

Signals through T Cell Receptor- ζ Chain Alone Are Insufficient to Prime Resting T Lymphocytes

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Summary

Activation studies performed with transfected T cell hybridomas and tumors revealed that chimeric molecules containing the CD3 ϵ or ζ chain intracytoplasmic portions can induce the complete effector functions normally seen only when the complete T cell receptor (TCR)/CD3 complexes of T lymphocytes are triggered. Therefore, the ζ chain, with its three antigen recognition activation motives, is thought to connect the antigen-binding T α chains with the intracellular signaling machinery of the T cell. Here we demonstrate that the cytoplasmic portion of the TCR- ζ chain is not sufficient to activate resting T lymphocytes when cells from transgenic mice expressing a chimeric ζ receptor are used. However, after (in vivo and in vitro) activation through their endogenous TCR/CD3 complexes, the preactivated T lymphocytes could be triggered through the ζ chimera to the same extent as when they were activated through their endogenous TCR/CD3 complexes. They were able to proliferate and elicit cytotoxic functions when triggered through their ζ chimeras. These results suggest that the triggering requirements for effector functions seem to be different in resting than in activated T cells.

TCR is a complex multisubunit structure composed of at least six different protein chains (TCR- α , TCR- β , CD3 γ , CD3 δ , CD3 ϵ , and ζ or its alternative splice-product η). The TCR- α and - β chains are clonotypically distributed and recognize peptide-Ag presented on MHC molecules by APC or target cells (1), while the CD3 components γ , δ , ϵ (2), and ζ (η) (3) are invariant proteins present in every TCR/CD3 complex. They are noncovalently associated with each other and TCR- α / β heterodimers and are necessary for correct assembly, transport, and surface expression of the whole TCR/CD3 complex (4). Since the TCR- α and - β proteins have no or little intracytoplasmic domains, the invariant CD3 and ζ (η) proteins are thought to couple the Ag-recognizing TCR- α / β proteins to an intracellular signaling machinery (5). Chimeric receptors, in which cytoplasmic domains of the ζ , η , CD3 ϵ , and Fc ϵ RI γ chains were fused to other extracellular domains (6–9), further supported the signal-transducing functions of these proteins. The chimeric receptors could be expressed in T cell hybridomas and tumors independently of TCR, and, upon cross-linking early and late signal-transducing events comparable to those observed after triggering of the whole multicomponent TCR/CD3 complexes, could be observed.

A conserved sequence motif in the cytoplasmic domains of ζ and η is required for signal transduction (6, 9–13). This Ag recognition activation motif (ARAM)¹ is built up of

paired tyrosines and leucines (Y-XX-L-X₇₋₈-Y-XXL) (10). Within the group of ARAM-carrying proteins (CD3 γ , δ , ϵ ; β subunit of the high affinity IgE receptor, ζ , η , Fc ϵ RI γ), the ζ and η chains are unique, because they contain multiple ARAMs (three or two, respectively). Due to this multimerization of ARAMs, as well as their functional superiority in chimeric receptor studies, it was thought that signals through ζ / η chains alone were sufficient to trigger T cells, while other ARAM-containing CD3 chains could then only enhance this central signal. However, since these conclusions were drawn from studies which were carried out with T cell hybridomas or tumors representing potentially fully differentiated cells, we wanted to investigate whether the ζ chain had similar properties in normal resting peripheral T lymphocytes. We, therefore, created transgenic mice that expressed a chimeric single chain TCR consisting of the intracellular and transmembrane portion of the ζ chain and an extracellular domain formed by a CD8 α hinge region and a single chain Ab (Fv) portion derived from an mAb specific for human (h) CD3 ϵ . Confirming the earlier studies using lymphoma systems, we previously demonstrated that this chimeric molecule can recognize Ag and subsequently transduce signals into transfected T cell lymphomas and hybridomas that lead to IL-2 and IL-3 production, indistinguishable from IL production induced by normal TCR–MHC interaction (14).

We demonstrate in this report that, in contrast to the hybridoma/lymphoma systems used in previous studies, the function of this protein is absolutely dependent upon the activation status of the T cell. The chimeric ζ chain cannot substitute for the complete TCR/CD3 complex in nor-

¹ Abbreviations used in this paper: ARAM, Ag recognition activation motives; h, human.

mal resting T cells, since they cannot be activated through the ζ chimera. In contrast, a triggering through the ζ chimera induces proliferation and effector functions only in previously activated T cells. Our results suggest that individual ARAM-containing chains in the TCR/CD3 complex may have qualitatively different functions, which can control the T cell triggerability in different physiological situations.

