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Reduced T-cell receptor CD3ζ-chain protein and sustained CD3ε expression at the site of mycobacterial infection

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SUMMARY

Control of mycobacterial infection by the cellular immune system relies both on antigen-presenting cells and on T lymphocytes. The quality of an effective cellular immune response is dependent on functional signal transduction residing in the cytoplasmic tails of the T-cell receptor CD3 components. In order to investigate potential effects of mycobacteria on T-cell receptor signalling, we examined the protein expression of T-cell signal transduction molecules (CD3ζ, ZAP-70, $p59^{fyn}$, $p56^{lck}$). In Western blots of peripheral blood mononuclear cells of *Mycobacterium tuberculosis* infected patients, only the CD3ζ-chain showed a marked reduction in protein expression. To investigate the situation *in situ*, immunoenzymatic and immunofluorescence stainings for CD3ε and CD3ζ expression were performed on sections of normal lymphoid tissue, *M. leprae* infected and sarcoid tissue. CD3ε and CD3ζ expression were similar with respect to intensity, localization and the number of cells stained in normal lymphoid tissue and in sarcoid granulomas. In contrast, the granulomas of *M. leprae* infected tissues showed a significantly reduced expression of CD3ζ compared to CD3ε. Using double immunofluorescence analysis, virtually no CD3ζ expression could be detected in comparison to the CD3ε expression in the lesions. Apparently, mycobacteria are capable of significantly reducing CD3ζ-chain expression, which may be restored by cytokines. IL-2-enhanced ζ-chain expression and T-cell effector functions, defined by interferon-γ release, in *M. tuberculosis*-specific and human leucocyte antigen-DR restricted $CD4^+$ T cells isolated from granuloma lesions from patients with pulmonary tuberculosis. Because CD3ζ is essential for CD3 signalling and for eliciting T-cell effector functions, reduced CD3ζ protein expression could result in altered signal transduction and inefficient T-cell effector functions. Alternatively, reduced CD3ζ-chain expression may protect T cells from repetitive TCR stimulation associated with anergy or apoptosis.

INTRODUCTION

Protective immunity to mycobacterial infection is considered to be a cell-mediated process, in which macrophages and $CD8^+$ or $CD4^+$ αβ T cells, $CD4^-$ $CD8^-$ αβ T cells, γδ T cells and, presumably, natural killer cells mediate either eradication or containment of viable bacilli.[1–9] Clinical manifestation of infection with mycobacteria is thought to result either from immune evasion of viable bacilli, from non-sufficient immune surveillance, or from immune effector mechanisms which are themselves contributing to tissue damage.[10] The process of phagocytosis, antigen degradation, presentation, and T-cell stimulation could be blocked at any point along this pathway, leading to ineffective activation of specific T cells and thus impairing the ability of the immune system to resolve infection. For instance, mycobacteria avoid triggering oxidative burst by entry into phagocytes via integrin family proteins,[11] phagosome lysosome fusion may be inhibited,[12] and the abundantly produced lipoarabinomannan has a number of immunosuppressive effects, such as scavenging of reactive

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Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase; BSA, bovine serum albumin; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; TBS, Tris-buffered saline; TCR, T-cell receptor.

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oxygen intermediates and inhibition of transcriptional activation of interferon-γ (IFN-γ) inducible genes.[13] Suppression on the level of the T-cell response in mycobacterial infections has been suggested to be mediated by increased production of soluble immunosuppressive factors, including transforming growth factor-β (TGF-β) or interleukin-10 (IL-10).[14–16] A key event in the induction of a T-cell response is the repetitive stimulation of the T-cell receptor (TCR)[17,18] by classical major histocompatibility complex (MHC) class I, MHC class II or non-polymorphic MHC antigen-presenting molecules (e.g. CD1b, CD1c), leading to the activation of several tyrosine kinases which are responsible for eliciting T-cell responses, e.g. proliferation and cytokine secretion.[19–22] Thus, variations in the expression of T-cell signal transduction molecules may be responsible for impaired immune function of T cells. In fact, alterations in expression of T-cell signal transducing molecules in correlation to immune dysfunction have been described *in situ* as well as in the systemic circulation in tumour-bearing patients,[23–25] in patients with systemic lupus erythematosus,[26] with rheumatoid arthritis,[27] and in human immunodeficiency virus (HIV)-infected individuals.[28] To elucidate whether mycobacterial infections may have an impact on T-cell signalling, we investigated protein expression of several key molecules of T-cell signal transduction, ZAP-70, the CD3ζ-chain, $p56^{lck}$ and $p59^{fyn}$ in healthy controls and in patients with tuberculosis, leprosy, or in patients with sarcoidosis, a granulomatous disease of unknown aetiology in which mycobacteria are thought not to be the causative agent.[29] Most studies examined alterations in T-cell signal transduction using Western blot analysis;[14,30] here, we examined the anatomy of CD3ζ-chain reduction *in situ*.

MATERIALS AND METHODS

Patients

Biopsies of patients suffering from sarcoidosis or tuberculosis, obtained for diagnostic purposes and no longer needed, were used in this study. Sarcoidosis was diagnosed and assessed by chest radiography, bronchoalveolar lavage, and transbronchial biopsy. Pulmonary tuberculosis was diagnosed by the clinical presentation of the patients and was proven by positive bacterial cultures. Blood samples from tuberculosis patients prior to any drug treatment and from healthy blood donors were obtained by venepuncture after informed consent and was approved by the local ethics committee. Skin biopsies of patients suffering from leprosy were obtained from patients of the leprosy eradication programme performed by the Department of Leprosy of the Ministry of Health and Welfare, Paraguay. Patients were classified according to the clinicopathological criteria of Ridley and Jopling, 1966.[31] All leprosy biopsies were taken from untreated patients, snap-frozen in liquid nitrogen and stored until use at -80°.

