The 26-kD Transmembrane Form of Tumor Necrosis Factor α on Activated CD4+ T Cell Clones Provides a Costimulatory Signal for Human B Cell Activation

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Summary

Interleukin 4 (IL-4) induces immunoglobulin (Ig)E and IgG4 synthesis in human B cells. In addition to IL-4, costimulatory signals provided by activated CD4+ T cells are required for productive IgG4 and IgE synthesis. Here we report that the 26-kD transmembrane form of tumor necrosis factor α (mTNF-α), which is rapidly expressed on CD4+ T cell clones after activation, contributes to the costimulatory signals resulting in IL-4-dependent Ig synthesis by B cells, including IgG4 and IgE production. mTNF-α expression was induced on T cell clones within 2 h after activation with concanavalin A. Peak expression was observed at 24 h, followed by a gradual decrease, but appreciable levels of mTNF-α were still detectable 72 h after activation. The presence of the 26-kD membrane form of TNF-α on activated T cell clones was confirmed by immunoprecipitation. Monoclonal antibodies (mAbs) recognizing mTNF-α, or the p55 TNF receptor, inhibited IgM, IgG, IgG4, and IgE synthesis induced by IL-4 and activated CD4+ T cell clones in cultures of highly purified surface IgD+B cells. The anti-TNF-α mAbs also blocked Ig production in cultures in which the activated CD4+ T cell clones were replaced by their plasma membranes. Furthermore, pretreatment of the plasma membranes with anti-TNF-α mAbs strongly reduced their capacity to stimulate B cells to produce Ig in the presence of IL-4, indicating that the anti-TNF-α mAbs blocked the effects of mTNF-α. Anti-TNF-α mAbs did not affect IgM, IgG, IgG4, or IgE synthesis induced by anti-CD40 mAbs and IL-4 in the absence of CD4+ T cells, supporting the notion that the anti-TNF-α mAbs indeed interfered with the costimulatory, contact-mediated signal provided by T cells, or their membranes. Collectively these results indicate that mTNF-α, which is rapidly induced after activation of CD4+ T cells, participates in productive T-B cell interactions resulting in IL-4-induced Ig production. This is a novel property of the T cell membrane form of TNF-α.

The requirement of IL-4 for IgE production has been established in both mice (1) and humans (2). Previous studies have shown that addition of IL-4 to human PBMC results in IgE production (2). However, IL-4 fails to induce IgE synthesis by highly purified B cells (2, 3), although it induces germline e transcription (4, 5). In addition to IL-4, contact-mediated costimulatory signals are necessary for the induction of IgE synthesis by B cells (2, 6–9). It has been shown that these costimulatory signals can be provided by activated CD4+ TCR α/β+, or TCR γ/δ+ T cell clones by direct T-B cell contacts (7, 8, 10). Plasma membranes prepared from activated CD4+ T cell clones can replace intact T cells in delivering this costimulatory signal in both human (6) and murine systems (11) irrespective of whether the CD4+ T cells are of the Th1 or Th2 type (12). Alternatively, the second signal required for IgE synthesis can be provided by mAbs to the CD40 antigen on B cells (13–15), by hydrocortisone treatment (16) or through EBV infection of B cells (17). Engagement of the CD40 molecule results in B cell activation, and recently the murine (18) and human (19–21) ligands for CD40 (CD40L),1 have been cloned. Both the human and mouse CD40L, when expressed on CV1/EBNA or COS-7 cells, induce proliferation and IL-4-dependent IgE synthesis by human and murine B cells (18–21). The CD40L is expressed as an early activation antigen by CD4+ T cell clones (18, 19, 21) as well as by CD8+ clones (19, 21). Thus, triggering B cells via CD40 either by anti-CD40 mAbs, or the CD40L expressed as a cell surface molecule, can induce B cell proliferation and Ig synthesis. However, the signals provided by the activated T cells do not seem to be confined to the CD40L–CD40 interaction since: (a) non-IL-4-producing T cells could directly induce germline e transcripts in B cells, whereas anti-CD40 mAb cannot (4, 5); (b) the anti-CD40-mediated IgE production is not susceptible to the

1 Abbreviations used in this paper: CD40L, CD40 ligand; mTNF-α, transmembrane form of TNF-α.
inhibitory effects of IFN-γ and IFN-α (13, 14), whereas these cytokines strongly abrogate IgE responses induced by T cell clones (2); (c) the anti-CD40 mAb and the T cell clones strongly synergize in the induction of IgE, IgG4, total IgG, and IgM synthesis (13); and (d) isotype switching can be induced by hydrocortisone or EBV in the presence of IL-4 (16, 17).

Based on these observations, we speculated that T cell activation–induced molecules other than CD40L may play a role in T–B cell interactions required for IL-4–dependent Ig synthesis. Kriegl et al. (22) have shown that TNF-α is expressed as a cell surface transmembrane protein of 26 kD on activated human monocytes or murine cells transfected with the human TNF-α gene. Subsequently, Kinkhabwala et al. (23) have shown that the transmembrane form of TNF-α (mTNF-α) is also expressed on normal activated human T cells. In addition, a TNF-α–related transmembrane molecule is expressed on murine T cells (24).