Materials and Methods

DNA Constructs and Microinjection. The plasmid pHbAPr-1-neo-FvCD3 ζ , used for the protoplast fusions, was previously described (14). To express the same FvCD3 ζ protein in transgenic mice, we cloned the 1.6-kb XhoI-HindIII(blunt) DNA fragment from pH β APr-1-neo-FvCD3 ζ into the XhoI-NdeI(blunt)-opened pBlue-script KS, which already contained a 1.1-kb HindIII-SalI polyA fragment from the plasmid pBG312 (16). Subsequently, we PCR-amplified a 600-bp fragment containing the TCR- α promoter region from pV $_{\alpha}$ C1 $_{\alpha}$ EnhH (K. Karjalainen, unpublished results) with the oligonucleotides 7698 and 7573. This PCR fragment was cut XhoI and SalI in the oligonucleotide regions and cloned into the XhoI-opened intermediate construction already containing the FvCD3 ζ portion and the polyA region. In front of this TCR α promoter region, we then cloned a 5.5-kb NotI-XbaI fragment from vector GSE1515 (16) containing the human CD2 3'UT region. After digestion with NotI and ApaI, the transgene DNA was separated from the vector sequences by electrophoresis, extracted from agarose gel slices, and resuspended in Tris-EDTA buffer (10 mM Tris, pH 7.4; 0.1 mM EDTA). Fertilized oocytes were obtained from BDF1 \times BDF1 mice, and DNA injection was performed as previously described (17).

To transfect the mouse EL4 thymoma with the cDNA encoding for the human CD3 ϵ chain, we PCR-amplified, with oligonucleotides 8003 and 8013, a 1-kb fragment from plasmid pDJ4 (18), containing the human CD3 ϵ -cDNA and digested in the oligonucleotide regions with HindI and SalI, and cloned it into the HindI-SalI-opened vector pH β APR-1-neo.

The sequences of the used oligonucleotide primers are: 5'ACTTAGTCGACTTCTGAGCCACTGGA3' (7698); 5'ATCTAGTCGACATGTCAGTCGGGCACTCAC3'(7573); 5'GTAACAGTCGAC-TGAAACAAAGATGCAGTCGGGC3'(8003); 5'TAGCTAAGC-TTCAGGGCCCTGTGCAACATTACC3' (8013) (see reference 18).

Cell Lines, mAbs, and Reagents. 14.3d, a mouse Th hybridoma (derived from the T cell clone V $_{2-15}$) (19), Jurkat, a human leukemic T cell line, and EL4, a mouse lymphoma (ATCC, TIB39), were maintained in IMDM, supplemented with 10% FCS, penicillin, streptomycin, and 2-mercaptoethanol. Chimera-transfected 14.3d clones were passaged in the above medium with the addition of Geneticin (Gibco Laboratories, Grand Island, NY) at 2 mg/ml and hCD3 ϵ -transfected EL4-clones at 0.7 mg/ml Geneticin for 14 d. The protoplast fusion was performed as previously described (14), and clones were obtained by limiting dilution and screened for protein expression by flow cytometry (see below).

The mAbs used in this study were anti-FvCD3 A1.3, a rat anti-mouse mAb (14), 2C11, a hamster anti-mouse CD3 ϵ mAb (20), a goat anti-rat Ig serum from Southern Biotechnology Associates (Birmingham, AL). The mAbs specific for CD4 (No. 09005) and L-selectin (Mel-14, No. 01264) were purchased from Pharmingen (San Diego, CA).

Flow Cytometry and Cell Sorting. Expression of cell surface proteins was assayed by indirect immunofluorescence. Cell suspensions of 1×10^5 viable cells were stained with 20 μ g/ml mAb that was

either direct labeled or biotinylated. After washing and the optional addition of the second step reagent, streptavidin-phycoerythrin (Becton Dickinson & Co., Mountain View, CA) cells were fixed in 1% paraformaldehyde before cytofluorometric analysis, using a FACScan[®] (Becton Dickinson & Co.).

Optionally, the stained cells were sorted under sterile conditions into pure FCS on a cell sorter (FACS[®] Vantage; Becton Dickinson & Co.).

Flow Cytometric Determination of [Ca²⁺]_i. 0.5×10^5 T lymphocytes were loaded with 5 μ M Indo-1-AM (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C in 5% FCS Hepes-buffered RPMI medium, washed once, and analyzed in the presence of different mAbs or sera on a cytofluorimeter (Elite Flow; Coulter Corp., Hialeah, FL) to detect Ca²⁺ fluxes. Only live (based on forward and side scatter criteria) and loaded cells (based on violet, 405 nm Ca²⁺-bound Indo-1, and blue, 525 nm free Indo-1) were included in the analysis.

