CD1-restricted CD4$^+$ T Cells in Major Histocompatibility Complex Class II–deficient Mice

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Summary

Rather unexpectedly, major histocompatibility complex class II–deficient mice have a significant population of peripheral CD4$^+$ T lymphocytes. We have investigated these cells at the population and clonal levels. CD4$^+$ T lymphocytes from class II–deficient animals are thymically derived, appear early in ontogeny, exhibit the phenotype of resting memory cells, are potentially functional by several criteria, and have a diverse T cell receptor repertoire. They do not include substantially elevated numbers of NK1.1$^+$ cells. Hybridomas derived after polyclonal stimulation of the CD4$^+$ lymphocytes from class II–deficient animals include a subset with an unusual reactivity pattern, responding to splenocytes from many mouse strains including the strain of origin. Most members of this subset recognize the major histocompatibility complex class Ib molecule CD1; their heterogenous reactivities and T cell receptor usage further suggest the involvement of peptides and/or highly variable posttranslational modifications.

Most CD4$^+$ T cells depend on an interaction with the MHC class II molecules expressed on thymic stroma to complete differentiation. As a consequence, mice that lack the conventional class II molecules (II° mice)[1] have a drastic reduction in the number of mature CD4$^+$ T cells in the thymus and peripheral lymphoid organs (1–3). Unexpectedly, though, a small population of CD4$^+$ T cells was detected in the spleen and lymph nodes of these animals. This population did not appear to result from nonspecific "leakage" because the cells had atypical properties: they expressed markers reminiscent of memory cells and were preferentially localized in the B cell areas of lymphoid organs.

The existence of CD4$^+$ T cells in II° mice contradicts previous dogma on positive selection, which states that CD8$^+$ cells result from a TCR/MHC class I molecule engagement in the thymus and react in the periphery to antigen presented by class I molecules, while CD4$^+$ cells emerge after a TCR/class II interaction and have class II–restricted antigen reactivities (reviewed in reference 4). Since the CD4$^+$ cells in the II° mice could not have been selected on conventional class II molecules, several questions arose: When and where are these cells generated? Does their cell surface phenotype show other atypical features? Are they functional? If so, what ligands do they recognize? Here, we address these questions at the population and clonal levels. Besides determining some important general properties of the peripheral CD4$^+$ T cell population in II° mice, we describe an interesting set of hybridomas derived from this population.

Materials and Methods

Mice. II° mice (1), originally on a mixed C57BL/6J (B6) × 129 background, were mostly from the fifth to eighth generation backcross to B6; invariant chain–deficient mice (Ii°) (5) were on a mixed B6 × 129 background; B cell–deficient mice (6), were bred onto the nonobese diabetic (NOD) genetic background; β2–microglobulin–deficient mice (β2m°) (7) were on a B6 × 129 background. Most mice were maintained in specific pathogen–free conditions at the Centre de Selection d'Animaux de Laboratoire (Orleans, France). DBA/2J, 129, and NOD/Lt mice were bred in our animal facilities. The BALB.Mls^a congenic strain carries the Mls^a locus (Mtv-7) of DBA/2 mice on the BALB/c genetic background and was obtained thanks to R. Zinkernagel (Institute for Experimental Immunology, Zürich, Switzerland) and R. McDonald (Ludvig Institute for Cancer Research, Epalinges, Switzerland). Mice deficient in the transporter

[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; β2m°, β2 microglobulin-deficient mice; Ii°, invariant chain deficient; II° mice, mice that lack the conventional class II molecules; TAP, transporter associated with antigen processing.

associated with antigen processing-1(TAP-1° mice; mixed B6 × 129 background) were provided by Drs. A. Berns (The Netherlands Cancer Institute, Amsterdam, The Netherlands), H. Ploegh, and H. Van Santen (Massachusetts Institute of Technology, Cambridge, MA). BALB/cBy and C3H/He mice were purchased from IFFA-CREDO (L'Arbresle, France); B10.BR, B10.A(4R), B10.D2, B10.S, B10.M, B10, and BALB.B mice were from Harlan Olac (Oxon, England); and SJL, B6.Tla^a, and B6 mice were from The Jackson Laboratory (Bar Harbor, ME). The strains derived from wild mice *Mus spretus* (SEG), *Mus spicilegus* (ZRU), *Mus musculus castaneus* (CTA), and *Mus macedonicus* (XBS) were kind gifts from Dr. F. Bonhomme (Université de Montpellier II, Montpellier, France).

Flow Cytometry. Single cell suspensions were stained with mAbs of the following specificities: KT3 (CD3ε), KT4 (Vβ4), MR9-4 (Vβ5), 44.22.1 (Vβ6), KJ16 (Vβ8.1/8.2), RR3.15 (Vβ11), IM7 (CD44), H57-597 (TCRαβ), for references see (1), MR10-2 (Vβ9 [8]), B21.5 (Vβ10 [9]), B20.1.1 (Vα2 [10]), KT50 (Vα8 [11]), M17/4 (LFA-1α [12]), YN1/1.7.4 (ICAM-1/CD54), MEL-14 (L-selectin/CD62L [13]), 16A (CD45RB [14]), PC61 (CD25 [15]), PK136 (NK1.1 [16]), and 12-15A-biotin (CD2 [17]). Before cells from newborn and young mice were stained, they were incubated with the 2.4G2 α-FcγR antibody (18) to block nonspecific background staining. Labeled cells were analyzed on FACScan® (Becton Dickinson & Co., Mountain View, CA), ODAM ATC 3000 (Bruker Spectrospin, Wissembourg, Germany), or Coulter Epic Elite (Coulter Corp., Hialeah, FL) flow cytometers, and sorted using the ATC or Elite.

Intrathymic FITC Injections. Intrathymic FITC injections were performed as described (19). Both thymic lobes of the 5-wk-old mice were injected with 10 μl of 1 mg/ml FITC in PBS. 24–36 h later, thymus and lymph node cells were prepared and stained with α-CD4 and α-CD8 reagents. The level of FITC label on thymocytes was analyzed to evaluate the efficiency of intrathymic injection, and only successfully injected mice were analyzed further. Events of each sample were collected ungated, and gated for $CD4^+$ and $CD8^+$ cells (at least 50–100 × 10^3 events per gated population), and analyzed for frequencies of $FITC^+$ cells. Cells from noninjected or intrathorax-injected (19) mice were used as negative controls.

Bromodeoxyuridine (BrdU) Labeling. 0.8 mg BrdU in PBS was injected intraperitoneally into mice every 12 h for 2 d. 12 h after the last injection, mice were killed and lymph node cells were prepared. The cells were fixed first in 70% EtOH, then 1% paraformaldehyde, and stained with CD4-PE and CD8-biotin followed by streptavidin-cytochrome, then stained to detect incorporated BrdU after a modification (20) of (21) using anti-BrdU-FITC (Becton Dickinson).

