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Functional Similarity and Differences Between Selection-Independent CD4$^-$CD8$^-$ $\alpha\beta$ T Cells and Positively Selected CD8 T Cells Expressing the Same TCR and the Induction of Anergy in CD4$^-$CD8$^-$ $\alpha\beta$ T Cells in Antigen-Expressing Mice[1]

Jordan Caveno, Yiqun Zhang, Bruce Motyka,[2] Soo-Jeet Teh, and Hung-Sia Teh[3]

In TCR-$\alpha\beta$ transgenic mice, CD4$^-$CD8$^-$ TCR-$\alpha\beta^+$ ($\alpha\beta$ DN) cells arise in the absence of positively selecting MHC molecules and are resistant to clonal deletion in Ag-expressing mice. In this study the activation requirements and functional properties of $\alpha\beta$ double-negative (DN) cells were compared with those of positively selected CD8$^+$ cells expressing equivalent levels of the same MHC class I-restricted transgenic TCR. We found that positively selected CD8$^+$ cells required a lower density of the antigenic ligand for optimal proliferative responses compared with $\alpha\beta$ DN cells derived from nonpositively selecting mice. However, when the CD8 coreceptor on CD8$^+$ cells was blocked with an anti-CD8 mAb, both $\alpha\beta$ DN and CD8$^+$ cells exhibited the same dose-response curve to the antigenic ligand and the same dependence on CD28/B7 costimulation. Positively selected CD8$^+$ cells also differed from $\alpha\beta$ DN cells in that they differentiated into more efficient killers and IL-2 producers after Ag stimulation, even after CD8 blockade. However, Ag-activated $\alpha\beta$ DN and CD8$^+$ cells were equally efficient in producing IFN-γ, suggesting that this functional property is independent of positive selection. We also found that $\alpha\beta$ DN cells recovered from the lymph nodes of Ag-expressing mice were functionally anergic. This anergic state was associated with defective proliferation and IL-2 production in response to Ag stimulation. These observations indicate that $\alpha\beta$ DN cells can be anergized in vivo by physiological levels of the antigenic ligand. ***The Journal of Immunology,*** **1999, 163: 1222–1229.**

CD4$^+$ and CD8$^+$ T cells are the products of positive selection of immature CD4$^+$CD8$^+$ thymocytes by MHC class II and class I molecules, respectively (1). They constitute the majority of peripheral TCR-$\alpha\beta^+$ T cells in normal mice. Although much is known regarding the mechanisms by which CD4$^+$ and CD8$^+$ T cells are positively selected, relatively little is known regarding the mechanisms by which CD4$^+$ and CD8$^+$ T cells acquire distinct functional characteristics. It is also unclear whether the positive selection process imparts signals that lead to the commitment of CD4$^+$ or CD8$^+$ T cells to the helper or cytotoxic lineage, respectively (2).

In addition to the major CD4$^+$ and CD8$^+$ subsets of $\alpha\beta$ T cells, a minor population of functionally mature CD4$^-$CD8$^-$ TCR-$\alpha\beta^+$ cells (herein referred to as $\alpha\beta$ DN[4] cells) exists in the thymus and peripheral lymphoid organs of normal mice (3, 4). This subset first appears late in thymic development (5). The TCR-$\alpha\beta$ on DN thymocytes is capable of transducing signals that lead to proliferation, lymphokine production, and cytolysis in vitro (6, 7). Studies of the methylation status of the CD8 gene in $\alpha\beta$ DN thymocytes indicate that they bear a demethylated CD8α gene, reflecting previous expression of CD8 (5). The number of $\alpha\beta$ DN thymocytes is also reduced by about 90% in β_2m$^{-/-}$ mice (8). These data suggest that the development of the $\alpha\beta$ DN thymocytes requires positive selection by MHC class I or class I-like molecules and the participation of the CD8 coreceptor in this selection process. The requirement for selection is further supported by the expression of the activation markers CD44 and CD69 by these cells (4). Positive selection by MHC class I molecules has also been postulated to be the basis for the development of an NK1.1$^+$ subset of $\alpha\beta$ DN thymocytes (8, 9). Some $\alpha\beta$ DN cells are autoreactive, as illustrated by the observation that cultured $\alpha\beta$ DN thymocytes of normal mice are cytolytic toward syngeneic target cells, a property not manifested by other thymic subsets (10).

The development of $\alpha\beta$ DN cells in TCR-$\alpha\beta$ transgenic mice is thymus dependent (11). However, their development is independent of positively selecting MHC molecules (11, 12), and they are resistant to clonal deletion in Ag-expressing mice (12, 13). These observations support the idea that $\alpha\beta$ DN cells in TCR transgenic mice are intrinsically indifferent to the positive and negative selection processes that affect CD4$^+$ and CD8$^+$ T cells. Further analysis of $\alpha\beta$ DN cells in TCR transgenic mice showed that these cells do not express endogenous TCRα genes, have maintained the TCR δ locus on both chromosomes, and can coexpress TCR $\alpha\beta$ and TCR $\gamma\delta$ on the cell surface (14). This finding led to the suggestion that $\alpha\beta$ DN cells in TCR transgenic mice may result from

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

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[2] Current address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

[3] Address correspondence to Dr. Hung-Sia Teh, Department of Microbiology and Immunology, 6174 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z3. E-mail address: teh@interchange.ubc.ca

[4] Abbreviations used in this paper: $\alpha\beta$ DN, CD4$^-$CD8$^-$ $\alpha\beta$ TCR$^+$; p2Ca, LSPFPFDL; H-Y peptide, KCSRNRQYL; T2-L^d, L^d transfectant of a peptide transporter-deficient cell line; β_2m, β_2-microglobulin.

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the premature expression of the α and β TCR transgenes in the $\gamma\delta$ lineage. Another similarity between $\alpha\beta$ DN cells from TCR transgenic mice and $\gamma\delta$ T cells in normal mice is that the development of $\gamma\delta$ T cells is not dependent on the expression of MHC class I molecules (15, 16).

In this study we have compared the activation requirements and functional properties of nonpositively selected $\alpha\beta$ DN cells with those of positively selected $CD8^+$ cells. These cells were isolated from 2C TCR transgenic mice with different MHC backgrounds. The $\alpha\beta$ 2C TCR is specific for the p2Ca peptide (derived from a mitochondrial protein) presented by L^d MHC class I molecules (17, 18) and is positively selected by K^b MHC class I molecules (19). The 2C TCR has a relatively high affinity ($\sim 1 \times 10^6\ M^{-1}$) for the p2Ca/$L^d$ ligand (20). We compared the activation requirements and functional properties of DN 2C TCR^+ T cells isolated from MHC class $I^{-/-}$ 2C mice with that of positively selected $CD8^+$ 2C TCR^+ T cells from H-2^b 2C mice. In this way we were able to determine which unique properties of $CD8^+$ T cells were conferred by the positive selection process. We also found that $\alpha\beta$ DN 2C TCR^+ cells isolated from Ag (H-2^d)-expressing mice were functionally anergic compared with those isolated from transgenic mice that did not express the H-2^d Ag. This observation indicates that physiological levels of the p2Ca/L^d ligand are sufficient to anergize $\alpha\beta$ DN cells.

