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1 **TNFα induced IKKβ complex activation influences epithelial, but not stromal**
2 **cell survival in endometriosis**

3

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16

17 **Running title;** IKKβ in endometriosis

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35 **Abstract;**

36

37 **Study Hypothesis**: Can the activity of the IκB kinase (IKKβ) complex in
38 endometriotic cells contribute to endometriotic lesion survival?
39 **Study answer**: There is a constitutive activity of the IKKβ catalytic complex in
40 peritoneal and deeply infiltrating lesions that can influence epithelial, but not stromal
41 cell viability.
42 **What is known already:** Endometriotic lesions exist in an inflammatory
43 microenvironment with higher local concentrations of cytokines, such as tumor
44 necrosis factor α (TNFα). TNFα stimulates the activation of the IKKβ complex, an
45 important nodal point in multiple signalling pathways that influence gene
46 transcription, proliferation and apoptosis. However, few data on the regulation of
47 IKKβ in endometriotic tissue are currently available.
48 **Study Design, size, duration:** A retrospective analysis of endometriotic tissue from
49 peritoneal, ovarian and deeply infiltrating lesions from 37 women.
50 **Participants/materials, setting, methods:** Basal and activated (phosphorylated)
51 IKKβ concentrations were analysed by Western blotting and immunohistochemistry.
52 The relationship between the expression and activation of these proteins and
53 peritoneal fluid (TNFα) concentrations, measured via ELISA, was examined. A
54 subsequent *in vitro* analysis of TNFα treatment on the activation of IKKβ and the
55 effect on epithelial and stromal cell viability by its inhibition with PS1145 was also
56 performed.
57 **Main results and role of chance**; Levels of the phosphorylated IKKβ complex in
58 endometriotic lesions had a significant positive correlation with peritoneal fluid TNFα
59 concentrations. Phosphorylated IKKβ complex was more prevalent in peritoneal and
60 DIE lesions compared to ovarian lesions. IKKβ was present in both epithelial and
61 stromal cells in all lesions but active IKKβ was limited to epithelial cells. TNFα
62 stimulated an increased expression of phosphorylated IKKβ and the inhibition of this
63 kinase with PS1145 significantly influenced ectopic epithelial cells viability but not
64 eutopic epithelial cells, or endometrial stromal cells.
65 **Limitations, reasons for caution**; *In vitro* analysis on epithelial cells was performed
66 with immortalized cell lines and not primary cell cultures and only low sample
67 numbers were available for the study.

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68 **Wider implications of the findings**; The regulation of aberrant signalling pathways
69 represents a promising yet relatively unexplored area of endometriosis progression.
70 The IKKβ complex is activated by inflammation and is critical nodal point of
71 numerous downstream kinase-signalling pathways, including NFκB, mTOR and
72 BAD. This study shows a significant relationship between peritoneal fluid TNFα and
73 IKKβ activation in epithelial cells that will have significant consequences for the
74 continued survival of these cells at ectopic locations through the regulation of
75 downstream pathways.

76 **Large scale data:** none

77 **Study funding/competing interests**; The study was funded by the Swiss National
78 Science Foundation (Grant Number. 320030_140774). The authors have no conflict
79 of interest to declare.

80

81

82 **Key Words;** endometriosis, kinase, signalling, IKK, TNF, inflammation,
83 transcription factor, DIE, peritoneal, endometrioma

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84 **Introduction**

85 Endometriosis is characterized by the growth of endometrial epithelial and stromal
86 cells outside the uterine cavity. It is an extremely prevalent disease occurring in 10-20
87 % of women of reproductive age and is accompanied by chronic pelvic pain and
88 subfertility. Although the exact pathogenesis is not yet clear Sampson's theory of
89 transplantation is commonly accepted (Sampson, 1928). This theory proposes that
90 viable endometrial epithelial and stromal cells are refluxed back through the Fallopian
91 tube into the peritoneal cavity during menstruation. Once in this ectopic environment
92 these cells avoid immune detection, invade the underlying mesothelial layer and
93 stimulate a chronic inflammatory response.

94

95 Numerous inflammatory cytokines and chemokines (Borrelli *et al.*, 2013; 2014) are
96 increased in the peritoneal fluid of women with endometriosis, which occurs through
97 the coordinated interaction of the refluxed endometrial and peritoneal immune cells.
98 Refluxed endometrial cells produce and secrete chemokines (Hornung *et al.*, 1997)
99 that attract leukocytes and activated peritoneal macrophages (Halme *et al.*, 1983). The
100 activated macrophages produce inflammatory cytokines, which in turn further
101 stimulate cytokine production by the endometrial cells, creating a feed forward
102 regulatory loop (Lebovic *et al.*, 2001) and the chronic inflammatory environment.
103 This inflammatory environment has the potential to both influence symptomology
104 (McKinnon *et al.*, 2015) and disease progression (Bruner-Tran *et al.*, 2013).

105

106 A chronic inflammatory environment can contribute to endometriotic lesion
107 progression through the activation of a series of intracellular kinase signalling
108 pathways (McKinnon *et al.*, 2016). The IκB kinase (IKK) complex represents a
109 significant, early nodal point in many of the kinase signalling pathways. In the nuclear
110 factor (NF)κB signalling pathway the IKK complex removes the inhibitory IκB
111 protein from NFκB allowing translocation into the nucleus and gene transcription
112 (Bonizzi and Karin, 2004) subsequently influencing the gene expression of many
113 cytokine and chemokines, immunoreceptors, cell adhesion molecules, stress response
114 genes, and growth factors (Pahl, 1999). IKKβ also interacts with the tubular sclerosis
115 (TSC2) protein in the mammalian target of rapamycin (mTOR) pathway influencing
116 cellular proliferation (Lee *et al.*, 2007) and phosphorylates Bcl2-antagonist of cell
117 death (BAD) pathway suppressing apoptosis (Yan *et al.*, 2013).

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118

119 The IKKβ complex consists of two catalytic subunits, IKKα and IKKβ and one
120 regulatory subunit (IKKγ) (Hinz and Scheidereit, 2014). The binding of extracellular
121 tumor necrosis factor (TNF)α to its cell membrane receptor TNFR (Haider and
122 Knöfler, 2009) stimulates the phosphorylation of both IKKα and IKKβ and activation
123 of the IKKβ complex. TNFα concentrations are increased in the peritoneal fluid of
124 endometriosis (Harada *et al.*, 1999) and are correlated with the severity of the disease
125 (Bedaiwy *et al.*, 2002) and thus TNFα stimulated IKKβ activity may have a
126 significant influence on the endometriotic lesions.

