Brief Definitive Report

Murine Thymic CD4+ T Cell Subsets: A Subset (Thy0) That Secretes Diverse Cytokines and Overexpresses the Vβ8 T Cell Receptor Gene Family

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Summary

We demonstrate here the presence of a distinct mature CD4+8- T cell subset in mouse thymus. This subset, termed "Thy0," is delineated by the absence of 3Gll expression from about half of the 6C10+/HSAlow/− fraction of CD4+8- thymic cells. Thy0 is detectable from the neonatal period and largely contributes the Th0-type diverse cytokine production previously reported for the HSAlow/−CD4+ thymic population. Further, cells expressing the T cell receptor Vβ8 gene family are found at increasing frequency in Thy0 with age, comprising 40–60% of Thy0 in adult BALB/c mice. This alteration of Vβ8+ cell frequency is unique to Thy0, since no other CD4+ subset in thymus or spleen shows such Vβ8 overusage. All functional CD4+ T cell subsets, including Thy0, show appropriate Vβ clonal deletion associated with endogenous superantigens. Thus, it appears that Thy0 is an intrathymically generated secondary cell subset produced after CD4+ T cell selection.

The thymus is the primary site for T lineage differentiation, and is responsible for generating functionally competent T cells. After initial generation of CD4+ and CD8+ T cells, further cell differentiation takes place in the thymus, during which time the functional competence to respond to antigen is increasing. This progression of cells into an immunocompetent state can be recognized by phenotypic alterations, such as loss of the heat-stable antigen (HSA) (1–3). However, it has never been established whether all (or any) HSAlow/− cells in thymus are comparable to peripheral naive T cells (3). Distinctive cytokine production similar to Th0 type cells by this mature thymic CD4+ T fraction was recently demonstrated by Bendelac and Schwartz (4, 5), distinguishing them from the majority of peripheral T cells. Thus, the question has arisen whether this intrathymic Th0 cell stage is an authentic intermediate in the pathway for peripheral CD4+ T cell generation, or instead, whether such cells are generated from naive cells in the thymus by secondary events (3, 4). Data we present here show that the HSAlow/− cell fraction of thymic CD4+ T cells can also be identified as a 6C10− population and, further, that it is comprised of two subsets resolvable by expression of 3Gll: one that resembles peripheral T cells and another that possesses a unique cell surface phenotype, cytokine potential, and TCR Vβ gene family representation.

Materials and Methods

Mice. BALB/cAnN, C57BL/6, and SJL mice were bred and maintained in our animal facility. 2–4-mo-old female BALB/c mice were used in most experiments, except where noted.

Immunofluorescence Staining and Flow Cytometry Analysis and Sorting. Cell preparation for immunofluorescence staining has been described previously (6). Antibodies used here for analysis and sorting are as follows: CD4 (GK1.5), CD8 (53-6), CD3 (500A-A2), HSA (J11d), 3Gll (SM3G11), 6C10 (SM6C10), Vβ8.1,2,3 (F23.1), Vβ8.1,2 (KJ16), Vβ3 (KJ25), Vβ11 (RR3-15), and Vβ6 (RR4-7). Most were purified in our laboratory and then fluorochrome labeled as described previously (7). FL-RR4-7 was purchased from PharMingen (San Diego, CA). Staining data analysis and cell sorting were carried out using a FACStar Plus® (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with two lasers, the second laser being a tunable dye laser. Most data were obtained by four-color immunofluorescence analysis as described to the legends to the figures. To quantitate the frequencies of Vβ cells in the rare HSA−3Gll−CD4+8- cell subset in the thymus, 2 × 105 cells were stained for each group, and data on 106 cells within the CD4+CD8− (HSA + 3Gll) cell gate were collected to the computer, so that 0.1% cells correspond to 100 positive cells (improving statistical validity).