Stimulation of T Lymphocytes. In each assay, 5×10^4 14.3d T hybridoma cells, or its transfectants, were stimulated by different amounts of human T cells, which were fixed before the experiment by a 30-s treatment with 0.05% glutaraldehyde. After coculturing in 200 μ l medium for 20 h, 50 μ l supernatant was collected and tested for its interleukin content, using the IL-2-dependent cell line cytotoxic T lymphocyte line and the IL-3-dependent cell line DA-1, as previously described (14).

When T lymphocytes from spleen or lymphnodes were used, we depleted the cell suspensions from B lymphocytes by incubating them with sheep anti-rat IgG (Dynabeads, catalogue number 110.08; DYNAL, Inc., Great Neck, NY) that were coupled before the experiment to the mAb 14.8 (21), recognizing the B cell Ag B220. The purity of T lymphocytes reached by this procedure was >95%.

These T cells were usually incubated on Ab-coated 96-well plates for 20 h at 2×10^4 cells/well before 1 μ Ci of [³H]thymidine was added for another 12-h period. Incorporation of radioactivity was measured by harvesting the cells and measuring their radioactivity content on a Betaplate system (1205; Wallacoy, Turku, Finland).

When T lymphocytes were prestimulated on 5 μ g/ml mAb 2C11, we incubated them for 2–3 d in 75-ml flasks (Costar Corp., Cambridge, MA), to which this mAb had been coated before. In the case where EL4-hCD3 ϵ were used to prime the T cells, we irradiated these stimulator cells with 25,000 rad and added them in a 1:1 ratio to the culture.

Cytotoxicity Assays. The 4-h ⁵¹Cr (Amersham International, Little Chalfont, UK)-release assay was performed as previously described (22).

Results and Discussion

The Level of ζ Chimera Cell Surface Expression Does Not Correlate with Function in T Cell Hybridomas. To find out which level of cell surface expression of the ζ chimera was necessary to induce effector functions, we transfect the mouse helper T cell hybridoma 14.3d with the previously described construct pH β APr-1-neo-FvCD3 ζ (14) that encodes for a chimeric TCR. This molecule consists of the cytoplasmic and transmembrane regions of the ζ chain, a 44-amino acid hinge region derived from CD8 α and a single chain Ab portion (FvCD3), which can recognize the native human CD3 ϵ protein on the surface of human T cells. Two clones representing both extremes of surface expression are described in Table 1. While 14.3d transfectant 1 shows only very low cell sur-

face expression of the ζ chimera, 14.3d transfectant 2 is a high expressor of the same protein as revealed by FACS[®] analysis with anti-Fv mAb. When both cells were stimulated with Ag (=hCD3)-expressing Jurkat cells, which were fixed before the assay, they nevertheless behaved similarly and could produce the same amounts of IL-3 (and IL-2; data not shown), while the untransfected 14.3d did not produce any interleukins (Table 1). All these hybridomas produced similar amounts of IL-2 and IL-3 and in the same range as after ζ chimera stimulation when they were triggered through their endogenous TCR, specific for hemagglutinin peptide complexed with I-E^d MHC class II molecules on APCs (data not shown).

These results clearly demonstrate that minimal amounts of the ζ chimera are sufficient to induce effector functions in T hybridomas in an Ag-dependent way and that overexpression of this signaling molecule does not enhance its stimulatory effects. To investigate if this TCR-like function of the ζ chain described in hybridomas could also be induced in resting T cells, we created transgenic mice that would express this FvCD3 ζ chain on normal T lymphocytes.

Expression of the ζ Chimera in Transgenic Mice. The transgene constructed for these experiments codes for the same single chain TCR (ζ chimera) as previously described (14), but now under the control of the hCD2 3'-flanking sequences and TCR- α promoter (Fig. 1). The hCD2 3'-flanking sequences have previously been described to direct high-level, position-independent, and T lineage-specific expression in transgenic mice (16). A total of three transgenic founder lines were obtained with similar expression patterns when the expression of the transgene was monitored with the mAb A1.3 (14), which recognizes the single chain Fv portion of the chimeric FvCD3 ζ receptor. This mAb stains T lymphocytes in

