Studies on the relation between the immune system and the reproductive system, i.e. ovarian function, are important for several reasons. First of all, immune responses regulate various reproductive functions, such as ovulation and menstruation, and deviations from normal immune responses may interfere with these processes. Secondly, immune responses vary with gender and the female reproductive phase, suggesting that factors associated with reproduction regulate immune responses. This was the focus of the present thesis. Data of animal studies and in vitro experiments on the humoral immune response are in general conclusive; estrogens (17β-estradiol) induce (auto) antibody production, whereas androgens (testosterone) have a suppressive effect on the production of antibodies. Although present evidence points towards an important role for 17β-estradiol and testosterone in antibody production, review of literature, as described in Chapter 1, on the cellular immune response at different reproductive phases and the effects of sex hormones on the various immune cells in vitro and in vivo are scarce and conflicting. The conflicting results between a number of in vivo experiments as well as various in vitro experiments may partly be explained by different experimental methods used; e.g. differences in parameters measured; differences in stimuli to activate the cells used and differences in isolation techniques of white blood cells.

The aim of the present thesis was therefore to rule out these differences and to set up a series of standardized experiments so that we were able to compare data collected in different reproductive conditions. We measured cytokine productive capacity of lymphocytes and monocytes, as a parameter of the immune response, in the follicular and the luteal phase, in oral contraceptive users and in males. The experimental set up was identical in all reproductive phases studied.

In the first part of this thesis we evaluated the percentage cytokine producing lymphocytes (Chapter 2), monocytes (Chapter 3) and natural killer (NK) cells (Chapter 4) in the follicular and the luteal phase of the ovarian cycle in humans and the effect of gender on percentage cytokine producing lymphocytes and monocytes (Chapter 6). In the second part of the thesis we studied the effect of progesterone in vivo (Chapter 5) and in vitro (Chapter 7).
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monocytes (Chapter 5).
In the second part of this thesis we investigated the influence of 17β-estradiol and progesterone in vitro on the percentage cytokine producing lymphocytes (Chapter 6) and in vitro and in vivo on the percentage cytokine producing monocytes (Chapter 7).

Cytokine production by lymphocytes and NK cells:
One of the major advances in our understanding of the regulation of immune responses has been the description of the T helper 1(Th1)/T helper 2 (Th2) paradigm1. Th1 and Th2 cytokine patterns have been implicated in various immune responses concerning infection, allergy and autoimmunity1. Although at present other cytokine subsets have been described, i.e. Th0 and Th3, in our studies we focused on Th1 and Th2.

In Chapter 2 we demonstrated a shift towards a Th2-type response in the luteal phase of the ovarian cycle compared to the follicular phase. This shift towards a Th2-type response was due to an increased percentage IL-4 producing lymphocytes after stimulation in the luteal phase. Since in the luteal phase 17β-estradiol and progesterone concentrations are higher as compared to the follicular phase, it is a logical assumption that these hormones might be able to induce an increase in percentage IL-4 producing lymphocytes. Therefore we set up an in vitro experiment to investigate the effects of these sex steroids on the cytokine productive capacity of lymphocytes. In Chapter 6 we presented data on the percentage IL-4 producing T-helper- and T-cytotoxic male and postmenopausal lymphocytes after incubation with different concentrations of 17β-estradiol and progesterone. These studies demonstrated no effect of either 17β-estradiol or progesterone on the percentage IL-4 producing lymphocytes of males and postmenopausal females.
A possible explanation for the discrepancy between in vivo and in vitro effects of sex hormones on lymphocyte IL-4 production might be that the in vivo effect is not a direct effect of the sex steroids on the immune cells, but is due to effects
of sex hormones on cells, which are not present in the in vitro setting. It has for instance been shown that a lot of other hormone-sensitive cells in the body produce cytokines, for instance trophoblast cells and endometrial stromal and epithelial cells. Another possible explanation is that the in vivo effect, increased cytokine productive capacity, is not an effect of sex steroids but is caused by other factors. It is for example possible that during the follicular phase an immunosuppressive factor is produced, inhibiting lymphocyte cytokine production in the follicular phase (see also below “Cytokine production by monocytes”).

In Chapter 5 we presented data on the effects of gender on cytokine productive capacity of lymphocytes. Together with a decrease in CD3⁺ cells (T-lymphocytes), a decrease in the percentage IL-2 producing lymphocytes after stimulation (i.e. type-1 cytokine) was found in men as compared with women, while no difference in IL-4, IL-10 and IFN-γ producing lymphocytes was found. Further in vitro studies from our lab demonstrated that testosterone did not affect IL-2 production of lymphocytes in vitro, suggesting no direct effect of testosterone on in vivo IL-2 production. The fact that IL-4, IL-10 and IFN-γ production did not differ between women in the follicular phase and men may indicate that testosterone does not affect lymphocyte production of these cytokines. Moreover, it is also not in line with a suppression of IL-4 in the follicular phase.