Anti-CD3 Stimulation and Cytokine Assay. 1–2 × 105 sorted cells were deposited into an anti-CD3 precoated 96-well culture plate (500A-A2, 0.1 µg/0.1 ml/well; no. 3598; Costar, Cambridge, MA) together with 105 irradiated (2,500 rad) syngeneic spleen...
Results and Discussion

3G11 ganglioside expression increases as T cells mature in the thymus (9). As shown in Fig. 1a, after selection into the CD4\(^+\)8\(^-\) stage in the thymus (referred to here as CD4\(^+\) T cells), a large fraction of CD4\(^+\) T cells (>80%) express 3G11 regardless of HSA levels. However, in contrast to peripheral CD4\(^+\) T cells, which are HSA\(^-\) and mostly 3G11\(^+\) (6), we found that between 30 and 50% of cells in the thymic HSA\(^-\)CD4\(^+\) T cell fraction are 3G11\(^-\) (Fig. 1a). Further, the level of several other cell surface molecules also differs within the HSA\(^-\) fraction depending on 3G11 expression. The HSA\(^-\)3G11\(^+\) subset exhibits a unique phenotype; that is, Mel-14\(^{low}\), Thy-1\(^{low}\), CD44\(^{high}\), and heterogeneous for CD3, often including cells with low CD3 expression. CD45RB levels are similar to (or slightly higher than) those seen on peripheral native T cells. In contrast, the HSA\(^-\)3G11\(^+\) subset bears the closest resemblance to the peripheral native CD4\(^+\) T cell phenotype with comparable levels of expression for all these molecules (not shown).

During the neonatal period when the generation and emigration of the peripheral naive T cell pool is expected to be most active, HSA\(^-\) T cells are rare in the thymus (Fig. 1b), while functional HSA\(^-\) T cells are already present in the periphery. In contrast with HSA, analysis for 6C10 expression clearly resolves a discrete functional cell population in the thymus at this time. We found that only 6C10\(^+\) cells, the majority of which are HSA\(^{low}\) with some HSA\(^-\) (Fig. 1c, boxed regions), respond to anti-CD3 stimulation whereas 6C10\(^+\) cells do not respond, consistent with a previous report using HSA fractionation (3). As Fig. 1d demonstrates, two subsets, 3G11\(^+\) and 3G11\(^-\), within this functionally mature 6C10\(^+\) (HSA\(^{low/-}\)) cell population can be resolved in the neonatal thymus, similar to adult. These two subsets were identifiable as early as day 1 after birth. Thus, our analysis reveals the continuing presence of two subsets within the functionally mature CD4\(^+\) T cell population in the thymus from the neonatal period to the adult.

We next investigated the cytokines produced by these mature thymic 6C10\(^+\) (and HSA\(^-\)) subsets (Table 1). As previously shown (5), stimulation of the HSA\(^{low/-}\) fraction of thymic CD4\(^+\) T cells resulted in secretion of high levels of IL-4, IL-5, and IFN-\(\gamma\), unlike peripheral T cells. We found that this striking ability to secrete diverse cytokines is largely contributed by the 3G11\(^-\) fraction. Upon anti-CD3 stimulation, 6C10\(^-\)3G11\(^-\) cells rapidly secrete diverse cytokines, regardless of the animal’s age. IFN-\(\gamma\) and IL-5 were also detected in 2-d culture supernatant. Based on this consistent Th0-like cytokine profile for the thymic 6C10\(^+\) (HSA\(^{low/-}\)) 3G11\(^-\)CD4\(^+\) subset, we propose to name it “Thy0” (for thymic Th0). In comparison, predominant IL-2 secretion was obtained from the 6C10\(^-\)3G11\(^+\) cell subset in neonatal mice. However, IL-4 was also detected from this fraction in adult mice. Further, some IL-5 (and IFN-\(\gamma\)) was also detectable, although only at later times after stimulation. Sorting for HSA\(^-\) cells, which are present at increased numbers in adult thymus, yielded essentially similar results (Table 1), distinguishing them from peripheral naive T cells.

