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Transglutaminase Type II Is a Key Element in the Regulation of the Anti-Inflammatory Response Elicited by Apoptotic Cell Engulfment[1]

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A key feature of the macrophage-dependent clearance of apoptotic cells is the down-regulation of proinflammatory cytokines. Deficiency in the phagocytosis of apoptotic cells is often associated with the development of inflammatory reactions, resulting in chronic inflammatory and autoimmune diseases. The molecular mechanisms that regulate the engulfment process and particularly the immunomodulatory factors involved are still largely unknown in mammals. We have previously reported that the ablation of transglutaminase type II (TG2) in mice results in the defective clearance of apoptotic cells associated with the development of splenomegaly, autoantibodies, and glomerulonephritis. In this study we have investigated the mechanisms at the basis of the development of inflammation/autoimmunity associated with the defective clearance of apoptotic cells characterizing TG2 knockout mice. To this aim we compared the macrophage response to apoptotic cell exposure in wild-type vs TG2-null mice. We demonstrated that the lack of TG2 results in an impaired capacity of macrophages to engulf, but not to bind, apoptotic cells, which is paralleled by an abnormal inflammatory response both in vivo and in vitro. We have identified a differential response in the release of several cytokines in TG2$^{-/-}$ vs wild-type mice. Particularly relevant is the finding that both TGF-β and IL-12 regulations were significantly altered in the absence of TG2. These results help explain the autoimmune phenotype developed by these mice and suggest that TG2 is a key regulatory element of the anti-inflammatory features of apoptosis. ***The Journal of Immunology,*** **2005, 174: 7330–7340.**

Phagocytosis is a phylogenetically ancient process that plays a key role in essential basic functions such as macromolecular uptake, host defense, and degradation of senescent cells (1, 2). Monocyte/macrophages are professional phagocytes that have evolved a specialized and complex machinery that provides for highly efficient ingestion of pathogens and initiation of the immune response (3, 4). Macrophages also play an important role in the recognition and clearance of apoptotic cells. An essential feature of this physiological process is the absence of an inflammatory response (5). Macrophages perform the functions of recognition, binding, and internalization of apoptotic cells without undergoing activation, as is the case in the clearance of infectious organisms (6). Activation consists of morphological and behavior changes in macrophages that result in the secretion of preformed and/or newly synthesized constituents, such as cytokines and chemokines. These, in turn, switch on the inflammatory response (7). In contrast, macrophages that ingest apoptotic cells fail to secrete chemoattractants such as IL-8 and MCP-1 (8, 9). Instead, these cells release TGF-β1, platelet-activating factor, and PGE_2, factors that dampen the inflammatory response (6, 10).

The efficient clearance of apoptotic cells is also necessary to prevent an immune response that may be triggered by self-Ags, which are normally not exposed on living cells (11, 12). It is widely accepted that a persistent state of inflammation in various organs is a consequence of the defective clearance of apoptotic cells (13, 14) and contributes to the development of autoimmune disorders (15–18).

The process of apoptotic cell removal includes a complex molecular machinery on both dying cells and phagocytes (reviewed in Refs. 19 and 20) and is based on two steps: recognition/binding (tethering) and engulfment (tickling) (21). In the engulfment pathway, several different mammalian proteins have been identified (CrkII, Dock180, Rac, and Elmo) (22, 23) that appear to be involved in the internalization process by promoting cytoskeletal rearrangement. However, the exact cascade of events as well as many of the intermediate factors involved are still unknown.

We recently reported that the ablation of transglutaminase type II (TG2)[3] in mice results in defective clearance of apoptotic cells associated with the development of splenomegaly, autoantibodies, and glomerulonephritis (24). TG2 is a multifunctional enzyme that belongs to a large family of calcium-dependent transamidating acyltransferase. TG2 catalyzes posttranslational modifications of proteins at the level of glutamine and lysine residues and is involved in a variety of biological functions, including cell death,

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[3] Abbreviations used in this paper: TG2, transglutaminase type II; sTNF-RI, soluble TNFR type I; WT, wild type.

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extracellular matrix stabilization, and signaling (25). The activation of TG2 during apoptosis leads to the assembly of detergent-insoluble polymeric protein structures (25). In addition, TG2 interaction with major cytoskeletal components results in cytoskeleton rearrangements (26, 27), although as a G protein it can also have a role in cell signaling (28).

To gain further insight into the reported role of TG2 in apoptosis-dependent phagocytosis and the prevention of inflammation/autoimmunity, we performed an in vitro and in vivo study on the macrophage response after exposure to apoptotic cells. Using highly sensitive cytokine protein arrays, we examined the production of macrophage cytokine under unstimulated conditions. Our results demonstrate that phagocytosis of apoptotic cells is followed by rapid decrease in proinflammatory cytokine release, a phenomenon that is impaired in the absence of TG2.

Materials and Methods

Animals

Wild-type (WT) and TG2 knockout ($TG2^{-/-}$) C57/BL6 mice (29) were used. Male mice were used for all experiments. Animals were 4–6 mo of age. All care and procedures were performed according to approved protocols and in accordance with institutional guidelines.

Experimental protocol

The effect of the absence of TG2 on the clearance of apoptotic cells was investigated in in vivo and in vitro experiments. For in vivo experiments, we induced apoptosis in the liver by injection of a single dose of lead nitrate ($PbNO_3$). Equal numbers ($n = 12$) of WT and $TG2^{-/-}$ mice were treated with $PbNO_3$; animals injected with vehicle only served as controls. Separate groups of WT and $TG2^{-/-}$ animals were used as the source of peritoneal macrophages for in vitro studies.

In vivo studies

Apoptosis was induced in vivo in the livers of WT and $TG2^{-/-}$ mice by a single i.v. injection of $PbNO_3$ (10 μmol/100 g body weight; CarloErba) dissolved in physiological saline. The injection of $PbNO_3$ produces a proliferative response and a doubling of liver weight in 3 days. This is followed by an involution phase characterized by a massive induction of apoptotic cell death and active clearance of the apoptotic cells, reaching a maximum on day 5 after treatment (30). Restoration of pretreatment liver weight occurs after 2–3 days.

Animals were killed at different times after $PbNO_3$ injection, and liver samples were collected. For histological examination, small fragments of liver tissue were formalin-fixed, embedded in paraffin, and stained with H&E. For the cytokine analysis, blood sera were collected and stored at −70°C.

Isolation of cells, apoptosis induction, and in vitro phagocytosis assays

Peritoneal macrophages were isolated from WT and $TG2^{-/-}$ mice. Macrophages were obtained by peritoneal lavage with PBS and enriched by adherence selection for 1 h at 37°C. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 for 48 h before use. Macrophages were plated in 2-well chamber slides (5×10^5/well) for light microscopic analysis, in 24-well plates containing coverslips (1×10^6/well) for scanning electron microscopy, and in 6-well plates (3×10^6/well) for cytokine assays. To study apoptotic cell uptake, human apoptotic lymphocytes were used. PBLs were isolated by differential centrifugation using Ficoll-Paque, resuspended, and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. After 1 h, nonadherent cells were collected; the majority consisted of lymphocytes, as determined by morphological examination of cytospin preparation. Most monocytes adhered to the culture flasks and remained behind. These lymphocytes were then cultured for 24 h before use.