Materials and Methods

Mice

2C $\beta_2m^{-/-}$ mice were produced by back-crossing H-2^b 2C TCR transgenic mice (21) (provided by Dr. Dennis Loh) to H-2^b $\beta_2m^{-/-}$ mice (22) (provided by Dr. Oliver Smithies). H-2^b TAP-$1^{-/-}$ mice (23) were obtained from The Jackson Laboratory (Bar Harbor, ME). H-$2^{b/d}$ 2C mice were produced by mating H-2^b 2C TCR transgenic mice with DBA/2 (H-2^d) mice. H-2^b H-Y TCR transgenic mice were produced as previously described (24, 25). All animals were maintained and bred in the animal facility of the Department of Microbiology and Immunology, University of British Columbia.

Cells and cell culture conditions

$CD4^-CD8^+$ cells were purified from the lymph nodes of 6- to 12-wk-old mice as follows. The cells were incubated with biotinylated anti-CD8β mAb 53.58 followed by positive selection using a MACS MS^+ separation column and MiniMACS magnet following the procedure described by the manufacturer (Miltenyi Biotech, Auburn, CA). This procedure yielded a population of cells of which >95% were $CD4^-8^+$ as determined by FACS. In this case, CD8 expression was detected by the anti-CD8α mAb 53.67. $CD4^-CD8^-$ (DN) cells were purified from the lymph nodes of 6- to 12-wk-old mice by first incubating the lymph node cells with a mixture of anti-CD4 (GK1.5) and anti-CD8α (53.67) mAbs and depletion of $CD4^+$, $CD8^+$, and Ig^+ cells with anti-mouse Ig-coated Dynabeads (Dynal, Oslo, Norway). The purified DN cells were >95% $CD4^-CD8^-Ig^-$. The TAP-deficient cell line, T2-L^d (derived from a human T × B hybridoma transfected with murine L^d) (26), was used as APCs for the p2Ca peptide. Spleen cells from TAP-$1^{-/-}$ mice were used for presentation of the H-Y peptide (27). All cells were cultured in I medium (Iscove's DMEM (Life Technologies, Burlington, Canada) supplemented with 10% (v/v) heat-inactivated FBS (Life Technologies), 100 U of penicillin G/ml, 100 μg of streptomycin/ml, and 5×10^{-5} M 2-ME).

The proliferative potential of the purified cells was determined by culturing 1×10^4 purified cells with 5×10^4 mitomycin-treated T2-L^d cells or 5×10^5 irradiated TAP-$1^{-/-}$ spleen cells as APCs and the indicated concentration of the antigenic peptide. The cells were incubated in 96-well round-bottom plates in 200 μl of I medium. Where indicated, a saturating concentration of anti-CD8α mAb (10 μg/ml) was used to block signaling through the CD8 coreceptor. In some cultures the CD28/B7 costimulatory pathway was blocked with 5 μg/ml of CTLA-4 Ig fusion protein (28). All cultures were set up in triplicate. Where indicated, the cultures were supplemented with 20 U/ml of rIL-2. The rIL-2 was provided in the form of spent culture medium of IL-2 gene-transfected X63/0 cells (29), which typically contained ~3000 U of IL-2/ml. One microcurie of [^{3}H]thymidine was added to the cultures in the last 6 h of a 72-h culture period.

Abs and flow cytometry

Abs and their specificities were as follows: 1B2, 2C TCR Id (30); 53.67, CD8α (American Type Culture Collection (ATCC), Manassas, VA); 53.58, CD8β (ATCC); GK1.5, CD4 (ATCC); PC61, CD25 (ATCC); Pgp-1, CD44 (ATCC); Mel-14, CD62L (ATCC); I3/2, pan-specific CD45;and 16A, CD45RB (PharMingen, San Diego, CA). All FITC and biotin conjugations of mAb, with the exception of FITC-goat anti-mouse Ig (obtained from Southern Biotechnology Associates, Birmingham, AL), and CD45RB (PharMingen) were performed in our laboratory. CD4-PE was obtained from Becton Dickinson (Mountain View, CA). Streptavidin-Tricolor (PharMingen) was used to detect biotinylated mAb. Cell staining and flow cytometric analysis were performed according to standard procedures. A FACScan equipped with LYSYS II software (Becton Dickinson) was used to acquire and analyze data. For three-color analysis, a total of 30,000 events were acquired.

Peptides

The following peptides were synthesized at the University of British Columbia: p2Ca (LSPFPFDL) (17), pMCMV (YPHFMPTNL) (20), and H-Yp (KCSRNRQYL) (27).

Cytokine assays

For cytokine production 1×10^5 purified $CD4^-CD8^+1B2^+$ or $CD4^-CD8^-1B2^+$ cells were stimulated with 5×10^4 mitomycin-treated T2-L^d cells and the indicated peptide in a volume of 0.20 ml in I medium. Supernatants from these cultures were harvested after 40 h. The amounts of IL-2 and IFN-γ in the culture supernatants were determined by ELISA. The capture and biotinylated mAbs were as follows: JES6-1A12 and JES6-5H4 for IL-2 and R4-6A2 and XMG1.2 for IFN-γ. The R4-6A2 hybridoma cell line was obtained from the ATCC, and the XMG1.2 cell line (31) was obtained from Dr. Tim Mossman, University of Alberta (Edmonton, Canada). The JES6-1A12 and JES6-5H4 mAbs were obtained from PharMingen.

Cytotoxic assay

Effector cells were produced by incubating 1×10^5 purified $CD4^-CD8^+$ $1B2^+$ cells or $CD4^-CD8^-1B2^+$ cells with 5×10^5 mitomycin-treated T2-L^d cells and 1 μM p2Ca in a volume of 2.0 ml of I medium in 24-well plates. The activated cells were split as necessary with I medium containing 20 U/ml of IL-2. After 6 days of culture, 5×10^4 activated cells were assayed for their ability to lyse ^{51}Cr-labeled T2-L^d target cells (1×10^4/well) with the indicated concentration of p2Ca peptide. In some experiments, the H-2^d mastocytoma, P815 (ATCC), was used as the target cells. Where indicated, a saturating concentration of anti-CD8α mAb (10 μg/ml) was used to block signaling through the CD8 coreceptor. The incubation time for the ^{51}Cr release assay was 3 h. Spontaneous release in all assays ranged from 10 to 15% of the maximum releasable counts.

