Interleukin-12 inhibits development of ectopic endometriotic tissues in peritoneal cavity via activation of NK cells in a murine endometriosis model

Hiroyuki Itoh · Toshihiro Sashihara ·
Akira Hosono · Shuichi Kaminogawa ·
Masayuki Uchida

Received: 27 September 2010 / Accepted: 11 November 2010 / Published online: 15 March 2011
© Springer Science+Business Media B.V. 2011

Abstract Involvement of impaired peritoneal immunosurveillance systems has been well established in the pathology of endometriosis. On the other hand, it has been observed that peritoneal administration of IL-12 suppress development of endometriotic lesions in a mouse endometriosis model. We investigated the effect of peritoneal administration of IL-12 on the peritoneal immunosurveillance system regarding NK cells in the mouse model. Treating the endometrial-tissue challenged mice with IL-12 for 5 consecutive days, from day -2 to day 2 (implantation of the endometrial tissues was done on day 0), cytotoxicity of splenic NK cells was enhanced immediately after the administration, on day 3, and development of the endometriotic lesions was reduced on day 21. In vivo NK cell depletion by administration of anti-IL-2R β mAb resulted in reduction of the cytotoxicity of splenic NK cells concomitant with a significant attenuation of suppressive effect of IL-12 on development of

endometriotic lesions. Therefore, it was suggested that IL-12 suppresses development of endometriotic lesions via activation of NK cells, and that NK cells are involved in the primary defense for the development of endometriotic lesions.

Keywords Endometriosis · Interleukin-12 · Natural killer cells · Murine model

Abbreviations

IL Interleukin
INF Interferon
NK Natural killer

Introduction

Endometriosis is a gynecological disorder characterized by that endometrial-like tissue grows outside the uterine cavity and causes pelvic pain and infertility in women of reproductive age (Giudice and Kao 2004). Approximately 6–10% of women of reproductive age are estimated to have endometriosis (Giudice and Kao 2004). Among several etiologies, currently the most widely accepted is the theory of retrograde menstruation, proposing that some of the endometrial debris exits the uterus through the fallopian tubes during a woman's menstrual flow and attaches itself to the abdominal cavity where it can proceed to invade the tissue as endometriosis (Sampson 1927). However, it still remains controversial why a part of

H. Itoh · T. Sashihara (✉) · M. Uchida
Food Science Institute, Division of Research
and Development, Meiji Dairies Corporation,
540 Naruda, Odawara, Kanagawa 250-0862, Japan
e-mail: toshihiro_sashihara@meiji-milk.com

A. Hosono · S. Kaminogawa
Department of Food Science and Technology,
College of Bioresource Sciences, Nihon University,
1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan

women do endometrial cells implant in ectopic locations and give rise to endometriosis, while retrograde menstruation occurs in over 90% of women with menstruation (Bartosik et al. 1986).

In peritoneal fluid of women with endometriosis, decreased natural killer (NK) cell activity (Oosterlynck et al. 1991; Ho et al. 1995), dysfunction of T lymphocytes (Ho et al. 1996; Szylo et al. 2003), infiltrating macrophage (Bacci et al. 2009) as well as aberrant concentrations of immune-related cytokines (Ho et al. 1997) have been reported in the past two decades. Thus, it has been proposed that an impaired immunological clearance of endometrial fragments shed into the peritoneal cavity may facilitate the implantation of endometrial cells in ectopic locations and result in endometriosis. However, a possibility can not yet be excluded that such alterations in the immune functions are the consequence of the presence of ectopic endometrial tissues in peritoneal cavity but not the cause of the disease. In fact, Kikuchi et al. reported resection of endometriotic lesions resulted in facilitation of differentiation of NK cells (Kikuchi et al. 1993). Thus, the role of these immune cells, especially NK cells in progression of endometriosis remains to be elucidated.