127

128 At present very little information is available on the expression and activity of this
129 important upstream nodal kinase in endometriotic cells. In this study we used a
130 combination of clinical and *in vitro* experiments to determine the presence and
131 importance of IKKβ in endometriosis. We found that peritoneal fluid TNFα
132 concentrations had a significant positive correlation with the activated IKKβ complex
133 and that this was most likely due to epithelial cell expression. Furthermore IKKβ
134 activity was important in regulating ectopic epithelial cell, but not stromal cell
135 survival. These results therefore suggest a significant role of IKKβ in endometriotic
136 epithelial cells that deserves further attention.

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137 **Methods;**

138 *Patient samples*

139 Prior to surgery the relevant institutional review board granted ethical approval and
140 informed consent was obtained from all patients. During surgery performed for
141 suspected endometriosis samples of endometrium, peritoneal fluid and endometriotic
142 lesions were collected. Endometrial biopsies were collected via soft curette (Pipelle
143 de Cornier, Laboratoire CCD, France) and stored in RNAlater (Thermo Fischer
144 Scientific, USA) at -80 °C as described previously (Santi *et al.*, 2011). Peritoneal
145 fluid was collected during the laparoscopic procedure from the cul-de-sac and
146 centrifuged to remove blood cells, aggregates and debris. Samples were excluded if
147 blood remained in the samples. The pelvic cavity was examined and any
148 endometriotic lesions were removed and the patient staged (no endometriosis, or stage
149 I-IV) according to the revised American Fertility Society staging system (rAFS)
150 ("Revised American Society for Reproductive Medicine classification of
151 endometriosis," 1997). The lesions were recorded as either peritoneal, ovarian or
152 deeply infiltrating endometriosis (DIE). All surgeries were performed during the
153 proliferative phase of the menstrual cycle and endometriosis was confirmed by
154 histological analysis.

155

156 Endometrial biopsies were collected from both women with (n = 8) and without
157 endometriosis (n = 7) and used for the isolation of primary cells via collagenase
158 digestion and size exclusion, as described previously (McKinnon *et al.*, 2012). Strong
159 yields were obtained for the primary stromal cells (ESC) from all women except one
160 without endometriosis, however only limited amounts of epithelial cells could be
161 successfully isolated. As endometrial epithelial cells are terminally differentiated and
162 do not propagate immortalized epithelial cells were acquired. Matching peritoneal
163 fluid of sufficient quality was not always available for cytokine measurement
164 therefore if peritoneal fluid was available the ectopic lesions were immediately frozen
165 and stored for stored fresh frozen for subsequent Western blot analysis. If peritoneal
166 fluid was not available, they were formalin fixed and paraffin embedded for
167 immunohistochemistry analysis.

168

169 *Cytokine measurement in peritoneal fluid*

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170 TNFα was measured by an enzyme-linked immunoabsorbent assay (ELISA) kit
171 (R&D Systems, Abingdon, England) using a high-sensitivity NADH cascade
172 amplified alkaline phosphatase with antigen-antibody incubations at 28 °C in a dry
173 incubator and at a dilution of 1:2 in the diluent provided. Peritoneal fluid progesterone
174 concentrations were also measured via a radioimmunoassay (Coata-count, DPC;
175 Buhlmann Laboratories, Allschwil, Switzerland) to confirm the patient cycle phase
176 (McKinnon *et al.*, 2014).

177

178 *Protein isolation and Quantification in ectopic lesions*

179 Approximately 30mg of fresh frozen ectopic tissue was used to prepare whole cell
180 extracts via homogenization with the FastPrep 120 tissue homogenizer (30 seconds at
181 4.0 m/s) in radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-Cl, pH 7.4,
182 150mM NaCl, 1mM EDTA, 1% v/v triton X-100, 1 % w/v sodium deoxycholate,
183 0.1% w/v sodium dodecyl sulfate and 1% v/v protease and phosphatase inhibitor
184 cocktail (Cell Signalling Technology, Danvers, Massachusetts)). Final protein
185 concentrations were determined by the bicinchoninic acid assay (QuantiPro BCA;
186 Sigma).

187

188 Proteins separation was performed by heating 20µg of total protein into LDS buffer
189 (Invitrogen) to 70 °C for 10 minutes and running it on a 4 -15 % Novex NuPAGE
190 Bis/Tris gel (Invitrogen). Proteins were transferred to a 0.45µm nitrocellulose
191 membrane in 4-morpholinepropanesulfonic acid buffer (MOPS; Invitrogen) pH 7.7
192 for 1 hour. Non-specific binding was blocked by incubation with 5% bovine serum
193 albumin (BSA) in phosphate buffered saline (PBS) containing 0.1% Tween 20
194 (PBST). Membranes were probed with rabbit anti-IKKβ antibody (1:1000) (Cell
195 Signalling Technology), and with rabbit anti-pIKKα/β(176/180)(1:1000) antibody
196 (Cell Signaling Technology), or mouse anti-actinβ antibody (Abcam, Cambridge,
197 UK) 1:5000. Secondary anti-rabbit (GE Healthcare, Opfikon, Switzerland) and anti-
198 mouse (Sigma) antibodies conjugated to horseradish perioxidase were diluted 1:50
199 000 and 1:200 000 respectively. Immuno-reactivity was determined with the
200 SuperSignal West Femto kit (Pierce; Thermo Scientific) using the Chemi-Doc XRS+
201 system (Bio-Rad Laboratories, AG, Cressier, Swituzerland). Band densitometry was
202 quantified with the Quantity One software and in each Western blot a calibrator
203 sample with strong IKKβ, and pIKKα/β expression was included to normalize