Antibodies

Antibodies used in this study were rabbit anti-CD3ε antiserum and APAAP-complex from DAKO (Glostrup, Denmark), anti-CD3ζ monoclonal antibody (mAb; clone 8D3, Pharmingen, Hamburg, Germany and clone TIA-2 (now named 2H2D9), Immunotech/Coulter, Krefeld, Germany), anti-ZAP kinase mAb, anti-$p59^{fyn}$ mAb and anti-$p56^{lck}$ mAb (Transduction Laboratories, Affiniti Research Products Ltd, Exeter, UK), Cy-3 conjugated goat anti-mouse immunoglobulin G (IgG) and fluoroscein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG from Dianova (Hamburg, Germany). Anti-CD3-phycoerythrin (PE) (clone UCHT1), anti-CD16-FITC (clone 3G8), anti-CD56-PE (clone B159), as well as isotype matched controls were obtained from Beckman/Coulter, Krefeld, Germany.

Immunoblotting

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) gradients followed by two washing steps. Frequency of CD3 positive cells, and CD16 CD56 double-positive staining cells was determined using an aliquot of this preparation by flow cytometry. Approximately 60–70% of the cells stained positive for CD3. Samples corresponding to 2×10^6 CD3 positive cells were analysed in Western blots. In order to reduce artefacts with respect to T-cell receptor regulation induced by anti-CD3 antibodies freshly isolated cells were implemented in *in vitro* assays and Western blot analysis without manipulation of positive or negative selection. Individual samples were adjusted to the same volume and were lysed for 20 min at 4° in 50 mM HEPES (pH 7·2), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM orthovanadate, 2·5% Triton-X-100, 200 μg/ml chymostatin (Boehringer Mannheim, Mannheim, Germany), 200 μg/ml trypsin–chymotrypsin inhibitor and 2 mM phenylmethylsulphonyl fluoride (PMSF) (both from Sigma, Deisenhofen, Germany). The lysates were subjected to 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and blotted onto polyvinylidendifluoride membranes (Immobilon-P, Millipore, Eschborn, Germany). Residual protein binding sites were blocked with 3% skim milk powder/1% bovine serum albumin (BSA) for 1 hr, incubated with primary antibody for 1 hr, washed in TBS/0·05% Tween-20, incubated with the peroxidase-conjugated secondary antibody and developed.

Immunoenzymatic staining

Cryostat frozen sections were fixed in acetone for 30 min, followed by fixation in chloroform for 30 min. After fixation the leprosy sections were preincubated with rabbit normal serum for 30 min to block non-specific binding. Paraffin sections were dewaxed (10 min xylene, 10 min acetone, 10 min 1 : 1 acetone and Tris buffered saline (50 mM Tris, 150 mM NaCl, pH 7·5)) and were cooked in a pressure cooker for 1 min for antigen retrieval before application of rabbit normal serum. Incubation with the primary antibody was performed for 30 min, and immunostaining was undertaken according to the APAAP (alkaline phosphatase anti-alkaline phosphatase) method with New Fuchsin development.[32] Finally, slides were counterstained with haematoxylin and mounted. Immunostainings were controlled by implementing the secondary reagents alone in order to confirm specificity or enzyme development alone to rule out endogenous enzyme activities.

Double immunofluorescence

Double immunofluorescence staining was performed after a blocking step with 10% TBS–BSA with the primary antibodies

for 30 min. After a washing step, the secondary antibodies were added and incubated for 30 min. Specimens were mounted in DABCO anti-fading solution (2·5% DABCO (1,4-diazabicyclo [2,2,2] octane) in 90% glycerol, pH 8·6) and viewed under a fluorescence microscope. Stainings were controlled by using only the secondary antibodies to detect unspecific and background fluorescence. Double exposures on film were performed using stained tonsillar tissue as a measure for the exposure time.

