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Prolonged Exposure of T Cells to TNF Down-Regulates TCRζ and Expression of the TCR/CD3 Complex at the Cell Surface

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A role for TNF-α in the pathogenesis of chronic inflammatory disease is now firmly established. Paradoxically, TNF also has potent immunomodulatory effects on CD4+ T lymphocytes, because Ag-specific proliferative and cytokine responses are suppressed following prolonged exposure to TNF. We explored whether TNF attenuated T cell activation by uncoupling proximal TCR signal transduction pathways using a mouse T cell hybridoma model. Chronic TNF exposure induced profound, but reversible, T cell hypersensitiveness, with TNF-treated T cells requiring TCR engagement with higher peptide concentrations for longer periods of time for commitment to IL-2 production. Subsequent experiments revealed that chronic TNF exposure led to a reversible loss of TCRζ chain expression, in part through a reduction in gene transcription. Down-regulation of TCRζ expression impaired TCR/CD3 assembly and expression at the cell surface and uncoupled membrane-proximal tyrosine phosphorylation events, including phosphorylation of the TCRζ chain itself, CD3ζ, ZAP-70 protein tyrosine kinase, and linker for activation of T cells (LAT). Intracellular Ca2+ mobilization was also suppressed in TNF-treated T cells. We propose that TNF may contribute to T cell hypersensitiveness in chronic inflammatory and infectious diseases by mechanisms that include down-regulation of TCRζ expression. We speculate that by uncoupling proximal TCR signals TNF could also interrupt mechanisms of peripheral tolerance that are dependent upon intact TCR signal transduction pathways. The Journal of Immunology, 2001, 166: 5495–5507.

The immune system has evolved to combat a wide variety of foreign pathogens. Although innate immunity may provide the first line of defense, the specificity of the adaptive immune system is generated through an extensive repertoire of lymphocyte Ag receptors. The TCR complex comprises clonotypic, disulfide-linked α and β Ag recognition subunits, made up of large extracellular ligand binding domains, and short intracellular domains devoid of signaling motifs (1). For full function, the TCR/CD3 assembly and expression at the cell surface and uncoupled membrane-proximal tyrosine phosphorylation events, including phosphorylation of the TCRζ chain itself, CD3ζ, ZAP-70 protein tyrosine kinase, and linker for activation of T cells (LAT). Intracellular Ca2+ mobilization was also suppressed in TNF-treated T cells. We propose that TNF may contribute to T cell hypersensitiveness in chronic inflammatory and infectious diseases by mechanisms that include down-regulation of TCRζ expression. We speculate that by uncoupling proximal TCR signals TNF could also interrupt mechanisms of peripheral tolerance that are dependent upon intact TCR signal transduction pathways. The Journal of Immunology, 2001, 166: 5495–5507.

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3 Abbreviations used in this paper: ER, endoplasmic reticulum; HCgp-39, human cartilage glycoprotein-39; hTNF, human TNF; ITAM, immunoreceptor tyrosine-based activation motif; mTNF, mouse TNF; NAC, N-acetylcysteine; RA, rheumatoid arthritis; TNF-R, TNF receptor; TBS-Tween, Tris-buffered saline, 1 mM EDTA (pH 8), and 0.1% Tween 20; LAT, linker for activation of T cells.
In chronic infectious and inflammatory diseases the immune system is persistently exposed to Ag as well as to numerous growth factors and cytokines. This cytokine environment is of functional importance, because cytokines such as IFN-γ, IL-12, and IL-4 regulate the maturation and differentiation of T cells in ways that profoundly influence their effector function (20). We have recently demonstrated that, in contrast to its acute proinflammatory and costimulatory effects, prolonged exposure to TNF suppresses T cell proliferative and cytokine responses following TCR ligation both in vitro and in vivo (21, 22). TNF has also been shown to suppress spontaneous murine models of type I diabetes in nonobese diabetic mice and lupus in NZB/W F1 mice, whereas TNF blockade enhances the frequency and severity of these diseases (23–28). More recently, acceleration and exacerbation of autoimmunity have been described in TNF- and TNF receptor (TNFR)−/− deficient mice (29, 30). Together, these data provide evidence for an immunomodulatory role of TNF during both the progression and evolution of autoimmune responses. Understanding the mechanisms of these effects could provide insight into how attenuation of T cell autoreactivity can subvert the expression of clinical autoimmune disease.

Although the mechanisms through which TNF impairs T cell activation have not been fully elucidated, the suppressive effects of TNF on intracellular Ca2+ mobilization in TCR transgenic T cells suggest that uncoupling of proximal TCR signal transduction pathways may be involved (22). Here, we show in a T cell hybridoma model that one mechanism by which prolonged exposure of T cells to TNF attenuates T cell activation is through down-regulation of TCR chain expression. As a consequence, assembly and cell surface expression of TCR/CD3 are impaired, and downstream signaling pathways are attenuated. We propose that through this mechanism prolonged exposure to TNF in vivo may lead to depressed T cell autoreactivity, impaired immunoregulatory function, and suppressed T cell effector responses to foreign pathogens, a T cell phenotype characteristic of chronic inflammatory diseases in mouse models and in man.