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204 concentrations across gels and determine protein concentrations relative to the
205 calibrator sample. The relative expression of each sample was expressed as a
206 percentage of the calibrator. Actinβ was used as a loading control.
207
208 *Immunohistochemistry*
209 Immunohisotchemistry was performed using serial sections of 4μm mounted onto
210 glass slides (Superfrost, Braunschweig, Germany), dewaxed in xylene and rehydrated
211 through a series of decreasing ethanol concentrations. Epitope retrieval was
212 performed with 10mM citrate buffer, pH 5.5 for 5 minutes in a 450W microwave.
213 Endogenous perioxidase activity was blocked with 3% hydrogen perioxide (H_2O_2)
214 and a blocking step performed with 3% BSA for 30 minutes in Tris buffered saline
215 (TBS; Tris 100mM, NaCl 0.15 M; pH 7.4). Rabbit anti-IKKβ antibody (1:100) and
216 rabbit anti-pIKKα/β (176/180) antibody (1:100) were diluted in 3% BSA in TBS and
217 incubated at 4 °C overnight in a humidified chamber. Slides were washed with TBS
218 and 0.1% Tween 20 (TBST) prior to incubation with an affinity purified, biotin
219 conjugated goat anti-rabbit antibody (Dako, Glastrup, Denmark) for 90 minutes at
220 room temperature. After a final wash slides were incubated with an avidin-biotin HRP
221 complex (Vectastain, ABC Kit, Vector Laboratories, Burlingham, CA, USA) for 45
222 minutes. The antigen-antibody complex was detected by incubation with 3,3'
223 diaminobenzidine substrate and slides were counterstained with hematoxylin and
224 mounted in Aquatex (Merck). The primary antibodies were excluded for the negative
225 controls. Images were photographed with a Nikon Eclipse E800 microscope (Nikon,
226 Japan). Semi-quantitative analysis of antibody staining in the epithelial and stromal
227 cells of the endometriotic lesions was determined by the allocation of scores between
228 0-3; 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), based on the intensity of
229 staining. The percentage of cells with positive immuno-reactivity was also determined
230 and allocated a score between 0-6 as described 0% = 0, 1-10% = 1, 11-30% = 2, 31-
231 50% = 3, 51-70% = 4, 71-90% = 5; > 91% = 6 in each cell type of the endometriotic
232 lesions. For the final immuno-reactive score the staining intensity and percentage of
233 positive cells was multiplied, as described previously (Samartzis *et al.*, 2012).
234
235 *Cell culture and TNFα treatment*
236 Isolated primary endometrial stromal cells (ESC) were maintained in Iscoves's
237 modified Eagle medium (IMEM) (Invitrogen) supplemented with 10% fetal calf

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238 serum (FCS) (Invitrogen) and 1% antibiotic/antimycotic (Invitrogen). The
239 immortalized epithelial cell lines, EM E6/E7 and EM'osis, were provided by
240 Professor Kyo, Kanazawa, Japan and were isolated from eutopic endometrium (Kyo
241 *et al.*, 2003) and an ectopic endometrioma (Bono *et al.*, 2012) respectively. These
242 cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Invitrogen)
243 with 10% FCS and 1% antibiotic/antimycotic. The 12Z cells were provided by
244 Professor Starzinski-Powitz, Goethe University and were originally isolated from a
245 peritoneal endometriotic lesion (Zeitvogel *et al.*, 2001). These cells were also
246 maintained in complete DMEM media.
247 To determine the influence of TNFα on pIKKα/β activity in all cell types the cells
248 were seeded into 6 well plates at approximately 3 $x10^5$ cells/ well. After reaching
249 approximately 80% confluence the media was changed to 0.5% FCS for overnight
250 incubation prior to treatment. Cells were treated either with control media (0.5% FCS
251 in normal media) or control media plus 10ng/ml and 100ng/ml recombinant human
252 TNFα (R&D systems, United Kingdom) for 6 hours. At the end of the treatment
253 period the cells were rinsed and collected in RIPA buffer.
254
255 *MTS assay and treatment with PS1145*
256 PS1145 is a small molecular weight compound that is a specific inhibitor of IKKβ
257 activity (Lam *et al.*, 2005). Inhibition of IKKβ activity with PS1145 was performed in
258 96 well plates seeded at a density of $6x10^3$/ well. Twenty-four hours prior to treatment
259 the cells were changed into serum free media and treatment media prepared by
260 diluting PS1145 into either serum free media at a final concentration of 1μM.
261 Subsequent concentrations were prepared by a 1:3 serial dilution (333.33nM,
262 111.11nM, 37.04nM, 12.3nM & 4.12nM). Cell viability was measured after 72 hours
263 by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).
264 Triplicate wells were used for each cell type and experiment. For the immortalized
265 epithelial cell lines the experiment was repeated three times and for the primary ESC
266 an experiment on each of the eight endometriosis and seven non-endometriosis
267 preparations was performed separately. A control (without PS1145) was included for
268 each experiment and designated as 100% viability and subsequent values expressed as
269 a percent of control.
270
271 *Statistical Analysis;*

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272 All statistical analyses were performed with Graphpad Prism version 6.0. The
273 correlation between the peritoneal fluid cytokines and IKKβ and pIKKα/β expression
274 was performed using the non-parametric Spearman's rank correlation coefficient.
275 Two groups comparisons were performed with a non-parametric Mann-Whitney U
276 test and the comparison of three or more groups with the non-parametric Kruskal-
277 Wallis One-way analysis (ANOVA) and Dunn's multiple comparison *Post hoc* test.
278 The interaction between two variables was determined via a two-way ANOVA with a
279 *post-hoc* Tukey's multiple comparison test to determine the difference between
280 individual groups or conditions.
281

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282 **Results;**

283 ***Patient data and characteristics***

284 In total, endometriotic tissue was removed from 37 endometriosis patients and
285 endometrial biopsies from eight women without endometriosis. Of these 37 women
286 we collected accompanying peritoneal fluid from 21 in order to compare peritoneal
287 fluid TNFα and ectopic IKKβ. Of the 21 samples 14 were collected from women
288 without any hormonal treatment, four were using combined oral contraceptives
289 (COC) and three were using GnRH analogues. No significant variation in TNFα,
290 IKKβ or pIKKα/β based on hormonal use (**Table 1**) was identified. Five of the lesions
291 were peritoneal, eleven ovarian and five DIE.

