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IL-11 SUPPRESSES VEGFR2 EXPRESSION AND HAMPERS ENDOTHELIAL CELL'S WOUND HEALING

Endothelial cells (EC) line the lumen of all blood vessels and are crucial for vascular integrity, haemeostasis, and inflammation. EC are also targets for infections such as human cytomegalovirus (hCMV), which can induce vascular injury and release of various cytokines including the closely related interleukin (IL) - 11 and IL-6. ***Objective.*** *To assess the effect of IL-11 and IL-6 on wound healing by EC.* ***Methods.*** *We report a follow-up study of our previous work on IL-11 and IL-6 responses to hCMV where the EC's wound healing capacity and expression of relevant gene transcripts in EC treated with IL-11 or IL-6 are assessed.* ***Results.*** *Treatment with IL-11, but not with IL-6, hampered the wound healing capacity, and this effect may be due to suppression of VEGF signaling caused by suppression of VEGFR2. The VEGFA levels remained unaltered.* ***Conclusions.*** *IL-11 hampers the regenerating wound healing capacity of EC, and this may be due to the reduced expression of VEGFR2.*

Keywords: *endothelial cell, interleukin-6, interleukin-11, cytomegalovirus, wound healing, VEGFR2.*

Endothelial cells (EC) line the lumen of all blood vessels. They contribute to the regulation of vascular integrity, haemeostasis, and inflammatory responses (Ribatti et al., 2021; Pate et al., 2010) and are susceptible to infection with human cytomegalovirus (hCMV) (Jarvis et al., 2007; Jeffery et al., 2013). hCMV can induce direct (Gustafsson et al., 2015) and indirect vascular injury via the induction of various cytokines (Clement & Humphreys, 2019). We and others have previously shown that hCMV infection results in the secretion of interleukin (IL) -11, an IL-6-type cytokine family member that also signals via gp130 (Putoczki & Ernst, 2010; Gustafsson et al., 2018). IL-11 is secreted by different human tissue cell types including EC (Gustafsson et al., 2018) and fibroblasts (Alarifi et al., 2020) and can protect EC from apoptosis (Mahboubi et al., 2001). To further in-

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vestigate if IL-11 may protect vascular tissue from damage, we treated experimentally scratched EC monolayers with recombinant human (rh) IL-11 and measured the regeneration capacity. Unexpectedly, we saw that IL-11 suppressed the healing capacity of EC, possibly influenced by suppression of *VEGFR2* gene expression as VEGFR2 has been shown to play a crucial role in EC healing (Santos et al., 2007). Together, our results suggest that IL-11 impairs EC regeneration, possibly influenced by an impairment of the VEGF-signaling pathway by suppression of VEGFR2 expression.

Materials and Methods. *Isolation and culture of human umbilical vein ECs.* Primary ECs were isolated from umbilical cords using collagenase (Sigma-Aldrich) as previously described (Cooke et al., 1993) and cultured at 37 °C and 5% CO_2 until confluent. Cells were cultured in Medium 199 (Gibco, Invitrogen, Life Technologies) supplemented with 20% Fetal Bovine Serum (FBS), 2.5 μg/mL amphotericin B, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 ng/mL EGF, 28 μg/mL gentamicin, and 1 μg/mL hydrocortisone (all Sigma). Primary cultures were dissociated with trypsin/EDTA (Sigma) and passaged into tissue culture in multi-well plates, which were pre-coated with gelatin (Sigma) (0.5% gelatin in PBS). The seeding density was based on $2.4*10^5$ cells/ 6-well or $1*10^5$ cells/12-well and yielded 80—90% confluent monolayers for infection overnight.

Wound healing scratch assay. For wound healing scratch assays, HUVECs were seeded in 12-well plates. When the monolayers reached around 80% confluence, the EC monolayers were scratched using a pipette tip, and the culture medium was replaced. The culture media used was as specified in the above paragraph but without FBS. EC were treated with a medium containing IL-6 or IL-11 (both from R&D Systems) and at 2.5 ng/mL based on our previous data on cell culture concentrations of IL-6 and IL-11 for EC inoculated with hCMV (Gustafsson et al., 2018), or without these cytokines (untreated). The wound gap areas were determined using the Lasso function in the software Photoshop CS6 (Adobe). The percent of gap closure was calculated by the formula:

Evaluation of gene expression by quantitative real-time PCR. The total RNA was isolated using the RNeasy Mini kit with QIAshredder (Qiagen) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and converted to cDNA using the SuperScript III First-Strand Synthesis System with Oligo(dT)20 or random hexamers (Invitrogen, Life Technologies) according to the manufacturer's instructions. ANGPT2, TEK, VEGFA, VEGFR1, and VEGFR2 were measured using the TaqMan Fast Universal PCR Master Mix with the relevant Applied Biosystems TaqMan MGB primers/probe mix. Reactions also included the primers/probe mix for 18S allowing measurement of the endogenous control in duplex (Applied Biosystems, Life Technologies). The PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems), and the data were analyzed using SDS 2.4 software. Relative expression was calculated by the comparative CT method.