In this report we demonstrate that the 26-kD mTNF-α expressed on activated CD4+ T cell clones provides a costimulatory signal required for IL-4–dependent Ig production including IgG4 and IgE synthesis by B cells. mAbs against both the mTNF-α and the p55 TNF receptor (TNFR) inhibited Ig production, indicating that in addition to CD40L–CD40 signaling, interactions between mTNF-α and its putative ligand, the p55 TNFR, are involved in induction of B cell activation by activated CD4+ Th cells.

Materials and Methods

Cells. Highly purified B cells were isolated from normal spleens obtained from patients undergoing splenectomy due to trauma. Splenocytes were obtained by aseptically squashing spleens through a sterile metal mesh. The splenocytes were then washed twice in medium and frozen in aliquots for subsequent use. When needed, splenocytes were thawed, washed twice in Yssel's medium, and stained for 30 min on ice with the following PE-conjugated mAbs: anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, and anti-CD56 (Becton Dickinson & Co., San Jose, CA). The cells were then washed once in medium, centrifuged over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) density gradient, and washed twice in PBS. The stained splenocytes were then negatively sorted with a FACStar Plus® (Becton Dickinson & Co.). An aliquot of the sorted cells was reanalyzed by FACS® after staining with CD20-FITC (anti-Leu-16) or isotype control mAb. The sorted cells were always >99% CD20+. In other experiments slgD+ B cells were obtained by two-color sorting of splenocytes labeled for negative sorting as above, followed by anti-hlgD-FITC mAb (Nordic, Tilburg, The Netherlands). slgD+ cells were obtained by sorting the PE-negative and FITC-positive cells. Reanalysis of the sorted cells showed that they were >99.5% slgD+.

The CD4+ TCR α/β+ T cell clone B21 (Th0), which is specific for tetanus toxoid, the non-IL-4–secreting T cell clone SP-A3, which is specific for HLA-DR4 (25), and HY06 (Th1) (26) were grown in Yssel's medium containing 1% heat-inactivated human serum and were used for coculture with B cells 4–6 d after restimulation with feeder cells and PHA, or for immunoprecipitation of mTNF-α as described in a following section.

Preparation of T Cell Membranes. Plasma membranes were prepared according to Brian (27) and Maeda et al. (28) from the T cell clones B21 and SP-A3. Briefly, B21 and SP-A3 cells were harvested 12–15 d after they had been activated by feeder cells and PHA, washed once in medium, and resuspended in Yssel's medium supplemented with 10% FCS and 10 μg/ml Con A (Sigma Chemical Co.) at 5 × 106 cells/ml. After 8–12 h of culture, the Con A in the culture was neutralized with 4 mg/ml α-methyl-D-mannoside, and 30 min later the cells were harvested, washed three times in PBS, resuspended at 5 × 106 cells/ml in cold homogenization buffer, and processed as described (27, 28). The plasma membranes were resuspended at 500 μg/ml (which is equivalent to ~2 × 108 cells/ml) and stored under liquid nitrogen.

Reagents. The following anti-TNF-α mAbs were used: 1825.12 (IgG1) and 6401.1 (IgG1), which recognize mTNF-α, were both kind gifts of Dr. Harvey Gaylord (R&D Systems, Minneapolis, MI). mAb 63-29 (IgG1) (IgG1) was a kind gift of Dr. R. Torensma (U-Gene Research, University of Utrecht, The Netherlands). anti–human TNF-α mAb was purchased from UBI (Lake Placid, NY). The anti-TNF-α-FITC mAb Fluorokine™ kit for cytofluorometric analysis was purchased from R&D Systems and the IgG1–FITC control mAb was from Becton Dickinson & Co. Polyclonal anti–human TNF-α was purchased from R&D Systems. The anti-TNF-α mAb CRTS was a kind gift of Dr. Wydenes (Centre de Recherche Transfusion, Sanguine Besançon, France). mAbs htr-9 and utr-I, directed to the p55 and p75 TNF receptors, respectively, were kind gifts of Dr. M. Brockhaus (Hoffman-La Roche, Basel, Switzerland). The hybridoma line producing the anti-CD40 mAb B9 (30) was a kind gift of Dr. J. Banchereau (Schering-Plough France, Dardilly, France). The mAb PDV5.2 directed against a framework determinant of human class II MHC molecules was kindly provided by Dr. H. Bruning (University Hospital, Leiden, The Netherlands). The anti-CD4 mAb RPA-T4 has been clustered in the IV International Workshop on Human Leukocyte Differentiation Antigens (31). The anti-CD4 mAb RIV-7 was kindly provided by Dr. H. Kreeftenberg (Ryks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands). mAb 25D2.11, specific for human IL-4, was a kind gift of Dr. J. Abrams (DNAX Research Institute, Palo Alto, CA). The hybridoma line producing the control mAb OX-8 specific for rat CD8 was a kind gift of Dr. Alan Williams (Medical Research Council, Oxford, UK). All mAbs used for functional studies were in purified form, sterile, and diluted in PBS. rhIL-4, IFN-α, and IFN-γ were provided by Schering-Plough Research (Bloomfield, NJ).

