Differentiation of Follicular Dendritic Cells and Full Antibody Responses Require Tumor Necrosis Factor Receptor-1 Signaling

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Summary

Using mice double deficient for tumor necrosis factor (TNF) and lymphotoxin alpha (LTα), we demonstrated that TNF and/or LTα are necessary for development of a normal splenic microarchitecture and for isotype switch after immunization with sheep red blood cells (SRBC). In the present study, we extended these observations by determining which TNF receptor (TNFR) is involved in morphological and functional differentiation of the spleen. Spleen morphology and antibody response were investigated in wild-type, $TNFR1^{-/-}$, $TNFR2^{-/-}$, and $TNF/LT\alpha^{-/-}$ mice immunized with SRBC. $TNF/LT\alpha^{-/-}$ mice, which have a complete disruption of the TNF/LTα signaling system including the LTβ-receptor pathway, displayed an abnormal microarchitecture, and isotype switch did not take place. $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice displayed a normal spleen microarchitecture and mounted an IgM and IgG antibody response to SRBC. However, the IgG production in $TNFR1^{-/-}$ mice was minimal, with titers leveling off 6 d after immunization. In this strain, immunofluorescence revealed a lack of follicular dendritic cells (FDC) network, detected with FDC-M1 as well as anti-CR1, and a lack of germinal centers, detected with peanut agglutinin. In conclusion, whereas normal splenic microarchitecture and isotype switch might require the LTβ receptor, differentiation of FDC network, development of germinal centers, and full IgG response depend on signaling via TNFR1.

We demonstrated previously that inactivation of TNF–lymphotoxin alpha (LTα) genes resulted in multiple abnormalities of the immune system with absence of LNs, lymphocytosis, hypoimmunoglobulinemia, an undifferentiated spleen, and defective antibody response to sheep red blood cells (SRBC) (1). LTα-deficient mice also display an abnormal morphogenesis of lymphoid organs and a defective antiviral IgG response (2, 3). TNF and LTα are members of a family of homologous proteins, including CD40 ligand, which is involved in T cell–B cell interaction (4). TNF and LTα form homotrimers that bind to two different receptors, TNFR1 and TNFR2 (4). LTα additionally binds, as a membrane-bound heterotrimer 1LTα/2LTβ, to the LTβ receptor (5).

In the present study, we investigated the splenic morphology and the antibody response after SRBC immunization in wild-type mice and in mice deficient in either TNFR1, TNFR2, or both ligands TNF and LTα. The data suggest that signaling via TNFR1 is important for the differentiation of follicular dendritic cells (FDC) in the spleen, the development of germinal centers, and T–cell dependent antibody response, whereas signaling via the LT-specific receptor is necessary for a normal microarchitecture and isotype switch.

Materials and Methods

Mice. Wild-type, $TNF/LT\alpha^{-/-}$ (1), $TNFR1^{-/-}$ (6), and $TNFR2^{-/-}$ mice (7) on a mixed 129SV × C57BL/6 background were bred under specified pathogen-free conditions.

Reagents. Primary antibodies for immunofluorescence were rat anti–mouse monoclonals: anti-B220 (clone RA3-6B2), anti-CD3 (clone OKT3), anti-FDC (clone FDC-M1), and anti-CR1 (clone 8C12). The second antibody was Cy3-labeled goat anti–rat (Jackson ImmunoResearch Laboratories, Inc., West Grove,

PA). Biotinylated peanut agglutinin (PNA), used as a marker for germinal centers, was detected by Cy3-labeled streptavidin (Jackson ImmunoResearch Laboratories, Inc.).

Immunization. Four mice of each strain, eight to twelve wk old, were injected intraperitoneally at day 0 with 2×10^8 sterile sheep erythrocytes (SRBC). Blood samples were taken at days 0, 6, and 15 from the retroorbital plexus. At day 15, the mice were killed by heart bleeding under methoxyflurane anaesthesia. The experiment was duplicated.

Histology. Spleen samples were fixed by immersion in 4% neutral buffered formalin. They were processed for paraffin sectioning according to routine techniques. Sections of 3-μm thickness were stained with hematoxilin-eosin.

