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GENERAL GYNECOLOGY

Dose–response effect of interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and interferon-γ on the in vitro production of epithelial neutrophil activating peptide-78 (ENA-78), IL-8, and IL-6 by human endometrial stromal cells

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Abstract
Purpose The production of epithelial neutrophil activating peptide-78 (NA-78) and the interleukins IL-8 and IL-6 by endometrial stromal cells is stimulated by pro-inflammatory interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α). IL-8 is suggested to play a role in the pathogenesis of endometriosis, and in these women the peritoneal fluid concentrations of ENA-78 and IL-8 are increased. TNF-α has been tested together with interferon-γ because of their cooperative stimulation of IL-6. The release of IL-8, however, is inhibited with increasing interferon levels. The aim of the study was the analysis of the production of ENA-78, IL-6 and IL-8 by cultured human endometrial stromal cells in the presence of varying concentrations of IL-1β, TNF-α, and interferon-γ.
Methods Eutopic endometrial tissue was obtained from seven cycling, endometriosis-free women undergoing laparoscopy for reasons of infertility or pain. The release of ENA-78, IL-8 and IL-6 by the isolated and monolayer cultured stromal cell fraction in the presence of IL-1β (0.08 to 50 ng/mL), TNF-α, and interferon-γ (both 20 to 500 ng/mL) was determined.
Results IL-1β stimulated the production of IL-8, IL-6, and ENA-78 dose dependently from 0.08 to 2.0 ng/mL (ENA-78) or to 10 ng/mL (IL-8, IL-6); at 50 ng/mL a decrease in release was observed for IL-8 and IL-6. TNF-α stimulation yielded a plateau between 20 and 100 ng/mL. Interferon-γ stimulated IL-6 and inhibited IL-8 production above 20 ng/mL. ENA-78 release was largely unaffected by interferon-γ.
Conclusions IL-1β and TNF-α stimulate stromal cytokine production cumulatively with different dose–response curves. The presence of interferon-γ has opposite effects on IL-8 and IL-6. TNF-α and interferon-γ should be investigated separately in future in vitro studies with endometrial cells and explants.

Keywords Endometrium · Cytokine stimulation · Epithelial neutrophil activating peptide-78 · Interleukins · Interferon

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Introduction

Epithelial neutrophil activating peptide-78 (ENA-78) and interleukin-8 (IL-8) are structurally and functionally related chemokines of the C-X-C type and for which angiogenic properties have been demonstrated [1]. The production of ENA-78 is stimulated by the addition of the pro-inflammatory cytokine IL-1 not only in monocytes and neutrophils [2], but also in stromal cells isolated from human endometrium [3]. Tumour necrosis factor-α (TNF-α), another proinflammatory cytokine, also stimulates ENA-78

in endometrial stromal cells [3] and TNF-α neutralising antibodies inhibit the expression of ENA-78 in eosinophils [4]. These results suggested an upregulation of ENA-78 and IL-8 in the inflammatory state, an observation which is clinically relevant in endometriosis. This pathology is characterised by endometrial tissue present outside the uterus and responding to hormonal and inflammatory stimulation, and which often results in dysmenorrhoea, debilitating pain and thus in a significant reduction in the quality of life [5]. Endometriosis is a chronic, oestrogen-dependent disease affecting approximately 10% of women during reproductive age. IL-6 and IL-8 have been suggested to play a role in the pathogenesis of the disease [6–8], and we as well as other investigators have found increased concentrations of ENA-78 [9, 10], IL-6 [11–13], and IL-8 [7, 14] in the peritoneal fluid of women with endometriosis.

Monolayer cultured eutopic endometrial stromal cells increased their output of IL-8 and ENA-78 by up to 500-fold in the presence of added IL-1β [3], and we were able to confirm this finding not only for stromal cells but also for the epithelial compartment—although to a less pronounced extent [15]. Such stimulation was similarly confirmed in the presence of TNF-α either added alone [16] or in combination with interferon-γ [15]. The two molecules were used together because of their cooperative stimulation of monocyte chemoattractant protein-1, macrophage colony stimulating factor, RANTES and IL-6 production in monocytes. In endometrial stromal cells, interferon-γ administered alone has similarly been shown to stimulate the production of IL-6 and several other cytokines in a dose-dependent manner, while the release of IL-8 was progressively inhibited by increasing interferon levels [17]. The role of interferon-γ (IFN-γ) on stimulated and non-stimulated endometrial stromal cells therefore remains unclear. An anti-proliferative action of this cytokine on epithelial cells has, however, been shown [18]. In the endometrium, IFN-γ is produced by natural killer cells [19]; it plays a role in angiogenesis and endometrial tissue modelling [19], and favours the establishment and maintenance of a successful pregnancy [17].

