Tumor necrosis factor α is an autocrine growth factor for normal human B cells

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ABSTRACT Transcription of the human tumor necrosis factor α (TNF- α) gene is one of the earliest events that occurs after stimulation of B or T cells via their antigen receptors. Antibody directed at surface immunoglobulin (anti-Ig) on B cells has previously been shown to induce a rapid burst of TNF- α gene transcription, which can be blocked by the immunosuppressants cyclosporin A (CsA) and FK506. Here, TNF- α gene transcription is shown also to be highly and rapidly induced in human B cells after stimulation via the CD40 and interleukin 4 pathways, which similarly is inhibited by CsA and a panel of CsA or FK506 analogues that block calcineurin phosphatase activity. Endogenous TNF- α produced after stimulation was involved in B-cell proliferation since anti-TNF- α monoclonal antibody inhibited both anti-Ig- and anti-CD40-induced B-cell proliferative responses. Moreover, addition of TNF- α during stimulation resulted in augmentation of B-cell proliferation, which was also inhibited by anti-TNF- α monoclonal antibody. Although lymphotoxin α (LT- α) mRNA is induced by both pathways, it is not blocked by CsA, whereas LT- β mRNA is constitutively expressed in B cells. Thus, TNF- α is a necessary autocrine growth factor for human B cells stimulated via two independent CsA-sensitive pathways and plays a role similar to that of interleukin 2 in T-cell proliferation. The autocrine nature of TNF- α in activated B cells implies a potential role for this cytokine in infection-related polyclonal B-cell expansion and in B-cell malignancies.

The human tumor necrosis factor α (TNF- α) gene is one of the earliest genes transcribed after stimulation of T or B cells through their antigen receptors (1, 2). Induction of TNF- α gene transcription occurs within 30 min of stimulation, does not require *de novo* protein synthesis, and can be blocked by the immunosuppressants cyclosporin A (CsA) and FK506 (1, 2). Induction of TNF- α gene expression after stimulation of T cells via the T-cell receptor occurs through the activation and nuclear translocation of a transcription factor, the κ 3 binding factor, either identical or closely related to the preformed subunit of nuclear factor of activated T cells (refs. 1 and 3; P. G. McCaffrey, A.E.G., and A. Rao, unpublished data). Once activated, the κ 3 binding factor binds to the TNF- α promoter element κ 3, which is required for the calcium-inducible, CsA-sensitive induction of TNF- α gene transcription (1).

Previous studies have demonstrated that exogenous TNF- α augments DNA synthesis and immunoglobulin production by mitogen-activated human B cells (5, 6). Moreover, elevated TNF- α levels have been observed in B-cell chronic lymphocytic leukemias (7), and T-cell membrane-bound TNF- α has been shown to be involved in human immunodeficiency virus (HIV)-induced polyclonal B-cell activation (8) and interleukin 4 (IL-4)-induced immunoglobulin production (9).

Crosslinking of surface Ig with anti-Ig antibody mimics antigen-mediated T-cell-independent activation of B cells (10). In contrast, T-cell-dependent B-cell proliferation is mediated at least in part, via CD40–CD40 ligand interactions and can be mimicked by an anti-CD40 antibody and IL-4 (11, 12). The CD40 ligand is a TNF family member expressed on activated T cells and CD40, its cognate receptor, is a member of the TNF receptor family (11). When CD40–CD40 ligand interactions are blocked *in vitro*, B cells cannot proliferate or produce immunoglobulin in response to T-cell signals (13–15). Because of the rapid expression of TNF- α gene transcription and protein production in B lymphocytes following anti-Ig stimulation (2), the questions of whether TNF- α gene transcription is induced following anti-CD40-mediated B-cell stimulation and the potential role of TNF- α in B-lymphocyte proliferation were investigated.