Functional assays

Granuloma associated lymphocytes (GAL) were obtained from pulmonary granuloma lesions by dissection of tissue samples in small (2 µm) pieces which were placed into 48-well plates and cultured with Dulbecco's modified Eagles minimal essential medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine and penicillin (all reagents from GIBCO-BRL, Heidelberg, Germany) supplemented with 50 ng human recombinant IL-7. T-cells which could be rapidly expanded after a 2-day culture period were analyzed for CD4, CD8 and TCR ζ-chain expression by flow cytometry. $CD4^+$ T cells were obtained by negative sorting, removing $CD8^+$ T cells using immunomagnetic beads (Miltenyi, Bergisch Gladbach, Germany). Pulmonary granuloma tissue was obtained by explorative thoracotomy from patients who underwent surgery to establish a definite diagnosis for pulmonary lesions, representing either a malignancy or tuberculosis. Samples used in this study were from patients with pulmonary tuberculosis (MHC typing: patient #1, human leucocyte antigen (HLA)-A3, 11, B32, 53, Cw4, 12, DRB1*01, 11, DQB1*03, 05; patient #2, HLA-A26, 29, B45, 52, Cw6, 12, DRB1*12, 15, DQB1*03, 06). HLA-DR-matched macrophages were implemented as antigen-presenting cells in a 24-hr IFN-γ-release assay. Briefly, macrophages were obtained from PBMC by adherence to plastic for 2 hr followed by three consecutive washing steps to assure minimal contamination with lymphocytes. Macrophages were infected with mycobacteria (virulent *M. tuberculosis* Erdman strain) 24 hr prior to the assay and infection evaluated by Ziehl–Neelsen staining. Cells were fixed with 1% formaldehyde, washed twice and incubated with $CD4^+$ GAL for 24 hr. The effector : target ratio was 10 : 1; T cells were adjusted to 5×10^5 cells/well in duplicates. Blocking antibodies (10 µg/well) included the anti-HLA-DR mAb L243 and (as a negative control) the anti-MHC class I mAb w6/32. The anti-CD3 directed mAb OKT3 (2 µg/ml, 50 µl/well) attached to plastic served as the positive control for IFN-γ secretion determined by enzyme-linked immunosorbent assay (ELISA; R & D Systems, Wiesbaden, Germany).

Flow cytometry

All antibodies were obtained from Beckman/Coulter, Krefeld, Germany, except for the anti-TCR ζ-chain mAb (clone 6B10.2) coupled to FITC, which was purchased from Santa Cruz Biotechnology, Heidelberg, Germany. T cells were stained with anti-CD4 (clone SFCI12T4D11) coupled to energy-coupled dye (ECD) and anti-CD8 mAb (clone B9.11) coupled to R-PE-cyanin 5 (PC5). TCR ζ-chain detection was carried out using the IntraPrep® permeabilization reagent (Beckman/Coulter) and the anti-TCR ζ-chain mAb 2H2D9 coupled to PE, or the clone 6B10.2 (see above). Appropriate murine isotype matched non-specific mAbs served as controls. Staining was visualized using a Coulter® Epics® XL flow cytometer with the XL software version 2.1.

RESULTS

Expression of ZAP-70, $p56^{lck}$, $p59^{fyn}$ and CD3ζ in PBMC from healthy donors and tuberculosis patients

Peripheral blood mononuclear cells of five age-matched healthy controls and five patients suffering from tuberculosis were analysed in Western blots with respect to the expression of ZAP-70, CD3ζ, $p56^{lck}$ and $p59^{fyn}$. As controlled by flow-cytometric analysis, CD3ε positive cells varied slightly between 60 and 70% of total cells. Samples corresponding to 2×10^6 CD3ε positive cells were applied per lane, to guarantee a comparable signal ratio for the investigated proteins. As shown in Fig. 1, protein expression levels of ZAP-70, $p56^{lck}$ and $p59^{fyn}$ appear to be similar in the patient and the control group whereas CD3ζ protein expression is significantly reduced in the tuberculosis patients compared to the controls.

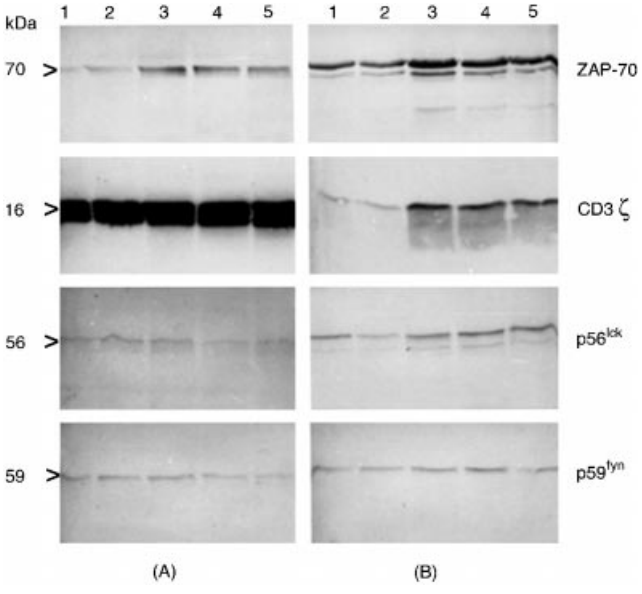

Figure 1. Reduced CD3ζ-chain expression in peripheral PBMCs from patients with tuberculosis. Freshly isolated PBMCs of five healthy donors and five patients with tuberculosis were adjusted to 2×10^6 CD3ε positive staining cells as determined by flow cytometry before Western blot analysis. Detection of individual T-cell signalling molecules was performed with specific mAbs as described in the material and methods section. (a) Results obtained from PBMC of five different age-matched healthy donors (lanes 1–5); (b) results of five different tuberculosis patients (lanes 1–5). The expression level of ZAP-70, $p56^{lck}$ and $p59^{fyn}$ appeared to be similar in both groups. In contrast, CD3ζ protein expression was significantly reduced in patients with tuberculosis. The additional band in $p56^{lck}$ or $p59^{fyn}$ was observed in some samples obtained from patients with tuberculosis. The nature of this double band has not yet been defined, it may be associated with the activation status of peripheral T cells from patients with tuberculosis: Upon activation, lck or fyn are enriched in membrane lipid rafts, phosphorylate TCR invariant chains and associate with ZAP-70. These different configurations of lck or fyn (e.g. non-associated or associated with ZAP-70) may provide a reason for a different behaviour in SDS–PAGE analysis and also in Western blot.