Materials and Methods

**Derivation, culture, and activation of T cell hybridomas**

Mouse T cell hybridomas specific for human cartilage glycoprotein-39 (HCgp-39) (HCgp-39) were derived following immunization of HLA-DRαβ1*0401, human CD4 double-transgenic, MHC class II (I-Ab−/−)-deficient mice with native Ag as previously described (31, 32). Peptide-specific T cell hybridomas were cloned by limiting dilution and propagated in complete medium (RPMI supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, 50 μg/ml gentamycin, 100 U/ml penicillin/streptomycin, 50 μg/ml 2-ME, 1 mM sodium pyruvate, and 25 mM HEPES) as previously described (32). To study the effects of chronic cytokine exposure, T cells were cultured in the presence of chronic recombinant mouse TNF (mTNF; B. Scallon, Centocor, Malvern, PA), human TNF (hTNF; Z. Kaymakcalan, BASF, Worcester, MA) or human IL-1α (IL-1; U. Gubler, Hoffmann-La Roche, Nutley, NJ) for the indicated periods of time. Cytokines were added to cultures every 3 days. T cells were resuspended in complete medium at 105 cells/ml before stimulation. Where indicated, N-acetyl-cysteine (NAC; Sigma, Poole, U.K.) was added to cultures at a final concentration of 1 mM together with TNF. For T cell activation, 1 × 106 T cells were stimulated for 24 h with peptides derived from HCgp-39 and 4 × 105 EBV-transformed B cells homologous for HLA-DRαβ1*0401 as APC in round-bottom 96-well plates or by plate-bound anti-CD3ε mAb. T cells were stimulated in the absence of cytokines, and all assays performed in duplicate. IL-2 production in culture supernatants was measured by immunooassay using rat anti-mouse IL-2 mAb pairs (PharMingen, San Diego, CA). The assay was developed using streptavidin–Europium detection system (Wallac Oy, Turku, Finland), and the results were analyzed on a Wallac fluorescence plate reader. Cytokines were added to cultures every 3 days. T cells were resuspended in complete medium at 105 cells/ml before stimulation. Where indicated, N-acetyl-cysteine (NAC; Sigma, Poole, U.K.) was added to cultures at a final concentration of 1 mM together with TNF. For T cell activation, 1 × 106 T cells were stimulated for 24 h with peptides derived from HCgp-39 and 4 × 105 EBV-transformed B cells homologous for HLA-DRαβ1*0401 as APC in round-bottom 96-well plates or by plate-bound anti-CD3ε mAb. T cells were stimulated in the absence of cytokines, and all assays performed in duplicate. IL-2 production in culture supernatants was determined by specific immunoassay using rat anti-mouse IL-2 mAb pairs (PharMingen, San Diego, CA). The assay was developed using streptavidin–Europium detection system (Wallac Oy, Turku, Finland), and the results were analyzed on a Wallac fluorescence plate reader. The data are presented as IL-2 fluorescence units ± SD. The HCQ6 mouse T cell hybridoma, specific for type II collagen and restricted to I-Ab+, was transduced with a chimeric cell surface receptor by retroviral gene transfer using the pBabe retroviral vector as previously described (33). The chimeric receptor comprises a single-chain Fv construct from the C2 mAb that is specific for native type II collagen coupled to the γ signaling subunit of FcεRI (C2/γ). This signaling subunit carries a single ITAM and is essential for transducing downstream signals necessary for IL-2 production in T cells. In addition, HCQ6 cells were transduced with a mutant receptor lacking the ITAM motif (C2/γ/VIC¬). C2 receptor expression was confirmed by flow cytometric analysis using a rabbit anti-C2 polyclonal Ab (33). The expression of C2/γ homodimers vs C2/γ-TCRζ heterodimers has been determined previously by immunoblotting of cell lysates with a rabbit polyclonal anti-C2 Ab under nonreducing conditions (33). Chronic culture in the presence of cytokines was performed as described above, and the chimeric receptor-mediated responses were tested by stimulating T cells with plate-bound native bovine collagen II (a gift from R. Williams, London, U.K.).

**Abs and flow cytometry**

The following mAbs were used for flow cytometric analysis: anti-mouse CD3ε–FITC, TCRβ–FITC, CD45–PE, and CD69–biotin (PharMingen) and anti-human CD4–FITC (Becton Dickinson, San Jose, CA). For flow cytometry, T cells were stained by standard methods and analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson). To study the proportions of apoptotic and dead cells, annexin V–FITC and propidium iodide staining was performed using a commercial kit (Alexis, San Diego, CA). Glutathione levels were determined in whole cells by flow cytometry after staining with 50 nM Cell Tracker Green 5-chloromethyl-fluorescein diacetate according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). For T cell activation, hamster anti-mouse CD3ε clones 145-2C11 and 500.A2 (PharMingen), mouse anti-human CD4 (OKT4) purified from a hybridoma supernatant, hamster Ig control (clone L2; R. Schreiber, Washington University School of Medicine, St. Louis, MO), and rabbit anti-C2 (33), followed by goat anti-hamster Ig (Cappel, SA Scientific, Eschwege, Germany) or goat anti-rabbit Ig (Pierce, Rockford, IL) were used for cross-linking. A blocking anti-HLA-DR mAb (L243) was provided by CellTech (Slough, U.K.). For immunoprecipitation and Western blotting experiments, polyclonal TCRζ antisera 98118 (J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and K2 (A. Kang, Veterans Administration Medical Center, Memphis, TN), monoclonal anti-TCRζ clones 6B10.2, which recognizes a transmembrane domain of the α chain, and goat anti-C2 polyclonal Ab (33). The expression of C2/γ, C2ε, C2α, C2β, and C2ζ was confirmed by Western blotting.

**Studies of signaling pathways**

T cells (5 × 106 cells/time point) were harvested into serum-free RPMI, washed twice, and rested on ice for 1 h. Cells were then incubated with 10 μg/ml of anti-mouse CD3ε with or without anti-human CD4 mAbs or hamster Ig control for 20 min on ice. After washing with ice-cold RPMI, T cells were resuspended in serum-free medium at 37°C before stimulation with 10 μg/ml of goat anti-hamster Ig for the indicated times. For peptide stimulation, 5 × 106 T cells were mixed with 20 × 105 APCs pulsed with 50 μg/ml of specific or control peptide for 4 h at 37°C. Cells were centrifuged for 30 s at 3000 rpm and incubated for various times at 37°C. T cell activation was terminated by adding ice-cold PBS containing 0.5 mM Na2VO4, 10 μg/ml aprotinin). For CD3ε and CDζ, at 3°C. For CD3ε the CD3ε immunoprecipitation, 0.5% Triton X-100 was used as a detergent to preserve the association between CD3 chains and TCRζ. Lysates were centrifuged at 10 min at 13,000 rpm to remove detergent-insoluble material. Postnuclear lysates were used for immunoprecipitation experiments or were resuspended into 2× SDS–PAGE sample buffer with or without 2-ME and studied by Western blotting.

