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Transferrin Receptor Expression as a Marker of Immature Cycling Thymocytes in the Mouse

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Dividing cells require iron and, therefore, express the transferrin receptor (CD71) on the cell surface to enable internalization of transferrin-bound iron. Since early T cell development is marked by intense proliferation, we questioned whether CD71 might serve as a marker of immature T cells. Therefore, we analyzed the expression of CD71 on fetal, neonatal, and adult thymocytes in correlation with cell size, cell cycle status, and expression of CD3, CD4, CD8, $\alpha\beta$TcR, and $\gamma\delta$TcR. Phenotypic analysis showed that only the large, immature $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ cells in fetal, neonatal, and adult thymus expressed CD71. In addition, DNA analysis showed that all $CD71^+$ large adult thymocytes were cycling. Downregulation of CD71 occurs when proliferation ceases, i.e., within the $CD4^+8^+3^-$ thymocyte subpopulation. The gradual changes in size and CD71 expression suggest a sequential development within this $CD4^+8^+3^-$ subpopulation from large $CD71^+$ via small $CD71^\pm$ to small $CD71^-$ cells. As a consequence, CD71 expression is downregulated, in adult T cell development as well as in ontogeny, before the $\alpha\beta$TcR appears on the cell surface of the thymocyte. Together, our findings show that CD71 is a marker of immature, proliferating T cells.

INTRODUCTION

The transferrin receptor (TfR)[2] is expressed on many cells that require iron for their development (1, 2). The murine TfR is a 200-kDa glycoprotein composed of two identical, disulfide-linked chains, each capable of binding transferrin, the iron-transport molecule (3). Binding of transferrin to its receptor followed by endocytosis of the receptor–ligand complex is a major cellular iron-uptake mechanism (1). The internalized iron is either stored in ferritin deposits or used as a substrate for the biosynthesis of iron-containing proteins. Iron-free apo-transferrin and the TfR are recycled to the cell surface.

The enzyme ribonucleotide reductase involved with DNA synthesis during cell proliferation is an example of an iron-containing protein (4). The biosynthesis of this enzyme as well as other iron-containing proteins during cell proliferation requires an

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[2] Abbreviations used: TfR, transferrin receptor; GD, gestational day; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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TABLE 1

Characteristics of the Monoclonal Antibodies

CD[a]	Antigen	Clone	Conjugation[b]	Reference
CD3	CD3	KT3	B	(16)
CD4	L3T4	GK1.5	PE	(17)
CD8	Lyt-2	53-6.7	F	(18)
CD71	TfR	ER-MP21	F	(15)
TcR1	$\gamma\delta$TcR	GL3	B	(19)
TcR2	$\alpha\beta$TcR	H57-597	B	(20)
—	—	ER-MP20	F	(21)

[a] Monoclonal antibodies are ordered by the cluster of differentiation (CD) nomenclature, when possible.
[b] mAb were used unconjugated or conjugated with FITC (F), biotin (B), or R-Phycoerythrin (PE).

increased iron uptake by the cell. Many cell types respond to this need by increasing the surface expression of the TfR. Cycling cells in normal tissues, e.g., cells of the basal layer of the epidermis, as well as in many neoplastic tissues express the TfR (2, 5, 6). Moreover, virtually all *in vitro* maintained cell lines show TfR expression (2). Thus, TfR expression is unequivocally associated with cell proliferation.

A phase of intense proliferation marks early T cell development in both fetal (7) and adult thymus (8, 9). In the adult thymus, for example, an overall expansion of around 10^5-fold was calculated when a daily entry of 100 prothymocytes was assumed (8). TfR expression by cycling thymocytes was found when the transferrin receptor was recognized as a human leucocyte differentiation antigen with its own cluster designation, i.e., CD71 (2). Flow cytometric analysis of adult human thymocytes showed a 14% mean reactivity with CD71 mAb. In addition, staining of human thymus sections with CD71 mAb showed a strong labeling of cells in the subcapsular area, where immature cycling thymocytes are located (10). These data suggest that indeed CD71 expression on human thymocytes is associated with proliferation. In contrast, flow cytometric analyses of mouse thymocytes with CD71 mAb showed less than 1% reactivity (11, 12). This does not seem to agree with the estimation that $\approx$20% of all thymocytes are actually dividing cells (8, 13, 14). The apparent discrepancy is solved in the present study by application of a new CD71 mAb, i.e., ER-MP21 (15), which stains 15–20% of all adult mouse thymocytes. Here we questioned whether CD71 expression by thymocytes could be used as a marker for immature proliferating cells. To that purpose we analyzed the cycle status as well as the developmental stage of CD71-expressing thymocytes in fetal, neonatal, and adult mice. We show that CD71 is mainly expressed by immature cycling thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes.

