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Regulation of TNF-α Release From Bone Marrow–Derived Macrophages by Vitamin D

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Abstract The calcium-regulating hormone 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] is recognized as an immunomodulator affecting the activities of macrophages and lymphocytes. We have shown that macrophages harvested from vitamin D–deficient mice (−D MPs) exhibit impaired phagocytic and tumoricidal activities as compared with control cells (+D MPs), and that bone marrow–derived macrophage (BMDM) differentiation is modulated by 1,25$(OH)_2D_3$. The release of tumor necrosis factor–α (TNF-α) by macrophages is considered a major mechanism by which these cells exert their tumoricidal function. This cytokine was also implicated in modulation of bone resorption. In the present study we examine the role of 1,25$(OH)_2D_3$ in TNF-α synthesis and release. BMDMs were harvested from +D and −D mice, cultured in vitro, and their conditioned media were analyzed for the presence of TNF-α. BMDMs did not release measurable amounts of TNF-α without stimulation. Addition of endotoxin (LPS) to the cultures resulted in a marked stimulation of TNF-α release. 1,25$(OH)_2D_3$ increased the stimulatory action of LPS, but failed to elicit a stimulatory effect in the absence of LPS. The use of another macrophage activator, interferon-γ (IFN-γ), yielded essentially similar results. +D and −D mice were injected with LPS and TNF-α levels in the serum were measured. A marked reduction (~fourfold) in the TNF-α levels was observed in the serum of −D mice as compared with +D mice. Western blot and immunoprecipitation analyses suggested that the main effect of 1,25$(OH)_2D_3$ is on TNF-α synthesis. Our findings suggest that 1,25$(OH)_2D_3$ plays a role in the regulation of TNF-α secretion by mononuclear phagocytes.

Key words: macrophage activation, interferon γ, endotoxin, bone resorption

The active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is well recognized for its central role in calcium homeostasis mediated by its interactions with specific receptors in the intestine, the kidney, and the bone [DeLuca, 1979; Minghetti and Norman, 1988; Stumpf et al., 1979]. A large amount of evidence accumulated in more than a decade indicates that the distribution of vitamin D receptors is more global than originally thought, and nearly every vertebrate cell type expresses this receptor [Minghetti and Norman, 1988]. Cells belonging to the immune system were shown to contain functional receptors and therefore it was proposed that 1,25$(OH)_2D_3$ may act as a natural immunomodulator [Manolagas et al., 1985; Rigby, 1988; Suda et al., 1986]. More than 20 years ago it was shown that vitamin D–deficient rachitic infants experience frequent respiratory infections [Stroder and Kasal, 1970] which could be caused by impaired functions of phagocytes. We therefore examined the role of vitamin D in macrophage activity and found that the ability of vitamin D–deficient mice to generate inflammatory responses is impaired [Bar-Shavit et al., 1981]. This included reduced macrophage phagocytic capability and neutrophil migration. More recently we demonstrated that macrophage activation is also impaired in vitamin D deficiency, and that macrophages harvested from vitamin D–depleted mice are less effective in their tumoricidal functions as compared to macrophages obtained from control littermates (vitamin D–replete mice) [Gavison and Bar-Shavit, 1989]. Extensive literature exists regarding the in vitro induction of monocytic differentiation in murine and human leukemic cell lines [Abe et al., 1981; Amento et al., 1984; Bar-Shavit et al., 1983; Mangelsdorf et al., 1984; McCarthy et al., 1983; Miyaura et al., 1981; Reitsma et al., 1984; Tanaka et al., 1982]. It is not clear if these findings represent effects of 1,25$(OH)_2D_3$ on trans-

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formed cells (since the hormone affects various types of transformed cells) [Cohen et al., 1988; Eisman et al., 1987, 1989], or interaction with authentic mononuclear phagocytes. Studies using peripheral blood monocytes and in vitro differentiating bone marrow–derived mononuclear phagocytes demonstrated, in general, that the hormone enhanced differentiation in this lineage [Clohisy et al., 1987; Gluck and Weinberg, 1987; McCarthy et al., 1983; Miyaura et al., 1982; Provvedini et al., 1986]. While these in vitro studies are of interest, it seems to us that studies involving cells obtained from vitamin D–deficient animals are more relevant physiologically. To this end we have used bone marrow cultures and compared the effects of vitamin D in vivo and in vitro [Abu-Amer and Bar-Shavit, 1993]. We found that the use of the bone marrow–derived cultures is superior to the use of peritoneal cells for examinations of the in vitro effects of $1,25(OH)_2D_3$, since the former cells are relatively immature and are better targets for the in vitro modulatory effects of $1,25(OH)_2D_3$ than the relatively mature peritoneal macrophages [Gavison and Bar-Shavit, 1989].