Apoptotic cell death was induced either by incubation with cycloheximide (10^{-2} M, 12 h) or by exposure to UV irradiation at 254 nm for 10 min, followed by 3 h of culture. The percentage of apoptotic cells was quantified by flow cytometric analysis using annexin V and propidium iodide staining. Under the conditions used, cells were <5% propidium iodide positive and were 50–80% annexin V positive. Experiments were performed using UV- and cycloheximide-treated lymphocytes independently.

For the in vitro phagocyte interaction assays, apoptotic lymphocytes were washed twice, resuspended in fresh medium, and then added to the macrophage monolayer (3/1). The cells were allowed to interact at 4°C (binding) or at 37°C (engulfment) for different intervals of time. Phagocytosis of apoptotic cells was determined under a light microscope by counting 400 macrophages and was expressed as the percentage of macrophages containing apoptotic bodies. For cytokine analysis, cell culture supernatants were centrifuged at 2000 rpm to remove particulate and debris, then stored at −70°C.

Scanning electron microscopy

Macrophages were fixed with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer at 4°C for 1 h. After washing in Millonig's phosphate buffer, cells were dehydrated in increasing acetone concentrations and then critical point-dried using liquid CO_2. Samples were sputter-coated with gold. Scanning electron microscopy was conducted using the Stereoscan 240 scanning electron microscope (Cambridge Instruments).

Measurement of cytokine release using a protein array system

Cytokines released by macrophages were measured in the culture medium or in mouse sera using a Mouse Cytokine Array I Kit (Panomics) consisting of 22 different cytokine and chemokine Abs spotted in duplicate onto a membrane.

The membranes were processed according to the instructions of the manufacturer. Briefly, membranes were incubated with 4 ml of 1× blocking buffer at room temperature for 1 h, washed twice with 1× wash buffer II, and incubated with 2 ml of sample (mouse serum or macrophage culture medium) for 2 h at room temperature. After decanting the samples, membranes were washed three times with 4 ml of 1× wash buffer I (5 min/wash), followed by two washes with 1× wash buffer II (5 min/wash). The membranes were then incubated with 1.5 ml of diluted biotin-conjugated Abs at room temperature for 1 h, and washing steps were repeated as described above. Membranes were incubated for 1 h at room temperature with a dilution of streptavidin-conjugated peroxidase. After a thorough wash, the membranes were exposed to the mixed detection buffers (Panomics), according to the instructions of the manufacturer, for 5 min in the dark before imaging. Spots were visualized using ECL (Amersham Biosciences). Membranes exposed to Kodak X-OMAT radiographic film were then processed. Each film was scanned, and spot densities were measured with Scion Image for Windows (National Institutes of Health). The densities were exported into Excel, and the background intensity was subtracted before analysis.

Measurement of TGF-β expression

TGF-β expression was evaluated by Western blot, immunohistochemical analysis, and ELISA.

Western blot analysis

Western blot analyses of TGF-β was performed on liver samples from WT and $TG2^{-/-}$ mice, both untreated and 3 or 5 days after $PBNO_3$ injection. Frozen tissue fragments were homogenized with lysis buffer (50 mM Tris-HCl, pH 8, containing 120 mM NaCl, 0.2 mM EDTA, 1% Nonidet, and protease inhibitor mixture). Aliquots of total protein extracts were run on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane overnight at 4°C in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Membranes were then incubated for 1 h at ambient temperature with rat anti-mouse mAb specific for TGF-β1 (BD Pharmingen) and with the appropriate secondary HRP-conjugated rabbit anti-rat Ab (Sigma-Aldrich). Detection was achieved using a preformed streptavidin-HRP complex (Amersham Biosciences), and the signal was developed using ECL detection system (Amersham Biosciences).

Immunohistochemical analysis

For immunohistochemistry, formalin-fixed, paraffin-embedded, thin liver sections from WT and $TG2^{-/-}$ mice (untreated or 3 or 5 days after $PBNO_3$ injection) were used. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 5 min. After rinsing in PBS, sections were incubated with normal goat serum for 5 min. Rat anti-mouse TGF-β mAb (BD Pharmingen) was applied at 1/10 dilution in PBS for 1 h at room temperature. A peroxidase-conjugated rabbit anti-rat IgG (Sigma-Aldrich) was used as secondary Ab. The immunoreaction product was revealed using aminoethylcarbazole (Biogenex) as chromogen substrate and 0.01% H_2O_2. Sections were counterstained in Mayer's acid hemalum.

ELISAs

The TGF-β concentrations in mouse sera and in the macrophage culture supernatants were determined by ELISA, using Quantikine immunoassays (R&D Systems). Samples were acid treated (to activate latent TGF-β) and neutralized before ELISA, according to the manufacturer's instructions.

Statistical analysis

Values were expressed as the mean ± SD. Analysis was performed by two-tailed Student's *t* test for comparisons between two groups. Statistical significance was set at $p < 0.05$.

Results

In vivo and in vitro effects of TG2 ablation on phagocytosis of apoptotic cells

We recently reported that the ablation of TG2 in mice is associated with the defective clearance of apoptotic cells (24). Light microscopic observations of the liver after apoptosis induction by lead nitrate treatment revealed that the deficiency in apoptotic cell clearance observed in the $TG2^{-/-}$ mice (Fig. 1*c*) was paralleled by an increased inflammatory response, as evidenced by the presence of large infiltrates of blood cells in the parenchimal tissue (Fig. 1*d*). This phenomenon was completely absent in control mice (Fig. 1, *a* and *b*).

To investigate the role of TG2 in the phagocytosis of apoptotic cells, in a first set of experiments we performed in vitro phagocytosis assays. Cultured peritoneal macrophages from WT mice exposed to apoptotic lymphocytes displayed a very efficient ability to recognize and phagocyte apoptotic cells, and we observed that the vast majority of macrophages had internalized two or more apoptotic cells after 20 min of incubation (Fig. 2*A*, *a* and *b*). When macrophages isolated from $TG2^{-/-}$ mice were cocultured with apoptotic lymphocytes, phagocytosis was impaired. We observed a marked reduction in their general ability to ingest apoptotic cells as well as in the average number of apoptotic cells ingested (Fig. 2*A*, *c* and *d*). The percentages of macrophages that contained apoptotic lymphocytes after 30 min of exposure in WT and $TG2^{-/-}$ mice were 80.2 ± 7.5 and 49.5 ± 3.6%, respectively. It is important to note that this deficiency appeared to be dependent on macrophages; in fact, no difference was observed in phagocytic efficiency when lymphocytes from $TG2^{-/-}$ mice were used (not shown). To discriminate between recognition (binding) and engulfment capacities, we performed an interaction assay, incubating apoptotic lymphocytes with macrophages at 4°C for 30 min. Under these conditions, binding without internalization occurred, as indicated by the peripheral association of apoptotic cells with macrophages. Macrophages from WT and $TG2^{-/-}$ mice exhibited similar binding capacities (one macrophage can bind one or more apoptotic cells; Fig. 2*Ba*), demonstrating that the lack of TG2 does not affect the ability to recognize and bind apoptotic cells, but only the capacity to internalize them.

