

Leukocyte Subpopulations in the Uteri of Leukemia Inhibitory Factor Knockout Mice During Early Pregnancy¹

Gemma Schofield³ and Susan J. Kimber²

Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

ABSTRACT

Leukemia inhibitory factor (LIF) is transiently expressed on Day (D) 1 of pregnancy by the uterine epithelium and on D4 specifically by the glandular epithelium. The *Lif* knockout female mice are infertile because of uterine defects that affect embryo implantation, but pregnancy can be rescued in these mice by injections of LIF on D4 of pregnancy. Many of the specific actions of LIF in the uterus are unknown, especially with regard to uterine cell biology. Leukocytes, such as macrophages, natural killer (NK) cells, and eosinophils, are present in the pregnant uterus and are thought to be beneficial, because alterations in their proportions can adversely affect pregnancy. Immunocytochemistry and cell counting were used to compare the distributions and dynamics of leukocyte subpopulations in wild-type and *Lif* knockout mice. The percentage of macrophages was reduced by more than half in the *Lif* knockout mice on D3 of pregnancy, and their distribution was disrupted, suggesting that LIF is a chemokine for these cells. The NK cells were detected as early as D3 of pregnancy, but the *Lif* knockout mice had double the percentage of NK cells compared to wild-type mice at this time, indicating that LIF restricts the migration of NK cells to the uterus. The *Lif* knockout mice also had significantly higher percentages of eosinophils in the outer stroma on D3, and in the midstroma on D4, of pregnancy, suggesting that LIF also may restrict eosinophil migration to the uterus. These alterations of the uterine leukocyte subpopulations in *Lif* knockout mice may disrupt pregnancy and contribute to failure of implantation.

cytokines, immunology, implantation, pregnancy, uterus

INTRODUCTION

Embryo implantation into the uterus takes place on the evening of Day (D) 4 of pregnancy and is dependent on the expression of leukemia inhibitory factor (LIF) [1–3]. Expression of LIF in the uterus is under the control of estrogen. Two transient peaks of LIF expression occur: the first on D1 of pregnancy from the uterine epithelium after ovulatory estrogen expression, and the second on D4 of pregnancy from the uterine glandular epithelium in response to nidatory estrogen [1]. The *Lif* knockout mice are infertile because of a failure of implantation, but pregnancy can be rescued by injections of LIF on D4, suggesting that

only this second peak of expression is essential for successful pregnancy [1]. The lack of LIF expression in the knockout mice has been shown to disrupt the expression of a number of molecules in the uterus, some of which have essential roles in embryo implantation and uterine stromal cell decidualization. These molecules include, for example, the secreted protein cochlin [4], the luminal epithelium-derived insulin-like growth factor regulatory protein (IGFBP3), immune response gene 1 (IRG1) [5] (although others have not demonstrated LIF dependence of IRG1 [6]), the epidermal growth factor (EGF) family-member amphiregulin [7], the homeobox transcription factor MSX1, and the WNT antagonist secreted frizzled-related protein-4 (SFRP4) [8]. Heparin-binding EGF is not expressed in the luminal epithelium, and cyclooxygenase-2 (COX-2; also known as prostaglandin-endoperoxide synthase 2 [PTGS2]) is aberrantly expressed around the blastocyst in *Lif* knockout mice [7]. Moreover, LIF seems to have a direct anti-decidualizing effect on stromal cells, as indicated by in vitro studies [9]. Mutations in *Lif* also have been postulated as the cause of some cases of infertility in women [10, 11]. However, little is known about the effect of LIF on the leukocyte subpopulations that are present during the pre- and peri-implantation periods.

During early pregnancy, an influx of leukocytes into the uterus occurs that has been likened to an inflammatory response, and although this may be partially stimulated by factors in seminal fluid [12], the expression of ovulatory estrogen also is required [13]. After implantation, the paternal antigens expressed by the embryo become exposed to the maternal blood system and, potentially, could stimulate an inflammatory response [14]. However, many of the leukocytes in the uterus during pregnancy are thought to be modified compared to those in the periphery and may even be beneficial to the developing embryo [15]. Macrophages enter the uterus in response to colony stimulating factor 1 (CSF1) expression from the luminal epithelium on D1 of pregnancy [16]. Mice lacking CSF1 expression have fewer macrophages in the uterus compared to wild-type mice, and the stromal macrophages are less mature than normal [17]. Pregnancy is affected in these mice, because they have reduced litter sizes [18]. Other cytokines are also involved in macrophage recruitment to the uterus, such as granulocyte-macrophage colony-stimulating factor (CSF2), interleukin (IL)-6, [19], and possibly, LIF.

Natural killer (NK) cells become the major leukocyte population in the uterus after implantation in both mice [20] and humans [21, 22]. Specific subsets of NK cells migrate to the uterus from the periphery using molecules such as L-selectin and α_4 -integrin and cell adhesion molecules on blood vessels that may include MADCAM-1 and VCAM-1 [23–25]. This also is true for humans, in which CD56^{BRIGHT} cells form only a small subset of NK cells in the periphery but are the major subpopulation of NK cells

¹Supported by a studentship from the Biotechnology and Biological Sciences Research Council UK and a BBSRC grant to S.J.K.

²Correspondence: Susan J. Kimber, Faculty of Life Sciences, University of Manchester, 3.239 Stopford Building, Oxford Road, Manchester M13 9PT, U.K. FAX: 0161 275 3915; e-mail: sue.kimber@man.ac.uk

³Current address: Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle upon Tyne, U.K.

Received: 9 August 2004.

First decision: 13 September 2004.

Accepted: 10 November 2004.

© 2005 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. <http://www.biolreprod.org>

in the uterus [21]. Recently, NK cells have been detected in the uterus using specific staining as early as D5 of pregnancy [26], although morphological studies have found granulated NK-like cells as early as D3.5 of pregnancy [27]. Additionally, these cells become localized to the mesometrial side of the uterus, as is typical of NK cells [20]. The NK cell-deficient mice are fertile but have disrupted decidualization that is odematous and hypocellular, and uterine blood vessel modification does not take place because of a lack of interferon- γ , which is normally produced by NK cells [28–30]. Some evidence indicates that increased numbers of NK cells are implicated in spontaneous abortion [31], but this also has been seen in women with decreased proportions of specific types of NK cells [32].

