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Do Structural Changes of T Cell Receptor Complex Occur in Tumor-bearing State?

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T cells in tumor-bearing mice and cancer patients were recently shown to be devoid of CD3-ζ chain, a signal-transducing invariant chain in T cell receptor (TCR) complex, and p56^{lck} tyrosine kinase. In the present study, we investigated the structure and function of TCR complex in T cells from BALB/c mice bearing CSA1M fibrosarcoma. The expressions of TCR chains and p56^{lck} in a T cell-enriched population from spleen were analyzed. Almost complete loss of CD3-ζ and p56^{lck} was observed in the preparation from tumor-bearing mice as assessed by immunoblotting analysis using whole cell lysates, whereas the amounts of other TCR chains were relatively unchanged. However, these changes were due to the increase of contaminating Mac-1$^+$ cells in the spleen of tumor-bearing mice because: 1) the removal of Mac-1$^+$ cells led to the restoration of CD3-ζ and p56^{lck}; and 2) CD3-ζ was clearly present when the preparation was solubilized with ionic detergent. Fc receptor γ chain detected in the preparation from tumor-bearing mice disappeared along with the removal of Mac-1$^+$ cells. These observations were further supported by the finding that addition of Mac-1$^+$ cells from tumor-bearing mice to normal T cells resulted in loss of CD3-ζ, leaving CD3-ε largely intact. When T cells from tumor-bearing mice were highly purified by depletion of Mac-1$^+$ cells, these T cells contained normal amounts of CD3-ζ at mRNA, protein, and surface levels, and expressed the properly assembled TCR complex on their cell surface. Moreover, stimulation of the TCR in these T cells by anti-TCR antibodies resulted in a comparable Ca^{2+} mobilization to that observed in normal T cells. These results suggest that no structural changes occur in TCR complex in our tumor-bearing mice, and that complete depletion of Mac-1$^+$ cells in important to assess the structure of TCR complex.

Key words: T cell receptor — CD3-ζ — p56^{lck} — FcεRIγ — Mac-1$^+$ cell

Impaired immune responses occur frequently during the tumor-bearing state.[1–9] For several years, we have been studying T cell and APC[5] functions in tumor-bearing mice,[10,11] and our basic conclusion is that T cell, especially CD4$^+$ T cell, function is suppressed in proportion to the progress of tumor-bearing stage in spite of an increase in tumor-antigen-presenting APC function. However, since T cells are activated through a complex cascade of signal transduction pathways, the precise molecular mechanism that underlies T cell dysfunction in the tumor-bearing state remains to be elucidated.

Recently, intriguing observations have been reported by two groups.[12–14] Mizoguchi *et al.*[12] demonstrated that T cells from mice bearing colon carcinoma expressed TCR devoid of CD3-ζ, and showed loss of protein tyrosine kinases, p56^{lck} and p59^{fyn}. Because CD3-ζ and these protein tyrosine kinases have important roles in the signal transduction pathways of TCR-mediated T cell activation,[15] the structural changes of the TCR in tumor-bearing mice resulted in a defective T cell function.[12] These observations were supported by the findings that the expressions of CD3-ζ and p56^{lck} were markedly decreased in tumor-infiltrating T cells from humans bearing renal cell or colorectal carcinoma.[13,14] Thus, alterations in signal-transducing molecules in T cells seem to be a generalized feature associated with the tumor-bearing state, which may account for impaired T cell function.

To investigate a possible mechanism responsible for T cell dysfunction in the tumor-bearing state, we checked the structure and function of TCR complex in T cells from our tumor-bearing mice. Surprisingly, our data demonstrated that loss of CD3-ζ and p56^{lck} in the T cell population was due to contamination with Mac-1$^+$ cells, which increased in the spleen from tumor-bearing mice.

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[5] Abbreviations used in this paper: APC, antigen-presenting cell; TCR, T cell receptor; FCM, flow cytometry; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FcR, Fc receptor; FcεRI, Fc receptor type I for IgE; C, complement; C′, activated complement; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Ab, antibody, mAb, monoclonal antibody.

MATERIALS AND METHODS

Mice Male BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Hamamatsu). These mice were used at 6 to 10 wk of age in all experiments.

Tumors CSA1M fibrosarcoma,[16] which was induced in BALB/c mouse with the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) and shown to produce no RSV virus, was used. This tumor cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of CSA1M tumor-bearing mice CSA1M tumor-bearing mice were prepared by i.d. inoculation of 2×10^5 viable CSA1M cells.

Cell preparations B cells were depleted from spleen cells by immunomagnetic negative selection. Spleen cells from tumor-bearing and age-matched control mice were incubated at 4°C with magnetic particles bound to goat anti-mouse IgG (Advanced Magnetic, Cambridge, MA). The magnetic particles attached to cells were then removed using a rare earth magnet (Advanced Magnetic) leaving behind surface IgG-negative cells. These B cell-depleted populations were called "first" preparations. In some experiments, B cell-depleted spleen cells were further depleted of Mac-1^+ cells by treatment with anti-Mac-1 (mAb MI/70) followed by incubation with goat anti-rat IgG-coated magnetic beads (Advanced Magnetic). The residual cells were >85% TCR-β^+ as determined by FCM (Fig. 2A). These T cell-enriched populations were called "second" preparations. Enrichment of Mac-1^+ cells from tumor-bearing mice was carried out by T cell depletion from the first preparation. The first preparation from tumor-bearing mice was depleted of T cells by treatment with anti-Thy-1 (mAb HO-13-4) followed by goat anti-mouse IgG-coated magnetic beads. The residual cells were >70% Mac-1^+ as determined by FCM. Preparation of Thy-1^- spleen cells was carried out by T cell depletion from spleen. Cell suspensions from normal mice were treated with anti-Thy-1 (HO-13-4) and C′. Two C′-killing cycles were performed successively. The residual cells were >90% surface IgM^+ as determined by FCM.

Immunofluorescence staining and FCM analysis The mAb used for staining were: FITC-conjugated anti-CD3-ε (145-2C11),[17] biotin-conjugated anti-TCR-β (H57-597),[18] and FITC-conjugated anti-Mac-1 (MI/70) (Serotec, Kidlington, GB). The staining procedure was essentially the same as described previously.[19] Single- and two-color FCM analyses were performed by a FACScan flow cytometer (Beckton Dickinson Immunocytometry Systems, Mountain View, CA).

