

Activated Macrophages Induce Structural Abnormalities of the T Cell Receptor-CD3 Complex

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Summary

The mechanism of the structural alterations of the T cell receptor (TCR)-CD3 complex, which appear to be greatly responsible for immunosuppression in the tumor-bearing status, was investigated in tumor-bearing mice. Splenic T cells from tumor-bearing hosts lost the expression of the CD3 ζ chain without being replaced by FcR γ , despite the normal expression of other components of the TCR complex. Tumor growth induced the accumulation of non-T, non-B cells in the spleen in correlation with the loss of ζ . Those cells were found to be macrophages that were able to induce the loss of ζ , as well as structural changes of CD3 $\gamma\delta\epsilon$, even in freshly isolated normal T cells by cell contact-dependent interaction. More importantly, macrophages activated with zymosan A + LPS but not residential macrophages were able to induce the similar abnormality of the TCR complex. These results indicate that macrophages in certain activation stages play a crucial role in causing an abnormal TCR complex in tumor-bearing conditions, as well as in regulating the structure of the TCR complex in immune responses.

Progression of tumor growth has been demonstrated to result in immunosuppression of T cell responses. The suppression has been attributed to the function of suppressor T cells, secretion of suppressive cytokines from tumor cells, or the accumulation of suppressive macrophages (1-3). Recently, structural abnormalities of the TCR-CD3 complex have been reported in splenic T cells from tumor-bearing mice (TBM) (4). These T cells lost the expression of the ζ chain and reduced the CD3 γ chain as well as TCR-associated tyrosine kinases, p56^{lck} and P59^{lyn}. Although similar abnormalities of the TCR complex have been observed in tumor-infiltrating and peripheral blood T cells from human cancer patients (5-7), the mechanisms of such alterations were unknown.

The TCR-CD3 complex is responsible for specific antigen recognition, which activates T cells to exhibit effector functions including tumor rejection. The antigen recognition signals by clonotypic TCR $\alpha\beta$ heterodimer are transduced into the cytoplasm through the CD3 complex, which is composed of six polypeptides: $\gamma\epsilon$, $\delta\epsilon$, and ζ dimers (8). The cytoplasmic domain of the ζ chain has been demonstrated to be capable of transducing signals independently of other TCR-CD3 components, and it has been thought to play a central role for signal transduction in T cell activation (9). ζ is also crucial for the cell surface expression of the TCR-CD3 complex (10). In ζ -deficient mice, the expression of the surface TCR complex is greatly reduced, and proliferative responses of thymocytes and peripheral T cells are impaired (11). Therefore, suppression of immune responses in tumor-

bearing hosts may be mostly attributable to the impairment of the TCR complex and its associated kinases.

We have investigated the mechanism of the structural alterations of the TCR complex in a tumor-bearing mouse model. Splenic T cells from TBM lost the CD3 ζ chain in spite of normal expression of other components of the TCR complex. We demonstrated that macrophages accumulated in the TBM spleen, as well as activated macrophages in certain stages, induced the loss of ζ even in freshly isolated normal T cells. Our results provide molecular approaches for understanding the regulation of the expression of the TCR-CD3 complex and immunosuppression in tumor-bearing conditions, as well as in normal immune responses.

Materials and Methods

Animals. To prepare TBM, 2×10^6 syngeneic colon carcinoma cells (colon 26) were implanted subcutaneously into 8-12-wk-old BALB/c mice (Japan SLC, Hamamatsu, Japan).