MATERIALS AND METHODS

Mice

Timed fetal (Gestational Days 14–18), neonatal (age $\leq$24 hr), and adult (6–10 weeks) BALB/c mice ($H\text{-}2^d$) were bred and maintained in the animal facilities of our department.

Antibodies

The antibodies used in this study are listed in Table 1. They were purified from hybridoma culture supernatant by affinity chromatography and conjugated with fluorescein isothiocyanate (FITC) or biotin for flow cytometric analysis. The CD4–R-Phycoerythrin conjugate (Becton–Dickinson, Mountain View, CA) was commercially obtained. The properties of the monoclonal antibodies used were described in detail in the indicated references (15–21). The mAb ER-MP20 (21) was used as an IgG2a isotype control mAb for ER-MP21. To avoid nonspecific staining, all conjugates were carefully titrated.

Immunofluorescence and Flow Cytometric Analysis

For flow cytometric analysis, thymocyte suspensions were prepared in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumine (BSA) and 2 m*M* sodium azide (NaN_3). Cells (10^5/10–20 μl) were incubated on ice for 30 min with the appropriate mAb or mixture of mAb (FITC-, biotin-, or R-Phycoerythrin conjugates). After three washes with PBS–BSA–NaN_3, the cells were further incubated for 30 min on ice, with appropriate dilutions of R-Phycoerythrin or Tricolor conjugated to Streptavidin (Caltag, San Francisco, CA). Finally, the cells were washed again three times and collected in a small volume for flow cytometric analysis. To identify dead cells in samples with two-color staining, propidium iodide was added just before acquisition at a final concentration of 5 μg/ml. Background fluorescence was determined by staining cells with second-step antibodies only or with the FITC-conjugated mAb ER-MP20.

Analytical flow cytometry was carried out on a FACScan (Becton–Dickinson). Dead cells were excluded during data analysis on the basis of forward and perpendicular light scatter (for three-color staining) or a combination of forward light scatter and propidium iodide staining (for two-color staining).

Cell Cycle Analysis of Sorted Thymocytes

Cell cycle analysis was performed on unfractionated and sorted large $CD71^+$ adult thymocytes. The latter population was purified using a FACS Vantage (Becton–Dickinson). For subsequent DNA analysis, cells were fixed with 70% ethanol and stained with 40 μg/ml propidium iodide, in the presence of 100 μg/ml RNase A. Cells were analyzed on a FACScan with linear amplification in the fluorescence channel. The proportion of cells in G_0/G_1, S, and G_2/M was calculated using the DNA Cell-Cycle Analysis Software (Becton–Dickinson).

RESULTS AND DISCUSSION

The expression of the transferrin receptor on adult thymocytes was analyzed using the CD71 mAb ER-MP21 (15). CD71 was found to be expressed with a weak to moderate intensity by 15–20% of adult thymocytes (Fig. 1A). In addition, we observed that most $CD71^+$ cells had a large cell size, as determined by forward light scatter (Fig. 1A). These data are in line with the idea that CD71 is expressed by cycling thymocytes, because (i) the number of $CD71^+$ cells corresponds with estimated numbers of cycling thymocytes (8, 13, 14) and (ii) the population of large thymocytes is known to contain virtually all cycling cells (22, 23). To substantiate this notion we subsequently analyzed the cell cycle status of adult large $CD71^+$ thymocytes.

