The Single Positive T Cells Found in CD3-$\zeta/\eta^{-/-}$ Mice Overtly React with Self–Major Histocompatibility Complex Molecules upon Restoration of Normal Surface Density of T Cell Receptor–CD3 Complex

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Summary

CD3-ζ/η–deficient mice have small thymuses containing cells that show a profound reduction in the surface levels of T cell receptors and terminate their differentiation at the $CD4^+CD8^+$ stage. Rather unexpectedly, $CD3^{-\text{ or very low}}$ single positive T cells accumulate over time in the spleen and lymph nodes of CD3-ζ/η–deficient mice after a process dependent on MHC expression. Fusion of these peripheral T cells with a CD3-ζ–positive derivative of the BW5147 TCR-α^-/β^- thymoma resulted in hybridomas that do express an heterogeneous set of T cell receptor α/β dimers at their surface and at density comparable to those found in hybridomas derived from wild-type peripheral T cells. We have investigated the specificities of these T cell receptors using spleen cells from congenic and mutant mouse strains, and showed that the majority of them readily recognized self-MHC class I or class II molecules. These results demonstrate that by increasing the density and/or output of the T cell receptors expressed in peripheral T cells, one can confer them with the capacity to respond to normal density of self-MHC molecules.

Most peripheral T cells express clonally variable antigen receptors (TCRs) made up of α and β chains and specific for peptide antigens bound to class I and II products of the MHC. Peripheral T cells derive from the thymus where their fate is determined on the basis of the specificity of the TCR-α/β heterodimers they express. For instance, $CD4^+CD8^+$ thymocytes appear to be rescued from programmed cell death and induced to differentiate into single positive cells only if their TCRs bind with low affinity to the self peptide/MHC complexes expressed on thymic stromal cells. Remaining $CD4^+CD8^+$ thymocytes with TCRs that do not react, or react with too high an affinity with self peptide/MHC complexes, either die by apoptosis or are censored by a variety of nondeletional mechanisms (for review see reference 1). This thymic selection process, often denoted as TCR-α/β–dependent selection, contributes to purge the periphery from autoreactive T cells and populate it with only those T cells capable of binding self-MHC products complexed to nonself peptides. The above findings have been incorporated into a "signal strength" model of TCR-α/β selection asserting that the signaling cassettes operated by the TCR and linked to positive and negative selection have different activation thresholds, the cassette for positive selection having a lower threshold of activation than that for negative selection (2–4). Furthermore, considering that most self peptide/MHC complexes are ubiquitously expressed by cells in the thymus and periphery (5), the inability of mature T cells to react detectably upon reencountering their corresponding selecting self peptide/MHC ligands has been generally accounted for by the fact that they have higher activation threshold and/or lower TCR-signaling output than immature $CD4^+CD8^+$ thymocytes (6).

Analysis of mice deprived of TCR-α polypeptide, ZAP-70 protein tyrosine kinase, as well as MHC class I and II products further supported the view that signaling via fully assembled TCR/CD3 complexes is required for TCR-α/β–dependent selection and the subsequent accumulation of single positive T cells in the periphery (for review see reference 7). We (8) and others (9, 10) have recently characterized mice lacking both the CD3-ζ and -η polypeptides. CD3-ζ and -η originate from a single gene (CD3-ζ/η) by way of alternative splicing, and contribute to the efficient transport of the TCR/CD3 complex to the cell surface and to its signaling function via their immunoreceptor tyrosine-based activation motifs (11–13). Studies using cell lines have shown that in the absence of CD3-ζ and -η, very low levels of subcomplexes made of TCR-α/β, CD3-δ/ϵ, and -γ/ϵ dimers can leak to the cell surface (14). After stimulation with high density of immobilized anti–CD3-ϵ mAbs, or

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high concentrations of antigen or superantigen (13, 15), these subcomplexes can still minimally signal via their CD3-δ/ε and -γ/ε autonomous transduction modules (13). CD3-ζ/η$^{-/-}$ mutant mice have small thymuses (2.4×10^7 cells on average) containing cells that show a profound reduction in the surface levels of TCR and are arrested at the CD4$^+$ CD8$^+$ stage of development. The CD3-ζ/η$^{-/-}$ mutation had no effect on the rearrangements of the TCR-β genes. In contrast, CD3-ζ/η$^{-/-}$ thymocytes showed low levels of TCR-α rearrangements and transcripts, providing a potential for the synthesis of only limited amounts of TCR-α polypeptides (7, 10). Complementation of CD3-ζ/η$^{-/-}$ mice with several matched pairs of TCR-α and -β transgenes does not lead to further maturation into CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single positive cells (Ardouin, L., A. Gillet, and M. Malissen, unpublished results). Therefore, the developmental blockade observed in CD3-ζ/η–deficient thymocytes does not primarily result from their limited content of rearranged TCR-α gene segments, but rather from the fact that the low density at which they express TCR-α/β / CD3-δ/ε / CD3-γ/ε subcomplexes at the cell surface prevents most of them from participating in TCR-α/β selection. Despite the almost complete absence of single positive thymocytes noted in CD3-ζ/η$^{-/-}$ mice, the spleen and lymph nodes of 3-mo-old CD3-ζ/η$^{-/-}$ mice contained a normal ratio of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells expressing barely detectable levels of TCR/CD3 complexes (8, 10). It is therefore possible that in CD3-ζ/η$^{-/-}$ mice, TCR/CD3 subcomplexes deprived of CD3-ζ/η dimers can be expressed at the cell surface in amounts not easily detectable by flow cytometry, but sufficient to drive MHC-dependent intrathymic (1) or extrathymic (16) selection events. Consistent with the view that the peripheral T cells found in CD3-ζ/η$^{-/-}$ mice have not survived by chance in a process independent of selection by MHC molecules, peripheral CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells were greatly diminished in CD3-ζ/η$^{-/-}$ × MHC class II$^{-/-}$ and CD3-ζ/η$^{-/-}$ × MHC class I$^{-/-}$ double mutants, respectively (17, and our unpublished observations).