Immunoblotting analysis Isolated cell populations (1×10^7) were pelleted by centrifugation and cell pellets were solubilized with 80 μl of ice-cold lysis buffer (1% Triton X-100, 50 m*M* Tris-HCl, 300 m*M* NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* EDTA) for 25 min at 4°C. The nuclear pellet was removed by centrifugation, and the lysate was mixed with 20 μl of 5 × sample buffer (5% SDS, 50% glycerol, 250 m*M* Tris-HCl, 0.1% Bromophenol Blue (BPB), and 15% 2ME) and boiled for 5 min. In some experiments, cell pellets were solubilized with 1× sample buffer and boiled for 10 min. The samples (1×10^6 cell equivalent/lane) were electrophoresed in 10% (for $p56^{lck}$), 14% (for TCR chains), or 18% (for FcεRIγ) SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA). Membranes were blocked in PBS containing 5% nonfat dried milk for 2 h, and blotted with various Abs. Membranes were then washed with PBS-0.1% Tween 20 and incubated with horseradish peroxidase-conjugated protein A (Amersham, Buckinghamshire, England). After washing with PBS-0.1% Tween 20, membranes were developed using the enhanced chemiluminescence (ELC) system (Amersham). Relative optical densities of bands on autoradiograms were analyzed with the Microcomputer Imaging Device software program (Imaging Research, St. Catharines, ON).

The Abs used for immunoblotting were: anti-TCR-α, H28-710, a hamster mAb against Cα[20]; anti-CD3-ε, No. 127, polyclonal anti-serum raised in a rabbit immunized with purified ε chains[21]; anti-CD3-ζ, No. 551[22] or No. 387,[23] polyclonal anti-serum raised in rabbits immunized with peptides corresponding to amino acids 151–164 or 132–144 or CD3-ζ chain, respectively; anti-$p56^{lck}$ (UBI, Lake Placid, NY); and a rabbit anti-FcεRIγ antiserum.[24]

Surface biotinylation and immunoprecipitation Cell surface biotinylation was performed as previously described.[25] Cells were then solubilized with 0.5 ml of lysis buffer. After preclearing with normal mouse IgG prebound to protein A-Sepharose (Pharmacia, Piscataway, NJ), the lysate was immunoprecipitated with anti-CD3-ζ (No. 551) or anti-CD3-ε (2C11) Abs prebound to protein A-Sepharose for 1 h at 4°C. The column was washed with the lysis buffer, and the eluates were subjected to two-dimensional nonreducing-reducing SDS-PAGE. The bands were electroblotted onto PVDF membrane. This was blocked in PBS containing 5% BSA, and biotinylated proteins were detected using streptavidin-biotinylated peroxidase complex (Amersham) and the ECL system.

RNA blot analysis Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method as described.[26] Samples were denatured at 65°C for 5 min in electrophoresis buffer containing 50% formamide, 6% formaldehyde, and 1 × MOPS buffer (20 m*M* MOPS, 15 m*M* NaOAc,

and 1 m*M* EDTA, pH 7.0). RNA was then isolated on 1% native agarose gel, and transferred to nylon filters. Filters were dried, stained with RNA staining solution (0.3 *M* NaOAc and 0.02% methylene blue), and photographed. The filters were then treated at 65°C for 30 min with GMC buffer (0.5 *M* Na_2PO_4, 1% BSA, 1 m*M* EDTA, and 7% SDS, pH 7.2) before hybridization. Hybridization was performed at 65°C for 18–24 h in the same solution containing [^{32}P]cDNA radiolabeled with a Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridization, filters were washed twice at room temperature in 2× SSC (300 m*M* NaCl, and 30 m*M* sodium citrate), 0.1% SDS, followed by one wash at 65°C in 0.2× SSC containing 0.1% SDS. As a probe, we used murine CD3-ζ cDNA[23] kindly provided by Dr. A. M. Weissman.

Pulse-chase metabolic labeling T cell-enriched populations (second preparations) from control and tumor-bearing mice were labeled with [^{35}S]methionine and [^{35}S]cysteine as previously described.[27] Pulse-labeled cells were chased for different periods of time at 37°C in regular culture medium (RPMI 1640 medium containing 10% FCS). At each time point, cells were pelleted by centrifugation and frozen at −70°C before analysis by immunoprecipitation.

Measurement of $[Ca^{2+}]_i$ Cells (1×10^7/ml) were incubated in PBS containing 1% FCS and 1m*M* $CaCl_2$ in the presence of 4.4 μM fura-2AM (Molecular Probes, Eugene, OR). Dye-loaded cells were washed and resuspended (5×10^6/ml) in PBS containing 1% FCS and 1 m*M* $CaCl_2$. Fluorescence intensity was measured with a fluorescence spectrophotometer (Hitachi F-3000, Tokyo), with excitation at 340 nm and emission at 510 nm. After the baseline had stabilized, anti-CD3-ε (2C11, 0.25 μg/ml) or anti-TCR-β (H57-597, 0.5 μg/ml) was added to the cells, followed by goat anti-hamster IgG (100 μg/ml, Cappel, Durham, NC). Maximum fluorescence (F_{max}) was determined by lysing the cells with