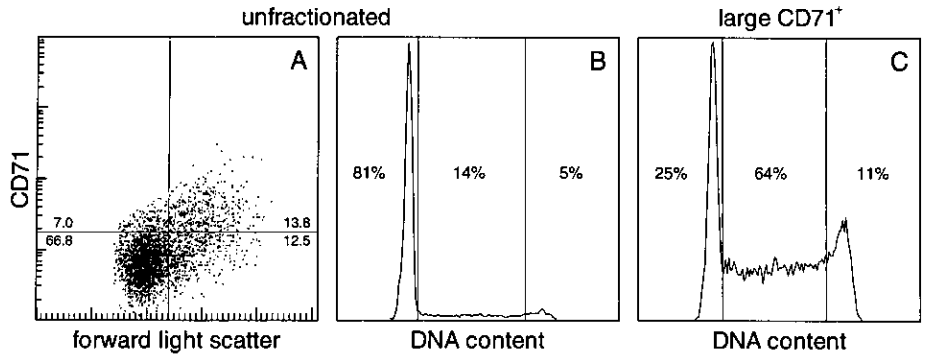


FIG. 1. Cell cycle analysis of large CD71$^+$ thymocytes from adult mice. Thymocytes were stained with CD71–FITC and CD71 expression was correlated with cell size, measured as forward light scatter (A). Sorted large CD71$^+$ cells (upper right quadrant in A) were compared with unfractionated cells for their cell cycle status. The DNA content, measured as propidium iodide fluorescence, of unfractionated (B) and large CD71$^+$ thymocytes (C) is shown in histograms with a linear scale. In DNA histograms, the left peak, the lower midsection, and the right peak represent the G_0/G_1, S, and G_2/M phases of the cell cycle, respectively.

The Transferrin Receptor Is Expressed by Large-Sized Dividing Thymocytes

Cell cycle analysis was performed on unfractionated and sorted large CD71$^+$ adult thymocytes, by using the DNA-specific dye propidium iodide. Fourteen and 5% of unfractionated thymocytes were in the S and G_2/M phases of the cell cycle, respectively (Fig. 1B). In contrast, 64 and 11% of the large CD71$^+$ cells were in the S and G_2/M phases, respectively (Fig. 1C). Since the S phase of a normal thymocyte cell cycle takes 50–70% of the whole cycle (24, 14), our results indicate that all large CD71$^+$ cells are cycling. It has been shown that large cycling thymocytes are phenotypically (and functionally) immature (8, 13, 14, 25, 26). Consequently, we assumed that large CD71$^+$ cells had an immature phenotype. This assumption was tested by establishing the developmental stage of the CD71-expressing thymocytes.

The Transferrin Receptor Is Expressed by Immature Thymocytes Only

T cell development in the murine thymus can be monitored by analyzing the surface expression of CD4, CD8, and the CD3/$\alpha\beta$TcR complex on the thymocyte surface. Phenotypically distinct subpopulations have been identified and the lineage relationship of these cells has been well established. A commonly accepted pathway for intrathymic $\alpha\beta$ T cell development is summarized in Fig. 4 (8, 13, 14, 27, 28). In this context, we determined the expression of CD71 by thymocyte subpopulations using three-color flow cytometry (Fig. 2). Cell size was included in our analysis, because thymocytes are heterogeneous in cell size (22) and immature cycling cells are contained in the large-sized fraction (22, 23).

We observed that large CD4$^-$8$^-$ thymocytes expressed CD71 with variable intensity, ranging from negative to high surface expression (Fig. 2C). CD4$^+$8$^+$TcR$^-$ thymocytes, identified as CD4$^+$TcR$^-$ cells, contained three subsets: large CD71$^+$ cells, small cells with intermediate CD71 expression, and small CD71$^-$ cells (Fig. 2D). The gradual change in CD71 expression and cell size of the three subsets suggests a sequence, with large CD71$^+$ cells developing through small CD71$^\pm$ cells into small CD71$^-$ cells. The