292

293 The remaining 16 samples without accompanying peritoneal fluid were kept for
294 immunohistochemistry. Of these, six women had no history of hormonal treatment,
295 five were using COC and five were using GnRH analogues. Three lesions were
296 peritoneal, seven ovarian and six DIE. No significant variation was observed between
297 either pIKKα/β, or IKKβ expression in both the epithelial and stromal cells based on
298 hormonal treatment (**Table II**).

299

300 ***IKKβ expression and activation in endometriotic tissue and its relationship to***
301 ***peritoneal fluid TNFα concentrations;***

302 Comparison of peritoneal fluid TNFα and endometriotic lesion IKKβ and pIKKα/β
303 showed a significant positive correlation between TNFα and pIKKα/β ($r = 0.6268$, $n =$
304 21, $p = 0.0024$) (**Figure 1A**), but not IKKβ ($r = 0.4216$, $n = 21$, p 0.0570) (**Figure**
305 **1B**), as determined by semi quantitative Western blot (**Figure 1C**). A significant
306 variation in pIKKα/β concentrations between lesions from different locations ($p <$
307 0.05) was observed with a *post-hoc* analysis confirming a significantly lower
308 expression in ovarian lesions (50 ± 8.7, n =11) compared to the peritoneal lesions (99
309 ± 9.7, n =5) ($p = 0.041$) (**Figure 1D**). No significant difference was observed with the
310 DIE lesions (91 ± 21.6, n = 5). In contrast, IKKβ expression showed no variation
311 between lesion types ($p = 0.4905$) with similar expression in the peritoneal (138 ±
312 29.3, n = 5), ovarian (128 ± 26.7, n = 11) and DIE lesions (80 ± 26.9, n = 5) (**Figure**
313 **1E**). The active to inactive ratio of IKKβ (pIKKα/β:IKKβ) varied significantly
314 between lesions ($p = 0.0123$) with both peritoneal (1.0 ± 0.39, n = 5) and DIE (1.7 ±

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315 0.54, n =5) higher than ovarian lesions (0.5 ± 0.09, n = 11) with a *post-hoc* analysis
316 showing a significant difference between DIE and ovarian lesions (p = 0.0168).
317
318 ***Cell specific activation of IKK in endometriotic lesions***
319 Immunohistochemistry identified a low but uniform expression of IKKβ in peritoneal
320 (**Figure 2A**), ovarian (**Figure 2B**) and DIE lesions (**Figure 2C**) in both stromal (*red*
321 *arrows*) and epithelial cells (*black arrows*). In contrast, pIKKα/β in peritoneal
322 (**Figure 2D**), ovarian (**Figure 2E**) and DIE (**Figure 2F**) lesions was predominantly
323 epithelial (*black arrows*), with significantly less stromal cells expression (*red*
324 *arrows*). Negative controls showed no expression in peritoneal (**Figure 2G**), ovarian
325 (**Figure 2H**), or DIE (**Figure 2I**) lesions. No statistically significant difference was
326 observed in IKKβ expression between either lesion location (p = 0.2420) or cell type
327 (p = 0.1972) (**Figure 2J**), although this could be due to a lack of power. pIKKα/β
328 expression was significantly different in cell types (p = 0.0198), but no statistically
329 significant difference could be observed between lesion type (p = 0.3402) (**Figure**
330 **2K**)possibly again due to lack of power.
331
332 ***IKKβ activity after TNFα treatment***
333
334 Western blot analysis of pIKKα/β after TNFα treatment of epithelial cell cultures
335 confirmed a low but positive expression in all cell lines examined **(Figure 3A).** Semi-
336 quantitation of band densitometry indicated that the 12Z cells showed the strongest
337 basal expression (no TNFα), but this did not vary after TNFα treatment (p = 0.2320).
338 The EM'E6/E7 cells showed lower basal expression and also no significant variation
339 after TNFα treatment (p = 0.4475). In contrast, TNFα treatment of EM'osis cells
340 significantly increased pIKKα/β above the no treatment control (46 ± 5.0, n = 3) at
341 concentrations of both 10ng/ml (141 ± 34.2, n = 3, p = 0.0173) and 100ng/ml (197 ±
342 23.3, n = 3, p = 0.0085) (**Figure 3B**). Similar Western blots were performed on
343 protein lysate isolated from stromal cells however no protein expression could be
344 observed in these preparations.
345
346 ***Cell specific influence of IKKβ inhibition on viability***
347

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348 In the epithelial cell cultures there was a significant influence of PS1145 on cell
349 viability based on cell type ($p < 0.0001$) (**Figure 4A**). No significant effect of PS1145
350 was observed on the EM E6/E7 at any concentration. For EM'osis cells there was a
351 significant increase in cell viability at the lowest concentrations (PS1145 2.43nM; 123
352 ± 0.2, n = 3, $p < 0.001$) that was gradually diminished as concentrations increased
353 (PS1145 1μm; 106 ± 6.3 $p > 0.05$), whereas 12Z cell viability was significantly
354 decreased at the lowest concentrations (PS1145 2.43nM; 70 ± 4.6, n = 3, $p < 0.0001$)
355 and remained significantly reduced through to the highest concentration (PS1145
356 1μm; 73 ± 2.6, n = 3, $P < 0.0001$). In contrast primary ESC isolated from women with
357 and without endometriosis showed no significant variation based on either PS1145 (p
358 = 0.8868) or cell type (p = 0.3516) (**Figure 4B**).
359
360
361