Further studies are needed to evaluate the exact relation between sex steroids and lymphocyte cytokine production; for instance, evaluation of lymphocyte cytokine production in other reproductive conditions, such as oral contraceptive users and postmenopausal women. Also, in vitro studies in which lymphocytes are incubated with more than one sex steroid can give insights into synergistic effects of the various hormones on lymphocyte cytokine production. Moreover, in the present thesis we evaluated only one parameter of the specific immune response, lymphocyte cytokine production after polyclonal stimulation. Although cytokines are important mediators of immune responses, they are not the only factors determining the nature and intensity of immune responses. Further studies are therefore needed to evaluate other parameters, such as toxicity or T-cell proliferation.

As NK-cells in the blastocyst and in the endometrium are sensitive to progesterone one could hypothesize that progesterone has a suppressive effect on NK cell cytokine production. In Chapter 4 we demonstrated that progesterone has a suppressive effect on IL-2 production of monocytes in vitro. Our experiments were performed under the same culture conditions, as described in the present thesis.

Cytokine production by monocytes

In Chapter 4 we demonstrated that monocytes produce the type-1 cytokine TNF-α and the type-2 cytokine IL-10. Our experiments were performed under the same culture conditions as described in the present thesis. Although the cytokine production of monocytes in vitro is not the same as in vivo, we believe that the results are valuable for understanding the role of cytokines in immune responses.

In Chapter 3 we presented data on the effects of gender on cytokine productive capacity of lymphocytes. Together with a decrease in CD3⁺ cells (T-lymphocytes), a decrease in the percentage IL-2 producing lymphocytes after stimulation (i.e. type-1 cytokine) was found in men as compared with women, while no difference in IL-4, IL-10 and IFN-γ producing lymphocytes was found. Further in vitro studies from our lab demonstrated that testosterone did not affect IL-2 production of lymphocytes in vitro, suggesting no direct effect of testosterone on in vivo IL-2 production. The fact that IL-4, IL-10 and IFN-γ production did not differ between women in the follicular phase and men may indicate that testosterone does not affect lymphocyte production of these cytokines. Moreover, it is also not in line with a suppression of IL-4 in the follicular phase.

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In vitro setting. It has for example cytokine productive cells in the body caused by increased production of cytokines but is caused by the follicular phase an increase cytokine production (when stimulated by monocytes). Cytokine production of cytokine productive cells (T-lymphocytes), after stimulation (i.e. when, while no difference was found. Further in vitro experiments did not affect IL-2 production. The effect of testosterone on TNF-α production did not differ between sex steroids. Moreover, in which lymphocytes may indicate that these cytokines. Moreover, the follicular phase.

In Chapter 3 we presented the data of the percentage cytokine producing monocytes within the ovarian cycle. The percentages of TNF-α and of IL-1β producing monocytes after stimulation were increased in the luteal phase as compared with the follicular phase. Since a concomitant increase in 17β-estradiol and progesterone plasma concentration in luteal phase might cause this studies are therefore also needed to investigate the effects of sex hormones on other parameters of the specific immune response, such as cell-mediated cytotoxicity or T-cell proliferation.

As NK-cells in the endometrium, play an important role in implantation of the blastocyst and in placentation we studied a possible effect of 17β-estradiol and progesterone on NK cell cytokine production. We investigated whether peripheral NK cell cytokine productive capacity differed within the ovarian cycle. In Chapter 4 we demonstrated that there was no difference in this capacity between the follicular and the luteal phase of the ovarian cycle in humans, suggesting no effect of ovarian factors on NK cell cytokine productive capacity. Therefore no experiments were set up to evaluate the in vitro effect of sex steroids on NK-cell cytokine production.

Cytokine production by monocytes:
In the past, the effects of gender and reproductive phase upon the specific immune response have gained much more attention than the effects on the non-specific immune response. However, a review of the literature (Chapter 1) and our experiments indicate that the effects of gender and the reproductive condition on the non-specific immune response are more obvious. This is not surprising, since it is the non-specific immune response that is involved in various reproductive processes, such as ovulation and menstruation. It is therefore much more important for the ovaries to regulate the non-specific immune response than the specific immune response.