Results

Development of DN 2C TCR^+ in non-Ag-expressing and Ag-expressing mice

The 2C TCR is positively selected by K^b (19), negatively selected by L^d (21), and not selected in H-2^b $\beta_2m^{-/-}$ mice (32), which lack MHC class I expression (22, 33). The CD4/CD8 phenotypes of thymocytes derived from 6- to 8-wk-old H-2^b, H-$2^{b/d}$, and H-2^b $\beta_2m^{-/-}$ 2C transgenic mice are shown in Fig. 1. These patterns are typical for thymocytes that expressed the transgenic 2C TCR in positively selecting, negatively selecting, and nonselecting MHC backgrounds, respectively (21, 32). The yields of thymocytes from H-2^b, H-$2^{b/d}$, and H-2^b $\beta_2m^{-/-}$ 2C TCR transgenic mice were $\sim 2 \times 10^7$, 1×10^7, and 15×10^7, respectively. The relatively low yield of thymocytes from H-2^b 2C mice is attributed to deletion caused by the relatively high affinity/avidity of the 2C TCR for the positively selecting ligand(s) in H-2^b mice (34). The total numbers of lymph node cells from H-2^b, H-$2^{b/d}$, and H-2^b $\beta_2m^{-/-}$ 2C mice were $\sim 3.3 \times 10^7$, 3.3×10^7, and 2.0×10^7, respectively. As expected, only lymph node cells from H-2^b 2C mice have a well-defined $CD4^-CD8^+$ population. DN cells from the lymph nodes of H-2^b, H-$2^{b/d}$, and H-2^b $\beta_2m^{-/-}$ 2C mice contained 24.4, 42.7, and 21.3% 2C TCR^+ cells, respectively (Fig. 1). The 2C TCR is detected by the anti-idiotypic mAb, 1B2 (30). The total numbers of

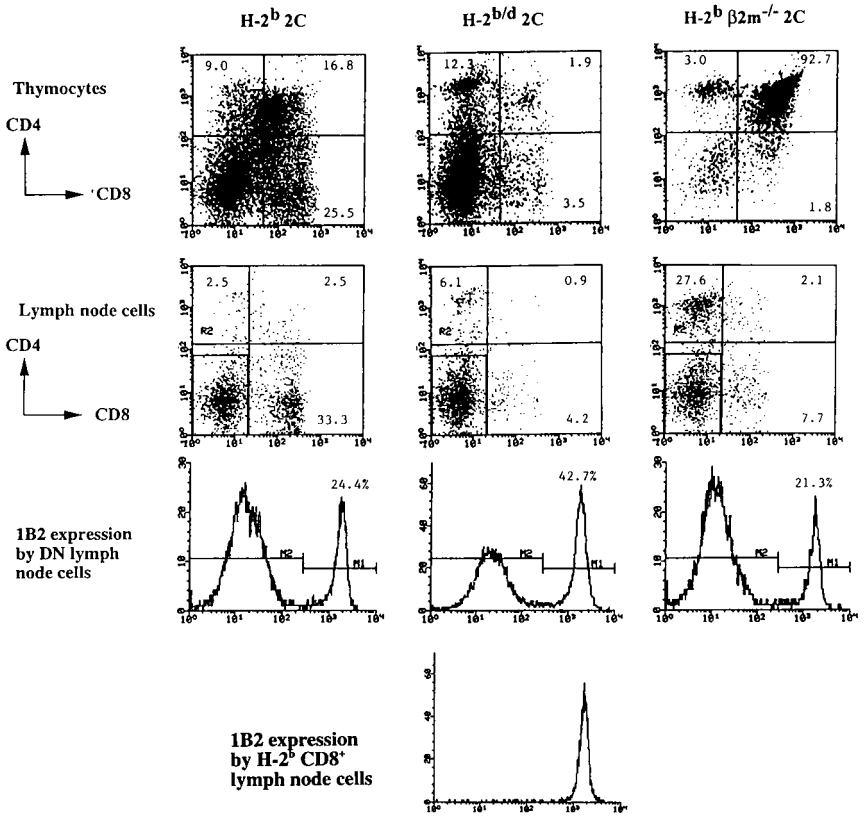

FIGURE 1. DN 1B2$^+$ cells are not positively selected and are not deleted in Ag-expressing mice. Thymocytes and lymph node cells from H-2^b, H-2$^{b/d}$, and H-2^b β_2m$^{-/-}$ 2C mice were incubated with anti-CD8α-FITC, anti-CD4-PE, and biotinylated 1B2 mAb. The cells were then washed and stained with streptavidin-Tricolor. The labeled cells were analyzed using the FACScan flow cytometer. A total of 30,000 events were acquired, and the data were analyzed with the LYSYS II software program. The dot plots indicate the CD4/CD8 phenotypes of thymocytes and lymph node cells from the 2C mice with the indicated MHC background. The numbers in each quadrant indicate the percentages of cells in that quadrant. The expression of 1B2 by gated CD4$^-$CD8$^-$ and CD4$^-$CD8$^+$ lymph node cells from the indicated mice are shown as histograms.

DN 1B2$^+$ cells that could be recovered from the lymph nodes of H-2^b, H-2$^{b/d}$, and H-2^b β_2m$^{-/-}$ 2C mice were about 8×10^6, 14×10^6, and 4×10^6, respectively. These results indicate that DN 2C TCR$^+$ T cells are produced in significant numbers in all three lines of 2C transgenic mice and are the most abundant in H-2$^{b/d}$ 2C mice.

Activation requirements for DN 1B2$^+$ and CD4$^-$CD8$^+$ 1B2$^+$ T cells

DN 1B2$^+$ cells from H-2^b β_2m$^{-/-}$ 2C mice are independent of MHC class I molecules for their development, whereas the development of CD8$^+$ 1B2$^+$ T cells is dependent on positive selection by K^b MHC class I molecules (19). Because these two populations of T cells express equivalent levels of the 2C TCR (Fig. 1), it was of interest to determine whether positively selected CD8$^+$ 1B2$^+$ cells differ from DN 1B2$^+$ cells with regard to their activation requirements and function. The proliferative response of these two population of T cells to Ag stimulation was determined by stimulating them with varying doses of the p2Ca peptide presented by the L^d-transfectant of the peptide transporter-deficient cell line, T2 (T2-L^d) (26). It was found that CD8$^+$ 1B2$^+$ cells proliferated more efficiently at low, but not at high, p2Ca/L^d ligand concentrations (Fig. 2). This proliferative response is specific for the p2Ca/L^d ligand, because the addition of another L^d-binding peptide, pMCMV (20), did not lead to any proliferative response above background levels (data not shown). Therefore, the expression of CD8 on positively selected cells led to a lowering of the activation threshold on positively selected cells. However, when the CD8 molecules on the CD8$^+$ T cells were blocked with an anti-CD8α mAb, the dose-response curve of CD8$^+$ cells to the p2Ca/L^d ligand was similar to that of DN cells.