Mononuclear phagocytes synthesize and secrete a variety of regulatory molecules. One of their important secreted products is tumor necrosis factor–α (TNF-α). This cytokine, which is produced mainly by activated macrophages, possesses a variety of biological activities. Originally, TNF-α was described as a factor capable of tumor cell lysis [Carswell et al., 1975; Mannel et al., 1980], but it is now also recognized as an important immunomodulator [Fiers, 1991]. In addition, it was demonstrated that TNF-α is a potent stimulator of bone resorption [Bertolini et al., 1986; Thomson et al., 1987].

We have demonstrated that macrophages harvested from vitamin D–deficient mice exhibit impaired tumoricidal capability. The key role TNF-α plays in this function, and the more global importance of this cytokine in immunomodulation and bone resorption, prompted us to examine, in the present study, the role played by vitamin D in regulation of TNF-α release and production.

MATERIALS AND METHODS

Mice

BALB/c male mice (HSD, Indianapolis, IN) were used. Vitamin D deficiency was obtained as described previously [Bar-Shavit et al., 1981; Gavison and Bar-Shavit, 1989].

Cells

Bone marrow–derived mononuclear phagocytes (BMDMs) were collected according to Tushinsky et al. [1982] using slight modifications [Abu-Amer and Bar-Shavit, 1993; Clohisy et al., 1987]. Briefly, cells were collected from mouse tibia and femurs. The nucleated cells (10^6/ml) were seeded into tissue culture dishes at a density of 0.34×10^6 cells/cm^2 in α-MEM containing 15% FCS and 10% L929 cell-conditioned medium as a source for macrophage colony stimulating factor (complete medium). After 24 hr (37°C, 5% CO_2), nonadherent cells were collected, pelletted, and resuspended in the above medium. These cells were used for the various experiments. Thioglycollate-induced peritoneal macrophages were obtained as previously described [Bar-Shavit et al., 1981; Gavison and Bar-Shavit, 1989] and cultured in α-MEM containing 10% FCS.

Protein

The cellular protein contents were measured according to Bradford [1976] using a microplate reader.

ELISA

The method of Sheehan et al. [1989] was followed. Briefly, ELISA plates (Nunc-Immunoplate-maxisorp) were coated with monoclonal anti-TNF-α antibodies (TN3-19.12, Genzyme, Cambridge, MA) (3.5 μg/ml) for 18 hr at 4°C, washed, and 3% BSA was added to block nonspecific binding. Plates were washed and samples or standards (50 μl/well) were added for 18 hr at 4°C, washed, and 0.1 ml of 1:500 rabbit anti-murine TNF-α polyclonal antibodies (Genzyme, Cambridge, MA) was added for 1 hr at room temperature. After extensive washes, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000, Genzyme, Cambridge, MA) was added for 1 hr at room temperature. Plates were then washed and alkaline phosphatase activity was measured using Dynatech plate reader. For the in vivo studies, +D and −D mice were injected intraperitoneally with LPS (1 mg/0.5 ml/mouse). TNF-α levels in blood were analyzed in triplicates as described [Sheehan et al., 1989].

Western Blot Analysis

Conditioned media of BMDMs were collected and proteins were precipitated with 10% TCA (1 hr on ice). The pellet was solubilized in 0.1 N

NaOH. This solution was mixed 1:1 with lysis buffer (1.0% NP-40, 50 mM Tris, 150 mM NaCl, pH 8.0). Cell monolayers were washed, collected, and lysed in ice-cold lysis buffer for 30 min. After removal of cellular debris, the preparations (5 μg protein/lane) were subjected to SDS-PAGE (12.5%) [Laemmli, 1970], proteins were transferred [Matlashewski et al., 1986; Towbin et al., 1979], and Western analysis was performed using polyclonal rabbit anti-murine TNF-α antibodies.

Metabolic Labeling and Immunoprecipitation

BMDM monolayers were washed with methionine-free medium and incubated in the presence of 100 μCi/ml [^{35}S] methionine (NEN Dupont, Dreiech, Germany) in the same medium for 6 hr. Cells were then collected and lysed in lysis buffer as described above. Cell lysates and conditioned media were immunoprecipitated with polyclonal rabbit anti-murine TNF-α antibodies, following pre-clearing with normal rabbit serum. Immune complexes were precipitated with protein A-Sepharose (Sigma, St. Louis, MO) [Harlow and David, 1988]. Immunoprecipitates were washed with 1.0% NP-40 containing Tris buffer, and electrophoresed on 12.5% SDS-PAGE. Autoradiography was performed at −70°C.

Statistical Analysis

The Mann–Whitney U-test (two tails) was used for the analyses. The experiments were performed in 4–6 replicates. Data are presented as mean ± SD.

RESULTS

In preliminary studies we have defined the optimal conditions for the measurements of TNF-α release. We have found that the presence of either FCS or BSA interfered with the ELISA and therefore the actual studies were performed in medium without FCS or BSA.