Cell Preparation. Lymphocytes from mesenteric lymph nodes and spleen, after depletion of erythrocytes, were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 μ M 2-ME, 10 mM HEPES, and 100 μ g/ml kanamycin (complete medium). Splenic T cells were enriched through nylon-wool columns. Flow cytometric analysis with anti-CD3 ϵ mAb showed that ~85% of the cells were T cells from normal mice and 30-40% from 6-9-wk TBM. The number of T cells from TBM was adjusted to that of normal T cells according to flow cytometric analysis. Intestinal intraepithelial lymphocytes (i-IEL) were isolated as previously described (12). Non-T, non-B cells were prepared from

spleen cells by cytotoxic depletion of T cells with anti-Thy1 mAb (J1j.10) and elimination of B cells with anti-Ig-coated magnetic beads. Peritoneal macrophages were obtained from peritoneal lavage followed by elimination of B cells with anti-Ig-coated magnetic beads. For activated macrophages, mice were inoculated intraperitoneally with 1 mg of zymosan A (Sigma Immunochemicals, St. Louis, MO), followed with 20 μ g of LPS (0127:B8, Sigma) 1 wk later, and the peritoneal macrophages were isolated 2 d later.

Flow Cytometric Analysis. Cells from spleen, lymph nodes and i-IEL were stained with mAbs of appropriate specificities and analyzed on a FACScan[®] flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using Lysis II software. The following mAbs were used: FITC-labeled anti-CD3 ϵ (145-2C11), PE-conjugated anti-CD45R/B220 (RA3-6B2), anti-HSA (M1/69), anti-MHC classII (anti-mouse I-A^{q,b,d}, I-E^{k,d}, 1E4) and FITC-conjugated anti-rat Ig (MAR18.5). Anti-Mac-1 (M1/70) and biotinylated anti-Gr-1(RB6-8C5) were generously provided by Dr. K. Okumura (Juntendo University, Tokyo, Japan).

Cell Surface Biotinylation, Immunoprecipitation, and Two-dimensional SDS-PAGE Analysis. Cells were surface labeled by biotinylation as previously described (13). Cells were then solubilized in a lysis buffer (1% digitonin, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSE, and 10 mM iodoacetamide). The lysates were immunoprecipitated with anti-CD3 ϵ mAb (145-2C11), anti- ζ mAb (H146.698A), and anti-FcR γ antiserum, which were kindly provided by Drs. J. Bluestone (University of Chicago, Chicago, IL), R. Kubo (Cytel, Inc., La Jolla, CA), and C. Ra (Juntendo University), respectively, analyzed on two-dimensional nonreducing-reducing SDS-PAGE (14% for the first dimension and 16% for the second dimension) or single-

dimensional SDS-PAGE (13%) under reducing condition, and transferred onto a polyvinylidene fluoride membrane. Biotinylated proteins on the membrane were detected using streptavidin-peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and an ECL system (Amersham, Buckinghamshire, England).

Results

Selective Loss of the ζ Chain in the Splenic T Cells of TBM. Splenic T cells were isolated from normal mice and TBM that had been subcutaneously implanted with syngeneic colon carcinoma, colon 26, 2–9 wk before use. Tumor growth was specifically localized on the back, and no visceral invasion or metastasis was observed. T cells were surface labeled by biotinylation, and the cell lysates were immunoprecipitated with anti-CD3 ϵ and anti- ζ mAb and analyzed by two-dimensional SDS-PAGE (Fig. 1). CD3 ζ homodimers, clearly detected as an off-diagonal spot in normal mice and 2-wk TBM, were diminished in 4-wk TBM and completely lost in 6-wk TBM (Fig. 1A). The disappearance of ζ in whole lysates was confirmed by Western blotting with anti- ζ mAb (data not shown). In spite of the disappearance of ζ , other components of the TCR-CD3 complex had not disappeared, even in the 6-wk TBM, and the expression level of the cell surface TCR complex was not altered (Fig. 1). Although Mizoguchi et al. reported that ζ was replaced by FcR γ in their TBM system (4), FcR γ was not detected within the TCR complex of TBM by immunoprecipitation with anti-CD3 ϵ mAb (Fig. 1), or even with anti-FcR γ Ab (data not

