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Peripheral CD4⁺ T Cell Maturation Recognized by Increased Expression of Thy-1/CD90 Bearing the 6C10 Carbohydrate Epitope

Ming Gui, David L. Wiest, Jin Li, Dietmar Kappes, Richard R. Hardy, and Kyoko Hayakawa

The SM6C10 IgM autoantibody recognizes a surface determinant, 6C10, that is highly expressed on all immature thymocytes. In contrast, its expression on peripheral T cells appears developmentally regulated, i.e., absent from most naive T cells in spleen of neonatal mice, but expressed on 40–80% of naive CD4⁺ T cells in adult. In this paper, we demonstrate that SM6C10 recognizes a carbohydrate epitope on the Thy-1 glycoprotein using immunoprecipitation analysis, by binding to affinity-purified Thy-1 in an ELISA, and by sensitivity to N-glycosidase-F treatment. Retroviral Thy-1 gene transduction experiments into Thy-1⁻ variant T cell lines and a pro-B cell line provide evidence that 6C10 glycosylated Thy-1 expression is not restricted to T cells but depends on the recipient cell. Therefore, differences in 6C10 levels among Thy-1⁺ T cells in mice likely reflect developmental regulation of posttranslational modification of the Thy-1 glycoprotein. The ability of naive CD4⁺ T cells to respond to anti-Thy-1 stimulation increases from neonate to adult, and 6C10⁻ naive cells from adult mice respond poorly compared with 6C10⁺ cells, similar to the cells in neonatal mice. These results suggest that there is functional maturation by peripheral CD4⁺ T cells that coincides with 6C10 glycosylated Thy-1 up-regulation, and natural autoantibody recognizes this 6C10 carbohydrate epitope. The Journal of Immunology, 1999, 163: 4796–4804.

The development and differentiation of T cells is accompanied by alteration of surface Ag expression, generating significant heterogeneity in the periphery (1–7). Although most commonly recognized phenotypic heterogeneity among CD4⁺ or CD8⁺ T cells is a result of antigenic activation (5), there are several surface proteins expressed differentially in the pool of cells considered to be mature Ag nonexposed T cells (“naive” T cells). In mice, these surface proteins include several GPI-linked molecules, such as Qa-2 (8), Ly-6C (6, 9), Ly-6A/E (6, 10), and the Thy-1/CD90-dependent 6C10 (3, 11–13). GPI-linked proteins are clustered in specialized glycolipid-enriched membrane microdomains called caveolae (14–18). Because several GPI-linked proteins associate with src-family tyrosine kinases, p56lk and p59fr, and cross-linking of these proteins leads to tyrosine phosphorylation of intracellular substrates (19–21), resulting in activation (22–25), they could function as signaling molecules on T cells or could modulate TCR-mediated cell activation (22, 26–30). Thus, there is increasing interest in the possibility that such phenotypic heterogeneity reflects functional diversification of peripheral T cells, predetermining cell fate following activation.

6C10 is of particular interest due to its association with CD4⁺ T cell function and with the GPI-linked protein Thy-1. The 6C10 determinant is recognized by the mouse IgM monoclonal anti-thymocyte/T cell autoantibody SM6C10 as a natural autoantibody (11). 6C10 expression is dependent on surface Thy-1/CD90 glycoprotein expression, and it is found at high levels on immature thymocytes of all inbred mouse strains, similar to Thy-1 (11). However, although Thy-1 is expressed by nearly all T cells in the spleen or lymph nodes, 6C10 is expressed only by a fraction of peripheral T cells, mostly by CD4⁺ T cells, and at lower level. Thus, in adult mice, 40–80% of CD4⁺ native T cells in spleen or lymph nodes are 6C10⁺. Interestingly, this 6C10⁺ CD4⁺ T cell population shows generally higher levels of several GPI-linked molecules (6) and greater responsiveness. Both 6C10⁻ and 6C10⁺ CD4⁺ naive T cells in the adult can proliferate and secrete IL-2 after activation (3). However, 6C10⁺ cells respond higher than 6C10⁻ cells in certain stimulation systems, such as with superantigen (31), by Con A in the presence of resting accessory B cells (3), or, as will be shown in this paper, by cross-linking Thy-1. Furthermore, 6C10⁺ is an obligatory phenotype for long-term memory Th cells (3), and it is absent from cells rendered anergic (3, 31). Thus, 6C10 is a unique phenotypic marker that distinguishes the functional status of CD4⁺ T cells both before and after activation.