Expression of CD3ε and CD3ζ in normal lymphoid tissue

Expression of CD3ε and the CD3ζ-chain was investigated in normal lymphoid tissue ($n = 10$) by immunoenzymatic staining of serial sections and by double immunofluorescence analyis of hyperplastic tonsillar tissue specimens. As shown in Fig. 2(a,b), for example, CD3ε and CD3ζ expression appeared to be equally intense, expressed on an equal number of cells and also localized to the same area in this tissue. This observation was substantiated by double immunofluorescence analyses (Fig. 3a–c). All CD3ζ expressing cells in tonsil tissue (red fluorescence, Fig. 3b) coexpress CD3ε (green fluorescence, Fig. 3a) as demonstrated by the double exposure of both fluorochromes (yellow fluorescence, Fig. 3c). Depending on the localization in the tissue, this congruent expression was up to 100% in the T-cell rich mantel zone of germinal centres whereas small numbers of CD3ε positive and CD3ζ negative cells could be discerned adjacent to the T-cell zone or in close

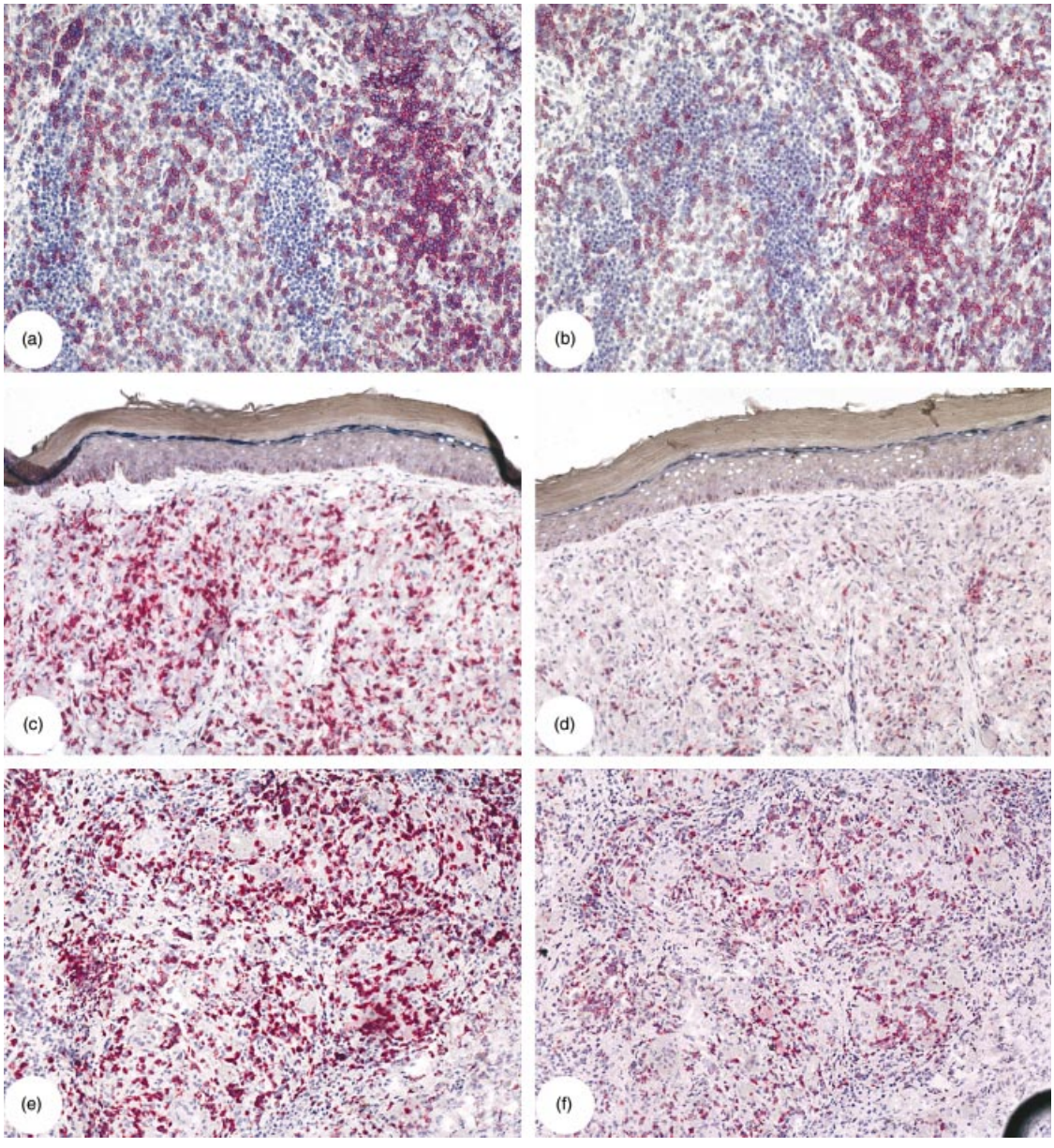

Figure 2. Reduced *in situ* CD3ζ expression in tissues with mycobacterial infection. Serial sections of a hyperplastic tonsil (a, b), a skin biopsy of a lepromatous leprosy patient (c, d) and a lymph node biopsy of a sarcoidosis patient (e, f) were stained for the presence of CD3ε (a, c, e) and CD3ζ (b, d, f) with the APAAP technique. In hyperplastic tonsil, the expression of CD3ε (a) and CD3ζ (b) was localized to the same region, expressed by an equal number of cells, and was of similar intensity. In the representative example of *M. leprae*-infected tissue, CD3ε (c) expression was distributed uniformly throughout the granuloma, whereas CD3ζ (d) expression was greatly reduced and limited to a few isolated cells throughout the granuloma. In granulomatous tissue of a sarcoid lymph node, CD3ε expression (e) was detected as intense staining of cells localized around epithelioid cells in the granuloma. CD3ζ staining (f) was not as intense as CD3ε staining and appeared not to stain as many cells but was localized to the same areas in the tissue.