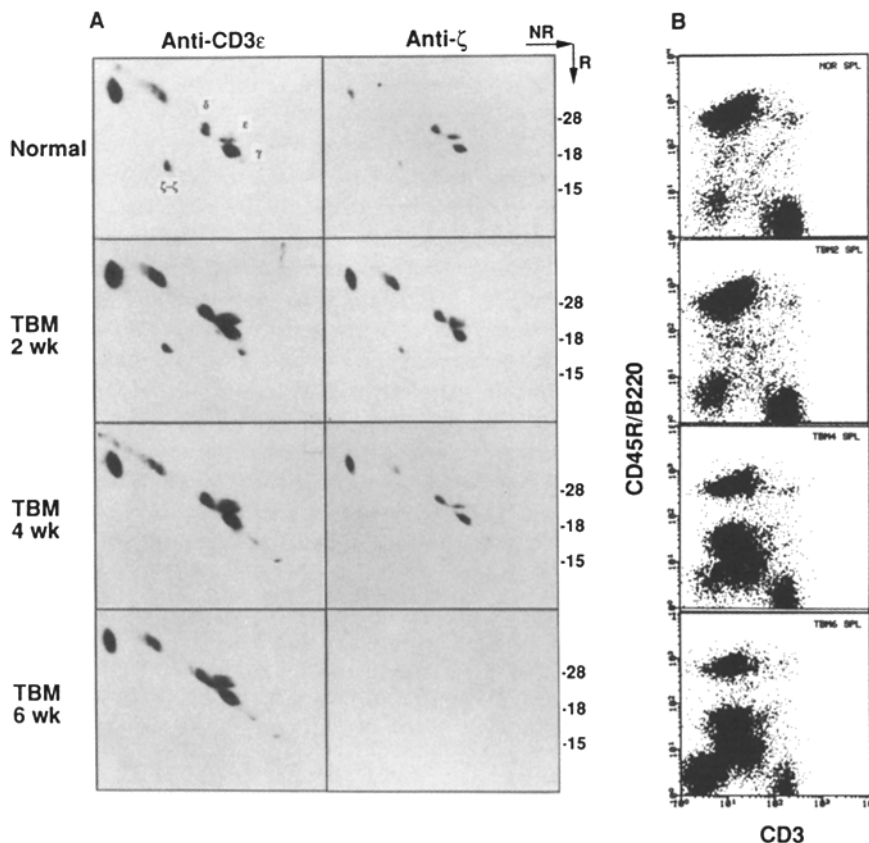


Figure 1. Time course of the disappearance of the CD3 ζ chain associated with the TCR-CD3 complex and flow cytometric analysis of spleen cells in 2–6-wk TBM. (A) The lysates of surface-biotinylated splenic T cells (4×10^7) were immunoprecipitated with anti-CD3 ϵ and - ζ mAbs and analyzed on 14% nonreducing (NR)–16% reducing (R) two-dimensional SDS-PAGE. The positions of CD3 $\gamma\delta\epsilon$ and ζ chains, as well as molecular size markers (in kilodaltons), are indicated. (B) Whole spleen cells were stained with FITC-labeled anti-CD3 ϵ and PE-conjugated anti-CD45R/B220 mAbs, and they were analyzed with a FACScan[®] flow cytometer.

shown) in our system. These results demonstrated that the ζ chain was selectively lost from the TCR-CD3 complex without being replaced by FcR γ in the spleen of TBM.

Progressive Increases of Non-T, Non-B Cells in the Spleen of TBM. To liberate T cells from in vivo influences of tumor, splenocytes from TBM were cultured in vitro for 48 h. Irrespective of T cell stimulation with PMA and Ca ionophore, the ζ chain remained undetectable in TBM (data not shown). Cell surface staining of TBM splenocytes revealed increases in non-T, non-B cell populations (T⁻B⁻) along with tumor progression (Fig. 1 B). The accumulation of T⁻B⁻ cells occurred only in the spleen but much less in lymph nodes and i-IEL and in parallel with splenomegaly (Fig. 2 B). In contrast with the complete loss of ζ in splenic T cells, ζ was detected within the TCR complex in both lymph nodes and i-IEL from the same mouse (Fig. 2 A). Therefore, the alteration of the TCR structure is induced in localized tissues and is not systemic, and the accumulation of T⁻B⁻ cells correlates with the loss of ζ .

