Differential Effects of Interleukin-15 and Interleukin-2 on Differentiation of Bipotential T/Natural Killer Progenitor Cells

By Georges Leclercq, Veronique Debacker, Magda De Smedt, and Jean Plum

From the Department of Clinical Chemistry, Microbiology and Immunology, University of Ghent, University Hospital, B-9000 Ghent, Belgium

Summary

Bipotential T/natural killer (NK) progenitor cells are destined to differentiate mainly into T cell receptor (TCR)αβ and TCRγδ cells in a thymic microenvironment, whereas extrathymically they selectively develop into NK cells. The exact environmental conditions that are required for differentiation into these three leukocyte populations are largely unknown. In this report, we have investigated and compared the effect of interleukin (IL)-15 and IL-2 in this process. The IL-15 receptor is composed of the γ and β chains of the IL-2 receptor (IL-2Rγ and IL-2Rβ) and of a specific α chain (IL-15Rα). Here, it is shown that IL-15 mRNA is mainly expressed in thymic epithelial stromal cells, whereas IL-2 mRNA is exclusively expressed in thymocytes. IL-2Rβ–expressing cells were present in the fetal thymus with a $CD25^-CD44^+FcγR^+HSA^{-/low}TCR^-$ phenotype, which is characteristic of progenitor cells. These cells also expressed IL-15Rα messenger RNA. Sorted $IL-2Rβ^+TCR^-$ cells differentiated into TCRαβ and TCRγδ cells after transfer to alymphoid thymic lobes, whereas culture of the same sorted cells in cell suspension in the presence of IL-15 resulted in the generation of functional NK cells. This shows that $IL-2Rβ^+TCR^-$ cells of the fetal thymus contain bipotential T/NK progenitors. Addition of low concentrations of IL-15 to fetal thymic organ culture (FTOC) resulted in an increase of all T cell subpopulations. The largest expansion occurred in the TCRγδ compartment. In contrast, low concentrations of IL-2 did not result in a higher total cell number and did not induce outgrowth of TCRγδ cells. High concentrations of IL-15 blocked TCRαβ development and shifted differentiation towards NK cells. Differentiation towards TCRγδ cells still proceeded. High concentrations of IL-2 similarly induced development into NK cells, but the cell number was fourfold lower than in IL-15 cultures. Importantly, blocking of IL-2Rα in IL-2-treated FTOC resulted in a drastic increase in cell number, indicating that IL-2Rα negatively regulates cell expansion. Collectively, these experiments provide direct evidence that IL-15 and IL-2 differentially affect the differentiation of bipotential T/NK progenitors.

IL-15 is a novel cytokine that was originally cloned from CV-1/EBNA, a simian kidney epithelial cell line (1). Although the amino acid sequence of IL-15 has no homology to IL-2, three-dimensional modeling of IL-15 predicts that it is a four-helix bundle type cytokine (1), and thus IL-15 is a member of the same cytokine family as is IL-2 (2). IL-15 also uses components of the IL-2 receptor (IL-2R): both the β and γ chains of the IL-2R are required for binding and signal transduction by this cytokine (3). In addition, a specific α chain of the IL-15R has been identified. This IL-15R

α chain (IL-15Rα)[1] is structurally similar to the IL-2R α chain, but with only limited sequence identity (4). The common use of the IL-2R β and γ chains by both IL-2 and IL-15 probably explains the finding that several biological

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activities of these cytokines are similar. These include induction of proliferation of phytohemagglutinin-stimulated human peripheral blood T lymphocytes (1) and induction of proliferation and differentiation of human B cells (5). In addition, as is the case for IL-2, IL-15 is a selective chemoattractant for human blood T lymphocytes (6) and is able to activate cytotoxic activity and cytokine production by human NK cells (7).

Although several biological activities of IL-15 and IL-2 are overlapping, there are some differences. It has been shown that production of GM-CSF by human NK cells in response to IL-2 is much larger than to IL-15 (7). Also, the proliferative response of human CD56^{bright} NK cells or anti-CD3–stimulated lymphocytes to IL-2 and IL-15 are distinct (7, 8). It has been recently shown that the interaction of IL-15 with IL-2Rβ/γ is less stable than is the case for IL-2 (8). In addition, IL-2Rα, which is able to bind IL-2 but not IL-15, negatively regulates peripheral expansion of T lymphocytes (9). IL-15 and IL-2 are also differentially produced. The highest amounts of IL-15 mRNA are expressed in epithelial and fibroblast cell lines, whereas it is absent in activated peripheral blood T lymphocytes (1). In contrast, the only cells known to produce IL-2 are T cells (10). The functional differences between IL-15 and IL-2, as well as the production of these two cytokines by mutually exclusive cell types, might result in the preferential usage of one of these cytokines in a particular system.

Whereas induction of proliferation and cytotoxic activity of peripheral blood T lymphocytes by IL-15 has been shown (1, 3), there are presently no studies that addressed the possible role of this cytokine in the differentiation of T progenitor cells in the thymus. Thymocytes differentiate from progenitor cells, which originate from stem cells of the fetal liver or the adult bone marrow. It has been shown in several independent studies that, both in mice and in humans, bipotential T/NK progenitor cells are present in the thymus. The phenotype of those bipotential T/NK progenitors has been defined as FcγRII/III$^+$CD3$^-$CD4$^-$ CD8$^-$CD25$^-$CD44$^+$ in the mouse (11–13) and as CD34^{bright} CD1$^-$CD2$^-$CD5$^-$CD3$^-$CD4$^-$CD8$^-$ in humans (14). From the next stage of differentiation, CD25$^+$CD44$^+$ cells in mice (15) and CD1$^+$CD2$^+$ CD5$^+$ cells in humans (14, 16), which both are still CD4$^-$CD8$^-$TCR$^-$, become T cell committed. Subsequently, these cells rearrange their TCR genes and become CD4$^+$CD8$^+$ thymocytes expressing the CD3/TCR complex. After negative and positive selection, they eventually become functionally mature CD4 or CD8 single-positive cells (SP) that emigrate from the thymus. Although bipotential T/NK progenitor cells are clearly present in the thymus, these cells preferentially differentiate in this lymphoid organ into T cells, whereas extrathymically, they mainly differentiate into NK cells (12, 14). This indicates that the signals which are required by the T/NK bipotential progenitor cells to differentiate into either direction are different.

In the murine fetal thymus, a substantial part of the progenitor cell population expresses the β chain of the IL-2R (this study and reference 17), as well as IL-15Rα (this study), which probably enables these cells to respond to IL-2 as well as to IL-15. We therefore examined and compared the effects of IL-15 and IL-2 on the differentiation of these progenitor cells. Our results show that there are important differences between IL-15 and IL-2, and that IL-2Rα negatively regulates IL-2–induced cell expansion.

Materials and Methods

Mice. BALB/c, originally purchased from Proefdierencentrum (Catholic University Leuven, Leuven, Belgium), were bred in our breeding facility. To obtain dated pregnant mice, mice were mated for 15 h and the fetuses were removed at the indicated fetal days (FD) (plug date = day 0).