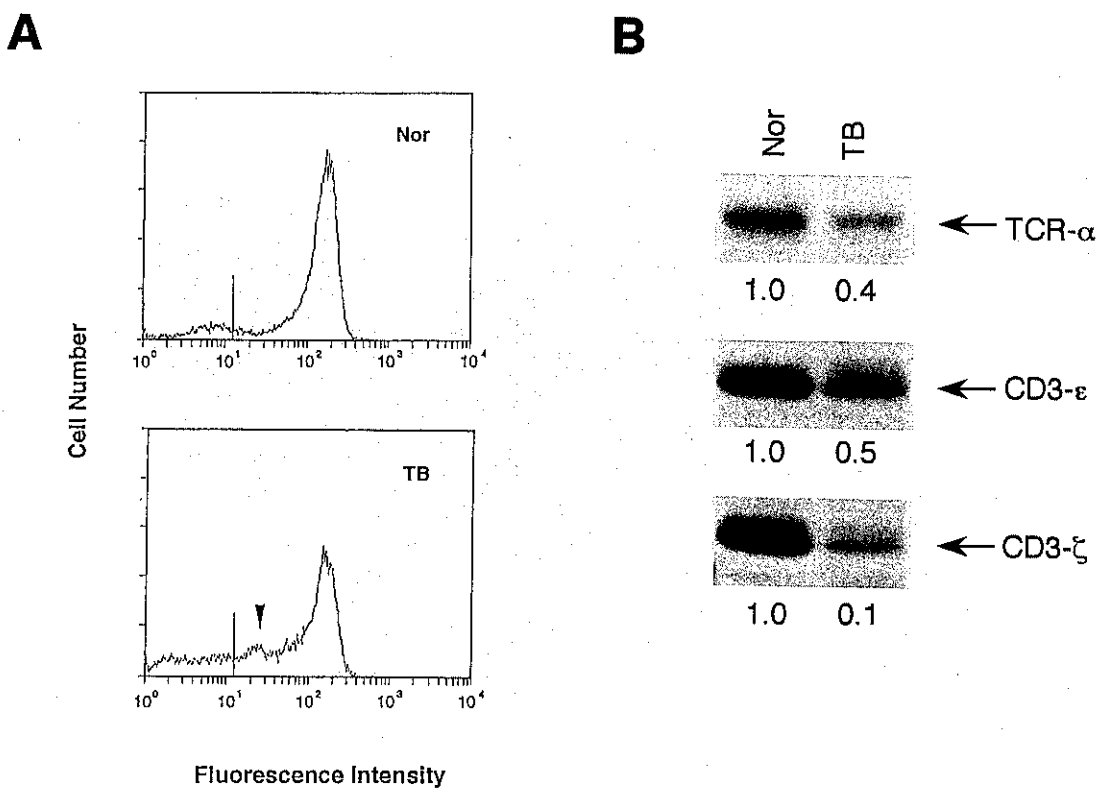

Fig. 1. Surface expression and total protein levels of TCR chains in the spleen from CSA1M tumor-bearing mice. A, B cell-depleted spleen cells from normal (Nor) or tumor-bearing (TB) mice were stained with FITC-conjugated anti-CD3-ε and analyzed by FCM. A $CD3^{intermediate}$ subpopulation (arrowhead) was detected in the preparation from tumor-bearing mice. B, Aliquots of cells shown in A were tested for their protein levels of TCR-α, CD3-ε, and CD3-ζ by immunoblotting analysis. Total cell lysates (1×10^6 cell equivalents/sample) were electrophoresed in 14% SDS-PAGE under reducing conditions and blotted with anti-TCR-α, anti-CD3-ε, and anti-CD3-ζ Abs. Relative band intensities were determined with a densitometer and are listed under each lane.

0.1% Triton X-100 and minimum fluorescence (F_{min}) by the addition of 10 μM $MnCl_2$. $[Ca^{2+}]_i$ was calculated by use of the following formula[28]:

$$[Ca^{2+}]_i = 224(F - F_{min}) / (F_{max} - F)$$

RESULTS

Total protein levels of various TCR chains in the spleen from CSA1M tumor-bearing mice We started our analysis to assess whether structural changes of TCR complex were observed in T cells from our tumor-bearing mice. BALB/c mice were inoculated i.d. with 2×10^5 viable CSA1M fibrosarcoma cells. These mice bore a solid tumor approximately 25 mm in diameter at 10–12 wk after the tumor cell inoculation. At this stage, mice were killed, spleens were removed, and splenic T cells were enriched by removing B cells with magnetic anti-mouse IgG beads (this population was called the "first" preparation). As shown in Fig. 1A, purities of T cells of these first preparations from tumor-bearing mice and age-matched control mice were 78% and 90%, respectively, as judged from staining with anti-CD3-ε mAb. These cells were lysed in lysis buffer containing 1% Triton-X 100. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with various anti-TCR Abs. The amount of CD3-ζ in the preparation from tumor-