The main role of eosinophils in the human uterus is thought to be the production of matrix metalloproteinases that influence menstruation [33, 34]. Large numbers of eosinophils are found in the murine uterus on D1 of pregnancy and are located throughout the stroma, but by D3 of pregnancy, they become restricted to the myometrium and the stromal-myometrial junction [35]. The *Il5* knockout mice have decreased numbers of eosinophils, and mice overexpressing IL-5 have increased numbers of eosinophils in the uterus [36]. However, no adverse effects on pregnancy are apparent in either case.

LIF could affect leukocyte subpopulations in the uterus either directly, because macrophages and eosinophils both express the LIF receptor [37], or indirectly. If these leukocyte subpopulations are disrupted in the absence of LIF, then pregnancy may be adversely affected, and this may contribute to the infertility phenotype seen in the *Lif* knockout mice. Therefore, in the present study, we compared the uterine leukocyte subpopulations during early pregnancy in *Lif* knockout mice with those in wild-type mice to evaluate the role of LIF in influencing leukocyte distributions and dynamics in early pregnancy.

MATERIALS AND METHODS

Breeding and Genotyping of LIF Knockout Mice

Mice were maintained under conditions required by the British Home Office Licence as described previously [9]. The *Lif* knockout mice were obtained from Dr. Andrew Sharkey (Department of Pathology, University of Cambridge, Cambridge, U.K.) from a colony produced by Dani et al. [38]. The *Lif* knockout mice on an MF-1 background between 7 and 9 wk of age were placed with MF-1 males overnight and checked for vaginal plugs the following morning, and this was designated as D1 of pregnancy. Pregnancy and/or ovulation also were confirmed by either identification of embryos in the reproductive tract or identification of corpora lutea. Because *Lif* knockout females are infertile, the colony was sustained by breeding from heterozygote females and homozygote knockout males. Genotyping for identification of *Lif* knockout mice was carried out by polymerase chain reaction on DNA samples from mice after weaning using the following primer sequences: To identify the wild-type *Lif* sequence, forward-primer 5'-cgcaatgacacctccaggtctagaag and reverse-primer 3'-ggcgctaggaatggctgacactc were used to produce a 300-base pair (bp) DNA fragment, and for the *Lif* knockout sequence, the 5' primer and knockout 3'-gctgcatctgcacgagactagta were used to produce a 490-bp DNA fragment.

Pontamine Blue Injections for Identification of Implantation Sites

Tail-vein injection of 0.5 ml of 2% Pontamine blue (Sigma, Dorset, U.K.) was carried out on mice between D4 and D6 of pregnancy. Mice were killed by cervical dislocation after 5 min, and the uterine horns were dissected and inspected for sites of embryo implantation.

Cryosectioning and Immunocytochemistry

All mice were killed by cervical dislocation before dissection of the uteri. Cryosections of the uterus (thickness, 7 μ m) were taken from the uteri of wild-type and *Lif* knockout mice from D2 to D6 of pregnancy for use in immunocytochemistry. On D5 and D6 of pregnancy, implantation sites were used, which were identified by pontamine blue injection and morphology. Sections were rinsed three times in PBS (Oxoid, Hampshire, U.K.) and then blocked in 20 μ l of normal goat serum (NGS; 1:20 dilution; Sigma) for 45 min. Primary antibodies (all from BD Biosciences Pharmingen, Oxford, U.K., unless otherwise stated) were then added and incubated overnight at 4°C. Neutrophils were detected using a rat immunoglobulin (Ig) G antibody to LY6G (2.25 μ g/ml), macrophages with a rat IgG antibody to F4/80 (1 μ g/ml; Serotec, Oxford, U.K.), NK cells with a mouse IgM antibody to CD57 (1.3 μ g/ml; clone NK-1; Neomarkers, CA), B cells with rat anti-CD19 (5 μ g/ml), and T cells with rat anti-CD3 (5 μ g/ml). Negative controls were carried out using rat IgG (Jackson ImmunoResearch, distributed by Stratech Scientific, Bedfordshire, U.K.) and mouse IgM (NeoMarkers) at the same concentration as the primary antibodies. Primary antibodies were removed with six rinses of PBS, and either an anti-rat IgG secondary antibody conjugated to fluorescein isothiocyanate (FITC; Sigma) or IgM conjugated to FITC (Sigma) were used to detect the primary antibodies. Sections were incubated in the secondary antibodies for 90 min at room temperature in the dark before six rinses in PBS and mounting in Vectashield containing 1.5 μ g/ml of diaminodiphenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA).

Diaminobenzidine Staining for Eosinophils

Uterine eosinophils were detected by a staining method that detects peroxidase within these cells [35]. One diaminobenzidine (DAB) tablet and one urea tablet (Sigma) were dissolved in 5 ml of PBS, and a 20- μ l drop was placed on each section for 10 min at room temperature in a dark, humid chamber. Next, they were rinsed three times in PBS and left in PBS for 10 min, and this washing step was then repeated. The sections were counterstained with hematoxylin for approximately 30 sec before washing in water for 1 min and mounting in Vectashield containing DAPI.

Dolichos Biflorus Agglutinin Lectin Staining for NK Cells

This method of staining for NK cells is adapted from a protocol described by Paffaro et al. [26]. Uterine sections were incubated with 1% H₂O₂ for 30 min to inactivate endogenous peroxidase and then rinsed six times with PBS before blocking in 20 μ l of NGS (1:20 dilution; Sigma) for 30 min. The NGS was removed, and 20 μ l of a 6.6- μ g/ml solution of Dolichos Biflorus Agglutinin (DBA) lectin conjugated to biotin (Sigma) were added and incubated at 4°C overnight. Sections were then rinsed six times in PBS and incubated in 20 μ l of a 40- μ g/ml solution of ExtraAvidin peroxidase conjugate (Sigma) for 30 min at room temperature in the dark. This was washed off in six changes of PBS before adding 20 μ l of 1 \times DAB solution for a further 30 min and then rinsing in six changes of PBS. The sections were counterstained with hematoxylin for approximately 30 sec before washing with tap water for 1 min and mounting in Vectashield containing DAPI. Controls were carried out by substituting PBS for H₂O₂ or DBA lectin.