To investigate whether the different phagocytic capacity observed in $TG2^{-/-}$ macrophages was related to a reduced rate of interaction with apoptotic cells, we conducted phagocytosis analysis at various time intervals. As reported in Fig. 2*Bb*, our results showed that the percentage of $TG2^{-/-}$ macrophages containing apoptotic cells was significantly lower and never reached WT levels. We also performed a time-course analysis of phagocytosis monitored by scanning electron microscopy. Cultured macrophages from WT mice were characterized by membrane ruffling with microvilli of variable lengths over the entire cell surface (Fig. 3). When apoptotic lymphocytes were added to the culture medium, they were rapidly bound by phagocytes, which immediately started to surround them (Fig. 3*a*). Internalization of apoptotic cells was completed in 15–20 min, demonstrating the ability of macrophages to clear apoptotic cells (Fig. 3*b*). At this stage, the cells showed globular protrusions, indicating the presence of engulfed corpses, whereas macrophages displayed a more flattened phenotype at longer time intervals (1 h after exposition of apoptotic cells), suggesting that the phagocytic process was completed (Fig. 3*c*). In contrast, macrophages from $TG2^{-/-}$ mice showed completely different behavior. In fact, apoptotic lymphocytes were noted to adhere to the surface of macrophages after a few minutes of coincubation (Fig. 3*d*); however, this first step, i.e., the tether mechanism, was not properly followed by the engulfment phase in the majority of macrophages, even after longer intervals (Fig. 3, *f* and *g*). Interestingly, increasing the exposure time (1–2 h; during which time, numerous apoptotic cells were still attached to macrophage surface), the shape of the macrophages changed to an activated phenotype, consisting of a round shape and extensive membrane ruffling (Fig. 3*g*).

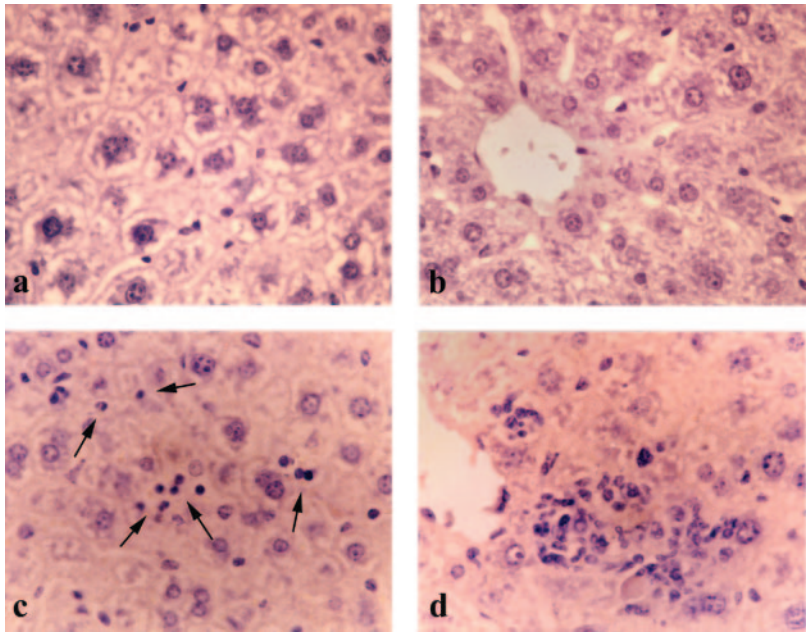

FIGURE 1. Light microscopy of WT (*a* and *b*) and $TG2^{-/-}$ (*c* and *d*) livers 5 days after $PbNO_3$ treatment. H&E-stained sections of $TG2^{-/-}$ hepatic parenchyma demonstrated the presence of numerous apoptotic cells (arrows; *c*) and massive inflammatory infiltrate (*d*), which were not present in WT liver (*a* and *b*). Original magnification, ×63.

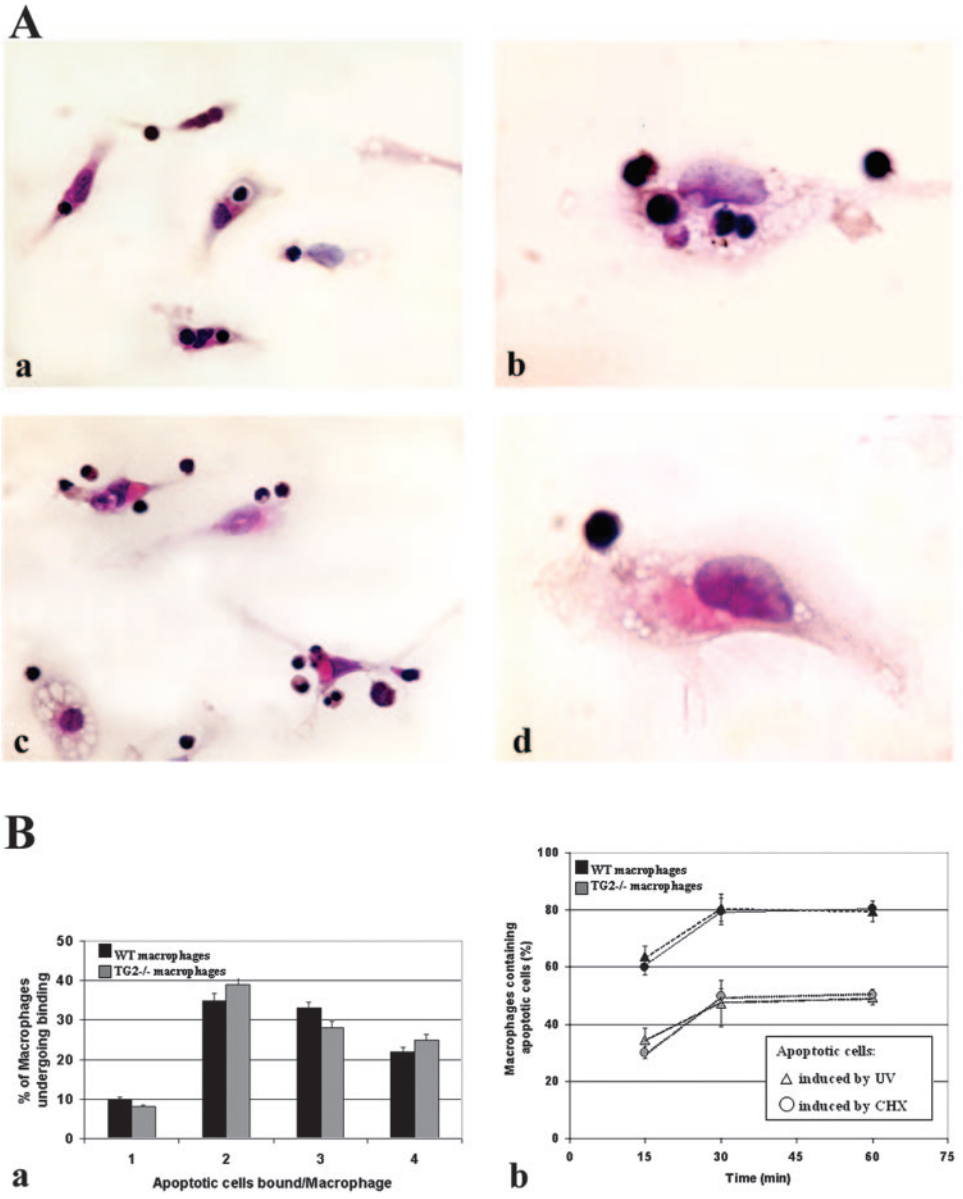

FIGURE 2. Binding and engulfment of apoptotic lymphocytes by macrophages isolated from WT and TG2$^{-/-}$ mice. *A*, Light micrographs of WT (*a* and *b*) and TG2$^{-/-}$ (*c* and *d*) macrophages incubated with apoptotic lymphocytes for 20 min. A phagocytosis assay was conducted in chamber slides, and cells were counterstained with H&E. *a*, WT macrophages that have phagocytosed apoptotic cells (condensed dark bodies) are visible; *b*, higher magnification shows that many apoptotic bodies are internalized by the same macrophage; *c*, TG2$^{-/-}$ macrophages display impairment of engulfment, but apoptotic cells were visible adhering to macrophage cell surface; *d*, higher magnification clearly shows the capacity of binding of TG2$^{-/-}$ macrophages. Original magnifications: *a*, ×40; *b*, ×100. *B*, Binding and engulfment capacity of macrophages were specifically investigated. The data represent the mean ± SEM of three independent experiments. *a*, The binding assay, performed by incubating macrophages for 30 min at 4°C, shows that the ability to bind apoptotic cells was not impaired in TG2$^{-/-}$ macrophages. *b*, Time course of phagocytosis, performed by exposing macrophages to apoptotic lymphocytes at 37°C for different periods of time. Apoptotic death in target cells was induced by both UV irradiation and cycloheximide (CHX) treatment. WT and TG2$^{-/-}$ macrophages display the same kinetic of phagocytosis, but the extent of engulfment was significantly lower ($p < 0.005$) in TG2$^{-/-}$ macrophages compared with that WT cells.