Here, using human splenic B cells, we show that similar to anti-Ig stimulation, anti-CD40+IL-4 induces rapid TNF- α gene transcription in a CsA-sensitive manner. Both anti-Ig and anti-CD40+IL-4-induced B-cell proliferation are inhibited by anti-TNF- α antibody. Furthermore, addition of TNF- α augments anti-Ig- and anti-CD40+IL-4-induced B-cell proliferation. Thus, TNF- α is a required autocrine B-cell growth factor. The understanding of TNF- α gene expression and receptor-mediated signal transduction could lead to different approaches to the control of normal and abnormal B-cell growth.

MATERIALS AND METHODS

Isolation of Splenic B Cells and *in Vitro* B Cell Activation. B cells were isolated from cadaveric human spleens as described (16) and were >95% B1⁺, <2% CD3⁺, and <1% CD14⁺, as determined by flow cytometry. Purified B cells were activated by crosslinking surface Ig (16) or by anti-CD40 monoclonal antibody (mAb) (5 μ g/ml) and IL-4 (10 ng/ml; Genzyme), and proliferation assays were performed using [³H]thymidine incorporation as an index of mitogenic activity (16). Recombinant TNF- α (Genzyme), human–mouse chimeric anti-TNF- α mAb (cA2 IgG) was constructed as a fusion protein (Centocor) and control fusion protein (Repligen) that was prepared as described (17) were added at the initiation of culture. Where indicated, 10 min before activation, cells were treated with either CsA (1 μ M) (Sandoz Pharmaceutical), FK506 (1 nM) (Fujisawa Pharmaceutical, Osaka), MeBm₂t¹-CsA (1 μ M), [Me-Ala⁶]CsA (1 μ M), rapamycin (0.5 μ M), or 15-O-demethyl-FK520 (referred to as FK520Me throughout this paper) (1 nM). MeBm₂t¹-CsA, [Me-Ala⁶]CsA, FK520Me, and rapamycin were synthesized as described (18).

RNA Isolation and PCR Analysis. B cells were cultured and activated as described above for the indicated time intervals.

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Abbreviations: TNF- α , tumor necrosis factor α ; LT- α and LT- β , lymphotoxins α and β ; IL-4, interleukin 4; CsA, cyclosporin A; mAb, monoclonal antibody.

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RNA was prepared and reverse transcribed, and PCR amplification of the cDNAs was performed with oligonucleotides for TNF- α , lymphotoxin α (LT- α) (Clontech), and LT- β (Biogen) as described (19, 20). A 20- μ l aliquot of each of the final reaction products was electrophoresed on a 2.5% agarose gel containing ethidium bromide.

RESULTS

B-Cell Activation via the CD40 Pathway Induces TNF- α mRNA in a CsA-Sensitive Manner. Highly purified human splenic B cells were stimulated with either recombinant human IL-4, anti-CD40 mAb, or anti-CD40 mAb and IL-4 in the presence or absence of CsA as indicated. RNA was isolated at 0.5, 3, 6, and 12 hr postinduction and reverse transcription PCR was performed. TNF- α mRNA was detectable at 3 hr (Fig. 1A, lane 4) and still evident at 6 hr (lane 6) after stimulation with IL-4; at both time points, pretreatment of the cells with CsA blocked induction of TNF- α mRNA transcription (lanes 5 and 7). After anti-CD40 stimulation, TNF- α mRNA was evident at 30 min (Fig. 1B, lane 2), was still detectable at 3 hr (lane 4), but was no longer detectable at 6 hr poststimulation (lane 6). Anti-CD40 induction of TNF- α mRNA transcription was in all cases blocked by pretreatment of the cells with CsA (Fig. 2B, lanes 3 and 5). Kinetics of amplified TNF- α mRNA expression after anti-CD40+IL-4 treatment reflected the additive effects of both stimuli. Transcription of TNF- α mRNA was strongly induced after 30 min (Fig. 1C, lane 2) and after 3 hr (lane 4), was still evident at 6 hr (lane 6), but was no longer detectable 12 hr after anti-CD40+IL-4 stimulation (lane 8). Induction of TNF- α mRNA transcription after stimulation of splenic B cells with anti-CD40+IL-4 was at all time points blocked by CsA (Fig. 1C, lanes 3, 5, and 7). The kinetics and CsA-sensitive nature of TNF- α mRNA expression induced by anti-CD40+IL-4 induction were similar to those observed after anti-Ig induction (compare Fig. 1C to 1D).