According to the signal strength model of TCR-α/β selection, any experiments aiming at increasing the density of self peptide/MHC complexes on antigen presenting cells (e.g., as a result of synthetic self-peptide loading) should trigger mature T cells previously selected on these ligands (18, 19). Similarly, boosting the signaling capacity and/or density of the TCR expressed by mature T cells should confer them with the capacity to react to normal density of self peptide/MHC complexes. Assuming that the single positive T cells found in the periphery of CD3-ζ/η$^{-/-}$ mice have been selected to survive via a process relying on TCR expression and in a peptide/MHC-dependent manner (see above), complementation of these cells with a CD3-ζ gene should dramatically increase the surface density and signaling capacity of their TCR-α/β dimers and provide a unique opportunity to investigate whether they have consequently gained an antiself reactivity. We therefore analyzed the specificity of the single positive T cells found in the periphery of CD3-ζ/η–deficient mice after introduction of a CD3-ζ gene and restoration of normal surface density of TCR/CD3 complexes harboring a complete set of immunoreceptor tyrosine-based activation motifs. Our results showed that most of them are reactive to self-MHC class I and II molecules.

Materials and Methods

Mice. The CD3-ζ/η–deficient mice have been described (8, 16, 20). These mice, originally on a C57BL/6 (H-2^b) background, were crossed with the C57BL/6-H-2^k congenic strain to produce CD3-ζ/η–deficient mice that carry the H-2^k haplotype. The MHC class II–deficient mice (II^0) (21) and β2-microglobulin–deficient mice (β2m^0; 22) have been bred for at least eight generations on a C57BL/6 genetic background. Mice were housed in a specific pathogen-free animal facility.

Cells. BW5147 TCR-α$^-$β$^-$ (hereafter referred to as BW$^-$) is a variant of the BW5147 thymoma lacking CD3-δ, CD3-ζ/η, and FcεRIγ transcripts as well as functional TCR α and β chain genes (13, 23, 24, 25).

Purification of Single Positive T Cells from Spleen and Lymph Nodes. Lymph node and spleen cells from several 3-mo-old CD3-ζ/η$^{-/-}$ or CD3-ζ/η$^{+/+}$ mice were pooled, and the single positive cells purified using a high gradient magnetic cell separation system (26). In brief, cells were first labeled with saturating levels of biotinylated antibodies against CD4 and CD8. After washing off the unbound mAbs, cells were stained with a fluorescein–streptavidin conjugate (Immunotech, Marseille, France). Finally, biotinylated superparamagnetic microparticles (Miltenyi Biotec., Sunnyvale, CA) were attached to the remaining free sites of the streptavidin conjugate bound to the cell surface, and the magnetic cell fractions isolated on a high gradient magnetic cell separation system separator. In our experience, this protocol leads to cell suspensions containing >96% labeled cells.

Preparation of T Cell Hybridomas. A CD3-ζ positive derivative of the BW$^-$ thymoma was constructed by protoplast fusion using plasmid pHβAPr-his–CD3-ζ (13). Histidinol-resistant clones were isolated and tested for the intracytoplasmic expression of CD3-ζ by Western blot analysis. A transfectant, hereafter denoted as BW$^-$ζ, and expressing levels of CD3-ζ polypeptide similar to the BWδζ transfectant (13), was selected and fused to purified peripheral single positive T cells (see above). After hypoxanthine/aminopterin/thymidine selection, the resulting T cell hybridomas were analyzed by staining for cell surface expression of CD3-ε. CD3-ε positive hybridomas were cloned and further characterized as described below.

Antibodies and Flow Cytometric Analysis. Before staining, spleen and lymph node cells were preincubated on ice for at least 10 min with polyclonal mouse and rat Ig to prevent FcR-mediated absorption of antibodies to cells. 2×10^5 cells were subsequently stained with saturating levels of antibodies for 30 min at 4°C. $2–10 \times 10^3$ gated events were acquired using a FACScan® flow cytometer and analyzed with Lysis II software. FITC-conjugated anti-CD4 antibody was purchased from CALTAG Labs. (South San Francisco, CA). Biotinylated FITC- or PE-conjugated antibodies against CD3, CD4, CD8, CD25, CD44, CD69, CD62, CD122, Fas, and NK1.1, were purchased from PharMingen (San Diego, CA). Biotinylated antibodies were revealed with streptavidin-tricolor (CALTAG Labs.). For flow cytometric analyses, T cell hybridomas were first incubated with saturating concentrations of mAb directed against CD3-ε (KT3), TCR-β (H57-597), TCR-δ (GL3), CD4 (RL172.4), CD8 (3.168.81), or the products of various Vα and Vβ gene segments, washed, and then stained

with saturating concentration of an F(ab′)2 fragment of a FITC-conjugated rabbit anti-mouse Ig (Cappel Labs., Malvern, PA).

