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Master's thesis

The effect of Interleukin 34 on human breast cancer cells

for acquisition of the academic degree
Diplom- Ingenieurin (Dipl.-Ing.^{in})
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by

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"Many of life's failures are people who did not realize how close they were to success when they gave up."

Thomas Edison

“Not art and science only, but patience will be required for the work.”

Johann Wolfgang von Goethe

“It does not matter how slowly you go as long as you do not stop.”

Confucius

Expression of thanks

First, I would thank my Supervisor and Group leader Ao. Univ.Mag.Dr.Dietmar Abraham for his supervising, supporting and for introducing me in his special working field.

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Finally, I dedicate this master's thesis to my loving parents Anna and Franz Hammerl, who supported me in my studies and made all this possible.
Mum, Dad I'm so thankful for all what you have done for me!

Abstract

Breast cancer is one of the most deadly cancer diseases in women. Immunohistochemical results of breast cancer tissues showed very high levels of tumour associated macrophages, which can support tumour growth within the stroma. The monocytic differentiation into tumour associated macrophages is regulated by the cytokine colony stimulating factor 1 (CSF-1), which regulates this process by binding to the receptor tyrosine kinase colony stimulating factor 1 receptor (CSF-1R).The proinflammatory cytokine Interleukin-34 (IL-34) was identified as a second ligand for the CSF1R being involved in the differentiation of M1 and M2 macrophages. IL-34 was also detected in breast cancer cells. However, its role remains unclear. Therefore, we hypothesized that IL-34 plays an important role in breast cancer.

The aim of this study was to identify the effect of IL-34 on triple negative MDA-MB-231, luminal A-type MCF-7 breast cancer cells and macrophages. THP-1 monocytes were differentiated into macrophage and these cells or their conditioned medium was used in (co-) culture experiments to analyse the crosstalk between macrophages and breast cancer cells. Gene expression of IL- 34, CSF-1, TNF-α and of macrophage polarisation markers IL-10 and IL-12 was quantified by qRT-PCR. Basic IL-34 signalling events were investigated in both breast cancer cell lines by immunoblotting. In addition, IL-34 mRNA expression was analysed in breast cancer patients.

The performed *in vitro* studies indicate that the interaction between IL-34/CSF-1 and TNF-α regulates the crosstalk between breast cancer cells, monocytes, and macrophages.

Zusammenfassung

Brustkrebs ist die häufigste bösartige Erkrankung der Frau. In immunhistochemischen Untersuchungen von Brustkrebsgewebe ist eine hohe Anzahl an Tumor-assoziierten Makrophagen gefunden worden, welche das Wachstum des Tumors fördern. Der Prozess der Differenzierung dieser Makrophagen wird durch das Zytokin “Colony stimulating factor 1” (CSF-1) stimuliert, der an den Tyrosin-Kinase-Rezeptor CSF-1R bindet. Kürzlich wurde das entzündungsfördernde Zytokin Interleukin 34 (IL-34) als zweiter Ligand für den CSF-1 Rezeptor entdeckt, welches auch am Differenzierungsprozess in M1 und M2 Makrophagen beteiligt ist. IL-34 wurde auch im Brustkrebgewebe identifiziert, aber mit noch unklarer Rolle. Deshalb haben wir die Hypothese aufgestellt, dass IL-34 eine wichtige Rolle beim Mammakarzinom spielt.

Ziel dieses Projekts war es, den Effekt von IL-34 auf triple -negative MDA-MB-231 Brustkrebszellen, luminal A MCF-7 Brustkrebszellen und auf Makrophagen zu untersuchen. THP-1 Monozyten wurden in Makrophagen differenziert und diese Zellen, oder deren konditioniertes Medium, wurden in (Ko-)Kulturexperimenten verwendet, um die Wechselwirkung zwischen Makrophagen und Brustkrebszellen zu analysieren. Die Gen Expression von IL- 34, CSF-1, TNF-α und von den Makrophagen-Polarisierungsmarkern IL-10 und IL-12 wurde mittels qRT-PCR quantifiziert. Grundlegende IL-34 Signalwege wurden in beiden Brustkrebszelllinien mittels Immunoblotting untersucht. Zusätzlich wurde die IL-34 mRNA Expression in Brustkrebspatientinnen analysiert.

Die Ergebnisse der Studie lassen darauf schließen, dass die Interaktion zwischen IL-34/CSF-1 und TNF-α die Wechselwirkung zwischen Brustkrebszellen, Monozyten und Makrophagen reguliert.

1 Introduction

Breast cancer is one of the most common cancer diseases in women and develops from normal breast tissue, mostly in cells of the milk ducts. The signs of breast cancer include lump in breast, changes in breast shape, dimpling of breast skin, nipple deposition. Several risk factors like sex, obesity, lack of physical exercise, alcohol consumption, diet with high amount of fat, caffeine and red meat, smoking, hormone status, ionizing radiation, menopause and age. Even the mortality rate of breast cancer is exceeding in pregnant women compared with those who never had been pregnant before. [1]

Of particular importance is a positive family history for breast cancer. It is one of the main risk factors, which is associated with an early age at outbreak, even bilateral breast cancer, higher stage, lymph node involvement and negative hormone receptors with a less favourable prognosis.[2] The diagnosis of the 18 different breast cancer subtypes is determined by taking a biopsy followed by immune histochemical analysis and analysis of gene expression changes. With this information medical treatment will be chosen e.g. anti-estrogen drugs, surgical removal of the breast, radiation therapy, chemotherapy, hormonal therapy or a targeted therapy.[1 2]

1.1 Breast cancer classification

The breast cancer classification is useful for prediction of the clinical outcome and response to certain therapies.

Different breast cancer subtypes have been identified including the estrogen receptor (ER)-negative, basal-like and human epidermal growth factor receptor-2 (HER2) positive tumours. The ER- positive subtypes include the luminal A and the luminal B tumour. All these subtypes differ in their prognosis and therapeutic targets.[3, 4]

The luminal cancer type has several molecular subtypes with low expression of hormone receptors and is derived from normal breast luminal epithelial cells, whereas the basal- like type is derived from normal basal breast epithelial cells (Table 1).[4]

The luminal A cancer type typically expresses luminal cytokeratins 8 and 18 and is the most common breast cancer type with estrogen and progesterone receptors and genes for estrogen-receptor activation. Whereas luminal A type of breast cancer lacks expression of HER2, the luminal B type can be derived into two different subgroups, which express HER2 or not.[4]

Because the Luminal A type has estrogen and progesterone receptors, it respond better to endocrine therapies and therefore has the best prognosis of all subtypes, whereas the

luminal B tumours have a worse prognosis, because they have a lower ER gene expressions and variable HER2 cluster.[3]

The HER2 subtype has a high expression of HER2, this type is negative for estrogen and progesterone receptors and has a poor prognosis.[1]

The basal-like subtype shows low or is lacking expression of HER2 and has normally no estrogen and progesterone receptors. The MDA-MB-231 breast cancer cell line show low HER2 gene expression and is ER negative and PR negative. Thus, MDA-MB-231 is a 'triple-negative' breast cancer cell line, which have the poorest prognosis for patients. In contrast, the MCF-7 breast cancer cell line is a luminal A type cell line, which have a better prognosis in patients. [1, 3]

Mutations in oncogenes, proto-oncogenes and tumour suppressor genes are potential biomarkers and targets for breast cancer therapies. Genes for apoptosis like the Induced myeloid leukemia cell differentiation protein gene (MCL1) and the BCL2 like 1 gene (BCL2L1), as well as Interleukin- 1 receptor- associated kinase 1 (IRAK1), Tumour necrosis factor (TNF) associated factor (TRAF) 6, the Ras/MAPK signalling pathway, inhibitor of nuclear factor kappa B kinase subunit gamma (IKBKG) for activation the NF-κB molecule and its signalling pathway are new potential targets for the triple negative breast cancer subtypes. [2]

NF-κB has a high activity in apoptosis, cell survival, cell proliferations pathways, cell adhesion and angiogenesis, it regulates tumour promoting molecules and increases the expression of proto-oncogenes, which stimulate the proliferation of breast cancer cells.[1]

Molecular subtype	**Subgroups**	**Definition**	**Explanatory note**
Luminal A		ER/PR positive HER2 negative	
Luminal B	HER2 negative	ER positive PR negative	luminal- B-like HER2 negative
	HER2 positive	ER positive HER2 positive	luminal- B- like HER2 positive
HER2- enriched		HER2 positive ER/PR negative	
Basal like		ER/PR negative HER2 negative	overlapping with triple negative breast cancer

Table 1: Definition of molecular subtypes of breast cancer [4]

1.2 Role of estrogen and progesterone

The estrogen hormone receptor alpha plays a big role in breast cancer cell development and genetic modifications have an effect in breast cancer tumourigenesis. So, polymorphisms of this receptor can increase the risk of estrogen associated breast cancer.[1] Estrogen promotes the development of breast cancer and stimulates the growth of breast cancer cell lines and is one of the most important biomarker for the classification of breast cancers, because ER positive tumours are present in 75% of all breast cancer patients.[2]

ER positive tumours are well differentiated, less aggressive are responsive to endocrine therapy and have better prognosis after surgery. It is one of the strongest single predictive factors in breast cancers. In contrast ER negative tumours have only a low response to endocrine therapy. [1]

Progesterone (PR) is required for active ER signalling and PR positive tumours react to an endocrine therapy better than PR negative tumours. Patients with a double positive tumour are mostly older, have a lower grade, a smaller tumour size and a lower mortality rate, in contrast to double negative tumours. These tumours are highly aggressive and have a lower survival rate and do not respond to an existing endocrine therapy; therefore double negative breast cancer is associated with a higher recurrence rate and a lower survival rate. These tumours are highly heterogeneous and can differentiate in many subgroups which are based on another biomarker, HER2. [2]

Invasive ductal breast cancers are frequently HER2-positive and more than a half of the tumours are ER- and PR- negative. Therefore, the HER2 status is used as an important biomarker for routine analysis before a potential therapy with *Trastuzumab*, which inhibits HER2 on breast cancer cells, is implemented. HER2-positive tumours have a good drug response to a higher dose of anthracycline related therapies.[1, 3, 4]

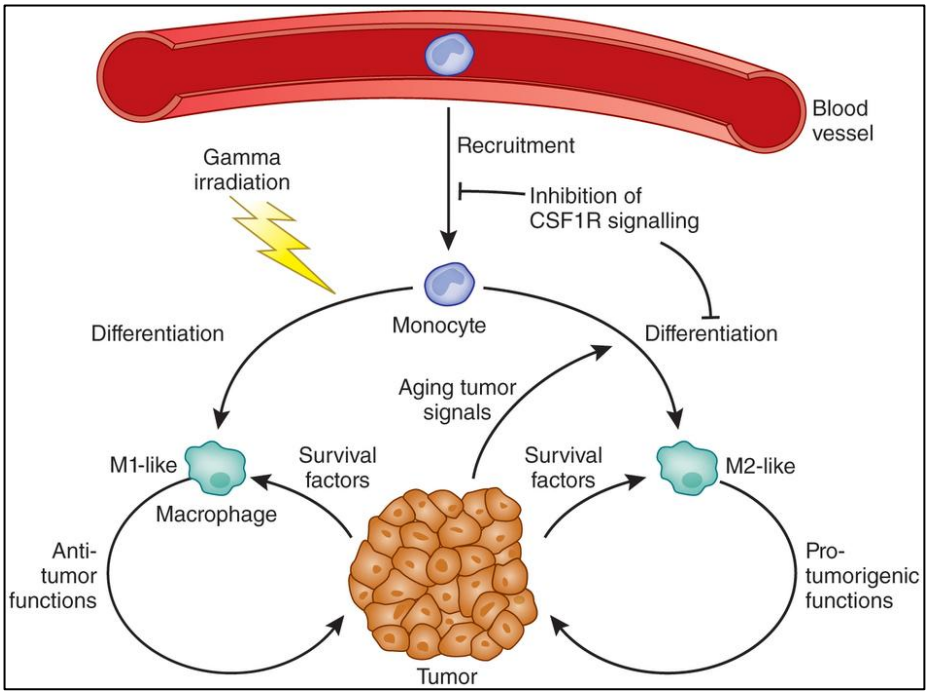
1.3 Tumour microenvironment of breast cancer cells

1.3.1 Tumour associated macrophages (TAMs)

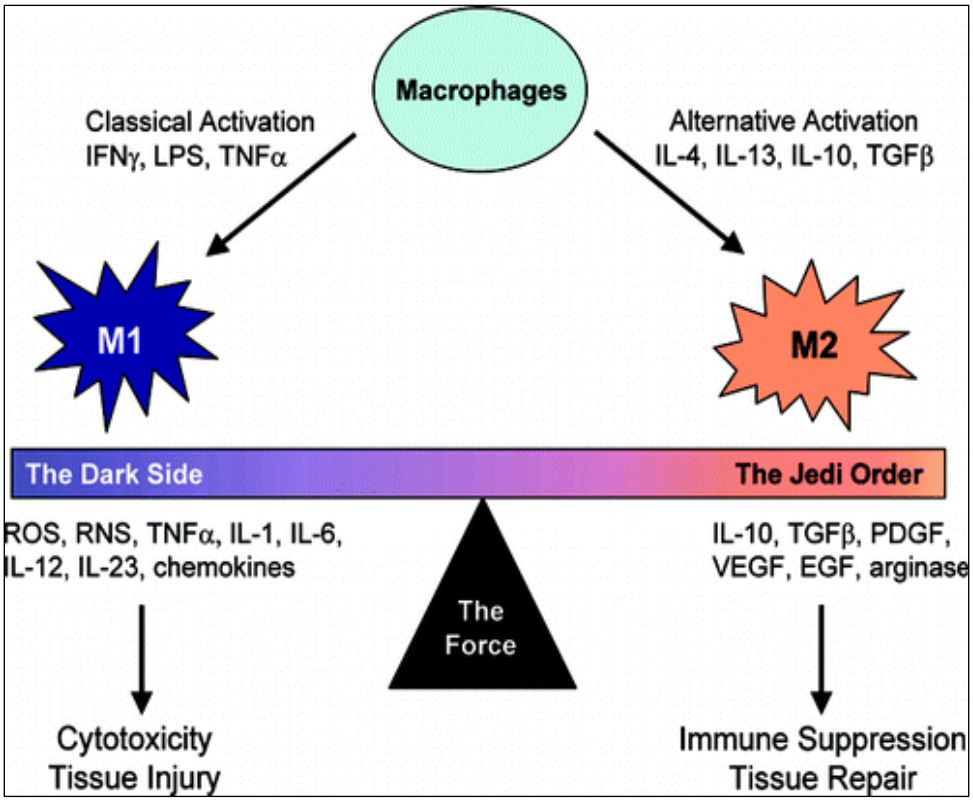
The immune system plays an important role in cancer, including breast cancer. Macrophages, which are raised from myeloid cells, can be found in almost all tissues and play a fundamental role in the innate and adaptive immune response to pathogens and are active in inflammation and infection processes.[5] Today it is well- known that macrophages can be activated by different cytokines and polarized to classically activated M1 macrophages or to alternatively activated M2 macrophages. Both have different functions to cancer cells and their environment.[6] M1 macrophages have a pro-inflammatory anti-cancer effect and express high levels of the effector molecules IL-12, IL-23, TNF-α, MHC I/II genes, as well as high levels of reactive oxygen and nitrogen species.[5] The M1 macrophages inhibit the proliferation of surrounding cells and have a high presence of antigens.

In contrast to the M1 macrophages, M2 macrophages express high levels of IL-10, IL-4, IL-13, Toll-like receptors, CSF-1, IL-34 and TGF-β (Figure 1). [7-11]

The M2 macrophages promote the proliferation of cells and tissue repair. The M2 phenotype can be found in many solid tumours, supporting tumour growth, tumour angiogenesis, metastasis and immune suppression.[5] These TAMs are also found in high density in breast cancers with poor prognosis and share many characteristics with the alternatively activated M2 macrophages.[5] In some breast cancers, TAMs constitute up to 50% of the cell mass. Therefore, solid tumours contain not only malignant cells, but also stromal host cells like macrophages, fibroblasts, adipocytes and hematopoietic cells, which are recruited from blood vessels.[5-8]



[i]**Figure 1: Scheme of polarisation of monocytes into M1-like or M2-like Macrophages**; M1-like Macrophages, which have anti-tumour functions or M2-like Macrophages with pro-tumourgenic



[ii]**Figure 2: Macrophage differentiation into M1 and M2 macrophages with classical or alternative activation**. M1 macrophage differentiation is activated by the classical pathway with IFN-gamma, LPS, TNF-α and lead to cytotoxicity and tissue injuries. M2 macrophage is activated by the alternative activation with IL-4, IL-13, IL-10 and TGF-beta and lead to immune suppression and tissue repair

1.4 Colony stimulating factor 1 receptor (CSF1R)

CSF1R is a cell surface receptor and responsible for the production, differentiation and function of macrophages. The CSF1R is a homodimeric tyrosine kinase transmembrane receptor and belongs to the family of the CSF-1 and PDGF receptor family of tyrosine kinases, the so called class III receptor tyrosine kinase (RTK) family and is also expressed on tumour associated macrophages. The resulting signalling leads to proliferation, differentiation and survival of the cells. The CSF1R consist of an extracellular glycosylated ligand-binding part, with five immunoglobulin (Ig) domains.[9, 10]

The trans-membrane domain divides the intracellular kinase domain in two parts and the kinase domain has 8 tyrosine phosphorylation sites.[9]

CSF1R and its ligand CSF-1 are expressed in normal breast tissue during lactation, pregnancy and puberty.[9, 10] However, the expression of CSF1R and CSF-1 were also reported at different levels in several types of cancer. The CSF1R is known to induce the activation of the extracellular signal regulated kinase 1/2 (ERK1/2) in breast cancer cells due to phosphorylation leading to cell proliferation.[10, 11] (Figure 2)

Recently, it was shown that besides CSF-1, IL-34 is an independent ligand for CSF1R.[12] Both are binding to the receptor and activate the receptor kinase via oligomerization and trans- phosphorylation. The CSF1R signalling is involved in activation or inactivation of several signalling proteins within the cellular cytosol, which can lead to cell proliferation, cell survival, CSF-1R internalization, differentiation and adhesion (Figure 2).[13]

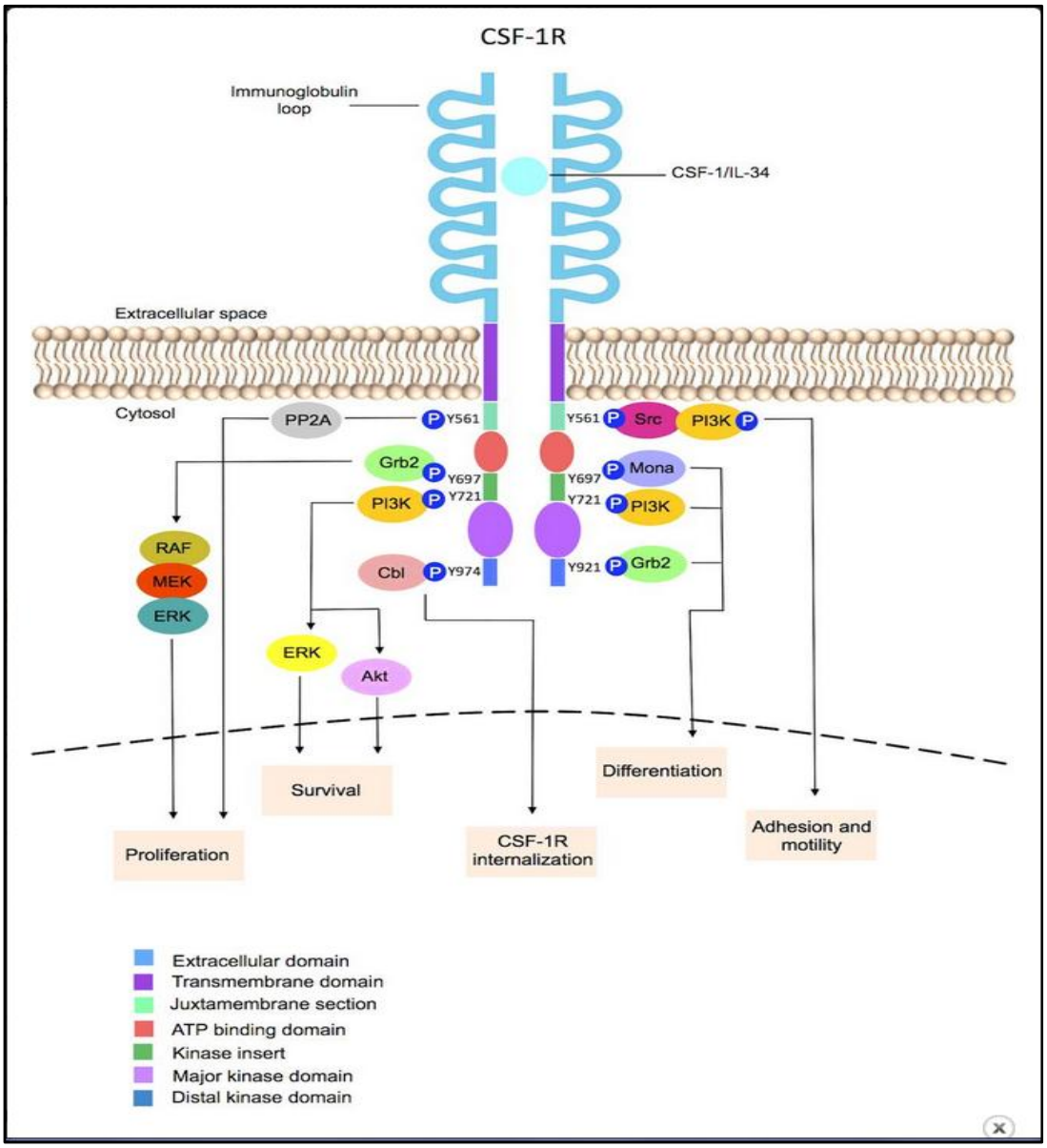


Figure 3: CSF-1R signaling[9]: CSF-1 or IL-34 activates CSF-1R chain dimerisation, with activation of a cross-tyrosine phosphorylation and association of signalling molecules with the receptor through their phosphotyrosine -binding domains. The resultant gene expression is responsible for cell proliferation, differentiation and survival.