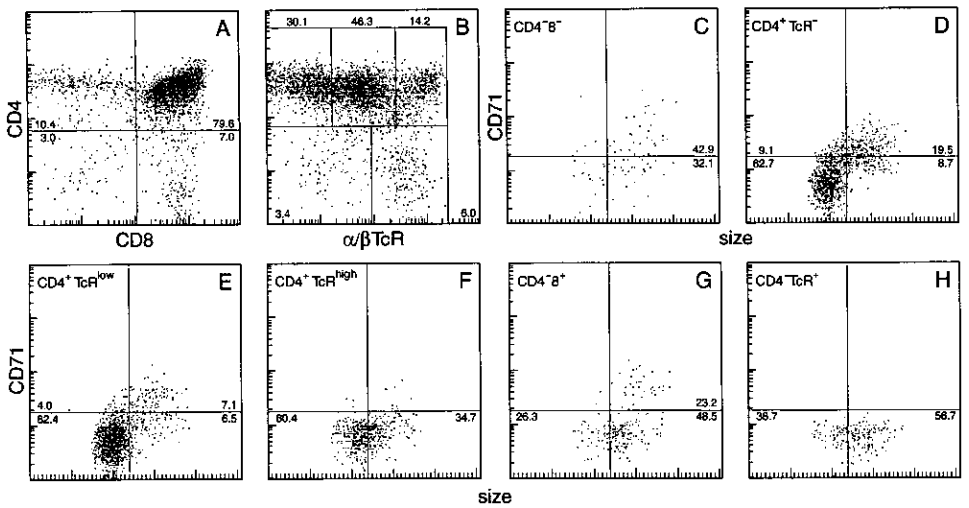


FIG. 2. CD71 expression by thymocytes from adult mice. Phenotypes of thymocytes were determined with CD71-FITC, CD4-R-Phycoerythrin, and either CD8-biotin or anti-$\alpha\beta$TcR-biotin, followed by streptavidin-Tricolor. CD4/CD8 and CD4/$\alpha\beta$TcR dotplots are shown for total thymocytes (A,B) and CD71/size dotplots for $CD4^-8^-$ (C), $CD4^+TcR^-$ (D), $CD4^+TcR^{low}$ (E), $CD4^+TcR^{high}$ (F), $CD4^-8^+$ (G), and $CD4^-TcR^+$ (H) cells. Thymocyte subpopulations were quantified by quadrant (A) or window analysis (B) and size of the thymocytes was measured as forward light scatter. Relative numbers of cells are indicated within the quadrants.

$CD4^+8^+TcR^{low}$ thymocytes, identified as $CD4^+TcR^{low}$ thymocytes, were mainly small and $CD71^-$, although a small but distinct population of large $CD71^+$ cells was also present (Fig. 2E). The mature $\alpha\beta$TcR expressing $CD4^+8^-$ thymocytes, identified as $CD4^+TcR^{high}$ cells, were all $CD71^-$ (Fig. 2F). In contrast, the $CD4^-8^+$ thymocytes were heterogeneous, containing $CD71^-$ thymocytes of intermediate cell size as well as $CD71^+$ thymocytes with a large cell size (Fig. 2G). However, when analyzing the $CD4^-TcR^+$ cells, representing the $CD4^-8^+TcR^{high}$ thymocytes (and also the $CD4^-8^-TcR^+$, both $\alpha\beta$ and $\gamma\delta$ thymocytes) no expression of CD71 was observed (Fig. 2H). This indicates that the $CD4^-8^+$ $CD71^+$ thymocytes represent the immature TcR-negative intermediates between $CD4^-8^-$ and $CD4^+8^+$ thymocytes.

Our analysis shows that, in adult thymus, CD71 is expressed by the majority of $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ immature blasts (Fig. 4). Likewise, cell cycle analysis studies have shown that cycling cells in adult thymus are confined to these subpopulations (8, 13, 14). The estimated numbers of cycling cells within the $CD4^-8^-$, $CD4^-8^+3^-$, and large $CD4^+8^+3^-$ thymocyte subpopulations (respectively, 60, $\approx$100, and 80%) are comparable with the frequencies of CD71-expressing cells that we observed (8, 13, 14, 26, 29). Together, these results indicate that CD71 is expressed by the cycling $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ immature blasts of the adult thymus. It also implicates that expression of CD71 marks the major expansion phase in early T cell development that starts within the $CD4^-8^-$ thymocyte population and ends at the level of the $CD4^+8^+3^-$ cells (8).

Thus, CD71 is downregulated as cell division ceases, before the CD3/$\alpha\beta$TcR complex is expressed (14, 27, 30). As a consequence, thymocytes with low or high CD3/$\alpha\beta$TcR expression do not express CD71.