Stimulation of T Cell Hybridomas. In brief, 0.25 ml microcultures were prepared containing 10^5 responding cells and 10^6 irradiated (20 Gy) splenocytes. For stimulation with the anti–CD3-ϵ antibody 2C11, the bottom of microtiter wells was precoated with 50 μl of a 10 μg/ml solution of antibody. Cultures were for 20 h, at which time culture supernatants were harvested and assayed for the level of IL-2 using the IL-2–dependent T cell line CTL-L. The amount of [^{3}H]thymidine incorporated into CTL-L cells was measured by direct β counting (Matrix 96 direct beta counter; Packard Instrs., Meriden, CT). Considering that the various hybridomas did not respond to the same extent to anti–CD3-ϵ stimulation, the levels of IL-2 production observed in response to irradiated splenocytes of H-2^b or H-2^k haplotypes were normalized for each hybridoma by using the following formula: ([^{3}H]thymidine incorporation in response to splenocytes of a given haplotype / [^{3}H]thymidine incorporation in response to immobilized anti–CD3-ϵ) × 100. The two percentages obtained for each hybridoma were plotted on a graph to reflect their respective abilities to respond to H-2^b or H-2^k splenocytes.

Results

Age-associated Accumulation of Peripheral Single Positive T Cells in CD3-ζ/η–deficient Mice. When analyzed by flow cytometry for the expression of CD4 and CD8 molecules, thymuses from 1- to 3-mo-old CD3-ζ/η$^{-/-}$ mice were found to contain dramatically fewer single positive cells than age-matched wild-type thymuses (Fig. 1). Moreover, most of these cells expressed residual levels of CD25 molecules and probably corresponded to "immature" single positive cells on the way to becoming double positive cells (20). Despite the near total absence of mature single positive thymocytes, Thy1$^+$-single positive T cells were found to accumulate over time in lymph nodes of CD3-ζ/η$^{-/-}$ mice (Fig. 1, *right*), but in smaller numbers than those from age-matched wild-type littermates (CD3-ζ/η$^{+/+}$, $n = 5$; average 13×10^6; CD3-ζ/η$^{-/-}$, $n = 7$, average 5.8×10^6). Similar to CD3-ζ/η$^{-/-}$ thymocytes, the single positive cells found in CD3-ζ/η$^{-/-}$ lymph nodes expressed levels of TCR/CD3 complexes barely detectable by flow cytometry (Fig. 2; 8–10, 17). When the lymph node T cell populations found in 3-mo-old wild-type and CD3-ζ/η$^{-/-}$ mice were compared for the expression of various cell surface markers, they both showed identical staining profiles for CD25, CD62, CD69, CD122, and Fas molecules as well as a NK1.1$^-$ phenotype (Fig. 2 and data not shown). The most striking difference was seen in the level of expression of CD44 which was highly expressed by all the single positive cells found in CD3-ζ/η$^{-/-}$ lymph nodes (Fig. 2). A corresponding population of Thy1$^+$, CD3$^-$ or very low single positive T cells was also present in the spleen of 3-mo-old CD3-ζ/η–deficient mice (data not shown). It should be noted that we have not been able to extend our analysis of CD3-ζ/η$^{-/-}$ over a longer time because mice die around 4 mo of age after developing a chronic intestinal inflammation, characterized by diarrhea and wasting, similar to that previously documented in cytokine or TCR mutant mice (27).

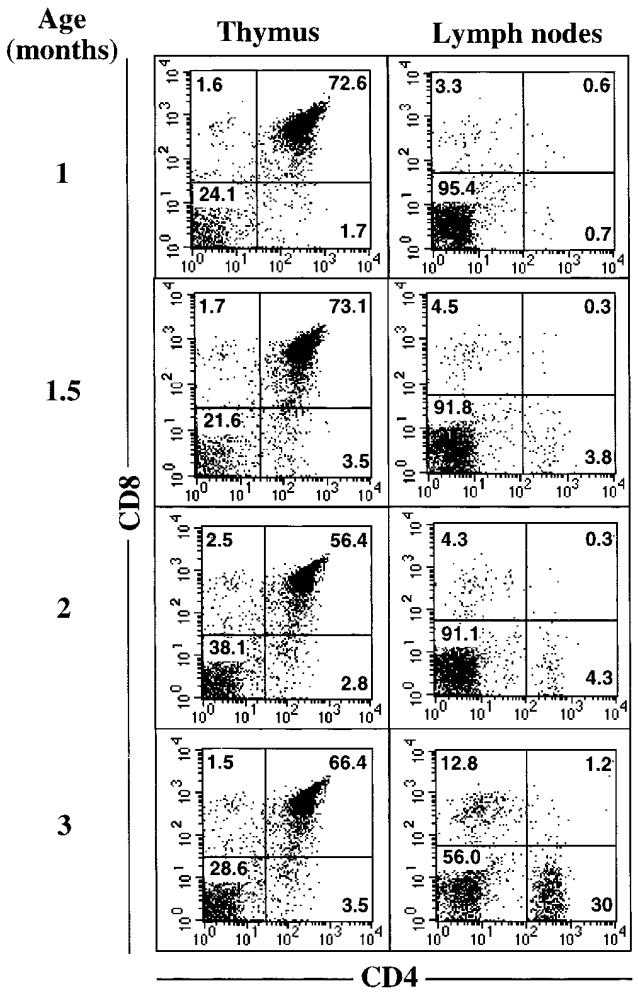

Figure 1. CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells accumulate over time in the lymph nodes of CD3-ζ/η–deficient mice. Cells from thymus and lymph nodes were isolated from 1- to 3-mo-old CD3-ζ/η–deficient mice and analyzed by two-color flow cytometry for the expression of CD4 versus CD8. The percentage of cells found in each quadrant is indicated. Most of the lymph node cells scoring as CD4$^-$CD8$^-$ were brightly stained with an anti-B220 antibody (RA3-6B2), and likely belong to the B cell lineage.