T Cell Activation. Spleen and lymph node cells were pooled and depleted of B cells by panning with polyclonal rabbit anti-mouse Ig antibodies coated on plastic petri dishes, and then depleted of $CD8^+$ cells after labeling with CD8-biotin and streptavidin-magnetic beads using the MACS (magnetic cell separation) magnet (Miltenyi Biotec, Bergish-Gladbach, Germany), following the manufacturers instructions. The cells were then sorted twice for $CD4^+$ cells, resulting in a >95% pure population. In some experiments, the depletion steps were omitted. The sorted $CD4^+$ cells were stimulated at 1.5–3 × 10^4 cells per well in anti-CD3–coated (KT3 antibody coated at 5 μg/ml or as indicated) round-bottom, 96-well plates in Hepes-buffered RPMI 1640 medium containing glutamine supplemented with 10% FCS, 20 μM 2-ME, 1 mM Na-pyruvate, and penicillin/streptomycin. 2 d later, the wells were pulsed with 1 μCi [^{3}H]TdR and were harvested 16–20 h later. The amount of incorporated [^{3}H]TdR was measured in a gas scintillation Matrix Direct Beta Counter 9600 (Packard Instrument Co., Meriden, CT) with a counting efficiency of 25% compared with liquid scintillation counting.

Analysis of Lymphokine Production. At the indicated times during in vitro stimulation, supernatants were harvested and dilutions were added to 10^4 CTLL cells per well. After 24 h of culture, wells were pulsed with 1 μCi [^{3}H]TdR for 3–6 h and harvested. IL-4 and IFN-γ were quantitated in an ELISA using the BVD4-1D11 (anti–IL-4) or AN-18 (anti–IFN-γ) antibodies to coat 96-well plates, followed by incubation with dilutions of culture supernatant. Bound lymphokine was revealed by a biotinylated second IL-4 (BVD6-24G2)– or IFN-γ (R4-GA2)–specific antibody, streptavidin–alkaline phosphatase and the enzyme substrate (104 phosphatase substrate tablets; Sigma Immunochemicals, St. Louis, MO).

T Cell Hybridomas. After 4–5 d of stimulation by plate-bound anti-CD3, $CD4^+$ T cell blasts were fused with TCR-$\alpha^-\beta^-$ BW5147 cells (22) according to standard protocols (23). Growing hybridomas were stained for TCR and CD4 expression, and only double-positive hybridomas were used for further analysis. For subsequent hybridoma stimulation assays, 3–5 × 10^4 hybridoma cells were cultured in 200 μl medium (supplemented as above) together with graded numbers of spleen (40–5 × 10^4 per well) or cell line (40–5 × 10^3 per well) stimulator cells. Anti-CD3 stimulation (see above) was used as a positive control. After 15–20 h, IL-2 release was evaluated in a CTLL assay as described above.

Cell Lines. L cells expressing Qa-1^b (as a cDNA of the T23 gene, clone 18.6.2A1 (24), $T10^{129}$ (25), or $T22^{129}$ genes (25), and RMA cells expressing CD1.1 (Teitell, M., H.R. Holcombe, M.J. Jackson, L. Pond, S. P. Balk, C. Terhorst, P.A. Peterson, and M. Kronenberg, manuscript submitted for publication) and the TL antigen (encoded by the *T18^d* gene [see reference 26]) were used to stimulate hybridomas as described above. Transfections of L cells were made with Qa-1^b– (24) and CD1.1– (Teitell, M., et al., manuscript submitted for publication) encoding plasmids using the calcium phosphate precipitation method.

TCR Sequencing. PCR sequencing of TCR-α and -β cDNAs from sorted populations of $CD4^+$ cells or from hybridomas was performed after amplification with degenerate Vα and Vβ primers as detailed previously (27). For Vα14-specific amplifications, the V region primer was 5′-GTCCTGACCTCGCATGCGA-CAACCACTTAAGGTGGTTC.

Results

A Peripheral Population of $CD4^+$ T Cells Independent of Conventional Class I and II Molecules. In adult II° mice, 5–15% of the usual number of $CD4^+$ cells can be found in the spleen and lymph nodes (Fig. 1 *A*), and 90% of these express the αβ TCR (references 1–3 and data not shown). As previously discussed (1), the simplest explanation for such cells is that they were positively selected on MHC class I molecules. To test this, we crossed the II° line with mice deficient in class I expression because of a disrupted β2m gene (7). The double mutants had a peripheral population of $CD4^+$ cells similar in numbers and phenotype to that of II° animals (Fig. 1 and data not shown). Such cells were also present in B cell–deficient/II° mice (data not shown), ruling out a dependence on molecules expressed on B cells, such as Igs or superantigens, as well as in Ii°/II° animals, ar-

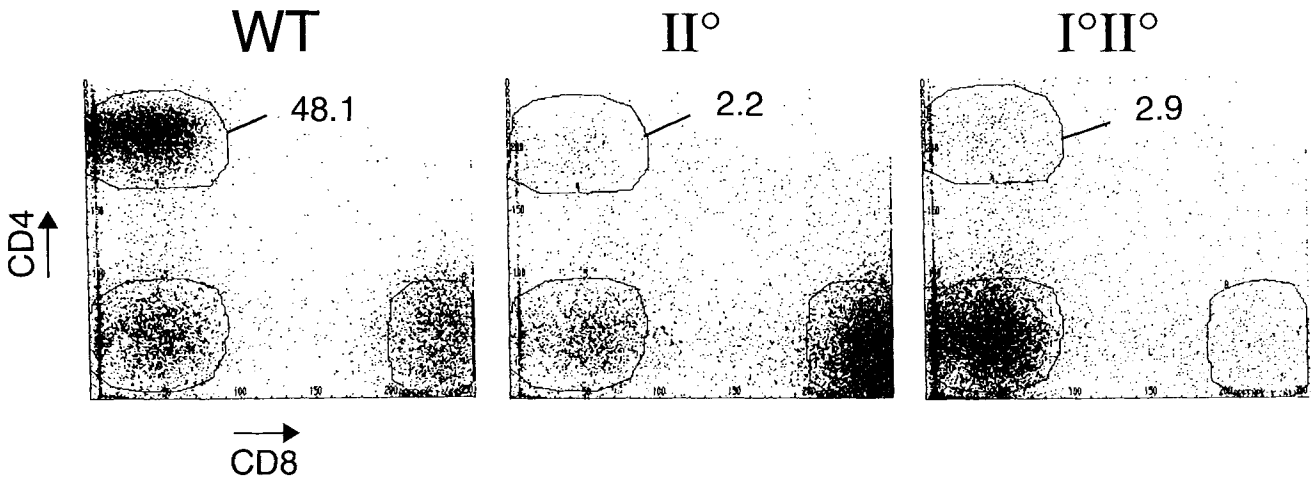

Figure 1. Peripheral CD4$^+$ T cells in MHC class II–deficient mice were not dependent on class I molecules. Lymph node cells from wild-type, class II–deficient (*II°*), and β2m/class II double-deficient (*I°II°*) littermate mice were stained for the CD4 and CD8 markers. Dot plots show typical distributions in several animals analyzed. The numbers show the percentage of CD4$^+$ cells in the indicated gates. The total number of cells in spleen or lymph nodes of the two mutants did not vary significantly from the wild type.

guing against their selection on a nonconventional class II–like molecule (Viville, S., C. Benoist, and D. Mathis, unpublished observation).

Population Dynamics of the CD4$^+$ T Cells in II° Mice. To study the ontogeny of this unusual population, we followed the appearance of CD4$^+$CD3$^+$ cells in the spleen and mesenteric lymph nodes of mutant and control animals from the 1st d after birth. A CD4$^+$CD3$^+$ population was clearly detectable in the two organs of both types of mice during the 1st wk of life. In both cases, the number of CD4$^+$CD3$^+$ cells increased ~10-fold during the subsequent 2 wk (quantitated in the spleen; Fig. 2). Once established, the II° CD4$^+$ population was maintained without changing size, even in mice >1 yr old (not shown).