Thymocytes of the $\gamma\delta$ T cell lineage and the $CD4^-8^-$ $\alpha\beta$ T cell lineage seem to be CD71-negative, as the adult $CD4^-8^-3^+$ subpopulation, containing cells of both these lineages (31, 32), did not stain with CD71 (data not shown). Accordingly, others reported the absence of cycling cells within this thymocyte subpopulation (8, 14, 33).

Next, we analyzed CD71 expression on developing thymocytes in the fetal and neonatal mouse to investigate whether CD71 is a marker of immature cycling cells, also in ontogeny. In addition, $\gamma\delta$ T cells are more easily studied in fetal thymus, because they are present in relatively high frequencies.

All Immature Thymocytes in Ontogeny Express the Transferrin Receptor

Fetal thymocytes from Gestational Days (GD) 14 and 16–18 as well as neonatal thymocytes were analyzed for CD71 expression and developmental status (Fig. 3). CD71 was expressed with similar intensity by all fetal thymocytes of GD16 and GD17 (Figs. 3A and 3B), as well as of GD14 (data not shown). From GD14 to GD17, $CD4^-8^-$ thymocytes developed through $CD4^-8^+$ intermediates into $CD4^+8^+$ thymocytes (Figs. 3F and 3G). Thymocytes in these subpopulations were all large in cell size and did not express the $\alpha\beta$TcR (data not shown). These observations show that large immature $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ fetal thymocytes of GD14–17 all expressed CD71 (Fig. 4). At these timepoints of fetal development, more than 80% of the cells are known to be actively cycling (7).

Downregulation of CD71 occurs around GD18 (indicated by reductions of both the relative number of positive cells and the staining intensity), when small $CD4^+8^+$ thymocytes developed (Figs. 3C and 3H). We observed that, in contrast to adult thymus, all small $CD4^+8^+TcR^-$ cells expressed CD71 at low levels (data not shown; Fig. 4). The subpopulation of small $CD4^+8^+TcR^{low}$ cells (like in adult thymus) did not express CD71 (data not shown).

Around birth, CD71 expression could only be observed in a minority of the cells, similar to the CD71 expression on adult thymocytes (compare Figs. 3D and 3E). At this stage in ontogeny, the only mature cells with high $\alpha\beta$TcR expression were $CD4^+8^-$ cells (Fig. 3I). Surprisingly, this $\alpha\beta TcR^+$ thymocyte subpopulation contained a subset of large $CD71^+$ cells (data not shown); this is likely related to the presence of cycling cells within the mature $CD4^+8^-$ thymocyte subpopulation in neonatal mice (34). Nonetheless, in general also during fetal T cell development CD71 expression is downregulated before the $\alpha\beta$TcR appears on the cell surface of the thymocyte, confirming our observations in the adult thymus.

Does this also apply to cells of the $\gamma\delta$ T cell lineage present in fetal thymus? In ontogeny, we have detected $\gamma\delta$TcR-expressing thymocytes at GD16, GD17, and GD18 (data not shown). We observed that all these fetal $\gamma\delta TcR^+$ thymocytes expressed CD71. This confirms a previous observation that fetal $\gamma\delta$ T cells are actively cycling (35).

Concluding Remarks

Together, our results indicate that CD71 is expressed by cycling thymocytes. Especially in early T cell development in both fetal and adult thymus, CD71 marks a major expansion phase of large immature thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes. The more mature thymocytes of the $\alpha\beta$ T cell lineage, i.e., the cells with low- and high-level $\alpha\beta$ TcR expression, do not express CD71. Yet,