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362 **Discussion**
363
364 In this study we examined the expression of the IKKβ protein kinase complex and the
365 activation of its catalytic subunits pIKKα/β in both endometriotic lesions, as well as
366 its influence on cell survival in *in vitro* models. The results show a significant
367 relationship between the phosphorylation of the IKKβ complex and peritoneal fluid
368 TNFα. Subsequent immunohistochemistry staining showed that although no
369 statistically significant difference in IKKβ expression was observed across all cells
370 and lesion types the phosphorylated IKKβ complex was predominantly epithelial.
371 This data was supported by the *in vitro* studies that confirmed pIKKα/β expression in
372 epithelial cell culture models, but not primary stromal cells and that inhibition of
373 IKKβ activity significantly influenced endometriotic epithelial cell viability, but not
374 eutopic epithelial cell viability, nor the viability of endometrial stromal cells from
375 women with and without endometriosis. These results therefore suggest that a
376 dysregulation of the IKKβ kinase occurs in ectopic epithelial cells that may be related
377 to the inflammatory microenvironment. Given the role of IKKβ in transmitting
378 extracellular signals into cell survival via kinase signalling pathways it may represent
379 a significant molecule in endometriosis pathogenesis.
380
381 At present there is very little known about the role of IKKβ in endometriosis. The
382 results from our clinical samples suggest that the constitutive IKKβ activity is
383 significantly different between peritoneal and DIE lesions compared to ovarian
384 lesions. This difference of expression was supported by our *in vitro* results that
385 showed TNFα stimulated an increase in pIKKα/β expression and that inhibition of
386 IKKβ activity increased the Em'osis cell viability. In contrast in the peritoneal derived
387 epithelia cells TNFα had a limited influence of pIKKα/β expression and inhibition of
388 IKKβ activity decreased cell viability. Unfortunately a DIE derived cell line was not
389 available.
390
391 Endometriosis is a significantly heterogeneous condition, although whether these
392 lesions have different pathologies (Nisolle and Donnez, 1997), or represent a
393 continuum of the same disease (Somigliana *et al.*, 2004) is still debated. These data
394 suggest a varied cellular response to inflammation may occur in different lesions. It
395 has previously been shown that rectovaginal septum lesions have a distinctly

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396 inflammatory phenotype (Bertschi *et al.*, 2013) and that concentrations of
397 inflammatory mediators are stronger in the peritoneal fluid of DIE compared to
398 lesions from other locations (Santulli *et al.*, 2012). As IKKβ can be associated with
399 TNFα both in ours and other studies (Lee *et al.*, 2007) it is possible that the increased
400 production of inflammation associated with DIE lesions is related to the higher IKKβ
401 activity.
402 The identification of a TNFα influenced activation of the IKKβ in endometriotic
403 tissue is significant because of the multiple downstream pathways it regulates **(Figure
404 5)** and the influence this can have on gene transcription, protein translation and both
405 cellular proliferation and apoptosis. Activation of IKKβ stimulates NFκB gene
406 transcription and a constitutive activation of NFκB has been observed in peritoneal
407 endometriosis (González-Ramos *et al.*, 2007). Multiple factors present in the
408 peritoneal fluid of women with endometriosis including cytokines and iron overload
409 (Alvarado-Díaz *et al.*, 2015) may lead to this constitutive activation. Furthermore an
410 increased NFκB activity has been linked to recurrence of ovarian endometrioma
411 (Shen *et al.*, 2008). Neither of these studies however examined IKKβ expression
412 directly. In the immortalized epithelial 12Z cells IKKβ inhibition attenuated
413 inflammatory cytokine secretion (Grund *et al.*, 2008) and in ectopic endometrial
414 stromal cells miR200a suppresses IKKβ (Dai *et al.*, 2012), raising the possibility of
415 suppressed IKKβ activity in stromal cells occurs via an epigenetic regulation. In
416 contrast to its role in inflammation via the NFκB pathway, IKKβ can also regulate
417 cellular proliferation and apoptosis through the mTOR and BAD pathways (Dunlop
418 and Tee, 2014) (Yan *et al.*, 2013). A dysregulation of mTOR has previously been
419 implicated in endometriosis pathogenesis of DIE lesions leading to increased
420 proliferation (Leconte *et al.*, 2011), as has a role for mTOR mediated autophagy
421 (Choi *et al.*, 2014) and BAD activation in ovarian endometriomas (Stickles *et al.*,
422 2015). Together this suggests that TNFα has the potential to modulate all of these
423 activities via IKKβ activation.
424
425 We found that the constitutive activation and influence on cell survival was largely
426 restricted to epithelial cells. Endometriotic lesions are a combination of epithelial and
427 stromal cells and an interdependency between the cells types is required for
428 endometriotic lesions to continue proliferating as tissue integrity of refluxed
429 endometrial matter is essential to endometrial tissue implantation (Nap *et al.*, 2003).

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430 We have also previously shown that the stromal cells produce significantly more
431 inflammatory cytokines than epithelial cells in response to stimulation (Bersinger *et*
432 *al.*, 2008). It could therefore be postulated that a paracrine regulation occurs in the
433 lesions through the stromal cells production of cytokines stimulating a constitutive
434 activation of the IKKβ complex in epithelial cells, which ultimately contributes to cell
435 survival. More research however is required to explore this hypothesis.
436
437 Furthermore, the activity of IKKβ in other cell types other than endometriotic cells
438 was not directly addressed in this study, but may give further insight into this
439 mechanism in normal tissue. For endometriosis, however whether this mechanism
440 also happens in healthy eutopic epithelial cells may be of less consequence. This is
441 because epithelial cells will only be present in the peritoneal cavity when
442 endometriosis is present, and when endometriosis is present there is a constant
443 inflammatory environment. We believe it is this confluence of ectopic epithelial cells
444 and constant inflammation that makes the contribution of TNFα stimulated IKKβ
445 activity significant. It may be such that this is a characteristic not inherent in the
446 endometrium, but rather acquired during the life of the lesion and contribute more to
447 progression through a constant stimulation of the inflammatory cascade. Further study
448 on whether there is a significant difference between the activation of IKKβ in the
449 eutopic endometrium of women with and without endometriosis would be an
450 interesting follow-up.
451
452 Whether other cell types also show a constitutive activity of IKKβ in the presence of
453 inflammation would also be interesting. In this study the images in Figure 2 indicate
454 cells proximal to the endometriotic lesion are largely negative for pIKKα/β
455 expression, providing circumstantial evidence for the preferential activation of
456 pIKKα/β in endometriotic epithelial cells. Previous studies suggest that cells proximal
457 to the lesion may have different characteristics to cells distal to the lesions (Young *et*
458 *al.*, 2014) and these cells thus may also be interesting to study, however we were
459 unable to collect this tissue due to our current ethical approvals. Future studies on this
460 topic may however be warranted.
461
462 Limitations of this study were the inclusion of women with hormonal treatment.
463 Previous research however has suggested there was no significant influence on NFκB