In Chapter 3 we presented the data of the percentage cytokine producing monocytes within the ovarian cycle. The percentages of TNF-α and of IL-1β producing monocytes after stimulation were increased in the luteal phase as compared with the follicular phase. Since a concomitant increase in 17β-estradiol and progesterone plasma concentration in luteal phase might cause this
increase in TNF-α and IL-1β production, we investigated the direct effects of progesterone and 17β-estradiol on monocyte cytokine productive capacity in vitro. In Chapter 6, we demonstrated that neither 17β-estradiol nor progesterone did influence the cytokine productive capacity of monocytes in vitro. A possible explanation for the difference between the in vivo and in vitro effects of sex steroids on the production of TNF-α and IL-1β production might be that this effect is not due to direct effects of the sex steroids on the immune cells, but may be due to effects on cells, which are not present in the in vitro situation.

However, in the same study, we also evaluated the correlation between endotoxin-stimulated TNF-α and IL-1β production and progesterone or 17β-estradiol concentrations in the luteal phase. We did not find a correlation between the concentration of sex hormones and cytokine production. Therefore, also an indirect effect of sex hormones on monocyte cytokine production is not very likely. Moreover, in women on oral contraceptive pills, endotoxin-stimulated monocyte TNF-α and IL-1β production was not different between pill intake (with high (synthetic) estrogen and progesterone) and the pill withdrawal week (low (synthetic) estrogen and progesterone). This suggests that, as with natural sex steroids, also synthetic sex steroids do not affect monocyte cytokine production.

In line with this lack of stimulating effect of female sex steroids on monocyte cytokine production are the data presented in Chapter 5. The percentage of endotoxin-stimulated TNF-α- and IL-1β producing monocytes are higher in males, in which progesterone and 17β-estradiol are very low, as compared to females in the follicular phase.

Although the percentage endotoxin-stimulated IL-12 producing monocytes did not vary in the menstrual cycle, the percentage IL-12 producing monocytes was higher in males as compared to females in the follicular phase. This might be the effect of testosterone, as a recent study of our research group demonstrated an increase in percentage IL-12 producing monocytes after incubation of female whole blood with endotoxin and physiological levels of testosterone6. Although in this experiment testosterone did affect IL-1β- it did not affect TNF-α- cytokine productive capacity. Sex steroids must be involved in the regulation of monocyte cytokine production. A possible mechanism can be that sex steroids are involved in the negative feedback loop for monocyte TNF-α and IL-1β production. This would mean that sex steroids influence TNF-α and IL-1β production as a result of their effect on the sex steroid concentration in the luteal phase.

### Table 1:

<table>
<thead>
<tr>
<th>Monocyte IL-1β, TNF-α, IL-12, IL-18 productive capacity</th>
<th>Follicular phase (F)</th>
<th>Luteal phase (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>17β-E</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>FG</td>
<td>+</td>
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</tbody>
</table>

* high synthetic estrogen

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the direct effects of procreative capacity in vivo nor progesterone effect in vitro. A possible explanation for in vitro effects of sex steroids might be that this effect might be on immune cells, but may be missing in vivo situation.

A correlation between endotoxin-stimulated or 17β-estradiol on cytokine production. Therefore, also an in vitro and production is not very high. Endotoxin-stimulated cytokine production between pill intake (with withdrawal week (low steroid, as with natural sex steroid on monocyte cytokine production. The percentage of monocytes are higher in males, compared to females in producing monocytes did producing monocytes was lower. This might be the group demonstrated an incubation of female steroid. Although in affect TNF-α cytokine

<table>
<thead>
<tr>
<th></th>
<th>Foll</th>
<th>Lut</th>
<th>Male</th>
<th>OC-stop</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1-β</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-12</td>
<td>=</td>
<td>=</td>
<td>+</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>17β-E</td>
<td>=</td>
<td>+</td>
<td>-</td>
<td>=</td>
<td>*</td>
</tr>
<tr>
<td>P</td>
<td>=</td>
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<td>T</td>
<td>=</td>
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<td>=</td>
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</tr>
<tr>
<td>FG</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</table>

Table 1: Monocyte IL-1β, TNF-α and IL-12 production at different reproductive conditions. (Foll = follicular phase, Lut = luteal phase, Male = male, OC-stop = pill free period in oral contraceptive use, OC = oral contraceptive use, 17β-E / P and T are the 17β-estradiol / progesterone and testosterone concentration and FG = the existence of follicular growth during this reproductive phase; + or = or - are more or equal or less as compared to the follicular phase.