**Immunoprecipitation and in vitro kinase assays**

After preclearing with protein A-agarose (Sigma), lysates were incubated with 5 μg of purified Ab or a 1/100 dilution of specific antisera for 1–2 h on ice, followed by the addition of 35 μl of protein A-agarose in each sample. Lysates were incubated for 2 h at 4°C with rotation, after which immunoprecipitates were washed three times with ice-cold lysis buffer and resuspended in 2× SDS–PAGE sample buffer with or without 2-ME. To immunoprecipitate cell surface CD3ε, the cells were stained with 10 μg/ml...
of the anti-CD3ε mAb for 20 min on ice before cell lysis. After three washes to remove the unbound mAb, the cells were lysed, and 35 μl of protein A-agarose was added directly. For Lck kinase assays, an additional wash in 20 mM Tris (pH 7.5) and 0.5 M LiCl and two additional washes in kinase buffer (20 mM Tris (pH 7.5), 10 mM MnCl₂, 0.1% 2-ME, and 100 μM NaVO₄) were performed. Thereafter, immunoprecipitates were resuspended in 50 μl of kinase buffer containing 5 μg of purified substrate (a GST fusion protein including a C-terminal fragment corresponding to aa 331–443 of human SAM-68; provided by W. Kolanus, University of Munich, Munich, Germany) and 10 μM unlabeled ATP. Five to 10 μCi of [γ-³²P]ATP (3000 Ci/mmol; Amersham) were added to each tube, and the reaction was allowed to proceed for 15 min at room temperature with agitation. The reactions were terminated by adding 4× SDS-PAGE sample buffer. Boiled samples were resolved on SDS-PAGE before staining with Coomassie Brilliant Blue R-250 to verify equal substrate loading and equal amounts of immunoprecipitating Ab per sample. Radioactivity was quantified using a phosphorimagery (FLA-2000; Fuji, Tokyo, Japan).

Results

Chronic TNF exposure induces nondeletional and reversible T cell hyporesponsiveness

To investigate the mechanisms for T cell hyporesponsiveness induced by TNF, we used a model of prolonged TNF exposure with HCGp-39 specific, HLA-DR4-restricted mouse T cell hybridomas. T cell hybridomas were chosen to study the direct effects of TNF on T cells in the absence of accessory cells and to undertake a more detailed kinetic analysis of the effects observed. Hybridoma clones expressing p55 and p75 TNF-R (as well as other cell surface activation Ags) at levels similar to those observed on chronically activated T lymphocytes were selected for the study (data not shown) (35, 36).

Fig. 1A shows that prolonged exposure of T cell hybridoma clones to mTNF or hTNF suppressed IL-2 production following stimulation with EBV-transformed human B cells pulsed with specific peptide. TNF could suppress T cell activation by up to 90% depending on the clone studied. Splenic APC derived from HLA-DR4 transgenic mice gave similar results, although the levels of IL-2 detected were somewhat lower (data not shown). Given that mTNF and hTNF attenuated T cell activation to a similar extent, and hTNF binds and signals through murine p55, but not p75 TNF-R, these results suggest that induction of T cell hyporesponsiveness is mediated by sustained signals transduced through the p55 TNF-R.

IL-1 shares many signaling pathways with TNF, and IL-2 production was noted for IL-1-treated 32A1 cells.

Suppression of T cell activation by chronic TNF treatment was both time and dose dependent. A detailed kinetic analysis revealed detectable depression of peptide-specific IL-2 production within 4 days of culture in the presence of hTNF (Fig. 1B). Moreover, a decrease in IL-2 production was observed at picomolar concentrations of hTNF (e.g., 0.6 ng/ml; <50 pM) after more prolonged culture, concentrations well within the range of TNF expression at sites of chronic inflammation (37). Finally, T cell hyporesponsiveness was reversible upon withdrawing TNF, because peptide-induced IL-2 production was restored to control levels after only 2 days of culture in the absence of TNF (Fig. 1C). Consistent with this result, TNF did not significantly affect cell viability under these experimental conditions (Fig. 1D).

Because TNF-treated T cell hybridomas reproduced many of the characteristics of T cells exposed to TNF in vivo reported previously (21, 22), this model system was used to explore in more detail the molecular basis for T cell hyporesponsiveness induced by TNF. For the purpose of these experiments, we selected experimental conditions that suppressed T cell activation by ~70–80%: 2.5 ng/ml hTNF for 8–14 days was used for clone 11A2 unless otherwise stated.
TNF-treated T cells require stronger and more sustained TCR engagement for commitment to IL-2 production.

We next studied T cell responses to a broad range of peptide and anti-CD3 mAb concentrations. T cells pretreated with mTNF or hTNF produced much lower levels of IL-2 regardless of the strength of the TCR stimulus (Fig. 2A). Indeed, suppression of responses could not be overcome with anti-CD3 concentrations up to 8 μg/ml (Fig. 2A) or with peptide concentrations up to 30 μg/ml (data not shown). In addition, these experiments demonstrated that stronger TCR engagement was required for TNF-treated T cells to produce detectable levels of IL-2 (Fig. 2A).

We then determined the time period of TCR engagement required for induction of IL-2 production. Control and TNF-treated cells were stimulated with peptide-pulsed APC as before, and at predefined time points TCR ligation by MHC/peptide complexes was interrupted by adding a blocking anti-HLA-DR mAb to the cultures. This mAb completely blocked IL-2 production when added at the beginning of stimulation (see zero time point in Fig. 2B). Using another approach, T cells were stimulated with plate-bound anti-CD3 mAb, and at predefined time points the cells were transferred to uncoated wells. All cultures were harvested after 24 h, and IL-2 production was determined. As shown in Fig. 2B, TNF-treated T cells required longer periods of TCR engagement than control cells for commitment to IL-2 production.