Toluidine Blue Staining for Mast Cells

A 20- μ l drop of acidified toluidine blue (0.5 g of toluidine blue [Sigma] and 5 ml of concentrated HCl in 95 ml of distilled H₂O) was added to uterine sections for 20 min and then rinsed off in tap water for 30 sec. Sections were then mounted in Vectashield.

Cell Counting

After immunocytochemistry or histological staining to detect different leukocyte subpopulations, cells in specific areas of uterine sections 100 \times 200 μ m² in size were counted as shown in Figure 1. Percentages of different leukocyte subpopulations were calculated for each area by counting the FITC-labeled cells and then dividing this by the total cell count obtained from counting the DAPI-stained nuclei of all the cells in that area. Leukocyte subpopulations as a percentage of total cells counted over all the areas examined also were calculated for each section. Cells that were only partially within the grid were included only if they fell on the upper or the left side, not if they fell on the lower or the right side. For each day of pregnancy, three animals were used, with three sections per animal for both wild-type and *Lif* knockout mice.

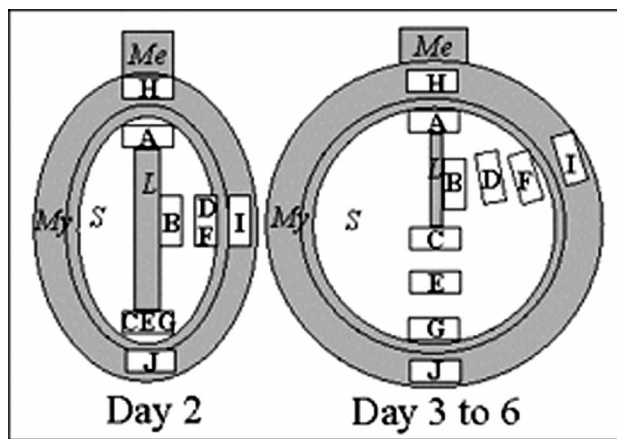


FIG. 1. The areas of transverse uterine sections used for cell counting (A–J) were chosen to cover as many different regions of the uterus as possible. The mesometrium (Me) was used for orientation, and bilateral symmetry was assumed throughout. Each area was $100 \times 200 \mu\text{m}^2$ in size. On D2 of pregnancy, the lumen (L) occupied much more space in the endometrium, and so only one area in place of C, E, and G and only one area in the place of D and F were counted. As pregnancy progressed, the size of the lumen changed. Therefore, the actual distances between the different areas altered, but the relative positions remained the same. My, Myometrium; S, stroma.

To determine whether the anti-CD57 antibody and DBA lectin used for NK cell detection recognized the same population of cells, cell counts were carried out on serial sections stained alternately with either anti-CD57 or DBA lectin and analyzed statistically. Sections also were compared visually for individual cells stained by both methods of NK cell detection.

Statistics

A repeated-measures ANOVA was used to test all data for significant differences between the wild-type mice and the *Lif* knockout mice and to see if any differences occurred between areas on different days of pregnancy. Differences were accepted as being significant at $P < 0.05$.

RESULTS

Preliminary Staining

Several leukocyte populations were analyzed by immunofluorescence in uterine sections. Neutrophils were detected from D1 of pregnancy. However, a sharp drop between D2 and D3 of pregnancy was observed (Fig. 2, A and B) and confirmed by flow cytometry, although the decrease was not as steep in the *Lif* knockout mice as in the wild-type mice (data not shown). From D3 onward, neutrophils were detected rarely.

The T and B cells were not detected by staining of uterine sections during early pregnancy in wild-type or *Lif* knockout mice (data not shown). Mast cells were detected on all the days of pregnancy analyzed (D2 to D6) by staining of uterine sections but were found only in very low numbers in the myometrium, and no obvious differences were seen between the wild-type and *Lif* knockout mice (data not shown).

Macrophages, NK cells, and eosinophils also were detected by immunofluorescence in uterine sections, and the dynamics of these cells as well as the differences between wild-type and *Lif* knockout mice were analyzed in more detail.

Distributions and Dynamics of Macrophages

Macrophages were detected in uterine sections by immunocytochemistry on all days of pregnancy analyzed (D1

to D6) in both wild-type and *Lif* knockout mice (Fig. 2, C and D). Cell counts showed that on D3 of pregnancy, 17% of the cells were macrophages in the wild-type uteri, and this was more than double the percentage in the *Lif* knockout mice ($P = 0.0003$). The areas around the lumen (areas A, B, and C; areas are as shown in Fig. 1) had the highest proportion of macrophages in the wild-type mice, but the percentages of macrophages in these areas in the *Lif* knockout mice were only one-third as high in comparison ($P = 0.013$, 0.014 , and 0.031 , respectively) (Figs. 2, C and D, and 3A). The areas in the midstroma (areas D and E) on D3 also had significantly fewer macrophages in the *Lif* knockout mice, with only half the percentage seen in the wild-type mice ($P = 0.034$ and 0.016 , respectively). A significant decrease of one-third in the total percentage of macrophages occurred in the wild-type mice between D3 and D4 ($P < 0.0001$), especially in the areas around the lumen. The stromal areas had significantly fewer macrophages than the myometrial areas on D4 of pregnancy in the wild-type mice ($P = 0.019$) (Fig. 3B). A significantly lower percentage of macrophages was found overall in the *Lif* knockout mice on D4 compared to the wild-type mice ($P = 0.016$), but differences in none of the individual areas reached significance. On D5, the areas around the lumen had the lowest proportion of macrophages in the wild-type mice, and no significant differences were found between wild-type and *Lif* knockout mice (Fig. 3C). On D6, the areas in the myometrium (areas H, I, and J) had significantly higher percentages of macrophages than the areas in the stroma ($P = 0.043$) (Fig. 3D) in the wild-type mice. The total percentage of macrophages was significantly lower in the *Lif* knockout mice compared to the wild-type mice ($P = 0.008$), as were those in areas C and I ($P = 0.038$ and 0.014 , respectively).

