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We have previously identified a mAb that binds to a molecule expressed preferentially on the surface of cycling thymocytes. In this study the molecule recognized by this mAb has been identified in the mouse as CD147 (basigin) by expression cloning. We show that CD147 expression correlates with cycling of immature thymocytes even in the absence of TCRβ selection and that ligation of this molecule on immature fetal thymocytes inhibits their further development into mature T cells. The Journal of Immunology, 2002, 168: 4946–4950.

Immature thymocytes differentiate along a defined pathway characterized by the differential expression of CD25 and CD44 (1). During this differentiation process, rearrangement and expression of the TCR genes (γ, δ, and β) are occurring in a temporal manner (2, 3). In order for a double-negative (DN)4 T lymphocyte to progress to the DN4 and subsequent stages of development, pre-TCR expression and signaling must occur (4, 5). The pre-TCR composed of a productive TCRβ chain, pTγ, and a functional CD3 complex is first expressed at the DN3 stage of development in ~25% of the cells (6, 7). Accompanying this selection step is a burst of proliferation, which serves to increase the population of immature cells with rearranged TCRβ genes available for further differentiation and TCRs rearrangement (8–10).

We have previously identified a mAb (RL73; Ref. 11) that bound an unidentified molecule expressed preferentially on the surface of cycling thymocytes (12, 13). In this study the molecule recognized by this mAb has been identified as CD147 by expression cloning. In addition, we show that CD147 expression correlates with cycling of immature thymocytes even in the absence of TCRβ selection and furthermore that ligation of this molecule on immature fetal thymocytes inhibits their further development into mature T cells.

Materials and Methods

Cell lines and culture conditions

The cell lines P815 and Raji were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture according to their specifications. The EBV nuclear Ag (EBNA)-1-expressing HEK-293 derivative was maintained according to the supplier’s instructions (Invitrogen, Leek, The Netherlands). Culture conditions for the EL-4 thymoma have been previously described (11).

Introduction of a stuffer in pEBS-PL

The CD2 cDNA coding region (14) was amplified by PCR with the primers 5′-CTCGTACACCCATGAGTTTCCAATGTAATTTG and 5′-CGGCTGCAGTATAATGAGGGGCAAAACAGTACG. The ligation product was phosphorylated using T4 kinase, purified by extraction with phenol-chloroform-isooamylalcool (50:50:1), precipitated with ethanol, and digested with the restriction enzyme XhoI. The cDNA was protected from the restriction enzyme digestion by the first-strand methylation (18). The cDNA was size selected by chromatography on Sephacryl S-1000 (Pharmacia Biotech). A linearized vector with compatible cohesive ends was prepared by digesting 50 μg of pEBS2CD2 with the restriction enzymes NheI and SalI. The vector and CD2 cDNA stuffer were separated by gel filtration on Sephacryl S-1000 (Pharmacia Biotech). The vector and size-fractionated cDNAs (≥1 kb) were ligated using a Rapid DNA Ligation kit (Roche Molecular Biochemicals, Meylan, France) according to the manufacturer’s instructions. The ligation product was precipitated with ethanol and introduced into competent DH10B bacteria (Life Technologies) by electroporation using a GenePulser II (Bio-Rad, Ivry sur Seine, France). The resultant library was amplified in 16 pools of 5 × 106 independent clones and purified using a Qiagen Maxi kit (Qiagen, Courtaboeuf, France).

HEK-293 EBNA cell transfection

Sixteen aliquots of 5 × 106 cells were transfected by electroporation with aliquots of plasmid DNA (25 μg) purified from the 16 amplified library pools using the following conditions: 280 V, 975 μF, infinite Ω (GenePulser II) with 150 μg Escherichia coli rRNA as carrier in 500 μl RPMI 1640 medium. Cells were expanded for 2 days before the first round of magnetic bead sorting.