The aim of this study was to find out whether it was indeed necessary to investigate the effects of TNF-α and interferon-γ on endometrial stromal cells separately, and to compare them with IL-1β mediated effects which have been shown to be more pronounced [3, 15]. We have thus decided to construct dose–response curves as a function of different concentrations of IL-1β, TNF-α and interferon-γ, administered either individually or in combination, to cultured stromal cells. We were able to show different stimulation or inhibition response patterns for IL-6, IL-8, and ENA-78.

Materials and methods

Endometrial tissue was biopsied with a soft suction curette (Pipelle-de-Cornier®, Laboratoire CCD, Paris, France) in the proliferative phase from pre-menopausal women undergoing laparoscopic investigations in our clinic. The study protocol was approved by the Ethical Committee of the University of Berne. Due to the large number of required cells, replicate cultures to set up, and measurements planned in this preliminary in vitro experiment, biopsied tissue samples from three of the seven patients were pooled prior to purification. For the same reason (amount of biological material required), it was not attempted to perform this experiment with purified epithelial endometrial cells.

In order to provide a homogeneous group of replicate tissue sample the absence of endometriosis was ascertained histologically and no reported gynaecological pathology, except the presence of abdominal pain, was allowed to be present. Also, as a large amount of biological material was required, the study was restricted to stromal cells which are easy to grow and to passage in monolayer cultures. As a consequence, a total of four experiments of isolation and cultures were performed (see next paragraph). Stromal cells were prepared according to Ryan et al. [20] in a protocol described recently [15] based on collagenase digestion and stepwise sieving down to 40 μm. Cultures were prepared by the dispersion of the cells in complete IMDM medium (Iscove's modified Dulbecco's medium) containing Hepes (25 mM), stabilised glutamine (Glutamax®), foetal bovine serum (10% v/v), penicillin, streptomycin, and fungizone (all from Gibco-Invitrogen, Paisley, Scotland) in 75 cm^2 culture flasks, and the medium (12.5 mL) was changed after 30 min of incubation to remove dead material, residual red blood and other non-attaching (including deteriorated stromal) cells. The attached endometrial stromal cells were grown to confluence at 37°C under 5% CO_2 in air in complete IMDM medium and passaged twice before starting the cytokine stimulation experiment. Then the cells were harvested by trypsinisation, and distributed into four 48-well (Nunclon Surface™, Roskilde, Denmark) culture plates (20,000 cells in 1.0 mL medium per well) in complete IMDM medium until they reached 80% of confluence.

The biopsied material obtained from the first three patients was pooled prior to the isolation of endometrial stromal cells, and duplicate culture wells were set up with this population. Preparations from the next four patients were set up individually but singly, which thus resulted in six culture wells per cytokine concentration combination. The following cytokines, all purchased from R&D Systems, Abingdon, Oxford, England, were introduced to the following final concentrations: recombinant human

IL-1β, Cat. No. 201-LB, 0, 0.08, 0.4, 2.0, 10, 50 ng/mL; recombinant human INF-α, Cat. No. 210-TA, 0, 20, 100, 500 ng/mL; recombinant human IFN-γ, Cat. No. 285-IF, 0, 20, 100, 500 ng/mL. Introducing all possible combinations of these concentrations, a total of 576 culture wells had been set up in this study. After 24 h of culture the supernatants were removed, centrifuged, and individually stored at −30°C until IL-6, IL-8 and ENA-78 protein concentrations were determined batchwise in these media.

ENA-78 and IL-8 concentrations in the conditioned media were determined by microplate ELISA as reported previously [15]. The assay for IL-6 was developed as it had been done for IL-8 using "matched pair" antibodies [21]; capture and biotinylated detection antibody concentrations for IL-6 were 2.0 and 0.25 µg/mL. All reagents (antibodies and recombinant standards) were obtained from R&D Systems, and the range of the standard curve for IL-6 was 12.5–800 pg/mL in serial 1:2 dilution steps. Culture supernatants were diluted between 1:2 and 1:500 in Blotto® (Pierce, USA; Socochim, Lausanne, Switzerland). All other assay parameters and conditions were unchanged from the protocol published for IL-8 [21]. Cytotoxicity of the culture conditions after 24 h was assessed by the determination of lactate dehydrogenase released (LDH) into the medium through loss of the membrane barrier function, using the reagent kit obtained from Roche (Rotkreuz, Switzerland, Cat. No. 47449 26001) on microplates. Culture media were diluted 1:2 in serum-free IMDM prior to assay. The protocol supplied by the manufacturer was followed with a 30 min incubation at 30°C, and the signal was normalised against blank medium containing 5% (v/v) foetal bovine serum from the same batch as the one used in culture. This had to be done due to the considerable but varying amounts of lactate dehydrogenase present in FCS.