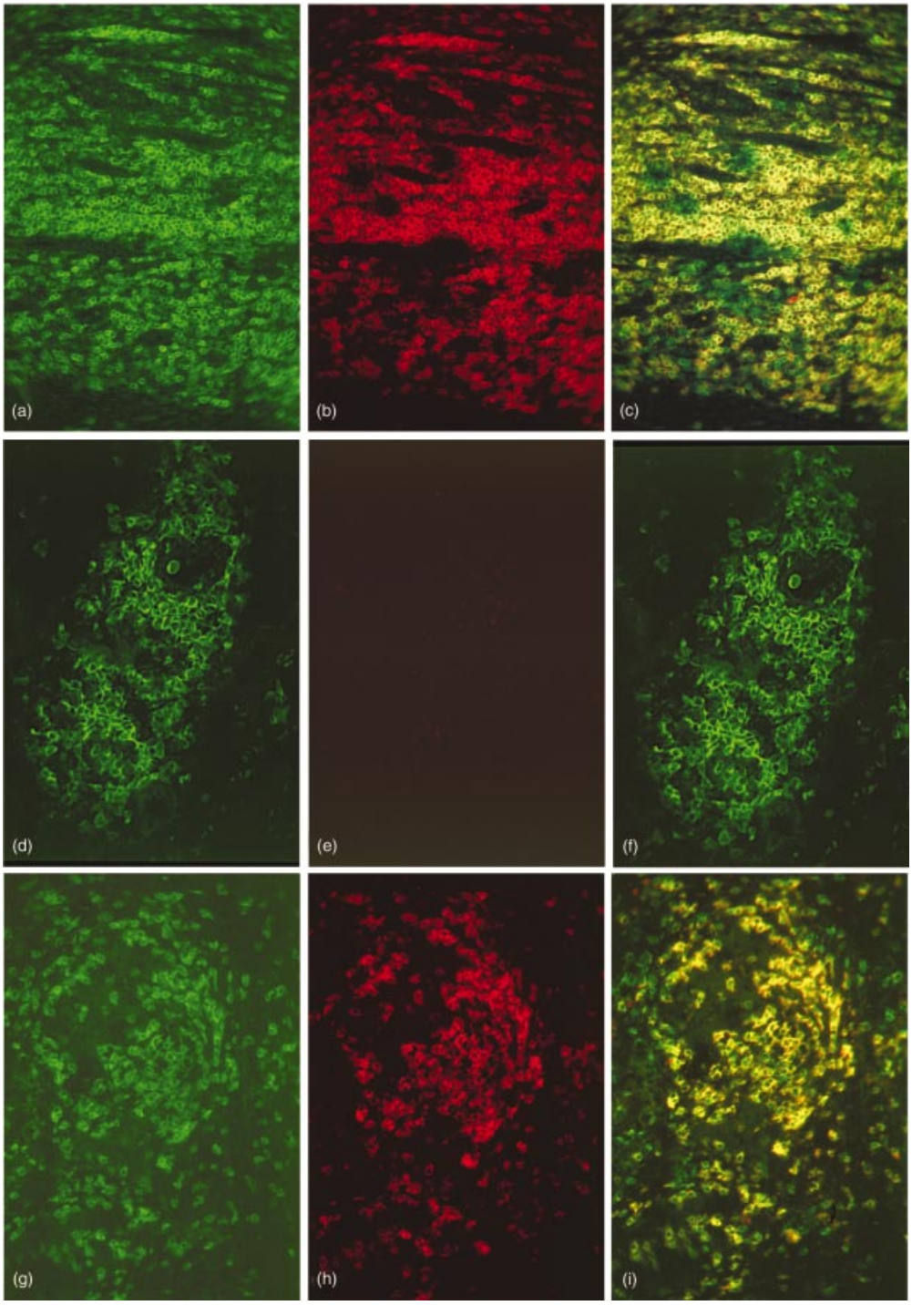

Figure 3. Double immunofluorescence analysis of CD3ε and CD3ζ expression *in situ.* Hyperplastic tonsil (a, b, c), a tuberculoid leprosy skin biopsy (d, e, f) and a sarcoid lymphnode (g, h, i) were stained for CD3ε (green fluorescence, a, d, g), CD3ζ (red fluorescence, b, e, h) and double exposed for colocalization of both CD3ε and CD3ζ (yellow fluorescence, c, f, i). In hyperplastic tonsil, CD3ζ expression colocalized 100% with CD3ε expression. In the tissue area chosen close to endothelium in this example, several cells exhibited CD3ε positivity and CD3ζ negativity. In an example of tuberculoid leprosy, CD3ε positive cells were detected throughout the granuloma (d) whereas CD3ζ expression was barely visible (e), resulting in no detection of CD3ε CD3ζ double positive cells (no yellow fluorescence (f). The sarcoid lymph node lesion exhibited CD3ε (g) and CD3ζ (h) expression to approximately the same extent. All CD3ζ cells were CD3ε positive (yellow fluorescence) and only a minor number of cells were CD3ε positive CD3ζ negative (i, green fluorescence).

proximity to the endothelium. CD3ε negative and CD3ζ positive cells were rarely observed (one in Fig. 3c).