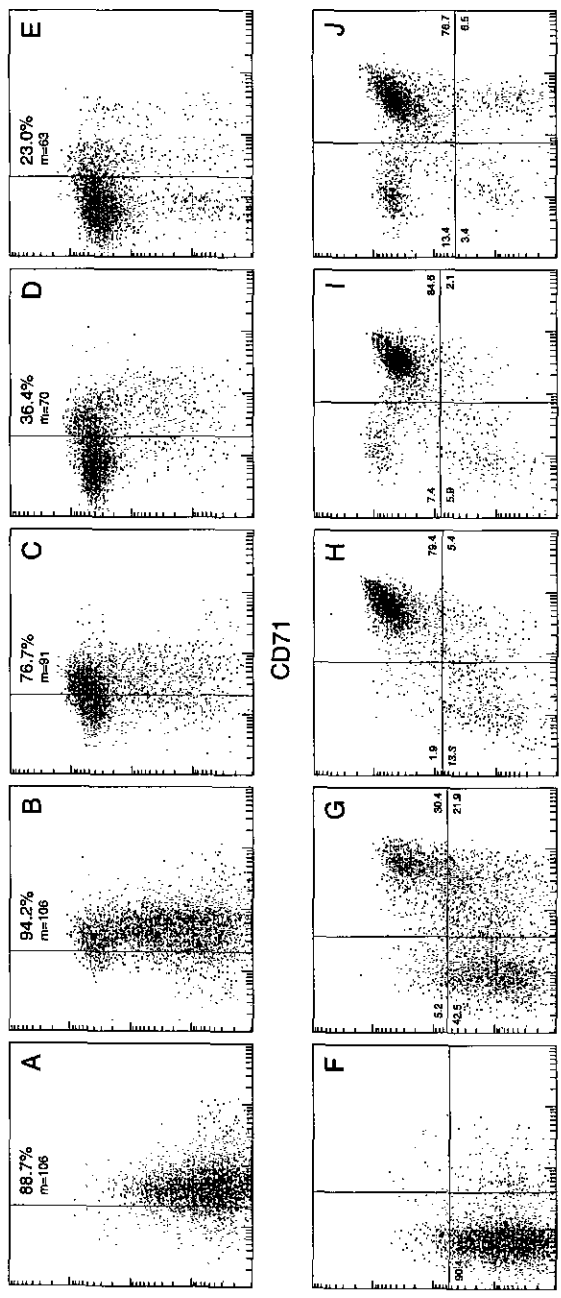
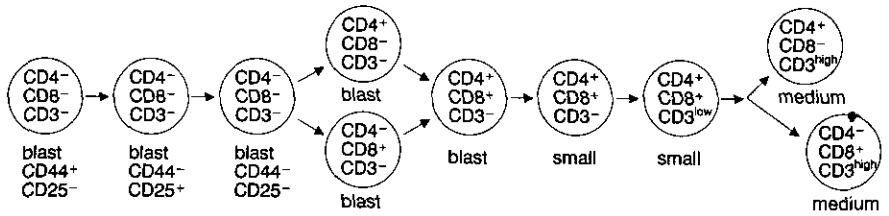


FIG. 3. CD71 expression and developmental status of thymocytes in ontogeny. Cells from fetal (GD16–18), neonatal, and adult thymus were stained with CD71–FITC and CD4–R-Phycoerythrin (A–E), or with CD8–FITC and CD4–R-Phycoerythrin (F–J). Figures indicate the relative number and mean fluorescence intensity (by channel number on a full scale of 256 channels). CD4/CD8 defined thymocyte subpopulations were quantified by quadrant analysis; relative numbers of cells are indicated within the quadrants.



Transferrin receptor expression

adult	⊢——variable——⊣			+	+	−	−	−	−
neonatal	⊢——variable——⊣			+	+	−	−	−/+	np
fetal	+	+	+	+	+	±	−	np	np

FIG. 4. Transferrin receptor expression in murine T cell differentiation. The development of thymocytes in the $\alpha\beta$ T cell lineage is shown, with emphasis on the early T cell development, based on our current results and results reported by others (8, 13, 14, 27, 28). The indicated subpopulations contain cells positive (+) or weakly positive (±) for CD71 or with no (−) CD71 expression at all. For fetal thymus the development of CD71 expression on cells from days 14–18 is given. Some of the mature thymocytes were not present (np) in neonatal or fetal thymus.

CD71 was expressed by fetal $\gamma\delta$TcR$^+$ and neonatal $\alpha\beta$TcR$^+$ CD4$^+$8$^-$ thymocytes, strongly suggesting that these cells are cycling.

The importance of CD71 for the uptake of iron by cycling thymocytes was recently established by treating fetal thymus organ cultures with our CD71 mAb ER-MP21 (36). We found that proliferation and maturation of $\alpha\beta$ T cells, but not $\gamma\delta$ T cells, was inhibited by CD71 mAb treatment, indicating that iron is an essential element for normal $\alpha\beta$ T cell development.

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