Although a more precise quantitation of the parameters required for productive TCR stimulation in control and TNF-treated cells is difficult, multiple experiments indicated that TNF-treated T cells required approximately four times more peptide or anti-CD3 mAb for up to 3 h longer to produce detectable levels of IL-2 (using a stimulation index of 3 as the detection limit). Collectively, these results indicated that chronic TNF exposure increases the threshold required for T cell activation and commitment to IL-2 production.

**Chronic TNF exposure down-regulates the expression of cell surface TCR/CD3 complex**

An increased activation threshold together with our previous results demonstrating suppressed intracellular Ca²⁺ mobilization by TNF (22) suggested that TNF might attenuate proximal TCR signal transduction pathways. To test this hypothesis, we first investigated cell surface TCR/CD3 expression after chronic TNF exposure. Indeed, flow cytometric analysis revealed a modest, yet reproducible, down-regulation of cell surface CD3e expression in TNF-treated cells, whereas the expression of CD3e was unaltered in cells chronically cultured in the presence of IL-1 (Fig. 3A); similar results were observed using anti-TCRβ mAbs (data not shown). Down-regulation of cell surface molecules was not a generalized characteristic of TNF-treated cells, because no significant effect on the expression of hCD4 or CD45 was noted (Fig. 3B), and the expression of the early activation Ag CD69 was consistently increased in TNF-treated cells. The effects of TNF on CD3e expression were dose dependent (Fig. 3C), and data from 10 experiments revealed that the median fluorescence intensities were 69.5, 77.8, and 82.4% of control in cells cultured in the presence of 2.5, 0.6, and 0.15 ng/ml of hTNF, respectively. It should be noted that at low TNF concentrations modest suppression of T cell IL-2 production (~20–30%) was observed in the absence of any detectable decrease in TCR/CD3 expression.

The above results suggested that either TNF may affect the expression of CD3e directly or the TCR/CD3 complex is not optimally assembled or transported to the cell surface. To investigate the first possibility, expression of CD3e was studied by Western blotting. As shown in Fig. 3D, chronic TNF exposure did not alter CD3e levels in whole cell lysates. Consistent with these results, similar levels of CD3e could be immunoprecipitated from whole cell lysates derived from control and TNF-treated cells (Fig. 3D). In contrast, cell surface immunoprecipitates derived following incubation of whole cells with the immunoprecipitating mAbs before cell lysis revealed a reduction in cell surface CD3e expression in TNF-treated cells compared with control cells (Fig. 3D). These data suggested that chronic TNF exposure altered the subcellular localization of CD3e and indicated to us that TNF perturbed TCR/CD3 complex assembly and/or transport to the cell surface.

**Time- and dose-dependent decrease in TCRζ chain expression by chronic TNF**

Because the association of TCRζ-ζ chain homodimers with the hexameric αβγδεδ complex is a critical step in the formation of
complete TCR/CD3 receptor complexes (5, 6), we explored the possibility that modulation of TCRζ chain levels by TNF could explain the differences in subcellular localization of CD3ε. Fig. 4A shows that TNF profoundly down-regulated the expression of TCRζ-ζ homodimers (32 kDa) in whole cell lysates from clone 11A2. The extent of TCRζ down-regulation by mTNF and that by hTNF were similar, whereas hIL-1 had no effect. The decrease in TCR ζ-chain expression was selective, because no decrease in the expression of protein tyrosine kinase ZAP-70 was observed in the same lysates (Fig. 4A), nor were the levels of Src kinases Lck and Fyn markedly altered by TNF (data not shown). Furthermore, streptavidin-HRP blotting of TCRζ immunoprecipitates derived from cell surface biotinylated T cells indicated that the expression of plasma membrane-associated TCRζ was substantially reduced in TNF-treated T cells (Fig. 4B).

A kinetic analysis revealed that TCRζ down-regulation could be detected by 4 days of culture in the presence of 2.5 and 0.6 ng/ml of hTNF, but could also be detected after culturing T cells at very low TNF concentrations for longer periods (e.g., 0.15 ng/ml for 16–20 days; data not shown). Nevertheless, as in the case of TCR/CD3 expression, an ~30% decrease in IL-2 production was observed without a detectable reduction in TCRζ expression, indicating that the correlation between TCRζ expression and IL-2 production may not be absolute, and that TNF may attenuate downstream signaling pathways. Consistent with the recovery of IL-2 production following TNF withdrawal (Fig. 1D), TCRζ down-regulation was reversible, because levels were restored toward normal upon withdrawing TNF (Fig. 4C).

Down-regulation of TCRζ by TNF could not be explained by dissociation of dimers into monomeric TCR ζ-chains, because comparable decreases were observed in the expression of the 16-kDa TCRζ monomer studied under reducing conditions (data not shown), nor could it be explained by modification of a specific epitope, because similar results were obtained using two different TCRζ-specific mAbs, derived from clones 6B10.2 and 8D3, recognizing transmembrane and C-terminal epitopes, respectively (data not shown). By contrast, steady state TCRζ mRNA levels determined by Northern blotting were reduced by ~30% in T cells cultured in the presence of 2.5 ng/ml TNF (Fig. 4D). Nonetheless, TCRζ mRNA expression was not substantially decreased in T cells treated with lower concentrations of TNF despite consistent reductions of TCRζ protein expression (see Fig. 8B below). These data indicate that in addition to its effects on TCRζ mRNA levels, TNF may down-regulate TCR ζ-chain expression through post-transcriptional and/or post-translational mechanisms.

We then investigated the association of TCRζ with the TCR/CD3 complex. Fig. 4E shows that the amount of dimeric TCRζ associated with CD3ε was significantly reduced in TNF-treated cells. In contrast, similar levels of CD3γ and CD3ε were present in CD3ε immunoprecipitates from control and TNF-treated cells (data not shown). These findings imply that the decrease in cell surface TCR/CD3 expression after chronic TNF exposure could arise as a direct consequence of a selective reduction in the expression of TCRζ.