Cytokine analysis

Evaluation of the cytokine expression profile in macrophage culture media or mouse sera was performed using a highly sensitive cytokine Ab array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay (picogram per milliliter range). The map of the 22 cytokines spotted on the membranes is reported in Fig. 4*A*. The detectable cytokines in our experimental systems were first evaluated by experiments using untreated WT and TG2$^{-/-}$ mice, both in vitro (on macrophage culture medium) and in vivo (on blood serum). The results reported in Fig. 4*B* show that ~50% of all available cytokines on the filters were detectable, even though some were at a very low level (Fig. 4*B*).

Macrophage cytokine response associated with apoptotic cell phagocytosis

Among the cytokines released in the supernatant by cultured macrophages (Fig. 5), four proinflammatory cytokines and chemokines showed major changes in response to apoptotic cell exposure: IL-12p70, RANTES, MCP-1, and MCP-5 (Fig. 5*B*). The modification in the levels of these cytokines was not due to the FBS present in the culture medium, because incubation with medium alone did not produce any detectable signal; in addition, no cytokines were detected in the supernatants when lymphocytes (viable or apoptotic) were incubated alone (data not shown). Thus, the cytokine expression profile reported in Fig. 5*A* reflects the macrophage production. Interestingly, the secretion of all cytokines considered was strongly inhibited 20 min after exposure of WT macrophages to apoptotic cells (Fig. 5).

Of note, the unstimulated macrophages obtained from TG2$^{-/-}$ mice produce similar amounts of cytokines as those detected in WT macrophages (Fig. 5*A*), but upon exposure to apoptotic cells, we found a completely different response (Fig. 5*B*). In fact, the above-described cytokines were still released in considerable amounts after exposure of the macrophages to apoptotic lymphocytes (3-fold higher compared with WT). In addition, the expression of IL-6 was detected only when TG2$^{-/-}$ macrophages were exposed to apoptotic cells (Fig. 5*A*).

In vivo cytokine response to apoptosis induction

To evaluate whether the impaired clearance of apoptotic cells observed in the livers of TG2$^{-/-}$ mice was reflected by cytokine

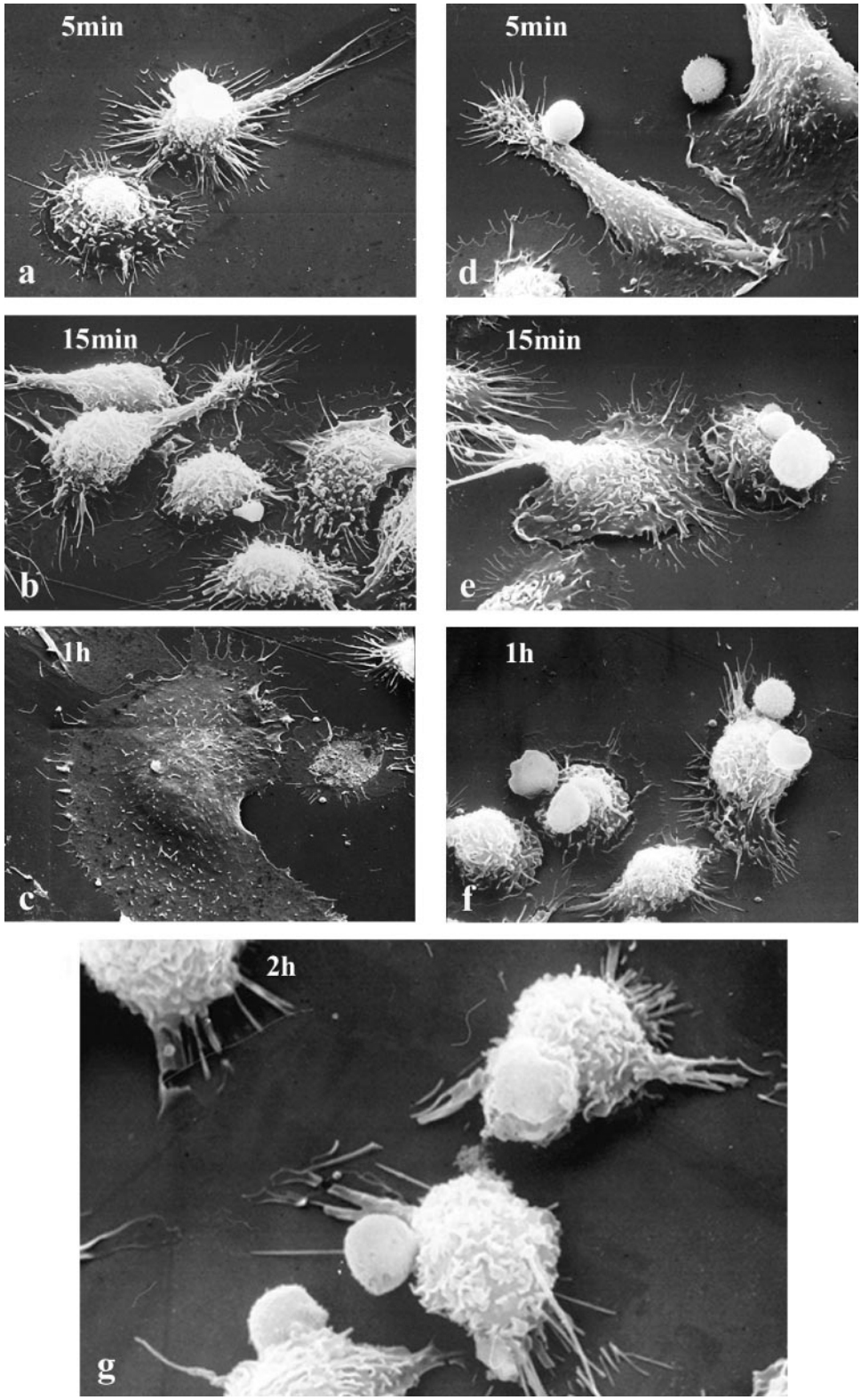

FIGURE 3. Scanning electron micrographs of peritoneal macrophages from WT (*a–c*) and TG2$^{-/-}$ (*d–g*) mice, cocultured with apoptotic lymphocytes for different interval times. WT macrophages in close association, and starting to engulf, apoptotic cells are visible after 5 min of incubation (*a*), and the binding is followed by engulfment within a few minutes (*b*); macrophages after 1 h of coincubation display a flattened morphology, suggesting that the disposal of apoptotic cells has been completed (*c*). TG2$^{-/-}$ macrophages rapidly bind apoptotic lymphocytes (*d*), but this phase is not followed by internalization, and apoptotic lymphocytes remain bound to macrophages for up 2 h (*e–g*); in this condition, TG2$^{-/-}$ macrophages acquire an activated phenotype, changing from an elongated to a round shape, and are less adherent to the culture plate, as is clearly visible at higher magnification (*g*). Original magnifications: *a–f*, ×2300; *g*, ×4000.