Cell sorting with magnetic beads

Aliquots of each transfected pool (5 × 105) were successively labeled with the mAb RL73.2 (11) at 5 μg/ml in DMEM-F12 supplemented with 1%
FACS sorting

After five rounds of positive selection with magnetic beads, 5 x 10^6 cells of pool 11 were labeled with the mAb RL73.2 (5 μg/ml) followed by FITC-labeled goat anti-rat Ig (1:200; Silenus Laboratories, Hawthorn, Australia). Positive cells were sorted using a FACS Vantage cell sorter (BD Biosciences, San Jose, CA) and expanded in culture as described above. Plasmid DNA was isolated by alkaline lysis, purified on QiaGen tip 20 columns (Qiagen), precipitated with ethanol together with 10 μg of glycerol as carrier, and electrophoresed into DH10B electrocomet competent cells (Life Technologies). Plasmid DNA was isolated from the pool of transformants or from 24 individual colonies. Transfectants were expanded for 12 days in culture medium supplemented with hygromycin (150 μg/ml). Purity was checked by labeling with RL73.2 and FITC-labeled goat anti-rat Ig.

DNA sequencing

The cDNA inserts were sequenced using a BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Les Ulis, France) and the sequence was analyzed using an ABI 377 sequencer (PE Applied Biosystems). DNA sequence contigs were assembled using the Sequencher 3 program (Gene Codes, Ann Arbor, MI) and compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool program.

Mice and cell suspensions

C57BL/6 female mice (Harlan Olac, Bicester, U.K.), TCR αβ-deficient mice (The Jackson Laboratory, Bar Harbor, ME), and pTcrαβ-deficient mice (a kind gift from H.-J. Fehling, Medical Faculty/University Clinics, Ulm, Germany) were used at 6 wk of age. Adult thymocyte suspensions were prepared by homogenization into HEPES-buffered DMEM/3% FCS. Immature DN thymocytes were purified as previously described (19). Cells were incubated with IgM anti-CD4 (clone 12/2.1) and anti-CD8 (clone 31 M) mAbs for 15 min at 4°C in HEPES-buffered DMEM/1% FCS. Rabbit complement (Saxone Europe, Suffolk, U.K.) and DNaseI (Boehringer Mannheim, Mannheim, Germany) were added and the incubation was continued for 45 min at 37°C. Dead cells were removed by centrifugation over a Lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient. Any further contaminating mature CD4+, CD8-, or CD3-expressing cells, including CD4+ CD8+ (DN) γδ T cells were removed by live gating on the FACS after staining with a lineage mixture of FITC-conjugated abs (anti-CD3, -CD4, -CD8, -CD11b, -TCRβ, -TCRγδ, -B220, -GR1, and NK1.1). DN4 thymocytes were prepared in a similar manner except that anti-CD25 (clone 7D4) was added to the mAb mix used for complement depletion and both anti-CD25-FITC and anti-CD44-FITC were added to the subsequent lineage mixture used in this case to gate out DN2 and DN3 subsets as well as contaminating mature cells. Fetal thymi were removed from timed (D16) pregnant female C57BL/6 mice maintained in the Pierre-Fabre Animal Facility (Saint-Julien en Genevois, France). Cell suspensions were prepared by squashing thymi between two microscope slide coverslips. Mature CD4-, CD8-, or CD3-expressing cells were removed from the analysis in the same manner as for adult DN preparations.

FACS analysis

Cell surface expression of CD147 by adult and fetal thymocyte subsets was performed using either three-color flow cytometry on a FACScan analyzer (BD Biosciences) or four-color flow cytometry on a FACS Calibur analyzer (BD Biosciences). All analysis was performed using CellQuest software (BD Biosciences). The following mAb fluorescent conjugates were used: anti-CD4-CyChrome, anti-CD44-CyChrome, anti-CD8-allophycocyanin, anti-CD3e-FITC (BD Pharmingen, San Diego, CA), anti-CD25-Cy5, anti-CD147-biotin, and the mixture of FITC conjugates described above (all purified and conjugated in this laboratory from culture supernatants). Biotin conjugates were revealed with PE-streptavidin (Caltag Laboratories, Burlingame, CA) or CyChrome-streptavidin (BD Pharmingen). All staining on fetal thymocytes was performed in the presence of culture supernatant of an anti-FcR mAb (2.4.G.2) to prevent nonspecific FcR binding. Combined surface and intracellular (c)TCRβ staining was performed as previously described (7, 20), using anti-TCRβ-FITC (BD Pharmingen). Cell cycle analysis was performed as previously described (20, 21).