Distributions and Dynamics of NK Cells

NK cells were not detected in uterine sections on D1 or D2 in wild-type or *Lif* knockout mice. However, from D3 onward, they were found throughout the stroma but not the myometrium, and by D7, they showed the typical mesometrial distribution associated with these cells. This was confirmed by statistical analysis of cell counts after alternate section staining with anti-CD57 and DBA lectin to show that both methods of NK cell detection recognized the same cell population (Fig. 2, E–H).

Cell counts showed that NK cells comprised 8% of the total cell population in wild-type mice on D3 (Fig. 3E) and were located mainly on the mesometrial side of the uterus (area A), which had a significantly higher percentage of NK cells than the other areas investigated ($P = 0.009$). However, *Lif* knockout mice had a significantly higher percentage of NK cells on the antimesometrial side of the uterus (areas C, E, and G) on D3 of pregnancy compared to the wild-type mice ($P = 0.034$, 0.021 , and 0.04 , respectively) (Fig. 3E). The total percentage of NK cells in wild-type animals more than doubled, to more than 16%, on D4 of pregnancy in wild-type mice ($P = 0.04$) (Fig. 3F), especially in areas A and B next to the lumen, which more than tripled in percentage of NK cells, and these areas also were significantly different from D3 onward ($P = 0.037$ and 0.007 , respectively). The *Lif* knockout mice showed a similar increase in percentage of NK cells on D4, but area A had a significantly higher percentage than that in the wild-type mice ($P = 0.027$) (Fig. 2, G and H, and 3F). On D5, the total percentage of NK cells in the wild-type mice de-

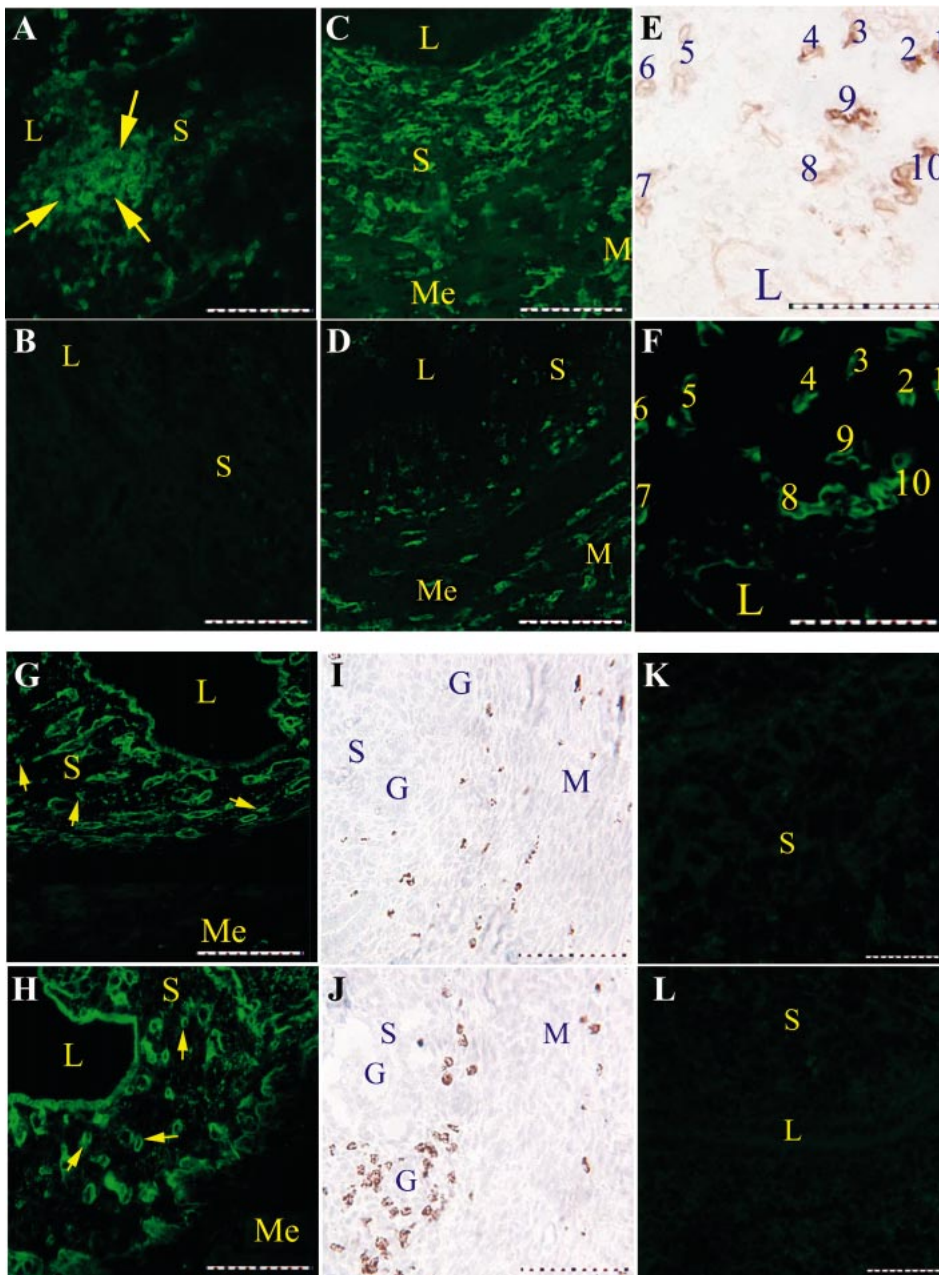


FIG. 2. Staining of uterine sections was carried out to detect different leukocyte subpopulations during early pregnancy. All images shown are of wild-type uterine sections unless stated otherwise. Neutrophils were detected from D1 to D3 of pregnancy in both wild-type and *Lif* knockout mice. Neutrophils are shown on D2 of pregnancy in a wild-type mouse (A); the arrows indicate a cluster of these cells adjacent to the lumen. No neutrophils were detected on D4 of pregnancy (B). Macrophages were detected throughout early pregnancy. Wild-type mice (C) had significantly higher percentages of macrophages in the mesometrial stroma on D3 of pregnancy compared to the *Lif* knockout mice (D). The NK cells were detected from D3 onward by both DBA lectin staining (E) and antibody staining to CD57 (F). Alternate sections from D5 of pregnancy also are shown, and some stained cells that appear in both sections are labeled 1–10 as an example. The percentage of NK cells in the mesometrial stroma on D4 of pregnancy in wild-type mice (G) was significantly higher than in the *Lif* knockout mice (H). Some NK cells are indicated by arrows. Eosinophils were detected throughout early pregnancy and were located preferentially in the myometrium and outer stroma. On D3 of pregnancy, wild-type mice (I) had fewer eosinophils in the outer stroma than the *Lif* knockout mice (J). Controls for antibody staining were consistently negative. The IgG control (K) and the IgM control (L) were both negative; D4 of pregnancy is shown. G, Gland; L, lumen; M, myometrium; Me, mesometrial; S, stroma. Bar = 100 μ m.