production, we analyzed the sera obtained from control and lead nitrate-injected mice (Fig. 6) by the cytokine Ab arrays method. Of note, the cytokine profile in mice sera (Fig. 6*A*) was comparable to that observed with isolated macrophages (compare with the profile in Fig. 5*A*). However, in addition to IL-12p70, RANTES, MCP-1, and MCP-5, two other cytokines, IL-12 and soluble TNFR type I (sTNF-RI), showed significant differences (Fig. 6). For each cytokine considered, similar values were found in WT and TG2-null control mice; once again, as observed in isolated macrophages, opposite trends were detected in response to apoptotic cell exposure (Fig. 6*A*). In fact, although a general reduction in the production of inflammatory cytokines (in particular, the chemokines MCP-1 and MCP-5 completely disappeared) was detected in WT serum 5 days after $PbNO_3$ injection (when there was the maximal

A

Cytokine position on membrane protein array.

	a	b	c	d	e	f	g	h
1	*Pos*	*Pos*	*Neg*	*Neg*	GCSF	GM-CSF	IL-2	IL-3
2	*Pos*	*Pos*	*Neg*	*Neg*	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
7	TPO	VEGF						*Pos*
8	TPO	VEGF						*Pos*

B

Comparative cytokine expression pattern under steady-state conditions in WT (black columns) vs TG2-/- mice (gray columns).

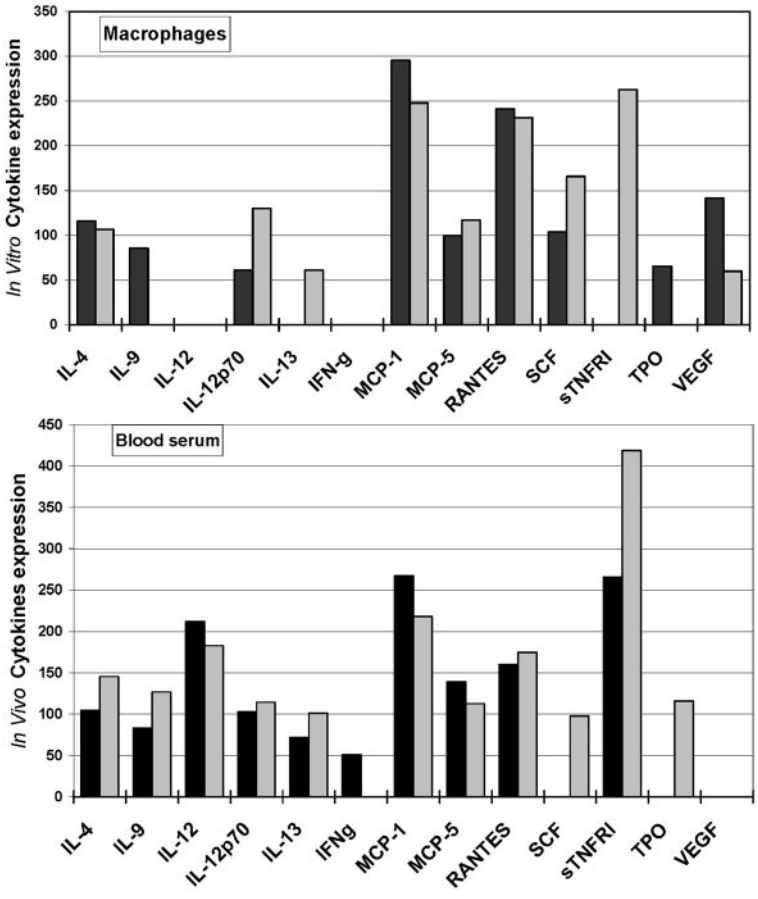

FIGURE 4. The cytokine level was measured in macrophage culture medium or in mouse sera using a cytokine protein array consisting of different cytokine and chemokine Abs spotted in duplicate onto a membrane. *A*, Map of the location of cytokine Abs spotted onto the cytokine array. A total of 22 cytokines were present. Pos, positive controls; Neg, negative controls; GCSF. SCF, Stem cell factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor. *B*, The steady-state basal expression of cytokines in WT (■) and TG2$^{-/-}$ (▨) mice was analyzed in vitro, in macrophage culture medium, and in vivo, in blood serum, using control untreated conditions. The detectable cytokines in our experimental systems were about one-half of all cytokines available in the array. Eleven and 12 different cytokines were detected, respectively. Values represent spot densities as measured by Scion Image. The data shown are representative of four independent experiments.

phagocytic activity of apoptotic cells) (24), the phenomenon was completely absent in TG2$^{-/-}$ mice. Interestingly, the proinflammatory cytokine IL-12 and sTNF-RI levels were significantly higher even when compared with those in untreated control animals (Fig. 6*B*).

Effect of TG2 on TGF-β production

It has been proposed that the anti-inflammatory immunosuppressive mechanism induced during macrophage ingestion of apoptotic cells leading to the production of anti-inflammatory cytokines is regulated by TGF-β. In view of the fact that TG2 has been shown to participate in the maturation of TGF-β (31), we have analyzed the expression of this cytokine in the livers of WT vs TG2$^{-/-}$ mice after lead nitrate treatment. Western blot analysis, performed on control and $PbNO_3$-treated animals (Fig. 7*A*), showed minimal expression in WT liver under normal conditions. An increased amount of TGF-β was observed after 3 days of treatment, which further increased on day 5. Interestingly, TG2$^{-/-}$ mice showed a higher steady-state TGF-β levels with respect to WT controls, and this difference was further enhanced after $PbNO_3$ treatment.

It has been demonstrated that macrophages are the major source of TGF-β after in vivo injection of apoptotic cells into peritoneum or lung (10). Consequently, we carried out immunohistochemical localization of TGF-β in $PbNO_3$-treated livers (Fig. 7*B*). Interestingly the enzyme showed a different localization in WT and TG2$^{-/-}$ mice. In WT livers, immunostaining was located at the

