Signals from the IL-9 Receptor Are Critical for the Early Stages of Human Intrathymic T Cell Development

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J Immunol 2000; 164:1761-1767; doi: 10.4049/jimmunol.164.4.1761
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Signals from the IL-9 Receptor Are Critical for the Early Stages of Human Intrathymic T Cell Development

Magda De Smedt,* Bruno Verhasselt,* Tessa Kerre,* Dominique Vanhecke,* Evelien Naessens,* Georges Leclercq,* Jean-Christophe Renauld,† Jacques Van Snick,† and Jean Plum²*  

Highly purified human CD34⁺ hemopoietic precursor cells differentiate into mature T cells when seeded in vitro in isolated fetal thymic lobes of SCID mice followed by fetal thymus organ culture (FTOC). Here, this chimeric human-mouse FTOC was used to address the role of IL-9 and of the α-chain of the IL-9 receptor (IL-9Rα) in early human T cell development. We report that addition of the mAb AH9R7, which recognizes and blocks selectively the human high affinity α-chain of the IL-9R, results in a profound reduction of the number of human thymocytes. Analysis of lymphoid subpopulations indicates that a highly reduced number of cells undergo maturation from CD34⁺ precursor cells toward CD4⁺CD8⁻CD1⁺ progenitor cells and subsequently toward CD4⁺CD8⁺ double positive (DP) thymocytes. Addition of IL-9 to the FTOC resulted in an increase in cell number, without disturbing the frequencies of the different subsets. These data suggest that IL-9Rα signaling is critical in early T lymphoid development. The Journal of Immunology, 2000, 164: 1761–1767.

The in vitro growth and differentiation of human T cell precursors requires a thymic microenvironment providing the epithelial cells, extracellular matrix components, and close range-acting cytokines. Direct evidence for the cytokine dependency of human T cell development relies on the immunological abnormalities in patients suffering from X-linked SCID (X-SCID)³. Affected boys have no or markedly reduced T cells, indicating a block in T cell differentiation (1). It has been shown that X-SCID is caused by mutations in the common γ-chain (γc/2). In these patients, there is a combined inability to respond to IL-2, IL-4, IL-7, IL-9, and IL-15 since the receptors for those cytokines include the γc chain as a functional component (3, 4). Analysis of gene-disrupted mice for those cytokines led to the conclusion that their importance in terms of T cell development is different. Based on the dramatically diminished T cell development in IL-7-deficient (5) or IL-7Rα-deficient mice (6), yet normal T cell development in mice deficient in either IL-2Rα (7), IL-2Rβ (8), IL-2 (9), IL-4 (10, 11), or both IL-2 and IL-4 (12), it seems likely that most of the defect in T cell development in patients with X-SCID is due to defective IL-7 signaling (13). Recently, it was shown that mice deficient in IL-15Rα had ~25% fewer cells in the thymus than the control littersates, indicating that the role of IL-15 may partially contribute to, but is dispensable for, the development of thymocytes (14). Mice deficient in IL-9 are not available at this time. Another way to address the relative importance of the cytokines in human T cell development is the human-mouse fetal thymus organ culture (FTOC) model. We have shown that human CD34⁺ fetal liver hemopoietic precursor cells are able to differentiate in a mouse thymic microenvironment (15, 16). This chimeric human-mouse FTOC provides us with a tool to examine the critical factors involved in the human T cell differentiation process. In a previous study, we have reported that IL-7 plays an essential role in the differentiation of human T cells (17). This was shown by either blocking IL-7 activity with neutralizing Abs against IL-7 or by blocking the IL-7Rα. A direct evidence for the essential role of IL-7 has now been given by coming across upon patients with a defective IL-7Rα expression, who suffer from a T⁻B⁻NK⁺ SCID (18). To date, humans or mice lacking IL-9 or IL-9Rα have not been reported. However, the analysis of such defect will be of interest, in view of the possibility that IL-9 might partially contribute to thymic development, given the responsiveness of fetal thymocytes to IL-9 (19) and the development of thymic lymphomas in IL-9 transgenic mice (20).