1.4.1 Colony- stimulating factor 1 (CSF-1)

The Colony- stimulating factor 1 is a homodimeric glycoprotein with a size of 70 kDa with 522 amino acids. The structure contains two small beta sheets and four alpha-helices in combination with disulphide bonds (Figure 3).[9-11]

The production of CSF-1 can be induced by fibroblasts, endothelial cells, monocytes, macrophages, osteoblast, microglia, keratinocytes, bone marrow stromal cells, natural killer cells, B- cells and T- cells.[14] CSF-1 stimulates the membrane bound CSF1R for regulating the proliferation, survival and differentiation of monocytic cell lineage by inducing the autophosphorylation of the internal receptor tyrosine kinase domain; therefore it activates various downstream signalling processes, including the activation of MAPK, PI3K, Src family kinases and Cbl.[14, 15] CSF-1 is responsible for macrophage accumulation in several tissues, and plays an important role in inflammation.[16, 17] In breast cancer the presence of CSF-1 regulates tissue macrophages. TAMs studies showed a connection with poor prognosis in breast and suggested that a high expression of CSF-1 and CSF1R can promote tumour growth. [1, 16, 17]

Normally breast tissue has no CSF1R expression and low values of CSF-1, except in stages of pregnancy, puberty and lactation. [16, 17]

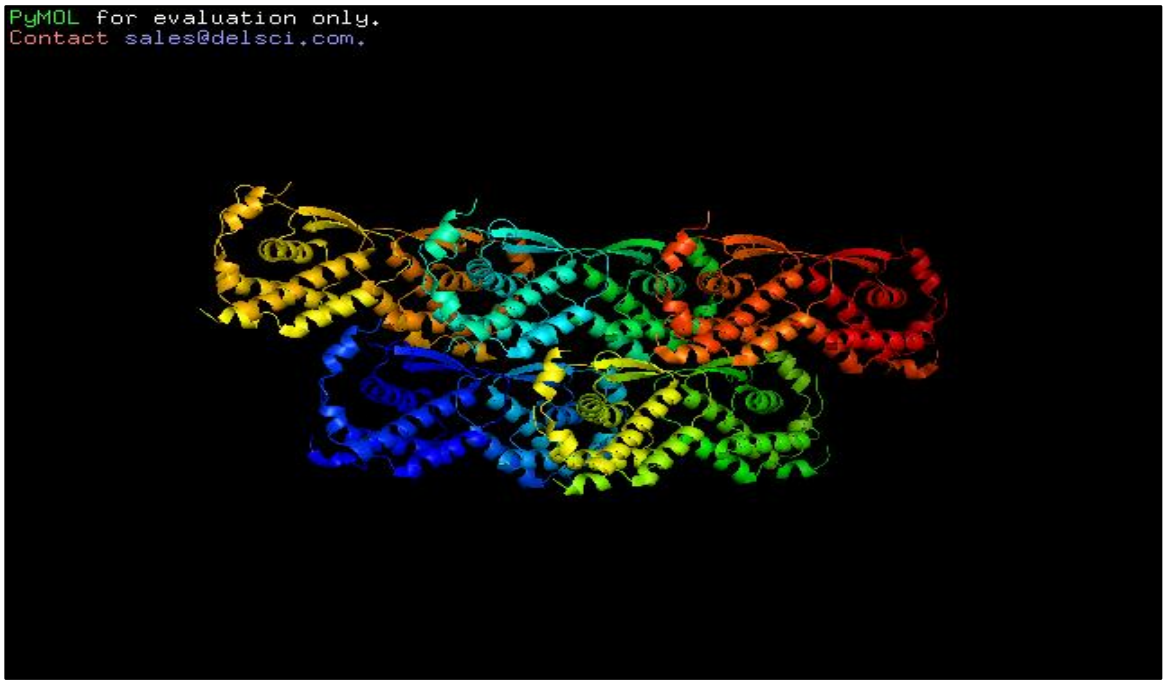


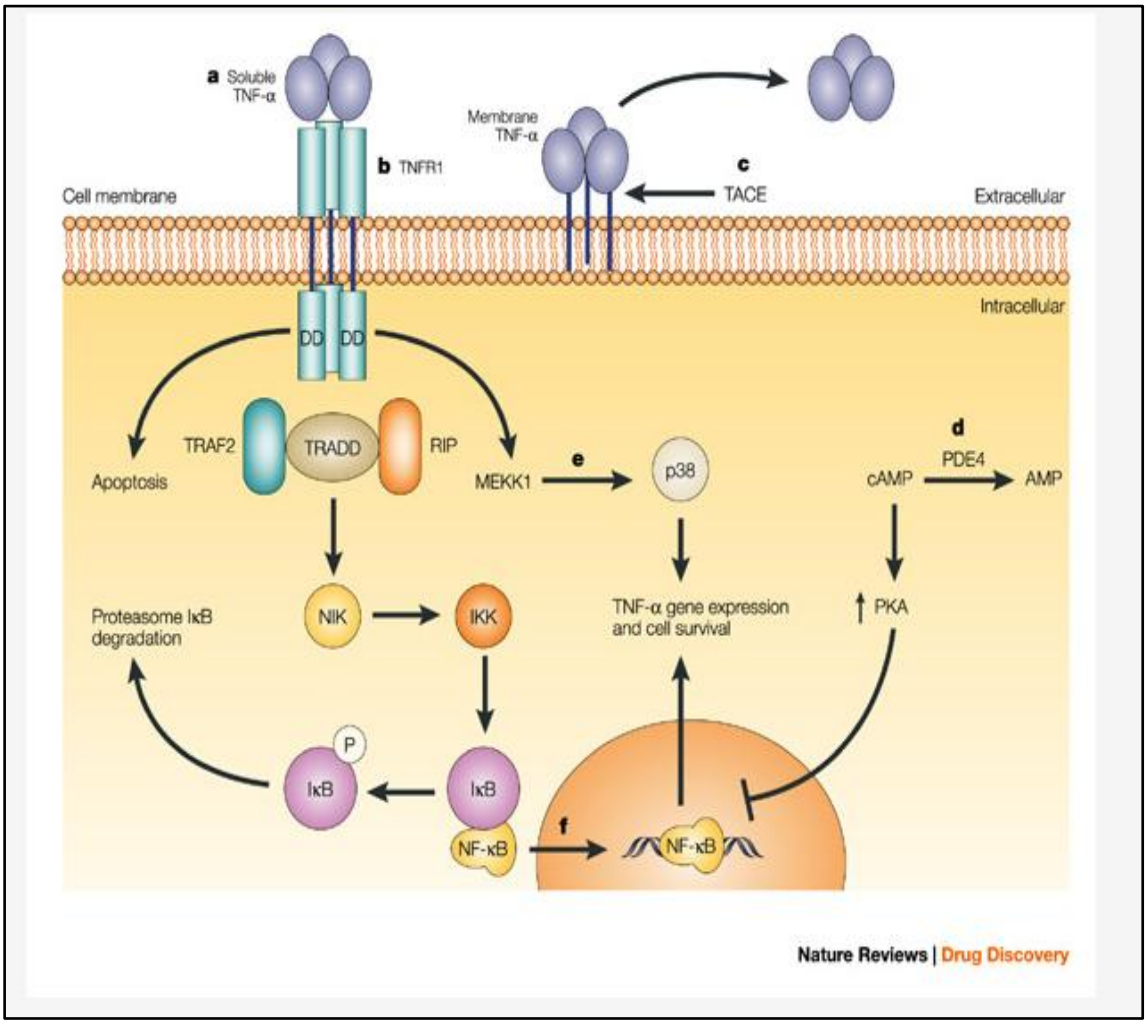
Figure 4: 3D simulated structure of cytokine CSF-1, designed by PyMOL programme; Designed in PyMOL with nucleotide sequence of CSF-1 (NM_000757.5)

1.4.2 Tumour Necrosis Factor Alpha (TNF-α)

The cytokine TNF-α is involved in inflammation and produced by macrophages and many other cells types e.g CD^{4+} lymphocytes, natural killer cells, neutrophils, mast cells and eosinophil cells. [18]

TNF-α has two trans-membrane glycoprotein receptors, TNFR1, also known as p60, p55, CD120a and TNFR2 (p80, p75, CD120b). Both contain cysteine rich repeats in their extracellular domains. TNFR1 is mostly active in tissue and mammalian cells, whereas TNFR2 is expressed normally in immune system cells. [18, 19]

Activation of TNFR1 by the ligand TNF-α stimulates certain pathways. The binding to TNFR2 has a reversible effect with on and off kinetics.[8] Binding to the two receptors causes different responses. For example, TNRF1 contains a signalling domain for cell death and is also responsible for inflammatory activities, cell growth, NF-κB and MAPK signalling pathways. TNFR2 is responsible for cell proliferation and cell survival.[8] The TNFR domain for death includes about 70 amino acids and leads to cellular apoptosis.[8, 18] The third signalling pathway includes the activation of the NF-κB (Figure 4). [20, 21]

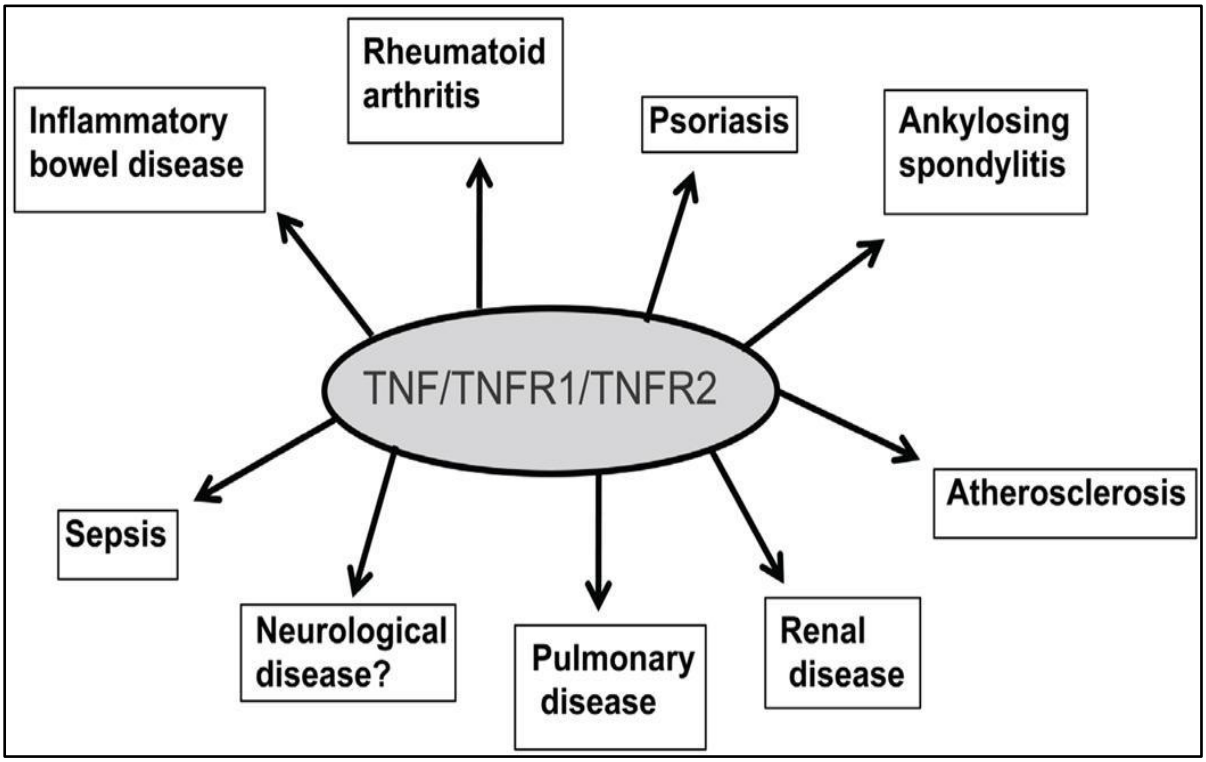


[iii]**Figure 5: TNF-α pathway;** Targets for TNF-α signalling pathway or protein expression

TNF-α has a major role in inflammation and is released immediately after trauma, infection and expose to bacterial lipopolysaccharides. Through these various functions, TNF-α is important for the pro-inflammatory cytokine cascade and cytokine production and in the development of chronic inflammatory diseases (Figure 5).[8]

In many cases inflammatory disorders increase the risk of cancer development. So, TNF-α is one of the key elements in inflammation associated cancers and is involved in promotion and progression of human cancer cells with a link to the NF-κB pathway. Furthermore, TNF-α is produced by tumours with poor prognosis and loss of hormones responsiveness. [15]

For example, in a recent study it was shown, that targeting transmembrane TNF-α with the monoclonal antibody *infliximab* suppresses breast cancer growth. Notably, TNF-α induces CSF- 1 and IL-34, shown in a recent study.[18, 19] [8]



[iv]**Figure 6: Scheme of diseases in relationship to TNF-α and TNF-α receptors.** TNF-α gene expression or modifications lead to certain human diseases

1.4.3 Interleukin 34 (IL-34)

From studies with CSF-1 and CSF1R knockout mice and the CSF1R ligand has been hypothesized in 2008, *Lin et al* identified the 39 kD protein interleukin 34 (IL-34) as a second ligand for the CSF1R. IL-34 contains 241 amino acids and is expressed in the spleen, heart, brain, liver, kidney, thymus, ovary, prostate and colon. IL-34 plays a role in monocyte and macrophage differentiation. [22, 23 24, 25] and leads either to immunosuppressive M1 macrophages with a high IL-10 and low IL-12 expression or to tumour associated M2 macrophages. IL-34 activates the ERK 1/2 MAPK pathway, similar to CSF-1. [12, 13, 26]

IL- 34 and CSF-1 are sharing the same receptor CSF1R, but their sequences are not homolog. [9, 27,26]. IL-34 has an important role in the development of microglia in the central nervous system and Langerhans cells and keratinocytes in the skin. [9, 13]

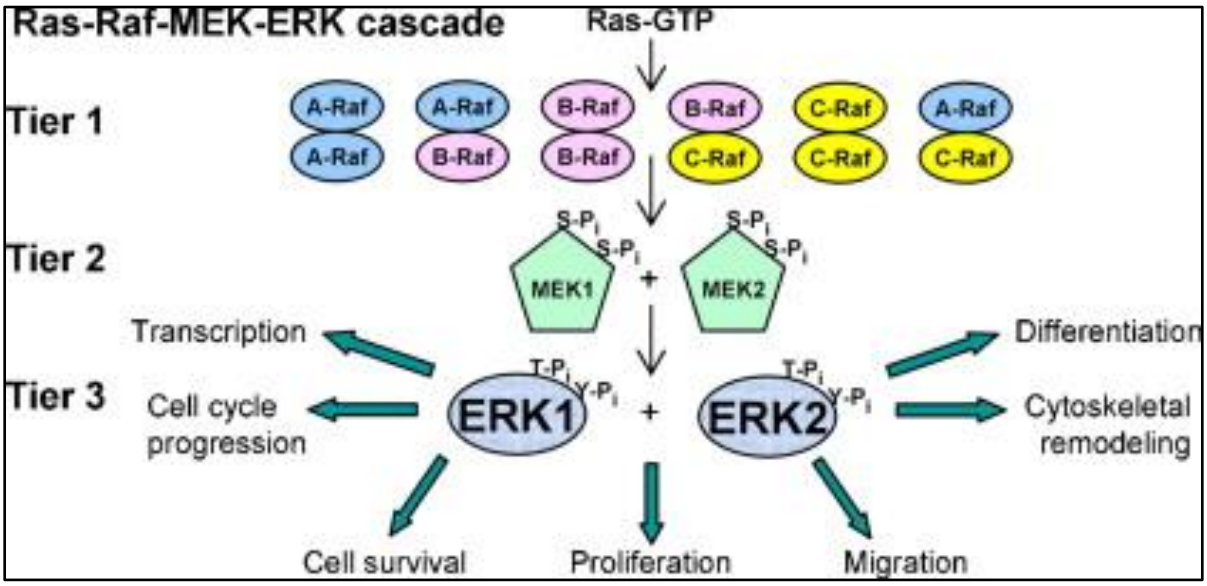
Lin et.al showed that IL-34 has a higher affinity to the CSFR than CSF-1. IL-34 forms a dimer and modulates cell activation due to binding on the D1- D3 site of the CSF1R, which leads to autophosphorylation of tyrosine residues of the receptor. The D2 and D3 domains of the CSF1R are flexible domains and allow IL-34 binding followed by changes of the conformational structure. [12, 14]

The IL-34 and CSF1R complex is regulated by the hydrophobic interactions between the receptor and its ligand. [12, 14, 23] The difference in the expression of CSF-1 and IL-34 might indicate that the two ligands do not have complementary roles in the body. Both ligands activate the MAPK ERK 1/2 which is caused by an overlapped manner. [13]

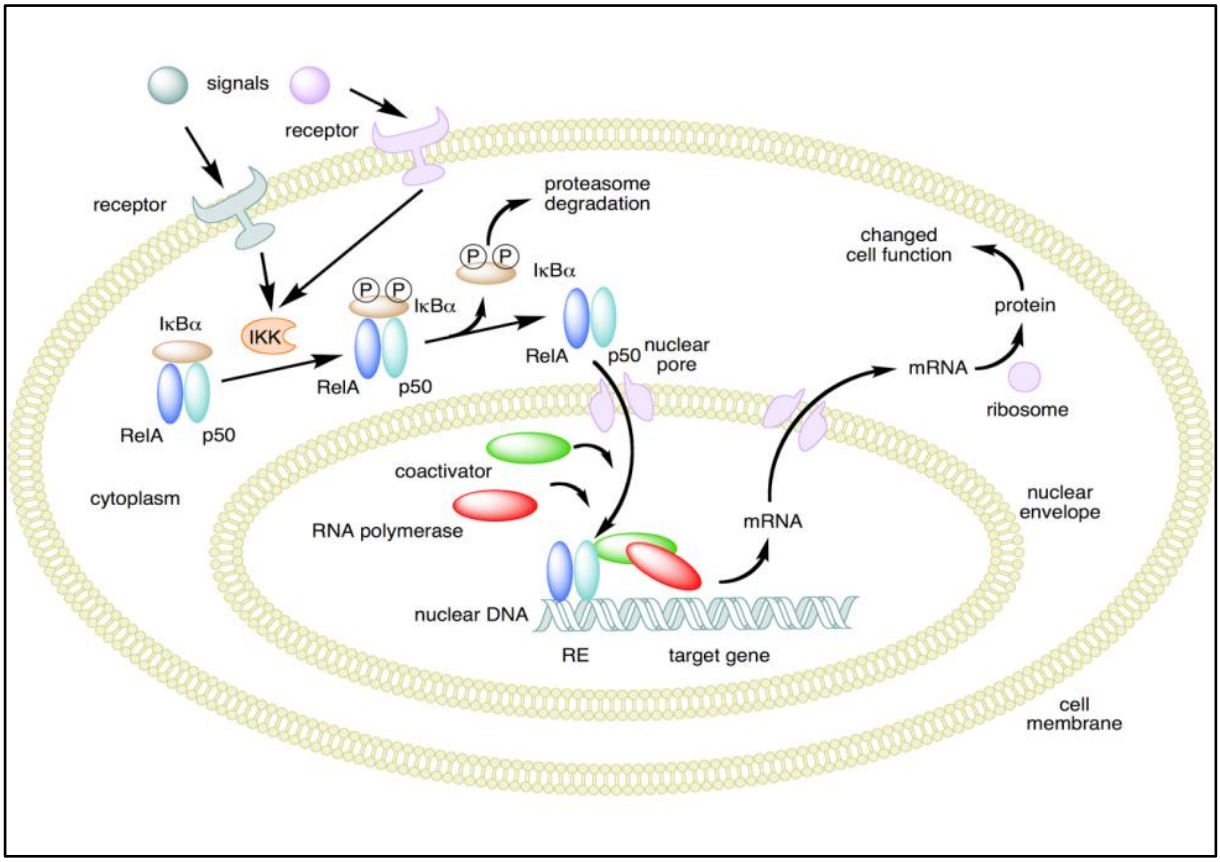
The pathophysiological role of IL-34 is poorly understood, but increased levels in serum of rheumatoid arthritis patients and in serum samples of coronary artery disease patients were found in a recent study.[27] Little is known about the role of IL-34 in cancer. A recent study showed that the expression of IL-34 and CSF-1 in breast cancer was simulated by applying ant-itumour drugs, which could increase the recruitment of stromal macrophage in the tumour tissue.[28] However, the role of IL-34 in the crosstalk between breast cancer cells and tumour associated cells is still unknown.