FTOC

C57BL/6 mice were bred in our facility under specific pathogen-free conditions. Fetal thymic organ culture (FTOC) was conducted as described (22). Briefly, fetal thymus from mice at day 14 of gestation were isolated and transferred to membranes of a Transwell system (Costar, Cambridge, MA). Eight to 10 lobes were cultured per well. The lower compartment was filled with complete DMEM-10 containing 2-ME and or without Abs. Both medium and Abs were changed every 2–3 days. After 7 days, thymus lobes were harvested and mechanically disrupted. Single cells were counted, stained, and analyzed by FACS as described above.

Results and Discussion

Cloning of the membrane protein recognized by the mAb RL73.2 and RL119

Expression cloning, a powerful approach to characterize surface Ags (23), was selected for the identification of the protein recognized by the mAbs RL73.2 and RL119. This Ag is constitutively expressed on the surface of both the murine thymoma cell line EL-4 and the murine mastocytoma P815 (11). Comparison by FACS staining indicated that the expression level was slightly

FIGURE 1. Isolation of the cDNA coding for the Ag recognized by the mAb 73.2 using expression cloning. A and B, 293-EBNA cells transfected with the library pool 11 and subjected to five rounds of selection with magnetic beads (A), followed by purification by flow cytometry (B), were stained with the mAb 73.2 and FITC-labeled goat anti-rat Ig. Open histograms represent control 293-EBNA cells transfected with pEB-CD2 subjected to the same labeling procedure. C–E, 293-EBNA cells were transfected with the plasmid pool isolated from pool 11 293-EBNA cells (C) or the clones 4, 9, and 13 (D and E) and expanded for 12 days in the presence of the selection antibiotic. Cells were then stained with mAb 73.2 (A–D) or mAb 119 (E) and FITC-labeled goat anti-rat Ig. Open histograms show control 293-EBNA cells transfected with pEB-CD2 subjected to the identical selection procedure.
higher on the P815 cells (data not shown); therefore, this cell line was chosen as the source of poly(A)/H11001 RNA used to generate the cDNA library.

The vector pEBS-PL used for the cDNA library, which comprises the EBV origin of replication (15), was used in conjunction with the cell line transfectant 293-EBNA. This acceptor cell line is permissive for vector replication and negative for the expression of the epitopes recognized by the mAbs RL73.2 and RL119 (Fig. 1).

A directional cDNA library of $10^7$ independent clones (average insert size, 1.6 kb) was generated and transfected into 293-EBNA cells. The 293 cells were subjected to five rounds of magnetic bead sorting for expression of the Ag recognized by the mAb RL73.2, followed by expansion in culture in the presence of the plasmid selection antibiotic. At this stage the 293-EBNA cells transfected with four of the library cDNA pools contained cells expressing the Ag recognized by the mAb RL73.2, with the largest fraction of positive cells (86%) in pool 11 (Fig. 1A and data not shown). Pool 11 was further enriched for positive cells by flow cytometry (Fig. 1B) and the plasmid DNA was isolated and introduced into bacteria by transformation. When plasmid DNA purified from the pooled bacterial colonies was transfected into 293-EBNA cells, a large fraction of cells expressed the Ag recognized by the mAb RL73.2 (Fig. 1C). The fact that only a fraction of the cDNA encodes for the Ag of interest was expected with the expression cloning procedure used, as cells permissive for the replication of plasmid with EBV replication origin are known to contain large numbers of episomes (24). Twenty-four individual plasmids were tested by transfection, stained with the mAb RL73.2 or RL199, and analyzed by flow cytometry. Three individual colonies contained cDNAs encoding for the Ag recognized by RL73.2 and RL119 (Fig. 1D), demonstrating directly that both mAbs recognize epitopes of the same Ag. The plasmid inserts were completely sequenced and compared with the cDNA sequences deposited in GenBank. This comparison revealed that the inserts from the three clones chosen contained the full-length coding region of the murine CD147 gene (Fig. 2 and data not shown; Ref. 25). Two of the clones were identical, whereas the third one differed at the 3' noncoding region.