Cyto- and chemokine (ENA-78, IL-6, IL-8) production data were plotted firstly as raw concentrations (in ng/mL or pg/mL), as measured in the cultures run with tissue pooled from three patients, in three-dimensional graphs as a function of the concentrations of two of the three stimulators/inhibitors (IL-1β, TNF-α, IFN-γ) at a time. 3D graphs were created using Microsoft Excel®. In this series each data column corresponded to a duplicate ELISA measurement from duplicate culture wells. For the combined analysis of the four separate cultures from the seven women, the readings were normalised with respect to the lowest IL-1β concentration added (0.08 ng/mL), and standard deviations were calculated with Prism® software (Graphpad, San Diego, CA, USA). The cultures run in the total absence of IL-1β and TNF-α could not be used for normalisation since ENA-78 and IL-8 was not detected under these conditions (division by zero). For this reason, no cross-sectional or grouped statistical analysis could be performed and it was decided to present the results in three-dimensional dose–response bar graphs as a function of the increasing concentrations of the stimulators/inhibitors added.

Results

The production of IL-8, IL-6, and ENA-78 by cultured endometrial stromal cells was stimulated by the presence of IL-1β at 0.08 ng/mL and higher, and by TNF-α at 20 ng/mL or higher. This effect was dose dependent and cumulative between IL-1β and TNF-α for all three endpoint markers (Fig. 1), and cumulative between IL-1β and IFN-γ for IL-6 only (at least up to 100 ng/mL IFN-γ, Fig. 2b). In Figs 1 and 2, each data column represents the mean of either four (IL-8, IL-6) or three (ENA-78) single cultures with cells from different patients. A non-linear, "hook" pattern was observed for IL-8 release, with considerably lower output of this chemokine in the presence of IL-1β at 50 when compared to 10 ng/mL and when TNF-α was present (Fig. 1a). This pattern was also seen for IL-6, though to a less pronounced extent (Fig. 1b). ENA-78 production, on the other hand, continued to rise with increasing IL-1β present in the medium, though we did not observe a difference in ENA-78 output between 2.0 and 10 ng/mL IL-1β (Fig. 1c) in normalised means over four culture experiments involving tissue from seven women. On the other hand, this "hook" pattern (lower production in presence of 50 when compared to 10 ng/mL of IL-1β) was observed for all three endpoint markers (IL-8, IL-6 and ENA-78) with increasing levels of interferon-γ in the absence of TNF-α (Fig. 2). When added to an in vitro concentration of 20 ng/mL or higher in the absence of IL-1β, IFN-γ inhibited the production of IL-8 (Fig. 3a) but stimulated the output of IL-6 and ENA-78 into the medium (Fig. 3b,c).

The cytotoxicity (LDH activity released into the medium) determined in the supernatants after culture did not vary between conditions as a function of the concentration (from absent to very high) of any of the cytokines (IL-1β, TNF-α, or IFN-γ) added to the medium (data not shown). Serum-free, blank media supplemented with cytokines at the highest concentration yielded an LDH reading below the detection limit of the assay.

Discussion

The presence of IL-8, IL-6, and ENA-78 in the human endometrium has been demonstrated immunohistochemically [3, 22, 23]; but while the two interleukins were predominantly (but not exclusively) located in the glandular epithelium [22, 23], ENA-78 was found mainly in