CD3ε and CD3ζ expression in mycobacterially infected tissue

Serial sections of different tissue specimens infected with *M. leprae* or *M. tuberculosis* were stained immunoenzymatically for CD3ε and CD3ζ expression. In 10 different cases of tuberculoid and 11 different cases of lepromatous leprosy, a striking reduction of CD3ζ positive cells was found as compared to CD3 positive cells (Fig. 2c,d). In some cases, no CD3ζ positive cells could be detected in granulomatous lesions. Reduced CD3ζ expression in comparison to CD3ε appeared to be even more evident in double immunofluorescence studies using the same conditions as for the tonsillar tissue (Fig. 3d–f). Substantial numbers of CD3ε positive cells were observed within the lesions (green fluorescence, Fig. 3d), whereas CD3ζ positive cells were scarcely discernible because of reduced intensity in staining and reduced numbers of positive cells (red fluorescence, Fig. 3e). Thus, no double positive cells (absent yellow fluorescence, only green fluorescence, Fig. 3f) in the double exposures could be detected. With respect to the different clinical forms of leprosy investigated, no evident differences could be observed between the tuberculoid and lepromatous forms of leprosy concerning reduced CD3ζ expression.

The analysis of *M. tuberculosis* infected tissues was performed on eight different lung tissues. Since these specimens were not enriched with cellular constituents, the difference between CD3ε and CD3ζ positive cells was not as evident as in specimens obtained from patients with leprosy. However, there was a clear trend to decreased expression of CD3ζ compared to CD3ε (data not shown).

CD3ε and CD3ζ expression in sarcoid granulomas

As a comparison to mycobacterially infected tissue, sarcoid granulomatous lesions, a disease in which mycobacteria are presumably not involved, were stained for CD3ε and CD3ζ expression. Immunoenzymatic staining of serial sections of five sarcoid lymphnodes showed that CD3ζ expression was reduced as compared to CD3ε expression (Figs 2e,f) with respect to the intensity of the staining, however, the localization of positive signals and the number of cells stained for either CD3ε or CD3ζ appeared to be approximately the same. Double immunofluorescence analysis indeed resulted in a similar image with respect to the presence of double positive cells, comparable to normal hyperplastic tonsil tissue. However, some CD3ε positive CD3ζ negative cells were present, but the majority of CD3ε positive cells stained also positive for CD3ζ (Fig. 3g–i). This observation appears to be associated with the localization of T cells within the granuloma. T-cell rich zones encircling an epithelioid cell granuloma contained predominantly CD3ε positive CD3ζ positive cells. In contrast, some CD3ε positive CD3ζ negative cells could be detected in the center of the epithelioid granuloma and on the edge of the T-cell zone. Additional analysis of three sarcoid granulomatous lesions in skin biopsies showed no reduction of CD3ζ expression as compared to CD3ε expression.

To summarize, there was a significant difference in the level of CD3ζ-chain expression between healthy PBMC, non-infected tissues or sarcoid tissue and mycobacterially infected tissues. No significant difference in CD3ζ-chain expression relative to CD3ε expression could be observed in healthy PBMC, tonsils and in sarcoid granulomas.

Enhanced immune effector functions in the presence of IL-2

Because no viable tissue was available from patients suffering from leprosy, we took advantage of surgically resected pulmonary granuloma lesions obtained from two patients with tuberculosis. Freshly isolated $CD4^+$ GAL were either cultured with or without IL-2 (3 days) followed by intracellular TCR ζ detection (Fig. 4). $CD4^+$ GAL cultured in the presence of IL-2 exhibited a stronger ζ-chain expression as determined by mean fluorescence intensity. These T-cell populations were also evaluated for differences in immune effector functions as determined by IFN-γ secretion upon antigen exposure. Both $CD4^+$ T-cell lines have been identified to recognize *M. tuberculosis*-associated antigens in the context of HLA-DR (manuscript in preparation). $CD4^+$ GAL cultured in the presence of IL-2 showed considerably higher IFN-γ secretion as compared to freshly isolated $CD4^+$ GAL without IL-2 (Table 1).

DISCUSSION

The salient finding of this report is the *ex vivo* analysis of reduced CD3ζ TCR protein expression in a comprehensive panel of human tissues infected with mycobacteria. In contrast, reduced CD3ζ TCR protein expression appears not to be present in hyperplastic tonsillar tissue containing T cells that have encountered antigenic stimulation, or in sarcoid lymph nodes, representing a granulomatous disease of unknown aetiology. Reduced TCR ζ-chain expression was observed both systemically in the peripheral blood of tuberculosis patients, as well as locally in the granulomatous lesions of tissue infected with *M. leprae*, but not in the hyperplastic tonsils nor in the sarcoid lymphnodes.