IL-9 was originally described as a murine T cell growth factor (21). Human and murine IL-9 are 126 amino acids long (21, 22). IL-9 is produced by activated T cells and supports the growth of Th clones but not cytolytic clones (23, 24). In the mouse, IL-9 has been reported to exert effects on erythroid progenitors, B cells, mast cells, and fetal thymocytes (19). Regarding mast cells, IL-9 has been shown to be identical to mast cell growth-enhancing activity, a factor present in conditioned medium derived from splenocytes (25). IL-9 also can synergize with IL-3 for maximal proliferation of mast cells (24). The action of IL-9 on thymocytes in vitro is interesting in view of the development of thymic lymphomas in IL-9 transgenic mice coupled to the observation that IL-9 is
a major anti-apoptotic factor for thymic lymphomas (26). Although murine IL-9 is active on human cells, human IL-9 is not biologically active on murine cells. IL-9 binds to the 64-kDa IL-9Rα binding protein, which is similar in size to γc (27). The functional IL-9 receptor, which binds IL-9 with a Kd of 100 pM, consists of IL-9Rα plus γc (28, 29).

In this study we addressed the role of IL-9 by comparing intrathymic human T cell development of CD34+CD38−Lin− hemopoietic progenitors from fetal liver or CD34+CD38−Lin− cord blood in FTOC that were treated with mAbs that block IL-9Rα. Our data indicate that blocking this receptor severely affects human T cell differentiation and is consistent with the notion that signaling through IL-7Rα and γc is not sufficient for normal T cell development in man.

Materials and Methods

Animals

C.B-17 SCID mice were obtained from our own specific pathogen-free breeding facility. For timed pregnancies, females were housed separately from the males until mating. The appearance of vaginal plugs after overnight mating was labeled as day 0 of pregnancy. Fourteen- to 15-day pregnant mice were sacrificed to obtain the embryos for preparation of the thymic lobes.

Antibodies

The mAb used were rat anti-mouse CD45 (CD45-cyochrome, 30F11.1, Pharmingen, San Diego, CA), and the following mouse anti-human mAb from Becton Dickinson Immunocytometry Systems (Mountain View, CA): CD3 (Leu-4 FITC or APC), CD4 (Leu-3a FITC, or APC), CD7 (Leu-9- FITC), CD8 (Leu2a FITC), CD19 (Leu-12), CD34 (HPCA-2 PE or APC), CD45 (HLe-1 FITC), and HLA-DR (PE). The following mAbs were from Ortho (Raritan, NJ): CD38 (OKT10 FITC or PE). The following were from Coulter (Miami, FL): CD1a, (T6 RDI), and TCR Panε (BMA031, PE). Monoclonal Abs to the human IL-9 receptor were obtained from DBA/2 mice immunized with P815 mastocytoma cells transfected with a pEF-BOS plasmid encoding the human IL-9 receptor. The Abs used in the present work included AH9R2 (IgG2a), AH9R4 (IgG2a), and AH9R7 (IgG2b). All specifically bind to the human IL-9 receptor but have different inhibitory activities. AH9R4 shows little or no inhibition whereas AH9R2 and AH9R7 have weak and strong inhibitory activities, respectively. Half-maximal inhibition of 100 pg/ml human IL-9 in a TS1H9RA3 assay (30) requires 10 μg/ml AH9R2 and only 0.05 μg/ml AH9R7 (30). In this assay, the murine helper T cell line TS1 (30) has been transfected with human IL-9 receptor α and cultured (300 cells/100 μl) in the presence of 100 pg/ml human IL-9 with or without the indicated Abs. Cell growth was evaluated by measuring hexosaminidase activity after 3 days. IgG2b, mouse mAb (IgG2b MOPC 195/s MOPC114/s) a mixture of IgG from two different tumor sources was used as IgG2b control (ICN, Biomedicals, Irvine, CA).