Immunofluorescence. Halves of the spleens were snap-frozen in isopentane maintained at the temperature of liquid nitrogen. 6-μm-thick sections were cut in a cryostat, air-dried, and stored at $-80°C$. Immediately before use, the sections were fixed in acetone for 10 min at 4°C. After a wash in phosphate-buffered saline (PBS), the sections were incubated for 16 h at 4°C with the primary rat antibodies or with biotinylated PNA. After two washes in PBS, incubation with labeled goat anti–rat antibody (preadsorbed with mouse serum) or with labeled streptavidine took place at room temperature for 1 h. The sections were examined with a laser scanner microscope (Carl Zeiss, Zurich, Switzerland).

Assay of Immunoglobulins. Serum titers of anti-SRBC–specific IgM, IgG1, and Ig2b were determined by a sandwich ELISA. Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 50 μl of a solubilized extract (3 mg/ml) from SRBC

prepared according to Kelly (8). Thereafter, plates were blocked with 2% BSA in PBS for 2 h at 37°C and incubated with serial dilutions of the immune sera overnight at room temperature. Bound antibodies were detected with biotinylated goat anti–mouse Ig isotype–specific antibodies for 4 h at room temperature. These plates were developed by the addition of streptavidin–alkaline phosphatase and then of substrate for 45 min each, and the reaction was stopped with 1.5 M NaOH. Absorbance was read at 405 nm.

Results and Discussion

Microarchitecture. In contrast to wild-type, $\text{TNFR1}^{-/-}$, and $\text{TNFR2}^{-/-}$ mice, the border of the white pulp was diffuse and the marginal zone was lacking in the spleens of $\text{TNF/LT}\alpha^{-/-}$ mice (Fig. 1). The periarterial lymphatic sheaths and the follicular area, identified by the high densities of T cells (CD3 positive) and B cells (B220 positive), respectively, were well delimited in wild-type, in $\text{TNFR1}^{-/-}$, and in $\text{TNFR2}^{-/-}$ mice (Fig. 2, *A* and *B*), but they were hardly recognizable in $\text{TNF/LTa}^{-/-}$ mice (Fig. 2, *C* and *D*). The same alterations of spleen microarchitecture as found in $\text{TNF/LT}\alpha^{-/-}$ mice have been reported by others in $\text{LT}\alpha^{-/-}$ mice (2, 3). It is likely that normal spleen morphogenesis, since it is maintained in mice lacking either of the two TNF receptors, requires $\text{LT}\alpha$ acting via a specific LT receptor.
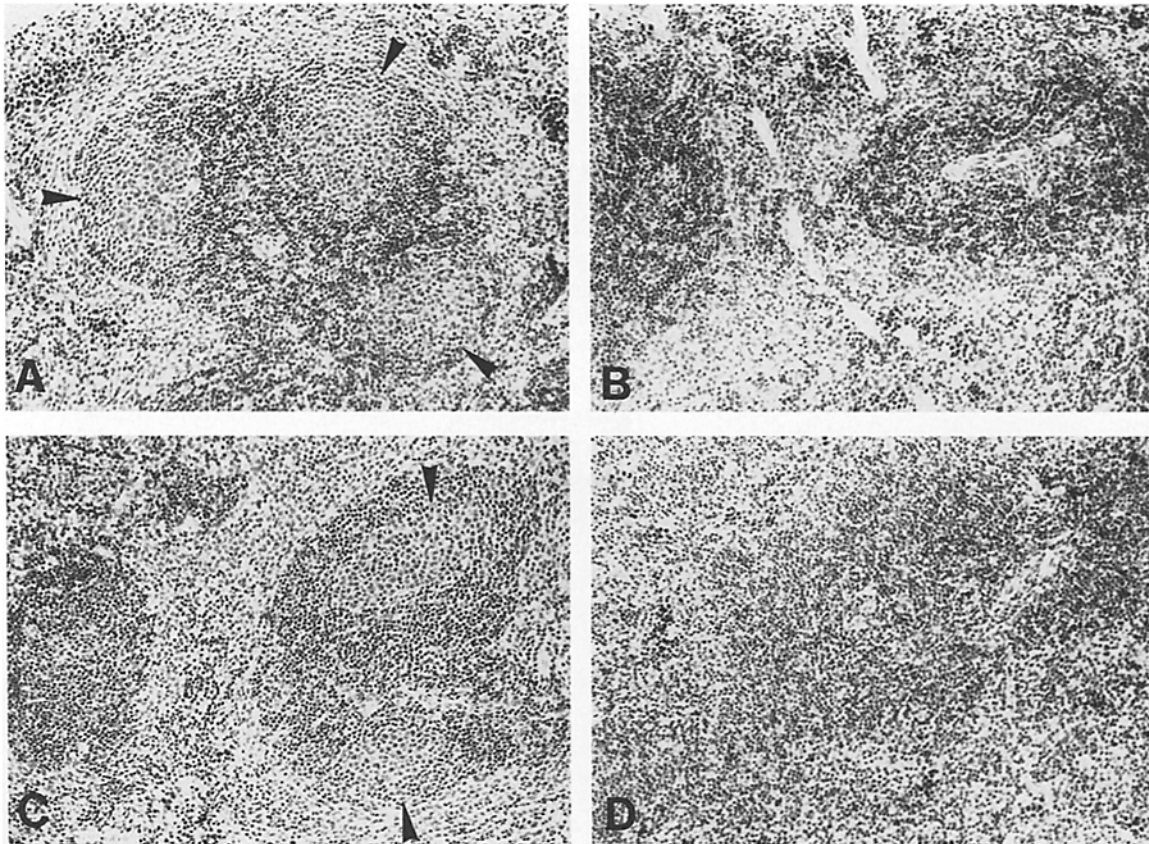