Normal Levels of TCR-αβ Heterodimer Can Be Rescued from CD3-ζ/η$^{-/-}$ Peripheral T Cells upon Transfer of a CD3-ζ Gene. To assess whether the single positive T cells accumulating at a low rate in the periphery of CD3-ζ/η$^{-/-}$ mice synthesized TCR-αβ heterodimers, we intended to fuse these cells with the BW$^-$ thymoma and convert them into T cell hybridomas. BW$^-$ is a variant of the BW5147 thymoma that lacks functional TCR-α and -β genes and has been extensively used to analyze the specificity of TCR expressed on heterogeneous populations of nontransformed T cells (e.g., references 28 and 29). Considering that BW$^-$ cells do not transcribe the CD3-ζ/η gene (13) and are, as such, unsuitable for experiments involving CD3-ζ/η–deficient T cells, we first transfected them with a plasmid directing the expression of CD3-ζ polypeptides. Because the CD3-η polypeptide does not appear endowed with unique signaling capacities relative to CD3-ζ (30, 31), we limited our effort to the construction of a derivative of BW$^-$, de-

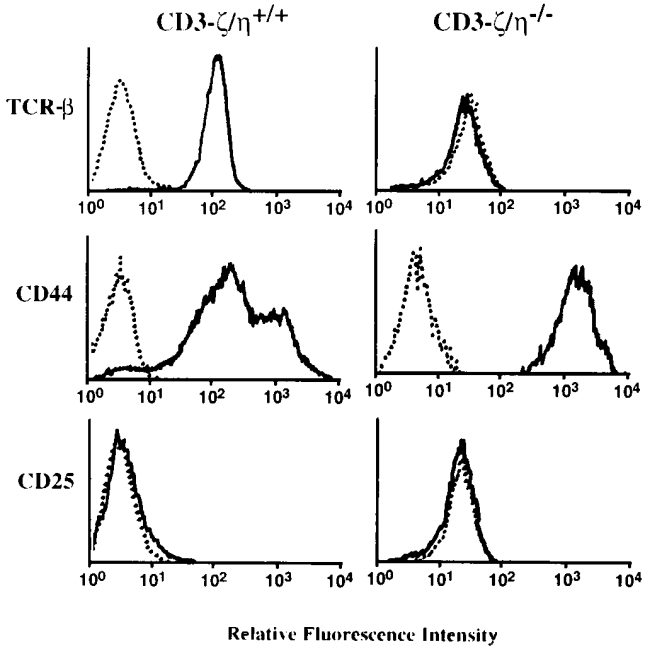

Figure 2. Most of the CD3$^-$ peripheral single positive T cells found in CD3-ζ/η–deficient mice display constitutively upregulated levels of CD44. Lymph node cells from 3-mo-old CD3-$\zeta/\eta^{-/-}$ and -$\zeta/\eta^{+/+}$ mice were analyzed by three-color flow cytometry for CD4 and CD8 expression in combination with various cell surface markers. Histograms show the expression of the markers indicated on the left on gated CD4$^+$CD8$^-$ cells from CD3-$\zeta/\eta^{-/-}$ and -$\zeta/\eta^{+/+}$ mice. Similar patterns were observed after gating on CD4$^-$CD8$^+$ cells. Each histogram is compared with a negative control histogram obtained after staining with isotype-matched negative control antibodies (*dotted lines*).

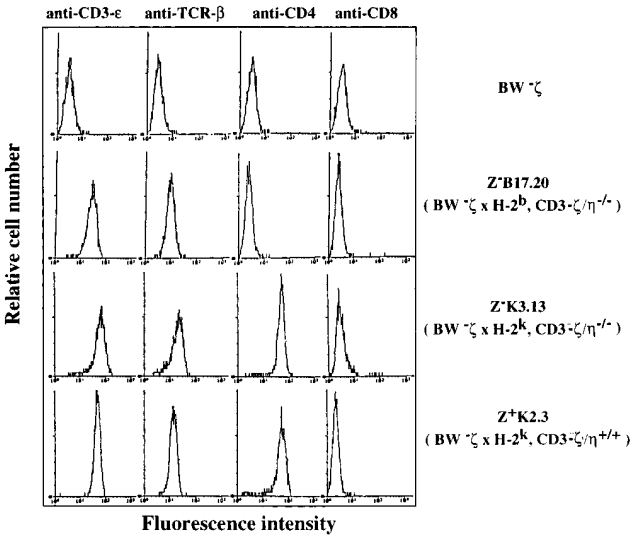

Figure 3. Fusion of CD3-$\zeta/\eta^{-/-}$ peripheral single positive T cells with BW$^-\zeta$, a CD3-ζ^+ derivative of the BW$^-$ thymoma, results in hybridomas expressing TCR-α/β complexes at their surface. BW$^-\zeta$ was fused to single positive T cells isolated from the spleen and lymph nodes of 3-mo-old CD3-$\zeta/\eta^{-/-}$ or -$\zeta/\eta^{+/+}$ mice. The BW$^-\zeta$ fusion partner and the resulting hybridomas were analyzed by flow cytometry after staining with the anti–CD3-ε antibody KT3, the anti–TCR-β antibody H57-597, the anti-CD4 antibody RL172.4, or the anti-CD8 antibody 3.168.81. Considering that BW$^-\zeta$ contributed *trans*-acting factors which inhibit the expression of the CD8-α gene in the resulting hybridomas (see text), the histograms obtained after staining with an anti–CD8-α antibody were used as genuine negative control histograms.