Splenocytes from TBM Cause the Structural Alterations of TCR in Normal T Cells. To examine the capability of splenocytes from TBM or tumor cells to induce the loss of ζ , we used in vitro culture by mixing these cells or culture supernatants with freshly isolated normal splenic T cells. Fig. 3 A clearly demonstrates that the coculture of T cells with TBM spleno-

cytes but not with tumor cells or supernatants resulted in the loss of ζ . It should be stressed that in addition to the disappearance of ζ , the mobility of the CD3 $\gamma\delta\epsilon$ chains were increased. Again, the expression level of the cell surface of the TCR complex was not changed during the period (data not shown). Surprisingly, these structural changes were induced by just performing surface biotinylation immediately after mixing normal T cells and TBM splenocytes. When normal T cells were separated from TBM splenocytes by pored membrane to prevent direct contact, no structural changes were observed after culture for 12 h (Fig. 3 B) or even 48 h (data not shown). These results suggest that direct cell contact with TBM splenocytes induced the TCR alterations in normal T cells.

Direct Interaction with Macrophages from TBM Induces the Structural Alterations of the TCR Complex. The T⁻B⁻ cells accumulated in the spleen of TBM were characterized by cell surface staining. Staining profiles revealed that CD3⁻B220⁻ cells were the major population in the TBM spleen and Mac-1⁺, MHC class II^{low}, HSA⁺ (Fig. 4), and Gr-1⁻ (data not shown). These phenotypes are consistent with those of tumor-induced macrophages (15). When macrophages were isolated from TBM and cocultured with normal T cells, they induced the structural changes of the TCR complex, the disappearance of ζ and mobility shifts of the CD3 $\gamma\delta\epsilon$ chains