The levels of TNF-α found in media conditioned by BMDMs in the absence of stimuli (LPS, IFN-γ) were near the sensitivity limit of the assay (30 pgs, 0.12 units). In order to determine the optimal duration of LPS stimulation, +D BMDMs were plated and incubated for 72 or 48 hr in complete medium, washed, and incubated for 6, 24, and 48 hr in the presence of different doses of LPS. Figure 1 shows that LPS induces TNF-α release from the cells in a dose-dependent manner. The levels of the cytokine in media collected after 6 and 24 hr LPS stimulation were not significantly different, while after 48 hr, probably due to a decreased viability of the cells and/or degradation of the cytokine, TNF-α levels were much lower. Therefore, in the following experiments we chose to perform our analyses from 6 hr LPS stimulation.

Next we examined the effect of $1,25(OH)_2D_3$ on LPS-induced TNF-α release from BMDMs. Figure 2 shows that the steroid alone was unable to induce TNF-α release. On the other hand, 50 nM of $1,25(OH)_2D_3$ increased the LPS-induced TNF-α release from BMDMs. This effect was observed with both +D and −D cells stimulated by 0.1–10 μg/ml of LPS. At the high dose of LPS (10 μg/ml) there was no significant

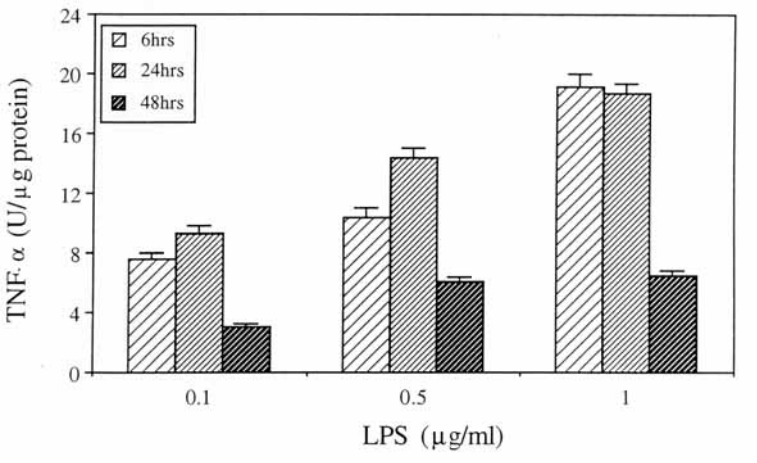

Fig. 1. TNF-α levels in conditioned media of +D BMDMs incubated for various periods with LPS. BMDMs were incubated for 48 hr (1.5×10^5/well) and incubated with LPS for an additional 48 hr, or incubated for 72 hr (10^5/well) and incubated with LPS for an additional 6 or 24 hr. TNF-α levels were measured by ELISA.

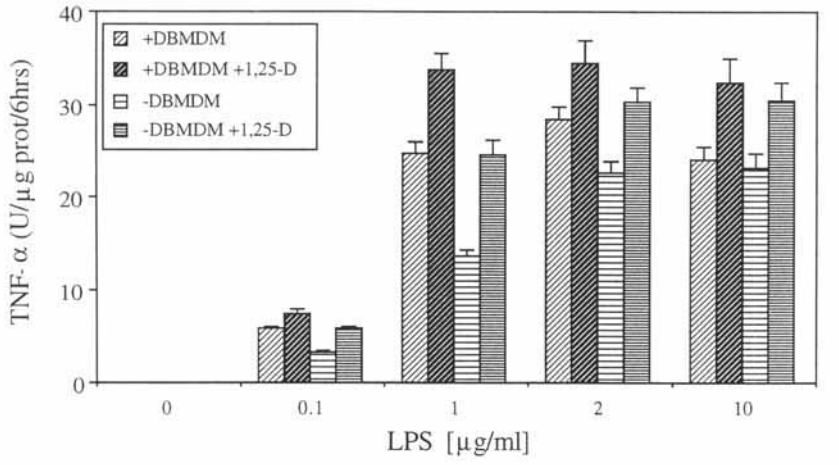

Fig. 2. The effect of $1,25(OH)_2D_3$ on the release of TNF-α from LPS-stimulated BMDMs. BMDMs were plated for 4 d in the presence or absence of 50 nM $1,25(OH)_2D_3$. Monolayers were washed and challenged with LPS for 6 hr. TNF-α levels were measured by ELISA.