Somigliana et al. reported using a mouse experimental model of endometriosis that peritoneal administration of interleukin (IL)-12p70 (IL-12) to endometriosis-induced mice exhibited suppressive effects of progression on endometriotic lesions (Somigliana et al. 1999). They showed that intra-peritoneal administration of IL-12 for 5 consecutive days (from day -2 to day 2 when endometrial tissue-challenge was done on day 0) resulted in reduction of development of endometriotic lesions on day 21. IL-12, a heterodimeric cytokine composed of p40 and p35 chains, is produced primarily by antigen-presenting cells such as macrophages that play an essential role in the interaction between the innate and adaptive immunity by stimulating production of IL-2 and interferon (IFN)- γ to activate not only NK cells but also cytotoxic T lymphocytes (D'Andrea et al. 1992; Heufler et al. 1996). In addition, Mazzeo et al. reported that IL-12 possesses the ability to modulate the NK cell-mediated recognition of endometrial targets and IL-12 treated NK cells exhibited enhanced recognition of endometrial cells in an in vitro experiment (Mazzeo et al. 1998). Therefore, it is deduced that a peritoneal

administration of IL-12 on endometriosis-induced mice affects endometriotic lesions via activation of these immune cells. However, there have been no direct evidences that show IL-12 reduces development of endometriotic tissues via activation of NK cells in vivo. In this study, we first investigated the effect of peritoneal administration of IL-12 on the peritoneal immune environment. Then, the role of NK cells on development of ectopic endometriotic lesion was assessed in the mouse endometriosis model.

Materials and methods

Mice

Female, 6-week old specific pathogen-free BALB/c mice that have been undergone ovariectomy at an age of 5 weeks old were purchased from Japan SLC (Shizuoka, Japan) and were maintained on a standard diet (CRF-1 diet; Oriental Yeast, Tokyo, Japan). The mice were housed in groups in a temperature- and humidity-controlled room, at $21 \pm 2^\circ\text{C}$ and $55 \pm 15\%$ humidity, on a 12/12 h reversed light/dark cycle. Food and tap water were provided ad libitum. The experimental protocols were approved by the Animal Care Committee of the Division of Research and Development, Meiji Dairies Corporation.

Induction of endometriosis

Induction of endometriosis was performed according to a method reported by Somigliana et al. with slight modifications (Somigliana et al. 1999). Briefly, endometriotic lesions were induced at day 0 by inoculating finely chopped endometrial fragments corresponding to about 50% of the endometrial tissue from both uterine horns of syngenic mice as described below into the peritoneal space. Mice challenged with endometrium have been subjected to ovariectomy through a small vertical midline laparotomic incision at day -7 (at 5-week old) and injected with oestrogens depot in castor oil (Wako, Osaka, Japan; $100 \mu\text{g/kg i.m.}$) on days -7, 0, 7, 14. Ovariectomy plus oestrogen supplementation was performed in order to abrogate differences related to the stage of the oestrous cycle.

Uterine samples to be inoculated were obtained from syngenic mice used as donor mice. Similarly to challenged mice, donor mice were ovariectomized and oestrogen-treated on day -7. On day 0, one donor mouse was killed for every two mice to be challenged with endometrium. Both uterine horns were removed using aseptic technique and subsequently placed in a sterile Petri dish containing sterile saline. Then, uterine samples were gently peeled in order to detach the uterine muscle from the endometrium. Following this procedure, they were transferred to a glass slide and finely chopped using two blunt scalpels. Endometrial fragments were suspended in 0.3 mL of sterile saline per challenged mouse and inoculated into the peritoneal cavity.

Assessment of endometriotic lesions

On day 21 after endometrium challenge, mice were sacrificed by cervical dislocation, the abdomen was inspected and endometriotic lesions were carefully excised from the surrounding tissue in order to assess their weight and surface area. The surface areas were measured with a caliper and calculated. When the lesion had a cyst appearance, measures were taken after aspiration of the cyst. The operator was blinded for the different conditions.

Recombinant murine IL-12 administration

Murine recombinant IL-12 (Wako) was administrated *i.p.* by daily injections of 0.15 µg/0.4 mL for 5 days, from day -2 through day 2. Control and other mice received only the vehicle following the same procedure.

NK cell depletion

NK cell depletion was performed as described elsewhere (Tanaka et al. 1991; Tanaka et al. 1993). Mice were injected intraperitoneally with 1 mg of anti-IL-2 receptor β chain monoclonal antibody (IL-2R β mAb; Rat IgG2b; Biolegend, San Diego, CA) in 0.5 mL of phosphate-buffered saline (PBS) only once one week prior to endometrial challenge (day -7).