We have also compared the proliferative responses of $\alpha\beta$ DN cells from H-2^b β_2m$^{-/-}$ 2C mice with those of $\alpha\beta$ DN cells from H-2^b 2C mice to the p2Ca/L^d ligand and found that the responses of these two populations to the p2Ca/L^d ligand were similar (Table I). This result provides further evidence that the $\alpha\beta$ DN cells from H-2^b β_2m$^{-/-}$ or H-2^b 2C mice are functionally equivalent and

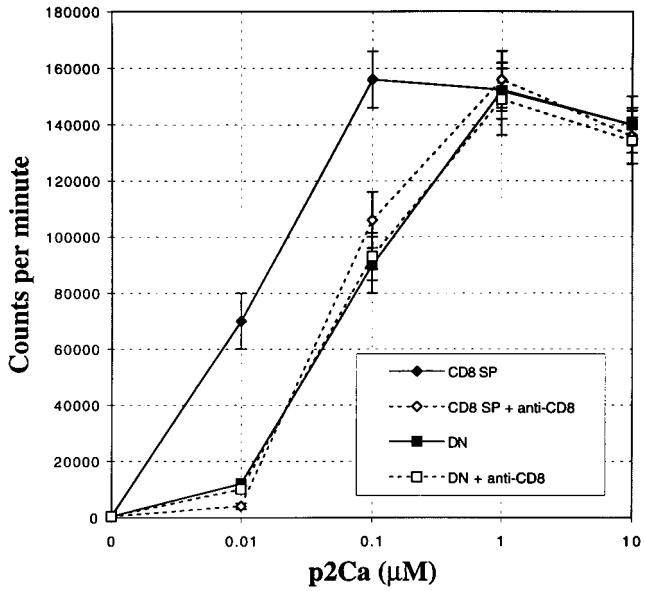

FIGURE 2. DN 1B2$^+$ cells have the same requirement for the p2Ca/L^d ligand as CD4$^-$CD8$^+$ 1B2$^+$ cells when the CD8 coreceptor is blocked. The responder cells (1×10^4/well) were either purified DN 1B2$^+$ lymph node cells from H-2^b 2C β_2m$^{-/-}$ mice or purified CD8$^+$ 1B2$^+$ lymph node cells from H-2^b 2C mice. They were incubated with mitomycin-C treated T2-L^d cells (5×10^4/well) and the indicated amount of p2Ca peptide. Anti-CD8α mAb was added to the indicated cultures at 10 μg/ml. The cultures were assayed for [^{3}H]thymidine incorporation in the last 8 h of a 72-h culture period. The error bars denote SDs.

Table I. *Similar proliferative response of DN cells from H-2^b 2C and H-2^b β_2m$^{-/-}$ 2C mice to the p2Ca/L^d ligand*[a]

p2Ca (μM)	H-2^b 2C DN	H-2^b β_2m$^{-/-}$ 2C DN
0	1,350 ± 454	1,005 ± 326
0.001	2,496 ± 845	3,675 ± 1,050
0.01	9,139 ± 1,761	15,155 ± 820
0.1	50,647 ± 2,854	30,334 ± 1,895
1.0	101,742 ± 6,737	109,081 ± 2,283
10	122,204 ± 1,771	101,845 ± 4,031

[a] Each culture well contained 1×10^4 purified CD4$^-$CD8$^-$1B2$^+$ lymph node cells from either H-2^b 2C or H-2^b β_2m$^{-/-}$ 2C mice and 5×10^4 mitomycin-treated T2-L^d cells and the indicated concentration of the p2Ca peptide. The cultures were set up in triplicates and assayed for [^{3}H]thymidine incorporation in the last 8 h of a 72-h culture period. The results of the proliferation assay are expressed as mean ± SD. Similar results were obtained from two separate experiments.

suggests that the presence of the positively selecting K^b molecules does not alter either the development or the function of $\alpha\beta$ DN cells.

We next determined whether optimal proliferative responses of DN 1B2$^+$ and CD8$^+$ 1B2$^+$ cells to stimulation by the p2Ca/L^d ligand are dependent on CD28/B7 costimulation. The contribution of the CD8 molecule on CD8$^+$ cells was controlled for by the addition of anti-CD8 mAb. CD28/B7 interaction was inhibited by adding an excess of the CTLA-4 Ig fusion protein, which binds B7.1 and B7.2 with higher affinity than CD28 (35). Under these conditions both CD8$^+$ 1B2$^+$ and DN 1B2$^+$ cells demonstrate the same dependence on CD28/B7 costimulation for optimal proliferative responses (Fig. 3). Thus, the requirement for CD28/B7 costimulation is dissociated from the positive selection process that operates on conventional positively selected CD8$^+$ T cells.

Cytotoxic responses of DN 1B2$^+$ and CD4$^-$CD8$^+$ 1B2$^+$ T cells

Ag-activated CD8$^+$ 1B2$^+$ cells differentiate into cytotoxic T cells specific for the p2Ca/L^d ligand. We compared the efficacy by which Ag-activated DN 1B2$^+$ and CD8$^+$ 1B2$^+$ T cells kill target cells expressing varying densities of the p2Ca/L^d ligand. The results in Fig. 4 showed that in the absence of anti-CD8 mAb, Ag-activated CD8$^+$ 1B2$^+$ cells killed target cells expressing the p2Ca/L^d ligand with a higher efficiency than Ag-activated DN 1B2$^+$ cells. When the CD8 coreceptor on CD8$^+$ cells was blocked with anti-CD8 mAb, this higher efficiency was only observed at higher density of the p2Ca/L^d ligand. The higher efficacy of Ag-activated CD8$^+$ 1B2$^+$ cells in killing target cells was also evident when an H-2^d mastocytoma cell line, P815, which expressed physiological levels of the p2Ca/L^d ligand, was used as the target cell (Fig. 5). This observation suggests that although both DN 1B2$^+$ and CD8$^+$ 1B2$^+$ T cells have the potential to differentiate into cytotoxic effector cells, the positive selection process may have facilitated the differentiation of CD8$^+$ 1B2$^+$ cells into more effective killers.

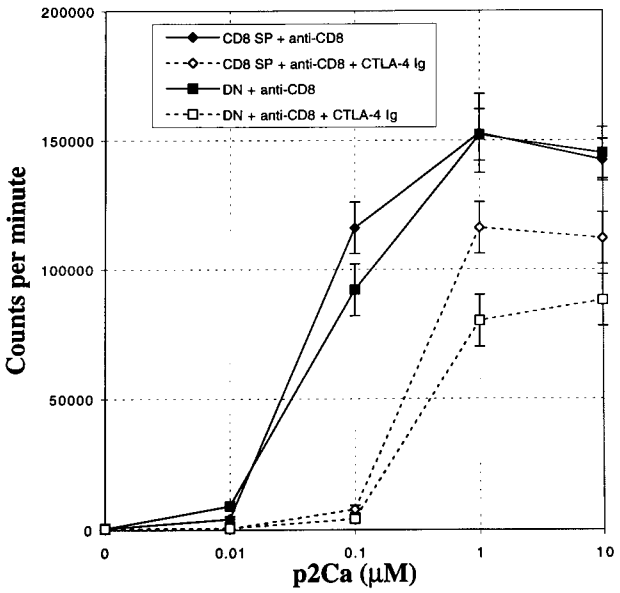

FIGURE 3. Ag-stimulated DN 1B2$^+$ cells are dependent on CD28/B7 costimulation for optimal proliferation. Culture and assay conditions are described in Fig. 2. CTLA-4 Ig was added at 5 μg/ml to the indicated cultures.