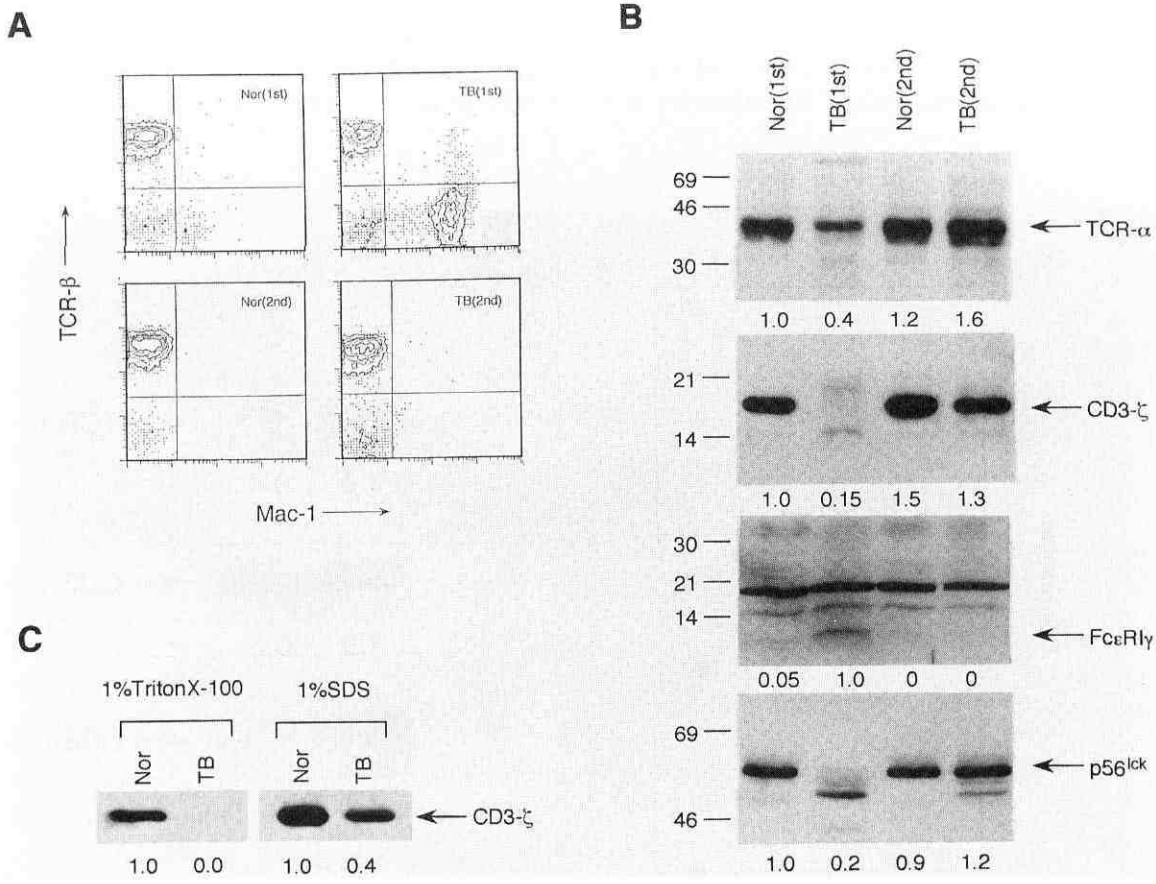

Fig. 2. The increase of Mac-1$^+$ cells in the spleen from tumor-bearing mice and its effect on immunoblotting analysis. A, B cell-depleted spleen cells (first (1st) preparations) and T cell-enriched spleen cells (second (2nd) preparations) from normal (Nor) or tumor-bearing (TB) mice were incubated with an anti-FcR mAb (2.4G2)[42] and then stained with FITC-conjugated anti-Mac-1, followed by biotin-conjugated anti-TCR-β plus phycoerythrin-conjugated streptavidin. Preparations of cells were performed as described in "Materials and Methods." B, Aliquots of cells shown in A were tested for protein levels of TCR-α, CD3-ζ, $p56^{lck}$, and FcεRIγ by immunoblotting analysis. Total cell lysates (1×10^6 cell equivalents for TCR-α, CD3-ζ, and $p56^{lck}$, and 8×10^6 cell equivalent for FcεRIγ) were electrophoresed in 10% (for $p56^{lck}$), 14% (for TCR-α and CD3-ζ), or 18% (for FcεRIγ) SDS-PAGE under reducing conditions and blotted with specific Abs. Molecular size standards are shown on the left (in kilodaltons). C, The first preparations from normal or tumor-bearing mice were solubilized with lysis buffer containing either 1% Triton X-100 or 1% SDS. These cell lysates were analyzed for the protein levels of CD3-ζ as described in B. Relative band intensities were determined with a densitometer and are listed under each lane.

bearing mice was markedly reduced compared to that from control mice, whereas the amounts of TCR-α and CD3-ε from tumor-bearing mice were less affected (Fig. 1B). These results suggest that structural changes of TCR complex seem to occur in T cells from our tumor-bearing mice.

Increase of Mac-1$^+$ cells in the spleen from tumor-bearing mice and its effect on immunoblotting analysis

In the course of performing the above experiments, we found that the preparation from tumor-bearing mice always contained a TCR/CD3^{intermediate} subpopulation (Fig. 1A). Since we did not block FcR-mediated binding of Abs for cell staining, we suspected that this TCR/CD3^{intermediate} subpopulation resulted from non-specific binding of anti-CD3 mAb with increased FcR-positive cells. To address this possibility, the first preparations from control and tumor-bearing mice were incubated with anti-FcR mAb to block FcR-mediated binding, then stained for TCR-β and Mac-1. As shown in Fig. 2A, immunofluorescence staining with this procedure resulted in complete disappearance of the TCR/CD3^{intermediate} subpopulation from the first preparation of tumor-bearing mice and demonstrated that Mac-1$^+$ cells were in fact increased in tumor-bearing mice. Although the percentages of splenic Mac-1$^+$ cells in the tumor-bearing state varied from mouse to mouse, tumor-bearing mice always contained increased Mac-1$^+$ cells in both percentage and absolute cell number compared to control mice. When we used these first preparations to assess protein levels of TCR chains and p56^{lck}, the results of Fig. 1 and those reported by others[12–14] were reproduced (Fig. 2B), i.e., in the preparation from tumor-bearing mice, complete absence of CD3-ζ and p56^{lck}, the presence of FcεRIγ, the γ chain of the high-affinity IgE receptor shown to be incorporated in TCR complexes in lieu of CD3-ζ,[29–31] and a partial reduction of TCR-α were observed. Although the reduction of TCR-α in the preparation from tumor-bearing mice could be due to a dilution effect caused by the presence of Mac-1$^+$ cells, the absence of CD3-ζ and p56^{lck} cannot be simply explained by this effect.

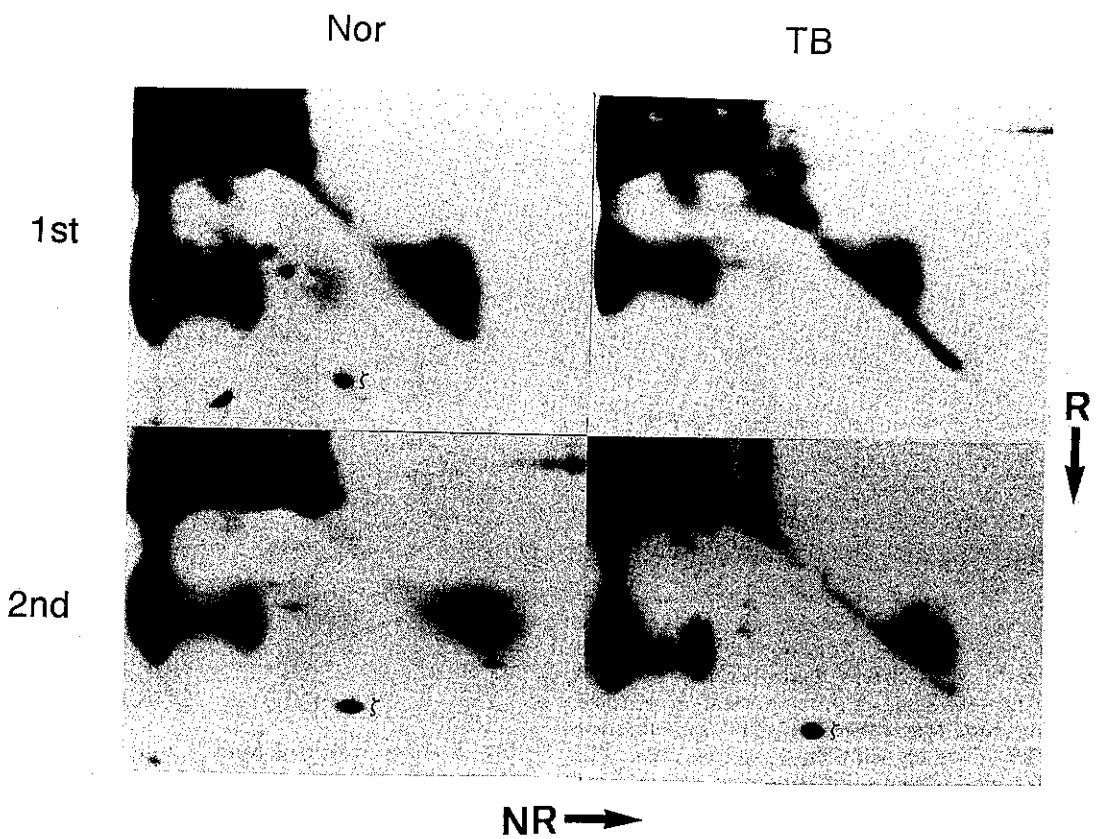

Fig. 3. Surface CD3-ζ expression on splenic T cells from tumor-bearing mice. Cell surface proteins from 1st and 2nd preparations of normal (Nor) or tumor-bearing (TB) mice were labeled with biotin and these cells were solubilized, immunoprecipitated with anti-CD3-ζ, and analyzed by two-dimensional nonreducing (NR)/resucing (R) SDS-PAGE. Positions of CD3-ζ chains are indicated.

