for certain, sometimes overlapping actions of TNF-α. However, mice deficient in either or both TNFRs do not appear to develop any serious pathology unless subjected to exogenous stress (eg, LPS challenge, bacterial infections).[20,22] The TTPKO mouse provides a useful model in which to study the role played by each TNFR in the development of the inflammatory response to chronic TNF-α stimulation.

The results presented here demonstrate that TNFR1 and TNFR2 play distinct roles in the development of the TTP deficiency–associated phenotype. The superimposition of TNFR1 deficiency on TTP deficiency resulted in prevention of the cachexia and arthritis characteristic of this syndrome, resulting in a phenotype that was very similar to that exhibited by the 3KO mice. The TTP/TNFR1KO mice, like the 3KO mice, developed normally and bred well, and we were able to produce a colony of homozygous mice. However, as seen in the 3KO mice, as the mice aged past approximately 6 months, they began to develop myeloid hyperplasia and extramedullary hematopoiesis with splenomegaly, and their growth rates dropped below those of control mice.

In contrast, the combined deficiency of TTP and TNFR2 resulted in a very different phenotype. These mice appeared similar to the TTPKO mice, with cachexia and growth impairment obvious at an early age. These animals also developed severe polyarticular arthritis of the paw joints, but the onset appeared to be earlier than in the TTPKO mice. TTPKO mice generally exhibit signs of discoloration and swelling in the paw joints at 8 to 10 weeks of age, but these signs were apparent as early as 3 to 4 weeks of age in the TTP/TNFR2KO mice. Histology of the paw joints confirmed the very early onset of erosive arthritis. As with all of the other genotypes lacking TTP, the TTP/TNFR2KO mice also developed myeloid hyperplasia and extramedullary hematopoiesis as they aged. Both the TTPKO and TTP/TNFR2KO mice were produced by crossing heterozygotes because both homozygous lines have very low breeding capabilities. In both cases, regardless of the severity of the postnatal syndrome, both TTPKO and TTP/TNFR2KO mice were born at the appropriate mendelian rates, suggesting that the excess TNF-α production observed in the absence of TTP did not significantly impair intrauterine development.

Two main conclusions can be drawn from these data. First, TNF-α, acting through TNFR1, appears to be responsible for the development of the cachexia and polyarticular arthritis seen in the TTPKO mice. Second, TNF-α acting through TNFR2 alone does not induce any of these inflammatory responses. In fact, the results presented here lend support to previous speculations about a potential protective role played by TNFR2. TNFR2 is a high-affinity receptor for TNF-α, and it is shed from the cell membrane upon stimulation of cells by TNF-α. It has been postulated that this soluble form of the receptor could sequester the circulating soluble TNF-α, thus limiting its availability to act through TNFR1.[16] The increased severity of the syndrome developed by the TTP/TNFR2KO mice supports this idea because the absence of TNFR2 could result in more circulating TNF-α, which could then act through TNFR1, thus exacerbating the TTP-deficiency syndrome.

All of the animals studied that were deficient in TTP displayed myeloid hyperplasia and extramedullary hematopoiesis after about 6 to 8 months of age, including those also deficient in one or both TNFRs. This appears to be an age-related phenomenon because the 3KO animals did not exhibit myeloid hyperplasia when examined at 82 days of age, in keeping with our previous results with the TNF-α antibody-treated TTPKO mice.[6] We have shown previously[13] that the absence of TTP results in increased stability and accumulation of GM-CSF mRNA and increased GM-CSF secretion. These phenomena occur whether or not there is excess effective TNF-α secondary to the absence of TTP because they occurred in the absence of either or both TNFRs. GM-CSF is a hematopoietic growth factor, but it also can play a role as a proinflammatory cytokine.[29] Our results support a role for GM-CSF in the development of the myelopoietic and possibly a component of the inflammatory syndrome seen in the TTPKO mice, both of which can occur even in the absence of TNF-α action.

We have previously suggested a model in which TTP regulates TNF-α production through a negative feedback loop, and we have shown that, as a part of that loop, TNF-α can induce the expression of TTP.[11] However, the results shown here suggest a distinct role for each TNFR in the development of the inflammatory response. These results suggest that the expression of TTP, TNF-α, and GM-CSF, all of which can be induced by TNF-α, can be differentially regulated by each TNFR. Only TNFR1 was able to mediate the TNF-α–induced expression of these 3 mRNAs, confirming its role as the major mediator of the inflammatory

response to TNF-α. However, our data also showed that, even though all of these mRNAs were still induced by TNF-α, in the absence of TNFR2 there was a clear shift to the right in the TNF-α concentration-dependence curve, indicating that TNFR2 is necessary for TNF-α to stimulate the same levels of mRNA accumulation seen in WT cells. These results agree with the previously described hypothesis proposing cooperation between the TNFRs. According to this hypothesis, the presence of the high-affinity TNFR2 would concentrate TNF-α on the cell surface, thus making it more accessible to TNFR1, which has a lower affinity for TNF-α.[18,25] In the absence of TNFR2, the concentration of TNF-α necessary to trigger a response through TNFR1 would increase, as shown in the experiments presented here.

Finally, our results suggest an involvement of TNFR1 in the TNF-α–induced regulation of mRNA stability. Our previous studies demonstrated that the absence of TTP resulted in increased stability of TNF-α and GM-CSF mRNAs.[11,13] TNF-α has been shown to stimulate the stabilization of at least GM-CSF mRNA.[14] Our data show that the combined absence of TTP and TNFR2 results in increased half-lives of both GM-CSF and TNF-α mRNAs compared with those seen in the absence of TTP alone. The increased production of active cytokines that results from this increased mRNA stability could explain, at least in part, the increased severity of the inflammatory syndrome observed in the TTP/TNFR2KO mice.

In conclusion, using the inflammatory disease model provided by the TTPKO mice, we have been able to differentiate the roles played by each TNFR in the regulation of key components of the inflammatory response, including TNF-α, GM-CSF, and TTP itself. The potential use of TNFRs as therapeutic targets has been proposed.[30,31] Our results indicate that there is a delicate balance between TNFR1 and TNFR2 signaling in mediating TNF-α actions. Thus, it is possible that therapies aimed at modifying cytokine mRNA expression or stability could be developed by separately targeting each type of TNFR.

Acknowledgments

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