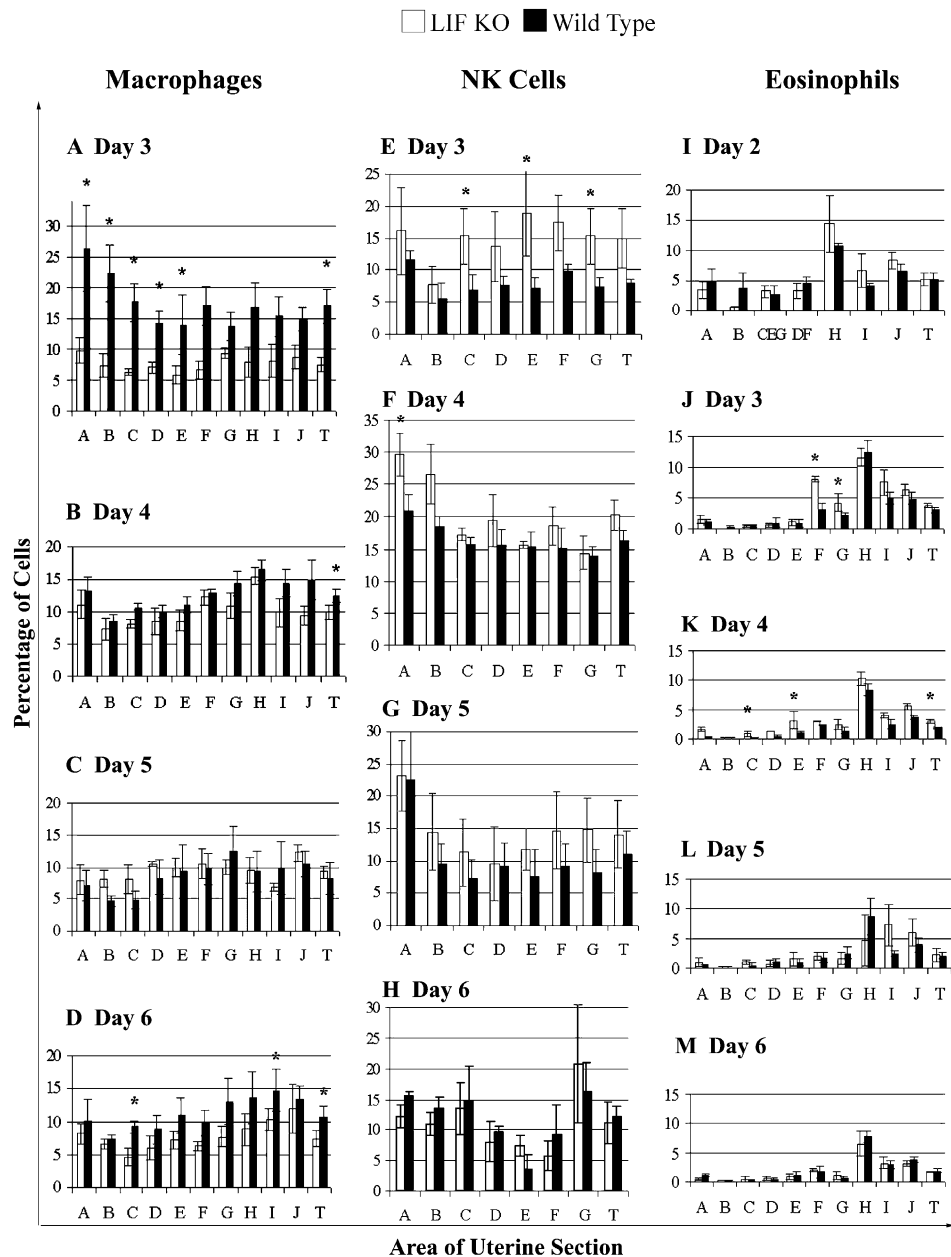
creased slightly, but they were still located mainly in the mesometrial region. Indeed, area A had at least double the percentage of NK cells compared to any other area ($P = 0.004$). The *Lif* knockout mice showed a similar decrease in the proportion of NK cells on D5, and no significant differences were found between them and the wild-type mice (Fig. 3G). On D6 in area A, the proportion of NK cells decreased to two-thirds of what it was on D4 in the wild-type mice ($P = 0.025$), but NK cells were still located mainly in the areas around the lumen (areas A, B, and C) (Fig. 3H). The midstromal region (area E) contained only 4% NK cells, which was significantly lower than the average ($P = 0.019$). No significant differences in NK cells were found between the wild-type mice and the *Lif* knockouts on D6 of pregnancy.

