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Inhibition of proliferation and differentiation during early T cell development by anti-transferrin receptor antibody*

Proliferating cells require iron and, therefore, express the transferrin receptor (CD71) that mediates cellular iron uptake. Cycling thymocytes, which have the $CD4^-8^-3^-$, $CD4^-8^+3^-$, or $CD4^+8^+3^-$ phenotypes, also express CD71. The importance of CD71-mediated iron uptake for proliferation and maturation of thymocytes was studied using fetal thymus organ cultures at day 14 of gestation and treating them for 7 days with a CD71 monoclonal antibody (mAb). The intracellular iron deficiency caused by this treatment, inhibits both proliferation and maturation of the thymocytes. Cell recovery was reduced by 60 %, but cells still expanded tenfold during the culture. Remarkably, the final maturation of αβ T cells was completely blocked as no thymocytes with low or high CD3/αβTcR expression developed. Moreover, only few cells reached the $CD4^+8^+3^-$ stage of T cell development. $CD4^-8^-3^-$ thymocytes, however, as well as its $CD44^-25^+$ subset developed in normal numbers, suggesting that $CD44^-25^+$ $CD4^-8^-3^-$ cells, or their immediate progeny, were most vulnerable to CD71 mAb treatment. The development of γδ T cells, which also express CD71, was not affected in these cultures. This suggests that γδ T cells are either less iron-dependent or possess alternative iron-uptake mechanisms. Thus, our observations indicate that CD71 treatment, causing decreased intracellular iron levels, severely inhibits the major proliferation phase from the $CD44^-25^+$ $CD4^-8^-3^-$ to the $CD4^+8^+3^-$ cells, and completely abrogates the final maturation of $CD4^+8^+3^-$ cells into αβTcR-expressing cells. In contrast, proliferation and differentiation of the earliest thymic precursors into $CD44^-25^+$ $CD4^-8^-3^-$ cells is not affected by CD71 treatment.

1 Introduction

The thymus provides a unique environment for the development of T lymphocytes, supporting both precursor cell proliferation and maturation [1]. These precursor cells, with a $CD4^-8^-3^-$ phenotype, differentiate in the thymus via $CD4^-8^+3^-$, $CD4^+8^+3^-$ and $CD4^+8^+3^{low}$cells into mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes, expressing the CD3/αβTcR complex at the cell surface [2–6]. In addition, the $CD4^-8^-3^-$ cells are heterogenous and can be divided into three subpopulations on the basis of expression of Pgp-1 (CD44) and the p55-chain of the interleukin-2 receptor (IL-2Rα or CD25): $CD44^+25^-$ cells develop via $CD44^-25^+$ into $CD44^-25^-$ cells [7–9]. Within this scheme of T cell development, a major expansion phase starts at the $CD44^-25^+$ $CD4^-8^-3^-$ stage of T cell development. This phase of intense proliferation ends within the $CD4^+8^+3^-$ thymocyte subpopulation, indicating that cell division ceases before the CD3/αβTcR complex is expressed on the surface of the $CD4^+8^+$ thymocyte [3, 10, 11].

Recently, we observed that this major expansion phase in early T cell development is marked by the expression of the transferrin receptor (CD71; [12]). Thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$ and $CD4^+8^+3^-$ phenotypes in both fetal and adult thymus expressed CD71 [12]. These subpopulations also contain almost all cycling cells present within the thymus [3, 5, 8, 10, 13], and through DNA analysis we found that CD71 expression on thymocytes is closely related to proliferation [12].

The transferrin receptor is involved with the uptake of iron delivered to the cell by the iron-transporting molecule, transferrin [14]. The internalized iron is either stored in ferritin deposits or used as a substrate for the biosynthesis of iron-containing proteins. Iron uptake by the proliferating cell is essential for the iron-containing enzyme ribonucleotide reductase involved with DNA synthesis [15]. However, CD71 is not only expressed by cycling cells, but also by cells that require iron for other iron-dependent proteins, such as the enzyme peroxidase in monocytes. Similarly, the expression of CD71 by thymocytes is probably not only related to cell division, but may also reflect the use of iron by iron-containing proteins involved with maturation of the thymocytes. Therefore, we analyzed the role of the transferrin receptor-mediated iron uptake in T cell development. By treating fetal thymus organ cultures with the CD71 mAb ER-MP21 [16], we observed that T cell

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proliferation was severely inhibited. Furthermore, we found that αβ T cell development was completely abrogated, whereas development of γδ T cells was not affected by the treatment. The data presented here indicate that CD71 treatment has a major effect on the $CD4^-8^-3^-$ thymocytes.

