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Monocyte-trophoblast interaction at the maternofetal interface

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Abstract

In this master's thesis the monocyte-trophoblast interaction at the maternofetal interface was investigated. Fractalkine (CX3CL1) is a chemokine and it is known for its dual nature with a membrane bound and a soluble form. The membrane bound form induces flow resistant adhesion of leukocytes to endo- and epithelial cells, while the soluble form results from metalloprotease dependent shedding and develops chemoattractive activity for monocytes, natural killer (NK) cells and T-cells.

The used cell line SGHPL-4 represents extravillous trophoblasts, that invade the decidualized uterus and remodel uteroplacental vessels. Within this study, CX3CL1 overexpressing SGHPL-4 cells and SGHPL-4 control cells were used.

Overexpression of CX3CL1 was confirmed via immunocytochemistry in a proportion of the cells and on protein and RNA level.

The disintegrin and metalloproteinase enzymes (ADAMs) cleave the membrane bound form of fractalkine to transfer it into the soluble form. With the metalloprotease inhibitor batimastat the activity of ADAMs was blocked and thus the enzymatic shedding of the membrane bound form to the soluble form. Therefore, with increasing concentration of batimastat the level of membrane bound CX3CL1 increased, whereas the release of the soluble form into the culture supernatant decreased.

The expression of the fractalkine receptor CX3CR1 was confirmed in parts of the extravillous trophoblast cell lines, in the overexpressing cells as well as in the control cells, by immunocytochemistry (ICC) and by western blot.

The first part of the hypothesis was that fractalkine would increase the adhesion of monocytes to the trophoblasts. This could be confirmed by adhesion assays, which revealed that CX3CL1 overexpression 2.4-fold increased the adhesion of the monocyte cell line THP-1 to SGHPL-4 CX3CL1 in comparison to the control cells.

The other part of the hypothesis was that fractalkine would activate monocyte cell lines. This part requires further investigations, since highest interleukin expression and release were shown in SGHPL-4 CX3CL1 overexpressing cells without THP-1 cell co-culture and in THP-1 cells that were incubated in the supernatant of SGHPL-4 control cells, which contained only minimal levels fractalkine.

This study suggests that fractalkine has an auto- or paracrine regulation on SGHPL-4 CX3CL1 overexpressing cells. The high interleukin production of the THP-1 cells cultured in the supernatant of SGHPL-4 control cells, needs further investigations, as in this supernatant a yet unidentified factor may activate the monocytes. This factor, however, seems to be absent in the supernatant of SGHPL-4 CX3CL1 overexpressing cells or is being suppressed by the overexpressed fractalkine.

As fractalkine has been found in many investigations around pregnancy complications further studies are required, as this topic could play a role in the better understanding of gestational problems.

Zusammenfassung

In dieser Masterarbeit wurde die Monozyten-Throphoblast Interaktion an der maternofetalen Barriere untersucht. Fraktalkin (CX3CL1) ist ein Chemokin, welches für seine doppelte Erscheinungsform, sowohl als Membran-gebundene als auch lösliche Form, bekannt ist. Die Membran-gebundene Form bewirkt die Adhäsion von Leukozyten an sowohl Endo- als auch Epithelzellen, während die lösliche Form durch Metalloproteinase induziertes Abspalten hervorgerufen wird und zur Aktivierung von Monozyten, natürlichen Killer Zellen und T-Zellen beiträgt.

In dieser Studie wurde mit der extravillösen Trophoblast Zelllinie SGHPL-4 gearbeitet. Extravillöse Trophoblasten wandern in die Dezidua ein und kleiden die uteroplazentaren Blutgefäße aus. Gearbeitet wurde mit zwei verschiedenen Versionen von SGHPL-4. SGHPL-4 CX3CL1 Zellen überexprimieren Fraktalkin, während die Kontrollzellen keine Überexpression zeigten.

Die Überexpression von CX3CL1 konnte mit Immuncytochemie (ICC) in einer gewissen Population von Zellen und auf Protein und RNA Ebene bestätigt werden.

Die Disintegrin und Metalloproteinasen (ADAMs) spalten die Membran-gebundene Form von Fraktalkin so, dass sie in die lösliche Form übergeht. Mit dem Metalloproteinase Inhibitor Batimastat wurde die Inhibierung der Spaltung der Membran-gebundenen Form in die lösliche Form nachgewiesen. Mit steigender Konzentration von Batimastat konnten ebenfalls steigende Konzentrationen der Membran-gebundenen Form und sinkende Konzentration der löslichen Form von Fraktalkin festgestellt werden.

Die Expression des Fraktalkin-Rezeptors CX3CR1 wurde ebenfalls in einer gewissen Anzahl von Zellen, sowohl in überexprimierenden als auch in Kontrollzellen, durch Immunocytochemie (ICC) und Western blot nachgewiesen.

Der erste Teil der Hypothese bestand darin, dass Fraktalkin die Adhäsion von Monozyten an Trophoblast Zellen erhöht. Dies konnte mit einem Adhäsionstest bestätigt werden, der ergab, dass die Adhäsion der Monozyten-Zellinie THP-1, welche den Fraktalin Rezeptor besaßen, bei SGHPL-4 CX3CL1 Zellen um das 2,4-fache höher war als bei den Kontrollzellen.

Der zweite Teil der Hypothese besagte, dass Fraktalkin Monozyten aktivieren würde. Dieser Teil der Hypothese sollte jedoch noch weiterer Forschung unterzogen werden, da die

Interleukin Produktion sowohl bei SGHPL-4 CX3CL1 Zellen, als auch bei den THP-1 Zellen, welche im Kulturüberstand von SGHPL-4 Kontroll-Zellen inkubiert wurden, am höchsten war.

Dies legt nahe, dass Fraktalkin eine auto- oder parakrine Regulation auf die SGHPL-4 CX3CL1 Zellen haben könnte. Die hohe Interleukin Produktion der THP-1 Zellen im Kulturüberstand von SGHPL-4 Kontroll-Zellen ist jedoch unklar und muss noch weiter untersucht werden. In dem Kulturüberstand könnte sich ein bisher noch nicht genau definierter Faktor befinden, der die Interleukin Produktion der Monozyten-Zelllinie hochreguliert. Dieser Faktor sollte jedoch nicht im Kulturüberstand der SGHPL-4 CX3CL1 zu finden sein, beziehungsweise könnte durch Fraktalkin unterdrückt werden.

Da Fraktalkin in Schwangerschaftsstudien immer häufiger gefunden und untersucht wurde, scheinen weitere Untersuchungen nötig zu sein um Probleme während der Schwangerschaft besser zu verstehen.

Introduction

Placenta

During pregnancy the placenta acts as an important gestation maintaining organ, fulfilling a wide spread panel of functions as a substitute for still immature embryonic and fetal organs. Beside metabolic, endocrine and transport functions, the placenta serves as a maternofetal barrier, by separating the maternal from the fetal bloodstream. (Sacks, Sargent, and Redman 1999; Mellembakken et al. 2002)

The cross-section dimension of the placenta at the end of gestation is about 15-25cm and the average thickness in the center of a placenta can be measured with 3cm. (Sadler and Langman 2008) Furthermore, the basic structural characteristics of the human term placenta (**Fig. 1**) is a disk-like thickening of the membranous sac. These membranes of the sac split into two different sheets: the chorionic plate and the basal plate, which surround the intervillous space. In the first trimester this space is filled with endometrial gland secretion and plasma. During the last two trimesters it is filled with maternal blood. The villi are complex tree-like structures of the chorionic plate that project into the intervillous space. They are covered with trophoblast cells, which have direct contact to the liquid in the intervillous space. At the barrier between villi and intervillous space the maternal-fetal gas and nutrition exchange occurs. (Benirschke and Kaufmann 2013)

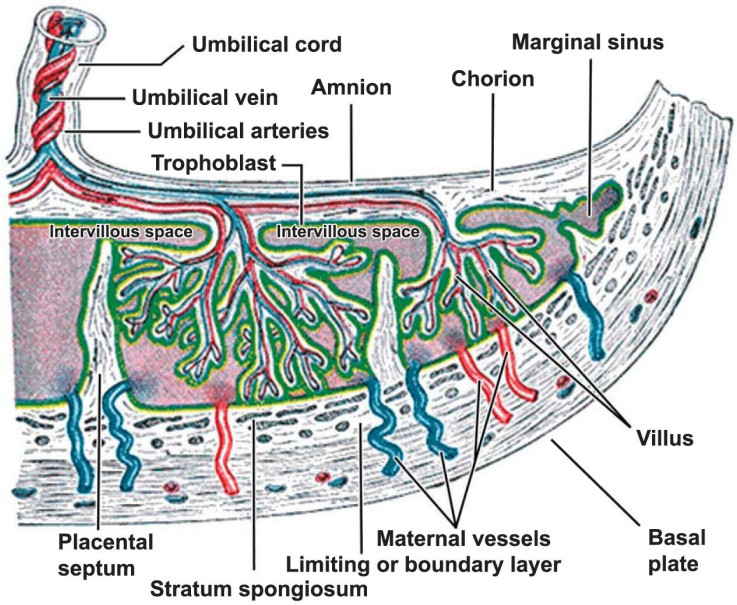

Fig. 1: Basic structure of a human term placenta. *The placenta has two different sheets: the chorionic plate and the basal plate, which surround the intervillous space. The villi are complex tree-like structures of the chorionic plate that project into the intervillous space. They are covered with trophoblast cells.*

The center of the villi is built out of mesenchyme with macrophages, fibroblasts and fetal blood vessels (**Fig. 2**). At the beginning of pregnancy the villi are covered by two cell layers: cytothropoblast-cells (Langhans-cells) and a continuous layer of the syncytiotrophoblast bordering the intervillous space. As the cytotrophoblast layer is merging and enlarging the syncytiotrophoblast as the pregnancy continues there is almost only one cell layer bordering between the maternal and fetal blood circulation. (Lüllmann-Rauch and Paulsen 2012)

Trophoblast

For the embryonic development and successful gestation outcome the proper development of the human placenta is very important. The surface of villi is covered with multinucleated syncytiotrophoblast (**Fig. 2**), which is continuously generated by cell division, differentiation and fusion of villous cytotrophoblasts with the syncytiotrophoblast. (Aplin 2010) Villi, which are connected to the basal plate of the human placenta, generate cell columns from which differentiated extravillous trophoblast cell types emerge by proliferation. During the first trimester of pregnancy, invasive endovascular cytotrophoblast cells block the maternal spiral arteries to prevent a premature flow of blood and therefore oxygen into the intervillous space. (Pijnenborg, Vercruysse, and Hanssens 2006) Mistakes in this process are associated with pregnancy complications due to the premature rise in oxygen levels. This can cause oxidative stress and harm placental villi. (Hustin, Jauniaux, and Schaaps 1990; Burton, Jauniaux, and Charnock-Jones 2010) The placenta switches from histiotrophic to haemotrophic nutrition around the 12th week of pregnancy with the establishment of the maternal-placental blood circulation by unblocking the maternal vessels, furthermore extensive arterial remodeling occurs. To ensure adapted fetal nutrient supply, reduced vessel contractility and constant oxygen delivery at low blood pressure the maternal spiral arteries are transformed into large diameter vessels. (Burton, Jauniaux, and Charnock-Jones 2010) Uterine natural killer (NK) cells and differentiated extravillous trophoblasts are thought to play an important role in vessel remodeling. (Robson et al. 2012) Insufficient extravillous trophoblast invasion, vessel remodeling and cytotrophoblast gene expression were linked with different pregnancy diseases for instance preeclampsia or severe intrauterine growth restriction. (Pijnenborg et al. 1991; Pijnenborg, Vercruysse, and Hanssens 2006; Zhou et al. 2013)

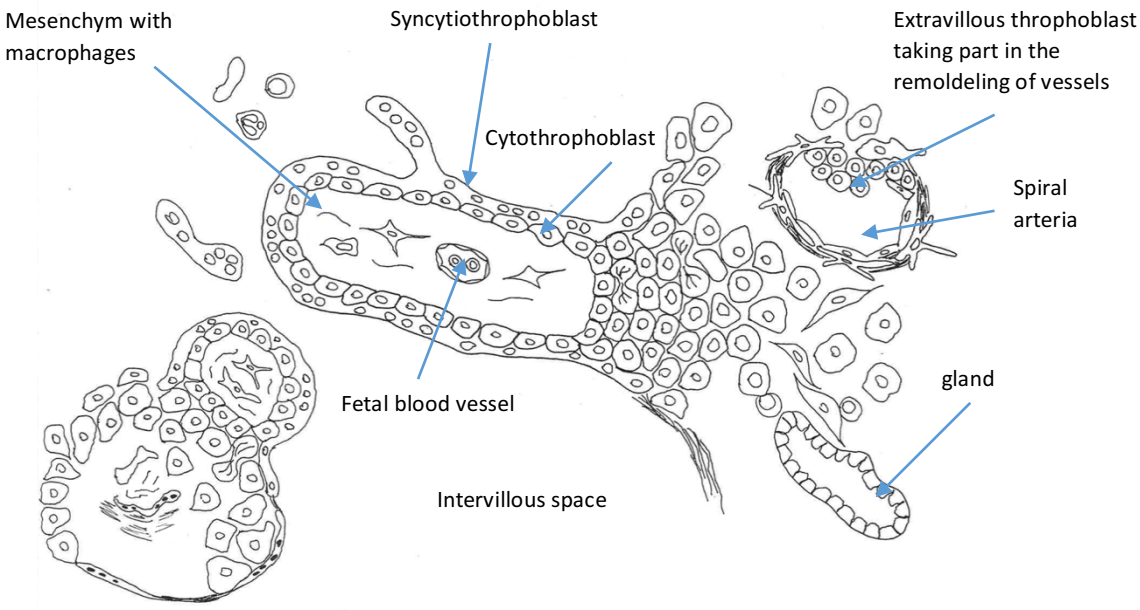

Fig. 2: Drawing of the structure of a villi and the different kinds of trophoblasts. *The center of the villi is built out of mesenchyme with macrophages, fibroblasts and fetal blood vessels. At the beginning of pregnancy the villi are covered by two cell layers: cytothropoblast-cells (Langhans-cells) and a continuous layer of the syncytiotrophoblast bordering the intervillous space. Extravillous trophoblasts invading the maternal side of the placenta and amongst others taking part in the remodeling of vessels.*

Monocytes

During pregnancy, the circulation of maternal blood through the placenta results in contact of maternal immune cells with the placenta cells. Circulating immune cells, especially monocytes, may be activated and therefore play an important role in gestation.