1.5 Protein expression in cell signalling

The serine/threonine ERK1/2 kinases are involved in cell growth and differentiation and mediate signals from receptor tyrosine kinase receptors via the including a receptor Ras-Raf-MEK-ERK pathway.[29] Activated ERK1/2 regulates many nuclear substrates and cytoplasmic substrates including regulatory molecules and transcription factors (Figure 6).
Therefore, high activity of Ras- Raf- MEK- ERK cascade is present in about one third of all human cancers and is a target for an anti-tumour therapy.[29] [20, 30]
Another important cell signalling pathway is the NF-κB pathway, which is responsible for DNA transcription controlling, cytokine production and cell survival. It can be found in every mammalian cell and plays an important role in the immune response, can lead to cancer, autoimmune diseases and inflammatory diseases.[20] Inducers of NF-κB activity are variable; one ligand for activation of NF-κB is TNF-α, which activates the IKK complex followed by activation of the IkB proteins by phosphorylation. This phosphorylation of IKB proteins leads to an ubiquitination and activates the NF-kB/Rel complex, which is involved in target gene expression inside the nucleus and can activate changes in the cell function.[21] (Figure 7)



[v]**Figure 7: Ras-Raf-MEK-ERK cascade;** Ras-Raf-MEK-ERK cascade in the cell signalling with certain cell processes for ERK1 and ERK2 activation. ERK1 lead to transcription, cell cycle progression and cells survival. Instead of ERK, which lead to cell differentiation, cytoskeletal remodelling and migration. Both are involved in cell proliferation.



[vi]**Figure 8: NF-κB cell signalling pathway with its action inside the cell.** The signal molecule activates the receptor in the cell membrane and lead to cell signalling cascade within the cytoplasm and the nucleus, which lead to activation of transcription of mRNA of the target gene.

2 Aims of the study

We hypothesized that IL- 34 plays an important role in breast cancer. Preliminary IL-34 data obtained from breast cancer patients in collaboration with Prof. Schreiber, Medical University Vienna, support this hypothesis.

The aim of the project was to identify the effect of IL-34 on triple negative MDA-MB-231, luminal-type MCF-7 breast cancer cell, and macrophage behaviour. The presence of macrophages was analysed in MDA-MB-231 and MCF-7 xenograft tumour tissue. The regulation of IL- 34, CSF-1 and TNF-α expression was studied in (co-)culture experiments using a phorbol-myristate-acetate (PMA)-induced cell differentiation model of monocytic/macrophage THP-1 cells and breast cancer cells. In addition, the effect of IL-34 on both breast cancer cell lines and macrophages was investigated by analysing IL-34 related signalling proteins.

3 Material and Methods

3.1 Cell lines

3.1.1 THP-1 (ATCC TIB-202)

The human monocytic leukemic cell line THP-1 was derived from the peripheral blood sample of a 1- year old male. This cell line was grown in suspension.

3.1.2 MDA- MB- 231 (ATCC HTB-26)

The adherent human triple negative mammary breast cancer cell line MDA-MB-231 was derived from a 51 years old female adult and is negative for the estrogen, progesterone and HER2 receptors.

3.1.3 MCF-7 (ATCC HTB-22)

The adherent human mammary breast cancer cell line MCF-7 was derived from a 69 years old female adult and has no HER2 receptor, but is positive for estrogen and progesterone.

3.2 Cell culture

Cell culturing is a laboratory technique for maintaining living cells for applications in research and clinical studies. In cell culture cells are grown under controlled conditions including temperature, pH, growth factors and nutrients outside of their natural environment, thus separated from their original tissue.

THP-1 cells were cultured in RPMI-1640 Medium, 10% heat inactivated foetal bovine serum and 100 U/mL penicillin/streptomycin(Sigma). The cells were cultured in suspension and were sub-cultured at a cell concentration of 10^6cell/mL after a centrifugation step and addition of fresh medium.

MDA-MB-231 and MCF-7 cells were cultured in DMEM-medium (Dulbecco's Modified Eagle Medium, Lonza Belgium), 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin/streptomycin (Sigma) and 0.1 M NEAA (Non- essential amino acids, Lonza Belgium). Sub cultivation was done at a ratio of 1:3.

All cells were cultured for their normal use in 10 cm cell culture dishes (Falcon) and were incubated in a humidified incubator (Binder) at 37°C and 5% CO_2.

Cell line	Medium	Supplements	Growth	Cancer type
THP-1	RPMI-1640	FBS, Pen/Strep	suspension	Leukemia
MDA-MB-231	DMEM	FBS, Pen/Strep, NEAA	adherent	Adeno-carcinoma
MCF-7	DMEM	FBS, Pen/Strep, NEAA	adherent	Adeno-carcinoma

Table 2: Overview of cell lines and their media

3.3 Sub-culturing of cells

3.3.1 Adherent cells

First, culture media and PBS were pre-warmed in a 37 °C water bath. Following removal of the old medium under sterile conditions, cells were washed twice with 10 mL PBS per 10 cm cell culture dish. Then 1 mL of 1x Trypsin-EDTA was added and the cells were incubated 5-10 minutes at 37°C in the humidified incubator. After the incubation step the trypsinisation was stopped by adding 9 mL of fresh medium per cell culture dish and the cells were transferred after pipetting several times from the cell culture dish to a fresh sterile 50 mL Falcon tube. Next, the cells were centrifuged at 1500 rpm and re-suspended in fresh medium in the volume of interest for the specific split ratio. Finally, new 10 cm cell culture dishes were pre-filled with 10 mL fresh medium and the cells were pipette into these cell culture dishes. These cell culture dishes were placed into the incubator at 37°C and 5% CO_2 concentration for further use.

3.3.2 Suspension cells

Suspension cells, like the THP-1 cells can be sub-cultured without the Trypsin-EDTA step. Cells were collected by centrifugation at 1500 rpm for 2 minutes at room temperature and resuspended in fresh medium in a volume of interest for the intended split ratio in a new 10 cm cell culture dish and incubated at 37°C with 5% CO_2.

3.4 Cell counting

For different assays different cell numbers were necessary, so first the cells were counted in a Neubauer chamber with 0.1 mm depth before seeding. For preparation of the counting, the cells were prepared by trypsinisation, pelleted by centrifugation and then resuspended in 1 mL fresh medium. For the counting dilution (1:100), 10 µL of the resuspended cell suspension were diluted in 990 µL PBS. The Neubauer chamber and the cover glass were first cleaned up with 70% ethanol and the cover glass was placed on the middle of the chamber. 10 µL of the diluted cell suspension were pipetted on each side of the chamber, which was filled up with the suspension by capillary force. Afterwards the cells were counted in the four square fields of both counting areas on the chamber and for cell concentration calculation the average number per field was determined. The number of cells in the cell suspension is calculated by N (numbers of cells) $x10^6$ cells/mL.

3.5 Cryoconservation

Cryoconservation is used for collection and storage of cells and tissue in liquid nitrogen at-196°C. Cell pellets were resuspended in special cryomedium to reduce the risk of cell damaging. The cryomedium is specific to each cell lines and contains the special growth medium for every cell line with 5% DMSO. The cells have to be stored in special cryotubes for storage in nitrogen. To reduce the risk of damaging the cells, the cells suspension in the cryotubes were frozen first in a styrofoam container at -80°C to allow a cooling rate of -1°C to -3°C per minute. Then cells were transferred in liquid nitrogen for long- term storage.

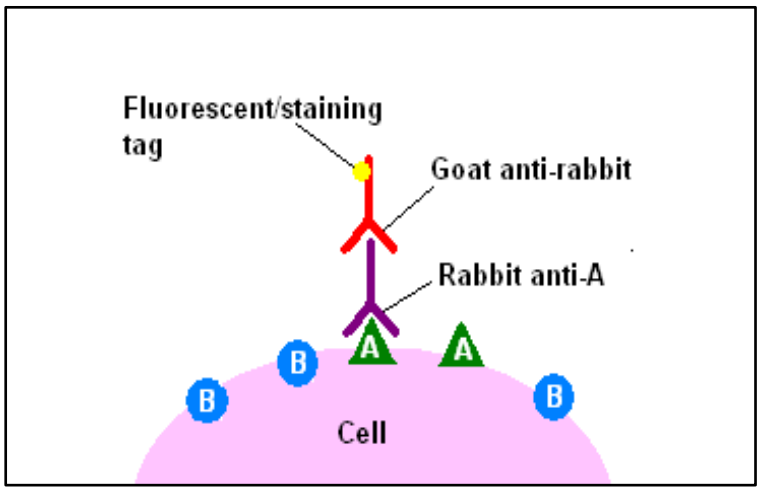
3.5.1 HE staining

Hematoxylin and eosin staining was used in histology. Hematoxylin, which is a dark blue stain, binds to DNA and RNA in the nucleus and in the cell. Eosin is a red stain, which binds to arginine and lysine amino acid residues, which can be found in cytoplasm filaments in muscle cells, intracellular membranes and extracellular fibres.

3.6 Immunohistochemistry staining

For staining, normally paraffin-fixed tissue sections have to deparaffinised with xylene treatment followed by decreasing alcohol solutions (100%, 96%, 70%) to expose the epitopes for antigen binding. To reduce background staining, the samples were treated with buffer systems, which block the reactive sites of the epitopes, where the first antibody may be able to bind. After the de-masking step the first antibody was used for recognising the target epitopes. The first antibody usually binds on the target of interest, whereas the second antibody is labelled with biotin and binds on the immune globulins of the first antibody. After binding with the labelled second antibody a fluorescence detection method by an enzyme was used, for this purpose the enzyme horseradish peroxidase was used for labelling. The enzyme is conjugated to Streptavidin, which binds to biotin and reacts by building an increasingly coloured product, afterwards these product were analysed under the light microscope.

For detecting certain antigens or proteins in cells or stroma of tissue sections of special antibodies are used. Immunohistochemistry staining is used for diagnostic research of abnormal tissues and detects special molecular markers in several cellular events.



[vii]**Figure 9: Immunhistochemistry staining procedure;** Rabbit anti- A binds to target protein of the cell membrane, after binding the goat anti- rabbit antibody with a fluorescent tag binds to the first antibody

3.6.1 F4/80 staining

F4/80 is an IHC staining method for detection macrophage cells in paraffin-fixed tissue sections. The monoclonal antibody F4/80(Serotec Cat. No. MCA497GA) reacts with the murine cell surface glycoproteins of blood derived macrophages between the tissue cells and dye the macrophages in a brownish colour.

3.7 Biological assays

3.7.1 Differentiation of THP-1 cells into macrophages and preparation of conditioned medium

For generating differentiated macrophages, THP-1 cells were cultivated in RPMI- 1640 with 1.5% heat inactivated foetal bovine serum, 10 mM Hepes, 1 mM pyruvate, 2.5 g/L D-glucose and 50 pM β-mercaptoethanol at a confluence of 10^6 cells/dish. Afterwards 150 nM phorbol myristate acetate (PMA) was added and the cells were incubated for 48 h.
For preparation of conditioned medium from differentiated THP- 1 cells, cells were incubated with 150 nM PMA for 48 h. Following a medium change to RMPI-1640 medium without PMA, cells were incubated for another 72 h.

3.7.2 THP-1 differentiation and polarisation to M1 or M2 macrophages

Human THP-1 cells were seeded at a cell density of 1.6 x 10^6 cells/ well in a 6- well dish at a final volume of 1 mL and were differentiated with 150 nM PMA in RPMI-1640 with 1.5% heat inactivated foetal bovine serum, 10 mM Hepes, 1 mM pyruvate, 2.5 g/l D-glucose and 50 pM β-mercaptoethanol for 48 h. Afterwards, cells were treated with recombinant protein at concentrations [IL-34 (125 ng/mL), IL-4 (20 ng/mL), IL- 13 (20 ng/mL) , CSF-1 (125 ng/mL) or LPS (100 ng/mL)] and were incubated for another 72 h. After the incubation step the medium was removed and RNA was isolated for determination of mRNA expression.

3.7.3 Co- culture assay of human breast cancer cells with differentiated THP-1 in 6-well dishes

Human THP-1 cells were seeded at a cell density of 10^5/6- well and were differentiated with 150 nM PMA in RPMI-1640 with 1.5% heat inactivated foetal bovine serum, 10 mM Hepes, 1 mM pyruvate, 2.5 g/l D- glucose and 50 pM β-mercaptoethanol for 48 h. Afterwards, 10^5 MCF-7 or MDA-MB-231 cells were added to each well and were incubated for another 72 h at 37°C. Finally, RNA Isolation of the differentiated THP-1 cells was isolated for mRNA analysis.

3.7.4 Co- culture assay of human breast cancer cells with differentiated THP-1 in 10 cm dishes

See 3.6.3

3.7.5 Treatment of differentiated THP-1 cells with conditioned medium from human breast cancer cells

For the preparation of conditioned medium from human breast cancer cells 10^6 MCF-7 and MDA-MB-231 cell per 10 cm dish were seeded in DMEM- medium and were incubated for 48 h. Meanwhile 10^6 THP-1 cells were differentiated as described in 3.6.1. Then the RMPI-1640 medium was removed from the cells and the conditioned medium of the human breast cancer cells was transferred to the differentiated THP-1 cells, followed by incubation of the cells for 72 h at 37°C.

3.7.6 Treatment of human breast cancer cells with differentiated THP-1 conditioned medium

Human THP-1 cells were seeded at a cell density of 10^6 10 cm dish and were differentiated by adding 150 nM PMA in the special RMPI- medium for 48 h. Afterwards, the medium was changed to fresh special RPMI- medium and was incubated for another 48h. Meanwhile the human MCF-7 and MDA-MB-231 breast cancer cells were seeded at a cell density of 10^6 cells per 10 cm dish in DMEM medium and were incubated for 24h. After the incubation step of the breast cancer cells, the DMEM medium was removed from cells and the conditioned medium from the differentiated THP-1 was transferred on the breast cancer cells. The cells were incubated for 72 h at 37°C and total RNA isolation was performed.

3.7.7 Antibody treatment of human breast cancer cell lines

For antibody treatment, human breast cancer cells were seeded at a cell density of 10^5/6-well dish in a final volume of 1 mL DMEM medium and were incubated for 24 h at 37°C. After 24 h antibodies at specific concentration [TNF-α (15 ng/mL), CSF-1R (20 ng/mL) and IL-34 (10 ng/mL)] were added to each well and were incubated for another 24 h at 37°C. Total RNA and protein isolation was performed.

3.7.8 Antibody assay of IL- 34 and conditioned medium of differentiated THP-1 on human breast cancer cells

Preparation of the conditioned medium from human THP-1 cells was performed as described in 3.6.1. In parallel, 10^5 human MDA-MB-231 and MCF-7 breast cancer cells were seeded in 6- well dishes in 1 mL DMEM medium and were incubated for 24 h at 37°C. After the incubation step, the DMEM medium from the human breast cancer cells was replaced and the conditioned medium from the differentiated THP-1 containing IL-34 antibody(R&D System) at a concentration of 10 ng/mL was transferred to the cells. Cells were incubated

with the conditioned medium-antibody-mixture for 24 h at 37°C, followed by total RNA isolation for mRNA expression analysis.

3.8 Analysis of mRNA expression in MCF-7 and MDA-MB-231 cells

3.8.1 Total RNA Isolation from adherent cells

Cells were lysed directly in the culture dish after removing the culture medium with 1 mL TRIzol Reagent and passing the cell lysate several times through a pipette. After 5 minutes incubation at room temperature, the cell lysate were transferred into fresh Eppendorf tubes. Due to of the mono-phasic solution of phenol and guanidine isothiocyanate of the TRIzol Reagent, the integrity of the mRNA is preserved, while disruption of the cells and dissolving of cell components occurs up in the lysate. After addition of chloroform and centrifugation at 12.000 g for 15 minutes at 4°C, lysate were separated into two phases, an upper aqueous and a red lower organic phase. The aqueous phase contains the RNA and was transferred into a fresh Eppendorf tube. For precipitation of the RNA, 500 µL of icecold isopropanol were added and the tubes with the mRNA isopropanol- mixture were vortexed for some seconds. The tubes were stored overnight at -20°C followed by centrifugation at 12.000 g for 30 minutes at 4°C for pelleting the mRNA precipitate. The RNA pellet was washed once with 1 mL 70% ethanol and centrifuged at 12.000 g for 8 minutes at 4°C. Then, the supernatant was removed from the pellet and was dried for 30 minutes at room temperature. Finally, the pellet was dissolved in 10 to 25 µL DEPC (Diethylpyrocarbonate) -treated water and the dissolved pellet incubated for 10 minutes on a heating block at 65°C. For the concentration and purity was determined using NanoDrop (Thermo Scientific). The A_{260}/ A_{280} ratio should be between 1.8 and 2.

3.8.2 Total RNA Isolation of suspension cells

Cells were centrifuged in the culture medium at 1200 rpm for 3 minutes at room temperature. The total RNA Isolation of suspension cells was performed as described in 3.7.1.

3.8.3 Complementary DNA (cDNA) synthesis

Complementary DNA is a synthetic DNA made from mRNA, using an enzyme called reverse transcriptase, originally isolated from retroviruses. In principles mRNA is used as a template and the reverse transcriptase synthesise a single-stranded DNA molecule. This single-stranded DNA is then used as a template for double stranded DNA synthesis.

The cDNA synthesis was performed with 2 µg RNA, addition of 1µg/µL oligo dT-primer and DEPC water in a final volume of 11.5 µL and incubation for 5 minutes at 70°C on a heating block. Then, the samples were cooled down another 5 minutes on ice. The condensed liquid was spun down and following reagents were added to the sample:

4 µL RevertAid^{Tm} M-MuLV Reverse Transcriptase 5x buffer

2 µL dNTP mix (10 mM)

1 µL RevertAid M-MuLV Reverse Transcriptase

0.5 µL Ribonuclease Inhibitor (40 U)

The Reaction mixture was incubated at 42°C for 60 minutes. The cDNA was stored at -20°C. For multiple reactions, a master mix was made by multiplying the number of reactions.

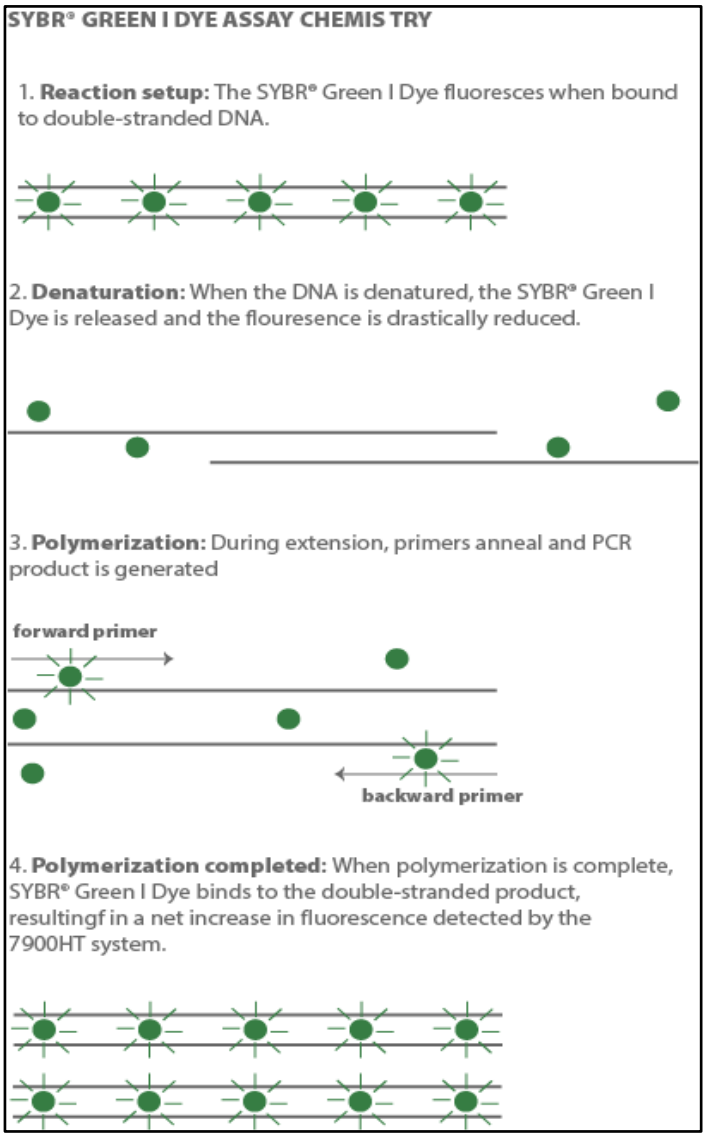
3.8.4 Real time qRT-PCR with the LightCycler 1.0 Instrument (Roche, Germany)

Real-time quantitative RT-PCR (pRT-PCR) is a technique using PCR for amplifying DNA and detection by using emitted fluorescence signal.

The LightCycler FastStart DNA Master SYBR Green is a ready-to-use “Hot Start” reaction mix for polymerase-chain-reactions (PCR) and contains a FastStart Taq DNA polymerase and a DNA double strand specific SYBR Green dye for detection. The Taq DNA polymerase was modified from a thermo stable recombinant Taq DNA polymerase and is inactive at room temperature, because of the heat- labile blocking groups on some of the amino acid residues of the enzyme. Thus, there is no activity when primers from non specific primer template form hybrids at lower temperature. The Taq DNA polymerase is activated by high temperature (e.g. 95°C for 10 minutes) with removing the blocking groups from the amino acids. For detection SYBR Green has been used, a specific dye for double stranded DNA, based on intercalation. The fluorescence is enhanced during each step of DNA synthesis since the SYBR Green dye binds to each the amplified PCR product and emits fluorescence. The detection and the collection of the data in the LightCycler machine occur in real-time. At low amplification cycles, the emitted fluorescence is still low and under the detection limit of the instrument, shown as a baseline. After several amplified cycles an exponential increase of the emitted fluorescence signal can be seen till a plateau is reached. The fluorescence signals of the samples were detected by the photo detector of the machine.

For specification of the amplificated products a melting curve analysis was performed. The relative quantification of the mRNA product was performed by measuring the specific signals of the housekeeping gene beta 2-microglobulin and normalising these signals to the signals of the amplificated products from the samples. Beta2-microglobulin is used as the housekeeping gene, because it is expressed in every nucleated cell as component of the major histocompatibility complex class I (MHC-I).