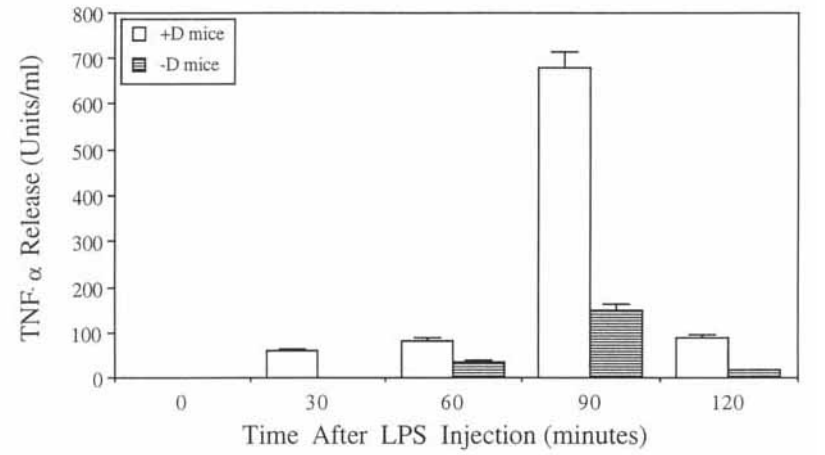

Fig. 3. The effect of $1,25(OH)_2D_3$ on the release of TNF-α from PMs. PMs were plated for 3 d in the presence or absence of 0.5–50 nM $1,25(OH)_2D_3$. Monolayers were washed and challenged with LPS for 6 hr. TNF-α levels were measured by ELISA.

difference between the ability of +D and −D BMDMs to release TNF-α. We have also assessed TNF-α release from peritoneal macrophages (MPs). In contrast to BMDMs, MP-derived conditioned medium contained TNF-α also in the absence of a stimulating agent (not shown). Similarly to the BMDMs, −D MPs released less TNF-α than their +D counterparts. +D and −D MPs were cultured for 3 d and then challenged with LPS for 6 hr. The presence of LPS markedly induced TNF-α release (not shown). In the absence of LPS the hormone did not affect TNF-α release from MPs (not shown). However, the hormone significantly increased the LPS-induced TNF-α release, in a dose-dependent manner (Fig. 3). The cytokine release was greater in +D than in −D MPs under similar conditions.

The involvement of vitamin D in IFN-γ induction of TNF-α release from BMDMs was determined next. Also in this case we found that the optimal stimulation time is 6 hr (not shown). We found that the addition of 1,000 U/ml of IFN-γ caused significant TNF-α release from both +D and −D BMDMs after 2 d in culture (not shown). This release was markedly increased at 4 and 6 d in culture. The addition of 100 and 500 U/ml of IFN-γ caused significant release only at 4 and 6 d in culture. As with LPS, the TNF-α release was less pronounced in −D than in +D BMDMs. The effect of $1,25(OH)_2D_3$ on the ability of IFN-γ to increase TNF-α release from BMDMs was

tested in 4 d cultures (Table I). Cells were grown with or without $1,25(OH)_2D_3$ (5 or 50 nM) and the various amounts of IFN-γ were added. At 500 U/ml of IFN-γ the hormone significantly increased TNF-α release. In the presence of 1,000 U/ml of IFN-γ, no $1,25(OH)_2D_3$ effect was observed.

In Table II we demonstrated that preincubation of MPs with 50 nM of $1,25(OH)_2D_3$ increased the stimulation of TNF-α release from both +D and −D MPs by IFN-γ.

The role of vitamin D in the regulation of TNF-α release was also demonstrated in vivo (Fig. 4). +D and −D mice were injected intraperitoneally with LPS (1 mg/0.5 ml/mouse). Without LPS injection (time 0), neither +D nor −D mice–derived sera contained detectable levels of TNF-α. Blood was collected 30–120 min postinjection and the serum levels of immunoreactive TNF-α were measured (Fig. 4). Consistent with the in vitro studies, sera derived from −D mice contained significantly less of the cytokine in response to LPS. For example, in blood collected 90 min postinjection, which contained the maximal levels in both +D and −D mice, the TNF-α levels were 680 and 150 U/ml in vitamin D–replete and –depleted mice, respectively.

Using Western blot analysis we demonstrated the presence of a 26 kDa species of TNF-α in the BMDMs (Fig. 5a). Cell-associated TNF-α level is lower in −D BMDMs than in +D BMDMs (lanes A and B, respectively). Activation with LPS (6 hr, 1 μg/ml) resulted in an increase in both cell types (lanes C and D). The presence of 50 nM of $1,25(OH)_2D_3$ for 4 d did not affect, or moderately increased, cell-associated TNF-α (lanes E and F). The highest cell-associated TNF-α in the BMDMs was observed when the cells were treated with both $1,25(OH)_2D_3$ and LPS (lanes G and H). In all conditions, TNF-α levels were higher in +D BMDMs. In Figure 5b we present the Western blot analyses of TCA precipitates of BMDM-derived conditioned media. Untreated BMDMs released a 17 kDa species of TNF-α. The level of this species is slightly higher in +D BMDMs than in −D BMDMs (lanes B and A, respectively). Following LPS stimulation, an increase of the cytokine release is observed in both cell types (lanes C and D), and in +D BMDMs (lane D) a higher molecular weight species of TNF-α (26 kDa ?) is also observed. The presence of 50 nM of $1,25(OH)_2D_3$ for 4 d (lanes E and F) slightly increased the release of TNF-α. Cells treated with both $1,25(OH)_2D_3$ and LPS (lanes