Our recent observation of a complete lack of 6C10⁻ cells in Thy-1 knockout mice demonstrated that the Thy-1 glycoprotein is absolutely required for 6C10 expression in mice (32). However, a fraction of peripheral Thy-1⁺ T cells lacks 6C10, and 6C10 is absent from peripheral T cells in neonates even though they express Thy-1 protein (6). Furthermore, 6C10 is not detectable on brain cells, activated NK cells, or on rat thymocytes, despite a high level of Thy-1 protein expression by all of these cell types (Ref. 11, and our unpublished observations). Thus, whereas 6C10 expression requires Thy-1, it is limited to certain Thy-1⁺ cell types, depending on tissue, species, and age.

Because of the difficulty to immunoprecipitate 6C10 from cell lysates using nonionic detergent such as Nonidet P-40 with this IgM autoantibody, and because of some discordance between 6C10 and Thy-1 surface expression, it has remained unclear whether 6C10 is an epitope on Thy-1 or else resides on a molecule distinct from, but associated with, Thy-1. Interestingly, previous immunoprecipitation analysis using anti-thymocyte autoantibodies...
that cross-block with SM6C10 and that show similar T cell reactivity suggested the presence of a 100-kDa molecule noncovalently associated with Thy-1 (33, 34). Because the issue of how GPI-anchored proteins such as Thy-1, associated only with the outer leaflet of the plasma membrane, can transduce signal into the cytoplasm remains unsettled, the possibility of transmembrane molecules associated with GPI-linked molecule(s) is a recurring attractive hypothesis, and this p100 molecule was proposed as a potential candidate to explain functional association (35). It was therefore critical to determine whether SM6C10 autoantibody reacts with p100 (or another Thy-1-associated protein), rather than directly with the Thy-1 epitope. In this study, we have attempted to discriminate between these possibilities and also to understand why 6C10 serves to mark the functional status of CD4+ T cells.

Materials and Methods

Mice

C57BL/6DN (B6), BALB/c, and B6.Thy-1-/- (36) mice were bred and maintained in the Institute for Cancer Research Animal Facility. Pigeon cytochrome c/V-E3 specific TCRβ transgenic mice, TCR “AND” (37), were purchased from The Jackson Laboratory (Bar Harbor, ME) and continuously crossed with Rag-2-/- mice, selecting offspring expressing transgene TCR and lacking B cells in the peripheral blood (by immunofluorescence analysis), to obtain transgene-expressing mice on a Rag-2-/- background.

Cell lines

EL-4 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Opti-MEM (Life Technologies, Rockville, MD) medium. AKR1 and AKR1-/-, 6.6 (38) were provided by Dr. R. Hyman (Salk Institute, La Jolla, CA) and maintained in Dulbecco’s medium (11). The mouse pro-B cell line ret20 was maintained in RPMI 1640 medium, as described previously (39).

Abs

Rat anti-mouse Thy-1, G7 (22), was purchased from PharMingen (San Diego, CA). Rat Abs to mouse Thy-1.2, (30-H12), heat stable Ag (HSA)/CD24 (30F1), T200CD45 (30F11), IgM (331.12), IgD (500-A2), L-selectin/CD62L (MEL-14), CD4 (GK1.5), IL-2 (JES6-12 and JES6-5H4), and mouse anti-rat κ (MAR18.5) were purified from ascites and fluorochrome coupled for flow cytometry or used for ELISA. Rat anti-immune IgM, M41, was purified by a protein G affinity column (Pierce, Rockford, IL), and the mouse IgMκ Abs, SM6C10 and SM3G11, were purified from ascites by protein L affinity columns (ACTIgen, Cambridge, U.K.) according to the manufacturer’s instruction. Purified TEP183 was purchased from Sigma (St. Louis, MO). In some experiments, SM6C10 was used in ascites form together with other ascites controls. Control hybridomas, SM3G11, SM4E9, and SM5D8 were all derived from the same cell fusion experiment as SM6C10 (11). Their IgM titers in ascites were all equalized based on an anti-μ/anti-κ ELISA sandwich assay to measure IgM content.

Immunochemoanalysis

Cell surface biotin-labeling, immunoprecipitation (i.p.), electrophoresis, and enhanced chemiluminescence procedures have all been described elsewhere (40). In brief, 2–10×10^6 cells were incubated with Sulfo-NHS-Biotin (Pierce) at 1 mg/ml in HBSS for 30 min at 4°C and then washed with HBSS with 25 mM lysine. Live biotinylated cells (>98%) were harvested by overlay of lymphocyte M and then lysed in 1% digitonin (Wako, Richmond, VA) lysis buffer in Tris (pH 7.4) with a protease inhibitor mixture at 2×10^6 cells/ml for 30 min at 4°C; subsequently, supernatant was obtained by centrifugation at 12,000 rpm for 15 min. For i.p. of Thy-1 or 6C10, MAR (for Thy-1), or anti-IgM (M41, for 6C10) was first coupled to Sepharose 4B beads (Sigma). A 20-μl-coupled bead aliquot was incubated with 5 μg of Ab or 1 μl of ascites, and then washed before incubation with 25–50 μl of cell lysate. After lysate incubation for 4 h at 4°C with rotation, beads were washed and resuspended in sample buffer for disso-