Measurement of cytotoxicity of NK cells

The cytotoxicity of splenic NK cells was assessed using flow cytometry (Johann et al. 1995); YAC-1 cells

were used as the target cells, and 5.0×10^5 YAC-1 cells/mL were labeled with 2.5 µg/mL of 3,3'-diiodatetradecyloxycarbocyanine perchlorate (Sigma–Aldrich, St. Louis, MO) by overnight incubation at 37 °C. The cells were washed 3 times with RPMI 1640 medium, and resuspended at a concentration of 2.5×10^5 cells/mL. Murine splenocyte effector cells (1×10^6 cells/well) were added to the target cells at 2.5×10^4 cells/well (40:1 ratio) in a total volume of 200 µl/well in a 96-well, round-bottomed plate (Corning Inc., Corning, NY). Samples were centrifuged ($30 \times g$, 1 min), and incubated for 4 h at 37 °C in a humidified 5% CO₂-air atmosphere. Fifteen minutes before the end of the incubation, 20 µl of propidium iodide (0.5 mg/mL in PBS; Sigma–Aldrich) was added to each well to label the dead cells. The level of target cell lysis was determined using a FACSCalibur flow cytometer with CellQuest software (BD Bioscience, Franklin Lakes, NJ), and the cytotoxicity of NK cells was expressed as the percentage of effector cell-specific lysis.

Flow cytometric analysis

Flow cytometric analysis of peritoneal cells was performed using a FACSCalibur after staining with anti-CD4-Phycoerythrin (PE), anti-CD8-Fluorescein Isothiocyanate (FITC), anti-DX5-PE, anti-CD45RB-FITC, anti-CD11b-PE, anti-CD11c-PE. The above Abs were purchased from BD Biosciences. Dead cells were eliminated by staining the cells with final concentration at 2 µg/mL of 7-amino-actinomycin (BD Bioscience) for 15 min.

Peritoneal cytokines and collection of peritoneal cells

For determination of peritoneal cytokine levels, 0.5 mL of peritoneal lavage fluid was collected after injecting 1 mL of PBS containing 1.0% fetal calf serum (Intergen, Purchase, NY). Cytokine levels in supernatant of the peritoneal lavage fluid were determined using Bio-plex cytokine assay kits (Bio-Rad, Hercules, CA). The operating procedures provided by the manufacturer were strictly followed. Peritoneal cells were harvested from the initially collected peritoneal lavage fluid after centrifugation, and then this was combined with the secondary collected peritoneal lavage fluid by additionally

injecting 4 mL of PBS containing 1.0% fetal calf serum.

Gene expression analysis

Total RNA was extracted from the peritoneal cells using the Trizol reagent (Invitrogen, Calsbad, CA). Total RNA (1 µg) was used for synthesis of the first strand of cDNA using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR were performed with an ABI prism 7300 (Applied Biosystems, Foster City, CA). The oligo-nucleotide primer pairs were synthesized as described elsewhere (Overbergh et al. 2003). The fluorescence intensity of each target gene was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) amplified under identical conditions.

Statistical analysis

Data were expressed as the mean $\pm$ SD. Statistical differences between the groups were analyzed by Mann–Whitney's *U* test or a one-way analysis of variance (ANOVA) followed, if justified, by Fisher's protected least significant difference (PLSD) test. Differences were considered significant when the *P*-value for the effect was less than 0.05.

Results

Effect of IL-12 on development of ectopic endometriotic lesion

We first performed a replication study as conducted by Somigliana et al. (1999) whether intraperitoneal administration of IL-12 from day -2 through day 2 reduces development of ectopic endometriotic lesions. The result showed that total weight of the endometriotic lesions tended to decrease, and the surface area was significantly reduced in the IL-12 group compared with the control group (Table 1). This result corresponded well with the findings of Somigliana et al. (1999). We examined peritoneal immune environment, however, no obvious differences between the groups on day 21 regarding peritoneal cytokine levels, peritoneal cell population, and cytotoxicity of splenic NK cells, were observed (data not shown).