Culture Conditions. 5,000 highly purified B cells were cocultured with an equal number of T cell clones B21 or SP-A3, which were harvested 4–6 d after stimulation with feeder cells and PHA, in a final volume of 0.2 ml of Yssel's medium supplemented with 10% FCS, 10 μg/ml ultra pure transferrin (Pierce Chemical Co., Rockford, IL), and 400 U/ml rhIL-4, as described (13). Cultures were set up in six to eight replicates in U-bottomed 96-well Linbro plates (Flow Laboratories Inc., McLean, VA) and incubated 12–14 d at 37°C in 5% CO2. At the end of the incubation period, the supernatants from each of the six to eight wells were harvested and pooled for isootype determination. In another series of experiments, the T cell clones were replaced by 5 μg of plasma membranes derived from the T cell clones, or by the anti-CD40 mAb B9.

Isotype Determination. Ig isotype production was determined by ELISA as previously described (2, 32). The sensitivities of the ELISA were determined with calibrated standards from Behring (Marburg, Germany), and found to be 0.2 ng/ml for IgE and IgG4, and 0.5–1 ng/ml for total IgG and IgM.

Radioiodination, Immunoprecipitation, and SDS-PAGE. B21 and HY06 cells were harvested 14 d after addition of feeder cells, washed once, and resuspended at 3 × 106 cells/ml in Yssel's medium con-
containing 10% FCS, 1 ng/ml 12–tetradecanoyl phorbol-13-acetate (TPA) (Calbiochem-Behring Corp., La Jolla, CA), and 500 ng/ml A23187 calcium ionophore (Calbiochem-Behring Corp.). After 7 h of culture, the cells were harvested, washed three times in PBS containing 10^{-6} M KI, and iodinated with 0.125 M lactoperoxidase-catalyzed reaction as previously described by Meuer et al. (33). After iodination, the cells were washed five times with cold PBS containing 0.02% NaN₃ and lysed on ice for 30 min in lysis buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 20 kU/ml aprotinin, 1 mM PMSF). The nuclei were removed by centrifugation, and the lysate was precleared three times (twice for 1 h and once overnight) with Pansorbin™ cells (Calbiochem-Behring Corp.) pelleted from 200 μl 10% suspension, precleared with rabbit-anti-mouse Ig (Pel-Freez Biologicals, Rogers, AR). Immunoprecipitation of radiolabeled surface proteins was carried out by incubation for 2.5 h at 4°C of each 100 μl of lysate with Pansorbin™ cells precleared with: rabbit anti-mouse Ig and mAbs 1825.12 and 6401.1, or rabbit anti-mouse Ig as control. The coated Pansorbin™ cells were washed six times in stringent wash buffer (lysis buffer containing 1% sodium deoxycholate) and boiled for 3 min in sample buffer containing 2-ME as reducing agent. The Pansorbin™ cells were removed by centrifugation and the samples were run in a 15% polyacrylamide gel (Integrated Separation Systems, Natick, MA) under reducing conditions at 200 V. After staining with Coomassie blue and destaining (50 and 5% methanol solutions, 7.5% glacial acetic acid), the gels were dried and autoradiographed using a Kodak X-Omatic AR5 film, in a cassette (Wolf X-Ray Corporation, New York, NY) mounted with a Cronex Lightening Plus intensifying screen (DuPont Co., Wilmington, DE).

Results

Anti-TNF-α or Anti-p55 TNFR mAbs Inhibit Ig Synthesis Induced by T Cell Clones. Addition of anti-human TNF-α mAbs to cultures of highly purified, negatively sorted B cells or sIgD⁺ B cells cocultured with activated CD4⁺ human T cell clones inhibited IL-4-induced Ig production (Table 1). The various anti-TNF-α mAbs had differential inhibitory effects. The mAbs 1825.12 and 6401.1 were most effective (Table 1) and were selected for subsequent experiments. Anti-TNF-α mAbs effectively inhibited IgG4 and IgE production by purified B cells, but only moderate or weak inhibition of total IgG production was observed, whereas IgM production was generally not affected in these cultures. However, the anti-TNF-α mAbs inhibited IgM, total IgG, IgG4, and IgE synthesis induced in cultures of sIgD⁺ B cells (Table 1, exp. 3).

In addition, the mAb htr-9, which recognizes the p55 TNFR, strongly inhibited IgG4 and IgE, and to a lesser extent total IgG, IgM, production in cocultures of sIgD⁺ B cells, and the T cell clone B21 (Table 1, exp. 3). In contrast, mAb utr-1, which recognizes the p75 TNFR, had no inhibitory effects (Table 1, exp. 3). As reported previously (6), anti-class II MHC and anti-CD4 mAbs inhibited the production of all isotypes.