Monocytes are generated from the bone marrow and represent about 5–10% of the circulating blood leukocytes. After circulating in the blood for a couple of days, they migrate into tissues to evolve into macrophages or dendritic cells. Monocytes play important roles in homeostasis, tissue repair and inflammation. Recent studies indicate that circulating monocytes are a heterogeneous population. (Gordon and Taylor 2005)

Classical monocytes are strong phagocytes that can produce cytokines in response to toll-like receptor dependent activation. Non-classical monocytes are weak phagocytes and are more efficient producers of pro-inflammatory cytokines after activation. (Gordon and Taylor 2005)

Although it is known that leukocyte numbers increase during pregnancy, little is known about monocyte activation during the course of gestation. (Faas, Spaans, and De Vos 2014)

CX3CL1

From the initial maternal blood flow into the intervillous space of the human placenta at the end of the first trimester of gestation on, the direct physical interaction between maternal circulating blood cells and the placental syncytiotrophoblast is enabled. (Huppertz et al. 2012; Jauniaux et al. 2000) The syncytiotrophoblast completely covers the placental villi towards the intervillous space and is thereby an important part of the placental barrier. The interaction between maternal circulating blood cells and the placental syncytiotrophoblast can be regulated by cytokines, chemokines and adherence molecules. The chemokine that recently attracted increasing attention due to its dual nature is fractalkine (**Fig. 3**). Fractalkine is a transmembrane molecule with 373 amino-acids. It consists of an extracellular N-terminal domain, a transmembrane alpha-helix and a short cytoplasmic tail. (Umehara et al. 2004; Jones, Beamer, and Ahmed 2010) Fractalkine has a membrane-bound and a soluble form. The membrane-bound form induces flow resistant adhesion of leukocytes to endo- and epithelial cells using its receptor CX3CR1. The soluble form results from metalloprotease dependent shedding and develops chemoattractive activity for monocytes, NK cells and T-cells. (Imai et al. 1997) Therefore, fractalkine establishes different forms of leukocyte recruitment, based on whether it is cleaved or not. (Schwarz et al. 2010)

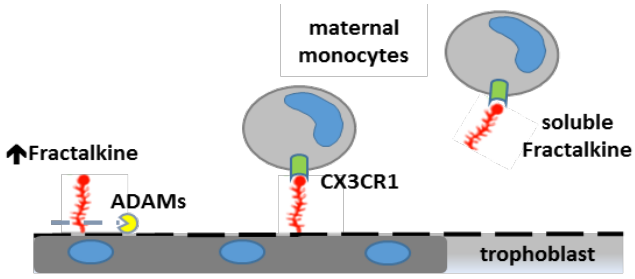

Fig. 3: Dual nature of fractalkine. *From left to right: The membrane bound form of fractalkine that is transferred into the soluble form by shedding activity of a metalloprotease. The membrane bound form that attaches a monocyte by connecting to the fractalkine receptor CX3CR1. The soluble form of fractalkine that is attaching to a floating monocyte in the maternal blood.*

CX3CR1

That CX3CL1 has the above mentioned biological effects is the result of its interaction with its receptor CX3CR1. (Imai et al. 1997; Kim et al. 2011; Mizoue et al. 1999) The CX3CR1 receptor can be sorted into the class of metabotropic receptors structurally. These are also known as G protein-coupled receptors or seven-transmembrane proteins. (Kim et al. 2011)

The seven α-helical structures of the polypeptide chain are extending across the cell membrane. Structurally, the receptor could be divided in an extracellular, transmembrane and intracellular part. The binding site for the ligand CX3CL1 is formed by external loops of the polypeptide chain. (Mizoue et al. 1999) CX3CR1 displays polymorphism, which may submit a statement for its varying affinity and reaction to CX3CL1. (Niessner et al. 2005)

ADAMs

The soluble form of CX3CL1 results from metalloprotease dependent shedding. Studies showed that the zinc-dependent family of metalloproteinase, among them, the disintegrin and metalloproteinase family proteins (ADAMs) are involved in the cleavage of several cytokines, receptors and adhesion molecules. (Huovila et al. 2005) Shedding of the chemokine CX3CL1 has been accredited to ADAM10 and ADAM17 activities. (Garton et al. 2001; Tsou, Haskell, and Charo 2001; Hundhausen et al. 2003) Wojdasiewicz et al. described a specific region for the cleaving action of these enzymes to transform the membrane bound form of fractalkine into the soluble form (**Fig. 4**). (Wojdasiewicz et al. 2014)

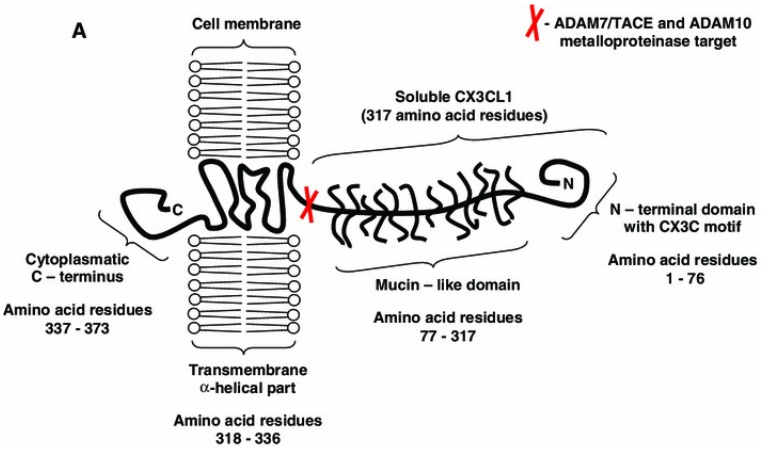

Fig. 4: The molecular structure of the membrane-bound form of CX3CL1 *(fractalkine) showing specific regions for cleaving action by the metalloproteinases ADAM7/TACE and ADAM10.*

Role of CX3CL1 in reproduction

Dysregulation of fractalkine expression is observed in several complications during pregnancy, e.g. diabetes mellitus and early-onset preeclampsia. (Szukiewicz et al. 2013; Siwetz, Dieber-Rotheneder, et al. 2015) Also, studies suggest that, together with other cytokines, fractalkine is involved in many processes of reproduction. It is said, that CX3CL1 is for example involved in the implantation of the blastocyst, invasion of trophoblasts into the spiral uterine arteries, responses to inflammatory and immunological factors in the utero-placental interface and many other processes still to be investigated. (Bowen et al. 2002; Hannan et al. 2006; Hannan and Salamonsen 2008)

The implantation of the blastocyst is initiated by attachment of the apical plasma membranes of mononucleated trophoblasts to the apical plasma membranes of the uterine epithelial cells. (Benirschke and Kaufmann 2013) Right before implantation, uterine epithelial cells become highly secretory and release several regulatory factors into the uterine lumen, where they can interact with the blastocyst even prior to the attachment process. (Dimitriadis et al. 2009) The assumption that CX3CL1 may be part of the molecular cocktail is based on immunohistochemistry of human endometrium during menstrual cycle, showing fractalkine expression in the luminal and glandular uterine epithelium. (Hannan et al. 2004) It is suggested that the released fractalkine together with other chemokines in the uterine lumen, may activate the blastocyst for attachment. This process could be initiated by the interaction of the chemokines with various receptors that have been detected in human blastocysts. (Dominguez et al. 2003)

Moreover, trophoblast invasion takes an important role during the development of the placenta and remodeling of uteroplacental arteries to adapt to pregnancy. (Benirschke and Kaufmann 2013) From the second week of pregnancy onwards mononucleated cytotrophoblasts (extravillous trophoblasts (EVT)) invade the uterine tissue up until the inner third of the myometrium. Expression analysis and other studies suggest a functional role of the CX3CL1/CX3CR1 axis in migration and invasion of EVTs. (Siwetz, Sundl, et al. 2015; Hannan et al. 2006; Hannan and Salamonsen 2008)

Hypothesis

The chemokine fractalkine was shown to be expressed in the plasma membrane of the syncytiotrophoblast in human placenta. By metalloprotease dependent shedding it is released into maternal circulation. This dual nature enables two ways of how placental fractalkine may interact with maternal leukocytes in the intervillous space. While released soluble fractalkine may activate passing maternal cells, membrane-bound fractalkine may mediate adhesion of leukocytes to the trophoblast.

In this study, experiments with an extravillous trophoblast cell line SGHPL-4 CX3CL1, that stably overexpressed fractalkine, were performed to test the hypothesis whether CX3CL1 (over-)expression in extravillous trophoblast cells mediate adherence and activation of monocyte cell line THP-1. First, the overexpression of the extravillous trophoblast cell line was characterized. Afterwards, the fractalkine-mediated adhesion of monocyte cell line (THP-1) to the CX3CL1 overexpressing extravillous trophoblast cells was compared to the control cells. Finally, monocyte activation – i.e. induction of proinflammatory cytokine expression and release – in response to membrane bound and soluble form of fractalkine was investigated on RNA and protein level.

Methods

Tissue and cell lines

Placenta

All women gave their informed consent, that their placenta could be used for scientific research after the abortion. Expert staff has collected the tissue shortly afterwards and delivered it safely and under optimal conditions to the Institute of Cell Biology, Histology and Embryology, MUG.

SGHPL-4

SGHPL-4 cells are derived from primary extravillous trophoblasts transfected with the early region of SV40. Characterization of these cells has confirmed an extravillous trophoblast-like phenotype including expression of cytokeratin-7, BC-1, HLA Class I, CD9, hPL and hCG. (Choy and Manyonda, 1998; Cartwright et al., 1999; Shiverick et al., 2001)

Our cell line was kindly provided by Dr. Florian Herse and Julianna Zadora from the Max-Delbrueck Center for Molecular Medicine (MDC), Berlin, Germany. We received a control cell line of the SGHPL-4 as well as a CX3CL1 transfected SGHPL-4 cell line.

SGHPL-4 cells were stored in liquid nitrogen, before they were thawed and seeded in a 75 cm^2 cell culture flask containing 15 ml pre-warmed Ham's F-10 medium (Merck) with additionally added 10 % FBS (Gibco) and 1 % Penicillin/Streptomycin (Biowest). Then they were incubated at 37°C in a CO_2-incubator (Heracell 150i (Thermo Scientific)). Cells were split with accutase (Biowest), a solution that detaches cells from the cell culture flask, when they were confluent. Cells, that were not used for experiments, were reseeded in cell culture flasks.

THP-1

THP-1 is a human, monocytic leukemia cell line and was cultured from the blood of a one year old boy. (Tsuchiya et al. 1980)

THP-1 suspension cells were stored in liquid nitrogen, before they were thawed and seeded in a 75 cm^2 cell culture flask containing 10 ml pre-warmed RPMI Medium 1640 (Gibco® by Life Technologies) with additional FCS and P/S (see chapter materials). They were incubated at 37°C in a CO_2-incubator (Heracell 150i (Thermo Scientific)).

Immunohistochemistry

Immunohistochemistry (IHC) is used to determine an antigen of interest in a specific tissue section using mono- or polyclonal antibodies by visualizing their binding reaction with one another. (Mulisch and Welsch 2015)

From placenta to sample

The method included collecting tissue, fixation, paraffin embedding, sectioning and finally incubating it with an antibody against the antigen of interest for visualizing the antigen in the cellular components under the microscope. (Mulisch and Welsch 2015)

Tissue processing

The cells' morphology was trying to be preserved by causing the least damage on the tissue as possible. Formalin fixation produces chemical cross-linkings of proteins within the tissue, so that all cellular processes get determined and degradation gets prevented. (Mulisch and Welsch 2015)

Villous tissue of first trimester placenta (gestational age: 7+0) were immediately fixed in 4% formalin. Samples were left in formalin for up to 48 hours (depending on the size). After fixation, samples were placed in tissue cassettes and processed overnight in an automatic processor (Tissue-Tek® VIPTM, Sakura®) for dehydration with a rising alcohol series (60%, 80%, 96% for 1 hour each and 100% for 3 hours) and afterwards soaked with paraffin wax. Then, fixed samples were embedded into paraffin blocks. (Mulisch and Welsch 2015)

Microtome

With a sledge microtome (MICROM HM440E) the placenta tissue sections were cut at a width of approximately 5µm from the paraffin blocks, collected onto microscope slides (Menzel-Gläser, Superfrost® Plus, Thermo Scientific), left on a rack at 54°C over night and stored in the dark at room temperature until staining. (Mulisch and Welsch 2015)

Pretreatment / antigen retrieval

To deparaffinize the sections a xylol substitute (Tissue Clear®, Tissue-Tek®, Sakura or Histolab-Clear, HistoLab®) was used. To eliminate all the paraffin left in the sections, they were treated 5 minutes for four times. Afterwards tissue sections were rehydrated with a series of solutions of descending alcohol concentration up to the hydration needed for the following treatments.

(Mulisch and Welsch 2015; Lang 2012) Although the fixation process is essential for the preservation of tissue morphology, the process can also have a negative impact on immunohistochemistry detection. Fixation can alter the protein biochemistry in terms of the epitope of interest being masked and no longer being able to be detected by the primary antibody. To avoid this alteration antigen retrieval (AGR) should be performed under preferably gentle conditions, so that the tissue morphology and antigens are not altered. (Mulisch and Welsch 2015) In these experiments the antigen retrieval was performed by incubating slides under pressure at 120°C (Decloaking Chamber, Biocare Medical) at pH 6 (Citrate buffer (0.01M; citric acid monohydrate)).

Chamberslides

SGHPL-4 cells were seeded at a density of 7.0 x 10^4 cells per chamber (Lab-Tek II Chamber Slide (Nalge Nunc International)). They grew for 48 hours in their usual F-10 culture medium, before F-10 was removed and the chambers were washed with PBS buffer. Afterwards the cells were left to dry and then frozen at -20°C until staining. For the immunohistochemical staining procedure the chamberslides had to be thawed and incubated in acetone for 10 minutes before rehydrated in PBS for 5 minutes. Now the usual procedure of immunohistochemistry could be performed (see chapter "Immunohistochemistry"), except for using TBS instead of TBS-T and adding of 10 % serum to the UltraVision Protein Block. (UltraVision LP detection system, Thermo Scientific)

Single cell paraffin embedding

SGHPL-4 cells were washed several times to remove potentially dead cells. Afterwards cells were detached from the flask with a cell scratcher and PBS and transferred into a 15 ml tube. The suspension was centrifuged at 1700 rpm at room temperature for 5 minutes and subsequently the PBS was removed. The cell pellet was resuspended and fixed in 4% PFA for 30 minutes at room temperature. Thereafter the suspension was centrifuged (1700 rpm, RT, 5 minutes), the 4% PFA was removed and the cell pellet was washed two times with PBS (centrifugation step in between). Now 5% gelatine was added (same amount as cell pellet), carefully resuspended and incubated for 30 minutes at 37°C. Afterwards the suspension was shortly centrifuged at 37°C and then cooled down at 4°C for 30 minutes. Thereafter 1 ml of

4% PFA was added and the gelatine pellet was fixed for 1 hour at 4°C. Then the gelatine pellet was freed and stayed floating in 4% PFA overnight. The next morning the pellet was washed with PBS before processed like tissue samples (see chapter “Tissue processing”).

Immunohistochemistry (IHC)/Immunocytochemistry (ICC)

In the indirect immune-histochemistry/-cytochemistry, used for this project, the primary antibody that bound the antigen of interest, was not labeled, but the secondary antibody, that bound the primary antibody, was marked. This labelling had a colorful visualization under the light microscope. (Mulisch and Welsch 2015)

Indirect immune-histochemistry/-cytochemistry was performed using the Ultravision LP detection system (Thermo Scientific) according to manufacturer’s instructions. The indirect method has the advantage that it can result in signal enhancement as more than just one of the polyclonal secondary antibodies can bind to the primary antibody. Also with one secondary antibody many antibodies that bind to different antigens can be localized as long as they were raised in the same species. (Mulisch and Welsch 2015)

At first the pretreated slides were incubated with a Peroxidase-Blocking Solution (Dako REAL™) for 10 minutes to block the endogen peroxidase. After washing the slides 3 times with aqua dest. slides were treated with an UltraVision Protein Block (UltraVision LP detection system, Thermo Scientific) for 5 minutes to block the unspecific bindings. After washing 3 times with TBS-T (20x TBS (pH 7.4-7.6) diluted 1:20 and added 0.05% Tween® 20 (Merck)) buffer, the slides were incubated with a primary antibody for 45 minutes, in case of a primary mouse AB followed by a primary antibody enhancer (UltraVision LP detection system, Thermo Scientific) for 10 minutes as a bridge antibody. After washing again 3 times with TBS-T an incubation with Large Volume HRP Polymer (UltraVision LP detection system, Thermo Scientific) took place for 15 min, followed by 3 times TBS-T washing. After the staining with Large Volume AEC Chromogen Single Solution (Thermo Scientific) for 10 min, slides were washed 3 times with aqua dest. and then incubated in basic hemalum (Mayer) for 12 minutes as a nucleus counterstaining. After washing 3 times with aqua dest., slides were dipped into ammonium water so that the nuclei staining changed color from red to blue due to the changing pH. Slides were mounted with Kaiser’s glycerol gelatine (Merck).

The only differences in immunocytochemistry is that instead of TBS-T TBS was used for washing steps and the UltraVision Protein Block (UltraVision LP detection system, Thermo Scientific) containd 10% human serum.

Cell culture experiments

In the cell culture different experiments with the SGHPL-4 cell lines and the THP-1 cell line were seeded with different conditions and special components. After harvesting the supernatants, the RNA lysates or the protein lysates, the samples could be analyzed on how the different conditions affected their growth, gene expression, protein synthesis and activation.

Batimastat experiment

In this experiment it should be tested whether the matrix metalloprotease (MMP) inhibitor bastimastat can increase the membrane-bound form of fractalkine. Based on recent research, the proportion of soluble and membrane-bound fractalkine seems to persist constant from early until term placenta. This ratio may be influenced by parameters such as gene expression and metalloprotease dependent shedding. The phenomenon of increased membrane-bound form of fractalkine in cells treated with batimastat was explained as a consequence of the damaged shedding activity of the metalloprotease, which happened due to batimastat's metalloprotease inhibition activity. (Bourd-Boittin et al. 2009; Ludwig et al. 2002; Siwetz et al. 2014)

For this experiment 3 wells of a 6 well plate were seeded with SGHPL-4 CX3CL1 overexpressing cells at a density of 6.0 x 10^5 cells per well. Different concentrations of batimastat were used (0μM, 5μM, 10μM (**Fig. 5**)) to see the interaction of the inhibitor with the shedding activity of the metalloprotease and the differences in the fractalkine forms in protein lysates and supernatants after 48h.