Table 1 Effect of IL-12 treatment on and the total weight and surface area of endometriotic lesions

Groups	Control (<i>n</i> = 4)	IL-12 (<i>n</i> = 4)	<i>P</i> -value
Total weight (mg)	23.8 $\pm$ 16.9	8.9 $\pm$ 4.9	0.0832
Total area (mm ²)	25.3 $\pm$ 21.4	8.3 $\pm$ 1.5	0.0209

Data were expressed as the mean with SD of individual mice

Immunological environment of peritoneal cavity of the endometriosis induced mice at day 3

In the afore-mentioned experiment, the last injection of IL-12 was done on day 2, whereas peritoneal immune environment was examined on day 21. It was deduced that effects of IL-12 on peritoneal immune environment was attenuated during the next 3 weeks. Therefore, we analyzed immunological environment in the peritoneal cavity on the next day after the final injection of IL-12 (day 3).

Population of cells in the peritoneal lavage fluid

Although the mean value for total cell number in the peritoneal lavage fluid was increased in the IL-12 group because a few individuals showed higher values, there were no significant differences in the values among the groups (Table 2). Population of CD4⁺, CD8⁺ and DX5⁺ cells were unexpectedly unaffected by both, implantation of endometrial tissues and IL-12 treatment. B220⁺ cells were significantly decreased in the control group in comparison to the normal group. On the contrary to B220⁺ cells, CD11b⁺ and CD11c⁺ cells were significantly increased in the control group compared with the normal group. Effect of IL-12 treatment on the cell population was not detected on day 3.

Peritoneal cytokine levels

Inflammatory cytokines such as IL-1 β and IL-6 levels were significantly higher in the control group compared with the normal group (Table 3). Of these, the enhanced IL-6 levels were reduced in the IL-12 group. IFN- γ levels were slightly increased in the IL-12 group, however, the difference was not significant. IL-2 and IL-12 levels were under the detection limit in all groups (data not shown).

Table 2 Population analysis of cells in peritoneal lavage fluid

Groups	Normal (<i>n</i> = 7)	Control (<i>n</i> = 8)	IL-12 (<i>n</i> = 7)
% CD4 ⁺	12.4 ± 0.8	10.3 ± 2.6	10.9 ± 1.8
% CD8 ⁺	3.5 ± 0.9	2.3 ± 0.6	4.1 ± 1.2
% B220 ⁺	32.6 ± 9.0 ^a	10.2 ± 3.7 ^b	14.9 ± 5.1 ^b
% DX5 ⁺	14.8 ± 7.1	14.9 ± 3.7	15.8 ± 2.0
% CD11b ⁺	29.8 ± 6.4 ^a	46.3 ± 5.5 ^b	38.5 ± 4.4 ^b
% CD11c ⁺	6.8 ± 2.5 ^a	16.1 ± 4.5 ^a	16.7 ± 2.2 ^b
Total cell number	5.55 ± 2.41	6.23 ± 2.20	9.01 ± 4.09

Data were expressed as the mean with SD of individual mice

Total cell number represents the cell number recovered from peritoneal lavage fluid ($\times 10^5$ cells)

Different superscripts denote significant differences between groups ($P < 0.05$)

Gene expression

Because effects of IL-12 on the Th1-related cytokine levels were not observed in the peritoneal lavage fluid, mRNA expression of these cytokines in the peritoneal cells were analyzed. The results showed that those of IL-2, IFN- γ , and T-bet (Th1-specific T box transcription factor), a master transcription factor required for differentiation of Th1 cells, were found to be significantly increased in the IL-12 group (Fig. 1). Therefore, IL-2 and IFN- γ levels were presumed to be enhanced by injection of IL-12 but were abolished after interaction with their specific receptors, therefore the levels were not elevated in the peritoneal lavage fluid (Fig. 1).

Cytotoxicity of splenic NK cells

Because substantial number of the peritoneal cell was not obtained, cytotoxicity of NK cells on day 3 was

alternatively determined using splenocytes. The result showed that the cytotoxicity was not different between the normal and control group, but it was significantly increased in the IL-12 group (Fig. 2). Cell population analysis of splenocytes revealed no significant difference in splenic DX5⁺ cell number as same as peritoneal cells (data not shown), the increase in the cytotoxicity of NK cells was found to be due to the enhancement of each DX5⁺ cell.