cation, and supernatant was applied to one-dimensional SDS-PAGE or two-dimensional electrophoresis (2-DE; NEPHGE/SDS-PAGE) (3) procedure. After electrophoresis, gels were blotted onto membrane (polyvinylidene difluoride, ChemiCell, Natick, MA) blocked with 5% nonfat dry milk (Bio-Rad, Hercules, CA) in PBS containing 0.2% Tween 20 (Sigma) for 1 h at room temperature (RT), incubated with HRP-coupled streptavidin conjugate (Southern Bio-technology Associates, Birmingham, AL) for 1 h at RT, washed extensively, and then incubated with Supersignal Western Blotting Substrate (Pierce) for luminescence development. Luminescence was detected by exposure of x-ray film for various times (from 5 to 15 min) for intensity comparison. Rainbow-colored high m.w. protein markers (Amersham, Arlington Heights, IL) and biotinylated broad range protein markers (New England BioLabs, Beverly, MA) were used as m.w. standards. OVA and carbonic anhydrase from Sigma were used to mark pl points on 2-DE. Peptide N-glycosidase-F (PNGase-F; Oxford GlycoSystems, Rosedale, NY) treatment of lysate was conducted according to manufacturer’s recommendation.

Thy-1 glycoprotein affinity purification

Thy-1 affinity purification was according to the method previously described by Chang et al. (41) with some modification. In brief, lysate was made from 1×10^7 thymocytes from either B6 or B6.Thy-1-/- mice at 2×10^6 cells/ml in Nonidet P-40 lysis buffer (1% Nonidet P-40 in 50 mM Tris-saline (pH 7.4), protease inhibitors, and 10 mM iodoacetamide) on ice for 45 min. Lysate was incubated with 1.5 ml of an anti-Thy-1.2- (30-H12) coupled Sepharose 4B slurry for 4 h at 4°C with rotation and then packed into an column. After extensively washing the column with 0.1% Nonidet P-40, bound material was eluted with 0.2 M glycine (pH 2.8). The 0.5-ml fractions were immediately neutralized by addition of 50 μl of 2 M Tris (pH 8.0). A pool of the first eight fractions was immediately applied to a Centricon-10 (Amicon, Beverly, MA) for concentration, followed by two resuspensions with 0.1 M PBS (pH 7.2), thus finally obtaining a 0.5-ml concentrated eluate in PBS. To examine the protein(s) in such eluates, samples were applied to 12% SDS-PAGE minigel and silver-stained. Purification of HSA/CD24 from B6 thymocytes Nonidet P-40 lysate was conducted by using a 30F1-coupled affinity column.

ELISA assay for detection of Thy-1/6C10

ELISA was conducted in a 96-well Immunoplate (Nalge Nunc Interna- tional, Roskilde, Denmark) as described previously, with some modifica- tion. For direct plate coat of Thy-1, 30–50 μl of eluate was used for plate coating, adjusted to 100 μl with PBS, by overnight incubation at 4°C. Coated wells were then blocked with 3% OVA, 30 min at RT, before subsequent steps with biotiniated anti-Thy1.2/30H12 and alkaline phosphatase (AP)-coupled avidin incubation. For ELISA sandwich assay, anti-Thy-1 (or anti-HSA) was coated at 1 μg/ml overnight at 4°C, followed by blocking with 3% OVA at RT, and then incubated with 30–50 μl of Thy-1 containing eluate overnight at 4°C. After washing, wells were again blocked with OVA, incubated with SM6C10Ab (2 μg/ml) at RT for 1.5 h, and subsequently incubated with biotiniated anti-IgM and AP-avidin.

Immunofluorescence staining and multicolor flow cytometry analysis and sorting

Four-color flow cytometry analysis and sorting were conducted using a FACStarPlus (Becton Dickinson, San Jose, CA) as described previously (3).

Anti-Thy-1 stimulation

Anti-Thy-1 stimulation was done in 96-well, U-bottomed plate (Coster, Cambridge, MA). Then 2–5×10^4 cell sorter-purified CD4 + T cells, or subsets, were incubated with G7 with or without PMA (Sigma) at different concentrations as described in the experiments. The 24-h culture superna- tant was harvested and tested for the presence of IL-2 by ELISA sandwich assay by using JES6-1A12 and biotin-JES6-5H4, in combination. Mouse IL-2 (R&D Systems, Minneapolis, MN) and supernatant from IL-2 se- creting cell line, 16H (42), were used as IL-2 standards. An OD of 0.5 corresponds to ~150 pg/ml. Cells were cultured for 6 more days, har- vested, and then counted by passing through the cell sorter. Live cells were discriminated by light scatter and by propidium iodide exclusion.