Taken together these results indicate that anti-TNF-α mAbs inhibit to variable extents IL-4-induced Ig production, including IgG4 and IgE synthesis in cultures of sIgD⁺ B cells or total B cells, although the inhibitory effects on IgG and particularly IgM production in the latter cultures were generally much weaker.

Anti-TNF-α mAbs Block Membrane-induced Ig Synthesis. The CD4⁺ Th clones used in these studies produce soluble TNF-α after activation (Yssel, H., R. de Waal Malefyt, J. E. de Vries, unpublished results). Since the anti-TNF-α mAbs also block the biological activity of soluble TNF-α, we replaced the intact activated CD4⁺ T cell clones by their plasma membranes to exclude that the inhibitory effect observed with anti-TNF-α mAb was due to the neutralization of soluble TNF-α released by the activated T cell clone. Anti-TNF-α mAbs inhibited Ig responses induced by membranes from B21 T cell clone (Tables 2 and 3), as well as the non-IL-4-producing clone SP-A3 (Table 2). The level of inhibition of IgG4 and IgE synthesis by mAb 1825.12 was in the same range as that obtained by an anti-IL-4 mAb, whereas mAb 6401.1 was less effective in blocking IgG4 and IgE synthesis (Table 2).

Anti-TNF-α mAbs also had considerable inhibitory effects on IgM and total IgG synthesis in these cultures, confirming the notion that mTNF-α is not only involved in T-B cell interactions resulting in switching to IgG4 and IgE synthesis, but also block the interaction of activated CD4⁺ T cell clones or their membranes and IgM- or IgG-committed B cells. Preincubation of the T cell membranes with anti-TNF-α mAbs significantly decreased the ability of the membranes to provide the costimulatory signals required for the induction of Ig synthesis, including IgG4 and IgE synthesis. Total IgG and IgM production was again affected to a lesser extent than IgG4 and IgE production (Table 3).

Anti-TNF-α mAbs Are Not Inhibitory for CD40-induced IgG4 and IgE Synthesis. The results above suggest that anti-TNF-α mAbs act at the T cell level, but it is well established that TNF-α also can act on B cells. We have previously shown that TNF-α enhances IL-4-dependent IgE production and germline c transcription in B cells (5). In addition, TNF-α produced by polyclonally activated B cells has been shown to be involved in B cell differentiation and IgG secretion in an autocrine fashion (34). Furthermore, IgG secretion by tonsillar B cells stimulated with Staphylococcus aureus cells and IL-2 were partially inhibited by polyclonal goat anti-human TNF-α (34). To determine whether the anti-TNF-α mAbs acted on B cells, these mAbs were tested in a T cell–free culture system, using soluble anti-CD40 mAb and IL-4 to stimulate highly purified B cells as described previously (13). In Table 4 it is shown that Ig production, including IgG4 and IgE, was not inhibited by anti-TNF-α mAbs, further supporting the hypothesis that the inhibition by anti-TNF-α mAb is at the T cell level.

Activated T Cell Clones Rapidly Express mTNF-α after Activation. A previous report has shown expression of mTNF-α on normal peripheral blood T cells after activation with TPA and ionomycin (23). After our observation that anti-TNF-α mAbs could abrogate IgE synthesis induced by activated T cell clones or their plasma membranes, we tested the T cell clones used in these experiments for surface TNF-α expression. The T cell clones were stimulated with Con A, and mTNF-α expression was measured at various time intervals by staining the cells with the Fluorokine™ kit FITC-labeled anti-TNF-α mAb, which binds to mTNF-α. The kinetics
Table 1. Effect of Anti-TNF-α mAbs on Ig Synthesis Induced by an Activated T Cell Clone and IL-4

| Exp. | IgM       | IgG       | IgG4      | IgE       |
|------|-----------|-----------|-----------|-----------|
| 1 B  | <1        | 2 ± 1     | <0.2      | <0.2      |
| B + IL-4 + B21 | 123 ± 28 | 232 ± 51 | 93 ± 15 | 97 ± 4   |
| + Control mAb   | 109 ± 55 | 205 ± 36 | 81 ± 5  | 90 ± 3   |
| + Anti-TNF-α 63.29 | 137 ± 117| 261 ± 47 | 69 ± 7  | 46 ± 13  |
| + Anti-TNF-α 1825.12 | 134 ± 43 | 208 ± 22 | 24 ± 15 | <0.2     |
| + Anti-TNF-α 6401.1 | 127 ± 33 | 198 ± 63 | 37 ± 17 | 31 ± 11  |
| + Anti-TNF-α DNAx | 156 ± 31 | 217 ± 82 | 51 ± 3  | 32 ± 4   |
| + Anti-TNF-α CRTS | 236 ± 90 | 222 ± 51 | 112 ± 35| 72 ± 6   |
| + Anti-TNF-α UBI   | 152 ± 87 | 248 ± 83 | 72 ± 8  | 51 ± 5   |
| + Anti-TNF-α polycl. | 214 ± 76 | 233 ± 38 | 129 ± 24| 46 ± 2   |
| + Anti-class II   | <1       | <0.2      | <0.2     | <0.2     |
| + Anti-CD4 (RIV-7) | 41 ± 9  | 23 ± 4    | 6 ± 1    | 7 ± 3    |