Cytokines. Purified simian rIL-15 and human rIL-2 were kindly provided by Dr. E. Thomas (Immunex Corp., Seattle, WA) and Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ), respectively. In a 24-h CTLL-2 proliferation assay, 500 ng/ml IL-15 induced the same proliferation as 1,000 U/ml IL-2.

Antibodies, Flow Cytometric Analysis, and Sorting. The mAbs used for staining, flow cytometric analysis, and sorting were described before (18, 19). To avoid aspecific binding of mAbs, the FcγR was blocked by preincubation of the cells with saturating amounts of anti-FcγRII/III mAb (clone 2.4G2) for 15 min at 4°C. FcγR-blocking was not done when cells had to be labeled with FITC-conjugated anti-FcγR mAb afterwards. Propidium iodide was added to the cells (2 μg/ml) just before analysis. Gating was done on propidium iodide–negative thymocytes to exclude dead cells. In the case of sorting of CD45-positive and -negative cells from FD17 thymus, thymic cell suspension was prepared by trypsinization of the thymic lobes (20). This treatment does not remove the CD45 antigen from the cell surface (data not shown).

The mAb used for the addition to fetal thymic organ culture (FTOC) was purified unlabeled anti–IL-2Rα (clone PC61) (kind gift of Dr. M. Nabholz, Epalinges, Switzerland).

Reverse Transcription (RT)-PCR and Southern Blotting. Trisol (Life Technologies, Gaithersburg, MD) was added to sorted cells and RNA was extracted according to the manufacturer's instructions. cDNA was synthesized with oligo(dT) as primer using the Superscript kit (Life Technologies). Oligonucleotides used for RT-PCR and hybridization were as described by Chun et al. (21) for recombination activating gene-1 (RAG-1). Other oligonucleotides used were as follows: for IL-15: AATGAAGTGCT-TTCTCTTGG (sense primer), GTGTTGATGAACATTTG-GAC (antisense primer), and AACAGAATCTGGATGCAAAG (probe); for IL-2: TGATGGACCTACAGGAGCTCCTGAG (sense primer), GAGTCAAATCCAGAACATGCCGCAG (antisense primer), and CACCTTCAAATTTTACTTGCCCAAG-CAGGCC (probe); for IL-15Rα: ATTGAGCATGCTGACAT-CCG (sense primer), TCTCTGTTATCCTGAGGGAG (antisense primer), and GTAACTCTGGCTTTAAGCGG (probe); and for hypoxanthin phosphoribosyltransferase (HPRT): GTAATGAT-CAGTCAACGGGGGAC (sense primer), CCAGCAAGCTTG-CAACCTTAACCA (antisense primer), and GCTTTCCCTG-GTTAAGCAGTACAGCCCC (probe). PCR amplification was performed using a 96-well thermocycler (Omnigene, Hybaid Teddington, UK) with 30 (RAG-1, IL-15, IL-2, and HPRT) or 33 cycles (IL-15Rα and HPRT) of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min. In each PCR, water and 50 ng mouse genomic DNA were included as negative controls. All primer pairs amplified cDNA only. For semiquantitative RT-PCR, four twofold (IL-15, IL-2, and HPRT) or threefold dilutions (IL-

15Rα and HPRT) of each cDNA sample were amplified. For Southern blotting, PCR products were separated on 2% agarose gels, denaturated, and transferred onto Nytran paper (Schleicher & Schuell, Inc., Keene, NH). The DNA was UV–cross-linked to the membrane, probed with a ^{33}P-labeled internal oligonucleotide, and the membrane was exposed to film (Amersham, Arlington Heights, IL).

Cell-mediated Cytotoxicity Assay. YAC-1 cells were used as target cells. Cytotoxicity was analyzed in a 6 h ^{51}Cr release assay using 1,000 target cells per well as described previously (22).

FTOC. Four to five thymic lobes from FD14 BALB/c mice were placed on the surface of polycarbonate filters (0.8-μm pore size; Nuclepore, Pleasanton, CA) that were supported on blocks of gelfoam (Upjohn Co., Kalamazoo, MI) in 1.8 ml of Iscove's modified Dulbecco's medium supplemented with 10% FCS (both from Life Technologies) in 12-well plates. Cytokines were added at different concentrations, as indicated. In the case of IL-2Rα–blocking experiments, anti–IL-2Rα mAb was added at 100 μg/ml. Cultures were grown in 7.5% CO_2 in humid air at 37°C for 11 d. Afterwards, the thymus lobes were disrupted using a small potter homogenizer. Cell viability was determined by trypan blue exclusion.

Recolonization Assays. Alymphoid lobes were prepared by culturing FD15 BALB/c thymic lobes in FTOC in the presence of 1.35 mM 2'-deoxyguanosine (Sigma Chemical Co., Filter Service, Eupen, Belgium) for 5 d. After extensive washing, single thymic lobes were put together with 1,000 sorted thymic cells in a total volume of 25 μl in the wells of a Terasaki plate. The plates were inverted to form hanging drops, and the cells were allowed to seed the lobes during 48 h. Subsequently, untransferred cells were removed by washing, and the lobes were cultured in FTOC

for the indicated time. No thymocytes could be detected after FTOC of deoxyguanosine-treated thymic lobes that were not repopulated with thymic cells.

Results

Expression of IL-2Rβ, IL-2Rα, and IL-15Rα on thymocytes. Freshly prepared thymocytes from FD15, FD17, 2-d-old, and adult mice were analyzed by flow cytometric analysis for the expression of IL-2Rβ and IL-2Rα (Fig. 1). The cell number per thymus was 50,000, 750,000, 10^7 and 10^8 for FD15, FD17, 2-d-old, and adult mice, respectively. Approximately 6% of FD15 thymocytes expressed IL-2Rβ. This percentage decreased to <1% in 2-d-old mice; in adult mice, it was ~2%. Expression of IL-2Rβ and IL-2Rα was almost, but not completely, mutually exclusive. In FD15 thymocytes, IL-2Rβ-positive cells were bright positive for FcγR and CD44, were mainly negative for TCRαβ or TCRγδ, and HSA was expressed at low levels or was absent. This indicates that IL-2Rβ is expressed on thymocyte progenitor cells. At FD17, part of the IL-2Rβ–positive cells became negative for FcγR, and part of the TCRγδ cells clearly expressed IL-2Rβ. In adult thymocytes, most IL-2Rβ–positive cells were mainly FcγR negative and TCRαβ positive.