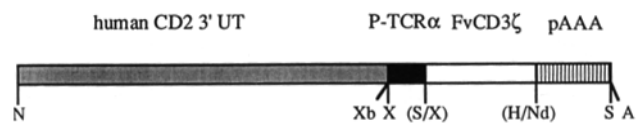


Figure 1. Restriction map of the transgenic construct FvCD3 ζ . FvCD3 ζ -cDNA (white box) was placed under the control of human CD2 3' UT region (grey box) and the mouse TCR- α promoter (black box). The poly A region is depicted as a striped box. A, ApaI; H, HindIII; N, NotI; Nd, NdeI; X, XhoI; Xb, XbaI.

whole blood, lymphnode, or splenocyte cell suspensions of transgenic mice only. Fig. 2 shows a typical FACS[®] staining performed on B220-depleted splenocytes. The lower staining shows that transgenic, as well as nontransgenic cells, display similar CD3 expression levels when analyzed with the mAb 2C11. When the staining was performed with the anti-Fv mAb A1.3, only transgenic cells could be labeled, while cells isolated from nontransgenic littermates remained negative (top). Further FACS[®] analysis with anti-CD4 and anti-CD8 mAbs showed that equal levels of FvCD3 ζ protein are expressed on both CD4⁺ and CD8⁺ lymphocyte populations (data not shown). FvCD3 ζ expression level is also comparable to the average T cell hybridoma transfectant described earlier.

Furthermore, the ζ chimera seemed not to disturb T cell development, since normal ratios of T lymphocytes could be found in the periphery and in the thymus (data not shown).

Triggering of the ζ Chimera Does Not Activate Resting T Lymphocytes. To assess the function of our chimeric FvCD3 ζ chain in resting lymphocytes, we incubated T lymphocytes (B220-depleted splenocytes, >95% pure) with different concentrations of plastic-coupled mAb 2C11 directed against the mouse CD3 ϵ molecule of the TCR/CD3 complex or the mAb A1.3 that recognizes specifically the Fv portion of the chimeric FvCD3 ζ protein.

As expected, the mAb 2C11 induces a proliferative response in an Ab dose-dependent manner (Fig. 3). The [³H]thymidine uptake shows a plateau at ~5 μ g/ml mAb, at which it starts to decrease. In contrast, when incubated with the anti-Fv mAb A1.3, there is no significant ³H incorporation, even at the highest Ab concentration.

Furthermore, it was impossible to stimulate the resting T lymphocytes through their FvCD3 ζ protein, even when the anti-Fv mAb was used in combination with an anti-CD4 mAb. While the CD4-specific mAb showed a clear costimulatory effect and synergized with low levels of the anti-CD3 mAb, it did not show any effect when used with the mAb A1.3 (Fig. 3). IL-2 or other mAbs which are thought to supply an additional costimulatory signal, such as mAbs directed against the coreceptors CD4, CD8, CD28, and CD45, behaved the same way and could not elicit any costimulatory effect when used together with the mAb A1.3 (data not shown).

We then monitored intracellular [Ca²⁺] levels of the resting lymphocytes during a trigger of the FvCD3 ζ molecule on their surface and were not able to detect an increase of [Ca²⁺] (Fig. 4, left). Even the addition of a cross-linking polyclonal

Table 1. IL-3 Production of the Hybridoma 14.3d and Two of Its Transfectants during Stimulation with Different Amounts of Fixed Jurkat Cells

Cells	Mean fluorescence anti-Fv	IL-3 [U/ml] production after stimulation with Jurkat cells ($\times 10^4$ Jurkat)			
		3	1	0.3	0.1
14.3d	3.6	0	0	0	0
14.3d I	9.5	11.5	7.5	3.9	1.8
14.3d II	160.2	13.8	7.4	3.6	2.1

Relation between surface expression level and function of the FvCD3 ζ -chimera in the T hybridoma 14.3d transfectants. Cells were incubated first with the biotinylated Fv-specific mAb A1.3 and then stained with streptavidin PE. Staining intensity, as measured on a FACScan[®], is expressed as mean fluorescence intensity. For IL-3 production, 5×10^4 cells were stimulated with different amounts of glutaraldehyde-fixed Jurkat cells, and the IL-3 in the culture supernatants were then determined in a bioassay.