For amplification 1 µL of a 1:10 pre-diluted (in DEPC water) cDNA sample was added to a 14 µL or 19 µL reaction mix (Table 3) in pre- cooled glass capillaries and put in the LightCycler machine. For multiple samples, a master mix was made. For the detection of IL-34, CSF-1, TNF-α, IL-10, IL-12, IL-4 and beta2M mRNA expression specific primers were designed. The PCR data from the LightCycler were analysed by the Data Analysis Version 3.5.3 (Roche, Germany).

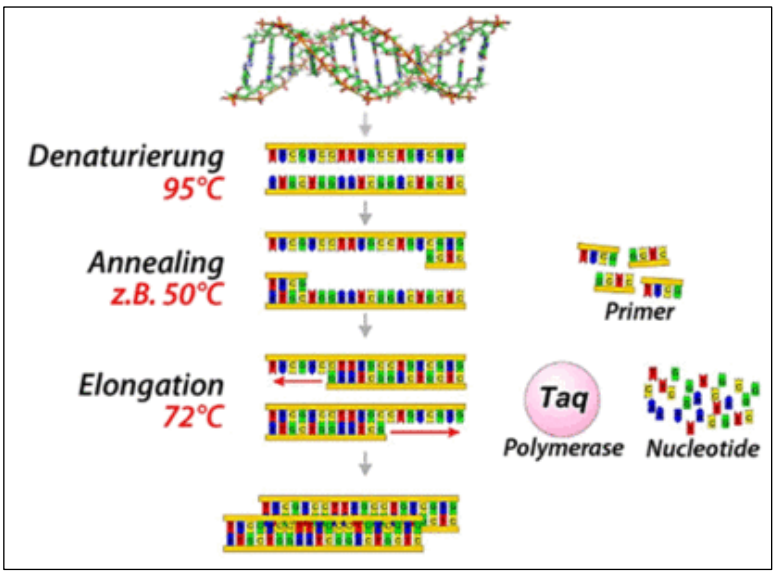


[viii]***Figure 1: Overview of SYBR Green based qPCR*** *reaction 1.) SYBR Green I dye binds to the minor groove of dsDNA. 2.) DNA denaturation causes fluorescence to decrease since SYBR Green I is released into solution. 3.) While DNA polymerase amplifies the template, SYBR Green I dye is incorporated into the newly synthesised dsDNA. 4.) Fluorescence is increased in the final product*

Components	Volume for one reaction [µL]
H_20, PCR grade	10.2
$MgCl_2$ (25 mM)	1.3
LightCyler FastStart DNA Master SYBR Green I	1.5
Forward primer (10 pmol)	0.5
Reverse primer (10 pmol)	0.5
Total volume	14

Table 3: Reaction mix components for LightCycler PCR

The cycle protocol consists of four programs. Program 1 is the pre-incubation step to remove the blocking groups from the polymerase denaturation step of the cDNA for 10 minutes at 95°C. The second Program is the amplification step of the target DNA and consist three segments, which were 45 times repeated. Therefore the first segment is for separation of the primer dimers, primer cDNA connections and melting of the cDNA for 10 seconds at 95°C. The second segment is the annealing step of the primers and takes 5 seconds at the primer specific temperature (Table 4). The third segment is the synthesising step at 72°C and depends on the fragment length. Program 3 is for getting the melting curve, which is used for product identification. Therefore the samples have to heat up to 95°C in 0.1°C per second steps. Program 4 is the cooling step to 40°.



[ix]**Figure 10: PCR reaction steps**. Denaturation temperature at 95°C, annealing temperature at 50°C and elongation at 72°C

Gene (human)	Forward Primer [5'- 3']	Reverse Primer [5'- 3']	Length [bp]	Annealing Temperature [°C]	Melting Temperature [°C]
IL-34	AATCCGTGTTGTCC CTGTTG	CAGTACAGCAGCT CCATGACC	112	64	86
CSF-1	GCTGTTGTTGGTCT GTCTC	CATGCTCTTCATA ATCCTTG	335	60	85
TNF-α	AGGGACCTCTCTCT AATCAG	AGCTTGAGGGTTT GCTAC	102	62	84
CSF-1R	CTGAGCAAGACCTG GAC	TGCTGTTCACCAG GATGCCAG		62	89
IL-4	ATGGGTCTCACCTC CCAACTGCT	CGAACACTTTGAA TATTTCTCTCTCAT	455		
IL-10	GCCTAACATGCTTC GAGATC	CTCATGGCTTTGT AGATGCC	346	56	82
IL-12	GAAGGCCAGACAAC TCTAG	CTATCAATAGTCA CTGCCCG	479	56	82
β-2M	GATGAGTATGCCTG CCGTGTG	CAATCCAAATGCG GCATCT	114	62	82

Table 4: Primer sequences used for qPCR with the LightCycler 1.0

3.8.5 Real- time Quantification

The real-time polymerase chain reaction is used to amplify cDNA, which is a routine tool for determining changes in mRNA levels of a target gene. Therefore, relative quantification gives information about the expression levels of a target gene versus the housekeeping gene. For calculating the expression levels LightCylcler melting curve was performed. The given Ct value within the LightCycler programme above the threshold is related to the initial DNA amount and assay sensitivity. The specific relative mRNA expression was calculated with the $2^{\Delta\Delta CT}$ method according to the housekeeping gene.

3.8.6 Agarose gel electrophoresis

To determine the size of separated DNA molecules and exclude primer dimers the method of agarose gel electrophoresis was used in some cases. DNA fragments were separated by charge and size in an electric field.

Therefore 1.5% agarose Gel was dissolved in 1x TAE buffer by using a microwave for heating up. 10 µL of the gel dye reagent was added immediately after agarose dissolution. Then the solution was poured in a specific moulded equipped with combs to forms shots.

The qRT-PCR samples were centrifuged shortly to collect the samples. 5 µL of 6x DNA Loading Dye (Thermo Scientific) were added to 15 µL sample and loaded into the stats of the gel. One stat was loaded with the DNA Gene ruler (100 - 10.000 bp, Thermo Scientific) for estimating the size of the samples. Gels were running at 100- 120 V for 1 h and observed under the UV illuminator for checking the size of the amplified PCR products.

3.9 Determination of ERK, pERK, NF-κB protein expression in MCF-7 and MDA-MB-231 cells

3.9.1 Protein Isolation

For protein isolation the cells were lysed in Frackelton lysis buffer (10 mM Tris, 30 mM sodium pyrophosphate, 50 mM sodium chloride and 1% TritonX-100) and freshly added 1 mM PMSF (phenylmethylsulfonyl fluoride), 100 µM Na_3VO_4 and 1x Complete^{TM}- EDTA- free protease inhibitor cocktail. The protein lysates were transferred into a fresh tube and the samples were shock frozen three times in liquid nitrogen followed by centrifugation with 15.000 rpm at 4°C for 10 minutes.

3.9.2 Protein quantification

For protein quantification a colourimetric assay (Bio-Rad Protein assay) was used. The Coomassie Brilliant Blue G-250 dye binds basic and aromatic amino- acids. The binding of the dye to a protein stabilises the negatively charged anionic dye form producing the blue colour, which can be detected at 595 nm. For measuring the protein concentration 1 µL of the samples and a standard curve series from 0 to 20 µL/mL bovine serum albumin were diluted in H_2O and measured with NanoDrop.

3.9.3 SDS polyacrylamide electrophoresis

The SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is an electrophoresis method, where the separation is caused by the size of the molecule through an electric field.

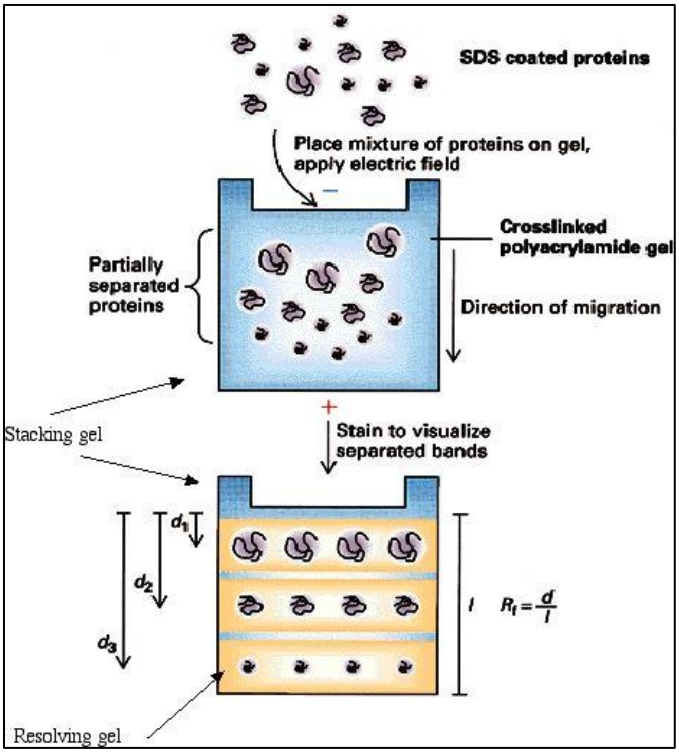
Polyacrylamides are built up through polymerisation of the monomeric acrylamide due to APS (Ammonium persulphate) and TEMED. In the electrophoresis gel larger molecules were strongly held back than smaller ones.

For the SDS-Page the samples have to pass the wide-meshed stacking gel, the gel contains chloride ions, which have a faster migration speed than the proteins. The running buffer for the electrophoresis contains chloride ions, which have a faster migration speed than the proteins. The separation gel has a denser network with a higher salt concentration and a higher pH value, than the stacking gel. So, first bigger protein molecules were held back at the gel borders of the stacking gel and the separation gel and the proteins get redefined according to their molecular weight.

The proteins have to denature under a certain temperature (e.g. 95°C for 5 minutes) and gets separated in the direction of the anode, depending on their molecular weight. The polypeptides of the denatured proteins get masked on their charge by the SDS (sodium

dodecyl sulphate) in the sample buffer, therefore the anionic components have a constant net charge and the separation is affected due to the molecular mass. The DTT (1M, Dithiothreitol) in the 5x FSB buffer reduced the disulphide bonds in the polypeptide chain. The sample buffer 5x FBS contains bromophenol blue dye, glycerol, SDS and Tris. Bromophenol blue dye indicates the electrophoresis front. The molecules of the dye are very small and are therefore totally unretarded. Glycerol gives the sample solution more density so it is easier to load the sample into the well. DTT reduces any disulphide bridges present that are holding together the protein tertiary structure, enabling SDS to bind strongly to the denatured protein.

For the SDS-Page the gel has to be prepared, afterwards the denatured samples were diluted in 3:1 5x FSB/ 1M DTT and were loaded to the chambers of the gel. The gel run first at 60 V till the samples reached the separation gel and then the voltage was increased up to 200 V for 30 to 60 min.



[x]**Figure 11: SDS-Page scheme for protein separation.** Smaller proteins were separated to the end of the gel, whereas bigger proteins stack on the top of the gel.

Stacking gel (Mini 3%)				
H_2O [mL]	**Acryl./Bisacryl (30:0.8) [mL]**	**Stacking buffer [mL]**	**10 % APS [µL]**	**TEMED [µL]**
1.7	0.42	0.38	25	2.5

Table 5: Preparation table of one Stacking gel

Separation gel					
% Gel	**H_2O [mL]**	**Acryl./Bisacryl. (30:0.8) [mL]**	**Separation buffer [mL]**	**10 % APS [µL]**	**TEMED [µL]**
6	2.6	1.0	1.345	5	5
8	2.3	1.3	1.345	5	5
10	2.0	1.7	1.345	5	5
12	1.6	2.00	1.345	5	5
14	1.3	2.3	1.345	5	5
16	1.0	2.7	1.345	5	5

Table 6: Preparation table of separation gels

For the preparation of other solutions see appendix (chapter 8).

3.9.4 Western blotting

Western blotting involves the transfer and immobilisation of proteins from a SDS- PAGE gel to a solid nitrocellulose membrane. The SDS-PAGE bound proteins are negatively charged and migrate to the positively charged anode. Thus, the gel with the proteins and the nitrocellulose membrane are arranged as a sandwich between the electrodes allowing the proteins to move from the gel to the membrane.

The Transblot SD Semi-Dry Transfer device from BioRad transfers the proteins in a horizontal orientation onto the membrane. The SDS gel and the nitrocellulose membrane are inserted between some sheets of pre-wetted filter papers and the gel membrane filter paper package was placed between the anode and the cathode and run for 75 minutes with 12 V. The transfer buffer (chapter 8) contains methanol to achieve efficient binding to the nitrocellulose and SDS improves the transfer of protein from the SDS gel. The nitrocellulose membrane is one of the high binding membranes and is made of 100% pure nitrocellulose on an inert synthetic support with small pore size for retaining the smaller molecules. The advantage of semi- dry blotting is the use of low voltages and minimal buffer volumes of the sandwich arrangement.

3.9.5 Ponceau S staining

The staining with Ponceau S solution is a fast and reversible staining method for estimating the location of protein bands on the nitrocellulose membrane. Ponceau S produces red stained bands and background can be easily removed by rinsing steps with water. Complete removal of the Ponceau S staining dye from the membrane can be done with the blocking solution (TBS buffer with Tween20 + 5% bovine serum albumin) used for the detection with specific primary antibodies. (For buffer preparation see Appendix)

3.9.6 Detection of specific proteins on the nitrocellulose membrane

After blocking with TBST + 5% bovine serum albumin (BSA) for 1 h at room temperature, the membrane was incubated with the specific primary antibody for detection of the protein of interest (Table 7) overnight with TBST at 4°C on a rotary shaker. Then the membrane was rinsed six times with TBST for 5 minutes and incubated with an HRP conjugated secondary antibody at room temperature for 2 hours. The labelled protein bands on the membrane were detected by chemiluminescence (Ace Glow, Peqlab, Erlangen, Germany), scanned using FUSION-FX7 (Vilber Lourmat, Marne-la-Vallée, France) and quantified by Fusion-CAPT-Software 16.07 (Vilber Lourmat).

Primary Antibody	**Dilution**	**Secondary Antibody**	**Dilution**
Phospho- p44/42 MAPK (Erk1/2) rabbit monoclonal antibody (Cell signalling)	1 : 2000	HRP- conjugated anti- rabbit IgG (Cell Signalling)	1 : 10 000
p44/42 MAPK (Erk1/2) rabbit monoclonal antibody (cell signalling #4695)	1 : 2000	HRP- conjugated anti- rabbit IgG (Cell Signalling)	1 : 10 000
Anti- NF-KB p65 (Ser536) rabbit IgG polyclonal antibody (Cell Signalling #3031)	1 : 1000	HRP -conjugated anti- rabbit IgG (Cell Signalling)	1 : 10 000
Anti- GAPDH monoclonal antibody (Abcam #9484)	1 : 20 000	HRP- conjugated anti- rabbit IgG (Cell Signalling)	1 : 10 000

Table 7: Antibodies used for western blotting

3.10 Bioinformatics

Bioinformatic tools were used for analysis of primer positions, target sequences, as well as for searching the different mRNA transcript variations of IL-34. This was done by NCBI searching tool.

The graphics of the cytokines in the theory part the pictures were designed with MEGA5, Pymol, Zinc Database and the sequence analysing tool Swiss Model was used for comparison.

Patient data (n=75) were done in cooperation with Prof. Schreiber from the Department of Obstetrics and Gynaecology at the Medical University of Vienna.

3.11 Statistical methods

Data of the *in vitro* experiments and the statistical graphics were done by *GraphPad prism* software.

4 Results

4.1 Human cells have 3 different IL-34 mRNA transcript variations

Searching for information about human IL-34 on NCBI- the national centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) revealed that IL-34 is located in the Chromosome 16 in cytoband q22.1 and is also called C16orf77 (Figure 13). Three different mRNA Transcript variants are listed up on the NCBI data searching tool. The IL-34 is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and frog and has certain functions, like cytokine activity, growth activity and is involved in inflammatory responses as described in the theory part before.

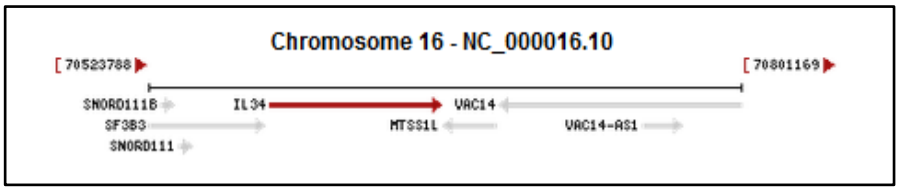
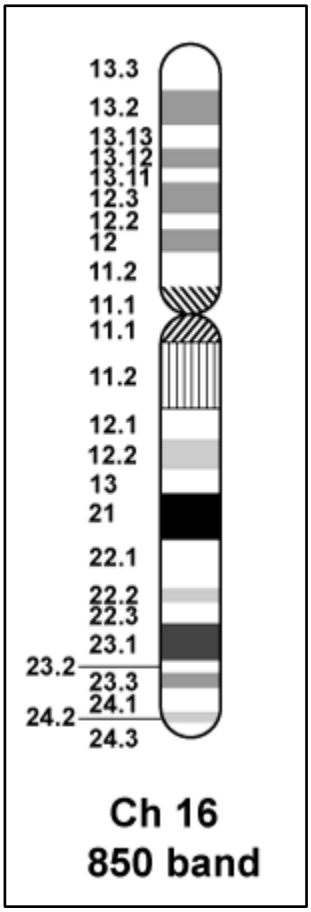


Figure 12: Chromosome 16 with IL-34; Chromosome 16 with red labelled position of the human Interleukin 34 on the left and Chromosome 16 on the right side with certain labelled Cytoband, within the Cytoband q22.1 where the locus of human Interleukin 34 takes place.(**Gene ID: 146433)**



For further information about IL-34 another searching tool, the human protein Atlas (http://www.proteinatlas.org/) was used to determine the protein expression of IL-34 in human tissues. The results show, that IL-34 is highly expressed in the brain, endocrine tissues, in bone marrow and immune system and skin, but also in other tissues. (Figure 14)

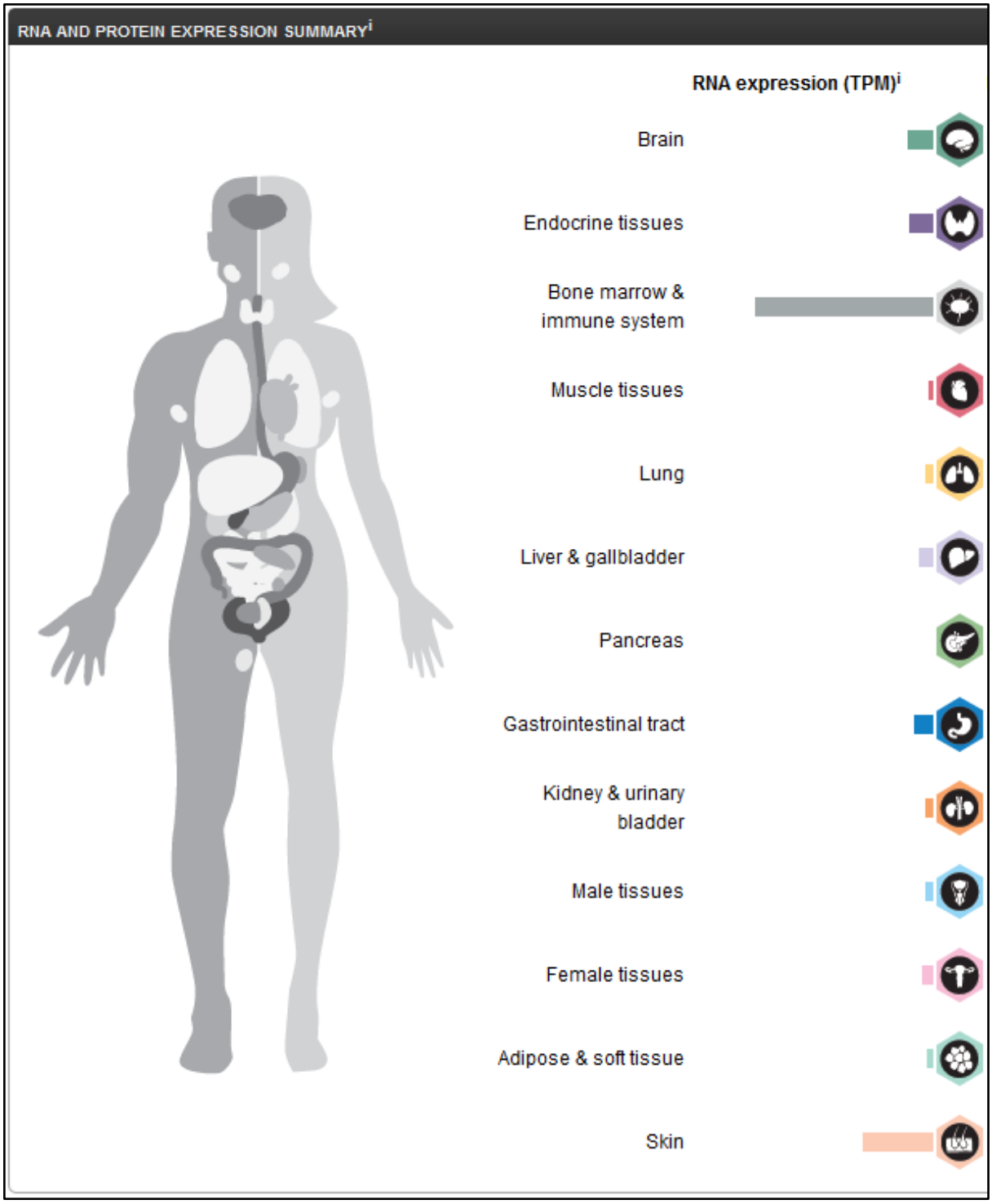


Figure 13: Graphic of RNA expression in human; RNA expression summary on Interleukin 34 in the human body. Therefore the RNA expression is reported as transcript per million (TPM) and showed a high expression rate in brain, endocrine tissues, bone marrow and immune system as well as in skin tissue. (ENSG00000157368-IL34/tissue)

Nucleotide sequences of three different mRNA transcripts of IL-34 (NM_152456.2 var 1; NM_001172771.1 var2; NM_001172772.1 var3) were used to investigate the forward and the reverse Primer of IL-34 mRNA expression for qRT-PCR purpose.