Chronic TNF exposure suppresses IL-2 production transduced through a chimeric receptor, which uses TCRζ for signaling, but not for assembly and expression

The results to date supported the idea that TNF suppressed T cell activation by down-regulating TCRζ expression, thereby perturbing the assembly and transport of stable TCR/CD3 complexes from the ER to the cell surface. To test the hypothesis that TNF
targeted specifically TCRz, as opposed to TCR/CD3 expression, we studied the responses of T cell hybridomas expressing a chimeric receptor that uses TCRz, but not CD3, molecules for signaling. Briefly, a panel of receptors and mutants was generated based on a prototype receptor comprising a single-chain Fv of C2 mAb specific for type II collagen coupled to the γ signaling subunit of FcεRI (C2/γ) (33). In T cell hybridomas these chimeric receptors are expressed on the cell surface as either C2/γ heterodimers or C2/γ/TCRz heterodimers. In HCQ6 T cells, which express relatively high levels of TCRz, the predominant form (∼80%) of the chimeric receptor is a C2/γ/TCRz heterodimer (33).

To examine responses transduced through a chimeric receptor complex with an absolute requirement for TCRz to signal, we studied HCQ6 cells expressing mutant C2/γ receptors lacking the ITAM motif (C2/γ/IC−; see schematic in Fig. 5A).

In contrast to its effects on TCR/CD3 expression, chronic TNF exposure had no effect on cell surface expression of the chimeric receptor on transduced HCQ6 cells (Fig. 5B). As expected, TNF reduced the expression of endogenous TCRz chain in HCQ6 cells (Fig. 5C). Furthermore, IL-2 responses to plate-bound collagen II were suppressed by TNF to the same extent as IL-2 responses to TCR ligation (Fig. 5D). These results indicated that TNF-induced changes in the expression of TCRz, but not the cell surface receptor complex, were sufficient to suppress IL-2 production.

**FIGURE 3.** Chronic TNF exposure down-regulates the expression of the cell surface TCR/CD3 complex. A, Flow cytometric analysis of CD3e expression on control 11A2 cells (thick line) and on cells chronically cultured with 2.5 ng/ml mTNF or hTNF or with 5 ng/ml hIL-1 (thin line). Shaded histograms represent staining with negative control mAb. B, Flow cytometric analysis of hCD4, CD45, and CD69 expression on control cells (thick line) and cells chronically cultured in the presence of 2.5 ng/ml hTNF (thin line). C, Flow cytometric analysis of CD3e expression on control cells (thick line) and on cells cultured with hTNF (thin line). D, CD3e expression in whole cell lysates from control and TNF-treated cells was studied by Western blotting under reducing conditions. CD3e was immunoprecipitated from whole cell lysates or from the cell surface of control cells and cells treated with 2.5 ng/ml hTNF, and CD3e expression was studied by Western blotting under reducing conditions. Representative results from eight (A), three (B), ten (C), and four (D) separate experiments are shown.
**FIGURE 4.** Down-regulation of TCRζ expression after chronic TNF exposure. Clone 11A2 was chronically cultured with 2.5 ng/ml mTNF or hTNF or with 5 ng/ml hIL-1. A, IL-2 production was investigated as described for Fig. 1. TCRζ-ζ and ZAP-70 expression in whole cell lysates was studied by Western blotting under nonreducing or reducing conditions, respectively. **B**, Following cell surface biotinylation, TCRζ was immunoprecipitated from whole cell lysates. Biotinylated cell surface TCRζ-ζ was detected by Western blotting with streptavidin-HRP under nonreducing conditions. **C**, Clone 11A2 was cultured in the presence or the absence of hTNF for 12 days, the cells were washed extensively, and the culture was continued for an additional 4 days in the absence of TNF (recovery). TCRζ-ζ expression in whole cell lysates was studied by Western blotting under nonreducing conditions. **D**, The levels of TCRζ and GAPDH mRNA in T cells were studied by Northern blotting. The values indicate the ratios of TCRζ:GAPDH compared with those in control cells, determined by phosphorimaging. In T cells treated with 2.5 ng/ml TNF, TCRζ mRNA was reduced by 32% (mean percent reduction in three experiments). **E**, CD3ε was immunoprecipitated from whole cell lysates, and the expression of CD3ε and TCRζ-ζ was studied by Western blotting under reducing or nonreducing conditions, respectively. Representative results from six (A), three (B and D), two (C), and four (E) separate experiments are shown.

**Chronic TNF exposure leads to quantitative, but not qualitative, changes in TCRζ phosphorylation**

The TCR ζ-chain is not only involved in TCR/CD3 assembly, but also initiates signal transduction cascades originating from the TCR (10). To study how long term TNF exposure affected early phosphorylation events, TCRζ-ζ was immunoprecipitated from control and TNF-treated cells after activation by anti-mouse CD3ε either alone or in combination with anti-human CD4 mAb. TCR ligation induced the characteristic pattern of phosphorylated ζ isoforms pp21 and pp23; activation with anti-CD3 alone mimicked a partial agonist signal (pp21 > pp23), whereas combined anti-CD3 and anti-CD4 stimulation induced a full agonist signal (pp23 > pp21; Fig. 6A). This pattern was mirrored by reductions in the amount of unphosphorylated TCRζ detected by immunoblotting with mAb 8D3, which does not recognize phospho-ζ (Fig. 6A). As shown in Fig. 6A, chronic exposure of T cells to TNF led to reductions in both pp21 and pp23 levels. Moreover, consistent with the reductions in cell surface CD3ε expression, levels of phosphorylated CD3ε were clearly decreased in TNF-treated T cells following TCR engagement (Fig. 6B). In CD3ε immunoprecipitates, levels of pp21/pp23 were also reduced in TNF-treated T cells.