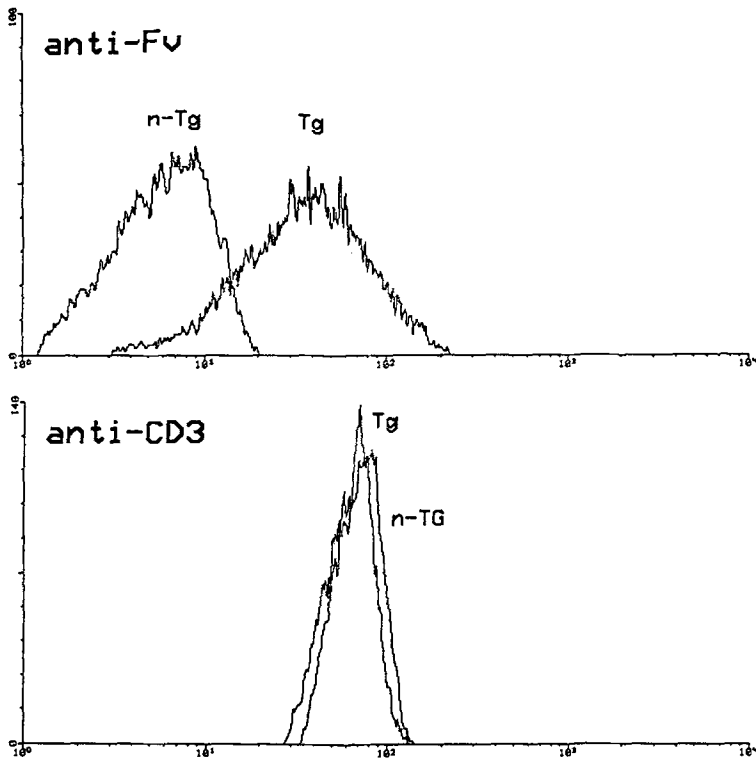


Figure 2. Expression of the transgene leads to detectable FvCD3 ζ protein cell surface expression. Splenocytes from a transgenic mouse were B cell-depleted, stained, and analyzed by two-color flow cytometry. Single-color histograms were obtained by gating on the CD3 $^{+}$ population in two-color stainings, using 2C11-FITC and A1.3 biotin plus streptavidin PE. The top panel shows the FvCD3 ζ expression level, while the lower panel compares TCR/CD3 expression of transgenic vs. nontransgenic lymphocytes.

goat anti-rat serum did not induce Ca^{2+} flux. The control experiment (Fig. 4, right) shows that these resting lymphocytes are very well able to flux Ca^{2+} when triggered through their TCR/CD3 complexes by the mAb 2C11, even without additional cross-linking. These data indicate that, unlike the full TCR/CD3 complex, the ζ chain alone is not sufficient

to transduce an activating signal into resting lymphocytes, as monitored by [3H]thymidine uptake and [Ca^{2+}] $_i$ measurement. Eventually, other components of the TCR/CD3 complex have to get involved to make the resting lymphocytes progress in the cell cycle and start to proliferate. The fact that not even early events, such as increases in [Ca^{2+}] $_i$, could be detected indicates that the ζ chain intracytoplasmic domain alone seems to be incapable of effectively signaling in resting T cells.

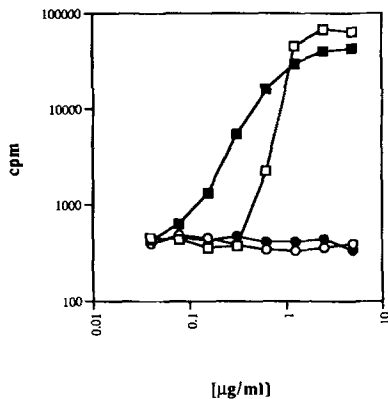


Figure 3. Triggering of FvCD3 ζ protein does not induce a proliferative response in resting T lymphocytes. B cell-depleted splenocytes were cultured for 20 h at 2×10^4 cells/well. [3H]thymidine was then added and the radioactive uptake measured after another 12 h. Cultures were performed in 96-well plates previously coated with either mAb 2C11 (open squares) or mAb A1.3 (open circles) at the indicated concentrations, or mAb 2C11 + mAb GK1.5 (filled squares) or mAb A1.3 + mAb GK1.5 (filled circles). The anti-CD4 mAb GK1.5 was used at 10 μ g/ml for the costimulation assays.

ζ Chimera Induces Proliferation in Preactivated T Lymphocytes. Since the ζ intracellular portions of various chimeric receptors were able to induce various effector functions (8, 9, 11, 13, 14) in T cell hybridomas and tumors, but not in resting T lymphocytes we wanted to investigate if this discrepancy was related to the different activation states displayed by lymphomas, hybridomas, or lines used in previous studies compared to the resting lymphocytes we use in this report.