### CD147 expression and cycling status of immature thymocytes

Previous experiments from this laboratory have shown that RL73.2 recognizes immature adult thymocytes that are predominantly cycling (12). As ligation of immature T cells with anti-CD147 mAbs has previously been shown to induce activation and cycling (11), and because CD147 expression and cell cycle status of fetal thymocyte subsets resemble those of adult thymocytes (Fig. 3), we decided to evaluate the influence of CD147 on thymic development. To this end, fetal thymi were removed at day 14–15 of gestation and cultured for 7 days in the presence of either anti-CD147 (RL73.2) or an isotype control Ab. Virtually no live cells were recovered from the anti-CD147-treated FTOCs, whereas control FTOCs developed normally (data not shown). The lack of cells in the anti-CD147-treated FTOCs was not due to nonspecific toxicity, because incubation of splenocytes and adult thymocytes with this Ab did not lead to an increase in cell death (data not shown).

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**Figure 2.** Sequence of the clone 4 encoding murine CD147. The nucleotide sequence of the insert is indicated together with the sequence of the encoded protein.

**Figure 3.** Expression of CD147 on adult or D16 fetal thymocytes after gating by staining with anti-CD147 (solid lines) or with an isotype control Ab (dotted lines). Right panels, Cell cycle analysis of DN3 and DN4 D16 fetal thymus subsets was performed by a standard propidium iodide staining protocol. Percentages represent the proportion of cells in S and G2/M phases of the cell cycle.

### Table 1. Cell recovery from FTOC in the presence of RL73 F(ab')2 or control Ab

<table>
<thead>
<tr>
<th>Control</th>
<th>RL73 F(ab')2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN1 b</td>
<td>300</td>
</tr>
<tr>
<td>DN2</td>
<td>16</td>
</tr>
<tr>
<td>DN3</td>
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</tr>
<tr>
<td>CD4 SP</td>
<td>560</td>
</tr>
<tr>
<td>CD8 SP</td>
<td>560</td>
</tr>
</tbody>
</table>

* Numbers are in thousands.

b Includes B cells, NK cells, etc.

* Includes immature, CD8+ TCR- thymocytes.

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**Figure 1.** Expression of CD147 on adult or D16 fetal thymocytes after gating by staining with anti-CD147 and anti-CD44 to define DN3 and DN4 subsets as described in Materials and Methods. Left panels, Histograms show staining with anti-CD147 (solid lines) or with an isotype control Ab (dotted lines). Right panels, Cell cycle analysis of DN3 and DN4 D16 fetal thymus subsets was performed by a standard propidium iodide staining protocol. Percentages represent the proportion of cells in S and G2/M phases of the cell cycle.
However, although the total CD8-positive (SP) populations, which were both reduced by 15-fold pronounced in the double-positive (DP) and mature CD4 relative to control cultures (Table I). This reduction was most pro-

To exclude the participation of FcR-mediated events from our analysis, FTOC was performed in the presence of F(ab')2 of RL73.2. Under these conditions absolute numbers of viable thy-
mocytes were still reduced in the F(ab')2-RL73.2-treated FTOCs relative to control cultures (Table I). This reduction was most pro-
nounced in the double-positive (DP) and mature CD4+ single-positive (SP) populations, which were both reduced by 15-fold (Fig. 4A). However, although the total CD8+ population appeared to be decreased to a lesser extent (4-fold), most of these cells did not express surface TCR (data not shown) and were therefore im-
mature SP cells.