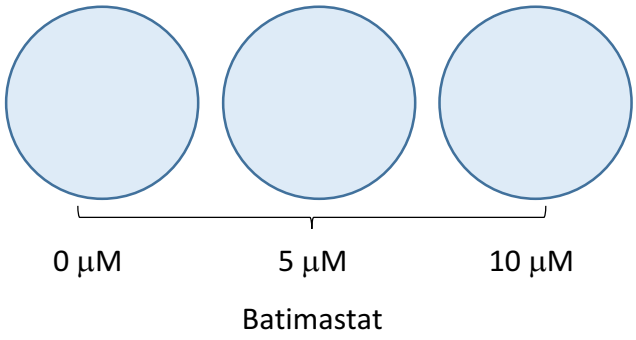

Fig. 5: Inhibition of shedding activity of the metalloprotease by batimastat. *A density of 6.0 x 105 SGHPL-4 cells per well in a 6 well plate were incubated with three different concentrations of batimastat (0 µM, 5 µM and 10 µM).*

After an incubation time of 48 hours the supernatants of each well were collected as well as protein and RNA lysates. All samples were further analyzed.

Monocyte activation by SGHPL-4 control cells vs SGHPL-4 CX3CL1 overexpressing cells

In this experiment the activation of the THP-1 monocyte cell line under the effect of fractalkine was tested. Therefore, the THP-1 cells were incubated for 24 h under different conditions in triplicates (**Fig. 6**). The activation by the soluble form of fractalkine was tested by letting the THP-1 cells (6.0 x 10^5/3ml/well in a 6 well plate) grow in the supernatants (SN) of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 overexpressing cells. Also, the THP-1 cells (3.0 x 10^5/well in 2 ml in a 12 well plate) incubated directly on the two different SGHPL-4 cells (2.0 x 10^5/well seeded and grown for two days) for testing the activation by the membrane bound form. As a background test the monocyte cell line was also incubated reclusively in the F-10 culture medium of the SGHPL-4 cells and the two SGHPL-4 cell lines were incubated solitarily in F-10 culture medium.

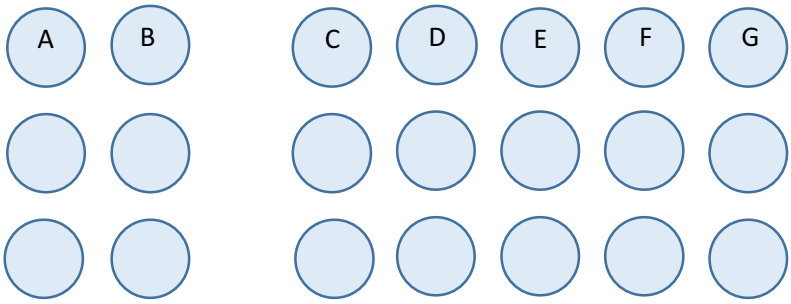

Fig. 6: Monocyte activation by soluble and membrane bound forms of fractalkine from SGHPL-4 control and CX3CL1 overexpressing cells. *All approaches as triplicates with an incubation time of 24 h: (A) Supernatant SGHPL-4 Control + THP-1; (B) Supernatant SGHPL-4 CX3CL1 + THP-1; (C) SGHPL-4 Control + THP-1; (D) SGHPL-4 CX3CL1 + THP-1; (E) THP-1 in F-10 medium; (F) SGHPL-4 Control in F-10 medium; (G) SGHPL-4 CX3CL1 in F-10 medium.*

After 24 hours of incubation time the supernatants of each well were collected for IL-6 and IL-8 ELISA, also RNA was collected for qPCR analysis of fractalkine and interleukins.

THP-1 adhesion assay

In this experiment, it should be tested whether THP-1 monocyte cells have a higher tendency to bind the SGHPL-4 CX3CL1 overexpressing cells due to its higher content of membrane bound fractalkine which interact with monocyte cell line due to the fractalkine receptor CX3CR1.

Both SGHPL-4 cell lines were seeded with a density of 2.0 x 10^5 cell per well as triplicates in a 12 well plate. After those cells grew to become almost confluent, the THP-1 cells were incubated with Cell Tracker Green CMFDA (Invitrogen) that gave them an unspecific green fluorescence at a wave length of 492/517 nm maxima. After the seeded SGHPL-4 cells were washed with PBS, 1.5 x 10^5 fluorescence stained THP-1 cells were resuspended in F-10 medium and incubated on the SGHPL-4 cells for 90 minutes. Afterwards the medium was removed and the wells were carefully washed three times with PBS to wash away all unbound monocyte cells. Then the cells were fixed with 4% paraformaldehyde for 30 minutes. After removing the paraformaldehyde PBS was added to the wells and the wells could be analyzed with the Cell-IQ®, which is a fully integrated, automated cell culture and analysis system for kinetic cell studies. THP-1 adhesion was evaluated by acquisition of extravillous trophoblast monolayer areas and bound THP-1 cells in phase contrast and green fluorescence channel. Pixel areas of bound THP-1 cells were related to pixel areas of trophoblast monolayers in 16 images per well.

Transfection of trophoblast cells

The K2® Transfection System (**Fig. 7**) includes the K2® Transfection Reagent (based on powerful cationic lipids) and the K2® Multiplier (decreases the cells' ability to detect foreign nucleic acids and increases the transfection efficiency).

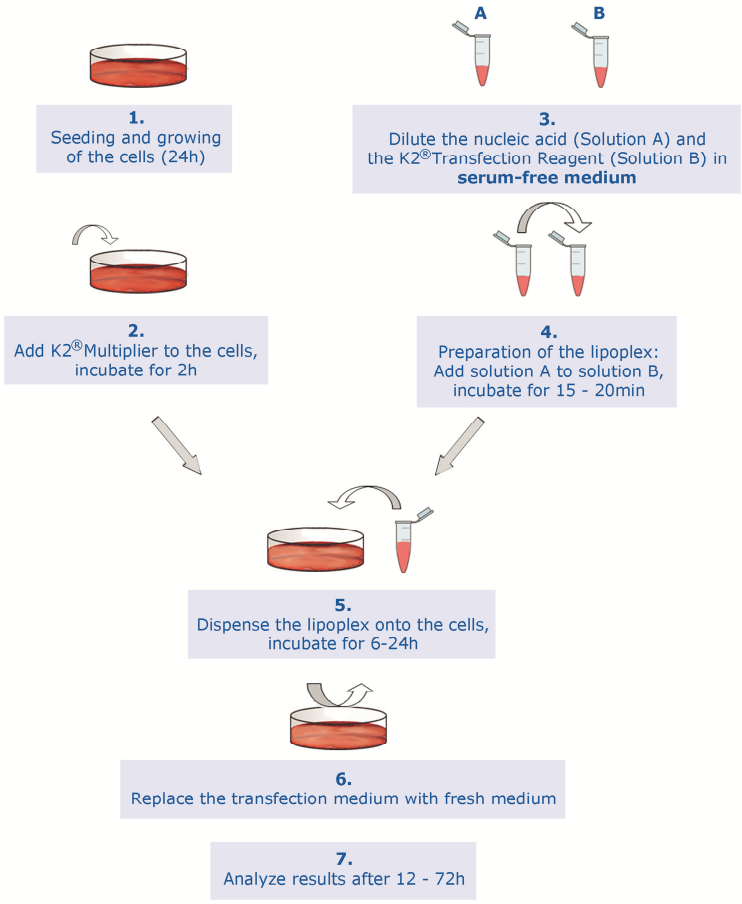

Fig. 7: Work plan of the K2® Transfection System. *Cells were seeded and grew for 24h. The K2® Multiplier was added for 2h, before the diluted nucleic acid and K2® Transfection Reagent was added. After 6-24h of incubation the transfection medium is replaced with fresh medium and the results can be analyzed after 12-72h.*

The SGHPL-4 control cells were seeded with a density of $3.0x10^5$ cells in wells of a 12 well plate and incubated for 24h at 37°C in a CO_2 incubator. All transfection solutions should be at room temperature. Two hours before adding the lipoplex of K2® Transfection Reagent and plasmid, 10 µl K2® Multiplier is added to the well of cells to be transfected. Following 4.0 µl K2® Transfection Reagent is diluted with 50 µl PBS and 1.0 µg of plasmid is diluted with 50 µl PBS as well. Both solutions were mixed gently by pipetting up and down once. The two solutions were combined, mixed by carefully pipetting up and down once and then incubated at room temperature for 15-20 minutes. After incubation, the mixture was immediately added to the cells. The well was agitated gently and afterwards incubated at 37°C in an CO_2 incubator. After 24h the transfection mixture was replaced with fresh growth medium. 24h-48h after adding the lipoplex the cells could be tested for reporter gen activity. ("K2 Transfection System, Biontex" 2017)

RNA Analysis

From cell culture experiments cells were harvested, RNA was isolated and the amount of RNA determined via NanoDrop. Then RNA was transcribed into complementary deoxyribonucleic acid (cDNA) with the reverse transcription. The cDNA is needed for performing a quantitative Polymerase Chain Reaction (qPCR) with which different parameters on RNA level can be analyzed.

RNA Lysate

The isolation of ribonucleic acid (RNA) is based on single-step method by Chomczynski and Sacchi. It contains a cell lysing step to set the cell components free and thereafter, the RNA gets separated from the other cell components (**Fig. 8**). (Chomczynski and Sacchi 1987)

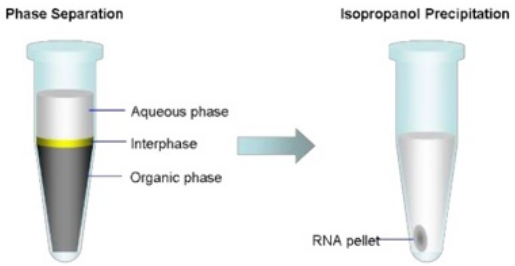

***Fig. 8: Phases of the RNA extraction. RNA is solubilized in the aqueous phase**, DNA is contained in the interphase and proteins and other cell components can be found in the organic phase. The RNA gets precipitated with isopropanol.*

Ribonucleic acid (RNA) was harvested with peqGOLD Trifast for cell lysing. The homogenized peqGOLD TriFast treated samples were vortexed and frozen at -20°C to set the cell components free. Afterwards they were thawed and 1/10 of the initial peqGOLD TriFast volume of the Phase Separation Reagent (BCP) was added to each sample. The samples were tipped over 20 times, incubated for 15 minutes at room temperature and centrifuged (15 minutes, 11300 rpm, 4 °C). Afterwards the solution had separated into three phases. The RNA was solved in the aqueous phase, the DNA was located in the interphase and the organic phase contained proteins and other cell components. (“peqGOLD Trifast” 2017)

Next, only the aqueous phase, containing RNA, was transferred into a new RNase free tube. The interphase should be avoided as it could contaminate the RNA with DNA. The RNA was

precipitated with 250 µl of Isopropanol for 10 minutes at RT and later centrifuged (10 minutes, 11300 rpm, 4 °C).

The supernatant was carefully removed and the pellet washed with 1 ml of ice-cold 70 % ethanol. After the next centrifugation step (4°C, 10 minutes, 11300 rpm), the ethanol was removed and the pellet dried. In the end, no residual liquid should be left in the tube.

The pellet was resuspended with 50 µl RNase-free water and for better solubility heated for 10 minutes at 55°C and 300 rpm on a shaker.

Nano Drop – RNA measurement

To determine the RNA concentration 1.5 µl of each sample was measured by NanoDrop Spectrophotometer.

The method was based on the measurement of the absorbance at a specific wavelength of interest. RNA has the highest absorbance at 260 nm. Lambeert Beer's Law was used to calculate the RNA concentration in each sample. ("NanoDrop Technologies, Inc." 2017)

The RNA is considered relatively pure with a 260 nm/280 nm ratio of 2.0. The presence of proteins, phenol or other components, which absorb at 280 nm, can be the reason of contamination and with that for lower values. Another indicator for the samples pureness is the ratio between 260 nm and 230 nm. The value of 2.0-2.2 can be contaminated with carbohydrates or residual phenols and decrease this value. ("NanoDrop Technologies, Inc." 2017)

Reverse Transcription

The reverse transcription is the transcription of RNA into complementary deoxyribonucleic acid (cDNA). ("Reverse Transcription Basics" 2017) The cDNA is needed for performing a quantitative Polymerase Chain Reaction (qPCR).

The general procedure of a reverse transcription is, that a short primer binds to the complementary RNA strand. A cDNA strand is synthesised by a RNA-dependent DNA polymerase, the reverse transcriptase. ("Reverse Transcription Basics" 2017) The RNA strand, of the now established cDNA/RNA hybrid, is hydrolysed and the DNA depending polymerase

completes the single cDNA strand to a double strand cDNA molecule, which is required for the qPCR.

At first, the necessary volume of each RNA sample of 1 µg was calculated and diluted with RNase-free water to a volume of 10 µl. High Capacity cDNA Reverse Transcription Kit (Thermo Scientific) was used and the necessary components of the mastermix for the reverse transcription were thawed on ice (except the reverse transcriptase) and mixed together. (**Table 1**) All operation steps were performed on ice. The reverse transcriptase should be added last and it should be kept on ice at all time.

Table 1: Components of the Reverse Transcription mastermix.

Components of the mastermix	µl/reaction
10x RT Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2

In mini-tubes 10 µl of the mastermix and 10 µl of the prediluted RNA (1 µg/10 µl) were merged. Before starting the thermo cycler, the reaction tubes were gently mixed. The reverse transcription was performed by a series of heating steps (**Table 2**).

After the program had ended, the samples were diluted to a final concentration of 1 ng/µl cDNA with RNase-free water.

Table 2: Program of the Reverse transcription.

time	temperature
10 minutes	25°C
120 minutes	37°C
5 minutes	85°C
forever	4°C

qPCR

Quantitative or real time polymerase chain reaction (qPCR) combines exponential PCR amplification of DNA templates and detection in a single step. It allows the measurement of the amplification product in real time and therefore, the determination of the initial amount of target gene mRNA in the tissue of interest. (Arya et al. 2005) Taq-Polymerase is a thermo stable polymerase from *Thermus aquaticus*, which performs the synthesis at over 60°C without denaturation.

The ready-to-use iTaq Universal SYBR Green Supermix (Bio Rad) contained next to other components the hot-start iTaq DNA polymerase and the SYBR® Green Dye, a double strand DNA (dsDNA) binding dye. It has a non-specific binding to the minor groove of dsDNA, which results in a fluorescence signal if bound (**Fig. 9**). (Arya et al. 2005) The thermo cycler (Bio Rad Laboratories 2000) detected the fluorescence in the exponential phase during each cycle.

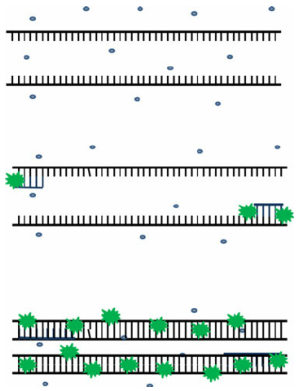

Fig. 9: Principle of the Taqman SYBR Green qPCR*. When SYBR Green binds to the dsDNA a fluorescence signal occurs.*

First, primers and the cDNA samples were thawed on ice. 2.5 µl of the primer mixture [800 nM] (**Table 3**) were transferred into every required well on a 96-well plate in duplicates. As a house keeping gene GAPDH (Microsynth) was used for all experiments.

Table 3: Primer that were used in the qPCR analysis.

Primer	Company	Sequences
GAPDH	**Microsynth**	**fwd: 5'-ACC CAC TCC TCC ACC TTT GA-3'** **rev: 5'-CTG TTG CTG TAG CCA AAT TCG T-3'**
CX3CL1 V1	**Microsynth**	**fwd: 5'-CAC CAC GGT GTG ACG AAA TG-3'** **rev: 5'-TCT CCA AGA TGA TTG CGC GT-3'**
CX3CL1 V2	**Microsynth**	**fwd: 5'-GCC ATG GCT CCG ATA TCT CT-3'** **rev: 5'-CTG TCT CGT CTC CAA GCA GC-3'**
IL-1A	**Microsynth**	**fwd: 5'-CTG CTG AAG GAG ATG CCT GA-3'** **rev: 5'-TGC CGT GAG TTT CCC AGA AG-3'**
IL-1B	**Microsynth**	**fwd: 5'-GCA GAA GTA CCT GAG CTC GC-3'** **rev: 5'-TCC TGG AAG GAG CAC TTC ATC T-3'**
IL-6	**Microsynth**	**fwd: 5'-AAC CCC CAA TAA ATA TAG GAC TGG A-3'** **rev: 5'-GGA CCG AAG GCG CTT GT-3'**
IL-8	**Microsynth**	**fwd: 5'-TCT GTG TGA AGG TGC AGT TTT G-3'** **rev: 5'-ATT TCT GTG TTG GCG CAG TG-3'**

The cDNA (1 ng/µl cDNA) was mixed with the Bio Rad – iTaq Universal SYBR Green Supermix in a fresh reaction tube (**Table 4**). The mastermix should be always kept on ice. 7.5 µl of the master mix were then added to each required well. The total volume in each well for the qPCR was 10 µl.