Endogenous TNF- α Is Required for Anti-Ig- and Anti-CD40-Induced B-Cell Proliferation. Since both anti-Ig- and anti-

FIG. 1. TNF- α gene expression is induced by anti-CD40 and IL-4 in a CsA-sensitive manner. Highly purified splenic B cells were cultured at a concentration of 2×10^6 cells per ml in 24-well plates in the presence of medium alone (lanes -) or in the presence of a stimulus (lanes +) as indicated. IL-4 (A), anti-CD40 (B), anti-CD40+IL-4 (C), or anti-Ig (D) was added in the presence or absence of 1 μ M CsA for the times indicated. RNA was isolated, reverse transcription was performed on 2 μ g of RNA, and PCR amplification using TNF- α primers was done. A TNF- α control (Ctrl) is included; the primers amplify a band of 610 bp in the control sample. Marker lane (M) is indicated.

FIG. 2. TNF- α is necessary for anti-Ig- or anti-CD40+IL-4-induced B-cell proliferation. Highly purified splenic B cells were activated for 72 hr with anti-Ig (A) or anti-CD40+IL-4 (B) in the presence of the indicated concentrations of the anti-TNF- α chimeric mAb or the chimeric control antibody as described. [3 H]Thymidine incorporation was assessed for the last 16 hr of a 72-hr culture.

CD40+IL-4-mediated stimulation induced TNF- α mRNA expression and since activation of B cells via both pathways results in B-cell proliferation, the question of whether TNF- α plays a role in such proliferative responses was examined. Highly purified splenic B cells were stimulated by either anti-Ig or anti-CD40+IL-4 in the presence of increasing concentrations of a blocking anti-TNF- α mAb or a control mAb. Addition of anti-TNF- α mAb reduced anti-Ig- (Fig. 2A) and anti-CD40+IL-4- (Fig. 2B) induced B-cell proliferation in a dose-dependent manner and was completely inhibitory at a concentration of 5 μ g/ml. Control mAb had no effect at any concentration used (Fig. 2). Trypan blue uptake was approximately the same for cells activated in the presence or absence of the TNF- α antibody or the control antibody, indicating that neither antibody significantly affected the viability of the cells (data not shown). Thus, the production of endogenous TNF- α after anti-Ig or anti-CD40+IL-4 stimulation is necessary for B-cell proliferation.

Exogenous TNF- α Augments Anti-Ig- and Anti-CD40-Induced B-Cell Proliferation. To determine whether TNF- α was also sufficient for B-cell proliferation, the effect of exogenous recombinant human TNF- α on resting B cells or on cells activated by anti-Ig or anti-CD40+IL-4 was examined. Human recombinant TNF- α was added in increasing concentrations (from 100 to 1500 units/ml) at the initiation of the culture with anti-Ig or anti-CD40+IL-4; it was determined that a dose of 500 units/ml produced the optimal effect in this system (data not shown), and this dose was thus used in all of the following experiments. Highly purified human splenic B cells were cultured with either medium, anti-Ig or anti-CD40+IL-4, in the presence or absence of TNF- α for 3 days. Addition of TNF- α alone did not increase B-cell proliferation compared to that observed in control cultures (Fig. 3A and B). In contrast, in the presence of anti-Ig (Fig. 3A) or anti-CD40+IL-4 (Fig. 3B), addition of TNF- α augmented B-cell proliferation by $\approx 50\%$ in the case of anti-Ig induction and by $\approx 25\%$ in the case of anti-CD40+IL-4 induction.