Evaluation of VEGFA secreted protein. VEGFA protein was measured in EC supernatants using a commercial ELISA kit (Human VEGFA Duo-Set ELISA, R&D Systems).

Statistics. The data were analyzed and illustrated in the GraphPad Prism v.5 software (GraphPad) using Student's t-test with two-tailed p-values and confidence interval or single factor ANOVA with $\alpha = 0.05$.

Ethics. This study was performed within the ethical standards of the Declaration of Helsinki. All donated umbilical cords included in this study were collected from the maternity ward at Karolinska University Hospital and de-identified with no possibility to trace back the original donor identities. Therefore, no ethical permit was required according to the Regional Ethical Review Board in Stockholm.

Results. *Effect of IL-11 and IL-6 on wound healing by EC.* We previously showed that EC can express

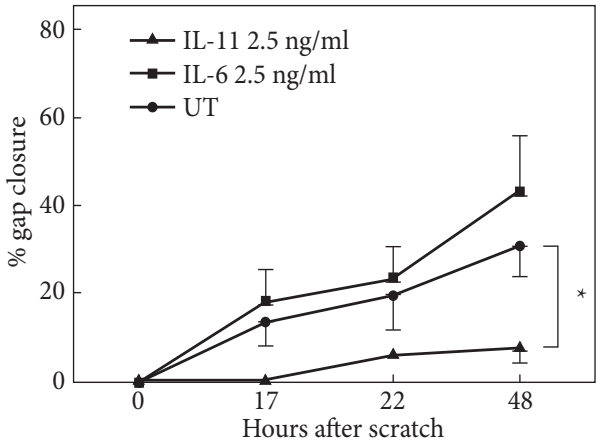

Fig. 1. Effect on wound healing capacity of untreated EC or EC treated with IL-11 or IL-6 in a concentration of 2.5 ng/mL. Images of wound sites were collected at time points 0 h, 17 h, 22 h, and 48 h, and the percent of the gap area closure at each time-point was determined by comparing with the gap area at 0 h. Data points represent means ±SEM for parallel cultures of EC from four independent donors analyzed using Single Factor ANOVA; *p<0.05

and secrete IL-11 in response to productive hCMV infection but not in response to several other stimuli such as cellular stressors or inflammatory mediators (Gustafsson et al., 2018). Here we have assessed the effect of IL-11 or IL-6 on wound healing by EC. EC exhibited a significant decrease in wound healing capacity in the presence of IL-11 compared to untreated EC, whereas IL-6 treatment had no significant effect but showed a trend of enhancing the wound healing capacity (Fig 1).

Effect of IL-11 and IL-6 on EC wound healing relevant gene expression. To test whether IL-11 can influence signaling pathways important in wound healing, the relative expression of ANGPT2, VEGFA, VEGFR1, VEGFR2 and TEK mRNA transcripts in EC treated for 24 hours with IL-11 or IL-6, or untreated was determined by quantitative PCR (qPCR). None of the markers were affected except VEGFR2, the expression of which was significantly suppressed by rhIL-11 treatment, but not with rhIL-6 treatment (Fig