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464 activation by oral contraceptives (González-Ramos *et al.*, 2007). We also observed no
465 statistically significant difference for IKKβ in this study, although the power of this
466 analysis was limited by the small sample size. It is possible that this may introduce a
467 variability in peritoneal fluid cytokine concentrations in endometriotic women, as
468 GnRHa analogues have been shown to have an influence on the inflammatory
469 environment (Nirgianakis *et al.*, 2013), however we postulate that a reduction in
470 inflammatory cytokines by hormonal treatment would also be reflected by a
471 subsequent reduction in IKKβ activation, maintaining any correlation between the
472 extra and intracellular environment. A further limitation of this study is the small
473 sample size. An expansion of the sample number would provide more definitive
474 information on both the influence of TNFα, as well as hormonal treatments, on IKKβ
475 activity. It is difficult to draw direct conclusions on the contribution of IKKβ to cell
476 survival in different lesions types as immortalized cell lines were used, however given
477 the similarity observed in the *in vitro* results to the clinical samples we can be
478 confident that IKKβ has a role in epithelial endometriotic cells.
479
480 In conclusion we have observed a significant relationship between TNFα and the
481 activation of IKKβ complex in the endometriotic microenvironment and that this
482 activation occurs predominantly in the epithelial cells of peritoneal and DIE lesions.
483 Furthermore IKKβ inhibition *in vitro* significantly influenced epithelial cell, but not
484 stromal cell behaviour. The regulation of kinase signalling pathways is a significant,
485 but under explored area of endometriosis pathogenesis and progression and may
486 represent potential non-hormonal treatment targets for endometriosis (McKinnon *et*
487 *al.*, 2016). Given the ability of the extracellular inflammatory environment to
488 influence IKKβ activity and its subsequent affect on downstream pathways this kinase
489 may be significant interest in endometriosis.
490
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498 culture. **NAB** assisted with sample collection and intellectual development of the
499 project. **MDM** assisted with sample collection and intellectual development of the
500 project. **BDM** conceived project, performed experiments and prepared the
501 manuscript.
502
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506 **Conflict of Interest:**
507 None
508

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649
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651 **Figure legends**

652

653 **Figure 1; IKKβ activation in endometriosis and its relationship to tumor necrosis**
654 **factor (TNF)α. (A)** A significant correlation (r = 0.6268, n = 21, p = 0.0024) was
655 present between the peritoneal fluid TNFα expression and the pIKKα/β(176/180)
656 protein complex in endometriotic lesions. **(B)** No significant association was observed
657 between the expression of endometriotic lesion IKKβ and peritoneal fluid TNFα
658 expression. **(C)** Western blot analysis of endometriotic tissue samples confirmed a
659 consistent presence of both IKKβ. In contrast the expression of pIKKα/β varied
660 significantly amongst samples. Actinβ was used as a loading control. **(D)** Analysis of
661 pIKKα/β expression indicated that high concentrations were identified in the
662 peritoneal and deep infiltrating endometriosis (DIE) lesions with lower concentrations
663 observed in the ovarian lesions. **(E)** Basal IKK was more uniform amongst all
664 samples, although with a slightly lower, but non-significant expression in the DIE
665 lesions. **(F)** Analysis of the pIKKα/β: IKKβ ratio confirmed a lower ratio of activation
666 in the ovarian lesions that was significantly lower than that observed in DIE lesions.
667 Protein concentration in all components was calculated as relative to the calibrator
668 sample and expressed as a percentage. Correlation was determined performed by
669 Spearman's Rank correlation coefficient and comparison between lesion location
670 performed by a non-parametric Kruskal-Wallis One-Way analysis (ANOVA) test
671 with a *post-hoc* Dunn's multiple comparison. * $p < 0.05$. (P) peritoneal, (O) ovarian
672 and DIE (deeply infiltrating endometriosis.

673

674 **Figure 2; Cell specific expression and activation of IKKβ in endometriotic**
675 **lesions.** Basal IKKβ expression was observed in both epithelial (*black arrows*) and
676 stromal cells (*red arrows*) of endometriotic lesions removed from the **(A)** peritoneal,
677 **(B)** ovarian, and **(C)** deep infiltrating endometriosis (DIE) regions. The expression of
678 the activated IKK complex (pIKKα/β) was limited predominantly to the epithelial
679 cells (*black arrows*), although some stromal cell expression was observed (*red*
680 *arrows*). This was consistent across lesions from the **(D)** peritoneal, **(E)** ovarian and
681 **(F)** and DIE lesions. Negative controls showed no expression in lesions from the **(G)**
682 peritoneal **(H)** ovarian, or **(I)** DIE region. A semi-quantitative analysis of the cell
683 specific expression indicated that no statistically significant variation in **(J)** IKKβ
684 expression was observed between epithelial and stromal cells, however pIKKα/β was

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685 significantly stronger in the epithelial cells for all lesions with a largest difference
686 observed in the DIE lesions. **(K)** Analysis of protein activation and expression
687 between cell type and lesion location was performed with a Two-way analysis of
688 variance (ANOVA) test with a *post-hoc* Tukey's multiple comparison. Scale bars =
689 50µm. * $p < 0.05$
690
691 **Figure 3; pIKKα/β after tumor necrosis factor (TNF)α treatment in**
692 **endometriotic epithelial cells. (A)** Western blot analysis confirmed the expression of
693 pIKKα/β in the 12Z, EM E6/E7 and EM 'osis cell lines both with and without TNFα
694 treatment. **(B)** Semi-quantitative analysis indicated TNFα did not significantly
695 influence either 12Z, or EM E6/E7 expression. There was however a significant
696 increase in pIKKα/β after both 10ng/ml and 100ng/ml TNFα in the EM'osis cell line.
697
698 **Figure 4; Influence of IKKβ inhibition on epithelial and stromal cell viability.**
699 **(A)** Inhibition of IKKβ activity with increasing concentrations of PS1145 had no
700 influence on the eutopic derived epithelial EM E6'E7 cells, significantly increased the
701 cell viability of the ovarian derived EM'osis cells, significantly reduced the viability
702 of the peritoneal derived 12Z cells. **(B)** PS1145 had no influence on stromal cells
703 from women with and without endometriosis. Analysis on the influence between cell
704 type and PS1145 concentrations on cell viability performed with a Two-way analysis
705 of variance (ANOVA) test with a post-hoc Tukey's multiple comparison test. ** $p <$
706 0.01, *** $p < 0.001$, **** $p < 0.0001$.
707
708 **Figure 5; The IKK complex and its influence on downstream signalling**
709 **pathways.** Binding of extracellular tumor necrosis factor (TNF)α to TNF receptor 1
710 (TNFR1) stimulates the phosphorylation and activation of IKKα and IKKβ that exist
711 as a complex along with IKKγ. Activation of IKKβ leads to the phosphorylation of
712 IκBα, which under basal conditions is bound to the p65 subunit of the NFκB complex.
713 Phosphorylation of IκBα removes it from the NFκB complex and initiates protesomal
714 degradation allowing NFκB translocation into the nucleus and gene transcription.
715 Activation of the IKK complex can also lead to an interaction with the tuberous
716 sclerosis (TSC)2 protein that exists in a heterodimer with TSC1. Inhibition of TSC2
717 activity increases the activity of the mammalian target of rapamycin (mTOR)
718 complex stimulating both protein translation and cellular proliferation. Activation of