To determine whether the ability of exogenous TNF- α to augment B-cell proliferation induced by anti-Ig or anti-CD40+IL-4 was specific, the ability of anti-TNF- α mAb to abrogate this effect was examined. Increasing concentrations

FIG. 3. Exogenous TNF- α is not sufficient for but augments anti-Ig- or anti-CD40+IL-4-induced B-cell proliferation. Purified human splenic B cells were cultured with either medium or anti-Ig (A) or soluble anti-CD40+IL-4 (B) in the presence or absence of recombinant TNF- α as indicated. Recombinant human TNF- α was added at the initiation of the cultures at the optimal concentration of 500 units/ml. Increasing concentrations of the anti-TNF- α chimeric mAb or the chimeric control antibody were added to splenic B cells stimulated with anti-Ig (C) or anti-CD40+IL-4 (D), as indicated. [^3H]Thymidine incorporation was assessed for the last 16 hr of a 72-hr culture.

of anti-TNF- α mAb inhibited the effect of exogenous TNF- α in augmenting B-cell proliferation in a dose-dependent manner (Fig. 3 C and D). Moreover, even in the presence of the exogenous TNF- α , the anti-TNF- α mAb at 5 $\mu\text{g/ml}$ essentially abrogated B-cell proliferation, indicating that the effects of endogenous and exogenous TNF- α were inhibited. Thus, although TNF- α can augment the proliferative response stimulated by the anti-Ig or anti-CD40+IL-4 pathways, it cannot by itself induce proliferation.

Induction of B-Cell Proliferation by Anti-Ig or Anti-CD40 Is CsA Sensitive and Requires Calcineurin Phosphatase Activity. Given that TNF- α was required for B-cell proliferation and that the induction of TNF- α gene transcription following anti-Ig or anti-CD40+IL-4 stimulation was CsA sensitive, the question of whether B-cell proliferation could be blocked by CsA was next examined. CsA was added to human splenic B-cell cultures 10 min before activating them with either anti-Ig or anti-CD40+IL-4. CsA inhibited anti-Ig- and anti-CD40+IL-4-induced B-cell proliferation (Fig. 4 A and B). When TNF- α was added with anti-Ig or anti-CD40+IL-4 at the initiation of the culture, CsA inhibited proliferation by 65% (Fig. 4 A and B).

Recent experiments with CsA and FK506 analogues demonstrated that calcineurin phosphatase activity is required for induction of TNF- α mRNA transcription via surface Ig in the human Burkitt lymphoma B-cell line Akata (3). The CsA analogues MeBm $_2$ t 1 -CsA and [Me-Ala 6]CsA and the FK506 analogues FK520Me and rapamycin show different binding affinities for calcineurin when complexed with cyclophilin A and FKBP-12, respectively, and differentially inhibit calcineurin's phosphatase activity (18, 21–23). The ability of these agents to inhibit cytokine gene transcription is strikingly correlated with their ability to inhibit calcineurin activity *in vitro* (3, 18, 21).

To determine whether induction of B-cell proliferation was dependent on calcineurin phosphatase activity, human splenic B cells were stimulated with anti-Ig or anti-CD40+IL-4 in the presence or absence of these compounds. MeBm $_2$ t 1 -CsA at a concentration of 1 μM inhibited anti-Ig-induced B-cell proliferation, as did 1 μM CsA. In contrast, an equivalent concentration of the inactive CsA analogue [Me-Ala 6]CsA did not affect anti-Ig-induced B-cell proliferation. Similarly, although 1 nM FK506 inhibited B-cell proliferation, the inactive FK520Me analogue had no effect even at 1000-fold higher concentration. Likewise, rapamycin did not block B-cell proliferation. Identical effects of these analogues on