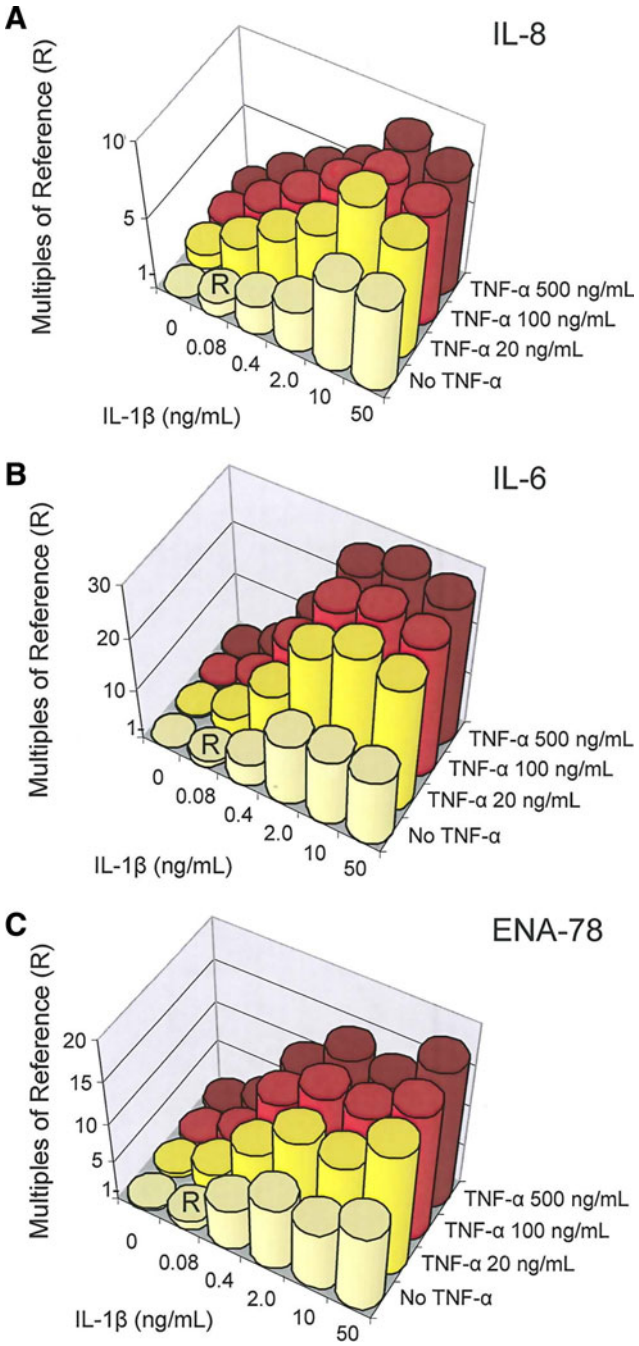

Fig. 1 Release of IL-8 (**a**), IL-6 (**b**), and ENA-78 (**c**), into the medium by endometrial stromal cells cultured for 24 h in the presence of IL-1β (0–50 ng/mL in fivefold dilution steps) and TNF-α (0, 20, 100, 500 ng/mL). No interferon was present in these cultures. Values were obtained from one duplicate well culture with cells obtained after extraction of pooled endometrial tissue from three patients plus four single well cultures with material extracted from individually extracted biopsies obtained in four individual women. Normalisation in each graph was set at 1.00 for an IL-1β level of 0.08 ng/mL and absence of added TNF-α (for explanation see "Materials and methods"); this reference point is marked (*R*) in the graph. Standard deviations ranged between CV = 16.5 and 80%; they are not shown here for the sake of not overloading the figures