Effect of NK cells depletion on endometriosis

In the above experiment, we confirmed that cytotoxicity of NK cells was enhanced by IL-12 treatment. To elucidate the role of NK cells on development of endometriotic lesions in this model, depletion of NK cells by injecting anti-IL-2R β mAb was attempted. After 4 weeks (day 21) of a peritoneal injection of anti-IL-2R β mAb, cytotoxicity of splenic NK cells was significantly attenuated compared to the IL-12 group, although it was not deleted completely (Fig. 3). Here, both of total weight and total area of endometriotic lesions were significantly reduced in the IL-12 plus anti-IL-2R β mAb group compared with the IL-12 group (Fig. 4). Regarding peritoneal cytokine levels, such as IL-1 β and IL-6, and the peritoneal cell populations, there were no significant differences between the groups (data not shown). However, INF- γ levels were significantly lowered in the IL-12 plus anti-IL-2R mAb group (data not shown). Therefore, it was strongly suggested that NK cells are involved in the primary defense for development of endometriotic lesions.

Table 3 Cytokine levels in the peritoneal lavage fluid on day 3

Groups	Normal (<i>n</i> = 7)	Control (<i>n</i> = 8)	IL-12 (<i>n</i> = 7)
IL-1 β	3.8 ± 3.4 ^a	21.1 ± 26.8 ^b	20.4 ± 24.2 ^b
IL-6	4.1 ± 9.0 ^a	73.4 ± 64.9 ^b	34.0 ± 12.0 ^a
IFN- γ	1.5 ± 2.0	2.5 ± 2.2	5.0 ± 3.8

Data were expressed as the mean with SD of individual mice (pg/mL)

Different superscripts denote significant differences between groups ($P < 0.05$)

Fig. 1 Relative mRNA expression of *Il-2* (a), *Ifn-γ* (b), and a master transcription factor required for differentiation of Th1 cells (*T-bet*) (c) in peritoneal cells. Data were quantitatively presented following normalization of the glyceraldehyde-3-

phosphate dehydrogenase (*Gapdh*) levels and were expressed as the mean with SD of individual mice. Different letters denote significant differences between groups ($P < 0.05$)

Fig. 2 Cytotoxicity of splenic natural killer cells on day 3. Splenocytes derived from each mice on day 3 were used as effector cells against YAC-1 target cells (E:T ratio = 40:1). Data were expressed as the mean with SD of individual mice. Different letters denote significant differences between groups ($P < 0.05$)

Discussion

In this study, on day 3, a part of the injected small pieces of translucent endometrial tissue were found to adhere to the peritoneum or surface of the intestinal

Fig. 3 Effect of anti-IL-2Rβ mAb on cytotoxicity of splenic natural killer cells on day 21. Splenocytes derived from each mice on day 21 were used as effector cells against YAC-1 target cells (E:T ratio = 40:1). Data were expressed as the mean with SD of individual mice. * $P < 0.05$

Fig. 4 Effect of anti-IL-2R β mAb on endometriotic lesions on day 21. Each value of total weight (a) and total surface area (b) of the endometriotic lesions is shown. The mean and SD of individual mice are indicated beside each value. * $P < 0.05$

organs, and they were turned to red lesions as often observed in human endometriosis. Although we could not count the number of the adhered lesions on the dissection on day 3 because it was too small and too many to count in each mouse, more than 20 of the small and red lesions were detected in each mouse. In addition, some of the small pieces of the endometriotic lesions aggregated to form large clusters of cysts on day 21, therefore, assessment of number of the endometriotic lesions was not performed as reported in other articles (Somigliana et al. 1999; Bacci et al. 2009). Yet, the number of endometriotic regions detected on day 21 was around 1 to 7 in each individual, while the number of endometriotic tissues injected on day 0 was 46 ± 5 pieces/mouse. This indicates that large parts of the injected endometrial tissues was eradicated by peritoneal immune surveillance systems during progression of endometriosis.

The present study was performed based on a working hypothesis that NK cells suppress growth of ectopic endometriotic tissues and that the suppressive effect of IL-12 owe to enhancement of NK cells. Because NK cells were known to be promoted to proliferate by IL-2 and IFN- γ (Weigent et al. 1983), we have expected that the number of NK cells was increased by IL-12. Analyses of peritoneal immune environment on day 3 showed that not only IL-12 but also IL-2 and IFN- γ levels in the peritoneal lavage

fluid were not detected, and population of CD4⁺ and NK cells was not altered. However, transcription of *Il-2* and *Ifn- γ* , and *T-bet*, which is a master transcription factor required for differentiation of Th1 cells (Szabo et al. 2000), were confirmed to be increased by IL-12. In addition, the cytotoxicity of splenic NK cells was enhanced by IL-12 although there were no differences in the number of splenic and peritoneal NK cells. Therefore, we prompted to delete NK cells to investigate whether development of the endometriotic lesions was affected.