We next sought to determine whether the CD4$^+$ cells in II° mice originate in the thymus (19). Thymocytes from 5 wk-old mutant and control mice were marked in situ by intrathymic injection of FITC. 30 h later, lymph nodes were analyzed for the presence of thymus-derived, FITC-labeled cells (Fig. 3 *A*). CD4$^+$ and CD8$^+$ cells from control animals and CD8$^+$ cells from mutants were all labeled to a similar extent. FITC$^+$ early thymic emigrants were also readily detectable among lymph node CD4$^+$ cells in II° mice, albeit at frequencies slightly lower than that of control CD4$^+$ and CD8$^+$ cells. The rate of export of CD4$^+$ cells from the II° thymus was ~1/50 of the control thymus, allowing an approximation of 1–2 × 10^4 cells per d (19).

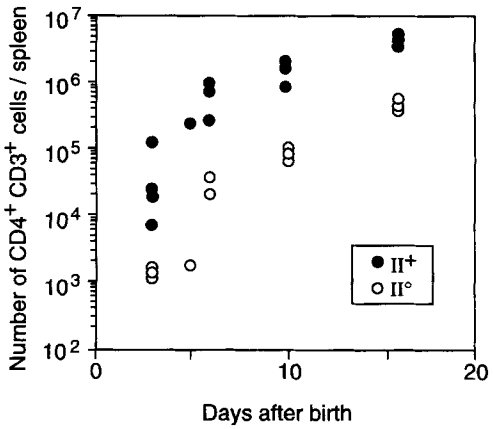

Figure 2. Early appearance of CD4$^+$ CD3$^+$ cells in the spleen of II° and control II$^+$ mice. On the indicated days, spleen cells were prepared from mixed litters of II° and wild-type heterozygote control mice, and they were analyzed for CD4, CD8, and CD3 expression. Each dot represents pooled cells or the mean value from at least three littermates of II° or II$^+$ phenotype.

To determine the proportion of cycling cells in the established CD4$^+$ population, we exposed control and mutant mice to BrdU continously for 48 h. As for the control II$^+$ (MHC class II–positive) mice, only a small proportion of lymph node CD4$^+$ cells in II° mice had incorporated BrdU during the labeling period (5–11%; see Fig. 3 *B*), indicating that only a minor fraction was actively dividing.

Altogether, these data indicate a stable population of CD4$^+$ T cells in II° mice. Its thymic input rate and proliferative turnover rate are not markedly different from those exhibited by the CD4$^+$ T cells of wild-type animals.

Cell Surface Marker Profiles. The initial description of CD4$^+$ T cells in II° mice indicated atypical cell surface antigen profiles: reduced levels of CD3/TCR and high levels of CD44. We extended this phenotypic analysis by using several additional antibodies that detect T cell differentiation and activation markers (Fig. 4).

The cells expressed normal levels of the T cell markers CD2 and CD28 (Fig. 4), as well as CD5 and Thy-1 (not shown). In contrast to control CD4$^+$ T cells, 75–80% of the CD4$^+$ cells from mutant mice had a phenotype characteristic of memory cells (for review, see reference 28): CD45RB^{low}, CD62L$^-$ and increased levels of CD54 (ICAM-1) and CD11a (LFA-1). Nevertheless, the majority of cells were in a resting state as evidenced by generally low

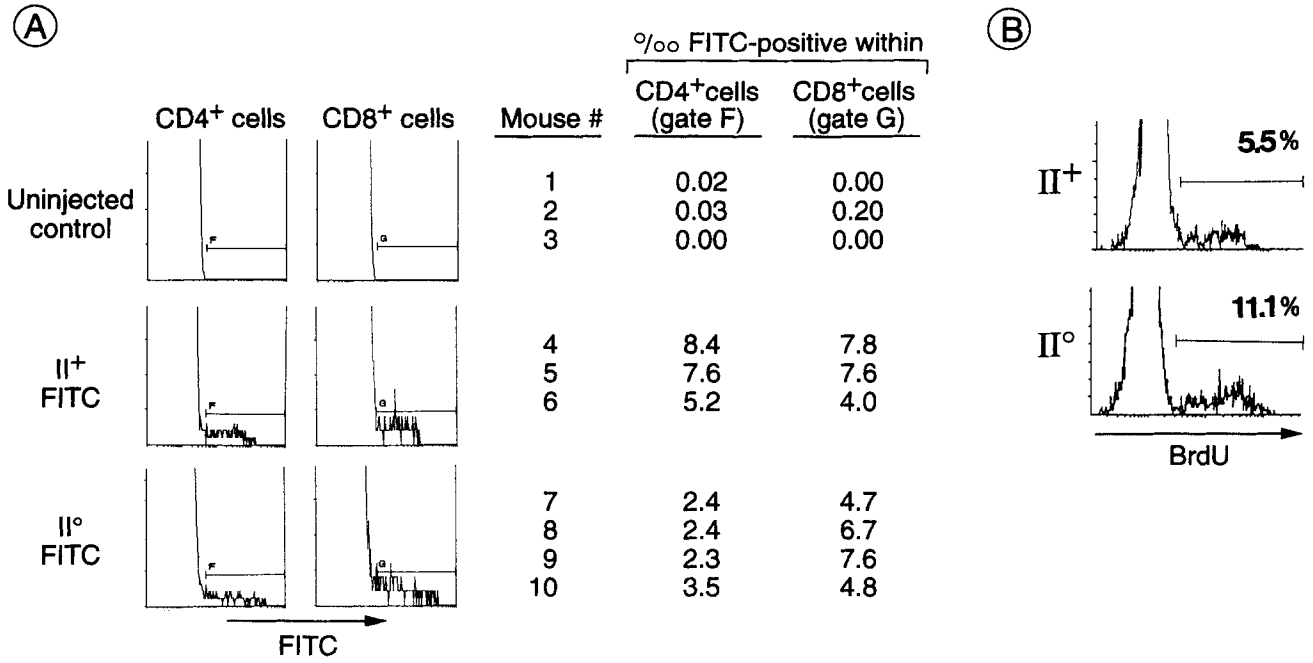

Figure 3. Thymic output and proliferative turnover of $CD4^+$ cells in II° and control mice. (*A*) Thymocytes of 5-wk old II° and II^+ mice were labeled in situ by injection of FITC into the thymus. 30 h later, lymph node cells were stained and analyzed for the presence of FITC-positive $CD4^+$ and $CD8^+$ cells. The histograms show representative green fluorescence profiles of gated $CD4^+$ or $CD8^+$ cells from an uninjected control mouse, as well as FITC-injected II^+ and II° mice. Gates for FITC-positive cells were set as shown (gates *G* and *F*), and values (0/00 = number of cells per thousand) from three to four individual mice in each group are shown on the right. (*B*) Adult mice were injected with BrdU at 12-h intervals during 2 d. Lymph node cells were stained for incorporated BrdU, CD4, and CD8 expression, and they were analyzed for the frequency of BrdU-labeled cells in each population. The histograms show BrdU staining of gated $CD4^+$ cells from control and II° mice.

CD69 and CD25 expression. The fraction of CD69-expressing $CD4^+$ cells in II° mice was somewhat elevated (20–30%, compared with 5–10% in II^+ $CD4^+$ cells), but still represented only a minority of the population, consistent with the cell cycle analysis mentioned above.