Hu IL-34 For1 primer (5'-AATCCGTGTTGTCCCTGTTG- 3') and the hu IL-34 Rev1 primer (5'- CAGTACAGCAGCTCCATGACC-3') were aligned with the transcripts variants. Both primer pairs were found after alignment only in mRNA transcripts variant 1 (NM_152456.2 var 1, Figure 16).

For further understanding between the 3 mRNA transcripts of IL-34 the Basic Local Alignment Searching Tool (BLAST, NCBI) for similarity was performed: (NM_152456.2 var 1; NM_001172771.1 var2; NM_001172772.1 var3). The results showed in alignment of IL-34 transcript 1 against transcript variant 2 99% identity with 3 gaps. The Alignment of IL-34 mRNA transcript 1 against IL-34 mRNA transcript variant 3 showed a 96% identity with 29 gaps (1%) (Figure 15).

Homo sapiens interleukin 34 (IL34), transcript variant 2, mRNA

Sequence ID: NM_001172771.1 Length: 1793 Number of Matches: 1

Range 1: 1 to 1793 GenBank Graphics ▼ Next Match ▲ Previous Match

NW Score	Identities	Gaps	Strand
3575	1793/1796(99%)	3/1796(0%)	Plus/Plus

Homo sapiens interleukin 34 (IL34), transcript variant 3, mRNA

Sequence ID: NM_001172772.1 Length: 1779 Number of Matches: 1

Range 1: 1 to 1779 GenBank Graphics ▼ Next Match ▲ Previous Match

NW Score	Identities	Gaps	Strand
3163	1721/1802(96%)	29/1802(1%)	Plus/Plus

Figure 14: Result of BLAST alignment of IL-34 mRNA transcript variant 1 with IL-34 transcript mRNA variant 2 showed 99% identity, whereas the comparison of IL-34 mRNA transcript 1 against the IL-34 mRNA transcript 3 showed only 96%.

```
1 catcagacgg gaagcctgga ctgtgggttg ggggcagcct cagcctctcc aacctggcac
       61 ccactgcccg tggcccttag gcacctgctt ggggtcctgg agccccttaa ggccaccagc
      121 aaatcctagg agaccgagtc ttggcacgtg aacagagcca gatttcacac tgagcagctg
      181 cagtcggaga aatcagagaa agcgtcaccc agccccagat tccgaggggc ctgccaggga
      241 ctctctcctc ctgctccttg gaaaggaaga ccccgaaaga cccccaagcc accggctcag
      301 acctgcttct gggctgccat gggacttgcg gccaccgccc cccggctgtc ctccacgctg
      361 ccgggcagat aagggcagct gctgcccttg gggcacctgc tcactcccgc agcccagcca
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      481 aggccgtgct taggcctctg tggacacact gctggggacg gcgcctgagc tctcaggggg
      541 acgaggaaca ccaccatgcc ccggggcttc acctggctgc gctatcttgg gatcttcctt
      601 ggcgtggcct tggggaatga gcctttggag atgtggccct tgacgcagaa tgaggagtgc
      661 actgtcacgg gttttctgcg ggacaagctg cagtacagga gccgacttca gtacatgaaa
      721 cactacttcc ccatcaacta caagatcagt gtgccttacg agggggtgtt cagaatcgcc
      781 aacgtcacca ggctgcagag ggcccaggtg agcgagcggg agctgcggta tctgtgggtc
      841 ttggtgagcc tcagtgccac tgagtcggtg caggacgtgc tgctcgaggg ccacccatcc
      901 tggaagtacc tgcaggaggt ggagacgctg ctgctgaatg tccagcaggg cctcacggat
      961 gtggaggtca gccccaaggt ggaatccgtg ttgtccctct tgaatgcccc agggccaaac
     1021 ctgaagctgg tgcggcccaa agccctgctg gacaactgct tccgggtcat ggagctgctg
     1081 tactgctcct gctgtaaaca aagctccgtc ctaaactggc aggactgtga ggtgccaagt
     1141 cctcagtctt gcagcccaga gccctcattg cagtatgcgg ccacccagct gtaccctccg
     1201 cccccgtggt cccccagctc cccgcctcac tccacgggct cggtgaggcc ggtcagggca
     1261 cagggcgagg gcctcttgcc ctgagcaccc tggatggtga ctgcggatag gggcagccag
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     1501 gggacctgaa ggtggatggg gacacagctc ctggcttctc ctggtgctgc cctcactgtc
     1561 cccccgccta aagggggtac tgagcctcct gtggcccgca gcagtgaggg cacagctgtg
     1621 ggttgcaggg gagacagcca gcacggcgtg gccattctat gacccccccag cctggcagac
     1681 tggggagctg ggggcagagg gcggtgccaa gtgccacatc ttgccatagt ggatgctctt
     1741 ccagtttctt ttttctatta aacaccccac ttcctttgga aaaaaaaaaa aaaaaa
//
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Figure 15 : IL-34 mRNA sequence; IL-34 mRNA transcript variant 1 with found position of the forward and the reversed Primer (red)

4.2 MDA-MB-231 and MCF-7 tumours contain different amount of tumour associated macrophages

To analyse the presence of macrophage in certain breast tumours, F4/80 staining was done in MDA-MB-231 and MCF-7 breast cancer xenograft tissue. Paraffin-embedded tumour tissue was obtained from an experimental performed previously in the laboratory. The stained slides were visually investigated under a microscope. (Figure 17)

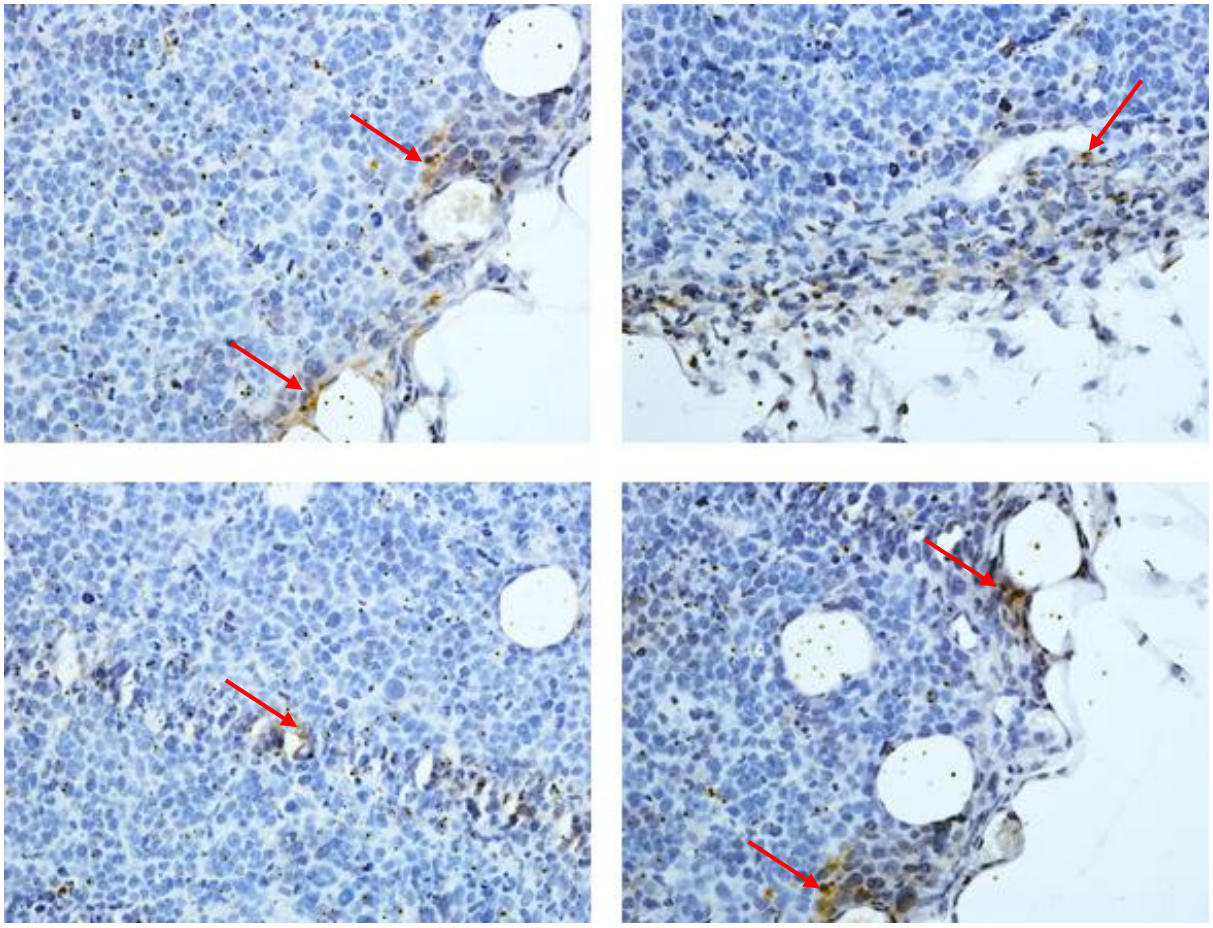


Figure 16: Macrophage immunohistochemistry of MCF-7 breast cancer xenograft tissue. Macrophages were stained with F4/80 antibody in tumour tissue 38 days after tumour cell transplantation into mice. Representative images of F4/80-stained tumor cell sections. Nuclei are stained with DAPI (blue).The brown labelled cells (arrows) show the macrophages within the tumor tissue.

F4/80 labelling of macrophages in human MCF-7 tumour showed a higher number of macrophages as compared to the centre of the tumour (Figure 17).

In MDA-MB-23 tumours the tumour associated macrophages were located in both, the centre of the tumour and the tumour boundary. Visually, there were overall more macrophages present in the tumour compared to MCF-7 tumours (Figure 18). This result indicates that the triple negative MDA-MB-231 tumour cells, which have a poor prognosis in patients, were associated with higher numbers of tumour associated macrophages as

luminal-type MCF-7 tumours. Macrophages play a crucial role in the tumour development, the tumour growth and are associated with poorer prognosis in breast cancer (Figure 18).

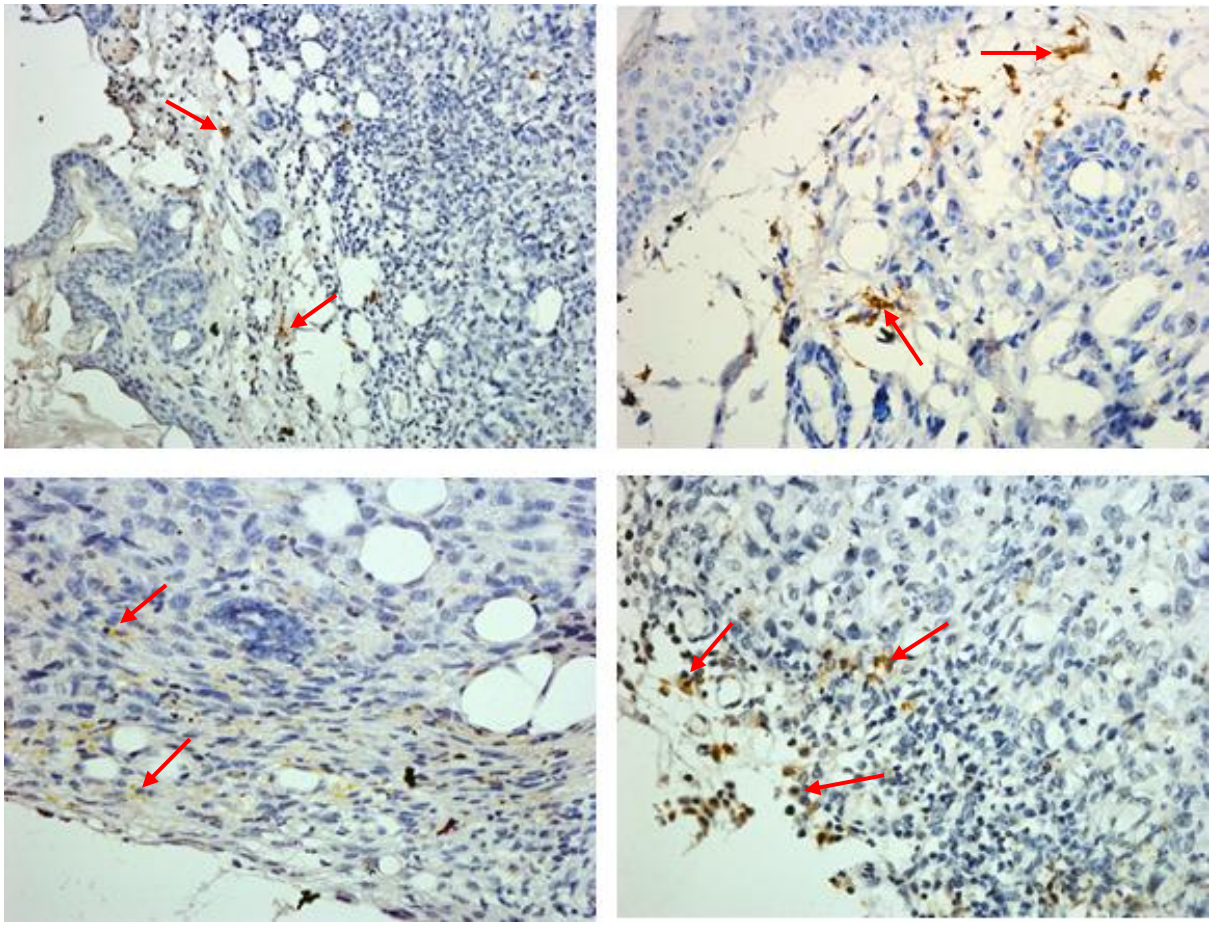
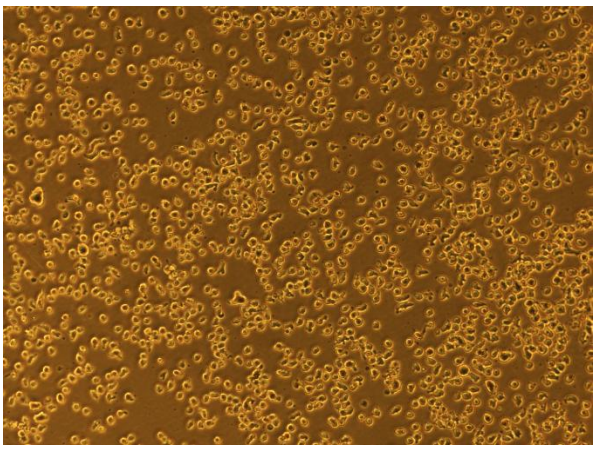


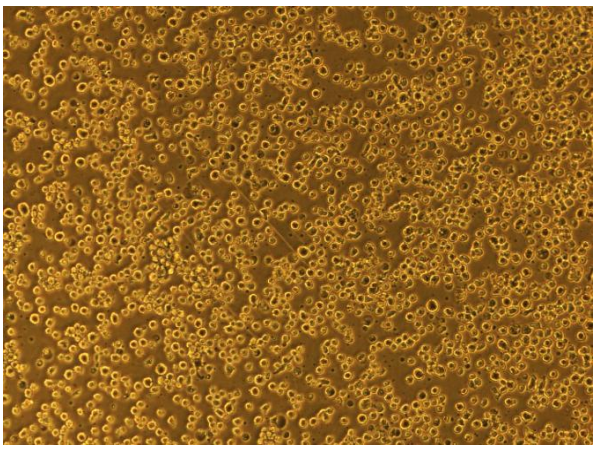
Figure 17: Macrophage immunohistochemistry of MDA-MB-231 breast cancer xenograft tissue. Representative images of F4/80-stained tumour cell sections. Nuclei are stained with DAPI (blue). Macrophages were labelled with F4/80 antibody (brown cells) within the tumour tissue 38 days after tumour cell transplantation into mice. Tumour associated macrophages can be found frequently within the tumour cell mass.

4.3 THP-1 differentiation and M1/M2-polarisation

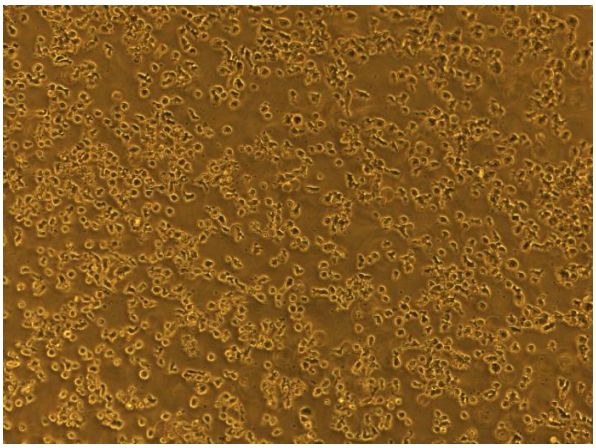
To further analyse the effect of IL-34 on macrophage polarisation, we compared the effect of IL-34 on macrophages with the known M2-polarising factors CSF-1, IL-13, and IL-4 and the M1-polarisng agent lipopolysaccaride (LPS). First, by using a phorbol-myristate-acetate (PMA)-induced cell differentiation model, monocytic THP-1 cells were differentiated into macrophages. The THP-1 cells were treated with 150 nM phorbol 12-myristate 13-acetate (PMA), which leads to differentiation into macrophages, followed by treatment with the polarising cytokines IL-34, CSF-1, IL-13 and IL-4. In addition treatment with LPS was performed, polarising macrophages towards a M1 phenotype. The M1 macrophage type is so-called classically activated macrophage type and has a higher concentration of IL-12. In contrast, alternatively activated M2 macrophages have a lower concentration of IL-12 and a higher concentration of IL-10. This macrophage-phenotype is anti-inflammatory and supports tumour growth.



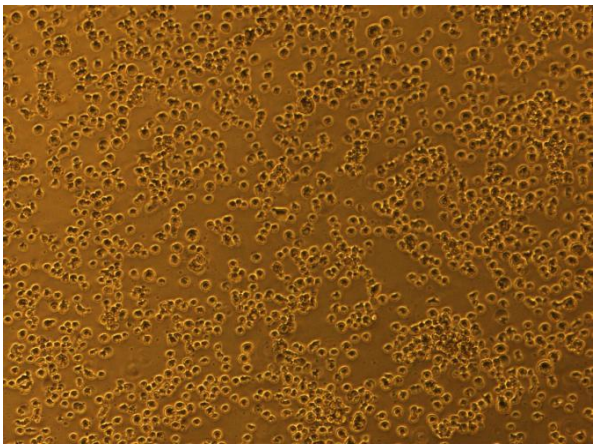
Control



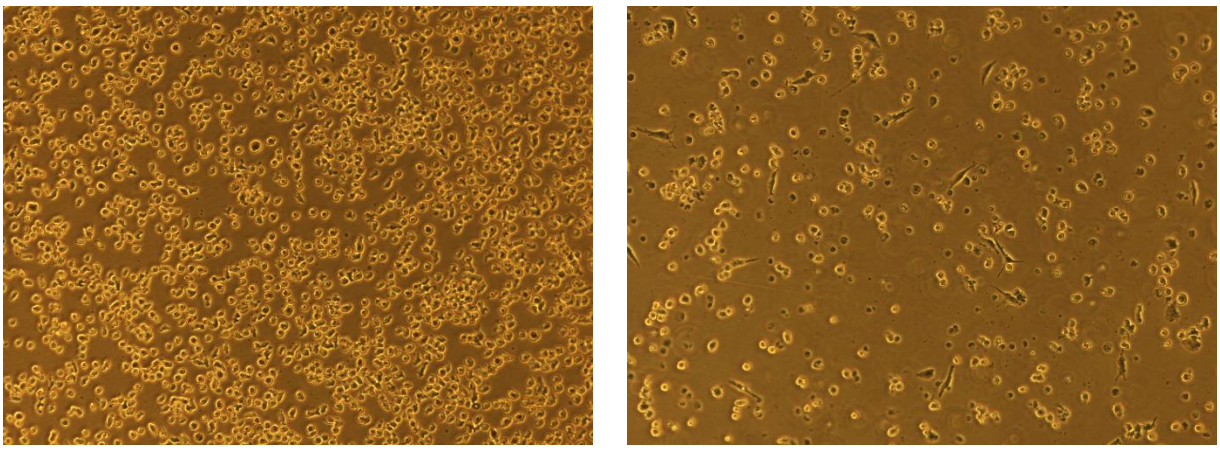
CSF-1



IL 13



IL 34



IL 4 **LPS**

Figure 18: PMA differentiated THP-1 macrophages treated with different recombinant proteins and LPS for differentiation into M2 and M1 macrophages, respectively. Inverted microscopy images are shown. The THP-1 cells were treated for 48h with PMA and further treated with the indicated recombinant proteins for 72h. Treatment with LPS resulted in clear morphological changes of macrophages, whereas the other treatments induced a higher proliferation rate.

The treatment of cells with CSF-1 resulted in a higher cell density, indicative of a higher proliferation rate and an adherent morphology. Likewise, treatment with IL-34, IL-4 and IL-13 showed similar effects (Figure 19). Treatment with LPS, however, resulted in a lower cell density and thus showed a lower proliferation. The morphology was characterised by adherent, elongated and spread out cells, which was in contrast to the rather round morphology of cells treated with M2-polarising agents (Figure 19).