TABLE I. The Effect of $1,25(OH)_2D_3$ on the Release of TNF-α From IFN-γ–Stimulated BMDMs*

	+DBMDMs		−DBMDMs	
IFN-γ (U/ml)	100	500	100	500
$1,25(OH)_2D_3$ (nM)				
0	1.1 ± 0.2	21.1 ± 1.2	1.2 ± 0.2	12.6 ± 0.6
5	1.4 ± 0.3[NS]	24.7 ± 1.2[a]	1.6 ± 0.2[a]	15.4 ± 0.9[a]
50	1.8 ± 0.3[a]	27.2 ± 1.6[b]	1.5 ± 0.1[NS]	19.2 ± 1.1[b]

*BMDMs were plated for 4 d in the presence or absence of $1,25(OH)_2D_3$ (5–50 nM). Monolayers were washed and challenged with IFN-γ for 6 hr. TNF-α levels were measured by ELISA. Data are expressed as U/mg protein/6 hr (mean ± SD).
[a]$P < 0.05$.
[b]$P < 0.01$.
[NS]Not significant. Compared to cells in the absence of $1,25(OH)_2D_3$.

TABLE II. The Effect of $1,25(OH)_2D_3$ on the Release of TNF-α From IFN-γ–Stimulated MPs*

	+DMPs		−DMPs	
$1,25(OH)_2D_3$ (nM)	0	50	0	50
IFN-γ (U/ml)				
10	3.7 ± 0.2	4.7 ± 0.3[a]	3.5 ± 0.2	3.8 ± 0.2[NS]
100	8.4 ± 0.4	9.6 ± 0.4[a]	6.2 ± 0.2	7.0 ± 0.1[a]
500	10.6 ± 0.5	12.6 ± 0.7[a]	7.0 ± 0.4	8.4 ± 0.5[a]
1,000	11.1 ± 1.3	13.4 ± 1.3[NS]	7.2 ± 0.5	8.5 ± 0.6[a]

*Peritoneal macrophages were plated for 3 d in the presence or absence of $1,25(OH)_2D_3$ (50 nM). Monolayers were washed and challenged with IFN-γ for 6 hr. TNF-α levels were measured by ELISA. Data are expressed as U/mg protein/6 hr (mean ± SD).
[a]$P < 0.05$.
[NS]Not significant. Compared to cells in the absence of $1,25(OH)_2D_3$.

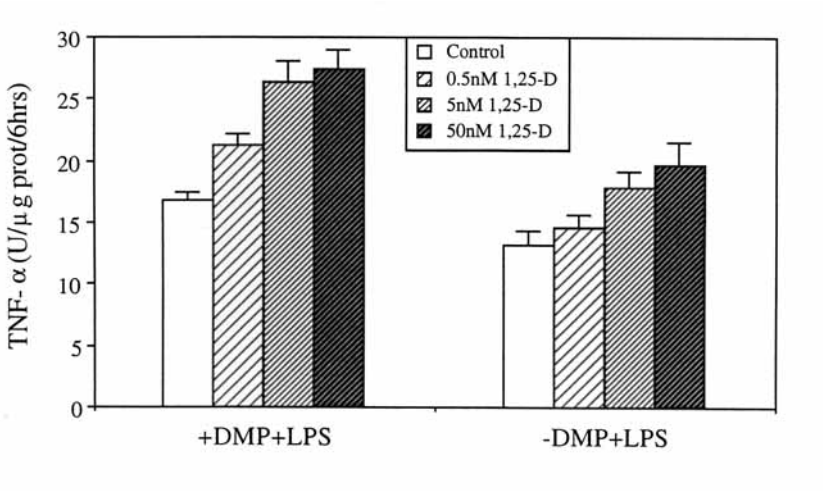

Fig. 4. The effect of vitamin D deficiency on the ability of LPS to increase TNF-α in vivo. +D and −D mice were injected with LPS (1 mg/0.5 ml/mouse). TNF-α levels were measured 0–120 min postinjection in the blood by ELISA.