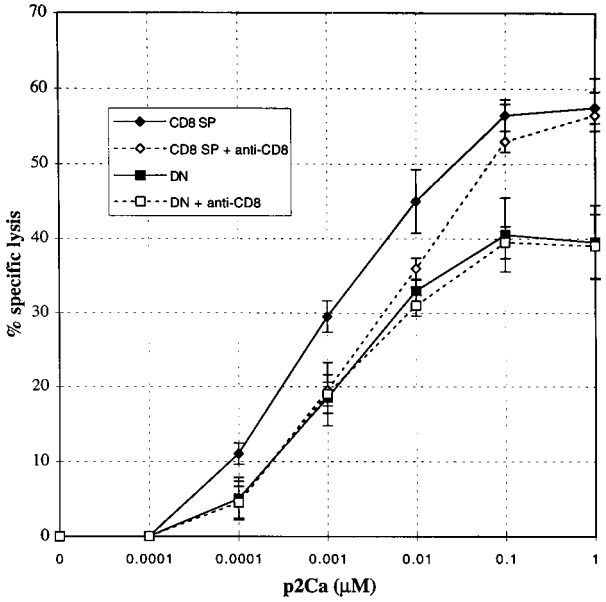

FIGURE 4. Ag-activated DN 1B2$^+$ cells differentiate into cytotoxic effector cells. Purified DN 1B2$^+$ lymph node cells from H-2^b β_2m$^{-/-}$ 2C mice or purified CD8$^+$ 1B2$^+$ lymph node cells from H-2^b 2C mice were cultured with T2-L^d and p2Ca as described in *Materials and Methods*. After 6 days of culture the Ag-activated cells (5×10^4/well) were assayed for their ability to lyse ^{51}Cr-labeled T2-L^d target cells (1×10^4/well) in the presence of the indicated concentration of exogenous p2Ca peptide. Where indicated, anti-CD8α mAb was added at 10 μg/ml during the cytotoxic assay.

Lymphokine production by Ag-activated DN 1B2$^+$ and CD4$^-$CD8$^+$ 1B2$^+$ T cells

CD8$^+$ T cells produce IFN-γ and IL-2 in response to Ag stimulation. We compared the amounts of IFN-γ and IL-2 produced by these two cell types in response to Ag stimulation. It was found that DN 1B2$^+$ cells were as efficient as CD8$^+$ 1B2$^+$ cells in producing IFN-γ in response to stimulation by the p2Ca/L^d ligand (Fig. 6*A*). By contrast, CD8$^+$ 1B2$^+$ cells were much more efficient than DN 1B2$^+$ cells in producing IL-2 in response to p2Ca/L^d stimulation (Fig. 6*B*). The anti-CD8 mAb inhibited IL-2 production by CD8$^+$ 1B2$^+$ cells only at low, but not high, p2Ca/L^d concentrations. This result suggests that the positive selection process may have contributed to the more efficient production of IL-2 by Ag-stimulated CD8$^+$ 1B2$^+$ cells.

DN 1B2$^+$ T cells from H-2$^{b/d}$ mice expressed high levels of CD44 and CD45RB

The above studies indicate that DN 1B2$^+$ cells from H-2^b β_2m$^{-/-}$ 2C mice are readily activated by the p2Ca/L^d ligand. DN 1B2$^+$ are

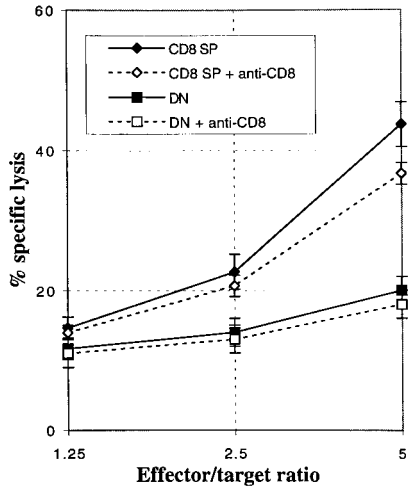

FIGURE 5. Less efficient killing of P815 by p2Ca/L^d-activated cells from H-2^b β_2m$^{-/-}$ 2C mice. p2Ca/L^d-activated CD8$^+$ 1B2$^+$ and DN 1B2$^+$ cells were generated as described in Fig. 4. The target cells in this experiment were ^{51}Cr-labeled P815 (H-2^d) mastocytoma cells (1×10^4/well). The cytotoxic assays were performed with the indicated E:T cell ratios in the presence or the absence of anti-CD8α mAb (10 μg/ml).

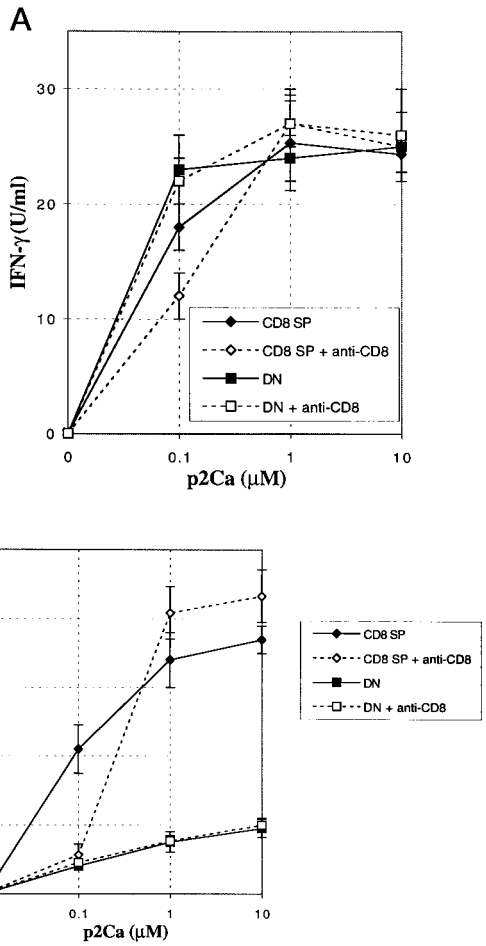

FIGURE 6. Ag-activated DN 1B2$^+$ cells produced normal levels of IFN-γ, but reduced levels of IL-2. For cytokine production 1×10^5 purified CD4$^-$CD8$^+$1B2$^+$ or DN 1B2$^+$ cells were stimulated with 5×10^4 mitomycin-treated T2-L^d cells and the p2Ca peptide in a volume of 0.20 ml in I medium. Where indicated, anti-CD8α mAb (10 μg/ml) was included at the beginning of the culture period. Supernatants from these cultures were harvested after 40 h. The amounts of IFN-γ (*A*) and IL-2 (*B*) in the culture supernatants were determined by ELISA as described in *Materials and Methods*. In these assays, 10 U of IFN-γ = 1 ng of IFN-γ and 16 U of IL-2 = 1 ng of IL-2.