Table 4: Components of the qPCR Mastermix.

Components	µl/reaction
Primer	2.5
1 ng/µl cDNA	2.5
SYBR Master Mix	5
Total Volume	**10**

After fixing the adhesive sheet onto the plate, it was centrifuged (1 minute, RT, 300 g) and afterwards put into the thermo cycler. The program for amplifying and detecting the cDNA was started and the data was later analyzed with the Bio Rad CFX Manager 3.1 program.

Interleukin 1

This cytokine exists in two forms, which are encoded by two genes. IL-1A and IL-1B are mainly produced by activated macrophages, but also by most nucleated cells. They are involved in different immune responses and inflammatory processes. Therefore, they are also involved in T-lymphocyte activation and proliferation and via IL-2 B-lymphocytes. (Schaller et al. 2008)

Interleukin 6

Interleukin 6 (IL-6) acts as both an anti-inflammatory myokine and a pro-inflammatory cytokine. (Ferguson-Smith et al. 1988) It is secreted by T-cells and macrophages to stimulate immune response, e.g. after infection or any tissue damage leading to inflammation. (van der Poll et al. 1997) IL-6 stimulates the inflammatory and auto-immune processes in many diseases such as diabetes, depression, SLE, prostate cancer, rheumatoid arthritis and many more.

Interleukin 8

After activation, monocytes can produce different cytokines that can be of pro-inflammatory nature. (Gordon and Taylor 2005) Interleukin 8 (IL-8) for example is a member of the CXC chemokine family and was primarily discovered as a potential chemoattractant for lymphocytes and neutrophils. (Matsushima et al. 1988; Matsushima, Baldwin, and Mukaida 1992) As indicated by recent studies IL-8 could also cause angiogenesis, tumor growth, invasion and potential metastasis in cancer. (Maeda et al. 1998)

Protein Analysis

From cell culture experiments cells were harvested, protein was isolated and the amount of protein in the lysate determined, which was used to calculate the right amount of lysate, that had to be applied to the western blot. With the western blot proteins of interest could be detected and their amount defined.

Protein Lysate

To gain protein lysate out of a cell line, first the medium of the cells was removed and the cells were washed with PBS buffer. Afterwards a mixture of RIPA buffer and 1x protein inhibitor cocktail (PIC) was added to the cells, which were brought into suspension by a cell scratcher. The suspension was then transferred into a tube, shortly vortexed and afterwards frozen, for a better breakage of the cells. Then the protein lysate was thawed again, centrifuged (8000rpm, 10min, 4°C) and the supernatant was transferred to a fresh tube.

Lowry-Protein Assay

The aim of the protein determination after lowry is to identify the amount of protein in each sample so that the same amounts of proteins can be used in further experiments and results can be compared.

The reaction mixture of solution A (100 parts), solution B (1 part) and solution C (1 part) (see chapter “materials”) was prepared. 3 µl of each sample and each protein standard were mixed with 375 µl of the incubation mixture, vortexed and incubated for 10 minutes at room temperature. Meanwhile the folin reagent was mixed equal parts water and folin (Ciocalteus Phenol Reagent (Merck)), of which 37.5 µl was added to each sample, vortexed again and incubated for 30 minutes at room temperature. Subsequently 190 µl in double determination were transferred into a 96 well plate (**Table 5**) and read out by the program ADAP at 620nm.

Table 5: Application of the Lowry protein determination.

	1	*2*	*3*	*4*	*5*	*6*
A	blank	blank	S1	S1	S9	S9
B	0.1 STD	0.1 STD	S2	S2	S10	S10
C	0.5 STD	0.5 STD	S3	S3	…	…
D	1 STD	1 STD	S4	S4	…	…
E	2 STD	2 STD	S5	S5		
F	4 STD	4 STD	S6	S6		
G	6 STD	6 STD	S7	S7		
H	8 STD	8 STD	S8	S8		

Western blot

The western blot is an immunological technique to detect protein that are solved in a solution. In addition to the samples a protein standard with defined molecular weight and concentration was separated, so that the blotted proteins could be identified and quantified. (Berg, Tymoczko, and Stryer 2002)

SDS Gel electrophoresis

To separate proteins by their molecular weight, a SDS page was performed. The strongly negative charged SDS molecule covers the intrinsic charge of the proteins and ensures the separation of the denatured proteins by molecular weight rather than by their charge. The negative SDS-protein complex moves in direction of the anode during the electrophorese process. (Watson et al. 2011)

The samples thawed on ice and were diluted with RNase-free water to the final concentration of 30 µg protein. The total volume of 30 µl (samples, RNase-free water, 7.5 µl 4x LDS and 10x Reducing agent DTT-1M) was heated at 95°C for 5 minutes.

The electrophoresis chamber was prepared with a gel (10%) and running buffer (1x MES in Aqua dest.). The gel was then loaded with one sample in each well. As a standard 4 µl of MagicMark XP Western Protein Standard (NOVEX) and 3 µl See Blue Pre-Stained Protein Standard (NOVEX) were applied into the first well. After electrophoresis of approximately one hour at 140 volt, the proteins were separated.

Blotting

After the SDS Gel electrophoresis the separated proteins were transferred onto a nitrocellulose membrane, because there the proteins could be detected much easier. (Mahmood and Yang 2012; Berg, Tymoczko, and Stryer 2002) All needed components for the electrophoretic transfer were soaked in transfer buffer (NuPAGE® Transfer buffer (20x) (novex by Life Technologies), 5 (v/v) % Methanol solved in Aqua.dest), except the membrane which got activated in aqua dest. first. The components for the blotting process needed to be arranged as it can be seen in **Fig. 10**. The membrane is laid on the filter paper. On top of the membrane the SDS-Gel is placed and then another filter paper. This is the sandwich between the anode and the cathode for the blotting process. The proteins were then blotted at 160 volt and 250 mA for 1.5 hours.

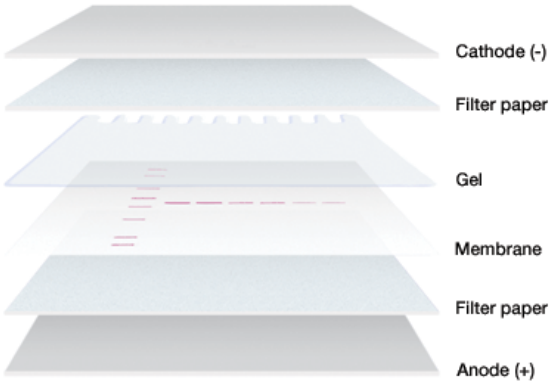

Fig. 10: Gel and membrane setup for electrophoretic transfer. *Anode – filter paper – membrane – SDS-Gel – filter paper – cathode. This is the sandwich between the anode and the cathode for the blotting process.*

As a control for the blotting efficiency, the membrane was stained with Ponceau red for 5 minutes. Afterwards it was washed with Aqua dest. and unspecific bindings were blocked for one hour with 5 (v/v) % milk powder (solved in TBST).

Primary antibody

The first antibody against the target antigen was incubated overnight at 4°C. (Watson et al. 2011) The membrane could be cut into different pieces if antibodies for different target proteins of different molecular weight were used (**Table 6**).

Table 6: Primary antibodies for western blot detection.

1st antibody	*concentration*	*Species*	*Company*
ß-actin	12.4 ng/ml	mouse	abcam
fractalkine	0.4 µg/ml	goat	R&D Systems
CX3CR1 receptor	2.0 µg/ml	rabbit	Acris
CX3CR1 receptor	2.0 µg/ml	rabbit	Sigma

Secondary antibody

The next day the membrane was washed three times for 10 minutes with TBS-T to wash off any unbound antibodies. Then specific secondary antibodies were put onto the membrane, to bind the primary antibody-antigen-complex. (Berg, Tymoczko, and Stryer 2002) It incubated for 2 hours at room temperature.

- anti-mouse antibody (Bio Rad) (diluted 1:3000 with TBS-T)
- anti-goat antibody (Dako) (diluted 1:3000 with TBS-T)
- anti-rabbit antibody (Bio-Rad) (diluted 1:3000 with TBS-T)

Detection

After washing three times for 10 minutes with TBS-T and then in TBS, the chemiluminescence development with WesternBright solutions (1:1 Peroxide and Quantum (advansta)) was performed to detect the proteins. Images were acquired with FluorChem Q System (Alpha Innotech) and band densities were analyzed with Image Studio Lite (Version 5.2).

ELISA

The used ELISAs were quantitative sandwich enzyme-linked immunosorbent assays (**Fig. 11**) with which specific factors in the supernatants from the cell culture could be analyzed.

A monoclonal antibody specific against the antigen of interest had been pre-coated onto the wells of the ELISA. Standards and samples were transferred into the wells and all antigens of interest present were bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific against the antigen of interest was added to each well. Afterwards any unbound antibody-enzyme reagent was removed by another washing step. Following the addition of a substrate solution to each well, a colour developed in proportion to the amount of the prior bound antigen of interest. The colour development was stopped and the intensity of colour was measured by Spark 10M Multimode Microplate Reader (Tecan). ("Quantikine ELISA, R&D Systems" 2017)

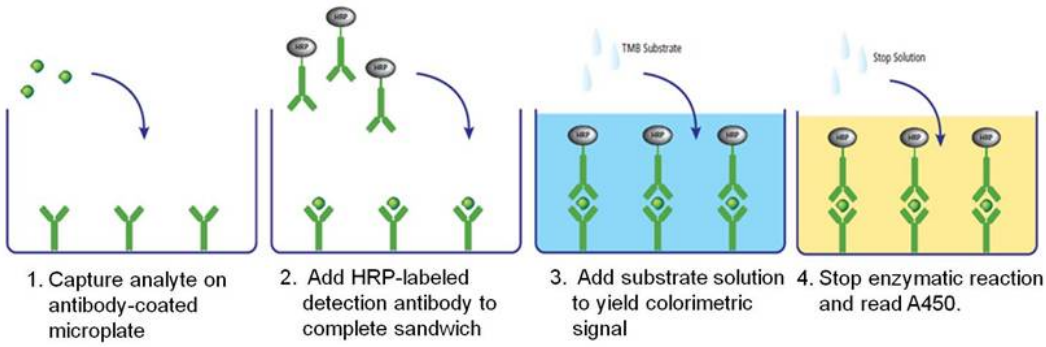

***Fig. 11: Reaction theme of a sandwich ELISA**. The primary antibody was already immobilized onto the wells. They did bind the target antigen in the initial step. Next, the enzyme-linked antibody did bind to the already bound antigen of interest and in the presence of a substrate a colorful product was catalyzed.*

Results & Discussion

Immunohistochemistry of CX3CL1 in human placenta

After first trimester placenta tissue samples were fixed, embedded in paraffin and cut into sections, an antigen retrieval with pH 6 and 120°C in the cooking pot was performed. The primary mouse antibody against fractalkine (R&D Systems) was used at a concentration of 0.5 μg/ml and the primary goat antibody against fractalkine (R&D Systems) was used at a concentration of 2 μg/ml. For each antibody a negative control was performed (Dako and Southern Biotechnology Associates, Inc.). The stained placenta sections were mounted with Kaiser's Glyceringelatine (Merck) and left to dry over night before the sections were microphotographed using the Axiophot microscope (Zeiss) with a AxioCam HRc camera (Zeiss).

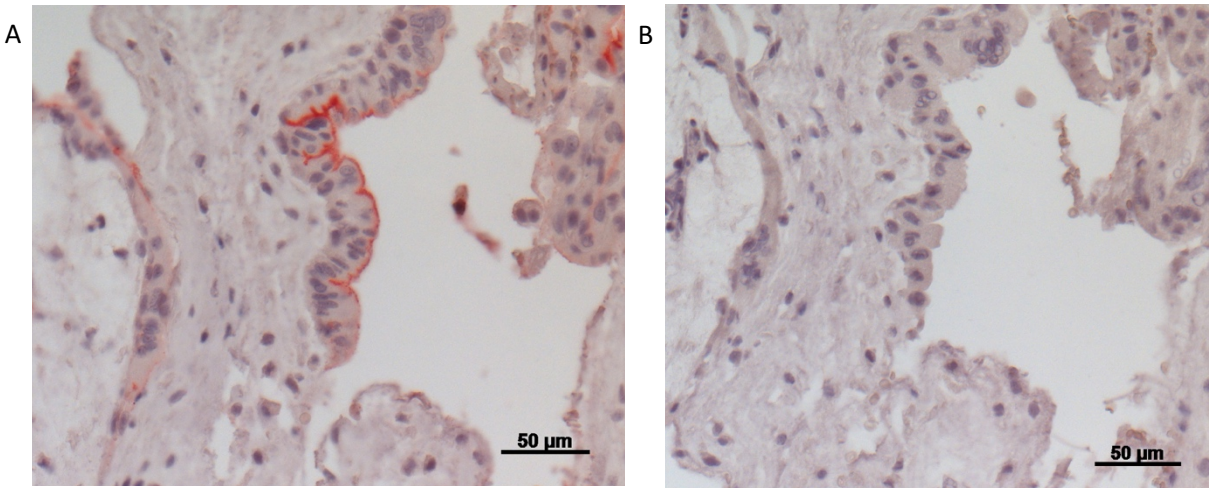

Fig. 12: First trimester placenta tissue sections stained for fractalkine using AGR: pH6 and 120°C. *(A) stained with anti-fractalkine mouse antibody (R&D Systems) (0.5 μg/ml) and (B) negative control with an anti-mouse IgG antibody (Dako). Axiophot microscope (Zeiss) with a AxioCam HRc camera (Zeiss).*

As previously shown by Siwetz et al., fractalkine is expressed in the syncytiotrophoblast of the villi in the placenta (**Fig. 12**). (Siwetz et al. 2014) The mouse anti-CX3CL1 antibody showed a colorful staining, whereas the goat anti-CX3CL1 antibody did not seem to be suitable for IHC staining. The mouse anti-CX3CL1 antibody showed a staining directly at the barrier of the fetal tissue and the maternal blood circulation. As CX3CL1 consists of an extracellular N-terminal domain, a transmembrane alpha-helix and a short cytoplasmic tail (Umehara et al. 2004; Jones, Beamer, and Ahmed 2010) the hypothesis was raised, that it could interact with components of the maternal blood, bind them to the fetal tissue and/or activate them.

Characterizing SGHPL-4 Control and CX3CL1 overexpressing cells

The cell line that was worked with in this thesis was an extravillous trophoblast cell line. Hence, the cells are not a suitable model for trophoblasts covering the placental villi, but rather resemble trophoblasts, lining for example the maternal spiral arteria luminal. For that reason, initial experiments were performed to characterize the SGHPL-4 cell line and determine the overexpression of the CX3CL1.

Chamber slide

The cells (8.0 x 10^4 cells/chamber) grew for 2 days on chamber slides, before they were fixed and afterwards stained with a goat antibody against fractalkine (2 µg/ml) (R&D Systems) and mounted with Kaiser's glycerin mounting medium (Merck). The pictures were taken at Axiophot microscope (Zeiss) with an AxioCam HRc camera (Zeiss).

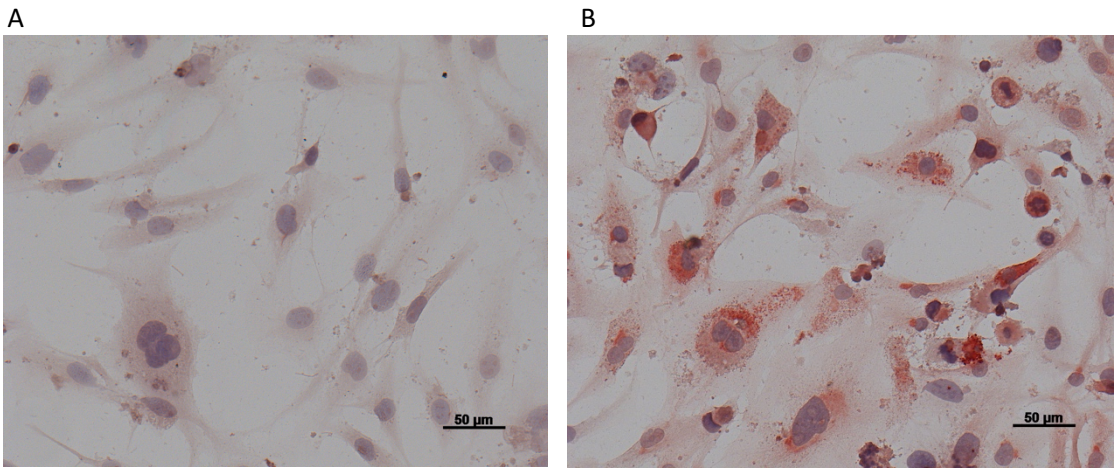

Fig. 13: Chamber slides of (A) SGHPL-4 control cells and (B) SGHPL-4 fractalkine overexpressing cells stained with goat-antibody against fractalkine (2 µg/ml)*. The control cells showed no specific staining, whereas the overexpressing cells had the specific staining. Axiophot microscope (Zeiss) with a AxioCam HRc camera (Zeiss).*

The control cells showed very weak to no staining of fractalkine. Only the counterstaining with hemalaun of the acid components of the cells were visible. However, the fractalkine overexpressing cells showed distinct staining of fractalkine and therefore confirmed the overexpression in a visible way (**Fig. 13**).