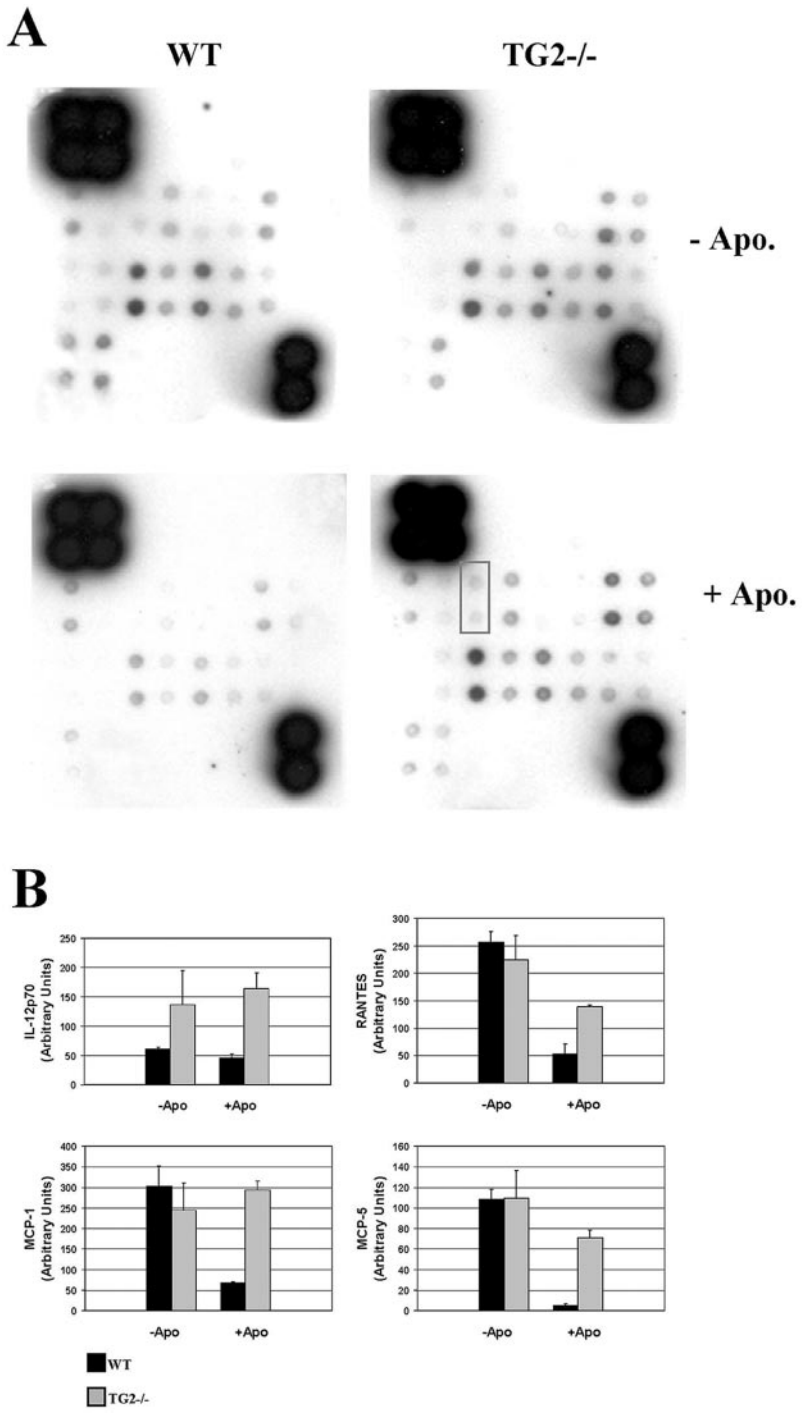

FIGURE 5. Cytokine analysis in the supernatants of cultured macrophages before and after exposure to apoptotic lymphocytes. *A*, Representative membrane protein arrays incubated with culture medium from WT and $TG2^{-/-}$ macrophages, untreated (−Apo) or incubated with apoptotic cells (+Apo). Differences in cytokine expression can be observed for each sample in the absence or the presence of apoptotic cells, and between samples from WT and $TG2^{-/-}$ macrophages in response to apoptotic cells. Squared spots show the expression of the cytokine IL-6 in $TG2^{-/-}$ macrophages after exposure to apoptotic cells. *B*, Quantitative analysis of cytokines IL-12p70, RANTES, MCP-1, and MCP-5. Similar profiles were detected in untreated conditions, comparing WT vs $TG2^{-/-}$. All proinflammatory cytokine release was strongly inhibited in WT macrophages in the presence of apoptotic cells ($p < 0.005$), although no significant changes were observed in $TG2^{-/-}$ macrophages in response to apoptotic cells. The data represent the mean ± SEM of four independent experiments.

sinusoidal level, where Kupffer cells (the tissue macrophages of the liver) are normally present (Fig. 7*B*, *a* and *c*); in contrast, in $TG2^{-/-}$ TGF-β was detected in hepatic parenchyma, particularly in hepatocytes facing the portal tract and in areas containing inflammatory infiltrates (Fig. 7*B*, *b* and *d*). To verify by ELISA whether this increased TGF-β production was reflected in the level of the circulating cytokines, we measured the level of TGF-β in the blood of control vs $PbNO_3$-treated mice (Fig. 7*Ca*). The basal level of circulating TGF-β in WT animals was not modified by apoptosis induction in the liver. Interestingly, in $TG2^{-/-}$ mice, differently from what was observed in liver, the basal level of circulating TGF-β was lower compared with that in WT mice (50%) and did not change after $PbNO_3$ treatment despite the accumulation of the cytokine in the liver (Fig. 7*Ca*).

The measurement of TGF-β levels in the culture medium of WT macrophages incubated with apoptotic cells (Fig. 7*Cb*) showed a significant increase after 60 min of exposure to apoptotic cells (no significant modifications were observed at 20 min). However, this increase was significantly less pronounced in $TG2^{-/-}$ macrophages, suggesting a defect in the regulation of this cytokine in the absence of TG2.

Discussion

An emerging feature of chronic inflammatory diseases is the persistent presence of apoptotic cells resulting from impaired phagocytosis (16, 18). We show in this study that macrophage uptake of apoptotic cells is dramatically affected in the absence of TG2,