FIG. 4. CsA and a panel of CsA and FK506 analogues inhibit B-cell proliferation via anti-Ig or anti-CD40+IL-4. Highly purified human splenic B cells were cultured with either anti-Ig (A) or anti-CD40+IL-4 (B) in the presence or absence of recombinant TNF- α (500 units/ml) and/or 1 μM CsA as indicated. Splenic B cells were also cultured with either anti-Ig (C) or anti-CD40+IL-4 (D) in the presence of 1 μM CsA, 1 μM [Me-Ala 6]CsA, 1 μM MeBm $_2$ t 1 -CsA, 1 nM FK506, 1 nM FK520Me, or 0.5 μM rapamycin, which were added 10 min before stimulation with anti-Ig or anti-CD40+IL-4. Cultures were continued for 3 days and [^3H]thymidine incorporation was assessed for the last 16 hr of culture.

B-cell proliferation were observed when stimulation was performed via the CD40 pathway (Fig. 4D). These data therefore suggest that calcineurin is a critical intermediate in the signal transduction pathway leading to TNF- α gene expression and B-cell proliferation stimulated by crosslinking surface Ig or by anti-CD40 or IL-4.

LT- α but Not LT- β mRNA Is Induced Following B-Cell Stimulation by Anti-Ig or Anti-CD40. The TNF- α gene shares a high degree of sequence similarity with the LT- α gene (reviewed in ref. 24), and the same cellular receptor is used by both lymphokines (25, 26). LT- α in its membrane-bound form is closely associated with a 33-kDa protein, LT- β (27). To determine the expression of these genes under the previously described culture conditions, PCR primers specific for LT- α and LT- β sequences were used. Similar to TNF- α mRNA, expression of LT- α mRNA was induced 30 min after anti-Ig or anti-CD40+IL-4 stimulation. However, in contrast to TNF- α mRNA, LT- α mRNA was detectable at all time points up to 12 hr after stimulation by anti-Ig or anti-CD40+IL-4 and was not affected by CsA (Fig. 5 A and B). In contrast to the expression of the TNF- α and LT- α genes, LT- β mRNA was not inducible by anti-Ig or anti-CD40+IL-4, but it was constitutively expressed at a low level in resting B cells and was not affected by CsA treatment (Fig. 5 C and D).

DISCUSSION

Stimulation of B cells via surface Ig initiates an ordered cascade of events that includes increases in intracellular calcium, activation of protein kinase C and calcium-dependent kinases, and activation of the calcium- and calmodulin-dependent phosphatase calcineurin (3, 11, 28). Interaction between CD40 and its receptor results in protein-tyrosine kinase activity, inositol triphosphate production, serine-threonine kinase activity (29), and an increase in the src type kinase lyn (30). The results presented here, that anti-CD40 and IL-4-induced TNF- α gene transcription and subsequent B-cell proliferation is CsA sensitive and dependent on calcineurin phosphatase activity, indicate that an increase in intracellular calcium also occurs postengagement

of CD40 and the IL-4 receptor and that this signal transduction pathway is mediated by calcineurin. It is therefore intriguing to speculate that TNF- α may be involved in other CD40-mediated processes and/or that calcineurin may be a critical intermediate in the signal transduction pathways leading to B-cell survival, Ig isotype class switching, and differentiation that are induced via CD40.

TNF- α is tandemly linked to LT- α (also named TNF- β) on human chromosome 6 and the two genes share $\approx 50\%$ sequence similarity (reviewed in ref. 24); the recently described LT- β , which forms a membrane complex with LT- α , is adjacent to the TNF LT locus (27). Although primarily a product of T cells, some Epstein-Barr virus (EBV)-transformed B cells and tonsillar B cells also secrete LT- α (31–35). Previous studies demonstrated a role for LT- α in B-cell proliferation (36, 37) and in the maintenance of EBV-transformed B-cell lines (38). In the present work, a mAb that specifically blocks TNF- α is shown to inhibit B-cell proliferation stimulated through two distinct pathways. Furthermore, the patterns of LT- α , LT- β , and TNF- α gene expression following anti-Ig and anti-CD40+IL-4 stimulation and CsA treatment are distinct. Thus, although these data do not rule out the possibility that LT- α and/or LT- β are involved in B-cell proliferation in certain cases, they do indicate that TNF- α , and not LT- α or LT- β , is an autocrine growth factor in the CsA-sensitive proliferation pathways set in motion by anti-CD40 and anti-Ig. In this respect, TNF- α functions in B-cell proliferation similarly to IL-2 in T-cell proliferation. These data are consistent with the previous observation that in an L-929 cytotoxicity assay, TNF- α bioactivity that was derived from anti-Ig-activated normal human splenic B cells was completely blocked by a neutralizing rabbit polyclonal anti-human TNF- α antibody (2).