RNA

The next step of the characterization of the SGHPL-4 cells, after confirmation via immunocytochemically visualization, was the examination of fractalkine expression on RNA level.

RNA lysates of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 cells were produced and after the nano-drop confirmation of sufficient RNA content, a reverse transcription was conducted to transfer the RNA into cDNA. With this cDNA a qPCR with specific CX3CL1 primers was run.

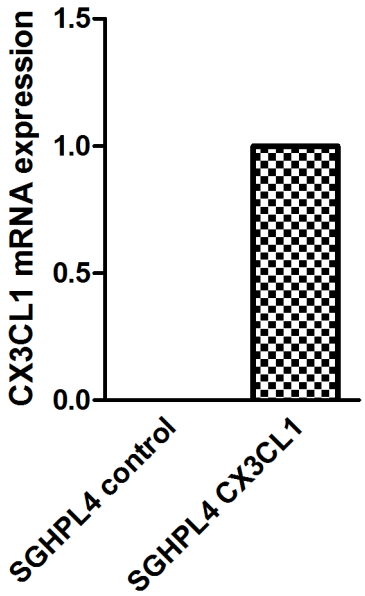

Fig. 14: RNA analysis of fractalkine in SGHPL-4 control cells and SGHPL-4 CX3CL1 cells. *An overexpression of fractalkine was found in the SGHPL-4 CX3CL1 cells on RNA level.*

The overexpression of fractalkine in the SGHPL-4 CX3CL1 cells was also confirmed on RNA level (**Fig. 14**). In comparison to that the SGHPL-4 control cells showed hardly any fractalkine expression on RNA level. This result confirmed the previous findings and showed that the control cells lacked any expression of fractalkine and the SGHPL-4 CX3CL1 cells are successfully transfected with the chemokine.

Protein

After the visualization of fractalkine via ICC on SGHPL-4 cells on the chamber slides and the examination of fractalkine on RNA level, the overexpression was confirmed on protein level.

Protein lysates of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 cells were produced and after the lowry assay, equal amounts of protein from the protein lysates and supernatants from the two SGHPL-4 cell lines and the recombinant fractalkine protein (R&D Systems) were separated in the SDS-gelelectrophoresis. The separated proteins were then blotted on a membrane and detected with a primary goat anti-fractalkine antibody (R&D Systems) and secondary anti-goat antibody (Dako). The detection was performed with bioluminescence and gave significant results (**Fig. 15**). Fractalkine was determined in three individual experiments.

The antibody detected band of the anti-CX3CL1 antibody (goat) at 95 kDa showed a significant overexpression of fractalkine in the SGHPL-4 CX3CL1 cells in comparison to the control cells (band densitometry gave a P value of less than 0.01). This was calculated with the “T-Test”. In the supernatant of the SGHPL-4 CX3CL1 cells fractalkine was found, too. This meant both forms of fractalkine could be detected by the goat anti-fractalkine antibody in the western blot. In the supernatant of the fractalkine overexpressing cells the soluble form was found and in the SGHPL-4 CX3CL1 cell protein lysate the membrane bound form was present. In comparison SGHPL-4 control cells and its supernatant showed hardly any reaction. The recombinant protein showed a clear band, but at a slightly different height than the other fractalkine bands. This could be traced back to the different length of the recombinant protein and the protein from SGHPL-4 cells. Other than the in vivo fractalkine, the recombinant protein consists of 314 amino acids with an additional 6-His tag and is therefore shorter than the 373 amino acids of the full-length fractalkine. This explains the different molecular weight of the recombinant protein at the western blot detection.

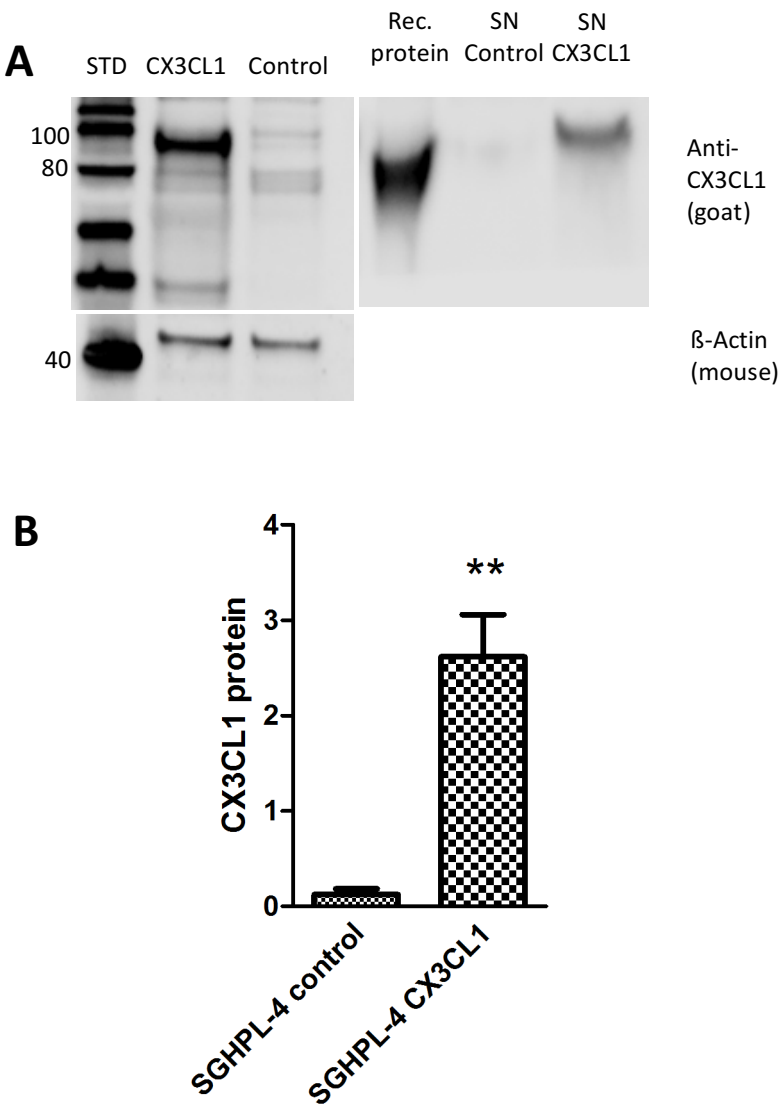

Fig. 15: Western blot analysis of SGHPL-4 control and SGHPL-4 CX3CL1 cells. *(A) Anti-CX3CL1 antibody (goat) (R&D Systems) – used concentration: 0.4 µg/mL. Clear bands shown at the SGHPL-4 CX3CL1 cells and the supernatant of the same cells as well as at the recombinant protein. Standard: MagicMark™ XP Western Standard (Invitrogen by Thermo Fisher Scientific) (B) The calculated ratio of the β-Actin (42kDa) and fractalkine (95 kDa) showed a significant overexpression of fractalkine in the CX3CL1 overexpressing cells in comparison to the control cells (P value ≤ 0.01).*

Interleukin analysis on protein level

The enzyme linked immunosorbent assays for interleukin 6 and interleukin 8 were performed with biological duplicates of supernatants (SN) of SGHPL-4 control cells, SN of SGHPL-4 CX3CL1 overexpressing cells, SN of THP-1 monocyte cell line, SN of THP-1 on SGHPL-4 control cells, SN of THP-1 on SGHPL-4 CX3CL1 overexpressing cells, SN of THP-1 in the SN of SGHPL-4 control cells and SN of THP-1 in SN of SGHPL-4 CX3CL1 overexpressing cells.

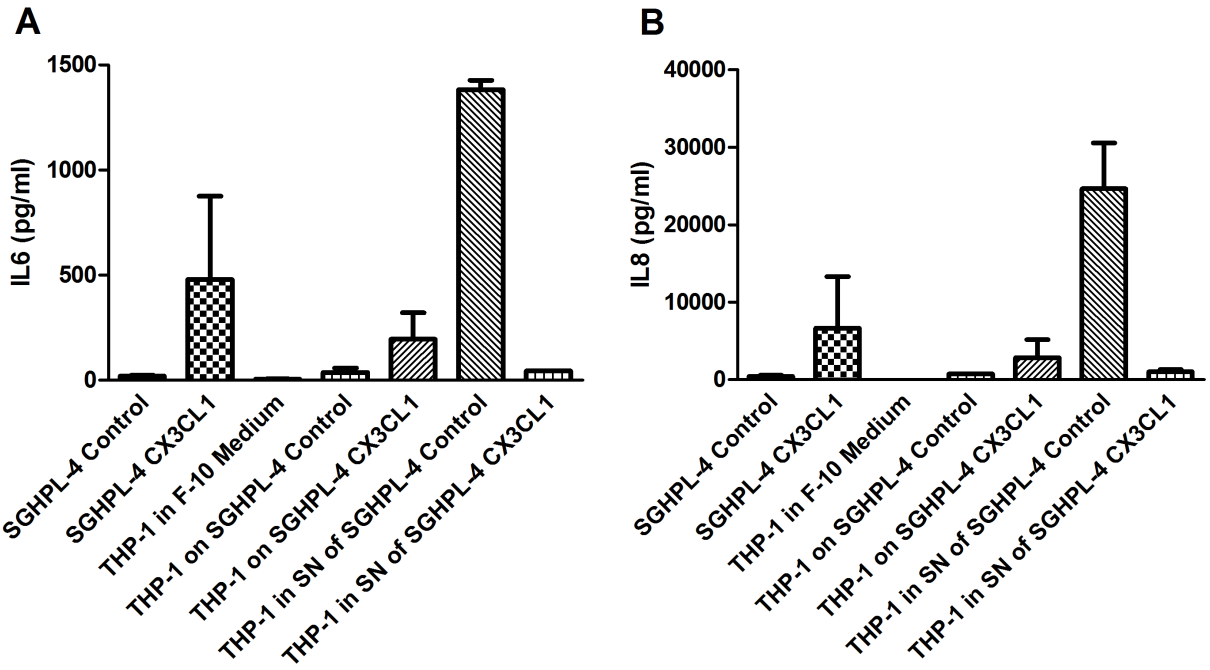

Fig. 16: Analysis of IL-6 and IL-8. The ELISA for (A) IL-6 and (B) IL-8 were performed with the supernatants (SN) of SGHPL-4 control cells, SN of SGHPL-4 CX3CL1 over expressing cells, SN of THP-1 monocyte cell line, SN of THP-1 on SGHPL-4 control cells, SN of THP-1 on SGHPL-4 CX3CL1 overexpressing cells, SN of THP-1 in the SN of SGHPL-4 control cells and SN of THP-1 in SN of SGHPL-4 CX3CL1 overexpressing cells.

The profile of IL-6 and IL-8 ELISA analysis did look surprisingly similar. But the overall expression of interleukin 8 was more than 10-fold higher than the expression of interleukin 6.

In comparison to the other samples the values of SN of THP-1, SN of SGHPL-4 control and SN of THP-1 on SGHPL-4 control were as expected low. Surprisingly also the values for SN of THP-1 in SN of SGHPL-4 CX3CL1 overexpressing cells were quite low. In these samples the amount of soluble fractalkine was the highest, and thus it was expected that the soluble form would activate the monocyte cell line. The third highest was the SN of THP-1 on SGHPL-4 CX3CL1 overexpressing cells. This was unexpected as it seemed like the fractalkine overexpressing cells produced the interleukins on their own and presence of THP-1 cells could have decreased the interleukin productions or reduced the amount rapidly. The values were still lower than in the SN of the SGHPL-4 CX3CL1 cells alone, which was not expected. These results indicated that the fractalkine, that was produced by the SGHPL-4 CX3CL1 cells, was used for auto regulation of the SGHPL-4 cells itself. However, this should be further analyzed on RNA level. Interesting however was that the SN of the THP-1 cells in the SN of SGHPL-4 control cells seemed to have the highest IL-6 and IL-8 production by far (**Fig. 16**). The IL-6 expression was 2.9-fold higher in controls than the expression in SGHPL-4 CX3CL1 cells, which was the second highest. Also, the IL-8 expression was 4.6-fold higher in controls than the expression of SGHPL-4 CX3CL1 cells, which was the second highest in IL-8 expression, too. As the expression of IL-6 and IL-8 was not nearly as high in the THP-1 cells in the SN of the SGHPL-4 CX3CL1 cells, there must be another factor present in the SN of SGHPL-4 control cells that is causing this activation. Another explanation would be that there is a mediating factor in the SN of the SGHPL-4 CX3CL1 cells that is suppressing the activation by inhibiting the substance directly or competing with it for binding sites. These assumptions, however, require further in-depth investigations.

Interleukin analysis on RNA level

The activation of the THP-1 monocyte cell line under the effect of membrane bound fractalkine was tested. Therefore, the THP-1 cells were co-cultured on the two different SGHPL-4 cell lines for 24 h and as a background test the monocytes were also incubated without trophoblasts in the F-10 culture medium. Moreover, the two SGHPL-4 cell lines were incubated without THP-1 monocytes in F-10 culture medium. After 24 h of incubation RNA was collected for qPCR analysis of interleukins in technical duplicates.

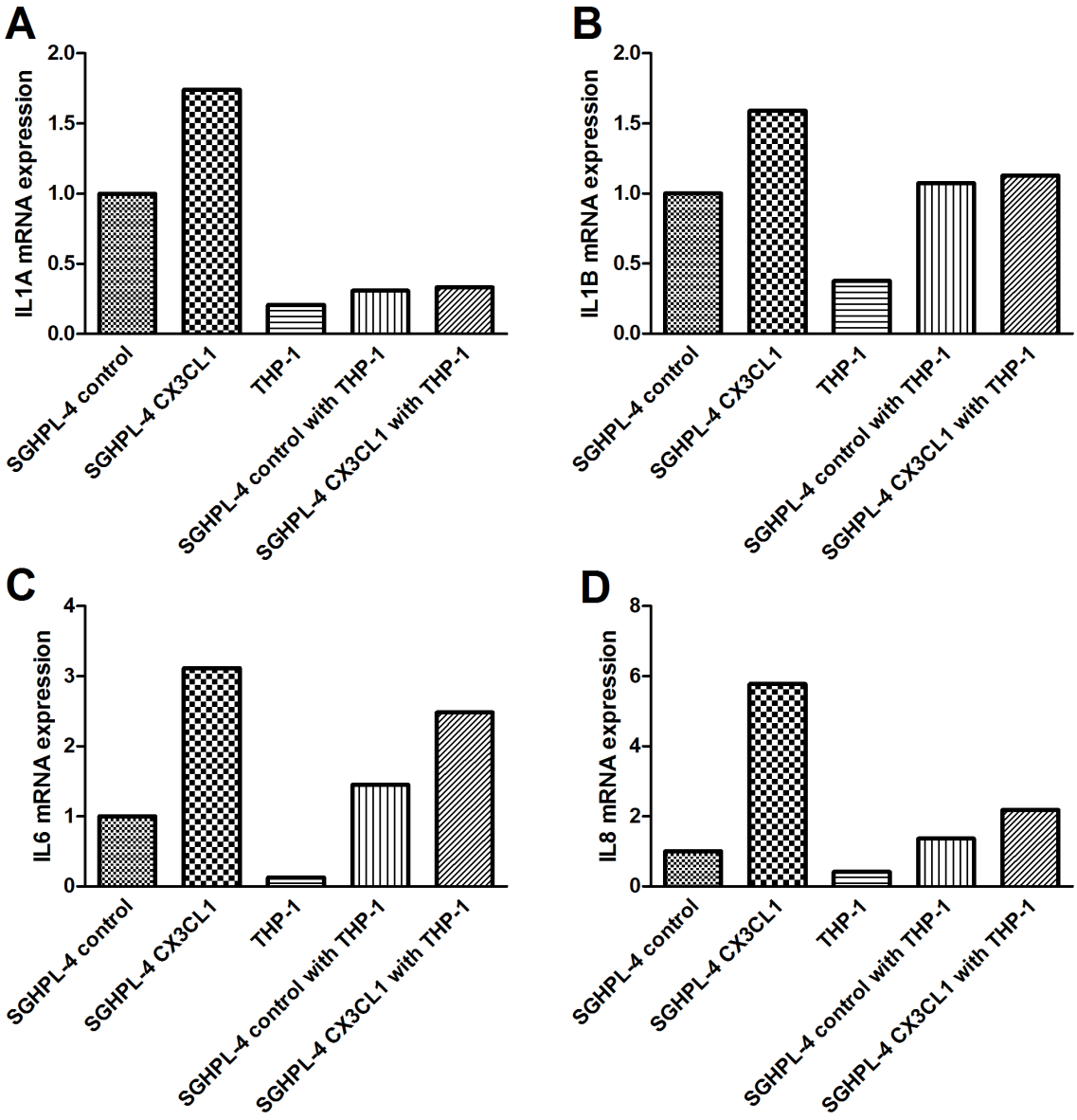

Fig. 17: qPCR analysis of interleukin mRNA expression in THP-1 cells and SGHPL-4 co-cultured. *(A) IL-1A, (B) IL-1B, (C) IL-6 and (D) IL-8 were analyzed in SGHPL-4 control, SGHPL-4 CX3CL1, THP-1, THP-1 with SGHPL-4 control and THP-1 with SGHPL-4 CX3CL1.*