We then studied TCRζ phosphorylation following activation by peptide-pulsed APCs by examining levels of phospho-ζ in ZAP-70 immunoprecipitates. A similar decrease in phosphorylated ζ was observed in TNF-treated cells following activation with the specific peptide (Fig. 6C). Interestingly, peptide 322–337 induced a partial agonist signal, similar to that observed with anti-CD3ε alone. Nevertheless, the ratio of pp23:pp21 in TNF-treated cells did not differ significantly from that in control cells regardless of the nature of TCR ligation (Fig. 6, A and C). Consistently, a comparable reduction in the level of unphosphorylated ζ was observed in control and TNF-treated cells following T cell activation (Fig. 6A). These results suggest that the phosphorylation process per se may proceed normally in T cells after chronic TNF exposure, and that reduced levels of phospho-ζ after TCR ligation could be explained by the lower levels of TCRζ protein in TNF-treated cells before activation.

Because the ratio of pp23:pp21 appeared to be unaltered in TNF-treated cells, we predicted that Lck kinase activity should not differ markedly between control and TNF-treated cells upon TCR engagement. To test this directly, we compared in vitro kinase activity in Lck immunoprecipitates from control and TNF-treated T cells after activation with anti-CD3 with or without anti-CD4 mAbs. Modest reductions in Lck kinase activity were observed at intermediate time points in TNF-treated cells, suggesting that the amplitude of kinase activity is marginally reduced (Fig. 6D). Although the reason for this small reduction is not understood, we envisage that loss of surface TCR/CD3 expression may lead to a
The downstream consequences of depressed TCRζ ligation (data not shown). In addition, Fig. 6 activation with either anti-CD3 these T cells was not as strong and was less consistent following anti-CD4 mAbs was optimal, because ZAP-70 phosphorylation in cells chronically cultured with hTNF was studied by Western blotting under nonreducing or reducing conditions, respectively. D, Control and hTNF-treated HQC6 cells were stimulated with immobilized anti-CD3ε mAb (0.5 μg/ml) or type II collagen (4 μg/ml), and IL-2 production was determined 24 h later. Representative results from seven separate experiments are shown.

A decrease in TCRζ expression uncouples ZAP-70 and LAT phosphorylation and attenuates Ca2⁺ responses after TCR ligation

The downstream consequences of depressed TCRζ expression were tested further by investigating the phosphorylation of ZAP-70, which is initiated by the recruitment of ZAP-70 to phosphorylated TCRζ-chains (16). As shown in Fig. 7A, chronic treatment of T cells with TNF resulted in a significant down-regulation of ZAP-70 tyrosine phosphorylation after T cell activation by anti-CD3ε plus anti-CD4 mAbs at all time points studied. A combination of anti-CD3ε and anti-CD4 mAbs was optimal, because ZAP-70 phosphorylation in these T cells was not as strong and was less consistent following activation with either anti-CD3ε mAb alone or specific peptide (data not shown). The extent of the decrease in ZAP-70 phosphorylation correlated closely with the duration of TNF exposure (8 vs 14 days), and with TCRζ expression in whole cell lysates before TCR ligation (data not shown). In addition, Fig. 6C shows reduced levels of phospho-ζ in ZAP-70 immunoprecipitates following TCR ligation in TNF-treated cells, indicating that less ZAP-70 is recruited to phosphorylated TCRζ chains. Finally, additional experiments demonstrated that ZAP-70 phosphorylation was depressed only under circumstances where TCRζ was significantly down-regulated (data not shown), further suggesting that decreased TCRζ-chain expression may account for attenuation of ZAP-70 phosphorylation.

LAT is a naturally occurring ZAP-70 substrate and links the phosphorylation of ZAP-70 to the activation of downstream Ras and Ca2⁺ signaling pathways (18, 19). Consistent with the reductions observed in ZAP-70 phosphorylation, tyrosine phosphorylation of p36LAT was depressed in cells chronically treated with TNF after TCR ligation by anti-CD3 mAb alone and in combination with anti-CD4 mAb (Fig. 7B). Furthermore, intracellular Ca²⁺ mobilization following TCR ligation was significantly attenuated in cells after chronic TNF exposure (Fig. 7C), to an extent remarkably similar to that previously reported in TNF-treated TCR transgenic T cells (22) as well as in T cells derived from TCRζ-deficient mice (38). Finally, we studied Ca²⁺ responses in HQC6 cells expressing the chimeric receptor C2/υ/ICζ, because this receptor requires endogenous TCRζ chains to signal (see Fig. 5A). As shown in Fig. 7C, we detected a clear reduction in the Ca²⁺ mobilization after cross-linking of the C2/υ/ICζ receptor in TNF-treated cells (Fig. 7D). Because these chimeric receptor complexes do not appear to associate with other CD3 invariant chains (33), these data suggest a more direct link between loss of TCRζ expression and attenuation of downstream signaling pathways. Nonetheless, the data cannot rule out the possibility that TNF uncouples additional downstream signaling pathways common to TCR and the C2 receptor independently of its effects on TCRζ.

Inhibition of TNF-induced TCRζ down-regulation by NAC is accompanied by partial, but not complete, reversal of T cell hyporesponsiveness

Alterations in intracellular redox balance following depletion of the abundant intracellular anti-oxidant glutathione have been shown to suppress T cell proliferation and TCR signaling pathways (39–41). To explore the possibility that TNF down-regulated
TCRζ expression and IL-2 production by reducing glutathione levels, we evaluated the effects of NAC, a biosynthetic precursor of glutathione that scavenges reactive oxygen species. Control or TNF-treated cells were cultured in the presence or the absence of 1 mM NAC for up to 20 days before stimulation. Under these conditions, NAC completely inhibited the reduction in glutathione levels observed following culture with TNF, as determined by flow cytometry (Fig. 8A). NAC had no consistent effects on TCRζ expression or IL-2 production by untreated T cells (data not shown). In contrast, data from multiple experiments with TNF-treated cells indicated that the effects of NAC on TCRζ expression varied according to the duration of the culture and TNF concentration. Specifically, inhibition of TNF-induced TCRζ down-regulation by NAC was observed after prolonged culture at intermediate and low doses of TNF, whereas the expression of ZAP-70 was not altered by NAC in the same cells (Fig. 8B; 20 days of culture). These results suggested that down-regulation of glutathione levels is not the only mechanism by which TNF influences TCRζ expression. Nevertheless, coculture of TNF-treated cells with NAC allowed us to investigate the relationship between TCRζ expression and T cell IL-2 production. Several important observations were noted from these experiments. Firstly, the effects of NAC on IL-2 production