Highly purified B cells (exp. 1 and 2) or slgD⁺ B cells (exp. 3) were cultured in the presence of B21 T cells and IL-4. Highly purified negatively sorted B cells (>98% CD20⁺) or sorted slgD⁺ B cells (>99.5% slgD⁺) from normal spleens were cocultured for 12-14 d with an equal number of B21 cells, 400 U/ml IL-4, and mAbs as indicated. All mAbs were added at the beginning of the culture at a concentration of 10 μg/ml. The supernatants from eight replicates were pooled and assayed for Ig isotype content by ELISA. Values represent means and standard deviations of triplicate wells of the ELISA.

of mTNF-α expression on T cell clone B21 are shown in Fig. 1. Induction of mTNF-α was observed already 2 h after stimulation. By 8 h 100% of the cells expressed mTNF-α. Peak expression was obtained between 24 and 36 h, after which mTNF-α expression decreased gradually (Fig. 1). Similar results were obtained with two other CD4⁺ T cell clones known to induce Ig synthesis by B cells in the presence of IL-4 (data not shown).

Expression of mTNF-α on T Cell Clones Is Not Affected by IFN-γ or IFN-α. IFN-γ and IFN-α have been shown to block IgE synthesis induced by activated T cells but not that induced by anti-CD40 mAbs (3, 13, 14), suggesting that these cytokines may interfere with costimulatory T cell signals. We therefore tested whether IFN-γ and IFN-α mediate their action by preventing mTNF-α expression on T cell clones. In Fig. 2 it is shown that IFN-γ and IFN-α did not affect mTNF-α expression, even when the T cell clones were preincubated with these cytokines for 24 h before Con A activation. This suggests that IFN-γ and IFN-α modulate IL-4-dependent IgE synthesis independently from mTNF-α expression.

Immunoprecipitation and SDS-PAGE of Radiolabeled TNF-α from Activated B21 T Cell Clone. Immunochemical studies have indicated that mTNF-α has a molecular mass of 26 kD
Table 2. Effect of Anti-TNF-α mAbs on Ig Synthesis Induced by T Cell Membranes and IL4

| Exp. | IgM       | IgG      | IgG4     | IgE       |
|------|-----------|----------|----------|-----------|
| 1    | B         | <1       | 10±3     | 1±0       | <0.2      |
|      | B + B21 membranes | 14±0     | 76±47    | 2±0       | <0.2      |
|      | B + B21 membranes + IL-4 | 145±62   | 178±28   | 12±3      | 82±24     |
|      | + Control mAb  | 184±55   | 199±35   | 10±4      | 89±34     |
|      | + Anti-TNF-α 1825.12 | 35±6     | 64±18    | <1        | 6±2       |
|      | + Anti-TNF-α 6401.1 | 75±18    | 90±24    | 5±2       | 35±14     |
|      | + Anti-IL-4     | 7±4      | 18±10    | 1±0       | 2±0       |
|      | + Anti-CD4 (Riv-7) | 25±3     | 69±31    | <0.2      | <0.2      |
|      | + Anti-CD4 (RPA-T4) | 21±7     | 23±1     | 1±0       | <0.2      |
|      | + Anti-class II MHC | 7±3      | 11±1     | 5±2       | <0.2      |
| 2    | B         | <1       | <1       | <0.2      | <0.2      |
|      | B + A3 membranes | 11±1     | 68±17    | <0.2      | <0.2      |
|      | B + IL-4 + A3 membranes | 183±31   | 306±53   | 204±23    | 151±13    |
|      | + Control mAb  | 150±23   | 296±41   | 193±11    | 145±17    |
|      | + Anti-TNF-α 1825.12 | 6±1     | <1       | <0.2      | <0.2      |
|      | + Anti-TNF-α 6401.1 | 93±25    | 175±40   | 107±2     | 73±8      |
| 3    | B         | <1       | <1       | <0.2      | <0.2      |
|      | B + IL-4 + A3 membranes | 61±5    | 338±60   | 222±21    | 88±20     |
|      | + Control mAb  | 59±5     | 340±49   | 254±34    | 131±48    |
|      | + Anti-TNF-α 1825.12 | 5±1      | 5±1      | <0.2      | <0.2      |
|      | + Anti-TNF-α 6401.1 | 25±1     | 190±42   | 60±17     | 37±17     |

Ig synthesis by highly purified B cells in the presence of plasma membranes of B21 of SPA3 T cells and IL-4 is blocked by anti-TNF-α mAbs. Highly purified B cells were cocultured with 10 μg/ml plasma membranes of Con A-activated B21 or A3 cells with or without 400 U/ml IL-4 and mAbs as indicated. The supernatants from each of six or eight replicate cultures in exps. 2 and 3 were pooled and then assayed by ELISA in triplicate wells. The cultures in exp. 1 were set up in triplicate and assayed individually by ELISA.