noted BW$^-\zeta$, and expressing only the CD3-ζ products (see Materials and Methods). As expected, transfection into BW$^-$ of the CD3-ζ gene alone did not lead to the appearance of surface molecules reactive with either an anti–CD3-ε or anti–TCR-β mAb (Fig. 3, *top*). Cells from spleen, inguinal, and mesenteric lymph nodes from 3-mo-old CD3-$\zeta/\eta^{-/-}$ mice of H-2^b (Z$^-$B) or H-2^k (Z$^-$K) haplotypes were isolated and separately fused with BW$^-\zeta$. Sets of control hybridomas were prepared similarly from spleen and lymph node cells from 3-mo-old wild-type littermates of H-2^b (Z$^+$B) or H-2^k (Z$^+$K) haplotypes. Once produced, T cell hybridomas from individual wells were expanded briefly and analyzed for expression of CD3 at their surface. 20% of the hybridomas from CD3-$\zeta/\eta^{+/+}$ mice expressed CD3 at their surface, the remaining CD3-negative hybridomas being likely TCR loss variants. Of the hybridomas derived from CD3-$\zeta/\eta^{-/-}$ mice, 5% did express CD3 at their surface and in amounts no lower than on hybridomas derived from CD3-$\zeta/\eta^{+/+}$ mice (compare hybridomas Z$^+$K 2.3, Z$^-$B17.20, and Z$^-$K3.13 in Fig. 3). The lack of detectable CD3 expression on a larger proportion of the hybridomas derived from CD3-$\zeta/\eta^{-/-}$ mice probably resulted from the fact that some of them had additionally lost the transfected CD3-ζ gene during their establishment. All the CD3$^+$ hybridomas (10 from CD3-$\zeta/\eta^{-/-}$, H-2^b animals, 8 from CD3-$\zeta/\eta^{-/-}$, H-2^k animals, and 10 from CD3-$\zeta/\eta^{+/+}$ animals of H-2^b or H-2^k haplotypes) were cloned and further analyzed using antibodies specific for the TCR-α/β, TCR-γ/δ, CD4, or CD8 molecules. All the CD3$^+$ hybridomas were readily stained with an antibody against the constant region of the TCR-β chain (Fig. 3 and data not shown). When further analyzed with a panel of anti-Vα and anti-Vβ mAb, they were found to express the products of gene segments belonging to the Vα2, Vα3.2, Vα8, Vβ2, Vβ6, Vβ8, Vβ12, or Vβ14 subfamilies. These results suggest that the CD3$^+$ hybridomas derived from CD3-$\zeta/\eta^{-/-}$ and CD3-$\zeta/\eta^{+/+}$ animals are not grossly skewed toward a peculiar Vα or Vβ usage. Approximately half of the CD3$^+$ hybridomas displayed a CD4$^+$CD8$^-$ phenotype (Fig. 3). Considering that BW5147 and its derivatives have been shown to contribute *trans*-acting factors capable of silencing the expression of the CD8-α gene (32), it is possible that the CD4$^-$CD8$^-$ phenotype of some of the remaining hybridomas resulted from their derivation from T cells belonging to the CD8$^+$ lineage.

Recognition of Conventional Self-MHC Molecules by the Majority of TCR-α/β$^+$ Hybridomas Derived from CD3-$\zeta/\eta^{-/-}$ Mice. To examine the specificities of the heterogeneous TCR-α/β dimers expressed on the Z$^-$B and Z$^-$K sets of hybridomas, we tested them for IL-2 production in response to spleen cells from C57BL/6 or C57BL/6-H-2^k mice. All the hybridomas were capable of IL-2 production in response to immobilized anti–CD3-ε mAb. Hybridomas from CD3-$\zeta/\eta^{-/-}$, H-2^b animals showed three distinct