An unusual population of thymocytes and peripheral T lymphocytes has recently been described. These cells are positive for the NK1.1 marker (29, 30), their selection is β2m dependent (31–34), and they preferentially express certain Vα and Vβ segments (29, 30, 35–37). The frequency of $CD4^+$ cells expressing the NK1.1 marker was higher in II° than II^+ mice: 15–25% of the $CD4^+$ cells in spleen or lymph node in II° mice were positive, while only 2–6% of control $CD4^+$ cells were. However, given that II° mice have 10–20-fold fewer $CD4^+$ cells than II^+ mice, the absolute number of $NK1.1^+$ $CD4^+$ cells in both animals was similar, a finding in agreement with previous results (32–34). We conclude, therefore, that the $NK1.1^+$ population is only a subset of the $CD4^+$ cells in II° mice. This point is further substantiated by the broad TCR repertoire of $CD4^+$ cells in II° mice (see below).

Functional Potential. The next priority was to establish to what extent the $CD4^+$ T cells from II° mice function like normal CD4 T cells. Purified $CD4^+$ cells from both mutant and control animals proliferated vigorously in response to plate-bound anti-CD3 (Fig. 5 *A*) or staphylococcal enterotoxin B in the presence of splenic APCs (not shown), the II° $CD4^+$ cells often slightly better. Anti-CD3 stimulation also led to upregulation of the early activation markers CD69 and CD25 on both mutant and control $CD4^+$ cells (not shown).

Stimulation with anti-CD3 induced the $CD4^+$cells from

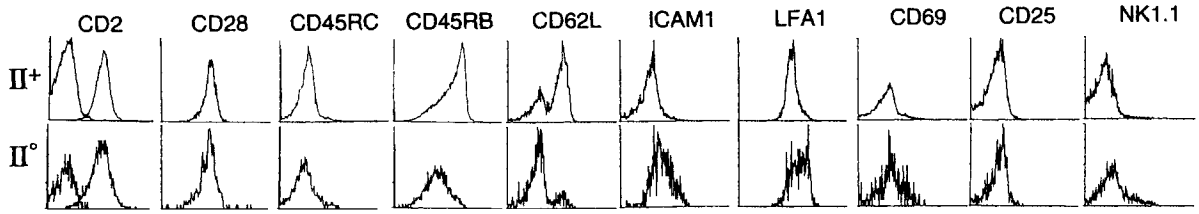

Figure 4. $CD4^+$ T cells in class II° animals display the phenotype of resting memory cells. Lymph node cells from adult II^+ and II° mice were stained for CD4 and CD8 in combination with various cell surface markers as detailed in Materials and Methods. Histograms, representative of several experiments, show the expression of the indicated markers on gated $CD4^+$ cells from both types of mice. A typical negative control is overlayed in the first panel on the left.

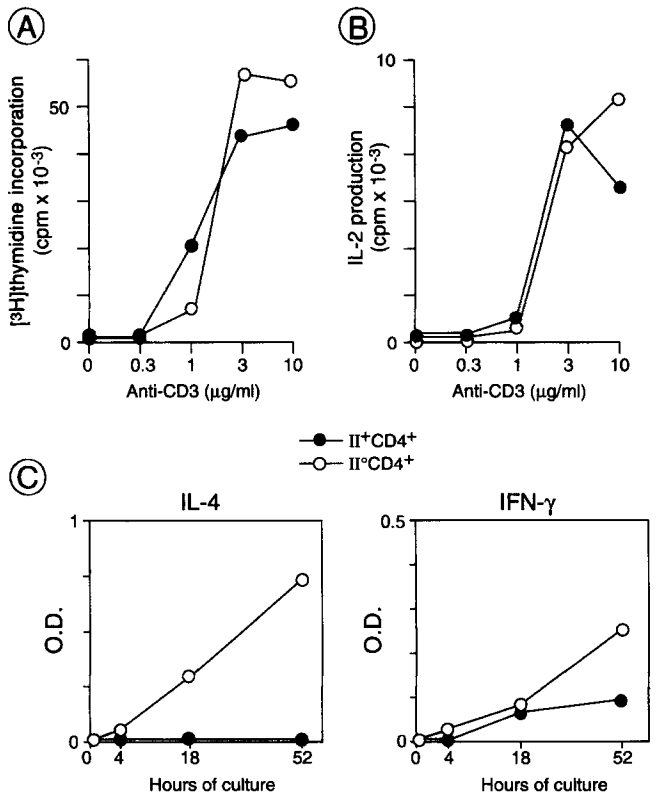

Figure 5. Responsiveness of purified CD4+ cells from class II° and control mice. Sorted CD4+ cells were activated with plate-bound anti-CD3. (*A*) Proliferation. Incorporation of [3H]TdR was measured on days 2–3. (*B*) IL-2 production. After 24 h of culture, supernatants were harvested and IL-2 production was estimated. IL-2 production is presented as [3H]TdR incorporation of the CTLL indicator cells. (*C*) Kinetics of IL-4 and IFN-γ production. After 4, 18, and 53 h of stimulation, supernatants were harvested and analyzed for IL-4 and IFN-γ content by ELISA. Relative amounts of cytokine are displayed as the read OD value for undiluted supernatant.

II° mice to produce similar amounts of IL-2 (Fig. 5 *B*), and more IL-3 (not shown), IL-4 , and IFN-γ (Fig. 5 *C*) than CD4+ cells from control mice. The main difference concerned IL-4, quite abundant in mutant CD4+ cells but very low to undetectable in wild-type cells, as expected for naive cells. Mutant-derived CD4+ cells secreted cytokines already at 4 h; in both cases, the levels of IL-4 and IFN-γ in the supernatant increased until day 2 (Fig. 5 *C*). Strikingly, the IL-4/IFN-γ ratio produced by the CD4+ cells derived from II° animals was several-fold higher than that of control CD4+ cells.

Furthermore, when Th activity of II° and control CD4+ cells was compared in a nonspecific B cell help assay in vitro, purified CD4+ cells from mutant and control mice induced B cell proliferation to similar levels (not shown).

Together, these findings argue that the CD4+ T cells in II° mice are functionally similar to control CD4+ cells but they secrete different ratios of lymphokines when activated.

TCR Repertoire. The TCR repertoire displayed by CD4+ cells from II° mice was first investigated by quantitating expression of various Vβs and Vαs on lymph node cells. Our initial studies (1), using conventionally housed mice at earlier stages of the backcross to B6, had shown some variability in Vβ usage between individual II° mice. This variability was not confirmed in the present study (Table 1). CD4+ cells in II° mice made broad use of Vβ segments, exhibiting an overall pattern comparable with that of CD4+ cells in control littermates. Some differences did emerge, however. Vβ5 and Vβ9 are much rarer in CD4+ than CD8+ cells of normal mice (Table 1 and reference 38); these Vβs were found in greater proportions in CD4+ cells of II° animals. Conversely Vβ4 is overrepresented in CD4+ vs CD8+ cells of normal mice (Table 1 and reference 39), and was used at a low frequency in CD4+ cells from II° animals. There are also a few peculiarities specific