The results showed that THP-1 cells expressed very little of the tested interleukins when incubated in F-10 culture medium. The SGHPL-4 control cells showed a little expression of the interleukins when cultured, but the CX3CL1 cells showed by far the highest expression of the interleukins on RNA level. In comparison to the SGHPL-4 control the RNA expression for IL-1A was 1.7-fold higher, for IL-1B 1.6-fold, for IL-6 3.1-fold and for IL-8 5.8-fold. This high expression was surprising, since expression of interleukins in THP-1 cells was expected to be upregulated by fractalkine. But the RNA of THP-1 and SGHPL-4 CX3CL1 co-cultured showed by far less of the investigated interleukins as the SGHPL-4 CX3CL1 cells alone (**Fig. 17**). This could mean that some of the fractalkine bound the receptor of the monocyte cell line and for that reason was not available for other activation processes of the SGHPL-4 cells itself (i.e. auto- and paracrine action). Maybe the THP-1 cells do express another factor that suppresses the interleukin production of the SGHPL-4 CX3CL1 cells in some way. Only 19% of the IL-1A, 71% of IL-1B, 80% IL-6 and 38% of IL-8 was detectable in SGHPL-4 CX3CL1 - THP-1 co-cultured in comparison to SGHPL-4 CX3CL1 solitarily. The THP-1 and SGHPL-4 control RNA showed a little rise (except for IL-1A) in comparison to the SGHPL-4 control cells. But as these cells do not express fractalkine at a significant level, the chemokine was not suspected to play a role in this rise of interleukins but other substances might.

Fractalkine seemed to have an auto- or paracrine autoregulation as interleukin 1A, 1B, 6 and 8 are so significantly higher expressed on RNA level in the SGHPL-4 CX3CL1 cells than in the SGHPL-4 control cells. Wojdasiewicz et al. already described in their paper that CX3CL1 synthesis and expression depends on several different factors, amongst others they mentioned auto- and paracrine regulation that could activate a network of intracellular messengers and transcription factors, leading to increased or reduced fractalkine production. (Wojdasiewicz et al. 2014; Cambien et al. 2001) Other factors mentioned were for example pro-inflammatory cytokines (Interleukin (IL)-1B, tumor necrosis factor (TNF)-α and interferon (IFN)-γ), tissue oxygen pressure and the presence of lipopolysaccharide. (Imaizumi, Yoshida, and Satoh 2004)

Also, the activation of the THP-1 cells under the effect of soluble fractalkine was tested. Therefore, the THP-1 cells were incubated in the two different supernatants of SGHPL-4 cell lines and as a background test the monocyte cell line was also incubated reclusively in the F-10 culture medium. After 24 h of incubation RNA was collected for qPCR analysis of interleukins. The amount of interleukins in the samples was determined in biological triplicates.

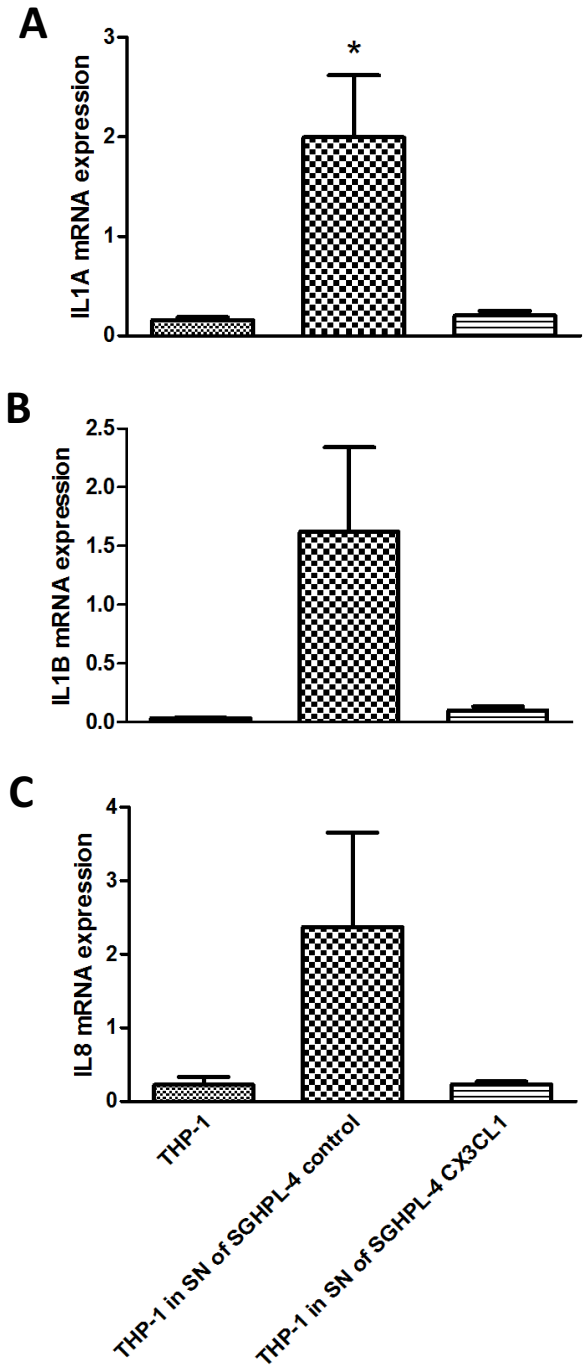

Fig. 18: qPCR analysis of interleukins in THP-1 cells cultured in supernatants of the two SGHPL-4 cell lines and in F-10 Medium. *(A) IL-1A, (B) IL-1B and (C) IL-8 were analyzed in THP-1 culture medium, THP-1 in SN of SGHPL-4 control, THP-1 in SN of SGHPL-4 CX3CL1. IL-1A of THP-1 in SN of SGHPL-4 control was significantly increased in comparison to the THP-1 cultured in F-10 medium (P value ≤ 0.05).*

As the Interleukin 6 results of the qPCR did not show any data, they were not analyzed in these studies. The interleukin qPCR analysis of THP-1 under influence of soluble fractalkine showed that THP-1 cell interleukin levels did not rise significantly while incubating in F-10 medium. Also, the rise of the interleukin levels in THP-1 cells incubating in the supernatants of SGHPL-4 CX3CL1 cells was almost the same as the levels in solitary medium. The surprising outcome was that the interleukin levels of THP-1 cells in the supernatants of SGHPL-4 control cells showed an increase for all tested interleukins (**Fig. 18**). In case of IL-1A the increase between THP-1 in SN of SGHPL-4 control and THP-1 cultured in F-10 medium was significant (P value $\leq$ 0.05). Data were tested with “ANOVA” and as a follow up test “Tukey”. The increase between THP-1 in SN of SGHPL-4 control and THP-1 in SN of SGHPL-4 CX3CL1 for IL-1A was 9.7-fold higher, for IL-1B 16.4-fold and for IL-8 10.0-fold. The increase was surprising as we expected the fractalkine in the supernatants to activate the monocyte cell line. CX3CL1 was already identified in the supernatant of the overexpressing cells by western blot in earlier experiments (see chapter “Characterizing SGHPL-4 Control and CX3CL1 cells – protein”). The supernatant of SGHPL-4 control cells did not contain nearly as much of the soluble form of fractalkine as the overexpressing cells. Thus, as three biological replicates of this experiment showed the same effect, one can conclude that the soluble form of fractalkine does not seem to activate the monocytes. As discussed above, the SGHPL-4 control cells may secrete another yet unidentified factor that seem to activate the THP-1 monocyte cell line to induce its interleukin production.

Currently unpublished data showed, that THP-1 monocyte cell line did not show any activation by increase of cytokine levels when being incubated with the recombinant protein fractalkine. This suggests that THP-1 cells do not get activated by fractalkine alone, but need one or more other co-factors for activation. This would explain why the THP-1 cells, that were incubated in the supernatant of the SGHPL-4 CX3CL1 cells, did not show an increased interleukin production.

Still another activation process without fractalkine is taking part when THP-1 cells incubated in the supernatant of SGHPL-4 control cells, which barely contained CX3CL1.

CX3CR1 in SGHPL-4

As the RNA analysis of the interleukins showed an increased production of interleukins in the SGHPL-4 CX3CL1 overexpressing cells, the assumption was, that these cells may also express the fractalkine receptor CX3CR1 for autocrine or paracrine activation.

For that reason, single cell paraffin embedding of the two SGHPL-4 cell lines was performed and after sectioning, deparaffinizing and performing AGR (pH6), the sections were immunocytochemically stained with a rabbit anti-CX3CR1 antibody (Sigma) (4 µg/ml) and a negative rabbit control (Neomarkers). Afterwards they were mounted with Kaiser's glycerin mounting medium (Merck).

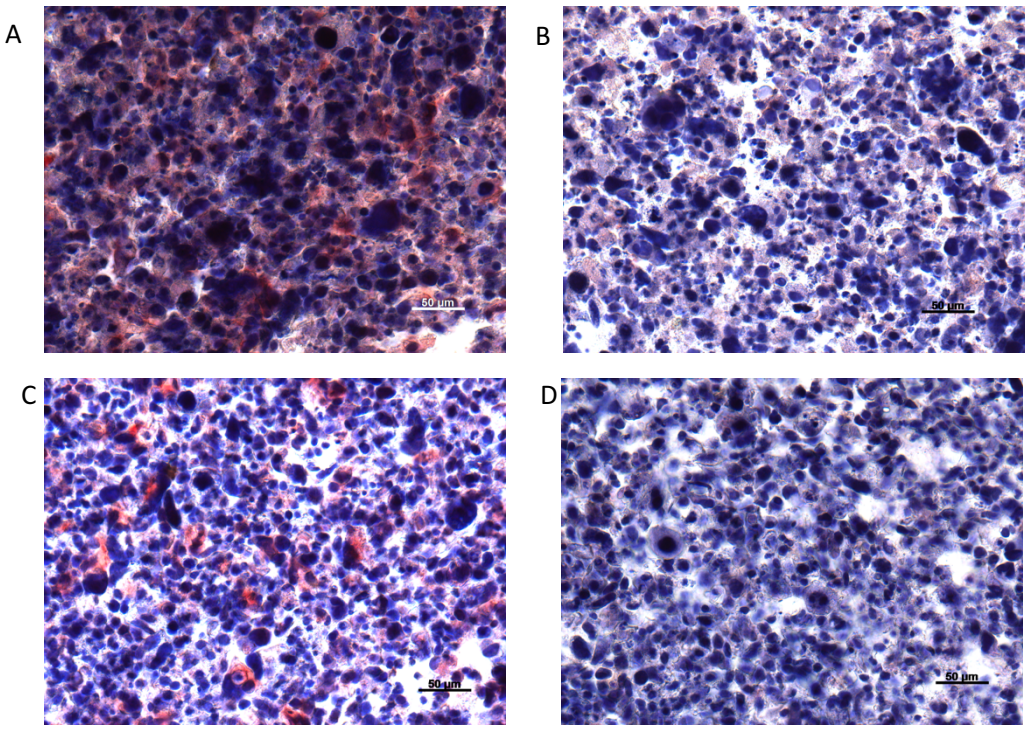

Fig. 19: Fractalkine receptor CX3CR1 staining of the single cell embedded SGHPL-4 control cells and SGHPL-4 CX3CL1 overexpressing cells*. pH 6 AGR was performed and the AB concentration was 4 µg/ml. A. SGHPL-4 control with CX3CR1 staining B. SGHPL-4 control with rabbit negative control staining C. SGHPL-4 CX3CL1 with CX3CR1 staining D. SGHPL-4 CX3CL1 with rabbit negative staining.*

The ICC staining of the fractalkine receptor CX3CR1 (red) showed that not all, but some of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 overexpressing cells expressed the receptor for fractalkine (**Fig. 19**). The negative control showed no staining.

It seemed like both SGHPL-4 cells expressed the receptor, a western blot was performed to confirm this finding. Two times equal amounts of protein lysates of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 cells were separated in the SDS-gelelectrophoresis. The separated

proteins were then blotted on a membrane and stained with two different primary rabbit anti-CX3CR1 antibodies (Sigma and Acris) and secondary anti-rabbit (Bio Rad) antibody.

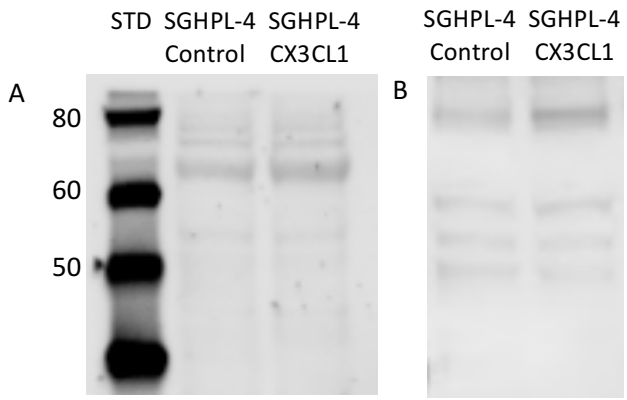

Fig. 20: Western blot of the fractalkine receptor CX3CR1 in SGHPL-4 control cells and SGHPL-4 CX3CL1 overexpressing cells*. A. Rabbit anti-CX3CR1 (Sigma) (2 µg/ml) B. Rabbit anti-CX3CR1 (Acris) (2 µg/ml)*

According to datasheets, both antibodies against CX3CR1 were described to work on immunoblotting and should give a band on around 50 kDa. The AB from Sigma detected a band that could be identified as the receptor protein at 65 kDa (**Fig. 20.A**), which would confirm the result from the immunocytochemical staining of the single cell embedding. Both SGHPL-4 cells featured the receptor protein. The other AB from Acris did show a band at the predicted 50 kDa, but next to that three other bands were visible, with the strongest one at 80 kDa. (**Fig. 20.B**). As the CX3CR1 antibody from Acris seemed to have a lot of unspecific binding the results were not taken into account.

As both SGHPL-4 cell lines appeared to have a certain percentage of cells that express the fractalkine receptor CX3CR1, it could be discussed whether CX3CL1 could have an auto- or paracrine function. This would explain the results of the interleukin qPCR which showed the highest interleukin release in SGHPL-4 CX3CL1 overexpressing cells. Wojdasiewicz et al. mentioned 2014 that CX3CL1 could have an autoregulatory function. (Wojdasiewicz et al. 2014) Kervancioglu Demirci et al. also mentioned that some trophoblast cell lines express both, CX3CL1 and its receptor. This makes it tempting to speculate on auto- or paracrine regulation of fractalkine and its receptor. Indeed, they found CX3CL1 and CX3CR1 expression in invading EVTs in human postpartum decidua sections via immunohistochemistry. (Kervancioglu Demirci, Salamonsen, and Gauster 2016)

Forms of fractalkine under batimastat

Protein lysates of the SGHPL-4 control cells and the SGHPL-4 CX3CL1 cells and from the Batimastat experiment (0 μM, 5μM and 10 μM) were produced and after the lowry assay, equal amounts of protein and respective supernatants were separated in the SDS-gelelectrophoresis. The separated proteins were then blotted on a membrane and stained with a primary goat anti-CX3CL1 antibody (R&D Systems) and secondary anti-goat antibody (Dako) for fractalkine detection and primary mouse anti-β-actin antibody (abcam) and secondary anti-mouse antibody (Bio Rad) for the control of equal protein loading.

