Brief Definitive Report

Altered Major Histocompatibility Complex Restriction in the NK1.1$^+$Ly-6C^{hi} Autoreactive CD4$^+$ T Cell Subset from Class II-deficient Mice

By Ilona Kariv, Richard R. Hardy, and Kyoko Hayakawa

From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Summary

We previously demonstrated selective enrichment of major histocompatibility complex (MHC) class II-specific autoreactive T cells in a subset of mouse CD4$^+$ thymocytes. Here we show that a significant fraction of these autoreactive cells in the normal adult thymus expresses NK1.1 and high levels of Ly-6C and also exhibits flexibility in MHC restriction. In normal mice, this NK1.1$^+$Ly-6C^{hi} subfraction accounts for 10–50% of the CD4$^+$ autoreactive subset and is enriched for MHC class II-restricted autoreactive cells as determined by mixed leukocyte reaction frequency analysis, similar to NK1.1$^-$Ly-6C$^-$CD4$^+$ autoreactive cells. In contrast, in the thymus of class II-deficient littermate mice, NK1.1$^+$Ly-6C^{hi} cells account for most of the mature heat stable antigen (HSA)$^-$CD4$^+$ fraction and exhibit MHC-restricted non-class II autoreactivity. Thus, NK1.1$^+$Ly-6C^{hi}CD4$^+$ T cells show flexibility in MHC class restriction, but their autoreactivity remains MHC dependent.

We previously reported that a portion of the mature heat stable antigen (HSA)$^-$CD4$^+$(8$^-$) T cell population in mouse thymus shows the functional capacity to secrete diverse cytokines and is enriched for MHC class II-specific autoreactivity (1,2). This "Thy0" subset, recognized as a 3G11$^-$ HSA$^-$ subfraction of CD4$^+$8$^{low/-}$ T cells, is detectable from early after birth and increases in frequency with age, coincident with an increase in the proportion of Vβ8$^+$ cells. Our previous study with TCR Vβ transgenic mice suggested a mechanism involving antigenic selection for generating Thy0, indicating that it is a secondary cell population produced in response to self/class II antigens present in the thymus (2).

Based on this model, we predicted a significant decrease in Thy0 in mice lacking class II, similar to what has been reported for peripheral CD4$^+$ T cells in these mice (3, 4). However, unexpectedly, we found that the lack of class II had little effect on the frequency of Thy0. Rather, analysis of Thy0 in these mice revealed the presence of MHC non-class II-restricted autoreactive CD4$^+$ T cells, a type of cell which is normally rare. We discuss this result in the context of a two-step selection model for α/β T cell development as proposed recently by Chan et al. (5) and extend it by introducing an additional differentiation pathway for autoreactive cells in thymus.

Materials and Methods

Mice. C57BL/6JN (B6), BALB/cAnN, and BALB.B mice were bred and maintained in the Institute for Cancer Research (ICR) animal facility. Mice lacking MHC class II produced by targeted mutation (here designated class II knockout [C2k/o]) were derived from mice originally described by Grusby et al. (3). One of the founder 129/Sv × C57BL/6J chimeric animals was backcrossed onto C57BL/6 five times and class II$^+$ and class II$^-$ littermates were purchased from GenPharm (Mountain View, CA). As described previously, the frequency of B cells in spleen of C2k/o mice was similar to that in class II$^+$ normal littermates (6). 2–4-mo-old female mice were used in most experiments.

Immunofluorescence Staining and Cell Sorting. Monoclonal antibodies used in this paper were: CD4 (GK1.5), CD8α (53-6.7), HSA (J11d, 30-F1), 3G11 (SM3G11), Ly-6C (AL-21), NK1.1 (PK136), CD3ϵ (500A-A2), and L-selectin (MEL-14). Antibody purification from ascites and coupling to fluorochromes (fluorescein, phycoerythrin, allophycocyanin) or to biotin have been described elsewhere (1). Four-color immunofluorescence analysis and cell sorting were carried out as described previously (1).