Retrovirus-mediated Thy1.2 gene transduction

The Thy-1-coding sequence was amplified by PCR using Tax polymerase from cDNA made using RNA isolated from T cells with primers engi- neered to contain EcoRI and XhoI restriction sites (sense 5′-GGGAAT TTGATGAACTGCGGGCT-3′, anti-sense 5′-CCGGCTAGGT CACAGGAAAATGATTCCGCTG-3′). The 505-n amplified fragment
was cloned into TA vector (Invitrogen, San Diego, CA) and verified by sequence analysis. The EcoRI/XhoI fragment released by double restriction digest was gel-purified and then ligated with EcoRI/XhoI cut pBMN-IRES-EGFP retroviral vector provided by Dr. G. Nolan (Stanford University, Palo Alto, CA). Clones containing appropriate size insert (released by double digest with EcoRI/XhoI) were again sequenced for verification. Purified plasmid was introduced into the Phoenix ecotropic packaging line (provided by Dr. G. Nolan) (43) by lipofection (LipofectAMINE, Life Technologies) following the manufacturer’s protocol; 1 day later, recipient cell lines were added together with polybrene (44) to facilitate viral entry. After 2 days of coculture, the infected cells were suspended by gentle agitation, washed with staining medium, and then stained with anti-CD4 (for AKR1-d.6.6) or AA4.1 (for ret2/0) to distinguish them from packaging cell lines, thymocytes, and Thy-1

The correlative 6C10 and Thy-1 expression in Fig. 1B may mean either that 6C10 is a Thy-1 epitope or that it is expressed on a Thy-1 associated molecule whose expression depends on surface Thy-1. To distinguish between these, we conducted i.p. analyses. Although i.p. of 6C10 has been difficult with Nonidet P-40 lysate, we found that digitonin lysate allows specific i.p. Thy-1+ T cell lines, thymocytes, and Thy-1– mutants were surface biotinylated and digitonin lysates were prepared for i.p. All Thy-1– (Thy-1+) cells are surface 6C10+ by immunofluorescence staining (11, 32). Fig. 2A, a–i, shows 10% SDS-PAGE analysis of T cell lines. Specific i.p. of Thy-1 from EL-4 (Thy-1.2) and AKR1 (Thy-1.1) with non-allele-specific or allele-specific Ab revealed a 25- to 30-kDa species under reducing conditions by SDS-PAGE that was not found with the Thy-1– variant AKR1-d. We could detect a specific band with SM6C10 from AKR1 in the same region, albeit weakly (requiring longer exposure), but not with AKR1-d, nor with control IgM (Fig. 2A, j–o). Some background in the region (and also around 55–70 kDa with variable intensity in samples) without SM6C10 Ab was also observed in the absence of lysate (data not shown), apparently due to nonspecific avidin binding to released immobilized Ab.

Fig. 2B shows thymocyte i.p. results, where Thy-1 was readily detectable by anti-Thy-1 (Fig. 2Bb). A species in the 26- to 29-kDa region was also immunoprecipitated with SM6C10 from thymocyte digitonin lysates at levels ~10- to 20-fold less than obtained with anti-Thy-1 (Fig. 2B, c vs e). Importantly, as shown by the next panel, this species was absent from i.p. of Thy-1– thymocytes (Fig. 2Bj), which showed only the background band seen with

were sorted by flow cytometry directly into wells containing culture medium. After expansion in culture, cells were reanalyzed to reveal ≥95% GFP positive cells.

Results

Induction of 6C10 expression and up-regulation of Thy-1 as a indication of peripheral CD4+ T cell development.

Although 6C10 is highly expressed on all immature CD4+8+ cells, it is down-modulated on more mature CD4+ single positive HSA– T cells in the thymus of both neonates and adults (Fig. 1A, left two panels). CD4+ T cells in the spleen of neonatal mice are mostly 6C10+ (Fig. 1A, right panel, thin line) similar to thymic HSA–CD4+ T cells. In contrast, the splenic CD4+ T cells established in adult animals are predominantly 6C10+ (40–80%) (Fig. 1A, right panel, thick line), as we have previously described (3). To test whether this 6C10 phenotypic change in the periphery is a developmental event independent of antigenic influence, we analyzed CD4+ T cells in spleens of Rag-2–/–AND TCRαβ 1-wk neonatal and 3-mo adult transgenic mice were stained and analyzed for 6C10 and Thy-1.2 expression of splenocytes in neonates and in adult, respectively.
control IgM Abs (Fig. 2B, h and i) or immobilized SM6C10 alone without lysate (Fig. 2B). No other specific bands were observed over the range from 10 to 220 kDa in SDS-PAGE (7%, 10%, and 15%) analysis. Pre-absorption of 6C10+ lysate with anti-Thy-1-coupled MAR beads, but not with uncoupled MAR beads, eliminated the 26- to 29-kDa band (Fig. 3), demonstrating association or identity of this material with the Thy-1 molecule. The use of octylglucoside in cell lysis, which prevents formation of detergent-resistant complexes by GPI-anchored proteins (45), again gave SM6C10 specific immunoprecipitation at the same location without generation of new specific bands (data not shown).

