

***In vivo* expression of interleukin-1 β (IL-1 β), IL-2, IL-4, IL-6, tumour necrosis factor- α and interferon- γ in the fetal murine thymus**

J. DEMAN, M. VAN MEURS,* E. CLAASSEN,* C. HUMBLET, J. BONIVER & M. P. DEFRESNE
Laboratory of Pathological Anatomy, University of Liège, Liège, Belgium, and *Department of Immunological and Infectious Diseases, C.E.Leiden, the Netherlands

SUMMARY.

Cytokines are known to play a role in T-cell lymphopoiesis as potent growth or differentiation factors, but many experiments focusing on their role in the thymus have been conducted only *in vitro*. We have thus used frozen sections obtained from fetal thymuses of normal C57BL/6 mice to investigate by immunohistochemistry the presence of interleukin-1 β (IL-1 β), IL-2, IL-4, IL-6, interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α). The results reveal that apart from IL-2, which was not detected, all these cytokines display a time-dependent expression pattern in the normal fetal thymus. First, production of IL-4, IL-6 and TNF- α is detected around days 13–14; this is followed by a second wave on days 16–17, with a production of IL-1 β , IL-4 and IL-6, and finally, just before birth (day 19), by a third wave of IL-1 β , IL-4, IL-6, IFN- γ and TNF- α production. This supports the hypothesis that cytokines play a role in T-cell lymphopoiesis.

INTRODUCTION

Interleukins are regulatory proteins secreted by white blood cells and a variety of other cells; their pleiotropic actions include modulation of inflammatory responses and numerous effects on cells of the immune system. They are also proposed to play a role in the thymus:¹ thymocytes and thymic epithelial cells have been shown *in vitro* to produce cytokines and to respond to their action. Overexpression of interleukin-4 (IL-4) in transgenic mice perturbs intrathymic differentiation;² anti-IL-2 receptor (IL-2R) treatment³ or an excess of IL-2⁴ or IL-7⁵ induce abnormal T-cell development in thymus organ cultures. By contrast, recent gene disruption experiments as reviewed⁶ suggest that IL-2, IL-4, IL-6, tumour necrosis factor (TNF)-Rp55, interferon- γ (IFN- γ) and IFN- γ -R are not essential to generation of normal thymocyte subpopulations.

Results obtained in knockout mice, however, are difficult to interpret, and it cannot be excluded that cytokines contribute to T-cell proliferation and/or maturation. *In situ* studies on cytokine production might contribute to identifying cytokines that play a role in T-cell development. Using *in situ* hybridization or polymerase chain reaction (PCR), we^{7,8} and others^{9–13}

have detected IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-7, IFN- γ , TNF- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA in the thymus during fetal development. Very few studies have sought to detect cytokine-containing cells by immunohistochemistry.¹³ In the present study, we have applied immunohistochemical staining procedures to mouse thymus sections isolated at different stages of fetal life, so as to demonstrate the presence of cytokines in the thymus.

MATERIALS AND METHODS

Mice

C57BL/Ka mice originating from Stanford University were raised in our animal colony. Mice were mated for one night and the fetuses removed on various days after the start of gestation (date of detection of a vaginal plug was counted as day 0).

Tissues

The thymuses were frozen in liquid nitrogen. Cryosections (6–8 μ m) prepared at -20° were placed on glass slides.

Antibodies

Interleukin-1 β was detected with VHp20 (IgG2a mouse anti-human IL-1 β) conjugated to biotin;¹⁴ IL-2 with S4B6 (IgG2a rat anti-mouse IL-2) conjugated to alkaline phosphatase;¹⁵ IL-4 with 11B11 (IgG rat anti-mouse IL-4) conjugated to biotin;¹⁶ IL-6 with 20F3 (IgG1 rat anti-mouse antibody);¹⁷ IFN- γ with DB1 antibody (IgG1 mouse anti-rat antibody) conjugated to β -galactosidase;¹⁸ TNF- α with IP-400 (IgG rabbit anti-mouse antibody).¹⁹

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Abbreviations: CD, cluster of differentiation; DN, double negative; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon- γ ; IL, interleukin; IL-R, interleukin receptor; TCR, T-cell receptor; TNF- α , tumour necrosis factor- α .

