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Transmembrane TNF-α Reverse Signaling Inhibits Lipopolysaccharide-Induced Proinflammatory Cytokine Formation in Macrophages by Inducing TGF-β: Therapeutic Implications

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TNF-α, a potent proinflammatory cytokine, is generated in a precursor form called transmembrane (m)TNF-α that is expressed as a type II polypeptide on the surface of certain cells. mTNF-α was shown to act both as a ligand by binding to TNF-α receptors, as well as a receptor that transmits outside-to-inside (reverse) signals back into the mTNF-α–bearing cells. In this study, we show that nonactivated macrophages express basal levels of mTNF-α and respond to anti–TNF-α Abs by triggering the MAPK kinase 4 signaling pathway. The pathway induces TGF-β. Based on inhibitory experiments, the production of TGF-β1 is regulated via Jun kinases, whereas that of other TGF-βs is regulated via p38 MAPKs. Exposure to LPS further induced the expression of mTNF-α, and triggering of mTNF-α strongly suppressed the LPS-induced proinflammatory response. Neutralizing TGF-β by Abs prevented the mTNF-α–mediated suppression of LPS-induced proinflammatory cytokine formation, indicating that the immune-suppressive effect of mTNF-α is mediated via TGF-β. Although apoptotic cells are also known to suppress LPS-induced proinflammatory cytokine formation in macrophages by upregulating TGF-β, we show that they do not use the mTNF-α signaling pathway. Because TGF-β possesses a wide range of immune-suppressive effects, our data indicate that upregulation of TGF-β synthesis by those TNF-α–targeting molecules, which are able to trigger mTNF-α, might contribute to their therapeutic effect in the treatment of certain inflammatory diseases such as Crohn's disease, Wegener's granulomatosis, or sarcoidosis. Additionally, none of the TNF-α–targeting molecules is expected to interfere with the immune-silencing effects of apoptotic cells. ***The Journal of Immunology*****, 2016, 196: 1146–1157.**

Tumor necrosis factor-α is a potent proinflammatory cytokine exerting pleiotropic effects on various cell types (1). It is generated in a precursor form called transmembrane TNF-α (mTNF-α) that is expressed as a cell surface II polypeptide consisting of 233 aa residues on activated macrophages and lymphocytes as well as other cell types (2–4). After being processed by metalloproteinases such as TNF-α–converting enzyme (5, 6), the soluble form of TNF-α of 157 aa residues is released and mediates its biological activities through type 1 and 2 TNFRs (7). Increasing evidences suggest that not only soluble TNF-α, but also its precursor form, mTNF-α, is involved in the regulation of the inflammatory response acting primarily locally. mTNF-α was shown to act both as a ligand by binding to TNFRs, as well as a receptor that transmits outside-to-inside (reverse) signals back into mTNF-α–bearing cells (8, 9). Under physiological conditions mTNF-α can be triggered by either soluble or cell-bound TNF-α receptors to initiate reverse signaling (9). Because it can also bind to both type 1 and 2 TNFRs, and these receptors are expressed on almost all nucleated cells (7), theoretically mTNF-α can be triggered by all cells, with which mTNF-α–bearing cells form a strong contact. mTNF-α exists as a homotrimer of the 26-kDa uncleaved monomers (8). Because its cytoplasmic domain does not possess any discernible enzymatic function, it is thought to signal through associated proteins that have not been identified yet. Previous studies have shown that triggering mTNF-α significantly interferes with the LPS-induced proinflammatory cytokine formation in macrophages and converts resistance to restimulation by LPS by activating ERKs (10, 11). However, the molecular mechanisms through which mTNF-α interferes with the LPS signaling have not been clarified so far.

In the present study we demonstrate that mTNF-α signaling inhibits LPS-induced proinflammatory cytokine formation by inducing the expression of TGF-β via activating the MAPK kinase (MKK) 4 signaling pathway. TGF-β is responsible then for activation of the previously described ERK kinase pathway. Previous studies have shown that apoptotic cells can also inhibit LPS-induced proinflammatory cytokine formation of macrophages by inducing TGF-β (12). However, we show that apoptotic cells do not activate the mTNF-α signaling pathway because their cell surface TNFRs are downregulated. Anti–TNF-α–targeting molecules are widely used in the treatment of chronic inflammatory

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; MKK, MAPK kinase; mTNF-α, transmembrane TNF-α.

diseases. However, although they neutralize TNF-α with the same efficiency, they show disease-specific effectiveness. In the present study, we show that etanercept, which failed in the therapy in Crohn's disease (13), Wegener's granulomatosis (14), and sarcoidosis (15), does not trigger TGF-β production in human macrophages, whereas golimumab and infliximab, which are effective in the therapy of these diseases (16–19), does trigger such TGF-β production. Because TGF-β possesses a wide range of immunesuppressive effects (20), our data indicate that upregulation of TGF-β synthesis by those TNF-α–targeting molecules, which are able to trigger mTNF-α, might contribute to their therapeutic effect in the treatment of inflammatory diseases, whereas none of them will interfere with the immune-silencing effects of apoptotic cells.

Materials and Methods

Reagents

Galardin, Ro-318220, and SB-203580 were obtained from Calbiochem, rTGF-β was from AbD Serotec, and FITC-labeled anti-mouse TNF-α and its FITC-labeled isotype control, PE-labeled anti-mouse TNFR type I/p55 and its PE-labeled isotype control Abs, were from BioLegend. TNFRII PE-conjugated Ab, anti-mouse TNF-α and anti-mouse TGF-β Abs were from R&D Systems, and DAPI was from Life and Technologies. TCS JNK60 was from Tocris Bioscience. Anti-mouse MKK3/4/7, p-MKK4/7, and p-MKK3 (Ser^{189})/MKK6 (Ser^{207}) Abs were from Cell Signaling and Technology. Ficoll-Paque Plus was obtained from Amersham Biosciences. CD14 microbeads, human specific, were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Human M-CSF was from PeproTech. All other reagents were obtained from Sigma-Aldrich (Budapest, Hungary).

Animals

In most of the experiments 2-mo-old NMRI male mice were used, whereas in some experiments TNF-α–deficient male mice (21) and their C57BL/6 wild-type littermates were used after being killed by ether anesthesia. Mice were maintained in a specific pathogen-free condition in the Central Animal Facility and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen.

Cell culture procedures

For bone marrow–derived macrophage (BMDMs), bone marrow was isolated from femurs with sterile physiological saline and cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% L929 conditioned media at 37°C in 5% CO_2 for 7 d before use. RAW264.7 cells were obtained from the American Type Culture Collection and were maintained in DMEM culture medium containing glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS.

Peripheral human blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus from buffy coats obtained from blood collected from healthy volunteers by the Blood Donation Center, Debrecen. Buffy coats not used up therapeutically were provided to us with the permit (RKEB 3582-2012) from the Regional Research Ethical Committee. $CD14^+$ cells were separated by magnetic sorting with MACS, followed by washing with PBS containing 0.5% BSA and 2 mM EDTA. Freshly isolated monocytes were cultured for a period of 5 d in IMDM supplemented with 10% AB serum and 5 ng/ml M-CSF to differentiate them to macrophages.

Immunohistochemistry

BMDMs and RAW264.7 macrophages (1×10^6) were washed in PBS, after which fixation in acetone (15 min at −20°C) was performed just before the immunolabeling. After rinsing in PBS, the samples were incubated at 4°C overnight with FITC-labeled anti-mouse TNF-α or FITC-labeled rat IgG1 isotype Abs. Then the samples were washed in PBS and incubated for 1 h at room temperature with DAPI (1:600). After rinse and cover, PBS-glycerol samples were examined using an Olympus microscope and analyzed by computer image analysis (magnification, ×600).

Thymocyte apoptosis induction in vitro

Thymocytes were isolated from 4-wk-old NMRI mice and cultured for 18 h (10^6 cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. This method typically resulted in >80% apoptotic cells (as assessed by propidium iodide/annexin V–FITC staining). In some experiments apoptosis was induced by 0.1 μM dexamethasone or by a combination of 5 ng/ml phorbol dibutyrate and 1 μg/ml ionomycin. Apoptotic cells that died spontaneously were used in the immune downregulation assays at a 10:1 (apoptotic cell/macrophage) ratio.

Determination of TNFR I and II expression on the apoptotic thymocyte cell surface

Control and apoptotic thymocytes (1×10^6) were washed with PBS, incubated with PE-labeled anti-mouse TNFR I and II Abs or their isotype controls for 30 min at room temperature, and then washed and and fixed in 1% paraformaldehyde. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

Immunofluorescence staining and confocal microscopy

BMDMs were plated in eight-well chamber slides (2.5×10^5/well) and cultured for 7 d before use. The cells were treated with 100 ng/ml LPS for 1 h. After exposure of macrophages to apoptotic cells stained overnight with 10 μM 5-(and -6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (1:5) for 30 min, cells were washed with HEPES buffer and fixed in methanol/acetone (1:1) for 10 min at −20°C. Macrophages were blocked with 10% normal donkey serum in HEPES for 2 h at 4°C, then washed with ice-cold HEPES buffer twice and stained with goat anti-mouse TNF-α primary Ab overnight at 4°C. After three washes, samples were incubated with Alexa Fluor 488–conjugated donkey anti-goat secondary Ab for 4 h. Cells were then stained with DAPI for 10 min, washed four times, left in HEPES buffer, and imaged immediately using a Zeiss LSM 510 confocal laser scanning microscope. For visualizing the distribution of mTNF-α, overview images and three-dimensional stacks were acquired at 1.5-μm optical thickness. Red fluorescence of 5-(and -6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine–stained apoptotic cells and blue fluorescence of nuclei were imaged for the same fields in multitrack mode to avoid spectral overlap.