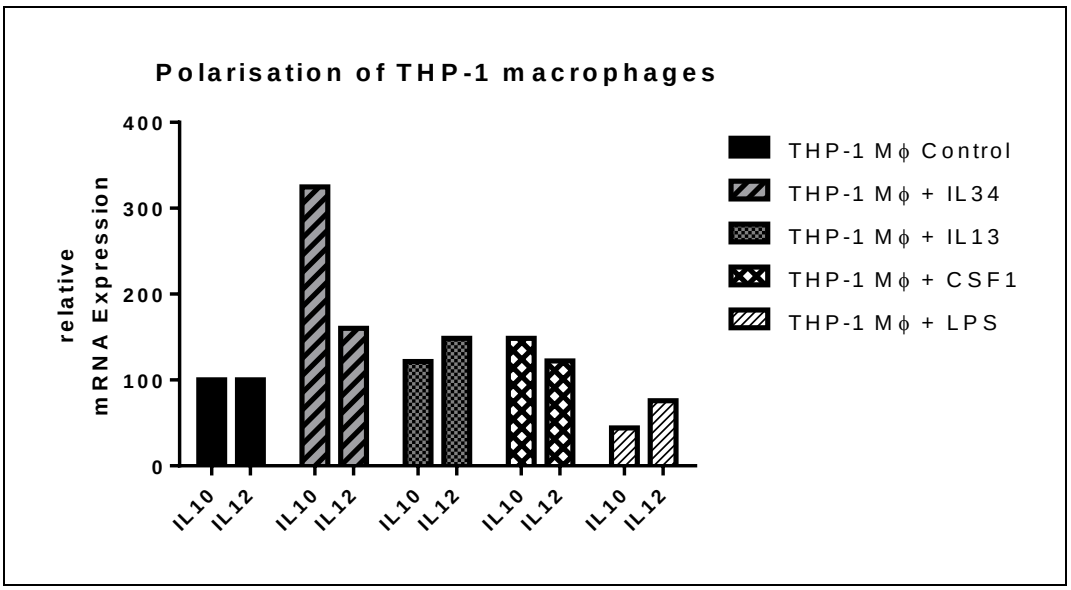


Figure 19: THP-1 macrophage polarisation. Polarised THP-1 macrophages express different IL-10 and IL-12 mRNA levels. Treatment with recombinant IL-34 induced the highest levels of IL-10 of all investigated factors.

Analysis of mRNA showed that the treatment with IL-34 induced a significantly increased expression of IL-10, suggesting that this cytokine drives macrophages towards a tumour associated M2 phenotype. Treatment with IL-13 and CSF-1 had no major effect on IL-10 expression. LPS treatment induced a marked reduction in IL-10 expression (Figure 19).
Determination of the IL-10/IL-12 ratio clearly showed that IL-34 polarises THP-1 macrophages to an M2-like phenotype. The effect of CSF-1 was less pronounced, while LPS-treated cells had a rather low IL-10/IL-12 ratio. (Figure 21)

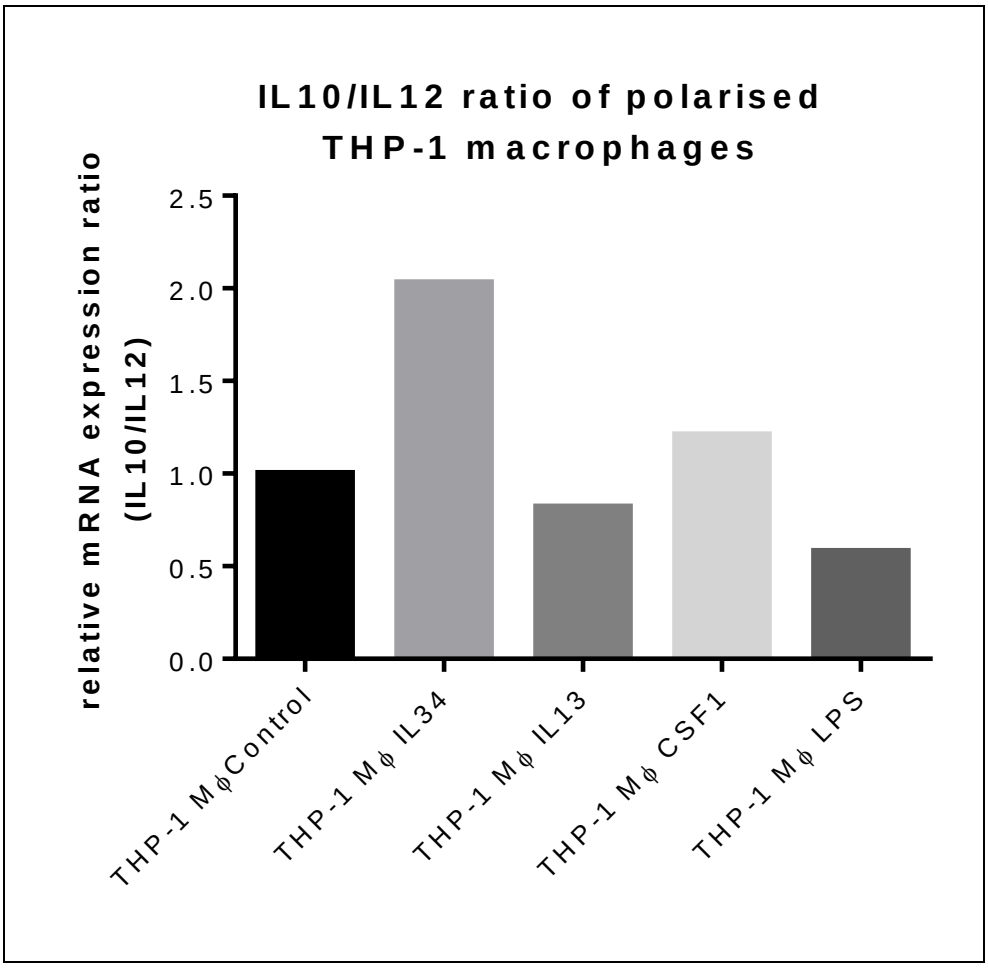


Figure 20: IL10/IL12 ratio in THP-1 macrophages stimulated with different recombinant proteins and LPS. Ratio of relative mRNA expression of IL-10 to IL-12 was highest following IL-34 treatment compared to the other treatments. (macrophages, Mϕ)

4.4 The mRNA expression of IL-34, CSF-1 and TNF-α is different in (co-) cultured MDA-MB-231 and MCF-7 breast cancer cells

To identify the role of IL-34 on human breast cancer cells, qRT-PCR measurements were done. First, the IL-34 mRNA expression levels in untreated MDA-MB-231 and MCF-7 breast cancer cells were investigated. The results of these measurements showed a higher IL-34 and CSF1R mRNA expression in the MCF-7 cell line, as compared to MDA-MB-231 cells (Figure 22).

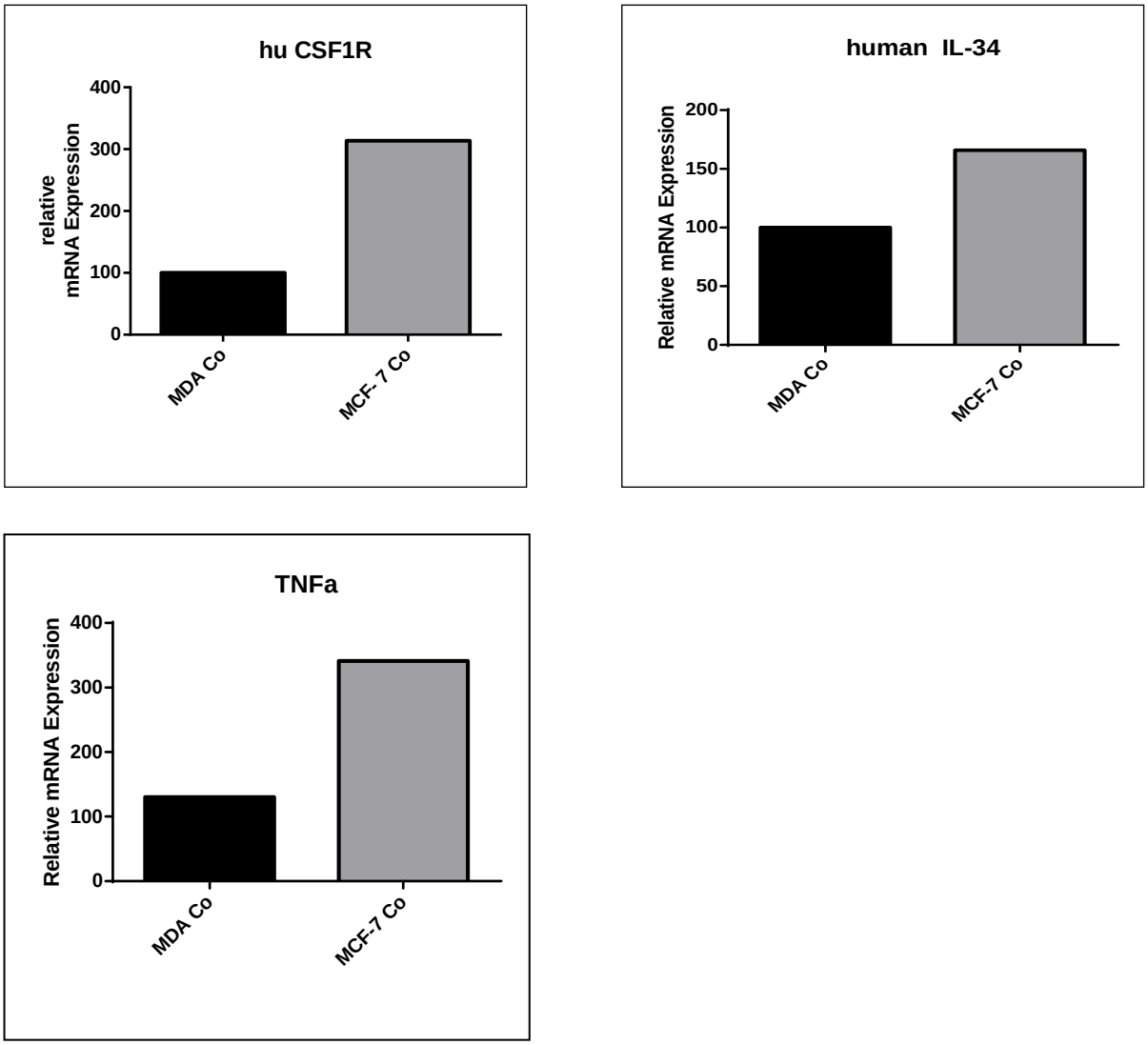


Figure 21: qRT-PCR results of IL-34 mRNA expression, CSF1R mRNA and TNF-α mRNA expression in MDA-MB-231 and MCF-7 breast cancer cells

This led to the hypothesis, that MCF-7 cells are more susceptible to the CSF1R ligand IL-34 than MDA-MB-231 cells, since IL-34 has a higher affinity to the CSF1R than CSF-1.

MDA-MB-231 breast cancer cells have a lower TNF-α mRNA expression compared to MCF-7 breast cancer cells TNF-α is expressed in TAMs and is affected by CSF1R signalling. Thus, the question arises, whether the breast cancer macrophage crosstalk affects the expression level of the CSF1R ligand IL-34 and CSF-1, which may lead to a M2- phenotype in TAMs. TNF-α is affected by these factors.

Of interest, MDA-MB-231 cells have a more aggressive phenotype than MCF-7 cells, which is associated with a poorer prognosis in patients (Figure 22).

For further understanding the regulation of IL-34 mRNA expression in human breast cancer cells and tumour associated macrophages co-culturing of PMA-differentiated THP-1 macrophages and human breast cancer cells were analysed. The results of this co-culture experiment showed a significant decrease of IL-34 mRNA expression in both MDA-MB-231 and MCF-7 cells co-cultured with differentiated THP-1 macrophages (Figure 23).

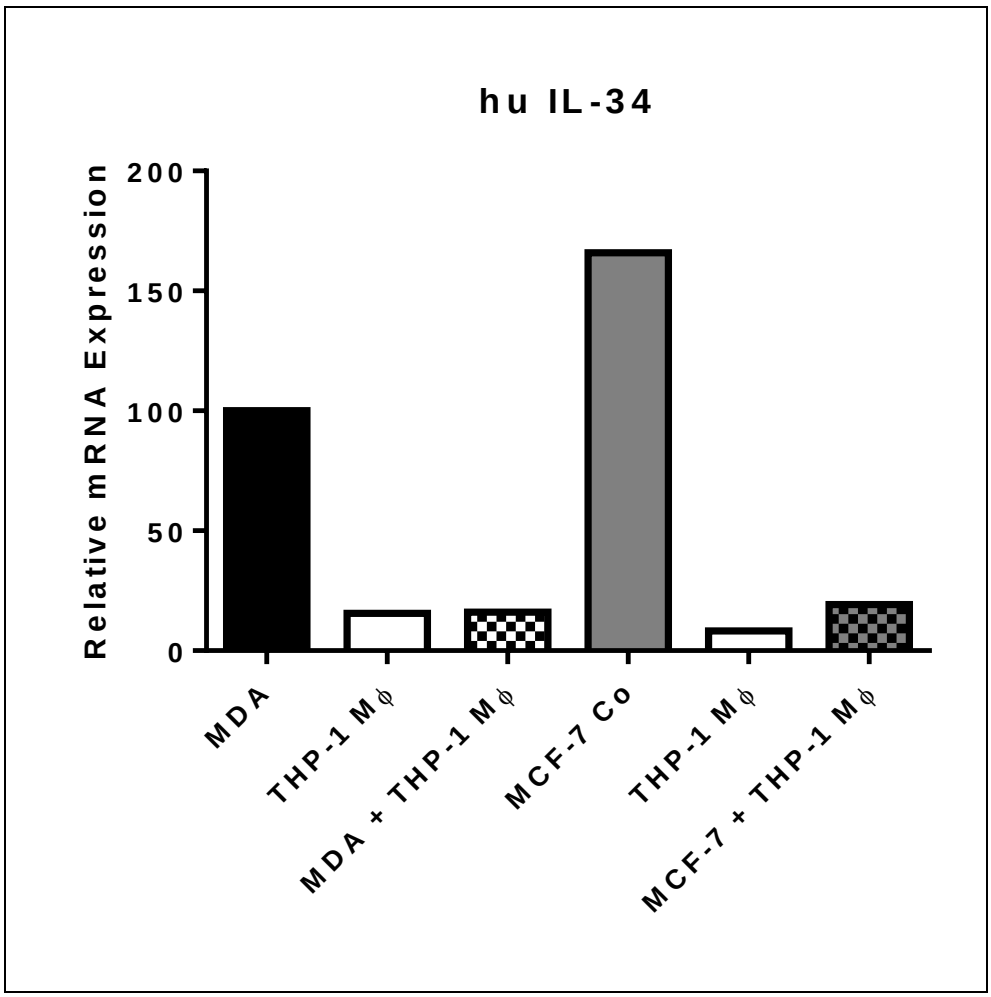


Figure 22: mRNA expression of human IL-34 in co-cultured cells; Decreased levels of IL-34 mRNA expression in both co-culture assays.(macrophages, Mϕ)

4.5 The mRNA expression of IL-34, CSF-1 and TNF-α is different in MDA-MB-231 and MCF-7 breast cancer cells treated with conditioned medium from differentiated THP-1 cells

To investigate the effect of secreted cytokines from differentiated THP-1 macrophages on the human breast cancer cell lines, conditioned medium assays were performed. The results of the IL-34 mRNA expression measurements by qRT-PCR showed a decrease in both human breast cancer cell lines treated with conditioned medium form THP-1 differentiated macrophages (Figure 23).

Next, the mRNA expression of TNFα and CSF-1 was investigated in co-culture of breast cancer cells were and macrophages. The mRNA expression of CSF-1 was very high in human MDA-MB-231 breast cancer cells, as compared to the MCF-7 breast cancer cells. Co-culturing of differentiated THP-1 macrophages and MDA-MB-231 or MCF-7 breast cancer cells reduced CSF-1 mRNA expression. As expected, differentiated THP-1 macrophages have a very low CSF-1 mRNA expression. (Figure 24)

Likewise, qRT-PCR measurements of CSF-1 also showed decreased CSF-1 mRNA levels in MDA-MB-231 cells treated with conditioned medium, whereas CSF-1 levels remained unchanged in conditioned medium treated MCF-7 cells (Figure 24).

Remarkably, the mRNA expression of TNF-α was significantly increased in co-cultured differentiated THP-1 macrophages and MCF-7 cells. In contrast, TNF-α mRNA levels remained low in MDA-MB-231/THP-1 macrophages co-cultures. Baseline levels of TNF-α were low in all investigated cell lines. These data indicate a breast cancer cell type specific regulation of TNF-α mediated by cancer cell macrophage crosstalk. (Figure 24)

Further analysis of mRNA levels of the proinflammatory cytokine IL-12 showed higher levels in MCF-7cells vs. MDA-MB-231 cells. Treatment with conditioned medium did not lead to major changes in both cell lines. (Figure 24)

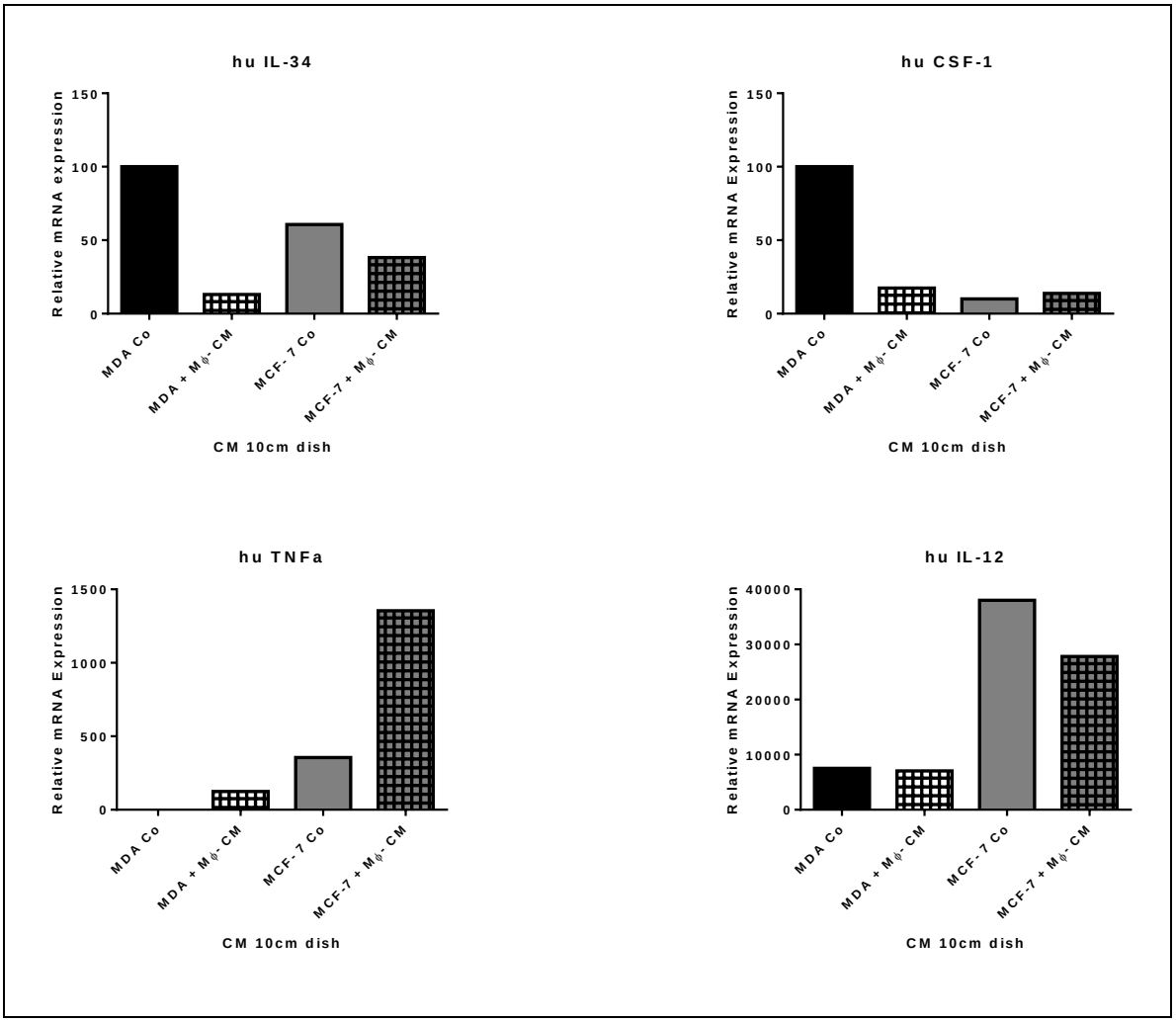


Figure 23: IL-34, CSF-1, TNF-α and lL-12 mRNA expression in MDA-MB-231 and MCF-7 breast cancer cells treated with conditioned medium of differentiated THP-1 (macrophages, Mϕ) cells

4.6 The mRNA expression of IL-34, CSF-1 and TNF-α in MDA-MB-231 and MCF-7 breast cancer cells following IL-34 antibody treatment

MDA-MB-231 and MCF-7 breast cancer cells were treated with anti-IL-34 antibody for 24 h The qRT-PCR results showed decreased IL-34, CSF-1 and TNF-α mRNA expression in antibody treated MDA-MB-231 cells (Figure 25) The opposite effect was observed in MCF-7 cells, in which IL-34, CSF-1 and TNF-α were up regulated following antibody treatment. These data suggest cell-type specific differences in gene expression mediated by an IL-34 feedback loop.

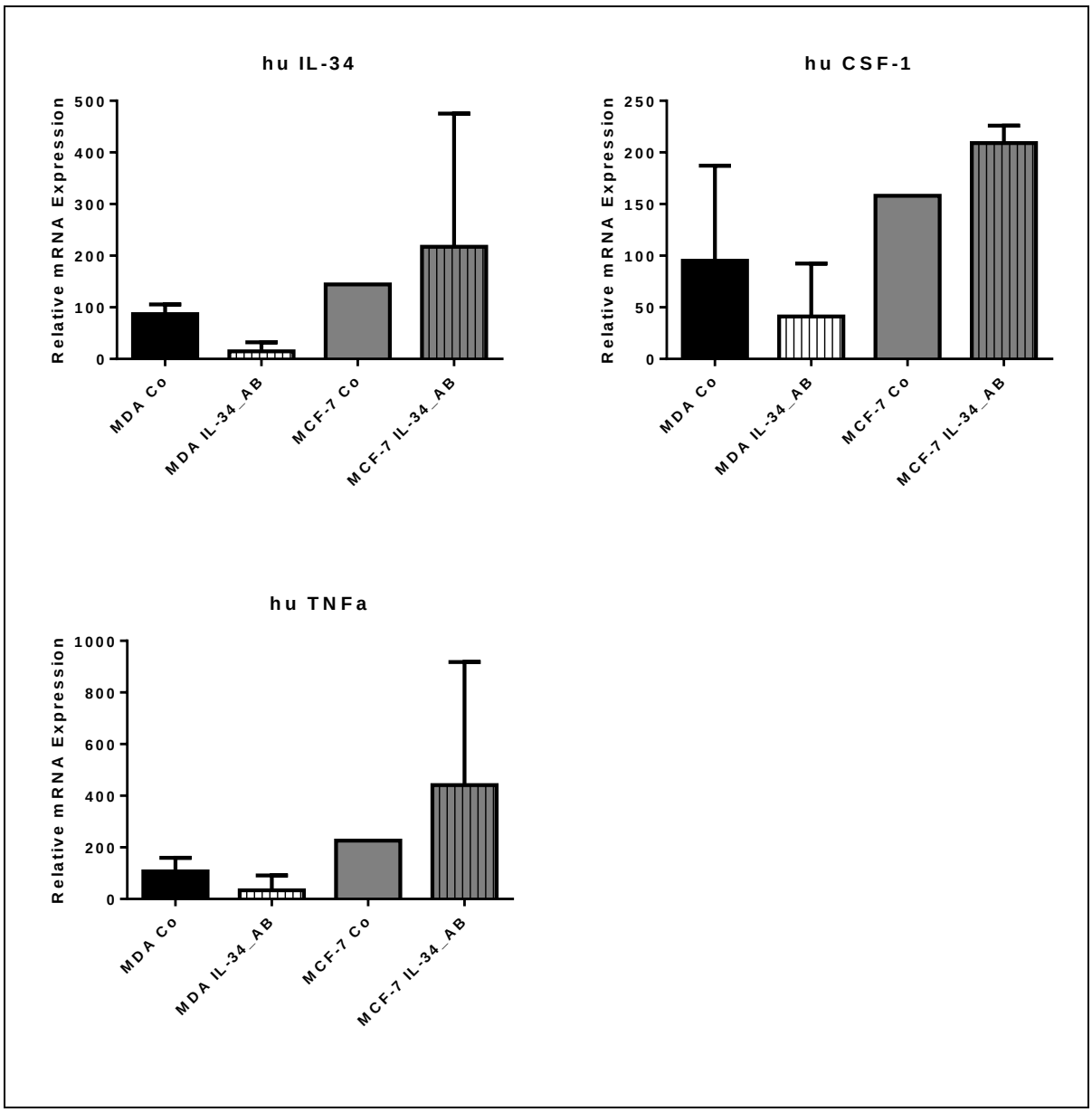


Figure 24: IL-34, CSF-1 and TNF-α mRNA expression of MDA-MB-231 and MCF-7 breast cancer cells following IL-34 antibody treatment

4.7 The mRNA expression of IL-34, CSF-1 and TNF-α in breast cancer cells following treatment with untreated and anti-IL-34 antibody pre-treated conditioned medium from monocytic, undifferentiated THP-1 cells.

Further analysis on the effect of differentiated (macrophages) and undifferentiated (monocytes) THP-1 cells on breast cancer cells showed differences in the IL-34 mRNA expression of MDA-MB-231 and MCF-7 breast cancer cells after treatment of conditioned medium from undifferentiated THP-1 cells, compared to the treatment with conditioned medium from differentiated THP-1 macrophages.

IL-34 mRNA expression of MDA-MB-231 breast cancer cells remained unchanged after treatment with conditioned medium from undifferentiated THP-1 cells. In contrast, the IL-34 mRNA expression in MCF-7 cells increased significantly after treatment with conditioned medium from undifferentiated THP-1 cells. However, IL-34 mRNA expression decreased in both breast cancer cells after treatment with conditioned medium from differentiated THP-1 macrophages. Conditioned medium from THP-1 monocytes pre-treated with an anti-IL-34 antibody resulted in decreased IL-34 mRNA expression compared to untreated T in both breast cancer cells (Figure 26).

The CSF-1 mRNA expression was increased in both breast cancer cell lines following treatment with conditioned medium from undifferentiated THP-1 cells. This effect was even stronger following treatment with conditioned medium from monocytic THP-1 cells pre-treated with anti-IL-34 antibody.
Interestingly, the TNF-α mRNA expression was significantly upregulated in MCF-7 breast cancer cells, which were treated with conditioned medium from undifferentiated THP-1 cells, whereas its level remained unchanged in all other groups (Figure 26).

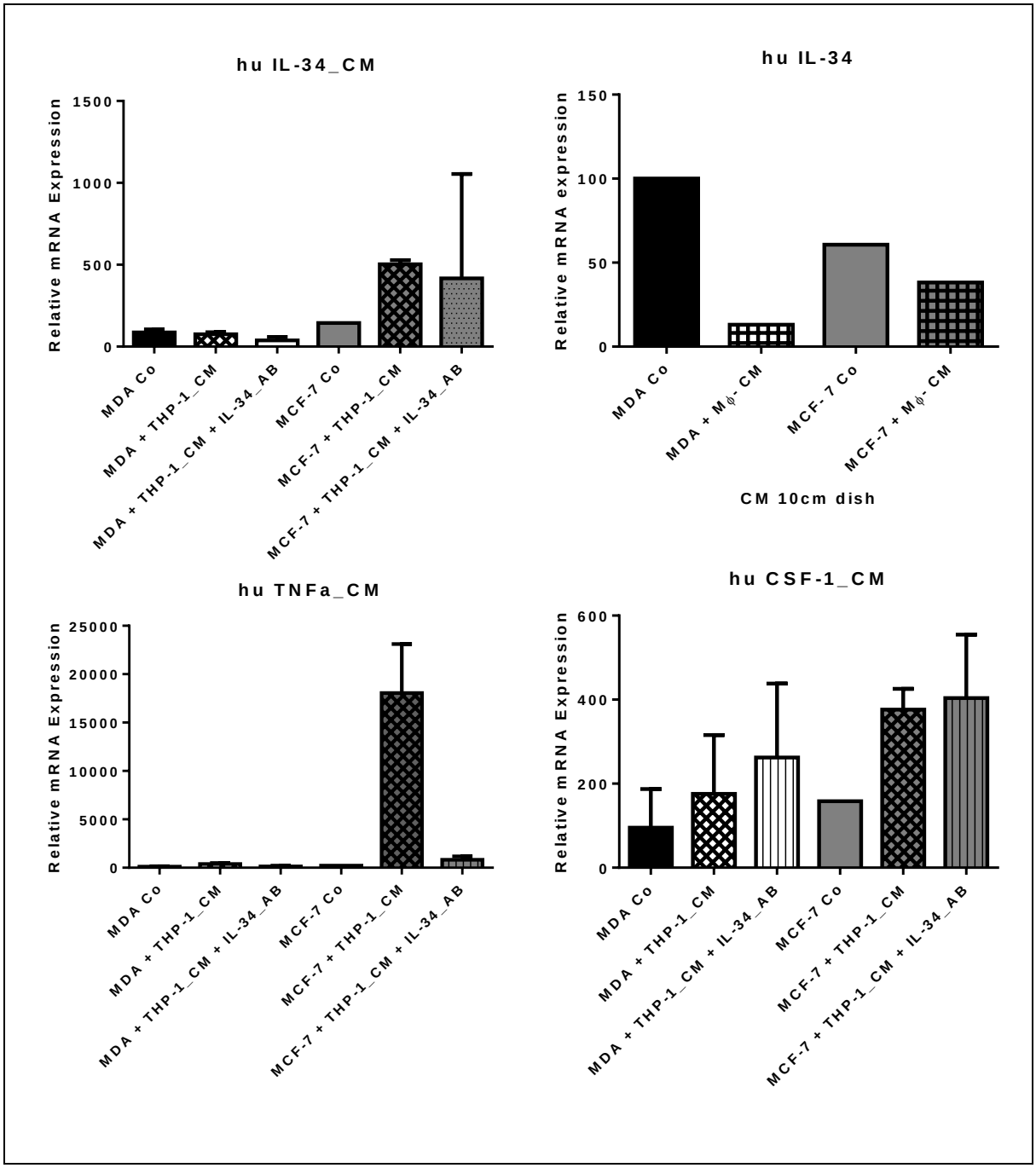


Figure 25: IL-34, TNF-α, CSF-1 mRNA expression in MDA-MB-231 and MCF-7 breast cancer cells following treatment with conditioned medium (CM) of undifferentiated, monocytic (THP-1) (macrophages) and differentiated (macrophages, Mϕ) THP-1 cells.