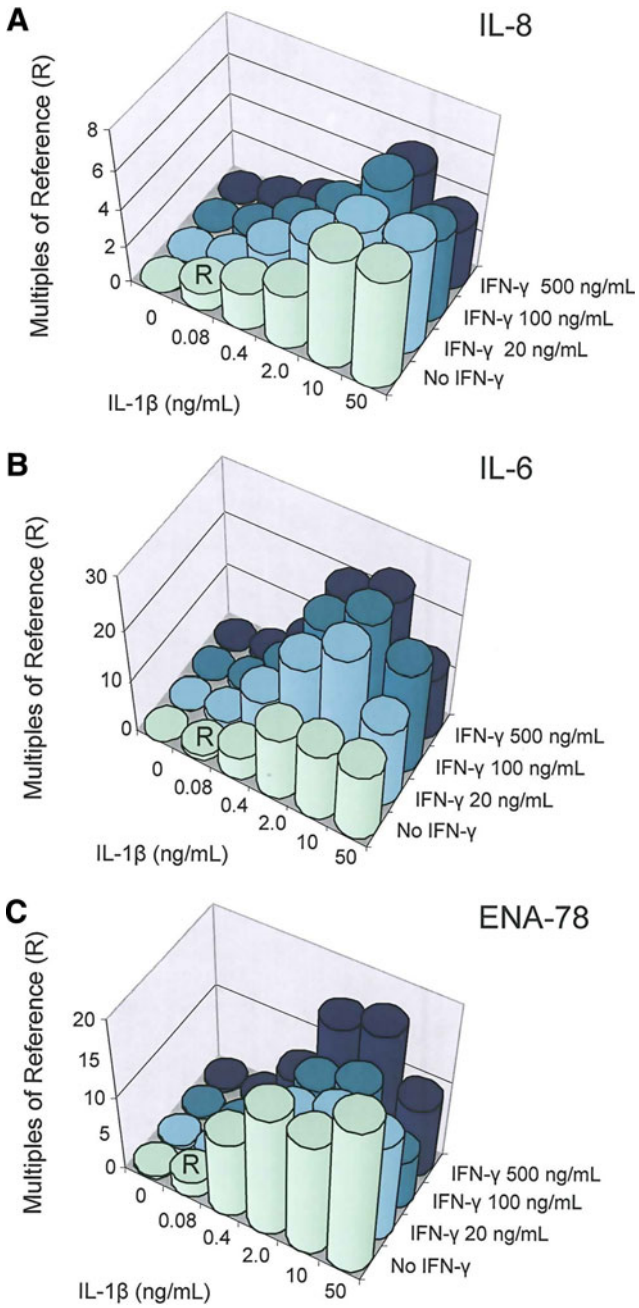

Fig. 2 Release of IL-8 (**a**), IL-6 (**b**), and ENA-78 (**c**) into the medium by endometrial stromal cells cultured for 24 h in the presence of IL-1β (0–50 ng/mL in five-fold dilution steps) and interferon-γ (0, 20, 100, 500 ng/mL). No TNF-α was present in these cultures. Values were obtained from one duplicate well culture with cells obtained after extraction of pooled endometrial tissue from three patients plus four single well cultures with material extracted from individually extracted biopsies obtained in four individual women. Normalisation in each graph was set at 1.00 for an IL-1β level of 0.08 ng/mL and absence of added IFN-γ (for explanation see Materials and methods); this reference point is marked (*R*) in the graph

the stroma [3]. Nevertheless, only low levels of ENA-78 have been detected in the supernatant of stromal cells cultured in the absence of stimulation by cytokines or