Correspondence, Dr. J. Deman, Laboratory of Pathological Anatomy, University of Liège, Liège, Belgium.

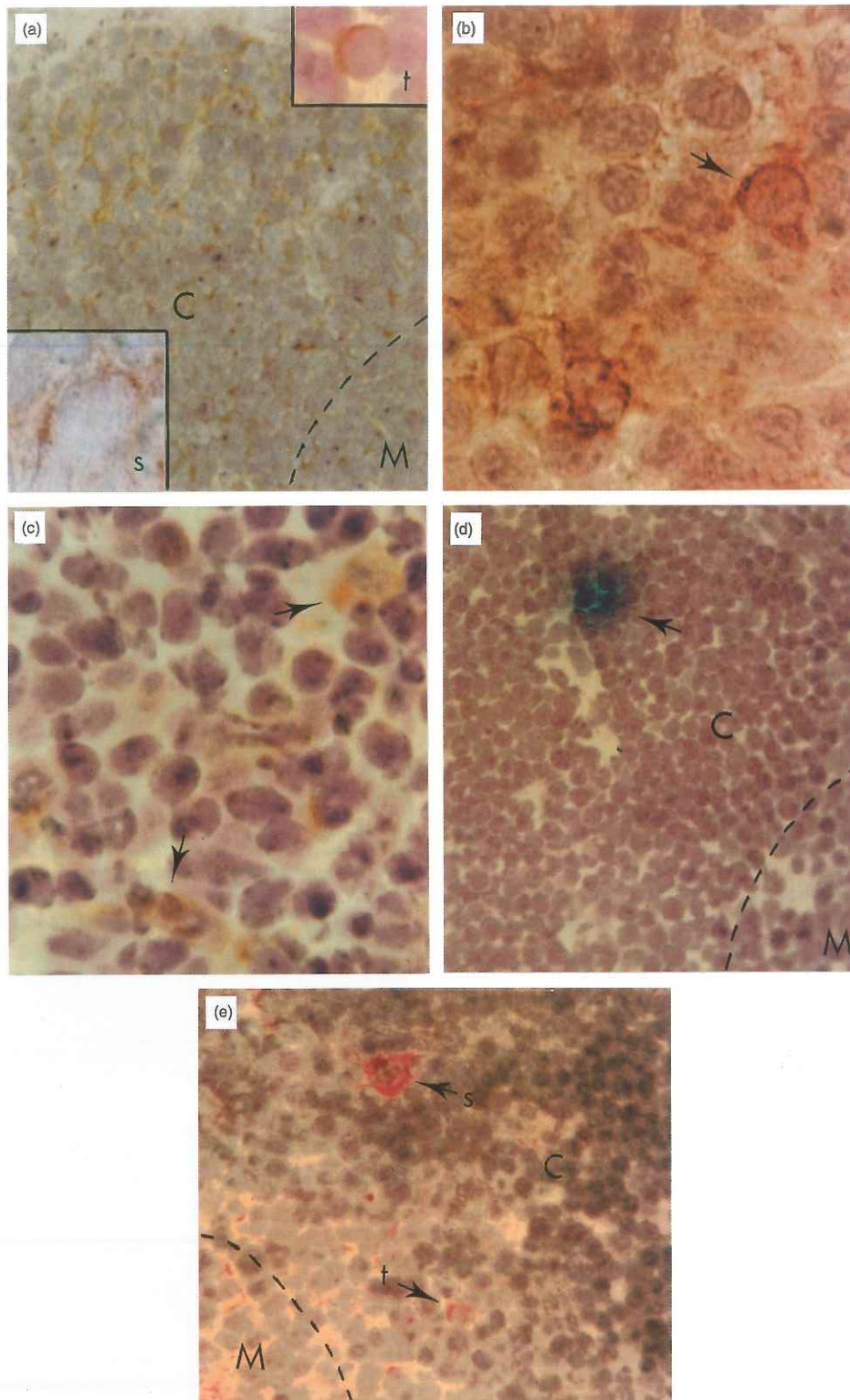


Figure 1. *In situ* expression of IL-1 β , IL-4, IL-6, IFN- γ and TNF- α in day 19 fetal thymus. (a) Thymic frozen sections were stained with a mouse anti-human IL-1 β antibody: IL-1 β is expressed either by thymocytes (t) or by stromal cells (s) and preferentially in the external area of the thymus (C: cortex, M: medulla) ($\times 328$). (b) Staining with a rat anti-mouse IL-4 antibody: IL-4 is detected in thymocytes (\rightarrow) ($\times 820$). (c) Staining with a rat anti-mouse IL-6 antibody: IL-6 is preferentially expressed by cells with a stromal morphology (\rightarrow) ($\times 820$). (d) Staining with a mouse anti-rat IFN- γ antibody: IFN- γ is detected in thymocytes (\rightarrow) and preferentially expressed in the external area of the thymus ($\times 328$). (e) Staining with a rabbit anti-mouse TNF- α antibody: TNF- α is expressed either by thymocytes (t) or by stromal cells (s) and preferentially expressed in the external area of the thymus ($\times 328$) (counterstained with haemalun (Mayer's)).

Immunostaining procedures

Cryostat sections (-20° , $8\ \mu\text{m}$) were kept overnight under high humidity at room temperature, then air-dried and stored in airtight boxes until used. For immunohistochemistry, slides were fixed for 10 min at room temperature in acetone containing 0.02% H_2O_2 and incubated horizontally overnight at 4° with anti-cytokine-specific antibody conjugates diluted to optimal concentration, as previously titrated, in phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA).

For the detection of IL-1 β and IL-4, the slides were then incubated with avidin-peroxidase (Sigma St Louis, MO); for IL-6, they were incubated with a rabbit anti-rat antibody conjugated with peroxidase (Sigma); for TNF- α they were incubated with a rat anti-rabbit antibody conjugated with alkaline phosphatase (Genzyme, Cambridge, MA) for 30–60 min. Preparations were stained by incubation in 3-amino-9-ethylcarbazole (AEC: Sigma A-5754) dissolved in *N,N*-dimethylformamide (DMF: BDH) and 0.05 M NaCl buffer (pH 5.0) containing 0.02% H_2O_2 for 10 min. After this treatment, stained material appears red.²⁰

Anti IL-2 antibodies were directly conjugated with alkaline phosphatase and revealed by a procedure entirely carried out at 37° : briefly, sections were incubated for 30 min in a Tris-HCl buffer (0.1 M, pH 8.5) containing Naphthol-AS-MX phosphate (Sigma N-4875), Fast Blue BB (Sigma F-3378), NaNO_2 (Merck 6549), and Levamisole (Sigma L-9756). Positive cells are stained in blue.²¹

Anti IFN- γ antibodies are conjugated to βGal and revealed by incubating the slides 30 min under high humidity at 37° with a solution containing Xgal (Boehringer Mannheim, 651745), 20% DMF (BDH), 1.1 mM magnesium chloride, 50 mM potassium ferricyanide, and 50 mM potassium ferrocyanide. Positive cells are stained in greenish blue.²²

Slides were then washed twice for 5 min each time in PBS, counterstained with haemalun (Mayer's) for 5 seconds, rinsed in tap water, and mounted in glycerin/gelatin.

Each experiment was carried out in duplicate on four separate animals at each gestational age. To estimate the number of positive cells, we scanned each section entirely. For a given cytokine, the intensity of the staining and the number of positive cells were identical for individual tissues from different animals of the same gestational age.

As positive controls we used spleens of mice immunized subcutaneously, with $50\ \mu\text{g}$ TNP-Ficoll prepared according to published methods.²³ The spleens were removed on day 4 (for all cytokines tested except IL-6) or on days 6–7 (for IL-6) after stimulation. As negative controls, we used slides incubated without the first antibody, subsequent immunohistochemical staining being done as described above, according to the second antibody used.