Determination of mTNF-α expression on the cell surface

BMDM and RAW264.7 cells were stimulated with 100 ng/ml bacterial LPS for the indicated time points after preincubation with galardin (5 μg/ml) for 4 h or alone. After the treatment, macrophages were washed (1× PBS), collected, blocked with 50% FBS for 30 min, stained with FITC-labeled anti-mouse TNF-α or FITC-labeled rat IgG1 isotype control Abs, and fixed in 1% paraformaldehyde. Stained cells were analyzed on a FACSCalibur (BD Biosciences). The results were analyzed by WinMDI 2.9 software.

Isolation of total RNA and quantitative RT-PCR

Total RNA was isolated from control and bacterial LPS-treated BMDMs and RAW264.7 macrophages (3×10^6 cells/sample) by TRI reagent. After various treatments, macrophages were washed with ice-cold PBS. RNA was extracted with TRI reagent. cDNA was synthesized with a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. TNF-α, TGF-β1, and IFN-α mRNA levels were determined with TaqMan PCR using FAM-MGB–labeled probes (Applied Biosystems) on a Roche (LC480) platform. Gene expression was normalized to GAPDH expression.

Phosphorylation changes in the MAPK signaling pathway following triggering mTNF-α

Anti–TNF-α Ab or its isotype control was coated to six-well plates to which BMDMs were added (3×10^6 cells/well) alone or in the presence of neutralizing TGF-β Ab (5 μg/ml). After 2 h incubation the relative levels of phosphorylation of MAPKs and other serine/threonine kinases were determined by using a human phospho-MAPK array (Proteome Profiler Ab array; R&D Systems). The pixel density in each spot of the array was determined by ImageJ software.

Western blot analysis to detect phosphorylation levels of MKK3/MKK4/MKK6/MKK7

Whole cell homogenate was used. 40 μg protein was run on a 12% polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes using the Bio-Rad electrophoresis and transfer system. The free binding sites of membranes were blocked with 5% non fat dry milk powder in 20 mM Tris, 0.1 M NaCl buffer with 0.1% Tween for 1 h at room temperature. Blots were then incubated overnight with primary Abs (MKK3/4/7, p-MKK4/7 and 3/6) at 1:500 dilution. To detect these signals, peroxidase-

labeled anti-rabbit IgGs (1:10,000) were used and the ECL was visualized using an ECL system (Amersham Biosciences). Equal loading of proteins was demonstrated by probing the membranes with β-actin Ab.

Determination of cytokine production

The cytokine content of the cell culture media in various experiments was analyzed by a mouse cytokine array (Proteome Profiler array; R&D Systems). The pixel density in each spot of the array was determined by ImageJ software. Alternatively, TNF-α, IL-6, MIP-2, and TGF-β cytokine levels measured with ELISA kits obtained from the R&D Systems. For TGF-β, the supernatants were acid-activated before the assay according to the manufacturer's instructions.

Statistical analysis

All data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± SD. The p values were calculated by using a two-tailed Student t test for two samples of unequal variance. The analysis of cytokine array experiments was carried out by ANOVA test. A p value < 0.05 was considered to be statistically significant.

Results

Stimulation of mouse BMDMs or RAW264.7 macrophages with LPS enhances the cell surface expression of mTNF-α

First, we investigated with confocal microscopy whether TNF-α is detectable in nonstimulated BMDMs or RAW264.7 macrophages. As shown in Fig. 1A, TNF-α is detectable in both BMDMs and RAW264.7 macrophages in intracellular vesicles. Although the protein could be readily seen, only a minimal amount of TNF-α mRNA was detected in nonstimulated cells. When, however, macrophages were exposed to LPS (100 ng/ml), a significant increase in TNF-α mRNA levels was detected in both cell types, reaching its maximum following 2 h of stimulation (Fig. 1B). Then the mRNA levels gradually decreased. In line with these observations, the cell surface levels of mTNF-α of both BMDMs and RAW264.7 macrophages (Fig. 1C), which were not detectable in nonstimulated cells, started to rise after LPS stimulation and became more readily detectable when the cell surface cleavage of mTNF-α was inhibited by galardin, a synthetic metalloproteinase inhibitor (22). Galardin treatment tested in RAW264.7 cells alone also increased the cell surface expression of mTNF-α (Fig. 1D), indicating that 1) the metalloprotease responsible for the cleavage of mTNF-α must be expressed in nonstimulated cells as well, and 2) although we could not detect the presence of mTNF-α without galardin, there must be some basal level of mTNF-α production in nonstimulated cells as well. The cell surface expression of mTNF-α of LPS-treated BMDMs and RAW264.7 cells reached its maximum at 1 h (Fig. 1D), and then it started to gradually decrease. In galardin-treated RAW264.7 cells, the increase in the cell surface mTNF-α expression could be detected as soon as 15 min after LPS stimulation, indicating that mTNF-α at the beginning must arrive from the already existing intracellular TNF-α pool. The sharp decrease of LPS-induced mTNF-α expression following 1 h of stimulation (Fig. 1D) despite the increase in TNF-α mRNA expression (Fig. 1B) indicates that expression of the metalloprotease, which is responsible for the mTNF-α cleavage, must be also induced by LPS stimulation in both types of macrophages. Galardin treatment strongly delayed the decrease in the mTNF-α expression of macrophages and also delayed and decreased the appearance of soluble TNF-α in the cell culture medium (Fig. 1D).

mTNF-α signaling activates the MKK3/6-, Jun kinase–, and PI3K-regulated pathways

Because our preliminary data indicated that mTNF-α must be present on the surface of nonstimulated macrophages as well, we decided to investigate mTNF-α signaling in BMDMs stimulated with the help of a phospho-MAPK array kit, which enables the parallel determination of relative levels of phosphorylation of MAPKs and those of several related proteins. The data received in the presence of coated anti-TNF-α Abs were compared with those that resulted in the presence of isotype control Abs. Because preliminary experiments, which we used as a read-out to detect the activation of mTNF-α signaling, demonstrated that soluble anti–TNF-α Abs are not able to inhibit LPS-induced IL-6 and MIP-2 production, we applied coated Abs to trigger mTNF-α signaling (Fig. 2A). Stimulation of mTNF-α by coated anti–TNF-α Abs led to activation of the MKK3/6-regulated MAPK, the JNK, the ERK kinase, and the PI3K pathways (Fig. 2B, 2C).

MKK3/6, Jun kinase, ERK kinase, and PI3K pathways are activated by TGF-β induced via the mTNF-α signaling pathway

Because these alterations in the phosphorylation status of various proteins were detected 2 h after macrophages were seeded onto the coated Abs, we could not exclude the possibility that mTNF-α signaling induced the expression of a cytokine that drives the above-mentioned three signaling pathways. Because TNF-α, IFN-α, and TGF-β were previously shown to activate the MKK3/6, Jun kinase, and PI3K pathways (23–26), we decided to test whether the expression of these cytokines is induced by triggering mTNF-α. Although we could not detect the induction of IFN-α or TNF-α at mRNA levels (data not shown), the induction of TGF-β was readily detected by a pan anti–TGF-β Ab at protein levels (Fig. 3A) and that of TGF-β1 at mRNA levels (Fig. 3B). To determine whether TGF-β is indeed responsible for the mTNF-α triggering–induced MKK3/6, JNK, ERK kinase, and PI3K pathway activation, the experiments described in Fig. 2 were repeated in the presence of neutralizing anti–TGF-β Abs as well. As shown in Fig. 3C, mTNF-α–induced phosphorylation of those proteins, which are known to be activated by the TGF-β signaling pathway (27) (Fig. 3D), was completely or partially prevented by administration of neutralizing TGF-β Abs. These data confirm that indeed the mTNF-α signaling–induced TGF-β is responsible for their phosphorylation.