also present in large numbers in L^d-expressing mice (Fig. 1). We have shown that even relatively low concentrations of the p2Ca/L^d ligand can activate DN 1B2$^+$ cells (Fig. 2). It is therefore not surprising to find that DN 1B2$^+$ cells from H-2$^{b/d}$ mice, which express the p2Ca/L^d ligand, have a cell surface phenotype that reflects past interaction with Ag. This is illustrated by the expression of high levels of CD44 on DN 1B2$^+$ cells from H-2$^{b/d}$ 2C mice (Fig. 7). However, DN 1B2$^+$ cells from H-2$^{b/d}$ 2C do not have the classical memory phenotype, in that they expressed very high levels of CD45RB (Fig. 7). The expressions of CD62L on DN 1B2$^+$ cells from H-2$^{b/d}$ and H-2^b β_2m$^{-/-}$ 2C mice also differed. Those from H-2^b β_2m$^{-/-}$ 2C mice expressed either an intermediate or a high level of the CD62L, whereas those from H-2$^{b/d}$ 2C mice expressed a uniformly high level of the CD62L. The data in Fig. 7 also indicate that DN cells from H-2$^{b/d}$ and H-2^b β_2m$^{-/-}$ 2C mice expressed equivalent levels of the 2C TCR, CD25, and total levels of CD45 (detected by the I3/2 mAb).

DN 1B2$^+$ T cells from H-2$^{b/d}$ mice are functionally anergic

The DN 1B2$^+$ cells that are present in H-2$^{b/d}$ mice are a potential source of autoreactive cells. The high expression level of CD44 on these cells also suggests that they have interacted with Ag in vivo. However, H-2$^{b/d}$ 2C mice do not exhibit any overt signs of autoimmune disease. It is therefore possible that the exposure of these cells to self Ag have rendered them anergic to further Ag stimulation. We tested this possibility by determining the proliferative response of DN 1B2$^+$ cells from H-2$^{b/d}$ 2C mice to the p2Ca/L^d ligand. The results in Fig. 8 indicate that these cells are hyporesponsive to the p2Ca/L^d ligand relative to DN 1B2$^+$ cells from H-2^b β_2m$^{-/-}$ 2C mice. This result indicates that these cells are functionally anergic.

A hallmark of T cell anergy is the inability to produce IL-2 in response to Ag stimulation. We found that DN 1B2$^+$ cells from H-2$^{b/d}$ 2C mice were also defective in IL-2 production relative to those derived from H-2^b β_2m$^{-/-}$ 2C mice (Fig. 9*A*). Previous studies have also shown that anergic T cells retain the ability to produce fairly normal levels of IFN-γ in response to Ag stimulation. This is also a feature of DN 1B2$^+$ cells from H-2$^{b/d}$ 2C mice (Fig. 9*B*). They produced similar amounts of IFN-γ as DN 1B2$^+$ cells from H-2^b 2C β_2m$^{-/-}$ mice when they were stimulated with a high density of the p2Ca/L^d ligand. However, they were less efficient in producing IFN-γ when they were stimulated with a low density of the p2Ca/L^d ligand.

In vivo induction of anergic in DN cells is dependent on the affinity/avidity of the TCR for the activating Ag

Previous studies in H-Y TCR transgenic mice suggest that DN cells derived from either female or male H-Y TCR transgenic mice were not male reactive (11, 13). Furthermore, there is no evidence that the DN H-Y TCR$^+$ cells were anergized as a result of in vivo exposure to the male Ag (11). The H-Y TCR is specific for the male peptide (H-Yp) presented by D^b. The sequence of the H-Y peptide for this transgenic TCR has been determined, and it was shown to bind poorly to the D^b molecule (27). As a result, the H-Y TCR functions like a low affinity/avidity TCR for the H-Yp/D^b ligand. We proceeded to determine whether DN H-Y TCR$^+$ cells from either female or male H-2^b H-Y TCR transgenic mice can be activated by high concentrations of the H-Yp/D^b ligand. The results in Fig. 10 indicate that DN H-Y TCR$^+$ cells from either