To examine expression of IL-15Rα, to which no antibody is available yet, we performed a semiquantitative RT-PCR. As a control, the housekeeping enzyme HPRT was stud-
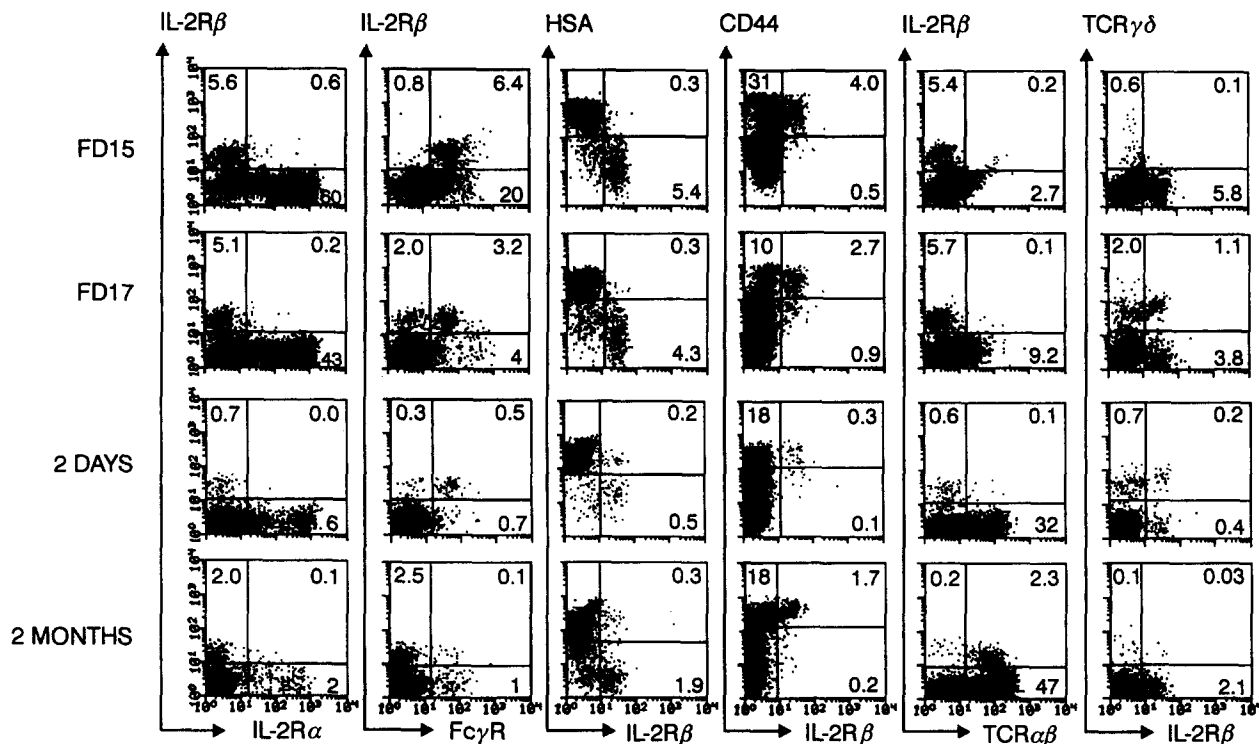
Figure 1. Distribution of IL-2Rβ and IL-2Rα on thymocyte subsets by flow cytometric analysis. Thymocytes from FD15, FD17, 2-d-old and 2-mo-old mice were labeled with mAbs against the indicated antigens. Propidium iodide was added before analysis. Dot plots are shown for propidium iodide–negative thymocytes. Percentages of each population are represented in each quadrant.
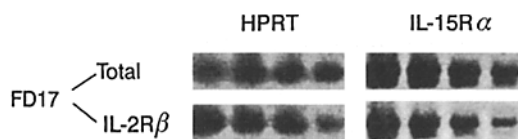
Figure 2. Expression of IL-15Rα in fetal thymus by semiquantitative RT-PCR analysis. mRNAs were prepared from unseparated or sorted IL-2Rβ⁺CD8⁻TCRαβ⁻TCRγδ⁻ cells from FD17 mice. The expression of IL-15Rα and HPRT mRNA was analyzed by semiquantitative RT-PCR and Southern blotting. For semiquantitative RT-PCR, four threefold dilutions of each cDNA sample were amplified.

Figure 3. RAG-1 expression in fetal thymic IL-2Rβ⁺ cells. mRNAs were prepared from unseparated thymocytes from adult mice or from sorted IL-2Rβ⁺ CD8⁻TCRαβ⁻TCRγδ⁻ cells from uncultured FD16 thymic lobes. mRNA was analyzed by RT-PCR and Southern blotting for the expression of RAG-1 and HPRT.

ied. The results show that an equivalent amount of HPRT mRNA was present in the populations tested. IL-15Rα mRNA was abundantly present in FD17 thymus, both in the total cell population and in IL-2Rβ⁺CD8⁻ TCRαβ⁻TCRγδ⁻ sorted cells (Fig. 2).

Differential Capacity of IL-2Rβ–positive Fetal Thymocytes to Differentiate into T Cells in a Thymic Environment and into Functional NK Cells in Cell Suspension in the Presence of IL-15. To examine whether IL-2Rβ⁺TCR⁻ cells were committed to the T cell lineage, we first tested if these cells contained RAG-1 mRNA. RT-PCR was performed using RAG-1–specific oligonucleotides. As a positive control, uncultured and unseparated thymocytes from adult mice were used. Fig. 3 shows that uncultured, sorted IL-2Rβ⁺CD8⁻TCRαβ⁻TCRγδ⁻ cells from FD16 thymuses did not express RAG-1 mRNA. This indicates that either these cells are not T progenitor cells or that these cells do not yet contain RAG-1 mRNA. Therefore, we examined if these cells had the potential to differentiate into T cells. For this purpose, highly purified IL-2Rβ⁺CD8⁻TCR⁻ cells (>99.5% purity) from FD15 or FD16 thymuses were transferred to alymphoid thymic lobes (1,000 cells per lobe) and analyzed after FTOC for 6 and 12 d. It is shown in Fig. 4 that CD4 and CD8 double positive (DP) cells were clearly present after 6 d, as were TCRαβ cells with intermediate TCR density and TCRγδ cells. After 12 d of FTOC, the percentage of CD4 and CD8 SP cells and of cells with high TCRαβ density was increased. The absolute cell number per lobe was 20,000 and 30,000 after 6 and 12 d of FTOC, respectively. Thymic lobes that did not receive sorted IL-2Rβ⁺ thymocytes did not contain thymocytes after FTOC (data not shown).