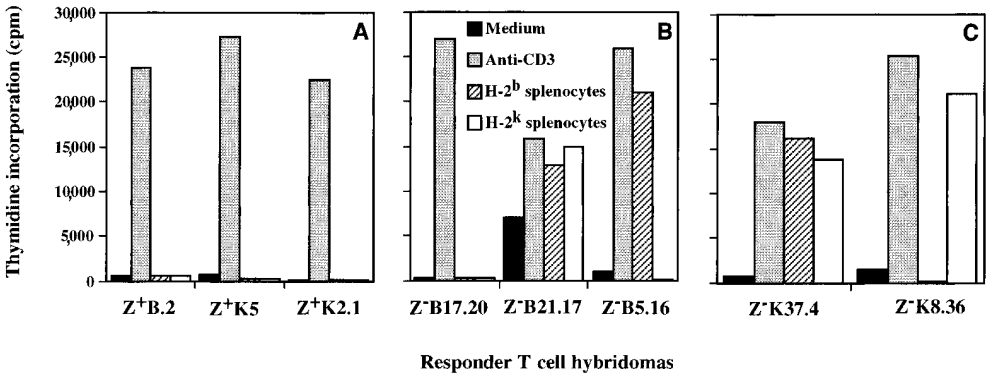

Figure 4. Specificity of hybridomas generated from single positive peripheral T cells isolated from wild-type mice of H-2^b or H-2^k haplotypes (*A*), CD3-$\zeta/\eta^{-/-}$ mutant mice of H-2^b (*B*), or H-2^k (*C*) haplotypes. IL-2 production of the various hybridomas was measured after incubation without any stimulus (*Medium*), with irradiated C57BL/6 (*H-2*^{b}) or C57BL/6-H-2^k (*H-2*^{k}) splenocytes (10^6 cells/well), or in wells coated with the anti–CD3-ϵ mAb 2C11 (10 μg/ml).

patterns of response. As shown in Fig. 4 *B*, one hybridoma (Z^-B17.20) failed to react with H-2^b or H-2^k splenocytes, while another one (Z^-B21.17) reacted with both H-2^k and H-2^b splenocytes and produced IL-2 when grown in medium alone. However, the most remarkable pattern of reactivity was observed with the eight remaining Z^-B hybridomas in that they reacted with C57BL/6 syngeneic spleen cells but failed to respond with spleen cells prepared from congenic C57BL-6-H-2^k mice. (This major reactivity pattern is illustrated by hybridoma Z^-B5.16 in Fig. 4 *B*). On the other hand, six of the eight hybridomas derived from CD3-$\zeta/\eta^{-/-}$, H-2^k animals showed a reciprocal pattern of reactivity. For example, as illustrated in Fig. 4 *C* for hybridoma Z^-K8.36, they did recognize C57BL/6-H-2^k cells, but not C57BL/6 cells. The two remaining hybridomas of the Z^-K set reacted with both spleen cell populations but, in contrast to hybridoma Z^-B21.17, did not produce any detectable IL-2 when cultured in medium alone (compare hybridomas Z^-K37.4 and Z^-B21.17 in Fig. 4). Despite the expression of TCR associated with the same complement of CD3 polypeptides and in amounts comparable to or even greater than those found at the surface of the Z^-B and Z^-K sets of hybridomas, none of the T cell hybridomas derived from CD3-$\zeta/\eta^{+/+}$ animals had autoreactivity (sets Z^+B and Z^+K in Fig. 4 *A*). The consistency of the latter result with previous reports obtained with the BW^- fusion partner (28, 29), clearly indicates that fusion with the $BW^-\zeta$ derivative does not in itself confer some artifactual reactivities to the resulting hybridomas. As summarized in Fig. 5, these results showed that the majority of the TCR-α/β^+ hybridomas established from CD3-$\zeta/\eta^{-/-}$ animals specifically reacted with syngeneic spleen cells.

To investigate the nature of the ligands recognized by the hybridomas derived from CD3-$\zeta/\eta^{-/-}$ mutant mice, we next tested the panel of Z^-B hybridomas with spleen cells from mutant MHC class II– or β2m–deficient mice (21, 22). Because both of these mutant mice were available only on a C57BL/6 background, we had to limit our analysis to the Z^-B set of hybridomas (Table I). If we omit the Z^-B21.17 hybridoma from further analysis because it produced IL-2 in the presence of medium alone and with all tested cells, six hybridomas were well stimulated by β2m–deficient spleen cells, but not by splenocytes from MHC class II–deficient mice, whereas the remaining Z^-B8.12 and Z^-B2.8 hybridomas showed a reciprocal pattern of reactivity. Consistent with the observation that normal