2 Materials and methods

2.1 Mice

Fetal (gestational day 14) and adult BALB/c ($H\text{-}2^d$) mice were bred and maintained in the animal facilities of our department.

2.2 Antibodies

The antibodies used in this study are listed in Table 1 [16–23]. They were purified from hybridoma culture supernatant by affinity chromatography and conjugated with FITC or biotin. The CD4-R-phycoerythrin was commercially obtained (Becton Dickinson, Mountain View, CA). To avoid aspecific staining, all conjugates were optimally titrated.

Table 1. Characteristics of the monoclonal antibodies

CD[a)]	Antigen	Clone	Conjugation[b)]	Reference
CD3	CD3	KT3	B	[17]
CD4	L3T4	GK1.5	PE	[18]
	MT4	H129.19	B	[19]
CD8	Lyt-2	53-6.7	F	[20]
CD71	TfR	ER-MP21	F	[16]
TcR1	γδTcR	GL3	B	[21]
TcR2	αβTcR	H57-597	B	[22]
–	–	ER-MP20	F	[23]

a) Monoclonal antibodies are ordered by the Cluster of Differentiation (CD) nomenclature, when possible.
b) mAb were used conjugated with FITC (F), biotin (B), or R-phycoerythrin (PE).

2.3 Fetal thymus organ culture

Fetal thymus lobes were placed in organ culture, as described by Mandel [24] and Jenkinson [25]. Briefly, four to five single thymus lobes were placed on the surface of polycarbonate filters (0.8-μm pore size; Poretics, Livermore, CA) supported by 7-mm-thick gelatin foam blocks (Upjohn Co., Kalamazoo, MI) in 2 ml of medium in 35-mm plastic petri dishes (Falcon; Becton Dickinson, Plymouth, GB). The culture medium consisted of Iscove's modified Dulbecco's medium containing 25 mM Hepes (GIBCO Life Technologies, Ltd, Paisley, GB), supplemented with penicillin, streptomycin, glutamin and 10% FCS (Hyclone, Logan, UT), heat inactivated (30 min, 56 °C). The cultures were grown at 37 °C in a humidified incubator with 8.5% CO_2 in air. Gestational day 14 fetal thymuses were cultured for 7 days (FTOC day 14 + 7).

2.4 Antibody blocking

Day 14 FTOC were treated with purified anti-transferrin receptor mAb ER-MP21 [16], in concentrations of 25, 50 and 100 μg/ml. Subsequent cultures were treated with the high dose of 100 μg/ml ER-MP21 only. Control cultures received PBS only, or the IgG2a isotype control antibody ER-MP20 [23] at a dose of 100 μg/ml. All cultures received the same amount of PBS and the contribution of PBS to the cultures never exceeded 10%. In FTOC day 14 + 12, mAb containing culture medium was replenished at day 6.

2.5 Immunofluorescence and flow cytometric analysis

Viable thymocyte suspensions were prepared by disrupting four to five lobes in a small volume of PBS containing 0.5% BSA and 2 mM sodium azide in a small potter homogenizer. Cells were washed, counted and subsequently used for analytical flow cytometry. Routinely, 10^5 cells were incubated with FITC-, biotin-, or R-phycoerythrin-conjugated mAb or an mAb mixture, followed by R-phycoerythrin or tricolor-conjugated streptavidin (Caltag, San Francisco, CA), as previously described [12]. For double staining using hybridoma supernatant and CD4-R-phycoerythrin, cells were successively incubated with supernatant, FITC-conjugated anti-rat antiserum, 3% normal rat serum, and CD4-R-phycoerythrin. Background fluorescence was determined by staining cells with streptavidin-fluorochrome conjugates only or with the FITC-conjugated mAb ER-MP20.