Alternatively, unsorted FD16 thymocytes or sorted IL-2Rβ⁺CD8⁻TCR⁻ cells from FD16 thymuses were cultured in cell suspension in the presence of IL-15 for 5 d. There was a 2.5-fold increase in the cell number of total FD16 thymic cells and a 12-fold increase in the cell number of sorted IL-2Rβ⁺CD8⁻TCR⁻ cells (data not shown). No TCRαβ⁺ or TCRγδ⁺ cells were present after IL-15 culture of the sorted IL-2Rβ⁺CD8⁻TCR⁻ cells (data not shown). The resulting cells were very efficient in lysing the NK-susceptible target cell line YAC-1. Freshly prepared uncultured cells did not kill YAC-1 cells to a significant extent (Fig. 5). No viable cells were left when FD16 thymic cells were cultured in the absence of exogenous IL-15 for 5 d (data not shown).
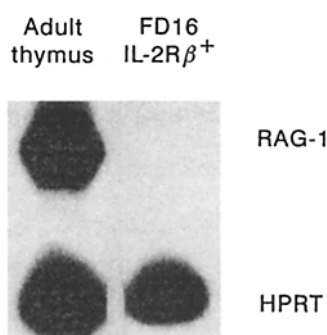
Differential Expression of IL-15 and IL-2 in the Thymus. An RT-PCR was performed to study expression of IL-15 mRNA in fetal and adult thymus. It is shown in Fig. 6 that IL-15 mRNA is clearly expressed in the thymus of fetal and adult mice. To examine which cell population of the thymus mainly expressed IL-15 mRNA, two types of experiments were done. In the first, FD15 thymic lobes were organ cultured in the presence of deoxyguanosine, which results in depletion of thymocytes and stromal cells of bone marrow origin. The majority of the cells that are still present after this treatment are epithelial stromal cells. Deoxyguanosine-treated thymic lobes clearly expressed IL-15 mRNA. In a second type of experiment, a semiquantitative RT-PCR was done on total as well as on CD45⁺ and CD45⁻ sorted cells from trypsinized FD17 thymic lobes. The control RT-PCR on HPRT showed that equivalent amounts of RNA were present in all three preparations. With regard to IL-15 mRNA expression, only low amounts were found in CD45⁺ cells, which contain thymocytes and stromal cells of bone marrow origin. CD45⁻ cells, which are epithelial stromal cells, expressed high levels of IL-15 mRNA. As a control, we also analyzed the expression of IL-2 mRNA in the same preparations. As expected, and in contrast to IL-15 mRNA expression, IL-2 mRNA could not be detected in deoxyguanosine-treated thymic lobes or in CD45⁻ sorted cells, but was present in CD45⁺ sorted cells.

Treatment of FTOC with IL-15 or IL-2. To study the effect of exogenous IL-15 on intrathymic differentiation, FD14 thymic lobes were cultured in FTOC in the presence of 20, 100, or 500 ng/ml IL-15 or 40, 200, or 1,000 U/ml IL-2 for 11 d. In a CTLL-2 proliferation assay, the activity of 500 ng/ml IL-15 was comparable with 1,000 U/ml IL-2 (data not shown). After 11 d of FTOC, the cell number per thymic lobe was increased more than twofold in the presence of 20 or 100 ng/ml IL-15, and fourfold in cultures with 500 ng/ml IL-15, as compared to the control culture (Table 1). Notwithstanding the considerable cell expansion in FTOC with 20 ng/ml IL-15, the percentages of the different subpopulations with regard to CD4 versus CD8, IL-2Rα versus IL-2Rβ, and TCRαβ expression were not drastically changed, showing that all these subpopulations increased in absolute cell number. The percentage of TCRγδ cells, both Vγ3⁺ and Vγ3⁻, doubled. This showed that the increase in the number of TCRγδ cells is higher than that of the other thymocyte subpopula-
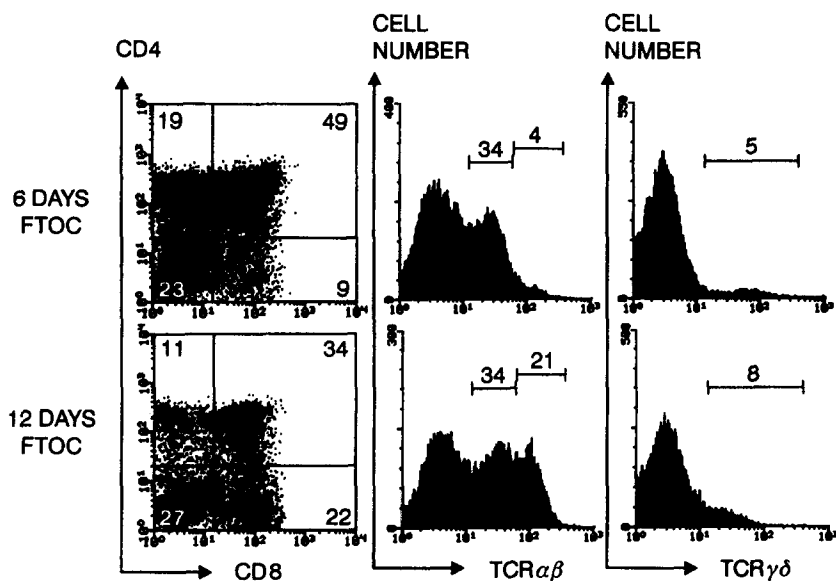
**Figure 4.** Fetal thymic IL-2Rβ⁺ cells differentiate into T cells in FTOC. IL-2Rβ⁺CD8⁻-TCRαβ⁻TCRγδ⁻ cells were sorted from FD16 thymic lobes and transferred to deoxyguanosine-treated thymic lobes. After FTOC for 6 and 12 d, cells were analyzed. Results shown are representative of two independent experiments.

tions. In the presence of 100 ng/ml IL-15, the percentages of CD4⁺CD8⁻ and CD4⁺CD8⁺ cells were only half of those of the control culture. Also, the TCRαβ^low and TCRαβ^high subpopulations were reduced to a similar extent. Given the increase in cell number per lobe in this culture condition, however, there was no change in absolute cell numbers of these subpopulations. A strong increase was found in cell numbers of TCRγδ thymocytes and of cells with an im-

mature phenotype: CD4⁻CD8⁻ IL2Rβ⁺FcγR⁺TCR⁻. In FTOC with 500 ng/ml IL-15, almost no CD4⁺CD8⁻ and CD4⁺CD8⁺ cells were present, and no significant number of TCRαβ^low or TCRαβ^high cells could be detected. 8% of the cells were CD4⁻CD8⁺, but these cells mainly expressed CD8αα instead of CD8αβ. The cell number of TCRγδ cells, mainly Vγ3⁺, increased significantly (Fig. 7). The CD8αα⁺ cells were predominantly TCRγδ cells (data not shown). The majority of the cells had a CD4⁻ CD8⁻ IL-2Rβ⁺FcγR⁺TCR⁻ phenotype. No Ig⁺ cells could be detected (data not shown), indicating that there was no outgrowth of B cells.

In contrast to IL-15–treated FTOCs, cultures with 40, 200, or 1,000 U/ml IL-2 did not result in a higher cell yield, as compared to the control culture (Table 1). With regard to phenotype, culture in the presence of 40 or 200 U/ml IL-2 resulted in a small increase of IL-2Rα⁺-IL-2Rβ⁺ cells and of FcγR⁺ cells. The cell number of TCRαβ^high cells decreased. There was no increase in the cell number of TCRγδ cells. Cultures with 1,000 U/ml IL-2 yielded essentially the same phenotype as did FTOC with 500 ng/ml IL-15. A small difference was that there was always a tendancy in IL-2–treated cultures for a higher percentage of IL-2Rα⁺ cells (Fig. 7).