To further characterize this developmental block, immature DN thymocytes were stained with mAbs recognizing CD25 and CD44, allowing precise discrimination of the four DN subsets. As shown in Fig. 4B, a large decrease in the most mature CD25+CD44+ (DN4) population was observed in cultures treated with the F(ab')2 of RL73.2 compared with control cultures (from 71 to 42%). This was accompanied by a relative enrichment in the most immature CD44−CD25− (DN1) population (from 18 to 42%), indicating a partial developmental block at this stage. However, when absolute numbers are calculated, the block in development starts to be 

prominent at the transition between the DN3 and DN4 stages of development (Table I).

During thymocyte development, discrete phases of proliferative expansion take place to amplify the pre-T cell pool. Early on, signaling through the IL-7R mediates survival/proliferation (26, 27). Following this cytokine-dependent stage, thymocytes that have successfully rearranged TCRβ are selected based on pre-TCR recognition and progress to the DP stage and beyond. Accompa-
nying this maturation is a second round of expansion, which roughly corresponds to the DN3 to DN4 transition (28). In the thymus, CD147 is most highly expressed on late immature thy-
mocytes (DN4), correlating with this proliferative burst (Fig. 3). It has been postulated that this proliferation is mediated by pre-TCR signaling, because in the absence of a functional pre-TCR very little, if any, proliferation occurs (4, 6). However, in more recent studies (8, 29), selection/survival and proliferation have been dis-
associated. Formation of a functional pre-TCR may mediate only survival/selection, whereas an as yet unidentified second signal may be required for induction of proliferation. In this work we show that, although the level of CD147 expression is identical on DN3 thymocytes of wild-type (wt) and pre-TCR-deficient mice (pTα and TCRβ knockout (KO)), the level expressed on most, but not all, DN4 cells is greatly decreased in the absence of a func-
tional pre-TCR (Fig. 5A). This result is consistent with the possi-

bility that CD147 expression is related to cycling, independent of whether or not a functional pre-TCR can be formed. Furthermore, the putative, second proliferative signal may be mediated through CD147. In support of this hypothesis is the finding that when DN4 cells are divided into icTCRβ+ and icTCRβ− subsets (although the majority of cycling cells are to be found within the icTCRβ+ subset), some cycling cells can still be observed in the icTCRβ− subset (7). However, if the DN4 subset is further subdivided into CD147+ or CD147− subsets and cell cycle analysis is performed on the icTCRβ+ or icTCRβ− cells in these subsets, we find that virtually all cycling cells in the icTCRβ− DN4 population are expressing CD147 (Fig. 5B). In addition, the small proportion of cycling cells detected in the CD147− DN4 population are all expressing icTCRβ protein (Fig. 5B) and therefore have presumably been TCRβ selected. In a previous study we have shown that around half the DN4 icTCRβ− cells express icTCRγδ, and the percentage of cycling cells is increased in icTCRγδ+ DN4 cells relative to icTCRγδ− DN4 cells (7). One interpretation of these results is that icTCRγδ itself mediates the small amount of prolif-
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eration observed, and that CD147 would not be required for
cycling. However, as all DN4 icTCR-γδ+ cells express CD147 and there is no difference in the level of expression of CD147 on DN4 thymocytes isolated from TCR-δ-deficient mice compared with wt mice (data not shown), this scenario is unlikely.

Taken together, these results support the hypothesis that expansion of DN4 thymocytes is independent of pre-TCR expression and point to a potential role for CD147 in the second phase of immature thymocyte expansion. The association of CD147 with cycling at this developmental stage raises the question as to the nature of the signal(s) leading to this pre-TCR-independent proliferation.

Acknowledgments

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References