Analytical flow cytometry was carried out on a FACScan (Becton Dickinson). Dead cells were excluded 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).

3 Results

3.1 CD71 expression in fetal thymus organ culture

Recently, we showed that *in vivo* CD71 was expressed by large, cycling thymocytes with $CD4^-8^-3^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ phenotypes, during both fetal and adult T cell development [12]. In the present study we determined the functional relevance of transferrin receptor-mediated iron uptake for proliferation and maturation of cells in this early phase of T cell development. To that purpose, fetal thymuses of gestational day 14 were cultured in the presence of the CD71 mAb ER-MP21. Using other target cells, such treatment resulted in growth inhibition of cells caused by iron starvation [16, 26]. We used FTOC, because (i) this system supports normal development of αβ and γδ T cells [27], and (ii) at the start of the culture, all day 14 fetal thymocytes were CD71-expressing $CD4^-8^-$ cells [12]. However, first we established whether *in vitro*, *i.e.* during 7 days of normal FTOC, CD71 was expressed by large immature thymocytes and was down-regulated when the αβTcR became expressed, as we observed *in vivo* [12]. We determined CD71 expression by thymocyte subpopulations after triple staining the cells with CD71, CD4 and either CD8 (Fig. 1) or the αβTcR (data not shown).

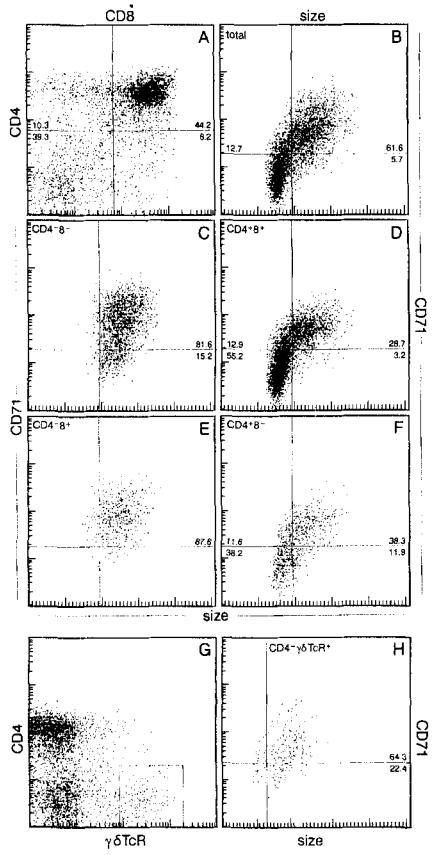

Figure 1. CD71 expression by thymocyte subpopulations in FTOC day 14 + 7. Thymocytes were stained with CD71-FITC, CD4-R-phycoerythrin, and either CD8-biotin (A-F) or anti-γδTcR-biotin (G,H), followed by streptavidin-Tricolor. CD71/size dot-plots are shown for total (B), $CD4^-8^-$ (C), $CD4^+8^+$ (D), $CD4^-8^+$ (E), and $CD4^+8^-$ (F) thymocytes. Marker lines separate small from large and $CD71^-$ from $CD71^+$ cells; relative numbers of cells are indicated within the quadrants. These CD4/CD8 subpopulations were defined by quadrant analysis as indicated in Fig. A. $\gamma\delta TcR^+$ thymocytes were identified as $CD4^-\gamma\delta TcR^+$ cells (G) and characterized by cell size and CD71 expression (H).

Our data indicate that in FTOC CD71 is expressed on immature $CD4^-8^-$, $CD4^-8^+3^-$, and $CD4^+8^+3^-$ blast cells, including γδ T cells, as observed *in vivo* in fetal and adult T cell development [12].

3.2 Proliferation of thymocytes is inhibited by CD71 mAb treatment

Day 14 fetal thymuses were cultured in the presence of different doses of purified ER-MP21. We observed that cell recovery in such cultures was significantly reduced, as ≈ 200,000 thymocytes per thymus lobe were recovered, *i.e.* only half the amount recovered from control cultures (Table 2). This reduction of 50 % in cell recovery seemed independent of the mAb concentration in the range studied. Despite the CD71 treatment, cells still proliferated and expanded at least tenfold from the start of the organ culture, as day 14 fetal thymus contains only about 20,000 cells. Thus, CD71 treatment causes an inhibition, but not a complete abrogation of cell proliferation in the organ cultures.