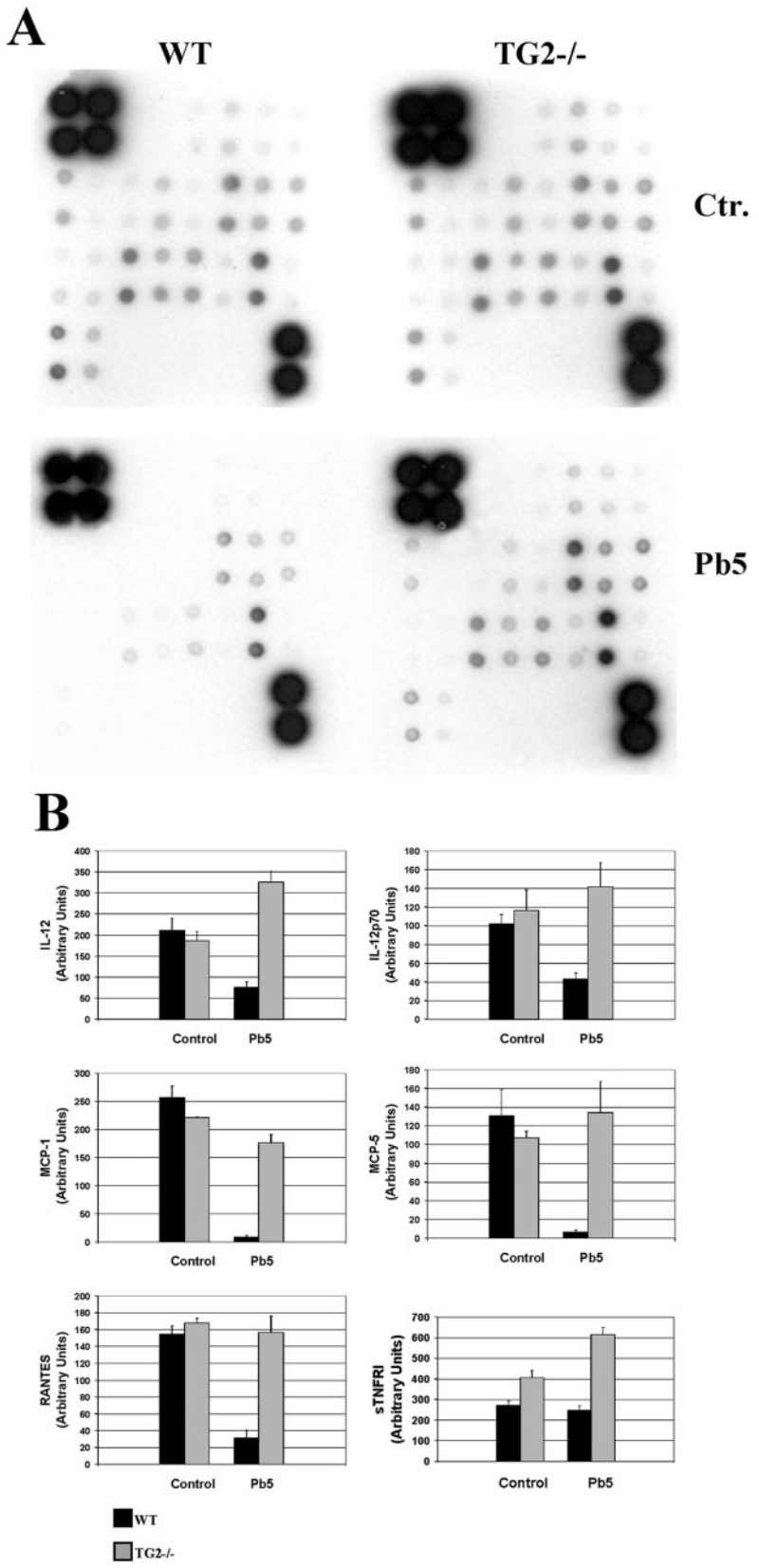

FIGURE 6. Cytokine analysis in sera of mice treated, or not, with lead nitrate. *A*, Representative membrane protein arrays incubated with serum from WT and $TG2^{-/-}$ mice, untreated (Ctr.) or treated for 5 days with $PbNO_3$ (Pb5). Differences in cytokine expression can be observed for each sample in the absence or the presence of treatment, and between samples from WT and $TG2^{-/-}$ mice in response to apoptotic cell induction. *B*, Quantitative analysis of cytokines IL-12, IL-12p70, MCP-1, MCP-5, RANTES, and sTNFRI. Similar profiles were detected in untreated conditions, comparing WT vs $TG2^{-/-}$. Apart from sTNFRI, the expression of which did not change, the release of all proinflammatory cytokines was significantly inhibited ($p < 0.005$) in WT mice in the presence of apoptotic cells, particularly that of MCP-1 and MCP-5. On the contrary, no significant changes were observed in $TG2^{-/-}$ mice for all cytokines analyzed, except for IL-12 and sTNFRI, which were significantly increased ($p < 0.05$). The data represent the mean ± SEM of four independent experiments.

whereas recognition and binding are not. In addition, we demonstrated that the defective clearance of apoptotic cells is associated with altered proinflammatory cytokine release. In particular, we show that the ablation of TG2, which is necessary for the correct uptake of apoptotic cells, also affects macrophage inactivation. Furthermore, our results indicate that the binding of apoptotic cells is not sufficient to inhibit the release of inflammatory cytokines. This suggests that the second step of phagocytosis (i.e. internalization) is required. This finding is in agreement with a recent study concerning inflammation in atherosclerosis, which demonstrates that