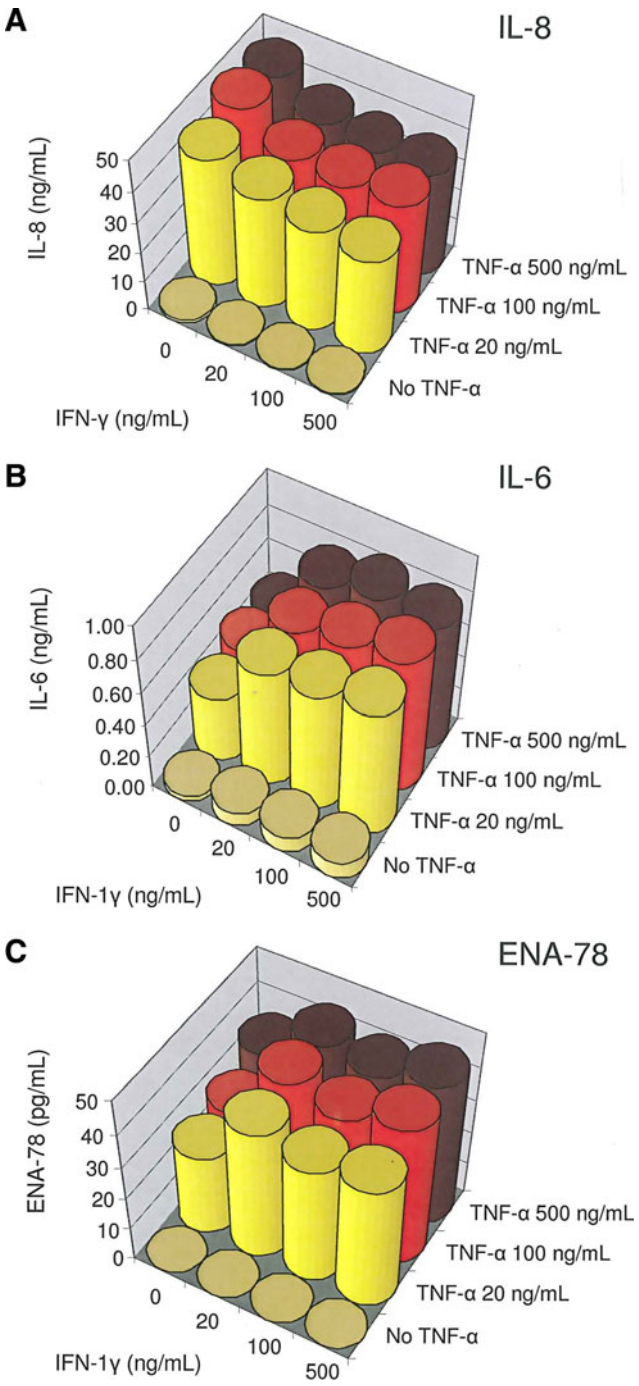

Fig. 3 Release of IL-8 (**a**), IL-6 (**b**), and ENA-78 (**c**) into the medium by endometrial stromal cells cultured for 24 h in the presence of interferon-γ and TNF-α (both at 0, 20, 100, 500 ng/mL and in all combinations). No IL-1β was present in these cultures, and for this reason the data are not normalised (for explanation see Materials and methods) and shown for the (duplicate well) culture experiment run with tissue extracted after pooling the biopsied material from three patients

lipopolysaccharide [3], and this pattern was the same as the one reported for IL-6 and IL-8 whose production was strongly stimulated by the presence of IL-1β or TNF-α in endometrial explant cultures [24], stromal monolayers [25, 26] and in a human stromal sarcoma cell line [27]. We have confirmed that cultured endometrial stromal, in contrast to epithelial, cells indeed only produced very small amounts of ENA-78 and IL-8 in the absence of in vitro stimulation, but that the addition of IL-1β or TNF-α with interferon (IFN-γ) increased this output to up to 500-fold the nonstimulated level [15]. TNF-α and interferon had been added in combination because of their co-operative action on several chemo- and cytokines, including IL-6. Such a cumulative effect, however, does not seem to occur in the case of IL-8 for which an inhibitory action of IFN-γ had been demonstrated [17]. For ENA-78, no information on possible stimulatory or inhibitory action of IFN-γ is available in the literature. We have therefore decided to investigate the dose-dependent effects of IL-1β, TNF-α and IFN-γ separated from each other as well as in combination.

Our results largely confirm the early findings on the opposite and specific effects of IFN-γ on the production of IL-6 and IL-8 [17] for the endometrium, which is contrary to the observed stimulation of IL-8 in keratinocytes [28]. On the other hand, we are reporting for the first time that the production of ENA-78 in the endometrium was not strongly influenced by IFN-γ, except maybe in the presence of very high levels of IL-1β (50 ng/mL, Fig. 2c). IFN-γ is an immunomodulator and a multifunctional cytokine produced by uterine natural killer, decidual inflammatory cells and later by the syncytiotrophoblast, and is essential for fertility and pregnancy maintenance [19]. Our results presented here indicate that the effect of IFN-γ on the recruitment of T cells and neutrophils, pertaining to the protective role of this cytokine, would not be mediated by ENA-78, but that these two immunomodulators play their role separately and independently.

Besides the above observation, ENA-78 showed a trend towards a stimulation of its release by IFN-γ in the absence of IL-1β (Fig. 3c). In cultures obtained from stromal cells from three out of six women we have, however, observed and a slight inhibition when IL-1β was present (not shown). Further studies with a larger number of replicate cultures and in the presence of very low IL-1β concentrations are required to determine whether this cytokine would modulate the action of IFN-γ in a negative feedback loop. Progesterone has also been demonstrated to stimulate the production of ENA-78 [3, 29]; but again we believe that this pathway did not play a role in our study since only biopsies taken in the proliferative phase of the menstrual cycle were used in culture. It may, however, be interesting to investigate the action of the mentioned cytokines on ENA-78 production in the presence and absence of the steroid hormone.

In conclusion, our study illustrates that, in spite of cumulative effects, the actions of cytokines such as IL-1β, TNF-α, and IFN-γ on the production and release of immunomodulatory and angiogenic markers in the endometrium follow separate patterns and may show non-linear, complex dose–response curves. It is therefore necessary to investigate the in vitro effects of bioactive substances on endometrial tissue in both separated and combined protocols. Moreover, these protocols will have to be tested on ectopic endometrium obtained from endometriosis patients. This is particularly relevant in the context of developing new, cyto- or chemokine targeted treatment approaches for this disease which is affecting a large number of women and to which increased research efforts will have to be made in the future.

Conflict of interest statement None.

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