Table 1. *TCR V Region Usage in II° and Control Lymph Node T Cells*

	Vβ4		Vβ5		Vβ6		Vβ8		Vβ9		Vβ10		Vβ11		Vα2		Vα8	
CD4																		
II+	7.3	6.7	2.3	2.8	8.4	8.8	18.2	16.8	0.8	1.1	4.7	4.0	4.6	4.3	10.3	—	2.1	—
	7.0	6.9	3.2	—	8.5	8.1	17.6	17.2	0.8	—	4.2	4.8	4.9	5.7	12.8	14.1	2.6	3.6
II°	**2.2**	**2.7**	**7.0**	**8.9**	5.8	6.1	19.9	17.6	**2.1**	**3.2**	1.8	2.3	4.2	3.4	**—**	**2.8**	—	1.1
	2.6	**4.2**	**6.1**	**—**	5.3	7.1	20.8	21.2	**2.3**	**—**	1.9	1.9	2.2	4.2	**2.1**	**4.4**	1.7	5.0
CD8																		
II+	3.8	3.4	14.7	14.2	6.7	7.6	16.8	16.8	2.7	2.3	4.5	4.9	6.4	6.2	7.1	—	3.0	—
	3.6	3.6	15.9	—	7.1	6.7	16.4	16.0	2.4	—	4.7	4.4	6.6	7.3	8.1	8.7	4.3	6.3
II°	4.3	4.3	17.8	17.2	8.3	8.2	15.7	15.8	1.9	2.1	3.6	3.3	6.3	6.3	—	5.5	—	1.7
	4.3	4.7	17.9	—	8.6	9.2	16.5	16.1	1.9	—	3.4	3.3	6.0	6.9	7.2	8.6	3.3	6.9

Lymph node cells from II° and II+ littermate control mice were stained and analyzed by flow cytometry for expression of the indicated TCR V segments. The percentage of positive cells within gated CD4+ and CD8+ populations of individual II+ and II° animals are shown. —, not done. Values in bold indicate ≥2.5-fold difference in V segment representation (mean value) between II+ and II° CD4+ cells.

to the II° $CD4^+$ population, notably the low Vα2 usage. Overall, the pattern of V segment usage by $CD4^+$ cells from mutant mice was subtly different from that exhibited by conventional MHC class I– or class II–restricted cells.

Additional evidence that the II° $CD4^+$ cells display a broad repertoire was obtained by sequencing VDJ regions from TCR-β chains (Bogue, M., C. Benoist, and D. Mathis, unpublished results). Vβ8.2 chains showed a high diversity in the VDJ joining region, displaying the usual spectrum of N nucleotides, J region usage, distribution of CDR3 lengths, and amino acid composition in the CDR3 loop.

High Frequency of Autoreactive Specificities among Hybridomas from II° Mice. Since the $CD4^+$ cells from II° mice represent a diverse population of potentially functional cells, it was of interest to identify their restricting (and possibly selecting) elements. We tried to derive allo- or autoreactive hybridomas on the grounds that such reactivities might give us a clue to their natural restricting elements, much as MHC class II–directed allo- and autoreactive cells from normal mice reflect the class II restriction of the bulk of the $CD4^+$ cells. To generate a panel of hybridomas, we purified $CD4^+$ T cells from several II° and II^+ littermates, stimulated them with anti-CD3, and fused them with TCR-$\alpha^-\beta^-$ BW5147 cells. We then challenged the hybridomas with splenocytes from several mouse strains, differing at MHC, minor histocompatibility, and/or endogenous murine tumor virus loci.

As indicated in Fig. 6, very few of the hybridomas from II^+ littermates responded to any spleen cell stimuli, save for a scattered few whose reactivities correlated with H-2 haplotype (e.g., XII44 and XIV18, reactive to $H\text{-}2^d$) or with endogenous murine tumor virus genes (e.g., XII49 and XIV50, Mls^a); only one (X17) was autoreactive (A^b-reactive). The reactivity pattern of the hybridomas derived from II° mice was very different: while there was no activation when they were cultured alone, >10% responded to syngeneic II° spleen cells in the absence of any added antigen. All of these autoreactive hybridomas, which originated from several mice, also displayed a broad reactivity pattern, since all but two were stimulated by spleen cells from every mouse strain tested. This suggested a nonpolymorphic target, clearly distinct from classical MHC class I and II molecules.

Recognition of Class I–like Ligands by the Autoreactive Hybridomas. To investigate the nature of the nonconventional ligand(s) recognized by the polyreactive hybridomas derived from mutant mice, we selected 11 for further analysis. Although a population of $CD4^+$ T cells was present in $\beta2m°/II°$ double-mutant animals, this did not entirely rule out peripheral reactivity to MHC molecules, and the nonpolymorphic nature of the ligands was reminiscent of nonclassical MHC molecules. We therefore considered the possibility that such molecules, either class II– or class I–like, might serve as ligands. Their expression might well depend, for the former, on the Ii involved in trafficking of conventional MHC class II molecules, or for the latter, on β2m. We thus tested the panel of hybridomas with spleen cells from mutant Ii° or β2m° mice (5, 7). All of the hybridomas were well stimulated by Ii-deficient APCs (not shown), while Ii° APCs were not recognized by control A^b-reactive hybridomas. On the other hand, 6 of the 11 hybridomas did not respond to splenocytes from β2m° mice (Fig. 7). This encouraged us to pursue the idea that nonpolymorphic class I–like molecules are recognized by the autoreactive hybridomas from II° mice.

Qa-1 Recognized by One of the Autoreactive Hybridomas. The reactivity of the β2m-dependent hybridoma VII11 appeared to correlate with the expression of the b allele of the class Ib molecule Qa-1 (Fischer-Lindahl, K., personal communication). For example, as shown in Fig. 6, splenocytes from the strains C3H/He ($H\text{-}2^k$) and B10.S ($H\text{-}2^s$) both express $Qa\text{-}1^b$ and elicited a response, while those from B10.BR ($H\text{-}2^k$) and SJL ($H\text{-}2^s$) show $Qa\text{-}1^a$ and did not. This correlation was extended by using a pair of congenic strains. B6 mice express the b allele of Qa-1, while $B6.Tla^a$ animals are congenic for the T region, including the Qa-1 encoding *T23* gene, and carry the $Qa\text{-}1^a$ allele. B6 but not $B6.Tla^a$ spleen cells were well recognized by the VII11.1 hybridoma (not shown). Finally, we directly tested whether Qa-1 was being recognized by stimulating the VII11.1 hybridoma with L cells transfected with the $T23^b$ gene (24). The hybridoma responded specifically to transfectants expressing $Qa\text{-}1^b$, but not to mock transfected cells nor to cells transfected with other Ib genes (Fig. 8).

CD1 Recognized by the Majority of the Autoreactive Hybridomas. For the other autoreactive hybridomas, the reactivities depicted in Fig. 6 were too broad to give much clue as to the targets involved. We could exclude the class Ib molecule Qa-2, as the stimulating ligand(s), was found to be present on splenocytes of B10.M mice (not shown) that carry a deletion encompassing the genes encoding surface-bound Qa-2 (40). Therefore, we tested the panel against a set of transfectants expressing other class Ib molecules: the T region–encoded antigens TL (26), T22, and T10 (25), and a molecule encoded outside the MHC, CD1 (Teitell, M., et al., manuscript submitted for publication). Both the β2m-dependent and -independent hybridomas were included in the screening because there is at least one example of a class Ib molecule that shows variable dependence on β2m for surface expression (41). The results are presented in Fig. 8. Strikingly, seven hybridomas, including two β2m-independent, specifically recognized transfectants expressing CD1.1; these reactivities could be detected with CD1.1-transfected RMA or L cells (not shown).

