Immune and Inflammatory Responses in TNFα-deficient Mice: A Critical Requirement for TNFα in the Formation of Primary B Cell Follicles, Follicular Dendritic Cell Networks and Germinal Centers, and in the Maturation of the Humoral Immune Response

By Manolis Pasparakis,* Lena Alexopoulou,* Vasso Episkopou,‡ and George Kollias*

*From the *Department of Molecular Genetics, Hellenic Pasteur Institute, 115 21 Athens, Greece; and ‡Mouse Embryology Lab, MRC Clinical Sciences Centre, Royal Postgraduate School Hammersmith Hospital, London W12 ONN, United Kingdom*

Summary

To investigate the role of TNFα in the development of in vivo immune responses we have generated TNFα-deficient mice by gene targeting. Homozygous mutant mice are viable and fertile, develop lymph nodes and Peyer's patches and show no apparent phenotypic abnormalities, indicating that TNFα is not required for normal mouse development. In the absence of TNFα mice readily succumb to *L. monocytogenes* infections and show reduced contact hypersensitivity responses. Furthermore, TNFα knockout mice are resistant to the systemic toxicity of LPS upon D-galactosamine sensitization, yet they remain sensitive to high doses of LPS alone. Most interestingly, TNFα knockout mice completely lack splenic primary B cell follicles and cannot form organized follicular dendritic cell (FDC) networks and germinal centers. However, despite the absence of B cell follicles, Ig class-switching can still occur, yet deregulated humoral immune responses against either thymus-dependent (TD) or thymus-independent (TI) antigens are observed. Complementation of TNFα functioning by the expression of either human or murine TNFα transgenes is sufficient to reconstitute these defects, establishing a physiological role for TNFα in regulating the development and organization of splenic follicular architecture and in the maturation of the humoral immune response.

Tumor necrosis factors (TNFα and TNFβ or lymphotoxin-α [LTα][1]) are known to be critically involved in the regulation of infectious, inflammatory and autoimmune phenomena (1). TNFα is produced in response to various stimuli mainly by macrophages and T cells and is shown to be bioactive both as a transmembrane protein and as a homotrimeric secreted molecule (2). LTα, originally identified as a major product of lymphocytes, exists as a secreted molecule only in a homotrimeric form (3), but it may also accumulate on the cellular membrane when complexed with LTβ, a type II transmembrane protein that is another member of the TNF ligand family (4). LTαβ signals exclusively through the LTβ receptor(s) (5) while TNFα and LTα share the same cell surface receptors, designated p55 and p75 TNF-R, which show common but also differential activities depending on the cell type in which they operate (6). p75 TNF-R signaling has been mainly implicated in lymphocyte proliferation (7, 8) whereas the p55 TNF-R is generally known to mediate apoptosis (9), a process in which the p75 TNF-R may also be involved (10, 11).

The generation of mice deficient for TNF-Rs and for LTα has confirmed the idea that these molecules serve specific and important immunoregulatory functions. Mice lacking the p55 TNF-R are resistant to the lethal effects of low doses of lipopolysaccharides (LPS), show increased susceptibility to infection with *Listeria monocytogenes* (12, 13) and enhanced reactivity against contact allergens (14), indicating the essential role of the p55 TNF-R in LPS-mediated systemic toxicity, anti-bacterial host defense mechanisms and contact hypersensitivity reactions. Surprisingly, targeted knockout of the murine LTα gene disrupts the development of lymph nodes and Peyer's patches and results in a disturbed splenic architecture characterized by the ab-

[1] *Abbreviations used in this paper:* CH, contact hypersensitivity; DAB, diaminobenzidine; ES, embryonic stem; FDC, follicular dendritic cell; GC, germinal centers; LH, *Limulus* hemocyanin; LTα, lymphotoxin-α; PNA, peanut agglutinin; S-AP, streptavidin-alkaline phosphatase; TD, T cell–dependent; TI, T cell–independent.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/10/1397/15 $2.00
Volume 184 October 1996 1397–1411

sence of distinct B and T cell areas (15). Interestingly, mice lacking the p55 TNF-R show a complete lack of Peyer's patches (16), yet they develop lymph nodes, suggesting that the organogenetic role of LTα involves signaling through the LTβ-R for the genesis of lymph nodes, and seemingly through the p55 TNF-R for the genesis of Peyer's patches. More recently, LTα-deficient mice were also found defective in the formation of germinal centers (GC) (17), but because of the inherent dominant perturbation of the lymphoid system in these mice, a direct role for LTα signaling in germinal center reactivity could not be demonstrated. Moreover, in analogous knockout systems, the presence of the p55 TNF-R, but not of the p75 TNF-R, was also found to be essential for the development of germinal centers (17), yet again, a conclusive answer as to whether either or both LTα and TNFα contribute to this phenomenon, could not be reached.

To obtain definitive answers on the individual role of TNFα in the development and physiology of the immune system, we have used gene targeting in embryonic stem (ES) cells to generate a TNFα-deficient mouse strain. Our findings demonstrate the important in vivo role of TNFα in LPS toxicity, contact hypersensitivity reactions and immune reactivity against pathogens. Furthermore, they establish a critical requirement for this factor in processes regulating the formation of primary B cell follicles, follicular dendritic cell (FDC) networks and germinal centers and in the physiological maturation of the humoral immune response.

Materials and Methods

Generation of TNFa Knockout Mice. The genomic locus containing the genes for TNFα and LTα was isolated from a 129/Sv mouse genomic library in λFIX™II (Stratagene Inc., La Jolla, CA) (18). The targeting vector was constructed by replacing with an MC1neopA cassette (Stratagene) the 438-bp NarI-BglII fragment containing 40 bp of the 5′ UTR, all the coding region, including the ATG translation initiation codon, of the first exon and part of the first intron of the muTNFα gene. The neo cassette was flanked by 4.5 and 1.2 kb of homologous sequences, respectively. A thymidine kinase (*tk*) gene under the control of the MC1 promoter (19) was inserted at the 5′ end of the targeting vector for negative selection against random integrations. The targeting vector (25 μg) was linearized and electroporated into CCE embryonic stem cells (20). Electroporation and selection were performed as previously described (21) and targeted clones were identified by Southern blot analysis using the external ScaI-EcoRI probe shown in Fig. 1 *a*. The frequency of homologous recombination was 1 in 48 double drug resistant colonies. Chimeric mice were produced by microinjection of embryonic stem cells from four different targeted clones into C57Bl/6 blastocysts. Male chimeras were mated to C57Bl/6 females and germ line transmission of the mutant TNFα gene in agouti progeny was confirmed by Southern-blot analysis of tail DNA. Germ line transmission was obtained from two out of four clones injected. Heterozygous mice were intercrossed and homozygous mice were obtained at the predicted frequency.

Northern Blot Analysis of Macrophage RNA. Mice were injected intraperitoneally with 1 ml of a 4% thioglycollate medium (Difco Labs., Detroit, MI) and 3 d later peritoneal exudate cells were harvested by peritoneal lavage with 5 ml RPMI 1640 medium supplemented with 5% FCS (GIBCO BRL, Gaithersburg, MD). Cells were cultured for 1 h at 37°C, 5% CO_2 in tissue culture petri dishes and were washed twice with medium to remove non adherent cells. Adherent macrophages were cultured with or without LPS (1 μg/ml) for 1 h and RNA was isolated with the method of guanidinium thiocyanate (22). Total RNA (15 μg) was resolved in 1% formaldehyde agarose gel, blotted onto Hybond N^+ nylon membrane (Amersham Corp., Arlington Heights, IL) and hybridized with a ^{32}P-labeled EcoRI-NarI genomic probe containing part of the first exon of the muTNFα gene. Membranes were stripped and rehybridized with a neo probe.

Measurement of Serum TNFa and IL-1β in LPS-induced Septic Shock. Mice were injected intraperitoneally with 1 mg LPS (*Salmonella enteritidis*, L-6011; Sigma Chem. Co., St. Louis, MO) and sera were collected 90 min and 4 h later. Levels of TNFα in the serum of wild-type and homozygous mutant mice 90 min after LPS challenge were measured with a muTNFα specific ELISA provided by Dr. Wim Buurman (University of Limburg, The Netherlands) (23). Levels of IL-1β were measured in sera collected 4 h after LPS injection using a murine IL-1β ELISA (Endogen Inc., Boston, MA).

LPS-induced Septic Shock. Mice (10–12 wk of age) were injected intraperitoneally with the indicated amounts of LPS (*Salmonella enteritidis*; Sigma L-6011) and D-Galactosamine (Sigma) in 0.2 ml of sterile phosphate-buffered saline.

Listeria Infection. Mice were infected intravenously or intraperitoneally with 10^4 CFU of *Listeria monocytogenes* strain L028 (24, 25). For bacterial counts mice were killed 4 d after infection and numbers of viable bacteria were determined by plating serial dilutions of liver and spleen homogenates in saline on BHI agar plates.

Contact Hypersensitivity Test. To test contact sensitivity to oxazolone, 100 μl of a 4% solution of 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, E-0753; Sigma) in acetone was applied on the shaved mouse abdomen and allowed to dry on the skin. 5 d later mice were challenged by painting both sides of one ear with 50 μl of 1% oxazolone in acetone, leaving the other ear as a control. Mice were killed 24 h after treatment and a disk of ear tissue was removed from both ears of each mouse using a 6-mm biopsy punch, and weighed. Swelling was measured as the difference in weight between the painted and the unpainted ear of each mouse. In each experiment, a group of non-sensitized mice was included to measure the swelling occurring after ear challenge without prior sensitization. The results are expressed as the weight gain of the challenged versus the non-challenged ear ± SE for each group and the data were analyzed by student's *t* test.