**FIGURE 6.** The effects of chronic TNF exposure on TCRζ and CD3e phosphorylation and on Lck kinase activity after TCR ligation. Clone 11A2 was chronically cultured in the presence or the absence of 2.5 ng/ml hTNF. The cells were activated by anti-CD3e with or without anti-CD4 mAbs or by APC pulsed with specific peptide for the indicated times at 37°C. TCR ζ-chain was immunoprecipitated using a TCRζ-specific antiserum (A) or a ZAP-70-specific Ab (C), whereas CD3e was immunoprecipitated using a hamster anti-CD3e Ab (B). Tyrosine phosphorylation was detected by Western blotting under reducing conditions. The blots were reprobed with anti-TCRζ mAb 8D3, which recognizes the unphosphorylated form of TCRζ (A), or with rabbit polyclonal anti-CD3e Ab (B). D, Lck kinase activity was determined by in vitro kinase assay on anti-Lck immunoprecipitates using GST-SAM68 C-terminal fragment as substrate. The values indicate the incorporation of radioactivity compared with that in nonstimulated control cells determined by phosphorimaging. Coomassie blue staining demonstrates comparable levels of the substrate and immunoprecipitating anti-Lck mAb heavy chain in each lane. Representative results from six (A) and two (B–D) separate experiments are shown.
by TNF-treated T cells correlated to changes in TCRζ expression, because T cell hyporesponsiveness was reversed only when down-regulation of TCRζ expression was inhibited by NAC (Fig. 8, B and C, and data not shown). Secondly, NAC only partially inhibited TCRζ down-regulation and suppression of IL-2 production at the highest concentration of TNF. Thirdly, ~40% suppression of IL-2 production was observed in T cells cultured in the presence of NAC and intermediate concentrations of TNF (0.6 ng/ml) despite TCRζ levels comparable to those in control cells. Although NAC may have a wide variety of cellular targets besides TCRζ, these data provide further evidence that prolonged exposure to TNF may uncouple additional TCR signaling pathways, perhaps downstream of TCRζ.

FIGURE 7. Down-regulation of ZAP-70 and LAT tyrosine phosphorylation and Ca^{2+} mobilization following TCR or C2 ligation in TNF-treated T cells. Clones 11A2 (A–C) and HCQ6 (D) were chronically cultured in the presence or absence of 2.5 ng/ml hTNF. A and B, T cells were activated by anti-CD3 with or without anti-CD4 mAbs for the indicated times at 37°C. ZAP-70 (A) or LAT (B) was immunoprecipitated using specific Abs, and tyrosine phosphorylation was detected by Western blotting under reducing conditions. The blots were reprobed with anti-ZAP-70 (A) or anti-LAT Abs (B) to verify equal loading in each lane. C and D, Cells loaded with fura-2 AM were stained with anti-CD3ε and anti-CD4 mAbs (C) or anti-C2 Abs (D) on ice. After establishing baseline fluorescence, cross-linking anti-hamster or anti-rabbit Ig was added, and fluorescence intensity was measured with a luminescence spectrometer. Representative results from six (A), two (B and D), and three (C) separate experiments are shown.
Our findings together with previously published data suggested that T cells to TNF down-regulated the expression of the TCR. Moreover, exposed to TNF cells, we set out to identify the most proximal signaling defects in T cells derived from sites of inflammation. More recently, experiments have demonstrated attenuated TCR signaling pathways in T cells derived from the synovial joints of patients with rheumatoid arthritis (RA) (48), from patients with chronic infections such as HIV (49), and from cancer patients (50) and ZAP-70 expression in whole cell lysates was studied by Western blotting under nonreducing or reducing conditions, respectively.

The results of our experiments revealed that chronic exposure of T cells to TNF, cell surface CD3ε expression was normal in TNF-treated cells, whereas the levels of CD3γ and CD3δ were unchanged. These findings together with the fact that TCRζ is synthesized at ~10% the rate of other TCR/CD3 chains in T cell hybridomas (7) are consistent with a model in which the association of TCRζ-ε homodimers with the αβγεδε hexameric complexes is a rate-limiting step in receptor assembly and transport to the cell surface.

The data also raise the critical question of whether loss of TCRζ expression could account for the paradox were understood. However, in these mice TCR/CD3 expression is normal, presumably because other TCRζ domains required for TCR/CD3 assembly and expression are retained, and ITAM-bearing CD3 chains can compensate for the lack of TCRζ signaling function. In contrast, TNF impairs TCR/CD3 assembly and cell surface expression, leading to a decrease in both phopho-TCRζ and phospho-CD3ε following activation. However, it is possible that TNF uncouples additional signaling pathways downstream of proximal tyrosine phosphorylation events, an idea supported by our studies of TNF-treated cells cultured with NAC. Nevertheless, our results are compatible with a model in which TNF targets TCRζ-chain expression, and attenuation of downstream signaling events occurs at least in part as a consequence of this primary defect.