Preparation of human fetal liver cells, cord blood cells, or thymocytes

Human fetal liver tissues from spontaneous termination of pregnancy or cord blood and human thymus tissue from children undergoing cardiac surgery were obtained and used following the guidelines of the Medical Ethical Commission of the University Hospital of Ghent. Human fetal liver cells were isolated by gentle disruption of the tissue in complete medium (IMDM medium/10% FCS, Life Technologies, Paisley, Scotland), followed by density gradient centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). Umbilical cord blood was obtained from term newborns and used within 24 h after collection for isolation of mononuclear cells as described before (31). Cells were washed and resuspended in 90% FCS/10% DMSO and cryopreserved in liquid nitrogen until use. For the preparation of thymocytes, the thymic tissue was cut into small pieces of 0.5 cm × 0.5 cm. One piece was extensively teased apart with cataract knives in serum-free RPMI 1640 at 4°C, and the freed cells were washed and either used for further purification or washed in serum-free RPMI 1640 medium (400 g for 5 min at 4°C) and finally resuspended in 1 ml Trizol (Life Technologies) for PCR analysis.

Preparation of CD34+ fetal liver, cord blood stem cells, and human thymocyte subsets

After thawing and washing the cells, fetal liver cells were labeled with glycoporin A and CD19 and immunofluorescently labeled with CD1, CD3, CD4, CD7, CD8, and CD38; cord blood cells were labeled with glycoporin A or CD19, and FITC-labeled CD7. Freshly prepared thymocytes were labeled with FITC-labeled CD3 and CD8. For immunomagnetic depletion, the cells were resuspended in 0.5 ml cold PBS/2% FCS and mixed with 0.5 ml prewashed (to remove the preservative) sheep anti-mouse Ig-coated Dynabeads (DynaI, Oslo, Norway) to obtain a ratio of cells/Dynabeads of 1:5. After 30 min at 4°C, the suspension was diluted by carefully adding 5 ml PBS/2% FCS, and the rosettes of cells with Dyna-beads were removed by placing the tube on a magnetic particle concentrator (Dynal). The supernatant containing the unlabelled and weakly stained cells was removed, cells were centrifuged (500 × g for 5 min at 4°C) and finally resuspended in 0.5 ml PBS/2% FCS, labeled respectively with CD34-PE or with FITC-labeled CD1, CD3, CD4, CD8, and PE-labeled CD34. Finally, cells were sorted for, respectively, CD34+CD38−Lin− fetal liver or CD34+CD38−Lin− cord blood stem cells on a FACS Vantage (Becton Dickinson). The human thymocytes were labeled with either CD4-PE and CD34-APC and allow FACS sorting of CD4 immure single positive with CD34-APC and CD1-PE to allow sorting of CD34+CD1+ and CD34+CD1− thymocytes. Sorted cells were checked for purity, which was always at least 99.0%.

Fetal thymic organ cultures

Thymic lobes were isolated from fetal day 14–15 SCID mice. Hanging drops were prepared in Terasaki plates by adding in each well 25 μl of complete medium containing 10,000 sorted CD34+CD38−Lin− fetal liver stem cells or CD34+Lin− cord blood stem cells or sorted single positive or CD34+CD1+ or CD34+CD1− thymocytes to one thymic lobe. The plates were immediately inverted to form hanging drops and incubated during 48 h in a humidified incubator (7.5% CO2 in air, 37°C) (32). After incubation, the cells were removed from the hanging drop, washed, and put on a nucleopore filter (Nucleopore, Costar, Cambridge, MA) resting on a Gelfoam sponge (Upjohn, Kalazomaz, MI). mAbs reacting with thymocytes were used at a concentration of 50 μg/ml. Antibodies were given from the start of the hanging drop culture throughout the FTOC.

Flow cytometry

Before labeling, cells were recovered from thymic lobes, suspended in PBS/1% BSA-0.1% NaN3, and the Fe receptors of the mouse thymocytes were blocked by preincubation for 15 min with saturating amounts of anti-FR3II/III mAb (clone 2.4G2) (33) to avoid the aspecific binding of Abs by the murine thymocytes. Subsequently, the cells were stained with a panel of mAbs, as indicated. All mAbs against human Ags were checked for negative staining on SCID thymocytes after blocking with 2.4G2 mAb. Isotype controls were also included in most staining series and were found to be negative. The cells were analyzed on a FACScalibur (Becton Dickinson) with an argon-ion laser tuned at 488 nm and red-diode laser at 635 nm. Forward light scattering, orthogonal scattering, and four fluorescence signals were determined on 10–20,000 cells and stored in list mode data files. Data acquisition and analysis were done with CellQuest software (Becton Dickinson). Viable human cells were gated by exclusion of mouse CD45-cyochrome-positive cells and propidium iodide (PI)-positive cells.