Figure 1. Histological preparations of spleens from wild-type (*A*), $\text{TNFR1}^{-/-}$ (*B*), $\text{TNFR2}^{-/-}$ (*C*), and $\text{TNF/LTa}^{-/-}$ (*D*) mice. Germinal centers (*arrowheads*) are present only in tissues from wild-type and $\text{TNFR2}^{-/-}$ mice. ×130.
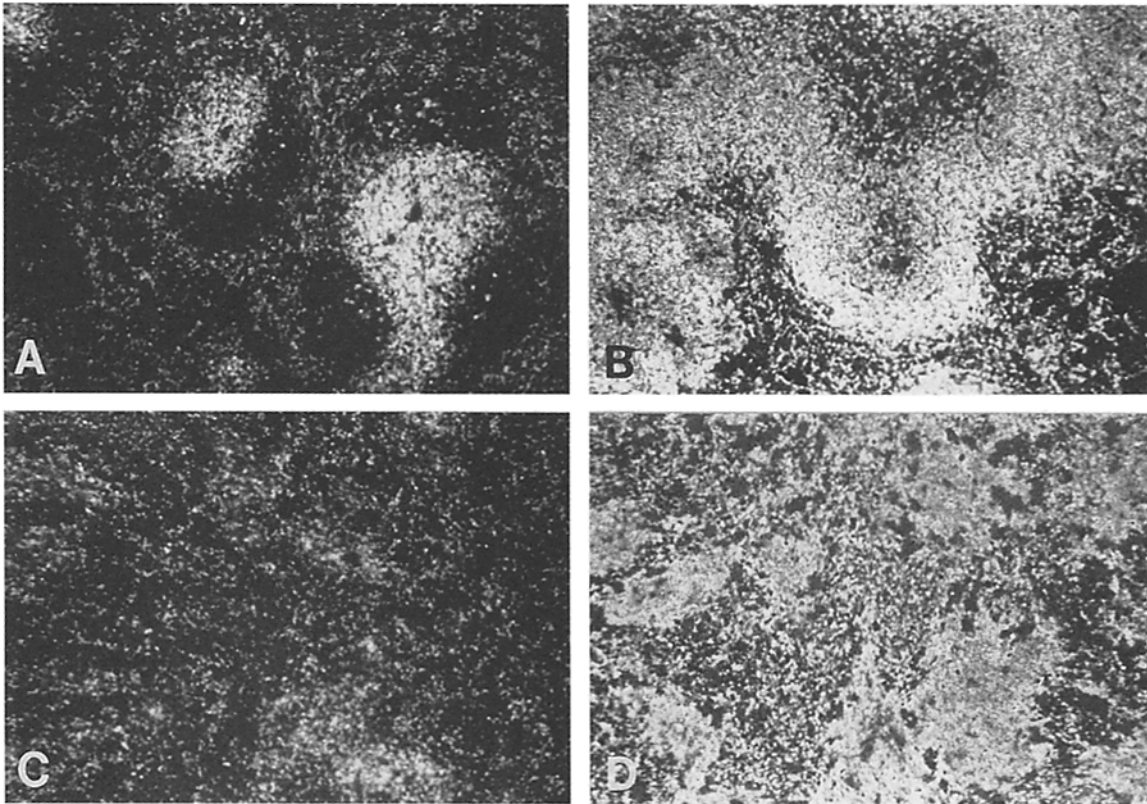
Figure 2. Distribution of T cells (CD3 immunofluorescence; *A* and *C*) and of B cells (B220 immunofluorescence; *B* and *D*) in the spleen. Similar patterns as shown for TNFR1$^{-/-}$ mice (*A* and *B*) were found in wild-type and TNFR2 mice. In those strains, the periarterial lymphatic sheaths display the highest incidence of T cells (*A*). B cells are located predominantly at the periphery of the white pulp (*B*). That organization is lost in TNF/LTα$^{-/-}$ mice (*C* and *D*). ×60.
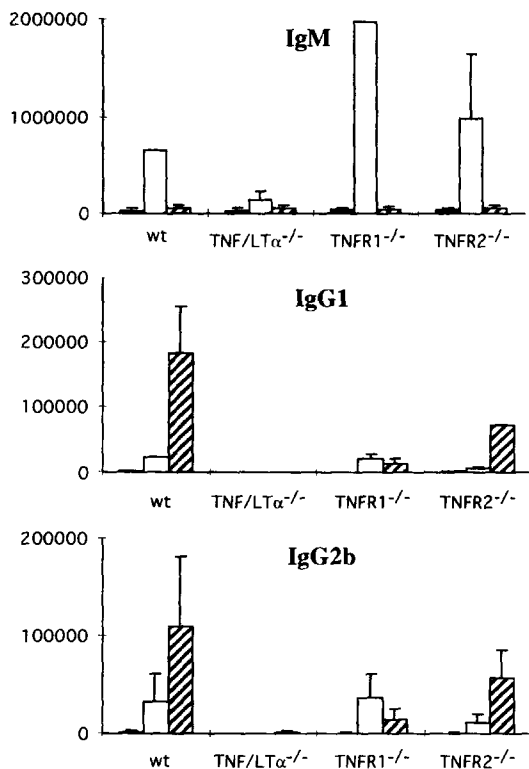
Humoral Response. Isotype switch was normal in wild-type, TNFR1$^{-/-}$ and TNFR2$^{-/-}$, but it did not take place in TNF/LTα$^{-/-}$ mice (Fig. 3) as previously reported (1). Similarly, specific IgGs were not produced in LTα$^{-/-}$ mice challenged with different antigens (3). Together these data suggest that isotype switch might require signaling via LTβ receptor. Deletion of TNFR1 did not affect the IgM response. However, a decrease of IgG level was observed between day 6 and day 15 in TNFR1$^{-/-}$ mice instead of the further increase found in the wild type and in TNFR2$^{-/-}$ mice (Fig. 3). The importance for long lasting IgG response of antigen persisting in the form of immune depots has been demonstrated (9). Thus, the absence of a sustained IgG antibody production suggested that the antigen source driving a prolonged humoral response might be defective in TNFR1$^{-/-}$ mice. FDC fulfill this function by providing antigen to activated B cells in germinal centers (10–13).