mTNF-α signaling induces the MKK4 signaling pathway

The same blots were then analyzed further to determine the kinases that belong directly to mTNF-α signaling. As shown in Fig. 4A, those kinases were selected to belong to the mTNF-α signaling pathway, the activation of which was not or was only partially affected by administration of neutralizing TGF-β Abs. Elements of the PI3K (TOR, Akt1, 2, and 3), the p38α/β, MAPK, and JNK pathways were found to be activated directly by mTNF-α. The MAPK pathways are evolutionally conserved, and it was shown that the p38α/β pathways can be activated by either MKK3/6 or MKK4/MKK7 (25, 26). Because MKK3 and MKK6 were not activated by the mTNF-α signaling pathway (Fig. 4A), whereas MKK4 and MKK7 can also activate Jun kinases (28), we decided to determine their phosphorylation levels following mTNF-α triggering. To avoid interference with the TGF-β signaling, the experiments were performed in the presence of neutralizing TGF-β Abs. As shown in Fig. 4B, the phosphorylation of MKK4 transiently increased, whereas that of MKK7 was not detectable during mTNF-α signaling. These data indicate that MKK4 mediates the effect of mTNF-α. In the absence of neutralizing TGF-β Abs, the induced phosphorylation levels of MKK4 remained high even at 2 h (Fig. 4C), in line with the fact that TGF-β also triggers the activation of MKK4 (29). Phosphorylation of MKK3/6 induced by TGF-β appeared with a delay as compared with that of

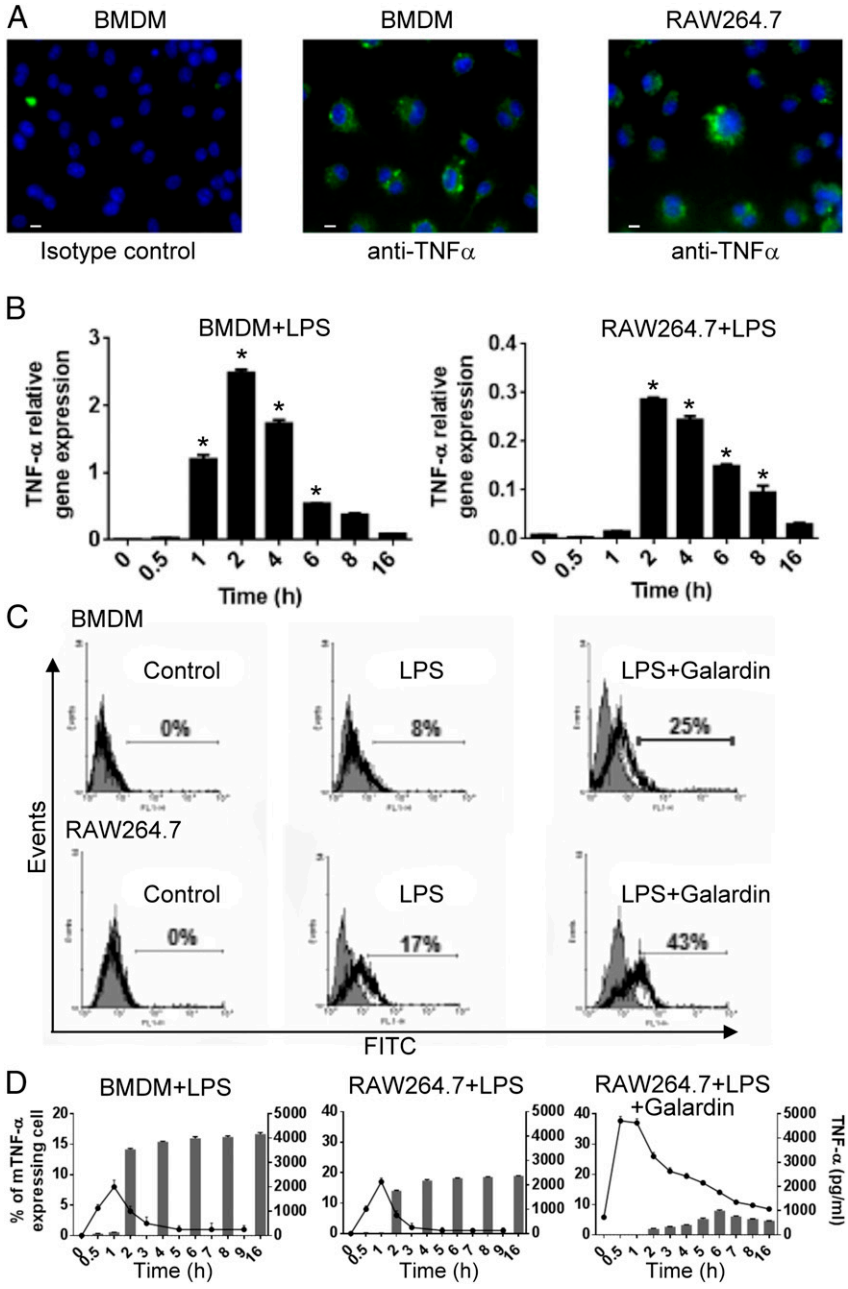

FIGURE 1. TNF-α is expressed by resting macrophages and is further induced by LPS. (**A**) TNF-α is expressed by resting BMDMs and RAW264.7 macrophages detected by confocal microscopy. Green indicates TNF-α; blue indicates cell nuclei stained with DAPI. Scale bar, 10 μm. (**B**) LPS induces the mRNA levels of TNF-α in both BMDMs and RAW264.7 macrophages. Macrophages were exposed to 100 ng/ml LPS for the indicated times followed by RNA isolation and reverse transcription. Gene expression levels of TNF-α were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean ± SD from three independent experiments. (**C**) LPS induces the expression of mTNF-α detected by FACS analysis at 1 h following LPS (100 ng/ml) exposure. Addition of galardin, a metalloprotease inhibitor (5 μg/ml for 2 h before LPS exposure), enhances the percentage of mTNF-α–expressing cells. (**D**) The time course of the percentage of mTNF-α–expressing macrophages (line graph) and the amount of soluble TNF-α in the cell culture medium (bar graph) following LPS (100 ng/ml) exposure in the presence or absence of galardin detected by FACS analysis and ELISA, respectively. The results are mean for the mTNF-α–expressing cells and mean ± SD for the ELISA data from three independent experiments. *$p < 0.05$ versus control.

MKK4 without alterations in MKK3 levels. The protein levels of MKK7, alternatively, gradually decreased, indicating that TGF-β downregulates MKK7 signaling (Fig. 4C). Next, we decided to investigate which of the mTNF-α activated signaling pathways contribute to the induction of TGF-β. The phosphorylation levels of both mitogen and stress-activated kinase 2 and ribosomal S6 kinase 2 known to be activated by p38 MAPKs were induced by mTNF-α signaling (Fig. 4A). These kinases can phosphorylate CREB at Ser^{133} (detected by the phosphokine array, Fig. 4A) and activate CREB-dependent gene expression (30, 31). Because the promoter of both TGF-β2 (32) and -β3 (33, 34) contains cAMP response element, we decided to investigate whether selective inhibition of p38 MAPKs by SB203580 (10 μM) could interfere with the mTNF-α–induced TGF-β production. As seen in Fig. 4D, inhibition of p38 MAPKs attenuated the mTNF-α–induced TGF-β production. The TGF-β1 promoter, alternatively, was reported to be regulated principally by AP-1 sites (34). In line with this observation, inhibition of Jun kinases by TCS JNK 60 also attenuated mTNF-α–induced TGF-β production (Fig. 4D) and completely prevented the mTNF-α–induced TGF-β1 mRNA formation (Fig. 4E). Inhibition of p38 α/β kinases by SB203580, alternatively, had no effect on the TGF-β1 mRNA production. However, when the p38 kinase α/β inhibitor was added together with the Jun kinase inhibitor, the mTNF-α–induced TGF-β production was completely prevented. These data indicate that the Jun kinase pathway regulates primarily TGF-β1, whereas the p38 kinase α/β pathway regulates the production of other TGF-βs (Fig. 4E). In striking contrast, inhibition of PI3K (which is known to activate the Akt pathway) by wortmannin resulted in increased TGF-β formation (Fig. 4D). This observation indicates that this pathway might initiate a negative autoregulatory loop in the regulation of TGF-β production (Fig. 4F). The mechanism of Akt activation was not investigated in our studies, but in other studies it was found that the MKK4 pathway can downregulate the levels of phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, leading to activation of Akt (35).