Immunization of Mice. To induce antibody responses to the T cell–dependent (TD) antigen SRBC, mice were immunized intraperitoneally with 10^8 SRBC on day 0 and day 15. Mice were bled on day 7, 14, and 23 for measurement of anti-SRBC serum antibodies. Sera obtained from similar groups of non immunized mice matched for genotype, age and sex were used as controls for pre-immune anti-SRBC values. For generation of antibody responses to a different TD antigen, mice were immunized intraperitoneally with 50 μg TNP-KLH in complete Freund's adjuvant on day 0 and boosted with an intraperitoneal injection of 5 μg TNP-KLH in PBS on day 28. Mice were bled 7, 20, and 35 d after primary immunization for measurement of anti-TNP serum antibodies. To induce antibody responses to T cell–independent (TI) antigens mice were immunized intraperitoneally with 50 μg of TNP-LPS (T-7268; Sigma) or 25 μg of TNP-Ficoll in PBS on

day 0 and bled 7 and 14 d later for measurement of anti-TNP serum antibodies.

ELISA for SRBC or TNP-specific Serum Antibodies. Sera from immunized mice were assayed using TNP-specific ELISA for IgM, IgE, IgG1, IgG2a, IgG2b, and IgG3 antibodies. In brief, 96-well flat-bottomed Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated by adding 100 μl/well of 10 μg/ml TNP-bovine serum albumin in carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed with deionized water and blocked with 2 mg/ml BSA (150 μl/well) in PBS for 1 h at 37°C. Plates were washed three times with PBS containing 0.05% Tween 20 and serum samples diluted in PBS containing 0.05% Tween 20, 1% BSA and 1 M NaCl were added in duplicate wells (100 μl/well) and incubated for 1 h at 37°C. For the determination of IgM antibodies, samples were incubated with Horseradish Peroxidase conjugated goat anti-mouse IgM (Sigma) (100 μl/well, 1:10,000 dilution in PBS containing 1% BSA) for 1 h at room temperature. For measurement of IgE antibodies, samples were incubated with a monoclonal rat anti-mouse IgE antibody (Serotec, Kidlington, England) (2 μg/ml in PBS 1% BSA, 100 μl/well) for 1 h at room temperature followed by incubation with a horseradish peroxidase conjugated goat anti-rat IgG antibody (Sigma). For the determination of IgG1, IgG2a, IgG2b, and IgG3 antibodies plates were incubated with peroxidase-labeled goat anti-mouse IgG subclass-specific antibodies (Southern Biotechnology, Birmingham, AL) (1:5,000 dilution in PBS containing 1% BSA). Measurements of TNP-specific total IgG antibodies were performed using a peroxidase-labeled goat anti-mouse IgG antibody (Sigma). All ELISAs were developed with 0.4 mg/ml *o*-phenyldiamine dihydrochloride (Sigma) in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% H_2O_2, stopped with 50 μl of 2 M H_2SO_4 and OD_{490} was measured using an MRX microplate Reader (Dynatech Labs., Chantilly, VA). Serum anti-SRBC antibodies were measured by SRBC-specific isotype ELISAs. Briefly, 96-well flat-

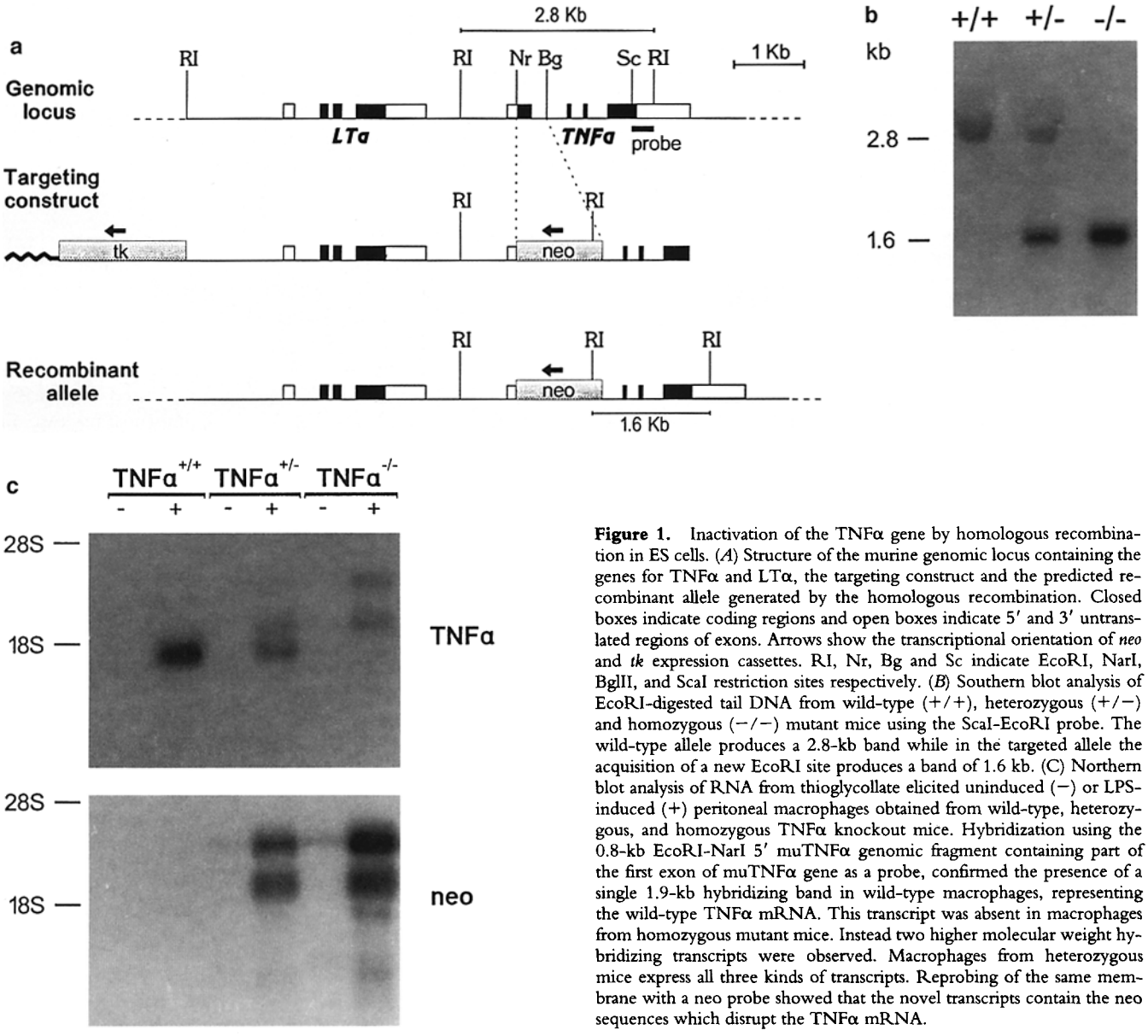


Figure 1. Inactivation of the TNFα gene by homologous recombination in ES cells. (*A*) Structure of the murine genomic locus containing the genes for TNFα and LTα, the targeting construct and the predicted recombinant allele generated by the homologous recombination. Closed boxes indicate coding regions and open boxes indicate 5′ and 3′ untranslated regions of exons. Arrows show the transcriptional orientation of *neo* and *tk* expression cassettes. RI, Nr, Bg and Sc indicate EcoRI, NarI, BglII, and ScaI restriction sites respectively. (*B*) Southern blot analysis of EcoRI-digested tail DNA from wild-type (+/+), heterozygous (+/−) and homozygous (−/−) mutant mice using the ScaI-EcoRI probe. The wild-type allele produces a 2.8-kb band while in the targeted allele the acquisition of a new EcoRI site produces a band of 1.6 kb. (C) Northern blot analysis of RNA from thioglycollate elicited uninduced (−) or LPS-induced (+) peritoneal macrophages obtained from wild-type, heterozygous, and homozygous TNFα knockout mice. Hybridization using the 0.8-kb EcoRI-NarI 5′ muTNFα genomic fragment containing part of the first exon of muTNFα gene as a probe, confirmed the presence of a single 1.9-kb hybridizing band in wild-type macrophages, representing the wild-type TNFα mRNA. This transcript was absent in macrophages from homozygous mutant mice. Instead two higher molecular weight hybridizing transcripts were observed. Macrophages from heterozygous mice express all three kinds of transcripts. Reprobing of the same membrane with a neo probe showed that the novel transcripts contain the neo sequences which disrupt the TNFα mRNA.

bottomed Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated by adding 100 μl/well of a solubilized extract from SRBC (26) (5 μg/ml in carbonate buffer, pH 9.6). After incubation at 4°C overnight, plates were washed and blocked with 1% BSA in PBS for 2 h at 37°C. Serum samples diluted in PBS containing 0.05% Tween 20, 1% BSA and 1 M NaCl were added in duplicate wells (100 μl/well) and incubated overnight at RT. Bound antibodies were detected as described above for the TNP-specific ELISAs.