Reduced CD3ζ-chain expression has been described *in situ* as well as in the systemic circulation in tumour-bearing patients,[23–25] in patients with systemic lupus erythematosus,[26] patients suffering from rheumatoid arthritis,[27] in HIV-infected individuals,[28] and has recently been shown in the periphery of lepromatous leprosy patients by Western blot analysis.[30] However, up to now, the *in situ* situation concerning TCRζ-chain expression has not been characterized in inflammatory diseases. The reduced protein expression of this crucial TCR signalling molecule appears to correlate with immune dysfunction. In the case of T lymphocytes recovered from human tumours or from the peripheral circulation of patients with advanced cancer, reduced CD3ζ expression was correlated with a decreased Ca^{2+} flux as well as impaired kinase activity.[33] In the context of mycobacterial infection, the reduced expression of the CD3ζ-chain is also likely to play an important role in influencing T-cell effector functions. Because CD3ζ expression is essential for TCR signalling and eliciting T-cell effector functions by linking the TCR complex phosphorylation to cytosolic events through association with and activation of ZAP-70,[34] this reduction could result in altered signal transduction and reduced T-cell effector functions, possibly culminating in the T-cell anergy often associated with mycobacterial infections. Indeed, freshly isolated

M. tuberculosis-specific and HLA-DR-restricted $CD4^+$ T cells from pulmonary granuloma lesions obtained from patients with tuberculosis showed enhanced IFN-γ secretion (Table 1) and a stronger TCR ζ-chain signal (Fig. 4) in the presence of IL-2. This notion has been confirmed in a recent study: peripheral T cells from patients with a *M. tuberculosis* infection which exhibited a negative delayed-type hypersensitivity (DTH)-response ('anergy') produced IL-10, no IFN-γ and showed a defective phosphorylation pattern of the TCR ζ-chain.[14]

The mechanism for reduced CD3ζ-chain expression following T-cell triggering is poorly understood. Previous reports have demonstrated that insufficient TCR triggering upon recognition of antagonists or partial agonist peptides may lead to a unique pattern of CD3ζ-chain phosphorylation and to insufficient activation of the ZAP-70 kinase.[35] Additionally, engagement of the TCR with its ligands leads to reduced CD3 expression, presumably resulting from decreased CD3ζ-chain expression.[36] However, these models are based on the fact that specific TCR–MHC/peptide engagement is required to ensure CD3ζ downregulation. Because the number of

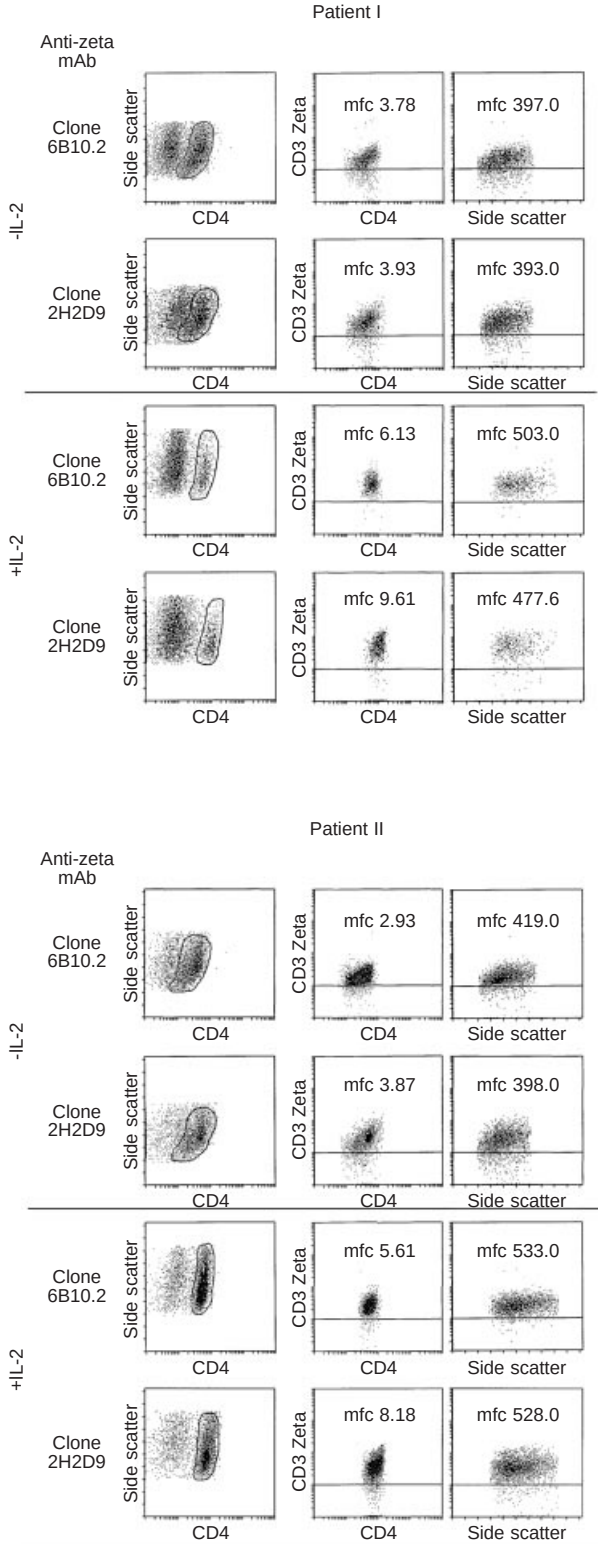

Figure 4. Enhanced TCR ζ−chain expression in *M. tuberculosis* granuloma-associated $CD4^+$ T lymphocytes induced by IL-2. GAL were freshly isolated from pulmonary lesions, as described in detail in the Materials and Methods section, cultured for 3 days in the presence or absence of IL-2 (1000 IU/ml), gated on $CD4^+$ T cells and stained for TCR ζ chain expression by flow cytometry using two different mAb as indicated. Differences in mean fluorescence channel (m.f.c.) intensity. CD4+T-cells correspond to effector cells described in Table 1. ss = side scatter.