TAP-independent Trafficking of the Target Ligands. Peptide loading and surface expression of classical MHC class I molecules are generally dependent on the TAP molecule (see reference 42 and references therein), but this is not always the case for nonclassical class I molecules (43–45). To determine whether the ligands recognized by our unusual $CD4^+$ T hybridomas depend on the *TAP* gene for expression, we stimulated the panel with spleen APC from mice carrying a debilitating mutation in the *TAP-1* gene (42). While the Qa-1 ligand seen by the VII11 hybridoma required TAP to be expressed, none of the ligands recog-

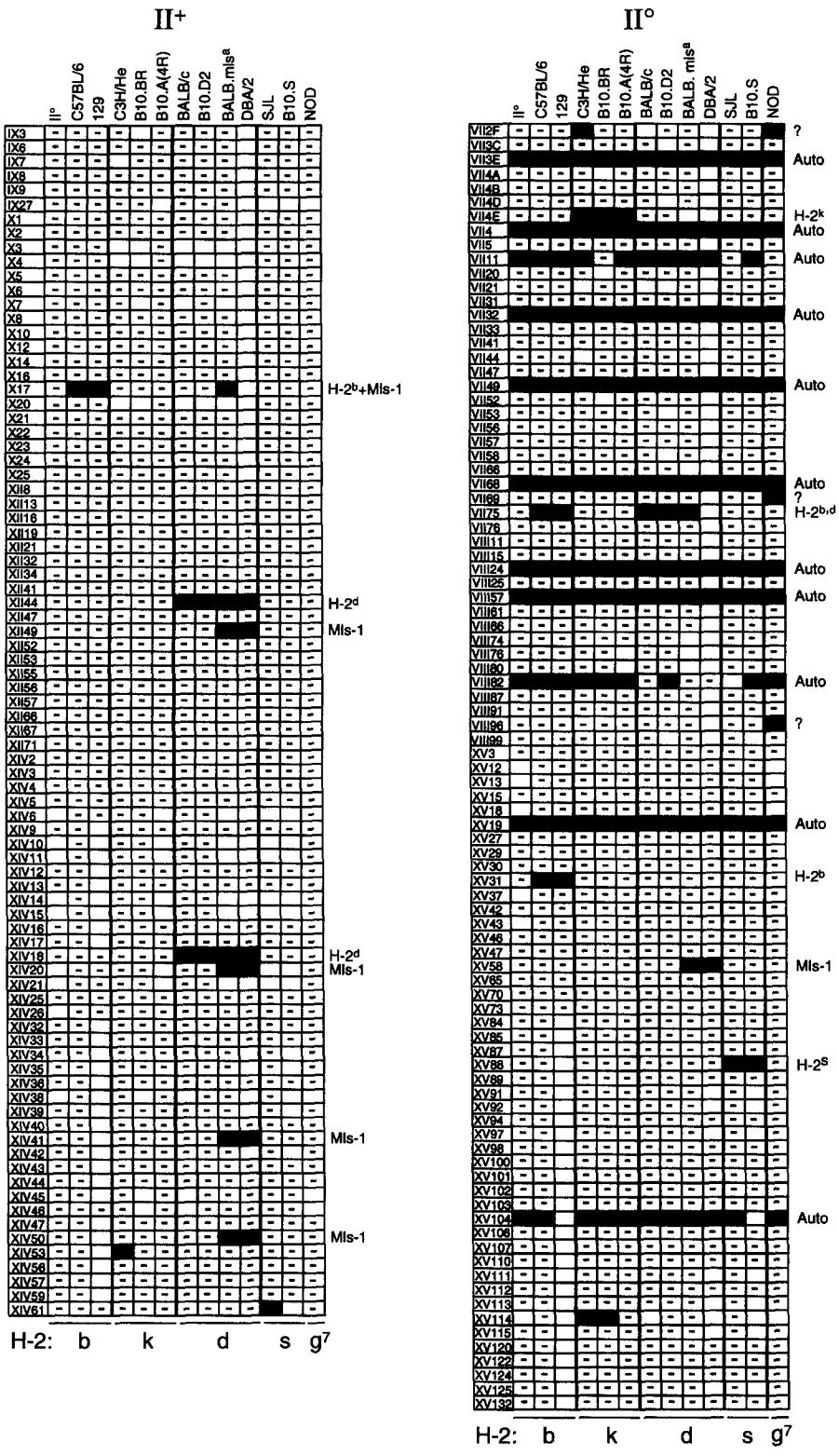

Figure 6. Allostimulation of CD4$^+$ hybridomas derived from II$^+$ and II° mice. Hybridomas were challenged with splenic APC from the indicated mouse strains. After 15–20 h of culture, supernatants were harvested and release of IL-2 was evaluated. Anti-CD3 stimulation was used as positive control for hybridoma function (not shown). Black boxes indicate positive reaction, – indicates a negative reaction, and empty boxes were not done. None of the hybridomas secreted IL-2 in the absence of APC. The presented results are a compilation of several experiments. Correlating loci are indicated on the right. The A^b specificity of VII75 and X17 has been confirmed by blocking their reactivities with specific mAbs, while the Mls-1 reactivity of X17 is caused by Vβ6 expression (not shown).

nized by the CD1-reactive hybridomas were TAP dependent (representative examples are shown in Fig. 9). This was true whether or not their expression was dependent on β2m.

Heterogeneity of the CD1-reactive Hybridomas. Given the high frequency of CD1-reactive clones among the autoreactive CD4$^+$ cells in II° mice, two questions arose. (*a*) Did these cells display a monomorphic specificity, perhaps accompanied by a constrained TCR repertoire? (*b*) Is the CD1 target recognized by itself or in conjunction with other molecules: peptides such as classical MHC molecules, or lipids as recently reported (46)?

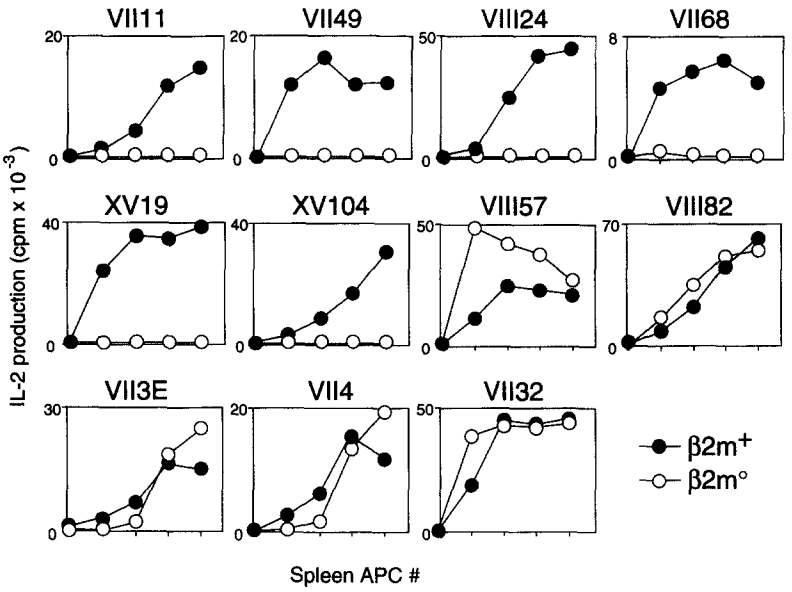

Figure 7. Recognition of a β2m-dependent ligand by several of the autoreactive hybridomas from II° mice. Broadly reactive hybridomas were stimulated with spleen cells from β2m° and wild-type littermate mice. Splenic APCs were added in serial twofold dilutions, from a maximum at 4×10^5 cells per well.