RESULTS

Patterns of IL-1 β , IL-2, IL-4, IL-6, TNF- α and IFN- γ expression in the fetal thymus as revealed by immunohistochemistry

Fetal thymuses collected at various stages of gestation were stained with anti-IL-1 β , anti-IL-2, anti-IL-4, anti-IL-6, anti-IFN- γ and anti-TNF- α antibodies. Intense IL-1 β reactivity was seen on days 16 and 19 (Fig. 1), mainly in the outer part of the thymus on day 16, in the cortex and subcapsular area when

morphologically distinct from the medulla. The labelled cells displayed a stromal or a thymocyte-like morphology (Fig. 1). No immunoreactivity was observed with anti IL-2 monoclonal antibodies. The number of IL-4-positive cells peaked on days 16–17; the positive cells displayed thymocyte morphology and were evenly distributed through the thymus. Weak staining was observed on days 13, 14 and 19 (Fig. 1); no positive cells were detected on days 15, 18 and 20. Intense IL-6 reactivity was detected on days 13, 16 and 19 (Fig. 1); the positive cells were mainly stromal cells with cytoplasmic processes, evenly distributed through the thymus (Fig. 1). No immunoreactivity was seen on days 15 and 18. Cells containing IFN- γ were present only on day 19 and around birth. The intensity of the staining was very high; stained cells were localized within the cortex (Fig. 1) and showed thymocyte-like morphology (Fig. 1). Weakly TNF- α -positive cells were detected in the external area of the thymus on day 13 of gestation, whereas strong staining was observed in both the cortex and the medulla on day 19

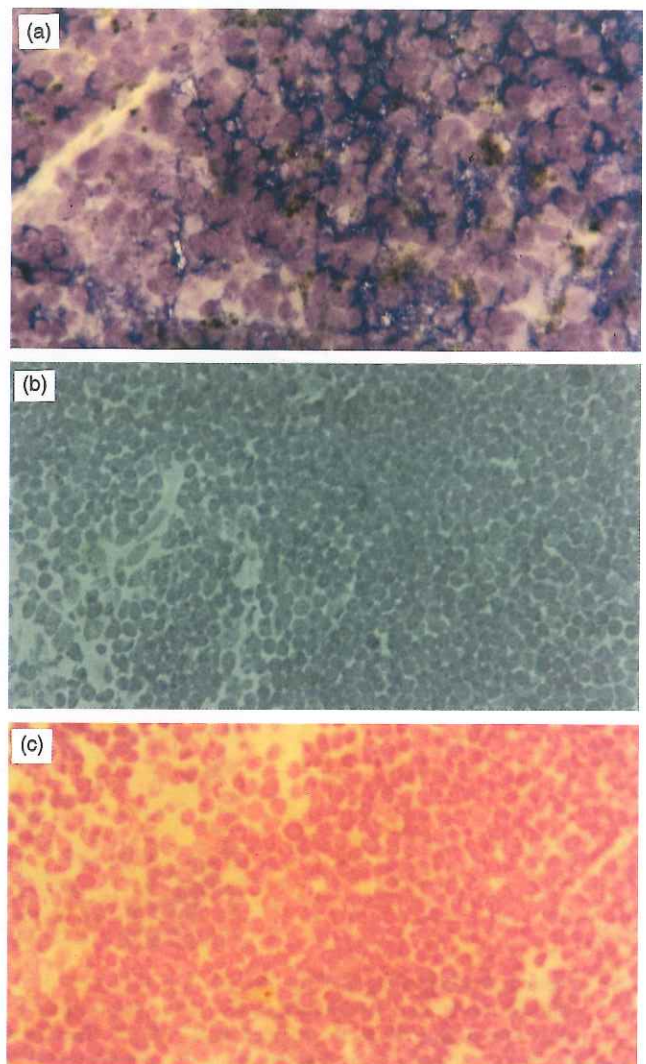


Figure 2. (a) *In situ* expression of IL-2 in a TNP-Ficoll-stimulated spleen, as a positive control; (b) lack of staining for IL-2 in day 19 fetal thymus; (c), thymic frozen sections treated without the first antibody as a negative control (counterstained with haemalun (Mayer's), $\times 260$).