The expression of TCRζ has been reported to be regulated at both transcriptional and post-transcriptional levels (9, 45). Precisely how chronic TNF exposure modifies TCRζ expression is still unclear, because although TCRζ mRNA was reduced following treatment of T cells with high TNF concentrations, we consistently observed loss of TCRζ protein at concentrations of TNF that had minimal effects on TCRζ mRNA levels. Therefore, additional post-translational effects may be involved, an idea further supported by the fact that down-regulation of TCRζ by TNF could be inhibited by NAC. Thus, changes in intracellular redox potential might affect the extent to which sulphydryl groups of proteins are maintained in the reduced state. According to this model, it is possible that in the oxidized state TCRζ is preferentially targeted for degradation in either lysosomal or ER/pre-Golgi compartments. Along similar lines, impaired association of TCRζ-ε homodimers with αβγεδε hexamers could lead to increased TCRζ degradation. Indeed, preliminary experiments indicate that TNF attenuates the expression of a molecular chaperone that may protect TCRζ-chains from degradation (P. Isomäki, B. Schraven, and A. P. Cope, unpublished observations). An alternative pathway of TCRζ degradation might involve caspase activation, because caspase-induced proteolysis of TCRζ has been reported in apoptotic Jurkat T cells (46). However, the kinetics of TNF-induced TCRζ down-regulation do not favor a role for caspases in our model. Finally, we are exploring the possibility that signaling pathways that lead to TCRζ degradation are NF-κB dependent, because NAC is a potent inhibitor of TNF-induced NF-κB activation (47).

TCRζ-chain expression appears to be targeted in several diseases in man. For example, decreased expression of TCRζ has been documented in hyporesponsive T cells derived from the synovial joints of patients with rheumatoid arthritis (RA) (48), from patients with chronic infections such as HIV (49), and from cancer patients (50). In addition, chronic TNF exposure appears to reproduce many of the TCR signaling defects observed to date in synovial joint T cells from RA patients, including attenuation of LAT phosphorylation and intracellular Ca2⁺ mobilization following TCR ligation (51, 52). Furthermore, we have recently observed a
significant reduction in cell surface CD3ε expression on synovial tissue T cells compared with peripheral blood T cells from the same patient (unpublished observations). Elevated levels of TNF have been described in joints of patients with RA (37), a TNF-driven disease in the majority of patients, in sera of HIV patients (53), and in tumors from cancer patients (54). In light of these findings, it is tempting to speculate that chronic TNF exposure in vivo could contribute to T cell hyporesponsiveness and down-regulation of TCRζ expression in these diseases, especially because proliferative responses of peripheral blood T cells from patients with RA were dramatically and rapidly restored after treatment with a neutralizing anti-TNF mAb (21).

Our findings raise questions about the pathophysiological significance of depressed TCRζ expression and T cell hyporesponsiveness. For example, they could reflect an extremely efficient mechanism for suppressing T cell autoreactivity perpetuated through persistent release of self Ags from inflamed tissue. The protective effects of sustained TNF expression in animal models of autoimmune disease such as type I diabetes and lupus would certainly favor this idea (23–25, 27). Indeed, recent studies in TNF-deficient mice indicate that failure to express TNF during the evolution of early T cell responses in vivo leads to progressive T cell autoreactivity and epitope spreading (55). Together, these data provide clear evidence for an immunomodulatory role for TNF during the induction and evolution of autoimmune T cell responses.

The possibility that defective T cell function could contribute to the inflammatory process should also be considered (42), especially in light of the disease-provoking effects of cyclosporin A that have been documented in animal models of autoimmunity (56). For example, attenuation of TCR signaling following ligation by self peptide/MHC complexes in vivo (a process termed homeostatic proliferation) (57) could shorten the half-life of the circulating T cell pool, leading to lymphopenia, a feature common to many chronic inflammatory diseases, but also a characteristic of mice injected with pharmacologic doses of rTNF (24). Through the same mechanism, it is not difficult to see how uncoupling of TCR responses would compromise not only host defense against foreign pathogens but also anti-tumor immunity. Less predictable is the capacity of TNF to influence the inflammatory process by altering the function of immunoregulatory Th2 or T regulatory (Tr1) cells. On the one hand, prolonged TNF exposure did not appear to preferentially suppress Th1 or Th2 cytokine responses of terminally differentiated TCR transgenic T cells derived from B10.D2 (Th1) or BALB/c (Th2) genetic backgrounds (22). In contrast, if the strength of the TCR signal influences the differentiation of Th subsets, as suggested previously (58), TNF might favor maturation of CD4+ T cells along the Th2 pathway. However, more recent data suggest that sustained TCR ligation is required for maturation and differentiation of Th2 cells (59). According to this model, TNF would favor the maturation of Th1 cells. Regardless of the Th subset affected, it is conceivable that chronic TNF exposure would lead to persistence of activated T cells at sites of inflammation, given that the expression and function of proximal signaling molecules such as TCRζ and ZAP-70 are essential for activation-induced cell death (60, 61). Whether hyporesponsive T cells contribute to the inflammatory process or are merely terminally differentiated bystander cells requires further investigation. For example, the findings that TNF increases the expression of CD69 on T cell hybridomas as well as on PBLs (62) are of particular interest given that anti-CD69 Abs can block cell contact-dependent proinflammatory cytokine production by macrophages by >80% (63). These observations suggest that hyporesponsive T cells could function as effector cells during the chronic inflammatory process through Ag-independent, cytokine-dependent mechanisms. We believe that a more comprehensive understanding of the TCR signaling defects in inflammatory diseases and identification of the most efficient ways to prevent or reverse such defects may help to define better the role of chronically activated, yet hyporesponsive, T cells in the pathogenesis of inflammatory diseases such as RA.

In summary, we report that sustained exposure to the proinflammatory cytokine TNF induces reversible and nondeletional hyporesponsiveness in T cells through mechanisms that disrupt membrane-proximal TCR signaling pathways. Although several major intracellular targets of chronic TNF signals may exist, the most proximal of these within the TCR signaling cascade appears to be the TCRζ chain. Because TNF can reproduce many characteristics of defective T cells in patients with inflammatory disease, we propose that chronic TNF exposure in vivo may contribute to the down-regulation of TCRζ expression documented in chronic inflammatory diseases as well as in cancer and chronic infection. This reciprocal effect of the inflammatory process on adaptive immunity may contribute to the immunosuppressive state of such diseases in man.

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