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719 the IKK complex also leads to an inactivation of the BH3 only BAD protein
720 inactivating TNFα stimulated apoptosis.
721
722

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723 **Table 1; Comparison of endometriotic lesion and peritoneal fluid protein**
724 **expression in samples removed from women subject to different hormonal**
725 **treatments. Data are Mean ± SEM**

	Hormone treatment				
	No hormone (n=14)	Combined oral contraceptive (n = 4)	GnRH analogue (n=3)	Total (n =21)	p
pIKKα/β (%)	66 ± 9.8	77 ± 27.8	92 ± 17.5	72 ± 8.5	0.4732
IKKβ (%)	120 ± 23.5	90 ±27.7	151 ± 27.7	119 ± 16.9	0.3819
TNFα (pg/ml)	2.1 ± 0.61	1.2 ± 0.53	1.5 ± 0.54	1.9± 0.43	0.7721

726 -IKKβ and pIKKα/β protein values were determined via Semi-quantitative Western
727 blot densitometry analysis and expressed as a percentage of a standardised control
728 sample included in each Western blot, as described in detail in the methods section.
729 -TNFα values represent peritoneal fluid TNFα concentrations determined by ELISA
730 and expressed as pg/ml.
731 -Analysis of significance was performed via the non-parametric One-way ANOVA
732 test (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple
733 comparison test) significance $p < .05$
734
735
736
737
738 **Table II: Comparison of protein expression in formalin fixed paraffin embedded**
739 **endometriotic tissue according to hormonal treatment. Data are mean ± SEM**

Hormonal use	No hormone (n= 6)	Combined oral contraceptive (n = 5)	GnRH analogue (n=5)	Total (n =16)	*P
pIKKα/β					

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Epithelial	1.7 ± 0.56	3.4 ± 1.78	6.2 ± 2.27	3.6 ± 0.98	0.108
Stromal	0.3 ± 0.33	0.8 ± 0.37	0.6 ± 0.60	0.5 ± 0.24	0.448
IKKβ					
Epithelial	2.3 ± 0.95	2.2 ± 1.11	3.0 ± 1.76	2.5 ± 0.70	0.949
Stromal	0.7 ± 0.42[BM1]	2.4 ± 1.75	0.0 ± 0.00	1.00 ± 0.58	0.327

740 -Values represent the scores derived from the semi-quantitative IHC analysis,
741 described in detail in the methods.
742 -Analysis of significance was performed via the non-parametric One-way ANOVA
743 test (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple
744 comparison test) significance $p < .05$
745
746
747

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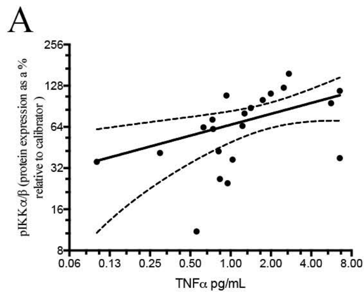
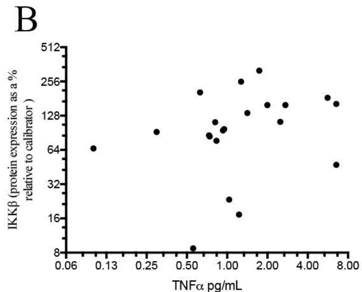
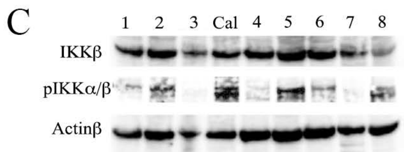
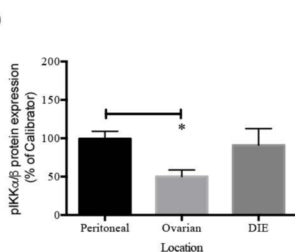
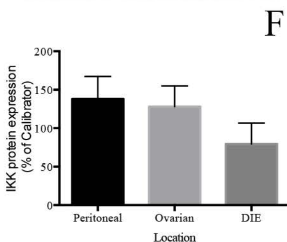
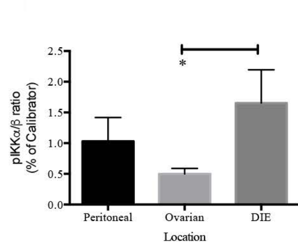

A
pIKKα/β (protein expression as a % relative to calibrator)
256
128
64
32
16
8
0.06
0.13
0.25
0.50
1.00
2.00
4.00
8.00
TNFα pg/mL

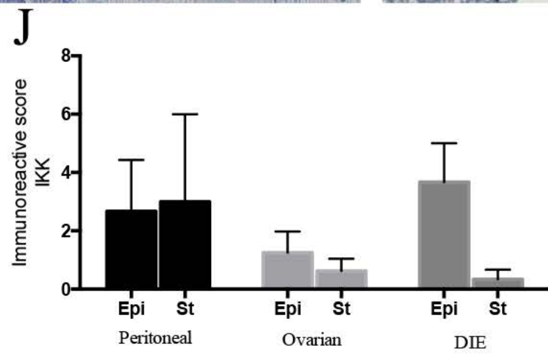

B
IKKβ (protein expression as a % relative to calibrator)
512
256
128
64
32
16
8
0.06
0.13
0.25
0.50
1.00
2.00
4.00
8.00
TNFα pg/mL