To exclude the possible contribution of Mac-1$^+$ cells in immunoblotting analysis, we further purified splenic T cells by depleting Mac-1$^+$ cells with immunomagnetic negative selection (this population was called the "second" preparation). The resulting cell populations were completely devoid of Mac-1$^+$ cells and predominantly consisted of TCR-$\alpha\beta^+$ T cells as shown in Fig. 2A. When we analyzed protein levels of TCR chains and p56^{lck} using whole cell lysates of the second preparations, surprising results emerged (Fig. 2B): the amounts of CD3-ζ and p56^{lck} from tumor-bearing mice were comparable with those from control mice and FcεRIγ chain was now completely absent. These results suggested that the striking absence of CD3-ζ and p56^{lck} in the first preparation from tumor-bearing mice was due to the presence of Mac-1$^+$ cells and that T cells from tumor-bearing mice contained normal amounts of CD3-ζ and p56^{lck}. The presence of FcεRIγ chain in the first preparation from tumor-bearing mice was presumably attributable to FcR in Mac-1$^+$ cells, not to TCR complexes that contained FcεRIγ chains.

Another observation supporting the above conclusion was the finding that the amounts of CD3-ζ and p56^{lck} in thymocytes and in B cell-depleted lymph node cells from tumor-bearing mice were almost identical to those from control mice, because these cells did not contain detectable Mac-1$^+$ cells (data not shown). Moreover, whereas CD3-ζ and p56^{lck} were undetectable from the first preparation of tumor-bearing mice under the conditions using non-ionic detergent (1% Triton X-100), solubilization of the same preparation using an ionic detergent (1% SDS) resulted in the appearance of CD3-ζ (Fig. 2C) and p56^{lck} (data not shown). Thus, these results demonstrated that CD3-ζ and p56^{lck} in T cells from tumor-bearing mice were not absent, but rather were masked or degraded during the process if immunoblotting analysis owing to the presence of Mac-1$^+$ cells.

Surface CD3-ζ expression on splenic T cells from tumor-bearing mice We next analyzed whether the presence of Mac-1$^+$ cells affected surface CD3-ζ expression on T cells as well. Spleen cells from control and tumor-bearing mice were first depleted of B cells, and aliquots of these B

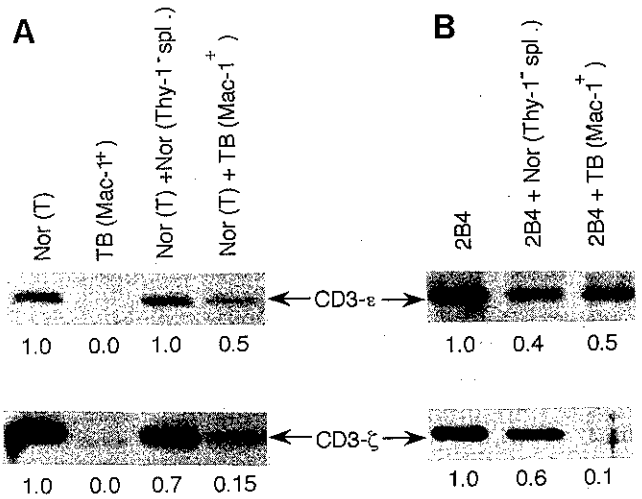

Fig. 4. The decrease of CD3-ζ from normal T cells by the addition of Mac-1$^+$ cells from tumor-bearing mice. A, Purified T cells (1×10^6/sample) from normal mice (Nor) were mixed either with Mac-1$^+$ cells (1×10^6/sample) from tumor-bearing (TB) mice or with Thy-1$^-$ spleen cells (Thy-1$^-$ spl.) (1×10^6/sample) from normal mice at 4°C after cell preparation. Immediately after mixing, cells were centrifuged and protein levels of CD3-ε and CD3-ζ were analyzed as described in Fig. 1B. B, T cell hybridoma, 2B4 cells (1×10^5/sample) were used as a source of T-lineage cells. Relative band intensities were determined with a densitometer and are listed under each lane.

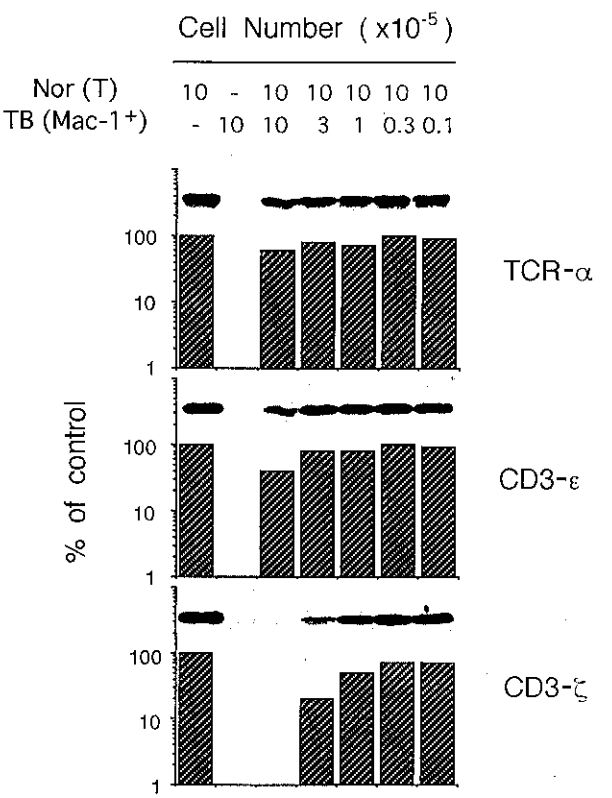

Fig. 5. Dose effect of Mac-1$^+$ cells on reduction of CD3-ζ from normal T cells in immunoblotting analysis. Various numbers of Mac-1$^+$ cells from tumor-bearing (TB) mice were mixed with normal T cells (Nor) (1×10^6/sample) and TCR chains from these mixed cell populations were analyzed as described in Fig. 2B. Relative band intensities were determined with a densitometer and calculated by taking the value of normal T cells without addition of Mac-1$^+$ cells as 100%.

cell-depleted cells were further purified by removing Mac-1$^+$ cells, as described above. Cell surface proteins from these two preparations were labeled with biotin, immunoprecipitated with anti-CD3-ζ Ab, and analyzed by two-dimensional nonreducing/reducing SDS-PAGE. As shown in Fig. 3, the analysis of the first preparation from tumor-bearing mice, which contained Mac-1$^+$ cells as well as T cells, revealed a complete absence of surface CD3-ζ. Again, removing Mac-1$^+$ cells from this preparation resulted in the appearance of CD3-ζ, suggesting that surface CD3-ζ was not lost in T cells. Thus, the apparent loss of surface CD3-ζ in the first preparation from tumor-bearing mice was due to the contamination with Mac-1$^+$ cells.