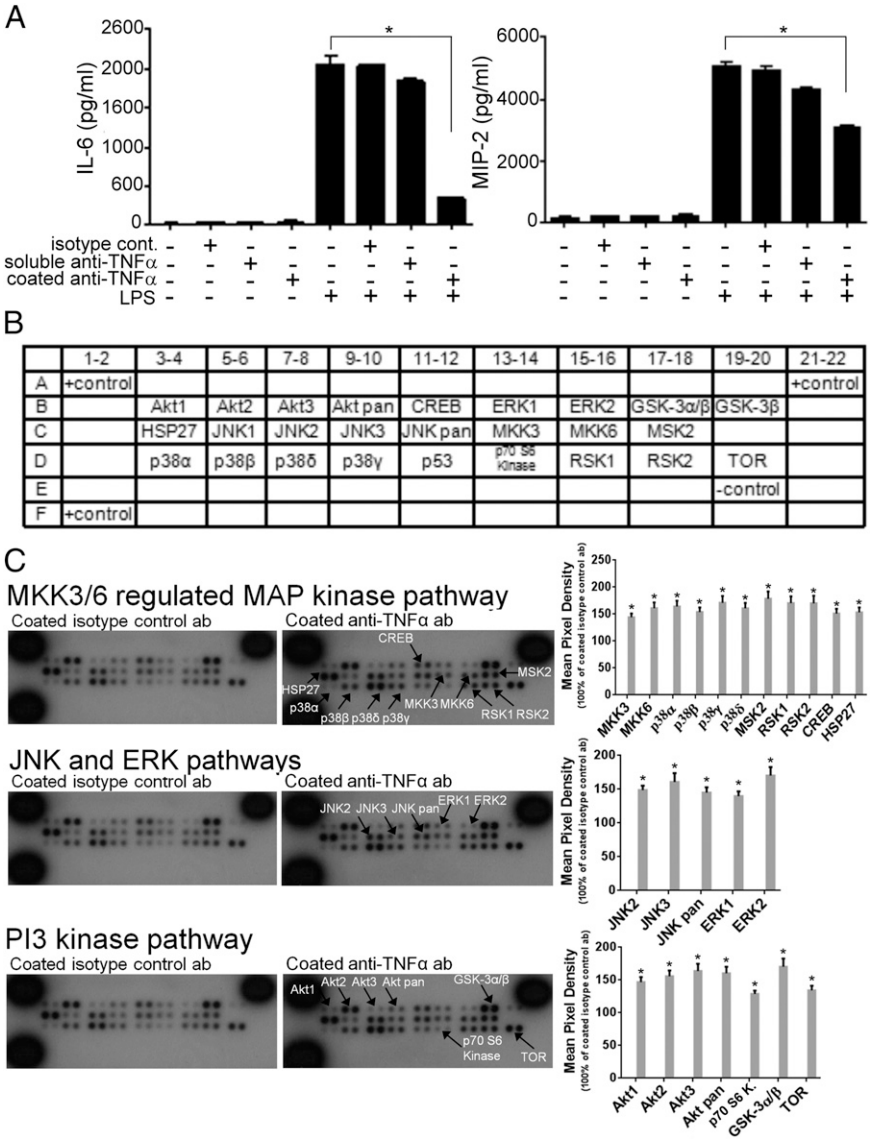

FIGURE 2. mTNF-α induces the MKK3/6-regulated MAPK, Jun kinase, ERK kinase, and PI3K pathways in mouse BMDMs. (**A**) Soluble anti–TNF-α Abs do not significantly inhibit LPS-induced IL-6 and MIP-2 production detected at 6 h by ELISA, whereas coated anti–TNF-α Abs inhibit such production. Macrophages were exposed to either soluble (2 μg/ml) or coated (2 μg/ml) anti–TNF-α Abs for 2 h before exposing macrophages to LPS (100 ng/ml). Cytokine levels were determined 6 h later. The results are mean ± SD from three independent experiments. $*p < 0.05$ versus respective LPS-exposed control. (**B**) Map of the 26 phosphorylated proteins detected on the membranes. (**C**) Phospho-MAPK panel of coated isotype control or coated anti–TNF-α Ab–exposed macrophages. BMDMs were exposed to coated isotype control or anti–TNF-α Abs for 2 h. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a phospho-MAPK array. Arrows highlight those proteins for which the phosphorylation was enhanced in BMDMs exposed to the coated anti–TNF-α Abs as compared with the isotype control. One representative array series of three is shown. Elevated phosphorylation is expressed as a percentage of mean pixel density of the respective isotype Ab control (mean ± SD from three independent experiments). $*p < 0.05$ versus respective control.

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22
A	+control										+control
B		Akt1	Akt2	Akt3	Akt pan	CREB	ERK1	ERK2	GSK-3α/β	GSK-3β	
C		HSP27	JNK1	JNK2	JNK3	JNK pan	MKK3	MKK6	MSK2		
D		p38α	p38β	p38δ	p38γ	p53	p70 S6 kinase	RSK1	RSK2	TOR	
E										-control	
F	+control										

mTNF-α signaling inhibits the production of a subset of LPS-induced proinflammatory cytokines

Because our preliminary experiments (Fig. 2A) indicated that mTNF-α signaling inhibits LPS-induced IL-6 and MIP-2 formation to a different degree, we decided to investigate the effect of mTNF-α signaling on the LPS-induced proinflammatory cytokine formation by simultaneously detecting the production of proinflammatory cytokines using a cytokine array (Fig. 5A). BMDMs generated from NMRI animals generally produced TIMP-1, MCSF-1, and JE proinflammatory cytokines at basal levels (Fig. 5B, 5C). Exposure to LPS did not affect the production of these cytokines. Although we exposed the cells always to the same amount of LPS, as shown in Fig. 5B and 5C, their response to LPS varied from isolation to isolation. A 2-h exposure of BMDMs to coated anti–TNF-α always attenuated the LPS-induced proinflammatory cytokine formation, but the effect was more dramatic when the cells showed a lower LPS response (Fig. 5C). However, not each cytokine demonstrated the same sensitivity to mTNF-α inhibition. LPS-induced G-CSF, I-309, IL-10, IL-23, IL-1α, IL-1β, and IL-16 production was nearly completely, IL-6, IP-10, MIP-1β, IL-1ra, M-CSF, and RANTES production was significantly inhibited (calculated from the pixel densities of cytokine arrays generated from four independent experiments), but MIP-1α, MIP-2, TIMP-1, JE, or KC productions were also reduced. To prove that coated anti–TNF-α Abs indeed act via mTNF-α, we also exposed TNF-α–null macrophages to LPS and checked whether anti–TNF-α Abs affect their cytokine production. As shown in Fig. 5D, coated anti–TNF-α Abs did not significantly alter LPS-induced proinflammatory cytokine production of macrophages isolated from TNF-α–null mice (21).

mTNF-α signaling inhibits LPS-induced proinflammatory cytokine formation by upregulating TGF-β

Next we decided to examine whether TGF-β is involved in the suppression of LPS-induced proinflammatory cytokine formation by mTNF-α by applying neutralizing anti–TGF-β Abs. As shown in Fig. 6A, neutralizing anti–TGF-β Abs did not affect LPS-induced proinflammatory cytokine production in the presence of isotype control Abs with the exception of IL-23, IL-1α, and IL-1β. However, they strongly interfered with the mTNF-α–mediated inhibition of LPS-induced proinflammatory cytokine formation with the exception of those three cytokines, which were affected by the neutralizing anti–TGF-β Abs in the isotype control Ab–treated samples as well. A summary of the inhibitory effect of mTNF-α signaling on LPS-induced IL-6 and MIP-2 production detected by ELISA and the effect of neutralizing TGF-β Abs from 10 independent experiments are shown in Fig. 6B. Additionally,