SM6C10 binds to affinity-purified Thy-1

Because the above experiments leave open the possibility that SM6C10 binds to a Thy-1-associated molecule that is not efficiently biotinylated, we conducted additional experiments to demonstrate its direct binding to Thy-1. For this purpose, affinity-purified Thy-1 from Nonidet P-40 thymocyte lysate, but not with uncoupled MAR beads, eliminated the 26- to 29-kDa band (Fig. 3), demonstrating association or identity of this material with the Thy-1 molecule. The use of octylglucoside in cell lysis, which prevents formation of detergent-resistant complexes by GPI-anchored proteins (45), again gave SM6C10 specific immunoprecipitation at the same location without generation of new specific bands (data not shown).

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![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Thy-1-dependent detection of 26- to 29-kDa species with SM6C10. Data are from a 10% SDS-PAGE analysis of T cell digitonin lysate i.p. A, T cell line lysates, Thy-1 phenotype as marked, were immunoprecipitated with rat Abs plus MAR beads (a–i) or MAR beads alone (−), or mouse IgM Abs plus anti-IgM beads (j–o) or anti-IgM beads alone (−). Exposure times for a–i and j–o are 15 s and 1 min, respectively. B, Thymocyte lysates from B6 (Thy-1+) or B6. Thy-1−/− (Thy-1−) mice were immunoprecipitated as described in A. Twenty times less lysate was used. a–f and g–l were from two separate experiments. Each exposure time was 1 min except for anti-CD45 (10 s). Numbers on the left indicate m.w. markers (kDa).

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Elimination of the SM6C10 20- to 29-kDa band after pre-absorption with anti-Thy-1-coupled beads. Thymocyte lysate was first titrated for Thy-1 absorption efficiency by varying lysate amount, to determine an appropriate ratio. After incubation of lysate with anti-Thy-1-coupled MAR beads (or with uncoupled MAR beads) for 30 min on ice, supernatant was divided for each immunoprecipitation as shown.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Silver staining of 12% SDS-PAGE of anti-Thy-1 affinity-purified material. Anti-Thy-1 column eluates from B6 (Thy-1+) or B6. Thy-1−/− (Thy-1−) thymi were applied to 12% SDS-PAGE minigel using reducing conditions. The arrow indicates material corresponding to the Thy-1 glycoprotein. Two bands at 50–70 kDa present in all lanes, including a lane without sample loading (−), were caused by reducing agent.
analysis of purified material that shows a single predominant band of m.w. equivalent to Thy-1, we conclude that SM6C10 recognizes a determinant on Thy-1, without any associated species.

The 6C10 carbohydrate epitope is expressed on several Thy-1 glycoforms

The Thy-1 glycoprotein contains a high proportion of carbohydrate chains at three N-linked glycosylation sites, with variable complexity at each site (46–48). Thus, in 2-DE (NEPHGE/SDS-PAGE) analysis (Fig. 6), Thy-1 from thymocyte lysate migrates as six to eight distinct isoelectric focusing species (Fig. 6a), as evident from comparison to the lysate negative control (Fig. 6c) (49). SM6C10 immunoprecipitated Thy-1 from thymocyte lysate with a similar extent of charge heterogeneity (Fig. 6b). Treatment of Thy-1 with PNGase-F generated a homogeneous 15-kDa polypeptide by elimination of carbohydrate (Fig. 7b), whereas SM6C10 no longer bound to such PNGaseF-treated Thy-1 (Fig. 7d). Thus,

FIGURE 5. SM6C10 binding to purified Thy-1 in an ELISA assay. A. Affinity-purified Thy-1 or HSA material from B6 thymocytes, or mock anti-Thy-1 material prepared from Thy-1−/− thymocytes (Thy-1−) were plate-coated for ELISA assay. Levels of binding were tested by biotin-anti-Thy-1.2 + AP-avidin (upper) or SM6C10 (or control TEPC183) + biotin-anti-IgM + AP-avidin (lower). * Subtracted OD data based on control TEPC183 binding to each coated plate, due to nonspecific IgM Ab binding to the coated plates. Purified Abs were used in all groups. B. Plates were precoated with anti-Thy-1 (G7) or anti-HSA (30F1), both rat IgG2c, and subsequently incubated with purified Thy-1 or HSA material. After washing, plates were then tested for reactivity to either purified SM6C10 or control TEPC 183 IgM, developed by biotin-IgM + AP-avidin. The OD 1 h after addition of substrate is shown.