Immunocytochemical Analysis of Splenic Structure. Spleens from nonimmunized mice were embedded in O.C.T. compound (BDH) and frozen in liquid nitrogen. Sections were cut on a cryostat, thaw-mounted on gelatinized slides, air dried, and then stored at −20°C. Immediately before use sections were fixed for 10 min in acetone containing 0.03% H_2O_2 to block endogenous peroxidase activity. For double immunostaining for IgM and IgD, sections were rehydrated in PBS and incubated with peroxidase-labeled goat anti-mouse IgM (Sigma) and biotin-conjugated rat anti-mouse IgD (Southern Biotechnology, Birmingham, AL) antibodies for 2 h at RT, followed by streptavidin-alkaline phosphatase (S-AP) (Vector Labs., Inc., Burlingame, CA). Bound peroxidase activity was detected by staining with diaminobenzidine (DAB; Sigma), alkaline phosphatase activity was visualized with NBT-BCIP (GIBCO BRL) and slides were embedded with glycerol-PBS. For anti-CD3 and anti-FDC staining sections were incubated with a rat anti-mouse CD3 monoclonal antibody (clone KT3 [27], kindly provided by Dr. S. Cobbold, Sir William Dunn School of Pathology, Oxford, UK) or with the FDC-M1 antibody (specific for mouse follicular dendritic cells [28], kindly provided by Dr. M. Kosco-Vilbois, Glaxo Institute for Molecular Biology, Switzerland). Subsequently, sections were incubated with biotin-conjugated anti-rat IgG antibody (Vector Labs., Inc.) followed by S-AP, and developed with NBT-BCIP.

For the analysis of germinal center formation mice were immunized with an intraperitoneal injection of 10^8 sheep red blood cells (SRBC) and spleens were removed 8 d later, embedded in O.C.T. compound (BDH), and frozen in liquid nitrogen. Cryostat sections were fixed in acetone and incubated with 0.5% H_2O_2 in methanol for 30 min at room temperature to block endogenous peroxidase activity. Sections were rehydrated with PBS, incubated with 20 μg/ml peanut agglutinin-horseradish peroxidase (Sigma) in PBS-BSA 0.1% for 2 h at room temperature, washed and stained with diaminobenzidine (Sigma). Sections were subsequently incubated with a biotin conjugated rat anti-mouse CD45R/B220 antibody (PharMingen, San Diego, CA) for 3 h at room temperature followed by S-AP (Vector Labs.). Bound alkaline phosphatase activity was visualized with NBT-BCIP (GIBCO BRL) and slides were embedded with glycerol-PBS. Slides were photographed using a Nikon Diaphot 300 microscope.

Table 1. *ELISA Analysis of TNFα and IL-1β Levels After LPS Induction*

Genotype	TNFα (ng/ml)	IL-1β (ng/ml)
Wild-type	7.88 ± 1.31	0.84 ± 0.22
TNFα$^{-/-}$	0.00 ± 0.00	0.74 ± 0.06

TNFα-deficient mice and wild-type controls (n = 7 for both groups) were injected intraperitoneally with 1 mg LPS and were bled 90 min and 4 h later. Serum levels of TNFα at 90 min and of IL-1β at 4 h after LPS injection were determined with specific ELISAs. The data are means ± SE per group.

Table 2. *Measurements of LPS-induced Lethality in TNFα Knockout Mice*

Dose		Lethality (deaths/total)	
LPS	D-Galactosamine	TNFα$^{+/+}$	TNFα$^{-/-}$
μg	*mg*		
1,200	—	3/5	3/5
100	—	0/3	0/3
100	20	5/5	0/5
10	20	5/5	0/5
1.0	20	5/5	0/5
0.1	20	5/5	0/5
0.01	20	2/5	0/5

Mice (10–12 wk of age) were injected intraperitoneally with the indicated amounts of LPS (*Salmonella enteritidis*, L-6011; Sigma) and D-Galactosamine (Sigma) in 0.2 ml of sterile phosphate-buffered saline. The dose of LPS was calculated per 20 g of body weight. Survival was monitored for 7 d.

Results and Discussion

Generation of TNFa-Deficient Mice. To investigate the individual role of TNFα in the regulation of the immune response we have generated TNFα-deficient mice by gene targeting in ES cells (Fig. 1, *a* and *b*). Disrupted TNFα mRNA expression in homozygous mutant mice was confirmed by Northern analysis of RNA isolated from LPS-stimulated macrophages (Fig. 1 *c*). The functional inactivation of the TNFα gene was further confirmed by the absence of TNFα protein in sera from LPS-injected homozygous knockout mice, as measured by a murine TNFα-specific ELISA (Table 1). Furthermore, no TNFα bioactivity could be detected in sera from LPS-injected TNFα$^{-/-}$ mice and supernatants from LPS-induced TNFα$^{-/-}$ macrophages, as measured by standard L-929 cytotoxicity assays (not shown). Homozygous TNFα$^{-/-}$ mice are viable and fertile and show no apparent phenotypic abnormalities, indicating that TNFα expression is not required for normal mouse development. However, in contrast to the LTα and p55TNF-R knockout mice, lymph nodes and Peyer's patches could readily be detected in TNFα knockout mice, suggesting that TNFα is not necessary for lymphoid organogenesis.

TNFa Knockout Mice are Resistant to the Systemic Toxicity of LPS. The critical role of TNFs in the lethal endotoxin shock syndrome has been addressed in several studies employing neutralizing anti-TNF strategies (29–31) or, more recently, in TNF-R knockout mice (12, 13). Using standard D-Galactosamine (D-GalN) sensitization in mice we could show that at doses as high as 100 μg of LPS, TNFα$^{-/-}$ mice remain completely resistant to shock and death, while at even 1,000-fold lower doses all normal control mice die

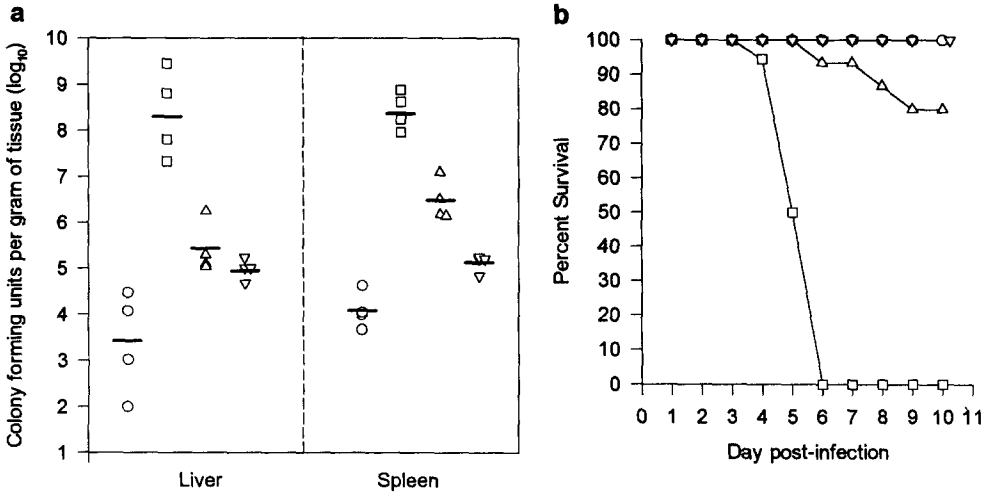


Figure 2. Role of TNFα in host defense to *L. monocytogenes*. $TNF\alpha^{+/+}$ (○), $TNF\alpha^{-/-}$ (□), $TNF\alpha^{-/-}$ × Tg A86 (△) and $TNF\alpha^{-/-}$ × Tg 1278 (▽). (*A*) Bacterial burden in liver and spleen. Mice were infected intraperitoneally with 10^4 CFU of *L. monocytogenes* and numbers of viable bacteria in liver and spleen were determined 4 d later. (*B*) Lethality after *L. monocytogenes* challenge. $TNF\alpha^{+/+}$ (n = 16), $TNF\alpha^{-/-}$ (n = 18), $TNF\alpha^{-/-}$ × Tg A86 (n = 15), and $TNF\alpha^{-/-}$ × Tg 1278 (n = 4) mice were inoculated intraperitoneally with 10^4 CFU of bacteria and survival was monitored for 10 d.

(Table 2). These results establish the pivotal role of TNFα in mediating endotoxin lethality in D-GalN sensitized mice. However, high doses of LPS alone (1,200 μg) are found to be equally lethal for both $TNF\alpha^{-/-}$ and control mice, indicating that other factors produced independently of TNFα, mediate toxicity in this model of endotoxic shock. Recent studies in interleukin-1β converting enzyme knockout mice suggest that IL-1 may play a role in these processes (32). Consistent with this hypothesis we could show that 4 h after LPS-challenge, a normal level of IL-1β production is detected in sera from $TNF\alpha^{-/-}$ mice (Table 1), suggesting that IL-1β is produced independently of TNFα upon high dose LPS stimulation.

Deficient Anti-Bacterial Responses in TNFa Knockout Mice Rescued by Expression of a Transmembrane TNFa Transgene. Earlier studies on TNF receptor-deficient mice (12, 13) have demonstrated the critical role of p55 TNF receptor signaling in host defense, in particular during infection with the intracellular bacterium *Listeria monocytogenes*. To establish the individual role of TNFα in this system, $TNF\alpha^{-/-}$ mice and wild-type controls were challenged with an intraperitoneal injection of 10^4 colony-forming units (CFU) of *Listeria monocytogenes*. 4 d after infection, TNFα-deficient mice exhibited 10,000–100,000 fold higher bacterial titers in their livers and spleens in comparison to controls (Fig. 2 *a*). Moreover, by day 6 after infection 18/18 of TNFα-deficient mice and 0/16 of the controls had died (Fig. 2 *b*). Similar results were obtained when bacteria were administered intravenously. These results demonstrate a critical role for TNFα in orchestrating host reactivity against pathogenic bacteria.