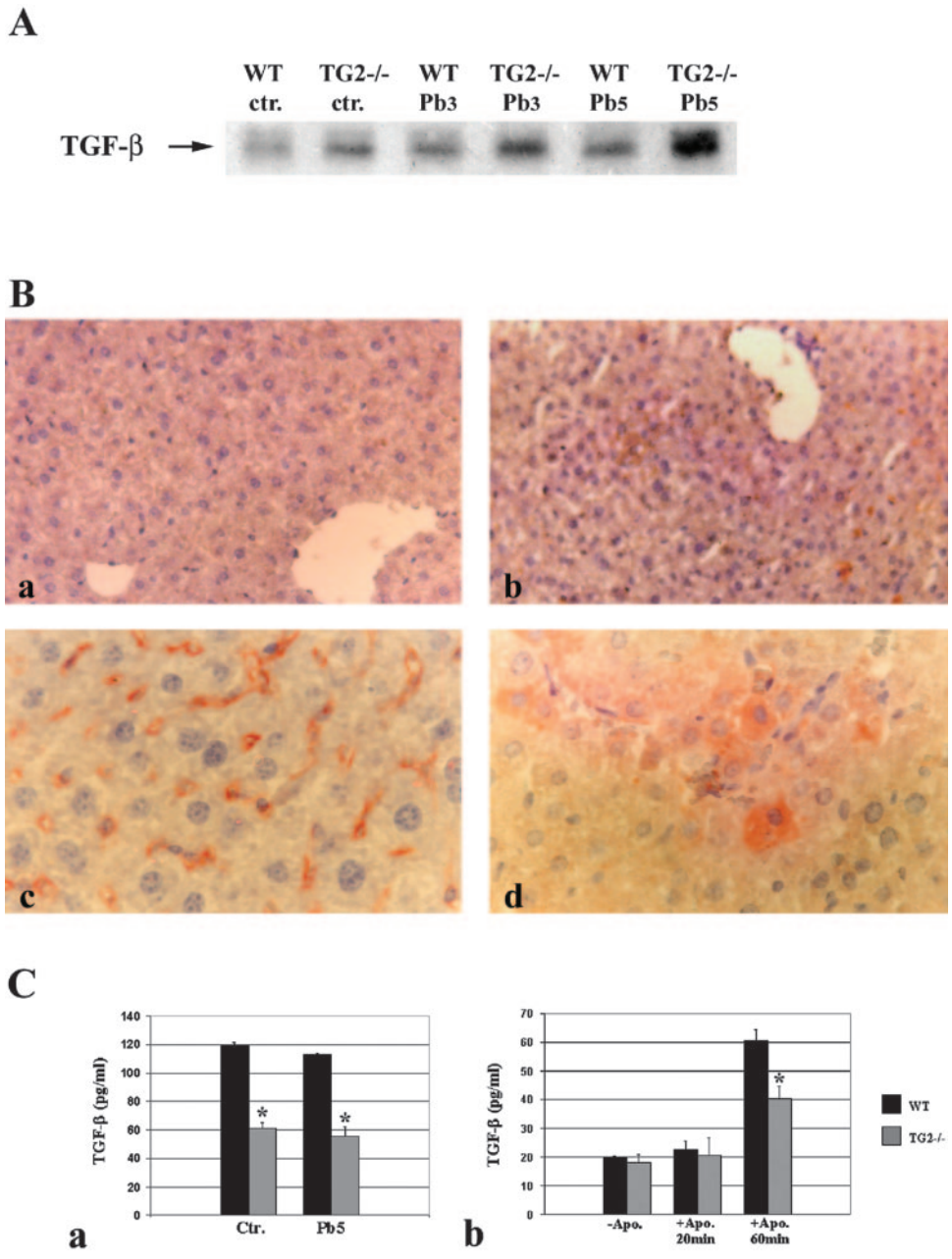

FIGURE 7. TGF-β expression in WT and TG2$^{-/-}$ mice. *A*, Western blot analysis of TGF-β expression in the livers of untreated control mice (ctr.) and mice 3 (Pb3) and 5 (Pb5) days after lead nitrate treatment. An increase in the amount of TGF-β protein correlates with apoptosis induction. A higher level of expression was found in TG2$^{-/-}$ mice compared with WT animals. The data represent one of three independent experiments with similar results. *B*, Immunohistochemical localization of TGF-β in liver of WT (*a* and *c*) and TG2$^{-/-}$ (*b* and *d*) mice. Three days after lead nitrate treatment, weaker staining was found in WT (*a*) compared with TG2$^{-/-}$ (*b*) livers. On day 5 after treatment, a striking difference was visible; strong staining of sinusoidal compartment was present in WT livers (*c*), whereas intense immunoreactivity was found in hepatocytes near areas of inflammatory infiltrates in TG2$^{-/-}$ liver. Original magnifications: *a* and *b*, $\times$40; *c* and *d*, $\times$63. *C*, The level of TGF-β in mouse blood serum and macrophage culture medium was evaluated by ELISA (■, WT; ▨, TG2$^{-/-}$). Data represent the mean $\pm$ SEM of three independent experiments. $*$, Significant compared with WT. *a*, Circulating TGF-β was measured in the sera of untreated (Ctr.) animals or on day 5 after $PbNO_3$ treatment (Pb5). TGF-β was significantly ($p < 0.005$) lower in TG2$^{-/-}$ vs WT mice; the cytokine level was not modified by $PbNO_3$ treatment in WT or TG2$^{-/-}$ mice. *b*, TGF-β release was measured in the culture medium of macrophages exposed (+Apo), or not (−Apo), to apoptotic cells. An increased amount of TGF-β was found after longer times of incubation; a significantly ($p < 0.05$) lower level was observed in TG2$^{-/-}$ compared with WT cultures.

the impairment of engulfment, and not binding, of apoptotic cells by macrophages caused by the presence of oxidized lipoproteins may promote inflammation (32).

A large body of work deals with the inhibition of cytokine release from macrophages that have ingested apoptotic cells (6, 33, 34). By contrast, Kobayashi et al. (35) reported that the interaction of phagocytes with apoptotic cells leads to the production of proinflammatory cytokines. Additional investigation by the same authors led to the conclusion that the production of inflammatory cytokines by macrophages can be induced by exposure to late apoptotic cells (36). This event normally does not occur in vivo, because apoptotic cells are cleared as soon as they appear, thus leading to an almost null response (37). Our work provides new insight into this controversy. In fact, considering that the macrophage cytokine expression levels are generally very low, methods used to measure their expression after apoptotic cell exposure need to be conducted under stimulatory conditions (i.e., after LPS treatment) and/or after prolonged incubation with target cells (6, 33, 34). For the first time, we analyzed cytokine release modulation under unstimulated conditions and after a very short time of exposure to apoptotic cells. Our results show that the basal level of cytokine expression is immediately inhibited by the internalization of apoptotic cells both in vivo and in vitro.

Another controversial issue concerns the level (transcriptional or translational) of regulation of cytokine release (38). Our results are consistent with a post-transcriptional regulation, because we found that the shutoff of inflammatory cytokine release occurs very rapidly. We hypothesize that the regulation could involve the stability or degradation of cytokine transcripts after the inhibition of their secretion. Indeed, it has been reported that production of inflammatory cytokines by macrophages requires activation of the p38 MAPK pathway. This promotes the stability and translability of inflammatory cytokine transcripts (39, 40), whereas selective inhibitors of p38 MAPK prevent proinflammatory cytokine release (41). In keeping with this, it is interesting to note that TG2 activity is required for activation of p38 MAPKs (42).

We previously reported that mice lacking TG2 are prone to develop inflammatory pathologies (24, 43). The data reported in this study demonstrate that no major differences in the basal levels of cytokines exist between WT and TG2$^{-/-}$ mice, with the exception of circulating TGF-β. However, our results indicate that a strong impairment of phagocytic clearance of apoptotic cells could account for the development of inflammatory disorders. In fact, we showed that the chemokines MCP-1 and MCP-5 are not attenuated in the absence of TG2. This event might result in the selective recruitment of monocytes into inflammatory sites, as observed in vivo in the livers of TG2$^{-/-}$ mice after apoptosis induction, where many inflammatory cells infiltrate the parenchimal tissue. Furthermore, we showed that in knockout mice, the expression of both IL-12 and sTNF-RI significantly increased. Interestingly, the dysregulation of IL-12 has been described in a wide range of autoimmune-prone mouse models (44–46).

The down-regulation of proinflammatory mediator release by macrophages in response to apoptotic cell ingestion has been largely attributed to TGF-β stimulation (10, 38). Accordingly, our data showed that in the livers of WT mice, TGF-β expression increases during the clearance of apoptotic cells, although it is minimally produced under normal conditions. Immunohistochemical analysis showed that the site of TGF-β expression is at the level of sinusoidal cells. Surprisingly, we found that TGF-β also increases in the livers of TG2$^{-/-}$ mice, and the up-regulation is even higher than that observed in WT livers. These results might appear to contrast with the unrepressed production of proinflammatory cytokines observed in TG2$^{-/-}$ mice. It is noteworthy that TGF-β in knockout mice is localized not in the sinusoidal compartment, but, rather, in the parenchimal cells. A similar expression pattern has been reported for the liver during inflammatory conditions, such as fibrosis in chronic liver disease and autoimmune hepatitis (47, 48), where a close correlation exists between this abnormal localization of TGF-β and the inflammatory activity in the tissue, thus confirming the ability of hepatocytes localized near areas of inflammatory infiltrates to synthesize TGF-β (47, 48). Our findings demonstrating a similar TGF-β localization confirm that the defective phagocytosis of apoptotic cells caused by TG2 ablation results in the imbalance of the inflammatory regulation at multiple levels. Particularly controversial is the role of the systemic level of TGF-β in inflammatory diseases. Local and circulating effects of TGF-β could be divergent (49, 50). In fact, due to the different systemic effects exerted by TGF-β and particularly its essential role in the maintenance of self-tolerance, its stimulation and secretion must be tightly regulated (51). Our data support this view; in fact, the clearance of apoptotic cells locally restricted to the liver does not affect the circulating level of TGF-β.

It has been reported that TGF-β is the only cytokine to be up-regulated in macrophages after the ingestion of apoptotic cells and that this is responsible for the proinflammatory cytokine down-regulation (38). It should be noted that in those studies TGF-β release was evaluated after a prolonged time of incubation with apoptotic cells. By contrast, the results of our experiments, performed a short time after apoptotic cell exposure, suggest that the inhibition of proinflammatory cytokines occurs rapidly and independently from TGF-β. These results are also in agreement with the hypothesis that TGF-β could act at a later time, amplifying the immunosuppressive response (52). As far as the role of TG2 on TGF-β expression is concerned, it must be stressed that the absence of TG2 causes a dysregulation of the steady-state level of the enzyme. In fact, compared with WT mice, TG2$^{-/-}$ mice displayed an increased level of TGF-β in liver and a lower amount in blood. The discrepancy between liver and circulating TGF-β levels suggests an impaired release of TGF-β. Regarding this point, it is important to note that TG2 may affect the vesicle traffic that is necessary for cytokine secretion, through modification of major components of the cytoskeleton (26, 53).

In conclusion, our study demonstrates that TG2 ablation in mice impairs the ability of macrophages to engulf dying cells, potentiating the susceptibility to inflammatory pathologies. Our data have profound clinical relevance, considering the recent proposal to use TG2 inhibitors for therapeutic applications in autoimmune and inflammatory diseases.

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Disclosures

The authors have no financial conflict of interest.

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