The heterogeneity of the hybridomas was evaluated by a number of criteria, as summarized in Fig. 10. TCR V region sequencing showed diverse usage of Vβ and Vα regions, with variable CDR3 lengths and amino acid compositions (not shown). It may be worth emphasizing that none of our CD1-reactive hybridomas expressed the invariant Vβ14–Jβ15 sequence characteristic of NK1.1 $CD4^+$ cells (37), as detemined by reverse transcription PCR with Vβ14-specific primers (not shown).

Heterogeneity was more evident in the responses to stimulation by APCs from different mutant or inbred mouse strains. As mentioned above (Fig. 7), two of the seven CD1-reactive hybridomas were capable of responding to APCs from β2m° mice. The μMT knockout mutation, which abrogates B cell development (6), prevented splenic APCs from stimulating two other hybridomas, indicating that B cells are necessary presenting cells in these instances. As already indicated in Fig. 6, most of the hybridomas responded to all of the nonmutant common laboratory strains. Clone VIII82, however, reacted very poorly to APCs from BALB/c mice, even though B6 and BALB/c mice have essentially identical CD1.1 molecules and CD1.2 molecules that differ only by one amino acid (47, 48). Finally, we tested the hybridomas against splenic APCs from various subspecies of Mus (Fig. 10). Here again, the responses led to splits. These results clearly indicate that the CD1-specific hybridomas are heterogeneous and do not recognize one simple ligand.

Discussion

A Diverse but Distinct Population. Our analysis of the $CD4^+$ T lymphocytes found in the periphery of II° mice indicated that these cells share many characteristics with the major $CD4^+$ T lymphocyte subset found in normal animals. By several criteria, the dynamics of the two populations appeared similar: ontogeny, thymic input, and division rate. Furthermore, the two seemed to have comparable functional potentials: they were stimulated to about the same degree with polyclonal reagents, secreted lymphokines, and they proliferated upon activation and provided "help" to B cells. Finally, both populations displayed a diverse repertoire of TCRs.

Nevertheless, the $CD4^+$ population from II° mice displayed some special features. These cells localized preferentially to the B rather than T cell areas in the peripheral lymphoid organs (1). Perhaps, most strikingly, the majority of them expressed cell surface markers reminiscent of resting memory cells, while in normal mice, only 10–20% of the $CD4^+$ T lymphocytes show this phenotype. Also atypical was their capacity to produce high amounts of lymphokines, particularly IL-4, upon activation. Lastly, although the II° $CD4^+$ cells, like their II^+ counterparts, displayed a diverse TCR repertoire, this repertoire did exhibit some particularities, notably altered levels of certain Vβs and a dearth of $V\alpha2^+$ receptors.

Altogether, one is left with the impression of a population sharing many features with the normal $CD4^+$ T lymphocyte population, yet not identical to it. We do not formally know whether these cells are a peculiarity of II° mice. They have not so far been identified in normal mice but could just be masked by the bulk $CD4^+$ T cell population.

The $CD4^+$ cells from II° mice were also distinct from the unusual population of $NK1.1^+$ mature $CD4^+8^-$ and $CD4^-8^-$ thymocytes and peripheral T lymphocytes discovered several years ago (35, 36). An enriched proportion of these cells displays $V\beta8^+$ TCRs, and almost all of them exhibit an invariant TCR-α chain, Vβ14-J15 (29, 30, 35–37). The majority shows a cell surface phenotype similar to memory cells, particularly high levels of $CD44^+$ expression (29). $NK1.1^+$ lymphocytes are the major lymphokine-producing cells in the thymus (49–51), and peripheral $NK1.1^+$ cells have recently been recognized as major producers of

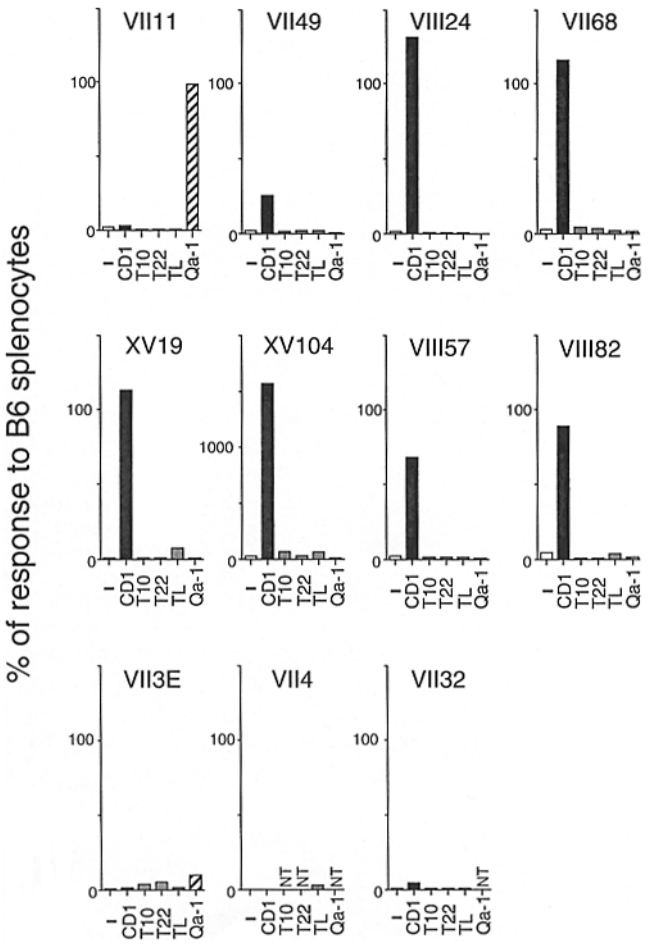

Figure 8. Activation by CD1 of most of the autoreactive hybridomas. Hybridomas were stimulated with transfectant cell lines expressing the indicated class Ib molecules and the respective untransfected controls. The stimulation obtained with B6 splenocytes was used as a reference value, and the responses to the transfectants are expressed as percentages of the response to B6 APC. The background value (−) obtained with the nontransfected control APC is shown.

IL-4 (52). The NK1.1$^+$ cells do not depend on MHC class II molecules for selection/expansion, but rather on expression of β2m (or MHC class I molecules) on bone marrow–derived cells (31–34). The CD4$^+$ population from II° mice does contain a component of NK1.1$^+$ cells; however, this is relatively minor, ~20–30%, with no enrichment in numbers. The bulk of the population has some features like NK1.1$^+$ cells , notably a memory phenotype and high production of lymphokines upon stimulation, but the differences between the two remain evident: a diverse vs restricted TCR repertoire and independence of vs dependence on expression of β2m. It is possible that the NK1.1$^+$ CD4$^+$ cells are a peculiar subset of the larger CD4$^+$ population we have defined in II° animals, perhaps expanded in response to a specific stimulus.

Recognition of MHC Class Ib Molecules. To study the reactivities of the CD4$^+$ lymphocytes from II° mice, we turned to an analysis at the clonal level. Initially, we attempted to derive antigen-specific hybridomas (not shown).