Blocking IL-2Rα Drastically Increases the Cell Number in IL-2–treated FTOC. A surprising finding was that FTOC treated with different concentrations of IL-2 never yielded more cells than control cultures, whereas IL-15 treatment resulted in a two- to fourfold increase in cell number (Table 1). Since IL-2, but not IL-15, is able to bind to the α chain of the IL-2R, we added anti–IL-2Rα mAb to IL-2–supplemented FTOC. The cell number of these cultures was increased more than twofold compared to FTOC in the presence of IL-2 only. The phenotype of the resulting cells was not drastically changed by addition of the anti–IL-2Rα mAb (Fig. 8).

% SPECIFIC ⁵¹Cr RELEASE
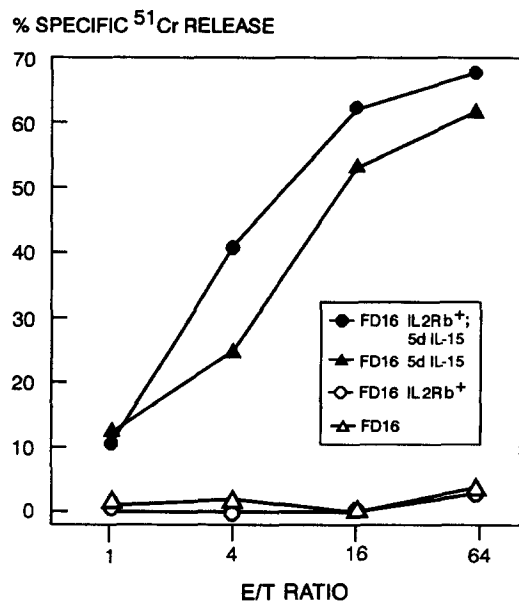
Figure 5. Fetal thymic IL-2Rβ⁺ cells cultured in cell suspension in the presence of IL-15 generate functional NK cells. Total or IL-2Rβ⁺ CD8⁻TCRαβ⁻TCRγδ⁻ sorted FD16 thymic cells were cultured in cell suspension in the presence of 500 ng/ml IL-15 for 5 d or were used uncultured. Lytic activity was tested against the NK-sensitive target cell line YAC-1. Results shown are representative of two independent experiments.
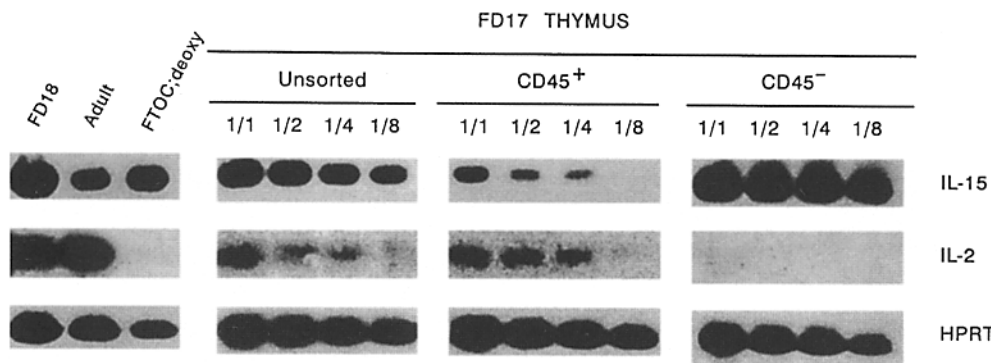
Figure 6. IL-15 and IL-2 mRNA expression in thymus. (*Left*) IL-15, IL-2, and HPRT mRNA expression analyzed with RT-PCR and Southern blotting in total thymic cells from FD18 or adult mice or in deoxyguanosine-treated FD15 thymic lobes. (*Right*) IL-15, IL-2, and HPRT mRNA expression analyzed with semiquantitative RT-PCR and Southern blotting in total, CD45$^+$, or CD45$^-$ sorted cells from the FD17 thymus. For semiquantitative RT-PCR, four twofold dilutions of each cDNA sample were amplified.

Cells from FTOC with Exogenous IL-15 Have Lost T Progenitor Potential. Given that FTOC with high concentrations of exogenous IL-15 yielded mainly CD4$^-$CD8$^-$CD25$^{+/-}$IL2Rβ^+FcγR$^+$TCR$^-$ cells, which are cells with an immature phenotype, we examined whether these cells could still differentiate into T cells. Sorted CD44$^+$ CD8$^-$ TCR$^-$ cells from FTOCs were transferred to deoxyguanosine-treated thymic lobes and organ cultured. Fig. 9 shows the phenotypic analysis of a representative experiment after 9 d culture. Sorted cells from control FTOC differentiated into T cells, as shown by the appearance of CD44$^-$CD25$^+$ and CD44$^-$CD25$^-$ cells in the CD8$^-$TCR$\alpha\beta^-$TCR$\gamma\delta^-$ population, and by the generation of CD4$^+$CD8$^+$ and SP cells. On the contrary, similarly sorted cells from IL-15 FTOC did not differentiate into the T lineage: they remained CD44 positive and CD4-CD8 double negative. The same results were obtained when the organ cultures were analyzed after 16 d (data not shown).

Addition of IL-15 to FTOC Shifts Differentiation towards Functional NK Cells. Since CD44$^+$CD8α^-TCR$^-$ cells iso-

lated from IL-15–supplemented FTOC did not have any T cell progenitor potential, we examined if these cells had lytic activity. CD44$^+$CD8$^-$TCR$^-$ cells were sorted from IL-15–supplemented FTOC and tested in a ^{51}Cr release assay. Fig. 10 shows that these cells killed the NK-sensitive YAC-1 target cell line very efficiently. Similarly sorted cells from control FTOC did not have any significant lytic activity.

Discussion

IL-15 and IL-2 both require the β and γ chain of the IL-2R for binding and signal transduction (3). This probably explains why several biological activities of those two cytokines are similar (1, 5–7). However, IL-15 and IL-2 are produced by mutually exclusive cell types (1, 10). In addition, only IL-2 is able to bind to IL-2Rα (3), whereas only IL-15 is able to bind to IL-15Rα (4). This indicates that there may be functional differences between those two cytokines. We have therefore examined and compared the effects of IL-15 and IL-2 on differentiation of thymic bipotential T/NK progenitor cells towards T cells and NK cells.