Table 2. Inhibition of proliferation in CD71 mAb-treated FTOC day 14 + 7

Treatment	Cell recovery[a)]	
PBS	429 ± 65	
ER-MP20, 100 μg/ml	390 ± 40	
ER-MP21, 100 μg/ml	171 ± 15	($p < 0.0005$)[b)]
ER-MP21, 50 μg/ml	230 ± 28	($p < 0.05$)
ER-MP21, 25 μg/ml	206 ± 29	($p < 0.01$)

a) Cell recovery is expressed in cells/lobe ($\times 10^{-3}$). Data represent the mean ± SEM of four to five experiments.

b) The cell recovery in ER-MP21 mAb-treated cultures differs significantly from the cell recovery in the ER-MP20 mAb-treated control cultures. The corresponding probability factors are indicated within parentheses.

After 7 days of culture, we found that $CD4^-8^-$ thymocytes developed into $CD4^+8^+$, $CD4^+8^-$ and $CD4^-8^+$ cells (Fig. 1A). At the same time, CD71 was down-regulated on developing small cells (Fig. 1B). Subpopulation analysis showed that most $CD4^-8^-$ thymocytes were large, $CD71^+$ cells (Fig. 1C). $CD4^+8^+$ thymocytes contained a major population of small $CD71^-$ cells and a minor population of large $CD71^+$ cells (Fig. 1D). The latter population did not express the αβTcR, whereas a subset of small $CD4^+8^+$ cells already did (data not shown). The $CD4^-8^+$ thymocytes were all large $CD71^+$ cells (Fig. 1E). These cells represent the immature $CD4^-8^+3^-$ cells, as mature $\alpha\beta TcR^{high}$ $CD4^-8^+$ cells had not yet developed under the *in vitro* conditions (data not shown; [12]). The $CD4^+8^-$ population, roughly selected using quadrant analysis (see Fig. 1A), contained both immature $\alpha\beta TcR^-$ and mature $\alpha\beta TcR^+$ cells (data not shown). The $\alpha\beta TcR^+$ subset within the $CD4^+8^-$ population does not express CD71 and has small to intermediate cell size (Fig. 1F). Most cells of the γδ T cell lineage, identified as $CD4^-\gamma\delta TcR^+$ cells, expressed CD71 and were large in cell size (Fig. 1G,H).

The optimal dose of the ER-MP21 mAb for functional studies was determined by establishing the dose of ER-MP21 mAb that results in complete saturation of CD71 molecules on the surface of the thymocytes in CD71 mAb-treated FTOC. The ER-MP21 mAb bound to the surface of the thymocyte during treatment was detected with a FITC-conjugated anti-rat antiserum using flow cytometry. We found that complete saturation was only obtained with the high dose of 100 μg/ml (data not shown). Therefore, the dose of 100 μg/ml was used in the remainder of this study.

3.3 Differentiation of $\alpha\beta TcR^+$, but not $\gamma\delta TcR^+$ thymocytes is inhibited by CD71 mAb treatment

In addition to an effect on T cell proliferation, we also anticipated an effect of CD71 treatment on T cell differentiation, because CD71 is expressed by three immature thymocyte subpopulations, linked to each other by a proliferation-driven differentiation process. In CD71 mAb-

treated cultures, cells were all large in size, whereas control cultures contained both small and large cells (Fig. 2A, *upper panels*). When thymocytes were stained simultaneously with CD4 and CD8 mAb, a dramatic inhibition of T cell differentiation was observed (Fig. 2A, *lower panels*). $CD4^+8^+$ and $CD4^+8^-$ thymocytes developed in very low numbers, causing a substantial (relative) increase in the number of $CD4^-8^-$ thymocytes as compared to the control culture. In absolute numbers, $CD4^+8^+$ and $CD4^+8^-$ cells were drastically reduced (Fig. 2B). The reduction of the $CD4^+8^+$ thymocytes was not only caused by the almost complete absence of small $CD4^+8^+$ cells, but also by a reduction in the number of large $CD4^+8^+$ cells (data not shown). The number of $CD4^-8^+$ cells, however, was only mildly reduced, whereas more $CD4^-8^-$ cells were recovered from CD71-treated cultures compared to control cultures. IL-$2R^+$ cells, representing mainly the $CD25^+$ $CD4^-8^-3^-$ thymocytes, were also recovered in slightly increased numbers (data not shown).