FIGURE 6. 6C10+ Thy-1 molecules in the thymus exhibit multiple isofocusing points similar to total thymocyte Thy-1 molecules. The 2-DE (NEPHGE/SDS-PAGE, 15%) analysis of anti-Thy-1.2 or SM6C10 immunoprecipitates. a and b, Digitonin lysate; c and d, Nonspecific binding by avidin to the reagents without lysate addition. Two circles in a and b indicate marker location, OVA (m.w. 45 kDa, pI 5.1) and carbonic anhydrase (m.w. 29 kDa, pI 7.0). Exposure time points 30 s (a and c) and 1 min (b and d).

FIGURE 7. Peptide N-glycosidase F treatment of Thy-1 abolishes SM6C10 i.p. Digitonin thymocyte lysate was treated with PNGase and immunoprecipitated with anti-Thy-1.2 or SM6C10. A 15% SDS-PAGE analysis is shown.
6C10 is a carbohydrate epitope present on several Thy-1 glycoforms on thymocytes.

**Recipient cell dependent induction of 6C10 by Thy-1 gene transduction**

Although 6C10 is expressed by all immature Thy-1+ thymocytes, it is limited to certain Thy-1+ cell types in the periphery and is not expressed by brain Thy-1, suggesting cell type or differentiation stage specific glycosylation. We have previously shown that Thy-1 gene transfection into a Thy-1+ variant T cell lines restores 6C10 expression (11). As Fig. 8, left, shows, retroviral Thy-1 gene transduction of the mouse pro-B ret2/0 line, originally 6C10– Thy-1−, resulted in 6C10 expression that was correlated with Thy-1, similar to most Thy-1+ T cell lines. Thus, 6C10 glycosylation is not restricted to T lineage cells. In turn, we found that some T cell lines failed to express 6C10 upon expression of Thy-1, reminiscent of the neonatal CD4+ T cell phenotype. Using the same retroviral Thy-1 transduction system, infection of the AKR1-d.6.6 T lymphoma, a spontaneous Thy-1− variant defective for expression of several surface Ags, resulted in a high level of surface Thy-1 expression but without detectable 6C10, and this lack of 6C10 was maintained in culture for >3 mo (Fig. 8, right). These data support the idea that 6C10 expression requires specific glycosylation machinery that is dependent on the cell’s developmental/differentiation stage and that such glycosylation can occur in non-T cells.

**Functional maturation by CD4+ T cells in association with 6C10+ phenotype**

To assess whether 6C10+ CD4+ T cells exhibit functional maturity more than 6C10− cells, we purified 6C10− 3G11+ and 6C10+ 3G11− naive CD4+ T cell fractions from adult spleen (Fig. 9A) and tested for IL-2 release and proliferation using various doses of anti-Thy-1 (G7) and PMA stimulation, a system to quantitate T cell responsiveness independent of accessory cells. More than 90–95% of cells were Thy-1+ in both fractions. As Fig. 9B shows, 6C10+ cells consistently showed higher IL-2 release and proliferation in comparison to 6C10− cells under all conditions employed. Anti-Thy-1-mediated cell aggregation (50) was also greater with 6C10+ T cell compared with 6C10−, suggesting that proximal anti-Thy-1 cross-linking events (51) also differ (not shown).

Using conditions that yielded the strongest anti-Thy-1 response, splenic naive 3G11+ CD4+ T cells purified from neonates (1 wk) and from adults (3 mo) of wild-type B6 and Rag-2−/− TCRαβ “AND” transgenic mice were compared for IL-2 secretion. As Fig. 9C shows, neonatal naive CD4+ T cells from both wild-type and transgenic mice responded poorly to anti-Thy-1 stimulation, compared with adult CD4+ T cells. Thus 6C10− cells show a consistently lower response to anti-Thy-1 activation. Levels of TCR/CD3 and CD45, which could potentially influence anti-Thy-1-mediated IL-2 secretion (27, 52), were comparable between 6C10− and 6C10+ naive cells (not shown). These results suggest that there is maturation that coincides with 6C10 glycosylated Thy-1 up-regulation by peripheral CD4+ T cells, generating functional and phenotypic heterogeneity.
Discussion