In contrast to T cells, NK cells do not rearrange TCR genes and develop normally in SCID mice (23) and RAG-deficient mice (24, 25). Nevertheless, there are several similarities between T and NK cells. These include cytoplasmic expression of CD3 proteins (26, 27), cell membrane expression of numerous cell surface antigens (reviewed in 28), and cytolytic activity. These phenotypic and functional similarities between T and NK cells indicate that these two lymphocyte subsets are related. Additional evidence for this has recently been obtained by the finding that both in mice and in humans, T and NK cells originate from a common precursor that is present in the thymus (12–14, 29, 30). Although the existence of bipotential T/NK progenitors implicates a direct lineage relationship between T and NK cells, the anatomical localizations for T and NK differentiation are not the same. The thymus is required for mainstream T cell development. Both thymic epithelial and mesenchymal cells are necessary for early differentiation of progenitor cells towards DP thymocytes (20), whereas thy-

Table 1. *Effect of Exogenous IL-15 and IL-2 on Cell Yield of Organ-cultured FD14 Thymic Lobes*

Cytokine added	Cells per lobe
None	282 ± 45*
IL-15	
20 ng/ml	738 ± 172
100 ng/ml	636 ± 46
500 ng/ml	1,132 ± 226
IL-2	
40 U/ml	285 ± 73
200 U/ml	313 ± 20
1000 U/ml	250 ± 16

*Data are the mean cell number (× 10³) per thymic lobe ±SD of three to six experiments.
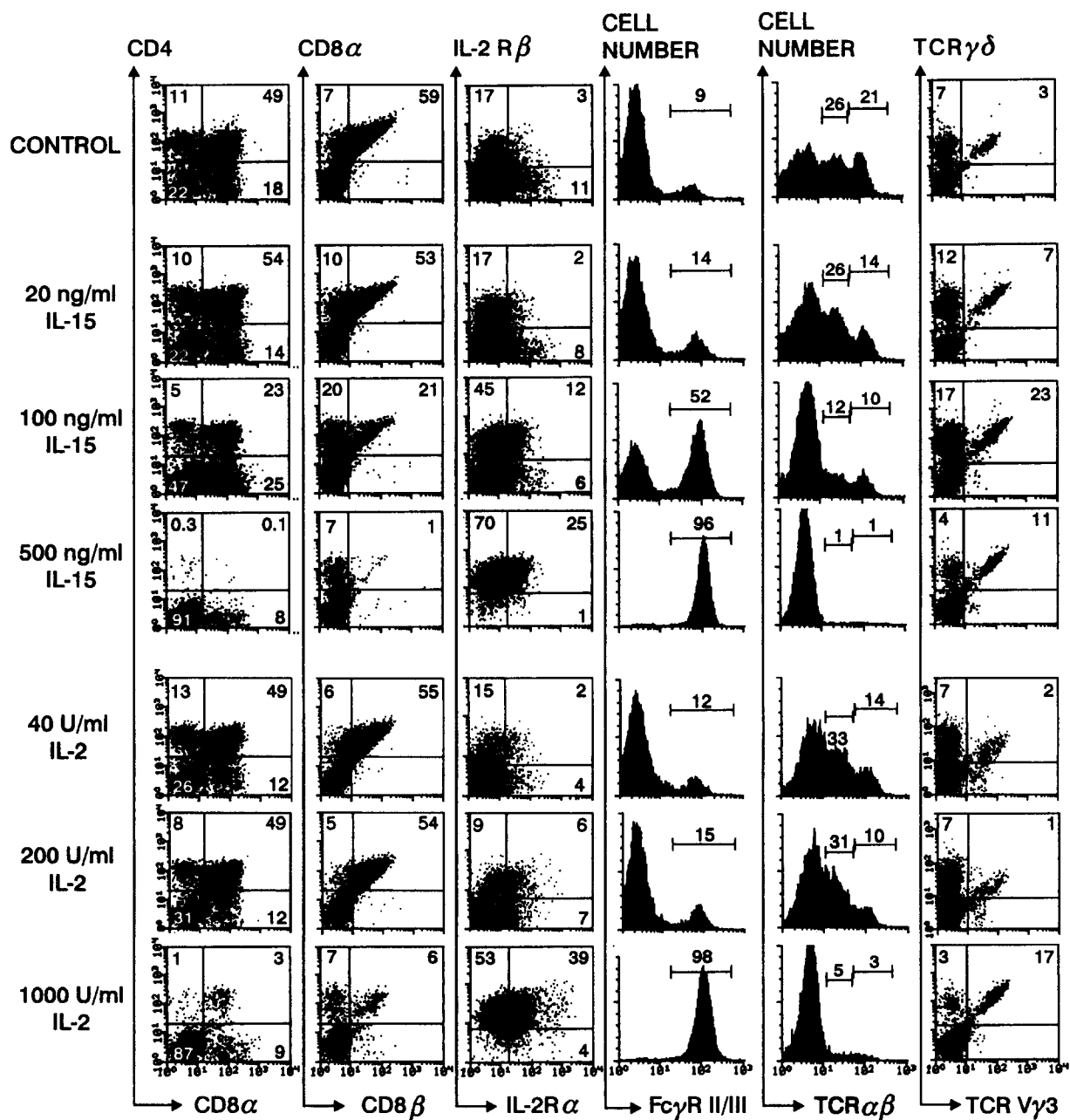
Figure 7. Phenotypic analysis of thymocytes after FTOC with IL-15 or IL-2. FD14 thymic lobes were organ-cultured without or with 20, 100, or 500 ng/ml IL-15 or 40, 200, or 1,000 U/ml IL-2 for 11 d. Cells were labeled with mAbs against the indicated antigens. Propidium iodide was added before analysis and propidium iodide–negative thymocytes were gated. Results shown are representative of three to six independent experiments.

mic epithelial cells provide unique signals for positive selection of DP thymocytes towards mature T lymphocytes (31). In contrast to T cell differentiation, NK cell differentiation clearly does not require the thymus, since athymic nude mice have normal numbers of NK cells. It is generally accepted that the bone marrow is one of the main sites for NK cell development (32, 33).

In the mouse, Rodewald et al. have shown that the bipotential T/NK progenitors in the fetal thymus are contained in the $Fc\gamma R^+$ population (12). This population,