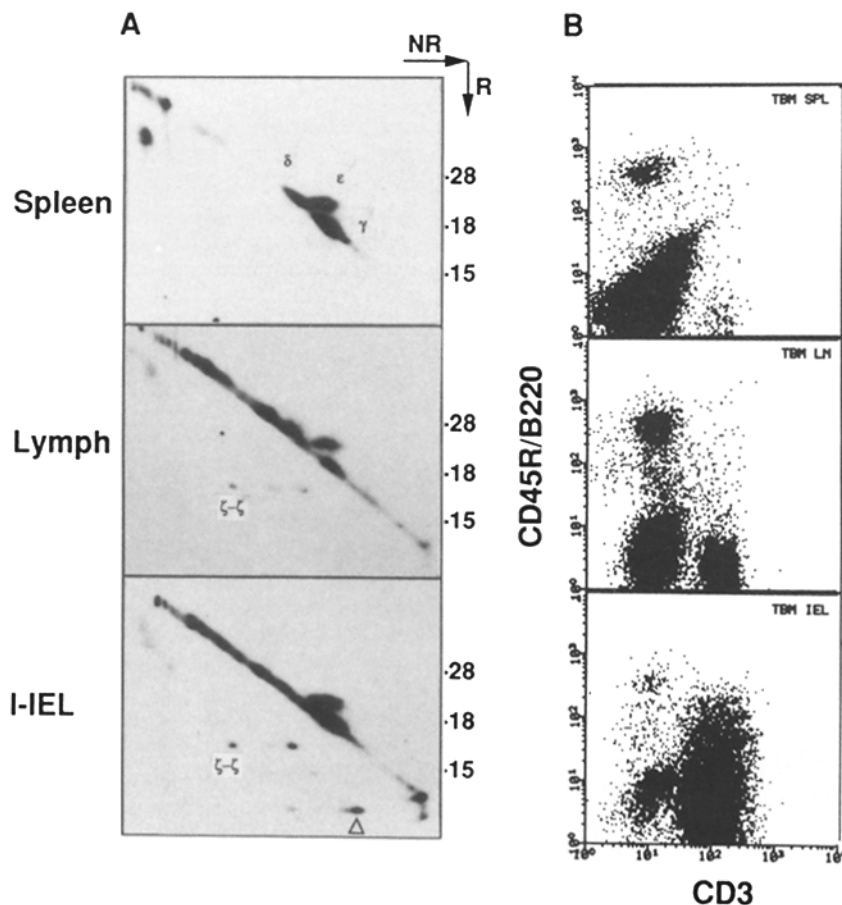


Figure 2. Structure of the TCR-CD3 complex and flow cytometric analysis of cells from spleen, lymph nodes, and i-IEL in 9-wk TBM. (A) Splenic T cells (1.5×10^7), whole lymphocytes (4×10^7 , ~40% were T cells), and i-IEL (1×10^7) were surface biotinylated, and the lysates were precipitated with anti-CD3 ϵ mAb and analyzed as in Fig. 1. FcR γ chain (Δ) was seen in i-IEL (14). The position of each CD3 chain and molecular size markers (in kilodaltons) are indicated. (B) Each population in A was stained with FITC-labeled anti-CD3 ϵ and PE-conjugated anti-CD45R/B220 mAbs and analyzed with a FACScan[®] flow cytometer.

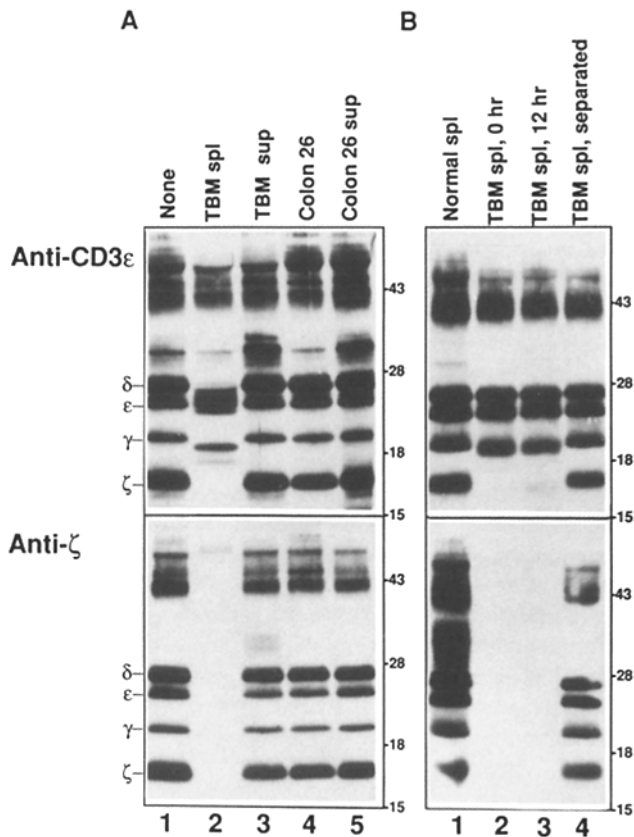


Figure 3. Structural alterations of the TCR complex in normal splenic T cells by coculture with TBM splenocytes. (A) Normal splenic T cells (1.5×10^7) were mixed with medium alone (lane 1), whole splenocytes from TBM (7.5×10^6 , lane 2), culture supernatant of TBM splenocytes (lane 3), colon 26 tumor cells (7.5×10^6 , lane 4), and culture supernatant of the tumor cells (lane 5), respectively. After a 12-h incubation, cells were surface-biotinylated and the lysates were immunoprecipitated with anti-CD3 ϵ and ζ mAbs, and they were analyzed on 13% reducing SDS-PAGE. *sup*, supernatant. (B) Whole spleen cells from normal mice (1.5×10^7 , lane 1) or from TBM (1.5×10^7 , lane 3) were co-cultured with normal splenic T cells (3×10^7) for 12 h. For lane 2, whole spleen cells from TBM (1.5×10^7) were mixed with normal splenic T cells (3×10^7), and the mixture was immediately harvested. In lane 4, TBM splenocytes (1.5×10^7) and normal splenic T cells (3×10^7) were cocultured for 12 h under separated condition by pored membrane. Cells were analyzed as in A. The positions of CD3 $\gamma\delta\epsilon$ and ζ chains, as well as molecular size markers (in kilodaltons), are indicated.

in a dose-dependent fashion; however, splenic macrophages, as well as peritoneal macrophages from normal mice, failed to induce these changes in T cells (Fig. 5).