A question arises whether the Thy0 subset might represent an intermediate differentiation stage between HSA\(^-\)3G11\(^+\) cells in the thymus and those in the periphery. One approach to determining the relationships of T lymphocyte subsets is to examine their respective TCR repertoires. If Thy0 is the direct precursor for the vast majority of peripheral CD4\(^+\) T cells, then its TCR gene usage would be expected to be similar and stable with age. However, we found (Fig. 2 and Table 2) that Thy0 in adult mice shows an increasing frequency of TCR V\(\beta8^+\) cells with age. In the thymus of adult BALB/c mice, 40–60% of Thy0 (3G11\(^+\)) are V\(\beta8^+\),...
Table 1. Diverse Cytokine Secretion from Thymic CD4+ Cells Predominantly Derives from 6C10- (HSA- or 3G11-) CD4-CD8- Subset (Thy0)

| Age   | CD4+ cell fractions     | IL-2 | IL-4 | IFN-γ | IL-5 |
|-------|-------------------------|------|------|-------|------|
|       |                         | U/ml | U/ml | pg/ml |
| 7 d   | 6C10-3G11+ thymus       | 35   | <10  | <20   | <40  |
|       | 6C10-3G11- thymus       | 153  | 215  | 165   | 1200 |
| 3 mo  | (Exp. 1) 6C10-3G11+ thymus | 123  | 192  | <20   | 440  |
|       | 6C10-3G11- thymus       | 113  | 229  | 707   | 8000 |
|       | (Exp. 2) HSA-3G11+ thymus | 490  | 280  | 210   | 890  |
|       | HSA-3G11- thymus        | 130  | 440  | 1010  | 5700 |
|       | HSA-3G11+ spleen*       | 900  | <10  | <20   | <40  |

3G11+ or 3G11- cells in the 6C10- (or HSA-) CD4+CD8- fraction in thymus of BALB/c mice were sorted (see Fig. 1) and stimulated with anti-CD3. 2- and 4-d culture supernatants were tested for the presence of indicated cytokines. Representative data are shown. Exclusive IL-2 secretion from the 6C10-3G11+ fraction was consistently found in eight experiments with young (<2-wk-old) mice. More than 20 experiments were performed with adult (>3 mo) thymus, where the thymic HSA-3G11- cell subset consistently showed the highest levels of IL-4, IFN-γ, and IL-5 secretion compared with cultures of HSA-3G11+ fraction.

* Data with HSA- spleen cells were obtained by sorting for 6C10-3G11+ cells (6, 9).

whereas only 25–30% of either 3G11+ thymic or splenic CD4+ T cells are Vβ8+. Reciprocally, the relative frequency of Vβ6 is decreased in Thy0. Vβ3+ or Vβ11+ cells, with potential for binding to self-ligands on BALB/c cells (endogenously expressed retroviral genome products) (10–12), are not detectable in any cell fraction. No other CD4+ or CD8+ cell subset in the thymus or peripheral lymphoid organs showed such a bias, with the exception of the thymic CD4+CD8-TCR-α/β- subset, as reported previously (Table 2, legend). The recently described Ly-6C+ CD4+ cell population with Vβ8+ cell overrepresentation (13) is contained within the Thy0 subset since we found that these cells are HSA-, 6C10-, and 3G11-, consistent with the Thy0 phenotype (our unpublished observations).

Table 2 also indicates that the frequency of Vβ8+ cells is dependent on strain background. In comparison with BALB/c, C57BL/6 mice show less increase of Vβ8+ cells in Thy0. Further, in SJL mice, which have a deletion of the Vβ8 gene family (14, 15), Thy0 frequency is reduced (10% of HSA- cell fraction in adult mice) and Vβ8+ cells are undetectable (as expected). Furthermore, we found that Vβ8 overrepresentation by Thy0 is age dependent (Fig. 3). In neonatal (<1

Figure 2. Elevated frequency of Vβ8+ cells in Thy0. Adult BALB/c thymocytes and spleen cells were stained with the PE-labeled anti-Vβ antibodies shown (except for RR4-7) together with APC-anti-CD4, FL-anti-CD8 plus FL-anti-HSA, and Bi-3G11+Tr-avidin. PE histograms of the 3G11+ or 3G11- cells in the CD4+CD8-HSA- fraction are shown. For Vβ6+ cell analysis, FL-RR4-7 was used together with APC-anti-CD4, PE-anti-CD8, and either Bi-SM3G11 plus Bi-HSA (for HSA-3G11- cell gate) or Bi-SM3G11 alone (for 3G11+ cell gate). The frequencies of Vβ+ cells in the 3G11+CD3+ thymic CD4+ cell fractions are the same regardless of HSA levels.
Table 2. Frequency of Expression of Various Vβ TCR Gene Families in HSA-3G11+ and 3G11- Thymic CD4+ T Cell Subsets