The IL-2R β is preferentially expressed in NK cells, but is not detected in a majority of resting T and B cells. It has been shown that a single injection of anti-IL-2R β mAb causes a long-term elimination of splenic NK cells, lasting for at least 5 weeks without affecting the function and proliferation of T and B cells (Tanaka et al. 1991; Tanaka et al. 1993). In this experiment, although splenic and peritoneal NK cells were not deleted completely, cytotoxicity of the splenic NK cells was suppressed in the IL-12 plus anti-IL-2R β mAb group. The suppressive effect of the development of endometriotic lesions by IL-12 was found to be attenuated in the IL-12 plus anti-IL-2R β mAb group. This result clearly indicates that peritoneal NK cells contribute to suppress the development of ectopic endometriotic lesions. Kikuchi et al. have reported that the decrease in NK cells in peritoneal

fluid of endometriotic patients was a consequence of the implantation of ectopic endometriotic lesions, because immature NK cells and, in addition, not only the percentage but also the absolute number of moderately differentiated NK cells increased by resection of endometriotic lesion in endometriotic patients (Kikuchi et al. 1993). Hill also reported that aberrant NK cell activity may be the result of an imbalance in the immune response to a chronic antigenic stimulus, such as ectopic endometrium, or to a previously initiated autoimmune event unrelated to endometriosis (Hill 1992). In this experiment, the number of the splenic and peritoneal NK cells, cytotoxicity of the splenic NK cells, and peritoneal NK-cells related cytokine levels were unaltered by implantation of endometrial tissues both on day 3 and 21. Therefore, results of the present experiment suggest that the reduction of cytotoxic activity of NK cells observed in the patients with endometriosis was not a consequence but a cause of the disease provoked by unknown reasons.

Regarding CD8⁺ cytotoxic T cells, there have been a few reports suggesting involvement of the cells in endometriosis. Steele et al. originally reported that T-lymphocyte-mediated cytotoxicity specifically to autologous endometrial cells was reduced in women with endometriosis and the impaired immune system allowed ectopic growth of endometriotic lesion (Steele et al. 1984). Thereafter, Ho et al. reported that CD8⁺ cells could be associated with the ectopic growth of endometriotic lesions because peritoneal IFN- γ and IL-2 levels were reduced in patients with endometriosis, and the reduced levels were recovered by gonadotropin-releasing hormone (Ho et al. 1996). However, to date, there are no reports that directly show involvement of the CD8⁺ cells in the onset/progression of endometriosis. Proliferation and cytotoxicity of the CD8⁺ cells are stimulated by IL-12 (Mehrotra et al. 1993), and a part of the cells generally expresses the IL-2R β (Tanaka et al. 1991). In this study, however, alteration in the peritoneal cell population of the CD8⁺ cells due to IL-12 or anti-IL-2R β mAb treatments was not observed both on day 3 and day 21. In addition, because the implanted endometrial tissues were obtained from syngenic mice, an acquired immune reaction by CD8⁺ cytotoxic T cells should not have occurred against the endometriotic tissues in the absence of immunization with the autologous endometrial antigen. Therefore, it was deduced that

development of the endometriotic lesions was not affected by CD8⁺ cells at least in this experiment.

In conclusion, it was strongly suggested that IL-12 suppressed development of endometriotic lesions via activation of NK cells, and that NK cells are involved in the primary defense against the development of endometriotic lesions.

Acknowledgments We thank Satoh, Asako for technical supports and valuable suggestions.