Normal Activated Macrophages Exhibit Similar Capacity to Induce the Alteration of the TCR Complex. We next analyzed whether normal macrophages in certain activated stages exhibit similar capability to induce the structural changes of the TCR complex, or if only macrophages from TBM are special. Peritoneal macrophages were activated with thioglycolate, zymosan A, and LPS, separately or together. Only macrophages activated with zymosan A + LPS induced the disappearance of ζ (Fig. 5 C), whereas other activated macrophages failed to induce such alterations. FACS[®] analysis revealed that zymosan A + LPS-treated peritoneal macrophages

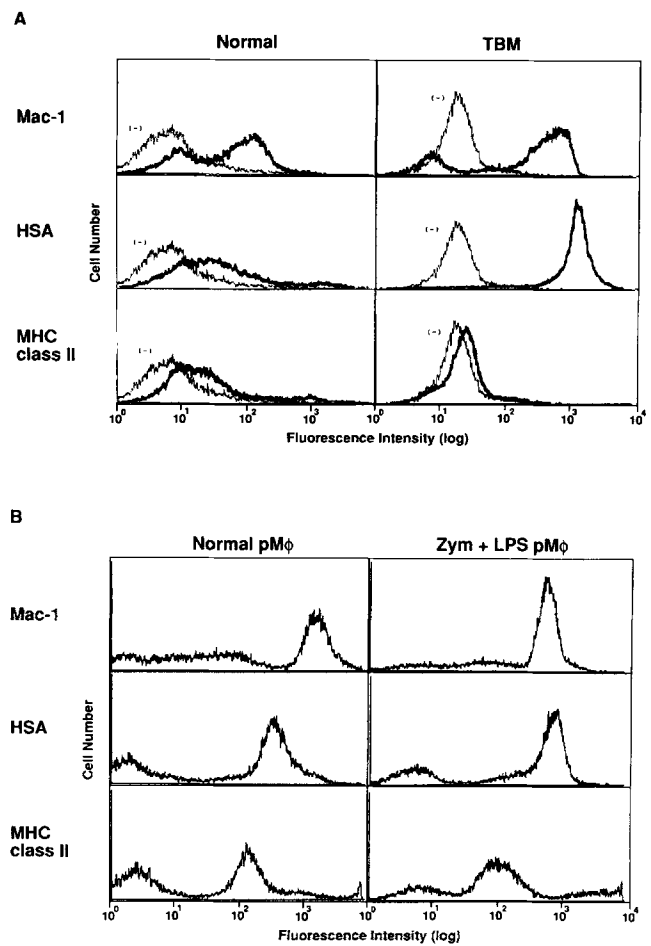


Figure 4. Flow cytometric analysis of T⁻B⁻ cells in the spleen of normal mice and TBM (A), and peritoneal macrophages from normal and zymosan A + LPS-treated mice (B). T⁻B⁻ cells and peritoneal macrophages were prepared as described in Materials and Methods. Cells were stained with mAbs against Mac-1, MHC class II, and HSA (*thick lines*) or with control Ab (*thin line*), followed by FITC-labeled anti-rat Ig mAb, and analyzed with a FACScan[®] flow cytometer.

showed similar phenotype of Mac-1⁺, HSA⁺, and MHC class II^{low} as macrophages observed in TBM (Fig. 4). These results clearly demonstrate that not only macrophages from TBM, but also normal macrophages in certain activated stages, can induce the structural alterations of the TCR complex in normal T cells.