RT-PCR and Southern blotting

Trizol (Life Technologies) was added to sorted cells or tissue fragments, and RNA was extracted according to the manufacturer’s instructions. cDNA was synthesized with oligo(dT) as a primer using the Superscript kit (Life Technologies). Oligonucleotides used for RT-PCR and hybridization were as follows: for human IL-9 primary PCR, AAGTGCACACTGAG TCAATGTGGAC (sense primer), TGATGGCTGTTCACAGGAAAA ATAT (antisense primer); and for the nested IL-9 PCR, CTTGTTGGTTCTT TAGTCTACAAGT (antisense primer) and TGGTTGGGACTTCC TCTGACA (sense primer) for hIL-9 PCR, ACCGTGCTCCAACAAAA CCTCTACA (sense primer) and ACCGTGCTCCAACATACCCA (antisense primer); and for hypoxanthin phosphoribosyltransferase (HPRT), AATTATGGACAGGACTGAACGT (sense primer) and TCAAATCCAA TATT (antisense primer). RT-PCR and Southern blotting were performed using a 96-well thermocycler (Omniyed, Hybridis, Ghent, U.K.) with 1 cycle at 94°C for 2 min, 35 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min for IL-9. All primer pairs amplified human cDNA. For semiquantitative RT-PCR, three 4-fold dilutions of each cDNA sample were amplified.
Results

Blocking IL-9Rα results in a drastic reduction in the cellularity in chimeric human-mouse FTOC

To establish the role of IL-9Rα in human T cell development, we investigated whether mAbs that bind to the human IL-9Rα are able to inhibit the proliferation and/or differentiation of human thymocytes in chimeric human-mouse FTOC. These Abs were added to the SCID thymic lobes when these were seeded with highly purified CD34+ precursor cells as well as during the FTOC thereafter. Addition of AH9R7 resulted in a dramatic decrease in the cell recovery from the thymus seeded with fetal liver stem cells (Table I). By 14–16 days of FTOC, at least a 90% reduction in human cellularity was observed. The drop was at least 70% by day 28. The reduction in cellularity of the human cells was at least 85% by day 17 and 96% by day 27 in FTOC initiated with cord blood stem cells.

The partially reduced or normal human cellularity observed in FTOC, seeded with fetal liver CD34+CD38- Lin- stem cells, treated with either AH9R2 or AH9R4, was in perfect agreement with the intermediate inhibition and lack thereof, observed with these Abs in a TS1H9RA3 proliferation assay (30), respectively (Fig. 1). Only the numbers of human thymocytes were decreased, whereas the numbers of murine thymocytes were unaffected. These findings strongly argue against a nonspecific effect, whereby precursor cells are eliminated through a cytotoxic effect of the mAbs, and are in favor for a causative relationship with the neutralization of the IL-9Rα binding capacity.

Blocking IL-9Rα results in a developmental block of the human hemopoietic precursor cells in chimeric human-mouse FTOC

As shown in Fig. 2A in the anti-IL-9Rα-treated FTOC, the frequency of CD34+ precursor cells is similar to that of the control culture and the frequency of the immature CD4+ progenitor cells is reduced. At that time point of the culture, all the CD4+ cells are CD3+ and belong to the CD4+ immature single positive subset (15, 16). As the absolute number of human cells is dramatically reduced (Table I), the net result is that both populations are reduced in absolute numbers in the anti-IL-9Rα-treated FTOC. However, the reduction is more pronounced for the CD4+ cell population. This indicates that especially the early steps of differentiation are affected by blocking IL-9. The block is not complete as a small proportion of the cells undergoes similar phenotypic changes as the control culture. Therefore, these data suggest that IL-9 plays a role in the maintenance and expansion of early T cell precursors at a stage between the transition of CD34+ cells toward CD4+ cells.