Natural IgM autoantibodies recognizing Thy-1 associated Ag(s) appear to be common (11, 53–57). However, chemical characterization of relevant self-Ags has been hampered for a long time by difficulties in obtaining clear immunoprecipitates with such IgM autoantibodies. In this paper, we used an alternative approach, purification of Thy-1, in combination with immunoprecipitation, to demonstrate that 6C10 is a carbohydrate epitope expressed on the Thy-1 glycoprotein. SM6C10 binds to affinity-purified Thy-1 glycoprotein as demonstrated by a “sandwich” ELISA. Lack of specific bands other than the 26- to 29-kDa region corresponding to Thy-1, and disappearance of the 26- to 29-kDa band by anti-Thy-1 pre-absorption in SDS-PAGE analyses, indicate that there is no distinct 6C10-reactive protein associated with Thy-1 under the conditions of our analysis. Analysis showing sensitivity of the 6C10 determinant to periodate treatment led us previously to suggest that this epitope has a carbohydrate nature (11); this is confirmed here by loss of reactivity after PNGase-F treatment. Furthermore, 6C10+ cells are absent in Thy-1 gene knockout mice (as we have recently shown) (32). Thus, the Thy-1 glycoprotein must be the predominant molecule that carries the 6C10 determinant in mice. Carbohydrates expressed on glycoproteins and/or glycolipids are frequent targets of natural autoantibodies (58), and we show in this paper that this also holds for the Thy-1 glycoprotein.

Thy-1/CD90 is a ubiquitous neural glycoprotein in most species, with extensive glycosylation, and is a major cell-surface glycoprotein of thymocytes in mice and rats (46). Whereas the polypeptide backbone is identical for Thy-1 in brain and thymus, the carbohydrate composition differs significantly (46, 47). Species specific glycosylation of Thy-1 is also well-documented (47, 59). Thus, the lack of 6C10 epitope expression in brain or on rat Thy-1 is not surprising. T cell differentiation associated differences in Thy-1 glycosylation have been also described previously. Decreased branching of the carbohydrate chains of Thy-1 with increased sialic acid addition were shown to accompany T cell differentiation from thymocytes to peripheral T cells (49, 60, 61). 6C10 becomes undetectable during maturation in the thymus, consistent with this differentiation related change in glycosylation. However, we found that 6C10 is re-expressed by peripheral T cells, specifically on adult CD4+ T cells. Our 2-DE analysis did not reveal specific association of 6C10 with acidic Thy-1 species and 6C10 seems relatively resistant to neuraminidase treatment (34), suggesting that the determinant may not be a specific sialylated form of Thy-1. Additionally, 6C10 expression was not affected by deficiency in either biosynthesis of complex oligosaccharides or fucose addition (our unpublished results, and from analysis of BW5147.PHA and BW5147.PL, 62, 63, respectively). These results suggest that the 6C10 epitope shared between thymocyte and peripheral T cell Thy-1 glycoprotein may be expressed on various Thy-1 glycoforms.

The question arises as to whether the re-expression of 6C10 on peripheral CD4+ T cells is due to a quantitative increase in Thy-1 glycoprotein bearing a similar ratio of glycoforms or, instead, reflects a shift in glycoform expression on a subset of Thy-1. That is, considering the close correlation between Thy-1 and 6C10 epitope levels, one might ask whether the apparent lack of 6C10 on some T cells might simply reflect a lower staining efficiency by SM6C10 compared with anti-Thy-1 peptide Abs. Answering this question by immunochromehcial means has been difficult because of the combination of lower Ag level in the periphery and lower Ab affinity. However, our retroviral transduction data revealed that T cells expressing even high levels of Thy-1 can lack 6C10, arguing against this interpretation. Of interest, this lack of 6C10 was observed in the Thy-1 gene transduced AKR1d.6.6 T lymphoma. This lymphoma is a spontaneous variant of the original Thy-1 deficient AKR1d, which shows several additional defects in expression of surface Ags, including absence of CD45 (R. Hyman, unpublished observations). Thy-1 transfection into the original AKR1d line results, in contrast, in 6C10+ Thy-1 expression as we have previously shown (11). This prompts us to suggest that 6C10+ Thy-1+ T cells in mice, whether CD4+ or CD8+, truly lack the specific 6C10 epitope due to alternative posttranslational modification, but that CD4+ T cells in particular regain the ability to synthesize and/or express 6C10+ Thy-1 glycoform(s) during peripheral development.