Autoreactivity by MLR Assay. The frequency of autoreactive cells was examined by MLR assay using 1 or 10 responder cells deposited by cell sorter, mixed with 7 × 10^5 irradiated spleen cells as stimulators per well. 96 wells were used for each subset and 3-d culture supernatant was tested by bioassay for the presence of IL-3 as described previously (2). Murine recombinant IL-3 was obtained from UBI (Upstate Biotechnology, Inc., Lake Placid, NY) and used

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as a standard to quantitative assay results. The cutoff level was determined from data with irradiated stimulatory cells alone. In parallel, anti-CD3 stimulation was carried out by using anti-CD3 precoated wells supplemented with 10^5 irradiated syngeneic spleen cells to obtain the frequency of anti-CD3 responding wells.

Results and Discussion

Fig. 1 shows the Thy0 cell subset in C2k/o (class II^-) mice and in wild-type littermates. In the thymus of C2k/o mice, only a few $CD4^+8^{low/-}$ cells were found, where most cells were $CD8^{low}$ rather than $CD8^-$, as reported previously (5). We analyzed the $CD4^+CD8^{low/-}$ cell fraction for expression of HSA and 3G11. As we showed elsewhere, the mature HSA^- fraction of $CD4^+$ T cells in normal thymus consists of two sets of cells that can be discriminated by expression of the 3G11 antigen. The majority, which express 3G11, are destined for export to the peripheral naive T cell pool and we have referred to this subset as Fr.I'. The remaining cells, that lack 3G11, we term Thy0 because of their intrathymic Th0 cytokine profile (1). The most significant distinction of the C2k/o mice was a virtual absence of HSA^-3G11^+ cells (Fr.I'), consistent with the lack of peripheral naive $CD4^+$ T cells (3, 4). On the other hand, immature HSA^+ cells and cells with the Thy0 phenotype were present. The frequency (and absolute number) of Thy0 in thymus of C2k/o mice was 75% of the level seen in wild-type mice (0.3 vs. 0.4%). These Thy0 cells in C2k/o mice produced a variety of cytokines, showed overrepresentation of TCR $V\beta8^+$ cells (data not shown), and exhibited autoreactivity as described below, all defining criteria for Thy0.

However, we found two unusual features of the Thy0 cells present in C2k/o mice: (*a*) a shift in cell surface phenotype, including predominant expression of NK1.1 and Ly-6C; and (*b*) altered MHC class restriction. Fig. 2 presents a comparison of cell surface phenotype for Thy0 from wild-type and C2k/o littermates in contrast with Fr.I'. As reported previously for Thy0 (1), these cells in both wild-type and C2k/o littermates expressed TCR/CD3 at similar low levels and most lacked L-selectin. However, we found that the majority of Thy0 cells in C2k/o mice are $NK1.1^+Ly\text{-}6C^{hi}$ (>90% of Thy0), in distinction to wild-type littermates where such cells were less frequent (<50% of Thy0). Most $NK1.1^+$ or $Ly\text{-}6C^{hi}$ cells in the $CD4^+8^{low/-}$ thymocyte population are contained in the Thy0 subset and the majority of $Ly\text{-}6C^{hi}$ Thy0 cells coexpressed NK1.1 (Kariv, J., R. R. Hardy, and K. Hayakawa, manuscript in preparation), similar to the $CD4^-CD8^-\alpha/\beta$ ($DN\alpha/\beta$) T cells of adult thymus (7–11). The extent of $V\beta8$ skewing was more prominent in this $NK1.1^+Ly\text{-}6C^{hi}$ fraction compared with the rest of Thy0, as expected from previous reports (12–14). Hereafter we refer to this $NK1.1^+Ly\text{-}6C^{hi}$Thy0 subfraction as "Thy0.2" and the remaining Thy0 cells as "Thy0.1".