Table 3. Effect of Pretreatment of T Cell Membranes with Anti-TNF-α mAbs on Ig Synthesis

|       | IgM       | IgG      | IgG4     | IgE       |
|-------|-----------|----------|----------|-----------|
| B     | <1        | <1       | 3±1      | <0.2      |
| B + B21 membranes | 3±1       | 27±17    | 4±1      | <0.2      |
| B + IL-4 + B21 membranes | 15±8     | 316±53   | 62±10    | 18±5      |
|      | + Control mAb  | 20±12    | 335±41   | 57±7     | 20±7      |
|      | + Anti-TNF-α 1825.12 | 1±1     | <1       | <0.2      | <0.2      |
|      | + Anti-TNF-α 6401.1 | 10±2     | 46±19    | 21±3     | 5±1       |
| Membranes pretreated with control mAb | 9±2       | 252±37   | 59±10    | 12±1      |
| Membranes pretreated with anti-TNF-α 1825.12 | 1±1       | 21±2     | 6±1      | 1±0       |
| Membranes pretreated with anti-TNF-α 6401.1 | 2±0       | 78±9     | <0.2     | 1±0       |

Inhibition of B21 membrane-induced IgE synthesis by anti-TNFα mAb and pretreatment of the membranes with anti-TNF-α mAbs. Plasma membranes from Con A-activated B21 cells were pretreated for 2 h with the indicated mAbs, washed three times, and resuspended in the original volume of medium. 5,000 highly purified B cells were cocultured with 2 μg of membranes in each of eight wells. IL-4 (400 U/ml) and mAbs were added to some of the cultures as indicated. The supernatants from eight wells were pooled after 14 d of culture and assayed for Ig content by ELISA. The standard deviation is of triplicate wells of the ELISA. One of two experiments shown.
Table 4. Effect of Anti-TNF-α mAb on Ig Synthesis Induced by Anti-CD40 mAbs and IL-4

| Exp. | IgM | IgG | IgG4 | IgE |
|------|-----|-----|------|-----|
| 1 B  | 1 ± 1 | 2 ± 1 | <0.2 | <0.2 |
| B + IL-4 | 1 ± 1 | 2 ± 1 | <0.2 | <0.2 |
| B + IL-4 + αCD40 | 13 ± 2 | 310 ± 45 | 139 ± 23 | 77 ± 1 |
| + Control mAb | 10 ± 2 | 275 ± 56 | 146 ± 35 | 89 ± 8 |
| + Anti-TNF-α 6401.1 | 10 ± 0 | 281 ± 47 | 122 ± 40 | 59 ± 5 |
| + Anti-TNF-α 1825.1 | 14 ± 1 | 228 ± 25 | 138 ± 13 | 53 ± 9 |
| 2 B  | 2 ± 1 | 3 ± 2 | 1 ± 0 | <0.2 |
| B + IL-4 | 2 ± 0 | 4 ± 1 | 1 ± 3 | <0.2 |
| B + IL-4 + αCD40 | 21 ± 5 | 267 ± 12 | 109 ± 15 | 56 ± 9 |
| + Control mAb | 18 ± 1 | 319 ± 44 | 122 ± 2 | 76 ± 7 |
| + Anti-TNF-α 6401.1 | 16 ± 2 | 275 ± 37 | 110 ± 17 | 54 ± 4 |
| + Anti-TNF-α 1825.12 | 14 ± 2 | 330 ± 21 | 132 ± 23 | 62 ± 7 |
| 3 B  | <1 | 1 ± 0 | <0.2 | <0.2 |
| B + IL-4 + αCD40 | 11 ± 3 | 48 ± 3 | 21 ± 1 | 10 ± 2 |
| + Control mAb | 12 ± 3 | 54 ± 1 | 27 ± 3 | 9 ± 1 |
| + Anti-TNF-α 6401.1 | 10 ± 2 | 40 ± 1 | 21 ± 4 | 13 ± 2 |
| + Anti-TNF-α 1825.12 | 12 ± 2 | 46 ± 1 | 23 ± 5 | 9 ± 1 |

Effect of anti-TNF-α mAbs on Ig synthesis induced in highly purified B cells by the anti-CD40 mAb 89 and IL-4. Highly purified negatively sorted B cells (>98% CD20⁺) were cultured in the presence of 400 U/ml IL-4 and 10 μg/ml mAb 89, specific for human CD40. The indicated mAbs were added at the beginning of the culture, and 12-14 d later the supernatants were assayed for Ig concentration by ELISA. The values represent means and standard deviations of triplicate wells of the ELISA.