We, therefore, prestimulated resting transgenic and nontransgenic T lymphocytes via their TCR/CD3 complexes by the mAb 2C11 coupled to plastic. After 2 d, the blasting cells were removed from the Ab and maintained for another 24 h in IL-2. Then, we restimulated the T blasts with either mAb against TCR/CD3 complexes, or with the mAb A1.3 recognizing the Fv portion of the ζ chimera. Fig. 5 A shows the proliferative response of transgenic lymphocytes to the different stimuli. Surprisingly, the Fv-specific Ab is now able to induce proliferation of prestimulated transgenic lymphocytes to the same extent and in the same Ab concentration-dependent fashion as the CD3-specific mAb 2C11. The proliferation reaches a plateau between 1 and 10 μ g/ml Ab and

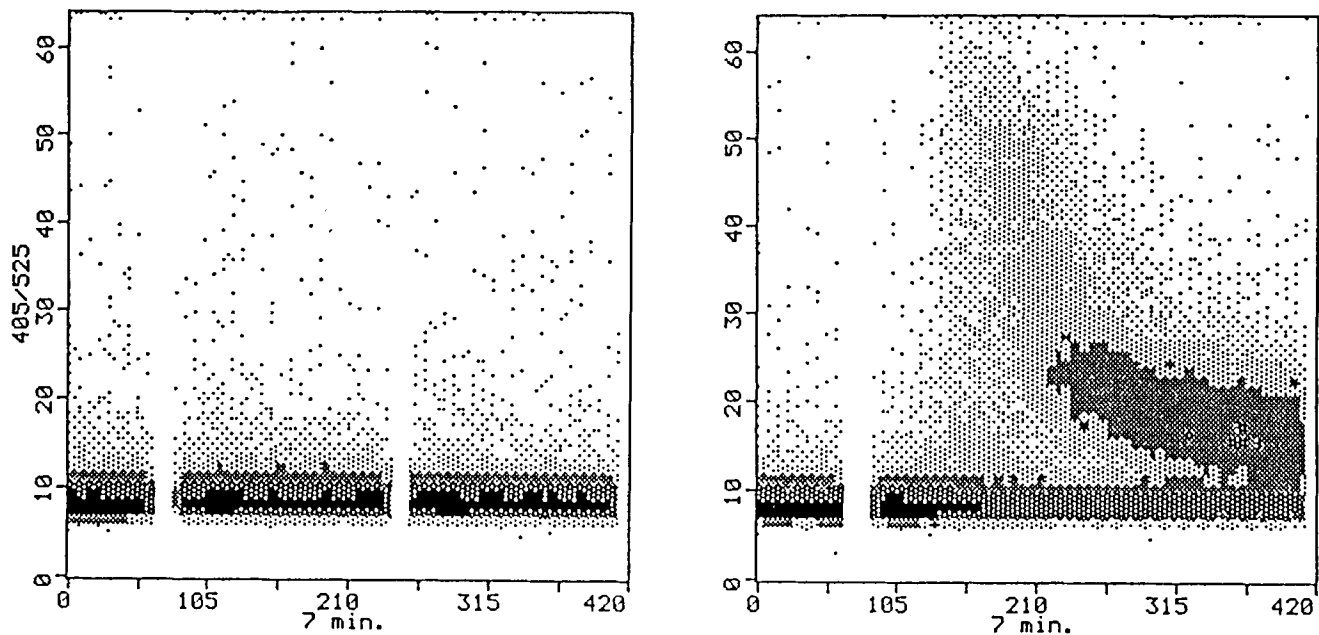


Figure 4. Cross-linking of FvCD3 ζ protein does not induce an increase of $[Ca^{2+}]_i$. Indo-1-loaded T lymphocytes were incubated with Fv-specific mAb A1.3 alone for 3 min and then cross-linked with a goat anti-rat Ig serum (*left*). In the control experiment (*right*), the cells were incubated with the mAb 2C11, without cross-linking.

matches the kinetics of proliferation induced by a TCR/CD3 trigger. In addition, the anti-Fv-induced proliferation (as well as the TCR/CD3-induced proliferation) can be elevated by the addition of 5 $\mu\text{g}/\text{ml}$ CD28-specific mAb to the cultures, and, in this respect, is also similarly susceptible to costimulatory signals, such as the TCR/CD3-induced proliferation. Fv mAb A1.3 does not induce proliferation in nontransgenic lymphocytes, and, as expected, they only proliferate when mAb 2C11 is used (Fig. 5 B).

These unexpected findings indicate that either the induction of proliferation of resting cells needs qualitatively different signals from that of preactivated cells, or that ζ chimera is differentially wired to the signal-transducing machinery in preactivated cells, compared to resting cells.