As we reported previously, the bulk Thy0 population in normal mice shows autoreactivity to self/class II antigens (2). Therefore, we next investigated the frequency of autoreactive cells in the Thy0.2 subfraction both from normal and C2k/o littermates in order to discern potential differences in reactivity between Thy0.2 and Thy0.1. An autologous mixed leukocyte reaction (AMLR) system (with 1 or 10 responder cell/well) was used as described previously (2). Regions for Thy0.2 cell sorting are marked in Fig. 2, selecting either for $Ly\text{-}6C^{hi}$ or $NK1.1^+$ Thy0. As with unfractionated Thy0 (2), 30% of Thy0.2 cells from wild-type mice of B6 background assayed at the single cell level showed significant IL-3 production induced specifically by stimulation with autologous/syngeneic cells (data not shown). When responder cells were

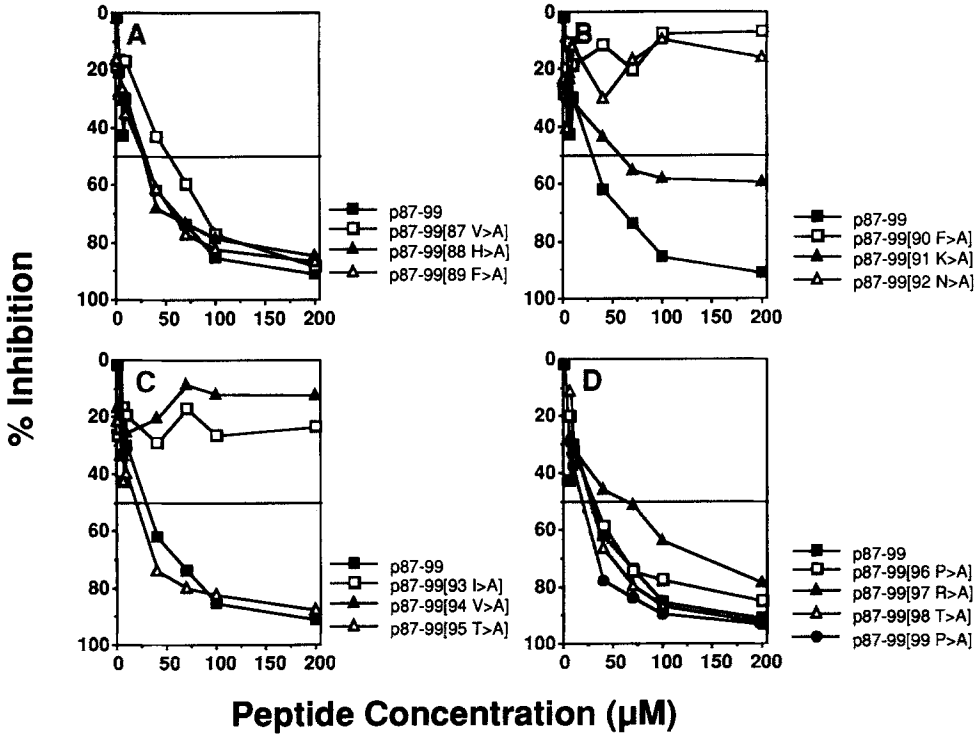

Figure 1. Thy0 subset generation in class II–deficient mice. Thymocytes of 3 mo class II^+ (wild-type) and class II^- littermates were analyzed for the presence of Thy0 by four-color immunofluorescence staining. Lower panels show the HSA and 3G11 profiles of the $CD4^+$ $CD8^{low/-}$ cells gated as shown by the boxed region of the upper panels. The HSA^- cell fraction is further divided into Fr.I' and Thy0 based on 3G11 expression (marked, *left*) (1). $CD4^+CD8^{low/-}$ cells and Thy0 cells are 8.0 and 0.4% in wild-type and 1.4 and 0.3% in class II^- mice (percentages of total thymocytes). Absolute numbers of thymocytes recovered were similar (1.5×10^8 cells/mouse). Analysis of four mice each yielded similar results.