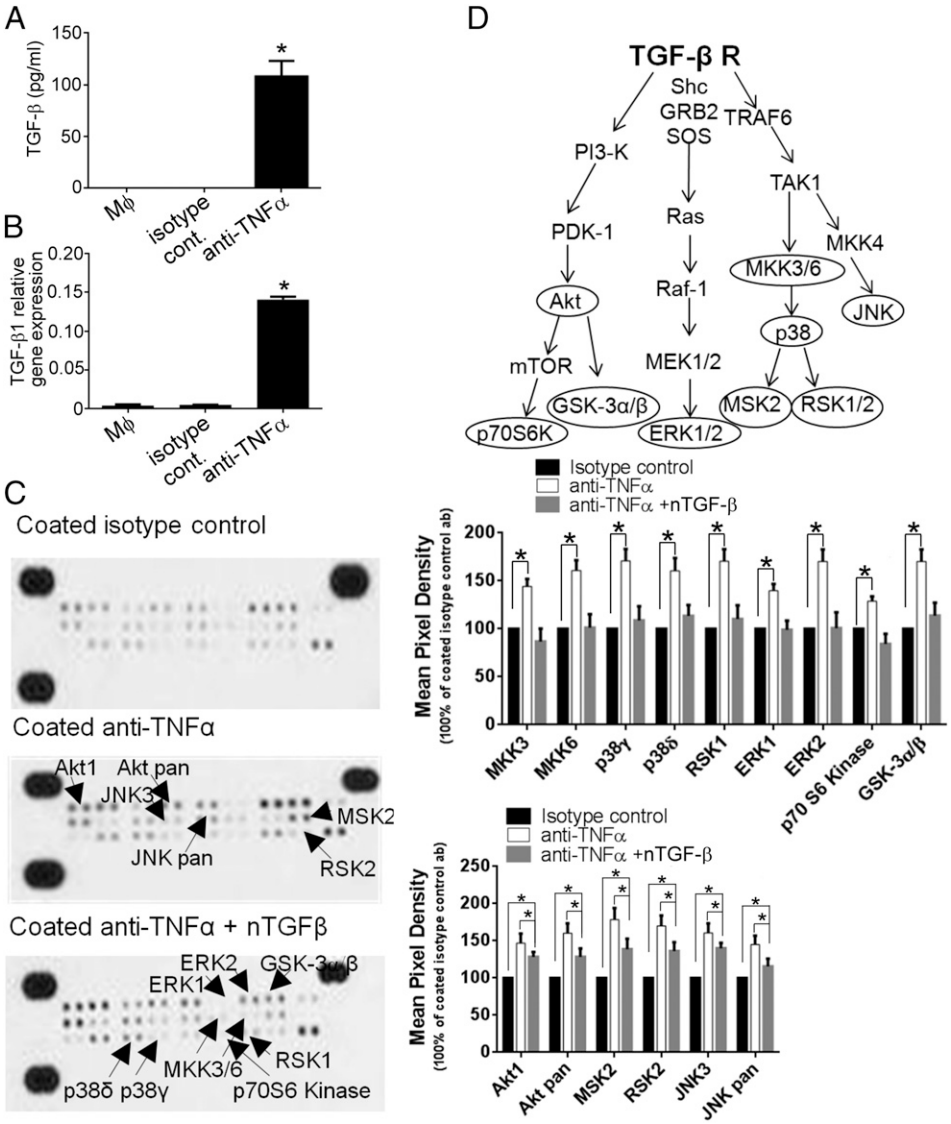

FIGURE 3. Triggering of mTNF-α induces TGF-β production in mouse BMDMs. (**A**) BMDMs were exposed to coated anti–TNF-α (2 μg/ml), and the TGF-β produced was detected 6 h later by ELISA using an anti–pan-TGF-β Ab. (**B**) BMDMs were exposed to coated anti–TNF-α Abs for 2 h followed by RNA isolation and reverse transcription. Gene expression levels of TGF-β1 were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean ± SD from three independent experiments. *$p < 0.05$ versus isotype Ab–exposed control. (**C**) Phospho-MAPK panel of coated isotype control–, coated anti–TNF-α Ab–, or coated anti–TNF-α and neutralizing anti–TGF-β Ab–exposed macrophages. BMDMs were exposed to coated isotype control (2 μg/ml), anti–TNF-α (2 μg/ml), or anti–TNF-α and neutralizing anti–TGF-β (5 μg/ml) Abs for 2 h. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a phospho-MAPK array. Arrows highlight those proteins for which the anti–TNF-α-enhanced phosphorylation was fully or partially prevented by neutralizing anti–TGF-β Abs. One representative array series of three is shown. Enhancement in phosphorylation is expressed as percentage of mean pixel density of the respective isotype Ab control (mean ± SD from three independent experiments). (**D**) Phosphorylated proteins identified by the usage of neutralizing TGF-β Abs are all known participants of the TGF-β signaling pathway. *$p < 0.05$ versus respective control.

preincubation with recombinant TGF-β for 2 h also attenuated LPS-induced proinflammatory cytokine formation in BMDMs in a cytokine-specific manner (Fig. 6C).

Apoptotic thymocytes do not use the mTNF-α signaling pathway to downregulate the LPS-induced proinflammatory response of macrophages

Whereas the phagocytosis of a variety of pathogenic targets, especially bacteria and virally infected cells, normally triggers a proinflammatory response in macrophages, ingestion of apoptotic cells by macrophages usually induces an anti-inflammatory phenotype. Apoptotic cells do not simply fail to provide proinflammatory signals; rather, they actively interfere with the inflammatory program. For example, preincubation of macrophages with apoptotic cells strongly suppresses the inflammatory response induced by LPS, and the mechanism involves release of TGF-β (12). Because most mammalian cells express TNFRs, and thymocytes express both TNFR I and II (36), we decided to investigate, by using macrophages from TNF-α–null mice (21), whether mTNF-α could contribute to the immune silencing effects of apoptotic cells. As seen in Fig. 7A, loss of TNF-α did not affect the basal proinflammatory cytokine formation of macrophages and the downregulation of them when they were engulfing apoptotic cells. Exposure of apoptotic cells to macrophages resulted in downregulation of the LPS-induced response of a number of proinflammatory cytokines in a TGF-β–dependent manner, but loss of mTNF-α tested on TNF-α–null macrophages did not affect their capability of responding to apoptotic cells (Fig. 7B). Additionally,