Decrease of CD3-ζ from normal T cells by the addition of Mac-1$^+$ cells from tumor-bearing mice Although the apparent structural changes of TCR complex in T cells from tumor-bearing mice can be explained by the presence of contaminating Mac-1$^+$ cells, it was still possible that T cells that had a defective TCR complex were removed along with Mac-1$^+$ cells during the process of immunomagnetic negative selection and that T cells with intact TCR complex remained after this process. To assess the role of Mac-1$^+$ cells more directly, we mixed T cells from normal mice and Mac-1$^+$ cells from tumor-bearing mice after cell preparation. Immediately after mixing, the cells were centrifuged and cell pellets were solubilized with lysis buffer containing 1% Triton X-100. We immunoblotted proteins from whole cell lysates of this mixed cell population. As a control for Mac-1$^+$ cells from tumor-bearing mice, T cell-depleted spleen cells from normal mice were used. As shown in Fig. 4A, Mac-1$^+$ cells did not contain detectable CD3-ε or CD3-ζ. Addition of Mac-1$^+$ cells from tumor-bearing mice to T cells resulted in a marked reduction of CD3-ζ, whereas large amounts of CD3-ε were retained. Fig. 4B shows the results of the experiments in which a T cell hybridoma, 2B4, was used as a source of T cells. The results clearly demonstrated a selective loss of CD3-ζ by the addition of Mac-1$^+$ cells to 2B4 cells.

To investigate further the decrease of CD3-ζ induced by Mac-1$^+$ cells, various numbers of Mac-1$^+$ cells from tumor-bearing mice were mixed with normal T cells and solubilized with lysis buffer containing 1% Triton X-100. TCR chains from these mixed cell populations were analyzed as shown in Fig. 5. When equal numbers of T cells (1×10^6) and Mac-1$^+$ cells (1×10^6) were mixed, complete absence of CD3-ζ was observed. A reduction of

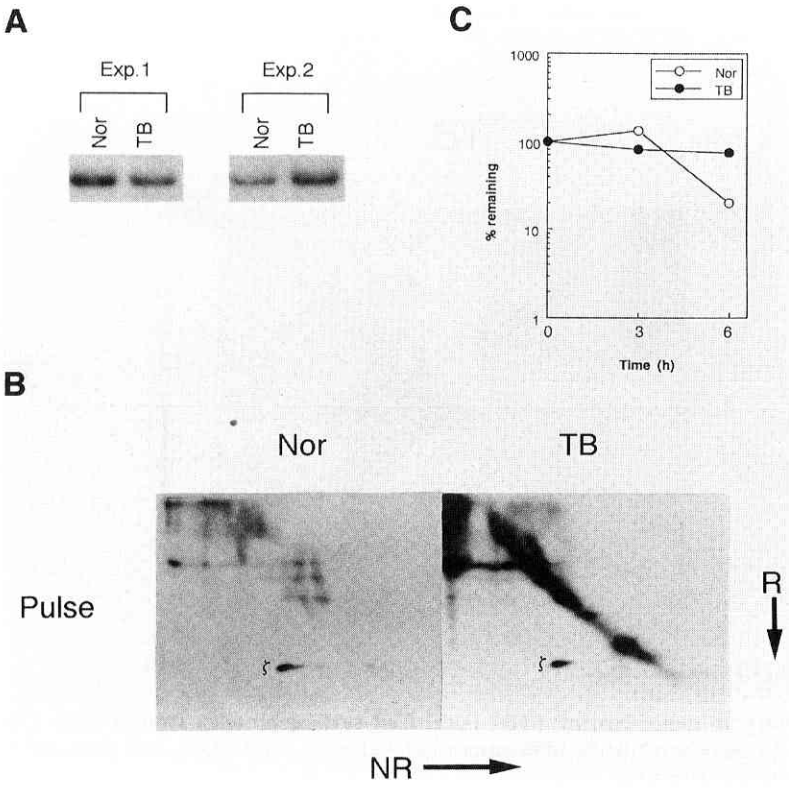

Fig. 6. Intracellular metabolism of CD3-ζ in T cells from normal (Nor) or tumor-bearing (TB) mice. A, Northern blot analysis of CD3-ζ mRNA produced in T cells (2nd preparations) from normal or tumor-bearing mice. Total RNA (10 μg/lane) was isolated on 1% agarose gel and transferred to nylon filters. The blots were hybridized with a ^{32}P-labeled CD3-ζ cDNA probe. Equal amounts of RNA per lane were confirmed by methylene blue staining before hybridization. B, Biosynthesis of CD3-ζ chain in T cells from normal or tumor-bearing mice. T cells were pulse-labeled at 37°C for 30 min with [^{35}S]methionine and [^{35}S]cysteine, solubilized, immunoprecipitated with anti-CD3-ζ, and analyzed by two-dimensional SDS-PAGE. C, Degradation of CD3-ζ chain in T cells from normal or tumor-bearing mice. T cells were pulse-labeled at 37°C for 30 min and chased for the indicated periods of time at 37°C in regular culture medium. Labeled proteins were analyzed as described in B. The relative amount of CD3-ζ chain remaining after chase (% compared to time 0) was determined by densitometric scanning.

CD3-ζ was apparent upon the addition of Mac-1^+ cells at doses from 1×10^6 down to 1×10^5. In contrast, the amounts of TCR-α and CD3-ε were less affected by the addition of Mac-1^+ cells. Taken together, these results confirmed that the presence of Mac-1^+ cells from tumor-bearing mice was responsible for the selective loss of CD3-ζ on immunoblotting analysis.

Structure and function of the TCR complex in T cells from tumor-bearing mice Since the data presented above clearly indicated that T cells from tumor-bearing mice contained normal protein levels of CD3-ζ (Fig. 2B), and normal surface CD3-ζ (Fig. 3), we anticipated that intracellular metabolism of CD3-ζ would be normal in these T cells. To check whether this is the case, the mRNA level, biosynthetic rate, and half-life of CD3-ζ chains were analyzed in purified T cells (the second preparations) from control and tumor-bearing mice. As shown in Fig. 6, the expression and degradation of CD3-ζ in T cells from tumor-bearing mice were not reduced compared to those from control mice. Moreover, the assembled TCR complex on the surface of these T cells was not changed (Fig. 7). Thus, no structural alteration was observed in the TCR complex in the tumor-bearing state.