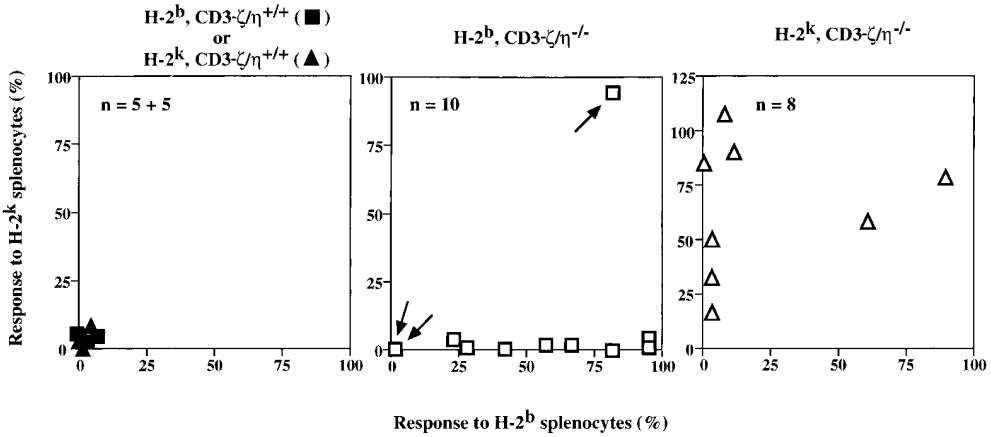

Figure 5. Most of the TCR-α/β^+ T cell hybridomas derived from the single positive cells found in the periphery of 3-mo-old CD3-$\zeta/\eta^{-/-}$ mice display antiself reactivity. T cell hybridomas derived from wild-type mice (CD3-$\zeta/\eta^{+/+}$) of H-2^b or H-2^k haplotypes (*left*) or CD3-$\zeta/\eta^{-/-}$ mutant mice of H-2^b (*middle*) or H-2^k haplotype (*right*) were tested individually for their ability to respond to irradiated spleen cells from wild-type mice of H-2^b (C57BL/6) or H-2^k (C57BL/6-H-2^k) haplotype. In parallel experiments, hybridomas were stimulated with an anti–CD3-ϵ mAb to assess for the integrity of their TCR signal transduction pathway (not shown). The IL-2 production values observed in response to H-2^b or H-2^k spleen cells were normalized as a percentage of the IL-2 production values observed upon anti–CD3-ϵ stimulation. The two percentages obtained for each hybridoma reflect its ability to respond to H-2^b (*abscissa*) or H-2^k (*ordinate*) spleen cells. In the middle panel, the Z^-B21.17 and Z^-B17.20 hybridomas are specified by one and two arrows, respectively.

Table 1. *Most of the Self-reactive CD3$^+$ Hybridomas Derived from H-2^b, CD3-ζ/η$^{-/-}$ Mice Recognize β2m– or MHC Class II–dependent Ligands*

Responder hybridomas	IL-2 secreted (thymidine incorporation) in response to				
	Medium	Immobilized anti–CD3-ε	H-2^b, β2m$^+$, II$^+$ splenocytes	H-2^b, β2m^0, II$^+$ splenocytes	H-2^b, β2m$^+$, II^0 splenocytes
Z$^-$B 8.12	214	14,459	3,952	761	3,368
Z$^-$B 2.8	159	13,598	12,863	1,052	13,302
Z$^-$B 53.4	228	13,455	7,625	7,010	308
Z$^-$B 5.16	692	14,766	20,071	19,762	327
Z$^-$B 103.1	56	11,923	4,955	4,766	53
Z$^-$B 35.18	99	10,883	10,290	15,234	440
Z$^-$B 170.3	181	15,651	3,512	4,141	191
Z$^-$B 190.28	111	7,177	4,186	8,365	152
Z$^-$B 21.1.7	19,115	20,040	15,096	13,961	12,046

Hybridomas (10^5) were cultured without any stimulus (*medium*), in wells coated with the anti–CD3-ε mAb 2C11 or with irradiated splenocytes (10^6) originating from wild-type, β2m–, or MHC class II–deficient mice of H-2^b haplotype. Cultures were for 20 h, at which time 50 μl of supernatant were collected and added undiluted to 50 μl of medium containing 5×10^3 cells of the IL-2 dependent line CTL-L. Growth of the CTL-L was estimated by [^{3}H]thymidine incorporation after pulsing the wells with 1μCi [^{3}H]TdR for 8 h. Mean values for duplicate samples are given.

CD4$^-$CD8$^+$ cells express MHC class I–restricted TCR and CD4$^+$CD8$^-$ cells express MHC class II–restricted TCR (33), four out of the six MHC class II–reactive Z$^-$B hybridomas were found to be CD4$^+$, whereas those activated by β2m–deficient spleen cells were found to be CD4$^-$CD8$^-$. Altogether, these reactivity patterns suggest that a large proportion of the TCR-α/β$^+$ hybridomas derived from CD3-ζ/η$^{-/-}$ mice reacted against conventional self-MHC molecules.

Discussion

Thymocytes of mice deficient in CD3-ζ/η gene express at their surface very low levels of TCR/CD3 subcomplexes deprived of the CD3-ζ/η transduction module, and as a result, appear to terminate their development at the CD4$^+$CD8$^+$ stage (8–10). Unexpectedly, a population of single positive T cells was found to accumulate over time in the spleen and lymph nodes of these mice. Although the reciprocal expression of the CD4 molecule and CD8-α/β isoform at their surface suggested that these peripheral T cells have matured via a thymus-dependent pathway (34, 35), it should be noted that, owing to the small size of the CD3-ζ/η$^{-/-}$ thymuses, it has not been possible yet to successfully thymectomize CD3-ζ/η$^{-/-}$ neonates to formally establish the thymic origin of these cells. Irrespective of their exact origin, the CD3$^{-\text{ or very low}}$ cells present in the spleen and lymph nodes of CD3-ζ/η$^{-/-}$ mice accumulate in a process dependent on the expression of MHC molecule (17), and as shown in that paper, do contain TCR-α/β heterodimers. Once expressed at the surface of T cell hybridomas, at density comparable to wild-type T cells and together with a complete constellation of CD3 subunits, the CD4-associated TCR-α/β dimers present in CD3-ζ/η$^{-/-}$ peripheral T cells showed a reactivity toward self-MHC class II products, whereas those found on CD4$^-$CD8$^-$ hybridomas and, presumably selected in association with CD8, mostly recognized self-MHC class I products. Importantly, none of the T cell hybridomas originating from wild-type peripheral T cells were found capable of reacting against self-MHC molecules. Three additional points should be made. First, it should be emphasized that the CD3-ζ/η–deficient mice used in the present study were constructed from an embryonic stem cell line derived from C57BL/6 mice (8), and maintained on either a C57BL/6 (H-2^b) or C57BL/6-H-2^k (H-2^k) background. Considering that the spleen cells used to stimulate the Z$^-$B and Z$^-$K sets of hybridomas originate from C57BL/6 or C57BL/6-H-2^k mice, this experimental setting permitted us to exclude that the patterns of reactivity displayed by these hybridomas are directed against minor histocompatibility antigen(s). Second, the peripheral T cells investigated here are distinct from the TCR-α/β$^+$ gut intraepithelial lymphocytes found in CD3-ζ/η$^{-/-}$ mice. In contrast to conventional mature T lymphocytes, the latter express the CD8-α/α isoform, bear TCR/CD3 complexes at their surface which incorporate FcεRIγ as one of their subunits, and are likely to be extrathymically derived (8, 9, 36). Third, it has recently been shown that negative selection of thymocytes occurs primarily in the medulla (37). Considering that CD3-ζ/η–deficient thymuses contain only residual foci of thymic medulla (Naquet, P., M. Naspetti, and M. Malissen, unpublished observations), negative selection may have been inoperative in these mice. Thus, the single positive cells found in their periphery may have undergone unopposed positive selection (see below) and some of them been autoreactive and exhibited overt signs of activation. However, the single positive cells found in the spleen and lymph nodes of 3-mo-old CD3-ζ/η$^{-/-}$ mice lack activation markers such