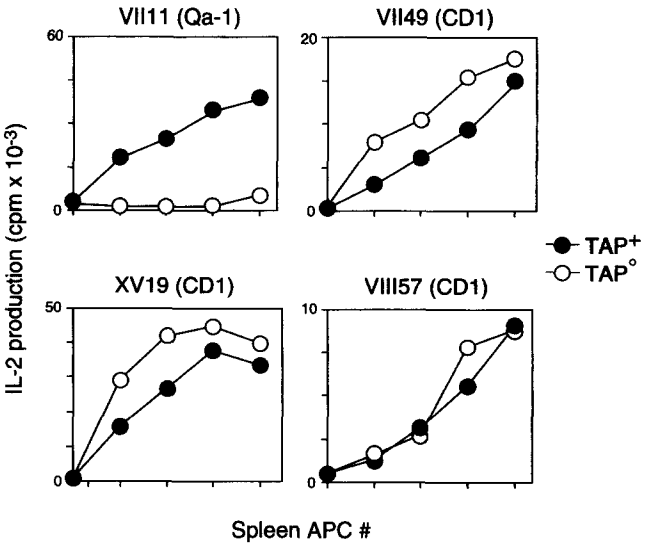

Figure 9. Differential TAP dependence of the class Ib ligands. Hybridoma cells were stimulated with splenic APC from TAP-1° and TAP-1$^+$ littermate mice. Splenic APCs were added as described in Fig. 7. While the Qa-1 recognized by VII11 was dependent on the presence of TAP-1 in the APCs, all CD1-reactive hybridomas were TAP independent; only a representative three are shown here.

II° mice were immunized with various proteins in complete Freund's adjuvant and sets of hybridomas derived from purified CD4$^+$ cells reactivated in vitro. The hybridomas were challenged with the protein antigen and with bacterial antigens such as heat shock protein 60 and purified protein derivative from mycobacteria, and preparations of heat-killed mycobacteria and listeria. No response to any of these challenges was elicited from the hybridomas. We

CD1-specific hybridoma	TCR α	TCR β	β2m°	μmT°	BALB/c	M.M. castaneus	M. spretus	M. spicilegus	M. macedonicus
VII49	8	9		■	■	■	■	■	■
VIII24	3.2	9		■	■	■			
VII68	4	11			■	■	■		■
XV19	nd	nd		■	■	■	■	■	
XV104	4/5	8.3				nd	nd	nd	nd
VIII57	2	5.1	■	■	■	■	■	■	
VIII82	10/11	nd	■	■		■	■	■	

Figure 10. Heterogeneity of the CD1-specific hybridomas. Results were compiled from several sets of experiments. Expression of TCR V segments was determined by sequencing of the TCR-α and -β chains and/or staining. For two hybridomas, two in-frame α chain sequences were found. A second TCR-α sequence was found for VII49 with an out of frame rearrangement. Surface expression of the V segment (κ chain for VIII24 and VIII82, β chain for VIII24, VII68, and VIII57) was verified by staining with V region–specific antibodies. Vβ9 expression of VII49 was determined by staining only. The VII57 Vα sequence is so far undescribed, but was tentatively assigned Vα since it had the highest homology (~80%) to members of this Vα family. The CD1-specific hybridomas were stimulated with splenic APCs from β2m°, B cell–deficient mice (μmT°), wild-type BALB/c mice, or several subspecies of Mus. *Black boxes*, positive reactions; *empty boxes*, no reaction; *nd*, not determined.

altered our strategy, therefore, this time preparing a large number of hybridomas after in vitro anti-CD3 activation of purified $CD4^+$ cells from II° and II^+ mice, and challenging them with splenocytes from a panel of mouse strains. We could identify hybridomas derived from both types of mice that exhibited anti-MHC or anti-Mls reactivities. Only in the case of II° mice, however, did we observe a significant percentage of hybridomas that were both auto- and polyreactive.

Focusing on 11 with this unusual pattern of reactivity, we explored their specificities using splenocytes from congenic and mutant mouse strains and cells transfected with MHC class I molecules. Many of the hybridomas reacted specifically to class Ib molecules: one recognized Qa-1, seven saw CD1, and three recognized unidentified ligands, all expressed independently of β2m.

One is led to ask whether reactivity to nonclassical class I molecules reflects the restricting specificity of the majority of the $CD4^+$ cells in II° mice, or is just a feature of a minor component of the population. Clearly, a large percentage of the autoreactive hybridomas recognized class Ib molecules. But most of them did not show reactivity to any of the APCs with which they were challenged. It is certainly possible that most of the $CD4^+$ cells in II° mice are restricted by class Ib molecules, including CD1, but that they react to these molecules in forms that are not present on splenocytes, e.g., in association with different ligands like peptides or lipids. The autoreactive clones could just represent the higher affinity fraction of a population of Ib-restricted cells, much like rare class II–autoreactive cells in wild-type mice betray the large class II–restricted population.

CD1 Recognition. Several recent findings have provoked interest in the class Ib molecule CD1. It is the only antigen-presenting Ib molecule known to have been conserved through evolution, and the homology between species is high (53). Interestingly, CD1 exhibits features similar to both class I and class II MHC molecules. On the one hand, the intron/exon structure of the CD1 gene and the corresponding domain structure of the protein are like those of standard class I genes and molecules, and there is sequence homology between the α3 domain of CD1 and the same domain of classical class I molecules (54). On the other hand, the α3 domain of CD1 has a similar degree of sequence homology with the β2 domain of class II molecules (54), and CD1's cell-type distribution (41, 53, 55) and antigen presentation pathway appear more characteristic of class II molecules (44). While murine CD1-restricted T cells have not yet been described, several human T cell clones recognizing CD1 have been isolated (44, 56–59): they are $CD4^-$ $CD8^-$ cells expressing either an αβ or γδ TCR. In one case, the presented antigen was of a novel type, mycolic acid, a unique lipid component of the mycobacterial cell wall (46). This observation reopens the question of whether CD1 can bind peptides at all, a question previously raised because sequence information suggested a very different structure in the ligand-binding region (60) and because surface expression of CD1 appeared independent of TAP molecules (44, 45). Indeed, TAP independence of murine CD1 was confirmed, at the functional level in our assay, and it has recently been demonstrated in transfectants (Teitell, M., et al., manuscript submitted for publication).

Our findings add several new features to the emerging portrait of this intriguing class Ib molecule. First of all, we found that $CD4^+$ T cells can also show CD1 reactivity. This may indicate that CD4 can serve as a coreceptor for CD1 recognition by direct CD1–CD4 contact. It has been demonstrated that CD8 can bind CD1 (Teitell, M., et al., manuscript submitted for publication), but the CD1 α3 domain similarity to both class I and II molecules may allow promiscuous binding to either coreceptor molecule. Secondly, we observed significant heterogeneity in the reactivity patterns and TCR usages of the different CD1-reactive hybridomas. This suggests that the target ligand may be composed of more than just the CD1 molecule itself, perhaps including peptides, lipids (46), or other posttranslational modifications. It seems unlikely, though, that a lipid moiety would be recognized by our hybridomas because the polymorphism we detect between very related Mus subspecies would be difficult to account for in this way. Finally, since the CD1-reactive hybridomas derive from the small $CD4^+$ population in II° mice, we have the means to dissect their function in vivo. Already, we know that they do not mount a response to several multiepitope protein antigens and are incapable of providing full B cell help, evidenced by a total lack of germinal centers in II° mice, no matter how or how much one stimulates them (1). We are left with several pressing questions: How are these autoreactive cells rendered and maintained innocuous in mice? What agents are capable of provoking them? What is the functional correlate of their impressive capacity to produce IL-4? What cells and molecules are responsible for their selection/expansion?

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