After 27 days of FTOC (Fig. 2B), the frequency of CD4CD8 double positive thymocytes and thymocytes expressing high density of CD3, or TCR-αβ, which defines thymocytes at a further stage of differentiation, were decreased. Taken together with the reduction in absolute numbers, these data show that a reduced number of cells is able to differentiate, when the IL-9Rα is blocked. Nevertheless, since precursor cells were able to differentiate, the block is not complete.

Additional evidence for an early block in the T cell developmental process was found by studying the influence of neutralizing anti-IL-9Rα Abs on the differentiation of different starting populations. Therefore, three populations were sorted from human thymus that are known to present successive steps in differentiation: CD34+CD1+, CD34+CD1+, and CD34+CD4+CD3- cells. As shown in Fig. 3, the first two populations were strongly inhibited (≥80%) by the treatment of the FTOC with the mAb, whereas this reduction was only 40% for the CD4+CD3- population. This shows again that the cells in early phases of differentiation are more dependent on IL-9Rα signaling. The differences in cell number between these populations are a consequence of the more rapid

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Table 1. Influence of anti-human IL-9Rα treatment on the development of human cells in chimeric human-mouse FTOC

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Treatment</th>
<th>Length of Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14 days</td>
</tr>
<tr>
<td>Fetal liver CD34+ cellsb</td>
<td>Control</td>
<td>48,787 ± 22,984</td>
</tr>
<tr>
<td></td>
<td>Anti-human IL9Rα (H7)</td>
<td>3,524 ± 1,544</td>
</tr>
<tr>
<td></td>
<td>% Control</td>
<td>7.8 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 days</td>
</tr>
<tr>
<td>Cord blood CD34+ cellsb</td>
<td>Control</td>
<td>74,443 ± 17,223</td>
</tr>
<tr>
<td></td>
<td>Anti-human IL9Rα (H7)</td>
<td>7,474 ± 1,874</td>
</tr>
<tr>
<td></td>
<td>% Control</td>
<td>10.0 ± 0.2</td>
</tr>
</tbody>
</table>

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* Cells were obtained as described in Materials and Methods, and numbers of human cells were calculated by multiplying the total number of viable cells counted under the microscope by the fraction of human CD45-FITC positive cells determined by FACS analysis. Numbers of cells are given as the mean for groups of 2–4 separate experiments (±SD) wherein a pool of at least three single lobes per experiment were analyzed.

* Differences between cell number in control and anti-human IL9Rα-treated FTOC were statistically significant, with \( p < 0.05 \) for 14 and 28 days of culture of CD34+ fetal liver cells and \( p < 0.05 \) for 17 and 27 days of culture of CD34+ cord blood cells by a paired t test on In-transformed values.

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FIGURE 1. Comparative analysis of the inhibition in cell number by mAbs binding IL-9Rα, which differ in neutralizing capacity. FTOC seeded with fetal liver stem cells was performed as described in Materials and Methods, in the presence of the mAbs binding IL-9Rα that are non inhibitory (AH9R4), weakly inhibitory (AH9R2), strongly inhibitory (AH9R7), or in the presence of a nonreactive control mouse monoclonal IgG2b Ab (control IgG2b) or without Ab (control). FTOC were examined after 16 days of culture.
kinetic of differentiation of the CD34+CD4+CD3- population, because these cells are already at a later stage of differentiation and differentiate more rapidly into CD4+CD8+ cells, which is accompanied with a significant cell increase. Finally, we addressed whether the addition of anti-IL9Rα-inhibiting mAbs is required during the full length of culture time of the FTOC, or only during part of the experimental procedure. The inhibition was less pronounced in the FTOC that were treated only in the hanging drop. Treatment after hanging drop resulted in a drastically reduced cell number and T cell development and was comparable to the inhibition that was obtained after continuous treatment during hanging drop and the FTOC afterwards (Table II).

Anti-IL-9R inhibition is restored by IL-9
To verify whether IL-9 was able to counteract the inhibition mediated by anti-IL-9Rα mAb, both reagents were added simultaneously to the FTOC. The mAb was used in different concentrations, and IL-9 was added at 10 ng/ml in the FTOC. As shown in Fig. 4, IL-9 prevented the inhibitory activity of AH9R7. These data argue in favor of an effect mediated by inhibition of the IL-9Rα and strongly argue against a nonspecific mechanism affecting the cell viability by the treatment with the mAbs.