Distributions and Dynamics of Eosinophils

Eosinophil staining showed that these cells were present in the uterus throughout the time period investigated (D2

to D6) (Fig. 2, I and J). Cell counting revealed that on D2 of pregnancy, 5% of the uterine cells were eosinophils in wild-type mice. The highest concentration of eosinophils was in area H in the mesometrial triangle, which contained 10% eosinophils, a significantly higher percentage than found in the other areas ($P = 0.026$) (Fig. 3I). No significant differences were found in the percentage of eosinophils between the *Lif* knockout and wild-type mice on D2 of pregnancy. On D3 of pregnancy, the total number of eosinophils dropped to 3% in wild-type mice, and few were detected in the stroma. Most areas contained less than 1% eosinophils. From D3 onward, eosinophils were mainly found in the myometrium, especially in the mesometrial triangle (area H) (Fig. 3J). However, on D3 in the *Lif* knockout mice, the areas in the outer stroma (areas F and G) had at least double the percentage of eosinophils seen in the wild-type mice ($P = 0.018$ and 0.048 , respectively) (Figs. 2, I and J, and 3J). The proportion and distribution of eosinophils in wild-type mice did not alter significantly

FIG. 3. Cell counting of different leukocyte subpopulations in specific areas of the uterus (A–J and T, the total cell count per section) were carried out as described in Figure 1. Bar charts A–D show the percentages of macrophages in the uterus from D3 to D6. Major differences were seen on D3 (A), when areas A to E in the stroma as well as the total had significantly higher percentages of macrophages in the wild-type mice compared to the *Lif* knockout mice. On D4 (B) and D5 (C), differences between the wild-type mice and the *Lif* knockout mice were less apparent, but by D6 (D), areas C and I and the total had significantly higher percentages of macrophages in the wild-type mice. Bar charts E–H show the percentages of NK cells from D3 to D6. The biggest differences can be seen on D3 of pregnancy (E), when areas C, E, and G all had significantly higher percentages of NK cells in the *Lif* knockout mice compared to the wild-type mice. By D4 (F), the difference was restricted to the areas around the lumen, with area A being significantly higher in the *Lif* knockout mice. Observations at D5 (G) and D6 (H) showed no significant differences. Bar charts I–M show the percentages of eosinophils from D2 to D6. Area H in the mesometrial triangle consistently contained the highest percentage of eosinophils in both wild-type and *Lif* knockout mice throughout pregnancy. No significant differences were seen between *Lif* knockout and wild-type mice until D3 (J) of pregnancy, when areas F and G in the outer stroma were higher in the *Lif* knockout mice. On D4 (K), areas C and E in the inner stroma contained higher percentages of eosinophils in the *Lif* knockout mice, but from D5 onward (L and M), no differences were seen. Asterisks indicate significant differences between *Lif* knockout and wild-type mice.



from D4 onward; the highest percentage of eosinophils was always located in area H in the mesometrial triangle. On D4, area H had more than 8% eosinophils, which was significantly higher than the percentage in the other areas ($P < 0.0001$) (Fig. 3K). In the *Lif* knockout mice on D4 of pregnancy, areas C and E in the antimesometrial stroma had significantly higher percentages of eosinophils ($P = 0.027$ and 0.034 , respectively). The overall proportion of eosinophils was significantly higher than that for the wild-type mice ($P = 0.034$) (Fig. 3K). On D5 (Fig. 3L) and D6 (Fig. 3M), no significant differences were found between the wild-type mice and the *Lif* knockout mice.

DISCUSSION

Previous work regarding LIF has focused on its role during the peri-implantation period rather than the preimplantation period, although even during the preimplantation period, LIF clearly has several functions. Expression of LIF on D4 of pregnancy from the uterine glandular epithelium

is essential for implantation to occur [1, 2], and its absence affects the expression of a variety of other molecules in both luminal epithelium and stroma [4, 5, 7, 8]. However, little information is available regarding any consequences from lack of LIF during the preimplantation period. We focused on the leukocyte populations in the uterus, and our comparison of *Lif* knockout and wild-type mice suggests that LIF has a greater effect on leukocyte dynamics and distribution before implantation than during the implantation period. Macrophages increase in number in the uterus during early pregnancy, and CSF1 expression from the uterine epithelium on D1 is important for their recruitment to the uterus [17]. However, even in the absence of CSF1 expression, macrophages are present, suggesting that other chemokines also are able to recruit these cells to the uterus. Like CSF1, LIF is expressed from the uterine epithelium on D1 of pregnancy, and both are under the control of estrogen. The *Lif* knockout mice analyzed in the present study showed a large decrease in the number of macrophages

compared to wild-type mice, especially on D3 of pregnancy, the earliest time point investigated, and this suggests that LIF acts as a chemokine for these cells. Additionally, LIF has been shown to be a chemokine for macrophages in other tissues, such as the nervous system, where it works in conjunction with other chemokines, such as IL-6 and oncostatin M, to recruit macrophages [39]. To our knowledge, no evidence suggests an effect of LIF on general stromal cell proliferation in the early preimplantation period [1] (unpublished data), but LIF could have a selective proliferative or survival effect on uterine macrophages. However, little in the literature regarding macrophages supports this idea. Interestingly, changes in macrophage proportions can have an effect on pregnancy [40], but whether the differences shown in the present study are sufficient to affect pregnancy is not known. The distribution of macrophages in the *Lif* knockout uteri also was disrupted: The high proportion of macrophages around the lumen on D3 and then in the myometrium from D4 onward that was seen in the wild-type mice was not seen in the absence of LIF. This suggests that LIF may act locally to produce specific areas concentrated in macrophages.

The NK cells were detected in the uterus as early as D3 by both DBA lectin staining and anti-CD57 staining. Recently, DBA lectin has been shown to detect only the NK cell subset of leukocytes in the uterus and not those elsewhere in the body. Immature uterine NK cells, which previously have been difficult to identify, are also recognized by DBA lectin, although it recognizes endothelial cells as well [26]. The present study has shown that anti-CD57 also can be used to identify these cells in mice. The first appearance of NK cells on D3 of pregnancy is earlier than previously reported [26], but this may result from the more sensitive detection methods used for the present study. The detection of NK cells on D3 correlates with the rise in progesterone expression from the corpora lutea, and this hormone may influence NK cell migration. The *Lif* knockout mice had higher percentages of NK cells in several areas of the uterus on D3 of pregnancy compared with wild-type females, suggesting that the recruitment of these cells is normally restricted by LIF. One possible role of LIF may be to reduce the expression of cell adhesion molecules on blood vessels that are used by NK cells for migration into the uterus, such as MADCAM-1 and VCAM-1 [23–25]. However, this remains to be investigated. Although the proportion of NK cells was altered in the *Lif* knockout mice, the overall distribution of NK cells was similar to that in the wild-type mice. The NK cells became located preferentially at the mesometrial side of the uterus, especially around the lumen, as has been shown previously [41]. Therefore, the present study shows that LIF has a role in limiting recruitment of NK cells to the uterus, but does not affect their distribution within the uterus.