FDC and Germinal Centers. We assessed the presence of FDC, using the anti-CR1 and FDC-M1 antibodies (Fig. 4), and of germinal center B cells, using PNA (Fig. 5). Both

Figure 3. Serum SRBC–specific immunoglobulin levels. Titers at day 0 (*filled bars*), 6 (*empty bars*) and 15 (*striped bars*) are expressed as the reciprocal value of the dilution showing an optical density of 0.1 over background. Mean values for four mice are given with the SD.
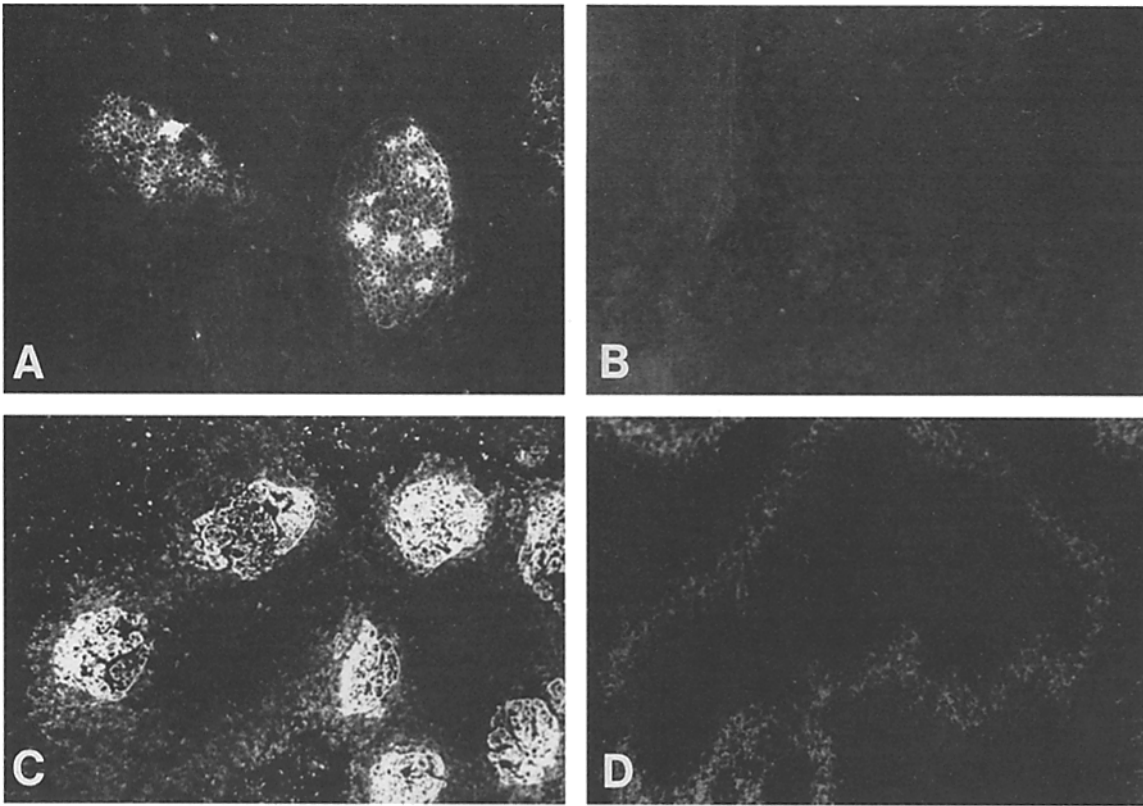
Figure 4. Detection of FDC in the spleen using the FDC-M1 (*A* and *B*) and anti-CR1 (*C* and *D*) antibodies. Similar patterns as in wild-type mice (*A* and *C*) were observed in TNFR2$^{-/-}$ mice. FDC were not detected in TNFR1$^{-/-}$ (*B* and *D*) nor in TNF/LT$\alpha^{-/-}$ (not shown) mice. ×120.
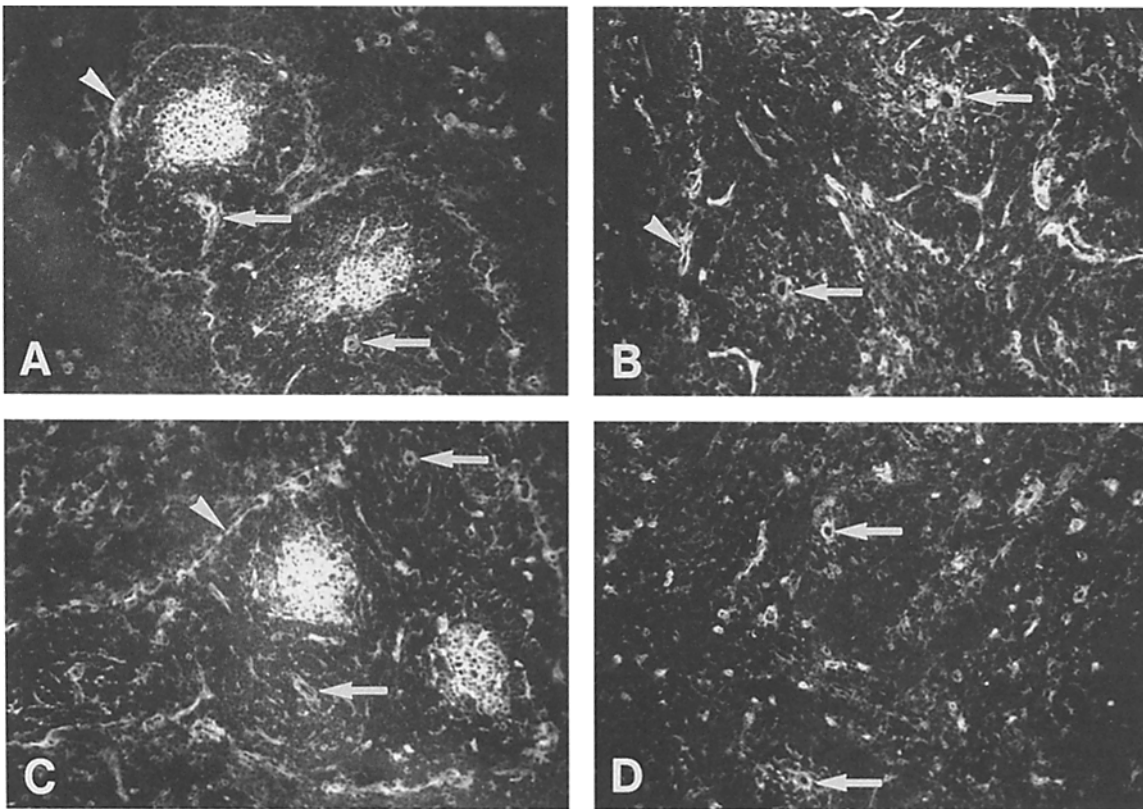