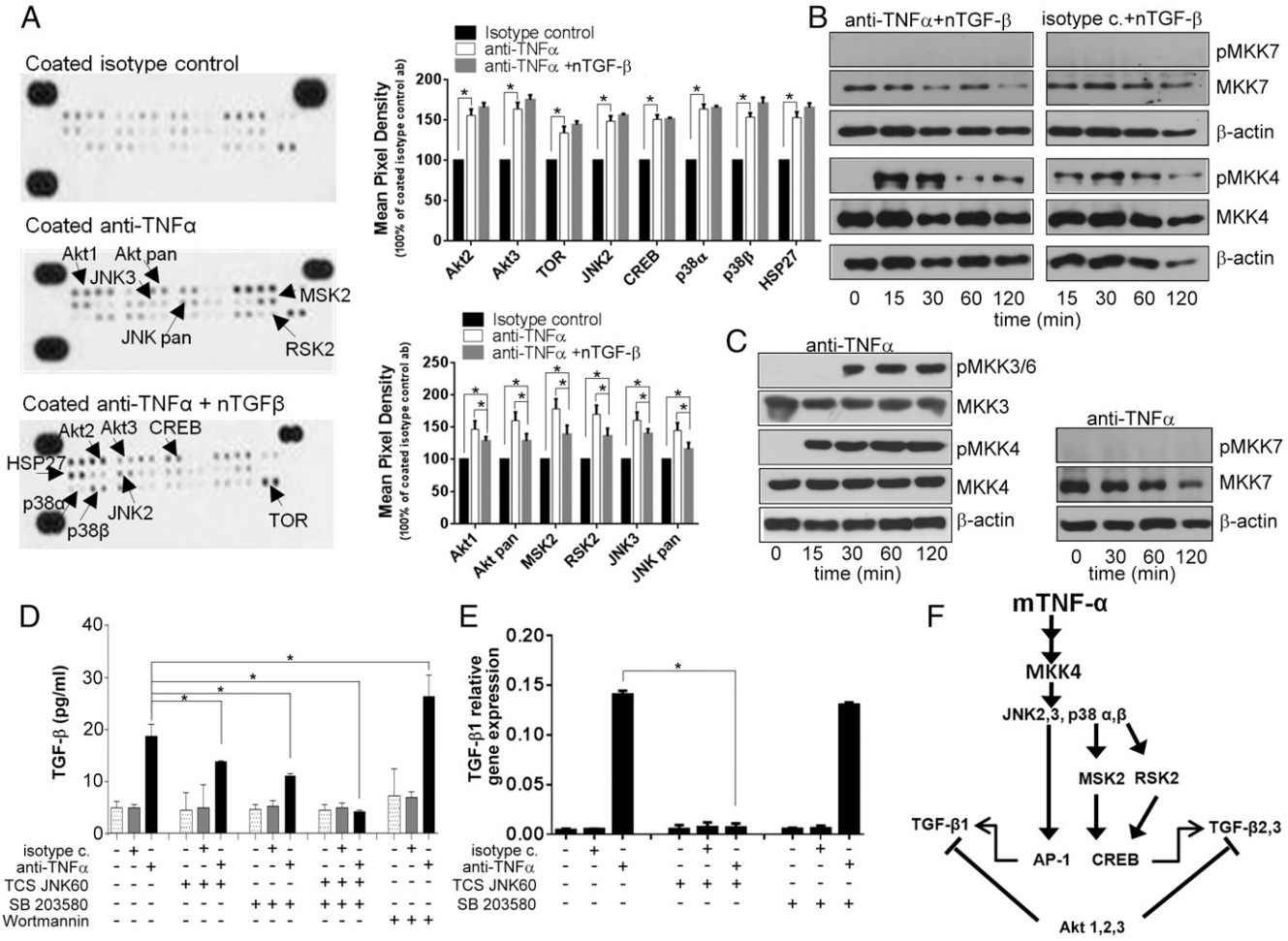

FIGURE 4. Triggering mTNF-α activates the MKK4-regulated MAPK pathway, leading to TGF-β production. (**A**) Phospho-MAPK panel of coated isotype control–, coated anti–TNF-α Ab–, or coated anti–TNF-α and neutralizing anti–TGF-β–exposed BMDMs. BMDMs were exposed to coated isotype control (2 μg/ml), anti–TNF-α (2 μg/ml), or anti–TNF-α and neutralizing anti–TGF-β (5 μg/ml) Abs for 2 h. Macrophages were then lysed, and the amount of phosphorylated proteins was detected by a phospho-MAPK array. Arrows highlight those proteins for which the anti–TNF-α-enhanced phosphorylation was not altered or was only partially prevented by neutralizing anti–TGF-β Abs. One representative array series of three is shown. Enhancement in phosphorylation is expressed as percentage of mean pixel density of the respective isotype Ab control (mean ± SD from three independent experiments). $*p < 0.05$ versus respective control. (**B**) Western blot analysis of the time-dependent phosphorylation levels of MKK4 and MKK7 in BMDMs exposed to coated anti–TNF-α Abs in the presence of neutralizing anti–TGF-β Abs (5 μg/ml). (**C**) Western blot analysis of the time-dependent phosphorylation levels of MKK4, MKK7, and MKK3/MKK6 in BMDMs exposed to coated anti–TNF-α Abs. β-Actin was used as loading control. (**D**) mTNF-α–induced TGF-β production is fully prevented by simultaneous inhibition of the Jun kinase and p38 MAPK pathways, whereas it is further induced when PI3K is inhibited. BMDMs were exposed to coated isotype control (2 μg/ml) or coated anti–TNF-α (2 μg/ml) Abs for 2 h in the presence or the absence of the indicated compounds SB203580 (10 μM), a p38 MAPK inhibitor, TCS JNK 60 (10 μM), a Jun kinase inhibitor, and wortmannin (1 μM), a PI3K inhibitor. TGF-β levels in the cell culture medium were determined by ELISA using an anti–pan-TGF-β Ab. The results are mean ± SD from three independent experiments. $*p < 0.05$ versus control. (**E**) mTNF-α–induced TGF-β1 mRNA production is fully prevented by inhibition of Jun kinases, whereas it is not affected by inhibition of p38 MAPKs. BMDMs were exposed to coated isotype control (2 μg/ml) or anti–TNF-α (2 μg/ml) Abs for 2 h in the presence or the absence of the indicated compounds followed by RNA isolation and reverse transcription. Gene expression levels of TGF-β1 were determined by quantitative RT-PCR using GAPDH as a normalizing gene. The results are mean ± SD from three independent experiments. $*p < 0.05$ versus anti–TNF-α–treated control. (**F**) Proposed signaling pathways induced by mTNF-α in macrophages leading to TGF-β production.

we did not observe a correlation between the location of TNF-α–containing vesicles of LPS-treated macrophages and the position of phagocytic portals for apoptotic cells (Fig. 7C). These data indicate that mTNF-α signaling does not play a role in the apoptotic cell-induced anti-inflammatory response. Thus, we checked whether apoptotic thymocytes still express TNFRs. As shown in Fig. 7D, thymocytes express both TNFR I and II, but as they enter apoptosis following serum withdrawal, they downregulate the expression of both TNFRs. The downregulation was not apoptotic signal specific, as thymocytes exposed to dexamethasone or phorbol dibutyrate/ionomycin also responded with full downregulation of their TNFRs within 5 h. Interestingly, apoptotic neutrophils known to encounter LPS-activated macrophages in an inflammatory situation also downregulate their TNFRs during apoptosis (37). These data indicate that apoptotic cells do not trigger an mTNF-α response for immune silencing (38).

TNF-α–targeting molecules selectively trigger TGF-β production in human macrophages

TNF-α–targeting molecules are widely used in the therapy of various inflammatory diseases such as rheumatoid arthritis, psoriatic arthritis, juvenile arthritis, Crohn's colitis, ankylosing spondylitis, and psoriasis. Etanercept is a recombinant human soluble fusion protein of TNFR2 coupled to the Fc portion of IgG (39), infliximab is a chimeric mAb with murine variable regions and human Ig G1 constant regions (40), and golimumab is a hu-

A

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+control											+control
B	BLC	C5/C5a	G-CSF	GM-CSF	I-309	Eotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1								
F	+control											-control

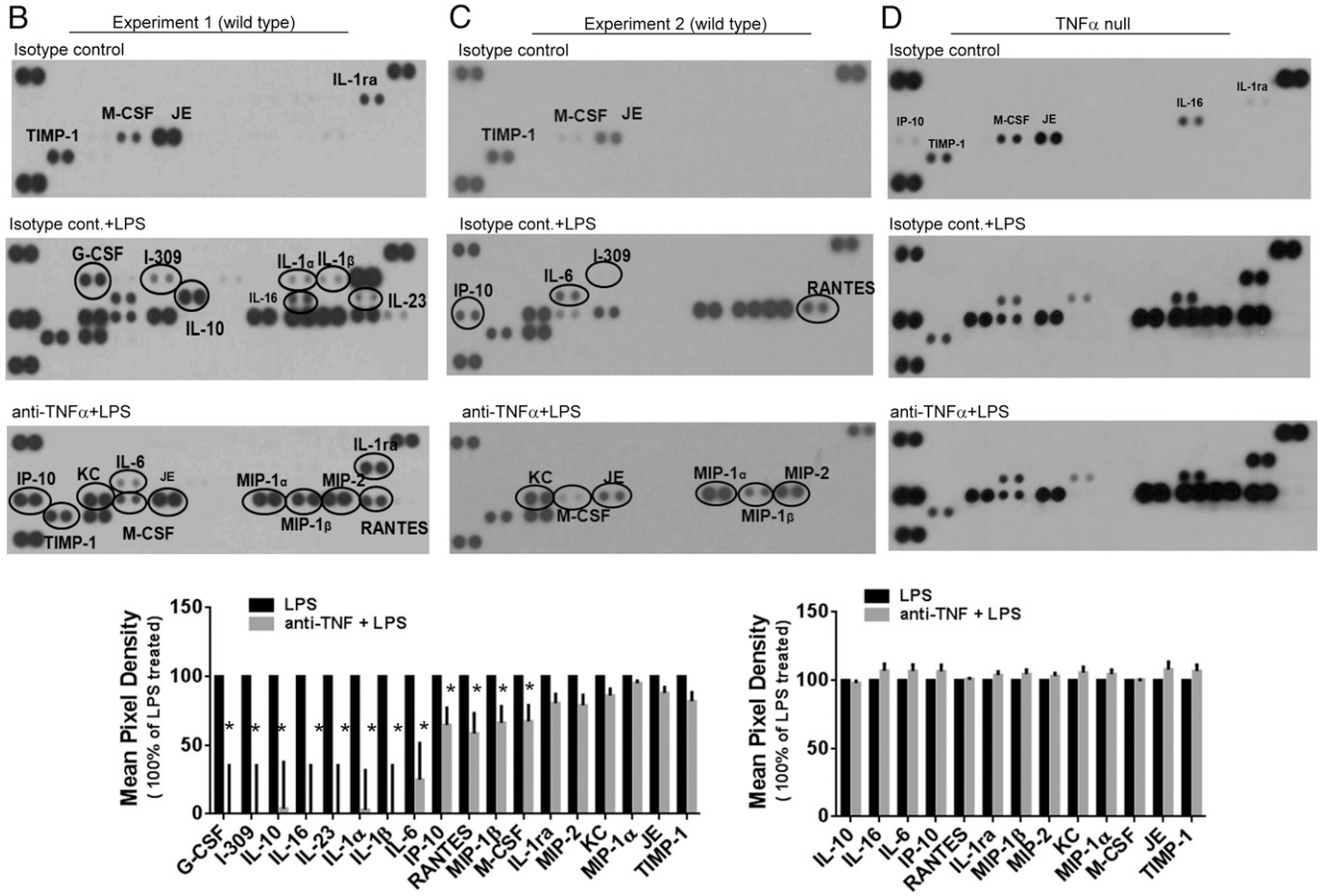

FIGURE 5. Triggering of mTNF-α selectively inhibits LPS-induced proinflammatory cytokine formation in mouse BMDMs. (**A**) Map of the 40 cytokines detected on the membranes. (**B** and **C**) Cytokine panel of isotype control–, isotype control and LPS–, or anti–TNF-α Ab and LPS-exposed wild-type macrophages from two independent experiments. Macrophages were exposed to coated isotype control or coated anti–TNF-α Abs for 2 h and then to LPS (100 ng/ml) for an additional 6 h. Supernatants were collected and cytokine levels were determined by cytokine array. Circles highlight those cytokines on the isotype control Ab/LPS–treated samples for which the expression was fully inhibited by mTNF-α signaling, whereas for cytokines on the anti–TNF-α Ab/LPS–treated samples, the LPS-induced expression was only attenuated by mTNF-α signaling. Alterations in cytokine levels are expressed as percentage of mean pixel density of the respective LPS-treated isotype Ab control (mean ± SD from four independent experiments). (**D**) One representative cytokine panel of isotype control–, isotype control and LPS–, or anti–TNF-α Ab and LPS–exposed TNF-α–null macrophages. Experiments were performed as above. Alterations in cytokine levels are expressed as percentage of mean pixel density of the respective LPS-treated isotype Ab control (mean ± SD from three independent experiments). *$p < 0.05$ versus isotype Ab/LPS–treated control.

man anti-TNFα IgG1κ mAb that can be administered by the patient (41). Although all compounds neutralize TNF-α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis (39–41), golimumab and infliximab are effective (16–19), whereas etanercept failed in the trials of Crohn's disease (13), Wegener's granulomatosis (14), and sarcoidosis (15). Thus we decided to investigate whether these compounds can trigger TGF-β production in human macrophages. Human macrophages generated from buffy coats in three separate experiments were exposed to either etanercept, infliximab, or golimumab all in 50 μg/ml concentration for 4 h, and then the TGF-β levels were determined in the cell culture fluid. In the case of nontreated macrophages, 39 ± 5 pg/ml TGF-β could be detected. Exposure to etanercept (TGF-β, 40 ± 4 pg/ml) did not affect the basal levels of TGF-β, whereas in the cultures of infliximab (78 ± 15 pg/ml) and in those of golimumab (96 ± 12 pg/ml) TGF-β could be detected, the levels of which were significantly higher ($p < 0.05$) than basal levels.