4.8 The mRNA expression of IL-34, CSF-1 and TNF-α in MDA-MB-231 and MCF-7 breast cancer cells following CSF1R antibody treatment

For further understanding whether expression of IL-34, CSF-1 and TNF-α is potentially regulated by a feedback mechanism related to CSF1R-signaling, we blocked CSF1R activity by treating breast cancer cells with an inhibitory antibody directed against CSF1R. The data show, that only IL-34 expression in MDA-MB-231 was down regulated following CSF1R-blockade. Expression of all other investigated factors in both cell lines remained unchanged. This suggests that IL-34 expression in MDA-MB-231 cells could be regulated by an autocrine CSF1R-dependend manner (Figure 27).

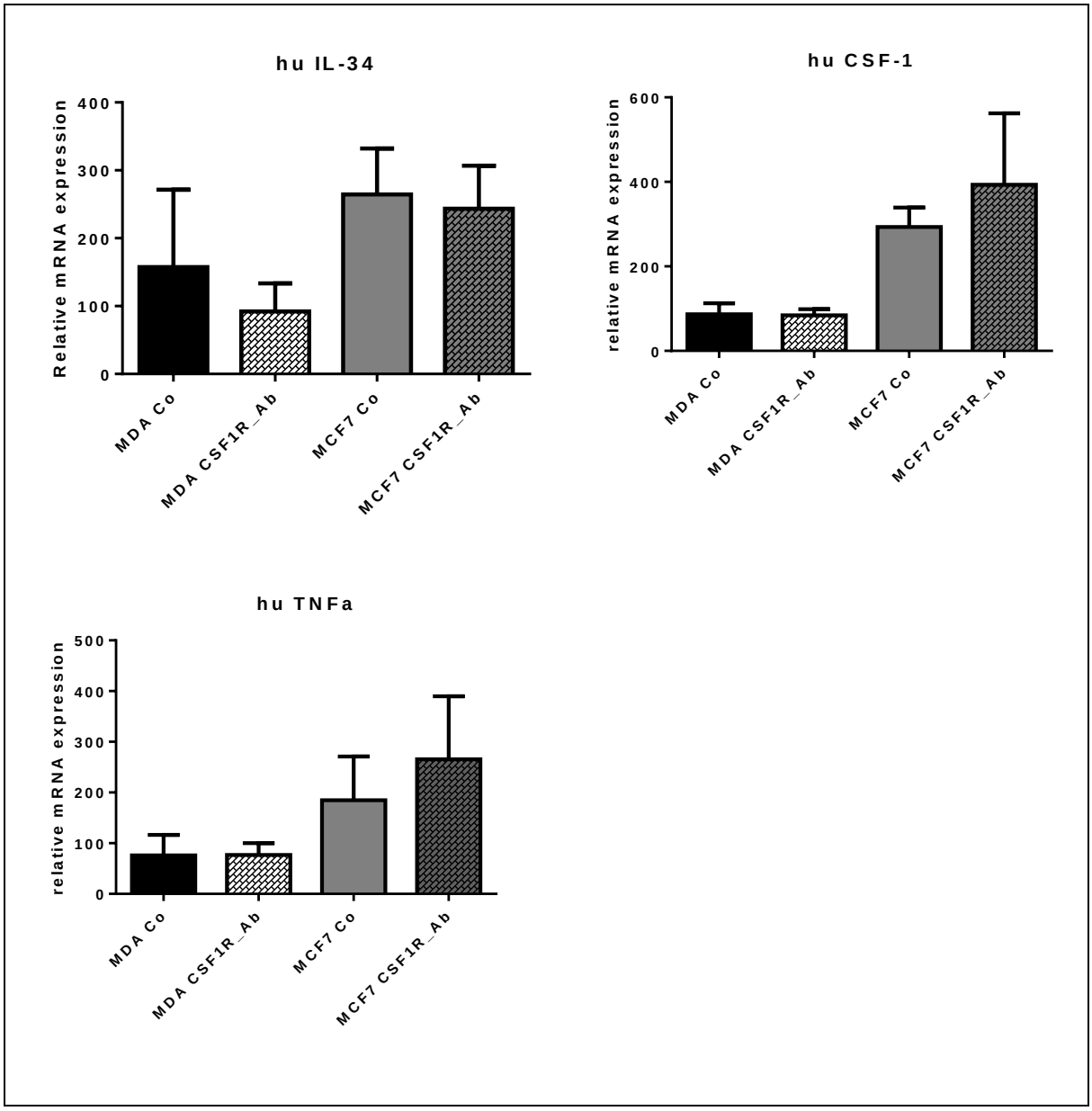


Figure 26: IL-34, CSF-1 and TNF-α mRNA expression in MDA-MB-231 and MCF-7 breast cancer cells treated with anti-CSF1R antibody (CSF1R_Ab).

4.9 The mRNA expression of IL-34, CSF-1 and TNF-α of MDA-MB-231 and MCF-7 breast cancer cells treated with TNF-α antibody

For analysing the potential role of TNF-α on IL-34 and CSF-1 expression in MDA-MB-231 and MCF-7 breast cancer cells, TNF-α antibody treatment was performed. In MDA-MB-231 cells IL-34 and TNF-α mRNA expression was up-regulated following TNF-α blockade, whereas CSF-1 levels were unaffected. No major changes were observed in MCF-7 cells. These data indicates cell type-specific differences in the regulation of IL-34, CSF-1 and TNF-α. (Figure 28)

Interestingly, TNF-α blockade lead to a significant increased CSF1R mRNA levels in MCF-7 cells and to moderately increased levels in MDA-MB-231 cells, indicating a crosstalk between TNF-α and CSF-1/IL-34 signalling pathways in breast cancer cells.

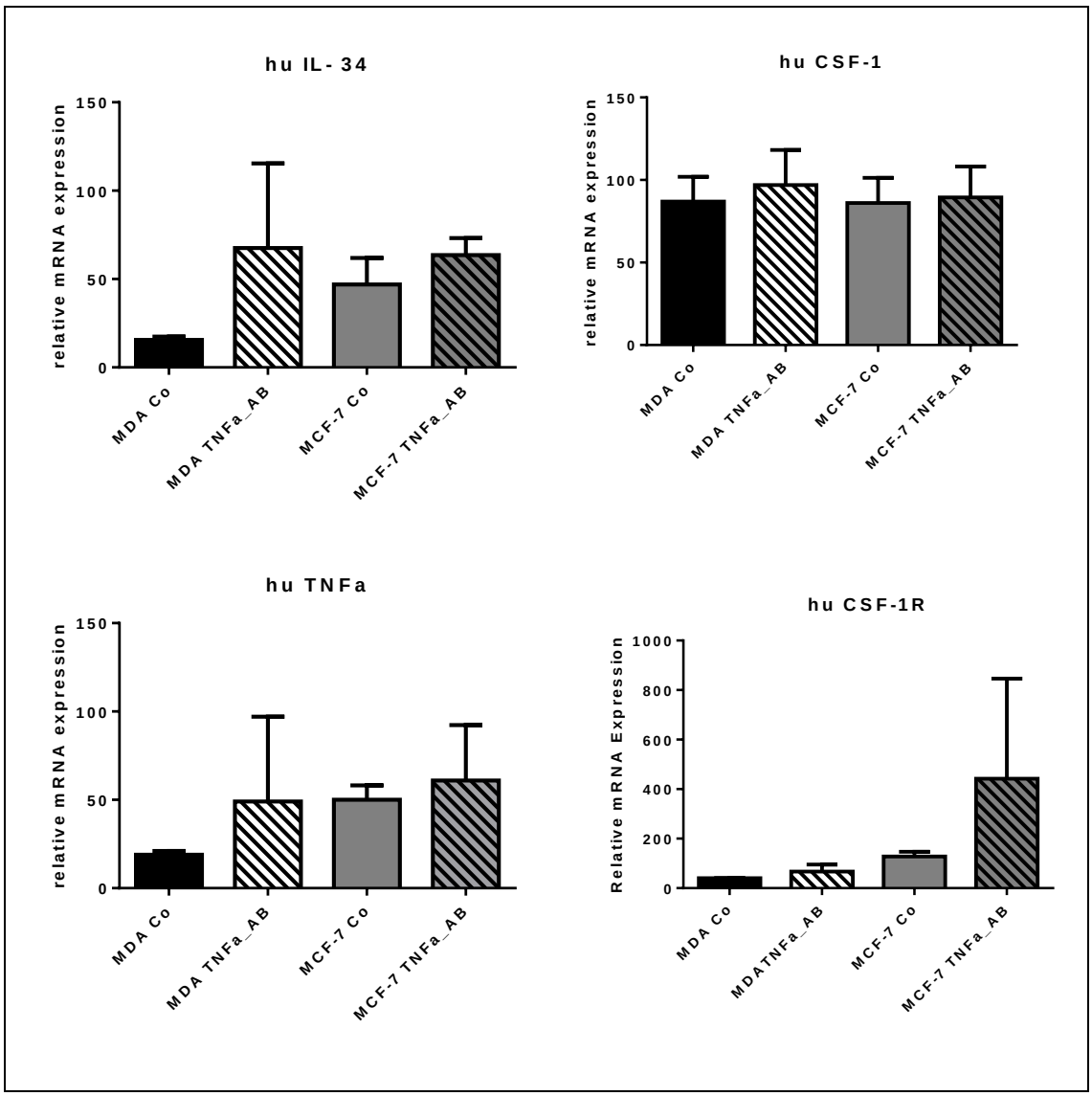


Figure 27: IL-34, CSF-1 and TNF-α mRNA expression with TNF-α antibody (TNFa_AB) treatment of MDA-MB-231 and MCF-7 breast cancer cells.

4.10 Protein expression of ERK, pERK and NF-KB in MDA-MB-231 and MCF-7 breast cancer cells treated with TNF-α or CSF1R antibody

The MAPK/ERK pathway is a mitogen activated kinase pathway that is regulated by phosphorylation of threonine and (or) tyrosine residues and can be active or inactive. ERK1/2 phosphorylation is important in intracellular cell signalling and has functions in cellular proliferation, differentiation, survival and has common effects in human cancers by catalysing the phosphorylation of more than hundreds of cytoplasmic and nuclear substrates. To investigate the effect of certain antibodies on ERK-activity in triple negative MDA-MB-231 and luminal-type MCF-7 breast cancer cells ERK1/2 and the phosphorylated ERK (pERK) protein levels were measured via immune blotting.

The NF-kB pathway is involved in gene expression by adaptive immune system, innate immune system, inflammation and other biological processes. Therefore pro-inflammatory cytokines stimulate the IKK complex, which stimulate and activate the IkB proteins inside the pathway, which leads to the activation of NF-kB/Rel complex. This active complex translocates to the nucleus inside the cell and induces gene expression in combination with other transcription factors.

The results of the immune blotting experiments showed a decrease of pERK levels in TNF-α and CSF1R antibody treated MDA-MB-231 breast cancer cells compared to the control. In MCF-7 breast cancer cells there was also a decrease in pERK protein levels, particularly in the cancer cells treated with CSF1R antibody (Figure 29).

NF-κB protein expression levels decreased in MDA-MB-231 breast cancer cells treated with TNF-α and CSF1R antibodies as compared to control. In MCF-7 cells, NF-κB protein expression was only reduced in CSF1R antibody treated cells.

These preliminary results indicate changes in ERK and NF-κB signalling pathways regulated by CSF1R and TNF-α signalling. Thus, further analysis of these pathways is warranted.

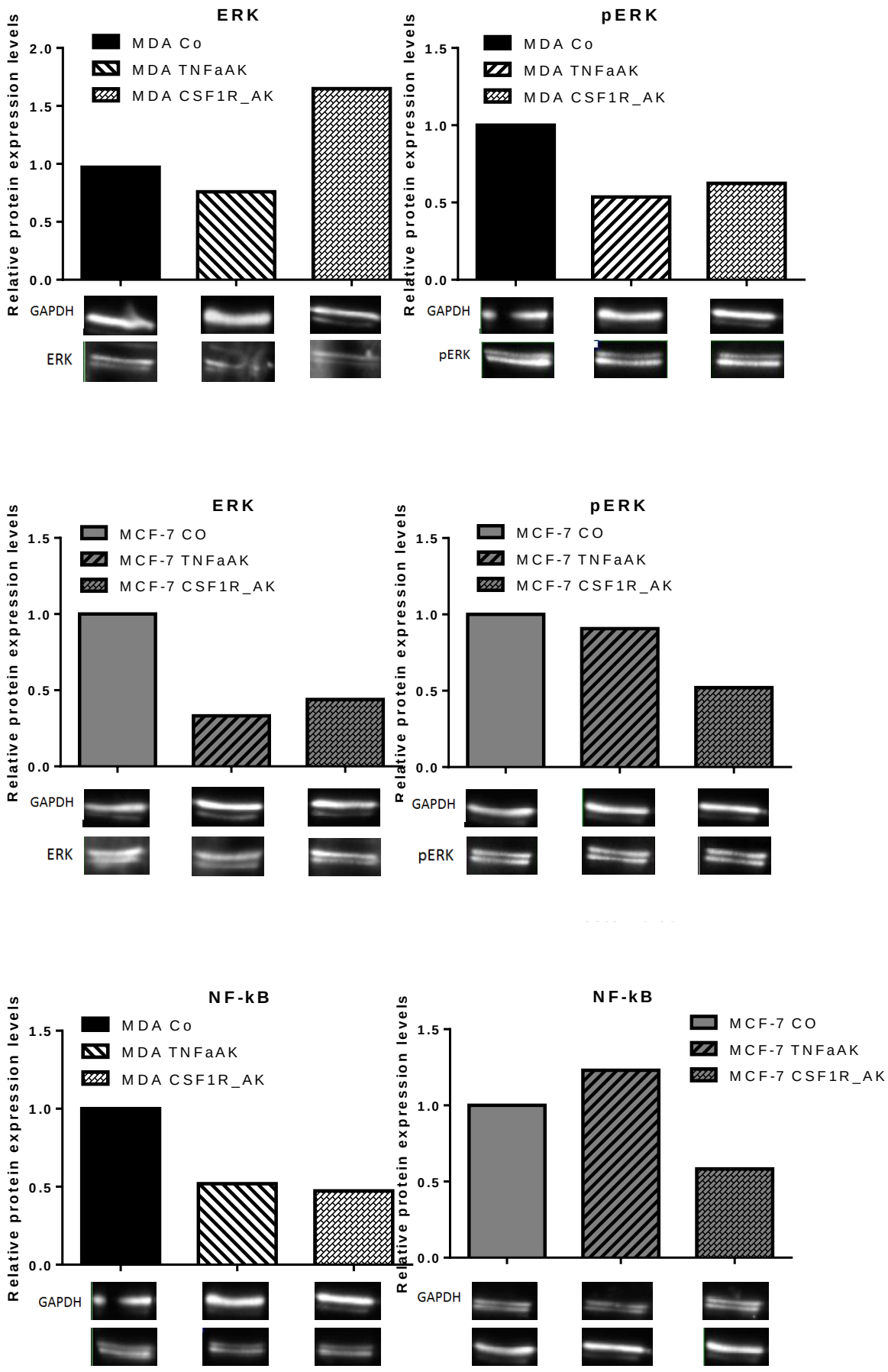


Figure 28: Protein expression in MDA-MB-231 and MCF-7 breast cancer cells after treatment with TNF-α and CSF1R antibody.

4.11 Clinical data of breast cancer patients

Clinical data of breast cancer patients were correlated with relative IL-34 mRNA expression (log2) (Figure 30), Analysis was done in cooperation with Prof. Schreiber from the Medical University of Vienna. In ductal tumour types a broad range of IL-34 expression was observed, which was in average higher than IL-34 mRNA levels found in lobular tumour types. The grading 1-2 of the tumour types showed no difference, compared to grade 3 tumours. The age of the breast cancer patients was associated with a significantly different IL-34 expression ($p<0.01$). Older patients showed a higher value of IL-34 than younger patients.

The menopausal status of breast cancer patients was also associated with significantly different post-menopausal status caused an increase of IL-34 mRNA expression as compared to a pre -menopausal status in breast cancer. Tumour size was not different with respect to IL-34.

The hormone status of breast cancer patients did not show a significant difference in IL-34 expression levels. Both, progesterone status and estrogen status were not significantly different with respect to IL-34 levels. The HER2 status showed an increase of mean IL-34 in HER2 negative breast cancer patients, however, they were not statistically different. These preliminary data suggest that IL-34 expression is potentially different in different breast cancer subtypes and also depends on age and meno-pausal status of patients. (Figure 30)

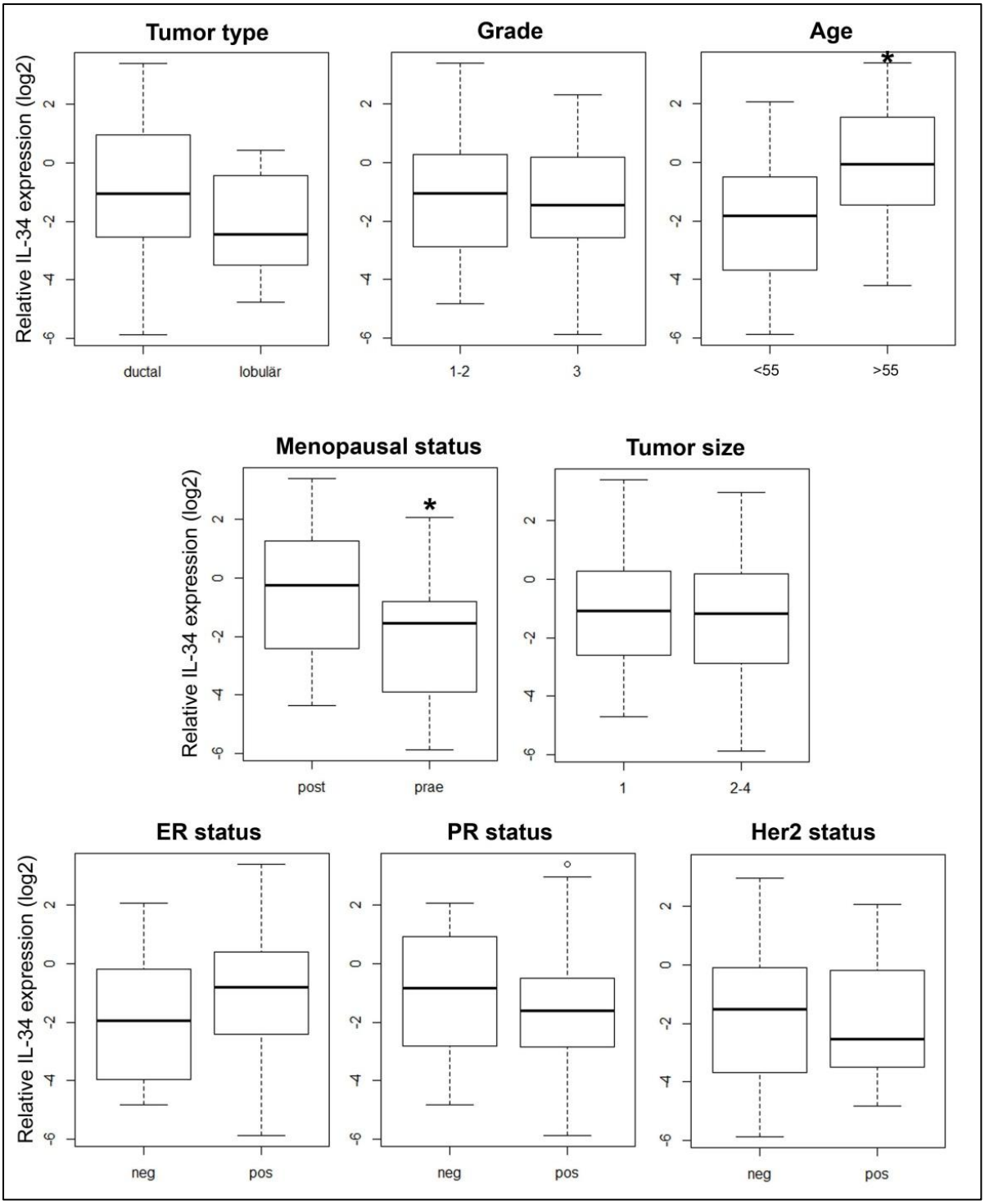


Figure 29: Clinical data of IL-34 expression in breast cancer patients

5 Discussion

Several reports have described the accumulation of tumour-associated macrophages (TAMs) in tissue during the tumour development and TAMs are considered to facilitate the metastatic process [31]

Immunohistochemical staining of TAMs in our MDA-MB-231 and MCF-7 breast cancer xenograft tissue supports these findings. Of interest, a higher number of TAMs was found in triple-negative MDA-MB-231 xenograft tumour tissue as compared to luminal-type MCF-7 breast cancer tissue, suggesting that TAMs are associated with the more malignant triple-negative breast cancer phenotype.

Differentiation of monocytes into macrophages is regulated by CSF-1 through interaction with its receptor tyrosine kinase CSF1R [32], which is also expressed by TAMs [33] CSF1R is also known to be expressed in breast cancer tissue and in different breast cancer cell lines, which mediates the CSF1 induced activation of extra cellular signal regulated kinase 1/2 (ERK 1/2) MAPK in breast cancer cells [10, 11]. Besides CSF-1, the cytokine IL-34 has been identified as an additional ligand for the CSF1R [44]. IL-34 has been shown to function as a specific and independent ligand of CSF1R, inducing the differentiation and proliferation through MAPK activation in macrophages. [12 13, 34]

Both ligands for the CSF1R have some functional overlaps, however, they also have some specific activities, especially in the differentiation and activation of the monocyte/macrophage lineage.[35] IL -34 was already shown to drive in vitro the polarisation of macrophages towards an M2 immunosuppressive phenotype and function [25]. The M2 phenotype of macrophages has the potential to promote cancer growth by releasing pro-angiogenic factors, such as VEGF, cytokines, and extracellular matrix-modifying factor, whereas classical activated M1 macrophages often demonstrate an antitumor behaviour[36]. Macrophages can be polarized into the M1 type by microbial lipopolysaccharides and in response to interferon-γ as an anti-inflammatory response. [37] Of note, TAMs often show M2 like polarisation features, enabling them to support tumour growth. [38]Several reports have shown that IL-34 plays a curial role in tumour development and tumour growth and that it can lead to cancer cell proliferation, progression and metastasis.[23, 24, 27] However, controversial roles of IL-34 in cancer have been reported. In leukaemia cell lines, IL-34 did not induce cell growth or proliferation, quite the contrary; it was able to induce differentiation of leukaemia cell lines from monoblastic precursor cells towards monocyte- and macrophage-like cells. [23]