Discussion

Alterations of the expression of the TCR complex and the associated kinases were first reported by Mizoguchi et al. (4). In the present study, we partly confirmed this phenomenon and extended the analysis to understand the mechanism. We observed the disappearance of the CD3 ζ chain along with tumor progression in a TBM model. Our main finding was that macrophages accumulated in the spleen of TBM induced the disappearance of ζ in all splenic T cells. This was clearly demonstrated by devising an *in vitro* culture system in which freshly prepared normal T cells lost the ζ chain by direct contact with macrophages from TBM. This finding explains the ob-

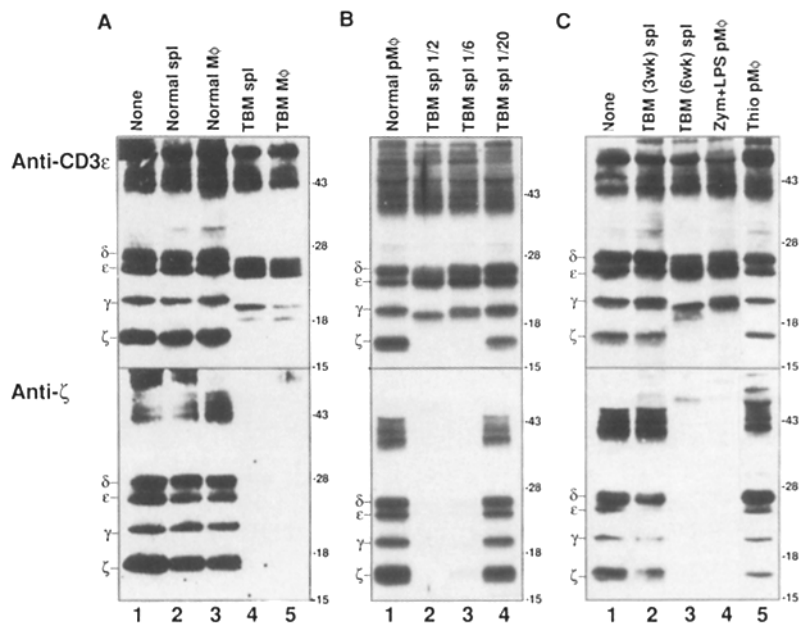


Figure 5. Structural alterations of the TCR complex in normal splenic T cells by coculture with macrophages in the spleen of TBM or zymosan A + LPS-treated peritoneal macrophages. (A) Medium alone (lane 1), splenocytes from normal mice (1×10^7 , lane 2), splenic macrophages from normal mice (1×10^7 , lane 3), splenocytes from TBM (1×10^7 , lane 4), and splenic macrophages from TBM (1×10^7 , lane 5) were mixed with normal splenic T cells (2×10^7). After a 30-min culture, cells were surface biotinylated and the lysates were precipitated with anti-CD3 ϵ and - ζ mAbs, and they were analyzed on 13% reducing SDS-PAGE. (B) Peritoneal macrophages (pM ϕ) from normal mice 2.5×10^6 , lane 1) and graded cell numbers of splenocytes from TBM (7.5×10^6 , lane 2; 2.5×10^6 , lane 3; 7.5×10^5 , lane 4) were mixed with normal splenic T cells (1.5×10^7). After a 1-h coculture, cells were analyzed as in A. (C) Medium alone (lane 1), splenocytes from 3-wk TBM (1.5×10^7 , lane 2), splenocytes from 6-wk TBM (1.5×10^7 , lane 3), peritoneal macrophages from zymosan A + LPS-treated mice (1.5×10^7 , lane 4), and peritoneal macrophages from thioglycolate-treated mice (1.5×10^7 , lane 5) were mixed with normal splenic T cells (1.5×10^7). After a 1-h coculture, cells were analyzed as in A. The positions of CD3 $\gamma\delta\epsilon$ and ζ chains, as well as molecular size markers (kilodaltons), are indicated.

ervation that the complete loss of ζ was observed only in T cells from the spleen but not from lymph nodes or i-IEL, because the extensive accumulation of macrophages was observed only in the spleen but not in the latter tissues. Recent analyses on human cancer patients that tumor-infiltrating T cells expressed less amount of ζ than peripheral blood T cells in the same individual (5, 6) could be attributable to macrophages (16) that interact with T cells in tumor lesions and may cause the alterations of the TCR structure. The lesser effect in peripheral blood T cells may be caused by the small number of such macrophages in peripheral blood. Since splenic T cells are separated from the subcutaneous tumors, tumor cells have a minimal influence on T cells but rather are indirectly involved by inducing functional changes in macrophages.