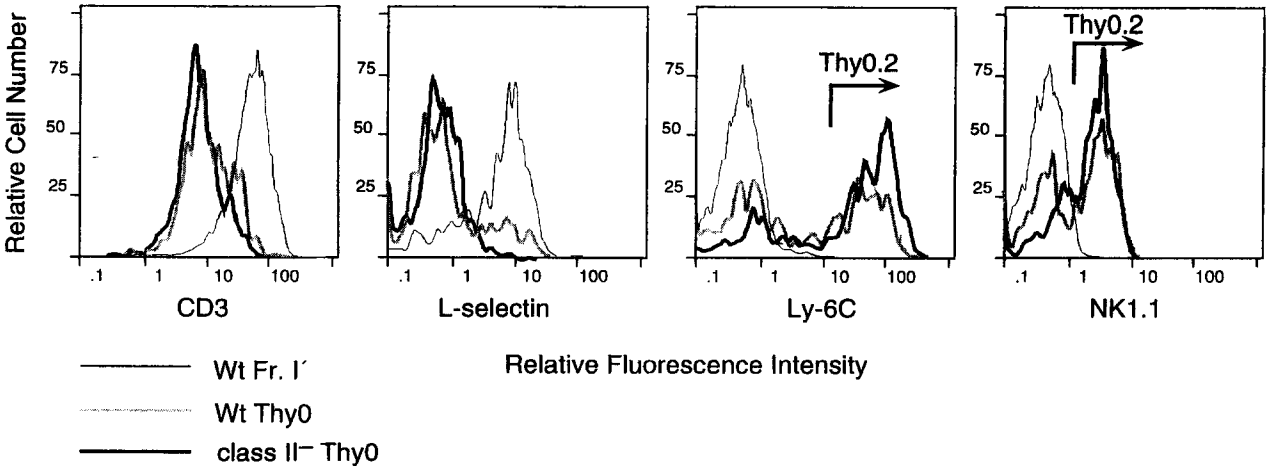

Figure 2. Thy0 cells in class II$^-$ mice are predominantly NK1.1$^+$Ly-6C^{hi} (Thy0.2). Histograms of CD3, NK1.1, Ly-6C and L-selectin levels in Fr.I' of wild-type, and in Thy0 from wild-type and class II$^-$ littermates are shown. Antibodies to these molecules were all phycoerythrin-coupled. The Thy0 cell population was gated as CD4$^+$, CD8$^{low/-}$, and (3G11/HSA)$^-$. Fr.I' was gated as CD4$^+$, (CD8/HSA)$^-$, and 3G11$^+$. Analysis of four class II$^-$ mice consistently showed that Thy0 was largely Thy0.2 (>90%), in contrast to wild-type mice (where Thy0.2 was 10–50% of Thy0).

increased to 10 cells/well (Fig. 3, data from Ly-6C^{hi} cells are shown), essentially 100% of wild-type Thy0.2 cultures were activated by autologous spleen stimulators (B6) (Fig. 3, *left*). Most of this autoreactivity involves MHC class II, since this AMLR was not observed when spleen cells from C2k/o (B6 Ia$^-$) or MHC incompatible mice (BALB/c) were used as stimulators. The complete abrogation rather than a reduction in IL-3 production in the 10 cell AMLR cultures without class II$^+$ stimulators also supports the notion that the majority of Thy0.2 cells express a class II–restricted autospecificity. Sorting based on NK1.1 expression yielded essentially the same results (data not shown). Therefore, our data indicate that the Thy0 subset in normal mice is enriched for class II–specific autoreactive cells, irrespective of some phenotypic heterogeneity in expression of NK1.1 or Ly-6C.