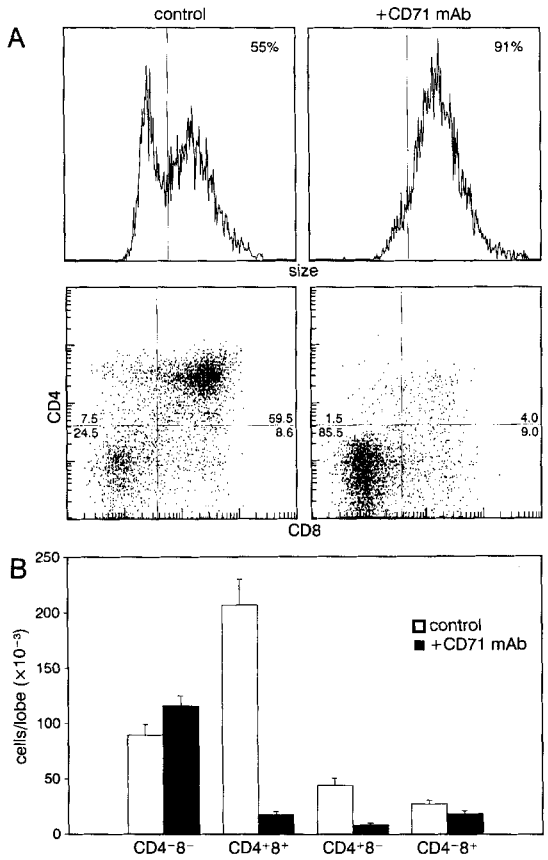

Figure 2. Inhibition of T cell development in FTOC day 14 + 7 by CD71 treatment. Cells from control and CD71 mAb-treated cultures were analyzed for cell size (A) and for CD4/CD8 expression (A,B). Cell size, as measured by forward light scatter, is depicted in histograms, showing the relative number of large cells (A, the upper panels). For CD4/CD8 phenotyping, cells were stained with CD4-biotin and CD8-FITC, followed by streptavidin-R-phycoerythrin. Subpopulations were quantified by quadrant analysis and relative numbers of cells are indicated in the two-color dotplots (A, lower panels). In B the absolute numbers have been depicted in a bar graph, with different bar patterns for the control and CD71 mAb treated cultures. Bars represent the mean ± SEM for eight (control) or nine experiments. Differences in cell recovery are statistically significant for $CD4^+8^+$ ($p = 0$) and $CD4^+8^-$ ($p = 0.0001$) thymocytes.

So far, these data indicate that, αβ T cell differentiation is severely impaired by CD71 mAb treatment. Indeed, $CD4^+8^-$ $\alpha\beta TcR^{high}$ and $CD4^+8^+$ $\alpha\beta TcR^{low}$ thymocytes, identified as $CD4^+3^{high}$ and $CD4^+3^{low}$ cells, respectively were completely absent in the CD71 mAb-treated cultures (Fig. 3A, B). Remaining $CD4^+8^+$ thymocytes were TcR^-. In contrast, there was a relative increase in the number of $CD4^-3^+$ thymocytes. As Fig. 3C and D indicate, these cells are all γδ T cells. Such a relative increase in the number of $\gamma\delta TcR^+$ thymocytes was observed in three different experiments (Table 3). Yet, in absolute numbers, the $\gamma\delta TcR^+$ population was similar in size in both CD71 mAb-treated and control cultures (Table 3).