Although it has been known that most CD4+ T cells in the thymus are not fully functionally competent despite their high TCR/CD3 expression (8, 64), it has not been clear whether the CD4+ T cells newly arrived in peripheral sites are all equally functionally mature. We found that 6C10− CD4+ naïve T cells in neonatal mouse spleen of transgenic mice with monomorphic TCR, consisting of cells newly exported from thymus, exhibit lower responsiveness to anti-Thy-1 stimulation compared with 6C10+ cells in adult mice. Aside from this ontogenic difference, we have previously described a pattern of 6C10+ to 6C10− transition by analyzing CD4+ T cells reconstituted by transfer of adult bone marrow cells into adult recipients (6); thus, this phenotypic change is not due to ontogenic differences in cell microenvironment. Rather, our data indicate that postthymic maturation continues in the periphery for CD4+ T cells as a programmed process, including up-regulation of 6C10+ Thy-1 and gain of function.

Why do 6C10+ CD4+ T cells respond better to anti-Thy-1 (and other) activation? Our data clearly exclude the possibility that 6C10 recognizes novel transmembrane signaling molecule. Acquisition of signaling capability in B cell or human T cell lines by transfection of the mouse Thy-1 gene has been reported previously, in which anti-Thy-1 cross-linking resulted in a calcium influx (26). In this work, it was suggested that if any transmembrane molecule was required for anti-Thy-1 signaling, then such a molecule must be conserved between species and be ubiquitously distributed. We show in this paper that 6C10 is a carbohydrate epitope of Thy-1, and that 6C10+ Thy-1 can be induced in non-T cells such as in pro-B cells by Thy-1 gene transduction, demonstrating that non-T cells can express the 6C10+ Thy-1 glycoform. This result strongly suggests that 6C10+ Thy-1 alone is important for cells to signal via anti-Thy-1 cross-linking.

Because the GPI-anchor is located entirely within the outer leaflet of the cell membrane bilayer, GPI-linked proteins possess increased mobility compared with transmembrane proteins, resulting in their rapid lateral redistribution. This has been considered a significant feature in cell-cell adhesion, and in transduction of extracellular stimuli (15). Internalization and concentration of GPI-linked small molecules appears to occur at a unique site, the caveolae (16). Caveolae are a specialized detergent-resistant membrane compartment where many signaling molecules appear to be concentrated, such as Src family tyrosine kinases (17, 18, 45, 65) and phosphatidylinositol bisphosphate (PIP2) (66). Thus, one proposed model has been the coupling of GPI-linked internalized molecules with the intracellular-signalignaling machinery at the caveolae (30). Because subtle differences in the carbohydrate side chains influence the physiological properties of plasma glycoproteins (59), it is possible that 6C10+ Thy-1 glycoforms may have an advantage in this lateral redistribution and invagination process, resulting in enhanced signal transduction.

Alternatively, higher anti-Thy-1 responsiveness by the 6C10+ Thy-1 cells may simply reflect functional maturity of CD4+ T cells. “Mature” CD4+ T cells may possess altered biosynthesis of
glycosidases and/or glycosyltransferases, resulting in changes in surface phenotype, such as production of 6C10 glycosylated Thy-1, and an altered signaling mechanism. Considering such T cell "maturation" may be more important in terms of understanding increasing T cell function in the immune system from newborn to adult. Besides their gain of 6C10, CD4+ T cells in adults also express higher levels of Qa2 and Ly6A/E when compared with neonates (Ref. 8, and our unpublished observations). As recent data suggests, dynamic accleration of activated Ag receptor complexes rafting to the signaling molecule rich caveolae appears to constitute an important step in T cell activation (65, 67). Thus, coordinate up-regulation of GPI-linked glycoproteins in mature CD4+ T cells may provide increased opportunity for signaling interactions, influencing the fate of cells during activation, either positively or negatively.

In summary, our work demonstrates that the 6C10 determinant, recognized by a natural autoantibody, is a carbohydrate epitope on the GPI-anchored glycoprotein Thy-1. Significantly, we show in this paper that the functional and phenotypic heterogeneity among naïve CD4+ T cells is due to continuing maturation of CD4+ T cells in the periphery, coincident with 6C10 glycosylated Thy-1 up-regulation. Whether the 6C10 carbohydrate epitope on Thy-1 has direct significance in T cell function or is simply an associated surface phenotype remains to be determined. A potential role for natural anti-Thy-1 autoantibody in T cell activation is another intriguing question to be answered. Nevertheless, our study introduces the concept of peripheral T cell maturation, demonstrating that most CD4+ T cells in the neonate and many of the CD4+ T cells in the adult have yet to reach full functional competence. Thus, it is important to consider that differences in the CD4+ T cell populations in APCs (68), contribute to distinctions between neonatal and adult immunity, in terms of tolerance induction, memory T cell generation, and generation of distinct effector cell types. Molecular understanding of maturation and determining why the capacity to express certain glycosylated Thy-1 is retained by memory Th cells but not by anergic T cells (3, 31) is important subjects for future investigation.

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