Thy0.2 from C2k/o mice was assessed for autoreactivity in parallel. Unlike Thy0.2 in wild-type mice, only 30–40% of cultures with cells from mutant mice showed IL-3 produc-

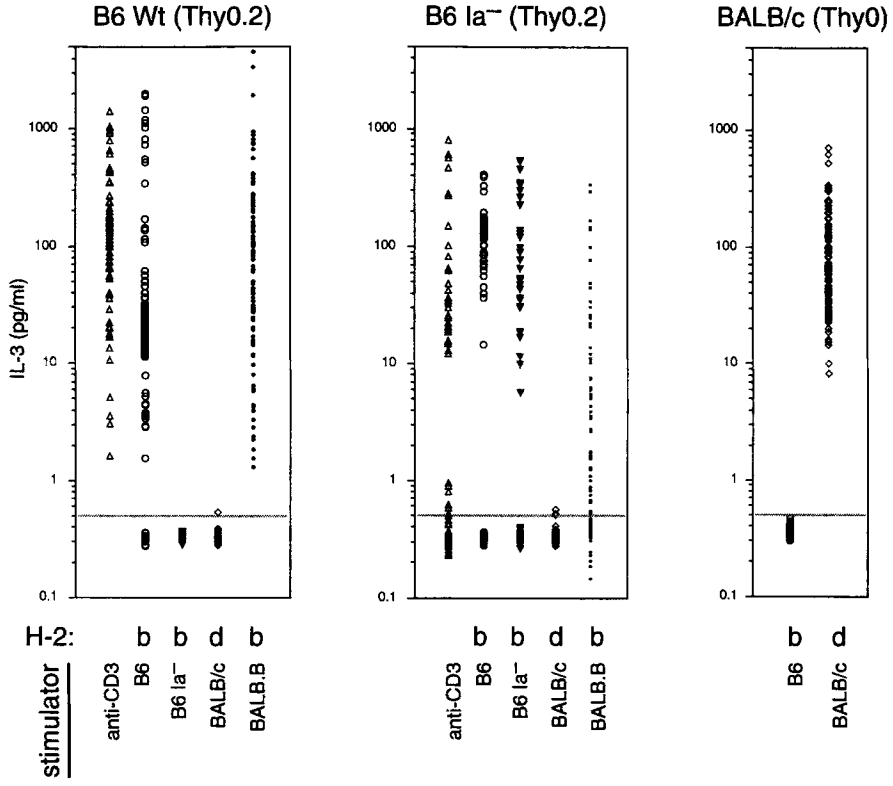

Figure 3. MHC class II–restricted autoreactivity for Thy0.2 in wild-type mice and its alteration to predominant non–class II MHC–restricted autoreactivity in class II deficient mice. Thy0.2 cells were sorted as CD4$^+$, (CD8/HSA)$^-$, 3G11$^-$, and Ly-6C^{hi} from class II$^+$ (B6Wt) and class II$^-$ (B6Ia$^-$) littermates. 10 responder cells were deposited per well. The number of positive wells in B6Wt responder cultures (*left*) for the various stimulators were: 96/96 (anti-CD3), 92/96 (anti-B6), 0/96 (anti-B6Ia$^-$), 2/96 (anti-BALB/c), and 96/96 (anti-BALB.B). In comparison, B6 Ia$^-$ (Thy0.2) responder cultures (*middle*) yielded: 39/96 (anti-CD3), 36/96 (anti-B6), 30/96 (anti-B6Ia$^-$), 2/96 (anti-BALB/c), and 57/96 (anti-BALB.B). Thy0 cells in BALB/c mice were derived by sorting without Ly-6C discrimination. Purity in the sorted sample was assessed by reanalysis of bulk cell fractions sorted under the cloning mode condition, revealing no contamination (0/5,000 cells, i.e., <0.02%) by other cell types. Data shown is representative of three experiments.