To dissect further the individual role of the soluble and transmembrane forms of TNFα in anti-bacterial host defense, we have sought to restore the TNFα deficiency of the $TNF\alpha^{-/-}$ mice by breeding them with TNFα transgenic mouse lines expressing either wild-type or uncleavable mutant TNFα proteins. Two different transgenic lines were used: Tg1278 mice (33) expressing a wild-type human TNFα transgene in several of their tissues including the macrophages, and TgA86 mice (unpublished data), similarly expressing in their tissues a mutant murine TNFα gene made by the deletion of the triplets encoding the first 12 amino acids of the mature TNFα protein. This latter transgene does not produce any measurable bioactive TNFα protein in sera from LPS-treated, double TgA86 × $TNF\alpha^{-/-}$ mice, or in supernatants from LPS-induced macrophages obtained from these mice (not shown). A similar mutant TNFα gene has been recently shown to produce bioactive transmembrane TNFα protein but no bioactive soluble TNFα when transfected in cultured cells (34, our unpublished results). Both double $TNF\alpha^{-/-}$ × Tg1278 and $TNF\alpha^{-/-}$ × TgA86 mice were able to control infection when challenged with 10^4 CFU *Listeria monocytogenes* as shown by 100–1,000-fold reduced bacterial counts measured 4 d after infection, in comparison to similarly treated $TNF\alpha^{-/-}$ mice (Fig. 2 *a*). Furthermore, by day 10 after in-

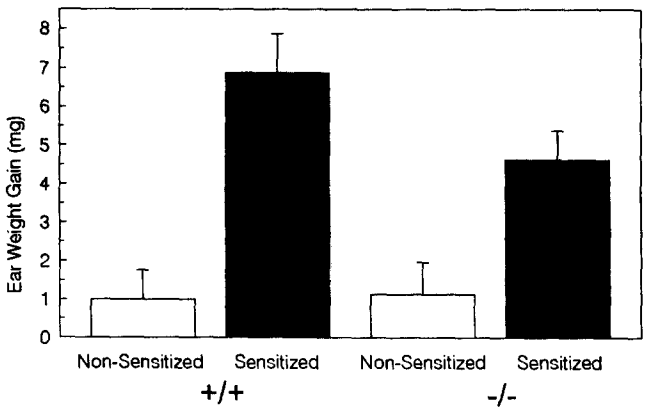


Figure 3. Hypersensitivity to contact allergen oxazolone. Wild-type controls (+/+) and TNFα knockout (−/−) mice (n = 8 for both groups) were either sensitized with application of oxazolone on the skin (*closed bars*) or left untreated (*open bars*). 5 d later mice were challenged by painting oxazolone on one ear leaving the other ear as a control. The contact hypersensitivity reaction was measured as the weight gain of the treated versus the untreated ear of each mouse 24 h later as described in the experimental procedures. The data are expressed as mean ± SE per group (sensitized, +/+ versus −/− mice, $P < 0.001$ by student's t test). This experiment was repeated twice with similar results.

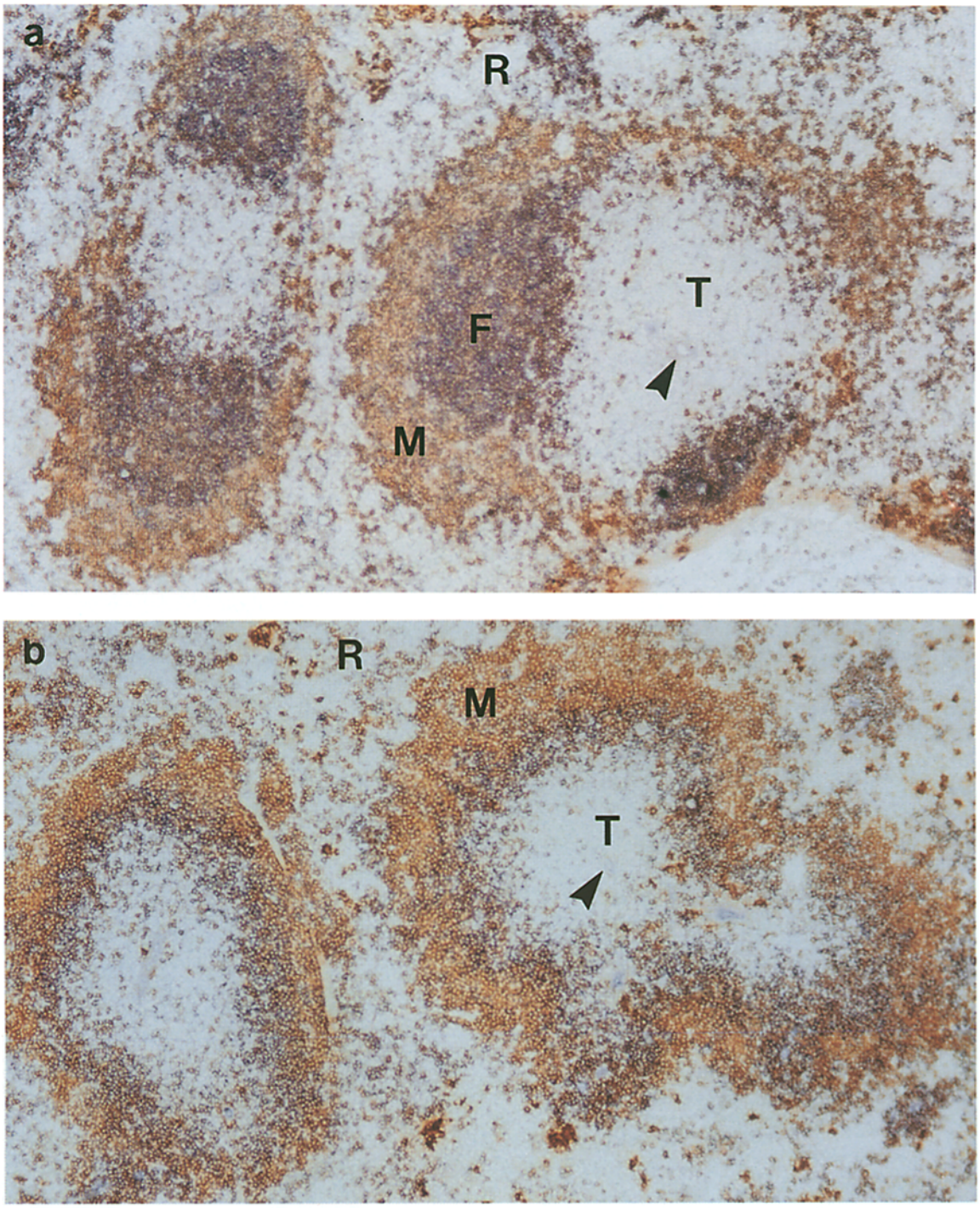
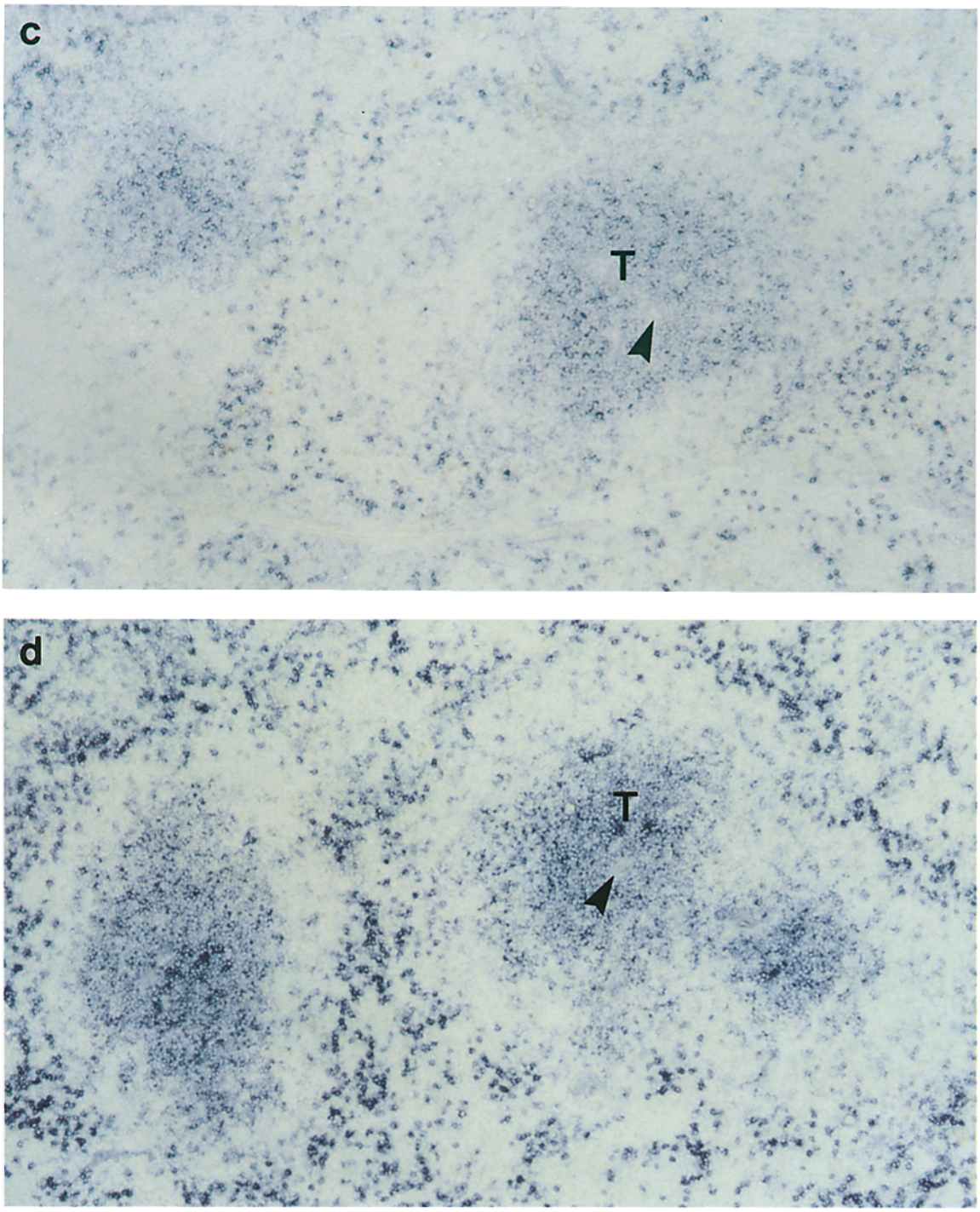