Table 3. Recovery of $\gamma\delta TcR^+$ thymocytes in CD71 mAb-treated FTOC day 14 + 7

	Relative (%)		Absolute ($\times 10^{-3}$/lobe)	
	Control	+ CD71 mAb	Control	+ CD71 mAb
Exp. 1[a)]	5.2	14.7	13.0	16.2
Exp. 2	5.4	12.8	26.1	24.6
Exp. 3	5.7	13.6	26.8	20.6

a) The relative number of $\gamma\delta TcR^+$ thymocytes was determined from histograms; the absolute recovery was calculated using the relative number and total cell recovery of the organ cultures.

Together these results indicate that (i) T cell differentiation in the αβ T cell lineage is completely abrogated by CD71 mAb treatment, (ii) thymocytes of the γδ T cell lineage are not affected, and (iii) within the $CD4^-8^-$ compartment, cells seem to develop in normal numbers, at least up to the $CD25^+$ stage.

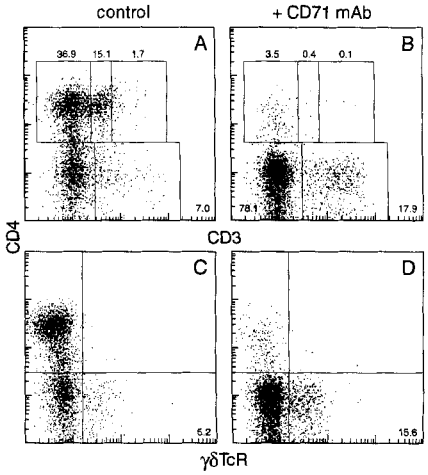

Figure 3. Inhibition of αβ T cell but not γδ T cell development in FTOC day 14 + 7 by CD71 treatment. Thymocytes from the control culture (A,C) or the CD71 mAb-treated culture (B,D) were stained by successive incubations with, either CD3 (A,B) or anti-γδTcR (C,D) supernatant, anti-hamster-FITC (Caltag, San Francisco), normal rat serum, and CD4-R-phycoerythrin. Subpopulations were quantified by window analysis for CD4/CD3 dotplots (A,B) and by quadrant analysis for CD4/γδTcR dotplots (C,D); relative numbers of cells are indicated in these dotplots.

3.4 Inhibition of differentiation occurs independently of inhibition of proliferation

The question arises whether the observed inhibition of differentiation is a mere consequence of the inhibition of proliferation, or whether differentiation itself also requires iron uptake by the cells. When inhibition of differentiation is a mere consequence of the inhibition of proliferation, it may be expected that a CD71 mAb-treated culture and a control culture with identical cell number would have the same developmental stage. To test this assumption, we performed two experiments. In the first experiment, we analyzed the cell recovery and the developmental stage of the organ cultures through a period from day 3 to day 7 of the culture (Fig. 4A). We found that cell number in the CD71 mAb-treated cultures increased more slowly with culture time, as compared with the control cultures. Comparison of the cell recovery shows that the control culture at day 4 and the CD71 mAb-treated culture at day 7 contained comparable cell numbers, 164000 and 178000 cells per lobe, respectively (see asterisks in Fig. 4A). Comparison of the developmental stage of the thymocytes in these particular samples showed that the CD71 mAb-treated culture contained more $CD4^-8^-$ and $CD4^-8^+$ cells, but fewer $CD4^+8^+$ cells (Fig. 4B). In addition, cells of the CD71 mAb-treated culture were all large in size, whereas the control culture also contained small cells (data not shown). This observation indicates that cells in CD71 mAb-treated cultures were generally less mature than cells in control cultures.