tion when activated by anti-CD3, even using 10 cells/well (Fig. 3, *middle*). This diminished responsiveness occurred despite levels of CD3 expression similar to Thy0.2 from wild-type B6 mice (Fig. 2). Most of these anti-CD3 responding cells from B6 background (H-2^b) mice appeared autoreactive, giving an MLR to B6 stimulator cells at a frequency similar to the anti-CD3 response. However, in clear distinction from Thy0.2 from wild-type mice, Thy0.2 cells from C2k/o mice did not show specificity to class II as revealed by response to autologous class II^- stimulatory cells (B6 Ia^-). Nevertheless, the response was still MHC restricted since there was no reactivity with allogeneic BALB/c cells, whereas there was a clear reaction with the MHC congenic BALB.B that possesses the same H-2^b haplotype as responder cells. The last panel in Fig. 3 provides a control for BALB/c stimulatory cells. Thus, Thy0.2 cells were generated in the absence of class II and remained both autoreactive and MHC restricted, but were not class II restricted.

These results could be explained by hypothesizing that Thy0.2 in wild-type and C2k/o mice arise through different developmental pathways. However, the corresponding populations show a remarkable number of shared features, such as a bias for the TCR Vβ8 gene family, identical cell surface phenotype and similar cytokine spectrum, so it is likely that the same mechanism accounts for the generation of Thy0.2 in both. Accordingly, we interpret our results by adapting the two-step selection model for thymic T cell development proposed by Chan et al. (5) (Fig. 4). We suggested previously (1, 2) that cells with relatively high avidity for self/MHC antigens can be positively selected at the $CD4^+8^+$ stage, but become activated by exposure to self/MHC immediately upon acquisition of functional maturity (marked by the loss of HSA), giving rise to the Thy0 subset, branching off the naive T cell maturation pathway (Fig. 4). Since the first step of positive selection of cells from the $CD4^+8^+$ cell pool is via TCR interaction with MHC, resulting in a stochastic downregulation of CD4 or CD8, then the early HSA^+ $CD4^+8^{low}$ stage is comprised of cells carrying affinity for either class I or class II, but are all MHC restricted (5). This accounts for the plasticity of Thy0 in terms of its class I or class II restriction and the lack of class II–restricted cells in the absence of class II. The non–class II MHC specificity in C2k/o mice is likely class I, since class I^- by class II^- double deficient mice completely lack $CD4^+8^{low/-}$ cells (5), presumably including Thy0. The apparent predominance of class II–restricted cells in Thy0 of normal adult mice may be explained by the second step selection stage (5), wherein class II–restricted cells possess an advantage conferred by high expression of CD4, leading to their preferential survival. Our observation of low responsiveness by the non–class II–restricted Thy0 cells in C2k/o mice supports this explanation.

Regardless of these alternative explanations, our report provides a clear demonstration that NK1.1 expression does not serve as a marker for TCR-α/β cells with affinity for a particular MHC class. The NK1.1 antigen is encoded by one of the NKR-P1 genes (15), which appear to play a role in signal transduction in activated NK cells (16). Because it is rarely found on TCR-α/β T cells, it was originally proposed

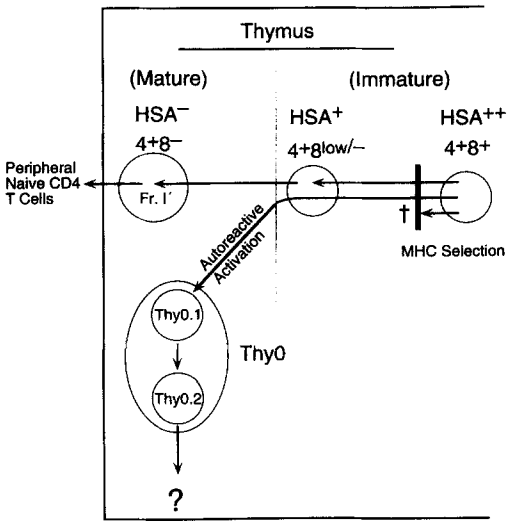

Figure 4. A model for development of an autoreactive α/β T cell subset in thymus. Cells with the potential for autoreactivity of self/MHC will be positively selected if their avidity and/or other apoptotic mechanism(s) is insufficient to result in their immediate death. The fraction of positively selected cells in the process of down regulating CD8 and still retaining HSA ($CD4^+8^{low/-}$ HSA^+) will include both MHC class I– and class II–restricted cells. Within this population, autoreactive cells become activated immediately upon reaching a functionally mature stage (from HSA^+ *to* HSA^-), where they are recognized as Thy0 by distinct cell surface phenotype and cytokine profiles. The eventual fate of Thy0 is yet to be determined.