Table 1. Enhanced immune effector functions in $CD4^+$ T cells from pulmonary tuberculosis granuloma lesions in the presence of IL-2

	Patient #1		Patient #2	
Culture in the presence of IL-2	−	+	−	+
APC alone IFN-γ (pg/ml/24 hr)	0	0	0	0
APC + GAL IFN-γ (pg/ml/24 hr)	0	0	192	326
APC + *M. tub.* IFN-γ (pg/ml/24 hr)	0	0	0	0
APC + *M. tub.* + GAL IFN-γ (pg/ml/24 hr)	769	2389	1032	3467
APC + *M. tub.* + GAL + anti-HLA-DR IFN-γ (pg/ml/24 hr)	0	36	276	233
APC + *M. tub.* + GAL + anti-MHC class I IFN-γ (pg/ml/24 hr)	548	1906	n.d.	n.d.
GAL + anti-CD3 IFN-γ (pg/ml/24 hr)	3695	2091	> 20 000	> 20 000

Freshly isolated (2 days) granuloma associated $CD4^+$ lymphocytes (GAL) were exposed for 24 hr to HLA-DR matched macrophages (antigen-presenting cells, APC) which had been infected with *M. tuberculosis* (*M. tub.*). The antigen-specific and HLA-DR-restricted $CD4^+$ T-cell response was confirmed by blocking with an anti-MHC class II (DR)-specific mAb. Blocking was not observed with the anti-MHC class I-directed mAb. Crosslinking the TCR with anti-CD3 served as the positive control. $CD4^+$ GAL correspond to the T-cell population shown in Fig. 4, which were either short-term (2 days) expanded in medium supplemented only with IL-7, or alternatively in the presence of high dose (1000 IU/ml) IL-2.

n.d. = not determined.

antigen-specific T cells in patients with tuberculosis may be low, it is unlikely that a general decreased CD3ζ-chain expression in the systemic circulation in patients with mycobacterial infection or even in patients with cancer,[23–25] stems from T-cell populations which have encountered their specific ligands. Thus, impaired T-cell function due to impaired CD3ζ-chain expression may be induced by (a) systemic factor(s) affecting the majority of circulating T cells. The search for these compounds has not been very successful up to now. We have tested human or viral IL-10 as well as TFG-β for the capacity to affect CD3ζ-chain expression in peripheral blood cells either from healthy blood donors or from patients with tuberculosis. Neither cytokine resulted in reduced TCR ζ-chain expression. In addition, 'chronic inflammation' which may also be present in lesions from patients with sarcoidosis did not lead to ablated CD3ζ-chain expression. The reduction of CD3ζ-chain expression has also been correlated with hydrogen peroxide production of stimulated macrophages.[37] In the experiments described, lipopolysaccharide activation of macrophages resulted in reduction of CD3ζ protein expression, which could be inhibited by the addition of catalase. However, in a different experimental setup with live *M. tuberculosis* we could not inhibit the mycobacterially induced effect on CD3ζ expression by the addition of catalase, arguing for the influence of other factors (data not shown).

An alternative model for TCR dynamics suggests that the TCR ζ-chain may physically dissociate from the rest of the TCR–CD3 complex, including CD3ε upon TCR triggering.[38] Although the ζ-chain represents a prerequisite to maintain stable CD3 cell surface expression, the TCR may be freely exchanged with newly synthesized CD3ζ molecules.[39] CD3ζ-chains are ubiquinated in a tyrosine-kinase dependent manner after TCR-ligation and subjected to proteolysis. Alternatively, different rates of TCR ζ-chain kinetics as compared with other TCR components also exist, presumably because of retention of intracellular TCR components rather than TCR internalization.[38] These data are particularly interesting in the context of the immunohistology data in this report: The TCR–CD3ε component was still detectable in the absence (or reduced level) of ζ-chain expression. Of note, ζ-chain downmodulation may not only indicate 'anergy', but also protection from overstimulation, a form of 'reversible tolerance' if a vast amount of antigen would potentially be deleterious to T-cell survival.[38–40]

The loss of signalling proteins is reversible but requires appropriate stimulation, e.g. IL-2[41–42] or CD3 and CD28 cross-linking.[43] In these cases, however, restoration of CD3ζ expression alone was not sufficient to restore specific cytolytic T-cell activity against the nominal target cells. It remains to be investigated whether restoration of CD3ζ expression in T cells affects bacterial survival in cells infected with mycobacteria. Interestingly however, clinical studies have shown that adjunctive IL-2 therapy in multidrug resistant tuberculosis may enhance the antimicrobial response.[44,45]

In conclusion, we have been able to show that mycobacterial infection significantly reduces the protein expression of the CD3ζ-chain. Because of the essential role of this signalling molecule in eliciting T-cell effector functions, this reduction of CD3ζ protein expression may represent a novel mechanism by which mycobacteria evade cellular immune surveillance.

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