References

- Bacci M, Capobianco A, Monno A, Cottone L, Di Puppo F, Camisa B, Mariani M, Brignole C, Ponzoni M, Ferrari S, Panina-Bordignon P, Manfredi AA, Rovere-Querini P (2009) Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. *Am J Pathol* 175:547–556
- Bartosik D, Jacobs SL, Kelly LJ (1986) Endometrial tissue in peritoneal fluid. *Fertil Steril* 46:796–800
- D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf SF, Trinchieri G (1992) Production of natural killer cell stimulatory factor (Interleukin 12) by peripheral blood mononuclear cells. *J Exp Med* 176:1387–1398
- Giudice LC, Kao LC (2004) Endometriosis. *Lancet* 364:1789–1799
- Heufler C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G (1996) Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon- γ production by T helper 1 cells. *Eur J Immunol* 26:659–668
- Hill JA (1992) Immunology and endometriosis. *Fertil Steril* 58:262–264
- Ho HN, Chao KH, Chen HF, Wu MY, Yang YS, Lee TY (1995) Peritoneal natural killer cytotoxicity and CD25⁺ CD3⁺ lymphocyte subpopulation are decreased in women with stage III-IV endometriosis. *Hum Reprod* 10:2671–2675
- Ho HN, Wu MY, Chao KH, Chen CD, Chen SU, Chen HF, Yang YS (1996) Decrease in interferon gamma production and impairment of T-lymphocyte proliferation in peritoneal fluid of women with endometriosis. *Am J Obstet Gynecol* 175:1236–1241
- Ho HN, Wu MY, Chao KH, Chen CD, Chen SU, Yang YS (1997) Peritoneal interleukin-10 increases with disease in activated CD4⁺ T lymphocytes in women with endometriosis. *Hum Reprod* 12:2528–2533
- Johann S, Blümel G, Lipp M, Förster R (1995) A versatile flow cytometry-based assay for the determination of short- and long-term natural killer cell activity. *J Immunol Methods* 185:209–216
- Kikuchi Y, Ishikawa N, Hirata J, Imaizumi E, Sasa H, Nagata I (1993) Changes of peripheral blood lymphocyte subsets before and after operation of patients with endometriosis. *Acta Obstet Gynecol Scand* 72:157–161

- Mazzeo D, Viganó P, Di Blasio AM, Sinigaglia F, Vignali M, Panina-Bordignon P (1998) Interleukin-12 and its free p40 subunit regulate immune recognition of endometrial cells: potential role in endometriosis. *J Clin Endocrinol Metabol* 83:911–916
- Mehrotra PT, Wu D, Crim JA, Mostowski HS, Siegel JP (1993) Effects of IL-12 on the generation of cytotoxic activity in human CD8⁺ T lymphocytes. *J Immunol* 151:2444–2452
- Oosterlynck DJ, Cornillie FJ, Waer M, Vandeputte M, Koninckx PR (1991) Women with endometriosis show a defect in natural killer activity resulting in a decreased cytotoxicity to autologous endometrium. *Fertil Steril* 56:45–51
- Overbergh L, Giulietti A, Valckx D, Decallonne B, Bouillon R, Mathieu C (2003) The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech* 14:33–43
- Sampson JA (1927) Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 14:422–469
- Somigliana E, Viganò P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P (1999) Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Hum Reprod* 14:2944–2950
- Steele RW, Dmowski WP, Mormer DJ (1984) Immunologic aspects of human endometriosis. *Am J Reprod Immunol* 6:33–36
- Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655–669
- Szylo K, Tchorzewski H, Banasik M, Glowacka E, Lewkowicz P, Kamek-Bortosinska A (2003) The involvement of T lymphocytes in the pathogenesis of endometriotic tissues overgrowth in women with endometriosis. *Mediators Inflamm* 12:131–138
- Tanaka T, Tsudo M, Karasuyama H, Kitamura F, Kono T, Hatakeyama M, Taniguchi T, Miyasaka M (1991) A novel monoclonal antibody against murine IL-2 receptor β -chain: characterization of receptor expression in normal lymphoid cells and EL-4 cells. *J Immunol* 147:2222–2228
- Tanaka T, Kitamura F, Nagasaka Y, Kuida K, Suwa H, Miyasaka M (1993) Selective long-term elimination of natural killer cells in vivo by anti-interleukin 2 receptor β chain monoclonal antibody in mice. *J Exp Med* 178:1103–1107
- Weigent DA, Stanton GJ, Johnson HM (1983) Interleukin 2 enhances natural killer cell activity through induction of gamma interferon. *Infect Immun* 41:992–997