Discussion

Innate responses orchestrate the immediate and early phases of host defense to microbes as well as to injury, initiating the inflammatory reaction and recruiting cells of the acquired immune system to the site of inflammation. Although inflammatory responses against invading microbial pathogens are critical mechanisms for survival, dysregulated inflammatory responses are detrimental to the host. Thus immune systems have evolved multiple strategies to regulate and maintain an adequate level of inflammation, including induction of negative feedback regulators for inflammation, such as TGF-β (20, 42). For example, exposure of macrophages to LPS

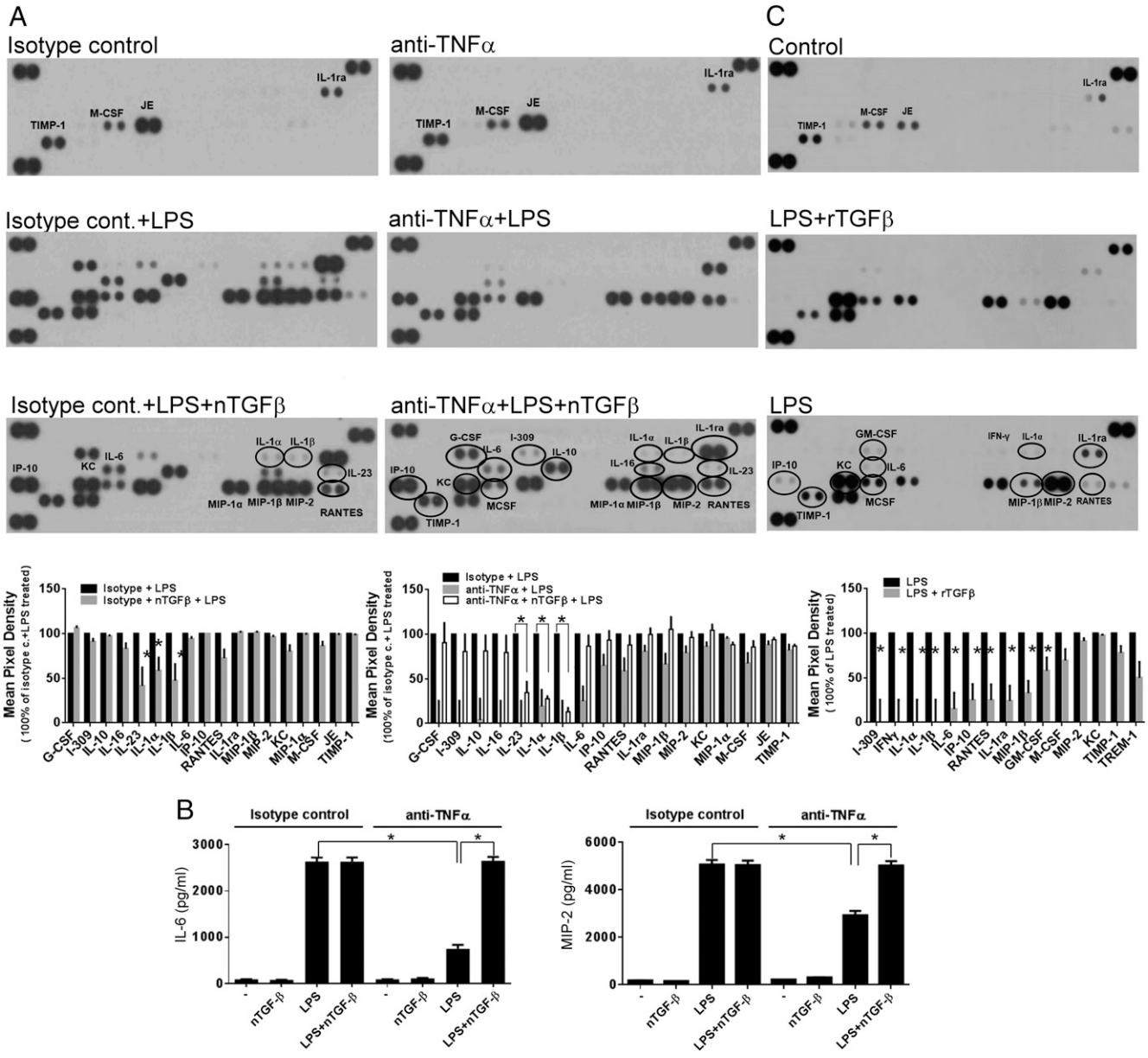

FIGURE 6. mTNF-α signaling interferes with the LPS-induced proinflammatory cytokine production via upregulating TGF-β. (**A**) One representative cytokine panel series of isotype control or anti–TNF-α Ab–exposed macrophages triggered by LPS in the presence and absence of neutralizing anti–TGF-β Abs. Macrophages were exposed to coated isotype control (2 μg/ml) or coated anti–TNF-α Abs (2 μg/ml) for 2 h and then to LPS (100 ng/ml) for an additional 6 h in the presence and absence of neutralizing anti–TGF-β Abs (5 μg/ml). Supernatants were collected, and cytokine levels were determined by cytokine array. Those cytokines are highlighted for which the mTNF-α–induced downregulation was prevented by neutralizing TGF-β. Alterations in cytokine levels are expressed as percentage of mean pixel density of isotype control Ab/LPS–treated macrophages (mean ± SD from three independent experiments). $*p < 0.05$ versus respective control. (**B**) Effect of neutralizing of TGF-β on the mTNF-α–mediated inhibition of LPS-induced IL-6 and MIP-2 production detected at 6 h by ELISA. Macrophages were exposed to either coated isotype control or anti–TNF-α Abs (2 μg/ml) for 2 h before exposing macrophages to LPS (100 ng/ml) in the presence and absence of neutralizing anti–TGF-β Abs (5 μg/ml). The results are mean ± SD from 10 independent experiments. $*p < 0.05$ versus respective LPS-exposed control. (**C**) One representative cytokine panel series of nontreated or LPS-exposed macrophages in the presence and absence of rTGF-β. Macrophages were exposed or not to 1 μg/ml rTGF-β for 2 h before addition of LPS (100 ng/ml) for an additional 6 h. Supernatants were collected, and cytokine levels were determined by cytokine array. Those cytokines are highlighted for which the LPS-induced expression is downregulated by rTGF-β. Alterations in cytokine levels are expressed as percentage of mean pixel density of LPS-treated macrophages (mean ± SD from three independent experiments). $*p < 0.05$ versus LPS-treated control.

induces the production of TGF-β with delayed kinetics as compared with that of the proinflammatory cytokines, and the TGF-β produced provides a protective mechanism against a second high dose of LPS challenge (42). Previous studies have demonstrated that mTNF-α signaling activated on the surface of macrophages also interferes with the LPS signaling and provides a protective mechanism against a high dose of LPS challenge (9–11).

In the present study, we investigated the mechanism of this anti-inflammatory signaling. We found that TNF-α is expressed, although at low levels, in unstimulated macrophages and some of it appears also on the cell surface as mTNF-α. Exposure to LPS increased both the synthesis of TNF-α and its cell surface expression, but this increase was only transiently due to the activation of the metalloprotease, which is responsible for its cleavage. Already the basal cell surface levels of mTNF-α allowed an ef-