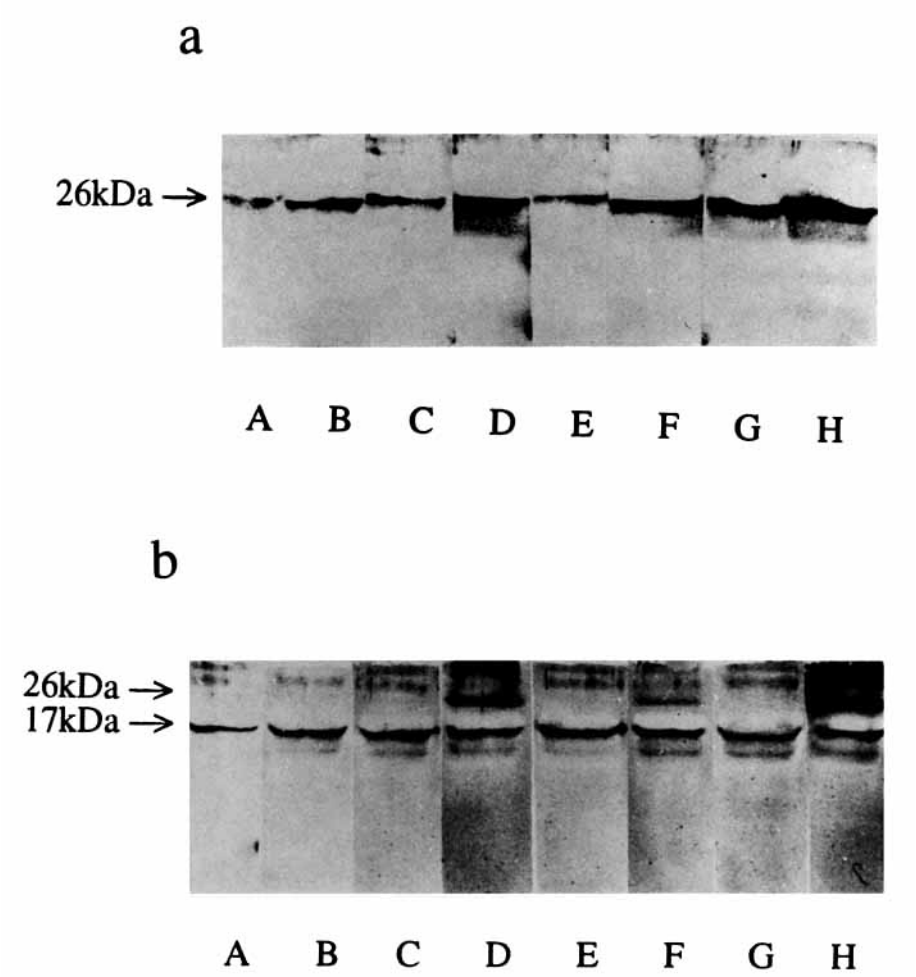

Fig. 5. Western blot analysis of TNF-α in BMDMs. Cell lysates (**a**) and conditioned media (**b**) were prepared, electrophoresed, transferred, and analyzed as described in Materials and Methods. **Lanes A, C, E,** and **G** were derived from −D BMDMs, and **lanes B, D, F,** and **H** were derived from +D BMDMs. Cells were incubated for 4 d in the presence or absence of $1,25(OH)_2D_3$ (50 nM), washed, and incubated for an additional 6 hr in the presence or absence of $1,25(OH)_2D_3$ (50 nM), washed, and incubated for an additional 6 hr in the presence or absence of LPS (1 μg/ml). Lanes A and B, without stimulation; lanes C and D, LPS stimulation; lanes E and F, $1,25(OH)_2D_3$ treatment; lanes G and F, LPS stimulation and $1,25(OH)_2D_3$ treatment.

G and H) released greater amounts of TNF-α than untreated cells, but approximately similar amounts to cells treated with LPS alone (lanes C and D).

In order to examine if $1,25(OH)_2D_3$ modulation of TNF-α involves regulation of protein synthesis, we have metabolically labeled cells and analyzed by SDS-PAGE immunoprecipitates obtained with anti-TNF-α. In Figure 6a we demonstrate that the 26 kDa species of the cytokine is constitutively synthesized in both +D and −D BMDMs. The synthesis is lower in −D than in +D BMDMs (Fig. 6a, lanes A and B, respectively). The addition of LPS resulted in a marked increase of TNF-α synthesis in both −D and +D BMDMs (lanes C and D, respectively). Incubation of the cells with 50 nM of $1,25(OH)_2D_3$ for 4 d also resulted in increased synthesis of TNF-α in both cell types (Fig. 6a, lanes E and F). Cells treated with both $1,25(OH)_2D_3$ and LPS (lanes G and H) synthesized higher levels of TNF-α than untreated cells, but not more than cells treated with LPS alone (lanes C and D). Analysis of the release of the de novo synthesized TNF-α (Fig. 6b) demonstrated similar interrelationships between the various treatments. In contrast to the cell-associated fraction, the size of the released TNF-α is 17 kDa.