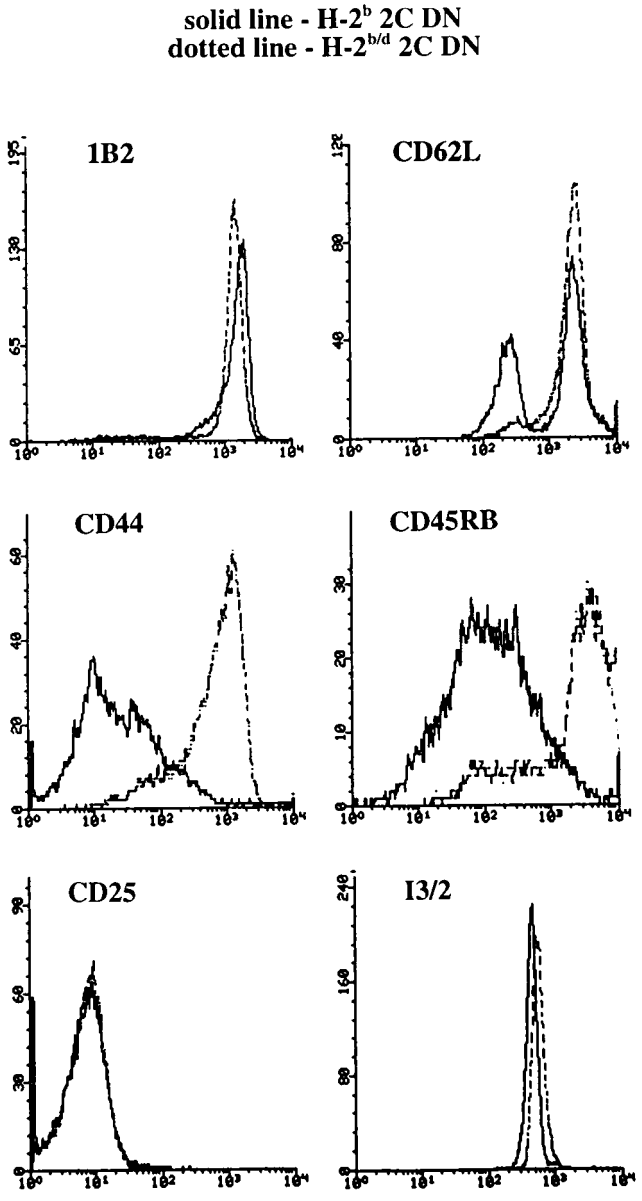

FIGURE 7. Up-regulation of CD44 and CD45RB in DN $1B2^+$ cells from H-$2^{b/d}$ 2C mice. DN cells from the lymph nodes of H-2^b 2C $\beta_2m^{-/-}$ and H-$2^{b/d}$ 2C mice were purified as described in *Materials and Methods*. The purified DN cells were incubated with anti-CD4-PE, anti-CD8α-FITC and biotinylated mAb specific for the 2C TCR (1B2), CD62L, CD44, CD45RB, or CD25. The cells were then washed and incubated with streptavidin-Tricolor and analyzed using FACScan. For analysis of total CD45 expression by these cells, the cells were incubated with anti-CD4-PE, anti-I3/2-FITC, and biotinylated 1B2 mAb. The cells were washed and then stained with streptavidin-Tricolor. The levels of these cell surface markers expressed by gated DN cells are indicated in the histograms. The two different methods for staining CD45RB (TriColor-labeled) and total CD45 (FITC-labeled) led to differences in the level of fluorescence for these two cell surface Ags and do not reflect higher expression of CD45RB relative to total CD45 levels. DN cells from the lymph nodes of H-2^b 2C mice expressed the same levels of these cell surface molecules as those from H-2^b $\beta_2m^{-/-}$ 2C mice (data not shown).

female or male mice mount similar proliferative responses to the H-Yp/D^b ligand. A high concentration of H-Yp was required for a proliferative response in the absence of exogenous IL-2. The proliferative response to the H-Yp/D^b ligand was enhanced by exogenous sources of IL-2. These results provide an independent assessment of the lack of anergy induction in DN cells from male H-2^b H-Y TCR transgenic mice. They also suggest that the in vivo induction of anergy in DN cells is determined by the relative affinity/avidity of the TCR for the activating Ag.

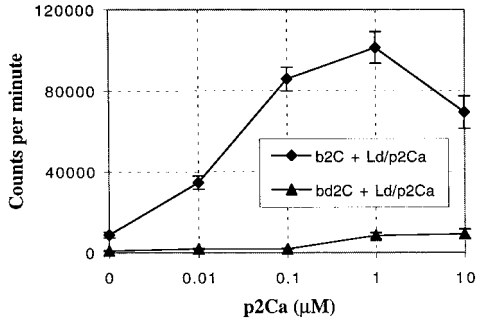

FIGURE 8. DN $1B2^+$ cells from H-$2^{b/d}$ 2C mice are hyporesponsive to the p2Ca/L^d ligand. DN from H-2^b $\beta_2m^{-/-}$ 2C (b2C) mice and DN cells from H-$2^{b/d}$ 2C (bd2C) mice were purified as described *in Materials and Methods*. The proliferative response of purified DN cells to the p2Ca/L^d ligand was determined as described in Fig. 2.

Discussion

In this study we determined whether the positive selection process that operates on $CD8^+$ T cells conferred unique activation requirements and/or functional properties specific for this cell type. This was done by comparing the activation requirements and functional properties of $CD8^+$ $1B2^+$ cells from positively selecting H-2^b mice with those of DN $1B2^+$ cells from nonselecting H-2^b $\beta_2m^{-/-}$ 2C mice. These two populations of T cells express equivalent levels of the transgenic 2C TCR. It was found that the expression of the CD8 coreceptor on positively selected cells leads to a lowering of activation threshold for proliferation (Fig. 2), cytotoxicity (Fig. 4), and IL-2 production (Fig. 6*B*). This lowering of threshold would enable positively selected $CD8^+$ T cells to respond to antigenic ligands that are present at densities insufficient to activate DN cells. Furthermore, $CD8^+$ $1B2^+$ cells differentiated into more potent cytolytic T cells and IL-2 producers even after the CD8 coreceptor was blocked. Whether these two latter features are the direct or indirect consequence of the positive selection process by MHC class I molecules remains to be determined. Significantly, Ag-activated DN $1B2^+$ cells also differentiated into fairly potent cytotoxic cells and efficient IFN-γ producers. Thus, positive selection signals do not appear to be essential for the acquisition of these two functions.

The mechanism by which DN $1B2^+$ cells develop in H-2^b $\beta_2m^{-/-}$ 2C mice remains to be determined. The bulk of the evidence suggests that DN TCR-$\alpha\beta^+$ cells in normal mice have previously express CD4 and CD8. Furthermore, a subset of these DN TCR-$\alpha\beta^+$ cells appears to be selected by MHC class I molecules. By contrast, the development of DN $1B2^+$ cells is independent of MHC class I selection, as they are found in large numbers in $\beta_2m^{-/-}$ 2C mice. DN $1B2^+$ cells are also unlikely to be derived from immature $CD4^+CD8^+$ thymocytes, because these cells are deleted in L^d-expressing mice. We therefore favor the suggestion that these cells belong to a lineage that is distinct from $\alpha\beta$ DN cells in normal mice (11). Similar to DN H-Y TCR^+ cells in H-Y TCR transgenic mice, the DN $1B2^+$ cells may, in fact, be of the $\gamma\delta$ lineage as a result of the premature expression of the α and β TCR transgenes in $\gamma\delta$ lineage cells (14). This raises the interesting prospect of using this system for detailed analysis of the signaling mechanisms that are required for $\gamma\delta$ T cell activation and the functions of Ag-activated $\gamma\delta$ T cells. One conclusion of this study is

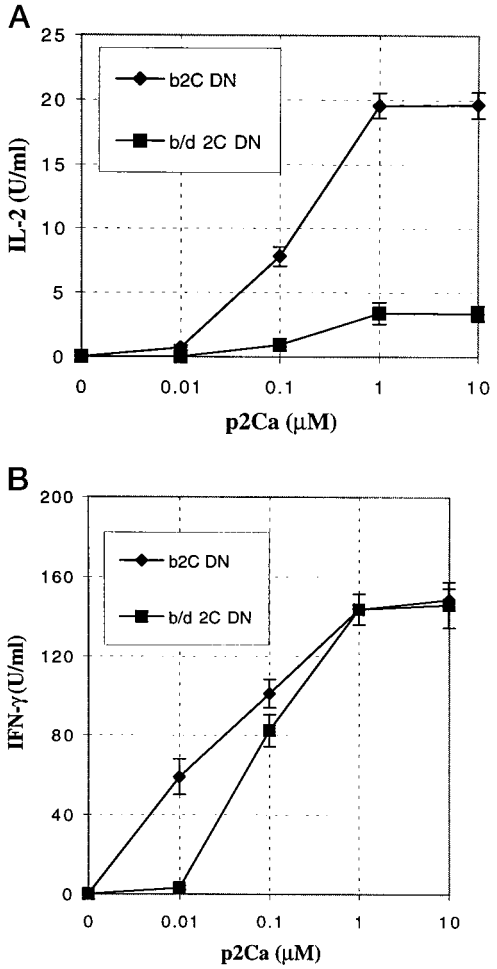

FIGURE 9. Ag-activated DN 1B2$^+$ cells from H-2$^{b/d}$ 2C mice are defective in IL-2 production, but produced normal levels of IFN-γ. DN 1B2$^+$ cells purified from the lymph nodes of H-2$^{b/d}$ 2C (bd2C) or H-2^b β_2m$^{-/-}$ 2C (b2C) were stimulated as described in Fig. 6. Culture supernatants were collected from the stimulated cells after a 40-h incubation period and were assayed for IL-2 (*A*) and IFN-γ (*B*) as described in Fig. 6.