Figure 4.

fection, most of the TNFα transgene-reconstituted knockout mice survive infection, while all TNFα-deficient mice die (Fig. 2 *b*). The small percentage of double TNFα$^{-/-}$ × TgA86 transgenic mice which finally succumb to Listeria infection may be associated with the incomplete clearance of bacteria observed in these mice in comparison to normal controls (Fig. 2 *a*). This apparently partial complementation of TNFα functioning may be due to deviations from normal patterns of TNFα expression inherent to transgenic systems. Taken together, these results demonstrate that TNFα is indispensable for immune homeostasis during anti-microbial responses. Furthermore, based on the shown inability of the human TNF to bind and signal through the murine p75TNF-R (35), the observed ability of human TNFα to rescue a compromised host defense in the TNFα$^{-/-}$ mice, confirms that TNFα signaling through the p55 TNF-R is an



essential component in this process. Most interestingly, our finding that transmembrane TNFα is sufficient to rescue deficient anti-bacterial responses in TNFα$^{-/-}$ mice, establishes a direct in vivo role for cell contact-mediated TNFα signaling in the regulation of host defense against pathogens.

TNFa Knockout Mice Show Impaired Contact Hypersensitivity Responses. A role for TNFα has been proposed in mediating contact hypersensitivity (CH) reactions. Studies in murine models of CH using neutralizing anti-TNF antibodies have been controversial, indicating either an enhancing (36–38) or an immunosuppressive (14) role for TNFα in CH reactions. However, mice deficient for the p55 TNF-R show enhanced contact hypersensitivity responses, suggesting an immunosuppressive role for this receptor in CH (14). In contrast, an enhancing role for TNFα-mediated p75 TNF-R signaling in contact sensiti-

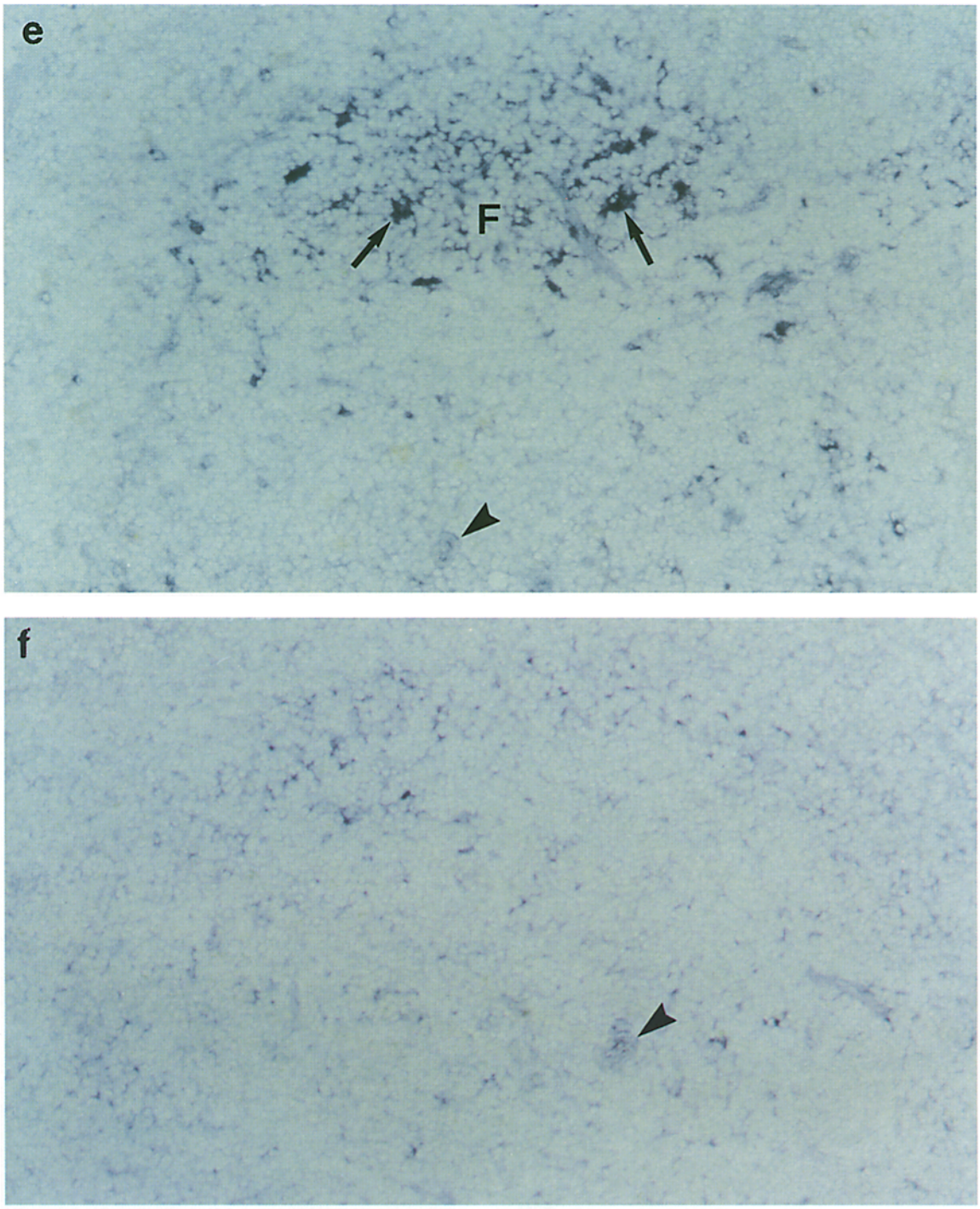


Figure 4. Absence of primary B cell follicles and FDC networks from the spleens of non-immunized TNFα knockout mice. Serial sections from normal mice stained with anti-IgM (*brown*) and anti-IgD (*blue*) (*a*), or with an anti-CD3 antibody (*blue*) (*c*) reveal the existence of clearly defined T cell–rich PALS (*T*), primary B cell follicles (*F*), marginal zone (*M*) and red pulp areas (*R*). Arrowheads denote central arterioles. Similar staining of serial sections from TNFα knockout mice (*b* and *d*) reveal the absence of primary B cell follicles while T cell–rich PALS and marginal zone areas appear normal. FDC networks (*arrows*) organized inside primary B cell follicles (*F*) are detected in sections from normal (*e*) but not from TNFα$^{-/-}$ mice (*f*) stained with the FDC-M1 antibody. Original magnifications ×100 for *a–d* and ×200 for *e* and *f*.

zation has been proposed in studies showing murine but not human TNFα to stimulate murine epidermal Langerhans cells (LC) to migrate from the skin to the draining lymph nodes after allergen application (38, 39). To address the role of TNFα in CH, we have characterized the response of TNFα knockout mice against the contact allergen oxazolone. Mice were sensitized by application of oxazolone on the abdominal skin, and 5 d later, they were

challenged by painting oxazolone on one ear. The CH response was measured 24 h later as the weight gain of the oxazolone-painted ear versus the untreated ear. In this assay, TNFα knockout mice showed a significant ($P < 0.001$) decrease in the hypersensitivity response, observed as a 33% reduction in ear swelling compared to the wild-type controls (Fig. 3). These results show that TNFα is necessary for optimal contact sensitization and establish an overall enhancing role for TNFα in contact hypersensitivity responses.

TNFa-deficient Mice Lack Primary B Cell Follicles and Organized FDC Networks. Splenic white pulp consists of three major compartments: (*i*) periarteriolar lymphoid sheaths (PALS) containing mainly T cells and interdigitating dendritic cells, (*ii*) primary B cell follicles consisting of a network of follicular dendritic cells (FDC) and naive recirculating IgM^+, IgD^+ B cells, and (*iii*) the marginal zone, a layer containing reticular cells, macrophages and resident IgM^+, IgD^- B lymphocytes that surrounds the PALS and follicular areas (Fig. 4 *a*, *c*, and *e*). Recent evidence, has implicated LTα and the p55TNF-R as important regulators of splenic microarchitecture. LTα knockout mice are found defective in all of the components which are characteristic of the presence of lymphoid tissue in the spleen. They lack distinct B and T cell areas and marginal zone markers (15, 17). In contrast to the LTα knockout mice, p55 TNF-R knockout mice show distinct B and T cell areas, yet upon immunization they lack organized FDC networks (40).