IL-9 augments the number of human cells in FTOC
Since the block of the IL-9Rα receptor inhibits early thymocyte generation, we investigated whether addition of exogenous IL-9 would increase cellularity in FTOC. Addition of human recombinant IL-9 (10 ng/ml) resulted in a significant increase in cell number without clearly affecting the frequencies of the cell populations.

**FIGURE 2.** Four-color FACS staining of thymocytes in control (IgG2b) and anti-IL-9Rα (AHR7)-treated FTOC initiated with fetal liver stem cells. A. Cells were labeled as described in Materials and Methods with anti-moCD45, anti-huCD45, anti-huCD4, anti-huCD34, anti-huCD3, anti-huCD8, and anti-huHLA-DR. Results of analysis are shown for one representative control (IgG2b) and anti-IL-9Rα-treated FTOC after 16 days of culture. Numbers correspond to the frequency of the cells found in the indicated region. B. Cells were labeled as described in Materials and Methods with anti-moCD45, anti-huCD45, anti-huCD34, anti-huCD3, anti-huCD4, anti-huCD8, and anti-huTCR-αβ. Results of analysis are shown for one representative control (IgG2b) and anti-IL-9Rα mAb-treated FTOC after 27 days of culture. Numbers correspond to the frequency of cells found in the indicated region.

**FIGURE 3.** Comparative analysis of the inhibition in cell number by mAbs binding IL-9Rα on FTOC initiated with thymocyte subpopulations that differ in maturation degree. Thymocytes were prepared and sorted and put in FTOC as described in Materials and Methods. FTOC were performed as described in Materials and Methods, in the presence or absence of the mAb as indicated. FTOC were examined after 11 days of culture. Note the 10-fold difference in scale of the y-axis of the left box and right box, respectively.
The ability of the SCID thymic lobes to support human T cell development, as shown in Fig. 5, mRNA was detected in the human thymus by a sensitive method. We have previously shown that in FTOC neutralizing IL-7R Abs, raises the question about the source of IL-9. Previously, presence of IL-9 in mouse thymus was shown by in situ hybridization (34). Because both murine and human IL-9 interact with the human IL-9R (35), IL-9 could be produced in our model either by human thymocyte precursors or by murine stromal cells. We looked whether IL-9 is produced in human thymus by a sensitive and specific RT-PCR for detection of RNA message for human IL-9. As shown in Fig. 5, mRNA was detected in the human thymus. Finally, we were able to demonstrate that mRNA for IL-9R is present on CD341 cells determined by FACS analysis.

IL-9Rα mRNA is present in human stem cells and IL-9 mRNA is produced intrathymically

The ability of the SCID thymic lobes to support human T cell differentiation, which can be blocked by neutralizing anti-IL-9Rα Abs, raises the question about the source of IL-9. Previously, presence of IL-9 in mouse thymus was shown by in situ hybridization (34). Because both murine and human IL-9 interact with the human IL-9R (35), IL-9 could be produced in our model either by human thymocyte precursors or by murine stromal cells. We looked whether IL-9 is produced in human thymus by a sensitive and specific RT-PCR for detection of RNA message for human IL-9. As shown in Fig. 5, mRNA was detected in the human thymus. Finally, we were able to demonstrate that mRNA for IL-9Rα is present on CD34+ thymocytes (Fig. 5).

Discussion

To our knowledge, our findings are the first direct proof that, in human, IL-9Rα signaling is important for T cell development. Four types of experiments indicate that IL-9Rα is critical for early T cell development.

First, treating the FTOC with a mouse mAb blocking human IL-9Rα decreased the number of human cells that develop in the mouse SCID thymus when seeded with CD34+ hemopoietic precursor cells from fetal liver. The inhibition appeared to be at the transition between CD34+ to CD34- CD1+ CD4+ CD3- immature thymocytes.