We finally assessed the function of the TCR in T cells from tumor-bearing mice. Stimulation of the TCR by antigen or by anti-TCR Abs directed against either TCR-$\alpha\beta$ or CD3-ε results in several signaling events.[32] Among them, increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) have been implicated as important early events in TCR-dependent T cell activation.[32] We compared Ca^{2+} mobilization in T cells from control and tumor-bearing mice in response to anti-TCR-β or anti-CD3-ε Abs. As shown in Fig. 8, the increase in the $[Ca^{2+}]_i$ in response to both anti-TCR-β and anti-CD3-ε Abs was comparable in T cells from control and tumor-bearing mice. Thus, the results suggested that engagement of the TCR in T cells from tumor-bearing mice evoked a normal signaling event.

DISCUSSION

The recent identification of several tumor antigens recognized by T cells in humans and in rodents provides strong evidence that T cells are involved in an immune response to tumors.[33] However, T cell-mediated immunity is considered to be impaired in the tumor-bearing state due to various suppressive mechanisms.[1-9] In fact, we have recently found that $CD4^+$ T cell function is suppressed in proportion to the progress of tumor-bearing stage.[10, 11] Investigating the molecular mechanism responsible for T cell dysfunction in the tumor-bearing state is, therefore, one of the important issues of tumor immunology, and may provide clues for cancer immunotherapy. In this regard, recent findings by Ochoa and colleagues[12, 13] are notable. According to their observations, T cells from tumor-bearing mice[12] and humans[13] had a defective TCR complex that was devoid

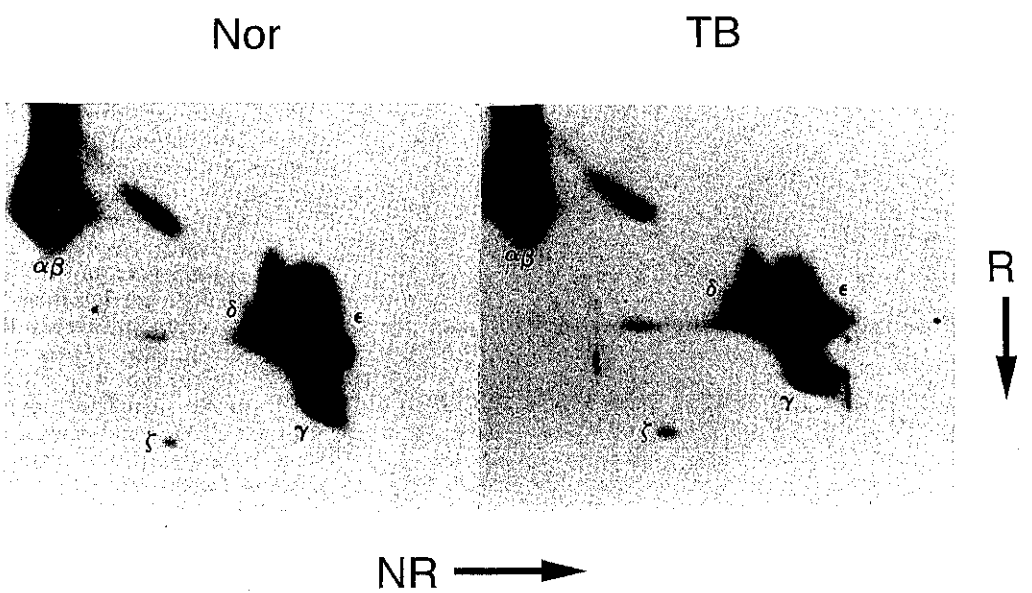

Fig. 7. TCR assembly in T cells from normal (Nor) or tumor-bearing (TB) mice. Cell surface proteins from T cells (2nd preparations) were labeled with biotin and these cells were solubilized, immunoprecipitated with anti-CD3-ε, and analyzed by two-dimensional SDS-PAGE. Positions of TCR chains are indicated.