as CD25 and CD69. Therefore, the TCR/CD3 subcomplexes that leak to the surface of these cells appear appropriately calibrated in relation to the CD3-$\zeta/\eta^{-/-}$ context, and it is only after increasing artificially their surface density and/or output that they react to self-MHC molecules.

One is led to ask whether our experimental protocol might have resulted in the preferential fusion to the $BW^{-}\zeta$ partner of only those peripheral T cells that were in the course of a self-MHC restricted response against infectious agents. To test this possibility, we conduced a set of preliminary experiments in which peripheral CD3-$\zeta/\eta^{-/-}$ cells were prealably stimulated through the synergistic action of phorbol 12-myristate 13-acetate and ionomycin, and found that the reactivity of the resulting hybridomas was still biased toward self-MHC molecules (data not shown). Thus, the unusual reactivity pattern observed for the CD3-$\zeta/\eta^{-/-}$–derived hybridomas described in this paper (Fig. 5) does not result from the fact that we have inadvertently favored the fusion of preactivated CD3-$\zeta/\eta^{-/-}$ peripheral T cells. However, it should be emphasized that these results do not permit us to determine whether the $\alpha\beta$-TCR rescued at the surface of the CD3-$\zeta/\eta^{-/-}$–derived hybridomas have previously undergone a process of MHC-dependent selection in the thymus or in the periphery (e.g., references 16 and 38). In any case, considering that the amount of MHC ligands, CD4/CD8 coreceptors, and adhesion molecules are not affected in CD3-$\zeta/\eta^{-/-}$ mice, we would like to suggest that in CD3-$\zeta/\eta^{-/-}$ mice, only those T cells displaying TCR with a high affinity for self peptide/MHC complexes may survive preferentially and accumulate. Accordingly, upon engagement with self peptide/MHC complexes, the small pool of high affinity TCR expected to be available at the surface of these CD3-$\zeta/\eta^{-/-}$ cells is likely to become rapidly exhausted, generating blunted intracellular signals (39), capable of triggering survival signals, but insufficient to reach the higher activation threshold needed for negative selection. In contrast, once expressed in a wild-type background, these very same TCR would be expected to trigger all the range of activation events up to negative selection.

Our results are reminiscent of those obtained in mice that lack β2m (40–42) and express MHC class I molecules at levels considerably lower than normal. In these mice, even the low levels of conformed class I heavy chains that can reach the surface appeared capable of selecting $CD8^{+}$ T cells, but in numbers lower than in wild-type mice. The few $CD8^{+}$ T cells selected in $\beta2m^{-/-}$ mice were capable to mount strong allospecific responses after in vivo priming. However, in marked contrast to alloreactive $CD8^{+}$ T cells generated in β2m-positive littermates, the alloreactive T cells induced in a $\beta2m^{-/-}$ context readily cross-reacted toward syngeneic cells derived from $\beta2m^{+/+}$ mice. As suggested by Glas et al. (40), the most likely interpretation of these data is that in $\beta2m^{-/-}$ animals, negative selection does not remove T cells capable of recognizing self peptide/MHC complexes once expressed at physiological levels. (Note that a more remote interpretation of these data would be that the repertoire of peptides bound to MHC class I molecules differs qualitatively between the $\beta2m^{-/-}$ and wild-type settings.) Therefore, the amount of selecting peptide/MHC ligands available in the thymus, as well as the density and output of the TCR/CD3 complexes expressed on thymocytes, contribute to set the signaling thresholds for TCR-α/β selection. Consequently, by readjusting selection windows, mutations in the CD3-ζ/η^{-} and β2m genes resulted in two phenotypes which display striking symmetrical relationships.

Finally, on a more practical ground, peripheral blood lymphocytes have been considered as primary targets for gene therapy of inherited and acquired disorders of the immune system. For instance, it has been suggested that transduction of a CD3-ϵ gene into the peripheral blood lymphocytes of an immunodeficient patient that lack CD3-ϵ chains should rescue a normal surface expression of their TCR/CD3 complexes (43). However, on the basis of our results, it can be predicted that when applied to mutant cells that have already undergone a phase of internal calibration in the primary and secondary lymphoid organs, gene therapy trials aiming at correcting defects in T cell recognition/activation events will result in the production of cells reactive against self-MHC products.

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