which has a $CD3^-CD4^-CD8^-CD25^-CD44^+HSA^{-/low}$ phenotype (13), is abundantly present during fetal life (12). It is shown in this report that $\sim 6\%$ of FD15 thymocytes expressed IL-2Rβ and that the majority of these cells had a progenitor-like phenotype, i.e., $Fc\gamma R^+$ and $CD25^- CD44^+HSA^{-/low}TCR^-$. All IL-2R$\beta^+$ cells coexpressed $Fc\gamma R$, but the IL-2Rβ^+ population (6% of total cell number) was only a fraction of the $Fc\gamma R^+$ population (26%). $IL-2R\beta^+CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ cells are not yet in the process of TCR rearrangement, since no RAG-1 mRNA
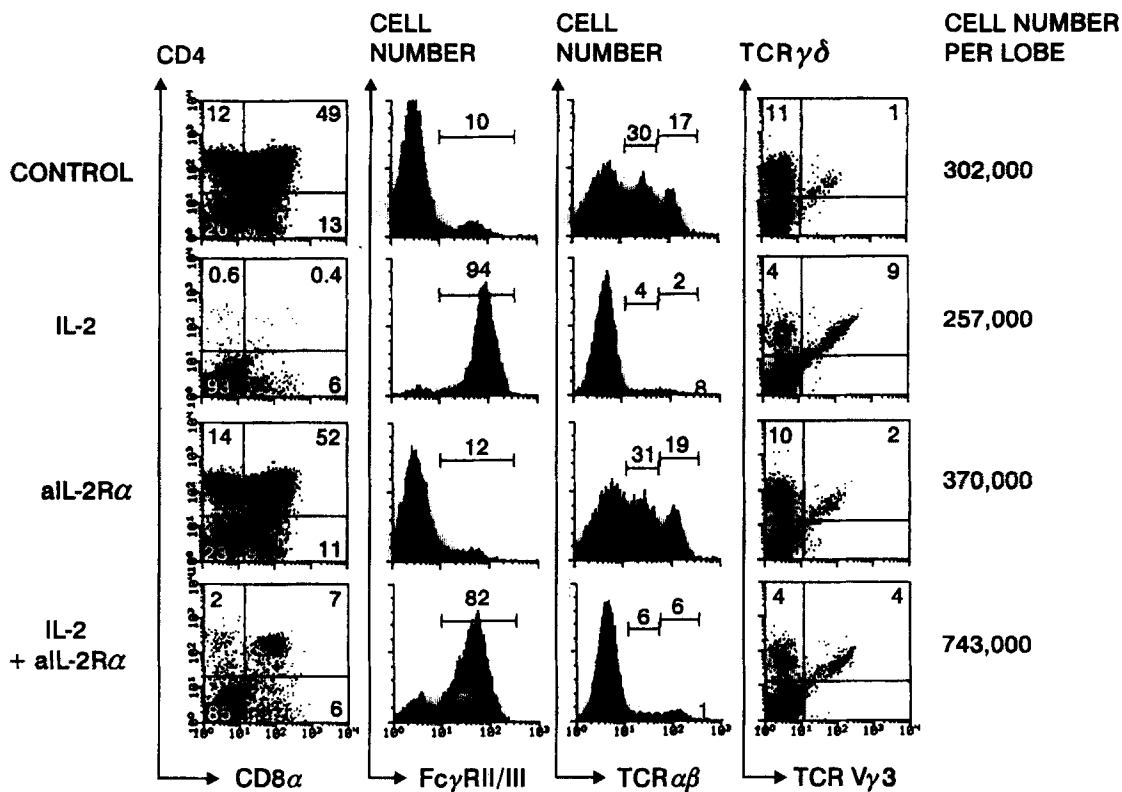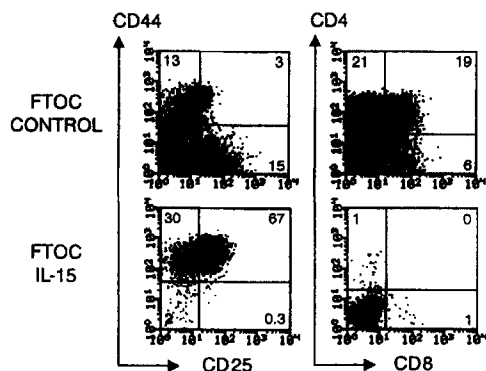
Figure 8. Blocking IL-2Rα drastically increases the cell number in IL-2–treated FTOC. FD14 thymic lobes were organ-cultured without the addition of cytokines or antibodies, with 1,000 U/ml IL-2, with 100 μg/ml anti–IL-2Rα mAb, or with IL-2 plus anti–IL-2Rα mAb for 11 d. Cells were labeled with mAbs against the indicated antigens. Propidium iodide was added before analysis, and propidium iodide–negative thymocytes were gated. Results shown are representative of two independent experiments.

could be detected. Upon transfer to deoxyguanosine-treated thymic lobes, highly purified IL-2Rβ⁺CD8⁻TCRαβ⁻TCRγδ⁻ cells differentiated into TCRαβ and TCRγδ cells, showing that these cells were indeed T progenitor cells. These results are in agreement with those of Falk et al. (17). Purified IL-2Rβ⁺CD8⁻TCRαβ⁻TCRγδ⁻ cells also

Figure 9. FTOC in the presence of IL-15 results in loss of T progenitor cells. FD14 thymic lobes were organ-cultured without or with IL-15 (500 ng/ml) for 11 d. Subsequently, CD44⁺CD8⁻TCRαβ⁻TCRγδ⁻ cells were sorted and transferred to deoxyguanosine-treated FD15 thymic lobes. Cells were analyzed after FTOC for 9 d. Cells shown in the CD44 versus CD25 dot plots were gated on CD8⁻TCRαβ⁻TCRγδ⁻ cells. Results shown are representative of two independent experiments.
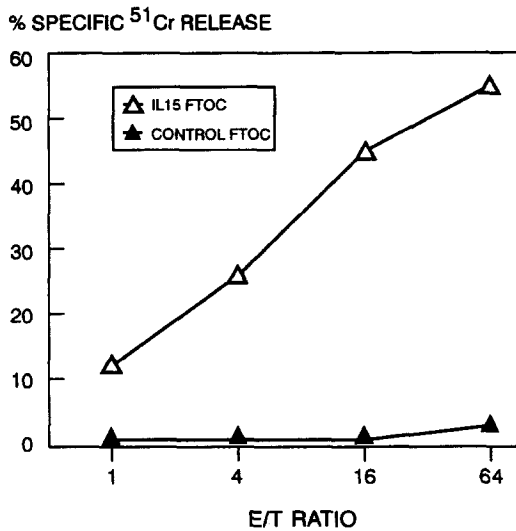
expressed IL-15Rα mRNA, indicating that these cells are able to respond to IL-15. Indeed, these cells proliferated extensively when cultured in cell suspension in the presence of IL-15 and, more importantly, in this culture condition, they differentiated into functional NK cells. Freshly sorted IL-2Rβ⁺ thymic cells did not have NK activity. These findings indicate that IL-2Rβ⁺ cells present in the fetal thymus contain progenitors for both T and NK cells. It still has to be determined whether IL-2Rβ⁺ cells are indeed bipotential T/NK cells or whether the IL-2Rβ⁺ cell population contains both T and NK progenitors.

Although bipotential progenitor cells can develop into T cells as well as NK cells, T cell differentiation mainly occurs in the thymus, whereas NK cell differentiation mainly occurs extrathymically. This indicates that cell–cell interactions and/or cytokines that direct these bipotential progenitor cells towards T or NK cell differentiation pathways are different. A central role for cytokines in the development of both T and NK cells has recently been demonstrated by the generation of mice deficient for the γ chain of the IL-2R, since no T or NK cells develop in these mice (34). Also in humans, mutations in the gene encoding the IL-2Rγ chain results in a severe block in T and NK development (35). The γ chain of the IL-2R is shared by IL-4R, IL-7R, IL-9R, and IL-15R (3, 36–39). Previously, it was proposed that IL-2 has a crucial role in thymic development. However,

% SPECIFIC ^{51}Cr RELEASE

Figure 10. Acquisition of lytic activity in FTOC with exogenous IL-15. FD14 thymic lobes were organ-cultured in the absence or presence of IL-15 (500 ng/ml) for 11 d. Subsequently, CD44+CD8− TCRαβ− TCRγδ− cells were sorted and examined for lytic activity against YAC-1 target cells. Results shown are representative of three independent experiments.