* high synthetic estrogen- and progesterone concentration.
Also our pilot experiments, in which we used whole blood of postmenopausal women, corroborate this hypothesis. This reproductive phase lacks all ovarian activity, i.e. there is absence of follicular growth and no sex hormone production. We found that the percentage of TNF-α and of IL-1β producing monocytes after stimulation was increased in these women as compared to the women in the follicular phase and similar to all other reproductive conditions tested. This increase in cytokine productive capacity can therefore not be explained by increased sex hormone concentrations. However, it can be explained by the absence of inhibiting factors produced by developing ovarian follicles. Moreover, a series of animal experiments from our laboratory also demonstrated that the in vivo endotoxin-induced inflammatory reaction is inhibited only in the follicular phase of the ovarian cycle in the rat. These experiments indicate that also in the rat, anti-inflammatory factors are produced during the follicular phase. These in vivo animal experiments also show that measuring monocyte cytokine production in vitro seems to reflect the nature and intensity of the inflammatory response in vivo. It should be noted, however, that sex hormones may be able to affect other parameters (for instance chemotaxis) or cells (for instance granulocytes) of the inflammatory response. In this respect it is interesting as to note, as described in Chapter 1, that progesterone has pro-inflammatory and estrogen anti-inflammatory effects on neutrophils.

To further establish this inhibiting effect of the follicular phase on monocyte cytokine production, we did the following experiment: we evaluated the percentage TNF-α producing monocytes in different reproductive conditions, i.e. follicular- and luteal phase, men and oral contraceptive users during pill intake, after in vitro stimulation of whole blood with increasing concentrations of endotoxin. The results are presented in Fig 1. As can be seen, the maximum percentage of TNF-α producing monocyte is significantly decreased during the follicular phase as compared to all other reproductive conditions. These experiments confirm the experiments in this thesis and are in line with the concept of an anti-inflammatory factor produced in the follicular phase of the ovarian cycle.

Figure 1:
Percentage TNF-α producing monocytes (n=5), of females using oral contraceptives, with endotoxin concentrations of reproductive conditions (Mann-Whitney-U-test).

These anti-inflammatory factors are produced during the follicular phase, since their primary function is to inhibit the inflammatory response in vivo. Therefore it can be concluded that in vivo the follicular phase acts as a factor the ovarian cycle. This is subject of future research.
The period of postmenopausal phase lacks all ovarian hormone production. Stimulating monocytes after stimulation to the women in the conditions tested. This cannot be explained by the follicular follicles. Moreover, demonstrated that the inflammatory cytokines indicate that also in the follicular phase. These finding monocyte cytokine activity of the inflammatory hormones may be able to pro-inflammatory cells (for instance it is interesting as to pro-inflammatory and pro-inflammatory factors might be produced by granulosa cells in the follicle wall; these cells not only have access to the peripheral circulation, but since their primary goal is to produce substances which are important for oocyte maturation, they have access to the inner space of the follicles. Preceding the process of ovulation, monocytes play an important role in the weakening of the follicle wall, a process which shows all signs of inflammatory reaction. Therefore it can also be argued that by producing an anti-inflammatory factor in the follicular phase, the ovaries control the timing of ovulation: by producing this factor the ovarian follicles inhibit the weakening of the follicle wall by monocytes before oocyte development is complete. Further research to identify this anti-inflammatory factor(s) is not only of scientific but also of clinical importance. This is subject of present studies in our research group.
Although the experiments described in this thesis do not unravel the complete regulation of immune responses by the reproductive condition and sex steroids, they show that for successful unravelling of this regulation, we should standardize our experiments in order to compare the various reproductive conditions. This thesis shows that only by comparing all reproductive conditions, it can be concluded that the cytokine productive capacity of monocytes (after in vitro stimulation) in follicular phase is different from all other reproductive phases tested. Thus, in trying to find the effect of reproduction on immune responses we should not only focus on the effect of sex steroids on immune responses, but we should also direct our focus towards the anti-inflammatory factor produced during the follicular phase and possibly other, yet unknown factors. Moreover, we should also focus on the effects of the reproductive condition and sex steroids on other parameters of the immune response.

Reference List

1. Mosmann TR, helper T cell secreted protein
4. Fukuda J, Nas production of Fertil.Steril. 20
6. King A: Uterine
7. Faas MM, Bakk the low-dose e Am.J.Reprod.Ir
10. Brannstrom M 2002;57:47-60
11. Gerard N, Caill and female reg
unravel the complete tion and sex steroids, we should standardize reductive conditions. This conditions, it can be cells (after in vitro reproductive phases immune responses we cytokine responses, but we immunity factor produced factors. Moreover, we tion and sex steroids on

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