As previously shown, eosinophils become located preferentially to the myometrium and the stromal-myometrial border during early pregnancy [36, 42]. The *Lif* knockout mice had higher percentages of eosinophils in the outer stroma on D3 and in the midstroma on D4, and the total percentages also were higher than those in the wild-type mice, suggesting that LIF restricts the migration of eosinophils into the uterus. As for NK cells, LIF may restrict the expression of molecules on blood vessels that facilitate recruitment of eosinophils. However, it also is possible that LIF induces inhibitory cytokines in the uterus or represses chemoattractant molecules.

Neutrophils also were affected by the lack of LIF in the

uterus, because the decrease that was seen between D2 and D3 in the *Lif* knockout mice was not as large as that seen in the wild-type mice. This suggests that LIF may normally promote the apoptosis of neutrophils in the uterus. Although to our knowledge no previous evidence supports this action of LIF on neutrophils, it can promote apoptosis in other cells types, such as mammary epithelial cells [43].

Alterations in the proportions of leukocytes have been shown to adversely affect pregnancy. For example, decreased numbers of NK cells during early pregnancy result in acellular and odematous decidual zones [20], and increased numbers of NK cells have been associated with embryo resorption [44]. Decreased numbers of macrophages in the uterus lead to decreased litter sizes and birth weights [17]. Although mice with altered proportions of single leukocyte subpopulations remain capable of producing viable offspring, it is unknown whether the combination of decreased macrophage and increased NK cell proportions seen in the *Lif* knockout mice is enough to severely perturb pregnancy, but it could at least be a contributing factor to the infertility seen in these mice. It is interesting to note that these differences precede D4 expression of LIF and, therefore, must be a result of D1 LIF expression, which has not been attributed previously with any major functions in the uterus during pregnancy. Previous work has indicated that ovulation in *Lif* knockout mice is normal [3] (unpublished data) and that progesterone levels in plasma are equivalent to those in wild-type mice [7]. Some of the differences seen on D6 of pregnancy could be a result of decidualization in wild-type mice, because this does not occur in the *Lif* knockout mice. Other work in the laboratory has shown differences in uterine cell density and stromal cell proliferation between wild-type mice and *Lif* knockout mice during early pregnancy (unpublished data), but these differences do not correlate with the differences in the leukocyte subpopulations reported in the present study.

Future work should test whether D1 injection of LIF into *Lif* knockout mice can reverse these differences from the wild-type mice in the proportions of leukocyte subpopulations. Dose-response experiments injecting LIF into the knockout uterus also would establish if LIF is a chemokine for macrophages as well as if it can restrict NK cell and eosinophil migration into the uterus. Additionally, immunocytochemistry for endothelial cell adhesion molecules involved in NK cell migration could be carried out to see if they are increased in the uterus of *Lif* knockout mice.

ACKNOWLEDGMENTS

We thank Andrew Sharkey (Cambridge University, Cambridge, U.K.) for provision of the *Lif* knockout mice and Dr. Chris Roberts (Manchester University, Manchester, U.K.) for statistical advice. We also thank Ali Fouladi-Nashta and Nahida Nijjar (Manchester University) for their assistance with genotyping of the *Lif* knockout mice.

REFERENCES

1. Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L, Stewart CL. Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology* 2000; 141: 4365–4372.
2. Bhatt H, Brunet LJ, Stewart CL. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc Natl Acad Sci U S A* 1991; 88:11408–11412.
3. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ. Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 1992; 359:76–79.