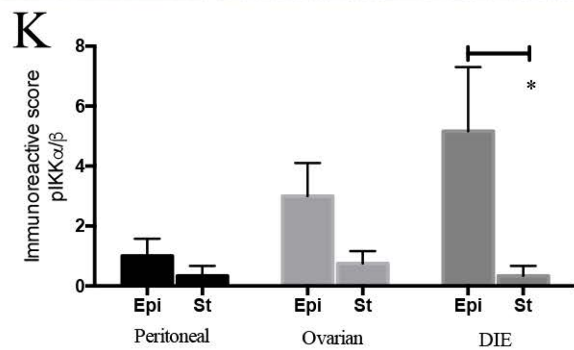

C
1
2
3
Cal
4
5
6
7
8
IKKβ
pIKKα/β
Actinβ


D
pIKKα/β protein expression (% of Calibrator)
200
150
100
50
0
*
Peritoneal
Ovarian
DIE
Location


E
IKK protein expression (% of Calibrator)
200
150
100
50
0
Peritoneal
Ovarian
DIE
Location


F
pIKKα/β ratio (% of Calibrator)
2.5
2.0
1.5
1.0
0.5
0.0
*
Peritoneal
Ovarian
DIE
Location


J
8
6
4
2
0
Immunoreactive score
IKK
Epi
St
Epi
St
Epi
St
Peritoneal
Ovarian
DIE


K
8
6
4
2
0
Immunoreactive score
pIKKα/β
*
Epi
St
Epi
St
Epi
St
Peritoneal
Ovarian
DIE

A

12Z EM E6/E7 EM'osis

TNF (ng/ml) 0 10 100 0 10 100 0 10 100

pIKKα/β

βActin

B

pIKKα/β (%) Relative to 12Z control

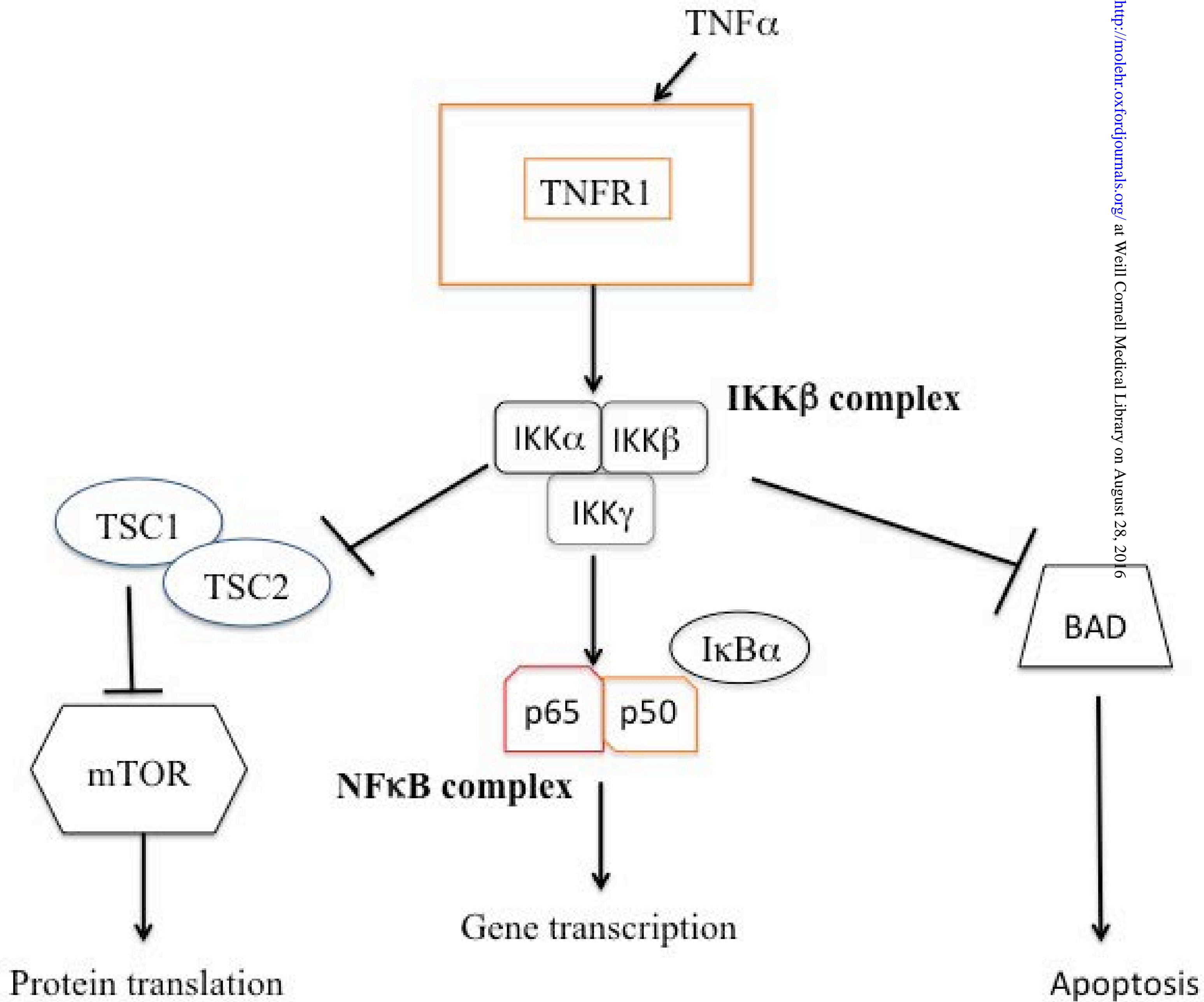
250
200
150
100
50
0

*
**

TNFα 0 10 100 0 10 100 0 10 100

12Z (peritoneal) EM E6/E7 (Eutopic) EM 'osis (Ovarian)

A
Cell Viability
(% of control)
200
150
100
50
0
***
***
***
**
****
**
****
**
**
****
2.43nM
8.1nM
27nM
90nM
300nM
1μM
PS1145 Concentration
EM E6/E7
Eutopic
EM'osis
Ovarian Ectopic
12Z
Peritoneal Ectopic
B
Cell Viability
(% of control)
200
150
100
50
0
2.43nM
8.1nM
27nM
90nM
300nM
1μM
PS1145 Concentration
Endometriosis
Non-Endometriosis



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