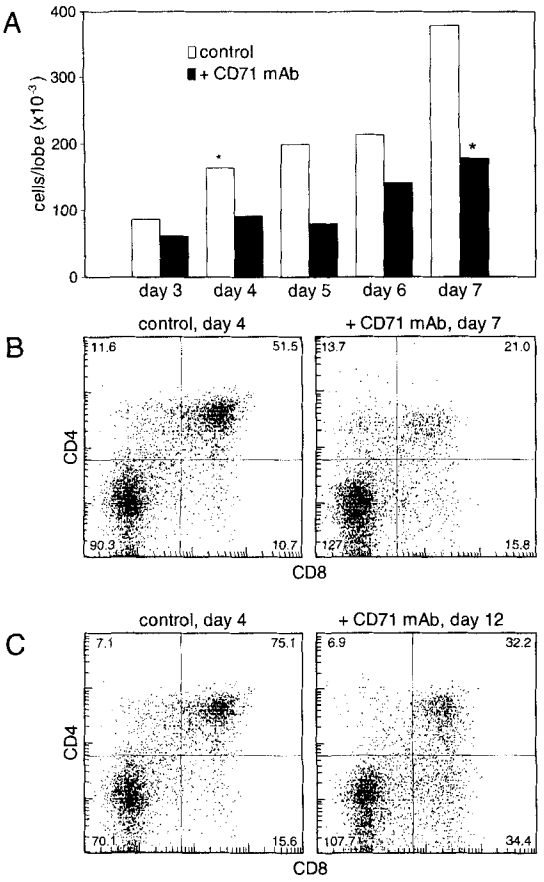

Figure 4. Inhibition of differentiation occurs independent of the inhibition of proliferation in CD71-treated FTOC day 14 + 7. In A, the cell recoveries at days 3 to 7 of the organ culture are shown for control and CD71 mAb treated cultures (indicated by bars with different bar patterns). Asterisks indicate the samples (of equal cell recovery) that were used for two-color staining with CD4 and CD8 mAb, presented in B. Cells from the control FTOC at day 4 and from the CD71 mAb-treated FTOC at day 7 were stained with CD4-biotin and CD8-FITC, followed by streptavidin-R-phycoerythrin. Subpopulations were quantified by quadrant analysis; the absolute numbers of cells ($\times 10^{-3}$) are indicated in the quadrants. In a second experiment, a control FTOC at day 4 was compared to a CD71 mAb-treated FTOC at day 12 (C). Cells were stained and analyzed as indicated for B.

In a second experiment, under identical experimental conditions, the control culture at day 4 and the CD71 mAb-treated culture at day 12 contained comparable cell numbers (168000 and 181000 cells per lobe, respectively; Fig. 4C). Comparison of the developmental stages of these cultures, once more showed that more $CD4^-8^-$ and $CD4^-8^+$ cells, but fewer $CD4^+8^+$ cells developed after CD71 mAb treatment and all cells were large in size (data not shown).

Together, these data suggest that (i) inhibition of differentiation is not just a consequence of inhibition of proliferation, and (ii) that terminal maturation of αβ thymocytes requires higher intracellular iron levels than proliferation only.

4 Discussion

Iron is essential to proliferation of cells. Therefore, cells express the transferrin receptor (CD71) on the cell surface during cell cycle [14]. Recently, we have also shown that cycling immature thymocytes are characterized by CD71 expression [12]. The importance of intracellular iron levels for maturational processes is much less known, however. In this study, we determined the importance of CD71 mediated iron uptake for both proliferation and maturation of thymocytes in FTOC.

Indeed, our data indicate that proliferation of thymocytes in FTOC is inhibited by CD71 mAb treatment. However, proliferation was only partially inhibited, and the number of cells still expanded tenfold from the start of the culture. Partial inhibition of proliferation was not caused by suboptimal mAb treatment, because all CD71 molecules were saturated with the CD71 mAb, even after 7 days of organ culture. Rather, this observation indicates that thymocytes can still internalize low levels of iron, likely, because the CD71 mAb ER-MP21 recognizes an epitope on CD71 different from the transferrin-binding site [16]. As a consequence, iron-loaded transferrin can still bind to the receptor and be internalized. However, for various cell types it was found that binding of the CD71 mAb severely decreases recycling of CD71 between the cell surface and the intracellular compartments and, consequently, renders cells iron deficient [16, 26, 28–30]. Likewise, thymocytes in FTOC became iron deficient, but, apparently, the low intracellular iron level could still support a low level of proliferation.