Discussion

IL-4 directs IgE switching in B cells (1, 2), but additional costimulatory signals are required for productive IgE synthesis. These signals can be delivered by activated CD4⁺ T cell clones, but not by resting CD4⁺ nor by activated CD8⁺ T cell clones (6). One such a costimulatory signal is provided by the CD40L, which has been recently cloned in mice (18) and humans (19–21).

Herein we show that the mTNF-α on activated CD4⁺ T cells is another costimulatory signal for IL-4-dependent Ig production by B cells. Membrane TNF-α is rapidly expressed on CD4⁺ T cell clones after activation. All T cell clones expressed this molecule 8 h after activation. mAbs that bind mTNF-α inhibited predominantly IgG4 and IgE synthesis and to a lesser extent total IgG production in cultures of purified B cells, activated CD4⁺ T cell clones, and IL-4. IgM production in these cultures was generally not affected by anti-TNF-α mAbs. However, in cultures of slgD⁺ B cells production of IgM, total IgG, IgG4, and IgE was inhibited, suggesting that inhibition of the interactions between mTNF-α and its ligand not only interfered with the signaling pathways required for IL-4-induced IgG4 and IgE switching, but also with productive T-B cell interactions resulting in IgM and total IgG production. The differential inhibitory effects of anti-TNF-α mAbs in cultures of total B cells versus slgD⁺ B cells are presently difficult to explain, but they may be related to differences in the relative contribution of mTNF-α and other T cell surface molecules (such as the CD40L) to activation of naive slgD⁺ B cells or total B cells. The experiments in which membranes of activated CD4⁺ T cell
Figure 1. Kinetics of surface TNF-α expression by the CD4+ T cell clone B21 activated with Con A. Resting B21 cells (12 d after last stimulation with feeder cells and PHA) were cultured in Yssel's medium supplemented with 10% FCS and 10 μg/ml Con A. At the indicated time intervals, aliquots of cells were harvested, washed twice in PBS, and stained with the following FITC-labeled antibodies: mouse IgG1 control (left), anti-human mTNF-α (middle), or the positive control anti-CD69 mAb (right). The cells were then washed and fixed in 1% paraformaldehyde. At the end of the 48-h period, all samples were analyzed by a FACScan® flow cytometer.

clones were used indicated that mTNF-α is also involved in the interaction between activated CD4+ T cell clones and IgM- or IgG-committed B cells. Anti-TNF-α mAbs blocked the production of all isotypes in these cultures.

Anti-TNF-α mAbs did not block Ig production induced by anti-CD40 mAbs and IL-4 in the absence of T cells, indicating that inhibition occurred at the Th cell and not at the B cell level. In addition, the observation that anti-TNF-α mAbs effectively blocked Ig production in cultures where intact CD4+ T cell clones were replaced by their plasma membranes is compatible with the idea that mTNF-α expressed on activated CD4+ T cell clones, rather than soluble TNF-α, produced by the T cell clones, was the target of the anti-TNF-α mAbs. This notion was further supported by the finding that pretreatment of the T cell plasma membranes with anti-TNF-α mAbs decreased their capacity to induce B cells to produce Ig.

The expression of mTNF-α on activated T cell clones was confirmed by immunoprecipitation of the 26-kD form of TNF-α, from the surface of radiolabeled T cell clones. These results are in agreement with other studies, which showed that this 26-kD polypeptide represented mTNF-α on monocytes, mouse cells transfected with the human TNF-α gene (22), and activated peripheral blood T cells (23). In addition, a distinct band of 17 kD was detected. This protein probably represents the mature form of TNF-α, since it has been shown that in addition to the 26-kD molecule, peptides of 17 kD representing the mature form of TNF-α could be immunoprecipitated from surface-labeled 3T3 cells, transfected with the human TNF-α gene (22). In contrast to the 26-kD molecule, the 17-kD band was not detected in immunoprecipitates from activated T cells (23). However, in the latter assays, a protein with a molecular mass of 50–60 kD, which was suggested to represent the trimeric TNF-α molecule, was detected (23). The possibility that TNF-α trimers bound to TNFRs on T cells could be responsible for delivering the
costimulatory signal to B cells seems remote, since it would imply that TNF-\(\alpha\) binding sites on TNFRs on T cells are different from those on B cells. To date such information is not available. Furthermore, pretreatment of the activated T cell clone B21 with a low pH glycine buffer, a treatment known to dissociate soluble ligands from their receptors (35), caused no significant decrease in anti-TNF-\(\alpha\) mAb binding to these cells, as judged by FACS analysis, while it readily removed FITC-labeled anti-CD4 mAb from precoated B21 cells used as controls (data not shown).

It has been suggested that the diversity of physiological actions mediated by TNF-\(\alpha\) may be a result of the differential actions of the soluble versus the transmembrane forms of TNF-\(\alpha\) (36). In addition, it has been reported that mTNF-\(\alpha\) and soluble TNF-\(\alpha\) can mediate distinct biological functions. For example, in vivo regression of some murine tumors is mediated by soluble, and not by mTNF-\(\alpha\) (37), whereas mTNF-\(\alpha\) on activated T cells, in contrast to soluble TNF-\(\alpha\), can trigger antileishmanial defense in macrophages by direct cell–cell contact (38, 39). It is therefore not surprising that in our studies the mTNF-\(\alpha\) expressed on activated CD4\(^+\) T cell clones, rather than the secreted cytokine, is involved in T-B cell interactions required for B cell activation and induction of Ig synthesis.