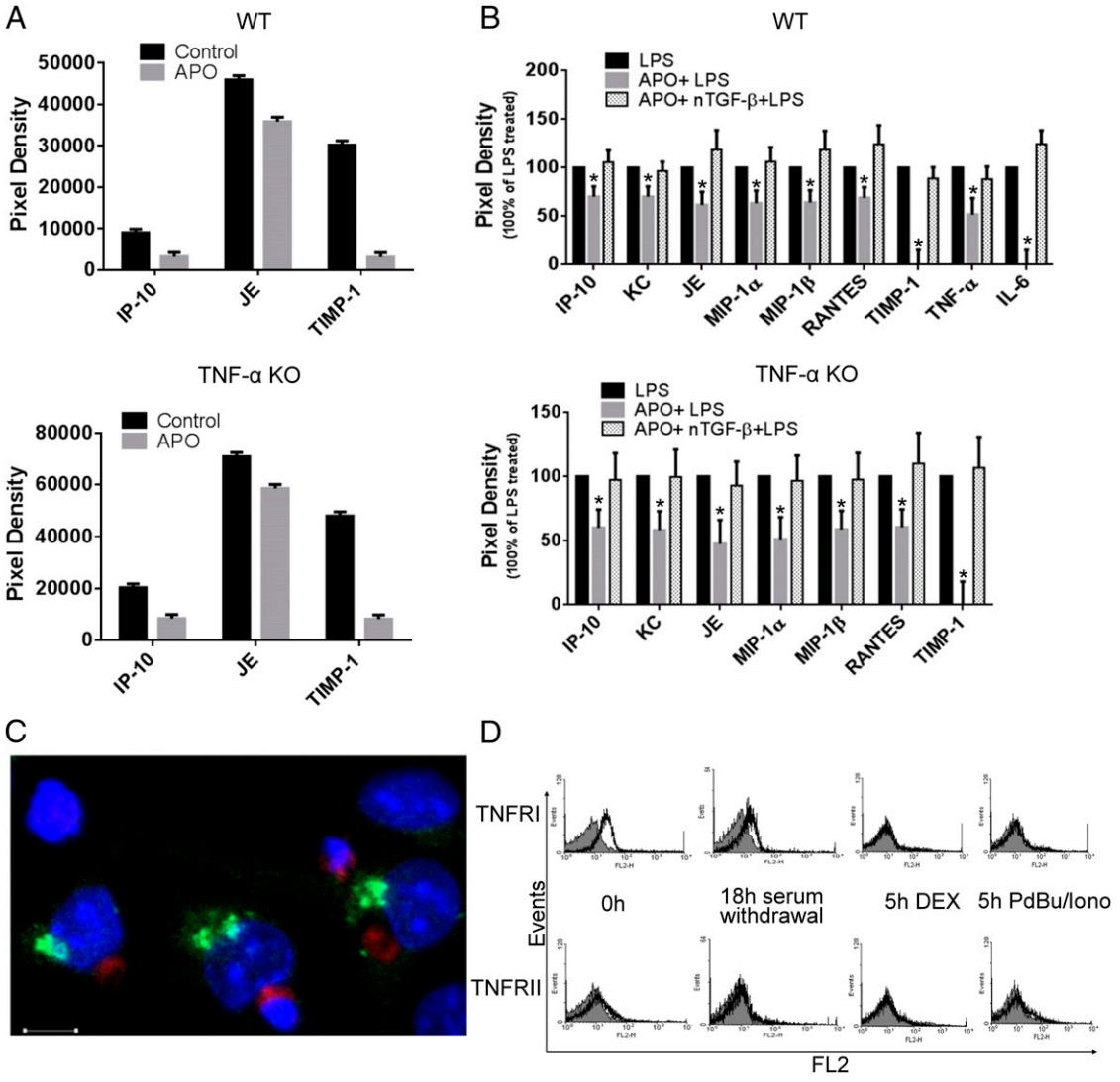

FIGURE 7. Apoptotic thymocytes do not use the mTNF-α signaling pathway to downregulate LPS-induced proinflammatory cytokine formation of macrophages. (**A**) Representative cytokine panel of control- and apoptotic cell–exposed wild-type or TNF-α null macrophages. (**B**) Representative cytokine panel of LPS-, LPS and apoptotic cell–, or LPS, apoptotic cell, and neutralizing TGF-β Ab–exposed wild-type or TNF-α–null macrophages. Macrophages either exposed or not to apoptotic thymocytes (10:1 apoptotic cell/macrophage ratio) for 2 h were washed and were or were not exposed to LPS (100 ng/ml) in the presence or absence of neutralizing TGF-β Abs (5 μg/ml). Supernatants were collected 24 h later, and cytokine levels were determined by cytokine array. The pixel density results are mean ± SD from three independent experiments. Alterations in cytokine levels are expressed as percentage of mean pixel density of LPS-treated macrophages (mean ± SD from three independent experiments). $^*p < 0.05$ versus respective LPS-treated control. (**C**) Distribution of TNF-α (green) protein in LPS-treated (100 ng/ml for 2 h) wild-type macrophages engulfing apoptotic cells (red) detected by confocal microscopy. Nuclei appear in blue color following with DAPI staining. Scale bar, 10 μm. (**D**) Apoptotic thymocytes downregulate their cell surface TNFR I and II. Thymocytes either dying following serum withdrawal for 18 h or induced to die by 0.1 μM dexamethasone or by 5 ng/ml phorbol dibutyrate/1 μg/ml ionomycin for 5 h were analyzed for their TNFR expression by FACS analysis.

ficient mTNF-α signaling, and we found that the signaling pathway led to the production of TGF-β. Induction of TGF-β production, however, is not a universal response of cells to mTNF-α signaling, because monocytes were shown to produce TNF-α and not TGF-β when their mTNF-α was triggered by the TNFRs of activated T cells (43). We have also identified the signaling pathway that regulates mTNF-α–induced TGF-β production in macrophages. This pathway involves the MKK4-initiated MAPK pathway, which leads the activation of both p38 MAPKs and JNKs. Whereas the Jun kinase pathway regulates the production of TGF-β1, the p38 MAPKs are responsible for the production of the rest of TGF-βs. In contrast, the Akt pathway also activated by mTNF-α seems to act as a negative autoregulatory loop in the control of mTNF-α–induced TGF-β production. Although previous studies have suggested that mTNF-α triggers the ERK kinase pathway (10, 11), our data indicate that activation of these kinases are only secondary and a consequence of TGF-β signaling. We have also demonstrated that mTNF-α signaling alone induces only a transient MKK4 and p38 MAPK activation when TGF-β is neutralized; however, the appearance of TGF-β sustains MKK4 and the consequent p38 MAPK signaling. We have confirmed that mTNF-α signaling indeed inhibits the LPS-induced proinflammatory cytokine formation, but we found that different subsets of cytokines show different susceptibility for this inhibition. We have also demonstrated that mTNF-α signaling interferes with the LPS signaling via the autocrine production of TGF-β. Because previous studies have shown that different elements of the LPS-induced signaling pathway are involved in the induction of individual cytokines (44), our data indicate that TGF-β signaling might selectively interfere with the various LPS-induced signaling pathways. Our data do confirm previous findings, in which it was demonstrated that in the presence of TGF-β the balance between the LPS-induced ERK and p38 MAPK activities is dysregulated

owing to simultaneous TGF-β–stimulated ERK activation. As a result, NF-κB–driven transcription was shown to be attenuated, whereas AP-1–driven transcription was shown to be enhanced (45).

We have also tested whether three TNF-α–targeting molecules introduced already in the therapy of various inflammatory diseases could also trigger mTNF-α–driven TGF-β production in human macrophages. We found that etanercept is not able to trigger such mTNF-α–driven TGF-β production, whereas infliximab and golimumab are able to do so, indicating that infliximab and golimumab trigger mTNF-α signaling. In support of our observation, infliximab in patients was reported to induce the transient phosphorylation of p38 MAPKs and Hsp70, elements of the mTNF-α signaling pathway (Fig. 4) (46).

Previous studies have suggested that the therapeutic efficacy of TNF-α–targeting molecules might be related not only to their capability of neutralizing TNF-α, but it might also be affected by their biological nature or whether they could also trigger mTNF-α signaling (47, 48). Indeed, although all three compounds neutralize TNF-α with the same efficiency and are similarly effective in the treatment of rheumatoid arthritis (39–41), etanercept, which in our assay failed to upregulate TGF-β, also failed in the trials of Crohn's disease (13), Wegener's granulomatosis (14), and sarcoidosis (15). Because in other biological assays etanercept responded similarly to infliximab and golimumab (47), our data indicate that its failure in the treatment of these diseases might be related to the lack of TGF-β induction. Interestingly, Crohn's disease, Wegener's granulomatosis, and sarcoidosis are all characterized by altered TGF-β signaling (49–51). Although in Crohn's disease TGF-β signaling was reported to be impaired due to elevated levels of TNF-α and IFN-γ, which interfere with the TGF-β signaling (49), administration of a diet rich in TGF-β2 was shown to be beneficial in its long-term maintenance (52). Thus, simultaneous neutralization of the proinflammatory and anti–TGF-β signaling effects of TNF-α and promotion of TGF-β signaling by elevating its levels might all contribute to the therapeutic effects of those anti–TNF-α targeting molecules, which also trigger TGF-β production.

Every day billions of cells die by apoptosis as part of the normal tissue homeostasis. Anti-inflammatory effects of apoptotic cells result in silent removal of dying cells as well as strongly contribute to the resolution of inflammation (53). We have investigated whether apoptotic cells known to interfere with the LPS signaling of macrophages also by upregulating TGF-β (12) do use mTNF-α signaling to act in this manner. Our data indicate that apoptotic cells cannot rigger the mTNF-α signaling pathway in macrophages, because they downregulate their TNFRs. As a result, none of the TNF-α–targeting molecules is expected to interfere with the anti-inflammatory effects of apoptotic cells. Collectively, our data indicate that induction of TGF-β, a strong anti-inflammatory cytokine (20), might be an essential contributing factor in determining the therapeutic efficacy of TNF-α–targeting molecules in chronic inflammatory diseases, such as Crohn's disease. Because in Crohn's disease patients, who do not respond to infliximab therapy, phosphorylation of both ATF-2 and Hsp70 was found to be impaired (52), it is very likely that in these patients the mTNF-α–signaling pathway is suppressed. In such patients as an additional therapy to the TNF-α–targeting molecules, triggering of receptors used by apoptotic cells to induce TGF-β production, such as stabilin-2 (54), could be considered.

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Disclosures

The authors have no financial conflicts of interest.

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