IL-2 knockout mice have normal thymic development (40). Also, IL-4 as well as IL-2 plus IL-4 knockout mice have normal numbers of functional T cells (41, 42). Recently, IL-2Rβ–deficient mice have been described (43). Although not addressed in this study, cells from these mice can be considered to be IL-15 unresponsive. The thymus from these mice reveals normal phenotypic features with regard to CD4-CD8 subpopulations up to 3 wk of age. In 6-wk-old mice, thymocyte cell numbers are decreased sevenfold with a drastic reduction of DN and DP thymocyte subpopulations. As peripheral T cells are spontaneously activated and autoimmunity develops in these mice, however, the observed reduction in thymocyte cell numbers was interpreted by the authors as being the result of general stress-induced changes rather than intrinsic problems with thymocyte development (43). The only cytokine with a crucial role in T cell development that has been described so far is IL-7. This has been demonstrated by generation of mice deficient for IL-7 (44) or deficient for the α chain of the IL-7R (45). Also, by the use of neutralizing anti–IL-7 mAbs, it has been demonstrated that IL-7 plays a central role in thymic development (46, 47).

Whereas IL-7 is an important cytokine for T cell differentiation, the crucial cytokines for NK cell development probably are IL-2 and/or IL-15. This has been shown by postnatal treatment of mice with anti–IL-2Rβ mAb, which results in long-term elimination of NK cells (48). NK cell activity is also markedly reduced in IL-2–deficient mice, but is still inducible (49). IL-15–deficient mice have not been described so far, but IL-15 has been demonstrated to induce activation and proliferation of human NK cells (7), indicating a potential role of this cytokine in NK cell development and function.

It is shown here that both IL-15 and IL-2 mRNA is expressed in fetal and adult thymuses. IL-15 mRNA was mainly present in CD45− cells, which are stromal epithelial cells. IL-2 mRNA was found exclusively in CD45+ cells. This is in agreement with previous reports showing that IL-15 is abundantly present in epithelial and fibroblast cell lines, but not in peripheral blood T lymphocytes (1), whereas the only cells that are known to produce IL-2 are T cells (10). Since it is shown in this report that IL-2Rβ as well as IL-15Rα are expressed by bipotential T/NK progenitor cells in the murine thymus, we examined whether addition of IL-15 to FTOC would have an effect on the choice that these progenitor cells make to differentiate in the direction of either T or NK cells. Since others have shown that there are some functional differences of IL-15 and IL-2 (7–9), we compared the effect of IL-15 addition to FTOC with that of IL-2. Addition of low concentrations of IL-15 to FTOC resulted in a 2.5-fold increase in total cell number, as compared to control cultures. All differentiation stages were expanded including TCR-negative, TCRαβ, and TCRγδ cells. This could be either a direct effect of IL-15 or an indirect effect caused by secondary cytokine production. FTOC in the presence of high concentrations of IL-15 resulted in a four times higher cell yield than control cultures. Differentiation towards TCRαβ cells did not occur, whereas TCRγδ cells still developed. The majority of the cells were IL-2Rβ+CD44+CD25+/−TCR−. Blocking IL-2Rβ with mAb neutralized the effects of IL-15 on FTOC; blocking IL-2Rα had no effect (data not shown). As the homogenous population of CD44+TCR− cells in IL-15–treated FTOC could be T cell progenitors, we transferred these cells to alymphoid thymic lobes and followed their differentiation. No T cell development could be detected. Contrary to the absence of T cell progenitor potential, these cells were functional NK cells, as shown in a ^{51}Cr release assay. Also, the finding that these cells only expressed FcγRIII (data not shown) indicates that these cells were indeed NK cells, since progenitor T cells express both FcγRIII and FcγRII (12). Collectively, our findings indicate that IL-2Rβ+ cells in the fetal thymus are bipotential T/NK progenitor cells, and that addition of high amounts of IL-15 to FTOC directs differentiation towards the NK pathway.

We also added IL-2 to FTOC. The main difference as compared to IL-15–treated FTOC was that no increase in cell number was obtained with any tested concentration of IL-2. With regard to the receptor chains that interact with IL-15 and IL-2, both IL-2Rβ and IL-2Rγ are required for the biological activity of these two cytokines (3). Only IL-2, however, is able to bind IL-2Rα (3), whereas only IL-15 is able to bind IL-15Rα (4). In addition, by the generation of IL-2Rα–deficient mice, has it been shown recently that IL-2Rα negatively regulates peripheral expansion of T lymphocytes (9). This prompted us to examine the effect of blocking IL-2Rα in IL-2–treated FTOC. The result was that the cell number increased 2.5-fold. This strongly indicates that the lower cell number obtained in IL-2–treated FTOC versus IL-15–treated FTOC is caused by negative regulatory signals delivered by IL-2Rα.

In early fetal thymuses, IL-2Rβ⁺ cells were mainly progenitor cells. From FD16-17, part of IL-2Rβ–expressing cells were TCRγδ cells. We have shown earlier that immature TCRγδ thymocytes, as defined by expression of HSA, are present from FD14, whereas mature, HSA negative, TCRγδ thymocytes appear from FD16 (50). During this transition from the immature to the mature phenotype, TCRγδ cells start to express IL-2Rβ (19). It has been shown by others that IL-2Rβ is an activation marker, since it is expressed on thymocytes after TCR ligation (51, 52). This indicates that mature, but not immature, fetal TCRγδ cells become activated in the thymus. This is supported by the finding that in addition to expression of IL-2Rβ, other cell surface markers that are characteristic for stimulation, such as FcγR and the B220 marker, become expressed during the transition from immature to mature fetal TCRγδ cells (22). As mature TCRγδ cells express IL-2Rβ, it was not surprising that an extensive proliferation of these cells was observed in FTOC supplemented with IL-15. Immature TCRγδ thymocytes did not expand to a significant extent (data not shown). Another difference between the effects of IL-15 and IL-2 in FTOC was that no or only a low expansion of TCRγδ cells was observed in IL-2–treated FTOC. Our results showing that exogenous addition of low amounts of IL-15, but not IL-2, induced expansion of TCRγδ cells, which were mainly TCR Vγ3 cells, is in line with reports showing that in utero treatment with anti–IL-2Rβ mAb completely abrogates development of Vγ3⁺ dendritic epidermal cells (53), which are the progeny of fetal TCR Vγ3 thymocytes (54), whereas IL-2–deficient mice still have Vγ3⁺ dendritic epidermal cells (55). This indicates a potential role for endogenous IL-15 in TCR Vγ3 cell development and/or peripheral expansion. Also, proliferation of other TCRγδ cells during salmonella infection, as well as the activation of IFN-γ production by these γδ T cells, has been shown to be mainly caused by IL-15 (56).

In summary, we have compared the effects of IL-15 and IL-2 on the differentiation of thymic bipotential T/NK progenitor cells towards TCRαβ, TCRγδ, and NK cells. It is shown that there are important functional differences between those two cytokines. It can be hypothesized from these results that IL-15 is an important cytokine in the development of TCRγδ cells and NK cells.

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