Several possible mechanisms have been proposed to explain the impaired immune responses in tumor-bearing hosts. Among them, the contribution of suppressor macrophages has been extensively studied (3, 14, 17). Macrophages play a major role in the host-defense mechanism through phagocytosis and production of inflammatory cytokines, as well as in antigen presentation to T cells. Tumor growth induces functional changes in macrophages from immunocompetent to immunosuppressive. One of the notable phenotypic changes is the downregulation of the surface expression of MHC class II molecules, as we observed in the accumulated macrophages in TBM. These macrophages have been demonstrated to suppress T cell functions by producing inhibitory mediators such as prostaglandin E₂ (3) and lipocortin (17). On the other hand, it has been reported that surface molecules on T cells, such as membrane-bound TNF- α , activate macrophages to induce effector functions (18). Taken together with our result that the cell contact between macrophages and T cells is required to induce the alteration of the TCR complex, it is likely that macrophages activated to certain stages in TBM interact with T cells and induce structural alterations of the

TCR complex by direct contact or by secreting short-lived mediators upon interactions.

More importantly, we found that the alterations of the TCR complex could be induced not only by macrophages from TBM but also by activated macrophages from normal mice. The findings that macrophages activated with zymosan A + LPS but not with thioglycolate induced the changes of the TCR complex by mixing with normal T cells imply that macrophages in certain activation stages have the capability to induce the structural alterations of the TCR complex by direct cell contact. Such activated macrophages may be readily induced in inflammatory lesions or upon bacterial infections, as well as in tumor-bearing conditions. Therefore, macrophages might regulate the structure of the TCR complex and related signaling functions in normal immune responses.

The intracellular events induced in T cells by suppressive macrophages remain unknown. Although Mizoguchi et al. reported that ζ was replaced by the FcR γ chain and the expression of CD3 γ was reduced in TBM, our results could not confirm these changes but rather demonstrated different alterations. In our model, ζ disappeared but was not replaced by FcR γ . In our in vitro analysis, TBM macrophages induced the loss of ζ in normal T cells just by mixing the two populations, without incubation. Our in vitro analysis clearly demonstrated the mobility shifts of CD3 $\gamma\delta\epsilon$, suggesting structural or conformational changes of each CD3 chain. A similar observation has been reported in invariant chain (Ii)-deficient mice, that the MHC class II $\alpha\beta$ dimers possessing unusual conformations in the absence of Ii exhibit a mobility shift in SDS-PAGE (19). Since the interaction of T cells with small number of macrophages induced only the disappearance of ζ and the mobility shift of other CD3 chains required more macrophages, the loss of ζ seems to be more sensitive than structural changes of other CD3 chains to the putative signals from macrophages. Alternatively, there may exist two different

mechanisms for induction of the ζ disappearance and the structural changes of other CD3 chains. Analysis of the structural basis of the CD3 chains' mobility shift could be a clue for analyzing the mechanism.

The ζ chain has been shown to regulate the intracellular assembly and cell surface expression of the TCR-CD3 complex. Previous studies in vivo and in vitro demonstrated that the TCR-CD3 components failed to express on the cell sur-

face in the absence of ζ (10, 11). Therefore, the mechanism for maintaining the normal level of cell surface TCR expression in the absence of ζ in TBM is not clear at the present time. Further analysis of the mechanism of the TCR complex alterations induced by activated macrophages may provide greater insight into the assembly and transport of the TCR-CD3 complex, as well as effective therapies for immunosuppression.

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