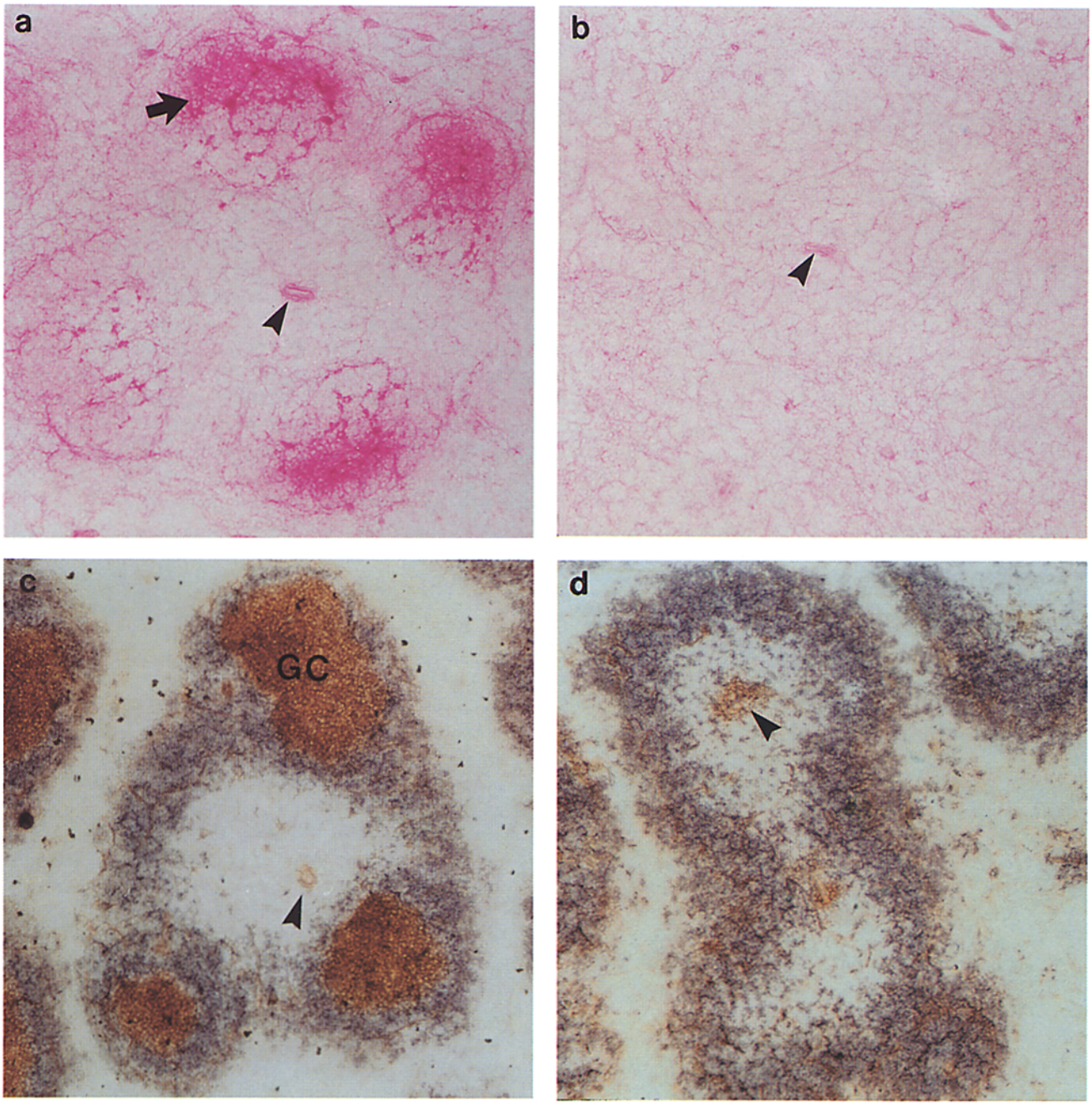
To determine whether TNFα is involved in these activities we have examined splenic structure in non-immunized TNFα-deficient mice. Double immunostaining of $TNF\alpha^{-/-}$ spleen sections with anti-IgM and anti-IgD antibodies revealed the absence of distinct primary B cell follicles. Instead, a thin layer of IgM^+, IgD^+ B cells was observed forming perimetrically underneath the marginal zone (Fig. 4 *b*). In addition, staining of spleen sections with the FDC-M1 antibody, a marker for follicular dendritic cells (28), failed to reveal the presence of FDC networks in TNFα knockout mice (Fig. 4 *f*). However, anti-CD3 staining showed the presence of normal T cell–rich PALS areas (Fig. 4 *d*). Moreover, similarly to the TNFα knockout mice, p55TNF-R-deficient mice (provided by Dr. Horst Bluethmann, Hoffman LaRoche, Switzerland) were also found to lack organized primary B cell follicles (data not shown). Taken together, these results show an absolute requirement for TNFα signaling through the p55TNF-R in the correct development and organization of structured B cell follicles and FDC.

Deficient Germinal Center Formation in TNFa Knockout Mice. To investigate the functional consequences of the primary B cell follicle deficiency, we have followed the ability of $TNF\alpha^{-/-}$ mice to develop FDC networks and germinal centers in their spleens after intraperitoneal immunization with T cell–dependent antigens i.e., SRBC, TNP-KLH in CFA, and *Limulus* hemocyanin (LH) in CFA. Immunocytochemical staining of frozen spleen sections with either FDC-M1 (Fig. 5 *a*) or peanut agglutinin (PNA), a specific marker of germinal center cells (41) (Fig. 5 *c*), showed the existence of typical FDC networks and germinal centers forming within follicles in wild-type control mice. In sharp contrast, a striking absence of both FDCs (Fig. 5 *b*) and typical germinal centers (Fig. 5 *d*) was observed in spleens from immunized $TNF\alpha^{-/-}$ mice, where only some PNA-positive cells could be detected around arterioles inside the T cell–rich PALS.

Complementation of TNFα functioning by the expression of a wild-type human TNFα transgene in double $TNF\alpha^{-/-}$ × Tg1278 mice is sufficient to partly reconstitute the formation of FDC networks and germinal centers. Most of the germinal centers seen in the spleens of such mice are found to form ectopically around arterioles and within the T cell–rich PALS (Fig. 5 *e*). In contrast, expression of murine transmembrane TNFα in the double $TNF\alpha^{-/-}$ × TgA86 mice is sufficient to completely rescue deficient FDC and germinal center formation in TNFα knockout mice (Fig. 5 *f*). It seems therefore that human TNFα signaling is sufficient to drive GC formation in the TNFα knockout mice. Since human TNFα only binds to the murine p55TNF-R and not to the murine p75TNF-R (35), this result indicates that signals through the p55TNF-R but not the p75TNF-R are essential for the formation of B cell follicles, FDC networks and germinal centers. The latter, is further supported by recent findings of morphologically normal GC in p75 TNF-R knockout mice (17).

The critical importance of LTα and p55 TNF-R signaling in germinal center formation has been recently established in mutant mice (17). However, a conclusive answer as to whether LTα mediates this effect through p55 TNF-R or LTβ-R signaling, could not be reached. As shown in this study, TNFα signaling through the p55TNF-R is required for the development of primary B cell follicles and germinal centers. Assuming that in the LTα-deficient mice, TNFα signaling through the p55 TNF-R remains unaffected, it may be postulated that LTα regulates GC formation through the LTβ receptor. This may be further supported by the observed differences in splenic architecture between the LTα knockout mice which completely lack distinct B and T cell areas, and the TNFα or the p55 TNF-R knockout mice which only lack organized B cell follicles.

The responsible mechanism(s) for these defects remains elusive, however, currently existing evidence suggests that it may lie in defective cell trafficking because of defective adhesion molecule expression, as was recently exemplified in p55 TNF-R knockout mice lacking expression of the adhesion molecule MAdCAM-1 in their splenic marginal zone (42). Trafficking or differentiation defects may also explain the absence of organized FDC networks which may in turn explain the impaired formation of primary B cell follicles. In addition to TNFα and LTα, the CD40L, yet another member of the TNF family of ligands, is also necessary for the formation of germinal centers mainly by regulating T cell–dependent B cell activation events (43, 44). Although TNFα is also known to mediate T-B cell interactions (45–48), promote differentiation of macrophages



(49, 50) and survival of Langerhans cells (51), and to induce adhesion molecule expression on follicular dendritic cells (52), it remains to be determined whether one or more of these properties are critically involved in the regulation of primary B cell follicles and germinal center formation.

T Cell–dependent and T Cell–independent Antibody Responses in TNFa Knockout Mice. To investigate the role of TNFα in TD humoral immune responses we tested the primary and secondary antibody response of TNFα-deficient mice to the TD antigen SRBC. Wild-type and TNFα knockout mice were immunized with SRBC on day 0 and 15, and serum antibody responses were determined on day 7, 14, and 23 using SRBC-specific ELISAs. As shown in Fig. 6, TNFα-deficient mice exhibit severely impaired IgG and IgE antibody responses but show a normal level of IgM antibodies. However, despite the observed absence of primary B cell follicles and germinal centers, Ig class-switching could still occur. Defective antibody responses could be reconstituted by either human or murine mutant transmembrane TNFα transgene expression (Fig. 6).