The findings of this study suggest that IL-34 is also regulating gene expression in breast cancer cells, in particular expression of TNF-α. We could observe differential regulation of TNF-α in triple-negative MDA-MB-231 and luminal-type MCF-7 breast cancer cells,

indicating that the effect of IL-34 may depend on the molecular subtype of breast cancer. In addition, conditioned medium from THP-1 monocytes and differentiated THP-1 macrophages regulated TNF-α expression to a different extent in breast cancer cells. On the other hand, cancer cells affected macrophage polarisation by expression of IL-34 and CSF-1. Both cytokines modified M2-related high IL-10/low IL-12 expression in macrophages, which is in line with reports showing the in vitro polarisation of human monocytes into M2 macrophages by IL-34 and that IL-34 appeared as efficient as CSF1 in inducing the generation of immunosuppressive macrophages [25]

These findings suggest a paracrine relationship between TAMs and breast cancer cells involving the IL-34/CSF-1/CSF-1R axis and TNF-α. It should be emphasized that the different effects caused by secreted factors from THP1 monocytes and macrophages clearly show that the differentiation status of monocytes/macrophages is a key factor for the regulation of the biological behaviour of breast cancer cells.

Importantly, this study also identifies MDA-MB-231 and MCF-7 breast cancer cells as a target for IL-34, showing that this cytokine is not merely regulating macrophages in the setting of cancer. Downregulation of pERK activity in breast cancer cells following treatment with an antibody directed against CSF1R suggests that an autocrine loop exists in breast cancer cells, which could be dependent on CSF-1 and/or IL-34. In recent studies it was shown IL-34 has a higher affinity to the CSF1R as CSF-1 [12] and our qRT-PCR data show different IL-34 mRNA expression levels in the MDA-MB-231 and MCF-7 breast cancer cells. The MDA-MB-231 breast cancer cell is a triple-negative breast cancer cell line, which is more aggressive and has a poorer prognosis in patients[2] whereas the MCF-7 breast cancer cells is a progesterone and estrogen receptor-positive cell with no HER2 receptor and a better prognosis.[4] Thus, the question arises, whether the level of IL-34 and CSF-1 has any importance for the malignant behaviour of breast cancer cells. Preliminary data of IL-34 expression in breast cancer patients show significantly different IL-34 mRNA expression levels dependent on age and menopausal status but no significant differences in lobular and ductal carcinomas. Nevertheless, the broad range of IL-34 expression in ductal carcinoma suggests that IL-34 high and low expressing subpopulations of breast cancer may exist, which may affect cancer growth. There is evidence showing the requirement of both paracrine and autocrine loops involving CSF1 and CSF1R for the invasion of human breast cancer cells MDA-MB-231 in vivo [39] Thus, the outcome of this study proposes a paracrine relationship between the breast cancer cells and macrophages involving the IL-34/CSF1/CSF1R axis.

Notably, it should be mentioned that conditioned medium from macrophages resulted in an upregulation of TNF-α gene expression. TNF-α is an important factor for inflammatory

processes and for cancer development and chronic diseases.[3] This pro-inflammatory cytokine has been shown to be produced by M1-polarized macrophages like many pro-inflammatory cytokines including IL-12. [36]It is well known that TNF-α can be toxic to tumour cells in high doses, however, a tumour-promoting function of TNF-α has been demonstrated as well. [40] The experiments of this study suggest that breast cancer progression could be regulated by the release of TNF-α from breast cancer cells. Finding in cancer patients showed that TNF-α expression is increased in the stroma of invasive breast carcinoma tissue compared with benign tissue[41] indicating an association of TNF-α with tumour progression and malignancy. Furthermore, serum TNF-α concentrations were positively correlated with tumour stage of breast cancer patients [42]. Of interest, our data show that the level of TNF-α is dependent on the molecular subtype of the breast cancer cell, suggesting that a potential stimulatory or inhibitory role for TNF-α in the growth of breast tumours may be effective only in a subset of patients with breast cancer. In support of this, our preliminary data from MDA-MB-231 and MCF-7 breast cancer treated with antibodies directed against TNF-α reveal differential effect on pERK and NF-kB levels following antibody treatment. Thus, it is tempting to speculate, that the IL-34/ TNF-α crosstalk affects the biological behaviour in subsets of breast cancer patients.

In summary, the findings of this study indicate that IL-34 is an important player in mediating interactions between breast cancer cells, monocytes, and macrophages. (Figure 31) schematically illustrates the regulation of breast cancer cell-monocyte/macrophage crosstalk by IL-34. This study indicates that factors secreted by monocytes and macrophages are differentially effecting gene expression of IL-34/CSF-1/CSF-1R in breast cancer cells. In addition, monocytes and macrophage can upregulate TNF-α expression in an IL-34-dependent manner in MCF-7 and MDA-MB-231 breast cancer cells, although to a different extent. Besides this paracrine regulation, TNF-α expression is also affected by IL-34 in an autocrine feedback loop in MDA-MB-231 but not MCF-7 cells. Thus, the expression of IL-34 and CSF-1 by breast cancer cells probably fosters the recruitment of monocytes. Moreover, IL-34 derived from breast cancer cells regulates TNF-α expression and the phenotype of macrophages, which affects tumour growth and progression as a result.

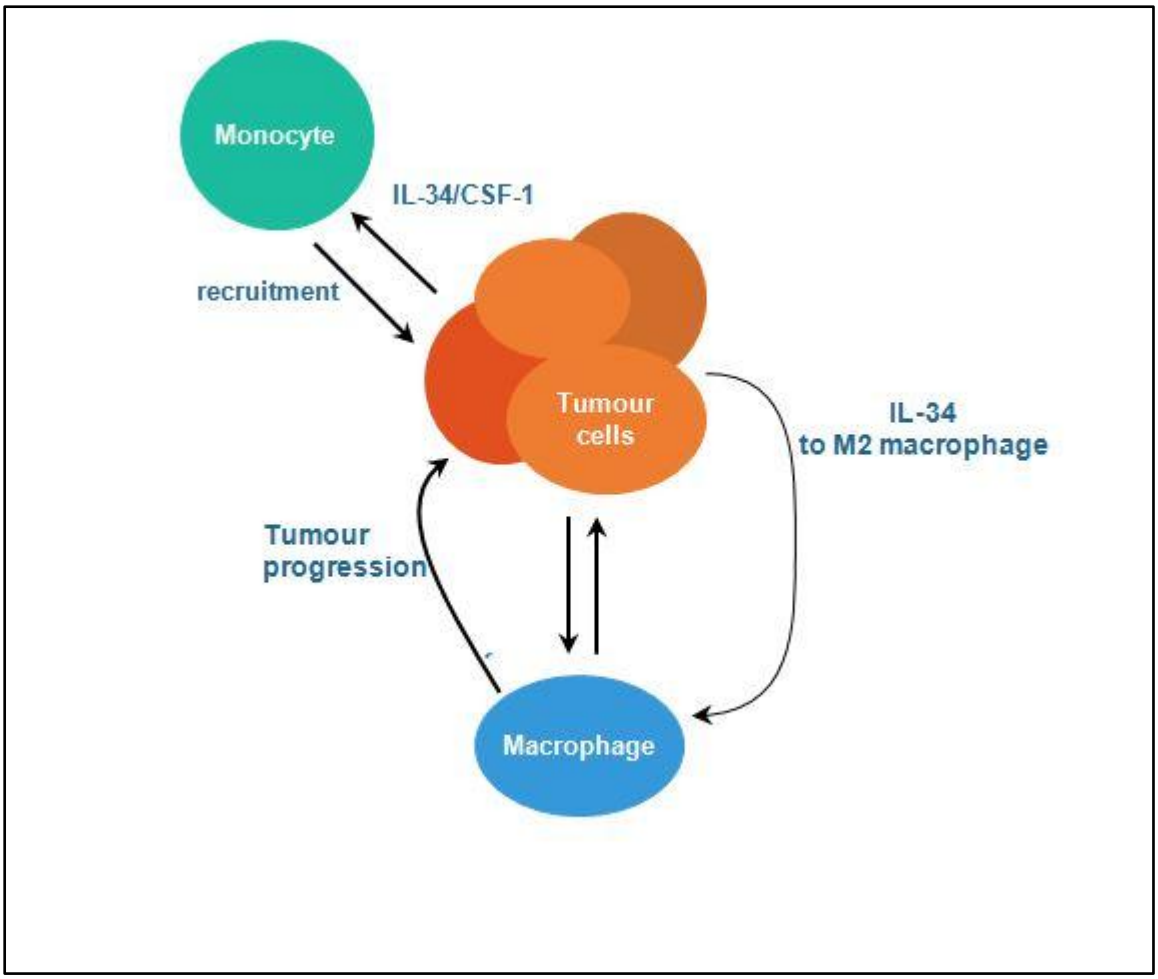


Figure 30: Conclusion of the effect of IL-34

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7 Appendix

7.1 Used buffer systems

7.1.1 SDS- acrylamide electrophoresis

Separation Buffer	
Tris	1.5 M
SDS	0.4 %
Fill up to 500 mL H_2O and set up the pH value to 8.8	

Table 8*: Separation Buffer for SDS- acrylamide electrophoresis*

Stacking Buffer	
Tris	0.5 M
SDS	0.4 %
Fill up to 500 mL H_2O and set up the pH value to 6.8	

Table 9*: Stacking Buffer for SDS- acrylamide electrophoresis*

10 x Running buffer	
Tris	0.25 M
Glycin	1.92 M
Fill up to 1000 mL H_2O	

Table 10*: 10x Running buffer for SDS- acrylamide electrophoresis*

1 x Running buffer	
10 x Running buffer	100 mL
20 % SDS	2.5 mL
Fill up to 1000 mL H_2O	

Table 11*: 1x Running buffer for SDS- acrylamide electrophoresis*

Acrylamide/ N,N'-Methylendiacrylamid	
N,N'-Methylendiacrylamid	0.8 %
Acrylamide	30 %
Fill up to 400 mL with H_2O.	

Table 12*: Acrylamide/N,N'-Methylendiacrylamid for SDS- acrylamide electrophoresis*

10 % Ammonium-Persulfate
Dissolve 1 g in 10 ml ddH_2O
20 % SDS
200 g (= 20 % w/v) dissolved in 1000 mL H_2O

Table 13*: 10% Ammonium-Persulfate for SDS- acrylamide electrophoresis*

7.1.2 Semi- dry blotting

Transfer buffer	
Gycine	39 mM
Tris	48 mM
20 % SDS	0.04 % v/v
Methanol	20 % v/v
Fill up to 1000 ml with H_2O.	

Table 14*: Transfer buffer for Semi-dry blotting*

7.1.3 Ponceau Staining

Ponceau S solution:	
Ponceau S	0.2 % w/v
TCA	3 % w/v
Fill up to 500 ml with H_2O.	

Table 15*: Ponceau S solution for Ponceau Staining*

7.1.4 Agarose gel electrophoresis

50x TAE electrophoresis buffer	
Tris	242 g
Glacial acetic acid	57.1 mL
0,5 M EDTA pH 8	100 mL
Fill up to 1000 ml with H_2O.	

Table 16*: 50x TAE buffer for Agarose gel electrophoresis*

1x TAE electrophoresis buffer	
50x TAE electrophoresis buffer	20 mL
Fill up to 1000 ml with H_2O.	

Table 17*: 1x TAE electrophoresis buffer*

8 Figures

9 Tables

10 Curriculum vitae

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