DISCUSSION

The ability of $1,25(OH)_2D_3$ to modulate TNF-α expression was studied by several investigators using transformed cell lines. It was demonstrated

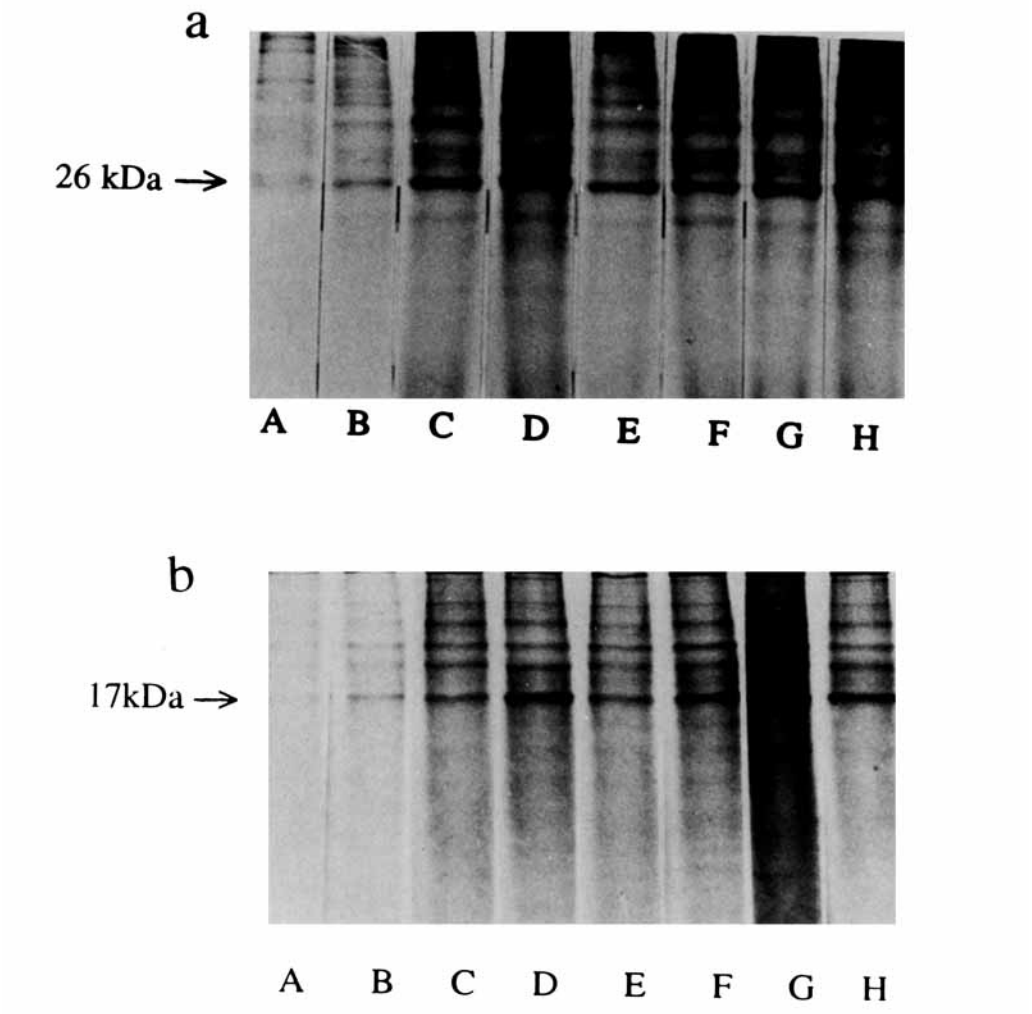

Fig. 6. Immunoprecipitation analysis of TNF-α in BMDMs. Cells were treated as in Figure 5, and the order of the lanes is similar to the order in Figure 5. The last 6 hr included metabolic labeling of the cells as described in Materials and Methods. Cell lysates (**a**) and conditioned media (**b**) were prepared and analyzed as described in Materials and Methods.

that $1,25(OH)_2D_3$ alone, or in combination with Phorbol 12-myristate 13-acetate (PMA), retinoic acid, granulocyte-macrophage colony-stimulating factor, or IFN-γ, increased TNF-α expression in human myeloid cell line (U937) [Bhalla et al., 1991; Blifeld et al., 1991; Kelsey et al., 1993; Taimi et al., 1993]. Prehn et al. [1992] demonstrated the potentiation of LPS-induced TNF-α expression by $1,25(OH)_2D_3$ in U937 cells. While these studies are of interest, they represent the interactions of $1,25(OH)_2D_3$ with transformed cells. However, in order to elucidate mechanisms underlying the interactions of the steroid with mononuclear phagocytes, authentic members of this series should be used. Studies with these cells yielded conflicting results. Some of them demonstrated an inhibitory effect of $1,25(OH)_2D_3$ on TNF-α production in peripheral blood mononuclear cells, in peripheral blood adherent cells, and in monocyte-enriched peripheral blood cells [Katakami et al., 1991; Muller et al., 1992; Zarrabeita et al., 1992]. On the other hand, in human monocyte-derived macrophages and in mouse peritoneal macrophages it was demonstrated that $1,25(OH)_2D_3$ stimulated TNF-α production [Bermudez et al., 1990; Gavison and Bar-Shavit, 1990; Kankova et al., 1991; Kreutz and Andreesen, 1990]. It is possible that in studies demonstrating inhibitory effects, the cultures (which were derived from peripheral blood) contained other cell types and the hormone effect was a combination of its interactions with the monocytic as well as other cell types.