4. Rodriguez CI, Cheng JG, Liu L, Stewart CL. Cochlin, a secreted von Willebrand factor type a domain-containing factor, is regulated by leukemia inhibitory factor in the uterus at the time of embryo implantation. *Endocrinology* 2004; 145:1410–1418.
5. Sherwin J, Freeman T, Stephens R, Kimber S, Smith A, Chambers I, Smith S, Sharkey A. Identification of genes regulated by Leukemia Inhibitory Factor in the mouse uterus at the time of implantation. *Mol Endocrinol* 2004; 18(9):2185–2195.
6. Chen B, Zhang D, Pollard JW. Progesterone regulation of the mammalian ortholog of methylcitrate dehydratase (immune response gene 1) in the uterine epithelium during implantation through the protein kinase C pathway. *Mol Endocrinol* 2003; 17:2340–2354.
7. Song H, Lim H, Das SK, Paria BC, Dey SK. Dysregulation of EGF family of growth factors and COX-2 in the uterus during the preattachment and attachment reactions of the blastocyst with the luminal epithelium correlates with implantation failure in LIF-deficient mice. *Mol Endocrinol* 2000; 14:1147–1161.
8. Daikoku T, Song H, Guo Y, Riesewijk A, Mosselman S, Das SK, Dey SK. Uterine Msx-1 and Wnt4 signaling becomes aberrant in mice with the loss of leukemia inhibitory factor or Hoxa-10: Evidence for a novel cytokine-homeobox-Wnt signaling in implantation. *Mol Endocrinol* 2004; 18(5):1238–1250.
9. Fouladi Nashta AA, Andreu CV, Nijjar N, Heath JK, Kimber SJ. Role of leukemia inhibitor factor (LIF) in decidualization of murine uterine stromal cells in vitro. *J Endocrinol* 2004; 181:477–492.
10. Giess R, Tanasescu I, Steck T, Sendtner M. Leukemia inhibitory factor gene mutations in infertile women. *Mol Hum Reprod* 1999; 5:581–586.
11. Vogiagis D, Marsh MM, Fry RC, Salamonsen LA. Leukemia inhibitory factor in human endometrium throughout the menstrual cycle. *J Endocrinol* 1996; 148:95–102.
12. Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biol Reprod* 1998; 58:1217–1225.
13. Finn CA, Pope M. Control of leukocyte infiltration into the decidualized mouse uterus. *J Endocrinol* 1986; 110:93–96.
14. Beer AE, Billingham RE. Immunobiology of mammalian reproduction. *Adv Immunol* 1971; 14:1–84.
15. Hunt JS, Petroff MG, Burnett TG. Uterine leukocytes: key players in pregnancy. *Semin Cell Dev Biol* 2000; 11(2):127–137.
16. Pollard JW, Lin EY, Zhu L. Complexity in uterine macrophage responses to cytokines in mice. *Biol Reprod* 1998; 58:1469–1475.
17. Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER. A pregnancy defect in the osteopetrotic (*op/op*) mouse demonstrates the requirement for CSF-1 in female fertility. *Dev Biol* 1991; 148:273–283.
18. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 1990; 345:442–444.
19. Robertson SA, O'Connell AC, Hudson SN, Seamark RF. Granulocyte-macrophage colony-stimulating factor (GM-CSF) targets myeloid leukocytes in the uterus during the post-mating inflammatory response in mice. *J Reprod Immunol* 2000; 46:131–154.
20. Croy BA, Chantakru S, Esadeg S, Ashkar AA, Wei Q. Decidual natural killer cells: key regulators of placental development (a review). *J Reprod Immunol* 2002; 57:151–168.
21. Trundle A, Moffett A, Koopman LA, Kopcow HD, Rybalov B, Boyson JE, Orange JS, Schatz F, Masch R, Lockwood CJ, Schachter AD, Park PJ, Strominger JL, Michou VI, Kanavaros P, Athanassiou V, Chronis GB, Stabamas S, Tsilivakos V, White CA, Johansson M, Roberts CT, Ramsay AJ, Robertson SA, Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, Croy BA, Miyazaki S, Tsuda H, Sakai M, Hori S, Sasaki Y, Futatani T, Miyawaki T, Saito S. Human uterine leukocytes and pregnancy. *Tissue Antigens* 2004; 63:1–12.
22. King A. Uterine leukocytes and decidualization. *Hum Reprod Update* 2000; 6:28–36.
23. Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC, Evans SS, Croy BA. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J Immunol* 2002; 168:22–28.
24. Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, Black GP, Ashkar AA, Kiso Y, Zhang J. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. *J Reprod Immunol* 2003; 59:175–191.
25. Kruse A, Martens N, Fernekorn U, Hallmann R, Butcher EC. Alterations in the expression of homing-associated molecules at the maternal/fetal interface during the course of pregnancy. *Biol Reprod* 2002; 66:333–345.
26. Paffaro VA, Bizinotto MC, Joazeiro PP, Yamada AT. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta* 2003; 24:479–488.
27. Stewart I, Peel S. Granulated metrial gland cells in the virgin and early pregnant mouse uterus. *J Anat* 1981; 133:535–541.
28. Greenwood JD, Minhas K, di Santo JP, Makita M, Kiso Y, Croy BA. Ultrastructural studies of implantation sites from mice deficient in uterine natural killer cells. *Placenta* 2000; 21:693–702.
29. Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S, Croy BA. Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biol Reprod* 1997; 56:169–179.
30. Ashkar AA, Croy BA. Interferon-gamma contributes to the normalcy of murine pregnancy. *Biol Reprod* 1999; 61:493–502.
31. Choudhury SR, Knapp LA. Human reproductive failure. I: immunological factors. *Hum Reprod Update* 2001; 7:113–134.
32. Quack KC, Vassiliadou N, Pudney J, Anderson DJ, Hill JA. Leukocyte activation in the decidua of chromosomally normal and abnormal fetuses from women with recurrent abortion. *Hum Reprod* 2001; 16:949–955.
33. Jeziorska M, Salamonsen LA, Woolley DE. Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biol Reprod* 1995; 53:312–320.
34. Salamonsen LA, Lathbury LJ. Endometrial leukocytes and menstruation. *Hum Reprod Update* 2000; 6:16–27.
35. Robertson SA, Mau VJ, Young IG, Matthaie KI. Uterine eosinophils and reproductive performance in interleukin 5-deficient mice. *J Reprod Fertil* 2000; 120:423–432.
36. Sferruzzi-Perri AN, Robertson SA, Dent LA. Interleukin-5 transgene expression and eosinophilia are associated with retarded mammary gland development in mice. *Biol Reprod* 2003; 69:224–233.
37. Sharkey AM, King A, Clark DE, Burrows TD, Jokhi PP, Charnock-Jones DS, Loke YW, Smith SK. Localization of leukemia inhibitory factor and its receptor in human placenta throughout pregnancy. *Biol Reprod* 1999; 60:355–364.
38. Dani C, Chambers I, Johnstone S, Robertson M, Ebrahimi B, Saito M, Taga T, Li M, Burdon T, Nichols J, Smith A. Paracrine induction of stem cell renewal by LIF-deficient cells: a new ES cell regulatory pathway. *Dev Biol* 1998; 203:149–162.
39. Tofaris GK, Patterson PH, Jessen KR, Mirsky R. Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. *J Neurosci* 2002; 22:6696–6703.
40. Robertson SA, Roberts CT, Farr KL, Dunn AR, Seamark RF. Fertility impairment in granulocyte-macrophage colony-stimulating factor-deficient mice. *Biol Reprod* 1999; 60:251–261.
41. Chantakru S, Kuziel WA, Maeda N, Croy BA. A study on the density and distribution of uterine Natural Killer cells at mid pregnancy in mice genetically-ablated for CCR2, CCR 5 and the CCR5 receptor ligand, MIP-1 α . *J Reprod Immunol* 2001; 49:33–47.
42. Wang D, Ishimura R, Walia DS, Muller H, Dai G, Hunt JS, Lee NA, Lee JJ, Soares MJ. Eosinophils are cellular targets of the novel uteroplacental heparin-binding cytokine decidual/trophoblast prolactin-related protein. *J Endocrinol* 2000; 167:15–28.
43. Kritikou EA, Sharkey A, Abell K, Came PJ, Anderson E, Clarkson RW, Watson CJ. A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development* 2003; 130:3459–3468.
44. Lala PK, Scodras JM, Graham CH, Lysiak JJ, Parhar RS. Activation of maternal killer cells in the pregnant uterus with chronic indomethacin therapy, IL-2 therapy, or a combination therapy is associated with embryonic demise. *Cell Immunol* 1990; 127:368–381.