Second, the results of using different mouse mAbs that all bind to human IL-9Rα, but differ in their neutralizing capacity, nicely fit with their effect on the cell number when added in FTOC. This indicates that the mAbs do not exert cytotoxic effects on IL-9Rα-bearing cells in vitro, but that the neutralization of the binding capacity of the receptor is essential to interfere with the normal T cell development process.

Third, the block that is mediated by the mAb can be overcome by exogenous IL-9. This finding argues strongly against a toxic effect of the mAb or an aspecific inhibition mediated by the mAb preparation.

Fourth, addition of exogenous IL-9 to the FTOC augments the cell number of the human cells obtained in FTOC, which signifies that IL-9Rα triggering favors either cell growth or cell survival. A model to explain the essential role of IL-9Rα signaling evolves from the comparison with the results obtained with IL-7. We have previously shown that in FTOC neutralizing IL-7Rα resulted in a similar inhibition.

Here, however, the means by which IL-9Rα signals was not apparent from blocking studies of IL-9. Neutralization of either mouse or human IL-9 or both did not result in the inhibition of T cell development in FTOC (data not shown). This quandary is compatible with the view that the experimental approach, in which neutralizing mAbs or polyclonal Abs against IL-9 were used, was not powerful enough to result in a significant neutralization of the cytokine.

Cumulatively, our findings suggest that we must add IL-9Rα as an essential signaling receptor for T lymphocyte differentiation in

Table III. Influence of IL-9 on the development of human cells in chimeric human-mouse FTOC

| Treatment | FTOC Day +28 | Number of cells |  |
|-----------|--------------|----------------|---|---|---|
| Control   | 99,013 ± 42,952 | 9 ± 4.4 | 56.3 ± 16.6 | 34.0 ± 19.4 | 0.7 ± 0.4 |
| IL-9      | 303,812 ± 32,085 | 2.9 ± 0.4 | 77.2 ± 5.3 | 19.7 ± 5.6 | 0.6 ± 0.16 |

* Human CD34+ fetal liver cells were obtained and cultured in FTOC for 27 days as described in Materials and Methods, and numbers of human cells were calculated by multiplying the total number of viable cells counted under the microscope by the fraction of human CD45-FITC positive cells determined by FACS analysis. The frequency of thymocyte subsets was obtained by FACS analysis. Numbers of cells are given as the mean for groups of three separate experiments (+ SD) where X (p) is the number of single lobes per experiment were analyzed.

* Differences between cell number in control and IL-9Rα-treated FTOC were statistically significant, with p < 0.05 for 28 days of culture of CD34+ fetal liver cells by a paired t test on In-transformed values.
addition to IL-7Rα, whereas we did not find direct evidence for IL-9 as the critical ligand. This opens the challenging perspective that another ligand for IL-9Rα might be critical.

Particularly seminal will be the study of mice in which the IL-9 and more importantly the IL-9Rα genes will be disrupted. Since mAbs that neutralize the mouse IL-9Rα binding are not available, we are not able to address whether in mouse this receptor is equally of importance in the development of thymocytes. We could not demonstrate any effect on FTOC seeded with mouse fetal liver precursor cells by blocking mouse IL-9 with polyclonal Abs (data not shown). These observations fit with the data of He et al. (36), who found that a mAb to IL-9 did not exert inhibitory effects when administrated in vivo in chimeric mice reconstituted with adult bone marrow. Lack of mAbs that block the murine IL-9Rα do not allow us to investigate the role of the IL-9Rα in murine FTOC.

Cumulatively, our findings reported here strongly favor the hypothesis that, in man, IL-9Rα signaling is essential for optimal survival and/or growth and/or differentiation during early steps of intrathymic T cell development. We propose that IL-9Rα signaling is not critical for the entry of CD34+ fetal liver precursor cells, because we obtained a similar block when the anti-IL-9Rα Abs were not given in the hanging drop, when the cells enter the thymic lobe, but only added in FTOC. The partial inhibition that was observed in the experiment when the Ab was given during the hanging drop procedure alone can be attributed to the fact that the Ab already binds to the IL-9Rα of the CD34+ fetal liver precursor cells in hanging drop without interfering with the entry of the cells but inhibiting the differentiation of the cells in FTOC. Another possibility is that enough Ab is carried over in the FTOC to exert an inhibitory function on T cell development.