that $NK1.1^+$ T cells might be related to natural killer cells (9). Furthermore, there is a recent suggestion that $NK1.1^+$ cells with restricted TCR Vβ usage constitute a unique α/β T cell lineage possibly carrying intrinsic affinity to MHC class I molecules (14). This stems from the findings that such cells, both $CD4^+8^{low/-}$ and $CD4^-8^{low/-}$, exhibit dependence for class I presentation by cells of hematopoietic origin, but not of thymic epithelium, as demonstrated with class I^- mutant mice (14, 17). Additionally, the lack of class II or CD4 expression has little effect on the frequency of $NK1.1^+$ T cells (14). These data were all interpreted as requiring a developmental pathway distinct from normal CD4 or CD8 cells. However, our data clearly demonstrate that $NK1.1^+$ cells can carry class II–restricted specificity. Indeed, class II–specific cells predominate at least for the $CD4^+$ $NK1.1^+$ thymic T cells in the normal class II^+ environment. We demonstrate here that functional (autoreactive) $CD4^+$ T cells can show flexibility in MHC restriction, i.e., not exclusively to class II, thereby explaining the presence of such cells in class II^- mice. These findings also serve as a warming of the risk of drawing conclusions for normal mice based exclusively on analyses of MHC-deficient mutant lines.

Admittedly, however, the puzzling question remains as to why Thy0.2 phenotype cells predominate in Thy0 from C2k/o mice and why cells comparable with Thy0.2 appear dependent on class I antigen expression (14), since the majority of Thy0.2 cells are normally class II specific. One possibility is that the distinctive Thy0.2 phenotype may result from certain type(s) of cell activation and that class I molecule(s) might

play a critical role in this process. In support of this notion, we have found that the proportion of Thy0.2 in Thy0 increases with age and that Thy0.2 cells are infrequent in Thy0 from neonatal mice. Furthermore, while class II specific autoreactivity and cytokine profiles are similar in Thy0.1 and Thy0.2 (almost all cells in both subfractions produce IL-2 and high levels of IFN-γ in response to anti-CD3 and at least 30% produce IL-4 as assessed by single cell cytokine analysis), there is a clear difference. Individual Thy0.2 cells produce less IL-3 than Thy0.1 cells (Kariv et al., manuscript in preparation). Finally, cells with features of Thy0.2 are also identified in Thy0 of the low Ly-6C expressing strain BALB/c as Ly-6C^{hi} cells, reminiscent of IFN-γ–treated BALB/c cells (18). These data lend support to the idea that Thy0.2 represents a further differentiated/activated stage of Thy0 cells. Therefore, it is tempting to speculate that certain class I molecule(s) dependent for expression of β_2-microglobulin might play a key role in the differentiation and survival of Thy0, as an alternative to "positive selection" by class I molecules(s) (17).

In conclusion, the autoreactive CD4$^+$ T cell subset we describe here exhibits flexibility in MHC class restriction. The developmental fate of these cells is not clear yet. Since the frequency of cells with such AMLR activity is normally insignificant within the splenic CD4$^+$ T cell population (2), we presume that the majority may eventually become silenced in the thymus or shortly after reaching peripheral sites. However, it is possible that some may be exported to the periphery where they could participate in immune responses (19) or trigger autoimmune disease. In this regard, their potential relationship to DNα/β cells certainly merits additional investigation.

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Address correspondence to Dr. Kyoko Hayakawa, Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burhome Ave., Philadelphia, PA 19111.

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