| Strain (n) | CD4+HSA- | Vβ8 (F23.1) | Vβ8.1,2 (KJ16) | Vβ6 (RR4-7) | Vβ3 (KJ25) | Vβ11 (RR3-15) |
|-----------|----------|-------------|----------------|-------------|-------------|---------------|
| BALB/c (16) |          |             |                |             |             |               |
| 3G11+     | 27.4 ± 4.5 | 20.0 ± 2.5  | 10.3 ± 0.9     | <1.0        | <1.0        |
| 3G11-     | 51.9 ± 7.6  | 38.0 ± 6.7  | 5.0 ± 0.8      | <1.0        | <1.0        |
| C57BL/6 (6) |          |             |                |             |             |               |
| 3G11+     | 21.9 ± 2.8  | 14.8 ± 0.9  | 10.3 ± 3.9     | 4.5 ± 0.6   | 5.0 ± 0.1   |
| 3G11-     | 31.4 ± 3.7  | 25.0 ± 2.6  | 6.3 ± 1.3      | 3.8 ± 0.5   | 4.0 ± 0.1   |
| SJL (4)   |          |             |                |             |             |               |
| 3G11+     | <1.0       | <1.0        | 6.0 ± 0.0      | <1.0        |
| 3G11-     | <1.0       | <1.0        | 23, 13         | 5.5 ± 0.6   | <1.0        |

2-4 mo-old BALB/c, C57BL/6, and SJL mouse thymocytes (numbers of mice in parenthesis) were stained as described in Fig. 2. Vβ6 in SJL is based on two animals. Data shown indicate standard deviation. Vβ8+ cell frequency of other cell subsets in BALB/c mice (data with seven adult mice) are: thymic CD8+ cells (HSA+, 32 ± 4%; HSA-, 35 ± 4%), thymic CD4-8-TCR-α/β+ cells (67 ± 7%), and splenic CD4+ subsets (Fr. I-V, 25-29%) (6).

Figure 3. Age-dependent alteration of Vβ8+ frequency in Thy0. BALB/c thymocytes of different ages were stained to obtain the Vβ8+ cell frequency (using F23.1) in the HSA-3G11+ (Thy0) and 3G11+ cell fractions as described in the legend to Fig. 2. For analysis of neonatal mice (<1 wk), FL-SM6C10 staining (in place of FL-anti-HSA) was also carried out. In both analyses (HSA and 6C10) the same results were obtained; i.e., no increase of Vβ8+ cells in the neonatal Thy0 fraction.

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regard, our finding may carry implications for the study of autoimmune lpr mice since T-lineage cells proliferating in mice with this defect (lpr cells) share features with DNo/β cells, as described elsewhere (18, 20).

CD4+ T cells play a key role in the immune system. Recent findings of their functional heterogeneity have evoked considerable attention as to how such subsets are generated and how they relate to CD4+ T cell differentiation and maturation (9). In these studies, defining the precursor population in the thymus that provides the naive CD4+ T cell repertoire in the periphery is an important goal. As yet, our data do not completely exclude the possibility that cells differentiate through the Thy0 stage, wherein the bias of TCR Vβ usage is contributed by only a fraction of cells within Thy0. However, it appears more likely that the 6C10-3G11+ subset represents the immediate precursor population for the peripheral naive cell pool. Although data from the thymic 6C10-3G11+ subset in adult mice did not show absolute IL-2 predominance, this could be explained by contamination of naive cells with an increasing proportion of secondary cells that have yet to lose 3G11. Finding IL-5 or IFN-γ during the later stage of HSA-3G11+ cell culture may imply a rapid progression of such cells into the Thy0 fraction. These arguments will be clarified by analysis at the single cell level by work that is in progress. Finally, as recent data have demonstrated, Thy0 can also be exported into the periphery while retaining CD4 expression (21). The functional significance of Thy0 poses an interesting puzzle for further investigation.

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