In the present study, we show that BMDMs release TNF-α to the medium only after the addition of stimulating agents, such as LPS and IFN-γ. While $1,25(OH)_2D_3$ failed to induce the cytokine release by itself, the steroid significantly enhanced the activities of LPS and IFN-γ. We also studied MPs, and found that these cells released TNF-α to the medium in the absence of activating agent. $1,25(OH)_2D_3$ failed to affect basal TNF-α release, but increased the LPS and IFN-γ enhancing effects. The in vitro effects on BMDMs are more pronounced than on MPs, reflecting the relatively immature state of the former cells. The involvement of the hormone in modulating TNF-α was also studied in vivo, and consistent with the in vitro experiments TNF-α levels measured in LPS-injected −D mice were lower than in +D mice. Similar findings were obtained by Kankova et al. [1991]. They, however, used the cytotoxicity assay, while we used the quantitative ELISA. The impressive difference between +D and −D mice in the in vivo experiment suggests that the data are physiologically significant. This difference became smaller with culture duration, suggesting that the impaired capability of the cells to release TNF-α was not due to an "intrinsic" defect of the cells, but probably due to their slower maturation. This conclusion is supported by the small or no difference between +D and −D cells under strong activation conditions and is consistent with our previous studies regarding BMDM production of H_2O_2 and surface antigens expression [Abu-Amer and Bar-Shavit, 1993]. Western blot analysis was performed to complement the data obtained from the ELISA. The major cell-associated TNF-α species was the 26 kDa, the membrane form and the precursor of the soluble 17 kDa form of TNF-α [Kriegler et al., 1988]. Increase in the cell-associated TNF-α levels was observed in the LPS- and in the $1,25(OH)_2D_3$-treated cells, and in each condition the levels were higher in +D BMDMs. In the media we identified the 17 kDa form of TNF-α. The 26 kDa form of TNF-α was also detected in the medium. Alternatively, the larger form might correspond to post-translationally modified and/or complex forms of the TNF protein [Descoteaux and Matlashewski, 1990] rather than the membrane form of TNF-α. It is of note, however, that Kelsey et al. [1993] suggested that surface TNF-α could be released from monocytic cells. In contrast to the ELISA, in the Western analysis, we could demonstrate that the hormone slightly stimulated TNF-α release. However, in the ELISA, which is more reliable in quantitative aspects, the sample was first bound to monoclonal antibodies and then identified by polyclonal antibodies. So, in general, these two assays were in agreement.

We used metabolically labeled cells and SDS-PAGE analysis of immunoprecipitates to shed light on the mechanism of the hormone modulation of TNF-α. In contrast to the slight effect (or no effect) of $1,25(OH)_2D_3$ on TNF-α release, the steroid effect on the synthesis of this cytokine was remarkable, and almost equaled the LPS effect. This was correct with both the cell-associated 26 kDa and the soluble released 17 kDa species. The autoradiograms also showed higher molecular weight bands, consistent with previous similar analyses by others [Zuckerman et al., 1989].

Therefore, we conclude that $1,25(OH)_2D_3$ treatment of the BMDMs results in a marked increase of the cytokine synthesis, enabling the cells to release higher levels of it in response to

activating stimulus. The BMDM, due to its relative immature state, is suitable for in vitro analyses of the mechanism(s) responsible for TNF-α modulation in mononuclear phagocytes.

Modulation of TNF-α by $1,25(OH)_2D_3$ is of significance to its role as an immunomodulator in light of the roles played by the cytokine as a mediator of the tumoricidal function of the macrophage and in inflammation, autoimmunity, infectious diseases, and septic shock [Fiers, 1990]. Moreover, this modulation may also reflect the interrelationships between the immune system and bone metabolism. Cytokines (such as TNF-α and IL-1) [Canalis, 1990] are known to affect bone metabolism. TNF-α stimulates bone resorption and bone cell replication [Canalis, 1990]. TNF-α also stimulates IL-1 production by macrophages, and IL-1 could mediate some of the TNF-α responses in bone. Indeed, there is a similarity in the effect of these two cytokines in bone. Thus, although direct effects of $1,25(OH)_2D_3$ on bone are well established [DeLuca, 1979; Minghetti and Norman, 1988; Stumpf et al., 1979], in light of our data, especially the in vivo studies, it is reasonable to propose that in addition to its recognized effects, $1,25(OH)_2D_3$ could also exert its impact on bone metabolism through modulation of molecules such as TNF-α. This hypothesis is strengthened by a recent report [Orcel et al., 1993] showing increased bone resorption in LPS-injected rats, possibly via TNF-α.

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