A major observation from our study is that the maturation of αβ T cells, but not γδ T cells, in the FTOC was inhibited

by CD71 mAb treatment. Thymocytes expressing the CD3/αβTcR complex at low or high level did not develop in the CD71 mAb-treated cultures. Similar to proliferation, the maturation of the cells was only partially inhibited: a small fraction of $CD4^-8^-$ cells, present in day 14 fetal thymus, could still develop into $CD4^+8^+3^-$ cells. Consequently, it seems that in CD71 mAb-treated cultures, thymocytes can only develop to the transition point where $CD3^-$ cells start to mature into $CD3^{low}$ cells. Normally, cell division stops at this transition, cells enter the G_0 phase of the cell cycle and become small in size [6, 8, 31]. In addition, down-regulation of CD71 also marks the transition of $CD3^-$ to $CD3^{low}$ cells [12]. Our present findings suggest that maturation of immature $\alpha\beta TcR^-$ thymocytes into αβTcR-expressing cells is critically dependent on the intracellular iron levels of the maturing thymocytes.

Despite the development of thymocytes up to the $CD4^+8^+3^-$ stage, our data suggest that CD71 treatment already affects thymocytes before the $CD4^+8^+3^-$ stage of T cell development. This suggestion was supported by the observation that all thymocyte subpopulations were severely reduced in cell number or even absent, with the exception of the $CD4^-8^-3^-$ cells. Additionally, we observed that in preliminary experiments using FTOC from day 16 embryos, proliferation and differentiation of cells were not inhibited by CD71 mAb treatment (data not shown). The most likely explanation of this preliminary finding is that day 16, in contrast to day 14, fetal thymocytes have passed the stage most sensitive to CD71 mAb treatment. Since day 16 fetal thymus, like day 14 fetal thymus, mainly contains $CD4^-8^-$ thymocytes [12], it seems likely that cells most sensitive for CD71 treatment belong to a subset within the $CD4^-8^-3^-$ thymocyte subpopulation.

Subsets of $CD4^-8^-3^-$ cells can be defined by expression of CD44 and CD25: $CD44^+25^-$ cells develop via $CD44^-25^+$ into $CD44^-25^-$ cells ([9, 32]; unpublished observations). Recently, the $CD25^+$ $CD4^-8^-3^-$ stage of adult thymocyte differentiation was identified as a possible major branching point for the development of the three T cell lineages, i.e. the αβ T cells, the γδ T cells and the $CD4^-8^-$ αβ T cells [9, 33]. The development of the $CD25^+$ $CD4^-8^-3^-$ cells is unaffected by CD71 mAb treatment. This is based on the observations, that (i) $CD44^-25^+$ cells develop in normal numbers in CD71 mAb treated cultures, and (ii) γδ T cells, developing from the $CD25^+$ cells, are not affected by CD71 treatment. Together, these data suggest that the $CD44^-25^+$ $CD4^-8^-3^-$ cells or their immediate progeny, the $CD44^-25^-$ $CD4^-8^-3^-$ cells, are probably most dependent on intracellular iron levels.

The development of γδ T cells, that also express CD71, was not affected by CD71 mAb treatment. This observation indicates that, in contrast to maturing αβ T cells, γδ T cells apparently internalize sufficient amounts of iron to support their normal development. Two mechanisms can provide an explanation for this difference: (i) γδ T cells internalize more iron because, despite CD71 mAb treatment, cells still have a high CD71 recycling rate, or (ii) γδ T cells have lower iron requirements for differentiation than αβ T cells.

In summary, our results indicate that iron deficiency, caused by CD71 mAb treatment, leads to an inhibition of αβ T but not γδ T cell development. Our observations indicate that CD71 treatment, causing decreased intracellular iron levels, severely inhibits the major proliferation phase from the $CD44^-25^+$ $CD4^-8^-3^-$ to the $CD4^+8^+3^-$ cells, and completely abrogates the final maturation of $CD4^+8^+3^-$ cells into αβTcR-expressing cells. In contrast, proliferation and differentiation of the earliest thymic precursors into $CD44^-25^+$ $CD4^-8^-3^-$ cells is not affected by CD71 treatment.

Iron deficiency, caused by malnutrition, is one of the most common health problems in third-world countries, and may lead to impaired cell-mediated immunity [34, 35]. As our data indicate, this may be caused by the inhibition of αβ T cell development within the thymus [36].

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