Humoral immune responses were also measured in wild-type, TNFα$^{-/-}$ and TNFα transgene-reconstituted TNFα$^{-/-}$ mice, immunized with a different TD antigen, 2,4,6-trinitrophenyl-conjugated keyhole limpet hemocyanin (TNP-KLH). IgG1, IgG2a, IgG2b, IgG3, and IgE responses were readily detectable in TNFα knockout mice, albeit at a

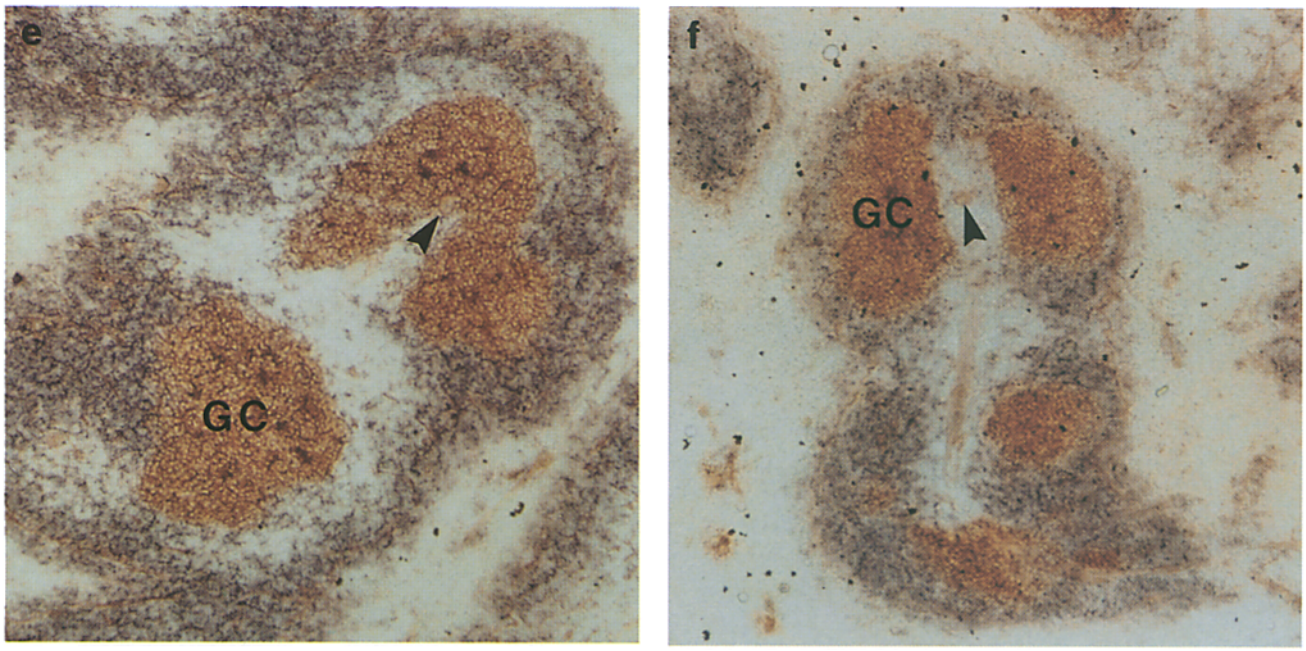


Figure 5. Follicular dendritic cell networks and germinal center formation in immunized TNFα$^{+/+}$, TNFα$^{-/-}$, and TNFα transgene-complemented TNFα$^{-/-}$ mice. Sections of spleens obtained 8 d after immunization with SRBC were stained with either FDC-M1 (*a* and *b*) or PNA (*brown*) and anti-B220 (*blue*) (*c–f*). In TNFα$^{+/+}$ control mice, clusters of FDC-M1-positive cells (*a*) or PNA-positive cells (*brown*) comprising the germinal centers (*GC*) (*c*), are detected. FDC networks (*b*) and germinal centers (*d*) are absent in spleens from TNFα$^{-/-}$ mice, where only some small patches of PNA positive cells are observed around arterioles (*d*). In homozygous knockout mice reconstituted with a human TNFα transgene, large germinal centers are observed forming ectopically inside the T cell–rich PALS and in close proximity to arterioles (*e*). Germinal center formation is completely restored in the spleens from transmembrane murine TNFα transgene-complemented TNFα$^{-/-}$ mice, where typical germinal centers are seen, forming inside the B220-positive follicles at the periphery of the T cell-rich PALS (*f*). Arrowheads denote central arterioles; original magnifications ×100.

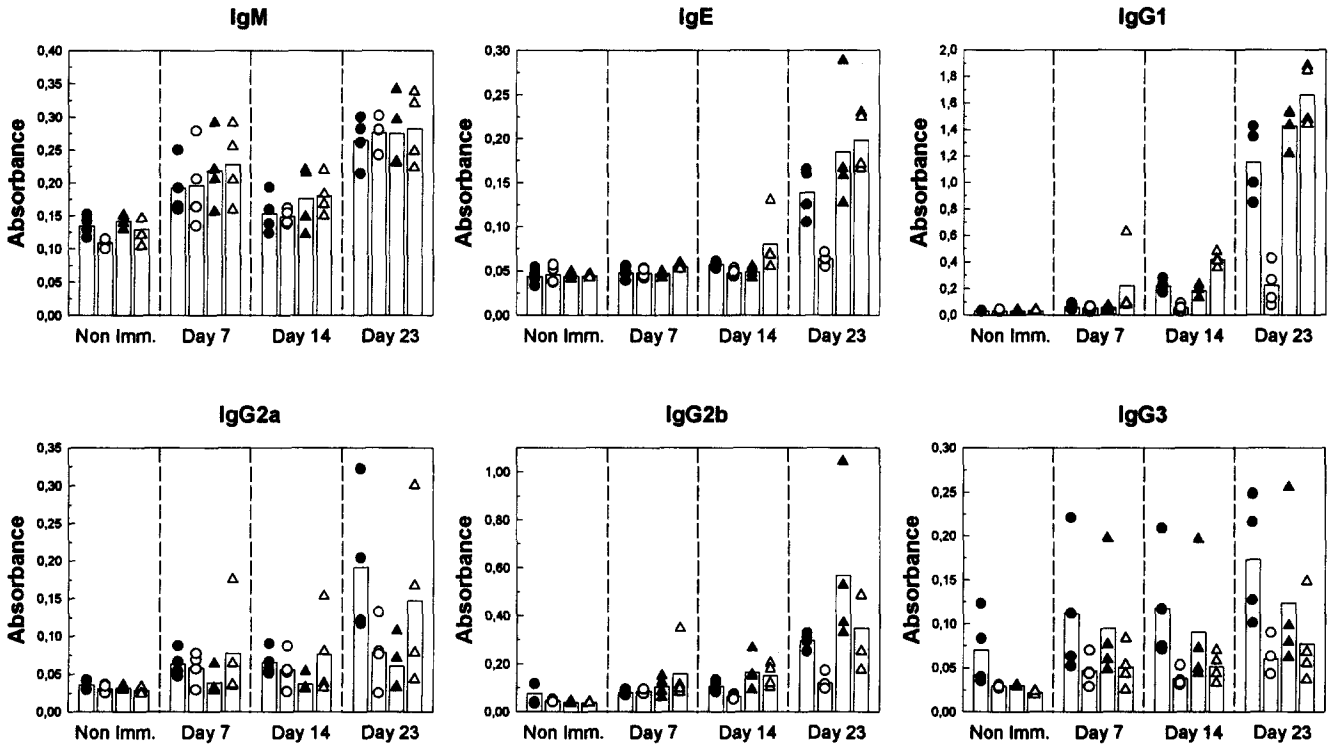


Figure 6. Antibody responses to the TD antigen SRBC. Wild-type (*closed circles*), TNFα$^{-/-}$ (*open circles*), TNFα$^{-/-}$ × Tg 1278 (*closed triangles*) and TNFα$^{-/-}$ × Tg A86 (*open triangles*) mice (n = 4 for all groups) were immunized intraperitoneally with 10^8 SRBC in PBS on day 0 and day 15 and bled on days 7, 14, and 23. Sera obtained from similar groups of non immunized mice matched for genotype, age and sex were used as controls for pre-immune anti-SRBC values. Serum levels of anti-SRBC antibodies were determined using specific ELISAs. Sera were used at a dilution of 1:100. Results are presented in means of duplicate data from individual mice. Open bars represent mean values for each group.