Figure 5. PNA binding in the spleen of wild-type (*A*), TNFR1$^{-/-}$ (*B*), TNFR2$^{-/-}$ (*C*), and TNF/LT$\alpha^{-/-}$ (*D*) mice. PNA-positive germinal center B cells are visible only in *A* and *C*. The labeled structure surrounding the white pulp in *A–C* (*arrowheads*) might represent the marginal sinus. Arrows point to central arterioles. ×120.

were detected in the spleens of wild-type and TNFR2$^{-/-}$ mice, but neither were detected in TNF/LT$\alpha^{-/-}$ nor TNFR1$^{-/-}$ mice. Likewise, routine histology did not reveal germinal centers in the latter strain (Fig. 1). The weak labeling with anti-CR1 in the follicular area likely represents binding to B cells (14).

These data provide the first evidence that a signal from the TNFR1 is required for the development of germinal centers and the generation of a full IgG-antibody response. Since mature FDC were not detected, we propose that the lack of germinal center formation in TNFR1$^{-/-}$ mice results from a deficient delivery of antigen to activated B cells. An interesting finding is that IgG switch was not affected. It appears then that the signals involved in the initial IgG switch are not sufficient for a sustained production of IgG, the latter being dependent on FDC. The proposition that FDC might be involved in isotype switch as well (15) is not supported by the present data.

It is a matter of discussion whether FDC represent a functional specialization of the reticular meshwork cells (15–19) or whether they are of bone marrow origin and

migrate to lymphoid organs as antigen-transporting cells (20). Therefore, it is difficult at the present time to propose a hypothesis about how signaling via TNFR1 might influence their differentiation.

Outlooks. Clearly, the TNFR1$^{-/-}$ mice represent an excellent tool to dissect further the mechanisms leading to IgG class switch, the selection of high-affinity antibody-producing B cells, and development of memory B cells. In this context, the available data strongly suggest a normal maturation of B cells in this strain. Indeed, TNFR1 deletion affects neither hematopoiesis (6, 21) nor the homing of B cells at the periphery of the periarterial lymphatic sheath in spleen (Fig. 2). Moreover, the IgM response to SRBC immunization as well as the IgG switch took place normally, indicating a normal interaction between B cells and T cells in TNFR1$^{-/-}$ mice (Fig. 3).

In conclusion, our data demonstrate that the differentiation of FDC networks, the development of germinal centers, and a sustained antibody response depend on a signal delivered through the TNFR1.

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