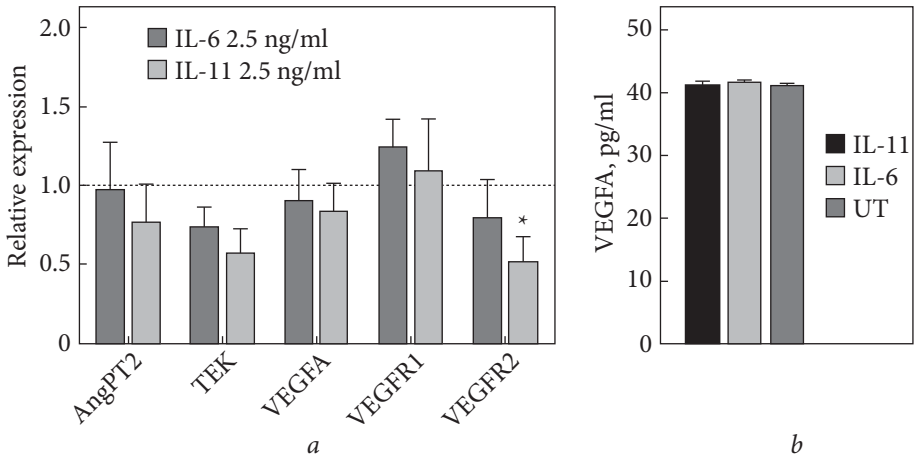

Fig. 2. Effect of IL-11 and IL-6 on the expression of wound healing relevant gene transcripts and secretion of VEGFA by EC. EC were left untreated (UT) or treated with IL-6 or IL-11 at 2.5 ng/mL and cultured for 24 h prior to the harvest of cells for mRNA analysis and of supernatants for VEGFA secretion analysis. (a) Relative mRNA expression of angiopoietin-2 (AngPT2), TEK receptor tyrosine kinas (TEK), vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor 1 (VEGFR1), and vascular endothelial growth factor receptor 2 (VEGFR2) was measured for EC cultures by qPCR. Target gene expression was normalized to 18S, and stimulation with IL-6 or IL-11 was compared to untreated cultures (dotted line). (b) The secretion of VEGFA was assessed in supernatants from EC collected after 24 hours of culture without stimulation (UT) or with stimulation with IL-11 or IL-6. Data points represent means ±SEM of parallel cultures of EC from four independent donors analyzed using t-tests. *p<0.05

2a). To further substantiate the notion that the effect is on the receptor side and not the ligand side of VEGF signaling, the secretion of VEGFA was assessed using ELISA after treatment for 24 hours. Indeed, no effect was seen on VEGFA secretion, further suggesting that the seen effect is on the VEGFR2 receptor side (Fig. 2b).

Discussion. hCMV infection is known to cause direct (Gustafsson et al., 2015) and indirect vascular injury (Clement & Humphreys, 2019; Styles et al., 2020) and to induce IL-11 expression in EC (Gustafsson et al., 2018). IL-11 is a pluripotent cytokine shown to enhance EC survival (Mahboubi et al., 2001) and promote compensatory proliferation in several cell types including hepatocytes (Nishina et al., 2012) and epithelial cells, protect mice from colitis (Nishina et al., 2023), and promote healing of oral mucosa in humans (Zhang et al., 2023). Therefore, we hypothesized that IL-11 would enhance recovery from experimentally induced EC injury using an *in vitro* scratch assay model. Unexpectedly, in our setting, rhIL hampered the capacity of EC to migrate into the scratched gap area, whereas IL-6 had no statistically significant effect but exhibited a trend to enhance the EC wound healing capacity. mRNA expression analysis revealed that IL-11, but not IL-6, suppressed VEGFR2 expression. For the ligand VEGFA, no effect of IL-11 or IL-6 was seen suggesting that the hampering effect lies in the suppression of the VEGFR2 receptor expression. VEGFR2 regulates VEGF signaling and is important in EC wound healing (Santos et al., 2007). Thus, the suppression of VEGFR2 may underlie the hampered wound healing on scratched EC monolayers. One reason for the discrepancy between our data and previous reports may be that whereas we used a serum-free medium in the experiments to see the pure effect of IL-11 or IL-6, Mahboubi (Mahboubi et al., 2001) and Nishina (Nishina et al., 2012) used the serum in their experiments. Hence, IL-11 may interact with a yet unknown serum factor resulting in enhanced survival and proliferation. Thus, this warrants further investigations.

Conclusions. IL-11 hampers regenerating the wound-healing capacity of EC and reduces the expression of VEGFR2.

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Conflicts of interest. The authors declare that there are no conflicts of interest.

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IL-11 ПРИГНІЧУЄ ЕКСПРЕСІЮ VEGFR2 ТА ПЕРЕШКОДЖАЄ ЗАГОЄННЮ РАН ЕНДОТЕЛІАЛЬНИМИ КЛІТИНАМИ

Ендотеліальні клітини (ЕК) вистилають просвіт усіх кровоносних судин і мають вирішальне значення для цілісності судин, гемостазу та запалення. ЕК також є мішенню для таких інфекцій, як цитомегаловірус людини (ЦМВ), які можуть індукувати пошкодження судин та вивільнення різних цитокінів, включаючи близькі до них інтерлейкіни (ІЛ) ІЛ-11 та ІЛ-6. **Мета.** Оцінити вплив ІЛ-11 та ІЛ-6 на загоєння ЕК. **Методи.** Ми доповідаємо про продовження нашої попередньої роботи з вивчення відповіді ІЛ-11 та ІЛ-6 на ВПГ, в якій оцінювали здатність ЕК до загоєння ран та експресію відповідних генних транскриптів в ЕК, оброблених ІЛ-11 або ІЛ-6. **Результати.** Лікування з ІЛ-11, але не ІЛ-6, пригнічувало здатність ЕК/ до загоєння ран, і цей ефект може бути зумовлений пригніченням сигналізації VEGF, спричиненим пригніченням VEGFR2. Рівень VEGFA залишався незмінним. **Висновки.** ІЛ-11 перешкоджає регенеративному загоєнню ран у разі ГЕК, що може бути пов'язано зі зниженою експресією VEGFR2.

Ключові слова: *ендотеліальна клітина, інтерлейкін-6, інтерлейкін-11, цитомегаловірус, загоєння ран, VEGFR2.*