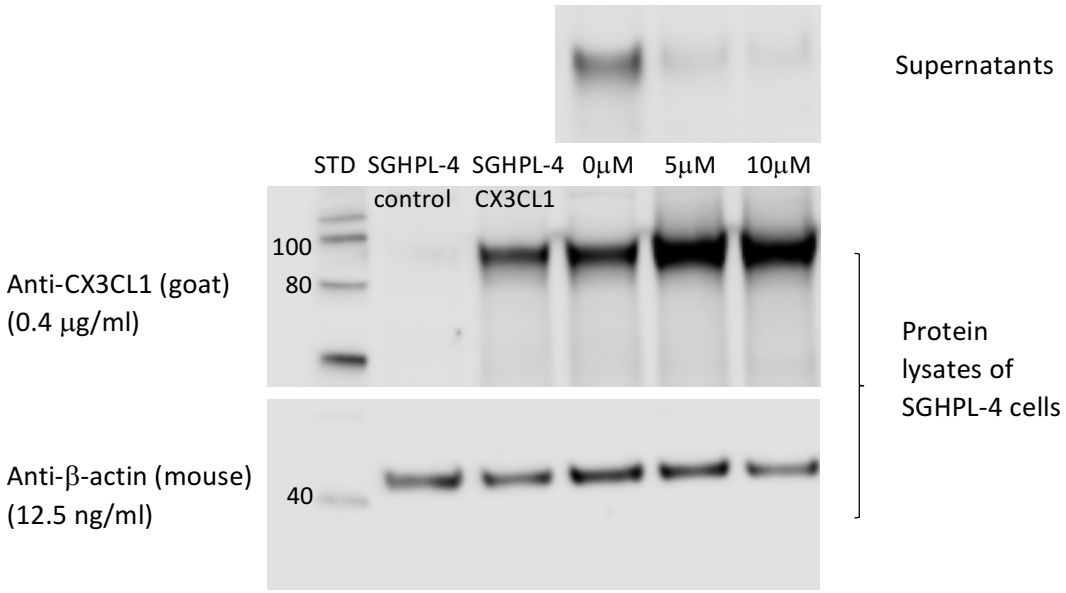

Fig. 21: Western blot of SGHPL-4 cell lines and the protein lysates and supernatants of the batimastat experiment. *The fractalkine concentration in each protein sample was determined regarding the amount of β-actin (42 kDa). With a rising concentration of batimastat the amount of CX3CL1 (95 kDa) in the protein increased also and connected with that the amount of soluble fractalkine in the supernatant decreased.*

The ratio of the fractalkine and β-actin detection showed an increase of membrane bound fractalkine in response to increasing concentration of batimastat. Treatment with 5 μM and 10 μM batimastat resulted in an increase of 60% and 110% of membrane bound CX3CL1 in the SGHPL-4 CX3CL1 overexpressing cells after 48 hours, compared to cells without treatment of the inhibitor (**Fig. 21**).

Connectedly the decreased release of soluble fractalkine could be shown by detecting the fractalkine amount in the supernatants. The treatment with 5 μM and 10 μM batimastat resulted in a decrease of soluble fractalkine in the supernatants. Only 17% and 10% of the total amount of soluble CX3CL1 could be found in the supernatants of the batimastat treated cells compared to the supernatants of the cells without treatment of batimastat.

The results of this experiment indicated that a dysfunction of the ADAMs could be followed by a changing of the ratio between the membrane bound and the soluble form of fractalkine. Recent studies have found increased expression of ADAMs in diabetic placentas. This increase may involve an increased shedding activity and therefore a higher concentration of the soluble form of fractalkine, which otherwise would have stayed in its membrane bound form. (Szukiewicz et al. 2013)

Siwetz et al. found an increased concentration of fractalkine together with up-regulated ADAM10 and ADAM17 expression in early-onset preeclampsia. This suggested the possibility of increased shedding activity and an increase of soluble fractalkine, which could lead to an increased attachment and possible activation between soluble CX3CL1 and fractalkine-receptor expressing maternal leukocytes. However, it should be noted that the soluble fractalkine could not only be of fetal origin but could also arise from endothelial cells of maternal origin. (Siwetz, Dieber-Rotheneder, et al. 2015)

In conclusion, many recent studies found different increases of ADAMs and soluble fractalkine during complications of pregnancies.

Adhesion assay

In this experiment, it should be tested whether THP-1 monocytes have a higher tendency to bind the SGHPL-4 CX3CL1 overexpressing cells than the SGHPL-4 control cells. Due to their higher content of membrane bound fractalkine, SGHPL-4 CX3CL1 can interact with monocyte cells because of the monocytes' fractalkine receptor CX3CR1.

After seeding SGHPL-4 control and CX3CL1 overexpressing cells at a density of $2.0x10^5$ cells per well and letting them grow for 48 h, culture medium of the SGHPL-4 cells was replaced by medium containing green fluorescence (CellTracker Green CMFDA (invitrogen)) labelled THP-1 cells ($4×10^5$ cells/well), and cells were co-cultured at 37°C for 90 minutes. After washing and fixing the cells with 4% formaldehyde, the different wells were analyzed with the Cell-IQ. The adhesion of the THP-1 monocyte cell line to the two SGHPL-4 cell lines was determined in triplicates.

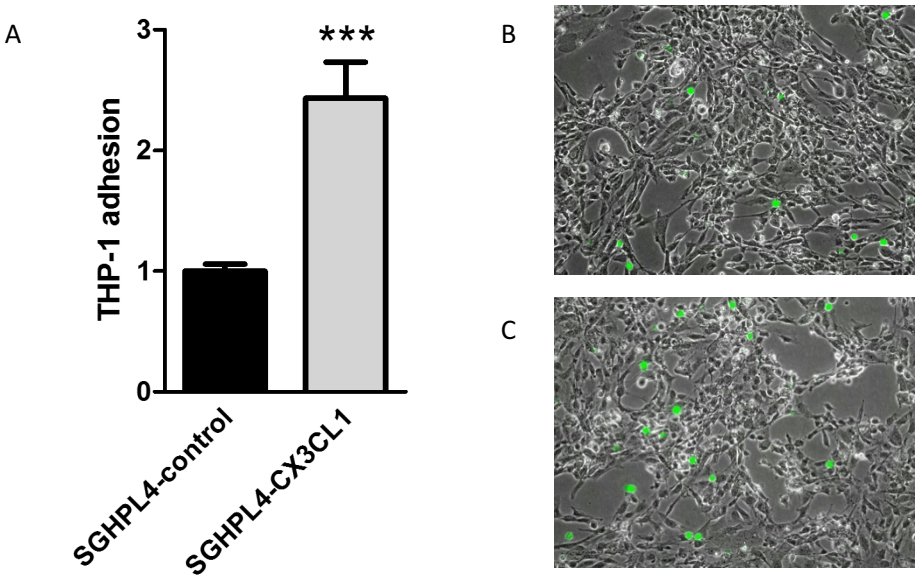

Fig. 22: Adhesion of THP-1 monocytes to SGHPL-4 control and SGHPL-4 CX3CL1 cells. *(A) The result showed clearly that the adhesion on the CX3CL1 overexpressing cells was 2.4-fold higher than on control cells. (B) Adhesion of THP-1 cells on SGHPL-4 control cells. (C) Adhesion of THP-1 cells in SGHPL-4 CX3CL1 cells.*

The area analysis by the Cell-IQ-software showed that the THP-1 adhesion to the extravillous trophoblast cells was 2.4-fold higher on the SGHPL-4 CX3CL1 cells than on the SGHPL-4 control cells (**Fig. 22**). Statistic testing showed a significant difference in adhesion behavior of the monocytes to the two cell lines (P value of less than 0.001).

As shown in previous studies by Siwetz et al. the THP-1 cell line expresses the fractalkine receptor CX3CR1 (Siwetz, Sundl, et al. 2015), which enables the monocyte cell line to attach to fractalkine in a highly specific manner. This experiment did show that fractalkine increased the attachment of the monocytes to the membrane bound form of CX3CL1.

Xiao et al. found that monocytes adhere poorly to unstimulated cells. That means that inflammatory cytokine activation up-regulates the adhesion of monocytes to placental trophoblasts almost 5-fold. Monocytes that tightly adhere to trophoblast cells are thought to transport infectious pathogens across the fetal-placental barrier, either by direct infection of the trophoblast or by transmigration of infected cells directly into the stroma of the villi. (Xiao et al. 1997)

Outlook - future experiments

Transfection for Correlative light and electron microscopy (CLEM)

SGHPL-4 and THP-1 interaction should be analyzed with CLEM. With correlative techniques one can combine information from light microscopy with the resolution of electron microscopy, investigate dynamic processes in the nanometer range and precisely locate rare events in cells and organisms. For this purpose, SGHPL-4 cells could be transfected with CX3CL1-DsRed plasmid and incubated with fluorescence stained THP-1 cells.

Under the fluorescence microscope the fluorescence of the two cell types could be identified and the exact location of the attachment between CX3CL1 and CX3CR1 could be localized by overlapping fluorescence. This location could be taken to the Core Facility Ultra Structure Analysis and with the help of Mag. Dr. rer. nat. Dagmar Kolb-Lenz and Dominique Pernitsch (BSc) the attachment of the cells could be investigated under the electron microscope to get further information about the possible dynamic processes like vesicle transport and binding processes.

For this future experiment the ability of SGHPL-4 cells to be transfected has already been analyzed. The control cell line was transfected with a plasmid harboring CX3CL1 that had a red fluorescence tag (CX3CL1-DsRed). The transfection showed a good transfection efficiency (**Fig. 23**) and the cells in which the transfection was successful could be clearly identified.

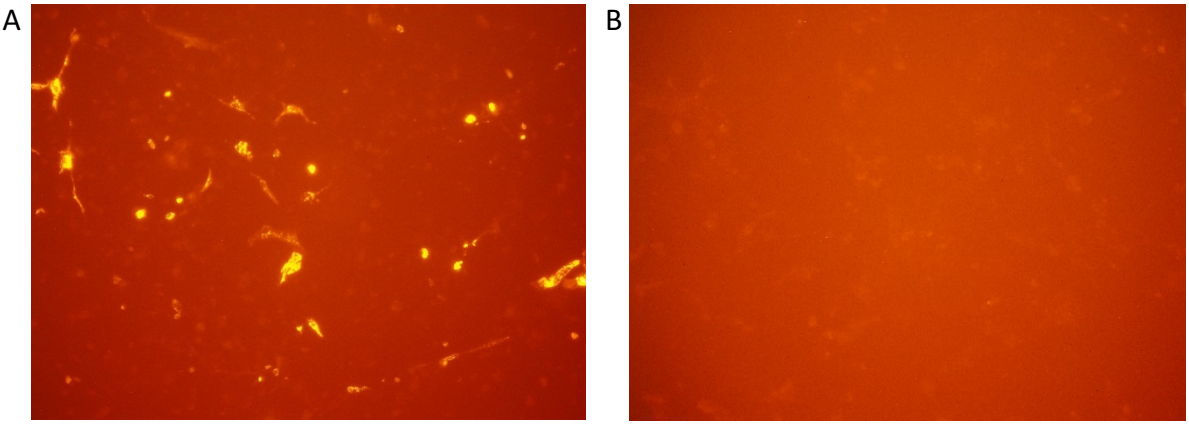

Fig. 23: Transfection of SGHPL-4 control cells *with (A) CX3CL1 with a red fluorescence tag (CX3CL1-DsRed) and (B) an empty vector. The transfection with the K2® Transfection System did show a good efficiency and the transfected cells could be identified under the fluorescence microscope.*

Double immunofluorescence staining

As CX3CL1 and its receptor CX3CR1 were both detected in the SGHPL-4 CX3CL1 overexpressing cells, it seems like there could be an auto- or paracrine regulation on the cells. This was shown in the RNA analysis of the interleukins. The immunocytochemistry showed that not all cells were expressing fractalkine, as well as not all cells were expressing its receptor. To identify whether the regulation of fractalkine is auto- or paracrine a double fluorescence staining on a chamber slide or the paraffin embedded cells could be performed. Under the fluorescence microscope it could be detected whether the same cells that express fractalkine also express its receptor or if fractalkine and receptor are expressed in different populations of the trophoblast cell line.

Batimastat adhesion assay

To test the hypothesis that CX3CL1 increases the adhesion percentage of THP-1 monocyte cell line to fractalkine expressing trophoblasts cells even further, the SGHPL-4 cells could be treated with batimastat before the green fluorescence labelled THP-1 cells would be incubated on the trophoblast cells. That way, even more membrane bound fractalkine should be present at the SGHPL-4 cells (Fig. 21) probably resulting in more THP-1 adhesion to the trophoblast cells. This emphasizes the role of posttranslational regulation of fractalkine for example by downregulation of the ADAMs. That would mean that the amount of membrane bound fractalkine would increase while the soluble form would decrease in response to upregulated ADAM activity.

Primary trophoblasts

The fractalkine expression was shown in the extravillous trophoblast cell line SGHPL-4 CX3CL1 overexpressing cells which also happen to express the fractalkine receptor CX3CR1. Siwetz et al. did describe that THP-1 monocyte cell line does express the fractalkine receptor CX3CR1 but not CX3CL1. On the other hand they have proven that the villous trophoblast cell line BeWo expresses fractalkine but not its receptor. (Siwetz, Sundl, et al. 2015) Kervancioglu Demirci et al. did a double staining of fractalkine and its receptor on a human term placenta. They detected CX3CL1 at the apical plasma membrane of the syncytiotrophoblast of the villi. CX3CR1 was localized on circulating maternal blood cells in the intervillous space. Occasionally

a close contact between the fractalkine positive syncytiotrophoblast and a CX3CR1 expressing maternal blood cell was observed, which suggests fetal-maternal interaction by the chemokine and its receptor. (Kervancioglu Demirci, Salamonsen, and Gauster 2016)

Expression of CX3CL1 and its receptor seem to be different between villous and extravillous trophoblasts. Primary term villous trophoblasts and differentiated BeWo cells were shown to express CX3CL1. (Siwetz, Sundl, et al. 2015) Its receptor, however, has been detected by IHC in endovascular extravillous trophoblasts, lining uteroplacental blood vessels. However, in interstitial extravillous trophoblasts, which invade the decidual stroma, but not the uteroplacental vessels, no receptor was found. (Hannan et al. 2006) Therefore, CX3CR1 may exist in some deme of invading trophoblasts. A trophoblast cell line (AC1M-88), which expressed the fractalkine receptor, showed enhanced migration when treated with recombinant CX3CL1. (Hannan et al. 2006) Furthermore, uterine glandular epithelia, stroma cells in the decidua and different uterine leukocytes were found to express CX3CL1 (Hannan et al. 2004) which may have a paracrine regulation on invading EVTs.

As no CX3CR1 expression was visible in the syncytiotrophoblast, that would mean that the villous trophoblast cells do not have any auto- or paracrine regulation and the fractalkine would probably serve to activate leukocytes with its soluble form and probably yet unknown cofactors or adhere leukocytes to the syncytiotrophoblast with its membrane bound form.

In further experiments it should be tested whether the cell line SGHPL-4 does represent the actual extravillous trophoblast cells in a human placenta as the cells that were worked with in this master's thesis were transfected to overexpress CX3CL1. The extravillous trophoblasts of the placenta should be isolated and tested for fractalkine and its receptor on RNA and protein level. Also, immunohistochemical/-cytochemical stainings on tissue, chamber slides or paraffin sections of the primary extravillous trophoblast cells should be performed to confirm whether CX3CL1 and its receptor are only expressed in a certain population of the cells.

If the primary extravillous trophoblast cells happen to confirm the findings of this master's thesis, that villous trophoblast cells only express fractalkine but not its receptor and extravillous trophoblast cells only express the receptor but do not express the fractalkine itself, it could be that the soluble form of fractalkine from the villous trophoblasts regulates the activation of the extravillous trophoblasts in the uteroplacental vessels. As the blood that comes into the placenta flows through the uterine arteria and did not enable contact with the

fractalkine of the villous trophoblasts yet, these EVT cannot be activated by fractalkine from the placenta. But extravillous trophoblast cells are not only able to bind the fractalkine from villous trophoblasts as other cells in the maternal body also express CX3CL1. Evidence was given by detected CX3CL1 in maternal plasma of a first trimester study (Hannan et al. 2014), in endothelial or epithelial cells and several reproductive tissues, including ovaries, uterus and testis. (Kervancioglu Demirci, Salamonsen, and Gauster 2016)

Recently it has been proven that also the uterine veins are remodeled by EVT. (Moser et al. 2017) These endovascular trophoblasts could indeed be regulated by the soluble fractalkine of the villous trophoblasts. An increased production of cytokines by the extravillous trophoblast cells of the placenta could have an impact on the maternal body. This might be a method for the fetal/gestational tissue to communicate with the mother's body, if any kind of inflammation or other complication occurs during pregnancy the rising level of interleukins might signal the maternal body to produce specific factors to counteract.