Previous studies demonstrated that certain polyclonal B-cell responses and specific antibody responses were sensitive to CsA (reviewed in ref. 39). A panel of CsA and FK506 analogues that block calcineurin phosphatase activity and TNF- α gene transcription also block B-cell proliferation. Interestingly, rapamycin blocks a non-calcineurin-dependent mitogenic event in the T-cell response that results in T-cell proliferation (23) but does not inhibit anti-Ig- or anti-CD40+IL-4-induced B-cell proliferation.

Although CsA is inhibitory, it does not totally block B-cell proliferation induced by anti-Ig and TNF- α or by anti-CD40+IL-4 alone or in combination with soluble TNF- α , as does the TNF- α mAb. TNF- α exists as both a 17-kDa soluble form and as a 26-kDa membrane-bound form on monocytes (40) and T cells (41). Taken together with the demonstration that TNF- α gene transcription is blocked by CsA, these results raise the possibility that preexisting membrane bound TNF- α exists on B cells and is responsible for the residual proliferation observed in the presence of CsA that is abrogated by anti-TNF- α mAb.

Historically thought to be a monocyte factor, TNF- α was subsequently shown to be a product of stimulated B (42–44) and T (31–35, 44, 45) cells and a variety of other cell types including mast cells (46), natural killer (NK) cells (47), and fibroblasts (4, 48). Since soluble TNF- α augments anti-Ig- or anti-CD40+IL-4-induced B-cell proliferation, these results indicate that TNF- α secreted during an active immunological response by surrounding T cells, monocytes, NK cells, and other B cells will result in expansion of the responding B-cell population.

The demonstrations that TNF- α was highly expressed in peripheral human B cells stimulated by virus (4) and that exogenous TNF- α augmented DNA synthesis in B cells (5) led us to speculate that autocrine TNF- α may be responsible for the elevated numbers of lymphocytes observed in some viral infections (4). The subsequent finding that the TNF- α gene was rapidly expressed in human B cells activated

FIG. 5. LT- α mRNA is induced by anti-Ig or by anti-CD40+IL-4 but is not blocked by CsA; LT- β mRNA is constitutively expressed in splenic B cells. Highly purified splenic B cells were cultured with anti-Ig or anti-CD40+IL-4 stimulation in the presence or absence of CsA for the times indicated. RNA was isolated, reverse transcription was performed on 5 μ g of RNA, and PCR amplification using LT- α (A and B) or LT- β (C and D) primers was done. LT- α and LT- β control (Ctrl) lanes are displayed, and the appropriate primers amplify a band of 617 and 240 bp, respectively. Marker lane (M) is indicated.

through surface Ig suggested that TNF- α may play an auto-crine role in B-cell proliferation observed in several chronic infectious disease states such as HIV-1 infection, malaria, and African trypanosomiasis, which are associated with chronic immune stimulation, polyclonal B-cell activation, and increased serum levels of TNF- α (2). The studies presented here provide the link between autocrine TNF- α production and antigen-mediated or T-cell-mediated B-cell stimulation and proliferation. Furthermore, these results suggest a role for calcineurin inhibitors and TNF- α blocking agents in the treatment of B lymphoproliferative disorders that are associated with increased levels of TNF- α .

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