that these putative γδ T cells, as a result of the lack of coreceptor expression, require relatively high ligand density for their activation. This requirement may render these cells ineffective for surveillance against ligands that are present at low density on cell surfaces. Interestingly, when the CD8 coreceptor is blocked, DN 1B2$^+$ cells have the same activation requirement as positively selected CD8$^+$ cells. This observation suggests that the coreceptor-independent signaling mechanisms that lead to T cell activation are highly conserved in different T cell lineages. Furthermore, this study also indicates that T cells of distinct lineages possess common functional properties, including IFN-γ production and cytotoxicity, suggesting that positive selection signals are not essential for the acquisition of these common functions.

We have shown that DN 1B2$^+$ T cells from Ag-expressing mice are anergic to further Ag stimulation. Furthermore, these anergic T cells are defective in IL-2 production. These observations indicate that physiological densities of the p2Ca/L^d ligand on APC are sufficient to anergize DN 1B2$^+$ T cells in Ag (L^d)-expressing mice. This observation contrasts with those previously described in H-Y TCR transgenic mice. In the H-Y system the majority of peripheral $\alpha\beta$ T cells in Ag-expressing mice are either of the DN or the CD8^{low} phenotype (13). The CD8^{low} population in Ag-expressing H-Y TCR transgenic mice are also γδ-like (14). We and others have found that the CD8^{low}, but not the DN, population was deleted as a result of the transgenic expression of higher levels of CD8 (36, 37). In male mice the CD8^{low} population expressed high levels of CD44, and they were shown to be autoreactive in adoptive transfer experiments into male *nu/nu* mice (11). Both the CD8^{low} and the DN populations from male H-Y TCR transgenic mice were not activated by male APC even when exogenous IL-2 was provided (11). However, these cells proliferated vigorously when they were stimulated with anti-TCR Abs and IL-2 (11, 38). This was used as an argument to support the idea that these cells were not intrinsically anergic. In this study we used an alternative approach to assess the functionality of DN cells derived from male transgenic mice. We showed that DN cells from either female or male transgenic mice were activated by high concentrations of the H-Yp/D^b ligand (Fig. 10) even in the absence of exogenous sources of IL-2. This provides more direct support for the conclusion that the DN cells from male transgenic mice were not functionally anergic. In our previous study we also showed that the transgenic expression of the CD8 molecule in male H-Y TCR transgenic mice led to impaired calcium mobilization in the DN cells (37). This was used to argue in favor of an inhibitory role for the CD8 coreceptor in DN cells. However, in light of our study with the DN 1B2$^+$ cells in Ag-expressing mice, we offer the following reinterpretation of our previously published data. Because the H-Y TCR effectively functions as a low avidity TCR for the H-Yp/D^b ligand (27), physiological densities of this ligand on APC are insufficient to activate and/or anergize DN cells. However, expression of the CD8 coreceptor on CD8^{low} cells enables them to be activated by physiological densities of the H-Yp/D^b ligand, as illustrated by the high level of CD44 expression on these cells (11). Transgenic expression of higher levels of CD8 coreceptor in male H-Y TCR transgenic mice led to the complete deletion of the CD8^{low}, but not the DN, population (36, 37). We propose that transgenic expression of CD8 will lead to the induction of anergy in DN cells from male H-Y TCR transgenic mice. This hypothesis is consistent with our observation that calcium mobilization is inhibited in DN cells from male H-Y/CD8α double transgenic mice

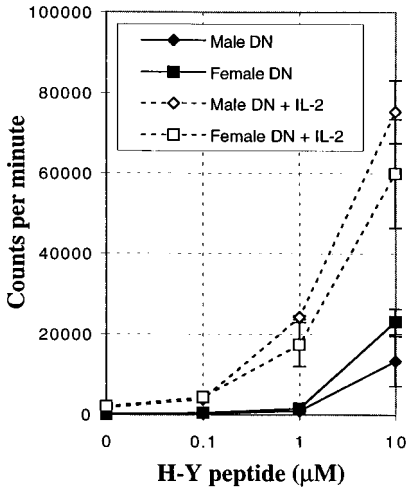

Figure 10. DN H-Y TCR$^+$ cells from male H-2^b H-Y TCR transgenic mice are not anergic. DN cells were purified from the lymph nodes of female or male H-2^b H-Y TCR transgenic mice. Both populations of DN cells expressed equivalent levels of H-Y TCR (data not shown). The DN cells (3×10^4/well) were stimulated with irradiated spleen cells (5×10^{10}) from H-2^b TAP-1$^{-/-}$ mice and the indicated concentration of the H-Y peptide. The proliferative response was determined by [^{3}H]-thymidine uptake after a 3-day culture period. Where indicated, IL-2 was added at 20 U/ml.

(37). In the 2C system, the 2C TCR has a high affinity for the p2Ca/L^d ligand (20). Our data suggest that physiological densities of the p2Ca/L^d ligand on APC are sufficient to anergize DN 1B2$^+$ T cells in Ag (L^d)-expressing mice, and this process is independent of CD8 coreceptor expression.

In this study we have shown that autoreactive DN 1B2$^+$ cells are anergized in Ag-expressing mice. Our ongoing studies revealed that this form of T cell anergy has the following unique feature. The anergic cells behave like Ag-primed cells, because they can be activated by a low affinity ligand to express high affinity IL-2R, and they proliferated vigorously in response to the low affinity ligand and exogenous sources of IL-2. Thus, the contribution of this unique form of anergy to autoimmune responses cannot be ignored. Furthermore, if these DN cells are, in fact, $\gamma\delta$ DN cells disguised as $\alpha\beta$ DN cells, then this system provides a convenient means of evaluating the role of $\gamma\delta$ cells in autoimmunity. This system is also amenable for the biochemical analysis of the signaling defects associated with this form of T cell anergy, because large numbers of anergic DN cells can be recovered from Ag-expressing mice.

Acknowledgments

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