ζ Chimera Mediates Cytotoxicity Only in Preactivated T Lymphocytes. To define more precisely the functional potentials of the FvCD3 ζ chimera, we performed killer assays with T lymphocytes isolated from B220-depleted transgenic or nontransgenic splenocytes. These lymphocytes were either primed with the potential target cells EL4-hCD3 ϵ , or activated by the mAb 2C11 coupled to plastic for 3 d and then tested in the cytotoxicity assay on the two different ^{51}Cr -labeled target cells, EL4 and EL4-hCD3 ϵ , respectively. The results in Fig. 6 show that exposure to the later target cells, EL4-hCD3 ϵ (C and D) or EL4 (data not shown), is not sufficient to prime transgenic (as well as nontransgenic) T lymphocytes for specific and significant target cell lysis. Since the level of expression of hCD3 ϵ on the EL4 transfectant, EL4-hCD3 ϵ , was very

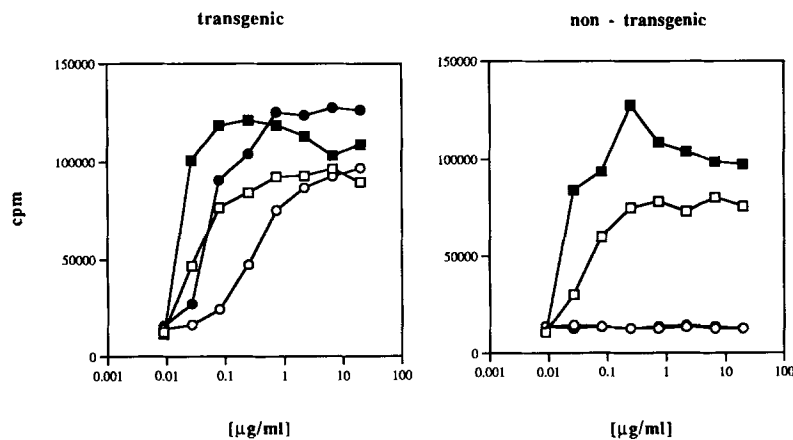


Figure 5. Cross-linking of the FvCD3 ζ chain does induce a proliferative response in previously activated T lymphocytes. B cell-depleted splenocytes from transgenic (*left*) or nontransgenic (*right*) littermates were preactivated for 48 h on 2C11 (5 $\mu\text{g}/\text{ml}$), removed from this mAb, and kept in IL-2-containing medium for 24 h. Subsequently, the T blasts were restimulated with different amounts of 2C11 (*squares*) or A1.3 (*circles*) coupled to plastic plates. These cultures were performed either in the presence (*filled symbols*) or absence (*open symbols*) of 5 $\mu\text{g}/\text{ml}$ anti-CD28-specific mAb.

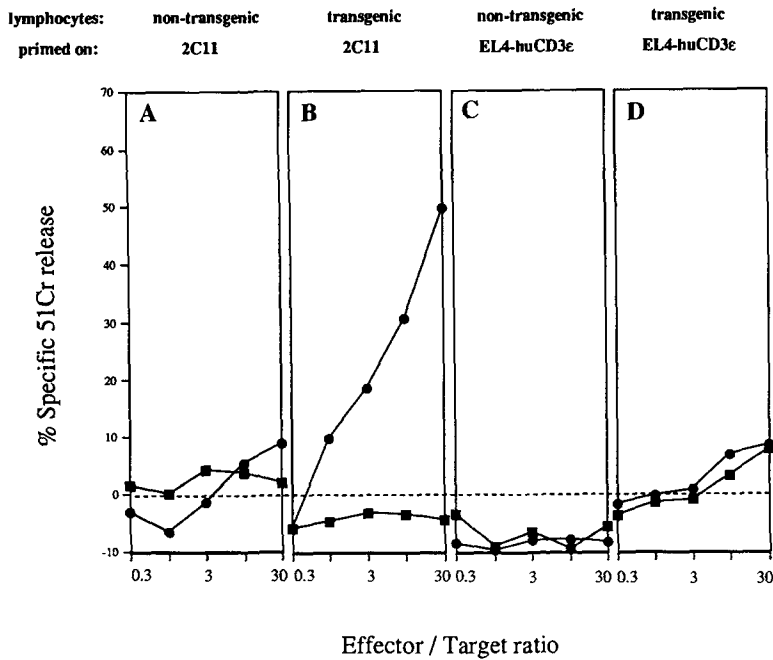


Figure 6. In fully activated T lymphocytes, the FvCD3 ζ chimera is able to induce antigen-specific cytotoxicity. B cell-depleted splenocytes from transgenic (B and D) or non-transgenic (A and C) littermates were primed either by the later targets EL4-hCD3 ϵ (C and D), or activated by the mAb 2C11 (A and B). The specific ^{51}Cr release was measured after a 4-h incubation period on either EL4 (squares) or EL4-hCD3 ϵ (circles).