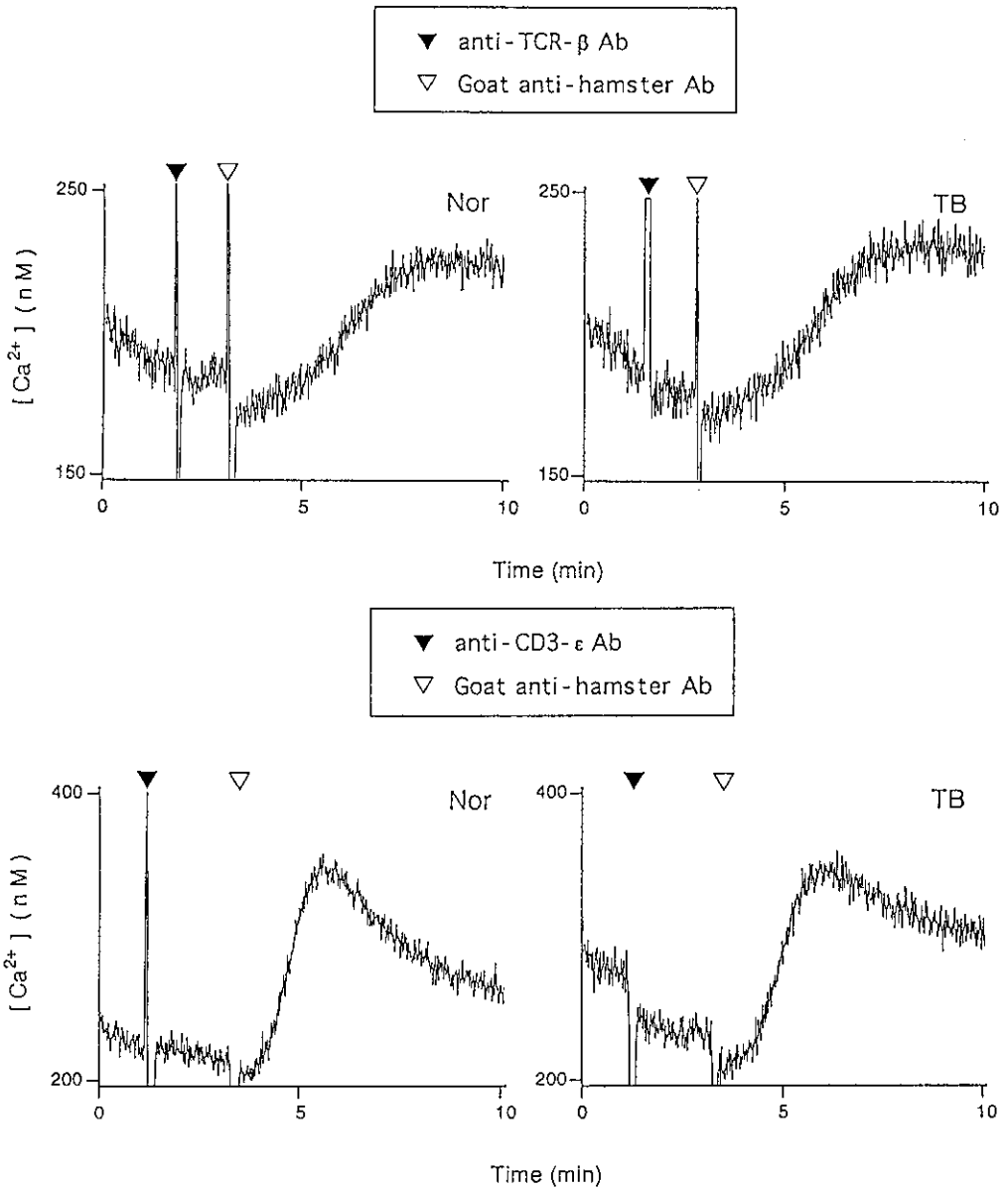

Fig. 8. Ca^{2+} response in T cells from normal (Nor) or tumor-bearing (TB) mice. T cells (2nd preparations) were loaded with Fura-2AM, and Ca^{2+} mobilizations in response to cross-linking of anti-TCR-β or of anti-CD3-ε were determined as described in "Materials and Methods." Closed triangles represent the addition of anti-TCR-β or anti-CD3-ε Abs and open triangles represent the addition of goat anti-hamster IgG.

of CD3-ζ. These T cells showed loss of $p56^{lck}$ tyrosine kinase as well. These structural changes resulted in a defective T cell function as assessed in terms of Ca^{2+} response.[12] Thus, impaired immune responses in tumor-bearing hosts are considered to be caused, at least in part, by the structural changes of the TCR.

Our initial goal in this study was to check the change of the TCR complex in T cells from our tumor-bearing mice, and to delineate the molecular mechanism underlying this change. However, we obtained a surprising result, which led us to reconsider the conclusion by Ochoa *et al.*[12] Although we observed loss of CD3-ζ and $p56^{lck}$ in T cell preparation from our tumor-bearing mice, this defect was due to the presence of contaminating Mac-1^+ cells since: 1) the removal of Mac-1^+ cells from the T cell preparation led to the restoration of CD3-ζ and

$p56^{lck}$ in immunoblotting analysis; 2) CD3-ζ was clearly visualized in an experiment in which a T cell preparation from tumor-bearing mice was solubilized with ionic detergent, not with non-ionic detergent; and 3) addition of Mac-1^+ cells from tumor-bearing mice to T cells from normal mice resulted in a marked reduction of CD3-ζ, while CD3-ε was little changed. When T cells from tumor-bearing mice were highly purified by depletion of Mac-1^+ cells, these T cells contained normal TCR complexes in the structure, and exhibited normal functional activity as monitored by the increase of intracellular Ca^{2+} after stimulation with anti-TCR Abs.

The mechanism responsible for the effect of Mac-1^+ cells, inducing loss of CD3-ζ and $p56^{lck}$, is not known. Several possible explanations can be considered to account for this effect. First, CD3-ζ and $p56^{lck}$ in T cells may move to the Triton X-100-insoluble fraction by translocation to the nucleus or interaction with the cytoskeleton after the T cells have interacted with Mac-1^+ cells. However, this does not seem likely, because there was no increase of CD3-ζ in the Triton X-100-insoluble fraction from the preparation of tumor-bearing mice compared to that from normal mice when these Triton X-100-insoluble fractions were further solubilized with SDS (data not shown). Second, it is well known that activated macrophages contain various proteolytic enzymes.[34] Solubilization of Mac-1^+ cells from tumor-bearing mice with Triton X-100 lysis buffer activates these proteolytic enzymes that are resistant to usual protease inhibitors, resulting in the degradations of CD3-ζ and $p56^{lck}$. In support of this notion, addition of lysate of Mac-1^+ cells from tumor-bearing mice to that of normal T cells caused a marked reduction of CD3-ζ (data not shown). Whatever the mechanism is, the preferential decrease of CD3-ζ and $p56^{lck}$ may be an artifact due to the procedure employed for detecting TCR chains and $p56^{lck}$ in the presence of Mac-1^+ cells in immunoblotting analysis.

We observed a marked increase of Mac-1^+ cells in our tumor-bearing mice (Fig. 2A). Although we did not characterize the nature of the Mac-1^+ cells in this study, there are many reports showing an increase of Mac-1^+ macrophages in the tumor-bearing state.[35–37] Tumor-derived cytokines seem to be responsible for the increase of macrophages. These macrophages may mediate down-regulation of the host immune responses by the production of several factors such as prostaglandin E_2. In this regard, we should evaluate the role of macrophages in immune defects in our tumor-bearing mice.

Recently, it has been reported that TCR-$\gamma\delta^+$ T cells in spleen and intestinal epithelial tissue express FcεRIγ as a subunit of TCR complex in lieu of CD3-ζ.[38–40] However, TCR-$\alpha\beta^+$ T cells that express FcεRIγ chain instead of CD3-ζ have not been reported so far, other than from tumor-bearing mice.[12] It is important to elucidate what factors in the tumor-bearing state are responsible for the change of usage from CD3-ζ to FcεRIγ in the TCR, since splenic T cells from tumor-bearing mice presumably expressed CD3-ζ before tumor cell growth. Our results, however, indicated that FcεRIγ detected in the T cell preparations from our tumor-bearing mice was due to contaminating Mac-1^+ cells (Fig. 2B). Thus, the existence of FcεRIγ in the TCR from freshly isolated TCR-$\alpha\beta^+$ T cells remains questionable, although its existence was clearly demonstrated in a certain T cell line.[29]

Normal structure and function of the TCR in T cells from our tumor-bearing mice are not necessarily at variance with our previous observation that $CD4^+$ T cell reactivity is decreased with the progress of the tumor-bearing state.[10, 11] It is now well established that stimulation of T cells with antigen requires a costimulatory signal in addition to a signal through the TCR,[41] whereas anti-TCR Abs are themselves mitogenic to T cells.[32] This qualitative difference between antigen stimulation and anti-TCR Ab stimulation may account for the failure to detect a functional impairment of T cells from tumor-bearing mice in this study. Nevertheless, our results are very informative in terms of immunotherapy for tumor-bearing hosts. If T cells from tumor-bearing hosts have a defective TCR complex in general, active immunotherapy with tumor antigens or gene therapy using T cells may not be effective.

We cannot conclude from this study that structural changes of the TCR in tumor-bearing hosts are universally attributable to the presence of Mac-1^+ cells, because we do not know whether Mac-1^+ are increased in mice bearing other tumors or in cancer patients. However, we do know from this study that the apparent loss of CD3-ζ chain can occur under some experimental conditions in T cells that contain intact TCR complexes. Further experiments are required to explain the mechanism of the T cell unresponsiveness in the tumor-bearing state, in addition to analyzing the structure of the TCR.

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