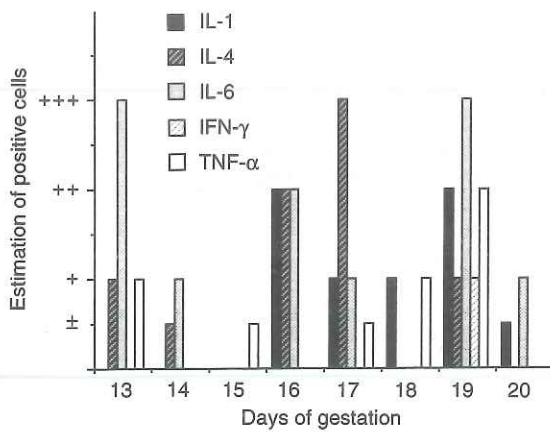


Figure 3. Semi-quantitative estimation of cytokines in the fetal thymus [\pm , 1 to 5 positive cells (per section); +, 5 to 10 positive cells (per section); ++, 10 to 50 positive cells (per section); +++, > 50 positive cells (per section)].

(Fig. 1). Stained cells displayed a stromal or a thymocyte-like morphology.

As positive controls, we stained spleens from TNP-Ficol-treated mice with the same antibodies (Fig. 2a): intense staining was observed for all antibodies tested. We observed no staining of thymus sections incubated with the anti-IL-2 antibody (Fig. 2b) or of controls not treated with the first antibody but stained according to the second antibody used (Fig. 2c).

In summary (Fig. 3): three waves of cytokine production are detected during thymic development: a wave of IL-4, IL-6 and TNF- α production around days 13–14, a wave of IL-1 β , IL-4 and IL-6 production on days 16–17, and a final wave just before birth (day 19) during which IL-1 β , IL-4, IL-6, IFN- γ and TNF- α are produced.

DISCUSSION

In order to investigate the possible role of cytokines in thymic development, we used immunohistochemistry to detect IL-1 β , IL-2, IL-4, IL-6, TNF- α and IFN- γ in the fetal thymus at different stages of ontogenic development. This system provides a tool for analysing both commitment during thymocyte differentiation and the evolving cytokine profile.

The data, summarized in Fig. 3, show three waves of cytokine production within the thymus during ontogeny. The first wave occurs on days 13–14, when IL-4, IL-6 and mostly TNF- α are detected. Haematopoietic stem cells having colonized the thymic anlage on day 11 display high proliferative activity on days 13 and 14;²⁴ they also rearrange the γ and δ genes and start to express T-cell receptor (TCR) $\gamma\delta$ on day 14.²⁴ IL-6 and TNF- α were detected on day 13 when triple-negative thymocytes (CD3⁻CD4⁻CD8⁻) are present.²⁴ These thymocytes or cells from the monocyte-macrophage lineage may be responsible for TNF- α production on day 13. TNF- α , alone or in combination with IL-4, can induce production of IL-6²⁵ and might thus contribute to causing the appearance of IL-6 on day 13. On the other hand, TNF- α can induce proliferation of stem cells and enhance proliferation of immature thymocytes stimulated *in vitro* by IL-7, the latter being detected from day 13.¹¹ Interleukin-6 might act as a second signal for TNF- α -induced thymocyte proliferation.²⁶ TNF- α and IL-6 might thus

account for the high proliferation rate observed on day 13. TCR $\gamma\delta$ double-negative (DN) thymocytes, which emerge on day 14 can produce IL-4 *in vitro*; some of them display constitutive expression of high-affinity IL-4 receptors.²⁴ Furthermore, it has been proposed on the basis of observations in young children with Down syndrome that IL-4 plays an important role in the proliferation of TCR $\gamma\delta$ DN thymocytes.²⁷ IL-4 inhibits production of cytokines such as GM-CSF, granulocyte colony-stimulating factor (G-CSF) and IL-8, involved in myelopoiesis.²⁸ This might be important in controlling the myelopoietic potential of stem cells in the thymus.²⁹ IL-4, furthermore, when added alone to 14-day-old fetal organ cultures³⁰ or in combination with phorbol myristate acetate (PMA) to cultures of DN thymocytes, almost completely inhibits expression of CD4 and CD8 molecules, which remain undetected until day 16 in the fetal thymus.²⁴