Human and murine IL-9 is mainly produced by activated T cells (37) raising questions regarding the cellular sources of IL-9 in the thymus, because activated T cells are absent at the start of FTOC. With RT-PCR, we were able to demonstrate IL-9 mRNA in human thymus (Fig. 5). We found that purifying stromal cells resulted in an increased IL-9 mRNA content per cell (data not shown). However, whether IL-9 is autotrophic or heterotrophic for CD34 precursor cells and whether stromal cells or fetal liver cell-derived cell components (e.g., dendritic cells) are producers of IL-9 is at present unclear. Techniques such as single cell assays for IL-9 mRNA expression or studies at the IL-9 protein level with high sensitivity are needed to allow a better insight in this matter.

Our findings allow us to propose a new mechanism of disease for SCID in humans. A lack of signaling through function deletion mutations of IL-2Rγc chain in human SCID is sufficient to cause the disease. The main function of the γc-chain is to recruit the tyrosine kinase JAK-3. However, theoretically an impairment of the function of a variety of cytokines such as IL-2, IL-4, IL-7, and IL-15, which all use the IL-2Rγc chain, could also cause the disease. Pallard et al. (38) confirmed our observation that Abs inhibiting IL-7Rα impair human T cell differentiation in FTOC. They extended these experiments by introducing chimeric receptors in T cell precursors and studying their ability to overcome the anti-IL-7Rα inhibition in FTOC. These experiments revealed that chimeric receptors containing the extracellular domain of the human IL-4 receptor and the transdomain and intracellular domain of hIL-7Rα chain enabled to rescue human T cell differentiation by IL-4, despite the anti-IL-7Rα inhibition. This was not the case when chimeric receptors were overexpressed that are no longer able to activate the PI-3K or the STAT5B pathway. IL-9Rα is associated with JAK-1 (30) as is the case for IL-7Rα. The signal transduction pathway triggered by IL-9Rα is surprisingly similar with that triggered by IL-7Rα, including STAT5 and PI3K (for review, Ref. 37). These findings are compatible with a synergy of the receptors resulting in a common signaling pathways and that a critical minimal threshold must be achieved by triggering both receptors, so that both receptors are not redundant but essential. Alternative explanations include the activation of specific intracellular mediators such as STAT1 and -3, which are activated by IL-9, or the possibility that IL-7Rα and IL-9Rα are expressed at distinct stages during T cell development. Unfortunately, lack of Abs against IL-9Rα that are capable to detect IL-9Rα positive cells by immunofluorescence do not allow us to test this hypothesis.

Cumulatively, our findings strongly suggest that it is important to consider not only lack of γc-chain, IL-7, and IL-7Rα, but also IL-9Rα to explain the human SCID pathology.

Acknowledgments

We thank Dr. Bart Vandekerckhove for reading the manuscript, Christian De Boever for artwork, Achiel Moerman for animal care, and the Department of Obstetrics, Cardiac Surgery, and Pathology for the supply of human tissue or blood. We thank Dr. D. De Bacquer of the Department of Health Sciences for statistical analysis.

References

2. Noegel, A. H., H. Li, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, S. W. Modis, O. W. McBride, and W. J. Leonard. 1993. Interleukin-2 receptor γc binding are not available, we are not able to address whether in mouse this receptor is equally of importance in the development of thymocytes. We could not demonstrate any effect on FTOC seeded with mouse fetal liver precursor cells by blocking mouse IL-9 with polyclonal Abs (data not shown). These observations fit with the data of He et al. (36), who found that a mAb to IL-9 did not exert inhibitory effects when administrated in vivo in chimeric mice reconstituted with adult bone marrow. Lack of mAbs that block the murine IL-9Rα do not allow us to investigate the role of the IL-9Rα in murine FTOC.

Cumulatively, our findings reported here strongly favor the hypothesis that, in man, IL-9Rα signaling is essential for optimal survival and/or growth and/or differentiation during early steps of intrathymic T cell development. We propose that IL-9Rα signaling is not critical for the entry of CD34+ fet