The regulation of SGHPL-4 control cells could be investigated by incubating the control cells with recombinant fractalkine or with the supernatant of forskolin treated BeWo cells, which where proven to express fractalkine but not its receptor. Thus, it could be tested whether fractalkine from villous trophoblasts could activate extravillous trophoblasts that only express the receptor for fractalkine.

Conclusion/summary

In summary, the overexpression of fractalkine in the SGHPL-4 CX3CL1 cells could be confirmed by ICC in a certain population of cells and on protein and RNA level.

The transition from the membrane bound form of CX3CL1 into the soluble form by the metalloprotease activity could be demonstrated on protein level by an experiment with the ADAM inhibitor batimastat. Therefore, with increasing level of batimastat the level of membrane bound CX3CL1 also increased and the level of soluble form decreased.

The expression of the fractalkine receptor CX3CR1 was confirmed in a population of the extravillous trophoblast cell line, in the overexpressing cells as well as in the control cells, by ICC and with western blot.

The first part of the hypothesis, that fractalkine increase the adhesion of monocytes to the trophoblasts, was confirmed by the adhesion assay. The Cell-IQ analysis revealed that the overexpression of fractalkine increased the adhesion of the fractalkine receptor included monocyte cell line THP-1 to the fractalkine overexpressing SGHPL-4 cells by a factor of 2.4.

The next step here could be an analysis of the bond between CX3CL1 and its receptor CX3CR1 by electron microscopy to investigate possible dynamic processes like vesicle transport and binding processes.

The other part of the hypothesis, that fractalkine would activate monocyte cell lines, needs to be part of further investigations. On protein level as well as on RNA level the highest interleukin expressions were shown in SGHPL-4 CX3CL1 overexpressing cells and in THP-1 cells that were incubated in the supernatant of SGHPL-4 control cells.

It seems like fractalkine has an auto- or paracrine regulation on the SGHPL-4 CX3CL1 overexpressing cells. To determine whether the regulation is auto- or paracrine an immunofluorescence double staining could be performed to see whether the same cells express both fractalkine and its receptor. Also, when THP-1 cells are incubated in the SN of SGHPL-4 CX3CL1 cells the interleukin expression decreases in comparison to SGHPL-4 CX3CL1 solitarily. It seems like the THP-1 cells inhibit the interleukin production of SGHPL-4 CX3CL1 cells by competing with it for the fractalkine binding site or by producing another co-factor that is interacting with the interleukin production of the trophoblast cells.

As for the high interleukin production of the THP-1 cells in SN of SGHPL-4 control cells, further experiments should be performed here, as in this supernatant there has to be a yet unidentified factor that activates the monocytes, but that does not exist in the SN of SGHPL-4 CX3CL1 overexpressing cells. Another theory could be that the substance exists in both supernatants but is suppressed by the overexpressed fractalkine in the SN of SGHPL-4 CX3CL1.

In addition to that, it should be investigated whether primary human trophoblast cells do have the same characteristics as the cell lines. By isolating villous and extravillous trophoblast cells and analyzing them by ICC, qPCR and western blot. It should be tested if villous trophoblast actually only express fractalkine and not the receptor and if extravillous trophoblasts that are not transfected only express the receptor, but not fractalkine itself (**fig. 24**).

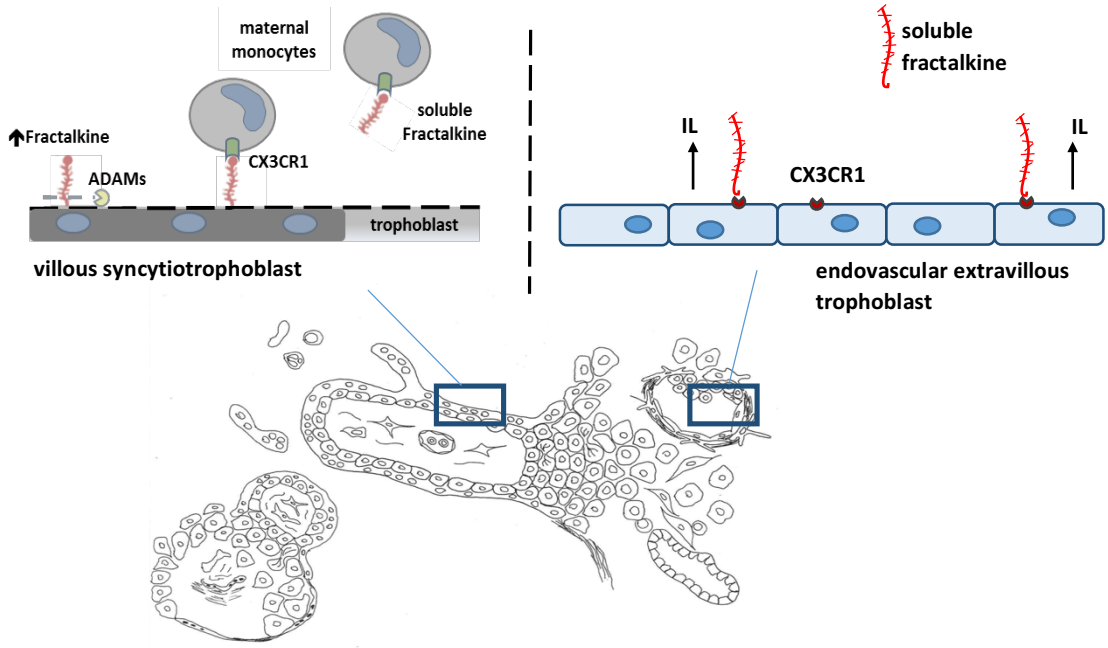

Fig. 24: Possible shema of trophoblast-fractalkine interaction. *Villous trophoblasts express fractalkine, which is present in membrane bound form and after ADAMs shedding soluble form. In endovascular extravillous trophoblasts (arterias and veins) the fractalkine receptor CX3CR1 is expressed, which can lead to the regulation of interleukin production when fractalkine of placental or maternal origin binds.*

An increased production of interleukins by the EVTs could have an impact on the maternal body. It might be a method for the gestational tissue to signal the maternal body, if any kinds of inflammation or other complications occur during pregnancy, so that the maternal body can counteract.

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Fig. 4: Wojdasiewicz, Piotr, Lukasz A. Poniatowski, Andrzej Kotela, Jarosław Deszczyński, Ireneusz Kotela, and Dariusz Szukiewicz. 2014. "The Chemokine CX3CL1 (Fractalkine) and Its Receptor CX3CR1: Occurrence and Potential Role in Osteoarthritis." *Archivum Immunologiae Et Therapiae Experimentalis* 62 (5): 395–403. doi:10.1007/s00005-014-0275-0.

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Fig. 10: http://www.bio-rad.com/de-at/applications-technologies/protein-blotting-methods, last visit 26.06.2017 19:00 PM

Fig. 11:
https://www.perkinelmer.com.cn/Resources/TechnicalResources/ApplicationSupportKnowledgebase/Microplates/absorbance_plates.xhtml, last visit 26.06.2017 19:30 PM

Fig. 24: Combination of

- Original drawing by Prof.in Astrid Blaschitz (retired, Institute of Cell Biology, Histology and Embryology, Medical University of Graz)
- Original figure by Assoz. Prof. Priv.-Doz. Mag.rer.nat. Dr.scient.med. Martin Gauster (Institute of Cell Biology, Histology and Embryology, Medical University of Graz)
- Figure by Jacqueline Serbin (BSc)

Tables

Abbreviations

AB	antibody
ADAM	a disintegrin and metalloproteinase
AGR	antigen retrieval
cDNA	complementary DNA
CX3CL1	fractalkine
CX3CR1	fractalkine receptor
DNA	deoxyribonucleic acid
dsDNA	double strand DNA
ELISA	enzyme linked immune sorbent assay
EVT	extravillous trophoblast
FBS	fetal bovine serum
Fig.	figure
h	hour
ICC	immunocytochemistry
IHC	immunohistochemistry
IFN	interferon
IL	interleukin
NK cells	natural killer cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative PCR
RNA	ribonucleic acid
rpm	rounds per minute
SLE	systemic lupus erythematosus
SN	supernatant
STD	standard
TBS(-T)	tris-buffered saline (with tween)
TNF	tumor necrosis factor

Materials

Cell lines and tissue

First trimester placenta (kindly provided by Dr. Glasner, Gynecologist (Graz))

SGHPL-4 (Dr. Florian Herse and Julianna Zadora from the Max-Delbrueck Center for Molecular Medicine (MDC), Berlin, Germany)

THP-1 (ATCC® TIB-202™)

Cell culture

Ham's F-10 Medium (Merck)

- W 1.2 g/L $NaHCO_3$
- W 10 mg/L Phenol red
- W stable glutamine
- Low endotoxine

Additional added P/S and FBS

RPMI Medium 1640 (1x) (Gibco by Life Technologies)

- L-Glutamine

Additional added P/S and FBS

P/S - Penecillin/Streptomycin (Biowest – The Serum specialist)

FBS – Heat Inactivated Fetal Bovine Serum (Gibco by Life Technologies)

Accutase (Biowest – The Serum Specialist)

PBS Buffer – pH 7.4 (1x) – Phosphate Buffered saline (Gibco by Life Technologies)

- $CaCl_2$
- $MgCl_2$

Immunohistochemistry

UltraVision LP Detection System (Thermo Scientific)

Antibody diluent with background reducing components (Dako)

Ultravision Hydrogen Peroxidase Block (Thermo Scientific)

Large Volume AEC Chromogen Single Solution (Thermo Scientific)

Hemalaun

- 1g Hematoxylin
- 0.2g $NaJO_3$
- 50g $KAI(SO_4)_2*12H_2O$
- 50g $C_2H_3Cl_3O_2$
- 1g $C_6H_8O_7*H_2O$

Kaisers Glyceringelatine (Merck)

Epitope Retrieval Solution (10x) – pH6 (Novocastro Leica)

Antibody

- Anti-hCX3CL1 (goat); Catalog number: AF365 (R&D Systems)
- Anti-CX3CL1 (mouse) (R&D Systems)
- Anti-CX3CR1 (rabbit); Catalog number: C8354 (Sigma)
- Negative control for mouse IgG (Dako)
- Negative control for rabbit IgG (Neomarkers)
- Negative control for goat IgG (Southern Biotechnology Associates, Inc.)

Histolab-Clear (HistoLab®)

TBS-T
- 50 ml TBS (20x)
- 950 ml aqua dest.
- 0.5 ml Tween® 20 (Merck)

Menzel-Gläser Superfrost® plus microscope slides (Thermo scientific)

Transfection of THP-1

Cell Tracker™ Green CMFDA (5-chloromethylfluorescin diacetate) (invitrogen)

Batimastat

Batimastat (Santa Cruz Biotechnology)

Protein Lysate

RIPA Buffer – Radioimmunoprecipitation assay buffer (Sigma Aldrich)

Protease Inhibitor Cocktail (solved from tablets (Roche))

Lowry

Solution A: 2% Na_2CO_3 in 0.1M NaOH

Solution B: 1% $CuSO_4$ x $5H_20$ in A.d.

Solution C: 2% $KNOC_4H_4O_8$ x $4H_20$ in A.d.

Folin – Ciocalteus Phenol Reagent (Merck)

Western blot

ddH2O

NuPAGE® LDS Sample Buffer (4x) (Invitrogen)

NuPAGE® Sample Reducing Agent (10x) (novex by Life Technologies)

NuPAGE® MES SDS Running Buffer (20x) (novex by Life Technologies)

NuPAGE™ 10% Bis-Tris Gel (Invitrogen by Thermo Fisher Scientific)

Page Ruler™ - Prestained protein ladder (Thermo Scientific)

MagicMark™ XP Western Standard (Invitrogen by Thermo Fisher Scientific)

NuPAGE® Transfer buffer (20x) (novex by Life Technologies)

Nitrocellulose Blotting Membrane; Amersham™ Protran™ 0.45μm NC (GE Healthcare Life Science)

Ponceau Red
- 0.1% Ponceau S
- 5.0 % acetic acid

Powdered milk (Carl Roth)

TBS-T
- 100 ml TBS (10x)
- 900 ml aqua dest.
- 0.5 ml Tween® 20 (Merck)

Primary antibodies
- anti-β-actin (mouse) (abcam)
- anti-fractalkine (goat) (R&D System)
- anti-CX3CR1 (CX3CL1 receptor) (rabbit) (Sigma)
- anti-CX3CR1 (CX3CL1 receptor) (rabbit) (Acris)

Secondary antibodies
- Rabbit anti-goat (Dako)
- Goat anti-mouse (Bio-Rad)
- Goat anti-rabbit (Bio-Rad)

Recombinant Human CX3CL1/Fractalkine (Full Length) Protein (R&D Systems)

WesternBright™ Peroxide and Quantum (advansta)

RNA Lysate

peqGOLD Trifast (VWR)

1-Bromo-3-Chloropropane (BCP) – Phase Separation Reagent – Molecular Biology Grade (Molecular Research Center Inc.)

2-propanol (Sigma-Aldrich)

70% EtOH (diluted from Ethanol Prima 96% (AustrAlco))

Nuclease-Free Water (Affymetrix®)

Reverse Transcriptase

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

qPCR

Primer
- GAPDH – fwd and rev (Microsynth)
  - fwd: 5'-ACC CAC TCC TCC ACC TTT GA-3'
  - rev: 5'-CTG TTG CTG TAG CCA AAT TCG T-3'
- IL1A – fwd and rev (Microsynth)
  - fwd: 5'-CTG CTG AAG GAG ATG CCT GA-3'
  - rev: 5'-TGC CGT GAG TTT CCC AGA AG-3'
- IL1B – fwd and rev (Microsynth)
  - fwd: 5'-GCA GAA GTA CCT GAG CTC GC-3'
  - rev: 5'-TCC TGG AAG GAG CAC TTC ATC T-3'
- IL6 – fwd and rev (Microsynth)
  - fwd: 5'-AAC CCC CAA TAA ATA TAG GAC TGG A-3'
  - rev: 5'-GGA CCG AAG GCG CTT GT-3'
- IL8 – fwd and rev (Microsynth)
  - fwd: 5'-TCT GTG TGA AGG TGC AGT TTT G-3'
  - rev: 5'-ATT TCT GTG TTG GCG CAG TG-3'
- CX3CL1 – V1 – fwd and rev (Microsynth)
  - fwd: 5'-CAC CAC GGT GTG ACG AAA TG-3'
  - rev: 5'-TCT CCA AGA TGA TTG CGC GT-3'
- CX3CL1 – V2 – fwd and rev (Microsynth)
  - fwd: 5'-GCC ATG GCT CCG ATA TCT CT-3'
  - rev: 5'-CTG TCT CGT CTC CAA GCA GC-3'

iTaq Universal SYBR Green Supermix (Bio Rad)

ELISA

Quantikine ELISA – Human CXCL8/IL-8 (R&D Systems – a biotechne brand)

Quantikine ELISA – Human CXCL6/IL-6 (R&D Systems – a biotechne brand)

Transfection of SGHPL-4 Control

K2® Transfection System (Biontex)
- K2® Multiplier
- K2® Transfection Reagent

Fixans:
- 2%PFA
- 2.5%GA
- 0.1M Na-Phosphat-puffer

Tools

Heracell 150i – CO_2 Incubator (Thermo Scientific)
MSC-Advantage – Safety bench (Thermo Scientific)
Heraeus™ Megafuge™ 40R Centrifuge (Thermo Fisher Scientific)
Tissue-Tek® VIP™ (Sakura)
Tissue Embedding System TES Valida (Medite)
HM 440 E Microtome (Microm)
Axiophot - microscope (Zeiss)
AxioCam HRc (Zeiss)
Decloaking chamber (Biocare Medical)
Lab-Tek II Chamber Slide w/Cover RS Glass Slide Sterile (Nalge Nunc International)
OD reader Anthos 2010
Thermomixer compact (Eppendorf)
Power Station 200 (Labnet International Inc.)
Power Pac 1000 (Bio Rad)
Vacupack plus (Krups)
MultiImage®III (Alpha Innotech)
NanoDrop® ND-1000 Spectrophotometer (peqLab – Biotechnologie GmbH)
DNA Engine Dyad®, Peltier Thermal Cyclers (Bio Rad)
CFX96™ Real-Time PCR Detection System (Bio Rad)
Spark 10M Multimode Microplate Reader (Tecan)
Olympus IX50 Inverted System Microscope
Olympus C5060 – ADU

Programs

ADAP Prisma (Biochrom)
ND-1000 V3.5.2
FluorChem Q System (Alpha Innotech)
Image Studio Lite (Version 5.2)
Bio Rad CFX Manager (Version 3.1)
AxioVision SE64 Rel. (Version 4.9)
CellIQ
- Cell-IQ Imagen
- Cell-IQ Analyser

GraphPad – Prism (Version 5.01)