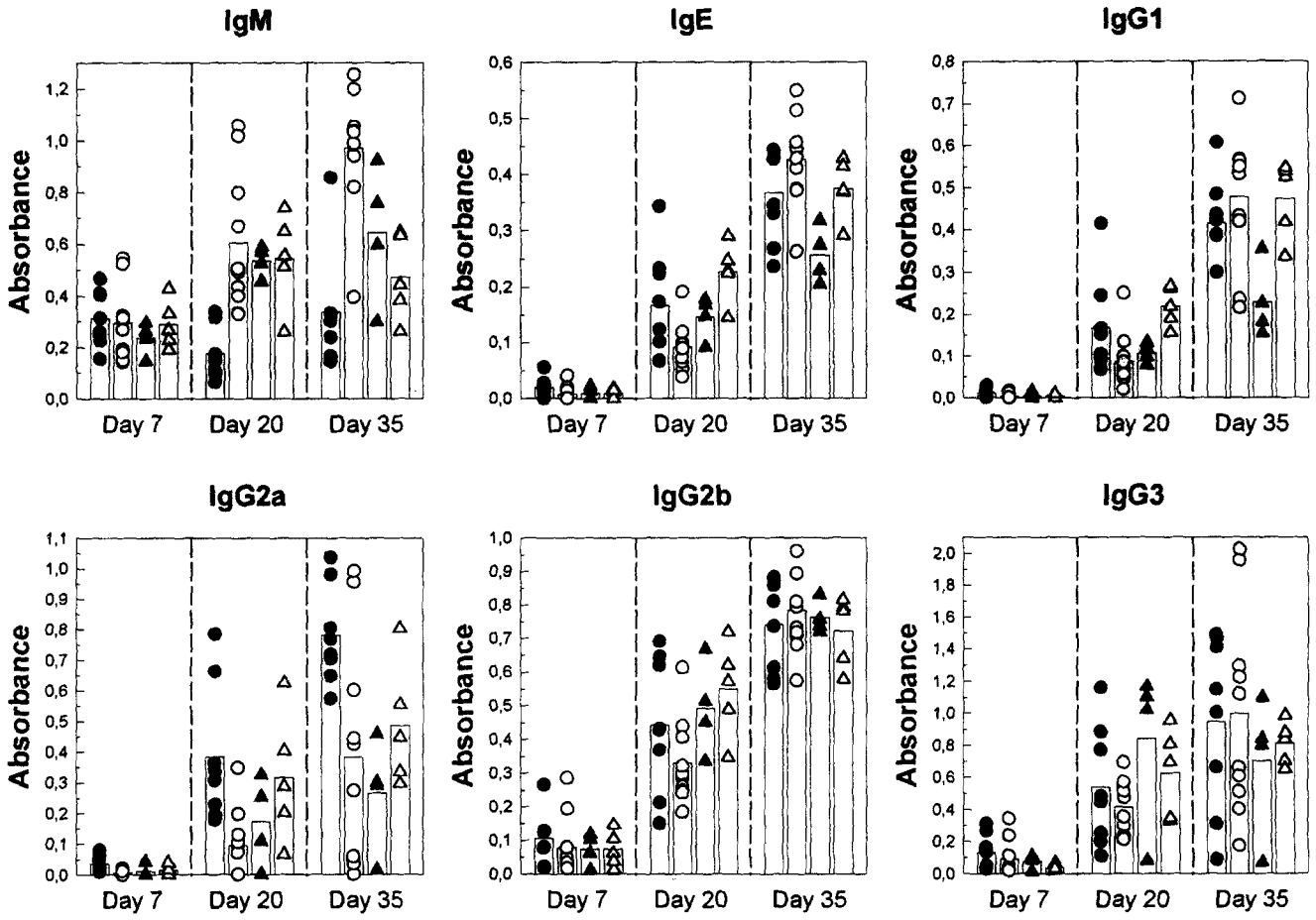


Figure 7. Antibody responses to the TD antigen TNP-KLH. Wild-type (*closed circles*, $n = 8$), TNFα$^{-/-}$ (*open circles*, $n = 10$), TNFα$^{-/-}$ × Tg 1278 (*closed triangles*, $n = 4$) and TNFα$^{-/-}$ × Tg A86 (*open triangles*, $n = 5$) mice were immunized intraperitoneally with 50 μg TNP-KLH in complete Freund's adjuvant on day 0, boosted on day 28 and bled on days 7, 20, and 35. Serum levels of anti-TNP antibodies were determined using isotype-specific ELISAs. Sera were diluted 1:1,000 for measuring IgM and IgE antibodies, and 1:20,000 for measuring IgG-subclass antibodies. Results are presented in means of duplicate data from individual mice. Open bars represent mean values for each group. Similar results were obtained when mice were immunized with 1 μg TNP-KLH in CFA.

lower level in comparison to wild-type controls, 20 d after the primary immunization (Fig. 7). However, upon secondary immunization the magnitude of the IgE and IgG isotype response was similar to wild-type controls. This was not the case in IgG2a responses which were found severely compromised in TNFα$^{-/-}$ mice in comparison to the normal controls suggesting a role for TNFα in directing this specific recombination. In addition, TNFα-deficient mice exhibit enhanced TNP-specific IgM antibody titters in comparison to the wild-type controls probably as a consequence of incomplete class switching. Reconstitution of the TNFα deficiency in the TNFα$^{-/-}$ × Tg1278 and TNFα$^{-/-}$ × TgA86 double transgenic mice, normalizes antibody responses (Fig. 7). Taken together, these results suggest that although isotype switching can still occur in the absence of germinal centers and FDCs, prolonged antibody responses are generally impaired in TNFα knockout mice, most probably because of a defect in sustained antigen presentation. Adjuvant-aided immunization (as seen in the TNP-KLH immunization) seems to compensate for this defect, probably by trapping the antigen in a local deposit and by reducing its rate of dispersal.

Among cytokines, NK cell–derived TNFα has been implicated in regulating humoral immune responses to TI antigens (53). The ability of TNFα knockout mice to produce antibodies in response to TI antigens, was measured after immunization of mice by either a type 1 (TNP-LPS) or a type 2 (TNP-Ficoll) TI antigen. In both cases, IgM antibody responses of TNFα$^{-/-}$ mice were found similar to wild-type controls (Fig. 8). However, anti-TNP IgG responses to TNP-LPS were found slightly decreased 14 d after immunization. As type I TI antigens are believed to be immunogenic mainly through polyclonal B cell activation (53), the decreased responses observed in TNFα knockout mice may reflect participation of TNF in this process. In contrast, TNFα knockout mice mounted enhanced IgG responses to TNP-Ficoll (Fig. 8 *b*) which may be due to the observed structural deficiencies in B cell compartments

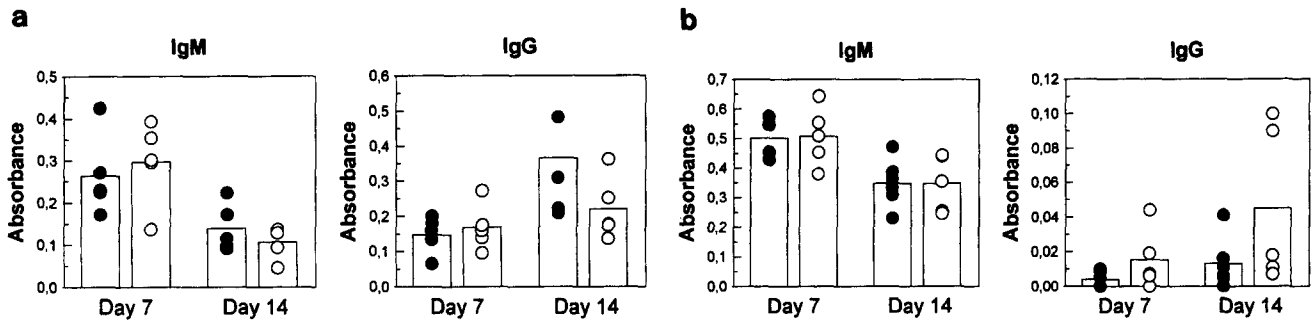


Figure 8. Antibody responses to TI antigens. TNFα knockout (*open circles*) and wild-type (*closed circles*) mice were injected intraperitoneally with 50 μg TNP-LPS (*a*) or 25 μg TNP-Ficoll (*b*) in PBS on day 0, and bled on days 7 and 14. Levels of IgM and IgG anti-TNP antibodies were determined using specific ELISAs. For all ELISAs 1:1,000 dilution of sera were used. Results are presented in means of duplicate data from individual mice. Open bars represent mean values for each group.

of TNFα knockout mice. Indeed, marginal zone B cells have been implicated in mediating responses to type II TI antigens (54, 55). It will be interesting to investigate whether additional structural perturbations may be localized in B cell containing areas other than follicles, as for example in the marginal zone.

In conclusion, our findings demonstrate the critical role of TNFα in LPS-mediated toxicity, anti-bacterial host defense and contact hypersensitivity reactions. Furthermore, they establish a critical requirement for this factor in processes regulating the formation of primary B cell follicles, FDC networks and germinal centers and in the physiological maturation of the humoral immune response. These results should prove useful in the further understanding of the biological consequences of a long term blockade of TNF functioning, as a means of treating chronic inflammatory and autoimmune pathology in human patients. TNFα knockout mice should also provide an important model for further analyzing the functions of B cell follicles and FDCs in peripheral B cell homing, survival, and differentiation.

We wish to thank Yong-Jun Liu for critical discussions, Horst Bluethmann for providing the p55TNF-R knockout mice, Wim Buurman for the murine TNFα-specific ELISA, Steve Cobbold for the KT3 anti-CD3 antibody, David Chaplin for helpful discussions on germinal center structure, Marie Kosco-Vilbois for the FDC-M1 antibody, Claude Leclerc for reagents and advice on immunization protocols, and Genevieve Millon for providing *L. monocytogenes* strain L028.

This project was supported in part by the Hellenic Secretariat for Research and Technology and European Union Grants, BIO-CT94-2092 and ERBCHRX-CT930182.

Address correspondence to Dr. George Kollias, Department of Molecular Genetics, Hellenic Pasteur Institute, 127 Vas Sophias Avenue, 115 21 Athens, Greece.

Received for publication 11 April 1996 and in revised form 16 July 1996.

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