On day 16 occurs a second wave of IL-6 production. At this stage IL-6, together with rather high levels of IL-4 and IL-1 β , may account for cells acquiring CD4 and CD8 molecules: IL-6 given to irradiated mice accelerates differentiation from Thy1⁺IL-2R⁺ DN cells into CD4⁺CD8⁺ cells.³¹ IL-4 is probably produced by the subset of $\gamma\delta$ DN cells still present on day 16. The other cell subsets present at this fetal age, CD4⁺CD8⁺TCR^{low} or TCR⁻ cells, are refractory to induction of cytokine gene expression in response to stimulation.³² The thymic stroma is probably responsible for IL-1 and IL-6 production. As expected, the increase in the proportion of CD4⁺CD8⁺ thymocytes on days 17–18 is paralleled by decreased cytokine production by thymocytes.

The appearance of CD4⁺CD8⁻TCR $\alpha\beta$ ⁺ thymocytes on day 18 precedes a third wave of cytokine production (IL-1 β , IL-4, IL-6, IFN- γ , TNF- α) detected on day 19 along with the emergence of CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ cells. Single-positive CD4⁺ thymocytes differ from their progeny, the naive peripheral CD4⁺ cells, in that they produce mainly IL-4, IL-10 and IFN- γ but no IL-2.³² They might thus be responsible for IL-4 and IFN- γ production on day 19. Interleukin-4 plays a pivotal role in the shift of Th1 cells to the Th2 phenotype, whereas IFN- γ probably plays a role in modulating the properties of stromal cells.³³ TNF- α is again detected at this fetal age: *in vitro*, TNF- α possibly produced by CD3⁺ DN cells enhances the proliferation of SP thymocytes in the presence of IL-7;¹¹ it probably accounts for the development of subsets of mature thymocytes. Interleukin-6 detected on day 19 is probably produced on one hand by TNF- α - and IL-4-stimulated lymphoid cells, and on the other hand by thymic epithelial cells under the influence of IL-1 β , IL-4 and IFN- γ . Interleukin-6 seems to be involved in the proliferation of thymocytes and to participate in T-cell activation via a TCR-independent pathway.²⁶ Finally, IL-1 β is present in the fetal thymus from day 16 until birth. *In vitro*, IL-1 is produced mainly by thymic stromal cells and to a lesser extent by thymocytes. IL-1 has also been detected in thymic epithelial cells *in vivo*.³⁴ In addition to its role in the regulation of cytokine production by thymic epithelial cells, IL-1 may be an important signal for growth and/or differentiation of T cells. This view is supported by the fact that CD4⁻CD8⁻ thymocytes display functional IL-1 receptors.³⁵

The role of IL-2 during ontogeny is more difficult to discuss, since cells containing transcripts for IL-2 are detected during fetal development^{8,9,13} but IL-2 protein has not been revealed by immunohistochemistry before day 5 after birth. This

discrepancy between the presence of mRNA and the absence of protein might be interpreted as follows: rapid degradation and/or secretion of the protein might result in its being extremely hard to identify by immunohistochemistry; yet this seems not to be the case, since we did detect IL-2 protein in the stimulated spleen and in the neonatal and adult thymus, protein synthesis can be controlled at a post-transcriptional level; perhaps some inhibitory mechanism blocks intrathymic translation of IL-2 mRNA. This observation suggests that IL-2 has little or no role in T-cell development and tallies with observations showing that IL-2-deficient mice display normal thymic development.⁶

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