high (data not shown), we expected that these cells could optimally cross-link the FvCD3 ζ molecules, and, therefore, induce priming of the transgenic lymphocytes. However, as above, no priming could be detected through the ζ chimera. In contrast, when transgenic T lymphocytes were preactivated through their TCR/CD3 complexes by the mAb 2C11, they lysed specifically the EL4-hCD3 ϵ targets, which were recognized by the Fv portion of the FvCD3 ζ molecule (Fig. 6 B). Targets (EL4) which didn't express this surface antigen (hCD3 ϵ) were not lysed. As expected, 2C11-activated non-transgenic T lymphocytes were not able to lyse either target (Fig. 6 A). Thus again, triggering of the FvCD3 ζ chain was

not sufficient for resting T lymphocytes to be primed (preactivated) and to reach their full effector functions, as compared to a prestimulation induced by a trigger of the complete TCR/CD3 complexes.

In Vivo-activated Cells Can Be Directly Triggered through ζ Chimera. Mel-14 (L-selectin) is a marker which has previously been reported to distinguish recently activated (negative) T cells from quiescent (positive) T cells (23). To analyze whether functionality of the ζ chain cytoplasmic portion was limited to an in vitro prestimulus through TCR/CD3 by mAbs, or if in vivo-activated T cells would display similar features, we sorted T lymphocytes from transgenic mice after a staining with mAbs against CD4 vs. Mel-14 and separated the Mel-14 $^{+}$ from the Mel-14 $^{-}$ population. With this procedure, we could enrich T lymphocytes responding to a stimulus through their FvCD3 ζ molecule, as shown in Table 2. While the Mel-14 $^{+}$ T cell population remained unresponsive to a trigger of their ζ chimera (upper row, 1.2%), the Mel-14 $^{-}$ fraction of the T lymphocytes responded to a significantly higher extent (lower row, 27.1%), as compared to a stimulus through their TCR/CD3 complex, which was taken as 100% in each of these two cell populations.

These data also indicate that in vivo-activated (Mel-14 $^{-}$) T lymphocytes are able to respond to a trigger through their ζ chimera. This reflects the in vitro findings described in the previous sections: Since the preactivation does not increase the level of surface expression of the ζ chimera (data not shown), one possible interpretation of the described phenomena could be that the ζ intracytoplasmic portion is differentially connected to the signal-transducing machinery in activated cells, as compared to resting lymphocytes. Our transgenic mice offer us the opportunity to reveal eventual signaling differences between resting and activated lymphocytes on the biochemical level.

Table 2. [^3H]thymidine Uptake in Counts per Minute of CD4 $^{+}$, Mel-14 $^{+}$ and CD4 $^{+}$, Mel-14 $^{-}$ T Lymphocytes after Stimulation with Anti-CD3 or Anti-Fv-Specific mAb

Cells	Anti-CD3 (10 $\mu\text{g}/\text{ml}$)	Anti-Fv (10 $\mu\text{g}/\text{ml}$)
Mel-14 $^{+}$	27,520 (100%)	340 (1.2%)
Mel-14 $^{-}$	14,158 (100%)	4,012 (27.1%)

Naturally activated Mel-14 $^{-}$ T lymphocytes can respond to a trigger through the FvCD3 ζ chimera. Splenocytes from transgenic mice were B cell depleted, stained with mAbs specific for CD4 and Mel-14 (L-selectin), and then sorted on a (FACS $^{\text{®}}$ Vantage, Becton Dickinson & Co.) cell sorter. The two sorted populations (CD4 $^{+}$, Mel-14 $^{+}$ and CD4 $^{+}$, Mel-14 $^{-}$) were >99% pure and plated at 2×10^4 cells/well in a 96-well plate coated with either the mAb 2C11 (anti-CD3) or A1.3 (anti-Fv) at 10 $\mu\text{g}/\text{ml}$. Proliferation was measured as [^3H]thymidine uptake after 20 h. The results shown are counts per minute mean values of triplicates with a standard deviation of <3%. The average background proliferation of both populations was <250 cpm.

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