The mTNF-\(\alpha\) is a type II glycoprotein (22) similar to the CD40L (18, 21). In addition, more recently, sequence homologies in the receptor-binding portion of the extracellular domains between both murine TNF-\(\alpha\) and the murine CD40L, and human TNF-\(\alpha\) and the human CD40L, have been reported (20, 40), suggesting a functional similarity between mTNF-\(\alpha\) and CD40L. The CD40 molecule is a member of the TNF receptor/nerve growth factor receptor family (41). Since CD40L–CD40 interactions result in B cell activation (18–21), the present data indicating that interactions of

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**Figure 2.** Effect of IFN-\(\gamma\) and IFN-\(\alpha\) pretreatment of B21 T cell clones on the expression of mTNF-\(\alpha\) after Con A stimulation. Resting (12 d after last stimulation with feeder cells and PHA) B21 cells were cultured at 10^6 cells/ml in Yssel's medium containing 1% human serum and 100 U/ml IFN-\(\gamma\) or IFN-\(\alpha\), or left untreated. After 24 h the cells were harvested, washed twice in PBS, and resuspended in the original volume of Yssel's medium containing 5 \(\mu\)g/ml Con A. Some cells were left untreated throughout the culture period (first row). 24 h later, the cultures were harvested, washed twice in RDF1 buffer (R&D Systems), and stained for 1 h with FITC-labeled anti-TNF-\(\alpha\) mAb (Fluokine kit; R&D Systems), mouse IgG1 isotype control (Becton Dickinson & Co.), or anti-CD69 mAbs. After washing in RDF1 buffer the cells were analyzed with a FACScan flow cytometer. The FACS profile from cells treated only with Con A (not pretreated with cytokines), stained with anti-TNF-\(\alpha\) mAb (middle, second row), was superimposed on the histograms of IFN-\(\gamma\)- and IFN-\(\alpha\)-pretreated cells stained with anti-TNF-\(\alpha\) mAb (middle, third and fourth rows, respectively).

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**Figure 3.** Immunoprecipitation of mTNF-\(\alpha\). Cell lysates from radiolabeled B21 (lanes A and B) and HY06 (lanes C and D) cells, activated with TPA plus A23187 calcium ionophore, were used to immunoprecipitate mTNF-\(\alpha\) with Pansorbin™ cells precoated with rabbit anti-mouse Ig and mAbs 1825.12 and 6410.1 (lanes A and D). Lanes B and C are from immunoprecipitates using rabbit anti-mouse Ig-coated Pansorbin™ as controls. Immunoprecipitated proteins were run on a 15% polyacrylamide mini-gel under reducing conditions. The dried gels were autoradiographed by exposing to Kodak x-ray films for 32 h.
mTNF-α with its putative ligand(s) on B cells are involved in B cells activation was not unexpected. The ligand(s) for mTNF-α are not known, although the most likely candidates are the two forms of TNFRs. Resting peripheral B cells express both forms of TNFRs in low amounts, and after activation the p75 TNFR is upregulated (42). Both forms of TNFRs can mediate signal transduction (43, 44). In our studies, the mAb htr-1, which recognizes the p55 TNFR, inhibited Ig synthesis in slgD⁺ cells stimulated with activated T cell clones and IL-4. This observation, together with the finding that anti-TNF-α mAb inhibits Ig synthesis at the T cell level, suggests that the p55 TNFR is a possible ligand for the mTNF-α expressed on activated T cell clones. It also suggests that this mAb binds to an epitope involved in the functional interaction between the mTNF-α on T cells and the p55 TNFR on B cells. The finding that the anti-p75 TNFR mAb utr-1 had no inhibitory effects on IL-4-dependent Ig synthesis implies that either the p75 TNFR is not a functional ligand for mTNF-α, or that this mAb binds to an epitope on the p75 TNFR that does not interfere with the binding of the mTNF-α.

IFN-γ and IFN-α are known to inhibit IgE synthesis when B cells are stimulated by T cell clones, but not by anti-CD40 mAbs (13, 14). These observations suggest that the inhibitory action of these cytokines is mediated via the costimulatory signals delivered by T cells. In our present studies, incubation of the T cell clones with IFN-γ and IFN-α did not prevent expression of mTNF-α, indicating that these cytokines do not inhibit IgE synthesis by modulating mTNF-α expression.

The finding that mTNF-α is involved in noncognate T-B cell interactions adds to the complexity of both the pleiotropic effects of this cytokine, and the mechanisms of T cell-induced B cell activation and differentiation. In addition, our data indicate that multiple T cell surface molecules play a role in T-B cell interactions resulting in Ig production.

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